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Bone morphogenetic protein-6 and parathyroid hormone-related protein coordinately regulate the hypertrophic conversion in mouse clonal chondrogenic EC cells, ATDC5

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Abstract

We evaluated the roles of bone morphogenetic protein (BMP)-6, BMP-4 and parathyroid hormone-related protein (PTHrP) in the hypertrophic conversion using mouse chondrogenic EC cells, ATDC5. In ATDC5 cells, the expression of BMP-6 and PTHrP receptor mRNAs increased in parallel with the progression of chondrogenic differentiation of these cells, exhibiting a time course similar to that of type II collagen, a phenotypic marker of proliferating chondrocytes, while BMP-4 mRNA was continuously expressed throughout the differentiation processes. The expression of type X collagen mRNA, a phenotypic marker of hypertrophic chondrocytes, was upregulated by BMP-6 and BMP-4, and downregulated by PTHrP(1–141). The expression of BMP-6 mRNA was upregulated while that of BMP-4 mRNA was downregulated by both BMP-6 and BMP-4. Moreover, the expression of BMP-6 mRNA was downregulated by PTHrP(1–141). Furthermore, even in the presence of PTHrP(1–141), BMP-6 increased the transcript level of type X collagen in a dose-dependent manner. These results indicate that transiently expressed BMP-6 promotes the hypertrophic conversion in association with the augmentation of BMP-6 gene expression by BMP signals and that both BMP-6 and PTHrP coordinately regulate the rate of the hypertrophic conversion of ATDC5 cells.

Keywords: Chondrogenic differentiation; Bone morphogenetic protein-6; Parathyroid hormone-related protein; ATDC5

1. Introduction

Chondrogenic differentiation, a key event during endochondral bone formation, consists of a series of events in which differentiated chondrocytes proliferate, mature and become hypertrophied. This sequential cellular event is under the regulatory control by a variety of growth/differentiation molecules, including bone morphogenetic proteins (BMPs) and parathyroid hormone-related protein (PTHrP) secreted from either chondrocytes or perichondrium in an autocrine/paracrine fashion [1–5]. Elucidating the molecular mechanisms that underlie the hypertrophic conversion of proliferating chondrocytes and uncovering the actions of these molecules during this distinct process is the current challenge.

Accumulating evidence shows that BMPs are po-

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tent stimulators of chondrogenic differentiation; BMP-4 upregulates the levels of type II collagen and aggrecan mRNAs in cultured articular chondrocytes [6]; and OP-1/BMP-7 promotes growth and maturation in chick sternal chondrocytes [3]. Among BMPs, BMP-6 is characteristically expressed in prehypertrophic chondrocytes [7,8], and induces chondrogenesis when ectopically expressed in nude mice transplanted with CHO cells overexpressing BMP-6 [7]. Although it is suggested that upregulation of the BMP-6 gene precedes hypertrophy of chondrocytes in vitro [1], little remains known regarding the effects of BMP-6 expressed in chondrocytes as well as the regulation of its gene expression.

PTHrP mRNA is expressed in perichondrium of long bones and proliferating chondrocytes [5], while PTHrP protein is expressed mostly at the interface of proliferating and hypertrophic chondrocytes, colocalizing with PTH/PTHrP receptor (-R) mRNA [4,5]. The observation that homozygous mice carrying either PTHrP or PTH/PTHrP-R null mutation each exhibit widespread abnormalities of cartilage development indicates that PTHrP restrains the rate of chondrogenic differentiation [8–11]. These observations collectively indicate that chondrogenic differentiation is under negative control by PTHrP/PTHrP-R signal.

We and others previously demonstrated that clonal mouse embryonal carcinoma (EC) cells, ATDC5, reproduce the multistep chondrogenic differentiation processes in vitro. Undifferentiated ATDC5 cells differentiate into chondrocytes through a cellular condensation stage, and differentiated ATDC5 cells then go through the sequential processes of proliferation, maturation, hypertrophic conversion and calcification [12–16]. Taking advantage of the sequential differentiation in ATDC5 cells occurring in a synchronous manner, we studied the effects of BMP-6, BMP-4 and PTHrP on the hypertrophic conversion in these cells.

