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Osteoclast Differentiation Factor Modulates Cell Cycle Machinery and Causes a Delay in S Phase Progression in RAW264 Cells

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Osteoclast differentiation factor (ODF) induces differentiation of mouse RAW264 cells to mature osteoclasts. To understand the mechanism controlling a coupling between withdrawal from the cell cycle and differentiation, we examined cell cycle progression and expression profiles of cell cycle regulatory genes at the initial phase in committed cells. ODF rapidly converted the hyperphosphorylated form of the retinoblastoma protein (pRb) into the hypophosphorylated form. The p21 protein was induced by ODF treatment in the same time course with that of dephosphorylation of pRb, followed by a sharp decline. After this period, a delayed entry of the S phase started accompanying the induction of CycD3 and cdk6 in differentiating cells. Hydroxyurea treatment indicated that the S phase entry was a prerequisite for osteoclast formation. Thus, ODF induces pleiotropic effects on cell cycle regulatory genes in RAW264 cells during the initial phase of the differentiation process to osteoclasts.

Osteoclasts are multinucleated giant cells and present only in bone with the capacity to resorb mineralized tissues. They are hematopoietic cells derived from colony-forming unit granulocyte-macrophage (CFU-GM) and branch from the monocyte-macrophage lineage early during the differentiation process (1, 2). Numerous studies showed that the presence of either marrow stromal cells or osteoblasts is required for this process and recently, the significance of this interaction between two cell systems was clarified at the molecular level (2). Namely, osteoclast differentiation factor (ODF) is a type II transmembrane protein of the tumor necrosis factor (TNF) ligand family and is expressed in stromal cells and osteoblasts (3–5). ODF binds the receptor called receptor activator of NF-κB ligand (RANK) that is expressed on osteoclast precursors and induces osteoclast formation (6). In this process, ODF does not function as a growth factor but plays a role as a differentiation factor at the final stage of the entire process (7, 8).

The mechanisms of the mammalian cell differentiation process have been studied extensively at the molecular level using a variety of cell lines (9, 10). In particular, cell cycle regulatory machinery at the G1/S transition has been a major target for such studies because it plays crucial roles in the temporal coupling between withdrawal from the cell cycle and differentiation. In this regard, while ODF has been shown to activate c-jun terminal kinase via one pathway and send signals to NF-κB via another pathway (11), little information is available on its effect on the cell cycle machinery.

To clarify which are the cell cycle regulatory factors that must be regulated to allow differentiation, it is essential to establish a suitable system to follow the differentiation process. Coculture systems which have been used in studying the differentiation as well as the maturation process of osteoclasts (2) may not be suitable for this end, because the presence of two different types of cells in the system could cause difficulties in analyzing the details involved in the process. In this regard, Hsu *et al.* (12) originally reported that the mouse monocyte/macrophage derived cell line RAW264 (13) could be induced to mature osteoclasts with purified ODF and without any stromal cells. We made use of this system and analyzed the initial phase of the differentiation process in terms of cell cycle progression and expression profiles of cell cycle regulatory genes. Here, we focused on genes that were known to participate in the G1/S transition step.

We found that the ODF treatment rapidly converted the hyperphosphorylated form of the retinoblastoma susceptibility protein (pRb) into the hypophosphorylated form, followed by rephosphorylation at a later stage. A delayed and prolonged duration of the S phase

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was detected in accordance with the phosphorylation profiles of pRb. Hydroxyurea treatment implicated this S phase entry was prerequisite for osteoclast formation. Furthermore, genes that were expressed differentially between proliferating and differentiating cells were identified.

MATERIALS AND METHODS

Construction and preparation of sODF. We cloned the extracellular domain (amino acid positions from 75 to 317) (3–5) into an expression vector pGEX-2TK and expressed them in *Escherichia coli.* The product (sODF) was purified using GST-affinity column (data not shown) and used for the following experiments. For a control experiment, an empty vector was expressed and the GST protein was purified and used as GST protein.

Cell culture and formation of osteoclasts. RAW264 mouse monocyte/macrophage line cells (13) were obtained from Human Science Research Resources Bank (HSRRB, Japan) and were maintained in Eagle medium supplemented with 10% fetal calf serum and 1% nonessential amino acids (GIBCO-BRL). For osteoclastogenesis in vitro, cells were serum-starved (0.5% serum) for 24 h and then 100 ng/ml of purified sODF was added together with 10% of fetal calf serum (HyClone, USA) (=0 h). After culturing for the indicated periods, cells were fixed and stained for tartrate resistant acid phosphatase (TRAP) as described previously (14).

