Differential Expressions of BMP Family Genes during Chondrogenic Differentiation of Mouse ATDC5 Cells

Haruhiko Akiyama1, Chisa Shukunami2, Takashi Nakamura1, and Yuji Hiraki2,*

1Department of Orthopedic Surgery, Graduate School of Medicine, and 2Department of Molecular Interaction and Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

ABSTRACT. Clonal cell line ATDC5 enables the monitoring of the early- and late-phase chondrogenic differentiation in a single culture. Undifferentiated ATDC5 cells differentiate into type II collagen expressing chondrocytes through a cellular condensation stage (early-phase differentiation) and then to type X collagen-expressing hypertrophic chondrocytes (late-phase differentiation). Progression of cellular differentiation was accelerated by the activation of bone morphogenetic protein (BMP) signaling. ATDC5 cells expressed transcripts for at least four members of the BMP family. The BMP-4 transcripts were expressed in all stages of differentiation, as were transcripts for BMP type IA receptor (ALK-3) and BMP type II receptor. In contrast, transcripts for Growth/Differentiation factor-5 (GDF-5) were induced during a cellular condensation, and those for BMP-6 were induced during the formation of cartilage nodules, and declined as the differentiated ATDC5 cells became hypertrophic, and BMP-7 transcripts were only detected after cells became calcified. Exogenously added BMP-4 indeed promoted the early-phase differentiation. Late-phase differentiation of cells was also stimulated by BMP-4 and BMP-6. Thus, the cumulative increase in BMP signaling promoted the sequential transitions of differentiation steps of cells. These results indicate that the coordinated expressions of endogenous BMPs are involved in the progression of chondrogenic differentiation in ATDC5 cells.

Key words: cartilage differentiation/BMP/ATDC5/endochondral bone formation

The bone morphogenetic protein (BMP) family plays a vital role in skeletal development and are capable of inducing ectopic bone formation through an endochondral pathway which includes a cascade of events such as the recruitment of progenitor cells, cellular condensation to form cartilaginous bone precursors, vascular invasion, the replacement of cartilage by bone, and marrow development (Rosen and Thies, 1992). Several BMP family genes are expressed at sites of bone formation with a unique spatiotemporal pattern, suggesting that the coordinated expression of these molecules is crucial for the cascade of events underlying endochondral bone formation. Transcripts for BMP-2 and BMP-4 genes are localized to precartilaginous mesenchymal condensation, and then the perichondrium (Lyons, et al., 1990). Growth/differentiation factor (GDF)-5 is expressed predominantly in the precartilaginous mesenchymal condensation and the cartilaginous cores of the developing long bone (Storm, et al., 1994). In contrast, BMP-6 and BMP-7 genes are expressed in mature chondrocytes (Carey and Liu, 1995; Vortkamp, et al., 1996; Vukicevic, et al., 1994).

In our previous studies (Shukunami, et al., 1997; Shukunami, et al., 1996), we demonstrated that the clonal cell line ATDC5 enables the monitoring of the multistep chondrogenic differentiation in a single culture. In the presence of insulin, chondrogenic differentiation is induced to form cartilage nodules through cellular condensation (early-phase differentiation). When the formation of cartilage nodules is completed, the cells are then converted to type X collagen-expressing hypertrophic chondrocytes followed by mineralization (late-phase differentiation). However, insulin by itself induced only a cellular condensation in the cultures of undifferentiated ATDC5 cells.

* To whom correspondence should be addressed.
Tel and Fax: +81–75–751–4633
E-mail: hiraki@frontier.kyoto-u.ac.jp
Abbreviations: ActR, activin receptor; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; FBS, fetal bovine serum; GDF, growth/differentiation factor; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; OP, osteogenic protein; PTH, parathyroid hormone; RT-PCR, reverse transcription-polymerase chain reaction.
Instead, exogenously added BMP-2 directly induced chondrogenesis by skipping out the condensation stage (Shukunami, et al., 1998). In order to decipher mechanism underlying the sequential differentiation of ATDC5 cells, we studied the temporal patterns during differentiation of cells in the presence of insulin and the actions of BMP signaling molecules.

Materials and Methods

Materials

Human recombinant BMP-6 was a generous gift from Dr. J. M. Wozney (Genetics Institute Inc., Cambridge, MA, USA). Xenopus recombinant BMP-4 homodimer and BMP-4/7 heterodimer were kindly provided by Takeda Chemical Industries Ltd. (Osaka, Japan).

