# Bone Morphogenetic Protein-2 Converts the Differentiation Pathway of C2C12 Myoblasts into the Osteoblast Lineage

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*Abstract*. The implantation of bone morphogenetic protein (BMP) into muscular tissues induces ectopic bone formation at the site of implantation. To investigate the mechanism underlying this process, we examined whether recombinant bone morphogenetic protein-2 (BMP-2) converts the differentiation pathway of the clonal myoblastic cell line, C2C12, into that of osteoblast lineage. Incubating the cells with 300 ng/ml of BMP-2 for 6 d almost completely inhibited the formation of the multinucleated myotubes expressing troponin T and myosin heavy chain, and induced the appearance of numerous alkaline phosphatase (ALP)-positive cells. BMP-2 dose dependently induced ALP activity, parathyroid hormone (PTH)-dependent 3′,5′-cAMP production, and osteocalcin production at concentrations above 100 ng/ml. The concentration of BMP-2 required to induce these osteoblastic phenotypes was the same as that required to almost completely inhibit myotube formation. Incubating primary muscle cells with 300 ng/ml of BMP-2 for 6 d also inhibited myotube formation, whereas induced ALP activity and osteocalcin production. Incubation with 300 ng/ml of BMP-2 suppressed the expression of mRNA for muscle creatine kinase within 6 h, whereas it induced mRNA expression for ALP, PTH/PTH-related protein (PTHrP) receptors, and osteocalcin within 24–48 h. BMP-2 completely inhibited the expression of myogenin mRNA by day 3. By day 3, BMP-2 also inhibited the expression of MyoD mRNA, but it was transiently stimulated 12 h after exposure to BMP-2. Expression of Id-1 mRNA was greatly stimulated by BMP-2. When C2C12 cells pretreated with BMP-2 for 6 d were transferred to a colony assay system in the absence of BMP-2, more than 84% of the colonies generated became troponin T-positive and ALP activity disappeared. TGF-$\beta$1 also inhibited myotube formation in C2C12 cells, and suppressed the expression of myogenin and MyoD mRNAs without inducing that of Id-1 mRNA. However, no osteoblastic phenotype was induced by TGF-$\beta$1 in C2C12 cells. TGF-$\beta$1 potentiated the inhibitory effect of BMP-2 on myotube formation, whereas TGF-$\beta$1 reduced ALP activity and osteocalcin production induced by BMP-2 in C2C12 cells. These results indicate that BMP-2 specifically converts the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage cells, but that the conversion is not heritable.

Several lines of evidence indicate that osteoblasts, chondrocytes, myocytes, and adipocytes are all derived from a common progenitor cell called undifferentiated mesenchymal cells (Taylor and Jones, 1979; Grigoriadis et al., 1988, 1990; Yamaguchi and Kahn, 1991). During the process of their differentiation, progenitor cells acquire specific phenotypes depending upon the differentiated cell types under the control of respective regulatory factors (for reviews see Rodan and Rodan, 1984; Owen, 1988; Wlodarski, 1990). The differentiation process of osteoblasts can be divided into at least two stages. One is the commitment of undifferentiated mesenchymal cells into osteoblast progenitors. The other is the maturation of osteoblast progenitors into osteoblasts which express the various phenotypes of bone-forming cells; production of a large amount of extracellular matrix proteins including type I collagen and osteocalcin, high levels of alkaline phosphatase (ALP)[1] activity,

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1. *Abbreviations used in this paper*: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; HLH, helix-loop-helix; MCK, muscle creatine kinase; MHC, myosin heavy chain; PTH, parathyroid hormone; Tn T, troponin T.

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and responsiveness to calcitropic hormones such as parathyroid hormone (PTH) and 1$\alpha$,25-dihydroxyvitamin $D_3$ (for reviews see Rodan and Rodan, 1984; Wlodarski, 1990). The regulatory mechanism of osteoblast differentiation at the maturation stage has been extensively studied using several osteoblast-like cell lines isolated from bone and bone marrow (McAllister et al., 1971; Heremans et al., 1978; Majeska et al., 1980; Sudo et al., 1983; Partridge et al., 1983; Benayahu et al., 1989). However, the mechanism of the commitment to osteoblast differentiation is not fully understood because of a limited number of suitable in vitro model systems reflecting the commitment of osteoblast differentiation from non-osteogenic cells.

The molecular mechanism of myogenic differentiation has been well characterized using in vitro systems (for reviews see Olson, 1990; Weintraub et al., 1991; Wright, 1992; Edmondson and Olson, 1993; Weintraub, 1993). The gene cloning of the muscle-specific regulatory factors, MyoD, myogenin, Myf-5 and MRF4/herculin/Myf-6 has contributed significantly to the understanding of muscle differentiation (Davis et al., 1987; Wright et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989; Rhodes and Konieczny, 1989; Fujisawa-Sehara et al., 1990; Miner and Wold, 1990; Braun et al., 1990). When the cDNAs of these regulatory proteins are introduced into pluripotent fibroblastic C3H10T1/2 cells, they differentiate into mature muscle cells (Davis et al., 1987; Wright et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989; Rhodes and Konieczny, 1989; Fujisawa-Sehara et al., 1990; Miner and Wold, 1990; Braun et al., 1990). These myogenic factors are called MyoD family and they are nuclear phosphorylated proteins that contain a common basic helix-loop-helix (bHLH) motif (Tapscott et al., 1988). The basic region of this motif is required for binding to a specific DNA sequence, CANNTG (E-box), which is present in the promoters and enhancers of various muscle-specific genes including muscle creatine kinase (MCK) (Murre et al., 1989*a*; Lassar et al., 1989; Davis et al., 1990). In addition, other members of helix-loop-helix (HLH) factors (E12/E47 and Id-1) play important roles in the process of binding to DNA as well as in the transcriptional activation of the muscle specific genes. E12 and E47, two differentially spliced products of the E2A gene, form heterodimers with MyoD family proteins, and these complexes bind to E-box with much higher affinity than homodimers of myogenic HLH factors (Murre et al., 1989*b*; Brennan and Olson, 1990). Another HLH protein, Id-1, is a negative regulator of myogenesis (Benezra et al., 1990). Id-1 lacks the basic region in the conserved motif which is necessary for binding to E-box (Benezra et al., 1990). It forms heterodimers with E12/E47 and prevents MyoD from forming complexes with E12/E47 in myoblasts (Benezra et al., 1990).

When demineralized bone matrix is implanted into muscular tissues, it induces the new bone formation at the implantation sites (Urist, 1965). This factor(s) present in bone matrix was named bone morphogenetic protein (BMP) (Urist, 1971). It was purified from demineralized bovine bone matrix (Wang et al., 1988; Luyten et al., 1989; Sampath et al., 1990), and human cDNAs of eight different BMPs have been cloned (Wozney et al., 1988; Celeste et al., 1990; Özkaynak et al., 1990, 1992). The deduced amino acid sequences of BMPs have indicated that BMP-2 through BMP-8 are members of the TGF-$\beta$ superfamily (Wozney et al., 1988; Celeste et al., 1990; Özkaynak et al., 1990, 1992), which is an important regulator of the differentiation of various types of cells and organs (for review see Roberts and Sporn, 1990).

