Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton

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Summary

Indian hedgehog (Ihh) is indispensable for development of the osteoblast lineage in the endochondral skeleton. In order to determine whether Ihh is directly required for osteoblast differentiation, we have genetically manipulated smoothened (Smo), which encodes a transmembrane protein that is essential for transducing all Hedgehog (Hh) signals. Removal of Smo from perichondrial cells by the Cre-LoxP approach prevents formation of a normal bone collar and also abolishes development of the primary spongiosa. Analysis of chimeric embryos composed of wild-type and Smo<sup>n/n</sup> cells indicates that Smo<sup>n/n</sup> cells fail to contribute to osteoblasts in either the bone collar or the primary spongiosa but generate ectopic chondrocytes. In order to assess whether Ihh is sufficient to induce bone formation in vivo, we have analyzed the bone collar in the long bones of embryos in which Ihh was artificially expressed in all chondrocytes by the UAS-GAL4 bigenic system. Although ectopic Ihh does not induce overt ossification along the entire cartilage anlage, it promotes progression of the bone collar toward the epiphysis, suggesting a synergistic effect between ectopic Ihh and endogenous factors such as the bone morphogenetic proteins (BMPs). In keeping with this model, Ihh signaling is further found to be required in BMP-induced osteogenesis in cultures of a limb-bud cell line. Taken together, these results demonstrate that Ihh signaling is directly required for the osteoblast lineage in the developing long bones and that Ihh functions in conjunction with other factors such as BMPs to induce osteoblast differentiation. We suggest that Ihh acts in vivo on a potential progenitor cell to promote osteoblast and prevent chondrocyte differentiation.

Key words: Osteoblast, Endochondral skeleton, Chondrocyte, Ihh, Smo

Introduction

Much of the vertebrate skeleton, including long bones in the limbs, is derived from a cartilage template. During limb development, mesenchymal cells that originate from the lateral plate mesoderm first condense and then form a cartilage anlage composed of chondrocytes that are surrounded by several layers of fibroblast-like cells, the perichondrium (Hinchcliffe, 1990). Chondrocytes within the cartilage anlage initially all proliferate; subsequently, cells residing at the middle of the anlage exit the cell cycle and undergo cellular hypertrophy (Poole, 1991). Following cartilage hypertrophy, osteoblasts first appear in the perichondrium adjacent to the hypertrophic zone where they form a bone collar (Caplan, 1987). Concomitantly, vasculature from the surrounding tissue invades and triggers the removal of hypertrophic cartilage (Gerber and Ferrara, 2000). The vasculature is also believed to bring in osteoblasts that synthesize and deposit bone matrix on the surfaces of the preexisting mineralized cartilage matrix, forming a network of irregular spicules termed the primary spongiosa. Thus, osteoblast differentiation in the limb, both in the bone collar and the primary spongiosa, is closely coupled with chondrocyte maturation.

Indian hedgehog (Ihh), one of the three mammalian homologues of the <i>Drosophila</i> Hedgehog (Hh) protein, has emerged as a key regulator of the developing endochondral skeleton. Ihh is primarily expressed by pre- and early hypertrophic chondrocytes, and it signals to both immature chondrocytes and overlying perichondrial cells (St-Jacques et al., 1999; Vortkamp et al., 1996). Studies in both chicken and mouse have established that Ihh controls the onset of chondrocyte hypertrophy primarily via parathyroid hormone-related protein (PTHrP) (Karp et al., 2000; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). The <i>Ihh</i> knockout studies also revealed that Ihh is a potent positive regulator of chondrocyte proliferation (St-Jacques et al., 1999). As with all Hh proteins, Ihh signaling in the receiving cells requires the seven-pass transmembrane protein encoded by smoothened (<i>Smo</i>). Recent genetic manipulations of <i>Smo</i> by both loss-of-function and gain-
of-function approaches have demonstrated that Ihh directly regulates chondrocyte proliferation (Long et al., 2001).

