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Clonal Osteoblastic Cell Lines from p53 Null Mouse Calvariae Are Immortalized and Dependent on Bone Morphogenetic Protein 2 for Mature Osteoblastic Phenotype

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p53 protein regulates cell cycle progression and its absence will result in unlimited cell divisions required for immortalization of cells. Immortalized osteoblastic cell lines were established from p53 null mouse calvariae of normal phenotype. The clonal murine cell lines demonstrated osteoblastic phenotype as exemplified by alkaline phosphatase enzyme activity. They also express bone morphogenetic protein 2 (BMP2) mRNA. Addition of recombinant BMP2 to these cells dramatically increased the alkaline phosphatase activity in a dose dependent manner. In the absence of BMP2 these cells do not undergo osteoblastic differentiation. Treatment of these cells with recombinant bone morphogenetic protein 2 stimulated differentiated osteoblast formation, as determined by mineralized nodule formation. Thus, these immortalized cells in culture represent osteoblast progenitors that lack p53 protein and respond to osteogenic stimuli. These cell lines offer a model system to study the role of p53 in osteoblastic differentiation and programmed cell death. Also these cells will be useful in studying the effects of p53 on transcriptional regulation of osteoblast specific gene expression. © 1997 Academic Press

p53 is an important biological molecule of multiple functions. Inactivation of wild type p53 protein is associated with malignant transformation. Solid tumors of colon, lung, breast, liver and bone carry mutations in p53 gene (1,2,3,4). p53 protein can act as an inducer of differentiation and also functions as a checkpoint that controls cell cycle arrest at the G_0/G1 stage in response to DNA damage. Previous work on the role of p53 in pre B-cell differentiation demonstrated that p53 protein can induce differentiation to a mature B-cell phenotype (5). Development of osteosarcoma in p53 transgenic mice has suggested the involvement of p53 in bone cell differentiation (6). Involvement of p53 in control of osteoblast differentiation has also been indicated by the fact that elevated levels of p53 is associated with reduced levels of bone gla protein expression in radiation induced murine osteosarcomas (7). p53 gene has also been reported to be rearranged in three human osteosarcoma cell lines and in mouse osteosarcoma, suggesting that p53 inactivation plays an important role in development of osteosarcomas (8).

The role of p53 in programmed cell death has also been documented (9). Absence of wild type p53 was found to be sufficient for immortalization of embryonic fibroblasts derived from p53 deficient mice (9). Mutations in the p53 gene were detected in immortalized rodent cells (10). SV40 T antigen and other oncogene immortalize cells by binding with and inactivating p53 (11,12,13,14). Development and characterization of p53 null mice proved that p53 protein is not necessary for embryonic development but lack of wild type p53 protein makes these animals prone to tumorigenesis (15,16). Immortalized fibroblast cell lines have been established from p53 deficient embryo to study the roles of p53 in cell proliferation and immortalization (16,17).

There is an ongoing interest in the role of p53 in apoptosis of cells, including bone cells. Osteoclast apoptosis has been reported to be critical for bone formation and is influenced by osteogenic factors like estrogen and TGFβ (18). In osteoblasts, high dose of TNF α or ceramide were found to induce apoptosis (19). So far the only osteoblastic cell line available to study the role of p53, is the human osteosarcoma cell line, SaOS-2, which also lacks the retinoblastoma protein. Thus

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using this cell line, one cannot get an effect of p53 alone. In order to establish a model osteoblast cell line lacking only p53, which could be useful for studying the effect of p53 protein on osteoblast apoptosis as well as on transcriptional regulation of bone forming proteins, we isolated and characterized immortalized cell lines from the calvariae of p53 null mouse. We report characterization of an immortalized osteoblast progenitor cell line that responds to osteogenic factors and undergo differentiation, *in vitro* , to form mineralized bone nodules.

MATERIALS AND METHODS

Establishment of clonal cell lines. Calvarial osteoblast cells were generated from the calvarial bone of a four month old p53 null mouse (15). The mouse did not develop any tumors evident at the time of sacrifice. The calvariae was digested sequentially with a mixture containing trypsin and collagenase, a routine method for isolation of primary cell population rich in osteoblastic phenotype (20,21). After plating the mixed cell population from 2nd through 6th rounds of digestions, a part of the cells was stored under liquid nitrogen and the rest of the cells were analyzed for their phenotype after generating single cell clonal populations by plating the cells at a dilution of 1000 cells per 100 cm. tissue culture plates.

