Development 134, 3133-3144 (2007) doi:10.1242/dev.002709

# Regulation of skeletogenic differentiation in cranial dermal bone

**Arhat Abzhanov[1,*], Stephen J. Rodda[2], Andrew P. McMahon[2] and Clifford J. Tabin[1,†]**

Although endochondral ossification of the limb and axial skeleton is relatively well-understood, the development of dermal (intramembranous) bone featured by many craniofacial skeletal elements is not nearly as well-characterized. We analyzed the expression domains of a number of markers that have previously been associated with endochondral skeleton development to define the cellular transitions involved in the dermal ossification process in both chick and mouse. This led to the recognition of a series of distinct steps in the dermal differentiation pathways, including a unique cell type characterized by the expression of both osteogenic and chondrogenic markers. Several signaling molecules previously implicated in endochondrial development were found to be expressed during specific stages of dermal bone formation. Three of these were studied functionally using retroviral misexpression. We found that activity of bone morphogenic proteins (BMPs) is required for neural crest-derived mesenchyme to commit to the osteogenic pathway and that both Indian hedgehog (IHH) and parathyroid hormone-related protein (PTHrP, PTHLH) negatively regulate the transition from preosteoblastic progenitors to osteoblasts. These results provide a framework for understanding dermal bone development with an aim of bringing it closer to the molecular and cellular resolution available for the endochondral bone development.

**KEY WORDS: Dermal bone, Intramembranous ossification, Cranial development, Mouse, Chick**

## INTRODUCTION

Mineralized skeleton provides the structural framework underlying the morphology of the head and trunk in most modern vertebrates. These skeletal elements arise in two developmentally distinct ways. Most of the trunk skeleton is produced from mesodermal progenitors via the formation of cartilage models that are replaced by bone in the process of endochondral ossification (Erlebacher et al., 1995; Olsen et al., 2000; Karsenty and Wagner, 2002; Kronenberg, 2003). By contrast, many of the bones of the craniofacial skeleton arise directly from cranial dermis via a process termed intramembranous bone formation (Fig. 1A) (Noden, 1983; Noden, 1991; Hall and Miyake, 1992; Dunlop and Hall, 1995; Jiang et al., 2002) (reviewed in Helms and Schneider, 2003). Intramembranous bones arise directly through differentiation of mesenchyme, initially compacted in sheets or membranes. Intramembranous bones are classified into three categories: the sesamoid bones, which form in tendons as a result of mechanical stress (such as the patella in the tendon of the quadriceps femoris); the periosteal bones, which form from connective tissue and add to the thickness of long bones; and the dermal bones, which form within the dermis of the skin.

Considerably more is known about endochondral ossification than dermal bone formation. During endochondral ossification, the cartilage precursor expands via the growth of proliferating chondrocytes (Kronenberg, 2003). However, shortly after formation of the skeletal condensation, the proliferating chondrocytes in the core start to mature into hypertrophic chondrocytes. During this differentiation process, cells transverse through several well-characterized intermediate cell types, including round proliferating chondrocytes, flattened proliferating chondrocytes, the so called 'pre-hypertrophic cells', which have dropped out of the cell cycle but not yet begun to undergo overt hypertrophy, and several types of hypertrophic cells. Each of these steps is characterized by the expression of discrete sets of molecular markers. All chondrocytes of the trunk skeleton express the *Coll II* (also known as *Col2a1* and *Col II*) gene, encoding collagen type II, and the transcription factor *Sox9* (Kronenberg, 2003; Harada and Roden, 2003; Zelzer and Olsen, 2003; Yamashiro et al., 2004; Aberg et al., 2005; Shibata et al., 2006). As the skeletal elements mature and growth plates form towards their distal ends, these two genes continue to be expressed at high levels in both proliferating and resting chondrocytes, whereas *Sox9* is downregulated during the transition to the hypertrophic chondrocytes. *Sox9* is required for chondrogenesis, and acts to induce the expression of such cartilage-specific markers as collagens II, IX and XI and aggrecan (Lefebvre et al., 1997; Lefebvre and de Crombrugghe, 1998; Bi et al., 1999; Healy et al., 1999; Mori-Akiyama et al., 2003). The hypertrophic state is characterized by an extracellular matrix containing unique components, such as collagen type X (Iyama et al., 1991; Kronenberg, 2003). The hypertrophic chondrocyte zone is later invaded by blood vessels from the perichondrium, which bring osteoblasts and hematopoietic cells. The invading osteoblasts replace hypertrophic chondrocytes, which undergo apoptosis, and form ossification centers (Olsen et al., 2000; Zelzer and Olsen, 2003).

These processes of proliferation, hypertrophy, apoptosis and bone replacement are tightly controlled by the activity of several signaling molecules (Kronenberg, 2003), such as bone morphogenic protein (BMP) family members, Indian hedgehog (IHH) and parathyroid hormone-related protein (PTHrP, PTHLH). BMPs have been shown to regulate the initiation of skeletal formation and to induce chondrocyte proliferation both in vitro and in vivo (Kronenberg, 2003; Zhou et al., 1997; Shum et al., 2003). Suppression of BMP activity with the antagonist noggin or with dominant-negative versions of BMP receptors leads to severely reduced bone growth (Capdevila and Johnson, 1998; Pathi et al., 1999; Zou and Niswander, 1996). IHH is another crucial coordinating signal regulating both cell proliferation and differentiation in long-bone

[1]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA.
[2]Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

*Present address: Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
†Author for correspondence (e-mail: tabin@genetics.med.harvard.edu)

**Accepted 28 June 2007**

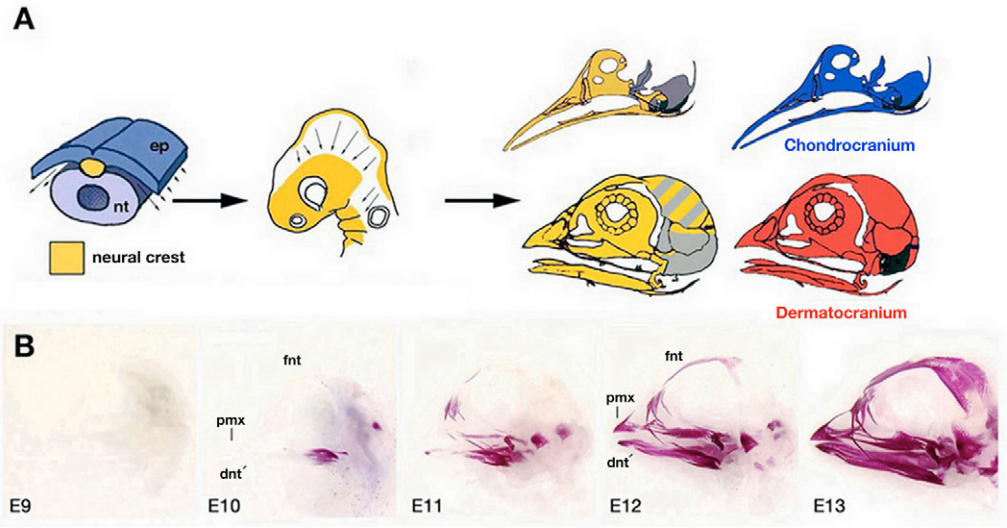


**Fig. 1. Origin and contribution of dermal bone in cranial skeletal development.** (**A**) Contribution of cranial neural crest cells (orange) and mesoderm (grey) to the cartilage (blue) and bone (red) components of the avian skull (see also Noden, 1986; Couly et al., 1993). Notice the heavy contribution of neural crest to the cranial dermal bones. (**B**) Bone mineralization in E9-E13 chick embryonic heads as revealed with alizarin red stain. dnt, dentary bone; ep, epidermis; fnt, frontal bone; nt, neural tube; pmx, pre-maxillary bone.

development. IHH stimulates proliferation of chondrocytes at the growth plate, indirectly suppresses chondrocyte hypertrophic differentiation and, later in development, is directly involved in osteoblast differentiation (Bitgood and McMahon, 1995; Vortkamp et al., 1996; St-Jacques et al., 1999; Long et al., 2004). In *Ihh*-null embryos, no endochondral bone skeleton develops in the trunk, whereas, in the skull, dermal bones form but are markedly reduced at birth (St-Jacques et al., 1999; Karp et al., 2000). *Ihh* is initially expressed throughout the chondrogenic condensations, where it promotes proliferation. Subsequently, the expression of Ihh is mostly limited to the pre-hypertrophic chondrocytes. In addition to its growth-promoting effect on chondrocytes, *Ihh* is necessary and sufficient to activate *PTHrP* expression in periarticular cells of the perichondrium (Schipani et al., 1997; St-Jacques et al., 1999; Long et al., 2004; Karp et al., 2000). In turn, *PTHrP* signaling through its receptor, *PTHrP-R*, acts to block hypertrophic differentiation (Vortkamp et al., 1996; Weir et al., 1996; Lanske et al., 1999). Together, IHH and PTHrP thus form a negative-feedback loop, which serves to regulate the onset of hypertrophic differentiation of chondrocytes (Vortkamp et al., 1996). Levels of IHH and PTHrP regulate the distance between the cells undergoing hypertrophy and the articular surface, the thickness of the growth plate (Vortkamp et al., 1996; Chung et al., 2001). BMP signaling also appears to play a role in the IHH-PTHrP regulatory loop, acting to induce the expression of IHH in differentiating chondrocyte cells released from PTHrP signaling (Minina et al., 2001).

At later stages of endochondral development, the hypertrophic cells are replaced by osteoblasts. Development of osteoblasts from mesenchymal cells depends on *Runx2* (formerly *Cbfa1*); no ossification occurs without its activity (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Komori, 2000; Yamaguchi et al., 2000). RUNX2 protein regulates several bone-specific genes, such as bone sialoproteins [*BSPI* (also known as *Spp1*, *Opn* and osteopontin – Mouse Genome Informatics) and *BspII*], *collagen type I* and osteocalcin (also known as *Bglap1* – Mouse Genome Informatics) (Ducy et al., 1997). RUNX2 also plays a more limited role in chondrocyte hypertrophy during long-bone development (Inada et al., 1999; Kim et al., 1999). The early chondrogenic markers *Coll II* and *Sox9*, discussed above, are also expressed in early osteogenic precursors, but are never found in differentiated osteoblasts (Yamashiro et al., 2004; Aberg et al., 2005).