2. Materials and methods

2.1. Materials

Human recombinant BMP-6 was a generous gift from Dr. J.M. Wozney (Genetics Institute, Cambridge, MA). Xenopus recombinant BMP-4 was a generous gift from Takeda Chemical Industries (Osaka, Japan) [17]. Human recombinant PTHrP(1–141), a full-length form of this molecule, was kindly donated by Dr. T.J. Martin (St. Vincent's Institute of Medical Research, Victoria, Australia).

2.2. Cells and culture conditions

ATDC5 cells were cultured as previously described [13–15]. Briefly, cells were maintained in a log growth phase in DMEM/Ham's F12 hybrid medium (Flow laboratories, Irvine, UK) containing 5% (v/v) FBS (Gibco BRL, Gaithersburg, MD), 10 μg/ml human transferrin (Boehringer Mannheim, Mannheim, Germany), and 3×10^{-8} M sodium selenite (Sigma Chemical Co., St. Louis, MO) (the maintenance medium) at 37°C in a humidified 5% CO_2/95% air atmosphere and were passaged every 3 days. Chondrogenesis and cartilage nodule formation could be induced only in a postconfluent phase when cells were cultured in the maintenance medium supplemented further with 10 μg/ml bovine insulin (Wako Pure Chemical, Osaka, Japan) (the differentiation medium). Cells were cultured in the differentiation medium for 21 days until the growth of cartilage nodules ceased. Induction of calcification was achieved when medium was replaced with α-MEM (Flow Laboratories) containing 5% (v/v) FBS, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite and 10 μg/ml bovine insulin (the calcification medium) and culture was continued at 37°C in a humidified 3% CO_2/97% air atmosphere. In the present study, we plated ATDC5 cells in a log growth phase in 6-multiwell plastic plates at an initial cell density of 6×10^4 cells/well and cultured these cells for a total of 42 days with medium replacement every other day. Cells were cultured for the initial 21-day period in the differentiation medium in a 5% CO_2/95% air atmosphere and then for the subsequent 21-day period in the calcification medium in a 3% CO_2/97% air atmosphere. For the examination of the effects of BMP-6, BMP-4 and PTHrP in differentiated ATDC5 cells, cells were cultured for the initial 21-day period in the differentiation medium in a 5% CO_2/95% air atmosphere and then cells were treated for the indicated time periods with various doses of the test substances in the absence of insulin.

2.3. RNA extraction and Northern analysis

Total RNA was prepared from the cultures as previously described [13–16]. For Northern hybridization, total RNA (20 μg) was denatured, separated by 1% agarose gel electrophoresis, and transferred onto Nytran membranes (Schleicher and Schuell, Dassel, Germany). Hybridization probes were prepared by the random-primer method with a BcaBEST labeling kit (Takara, Shiga, Japan). The following cDNA fragments were used as hybridization probes: a 0.42-kb fragment of BMP-6 mRNA; a 0.6-kb fragment of BMP-4 mRNA; a 0.42-kb fragment of PTH/PTHrP receptor mRNA; a 1.4-kb *Eco*RI fragment of pKT1180 for α1(II) collagen mRNA [18]; and a 0.65-kb *Hin*dIII fragment of pSAm10h for α1(X) collagen mRNA [19]. After hybridization, the membranes were exposed to X-Omat films (Kodak, Rochester, NY) at −80°C with Cronex lightening plus intensifying screens (DuPont, Boston, MA).

3. Results

3.1. Expression patterns of BMP-6,BMP-4 and PTH/PTHrP-R mRNAs during the processes of chondrogenic differentiation of ATDC5 cells

We previously showed that chondrogenic differentiation of ATDC5 cells took place in the presence of insulin and that transitions of the differentiation stages occurred in a synchronous manner as evidenced by the orderly expression of cartilage phenotypic marker genes [13]. In the presence of insulin, ATDC5 cells reached confluence 5 days after plating, and initiated chondrogenesis. The process proceeded orderly in a synchronous manner as evidenced by the expression of phenotypic marker genes with cartilage characteristics, as previously reported [13]. Cellular condensation occurred on day 7, and the formation and growth of cartilaginous nodules were observed from day 9 to day 21 followed by a calcification process which began on day 35. We extracted total RNA at the time points indicated, and analyzed the transcript levels of BMP-6, BMP-4 and PTH/PTHrP-R genes as well as those of type II collagen and type X collagen genes, phenotypic markers of proliferating and hypertrophic chondrocytes, respectively (Fig. 1). The expression of BMP-6 and PTH/PTHrP-R mRNAs were first observed on day 7, increased in parallel with the progression of chondrogenic differentiation of ATDC5 cells, exhibiting a time course similar to that of the type II collagen mRNA expression, and then declined until the cells became calcified. BMP-4 mRNA was expressed at the undifferentiated stage and its level declined as the cartilage nodules stopped growing after 2 weeks in culture, but was maintained constitutively. BMP-2 was not expressed throughout chondrogenic differentiation processes in ATDC5 cells (data not shown).

