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# Generation of Mouse Osteoclastogenic Cell Lines Immortalized with SV40 Large T Antigen

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## ABSTRACT

**Progress in the field of osteoclast gene regulation has been hampered significantly by the lack of such cell lines. In this study, mouse osteoclast precursor cells were elicited in an osteoclast-inductive coculture system and immortalized using SV40 large T antigen. One of the osteoclast precursor cell lines (MOCP-5) forms 95% tartrate-resistant acid phosphatase positive ($TRAP^+$) multinuclear osteoclast-like cells (OCLs) in the coculture system. The yield of $TRAP^+$ OCLs was 4.5–7 × $10^4$ cells per 10 $cm^2$ dish. Expression of SV40 large T antigen was visualized in the nucleus of MOCP-5 cells and OCLs by immunohistochemistry. MOCP-5 cells were positive for MoMa-2 antigen and nonspecific esterase but negative for F4/80 antigen. OCLs derived from MOCP-5 cells were able to form extensive resorption bone pits on bone slices. The resorbing activity of the OCLs was comparable to that of authentic mouse osteoclasts. Pit formation was inhibited by salmon calcitonin (CT). Acid production by OCLs was demonstrated by vital staining with acridine orange. The OCLs expressed cathepsin K and CT receptors. MOCP-5 cells could be transfected by a construct that carries the β-galactosidase gene. Transfected MOCP-5 cells expressing β-galactosidase retain the ability to differentiate into OCLs, indicating a useful model for osteoclast gene regulation. To date, the MOCP-5 cell line has been maintained in continuous culture for 23 months and has maintained the capacity to differentiate into osteoclasts throughout this time. In summary, these data show that a stable immortalized osteoclast precursor cell line has been established and that the immortalization with SV40 large T oncogene does not prevent osteoclast precursor cell differentiation. (J Bone Miner Res 1998;13:1112–1123)**

## INTRODUCTION

Osteoclasts, the bone-resorbing cell type, play a key role in normal bone remodeling and in skeletal osteopenia of arthritis, osteoporosis, and periodontal disease. Osteoclasts are derived from hematopoietic stem cells and formed by the fusion of mononuclear osteoclast precursors.[1–3] Unlike most other cell types, cells in the osteoclast lineage do not appear to give rise to tumors, and successful oncogene immortalization of cells in the osteoclast lineage has not been reported. Progress in the field of osteoclast-gene regulation associated with osteoclast differentiation has been hampered significantly because of the lack of suitable osteoclastogenic cell lines. Development of a stable cell line with characteristics of osteoclast precursors would therefore represent a major advance in the study of the molecular and cell biology of the osteoclasts.

As a model system for osteoclast differentiation, the coculture of mouse stromal cells and bone marrow has promoted the understanding of osteoclast biology.[4–10] However, in the coculture system, osteoclasts remain a minor component of a heterogenous population, and furthermore the system is not generally suited for osteoclast gene transfection studies.

To seek an osteoclastogenic cell line, investigators have used hematopoietic cell lines for osteoclast formation. For example, FDCP-mix (interleukin-3–dependent hematopoietic stem cells),[11] and HL-60 (a promyelocytic cell line)[12] have the potential to differentiate into osteoclast-like cells (OCLs). Brandi and coworkers[13] have used FLG 29.1, a

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myelo-monocytic leukemia cell line as a model for osteoclast precursors. Shin and coworkers[14] reported that the cell line derived from the murine macrophage cell line, BDM-1, could form OCLs when cocultured with primary osteoblasts. However, although the cell lines described above have some characteristics of osteoclasts, some of them do not resorb bone, or the number of OCLs was extremely low. None of the cell lines form large enough numbers of osteoclasts to be suitable for biochemical or molecular biological studies, including gene transfections.

Some investigators have employed in vivo immortalization to develop osteoclastogenic cell lines. Chambers et al.[15] established murine osteoclastogenic cell lines from transgenic mice that express a temperature-sensitive mutant of SV40 T antigen. However, only 5–8% of cells present after culture were osteoclastic. Their results suggest that the cell lines generated consisted of precursors that are not yet committed between osteoclasts and macrophages. Boyce et al.[16] produced two lines of transgenic mice in which expression of SV40 large T antigen oncogene was targeted to osteoclasts using the tartrate-resistant acid phosphatase (TRAP) gene promoter. However, the osteoclasts formed in these mice undergo apoptosis. A stable immortalized osteoclast cell line has not yet been developed from the transgenic mice.

In other tissues, some cells have been immortalized in vitro by the introduction of immortalizing oncogenes.[17–20] However, for the immortalization of a cell as rare as the osteoclast, a system that favors the immortalization of osteoclastic precursors over other cell types is needed. The preponderance of osteoclast cells in the coculture system of MS12 osteoclast-inductive stromal cells and bone marrow (Y.-P. Li, unpublished data) suggests that the system can provide conditions in which osteoclast precursors may be selectively immortalized. In the present study, we have employed an immortalizing method by which osteoclast precursor cells in the coculture system were immortalized to develop osteoclast-committed precursor cell line, MOCP-5. The OCLs derived from MOCP-5 cells satisfy major criteria of authentic osteoclast, including TRAP positivity, acid production, presence of calcitonin (CT) receptors, expression of the osteoclast-specific enzyme cathepsin K, and the ability to form resorption pits on dentin slices. Moreover, the cells even after transfection with a reporter gene retain the ability to differentiate into OCLs.

## MATERIALS AND METHODS

### *Establishment of osteoclastogenic cell lines*

The mouse MS12 coculture system was employed for osteoclast formation. In this system the responding cells were cocultured with osteoclast-inductive MS12 stromal cells (Y.-P. Li, unpublished data) as a feeder cell in the presence of vitamin $D_3$ ($VD_3$) in alpha modified essential medium ($\alpha$-MEM) containing 15% fetal bovine serum (FBS) in a humidified atmosphere of 5% $CO_2$ in air. Mouse bone marrow cells in the MS12 coculture system were immortalized using a recombinant retrovirus containing SV40 large T antigen cDNA and the neomycin resistance gene. The retrovirus was produced by $\Psi$-tex36 cells,[21] kindly provided by Dr. Brown M, Dana Farber Cancer Institute. Six-week-old mouse bone marrow cells were cocultured with MS12 stromal cells in the presence of $VD_3$ for 3 days, and were then incubated for 24 h with the $\Psi$-tex36-conditioned medium (50%, v/v) plus 8 $\mu$g/ml Polybrene (Aldrich Chemical Co., St. Louis, MO, U.S.A.). Treated bone marrow cells were cultured in the presence of the antibiotic G418 (200 $\mu$g/ml). The G418-resistant cells were passaged five times in 400 $\mu$g/ml G418, and were cloned by limited dilution. The cells derived from cloned cell lines were examined for TRAP activity using a TRAP stain kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) before and after coculture with MS12 cells. The four clonal cell lines that produced TRAP-positive ($TRAP^+$) OCLs were selected for further analysis. One of the four clonal cell lines is designated MOCP-5. The cells were routinely subcultured by scraping with a plastic scraper (Costar, Cambridge, MA, U.S.A.) after treatment with 0.05% trypsin and 0.02% EDTA for 5 minutes.