In contrast to endochondral differentiation, the process of dermal bone formation is poorly understood. Several recent studies have demonstrated that many of the molecules associated with endochondral development are also present during intramembranous bone development, thus suggesting certain similarities in the development of these tissues; however, closer analyses also revealed some specific differences between appendicular and dermal bone development, including differences in their respective matrix composition and structure (Bitgood and McMahon, 1995; Vortkamp et al., 1996; St-Jacques et al., 1999; Long et al., 2004; Scott and Hightower, 1991; Zhao et al., 2002; Holleville et al., 2003; Vega et al., 2004). The different cellular events leading up to dermal and endochondral bone formation, the differences between these tissues themselves, as well as distinct ontogenetic origins of the dermal and endochondral bones, all suggest that a thorough detailed analysis specifically of dermal bone development is required to understand the formation of this important tissue type.

In this study, we characterized the expression of a number of molecular markers during the development of frontal and dentary bones in chick embryos (the proximal part of the dentary bone is occasionally referred to as surangular). This has allowed us to define a series of distinct cellular steps involved in dermal bone formation, including a novel transitional cell type, a chondrocyte-like osteoblast, characterized by the co-expression of both osteogenic and chondrogenic markers in both chicks and mice. The presumptive role that we assigned to the novel ‘chondrocyte-like osteoblast’ as a precursor to the mature osteoblasts was verified in mice by recombinase-based fate mapping. With this context, we analyzed the expression domains of several growth factors known to play key roles in regulating endochondral ossification, including *Bmp2*, *Bmp4*, *Ihh* and *PTHrP*, assigning their expression to specific cell types. Their functions during dermal bone formation were assayed using retrovirus-based constructs in chicken embryos. We found that BMPs play an important role in dermal bone development, regulating the earliest cell differentiation decisions. IHH and PTHrP

act at a later step to negatively regulate the formation of osteoblasts from osteoprogenitors. In the case of IHH, the conclusions based on these gain-of-function studies were verified by analysis of mice deficient for the *Ihh* gene.

## MATERIALS AND METHODS

### Chicken embryo manipulations

Eggs were obtained from SPAFAS (Norwich, CT) and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). The RISAP, RCAS(A)::noggin, RCAS(A)::*Bmp4*, RCAS(B)::*Bmp2* and RCAS(A,E)::*Ihh* constructs have been previously described (Capdevila and Johnson, 1998; Vortkamp et al., 1996; Chen et al., 1999; Duprez et al., 1996; Yu et al., 2002). The RCAS::*PTHrP* construct was from H. Kempf (A. Lassar's laboratory, Harvard Medical School, Boston, USA).

### Mouse work

*Col2::Cre3* transgenic mice have been described previously (Long et al., 2001). For timed pregnancies, we used the plug date as 0 days post-coitum (dpc). Activity of *Col2::Cre3* was assessed by mating the *Col2::Cre3* mouse line (Long et al., 2001) to the *Rosa26-lacZ* reporter mouse (Soriano, 1999), after which embryos were collected at 16.5 dpc and β-galactosidase activity detected as described previously (Whiting et al., 1991).

### In situ hybridizations and bone/cartilage staining of embryos

Heads of chick embryos were collected and fixed in 4% paraformaldehyde (PFA) overnight, washed with 30% sucrose, and frozen in OCT for coronal sections. Older embryos we collected and fixed in 4% PFA and then dehydrated in 95% ethanol for 2 days before staining with Alcian blue to reveal cartilage and alizarin red to reveal bone.

We used the following in situ hybridization probes for chicken: *Bmp4* (1.2 kb), *PTHrP-R* (~970 bp), *Runx2* (~700 bp, gift from Dr Helms, Stanford University, Stanford, USA), *Ptc1* (~3 kb), *Gli* (*Gli1*; ~1.5 kb), *Bmpr1a* (~1.6 kb, gift from Dr Niswander, University of Colorado, Denver, USA), *Bmpr1b* (~1.6 kb, 5′ UTR, gift from Dr Niswander), *Sox9* (~1.5 kb; gift from Dr Sharpe, King's College, University of London, London, UK), *Bmp2* (~1.9 kb), *Bmp7* (~750 bp, gift from Dr Niswander), *Bmp2* (~780 bp, gift from Dr Niswander), *Bmp6* (~2 kb), *Bmp5* (~1.6 kb), *Coll II* (450 bp, amplified from AA182-AA616 region of the GenBank sequence #M74435), *Opn* (~640 bp, AA34-249), *Coll IX* (*Col9a1*; ~500 bp, gift from Dr Olsen, Harvard Dental School, Boston, USA) and *Ihh* (~1.6 kb). We used the following in situ hybridization probes for mouse: *Coll IIa1* (~400 bp from the 3′ UTR; gift from Dr Olsen), *Ptc2* (*Ptch2*; 2 kb), *Opn* (~950 bp, gift from Dr Rosen, Harvard Dental School, Boston, USA), *Ihh* (700 bp), *Osc* (*Lss*; ~500 bp), *PTHrP* (~500 bp), *PTHrP-Rec* (*Pthr1*; ~700 bp), *Osx* (*Sp7*; ~1 kb), *Bmp7* (~2.1 kb), *Bmp2* (~1.2 kb) and *Bmp4* (~1 kb).

### Isolation of osteoblastic cells and immunohistochemical procedures

Bone cells were isolated from frontal bones of embryonic day (E)13 chick or 17.5 dpc mouse embryos by sequential enzymatic digestion as previously described (Yokose et al., 1996; Ishizuya et al., 1997). More specifically, the calvaria were minced and incubated at room temperature for 15 minutes with gentle shaking in a mix of 0.1% collagenase P, 0.05% trypsin and 4 mM EDTA in calcium and magnesium-free phosphate buffered saline. This enzymatic procedure was repeated a total of three times. The resultant supernatant was forced through a 40 μm nylon cell strainer (BD Falcon, Bedford, USA). The cells were placed on a slide and used for in situ hybridization.

### Double fluorescent in situ hybridization (FISH) protocol

The slides with dissociated cells or embryonic head sections were post-fixed in 4% paraformaldehyde for 10 minutes, washed in PBT and treated with acetylation solution (acetic anhydride in 0.1 M triethanolamine). Hybridization was performed at 65°C overnight. After hybridization, the slides were washed twice with 0.2× SSC at 65°C. Following the wash, the slides were incubated in TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20). Then, the slides were blocked before antibody (anti-DIG or anti-FITC POD-conjugated antibody) was applied. Following the antibody exposure, we performed the tyramide amplification reaction following the manufacturer's instructions (PerkinElmer Life Sciences, Boston). The red Cy-3 and Oregon Green signals were obtained with the TSA-Plus Fluorescence Palette System (PerkinElmer Life Sciences, Boston) and TSA Kit#9 (Molecular Probes, Oregon). We empirically determined that the embryonic head sections needed to be treated with 1% $H_2O_2$ for 30 minutes prior to the TNT buffer wash to suppress the endogenous peroxidase activity.

## RESULTS

### Expression of molecular markers during the development of dermal bone

To identify the cellular transitions in the development of dermal bone, we analyzed the expression of a number of chondrogenic and osteogenic markers in chick and mouse embryos. In the chick embryo, pre-skeletal, alkaline phosphatase-positive, mesenchymal condensations can be detected at stage HH30 [Hamburger-Hamilton stage 30 or embryonic day 7 (E7)]; mineralized bone matrix deposition in the skull begins at around HH36 (E10); osteogenesis is well underway at HH39 (E13) and most of the skeletal structures are in place by HH41 (E15) (Fig. 1B) (Hamburger and Hamilton, 1951; Hall and Miyake, 1992; Dunlop and Hall, 1995; Eames and Helms, 2004). In mouse embryos, definitive bone mineralization begins at around 15.0 days post coitum (dpc) and is well underway by 17.5 dpc (Kaufman, 2003). Expression patterns of a variety of skeletogenic cell type markers were examined in both species on both dentary and frontal bones, with the purpose of characterizing dermal bone cells from morphologically distinct structures. These markers included *Sox9*, *Coll II*, *Coll IX* (also known as *Col9a1*) and aggrecan, which are markers for cells undergoing various stages of chondrogenesis in the trunk skeleton (Harada and Roden, 2003; Lefebvre and de Crombrugghe, 1998; Bi et al., 1999). We also explored the expression of *Runx2*, *Osx* (also known as *Sp7* – Mouse Genome Informatics), *Opn* and *BspII*; the presence of these markers is characteristic of the osteoblastic lineage (Harada and Roden, 2003; Zelzer and Olsen, 2003; Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Some of these markers are known to be expressed in both chondrogenic and osteogenic cells, especially at early developmental points. In particular, *Runx2* is known to be expressed in, and to be important for the differentiation of, early chondrocytes as well as osteoblasts, whereas *Sox9* is known to be expressed in common progenitors of both cartilage and bone (Stricker et al., 2002; Akiyama et al., 2005; Shibata et al., 2006). However, the particular combinations of markers that we examined were highly diagnostic of the specific bone versus cartilage cell identity. We examined the expression of these multiple skeletogenic genes during dermal bone formation in coronal sections of HH39 chick embryos and 16.5 dpc mouse embryos (Fig. 2A-O). We first focused on the developing dermal dentary bone in sections also featuring Meckel's cartilage of the lower jaw, as an internal control. As expected, the neural crest-derived Meckel's cartilage cells expressed the chondrogenic markers *Coll II* and *Coll IX* but not osteogenic markers (*Runx2*, *Opn*, *Osx*, *BspII*) (Fig. 2A-E, see Fig. S1 in the supplementary material; and data not shown) (Noden, 1991; Couly et al., 1993; Le Douarin et al., 1993). Expression of *Sox9* was weak at HH39 in chick embryos but was much stronger at the earlier stages of development (data not shown). Turning to the intramembranous (dermal) dentary bone, we observed cells that expressed *Coll II* and *Coll IX*, and also *Runx2*, *Osx* and osteopontin, both in chicks and mice (Fig. 2A-E,L-N; see Fig. S1 in the supplementary material; data not shown). Expression of the typically chondrogenic markers *Coll II* and *Coll IX* in particular was unexpected because dermal bone is not believed to arise via a

