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Original Article

BMP4 promotes chondrocyte proliferation and hypertrophy in the endochondral cranial base

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ABSTRACT Defects in the growth and development of the endochondral bones that comprise the cranial base contribute to several craniofacial dysmorphic syndromes. Since Bone Morphogenetic Protein (BMP) signaling regulates chondrocyte differentiation and endochondral ossification in developing long bones, we have tested the hypothesis that BMP signaling also participates in regulating development of the cranial base. During *in vivo* developmental progression of the cranial base in mice, a burst of skeletal growth and chondrocyte maturation was identified in the perinatal period. Using a novel serum-free organ culture system, cranial base structures were cultured as explants in the presence of BMP4 or noggin, and analyzed for morphological and molecular changes. Growth of perinatal cranial base explants was inhibited by treatment with noggin, a BMP inhibitor. Exogenous BMP4 promoted cartilage growth, matrix deposition and chondrocyte proliferation in a dose dependent manner. Correspondingly, expression level of the cartilage markers Sox9 and collagen type II were also increased. Alkaline phosphatase and collagen type X expression were up-regulated and expressed in ectopic hypertrophic chondrocytes after treatment of the cultures with 100 ng/ml BMP4 for seven days. This increase in chondrocyte hypertrophy was accompanied by increased indian hedgehog (Ihh) and parathyroid hormone/parathyroid hormone related peptide (PTH/PTHrP) receptor (PPR) expression, but not increased PTHrP expression. We conclude that endogenous BMPs are required to maintain cartilage growth, and exogenous BMP4 can enhance cartilage maturation and induce ectopic chondrocyte hypertrophy in the cranial base. Therefore, appropriate levels of BMP signaling are important for normal cranial base development.

KEY WORDS: *bone morphogenetic proteins, cranial base, synchondrosis, endochondral ossification, chondrocyte hypertrophy*

Introduction

Congenital skeletal malformations occur in 2% of all live births and the majority of these involve the craniofacial complex. The craniofacial skeleton is composed of the neurocranium that surrounds the brain and the viscerocranium that encompasses the facial bones. The neurocranium is further subdivided into the cranial base (chondrocranium) and the calvaria, which develop by endochondral and intramembranous ossification, respectively. Defects in the growth and development of the cranial base contribute to the abnormal phenotype of several human malformation syndromes including Apert (MIM 101200), Crouzon (MIM 123500), achondroplasia (MIM 100800), Seckel (MIM 210600), cleidocranial dysplasia (MIM 119600), and mandibulofacial dysostosis (MIM 154500) (Stewart *et al.,* 1977; Horowitz, 1981; Peterson-Falzone and Figueroa, 1989; Jensen and Kreiborg, 1993; Kjaer *et al.,* 2001). Furthermore, because of the interaction of the cranial base with other skeletal and soft tissue structures, defects in the growth and development of structures such as the calvarial sutures can cause malformations in the cranial base (Mooney *et al.,* 1993; Stelnicki *et al.,* 1998; Perlyn *et al.,* 2001).

Abbreviations used in this paper: BMP, bone morphogenetic protein; Ihh, Indian hedgehog; PPR, parathyroid hormone / parathyroid hormone related peptide receptor; PTH, parathyroid hormone; PTHrP, parathyroid hormone related peptide; SFM, serum free medium.

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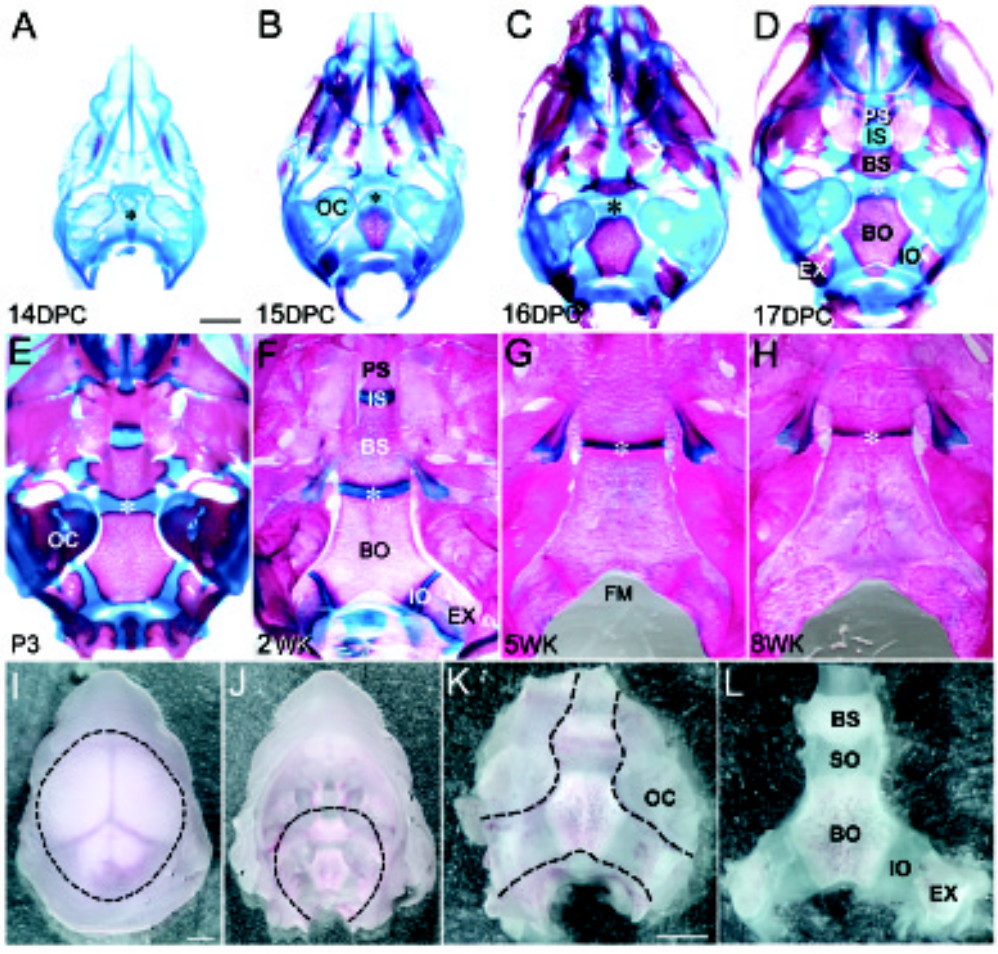

