0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 1
Printed in U.S.A.

Immortalized Murine Osteoblasts Derived from BMP 2-T-Antigen Expressing Transgenic Mice*

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ABSTRACT

Osteoblast cell lines capable of undergoing bone formation *in vitro* would provide useful models for understanding gene expression during bone cell differentiation. To that end, transgenic mice were produced using a 2.9-kilobase bone morphogenetic protein 2 (BMP-2) promoter fragment, driving simian virus 40 T antigen as the transgene. The expression of simian virus 40 T antigen driven by the BMP-2 promoter immortalizes the cells. From the calvaria of the transgenic mouse, several osteoblastic cell lines were isolated and cloned. One clonal osteoblast cell line, called 2T3, has been characterized and shown to produce mineralized bone nodules. Recombinant human BMP-2 (rhBMP-2) accelerates the formation of these mineralized bone nodules. 2T3 cells express alkaline phosphatase, collagen type I, osteocalcin, and endogenous BMP-2 messenger RNA (mRNA) in a similar chronological order as normal freshly isolated fetal rat calvarial cells during early nodule formation and subsequent mineralization. The 2T3 cells also exhibit extensive growth and multilayering during differentiation, as demonstrated by growth curves and transmission electron microscopy. As with freshly isolated fetal rat calvarial cells, 1,25-dihydroxyvitamin D_3 inhibited alkaline phosphatase activity and alkaline phosphatase mRNA expression, but stimulated osteocalcin mRNA expression. rhBMP-2 also accelerated the expression of alkaline phosphatase activity and mRNA, osteocalcin mRNA, and BMP-2 mRNA in 2T3 cells along with the formation of larger and more mineralized bone nodules. The 2T3 cell exhibits autoregulation of endogenous BMP-2 by rhBMP-2 ligand. This is shown at the mRNA and transcriptional levels. The 2T3 osteoblast cell line offers a system for examining autoregulation of the BMP-2 gene and downstream gene expression during osteoblast differentiation. 2T3 cells are reclonable and maintain their differentiation capabilities. (*Endocrinology* **137:** 331–339, 1996)

THERE IS a need for new *in vitro* models of osteogenesis that can be manipulated experimentally to dissect out components of the complex program of bone cell differentiation. A variety of osteosarcoma models have yielded useful information on limited aspects of bone cell differentiation (1). However, due to the transformed and tumorigenic properties of many of these cells, it is difficult to determine what stage(s) of the osteoblast differentiation program they may represent. In contrast, cultures of primary fetal rat calvarial (FRC) osteoblasts can undergo an ordered program of bone cell differentiation, leading to the production of nodules of mineralized bone (2–8).

Prolonged cultures of freshly isolated primary FRC osteoblasts are an excellent *in vitro* system for studying the role of BMP-2 and other BMPs and growth factors in bone cell differentiation (9–11). This system, however, has several limitations. First, these cultures may represent a mixed population of osteoblasts at different stages of differentiation (4, 7, 12). There may be overlapping programs of gene expression that are difficult to dissect out due to this heterogeneity. The second major difficulty with the primary cell culture system is the limited availability of the cells for extensive analysis of gene expression patterns during bone cell differentiation. Cell lines that can undergo extensive programmed bone cell differentiation in a similar manner to these normal cells in a reasonable time period would be most useful.

To overcome some of these difficulties in studying the role of BMP-2 in osteoblast differentiation and to permit extensive analysis of the gene expression patterns, we produced transgenic mice expressing T antigen (T-Ag) under the control of the BMP-2 promoter, with the goal of establishing immortalized osteoblast cell lines. Immortalization of rare cell types representing particular developmental stages by targeting expression of oncogenes with tissue-specific promoters in transgenic mice has been used effectively (13–15). We have isolated and characterized the mouse BMP-2 promoter from a 129 mouse genomic library (gift from Tom Doetschman, Cincinnati, OH) and have identified various response elements within sequences upstream of its transcription start sites (Ghosh-Choudhury, N., M. A. Harris, J. Wozney, G. R. Mundy, S. E. Harris, submitted). A 2.9-kilobase (kb) DNA fragment containing the transcription start site and 2.7-kb upstream sequence of BMP-2 promoter (−2736/114) was used to drive the expression of simian virus 40 (SV40) T-Ag in transgenic mice. Clonal cell lines were derived from the calvarial cells of these transgenic mice. This

Received February 9, 1995.
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* This work was supported in part by NIH Grant AR-39529 and Grant 2947 from the Council for Tobacco Research (to S.E.H.), and NIH Grant EY-09213 (to J.J.W.).

report describes the characteristics of the BMP-2 T-Ag-3 or 2T3 cell line and demonstrates that it is a cell of the osteoblast lineage capable of extensive bone-forming capacity under the appropriate culture conditions. This report also demonstrates the feasibility of isolating cell lines from transgenic mouse containing bone-selective promoter constructs driving T-Ag. One of the many uses of this cell line is demonstrated by stably transfecting the cells with the BMP-2 promoter-luciferase constructs and isolating clonal cell lines expressing high levels of luciferase enzyme under the control of BMP-2 promoter. These second generation clonal cell lines have been used to show autoregulation of BMP-2 promoter by recombinant human BMP-2 (rhBMP-2). Regulation of BMP-2 promoter by various other osteogenic factors and hormones can thus be studied using this approach by a very simple assay system.

Materials and Methods

Transgene construction

A 2.9-kb *Eco*RI/*Bam*HI fragment containing the mouse BMP-2 gene promoter region from −2736 to 114 relative to the transcription initiation site was inserted into the plasmid α-Gly-Tag (14), which had been digested with *Eco*RI and *Bam*HI to remove the glycoprotein hormone α-subunit promoter. This plasmid contains SV40 early region from the *Bgl*II to *Bam*HI site, which contains the protein-coding region for large T and small t antigens, including the translation initiation and transcription termination sites, but lacking the SV40 early promoter/enhancer. The BMP-2-Tag transgene was excised by digestion with *Eco*RI and *Sal*I and purified by agarose gel electrophoresis and use of the Geneclean kit (BIO 101, La Jolla, CA).