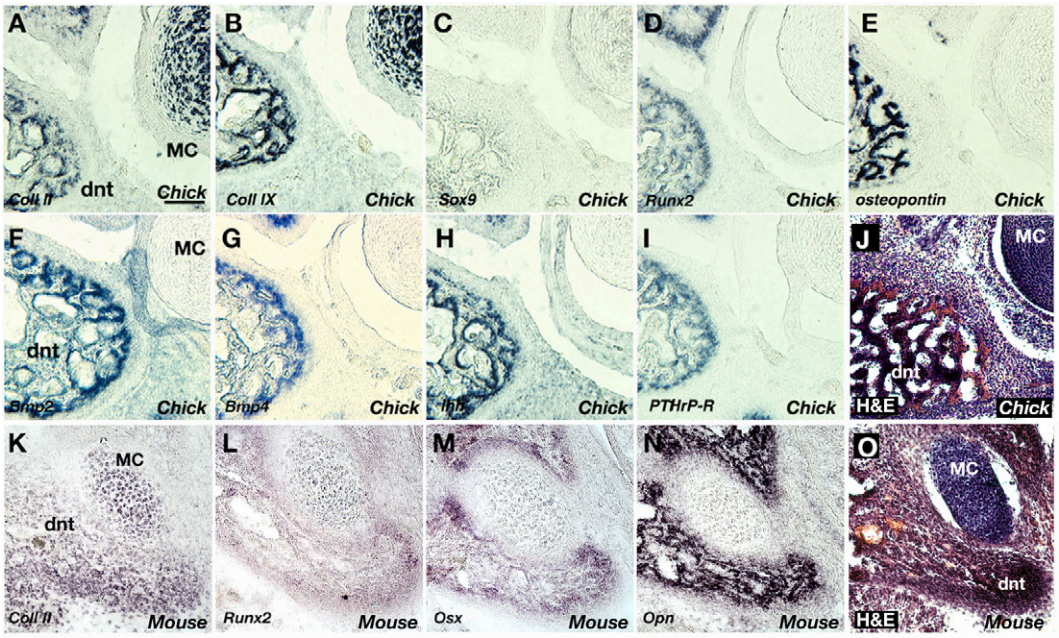


**Fig. 2. Expression domains of skeletogenic markers in the developing dentary bone.** (A-J) HH39 chick embryos; (K-O) 16.5 dpc mouse embryos. Expression of *Coll II* (**A**) and *Coll IX* (**B**) is detected in cells of both Meckel's cartilage and dentary bone. (**C**) Expression of *Sox9* can be seen in cells of Meckel's cartilage but not in the dentary bone. (**D**) Expression of *Runx2* is limited to the cells of the dentary bone. (**E**) Osteopontin is expressed in cells of dentary bone but is restricted to the more mature cells. (**F**) *Bmp2*, (**G**) *Bmp4*, (**H**) *Ihh* and (**I**) strong expression of *PTHrP-R* are detected in the dentary bone. (**J**) H&E staining of the dentary bone and Meckel's cartilage from the embryo in A-I. In mice, (**K**) *Coll II*, (**L**) *Runx2* and (**M**) *Osx* are expressed in the peripheral cells, whereas (**N**) the more mature osteoblast marker *Opn* is expressed in cells surrounded by mineralized matrix (H&E stain; **O**). dnt, dentary bone; MC, Meckel's cartilage. Scale bars: 200 μm.

cartilage template. Other cartilage markers known to be expressed in the cartilage, such as *Sox9* and aggrecan, were not expressed in this tissue. We observed very similar expression profiles for these skeletogenic markers in the developing dermal frontal bone of HH39 (E13) chick embryos (data not shown).

## Identification of major cell types in the developing dermal bone

To identify which markers were co-expressed in the same dermal bone cells, and thereby to define cell types within the developing dermal bones, we used fluorescent double in situ hybridization on embryonic sections at HH39 (E13) (Fig. 3A-X). These data suggest the existence of four distinct cellular types in the process of dermal ossification (see Fig. 8A). The developing dermal elements can first be divided into two domains, expressing the early osteogenic marker *Runx2* and expressing the later osteoblastic marker osteopontin. The expression patterns of these two markers were mutually exclusive (Fig. 3G-I). *Runx2*-positive cells lied peripherally. These were also regions of active proliferation based on BrdU and PCNA staining (data not shown). The osteopontin-expressing cells could be further subdivided into two distinct domains. These cells also expressed the marker *BspII* (Fig. 2S-U; data not shown), whereas more-peripheral cells within the osteopontin-positive domain expressed *Coll II* and *Coll IX* (Fig. 3A-C; data not shown); the expression of these markers, *BspII*, *Coll II* and *Coll IX*, identified mutually exclusive domains of the osteopontin-positive cells (Fig. 2S-U; data not shown). The more medial of the *Runx2*-positive cells also expressed *Coll II* and *Coll IX* (Fig. 3J-L), whereas the most peripheral *Runx2*-expressing cells did not. Our analysis thus allows us to define four transitional cell types during dermal bone formation (Fig. 8A). The most peripheral cells at the surface of the dermal elements, and hence presumably the most immature, express *Runx2*, but not *Coll II* or *Coll IX*. These preosteoblast cells gave rise to a secondary preosteoblast cell characterized by the activation of *Coll II* and *Coll IX* in addition to *Runx2* expression. The next differentiation step involved the shut-off of *Runx2* expression and the activation of osteopontin expression, while maintaining *Coll II and Coll IX* expression. This is a cell type characterized by the expression of both chondrocyte (*Coll II* and *Coll IX*) and osteoblast (osteopontin) markers. We detected no *Sox9* or aggrecan expression in these cells, which makes them distinct from both chondrocytes and osteoblasts observed in the trunk (Fig. 2A-C; data not shown). We have termed these 'chondrocyte-like osteoblasts', or CLO cells. Finally, these cells differentiated into mature osteoblasts, expressing *BspII* as well as osteopontin, but no longer expressing *Coll II* or *Coll IX*.

To verify the observed overlap in expression, we dissociated dermal bone tissue from HH39 chick embryos and from 17.5 dpc mouse embryos. As expected from the results of in situ hybridization in sections, when the dissociated cells were probed for *Coll IX* and osteopontin (or *Coll II* with either *Osx* or *Osc* in mouse embryos) expression, we identified four classes of cells: cells expressing osteopontin but not *Coll IX*, cells expressing osteopontin and *Coll IX*, cells expressing *Coll IX* but not osteopontin and cells expressing neither; representing, respectively, the most differentiated to least differentiated cell types. We found that approximately 40% (43±6%; $P \leqslant 0.003$) of cells that had been dissociated from chick frontal bone ($n$=500) co-expressed *Coll* IX and *Opn* (Fig. 4A-D). Even fewer cells (17±8%; $P \leqslant 0.0043$) co-expressed *Runx2* and *Opn* (Fig. 4E-H). By contrast, co-expression of *Coll IX* with *Runx2*, a very early osteoblastic marker, was close to 70% (71±7%; $P \leqslant 0.005$, $n$=500) in HH39 chick frontal bone cells (Fig. 4I-L). Similarly, in mouse,

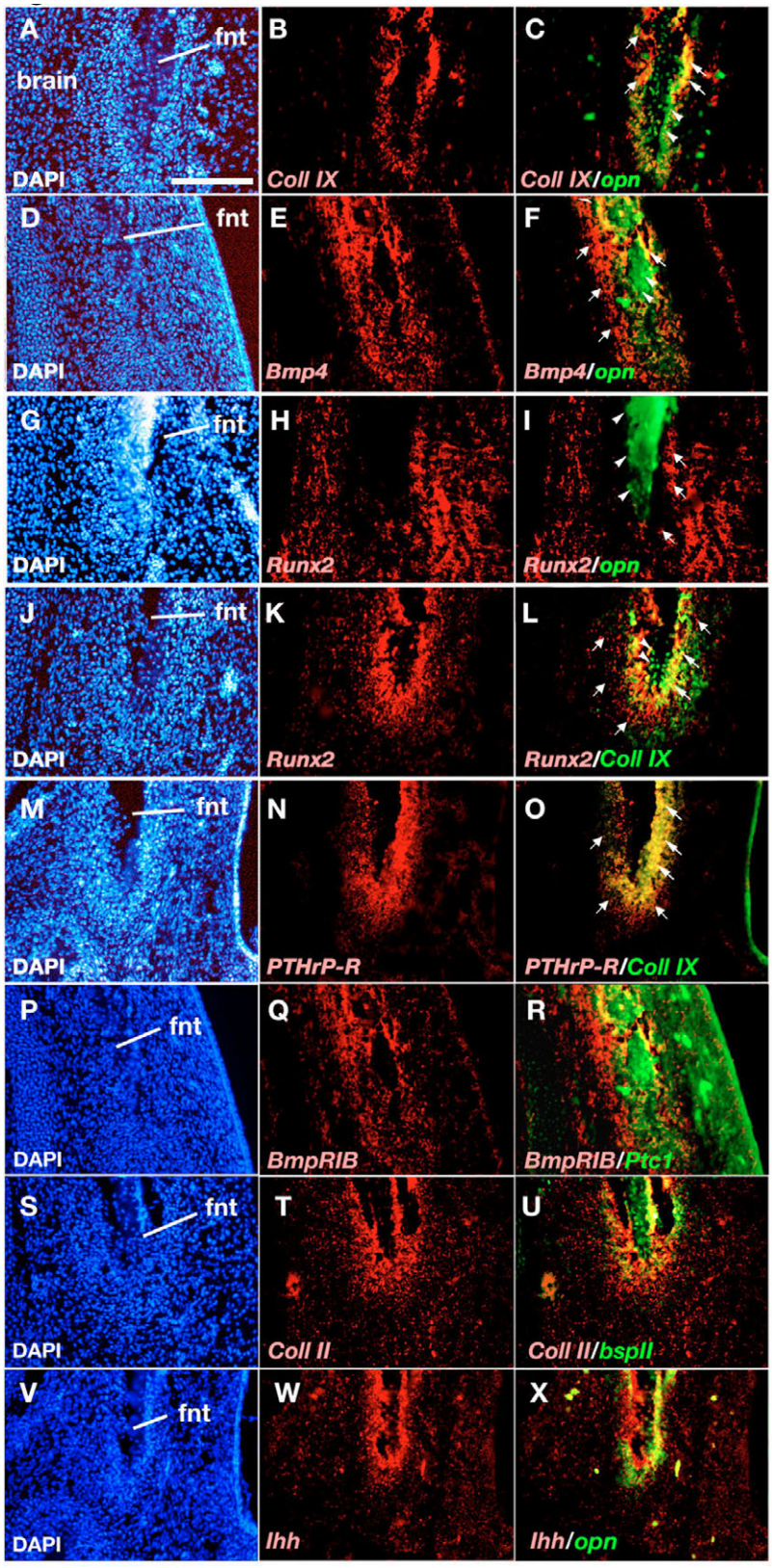


**Fig. 3. Fluorescent double in situ hybridizations showing expression of *Coll IX*, *Coll II* and *Opn* relative to each other and to the domains of *Runx2*, *PTHrP-R*, *Bmp4* and *Bmpr1b* expression.** (**A-C**) Direct comparison of the *Opn* and *Coll IX* expression domains. (C) There are cells that express *Coll IX* only (red; broad arrows), *Opn* only (green; arrowheads) or that co-express *Opn* and *Coll IX* (yellow; narrow arrows). (**D-F**,**J-O**) Considerable overlap is detected between *Opn* and *Bmp4* (D-F), between *Runx2* and *Coll IX* (J-L), and between *Coll IX* and *PTHrP-R* (M-O). (**G-I**) Expression domains of *Opn* (green) and *Runx2* (*Cbfa1*; red) have very little or no overlap. The same is true for the expression domains of *Coll II* versus *BspII* (**S-U**) and *Ihh* versus *Opn* (**V-X**). Some overlap is detected for the expression domains of *Bmpr1b* versus *Ptc1* (**P-R**). Scale bar: 200 μm.

approximately 35% (34±7%; $P \leqslant 0.035$; $n$=500) of the cells co-expressed *Coll II* and *Osx*, whereas less than 10% (7±2%; $P \leqslant 0.002$; $n$=500) co-expressed *Coll II* and *Osc*, and 28% (27±8%; $P \leqslant 0.01$; $n$=500) co-expressed *Runx2* and *Coll II* (see Fig. S2A-P in the supplementary material).

