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Original Article

Signalling via type IA and type IB Bone Morphogenetic Protein Receptors (BMPR) regulates intramembranous bone formation, chondrogenesis and feather formation in the chicken embryo

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ABSTRACT Bone morphogenetic proteins (BMPs) signal via complexes of type I and type II receptors. In this study, we mapped the expression of type IA, type IB and type II receptors during craniofacial chondrogenesis and then perturbed receptor function *in vivo* with retroviruses expressing dominant-negative or constitutively active type I receptors. *BmprIB* was the only receptor expressed within all cartilages. *BmprIA* was initially expressed in cartilage condensations, but later decreased within cartilage elements. *BmprII* was expressed at low levels in the nasal septum and prenasal cartilage and at higher levels in other craniofacial cartilages. The maxillary prominence, which gives rise to several intramembranous bones, expressed both type I receptors. Misexpression of dnBMPRIB decreased the size of cartilages and bones on the treated side. In contrast, dnBMPRIA had no effect on the skeletal phenotype. The phenotypes of caBMPRIA and caBMPRIB were similar ; both led to overgrowth of cartilage elements, thinner bones with fewer trabeculae and inhibition of feather development. Infection with constitutively active viruses resulted in ectopic expression of *Msx1, Msx2* and *Fgfr2* throughout the maxillary mesenchyme. These data suggest that the pattern of trabeculation in membranous bones derived from the maxillary prominence was related to the change in expression pattern and that *Msx* and *Fgfr2* genes were downstream of both type I BMP receptors. We conclude that the requirement for the type IB is greater than for the type IA receptor but, when active, both receptors play similar roles in regulating bone, cartilage and feather formation in the skull.

KEY WORDS: *Intramembranous bone, BMP receptor, retrovirus, cartilage, chicken embryo*

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily of growth factors and are used in many aspects of embryonic development including apoptosis, chondrogenesis, and patterning (Hogan, 1996). In the developing chicken face, *Bmp2*, *Bmp4,* and *Bmp7* are expressed in both the ectoderm and mesenchyme (Francis-West *et al.*, 1994; Wang *et al.*, 1999) in specific patterns. The addition of exogenous BMP2, BMP4, and BMP7 protein to the developing chick face induces the formation of supernumerary bones in the maxillary region and secondary growth sites branching off Meckel's cartilage (Barlow and Francis-West, 1997; Wang *et al.*, 1999). These data argue for an important role for endogenous members of the BMP family in facial development, but the mechanism of signal transduction is not clear.

BMPs bind to two type I receptors (IA, also known as Alk-3, Brk-1; IB, also known as Alk6, Brk2) and one type II receptor. Ligand binding can form heterotrimeric or heterotetrameric complexes with types I and II transmembrane serine threonine kinases (ten Dijke *et al.*, 1996). The type I receptor phosphorylates downstream targets, including members of the Smad family (Smad 1, 5, 8; Miyazono, 1999). Despite sequence divergence between *Bmp4* and *Bmp7*, both of these growth factors share the same receptors (Koenig *et al.*, 1994; ten Dijke *et al.*, 1994). Other members of the BMP family, growth/differentiation factors GDF5 and GDF7, also bind to BMP receptors (Yamashita *et al.*, 1996). Thus, there is some promiscuity in ligand- binding characteristics of BMP receptors.

Abbreviations used in this paper: BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; dnBMPR, dominant negative BMP receptor; caBMPR, constitutively active BMP receptor; *Msx*, muscle segment homeobox-containing gene; *Fgfr* , fibroblast growth factor receptor gene; GDF, growth and differentiation factor; KOH, potassium hydroxide.

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The ubiquitous early expression of *BmprIA* (Dewulf *et al.*, 1995) and *BmprII* (Roelen *et al.*, 1997) reported in mice together with the observation that knock out mice lacking *BmprIA* (Mishina *et al.*, 1995) or *BmprII* (Beppu *et al.*, 2000) die prior to gastrulation confirms that type IA and type II receptors are fundamentally important for early development. In contrast, both the tissues expressing *BmprIB* (Dewulf *et al.*, 1995) and the phenotypes of *BmprIB -/-* mice are much more limited. Mice lacking the cartilage-specific isoform of *BmprIB* do not form the distal phalanges of the foot and handplate (Baur *et al.*, 2000). A similar distal limb truncation has been reported in mice lacking exon 1 of *BmprIB* (Yi *et al.*, 2000). The lack of abnormalities in the rest of the embryo suggests that the type IA receptor can take over for the IB receptor during differentiation of the craniofacial skeleton, most of the axial skeleton, and the long bones of the appendicular skeleton. It seems likely, therefore, that the type I BMP receptors share similar functions in the differentiation of craniofacial skeletal tissue.

Another approach used to determine the redundant or unique functions of BMP receptors has been to express constitutively active (ca) or dominant negative (dn) forms of BMPRIA and BMPRIB in chicken limb buds. The constitutively active receptors can stimulate Smad1 phosphorylation in the absence of a BMP ligand (Hoodless *et al.*, 1996), where as dominant negative receptor constructs can bind ligand but are unable to phosphorylate downstream targets (Zou and Niswander, 1996). Retroviruses expressing mutated forms of BMPRIA and BMPRIB injected into the limb-bud region of chick embryos (Zou and Niswander, 1996; Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996; Zou *et al.*, 1997) give rise to a variety of stage-specific and viral-specific phenotypes. Infection of stage-20 limb buds showed that both constitutively active receptors lead to increased chondrogenesis, indicating that at this stage of development they have overlapping functions. In contrast, injections done before stage 17 gave different results for the caBMPRIA (CaA) and caBMPRIB (CaB) receptors. The CaA virus delayed the maturation of cartilage during endochondral bone formation, whereas CaB had no effect on the rate of differentiation but instead gave rise to thin limbs due to increased programmed cell death. The requirement for BMP signaling is shown in the dominant-negative results. Infection of dnBMPRIB (DnB) decreased the size of the elements and decreased programmed cell death (Kawakami *et al.*, 1996; Zou and Niswander, 1996; Zou *et al.*, 1997). Two different studies found no phenotype for dnBMPRIA (DnA) (Zou *et al.*, 1997; Kawakami *et al.*, 1996), whereas a third study, using a different viral system, showed that DnA inhibited programmed cell death (Yokouchi *et al.*, 1996). These studies suggest that both the type IB and the type IA receptor could be involved in the cell-death pathway and that the type IB and type IA receptors mediate different aspects of chondrogenesis.

The avian facial skeleton differs from the appendicular and axial skeleton in that most of the bones and cartilages are derived from cranial neural crest cells rather than mesoderm (Köntges and Lumsden, 1996). The difference in embryonic origin may mean that BMP receptors function differently in the face compared with elsewhere in the body. Facial mesenchyme is also unique in that the majority of the bones differentiate directly from mesenchyme without a cartilage template (intramembranous bone formation). The components of the embryonic face (facial prominences) each give rise to distinct skeletal elements. Three facial prominences, frontonasal mass, lateral nasal prominence, and mandibular prominence, differentiate into cartilage and bone, with cartilage differentiating between stage 24 and stage 28 (Matovinovic and Richman, 1997) and bone condensing at stage 31 (Murray, 1963). The frontonasal mass gives rise to the interorbital septum, prenasal cartilage, premaxilla, and egg tooth; the lateral nasal prominence forms the nasal conchae and nasal bones; and the paired mandibular prominences form Meckel's cartilage and all the mandibular bones. The maxillary prominence is unique in that it gives rise only to membranous bone. The bones derived from the maxillary prominence support the side of the beak and roof of the oral cavity and include the pterygoid, quadratojugal, jugal, maxillary and palatine bones. The role of chicken BMP receptors during the aforementioned critical stages of skeletal differentiation has not been studied in the chicken craniofacial region.

