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ARTICLES

Noggin Expression in a Mesodermal Pluripotent Cell Line C1 and Its Regulation by BMP

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Abstract Osteoblasts and chondrocytes are derived from mesodermal stem cells and their differentiation is under the control of coordinated interaction among signaling molecules. Noggin is one of the signaling molecules which bind to and inactivate BMPs to induce neural tissues and dorsal mesoderm in Xenopus. However, its expression and regulation in mammalian cells has not been known. In this study, we investigated expression of noggin in murine pluripotent mesodermal cell line, C1. Noggin expression was very low in these C1 cells before they were induced to differentiate. When C1 cells were induced to differentiate into chondrocytes in aggregate cultures in the presence of dexamethasone(dex), noggin expression was significantly increased. In a sharp contrast, when the C1 cells were induced to differentiate into osteoblastic cells by the treatment with β glycerophosphate (βGP) and ascorbic acid (AA), noggin mRNA expression remained to be barely detectable. Noggin expression was also observed in the developing cartilage of vertebrae in 15.5 dpc mouse embryos. The noggin mRNA level in C1 cells in monolayer cultures was enhanced significantly by the treatment with BMP4/7 in a dose-dependent manner with a maximal effect at 100 ng/ml. The BMP4/7 effect on noggin expression was time dependent starting within 12 h and peaked at 24 h. These results indicate that noggin is expressed in the pluripotent mesodermal cell line C1 and that its expression is regulated by BMP. J. Cell. Biochem. 73:437–444, 1999.

Key words: noggin; BMP; skeletogenesis; embryos; differentiation

Recent studies on skeletal development have revealed that several key molecules are shared in the regulation of embryonic patterning and the control of differentiation of bone cells and formation of skeletal tissues. Bone morphogenetic proteins (BMPs) act as a signal for the formation of ventral tissues in Xenopus embryos [Dosch et al., 1997; Graff, 1997], while they are also involved in regulation of early development of axial skeleton [Nifuji et al., 1997]. In fact, BMP2, 4, 5, 6, and 7 are expressed in the developing skeletal tissues, and subcutaneous implantations of BMP2 and 4 have been shown to induce ectopic formation of cartilage and bone [Reddi, 1997; Lyons et al., 1995; Zou et al., 1997; Dudley and Robertson, 1997; Wozney et al., 1988]. Another example of signaling molecule is fibroblast growth factor which induces mesoderm when its mRNA is injected into early Xenopus embryos [Amaya et al., 1993], while constitutive activation of FGF receptors in vivo suppresses chondrogenesis and gives rise to severe skeletal abnormalities in humans and mice [Webster and Donoghue, 1997; De Moeriooze and Dickson, 1997]. These observations indicate that molecules important for embryonic patterning also function as crucial regulatory factors in the differentiation of cartilage and bone cells.

Noggin is a relatively new member of signaling molecules in development, which was originally discovered based on its capability to dorsalize ventral mesoderm in Xenopus embryos [Smith and Harland, 1992]. Noggin is expressed in the Spemann's organizer and acts as a neural inducer [Lamb et al., 1993; Smith et al., 1993]. This function of noggin is exerted via inactivation of BMPs during the establishment

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of dorsoventral pattern formation in Xenopus [Zimmermann et al., 1996]. In mammals, BMPs are crucial regulatory molecules of cartilage and bone development. Recently, it has been shown that noggin is also required for proper development of skeleton [Brunet et al., 1998], however, relationship between noggin and BMPs during skeletogenesis has not yet been elucidated.

Bone formation involves either direct conversion of mesodermal stem cells into osteoprogenitors or conversion via intermediate stages where cartilaginous matrix is replaced by bone. Clonal cell lines capable of undergoing in vitro osteogenic and chondrogenic differentiation offer a unique opportunity to study the relative contribution of the genetic and epigenetic factors in skeletal differentiation. A pluripotent mesodermal cell line, C1, serves as an unique in vitro model for the study of the molecular basis of skeletal cell differentiation [Poliard et al., 1995]. In the exponential phase, C1 cells remain undifferentiated. When these cells are allowed to form cell aggregates in the presence of β-glycerophosphate and ascorbate(βGP + AA), they form mineralized matrix and express features of osteoblast-like cells. When C1 cells are allowed to aggregate in the presence of dexamethasone-(Dex), they differentiate into chondrocyte-like cells with selective accumulation of a matrix composed of type II collagen and aggrecan [Poliard et al., 1995].

