Regulation of skeletogenic differentiation in cranial dermal bone

Arhat Abzhanov1,*, Stephen J. Rodda2, Andrew P. McMahon2 and Clifford J. Tabin1,†

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 endochondral 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 morphogenetic 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 I 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., 1998; De Crombrugghe, 1998; Bi et al., 1999; Healy et al., 1999; Morikawa and Takahashi, 2005; Iyama 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 morphogenetic 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

1Department of Genetics, Harvard Medical School, Boston, MA 02115, USA.
2Department 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
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 PTHR signaling 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, PTHR signaling through its receptor, PTHR-R, acts to block hypertrophic differentiation (Vortkamp et al., 1996; Weir et al., 1996; Lanske et al., 1999). Together, Ihh and PTHR 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 PTHR 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 PTHR 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 Cbfal); 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 [BSP1 (also known as Sppl, 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 PTHR, 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 PTHR
 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, Gli1 (Gli1); ~1.5 kb), Bmp7a (~1.6 kb, gift from Dr Niswander, University of Colorado, Denver, USA), Bmp7b (~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), Bmp5 (~2 kb), Coll II (~450 kb, amplified from AA182-AA616 region of the GenBank sequence #M744335), Opn (~640 bp, AA34-249), Coll IX (Cola1); ~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 I (~400 bp from the 3′ UTR; gift from Dr Olsen), Ptc2 (Ptc2; 2 kb), Opn (~950 bp, gift from Dr Rosen, Harvard Dental School, Boston, USA), Ihh (760 bp), Osc (Lux; ~500 bp), PTHrP (~500 bp), PTHrP-Rec (Phe1; ~700 bp), Oss (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; Iishiuya 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 F, 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 Kit9 (Molecular Probes, Oregon). We empirically determined that the embryonic head sections needed to be treated with 1% H2O2 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, Oss (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, Oss, 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, Oss 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
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 Dlx or Oso 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=0.003) of cells that had been dissociated from chick frontal bone (n=500) co-expressed Coll IX and Ospn (Fig. 4A-D). Even fewer cells (17±8%; P=0.0043) co-expressed Runx2 and Ospn (Fig. 4E-H). By contrast, co-expression of Coll IX with Runx2, a very early osteoblastic marker, was close to 70% (71±7%; P=0.005, n=500) in HH39 chick frontal bone cells (Fig. 4I-L). Similarly, in mouse,
approximately 35% (34±7%; \( P\leq 0.035; n=500 \)) of the cells co-expressed Coll II and Osx, whereas less than 10% (7±2%; \( P\leq 0.002; n=500 \)) co-expressed Coll II and Osc, and 28% (27±8%; \( P\leq 0.01; n=500 \)) 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,
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 \textit{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 \beta-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 \beta-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 \beta-galactosidase-positive cells using molecular markers. The relative proportion of the \beta-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
Roles of signals in dermal bone development

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 Omp 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. Bmp1b 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 (Pich1) 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 injection, 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, Omp 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,
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. 5I). 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).

**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.
Roles of signals in dermal bone development

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 Ocn, 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 skeleton.

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...
differentiated chondrocytes and never overlap with the expression of more-mature osteoblastic markers, such as Opm. 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 ‘ostochondro-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 and Neff, 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 Silx9 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 PTHR, 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. This might reflect incomplete recombination due to less than uniform expression of the Coll2:-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 PTHR, 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 the mature dermal osteoblasts suggests that there was sufficient proliferation in the absence of IHH to form osteoblasts in vivo (Fig. 7G-N). The Ihh-null allele thus mimics the condition of Ihh-/- mutants. It suggests that Ihh is required for the maintenance of preosteoblasts for osteoblast differentiation. Ihh-null mice, however, develop normally in the absence of Ihh (St-Jacques et al., 1999).

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 provides 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 P01 DK56246 from the NIH. A.A.A. was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG1618. S.I.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