Fig. 1. *In vivo* development of the mouse cranial base and isolation for culture. *Mouse cranial bases were stained with alcian blue for cartilage and alizarin red for calcified cartilage and bone at 14 dpc* **(A)**, *15 dpc* **(B)**, *16 dpc* **(C)**, *17 dpc* **(D)**, *P3* **(E)**, *2 week* **(F)**, *5 week* **(G)** *and 8 week* **(H)** *stages of development. The spheno-occipital synchondrosis in each panel was marked with an asterisk. In preparation for culture, the calvaria was removed along the dashed line and to the foramen magnum* **(I)**. *Brain and associated tissues were removed to expose the cranial base* **(J)**. *The posterior portion of the cranial base was isolated along the dashed lines and further trimmed to remove the otic capsules (OC) and other connective tissues* **(K)**. *The cranial base consisting of the basisphenoidal (BS), basioccipital (BO), and exoccipital bones (EX), with intercalating spheno-occipital (SO) and intraoccipital (IO) synchondroses, was explanted into culture* **(L)**. *PS, presphenoid; FM, foramen magnum; IS, intrasphenoidal synchondrosis. Scale bars in A for A-H, in I for I-J, and in K for K-L represent 1 mm.*

Normal development of the cranial base requires the coordinated growth and maturation of multiple skeletal elements. The embryonic cranial base is a cartilaginous structure formed by the fusion of the parachordal plates around the notochord. During the fetal period, ossification centers form within the cartilage, and the segments separating these centers are referred to as synchondroses. In the postnatal period, endochondral ossification of the synchondroses contributes to the expansion of the ossification centers and growth of the cranial base. Therefore the synchondroses are analogous structures to the growth plates of long bones in that they allow for the rapid endochondral growth of the bone and eventually close when the bone approaches its final size. Also similar to growth plates, chondrocytes of the synchondroses are distributed into the reserve, proliferating and hypertrophic zones. However, in the synchondroses, two proliferating and hypertrophic zones flank a central reserve zone. In humans, the anterior intraoccipital synchondroses begin fusing by 1-3 years of age and the spheno-occipital synchondrosis fuses at about puberty (Ingervall and Thilander, 1972; Madeline and Elster, 1995). Reduced growth of synchondroses, as seen in Apert Syndrome, can cause hypoplasia of the cranial base and associated craniofacial malformations.

The growth and development of cartilage is regulated by cell-cell and cell-matrix interactions, biomechanical stimuli and multiple growth and differentiation factors including bone morphogenetic proteins (BMPs) (for reviews, see (Erlebacher *et al.,* 1995; Shum and Nuckolls, 2002). BMPs were first coined due to their function in demineralized bone matrix that induced endochondral bone formation when injected into muscular sites (Urist, 1965). Since then, a large number of related molecules have been identified and the transforming growth factor beta superfamily, which includes the BMPs, was formed (Ducy and Karsenty, 2000). BMPs regulate chondrocyte cell fate determination, differentiation, proliferation, maturation, hypertrophy and apoptosis (Ducy and Karsenty, 2000; Hoffmann and Gross, 2001; Pizette and Niswander, 2001). The binding of soluble and extracellular matrix associated BMPs activates the serine/threonine kinase activity of their transmembrane receptors (Miyazono *et al.,* 2001). The intracellular signaling cascade in chondrogenic cells leads to increased expression of Sox9, a key transcription factor in the regulation of chondrocyte specific genes (de Crombrugghe *et al.,* 2000). Noggin, a developmentally relevant BMP binding protein that interferes with receptor activation, has become a useful experimental tool for testing the significance of endogenous BMPs in developmental phenomena (Pizette and Niswander, 2001; Reddi, 2001).

Chondrocyte hypertrophy, the final stage of chondrocyte differentiation in preparation for bone formation, is associated with alkaline phosphatase (AP) expression and a shift from collagen type II (Col2) to collagen type X (Col10) expression. Chondrocyte proliferation is maintained and hypertrophic differentiation is inhibited by indian hedgehog (Ihh) and parathyroid hormone related peptide (PTHrP) (Lanske *et al.,* 1996; Vortkamp *et al.,* 1996; Karp *et al.,* 2000).

Organ culture of explants of the cranial base synchondroses have been used previously to investigate the determinants of cranial base development (Gakunga *et al.,* 2000). We have modified this culture system to analyze the functions of BMP4 in the regulation of cranial base development. We demonstrated that noggin inhibited cartilage growth and BMP4 elicited dose-dependent increases in cartilage growth, chondrocyte proliferation and chondrocyte hypertrophy in

the cranial base. By analyzing gene expression of cranial base chondrocytes over a time course of BMP4 treatment, we found that early effects on chondrocyte proliferation were not associated with increased PTHrP or Ihh expression. However, prolonged effects of BMP4 resulted in the expansion of hypertrophic cartilage zone and ectopic hypertrophy that were associated with up-regulated expression of alkaline phosphatase, Col10, Ihh and PTH/PTHrP receptor (PPR). Furthermore, this culture system provides the opportunity for *in vitro* studies of cranial base development, and allows for further investigation of the similarities and differences in the molecular mechanisms regulating endochondral ossification in the growth plates and synchondroses.