### *Preparation of OCLs*

MOCP-5 cells ($5 \times 10^2$ cells/well) were cocultured with $2 \times 10^4$ MS12 stromal cells in the presence of $10^{-8}$ M $VD_3$ for 9 days in 24-well plates. $TRAP^+$ OCLs derived from MOCP-5 were enriched by incubation with 0.2% trypsin for 4 minutes (Fig. 1C). After enzyme treatment, more than 95% of the MS12 stromal cells were detached from culture dishes, but all $TRAP^+$ OCLs remained bound. The enriched $TRAP^+$ OCLs were then detached with a plastic scraper after treatment with 0.05% trypsin and 0.02% EDTA for an additional 5 minutes. The OCL preparation from all 24 wells was pipetted onto dentin slices for dentin resorption assays.

### *Preparation of mouse osteoclasts*

Mouse osteoclasts (MOCs) were prepared from 3-day-old strain 129 mice as previously described with modification.[22] The femorae, tibiae, and humeri were cleaned of soft tissues, cut crosswise at the diaphysis, split longitudinally, and cultured in $\alpha$-MEM containing 10% FBS (pH 7.0). The resulting suspension containing bone fragments and cells was gently pipetted and allowed to settle for 10 s to eliminate large bone fragments. An aliquot (1/10) of cell suspension prepared from 10 animals was pipetted onto dentin slices for dentin resorption assays.

### *Assay for dentin resorption*

Dentin resorption assays were performed according to the method of Tamura et al.[22] with slight modifications. The OCL preparation from one 24-well plate, or an aliquot (1/10) of MOC preparation was pipetted onto one dentin slice (4 mm diameter, ~200 $\mu$m thick) and placed in a 96-well culture plate with 0.2 ml of $\alpha$-MEM containing 10% FBS (pH 6.9). After a settling period of 60 minutes at 37°C, the slices were removed and gently washed in fresh $\alpha$-MEM, leaving an adherent cell population enriched in

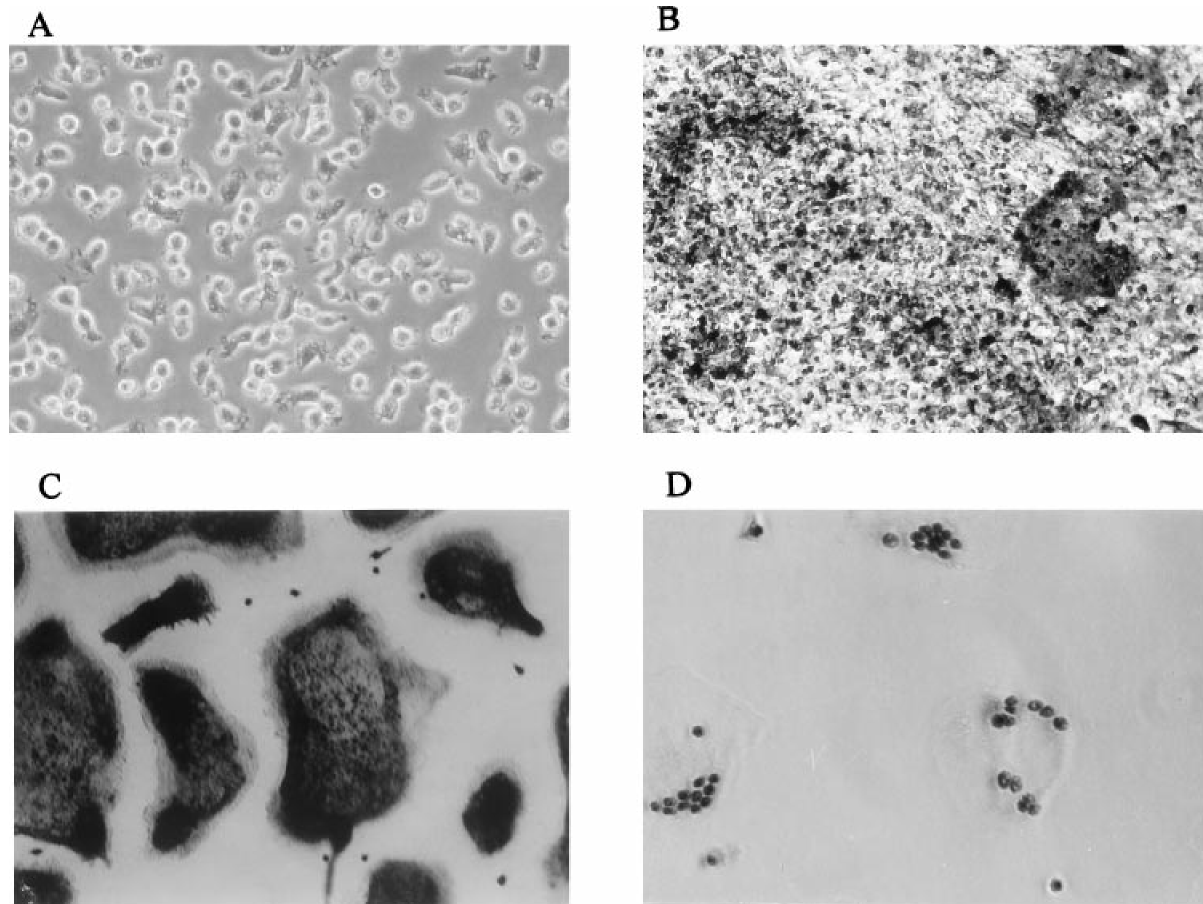


**FIG. 1.** The morphology and properties of MOCP-5 cells. (A) Morphology of the undifferentiated MOCP-5 cell line. (B) After 9 days of coculture with MS12 cells, more than 95% TRAP$^+$ preosteoclasts and multinuclear OCLs are present. (C) TRAP$^+$ OCLs differentiated from MOCP-5 cells enriched by limited 0.2% trypsinization. Note that most TRAP$^+$ OCLs were spread out on the plastic dish. (D) Expression of SV40 large T antigen visualized in nuclei of OCLs using a monoclonal antibody to large T antigen and immunohistochemical staining. Original magnification (A, C, D) ×200, (B) ×100.

osteoclasts. The slices were then placed into wells of 24-well plates (1 slice/well) containing $\alpha$-MEM with 10% FBS (pH 6.9) either with or without $10^{-8}$ M salmon calcitonin (sCT; Sigma Chemical Co.). After 3 days of incubation, the slices were placed for 10 minutes in 1 M $NH_4OH$ and were sonicated to remove the cells. The cell-free slices were stained in 1% toluidine blue in 1% sodium borate for 1 minute[23] or by immunostaining using an anticollagen type I polyclonal antibody as described by Shibutani and Heersche.[24] Using antibody staining, resorption pits are visualized as gray-black stain areas. Some of the resorption lacunae were further confirmed by scanning electron microscopy (SEM). Dentin slices were prepared for SEM examination by sonication in distilled water for 5 minutes, and dehydration through a graded ethanol series. Slices were coated with gold and examined with a Hitachi S-520 SEM operating at 15 kV (Hitachi, Tokyo, Japan). All three methods described above gave comparable results.

For enumeration of TRAP$^+$ OCLs on dentin slices, three of the slices from each group were fixed in 5% formalin and stained for 15 minutes for TRAP activity after 1 day of culture. The total number of TRAP$^+$ OCLs (three or more nuclei per cell) on the slices was counted under light microscopy.

### *Quantitation of resorption pits*

The total surface area of pits on each dentin slice was measured under a 10× objective light microscope equipped with an eyepiece micrometer as described by Tamura et al.[22] Triplicate slices were assessed per experiment. Data are expressed as mean ± SEM. The statistical significance between the control and the experimental groups was assessed using Student's *t*-test ($p < 0.05$ was considered to be significant).