Recently, we reported that recombinant human BMP-2 stimulates osteoblastic maturation in osteoblast progenitor cells, ROB-C26, and induces the pluripotent fibroblastic cell line, C3H10T1/2, to differentiate into osteoblast-like cells (Katagiri et al., 1990*a*; Yamaguchi et al., 1991). These results suggest that BMP-2 not only stimulates the maturation of committed osteoblast progenitors, but also induces the commitment of undifferentiated non-osteogenic cells into osteoblasts. We also reported that BMP-2 inhibited myotube formation in ROB-C26 cells and L-6 myoblasts in vitro (Yamaguchi et al., 1991). These results led us to examine the possibility that BMP-2 regulates the differentiation pathway of myogenic cells around the sites of BMP-2 implantation.

We report here that BMP-2 inhibits myotube formation of myoblastic C2C12 cells by suppressing myogenin mRNA and inducing Id-1 mRNA expression. More importantly, BMP-2 converts the differentiation pathway of C2C12 cells into that of osteoblast lineage.

## Materials and Methods

### Bone Morphogenetic Protein-2 and Transforming Growth Factor-$\beta$1

Bioactive recombinant human BMP-2 (BMP-2) was produced and purified from the conditioned medium of CHO cells as described previously (Wang et al., 1990). The purity of the protein was over 90% as judged by silver staining on SDS-polyacrylamide gels and amino-terminal sequencing. Recombinant human TGF-$\beta$1 was purchased from R & D Systems, Inc. (Minneapolis, MN).

### Cell Culture

The mouse myoblast cell line, C2C12 (Blau et al., 1983), was purchased from the Amer. Type Culture Collection. C2C12 cells were maintained in DMEM (Sigma Chem. Co., St. Louis, MO) containing 15% FBS (GIBCO, Grand Island, NY) and antibiotics (100 U/ml of Penicillin-G and 100 $\mu$g/ml of Streptomycin) (growth medium) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Primary muscle cells were prepared from thigh muscles of 1-d-old mice (ddY strain) as described previously (Yaffe, 1973). The cells were inoculated at $2 \times 10^4$ cells/cm$^2$ and cultured with growth medium. To examine the effects of BMP-2 and TGF-$\beta$1 on muscle and osteoblast differentiation in C2C12 cells and primary muscle cells, the growth medium was replaced on day 1 and DMEM containing 5% FBS (low mitogen medium) with various concentrations of these growth factors.

### Histochemical Analyses

To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 20 min with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate (Sigma Chem. Co.), 0.5% *N,N*-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml of fast blue BB salt (Sigma Chem. Co.) in 0.1 M Tris-HCl, pH 8.5, at room temperature.

To analyze expression of troponin T (Tn T) and myosin heavy chain (MHC) immunohistochemically, the cells were fixed for 10 min at room temperature with 3.7% formaldehyde. They were washed with PBS and treated for 1 min with an acetone/ethanol mixture (50:50, vol/vol) at −20°C to permeate the cell membrane. After incubating the cells for 10 min with 10% normal rabbit serum to block non-specific binding, they were incubated for 1 h with mouse anti-Tn T monoclonal antibody (NT-302; provided by Dr. T. Obinata, Chiba University, Japan) (Abe et al., 1986) or mouse anti-MHC monoclonal antibody (MF-20; purchased from Developmental Studies Hybridoma Bank, Iowa City, IA) (Bader et al., 1982) at room temperature. The cells were washed with PBS, and then incubated for



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15 min with a secondary antibody (biotinylated rabbit anti-mouse immunoglobulins). The cells were washed with PBS, and then incubated with peroxidase-conjugated streptavidin. The reaction products were visualized using an AEC substrate kit (Histofine, Nichirei Co., Tokyo, Japan). Occasionally, the Tn T-positive myotubes were counted in 11.6 $mm^2$ at arbitrarily selected area of each culture after immunohistochemical staining.

## Colony Formation Assay

After C2C12 cells were cultured for 6 d with or without 300 ng/ml of BMP-2 as described above, they were replated at 500 cells/well in 6-well plates and maintained for 9 d in DMEM containing 15% FBS with or without 300 ng/ml of BMP-2. To stimulate myotube formation, the cells were incubated for additional 3 d in DMEM containing 5% FBS in the presence or absence of 300 ng/ml of BMP-2. The colonies formed were fixed and double stained for Tn T and ALP activity as described above. The colonies formed in each culture were divided into four groups by the expression pattern of Tn T immunoreactivity and ALP activity: Tn $T^+/ALP^-$; colonies containing numerous Tn T-positive myotubes but no ALP-positive cells, Tn $T^-/ALP^+$; colonies containing numerous ALP-positive cells but no Tn T-positive cells, Tn $T^+/ALP^+$; colonies containing both Tn T-positive and ALP-positive cells at various proportions, and Tn $T^-/ALP^-$; colonies containing neither Tn T-positive cells nor ALP-positive cells.

## Alkaline Phosphatase Activity

After removing the culture medium, cell layers were washed with PBS, and then sonicated for 10 s at 0°C three times in 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.5. ALP activity in the cell lysate was assayed at 37°C in the buffer containing 0.1 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$, pH 10.5, for 30 min using *p*-nitrophenylphosphate as a substrate. The enzyme activity was expressed as micromoles of *p*-nitrophenol produced per min per mg of protein. The protein content was determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

## Osteocalcin Production

The amount of osteocalcin secreted into the culture medium was determined by RIA using a mouse osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA).

## 3',5'-cAMP Production in Response to PTH

To measure responsiveness to PTH, the amount of cAMP produced by the cells was assayed. Cells were cultured for 20 min at 37°C with DMEM containing 0.5% BSA and 1 mM 3-isobuthyl-1-methylxanthine. Thereafter, the cells were incubated for 10 min at 37°C with 200 ng/ml of human PTH(1-34) (provided by Dr. M. Hori, Asahi-Kasei Co., Shizuoka, Japan) dissolved in the same media. The cAMP produced by the cells was extracted with ethanol containing 3 mM HCl at 4°C for 2 h. The extract was dried by boiling, and dissolved in 3 mM HCl. The content of cAMP was determined using a cAMP RIA kit (Yamasa Co., Chiba, Japan).

## RNA Preparation and Northern Blots

Total cellular RNA was extracted from C2C12 cells according to an acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Ten or twenty micrograms of total RNA was electrophoresed in 1.2% agarose-formaldehyde gels, and transferred onto Hybond-N membranes (Amersham International, Amersham, UK). The membranes were prehybridized for 3 h at 42°C in 50% formamide; 5 × SSC; 5 × Denhardt's solution; 50 mM Tris-HCl, pH 7.5; 0.1% SDS; and 250 μg/ml of salmon sperm DNA. Thereafter, the membranes were hybridized with $^{32}P$-labeled cDNA probes overnight at 42°C. The labeled probes were prepared using a DNA random priming kit (Takara Shuzo Co., Kyoto, Japan) and [α-