Cell culture

ATDC5 cells were maintained in a medium consisting of a 1:1 mixture of DME and Ham’s F-12 (DME/F12) medium (Flow Laboratories, Irvine, U.K.) containing 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), and ITS, i.e., 10 μg/ml bovine insulin (I: Wako Pure Chemical, Osaka, Japan), 10 μg/ml human transferrin (T: Boehringer Mannheim, Gaithersburg, MD, USA), and 3×10−8 M sodium selenite (S: Sigma, St. Louis, MO, USA), as previously described (Shukunami, et al., 1997; Shukunami, et al., 1996). In brief, the inoculum size of the cells was 6×103 cells/well in 6-multiwell plates (Corning Glass, Corning, NY, USA) at 37°C with an appropriate cDNA probe (106 cpm/ml) in a 0.1% SDS, then washed for 30 min at 55°C in 0.1% SDS, and then exposed to X-Omat film (Eastman Kodak Co., Rochester, NY, USA) at −80°C with a Cronex lightening plus intensifying screen (DuPont, Boston, MA, USA).

RNA extraction and hybridization analysis

Total RNA was prepared from the cultures by the single-step method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). For northern hybridization, 20 μg of total RNA was denatured with 6% formaldehyde, separated by 1% agarose gel (Seakem GTG, Corning, NY, USA) at 37°C, and the CO2 concentration was shifted to 3% for the facilitation of cellular hypertrophy and mineralization in culture.

RT-PCR

The RT-PCR was performed as described previously (Shukunami, et al., 1996). Briefly, first-strand cDNA was synthesized using SuperScript II Rnase H reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA) with total RNA extracted from ATDC5 cells. The PCR conditions were 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min for 30 cycles, and final extension at 72°C for 5 min. The primers specific for mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene (Clontech, Palo Alto, CA, USA) were used as a positive control for cDNA amplification. The following specific primers were used: 5’-CTCTTCAACCTCAGCAGCATCC-3’ (sense primer) and 5’-TGGCAGTAGAAGGCCTGTAAGC-3’ (antisense primer) for the detection of BMP-4 mRNA; 5’-TGACACGAGCACACATCTCC-3’ (sense primer) and 5’-AGAAGGCACCTCCACAGAGC-3’ (antisense primer) for BMP-6 mRNA; 5’-GCTGAACCTCGGAGTCTACG-3’ (sense primer) and 5’-AGCCCTGTTCTGCTGATG-3’ (antisense primer) for GDF-5 mRNA; 5’-GTGGCACCTCTTCTTCTGTGCG-3’ (sense primer) and 5’-GACGGACTACAGGAGATG-3’ (antisense primer) for BMP-7 mRNA; 5’-GGCCCTGTTCTGCTGATG-3’ (sense primer) and 5’-CCTACAGCTCTGCTGTCG-3’ (antisense primer) for BMP-8 mRNA; 5’-GACATCCACATTGGTGACGG-3’ (sense primer) and 5’-GGAGGTGATGAACTGTCGCG-3’ (antisense primer) for type IB activin receptor (ActR-IB or ALK-4) mRNA; 5’-ACAAGCCACTGTCAGAGGCC-3’ (sense primer) and 5’-CAAGTTCAGATTTGAGAGG-3’ (antisense primer) for BMP-2 mRNA; 5’-GCCGGGTGATTTGAGAGG-3’ (sense primer) and 5’-CTCTTGCTTCTTCTTGCG-3’ (antisense primer) for type IB activin receptor (ActR-IB) mRNA. For the detection of mRNAs for the Smad family members, the following primers were used: 5’-CCTGCGGCAATGAAAGCCGC-3’ (sense primer) and 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for the detection of Smad1 mRNA; 5’-ACCAATCTCGGTGTCTTCTTCTG-3’ (sense primer) and 5’-CTGGCACTCTGTCGACTTCTG-3’ (antisense primer) for Smad3 mRNA; 5’-CACAGCCACACACCTTACCC-3’ (sense primer) and 5’-CAACCCTTGCTTCTGTCTTAC-3’ (antisense primer) for Smad5 mRNA; 5’-AGAGGCAGAGCCAGAGCCGC-3’ (sense primer) and 5’-CACTGAGGAGAGGAGGG-3’ (antisense primer) for Smad4 mRNA; 5’-ACCCAGCACTCTCAGCAGG-3’ (sense primer) and 5’-GGCCTTGTCTG-3’ (antisense primer) for Smad2 mRNA; 5’-AGAGGCAGAGCCAGAGCCGC-3’ (sense primer) and 5’-CACTGAGGAGAGGAGGG-3’ (antisense primer) for Smad3 mRNA; 5’-CACAGCCACACACCTTACCC-3’ (sense primer) and 5’-CAACCCTTGCTTCTGTCTTAC-3’ (antisense primer) for Smad5 mRNA; 5’-ACCAAGCCACTGTCGACTTCTTCC-3’ (sense primer) and 5’-GGAGGTGATTTGAGAGG-3’ (antisense primer) for Smad6 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad2 mRNA; 5’-ACCAAGCCACTGTCGACTTCTTCC-3’ (sense primer) and 5’-GGAGGTGATTTGAGAGG-3’ (antisense primer) for Smad5 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad6 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad3 mRNA; 5’-ACCAAGCCACTGTCGACTTCTTCC-3’ (sense primer) and 5’-GGAGGTGATTTGAGAGG-3’ (antisense primer) for Smad6 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad6 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad6 mRNA; 5’-ATCGTTGGGCTCTTCTCTGACG-3’ (antisense primer) for Smad6 mRNA.
Coordinated Bmp Expression during Chondrogenesis