Ihh is also indispensable for osteoblast differentiation in the endochondral skeleton. This is evidenced by the lack of a bone collar in the long bones of Ihh null (Ihh<sup>−</sup>/−) mice (St-Jacques et al., 1999). Furthermore, bone collar forms adjacent to ectopic hypertrophic chondrocytes in an Ihh-dependent fashion in growth plates containing both wild-type and PTH/PTHrP receptor (also known as Pthr1) null chondrocytes (Chung et al., 1998; Chung et al., 2001). However, it is not clear whether the regulation of osteoblast formation by Ihh indicates a direct requirement for Ihh signaling in osteoblast differentiation, or occurs merely as a secondary consequence of Ihh signaling in chondrocytes. In addition, since the long bones in Ihh<sup>−</sup>/− embryos fail to develop a marrow cavity when they die at birth, it has not been possible to determine whether Ihh signaling is required for the formation of the primary spongioza.

The bone morphogenetic protein (BMP) family of proteins has long been implicated in osteogenesis. BMPs are capable of inducing bone de novo when implanted ectopically in vivo (Wozney et al., 1988); several BMPs are expressed by chondrocytes and the perichondrium during normal development (Pathi et al., 1999). A direct role of BMPs in osteoblast differentiation however remains to be established, as bone induction by ectopic BMPs appears to be mediated by a cartilage intermediate, recapitulating endochondral bone formation. Moreover, genetic removal of BMP signaling has not be informative in this regard because of complications such as early lethality or potential functional redundancy among family members (Hogan, 1996).

We have studied the requirement for Ihh signaling in osteoblast development in the long bones. The data suggest that Ihh plays a direct role in promoting osteogenesis, most probably in conjunction with BMPs.

### Materials and methods

#### Genetically modified mouse strains and embryonic stem (ES) cell lines

Mouse strains of Smo<sup>+/+</sup> and Smo<sup>−/−</sup>, Colat<sup>(II)-Cre</sup>3, Colat<sup>(II)-Cre</sup>10, Colat<sup>(II)-Cre</sup>15, Col2-Gal4 and UAS-Ihh have been described previously (Long et al., 2001). Smo<sup>+/+</sup> and Smo<sup>−/−</sup> ES cell lines carrying the Rosa26-lacZ reporter allele (Zambrowicz et al., 1994) have been described previously (Byrd et al., 2002). To produce chimeras with various degrees of ES cell contributions, the number of ES cells injected into the blastocoelic cavity was varied from five to ten. At least two independently established ES cell lines of each genotype yielded an identical phenotype. Resultant chimeric mice were sacrificed at various ages. All the observations in the Results were verified in at least five different chimeric mice. The degree of chimerism was estimated by staining for β-galactosidase activity.

#### Morphological analysis

Whole-mount staining of skeletons were performed according to the method of McLeod with modifications (McLeod, 1980). For chimeric analyses, embryos were sacrificed at various ages, dissected, and fixed in 4% paraformaldehyde/PBS at 4°C for 4 hours. For detection of β-galactosidase activity, tissues were stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) as described previously (Rossert et al., 1995). Subsequently, stained tissues were processed, embedded in paraffin wax, and sectioned at 6 μm. Sections were stained with Hematoxylin and Eosin (H&E) or Nuclear Fast Red for histological analyses. For detection of mineralization, sections were stained with 1% silver nitrate (von Kossa method) and counterstained with Methyl Green, Nuclear Fast Red, or H&E. To detect cartilage matrix, sections were stained with Safranin-O. For histological analyses of postnatal limbs, after fixation in 10% formalin/PBS at room temperature overnight, the samples were decalcified in 20% EDTA at room temperature for 3–4 days with changes of the solution daily. Decalcified limbs were subsequently processed and sectioned in paraffin wax at 6 μm.

### In situ hybridization

Tissues were fixed overnight either in 4% paraformaldehyde/PBS at 4°C or in 10% formalin/PBS at room temperature, processed, embedded in paraffin wax, and sectioned at 6 μm. As for histological analyses, postnatal limbs were decalcified in EDTA (see above) prior to processing and sectioning. In situ hybridization was performed as described previously (Lee et al., 1995) by using complementary 35S-labeled riboprobes.