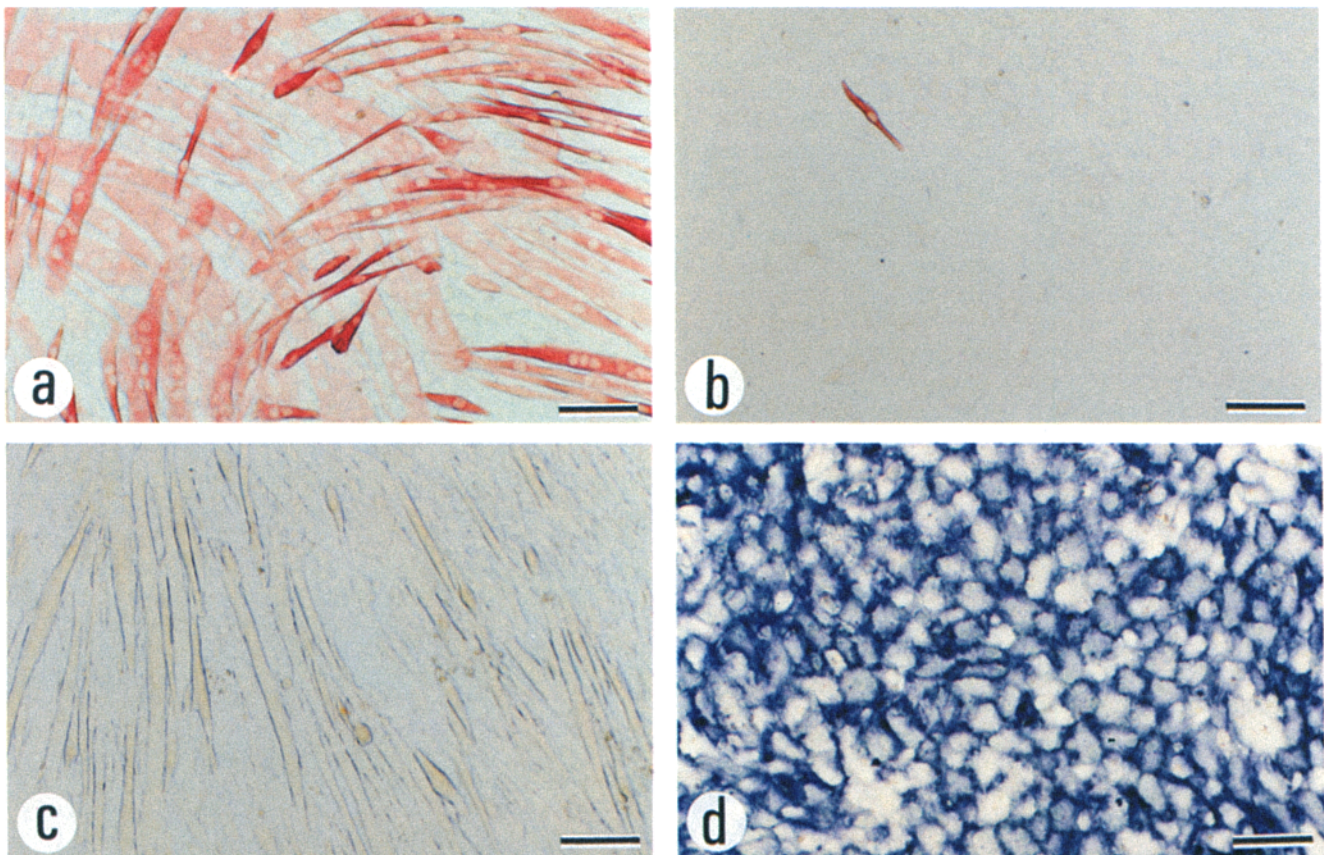


*Figure 1.* The effects of BMP-2 on induction of Tn T and ALP activity in C2C12 cells. The cells were cultured for 6 d with (*b* and *d*) or without (*a* and *c*) 300 ng/ml of BMP-2 as described in Materials and Methods. The cultured cells were fixed and stained for Tn T (*a* and *b*) and ALP (*c* and *d*) as described in Materials and Methods. Bars, 100 μm.

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[^{32}P]dCTP (New England Nuclear, Boston, MA). The membranes were washed three times at room temperature in 2 × SSC containing 0.1% SDS and twice at 55°C in 0.1 × SSC containing 0.1% SDS and autoradiographed at −80°C. Sometimes, the hybridized probes were removed from the membranes by boiling them in 0.5% SDS, and rehybridized. The cDNA probes were those for the rat ALP (Noda et al., 1987), rat PTH/PTHrP receptors (Jüppner et al., 1991), rat osteocalcin (Celeste et al., 1986), mouse MCK, mouse MyoD (Davis et al., 1987), mouse myogenin (Fujisawa-Sehara et al., 1990), mouse Id-1 (Hara et al., 1994), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

## Results

### *Effects of BMP-2 on the Muscle and Osteoblast Differentiation*

C2C12 cells generated numerous multinucleated myotubes on day 6, when they were cultured in low mitogen medium. The myotubes formed were positive for both Tn T (Fig. 1 *a*) and MHC (data not shown) by immunohistochemical staining. An enzyme histochemical study showed that these cells were negative for ALP (Fig. 1 *c*). BMP-2 exposure (300 ng/ml) for 6 d inhibited the formation of multinucleated myotubes (Fig. 1 *b*). The cells remained as unfused mononuclear polygonal cells in the presence of BMP-2. These mononuclear cells were negative for both Tn T (Fig. 1 *b*) and MHC (data not shown). Incubation with BMP-2 (300 ng/ml) for 6 d strikingly induced ALP-positive cells (Fig. 1 *d*). Over 90% of the mononuclear cells were ALP positive (Fig. 1 *d*). A few multinucleated myotubes that formed in the presence of BMP-2 were negative for ALP.

Fig. 2 shows the dose-dependent effects of BMP-2 on differentiation of C2C12 cells cultured for 6 d in low mitogen medium. Incubating C2C12 cells with BMP-2 dose dependently decreased the number of myotubes, which slightly decreased at 100 ng/ml and almost completely disappeared at concentrations above 300 ng/ml of BMP-2 (Fig. 2 *a*). C2C12 cells cultured without BMP-2 expressed extremely low or undetectable levels of osteoblastic phenotypes such as ALP activity, PTH responsiveness, and osteocalcin production (Fig. 2). ALP activity was slightly induced at 100 ng/ml and markedly increased at more than 300 ng/ml of BMP-2 (Fig. 2 *b*). BMP-2 at concentrations of more than 300 ng/ml induced high levels of cAMP in response to PTH; an 8.1-fold increase at 300 ng/ml and a 13.6-fold increase at 1,000 ng/ml (Fig. 2 *c*). BMP-2 slightly induced osteocalcin production at 100 ng/ml and markedly induced it at concentrations above 300 ng/ml (Fig. 2 *d*). The concentration of BMP-2 required to induce osteoblastic phenotypes was almost identical to that required to inhibit myotube formation (Fig. 2).

A time course study in the control culture with low mitogen medium showed that myotubes first appeared on day 3 and that the number increased time dependently until day 6 (Fig. 3 *a*). However, 300 ng/ml of BMP-2 almost completely abolished myotube formation in C2C12 cells during the entire culture period (Fig. 3 *a*). When C2C12 cells were cultured in the presence of 300 ng/ml of BMP-2, both ALP activity and osteocalcin production began to appear within 48 h, and then increased time dependently until day 6 (Fig. 3, *b* and *c*).