In the present work, we show that noggin is expressed in the mesodermal C1 cells and its expression is enhanced in these C1 cells during their chondrogenic differentiation, but not during the osteogenic differentiation. We also show that noggin expression in the C1 cells was increased by the treatment with BMP4/7.

MATERIALS AND METHODS

Cell Cultures and Growth Factors

C1 cells were routinely cultured in DMEM supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. For the experiments of chondrogenic and osteogenic differentiation, C1 cells were seeded at 5×10^5 cells per 10 ml of DMEM supplemented with 10% FBS onto bacteriological dishes to form cell aggregates. After 10 days, cells were refed with DMEM containing 1% FBS and induced to differentiate along the osteogenic pathway by the addition of 7 mM βGP + 50 ug/ml AA or along the chondrogenic pathway by the addition of 10^{-6} M dexamethasone (Dex) into the media [Poliard et al., 1995]. For the experiments of noggin induction by BMP, C1 cells were plated in tissue culture dishes at 1×10^4 cells/cm^2 to form monolayers. After 2 days of incubation, medium was changed to DMEM supplemented with 1% FBS and the cells were cultured in the presence of BMP or other agents for the indicated periods.

Recombinant Xenopus BMP4/7, which was produced by using a baculovirus system [Aono et al., 1995] and purified by RP-HPLC, was obtained from Takeda Chemical Industries. Homodimers of human BMP2 and human OP1(BMP7) were kindly provided by Drs. John Wozney (Genetics Institute, Cambridge, MA) and Kuber Sampath (Creative BioMolecules, Hopkinton, MA), respectively. Both BMP2 and BMP7 were expressed in CHO cells and the final purification was performed by RP-HPLC.

RNA Preparation and Northern Blot Analysis

RNA preparation and Northern blot analysis were performed as described previously [Murakami et al., 1997]. Briefly, total cellular RNA was extracted according to the single step of acid guanidium thiocyanate-phenol-chlroform method. Total RNA was fractionated by electrophoresis on 1% agarose containing 0.22 M formaldehyde and transferred onto nylon filters by electroblotting. The filters were prehybridized overnight at 42°C in the hybridization solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS, and 100 ug/ml herring sperm DNA. Hybridization was carried out at 42°C in the fresh hybridization supplemented with ^{32}P-labeled probe.

Probes

Murine noggin partial cDNA was obtained by RT-PCR using total RNA derived from C1 cells as a template. The upstream primer sequence was GAGATCAAAGGGCTGGAGT and the downstream primer sequence was GCAGGAACACTTACACTCG. The amplified fragment was cloned into pCR vector (Stratagene, La Jolla, CA) and sequenced. The other probes were 1 kb mouse OP1 (BMP7) cDNA insert [Ozkaynak et al., 1990], mouse BMP4 DNA [Jones et al., 1991], 0.4 kb mouse type II collagen cDNA insert [Metsaranta et al., 1991], 0.5 kb rat osteocalcin(OC) cDNA insert [Yoon et al., 1988], and 1.2 kb human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA insert [Tso et

al., 1985]. The cDNA fragments was radiolabelled by Oligolabelling Kit (Pharmacia, Gaithersburg, MD) according to the manufacturer's protocol.