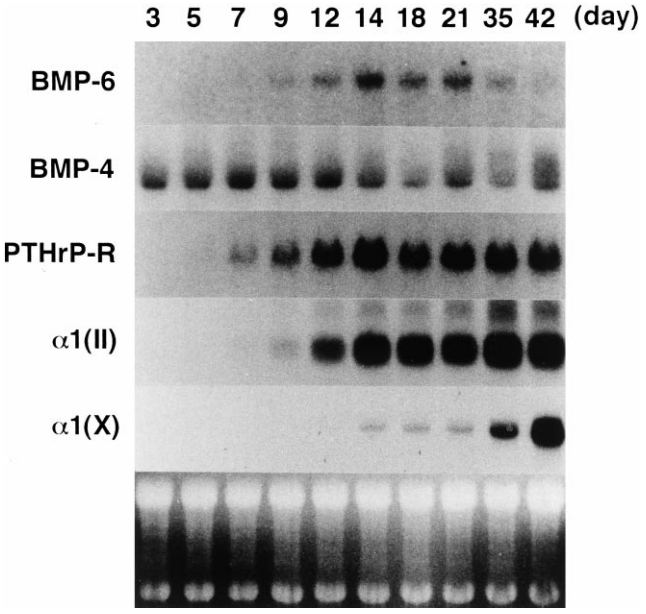

Fig. 1. Expression patterns of BMP-6, BMP-4 and PTH/PTH-R mRNAs during the processes of chondrogenic differentiation in ATDC5 cells. Undifferentiated ATDC5 cells were plated as described in Section 2 and cultured for 42 days. Total RNA was prepared from ATDC5 cells on days 3, 5, 7, 9, 12, 14, 18, 21, 35 and 42, and subjected to Northern analysis (20 μg of total RNA per lane). The transcript levels of type II collagen and type X collagen were also examined. The same filter was hybridized with these ^{32}P-labeled probes. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

3.2. Effects of exogenously administered BMP-6 and BMP-4 on the expression of type X collagen mRNA in differentiated ATDC5 cells

Previous studies showed that BMP-6 and BMP-4

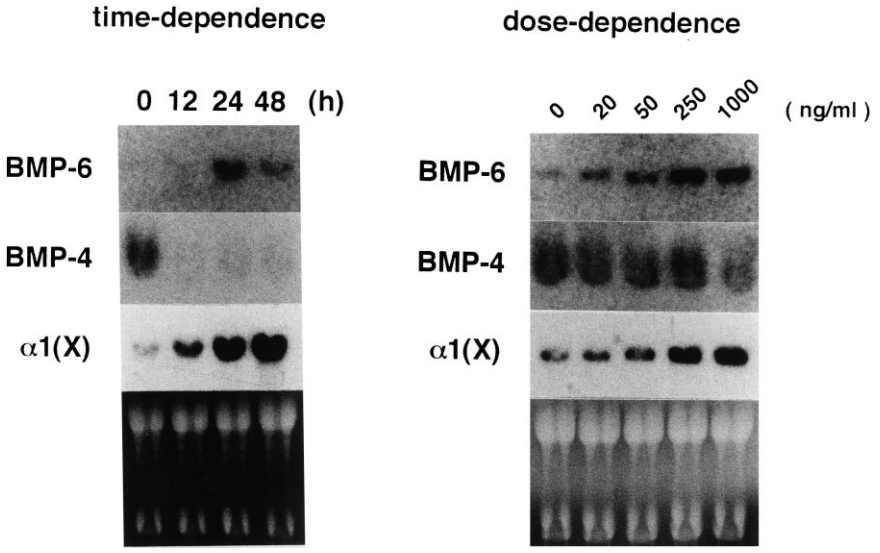

Fig. 2. Effect of exogenously administered BMP-6 on the expression of BMP-6, BMP-4 and type X collagen mRNA in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described in Section 2 for 21 days in the differentiation medium and were then exposed for the indicated time periods to 1000 ng/ml of BMP-6 (time-dependence), and an additional 48 h to either vehicle or BMP-6 (20, 50, 250 and 1000 ng/ml) (dose-dependence). Total RNA were prepared and subjected to Northern analysis (20 μg of total RNA per lane). The same filter was hybridized with these ^{32}P-labeled probes. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

