

# Commitment and Differentiation of Osteoclast Precursor Cells by the Sequential Expression of c-Fms and Receptor Activator of Nuclear Factor $\kappa$ B (RANK) Receptors

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## Summary

Osteoclasts are terminally differentiated cells derived from hematopoietic stem cells. However, how their precursor cells diverge from macrophagic lineages is not known. We have identified early and late stages of osteoclastogenesis, in which precursor cells sequentially express c-Fms followed by receptor activator of nuclear factor  $\kappa$ B (RANK), and have demonstrated that RANK expression in early-stage of precursor cells (c-Fms<sup>+</sup>RANK<sup>-</sup>) was stimulated by macrophage colony-stimulating factor (M-CSF). Although M-CSF and RANKL (ligand) induced commitment of late-stage precursor cells (c-Fms<sup>+</sup>RANK<sup>+</sup>) into osteoclasts, even late-stage precursors have the potential to differentiate into macrophages without RANKL. Pretreatment of precursors with M-CSF and delayed addition of RANKL showed that timing of RANK expression and subsequent binding of RANKL are critical for osteoclastogenesis. Thus, the RANK-RANKL system determines the osteoclast differentiation of bipotential precursors in the default pathway of macrophagic differentiation.

Key words: osteoclastogenesis • commitment • macrophage • RANK ligand • M-CSF

Bone is continuously forming and being resorbed. This process is accomplished by precise coordination of two cell types: osteoblasts, which deposit the calcified bone matrix, and osteoclasts, which are derived from hematopoietic stem cells (1–5) and resorb the bone tissue. Osteoclasts are large multinucleated cells (MNCs)<sup>1</sup> located on endosteal bone surfaces and the periosteal surface beneath the periosteum. Mononuclear osteoclasts differentiate from the same precursor cells as macrophages (6–8) and fuse to form multinuclear osteoclasts. However, how osteoclasts differentiate from precursors and their relationship to macrophage precursors is still controversial. It has been shown that mice lacking c-Fos are osteopetrotic due to a differentiation block in bone-resorbing osteoclasts, even though macrophage differ-

entiation is intact and osteoclast differentiation can be rescued by introduction of Fos protein (9, 10).

Osteoclasts characteristically show a high level of cell adhesion, and their growth and differentiation are anchorage dependent. In this aspect, assays of osteoclast precursor cells require coculture with stromal cells rather than colony formation in semisolid cultures. Osteoclastogenesis in culture depends on interaction with stromal cells, which provides the microenvironment essential for this process (11, 12). One of the critical factors produced by stromal cells is M-CSF (13–16). A lack of osteoclasts is observed in osteopetrotic *op/op* mutant mice, which lack functional M-CSF (17–19). It is thought that the M-CSF receptor, c-Fms tyrosine kinase, might be expressed on osteoclast precursor cells. Hofstetter et al. (20) reported that c-Fms mRNA was detected in cells at late stages of osteoclastogenesis and in mature osteoclasts. Another factor important for osteoclastogenesis is osteoprotegerin ligand (OPGL)/osteoclast differentiation factor (ODF), which was cloned from an osteoblastic cell line (21, 22). A new member of the TNFL family, designated TNF-related activation-induced cytokine (TRANCE) or receptor activator of nuclear factor (NF)- $\kappa$ B (RANKL)

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<sup>1</sup>Abbreviations used in this paper: BM, bone marrow; Dex, dexamethasone; Epo, erythropoietin; L, ligand; MNCs, multinucleated cells; NF, nuclear factor; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor  $\kappa$ B; RT, reverse transcriptase; s, soluble; SCF, stem cell factor; TRAFs, TNFR-associated factors; TRAP, tartrate-resistant acid phosphatase.

was cloned independently. Its predicted amino acid sequence is identical to that of OPGL/ODF. TRANCE was identified as an activator of c-Jun NH<sub>2</sub>-terminal kinase (JNK) in T cells (23), and RANKL was shown to be a ligand for RANK, a new member of the TNFR family derived from dendritic cells (24). In vitro results suggest that signaling mediated by TNFR-associated factors (TRAFs) is important for the activation of the stress kinase pathway (SAPK/JNK) and the transcription factor NF- $\kappa$ B (25–27). In addition, the RANK intracellular domain contains two distinct TRAF binding domains (26, 28), and TRAF6 deficiency results in osteopetrosis (29). Mice in which both the p50 and p52 subunits of NF- $\kappa$ B are disrupted fail to generate mature osteoclasts and B cells (30, 31). Moreover, RANKL-deficient mice show severe osteopetrosis and a defect in tooth eruption (32).

Here, to clarify the commitment and differentiation pathway of osteoclasts, we have identified the early and late stages of osteoclast precursor cells using anti-c-Kit, Mac-1, c-Fms, and RANK mAbs. We have observed sequential expression of c-Fms and RANK and analyzed the function of each factor in osteoclastogenesis.

## Materials and Methods

**Mice.** 8–10-wk-old female C57BL/6 mice were purchased from Japan SLC. Bone marrow (BM) cells were freshly prepared from femur and tibia and used as a source of hematopoietic precursors.

**Reagents.** 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-[OH]<sub>2</sub>D<sub>3</sub>) was provided by Dr. Ishizuka (Teijin Institute for Biomedical Research, Tokyo, Japan). Recombinant M-CSF and anti-mouse M-CSF neutralizing antibody were purchased from R & D Systems, Inc. Recombinant IL-7 and recombinant IL-3 were provided by Toray Industries Inc. Recombinant stem cell factor (SCF) was a gift from Chemo-Sero-Therapeutic Co., Ltd. Erythropoietin (Epo) was a gift from Snow-Brand Milk Product Co.

**Preparation of Mouse BM Mononuclear Cells.** Mice were killed by cervical dislocation, and the tibiae and femora were removed and dissected free from adhering soft tissues. The bone ends were cut off with a scalpel, and the marrow was flushed with  $\alpha$ -modified ( $\alpha$ -)MEM (GIBCO BRL) containing 10% FCS (JRH Biosciences). Mononuclear cells were isolated by centrifugation of total BM cells on Lymphoprep™ (Nycomed Pharmaceuticals) according to the manufacturer's instructions.

**Preparation of Soluble RANKL.** A DNA fragment encoding the extracellular domain (Asp<sub>76</sub>–Asp<sub>316</sub>) of RANKL was prepared using reverse transcriptase (RT)-PCR from total RNA of ST2 stromal cells that were cultured in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 4 d. PCR primers were as follows: 5'-CCGCTCGAGCGTC-TATGTCCTGAACCTTGA-3' (sense) and 5'-CCCAAGCTTG-ATCCTAACAGAATATCAGAAGACA-3' (antisense).

The PCR product was digested with HindIII and XhoI and ligated into the HindIII and XhoI sites of the pSecTag2 vector (Invitrogen Corp.) to yield pSecTag2-soluble (s)RANKL containing His<sub>6</sub> and myc tags. pSecTag2-sRANKL was transfected into COS7 cells cultured in DMEM (Life Technologies, Inc.) containing 10% FCS, and the supernatant was collected every 4 d for 12 d. sRANKL was purified from the supernatant using TALON and TALON Superflow Metal Affinity Resin (Clontech). The supernatant was applied to a chromatography column (Bio-Rad

Labs.) filled with affinity resin, and the trapped proteins were eluted in 125 nM imidazol and fractionated. The high concentration fraction was collected and applied to a PD-10 column (Amersham Pharmacia Biotech, Inc.), eluted in PBS to eliminate the imidazol, and fractionated. Fractions containing a large amount of sRANKL were concentrated by a centricon concentrator (Amicon, Inc.).

**Cell Lines.** Mouse BM-derived stromal cell line ST2 (a gift from Dr. Hayashi, Tottori University, Yonago, Japan) was maintained in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS with 10<sup>-5</sup> M of 2-ME (GIBCO BRL) at 37°C in humidified 5% CO<sub>2</sub> air. Cells were harvested every 3 d using 0.05% trypsin-EDTA (GIBCO BRL), and 10<sup>5</sup> cells were passaged on 100-mm culture dishes (Falcon 3003; Becton Dickinson Labware).

The newborn calvaria-derived mouse stromal cell line OP9 (a gift from Dr. Kodama, Bayer Yakuhin Ltd., Kyoto, Japan) was maintained in  $\alpha$ -MEM supplemented with 20% FCS at 37°C in humidified 5% CO<sub>2</sub> air. Cells were harvested every 3 d using 0.05% trypsin-EDTA, and 1.5  $\times$  10<sup>5</sup> cells were resuspended on 100-mm cell culture dishes.

**Immunofluorescence Staining and Cell Sorting.** The cell staining procedure for flow cytometry was performed as previously described (33). The mAbs used in immunofluorescence staining were anti-c-Kit antibody (ACK2; a gift from Dr. S.-I. Nishikawa, Kyoto University, Kyoto, Japan), anti-Mac-1 (CD-11b) antibody (M1/70; PharMingen), anti-c-Fms antibody (AFS98; a gift from Toray Industries Inc.), anti-RANK antibody (muRANK-M395; a gift from Immunex Corp.), anti-CD51 antibody (H9.2B8; PharMingen), and anti-B220 antibody (RA3-6B2; PharMingen). The mAbs used were either biotinylated or fluoresceinated. Biotinylated mAbs were detected with streptavidin-conjugated allophycocyanin (Caltag Labs.) or streptavidin-conjugated Red613 (GIBCO BRL). The following rat Igs were used as isotype controls: biotinylated IgG2a and IgG2b and fluoresceinated IgG2a and IgG2b (PharMingen). Cells were incubated for 15 min on ice with CD16/32 (Fc $\gamma$ III/II receptor; 1:100; FcBlock™; PharMingen) before staining with the first antibody. 10<sup>6</sup> cells/100  $\mu$ l were suspended in 5% FCS/PBS (washing buffer). Cells were stained with the first antibody, incubated for 30 min on ice, and washed twice with washing buffer. The secondary antibody was added, and the cells were incubated for 30 min on ice. After incubation, cells were washed twice with washing buffer and suspended in washing buffer for FACS® analysis. The stained cells were analyzed and sorted by FACSVantage™ (Becton Dickinson).