#### Cell culture and northern blot analysis

MLB13MYC clone 17 (Rosen et al., 1994) was obtained from Genetics Institute (Cambridge, MA) and was grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin at 37°C. To perform an osteoblast differentiation assay, one day after plating, cells were treated with recombinant Bmp2, 500 ng/ml, or the N-terminal fragment of Shh, 500 ng/ml, in the presence or absence of Hh-signaling inhibitors for 1 or 2 days. Recombinant Bmp2 was obtained from Genetics Institute (Cambridge, MA), and the recombinant N-terminal fragment of Shh and the recombinant N-terminal fragment of Ihh were obtained from Curis (Cambridge, MA). Anti-Shh antibody 5E1 (Roelink et al., 1995) (Developmental Studies Hybridoma Bank, University of Iowa) was used at 40 μg/ml. Recombinant Noggin (Regeneron Pharmaceuticals, NY) was used at 1 μg/ml. Total cellular RNA was isolated using Trizol reagent (Gibco-BRL, Gaithersburg, MD). For northern analysis, 30 μg of total RNA was run on a 1.2% gel, blotted onto a nylon membrane, and hybridized with 32P-labeled cDNA probes for osteocalcin, Cbfal (gift from G. Karsenty, Baylor College of Medicine, Houston, TX), Ptc1 (R. Johnson, Stanford University, Stanford, CA), Ihh (Echelard et al., 1993) and Id1 (R. Benezra, Memorial Sloan-Kettering, New York City, NY). Northern analysis for each probe was performed three times.

### Results

#### Direct Ihh requirement for osteoblast differentiation in the bone collar

In order to determine whether the lack of osteoblasts in the long bones of Ihh<sup>−/−</sup> embryos is secondary to the lack of Ihh signaling in chondrocytes, we have examined the bone collar in embryos in which Smo has been ablated in chondrocytes by three independent Colat<sup>(II)-Cre</sup> lines, namely Cre15, Cre3 and Cre10 (Long et al., 2001). Previous studies have demonstrated an increasing efficiency from Cre15 to Cre3 to Cre10 in removing Smo from chondrocytes at E14.5. In Ihh<sup>−/−</sup> mutants, no bone collar is formed in the long bones because of an early arrest in the osteoblast lineage (Fig. 1D) (St.-Jacques et al., 1999). In contrast, bona fide bone collars, as demarcated by Alizarin Red and von Kossa staining, are evident at E18.5 in the long bones of all Colat<sup>(II)-Cre</sup>; Smo<sup>−/−</sup> embryos, including those generated by the Cre10 line which abrogates Ihh signaling in all chondrocytes by E13.5 prior to normal bone collar formation (Fig. 1B,C,F,G, and data not shown) (Long et al., 2001). These results indicate that Ihh signaling in chondrocytes is not necessary for osteoblast differentiation in
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The bone collar formed in the Cre3; Smo\textsuperscript{nc} and Cre10; Smo\textsuperscript{nc} embryos does exhibit several defects. At E18.5, the bone collar normally adheres tightly to the underlying cartilage at the metaphysis and constitutes the immediate boundary of the bone marrow cavity at the diaphysis (Fig. 1A,E). While this remains true in Cre15; Smo\textsuperscript{nc} embryos (Fig. 1B,F), it is not the case in Cre3; Smo\textsuperscript{nc} (data not shown) and Cre10; Smo\textsuperscript{nc} (Fig. 1C,G) embryos. In these genotypes, the bone collar of long bones such as the tibia is separated from the cartilage at the metaphysis and from the bone marrow cavity at the diaphysis by several layers of cells continuous with the perichondrium. Interestingly, the intervening cells appear to assume the morphology of chondrocytes particularly in the diaphyseal region (Fig. 1H), and, indeed, they express mRNA for type II collagen (Fig. 1J). In addition, whole-mount staining indicates that the bone collars of the tibia and fibula in Cre3; Smo\textsuperscript{nc} (data not shown) and Cre10; Smo\textsuperscript{nc} (Fig. 1C) embryos are discontinuous and interspersed with cartilage. Von Kossa staining of sections confirmed that the bone collar is absent from certain regions along the surface of the long bone (Fig. 1G,H).

