Journal of Cellular Biochemistry 90:1112–1127 (2003)

BMP Treatment of C3H10T1/2 Mesenchymal Stem Cells Induces Both Chondrogenesis and Osteogenesis

Colleen M. Shea,[1,2] Cory M. Edgar,[1] Thomas A. Einhorn,[1] and Louis C. Gerstenfeld[1]*

[1]Department of Orthopaedic Surgery, Orthopaedic Research Laboratory, Boston University Medical Center, Boston, Massachusetts

[2]Department of Periodontology, Harvard School of Dental Medicine, Boston, Massachusetts

Abstract The molecular mechanisms by which bone morphogenetic proteins (BMPs) promote skeletal cell differentiation were investigated in the murine mesenchymal stem cell line C3H10T1/2. Both BMP-7 and BMP-2 induced C3H10T1/2 cells to undergo a sequential pattern of chondrogenic followed by osteogenic differentiation that was dependent on both the concentration and the continuous presence of BMP in the growth media. Differentiation was determined by the expression of chondrogenesis and osteogenesis associated matrix genes. Subsequent experiments using BMP-7 demonstrated that withdrawal of BMP from the growth media led to a complete loss of skeletal cell differentiation accompanied by adipogenic differentiation of these cells. Continuous treatment with BMP-7 increased the expression of Sox9, Msx 2, and c-fos during the periods of chondrogenic differentiation after which point their expression decreased. In contrast, Dlx 5 expression was induced by BMP-7 treatment and remained elevated throughout the time-course of skeletal cell differentiation. Runx2/Cbfa1 was not detected by ribonuclease protection assay (RPA) and did not appear to be induced by BMP-7. The sequential nature of differentiation of chondrocytic and osteoblastic cells and the necessity for continuous BMP treatment to maintain skeletal cell differentiation suggests that the maintenance of selective differentiation of the two skeletal cell lineages might be dependent on BMP-7-regulated expression of other morphogenetic factors. An examination of the expression of Wnt, transforming growth factor-β (TGF-β), and the hedgehog family of morphogens showed that Wnt 5b, Wnt 11, BMP-4, growth and differentiation factor-1 (GDF-1), Sonic hedgehog (Shh), and Indian hedgehog (Ihh) were endogenously expressed by C3H10T1/2 cells. Wnt 11, BMP-4, and GDF-1 expression were inhibited by BMP-7 treatment in a dose-dependent manner while Wnt 5b and Shh were selectively induced by BMP-7 during the period of chondrogenic differentiation. Ihh expression also showed induction by BMP-7 treatment, however, the period of maximal expression was during the later time-points, corresponding to osteogenic differentiation. An interesting phenomenon was that BMP-7 activity could be further enhanced twofold by growing the cells in a more nutrient-rich media. In summary, the murine mesenchymal stem cell line C3H10T1/2 was induced to follow an endochondral sequence of chondrogenic and osteogenic differentiation dependent on both dose and continual presence of BMP-7 and enhanced by a nutrient-rich media. Our preliminary results suggest that the induction of osteogenesis is dependent on the secondary regulation of factors that control osteogenesis through an autocrine mechanism. J. Cell. Biochem. 90: 1112–1127, 2003.

Key words: BMP; mesenchymal stem cells; chondrocyte; osteoblast; Wtns

The skeletal system is derived from common mesenchymal progenitors that can differentiate into osteoblasts, chondroblasts, myoblasts, adipocytes, and a variety of fibroblast cell types. Cells cultured from multiple species of calvaria and bone marrow stroma have been shown to have the potential to differentiate into various phenotypes: bone, cartilage, muscle, and fat [Friedenstein et al., 1987; Grigoriadis et al., 1988; Gerstenfeld et al., 1989]. Similarly, several mesenchymal stem cell lines have been induced to differentiate along these lineages following treatment with different morphogenetic factors or pharmacological agents

Grant sponsor: NIAMS (to L.C.G.); Grant number: AR 47045; Grant sponsor: Department of Orthopaedic Surgery, Boston University School of Medicine (Institutional support); Grant sponsor: NIDCR (Colleen M. Shea is a recipient of a K16 Dentist Scientist Award); Grant number: DE00275.

*Correspondence to: Louis C. Gerstenfeld, Department of Orthopaedic Surgery, Orthopaedic Research Laboratory, Boston University Medical Center, 715 Albany Street, R-205, Boston, MA 02118. E-mail: lgersten@bu.edu

Received 11 September 2003; Accepted 12 September 2003

DOI 10.1002/jcb.10734

[Yamaguchi et al., 1991; Umezawa et al., 1992; Katagiri et al., 1994; Poliard et al., 1995; Asahina et al., 1996; Fujii et al., 1999]. One such mesenchymal stem cell line, C3H10T1/2, was established from an early mouse embryo that was cloned and selected due to its high degree of sensitivity to post-confluence inhibition [Reznikoff et al., 1973]. C3H10T1/2 cells, along with several myogenic and bone marrow stromal cell lines, have been used extensively as in vitro models to examine mesenchymal differentiation into various phenotypic lineages by different inductive mediators [Beresford et al., 1992; Ahrens et al., 1993; Katagiri et al., 1994; Denker et al., 1995; Atkinson et al., 1997; Denker et al., 1999]. For example, depending on the concentration used, 5-azacytidine stimulated C3H10T1/2 to differentiate into myotubes, adipocytes, or chondroblasts [Taylor and Jones, 1979]. Many studies have also shown that multiple isotypes of BMP, including BMP-2 and BMP-7, induce these cells to undergo osteogenic differentiation [Katagiri et al., 1990; Wang et al., 1993; Puleo, 1997; Ducy et al., 1997].

Cellular differentiation along the cartilage or bone lineages is a multistep process starting from the undetermined mesenchymal stem cell. The inductive influences and mechanisms of action involved in mesenchymal stem cell differentiation have been examined intensely during the past decade. An important determinant of differentiation of stem cells in vivo is the autocrine influence of the cells within a developing tissue. These neighboring cells may alter the differentiation of uncommitted cells through the production of diffusible factors. Additionally, local regulation may result from interactions between cells, the extracellular matrix, or contact with the neighboring cells themselves [Gerstenfeld et al., 2002]. There is also an increasing understanding that the differentiation of stem cells is affected by their nutrient and oxygen environments [Bruder et al., 1994; Csete et al., 2001]. Therefore, in vitro studies mimicking multiple aspects of the in vivo environment will lead to a better understanding of how the process of differentiation progresses.

Bone morphogenetic proteins (BMPs) have been the subject of intensive study because of their known biological activities in the promotion of mesenchymal stem cell differentiation into bone [Katagiri et al., 1990; Wang et al., 1993; Puleo, 1997]. Initial studies of BMP actions on stem cell differentiation focused on phenotypic characteristics of skeletal cells, such as alkaline phosphatase activity, extracellular matrix mineralization, and the expression of various extracellular matrix proteins such as collagen type II or osteocalcin, the terminal phenotypic markers of the differentiated state of these cells [Asahina et al., 1993]. More recent studies have focused on the signal transduction mechanisms by which BMPs mediate their biological activities into cells as well as the relationship of BMPs to the induction of specific transcription factors, such as Sox9, Runx2, Dlx 5, and c-fos, that are known to determine the commitment of the mesenchymal cells into the chondrogenic or osteogenic lineages [Ohta et al., 1992; Ducy et al., 1997; Zehentner et al., 1999; Healy et al., 1999; Miyama et al., 1999; Zeng et al., 2002]. To date, however, the sequence of expression of these various phenotypic properties used to define the commitment and differentiation of skeletal cells after treatment with BMP has produced varying temporal profiles and remains incomplete.

In order to elucidate the temporal pattern of the induction of key transcriptional events relative to end-point expression of differentiated function during the differentiation of uncommitted cells into skeletal lineages, the mesenchymal stem cell line C3H10T1/2 was treated with BMP-7 or BMP-2. The mRNA expression of specific transcription factors that are associated with skeletal cell differentiation and the end stage expression of genes regulated by these factors were examined. We then examined the time frame of BMP-7 addition in relationship to the maintenance and continued differentiation of these mesenchymal stem cells. In conjunction with these studies, the pattern of BMP-7 induced expression of the four families of known morphogenetic proteins (BMPs, GDFs, Wnts, and hedgehog proteins) was determined to establish how these factors are expressed in response to BMP-7 and identify those factors that are providing co-regulatory autocrine signals during BMP-induced differentiation. Finally, we examined role of the local nutrient environment as a determinant or co-modifier of skeletogenic differentiation.

