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BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation

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SUMMARY

During endochondral ossification, two secreted signals, Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP), have been shown to form a negative feedback loop regulating the onset of hypertrophic differentiation of chondrocytes. Bone morphogenetic proteins (BMPs), another family of secreted factors regulating bone formation, have been implicated as potential interactors of the Ihh/PTHrP feedback loop. To analyze the relationship between the two signaling pathways, we used an organ culture system for limb explants of mouse and chick embryos. We manipulated chondrocyte differentiation by supplementing these cultures either with BMP2, PTHrP and Sonic hedgehog as activators or with Noggin and cyclopamine as inhibitors of the BMP and Ihh/PTHrP signaling systems. Overexpression of *Ihh* in the cartilage elements of transgenic mice results in an upregulation of *PTHrP* expression and a delayed onset of hypertrophic differentiation. Noggin treatment of limbs from these mice did not antagonize the effects of *Ihh* overexpression. Conversely, the promotion of chondrocyte maturation induced by cyclopamine, which blocks Ihh signaling, could not be rescued with BMP2. Thus BMP signaling does not act as a secondary signal of Ihh to induce *PTHrP* expression or to delay the onset of hypertrophic differentiation. Similar results were obtained using cultures of chick limbs.

We further investigated the role of BMP signaling in regulating proliferation and hypertrophic differentiation of chondrocytes and identified three functions of BMP signaling in this process. First we found that maintaining a normal proliferation rate requires BMP and Ihh signaling acting in parallel. We further identified a role for BMP signaling in modulating the expression of *Ihh*. Finally, the application of Noggin to mouse limb explants resulted in advanced differentiation of terminally hypertrophic cells, implicating BMP signaling in delaying the process of hypertrophic differentiation itself. This role of BMP signaling is independent of the Ihh/PTHrP pathway.

Key words: BMP, Ihh, PTHrP, Chondrocyte, Proliferation, Hypertrophic differentiation, Endochondral ossification, Mouse, Chick

INTRODUCTION

During embryonic development, the bones of the axial and appendicular skeleton as well as most of the facial bones are formed by endochondral ossification. This process starts with mesenchymal cells that condense and differentiate into two types of cells: chondrocytes that form cartilage elements and perichondrial cells that surround the cartilage model. Starting from the center of the cartilage elements, chondrocytes undergo several steps of maturation from proliferating chondrocytes to non-proliferating hypertrophic cells. During normal development only terminally differentiated chondrocytes are replaced by bone. In parallel to the onset of hypertrophic differentiation, the perichondrium flanking this region differentiates into an osteoblast-forming periosteum. Hypertrophic differentiation and periosteum formation are tightly linked throughout development. As a front of differentiation and ossification spreads from the center to the ends of the skeletal elements, the proliferative and hypertrophic zones are ultimately reduced to narrow bands termed growth plates, located near the ends of the skeletal elements. Longitudinal growth of bones is dependent on proliferation and hypertrophic differentiation of chondrocytes in the growth plate. As terminally hypertrophic cartilage is continuously replaced by bone, the tight regulation of the various steps of chondrocyte differentiation is critical for balancing growth and ossification of the skeletal elements (Erlebacher et al., 1995; Hinchcliffe and Johnson, 1980).

Indian hedgehog (Ihh), a member of the conserved Hedgehog family of signaling factors, regulates several aspects of endochondral ossification. During mouse embryonic development, *Ihh* expression is first detected at embryonic stage 11.5 (E11.5) in chondrocytes of the early cartilaginous condensation. With the initiation of hypertrophic differentiation

Ihh expression becomes restricted to the prehypertrophic chondrocytes (Bitgood and McMahon, 1995; Vortkamp et al., 1996; Vortkamp et al., 1998). Another secreted signaling molecule that regulates endochondral ossification is parathyroid hormone-related protein (PTHrP; Pthlh – Mouse Genome Informatics), which is expressed in the periarticular region of the developing cartilage elements. PTHrP signals through the PTH/PTHrP receptor (PP-R; Pthr – Mouse Genome Informatics), which is expressed at low levels throughout the growth plate and at high levels in the transitional region where the switch from proliferating into hypertrophic chondrocytes takes place (Amizuka et al., 1994; Lee et al., 1995). Targeted disruption of *PTHrP* or *PP-R* in mice results in a dwarfism phenotype, due to a reduced zone of proliferating cells and an advanced onset of hypertrophic differentiation (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996). Conversely, activation of PTHrP signaling leads to a delay of hypertrophic differentiation (Schipani et al., 1995; Weir et al., 1996; Schipani et al., 1997).

Several lines of evidence indicate that Ihh and PTHrP interact in a negative feedback loop regulating the onset of hypertrophic differentiation. Ectopic expression of *Ihh* in developing limbs of chicken embryos results in a delay of hypertrophic differentiation and in strong upregulation of *PTHrP* in the periarticular region of the infected cartilage elements. Furthermore, limb explants of *PTHrP*$^{-/-}$ mice that were treated with Hedgehog protein in culture demonstrated that PTHrP is necessary to mediate the effect of Ihh on chondrocyte differentiation (Vortkamp et al., 1996). It has been proposed that Ihh, which is expressed in the prehypertrophic chondrocytes, signals to the periarticular region to induce *PTHrP* expression. PTHrP in turn signals back to its receptor and prevents hypertrophic differentiation of additional chondrocytes. The expression level of the Ihh/PTHrP system thus regulates the distance between the joint region and the onset of hypertrophic differentiation (Vortkamp et al., 1996). This model has been supported by examination of *Ihh* deficient mice, which lack *PTHrP* expression and exhibit premature hypertrophic differentiation (St-Jacques et al., 1999). Moreover, experiments in chimeric mice carrying either *PP-R*$^{-/-}$ cells or *PP-R/Ihh* double mutant cells in a wild-type background strongly confirm the negative feedback relationship between Ihh and PTHrP in regulating chondrocyte differentiation. Chimeras carrying *PP-R*$^{-/-}$ chondrocytes display advanced hypertrophic differentiation of mutant cells close to the joint region, which transiently express *Ihh*. This results in increased periarticular *PTHrP* expression and a subsequent delay in hypertrophic differentiation of wild-type cells. This delay is abolished in chimeras carrying double mutant cells, which cannot express *Ihh* and subsequently induce *PTHrP* expression (Chung et al., 1998; Chung et al., 2001).

In addition to regulating chondrocyte differentiation, the analysis of *Ihh* knockout mice revealed that Ihh signaling regulates chondrocyte proliferation independent of PTHrP. Furthermore endochondral bones of *Ihh*$^{-/-}$ mice do not ossify indicating that Ihh is required for the ossification process (Karp et al., 2000; St-Jacques et al., 1999).

An important issue in considering the roles of signaling factors during bone development is whether their various functions are direct or indirect consequences of their binding to a target cell. It has been shown by antibody staining that PTHrP can travel through the growth plate and directly act on its target cells expressing *PP-R* (Tsukazaki et al., 1995; Lee et al., 1996). By contrast, it is currently unclear whether Ihh does act as a long range signal or if the Ihh response is mediated through secondary signaling molecules. If Ihh does act indirectly, possible candidates for secondary signals include members of the conserved family of bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGFβ) superfamily of secreted proteins (Hogan, 1996; Kingsley, 1994a; Kingsley, 1994b). Several Bmp genes are expressed in the developing cartilage elements and are thus good candidates for serving as secondary signals of Ihh. In mice, *Bmp2*, *Bmp3*, *Bmp4*, *Bmp5* and *Bmp7* are expressed in the region of the perichondrium that flanks the *Ihh* expression domain (Pathi et al., 1999; Zou et al., 1997; Daluiski et al., 2001; Haaijman et al., 2000). In addition, *Bmp7* is expressed in the proliferating chondrocytes (Haaijman et al., 2000). BMPs signal through serine/threonine kinase receptors type I and type II, which heterodimerize to transduce the BMP signal (Massague, 1998). In the developing cartilage elements Bmp receptor IB (*BmpR-IB*; *Bmpr1b* – Mouse Genome Informatics) is expressed in the perichondrium, whereas Bmp receptor IA (*BmpR-IA*; Bmpr1b – Mouse Genome Informatics) and Bmp receptor II (*BmpR-II*; Bmpr2 – Mouse Genome Informatics) are expressed in the prehypertrophic chondrocytes (Zou et al., 1997; Yi et al., 2000; Haaijman et al., 2000). In addition, Bmp receptors as well as *Bmp2*, *Bmp4* and *Bmp7* are expressed in the periarticular region (Macias et al., 1997; Pathi et al., 1999; Zou et al., 1997; Solloway et al., 1998). Several lines of evidence support a possible interaction between BMP and Ihh signaling. It has been shown in chicken embryos that ectopic expression of *Ihh* in developing cartilage upregulates *Bmp2* and *Bmp4* expression in the perichondrium (Pathi et al., 1999). Furthermore, retroviral misexpression of a constitutively activated *BmpR-IA* during chick limb development results in an upregulation of *PTHrP* expression and a block of chondrocyte differentiation, thus mimicking the effect of retroviral misexpression of *Ihh*. On the basis of these results, it has been proposed that Ihh signaling regulates *Bmp* expression locally, which in turn influences PTHrP production at a distance, thereby regulating the rate of chondrocyte differentiation (Zou et al., 1997).

