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

Fanxin Long[1,*,†], Ung-il Chung[2,*,‡], Shinsuke Ohba[3], Jill McMahon[1], Henry M. Kronenberg[2] and Andrew P. McMahon[1,§]

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA
[2]Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
[3]Department of Bone and Cartilage Regenerative Medicine, University of Tokyo School of Medicine, Tokyo, Japan

*These authors contributed equally to this work
†Present address: Department of Internal Medicine, Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MO
‡Present address: Department of Bone and Cartilage Regenerative Medicine, University of Tokyo School of Medicine, Tokyo, Japan
§Author for correspondence (e-mail: amcmahon@mcb.harvard.edu)

Accepted 2 December 2003

Development 131, 1309-1318
Published by The Company of Biologists 2004
doi:10.1242/dev.01006

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 $Smo^{n/n}$ cells indicates that $Smo^{n/n}$ 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, Hh 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 *Drosophila* 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 *Ihh* 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 (*Smo*). Recent genetic manipulations of *Smo* 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*$^{n/n}$) 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*$^{n/n}$ 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 spongiosa.

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*$^{n/+}$ and *Smo*$^{c/c}$, *Col*α*1(II)-Cre3*, *Col*α*1(II)-Cre10*, *Col*α*1(II)-Cre15*, *Col2-Gal4 and UAS-Ihh* have been described previously (Long et al., 2001). *Smo*$^{n/n}$ and *Smo*$^{n/+}$ ES cell lines carrying the *Rosa26-lacZ* reporter allele (Zambrowicz et al., 1997) 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 blastocoele 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 ^{35}S-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 ^{32}P-labeled cDNA probes for *osteocalcin*, *Cbfa1* (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*$^{n/n}$ 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 *Col*α*1(II)-Cre* 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*$^{n/n}$ mutants, no bone collar is formed in the long bones because of an early arrest in the osteoblast lineage (Fig. 1D) (St.-Jacque 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 *Col*α*1(II)-Cre; Smo*$^{n/c}$ 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