Results

The cranial base develops from endochondral ossification

The cranial base develops from a cartilaginous template that is replaced by bone through the process of endochondral ossification (Fig. 1). The basioccipital ossification center was the first to form, starting at 14 days post coitum (dpc), followed by the paired exoccipitals, basisphenoid and finally the presphenoid formed between 16 dpc and 17 dpc (Fig. 1A-D). During the first week of postnatal development, there was accelerated growth and ossification of the cranial base and rapid ossification of the otic capsule (Fig. 1E and F). The intraoccipital synchondroses were completely ossified by 5 weeks of postnatal development whereas the intrasphenoidal and the spheno-occipital synchondroses retained a small amount of cartilage into adulthood (8 weeks) (Fig. 1G and H). Until 1 week, the cranial base grew in all dimensions, however after 1 week, growth was predominantly in the anterior-posterior dimension with little increase in width (Fig. 1F-H).

We have characterized an organ culture system to support cranial base growth and development in serum-free medium (SFM). Portions of the cranial base including the basisphenoid, basioccipital, and anterior portions of the exoccipital ossification centers with their intervening synchondroses (anterior intraoccipitals and spheno-occipital), along with the posterior half of the intrasphenoidal synchondroses were isolated (Fig. 1I-L) and cultured submerged in multiwell plates. Since culturing the explants submerged in multiwell plates or in a Trowell type system (Slavkin *et al.,* 2000) gave similar results (data not shown), we used the simpler submerged configuration for the rest of our experiments.

The cranial base explanted into serum-free culture approximates in vivo *development*

In order to determine what stage of development was most suitable for *in vitro* analysis, we dissected and cultured cranial bases from 14 and 17 dpc fetuses and from pups 3 days after birth (P3). The explants were cultured under various medium formulations. After fixation and whole-mount staining, morphometric analysis of cartilage and bone tissue growth was evaluated. The growth and development of the explants were compared to an *in vivo* series of development at 17 dpc, newborn and P3 (Fig. 2A-C). Cranial base explants from 14 dpc embryos tended to malform in the culture and there was little advance of chondrocyte hypertrophy (data not shown). Since the 17 dpc explants remained relatively flat and exhibited growth in cartilage and calcified tissue (Fig. 2D-I, M), they were best for analysis in culture. Explants isolated from P3 animals showed cartilage growth in culture (Fig. 2J-L, N) although advance of chondrocyte hypertrophy was more evident in the 17 dpc cultures (Fig. 5, 6). Explants cultured for seven or 12 days in SFM or medium supplemented with 10% fetal bovine serum (FBS) exhibited increases in cartilage and calcified tissue (Fig. 2). The SFM cultures exhibited a greater increase in the bone/cartilage ratio than did the FBS cultures, a change more similar to that occurring *in vivo* (Fig. 2M). In medium supplemented with insulin, transferrin, selenium and pyruvate (ITSP), cultures exhibited an abnormal expansion of cartilage tissue, unlike *in vivo* development

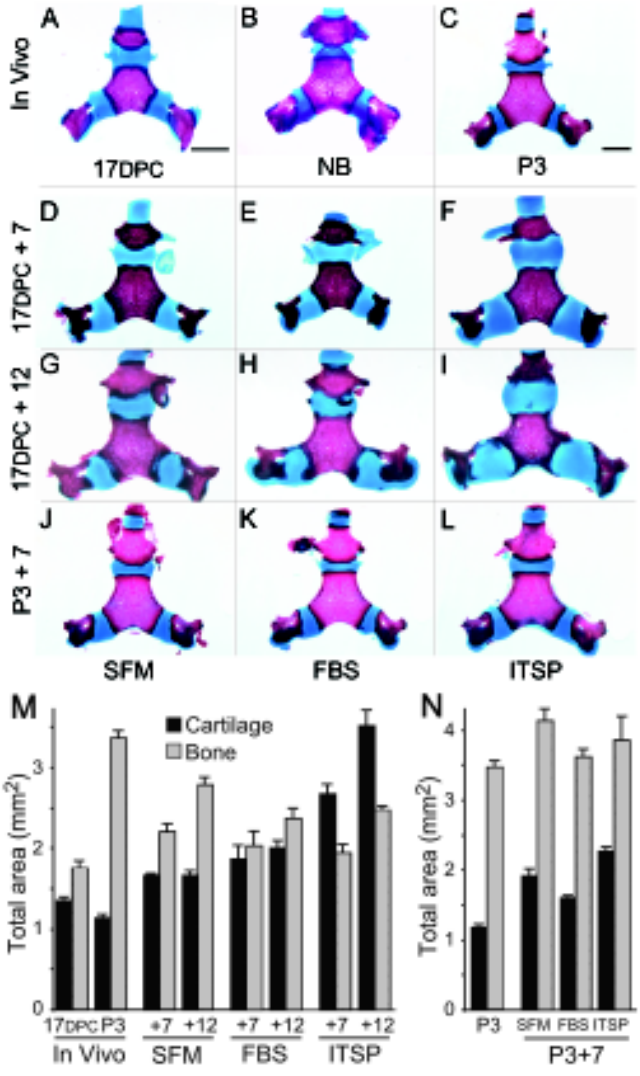

Fig. 2. Cranial base explants cultured under serum-free conditions approximated *in vivo* development. *Cranial bases were stained with alcian blue for cartilage and alizarin red for calcified cartilage and bone. 17 dpc* **(A, D-I)** *or P3* **(C, J-L)** *cranial bases were cultured in the absence of serum (SFM; D,G,J), or in 10% fetal calf serum (FBS; E,H,K), or in the presence of serum substitutes (ITSP; F,I,L) for seven (D-F, J-L) or 12 (G-I) days. The area of the basioccipital ossification (gray bars), and the aggregate area of cartilaginous synchondroses (black bars) including the spheno-occipital and intraoccipital synchondroses were determined for each group using morphometric analyses* **(M,N)***. The scale bar in A represents 1 mm and applies to all of the 17 dpc and newborn (NB) specimens (B, D-I). The scale bar in C represents 1 mm and applies to all of the P3 specimens (J-L).*