were expressed in differentiated chondrocytes or perichondrium [1,7,20,21]. However, the effects of BMP-6 and BMP-4 on the hypertrophic conversion of chondrocytes remain to be elucidated. We assessed

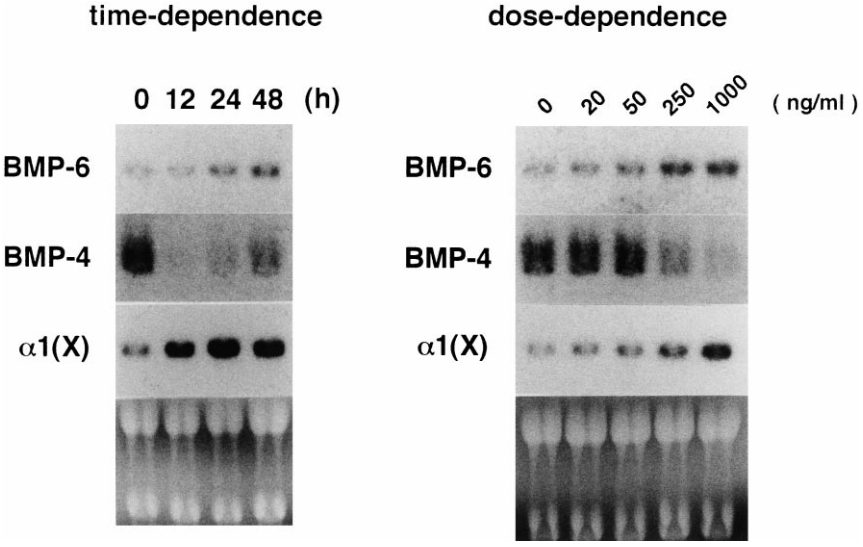

Fig. 3. Effect of exogenously administered BMP-4 on the expression of BMP-6, BMP-4 and type X collagen mRNA in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described in Section 2 for 21 days in the differentiation medium and were then exposed for the indicated time periods to 1000 ng/ml of BMP-4 (time-dependence), and for an additional 48 h to either vehicle or BMP-4 (20, 50, 250 and 1000 ng/ml) (dose-dependence). Total RNA were prepared and subjected to Northern analysis (20 μg of total RNA per lane). The same filter was hybridized with these ^{32}P-labeled probes. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

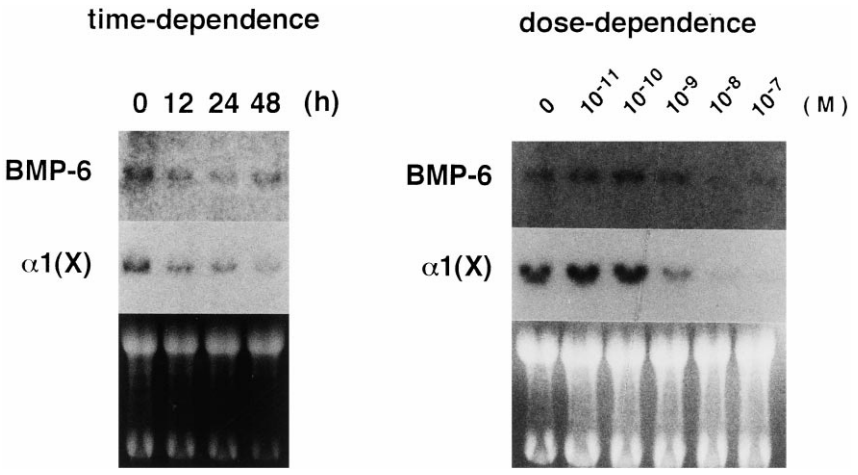

Fig. 4. Effect of exogenously administered PTHrP(1–141) on the expression of BMP-6 and type X collagen mRNAs in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described in Section 2 for 21 days in the differentiation medium and were then exposed for the indicated time periods to 10^{-8} M of PTHrP(1–141) (time-dependence), and for an additional 48 h to either vehicle or PTHrP(1–141) (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M) (dose-dependence). Total RNA were prepared and subjected to Northern analysis (20 μg of total RNA per lane). The same filter was hybridized with these ^{32}P-labeled probes. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