In the developing embryo BMP signaling can be antagonized by several secreted factors including Noggin (Smith and Harland, 1992; Smith et al., 1993). Biochemical analysis demonstrated that Noggin directly binds to BMP proteins thus preventing them from binding to their receptors (Zimmerman et al., 1996; Holley et al., 1996; Smith, 1999). It has been shown that Noggin can inhibit various members of the BMP family, including at least BMP2, BMP4 and BMP7 (Zimmerman et al., 1996; Kawabata et al., 1998). Noggin protein therefore provides a powerful tool to reduce BMP signaling in experimental systems.

In this study we have analyzed a potential interaction of the Ihh/PTHrP and BMP signaling pathways using a culture system for embryonic mouse and chick limb explants, which was supplemented with activators and inhibitors of both signaling pathways. To block Ihh signaling, we used the alkaloid cyclopamine, which has previously been demonstrated to inhibit Hedgehog signal transduction in target cells (Incardona et al., 1998; Cooper et al., 1998; Taipale et al., 2000). We did not find evidence for BMPs acting as secondary

signals of Ihh in mediating the induction of *PTHrP* expression. Instead we found that BMP signaling acts at various steps of chondrocyte differentiation and interacts with Ihh signaling in different ways. First, BMP and Ihh signals act in parallel in regulating chondrocyte proliferation. In addition, BMP signaling modulates the expression level of *Ihh*, thereby integrating the regulation of chondrocyte proliferation and the onset of hypertrophic differentiation. Third, we identified a negative role for BMP signaling in regulating the process of hypertrophic differentiation by delaying the maturation of terminally hypertrophic cells.

MATERIALS AND METHODS

Organ cultures of embryonic limb explants

Forelimbs of mouse and chicken embryos were stripped of skin and muscles and cultured for 2 or 4 days in BGJ-B medium (GibcoBRL) with Antibiotic/Antimycotic (Life Technologies) and 0.1% BSA in organ culture dishes under humidified conditions (Vortkamp et al., 1996; Lanske et al., 1996). Cultures were supplemented with 500 ng/ml recombinant human BMP2 (Genetic Institute, Boston, MA), 5 μg/ml recombinant murine Shh-N (Ontogeny, Cambridge, MA), 3×10^{-7} M human 1-34 PTHrP (Sigma), 10 μM cyclopamine (Incardona et al., 1998), or 500 ng/ml recombinant *Xenopus* Noggin protein. Noggin protein was isolated from stably transfected CHO cells (Lamb et al., 1993) and analyzed by antibody staining and mass spectrometry. Concentrations of the various growth factors have been carefully titered to establish the minimal concentration necessary to induce a specific effect on chondrocyte differentiation.

All mouse experiments were carried out on E14.5 and E16.5 limbs, which were cultured for 2 and 4 days. If not otherwise mentioned, each combination of growth factors was repeated at least six times. Parallel experiments showed comparable results. Over the course of the experiment (2-4 days), the severity of the phenotype of each manipulation increased. Limb explants of E14.5 and E16.5 embryos underwent similar changes in chondrocyte differentiation upon growth factor treatment. As the effect on hypertrophic differentiation was most obvious in limb explants of E14.5 embryos, figures in this publication display experiments of this stage.

To establish the effect of single factors the right limb was cultured in supplemented medium and compared with the left one cultured in control medium. For all co-treatment experiments, double treated explants were compared with controlateral limbs treated with each of the single factors. For each growth factor combination, at least one limb was hybridized with a probe for *ColII*, a general marker of chondrocytes, to confirm that the cells were still alive after culture.

Chick experiments were carried out using limb explants of stage Hamburger Hamilton 32 (HH 32) (Hamburger and Hamilton, 1951) chick embryos. Two parallel cultures were treated with each combination of growth factors for 2 days.

Wild-type mice (NMRI) and pathogen free white leghorn chicken eggs were derived from Charles River (Sulzfeld, Germany). *ColII-Gal4* and *UAS-Ihh* transgenic mice were identified by PCR of tail DNA (Long et al., 2001).

Analysis of limb explants

After culture, limb explants were fixed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin. Serial sections of 5 μm were processed for radioactive in situ hybridization using [P^{33}]-UTP labeled antisense riboprobes. Hybridization was carried out at 70°C in 50% formamide as previously described (Vortkamp et al., 1996). Sections were counterstained with Toluidine Blue O (Sigma). Probes for in situ hybridization were as follows: chick *Ihh* (Vortkamp et al., 1996); chick *PTHrP* (Thiede and Rutledge, 1990); rat *ColII* (Kohno et al., 1984); rat *PTHrP* [(Karaplis et al., 1990); a 440 bp *Sma*I fragment was cloned into pBSK]; mouse *ColX* (Jacenko et al., 1993); mouse *Ihh* (Bitgood and McMahon, 1995); mouse *Osp* (Kim et al., 1999); mouse *Bmp3*; mouse *Bmp4*; and mouse *Bmp7* (Bitgood and McMahon, 1995).

For proliferation analysis, limb explants were incubated with 5-bromo-2-deoxy-uridine (BrdU) (BrdU labeling and detection kit II, Roche, Germany; dilution of BrdU 1:100) for 2 hours before harvesting. Limbs were embedded in paraffin and sectioned. Proliferating cells were detected by antibody staining according to the manufacturer.

RESULTS

BMP signaling regulates multiple steps of chondrocyte differentiation

To analyze the effect of BMP signaling on chondrocyte differentiation, limb explants were cultured in the presence of BMP2 or Noggin protein. After 4 days of culture, untreated limbs showed an increase in total length of the skeletal elements (Fig. 1A,C). Treatment with BMP2 resulted in a further increase in length if compared with untreated cultures (Fig. 1C,D). After treatment with BMP2, markers for prehypertrophic and hypertrophic chondrocytes, *Ihh* and *ColX*, respectively, were expressed in a normal pattern, but in expanded domains compared with untreated control limbs (Fig. 1G,K,H,L). As the cartilage elements themselves were longer, the relative length of the different zones of proliferating, prehypertrophic and hypertrophic chondrocytes appeared normal. BMP2, therefore, promotes the expansion of all zones of differentiation in the developing skeletal elements. By contrast, treatment with the BMP antagonist Noggin resulted in an inhibition of limb growth in culture (Fig. 1B). However, despite the smaller size of the cartilage elements, *ColX* expression indicated that the chondrocytes have undergone hypertrophic differentiation. Moreover, the two zones of *ColX*-expressing hypertrophic cells were clearly separated from each other (Fig. 1J). They flanked a distinct zone of terminally hypertrophic cells. Consequently, the less differentiated regions of chondrocytes were smaller than in non-treated limbs. Noggin treatment also resulted in reduced expression of *Ihh*. Furthermore, the distance between the *Ihh* expression domain and the periarticular region, which demarcates proliferating chondrocytes at E14.5, was reduced in size (Fig. 1F). These effects of Noggin could be antagonized by BMP2 in cultures double treated with both factors (n=4, data not shown).

It has previously been hypothesized that BMP signaling acts as a secondary signal downstream of Ihh, mediating the delay in hypertrophic differentiation. Consistent with this, the reduced zone of proliferating and prehypertrophic chondrocytes and the expanded region of terminal hypertrophic cells after Noggin treatment could be interpreted as advanced hypertrophic differentiation, caused by the absence of BMP signaling. Alternatively, as blocking of BMP signaling by Noggin treatment leads to reduced expression of *Ihh*, the advanced onset of hypertrophic differentiation could be a secondary consequence of the reduced Ihh signal.

Overexpression of *Ihh* in transgenic mice delays hypertrophic differentiation

To analyze the epistatic relationship between Ihh/PTHrP and BMP signaling, we used a transgenic mouse system allowing