Production and identification of transgenic mice

The BMP-2-T-Ag transgene at a concentration of 2 μg/ml was microinjected into the male pronucleus of fertilized one-cell mouse embryos essentially as previously described (16). The microinjected embryos were reimplanted into the oviducts of pseudopregnant B6D2F1 female mice. The presence of the transgene in the resulting offspring was identified by Southern blot analysis (17) of DNA purified from a small piece of tail obtained at the time of weaning (16). Transgenic mice of subsequent generations were identified by slot blot analysis with a T-Ag probe or by polymerase chain reaction analysis using T-Ag-specific primers flanking the T-Ag intron. Probes for Southern and slot blot analyses were generated by random oligonucleotide labeling (Pharmacia P-L Biochemicals, Piscataway, NJ), using [α-^{32}P]deoxy-CTP (DuPont-New England Nuclear, Boston, MA).

Cell lines from calvariae of BMP-2 T-Ag-3

Calvarial osteoblasts were isolated from BMP-2 T-Ag-3 transgenic founder mice at 7 weeks of age, using sequential digestions with trypsin and collagenase, as described previously (2, 8). Calvarial osteoblasts were plated initially in 100-cm tissue culture plates at a dilution of 1000 cells/plate. This allowed the cells from this population to spread in the tissue culture plates as single cells, and with time, each of these cells generated different clonal cell lines.

SV40 T-Ag expression

2T3 cells and control stromal ST2 cells were grown on 96-well tissue culture dishes to a subconfluent state in α-Minimum Essential Medium (αMEM) containing 7% fetal calf serum (FCS). They were washed with PBS and fixed. Polyclonal rabbit antibody to SV40 T-Ag (PAb416) (18) was used first, followed by a secondary horse radish peroxidase-conjugated immunoglobulin G for staining.

Assays of cell growth and mineralized bone nodule formation

Mineralization/bone nodule assay. 2T3 cells (passage 3–20) were plated at 10,000 cells/well in 24-well (1.5-cm diameter/well) tissue culture plates using 1.0 ml αMEM containing 7% FCS. They were allowed to grow to confluency (day 0), and the medium was then changed to the differentiation medium (7% FCS in αMEM containing 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate). Recombinant human BMP-2 (rhBMP-2) and/or 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] were added at various concentrations to triplicate wells. Media were changed every 2–3 days. At various times (*i.e.* 4, 9, 12, and 16 days) plates were fixed with 10% formalin for 20 min, washed with distilled water followed by ethanol, and air dried. The plates were then stained with the Van Giesen stain (matrix-collagen) and the Von Kossa stain (mineral), as described previously (5).

Mineralized bone nodules were then quantitated for average area (S) of mineralized nodule, total number (N) of mineralized nodules per square centimeter, and total area of mineralized nodules per cm^2, using Java Image Analysis Software (Jandel Scientific, Corte Madera, CA) (5, 6).

Quantitation of multilayering (growth) during 2T3 bone cell differentiation. Duplicate 24-well plates were set up as described above. Cell number was determined in triplicate wells at various time points as previously described (5). In brief, the cell layer was washed with PBS, and the cells were then incubated with 0.1–0.5 ml 0.05% trypsin-25 mM EDTA (GIBCO, Gaithersburg, MD) at 37 C for 10–40 min (until the cells in wells visibly rounded up). An equal volume of FCS was then added, and the cells were dispersed to a single cell suspension with 25 up/down strokes using a 1.0-ml automatic pipetting device. Cell number was determined using a hemocytometer.

Transmission electron microscopy of 2T3 mineralized bone nodules

2T3 cells were cultured in 24-well plates for 10 days in differentiation medium, with or without rhBMP-2. The cells were washed with PBS, fixed with 0.01 M Na cacodylate buffer (pH 7.2) containing 2% glutaraldehyde, and stored at 4 C for up to a week. The cells were then postfixed with 1% osmium tetroxide in 0.1 M Na cacodylate buffer for 1 h at room temperature. The specimens were dehydrated in an ethanol series, embedded in Polybed 812 resin (Polysciences, Warrington, PA), polymerized for 72 h at 60 C, thin-sectioned with a Sorvall MT 5000 ultramicrotome (Sorvall, Norwalk, CT), and viewed in a JEOL 1200 EX transmission electron microscope (JEOL, Peabody, MA).

Polyadenylated [poly(A)] RNA preparation and Northern analysis

One × 10^6 2T3 cells were plated in T150 tissue culture flasks using αMEM containing 7% FCS. After the cells reached confluence (day 0), the medium was changed to differentiation medium. To some flasks rhBMP-2 at 10 ng/ml or 1,25-$(OH)_2D_3$ at 10^{-8} M was added on day 0. At various times, the medium was removed, and the total RNA was isolated using 10 ml RNAzol B (Cinna/Biotex Laboratories, Houston, TX)/T-150 flask, followed by chloroform addition, centrifugation, and precipitation of the RNA with isopropanol (5). Poly(A) RNA was isolated using 1.0 ml oligo(dexoxythymidine)-cellulose columns (push columns) from Stratagene (San Diego, CA). Three to five μg poly(A) RNA were then separated by electrophoresis according to size in denaturing agarose gels and transferred to a Nytran filter (Schleicher and Schuell, Keene, NH). The filters were hybridized with osteocalcin, alkaline phosphatase, BMP-2, T-Ag [750-base pair (bp) fragment], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) complementary DNA probes, as described by Harris *et al.* (5). The data were quantitated using a Betascope Analyzer (Betagen Corp., Waltham, MA), and the fold change in mRNA levels was expressed relative to the GAPDH mRNA levels at various times during the differentiation process or after treatment with 1,25-$(OH)_2D_3$.

Stable transfection of 2T3 cells with BMP-2 (−2736/114)-luciferase constructs

2T3 cells in 10-cm tissue culture plastic plates were stably transfected with 10 μg plasmid containing luciferase reporter gene driven by −2736/114 bp of BMP-2 promoter and 1 μg pSV2neo plasmid for G418-resistant clone selection. The transfection was carried out by the calcium phosphate precipitation technique, as described previously (19). The stable clonal cell lines were generated using MEM containing 7% FCS and 200 μg/ml G418, essentially as described previously (20). Luciferase enzyme activity was measured in 15 different clonal cell lines. One cell line was chosen for the studies reported here, because assays for luciferase activity have shown little variability over 20 passages. This cell line is referred to as 2T3-BMP-2-LUC. For a control group, stable clonal cell lines were established as described above, using the promoterless luciferase vector, pGL2basic (Promega Corp., Madison, WI), in the place of BMP-2 promoter-luciferase plasmid. The control cell line used in this study is referred to as 2T3-basic LUC. Both of these cell lines were plated in 35-mm tissue culture dishes and treated with 0, 2, 5, 10, and 20 ng/ml rhBMP-2 for 48 h in MEM containing 2% FCS. Each concentration of rhBMP-2 was used in triplicate. The experiments were repeated three times. Luciferase activity was measured using a kit from Promega and a luminometer. Other 2T3 BMP-2-LUC clones were also tested.