by Northern analysis the effects of BMP-6 and BMP-4 on the expression of type X collagen mRNA in differentiated ATDC5 cells. As shown in Figs. 2 and 3, both BMP-6 and BMP-4 upregulated the expression of type X collagen mRNA in time- and dose-dependent manners. These observations suggest that they are potent stimulators of the hypertrophic conversion of ATDC5 cells.

3.3. *Regulation of the expression of BMP-6 and BMP-4 mRNAs by BMP signals in differentiated ATDC5 cells*

BMP signals mediated by BMP-6 and BMP-4 stimulate the hypertrophic conversion of ATDC5 cells. Next, we examined the regulation of the amplitude of BMP signals by BMP-6 (Fig. 2) and BMP-4 (Fig. 3). Exogenously administered BMP-6 and BMP-4 upregulated the transcript level of BMP-6 gene in time- and dose-dependent manners and downregulated that of BMP-4. These results indicate that the amplitude of endogenous BMP-4 signal was maintained relatively constant by the negative-feedback mechanisms by BMP signals and that BMP-6 gene expression was augmented by BMP signals in differentiated ATDC5 cells.

3.4. *Effects of exogenously administered PTHrP(1–141) on the expression of type X collagen and BMP-6 mRNAs in differentiated ATDC5 cells*

PTHrP is inhibitory to chondrogenic differentia-

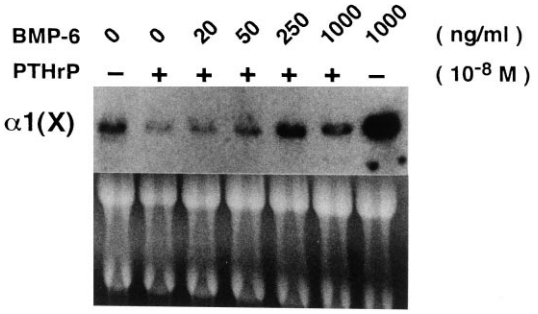

Fig. 5. Reversal by BMP-6 of the inhibitory action of PTHrP(1–141) on the expression of type X collagen mRNA in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described in Section 2 for 21 days in the differentiation medium and were then treated for an additional 48 h with the submaximal dose of PTHrP(1–141) (10^{-8} M), either alone or in the presence of varying doses of BMP-6 (20, 50, 250 and 1000 ng/ml). Total RNA were prepared and subjected to Northern analysis (20 μg of total RNA per lane). The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Two independent experiments were performed and gave similar results.

tion in vitro and in vivo [5,8–10,22,23]. Shukunami et al. reported that differentiated ATDC5 cells express PTHrP and PTH/PTHrP-R [14]. Thus, we examined the effect on the expression of type X collagen mRNA by PTHrP(1–141). Treatment of differentiated ATDC5 cells with PTHrP(1–141), a full-length form of the PTHrP molecule, resulted in downregulation of the expression of type X collagen mRNA in time- and dose-dependent manners (Fig. 4), indicating that PTHrP(1–141) inhibited the hypertrophic conversion of ATDC5 cells.

We hypothesized that one of the possible mechanisms underlying this inhibitory action of PTHrP(1–141) on the hypertrophic conversion may be the modulation of BMP-6 expression in ATDC5 cells. As shown in Fig. 4, indeed, PTHrP(1–141) downregulated the steady-state transcript level of BMP-6 gene in time- and dose-dependent manners.