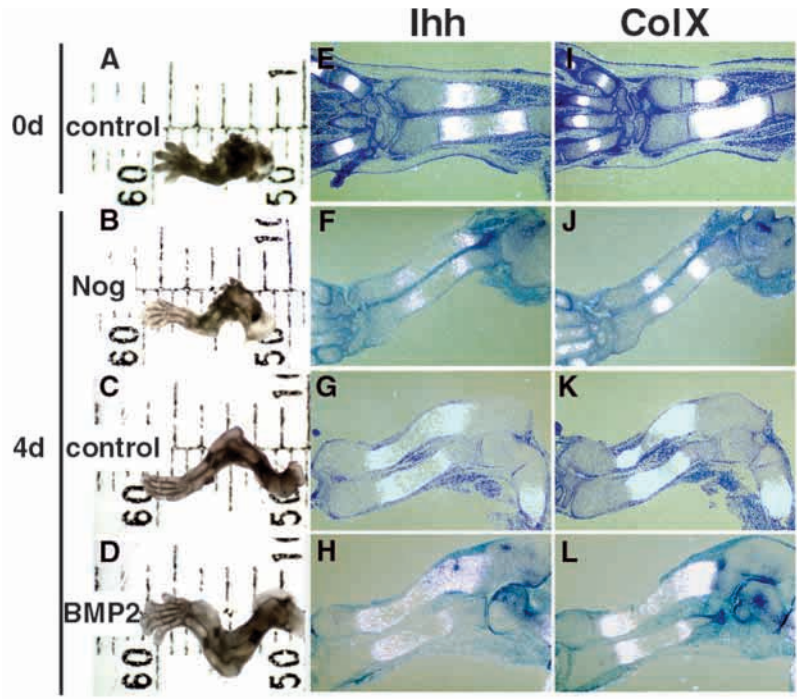

Fig. 1. BMP signaling regulates chondrocyte differentiation. (A-D) Forelimbs of E14.5 mouse embryos were cultured for 4 days and photographed before (A) and after (B-D) culture. Serial sections of these limbs were hybridized with riboprobes for *Ihh* (E-H) or *ColX* (I-L). Uncultured limbs (A) display the characteristic expression domains of *Ihh* in prehypertrophic (E) and *ColX* in hypertrophic (I) chondrocytes. Limbs cultured for 4 days increase in size (C) but display the normal distribution of *Ihh*- (G) and *ColX*- (K) expressing cells. Treatment with BMP2 results in a further enlargement of the cartilage elements (D) and an increased size of the *Ihh* (H) and *ColX* (L) expression domains. By contrast, Noggin treatment blocks limb growth (B) and results in reduced expression of *Ihh* (F) and *ColX* (J). (A-D) Ruler indicates relative size units. In E-L ulna is upwards and radius is downwards.

overexpression of the chicken *Ihh* gene under the *ColII* promoter. We generated two transgenic mouse lines, one of which misexpresses the yeast transcriptional activator *Gal4* under the *ColII* promoter (*ColII-Gal4*). The second line carries the chick *Ihh* gene downstream of the yeast Gal4-binding site *UAS* (*UAS-Ihh*). Mating the two strains results in 25% embryos misexpressing *Ihh* under the *ColII* promoter (*ColII/Ihh* embryos) (Long et al., 2001). Transgenic embryos survive until at least E18.5 and display severe defects in the developing skeleton. In the limbs, the skeletal elements are broadened and joints of elbow and phalanges are missing or severely malformed. At the molecular level, we found reduced expression of *ColX* and endogenous *Ihh* in the developing cartilage elements, whereas *PTHrP* was highly expressed in the periarticular region (Fig. 2A,E and data not shown). The relative expression of these genes was maintained after culture (Fig. 3A-D, Fig. 5D). The distance between the joint region and the onset of hypertrophic differentiation, demarcated by the distal end of the *Ihh* expression domain, is enlarged relative to the size of skeletal elements when compared to wild type embryos, indicating a delay in the onset of hypertrophic differentiation (Fig. 2A,E, Fig. 3A,C) (Long et al., 2001). All single transgenic *ColII-Gal4* and *UAS-Ihh* or wild-type siblings appeared normal by expression analysis (data not shown).

Bmp expression is upregulated in *ColII/Ihh* mice

It has previously been shown in chicken embryos that ectopic misexpression of *Ihh* results in upregulation of *Bmp2* and *Bmp4* expression (Pathi et al., 1999). To test if *Bmp* expression is similarly regulated in mice, we analyzed the expression of different *Bmp* genes in *ColII/Ihh* transgenic embryos. We found that at stage E14.5 *Bmp3*, *Bmp4* and *Bmp7* were strongly upregulated in the perichondrium, compared with wild-type embryos (Fig. 2B-D,F-H). In addition, *Bmp4* and *Bmp7* expression was increased in proliferating chondrocytes (Fig. 2C,D,G,H). Therefore, as in chick embryos, overexpression of *Ihh* in mice leads to upregulation of BMP signaling.

Interaction of Ihh and BMP signaling during chondrocyte differentiation

It has been proposed that at least some of the effects of Ihh on hypertrophic differentiation are mediated by BMPs (Pathi et al., 1999; Zou et al., 1997). To test whether BMP signaling is necessary for the observed delay in hypertrophic differentiation induced by *Ihh* overexpression, we treated limbs of *ColII/Ihh* embryos with Noggin protein to block BMP signaling. If BMP signaling acted as a secondary factor in the Ihh pathway we would expect an acceleration of hypertrophic differentiation. Surprisingly, hybridization with the hypertrophic marker *ColX*

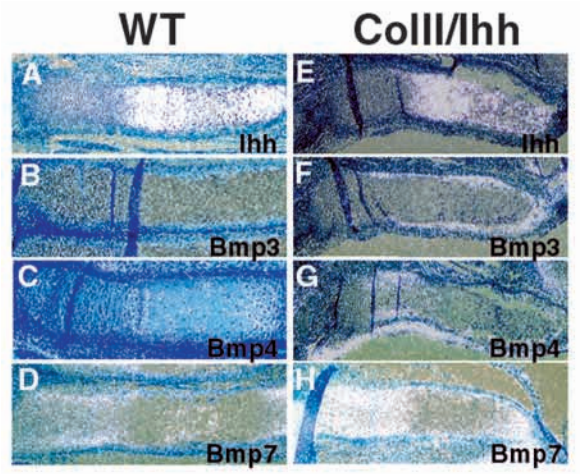

Fig. 2. Overexpression of *Ihh* results in upregulation of Bmp genes. Forelimbs of wild-type (A-D) and *ColII/Ihh* mouse embryos (E-H) at stage E14.5 were sectioned and hybridized with riboprobes for *Ihh* (A,E), *Bmp3* (B,F), *Bmp4* (C,G) or *Bmp7* (D,H). In limbs of *ColII/Ihh* mice, endogenous *Ihh* expression is reduced compared with that in the wild-type mice (A,E). *Bmp3* (F), *Bmp4* (G) and *Bmp7* (H) are upregulated in perichondrium of *ColII/Ihh* mouse limbs compared with that of wild-type limbs (B-D). In addition, in *ColII/Ihh* transgenic mice *Bmp4* (C,G) and *Bmp7* (D,H) are upregulated in proliferating chondrocytes. All panels show sections through the radius.

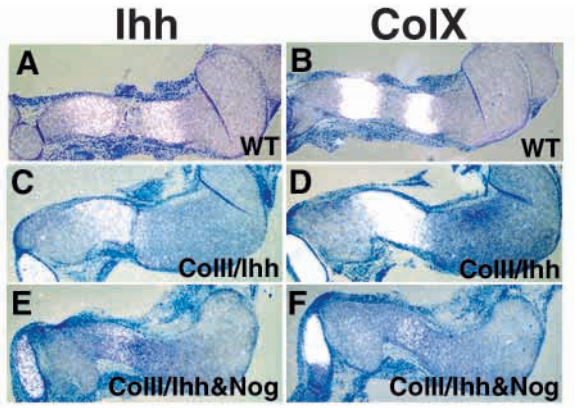

Fig. 3. A block of BMP signaling cannot overcome the Ihh-induced delay in hypertrophic differentiation. Forelimbs of E14.5 embryos from wild-type (A,B) or transgenic *ColII/Ihh* mice (C-F) were cultured for 4 days in control medium (A-D) or treated with Noggin protein (E,F). Serial sections of these limbs were hybridized with riboprobes for *Ihh* (A,C,E) or *ColX* (B,D,F). (A-D) Untreated limbs of *ColII/Ihh* embryos display a reduced expression domain of *Ihh* (C) and *ColX* (D), compared with untreated limbs of wild-type embryos (A,B). (E,F) Forelimbs of *ColII/Ihh* embryos treated with Noggin show a further reduction of *Ihh* (E) and *ColX* (F) expression. All panels show sections through the humerus.

revealed that Noggin treatment of limbs from *ColII/Ihh* embryos results in a further reduction of hypertrophic chondrocytes in cultures of both, E14.5 and E16.5 limbs. Consistent with this observation, hybridization with *Ihh* indicates a further reduction of endogenous *Ihh* expression (Fig. 3).