Results

BMP-2 T-Ag transgenic mice

A hybrid gene containing approximately 2.7 kb of 5′-flanking sequences of the mouse BMP-2 gene linked to the protein-coding sequences of the SV40 T-Ag oncogene (Fig. 1) was used to generate transgenic mice. Six of 13 pups born were transgenic. A BMP-2 T-Ag-3 (male) mouse was killed at 7 weeks of age for isolation of cell lines (see below).

No bone tumors were noted in any of the founder mice. Therefore, we isolated osteoblasts from apparently normal bone tissue. Osteoblast-like cells were isolated from the calvaria of a BMP-2 T-Ag-3 mouse, and 30 different clonal cell lines were derived. Some of these cell lines were clearly distinct from others in appearance. For example, 2T3 cells, described in this report, were phenotypically different from some of the other cell lines based on their orthogonal appearance and their tendency to pile up, two important characteristic features of primary FRC cells. Therefore, we selected this cell line for more extensive characterization to determine whether these cells have the capacity to form bone-like structures on prolonged culture.

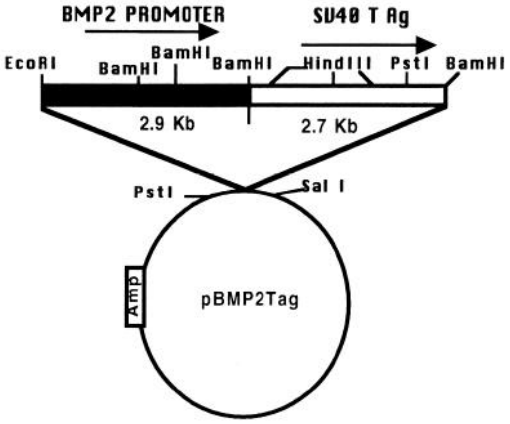

FIG. 1. The BMP-2 T-Ag transgene contains the mouse BMP-2 gene promoter region from −2736 to 111 relative to the transcription initiation site linked to the SV40 T-Ag gene.

Characterization of 2T3 cells

Southern analysis of 2T3 DNA, probed with a T-Ag probe (750-bp polymerase chain reaction product spanning 4947–4197 nucleotides of T-Ag) (14) and a BMP-2 promoter probe, confirmed that the transgene is intact (Fig. 2). For example, a *Pst*I digest of 2T3 DNA and hybridization with both T-Ag probe and the BMP-2 probe produced a 6-kb band. The 9.2-kb *Pst*I fragment detected with the 2.8-kb BMP-2 probe represents the endogenous BMP-2 gene. The 0.5-kb *Hin*dIII fragment detected with T-Ag probe represents the 3′-region to T-Ag. The intensity of hybridization of the BMP-2 probe (−2736 to 114) to the transgene compared to the endogenous BMP-2 gene indicated that there are two or three copies of the BMP-2 T-Ag fragment per diploid genome of 2T3 cells.

Northern analysis of RNA from 2T3 cells using a T-Ag probe demonstrated expression of T-Ag mRNA. The level of T-Ag mRNA increased 5-fold when the cells were cultured for extended periods (6 days) in differentiating medium (Fig. 3, *left*). Differentiation medium allows formation of bone-like structures in primary FRC cells (8). This culture condition also resulted in induction of endogenous BMP-2 mRNA in 2T3 cells (see Fig. 8). These results demonstrate that the integrated BMP-2 T-Ag DNA is also responsive to similar factors that regulate the endogenous BMP-2 gene. The expression of T-Ag protein in the 2T3 cell line was confirmed by immunocytochemistry using biotinylated T-Ag antibody and staining with horse radish peroxidase (Fig. 3, *right*).

Chromosome spreads of 2T3 cells were prepared, and the cells were found to be aneuploid, with an average chromosome number of 63 (data not shown).

To assess the tumorigenic potential of the cell, 1×10^6 2T3 cells were plated on soft agar (10-cm dish) in αMEM containing 10% FCS. After 2 weeks, no colonies were observed.

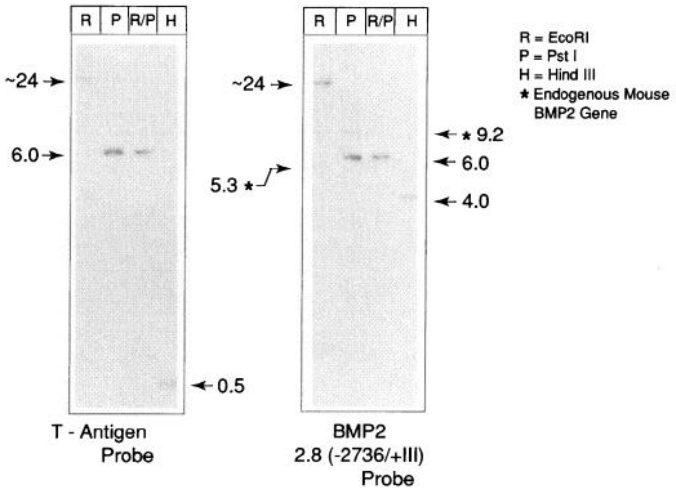

FIG. 2. BMP-2 T-Ag gene dosage. Southern blot of 2T3 DNA (10 μg/lane) probed with mouse BMP-2 probe (−2736 to 111) and parallel 2T3 DNA samples were probed with a T-Ag probe. Note the hybridization to the endogenous BMP-2 gene (*Eco*RI, 5.3 kb; *Pst*I, 9.2 kb) and the BMP-2 T-Ag DNA (*Eco*RI, 24 kb; *Pst*I, 6 kb) using the 2.8-kb BMP-2 probe.