The present study first mapped the patterns of expression specifically during stages when active chondrogenesis and osteogenesis occurred. We then addressed the function of type IA and type IB BMPRs in facial development by targeted viral misexpression of constitutively active or dominant-negative BMP receptors. Such viral constructs bypass the inhibitory effects of BMP antagonists, some of which are expressed in craniofacial tissues (A. M. Ashique, unpublished data). We compared the phenotype of embryos at several stages to determine whether there were differences in function of the type IA or type IB receptors during craniofacial development. We found that in some cases the phenotypes were distinct, while in other situations the effects were similar. Our data with dominant-negative viruses suggested that the requirement for IA was not as great as that for the IB receptor, but that active forms of the receptors had similar roles in chondrogenesis, osteogenesis, and feather formation.

Results

Expression of BMP Receptors During Craniofacial Chondrogenesis

At stage 28, *BmprIB* expression was largely restricted to regions that will shortly undergo chondrogenesis such as the condensing nasal conchae lateral to the nasal slits, the condensing Meckel's cartilage in the mandible, and the prenasal cartilage in the frontonasal mass. (Fig. 1A). There was also expression in non chondrogenic mesenchyme at the caudal edge of the maxillary prominences. *BmprIA* was expressed at high levels throughout most of the mesenchyme. *BmprIA* was expressed in a domain complementary to that of *BmprIB* in the mandible (compare Fig. 1 A,B). *BmprIA* transcripts were also concentrated at the cranial edge of the frontonasal mass and caudal mesenchyme of the maxillary prominences. *BmprII* transcripts were ubiquitously expressed at stage 28 in the epithelium and mesenchyme (data not shown). By stage 30, cartilage differentiation was underway in the chondrocranium (Fig. 1 C,D). *BmprIB* was expressed within cartilage elements and the developing perichondrium of the nasal septum, nasal conchae, and Meckel's cartilage (Fig. 1 E,H). There was little expression within the maxillary mesenchyme; however, the medial edge epithelium expressed *BmprIB* transcripts. There are two isoforms of *BmprIB* in mouse embryos (Baur *et al.*, 2000). Although it is not known if multiple isoforms exist in chicken embryos, it is likely that our probe recognized all expressed forms of the receptor. In contrast to *BmprIB*, *BmprIA* was expressed at lower levels in the cartilage compared with the surrounding mesenchyme (Fig. 1 F,I). *BmprII* had high levels of expression in the nasal conchae, orbital cartilage, Meckel's cartilage, and the hyoid cartilages. The nasal septal cartilage had low expression of *BmprII* except in a column of cells in the midline (Fig. 1 G,J).

The egg tooth is an epithelial specialization that forms at the tip of the upper beak at stage 30 (Fig. 1 H-J) and first becomes keratinized at stage 32. The outer cornified intermediate and basal layers of the egg tooth expressed *BmprIA*, and *BmprII* (Fig. 1 L,M). In contrast, *BmprIB* was not expressed in the egg tooth (Fig. 1K). Patterns of gene expression similar to those seen at stage 30 were observed in the stage-32 cartilage elements.

Onset of Viral Expression. In order to determine the extent of viral infection in relation to the onset of skeletal differentiation, a series of embryos were hybridized to the *pol* probe at different times after injection. At 72 h post injection at stage 10, there were foci of viral expression within facial mesenchyme, but by stage 28 and stage 29 nearly all the mesenchyme on the injected side was expressing the virus (data not shown, viral infected cartilage visible in Fig. 2 A-C). The later the injections were carried out, the more localized the virus was spread, and embryos injected at stage 16 or stage 20 had primarily unilateral spread (Figs. 2A, 4K). All the viral constructs showed a similar spread of the virus. The relative abundance of viral message compared with endogenous message was demonstrated by sections hybridized to the *BmprIB* probe. The expression of endogenous *BmprIB* did not become visible until after 10 days with radiolabeled probe; however, viral transcripts for *RIB* could be detected after 3 days (Fig. 4J).

Effects of Overexpression of Wild-Type BMPRIB

In general, the wtBMPRIB virus produced a similar but less severe phenotype compared with the CaB-infected embryos; therefore, these results will not be described in detail. Injection of wtBMPRIB virus particles at stage 10 and at stage 14 resulted in a mild deviation

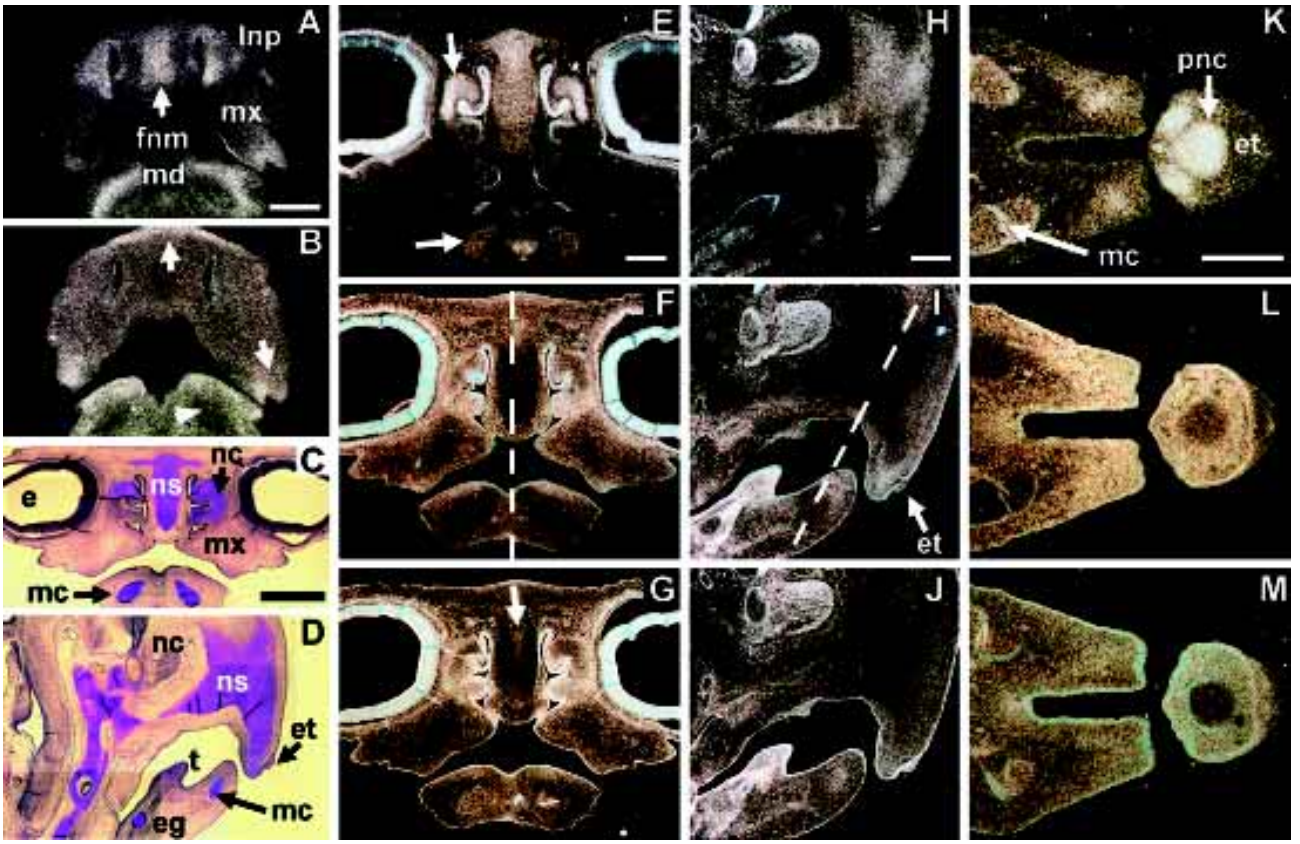
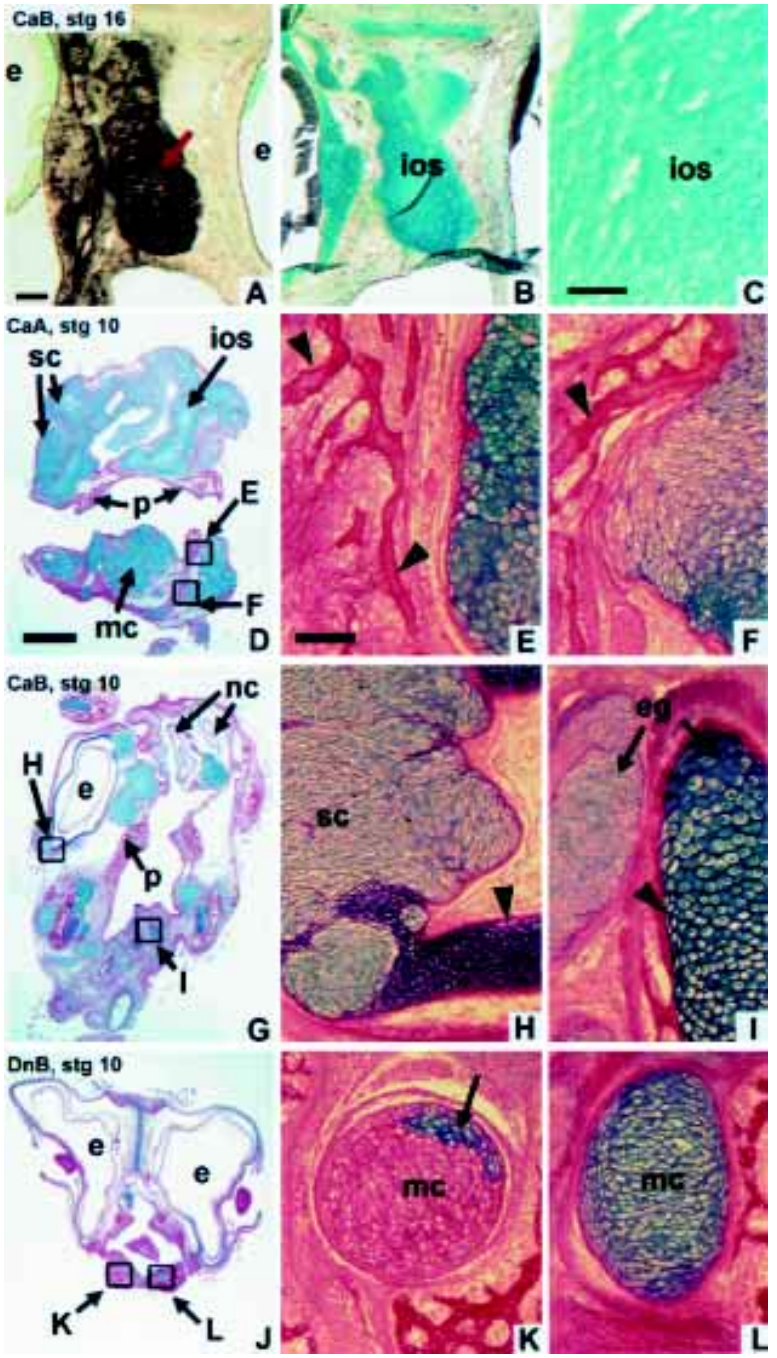


