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Monokine induced by interferon-gamma is induced by receptor activator of nuclear factor κB ligand and involved in osteoclast adhesion and migration

조선대학교 대학원

생물신소재학과

곽 한 복

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파골세포의 부착 및 이동과 RANKL에 의해 유도되는 Monokine induced by interferon-gamma (MIG)의 연관성에 대한 연구

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이 논문을 이학 박사학위신청 논문으로 제출함.

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ABBREVIATIONS

RANK : Receptor activator of nuclear factor κB

RANKL : RANK ligand

M-CSF : Macrophage-colony stimulating factor

TNF : Tumor necrosis factor

GM-CSF : Granulocyte macrophage-colony-stimulating factor

TRAP : Tartrate-resistant acid phosphatase

MMP : Metalloproteinases

MIG : Monokine induced by interferon-gamma

TRITC : Tetramethylrhodamine isothiocyanate

FITC : Fluorescein isothiocyanate

GFP : Green fluorescence protein

STAT1 : Signal transducer and activator of transcription 1

JAK : Janus kinase

MAPK : Mitogen-activated protein kinase

ERK : Extracellular signal-regulated kinase

JNK : c-Jun N-terminal kinase

PI3-kinase : Phosphoinositide-3OH kinase

NF-κB : Nuclear factor kappa B

DAPI : 4,6-diamidino-2-phenylindole

ELISA : Enzyme-linked immunosorbent assay

CHX : Cyclohexamide

FACS : Fluorescence-activated cell sorter

BSA : Bovine serum albumin

α-MEM : α-Minimum essential medium

ABSTRACT

Monokine induced by interferon-gamma is induced by receptor activator of nuclear factor κB ligand and involved in osteoclast adhesion and migration

Role of MIG induction by RANKL in adhesion and migration of osteoclasts

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Bone remodeling, a coupled process involving bone resorption and formation, is initiated by mechanical signals and is thought to be regulated by endocrine factors and by local inflammatory cytokines. Bone loss is accompanied by differentiation of osteoclasts from monocyte/macrophage lineage of hematopoietic cells. The TNF receptor family member RANK, its ligand RANKL are the crucial factors responsible for inducing osteoclast differentiation, survival, and bone resorption. RANKL is required for osteoclast differentiation through expression of various genes.

Information about genetic programs and coordinated gene expression can be obtained effectively by the use of cDNA microarray technology that allows simultaneous measurements of the expression levels of several thousand genes. In a cDNA microarray study to identify genes targeted by RANKL, I found that monokine induced by interferon-γ (*MIG*) gene was up-regulated in osteoclast precursors. MIG was well known as a CXC chemokine that is produced mainly by activated macrophages and binds to CXCR3, which is functionally expressed on activated T cells, B cells, and endothelial cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR), western blot analysis, and enzyme-linked immunosorbent assay (ELISA) were used to verify increased expression of *MIG* gene identified by the microarray analysis. RANKL induction of *MIG* required the activity of nuclear factor kappa B (NF-κB), whose binding site is present in MIG promoter. MIG induction by RANKL was also dependent on p38 mitogen-activated protein (MAP) kinase and signal transducers and activators of transcription (STAT) 1. RANKL stimulated the phosphorylation of serine 727 of STAT1, which required p38 MAPK activity. Moreover, the expression of *MIG* mRNA was observed in RANKL-stimulated wild-type osteoclast precursors, but not in those cells derived from STAT1-deficient mice

MIG secreted upon RANKL treatment could stimulate the migration and adhesion of osteoclast precursors and osteoclasts that were primed to express CXCR3, the MIG receptor, by macrophage-colony stimulating factor (M-CSF). I provide the

first evidence demonstrating that RANKL stimulates the serine phosphorylation of STAT1 through the p38 MAPK pathway, causing *MIG* gene transcription and secretion, which may have a role in recruiting CXCR3-positive osteoclast precursors and osteoclasts to bone remodeling or inflammatory sites.

I. Introduction

I.1. Osteoclasts

Bone is constantly being resorbed by osteoclasts and then filled in by osteoblasts, and the bone homeostasis is regulated through bone remodeling by coordinated activity of osteoclasts and osteoblasts (Suda *et al*., 1999; Teitelbaum *et al*., 2003). Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage lineage that are responsible for bone resorption (Figure 1). The progenitor cells are recruited from the hemopoietic tissues such as bone marrow and splenic tissues to bone via the circulating blood stream. They proliferate and differentiate into osteoclasts through a mechanism involving cell-to-cell interaction with osteoblastic cells (Roodman, 1996; Martin *et al*., 1994). Osteoclasts have several unique characteristics for resorbing bone. The most characteristic morphological features of osteoclasts are the presence of ruffled borders and clear zones (Teitelbaum, 2000). The specialized ruffled border and clear zone areas serve in the attachment of osteoclasts to the bone surface. The resorbing area under the ruffled border of osteoclasts is acidic. Acid is released into the resorption lacuna between the ruffled border membrane of the osteoclast and the bone. Protons are provided by carbonic anhydrase II (CA II) in bone-resorbing osteoclasts (Lehenkari *et al*., 1998). The vacuolar type proton ATPase exists in the ruffled border membranes of osteoclasts

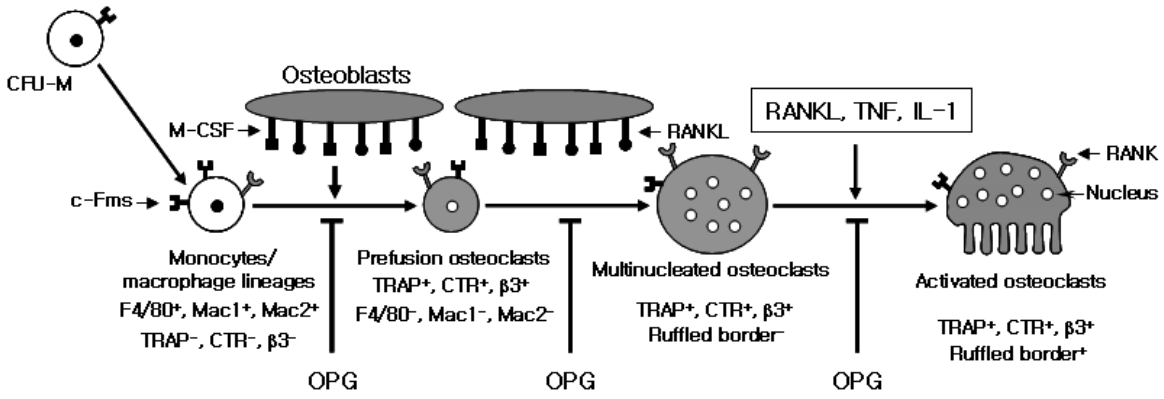


Figure 1. The differentiation pathway of osteoclast progenitors into activated osteoclasts

Osteoclasts are specialized cells derived from the monocyte/macrophage lineage. RANKL and M-CSF are essential for osteoclastogenesis. OPG can bind and neutralize RANKL, and can negatively regulate both osteoclastogenesis and activation of mature osteoclasts. CFU-M : Colony forming unit macrophage, TRAP : Tartrate resistant acid phosphatase, CTR : Calcitonin receptor, F4/80, Mac1, and Mac2 : marker of macrophages

(Laitala *et al*., 1994). The transport of protons into resorption lacunae is mediated by this vacuolar type proton ATPase. Cystein proteinases such as cathepsin K and metalloproteinases (MMPs) such as MMP-9 of osteoclasts are also secreted into a resorbing area to degrade the organic matrix of bone (Wittrant *et al*., 2003; Parikka *et al*., 2001). Activation of osteoclasts under pathological conditions leads to the development of bone loss diseases, such as osteoporosis and rheumatoid arthritis.

I.2. Receptor activator of nuclear factor κB ligand (RANKL)

A tumor necrosis factor (TNF) family molecule, receptor activator of nuclear factor κB ligand (RANKL; also known as OPGL, TRANCE, ODF), is expressed as a membrane-bound protein in osteoblast/stromal cells or released as soluble factor by activated T cells (Teitelbaum *et al*., 2003; Theill *et al*., 2002). In the case of soluble RANKL, membrane release requires the metalloprotease TNF-α converting enzyme (TACE). The *rankl* gene encodes a TNF superfamily molecule of 316 amino acids (38 kDa), and three RANKL subunits assemble to form the functional trimeric molecule (Theill *et al*., 2002). RANKL stimulates its specific receptor, the receptor activator of NF-κB (RANK), which expressed on osteoclast precursors and osteoclasts, to promote differentiation, fusion, survival, and bone resorption (Suda *et al*., 1999). Also, RANKL has been identified as a crucial factor in early lymphocyte development and dendritic cell function (Anderson *et al*., 1997).

I.3. RANK signaling in osteoclasts

Anderson *et al*. reported RANK as a novel member of the TNF receptor family that is functionally involved in T cell development and dendritic cell function (Anderson *et al*., 1997). The mouse *RANK* cDNA encoded a type I transmembrane protein of 625 amino acid residues. RANK is ubiquitously expressed in human tissues, its cell surface expression is limited to dendritic cells, osteoclasts, $CD4^+$ T cells, foreskin fibroblasts, chondrocytes, and certain Hodgkin lymphomas (Anderson *et al*., 1997; Hsu *et al*., 1999; Fiumara *et al*., 2001). RANKL induces the differentiation of osteoclasts in the presence of M-CSF. Indeed, RANKL mediates osteoclast differentiation by a number of pathways. The interaction of RANKL with RANK leads to the recruitment of TRAFs 1, 2, 3, 5, and 6 (Boyle *et al*, 2003). RANK signaling is mediated by cytoplasmic factors that activate downstream signaling pathways that direct osteoclast-specific gene expression leading to differentiation and activation. TRAF proteins play an important role in the activation of downstream signaling pathways, including NF-κB, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) (Lee *et al*., 2003). In particularly, TRAF6, c-Fos, and calcium signaling pathways are essential for the induction of nuclear factor of activated T cell 2 (NFAT2, also known as NFATc1), which plays an important role in osteoclastogenesis (Takayanagi *et al*., 2002).

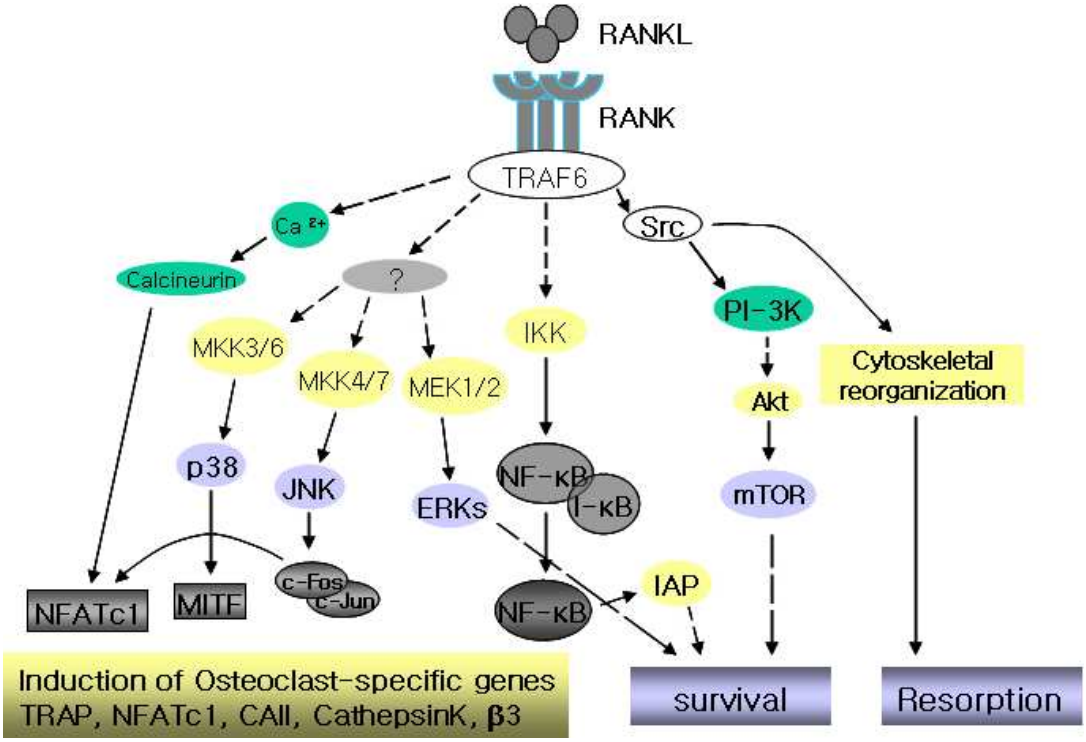


Figure 2. RANK signaling network in osteoclast

Activation of RANK by RANKL leads to the expression of osteoclast-specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival. TRAF6 plays essential roles in RANK signaling by activating various kinases via its multiple domains. The cytoplasmic domain of TRAF6 is capable of activating downstream signals to JNK, ERK, p38 MAPK, and transcription factors such as NF-κB and NFATc1.