The bone collar defects observed in Cre3; Smo\textsuperscript{nc} and Cre10; Smo\textsuperscript{nc} embryos prompted us to examine whether Cre is expressed in cells of the osteoblast lineage in these mice. In assays using the Rosa26 reporter strain, both Cre3 (data not shown) and Cre10 (Fig. 1K-M) lines elicit evidence of strong Cre activity not only in chondrocytes but also in the perichondrium, the periosteum and in osteoblasts in the bone collar and primary spongiosa (arrowheads in Fig. 1L and M, respectively). Therefore, it is probable that Cre activity in some
cells of the osteoblast lineage leads to removal of Smo and results in the bone collar defects.

In order to assess directly whether Ihh signaling in the perichondrium is indeed compromised in Cre3; Smo<sup>n/c</sup> and Cre10; Smo<sup>n/c</sup> embryos, we examined expression of patched 1 (Ptc1; also known as Ptch) and Gli1, both known transcriptional targets of the hedgehog pathway (McMahon et al., 2003), by in situ hybridization. In the wild-type embryo at E14.5, both Ptc1 and Gli1 are highly expressed in the perichondrium abutting Ihh-expressing chondrocytes, with Gli1 expressed in a broader domain (Fig. 2A,D). In Cre3; Smo<sup>n/c</sup> (data not shown) and Cre10; Smo<sup>n/c</sup> (Fig. 2C,F) embryos however, neither Ptc1 nor Gli1 is expressed in a normal perichondrial domain, which is identifiable by a darker counterstaining resulting from a more compact organization of perichondrial cells (denoted by arrows in Fig. 2C,F). Interestingly, both genes are expressed at high levels in cells outside this normal domain of perichondrium, indicating robust Ihh signaling at this new position. Therefore, in Cre3; Smo<sup>n/c</sup> and Cre10; Smo<sup>n/c</sup> embryos, removal of Smo from the inner perichondrium renders cells outside the normal Ihh signaling domain responsive to Ihh. The extended range most probably reflects the absence of Ptc1-mediated ligand sequestration in the normal inner perichondrium, as Ptc1 restricts the movement of Hh proteins (Chen and Struhl, 1996; Lewis et al., 2001). The Cre15; Smo<sup>n/c</sup> embryos, in contrast, express Ptc1 and Gli1 in a normal perichondrial domain, albeit at a higher level than wild-type embryos, most probably reflecting increased levels of Ihh caused by diminished expression of Ptc1 in chondrocytes (Fig. 2B,E). These results therefore correlate displacement of the Ihh-responsive domain in the perichondrium with the observed displacement of the bone collar with respect to the cartilage in Cre3; Smo<sup>n/c</sup> and Cre10; Smo<sup>n/c</sup> embryos.

To determine whether the alteration of Ihh signaling in the perichondrium directly affects osteoblast differentiation, we next examined the expression of Cbfa1, a gene whose activity is essential for osteoblast formation, in Cre3; Smo<sup>n/c</sup> and Cre10; Smo<sup>n/c</sup> embryos. At E14.5, Cbfa1 is expressed by both mature chondrocytes and perichondrial cells (Fig. 2G). Interestingly in Cre3; Smo<sup>n/c</sup> (data not shown) and Cre10; Smo<sup>n/c</sup> (Fig. 2H) embryos, Cbfa1 is not expressed in the normal perichondrial region where Ihh responsiveness is abolished (denoted by arrows), but rather is expressed in more peripheral cells where robust Ihh signaling is observed. Therefore, Ihh signaling is not only required for expression of Cbfa1 in inner perichondrial cells, but is also sufficient to induce ectopic Cbfa1 expression in outer cells. Furthermore, the displacement of the Cbfa1 expression domain provides a molecular basis for the displacement of the bone collar.