The effects of BMP-2 were reproducible in primary muscle cells isolated from thigh muscles of 1-d-old mice as well. When primary muscle cells were treated with BMP-2 for

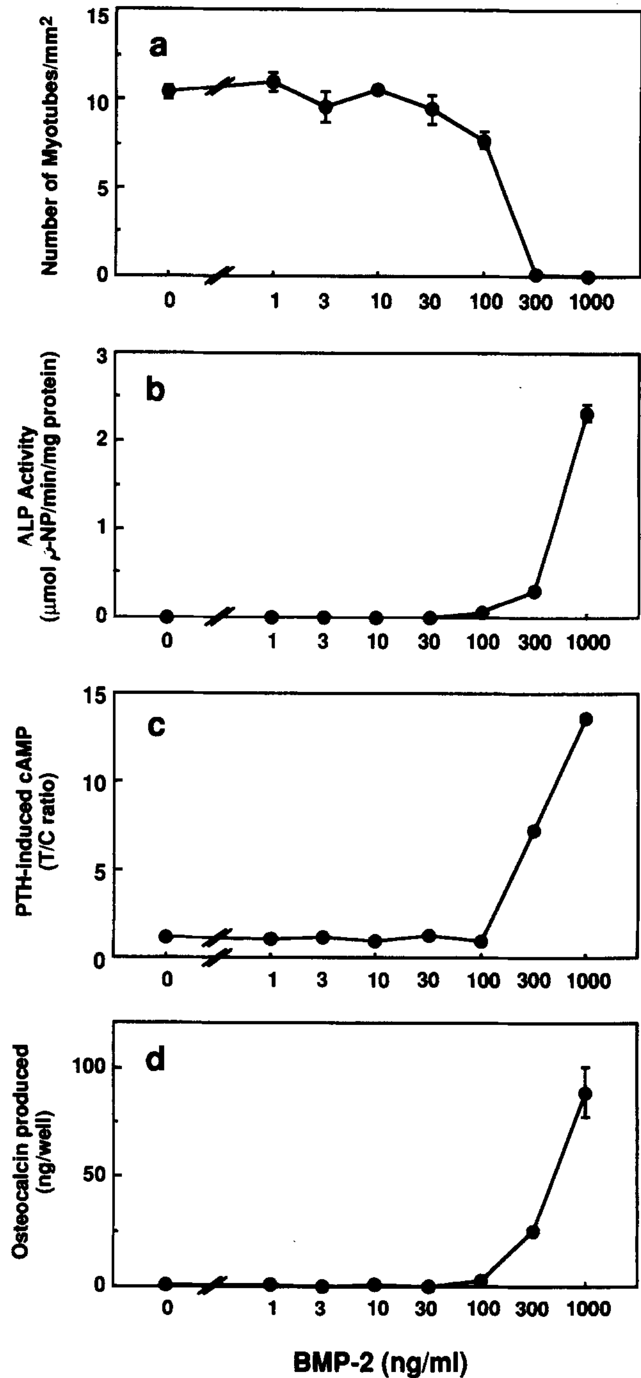


*Figure 2.* Dose-response effects of BMP-2 on myotube formation, ALP activity, PTH-dependent cAMP accumulation, and osteocalcin production in C2C12 cells. The cells were cultured for 6 d with graded concentrations of BMP-2 as described in Materials and Methods. (*a*) The number of myotubes was counted after staining for Tn T as described in Materials and Methods. (*b*) The ALP activity of the cell lysate was measured using *p*-nitrophenylphosphate as a substrate. (*c*) The amounts of cAMP accumulation in response to PTH were measured by RIA. (*d*) The amounts of osteocalcin secreted into the culture media were determined by RIA. Data are means ± SEM of three wells.

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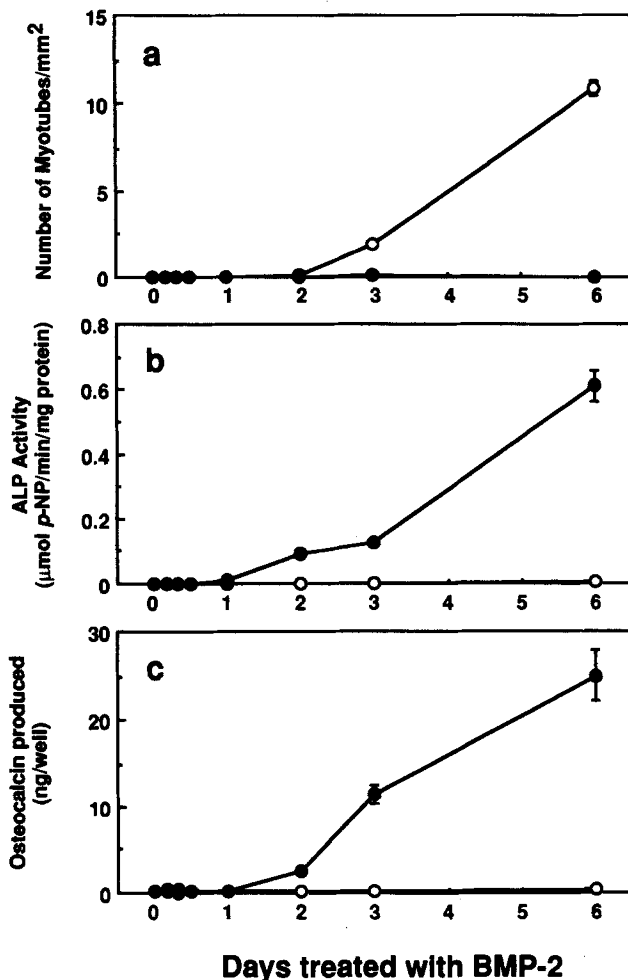


*Figure 3.* Time course of changes in myotube formation, ALP activity, and osteocalcin production in C2C12 cells. Cells were cultured with (●) or without (○) 300 ng/ml of BMP-2 for the indicated times. (*a*) The number of myotubes was counted after staining for Tn T as described in Materials and Methods. (*b*) The ALP activity of the cell lysate was measured using *p*-nitrophenylphosphate as a substrate. (*c*) The amounts of osteocalcin secreted into the culture media were determined by RIA. Data are means ± SEM of three wells.

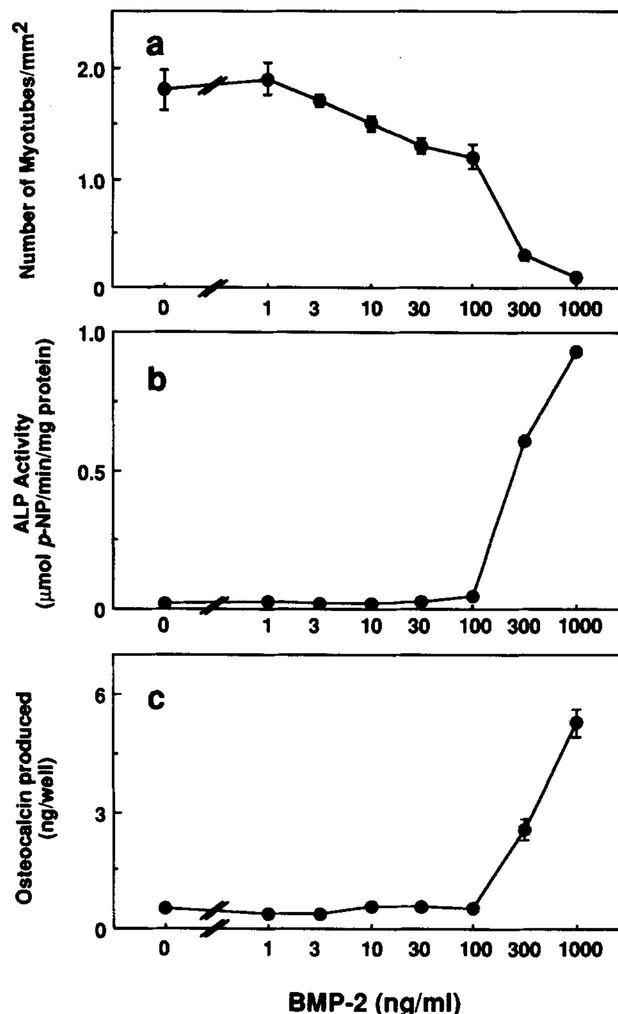


*Figure 4.* Dose-response effects of BMP-2 on myotube formation, ALP activity, and osteocalcin production in primary muscle cells. The cells were cultured for 6 d with graded concentrations of BMP-2 as described in Materials and Methods. (*a*) The number of myotubes was counted after staining for Tn T as described in Materials and Methods. (*b*) The ALP activity of the cell lysate was measured using *p*-nitrophenylphosphate as a substrate. (*c*) The amounts of osteocalcin secreted into the culture media were determined by RIA. Data are means ± SEM of three wells.