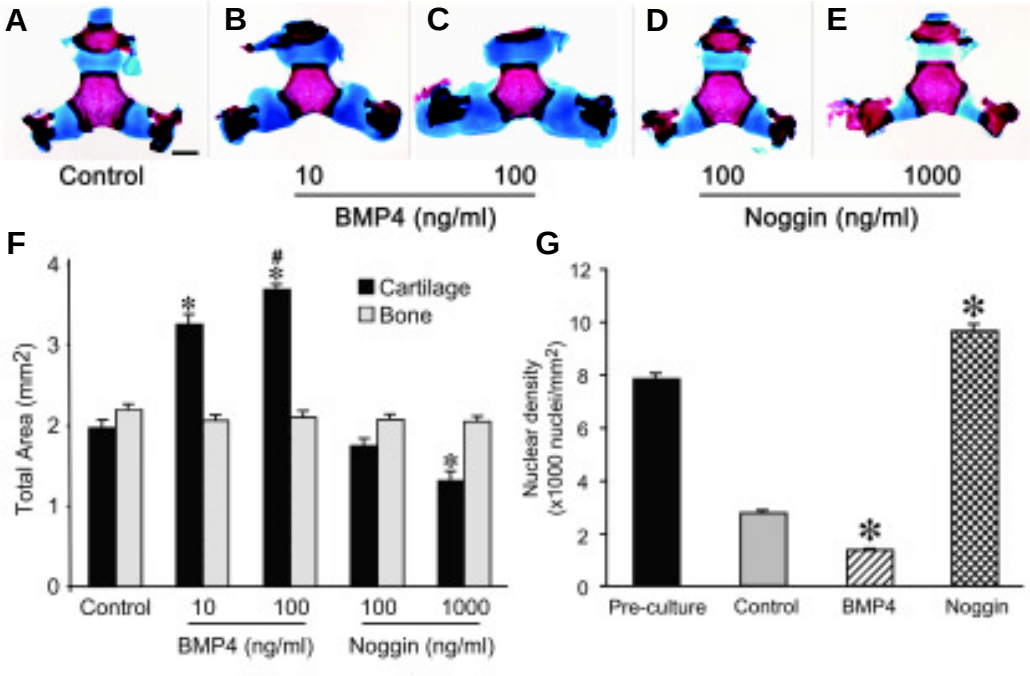

Fig. 3. BMP4 enhanced whereas noggin inhibited cartilage matrix accumulation. *Seventeen dpc stage cranial bases were cultured in SFM* **(A)**, *or exposed to 10* **(B)** *or 100 ng/ml* **(C)** *BMP4, or 100* **(D)** *or 1000 ng/ml* **(E)** *noggin. After seven days in culture, explants were stained with alcian blue for cartilage and alizarin red for calcified cartilage and bone. The area of the basioccipital ossification (gray bars), and the aggregate area of cartilaginous synchondroses (black bars) including the spheno-occipital and intraoccipital synchondroses were determined for each group using morphometric analyses* **(F)**. *Nuclear density measurements were obtained from sections stained with Hoechst for the nuclei for each group* **(G)**. **p<0.05 when compared with controls; #p<0.05 when compared with 10 ng/ml BMP4 dose.*

(Fig. 2M). We determined that SFM supported cartilage growth and development of cranial bases that mimicked *in vivo* development and it provided a more defined culture environment for subsequent experiments with growth factor supplements. By comparing the size of the basioccipital ossification center and the surrounding synchondroses at 17 dpc, 17 dpc plus 7 days of organ culture, and P3, we estimate that the 7-day culture period encompasses the extent of endochondral ossification that occurs in approximately two days of *in vivo* development.

BMP4 promotes whereas noggin inhibits cartilage growth in cranial base cultures

BMP4 is known to regulate cartilage and bone development at other sites in the skeleton, however the role of BMPs in cranial base development has not been investigated. Therefore, using exogenous BMP4 and noggin, we studied the function of BMP signaling in cranial base cultures. Treatment of 17 dpc cranial bases with BMP4 for seven days resulted in a dose-dependent increase in cartilage tissue (Fig. 3A-C, and F). Alcian blue stain was more intense, and the amount of cartilage tissue area increased by 50 and 70% at 10 and 100ng/ml BMP4, correspondingly ($p<0.05$). In contrast, noggin at 1μg/ml decreased the amount of cartilage and the intensity of alcian blue staining (Fig. 3E-F). Noggin at 10-fold lower dosage had only a modest effect (12% decrease, $p<0.05$) on cartilage growth (Fig. 3D and F). Interestingly, none of the treatment groups exhibited changes in bone development in comparison to control cultures.

From histological sections, we evaluated the number of chondrocytes per unit area (mm^2) in the reserve zone (center of the synchondrosis) as an indication of matrix deposition (Fig. 3G).

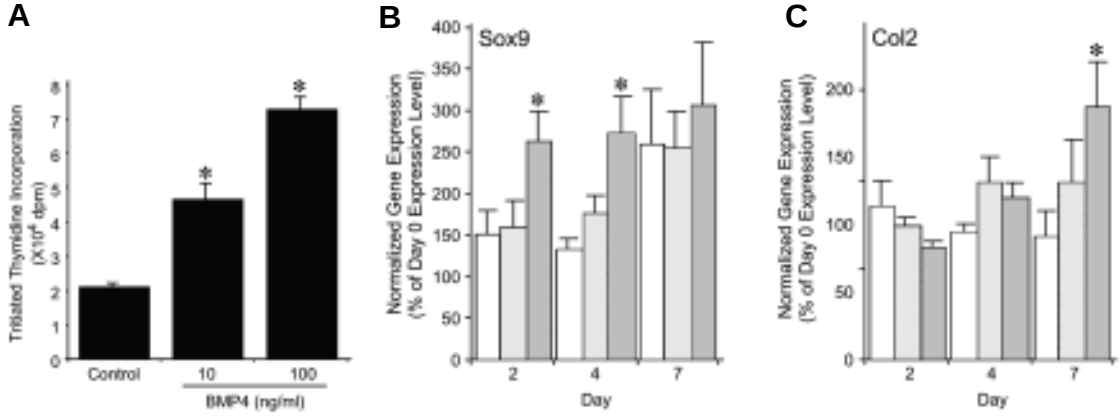

Fig. 4. BMP4 enhanced cell proliferation and cartilage marker gene expression. *To assay for the rate of proliferation, 17 dpc cranial bases were cultured and labeled with tritiated thymidine. Tritiated thymidine incorporation into the synchondroses of cranial base explants was analyzed* **(A)**. *Seventeen dpc stage cranial bases were cultured for two, four or seven days after which the synchondroses were harvested and analyzed for* Sox9 **(B)** *and* Col2 **(C)** *expression by real time RT-PCR. Expression of these cartilage markers was normalized for* β-actin *expression. The level of each gene at the beginning of culture (Day 0) was designated at 100%. Cultures were treated with 10 (gray bars) or 100 ng/ml BMP4 (black bars) and compared with controls (white bars). *p<0.05 when compared with controls.*