(sense primer) and 5'-CACAGCCGATCTTGCTCCG-3' (antisense primer) for Smad7 mRNA, and 5'-CAAAGTGCAAGTGCCCTGCT-3' (sense primer) and 5'-GACGCTGACGACAGTGCCCG-3' (antisense primer) for Smad8 mRNA. Aliquots (8 µl) of each PCR reaction products were resolved on 3% NuSieve 3:1 agarose gels (FMC Bioproducts) alongside the markers. The amplified products were stained with ethidium bromide. The amplified PCR products were subcloned pCRII (Invitrogen, San Diego, CA, USA), and the nucleotide sequences of all cDNA fragments were verified by an ALP Express Sequencer (Amersham-Pharmacia, Uppsala, Sweden). No products were observed when the reverse transcriptase was omitted during the synthesis of cDNA (data not shown).

**Alizarin red staining**

ATDC5 cells were cultured in DME/F12 medium containing 5% FBS and ITS for 21 days. Then the culture medium was switched to αMEM containing 5% FBS and ITS, and the cultures were continued for an additional 21 days. After a total of 42 days, the cultures were incubated for an additional 8 days with or without 1,000 ng/ml of BMP-7 in αMEM containing 5% FBS and ITS. Cells were rinsed three times with PBS and fixed with 95% methanol for 30 min. They were stained with 1% alizarin red S (Wako Pure Chemicals) at pH 6.4 for 5 min and washed with distilled water, as described (Shukunami, et al., 1997).

![Image](https://via.placeholder.com/150)

**Fig. 1.** Expression of the BMP family genes during the chondrogenic differentiation of ATDC5 cells. Cells were plated at 6×10^4 cells per well in 6-multiwell plates, and maintained in DME/F12 medium containing 5% FBS and ITS. The culture medium was then changed to αMEM containing the same supplements to facilitate cellular hypertrophy and mineralization on day 21. Total RNA was prepared on the indicated days of culture. In (a), the same membrane filter was hybridized with the probes of mouse BMP-4, mouse GDF-5, mouse BMP-6, rat type II collagen and mouse type X collagen cDNAs. The equivalent loading of RNA (20 µg/lane) was verified by ethidium bromide staining, as shown in the bottom panel. In (b), reverse-transcribed cDNAs were amplified by PCR for the detection of BMP-7 mRNA in comparison with G3PDH mRNA. Aliquots (8 µl) of the PCR products were resolved on 3% agarose gels alongside the markers.
Results

Expression of BMP-4, BMP-6, BMP-7 and GDF-5 mRNAs during chondrogenic differentiation

The transcripts for the BMP family genes are expressed spatially and temporally at the sites of skeletogenesis such as limb buds and vertebrae during embryonic development (Francis West, et al., 1995; Lyons, et al., 1990). ATDC5 cells express type I collagen in the undifferentiated stage. However, the cells undergo differentiation to become type II collagen-expressing chondrocytes in the presence of insulin (Shukunami, et al., 1996). As shown in Fig. 1a, ATDC5 cells expressed transcripts for BMP-4 in the undifferentiated (day 3) and condensation stages (day 5), prior to conversion into chondrocytes. When the growth of differentiated cells ceased by day 21 of culture, cells in cartilage nodules then undergo late-phase differentiation to become hypertrophic and calcifying chondrocytes (Shukunami, et al., 1997). The BMP-4 mRNA level declined as the cartilage nodules stopped growing after two weeks. The GDF-5 transcripts were first detected in a cellular condensation stage on day 5 and continuously expressed in these cells, and declined as differentiated cells began to calcify.