In Situ Hybridization

In situ hybridization of paraffin sections were carried out based on the protocol kindly provided by Dr. Ebensperger (University of California, San Francisco). Briefly, embryos were fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin wax. Sections were made in 6 μm thickness and placed on the 3-aminopropyltriethoxysilan coated slides. The sections were dewaxed in xylene and rehydrated in PBS and treated with 10 μg/ml proteinase K (Sigma, St. Louis, MO) for 5 min. The sections were then re-fixed in 4% paraformaldehyde in PBS and washed twice in PBS, followed by twice washing with Tris-glycine buffer. Hybridization was performed at 65°C overnight in hybridization buffer (40% formamide, 5× SSC, 1× Denhardt's solution, 100 μg/ml herring sperm DNA, 100 μg/ml tRNA) containing 1 ng/μl of the digoxigenin-labelled mouse noggin. Labeling of RNA probes with digoxigenin-11-UTP was performed as described [Nifuji et al., 1997]. The slides were washed three times in 5× SSC, twice in 20% formamide/0.5× SSC, and treated with 10 μg/ml RNase A. After washing twice in 2× SSC, the sections were incubated with anti-digoxygenin-antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) overnight at 4°C. Then the sections were washed five times with TBS. To visualize, the sections were placed in BM purple AP substrate. After stopping the reaction, they were dehydrated and mounted in Glycerogel (Dako Co., Carpinteria, CA).

RESULTS

Noggin Expression is Enhanced During Chondrogenic Differentiation of C1 Cells in Aggregate Culture

We first examined if noggin is expressed in the mammalian mesodermal pluripotent cells, C1. In their exponential growth phase in monolayer cultures, noggin transcripts were not detected (data not shown). When the C1 cells were allowed to become confluent in monolayer cultures (data not shown) or to form cell aggregates, noggin expression was observed although at a very low level (Fig.1; left most lane, day 0). In C1 cells, treatment with Dex in aggregate culture induces chondrogenic differentiation. Under this condition, expression of the genes encoding type II, IX, X, and XI collagens, aggrecan, and link protein was observed [Poliard et al., 1995]. Dex treatment in C1 cells enhanced noggin mRNA expression within 8 days. Noggin mRNA level peaked on day 18. Noggin expression was declined thereafter but still higher than the basal level at least up to day 27. The changes in noggin mRNA levels were specific since GAPDH mRNA levels were similar during these experimental periods (Fig. 1). We also examined whether the enhancement of noggin mRNA expression could be observed in another condition which induces differentiation of the

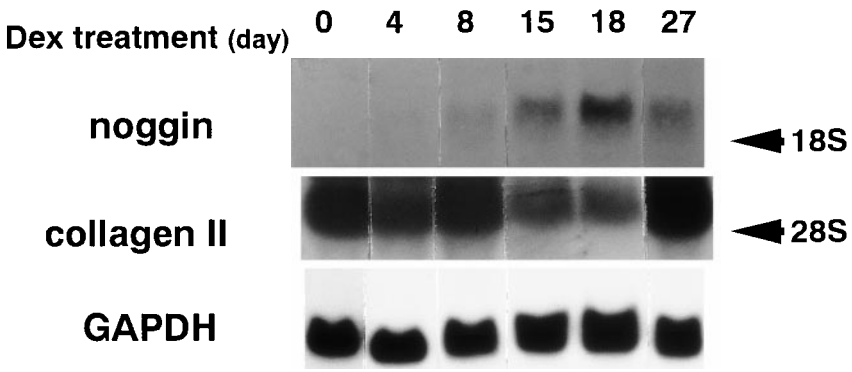

Fig. 1. Expression of noggin in C1 cell aggregates was enhanced by the treatment with dexamethasone. C1 cells were cultured in bacterial petri dishes to form cell aggregates in DMEM supplemented with 10% FBS. Ten days later, medium was changed to fresh one supplemented with 1% FBS containing 10^{-6} M dexamethasone (Dex). Northern blot analysis was conducted as described in Materials and Methods. Noggin expression was increased by Dex and its enhancement of noggin expression peaked on day 18 of dex treatment. Type II collagen and GAPDH cDNAs were used to probe the same filter. Quantification was conducted by using a densitometer. The ratios of the noggin mRNA levels normalized against GAPDH mRNA levels for day 0, 4, 8, 15, 18, and 27, were 0, 0.04, 0.37, 0.78, 1, and 0.50, respectively.