Nuclear density at the beginning of culture was 8000 nuclei/mm^2, decreasing to 3000 nuclei/mm^2 after 7 days in control cultures. Chondrocytes reside in lacunae within the matrix, and since we observed very few vacant lacunae in our sections, we attribute the decreased nuclear density to increased matrix deposition. Treatment of cranial base cultures with 100ng/ml BMP4 for 7 days resulted in further decrease in the nuclear density to 1800 nuclei/mm^2. In contrast, noggin blocked matrix deposition and maintained nuclear densities at levels comparable to the pre-culture specimens.

BMP4 promotes chondrocyte proliferation and cartilage marker gene expression

In order to analyze the effects of BMP4 on chondrocyte proliferation, DNA synthesis in the synchondroses of 17 dpc cranial base cultures treated with BMP4 for 48 hours was monitored by [^{3}H]-thymidine labeling. At 10 ng/ml, BMP4 increased chondrocyte proliferation by 123%, and 100 ng/ml of BMP4 increased proliferation by 251% (Fig. 4A).

In order to investigate changes in chondrocyte gene expression caused by BMP4 treatment of cranial bases, we collected RNA from a time course of BMP4 treated cultures and quantitated Sox9 and Col2 message levels by real-time RT-PCR. Sox9 expression increased over seven days in control cultures, and this increase was accelerated to approximately two days in the cultures treated with BMP4 at 100 ng/ml (Fig. 4B). Col2 expression remained relatively constant over the time course of BMP treatment, but was found to increase at day seven in cultures treated with BMP4 at 100 ng/ml (Fig. 4C).

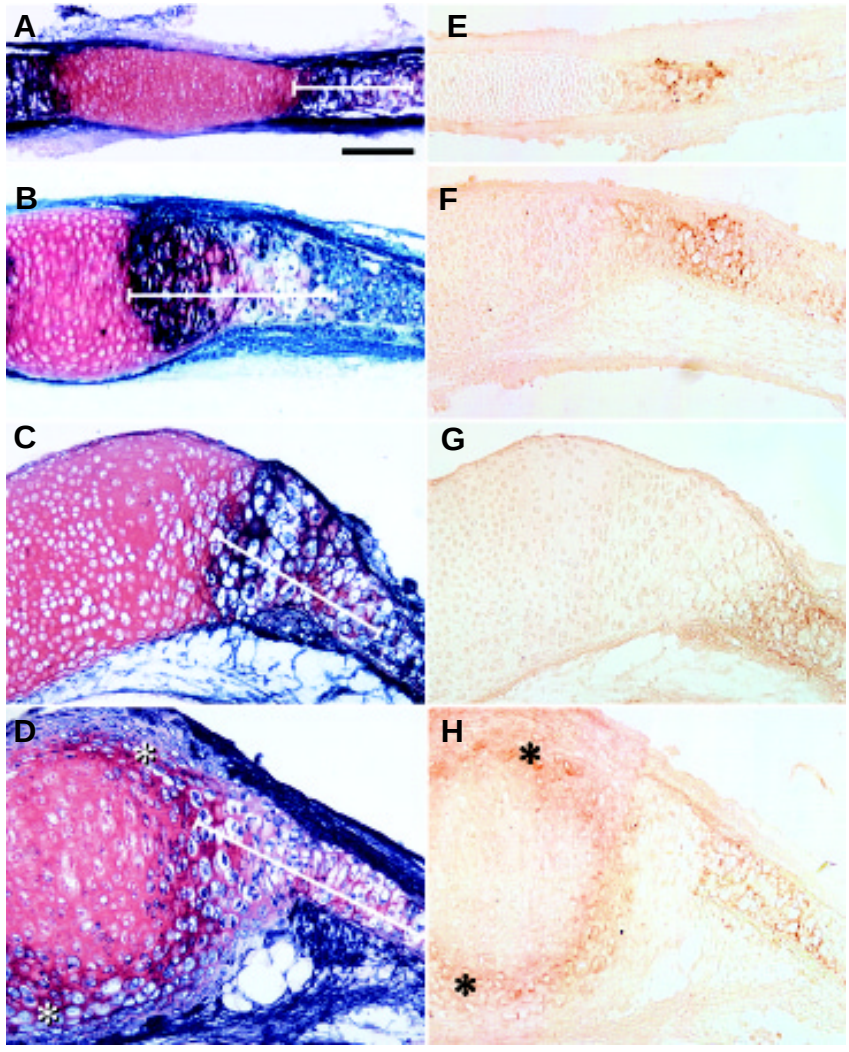

Fig. 5. BMP4 promoted chondrocyte hypertrophy. *Seventeen dpc stage cranial bases* **(A,E)** *were cultured for seven days in SFM* **(B,F)**, *or exposed to 10* **(C,G)** *or 100 ng/ml BMP4* **(D,H)**. *Mid-sagittal sections of the spheno-occipital synchondrosis were stained with Safranin O (red) for cartilage and alkaline phosphatase activity (blue) (A-D), or immunostained for type X collagen (brown) (E-H). Hypertrophic zones are marked by white brackets (A-D), and ectopic hypertrophy in BMP4-treated tissues is indicated by asterisks. Scale bar in A for all panels represents 100 μm.*

BMP4 accelerates chondrocyte hypertrophy

During endochondral ossification, chondrocyte hypertrophy is associated with expression of AP and Col10. Histochemical analysis of AP and Col10 in cranial bases cultured for seven days in SFM, revealed an increase in the thickness of the synchondroses and an increase in the zone of hypertrophic chondrocytes at both of their cartilage/bone interfaces (Fig. 5A, B, E, F). In the presence of 10ng/ml BMP4, we observed an additional increase in the thickness of the cartilage, consistent with the results of alcian blue area measurements (Fig. 3B, 5C, and 5G). Chondrocyte hypertrophy in these cultures was increased proportional to the increase in overall size of the synchondrosis. However, in the presence of 100ng/ml of BMP4, the increase in cartilage thickness was accompanied by an increase in chondrocyte hypertrophy adjacent to the bone and ectopic hypertrophy surrounding the central reserve zone of the synchondrosis (Fig. 5D and 5H).