I.4. Osteoprotegrin (OPG)

Osteoprotegrin (OPG, also known as osteoclastogenesis inhibitory factor, OCIF), a secreted glycoprotein of the TNF receptor superfamily, specifically binds to RANKL and inhibits osteoclast differentiation (Lacey *et al*., 1998; Yasuda *et al*., 1998). Unlike other members of the TNF receptor superfamily, OPG does not have a transmembrane domain. OPG is a soluble decoy receptor for RANKL that is released from stromal/osteoblast cells. Also, OPG is widely expressed in a variety of tissues, including the kidney, lung, liver, brain, placenta, and heart (Yasuda *et al*., 1998). In fact, OPG exists functionally as a dimer. It has been shown that dimeric OPG prevents the interaction between RANKL and its receptor RANK. OPG has also been shown to inhibit the activity and survival of osteoclasts *in vitro* and *in vivo* (Simonet *et al*., 1997). The presence of severe osteoporosis with fractures has been reported in OPG deficient mice and the development of osteopetrosis is observed in transgenic mice overexpressing OPG or in RANKL and RANK deficient mice (Dougall *et al*., 1999; Khosla, 2001).

I.5. Macrophage-colony stimulating factor (M-CSF)

Macrophage colony-stimulating factor (M-CSF; also called CSF-1) is a key regulator of monocytic lineage development and subsequent differentiation of

osteoclast (Tanaka *et al*., 1993). In bone marrow, M-CSF stimulates proliferation and differentiation of committed progenitors, leading to the production of blood monocytes and tissue macrophages (Barreda *et al*., 1995). Expression of the receptor for M-CSF (c-Fms) is a crucial feature of an osteoclast precursor. The addition of M-CSF to osteoclast precursors induces the expression of RANK, thereby providing signals required for their proliferation, survival, and differentiation to osteoclasts (Arai *et al*., 1999; Miyamoto *et al*., 2000). Upon ligand binding the c-Fms undergoes dimerization and subsequent auto- and trans-phosphorylation of tyrosine residues in cytoplasmic domains of the receptor. (Roussel *et al*., 1990). c-Fms is a transmembrane tyrosine-specific protein kinase receptor, whose intracellular signaling involves signaling pathways, including PI-3K, Src family kinases, STAT molecules, and mitogen-activated protein (MAP) kinase activation (Insogna *et al*., 1997; Csar *et al*., 2001). The phosphorylation of the c-Fms is thought to create docking sites for downstream signal transduction molecules containing SH2 domains (Kmiecik *et al*., 1987; Anderson *et al*., 1990).

I.6. Chemokines

Chemokines are important chemotactic cytokines that play critical roles in leukocyte trafficking in normal and inflammatory conditions (Rossi *et al*., 2000; Mellado *et al*., 2001). Chemokines are a group of small, structurally related

chemoattractant molecules that regulate cell trafficking through interactions with a set of receptors. The CXC, CC, CX3C, C chemokines are distinguished by the presence of one, none, or three amino acids, respectively, between the first two cysteines (Basu *et al*., 2002). Chemokines are often released from activated macrophages under inflammatory process and viral infection. Interferon-gamma (IFN-γ), an important regulator of macrophage function, induces the CXC chemokines including *MIG* and *IP-10* in mouse macrophages (Farber, 1990). IFN-γ induction of *MIG* is mediated by the transcription factor, signal transducers and activators of transcription (STAT)-1, which binds to γ-RE site of MIG promoter (Hiroi *et al*., 2003). In particular, the CXC chemokine subfamily includes CXCL1 [growth-related oncogene-α (GRO-α)], CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL4 [platelet factor-4 (PF-4)], CXCL5 [epithelial neutrophil-activating peptide-78 (ENA-78)], CXCL6 [granulocyte chemotactic protein-2 (GCP-2)], CXCL7 [neutrophil-activating peptide-2 (NAP-2)], CXCL8 [interleukin-8 (IL-8)], CXCL9 (*MIG*), CXCL10 (*IP-10*), CXCL11 [IFN-inducible T-cell α chemoattractant (*I-TAC*)], CXCL12 [stromal derived factor-1 (*SDF-1*)], CXCL13 [B-cell chemoattractant-1 (*BCA-1*)], CXCL14 [breast and kidney-expressed chemokine (*BRAK*)], and *CXCL16* (Zlotnik *et al*., 2000; Rossi *et al*., 2000). All of the genes encoding known CXC chemokines are clustered on human chromosome 4, with the exception of the *CXCL12* gene, which is mapped to 10q11.21, the *CXCL14* gene, which is mapped to 5q31.1, and the *CXCL16* gene, which is mapped to 17p13.

I.7. Roles of chemokines

The primary roles of chemokines are to activate integrins for leukocyte adherence on endothelial cells and to induce chemotaxis of leukocytes in tissue microenvironments. In addition to chemotaxis, chemokines are also involved in the regulation of T cell differentiation, proliferation, apoptosis, angiogenesis and metastatic processes. Moreover, chemokines can control the generation of soluble inflammatory products such as free radicals, nitric oxide (NO) (Zlotnik *et al*., 2000), cytokines (Yu *et al*., 2004) and matrix metalloproteases (Yu *et al*., 2003). Binding of chemokines to their receptors triggers the activation of several molecules and signaling pathways, including the activation of the Jaks/STAT pathway, activation of small GTPases of the Rho family, and activation of phosphatidylinositol 3-kinase (PI-3K) and MAP kinase pathways (Rossi *et al*., 2000; Loetscher *et al*., 2000). Cells respond to chemokines by altering their morphology during migration in which cell polarization with redistribution of several membrane receptors and rearrangement of actin cytoskeleton takes place (Francisco *et al*., 1999). Cell migration also involves sequential adhesion and detachment steps, and transient up-regulation of the activity of cell adhesion receptors mediated by chemokines is likely to play an important role during migration. Hence, several chemokines including the CXC chemokine SDF-1α and the CC chemokines monocyte chemoattractant protein-1 (CCL2) and RANTES (regulated on activation normal T cell expressed and secreted) (CCL5) modulate the

adhesive activity of integrins VLA-4 and VLA-5 in lymphocytes and in bone marrow hematopoietic progenitors and myeloma cells (Carr *et al*., 1996; Ria *et al*., 2002).

I.8. Receptors for chemokines

Chemokines act via seven-transmembrane-domain receptors (Mellado *et al*., 2001; Thelen, 2001), which form a distinct group of structurally related proteins within the superfamily of receptors that signal through heterotrimeric GTP-binding proteins. Most chemokines share the ability to bind to chemokine receptors that trigger these downstream cascades, rapidly activating phosphoinositide-specific phospholipase C-β_2 (PLC-β_2) and PLC-β_3 isoenzymes, which lead to inositol-1,4,5-triphosphate formation and to a transient rise in the concentration of intracellular free calcium (Ca^{2+}). This pathway has been widely used to test the responsiveness of chemokine receptors to different chemokines (Wang *et al*., 1999). Several other chemokines have been shown to inhibit adenylate cyclase and to activate mitogen/extracellular signal-regulated kinase (MEK)-1 and/or extracellular signal-regulated kinase (ERK)-1/2. In addition, these responses stimulate tyrosine phosphorylation of focal adhesion complex components and activate nuclear factor-κB (NF-κB) as well as STAT1 and STAT3 (Woo *et al*., 2002; Han *et al*., 1999). Thus, chemokines can couple to distinct signaling pathways that have been demonstrated to mediate not only migration, but also cell growth and transcriptional activation.

I.9. Monokine induced by interferon-gamma (MIG)

MIG, a chemokine of the CXC subfamily, was identified by differential screening of a cDNA library prepared from lymphokine-activated macrophages. The *MIG* gene is expressed in monocytes/macrophages, hepatocytes, fibroblasts, keratinocytes, and endothelial cells in response to IFN-γ (Farber, 1993). In mice, systemic administration of IFN-γ and infection with protozoa or virus was associated with induction of the *MIG* gene in a variety of tissues, including liver, spleen, heart, and lung (Amichay *et al*., 1996). Functionally, MIG has been shown to target activated T cells. MIG was also reported to inhibit angiogenesis *in vivo* (Piali *et al*., 1998). The receptor for MIG, CXCR3, is functionally expressed on activated T cells, B cells, and endothelial cells (Liao *et al*., 1995; Romagnani *et al*., 2001). MIG is chemotactic factor for activated T cells and natural killer cells and induces the adhesion of interleukin-2 (IL-2)-stimulated T lymphocytes through its receptor CXCR3 (Qin *et al*., 1998; Piali *et al*., 1998).

I.10. Purpose of this paper

There have been a few studies that investigated chemokines in relation to the regulation of bone. Several chemokines have been shown to be highly expressed in bone erosive lesion, suggesting a potential role of the chemokines in bone remodeling

process. MIP-1α and MIP-1β produced by multiple myeloma cells were shown to play critical roles in the development of lytic bone lesions by enhancing both osteoclast formation and bone resorption (Abe *et al*., 2002). Bendre *et al.* reported that IL-8, a member of the chemokines, stimulated osteoclastogenesis and bone resorption in metastatic bone disease (Bendre *et al*., 2003). Osteoclast itself has been shown to highly express CXC chemokines IP-10 and SDF-1 during the differentiation and maturation process (Grassi *et al*., 2003). Besides RANKL, the bone remodeling process is accompanied by complex changes in the expression levels of various genes in osteoblasts and osteoclasts. Identifying the genes that are associated with osteoclast differentiation can help elucidate the molecular mechanisms that underlie bone remodeling. I have tried to detect differentially-expressed genes during osteoclast differentiation using cDNA microarray technique. Microarray-based global profiling of gene expression, in combination with bioinformatic tools, can yield valuable insights into cell or tissue specific functions (Lee *et al*., 2002, 2003). In this effort, I found several up-regulated genes in RANKL-treated osteoclast precursors (Table 1). Among those genes, *MIG* was selected for further investigation in this study. *MIG*, which is also called CXCL9, is a member of the CXC chemokine family.

In this study, I found that the chemokine *MIG* is up-regulated by RANKL in osteoclast precursors. STAT1 played a critical role in the induction of *MIG* by RANKL, and the RANKL-stimulated serine 727 phosphorylation of STAT1 through the p38 MAPK was the signaling event crucial for *MIG* expression. In addition, the expression

of CXCR3 was also significantly elevated in M-CSF-dependent osteoclast precursors. Therefore, I present the evidence that expression of MIG induced by RANKL can stimulate the adhesion and migration of M-CSF-dependent osteoclast precursors and differentiating osteoclasts.

II. Materials and methods

II.1. Reagents and mice

Recombinant human sRANKL and human M-CSF were purchased from PeproTech EC (London, UK). A polyclonal antibody to MIG was obtained from R&D Systems (Minneapolis, MN). Phospho-serine-STAT1, phospho-tyrosine-STAT1, and STAT1 antibodies were obtained from Upstate Biotechnology (Waltham, MA). Anti-phospho-ERK, anti-ERK, phospho-FAK, and anti-FAK were purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody to actin was purchased from Santa Cruz Technology (Santa Crux, CA). A polyclonal antibody to CXCR3 was from Zymed (South San Francisco, CA), and antibody to Pyk2 was from BD Biosciences Pharmingen (San Jose, CA). SB203580, PD98059, and SP600125 were purchased from Calbiochem (La Jolla, CA). NF-κB specific inhibitor SN50 was from BIOMOL (Plymouth Meeting, PA). 4,6-Diamidino-2-phenylindole (DAPI) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated mouse antibody were purchased from Sigma. Transwell (24-mm diameter, 6 μm pore size) were from Corning Costar (Cambridge, MA). Green fluorescence protein (GFP) transgenic mice were a gift from Hyun-Man Kim (Seoul National University). Homozygous STAT1-deficient (*stat1*$^{-/-}$) mice in which the *stat1* gene has been deleted by homologous recombination and wild type 129/S6 mice were obtained from Taconic (Germantown,

NY). ICR mouse strain was used as another mouse source to prepare the osteoclast precursors.

II.2. Preparation of osteoclast precursors and mature osteoclasts

Osteoclast precursors were generated from mouse bone marrow cells as previously described (Lee *et al*., 2001). Bone marrow cells were obtained by flushing tibiae and femora from 6- to 7-week-old mice, suspended in α-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in the presence of 1:10 dilution of CMG14-12 culture supernatant to provide M-CSF (Takeshita *et al*., 2000) and cultured for 3 days at 37°C in a humidified atmosphere of 5% CO_2. Nonadherent cells were removed by washing with PBS. Adherent cells were used as osteoclast precursors.

Mature osteoclasts were purified from cocultures of mouse bone marrow cells and calvarial osteoblasts. Briefly, calvarial osteoblasts were obtained by growing calvarial cells from ICR newborn mice for 3 days in α-MEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Bone marrow cells and osteoblasts were seeded on a 90-mm culture dish and incubated for 6 days in the presence of 10^{-8} M 1α,25-dihydroxyvitamin D_3 (VtD_3) and 10^{-6} M prostaglandin E_2 (PGE_2). Cells were detached by treating with 0.1% collagenase (Wako) at 37°C for 10 min, replated on 6-well plates, and cultured for 6 h. The dishes were then treated with 0.1% collagenase at

37°C for 20 min and intensely pipeted to remove osteoblasts. The remaining cells were considered mature osteoclasts.