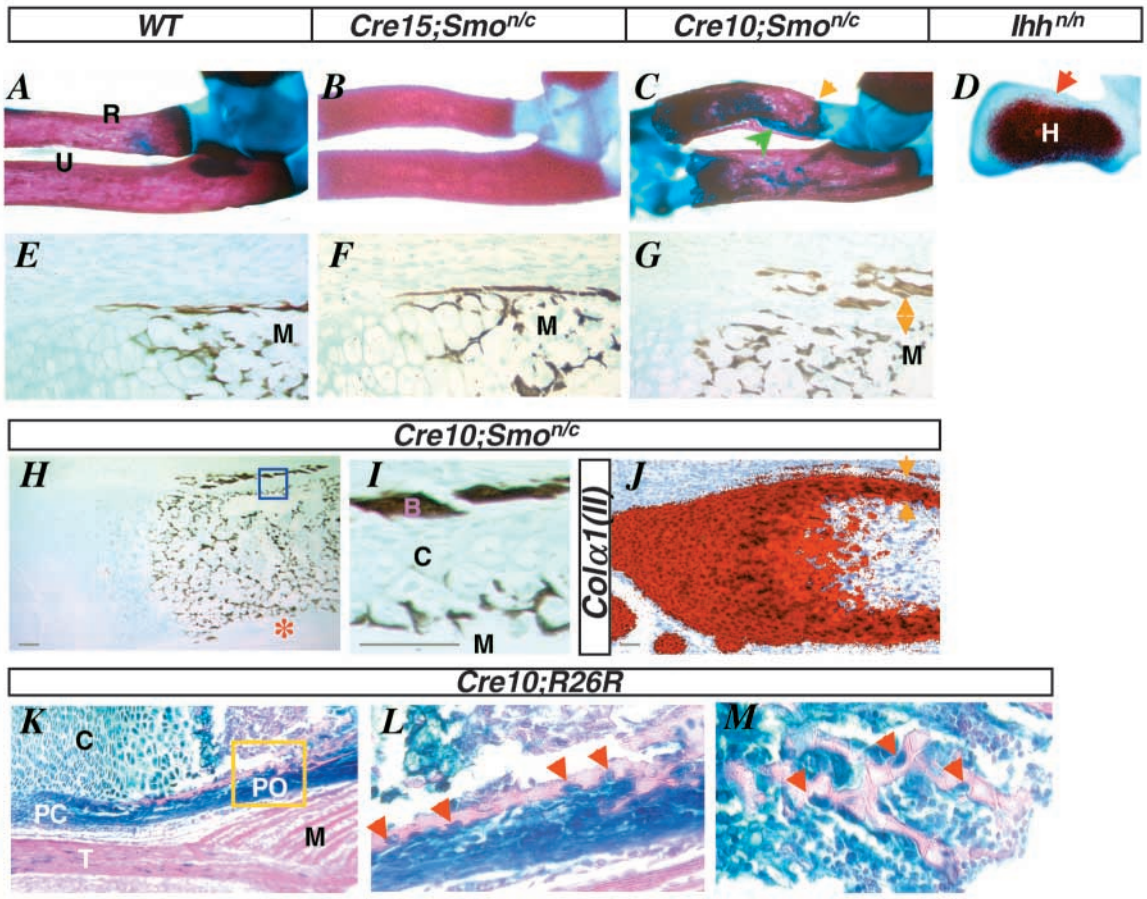

Fig. 1. Morphological analyses of the bone collar. (A-D) Whole-mount skeletal staining of (A-C) the radius (R) and ulna (U) and (D) the tibia at E18.5. Alizarin Red stains the bone red and Alcian Blue stains the cartilage blue. (C) The bone collar in *Cre10; Smo$^{n/c}$* is separated from the cartilage (orange arrowhead) and contains cartilage in certain areas (green arrowhead). (D) No bone collar (red arrowhead) is formed in the long bones of *Ihh$^{n/n}$* embryos. The mineralized hypertrophic cartilage (H) is stained dark red. (E-I) Mineralization revealed by von Kossa staining (black) on sections of E18.5 tibia counterstained with Methyl Green. Genotypes for E-G correspond to A-C, respectively. (G) The bone collar in *Cre10; Smo$^{n/c}$* is separated from the marrow cavity (M) by several layers of cells (orange double arrowhead). (H,I) These cells assume the morphology of chondrocytes (I, a higher magnification of the boxed region in H. B, bone; C, chondrocytes; M, marrow). The asterisk in H denotes a region where the bone collar is missing. (J) In situ hybridization using ^{35}S-labeled riboprobes against *Colα1(II)* on a section adjacent to that in H. High levels of expression (signal in red) are detected in cells separating the bone collar and the marrow cavity in *Cre10; Smo$^{n/c}$* embryos (between arrows). (K,L,M) Analyses of Cre activity of the *Cre10* line using Rosa26 reporter (*R26R*) mice. Evidence of Cre activity (strong blue staining) is detected in chondrocytes (C) as well as in the perichondrium (PC) and the periosteum (PO), whereas muscles (M) and tendons (T) are negative. A higher magnification (L) of the boxed region in K shows strong Cre activity in osteoblasts of the bone collar (red arrowheads). Strong Cre activity is also evident in osteoblasts of the primary spongiosa (red arrowheads in M).

the bone collar and that Ihh probably regulates osteogenesis directly.

The bone collar formed in the *Cre3; Smo$^{n/c}$* and *Cre10; Smo$^{n/c}$* 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$^{n/c}$* embryos (Fig. 1B,F), it is not the case in *Cre3; Smo$^{n/c}$* (data not shown) and *Cre10; Smo$^{n/c}$* (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. 1I), 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$^{n/c}$* (data not shown) and *Cre10; Smo$^{n/c}$* (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$^{n/c}$* and *Cre10; Smo$^{n/c}$* 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

