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Original Article

The *in vivo* and *in vitro* effects of Bone Morphogenetic Protein-2 on the development of the chick mandible

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ABSTRACT **During embryonic development, neural crest derived mesenchymal (ectomesenchymal) cells in the chick mandible give rise to cartilage and membrane bone. Signaling molecules involved in the development of the mandible are less understood. To examine whether BMP-2 is involved in morphogenesis and growth of the mandible *in vivo*, agarose beads, loaded with BMP-2 at concentrations of 5 to 150 ng/μl were implanted into the mandible at HH stage 22 and embryos were maintained in shell-less culture. To examine whether BMP-2 is involved in osteogenic or chondrogenic differentiation, mandibular ectomesenchyme from HH stage 22 embryos was cultured in the absence of mandibular epithelium, but in the presence of BMP-2 or BMP-2 and/or type IV collagen. Chondrogenesis and osteogenesis were examined by histological, histochemical and immunohistochemical methods. Implantation of BMP-2-containing beads *in vivo* retarded mandibular growth and morphogenesis in a dose-dependent manner. BMP-2 induced localized death of ectomesenchymal cells in the vicinity of the implanted bead and in proportion to the concentration of BMP-2 applied. Neither BMP-2 alone, nor BMP-2+collagen type IV, was sufficient to initiate osteogenesis *in vitro* in the absence of epithelium. BMP-2 inhibited chondrogenesis both *in vivo* and *in vitro*. Cartilage morphology was rod-like in the absence of BMP-2 but nodular in ectomesenchyme cultured in the presence of BMP-2. These results are discussed in relation to the stimulatory and inhibitory effects of BMPs on skeletal development.**

KEY WORDS: *bone morphogenetic protein, mandible, chondrogenesis, osteogenesis*

Introduction

The mandibular skeleton of the chick is derived from migratory neural crest cells. Ectodermal in origin, but mesenchymal in nature, they are known as ectomesenchymal cells. During early stages of development (beginning at 5.5 days of incubation; Hamburger and Hamilton (1951) stage 26), ectomesenchymal cells produce a single cartilaginous rod — Meckel's cartilage — in the core of each half of the mandibular arch. From 7 to 7.5 days of incubation (HH stage 31) onwards, ectomesenchymal cells give rise to membrane bone by direct osteogenesis. By day 14, membrane bone surrounds Meckel's cartilage along the length of the lower jaw (Romanoff, 1960; Murray, 1963).

It has been shown previously that epithelial-mesenchymal interactions play key roles in inducing both chondrogenesis and osteogenesis from mandibular ectomesenchyme (Hall, 1987b, 1988a, 1994). The epithelial-mesenchymal interaction that initiates chondrogenesis in the chick occurs during migration of neural crest cells from the neural tube (Hall and Tremaine, 1979; Bee and Thorogood, 1980). As evident from chondrogenesis in clonal cell culture, by the time they reach the mandibular region, a sub population of cells can undergo chondrogenesis independent of the mandibular environment (Ekanayake and Hall, 1994a). There is a lag of about 4 days between the time neural crest cells populate the mandibular region (HH stage 15-16) and when they express the chondrocyte phenotype (HH stage 26). The molecules associated with determination of these cells as prechondrogenic, or that allow them to differentiate, are not completely known, but include *Msx*-1, BMP-2, -4, and -5, TGF-ß1, N-CAM and tenascin (Hall, 1994; Richman, 1994; Hall and Miyake, 1995).

Unlike chondrogenesis, osteogenesis depends on interactions between mesenchyme and mandibular epithelium that continue to HH stage 24 (Hall, 1978). When mandibular ectomesenchyme from embryos younger than HH stage 24 is cultured in the absence of mandibular epithelium, cartilage forms, but, bone does not. In the presence of epithelium both bone and cartilage form (Hall, 1978; Hall *et al.*, 1983). Although molecules involved in the osteoinductive interaction are not completely known, signals localized in the basement membrane mediate the interaction which is therefore matrix-mediated (Hall *et al.*, 1983), and epidermal growth factor and NaF can promote osteogenesis *in vitro* (Hall, 1987a, 1992, 1994).

Growth of the mandibular process depends both on an epithelium being present and on interaction between epithelium and

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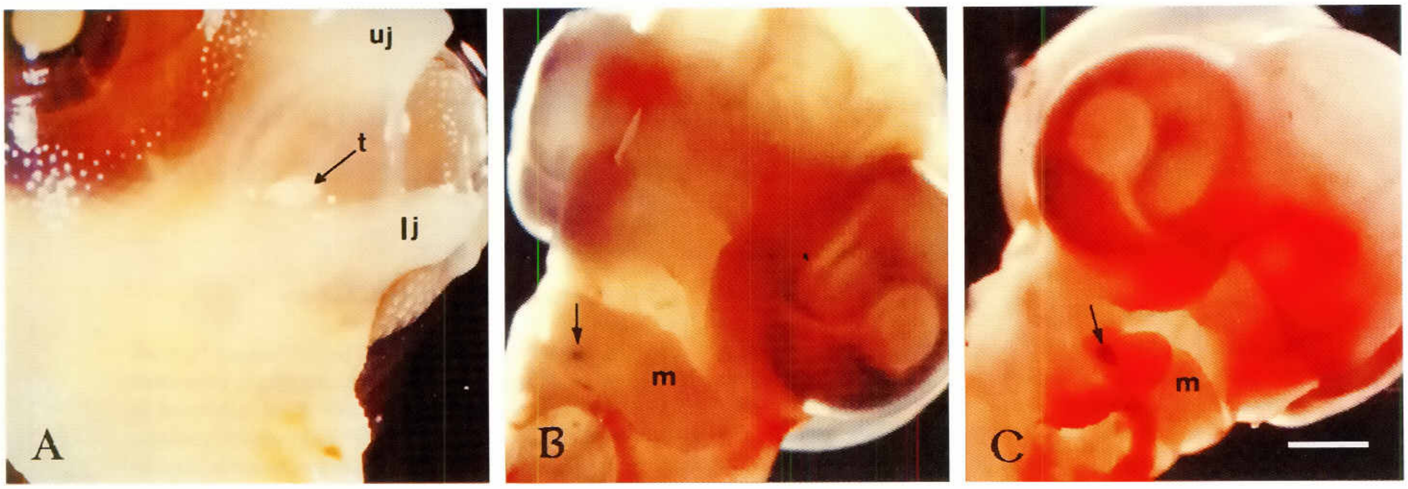

Fig. 1. Effect of local application of BMP-2 on mandibular development *in vivo*. *Affi-Gel blue agarose beads loaded with BMP-2 were implanted into one side of the mandible at HH stage 22 and embryos were maintained in shell-less culture.* **(A)** *The face of an embryo that received a bead loaded with BMP-2 at a concentration of 50 ng/µl, and was cultured for 6 days. The mouth remains open due to the asymmetry of the lower jaw as seen in C. Abbreviations: lj, lower jaw; t, tongue; uj, upper jaw.* **(B)** *The face of a control embryo that received a bead without BMP-2, and was cultured for 1 day. Note the location of the bead (arrow). Both sides of the mandibular process are equal in size.* **(C)** *The face of an embryo that received a bead loaded with BMP at a concentration of 75 ng/µl, and was cultured for 1 day. The implanted side of the mandibular process is smaller than the unimplanted side. Arrow indicates the location of the bead. Bar: A, 1 mm; B-C, 0.8 mm.*

mesenchyme (Wedden, 1987; Richman and Tickle, 1989; Mina *et al.,* 1994; Richman, 1994). *Msx*-1 is upregulated by epithelial-mesenchymal interaction (Takahashi *et al.*, 1991; Phippard *et al.*, 1996; Watanabe and Le Douarin, 1996), downregulated where such interactions fail to occur, as in the diastema of rodent dentition (Tureckova *et al.*, 1995), and may mediate mandibular growth (Mina *et al.*, 1995). Signals regulating growth are reciprocal: mandibular epithelium regulates proliferation of mesenchyme, while mesenchyme regulates epithelial proliferation (Hall and Coffin-Collins, 1990; Minkoff, 1991).

Recent studies indicate that BMP-2 and BMP-4, the BMPs related to the *Drosophila* decapentaplegic (DPP) gene, play important roles in epithelial-mesenchymal interactions during embryonic development (Lyons *et al.*, 1990; Jones *et al.*, 1991; Vainio *et al.*, 1993; Bitgood and McMahon, 1995; Phippard *et al.*, 1996), are involved in induction of mesoderm and neural ectoderm (see, for example, Jones *et al.*, 1992; Hawley *et al.*, 1995; Liem *et al.*, 1995), and, along with other growth factors, can regulate the fate of neural crest cells and their derivatives (Hall and Ekanayake, 1991; Reissmann *et al.*, 1996; Shah *et al.*, 1996).

BMPs were initially identified as the cartilage and bone inducing activity present in demineralized bone matrix (Urist, 1965; Sampath and Reddi, 1981; Wang *et al.*, 1988, 1990; Wozney *et al.*, 1988; Celeste *et al.*, 1990, 1994; Ozkaynak *et al.*, 1992). Eight of the nine BMPs reported to date (BMP-2-9) belong to the TGF-ß superfamily. [BMP-1 has now been shown to be a type 1 procollagen C-proteinase that cleaves the COOH-propeptides of procollagens I-III (Kessler *et al.*, 1996)]. When implanted at ectopic sites in pre- and postnatal animals, BMP-2 and -4, as well as other TGF-ß-related BMPs, induce cartilage which undergo endochondral ossification (Wang *et al.*, 1990; Wozney, 1992, 1993; Hirota *et al.*, 1994; Watanabe and Le Douarin, 1996).

When introduced *in vitro*, DPP-related BMPs stimulate cartilage formation by chick limb bud mesenchymal cells and established lines of multipotential progenitor cells (Chen *et al.*, 1991a,b, 1992; Ahrens *et al.*, 1993; Wang *et al.*, 1993; Luyten *et al.*, 1994), and enhance chondrogenesis by chondrocyte cultures. BMPs also enhance osteoblast differentiation in established lines of multipotential progenitor cells and primary cultures of rat osteoblasts (Yamaguchi *et al.*, 1991; Ohta *et al.*, 1992; Ahrens *et al.*, 1993; Wang *et al.*, 1993; Amédée *et al.*, 1994; Rickard *et al.*, 1994; Centrella *et al.*, 1995; Yamaguchi, 1995).

The first mutant involving BMP has been identified in the *short ear* mouse, in which a large portion of the BMP-5 gene is deleted. Action at the prechondrogenic condensation stage results in short ears, missing ribs and absence of the xiphoid process of the sternum (Kingsley *et al.*, 1992; King *et al.*, 1994; Kingsley, 1994; Hall and Miyake, 1995). The *Talpid (ta^3)* mutation in the chick has

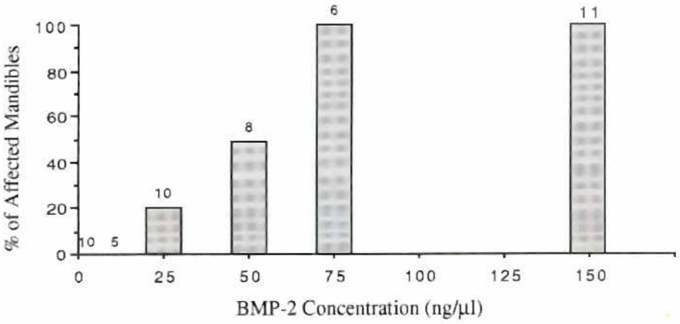

Fig. 2. Percentage of embryos with affected mandibles following implantation of agarose beads loaded with different concentrations of BMP-2 as evident by whole-mount analysis. *Mandibular malformation is proportional to BMP concentration. All control embryos that received a bead without BMP-2 or a bead loaded with BMP-2 at 10 ng/µl are unaffected. Sample size is shown above each bar.*