C1 cells to become osteoblast-like cells. For this purpose, these cells were cultured in aggregates and were treated with β-glycerophosphate and ascorbate(βGP + AA). Under this condition, noggin expression remained barely detectable until day 27 (Fig. 2). In contrast, osteocalcin mRNA expression was increased within 4 days by the treatment with βGP + AA as reported before [Poliard et al., 1992] (Fig. 2). Osteocalcin mRNA levels were high on days 8 and 15 while noggin mRNA levels were not detectable on either day 8 or day 15 (data not shown). Thus, enhancement of noggin mRNA expression was specifically observed in the case of chondrogenic differentiation of the C1 progenitor cells in cell aggregates culture.

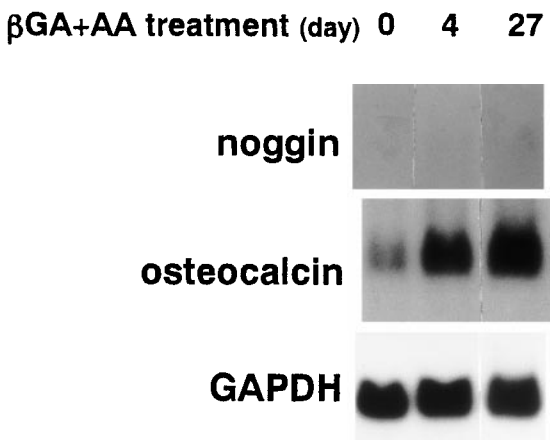

Fig. 2. Osteogenic differentiation of C1 cells does not associate with noggin expression. Cell aggregates were formed and were treated with βGP + AA to induce osteogenic differentiation as described in Materials and Methods.

Noggin mRNA was Expressed in Developing Cartilage in Mouse Embryos

We further examined whether noggin mRNA is expressed in chondrogenic cells in vivo. In situ hybridization was carried out on the sections prepared from 15.5 day post coitum (dpc) mouse embryos. Noggin transcripts were expressed in the developing cartilage of vertebrae in the mouse embryos (arrowhead in Fig. 3).

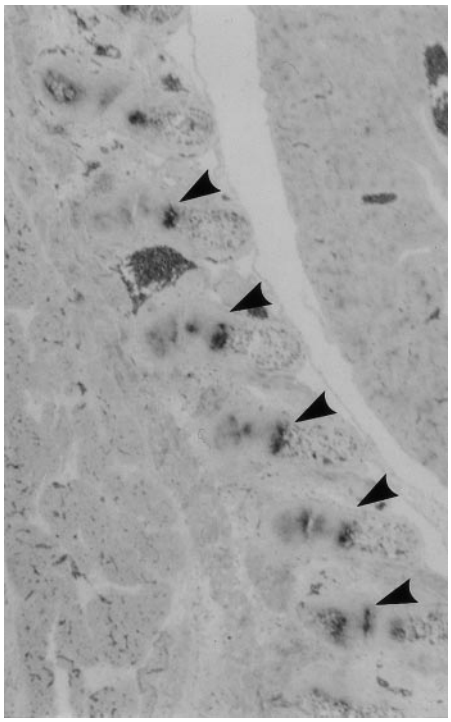

Fig. 3. Noggin expression in the vertebrate of 15.5 dpc mouse embryo. In situ hybridization was performed on the saggital section of 15.5 dpc mouse embryos using noggin cDNA. Arrowheads indicate noggin expression observed in the cartilaginous tissue of developing vertebrae (40×).

Noggin Expression was Enhanced by BMP4/7 in the C1 Cell Monolayer Culture

The enhancement of noggin expression during chondrogenic differentiation of C1 cells and noggin expression in the developing cartilage prompted us to investigate whether noggin expression is regulated by local factors known to regulate skeletal cell growth and differentiation. BMPs induce ectopic cartilage and subsequent bone formation in vivo at ectopic sites such as muscle or subcutaneous tissues and hence are considered to induce differentiation of pluripotent mesenchymal stem cells. Recently, several reports have demonstrated that heterodimeric forms of BMP are more potent in biological activity than the homodimers [Israel et al., 1996; Aono et al., 1995]. Many BMP isoforms are known to be co-expressed during embryogenesis, raising the possibilities that heterodimeric forms may be physiologically functional [Dudley et al., 1997]. We, therefore, examined the effect of BMP4/7 heterodimer, and BMP7 or BMP2 homodimer on noggin mRNA expression in C1 cells. For this purpose, we cultured C1 cells in the presence of recombinant protein of BMPs in monolayer cultures, which prevents these cells from differentiation.