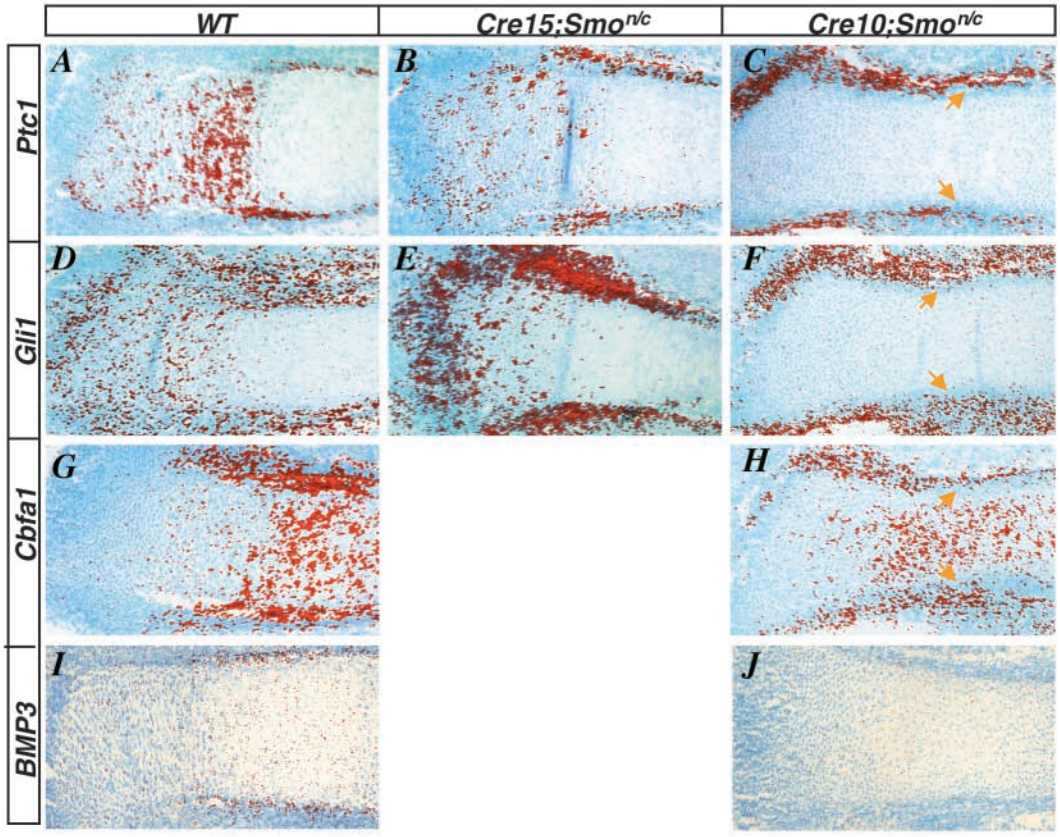

Fig. 2. In situ hybridization of ^{35}S labeled riboprobes to limb elements following Smoothened removal. (A-J) E14.5 tibial elements of indicated genotypes hybridized with probes as indicated. Signals are shown in red on sections counterstained with Toluidine Blue. The orange arrows denote the perichondrium not expressing the various genes in *Cre10; Smo$^{n/c}$* embryos.

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$^{n/c}$* and *Cre10; Smo$^{n/c}$* 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$^{n/c}$* (data not shown) and *Cre10; Smo$^{n/c}$* (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$^{n/c}$* and *Cre10; Smo$^{n/c}$* 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$^{n/c}$* 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$^{n/c}$* and *Cre10; Smo$^{n/c}$* 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$^{n/c}$* and *Cre10; Smo$^{n/c}$* embryos. At E14.5, *Cbfa1* is expressed by both mature chondrocytes and perichondrial cells (Fig. 2G). Interestingly in *Cre3; Smo$^{n/c}$* (data not shown) and *Cre10; Smo$^{n/c}$* (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$^{n/c}$* 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