Conversely, we tested whether a block of Ihh signaling, which results in advanced hypertrophic differentiation, can be rescued by treatment with BMPs. It has recently been demonstrated that the alkaloid cyclopamine inhibits the Hedgehog signaling pathway, acting on the target cells (Incardona et al., 1998; Cooper et al., 1998; Taipale et al., 2000). Treatment of limb cultures with cyclopamine resulted in an enlargement of the skeletal elements, due to a strong increase in hypertrophic differentiation as indicated by the expanded expression domains of *Ihh* and *ColX* (Fig. 4A,B,G,H). This effect is due to a loss of downstream *PTHrP* expression, as cyclopamine treatment blocks the expression of *PTHrP* (Fig. 5G). Moreover, the effect of cyclopamine on chondrocyte differentiation can be rescued by simultaneous treatment with PTHrP (Fig. 4C-H). To test whether BMPs are acting as secondary signals in the Ihh/PTHrP pathway, we attempted a similar rescue of the cyclopamine induced effect with BMP2 protein. However, treatment of limb cultures with a combination of cyclopamine and BMP2 protein resulted in limbs that were even bigger than those treated with either factor alone (data not shown). Section in situ hybridization revealed an extended zone of *ColX*-expressing hypertrophic chondrocytes in double treated limbs comparable with that of limbs treated with cyclopamine only (Fig. 4H,J,L). Surprisingly, the expression domain of *Ihh* in double treated cultures extended towards the periarticular region relative to

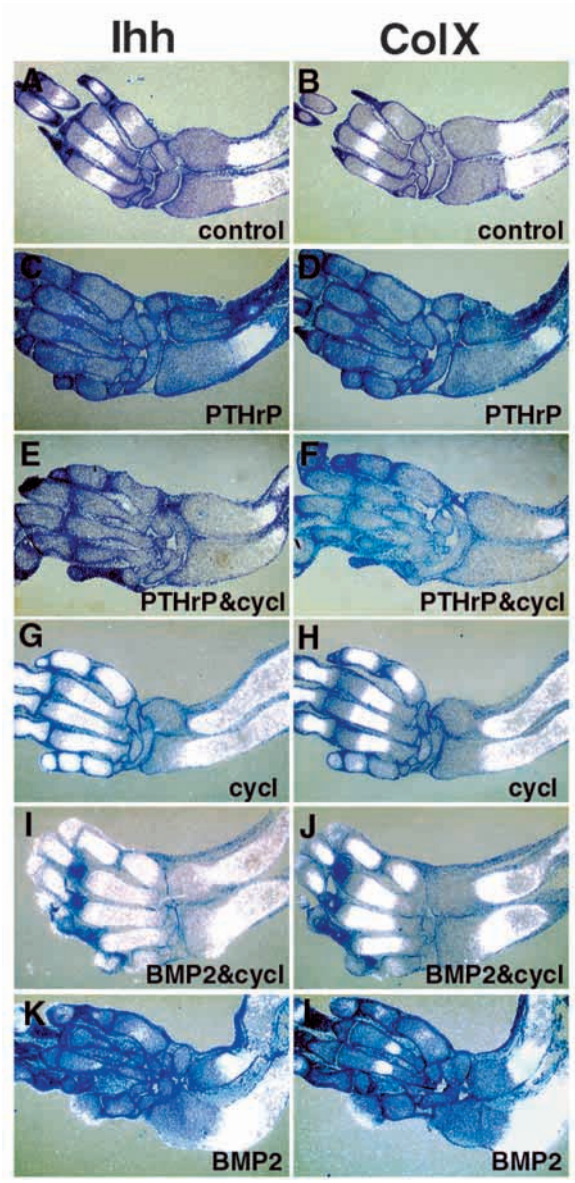

Fig. 4. BMP2 does not rescue the advanced onset of hypertrophic differentiation induced by a loss of Ihh signaling. Forelimbs of E14.5 embryos were cultured for 4 days in control medium (A,B) or in medium supplemented with PTHrP (C,D), PTHrP and cyclopamine (E,F), cyclopamine (G,H), BMP2 and cyclopamine (I,J) or BMP2 (K,L). Serial sections were hybridized with riboprobes for *Ihh* (A,C,E,G,I,K) or *ColX* (B,D,F,H,J,L). (A-D) Treatment with PTHrP results in a delay of hypertrophic differentiation, as seen by the reduced expression of *Ihh* (A,C) and *ColX* (B,D), and the increased distance between the *Ihh* expression domain and the joint region. Treatment with cyclopamine results in an advanced onset of hypertrophic differentiation as can been seen by the enlarged domain of *Ihh* and *ColX* expression (G,H). (E,F) PTHrP can rescue the advanced onset of hypertrophic differentiation in explants co-treated with PTHrP and cyclopamine. (I,J) Co-treatment with BMP2 and cyclopamine does not rescue the advanced onset of hypertrophic differentiation induced by cyclopamine, but leads to a further enlargement of the *Ihh* expression domain (I) compared with limbs treated with either cyclopamine (G) or BMP2 (K). Limbs treated with cyclopamine and BMP2 plus cyclopamine were derived from the same embryo. In all panels ulna is upwards and radius is downwards. cycl, cyclopamine.

the domains seen after either cyclopamine or BMP2 treatment (Fig. 4G,I,K; 2/6 and 10/10 in 2 and 4 day cultures, respectively). Summarizing these experiments strongly indicate that BMP signaling does not act downstream of Ihh in regulating the onset of hypertrophic differentiation.

PTHrP expression is not regulated by BMP signaling

It has previously been demonstrated that the effect of Ihh signaling on chondrocyte differentiation is mediated by the induction of *PTHrP* expression in the periarticular region (Vortkamp et al., 1996). Misexpression of activated Bmp receptors raised the possibility that BMP signaling mediates the upregulation of *PTHrP* expression by Ihh (Zou et al., 1997). To test this hypothesis we analyzed the expression of *PTHrP* in single and double treated cultures. As expected, in skeletal elements of *ColII/Ihh* embryos, we found strong expression of *PTHrP* in the periarticular region (Fig. 5D). Conversely, treatment of wild-type limbs with cyclopamine resulted in a complete block of *PTHrP* expression (Fig. 5G). After treatment of wild-type limbs with BMP2, we did not find significant upregulation of *PTHrP* compared with untreated cultures; however, treatment with Noggin resulted in a slight decrease in *PTHrP* expression (Fig. 5A,B,E). To differentiate whether this decrease is an effect of the reduced Ihh signal in these cultures (see Fig. 1F) or a direct effect of blocking BMP signaling, we treated limbs of *ColII/Ihh* embryos with Noggin protein. In these cultures we still found strong expression of *PTHrP* in the joint region, comparable with that of untreated cultures of mutant embryos (Fig. 5C,D). Conversely, BMP2 did not rescue *PTHrP* expression in wild-type explants treated with cyclopamine (Fig. 5F). Our results therefore strongly suggest that BMP signaling is not mediating the induction of *PTHrP* expression by Ihh.

Interaction of Ihh and BMP signaling in chick embryos

The experiments that first indicated a possible role for BMPs in regulating *PTHrP* expression, were carried out by viral misexpression of an activated *BmpR-IA* in chick embryos (Zou et al., 1997). Thus, one possible explanation for the opposite conclusions revealed in those earlier studies could be that the signaling systems interact in different ways in mouse and in chick. We therefore examined the interactions between Hedgehog and BMP signaling using cultures of stage HH 32 chick limbs. We found that limbs treated at this stage of development reacted in a similar way to mouse limbs. Treatment with Shh protein resulted in a delay of chondrocyte differentiation, whereas cyclopamine accelerated hypertrophic differentiation as assayed by the expression of the prehypertrophic and hypertrophic markers, *Ihh* and *ColX* (Fig. 6A,G,M and data not shown). In limbs treated with a combination of Shh and Noggin protein we did not find increased chondrocyte differentiation compared with treatment with Shh alone (Fig. 6C,E,G). In addition, *PTHrP* was strongly expressed in the periarticular region in spite of the block in BMP signaling (Fig. 6D,F,H). Conversely, cultures treated with a combination of cyclopamine and BMP2 displayed the same acceleration of hypertrophic differentiation seen with cyclopamine alone (Fig. 6J,K,M) and *PTHrP* was not induced by BMP treatment in the absence of Ihh signaling (Fig. 6J,L,N). Therefore, in chick as in mice, BMP2 does not appear to mediate the effect of Ihh on *PTHrP* expression and hypertrophic differentiation.

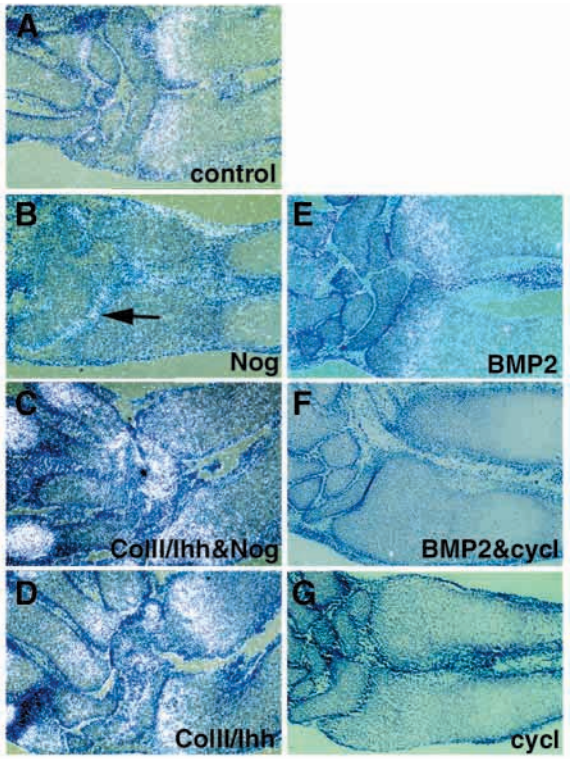

Fig. 5. BMP2 signaling does not act as a secondary signal of Ihh to induce *PTHrP* expression. Forelimbs of E14.5 mouse embryos were cultured for 4 days in control medium (A,D), or in medium supplemented with Noggin (B,C), BMP2 (E), BMP2 and cyclopamine (F), or cyclopamine (G). Limbs in A,B,E-G were derived from wild-type embryos and limbs in C,D from *ColII/Ihh* embryos. Sections were hybridized with a riboprobe for *PTHrP*. (A,B) Treatment of wild-type limbs with Noggin results in a reduction of *PTHrP* expression (B, arrow) compared with untreated control limbs (A). (C,D) High expression of *PTHrP* in limbs of *ColII/Ihh* embryos (D) is not reduced after Noggin treatment (C). (E) BMP2-treated limbs show normal expression of *PTHrP*. (F,G) Cyclopamine treatment results in a block of *PTHrP* expression (G), which cannot be rescued by BMP2 in double treated cultures (F). In all panels, ulna is upwards and radius is downwards. cycl, cyclopamine; Nog, Noggin.

