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Original Article

Convergence of the BMP and EGF signaling pathways on Smad1 in the regulation of chondrogenesis

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ABSTRACT Bone morphogenetic protein 4 (BMP4) induces, whereas epidermal growth factor (EGF) inhibits chondrogenesis. We hypothesize that BMP4 and EGF mediated intracellular signals are both coupled in the regulation of Meckel's cartilage development. Two chondrogenic experimental model systems were employed to test the hypothesis: (1) an *ex vivo*, serum-free, organ culture system for mouse embryonic mandibular processes, and (2) a micromass culture system for chicken embryonic mandibular processes. Chondrogenesis was assayed by alcian blue staining and expression of *Sox9* and *type II collagen*. Exogenous EGF inhibited and BMP4 induced ectopic cartilage in a dose-dependent manner. When BMP4- and EGF-soaked beads were implanted in juxtaposition within embryonic day 10 mouse mandibular processes, the incidence and amount of ectopic cartilage, and *Sox9* and *type II collagen* expression induced by BMP4, were significantly reduced as the concentration of EGF was increased. Similarly, in chicken serum-free micromass cultures, expression of a constitutively active BMP receptor type IB by replication competent avian retrovirus system promoted the rate and extent of chondrogenesis; however, exogenous EGF attenuated this effect. In micromass cultures, BMP signaling resulted in nuclear translocation and accumulation of the signaling molecule Smad1, whereas the addition of EGF inhibited this event. Our results suggest that BMP4 and EGF function antagonistically, yet are coupled in the regulation of initial chondrogenesis. Smad1 serves as a point of convergence for the integration of two different growth factor signaling pathways during chondrogenesis.

KEY WORDS: *Sox9, type II collagen, mandible, organ culture, micromass culture*

Introduction

Ectopic bone and cartilage formation are associated with a number of human genetic disorders such as fibrodysplasia ossificans progressiva, Albright hereditary osteodystrophy, chondrodystrophy, osteoma cutis and myositis ossificans (Jones, 1997). Extraskeletal ossifications and calcification are also found in pathological conditions such as in osteosarcoma and chondrosarcoma, or arise as orthopedic post-operative complication in joint arthroplasty. Although the etiology of some of these diseases and conditions has been documented, the causal factors and mechanisms of action remain elusive. Pathogenesis may involve deregulation or uncoupling of signal transduction pathways involved in skeletogenesis at the level of the ligands, receptors, cytoplasmic transducers, or transcription factors. Therefore, investigations into molecular controls for normal and abnormal craniofacial and skeletal development may contribute to the prevention, diagnosis and treatment of these diseases and disorders.

The onset and progression of chondrogenesis are hallmarked by three cardinal stages: (i) cessation of cell proliferation, (ii) cell

Abbreviations used in this paper: BMP, bone morphogenetic protein; EGF, epidermal growth factor; Col2a1, type II collagen; ca-BMPR-IB, constitutively active bone morphogenetic protein receptor type IB; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; DIG, digoxigenin; RCAS, replication competent avian leukemia virus long terminal repeat with a splice acceptor; E, embryonic day; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; EDTA, ethylenediaminetetraacetic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; NIH, National Institutes of Health; PTHrP, parathyroid hormone related peptide; TGF-alpha, transforming growth factor-alpha; FOP, fibrodysplasia ossificans progressiva.

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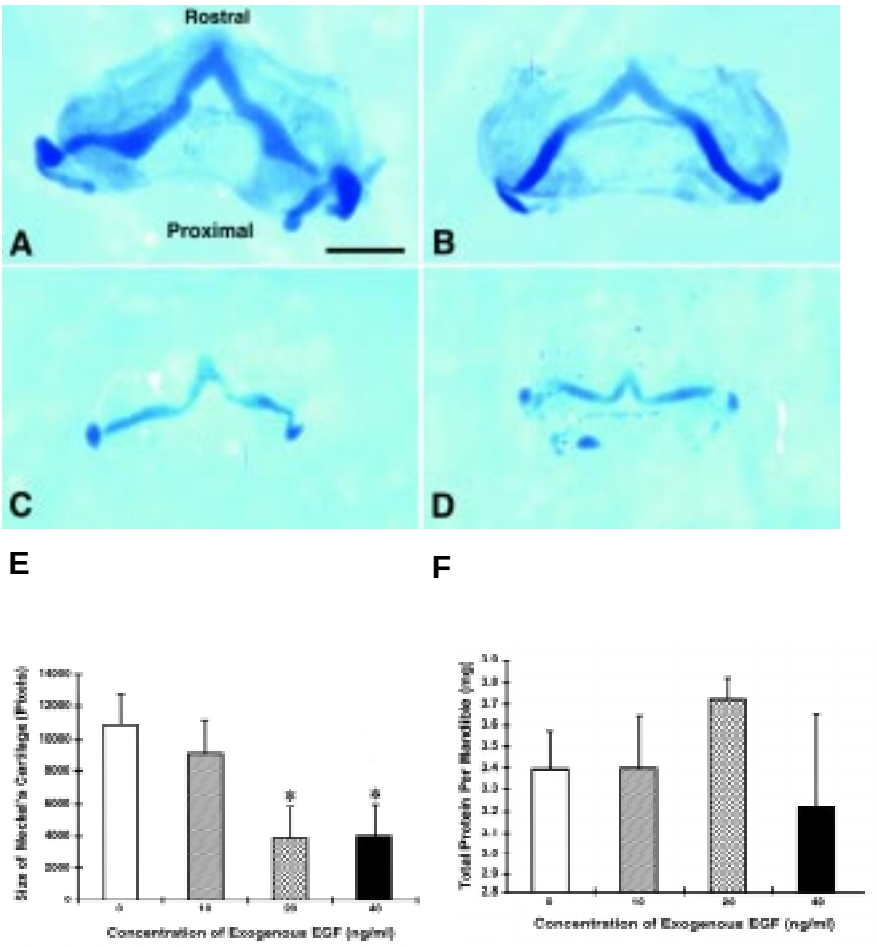


Fig. 1. Exogenous EGF inhibited cartilage formation without affecting total protein content in mouse embryonic mandibular explants. *Meckel's cartilage was identified by whole-mount alcian blue staining of E10 mouse mandibular processes cultured in serumless, chemically-defined medium for 6 days in the absence* **(A)***, or presence of 10* **(B)**, *20* **(C)** *or 40* **(D)** *ng/ml EGF. Bar, 0.4 mm. Morphometric analyses of the size of Meckel's cartilage in mandibular explants* **(E)** *cultured in the absence (open bar; N=12), or presence of 10 (hatched bar; N=12), 20 (cross hatched bar; N=10) or 40 (solid bar; N=10) ng/ml EGF were consistent with the morphological findings. *p<0.01 when compared with 0 and 10 ng/ml EGF. Total protein content in mandibular explants* **(F)** *cultured in the absence (open bar), or presence of 10 (hatched bar), 20 (cross hatched bar) or 40 (solid bar) ng/ml EGF was comparable among groups; N=3 for all groups.*

rounding and aggregation/condensation, and (iii) cytodifferentiation, including cartilage-specific transcriptional activation and extracellular matrix synthesis, secretion and deposition. These sequential events are dependent on cell-cell interactions, cell-extracellular matrix interactions, and autocrine/paracrine growth factor regulations (Daniels and Solursh, 1991; Hill and Logan, 1992; Hall and Miyake, 1995; Olsen *et al.*, 1996; Lefebvre and de Crombrugghe, 1998). A number of growth and differentiation factors have been demonstrated to alter the rate and extent of chondrogenesis, including bone morphogenetic proteins (BMPs) and epidermal growth factor (EGF).

BMPs, originally identified and isolated from semi-purified bone matrix (Urist, 1965; Wozney *et al.*, 1988) are strong inducers of bone and cartilage formation, *in vivo* and *in vitro*, in embryonic and postnatal development (Hogan, 1996; Vortkamp, 1997; Reddi, 1998; Wozney, 1998). BMPs signal through multimeric transmembrane serine/threonine kinase receptors, the activities of which are further modulated by cytoplasmic signaling molecules, the Smads (Derynck *et al.*, 1998; Kawabata *et al.*, 1998). Besides the osteogenic and chondrogenic potentials, BMPs are pleiotropic molecules and function in tissues in which epithelial-mesenchymal interactions mediate morphogenesis and differentiation (Nuckolls *et al.*, 1998). Within the murine and avian facial mesenchyme, BMP2 and 4 exhibit restrictive expression patterns (Francis-West *et al.*, 1994; Bennett *et al.*, 1995; Wall and Hogan, 1995; Helms *et al.*, 1997). They function primarily in controlling outgrowth of facial primordia (Francis-West *et al.*, 1994), skeletogenesis (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997), and odontogenesis (Vainio *et al.*, 1993; Neubüser *et al.*, 1997; Tucker *et al.*, 1998a,b; Wang *et al.*, 1998).