Alkaline phosphatase enzyme assay. Alkaline phosphatase specific activity was measured according to methods described previously (22). Briefly, cells were plated in 96 well plates at a density of 3000 cells per well and were allowed to grow for 48 h. Cells were treated with or without increasing amount of recombinant BMP2 for additional 48 h. The cells were harvested using 3 cycles of freezing and thawing in 100 μl 0.05% Triton X-100. Alkaline phosphatase enzyme activity was measured in 20 μl cell lysate using AMP buffer (22). Protein content was measured in 20 μl cell lysate and BioRad (Richmond, CA.) protein assay reagent. Specific activity was calculated using the protein content of the samples and the time of incubation for alkaline phosphatase enzyme activity (15 mins.).

Staining for alkaline phosphatase. The cells were plated in 35mm tissue culture dishes for 24 h and one set of cells were treated with 40 ng/ml recombinant BMP2 for 48 h. The cells were then fixed with 10% formalin solution and were stained using 5-bromo 4-chloro 3-indolyl phosphate and nitro blue tetrazolium (Sigma Chemicals), in a buffer containing 100mM Tris.Cl pH 9.5, 5mM $MgCl_2$, 100 mM NaCl, for 30 mins. at room temperature. The stain was removed by washing with water and the cells were photographed and the stained cells were quantitated by using Java Image Analysis Software (Jandel Scientific, CA.).

Northern analysis. Total RNA was isolated from cells growing in a T150 tissue culture flask using RNAzol B (Cinna/Biotex Laboratories, Houston, Tx.) reagent (21). Poly (A) RNA was isolated using 1.0 ml oligo (dT)-cellulose columns (Stratagene, San Diego, CA.). 3-5 μg poly(A) RNA were used for Northern blot analysis using BMP2 or p53 cDNA as probes (21).

Mineralized bone nodule formation. Cells were plated at 10,000 cells/well in 24-well (1.5 cm diameter / well) tissue culture plates in a medium containing alpha minimum essential medium, 7% fetal calf serum. Following confluency the growth medium was supplemented with 100 μg /ml ascorbic acid and 5mM β-glycerophosphate. Recombinant BMP2 was added at 10, 20 or 40 ng/ml concentration in appropriate wells. Media were changed every 3 days. The cells were fixed at 6, 10 and 15 days and stained for mineralized nodule formation using methods described previously (23). Mineralized bone nodules were quantitated for their average area using Java Image Analysis Software (Jandel Scientific, Corte Madera, CA.).

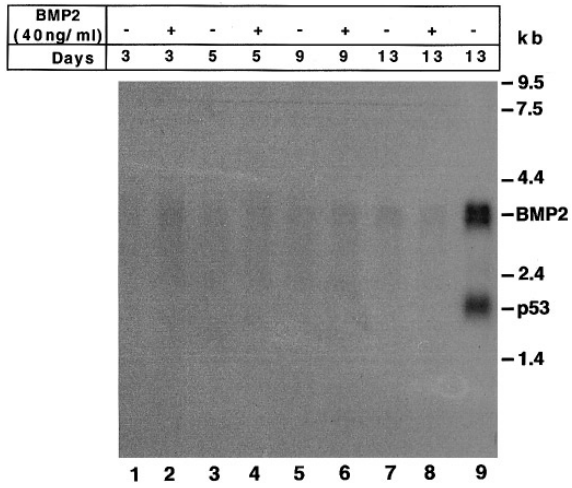

FIG. 1. BMP2 and p53 gene expression by Northern Analysis. Poly (A) RNA isolated from p53 KOB 1 cells (lanes 1–8) and an established murine osteoblast cell, 2T3 (lane 9) were analyzed for the presence of BMP2 and p53 mRNA expression by Northern blot technique, using BMP2 and p53 cDNA as probes. The RNA size markers (Gibco, BRL) and the positions for BMP2 and p53 mRNAs are indicated at right hand side. Lanes 1,3,5,7 and 9 represent untreated and lanes 2,4,6 and 8 represent cells treated with 40 ng/ml BMP2 for 3,5,9 and 13 days, respectively.