Fig. 1. Normal expression of BMP receptors during chondrogenesis. *Sections in A,B,E-M were photographed in dark field; silver grains appear white on a black background. Sections in A and B are frontal sections from a stage-28 embryo, sections in E-J are from 2 stage-30 embryos, and sections in K-M are coronal sections from a stage-32 embryo. Planes of section are indicated for stage-30 sections in F and I. Coronal sections in K-M pass through the tips of the upper and lower beaks. The upper beak extends out and curves over the lower beak at stage 32.* **(A)** *Increased* RIB *signal in chondrogenic areas of the lateral nasal, frontonasal mass and mandible.* **(B)** *Section adjacent to A showing generally higher levels of* RIA *expression compared with* RIB. *The cranial edge of the frontonasal mass and the caudal edges of maxillary prominences have higher expression than adjacent mesenchyme (arrows). The area where Meckel's cartilage will form has relatively lower levels of transcript (arrowhead).* **(C,D)** *Toludine blue-stained sections of stage-30 embryos showing location of cartilage elements. No bone has differentiated at this stage.* **(E,H,K)** BmprIB *expression is concentrated within all visible cartilage elements. There is no expression in the stage-32 egg tooth (K).* **(F,I,L)** BmprIA *expression is lower within the cartilage elements than in the surrounding mesenchyme. There is high expression within the basal ectodermal layer of the egg tooth (L).* **(G,J,M)** BmprII *expression is lower in the nasal septum and prenasal cartilage (M) than in other cartilages. There is a column of cells in the midline of the nasal septum that has a slightly higher level of expression (arrow in G). The egg tooth has expression of the type II receptor in the basal and intermediate layers (M). Scale bars, 500 μm for A, B, E-M. Scale bars for C, D, 1 mm. Abbreviations: e, eye; eg, entoglossum; et, egg tooth; fnm, frontonasal mass; lnp, lateral nasal prominence; mc, Meckel's cartilage; md, mandible; mx, maxilla; nc, nasal conchae; ns, nasal septum; pnc, prenasal cartilage; t, tongue.*



of the upper beak away from the injection site due to slightly enlarged cartilages on the injected side (21/25 had the phenotype; data not shown).

DnB and DnA Virus Infections have Different Effects on Skeletal Differentiation. In order to determine whether there was a requirement for the type IA or type IB receptor in skeletogenesis, embryos were injected with either DnA or DnB virus. Despite viral expression, there was no phenotype produced with the dnBMPRIA in any embryo (data not shown). In contrast, the embryos injected with DnB virus had a consistent phenotype. Embryos injected at stage 10 had smaller, thinner cartilage elements and, consequently, shortening or deviations of the beaks. The cartilage differentiation was not as advanced as non infected cartilage, judging by the lack of alcian blue staining (Fig. 2 K,L). Bone formation occurred, although some specimens were partly or completely missing the maxillary bone and some had a thinner right palatine bone (Fig. 3 C,D; compare with normal specimen in 3B; Table 1). This demonstrated that intramembranous bone formation not associated with a cartilage element could be inhibited. The mandibular bones formed in all specimens, but were proportional to the overall reduced size of the mandible. When infections were done at later stages of development, there was very little effect on morphogenesis of the skeleton (Table 1).

CaBMPRIA and caBMPRIB Produce Enlarged Cartilage and Proportionately Increased Bone Formation. In order to compare the potential functions of the type I receptors, CaA and CaB viruses were injected into the facial region. The effects of the two constitutively active type I viruses were very similar. The onset of the phenotype was relatively rapid. By the time cartilage had differentiated (3-4 days after injection, Fig. 2B), the virus had disrupted the smooth outline of the cartilage elements. There were numerous closely packed chondrocytes in a disorganized matrix (Fig. 2C). The location of the disruption in cartilage was closely correlated with the spread of the virus. Both CaA and CaB infections led to irregular-shaped cartilage elements due to focal expansion in the cartilage (Fig. 2 D,I). The earlier in development the infections were done, the more severe the disruption of morphology. Embryos injected at stage 10 had virtually no individual cartilage elements by the time they were sacrificed (Figs. 2 D,G; 3 E,F; compare with normal chondrocranium in 3A). There were many extra buds of cartilage that were connected to the major cartilage element. The early phenotypes suggested that