EGF functions as an inhibitor of chondrogenesis in the chick mandibular and limb mesenchyme (Coffin-Collins and Hall, 1989; Dealy *et al.*, 1998). In the mouse embryo, exogenous EGF inhibited cartilage formation. Further, antisense oligonucleotides treatment of embryonic mouse mandibular explants resulted in endogenous cartilage dysmorphogenesis, suggesting that endogenous autocrine and/or paracrine EGF and EGF-like proteins regulate the size and shape of cartilage (Shum *et al.*, 1993). Targeted disruption of the EGF receptor in mice also caused cartilage dysmorphogenesis (Miettinen *et al.*, 1999). However, it remains as yet unclear what are the mechanisms of action for the inhibitory effects of EGF on endogenous or ectopically-induced chondrogenesis. It is possible that EGF blocks cartilage development by functioning in all three stages of chondrogenesis, that is, promote cell proliferation, inhibit cell rounding and aggregation, and inhibit cytodifferentiation. EGF is a well-documented mitogen to many cell types (Boonstra *et al.*, 1995; Riese and Stern, 1998) including chondroprogenitor cells and chondrocytes (Gospodarowicz and Mescher, 1977; Kato *et al.*, 1983; Hall and Coffin-Collins, 1990; Vivien *et al.*, 1990; Ribault *et al.*, 1997). EGF has also been shown to act on the cytoskeleton and inhibit cell aggregation (Johtoh and Umezawa, 1992; Hazan and Norton, 1998).

In this investigation, we propose that EGF inhibits chondrogenesis by functioning specifically as an inhibitor of chondrocyte differentiation, distinct from its effects on cell proliferation and the cytoskeleton. Since BMPs are well-documented differentiation factors leading to skeletogenesis, we hypothesize that EGF acts as an inhibitor of both endogenous as well as ectopically-induced chondrocyte differentiation by antagonizing the activity of BMPs, and mediated by the convergence on and competition for the Smad1 signaling pathway. To address this issue, we have employed a mandibular process organ culture system in which BMP4-soaked beads were implanted with or without EGF-soaked beads implanted in juxtaposition, and cultured in serumless, chemically-defined medium. To further address the hypothesis, we have additionally utilized an avian micromass cell culture system in which constitutively active *BMP receptor type*

IB (ca-BMPR-IB) was delivered by retroviral-mediated gene delivery strategy, in the presence or absence of exogenous EGF. We discovered that in both experimental model systems, BMP signaling induced ectopic chondrogenesis and exogenous EGF abolished this effect in a dose-dependent manner as assayed by quantitative alcian blue staining and *type II collagen (Col2a1)* and *Sox9* expression levels. Further, in micromass cell cultures BMP signaling resulted in nuclear translocation and accumulation of Smad1, whereas the addition of EGF inhibited this event. We conclude that EGF inhibits ectopic chondrogenesis induced by BMP4 through dysregulation or uncoupling of the Smad1 signaling pathway.

Results

Exogenous EGF inhibits cartilage formation

Embryonic mouse mandibular processes were explanted into serumless, chemically-defined medium and cultured for 6 days in the presence or absence of 10, 20 or 40 ng/ml exogenous EGF. These EGF concentrations were selected because similar doses have been previously tested to elicit chondrogenic responses (Coffin-Collins and Hall, 1989; Shum *et al.*, 1993). In the absence of exogenous EGF, Meckel's cartilage consisted of three components: i) a central rostral triangular piece, ii) bilateral rod-shaped pieces, and iii) proximal pieces of cartilage (Fig. 1A). With increasing concentrations of exogenous EGF, endogenous cartilage appeared progressively smaller and less mature (Fig. 1B-D). Cartilage components appeared shorter, thinner, and were less distinct. Further, the intensity of alcian blue staining, which is indicative of the amount of chondroitin sulfate proteoglycans within the cartilage, was reduced in the presence of exogenous EGF and suggested that the cartilage was less mature.

To measure these morphological alterations, we performed morphometric analyses of the size of cartilage under various treatment conditions (Fig. 1E). Consistent with morphological observations, the addition of 10, 20 and 40 ng/ml exogenous EGF significantly reduced the size of cartilage when compared with control values ($p<0.01$). Moreover, in the presence of 20 and 40 ng/ml EGF, the size of cartilage was also significantly smaller than that at 10 ng/ml EGF ($p<0.01$), suggesting that the reduction in size of cartilage was dependent on the concentration of exogenous EGF.

The morphological results of the alcian blue stained specimens (Fig. 1A-D) showed that the size of the mandibular process explants in the presence of exogenous EGF was smaller; moreover, the detected reduction in size of cartilage reflected relative changes in association with the entire explant. However, total protein content of mandibular explants retained on supporting filters was similar between various treatment groups ($p>0.05$) (Fig. 1F). Exogenous EGF stimulated mesenchymal outgrowth from the explants onto the supporting filters. Therefore, explants recovered from filters for alcian blue staining appeared smaller, whereas explants retained on the supporting filters and associated cell outgrowths demonstrated no changes in total protein content. This observation is consistent with previous reports (Shum *et al.*, 1993).

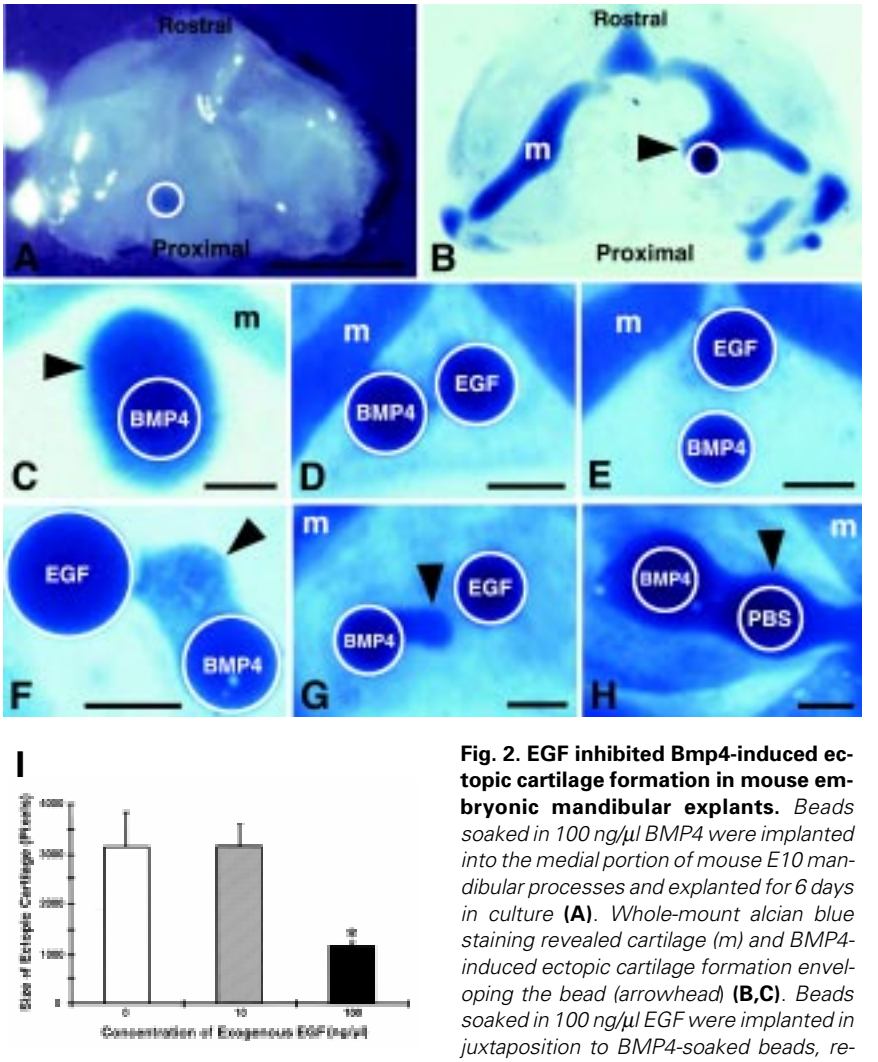


Fig. 2. EGF inhibited Bmp4-induced ectopic cartilage formation in mouse embryonic mandibular explants. *Beads soaked in 100 ng/μl BMP4 were implanted into the medial portion of mouse E10 mandibular processes and explanted for 6 days in culture* **(A)**. *Whole-mount alcian blue staining revealed cartilage (m) and BMP4-induced ectopic cartilage formation enveloping the bead (arrowhead)* **(B,C)**. *Beads soaked in 100 ng/μl EGF were implanted in juxtaposition to BMP4-soaked beads, resulting in an abrogation of ectopic cartilage formation* **(D,E)**, *or an inhibition of the progress of ectopic chondrogenesis (arrowhead) leaving a cartilage-free gap between the BMP4- and EGF-soaked beads* **(F,G)**. *Control PBS-soaked beads had no effect on BMP4-induced chondrogenesis (arrowhead)* **(H)**. *All beads were circled. Bar (A,B), 0.5mm; (C-H), 100 μm. Morphometric analyses of the size of BMP4-induced ectopic cartilage in mandibular explants* **(I)** *cultured in the absence (open bar; N=8), or presence of 10 (hatched bar; N=7), or 100 (solid bar; N=6) ng/μl EGF-soaked beads in juxtaposition were consistent with the morphological findings. *p<0.01 when compared with 0 and 10 ng/μl EGF.*