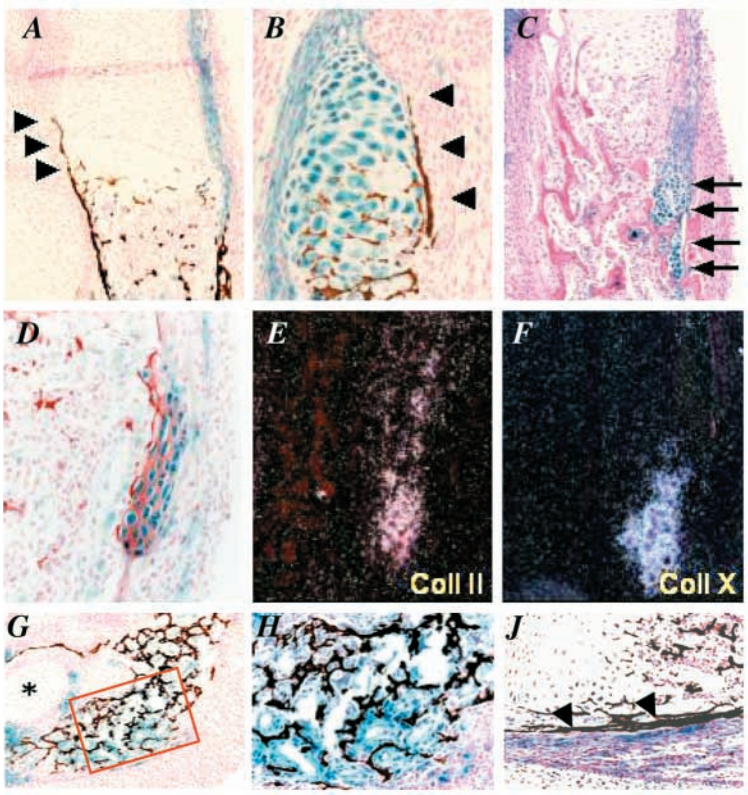

Fig. 3. Analysis of skeletal elements in chimeric embryos. Contribution of *Smo*$^{n/n}$ (A-H) or *Smo*$^{n/+}$ (J) cells to the bone collar of long bones (A-F,J) and to the mandible (G,H) in chimeric embryos at E17.5. *Smo*$^{n/n}$ and *Smo*$^{n/+}$ cells are stained blue as a result of the expression of β-galactosidase and the sections are counterstained with Nuclear Fast Red (A,B,G,H), H&E (C,J), or Methyl Green (D). Mineralized tissues are stained black by the von Kossa method (A,B,G,H,J). (A,B) The bone collar forms on one side of the tibia (A, arrowheads) or the fibula (B, arrowheads), but fails to develop from the perichondrium on the opposite side which is composed of mutant cells. (C) The *Smo*$^{n/n}$ perichondrial cells differentiate into chondrocytes (arrows). (D) Safranin-O stains red the cartilaginous matrix deposited by chondrocytes differentiated from the *Smo*$^{n/n}$ perichondrial cells. (E,F) Dark-field images of in situ hybridization using ^{35}S-labeled riboprobes for *Colα1(II)* (E) and *Col α1(X)* (F) on sections similar to that in D. (G,H) *Smo*$^{n/n}$ cells contribute to the osteoblast population of the mandible. Asterisk in G denotes Meckel's cartilage. The boxed region in G is shown at a higher magnification in H. (J) In chimeras composed of *Smo*$^{n/+}$ and wild-type cells, the *Smo*$^{n/+}$ cells contribute to the bone collar (black arrowheads).

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*$^{n/n}$ 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*$^{n/n}$ 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*$^{n/n}$ origin (Fig. 3B). As expected, the *Smo*$^{n/+}$ 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*$^{n/n}$ 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*$^{n/n}$ 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*$^{n/c}$ 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*$^{n/c}$ (data not shown) and *Cre10; Smo*$^{n/c}$ (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*$^{n/c}$ and *Cre10; Smo*$^{n/c}$ embryos (data not shown). In contrast, the *Cre15; Smo*$^{n/c}$ 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*$^{n/c}$ 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*$^{n/c}$ 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*$^{n/c}$ 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