II.3. Reverse transcription–polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was performed as previously described (Kim *et al*., 2003). Total RNA was prepared using TRI Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Two micrograms of total RNA were reverse-transcribed with SuperScriptII reverse transcriptase (Invitrogen) and 1/10 of the reverse-transcribed cDNA was amplified by PCR. For PCR amplification, the following primers were used: MIG sense, 5'-ATGAAGTCCGCTGTTCTTTTCCT-3'; MIG antisense, 5'-AGTCTTCCTTGAACGACGACGA-3'; CXCR3 sense, 5'–GCCACCCATTGCCAGTACAAC-3'; CXCR3 antisense, 5'-TCCCACAAAGGCATAGAGCAGC-3'; Fra II sense, 5'-CCCTATCCACGCTCACATCCC–3'; Fra II antisense, 5'-CGTTCCTCGGGGCTGATTTT-3'; MMP-9 sense, 5'-CTGTCCAGACCAAGGGTACAGCCT-3'; MMP-9 antisense, 5'-GAGGTATAGTGGGACACATAGTGG-3'; Integrin α4 sense, 5'-TCCAAAAATCCCCTATCCTCTC-3'; Integrin α4 antisense, 5'-AAGCCATCCTGCTGCAAAC-3'; Integrin α5 sense, 5'-

CATTTCCGAGTCTGGGCCAA-3'; Integrin α5 antisense, 5'-GGAGGCTTGAGCTGCTTT-3'; Integrin β1 sense, 5'-TGTTCAGTGCAGAGCCTTCA-3'; Integrin β1 antisense, 5'-TGTTCAGTGCAGAGCCTTCA-3'; Integrin β3 sense, 5'-TGACTCGGACTGGACTGGCTA-3'; Integrin β3 antisense, 5'-CACTCAGGCTCTTCCACCACA-3'; Integrin αv sense, 5'-AACATCACCTGGGGCATTCA-3'; Integrin αv antisense, 5'-CGTCAGTGTGGGCGAAGTAAA-3'; GAPDH sense, 5'-CAAGGCTGTGGGCAAGGTCA-3'; GAPDH antisense, 5'-AGGTGGAAGAGTGGGAGTTGCTG-3'. PCR consisted of 20 to 25 cycles of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. The PCR product were separated on 1-1.5% agarose gels and stained with ethidium bromide (Et-Br).

II.4. Enzyme-linked immunosorbent assay (ELISA)

MIG was detected in culture supernatants by enzyme-linked immunosorbent assay (ELISA), according to the instructions of the manufacturer (R&D Systems). In brief, an anti-MIG capture antibody (R&D Systems) was diluted to 1 μg/ml in phosphate-buffered saline (PBS) and then added to wells of a microtiter plate. After overnight incubation at room temperature, the capture antibody was removed, and

nonspecific binding was blocked by adding 200 µl blocking buffer [3% bovine serum albumin (BSA) in PBS]. The plate was incubated at room temperature for 2 h, after which wells were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). Sample or standard was added, and the plate was incubated overnight at 4°C. After incubation, the plate was washed 3 times with PBS-T, a biotinylated detection antibody was diluted to 1 µg/ml in PBS and added to the wells, and the plate was incubated for 2 h at room temperature. After the plate was washed 3 times with PBS-T, streptavidin-conjugated horseradish peroxidase (HRP) was added to the wells. The plate was incubated at room temperature for 20 min and then washed 5 times with PBS-T. One hundred microliters substrate solution was added to each well, and the plate was incubated for 20 to 30 min. The color reaction was stopped by the addition of 50 µl stopping solution (1 M H_2SO_4), and the optical density was read with a microtiter ELISA plate reader at 450 nm.

II.5. Western blotting

Cells were lysed in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease inhibitors [leupeptin, aprotinin, and phenylmethylsulfonyl fluoride (PMSF)] and phosphatase inhibitors (sodium orthovanadate and sodium fluoride). Protein concentrations of cell lysates were determined using DC Protein

Assay Kit (Bio-Rad, Hercules, CA). 20 to 30 μg of cellular proteins were resolved by 10% to 15% sodium dodecyl sulfate-polyacrylamide gel electorphoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). After blocking with 5% skim milk, the membrane was probed with anti-MIG, anti-phospho-serine-STAT1, anti-phospho-tyrosine-STAT1, anti-phospho-FAK, anti-phospho-Pyk2, and anti-phospho-ERK. The same membrane was stripped and reprobed with anti-STAT1, actin, FAK, Pyk2, and ERK.

II.6. Immunohistochemistry

Osteoclast precursors were starved for 12 h in serum-free α-MEM and then stimulated with vehicle, 100 ng/ml RANKL, 50 ng/ml M-CSF, or 50 ng/ml M-CSF plus 100 ng/ml RANKL for 20 min. Cells were fixed in 4% formaldehyde in PBS for 10 min and permeabilization with 0.1% Triton X-100 in PBS for 5 min followed by a block of 10% BSA in PBS for 30 min. Cells were incubated with an antibody specific for STAT1. After washing with PBS, TRITC-conjugated mouse antibodies were applied. After washing with PBS, Cells were stained with DAPI.

II.7. Fluorescence microscopy

In adhesion experiments with GFP-expressing cells, the bound cells were detected by confocal microscopy. All images are shown at original magnification, x 200. Confocal equipment comprised an LSM 5 PASCAL microscope equipped with a Plan-Neofluor 20 x/0.5 objective lens, an LSM 5 PASCAL photomultiplier tube, and Laser Scanning Microscope LSM 5 PASCAL software version 3.0 (all from Carl Zeiss, Jena, Germany).

II.8. Flow cytometry

Flow cytometry analyses were performed as previously described (Jinquan *et al*., 1999). Briefly, osteoclast precursors were stimulated with 50 ng/ml M-CSF or 50 ng/ml M-CSF plus 100 ng/ml RANKL for the indicated times. Cells were washed with FACS buffer (PBS containing 1% BSA and 0.1% sodium azide) and then incubated with anti-CXCR3 antibody (Zymed, South San Francisco, CA) for 1 h at 4°C. The cells were washed with FACS buffer 3 times and then incubated with a secondary antibody labeled with FITC for 1 h at 4°C. The cells were fixed with 3.7% formaldehyde and subjected to flow cytometry.

II.9. Migration assay

Cell migration assays were performed as previously described with some modification (Kim *et al*. 1998). Osteoclast precursors were cultured in the presence 50 ng/ml M-CSF or 50 ng/ml M-CSF plus 100 ng/ml RANKL for 60 to 72 h. Cells were harvested by treatment with cell dissociation solution (Sigma, St. Louis, MO) and then resuspended in serum-free α-MEM. Cells were seeded in the upper well of the Boyden chamber with polycarbonate filters containing 8 μm pore membranes (Corning Costar, Cambridge, MA). The lower well was loaded with α-MEM in the presence or absence of recombinant MIG. In addition, to determine the effect of conditioned media on cell migration, wild type and STAT1-deficient osteoclast precursors were incubated in serum-free α-MEM in the presence of M-CSF or M-CSF plus RANKL for 24 to 30 h. The conditioned media were collected and loaded into the lower well as chemoattractant. After 6 to 8 h of incubation, the migrated cells were fixed in 3.7% formaldehyde for 10 min, stained with tartrate-resistant acid phosphatase (TRAP, Sigma), and counted.

II.10. Adhesion assay

Adhesion assays were performed as described previously (Jinquan *et al*., 1999). Briefly, 96-well microtiter plates were coated with 20 μg/ml fibronectin or 10 μg/ml vitronectin in PBS for 14 to 16 h at 37°C. The plates were washed with PBS and

incubated with PBS containing 0.2% BSA for 1 h at 37°C. Osteoclast precursors were cultured in the presence of 50 ng/ml M-CSF or 50 ng/ml M-CSF plus 100 ng/ml RANKL for 60 to 72 h. Cells were detached by treatment with cell dissociation solution (Sigma) and suspended in serum-free α-MEM. Cells were seeded to each well of fibronectin-coated 96-well plates and incubated for 10 min at 37°C in the presence of MIG or PBS. Nonadherent cells were removed by washing with PBS. Adherent cells were fixed in 3.7% formaldehyde for 10 min, and were stained with hematoxylin or TRAP solution. The stained cells were counted.

II.11. Homophilic adhesion assay

For homophilic adhesion, osteoclast precursors obtained from GFP transgenic mice were cultured in the presence of 50 ng/ml M-CSF plus 100 ng/ml RANKL for 60-72 h. Osteoclast precursors from wild-type mice were cultured to glass coverslip in a 24-well plates and incubated in the presence of 50 ng/ml M-CSF plus 100 ng/ml RANKL for 60 to 72 h. GFP cells were transferred to cultures of non-GFP cells and incubated for 1 h in the presence or absence of MIG (100 ng/ml). Also, osteoclast precursors from GFP transgenic mice were treated with RANKL and M-CSF for 60 to 72 h and then stimulated with MIG (100 ng/ml) for 6 h. Cells from GFP-transgenic mice were transferred to cultures of non-GFP cells and incubated for 1 h. Nonadherent

cells were removed by washing with PBS. Adherent GFP cells were scored.

II.12. Statistical analysis.

Each experiment was performed 3 to 5 times, and all quantitative data are presented as mean ± S.D. Statistical differences were analyzed by Students *t*-test.

III. RESULTS

III.1. RANKL-induced *MIG* mRNA expression

III.1.1. RANKL induces *MIG* mRNA expression in osteoclast precursors

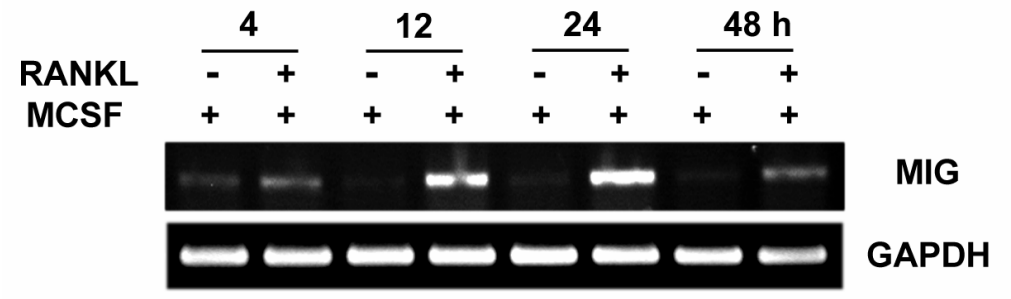
In order to identify genes regulated by RANKL in osteoclast precursors, total RNA was isolated at 24 h from M-CSF plus RANKL-treated osteoclast precursors and used for hybridization with the cDNA microarray. Seventeen genes were identified to be regulated by RANKL (Table 1). One of several genes up-regulated in response to RANKL was turned out *MIG*. With this array result, the induction of *MIG* expressed by RANKL was confirmed by RT-PCR analysis. The expression level of *MIG* mRNA was examined at 4, 12, 24, and 48 h after 50 ng/ml M-CSF or 50 ng/ml M-CSF plus 100 ng/ml RANKL stimulation. The increase in *MIG* mRNA level was evident at 12 h and maximal at 24 h after M-CSF plus RANKL stimulation (Figure 3A). The induction of *MIG* mRNA was dependent on RANKL concentrations (1, 5, 10, 30, 50, or 100 ng/ml) (Figure 3B), expression being evident in the presence of as little as 5 ng/ml RANKL. Also, I examined the induction of *MIG* mRNA in mature osteoclasts. However, RANKL did not induce *MIG* mRNA in mature osteoclasts (Figure 3C).