### *mRNA phenotypic assessment*

TRAP$^+$ OCLs that differentiated from the MOCP-5 cells were examined further by mRNA phenotypic assessment. MOCP-5 cells ($2 \times 10^4$ per 10 cm dish) and MS12 cells ($1 \times 10^6$ per 10 cm dish) were cocultured in $\alpha$-MEM

containing 10% FBS in the presence of $10^{-8}$ $VD_3$ at 37°C for 9 days. After culturing, the OCL population was enriched by limited trypsin digestion as described above and washed in PBS. The purified OCLs were used for the preparation of total RNA by the method of Chomczynski and Sacchi.[25] Northern blot analysis of murine cathepsin K and CT receptor mRNA was performed as described.[26] Total cell RNA was electrophoresed (15 $\mu$g/lane) and hybridized with a $^{32}P$-labeled fragment of 1.6 kb of human cathepsin K cDNA[27] or 500 bp human CT receptor cDNA (ATCC#105683) as probes.

### *Assay of DNA transfection and* $\beta$-galactosidase

Transfection of MOCP-5 cells was performed using lipofectin (Life Technologies Inc., Grand Island, NY, U.S.A.) as described by the manufacturer. One day before transfection, MOCP-5 cells ($1 \times 10^5$ per dish) that had undergone 4 days of culture in the MS12 coculture system were replated in 60 mm culture dishes with MS12 ($1 \times 10^6$ per dish) as feeder cells in $\alpha$-MEM containing 10% FBS and $10^{-8}$ M $VD_3$. pSV-$\beta$-galactosidase (Promega Biotech Co., Madison, WI, U.S.A.), which carries the $\beta$-galactosidase gene downstream of the LTR of the Rous sarcoma virus, was transfected. After an additional 3 days of culture in this system, a $\beta$-galactosidase assay was performed using a kit (Invitrogen, San Diego, CA, U.S.A.) as described by the manufacturer. To establish stable transfected cell lines, MOCP-5 cells were cotransfected with 5 $\mu$g of pSV-$\beta$-galactosidase constructs and one tenth that amount of pZeoSV (Invitrogen). The cells were allowed to recover for 48 h and then selected with 200 $\mu$g/ml of the Zeocin (Invitrogen) for 2 weeks. The surviving colonies of cells carrying these constructs were pooled, expanded, induced to osteoclasts in the coculture system, and assayed for $\beta$-galactosidase activity on day 4 as described above.

### *Immunohistochemistry*

Two rat monoclonal antibodies (F4/80 and MoMa-2, Biosource directed against mouse macrophage[28,29]) and a rabbit anticathepsin K antiserum were used to examine the phenotype of MOCP-5 cells. Anti-cathepsin K antisera was produced in rabbits by immunizing with recombinant cathepsin K protein expressed in *E. coli* using the QIA express system (Qiagen, Chatsworth, CA, U.S.A.). The DAB peroxidase substrate kit and the TMB substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.) were used for staining cells. DAB yields a reddish brown stain, and TMB yields a blue stain. F4/80[28] and MoMa-2[29] are known to bind to antigens of murine monocytes and macrophages. Antibody reactivity was visualized using an ABC kit according to the manufacturer's instructions (Vector Laboratories). After coculture with MS12 stromal cells in the presence of $VD_3$, OCLs were reacted with the above antisera. Expression of SV40 large T antigen was visualized in the nucleus of MOCP-5 cells and OCLs by immunohistochemical staining using a monoclonal antibody (Oncogene Science, Inc, Cambridge, MA, U.S.A.) and a mouse ABC kit (Vector Laboratories). AEC peroxidase substrate (Vector Laboratories) was used for staining cells; AEC yields a red stain.

### *Enzyme histochemistry*

TRAP was used as a marker for osteoclasts. Preosteoclasts and OCLs derived from MOCP-5 cells were fixed and stained for TRAP activity using a commercial kit (Sigma Chemical Co.) according to the manufacturer's instructions. $TRAP^+$ cells appeared as dark red cells. Nonspecific esterase (NSE) was used as a marker for mononuclear phagocytes. For determination of NSE-positive cells, cultures were fixed and stained using a commercially available kit (Sigma Chemical Co.) according to the manufacturer's protocol. The presence of a black precipitate indicated positive reactivity.

### *Assessment of acid production*

Acid production was determined using acridine orange, according to the method of Baron et al.[30] with a slight modification. OCL preparations and MOC preparations were placed on dentin slices and incubated for 3 h in a $CO_2$ incubator. They were subsequently incubated in $\alpha$-MEM containing 5 $\mu$g/ml of acridine orange (Sigma Chemical Co.) for 15 minutes at 37°C, washed, and chased for 10 minutes in fresh media without acridine orange. The OCL and MOC preparation was observed under a fluorescence microscope with a 490 nm excitation filter and a 525 nm arrest filter. In other experiments, the OCL preparation was further incubated in $\alpha$-MEM containing 20 mM $NH_4Cl$ and 5 $\mu$g/ml of acridine orange for 15 minutes and then reexamined. The preparation was then returned to a medium with 5 $\mu$g/ml of acridine orange alone, incubated another 15 minutes, washed, chased in medium without acridine orange, and reobserved.

## RESULTS

### *Establishment of osteoclast precursor cell lines*

Osteoclast precursors are rare in bone marrow cells. To ensure successful immortalization, we have used the MS12 coculture system through which osteoclast precursors can be generated for immortalization. Bone marrow cells from 6-week-old mice were cocultured with MS12 cells in the present of $VD_3$ for 3 days. The treated cells then were induced to immortalize by infection with a recombinant retrovirus containing SV40 large T antigen cDNA for 24 h. The cells were cloned by limited dilution. Seventy-two MS12-conditioned medium-dependent cell lines were established. Of the 72 cell lines examined, none was $TRAP^+$ when they were cultured alone. However, when cocultured with MS12 cells in the presence of $10^{-8}$ M $VD_3$, four cell lines were induced to generate $TRAP^+$ presumptive OCLs and preosteoclasts (Fig. 2). Other cell lines failed to differentiate to $TRAP^+$ OCLs or $TRAP^+$ preosteoclasts.

One of the osteoclast precursor cell lines, MOCP-5 (Fig. 1A), forms $> 95\%$ $TRAP^+$ OCLs and preosteoclasts (Fig. 1B). The cells that differentiated into preosteoclasts or osteoclasts and that were $TRAP^+$ could be enriched to 95% purity by limited trypsinization (Fig. 1C). Expression of large T antigen was detected by immunocytochemistry with strong nuclear staining of both MOCP-5 cells and the

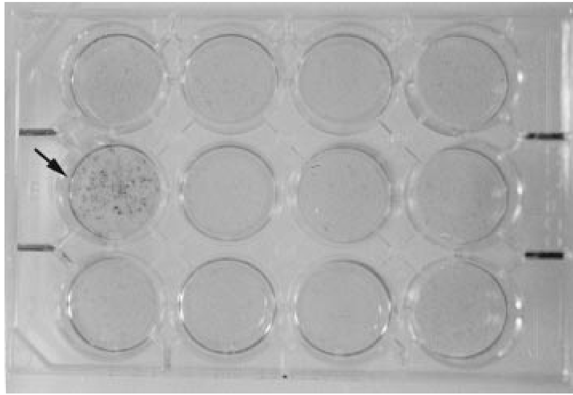

**FIG. 2.** Generation of osteoclastogenic cell lines immortalized by SV40 large T antigen. The G418-resistant cells were cloned by limiting dilution and then tested for their ability to generate osteoclasts. Note that one cell line, termed MOCP-5, could be induced to generate TRAP$^+$ multinuclear OCLs (the arrow indicates MOCP-5).