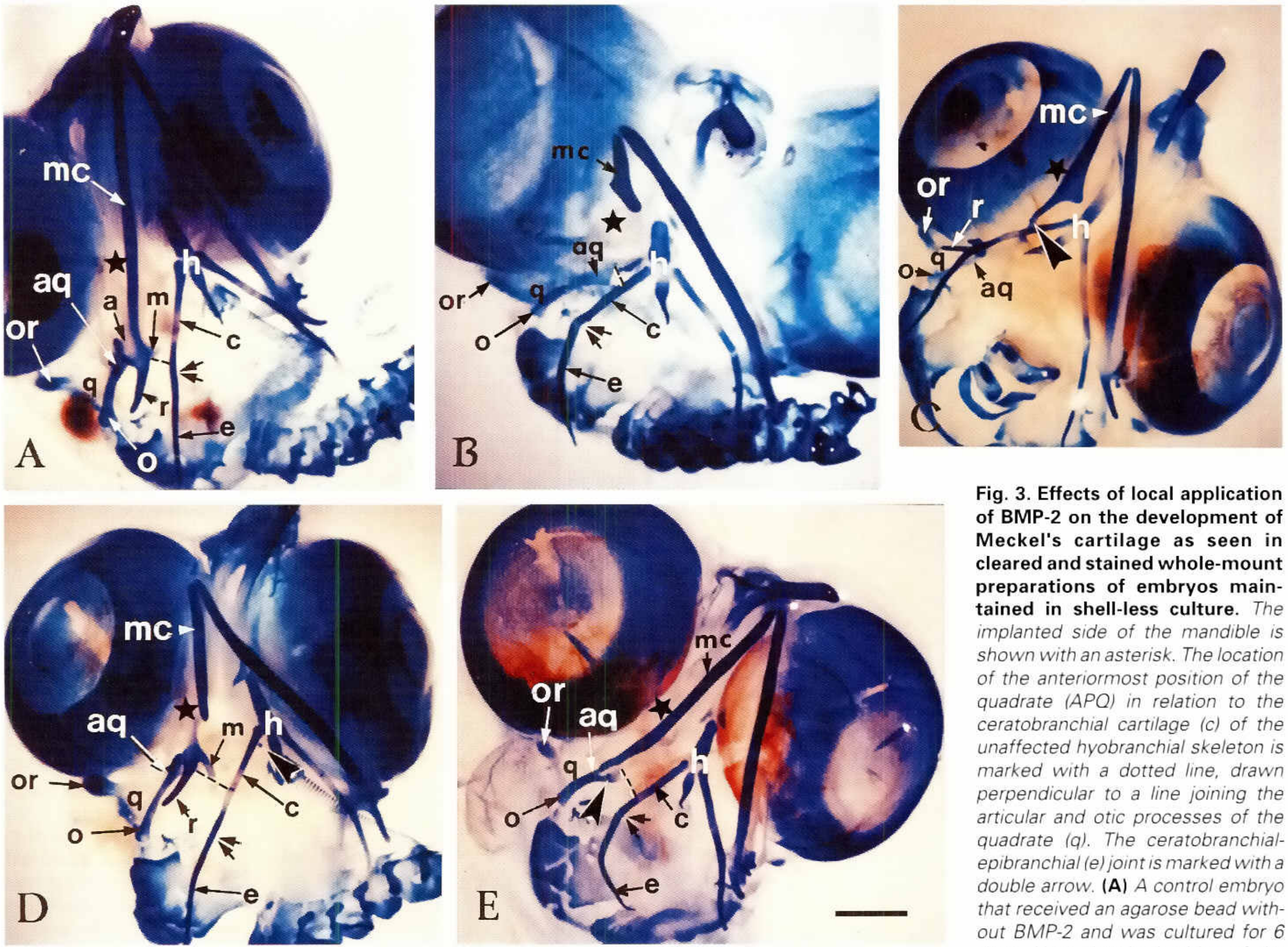

Fig. 3. Effects of local application of BMP-2 on the development of Meckel's cartilage as seen in cleared and stained whole-mount preparations of embryos maintained in shell-less culture. *The implanted side of the mandible is shown with an asterisk. The location of the anteriormost position of the quadrate (APQ) in relation to the ceratobranchial cartilage (c) of the unaffected hyobranchial skeleton is marked with a dotted line, drawn perpendicular to a line joining the articular and otic processes of the quadrate (q). The ceratobranchial-epibranchial (e) joint is marked with a double arrow.* **(A)** *A control embryo that received an agarose bead without BMP-2 and was cultured for 6 days. Note that both sides of the mandible are equal in size. Implanting the bead has not affected development of Meckel's cartilage (mc). The anteriormost position of quadrate is close to the ceratobranchial-epibranchial joint.* **(B,C,D and E)** *Embryos that received an agarose bead loaded with BMP-2 at a concentration of 150, 75, 50, or 25 ng/µl, respectively. Embryos in* **(C-E)** *were maintained in shell-less culture for 6 days, the embryo in* **(B)** *for 4 days. Note that in* **(B)** *Meckel's cartilage (mc) on the implanted side is drastically shortened. Articulation processes at the proximal end of the cartilage are almost completely missing. The quadrate (q) is shifted forward. The APQ is more anterior, further from the ceratobranchial-epibranchial joint. Bone has not differentiated at this stage. In* **(C)** *the implanted bead is visible (arrowhead). Meckel's cartilage (mc) is shorter than on the unimplanted side because the proximal region has failed to form. The retroarticular process (r) at the proximal tip of Meckel's cartilage is present, but malformed.* **(D)** *The implanted bead can be seen (arrowhead), however, it has fallen from the implanted position to a place near the hyobranchial skeleton (h) during clearing and staining. A portion of the shaft of Meckel's cartilage (mc) is missing. The proximal articulation processes of Meckel's cartilage are all present. Morphology of the quadrate (q) has not been affected, although the quadrate has moved forward, placing the APQ at a more anterior level, in line with the middle of the ceratobranchial cartilage (c).* **(E)** *The bead (arrowhead) can be seen at the proximal end of Meckel's cartilage (mc). The shaft of Meckel's cartilage is less affected. The articulation processes at the proximal end of Meckel's cartilage are all missing. However, the morphology of the adjacent quadrate (q) is not affected. The quadrate is slightly shifted forward and the APQ is somewhat further from the ceratobranchial-epibranchial joint than in the control embryo shown in* **(A)**. *In all figures: a, articular facet of Meckel's cartilage; aq; articular process of quadrate; c, ceratobranchial cartilage; e, epibranchial cartilage; h, hyobranchial skeleton; m, medial process of Meckel's cartilage, o, otic process of quadrate; or, orbital process of quadrate; q, quadrate; r, retroarticular process of Meckel's cartilage. Bar: A,C,D, 2.5 mm; B, 1.6 mm; E, 2.2 mm.*

also recently been shown to be defective in BMPs and *sonic hedgehog*, a patterning gene upstream of BMP (Francis-West *et al.*, 1995). *Sonic hedgehog* is also expressed in the mouse mandible concomitant with epithelial-mesenchymal interaction (Kronmiller *et al.*, 1995).

Most information on the role of BMPs on embryonic development comes from the localization of expression sites of mRNAs by *in situ* hybridization. In addition to localization in developing cartilage and bone, BMPs, especially BMP-2 and 4, are expressed at a number of embryonic sites where epithelial-mesenchymal interactions occur (Lyons *et al.*, 1989, 1990, 1995; Jones *et al.*, 1991; Francis-West *et al.*, 1994; Bitgood and McMahon, 1995; Dudley *et al.*, 1995; Liem *et al.*, 1995; Luo *et al.*, 1995). Exogenous introduction of BMP-4 induces expression of transcription factors by dental mesenchyme in a manner similar to that caused by dental epithelium (Vainio *et al.*, 1993). Moreover, BMP-2 is expressed in the

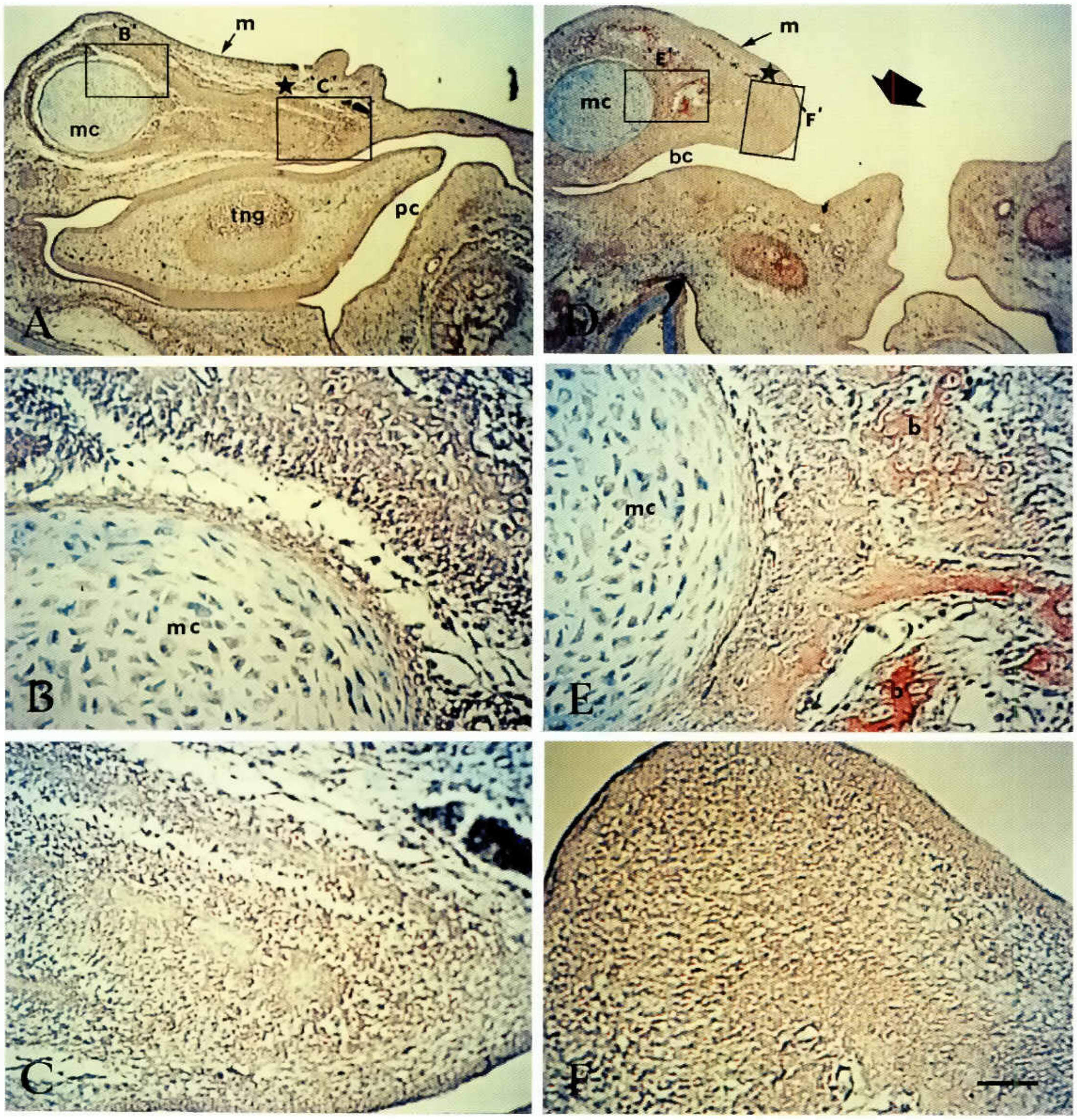

Fig. 4. Histological analysis of the head of an embryo with a bead loaded with 75 ng/μl BMP-2 implanted on one side of the mandible (asterisk) and maintained in shell-less culture for 8 days. *Age of the embryo is equivalent to 12 days in ovo. The head of the embryo was serially sectioned and stained with Alcian blue for cartilage and direct red for bone.* **(A)** *A section through the pharynx showing the proximal end of the mandible (m). Note that Meckel's cartilage (mc) is present only on one side of the jaw. Areas marked with 'B' and 'C' are enlarged in* **(B and C)**. *tng, tongue; pc, pharyngeal cavity.* **(B)** *Unimplanted side of the mandible with Meckel's cartilage (mc). Mandibular bones are absent at the level of pharynx.* **(C)** *Implanted side of the mandible. Both cartilage and bone are absent. Only loosely arranged mesenchymal cells are present in the area.* **(D)** *A section through a more distal region of the mandible (m) of the same embryo. Meckel's cartilage (mc) is present only in the unimplanted side, producing an asymmetrical jaw, leaving the mouth open (arrow). The areas marked with 'E' and 'F' are enlarged in E and F. bc, buccal cavity.* **(E)** *Unimplanted side of the mandible showing Meckel's cartilage (mc) and differentiating membrane bone (b) around it.* **(F)** *Implanted side of the mandible. Neither cartilage nor bone are present. Bar: A,D, 250 μm; B,E,F, 38 μm; C, 50 μm.*

basement membrane of both embryonic chick and mouse mandibular epithelium or cranial ectoderm at times consistent with a role in epithelial-mesenchymal signaling (Hall, 1988a, 1994; Lyons *et al.*, 1989; Francis-West *et al.*, 1994; Bennett *et al.*, 1995; Liem *et al.*, 1995). Furthermore, BMP-3, -4, and -7 bind with high affinity to type IV collagen, the main collagen in epithelial basement membrane (Paralkar *et al.*, 1990, 1992; Vukicevic *et al.*, 1994).