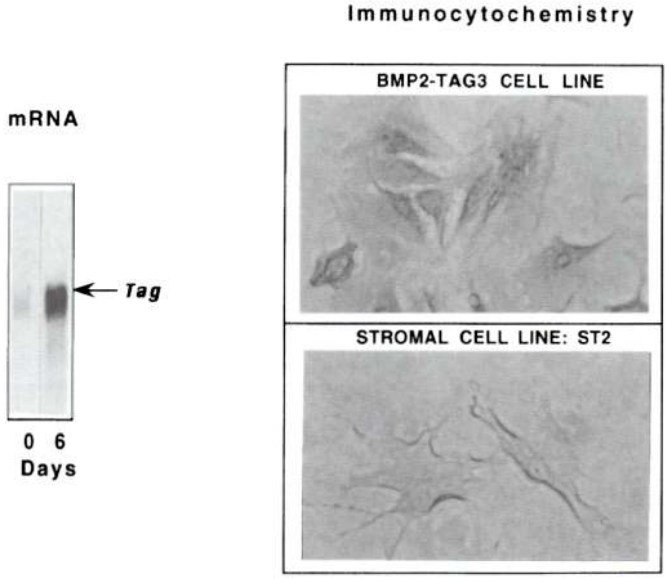

FIG. 3. SV40 T-Ag expression in BMP-2 T-Ag 3 (2T3) cells. *Left panel*, T-Ag mRNA in 2T3 cells. 2T3 cells were grown to confluency (day 0), and poly(A)$^+$ RNA was isolated. The cells were then changed to differentiation medium containing 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate and cultured for 6 days, and poly(A$^+$) RNA was isolated. The two poly(A) RNA preparations (days 0 and 6) were analyzed by Northern analysis using a 750-bp T-Ag probe. *Right panel*, Subconfluent 2T3 cell cultures were fixed and stained with a polyclonal antibody to SV40 T-antigen protein. The stromal cell line ST2 was used as a negative control (see *Materials and Methods*).

The prostate adenocarcinoma cell line, PAIII, served as a positive control, because these cells produce tumors (21, 22). PAIII cells formed many large colonies on soft agar after this 2-week period. 2T3 cells (2×10^6 cells) were injected into nu/nu mice over the calvariae and sc. After 4 months, no tumors were observed.

2T3 cells form mineralized bone-like structures in prolonged cultures and rhBMP-2 accelerates their formation

2T3 cells, when grown to confluency and then cultured in differentiation medium, formed mineralized bone nodules within 9–16 days. 2T3 cells up to 30 passages maintained this property. 2T3 cells have been recloned, and the new subclones maintain similar mineralized bone nodule-forming capacity (data not shown).

Figure 4 shows the modified Van Giesen-Von Kossa staining pattern of 2T3 cells after 16 days of culture. The multilayer nature of the cultures is apparent. The beginning of mineralized nodule formation in the control cultures is shown (*left panel*). rhBMP-2 greatly accelerates both the size and the number of nodules as well as their subsequent mineralization (black stain, *right panel*; see also Fig. 6, A and B).

Figure 5 (*upper left panel*) shows a phase contrast photograph of mineralized bone nodules formed by 2T3 cells in the presence of 10 ng/ml rhBMP-2 at 12 days. Using Nomarski optics (*lower left panel*), the bone-like fractal quality of the mineralized nodules can be seen, indicative of a highly ordered program of bone cell differentiation similar to bone formation *in vivo* and primary cultures of FRC cells. FRC cultures form similar bone-like fractal structures under the same culture conditions (12 days; Fig. 5, *right panel*). The temporal and spatial aspects of this *in vitro* differentiation program are thus similar between the primary cultures and 2T3 cell cultures. The 2T3 cells, however, are clonal in origin.

Time-dependent mineralized bone nodule formation in prolonged cultures of 2T3 cells treated with and without rhBMP-2

Figure 6A shows the appearance of 2T3 cultures over time with or without rhBMP-2 treatment, stained with a modified Van Giesen and Von Kossa stain for collagen (red), secretory osteoblasts (yellow), and mineralized structures (black) (8). There is an increase in thickness of the cultures over time. This is a reflection of the multilayers of cells and matrix (osteoid) formation occurring during differentiation. This multilayering process is required for extensive bone cell differentiation, as seen in the primary FRC cultures (2, 7, 12). rhBMP-2 accelerates the formation of the multilayers and accelerates the appearance of mineralized bone formation in 2T3 cultures (Fig. 6,A and B), as it does in primary FRC cultures (6, 7).

A representative experiment from passage 10 is quantitated (black) for the mineralized nodule number (N), total area of mineralized nodules per cm^2 (A), and average mineralized bone nodule area (S) in Fig. 6B. Mineralized bone nodules could be detected starting from day 6 of rhBMP-2

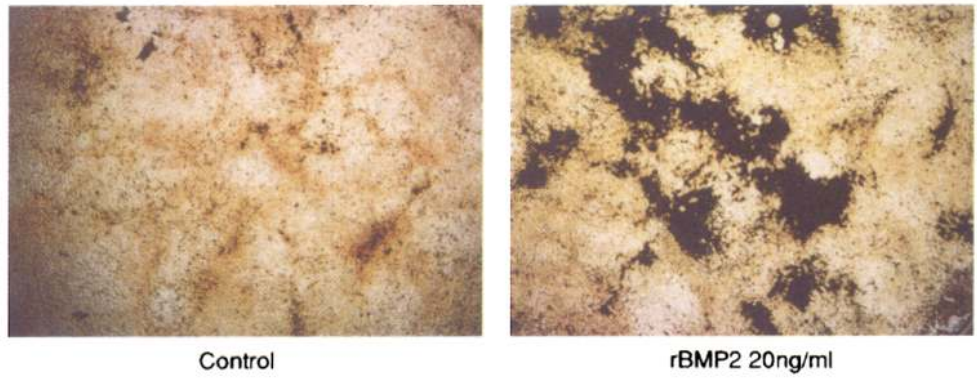

FIG. 4. Mineralized bone nodule formation by 2T3 cells after 16 days of culture in differentiation medium in the presence or absence of rhBMP-2 at 20 ng/ml. The cultures were stained with a Van Giesen stain for matrix and collagen and a Von Kossa stain for mineralized structures. Note the darker yellow-brown multilayered nodules and the black mineralized bone-like material. rhBMP-2 (20 ng/ml) greatly accelerates nodule formation and mineralization of these 2T3 cell cultures.