MATERIALS AND METHODS

Cell Cultures

C3H10T1/2 mesenchymal stem cells were obtained from the American Type Culture

Collection (Rockville, MD). C3H10T1/2 cells were plated at 2×10^5 per 100 mm diameter culture dish, maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD), and grown at 37°C in a 5% CO_2 humidified incubator. After 48 h, designated "day 0," the cells were treated with BMP-7 or BMP-2 (generously provided by Stryker Biotech, Hopkinton, MA through a Materials Transfer Agreement). BMP-7 was added at 0, 80, or 250 ng/ml, or BMP-2 was added at 200 ng/ml, and cells were fed every 3 days with fresh media with or without BMP-7 or BMP-2. Total RNA was collected at days 0, 1, 2, 4, 8, 12, and 16 after the addition of BMP. For the withdrawal study, BMP-7 was added at 250 ng/ml for either the first 4 or 8 days and then fed every 3 days with DMEM plus supplements without BMP. RNA was collected at days 4, 8, 12, and 16. In studies of the effect of the nutrient environment, cells were maintained in either DMEM plus supplements as above with or without 50 μg/ml ascorbic acid or in the nutrient-rich growth media BGJb (Fitton-Jackson Modification) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 12.5 μg/ml ascorbic acid. RNA from days 4, 8, and 12 were collected and compared.

Alkaline Phosphatase, Growth, and Mineral Accumulation

C3H10T1/2 cells were plated into 12-well tissue culture dishes at a density of 10^4/well and grown as above. Alkaline phosphatase (APase) activity and cell number were determined sequentially from individual wells. APase activity was measured by *p*-nitro-phenol phosphate conversion and relative cell number was determined by the uptake of crystal violet dye [Kostenuik et al., 1997]. APase activities were normalized to the relative cell numbers. Mineral content was then determined by staining with 1 ml 2% alizarin red dye for 10 min, rinsing, and eluting with 0.5 N HCl/5% SDS. The eluted stain was then measured spectrophotometrically at 415 nm and total mineral content was determined.

RNA Isolation From Cell Cultures

Total RNA was extracted by the acid guanidine thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987] using 1 ml of TRIZOL Reagent (Gibco-BRL) according to the manufacturer's instructions. RNA was then quantified by spectrophotometry based on the OD_{260} nm reading and sample integrity was monitored by visualization of ribosomal RNAs with ethidium bromide after denaturing gel electrophoresis.

Ribonuclease Protection Assays (RPAs)

Template sets for the RPAs were purchased from Pharmingen Corp. (San Diego, CA). Template sets contained DNA templates which were used for the T7 RNA polymerase-directed synthesis of high specific-activity, ^{32}P-labeled, antisense RNA probes that were hybridized with target mRNAs specific for the genes included in the template set. Commercially prepared template sets included those for matrix proteins, including collagen types I, II, and X, bone sialoprotein, osteopontin, and osteocalcin; transcription factors, including Sox9, Dlx 5, Msx 2, c-jun, c-fos, Fra 1, Fra 2, and Runx2; BMPs 1, 2, 3, 3B, 4, 5, 6, 7, 8A, and 8B; GDFs 1, 3, 5, 6, 8, and 9; and Wnts 1, 2, 3, 3a, 4, 5b, 6, 7a, 7b, 8d, 10a, 10b, 11, 13, and 15. All template sets included the L32 probe as an internal standard. RNase protection assays were performed using a RiboQuant™ Ribonuclease Protection Kit (PharMingen) following the manufacturer's instructions. Analysis of the protected product was resolved on a denaturing 6% polyacrylamide gel according to their size [Gerstenfeld et al., 1989]. The identity and quantity of each mRNA species in the original RNA sample was then determined based on the signal intensities given by the appropriately sized, protected probe fragment bands. RNase protected bands were quantified using an image analyzer (Alpha Innotech Image Analysis System, Alpha Innotech, San Leandro, CA). The housekeeping gene *L32* was used for the normalization of samples and allowed correction of technique errors. Each RPA was performed a minimum of two times.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Aggrecan, adipsin, Indian hedgehog (Ihh), and sonic hedgehog (Shh) expression were assayed by RT-PCR. The primer sets used for aggrecan were reverse 5′-TGACAACTTCT-TTGCCACCG-3′ and forward 5′-CTCAGCAT-CCTGGGCACATTA-3′, adipsin reverse 5′-CC-TCAGGATGTCATGTTACCA-3′ and forward

5′-AATCTCAGGAGCTTCTGGGAG-3′, Ihh reverse 5′-GCCTGCAGGGAAGGTCATGT-3′ and forward 5′-GTCTCTTGCTAGAAGAGAGC-3′, and Shh reverse 5′-ACTGCTCGAC-CCTCA-TAGTG-3′ and forward GGCAGATATGAAGG-GAAGAT-3′. For semi-quantitative RT-PCR, initial reverse transcriptase reactions were carried out followed by sequence amplification for five cycles. The resultant cDNAs were then serially diluted three times and each of the dilutions was PCR amplified for an additional 24 cycles. Products were resolved on a 1.4% agarose gel stained with Gel Star (FMC BioProducts, Rockland, ME).

RESULTS

Temporal Expression Pattern of Chondrogenic and Osteogenic Marker Genes in Response to BMP Treatment

The expression of specific extracellular matrix protein genes that are associated with the differentiation of the chondrogenic and osteogenic lineages were examined over a 16-day time-course after the addition of 0, 80, or 250 ng/ml BMP-7 to C3H10T1/2 cells (Fig. 1A,B). Two genes associated with the progression of chondrogenic differentiation (collagen types II and X) and four genes associated with osteogenic differentiation (collagen type I, osteopontin [OPN], osteocalcin [OC], and bone sialoprotein [BSP]) were examined. Type II collagen was present at the onset of treatment; however, its expression was only maintained if the cells were treated with exogenous BMP-7. In the high-dose BMP-7 treated cells, type II collagen expression increased dramatically and reached peak expression by 4 days after the initiation of BMP-7 treatment and was maintained at this high level until day 8, after which it sharply declined. The low-dose BMP-7 treated cells maintained their type II collagen expression longer than the untreated cells but never obtained the same maximal level of expression seen in the 250 ng/ml dose group. Type X collagen expression was also present in the BMP-7 treated groups with a similar profile of induction as that for type II collagen with maximal levels at day 4 in both the 80 ng/ml and 250 ng/ml BMP-7-treated cells. Once again, the highest and more persistent levels were present in the 250 ng/ml BMP-7 dose group.

The bone-related matrix genes are also depicted in Figure 1. Osteocalcin expression was only present in the BMP-7 treated groups on and after 8 days of treatment. While the lower dose of BMP-7 also induced osteocalcin expression at day 8, its relative expression level was half of that seen in the high dose group. Furthermore, these cells were unable to maintain osteocalcin expression at later times in culture. In contrast, type I collagen was expressed at very high levels in both treated and untreated cultures from the beginning of the experiment. It is interesting to note that as the osteocalcin levels increased, there was a reciprocal decrease in type I collagen expression in the BMP-7 treated cultures. High levels of osteopontin expression were seen as well in untreated C3H10T1/2 cells with a peak at day 1 for all the culture groups. This gene, however, showed a biphasic expression pattern of diminished to near absent expression at day 4 in the treated groups during the period of maximal cartilage gene expression. A second peak of osteopontin expression was present at later times with the maximal levels of expression in the cultures treated with BMP-7. Interestingly, bone sialoprotein was not detected in any of the cultures.

A similar time-course experiment was carried out with 200 ng/ml BMP-2; these results are shown in Figure 1C. BMP-2 also stimulated the induction of both chondrogenic- and osteogenic-associated genes although chondrogenic markers were induced later and remained elevated throughout the period of osteocalcin expression.