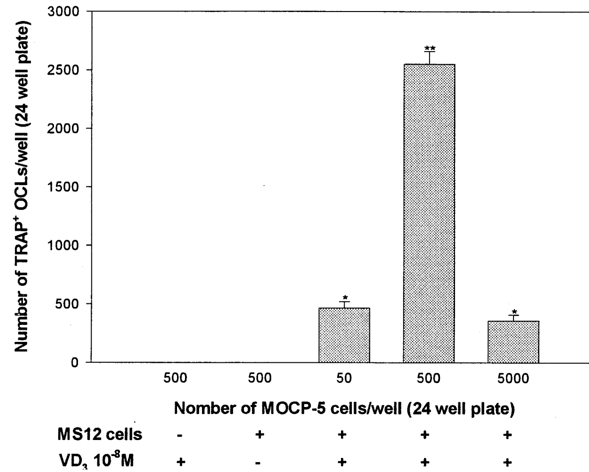


**FIG. 3.** Dose effect of the number of MOCP-5 cells on the formation of TRAP$^+$ OCLs. Several concentrations of MOCP-5 cells were cocultured with MS12 ($2 \times 10^4$ per well, 24-well plate) in the presence of $10^{-8}$ M $VD_3$. After the cells had been cultured for 9 days, TRAP$^+$ OCLs were counted. Data are expressed as the mean ± SEM for four cultures. Significant difference was based on Student's *t*-test, $**p < 0.01$, $*p < 0.05$ versus control (in the absence of the MS12 cells).

TRAP$^+$ OCLs derived from them (Fig. 1D). TRAP$^+$ OCL attachment to plastic is resistant to 4 minutes of 0.2% trypsin digestion. In contrast, MS12 cells were detached by digestion. Thereby, TRAP$^+$ OCLs could be enriched using the limited trypsin digestion (Fig. 1C). The enriched TRAP$^+$ OCLs could be harvested with a plastic scraper after treatment of 0.05% trypsin and 0.02% EDTA for 5 minutes. Note that most TRAP$^+$ OCLs formed on the plastic dish spread out (Fig. 1C). The phenomenon was observed in the marrow-stromal cell coculture system as well.[31] MOCP-5 cells have been routinely characterized by their ability to produce OCLs every three passages. To date, the MOCP-5 cell line has been maintained in continuous culture for 23 months and has fully maintained the capacity to differentiate into OCLs throughout this time. The results reported in this paper are from passages 28–42. Our results demonstrate that MOCP-5 cells have been immortalized but maintain the ability to generate the differentiated phenotype.

### *Growth properties and morphology*

The MOCP-5 cells were plastic adherent and grew on tissue culture dishes with a doubling time of about 2 days. In monolayer, these cells were round in shape and small in size (Fig. 1A). The mononuclear cells have a low nuclear:cytoplasmic ratio. The doubling time of MOCP-5 cells was decreased to about 20 h when the cells were cultured in the MS12 coculture system. This indicates that contact between MOCP-5 and MS12 stromal cells is not only necessary for MOCP-5 to differentiate into osteoclast cells but is also important for their proliferation. The optimal number of seeded MOCP-5 cells was $5 \times 10^2$ cells per well in a 24-well plate (Fig. 3). TRAP$^+$ OCL formation did not occur if the MOCP-5 cells were cultured in the absence of MS12 stromal cells and also was dependent upon the presence of $VD_3$ (Fig. 3). When MOCP-5 cells ($1 \times 10^3$ per well, 12-well plate) were cocultured with MS12 stromal cells ($4 \times 10^4$ per well) in the presence of $10^{-8}$ M $VD_3$, TRAP$^+$ OCLs were detected after 3 days of culture and then gradually increased from day 3–9 (Fig. 4). Interestingly, when the same number of MOCP-5 cells that had been previously cultured for 4 days in MS12 coculture system were tested, the time for the maximum formation of osteoclast was 4–5 days (Fig. 4). The period of preosteoclast (TRAP$^+$ mononuclear cell) production from MOCP-5 was about 3–4 days. The yield of TRAP$^+$ OCLs was $4–7 \times 10^4$ cells per 10-cm dish. The yield of TRAP$^+$ mononuclear cells was $6.5 \times 10^6$ cells per 10-cm dish. The differentiation efficacy of MOCP-5 cells into TRAP$^+$ OCLs was estimated by counting numbers of cells or number of cell nuclei.[14] The proportion of the total number of TRAP$^+$ cells to the number of MOCP-5 cells was 95%. Of the total number of nuclei in the culture, 28% were contained the TRAP$^+$ OCLs.

### *Immunohistochemistry and enzyme histochemistry*

To obtain further details of the characteristics of MOCP-5 as an osteoclast precursor cell line, we examined, using immunocytochemical staining, the expression of two surface antigens (i.e., MoMa-2 and F4/80) that are known as macrophage markers. As shown in Fig. 5A, MOCP-5 cells expressed MoMa-2 antigens. However, TRAP$^+$ OCLs were MoMa-2 negative (Fig. 5A). Both MOCP-5 cells and TRAP$^+$ OCLs were F4/80 negative (Fig. 5B). Cathepsin K, a novel cysteine proteinase, has been described recently by five groups, including us,[26,32–35] who demonstrated its specific production by osteoclasts. Expression of cathepsin K was detected in both TRAP$^+$ OCLs and TRAP$^+$ mononuclear cells (preosteoclast cells) by immunohistochemical localization (Fig. 5C). Expression of SV40 large T antigen

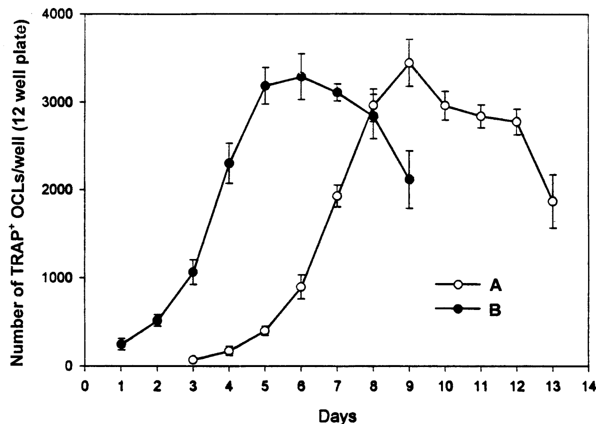


**FIG. 4.** Time course for the formation of $TRAP^+$ OCLs. (A) Undifferentiated MOCP-5 cells ($1 \times 10^3$ per well, 12-well plate) were cocultured with MS12 stromal cells ($4 \times 10^4$ per well) in the presence of $10^{-8}$ M $VD_3$. $TRAP^+$ OCLs were first detected after 3 days of culture and gradually increased from days 3–9. (B) The same number of MOCP-5 cells that had been cocultured with MS12 cells for 4 days were then replated as in (A), and the time for maximum formation of OCLs was reduced to 4–5 days. Data are expressed as the mean ± SEM for three cultures.

was also visualized in the nuclei of both undifferentiated and differentiated MOCP-5 cells by immunohistochemistry (Fig. 1D).

The MOCP-5 cells initially were TRAP negative. Differentiated MOCP-5 cells demonstrated TRAP activity after 3 days of coculture with MS12 cells in the presence of $10^{-8}$ M $VD_3$, and this activity was greatly enhanced after 5 days coculture, reaching a peak on day 9. As shown in Fig. 4B, more than 95% of MOCP-5 cells were $TRAP^+$ on day 9. Undifferentiated MOCP-5 cells were NSE positive (Fig. 5D), a marker enzyme for the monocyte-macrophage lineage. NSE activity was greatly reduced in $TRAP^+$ mononuclear preosteoclasts and OCLs (Table 1). These results suggest that the MOCP-5 cell line shows a common lineage with macrophage but is committed to osteoclast differentiation.