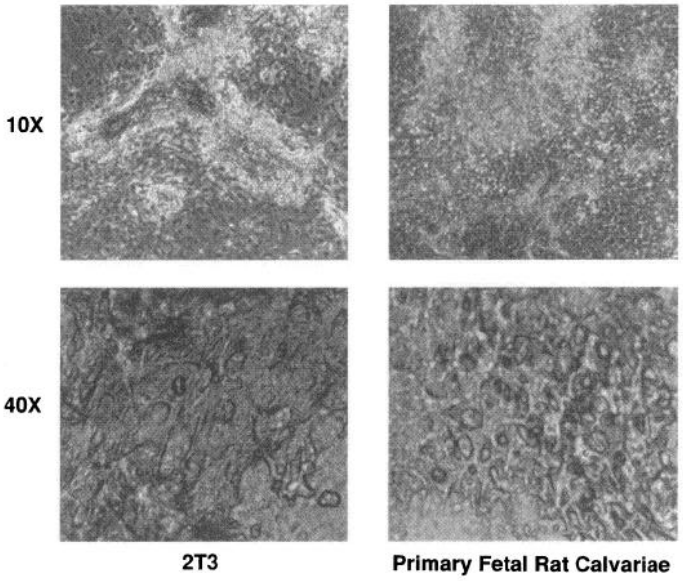

FIG. 5. Phase contrast photomicrograph and Nomarski photograph of 2T3 cultures at 12 days compared to similar primary FRC cultures. Note the fractal quality (sponge-like structure) of the mineralized nodules formed by both 2T3 cells and primary FRC cultures.

treatment, and they continued to increase in number and area with days in culture. When the 2T3 cell cultures were treated with rhBMP-2, the rate of bone nodule formation was accelerated. rhBMP-2 at a concentration of 10 ng/ml increased the number (N) and total area of the nodules (Fig. 6B). At 5 ng/ml rhBMP-2, a large number of smaller nodules was produced compared to control cells. At 5 ng/ml, the total area was similar to the controls (Fig. 6B). In the FRC primary osteoblast population, rhBMP-2 induced increases in number and total area at concentrations of 10–50 ng/ml (7, 8). These experiments with 2T3 were performed at least five times with different passages of cells and different lots of rhBMP-2, and similar results were obtained.

Growth and multilayering during differentiation of 2T3

Ten thousand 2T3 cells per well were plated in 24-well dishes and grown to confluency (day 0). Growth and multilayering during the differentiation process were then monitored during prolonged culture in differentiation medium.

The number of cells increased approximately 7-fold during the first 5 days and then remained constant up to 11 days (Fig. 7A). This multilayering (growth) process is a critical parameter seen in FRC cell cultures capable of *in vitro* bone formation (23, 24). Factors such as retinoic acid or vitamin D_3 that block multilayering also block *in vitro* bone formation in both 2T3 and FRC cultures (data not shown). The 2T3 cultures, thus, behave in a similar manner as the fetal rat primary cultures in this multilayering process. The growth curves were carried out twice, and similar profiles were obtained.

By transmission electron microscopy, 2T3 nodules in the presence and absence of rhBMP-2 at 10 days of culture were similar in appearance to those with primary fetal calvarial cultures during the early phases of bone nodule formation (3). Note the large extracellular membrane containing mineralized area (Fig. 7B, *arrow*) near the bottom of the cell layers in the rhBMP-2-treated 2T3 cultures. In the control, this equivalent area is just beginning to mineralize, as noted by the large abundance of collagen fibers (*arrow, left panel* of Fig. 7B).

Expression of collagen type I, alkaline phosphatase, and osteocalcin mRNA in prolonged cultures of 2T3 cells in differentiation medium in the presence and absence of rhBMP-2

Poly(A) RNA was isolated from 2T3 cell cultures at 0, 2, 5, 7, 11, and 16 days after confluency and the addition of differentiation medium with or without rhBMP-2 (10 ng/ml). Collagen $\alpha(1)$ type I, alkaline phosphatase, and osteocalcin mRNA levels were analyzed by Northern hybridization reaction to poly(A)$^+$ mRNA (Fig. 8). mRNA levels were normalized to GAPDH levels, and the level on day 0 was then set at 1.0. rhBMP-2 was added on day 0. The fold change in mRNA was calculated as described in *Materials and Methods*.

Collagen type I $\alpha(1)$, alkaline phosphatase, and osteocalcin mRNA

Collagen $\alpha(1)$ type I mRNA levels were high on day 0, increased 3-fold by day 2, and subsequently declined by day 5. rhBMP-2 was capable of maintaining the higher level of collagen type I mRNA than controls at 5 days, although the level of collagen type I mRNA in the rhBMP-2-treated sample is lower than the day 3 value (Fig. 8).

After 2 days of culture, there was a spontaneous 38-fold increase in alkaline phosphatase mRNA that subsequently decreased. rhBMP-2 treatment for 48 h (days 0–2) accelerated the rate of accumulation of alkaline phosphatase mRNA over the control value (64-fold). rhBMP-2 maintained high induced levels on day 5, whereas in the control cultures the level of alkaline phosphatase mRNA decreased. However, alkaline phosphatase expression on day 5 in the presence of rhBMP-2 was reduced 2-fold compared to that on day 2.

Similar to the primary FRC system, in 2T3 cells, osteocalcin mRNA appeared later than alkaline phosphatase mRNA and increased continually up to 16 days, consistent with mineralized nodule formation. Results from the first 5 days are shown in Fig. 8.

The addition of rhBMP-2 resulted in an early accelerated increase in osteocalcin mRNA on days 0–5. Osteocalcin mRNA expression was elevated throughout the entire culture period in rhBMP-2-treated 2T3 cells. These results are consistent with the effect of rhBMP-2 on acceleration of mineralized bone nodule formation as well as a net increase in mineralized bone formed in rhBMP-2-treated cultures (see Fig. 6).

Endogenous BMP-2 mRNA expression in 2T3 cells during prolonged culture and the effect of rhBMP-2

As shown in Fig. 9, BMP-2 mRNA levels spontaneously increased early in the prolonged cultures of 2T3 cells and subsequently decreased slightly between days 10–16. rhBMP-2 accelerated the induction of endogenous (2T3) BMP-2 mRNA between 2–16 days, as shown in Fig. 9. In