To examine the action(s) of BMP-2 on mandibular development, morphogenesis, and growth *in vivo*, agarose beads loaded with recombinant human BMP-2 were implanted into one side of the mandibular arch of chick embryos at HH stage 22. Human, mouse and chick BMP-2 share some 80% amino acid identity (Francis *et al.*, 1994). The embryos were established in shell-less culture, and examined up to 8 days later for skeletal tissue differentiation, morphogenesis, growth, and cell death in the mandible. To examine whether BMP-2 could substitute for the mandibular epithelium and induce osteogenesis in mandibular ectomesenchyme or enhance chondrogenesis from prechondrogenic cells, mandibular ectomesenchyme from HH stage 22 chick embryos was cultured, without epithelium, on a substrate coated with BMP-2 alone, or with BMP-2 and type IV collagen.

Results

Effect of local application of BMP-2 in vivo

Whole-mounts

BMP-2 was applied locally by implanting agarose beads loaded with BMP-2 into one side of the mandibular arch. The bead was placed in the proximal region of the mandibular process and embryos were maintained in shell-less culture for up to 8 days. As controls, agarose beads loaded with BSA in PBS were implanted.

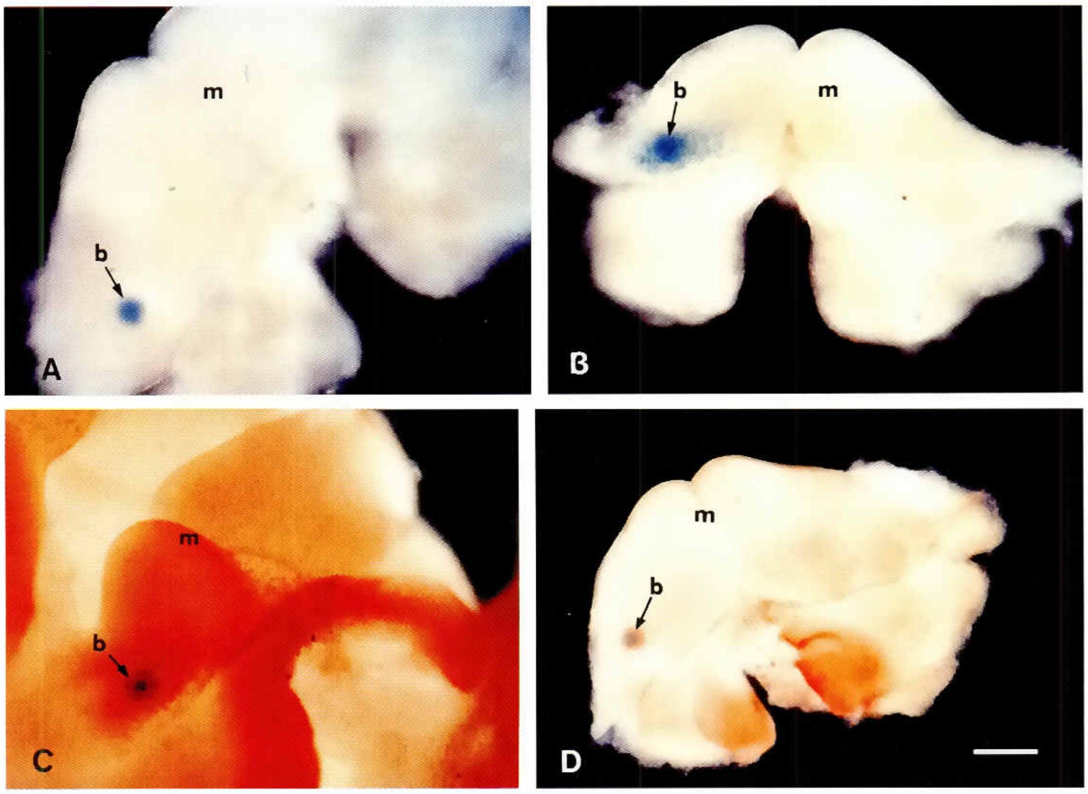

Fig. 5. Cell death analysis by Nile blue sulfate (A and B) and neutral red (C and D) of embryos implanted with control or BMP-2-loaded beads and maintained in shell-less culture for 1 day. (A) *The mandible of a control embryo with an implanted bead (b) without BMP-2. There is no cell death around the bead.* **(B)** *The mandible of an embryo with an implanted bead loaded with 50 ng/µl BMP-2. Blue granular staining indicates cell death, which is localized to the site of the bead.* **(C)** *The mandible of an embryo with an implanted bead loaded with 75 ng/µl BMP-2. Red granular staining adjacent to the bead (b) indicates cell death. The implanted side of the mandible is much smaller than the unimplanted side.* **(D)** *The mandible of an embryo with an implanted bead (b) loaded with 25 ng/µl BMP-2. Cell death is evident, but to a lesser degree than in embryos treated with higher concentrations of BMP-2 (compare* **D** *to* **B** *and* **C***). There is no recognizable size difference between implanted and unimplanted sides of the mandible. Bar: A, 312 µm; B,D, 390 µm; C, 500 µm.*

As beads were only implanted on one side, the contralateral arch served as an internal control.

Morphogenesis and growth of the mandibles in control embryos was normal. However, embryos that received agarose beads loaded with BMP-2 had jaw malformations on the implanted side; the unimplanted side was normal. The implanted side of the jaw was shorter, the mandible deviated to one side, and the mouth was open (Fig. 1A). The upper jaw and the rest of the face was normal, i.e. the effect was localized to the region implanted with the bead. The effect of BMP was directly related to the concentration used to load the beads (Fig. 2). At concentrations of 75-150 ng/µl, all embryos were affected. At 50 ng/µl, 50% of implanted embryos had malformed jaws. At concentrations of 10 ng/µl or less, all embryos had normally developing jaws.

The effect of BMP-2 was apparent within one day. At this time, the left and right mandibular processes of embryos implanted with beads loaded with BSA were equal in size. In embryos with a bead loaded with a high concentration of BMP-2, the implanted side of the mandibular process was very small in comparison with the unimplanted side (compare Fig. 1B and C). Implantation of beads treated with either BSA or BMP-2 did not cause an inflammatory response by mandibles (Fig. 1B and C). Vascular system is not well developed in the mandible at the age (HH stage 22) that the implantations were performed.

Older embryos that were cleared and stained with Alcian blue and alizarin red as whole-mounts indicated that Meckel's cartilage was largely affected by exogenous BMP-2. Meckel's cartilages in control embryos implanted with beads loaded with BSA were normal (Fig. 3A). Embryos implanted with BMP-2-loaded beads had major deficiencies in Meckel's cartilages and other elements of the mandibular arch skeleton on the implanted but not on the unimplanted side. The effect was concentration dependent (compare Fig. 3B and D). Variation in the degree of malformation among individuals in the same treatment group was attributed to variation in the size of individual agarose beads.

In all affected embryos, Meckel's cartilage in the implanted side was shorter than on the unimplanted side. The bead was implanted proximally and malformation was most evident at the proximal end of Meckel's cartilage. In extreme cases (embryos with beads treated with BMP-2 at 150 ng/µl), the articulating elements of Meckel's cartilage (retroarticular process, medial process and the facet that articulates with the articular process of the quadrate) were completely missing and Meckel's cartilage was a very small rod restricted to the distal end of the jaw (Fig. 3B). In embryos that received beads loaded with 50-75 ng/µl BMP, Meckel's cartilage in the implanted side was shorter than on the unimplanted side. The articulation elements and shaft of the cartilage adjacent to the bead were malformed to different degrees (Fig. 3C,D). In embryos that received beads treated with 25 ng/µl BMP-2, effects on the length of Meckel's cartilage were less dramatic. However, the articulation was malformed (Fig. 3E).

Although morphology of the quadrate that articulate with Meckel's cartilage was unaffected, it appeared to be displaced anteriorly, forming an abnormal articulation with the shortened and deformed Meckel's cartilage.

Displacement of the quadrate was examined by comparing the location of the anteriormost position of the quadrate (APQ) relative to the ceratobranchial-epibranchial joint of the adjacent

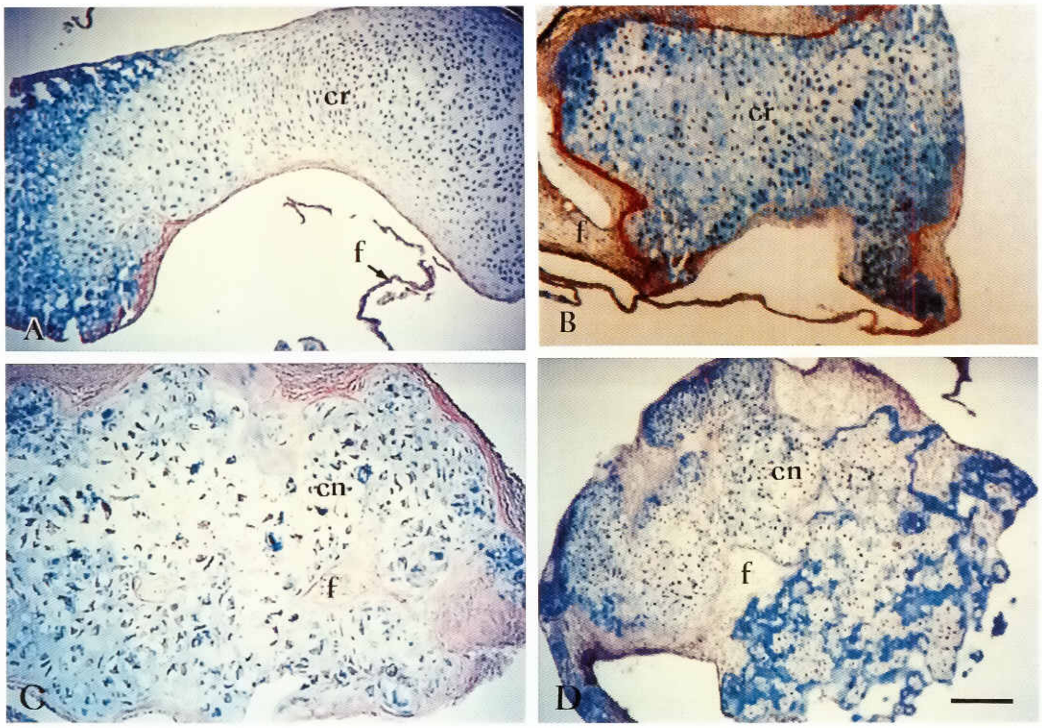

Fig. 6. Histological analysis by Alcian blue and direct red staining of HH stage 22 mandibular ectomesenchymal explants cultured with or without BMP-2 for 15 days. (A) *Control, cultured on tissue culture plastic. Ectomesenchymal cells have given rise to a rod shaped cartilage (cr), mimicking in vivo development. Membrane bone is absent. A thin layer of fibroblasts (f) is present at the periphery.* **(B)** *Control, cultured in culture plates coated with type IV collagen. These explants also produce a rod-like cartilage (cr). Membrane bone is also absent, but there are more fibroblasts (f) at the periphery.* **(C)** *Ectomesenchyme cultured in plates coated with BMP-2. Cells have produced cartilage nodules (cn). The periphery and internodular spaces are occupied by fibroblasts (f). Membrane bone is absent.* **(D)** *Ectomesenchyme cultured in plates coated with BMP-2 and type IV collagen. Similar to C, cells have produced nodular cartilage (cn) interspersed with fibroblasts (f). Membrane bone is absent. Bar: A, 130 μm; B, 155 μm; C, 80 μm; D, 300 μm.*

hyobranchial skeleton among control and BMP-treated embryos. The APQ was projected onto the ceratobranchial cartilage by drawing a line from the APQ perpendicular to the axis that goes through the articular and orbital processes of the quadrate, as shown in Figure 3A,B,D and E. In control embryos, APQ is at a level very close to the hyobranchial-epibranchial joint. In BMP-2 treated embryos, the APQ is projected more anteriorly on the ceratobranchial cartilage. Anterior displacement of the quadrate was greater with increasing concentrations of BMP-2. The adjacent hyobranchial skeleton, including the median copula and laterally extended ceratobranchial and epibranchial cartilages, were unaffected by BMP-2.

In neither control nor in BMP-2-treated embryos could mandibular bones be visualized by alizarin red-staining of whole-mounts of embryos maintained in shell-less culture up to 8 days. The Ca^{++}-deficient environment in shell-less culture is known to delay mineralization (Jacenko and Tuan, 1986b; Tuan *et al.*, 1991).