### *Bone resorption*

Figure 6A shows that OCLs derived from MOCP-5 cells formed extensive bone-resorbing pits. Pit formation by OCLs was inhibited by salmon CT (Fig. 6B). Several parameters have been employed in studies of osteoclasts to quantitate pit formation: number of pits, surface area of pits, and depth or volume of pits.[36] The number of pits per slice and the number of pits per osteoclast have recently been found to be useful parameters of the resorbing activity of osteoclasts.[37,38] However, since resorption pits formed by OCLs in these experiments were excessive, and it was difficult to distinguish an individual pit from neighboring pits (Fig. 6A), neither of these was a suitable parameter in our assay system. It has been shown that the resorbed surface area is closely correlated with the number of resorption pits.[23] Therefore, in the present study, the total resorbed surface area was used to quantitate resorption.

The resorbing activity of OCLs was compared with that of MOCs. In our OCL preparations, more than 500 $TRAP^+$ OCLs and 2500 $TRAP^+$ mononuclear preosteoclasts were placed on each dentin slice. As shown in Fig. 7A, the total area resorbed by about 500 OCLs after 72 h incubation was ~7.5 $mm^2$ and equaled 42% of the entire surface. Using fresh mouse long bone osteoclast preparations, however, only about 45 MOCs were placed on each slice. The plane area resorbed by about 45 MOCs was 0.38 $mm^2$, and it was 2.4% of the entire surface. These results indicate that the resorbing activity of the MOCP-5–derived OCLs (0.015 $mm^2$/OCL) was comparable to that of authentic MOCs (0.008 $mm^2$/cell). The extent of resorption varied from small, discrete pits to very large, overlapping excavations. This suggests that both OCLs and mononuclear preosteoclast cells contribute to dentin resorption (Fig. 7A). Adding $10^{-8}$ M sCT caused a marked decrease in pit formation by both $TRAP^+$ OCLs and MOCs (Fig. 7). Mouse stromal cells cultured alone were unable to excavate pits in the dentin (data not shown). Some resorption lacunae were confirmed by using a Hitachi S-520 SEM. Typical resorption pits were observed on the dentin surface by SEM. Exposed collagen fibrils are apparent in the base of the lacunae (Fig. 6C).

### *Expression of cathepsin K and CT receptor in differentiated MOCP-5 cells*

Expression of cathepsin K in OCLs was further confirmed by Northern blot analysis. Strong and specific signals were detected on preparation of OCLs. The arrow indicates the 1.6 kb band of cross-hybridization (Fig. 8A). In contrast, no signal was detected on preparations of undifferentiated MOCP-5 cells, MS12 stromal cells, and other tissues, except that a very low expression of cathepsin K was detected on the preparation of kidney. This result demonstrated that mouse cathepsin K was specifically and highly expressed in OCLs.

CT receptor is another marker for differentiated osteoclasts. Northern analysis using a human CT receptor cDNA probe demonstrated that CT receptor was expressed on OCLs. The arrow indicates the 4.2 kb band of cross-hybridization (Fig. 8B). In contrast, expression of CT receptor was not detected in undifferentiated MOCP-5 cells, MS12 stromal cells, and other tissues (Fig. 8B).

### *DNA transfection*

To test if MOCP-5 cells could be used for gene transfection studies, the cells were transfected with a construct that carries the $\beta$-galactosidase gene. The strong blue staining indicated that the transfection was successful (Fig. 9). It is notable that both transient and stably transfected MOCP-5 cells that expressed $\beta$-galactosidase retain the ability to differentiate into OCLs (Fig. 9B). That MOCP-5 cells can be transfected suggests that they can be used to study osteoclastgene regulation. As show in Fig. 9C, a stable transfected cell line that expressed $\beta$-galactosidase was established.

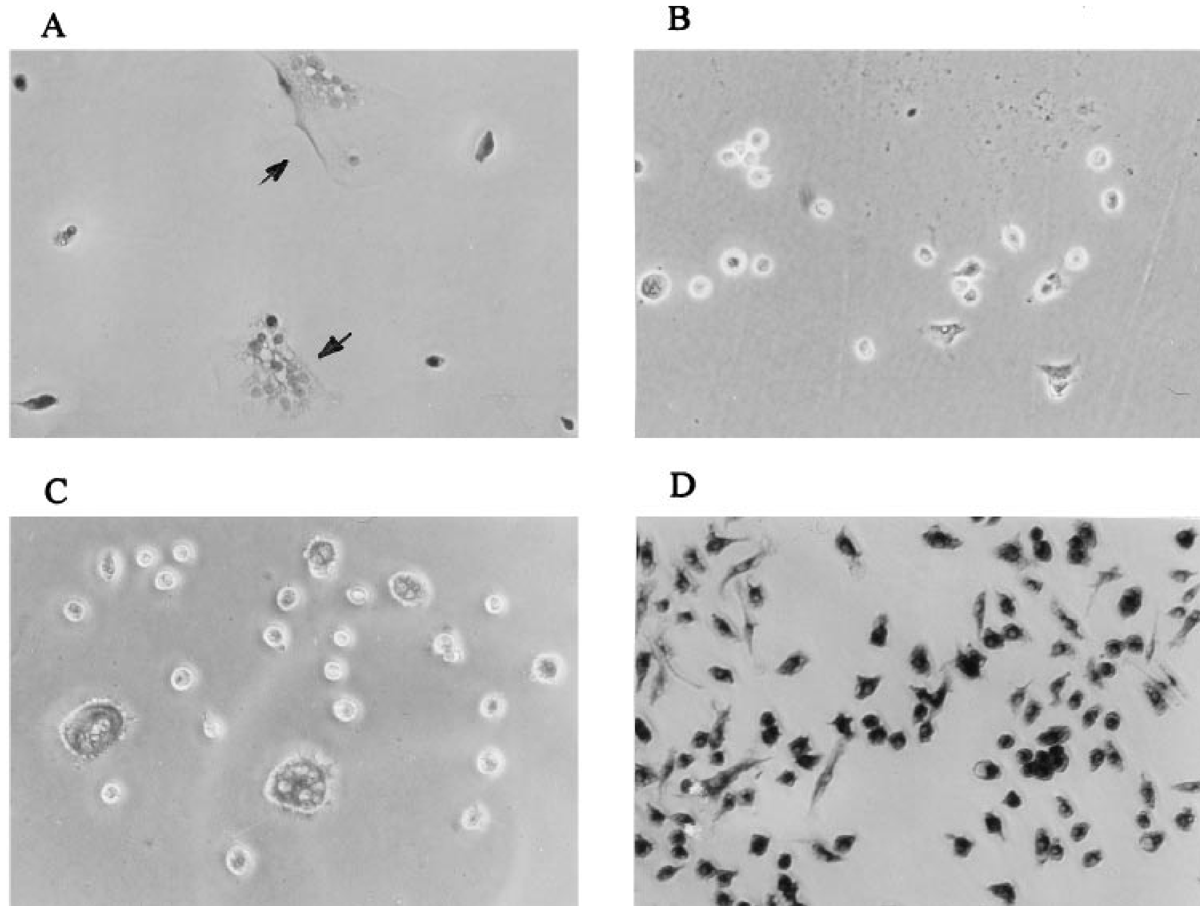


**FIG. 5.** Phenotypic analysis of MOCP-5 cells by enzyme and immunohistochemistry. (A) Mononuclear MOCP-5 cells were MoMa-2 antigen positive, but OCLs were negative. The arrow indicates the OCLs. (B) Undifferentiated MOCP-5 cells were not labeled by F4/80 antibody. (C) Expression of cathepsin K in preosteoclasts and OCLs, as indicated by reddish brown stain. (D) Undifferentiated MOCP-5 cells were NSE positive. (A–D) original magnification, ×200.