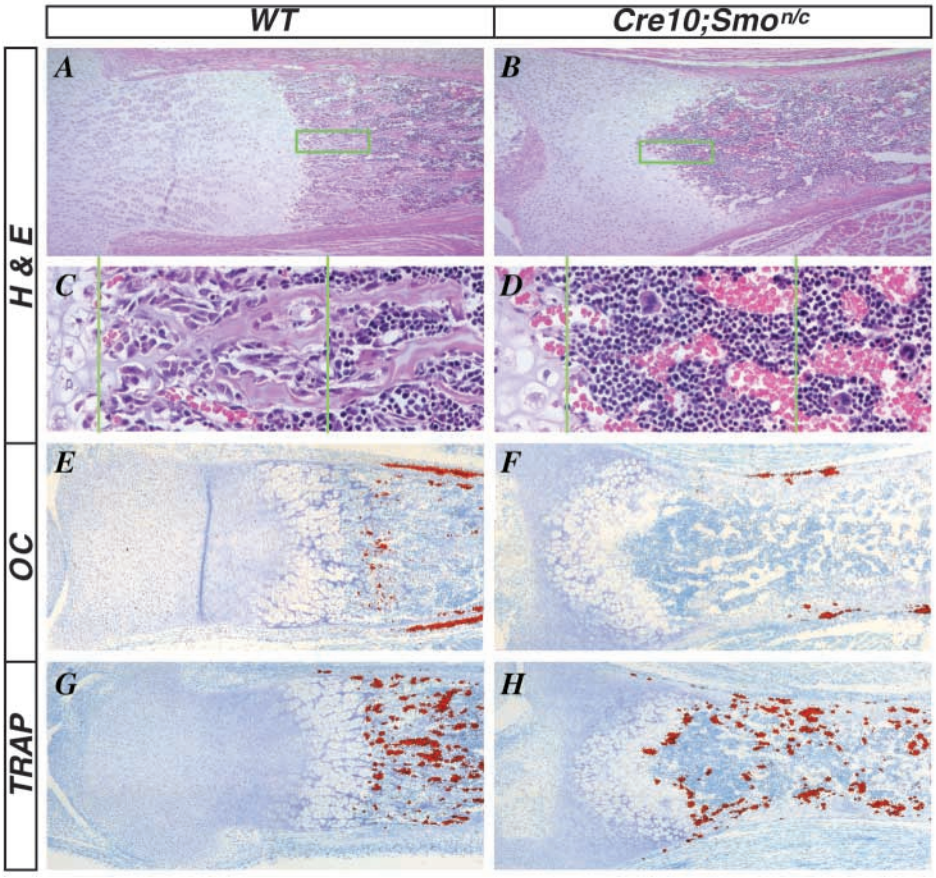

Fig. 4. Lack of primary spongiosa in *Cre10; Smo$^{n/c}$* embryos. (A-D) H&E staining of longitudinal sections through the tibia at P3. The boxed regions in A and B are shown at a higher magnification in C and D. The straight lines in C and D define a region of the same length that in the wild type (C) but not in the mutant (D) is populated by cells with morphology that is distinct from that of the marrow cells. (E-H) In situ hybridization using ^{35}S labeled riboprobes for *osteocalcin* (E,F) and *TRAP* (G,H) on longitudinal sections of the tibia at P3. Signals are shown in red and the sections are counterstained with Toluidine Blue.