Exogenous EGF inhibits BMP4-induced ectopic cartilage formation

The mandibular explant culture system is readily accessible to micromanipulation. Beads soaked in 100 ng/μl BMP4 were implanted into the medial aspect of the developing mandible; the anatomical position of the tuberculum impar or presumptive tongue region in which no endogenous chondrogenesis occurs. This concentration of BMP4 at 100 ng/μl was similar to previous studies (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997). The explants were allowed to grow for 6 days (Fig. 2A). BMP4 induced

ectopic cartilage formation was detected in 83.3% of the explants (Table 1). Ectopic cartilage was observed to fuse with endogenous cartilage formation patterns resulting in expansion or bifurcation morphology, or as isolated ectopic cartilage pieces (Fig. 2B,C).

EGF inhibited ectopic chondrogenesis. Beads soaked in 10 or 100 ng/μl EGF were implanted in juxtaposition to the BMP4-soaked beads and cultured explants were examined after 6 days in culture. EGF reduced the incidence of BMP4-induced chondrogenesis (Table 1). EGF abrogated BMP4-induced chondrogenesis (Fig. 2D,E) or inhibited the growth of ectopic cartilage such that there was cartilage-free gaps detected between the BMP4- and the EGF-soaked beads (Fig. 2F,G). In contrast, control PBS-soaked beads had no effect on the growth and progression of BMP4-induced cartilage formation such that the ectopic cartilage completely enveloped the PBS-soaked bead before merging with endogenous cartilage (Fig. 2H). Further, comparisons between the size of ectopic cartilage induced by BMP4 in the presence or absence of EGF showed that at 100 ng/μl EGF the size of the induced cartilage was significantly smaller ($p<0.01$) (Fig. 2I). These results show a dose-dependent inhibition of BMP4-induced

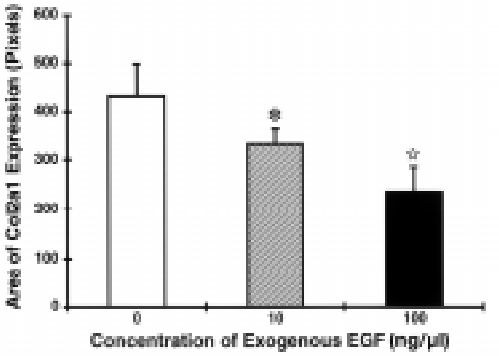


Fig. 3. EGF inhibited Bmp4-induced *Col2a1* expression in mouse embryonic mandibular explants. *Beads soaked in 100 ng/μl BMP4 were implanted into the medial portion of mouse E10 mandibular processes and beads soaked in 100 ng/μl EGF were implanted in juxtaposition. The mandibular processes were cultured for 2 days. Whole-mount in situ hybridization for Col2a1 expression was performed and morphometric analyses of the area of BMP4-induced Col2a1 expression cultured in the absence (open bar), or presence of 10 (hatched bar), or 100 (solid bar) ng/μl EGF-soaked beads were conducted. *$p<0.01$ when compared with 0ng/μl EGF, and ☆ $p<0.01$ when compared with 10 ng/μl. N=8 for all groups.*

ectopic cartilage by EGF. EGF at 10 ng/μl reduced the incidence of ectopic cartilage formation, yet in cases in which cartilage was formed, the size was similar to that of unsupplemented controls. At 100 ng/μl EGF both the frequency and size of ectopic cartilage were significantly reduced.

Exogenous EGF inhibits BMP4-induced Col2a1 and Sox9 expression

Col2a1 is a cartilage specific structural marker (Sasano *et al.*, 1992). *Sox9* is a Sry-box containing transcription factor, the earliest known marker for chondrogenesis (Wright *et al.*, 1995; Ng *et al.*, 1997; Zhao *et al.*, 1997; Bi *et al.*, 1999). Mandibular explants were implanted with BMP4-soaked beads with or without EGF-soaked

TABLE 1

INCIDENCE OF ECTOPIC CARTILAGE FORMATION INDUCED BY BMP4 IN THE PRESENCE OF EGF

Concentration of EGF (ng/μl)	0	10	100
	20/24 (83.3%)	7/27 (25.9%)	7/28 (25.0%)

beads implanted in juxtaposition and then cultured for 24 h for *Sox9* assays or 48 h for *Col2a1* assays.

Consistent with morphological results, there was a dose-dependent inhibition of BMP4-induced ectopic *Col2a1* expression by EGF. In the presence of exogenous EGF, there was a progressive decrease in the incidence of ectopic *Col2a1* expression inducible by BMP4; from 88.9% in the absence of EGF, to 66.7 and 50% for 10 and 100 ng/μl EGF, respectively (Table 2). In addition, in specimens in which ectopic *Col2a1* expression remained in the presence of EGF, there was a progressive and significant reduction in the area of gene expression ($p<0.01$) (Fig. 3).

Similarly, there was a dose-dependent inhibition of the incidence as well as the area of BMP4-induced *Sox9* expression by EGF. In the absence of EGF, BMP4-soaked beads induced a ring of *Sox9* expression in the vicinity of the beads (Fig. 4A,B), consistently and reproducibly in all specimens (Table 3). Endogenous expression of *Sox9* (which served as a morphogenetic template for Meckel's cartilage) was observed in these specimens (Fig. 4A). In the presence of exogenous EGF, there was a progressive decrease in the incidence of BMP4-induced ectopic *Sox9* expression; from 100% in the absence of EGF to 66.7 and 43.8% for 10 and 100 ng/μl EGF, respectively (Table 3). In addition, EGF completely abolished *Sox9* expression in the vicinity of the BMP4-soaked beads (Fig. 4C). EGF eliminated the *Sox9* expression domain around the BMP4-soaked bead that was closest to the EGF-soaked beads, resulting in a crescent-shaped *Sox9* expression pattern (Fig. 4D,E). PBS-soaked bead control had no effect on the incidence or the pattern of BMP4-induced *Sox9* expression (Fig. 4F). EGF reduced the size of ectopic *Sox9* expression area induced by BMP4 in a dose-dependent manner ($p<0.01$) (Fig. 4G). These results support the morphological findings that EGF inhibited ectopic chondrogenesis.

BMP4-soaked beads induced ectopic cartilage formation in chick mandibular process explant

Beads soaked in 100 ng/μl BMP4 were implanted into the medial portion of chicken mandibular explants and cultured for 6 days. BMP4-soaked beads induced ectopic cartilage formation but PBS-soaked beads have no effect on chondrogenesis (Fig. 5). These results were consistent with our observations using embry-

TABLE 2

INCIDENCE OF ECTOPIC COL2A1 EXPRESSION INDUCED BY BMP4 IN THE PRESENCE OF EGF

Concentration of EGF (ng/μl)	0	10	100
	8/9 (88.9%)	8/12 (66.7%)	8/16 (50.0%)