III.1.2. RANKL alone induces *MIG* mRNA expression in osteoclast precursors

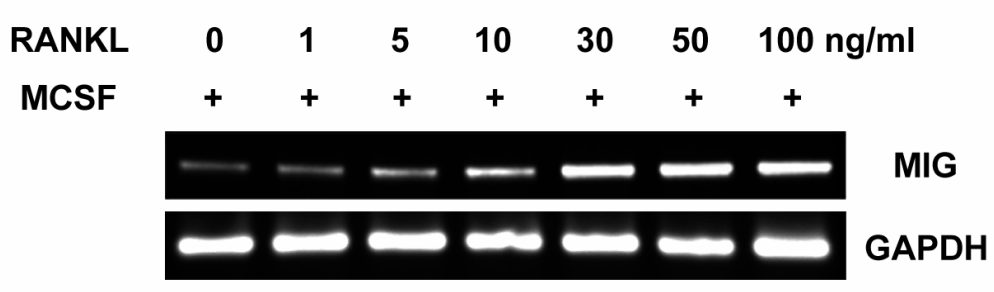
Table 1. Increased and decreased genes in osteoclast precursors after treatment with M-CSF and RANKL

Gene	Properties	Score
HSPC244	Haematopoietic stem/progenitor cell-related gene	Decrease
Rheb	Small GTPase	-
Nedd4	E3 ligase, ubiquitination	-
D-AKAP2	Dual-specific A kinase anchoring protein 2, Its interaction with protein kinase A	Decrease
CD27	TNFR-family member	Decrease
JunB	AP-1 component	Increase
E13	Isoform of the calcitonin receptor	Increase
Tspan5	Tetraspanin superfamily	Decrease
Anxa7	Synexin, family of Ca^{2+}- and phospholipid-binding proteins	Decrease
Trp63	Integral membrane proteins	Increase
MIG	CXCL9, CXC chemokine	Increase
mPLXNB1	Unknown	-
mCD72	45-kDa type II membrane protein	Increase
Sema4d	Semaphorin family, Neuronal development	Increase
c-Fos	AP-1 component	Increase
mPpargc1	Nuclear hormone receptors	Increase
mPrrx1	Unknown	Decrease
mMIZzf	Unknown	Decrease
mPlec1	Unknown	Increase
mRab3d	Subfamily of GTPases	Decrease

A


4
12
24
48 h
RANKL
MCSF
MIG
GAPDH

B


RANKL
0
1
5
10
30
50
100 ng/ml
MCSF
MIG
GAPDH

C

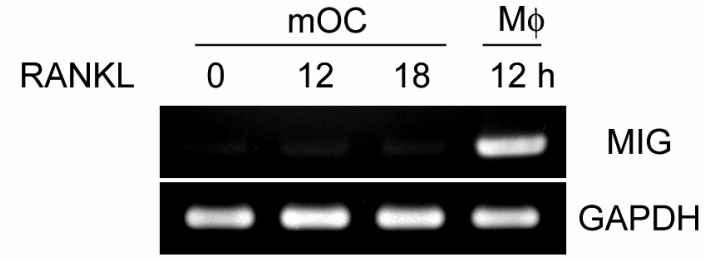

mOC
Mϕ
RANKL
0
12
18
12 h
MIG
GAPDH

Figure 3. Induction of *MIG* mRNA expression by RANKL

(A) Time course of *MIG* mRNA induction by RANKL. Osteoclast precursors were stimulated with M-CSF (50 ng/ml) or M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for the indicated time. Total RNA was extracted from the treated cells. RNA was reverse transcribed and PCR amplified with *MIG* or *GAPDH* primers. (B) RANKL dose-dependent increase in *MIG* mRNA levels. Osteoclast precursors were treated with the indicated concentration of RANKL in the presence of M-CSF (50 ng/ml) for 12 h. (C) RANKL induction of *MIG* mRNA in mature osteoclast. Mature osteoclasts were stimulated with RANKL (100 ng/ml) for the indicated time in the presence of M-CSF. Total RNA was extracted from the treated cells. RNA was reverse transcribed and PCR amplified with *MIG* or *GAPDH* primers. PCR product were separated on a 1.2% agarose gel and stained with ethidium bromide.

RANKL and M-CSF make distinct contributions to osteoclast formation: M-CSF provides osteoclast precursors through induction of survival, proliferation, and expression of RANK, whereas RANKL induces osteoclast differentiation in these precursors. Moreover, M-CSF supports not only osteoclast differentiation but also the survival of mature osteoclasts (Jimi *et al*., 1999; Fuller *et al*., 1993). I therefore examined the effects of M-CSF on *MIG* mRNA expression in osteoclast precursors. The up-regulation of *MIG* mRNA by RANKL was observed in the absence of concurrent M-CSF treatment (Figure 4A). M-CSF itself did not increase *MIG* mRNA and also did not have any synergistic effect on the RANKL-induction of *MIG* gene expression (Figure 4B).

III.1.3. RANKL directly induces *MIG* mRNA expression

Osteoprotegrin (OPG) is a secreted receptor of the tumor necrosis factor receptor family without transmembrane domain. It binds directly to RANKL on the osteoblasts/stromal cell surface, inhibiting RANKL-mediated signaling (Simonet *et al*., 1997; Kong *et al*., 1999). Therefore, to examine the impact of OPG on RANKL-induced *MIG* mRNA expression, osteoclast precursors were pretreated with or without 500 ng/ml OPG and incubated with 50 ng/ml M-CSF plus 100 ng/ml RANKL. The induction of *MIG* mRNA by RANKL was blocked by the addition of OPG (Figure 5A). These data indicate that RANKL induces *MIG* mRNA expression through RANK-

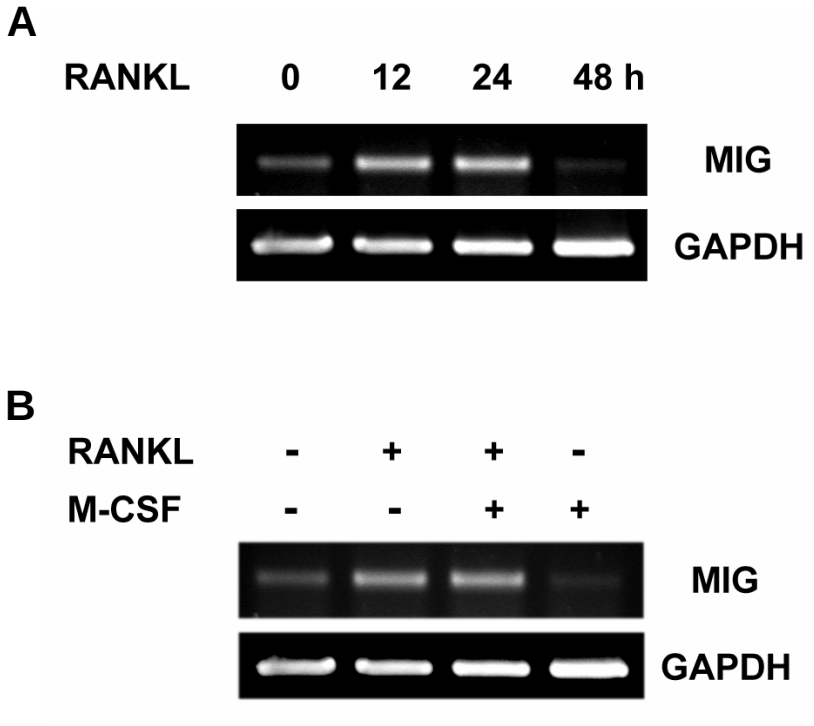


Figure 4. RANKL induces *MIG* mRNA expression in the absence of M-CSF

(A) RANKL induction of *MIG* mRNA in the absence of M-CSF. Osteoclast precursors were stimulated with RANKL (100 ng/ml) for the indicated time in the absence of M-CSF. (B) Lack of synergy between RANKL and M-CSF in *MIG* induction. Osteoclast precursors were incubated with M-CSF (50 ng/ml), RANKL (100 ng/ml), or both for 24 h. PCR product were performed as in Figure 3.

RANKL signaling. To exclude the possibility that RANKL could affect the expression of *MIG* through an indirect manner, osteoclast precursors were stimulated with RANKL in the absence or presence of cyclohexamide to block new protein synthesis. The expression pattern of *MIG* mRNA was not affected by the presence of cyclohexamide (Figure 5B). The stimulation of *MIG* mRNA expression by RANKL is not induced by increased synthesis of a regulatory protein. These results suggest that RANKL directly induces *MIG* mRNA expression in osteoclast precursors.

III.1.4. RANKL increases the level of MIG protein in osteoclast precursors

In line with the expression pattern of *MIG* mRNA, the effect of RANKL on MIG protein expression was studied. Osteoclast precursors were treated with 50 ng/ml M-CSF plus 100 ng/ml RANKL, and the expression of MIG was determined by Western blotting. The protein level of MIG was increased at 12 and 24 h after RANKL treatment (Figure 6A). Then, the dose-dependent effect of RANKL on MIG protein expression in osteoclast precursor was assessed using Western blot analysis. Treatment of cells with 10, 30, 50, and 100 ng/ml RANKL for 24 h resulted in a dose-dependent increase in MIG protein levels (Figure 6B). Osteoclast precursors were stimulated with RANKL for 24 h, and the concentrations of MIG levels in the culture supernatant were determined by ELISA. When the MIG concentration in the culture supernatant was measured by ELISA, 40 to 50 ng/ml was detected (Figure 6C).

III.1.5. RANKL induces *MIG* expression through NF-κB and p38 MAPK-dependent pathways in osteoclast precursors

Stimulation of RANK has been shown to activate the NF-κB transcription factor and the ERK, JNK, and p38 MAPKs in osteoclastogenesis (Lee *et al*., 2003). To investigate whether these signaling molecules are involved in the up-regulation of *MIG* mRNA by RANKL, I examined the effect of signaling pathway inhibitors on *MIG* mRNA expression. Osteoclast precursors were treated with NF-κB peptide inhibitor SN50, p38 inhibitor SB203580, MEK (ERK upstream kinase) inhibitor PD98059, or JNK inhibitor SP600125 prior to RANKL stimulation and *MIG* mRNA expression levels were determined by RT-PCR and Western blotting. As shown in Figure 7, the RANKL induction of *MIG* mRNA expression was abolished in the presence of SN50 and SB203580 at both mRNA and protein levels. Inhibition of JNK had no effect on *MIG* mRNA expression while inhibition of ERK resulted in a slight increase in the MIG protein level (Figure 7B). These results indicate that NF-κB and p38 MAPK play a crucial role for the expression of *MIG* mRNA in RANKL-stimulated osteoclast precursors.

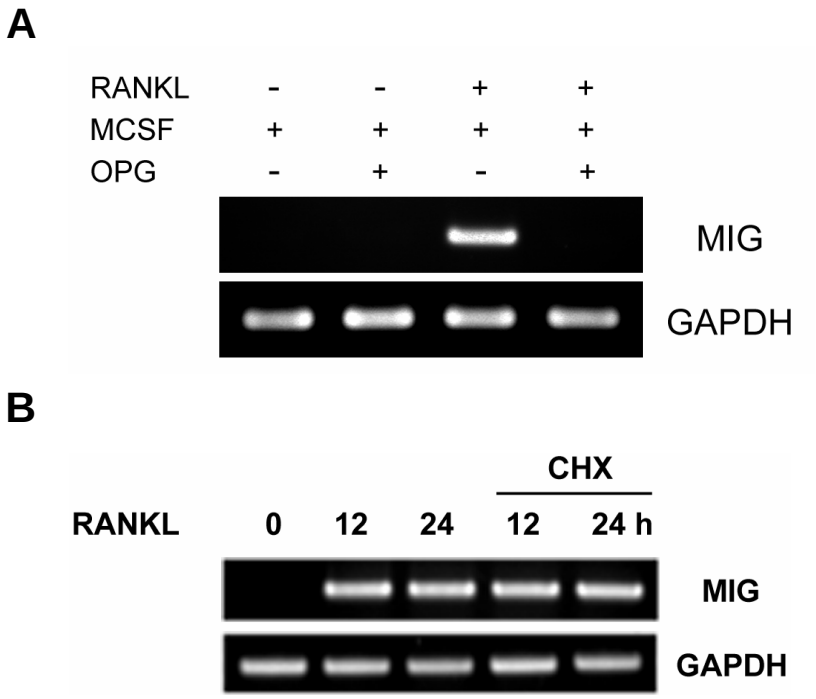
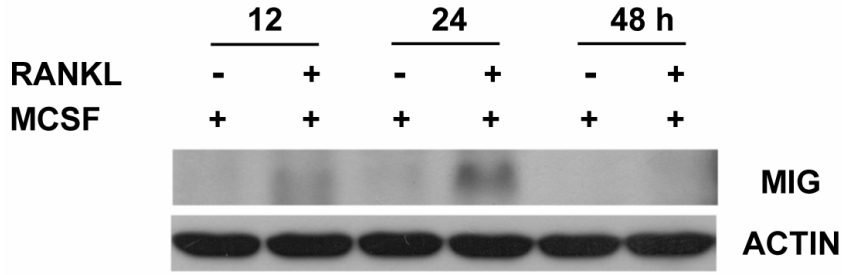


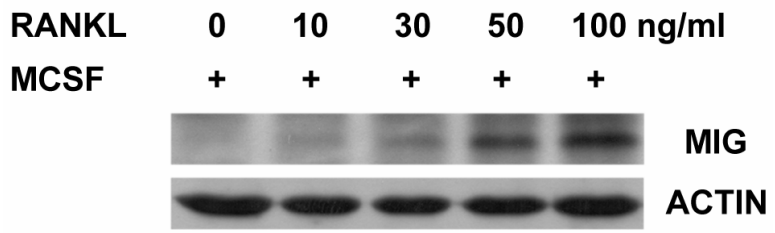
Figure 5. RANKL directly promotes *MIG* mRNA expression

(A) Inhibition of RANKL induction of *MIG* mRNA by Osteoprotegrin (OPG). Osteoclast precursors were pretreated with or without OPG (500 ng/ml) and incubated with M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 12 h. (B) Effect of cycloheximide (CHX) on RANKL induction of *MIG* mRNA. Osteoclast precursors were pretreated with or without 1 μg/ml cycloheximide and were further stimulated with RANKL (100 ng/ml) for the indicated time.