**Cytochemical Staining.** Sorted cells were centrifuged onto microscope slides using a cytospin centrifuge (Shandon Southern Products) and stained with May-Grünwald-Giemsa staining solution (Merck KGaA).

**Osteoclast Formation Assay.** 5  $\times$  10<sup>3</sup> ST2 cells were plated in 96-well plates (PRIMARIA; Becton Dickinson Labware) 1 d before coculture with sorted BM cells. Cultures were maintained in  $\alpha$ -MEM/10% FCS with 10<sup>-8</sup> M 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 10<sup>-7</sup> M dexamethasone (Dex). Tartrate-resistant acid phosphatase (TRAP) staining was performed on day 4, and the TRAP solution assay (TRAP activity) was performed on day 7 or 10. Cocultures were scaled down to 100, 50, 40, 30, 20, 10, 8, 6, 5, 4, 2, and 1 cell in 100  $\mu$ l for a limiting dilution assay.

To assay osteoclast formation without a stromal layer, sorted BM cells were cultured in  $\alpha$ -MEM/10% FCS in the presence of recombinant M-CSF (100 ng/ml) and sRANKL (25 ng/ml).

**TRAP Staining.** After aspiration of medium, cells were fixed with 1% glutaraldehyde (Wako Pure Chemical Industries, Ltd.) in PBS for 15 min at room temperature. Cells were stained for TRAP using a histochemical kit (no. 387; Sigma Chemical Co.)

according to the manufacturer's instructions. TRAP<sup>+</sup> cells were scored as osteoclasts microscopically.

**TRAP Solution Assay (TRAP Activity).** In the TRAP solution assay, enzyme activity was examined by the conversion of  $\alpha$ -naphthyl phosphate (4 mmol/liter; Sigma Chemical Co.) to  $\alpha$ -naphthol in the presence of 2 mol/liter 1-tartrate solution (Sigma Chemical Co.) in each well. Absorbance was measured at 405 nm using a microplate reader (model 550; Bio-Rad Labs.).

**Analysis of Differentiation in Cultured Cells.** c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells were cultured for 2 d in  $\alpha$ -MEM/10% FCS in the presence of SCF (100 U/ml), and c-Fms expression was examined. Sorted c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells derived from BM were cultured for 3 d in  $\alpha$ -MEM/10% FCS plus either M-CSF (30 ng/ml) or IL-3 (100 U/ml), and the expression of RANK was analyzed. To investigate whether c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells and c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells differentiate into B cells, these cells were cocultured with OP9 stromal cells for 10 d in RPMI 1640/10% FCS in the presence of IL-7 (20 U/ml). After 10 d in culture, the expression of B220 was analyzed using FACS<sup>Vantage</sup><sup>TM</sup>.

**RT-PCR Analysis.** An RNeasy mini Kit (QIAGEN GmbH) was used for isolation of total RNA from total BM cells or fractionated BM cells. Total RNA was reverse transcribed using an RT for PCR kit (Clontech). The cDNAs were amplified using an Advantage polymerase mix (Clontech) in a GeneAmp PCR system (model 9700; Perkin-Elmer Corp.) for 25–30 cycles. Sequences of gene-specific primers for RT-PCR were as follows: 5'-mRANK, CCAGGGGACAACGGAATCAG; 3'-mRANK,

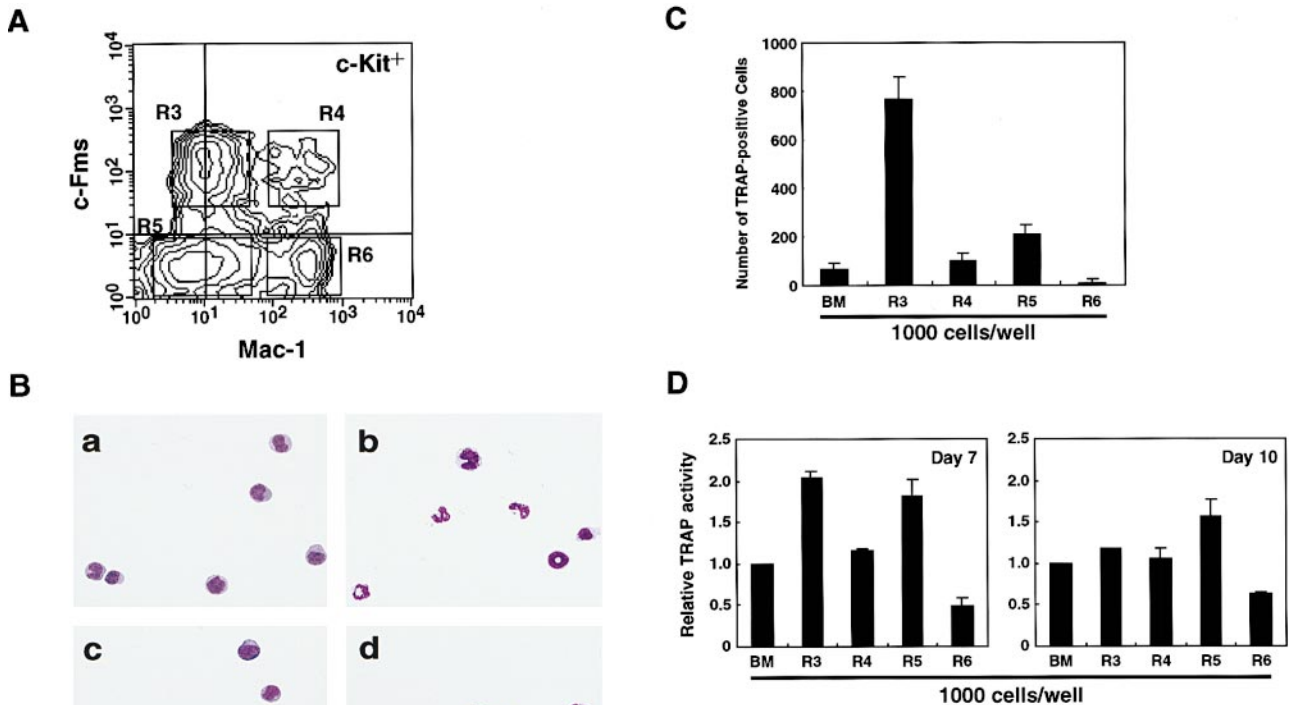
GGCCGGTCCGTGTACTCATC; 5'-m $\beta$ -actin, TCGTGCGT-GACATCAAAGAG; and 3'-m $\beta$ -actin, TGGACAGTGAGGC-CAGGATG. Each cycle consisted of 30 s denaturation at 94°C and 4 min annealing/extension at 70°C.

**In Vitro Colony Assay.** To compare CFU-C (culture) activity between c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>±</sup> cells, 10<sup>3</sup> cells were cultured in 1 ml of culture medium containing  $\alpha$ -MEM, 1.2% methylcellulose (1,500 centipoise; Aldrich Chemical Co.), 30% FCS, 1% deionized BSA, 50 mM 2-ME, 100 U/ml IL-3, 2 U/ml Epo, and 100 ng/ml SCF. After 7 d in culture, aggregates of 50 or more cells were counted as a single colony.

For in vitro osteoclast colony formation assay, 10<sup>3</sup> R3 cells were plated in methylcellulose medium. In brief, cells were embedded in 1 ml of 1.2% methylcellulose, 30% FCS, 1% deionized BSA, 50 mmol/liter 2-ME, and 100 ng/ml M-CSF in the presence or absence of sRANKL (25 ng/ml) in  $\alpha$ -MEM. The culture dishes were incubated in humidified atmosphere at 37°C with 5% CO<sub>2</sub>. After 7 d, the colonies were counted, picked up, and stained for nonspecific esterase, May-Grünwald-Giemsa, and TRAP.

## Results

**Isolation of Osteoclast Precursor Cells from Mouse BM Mononuclear Cells.** To characterize and isolate osteoclast precursor cells, expression of cell surface markers was analyzed (Fig. 1 A). Of the c-Kit<sup>+</sup> BM mononuclear cells, 37.5% were



**Figure 1.** Differentiation of TRAP<sup>+</sup> cells from fractionated mouse BM mononuclear cells. (A) Expression of c-Fms and Mac-1 (CD11b) on BM mononuclear cells was analyzed by FACS<sup>®</sup> gated with c-Kit<sup>+</sup> cells. c-Kit<sup>+</sup> cells were subdivided further into four fractions based on expression of c-Fms and Mac-1. R3, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup>; R4, c-Kit<sup>+</sup>Mac-1<sup>high</sup>c-Fms<sup>+</sup>; R5, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup>; and R6, c-Kit<sup>+</sup>Mac-1<sup>high</sup>c-Fms<sup>-</sup>. (B) Fractionated cells were examined by May-Grünwald-Giemsa staining. (a) R3; (b) R4; (c) R5; and (d) R6. Scale bar, 50  $\mu$ m. (C) 10<sup>3</sup> cells of each fraction were cocultured with ST2 stromal cells in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) and Dex (10<sup>-7</sup> M), and the number of TRAP<sup>+</sup> cells was determined on day 4. (D) Relative TRAP activities were measured on days 7 and 10 of coculture with ST2 stromal cells. TRAP activity from unfractionated BM cells was set to 1.0.

