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Distinct Functions of BMP4 and GDF5 in the Regulation of Chondrogenesis

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Abstract Bone morphogenetic protein 4 (BMP4) and growth/differentiation factor 5 (GDF5) are closely related protein family members and regulate early cartilage patterning and differentiation. In this study, we compared the functional outcome of their actions systematically at various stages of chondrogenesis in mouse embryonic limb bud mesenchyme grown in micromass cultures. Overall, both growth factors enhanced cartilage growth and differentiation in these cultures. Uniquely, BMP4 not only accelerated the formation and maturation of cartilaginous nodules, but also induced internodular mesenchymal cells to express cartilage differentiation markers. On the other hand, GDF5 increased the number of prechondrogenic mesenchymal cell condensation and cartilaginous nodules, without altering the overall pattern of differentiation. In addition, GDF5 caused a more sustained elevated expression level of Sox9 relative to that associated with BMP4. BMP4 accelerated chondrocyte maturation throughout the cultures and sustained an elevated level of Col10 expression, whereas GDF5 caused a transient increase in Col10 expression. Taken together, we conclude that BMP4 is instructive to chondrogenesis and induces mesenchymal cells toward the chondrogenic lineage. Furthermore, BMP4 accelerates the progression of cartilage differentiation to maturation. GDF5 enhances cartilage formation by promoting chondroprogenitor cell aggregation, and amplifying the responses of cartilage differentiation markers. These differences may serve to fine-tune the normal cartilage differentiation program, and can be exploited for the molecular manipulation in biomimetics. J. Cell. Biochem. 91: 1204–1217, 2004. Published 2004 Wiley-Liss, Inc.†

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During evolution and the refinement of more complex physiology, cellular and developmental events are increasingly controlled by stringent yet versatile signaling networks. Large protein families can produce such highly regulated signaling networks in two manners: family members can have redundant functions that produce a backup system, and have distinct functions that generate fine-tuning to the balance of signaling. The end result is that combinatorial signaling at specific stages and sites of development ensures developmental fidelity. The mammalian genome consists of many genes encoding proteins that belong to large families. These proteins are closely related to each other in both structure and function. One such large family that consists of members with pleiotropic functions is the transforming growth factor beta (TGF-β) family. To date, there are more than 25 family members, of which bone morphogenetic proteins (BMPs) and growth/differentiation factors (GDFs) account for 14 members, and are considered a subfamily of the TGF-βs [Ducy and Karsenty, 2000].

BMPs were first discovered when demineralized bone matrix materials injected into muscle compartments resulted in ectopic endochondral bone formation [Urist, 1965]. Since then, BMPs have been documented to be multifunctional and participate in the developmental events of many tissues and organs. One of the key functions of BMPs is to regulate limb bud

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development. BMPs are instrumental to the formation of the limb and are intimately involved in multiple stages of limb development, including patterning, outgrowth, apical ectodermal ridge regression, digit formation, chondrogenesis, digit identity specification, interdigital apoptosis, and joint formation [for reviews, see [Dahn and Fallon, 1999; Merino et al., 1999a; Capdevila and Izpisua Belmonte, 2001; Pizette and Niswander, 2001] and references therein]. However, the complex events of patterning, cell fate determination, and differentiation of limb bud mesenchyme are independent as well as interdependent of each other. Furthermore, several BMPs and their cognate receptors are present throughout limb bud development [Hogan, 1996; Cheifetz, 1999]. Therefore, it may be difficult to specifically assign direct functions to a single BMP. BMP4 and GDF5 are closely related protein family members that share some common receptors. However, they also bind to and elicit responses from unique receptor combinations. In vivo and in vitro studies have shown that both BMP4 and GDF5 are instrumental to skeletogenesis [Storm et al., 1994; Duprez et al., 1996; Francis-West et al., 1999; Storm and Kingsley, 1999; Tsumaki et al., 1999, 2002; Coleman and Tuan, 2003a]. In these studies, the primary functions of BMP4 and GDF5 on precartilage mesenchymal cells or chondroprogenitor cells have not been clearly delineated from an overall enhancement of cartilage production. Therefore, a direct comparison of these two closely related factors during the multiple stages of early cartilage differentiation in primary mesenchyme remains to be characterized.

In this investigation, we employed a micromass culture system in which limb bud mesenchyme was dissociated and plated at high density to allow for cell–cell interactions and cell aggregation in a manner similar to in vivo development. However, these dissociated cells would not reconstitute a patterned limb bud, and thereby limb bud patterning is effectively uncoupled from chondrogenesis. This system allows us to isolate the cellular functions of BMP4 and GDF5 during chondrogenesis in primary mesenchyme. Our specific aim is to take advantage of this system and address the distinct functions of BMP4 and GDF5 in their regulation of cartilage formation without the confounding variables of signaling that is dependent on developmental patterning. Using this culture system, and markers and assays specific for the progressive stages of chondrogenesis, we determine that both BMP4 and GDF5 enhance cartilage differentiation but they function by different cellular mechanisms. BMP4 is instructive to chondrogenesis. BMP4 induces mesenchymal cells to become chondroprogenitors and promotes their differentiation into mature chondrocytes. Similarly, GDF5 also enhances chondrocyte differentiation, but in contrast, does so primarily by promoting cell condensation.

MATERIALS AND METHODS

Cell Culture

Animal studies conformed to approved Animal Study Protocols from the National Institutes of Health. Timed pregnant Swiss Webster mice (Harlan Bioproducts for Science, Indianapolis, IN) were obtained and embryos were collected at embryonic day 10 (E10). Micromass culture of embryonic limb bud mesenchymal cells was performed as described previously [Ahrens et al., 1977] with minor modifications. Briefly, whole limb buds were dissociated into single cell suspension at 1.5×10^7 cells/ml, plated as 10 μl drops and cultured in 45% Dulbecco's Modified Eagle Medium (D-MEM), 45% F12 Medium (Invitrogen Life Technologies, Gaithersburg, MD), and 10% fetal calf serum (HyClone, Logan, UT), supplemented with 100 μg/ml ascorbic acid and 1 mM sodium pyruvate. After 24 h of initial plating, cultures were treated with BMP4 or GDF5 (R&D Systems, Minneapolis, MN). Total cell number was determined by dissociating the cultures and counting the cells using a Beckman Coulter Z1 Cell and Particle Counter (Beckman Coulter, Fullerton, CA). Cell counting was conducted in triplicate samples.