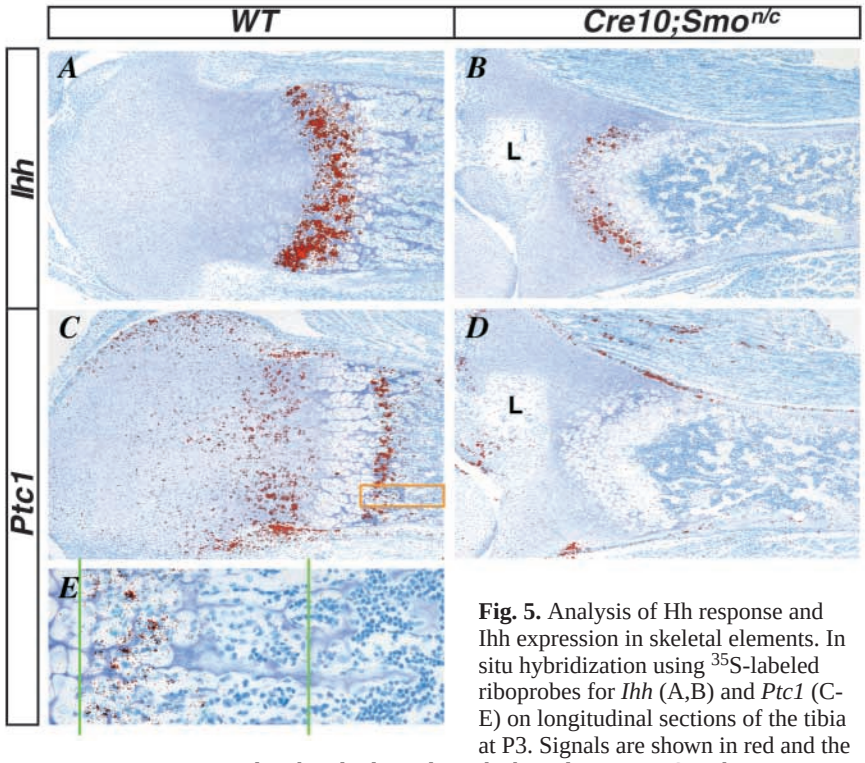

Fig. 5. Analysis of Hh response and Ihh expression in skeletal elements. In situ hybridization using ^{35}S-labeled riboprobes for *Ihh* (A,B) and *Ptc1* (C-E) on longitudinal sections of the tibia at P3. Signals are shown in red and the sections are counterstained with Toluidine Blue. The boxed region in C is shown at a higher magnification in E. The straight lines in *E* demarcate the region populated by cells with morphology that is distinct from that of the marrow cells, similar to that defined in C and D of Fig. 4.

of the tibia in *Cre10; Smo$^{n/c}$* 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, *Pct1* 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$^{n/c}$* 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$^{n/n}$* chimeric embryos. Although *Smo$^{n/n}$* cells (stained blue in Fig. 6) contribute to blood vessels in the bone marrow at E17.5, no *Smo$^{n/n}$* 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$^{n/+}$* cells contribute extensively to the primary spongiosa in *WT↔Smo$^{n/+}$* 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-*

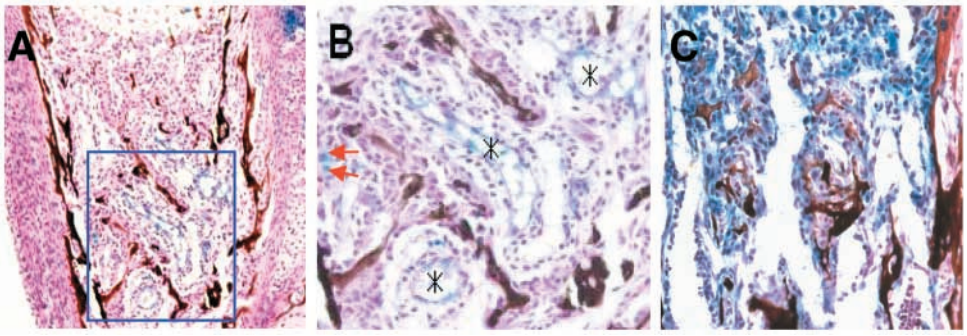

Fig. 6. Analysis of the primary spongiosa in chimeric embryos. *Smo*$^{n/n}$ cells fail to contribute to the primary spongiosa in chimeric embryos (A,B) whereas *Smo*$^{n/+}$ cells contribute extensively (C). Longitudinal sections of the tibia at E17.5 are stained with H&E. *Smo*$^{n/n}$ or *Smo*$^{n/+}$ cells are stained blue as a result of the expression of β-galactosidase. The boxed region in A is shown at a higher magnification in B. Asterisks denote the blood vessels. Arrows in B point to occasional *Smo*$^{n/n}$ cells in the marrow cavity.

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 *Colα1(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