RESULTS

Clonal osteblast cell line are useful to study the regulatory mechanism that drive their differentiation. In order to establish clonal osteoblastic cell lines, we used mice containing a homozygous null p53 genotype. The calvarial bone was digested with collagenase and trypsin and the pooled population of cells were plated. From this culture, fifteen clonal cell lines (p53 KOB 1-15) were generated. Three of these fifteen cell lines (p53 KOB 1-3) were characterized for their osteoblastic phenotype. All three cell lines were found to undergo up to 30 cell divisions without changing their orthogonal morphology. These cells could be stored under liquid nitrogen for up to one year and were found to maintain their original phenotype and morphology. They do not form colonies on soft agar, thus suggesting that they are not transformed.

One property of cells in the osteoblast lineage is the expression of certain specific genes such as bone morphogenetic proteins (BMPs) (21). Fig. 1 shows that p53KOB-1 cells express very low levels of BMP-2 mRNA. Treatment of these cells with 40 ng/ml recombinant BMP2 for up to 13 days has no effect on BMP2 mRNA expression. To confirm the absence of p53, we probed the same RNAs with p 53 cDNA. The data showed no expression of p53 mRNA in p53KOB-1 cells while cultures of p53 +/+ murine osteoblasts (2T3, 21) showed high levels of expression of both BMP2 and p53 mRNAs (lane 9, Fig. 1).

These cells were characterized for alkaline phospha-

tase (ALP) expression, both by enzymatic assay (Fig. 2a) and by colorimetric staining (Fig. 2b,c). Extracts of p53KOB-1 cells showed very low level of alkaline phosphatase activity. Stimulation of these cells with recombinant BMP2 increased the ALP activity in a dose-dependent manner (Fig. 2a). To confirm this observation, we also stained control and BMP2-treated p53KOB-1 cells for the expression of ALP protein. As shown in the Fig. 2b, recombinant BMP2 increased the number and area of alkaline phosphatase positive cells. Quantitation of the area of ALP positive cells, showed that BMP2 stimulation of p53KOB-1 cells increased the ALP stained area 118 fold when compared to the untreated p53 KOB 1 cells (Fig. 2c).

Differentiation to form mineralized bone is a phenotype of osteoblastic cells. We have shown earlier that calvarial-derived osteoblasts undergo mineralization in the presence of differentiation medium containing β-glycerophosphate and ascorbic acid (21,23). To test whether p53KOB-1 cells can form mineralized bone nodules *in vitro*, we grew these cells in the presence of β-glycerophosphate and ascorbic acid. As shown in Fig. 3a, these cells did not form mineralized bone nodules spontaneously (Fig. 3a, 0 ng/ml BMP2). However exogenously added BMP2 dose dependently induced differentiation of these cells, as indicated by the formation of mineralized bone nodules (black staining, Fig.3a, 10, 20 and 40 ng/ml BMP2). The induction of mineralized nodule formation could be visualized as early as 6 days, by 20 and 40 ng/ml BMP2. With lower levels of BMP2 (10 ng/ml), it required longer periods (10 days), to initiate osteoblastic differentiation in these cells (Fig. 3a). Quantitation of the nodule area in control and BMP2 treated cells, showed that BMP2, dose dependently, increased nodule formation in these p53 negative osteoblastic cells (Fig. 3b). The quality of mineralized nodules formed by p53 KOB 1 cells treated with 40 ng/ml recombinant BMP2 (Fig. 3c), compares with that formed by primary fetal rat calvarial osteoblastic cells (21,23).