Tissue organization evident by histological analysis

Histological examination of serial sections of the heads of embryos implanted with beads loaded with BMP-2 provided information about the effect of BMP-2 on cell and tissue organization in the mandible. The results are in accordance with findings from the whole-mounts.

In the unimplanted side, a rod-like cartilage was present, running along the whole length of the mandible. In transverse sections stained with Alcian blue, it appeared as a blue-stained circular cartilage consisting of polygonal cells embedded in extracellular matrix. Figure 4 illustrates two such transverse sections taken at the level of the pharynx and the buccal cavity of a BMP-2-treated embryo, showing both the unimplanted and implanted sides. Cartilage was absent in the BMP-2-treated side of the mandible in the proximal region where the bead had been implanted (Fig. 4A,C,D and F). In this region cells appeared mesenchymal and loosely organized. They were not organized into any particular structure (Fig. 4C,F).

Membrane bone was also absent in the side of the mandible implanted with a BMP-soaked bead, where cartilage was absent. In contrast, developing membrane bone (red staining areas in Fig. 4D and E) could be seen in the unimplanted side. Bone surrounded the cartilage along most of its length except for the most proximal (pharyngeal) region

Cell death

Since BMP-2 reduces the size of the mandibular process within one day after implantation (Fig. 1C), we examined whether malformation of Meckel's cartilage and surrounding bones were due to death of ectomesenchymal cells. Staining BMP-2-treated embryos with vital dyes revealed dead cells in the area adjacent to the bead but not in the unimplanted side of the mandible in the same embryo. Nor was cell death evident in the mandibles of embryos implanted with beads soaked in PBS containing BSA (compare Fig. 5A and B), i.e., cell death is not caused by injury associated with implanting the bead, but by BMP-2 adsorbed into the bead. The amount of cell death was in proportion to the concentration of BMP-2 applied (Fig. 5B-D). Similar results were obtained using either neutral red and Nile blue sulfate to visualize cell death.

Effect of BMP-2 on chondrogenesis in vitro

Histology and immunohistochemistry of control cultures

Mandibular ectomesenchymal explants cultured on either tissue culture plastic or type IV collagen-coated substrate were used as controls. Histological examination revealed that in both controls explants produced cartilage characterized by spherical cells with Alcian blue staining matrix (Fig. 6A,B). Immunohistochemical

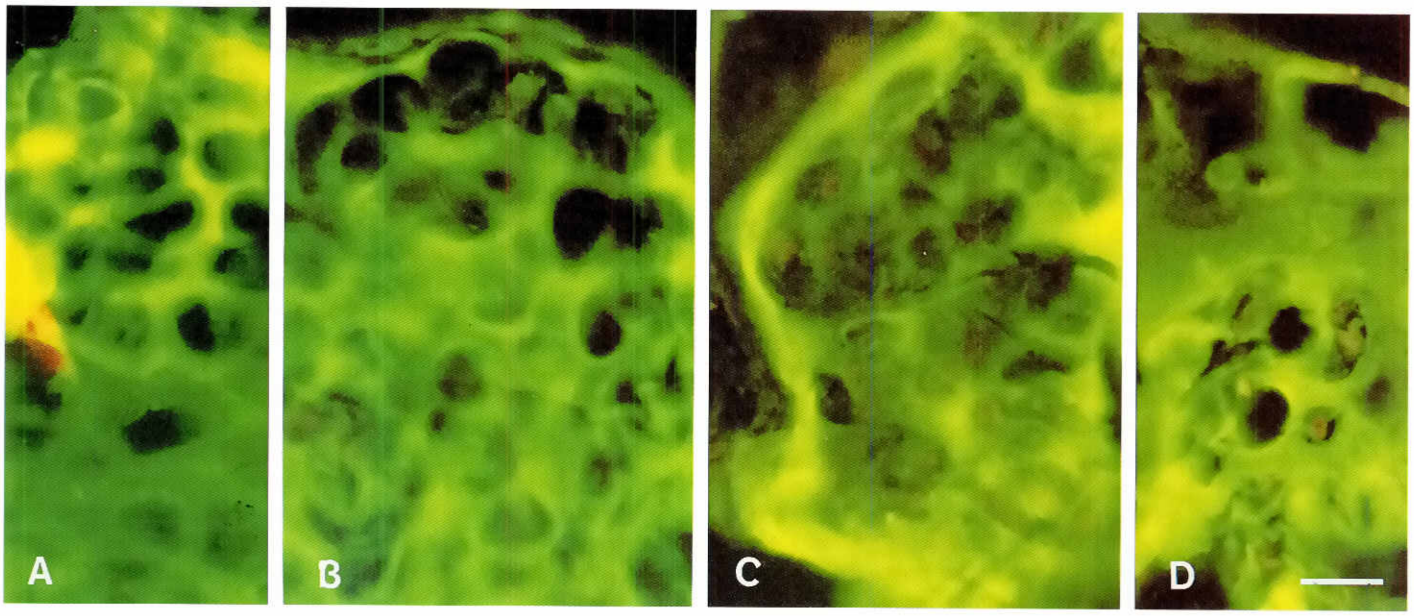

Fig. 7. Expression of type II collagen by mandibular ectomesenchymal cells undergoing chondrogenesis in explant culture. *Cryosections of 6-day-old cultures were stained with a monoclonal antibody against chicken type II collagen followed by FITC-conjugated secondary antibody. Positive staining is indicated by yellowish green fluorescence. All four groups of cultures express type II collagen in the cartilage matrix.* **(A)** *Control, cultured on plastic.* **(B)** *Control, cultured on type IV collagen.* **(C)** *Ectomesenchyme cultured in plates coated with BMP-2.* **(D)** *Ectomesenchyme cultured in plates coated with BMP-2 and type IV collagen. Bar, 25 µm.*

staining with anti-chicken type II collagen antibody revealed this cartilage specific collagen in the matrix (Fig. 7A,B). In both control groups, cartilage morphology was rod-like, similar to the normal morphology of Meckel's cartilage *in vivo*. Rod-like morphology was more prominent in explants cultured on plastic, in which almost the whole explant was comprised of cartilage. Non-chondrogenic cells, appearing fibroblastic, were limited to a thin layer around the cartilage rod (Fig. 6A). In explants cultured on type IV collagen-coated substrata, more fibroblast-like cells surrounded the cartilage rod than in explants cultured on plastic.

BMP-2 treated explants

Explants cultured in culture plates coated with either BMP-2 alone or BMP-2+type IV collagen also produced cartilage, evident as cells surrounded by Alcian blue staining matrix (Fig. 6C,D) containing type II collagen (Fig. 7C,D). However, in both cultures, chondrocytes were not organized into a rod-like morphology. Rather, they existed as a large number of cartilage nodules (compare Fig. 6C and D with 6A and B). Since culturing mesenchyme on type IV collagen alone did not influence rod-like morphology, disruption of the rod-like cartilage morphology seen in cultures treated with BMP-2 alone or BMP-2+type IV collagen was due to BMP-2. In these cultures internodular spaces as well as peripheral region were occupied by fibroblasts. There were more fibroblasts in these cultures than in control explants cultured on plastic (compare Fig. 6C and D with 6A and B).

Collagen type X expression

Expression of type X collagen is an indication of maturation of cartilage, which *in vivo*, precedes endochondral ossification. Although chondrocytes in Meckel's cartilage do not express type X collagen *in vivo*, they express type X collagen *in vitro* in response to ascorbic acid, a commonly used supplement in the medium to enhance chondrogenesis (Ekanayake and Hall, 1994b). *In vitro* expression of type X collagen is then followed by mineralization of cartilage matrix.

To determine whether exogenous BMP-2 affected expression of type X collagen *in vitro*, we examined 15-day-old explant cultures using a monoclonal antibody raised against chicken type X collagen. BMP-2 did not affect expression of type X collagen. In the two groups of control cultures, positive antibody staining was present in the extracellular matrix throughout the cartilage rod (Fig. 8A,B). In the two groups of BMP-2 treated cultures (BMP-2 with and without type IV collagen), type X collagen was present in the extracellular matrix of chondrocytes arranged into nodules throughout the culture (Fig. 8C,D). Figure 3E-H shows phase contrast images of Figure 8A-D, respectively.

Mineralization of cartilage

Mineralization of cartilage was examined by histochemical staining with $AgNO_3$ following Von Kossa's method. Dark granular patches indicate positive staining. All four culture groups indicated mineralization of cartilage matrix beginning at 11-12 days of culture. Mineralization spread over a larger area of cartilage with time. By day 15 a considerable portion of the cartilage rod was mineralized in both control groups (Fig. 9A,B). Cartilage nodules produced by explants cultured on BMP-2 alone or BMP-2+type IV collagen were either completely or partially mineralized by day 15 (Fig. 9C,D).

Exogenous BMP-2 does not induce osteogenesis of mandibular ectomesenchyme in vitro

Treatment of mandibular ectomesenchyme with BMP-2 in the absence of epithelium did not induce bone formation. Neither groups of control explant cultures (mesenchyme alone cultured on plastic or on type IV collagen), nor those explants cultured in plates coated with either BMP-2 alone or BMP-2+type IV collagen, produced bone, as evident by histological analysis of serial sections using direct red to visualize bone (Fig. 1A-D). Histochemical staining by Von Kossa's method also indicated the absence of membrane bone in these specimens.

Discussion

Effects of BMP-2 implanted in vivo

To examine whether chondrogenesis of mandibular ectomesenchyme *in vivo* would be affected by exogenous BMP-2, agarose beads loaded with BMP-2 were implanted into the proximal region on one side of the mandible. Agarose beads have been previously used as a vehicle for slow release of peptides (Schreiber *et al.*, 1986; Hayamitzu *et al.*, 1991; Vainio *et al.*, 1993; Gañán *et al.*, 1996). Our results indicate that exogenous BMP-2 suppressed the development of Meckel's cartilage in the area adjacent to the implanted bead in a dose-dependent manner. Highest activity was seen with 75-150 ng/μl. BMP-2 had no affect on cartilage development at concentrations below 10 ng/μl.

The development of membrane bone was also suppressed by exogenous BMP-2. It is important to note that, although BMP-2 caused retardation of the mandibular skeleton, it did not affect the development of the quadrate which is also a derivative of the first visceral arch. The adjacent hyobranchial skeleton was also unaffected. Therefore, BMP-2 acts within a limited concentration range and over a limited area. Francis-West *et al.* (1994) have reported that both BMP-2 and BMP-4 are expressed in a temporal sequence in the epithelium and mesenchyme mainly in the *distal region* of the mandibular primordia suggesting a possible role in mandibular development. Present study indicates that when BMP-2 is introduced in the *proximal region* of the mandibular primordium, it exerts a negative effect on chondrogenesis and osteogenesis as well as growth and morphogenesis of the mandible as a whole. Both temporal and spatial patterns of expression of BMP-2 influence mandibular development.

Mechanism of in vivo action of exogenous BMP-2

Complete absence of cartilage and bone in the area where a bead loaded with BMP-2 was implanted could be explained by either the death of ectomesenchymal cells or the inhibition of differentiation of ectomesenchymal cells into cartilage and bone caused by introduced BMP-2. No evidence for premature differentiation and removal of cartilage was found.

There are now a number of reports of BMP either inhibiting or slowing differentiation or initiating cell death or apoptosis. BMPs have been reported to cause cell death in neural crest cells. BMP-4, expressed in rhombomeres 3 and 5, upregulates *Msx*-2 and initiates apoptosis of neural crest cells in these rhombomeres, but not in adjacent, even-numbered rhombomeres (Graham *et al.*, 1994; Lumsden and Graham, 1996). Interdigital apoptosis in the limb buds of developing chick embryos is also mediated by BMPs (Gañán *et al.*, 1996; Zou and Niswander, 1996). The diastema of the rodent dentition, i.e. the area of the jaw in which teeth fail to develop, is a region in which BMP-2 and -4 and *Msx*-2 are downregulated as tooth primordia are removed by apoptosis (Tureckova *et al.*, 1995).

BMP-2 has also been reported to promote expression of the inhibitor of differentiation *(Id)* gene in osteoblast-like cells (Ogata *et al.*, 1993) and to inhibit myogenesis but promote chondrogenesis from chick limb bud cells (Duprez *et al.*, 1996b). Of interest is the finding that overexpression of BMP-2 and BMP-4 in chick limb buds *in ovo* enhanced chondrogenesis by enhancing recruitment of adjacent cells, but inhibited chondrocyte hypertrophy and osteogenesis (Duprez *et al.*, 1996a), a finding that highlights the divergent actions that the same application of BMP can have on cell types at different stages of differentiation.