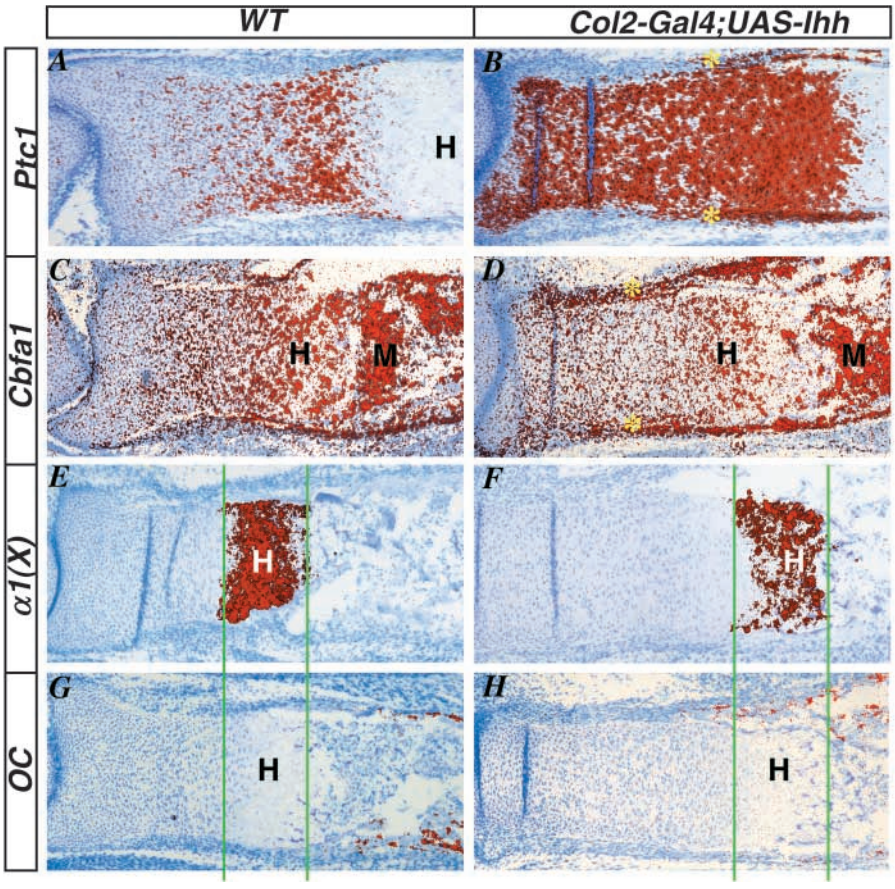

Fig. 7. Analysis of bone formation in the perichondrium following activation of Ihh signaling. (A-H) In situ hybridization using ^{35}S-labeled riboprobes on longitudinal sections of the tibia at E16.5; probes and genotypes as indicated. Signals are shown in red and the sections are counterstained with Toluidine Blue. H, hypertrophic chondrocytes; M, marrow cavity. Asterisks denote the perichondrium in which *Ptc1* or *Cbfa1* are up-regulated.

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 *Cbfa1* 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 *Cbfa1* 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 *Cbfa1* 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 *Cbfa1* 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.

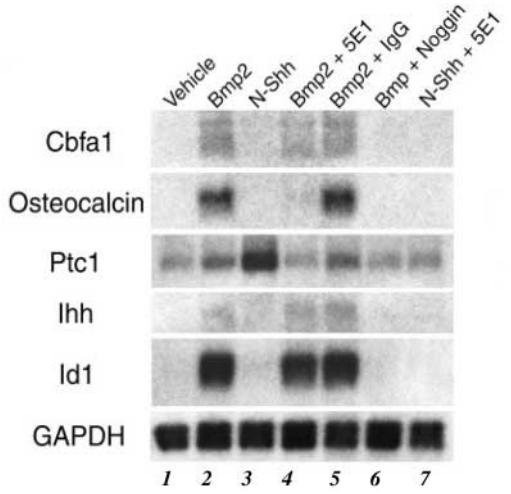

Fig. 8. Interaction of Hh and BMP pathways during osteoblast differentiation *in vitro*. Total RNA extracted from MLB13MYC clone 17 cells treated with various reagents were subjected to northern analyses (see Materials and methods). GAPDH is used as a control for loading and transfer efficiency.

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 $Ihh^{n/n}$ mutants, all *Col2-Cre;* $Smo^{n/c}$ 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 $Ihh^{n/n}$ 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;* $Smo^{n/c}$ and *Cre10;* $Smo^{n/c}$ embryos, where the perichondrium is devoid of *Smo* activity, no bone collar forms. Moreover, in chimeric embryos $Smo^{n/n}$ 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;* $Smo^{n/c}$ and *Cre10;* $Smo^{n/c}$ 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;* $Smo^{n/c}$ and *Cre10;* $Smo^{n/c}$ 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;* $Smo^{n/c}$ 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 $Smo^{n/n}$ 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 *Colα1(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 *Colα1(II)* promoter in the perichondrium of the transgenic lines does not faithfully represent expression of the endogenous gene, another *Colα1(II)-Cre* transgenic line independently developed using a longer version of the *Colα1(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;* $Smo^{n/c}$ and *Cre10;* $Smo^{n/c}$ but not *Cre15;* $Smo^{n/c}$ 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;* $Smo^{n/c}$ and *Cre10;* $Smo^{n/c}$ 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 spongiosa, 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 frontier of the bone collar, it does not induce bone throughout the perichondrium. A similar result was observed in studies in which chimeric growth plates containing both wild-type and PTH/PTHrP receptor null chondrocytes were generated (Chung et al., 1998; Chung et al., 2001). Bone 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.