Preparation of RNA and RT-PCR. After the preparation of total RNA and complementary DNA (cDNA), RT-PCR reactions were carried out under the conditions described previously, in which a linear amplification was achieved (15). Primers covering the unique region of each of the following mouse genes were designed: GAPDH (16), p21 (17), and p27 (18). Products were separated by agarose gel electrophoresis. Primers for GAPDH were included in each experiment as an internal control.

Western blot analysis and antibodies. Western blotting was essentially carried out as described previously (19). Antibodies for cyclin D1 (sc-450), D2 (sc-452), D3 (sc-453), and E (sc-481) were purchased from Santa Cruz Biotechnology (CA). Rabbit polyclonal antibodies for cdk4 (sc-260), cdk6 (sc-177), and p27 (sc-527) were also obtained from Santa Cruz Biotechnology. Anti-p53 monoclonal antibody (OP03, Oncogene Science Inc., USA), anti-pRb monoclonal antibody (14001A, Pharmingen, USA) and anti-β-actin monoclonal antibody (A-5316, Sigma, USA) were used. Anti-rat IgG and -mouse IgG were obtained from Cappel (USA) and Amersham-Pharmacia-Biotech, respectively. Anti-BrdU monoclonal antibody (RPN202, Amersham-Pharmacia-Biotech) was used.

BrdU incorporation and flow cytometry. These were also carried out as described previously (19). Cells were grown on coverslips and pulsed for 30 min prior to harvesting at the indicated time by adding bromodeoxyuridine (BrdU; Amersham-Pharmacia-Biotech) directly to the culture medium to a final concentration of 100 μM. The cells were then harvested, fixed with glycine buffer (50 mM glycine, pH 2.0 containing 70% ethanol) and stained with anti BrdU mouse monoclonal antibody (Amersham-Pharmacia-Biotech) and fluorescein isothiocyanate (FITC)-conjugated anti mouse IgG antibody (Amersham-Pharmacia-Biotech). BrdU positive cells were counted under a confocal microscopy (LSM410, Carl Zeiss). For fluorescence-activated cell sorting (FACS) analysis, cells were harvested and finally suspended in PBS containing 0.1% sodium citrate, 0.1% Triton X-100, and 1 μ g/ml of RNase. Following incubation at 37°C for 30 min, cells were stained with 50 μ g/ml of propidium iodide at 4°C in the dark and DNA content was analyzed on a FACScan station with Cell Quest software (Becton Dickinson). Measurements from about 20,000 cells were saved in the list mode and displayed as a frequency distribution histogram of propidium iodide fluorescence captured at 585 ± 21 nm. Analysis of histograms was performed using ModFIT LT program (Verity Software House Inc.). The coefficient of variation of the diploid peak was always smaller than 8%.

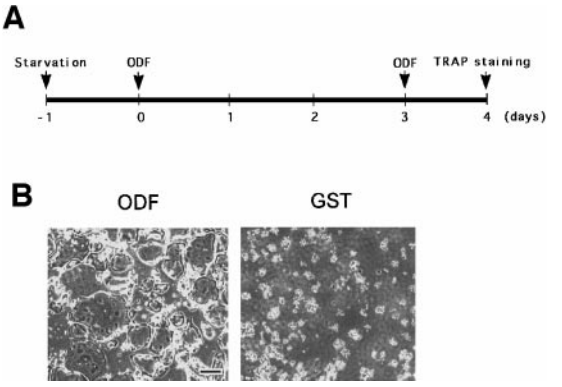

FIG. 1. Osteoclastogenesis *in vitro.* (A) The outline of the culture system. (B) Cultured mouse RAW264 cells in the presence of sODF (left) and GST protein (right). Cells were cultured for 4 days as described in (A), fixed, and stained for TRAP. Bar, 200 μm.

Hydroxyurea treatment. Hydroxyurea (HU) (Sigma) was directly added to the medium of either GST- or ODF-treated cells to a final concentration of 1 mM (20) and incubated for the incubation periods as indicated in each experiment.