Treatment with 40 ng/ml of BMP4/7 for 24 h increased noggin expression more than 20-fold in C1 cell monolayer cultures (Fig. 4). BMP7(OP1) at 100 ng/ml weakly enhanced

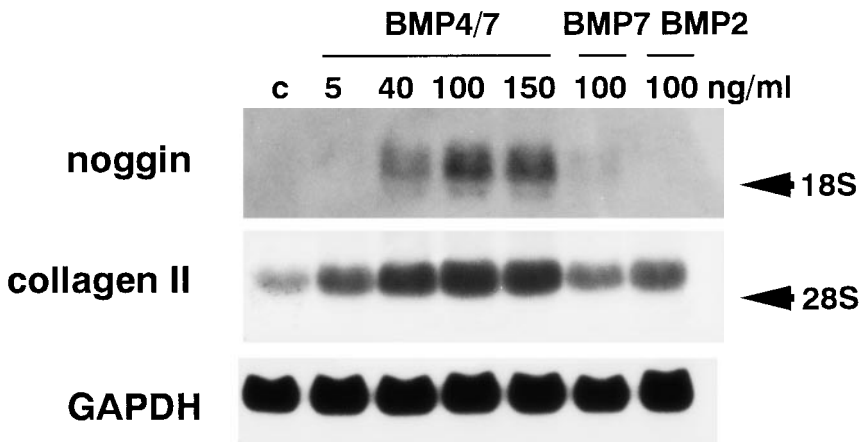

Fig. 4. Dose dependent enhancement of noggin and type II collagen expression by BMP4/7 in C1 cells. C1 cells were plated at 1 $\times 10^4$ cells/cm^2. After 2 days of incubation, medium was changed to DMEM supplement with 1% FBS plus indicated doses of BMP4/7, BMP7, and BMP2. Total RNA was extracted 24 h later and subjected to Northern blot. Quantification was conducted by using a densitometer. The ratios of the noggin mRNA levels normalized against GAPDH mRNA levels for control, 5, 40, 100, and 150 ng/ml BMP4/7, 100 ng/ml BMP7, and 100 ng/ml BMP2 were 0, 0.04, 0.67, 0.96, 1, 0.017, and 0.03, respectively. The ratios of the type II collagen mRNA levels normalized against GAPDH mRNA levels for control, 5, 40, 100, and 150 ng/ml BMP4/7, 100 ng/ml BMP7, and100 ng/ml BMP2 were 0.34, 0.57, 0.87, 0.97, 1, 0.45, and 0.54, respectively. Enhancement of noggin and type II collagen mRNA expression by BMP 4/7 heterodimer was observed in a dose dependent manner.

noggin expression. Interestingly, BMP2 at 100 ng/ml, which exhibits similar biological activities to BMP4 in other assay systems, did not enhance noggin mRNA expression. GAPDH mRNA levels were not altered by the treatment with any of these BMPs, serving as control.

The BMP4/7 effect on noggin mRNA was dose-dependent, and was maximal at 100 ng/ml. Time course experiment showed that noggin expression was enhanced within 12 h and peaked at 24 h by the treatment with BMP4/7 at 40 ng/ml (Fig. 5). Treatment with other signaling molecules such as TGFβ1(100 ng/ml), FGF4 (20 ng/ml), or sonic hedgehog (50 ng/ml) did not enhance noggin expression in C1 cells (data not shown). Thus BMP4/7 heterodimer and BMP7 homodimer were specific modulators for noggin expression in the C1 cells in monolayer cultures.

