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...
bone and cartilage formation, in vivo and in vitro, in embryonic and matrix (Urist, 1965; Wozney et al., 1988) are strong inducers of growth factor (EGF). Including bone morphogenetic proteins (BMPs) and epidermal demonstrated to alter the rate and extent of chondrogenesis, 1998). A number of growth and differentiation factors have been and Miyake, 1995; Olsen et al., 1996; Lefebvre and de Crombrugghe, 1991; 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

**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 (open bar; N=12), or presence of 10 (hatched bar; N=10) or 20 (cross hatched bar) 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 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.
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).

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 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.
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

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

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Table 1: Incidence of ectopic cartilage formation induced by BMP4 in the presence of EGF

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

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Table 2: Incidence of ectopic Col2a1 expression induced by BMP4 in the presence of EGF

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 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.
BMP and EGF converge on Smad1 to regulate chondrogenesis

Fig. 4. EGF inhibited Bmp4-induced Sox9 expression in mouse embryonic mandibular explants. Beads soaked in 100 ng/ml BMP4 were implanted into the medioventral 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/ml 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=8), 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/ml EGF, and + p<0.01 when compared with 10 ng/ml EGF.

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 develop 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**

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<td>INCIDENCE OF ECTOPIC Sox9 EXPRESSION INDUCED BY BMP4 IN THE PRESENCE OF EGF</td>
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<td></td>
<td>10/10</td>
<td>10/15</td>
<td>7/16</td>
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<td></td>
<td>(100%)</td>
<td>(66.7%)</td>
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Fig. 6. Ectopic chondrogenesis by BMP4 in chick mandibular micromass cultures. (A) Control PBS-soaked beads were implanted into the medioventral portion of mouse E10 mandibular processes and explanted for 1 day in culture. 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/ml 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=8), 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/ml EGF, and + p<0.01 when compared with 10 ng/ml EGF.
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. 8K).

To confirm the identity of micromass cells showing nuclear accumulation of Smad1 to be chondrocytes, we double-stained for FLAG-tagged-Smad1 and in the presence of BMP4-induced ectopic cartilage development. The concomitant treatment with BMP4 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 cartilage development 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.

**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 BMP4 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
BMP and EGF converge on Smad1 to regulate chondrogenesis

BMP and EGF converge on Smad1 to regulate chondrogenesis (Neubüser et al., 1997), to regulate Barx1 expression (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 fibroblasts (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
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

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**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.

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**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.
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 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 fibromatosus 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.
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., 1998). The alcian blue stained cartilage matrix was solubilized from the specimens by incubation in 4M guanidine hydrochloride overnight at 4°C. The absorbance at 955 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, which for Sox9 was used 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-indolylophosphate (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 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 extract 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 2×10^7 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 (Southland 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 2×10^5 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 K3Fe(CN)6, 35 mM K4Fe(CN)6, 2 mM MgCl2, 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 filtered according to Morgan and Fekete (1996). Briefly, QTS equal cell cultures at 20% confluence were plated and infected with a serial diluted series of concentrated RCAS-cabMPPR-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, IOW) 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 2×10^7 cells/ml in micromass culture were infected with 1 µl of RCAS-cabMPPR-IB (titer of 5×10^5 pfu/ml) or 1.25 µl of no insert control RCAS (titer of 4×10^6 pfu/ml) and cultured at 37°C, 5% CO2 for up to 5 days in serum-free 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 2×10^5 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-indolylophosphate/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. Amplifiers designed for Sox9 were 5′-GGAAAGGCG-GAGGACAAAT-3′ and 5′-TACTTGTAGTGGGGTGTC-3′ based on the chicken Sox9 cDNA sequence (Uwano et al., 1995; Accession U12533), that for Col2a1 were 5′-CCGGACAGGAGCAGGAGAG-3′ and 5′-CCTCTCCTTGGGACCCATCCAACC-3′ based on the chicken Col2a1...
BMP and EGF converge on Smad1 to regulate chondrogenesis

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.

References


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⼤o, 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).

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