Murine embryonic cell line C3H10T1/2 clone eight cells were cultured as described previously [Haas and Tuan, 1999] and maintained in D-MEM supplemented with 10% fetal calf serum. Cultures were treated similarly as those derived from embryonic limb buds.

Alcian Blue, Alkaline Phosphatase, and Peanut Agglutinin Staining

The presence of sulfated proteoglycans indicative of cartilage formation was detected by alcian blue staining [Lev and Spicer, 1964]. Staining and quantitation of staining

intensity were performed as described previously [Nonaka et al., 1999] with minor modifications. Briefly, cultures were fixed in 2% acetic acid in ethanol for 15 min at room temperature, rehydrated and stained overnight at 4°C with 0.5% Alcian Blue 8GX (Sigma, St. Louis, MO) in 0.1 M HCl. The alcian blue stained cartilage matrix was then solubilized with 4 M guanidine hydrochloride. The absorbance at 595 nm was measured with a MRX Microplate Reader (Dynatech Laboratories, Inc., Chantilly, VA).

The presence of alkaline phosphatase (AP), indicative of cartilage maturation, was detected by AP staining [Mello and Tuan, 1999]. Cultures were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) for 10 min at room temperature. Cultures were washed and permeabilized with 0.2% Triton X-100 in Tri-buffered saline (TBS, pH 7.4) and stained with an AP solution containing 0.4 mg/ml nitro blue tetrazolium (NBT), 0.19 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 50 mM $MgSO_4$ in 100 mM TBS at pH 9.5.

Mesenchymal cell aggregates were detected by peanut agglutinin (PNA) staining method [Zimmermann and Thies, 1984]. Cultures were fixed in 4% PFA, washed, blocked, and incubated with 50 μg/ml biotinylated anti-PNA (Vector Laboratories, Burlingame, CA) at 4°C overnight. The positive reaction was developed using an ABC kit (Vector Laboratories, Burlingame, CA).

In Situ Hybridization

In situ hybridization was performed as described previously [Semba et al., 2000] with minor modifications. Briefly, cultures were fixed in 4% PFA, permeabilized with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN), postfixed, prehybridized, and hybridized overnight with 1 μg/ml digoxigenin (DIG)-labeled riboprobes at 70°C. The cultures were then washed, blocked, and incubated with anti-DIG alkaline phosphatase conjugated antibody (Roche, Indianapolis, IN) at a dilution of 1:1,500 overnight at 4°C. The cultures were washed and the color reaction was developed using NBT/BCIP (Roche, Indianapolis, IN). Sense and antisense riboprobes for Col2a1 were prepared as described [Semba et al., 2000].

The number of nodules expressing Col2a1 or positive for PNA staining as described in the previous section was counted under a light microscope within an area of 1.5×1.5 mm^2 located at the center of the culture. The number of alcian blue positive nodules was counted for each quardrant of the culture. Nodule counting was conducted in at least triplicate.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from micromass cultures using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to specifications from the manufacturer. Semi-quantitative RT-PCR was performed to evaluate the expression level of Sox9, Col2a1, aggrecan, or collagen type X (Col10) relative to that of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described [Sabath et al., 1990; Elima et al., 1993; Takahashi et al., 1998].

Luciferase Assay

Cultures were transfected with 1.5 μg of the Col2a1 promoter luciferase reporter; pKN185 [Krebsbach et al., 1996] or p4x48/Col2a1 [Lefebvre et al., 1996], and 0.05 μg renilla luciferase control reporter, pRL-CMV (Promega Corporation, Madison, WI), using 5 μl of Fugene reagent (Roche, Indianapolis, IN) according to specifications from the manufacturer. pKN185 contains the native proximal Col2a1 promoter and enhancer, whereas p4x48/Col2a1 contains the native sequences as well as a four times tandem repeat of the Sox9 response element. One day later, cultures were treated with growth factors and luciferase activities were assayed another 24 h later using the Dual Luciferase Reporter System (Promega) according to protocols supplied from the manufacturer, and analyzed using a Fluoroskan Accent FL Fluorescent Plate Reader (Labsystem, Franklin, MA).

Statistical Analysis

Numerical data were analyzed by computer software package for statistical analysis (Excel 2001, Microsoft Co., Redmond, WA). All values are reported as mean ± SE of the mean. Statistical significance was determined using Student's *t*-test using a confidence level of 95% ($P < 0.05$).

RESULTS

BMP4 and GDF5 Increased Total Cell Number

In order to compare and contrast the functions of BMP4 and GDF5 systematically in their regulation of cartilage formation, we first analyzed their effects on cell proliferation. Total

cell number was determined in limb bud micromass after 24 h of culture in the absence of growth factors, or in the presence of BMP4 or GDF5 at 10, 100, or 500 ng/ml (Fig. 1). These concentrations were tested because similar doses have been used previously to elicit a chondrogenic response [Chimal-Monroy and Diaz de Leon, 1997; Francis-West et al., 1999]. In the absence of exogenous growth factor supplements, the total cell number after 24 h of culture (194.7 ± 7.8%) almost doubled that of initial plating, which was designated as 100%. In the presence of BMP4, total cell numbers were not significantly different from controls, except for a modest increase at the 100 ng/ml dose (control, 197.4 ± 7.8%; BMP4, 215.7 ± 0.06%). In the presence of GDF5, we detected significant increases in total cell number in all concentrations tested, albeit no dose-dependency was correlated (control, 197.4 ± 7.8%; 10 ng/ml GDF5, 217 ± 0.98%; 100 ng/ml, 228.6 ± 3.16%; 500 ng/ml, 222.3 ± 2.8%).

GDF5 Promoted Mesenchymal Cell Condensation and Subsequent Cartilage Nodule Formation More Significantly Than BMP4

Next, we investigated the differential functions of BMP4 and GDF5 on the early formation of cartilage by morphometric analyses. High density micromass cultures derived from primary limb bud mesenchyme spontaneously differentiate by mesenchymal condensation and subsequent differentiation into cartilaginous nodules, in a manner that closely resembles in vivo chondrogenesis [Ahrens et al., 1977; Kaplowitz et al., 1982; Edwall-Arvidsson and Wroblewski, 1996]. Mesenchymal cells isolated from mouse E10 limb buds that corresponded to Theiler Stage 16 were in a prechondrogenic and precondensation stage at the time of isolation. After 1 day of culture, PNA staining showed distinct positive signal over cellular aggregates within the cultures (Fig. 2A,E). At lower magnification, this punctate pattern can be quantified by counting the number of PNA positive foci in a defined area in the culture. BMP4 at 10 or 100 ng/ml significantly increased the number of mesenchymal condensations by approximately 50% (Fig. 2B–C,I). Interestingly, at the highest concentration, BMP4 inhibited cell aggregation (Fig. 2D,I). In the presence of 10 ng/ml GDF5, the number of mesenchymal condensations were unaffected (Fig. 2F,I). However, at 100 or 500 ng/ml, GDF5 treatment resulted in similar significant increases in the number of condensations (Fig. 2G–I).