Treatment With BMP4/7 Enhances Expression of type II Collagen in C1 Cells

Since noggin expression was enhanced during the chondrogenic differentiation of C1 cells and was upregulated by BMP4/7, we next examined whether treatment with BMP4/7 regulates the expression of type II collagen in C1 cells. Expression of type II collagen gene was enhanced by the treatment with BMP4/7 in a dose dependent manner (Fig. 4). Time course experiments showed that BMP4/7 enhanced

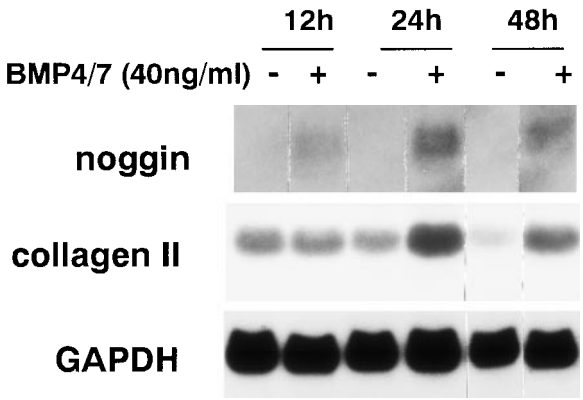

Fig. 5. Time course of the effects of BMP4/7 on noggin and type II collagen expression in C1 cells. C1 cells were plated as described in Materials and Methods. After treatment of C1 cells with 40 ng/ml of BMP4/7 for 12, 24, and 48 h, total RNA was extracted and subjected to Northern blot. Quantification was conducted by using a densitometer. The ratios of the noggin mRNA levels normalized against GAPDH mRNA levels for 12 h control, 12 h BMP4/7, 24 h control, 24 h BMP4/7, 48 h control, and 48 h BMP4/7 were 0.01, 0.31, 0, 1, 0, and 0.72, respectively. The ratios of the type II collagen mRNA levels normalized against GAPDH mRNA levels 12 h control, 12 h BMP4/7, 24 h control, 24 h BMP4/7, 48 h control, and 48 h BMP4/7 were 0.39, 0.31, 0.25, 1, 0.19, and 0.62, respectively. Noggin gene expression was enhanced by BMP 4/7 within 12 h and peaked at 24 h.

type II collagen gene expression after 24 h of treatment (Fig. 5). Thus, BMP4/7 upregulates expression of both noggin and type II collagen genes in a similar time course and a dose dependent profile in the C1 cells in monolayer cultures.

DISCUSSION

We showed that noggin mRNA expression was enhanced during chondrogenic differentiation of mammalian pluripotent mesodermal C1 cells in vitro and that the expression of its transcripts was upregulated by the treatment with BMP4/7 and BMP7. Noggin mRNA was also observed in the developing cartilage. These data indicate that noggin is expressed in mammalian mesodermal cells and its expression is associated with that of the phenotypes of the chondrogenic cells.

In our previous study, we described three steps in the differentiation of C1 mesodermal stem cells [Poliard et al., 1995]. First, in sparse monolayer cultures, C1 cells do not exhibit any phenotypic markers at this stage, and noggin transcripts were not detectable. In the second stage, genes encoding proteins related to both chondrogenic and osteogenic phenotypes are expressed after forming aggregation and establishment of cell contacts. Noggin mRNA was detectable at this stage although at a very low level. In the third stage, treatment with Dex in cell aggregates selectively enhances expression of cartilage specific differentiation related makers such as type II, and XI collagen followed by accumulation of type IX and X collagen expression, while expression of the osteoblastic differentiation markers such as type I collagen and osteocalcin is suppressed during this chondrogenic differentiation in C1 cells [Poliard et al., 1995]. Noggin expression was enhanced by 8 days of the Dex treatment. The level of noggin expression peaked on day 18 of the Dex treatment, a relatively late stage of chondrogenic differentiation of C1 cells [Poliard et al., 1995]. This indicates that noggin mRNA expression increases along with the chondrogenic differentiation of C1 cells.

In contrast to the noggin expression in C1 cells under the condition to differentiate into chondrocytes, its expression remained at very low levels under the condition which promotes osteogenic differentiation of C1 cells, indicating that noggin expression is specifically associated with chondrogenesis. Indeed, in situ hybridization analysis of the noggin mRNA expression in embryonic skeletons in vivo revealed that noggin mRNA was expressed in cartilage (Fig. 3), but not in the region of ossification such as periosteum of long bones or calvariae (data not shown).