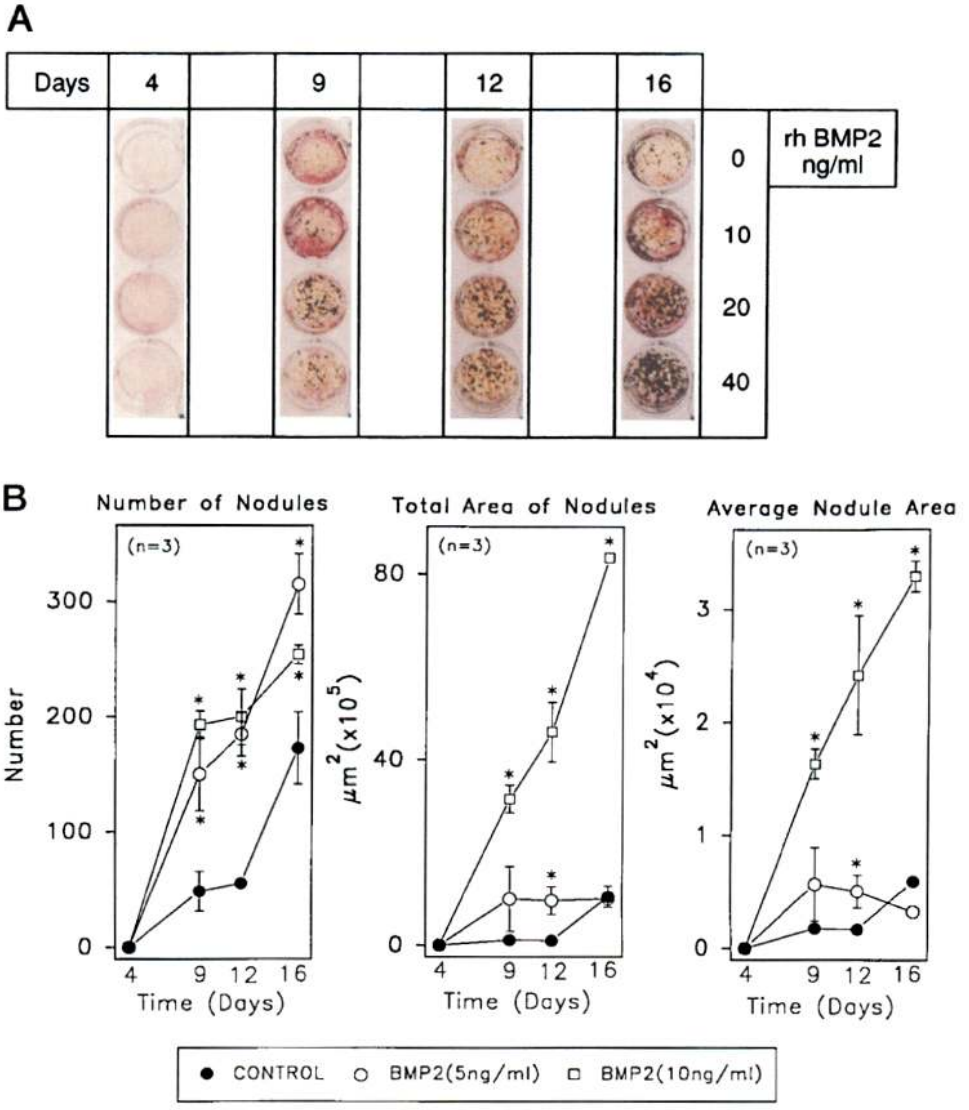

FIG. 6. A demonstration of the time-dependent formation of mineralized bone nodules and quantitation of the mineralized bone nodule formation process in prolonged cultures of 2T3 cells in the absence and presence of increasing concentration of rhBMP-2. A, 2T3 plates stained with Van Giesen and Von Kossa stain after 4, 9, 12, and 16 days of culture with 0, 10, 20, and 40 ng/ml rhBMP-2. B, Quantitation of mineralized nodule formation over time with 0, 5, and 10 ng/ml rhBMP-2. The number (N), mean area (S), and total area (A = N × S) per cm^2 are presented. A Java image analyzer was used to quantitate the variables (5). *, Significantly different from the control at that time point ($P \leq 0.05$).

three independent 2T3 experiments, we showed that rhBMP-2 accelerates endogenous BMP-2 mRNA 2- to 5-fold between 5–10 days of culture. rhBMP-2 stimulation of BMP-2 mRNA and transcription (autoregulation) has recently been documented in the primary FRC cells (6). These results on autoregulation of the BMP-2 gene in 2T3 cells are consistent with results in primary cultures (also see below).

Effect of 1,25-$(OH)_2D_3$ on alkaline phosphatase and osteocalcin mRNA in 2T3 cultures

Confluent cultures of 2T3 cells on day 0 were shifted to differentiation medium containing 10^{-8} M 1,25-$(OH)_2D_3$ and grown for 2 days. Poly(A) RNA was prepared on days 0 and 2 and analyzed by Northern analysis for alkaline phosphatase and osteocalcin mRNA levels. In control cultures, alkaline phosphatase levels increased 5.4-fold by 2 days (Fig. 10). However, in the culture treated with 1,25-$(OH)_2D_3$, there was a 40% reduction of alkaline phosphatase mRNA expression (Fig. 10, *upper panel*). These results in 2T3 cells are similar to the effect of 1,25-$(OH)_2D_3$ on alkaline phosphatase activity in FRC primary cultures (25).

Osteocalcin mRNA increased spontaneously 3- to 4-fold after 2 days of culture in untreated cells, whereas cultures treated for 2 days with 10^{-8} M 1,25-$(OH)_2D_3$ showed a 9-fold increase in osteocalcin mRNA (Fig. 10, *middle panel*). Similar results were observed in two independent experiments. This indicates that the osteocalcin gene is still functionally responding to the vitamin D receptor, although 1,25-$(OH)_2D_3$ at this concentration (10^{-8} M) inhibits the long term multilayering and growth required for differentiation. Osteocalcin production is associated with the mineralization phase, after much of the multilayering and growth has occurred. Premature treatment (before multilayering) of 2T3 cells with 1,25-$(OH)_2D_3$ suppresses multilayering and completely suppresses *in vitro* bone formation (data not shown). This is identical with the effect of 1,25-$(OH)_2D_3$ on long term multilayering in primary FRC cultures (12, 23).