Ihh and BMP independently regulate chondrocyte proliferation

In addition to the role of Ihh in regulating chondrocyte differentiation, the *Ihh* knockout phenotype revealed a role for Ihh in regulating chondrocyte proliferation (St-Jacques et al., 1999; Karp et al., 2000). The different lengths of the cartilage elements in BMP- and Noggin-treated cultures indicates that BMP signaling also regulates chondrocyte proliferation. To investigate this hypothesis, we labeled limbs in culture with BrdU and detected proliferating cells by antibody staining. After treatment with BMP2, the zones of proliferating chondrocytes were increased, whereas Noggin treatment results in a block of chondrocyte proliferation (Fig. 7A,B,E). Limbs of *ColII/Ihh* embryos show a high level of chondrocyte proliferation, demonstrating a continuous role of Ihh in regulating this process

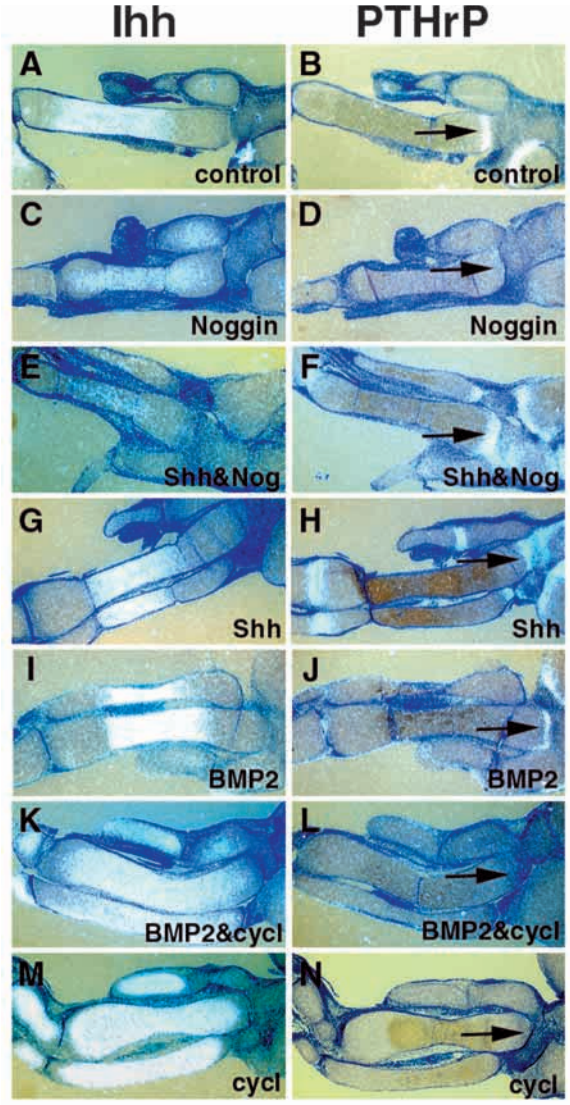

Fig. 6. Interaction of BMP and Ihh signaling in chick embryos. Wings of HH32 chick embryos were cultured for 2 days in control medium (A,B) or medium supplemented with Noggin (C,D), Shh and Noggin (E,F), Shh (G,H), BMP2 (I,J), BMP2 and cyclopamine (K,L) or cyclopamine (M,N). Parallel sections were hybridized with riboprobes for chick *Ihh* (A,C,E,G,I,K,M) or chick *PTHrP* (B,D,F,H,J,L,N). Noggin treatment results in small cartilage elements that show a reduced level of *Ihh* (C) and *PTHrP* (D) expression compared with untreated cultures (A,B). Treatment with Shh leads to a delay in hypertrophic differentiation as seen by the smaller domain of *Ihh* expression and the increased distance between the *Ihh* expression domain and the joint region (G) if compared with untreated explants (A). Shh-treated limbs show high expression of *PTHrP* in periarticular chondrocytes (H). Co-treatment of limbs with Shh and Noggin leads to a further reduction of *Ihh* expression (E) and does not block the expression of *PTHrP* (F). BMP2-treated limbs show high expression of *Ihh* (I) and *PTHrP* (J). Treatment with cyclopamine results in an advanced onset of hypertrophic differentiation, as seen by the expanded domain of *Ihh* expression and reduced distance between the *Ihh* expression domain and the joint region (M) compared with control limbs (A). Furthermore, *PTHrP* expression is blocked by cyclopamine treatment (N). Both effects cannot be rescued by BMP2 in explants co-treated with BMP2 and cyclopamine (K,L). Arrows point to the *PTHrP* expression domain. All panels display sections through metatarsals. cycl, cyclopamine; Nog, Noggin.

(Fig. 7D) (Long et al., 2001)). To reveal the epistatic relationship between the two signaling systems, we analyzed double treated limb explants. Like treatment of wild-type limbs, Noggin treatment of limbs that overexpress *Ihh* resulted in a block of chondrocyte proliferation (Fig. 7B-D). Therefore, Ihh does not act downstream of BMP signals. Similarly, after double treatment with cyclopamine and BMP2, chondrocyte proliferation is blocked as seen in cultures treated with cyclopamine alone (Fig. 7E-G). Thus, BMP2 cannot overcome the block of chondrocyte proliferation induced by loss of Ihh signaling and hence BMPs do not act downstream of Ihh. Taken together, these experiments indicate that the two signaling pathways act in parallel in regulating chondrocyte proliferation.

Terminal hypertrophic differentiation is negatively regulated by BMP signaling

As endochondral differentiation progresses, hypertrophic cells further differentiate into terminally hypertrophic chondrocytes, which produce mineralized matrix. At E14.5, the distance between the *ColX*-expressing hypertrophic zones in the center of the skeletal elements reflects the rate of terminal hypertrophic differentiation. Although after Noggin treatment the zones of *Ihh* and *ColX* expression are significantly decreased in size, we observed a distinct separation of the *ColX*-expressing hypertrophic domains (Fig. 1E,F,I,J, Fig. 8A,G). To ask if this could reflect an additional effect of BMP signaling on terminal hypertrophic differentiation, we hybridized Noggin-treated limbs with osteopontin (*Osp*), a gene expressed in terminally differentiated chondrocytes (Nakase et al., 1994). We found strong upregulation of *Osp* in these cultures relative to controls, indicating a role of BMP signaling in regulating the differentiation process itself (Fig. 8B,H). To analyze the role of BMP signaling during hypertrophic differentiation in relation to that of Ihh, we co-treated limb cultures with cyclopamine and Noggin. We detected a strong reduction of the *Ihh* expression level, which resembles that of Noggin-treated explants (data not shown). Most importantly we observed a reduced size of the *ColX* expression domains flanking a large region of strong *Osp* expression (Fig. 8E-H). By contrast, after cyclopamine treatment the expression of *Osp* resembles that of untreated cultures (Fig. 8B,D). Therefore, BMP signaling seems to negatively regulate terminal hypertrophic differentiation independent of Ihh signaling.

To confirm this result we analyzed limbs treated with a combination of Noggin and PTHrP. Treatment with PTHrP results in a delayed onset of hypertrophic differentiation as indicated by reduced expression of *ColX* and *Ihh*, and by a consequent enlargement of the distance between the *Ihh* expression domains and the ends of the skeletal elements. This block of expression can be recognized after 2 days (Fig. 8A,K) and is more obvious after 4 days in cultures (Fig. 4A-D). As expected, *Osp* is downregulated and only expressed in a small region in the center of the cartilage elements (Fig. 8L). In Noggin and PTHrP double treated cultures we found a strong reduction of *Ihh* and *ColX* expression already after 2 days and a complete loss of expression of both genes after 4 days of culture (Fig. 8I;