A


12
24
48 h
RANKL
- + - + - +
MCSF
+ + + + + +
MIG
ACTIN

B


RANKL
0 10 30 50 100 ng/ml
MCSF
+ + + + +
MIG
ACTIN

C

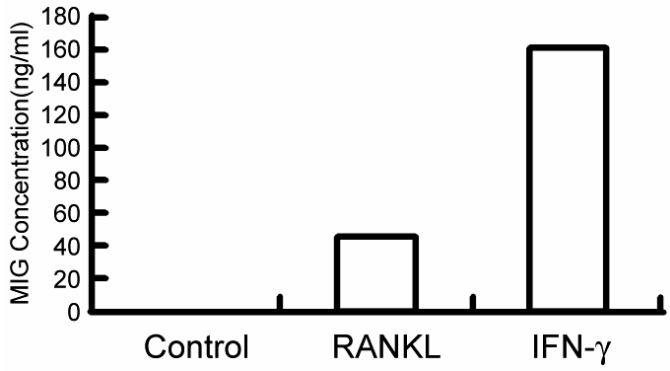

MIG Concentration(ng/ml)
180
160
140
120
100
80
60
40
20
0
Control
RANKL
IFN-γ

Figure 6. MIG protein expression stimulated by RANKL

Osteoclast precursors were treated with M-CSF (50 ng/ml) or M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for the indicated time (A) or were incubated with M-CSF (50 ng/ml) plus the indicated concentration of RANKL for 24 h (B). Cell lysates were prepared and subjected to Western blot analysis with anti-MIG antibody. The same membrane was stripped and reprobed with anti-actin antibody and visualized by ECL. (C) Osteoclast precursors were treated with RANKL (100 ng/ml) or IFN-γ (1 ng/ml) for 24 h. MIG concentrations in culture supernatants were determined using ELISA as described in "Materials and methods."

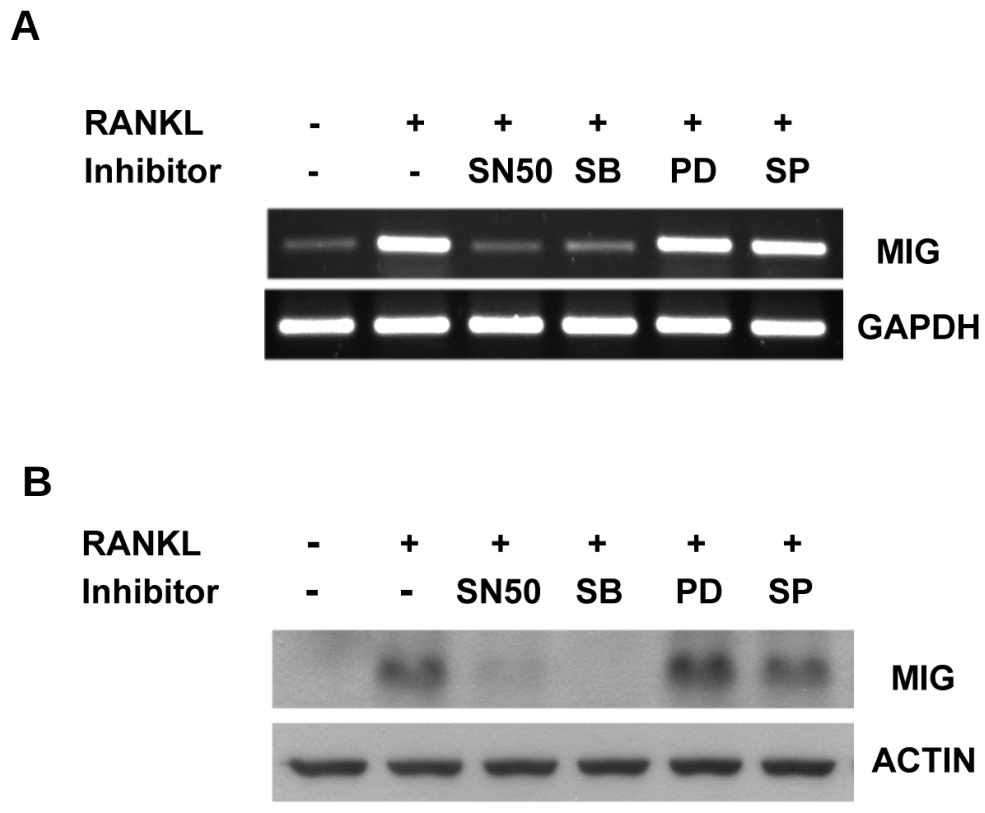


Figure 7. Effects of signaling inhibitors on expression of MIG induced by RANKL

Osteoclast precursors were pretreated with SN50 (30 μg/ml), SB203580 (20 μM), PD98059 (5 μM), or SP600125 (20 μM) for 30 min and were stimulated in the presence of M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 12 h. Expression levels of MIG were analyzed by RT-PCR (A) or Western blot analysis (B). SN50 : NF-κB inhibitory peptide; SB203580 : p38 MAPK inhibitor; PD98059 : MEK inhibitor; SP600125 : JNK inhibitor.

III.2. Mechanism of MIG expression induced by RANKL

III.2.1. RANKL induces STAT1 serine phosphorylation

In the promoter of *MIG* gene, NF-κB and STAT1 binding sites are present and a synergistic regulation of *MIG* transcription through NF-κB and STAT1 has been reported (Ohmori *et al*., 1995, 1997). Given that the NF-κB activation by RANK has been well demonstrated in many studies and that SN50 blocked RANKL induction of *MIG* mRNA (Figure 7), it is reasonable to conclude that RANK activation of NF-κB is required for *MIG* induction in osteoclast precursors. In contrast, there has been no investigation reported on the regulation of STAT1 by RANKL. Therefore, I explored the possibility that RANKL modulates STAT1 for MIG expression in osteoclast precursors. The tyrosine phosphorylation and subsequent SH2 domain-mediated dimerization of STAT are considered as an essential prerequisite for its biological activity because dimerization enables STAT molecules to enter the nucleus and bind DNA (Darnell *et al*., 1997). However, serine phosphorylation of STATs also has profound effects on their target gene transcription in certain cases (Decker *et al*., 2000). Therefore, I examined the phosphorylation of STAT1 in RANKL-stimulated osteoclast precursors. As shown in Figure 8A, the serine phosphorylation of STAT1 increased from 10 min till 30 min and returned to the basal level at 60 min in response to RANKL stimulation. The increase in STAT1 serine phosphorylation was strikingly

enhanced by RANKL in a dose-dependent manner (Figure 8B). However, RANKL did not increase the tyrosine phosphorylation level of STAT1 (Figure 8A and B).

III.2.2. M-CSF stimulates STAT1 phosphorylation in osteoclast precursors

Previous reports have demonstrated that c-Fms signaling leads to the activation of MAP kinases and the transcriptions factors NF-κB and AP-1 in macrophages. Subsequent studies sought to determine whether engagement of M-CSF also leads to the activation of the transcription factor STAT1. Osteoclast precursors were stimulated with M-CSF for various times. Whole cell lysates were prepared, and lysates were then analyzed by western blotting. STAT1 activation was measured using specific antibodies that discriminate between STAT1 phosphorylated at serine 727 and tyrosine 701. As shown in Figure 9, M-CSF stimulated both phosphorylation of STAT1 at serine 727 and tyrosine 701 in a time-dependent manner. Also, M-CSF-induced both phosphorylation of STAT1 at serine 727 and tyrosine 701 significantly increased with increasing M-CSF concentrations with no change in non-phosphorylated levels of STAT1.

III.2.3. STAT1 localization between cytoplasm and nucleus in the absence of RANKL signaling

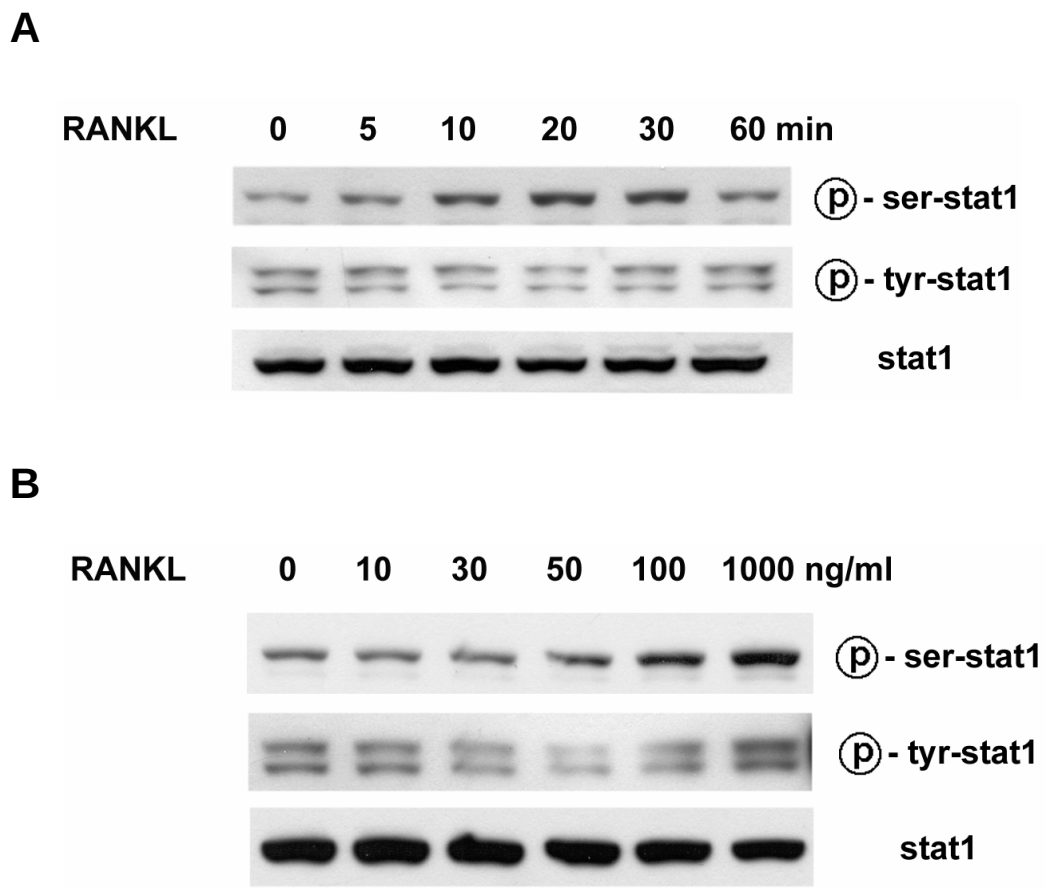


Figure 8. Stimulation of STAT1 serine phosphorylation by RANKL

(A) Time course of STAT1 phosphorylation by RANKL. Osteoclast precursors were serum starved for 12 h and were stimulated with RANKL (100 ng/ml) for the indicated time. Cell lysates were subjected to Western blotting for the phosphorylation of STAT1 at serine 727 and tyrosine 701 using anti-phosphospecific STAT1 antibodies (top and middle rows). The same membrane was stripped and reprobed with anti-STAT1 antibody (bottom row). (B) The phosphorylation of STAT1 dependent on RANKL dose. Osteoclast precursors were serum starved as in (A). Cells were stimulated with various

concentrations of RANKL for 10 min as indicated. Western blot was subjected as described above.