6 d, the number of myotubes was decreased dose dependently (Fig. 4 *a*). At 300 ng/ml or higher of BMP-2, myotubes disappeared almost completely (Fig. 4 *a*). Primary muscle cells cultured without BMP-2 expressed essentially no osteoblastic phenotype, but the cells exposed to BMP-2 at concentrations higher than 300 ng/ml expressed high levels of ALP activity and osteocalcin production (Fig. 4, *b* and *c*). The effective dose levels of BMP-2 to inhibit myogenic phenotype and to induce osteoblast phenotype were almost identical between primary muscle cells and C2C12 cells.

To determine whether BMP-2 expresses osteoblast phenotypes at the transcriptional level, mRNA expression for myogenic and osteoblast differentiation was examined by Northern blotting. When C2C12 cells were cultured with low mitogen medium, they expressed MCK mRNA at 48 h and the expression increased in a time-dependent manner (Fig. 5, MCK, lane *A*). BMP-2 at 300 ng/ml diminished the expression of MCK mRNA almost completely (Fig. 5, MCK, lane *B*). The untreated C2C12 cells did not express any mRNAs for ALP, PTH/PTHrP receptors, and osteocalcin during the entire culture period. BMP-2 at 300 ng/ml induced the expression of ALP and osteocalcin mRNAs within 48 h (Fig. 5, ALP and osteocalcin, lane *B*). BMP-2 (300 ng/ml) also induced a weak but detectable level of PTH/PTHrP receptors mRNA within 48 h (data not shown). The expressed levels of mRNAs of these osteoblast phenotypes increased in a time-dependent manner.

### *Effects of BMP-2 on mRNA Expression of HLH Proteins*

To investigate the mechanism of the inhibitory effect on the myogenic differentiation of BMP-2, the mRNA expression of HLH proteins such as myogenin, MyoD, and Id-1 was exam-

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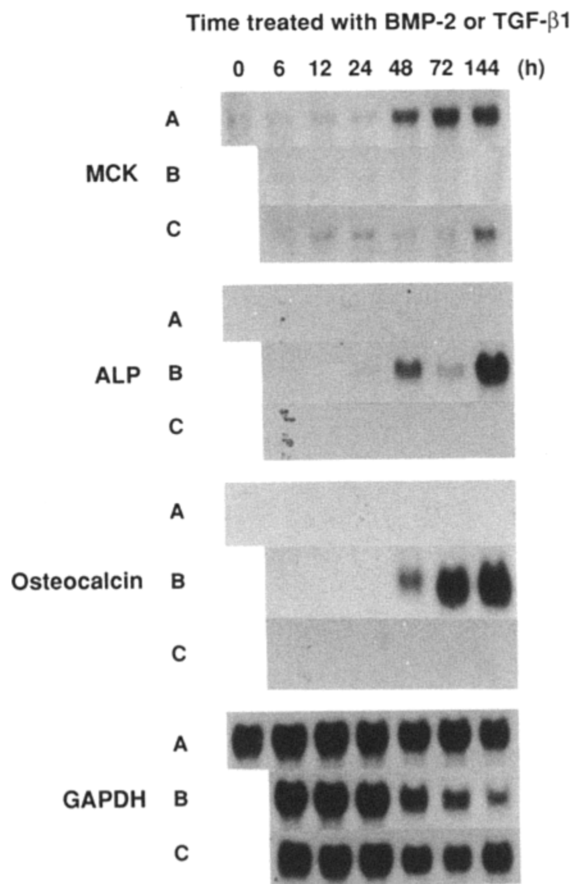


*Figure 5.* Northern blots of the expression of phenotypes of myogenic and osteoblast differentiation in C2C12 cells. Cells were cultured with or without BMP-2 (300 ng/ml) or TGF-$\beta$1 (5 ng/ml) for the indicated periods. Total RNA from each culture (20 $\mu$g) was separated in a formaldehyde-agarose gel and sequentially hybridized with the respective cDNA probes as described in Materials and Methods. *A*, control culture; *B*, BMP-2-treated culture; *C*, TGF-$\beta$1-treated culture.

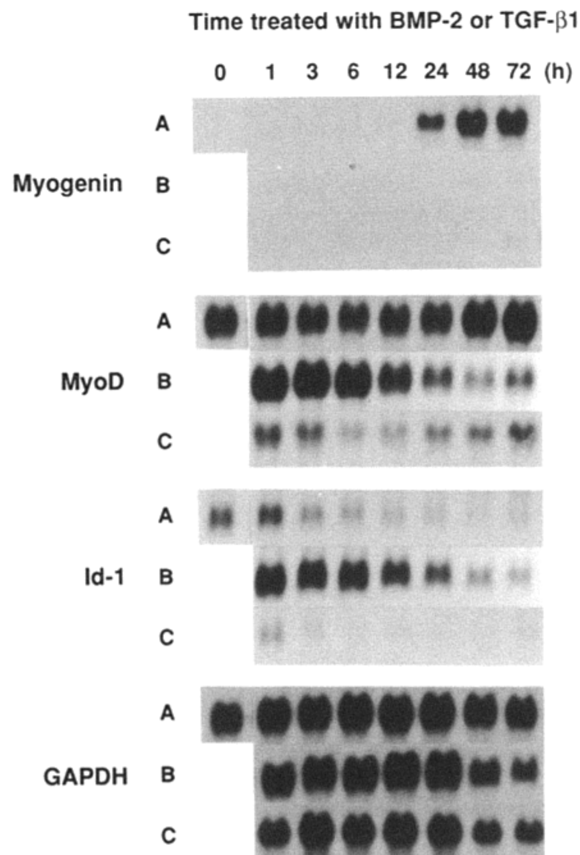


*Figure 6.* Expression of the mRNA of HLH proteins which regulate myogenic differentiation in C2C12 cells. The cells were cultured in the presence or absence of BMP-2 (300 ng/ml) or TGF-$\beta$1 (5 ng/ml) for the indicated periods. Total RNA prepared from each culture (10 $\mu$g) was separated in a formaldehyde-agarose gel and sequentially hybridized with the respective cDNA probes as described in Materials and Methods. *A*, control culture; *B*, BMP-2-treated culture; *C*, TGF-$\beta$1-treated culture.

ined. These proteins are important regulators of myogenic differentiation. C2C12 cells cultured with growth medium expressed substantial levels of mRNA for MyoD and Id-1, but none for that of myogenin (Fig. 6, 0 h). When the medium was changed from growth to low mitogen medium in the absence of BMP-2, myogenin mRNA was expressed at 24 h and it increased thereafter. In contrast, Id-1 mRNA was downregulated within 3 h (Fig. 6). The expression of MyoD mRNA was stimulated within 24 h in the control culture (Fig. 6). BMP-2 (300 ng/ml) almost completely inhibited the increase in myogenin mRNA expression in C2C12 cells. The expression of MyoD mRNA was transiently stimulated at 1-3 h but decreased thereafter by BMP-2. BMP-2 also increased the level of Id-1 mRNA within 1 h, but decreased it gradually thereafter. The expression profile of Id-1 mRNA induced by BMP-2 was identical to that of MyoD. The expression level of Id-1 was always higher in BMP-2-treated cells than in the corresponding control cells.