BMP4 signaling modulates chondrocyte gene expression

In order to investigate changes in gene expression during chondrocyte maturation caused by BMP4 treatment of cranial bases, we collected RNA from a time course of BMP4 treated cultures and quantitated Col10 message levels by real-time RT-PCR (Fig. 6A). Furthermore, to investigate interactions between the BMP signaling pathway and the PTHrP/Ihh pathway, we also quantitated expression of PTHrP (Fig. 6B), PPR (Fig. 6C) and Ihh (Fig. 6D). Consistent with the results of immunohistochemistry, Col10 expression was increased at day seven only in the higher concentration of BMP4, but expression of this gene was down regulated by BMP4 at earlier time points. We detected no effect of BMP4 on PTHrP expression, which was decreased in all cultures relative to the day 0 expression level. PPR expression was increased in cultures treated with BMP4 at 100 ng/ml for seven days. Similar to Col10, expression of Ihh was down regulated by BMP4 on day two and four, and then up regulated by the higher concentration of BMP4 by day seven. Expression of Ihh in the cultures remained lower than the day 0 specimens.

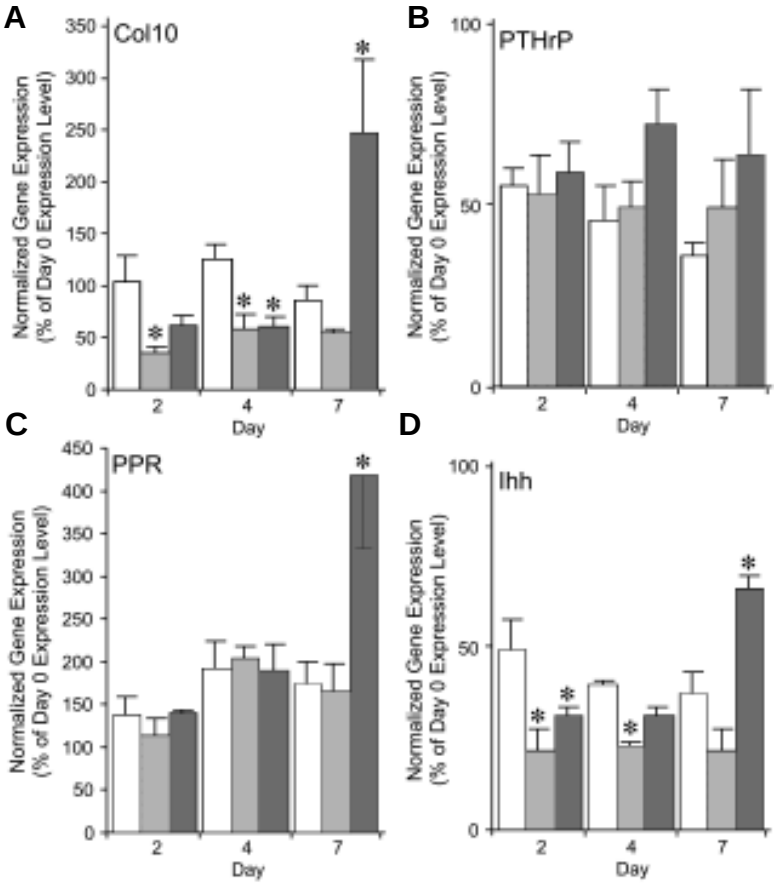

Fig. 6. BMP4 modulated cartilage gene expression. *Seventeen dpc stage cranial bases were cultured for two, four or seven days after which the synchondroses were harvested and analyzed for* Col10 **(A)**, PTHrP **(B)**, PPR **(C)** *and* Ihh **(D)** *expression by real time RT-PCR. Expression of these cartilage markers was normalized for* β-actin *expression. The level of each gene at the beginning of culture (Day 0) was designated at 100%. Cultures were treated with 10 (gray bars) or 100 ng/ml BMP4 (black bars) and compared with controls (white bars). *p<0.05 when compared with controls.*

Discussion

By analyzing the time course of normal growth and development of the endochondral cranial base of mice, we have identified the perinatal period as a time of rapid cranial base growth and endochondral ossification. Using a serum-free organ culture system, we have demonstrated that, during this time of accelerated chondrocyte maturation, the cranial base synchondroses are responsive to BMP4, and endogenous BMPs are essential for normal development. We have characterized the effects of BMP4 on chondrocyte proliferation, hypertrophy and gene expression in cranial base cartilage. BMP4 promoted chondrocyte proliferation in our cultures, which is consistent with previous studies that have shown increased chondrocyte proliferation by BMPs or inhibition of proliferation by noggin (Enomoto-Iwamoto *et al.,* 1998; Venezian *et al.,* 1998; De Luca *et al.,* 2001; Minina *et al.,* 2001).