3.5. *Reversal by BMP-6 of the inhibitory action of PTHrP(1–141) on the expression of type X collagen mRNA in differentiated ATDC5 cells*

Our results that the expression of BMP-6 and PTH/PTHrP-R mRNAs increased in parallel with the progression of chondrogenic differentiation and that BMP-6 is stimulatory while PTHrP is inhibitory to the hypertrophic conversion of differentiated ATDC5 cells, raise the possibility that BMP-6 and PTHrP coordinately regulate the rate of the hypertrophic conversion of these cells. The differentiated ATDC5 cells were treated for 48 h with a submaximal dose of PTHrP(1–141) (10^{-8} M), either alone or in the presence of varying doses of BMP-6, and total RNA was extracted (Fig. 5). Even in the presence of PTHrP(1–141) (10^{-8} M), BMP-6 increased the transcript level of type X collagen gene in a dose-dependent manner. However, excess BMP-6 (1000 ng/ml) did not override the inhibitory action of PTHrP(1–141) (10^{-8} M).

4. Discussion

Previous in vitro and in vivo studies provide evidence that BMPs play important roles in chondrogenic differentiation [1–3,7,16,21]. The present study has demonstrated that BMP-6 stimulated the hypertrophic conversion of ATDC5 cells as assessed by Northern analyses of the gene expression of type X collagen, a cartilage phenotypic marker gene specifically expressed in hypertrophic chondrocytes. The expression of BMP-6 mRNA in ATDC5 cells was differentiation stage-related; it increased in parallel with the progression of chondrogenic differentiation and decreased as cells became hypertrophied. The distinct expression pattern of BMP-6 mRNA in ATDC5 cells is comparable with the localization of BMP-6 gene transcripts in vivo [1,7,8], and suggests that the action of BMP-6 is also differentiation stage-related in chondrogenic differentiation. In this study, we showed that exogenously administered BMP-6 upregulated the transcript level of type X collagen gene in ATDC5 cells. This observation thus supports the notion that BMP-6 expressed in chondrocytes prior to the hypertrophic conversion stimulates the cellular hypertrophy in vivo.

Solloway et al. recently reported that BMP-6 null mice were not compromised in any of the skeletal tissues in which it is expressed, with the exception of the sternum which showed a delay in the hypertrophic conversion of proliferating chondrocytes and the subsequent ossification [24]. This result suggests that a null mutation of BMP-6 might be largely compensated for or masked by related BMPs expressed in bone rudiments, such as BMP-2 or BMP-4. This hypothesis is consistent with our observations in this study that BMP-4 also upregulated the expression of type X collagen mRNA and that BMP-6 downregulated the expression of BMP-4 mRNA in differentiated ATDC5 cells. In addition, BMP signals mediated by BMP-6 and BMP-4 upregulated the expression of BMP-6 mRNA. These results suggest that although the regulatory mechanisms of BMP-4 mRNA expression as well as the protein levels of BMP-6 and BMP-4 remain to be elucidated, the gross amplitude of differentiation signals increases along with the transient expression of BMP-6 and the constitutive expression of BMP-4 in ATDC5 cells.

Little is known regarding the receptor specific to BMP-6. Zou et al. showed that BMP receptor (BMP-R) type IA was highly expressed in prehypertrophic chondrocytes [21], raising the possibility that these cells were the direct targets of BMP signals including BMP-2, BMP-4, OP-1/BMP-7 and BMP-6. Since dif-

ferentiated ATDC5 cells also express BMP-R type IA mRNA (H. Akiyama et al., unpublished data), it may be conceivable that the stimulatory effect of BMP-6 on the hypertrophic conversion is mediated by BMP-R type IA in ATDC5 cells. Verification of this hypothesis needs further study.

The rate of the hypertrophic conversion of proliferating chondrocytes is required for the distinct regulatory mechanisms. We previously indicated that acquisition of PTH/PTHrP responsiveness is closely associated with chondrogenic differentiation [14], and in this study we found that PTHrP(1–141) markedly downregulated the transcript level of type X collagen gene in differentiated ATDC5 cells. Moreover, BMP-6 reversed the inhibitory action of the submaximal dose of PTHrP(1–141) (10^{-8} M) on the expression of type X collagen in a dose-dependent manner in differentiated ATDC5 cells. Furthermore, PTHrP(1–141) downregulated the transcript level of BMP-6 gene in these cells. Taken together, these results provide evidence for the hypothesis that both BMP-6 and PTHrP signals coordinately regulate the rate of the hypertrophic conversion.

In conclusion, we have shown that the transient augmentation of BMP signals stimulates the hypertrophic conversion of ATDC5 cells and that one of the possible mechanisms for the inhibitory action of PTHrP on this cellular event is mediated by the downregulation of BMP-6 expression.

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