DISCUSSION

Establishment of immortalized cell lines of specific origin is important to study growth and development of these cells in vivo and test hypothesis related to specific genes thought to play a role in differentiation. The availability of cultures of nontransformed immortalized osteoblasts will help to systematically study the biochemistry and molecular biology of osteoblast differentiation. The role of p53 as a tumor suppresser has been extensively studied. Absence of wild type p53 is associated with a variety of tumors and tumor-derived cell lines. However, p53 knockout mice do not have any gross abnormality at birth, although they become susceptible to tumor formation at later stages in their life. Since the early stage of these p53 null mice are normal, it serves as model system, to isolate cell lines of tissue specific origin. Immortalized fibroblasts have been isolated from these mice and it has been demonstrated that the absence of p53 is sufficient for immortalization (9). Here, we report isolation of immortalized cell lines of osteoblast lineage from p53 null mice. These cells are nontransformed and can be passaged indefinitely. These cells express osteoblast specific markers that include BMP-2 and alkaline phosphatase gene. However endogenous BMP-2 expression is very low and exogenously added recombinant BMP-2 did not autoregulate its expression, as had been observed for the primary osteoblastcultures and 2T3 cells (21,24). In this respect, these cells behave differently than primary rat osteoblasts, which show BMP2-induced increase of endogenous BMP2 mRNA (24). SaOS-2 human osteosarcoma cells, negative for funstional p53 and Rb proteins, also contain very low levels of BMP2 mRNA (data not shown). Thus low levels of BMP2 mRNA, found in the p53 KOB1 cells, are not exclusive for this p53 negative cell line alone. The osteoblast like cells and the primary cultures from fetal rat calvarial digest, known to be rich in the osteoblast populations, express high levels of alkaline phosphate enzyme activity. In contrast, the p53 KOB 1 cells, express very low levels of alkaline phosphatase enzyme activity (Fig. 2). However, in these cells, low basal levels of ALP activity could be increased dramatically in response to recombinant BMP2. BMP2-induced increases in ALP activity has previously been shown for primary rat and established mouse osteoblastic cell lines (21). The p53 $-/-$ osteoblast progenitors, also undergo BMP2 induced mineralized bone nodule formation, a phenotype, evident in other osteoblastic cells of rat or mouse origin. The failure to spontaneously undergo osteoblastic differentiation and strong response to exogeneously added BMP2 towards ALP activity and osteoblastic differentiation suggests that these cell lines represent osteoblast progenitor phenotype. The enhanced response of these cell lines, towards exogeneously added BMP2, also shows that these cells express cell surface receptors for BMP2. Thus we have successfully established immortalized clonal osteoblast progenitor cell lines with a p53 null ($-/-$) genotype which have potential to differentiate into mature osteoblasts in the presence of BMP2. It will be of interest to study the effects of other osteogenic compounds on these p53 negative osteoblast progenitors towards the mature osteoblastic phenotype.

Since p53 is known to regulate transcription of a number of genes including osteocalcin and BMP2 (25,26), p53KOB-1 cells will serve as a model system to explore into the mechanisms of regulation of expression of these genes by p53. To date, the only cell system available to study such gene regulations at the transcriptional level, is the human osteosarcoma cell line, SaOS-2. This cell line lacks p53, and thus was the cell of choice to study the effect of p53 on gene expression.