TABLE 1. EXPRESSION OF OSTEOCLAST AND MACROPHAGE-ASSOCIATED PHENOTYPE MARKERS DURING DIFFERENTIATION OF MOCP-5 OSTEOCLAST PRECURSORS INTO OSTEOCLASTS

| | *MOCP-5* | *Preosteoclast* | *OCLs* |
|---|---|---|---|
| CTR | − | + | + |
| TRAP | − | + | + |
| Cathepsin K | − | + | + |
| MoMa-2 | + | −/+ | − |
| NSE | + | −/+ | − |
| F4/80 | − | − | − |

### *Evidence that OCLs actively produce acid compartments*

Acid production is one of major functions of the osteoclast.[30] Osteoclasts release acid from their ruffled border to dissolve the inorganic phase of bone. The presence of acid compartments in the OCLs was demonstrated by vital staining with acridine orange under a fluorescence microscope. Upon incubation with acridine orange, intense orange fluorescence was seen as large uniformly brilliant orange discs in OCLs (Fig. 10A). The acridine orange-positive fluorescence in the OCLs diminished with the addition of $NH_4Cl$ indicating specific staining of acridine orange (Fig. 10B). These data demonstrate that OCLs, like authentic MOCs (Fig. 10C), actively produce acid compartments.

## DISCUSSION

One of the major problems in bone cell biology research has been the lack of a stable osteoclastogenic cell line. Several cell lines capable of osteoclastic differentiation have previously been reported. However, such cell lines demonstrate at best only occasional bone resorption, suggesting either that osteoclastic differentiation was incomplete or that osteoclasts represent a very small proportion of the cells present.

We have used the SV40 large T antigen gene for immortalizing osteoclast precursor cells. A stable osteoclast precursor cell line, MOCP-5, was developed. It was not unexpected that fully differentiated $TRAP^+$ osteoclast cell lines could not be immortalized in the study, since postmitotic

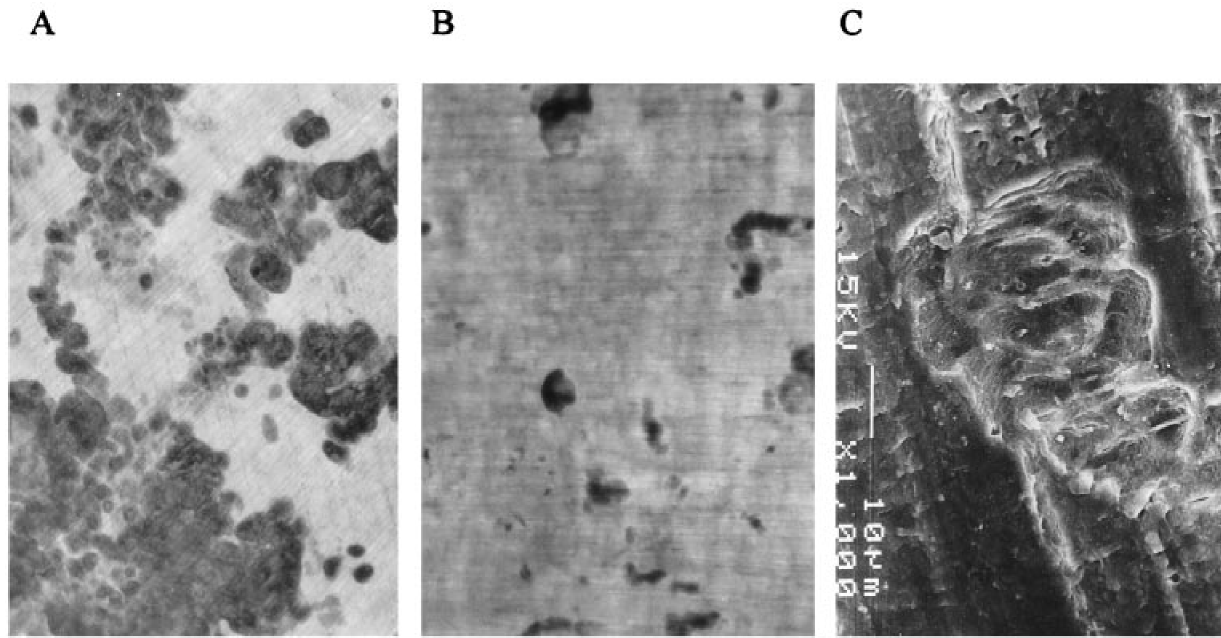


**FIG. 6.** Demonstration of resorption pits formed by TRAP$^+$ OCLs. Preformed OCLs were applied to dentin slices and cultured for 3 days in the absence (A) or presence (B) of $10^{-8}$ M sCT. Cells were then removed and resorption pits stained using an anticollagen type I polyclonal antibody. (C) Some resorption lacunae on dentin slices were confirmed using SEM. A number of typical resorption pits were observed on the dentin surface. Exposed collagen fibrils are apparent in the base of the lacuna. Original magnification (A, B) ×200, (C) ×1000.

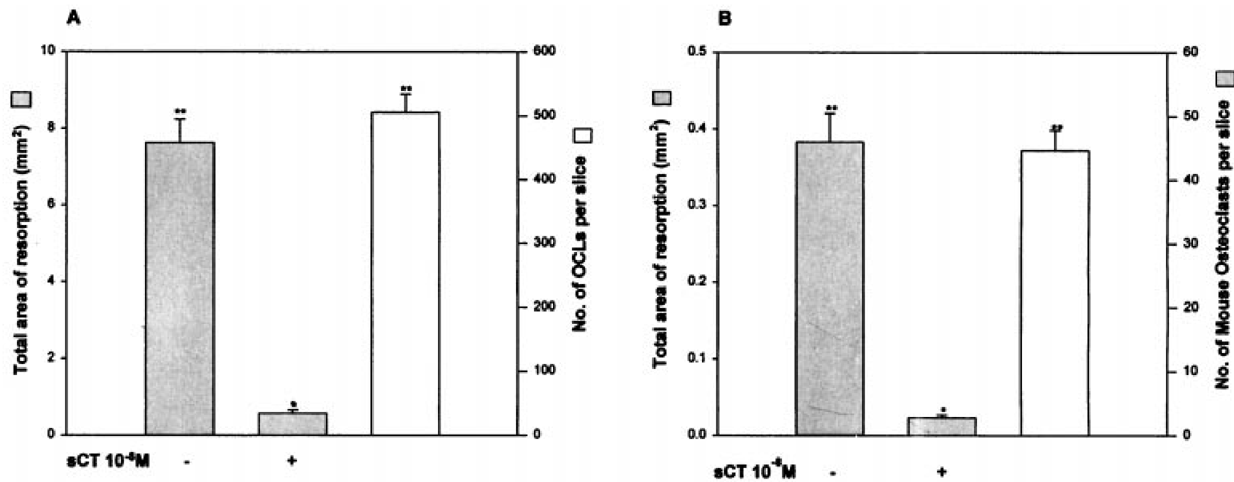


**FIG. 7.** Comparison of dentin pit formation by MOCP-5 derived OCLs and isolated mouse long bone osteoclasts. (A) OCLs or (B) mouse osteoclast preparations were cultured on dentin slices for 3 days. The plane area resorbed was measured as described in the Materials and Methods. Numbers of OCLs or MOCs were counted after 20 h of culture as described in the Materials and Methods. Data are expressed as mean ± SEM of quadruplicate cultures. Significant difference was determined by Student's $t$-test: $^{**}p < 0.01$, $^{*}p < 0.05$ versus control (in the absence of the MS12 cells).