Fig. 2. Effects of BMPR viruses on cartilage and bone differentiation. (A) *Frontal section of a stage-32 embryo injected at stage 16 into the presumptive maxillary region, hybridized to a* pol *probe. Black silver grains indicate expression of virus primarily on the left side of the embryo.* **(B)** *Near adjacent section stained with alcian blue to show the cartilage.* **(C)** *Higher power magnification of nasal septal cartilage showing closely packed chondrocytes in disorganized matrix.* **(D)** *Frontal section of a stage-38 embryo. A disrupted pattern to cartilage and bone is evident throughout section.* **(E)** *Higher power magnification of area indicated by the box in D showing enlarged lacunae in the cartilage with poorly stained matrix and disorganized but well-stained bone trabeculae (arrow heads).* **(F)** *Higher power of box in D showing very cellular cartilage with poorly stained matrix and adjacent bone (arrow head).* **(G)** *Similar phenotype in a frontal section of a stage-38 CaB- injected specimen, although viral spread is more limited to the right side of embryo.* **(H)** *Scleral cartilage with focal viral infection compared to non-infected cartilage (arrowhead).* **(I)** *Comparison between non-infected and infected cartilage on the right and left side of an embryo.* **(J)** *Stage-38 embryo showing lack of alcian blue staining of Meckel's cartilage and smaller bones on the right side compared to the left side.* **(K)** *Right-side enlargement of Meckel's cartilage showing one small region with normal alcian blue staining (arrow).* **(L)** *Normal staining of left Meckel's cartilage with alcian blue. Scale bars, 200 μm for A,B; 50 μm for C; 100 μm for E,F,H,I; 2 mm for D,G,J. Abbreviations: e, eye; eg, entoglossum; ios, interorbital septum; mc, Meckel's cartilage; nc, nasal conchae; p, palatine bone; sc, scleral cartilage.*

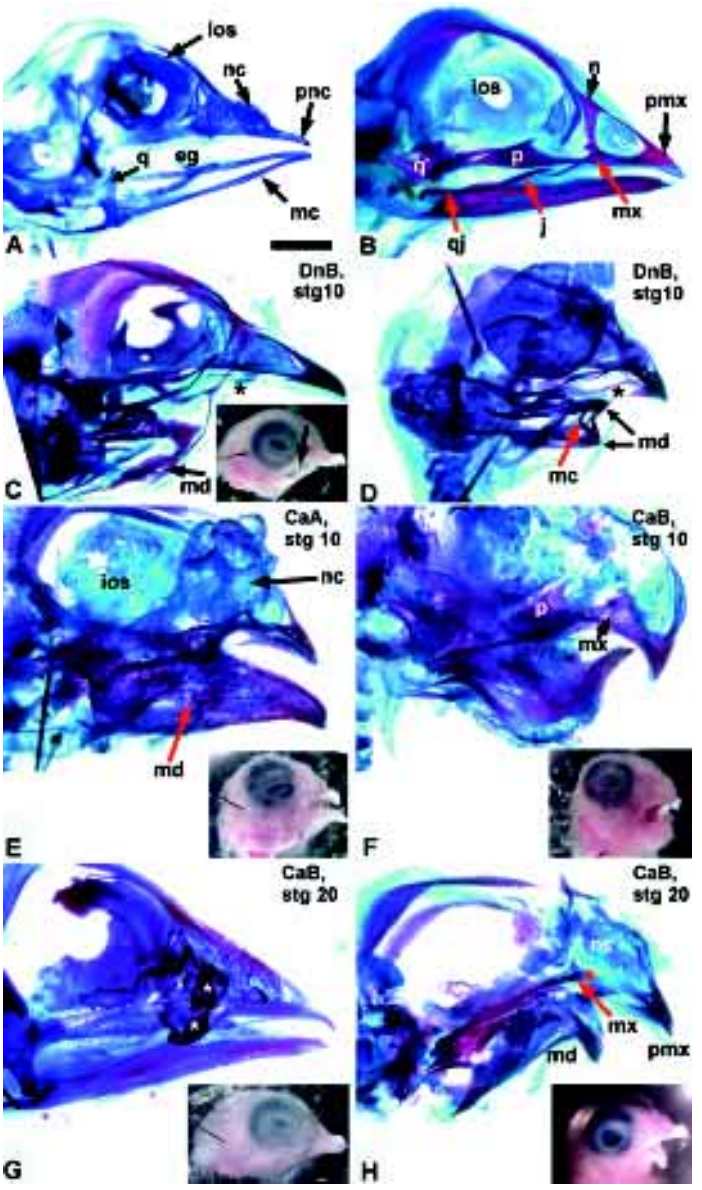


Fig. 3. Effects of mutant BMP receptor viral expression on the three-dimensional morphogenesis of the craniofacial skeletal. *All embryos were fixed at stage 38 and stained with alizarin red for bone and alcian blue for cartilage. Insets are external views of the same embryo.* **(A)** *Embryo stained only for cartilage to clearly indicate the elements that are still cartilaginous at stage 38.* **(B)** *Same-stage embryo stained for cartilage and bone showing all the bones that have differentiated by this stage. Part of the quadrate has ossified.* **(C,D)** *Embryos showing truncated mandibular development with missing maxillary bone (*). Premaxilla and nasal capsule has formed in both specimens, but the interorbital septum is underdeveloped (C). Cleft of the upper beak is visible in the external view (arrow, C'). The eye is not removed in D.* **(E,F)** *Similar phenotype in CaA- and CaB-injected embryos. There is a large expansion of conchal cartilage and a poorly formed interorbital septum; however, relatively normal premaxilla has formed in both cases. An enlarged mandible with increased bone covers the greatly distorted and expanded Meckel's cartilage. Keratin deposits replace feathers in the surface views (E', F').* **(G)** *Relatively normal development of the upper beak except for ectopic cartilage lateral and superior to the jugal bone (*). Normal lower beak and normal external phenotype.* **(H)** *Widespread infection has expanded Meckel's cartilage and the surrounding bone of the mandible. The interorbital septum was fragile and was lost during transfer of the specimen. The distal end of the maxillary bone is missing in this specimen (asterisk), but no cleft upper beak has formed in this embryo. Scale bar, 4 mm. Abbreviations: eg, entoglossum; ios, interorbital septum; j, jugal; pmx, premaxilla; mc, Meckel's cartilage; md, mandible; mx, maxillary bone; n, nasal bone; nc, nasal cartilage; p, palatine bone; pnc, prenasal cartilage; q, quadrate; qj, quadratojugal.*

separate ectopic condensations did not form. Instead, multiple polyps of cartilage connected to the main cartilage element enlarged, progressively obscuring the normal morphology. In sections, the stage-38 viral-infected cartilage was highly cellular, with enlarged lacunae and poorly differentiated matrix, as shown by less intense alcian blue staining (Fig. 2 E,F,H,I). The constitutively active viruses did not directly promote osteogenesis. Rather, bone was spread out over a larger area to cover the enlarged cartilage (Fig. 3 E,F). This was especially evident in the mandible, where a very thin layer of bone covered the greatly enlarged Meckel's cartilage (Fig. 1D). In order to clarify what was a primary effect on bone and what was secondary to changes in cartilage, we examined the maxillary bones. In all embryos injected with CaA or CaB virus, the maxillary derivatives continued to form; however, the trabeculae were less densely distributed than in normal bone. The intensity of staining with picosirius red was similar in viral-infected bone and in neighboring bone not infected with virus (Figs. 2 E,F; 3 E,F, Table 1).

Embryos injected into the maxillary prominence were predicted to have a more localized effect on the maxillary-derived bones. One of two phenotypes was produced with CaA or CaB injections. In embryos that had very localized viral spread as judged by morphology, there was induction of a separate, supernumerary cartilage just above the jugal bone (Fig. 3G; CaB 6/11, CaA 3/5, Table 1). This extra cartilage in these maxillary-injected specimens was the only clear case where the virus induced a new cartilage condensation, rather than expanded existing condensations. Embryos with more widespread infection had en-

TABLE 1

SUMMARY OF EFFECTS ON BONE AND CARTILAGE FORMATION IN STAGE-38, VIRAL-INJECTED EMBRYOS

Virus type	Total N	N with phenotype	Stage injected	Phenotype summary
DnB	25	10	10 *	Cartilage is thin, underdeveloped in 8/10 specimens. Maxilla partly or fully inhibited from forming in 5/10 specimens. Palatine bone partly missing in 3/10 specimens
DnB	18	2	20*	Size of maxilla reduced in 2 specimens
CaB	6	5	10	Cartilage overgrowth, disruption in trabeculation of membranous bones on side of injection, 5/5 specimens.
CaB	11	11	20	Trabeculation in membranous bones less dense on side of injection, extra cartilage above jugal bone in 6/11 specimens. 2/11 had enlarged Meckel's cartilage.
CaA	4	4	10	Enlargement of Meckel's cartilage with complete disruption of normal bone morphology on injected side.
CaA	12	5	20	Extra cartilage present above jugal in 3/5 specimens.