We observed that within one day after implantation, BMP-2 drastically reduces the size of the mandibular process. This indicates that BMP-2 reduces cell number. Cell death studies using neutral red and Nile blue sulfate show that in fact BMP-2 causes death of cells adjacent to the bead and that cell death is proportional to the concentration of BMP-2 applied. Control embryos that received beads without BMP-2 did not show cell death.

Neutral red and Nile blue sulfate have been previously widely used to study programmed cell death during development of chick embryo (Saunders and Gasseling, 1962). The death of ectomesenchymal cells caused by BMP-2 is a likely reason for suppression of development and growth of the skeleton as a whole in the implanted side of the mandible. Given that Meckel's cartilage plays an important role in regulating mandibular growth (Hall, 1978, 1987b, 1988b, 1990, 1994; Richman, 1994), later deficiencies in mandibular growth may result from primary inhibition of development of Meckel's cartilage.

In contrast to previous reports indicating cartilage and bone inducing activity of BMPs in postnatal animals (Wang *et al.*, 1990; Wozney, 1992, 1993; Sasano *et al.*, 1993; Hirota *et al.*, 1994), our study shows that exogenous BMP-2 does not induce chondrogenesis *in vivo* or *in vitro* from chick embryonic ectomesenchymal cells at stage 22.

BMP-2 and -4 have been reported to have varying functions during embryonic development, depending on the developmental stage of the embryo. For example, BMP-4 plays a role in gastrulation and mesoderm formation in mouse (Winnier *et al.*, 1995) and dorso-ventral patterning of *Xenopus* at the gastrula stage (Dale *et al.*, 1992; Jones *et al.*, 1992). Somewhat later during development, BMP-2 and 4 are expressed in regions of inductive interactions between epithelium and mesenchymal tissues (Lyons *et al.*, 1989, 1990; Jones *et al.*, 1991) and are involved in patterning the chick limb (Lyons *et al.*, 1990; Jones *et al.*, 1991; Francis *et al.*, 1994). Once cytodifferentiation occurred, both BMP-2 and 4 are found in more mature perichondrium, periosteum and in odontoblasts (Wozney, 1992). Given these diverse roles of DPP-related BMPs, it is not surprising to see effects of BMP-2 on embryonic ectomesenchymal cells not seen in adults.

Exogenous BMP-2 and epithelial-mesenchymal interactions

Epithelial-mesenchymal interactions that occur between mandibular ectomesenchyme and the overlying epithelium are essential for the development of membrane bones in the chick mandible (Hall, 1987b). These interactions are completed by HH stage 24, after which mesenchyme no longer requires the epithelium for osteogenic differentiation (Hall *et al.*, 1983; Hall, 1987b). Signaling molecules involved in these interactions are little understood, however, epithelial basement membrane mediates these interactions (Hall *et al.*, 1983).

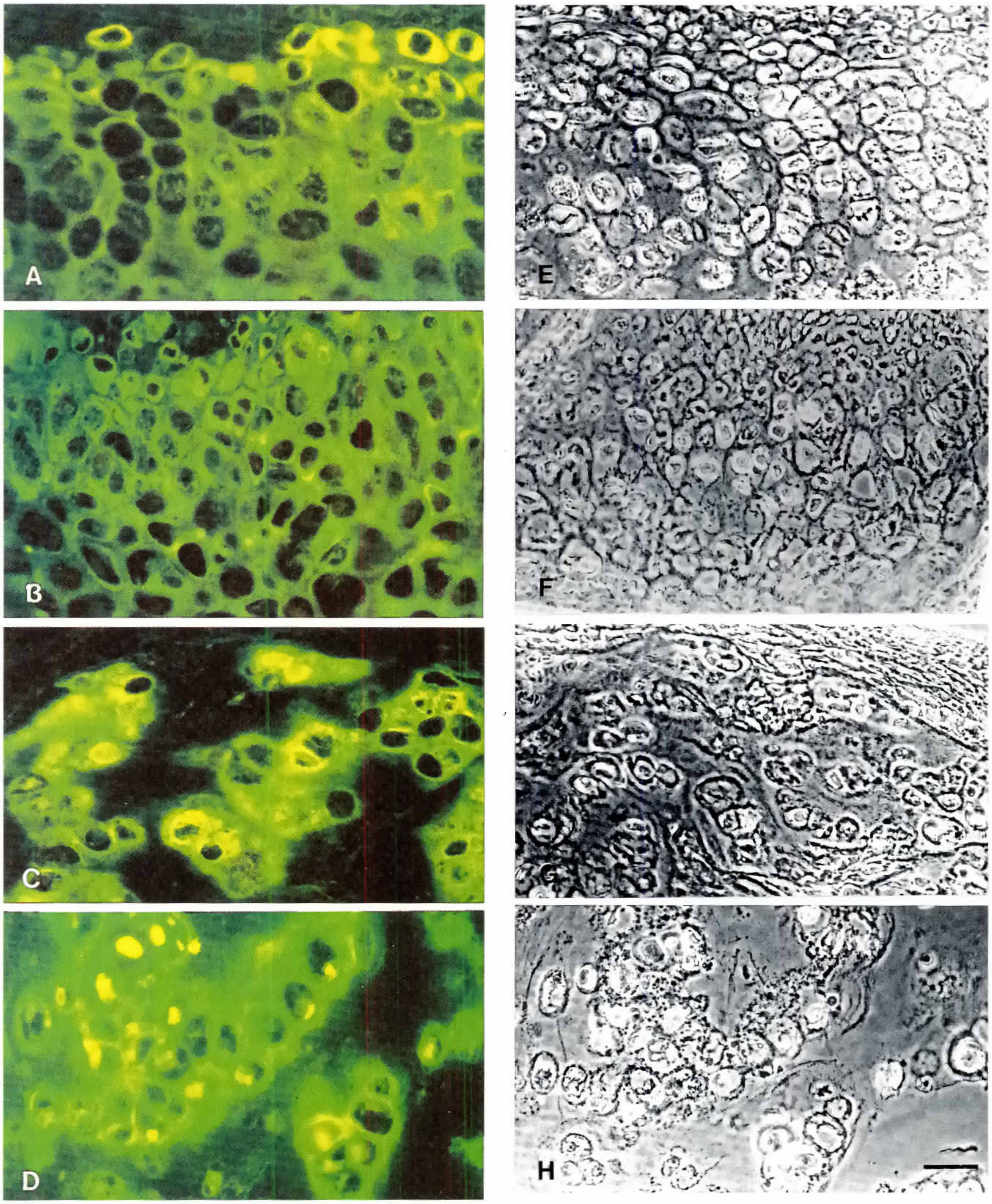

Fig. 8. Expression of type X collagen by mandibular ectomesenchymal cells in explant culture either with or without BMP-2. (A-D) *Sections of 15 day-old cultures, stained with a monoclonal antibody against chicken type X collagen followed by FITC-conjugated secondary antibody.* **(E-H)** *Phase contrast images of* **(A-D)** *respectively. BMP-2-treated and control cultures express type X collagen.* **(A)** *Control, cultured on tissue culture plastic.* **(B)** *Control, cultured in culture plates coated with type IV collagen.* **(C)** *Ectomesenchyme cultured in plates coated with BMP-2.* **(D)** *Ectomesenchyme cultured in plates coated with both BMP-2 and type IV collagen. Bar: A-D, 30 μm; E-H, 33 μm.*

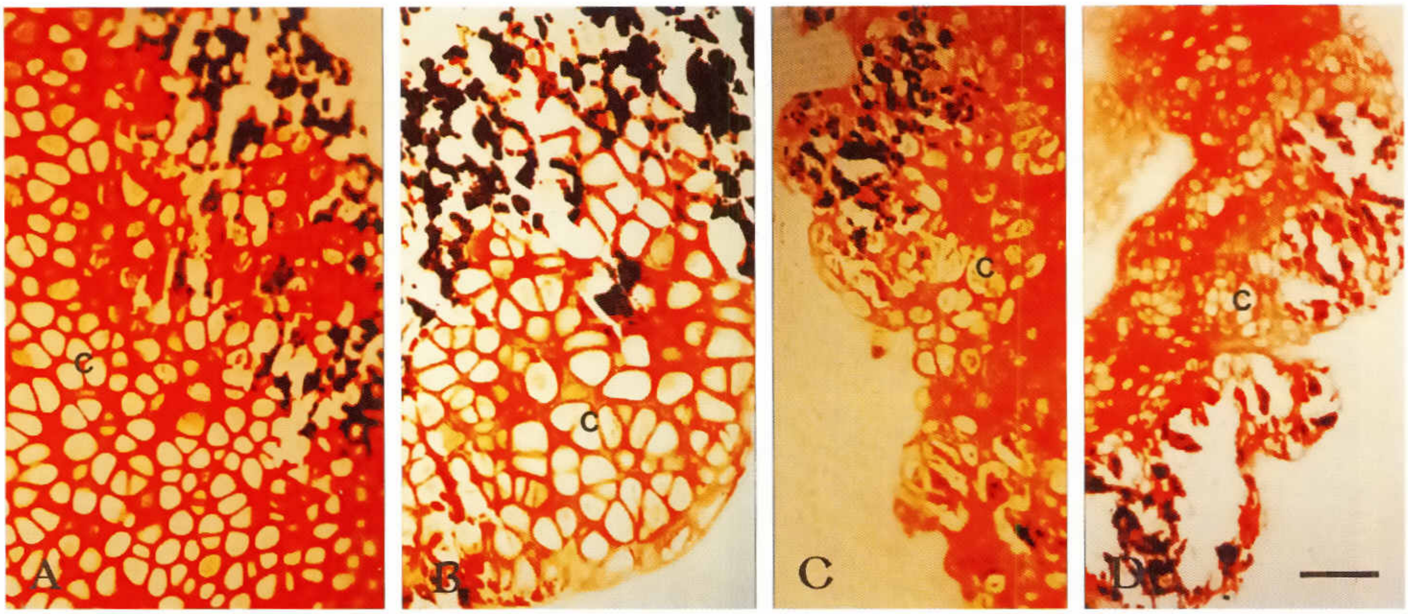

Fig. 9. Histochemical analysis of cultures for mineralization using Von Kossa's method. *Dark granules indicating mineralization of cartilage matrix are evident in all four groups.* **(A)** *Control, cultured on tissue culture plastic.* **(B)** *Control, cultured on type IV collagen.* **(C)** *Ectomesenchyme cultured in plates coated with BMP-2.* **(D)** *Ectomesenchyme cultured in plates coated with both BMP-2 and type IV collagen. Bar: A-C, 63 μm; D, 100 μm.*

We examined whether BMP-2 could replace mandibular epithelium and induce osteogenic differentiation of mandibular ectomesenchymal cells by culturing ectomesenchymal explants from a stage prior to *in vivo* epithelial-mesenchymal interaction on a substrate coated with BMP-2. In addition, for the reasons that: (1) BMPs bind to type IV collagen with high affinity (Paralkar *et al.*, 1990, 1991, 1992; Vukicevic *et al.*, 1994); and (2) that type IV collagen is the main collagen in basement membranes (Linsenmayer, 1981), we cultured some ectomesenchymal explants on a substrate coated with both type IV collagen and BMP-2. However, neither of these culture conditions induced formation of bone by mandibular ectomesenchymal cells. Mineralized tissue present in both control and BMP-treated specimens is not bone, but mineralizing cartilage, evident by immunohistochemical localization of type X collagen and visualization of mineralized cartilage matrix.

BMP-2 and BMP-4 have been localized in embryonic sites such as tooth, hair follicle, gut and urinary bladder where epithelial-mesenchymal interactions occur (Lyons *et al.* 1990; Jones *et al.*, 1991). Furthermore, exogenous BMP-4 induces expression of *msx*-1, *msx*-2 and *Egr*-1 in dental mesenchyme in a manner similar to that caused by dental epithelium (Vainio *et al.*, 1993), while BMP and Msx are both downregulated in the tooth-free diastema (Tureckova *et al.*, 1995). While previous studies suggest that BMP-2 and BMP-4 may play a role in epithelial-mesenchymal interactions, the present study indicates that BMP-2 alone or BMP-2+type IV collagen are not sufficient to replace mandibular epithelium.