RESULTS

Osteoclastogenesis in Vitro

Mouse RAW264 cells, derived from the monocyte/macrophage lineage (13) and known to express RANK (12), were stimulated with sODF under the conditions described under Materials and Methods (Fig. 1A). After four days, plates became fully covered by giant multinucleated cells (Fig. 1B). It was suggested that these cells were osteoclasts by the following criteria in addition to their morphological characteristics. First, the cells were TRAP positive, second, they showed pit forming activity on dentine slices and finally, the expression of differentiation marker genes such as calcitonin receptor was detected (data not shown). In contrast, RAW264 cells just proliferated and osteoclasts were not observed with the GST protein (Fig. 1B).

Cell Cycle Progression on ODF Stimulation

We first examined the effect of ODF stimulation on cell cycle progression by measuring the BrdU incorporation and using flow cytometry. BrdU incorporation was measured every 3 to 4 h after ODF stimulation in comparison with GST-treated cells (hereafter these cells will be referred to as control cells); at each point, cells were labeled with BrdU for 30 min. As shown in Fig. 2A, the incorporation became evident after 8 h in control cells and peaked at 11 h, followed by a sharp decline; the incorporation started to increase again at 21 h. In contrast, the population of cells labeled with BrdU started to appear after 11 h in ODF-treated cells

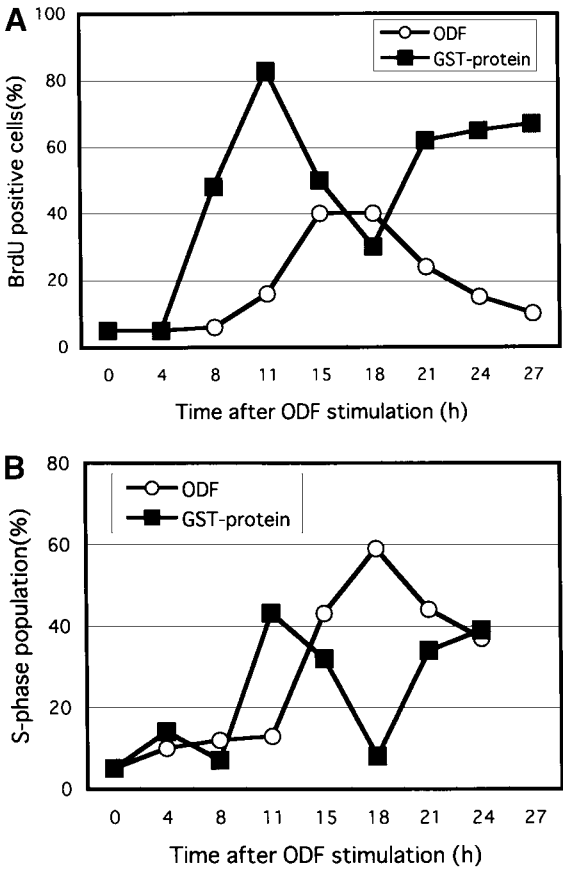

FIG. 2. Cell cycle progression in ODF-treated RAW264 cells. (A) BrdU incorporation was measured every 3–4 h. ODF-treated cells were stained with anti-BrdU antibody and positive cells were counted against the total population (%) (open circles). RAW264 cells treated with GST protein alone were used as control cells (closed circles). (B) Flow cytometric analyses of ODF-treated and control cells. Control cells (closed circles) and ODF-treated cells (open circles).

and gave a broad peak, followed by a decline at 21 h. Flow cytometric analysis of the cell population in the S phase gave essentially identical patterns observed by BrdU incorporation in both conditions (Fig. 2B). These findings strongly suggested that ODF caused a delayed entry and prolonged duration of the S phase in cells to commit to osteoclastogenesis; in other words, ODF stimulation did not block S phase entry, but rather induced a different mode of S phase progression.

pRb Phosphorylation in ODF-Treated Cells

We then examined the phosphorylation profiles of pRb during the period of the first 24 h in control and ODF-treated cells (Fig. 3A). Phosphorylation of pRb is essential for initiating a series of reactions required for the G1/S transition (21) and hence, the phosphorylation state of pRb is believed to be an important determinant of cell proliferation or arrest.

pRb was found to be fully phosphorylated in RAW264 cells at 0 h even after serum starvation for 24 h. The hypophosphorylation form then became evident at 18 h where the incorporation rate of BrdU reached the bottom. In contrast, a dephosphorylation reaction of pRb took place by ODF stimulation; the hypophosphorylated form of pRb was the major one at 4 h. pRb was completely converted to the hyperphosphorylated form at 15 h, and this form continued through the period (even up to 40 h; data not shown). Such phosphorylation profiles of pRb, seen in both conditions, correlated well with the population of cells entering the S phase observed by flow cytometry (Fig. 2).