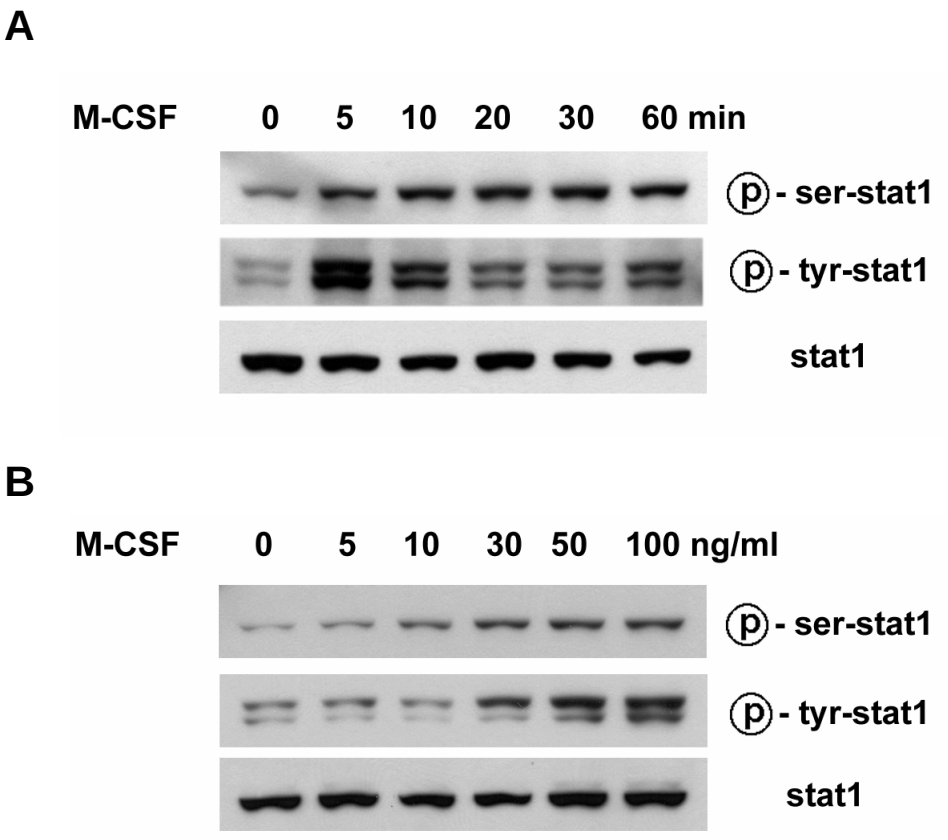


Figure 9. Time- and dose-dependent stimulation of STAT1 phosphorylation by M-CSF

(A) Time course of STAT1 phosphorylation by M-CSF. Osteoclast precursors were serum starved for 12 h and were stimulated with M-CSF (50 ng/ml) for the indicated time. Cell lysates were subjected to Western blotting for the phosphorylation of STAT1 at serine 727 and tyrosine 701 using anti-phosphospecific STAT1 antibodies (top and middle rows). The same membrane was stripped and reprobed with anti-STAT1 antibody (bottom row). (B) The phosphorylation of STAT1 dependent on M-CSF dose. Osteoclast precursors were serum starved as in (A). Cells were stimulated with various

concentrations of M-CSF for 10 min as indicated. Western blot was subjected as described above.

STAT proteins serve as a link between the cell surface and the nucleus, and this requires their physical movement from the cytoplasm to the nucleus. STAT proteins transduce the signals of cytokines and growth factors from their cognate receptors on the cell surface to the nucleus. This process involves sequential events including the specific tyrosine phosphorylation, dimerization, and the nuclear translocation, which is a key control for their transcriptional activity (Bromberg *et al*., 1996; John *et al*., 2002). The basal level of STAT1 tyrosine phosphorylation was more consistently observed than that of serine phosphorylation (Figure 8). Therefore, my primary goal was to investigate whether the basal level of STAT1 tyrosine phosphorylation is transported into the nucleus. I therefore examined immunohistochemistry the subcellular localization of STAT1. I found that M-CSF induces phosphorylation at serine and tyrosine of STAT1 (Figure 9). As expected, STAT1 was translocated into the nucleus of osteoclast precursors in response to M-CSF or M-CSF plus RANKL. However, RANKL alone did not induce translocated into the nucleus of STAT1 (Figure. 10A-D). Staining with DAPI indicated that STAT1 was preferentially localized in the nucleus (Figure 10E-H). These data provide strong evidence that the tyrosine phosphorylation of STAT1 observed in osteoclast precursors plays an important role in the expression of MIG after RANKL treatment.

III.2.4. p38 MAP kinase mediates RANKL-induced serine phosphorylation of STAT1

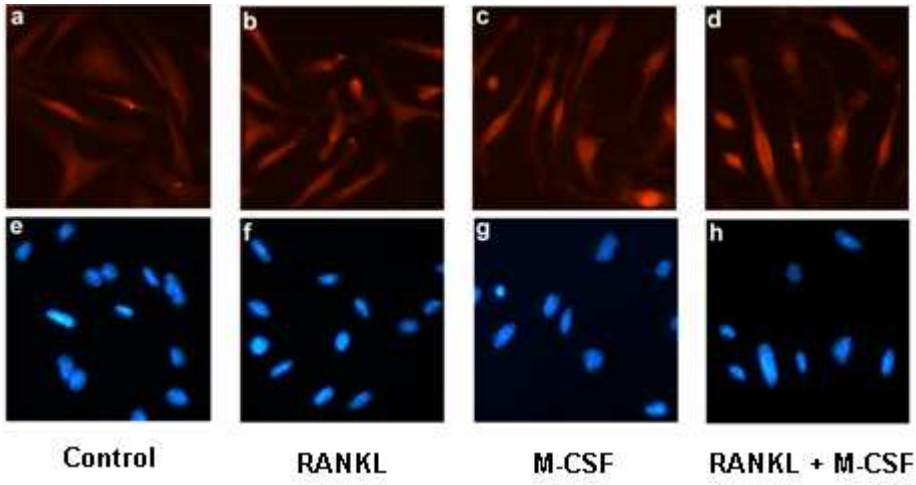


Figure 10. Immunolocalization of STAT1 in osteoclast precursors

Osteoclast precursors were serum starved for 12 h and were stimulated with RANKL (100 ng/ml), M-CSF (50 ng/ml), or M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 20 min. Cells were immunostained with antibody for STAT1 (red, a-d). Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (e-h).

The p38 MAPK signaling pathway has been implicated in the serine phosphorylation of STAT1 in some cell types treated with certain stimuli (Huang *et al*., 2004), and the RANKL-induced expression of *MIG* was suppressed by the p38 inhibitor SB203580. To evaluate the role of p38 MAPK in RANKL-induced STAT1 serine phosphorylation, osteoclast precursors were stimulated with RANKL in the presence and absence of the specific p38 inhibitor SB203580. Osteoclast precursors were treated with RANKL for 10 min, lysed, and subjected to Western blotting using phospho-specific antibodies for STAT1. As shown in Figure 11, SB203580 greatly attenuated the serine phosphorylation of STAT1 by RANKL. Treatment with the SB203580 dose not affects tyrosine phosphorylation of STAT1. These results suggest that the p38 pathway may mediate the RANKL induction of *MIG* gene expression through the serine phosphorylation of STAT1.

III.2.5. RANKL induction of *MIG* is absent in osteoclast precursors from STAT1-deficient mice

As RANKL stimulates serine phosphorylation of STAT1 through p38-dependent way (Figure 11) and the p38 inhibitor SB203580 blocked RANKL induction of *MIG* (Figure 7), I next examined the requirement of STAT1 in the *MIG* induction by using STAT1-deficient mice. Osteoclast precursors were derived from bone marrow cells of the wild type and STAT1-deficient mice and were treated with RANKL in the

presence of M-CSF. The expression of *MIG* mRNA was observed in M-CSF plus RANKL-treated wild type osteoclast precursors, but not in those cells derived from STAT1-deficient mice (Figure 12, first panel). The lack of *MIG* mRNA induction in STAT1-deficient osteoclast precursors were not due to general defect in cellular responses to RANKL because the expression level of *MMP9* and *Fra II* was increased in the STAT1-deficient osteoclast precursors as much as in the wild type osteoclast precursors upon treatment with RANKL (Figure 12, second and third panels). These results demonstrate that STAT1 plays an essential and specific role in RANKL-induced expression of *MIG* mRNA in osteoclast precursors.

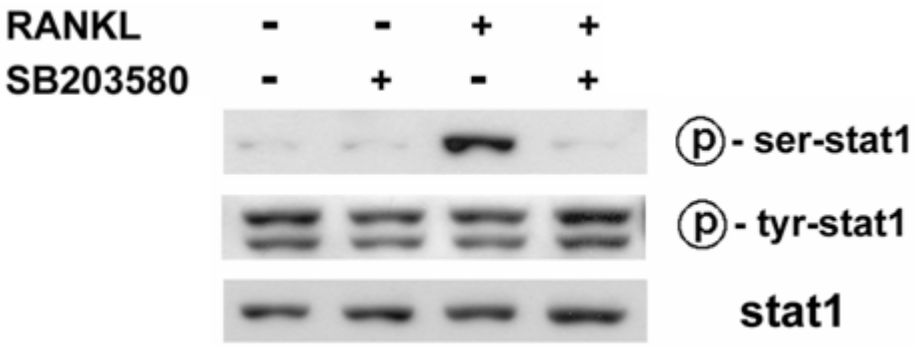


Figure 11. Inhibition of phosphorylation of STAT1 at serine 727 by p38 MAPK inhibitor SB203580

Effects of p38 inhibitor on RANKL-induced phosphorylation of STAT1. Osteoclast precursors were deprived of serum for 12 h and were pretreated with SB203580 (20 μM), followed by stimulation with RANKL (100 ng/ml) for 10 min. Cell lysates were prepared and subjected to Western blot analysis, as described in "Materials and methods."

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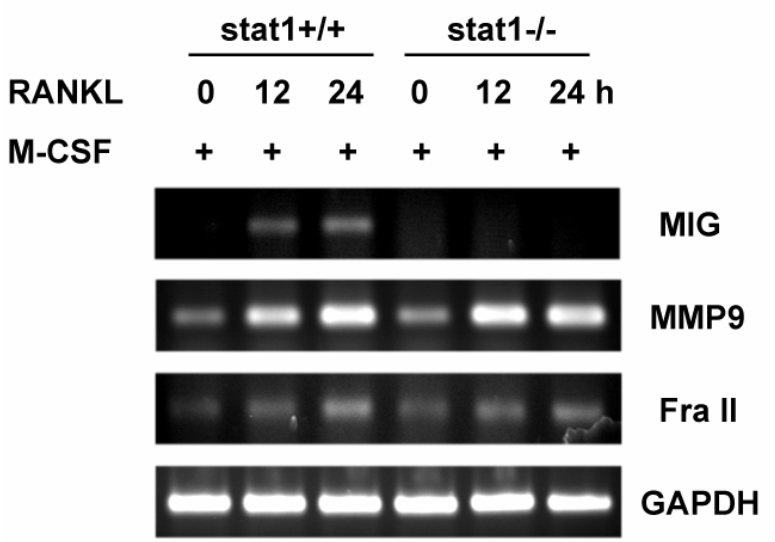


Figure 12. Lack of *MIG* induction by RANKL in STAT1-deficient osteoclast precursors

Osteoclast precursors from wild-type and STAT1-deficient mice were stimulated with M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for the indicated time. RT-PCR was performed with isolated RNA using primers specific for each of *MIG*, *MMP-9*, *Fra II*, and *GAPDH*. PCR products were separated on a 1.2% agarose gel and were stained with ethidium bromide.

III.3. Expression of CXCR3 on osteoclast precursors and TRAP-positive osteoclasts

III.3.1. M-CSF induces the expression of CXCR3, the receptor for MIG

CXCR3 mediates chemotaxis in response to its ligands, MIG, IP-10, and I-TAC (Lu *et al*., 1999). CXCR3 is expressed on the surface of a number of cell types, including activated T cells and B cells, and subsets of inflammatory dendritic cells, macrophages, and NK cells. Therefore, I hypothesized that MIG induced by RANKL in osteoclast precursors may play a role in recruiting more osteoclast precursors to form multinucleated cells through fusion. The basic requirement for this hypothesis would be expression of the receptor for MIG in osteoclast precursors or osteoclasts. I therefore examined whether the expression of CXCR3, the receptor for MIG, is regulated in osteoclast precursors by M-CSF or M-CSF plus RANKL. The *CXCR3* mRNA level was significantly increased from 12 h and reached maximum at 48 h in the presence of M-CSF (Figure 13). Cotreatment with RANKL delayed the M-CSF induction of *CXCR3* mRNA expression without reducing the maximum response level (Figure 13).

III.3.2. Up-regulation of CXCR3 surface expression by M-CSF

To determine whether the cell surface expression of CXCR3 was also

increased by M-CSF or M-CSF plus RANKL, I checked the CXCR3 receptor expression on the surface by flow cytometry. As shown in Figure 14, unstimulated bone marrow-derived osteoclast precursors contained very low number of CXCR3-positive cells (about 1.14%). The population of CXCR3-positive cells increased to 33.64% and 75.21% at 48 h and 72 h, respectively, after M-CSF treatment. The concomitant presence of RANKL did not influence the surface expression of CXCR3 (Figure 14). These results demonstrate that the chemokine receptor CXCR3 is expressed on the surface of both osteoclast precursors (cells incubated with M-CSF) and TRAP-positive osteoclasts (cells incubated with M-CSF plus RANKL for 48 h). These results suggest the possibility that MIG produced by RANKL might play a role in an autocrine or paracrine mode during osteoclastogenesis.

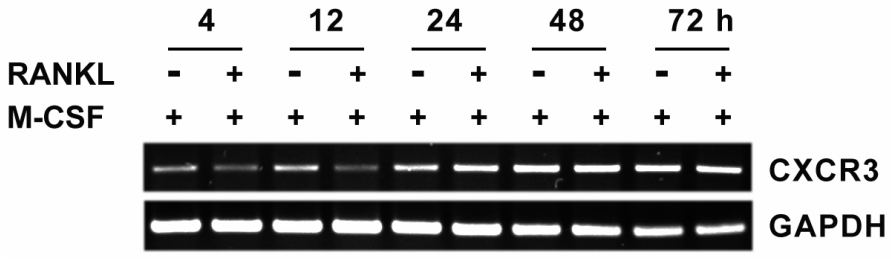


Figure 13. Expression of *CXCR3* mRNA induced by M-CSF

Osteoclast precursors were stimulated with M-CSF (50 ng/ml) or M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for the indicated time. Total RNA was extracted from the treated cells and subjected to RT-PCR analysis with *CXCR3* or *GAPDH* primers. PCR products were separated on a 1.2% agarose gel and were stained with ethidium bromide.