There has been no reports on modulators of noggin expression. In this study, we showed that BMP4/7 and BMP7 enhanced noggin expression in C1 cells in monolayer cultures. Enhancement of noggin expression is not observed with the treatment of other diffusible molecules such as BMP2, TGFβ, SHH, and FGF4. Previously it was shown that BMP7 transcripts were expressed in the mesenchyme surrounding the perichondrium and to stimulate epiphyseal cartilage formation [Haaijman et al, 1997]. BMP7 protein was also shown to induce chondrogenic differentiation of mesenchymal osteoprogenitor cells, suggesting that BMP7 regulates positively early chondrogenesis [Asahina et al., 1993]. Our results also support functions of BMP7 in the chondrogenic differentiation, since treatment with BMP4/7 and BMP7 proteins enhanced expression of both noggin and type II collagen mRNA in C1 cells in monolayer cultures. Moreover, expression of both BMP4 and BMP7 transcripts was observed in C1 cells in aggregates cultures and their expression preceded that of noggin during Dex treatment (data not shown). Thus, we speculate that during the pathway C1 cells differentiation, BMPs could regulate genes which are specifically expressed in chondrogenic differentiation. Other BMP isoforms, such as BMP6, may be also expressed during C1 differentiation and may induce noggin expression, although these points need further elucidations.

BMP4/7 heterodimers were previously shown to have a potent activity in the mesoderm inducing assay in Xenopus when compared to BMP4 and BMP7 alone [Suzuki et al., 1997]. It has been also demonstrated that the heterodimeric forms of BMP are more potent than the homodimers in ectopic bone formation activity [Israel et al., 1996; Aono et al., 1995]. In our study there is slight effect of BMP7 and no effect of BMP2 on noggin expression at the doses chosen. Although we do not have complete dose-dependence data for BMP7 and BMP2, BMP 4/7 heterodimer may be more potent than BMP 7 or BMP2 alone in terms of the enhancement of noggin gene expression in C1 cells. If so, it would support the idea that heterodimeric form may function more effectively than the homodimers. Indeed our recent report also showed that BMP4/7 was more effective than BMP2 in the stimulation of CBFA1 expres-

sion in ROS17/2.8 and C2C12 cells [Tsuji et al., 1998].

Although noggin enhancement by BMP2 was not observed, enhancement of type II collagen expression by BMP2 was observed in C1 cells. This result implicates that regulation of noggin and type II collagen may occur independently during chondrogenic differentiation. It is also possible that the amounts of BMP protein required for the enhancement of noggin and collagen gene expression are not the same. Although not tested in this study, higher doses of BMP2 might affect noggin expression in C1 cells.

Recently, antagonistic effects of BMPs and noggin were reported to be involved in neural induction and mesoderm dorsalization in Xenopus [Zimmermann et al., 1996]. The opposing effects of these molecules were explained by direct binding of noggin to BMPs to block its binding to the cognate receptors. If this is the case for cartilage development, noggin may also counteract against BMP activity during the chondrogenesis. Indeed, noggin null mutant mice have demonstrated failure in joint formation and overgrowth of cartilage, suggesting that the lack of noggin protein results in the increase in BMP activity in the skeletal tissue [Brunet et al., 1998]. BMP activation of noggin expression, shown in our study, could serve as a negative feed back mechanism to maintain appropriate development of cartilage by balancing the activities between BMP and its antagonists. Noggin may also favor for the maintenance of cartilaginous tissue by preventing its transition to bone as noggin was expressed under the chonderogenic condition but not under the osteogenic condition in C1 cells.

Although we showed that noggin was selectively expressed in chondrogenic differentiation in C1 mesodermal cells and that noggin expression was regulated by BMP, functional roles of noggin and epistatic relationship between BMPs and noggin during chondrogenesis remained to be investigated further in detail. BMP7 null mutant mice did not show such a severe phenotype in cartilage as was shown for noggin mutant, suggesting that the other BMP isoforms, whose expression could overlap with that of BMP7, may interact with noggin [Dudley et al., 1995, 1997]. It is possible that coordinated interaction between BMPs and noggin may be a prerequisite condition for the normal differentiation of skeletal tissues.

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