Temporal Transcription Factor Expression Patterns Following BMP-7 Treatment in Culture

In order to assess the transcriptional mechanisms by which BMP-7 induced skeletal cell differentiation, we next assessed the mRNA expression of various transcription factors that have been associated with skeletal cell differentiation (Fig. 2A,B). Sox9, a transcription factor associated with chondrogenesis, showed a similar pattern of expression to that of type II collagen. In the two BMP-7-treated groups, Sox9 expression increased in a dose-dependent manner with the highest levels at days 4 and 8, coinciding with maximal type II collagen expression. Dlx 5 expression was found to increase at day 4 of the culture period in the high dose BMP-7 group. A lesser increase was noted also at day 4 in the lower dose group, whereas no expression was noted in the untreated cells. Msx 2 expression was found to be high initially in all groups, with an increase in expression in the

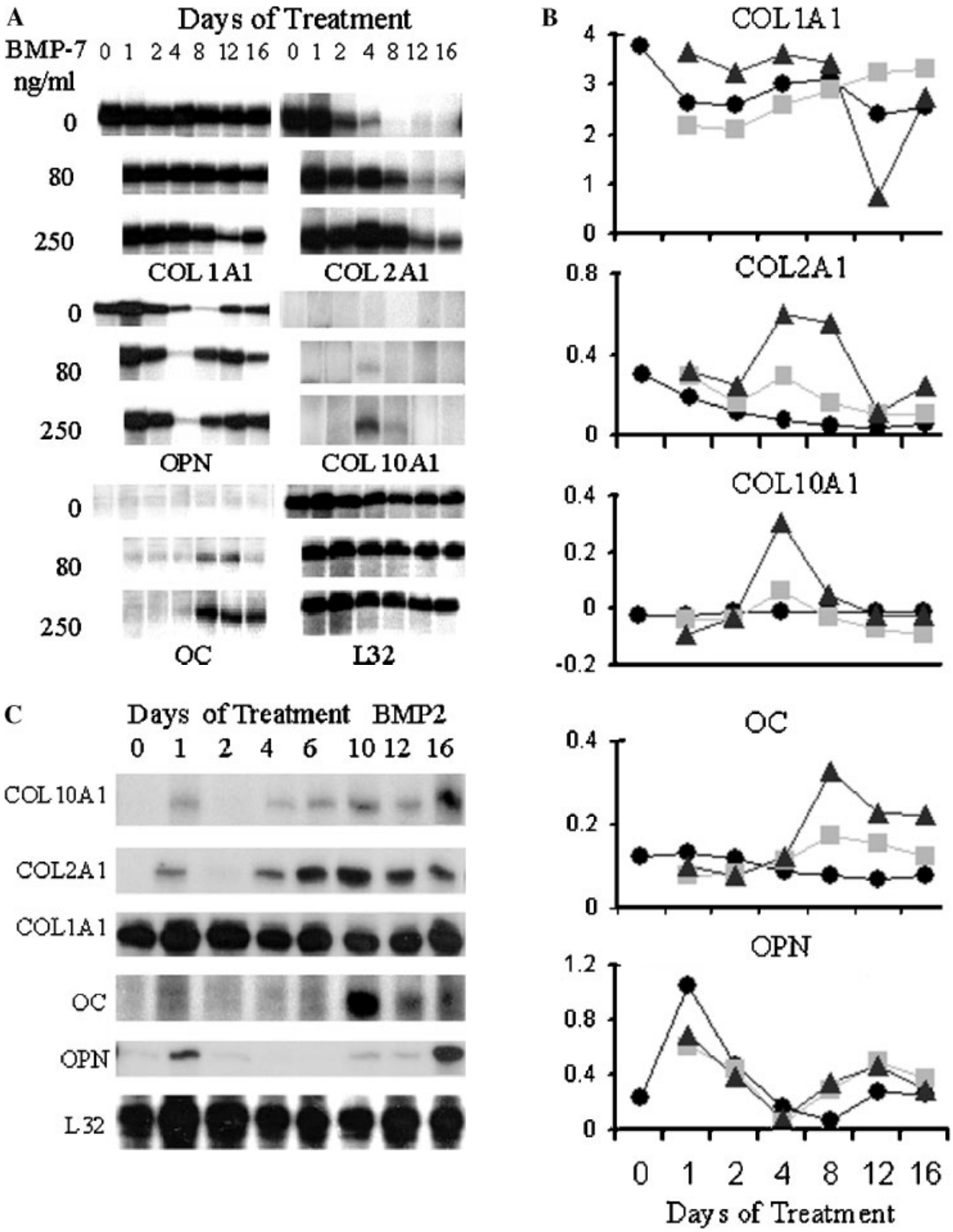

Fig. 1. BMP-7 induces chondrogenesis and osteogenesis in C3H10T1/2 cells in a dose-dependent manner. C3H10T1/2 cells were grown in DMEM with 10% FBS and treated with BMP-7 at 0, 80, or 250 ng/ml over a 16 day period. **Panel A**: Expression of the cartilage-related genes, collagen types II (COL2A1) and X (COL10A1), and the bone-related genes, osteocalcin (OC), type I collagen (COL1A1), and osteopontin (OPN), were determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. **Panel B**: Graphic representation of the relative densities (Y-axis) from the RPAs in Panel A as compared to the housekeeping gene, *L32*. (●) No BMP-7, (■) BMP-7 80 ng/ml, (▲) BMP-7 250 ng/ml. **Panel C**: RPA expression of cartilage- and bone-related genes in C3H10T1/2 cells treated with 200 ng/ml BMP-2 over a 16 day period.

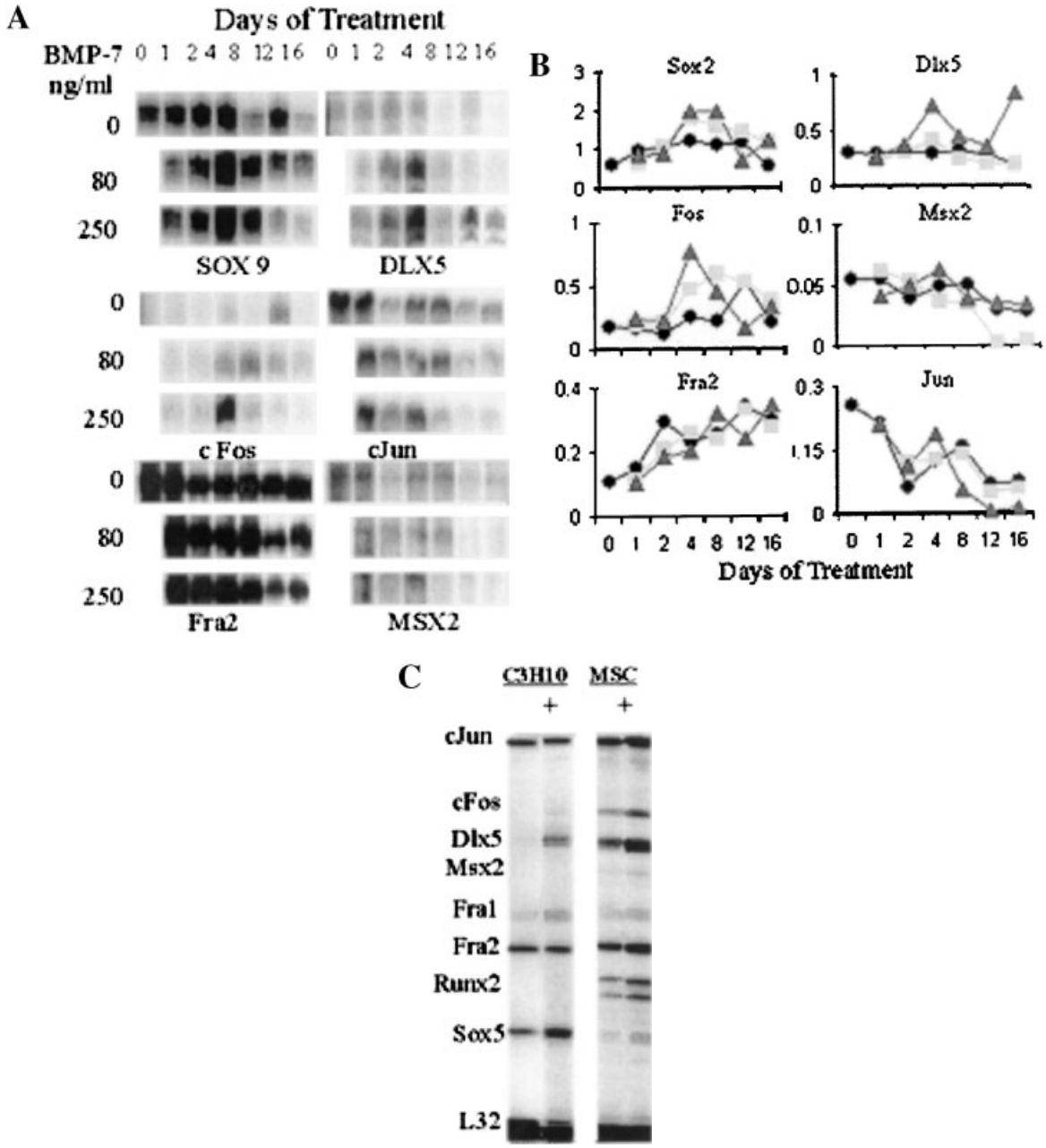

Fig. 2. Effect of BMP-7 treatment on cartilage- and bone-related transcription factors. C3H10T1/2 cells were grown in DMEM with 10% FBS and treated with BMP-7 at 0, 80, or 250 ng/ml over a 16 day period. **Panel A**: Expression of the transcription factor genes, Sox9, Dlx 5, Msx 2, c-jun, c-fos, and Fra 2, was determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. **Panel B**: Graphic representation of the relative densities (Y-axis) from the RPAs in Panel A as compared to the housekeeping gene, *L32*. (●) No BMP-7, (■) BMP-7 80 ng/ml, (▲) BMP-7 250 ng/ml. **Panel C**: Comparison of transcription factor expression of C3H10T1/2 (C3H) versus marrow stromal cells (MSC). Cells were grown in DMEM with 10% FBS and treated with no BMP-7 (−) or BMP-7 at 250 ng/ml (+). Day 16 samples are shown. Transcription factor gene expression was determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown.