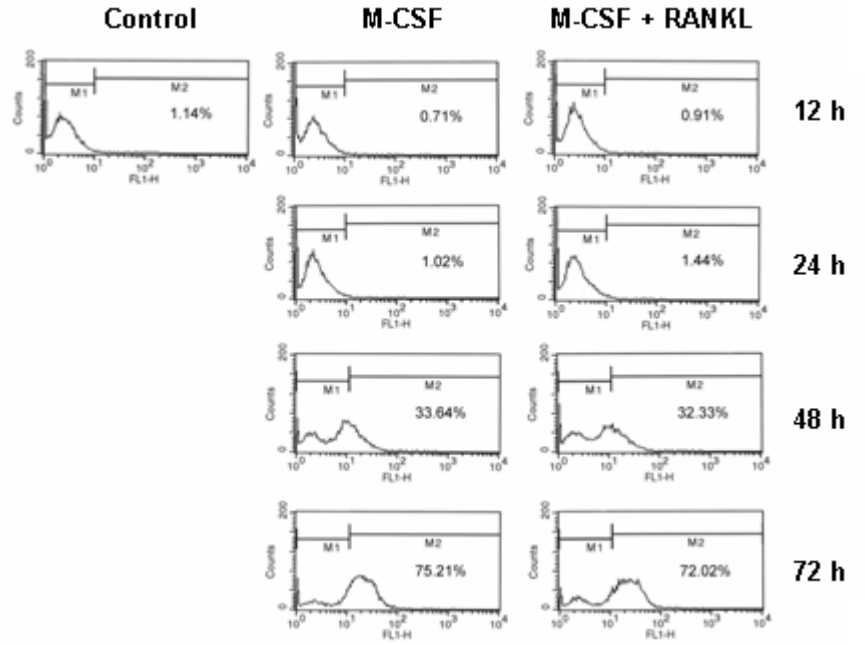


Figure 14. Expression of CXCR3 in osteoclast precursors and osteoclasts incubated with M-CSF

Osteoclast precursors were stimulated with M-CSF (50 ng/ml) or M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for the indicated time. The presence of CXCR3 on the cell surface was analyzed by flow cytometry, as described in "Materials and methods."

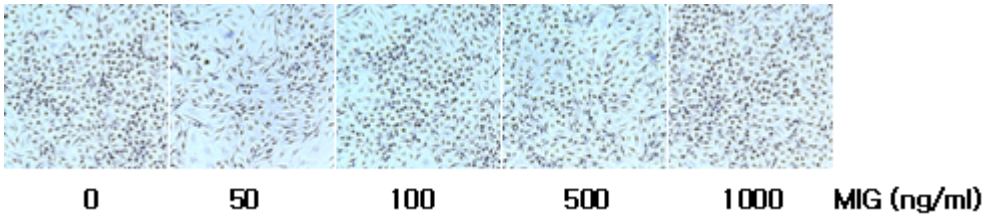
III.4. Roles of MIG in osteoclast precursors and osteoclasts adhesion and migration

III.4.1. Effect of MIG on osteoclast differentiation

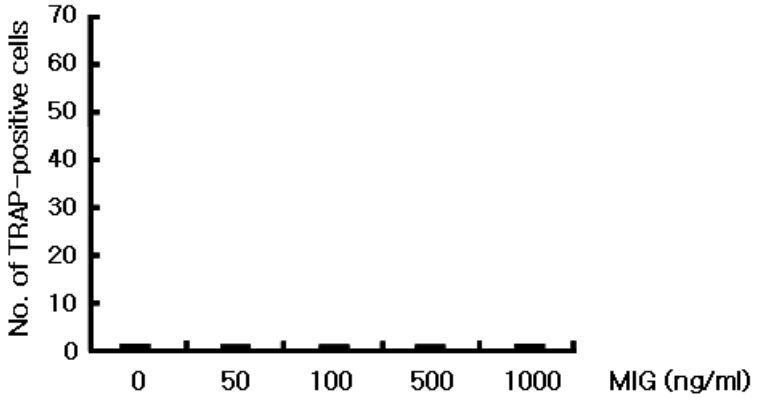
It has been reported that several chemokines may regulate the migration and differentiation of osteoclasts, including MIP-1-α, -β, -γ, IL-8, and MCP-1 (Abe *et al*., 2002; Okamatsu *et al*., 2004; Kim *et al*, 2005). I investigated the role of endogenously produced MIG in RANKL-treated osteoclast precursors. The effect of adding MIG on osteoclast differentiation was determined. Osteoclast precursors were cultured in the presence of M-CSF with various concentrations of MIG (50, 10, 500, or 1000 ng/ml) for 6 days (Figure 15A). However, TRAP-positive osteoclasts were not induced by MIG alone. Also, osteoclast precursors were cultured in the presence of various concentrations of neutralizing anti-MIG antibody (0.1, 0.5, 1, or 5 μg/ml) with 30 ng/ml M-CSF plus 100 ng/ml RANKL for 6 days (Figure 15B). A neutralizing anti-MIG antibody does not inhibit osteoclast differentiation induced by RANKL. These results suggest that MIG induced by RANKL in osteoclast precursors can not stimulate differentiation of osteoclasts.

III.4.2. MIG stimulates the adhesion of osteoclast precursors and osteoclasts

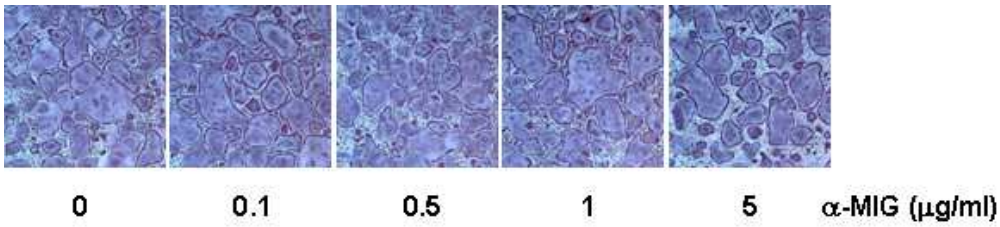
A


0
50
100
500
1000
MIG (ng/ml)

B


No. of TRAP-positive cells
70
60
50
40
30
20
10
0
0
50
100
500
1000
MIG (ng/ml)

C


0
0.1
0.5
1
5
α-MIG (μg/ml)

D

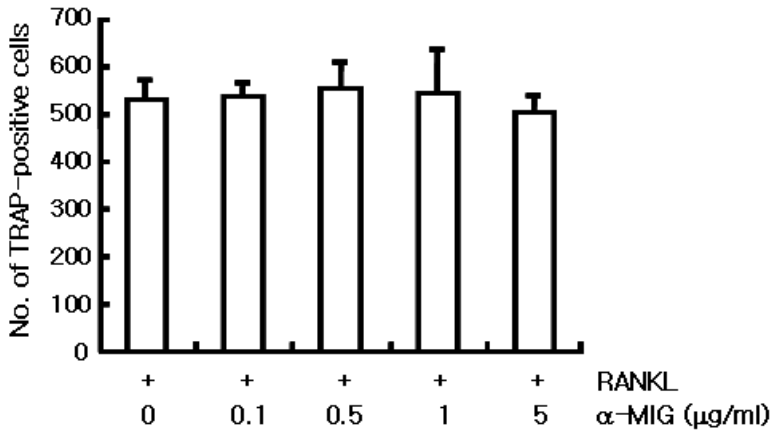


Figure 15. MIG had no effect on osteoclast differentiation

Osteoclast precursors were cultured in 48-well plates for 6 days in the presence of M-CSF (30 ng/ml) and MIG at the indicated concentrations. (A) Cells were fixed and then stained for TRAP. (B) TRAP-positive cells were counted as osteoclasts. Osteoclast precursors were cultured in 48-well plates for 6 days in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) with anti-MIG antibody (0.1, 0.5, 1, or 5 μg/ml). (C) Cells were stained as in (A). TRAP-positive cells were counted as osteoclasts.

It has been reported that MIG increases the adhesion of CXCR3-expressing hematopoietic progenitor cells in the receptor-dependent manner (Jinquan *et al*., 2000). Because M-CSF treatment induced surface expression of CXCR3 in osteoclast precursors (Figure 14), I investigated whether MIG can increase the adhesion of these cells. Osteoclast precursors treated with M-CSF for 60 to 72 h were allowed to attach to fibronectin-coated substratum in the presence or absence of MIG. The presence of MIG increased the adhesion of osteoclast precursors in a dose-dependent manner (Figure 16A). I next examined the effect of MIG on the adhesion of TRAP-positive osteoclasts. Osteoclast precursors were treated with RANKL and M-CSF for 60 to 72 h and were plated on vitronectin-coated wells. The adhesion of TRAP-positive osteoclasts to vitronectin was significantly increased by MIG (Figure 16B).

III.4.3. MIG induces homophilic adhesion

During osteoclast differentiation, the mononuclear osteoclast precursors fuse to form syncytia of multinucleated osteoclasts. To determine whether MIG can induce the adhesion between cells (homophilic adhesion) during osteoclast differentiation was tested. Osteoclast precursors from wild-type and green fluorescence protein (GFP)-transgenic mice were treated with RANKL and M-CSF for 60-72 h. Cells from GFP-transgenic mice were then transferred to cultures of non-GFP cells and incubated for 1 h in the presence or absence of MIG. After removing unbound cells, the bound GFP

cells were scored. The homophilic adhesion was significantly increased by MIG (100 ng/ml) (Figure 17). Also, osteoclast precursors from GFP-transgenic mice were treated with RANKL and M-CSF for 60 to 72 h and then stimulated with or without MIG (100 ng/ml) for 6 h. Cells from GFP-transgenic mice were transferred to cultures of non-GFP cells and incubated for 1 h in the absence of cytokines. Nonadherent cells were removed by washing with PBS. Adherent cells were scored. The appearance of homophilic adhesion occurred more in MIG-stimulated cells than in unstimulated cells (Figure 18). These results clearly show that MIG can stimulate the homophilic adhesion of both osteoclast precursors and differentiating osteoclasts that were induced to express CXCR3 by treatment with M-CSF.

III.4.4. Expression of integrin subunits in CXCR3-expressing osteoclast precursors stimulated by MIG

Modulation of VLA-4 ($\alpha4\beta1$)- and VLA-5 ($\alpha5\beta1$)-dependent cell adhesion by chemokines, including MIG and SDF-1α, has also been shown for hematopoietic progenitor cells, lymphocytes, and leukemic cells (Hidalgo *et al*., 2001), suggesting the presence of common pathways for integrin activation in different cell types. Moreover, it has been reported that VLA-4 and VLA-5, the receptor for fibronectin, mediate melanoma cell adhesion to fibronectin (Mould *et al*., 1997; Danen *et al*., 1995). Because I had already demonstrated that MIG induces osteoclast precursors adhesion

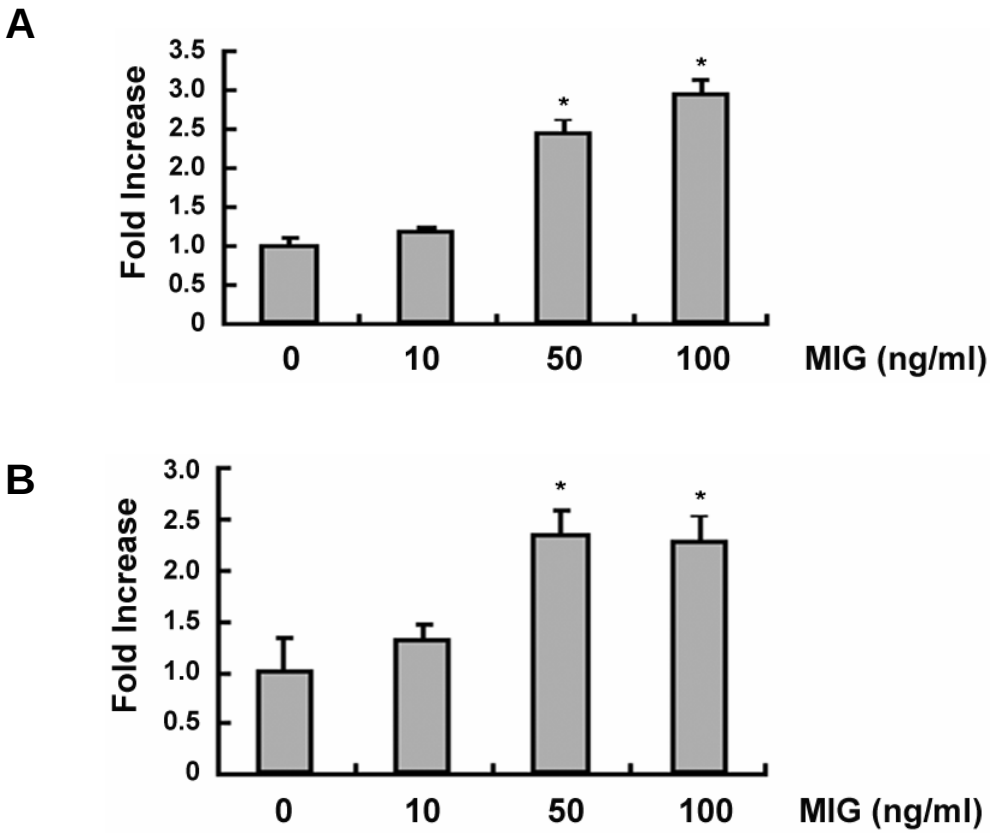


Figure 16. Effect of MIG on adhesion of osteoclast precursors and TRAP-positive osteoclasts

(A) Osteoclast precursors were treated with M-CSF (50 ng/ml) for 60 to 72 h. Cells were incubated for 10 min on fibronectin-coated culture plates supplemented with the indicated concentration of MIG. Nonadherent cells were washed with PBS, and adherent cells were stained with hematoxylin and counted under a light microscope. *Significant difference from the medium control ($P < 0.01$). (B) Osteoclast precursors

were treated with M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 60 to 72 h. Cells were added to the vitronectin-coated culture plates, supplemented with the indicated concentration of MIG, and incubated for 10 min. Adhesion assays were performed as described. *Significant difference from the medium control ($P < 0.01$).