Although chondrocyte proliferation in developing growth plates is maintained by PTHrP and Ihh signaling (for reviews see (Juppner, 2000; Kronenberg and Chung, 2001; Vortkamp, 2001), our data suggest that the regulation of chondrocyte proliferation by BMP4 does not involve the modulation of PTHrP or Ihh expression. BMP4 treatment of cranial base cultures did not have a significant effect on the expression of PTHrP. BMP4 treatment of cranial base organ cultures caused a decrease in Ihh expression at days two and four, followed by an increase at day seven, only at the higher dose of BMP4 (100 ng/ml). Similarly, long bone cultures treated with 500 ng/ml of BMP2 exhibited an increase in the domain of Ihh expression after four days (Minina *et al.,* 2001). However, these changes likely reflect changes in the relative proportions of proliferating and post-mitotic chondrocytes rather than a direct effect of BMP4 on Ihh expression. Since Ihh and Col10 are expressed in post-mitotic chondrocytes, the decrease in expression of these genes at days two and four may reflect a higher relative proportion of chondrocytes undergoing proliferation to those undergoing hypertrophic maturation. The later increase in Ihh and Col10 expression suggests that after the early phase of chondrocyte proliferation, the relative proportions of proliferating and maturing chondrocytes shift to favor those initiating hypertrophy. The expanded zone of hypertrophic chondrocytes relative to the reserve and proliferating zones was evident in Fig. 5D. By examining the effects of BMP4 treatment on overall Ihh expression at multiple time points, we found that decreased or increased Ihh expression correlated with the state of chondrocyte differentiation. This may help to explain differences between previous studies regarding the effects of BMP signaling on Ihh expression (Zou *et al.,* 1997; Minina *et al.,* 2001). The mechanisms by which BMP4 regulates chondrocyte proliferation in the cranial base are not yet characterized. One possible mediator of the effects of BMP4 on cranial base chondrocyte proliferation is the homeobox transcription factor Msx1, which is a regulator of the cell cycle and is up-regulated by BMP signaling in the craniofacial region (Hu *et al.,* 2001).

The increase in chondrocyte proliferation was accompanied by increased matrix accumulation, as demonstrated by alcian blue and Safranin O staining for cartilage matrix, and the decrease in nuclear density. The higher concentration of BMP4 promoted increased transcription of Col2, Col10 (Fig. 4 and 6) and aggrecan (data not shown) by day seven. This increase in matrix gene expression was accompanied by an increase in the overall size of the synchondroses, consistent with increased matrix protein accumulation. Interestingly, the lower concentration of BMP4 produced an increase in synchondrosis size but not a statistically significant increase in Col2 or Sox9 transcription. This may suggest that BMP4 affects post-transcriptional events that promote matrix accumulation and assembly at concentrations that are below a threshold that would affect matrix gene regulation.

Previous studies have characterized the regulation of the Col2 gene by BMPs through the modulation of Sox9 expression and activity (Healy *et al.,* 1999; Zehentner *et al.,* 1999; Semba *et al.,* 2000). Sox9, in a complex with L-Sox5 and Sox6, binds to regulatory sequences in the first intron enhancer of the Col2 gene (Lefebvre *et al.,* 1998; Lefebvre *et al.,* 2001). In cranial base organ cultures treated with BMP4 at 100ng/ml, Sox9 expression was elevated on days two and four, while increased Col2 expression was not detected until day seven. This time lag between peak Sox9 expression and Col2 expression is consistent with that seen in limb

bud development *in vivo* (Takahashi *et al.,* 1998). Sox9 requires post-translational activation (Huang *et al.,* 2000; Huang *et al.,* 2001), and the complex of Sox proteins may require additional signaling cues to assemble and activate target gene transcription. In our experiments, Col2 expression was elevated at seven days of BMP4 treatment. Increased BMP signaling may induce a cascade of downstream signaling events that, within several days, leads to the formation of transcriptionally active complexes necessary for the up-regulation of target genes. However, since cartilage matrix deposition was increased at earlier time points, this suggests that Col2 message level per cell is not rate limiting for matrix accumulation.

In addition to its effects on chondrocyte proliferation and matrix accumulation, BMP4 treatment of cranial bases at 100 ng/ml increased the proportion of hypertrophic chondrocytes. This BMP treatment promoted ectopic chondrocyte hypertrophy in a zone surrounding the center of the synchondroses as demonstrated by increased staining for alkaline phosphatase and Col10. Elevated Col10 message was detected in cranial bases treated with 100ng/ml BMP4 for seven days. Due to the expansion of prehypertrophic and hypertrophic zones in the synchondroses, significantly elevated levels of Ihh and PPR message were also detected. The balance of chondrocyte proliferation and hypertrophic maturation in the developing growth plates is maintained by signaling between the postmitotic, prehypertrophic chondrocytes that express Ihh and the periarticular perichondrium, which is the source of PTHrP secretion (Lee *et al.,* 1995; Vortkamp *et al.,* 1996). Although PTHrP expression in the cranial base synchondroses has not been thoroughly analyzed, these structures may be similar to postnatal growth plates where PTHrP is expressed by the growth plate chondrocytes (van der Eerden *et al.,* 2000; Kindblom *et al.,* 2002). The centrally located reserve zone of the synchondrosis provides the source of cells for both of the expanding hypertrophic zones. During postnatal development, growth of the synchondrosis eventually depletes the pool of cells in the reserve zone leading to closure of the synchondrosis and fusion of the bones of the cranial base. BMP4 may accelerate this process leading to the decrease in reserve zone and encroachment of the hypertrophic zone as observed in our cultures. This decrease in the pool of reserve chondrocytes, which may be the primary source of PTHrP expression, may diminish the action of Ihh, which would normally upregulate PTHrP expression and inhibit hypertrophic differentiation. Although increased chondrocyte hypertrophy is observed in long bones treated with BMPs, ectopic hypertrophy has not been reported. Ectopic hypertrophy in the cranial base may be due to the unique structure of the centrally located reserve zone that supports two hypertrophic zones.

In summary, our most significant finding is to have defined the two responses of cranial base synchondroses to BMP4 signaling; an initial phase of increased chondrocyte proliferation and matrix deposition, followed by accelerated hypertrophic maturation. The increase in proliferation did not involve a direct modulation of PTHrP or Ihh expression by BMP4 signaling. The later phase of increased chondrocyte hypertrophy and ectopic hypertrophy were accompanied by increases in Ihh, PPR and Col10 expression, but not that of PTHrP. We suggest that the unique structural arrangement of the zones of chondrocytes in the synchondroses contributes to differences in the response of synchondroses and growth plates to BMP signaling. Finally, our serum-free culture system of cranial base synchondroses provides the opportunity for *in vitro* manipulations and comparisons with growth plate development in the long bones.