*Stage-10 embryos injected into the mesenchyme to the right of the mesencephalon;−stage-20 injections were done into the right maxillary prominence.

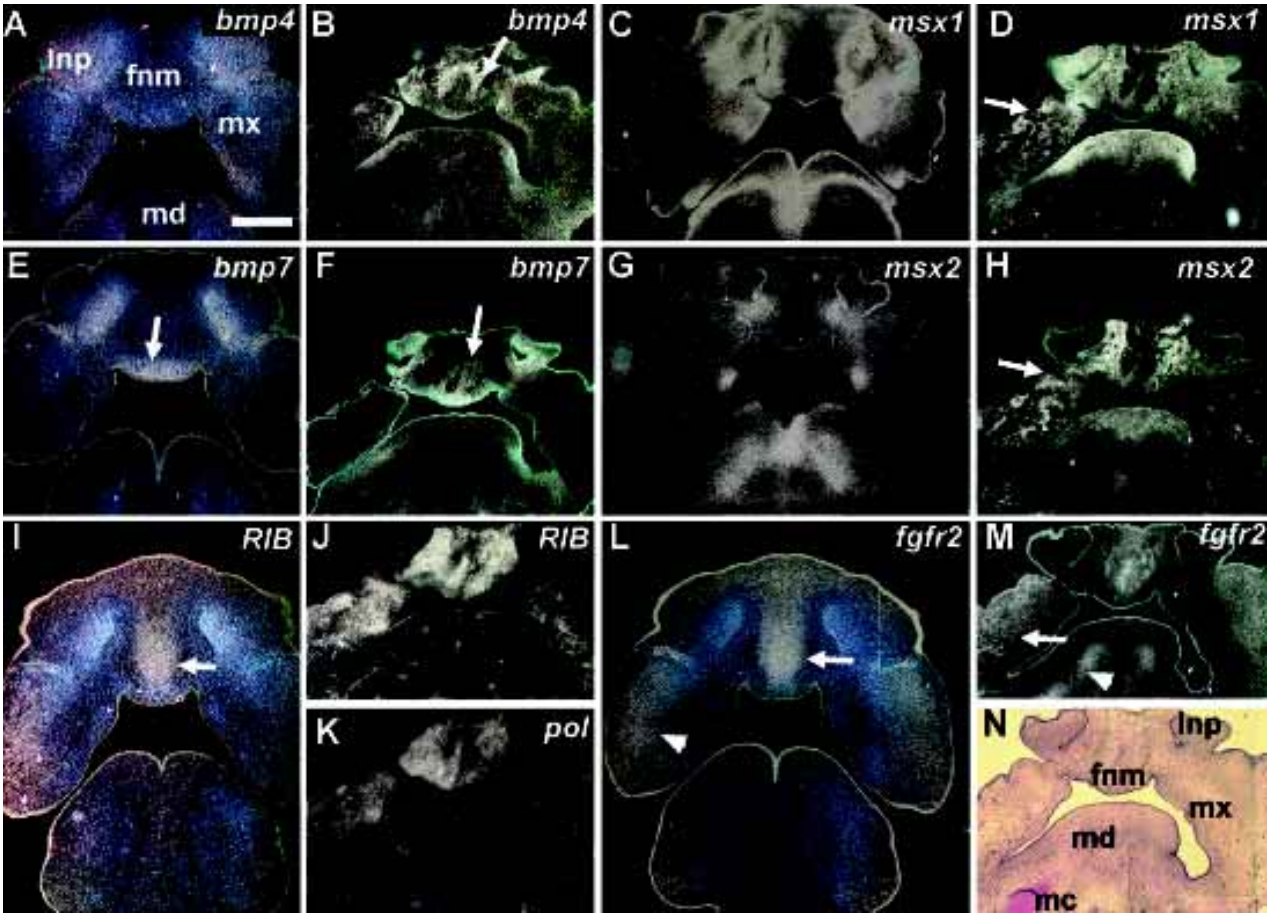


Fig. 4. Expression of genes in normal and viral injected stage 29 embryos. *Sections of non-injected control embryos (A,E,I,L) pass through the chondrogenic regions of the lateral nasal prominences and frontonasal mass but do not include Meckel's cartilage. The embryo in B,D,F,H,J,M,K and N was injected with the caBMPRIB virus at stage 14. The expression in the mandibular prominence is normal for all genes, but the virus has changed gene expression in the frontonasal mass and maxillary prominences on the right side of the embryo.* **(A,B)** *Ectopic expression induced in the frontonasal mass (arrow) does not overlap the region with the highest viral expression (K).* **(C,D)** *Dispersed expression in the right maxillary prominence compared to left side in a viral-infected embryo. Mandibular expression is similar to that observed in the non-injected embryo.* **(E,F)** *Normal expression of* Bmp7 *is found at the caudal edge of the frontonasal mass (arrow) and is still present in the viral-infected specimen. There is also ectopic expression in the frontonasal mass in the CaB-injected embryo (arrow), complementary to the expression of* Bmp4 *(compare B,F).* **(G,H)** *Dramatic redistribution of transcripts in the maxillary prominence of CaB-injected embryo (arrow). There is also more widespread expression in the frontonasal mass mesenchyme.* **(J)** *The expression of* BmprIB *is similar to that of the* pol *gene* **(K)** *after 5 days exposure. Such a short exposure does not allow endogenous* BmprIB *expression to be detected in Meckel's cartilage (N).* **(L,M)** *Expression in viral-infected frontonasal mass is not as consolidated as in control embryos. Note more diffuse expression in the right maxillary prominence (arrow in M) compared to the left. Meckel's cartilage is condensing in the mandible and expresses* Fgfr2 *(arrowhead in M).* **(N)** *Toluidine blue-stained section showing no obvious condensation in the center of the frontonasal mass, but Meckel's cartilage is visible. Scale bar, 500 μm. Abbreviations: fnm, frontonasal mass; lnp, lateral nasal prominence; mc, Meckel's cartilage; md, mandibular prominence; mx, maxillary prominence.*

largement of cartilage, a proportional expansion of bone covering Meckel's cartilage, and greater porosity in the maxillary bones, just as at earlier stages of infection (Fig. 3H). Thus, a relatively late onset of infection was still able to affect cartilage formation.

Constitutively Active Viral Expression Leads to Ectopic Expression of *Bmp4, Bmp7, Fgfr2, Msx1* and *Msx2.* The expression patterns of several genes that are expressed in the facial prominences and involved in intramembranous bone formation (Iseki *et al.*, 1997; Kim *et al.*, 1998) were examined in order to see if they were regulated by BMP receptor signaling. No embryos injected with dnBMPRIB had changes in the levels of transcripts for the genes examined. Due to the similarity in skeletal phenotype, we did not examine CaA-injected embryos; however, embryos infected with CaB had ectopic expression of several genes. We chose to infect embryos at stage 14 with the CaB virus so that the maxillary and frontonasal mass prominences would be affected. We examined two of the ligands for BMP receptors, *Bmp4* and *Bmp7*, to see whether there was a feedback loop between receptor signaling and expression of ligands. *Bmp4* was normally expressed at low levels in the mesenchyme at stage 29 (Fig. 4A); however, the virus induced ectopic expression in the center of the frontonasal mass (Fig. 4B). Some regions of ectopic *Bmp4* expression at the lateral edges of the frontonasal mass and in the maxilla overlapped the expression of the virus (compare Fig. 4B,K). In the center of the frontonasal mass, *Bmp4* expression appeared to be induced in cells less heavily infected with virus (arrow in Fig. 4B). *Bmp7* was normally expressed along the caudal edge of the frontonasal

mass (Fig. 4E), but an additional expression domain was induced in the center of the frontonasal mass by the constitutively active virus (Fig. 4F). Virally induced *Bmp4* expression patterns were complementary to those of *Bmp7.*