high dose group at day 4, followed by a drop in expression in all groups at the later time-points. Numerous members of the AP 1-related transcription factor family have been associated with skeletal cell differentiation and were also examined in this model system (Fig. 2). C-jun showed decreased expression over time in all groups of cultured cells; however, the presence of BMP-7 increased the rate of decrease of this factor's expression. C-fos exhibited almost no

expression in the control cultures but showed a very strong peak of expression in the BMP-7 treated groups during the period of maximal chondrogenic expression. In contrast, Fra 2 expression was initially high in all groups of cultured cells with a slight increase in expression over time in all groups. Fra 1 was also expressed initially by all groups but decreased to near absent levels by day 2, with no differences between groups (data not shown). Interestingly, Runx2/Cbfa1 was not detected during the entire culture period. By contrast, marrow stromal cells grown under the same conditions expressed Runx2/Cbfa1 and expressed higher levels in the presence of BMP-7 (Fig. 2C). Comparison of the time-course of induction of these same transcription factors by BMP-2 showed similar results to BMP-7 except that Sox9 showed a higher and more prolonged level of expression throughout the time-course of treatment (data not shown).

Continuous BMP-7 Treatment Is Needed to Maintain Skeletal Cell Differentiation

The duration of BMP-7 exposure that was needed to maintain differentiation was next assessed in these cultures. Cell cultures were either maintained continuously in the presence of 250 ng/ml of BMP-7 or withdrawn from BMP-7 treatment at either day 4 or day 8 and maintained in control media for the remainder of the study. RNA was then collected at days 4, 8, 12, and 16. With continuous BMP-7 treatment, C3H10T1/2 displayed a pattern of extracellular matrix gene expression similar to that seen during the time-course studies, with chondrogenesis always preceding osteogenesis (Fig. 3A). High levels of types II and X collagen were expressed on days 4 and 8, with decreasing levels thereafter, followed by increasing amounts of osteocalcin RNA on days 8, 12, and 16. However, when BMP-7 was removed from the culture medium at either day 4 or day 8, the C3H10T1/2 cells were unable to maintain either the chondrogenic or osteoblastic phenotype. Minimal levels of type II collagen were expressed, and neither type X collagen nor osteocalcin expression were detected in the absence of continuous BMP-7 treatment. Chondrogenic differentiation was independently assessed by measuring the expression levels of the large proteoglycan aggrecan mRNA by RT-PCR (Fig. 3B). Consistent with the observation of the type II collagen mRNA levels, when BMP-7 was withdrawn at either day 4 or day 8, aggrecan mRNA failed to be maintained. It is interesting to note, however, that while both Sox9 and type II collagen expression were initially observed in the untreated C3H10T1/2 cultures, aggrecan was not, suggesting that Sox9 and collagen type II may be expressed in the stem cells before full commitment to the chondrogenic lineage, while aggrecan is only seen in fully committed chondrocytes.

Within 48 h of BMP-7 withdrawal at either 4 or 8 days, numerous cells containing multiple refractile vacuoles with an adipocyte-like morphology were seen in the cultures (Fig. 3C). The adipogenic phenotype of these cells was confirmed by assaying for the expression of adipsin mRNA (Fig. 3D). As can be seen from these data, adipsin was present at early time-points but as the cells continued to grow in culture, its expression decreased. The transient exposure of these cells to BMP-7 followed by its removal led to very high levels of adipogenic differentiation and the level of adipocyte formation increased as the transient period of BMP-7 exposure increased. Thus, in order to maintain their commitment to either the chondrocyte or osteoblast lineage, C3H10T1/2 cells needed to be maintained in BMP-7 for greater than 8 days, and the removal of BMP-7 after a 4 or 8 day exposure led to the default induction of adipogenesis.

Analysis of BMP-7 Regulation of Other Morphogenetic Factors

The sequential nature of skeletal cell differentiation, the necessity for continuous BMP-7 treatment to maintain skeletal cell differentiation, and the extended period needed for BMP-7 to induce osteogenic differentiation (8 days or more), suggested that induction and maintenance of skeletal cell differentiation might be dependent on BMP-7 regulated expression of other morphogenetic factors over the time-course of the treatment of these cells. We next examined the expression of a number of families of known morphogenetic proteins that are associated with skeletal lineage progression, including BMPs 1–8, GDFs 1, 3, 5, 6, 8, and 9, Wnts 1–15, and two members of the hedgehog family, Ihh and Shh.

An analysis of the expression of Wnts 1–15 showed 1, 2, 3, 3a, 4, 6, 7a, 7b, 8d, 10a, 10b, 13, and 15 were not expressed or regulated during the BMP-7 induction of skeletogenic