### *The Fate of the Phenotype of the Colonies Formed from C2C12 Cells Pretreated with or without BMP-2*

To determine whether the effects of BMP-2 on the muscle and osteoblastic differentiation are heritable, the colony formation assay was performed by replating C2C12 cells pretreated for 6 d with or without 300 ng/ml of BMP-2. The colonies were maintained for 12 d in the presence or absence of 300 ng/ml of BMP-2. The colonies formed in each culture were divided into four groups (Tn $T^+$/$ALP^-$, Tn $T^-$/$ALP^+$, Tn $T^+$/$ALP^+$, and Tn $T^-$/$ALP^-$) by the expression pattern of Tn T immunoreactivity and ALP activity. The phenotype of the colonies depended on the presence or absence of BMP-2 in the colony assay but not in the pretreatment culture (Fig. 7 and Table I). Almost all (over 83.9%) of the colonies formed from C2C12 cells pretreated with or without BMP-2 were Tn $T^+$/$ALP^-$, when they were maintained in the absence of BMP-2 in the colony assay. However, more than 84% of the colonies became Tn $T^-$/$ALP^+$ by culturing C2C12 cells with BMP-2 in the colony assay, irrespective of the presence or absence of BMP-2 in the pretreatment cul-

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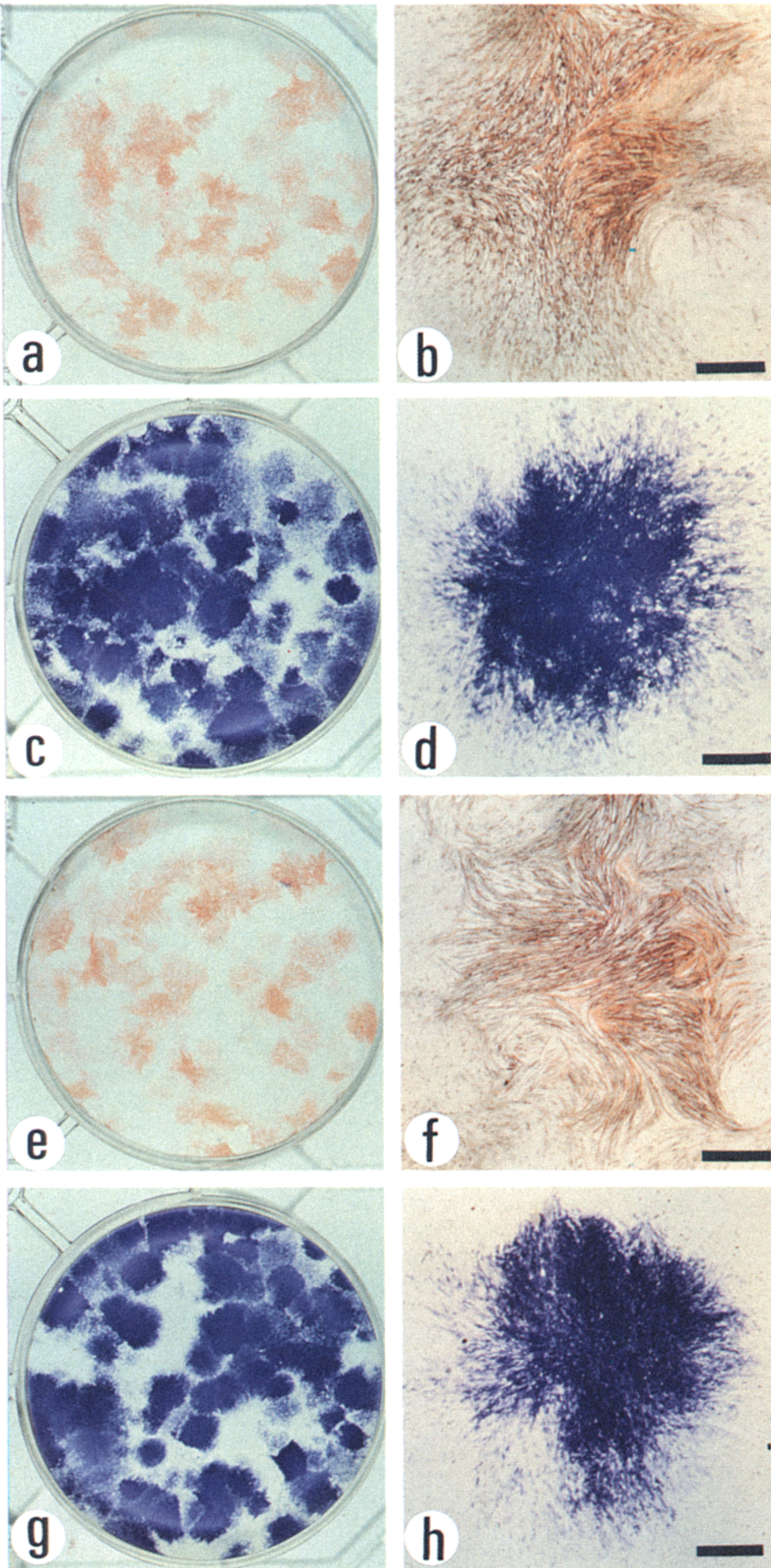


*Figure 7.* Effects of BMP-2 on the muscle and osteoblastic properties in the colony formation assay. C2C12 cells cultured for 6 d with (*e–h*) or without (*a–d*) 300 ng/ml of BMP-2 were replated at 500 cells/well in 6-well plates and maintained for 9 d in DMEM containing 15% FBS in the presence (*c*, *d*, *g*, and *h*) or absence (*a*, *b*, *e*, and *f*) of 300 ng/ml of BMP-2. To stimulate myotube formation, the colonies were incubated for 3 additional d in DMEM containing 5% FBS in the presence (*c*, *d*, *g*, and *h*) or absence (*a*, *b*, *e*, and *f*) of 300 ng/ml of BMP-2. The colonies were double-stained for Tn T expression and ALP activity as described in Materials and Methods. The right panels (*b*, *d*, *f*, and *h*) show the typical colonies corresponding to each well in the left panels (*a*, *c*, *e*, and *g*), respectively. Bars, 1 mm.