## *Coll II*-expressing cells contribute to bone in mice

Based on their spatial arrangement within the developing skeletal elements, we postulated that the cell types identified in this analysis form a linear cascade of cell differentiation. It was, however, formally possible that the CLO cells were an independent,

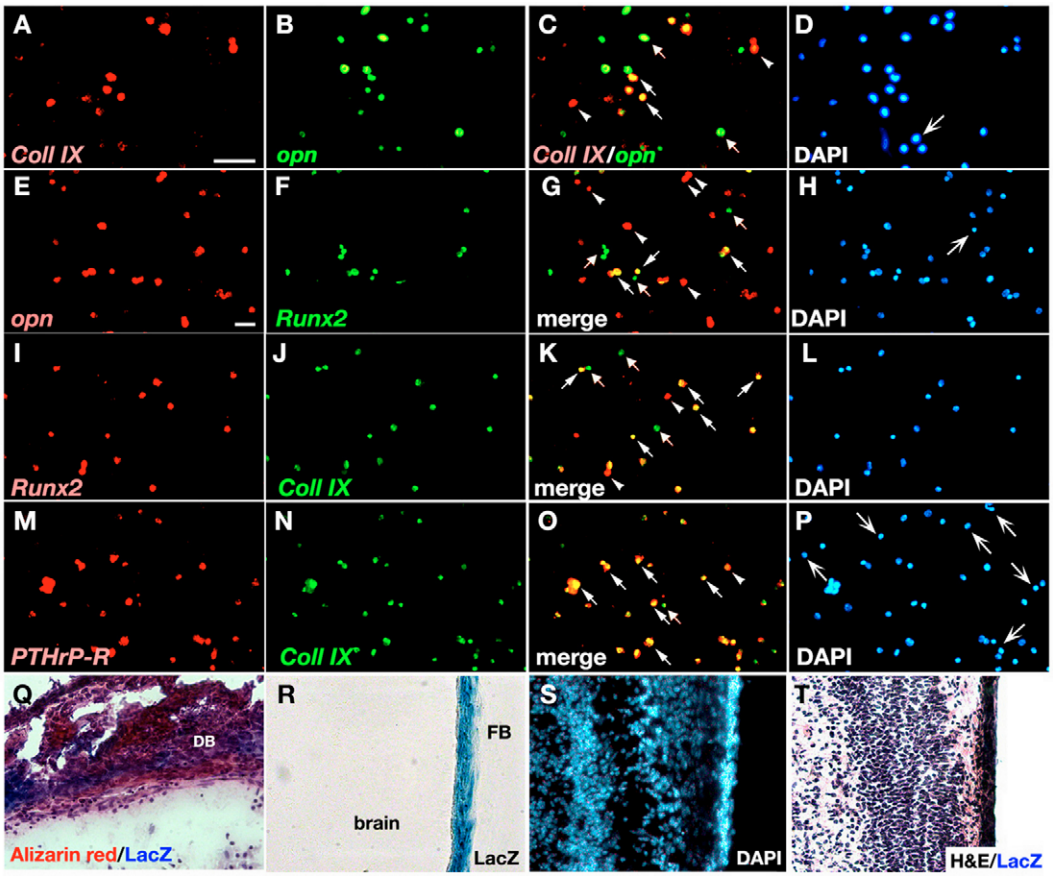


**Fig. 4. Analysis of dissociated frontal bone cells of HH39 chick embryos reveals distinct cell populations defined by the expression of skeletogenic markers.** (**A-D**) Four populations of cells were identified: cells expressing only *Coll IX* (arrowheads in C), only *Opn* (broad arrows in C), both *Coll IX* and *Opn* (narrow arrows in C), and cells expressing neither marker (arrow in D). FISH revealed a similar situation with *Opn* and *Runx2* (**E-H**), with *Runx2* and *Coll IX* (**I-L**), and with *PTHrP-R* and *Coll IX* (**M-P**). (M-P) Despite the high degree of co-expression between *Coll IX* and *PTHrP-R* that was observed, some slides showed many cells expressing neither marker (arrows, P), suggesting that many of these cells were early osteoblasts not expressing *Coll IX*. (**Q**) Analysis of *Col2::Cre3*×*Rosa26::lacZ* dentary bones with both alizarin red and β-galactosidase staining revealed a considerable contribution of *Coll II*-expressing cells to the developing dentary bone. (**R-T**) Similar analysis of β-galactosidase and H&E stainings of the developing frontal bone in 17.5 dpc *Col2::Cre3*×*Rosa26::lacZ* embryos showed many β-galactosidase-positive cells within this intramembranous bone. Db, dentary bone; FB, frontal bone. Scale bars: 50 μm.

coexisting transient cell type and not the precursors of the mature osteoblasts. Recombinase-based fate mapping in mice provided the opportunity to directly test the relationship between these cells. Mice expressing Cre recombinase from a *Coll II* regulatory element (*Col2::Cre3*) were crossed to *Rosa26::lacZ* reporter lines. In these reporters, the *Rosa26* promoter is ubiquitously active, but the reporter, *lacZ*, is not expressed due to a floxed transcriptional stop cassette. In mice carrying both the *Col2::Cre3* transgene and one of the *Rosa26* reporters, Cre activity in the *Coll II*-expressing cell population resulted in irreversible activation of the reporter gene irrevocably marking the *Coll II*-expressing cells and, importantly, their descendants. A previous similar study only studied up to 15 dpc (Ovchinnikov et al., 2000). In our study, many β-galactosidase-positive cells could be observed deep inside the mineralized parts of the dentary and frontal bones, demonstrating that a significant portion of the mature osteoblasts (estimated 5-20% of osteoblasts depending on specific bone) are indeed derived from *Coll II*-positive CLO cells (Fig. 4Q-T; data not shown). It should be noted, however, that none of the dermal bones were completely β-galactosidase-positive, and often had a 'salt-and-pepper' appearance. Unfortunately, our in situ hybridization protocols did not allow us to confirm the identity of β-galactosidase-positive cells using molecular markers. The relative proportion of the β-galactosidase-positive osteoblasts differed from bone to bone (Fig. 4Q-T). This might reflect variation in the timing and/or extent of expression of the particular *Col2::Cre3* transgene we used, or alternatively might indicate an alternative differentiation pathway (see below). In either case, however, it is clear that the (*Coll II*-positive) CLO cells are indeed precursors of mature osteoblasts.

## Dermal bone osteoblasts express signaling molecules with known association to chondrogenesis

Having identified markers for the various stages of dermal bone differentiation, we next wanted to examine the expression patterns of various secreted proteins known to modulate endochondral differentiation. The cells of the dentary bone expressed high levels of *Bmp2*, *Bmp4*, *Bmp7* and *Ihh*, and weak but detectable levels of *PTHrP* (Fig. 2F-I; data not shown). We used double in situ hybridization to identify the cell types expressing each of these factors. Our analysis showed that *Bmp4* was expressed in a very similar pattern to *Coll II* and *Coll IX*, overlapping with the same

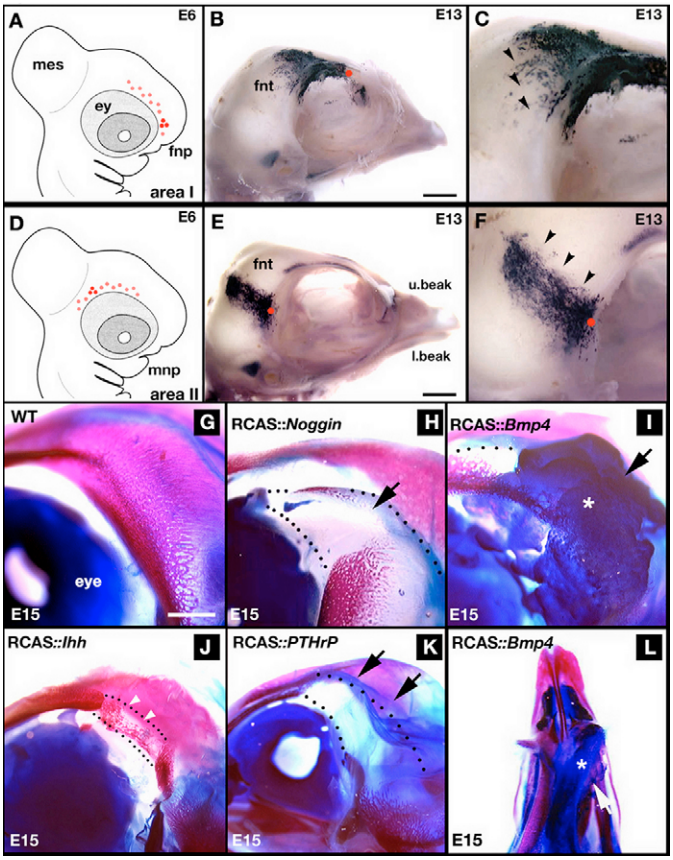


**Fig. 5. Analysis of chick frontal bone development with the RISAP replication-incompetent virus.** (**A-F**) Viral injections into condensations in areas 'I' and 'II' (dark red spots) labeled large portions of the developing frontal bone (A,D), with cells contributing to the anterior (B) and posterior (E) halves of the frontal bone, respectively. Expansion of the two condensations results in a relatively sharp boundary between the anterior and posterior parts, but no suture forms (arrowheads; C,F). Notice that cells from the anterior condensation contribute to the more posterior part (C). (G-L) Skeletal phenotypes in frontal bones after condensations were infected with the RCAS-based viral constructs at E6. Bone and cartilage mineralized structures were revealed with alizarin red (bone) and Alcian blue (cartilage) histological stains. (**G**) Normal ossification pattern of the posterior frontal bone in E15 embryos. The top, side and back of the skull are covered with membranous bone. Infection with RCAS::noggin led to a loss of bone mineralization in the posterior (**H**) parts of the frontal bone. By contrast, infection of the posterior (**I**) and anterior (**L**) frontal bone with RCAS::*Bmp4* led to a loss of mineralized bone material and its replacement with cartilage. (**J**) RCAS::*Ihh* misexpression resulted in a significant decrease of frontal bone mineralization, a phenotype that was similar to that of RCAS::*PTHrP* misexpression (**K**). Scale bars: 0.7 cm in B,E; 2 mm in G.

subset of osteopontin-expressing CLO cells and *Coll II*, *Coll IX*, *Runx2*-positive preosteoblasts) (Fig. 3D-F; data not shown). *PTHrP* was expressed in the same two cell types, the *Coll II*- and *Coll IX*-expressing preosteoblasts and CLO cells (data not shown). By contrast, *Ihh* was co-expressed with *Opn* in both the CLO cells and the more mature osteoblasts (Fig. 3V-X).