We next attempted to determine in the Cre10; Smo<sup>n/c</sup> embryos whether the ‘outer periphery’ cells beyond the normal perichondrium manifest the entire program of gene expression of perichondrial cells, since these cells express Cbfa1 in response to Ihh and evidently give rise to the bone collar. Bone morphogenetic protein 3 (Bmp3) is normally expressed in the perichondrium at E14.5 in a highly specific manner, and therefore serves as a useful marker for the perichondrium (Fig. 2I). Whereas Bmp3 expression is abolished in the perichondrium where Smo expression is abrogated, Bmp3 expression is not induced in the ‘outer periphery’ cells (Fig. 2J). Therefore, these cells appear to be distinct from the bona
fide perichondrium and moreover, osteogenesis in a bone collar can be induced without Bmp3 expression.

To further corroborate the direct role for Ihh signaling in osteoblast differentiation, we analyzed chimeric embryos derived from Smo\(^{+/−}\) and wild-type ES cells. Whereas mutant cells (stained blue in Fig. 3A,B) can contribute to the perichondrium of long bones at E17.5, they fail to differentiate into osteoblasts. Rather, Smo\(^{+/−}\) cells in the perichondrium assume the morphology of chondrocytes (Fig. 3C), deposit cartilaginous matrix (Fig. 3D) and express chondrocytic markers such as collagens type II and X (Fig. 3E,F). Adjacent wild-type perichondrial cells undergo normal osteoblast differentiation even when the neighboring chondrocytes are entirely of the Smo\(^{+/−}\) origin (Fig. 3B). As expected, the Smo\(^{+/−}\) cells contribute normally to the bone collar of chimeric embryos (Fig. 3J, arrowhead). These results suggest a direct cell-autonomous requirement for Smo activity to promote osteoblast development and prevent chondrocyte formation in a perichondrially located precursor cell.

In contrast to the direct requirement for Hh signaling in osteogenesis in the long bones, Smo\(^{+/−}\) cells contributing to the mandible do differentiate into mature osteoblasts and synthesize a mineralized bone matrix that stains by the von Kossa method (Fig. 3G,H). Consistent with this result, the mandible of Ihh\(^{+/−}\) embryos forms, although Ptc1 expression in the mandible is abolished in the absence of Ihh signaling (data not shown).

**Direct requirement for Hh signaling in the formation of primary spongiosa**

We next examined development of the primary spongiosa in Colα1(II)-Cre; Smo\(^{+/−}\) animals. In the wild-type pups at postnatal day 3 (P3), the primary spongiosa forms a network of irregular pink-stained (Hematoxylin and Eosin) bone spicules that extend from the terminal hypertrophic chondrocytes into the bone marrow (Fig. 4A,C). Before reaching the marrow cells that are characterized by darkly stained nuclei and little cytoplasm, the bone spicules are interspersed with cells of lightly stained nuclei and basophilic cytoplasm, presumably cells of the osteoblast lineage (note region between the green lines, Fig. 4C). In contrast, the Cre3; Smo\(^{+/-}\) (data not shown) and Cre10; Smo\(^{+/-}\) (Fig. 4B,D) pups at P3 completely lack the matrix of the primary spongiosa as well as the non-marrow cells normally adjacent to the matrix (note region between the green lines, Fig. 4D). Consequently, hypertrophic chondrocytes directly abut the marrow cells. A similar defect in the primary spongiosa was observed at E18.5 with the Cre3; Smo\(^{+/-}\) and Cre10; Smo\(^{+/-}\) embryos (data not shown). In contrast, the Cre15; Smo\(^{+/-}\) embryos develop a normal primary spongiosa (data not shown).

We next determined whether the lack of matrix in the region of the primary spongiosa in the Cre10; Smo\(^{+/-}\) animals is associated with a lack of osteoblasts or with an excessive number of osteoclasts. In situ hybridization analyses for the osteoblast marker TRAP (tartrate-resistant acid phosphatase) demonstrate that the mutant animals (Fig. 4H) have normal numbers of osteoclasts immediately adjacent to the hypertrophic chondrocytes and normal numbers adjacent to the bone collar, with no evidence for an increase in the number of osteoclasts in the area adjacent to the growth plate (compare Fig. 4G and H). In contrast, the Cre10; Smo\(^{+/-}\) animals have few or no mature osteoblasts in the marrow cavity that express osteocalcin (also known as Bglap1) even though osteocalcin-positive cells are present in the bone collar (Fig. 4F). Thus the lack of primary spongiosa in the Cre10; Smo\(^{+/-}\) animals is most probably due to the lack of mature osteoblasts.