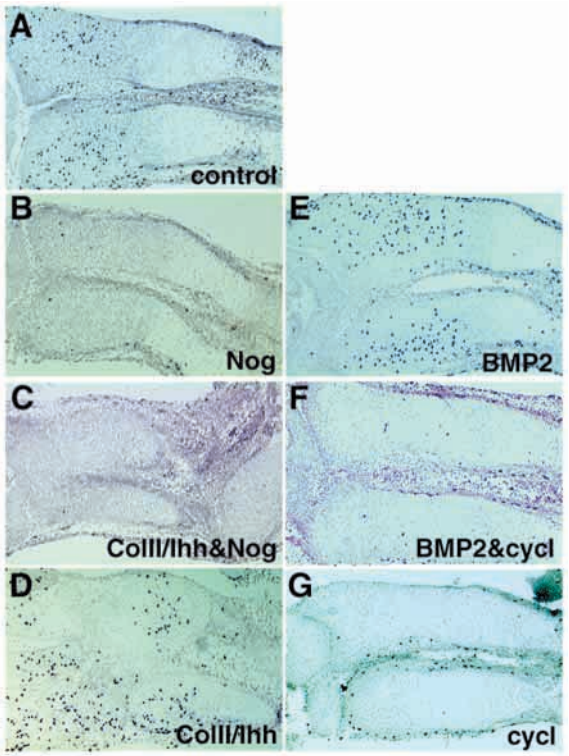

Fig. 7. BMP and Ihh signaling regulate chondrocyte proliferation in parallel pathways. Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A,D) or in medium supplemented with Noggin (B,C), BMP2 (E), BMP2 and cyclopamine (F) or cyclopamine (G). Limbs in A,B,E-G were derived from wild-type mice, and limbs in C,D from ColII/Ihh embryos. Proliferating cells were labeled with BrdU and detected by antibody staining. (A,B) Noggin treatment results in a block of chondrocyte proliferation (B) compared with untreated limbs (A). Explants from ColII/Ihh embryos show a high level of chondrocyte proliferation (D), which is blocked after Noggin treatment (C). (E-G) BMP2 treatment results in an increased zone of proliferating cells (E) but cannot overcome the cyclopamine-induced block of chondrocyte proliferation (G) in explants double treated with BMP2 and cyclopamine (F). In all panels radius is upwards and ulna is downwards. cycl, cyclopamine; Nog, Noggin.

data not shown). Nevertheless, *Osp* was upregulated in the center of the skeletal elements (Fig. 8J). Such a phenotype would be expected if the onset of hypertrophic differentiation is blocked by PTHrP and simultaneously, hypertrophic cells present at the beginning of the culture undergo accelerated differentiation into terminally hypertrophic chondrocytes in response to Noggin treatment. Together these results strongly suggest that the process of terminal hypertrophic differentiation is negatively regulated by BMP signaling independent of the Ihh/PTHrP system.

DISCUSSION

For this study, we have used a culture system of embryonic mouse and chick limbs to analyze the role of BMP signaling during the early stages of endochondral ossification. We found that BMP signals act at various steps during this process, regulating chondrocyte proliferation, *Ihh* expression and the process of terminal hypertrophic differentiation. Ihh signaling on the other hand has been shown to regulate chondrocyte proliferation and the onset of hypertrophic differentiation (Vortkamp et al., 1996; St-Jacques et al., 1999; Long et al., 2001). As the two signaling systems act at distinct steps of the process of chondrocyte development, they do not interact in a true epistatic relation. However, Ihh and BMP signaling each reciprocally induce the expression of signaling factors from the other pathway, thereby coordinating the regulation of different steps of the differentiation process.

BMP signals do not act as secondary signals of Ihh in regulating the onset of hypertrophic differentiation

It has previously been demonstrated that two secreted factors, Ihh from the prehypertrophic chondrocytes and PTHrP from the periarticular region, interact in a negative feedback loop to regulate the onset of hypertrophic differentiation (Vortkamp et al., 1996; St-Jacques et al., 1999; Karp et al., 2000). These interactions require the transport of both signals through the growth plate. PTHrP has been shown to travel through the growth plate. In addition direct signaling through PP-R is necessary to block hypertrophic differentiation of chondrocytes (Tsukazaki et al., 1995; Lee et al., 1996; Chung et al., 1998; Chung et al., 2001). Ihh was recently demonstrated to travel over several cell diameters in the developing endochondral skeleton (Gritli-Linde et al., 2001). However patched (*Ptc*; *Ptch* – Mouse Genome Informatics), a direct target of Hedgehog signaling (Marigo et al., 1996), is not upregulated in the periarticular region, suggesting that Ihh does not directly regulate *PTHrP* expression. As misexpression of constitutively activated *BmpR-IA* in the developing cartilage of chick embryos results in a block of hypertrophic differentiation and an increase in *PTHrP* expression it has been hypothesized that BMPs act downstream of Ihh in mediating the induction of *PTHrP* expression (Zou et al., 1997).

To test this hypothesis, we treated limb explants of E14.5 or E16.5 mouse embryos with combinations of inducers (BMP2, Ihh and PTHrP) and inhibitors (Noggin, cyclopamine) of the two signaling pathways. We could not detect a direct epistatic interaction between the two signaling systems in regulating the onset of hypertrophic differentiation. Treatment with Noggin, a potent inhibitor of BMP signaling (Zimmerman et al., 1996; Smith, 1999), could not antagonize the Ihh-induced delay of hypertrophic differentiation or the upregulation of *PTHrP* in limb cultures of mice overexpressing *Ihh* under the *ColII* promoter. Correspondingly, BMP2 could not rescue the cyclopamine-induced advanced onset of hypertrophic differentiation or induce the expression of *PTHrP*, which is lost by blocking the Ihh signaling pathway. We can therefore conclude that BMP signaling, at least that of BMP2, does not act downstream of Ihh to regulate *PTHrP* expression and the onset of hypertrophic differentiation. Other members of the BMP family such as BMP7, which is expressed in the proliferating chondrocytes between *Ihh* and *PTHrP*, might serve as mediators of the Ihh signal. However, as BMPs are members of a highly conserved gene family and can substitute for each other in many assays (Monsoro-Burq et al., 1996; Kawabata et al., 1998), it is not likely that a close relative of BMP2, like BMP7, acts as the proposed secondary signal. Further studies will be necessary to test if other growth factors, regulated by Ihh, can function to propagate the Ihh signal or if

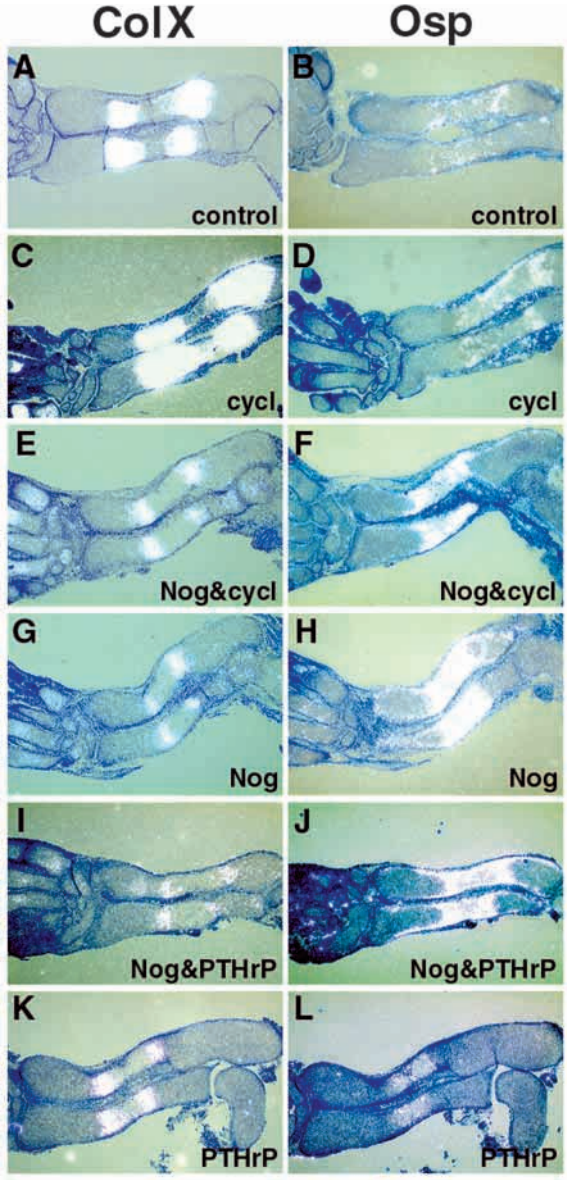

Fig. 8. BMP signaling delays terminal hypertrophic differentiation independent of the Ihh/PTHrP system. Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A,B) or in medium supplemented with cyclopamine (C,D), Noggin and cyclopamine (E,F), Noggin (G,H), Noggin and PTHrP (I,J) or PTHrP (K,L). Serial sections were hybridized with riboprobes for *ColX* (A,C,E,G,I,K) or *Osp* (B,D,F,H,J,L). (A-D) Cyclopamine treated limbs show increased expression of *ColX* (C) but normal expression of the marker for terminally differentiated cells, *Osp* (D) compared with untreated limbs (A,B). (E-H) Noggin treatment results in reduced expression of *ColX* (G) and significantly increased expression of *Osp* (H), similar to co-treatment with Noggin and cyclopamine (E,F). (I-L) Limbs treated with PTHrP show reduced expression of *ColX* (K), which is further reduced in explants double treated with Noggin and PTHrP (I). The low expression of *Osp* after PTHrP treatment (L) is increased in limbs co-treated with Noggin and PTHrP (J). In all panels, ulna is upwards and radius is downwards. cycl, cyclopamine; Nog, Noggin.

Ihh itself can reach the joint region to directly regulate *PTHrP* expression.