To get an indication of the roles that these various factors might play, we next examined the expression patterns of the receptors through which each is known to act, to identify potential target cell types. *Bmpr1b* was expressed at a low level throughout the forming dermal elements, suggesting that BMP signaling might play a role at multiple stages of dermal osteogenesis (Fig. 3P-R). By contrast, *PTHrP-R* was expressed specifically in the CLO cells, based on its co-expression with osteopontin, *Coll II* and *Coll IX* (Fig. 3M-O; data not shown), indicating that this is the cell type that is the likely target of *PTHrP* signaling. Finally, the IHH receptor *Ptc1* (*Ptch1*) and the downstream transcription factor *Gli1*, both known transcriptional targets of hedgehog signaling, were co-expressed with osteopontin both in CLO cells and *Coll II*- and *Coll IX*-positive preosteoblasts, suggesting that these cells were actively responding to IHH signaling (Fig. 3P-R; see Fig. S3 in the supplementary material; data not shown).

## Targeting the frontal bone condensations with RCAS viral constructs

To examine the roles of the various signaling molecules during dermal bone formation, we wanted to use the ability of retroviral vectors to misexpress genes in the developing chick. To develop protocols for specifically targeting the relatively accessible frontal bone, we used a replication-defective RISAP vector (which does not spread to adjacent cells following initial infection) (Chen et al., 1999). Our fate-mapping analysis based on infections with this vector indicated that the avian frontal bone forms by the fusion of cells derived from at least two distinct regions of the craniofacial mesenchyme, which we refer to as area I and area II, at E6; these areas contribute to the anterior and to the posterior frontal bone, respectively (Fig. 5A-F). For our functional analyses, in which we used the replication-competent retroviral vectors (RCAS), the frontal bone is particularly convenient because it is relatively easily isolated from other skeletal structures and infections in the condensation areas 'I' and 'II' do not affect embryonic survival rates (data not shown). We infected only the right side of the embryonic head, with the left acting as an internal control. Embryos were infected at E6 and collected at HH41 when all ossified cranial skeletal structures could be detected with alizarin red (bone) and Alcian blue (cartilage) histological stains and easily identified (Fig. 5G).

## Roles for BMP signaling during intramembranous bone development

We first addressed whether signaling by BMP proteins is required for proper frontal bone development by blocking their activity with noggin, a specific inhibitor of BMP2 and BMP4 (Fig. 5H). Similar misexpression experiments were conducted previously, but cell types were not analyzed (Warren et al., 2003; Murtaugh et al., 1999; Abzhanov et al., 2004; Wu et al., 2006). We found that, in response to noggin infection, there was a dramatic decrease in the ossified (mineralized) bone material in both anterior ($n$=6) and posterior ($n$=9) parts of the frontal bone, as indicated by staining with alizarin red (Fig. 5H, black arrow; data not shown). This experiment suggested that BMP2 and/or BMP4 activity is required for proper dermal frontal bone ossification.

To determine whether any of the early steps in dermal bone formation occur in the absence of BMP signaling, we examined the effect of noggin misexpression at HH39 by using the molecular markers for the various cell types that we had identified. By HH39, the frontal bone on the uninfected contralateral side was already ossified and contained cells expressing *Runx2*, *Coll II*, *Coll IX*, *Opn* and *BspII* (data not shown). However, following noggin misexpression, in most cases (4 out of 5), very little or no skeletogenic condensation formed and the infected cells failed to express markers for any of the four cell types (*Coll II*, *Coll IX*,

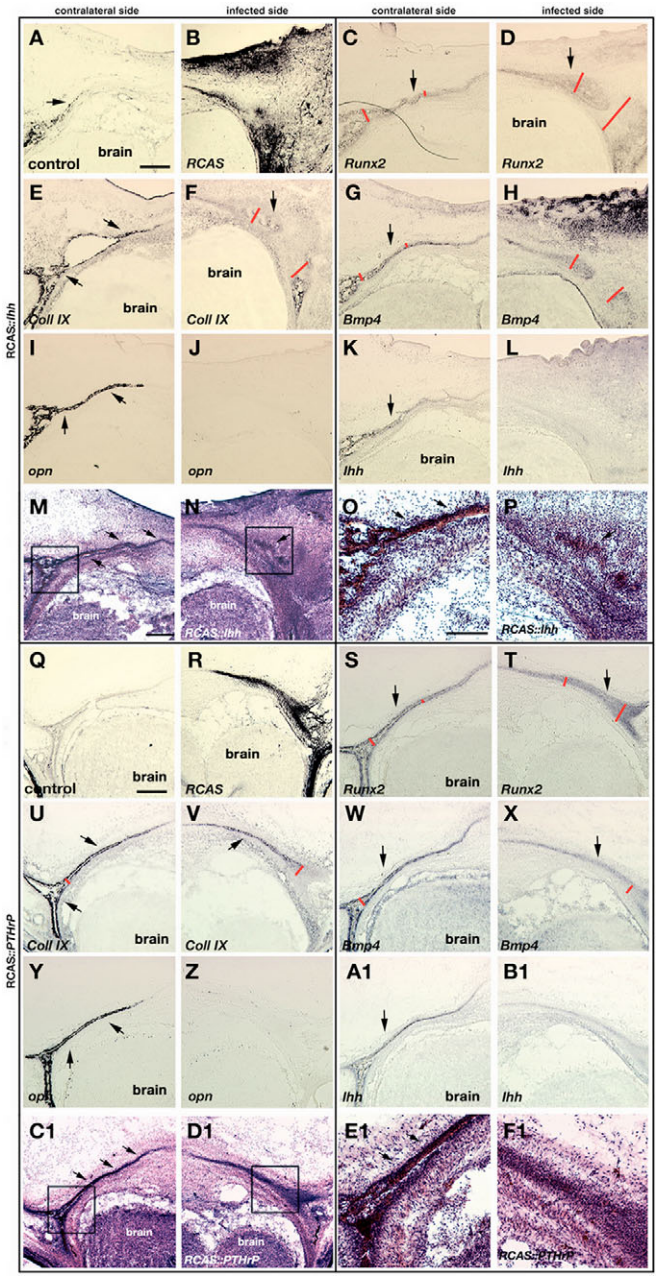


**Fig. 6. Molecular and histological analysis of RCAS::*Ihh* and RCAS::*PTHrP*-infected frontal bones.** (**A-P**) Analysis of the RCAS::*Ihh*-infected frontal bones with molecular markers and H&E staining shows that infected bone failed to produce differentiated osteoblasts (I,J) and to mineralize (M-P). Framed areas in M and N are shown amplified in O and P, respectively. (**Q-F1**) Misexpression of RCAS::*PTHrP* resulted in a very similar phenotype, with a loss of *Opn* expression in the infected skeletal element (Y,Z). Framed areas in C1 and D1 are shown amplified in E1 and F1, respectively. Scale bars: 1 mm.

*Runx2*, *BspII* or *Opn*) (data not shown). This observation suggests that BMP2 and/or BMP4 activities are required at the earliest stages for the proper formation of the frontal bone condensation and preosteoblastic progenitors.

Although noggin misexpression indicates a role for BMPs in allowing dermal bone formation to take place, it acts so early that we could not ascertain a role for BMPs in later cell types. To address this issue, we infected frontal bone primordia with RCAS::*Bmp4* and RCAS::*Bmp2* viruses (Fig. 5I; data not shown). Infection with either virus caused large areas of frontal bone to be replaced with cartilage material in both the anterior (*Bmp4*, *n*=5; *Bmp2*, *n*=8) and the posterior (*Bmp4*, *n*=11; *Bmp2*, *n*=5) part of the frontal bone, as determined with histological stains (Fig. 5I, arrow and asterisk; data not shown). In many cases, anterior frontal bone was replaced by a cartilage of a comparable size and shape with that of the contralateral uninfected frontal bone (Fig. 5L, asterisk). In the posterior frontal bone, misexpression of RCAS::*Bmp4* caused a transformation of the dermal bone condensation into a cartilaginous one, as revealed by suppression of the bone-specific *Runx2*, *Opn* and *BspII*, and by the upregulation of *Sox9*, *Coll II*, *Coll IX* and aggrecan, all markers of cartilage formation (data not shown). Induction of *Sox9* is important because it was previously shown that ectopic *Sox9* expression is sufficient to inhibit *Runx2* expression and induce chondrogenesis (Eames and Helms, 2004); the primordium of the infected anterior part of the frontal bone fuses with an expanded nasal cartilage early in development and fails to ossify. These results suggest that BMP signaling can act as a switch on the early neural crest skeletogenic condensation, promoting a chondrogenic fate at the expense of the initiation of dermal bone development.

Thus, BMP signaling appears to play multiple roles at various stages of membranous bone development. In our misexpression studies with noggin and BMP4, we identified roles for BMP activity at the early stages of condensation and commitment to osteogenic fate. To assess whether BMP signaling also plays an essential role at later stages of dermal bone formation, we infected the developing frontal bone with noggin virus at HH33 (E8), after skeletal condensation is fully formed and dermal bone differentiation is actively taking place. In spite of widespread infection of the frontal bone, we observed no defect in the subsequent formation of this element, with little or no loss of mineralization (data not shown).