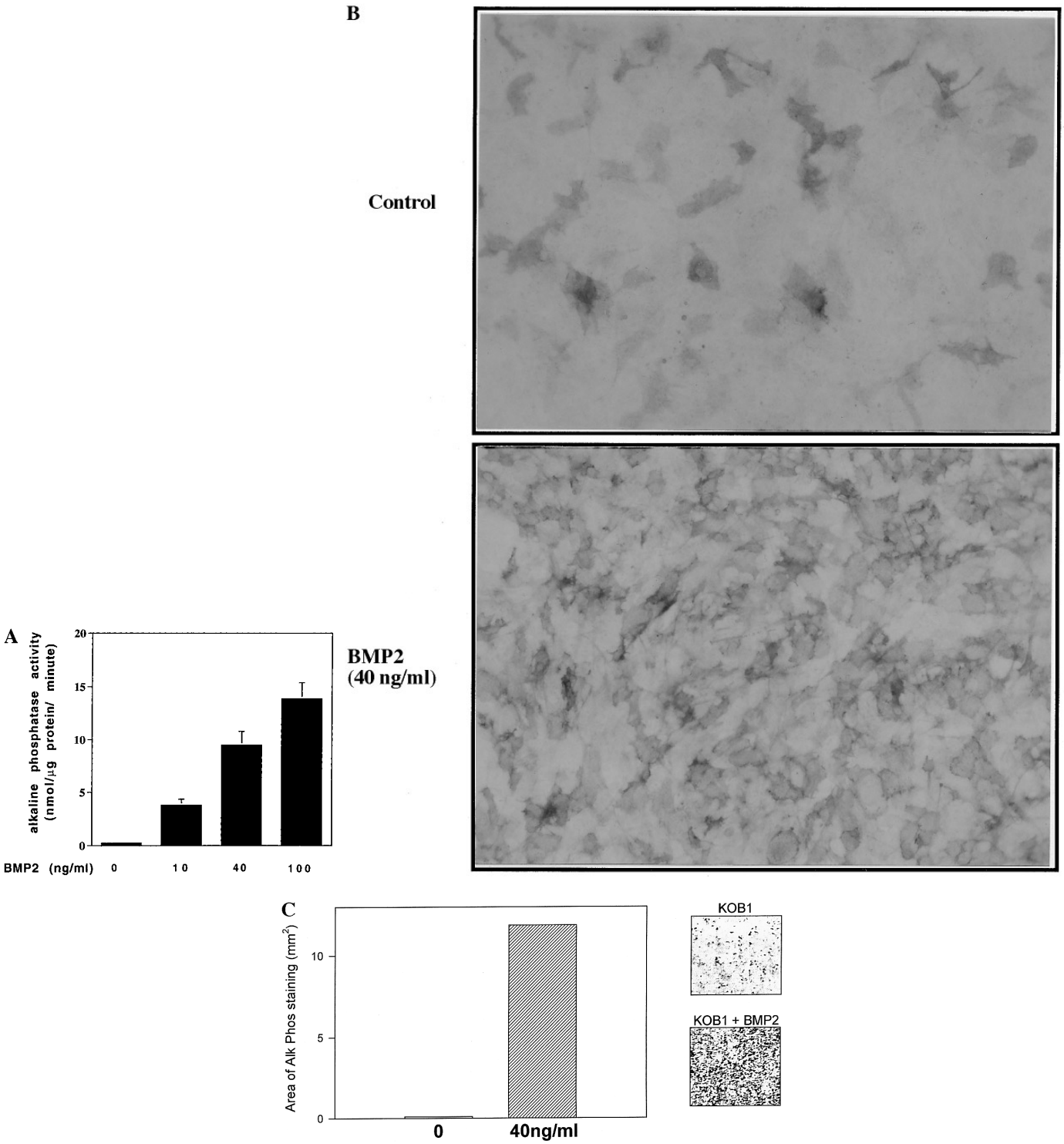

FIG. 2. Expression of alkaline phosphatase (ALP) activity by p53 KOB 1 cells. (a) ALP specific activity (nmol/μg protein/min.) was measured in the cells untreated (0 ng/ml BMP2) or treated with 10, 40 and 100 ng/ml BMP2 using methods described in text. (b) ALP positive cells were stained by method described in text. Top panel represents the control (untreated) cells and the bottom panel represents cells treated with 40 ng/ml BMP2 for 48 h. (c) The ALP positive cells from (b) were quantitated using JAVA image analyzing software. A representative area from the control and BMP2 treated plates, shown in (b) and also scanned in the right panel here, is quantitated for cells stained blue, representing ALP expression in those cells.