**Table I.** Proportion of Osteoclast Precursor Cells

Fraction	BM mononuclear cells	Gated with c-Kit <sup>+</sup> cells
	%	%
R3	0.53 ± 0.04	13.4 ± 0.6
R4	0.20 ± 0.09	4.8 ± 1.9
R5	1.99 ± 0.08	50.0 ± 3.7
R6	0.72 ± 0.13	18.6 ± 1.3

BM mononuclear cells were stained with anti-c-Kit, anti-Mac-1, and anti-c-Fms antibodies and were gated with c-Kit<sup>+</sup> cells. c-Kit<sup>+</sup> cells were subdivided into four fractions on the basis of expression of Mac-1 and c-Fms. R3, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup>; R4, c-Kit<sup>+</sup>Mac-1<sup>high</sup>c-Fms<sup>+</sup>; R5, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup>; and R6, c-Kit<sup>+</sup>Mac-1<sup>high</sup>c-Fms<sup>-</sup>. Numbers represent the mean percentage ± SD.

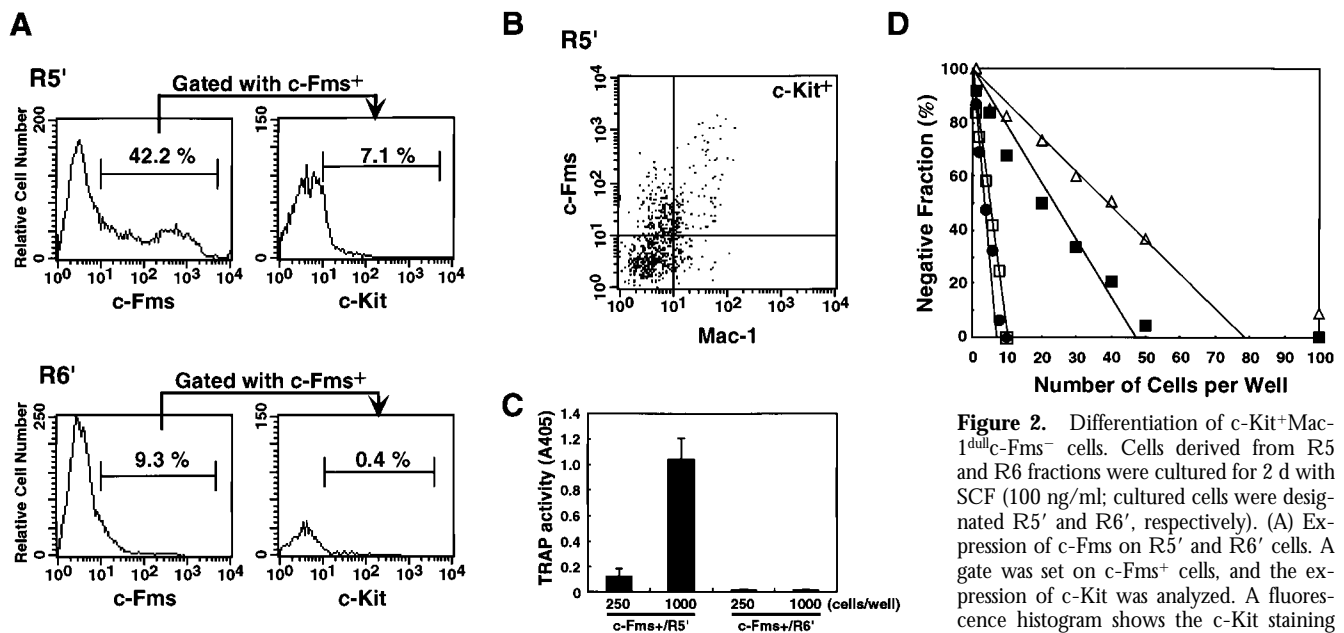
c-Fms<sup>+</sup> and 56.1% were Mac-1<sup>+</sup>. Using contour blot analyses, four populations of c-Kit<sup>+</sup> cells were detected: Mac-1<sup>dull</sup>c-Fms<sup>+</sup> (R3), Mac-1<sup>high</sup>c-Fms<sup>+</sup> (R4), Mac-1<sup>dull</sup>c-Fms<sup>-</sup> (R5), and Mac-1<sup>high</sup>c-Fms<sup>-</sup> (R6). These cells were fractionated, and further analyses were performed. The percentages of these populations in both BM mononuclear cells and c-Kit<sup>+</sup> cells is shown in Table I. The morphology of cells of each fraction was examined by May-Grünwald Giemsa staining (Fig. 1 B). R3 and R5 cells were immature cells showing a large N/C ratio, whereas most of the R4 and R6 cells were mature cells, including macrophages or neutrophils.

TRAP is an enzyme highly expressed in both immature and mature osteoclasts. To determine which fractionated cells differentiate into TRAP<sup>+</sup> cells, 10<sup>3</sup> cells of each frac-

tion were cocultured with an ST2 stromal cell line in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and Dex (Fig. 1, C and D). To detect TRAP<sup>+</sup> mononuclear or multinuclear osteoclasts, TRAP staining was performed on day 4 (Fig. 1 C) and TRAP activity (TRAP solution assay) was measured on days 7 and 10 (Fig. 1 D). The number of TRAP<sup>+</sup> cells in the R3 fraction was 10-fold higher than observed in unfractionated cells (Fig. 1 C). Relative TRAP activity (TRAP activity in each cell fraction/TRAP activity in unfractionated BM mononuclear cells) was highest in fraction R3 on day 7. In contrast, on day 10, the highest TRAP activity was detected in fraction R5 (1.55 ± 0.20) compared with R3 (1.17 ± 0.01). The R4 and R6 fractions showed low TRAP activities on both days 7 and 10.

These data suggest that the R3 fraction contains a higher proportion of osteoclast precursor cells than do other populations (R4, R5, and R6) and that R5 cells also contain osteoclast precursor cells that are less mature than R3 cells.

**Induction of c-Fms Expression during Osteoclast Differentiation.** To investigate whether R5 cells (c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup>) differentiate to R3 cells (c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup>), the expression of c-Fms was analyzed after cultivation (Fig. 2 A). c-Fms<sup>-</sup> cells in R5 or R6 were sorted and cultured in SCF (100 ng/ml), as they expressed c-Kit receptors. After 2 d in culture, 42.2% of cultured R5 cells (R5') expressed c-Fms and 7.1% of c-Fms<sup>+</sup> cells were also c-Kit<sup>+</sup>. In contrast, of cultured R6 cells (R6'), 9.3% were c-Fms<sup>+</sup> cells and 0.4% were c-Kit<sup>+</sup>c-Fms<sup>+</sup> cells. Moreover, of R5' cells, c-Kit<sup>+</sup>c-Fms<sup>+</sup> cells were mainly Mac-1<sup>dull</sup> (80.3%; Fig. 2 B). To determine if c-Fms<sup>+</sup> cells in R5' or R6' could undergo osteoclastic differentiation, both cell fractions were cocultured with ST2 stromal cells for 4 d in the presence of both 1,25-



**Figure 2.** Differentiation of c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells. Cells derived from R5 and R6 fractions were cultured for 2 d with SCF (100 ng/ml); cultured cells were designated R5' and R6', respectively. (A) Expression of c-Fms on R5' and R6' cells. A gate was set on c-Fms<sup>+</sup> cells, and the expression of c-Kit was analyzed. A fluorescence histogram shows the c-Kit staining profile of the fraction gated with c-Fms<sup>+</sup>.

(B) Expression of Mac-1 and c-Fms on c-Kit<sup>+</sup> cells. (C) c-Fms<sup>+</sup> cells were sorted from R5' or R6'. 10<sup>3</sup> or 2.5 × 10<sup>2</sup> cells of each fraction were cocultured with ST2 stromal cells and 1,25-(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 4 d, and TRAP activity was measured. (D) Limiting dilution analysis of unfractionated BM mononuclear cells (Δ), R3 (●), R5 (■), and c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5' cells (□). Cells were cocultured with ST2 stromal cells for 4 d, and the percentages of TRAP<sup>+</sup> cells were determined.

(OH)<sub>2</sub>D<sub>3</sub> and Dex and assayed for TRAP activity (Fig. 2 C). Although c-Fms<sup>+</sup> cells of R5' differentiated to osteoclasts, c-Fms<sup>+</sup> cells of R6' did not differentiate into TRAP<sup>+</sup> cells. R6' cells were mature granulocytes and macrophages.

To analyze the proportion of osteoclast precursor cells in unfractionated BM cells, R3, R5, or c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5' cells, each fraction was cultured for 4 d on ST2 stromal cells and scored for TRAP<sup>+</sup> cells by limiting dilution analysis (Fig. 2 D). Limiting dilution of BM cells, R3, R5, and c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5' cells revealed 2.0% (1/49.3; unfractionated BM), 20.4% (1/4.9; R3), 3.4% (1/29.8; R5), or 16.4% (1/6.1; c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5') TRAP<sup>+</sup> cells. R3 or c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5' cells contained 10-fold more osteoclast precursor cells than did unfractionated BM cells. These data suggest that c-Fms<sup>-</sup> cells differentiate to osteoclasts through the induction of c-Fms.