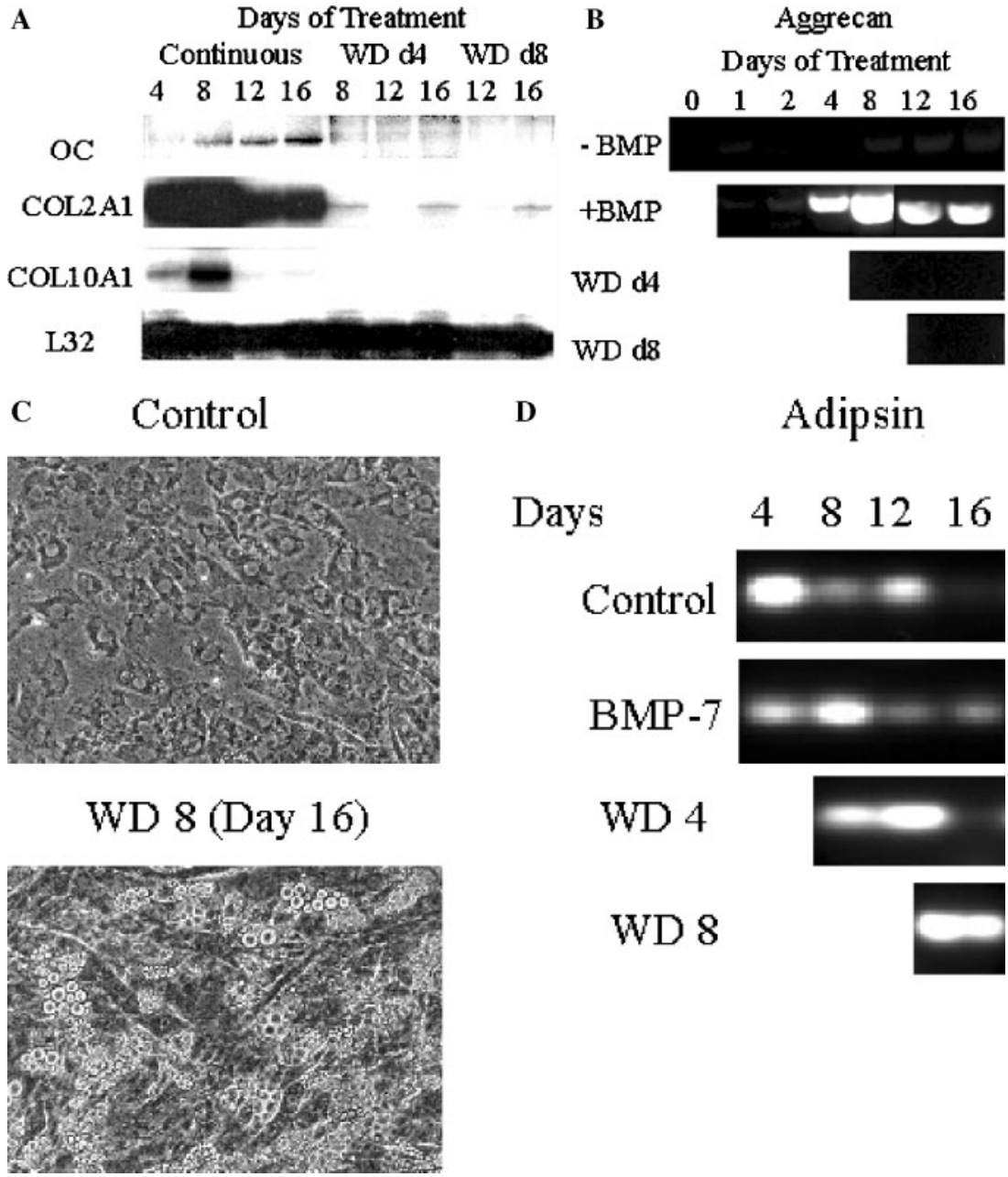

Fig. 3. Effect of BMP-7 withdrawal on cellular differentiation. C3H10T1/2 cells were grown in DMEM with 10% FBS over a 16 day period. BMP-7 (250 ng/ml) treatment was continuous or withdrawn from the cultures at days 4 (WD d4) or 8 (WD d8) and then fed with media until RNA was collected. **Panel A**: Expression of collagen types II (COL2A1) and X (COL10A1) and osteocalcin (OC) were determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. **Panel B**: Expression of the cartilage-related gene, aggrecan, was determined by RT-PCR analysis. Amplified RNA samples were resolved on a 1.4% agarose gel and stained with Gel Star. **Panel C**: Photomicrographs from the phase-contrast analysis of the effects of withdrawal of BMP-7 from C3H10T1/2 cells (magnification × 200). Day 16 cells are shown. **Panel D**: Expression of the fat-related gene, adipsin, was determined by RT-PCR analysis. Amplified RNA samples were resolved on a 1.4% agarose gel and stained with Gel Star.

differentiation of this cell line (Table I). Wnt 11 showed a unique pattern of expression within these cultures (Fig. 4A). Over time in culture in the absence of BMP-7 treatment, Wnt 11 expression increased approximately eightfold over its baseline levels. In contrast, BMP-7 treatment led to a dose-dependent inhibition of Wnt 11 expression with the highest doses of BMP-7 resulting in complete suppression of Wnt 11. In contrast, Wnt 5b was induced by BMP-7

TABLE I. Factors not Expressed or Regulated by BMP-7 Treatment of C3H10T1/2 Cells

Wnts		BMPs		GDFs
1	7a	2	8a	3
2	7b	3	8b	5
3	8d	3b		6
3a	10a	5		8
4	13	6		9
6	15	7		

treatment over time in these same cultures and showed the greatest levels of expression during the chondrogenic period of skeletal cell differentiation.

We next examined the expression of two classes of morphogenetic factors that are part of the larger TGF-β superfamily, BMPs and GDFs. Of the eight BMPs and six GDFs that were examined, only BMP-4 and GDF 1 were expressed by these cells in either BMP-7 treated or untreated conditions. BMP-4 was expressed

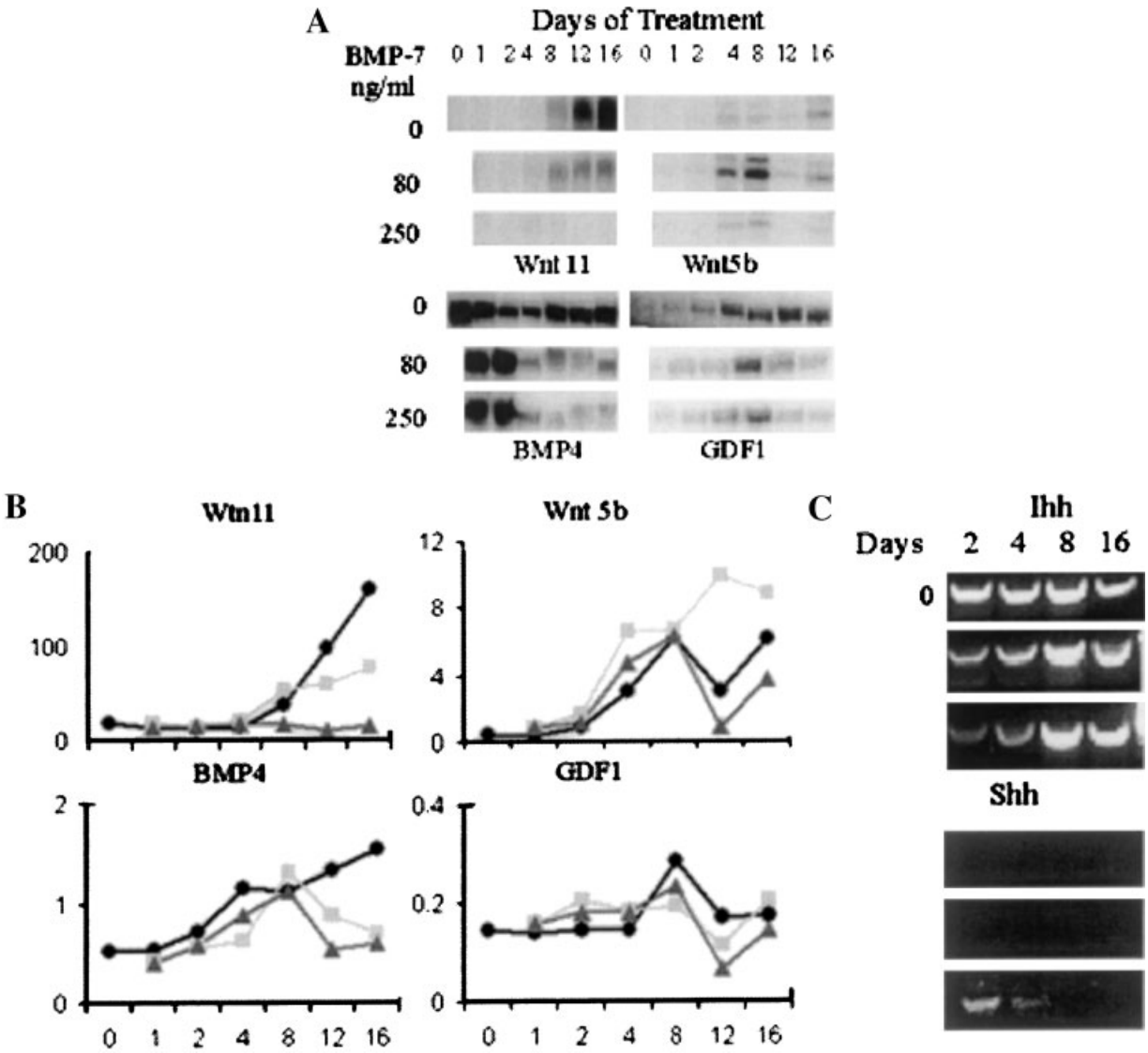

Fig. 4. Effect of BMP-7 treatment on the endogenous expression of Wnts, BMPs, GDFs, and Hedgehog proteins. C3H10T1/2 cells were grown in DMEM with 10% FBS and treated with BMP-7 at 0, 80, or 250 ng/ml over a 16 day period. **Panel A**: Expression of Wnts, BMPs, and GDFs were determined by quantitative RPA analysis. Of those examined, only Wnts 11 and 5b, BMP-4, and GDF 1 were regulated. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. **Panel B**: Graphic representation of the relative densities (Y-axis) from the RPAs in Panel A as compared to the housekeeping gene, *L32*. (●) No BMP-7, (■) BMP-7 80 ng/ml, (▲) BMP-7 250 ng/ml. **Panel C**: Expression of Ihh and Shh were determined by RT-PCR analysis. Amplified RNA samples were resolved on a 1.4% agarose gel and stained with Gel Star.

at very high levels at the time the cultures were initiated, and in the absence of BMP-7, its level of expression did not change throughout the 16 days of culture. In contrast, BMP-4 showed a dose-related suppression in expression at the later time-points in the treated groups (Fig. 4). Unlike BMP-4, GDF 1 expression was not initially seen but its expression increased over time in all groups until day 8, with no difference between groups. Subsequently, its expression continued to increase throughout the time-course in the untreated cultures, while in the treated groups its expression decreased in a BMP-7 dose-dependent manner.

The final group of morphogens that were examined in these studies included two members of the hedgehog family, Indian and Sonic hedgehog (Fig. 4C). While Ihh was present throughout the experimental period, it was not regulated by BMP-7 treatment. Sonic hedgehog, however, was induced in the early stages of treatment with the higher dose of BMP-7 and showed a very restricted pattern of expression during the period of active chondrogenesis.