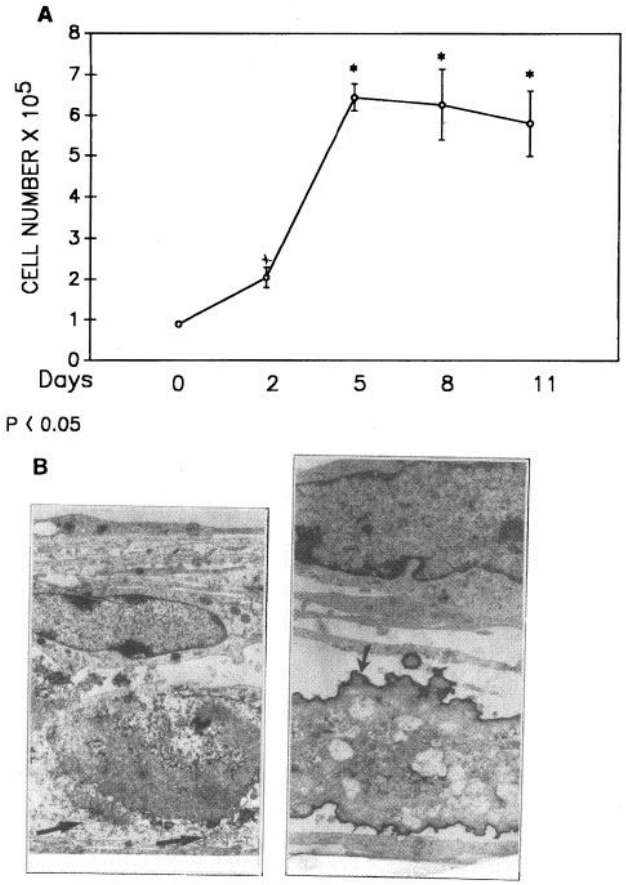

FIG. 7. A, Growth curve (multilayering) of 2T3 cells during differentiation and bone nodule formation. *, Significantly different from the day 0 value ($P \leq 0.05$). B, Transmission electron micrograph through 2T3 cultures at 10 days in the absence (control) and presence of 40 ng/ml rhBMP-2.

rhBMP-2 autoregulates transcription of −2736/114 BMP-2 promoter region stably integrated into chromatin of 2T3 cells

2T3-BMP-2-LUC cells, when treated with increasing amounts of rhBMP-2 (2–20 ng/ml), showed a dose-dependent increase in luciferase activity (Fig. 11). The optimum increase was noted after 10 ng/ml rhBMP-2 treatment for 48 h. A further increase in added rhBMP-2 (20 ng/ml) did not increase BMP-2 promoter activity. The control cell line, 2T3-basic LUC (empty vector control), had a 100-fold lower luciferase activity. This stably integrated control vector showed no response to rhBMP-2 (Fig. 11, *inset*). Two other 2T3-BMP-2-LUC clones also showed an approximately 2-fold change in luciferase activity with added rhBMP-2.

Discussion

We have produced transgenic mice that express SV40 T-Ag under the control of the BMP-2 promoter. From the calvariae of one of these mice, we have isolated and cloned osteoblast progenitors capable of bone formation *in vitro* under the appropriate conditions. These cell lines are immortalized, and the 2T3 cell line has undergone more than 40 passages.

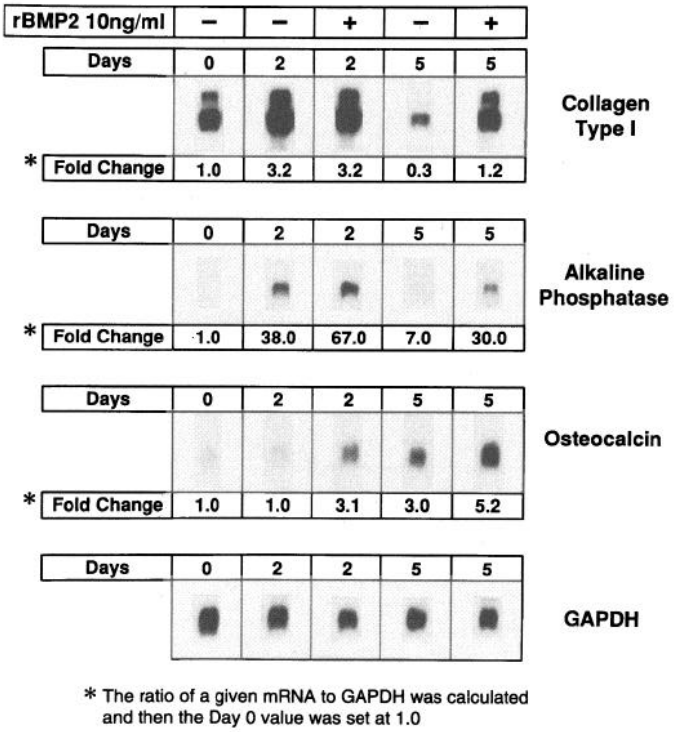

FIG. 8. Northern analysis of collagen $\alpha(1)$ type I, alkaline phosphatase, and osteocalcin mRNA expression during prolonged cultures of 2T3 cells in differentiation medium. On day 0 of culture, rhBMP-2 was added at 10 ng/ml. Poly(A)$^+$ RNA was isolated and analyzed as described in *Materials and Methods*. The fold change indicated is first normalized to the GAPDH level, and then day 0 is set at 1.0.

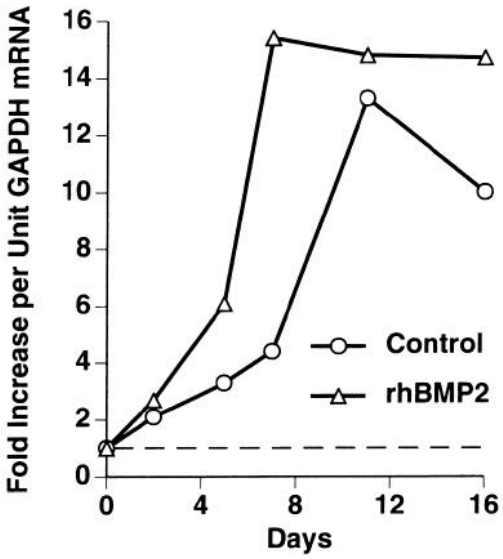

FIG. 9. Endogenous BMP-2 expression in 2T3 cells during prolonged culture in the presence (△) or absence (○) of rhBMP-2. Poly(A)$^+$ RNA was isolated and analyzed as described in *Materials and Methods*.

They can be diluted to single cell cloning density and recloned, and still maintain their differentiation properties.