When the regions of cellular condensation are formed in ATDC5 cell cultures from day 3 to day 6, type II collagen-expressing chondrocytes are generated in the condensing regions (Shukunami, et al., 1996). Chondrocytes thus formed gradually proliferate to expand cartilage nodules in the culture for another 10 days, as previously reported (Shukunami, et al., 1996). As the number of chondrocytes increased during this time period, the type II collagen mRNA level increased and became maximal on day 14 or later. Type X collagen-expressing hypertrophic chondrocytes then appeared in cartilage nodules, and markedly increased in number from day 21 when the growth of chondrocytes in the nodules completely ceased.

Interestingly, the BMP-6 and BMP-7 genes exhibited a distinct temporal pattern of expression along with the phenotypic transitions of ATDC5 cells. Transcripts for the BMP-6 gene were absent until mature chondrocytes appeared in the culture. The BMP-6 mRNA level increased in parallel with the induction of type II collagen mRNA level in the culture, and peaked at around day 14 (Fig. 1a). The level of BMP-6 mRNA was kept high during the maturation of chondrocytes (from day 14 to day 21), and then declined until the cells became calcified. In contrast, transcripts for the BMP-7 gene were not detected until the cells became calcified on day 42 or later (Fig. 1b). However, the level of BMP-7 mRNA was so low as to be detected by northern blotting.

Expression of BMP receptor mRNA during differentiation

As shown in Fig. 2a, ATDC5 cells expressed the transcripts for the ALK-3 gene, encoding the BMP type IA receptor. The level of these transcripts did not change significantly during chondrogenic differentiation of ATDC5 cells. Similarly, transcripts for the BMP type II receptor gene were detected in all stages of differentiation of ATDC5 cells, although the level of the transcripts upregulated during maturation of differentiated chondrocytes from day 10 to day 21 of culture. However, mRNA for these receptors were detected in all stages. The transcripts for the BMP type IB receptor (ALK-6) gene were not detected in the ATDC5 cells (data not shown). Transcripts for the type IB and type IIB receptors for activin (AcIR-IB and AcIR-IIB) were also found in all stages of differentiation of ATDC5 cells (Fig. 2b). ATDC5 cells similarly expressed transcripts for the type I receptor for activin (AcIR-I or ALK-2) (data not shown).

The Smad family proteins play an important role in the transduction of BMP signals from the cell-surface receptors (Heldin, et al., 1996). As shown in Fig. 3, ATDC5 cells clearly expressed transcripts for the Smad family genes in all stages of differentiation.

Effect of the treatment with recombinant BMP-4 on the early-phase differentiation in ATDC5 cells

BMP-4 is expressed in undifferentiated ATDC5 cells, indicating that BMP-4 participates in the early-phase differentiation of these cells. When ATDC5 cells were cultured in DME/F12 medium containing 5% FBS and ITS for 11 days, cartilage nodules were apparently formed (Fig. 4a), as reported previously (Shukunami, et al., 1996). Recombinant BMP-4 (1,000 ng/ml) was added to the confluent monolayer culture in the medium containing 5% FBS and ITS on day 5, and the culture was maintained for additional 6 days. By skipping out the condensation stage, almost all cells in culture underwent differentiation and formed a nearly continuous sheet of chondrocytes (Fig. 4a) accompanying alcian blue-positive matrix. As shown in Fig. 4b, recombinant BMP-4 apparently induced the expression of type II collagen gene within 24 hours in a dose dependent manner in the confluent monolayer culture of ATDC5 cells.