Role of Growth Regulation and BMP-7 Function

The last component of these studies focused on the role of the nutrient environment on the induction of chondrogenesis and osteogenesis by BMP-7. In these studies, the cells were grown in DMEM (nutrient-poor conditions) or BGJb (nutrient-rich conditions). The addition of ascorbic acid to DMEM, induced minimal differences compared with RNA from cells grown in DMEM only (data not shown). However, this response was synergistically enhanced when the cells were grown in the nutrient-enriched media BGJb (Fig. 5). This induction was over 60% greater in the BGJb media than in the DMEM media at all three time-points for type II collagen and over 700% greater for type X collagen at day 4. Osteocalcin was induced in BMP-7 treated cells grown in DMEM in a similar pattern as in the previous study, with maximal induction at day 8 and a slight decrease thereafter. In the BGJb-grown cells, the level of osteocalcin expression was similar to the DMEM-grown cells at day 8, but at the later times when the level of osteocalcin expression was maximal, a 100% increase was observed in the cultures maintained in the nutrient-rich BGJb media. Type I collagen expression was also greater in the BGJb treated

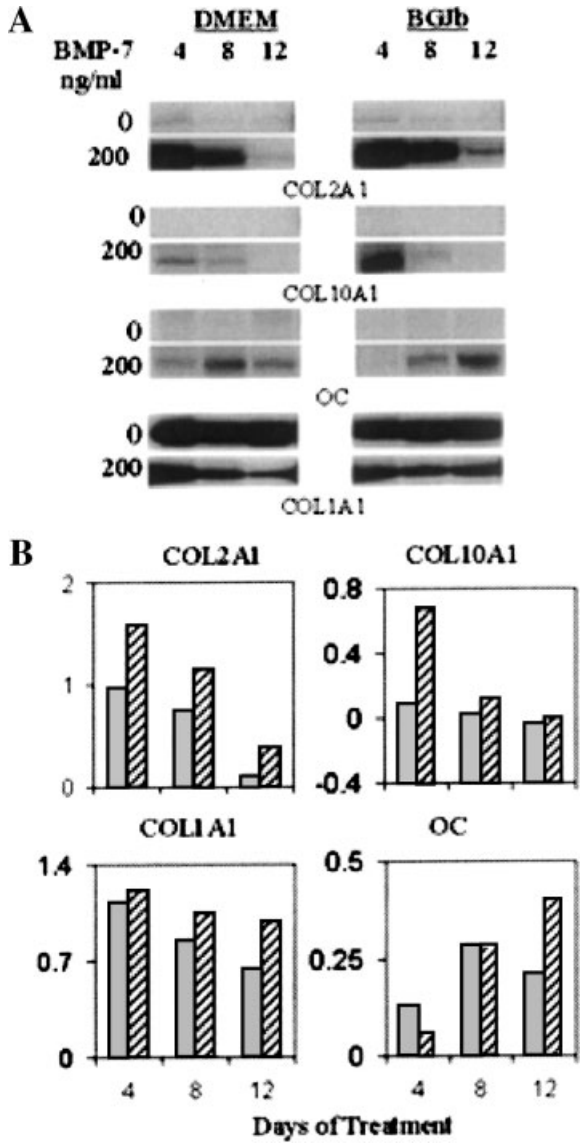

Fig. 5. Effect of nutrient-rich media on BMP-7 promotion of skeletal cell differentiation. C3H10T1/2 cells were grown in DMEM or BGJb with 10% FBS and treated with BMP-7 at 0 or 250 ng/ml over a 12 day period. **Panel A**: Expression of matrix genes [collagen types II (*COL2A1*) and X (*COL10A1*), osteocalcin (OC), and collagen type I (*COL1A1*)] and transcription factor genes (*Sox9*, *Dlx 5*, c-*jun*, and c-*fos*) were determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. **Panel B**: Graphical representation of the effect of BGJb growth media on transcription factor expression and, matrix gene expression. C3H10T1/2 cells were grown in DMEM or BGJb with 10% FBS and treated with BMP-7 at 0 or 250 ng/ml over a 12 day period. Expression of the mRNA depicted in panel A were determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved on a 6% sequencing gel are shown. Graphic representation of the relative densities of only the BMP-7 treated samples from the RPA as compared to the housekeeping gene, *L32*. Solid bars represent DMEM + BMP-treated samples. Slashed bars represent BGJb + BMP-treated samples.

cells for all time-points. Thus, the nutrient-rich growth media BGJb appeared to enhance the mechanisms of both chondrogenesis and osteogenesis in C3H10T1/2 cells when treated with BMP-7, and this effect was not due to ascorbic acid.

The pattern of bone-related transcription factor expression was examined concurrently with matrix genes in the two different growth medias (Fig. 5). In the BMP-7-treated cells grown in BGJb, Sox9 was expressed in greater quantities as compared to cells grown in DMEM at days 4, 8, and 12. The expression decreased over time, following the same temporal pattern of type II collagen expression. Dlx 5 was expressed in the greatest amount at day 4 in the BMP-7 treated cells grown in BGJb with a decrease in expression at days 8 and 12, following a similar pattern as the DMEM-treated cells but with a higher peak expression. The expression of c-jun exhibited an interesting result. Although high levels of c-jun were present in untreated cells grown in both DMEM and BGJb, much lower levels of expression were detected in BMP-7 treated samples. This level of c-jun expression decreased over time but was always approximately twice as high in the BGJb grown cells. C-fos showed expression at day 4 and then dropped to negligible at days 8 and 12 in the BMP-7 treated cells grown in either DMEM or BGJb. Fra 2 was strongly expressed in both DMEM and BGJb cells grown with or without BMP-7 and no major differences in expression were noted (data not shown).

Finally, we assessed the relative levels of growth, alkaline phosphatase activity, and mineralization under these different growth conditions (Fig. 6). The stimulatory effect of BMP-7 was found to result in increased growth of C3H10T1/2 cells over time as compared to

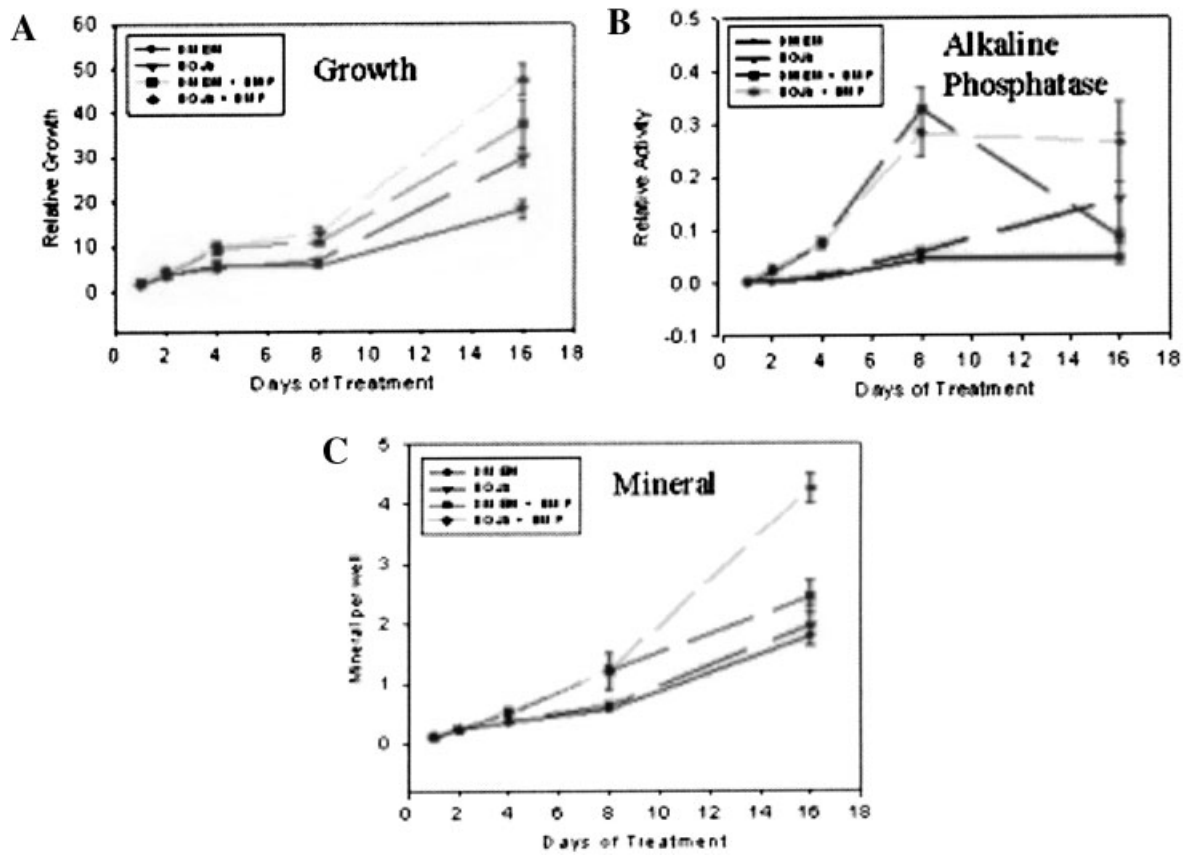

Fig. 6. Effect of BMP-7 treatment on growth, alkaline phosphatase, and mineral content in C3H10T1/2 cells grown in DMEM or BGJb. C3H10T1/2 cells were grown in DMEM or BGJb with 10% FBS and treated with BMP-7 at 0 or 250 ng/ml over a 16 day period. **Panel A**: Growth was determined by crystal violet staining. **Panel B**: Alkaline phosphatase was determined by substrate activity. **Panel C**: Mineral content was determined by alizarin red staining. All measurements were determined by spectrophotometric analysis. Error bars represent standard deviation of 6-wells. (◆) DMEM only, (■) BGJb only, (▲) DMEM + 250 ng/ml BMP-7, (●) BGJb + 250 ng/ml BMP-7.

untreated cells. The nutrient-rich growth media BGJb synergistically enhanced the growth effect of BMP-7, especially at day 8 and later. Similarly, increased mineralized matrix was found in those cultures treated with BMP-7, especially when grown in BGJb. Alkaline phosphatase activity peaked at day 8 in BMP-7 treated cultures, yet this high level of activity was only maintained in cells grown in BGJb at day 16.