**Differentiation Capacity of Osteoclast Precursor Cells.** c-Fms expression increased during cultivation. As c-Kit<sup>+</sup>c-Fms<sup>+</sup> R5' cells contained the same proportion of osteoclast precursor cells as did R3 cells, we speculated that R5 cells are less mature than R3 cells. To examine the capacity of those osteoclast precursor cells to differentiate to other lineages, a colony assay using R3 or R5 cells was performed (Table II). 10<sup>3</sup> cells from each fraction were cultured with methylcellulose semisolid medium in the presence of SCF (100 ng/ml), IL-3 (100 U/ml), and Epo (2 U/ml) for 7 d. The numbers of colonies (CFU-GEMM [granulocyte, erythrocyte, macrophage, megakaryocyte] burst forming unit-erythrocyte, CFU-GM [granulocyte, macrophage], CFU-G, and CFU-M [macrophage]) were counted under an inverted microscope. Most colonies derived from R3 cells were macrophage colonies. In contrast, R5 cells contained more CFU-GM than CFU-M. Moreover, CFU-GEMM or burst forming unit-erythrocyte-derived colonies were observed in R5 cells.

To analyze the ability of R3 or R5 to differentiate into a B cell lineage, 10<sup>4</sup> cells of each fraction were cocultured with the OP9 stromal cell line and IL-7 (20 U/ml; Table III). After 10 d in culture, the number of expanded nonadherent cells was 73-fold more in R5 (7.9 ± 0.6 × 10<sup>6</sup> cells) than in R3 (1.0 ± 0.2 × 10<sup>5</sup> cells), and 47.0% of R3 or 93.9% of R5 cells were B220<sup>+</sup> cells. These data suggest that R5 cells are more immature than R3 cells and that R5 cells can differentiate not only into osteoclast but also into myeloid, erythroid, or B cell lineages.

**Table II.** Colony Assay on Osteoclast Precursor Cells

Fraction	No. colonies per 10 <sup>3</sup> cells				
	CFU-GEMM	BFU-E	CFU-GM	CFU-G	CFU-M
R3	0 ± 0	0 ± 0	3.7 ± 0.6	1.7 ± 0.6	20.3 ± 3.8
R5	2.0 ± 1.0	2.3 ± 1.2	39.0 ± 6.6	14.7 ± 3.1	23.0 ± 4.4

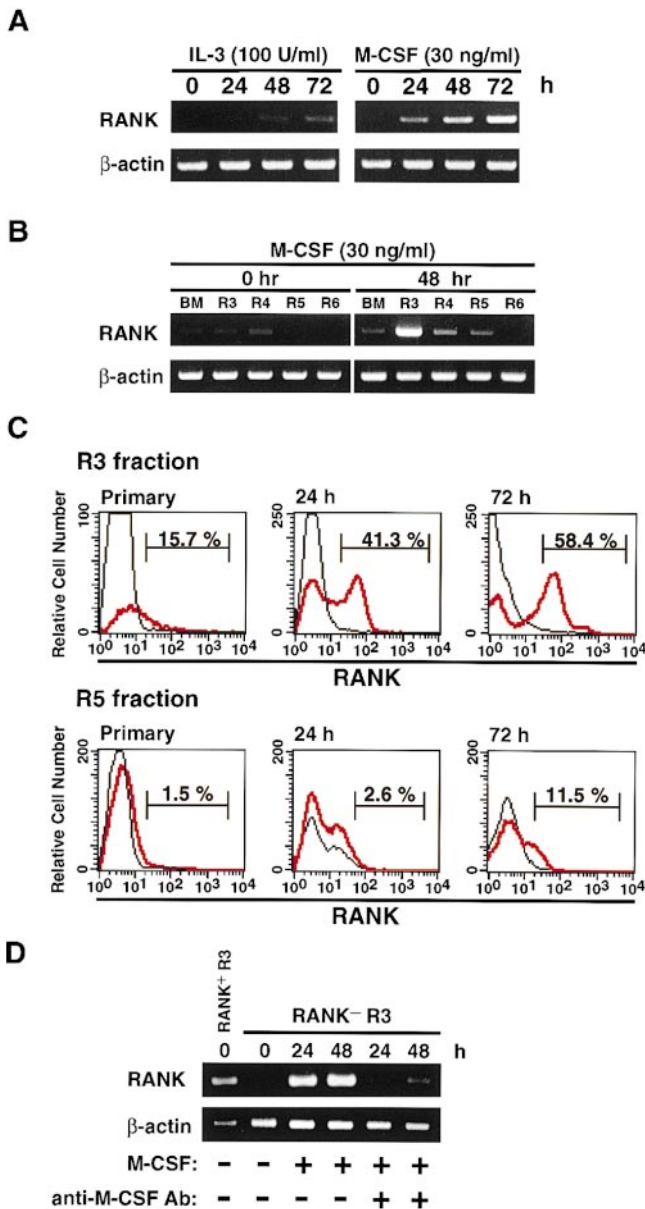
10<sup>3</sup> cells of each fraction were cultured in methylcellulose medium containing IL-3, SCF, and Epo. After 7 d in culture, a colony containing >50 cells was scored as a single colony. The data shown represent the mean colony number ± SD of triplicate samples. BFU-E, burst forming unit-erythrocyte.

**Table III.** Differentiation of B Cells

Fraction	No. nonadherent cells	B220 <sup>+</sup> cells
		(total no. B220 <sup>+</sup> cells)
		%
R3	1.0 ± 0.2 × 10 <sup>5</sup>	47.0 (5.0 ± 1.0 × 10 <sup>4</sup> )
R5	7.9 ± 0.6 × 10 <sup>6</sup>	93.9 (7.4 ± 0.5 × 10 <sup>6</sup> )

10<sup>4</sup> cells of each fraction were cocultured with OP9 stromal cells with IL-7 (20 ng/ml) for 10 d. After cultivation, nonadherent cells were collected and analyzed for the expression of B220. Numbers represent mean ± SD.

**Induction of RANK Expression with M-CSF in Osteoclast Precursor Cells.** Expression of RANK in fractionated cells was examined by RT-PCR and FACS<sup>®</sup>. To examine whether M-CSF induces RANK mRNA expression, unfractionated BM mononuclear cells were cultured for 72 h in the presence of IL-3 (100 U/ml) or M-CSF (30 ng/ml). Both IL-3 and M-CSF are able to support the differentiation of macrophagic differentiation. The expression of RANK mRNA was detected in a 24-h incubation with M-CSF and a 72-h incubation with IL-3 (Fig. 3 A). Subsequently, fractionated cells were cultured with M-CSF for 48 h. Before induction with M-CSF, very low levels of RANK mRNA were detected in all fractionated cells except R6 cells (Fig. 3 B). After incubation for 48 h, the expression of RANK mRNA was obvious in the R3 fraction. FACS<sup>®</sup> analyses demonstrated that 5.4% of unfractionated BM cells were RANK<sup>+</sup> (data not shown). Of R3 cells, 15.7% were RANK<sup>+</sup>, and 1.5% of R5 cells were RANK<sup>+</sup> (Fig. 3 C). The expression of RANK protein in R3 or R5 cells was analyzed by FACS<sup>®</sup> after incubation with M-CSF for 24 or 72 h (Fig. 3 C). The percentage of RANK<sup>+</sup> cells in R3 increased with longer incubations with M-CSF (41.3% for a 24-h incubation and 58.4% for a 72-h period). Although a similar increase was also observed in R5 cells, the overall percentage of RANK<sup>+</sup> R5 cells was lower than that of R3 cells (2.6% after a 24-h incubation and 11.5% after 72 h). To characterize RANK<sup>+</sup> or RANK<sup>-</sup> cells in the R3 fraction cultured with M-CSF, cells were sorted with a RANK mAb, and the expression of c-Kit, Mac-1, and c-Fms was analyzed. After 24 h in culture with M-CSF, sorted RANK<sup>+</sup> cells



**Figure 3.** Expression of RANK on unfractionated BM cells or fractionated cells. Expression of RANK mRNA was analyzed by RT-PCR. (A) BM mononuclear cells were cultured with IL-3 (100 U/ml; left panel) or M-CSF (30 ng/ml; right panel), and the expression of RANK mRNA was examined before and after 24, 48, or 72 h of culture. (B) Fractionated BM cells were cultured for 48 h with M-CSF (30 ng/ml), and the expression of RANK was examined before and after 48 h of culture. (C) Expression of RANK protein on BM R3 or R5 cells was analyzed by FACS<sup>®</sup>. R3 (upper panels) or R5 (lower panels) cells were cultured for 24 or 72 h with M-CSF (30 ng/ml), and the expression of RANK was analyzed. Red line, anti-RANK; black line, rat IgG2a. (D) R3 cells (c-Kit<sup>+</sup>Mac-1<sup>high</sup>c-Fms<sup>+</sup>) were divided into RANK<sup>+</sup> or RANK<sup>-</sup> cells, and RANK<sup>-</sup> R3 cells were cultured for 24 or 48 h in the presence of M-CSF (30 ng/ml) with or without anti-mouse M-CSF neutralizing antibody (10 μg/ml). The expression of RANK was analyzed by RT-PCR.