Epithelial-mesenchymal interactions occur as a cascade of mutual signaling between the epithelium and the mesenchyme, causing gradual developmental progression of both tissues. Molecules involved in this cascade of signaling are just beginning to be understood. The hedgehog family of proteins have been shown recently to be expressed early in this cascade and may in turn regulate expression of some BMPs (Bitgood and McMahon, 1995). BMPs may act as heterodimers as these have been shown to be more potent inducers than the respective homodimers (Aono *et al.*, 1995; Lyons *et al.*, 1995). BMP-2 and BMP-4 as well as other BMPs, are known to promote the osteoblastic phenotype while suppressing the myoblastic phenotype in established lines of multipotential progenitor cells (Yamaguchi *et al.*, 1991; Ohta *et al.*, 1992; Ahrens *et al.*, 1993; Wang *et al.*, 1993; Katagiri *et al.*, 1994; Yamaguchi, 1995). BMPs also enhance the osteoblastic phenotype in primary cultures of rat calvarial osteoblasts and bone marrow cells (Amédée *et al.*, 1994; Rickard *et al.*, 1994; Aono *et al.*, 1995; Centrella *et al.*, 1995; Harris *et al.*, 1995). However, it is important to note that these cell types are at a developmentally more advanced status than uninduced, embryonic, mandibular ectomesenchymal cells. Carrington (1994) suggested that the effect of BMPs may depend on the developmental status of the responding cell. Indeed, in some cases, BMP inhibits osteoblastic differentiation (Ogata *et al.*, 1994).

Effect of BMP-2 on chondrogenesis in vitro

Both control explants and those explants cultured on a substrate coated with BMP-2 produced cartilage. However, the morphology of the cartilages was different. In control cultures, it was rod-like, resembling Meckel's cartilage *in vivo*. Rod-like cartilage morphology was abolished in cultures treated with either BMP-2, or BMP-2+type IV collagen. In the latter case, nodules of cartilage with intervening fibroblasts formed. Formation of nodules is not caused by type IV collagen, since the explants cultured on type IV collagen alone did not produce cartilage nodules.

Mandibular ectomesenchyme at HH stage 22 contains prechondrogenic cells, which can undergo chondrogenesis even under clonal culture conditions (Ekanayake and Hall, 1994a). Therefore, it was not surprising to see chondrogenesis in control cultures. However, disruption of the rod-like morphology of Meckel's cartilage by BMP-2 is both surprising and interesting.

Mandibular ectomesenchymal cells have been shown to produce cartilage nodules in micromass culture (Ekanayake and Hall, 1994a,b; Langille, 1994). When cultured as micromasses, mesenchyme is first dissociated into single cells in a suspension and plated as drops of cells at a high density. During this process, organization of subpopulations of cells such as prechondrogenic cells, premyogenic cells and fibroblasts is disrupted and cells are plated as a mixture. Therefore, rather than forming a continuous rod of cartilage as *in vivo*, chondrogenic cells aggregate to form nodules, separated by non-chondrogenic cells. In contrast, when ectomesenchyme is cultured as an explant, cellular order is maintained undisrupted and cells produce a rod-like cartilage.

That BMP-2 alters the rod-like morphology of cartilage to nodules in explant culture may indicate that there are more non-chondrogenic cells in these cultures than in control cultures. Non-chondrogenic cells would disrupt the continuity of a cartilage rod, forcing groups of chondrocytes to form nodules. BMP-2 may suppress chondrogenesis of some cells in the prechondrogenic population. Langille (1994) demonstrated that BMP-7 promotes chondrogenesis of rat mandibular mesenchyme maintained in micromass cultures. BMP-3 and 4 stimulate cartilage formation from HH stage 24-25 chick limb bud mesenchymal cells *in vitro* (Carrington *et al.*, 1991; Chen *et al.*, 1991b), while BMP-2 and 4 promote chondrogenesis of established cell lines of multipotential progenitors (Ahrens *et al.*, 1993; Wang *et al.*, 1993). Luyten *et al.* (1994) suggested the presence of functional receptors for BMP-4 in articular chondrocytes. The possibility that BMP acts through cell surface molecules such as N-CAM, which is known to modulate skeletal morphogenesis (Hall and Miyake, 1995; Fang and Hall, 1995, 1996) is worth exploring.

Chondrogenesis inhibitory action of BMP-2 seen in the present study could be due to one or more of:

1) the developmentally immature status of the ectomesenchymal cells used,
2) BMP-2 may play an inhibitory role at the condensation stage even though it enhances chondrogenesis later in differentiation (Hall and Miyake, 1995), and/or,
3) BMP-2 may mediate the known inhibition of chondrogenesis by mandibular epithelium (Coffin-Collins and Hall, 1989; Mina *et al.*, 1994).

Materials and Methods

BMP bead implantation and shell-less culture of chick embryos

Fertilized eggs of the domestic fowl *(Gallus domesticus)* obtained from Lone Pine Farm (Truro, Nova Scotia, Canada) were incubated in a 'model 50 Humidaire' incubator at 37°C and 95% relative humidity for approximately 4 days to obtain embryos at HH stage 22.

Embryos were taken from their shells, established in shell-less culture (Tuan and Lynch, 1983) and beads were implanted. To prepare for shell-less culture, eggs were carefully cracked open into sterile 100 mm diameter culture plates so that the yolk remained intact with the embryo on top. Shells were discarded. Shell-less embryos were kept in an incubator at 37°C with 95% humidity and 5% CO_2 until bead implantation. In shell-less culture, embryos are easily accessible and beads could be implanted under a stereo microscope, without damaging adjacent tissues. Since chick embryo usually lies on the yolk with the right side of the body uppermost, beads with or without BMP-2 were implanted in the proximal region of the right half of the mandibular arch through a hole made in the amniotic membrane. As even a small amount of bleeding can cause death, extra caution was taken not to damage the heart and the main blood vessels that are in the proximity to the developing mandible. Once the bead was in place, embryos were returned to the incubator, maintained as shell-less cultures, and examined daily over an 8-day period.

Treatment of beads with BMP-2

Recombinant human BMP-2 protein was donated by The Genetics Institute Inc. (Cambridge, MA, USA). Because human, mouse and chick BMP-2 share some 80% amino acid identity, the human protein can be used in the other species. In the present study, a wide range of concentrations of BMP-2, including those concentrations that have been shown to be physiological in other systems such as tooth and limb primordia in the developing mice and the chick, were used to treat the beads. (Vainio *et al.*, 1993; Gañán *et al.*, 1996). Stock solution (1.33 mg/ml of 0.5 mM arginine-HCl, 10 mM histidine; pH 5.6 Buffer) was diluted to 5-150 ng/µl PBS (pH 7.3) containing 0.1% BSA. However, it should be noted that the actual concentration of BMP released by the implanted bead to the tissues is much less than the concentration used to treat the beads. Agarose beads have been used previously as a vehicle for slow release of peptides (Schreiber *et al.*, 1986; Hayamitzu *et al.*, 1991; Vainio *et al.*, 1993; Gañán *et al.*, 1996).

Affi-Gel blue agarose beads (100-200 mesh, 75-150 µm diameter; Biorad) in PBS (pH 7.3) were counted under a stereo microscope (200 beads/vial), washed once with PBS, and pelleted. Beads were incubated with solutions of BMP-2 (200 beads per 10 µl) and incubated at 37°C for 30 min (Vainio *et al.*, 1993). Control beads were treated in the same way but incubated in PBS containing 0.1% BSA (vehicle) without BMP-2. Treated beads were either used immediately or stored at 4°C for no more than one week before being implanted. Storage had no effect on activity. Because bead size varied between 75 and 150 µm diameter, beads in the mid-diameter range were selected under a microscope prior to implantation. Beads were rapidly washed in culture medium and implanted using a pair of very fine forceps (Watchman #5).

Isolation and explant culture of mandibular ectomesenchyme

Mandibular processes were dissected from HH stage 22 embryos and treated with a mixture of trypsin and pancreatin (13:2 ratio) at a concentration of 1.5% in Ca^{++}- and Mg^{++}-free Tyrode's solution at 4°C for 1.25 h to facilitate removal of the epithelia (Tyler and Hall, 1977). The epithelium was manually removed from each mandibular process under a dissecting microscope and discarded. The remaining mandibular ectomesenchyme was placed, one mesenchyme per well, as an explant in 24-well culture plates previously coated with either BMP-2 alone or BMP-2+type IV collagen as described below. Some ectomesenchymal explants were maintained as controls and cultured in either uncoated plates or on plates coated with type IV collagen. Each culture group contained approximately 24 ectomesenchymal explants. Culture medium used was Ham's F12 and BGJ_b in a ratio of 3:1, supplemented with 10% fetal bovine serum (FBS). Medium was changed daily. Because ascorbic acid and ß-glycerophosphate are required for optimal collagen synthesis and matrix mineralization (Tenenbaum and Heersche, 1982), medium was supplemented with recommended levels of ascorbic acid (75 µg/ml) and ß-glycerophosphate (10 mM), from the fourth day of culture on. Explants were maintained for 15 days.

Coating culture plates with BMP-2

Tissue culture plates coated with type IV collagen (Bio-coat Cell ware) were purchased from Collaborative Biomedical Products. Stock solution of BMP-2 (1.33 mg/ml of 0.5 mM arginine-HCl, 10 mM histidine; pH 5.6 Buffer) was diluted to 25 µg/ml in culture medium with 10% FBS. 250 µl of this solution was placed in each well of 24-well tissue culture plates and incubated for 1 h at 37°C. The BMP-2 solution was then aspirated out, plates were rinsed once with culture medium and returned to a 37°C incubator with fresh medium until explants were placed in the wells. Both regular 24-well tissue culture plates and type IV collagen coated Bio-coat 24-well tissue culture plates were coated with BMP-2 in this manner.

Analysis of specimens

Histology

Explant cultures were scraped from the bottom of the culture plates, fixed in neutral-buffered formalin (NBF), dehydrated in ethanol, embedded in Paraplast, and serially sectioned at 6 μm. Sections were stained using a multiple staining procedure consisting of celestine blue, hematoxylin, Alcian blue and direct red to examine for the presence of cartilage and bone (Hall, 1986). Alcian blue binds with sulfated proteoglycan in cartilage matrix (Lev and Spicer, 1964). Direct red parallels the distribution of alkaline phosphatase as a marker for osteogenesis. Embryos from shell-less culture were separated from the yolk, decapitated, fixed in NBF and processed for paraffin sectioning. Six μm sections were stained with the same multiple staining procedure.

Immunohistochemistry of type II and type X collagens

Both cryosections and wax sections were used for immunohistochemistry. For cryosectioning, unfixed cultures were quick frozen in O.C.T. compound (Tissue-Tek 4583, Miles Scientific Division, Naperville, IL, USA) and sectioned (6 μm) in a cryostat. Sections were fixed in 100% acetone for 10 min., air dried, and stored at -20°C until use. Acetone-fixed sections were mostly used for collagen II staining. For wax embedding, specimens were fixed in NBF at 4°C, dehydrated in ethanol at 4°C, embedded in X-tra (low melting point) Paraplast and sectioned. Mineralized cultures were demineralized prior to wax embedding following Bourque *et al.* (1993). Briefly, specimens were fixed overnight at 4°C in a freshly prepared periodate-lysine-paraformaldehyde fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate), washed in phosphate buffered saline (PBS), decalcified in a solution of ethylenediamine tetraacetic acid (EDTA, 0.5 M) and glycerol (15%) at pH 7.3 for 4-6 days at 4°C, washed in PBS, dehydrated in ethanol, embedded in X-tra Paraplast and sectioned. Wax sections were dewaxed in Histoclear and rehydrated in a series of ethanols prior to antibody staining.

Antibody staining

Cryosections and rehydrated wax sections were rinsed in PBS and digested for 30 min at 37°C with bovine testicular hyaluronidase at 0.5 mg/ml in 10 mM potassium and sodium phosphate buffer (Jacenko and Tuan, 1986a). Sections were then incubated with monoclonal antibodies to either chicken type II or X collagen raised in mice (donated by Dr. T. Linsenmayer, Tufts University, Boston, MA, USA) for 2 h at room temperature followed by fluorescein-conjugated rabbit anti-mouse IgG (Sigma) for 30 min at room temperature and mounted in Vectarshield (Dimensions Lab.).

Histochemical staining for mineralized tissue

To distinguish mineralized tissues, paraffin sections of explant cultures were stained histochemically with Von Kossa's method (Page, 1982). In short, sections were stained with 1% $AgNO_3$, exposed to strong light to develop positive silver staining, and counter stained with safranin 'O'.

Whole-mount staining

Heads of embryos implanted with beads were fixed in NBF, stained with Alcian blue to visualize cartilage, digested with trypsin, stained with alizarin red S to visualize bone, transferred to glycerine for clearing and storing (Hanken and Wassersug, 1981), examined, and photographed.

Cell death studies by Nile blue sulfate and neutral red staining

One day after implanting beads, some embryos were detached from the extra-embryonic membranes and underlying yolk, rinsed briefly in Ringer's saline and stained for 60 min at room temperature with either Nile blue sulfate or neutral red, both at a concentration of 0.01% in Ringer's saline. Embryos were transferred to Ringers saline, destained at 4°C overnight to remove stain taken up by viable cells (Saunders and Gasseling, 1962), examined, and photographed.