Expression Profiles of Cell Cycle Regulatory Genes

The above findings indicated that the modulation of the phosphorylation profile of pRb could reflect the functional mechanism of ODF. Since G1 cyclins/cdk complexes and their regulators, cdk inhibitors, play roles for this reaction (21, 22), we examined the expression profiles of these genes at the mRNA and protein levels within the first 24 h after ODF stimulation and compared with those in control cells.

Of cyclins, we examined CycD1, D2, D3, and E that had been believed to function in the G1 phase and also at the G1/S transition phase (Fig. 3B). Little expression of CycD1 was detected in both conditions at the mRNA and protein levels (data not shown). However, a significant induction of the CycD2 and D3 proteins was detected in ODF-treated cells while the induction was weak and transient in control cells; in particular, the induction of the CycD3 protein appeared to be more stringently regulated around at 11 h. The CycE protein was barely detected during the initial phase in both conditions, but it became evident at 24 h in control cells.

Next, we examined cdk2, 4, and 6 (Fig. 3C). Among these kinases, cdk2 and cdk6 showed characteristics of ODF-dependency, although their profiles were different each other. The cdk2 protein level was suppressed in the early phase (4 and 11 h) in ODF-treated cells; otherwise the protein levels were comparable in both conditions. The cdk6 protein, on the other hand, was significantly induced at 11 h in ODF-treated cells. Cdk4 was observed to be expressed constantly in both conditions for the first 24 h.

Of the Cip1/Kip1 family of cdk inhibitors, the p21 protein was barely detected in control cells while a significant induction was detected during the early phase until 11 h in ODF-treated cells, followed by an abrupt decline (Fig. 3D). In this regard, the bands were always detected as doublets, but it remains to be clarified whether this reflects any differences in their physiological conditions such as phosphorylation level. Expression profiles of p21 mRNA showed good correlation with that of protein (Fig. 3E). On the other hand, the p27 protein showed a constant expression throughout this period in both conditions (Fig. 3D), although the