Effect of the treatment with recombinant BMP-4 and BMP-6 on the late-phase differentiation

When type II collagen-expressing differentiated ATDC5 cells cease to grow in cartilage nodules, type X collagen-expressing cells begin to appear in the nodules (Shukunami, et al., 1997). We assessed by northern analyses the effects of recombinant BMP-4 and BMP-6 on the late-phase differentiation of ATDC5 cells. The cells were first cultured in DME/F12 medium containing 5% FBS and ITS for 21 days

H. Akiyama et al.
Coordinated *Bmp* Expression during Chondrogenesis

until the cells ceased to grow. Culture medium was then replaced with fresh medium containing various concentration of recombinant BMP-4 or BMP-6. Cultures were incubated for the indicated time periods (Fig. 5). Northern analyses clearly indicated that both BMP-4 (Fig. 5a) and BMP-6 (Fig. 5b) stimulated the expression of type X collagen mRNA in the cells in time- and dose-dependent manners.

**Fig. 2.** Expression of the *BMP* receptor genes during the chondrogenic differentiation of ATDC5 cells. Cells were plated and grown as described in the legend to Fig. 1. Total RNA was prepared on the indicated days of culture. In (a), the same membrane filter was hybridized with the probes of mouse *ALK-3*, or mouse *BMPR-II*. The equivalent loading of RNA (20 μg/lane) was verified by ethidium bromide staining, as shown in the bottom panel. In (b), reverse-transcribed cDNA was amplified by PCR for the detection of *ActR-IB* or *ActR-IIB* mRNA in comparison with *G3PDH* mRNA. Aliquots (8 μl) of the PCR products were resolved on 3% agarose gels alongside the markers.

**Effect of the treatment with recombinant BMP-4/7 heterodimer on matrix mineralization**

We previously reported that differentiated ATDC5 cells underwent matrix mineralization in the cartilage nodules as a consequence of the late-phase differentiation (Shukunami, et al., 1997). In this stage on day 42 or later, the BMP-7 transcripts were expressed in these cells, indicating that BMP-4/7 heterodimer may additionally participate in the progression of the final stage of differentiation in culture. We assessed the effects of recombinant BMP-4/7 on matrix mineralization and the expression of ALP mRNA in these
the PCR products were resolved on 3% agarose gels alongside the markers.

Fig. 3. Expression of the genes for the Smad family members during the chondrogenic differentiation of ATDC5 cells. Cells were plated and grown as described in the legend to Fig. 1. Total RNA was prepared on the indicated days of culture. Reverse-transcribed cDNAs were amplified by PCR for the detection of Smad1, Madr2, Smad3, DPC4, Smad5, Smad6, Smad7, or Smad8 mRNA in comparison with G3PDH mRNA. Aliquots (8 μl) of the PCR products were resolved on 3% agarose gels alongside the markers.

cells. Differentiated cultures of ATDC5 cells were incubated with BMP-4/7 (1,000 ng/ml) from day 42 to day 50. As shown by alizarin red staining (Fig. 6a), mineralization was clearly promoted by the treatment. The induction of a high level of ALP activity in chondrocytes is closely associated with matrix mineralization (Shukunami, et al., 1997). When the ATDC5 cell culture reached the initial calcifying stage on day 42, the culture was further incubated for the indicated time periods in the presence of various concentrations of recombinant BMP-4/7. As indicated by northern blot analysis, the treatment with BMP-4/7 resulted in an increase of the level of ALP mRNA in a dose-dependent manner within 12 hours (Fig. 6b).

**Discussion**

ATDC5 cells retain the properties of chondroprogenitor cells well (Shukunami, et al., 1996). Taking on a fibroblastic morphology, undifferentiated cells express the type I collagen gene transcripts, and they actively proliferate with a short doubling time of 16 hours. In the presence of insulin, regions of cellular condensation appear transiently in the culture, from which proliferating chondrocytes (with a longer doubling time of 48 hours) are generated to form cartilage nodules (Shukunami, et al., 1996). When the formation of cartilage nodules is completed, the cells then become hypertrophic and calcified as the late-phase differentiation progresses (Shukunami, et al., 1997). These orderly transitions of cellular phenotype were positively and negatively modulated by the exogenous growth and differentiation factors added to the culture (Shukunami, et al., 1998). Recombinant BMP-2 positively regulated the phenotypic transitions during early-phase and late-phase differentiation. Parathyroid hormone (PTH) negatively regulated the progression of differentiation (Shukunami, et al., 1997; Shukunami, et al., 1996). These results strongly suggested the presence of a complex network of endogenous growth/differentiation signaling molecules underlying the orderly transition of cellular phenotype in the ATDC5 cultures.