DISCUSSION

Our findings indicate that the murine mesenchymal stem cell line C3H10T1/2 can be induced to become both chondrogenic and osteogenic when culture conditions are supplemented with BMP. This induction is dose-dependent and occurs in a temporal pattern such that the chondrogenic differentiation precedes osteogenesis by approximately four days. This finding is reminiscent of the endochondral ossification process whereby mesenchymal cells are recruited to proliferate and differentiate into chondrocytes, forming the cartilaginous matrix which is later replaced by bone. A similar sequence and timing of events was shown to occur in vivo with subcutaneous implantation of BMP-7 [Sampath et al., 1992). The findings of this study are similar to those of a study by Ducy et al. but differ from the study of Asahina et al. In our study, a BMP-7 dose of 250 ng/ml or a BMP-2 dose of 200 ng/ml induced both chondrogenesis and osteogenesis, although 80 ng/ml of BMP-7 produced only a small amount of type II collagen and osteocalcin mRNA. Ducy et al. [1997] treated C3H10T1/2 cells with 200 ng/ml of BMP-7 and induced osteocalcin expression, while Asahina et al. [1996] induced adipogenesis with 80 ng/ml and chondrogenesis with 500 ng/ml, but no osteogenesis. The differences between these studies may reside in the various technical procedures used to assay mRNA as the ribonuclease protection assay is considered more sensitive than Northern blot analysis. Variations in the concentration of BMP used to induce differentiation may also be a critical factor in determining the pathway of differentiation. The results of our study confirm a previous report where BMP-2 treatment of C3H10T1/2 cells favored chondrogenic and osteogenic differentiation while lower concentrations induced adipogenesis [Wang et al., 1993]. This suggests that the local environment and the surrounding concentration of BMPs as well as other factors play an important role in inducing differentiation of uncommitted progenitors. It is interesting to note that the apparent default lineage into which mesenchymal cells differentiate in the absence of sustained BMP levels is adipocytic. The presence of more fat-filled marrow spaces in aging animals may suggest that with increasing age, animals possess a diminished ability to produce or respond to BMPs.

A central aspect of our studies was to define how variations in BMP concentrations, the duration of BMP-7 exposure, and the nutrient environment interact to promote skeletal cell differentiation. Our results clearly show that cells need to be continuously exposed to BMP-7 for greater than 8 days in order to maintain the differentiated chondrogenic and osteoblastic phenotype. Similarly, Puleo [1997] found that C3H10T1/2 cells maintained and more fully expressed their osteoblastic characteristics only with progressively longer durations of exposure to BMP-2 for up to 20 days. These results suggest that, unlike hormones which act as on/off modifiers, BMPs are important throughout the differentiation process and their actions are sequential in nature, with continuous modifications occurring throughout the process. Consistent with these studies are those examining BMP expression during fracture repair. During the bone repair process, BMPs are expressed at elevated levels over 28 days of repair [Cho et al., 2002] suggesting the need for these morhphogens to be continuously present to drive skeletogenic differentiation throughout the repair process. These results again point out the importance of the local environment in differentiation. Finally, it is interesting to note that if BMP-7 was removed, the cells converted to an adipogenic phenotype. Such an observation is consistent with previous reports.

One of our most interesting findings was that chondrogenesis appeared earlier than osteogenesis. This initial induction of cartilage-related genes occurred by day 4 of treatment with BMP-7. Therefore, in this mesenchymal cell system, it appears that BMP-7 is able to induce chondrogenesis and allow the further differentiation of chondrocytes to hypertrophic chondrocytes, similar to the pattern that occurs during the endochondral sequence. Asahina et al. [1993] were able to induce a similar pattern of chondrogenesis and osteogenesis in rat calvarial cells, yet BMP-7 was inductive for

osteogenesis only when added to the culture after day 7.

The induction of chondrogenesis and osteogenesis was enhanced when cells were grown in the nutrient-rich media BGJb. BGJb has been shown to promote the growth of cultured bone cells and was used in the present study to examine the effect of the local environment on the BMP-induced promotion of differentiation [Wergedal and Baylink, 1984]. The pattern of induction was similar but the levels of expression of all extracellular matrix genes were greater in cells grown in BGJb. BGJb media containing ascorbic acid was able to enhance the expression of cartilage- and bone-related genes; however, this nutrient effect was not related to ascorbic acid in the media since the cells grown in DMEM in the presence of ascorbic acid showed minimal differences in the pattern or levels of expression. Thus, the primary inductive effect of the more nutrient-rich media appears to be a result of the nutrient effects leading to enhanced growth. BMPs are known stimulators of proliferation. The de novo initiation of endochondral bone formation occurs by BMP-stimulated proliferation and migration of undifferentiated bone cell precursors and the subsequent commitment of these cells into differentiated cartilage- and bone-forming cells [Wozney, 1992; Reddi and Cunningham, 1993]. In our study, BMP-7 induced increased growth of C3H10T1/2 cells early in the culture, with twice the crystal violet absorbance found at days 4 and 8 in BMP-7 treated cells grown in either DMEM or BGJb. After day 8, however, enhanced growth was noted in the BMP-7 treated cells grown in BGJb. This finding supports the hypothesis that the media provided an environment that stimulated both growth and the increased expression of the differentiated phenotype and that the two processes appear to occur concurrently, suggesting that the mechanisms of skeletal cell differentiation are integrated in some manner with those related to growth control. What is yet to be determined is if the two processes are causally linked.

A number of features regarding the manner in which BMP-7 induced skeletal cell differentiation suggested that some of the control resides in the sequential regulation by BMP of other morphogenic factors that regulate differentiation in an autocrine manner. The length of the temporal profile of skeletal cell differentiation and the long period of time needed to induce differentiation, and because BMP-7 needed to be present continuously to induce this differentiation, suggest that BMP-7 actually may work through the concerted regulation of a network of other morphogenic factors. Many of the transcription factors examined were present early in the study suggesting that although they are present, they are not regulating the differentiation process. However, Sox9 expression mimicked the expression of collagen type II, and Dlx 5 and c-fos showed an increase in expression at day 4 in the BMP-7-treated cultures. Interestingly, an examination of autocrine factor expression revealed that Wnt 11 RNA was completely suppressed in cells treated with 250 ng/ml BMP-7 as compared to untreated cells. Wnt 11 was initially expressed in the untreated cells at day 8 of the culture period, the same time as which osteocalcin was first expressed in treated cells. The role of Wnt 11 as an inhibitor of osteogenesis has not been established, yet it has been suggested to play a role in chondrocyte dedifferentiation [Lako et al., 1998].

During this study, BMP-7 induced the increased expression of collagens and other cartilage- and bone-related proteins. This accumulated matrix, along with the continued presence of BMP-7, most likely provided a permissive environment that allowed the differentiation process of mesenchymal cells to proceed into committed chondrocytes or osteoblasts. In a study by Gerstenfeld et al. [2002], C3H10T1/2 cells co-cultured in trans-well devices with chondrocytes were induced to express osteogenic genes without chondrogenesis, suggesting that factors produced by chondrocytes directly induce osteogenesis. In our system, it is possible that chondrogenesis was a necessary step in the differentiation of osteoblasts whereby undifferentiated cells treated with BMP-7 first became chondrocytes which then produced autocrine factors that subsequently promoted osteogenic differentiation. Such an interpretation of the results is further supported by the sequential expression of chondrogenic and then osteogenic genes. Additionally, a more nutrient-rich environment upregulated chondrogenic gene expression, leading to a more enhanced osteogenic response.