Because the experiments by Zou et al., showing *PTHrP* regulation by an activated BMP receptor, were carried out in chick embryos (Zou et al., 1997), it was important to confirm that our results did not reflect a species-specific difference between chick and mouse bone development. We therefore repeated the culture experiments using limb explants of stage HH 32 chick embryos. Similar to the mouse experiments, we did not find evidence for BMP signaling acting downstream of Ihh in regulating *PTHrP* expression and hypertrophic differentiation. After overexpression of constitutively activated *BmpR-IA*, the infected chicken limbs were analyzed at comparable stages with our study. However, the virus was injected at stage HH14 into the presumptive limb field, leading to the activation of BMP signaling before the onset of hypertrophic differentiation (Zou et al., 1997). Our studies using the limb culture system allowed us to activate BMP signaling specifically at later stages of chondrocyte development after the establishment of the different regions of the growth plate is completed. Therefore the effect seen in the studies by Zou et al. (Zou et al., 1997) might detect a different role for BMP signals at earlier stages of skeletal development. Continuous activation of BMP signaling might keep all chondrocytes in a proliferating state, thereby preventing the initiation of the hypertrophic differentiation process.

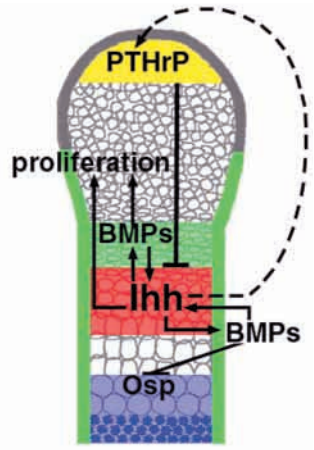

Fig. 9. Interaction of BMP and Ihh signaling. During endochondral ossification *Ihh* is expressed in the differentiating chondrocytes (red). Ihh induces the expression of *PTHrP* in periarticular chondrocytes (yellow) independently of BMP signaling. The range of PTHrP signaling keeps chondrocytes in a proliferating state and determines the distance from the joint region at which chondrocytes can undergo hypertrophic differentiation. Ihh in addition regulates the expression of Bmp genes in the perichondrium/periosteum and in part of the proliferating chondrocytes (green). BMP and Ihh signaling together upregulate chondrocyte proliferation, thereby pushing cells out of the range of PTHrP signaling. These chondrocytes are released from the block of differentiation and upregulate *Ihh* expression upon BMP signaling. BMP signaling furthermore negatively regulates the development of terminally differentiated hypertrophic chondrocytes (blue). By regulating chondrocyte proliferation, *Ihh* expression and terminal hypertrophic differentiation, BMP signaling integrates the different steps of endochondral ossification.

Stage-specific analysis of Ihh signaling

The organ culture system used in this study allows us to analyze stage-specific functions of signaling factors. As targeted deletion of *Ihh* disrupts cartilage development at the initial proliferation stage, the normal pattern of the various zones of chondrocyte differentiation in the growth plate is never established properly (St-Jacques et al., 1999). The interaction of signaling systems regulating the later stages of chondrocyte differentiation is thus difficult to address in these mice. We have mimicked the loss of Ihh by treating limb explants with cyclopamine, which is a potent inhibitor of the

Hedgehog signaling pathway (Incardona et al., 1998). As in *Ihh*$^{-/-}$ mice, cyclopamine treatment leads to reduced expression of the Hedgehog receptor *Ptc* (St-Jacques et al., 1999) (H. M. W. and A. V., unpublished) and to a block in *PTHrP* expression. Furthermore, the effect of cyclopamine on chondrocyte differentiation can be rescued by PTHrP, confirming that the cyclopamine-induced phenotype is a specific consequence of the loss of Ihh signaling. Cyclopamine treatment therefore allowed us to address the interaction of Ihh with that of BMP signaling during defined stages of chondrocyte differentiation.

BMP signaling regulates several aspects of chondrocyte development

We have identified several Ihh-independent functions of BMP signaling. One of these is to regulate the process of terminal hypertrophic differentiation. Blocking of BMP signaling by Noggin results in an increased number of *Osp*-expressing, terminal hypertrophic cells. By contrast, cyclopamine treatment, which leads to an advanced onset of hypertrophic differentiation, does not increase *Osp* expression. Double treatment experiments support the idea that BMP and Ihh/PTHrP signaling control different aspects of hypertrophic differentiation: the Ihh/PTHrP system regulates the onset of hypertrophic differentiation, whereas BMP signaling controls the pace of the differentiation process itself. The enlargement of the hypertrophic region seen in similar experiments treating cultures of mouse metatarsals with BMP2 (De Luca et al., 2001) might thus reflect the ability of BMP2 to slow down the differentiation process in addition to increasing the number of cells undergoing hypertrophic differentiation.

We and others have shown that Ihh and BMP signaling regulate chondrocyte proliferation (St-Jacques et al., 1999; Karp et al., 2000; De Luca et al., 2001; Long et al., 2001). Treatment of *ColII/Ihh* mice with Noggin or co-treatment of wild-type limbs with BMP2 and cyclopamine revealed that the two signals are necessary in concert for proper chondrocyte proliferation. Blocking either pathway results in inhibition or severe reduction of chondrocyte proliferation, demonstrating that the two signaling system act in parallel.

A third function of BMP signaling identified in this study is the regulation of *Ihh* expression. After treatment with only BMP2 we observed a slight enlargement of the *Ihh* expression domain that was proportional to the expansion of the region of proliferating chondrocytes. Correspondingly, treatment with Noggin results in reduced *Ihh* expression. Double treatment with cyclopamine and BMP2 leads to a greater increase in *Ihh* expression than does treatment with cyclopamine alone, whereas double treatment of cyclopamine and Noggin reduces the cyclopamine-induced upregulation of *Ihh*. These experiments demonstrate that *Ihh* expression is regulated by BMP signaling. In this respect it is interesting to note that in the presence of cyclopamine BMP2 induces *Ihh* expression closer to the periarticular region as treatment with cyclopamine alone. One possible way to interpret this result is that BMPs upregulate the expression of *Ihh* in absence of the Ihh/PTHrP pathway. This could furthermore mean that PTHrP not only blocks chondrocyte differentiation but simultaneously determines the competence of chondrocytes to react to BMP signals with the upregulation of *Ihh* expression. Whether the induction of *Ihh* expression is a direct consequence of BMPs regulating *Ihh* expression on a cellular level or if it is a secondary consequence of BMPs regulating chondrocyte differentiation remains to be addressed in future experiments.

Another interesting aspect of our results is the finding that BMP signaling integrates chondrocyte proliferation and differentiation during bone development. As the distance between the joint region and the onset of hypertrophic differentiation is determined by the Ihh/PTHrP system, one could expect that upregulation of chondrocyte proliferation does not increase this distance and that instead chondrocytes undergo accelerated differentiation. However, treatment of explants with BMP2 protein results in an enlargement of both, the zone of proliferating and the region of hypertrophic chondrocytes. The negative effect on the onset of hypertrophic differentiation seems to be mediated by the upregulation of *Ihh* expression by BMP signaling. Furthermore, as discussed above, BMP signals delay the hypertrophic differentiation process itself. By acting at several stages of chondrocyte differentiation BMP signaling seems to regulate the size of a skeletal element without disturbing the differentiation process relative to developmental age. A slight increase of BMP signaling would thus result in slightly larger bones, which would however develop at an unaltered pace. Similarly, a slight decrease in BMP signaling would produce the reverse effect leading to shorter bones, also without a change in the rate of development. Such an effect can be seen in several of the *Bmp* mutant mice like the short ear mutant (*Bmp5*) (King et al., 1994) or mice carrying a targeted disruption of *Bmp6* or *Bmp7* (Dudley et al., 1995; Luo et al., 1995; Solloway et al., 1998), which display mild skeletal phenotypes, including shorter bones, without severe disturbance of the overall differentiation process.

The Bmp gene family

Analysis of mutations in single Bmp genes has not given significant insight into their role during skeletal differentiation. Targeted disruption of either *Bmp2* or *Bmp4* leads to early embryonic lethality (Winnier et al., 1995; Zhang and Bradley, 1996), whereas mutations in *Bmp5*, *Bmp6* or *Bmp7* only display mild skeletal phenotypes (Dudley et al., 1995; King et al., 1994; Kingsley et al., 1992; Luo et al., 1995; Solloway et al., 1998), indicating a highly redundant role of Bmp genes in regulating bone development. In this study we have attempted to analyze the role of BMP signaling during chondrocyte maturation without differentiating between individual Bmp genes. As BMP proteins can substitute for each other in experimental systems, we have used BMP2 protein to mimic the role of several BMPs. Similarly, Noggin has been shown to inhibit signaling of various members of the BMP family (Zimmerman et al., 1996; Kawabata et al., 1998).

Several Bmp genes are expressed in specific regions of the developing cartilage elements and might thus be important for different aspects of chondrocyte differentiation during normal development. *Bmp7* is expressed in the proliferating chondrocytes distal to *Ihh* (Haaijman et al., 2000; Solloway et al., 1998) and may be responsible for regulating chondrocyte proliferation and *Ihh* expression. Other Bmps, including *Bmp2*, *Bmp3*, *Bmp4* and *Bmp7*, are expressed in the perichondrium/periosteum (Pathi et al., 1999; Zou et al., 1997; Daluiski et al., 2001; Haaijman et al., 2000) (data shown in this manuscript),

and may signal back to adjacent chondrocytes to regulate chondrocyte proliferation, chondrocyte differentiation or *Ihh* expression. *Bmp6* is expressed in the prehypertrophic and hypertrophic chondrocytes (Vortkamp et al., 1996; Solloway et al., 1998) and is a good candidate to regulate the process of hypertrophic differentiation.