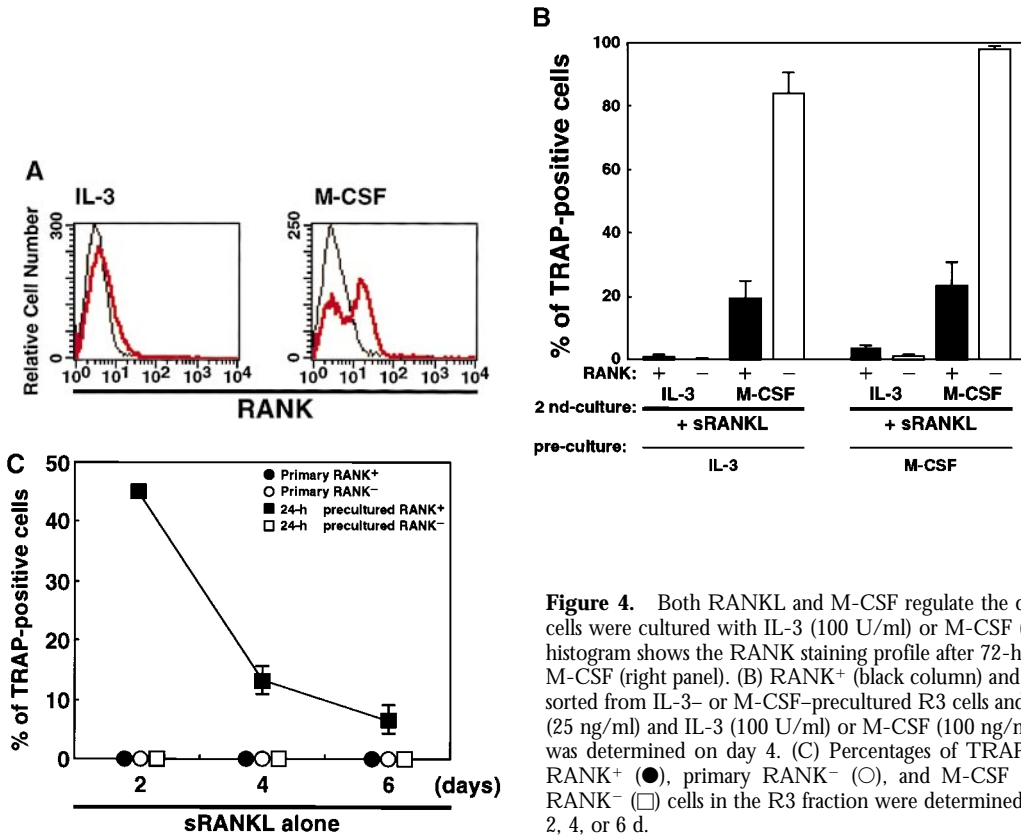
were c-Kit<sup>-</sup>Mac-1<sup>high</sup>c-Fms<sup>+</sup>, and sorted RANK<sup>-</sup> cells were c-Kit<sup>-</sup>Mac-1<sup>high</sup>c-Fms<sup>+</sup> (data not shown). RANK expression in R3 cells was induced by stimulation of M-CSF; however, R3 cells originally contained RANK<sup>+</sup> cells.

To examine whether M-CSF induces the expression of RANK on RANK<sup>-</sup> R3 cells, R3 cells were subdivided into RANK<sup>+</sup> and RANK<sup>-</sup> fractions. RANK<sup>-</sup> R3 cells were cultured in the presence of M-CSF with or without anti-mouse M-CSF neutralizing antibody, and the expression of RANK was analyzed by RT-PCR. The expression of RANK was detected in freshly isolated RANK<sup>+</sup> R3 cells and cultured RANK<sup>-</sup> R3 cells. However, RANK expression was significantly reduced by the addition of mouse M-CSF neutralizing antibody to the culture (Fig. 3 D). These results indicate that M-CSF stimulates the expression of RANK on osteoclast precursor cells.

*M-CSF and RANKL Cooperate in the Differentiation of Osteoclasts.* Next we analyzed how osteoclast precursor cells differentiate into TRAP<sup>+</sup> cells in the presence of M-CSF and sRANKL rather than ST2 stromal cells. Fractionated R3 cells were cultured for 72 h in IL-3 (100 U/ml) or M-CSF (30 ng/ml). As shown in Fig. 4 A, M-CSF induced RANK expression more efficiently than did IL-3. RANK<sup>+</sup> or RANK<sup>-</sup> cells precultured with IL-3 or M-CSF for 72 h were sorted and cultured in the presence of sRANKL and either IL-3 or M-CSF. The percentage of TRAP<sup>+</sup> cells is shown in Fig. 4 B. Both IL-3- and M-CSF-precultured cells differentiated into TRAP<sup>+</sup> cells in the presence of sRANKL and M-CSF. Moreover, RANK<sup>-</sup> cells in each preculture condition showed a higher percentage of TRAP<sup>+</sup> cells than RANK<sup>+</sup> cells in the presence of sRANKL and M-CSF. M-CSF-precultured cells showed a higher percentage of TRAP<sup>+</sup> cells than did IL-3-precultured cells.

To examine whether RANK<sup>+</sup> cells differentiate to TRAP<sup>+</sup> cells in the presence of sRANKL alone, primary R3 cells or R3 cells precultured with M-CSF were cultured for 2, 4, or 6 d and analyzed for TRAP staining. RANK<sup>+</sup> cells precultured for 24 h differentiated into TRAP<sup>+</sup> cells, and the percentage of TRAP<sup>+</sup> cells decreased in the presence of sRANKL alone (Fig. 4 C). However, primary R3 cells (RANK<sup>+</sup> or RANK<sup>-</sup>) or RANK<sup>-</sup> cells precultured for 24 h did not differentiate into TRAP<sup>+</sup> cells with sRANKL alone. No TRAP<sup>+</sup> cells were observed when cells were cultured for 6 d with M-CSF alone (data not shown). These data suggest that M-CSF affects not only the survival factor but also the competence factor for osteoclast precursor cells during their differentiation.

*The Timing of RANKL Binding to Osteoclast Precursor Cells Is Critical for Osteoclastogenesis.* To clarify the synergistic effect of M-CSF and sRANKL, the relationship between the onset of RANK expression and osteoclast differentiation was examined. Fig. 5 shows the flow chart of cell sorting and conditions of further cultivation. Sorted RANK<sup>+</sup> or RANK<sup>-</sup> cells from the primary R3 fraction were cultured for 2, 4, and 6 d with both sRANKL and M-CSF (Fig. 6, A and B). On days 2 and 4, the percentage of TRAP<sup>+</sup> cells was higher in RANK<sup>-</sup> cells (11.8 ± 1.9% on day 2 and 97.5 ± 1.4% on day 4) than in RANK<sup>+</sup> cells (7.0 ± 1.7% on day 2 and 42.5 ± 6.1% on day 4). On day 6, however, the percentage of TRAP<sup>+</sup> cells was similar in RANK<sup>+</sup> and RANK<sup>-</sup> cells. Interestingly, the percentage of TRAP<sup>+</sup> cells in R3 cells precultured for 24 h with M-CSF was similar be-

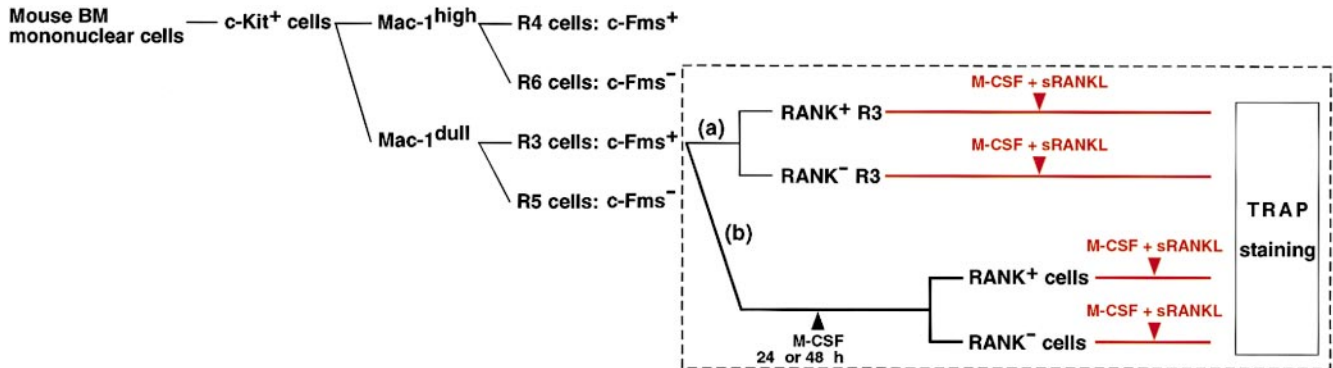


**Figure 4.** Both RANKL and M-CSF regulate the differentiation of osteoclasts. (A) R3 cells were cultured with IL-3 (100 U/ml) or M-CSF (30 ng/ml) for 72 h. A fluorescence histogram shows the RANK staining profile after 72-h cultivation with IL-3 (left panel) or M-CSF (right panel). (B) RANK<sup>+</sup> (black column) and RANK<sup>-</sup> (white column) cells were sorted from IL-3- or M-CSF-precultured R3 cells and were then cultured with sRANKL (25 ng/ml) and IL-3 (100 U/ml) or M-CSF (100 ng/ml). The percentage of TRAP<sup>+</sup> cells was determined on day 4. (C) Percentages of TRAP<sup>+</sup> cells differentiated from primary RANK<sup>+</sup> (●), primary RANK<sup>-</sup> (○), and M-CSF 24-h-precultured RANK<sup>+</sup> (■) or RANK<sup>-</sup> (□) cells in the R3 fraction were determined after cultivation with sRANKL for 2, 4, or 6 d.