We next assessed whether the defects in osteoblast differentiation in the primary spongiosa correlate with disruption of Hh signaling. At P3, the growth region cartilage
of the tibia in Cre10; Smo<sup>n/c</sup> animals is considerably shorter than in the wild type, because of a decrease in chondrocyte proliferation (Long et al., 2001). However, Ihh is characteristically expressed by pre- and early hypertrophic chondrocytes in the mutant (Fig. 5B) as in wild-type littermates (Fig. 5A). In wild-type pups, Ptc1 is expressed strongly at the chondro-osseous junction by a subpopulation of the cells interspersed among the bone spicules (Fig. 5C,E). In the Cre10; Smo<sup>n/c</sup> littermates however, no Ptc1 expression is detected in the similar region abutting the terminal hypertrophic chondrocytes (Fig. 5D), consistent with the loss of these cells. Therefore, Hh signaling in cells at the chondro-osseous junction correlates with normal development of the primary spongiosa.

To confirm that Hh signaling is required for osteoblast differentiation in the primary spongiosa, we analyzed WT→Smo<sup>nh</sup> chimeric embryos. Although Smo<sup>nh</sup> cells (stained blue in Fig. 6) contribute to blood vessels in the bone marrow at E17.5, no Smo<sup>nh</sup> osteoblasts are observed within the primary spongiosa (Fig. 6A,B), although mutant cells are occasionally found in the bone marrow cavity (arrows, Fig. 6B). Whether these cells belong to the hematopoietic lineage or to mesenchymal precursors of the osteoblast lineage is unclear. In contrast, Smo<sup>n/+</sup> cells contribute extensively to the primary spongiosa in WT→Smo<sup>n/+</sup> chimeric embryos (Fig. 6C).

**Ectopic Ihh promotes bone collar formation**

In order to determine whether Ihh is sufficient to induce osteoblast differentiation in the developing long bone, we analyzed Col2-
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Gal4; UAS-Ihh embryos (Long et al., 2001). In these embryos Ihh is ectopically expressed throughout the cartilage as evidenced by the strong up-regulation of Ptc1 in both chondrocytes and the perichondrium (Fig. 7B, perichondrium denoted by asterisks). Whole-mount staining of the skeletons revealed ectopic bone formation at the knee and elbow regions of the Col2-Gal4; UAS-Ihh embryos at E18.5 (data not shown). However, as the knee and elbow joints fail to develop in these embryos (data not shown), it is possible that ectopic osteogenesis there is secondary to the failure in joint formation. We therefore focused our analyses on the distal portion of the tibia where joints form normally.

We first examined the expression of Cbfa1 in the Col2-Gal4; UAS-Ihh and the wild-type embryos. At E16.5, Cbfa1 is normally highly expressed in the pre-hypertrophic and hypertrophic chondrocytes and at a lower level in the perichondrium (Fig. 7C). In the double transgenic littermates, Cbfa1 expression in the perichondrium is up-regulated and expression extended to reach the epiphysis (Fig. 7D, perichondrium denoted by asterisks). In contrast, Cbfa1 expression in chondrocytes is not significantly altered (Fig. 7D). Thus, ectopic activation of the Hh pathway induces expression of Cbfa1 in the perichondrium.