Muscle segment homeobox-containing genes (*Msx1* and *Msx2*) were expressed in the stage-29 facial prominences in discrete patterns, similar to but more defined than at earlier stages (Barlow and Francis-West, 1997). *Msx1* was expressed at the lateral edges of the frontonasal mass, the lateral nasal prominences, the medial tips of the mandible, and the cranio-medial one-third of the maxillary prominences (Fig. 4C). The *Msx2* gene was expressed at the corners of the frontonasal mass where fusion of the primary palate is taking place and in a small patch of mesenchyme just below the budding palatal shelves (Fig. 4G). The expression of *Msx1* and *Msx2* is up regulated by exogenous BMPs in avian facial tissues (Barlow and Francis-West, 1997; Wang *et al*., 1999). We wanted to see whether BMP receptor signaling could be mediating the coordinated increase in *Msx* expression observed in previous studies. In embryos infected with CaB, *Msx1,* and *Msx2,* transcripts were ectopically induced in regions that normally do not express the gene, such as the center of the frontonasal mass (3/3; Fig. 4 D,H). More remarkable was the distinct change in expression of both *Msx* genes in the maxillary prominences on the infected side. Instead of localized expression, small foci of expression were distributed across the maxillary mesenchyme (3/3, Fig. 4 D,H).

Finally, we examined expression of *Fgfr2,* a gene that is also expressed at high levels in membranous bone of the calvaria (Iseki *et al*., 1997), in facial mesenchyme, and in condensing cartilage (Wilke *et al*., 1997). In stage-29 embryos, *Fgfr2* transcripts normally encompass the chondrogenic regions in the center of the frontonasal mass and mandibular prominences (Fig. 4L and data not shown). We found that in CaB-infected embryos, there was a less regular edge to the infected, chondrogenic region in the frontonasal mass (3/3, Fig. 4M). *Fgfr2* was also expressed in the lateral aspect of the maxilla (Fig. 4L; Matovinovic and Richman, 1997). In the maxillary prominence of CaB-infected embryos the transcripts extended more medially (Fig. 4M, arrow). The changes in *Fgfr2* expression were correlated with cartilage hyperplasia of the nasal septum and change in the porosity of intramembranous bone.

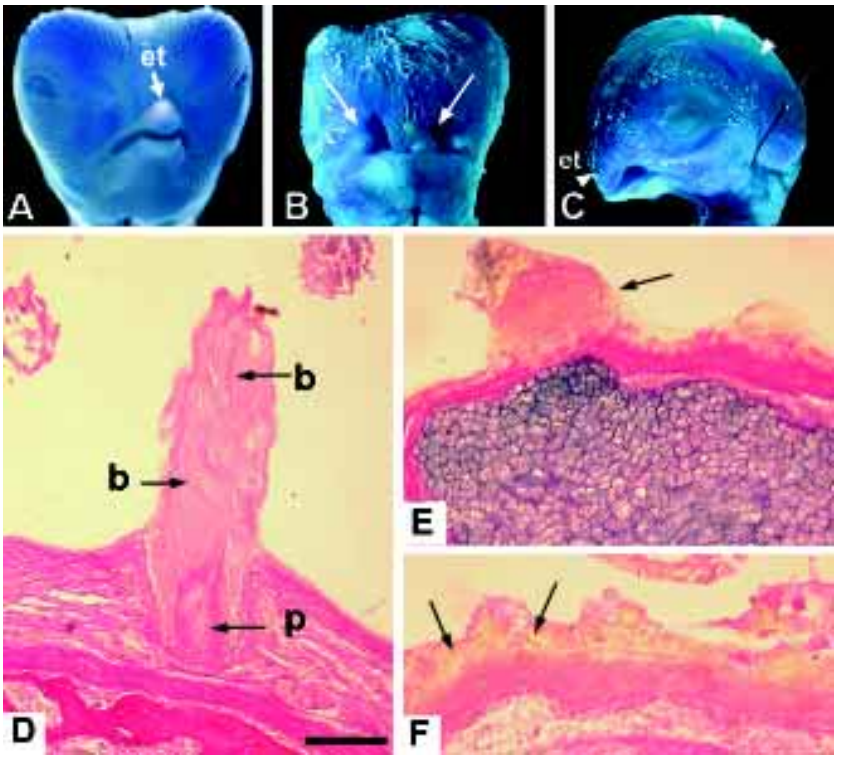


Fig. 5. Feather formation disrupted by misexpression of caBMPRIB. (A) *Normal pattern of feathers and egg tooth at stage 38.* **(B)** *Frontal view and* **(C)** *sagittal external views of a stage-38 embryo, injected at stage 10, with widespread viral infection. There is thickening of upper and lower beaks, bilateral notching of the upper beak (arrows in B) and disruption of the feathers and egg tooth (arrow heads in C). Keratinization has occurred randomly on the ectoderm instead of in the feathers.* **(D)** *Section of normal feather showing pulp and formation of barb ridges.* **(E)** *Disrupted feather forms keratin but no pulp or barb ridges. Hyperplastic cartilage lies underneath.* **(F)** *Random deposition of keratin in the surface ectoderm (arrows). Scale bar, 100 μm. Abbreviations: b, barb ridges; et, egg tooth; p, pulp.*

CaA and CaB Activity Suppresses Feather Formation. Infection of the craniofacial region with CaA or CaB resulted in retardation of feather formation (50% of embryos) or complete elimination of the feathers covering the head (compare Fig. 5A to B,C). Embryos with this phenotype generally had enlarged heads and widespread changes in cartilage morphology (Fig. 3 E,E'). The egg tooth was reduced in size or absent in 50% of embryos with no cranial feathers (Fig. 5 B,C). Sections revealed that disorganized plaques of keratin were present on the surface of the epithelium instead of organized feather barbs (compare Fig. 5D with E,F).

Discussion

Cartilage Element-Specific Expression of the Type II BMP Receptor

We expected to see the type II receptor expressed in all cartilages since a type I-II dimer is required for activation of downstream target genes. Remarkably, we observed lower expression of the type II receptor in the nasal septum compared with other cartilages. The difference in distribution of the type II receptor may correlate with the unique growth characteristics of the nasal septum. Proliferation in chicken nasal septal cartilage has not been analyzed; however, the equivalent stage and anatomical site has been studied in rat embryos (Diewert, 1980). The rat data show that at E 16 very little proliferation occurs in the nasal septal cartilage compared with Meckel's cartilage. Instead, growth of the nasal septal cartilage occurs via deposition of extracellular matrix. Our expression data suggest that chicken craniofacial cartilages may use mechanisms of growth similar to those used in the rat. It is also possible that expression of Bmp type II receptors distinguishes cartilage that grows via proliferation (e.g., Meckel's cartilage) from cartilage that grows by deposition of extracellular matrix (nasal septal cartilage).

Overlapping and Unique Function of Type IA and Type IB Receptors in Regulating Size of Cartilage Elements

Expression of DnA had no effect on cartilage formation or differentiation, whereas DnB decreased the size of cartilage condensations and delayed differentiation. A similar lack of phenotype is observed with DnA in the chicken limb bud (Kawakami *et al.*, 1996; Zou *et al.*, 1997; Yokouchi *et al.*, 1996). Although dominant-negative receptors could be expected to interfere with either type IA or IB signaling, there is a difference in affinity of the receptors for BMP ligands (reviewed in Yamashita *et al.*, 1996). DnA virus may therefore not be as effective in binding Bmp2, Bmp4, Bmp7, or GDF-5 as DnB. There are differences in the expression patterns of Bmps during chondrogenesis. The presumptive perichondrium expresses *Bmp2, -4,* and *-7,* whereas only *Bmp2* is expressed within the cartilage (data not shown). On the basis of this expression data, the inhibitory effects of the DnB virus may therefore be on the recruitment of cells from the perichondrium rather than on the amount of extracellular matrix produced by the chondrocytes. This is consistent with our observation that smaller, less differentiated cartilage elements formed in DnB-infected embryos. Cellular density was similar in the viral infected cartilage and the non-infected cartilage. In addition, the DnB virus did not prevent condensation formation.