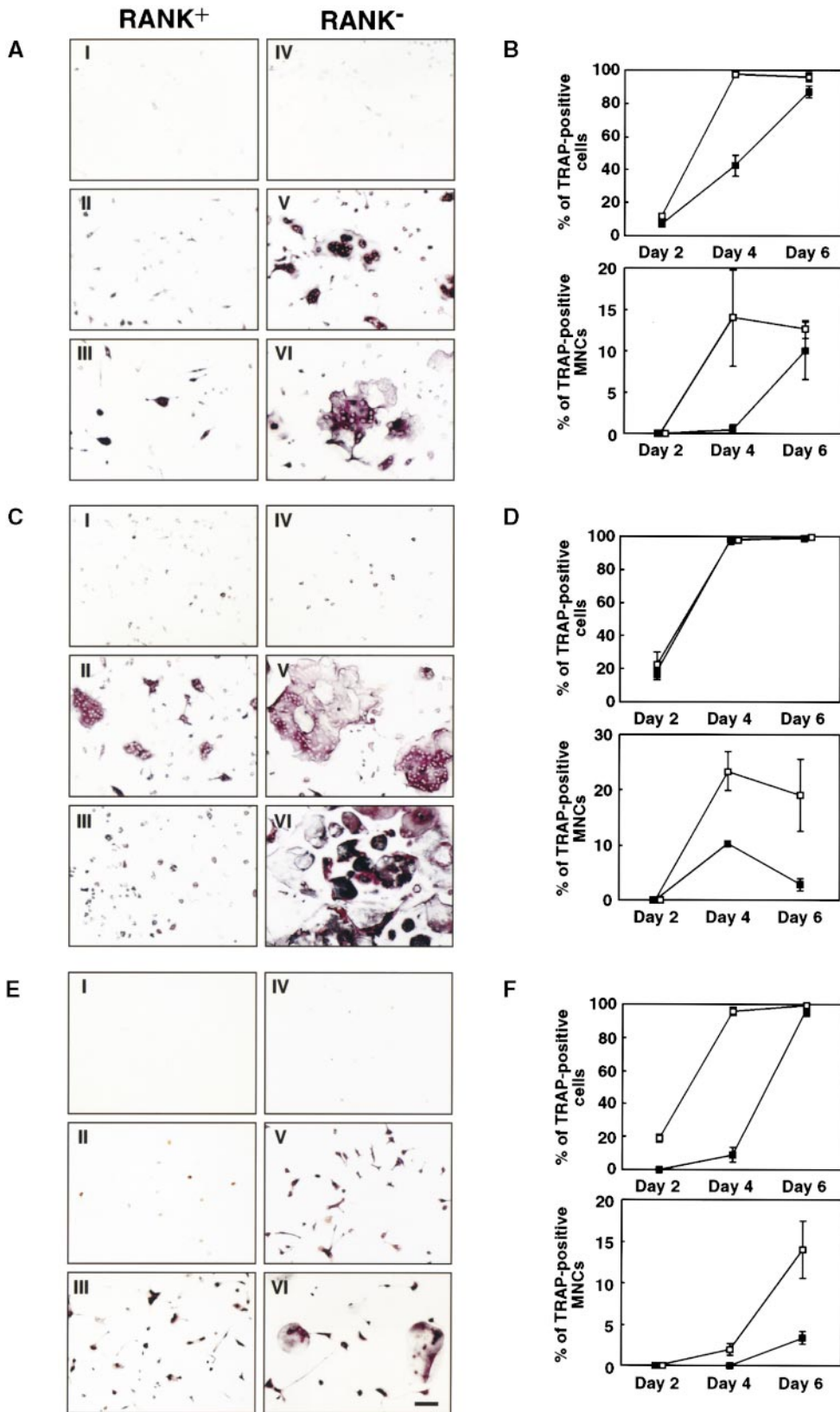
tween RANK<sup>+</sup> and RANK<sup>-</sup> cells (Fig. 6, C and D), whereas in the case of R3 cells that were not precultured, RANK<sup>+</sup> cells did not efficiently differentiate into TRAP<sup>+</sup> cells (Fig. 6, A and B). RANK<sup>-</sup> cells showed a higher percentage of MNCs, which are fully matured osteoclasts, than did RANK<sup>+</sup> cells. These MNCs from RANK<sup>-</sup> cells were extremely large and contained a large number of nuclei.

Growth of RANK<sup>+</sup> and RANK<sup>-</sup> cells after 72 h of preculture with M-CSF was less than that of primary or 24-h-precultured R3 cells (Fig. 6 E). Although the percentage of TRAP<sup>+</sup> cells was higher in RANK<sup>-</sup> cells than

RANK<sup>+</sup> cells on day 4 ( $9.16 \pm 4.3\%$  in RANK<sup>+</sup> and  $95.6 \pm 1.8\%$  in RANK<sup>-</sup> cells), the percentage was similar on day 6 ( $97.1 \pm 0.5\%$  for RANK<sup>+</sup> and  $99.5 \pm 0.1\%$  for RANK<sup>-</sup> cells; Fig. 6 F). A delay in differentiation of TRAP<sup>+</sup> cells from RANK<sup>+</sup> cells was observed. Also RANK<sup>-</sup> cells showed a higher percentage of TRAP<sup>+</sup> MNCs than did RANK<sup>+</sup> cells, but this number was lower than that observed in RANK<sup>-</sup> cells precultured for 24 h. Moreover, the size of MNCs was reduced and the number of nuclei contained in MNCs was smaller in RANK<sup>-</sup> cells precultured for 72 h than in cells precultured for 24 h.



**Figure 5.** Flow chart of FACS<sup>®</sup> analysis and the points for further examination. Mouse BM c-Kit<sup>+</sup> cells were divided into four fractions (R3, R4, R5, and R6) as described in the Fig. 1 legend. The box enclosed with dashed lines indicates further analysis performed in Fig. 6. (a) R3 cells (c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup>) were subdivided into RANK<sup>+</sup> or RANK<sup>-</sup> cells, and each fraction was cultured in the presence of M-CSF and sRANKL. (b) R3 cells were cultured for 24 or 72 h with M-CSF and were subdivided into RANK<sup>+</sup> or RANK<sup>-</sup> cells. 24- or 72-h-precultured RANK<sup>+</sup> and RANK<sup>-</sup> cells were cultured with both M-CSF and sRANKL. Red line, cultivation with M-CSF and sRANKL; dark blue line, cultivation with M-CSF.



**Figure 6.** Differentiation of TRAP<sup>+</sup> cells from RANK<sup>+</sup> or RANK<sup>-</sup> cells. R3 cells were pre-cultured with M-CSF (30 ng/ml) for 24 or 72 h. These precultured cells and primary R3 cells were then divided into RANK<sup>+</sup> or RANK<sup>-</sup> fractions. Primary and cultured RANK<sup>+</sup> or RANK<sup>-</sup> cells were cultured with sRANKL (25 ng/ml) and M-CSF (100 ng/ml). (A and B) Primary R3 cells. (C and D) 24-h M-CSF-precultured cells. (E and F) M-CSF 72-h-precultured cells. TRAP staining (A, C, and E) was performed on day 2 (I and IV), day 4 (II and V), or day 6 (III and VI), and the percentage of TRAP<sup>+</sup> cells or TRAP<sup>+</sup> MNCs (B, D, and F) was scored at the same time. I, II, and III were derived from RANK<sup>+</sup> cells; IV, V, and VI were from RANK<sup>-</sup> cells. Scale bar, 100  $\mu$ m. Upper panels of B, D, and F represent percentages of TRAP<sup>+</sup> cells that include both mononuclear cells and MNCs in the total cells in the well. Lower panels of B, D, and F represent percentages of TRAP<sup>+</sup> MNCs in total TRAP<sup>+</sup> cells in the well. Percent of TRAP<sup>+</sup> cells or TRAP<sup>+</sup> MNCs derived from RANK<sup>+</sup> (■) or RANK<sup>-</sup> (□) cells is shown.

In all culture conditions, especially 24-h preculture with M-CSF, the potential of cell growth of RANK<sup>-</sup> cells was greater than that of RANK<sup>+</sup> cells. Also, RANK<sup>-</sup> cells formed a large number of MNCs, which contained high

numbers of nuclei because of the increment of cell density induced by cell proliferation. In addition, when RANK<sup>+</sup> and RANK<sup>-</sup> cells were precultured with M-CSF for 24 h, a higher number of TRAP<sup>+</sup> cells was observed in the lat-