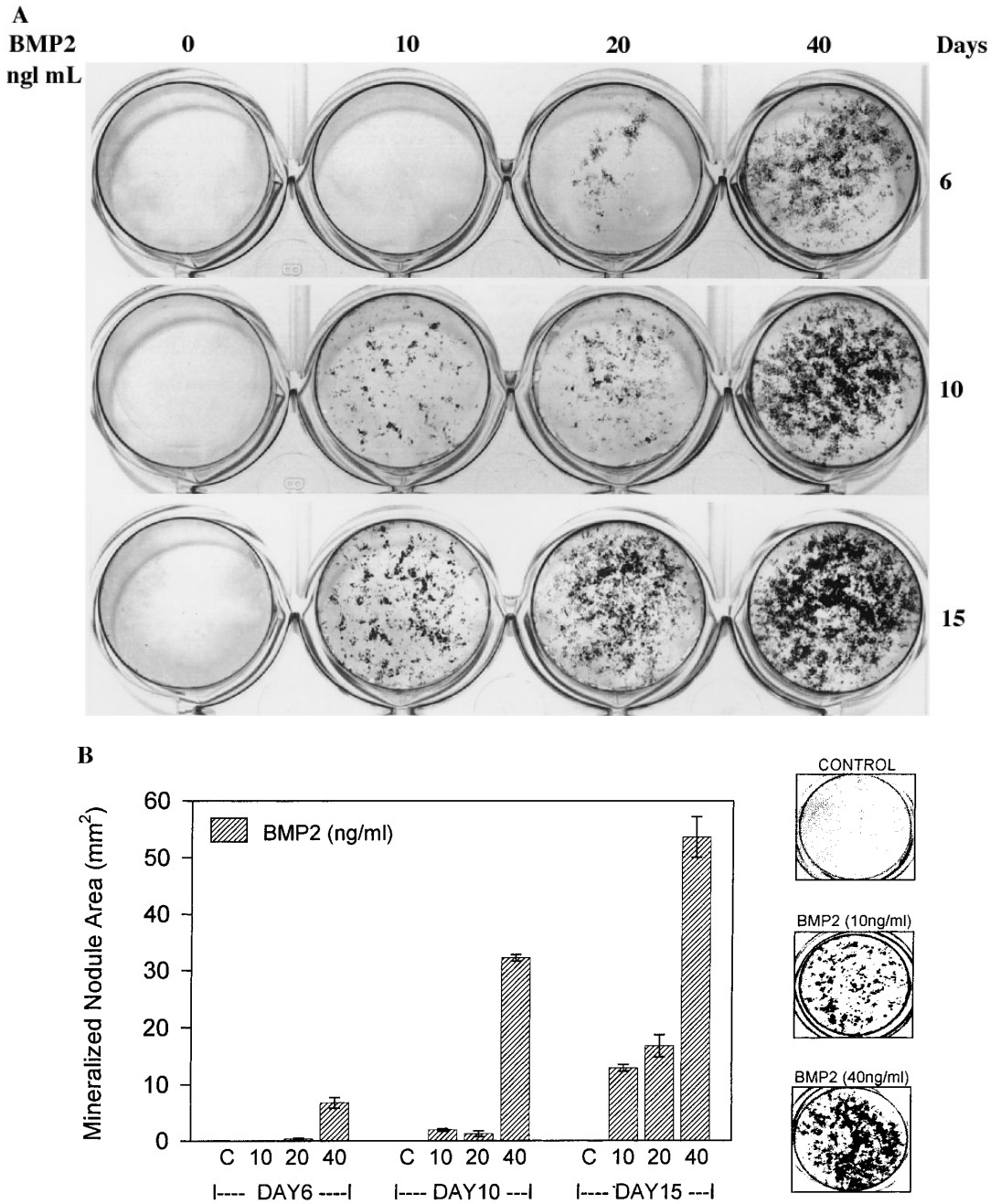

FIG. 3. Mineralized bone nodule formation by p53 KOB 1 cells. (a) The mineralized nodule formation assay was carried out as described in Methods. The cells either untreated (0 ng/ml BMP2) or treated with 10, 20 or 40 ng/ml BMP2 were analyzed for bone nodule formation and cells were fixed and stained for calcium phosphate using Von Kossa stain at 6, 10 or 15 days. (b) Quantitation of the mineralized nodules formed by p53 KOB 1 cells treated with BMP2. The untreated (C, control) cells did not form mineralized nodules even after 15 days. The exogeneously added BMP2-induced nodule formation is quantitated for the area of mineralized nodules/mm^2 of the wells. The quantitation was performed by measuring the black, Von Kossa, stain using Java image analyzing software. Representative wells are scanned at the right hand side of the plot. (c) Light microscopic photograph of untreated (control) p53 KOB1 (top) and cells treated with 40 ng/ml BMP2 (bottom), after fixing and staining with Von Kossa reagent, as described in Methods. The photographs were taken at 40× magnification with cells that were in culture for 15 days in differentiating medium (α-MEM, 7% FCS, 5mM β-glycerophosphate, 100 μg/ml ascorbic acid).

However, the interpretation of the data obtained from such studies, are not straight forward, since the SaOS-2 cells also lack functional retinoblastoma (Rb) protein. Rb, like p53, can influence transcription of a variety of genes, including cell cycle regulatory intermediates (27). Thus, the effects of p53 on gene transcription, obtained in a Rb (−/−) and p53 (−/−) genetic background, may not reflect the regulatory mechanism in-