## IHH and PTHrP signaling regulate the preosteoblast-to-osteoblast transition

Misexpression of *Ihh* indicated that this factor acts at a later stage of dermal bone formation. Analyses of RCAS::*Ihh*-injected frontal bone at HH41 showed a significant decrease in bone mineralization in anterior (*n*=5/5) and posterior (*n*=8/8) infections; however, in these cases, the dermal bone was not replaced by cartilage (Fig. 5J). Molecular analyses at HH39 (E13) indicated that IHH completely inhibited the expression of osteopontin, *Ihh* and *BspII*, which, together, are markers of CLO cells (osteopontin, *Ihh*) and later-stage mature osteoblasts (osteopontin, *BspII* and *Ihh*); by contrast, earlier-stage cells, which express *Runx2*, *Coll II* and *Coll IX*, were unaffected (Fig. 6A-L; data not shown). In conjunction with our expression data, showing that CLO and mature osteoblast cells express *Ihh* whereas the earlier-stage preosteoblasts express the IHH receptor, this data suggests that IHH acts as a feedback-inhibitor of early stages of differentiation, a role very much analogous to that which it plays during limb cartilage development (Fig. 6M-P). In endochondral ossification, however, IHH acts indirectly on cartilage differentiation through PTHrP upregulation. That does not seem to be the case here, because PTHrP, normally expressed in CLO cells, was downregulated in the dermal bone condensation following IHH misexpression, consistent with a block in differentiation at the preosteoblast stage (data not shown). We did, however, see scattered upregulation of PTHrP in mesenchyme outside of the condensation in response to *Ihh* misexpression (see Fig. S4 in the supplementary material).

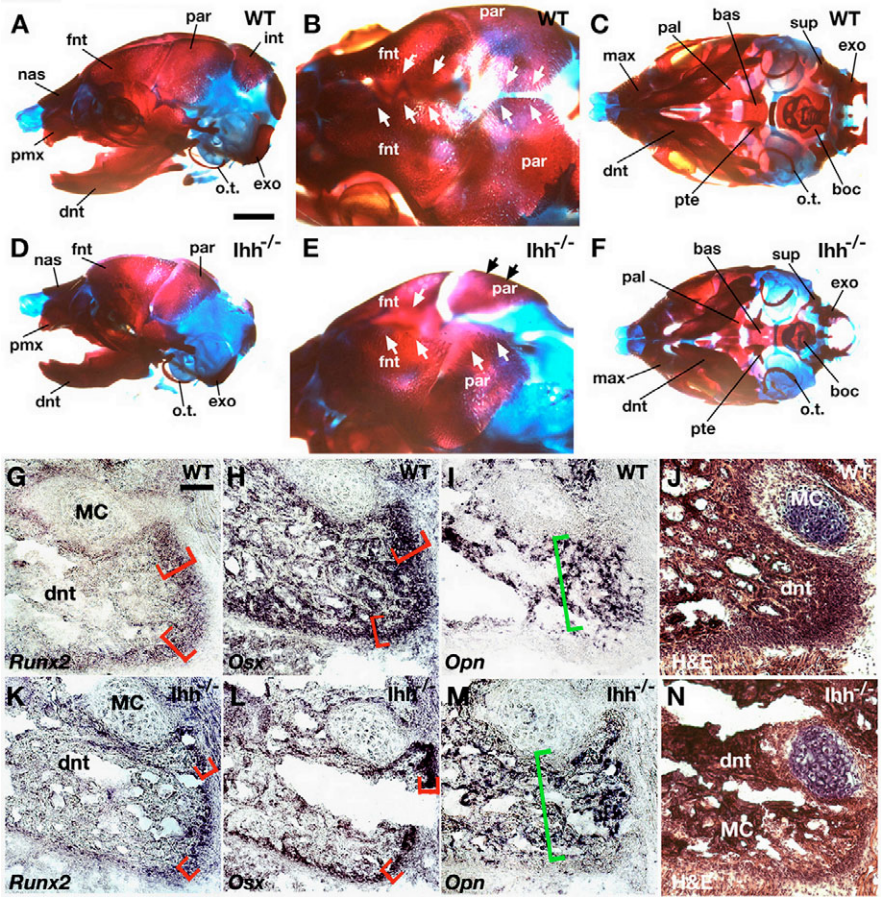


**Fig. 7. Analysis of 18.5 dpc *Ihh*$^{-/-}$ mutant mouse embryos.** The skull of wild-type (WT) embryos (**A-C**) is larger than that of mutant siblings (**D-F**). Notice the presence of the ossified basal cranial bones in the mutant. Arrows indicate the leading edge of the mineralized dermal calvarial bone. Expression of the early osteoblastic markers, such as *Runx2* (**G**) and *Osx* (**H**), is severely reduced in the *Ihh*$^{-/-}$ condition (**K** and **L**, respectively). Red brackets indicate area occupied by undifferentiated proliferating osteoblasts. The expression of *Opn* (**I**,**M**), a marker for more mature osteoblasts, is unaltered or slightly expanded in the mutant condition. Green brackets indicate area occupied by differentiated mature osteoblasts. (**J**,**N**) H&E stainings of wild-type and mutant dentary bones, respectively. bas, basisphenoid; boc, basioccipital; dnt, dentary; exo, exoccipital; fnt, frontal bone; int, interparietal; max, maxillary; MC, Meckel's cartilage; nas, nasal; pal, palatal; par, parietal; pte, pterygoid; o.t., os tympanicum. Scale bars: 1 mm in A; 200 μm in G.

Although PTHrP and IHH do not seem to form a common feedback loop in regulating dermal differentiation, *PTHrP* misexpression gives a very similar phenotype to *Ihh* misexpression in dermal bones, as it does in endochondral ossification. Misexpression with RCAS::*PTHrP* led to a decrease in bone mineralization at HH41 (anterior, $n$=9/9; posterior, $n$=11/11) (Fig. 5K, black arrows; data not shown). Frontal bones analyzed at a molecular level at HH39 showed that, as did IHH, PTHrP completely inhibited expression of *Opn*, *BspII* and *Ihh*, whereas the expression domains of *Runx2*, *Coll II*, *Coll IX* and *Bmp4* expanded (Figs 6C-J; data not shown). Consistent with it acting within the dermal bone differentiation pathway, PTHrP failed to induce the chondrogenic marker *Sox9*, its known target in the endochondral bone (Huang et al., 2001) (data not shown). Thus, as does IHH, PTHrP acts during dermal bone formation as a feedback-inhibitor preventing the differentiation of the preosteoblasts to the CLO cell state (Fig. 6C1-F1).

## Molecular analysis of *Ihh*$^{-/-}$ mouse embryos verifies the role of IHH in regulating dermal preosteoblast differentiation

Misexpression in chick embryonic heads suggests that IHH functions to block the transition from preosteoblasts to osteoblasts. This observation suggests that loss of IHH activity should result in premature ossification via the accelerated differentiation of preosteoblasts, and in smaller dermal bone structures via the depletion of the pool of immature osteoprogenitors. A previous report described the phenotype of mice carrying a targeted null mutation in *Ihh* (*Ihh*$^{-/-}$). These mice indeed displayed a smaller but otherwise unaffected skull and endochondrally derived trunk bones were missing (St-Jacques et al., 1999).

The skulls of these mutant mice were not, however, characterized in depth. We, therefore, reanalyzed the cranial skeletons of wild-type and *Ihh*$^{-/-}$ mutant 18.5 dpc embryos. We found that the mutant skulls were approximately 10% shorter and individual cranial bones were also smaller ($n$=4) (Fig. 7A-F). Interestingly, we found that the endochondrally derived cranial base bones, such as basioccipital, basisphenoid and others, were clearly present, albeit also proportionally smaller, in the mutant embryos (Fig. 7A-F) (Hanken and Hall, 1993). This could suggest either that there is a significant unrecognized dermal bone contribution to these structures or that intramembranous ossification can compensate for the reduction of ossification, via the endochondral pathway. Alternatively, the endochondrally derived bones of the cranial base could differ in their requirement for IHH activity from those in the limb and axial skeleton. This observation is important because a strong phenotype in the cranial base would affect morphogenesis of the calvarial bones. By contrast, the jawbones are not expected to be significantly affected by the changes in the calvarial base. Nonetheless, dentary bones in *Ihh*$^{-/-}$ mutants were approximately 20% shorter, suggesting a localized phenotype ($n$=4) (Fig. 7A-F). This was not due to the shortening of the Meckel's cartilage (length of Meckel's cartilage in wild-type mice was 3.7 mm±10%, $P \leqslant 0.0058$, $n$=7; in *Ihh*$^{-/-}$ mutants it was 3.5 mm±14%, $P \leqslant 0.007$, $n$=8). Moreover, cells of this cartilage did not hypertrophy or express *Ihh* before or during the stages studied to be affected by the mutation.

To investigate whether differentiation of the intramembranous osteoblasts was altered, we analyzed the expression of key skeletogenic markers (Fig. 7G-N). By 18.5 dpc, the dentary bones in mutants showed a significant downregulation of the preosteoblastic and early-osteoblastic markers, such as *Runx2* and