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References

AHRENS, M., ANKENBAUER, T., SCHRÖDER, D., HOLLNAGEL, A., MAYER, H. and GROSS, G. (1993). Expression of human bone morphogenetic proteins-2 or 4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell Biol. 12:* 871-880.

AMÉDÉE, J., BAREILLE, R., ROUAIS, F., CUNNINGHAM, N., REDDI, H. and HARMAND, M-F. (1994). Osteogenin (bone morphogenetic protein 3) inhibits proliferation and stimulates differentiation of osteoprogenitors in human bone marrow. *Differentiation 58:*157-164.

AONO, A., HAZAMA, M., NOTOYA, K., TAKETOMI, S., YAMASAKI, H., TSUKUDA, R., SASAKI, S. and FUJISAWA, Y. (1995). Potent ectopic bone inducing activity of Bone Morphogenetic Protein-4/7 heterodimer. *Biochem. Biophys. Res. Commun. 210:* 670-677.

BEE, J. and THOROGOOD, P. (1980). The role of tissue interactions in the skeletogenic differentiation of avian neural crest cells. *Dev. Biol. 78:* 47-62.

BENNETT, J.H., HUNT, P. and THOROGOOD, P. (1995). Bone morphogenetic protein-2 and -4 expression during murine orofacial development. *Arch. Oral Biol. 40:* 847-854.

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.

BOURQUE, W.T., GROSS, M. and HALL, B.K. (1993). A Histological processing Technique that preserves the integrity of calcified tissues (bone, enamel), yolky amphibian embryos and growth factor antigens in skeletal tissue. *J. Histochem. Cytochem. 41:* 1429-1434.

CARRINGTON, J.L. (1994). Bone morphogenetic proteins and the induction of embryonic and adult bone. In *Bone*, Vol. 8 (Ed. B.K. Hall). CRC Press, Boca Raton, pp. 85-108..

CARRINGTON, J.L., CHEN, P., YANAGISHITA, M. and REDDI, A.H. (1991). Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells *in vitro. Dev. Biol. 146:* 406-415.

CELESTE, A.J., IANNAZZI, J.A., TAYLOR, R.C., HEWICK, R.M., ROSEN, V., WANG, E.A. and WOZNEY, J.M. (1990). Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA 87:* 9843-9847.

CELESTE, A.J., SONG, J.J., COX, K., ROSEN, V. and WOZNEY, J.M. (1994). Bone Morphogenetic Protein-9, a new member of the TGF-β superfamily. *J. Bone Miner. Res. 9 (Suppl. 1):* S136.

CENTRELLA, M., CASINGHINO, S., KIM, J., PHAM, T., ROSEN, V., WOZNEY, J. and McCARTHY, T.L. (1995). Independent changes in type I and type II receptors for Transforming Growth Factor β induced by bone morphogenetic protein 2 parallel expression of the osteoblastic phenotype. *Mol. Cell. Biol. 15:* 3273-3281.

CHEN, T.L., BATES, R.L., DUDLEY, A., HAMMONDS Jr, R.G. and AMENTO, E.P. (1991a). Bone morphogenetic protein-2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells: comparison with TGF-β1. *J. Bone Miner. Res. 6:* 1387-1393.

CHEN, P., CARRINGTON, J.L., HAMMONDS, R.G. and REDDI, A.H. (1991b). Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B (BMP 2B) and modulation by transforming growth factor β1 and β2. *Exp. Cell Res. 195:* 509-515.

CHEN, P., CARRINGTON, J.L., PARALKAR, V.M., PIERCE, G.F. and REDDI, A.H. (1992). Chick limb bud mesodermal cell chondrogenesis: inhibition by isoforms of platelet-derived growth factor and reversal by recombinant bone morphogenetic protein. *Exp. Cell Res. 200:* 110-117.

COFFIN-COLLINS, P.A. and HALL, B.K. (1989). Chondrogenesis of mandibular mesenchyme from the embryonic chick is inhibited by mandibular epithelium and by epidermal growth factor. *Int. J. Dev. Biol. 33:* 297-311.

DALE, L., HOWES, G., PRICE, B.M.J. and SMITH, J.C. (1992). Bone morphogenetic protein-4: a ventralizing factor in early *Xenopus* development. *Development 115:* 573-585.

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.

DUPREZ, D., BELL, E.J. DE H., RICHARDSON, M.K., ARCHER, C.W., WOLPERT, L., BRICKELL, P.M. and FRANCIS-WEST, P.H. (1996a). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev 57:* 145-157.

DUPREZ, D., COLTREY, M., AMTHOR, H., BRICKELL, P.M. and TICKLE, C. (1996b). Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol. 174:* 448-452.

EKANAYAKE, S. and HALL, B.K. (1994a). Formation of cartilaginous nodules and heterogeneity in clones of HH 17 mandibular ectomesenchyme from the embryonic chick. *Acta Anat. 151:* 71-79.

EKANAYAKE, S. and HALL, B.K. (1994b). Hypertrophy is not a prerequisite for type X collagen expression or mineralization of chondrocyte derived from cultured chick mandibular ectomesenchyme. *Int. J. Dev. Biol. 38:* 683-694.

FANG, J. and HALL, B.K. (1995). Differential expression of neural cell adhesion molecule (N-CAM) during osteogenesis and secondary chondrogenesis in the embryonic chick. *Int. J. Dev Biol. 39:* 519-528.

FANG, J. and HALL, B.K. (1996). *In vitro* differentiation potential of the periosteal cells from a membrane bone, the quadratojugal of the embryonic chick. *Dev. Biol. 180:* 701-712.

FRANCIS, P.H., RICHARDSON, M.K., BRICKELL, P.M. and TICKLE, C. (1994). Bone Morphogenetic proteins and a signaling pathway that controls patterning in the developing chick limb. *Development 120:* 209-218.

FRANCIS-WEST, P.H., ROBERTSON, K.E., EDE, D.A., RODRIGUEZ, C., IZPISUA-BELMONTE, J.C., HOUSTON, B., BURT, D.W., GRIBBIN, C., BRICKELL, P.M. and TICKLE, C. (1995). Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in Talpid (ta^3) limb buds: their relationships in the signaling cascade involved in limb patterning. *Dev. Dynamics 203:* 187-197.

FRANCIS-WEST, P.H., TATLA, T. and BRICKELL, P.M. (1994). Expression patterns of the Bone Morphogenetic Protein genes *Bmp-4* and *Bmp-2* in the developing chick face suggest a role in outgrowth of the primordia. *Dev. Dynamics 201:* 168-178.

GAÑÁN, Y., MACÍAS, D., DUTERGUE-COQUILLAND, M., ROS, M.A. and HURLÉ, J.M. (1996). Role of TGFβs and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development 122:* 2349-2357.

GRAHAM, A., FRANCIS-WEST, P.H., BRICKELL, P. and LUMSDEN, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature 372:* 684-686.

HALL, B.K. (1978). *Developmental and Cellular Skeletal Biology.* Academic Press, New York.

HALL, B.K. (1986). The role of movement and tissue interactions in the development and growth of bone and secondary cartilage in the clavicle of the embryonic chick. *J. Embryol. Exp. Morphol. 93:* 133-152.

HALL, B.K. (1987a). Sodium fluoride as an initiator of osteogenesis from embryonic mesenchyme *in vitro. Bone 8:* 111-116.

HALL, B.K. (1987b). Tissue interactions in the development and evolution of the vertebrate head. In *Developmental and Evolutionary Aspects of the Neural Crest* (ED. P.F.A. Maderson). Wiley Interscience, New York, pp. 215-259.

HALL, B.K. (1988a). The embryonic development of bone. *Am. Sci. 76:* 174-181.

HALL, B.K. (1988b). Mechanisms of craniofacial development. In *Craniofacial Morphogenesis and Dysmorphogenesis* (Eds. K.W.L. Vig and A.P. Burdi). Craniofacial Growth Monograph Series, Vol. 21. Center for Human Growth and Development, Univ. Michigan, Ann Arbor, pp. 1-21.

HALL, B.K. (1990). Genetic and epigenetic control of vertebrate development. *Neth. J. Zool. 40:* 362-361.

HALL, B.K. (1992). Cell-cell interactions in craniofacial growth and development. In *The Biological Mechanisms of Tooth Movement and Craniofacial Adaptation* (Ed. Z. Davidovitch). The Ohio State University, Columbus, pp. 11-17.

HALL, B.K. (Ed.) (1994). Embryonic bone formation with special reference to epithelial-mesenchymal interactions and growth factors. In *Bone, Volume 8: Mechanisms of Bone Development and Growth.* CRC Press, Boca Raton, pp. 137-192.

HALL, B.K. and COFFIN-COLLINS, P.A. (1990). Reciprocal interactions between epithelium, mesenchyme and epidermal growth factor (EGF) in the regulation of mitotic activity of mandibular epithelium and mesenchyme in the embryonic chick. *J. Craniofac. Genet. Dev. Biol. 10:* 241-261.

HALL, B.K. and EKANAYAKE, S. (1991). Effects of growth factors on the differentiation of neural crest cells and neural crest cell-derivatives. *Int. J. Dev. Biol. 35:* 367-387.

HALL, B.K. and MIYAKE, T. (1995). Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int. J. Dev. Biol. 39:* 881-893.

HALL, B.K. and TREMAINE, R. (1979). Ability of neural crest cells from the embryonic chick to differentiate into cartilage before their migration away from the neural tube. *Anat. Rec. 194:* 469-476.

HALL, B.K., VAN EXAN, R.S. and BRUNT, S.L. (1983). Retention of epithelial basal lamina allows isolated mandibular mesenchyme to form bone. *J. Craniofac. Genet. Dev. Biol. 3:* 253-267.

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

HANKEN, J. and WASSERSUG, R. (1981). The visible skeleton; A new double stain technique reveals the nature of the hard tissue. *Funct. Photo. 16:* 22-26.

HARRIS, S.E., FENG, J.Q., HARRIS, M.A., GHOSH-CHOUDHURY, N., DALLAS, M.R., WOZNEY, J. and MUNDY, G.R. (1995). Recombinant bone morphogenetic protein 2 accelerates bone cell differentiation and stimulates BMP-2 mRNA expression and BMP-2 promoter activity in primary fetal rat calvarial osteoblast cultures. *Mol. Cell. Differ. 3:* 137-155.

HAWLEY, S.H.B., WÜNNENBERG-STAPLETON, K., HASHIMOTO, C., LAURENT, M.N., WATABE, T., BLUMBERG, B.W. and CHO, K.W.Y. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev. 9:* 2923-2935.

HAYAMITZU, T.F., SESSIONS, S.K., WANEK, N. and BRYANT, S.V. (1991). Effects of localized application of transforming growth factor β1 on developing chick limbs. *Dev. Biol. 145:* 164-173.

HIROTA, S., TAKAOKA, K., HASHIMOTO, J., NAKASE, T., TAKEMURA, T., MORII, E., FUKUYAMA, A., MORIHANA, K., KITAMURA, Y. and NOMURA, S. (1994). Expression of mRNA of murine bone-related proteins in ectopic bone induced by murine bone morphogenetic protein-4. *Cell Tissue Res. 277:* 27-32.

JACENKO, O. and TUAN, R.S. (1986a). Calcium deficiency induces expression of cartilage-like phenotype in chick embryonic calvaria. *Dev. Biol. 115:* 215-232.

JACENKO, O. and TUAN, R.S. (1986b). Changes in extracellular matrix in chick embryonic bone during induced calcium deficiency. In *Progress in Developmental Biology*, Part B (Ed. H.C. Slavkin). Alan R. Liss Inc., New York, pp. 401-404.

JONES, C.M., LYONS, K.M. and HOGAN, B.L.M. (1991). Involvement of bone morphogenetic protein-4 (BMP-4) and *Vgr-1* in morphogenesis and neurogenesis in the mouse. *Development 111:* 531-542.

JONES, C.M., LYONS, K.M., LAPAN, P.M., WRIGHT, C.V.E, and HOGAN, B.L.M. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. *Development 115:* 639-647.

KATAGIRI, T., YAMAGUCHI, A., KOMAKI, M., ABE, E., TAKAHASHI, N., IKEDA, T., ROSEN, V., WOZNEY, J. M., FUJISAWA-SEHARA, A. and SUDA, T. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol. 127:* 1755-1766.

KESSLER, E., TAKAHARA, K., BINIAMINOV, L., BRUSEL, M. and GREENSPAN, D. S. (1996). Bone morphogenetic protein-1: the type I procollagen C-proteinase. *Science 271:* 360-362.