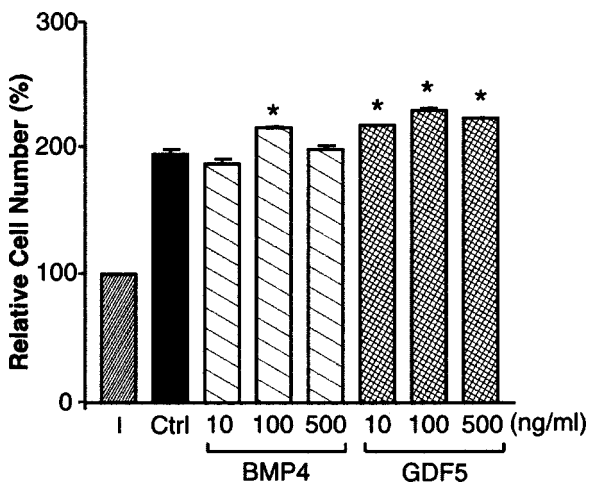
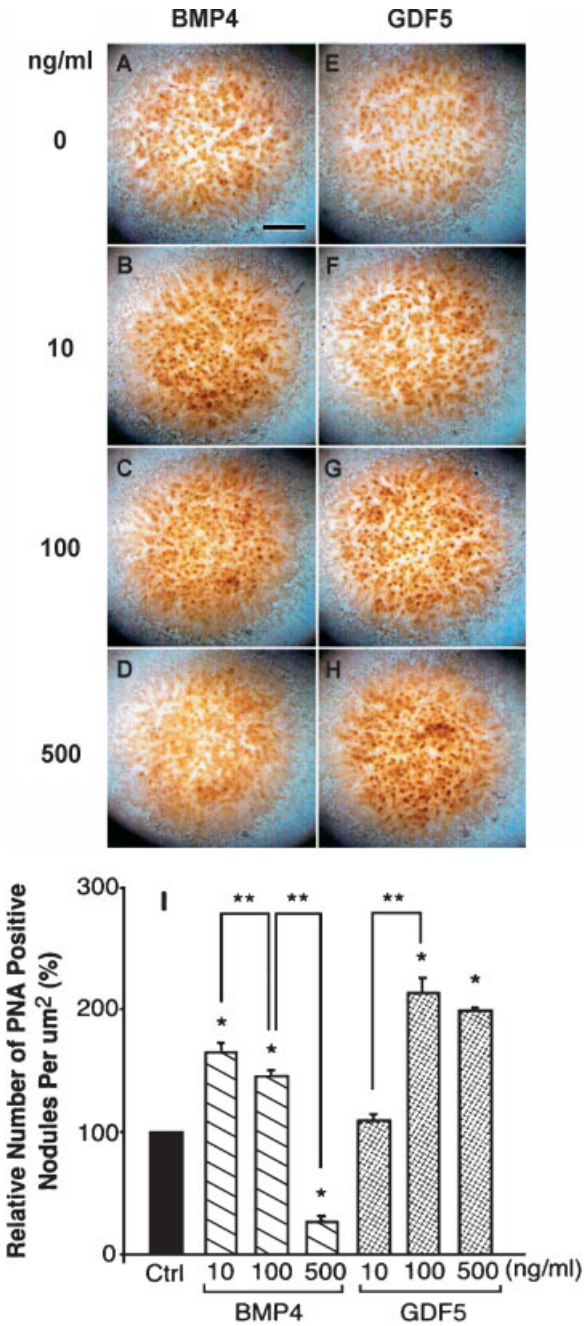


Fig. 1. BMP4 and GDF5 increased total cell number of micromass cultures. Limb bud mesenchymal cells were initially plated for 24 h, at which the total cell number was designated as 100% (I). Cultures were allowed to develop for an additional 24 h in the absence of growth factor supplement (Ctrl), or in the presence of three different doses of BMP4 or GDF5. Control cultures (Ctrl) showed a doubling of total cell number. BMP4 or GDF5 treatment resulted in slight increases in total cell number. Data represent mean ± SE of the mean for five samples in each group. $*P < 0.05$ when compared with control.

Most chondrogenic cell condensations eventually became cartilaginous nodules, which can be identified by alcian blue staining after 3 days of culture. Both BMP4 (Fig. 3B–D,I) and GDF5 (Fig. 3F–I) treatment resulted in significant, dose-dependent increases in alcian blue staining when compared with controls (Fig. 3A,E). It appears that these increases were in part due to an increase in the intensity of staining of the nodules, which was most evident at the high doses (Fig. 3D,G–H). This suggests that the growth factors promoted sulfated matrix deposition in micromass cultures. When the same dose of BMP4 and GDF5 were compared, GDF5 increased alcian blue staining to a significantly greater extent than BMP4. We have also cultured limb mesenchyme in micromass with the initial plating cell density reduced to half. In these cultures, cartilaginous nodules were formed but in a much reduced number when compared with normal cell density (Fig. 3J,L), consistent with previous observations [Rodgers et al., 1989]. When cultures were treated with 100 ng/ml BMP4 or GDF5, increased staining was observed. While BMP4 enhanced the intensity of staining of the nodules, GDF5 also

significantly increased the number of nodules formed ($P < 0.05$); control was 28.25 ± 3.34, BMP4-treated was 31.25 ± 0.83, and GDF5-treated was 46 ± 5.79 nodules per quandrant of culture (Fig. 3K,M). Taken together, these results suggest that both growth factors promoted matrix deposition. In addition, GDF5 promoted mesenchymal condensation and subsequent nodule formation.