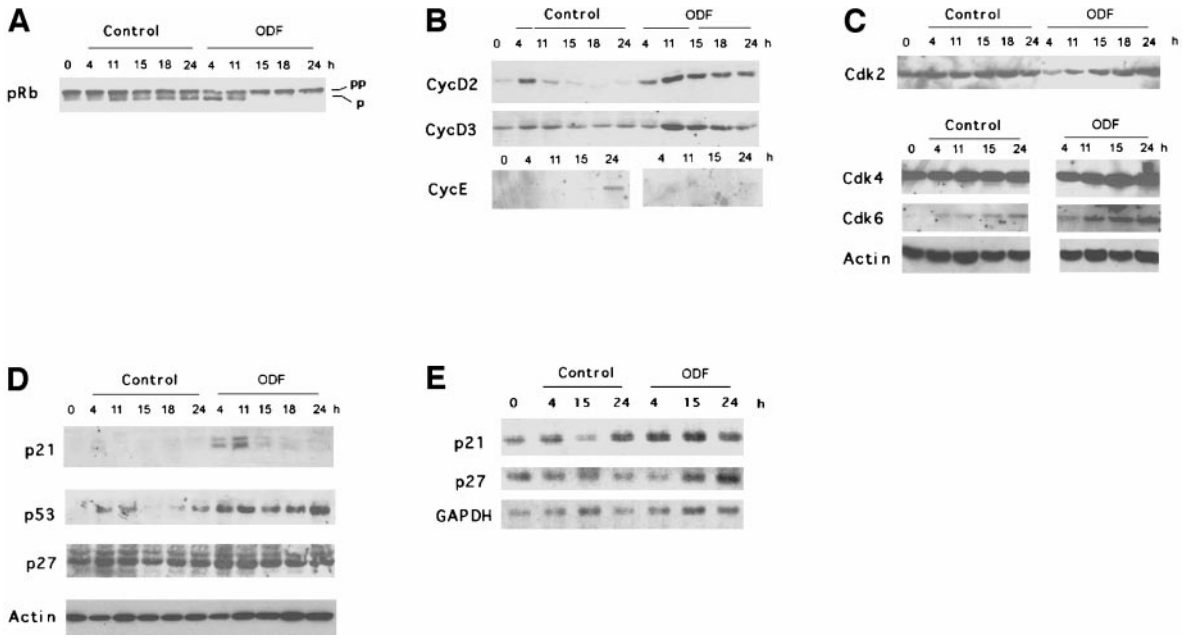

FIG. 3. Expression profiles of cell cycle regulatory genes. RAW264 cells were incubated with a low concentration of fetal calf serum (0.5%) and either GST protein or ODF was added with 10% fetal calf serum (=0 h). Cell extracts and total RNA were prepared from cells at indicated times for Western blot analysis (A–D) and RT-PCR (E), respectively, as described in materials and methods. (A) pRb; (B) cyclin: CycD2, D3, and E; (C) cdk: cdk2, 4 and 6; (D) CDK inhibitors: p21, p27 and p53; (E) RT-PCR products of p21 and p27. P and pp in (A) indicate hypophosphorylated and hyperphosphorylated forms of pRb, respectively. Actin was blotted to quantitate proteins applied. GAPDH was used as an internal control for RT-PCR in (E).

expression profile of p27 mRNA did not correlate with that of protein in ODF-treated cells (Fig. 3E). The INK4 family, on the other hand, did not show differential expression profiles between proliferating and differentiating cells (data not shown).

The induction of p21 detected in ODF-treated cells suggested the preceding activation of p53, because it was well established that p53 is a positive regulator of p21 mRNA expression (23). We, therefore, examined whether p53 was induced by ODF-treatment. As shown in Fig. 3D, the induction of p53 protein was clearly demonstrated during the initial phase in ODF-treated cells. Taken together, a delayed entry and prolonged duration of the S phase was prominent in ODF-treated cells and in accordance with this phenomenon, expression profiles of cyclin, cdk and cdk inhibitor genes as well as phosphorylation profiles of pRb were found to be regulated by ODF.

Effect of HU Treatment on Osteoclast Formation

To clarify whether the unique S phase progression detected in ODF-treated cells was a pivotal process for osteoclastogenesis, we treated RAW264 cells with HU in the presence or absence of ODF (Fig. 4). The addition of HU at 0 h and its maintenance throughout the following incubation period for 24 h caused cell detachment into media in both conditions. However, if HU was added at 24 h when most cells were in the S phase and the condition was maintained for the following 3 days, osteoclast formation was detected as in the normal time course, although the numbers of osteoclasts were reduced to 10%. If HU was added at 48 and 72 h after ODF stimulation, the matured cell numbers increased and the population reached to 40 and 90%, respectively, of the normal level at 4 days after ODF-stimulation. Incubation for further two to three days did not show any significant differences. These findings strongly suggested that the entry to the S phase was prerequisite for osteoclast formation and once passed this stage, DNA replication was not required.

DISCUSSION

In the present study, we focused on the initial stage of the differentiation process in which mouse RAW264 cells were induced to become osteoclasts by ODF stimulation. We added ODF together with 10% fetal calf serum after serum starvation (0.5%) for 24 h, but this condition was not essential for osteoclast formation; ODF could induce osteoclasts even from asynchronously proliferating RAW 247 cells (data not shown). In all cases, ODF treatment could induce differentiation to osteoclasts in the presence of 10% serum. This might be a characteristic feature of this differentiation system, given that other systems such as C2C12 cells in which differentiation toward muscle cells can be