We gratefully acknowledge all those investigators mentioned earlier who supplied reagents for these studies. Work in A.P.M.'s and H.M.K.'s laboratories was supported by a program grant from the NIH (P01 DK 56246). F.L. was supported by a postdoctoral fellowship from the NIH (GM19621). We thank Paul Cassidy for help with manuscript preparation.

References

Byrd, N., Becker, S., Maye, P., Narasimhaiah, R., St-Jacques, B., Zhang, X., McMahon, J., McMahon, A. and Grabel, L. (2002). Hedgehog is required for murine yolk sac angiogenesis. *Development* **129**, 361-372.

Caplan, A. I. and Pechak, D. G. (1987). The cellular and molecular embryology of bone formation. In *Bone and Mineral Research* vol. 5 (ed. W. A. Peck), pp. 117-183. New York, NY: Elsevier.

Chen, Y. and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87**, 553-563.

Chung, U. I., Lanske, B., Lee, K., Li, E. and Kronenberg, H. (1998). The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc. Natl. Acad. Sci. USA* **95**, 13030-13035.

Chung, U. I., Schipani, E., McMahon, A. P. and Kronenberg, H. M. (2001). Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J. Clin. Invest.* **107**, 295-304.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Gerber, H. P. and Ferrara, N. (2000). Angiogenesis and bone growth. *Trends Cardiovasc. Med.* **10**, 223-228.

Gong, Y., Slee, R. B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A. M., Wang, H., Cundy, T., Glorieux, F. H., Lev, D. et al. (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* **107**, 513-523.

Hinchcliffe, J. R. and Johnson, D. R. (1990). *The Development of the Vertebrate Limb.* Oxford, UK: Clarendon Press.

Hogan, B. L. (1996). Bone morphogenetic proteins in development. *Curr. Opin. Genet. Dev.* **6**, 432-438.

Karp, S. J., Schipani, E., St-Jacques, B., Hunzelman, J., Kronenberg, H. and McMahon, A. P. (2000). Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone-related protein-dependent and -independent pathways. *Development* **127**, 543-548.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A. and Suda, T. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol.* **127**, 1755-1766.

Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H., Glass, D. A., 2nd, Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F. et al. (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J. Cell Biol.* **157**, 303-314.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H., Ho, C., Mulligan, R. C. et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**, 663-666.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**, 453-463.

Little, R. D., Carulli, J. P., Del Mastro, R. G., Dupuis, J., Osborne, M., Folz, C., Manning, S. P., Swain, P. M., Zhao, S. C., Eustace, B. et al., (2002). A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am. J. Hum. Genet.* **70**, 11-19.

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599-612.

Long, F., Zhang, X. M., Karp, S., Yang, Y. and McMahon, A. P. (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* **128**, 5099-5108.

McLeod, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301.

McMahon, A. P., Ingham, P. W. and Tabin, C. J. (2003). Developmental roles and clinical significance of hedgehog signaling. *Curr. Top. Dev. Biol.* **53**, 1-114.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D. H. and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc. Natl. Acad. Sci. USA* **100**, 9360-9365.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R. and de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17-29.

Ovchinnikov, D. A., Deng, J. M., Ogunrinu, G. and Behringer, R. R. (2000). Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26**, 145-146.

Pathi, S., Rutenberg, J. B., Johnson, R. L. and Vortkamp, A. (1999). Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. *Dev. Biol.* **209**, 239-253.

Poole, A. R. (1991). The growth plate: Cellular physiology, cartilage assembly and mineralization. In *Cartilage: Molecular aspects* (ed. B. K. a. N. S. A. Hall), pp. 179-211. Boca Raton, FL: CRC Press.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rosen, V., Nove, J., Song, J. J., Thies, R. S., Cox, K. and Wozney, J. M. (1994). Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. *J. Bone Miner. Res.* **9**, 1759-1768.

Rossert, J., Eberspaecher, H. and de Crombrugghe, B. (1995). Separate cis-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. *J. Cell Biol.* **129**, 1421-1432.

St-Jacques, B., Hammerschmidt, M. and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13**, 2072-2086.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528-1534.

Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G. and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **94**, 3789-3794.