BMP4 and GDF5 Promoted Cartilage Gene Expression

In addition to morphometric analyses, we have also compared BMP4 and GDF5 in their regulation of cartilage gene expression. Micromass cultures were transfected with two different Col2a1 luciferase reporters (Fig. 4A). Both BMP4 and GDF5 transactivated the reporters to similar extents and with similar profiles, suggesting that these growth factors utilized similar mechanism in activating Col2a1 expression. Since one of the reporters, p4x48/Col2a1, has been engineered to be specifically responsive to Sox9 binding and transactivation [Lefebvre et al., 1996], our results further suggested that BMP4 and GDF5 activation of Col2a1 were mediated similarly by Sox9.

We next examined the accumulation of cartilage marker transcripts, such as Sox9, Col2a1, and aggrecan using RT-PCR. We detected increased expression in all markers induced by BMP4 or GDF5 in micromass cultures (Fig. 4B). These increases were dose-dependent, except for the expression of aggrecan induced by GDF5. The extent of the increases were similar for both growth factors. Interestingly, when we examined the temporal profile of Sox9 expression using time points at 4, 8, 24, 48, and 72 h of treatment, we revealed differences between BMP4 and GDF5 actions. In control cultures, Sox9 expression transiently increased at 24 and 48 h time points, which coincided with chondroprogenitor cell condensation and the early phases of chondrogenesis. BMP4 and GDF5 induction of Sox9 expression was first detectable after 24 h of treatment (Fig. 4C). BMP4 induction of Sox9 peaked at 24–48 h of treatment, after which the level returned to that of

Fig. 2. BMP4 and GDF5 promoted mesenchymal cell condensation at different levels. Limb bud mesenchymal cells were cultured for 24 h in the absence of growth factor supplement (**A**, **E**), or in the presence of three doses of BMP4 (**B–D**) or GDF5 (**F–H**). Precartilaginous cell aggregates that represent initial nodule formation were identified by peanut agglutinin staining. Peanut agglutinin positive nodules were counted within a defined area at the center of the culture and expressed as a percentage of the untreated control (Ctrl) (**I**). BMP4 at 10 and 100 ng/ml promoted mesenchymal cell condensation but the higher dose resulted in an inhibition of PNA positive aggregates. GDF5 at 100 and 500 ng/ml significantly promoted cell condensation. Numerical data represent mean ± SE of the mean for three samples in each group. *$P < 0.05$ when compared with control, **$P < 0.05$ when compared among doses. Scale bar in (A) for all images is 1 mm.

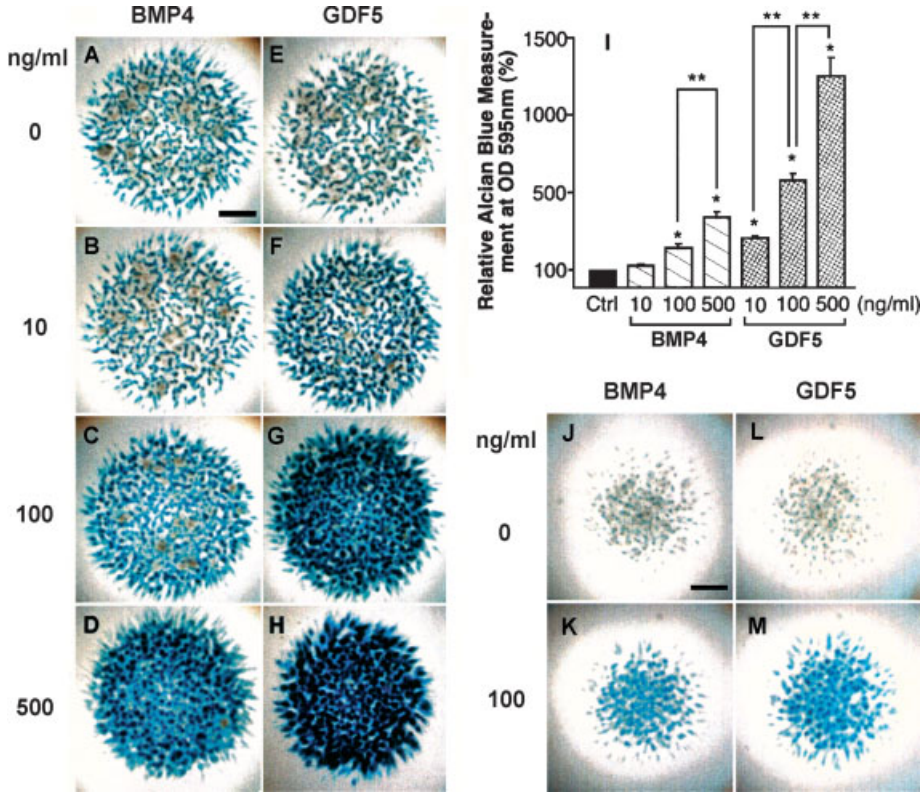


Fig. 3. GDF5 promoted cartilage nodule formation and sulfated proteoglycan deposition more significantly than BMP4. Limb bud mesenchymal cells were cultured for 3 days in the absence of growth factor supplement (**A**, **E**), or in the presence of three doses of BMP4 (**B–D**) or GDF5 (**F–H**). Cartilaginous nodules were identified by alcian blue staining. Alcian blue stain was solubilized, quantitated, and expressed as a percentage of the untreated control (Ctrl) (**I**). GDF5 was more potent in enhancing alcian blue staining when compared with BMP4. Limb bud mesenchymal cells were also cultured for 3 days at half the normal plating density in the absence of growth factors (**J**, **L**) or in the presence of 100 ng/ml BMP4 (**K**) or GDF5 (**M**), and stained for alcian blue. GDF5 significantly increased the number of alcian blue positive cartilaginous nodules (46 ± 5.79 nodules per quandrant of culture) as well as enhanced staining when compared with control (28.25 ± 3.34). In contrast, BMP4 enhanced staining without significantly altering the number of nodules (31.25 ± 0.83). Numerical data represent mean $\pm$ SE of the mean for six samples in each group. $^{*}P < 0.05$ when compared with control, $^{**}P < 0.05$ when compared among doses. Scale bar in (A) for all images is 1 mm.

control values. In contrast, GDF5 induction of Sox9 peaked at 48 h time point, after which expression was reduced but remained significantly elevated over control values. These differences in Sox9 expression profiles produced by BMP4 and GDF5 were not reflected in the expression profiles of Col2a1, which were similar for the two factors (Fig. 4D).