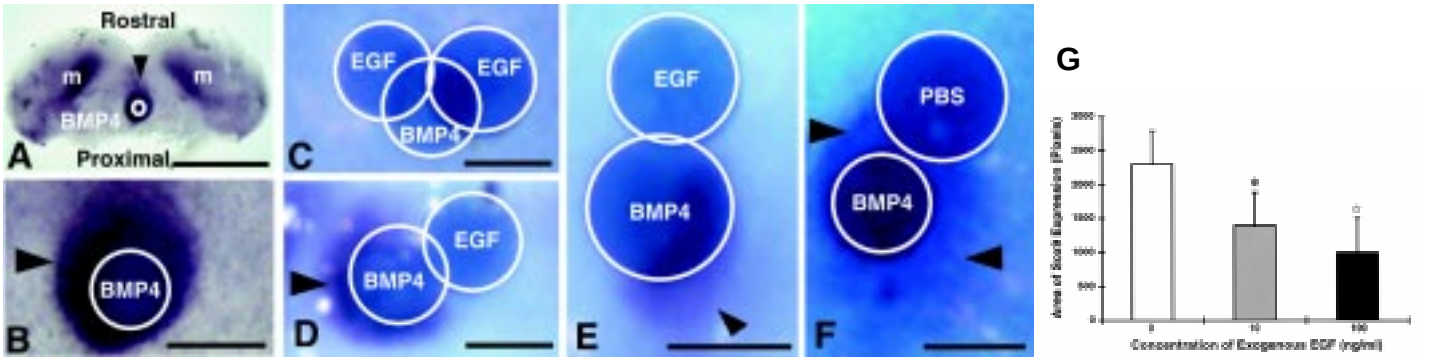


Fig. 4. EGF inhibited Bmp4-induced *Sox9* expression in mouse embryonic mandibular explants. *Beads soaked in 100 ng/μl BMP4 were implanted into the medial portion of mouse E10 mandibular processes and explanted for 1 day in culture* **(A)**. *Whole-mount in situ hybridization for Sox9 expression revealed the future position of Meckel's cartilage (m) and BMP4-induced gene expression enveloping the bead (arrowhead)* **(A,B)**. *Beads soaked in 100 ng/μl EGF were implanted in juxtaposition to BMP4-soaked beads, resulting in an abrogation of BMP4-induced Sox9 expression* **(C)**, *or an inhibition of Sox9 expression closer to the EGF-soaked beads leaving a crescent-shaped gene expression pattern around the BMP4-soaked bead (arrowhead)* **(D,E)**. *Control PBS-soaked beads had no effect on BMP4-induced Sox9 expression (arrowhead)* **(F)**. *All beads were circled. Bar (A), 0.6 mm; (B-F), 100 μm. Morphometric analyses of the area of BMP4-induced Sox9 expression in mandibular explants* **(G)** *cultured in the absence (open bar; N=10), or presence of 10 (hatched bar; N=9), or 100 (solid bar; N=7) ng/ml EGF-soaked beads in juxtaposition were consistent with the morphological findings. * p<0.01 when compared with 0 ng/μl EGF, and ☆ p<0.01 when compared with 10 ng/μl EGF.*

onic day 10 mouse mandibular explants (Fig. 2B). Since the chick mandibular process responded similarly to that of the mouse with respect to the induction of ectopic chondrogenesis by BMP4, we further utilized the avian model system to extend our investigations of the effects of BMP4 and EGF at the cellular and molecular levels of analysis.

Exogenous EGF reduced chondrogenesis and Sox9 and Col2a1 expression enhanced by constitutively active BMP receptor IB in chick mandibular micromass cultures

To examine the interactions between BMP4 and EGF during chondrogenesis at cellular and molecular levels, we employed the chick mandibular cell micromass culture system. This is a primary cell culture system that undergoes chondrogenesis even in serum-free conditions and allows for the ectopic gene expression by RCAS gene delivery strategy. As controls to monitor the timing and spread of gene expression by the RCAS method, we infected cultures with retrovirus expressing *alkaline phosphatase*. Gene expression was detected in the majority of the cells in culture 24 h after infection (Fig. 6A,B).

Chick mandibles were dissociated, placed into micromass cultures, and allowed to developed in serum-free conditions. In untreated controls, a small number of alcian blue positive chondrogenic nodules of various sizes and intensities were detected on day 5 (Fig. 6C). In these cultures cell condensation and the early formation of chondrogenic nodules were evident on day 2 and 3, respectively (data not shown). Infection with retrovirus carrying vector only [with no insert (Fig. 6E)], treatment with 300 ng/ml exogenous EGF (Fig. 6D), or a combination of mock retroviral infection and EGF supplementation (Fig. 6F) yielded similar patterns and amounts of chondrogenesis as compared with untreated controls (Fig. 6C-F). The concentration of EGF at 300 ng/ml was selected based upon empirical determinations to maximize the response in chondrogenic assays. Higher doses were found to result in toxicity (data not shown). When cultures were infected with retrovirus carrying *caBMPR-IB*, there was an apparent increase in the number, size, and intensity of alcian blue positive nodules (Fig. 6G). These changes resulted in individual nodules coalescing together to form continuous pieces of cartilage.

These results of enhanced chondrogenesis by RCAS-*ca-BMPR-IB* were consistent with previous reports using serum-containing medium (Zou *et al*., 1997). When *caBMPR-IB* expressing cultures were exposed to 300 ng/ml exogenous EGF, chondrogenesis was less than that observed in the absence of EGF, even though it appeared more when compared with untreated control (Fig. 6H). To measure these observations, we solubilized the alcian blue stained matrix and subjected it to absorbance measurement at a wavelength of 595 nm. Consistent with morphological findings, the amount of alcian blue matrix, thereby the degree of chondrogenesis, was comparable among untreated controls, cultures treated with 300 ng/ml EGF, cultures infected with retrovirus carrying vector only with no insert, or a combination of mock retroviral infection and EGF supplementation. Cultures infected with *caBMPR-IB* demonstrated an 8-fold increase in chondrogenesis ($p<0.01$) when compared with the previous groups, whereas the addition of 300 ng/ml exogenous EGF reduced the level of BMP-enhanced chondrogenesis to only 3.8-fold over controls ($p<0.01$) (Fig. 6I).

Since the rate and extent of chondrogenesis are in part dependent on cell density, we examined whether the promotion of chondrogenesis by *caBMPR-IB* was a direct effect of BMP signaling or a secondary effect from increase in cell number. We performed a cell proliferation assay and observed that cultures infected with retrovirus carrying *caBMPR-IB* exhibited similar levels of cell proliferation as the untreated controls on day 2 of culture

TABLE 3

INCIDENCE OF ECTOPIC SOX9 EXPRESSION INDUCED BY BMP4 IN THE PRESENCE OF EGF

Concentration of EGF (ng/μl)	0	10	100
	10/10 (100%)	10/15 (66.7%)	7/16 (43.8%)

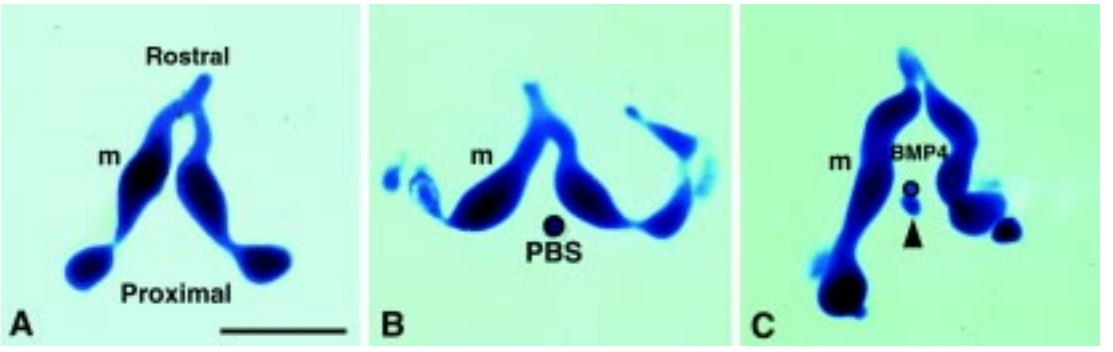


Fig. 5. Bmp4-soaked beads induced ectopic cartilage formation in chick mandibular explants. *Beads soaked in 100 ng/μl BMP4 were implanted into the medial portion of chick HH stage 24 mandibular processes and allowed to develop in vitro for 6 days. Chondrogenesis was assayed by whole-mount alcian blue staining* **(A-C)**. *BMP4 induced ectopic cartilage formation (arrowhead) (C), whereas control PBS-soaked beads had no effect on chondrogenesis (B). All beads were circled. m, Meckel's cartilage. Bar (A-C), 0.7mm.*

(Fig. 6J). Consistent with EGF being a well documented mitogen, cultures exposed to 300 ng/ml exogenous EGF showed a 14.7 to 18.3% increase in proliferative rate (Fig. 6J). However, the proliferative rates in cultures infected with RCAS-*caBMPR-IB* were comparable to untreated controls ($p>0.05$) (Fig. 6J). Therefore, we conclude that BMP signaling had no effect on the rate of cell proliferation, and resulted in no change in cell density. The enhancement of chondrogenesis by *caBMPR-IB* was attributable to a direct effect on BMP signaling.

To determine whether the antagonistic action of EGF and BMP signaling influences transcription activities, we performed semi-quantitative RT-PCR of *Sox9* and *Col2a1* transcript expression in micromass cultures in the presence or absence of retroviral delivered *caBMPR-IB* and exogenous EGF. Consistent with the qualitative and quantitative alcian blue results, relative *Sox9* transcripts were elevated by 4.4-fold in cultures infected with retrovirus carrying *caBMPR-IB* ($p<0.01$), whereas this increase was diminished to 2.7-fold when the cultures were treated with 300 ng/ml EGF ($p<0.01$) (Fig. 7A). Similarly, relative *Col2a1* transcript expression was increased by 3.4-fold in cultures infected with retrovirus expressing *caBMPR-IB* ($p<0.01$), whereas this increase was reduced to 2.8-fold when the cultures were also treated with 300 ng/ml EGF ($p<0.01$) (Fig. 7B). The magnitudes of response were similar in the alcian blue, *Sox9* and *Col2a1* assays. These molecular data confirmed and supported the morphological findings.