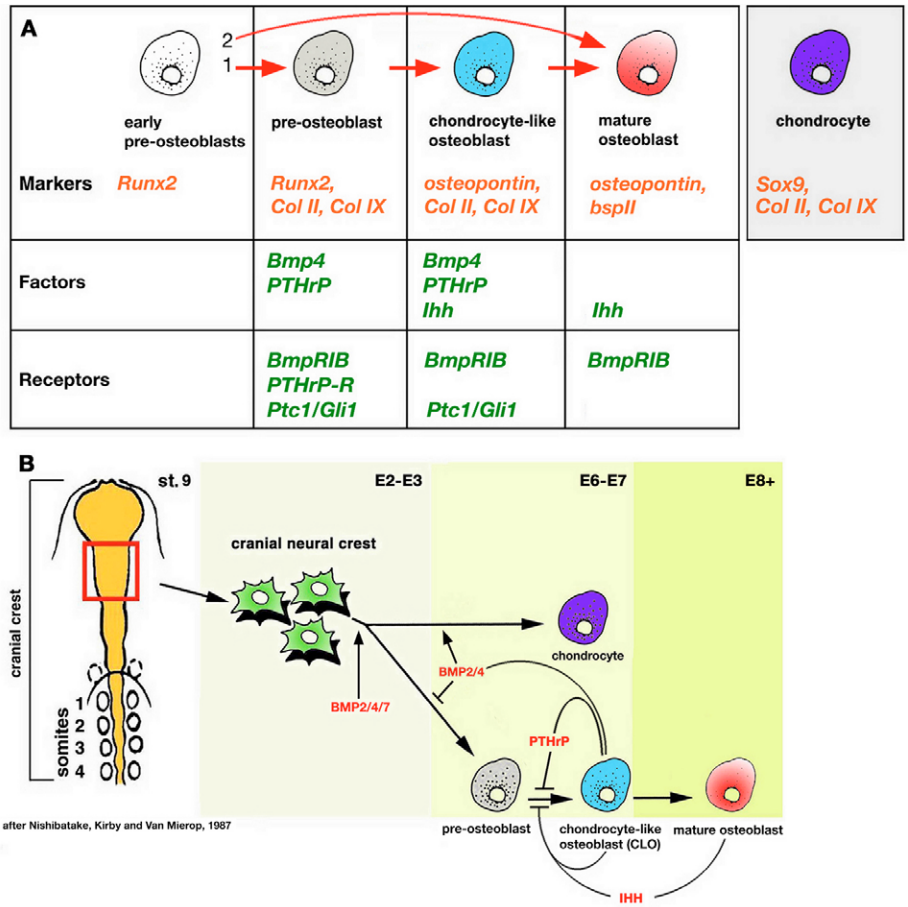


**Fig. 8. Regulation of osteoblastic differentiation in cranial dermal bone.** (**A**) Table summarizing the expression pattern data for the developing dentary and frontal bones. Four major cell types could be distinguished based on skeletogenic markers: proliferating cells at the periphery of the osteogenic front, cells expressing only *Runx2*, preosteoblastic progenitors and surrounding cells expressing *Runx2*, *Coll II*, *Coll IX*, *Ptc1*, *Gli1*, *PTHrP-R* and *Bmp4*. (1) These cells differentiate into the CLO (chondrocyte-like osteoblast) cells expressing a unique combination of *Opn*, *Coll II* and *Coll IX* (but not *Sox9* or aggrecan) as well as *Ihh*, *Ptc1*, *Gli1*, *Bmp4* and *PTHrP*. As these cells develop into mature osteoblasts, they downregulate *Coll II* and *Coll IX* expression and, out of the analyzed markers, they express only *Opn*, *Bspll* and *Ihh*. (2) Alternatively, some cells could differentiate directly into mature osteoblasts. (**B**) During craniofacial development, mesencephalic cranial neural crest cells migrate to populate mesenchyme of the future face and skull. Cells of the early cranial skeletogenic condensations depend on BMP2/4/7 activities to form preosteoblastic progenitors, whereas high levels of BMP2 and/or BMP4 alone induced a chondrogenic fate. Differentiation into the chondrocyte-like osteoblasts is regulated by both IHH and PTHrP activities.

*Osx*, whereas expression of later markers, such as *Opn* and *Osc*, was unaltered or increased. This is exactly what would be expected if the loss of *Ihh* relieved a block in the differentiation of osteoblasts into CLO cells and mature osteoblasts. As a result, the osteogenic front containing immature osteoblasts was 60-70% thinner in mutant embryos, thus explaining much of the bone size reduction (Fig. 7G,H,K,L, red brackets). Osteoblast proliferation was only slightly downregulated in the mutants (data not shown). This dramatic decrease in the number of preosteoblasts in *Ihh* homozygous mutants strongly supports our model of IHH as a negative regulator of osteoblast differentiation during dermal bone development.

## DISCUSSION

### Dermal bone osteoblasts differentiate via a novel transient cell type

Our in situ hybridization analyses show that many of the dermal bone cells express combinations of skeletal markers not seen during endochondral bone development. It is known that some of these markers, taken individually, can be shared by both chondrogenic and osteogenic progenitors, especially during early differentiation decisions. For example, *Sox9*, generally thought of as a cartilage marker, is expressed by all early osteochondral progenitors and in all cranial neural crest cells, including those giving rise to osteoblasts (Ng et al., 1997; Zhao et al., 1997). Similarly, although considered a bone marker, *Runx2* expression in early chondrocyte progenitors also appears important for their differentiation (Stricker et al., 2002; Komori et al., 1997; Otto et al., 1997). However, as cells become differentiated, such markers become highly cell type specific. For example, *Sox9*, *Coll II* and *Coll IX* are expressed only in differentiated chondrocytes and never overlap with the expression of more-mature osteoblastic markers, such as *Opn*. Thus, cell types in which such unusual, albeit transient, combinations of both osteoblast and chondrocyte markers are found are unique to the developing dermal bone.

These cell types had not been previously recognized as such. Expression of the chondrogenic markers *Coll II*, *Coll IX* and aggrecan in the developing dermal bone was previously reported as a result of northern blot and immunohistochemical analysis. However, in the absence of osteogenic markers and single-cell resolution, it was concluded that *Coll II*, *Coll IX* and aggrecan expression was restricted to typical cartilage cells. Consequently, dermal bones were suggested to develop through a transient chondrogenic phase similar to the prechondrogenic mesenchyme (Ting et al., 1993; Nah et al., 2000). Also, the idea of an ‘osteo-chondro-progenitor’ (osteochondral progenitor) has been around for some time, but this concept referred to very early populations of cells giving rise to both chondrocytes and osteoblasts, whereas, in our study, we refer to a population of cells expressing markers indicative of a differentiated osteoblastic state that are fated to give rise only to mature osteoblasts (Gerstenfeld and Shapiro, 1996; Akiyama Ddagger et al., 2005; Smith et al., 2005). Interestingly, although both *Coll II* and *Coll IX* RNA are readily observed in these cells, we were unable to detect their translation products with specific antibodies, indicating a post-transcriptional regulation of collagen production specifically in these cells.

### A model for dermal bone development

Cranial neural crest cells migrated to the facial region, in which, in response to ecto-mesenchymal interactions, they differentiated into skeletogenic progenitor cells, which formed condensations capable

of differentiating along either the chondrogenic or osteogenic lineage (Fig. 8B). Our noggin misexpression experiments indicate that BMP signaling is required for these bi-potential condensations to form. In addition, the effect of BMP misexpression indicates that further BMP signaling acts to direct cells towards a chondrogenic pathway at the expense of dermal osteogenesis, by inducing *Sox9* expression and inhibiting the expression of *Runx2* and osteopontin. The dermal condensations that committed to an osteogenic fate first expressed *Runx2* in the early preosteoblasts of the proliferating osteogenic fronts and subsequently expressed *Runx2*, *Coll II* and *Coll IX*. These cells also expressed receptors for IHH and PTHrP, and, accordingly, are targets for the action of those factors. These preosteoblasts differentiated into CLO cells by downregulating *Runx2* expression and by expressing osteopontin in addition to *Coll II* and *Coll IX*. The CLO cells finally differentiated into mature osteoblasts expressing *BspII*, osteopontin and osteocalcin. Our recombinase-based fate mapping verified that the *Coll II*-expressing cells were indeed precursors of the mature dermal osteoblasts. Not all of the mature osteoblasts expressed the *lacZ* lineage markers in our experiments. Although this might reflect incomplete recombination due to less than uniform expression of the *Col2::Cre3* transgene, it also remains possible that there is an alternative differentiation pathway in which mesenchymal cells differentiate into mature osteoblasts without ever expressing *Coll II* (Fig. 8A).

The transition of the proliferating preosteoblasts to CLO cells appears to be particularly important, moving from an expanding to a differentiating phase. This transition was regulated by two different factors, IHH and PTHrP, in apparently parallel pathways. Both factors were expressed by CLO cells and acted, presumably through their respective receptors, on preosteoblasts in the preceding stage in order to limit the differentiation of these cells. The role of IHH in maintaining dermal osteogenic cells in a proliferative state is consistent with the observation of the significantly smaller-than-normal size skulls and individual skull bones in the *Ihh* knockout embryos (Fig. 6) (St-Jacques et al., 1999). Moreover, molecular analysis of $Ihh^{-/-}$ mutants suggests that the pool of preosteoblasts is depleted in these animals, whereas overall osteoblast differentiation was normal (Fig. 7G-N). The fact that there was sufficient proliferation in the absence of IHH to form skeletal elements with normal (albeit smaller) morphology (Fig. 7A-F) (St-Jacques et al., 1999; Suda et al., 2001; Long et al., 2001) might be explained by the partially redundant role of PTHrP in negatively regulating the same cellular transition. This negative regulation by IHH and PTHrP is analogous to their roles in controlling the pre-hypetrophic-to-hypertrophic chondrocyte transition.

Taken together, the studies reported here define a series of cellular transitions that underlie dermal bone formation. In particular, we have identified four distinct stages of differentiation, namely the early preosteoblast, preosteoblast, chondrocyte-like osteoblast and mature osteoblast stages (Fig. 8A). The progression of cell types that we have identified provided us with a context for investigating the regulation of dermal bone formation. Further, our studies provide evidence for the roles of several key signals in regulating these transitions. This model should provide a useful framework for future studies addressing the mechanism of craniofacial skeletal development.

Work on this project in both the C.J.T. and A.P.M. laboratories was funded by a program project grant PO1 DK56246 from the NIH. A.A. was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG1618. S.J.R. was supported by postdoctoral fellowships from the NHMRC of Australia (#301299) and the Arthritis Foundation (#401683).

**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/17/3133/DC1

**References**

**Aberg, T., Rice, R., Rice, D., Thesleff, I. and Waltimo-Siren, J.** (2005). Chondrogenic potential of mouse calvarial mesenchyme. *J. Histochem. Cytochem.* **53**, 653-663.

**Abzhanov, A., Kuo, W. P., Hartmann, C., Grant, R., Grant, P. R. and Tabin, C. J.** (2004). The Calmodulin pathway and the evolution of elongated beak morphology in Darwin's Finches. *Nature* **442**, 563-567.

**Akiyama, H., Kim, J.-E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., Nakamura, T. et al.** (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci. USA* **102**, 14665-14670.

**Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B.** (1999). Sox9 is required for cartilage formation. *Nat. Genet.* **22**, 85-89.

**Bitgood, M. J. and McMahon, A. P.** (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

**Capdevila, J. and Johnson, R. L.** (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* **197**, 205-217.

**Chen, C. M., Smith, D. M., Peters, M. A., Samson, M. E., Zitz, J., Tabin, C. J. and Cepko, C. L.** (1999). Production and design of more effective avian replication-incompetent retroviral vectors. *Dev. Biol.* **214**, 370-384.

**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.

**Couly, G. F., Coltey, P. M. and Le Douarin, N. M.** (1993). The triple origin of skull in higher vertebrates: a study of quail-chick chimeras. *Development* **117**, 409-429.

**Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G.** (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-754.

**Dunlop, L. T. and Hall, B. K.** (1995). Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. *Int. J. Dev. Biol.* **39**, 357-371.