The question arises, however, that although these cells were induced to express osteocalcin, why was bone sialoprotein never detected?

Osteocalcin has been considered the protein whose presence establishes the differentiated state of the osteoblast [Hauschka et al., 1983]. However, some investigators have suggested that hypertrophic chondrocytes also express osteocalcin [Lian et al., 1993; Neugebauer et al., 1995; Gerstenfeld and Shapiro, 1996]. In light of the absence of bone sialoprotein, it is possible that we are observing the terminal differentiation of chondrocytes. This would also explain the absence of Runx2/Cbfa1. It should be noted, however, that in our previous studies with chondrocytes co-cultured with C3H10T1/2 cells, *Runx2* gene expression was detected by RT-PCR [Gerstenfeld et al., 2002]. One possibility is that the RT-PCR was much more sensitive than the RPA in detecting very low levels of this transcription factor and that it does not need to be present in quantitatively high levels to drive osteoblast differentiation. Another possibility is that a different slice variant or isotype of the Runx gene was present in these cells [Harada et al., 1999]. In our previous studies, an RT-PCR primer set that detects the conserved Runx domain was used while the RPA template in these studies was selectively directed to the more restricted and proposed osteoblast specific isotype of the Runx2 transcript. The absence of BSP expression and the low levels of Runx2 expression these studies suggest that the use of specific cell lines to assess the in vitro biological effects of BMPs must be interpreted with considerable caution.

In summary, the results of this study stress the importance of the local environment in controlling differentiation. This is not surprising considering the many processes in which the surrounding cells and matrix act to control the effects on other cells. One such example is the infection process. During infection, cell stimulation and eventual cell death leads to the release of multiple mediators which act as mitogens and morphogens to recruit and stimulate the maturation of particular cell types which will lead to the control and elimination of the infectious agent. The mediators present play a critical role in determining the cellular response in the local area.

Some important findings in this study have been elucidated. BMP-7 was able to induce both chondrogenesis and osteogenesis in the mesenchymal stem cell line C3H10T1/2 in a temporal manner. The induction of both was dose-dependent. The observed pattern mimics that present during the endochondral ossification process. Additionally, the use of a nutrient-rich media enhanced the expression of chondrogenic and osteogenic genes. The transcriptional regulators that have previously been associated with osteogenesis were present early in the culture, and their presence may be important in the early stages of differentiation, yet the presence of BMP-7 in the media appeared to have the greatest importance in maintaining the differentiated phenotype. Along with the long period of time that BMP-7 needed to be present in order to maintain differentiation, the role of endogenous factors, including Shh, Wnt 5b, Wnt 11, BMP-4, and GDF 1, appear to be of particular importance in BMP-7 stimulation of differentiation. The temporal pattern of results also suggest a possible role for chondrogenic differentiation and the presence of chondrocyte-associated factors in the environment that enable other undifferentiated cells to commit to the osteogenic phenotype.

REFERENCES

Ahrens M, Ankenbauer T, Schroder D, Hollnagel A, Mayer H, Gross G. 1993. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol 12:871–880.

Asahina I, Sampath TK, Nishimura I, Hauschka PV. 1993. Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123:921–933.

Asahina I, Sampath TK, Hauschka PV. 1996. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47.

Atkinson BL, Fantle KS, Benedict JJ, Huffer WE, Gutierrez-Hartmann A. 1997. Combination of osteoinductive bone proteins differentiates mesencyhmal C3H10T1/2 cells specifically to the cartilage lineage. J Cell Biochem 65:325–339.

Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells rat marrow stromal cells. J Cell Sci 102:341–351.

Bruder SP, Fink DJ, Caplan AI. 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem 56:283–294.

Cho T-J, Gerstenfeld LC, Einhorn TA. 2002. Differential temporal expression of members of the TGF-β superfamily during murine fracture healing. J Bone Min Res 17:513–520.

Chomczynski P, Sacchi N. 1987. Single-step method of extraction of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159.

Csete M, Walikonis J, Slawny N, Wei Y, Korsnes S, Doyle JC, Wold B. 2001. Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. J Cell Physiol 189:189–196.

Denker AE, Nicoll SB, Tuan RS. 1995. Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-β1. Differentiation 59:25–34.

Denker AE, Haas AR, Nicoll SB, Tuan RS. 1999. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. Differentiation 64:67–76.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Friedenstein AJ, Chailakhyan RK, Gerasimov UV. 1987. Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 20:263–272.

Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, Kawabata M, Kato M, Ichijo H, Miyazono K. 1999. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol Biol Cell 10:3801–3813.

Gerstenfeld LC, Shapiro FD. 1996. Expression of bone-specific genes by hypertrophic chondrocytes: Implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. J Cell Biochem 62:1–9.

Gerstenfeld LC, Finer MH, Boedtker H. 1989. Quantitative analysis of collagen expression in embryonic chick chondrocytes having different developmental fates. J Biol Chem 264:5112–5120.

Gerstenfeld LC, Cruceta J, Shea CM, Sampath K, Barnes GL, Einhorn TA. 2002. Chondrocytes provide morphogenic signals that induce osteogenic differentiation of mesenchymal stem cells. J Bone Miner Res 17:221–230.

Grigoriadis AE, Heersche JN, Aubin JE. 1988. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: Effect of dexamethasone. J Cell Biol 106:2139–2151.

Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. 1999. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 274:6972–6978.

Hauschka PV, Frenkel J, DeMuth R, Gundberg CM. 1983. Presence of osteocalcin and related higher molecular weight 4-carboxyglutamic acid-containing proteins in developing bone. J Biol Chem 258:176–182.

Healy C, Uwanogho D, Sharpe PT. 1999. Regulation and role of Sox9 in cartilage formation. Dev Dyn 215: 69–78.

Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T. 1990. The non-osteogenic pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kostenuik PJ, Halloran BP, Morey-Halton ER, Bikle DD. 1997. Skeletal unloading inhibits in vitro proliferation and differentiation of rat osteoprogenitor cells. Am J Physiol 273:E1133–E1139.

Lako M, Strachan T, Bullen P, Wilson DI, Robson SC, Lindsay S. 1998. Isolation, characterization, and embryonic expression of *WNT11*, a gene which maps to 11q13.5 and has possible roles in the development of skeleton, kidney, and lung. Gene 219:101–110.

Lian JB, McKee MD, Todd AM, Gerstenfeld LC. 1993. Induction of bone-related proteins, osteocalcin and osteopontin, and their ultrastructural localization with development of chondrocyte hypertrophy in vitro. J Cell Biochem 52:206–219.

Miyama K, Yamada G, Yamamoto TS, Takagi C, Miyado K, Sakai M, Ueno N, Shibuya H. 1999. A BMP-inducible gene, dlx5, regulates osteoblast differentiation and mesoderm induction. Dev Biol 208:123–133.

Neugebauer BM, Moore MA, Broess M, Gerstenfeld LC, Hauschka PV. 1995. Characterization of structural sequences in the chicken osteocalcin gene: Expression of osteocalcin by maturing osteoblasts and by hypertrophic chondrocytes in vitro. J Bone Miner Res 10:157–163.

Ohta S, Hiraki Y, Shigeno C, Suzuki F, Kasai R, Ikeda T, Kohno H, Lee K, Kikuchi H, Konishi J, et al. 1992. Bone morphogenetic proteins (BMP-2 and BMP-3) induce the late phase expression of the proto-oncogene c-fos in murine osteoblastic MC3T3-E1 cells. FEBS Lett 314: 356–360.

Poliard A, Nifuji A, Lamblin D, Plee E, Forest C, Kellermann O. 1995. Controlled conversion of an immortalized mesodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. J Cell Biol 130: 1461–1472.

Puleo DA. 1997. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. J Cell Physiol 173:93–101.

Reddi AH, Cunningham NS. 1993. Initiation and promotion of bone differentiation by bone morphogenetic proteins. J Bone Miner Res 8(Suppl 2):S499–S502.

Reznikoff CA, Brankow DW, Heidelberger C. 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res 33:3231–3238.

Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RHL, et al. 1992. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 267:20352–20362.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Umezawa A, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata J. 1992. Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. J Cell Physiol 151:197–205.

Wang EA, Israel DI, Kelly S, Luxenberg DP. 1993. Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors 9:57–71.

Wergedal JE, Baylink DJ. 1984. Characterization of cells isolated and cultured from human bone. Proc Soc Exp Biol Med 176:60–69.

Wozney JM. 1992. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 32:160–167.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Khan AJ, Suda T, Yoshiki S. 1991. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.

Zehentner BK, Dony C, Burtscher H. 1999. The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 14:1734–1741.

Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. 2002. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. Genes Dev 16:1990–2005.