BMP4 Induced Cartilage Formation From Mesenchymal Cells and Cartilage Maturation

In order to distinguish whether increases in cartilage gene expression induced by BMP4 and GDF5 were due to increased expression in a subpopulation of cells in the culture, or more generally to all the cells, we performed in situ hybridization on the micromass cultures for Col2a1 after 1 day of culture. In control cultures, Col2a1 expression was detected in mesenchymal cell condensation (Fig. 5A,E), a pattern reminiscent of that of PNA staining (compare with Fig. 2A,E), suggesting that these cell aggregates were chondrogenic. Both BMP4 and GDF5 increased Col2a1 expression in micromass cultures, consistent with our RT-PCR data. However, the resulting patterns from the two growth factors were different. It appeared that BMP4 induced a modest increase in Col2a1 signal within the cell aggregates destined to be cartilaginous nodules (Fig. 5B–D). In addition, signal was also detected at the internodular spaces where the cells were not organized into aggregates. This was more evident at higher magnification and at higher doses (Fig. 5I–J). Col2a1 signal was observed in the overall culture, albeit a higher level was seen within

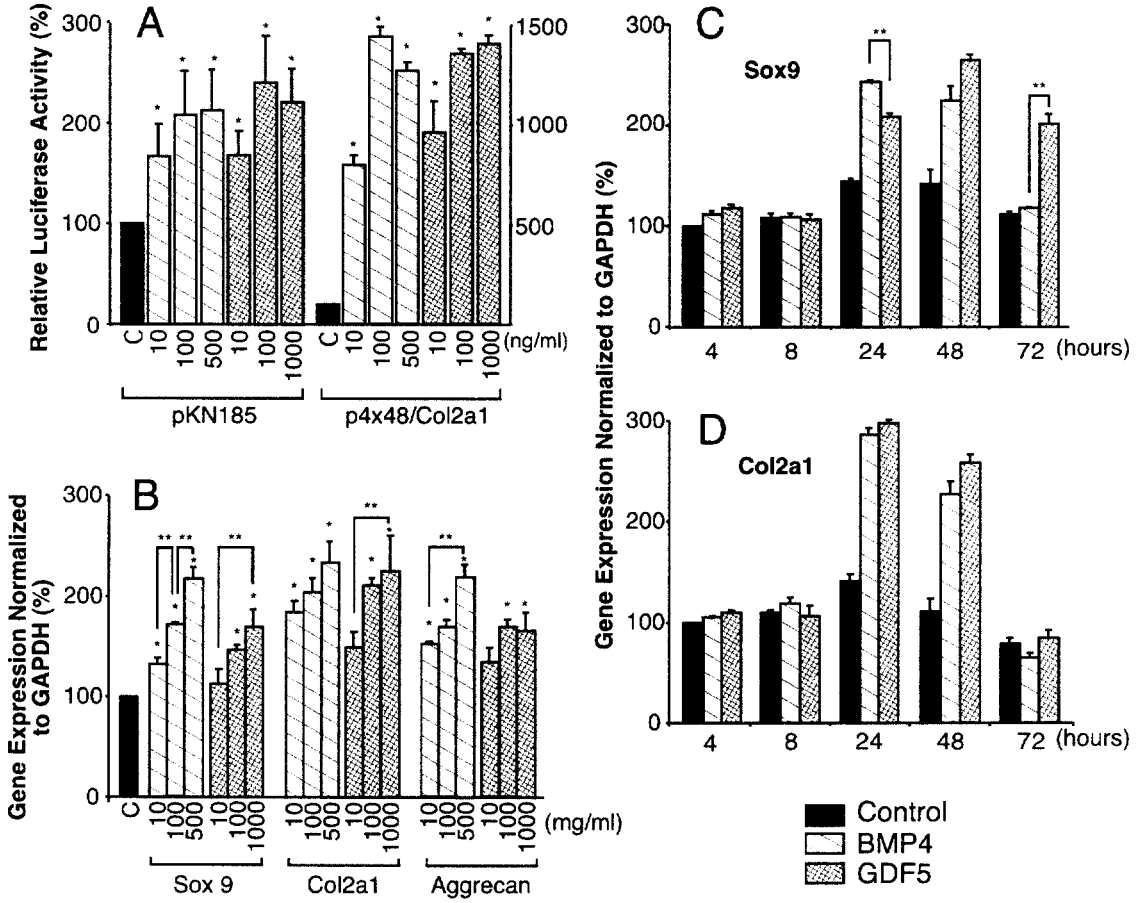


Fig. 4. BMP4 and GDF5 elevated the expression of Col2a1 and Sox9. Limb bud mesenchymal cells were transfected with Col2a1 promoter luciferase reporter; pKN185 or p4x48/Col2a1 for 24 h after which the cells were untreated (Ctrl) or treated with three doses of BMP4 or GDF5. Luciferase activities of Col2a1 promoters were assayed 24 h later and normalized with activities from control reporter (**A**). Luciferase activities of untreated cells were designated as 100%. Left and right ordinates are for luciferase activities for pKN185 and p4x48/Col2a1, respectively. Limb bud mesenchymal cells were also treated with BMP4 or GDF5 and the expression level of the cartilage markers Sox9, Col2a1, and aggrecan were assayed by RT-PCR (**B**). Marker gene expression was normalized against GAPDH and the expression level of untreated control cells (Ctrl) was designated as 100%. A time course of Sox9 (**C**) or Col2a1 (**D**) expression was monitored at 4, 8, 24, 48, and 72 h after cells were treated with 100 ng/ml BMP4 or GDF5 was performed. Peak expression of Sox9 induced by BMP4 was at 24 h, whereas that by GDF5 was at 48 h. Data represent mean ± SE of the mean for nine samples in each group. $^{*}P < 0.05$ when compared with control, $^{**}P < 0.05$ when compared among doses.

the cell aggregates. In contrast, GDF5 induced a strong Col2a1 signal within the cell aggregates, leaving the internodular areas relatively signal free (Fig. 5F–H,K–L). In experiments in which micromass cultures were maintained in serum-free medium, the differences between BMP4 and GDF5 induced chondrogenesis were accentuated. BMP4 treatment resulted in micromass cultures that were alcian blue positive throughout most of the culture (Fig. 5M–N), whereas GDF5 treatment caused increase in the number of cartilaginous nodules, as well as increased intensity of alcian blue stain in the nodules (Fig. 5O-P).