Materials and Methods

Animals, microdissection and organ culture

Timed pregnant Swiss Webster animals were purchased (Harlan), housed and handled according to approved Animal Study Protocols from the National Institutes of Health. Cranial base structures were dissected from fetuses and cultured in 24-well plates. Cultures were maintained in 1ml BGJb medium (Gibco Invitrogen), supplemented with 2 mg/ml bovine serum albumin (BSA; Sigma), 100 μg/ml ascorbate, 1 mM beta-glycerophosphate, antibiotics and antimycotics (Gibco Invitrogen). Serum containing medium consisted of 10% fetal bovine serum (Hyclone), and a mixture of insulin, transferrin, selenium and pyruvate (ITSP; Gibco Invitrogen) at 10 μg/ml, 5.5 μg/ml, 6.7 ng/ml, and 1 mM respectively. Medium was changed every two days. Experimental cultures were exposed to BMP4 (R and D Systems) at 10 or 100ng/ml, or noggin (R and D Systems) at 100 or 1000 ng/ml. These concentrations were selected based on previous published work (Semba *et al.,* 2000; Minina *et al.,* 2001).

Histology, immunohistochemistry and morphometry

For whole mount staining, tissues were harvested, processed and stained with 1% alcian blue (Sigma) and 2% alizarin red (Sigma) according to protocols previously described (Kaufman, 1994). Alcian blue stained cartilage matrix, and alizarin red stained calcified tissue (henceforth referred to as “bone” although it also included calcified cartilage). For histological sectioning, tissues were collected, fixed in fresh 4% paraformaldehyde (w/v in PBS) at pH7.4 overnight at 4°C and processed for cryosectioning. Ten-micron thick frozen sections were collected. Sections were stained in 0.4mg/ml nitro blue tetrazolium chloride and 0.19mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche) for alkaline phosphatase activities, and 0.02% Safranin O (Sigma) for cartilage as previously described (Luna, 1992). Immunohistochemistry was performed as previously described (Shum *et al.,* 1993). Rabbit polyclonal antibodies directed against Col10 (a gift from S. Robins; (Rucklidge *et al.,* 1996)) was used at 1:500 dilution. Negative controls include no primary antibody, or the use of normal rabbit serum in place of the primary antibody. Images of stained specimens were captured using a Polaroid DMCle Digital camera (Polaroid), and area measurements were performed using NIH Image 1.62 or OpenLab 3.0.3 software (Improvision). Nuclear counts were conducted by color density thresholding using Hoechst stained images and analyzed with OpenLab 3.0.3. All measurements were collected from at least three samples.

Quantitation of gene expression

Specimens were rinsed in phosphate buffered saline (PBS) and microdissected to isolate only the spheno-occipital and the two intraoccipital synchondroses. At least three cranial bases were analyzed for each gene, and in some cases, the synchondroses from two cranial bases were pooled for each of the three samples. Total RNA was extracted using an RNeasy kit (Qiagen) and analyzed by real time RT-PCR with SYBR green detection using the iCycler (BioRad). Amplimer pairs for Sox9, Col2, aggrecan, Col10, Ihh, PTHrP and PPR were
5’-ACT CCC CAC ATT CCT CCT CCG GCA T-3’ and
5’-GTG CTG CTG ATG CCG TAA CTG CCA G-3’,
5’-ACA GCA TCG CCT ACC TGG ACG AAG C-3’ and
5’-TGG GTC CTT TGG GTT CGC AAT GG -3’,
5’-GAG ACC CAG ACA GCA GAA ACA ACC-3’ and
5’-ATG CCA GAT CAT CAC CAC ACA GTC-3’,
5’-GGC AGC AGC ATT ACG ACC CAA GAT-3’ and
5’-GAA TAA CAG ACA CCA CCC CCT CAC-3’,
5’-GCT CTG GCT GCG ATT CTT CAC ACG-3’ and
5’-CAG AGA CTC CGC CCA TTG ACA GCA-3’,
5’-TGG AGT GTC CTG GTA TTC CTG CTC-3’ and
5’-CCA CCT TGT TGG TTT CCT GAG TTA-3’, and

5'-GGC AGT ACC TTG TCC CGA TTA CAT-3' and
5'-TAC AGT CCC TCC ACC AGA ATC CAG-3', respectively. Concomitant PCR for mouse *β-actin* (primers from Clontech) was performed for each RT reaction as a standard. Expression of cartilage markers were determined after normalization for *β-actin* expression and graphed as percentage of day 0 value.

Tritiated [^{3}H]-thymidine incorporation

Cell proliferation was assessed in the organ cultures by measuring [^{3}H]-thymidine incorporation. After 24 hours of culture, [^{3}H]-thymidine (Amersham Pharmacia Biotech) was added to the culture medium at a concentration of 5 mCi/ml and labeling continued for an additional 18 hours. Some explants were inactivated by freeze/thaw prior to culture, to serve as a negative control. The synchondroses were dissected from each of the explants and washed extensively in PBS and 10%TCA, after which the tissues were solubilized in 0.1 ml of 0.1 N NaOH solution at 60°C for 2 hours. The amount of incorporated isotope was determined by liquid scintillation counting.

Data analyses

Data were presented as mean ± standard error of the mean (SEM). Morphometric data were analyzed using Student's t-test. Data collected at a single time point ([^{3}H]-thymidine incorporation) and multiple time points (real time RT-PCR) were analyzed using one-way ANOVA and two-way ANOVA, respectively. At each time point, groups receiving treatment were compared with control using Dunnett's test. Statistical significance was taken at P values of less than 0.05 ($P<0.05$).

Electronic Supplementary Material for this paper, consisting of a "QuickTime" movie is available at the following address:

http://www.ijdb.ehu.es/abstract.0306/esm2.htm

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