Nuclear translocation of Smad1 induced by BMP4 was inhibited by EGF

Chick mandibular micromass cells were transiently transfected with pRK7-*LacZ* as control or pCMV-N-terminal-FLAG-tagged-*Smad1*. Transfection efficiency was 13% in micromass culture assayed by X-gal staining 24 h after pRK7-*lacZ* transfection (Fig. 8A,B). Immunofluorescence for Smad1 in FLAG-tagged-*Smad1*-transfected cells revealed some nuclear localization in addition to cytoplasmic distribution in cells with untreated condition (Fig. 8C,D), and a predominantly nuclear localization in cells transfected with FLAG-tagged-*Smad1* and in the presence of retroviral delivered *caBMPR-IB* (Fig. 8G,H). In contrast, exposure of cells to 300 ng/ml exogenous EGF resulted in the exclusion of FLAG-tagged-*Smad1* from the nuclei (Fig. 8E,F), and reduced the increased *caBMPR-IB*-induced nuclear accumulation of *Smad1* (Fig. 8I,J). Quantitation of the percentage of cells showing nuclear accumulation of transfected FLAG-tagged-*Smad1* showed a 3.5-fold increase in cultures infected with retrovirus carrying *caBMPR-IB* ($p<0.01$); this increase was diminished to 2.1-fold when the cultures were treated with 300 ng/ml EGF ($p<0.01$) (Fig. 8K).

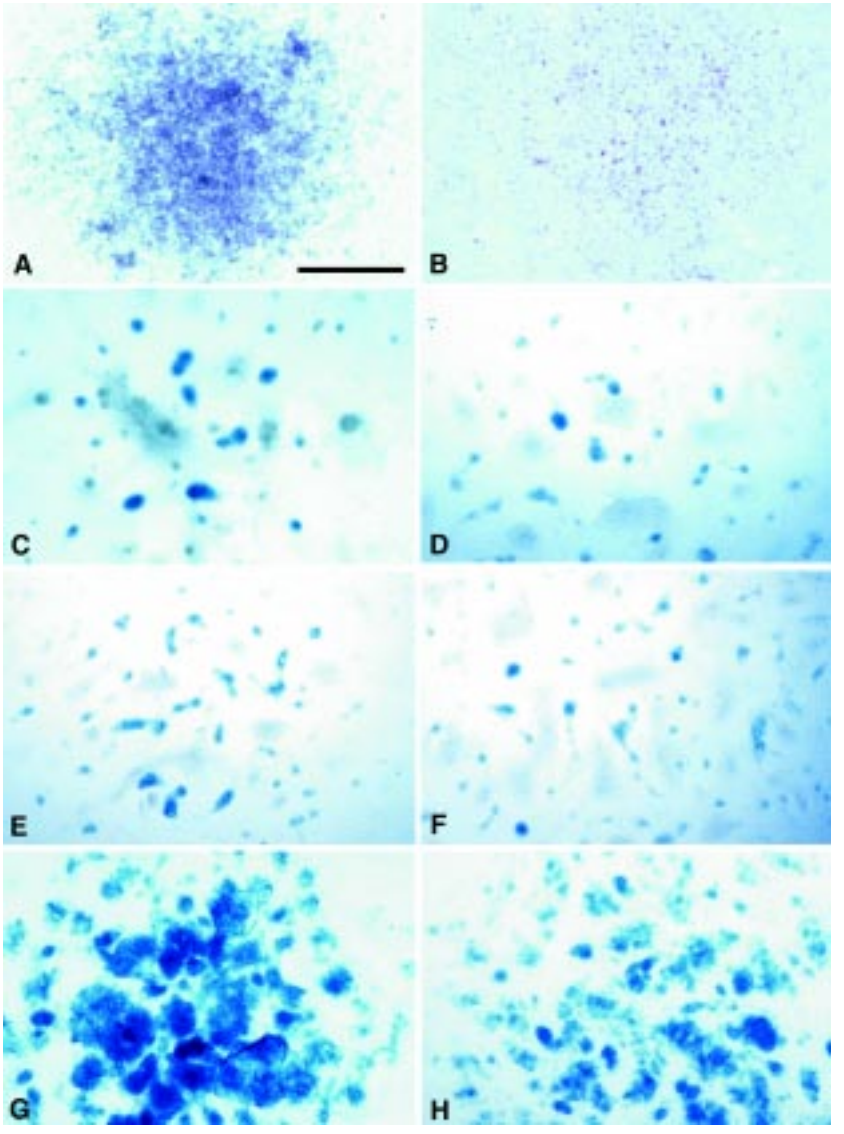
To confirm the identity of micromass cells showing nuclear accumulation of Smad1 to be chondrocytes, we double-stained for type II collagen in cells exhibiting predominantly nuclear localization of FLAG-tagged-Smad1. We observed strong positive *type II collagen* immunoreactivity in cells that were also immunopositive for Smad1 (Fig. 9). These results provide additional evidence that Smad1 mediates the effects of BMP4 in chick mandibular micromass cells leading to enhanced chondrogenesis. Moreover, this BMP4 action was antagonized or uncoupled by EGF which prevented nuclear translocation of Smad1 and reduced or inhibited chondrogenesis.

Discussion

We report the convergence or coupling of BMP and EGF signaling pathways on Smad1 during initial chondrogenesis (Fig. 10). Using various chondrogenesis assays in both the embryonic mouse as well as chick, we first documented morphologically (Figs. 2,5,6) and then molecularly (Figs. 3,4,7) that exogenous BMP4 induced ectopic cartilage development. The concomitant treatment with EGF reduced or abolished the BMP4-induced ectopic cartilage development (Figs. 2,3,4,6,7). At the cellular level, we demonstrate the nuclear translocation of Smad1; BMP4 and EGF treatment resulted in the accumulation of Smad1 in the nucleus and cytoplasm, respectively (Fig. 8). We conclude that the antagonistic functions of BMP4 and EGF on endogenous as well as ectopic chondrogenesis are integrated or coupled at the level of the signaling molecule, Smad1. This represents the first documentation of Smad1 signal integration in a developing biological system.

Combinatorial signaling during embryogenesis

The combinatorial actions of signaling growth factors, whether antagonistic or synergistic, emerge as the common mechanism for the control and regulation of diverse functions from a limited number of molecules. These combinations occur at the level of the ligands, receptors, cytoplasmic transducers, and transcription factors. Vertebrate organogenesis is often specified by the combination of different signaling molecules produced in wide yet overlapping domains rather than by a single localized inducer. For example, BMPs are multifunctional regulators of vertebrate development (Hogan, 1996). Signals elicited by BMPs are antagonized by FGFs to define the site of *Pax9* expression during tooth



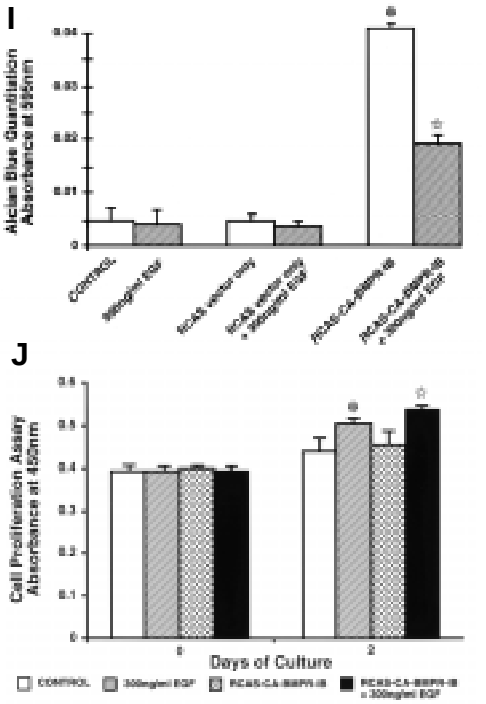


Fig. 6. EGF antagonizes the effect of constitutively active BMP receptor in the promotion of chondrogenesis in chick mandibular micromass cell cultures. *High density micromass cultures derived from HH stage 24 chick mandibular processes were infected with RCAS-alkaline phosphatase to monitor the timing and spread of infection. Twenty-four hours after infection, increased staining for alkaline phosphatase was observed in infected cultures* **(A)** *in comparison to uninfected control cultures* **(B)**. *Micromass cultures on day 5 were also stained with alcian blue to reveal the formation of cartilaginous nodules (C-H). Cultures were uninfected* **(C)**, *or treated with 300 ng/ml EGF* **(D)**, *or infected with no insert control RCAS vector only* **(E)**, *or infected with no insert control RCAS vector and treated with 300 ng/ml EGF* **(F)**, *or infected with RCAS-ca-BMPR-IB only* **(G)**, *or infected with RCAS-ca-BMPR-IB and treated with 300 ng/ml EGF* **(H)**. *Bar (A-H), 1mm. Alcian blue quantitation confirmed that exogenous EGF reduced the amount of chondrogenesis enhanced by constitutively active BMP receptor in chick mandibular micromass culture* **(I)**. **p<0.01 when compared with all other treatment conditions, and ☆ p<0.01 when compared with RCAS-ca-BMPR-IB infected cultures. N=3 for all groups. Exogenous EGF promoted chick mandibular mesenchymal cell proliferation but constitutively active BMP receptor had not effect* **(J)**. **p<0.01 when compared with uninfected control, and ☆ p<0.01 when compared with RCAS-ca-BMPR-IB infected cultures. N=4 for all groups.*