C3H10T1/2 is a multipotential mesenchymal cell line that is capable of differentiating into cells of the bone, cartilage, muscle, and fat lineages under defined conditions [Gazit et al., 1993]. When these cells were treated with BMP4 or GDF5, the cultures exhibited increased alcian blue staining (Fig. 5Q–V). BMP4 was effective even at the relatively low concentration of 10 ng/ml. At the same concentration, GDF5 has minimum effects, although there were increased staining at higher doses. BMP4 induced a stronger and more uniform staining when compared with GDF5. On the other hand, GDF5 treated cultures displayed patchy stain-

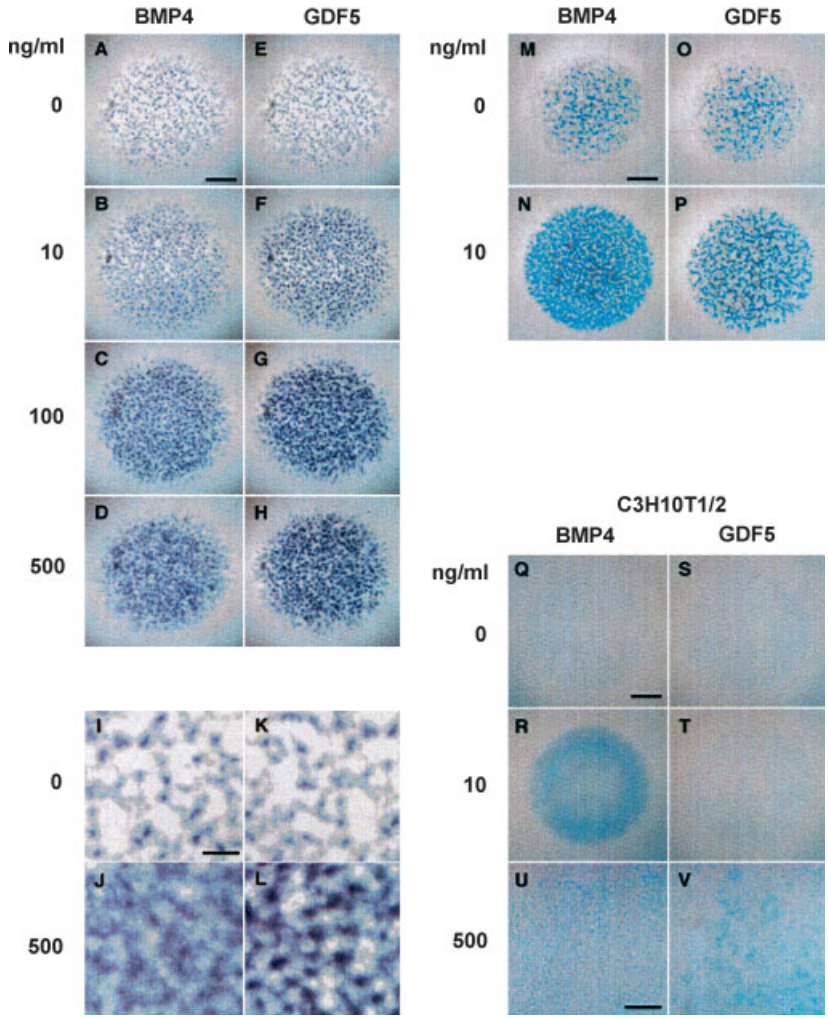


Fig. 5. BMP4 induced cartilage differentiation from mesenchymal cells. Limb bud mesenchymal cells were cultured for 24 h in the absence of growth factors (**A**, **E**), or in the presence of three doses of BMP4 (**B–D**) or GDF5 (**F–H**). Cartilage differentiation was monitored by the expression of Col2a1 identified by in situ hybridization. Treatment with either BMP4 or GDF5 resulted increases in Col2a1 expression. Upon higher magnification, Col2a1 expression was enhanced at cartilaginous nodules, and was detected also at internodular spaces (**J**) when compared with control (**I**). In contrast, micromass cultures treated with GDF5 only exhibited enhancement of nodular expression of Col2a1 (**L**), whereas internodular domains remain negative for Col2a1 as observed in the control (**K**). Under serum-free culture conditions, the difference between BMP4 and GDF5 was accentuated. BMP4 at 10 ng/ml produced micromass cultures that displayed alcian blue staining throughout most of the culture (**N**), whereas GDF5 at the same concentration increased the number of alcian blue positive nodules without increasing staining at internodular spaces (**P**) when compared with controls (**M**, **O**). Mesenchymal cell line C3H10T1/2 is also responsive to BMP4 and GDF5. BMP4 enhanced overall alcian blue staining in these cells cultured as micromass for 4 days (**R**, **U**), whereas GDF5 produced minimal effects but produced a more patchy pattern reminiscent of nodule formation (**T**, **V**) when compared with controls (**Q**, **S**). Scale bar in (A) for (A–H) and (M–T) is 1 mm, and in (I) for (I–L) and (U–V) is 250 μm.

ing pattern that at higher magnification shown to be intensely positive cells scattered among the culture.

In order to pursue whether the effects of BMP4 and GDF5 on early chondrogenesis can extend to the full cartilage development program in vitro, we performed analyses for alkaline phosphatase and Col10 expression, markers for chondrocyte maturation, on day 4 of culture. Alkaline phosphatase staining of control cultures showed staining confined to the perimeter of each of the cartilaginous nodules (Fig. 6A,C). In the presence of 100 ng/ml BMP4, alkaline phosphatase staining increased strongly in intensity and can be observed throughout the culture (Fig. 6B). In