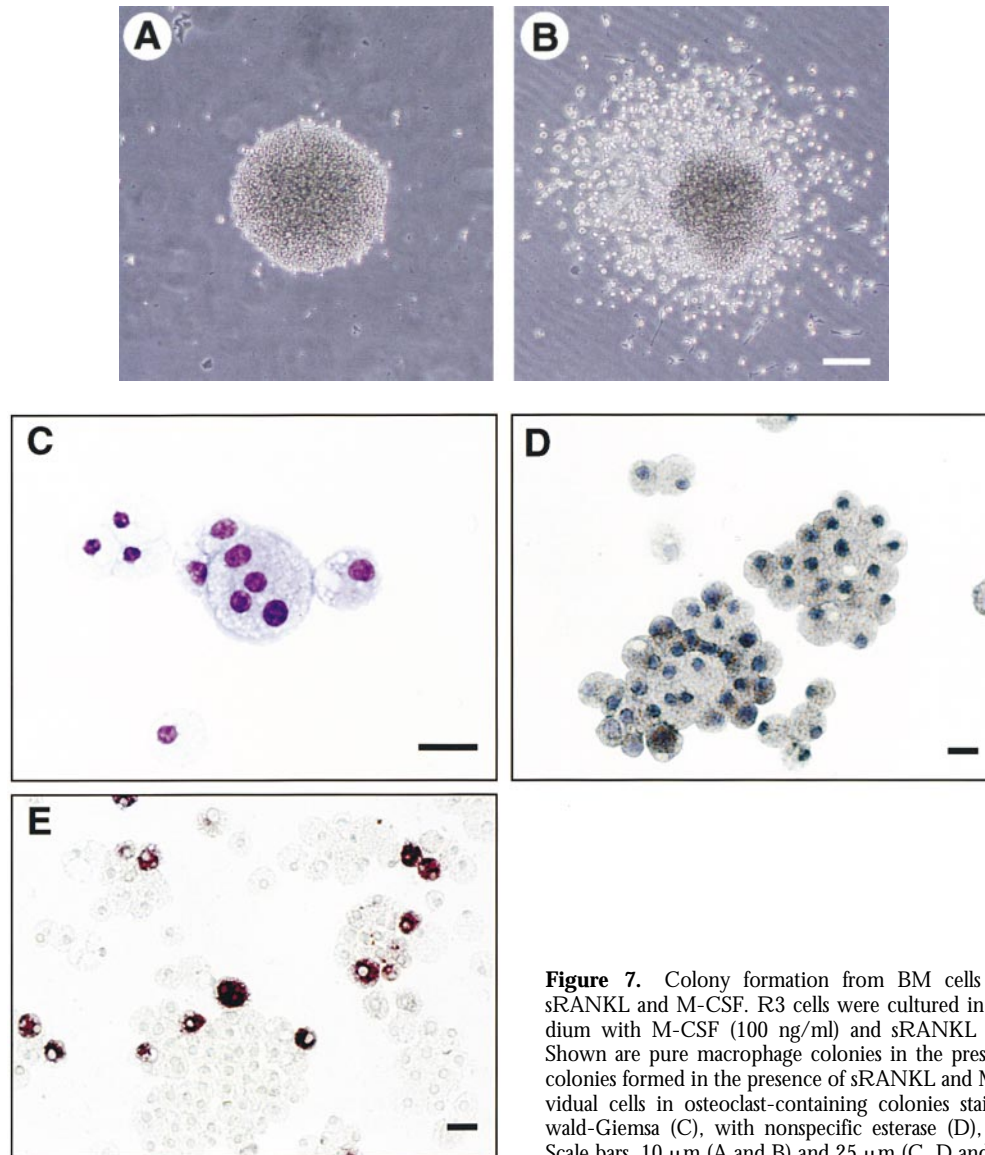
ter, suggesting that RANK expression is necessary but not sufficient for osteoclast differentiation and that the timing of RANKL binding to RANK<sup>+</sup> cells may be critical for commitment of osteoclast cells.

*The Osteoclast Precursor Cell Is Still Bipotential at a Late Stage of Osteoclast Differentiation.* Fractionated cells (R3 or R5) can differentiate to lineages other than osteoclast (Tables II and III); however, it is not clear whether a single osteoclast precursor cell is committed to differentiate into a TRAP<sup>+</sup> cell or whether it can differentiate into other lineages. To understand the mechanism of osteoclast differentiation, R3 cells were cultured in methylcellulose instead of liquid culture (Fig. 7). 10<sup>3</sup> R3 cells were cultured with methylcellulose medium containing M-CSF (100 ng/ml) in the presence or absence of sRANKL (25 ng/ml) for 7 d. In the absence of both cytokines, colony formation was not detected, whereas 139 ± 14.5 and 131 ± 1.7 colonies were observed in M-CSF alone and in M-CSF and sRANKL, respectively (Table IV).

Whereas colonies formed in the presence of M-CSF alone were tightly compacted (Fig. 7 A), colonies observed in M-CSF and RANKL were of mixed type (Fig. 7 B). Individual colonies in the presence of M-CSF and sRANKL contained not only nonspecific esterase-positive macrophages but also TRAP<sup>+</sup> cells (Fig. 7, C–E). The percentage of TRAP<sup>+</sup> cells in 20 colonies was 0 ± 0% (M-CSF alone) and 43.8 ± 30.4% (13.3–94.2%; M-CSF and sRANKL; Table IV). Any homogeneous colonies consisting of all TRAP<sup>+</sup> cells were not observed. These data strongly suggest that single precursor cells can differentiate into TRAP<sup>+</sup> cells and macrophages at a late stage of osteoclast differentiation.

## Discussion

Here we identify early and late stages of osteoclast precursor cells and describe the differentiation pathway of osteoclasts from hematopoietic precursor cells by coculture of



**Figure 7.** Colony formation from BM cells in the presence of sRANKL and M-CSF. R3 cells were cultured in methylcellulose medium with M-CSF (100 ng/ml) and sRANKL (25 ng/ml) for 7 d. Shown are pure macrophage colonies in the presence of M-CSF (A), colonies formed in the presence of sRANKL and M-CSF (B), and individual cells in osteoclast-containing colonies stained for May-Grünwald-Giemsa (C), with nonspecific esterase (D), and for TRAP (E). Scale bars, 10 μm (A and B) and 25 μm (C, D and E).

**Table IV.** Differentiation of Single Osteoclast Precursor Cell

	No. of colonies	TRAP <sup>+</sup> cells
		%
No factor	0 ± 0	ND
M-CSF alone	139 ± 14.5	0 ± 0
M-CSF + sRANKL	131 ± 1.7	43.8 ± 30.4

10<sup>3</sup> R3 cells were cultured in methylcellulose medium with M-CSF (100 ng/ml) in the presence or absence of sRANKL (25 ng/ml). After 7 d of cultivation, the number of colonies (containing >50 cells) was determined, and the percentage of TRAP<sup>+</sup> cells in each colony was calculated. The data shown represents the mean number of colonies ± SD.

those cells with the ST2 stromal cell line and 1,25-(OH)<sub>2</sub>D<sub>3</sub>. We also analyze the osteoclast commitment process by substituting M-CSF and sRANKL for the stromal cells.

**Identification of Osteoclast Precursors.** Hematopoietic precursor cells exist in the c-Kit<sup>+</sup> fraction (34, 35). This fraction was clearly subdivided by the expression of c-Fms and Mac-1. Mac-1<sup>+</sup> cells contain mainly mature granulocytes and macrophages, whereas Mac-1<sup>dull</sup> cells are multipotential progenitor cells (36, 37). We demonstrate that c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells in murine BM are early stage precursors of osteoclasts using a limiting dilution method. These cells are shown to be derived from c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells. We show directly that c-Fms<sup>-</sup> cells differentiate into c-Fms<sup>+</sup> cells after 2 d in culture with SCF. It has been previously reported that c-Fms is a key determinant in the differentiation of monocyte-macrophage lineage cells (38). c-Fms expression is regulated by a tissue-specific promoter. Although the precise mechanism of c-Fms expression is not known, transcription factors c-ets-1, c-ets-2, and PU.1 mediate induction of c-Fms (39–41). Thus, it is reasonable that c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells differentiate into TRAP<sup>+</sup> osteoclasts after 4 d, whereas c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells require >7 d. In addition, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells express the RANK mRNA and protein in 24 h in the presence of M-CSF. Lacey et al. (21) demonstrated that osteoclast precursor cells were identified by sRANKL-FITC. They sorted sRANKL-FITC<sup>+</sup> cells after cultivation of mouse BM cells in the presence of M-CSF and sRANKL for 1 d, and these precursor cells were therefore regarded as RANK<sup>+</sup> cells. These sRANKL-FITC<sup>+</sup> cells formed a larger number of multinucleated TRAP<sup>+</sup> cells than sRANKL-FITC<sup>-</sup> cells. In contrast to this finding, we found that a large number of multinucleated TRAP<sup>+</sup> cells were derived from RANK<sup>-</sup> rather than RANK<sup>+</sup> cells, regardless of precultivation with M-CSF (Fig. 6). By cultivation with M-CSF and sRANKL for 1 d before cell sorting, it is speculated that sRANKL-FITC<sup>+</sup> cells have already been committed to osteoclast lineage. We show a sequential change of phenotype in the differentiation pathway of osteoclasts (Fig. 8 A).