cells are resistant to transfection by the retrovirus containing SV40 large T antigen. TRAP$^+$ OCLs derived from MOCP-5 cells possessed the major phenotypic criteria for authentic osteoclasts. These included TRAP activity, expression of CT receptors and cathepsin K, acid production, and the ability to form resorption pits on dentin slices and cortical bone (Y.-P. Li, unpublished data). Moreover, MOCP-5 cells could be transfected with a construct that carries the $\beta$-galactosidase gene. Transfected MOCP-5 cells that expressed $\beta$-galactosidase retain the ability to differentiate into OCLs. Currently, there is no such osteoclast precursor cell line that can be used for osteoclast-specific

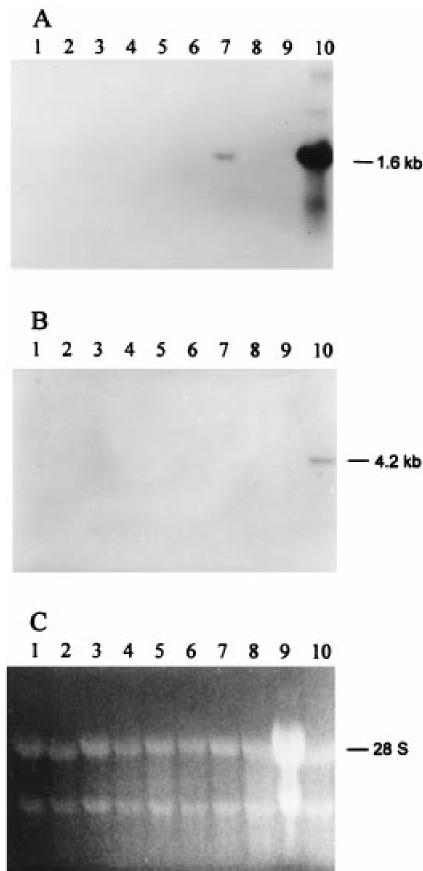


**FIG. 8.** Northern hybridization of human cathepsin K cDNA and CT receptor cDNA to total cell RNA from cell lines and mouse tissues: lane 1, testes; lane 2, brain; lane 3, skeletal muscle; lane 4, liver; lane 5, ROS 17/2.8; lane 6, U-937 cells; lane 7, kidney; lane 8, MS12 stromal cells; lane 9, Undifferentiated MOCP-5; lane 10, Purified OCLs differentiated from MOCP-5. (A) Autoradiograph after hybridization with human cathepsin K cDNA probe; the arrow indicates the 1.6 kb band. (B) Same membrane was stripped and rehybridized with a human CT receptor cDNA probe. The arrow indicates the 4.2 kb band of cross-hybridizing CT receptor mRNA. (C) Ethidium bromide stained gel showing equivalent RNA loading.

gene regulation by the transfection approach. To our knowledge, this is the first osteoclast precursor cell line immortalized in vitro and the first report of a successful transfection using a mammalian osteoclast precursor cell line.

The variability in the size of the resorption lacunae (Fig. 6A) suggests that they were excavated by mononuclear preosteoclasts as well as by OCLs. This observation is not surprising, since resorbing mononuclear osteoclasts have been detected in sections of human osteophytic bone[39] and characterized as a function of human osteoclast precursors.[40] In addition, Iotsova et al. recently reported that the NF-kB1$^{-/-}$, NF-kB2$^{-/+}$ mice have only mononuclear immature osteoclasts, yet exhibit a normal bone phenotype, implying that their immature osteoclasts are capable of remodeling bone.[41]

Osteoclast precursors in bone marrow are relatively rare. Our strategy to immortalize such rare cells was to enrich these target cells in an induction system before immortalization. The strategy was successful, as demonstrated by detection of SV40 large T antigen protein both in nuclei of OCLs derived from MOCP-5 cells (Fig. 1D) and in the stable osteoclast precursor phenotype of MOCP-5 cells. Using this technique, cell lines of rare cells such as osteoclast precursors have been obtained. A similar strategy may be used for other cell types if suitable enrichment and induction models are available.

Boyce et al.[16] failed to develop an immortalized osteoclastic cell line from transgenic mice in which expression of the SV40 large T antigen oncogene was targeted to osteoclasts using the TRAP gene promoter. They suggested that SV40 T antigen is insufficient to immortalize osteoclast lineage cells. However, our results demonstrated that SV40 large T antigen expression in osteoclast precursor cell is sufficient by itself for immortalization. MOCP-5 cells can be grown indefinitely while retaining their differentiation capacity. In most tissues, when cells are transformed or immortalized, the cells will usually represent "frozen" stages of differentiation at which the cells are transformed or immortalized and lose further differentiation ability. Considering that expression of SV40 large T antigen might hinder cell differentiation, others have used a temperature-sensitive mutant of the SV40 large T antigen to immortalize cells.[15] However, our results demonstrate that expression of wild type SV40 large T oncogene in mouse osteoclast precursor cells allows unrestricted cell growth without hindering the potential for differentiation. The mechanism by which immortalized cells traverse the proliferation stage and differentiation in the MS12 coculture system remains a question.

Immortalization of MOCP-5 cells by SV40 large T antigen is not fully understood. SV40 large T antigen is a multifunctional regulatory protein that plays a key role in the viral life cycle and can stimulate cell proliferation. Several studies have indicated that large T antigen can regulate cell growth, in part, by binding and inactivating the tumor suppressors proteins pRB and p53.[42,43] In addition, large T antigen can transactivate cellular genes, and this process is mediated by specific protein–protein interactions with multiple components of the transcription machinery.[44–47] These alterations in the transcriptional activity of specific cellular genes most likely play an important role in the growth-promoting activity induced by large T antigen.

The origin of the osteoclast has been a subject of intense investigation and controversy. Transplantation of hematopoietic tissue studies[48–50] has clearly indicated that the osteoclast precursor is a mononuclear cell that is hematopoietic in origin. There have existed questions about the specific cell lineage that gives rise to the osteoclast. A number of studies first performed in vivo[51] and in vitro[10,52,53] suggest that osteoclasts are derived from cells of the mononuclear phagocyte system. However, other researchers[54,55] have argued that osteoclasts are derived from a unique lineage that is a distinct branch of the