bud initiation (Neubüser *et al.*, 1997), to regulate *Barx1* expression during facial primordia patterning (Barlow *et al.*, 1999), to specify tooth identity (Tucker *et al.*, 1998a,b), to specify the erythropoietic lineage (Xu *et al.*, 1999), to regulate limb bud patterning and digit formation (Niswander and Martin, 1993; Ganan *et al.*, 1996; Buckland *et al.*, 1998), and to regulate the amount of cellular retinoic acid binding protein produced by fibroblast (Means and Gudas, 1996) or *PTHrP* expression in hypertrophic chondrocytes in cultures (Terkeltaub *et al.*, 1998). BMP signaling is also antagonized with Shh to regulate cartilage formation (Watanabe *et al.*, 1998). In this report we show that BMP4 signaling and EGF signaling have opposing effects during mouse and chick mandibular chondrogenesis. Similar to our observations, EGF was previously found to suppress the BMP-induced differentiation of an osseous cell line derived from fetal rat calvaria (Bernier and Goltzman, 1992).

Combinatorial signaling achieves two purposes: 1) allows for a single pleiotropic molecule to be functional at various times and positions during embryogenesis, such that the specificity of morphogenesis and differentiation are a summation of multiple inputs, and 2) allows for the establishment of tissue boundaries. Within the developing mandibular process, BMP4 has been demonstrated to be involved in odontogenesis, including the initiation and progression through the bud and cap stages, enamel knot formation and regression, and specification of tooth types (Peters and Balling, 1999; Tucker and Sharpe, 1999). At the same developmental stages, BMP4 also functions during chondrogenesis and osteogenesis (Hogan, 1996; Vortkamp, 1997; Reddi, 1998). On the

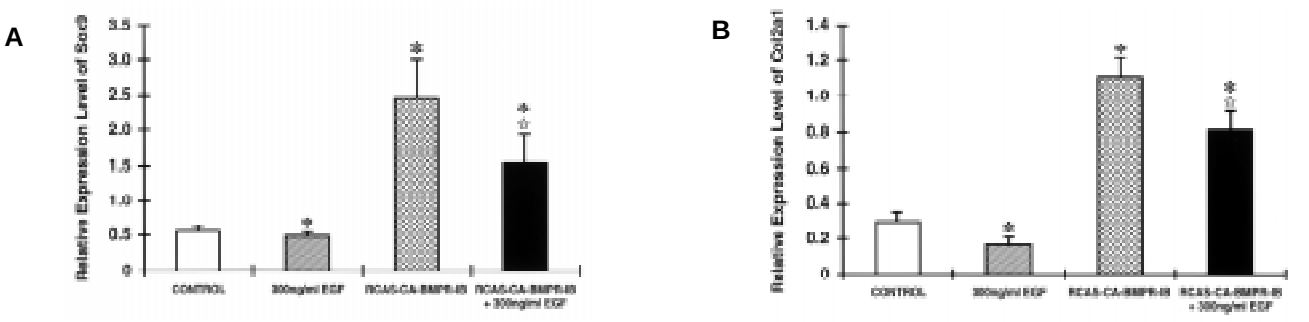


Fig. 7. Exogenous EGF reduced *Sox9* (A) and *Col2a1* (B) expression enhanced by constitutively active BMP receptor in chick mandibular micromass cultures. *Semi-quantitative RT-PCR was performed on day 1 or 2 of culture for Sox9 or Col2a1 expression, respectively. Constitutively active BMP receptor enhanced gene expression by 3-4 fold, whereas treatment with EGF reduced both endogenous as well as BMP-enhanced gene expression. #p<0.01 when compared with uninfected control, and ☆ p<0.01 when compared with RCAS-ca-BMPR-IB infected cultures. N=8 for all groups.*

other hand, EGF is also involved in multiple stages of odontogenesis (Slavkin *et al.*, 1994) and chondrogenesis (Coffin-Collins and Hall, 1989; Shum *et al.*, 1993; Miettinen *et al.*, 1999).

BMP4 and *EGF* have expression patterns that are partially overlapping with each other and with other growth and differentiation factors. Curiously, these molecules may be coupled or integrated. For example, the size and shape of Meckel's cartilage during embryonic mouse and chick mandible development may be determined in part by the expression domain of *BMP4* and *EGF*. In particular, the expression of *TGF-alpha*, which also binds to the *EGF* receptor, at the tuberculum impar (Yamane *et al.*, 1997) may serve to exclude the future tongue region from producing cartilage. It is likely that these morphoregulatory molecules additionally couple with other signaling molecules to further define a multitude of differentiative capacities within the developing mandible.

Relevance of Smad1 as a point of convergence of diverse signaling pathways

Our studies not only observed the antagonistic functions of BMP4 and EGF during endogenous as well as ectopic chondrogenesis, but also demonstrated the molecular integration of these two signaling pathways at the level of Smad1. Previously, the opposing actions of BMP and EGF were elucidated at the biochemical level (Kretzschmar *et al.*, 1997). BMP signaling resulted in the phosphorylation of carboxy terminal residues of Smad1 leading to nuclear translocation and downstream activation, whereas

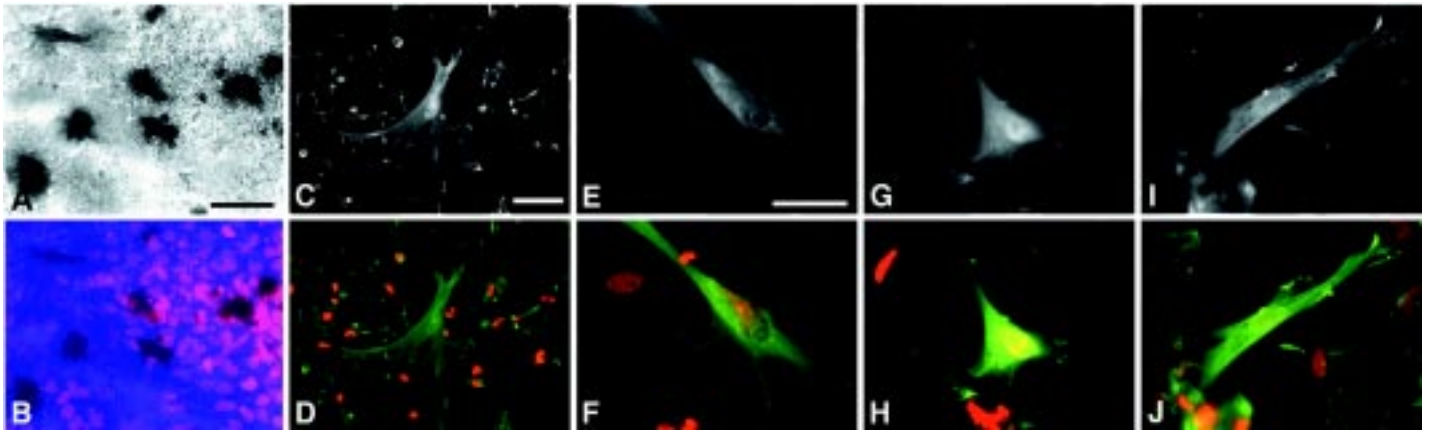


Fig. 8. Nuclear accumulation of Smad1 was enhanced by constitutively active BMP receptor and inhibited by exogenous EGF in chick mandibular micromass cultures. *Chick mandibular micromass cultures were transiently transfected by electroporation with pRK7-lacZ* **(A,B)** *and beta-galactosidase staining was performed 24 h later to monitor for transfection efficiency* **(A)**. *Cultures were also transiently transfected by electroporation with FLAG-tagged Smad1* **(C-J)**. *These cultures were untreated* **(C,D)**, *or additionally treated with 300 ng/ml exogenous EGF for 1 h* **(E,F)**, *or infected with RCAS-ca-BMPR-IB only* **(G,H)**, *or infected with RCAS-ca-BMPR-IB and treated with 300 ng/ml exogenous EGF for 1 h* **(I,J)**. *Immunostaining was performed using anti-FLAG monoclonal antibody and rhodamine-conjugated secondary antibody* **(C,E,G,I)**. *Specimens were counterstained with Hoecht 33342 to visualize the nuclei* **(B,D,F,H,J)**. *Bar, 100 μm. Exogenous EGF resulted in the exclusion of FLAG-Smad1 from the nuclei and inhibited caBMPR-IB-induced nuclear accumulation of Smad1* **(K)**. **p<0.01 when compared with uninfected control, and ☆ p<0.01 when compared with RCAS-ca-BMPR-IB infected cultures. N=7 for all groups.*