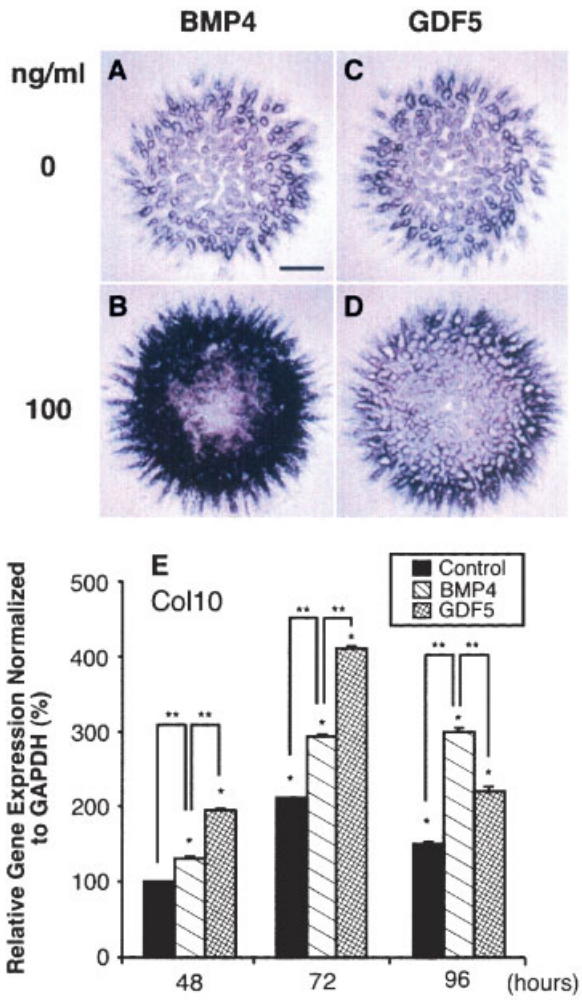


Fig. 6. BMP4 induces cartilage maturation. Limb bud mesenchymal cells were cultured for 4 days in the absence of growth factors (**A**, **C**), or in the presence of 100 ng/ml BMP4 (**B**) or GDF5 (**D**). Cartilage maturation was monitored by alkaline phosphatase staining. BMP4 induced nearly the entire culture to be alkaline phosphatase positive such that the internodular spaces were obliterated (B) GDF5 treatment (D) retained the perinodular pattern of alkaline phosphatase staining similar to control (C) and produced enhanced alkaline phosphatase staining secondarily through increase in the number of nodules. Scale bar in (A) for all images is 1 mm. Cartilage maturation was further assayed by RT-PCR for the expression of Col10 normalized for GAPDH expression in micromass cultures in the absence of growth factors or in the presence of 100 ng/ml BMP4 or GDF5 (**E**). Both BMP4 and GDF5 caused elevated Col10 expression after 48 and 72 h of treatment, with GDF5 consistently being more effective than BMP4. However, by 96 h of treatment, BMP4 was able to sustain the increase on Col10, whereas GDF5 became less effective. Data represent mean ± SE of the mean for nine samples in each group. $*P < 0.05$ when compared with control, $**P < 0.05$ when compared among doses.

contrast, GDF5 appeared to also increase alkaline phosphatase staining (Fig. 6D). However, upon close examination, staining in the presence of GDF5 resembled that of controls, that is, limited to the margins of the cartilaginous nodules. The apparent increase in staining in GDF5-treated cultures may be due to an increase in the number of nodules formed. Col10 expression was monitored by RT-PCR on samples harvested after 2, 3, or 4 days of culture (Fig. 6E). Modest increases over controls were detected after 2 days of cultures in the presence of 100 ng/ml BMP4 or GDF5, with GDF5 eliciting a stronger response than BMP4. Similar observations were made after 3 days of culture with all values proportionally higher than those from day 2. After 4 days of culture, the significant increase in Col10 expression resulted from BMP4 treatment was sustained at a level similar to day 3. However, Col10 level returned to control values in GDF5-treated cultures by day 4. Our data suggest that BMP4 may induce and accelerate cartilage maturation, whereas GDF5 may increase the expression of maturation markers but probably secondarily through the increase in nodule formation.

DISCUSSION

Chondrogenesis is a multistep process during which uncommitted mesenchymal cells undergo cell fate determination to become chondroprogenitors that differentiate into chondrocytes. A repertoire of extracellular ligands serves to coach this series of developmental events. They elicit both positive and negative signals. Large protein families, such as that of TGFβs, often consist of multiple members that share similar and redundant functions. The biochemical dissection of their molecular signaling pathway can be conducted in many cell lines. However, the functional distinction of any two members in tissues and organs are often complicated by their pleiotropic engagement in both cartilage patterning and chondrocyte differentiation. Therefore, in our study, we employed dissociated embryonic limb bud mesenchyme, and placed them in a culture system that mimics chondrogenesis in vivo without allowing for limb bud patterning [Ahrens et al., 1977; Kaplowitz et al., 1982; Edwall-Arvidsson and Wroblewski, 1996]. We addressed the issue of how BMP4 and GDF5 differ in their regulation of multiple steps during early chondrogenesis. The most significant finding from our study is to have identified and distinguished the primary major cellular effects of BMP4 and GDF5 regulation of chondrogenesis. Whereas

both serve to increase the rate and extent of cartilage formation, BMP4 functions primarily as an instructive factor, inducing uncommitted mesenchymal cells to become chondroprogenitors. In contrast, GDF5 functions as a permissive factor, facilitating the manifestation of the cartilage phenotype of chondroprogenitor cells.

Mesenchymal Cell Fate Determination

The expression profiles of Sox9 and Col2a1 in the presence of BMP4 or GDF5 supports the assertion that BMP4 induces, whereas GDF5 enhances chondrogenesis. Although, the level of transactivation of Col2a1 promoters and the accumulation of Sox9 and Col2a1 transcripts were similar under BMP4 or GDF5, the temporal patterns of Sox9 expression were different with the two factors. Our data suggest that BMP4 functions at an early phase of chondrogenesis, presumably cell fate determination, and GDF5 has sustained effects at later stages of chondrocyte differentiation. More significantly, BMP4 induced Col2a1 expression throughout the limb bud micromass cultures such that internodular areas that were normally non-chondrogenic became positive for Col2a1. This was most evident at the highest dose of BMP4. Interestingly, this high dose of BMP4 appeared to have an inhibitory effect on mesenchymal cell condensation, suggesting that BMP4 might be able to induce chondrogenesis in a manner that would allow for bypassing the condensation stage. Indeed, in several cell lines including C3H10T1/2 in this study, BMP4 induces cartilage marker expression without promoting detectable cell condensation [Denker et al., 1999; Valcourt et al., 1999; Hatakeyama et al., 2003]. In our study, the inductive effect was more obvious in serum-free cultures of limb bud mesenchyme in which a low concentration of BMP4 already induced chondrogenesis in internodular areas. This piece of data suggests that serum factors may directly antagonize the inductive function of BMP4, or that serum factors may serve to maintain non-chondrogenic cells in a proliferative state and delay differentiation. Therefore, although it appears that there were no quantitative differences in Col2a1 expression stimulated by BMP4 or GDF5, we detected that the distribution of Co2a1 expression was distinct under the two growth factor. GDF5 increases Col2a1 expression exclusively in chondrogenic nodules, whereas BMP4 increases Col2a1 expression throughout the culture.