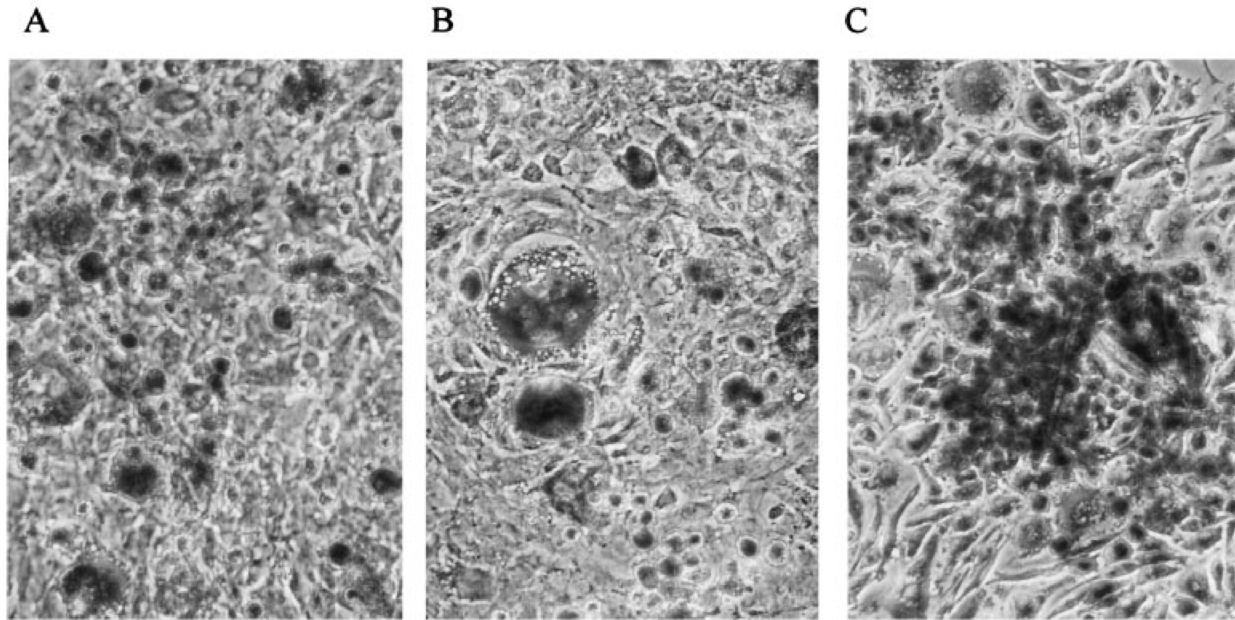


**FIG. 9.** Expression of $\beta$-galactosidase in MOCP-5 cells transfected with pSV-$\beta$-galactosidase. (A) $\beta$-galactosidase assay of transfected MOCP-5 cells 2 days after transfection. (B) Transfected MOCP-5 cells 3 days after transfection. Some transfected cells have fused to form multinucleated osteoclasts. (C) A stably transfected MOCP-5 cell line expressing $\beta$-galactosidase. Original magnification, ×200.

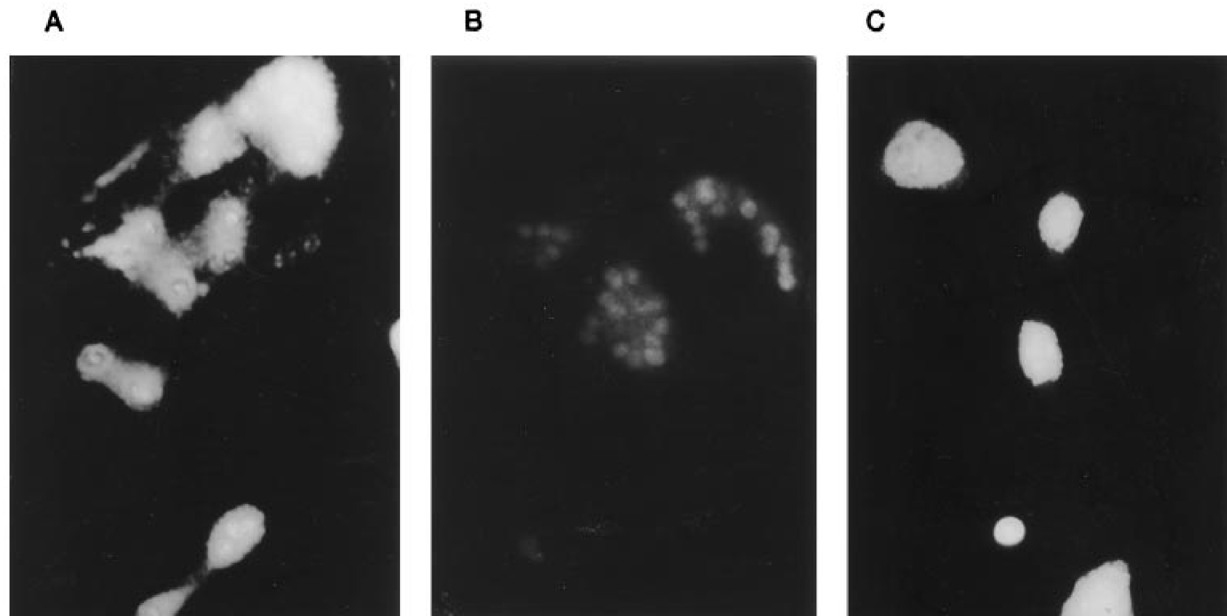


**FIG. 10.** OCLs and MOCs acid compartments demonstrated by vital staining with acridine orange. OCL preparations were place on dentin slices and incubated with acridine orange either without (A) or with (B) $NH_4Cl$. Note that intense orange fluorescence was seen as large uniformly brilliant orange discs (A). Orange-positive fluorescence diminished with $NH_4Cl$, indicating specific staining of acridine orange (B). When MOCs applied to dentin slices, similar brilliant orange discs were observed (C). Original magnification (A–C), ×200.

monocyte-macrophage pathway. Recently, Grigoriadis et al. reported that the lack of the c-*fos* gene product causes a lineage shift between osteoclast and macrophage differentiation.[56] Tondravi et al. reported that PU.$1^{-/-}$ mice are not only devoid of osteoclasts but also lack macrophages.[57] These data from in vivo studies support the concept that osteoclasts share the lineage of the monocyte-macrophages. Interestingly, undifferentiated MOCP-5 cells

were negative for F4/80 immunoreactivity yet were positive for the macrophage markers MoMa-2 and NES activity (Table 1). These antigens or enzymes are generally agreed to be macrophage markers. The phenotypic assays indicate that the MOCP-5 cell line derives from the monocyte-macrophage lineage but is committed to the osteoclast lineage. Our results suggest that osteoclasts are derived from more differentiated tissue-specific cells in the monocyte-macrophage lineage.

The MOCP-5 cell line we have developed offers several important advantages over previously described model systems for osteoclasts. First, the resorption activity of OCLs differentiated from MOCP-5 cells is much higher than that of other cell lines previously reported.[3,11] Second, the efficiency of osteoclast formation of MOCP-5 cells is much higher than that of other systems and cell lines.[4,6,58] The procedure for producing this line allows us to easily obtain larger amounts of homogeneous OCLs in a few days without having to begin with mouse bone marrow cells. Third, the osteoclastic fate of the MOCP-5 cell line determined, and more than 95% of MOCP-5 cells, can differentiate into $TRAP^{+}$ OCLs or preosteoclasts under the appropriate conditions (Fig. 2B). This feature is important for simplifying the differentiation system and eliminating the effect of cells of other lineages. Fourth, the MOCP-5 cell line is immortalized by wild type SV40 large T antigen. MOCP-5 cells retain their native properties, and the entire experimental protocol for osteoclast differentiation can be performed at 37°C. Fifth, most interestingly, the MOCP-5 cells may be stably or transiently transfected by a reporter gene without hindering cell differentiation.

In conclusion, we have employed an in vitro immortalizing method to develop an osteoclast precursor cell line. The expression of SV40 large T oncogene in MOCP-5 cells induces cell proliferation without hindering cell differentiation. MOCP-5 cells can also be transfected without losing the ability to differentiate into OCLs. The MOCP-5 cell line represents a useful model for osteoclast formation, osteoclast-expressed gene regulation, and further studies on osteoclast functions.

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