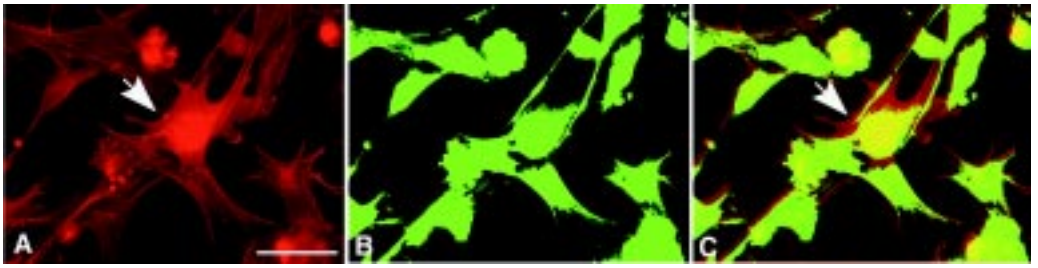


Fig. 9. Type II collagen was detected in cells showing predominantly nuclear localization of FLAG-Tagged Smad1. (A) *Nuclear accumulation of FLAG-tagged Smad1 was enhanced by caBMPR-IB. FLAG-tagged Smad1 transfected cells were infected with RCAS-caBMPR-IB and incubated for an additional 48 h. FLAG-tagged Smad1 expression was visualized with rhodamine (red)-conjugated secondary antibody (arrow).* **(B)** *Type II collagen expression was detected by FITC (green)-conjugated secondary antibody in the same cell.* **(C)** *Arrowhead indicated double positive nuclei (yellow). Bar, 100 mm.*

EGF signaling produced phosphorylation of the linker region of Smad1 instead, which was inhibitory to nuclear accumulation. The control of cell fate may be achieved by subcellular localization of Smad1. Similarly, in the chondrogenic cell line C5.18, BMP signaling mediated by Smad1 has been shown to activate *Sox9* response elements on the *type X collagen* promoter (Harada *et al.*, 1998). Since chondrogenesis was dependent on the concentration of either BMP4 or EGF in our experiments (Figs. 2,3,4; Tables 1,2,3), we suggest that a nuclear gradient of Smad1 accumulation may exist; that is, increasing concentration of EGF resulted in increasing concentration of linker-phosphorylated Smad1 and decreasing concentration of nuclear-localized Smad1. A nuclear gradient of Smad activity has also been proposed to explain the concentration dependent patterning of the *Xenopus* ectoderm by BMP4 and Smad1 (Wilson *et al.*, 1997). In our investigations, we suggest that a gradient of Smad1 activity produces transcriptional activation of cartilage marker genes such as *Sox9* and *Col2a1* at varying amounts (Table 2,3). However, *Sox9* and *Col2a1* appeared to be operative when a threshold level was attained. In our experiments, low levels of *Sox9* induced by 100 ng/μl BMP4 in the presence of 10 or 100 ng/μl EGF resulted in a much lower incidence of actual cartilage formation than the molecular marker expressions (Table 1,2,3). Consistent with our observations, high levels of *Sox9* expression was associated with all sites of cartilage formation, whereas low levels were detected in non-cartilaginous tissues

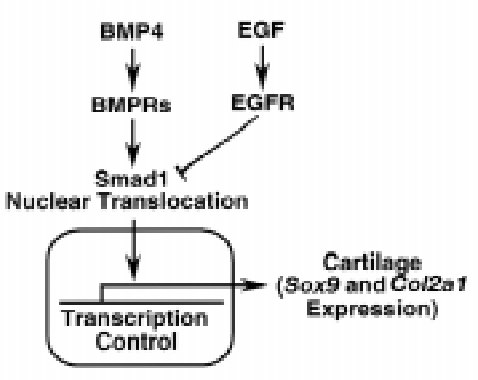


Fig. 10. BMP4 induces cartilage formation in organ culture and promotes chondrogenesis in micromass cultures, whereas the addition of EGF counteracts this effect in both systems. *These two pathways converge on the signaling molecule Smad1. BMP4 causes Smad1 to translocate into the nucleus to activate transcription of cartilage specific genes such as Sox9 and Col2a1, whereas EGF reduces BMP4-induced nuclear accumulation of Smad1.*

such as notochord, neural tube, heart and lung (Ng *et al.*, 1997; Zhao *et al.*, 1997). It is likely that similar activities may involve Smad5 which also transduces BMP signals (Tamaki *et al.*, 1998; Macias-Silva *et al.*, 1998), and mediates BMP-induced osteoblast differentiation in C2C12 cells (Yamamoto *et al.*, 1997; Nishimura *et al.*, 1998).

Potential therapeutic applications of EGF as an inhibitor of ectopic chondrogenesis

Our findings demonstrate that EGF inhibited BMP4-induced ectopic chondrogenesis. We further show that these diverse signal pathways converged prior to activation of cartilage specific genes and manifestation of the cartilage phenotype. This integration or coupling between BMP4 and EGF signaling may provide a unique opportunity to explore innovative therapies for human skeletal disorders. In particular, fibrodysplasia ossificans progressiva (FOP), a rare genetic disorder characterized by congenital malformation of the great toes, progressive heterotopic ossification of the neck, spine and shoulder girdle, thus severely limiting mobility, is directly attributable to BMP4 deregulation. In this disorder, BMP4 expression level was found to be elevated in lymphoblastoid cell lines derived from FOP patients (Shafritz *et al.*, 1996; Lanchoney *et al.*, 1998). Preosseous fibromatous lesions from patients were immunopositive for BMP2 and BMP4 (Gannon *et al.*, 1997). Overexpression of BMP4 could potentially be counteracted by EGF treatment.

In summary, we provided evidence that EGF acts as an anti-differentiation factor for BMP4 during endogenous as well as ectopic chondrogenesis. These signal transduction pathways converge and integrate at the level of Smad1 subcellular localization. These coupled interactions may further provide an opportunity for the prevention and therapy of human skeletal diseases.

Materials and Methods

Mandibular process explant culture and bead implantation

Timed pregnant Swiss Webster mice were obtained (Harlan Bioproducts for Science, Indianapolis, IN) and embryos at embryonic day 10 (E10), Theiler stage 16 (Theiler, 1989) were collected. Fertilized chicken eggs were obtained (Truslow Farms Inc., Chestertown, MD), and embryos at Hamburger and Hamilton (1992) stage 24 were harvested. Organ culture of mandibular processes was performed according to previously described methods (Shum *et al.*, 1993). Mandibular processes were cultured using serumless, chemically-defined BGJb medium (Life Technologies Inc., Gaithersburg, MD) supplemented with 100 μg/ml ascorbic acid. Exogenous EGF (ICN Biomedicals Inc., Aurora, OH) was added directly into the culture medium at a concentration of 300 ng/ml. The culture medium was changed every other day.

Affi-Gel blue agarose beads (Bio-Rad Labs., Hercules, CA) with a diameter of 100-120 μm were selected and soaked in 10 or 100 ng/μl human recombinant BMP4 (Genetics Institute Inc., Cambridge, MA), 100 ng/μl mouse submaxillary gland EGF (ICN Biomedicals Inc., Aurora, OH), or PBS for 6 h at room temperature. BMP4-, EGF- or control PBS-soaked beads were implanted into the mandibular processes using a mouth-controlled micropipette under the stereomicroscope.

Whole-mount alcian blue staining and quantitation

The presence of sulfated proteoglycans indicative of cartilage formation was detected by alcian blue staining. Mandibular explants were stained with Alcian Blue 8GX (Sigma, St. Louis, MO) at 0.04% in acid ethanol followed by tissue clearing with a graded series of potassium hydroxide and glycerol (Shum *et al.*, 1993). Micromass cultures were fixed with acid ethanol for 15 min at room temperature, hydrated and stained overnight at 4°C with 0.5% Alcian Blue 8GX in 0.1M HCl. The amount of alcian blue staining was quantitated by a colorimetric assay (Paulsen *et al.*, 1988). The alcian blue stained cartilage matrix was solubilized from the specimens by incubation in 4M guanidine hydrochloride overnight at 4°C. The absorbance at 595 nm was measured with a MRX Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA).