In order to determine whether Cbfa1 expression, induced by ectopic Ihh, leads to differentiation of mature osteoblasts, we next examined the expression of osteocalcin. In the tibia of E16.5 wild-type embryos, a morphologically identifiable bone collar is only present at the level of the diaphysis, and osteocalcin is only detected in the diaphyseal bone collar (Fig. 7G). No osteocalcin expression is detected in the perichondrium at the level of hypertrophic cartilage expressing Col1(X) (Fig. 7E). In Col2-Gal4; UAS-Ihh littermates however, bone is readily identifiable at a level proximal to the hypertrophic zone, and osteocalcin expression is evident in cells associated with the precocious bone collar (Fig. 7F,H). Interestingly, unlike the up-regulation of Cbfa1, the precocious bone collar does not extend throughout the perichondrium (compare Fig. 7D and H). Thus ectopic Ihh promotes bone collar formation in the long bone but it alone is not sufficient to induce osteogenesis throughout the perichondrium.

To examine the relationship between Hedgehog signaling and other osteogenic
factors such as BMPs, we performed cell culture studies using a mouse limb bud cell line MLB13MYC clone 17 that is known to undergo osteoblast differentiation upon treatment with BMPs (Rosen et al., 1994). BMP2 induces expression of both Cbfal and osteocalcin after 2 days (Fig. 8, lane 2). Interestingly, Bmp2 also induces expression of Ihh and up-regulates Ptc1 (Fig. 8, lane 2). The N-terminal fragment of Shh (N-Shh, an established surrogate for Ihh) however, does not itself induce either Cbfal or osteocalcin in this cell line, even though it greatly upregulates Ptc1 (Fig. 8, lane 3). Importantly, a blocking antibody against Shh and Ihh, 5E1 (Fig. 8, lane 7) inhibits the induction of osteocalcin and Cbfal by Bmp2 either completely or partially (Fig. 8, lane 4) whereas an unrelated monoclonal IgG has no effect (Fig. 8, lane 5). The block of BMP2-induced Cbfal and osteocalcin expression is specific as the induction of Id1 (also known as Idb1), a target of BMP signaling (Katagiri et al., 1994), is not inhibited by 5E1 (Fig. 6A, lane 4) whereas noggin, a potent inhibitor of BMPs, blocks Id1 expression (Fig. 8, lane 6). These results indicate that Hh signaling is required for Bmp2-induced osteoblast differentiation in MLB13MYC clone 17 cells, and Hh and BMP pathways cooperate to establish the mature osteoblast phenotype.

Discussion

By genetically removing Smo from subsets of cells through the use of both the Cre-LoxP system and the generation of chimeric mice, we have demonstrated a direct role for Hh signaling in osteoblast differentiation within the bone collar. In contrast to Ihh mutants, all Col2-Cre; Smo\textsuperscript{apc} embryos, including those generated by the Cre10 line where Smo is removed from all chondrocytes by E13.5 (Long et al., 2001), develop a bone collar in long bones. Thus, the bone defect in the Ihh\textsuperscript{apc} mutant is unlikely to be a secondary consequence of the lack of Hh signaling in chondrocytes, but rather the loss of direct Hh signaling in the perichondrium. Consistent with this notion, in Cre3; Smo\textsuperscript{apc} and Cre10; Smo\textsuperscript{apc} embryos, where the perichondrium is devoid of Smo activity, no bone collar forms.

Moreover, in chimeric embryos Smo\textsuperscript{apc} cells, although contributing to the perichondrium, fail to undergo osteoblast differentiation. An ectopic, discontinuous bone collar is formed from more peripheral and Ihh-responsive cells in the Cre3; Smo\textsuperscript{apc} and Cre10; Smo\textsuperscript{apc} embryos. Thus at least certain cells outside the normal perichondrium are competent to undergo osteogenesis and Hh signaling is sufficient to trigger this differentiation pathway.