**Duprez, D. M., Kostakopoulou, K., Francis-West, P. H., Tickle, C. and Brickell, P. M.** (1996). Activation of Fgf-4 and HoxD gene expression by BMP-2 expressing cells in the developing chick limb. *Development* **122**, 1821-1828.

**Eames, B. F. and Helms, J. A.** (2004). Conserved molecular program regulating cranial and appendicular skeletogenesis. *Dev. Dyn.* **231**, 4-13.

**Erlebacher, A., Filvaroff, E. H., Gitelman, S. E. and Derynck, R.** (1995). Toward a molecular understanding of skeletal development. *Cell* **80**, 371-381.

**Gerstenfeld, L. C. and Shapiro, F. D.** (1996). Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J. Cell. Biochem.* **62**, 1-9.

**Hall, B. K. and Miyake, T.** (1992). The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anat. Embryol.* **186**, 107-124.

**Hamburger, V. and Hamilton, H. L.** (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

**Hanken, J. and Hall, B. K.** (1993). *The Skull: Patterns of Structural and Systematic Diversity, Vol. 2*. Chicago, London: The University of Chicago Press.

**Harada, S.-I. and Roden, G. A.** (2003). Control of osteoblast function and regulation of bone mass. *Nature* **423**, 349-355.

**Healy, C., Uwanogho, D. and Sharpe, P. T.** (1999). Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* **215**, 69-78.

**Helms, J. A. and Schneider, R. A.** (2003). Cranial skeletal biology. *Nature* **423**, 326-331.

**Holleville, N., Quilhac, A., Bontoux, M. and Monsoro-Burq, A. H.** (2003). BMP signals regulate Dlx5 during early avian skull development. *Dev. Biol.* **257**, 177-189.

**Huang, W., Chung, U. I., Kronenberg, H. M. and de Crombrugghe, B.** (2001). The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones. *Proc. Natl. Acad. Sci. USA* **98**, 160-165.

**Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N. et al.** (1999). Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev. Dyn.* **214**, 279-290.

**Ishizuya, T., Yokose, S., Hori, M., Noda, T., Suda, T., Yoshiki, S. and Yamaguchi, A.** (1997). Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. *J. Clin. Invest.* **99**, 2961-2970.

**Iyama, K., Ninomiya, Y., Olsen, B. R., Linsenmayer, T. F., Trelstad, R. L. and Hayashi, M.** (1991). Spatiotemporal pattern of type X collagen gene expression and collagen deposition in embryonic chick vertebrae undergoing endochondral ossification. *Anat. Rec.* **229**, 462-472.

**Jiang, X., Iseki, S., Maxson, R. E., Sucov, H. M. and Morris-Kay, G. M.** (2002).

Tissue origins and interactions in the mammalian skull vault. *Dev. Biol.* **241**, 106-116.
**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.
**Karsenty, G. and Wagner, E. F.** (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2**, 389-406.
**Kaufman, M. H.** (2003). *The Atlas of Mouse Development.* Amsterdam, Boston, London, New York: The Elsevier Academic Press.
**Kim, I. S., Otto, F., Zabel, B. and Mundlos, S.** (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech. Dev.* **80**, 159-170.
**Komori, T.** (2000). A fundamental transcription factor for bone and cartilage. *Biochem. Biophys. Res. Commun.* **276**, 813-816.
**Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M. et al.** (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-764.
**Kronenberg, H. M.** (2003). Developmental regulation of the growth plate. *Nature* **423**, 332-336.
**Lanske, B., Amling, M., Neff, L., Guiducci, J., Baron, R. and Kronenberg, H. M.** (1999). Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. *J. Clin. Invest.* **104**, 399-407.
**Le Douarin, N. M., Ziller, C. and Couly, G. F.** (1993). Patterning of neural crest derivatives in the avian embryo: in vivo and in vitro studies. *Dev. Biol.* **159**, 24-49.
**Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N. and de Crombrugghe, B.** (1997). Sox9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha(II) collagen gene. *Mol. Cell. Biol.* **17**, 2336-2346.
**Lefebvre, V. and de Crombrugghe, B.** (1998). Toward understanding SOX9 function in chondrocyte differentiation. *Matrix Biol.* **16**, 529-540.
**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.
**Long, F., Chung, U. I., Ohba, S., McMahon, J., Kronenberg, H. M. and McMahon, A. P.** (2004). Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development* **131**, 1309-1318.
**Minina, E., Wenzel, H. M., Kreschel, C., Karp, S., Gaffield, W., McMahon, A. P. and Vortkamp, A.** (2001). BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. *Development* **128**, 4523-4534.
**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.
**Murtaugh, L. C., Chyung, J. H. and Lassar, A. B.** (1999). Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* **13**, 225-237.
**Nah, H. D., Pacifici, M., Gerstenfeld, L. C., Adams, S. L. and Kirsch, T.** (2000). Transient chondrogenic phase in the intramembranous pathway during normal skeletal development. *J. Bone Miner. Res.* **15**, 522-533.
**Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S. and Koopman, P.** (1997). SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183**, 108-121.
**Nishibatake, M., Kirby, M. L. and Van Mierop L. H.** (1987). Pathogenesis of persistent truncus arteriosus and dextroposed aorta in chick embryo after neural crest ablation. *Circulation* **75**, 255-264.
**Noden, D. M.** (1983). The role of the neural crest in patterning of avian cranial skeletal, connective and muscle tissues. *Dev. Biol.* **96**, 144-165.
**Noden, D. M.** (1986). Origins and patterning of craniofacial mesenchymal tissues. *J. Craniofac. Genet. Dev. Biol. Suppl.* **2**, 15-31.
**Noden, D. M.** (1991). Cell movements and control of patterned tissue assembly during craniofacial development. *J. Craniofac. Genet. Dev. Biol.* **11**, 192-213.
**Olsen, B. R., Reginato, A. M. and Wang, W.** (2000). Bone development. *Annu. Rev. Cell Dev. Biol.* **16**, 191-220.
**Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R. et al.** (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765-771.
**Ovchinnikov, D., 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.
**Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C. S., Lee, K., Pirro, A., Kronenberg, H. M. and Juppner, H.** (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. *Proc. Natl. Acad. Sci. USA* **94**, 13689-13694.
**Scott, C. K. and Hightower, J. A.** (1991). The matrix of endochondral bone differs from the matrix of intramembranous bone. *Calcif. Tissue Int.* **49**, 349-354.
**Shibata, S., Suda, N., Suzuki, S., Fukuoka, H. and Yamashita, Y.** (2006). An in situ hybridization study of Runx2, Osterix, and Sox9 at the onset of condylar cartilage formation in fetal mouse mandible. *J. Anat.* **208**, 169-177.
**Shum, L., Wang, X., Kane, A. A. and Nuckolls, G. H.** (2003). BMP4 promotes chondrocyte proliferation and hypertrophy in the endochondral cranial base. *Int. J. Dev. Biol.* **47**, 423-431.
**Smith, N., Dong, Y., Lian, J. B., Pratrap, J., Kingsley, P. D., van Wijnen, A. J., Stein, J. L., Schwarz, E. M., O'Keefe, R. J., Stein, G. S. et al.** (2005). Overlapping expression of Runx1(Cbfa2) and Runx2(Cbfa1) transcription factors supports cooperative induction of skeletal development. *J. Cell. Physiol.* **203**, 133-143.
**Soriano, P.** (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
**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.
**Stricker, S., Fundele, R., Vortkamp, A. and Mundlos, S.** (2002). Role of Runx genes in chondrocyte differentiation. *Dev. Biol.* **245**, 95-108.
**Suda, N., Baba, O., Udagawa, N., Terashima, T., Kitahara, Y., Takano, Y., Kuroda, T., Senior, P. V., Beck, F. and Hammond, V. E.** (2001). Parathyroid hormone-related protein is required for normal intramembranous bone development. *J. Bone Miner. Res.* **16**, 2182-2191.
**Ting, K., Petropulos, L. A., Iwatsuki, M. and Nishimura, I.** (1993). Altered cartilage phenotype expressed during intramembranous bone formation. *J. Bone Miner. Res.* **8**, 1377-1387.
**Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A. et al.** (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* **119**, 555-566.
**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.
**Warren, S. M., Brunet, L. J., Harland, R. M., Economides, A. N. and Longaker, M. T.** (2003). The BMP antagonist noggin regulates cranial suture fusion. *Nature* **422**, 625-629.
**Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. and Broadus, A. E.** (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc. Natl. Acad. Sci. USA* **93**, 10240-10245.
**Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W., Stott, D. and Allemann, R. K.** (1991). Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. *Genes Dev.* **5**, 2048-2059.
**Wu, P., Jiang, T., Shen, J., Widelitz, R. B. and Chuong, C.-M.** (2006). Morphoregulation of avian beaks: comparative mapping of growth zone activities and morphological evoluton. *Dev. Dyn.* **235**, 1400-1412.
**Yamaguchi, A., Komori, T. and Suda, T.** (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr. Rev.* **21**, 393-411.
**Yamashiro, T., Wang, X. P., Li, Z., Oya, S., Aberg, T., Fukunaga, T., Kamioka, H., Speck, N. A., Takano-Yamamoto, T. and Thesleff, I.** (2004). Possible roles of Runx1 and Sox9 in incipient intramembranous ossification. *J. Bone Miner. Res.* **19**, 1671-1677.
**Yokose, S., Ishizuya, T., Ikeda, T., Nakamura, T., Tsurukami, H., Kawasaki, K., Suda, T., Yoshiki, S. and Yamaguchi, A.** (1996). An estrogen deficiency caused by ovariectomy increases plasma levels of systemic factors that stimulate proliferation and differentiation of osteoblasts in rats. *Endocrinology* **137**, 469-478.
**Yu, M., Wu, P., Widelitz, R. B. and Chuong, C. M.** (2002). The morphogenesis of feathers. *Nature* **420**, 308-312.
**Zelzer, E. and Olsen, B. R.** (2003). The genetic basis for skeletal diseases. *Nature* **423**, 343-348.
**Zhao, Q., Eberspaecher, H., Lefebvre, V. and De Crombrugghe, B.** (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev. Dyn.* **209**, 377-386.
**Zhao, Q., Brauer, P. R., Xiao, L., McGuire, M. H. and Yee, J. A.** (2002). Expression of parathyroid hormone-related peptide (PthrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process. *J. Anat.* **201**, 137-151.
**Zhou, H., Choe, K. M., Lu, Y. and Niswander, L.** (1997). BMP signaling and vertebrate limb development. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 269-272.
**Zou, H. and Niswander, L.** (1996). Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* **272**, 738-741.