Mesenchymal Cell Condensation

One of the cardinal cellular features of early chondrogenesis is cell aggregation into precartilaginous nodules, which is facilitated by cell–cell interactions regulated primarily by the expression of N-cadherin in chondroprogenitors [DeLise and Tuan, 2002a,b]. We showed definitively that both BMP4 and GDF5 promoted mesenchymal cell condensation by PNA staining for cell aggregations, the number of which increased dose-dependently. BMP4 was more potent at lower concentrations, whereas GDF5 was more effective at high concentrations. These condensations were precartilaginous nodules that also expressed Col2a1 in double staining experiments (data not shown). Although, BMP4 at 10 and 100 ng/ml promoted cell condensation by PNA staining, the number of alcian blue positive nodules did not appear to increase. This is probably due to the inductive effect of BMP4 on internodular mesenchymal cells that allow condensations to coalesce. In response to GDF5, mesenchymal cell line C3H10T1/2 exhibited patchy alcian blue positive areas reminiscent of nodule formation, although these patches were less well defined than those observed with primary limb bud mesenchyme. Our quantitative and qualitative results are consistent with observations in chick and rat limb bud mesenchyme in culture [Hotten et al., 1996; Francis-West et al., 1999; Coleman and Tuan, 2003a]. The increase in cell condensations could be due to the initial increase in cell number. However, the increases in cell number were modest even at the highest dose of GDF5, and could not account for the large increase in the number of cell condensations. It is yet unclear how GDF5 specifically enhances cell condensation. It is known that Sox9 can bind to DNA sequences of the N-cadherin promoter, thus transactivating the expression of N-cadherin [Panda et al., 2001]. However, since BMP4 and GDF5 can induce Sox9 expression equally well, this may be an inviting but not an exclusive explanation. Alternatively, BMP antagonists may play a major role in modulating the functions of BMP4 and GDF5 at this stage. Most notably is the high expression of noggin and chordin in condensing mesenchyme that may serve to limit the relative activities of BMPs [Brunet

et al., 1998; Nifuji and Noda, 1999; Scott et al., 2000].

Chondrocyte Maturation

Both BMP4 and GDF5 promoted sulfated matrix accumulation as shown by alcian blue staining with GDF5 being more potent. This is consistent with the overall expansion of cartilage when BMP4 or GDF5 is overexpressed [Duprez et al., 1996; Hotten et al., 1996; Buckland et al., 1998; Francis-West et al., 1999; Storm and Kingsley, 1999; Merino et al., 1999b; Tang et al., 2000]. In contrast, BMP4 was more effective in promoting aggrecan synthesis. Taken together, these results suggest that different growth factors may have different effectiveness in the regulation of matrix molecules. Their combinatorial and concerted functions would allow for balanced matrix assembly and cartilage homeostasis.

Late stages of cartilage development may progress to maturation. We showed that alkaline phosphatase staining could be detected on the margins of cartilage nodules, reminiscent of alkaline phosphatase expression at the perichondrium [Vakeva et al., 1990]. GDF5 did not alter the spatial pattern of alkaline phosphatase, that is, expression remained restricted at the perimeter of the cartilaginous nodules. In contrast, BMP4 treatment resulted in alkaline phosphatase staining throughout the culture, suggesting that BMP4 induces chondrocyte maturation at both internodular and nodular areas. Consistently, BMP4 induced sustained and elevated expression of Col10, whereas GDF5 promoted only a transient increase in Col10 expression. This transient increase may be secondary to the increase in cartilaginous nodules. In chick micromass cultures, overexpressing of BMP2 or BMP6 also resulted Col10 expression throughout the entire culture [Kameda et al., 2000]. Our results are consistent with data showing that BMP2 (closest homolog to BMP4), but not GDF6 or GDF7 (closest homologs to GDF5), was able to induce chondrocyte hypertrophy [Enomoto-Iwamoto et al., 1998; Valcourt et al., 1999, 2002; De Luca et al., 2001; Gooch et al., 2002]. We speculate from evidence in the literature that BMP induction of chondrocyte maturation may be mediated by Smad1 and Smad5, and their subsequent activation of the Col10 promoter [Volk et al., 1998; Leboy et al., 2001], and that the failure of GDF5 to sustain the expression of Col10 is due to the inability of GDF5 to cause significant Smad1 and Smad5 phosphorylation [Aoki et al., 2001]. Inhibitory Smads, such as Smad6 and Smad7, may also function in a negative feedback loop to regulate the activities of BMPs and GDFs [Takase et al., 1998; Valcourt et al., 2002; Li et al., 2003; Nakahara et al., 2003].

In contrast, in long-term micromass cultures of limb bud mesenchymal cells derived from the chick, we showed that 21-day exposure to GDF5 resulted in chondrocyte hypertrophy [Coleman and Tuan, 2003b]. This is a function of GDF5 on late chondrocyte maturation, a function that is associated with its expression during joint formation and endochondral bone formation. There may also be intrinsic differences in hypertrophic and osteogenic potential of mouse and chick cells in cultures. Whereas mouse limb bud cells were positive for alkaline phosphatase after 4 days in culture, it took 21 days for those derived from the chick to exhibit this signal.

Biomimetics

Due to their osteochondro-inductive capacity, the use of BMPs for orthopedic applications, such as skeletal repair and regeneration, and in dental applications such as the treatment of periodontal diseases is promising [Li and Wozney, 2001; Reddi, 2001]. Studies have shown that several BMPs could be used for cartilage tissue engineering [Hunziker et al., 2001; Gooch et al., 2002; Kaps et al., 2002] but it remains debatable that which one is optimum. However, since growth and differentiation are multistep progams, it is useful to apply a combinatorial approach in biomimetics rather than relying on a single factor. Therefore, the comparison and delineation of growth factor functions, especially those that are closely related molecularly, would allow for selective application based on the cell types, stages of development, and the desired outcome. The unique combination of growth factors produces the regulated emergence of specific tissues, such as that of cartilage. Moreover, embryonic mesenchymal cell differentiation could serve as a useful model for tissue engineering. Information derived from the functional dissection of closely related growth factors during the developmental program of cartilage formation can form the basis of molecular formulation to produce predictable lineage outcome for their

potential and targeted therapeutic use in tissue repair, regeneration, and engineering.

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