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*Table I. The Fate of the Phenotype of the Colonies Formed from C2C12 Cells Pretreated with or without BMP-2*

| Pretreatment with BMP-2 for 6 d | Presence or absence of BMP-2 in the colony assay (on day 12) | Phenotype of colonies | | | | |
|---|---|---|---|---|---|---|
| | | Tn T⁺/ALP⁻ | Tn T⁻/ALP⁺ | Tn T⁺/ALP⁺ | Tn T⁻/ALP⁻ | Total |
| | | Number of colonies/well (% of total colonies) | | | | |
| − | − | 93 (88.5) | 1 (1.0)* | 0 (0.0) | 11 (10.5) | 105 |
| | + | 0 (0.0) | 82 (93.2) | 2 (2.3)‡ | 4 (4.5) | 88 |
| + | − | 73 (83.9) | 0 (0.0) | 2 (4.6)§ | 10 (11.5) | 87 |
| | | + | 7 (1.2)* 85 | 72 (84.7) | 4 (4.7)‡ | 8 (9.4) |

The colony assay was performed by the method described in Materials and Methods using C2C12 cells pretreated for 6 d with or without 300 ng/ml of BMP-2. The colonies were divided into four groups by the expression pattern of ALP activity and Tn T immunoreactivity. The number of colonies was counted as described in Materials and Methods. Data are expressed as the number of colonies in a well. The data in the parentheses are the percentages of the colonies to total colonies.
* The colonies consisted of a small number of ALP-positive or Tn T-positive cells.
‡ The colonies consisted of a large number of ALP-positive cells and a small number of Tn T-positive cells.
§ The colonies consisted of a large number of Tn T-positive cells and a small number of ALP-positive cells.

ture. A small number of the colonies formed in each culture were Tn T⁻/ALP⁻ (~11.5%) or Tn T⁺/ALP⁺ (~4.7%) (Table I). The proportion of these colonies to total colonies were similar between the four groups.

### *Effects of TGF-β1 on Muscle and Osteoblast Differentiation*

Like BMP-2, treatment with TGF-β1 for 6 d partially reduced the number of myotubes of 0.5 ng/ml and almost completely inhibited its formation at 5 ng/ml in C2C12 cells (Fig. 8 *a*). TGF-β1 markedly suppressed the expression of myogenin and MyoD mRNAs without the transient stimulation of Id-1 mRNA (Fig. 6). TGF-β1 (0.5 and 5 ng/ml) did not induce ALP activity, osteocalcin production (Fig. 8, *b* and *c*), or the mRNA for these proteins (Fig. 5, lane *C*). When 0.5 ng/ml of TGF-β1 was added to the culture in the presence of 100 ng/ml of BMP-2, it potentiated the inhibitory effect of BMP-2 on myotube formation in C2C12 cells (Fig. 8 *a*). In contrast, TGF-β1 inhibited ALP activity at 5 ng/ml and osteocalcin production at 0.5 ng/ml, both induced by 300 ng/ml of BMP-2 (Fig. 8, *b* and *c*).

## Discussion

This study demonstrated that BMP-2 not only inhibits the myogenic differentiation of C2C12 myoblasts but also converts their differentiation pathway into that of osteoblast lineage. The C2C12 cells differentiated into multinucleated myotubes when the concentration of FBS in the culture medium was reduced from 15 to 5%. Under the culture condition for myogenic differentiation, 300 ng/ml of BMP-2 almost completely inhibited the formation of myotubes which expressed Tn T and MHC. Under the same conditions, BMP-2 induced the expression of typical osteoblast phenotypes such as ALP activity, cAMP production in response to PTH, and osteocalcin production in C2C12 cells. Reduction of the FBS concentration in the medium was required for inducing myogenic differentiation in C2C12 cells, but not for osteoblast differentiation by BMP-2, since ALP-positive cells were similarly induced in the presence of 15% FBS (unpublished observation). These results clearly indicate that the C2C12 myoblasts treated with BMP-2 converted the differentiation pathway into that of osteoblast lineage. Similar effects of BMP-2 were recognized in primary muscle cells isolated from thigh muscles of 1-d-old mice as well. It is therefore likely that the in vitro culture system using C2C12 myoblasts provides a useful experimental model for investigating the mechanism of ectopic bone formation induced by implanting BMP into muscular tissues.

When C2C12 cells were incubated for 6 d with 300 ng/ml of BMP-2, over 90% of the cells expressed ALP activity without Tn T expression. However, when they were further maintained for 12 d in the absence of BMP-2, almost all of the colonies expressed Tn T but not ALP activity. These results indicate that C2C12 cells which had been induced to differentiate into osteoblastic lineage cells by BMP-2 are capable of differentiating into mature muscle cells again when BMP-2 is removed. Furthermore, a large number of ALP-positive colonies appeared when they were maintained with BMP-2. These results indicate that BMP-2 is necessary not only for inhibiting terminal differentiation of muscle cells and inducing osteoblast differentiation, but also for maintaining the differentiation pathway converted to the osteoblast lineage. It was also suggested that the conversion of the differentiation pathway caused by BMP-2 was not heritable. The relevance of these findings to development as well as the mechanism of regulation of muscle and osteoblast differentiation by BMP-2, however, need further investigation.

C2C12 is a subclone isolated from parental C2 myoblasts, which were established from the regenerating thigh muscle of an adult mouse (Blau et al., 1983; Yaffe and Saxel, 1977). The cells are thought to be derived from satellite cells, which are mononucleated cells lying along the muscle fibers in the normal tissue, and exist throughout the adult life as a potential source of new myoblasts. When muscular tissues receive injury or some dystrophic stimuli, the satellite cells divide and differentiate into myoblasts and fuse to form muscle fibers (for review see Stockdale, 1992). In our preliminary experiments using parental C2 cells, BMP-2 also inhibited myotube formation and induced ALP activity and osteocalcin production. These characteristics of C2C12 and C2 cells indicate that the satellite cells are potential progenitors differentiating into osteoblasts in response to BMP-2 during ectopic bone formation at muscular tissues.

This study showed that the expression of MyoD mRNA was reduced in C2C12 cells incubated with BMP-2 for more than 24 h. Furthermore, no expression of myogenin mRNA

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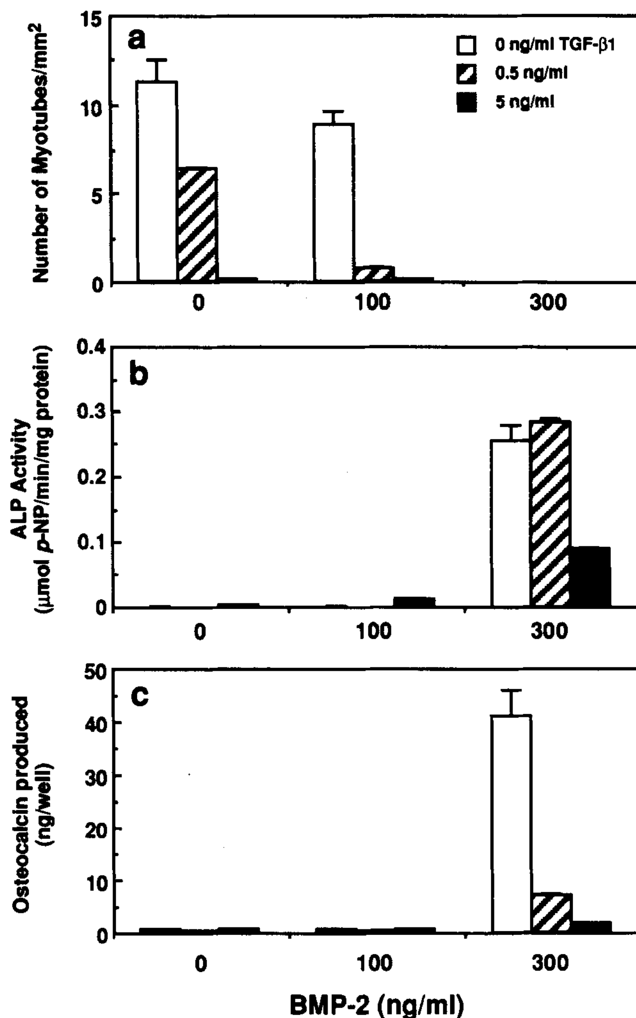