In the developing cartilaginous bone precursors, BMP-4 mRNA is expressed first in the perichondral mesenchymal cells and late in perichondrium from which undifferentiated mesenchymal cells are recruited (Wozney, 1993). The BMP-4 transcripts were constitutively expressed in ATDC5 cells irrespective of their differentiation stages. However, the mRNA level slightly decreased prior to the induction of the late-phase differentiation (day 21 or later). In contrast, the GDF-5 mRNA were expressed after the onset of cellular condensation. As shown in Fig. 2, ATDC5 cells also expressed the BMP signaling counterparts, BMP type I receptor (ALK-3) and BMP type II receptor, in all stages of differentiation. Intracellular signal transducing components Smads were also expressed in the cells (Fig. 3). Thus, ATDC5 cells retained their responsiveness to BMP-4 signaling.

Zou and coworkers demonstrated the distinct functions of BMP receptors type IA and type IB (Zou, et al., 1997). BMP type IB receptor (ALK-6) expression preceded the early chondrogenic differentiation. Signaling from the receptor is necessary for the early steps of mesenchymal condensation and cell death. In contrast, BMP type IA receptor (ALK-3) was essential for proper progression of the cartilage differentiation program. In agreement with these observations, ATDC5 cells properly kept track of the early and late-phase differentiation program despite the lack of BMP type IB receptor expression. ATDC5 cells are committed chondroprogenitor cells. In fact, when the confluent cultures of undifferentiated ATDC5 cells were treated with recombinant BMP-4, almost all of the cells were induced to become chondrocyte-like without the formation of cellular condensation (Fig. 4a). GDF-5 has also been shown to stimulate chondrogenic differentiation (Tsumaki, et al., 1999). ATDC5 cells expressed GDF-5 mRNA as well as mRNAs for its type II receptors, BMP type II receptor and ActR-II (Nishitoh et al., 1996). However, its type I receptor (ALK-6) mRNA was not detected in these cells. Thus, it remained to be elucidated the specific role of GDF-5 in differentiation of ATDC5 cells.

Interestingly, BMP-6 and BMP-7 genes exhibited the distinct and transient patterns of expression along with the late-phase differentiation of ATDC5 cells. The expression
Coordinated *Bmp* Expression during Chondrogenesis

The stimulatory effects of recombinant BMP-6 on the late-phase differentiation of ATDC5 cells is similar to those of BMP-4 (Fig. 5a). Differentiated ATDC5 cells express ALK-3, but do not express ALK-6. Thus, the stimulatory effects of BMP-6 on differentiation of ATDC5 cells may possibly be mediated by ALK-3 in ATDC5 cells.

We previously showed that the ATDC5 cultures underwent matrix mineralization in the differentiated cartilage nodules without β-glycerophosphate supplement (Shukunami, et al., 1997). BMP-7 mRNA was expressed in a matrix mineralization stage in these cells, and recombinant BMP-4/7 actually promoted a marked upregulation of ALP mRNA and matrix mineralization in these cells (Fig. 6), suggesting that BMP-7 plays a role in the progression of matrix mineralization.
mineralization in ATDC5 cells.

Chondrogenic differentiation includes a series of cellular events, which enable cells to undergo orderly transitions of the cartilage phenotype, as characterized by the sequential expression of unique cartilage matrix. The present results indicated that, in addition to the basal expression of BMP-4, the differentiation stage-specific expression of BMP-6 and BMP-7 contributed to the progressive multistep differentiation after induction of cellular condensation by insulin in ATDC5 cells. Thus, the mouse clonal cell line ATDC5 is a useful in vitro model for molecular analysis of the multistep chondrogenic differentiation during endochondral bone formation.

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Coordinated Bmp Expression during Chondrogenesis

Fig. 6. Effects of recombinant BMP-4/7 on the matrix mineralization of ATDC5 cells. Cells were plated at 6×10^4 cells per well in a 6-assay-well plate and cultured for 42 days as described in the methods section. (a) Cells were then incubated in the absence or the presence of recombinant BMP-4/7 (1,000 ng/ml) for an additional 8 days and stained with 1% alizarin red S. (b) After 42 days, medium was replaced with the fresh medium containing various doses of BMP-4/7. For the time-dependence, cells were treated with 1,000 ng/ml of BMP-4/7 for the various time periods indicated. For the dose-dependence, cells were treated with 100 or 1,000 ng/ml of BMP-4/7 for a total of 48 hours. Total RNA was prepared and subjected for northern analysis of ALP mRNA. The equivalent loading of RNA (20 μg/lane) was verified by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

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