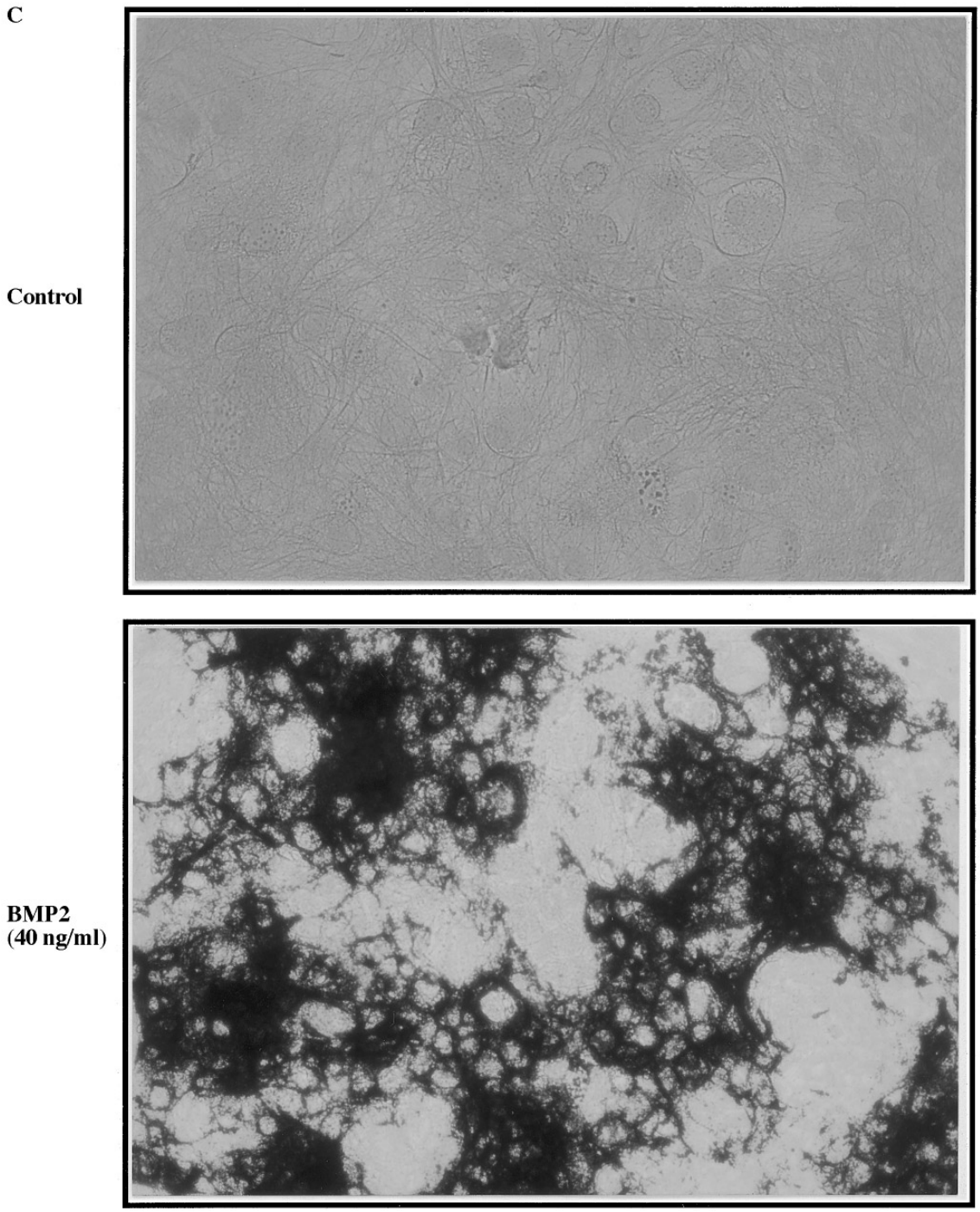

FIG. 3—*Continued*

volving p53 alone, since both p53 and Rb interact in growth control. Therefore, we predict that p53 negative p53KOB-1 pre-osteoblastic cells will be appropriate for studying the role of this tumor suppresser at the level of gene regulation. In particular, the genes that are expressed in a regulated fashion during osteoblast differentiation, in the presence of BMP2 and other bone inducing factors, could be studied in this cell system, more successfully. Another important use for this cell line will be in the area of understanding the role of p53 in apoptosis of the osteoblastic cells. This is an area still very poorly explored, mainly because of the lack of suitable *in vitro* cell systems. Since we have developed the immortalized osteoblast progenitor cell line

that does not express p53 protein but can undergo osteoblastic differentiation, we propose, that these cells can be used to study the role of p53 protein in the programmed cell death in osteoblasts. Altogether we have isolated and characterized an osteoblastic progenitor cell line that can undergo osteoblast differentiation in the presence of recombinant BMP2 and can be used to study the complex differentiation program involved in the generation and development of mature osteoblast phenotype. These cells will also be useful in identifying new proteins that regulate osteoblast differentiation.

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