Whole-mount in situ hybridization

Probes for mouse *Sox9* (Wright *et al.*, 1995; 926-1683nt), and *Col2a1* (Metsaranta *et al.*, 1991; GenBank M65161: 29648-31343nt) were obtained by RT-PCR method and confirmed by direct sequencing. Digoxigenin (DIG)-labeled sense and antisense riboprobes for *Sox9* and *Col2a1* were prepared by *in vitro* transcription using RNA Transcription Kit (Stratagene, La Jolla, CA) according to specifications from the manufacturer. Whole-mount *in situ* hybridization was performed as previously described (Rosen and Beddington, 1993) with modifications. Specimens were fixed overnight in 4% paraformaldehyde in PBS at 4°C, bleached for 1 h with 6% hydrogen peroxide in PBS containing 0.1% Tween 20 at room temperature, and permeabilized with 10 μg/ml proteinase K for 15 min at room temperature. Following post-fixation and prehybridization, the specimens were hybridized overnight with 1 μg/ml DIG-labeled riboprobes at 70°C. The specimens were washed extensively after which endogenous alkaline phosphatase activities were blocked by overnight incubation in 0.48 mg/ml levamisol (Sigma, St. Louis, MO), and incubated with anti-DIG alkaline phosphatase conjugated antibody (Boehringer Mannheim Corp., Indianapolis, IN) at a dilution of 1:2000 for 90 min at 4°C. The color reaction was developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Sigma, St. Louis, MO).

Morphometric analyses

The size of cartilage visualized by alcian blue staining or the area of gene expression visualized by whole-mount *in situ* hybridization in the mandibular processes was quantitated on photographs of processed specimens taken at identical magnification. The photographs were optically scanned, the outlines of stained areas were traced, and the relative area determined by NIH Image Version 1.6.1 (NIH, Bethesda, MD). Signal to background was determined by density slice method residing within the analysis software package.

Protein determination

Six day cultured mandibular explants were lysed in 100 μl of 4M guanidine hydrochloride. The lysate was analyzed using the BCA Protein Assay Kit (Pierce, Rockford, IL). A standard curve was generated using known concentrations of bovine serum albumin diluted in 4M guanidine hydrochloride. The amount of total protein in each explant was calculated from the optical density reading plotted against the standard curve and the dilution factor.

Chick embryo mandibular cell micromass culture

Chick mandibular processes at stage 24 were isolated and dissociated in 0.25 mg/ml trypsin EDTA (Life Technologies, Inc., Gaithersburg, MD) and 0.25 mg/ml collagenase (Washington Biochemical Corp., Lakewood, NJ) in 0.1M PBS for 10 min at 37°C. Ten microliters of the cell suspension at $2x10^7$ cells/ml were plated in serum-containing medium for 1 h to allow for initial cell attachment (Stott *et al.*, 1998) and then cultured in serum-free, chemically-defined medium as described (Southerland and Lucas, 1995). Exogenous EGF (ICN Biomedicals Inc., Aurora, OH) was added directly into the serumless culture medium at a concentration of 300 ng/ml. Culture medium was changed every other day.

pRK7-LacZ transfection in chick mandibular micromass cultures and X-gal staining

Ten nanograms of pRK7-*LacZ* were electroporated into chick mandibular micromass cell and plated at a density of $2x10^7$ cells/ml. After 24 h of culture in serum-free medium, cells were fixed in 4% paraformaldehyde for 10 min. Specimens were washed in PBS and incubated for 12 h at 37°C in 1 mg/ml X-gal solution, with 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet P-40 (Takahashi *et al.*, 1998) to confirm the transfection efficiency.

Retrovirus production and gene delivery systems

Medium containing retrovirus was produced in chicken embryonic fibroblasts, concentrated and tittered according to Morgan and Fekete (1996). Briefly, QT6 quail cells at 20% confluence were plated and infected with a serial diluted series of concentrated RCAS-*caBMPR-IB* (Zou *et al.*, 1997) or no insert control RCAS retroviral stock. After 48 h, the cells were fixed in 4% paraformaldehyde and processed for immunocytochemistry for retroviral coat protein, using anti-gag monoclonal antibody, AMV-3C2 (Developmental Study Hybridoma Bank, The University of Iowa, IW) at 1:100 dilution for 12 h at 4°C, and rhodamine-conjugated anti-mouse IgG secondary antibody (Jackson Immuno Research, West Grove, PE) at 1:100 dilution for 2 h at room temperature.

Ten microliters of chick mandibular cells at $2x10^7$ cells/ml in micromass culture were infected with 1 μl of RCAS-*caBMPR-IB* (titer of $5x10^9$ pfu/ml) or 1.25 μl of no insert control RCAS (titer of $4x10^9$ pfu/ml) and cultured at 37°C, 5% CO_2 for up to 5 days in serumless medium. After 1,2 or 5 days of culture with medium change every other day, specimens were processed for semi-quantitative RT-PCR analysis for *Sox9*, *Col2a1* and *β-actin* expression level, or for whole-mount alcian blue staining for detection of cartilage formation, respectively. In experiments examining the nuclear translocation of Smad1, ten nanograms of pCMV-N-terminal-FLAG-tagged-Smad1 were electroporated into chick mandibular cells, plated at a density of $2x10^7$ cells/ml and then immediately infected with retrovirus. Exogenous EGF at 300 ng/ml was then added in the serum-free medium and immunostaining for FLAG-tagged-Smad1 was performed after 48 h. To monitor the timing and spread of ectopic gene expression by RCAS gene delivery strategy, chick mandibular micromass cultures were infected with retrovirus expressing alkaline phosphatase (RCAS-AP). Twenty four hours after infection, the cells were fixed in 4% paraformaldehyde for 10 min. Specimens were washed in PBS and incubated for 20 min at 37°C in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma, St. Louis, MO) for color development.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from day 1 (for *Sox9* assay) or day 2 (for *Col2a1* assay) micromass cultures using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to specifications from the manufacturer. After DNase treatment of total RNA, one microgram of total RNA from each sample was transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD). Semi-quantitative RT-PCR was performed to evaluate the expression level of *Sox9* or *Col2a1* relative to that of *β-actin*. Amplimers designed for *Sox9* were 5'-GGAAAGCGA-CGAGGACAAAT-3' and 5'-TACTTGTAGTCGGGGTGGTC-3' based on the chicken *Sox9* cDNA sequence (Uwanogho *et al.*, 1995; Accession U12533), that for *Col2a1* were 5'-CCCGCAGGA-GAGCAGGGACAGCG-3' and 5'-CCTCTCCTTTGGCACCATCCAAACC-3' based on the chicken *Col2a1*

cDNA sequence (Nah and Upholt, 1991; Accession M74435), and that for *β-actin* were 5'-TGCTGATGCCGTAACTGCC-3' and 5'-TGAGGTAGTCCG-TCAGGTCC-3' based on the actin cDNA sequence (Alonso *et al.*, 1986). Thirty three cycles of amplification for Sox9 annealing at 60°C for 30 sec, 35 cycles for *Col2a1* annealing at 56°C for 30 sec, and 28 cycles for *β-actin* annealing at 57°C for 30 sec were empirically determined to optimize for signal and amplification linearity. PCR amplifications were carried out using Ready To Go PCR Beads (Amersham Pharmacia Biotech, Arlington Heights, IL) and GeneAmp PCR System 2400 (Perkin Elmer, Branchburg, NJ). PCR products were separated by electrophoresis, stained with ethidium bromide, imaged with charged coupled device camera, then pixel depth was converted to optical density and quantitated with the aid of NIH Image Version 1.6.1 (NIH, Bethesda, MD). The identities of the PCR fragments from total RNA were confirmed by direct sequencing.

Immunocytochemistry for FLAG-tagged Smad1 and type II collagen

Cultures were fixed with 4% paraformaldehyde in PBS for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 30 min, and blocked in 10% normal goat serum, 5% bovine serum albumin, 0.025% Triton X-100 in PBS for 60 min. The cells were incubated with anti-FLAG M2 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:360 dilution for overnight at room temperature and then with rhodamine conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA) at a 1:200 dilution for 2 h at room temperature. The specimens were counterstained with Hoechst 33342 (Molecular Probes, Inc. Eugene, OR) to visualize the nuclei. The percentage of cells showing nuclear accumulation of Smad1 was determined by visual inspection and cell counting of stained specimens. Nuclear accumulation of Smad1 was defined by the apparent more intense staining in the nucleus than that in the cytoplasm. Chick mandibular micromass cultures immunostained for FLAG-tagged Smad1 were also double-stained using anti-type II collagen antibody and FITC-conjugated secondary antibody. Immunohistochemical procedure was performed as previously described (Mizoguchi *et al.*, 1993).

Cell proliferation assay

The total number of viable cells in each mandibular micromass culture at the beginning and day 2 of culture was determined using a colorimetric assay, the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to specifications from the manufacturer. The reaction was allowed to go for 4 h and the absorbance at 450 nm was measured with a MRX Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA).

Statistical analysis

All experiments were performed at least in triplicate. Numerical data were subject to statistical analyses using Student's t-test. Statistical significance was deduced at $p<0.05$.

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