Our expression data supports the idea that at early stages (stage 28), both type IA and IB receptors are used during the formation of cartilage condensations. However, once cartilage was differentiated, *BmprIA* is downregulated and *BmprIB* continues to be expressed within the cartilage. Differentiation and interstitial growth may be more dependent on the IB receptor. The two BMPRIB-knockout experiments show that *in vivo* the role of the BMPRIB is much more limited than either our expression data or our dominant-negative virus experiments would suggest (Baur *et al.*, 2000; Yi *et al.*, 2000). The homozygous null embryos develop normally with the exception of the distal phalanges. Thus, it is possible that the type IA receptor can take over the function of the type IB receptor *in vivo.* Once condensations form in the *RIB*- knock out mice, other receptors may take over the role of the type IB receptor in cartilage differentiation. The phenotype we observed in the dominant-negative RIB-infected embryos was more severe than that of the RIB-knock out mice, but this may be due to some inhibition of RIA during the earlier stages of chondrogenesis.

We report that the effect of CaA and CaB on chondrogenesis was to greatly increase the size of the cartilage, probably via increased proliferation, and to delay differentiation. Other studies have shown that the CaB virus increases proliferation and accelerates differentiation of cartilage, whereas CaA virus delays differentiation (Zou *et al.*, 1997). We did not observe any differences in the alcian blue staining between the CaA- and CaB-infected embryos, The similarity in phenotype in our studies between the two active viral constructs suggests that during normal development activated forms of the two type I receptors share similar functions in craniofacial chondrogenesis. In the CaA or CaB viral-infected cartilage, type I receptors continued to signal beyond the condensation stage and this may have sustained the immature cartilage phenotype. A second explanation for the similarity in phenotypes is that increased receptor signaling may have activated a feedback mechanism that up regulated expression of ligands. Exogenous BMPs have been shown to upregulate their own gene expression (Barlow and Francis-West, 1997), and the expression of constitutively active BMP receptors in our study led to ectopic expression of *Bmp4* and *Bmp7.* The ectopic expression of *Bmp4* and *Bmp7* may be part of the mechanism by which chondrogenesis is promoted outside the normal confines of the skeletal elements in embryos infected with constitutively active BMP receptors.

Intramembranous Bone Formation Requires BMP Receptor Signaling

The formation of intramembranous bone was inhibited by expression of DnB but not of DnA. This was best seen in the maxilla of DnB-infected embryos, where the bones were smaller or did not form. A similar inhibition of bone formation was obtained with a truncated BMPRIB expressed in NIH 2T3 cells (Chen *et al.*, 1998). The truncated BMPRIA had the opposite effect of stimulating bone matrix formation. Thus, in these experiments the type IB receptor has a different role from that of the type IA receptor. These results also demonstrate that even though dominant receptors should be able to block all receptor signaling, there are observed differences in the effects of type IA versus type IB dominant-negative constructs on bone formation. It is interesting that there are no reported skull defects in *BmprIB -/-* mice (Yi *et al.*, 2000; Baur *et al.*, 2000). The lack of a membranous bone phenotype could be due to compensation by the type IA receptor or due to signaling via other receptors such as the activin type I, II, and IIb receptors. Our data suggest that a transgenic mouse expressing a dominant-negative form of BMPRIB may give results different from those obtained with knockout mice. Our expression results, together with the Dn virus data, suggest that although both the type IA and type IB receptors were expressed in maxillary mesenchyme, the type IB receptor may have been primarily involved in the initiation of bone condensations.

The expression of CaB and CaA had similar effects on intramembranous bone formation in our study. Some of the effects were secondary to the enlargement of cartilage. Since cartilage differentiates several days before bone, this was not unexpected. In the maxillary bones, where effects are likely to be more direct, there was a disruption in trabeculation. A similar alteration in the pattern of osteogenesis is observed in long bones of the chick limb (Zou *et al.*, 1997). Examination of cell cultures infected with CaB shows more clearly that this receptor promotes bone formation in the absence of ligand (Chen *et al.*, 1998). In a three-dimensional model system such as the one we used, the ability to quantify bone is limited. However, on the basis of the histochemical staining properties, there did not appear to be substantial changes in the quality of bone.

The effect of local infection of the stage-20 maxilla with CaA or CaB was often to form an ectopic cartilage within the maxillary region. Cultures of maxillary mesenchyme grown in serum-containing medium do not form cartilage; however, in defined medium, small nodules of cartilage do develop (Richman and Crosby, 1990). These data suggest that maxillary mesenchyme has the potential to form cartilage but that this is normally inhibited in the presence of serum. Active BMP receptor signaling bypassed the inhibitory influences present in the maxilla and persuaded mesenchyme cells to form cartilage *in vivo*.

BMP Receptor Pathways Mediate Membranous Bone Formation via the* Msx *and* Fgfr2 *Genes

Our data suggest that there is an interaction between Bmp receptor signaling and Fgfr2 signaling. *Fgfr2* is ectopically expressed in the medial maxillary prominence in embryos with

constitutively active viral infection. This region of the maxilla gives rise to the palatal shelves. Other expression data also show that *Fgfr2* is expressed in the membranous bone of the skull. (*Cek-3,* Iseki *et al*., 1997). The important role that this receptor plays in membranous bone formation is demonstrated in syndromes with constitutively active mutations in *FGFR2* (Crouzon and Pfeiffer syndromes Cohen, 2000). The phenotype includes severe midface hypoplasia and craniosynostosis. It is not clear from our data whether misexpression of constitutively active type I BMP receptors is affecting the function of *Fgfr2.* However, having *Fgfr2* in ectopic regions may result in a localized gain of function.

Our study is the first to link BMP receptor signaling and changes in *Msx* gene expression to the effects on intramembranous bone formation. The link between *Msx2* and intramembranous bone formation has been made in the developing calvaria. Haploinsufficiency of MSX2 in humans leads to deficient ossification of the calvaria (Wilke *et al*., 2000), whereas increased activity of the *MSX2* gene leads to craniosynostosis (Jabs *et al*., 1993). In mice, the lack of *Msx2* leads to a deficiency in the calvaria (Satokata *et al*., 2000). Over expression of the murine *Msx2* gene in the osteogenic front of the sutures leads to increased bone formation in the sutures, although synostosis did not occur (Liu *et al*., 1999). Culture of osteoblasts from these mice shows that *Msx2* is downregulated in differentiated bone nodules (Dodig *et al*., 1999). *Msx2* overexpression in chicken calvarial cells delays differentiation and instead increases the osteoblastic population (Dodig *et al*., 1999). *Msx1* is also associated with membranous bone formation, although primarily in the facial bones rather than in the sutures. *Msx1* knockout mice have cleft palate, deficient mandibles, and absent premaxillas (Satokata and Maas, 1994). Thus, *Msx1* has some unique functions in bone formation in the face, but in the sutures, *Msx2* can compensate for the lack of *Msx1*. In *Msx1*$^{-/-}$, *Msx2*$^{-/-}$ double mutants, the phenotype is additive in that the mandible, maxilla, and calvaria are all severely hypoplastic (Satokata *et al*., 2000). Kim *et al*. (1998) have shown that BMP application increases expression of *Msx1* and *Msx2* and increases the amount of tissue between sutures. They do not identify the cells contributing the increased tissue; however, they do show that *Msx1* is downstream of BMP in a region that has intramembranous bone.