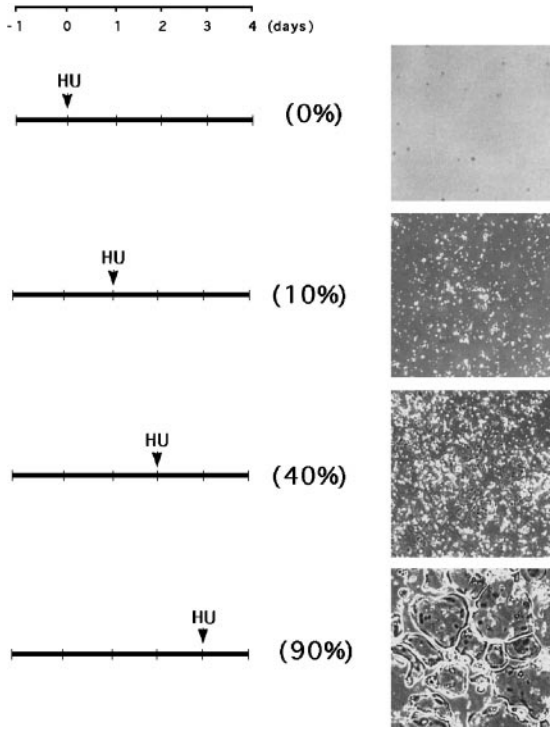

FIG. 4. Effects of HU addition on osteoclast formation. Time course of HU treatment and its effect on osteoclast formation is summarized. Culture conditions including ODF treatment were as described in the legend to Fig. 1. Morphology of cells after HU treatment (right side): from the top, HU added at 0, 24, 48, and 72 h. Numbers of detected osteoclasts at 4 days after the first ODF stimulation are shown as the % of cell numbers formed under the normal conditions.

triggered by serum starvation alone (10) or adipocyte differentiation of 3T3-L1 cells in which cells have to be rendered confluent and arrested during the G1 phase before committing to differentiation (24). ODF treatment was thus expected to induce a suppression or blockage of proliferative signals stimulated by serum, and we focused our analyses on the cell cycle regulatory system in RAW264 cells.

An early response detected only in ODF-treated cells was dephosphorylation of pRb. Since phosphorylation of pRb is well known to be crucial for the G1/S transition, dephosphorylation induced by ODF is presumed to induce cell cycle adjustment or arrest at the stage before the G1/S transition. In fact, a delay of cell cycle progression was observed in ODF-treated cells as seen in Fig. 2. In addition to dephosphorylation of Rb, the transient induction of p21 and suppression of cdk2 could function together favorably to suppress the S phase entry. This hypophosphorylated form of pRb was then observed to shift to the hyperphosphorylated form at 15 h in accordance with the accumulation of BrdU-positive cells. At this point, the induction of CycD2/D3 and cdk6 was evident and these factors might be invovled in the phosphorylation of Rb. Taken together, the present findings implieded that ODF induced pleiotropic effects on expressions of cell cycle regulatory genes, resulting in a delayed S phase progression in RAW264 cells.

HU treatment before and during the period of a delayed entry and prolonged duration of the S phase blocked the osteoclast formation, but treatment after this period (48 and 72 h) caused only a slight effect on the osteoclast formation. Therefore, the entry to and the completion of the S phase might be essential to accomplish the whole differentiation process. In this regard, full formation of osteoclasts was not observed by HU treatment even at 24 and 48 h. We found that the efficiency of osteoclast formation was very sensitive to inoculated cell numbers. We therefore presumed that HU treatment caused severe damage to cells that had not completed the S phase and thus reduced viable cell numbers, resulting in fewer osteoclasts. The mechanism involved in the S phase progression in ODF-treated cells, however, may not be identical with that seen in mitotic cells. For example, in contrast to control cells, CycE protein was barely detected in ODF-treated cells. Phosphorylation of pRb by the CycE/cdk2 complex has been reported to be required for complete phosphorylation of pRb (27), inactivation of E2F binding and activation of E2F transcription, which are all essential for successful mitotic cell cycle progression.

Osteoclasts are multinucleated giant cells sometimes containing up to 100 nuclei (1, 2). These multinuclei are reported to be formed by fusion of mononuclear precursor cells (25), not by simple endoreduplication or endomitosis. In this regard, during the early phase of adipocyte differentiation of 3T3-L1 cells, another model system of differentiation, the cells are reported to transiently reenter the cell cycle, resulting in an increase in DNA synthesis and several rounds of cell division (26). This phase, called mitotic clonal expansion, was reported to be essential to complete the differentiation process. pRb in that system becomes hyperphosphorylated during the early phase where the time course resembles that of clonal expansion. Taken together, each differentiation system seems to have its own unique mechanism controlling a coupling between withdrawal from the cell cycle and differentiation. To gain further insight on the process to osteoclast formation, the system described in this study was shown to be very useful for the purpose.

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