*Figure 8.* Effects of combination treatment of BMP-2 and TGF-$\beta$1 on myotube formation, ALP activity, and osteocalcin production in C2C12 cells. The cells were cultured for 6 d with or without BMP-2 (0, 100, 300 ng/ml) and/or TGF-$\beta$1 (0, 0.5, 5 ng/ml) as described in Materials and Methods. (*a*) The number of myotubes was counted after staining for Tn T as described in Materials and Methods. (*b*) The ALP activity of the cell lysate was measured using *p*-nitrophenylphosphate as a substrate. (*c*) The amounts of osteocalcin secreted into the culture media were determined by RIA. Data are means ± SEM of three wells.

was induced by BMP-2. Several lines of evidence have demonstrated that among various myogenic factors, myogenin plays an important role in terminal differentiation of committed myoblasts into mature muscle cells. Adding an antisense oligonucleotide for myogenin blocked the myogenic differentiation of myoblasts in vitro (Brunetti and Goldfine, 1990). Furthermore, skeletal muscles developed abnormally in the myogenin knockout mouse (Hasty et al., 1993; Nabeshima et al., 1993). Although mice lacking either the MyoD or the Myf-5 gene developed well with normal skeletal muscles, it has been suggested that MyoD and Myf-5 share a redundant function required for generating the muscle cell identity and activating myogenin (Braun et al., 1992; Rudnicki et al., 1992, 1993). We demonstrated that BMP-2 transiently stimulated the expression of MyoD mRNA within 3 h. In a time course experiment, the change of expression levels of MyoD was almost identical to that of Id-1 in the cells incubated with BMP-2. Jen et al. (1992) reported that overexpression of Id-1 in C2C12 cells inhibited myogenesis by binding to E12/E47 and preventing the formation of heterodimers between E12/E47 and myogenic HLH proteins. These suggest that the activity of MyoD transiently stimulated by BMP-2 is blocked by Id-1, which is concurrently induced by BMP-2. Consequently, C2C12 cells treated with BMP-2 might fail to express myogenin. The cooperative regulation in mRNA expression between the positive (myogenin) and the negative regulators (Id-1) induced by BMP-2 may play, at least in part, an important role in inhibiting myogenic differentiation.

Myogenic differentiation is also inhibited by other growth factors such as TGF-$\beta$ and basic FGF (bFGF) (Massagué et al., 1986; Spizz et al., 1986, 1987; Florini et al., 1986; Clegg et al., 1987; Vaidya et al., 1989; Brunetti and Goldfine, 1990; Brennan et al., 1991; Martin et al., 1992; Hardy et al., 1993). These growth factors inhibit the expression of MyoD and myogenin mRNAs (Vaidya et al., 1989; Brunetti and Goldfine, 1990). In addition, bFGF induces the phosphorylation by protein kinase C at a conserved site of the DNA-binding domain of myogenin, which causes a loss of DNA-binding activity of myogenin (Li et al., 1992). It has also been reported that TGF-$\beta$ inhibits the activity of myogenic factors through a mechanism independent of both DNA-binding and Id-1 induction (Brennan et al., 1991). Like these growth factors, BMP-2 completely inhibited expression of myogenin in C2C12 cells. Contrary to TGF-$\beta$, BMP-2 induced a transient stimulation of expression levels of Id-1 mRNA in C2C12 cells. When C2C12 cells were incubated with a suboptimal dose of BMP-2 and TGF-$\beta$1, myotube formation was inhibited more strikingly than the cells were treated with either factor. These results suggest that BMP-2 inhibits myogenic differentiation through a different mechanism from that of bFGF and TGF-$\beta$. BMP-2 may change the DNA binding and/or transcriptional activities of MyoD in C2C12 cells.

Recently, Murray et al. (1993) reported that partially purified BMP from demineralized bone inhibited myogenic differentiation in C2C12 and L6 myoblasts. They showed that the BMP fraction suppressed the mRNA expression of myogenic factors after incubation for 1–2 wk, but there was no induction of osteoblast and chondroblast differentiation in these cells (Murray et al., 1993). The cause of the failure to induce osteoblast differentiation in their study is unknown. It may be due to the purity of the BMP (recombinant vs native).

Since BMP-2 is a member of the TGF-$\beta$ superfamily, it was important to compare the effects of BMP-2 and TGF-$\beta$1 on osteoblast differentiation in C2C12 cells. BMP-2 induced C2C12 cells to differentiate into osteoblast-like cells, but TGF-$\beta$1 did not. These results are consistent with our previous experiments in which osteoblast differentiation of non-osteogenic C3H10T1/2 fibroblasts and osteoblast progenitor ROB-C26 cells was induced by BMP-2 but not by TGF-$\beta$1 (Katagiri et al., 1990*a*; Yamaguchi et al., 1991). Furthermore, TGF-$\beta$1 reduced ALP activity and osteocalcin production induced by BMP-2 in C2C12 cells. TGF-$\beta$1 even suppressed ALP activity and osteocalcin production in some osteoblast-like cells in vitro (Noda and Rodan, 1986; Elford



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et al., 1987; Rosen et al., 1988; Noda, 1989; Katagiri et al., 1990*b*). Although TGF-$\beta$ induced new bone formation when injected at the periosteal region in vivo (Noda and Camilliere, 1989; Joyce et al., 1990), it did not induce ectopic bone formation by implantation at a muscular site (Sampath et al., 1987). These results indicate that the effect of BMP-2 on osteoblast differentiation differ from that of TGF-$\beta$1. The differences in the effects between BMP-2 and TGF-$\beta$ may be caused by differences in their receptors and signal transduction systems.

Several investigators have reported that Id-1 and/or E12 mRNAs are expressed in osteoblast-like cells in vitro, and that Id-1 expression is downregulated during the maturation of osteoblasts as well as the differentiation of myoblasts (Murray et al., 1992; Kawaguchi et al., 1992). In addition, transfection of an Id-1 expression vector into osteoblast-like cells inhibited ALP activity (Murray et al., 1992). Since the Id-1 protein is a negative regulator for bHLH factors in myoblasts, these results suggest that some novel HLH proteins are involved in the regulatory mechanism of osteoblast differentiation. In the present study, however, treatment of C2C12 cells with BMP-2 markedly induced the expression of osteoblast phenotypes with a concomitant, but transient increase in Id-1 mRNA expression compared with the control culture. The stimulation of Id-1 mRNA by BMP-2 has also been reported in the osteoblastic cell line, MC3T3-E1, and in the pluripotent fibroblastic cell line, C3H101/2 (Ogata et al., 1993). In these cells, BMP-2 stimulated or induced the expression of osteoblast phenotypes (Katagiri et al., 1990*a*; Takuwa et al., 1991). These results suggest that osteoblast differentiation shares a common mechanism with muscle differentiation in some aspects, but also involves some other mechanisms from muscle differentiation. To the understanding of the conflicting regulatory mechanisms of osteoblast differentiation, identification of the osteoblast regulatory factors induced by BMP-2 is an important project in bone cell biology.

In conclusion, BMP-2 inhibits myotube formation, induces Id-1 expression, and suppresses myogenin expression. Also, BMP-2 converts the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage. These results indicate that BMP-2 is a potent regulator in determining the osteoblast differentiation of not only pluripotent immature mesenchymal cells, but also that of committed myoblasts. Osteoblast and muscle differentiations may be contradictory pathways to each other. BMP-2 appears to have capacities to withdraw the committed myoblasts from the differentiation pathway into myogenic lineage cells and to induce them to differentiate into the osteoblast lineage.

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