KING, J.A., MARKER, P.C., SEUNG, K.J. and KINGSLEY, D.M. (1994). BMP-5 and the molecular, skeletal, and soft-tissue alterations in *short ear* mice. *Dev. Biol. 166:* 112-122.

KINGSLEY, D.M. (1994). 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 TGFβ superfamily. *Cell 71:* 399-410.

KRONMILLER, J.E., NGUYEN, T., BERNDT, W. and WICKSON, A. (1995). Spatial and temporal distribution of *Sonic Hedgehog* mRNA in the embryonic mouse mandible by reverse transcription/polymerase chain reaction and *in situ* hybridization analysis. *Arch. Oral Biol. 9:* 831-838.

LANGILLE, R.M. (1994). *In vitro* analysis of the spatial organization of chondrogenic regions of avian mandibular mesenchyme. *Dev. Dynamics 20:* 55-62.

LEV, R. and SPICER, S.S. (1964). Specific staining of sulfate groups with alcian blue at low pH. *J. Histochem. Cytochem. 12:* 309.

LIEM, K.F., TREMMI, G., ROELINK, H. and JESSELL, T.M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell 82:* 969-979.

LINSENMAYER, T.F. (1981). Collagen. In *Cell Biology of Extracellular Matrix* (Ed. E.D. Hay). Plenum Press, New York, pp. 5-32.

LUMSDEN, A. and GRAHAM, A. (1996). Death in the neural crest: implications for pattern formation. *Semin. Cell Dev. Biol. 7:* 169-174.

LUO, G., HOFMANN, C., BRONCKERS, A.L.J.J., 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.

LUYTEN, F.P., CHEN, P., PARALKAR, V. and REDDI, H. (1994). Recombinant bone morphogenetic protein-4, transforming growth factor-ß1, and activin A enhance the cartilage phenotype of articular chondrocytes *in vitro. Exp. Cell Res. 210:* 224-229.

LYONS, K.M., HOGAN, B.L.M. and ROBERTSON, E.J. (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech. Dev. 50:* 71-83.

LYONS, K.M., PELTON, R.W. and HOGAN, B.L.M. (1989). Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that Transforming growth factor-b like genes coordinately regulate aspects of embryonic development. *Genes Dev. 3:* 1657-1668.

LYONS, K.M., PELTON, R.W. and HOGAN, B.L.M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for Bone morphogenetic protein-2a (BMP-2a). *Development 109:* 833-844.

MINA, M., GLUHAK, J., UPHOLT, W.B., KOLLAR, E.J. and ROGERS, B. (1995). Experimental analysis of *Msx*-1 and *Msx*-2 gene expression during chick mandibular morphogenesis. *Dev. Dynamics 202:* 195-214.

MINA, M., UPHOLT, W.B. and KOLLAR, E.J. (1994). Enhancement of avian mandibular chondrogenesis *in vitro* in the absence of epithelium. *Arch. Oral Biol. 7:* 551-562.

MINKOFF, R. (1991). Cell proliferation during formation of the embryonic facial primordia. *J. Craniofac. Genet. Dev. Biol. 11:* 251-261.

MURRAY, P.D.F. (1963). Adventitious (secondary) cartilage in the chick embryo and the development of certain bones and articulations in the chick skull. *Aust. J. Zool. 11:* 368-430.

OGATA, T., WOZNEY, J.M., BENEZRA, R. and NODA, M. (1993). Bone morphogenetic protein-2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix-loop-helix molecule in osteoblast-like cells. *Proc. Natl. Acad. Sci. USA 90:* 9219-9222.

OGATA, T., WOZNEY, J.M., RODAN, G.A. and NODA, M. (1994). Bone morphogenetic protein-2 (BMP-2) acts both synergistically with and antagonistically against retinoic acid in regulating expression of phenotypic genes in osteoblast-like cells. *Endocrinol. J. 2:* 237-240. .

OHTA, S., HIRAKI, Y., SHIGENO, C., SUZUKI, F., KASAI, R., IKEDA, T., KOHNO, H., LEE, L., KIKUCHI, H., KONISHI, J., BENTZ, H., ROSEN, D.M. and YAMAMURO, T. (1992). Bone Morphogenetic proteins (BMP-2 and BMP-3) induce the late phase expression of the proto-oncogene c-fos in murine osteoblastic MC3T3-E1 cells. *FEBS Lett. 314:* 356-360.

OZKAYNAK, E., SCHNEGELSBERG, P.N., JIN, D.F., CLIFFORD, G.M., WARREN, F.D., DRIER, E.A. and OPPERMANN, H. (1992). Osteogenic protein-2: a new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. *J. Biol. Chem. 267:* 25220-25227.

PAGE, K. (1982). Bone and the preparation of bone sections. In *Theory and Practice of Histological Techniques* (Eds. J.D. Bancroft and A. Stevens). Churchill Livingstone Press, New York, pp. 324-325.

PARALKAR, V.M., NANDEDKAR, A.K.N., POINTER, R.H., KLEINMAN, H.K. and REDDI, A.H. (1990). Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. *J. Biol. Chem. 265:* 17281-17284.

PARALKAR, V.M., VUKICEVIC, S. and REDDI, A.H. (1991). Transforming growth factor ß type 1 binds to collagen IV of basement membrane matrix: implications for development. *Dev Biol. 143:* 303-308.

PARALKAR, V.M., WEEKS, B.S., YU, M.Y., KLEINMAN, H.K. and REDDI, A.H. (1992). Recombinant human bone morphogenetic protein 2B stimulates PC12 cell differentiation: potentiation and binding to type IV collagen. *J. Cell Biol. 119:* 1721-1728.

PHIPPARD, D.J., WEBER-HALL, S.J., SHARPE, P.T., NAYLOR, M.S., JAYATALAKE, H., MAAS, R., WOO, I., ROBERTS-CLARK, D., FRANCIS-WEST, P.H., LIU, Y-H., MAXSON, R., HILL, R.E. and DALE, T.C. (1996). Regulation of *Msx*-1, *Msx*-2, BMP-2 and BMP-4 during foetal and postnatal mammary gland development. *Development 122:* 2729-2737.

REISSMANN, E., ERNSBERGER, U., FRANCIS-WEST, P.H., RUEGER, D., BRICKELL, P.M. and ROHRER, H. (1996). Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development 122:* 2079-2088.

RICHMAN, J.M. (1994). Morphogenesis of bone. In *Bone Volume 9: Differentiation and Morphogenesis of Bone* (Ed. B.K. Hall). CRC Press, Boca Raton, pp. 65-118.

RICHMAN, J.M. and TICKLE, C. (1989). Epithelia are interchangeable between facial primordia of chick embryos and morphogenesis is controlled by the mesenchyme. *Dev. Biol. 136:* 201-212.

RICKARD, D.J., SULLIVAN, T.A., SHENKER, B.J., LEBOY, P.S. and KAZHDAN, I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev. Biol. 161:* 218-228.

ROMANOFF, A.L. (1960). *The Avian Embryo. Structural and Functional Development.* The Macmillan Company, New York.

SAMPATH, T.K. and REDDI, A.H. (1981). Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. *Proc. Natl. Acad. Sci. USA 78:* 7599-7602.

SASANO, Y., OHTANI, E., NARITA, K., KAGAYAMA, M., MURATA, M., SAITO, T., SHIGENOBU, K., TAKITA, H., MIZUNO, M. and KUBOKI, Y. (1993). BMPs induce direct bone formation in ectopic sites independent of the endochondral ossification *in vivo. Anat. Rec. 236:* 373-380.

SAUNDERS, J.W. and GASSELING, M.T. (1962). Cellular death in morphogenesis of the avian wing. *Dev. Biol. 5:* 147-178.

SCHREIBER, A.B., WINKLER, M.E. and DERYNCK, R. (1986). Transforming growth factor α: a more potent angiogenic mediator than epidermal growth factor. *Science 232:* 1250-1253.

SHAH, N.M., GROVES, A.K. and ANDERSON, D.J. (1996). Alternate neural crest cell fates are instructively promoted by TGFß superfamily members. *Cell 85:* 331-343.

TAKAHASHI, Y., BONTOUX, M. and LE DOUARIN, N.M. (1991). Epithelio-mesenchymal interactions are critical for *Quox*-7 expression and membrane bone differentiation in the neural crest derived mandibular mesenchyme. *EMBO J. 10:* 2387-2393.

TENENBAUM, H.C. and HEERSCHE, J.N.M. (1982). Differentiation of osteoblasts and formation of mineralized bone *in vitro. Calcif. Tissue Int. 34:* 76-79.

TUAN, R.S. and LYNCH, M. (1983). Effect of experimentally induced calcium deficiency on the developmental expression of collagen types in chick embryonic skeleton. *Dev. Biol. 100:* 374-386.

TUAN, R.S., ONO, T., AKINS, R.E. and KOIDE, M. (1991). Experimental studies on cultured, shell-less fowl embryos; calcium transport, skeletal development, and cardiovascular functions. In *Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles* (Eds. D.C. Deeming and M.W.J. Ferguson). Cambridge University Press, Cambridge, pp. 419-433.

TURECKOVA, J., SAHLBERG, C., ABERG, T., RUCH, J.V., THESLEFF, I. and PETERKOVA, R. (1995). Comparison of expression of the *msx*-1, *msx*-2, *BMP*-2 and *BMP*-4 genes in the mouse upper diastemal and molar tooth primordia. *Int. J. Dev. Biol. 39:* 459-468.

TYLER, M.S. and HALL, B.K. (1977). Epithelial influences on skeletogenesis in the mandible of the embryonic chick. *Anat. Rec. 188:* 229-240.

URIST, M.R. (1965). Bone: formation by autoinduction. *Science 150:* 893-899.

VAINIO, S., KARAVANOVA, I., JOWETT, A. and THESLEFF, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell 75:* 45-58.

VUKICEVIC, S., LATIN, V., CHEN, P., BATORSKY, R., REDDI, A.H. and SAMPATH, T.K. (1994). Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes. *Biochem. Biophys. Res. Commun. 198:* 693-700.

WANG, E.A., ISRAEL, D.I., KELLY, S. and LUXENBERG, D.P. (1993). Bone Morphogenetic Protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors 9:* 57-71.

WANG, E.A., ROSEN, V., CORDES, P., HEWICK, R.M., KRIZ, M.J., LUXENBERG, D.P., SIBLEY, B.S. and WOZNEY, J.M. (1988). Purification and characterization of other distinct bone inducing factors. *Proc. Natl. Acad. Sci. USA 85:* 9484-9488.

WANG, E.A., ROSEN, V., D'ALESSANDRO, J.S., BAUDUY, M., CORDES, P., HARADA, T., ISRAEL, D.I., HEWICK, R.M., KERNS, K.M., LAPAN, P., LUXENBERG, D.P., McQUAID, D., MOUTSATSOS, I.K., NOVE, J. and WOZNEY, J.M. (1990). Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. USA 87:* 2220-2224.

WATANABE, Y. and LE DOUARIN, N.M. (1996). A role for BMP-4 in the development of subcutaneous cartilage. *Mech. Dev. 57:* 69-78.

WEDDEN, S.E. (1987). Epithelial-mesenchymal interactions in the development of chick facial primordia and the target of retinoid action. *Development 99:* 341-352.

WINNIER, G., BLESSING, M., LABOSKY, P.A. and HOGAN, B.L.M. (1995). Bone Morphogenetic Protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev. 9:* 2105-2116.

WOZNEY, J.M. (1992). The Bone Morphogenetic Protein family and osteogenesis. *Mol. Reprod. Dev. 32:* 160-167.

WOZNEY, J.M. (1993). Bone morphogenetic proteins and their gene expression. In *Cellular and Molecular Biology of Bone* (Ed. M. Noda). Academic Press, San Diego, pp. 131-167.

WOZNEY, J.M., ROSEN, V., CELESTE, A.J., MITSOCK, L.M., WHITTERS, M.J., KRIZ, R.W., HEWICK, R.M. and WANG, E.A. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science 242:* 1528-1534.

YAMAGUCHI, A. (1995). Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor b superfamily. *Semin. Cell Biol. 6:* 165-173.

YAMAGUCHI, A., KATAGIRI, T., IKEDA, T., WOZNEY, J.M., ROSEN, V., WANG, E.A., KAHN, A.J., SUDA, T. and YOSHIKI, S. (1991). Recombinant human bone morphogenetic protein-2 stimulates osteoblast maturation and inhibits myogenic differentiation *in vitro*. *J. Cell Biol. 113:* 681-687.

ZOU, H. and NISWANDER, L. (1996). Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science 272:* 738-741.

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