However, owing to the proposed redundancy of BMP function, it is likely that for some of the processes addressed in this paper, the overall level of BMP signaling is more critical than the signal from a single factor. BMPs signal through heterodimers of BMP receptors type I and type II, which in turn activate members of the Smad family of transcription factors. Smad1, Smad5 and Smad8 have been shown to act downstream of BMP receptors. Upon activation, these Smad proteins can form heterodimers with either Smad4 or Smad6 to direct transcription of target genes (Massague, 2000; ten Dijke et al., 2000). To fully understand the role of Bmp genes during bone development, it will be necessary to analyze how combinations of BMP signals are interpreted by BMP receptors and Smad activation pathways.

Interaction of Ihh and BMP signals

Our results, together with results from previous studies, suggest the following model for the regulation of chondrogenesis by the Ihh/PTHrP and BMP signaling pathways (Fig. 9). Ihh produced by prehypertrophic chondrocytes promotes proliferation of the adjacent chondrocytes and, in addition, induces the expression of several Bmp genes in the perichondrium and in the proliferating chondrocytes. Ihh, furthermore, induces the expression of *PTHrP* in the periarticular region. PTHrP, in turn, negatively regulates the onset of hypertrophic differentiation. The range of PTHrP activity determines the distance from the joint region at which chondrocytes initiate the hypertrophic differentiation program and thereby the size of the domain of chondrocytes that are competent to proliferate. Ihh and BMP signaling together regulate the level of chondrocyte proliferation thereby pushing some cells out of the PTHrP signaling range. These cells are then released from the block of hypertrophic differentiation and activate the expression of *Ihh*, which, as discussed above, might be directly or indirectly regulated by BMP signaling. As Ihh signaling regulates the expression of both, Bmp genes and *PTHrP*, it tightly controls its own activation.

Furthermore, BMP signaling negatively regulates the differentiation of terminal hypertrophic chondrocytes. This delay in terminal hypertrophic differentiation might be necessary for chondrocytes to acquire the enlarged size of hypertrophic cells and to undergo the accompanying changes in gene expression and matrix composition. By regulating chondrocyte proliferation, *Ihh* expression and the pace of hypertrophic differentiation, BMP signaling might thus keep the overall pace of cartilage development in phase.

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REFERENCES

Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman, D. and Karaplis, A. C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol*. **126**, 1611-1623.

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.

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

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

Cooper, M. K., Porter, J. A., Young, K. E. and Beachy, P. A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607.

Daluiski, A., Engstrand, T., Bahamonde, M. E., Gamer, L. W., Agius, E., Stevenson, S. L., Cox, K., Rosen, V. and Lyons, K. M. (2001). Bone morphogenetic protein-3 is a negative regulator of bone density. *Nat. Genet.* **27**, 84-88.

De Luca, F., Barnes, K. M., Uyeda, J. A., De-Levi, S., Abad, V., Palese, T., Mericq, V. and Baron, J. (2001). Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. *Endocrinology* **142**, 430-436.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Erlebacher, A., Filvaroff, E. H., Gitelman, S. E. and Derynck, R. (1995). Toward a molecular understanding of skeletal development. *Cell* **80**, 371-378.

Gritli-Linde, A., Lewis, P., McMahon, A. P. and Linde, A. (2001). The whereabouts of morphogen: Direct evidence for short-and graded long-range activity of Hedgehog signaling peptides. *Dev. Biol.* **236**, 364-386.

Haaijman, A., Burger, E. H., Goei, S. W., Nelles, L., ten Dijke, P., Huylebroeck, D. and Bronckers, A. L. (2000). Correlation between ALK-6 (BMPR-IB) distribution and responsiveness to osteogenic protein-1 (BMP-7) in embryonic mouse bone rudiments. *Growth Factors* **17**, 177-192.

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

Hinchcliffe, J. R. and Johnson, D. R. (1980). *The Development of the Vertebrate Limb*. New York: Oxford University Press.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Holley, S., Neul, J., Attisano, L., Wrana, J., Sasai, Y., O'Connor, M., De Robertis, E. and Ferguson, E. (1996). The Xenopus dorsalizing factor noggin ventralizes Drosophila embryos by preventing DPP from activating its receptor. *Cell* **86**, 607-617.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Jacenko, O., LuValle, P. A. and Olsen, B. R. (1993). Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to-bone transition. *Nature* **365**, 56-61.

Karaplis, A. C., Yasuda, T., Hendy, G. N., Goltzman, D. and Banville, D. (1990). Gene-encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue. *Mol. Endocrinol.* **4**, 441-446.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

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.

Kawabata, M., Imamura, T. and Miyazono, K. (1998). Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* **9**, 49-61.

Kim, I. S., Otto, F., Zabel, B. and Mundlos, S. (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech. Dev.* **80**, 159-170.

King, J. A., Marker, P. C., Seung, K. J. and Kingsley, D. M. (1994). BMP5

and the molecular, skeletal, and soft-tissue alterations in short ear mice. *Dev. Biol.* **166**, 112-122.

Kingsley, D. M. (1994a). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133-146.

Kingsley, D. M. (1994b). What do BMPs do in mammals? Clues from the mouse short-ear mutation. *Trends Genet.* **10**, 16-21.

Kingsley, D. M., Bland, A. E., Grubber, J. M., Marker, P. C., Russell, L. B., Copeland, N. G. and Jenkins, N. A. (1992). The mouse short ear skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF beta superfamily. *Cell* **71**, 399-410.

Kohno, K., Martin, G. R. and Yamada, Y. (1984). Isolation and characterization of a cDNA clone for the amino-terminal portion of the pro-alpha 1(II) chain of cartilage collagen. *J. Biol. Chem.* **259**, 13668-13673.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

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

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

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., Kronenberg, H. M. and Segre, G. V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology* **137**, 5109-5118.

Long, F., Zhang, X., Karp, S. and McMahon, A. (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* (in press).

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A. and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124**, 1109-1117.

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J. (1996). Conservation on hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233.

Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.

Massague, J. (2000). How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169-178.

Monsoro-Burq, A. H., Duprez, D., Watanabe, Y., Bontoux, M., Vincent, C., Brickell, P. and Le Douarin, N. (1996). The role of bone morphogenetic proteins in vertebral development. *Development* **122**, 3607-3616.

Nakase, T., Takaoka, K., Hirakawa, K., Hirota, S., Takemura, T., Onoue, H., Takebayashi, K., Kitamura, Y. and Nomura, S. (1994). Alterations in the expression of osteonectin, osteopontin and osteocalcin mRNAs during the development of skeletal tissues in vivo. *Bone Miner. Res.* **26**, 109-122.

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., Kruse, K. and Juppner, H. (1995). A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science* **268**, 98-100.

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.

Smith, W. C. (1999). TGF beta inhibitors. New and unexpected requirements in vertebrate development. *Trends Genet.* **15**, 3-5.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.

Smith, W. C., Knecht, A. K., Wu, M. and Harland, R. M. (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing Xenopus mesoderm. *Nature* **361**, 547-549.

Solloway, M. J., Dudley, A. T., Bikoff, E. K., Lyons, K. M., Hogan, B. L. and Robertson, E. J. (1998). Mice lacking Bmp6 function. *Dev. Genet.* **22**, 321-339.

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.

Taipale, J., Chen, J. K., Cooper, M. K., Wang, B., Mann, R. K., Milenkovic, L., Scott, M. P. and Beachy, P. A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406**, 1005-1009.

ten Dijke, P., Miyazono, K. and Heldin, C. H. (2000). Signaling inputs converge on nuclear effectors in TGF-beta signaling. *Trends Biochem. Sci.* **25**, 64-70.

Thiede, M. A. and Rutledge, S. J. (1990). Nucleotide sequence of a parathyroid hormone-related peptide expressed by the 10 day chicken embryo. *Nucleic Acids Res.* **18**, 3062.

Tsukazaki, T., Ohtsuru, A., Enomoto, H., Yano, H., Motomura, K., Ito, M., Namba, H., Iwasaki, K. and Yamashita, S. (1995). Expression of parathyroid hormone-related protein in rat articular cartilage. *Calcif. Tissue Int.* **57**, 196-200.

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.

Vortkamp, A., Pathi, S., Peretti, G. M., Caruso, E. M., Zaleske, D. J. and Tabin, C. J. (1998). Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mech. Dev.* **71**, 65-76.

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.

Winnier, G., Blessing, M., Labosky, P. A. and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9**, 2105-2116.

Yi, S. E., Daluiski, A., Pederson, R., Rosen, V. and Lyons, K. M. (2000). The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. *Development* **127**, 621-630.

Zhang, H. and Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* **122**, 2977-2986.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zou, H., Wieser, R., Massague, J. and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11**, 2191-2203.