Our data showing ectopic expression of *Msx1* and *Msx2* in the maxilla due to infection with constitutively active viruses are consistent with the idea that both Msx genes have roles in intramembranous bone formation. Previously, a decrease in *Msx2* expression in the interdigital region of the limb was observed following infection with DnB virus (Zou and Niswander, 1996). While we do not see a similar effect in the face with DnB virus, we are the first to show a relationship between active BMP receptor signaling and *Msx1*, *Msx2* gene expression. We can conclude from our data that a large disruption in the expression domain of *Msx2* and *Msx1* does little to affect the overall morphology of maxillary bones. Rather, the effect of misexpression may be to delay the rate of osteoblast differentiation (as with overexpression of *Msx2*; Dodig *et al*., 1999). It will be interesting to trace the relationship between BMP type I receptors, intermediary signals, and *Msx* gene transcription. There is at least one homeobox gene that specifically interacts with BMP receptor-activated Smads (Smad1-*Hoxc8;* Shi *et al*., 1999), and it is possible that others will be discovered in the near future.

Finally, our data offer a mechanism by which exogenous BMPs induce Msx expression (Barlow and Francis-West, 1997; Wang *et al*., 1999). All facial mesenchyme expresses at least one type I BMP receptor along with the type II receptor (also at earlier stages, M.A. Ashique unpublished data). Our findings fit with the ability of BMPs to induce or expand Msx expression in a variety of facial tissues and highlight the ability of both receptors to regulate *Msx* expression *in vivo*.

Increased BMP signaling inhibits formation of ectodermal specializations

The ectopic expression of caBMPRIA or IB reduced or eliminated feather formation and instead caused random keratin deposits in the epithelium. Our expression data showed that the type IB receptor was not expressed in surface ectoderm or in the egg tooth. Infection with CaB virus showed that excess RIB signaling disrupted ectodermal patterning, although differentiation was not affected. A similar result was obtained when BMP2 was applied to the healing epidermis of a lamb fetus. A scar formed due to increased keratinization (Stelnicki *et al*., 1998). In an avian model, the application of BMP4 suppresses differentiation of feather buds (Jung *et al*., 1998). Our data confirm that both BMP type I receptors are able to mediate this response. A different gene pathway is also involved in feather germ formation. The *notch* receptor and its ligands, *delta* and *serrate,* are expressed in a dynamic fashion during patterning of the feather array and differentiation of the feather buds (Crowe *et al*., 1998). Misexpression of Delta-1 leads to an inhibition of *Bmp2*, *Bmp4,* and *Bmp7* expression and a loss of feathers in virally infected skin (Crowe *et al*., 1998). Interestingly, the phenotype differs slightly from that of CaA- or CaB-infected embryos. The ectoderm in our experiments formed randomly distributed keratin plaques, whereas Delta-1 expression leads to smooth skin without accumulations of keratin.

We conclude from our experiments that the embryo can use either the type IA or type IB receptor for chondrogenesis, osteogenesis, and formation of ectodermal specializations. *In vivo,* however, there may be a preference for using one receptor over another in certain locations and at certain stages of development. BMP signaling is critical for embryo development, and therefore it is necessary to build some redundancy into the system. In the present study, we have demonstrated that type I receptors are functionally equivalent when active, but, when activity is decreased, it is primarily the type IB receptor that is required for chondrogenesis and osteogenesis.

Materials and Methods

Chicken Embryos

Fertilized white leghorn chick eggs were obtained from Coastline Chicks, Abbotsford, B.C., Canada, and specific-pathogen- Hyline chick fertilized eggs were obtained from Hyvac Corp., Adel, Iowa, USA. Eggs were incubated at 37.5°C until they reached the desired stage of development.

In Situ *Hybridization*

The following chicken cDNAs were kindly provided by the following individuals: RCASBP *pol* (L. Niswander); *Msx1* and *Msx2* (S.E. Wedden); *Fgfr2* (*Cek-3,* E. Pasquale); *BmprIA* and *BmprIB* (L. Niswander); *BmprII* (T. Nohno); *Bmp2* and *Bmp4* (P. Brickell, P. Francis-West); and *Bmp7* (B. Houston). Antisense riboprobes labeled with [^{35}S]-UTP were prepared as described by Matovinovic and Richman (1997).

Autoradiographic In Situ *Hybridization*

Specimens used for *in situ* hybridization were dissected in fresh phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA), and processed into wax. RNA *in situ* hybridization

with ^{35}S-labeled RNA probes on wax sections were done as described by Matovinovic and Richman (1997). Serial sections were divided up among several slides so that expression patterns for different genes could be compared within the same embryo. Slides were left to develop between 1 and 2 weeks. Following hybridization and development of the emulsion, sections were counter stained with 0.05% malachite green. Photographs were taken on a Zeiss compound microscope (Germany) under dark-field or brightfield illumination with a Minolta RD175 digital camera. Plates were composed with Adobe Photoshop (Adobe Systems Inc., CA).

Histology

Stage-30 to -32 embryos were fixed in 4% PFA overnight, embedded in wax, sectioned, and stained. Sections were dewaxed, processed to water and stained in 1/3 diluted hematoxylin stain (Gill's formulation #2: 0.4% hematoxylin, 0.04% sodium iodate, 7% aluminum sulfate, 4% glacial acetic acid, 25% ethylene glycol) for 1 min. Gentle rinsing under tap water was done for 10 min, and then the sections were stained in alcian blue (0.5% alcian blue 8G, 0.005% thymol, 0.5% glacial acetic acid) for 10 min. Sections were then rinsed in water for 5 min, dehydrated, and mounted.

The stage-38 embryos were first decalcified in a solution of 10% formaldehyde and 5.5% EDTA for at least 6 months. Embryos were then embedded and sectioned and stained with 0.1% picosirius red for bone (0.1% sirius red F3B in saturated picric acid) and 1% alcian blue for cartilage (1% alcian blue in 1% acetic acid).

Virus Infection

Replication-competent RCASBP virus was grown up using methods described by Zou *et al.* (1997). RCASBP (A) virus containing genes for the following receptors were injected into embryos: human wild-type (wt) BMPR-1A, human dominant-negative (DnA) BMPR-1A, human constitutively active (CaA) BMPR-1A (human alk-3 cDNAs originally provided by P. ten Dijke), chick wtBMPR-1B, chick dnBMPR-1B (DnB), chick caBMPR-1B (CaB; Zou and Niswander, 1996; all BMP receptor viruses generously provided by L. Niswander). Human placental alkaline phosphatase (PLAP) cloned into RCASBP (E) was provided by S. Hughes. A Picospritzer (General Valve Corp.) with electrolytically pulled needles was used to deliver the virus to the embryo. In stage-15 and -20 embryos, the vitelline membrane was torn prior to injection.

In order to determine injection sites that would give reproducible viral spread, several experiments were done using the PLAP-containing virus. The stage-10 embryos injected adjacent to the mesencephalon resulted in viral infection in the upper and lower beaks with occasional bilateral spread. Stage-16 embryos injected into the presumptive maxillary region or stage-20 embryos injected directly into the maxillary prominence gave unilateral spread in the upper beak. All embryos injected with the PLAP virus had a completely normal phenotype, thus confirming that the virus itself does not affect development. A second group of embryos was injected with DnB or CaB virus at stage 14/15 and then allowed to develop for 3-4 days. These embryos were sectioned and hybridized to the *pol* probe in order to detect viral spread. The viral spread was primarily unilateral and in the upper beak only.

A third group of virus-infected embryos was incubated until they reached stage 38, and then skeletal morphology was assessed. Approximately 50% of embryos used for the characterization of the stage-38 phenotype were pathogen-free. There was no difference in the morphology between pathogen-free and pathogen-containing chick embryos.

Skeletal Staining

Stage 38 embryos with skin and eyes removed, were fixed in 100% ethanol for 4 days followed by 100% acetone for 4 days. Embryos were rinsed in water briefly and then stained for 10 days in alizarin red/ alcian blue solution (1 vol. 0.3% alcian blue 8GX in 70% ethanol, 1 vol. 0.1% alizarin red S in 95% ethanol, 1 vol. acetic acid, 17 vol 70% ethanol). Specimens were then cleared at room temperature in 20% glycerol/ 2% KOH solution for 1 week. Specimens were processed through an increasing series of glycerol/H_2O solutions to clear them, and then they were stored in 80% glycerol/20% H_2O.

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