Although an mAb against c-Fms suppresses osteoclastogenesis (42), most osteoclast precursors in BM are c-Kit<sup>+</sup>

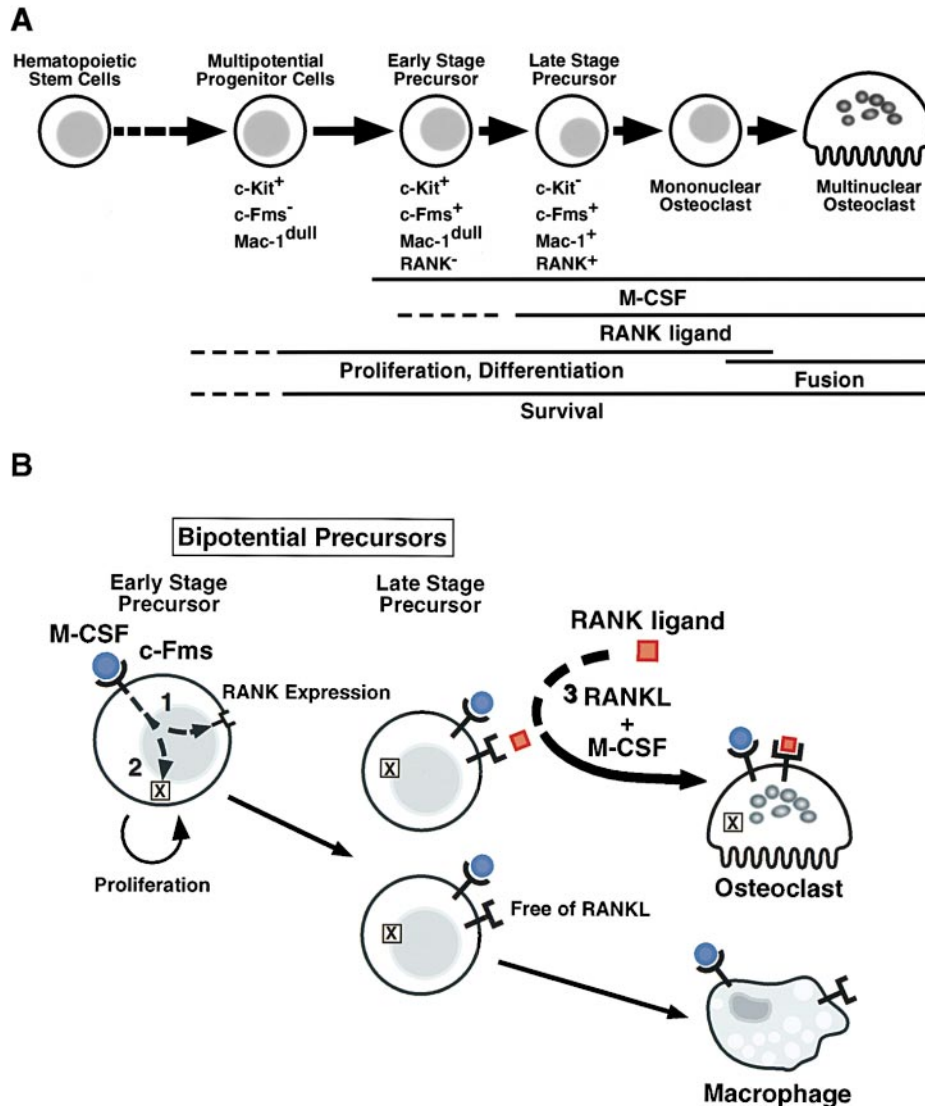
c-Fms<sup>-</sup>, and their numbers decrease along with expression of c-Fms (43). This observation is based on an assay of TRAP<sup>+</sup> cells on day 6 of culture, which is consistent with our observation. We show that a later stage of osteoclast precursor cells (c-Fms<sup>+</sup>RANK<sup>+</sup>) differentiates into TRAP<sup>+</sup> osteoclasts in 2 d. It was reported that osteoclast precursor cells were significantly higher in the c-Kit<sup>low</sup> fraction, whereas myeloid cells of other lineages were higher in the c-Kit<sup>high</sup> fraction (44). However, the c-Kit<sup>high</sup> fraction contained a similar number of osteoclast precursor cells (45).

We showed that c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>-</sup> cells can differentiate not only into the myeloid lineage but also into B220<sup>+</sup> B cells in the presence of IL-7. On the other hand, c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells differentiate into osteoclasts at a frequency of 1:5 on stromal cells. Stromal cells such as ST2 express RANKL, and its expression can be upregulated by the bone-resorbing factors 1,25-(OH)<sub>2</sub>D<sub>3</sub>, IL-11, prostaglandin E2, and parathyroid hormone (46). However, the ST2 coculture system with 1,25-(OH)<sub>2</sub>D<sub>3</sub> does not maximally stimulate osteoclastogenesis, as ST2 also produces the inhibitory molecule OPG/OCIF, which belongs to the TNFR family (47, 48).

**Commitment of Osteoclasts.** We investigated the commitment process of c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells to osteoclasts using M-CSF and sRANKL instead of stromal cells. Our data suggest that M-CSF stimulates c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells to induce RANK mRNA and protein in 24 h more efficiently than does IL-3. These cells can differentiate into osteoclasts in the presence of both M-CSF and sRANKL. With M-CSF only, they differentiate into macrophages but not into osteoclasts. RANKL is a differentiation factor for osteoclasts but not an exclusive osteoclast commitment factor, as RANK is expressed not only in osteoclasts but in T cells and dendritic cells (24). Mice with a disrupted RANKL (*opgl*) gene show severe osteopetrosis and lack all lymph nodes (32), suggesting that RANKL-RANK signaling plays several roles in organogenesis.

Preculture of c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells with M-CSF for 24 or 72 h results in different fates for osteoclast precursors. RANK<sup>+</sup> cells after 24-h preculture differentiate into osteoclasts more efficiently than after 72-h preculture. RANK<sup>+</sup> cells may autonomously differentiate into the macrophage lineage in the absence of sRANKL for 72 h. By contrast, in the presence of both sRANKL and M-CSF after 24-h preculture with M-CSF, RANK<sup>+</sup> cells efficiently differentiate into osteoclasts. RANK<sup>-</sup> cells, after preculture with M-CSF, may differentiate into RANK<sup>+</sup> cells if they are continuously exposed to M-CSF. Once the cells express RANK, existing RANKL binds to the receptors. This is the most productive system for osteoclast differentiation, which may take place in vivo on bone surfaces. Thus, RANK<sup>-</sup> cells differentiate into osteoclasts more efficiently than RANK<sup>+</sup> cells. Moreover, as their proliferative activity is higher, multinuclear osteoclast formation from RANK<sup>-</sup> cells is greater than that of RANK<sup>+</sup> cells. This is dependent upon the cell density (data not shown).

c-Kit<sup>+</sup>Mac-1<sup>dull</sup>c-Fms<sup>+</sup> cells differentiate exclusively into osteoclasts (~100%) in the presence of both M-CSF and



**Figure 8.** Model of osteoclast differentiation derived from hematopoietic stem cells. (A) Sequential phenotypic progression of osteoclasts and relevant factors. (B) Determination of osteoclastic differentiation in the presence of M-CSF and RANKL.

sRANKL. As even RANK<sup>+</sup> cells can differentiate into macrophages, we conclude that the stage of commitment of osteoclasts is late in the differentiation process. A clonal assay using colony formation in semisolid methylcellulose medium provided us with precise identification of constituent cells and revealed no pure osteoclast colonies in the presence of both M-CSF and sRANKL. A single colony contained 13.3–94.2% TRAP<sup>+</sup> osteoclasts. This observation suggests the very low incidence of osteoclast colony forming cells, which are exclusively committed to osteoclasts. The growth of osteoclasts in methylcellulose is poorer than that observed in a liquid culture system, which enables osteoclasts to attach to the culture dish. Cell anchoring or polarity is critical for osteoclast differentiation. A study of cell adhesion molecules expressed by osteoclasts is underway.

**Function of M-CSF in Osteoclastogenesis.** Our findings suggest that M-CSF plays three roles in osteoclastogenesis: (a)

it induces RANK, (b) it is a competence factor for differentiation, and (c) it stimulates cell survival and proliferation (Fig. 8 B). Although upregulation of RANK by M-CSF is higher than that by IL-3 (Figs. 3 A and 4 A) and GM-CSF (data not shown), RANK induction is not M-CSF specific. Even though expression of RANK on RANK<sup>-</sup> cells was upregulated in the presence of M-CSF and its expression was significantly inhibited by addition of neutralizing M-CSF antibody (Fig. 3 D), further analysis in association with the signaling pathway would be necessary to elucidate the mechanism of c-Fms–RANK interaction.

As shown in Fig. 4 C, TRAP<sup>+</sup> cells differentiated from M-CSF–precultured RANK<sup>+</sup> cells but not from primary RANK<sup>+</sup> cells in the presence of sRANKL alone, suggesting that unknown events induced by M-CSF are competent for differentiation to osteoclasts. Without M-CSF, RANKL–RANK could not induce osteoclasts even in the presence

of IL-3. It will be interesting to define the molecular event induced by M-CSF in precursors. As RANK belongs to the TNFR family and does not stimulate cell proliferation, M-CSF-precultured RANK<sup>+</sup> cells could not survive and proliferate without the continuous presence of M-CSF. It has been shown that RANKL stimulates mature osteoclasts to activate bone resorption (49). In the absence of RANKL, precursor cells autonomously differentiate into macrophages in the presence of M-CSF, a situation regarded as the default pathway of macrophagic differentiation. The determination of osteoclastic differentiation is achieved to avoid the default pathway of macrophagic differentiation from the signal of the RANK-RANKL system (Fig. 8 B).

Spontaneous recovery of adult *op/op* mice suggests the

presence of unknown molecules (50, 51). The osteoclast defect in M-CSF mutant osteopetrotic mice can be rescued by overexpression of the antiapoptotic protein Bcl-2 in the monocyte lineage (14). Although M-CSF is an indispensable factor for osteoclastogenesis, other molecules may induce RANK less efficiently than M-CSF. It is noteworthy that the intracellular domain of RANK directly binds TRAF2, TRAF5, and TRAF6 (26, 28), and TRAF6 deficiency results in osteopetrosis (29).

In conclusion, we have identified osteoclast precursor cells and clarified the function of M-CSF and RANKL. The cooperation of these two factors is critical for osteoclast differentiation. Osteoclastogenesis is thus a unique model system in the lineage determination of blood cell differentiation.

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