Interestingly in Cre3; Smo\textsuperscript{apc} and Cre10; Smo\textsuperscript{apc} embryos, ectopic chondrocytes separate the bone collar from the underlying cartilage and bone marrow cavity. It is possible that this simply results from compromised cartilage removal due to the lack of Ihh signaling in chondrocytes, however, we consider this unlikely since Cre15; Smo\textsuperscript{apc} embryos are normal in this regard. Alternatively, the ectopic chondrocytes could be derived from perichondrial cells that fail to undergo osteoblast differentiation in the absence of a direct Ihh input. This interpretation is consistent with data from the chimeric embryos in which Smo\textsuperscript{apc} perichondrial cells also differentiate into chondrocytes. Therefore, certain progenitor cells residing within the perichondrium are capable of developing into either osteoblasts or chondrocytes, depending upon the presence or absence of Ihh signaling. Interestingly in this regard, in the absence of Osx the perichondrium also undergoes chondrocyte differentiation (Nakashima et al., 2002) whereas loss of Sox9 activity in the cranial neural crest cells appear to result in ectopic osteoblast development (Mori-Akiyama et al., 2003). It is not known whether Ihh, Osx and Sox9 interact to regulate cell fate. The molecular identity of the bipotential progenitor cells in the perichondrium also is not clear at present, but they evidently express collagen type II transiently since the Col1a1(II)-Cre transgene in the Cre3 and Cre10 lines is expressed in both lineages. The transgene in the Cre15 line is expressed later during development and apparently mostly in the chondrocyte lineage. Although it remains possible that the transient activity of the Col1a1(II) promoter in the perichondrium of the transgenic lines does not faithfully represent expression of the endogenous gene, another Col1a1(II)-Cre transgenic line independently developed using a longer version of the Col1a1(II) regulatory sequences (Ovchinnikov et al., 2000) also expresses Cre in both the chondrocyte and the osteoblast lineages, and generates a similar bone phenotype when crossed to the Smo conditional allele (data not shown).

Our experiments also demonstrate that Hh signaling is required for development of the primary spongiosa. Both Cre3; Smo\textsuperscript{apc} and Cre10; Smo\textsuperscript{apc} but not Cre15; Smo\textsuperscript{apc} embryos fail to develop primary spongiosa. Since Cre3 and Cre10 remove Smo from the perichondrium whereas Cre15 does not, it is likely that osteoblasts forming the primary spongiosa originate in the perichondrium. In addition, the long bones of the Cre3; Smo\textsuperscript{apc} and Cre10; Smo\textsuperscript{apc} embryos also lack certain morphologically distinct cells at the cartilage-marrow junction. These cells are probably in the osteoblast lineage as they physically associate with nascent bone spicules. Of note, within this group of cells a subpopulation immediately adjacent to the last row of hypertrophic chondrocytes expresses high levels of Ptc1, indicative of a robust response to Ihh. Therefore, it is possible that during normal development of the
primary spongyia, osteoprogenitors derived from the perichondrium undergo osteoblast differentiation at the chondro-osseous junction upon sensing Ihh. In the mutant embryos, the progenitor cells devoid of Smo do not respond to Ihh and thus fail to become osteoblasts. Alternatively, the perichondrium-derived osteoprogenitor cells in the absence of Ihh signaling could fail to migrate to the potential chondro-osseous junction. The present study does not distinguish between these possibilities.

Interestingly, although Hh signaling is indispensable for osteogenesis in the limb, it is apparently not necessary for the development of certain other bones such as the mandible. The mandible is of neural crest origin and undergoes intramembranous ossification. It remains to be determined whether all bones of this origin develop independent of Hh signaling.

Hh signaling appears to cooperate with other molecules to induce osteoblast differentiation. Although Ihh, when ectopically expressed in all chondrocytes, advances the perichondrial region to promote osteoblast specification, the cartilage collar extended up toward the epiphysis when Ihh was expressed by mutant chondrocytes near the epiphysis, but not when the chondrocytes were mutant for both the PTH/PTHrP receptor and Ihh. These results suggest that other signals must combine with Ihh to generate mature osteoblasts. The studies using MLB13MYC clone 17 cells suggest that BMPs require concomitant Ihh signaling to induce osteoblasts in cells derived from the limb bud. In addition to BMPs, Wnt signaling has recently been implicated in osteogenesis (Gong et al., 2001; Kato et al., 2002; Little et al., 2002). It remains to be determined whether the Hh, BMP and Wnt pathways also interact in normal osteoblast differentiation.

In summary, our data support a model in which Ihh acts on a bipotential osteochondroprogenitor cell that is located within the perichondrial region to promote osteoblast specification while inhibiting the alternative chondrocyte pathway.

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References