The clonal mouse cell line described in this report, 2T3, has been shown to undergo an ordered program of bone cell differentiation similar to the primary FRC cell cultures. We demonstrate that 2T3 cells can grow in multilayers and form

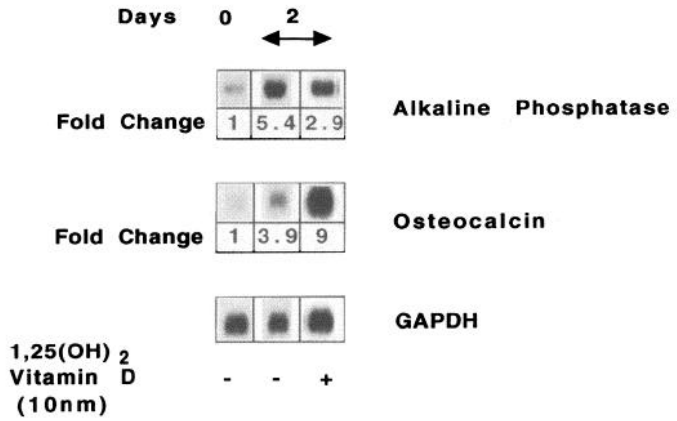

FIG. 10. Effects of 1,25-$(OH)_2D_3$ on alkaline phosphatase mRNA and osteocalcin mRNA expression in 2T3 cultures (2 days of treatment after confluency). Northern analysis of the alkaline phosphatase and osteocalcin mRNA from 2T3 cells treated or untreated with 10^{-8} M 1,25-$(OH)_2D_3$ for 2 days in differentiating medium. The fold change in mRNA was calculated as described in *Materials and Methods*.

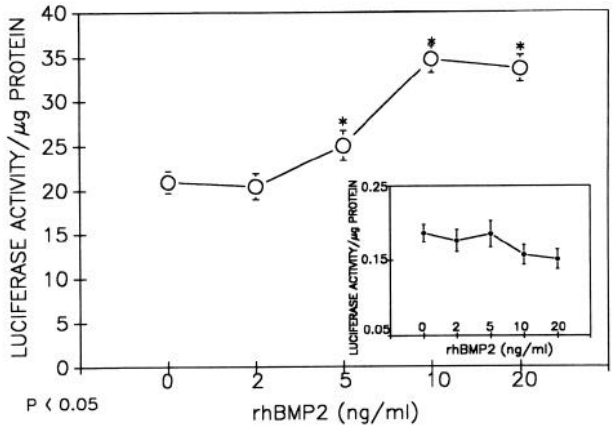

FIG. 11. Autoregulation of BMP-2 promoter by rhBMP-2. 2T3-BMP-2-LUC cells and 2T3-basic LUC cells (*inset*) were treated with 0, 2, 5, 10, and 20 ng/ml rhBMP-2 for 48 h. The cells were subsequently lysed and assayed for luciferase enzyme and protein content. The luciferase enzyme activity was normalized to the protein content. Due to the 100-fold lower basal luciferase enzyme activity in 2T3-basic LUC cells, the graph was plotted using a lower range of ordinate values and is presented as an *inset*.

nodules that subsequently mineralize into bone-like fractal structures. The 2T3 cells express osteocalcin and alkaline phosphatase in a time-dependent manner, and the two mRNAs are regulated by 1,25-$(OH)_2D_3$ in a manner identical to that observed in primary FRC osteoblasts.

rhBMP-2 accelerates the differentiation process of 2T3 cells, as demonstrated by increases in the number, size, and area of mineralized bone nodules formed over time. rhBMP-2 accelerated the appearance of alkaline phosphatase and osteocalcin mRNA. rhBMP-2 also enhances expression of the endogenous BMP-2 gene, thus demonstrating autoregulation of the BMP-2 gene by rhBMP-2.

We have stably transfected BMP-2 promoter-luciferase construct in 2T3 cells and shown that rhBMP-2, in a dose-dependent manner, stimulates BMP-2 promoter activity in the context of chromatin structure in 2T3-BMP-2-LUC cells. This advance indicates that we can now map the rhBMP-2-responsive *cis* DNA elements in the BMP-2 promoter integrated into chromatin. Previous assays on mapping rhBMP-2 response regions have been dependent on transient assays (6).

Many of the properties of 2T3 cells are common to primary FRC osteoblasts (2, 4–8). The timing in 2T3 cells is similar, because mineralized bone nodules can be detected as early as 6 days after confluency (day 0). The fractal bone-like character of the mineralized nodules of 2T3 cultures is identical to that formed in the primary FRC cell system (6, 7).

Although 2T3 cells are aneuploid, they do not grow on soft agar or form tumors in nu/nu mice by 4 months. T-Ag expression driven by the −2736 to +114 BMP-2 promoter fragment does occur during bone cell differentiation and could influence p53 and/or Rb expression and function in these cells. T-Ag expression does not affect mineralized nodule formation or endogenous BMP-2 mRNA expression. The BMP-2 promoter is a fairly weak promoter compared to the osteocalcin promoter. This is reflected in the fact that no bone tumors have been observed in any of the BMP-2 T-Ag mice, whereas osteocalcin promoter T-Ag mice form extensive osteosarcomas (Hundley J., and J. J. Windle, unpublished). 2T3 cells form mineralized bone nodules spontaneously, and rhBMP-2 increases both the rate and net amount of bone formation *in vitro*, at least at levels above 10 ng/ml.

If levels of p53 or Rb are altered by the presence of BMP-2 promoter-driven T-antigen, they do not greatly affect the overall bone cell differentiation program in 2T3 cells. We are presently investigating the role of p53 in BMP-2 expression using various mutant p53 expression vectors stably transfected into 2T3 cells. We believe that T-Ag is produced in these cells at sufficient levels to permit immortalization, but not at high enough levels to drastically alter the differentiation program. This again is reflected in the timing of expression of various differentiation markers in 2T3 cells and is similar to that observed in primary rodent osteoblast cultures (6, 8).

An issue of considerable relevance to our studies is the stages in normal osteoblast differentiation that 2T3 cells may represent. Clearly, as the cells grow, they express very low levels of alkaline phosphatase and osteocalcin. After reaching confluency, they begin an ordered program of multilayering and expression of alkaline phosphatase mRNA, followed by osteocalcin mRNA. Therefore, 2T3 cells are probably representative of an early osteoblastic lineage with the capability of differentiating to form fractal bone-like mineralized structures. We did not observed chondrocytes or adipocytes from 2T3 cells under the present culture conditions at any time.

In our hands, cultures of 2T3 cells seem to exhibit all of the functional properties of primary osteoblast cultures. However, 2T3 cells are available in unlimited supply and can be readily transfected with various genes and then recloned to study the consequences of various manipulations. They represent a valuable resource for studying the program of bone cell differentiation and offer a tool with which to study BMP-2 action from the BMP-2 receptor(s) to the BMP-2 gene as well as downstream genes activated by rhBMP-2.

Acknowledgment

We are indebted to Ms. Thelma Barrios for preparation of the manuscript.

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