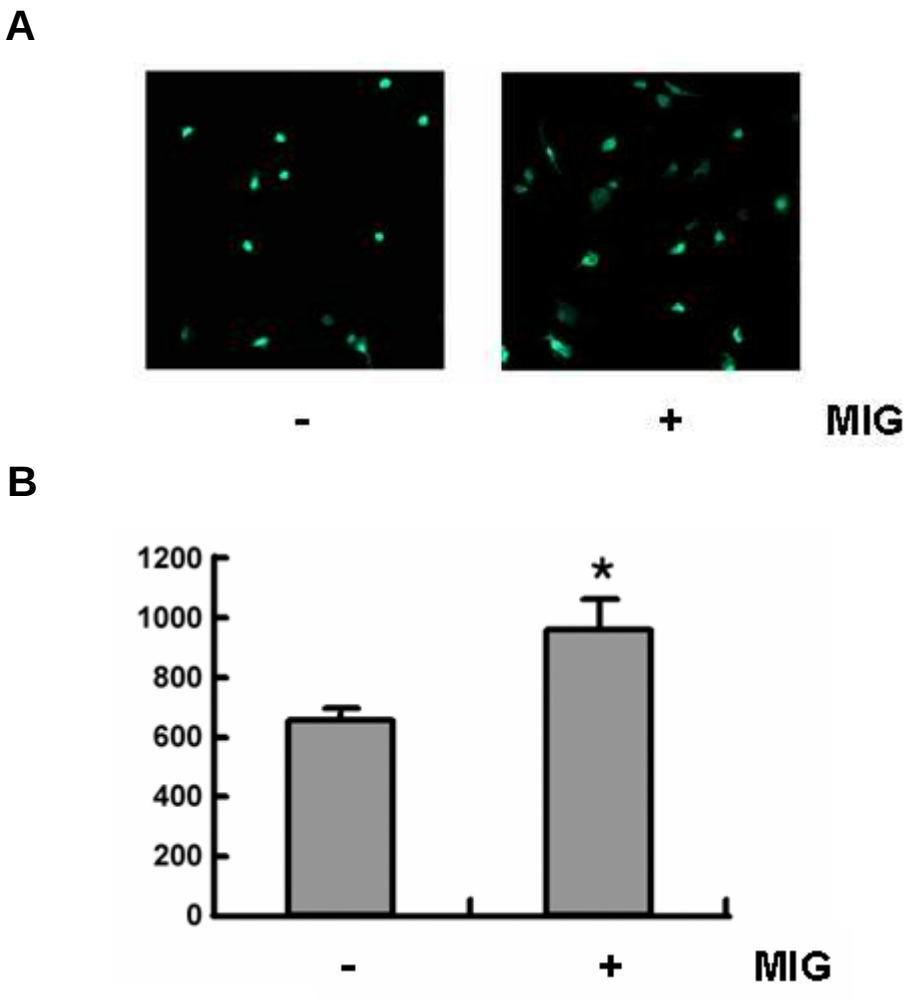


Figure 17. MIG induces homophilic adhesion

Osteoclast precursors from wild-type mice and GFP-transgenic mice were treated with M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 60 to 72 h. GFP-expressing cells were then transferred to dishes containing non-GFP cells and were incubated for 1 h in the presence or absence of MIG (100 ng/ml). Plates were washed to remove unbound cells, and the number of bound cells was scored under a fluorescence microscope. *Significant difference from the medium control ($P < 0.01$).

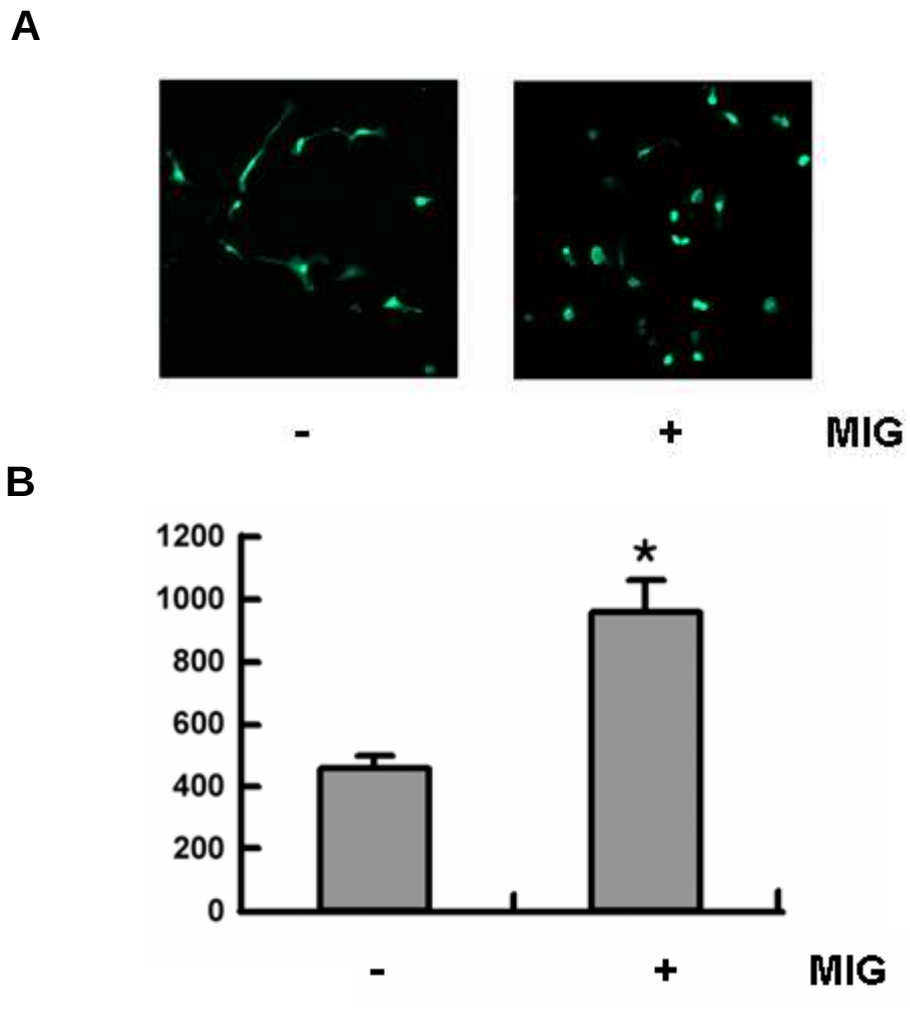


Figure 18. Effect of MIG on homophilic adhesion of TRAP-positive osteoclasts

Osteoclast precursors from wild-type mice and GFP-transgenic mice were treated with M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 60 to 72 h. GFP-expressing cells were stimulated with MIG for 6 h and then transferred to dishes containing non-GFP cells. Dishes were washed to remove unbound cells, and the number of bound cells was scored under a fluorescence microscope. *Significant difference from the medium control ($P < 0.01$).

to fibronectin and induces osteoclasts adhesion to vitronectin (Figure 16). To define which integrin subunit was involved in the MIG-induced osteoclast precursors adhesion to fibronectin and vitronectin, the expression of VLA-4 and VLA-5 integrin subunits, major candidates bind to fibronectin and vitronectin, was examined in osteoclast precursors stimulated by MIG. mRNA for integrin subunits, $\alpha 4$, $\alpha 5$, αv, $\beta 1$, and $\beta 3$ were detected by using RT-PCR. Osteoclast precursors were treated with M-CSF for 60 h. Cells were stimulated for various times with MIG. The results of the RT-PCR analyses shown in Figure 19 indicated that MIG can significantly increase the expression of $\alpha 4$, $\alpha 5$, and αv integrins in CXCR3-expressing osteoclast precursors. These results indicate that MIG may contribute to osteoclast precursor adhesion through the induction of $\alpha 4$, $\alpha 5$, and αv integrin expression in osteoclast precursors.

III.4.5. MIG activates FAK and ERK in CXCR3-expressing osteoclast precursors

Depending on the cell type and the stimulus, both focal adhesion kinase (FAK) and the closely related praline-rich tyrosine kinase-2 (PYK2) have been shown to mediate signals from integrins that can lead to MAPK activation (Schlaepfer *et al*., 1998; Andrey *et al*., 2001). In particular, FAK has been shown to be a critical point of convergence in the action of multiple signaling pathway initiated by integrins (Richardson *et al*., 1997). Moreover, the phosphorylation of ERK was up-regulated on

melanoma cells by MIG. The ERK can also be activated by cell adhesion. To study whether MIG could activate ERK and FAK on osteoclast precursors, I stimulated osteoclast precursors for different times with MIG, and changes in the phosphorylation of FAK, Pyk2, and ERK were analyzed by Western blotting using antibodies recognizing the phosphorylated form of these kinases. In Figure 20, the results of western blotting assays revealed that MIG induces ERK and FAK phosphorylation in CXCR3-expressing osteoclast precursors. However, MIG did not lead to increased phosphorylation of Pyk2. These results demonstrate that MIG stimulates a rapid increase in the phosphorylation of FAK and ERK.

III.4.6. MIG induces the migration of TRAP-positive osteoclasts

Chemokines coordinate leukocyte migration required for development, differentiation, tissue localization, immune surveillance, and effector function. As chemoattractants, they play an essential role in the innate and acquired immune response. To resorb bone, osteoclasts should be recruited to local site from bone marrow or peripheral circulation. MIG is known to function as a chemotactic factor for T cells in inflammatory reactions (Liao *et al*., 1995). To determine whether MIG could induce migration of osteoclasts, I carried out migration assays. As shown in Figure 20, osteoclast migration was significantly increased upon treatment with MIG (100 ng/ml). However, CXCR3-negative cells did not migrate to MIG (Figure 22). These results demonstrate that the

expression of CXCR3 was essential for MIG-induced migration.

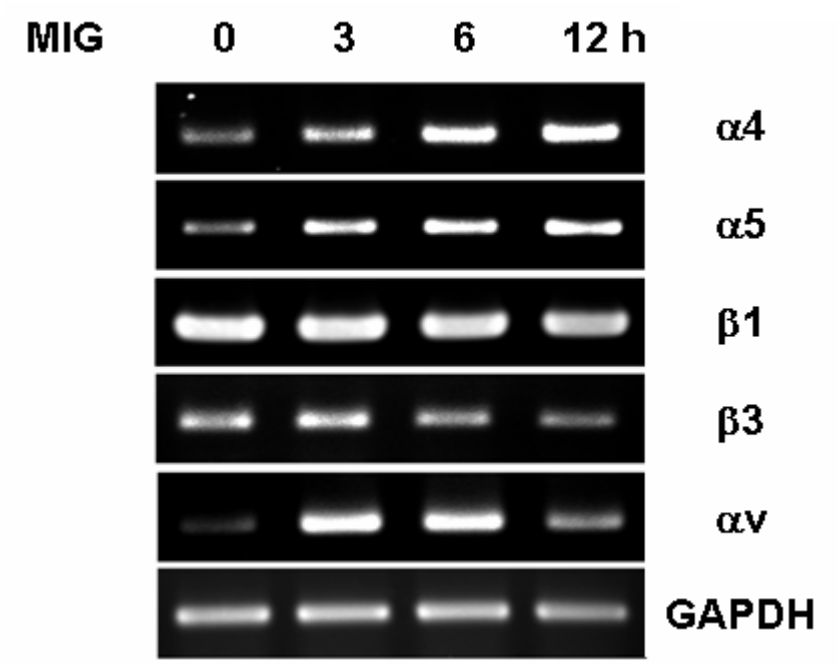


Figure 19. Expression of integrin subunits in osteoclast precursors stimulated by MIG

Osteoclast precursors were stimulated with M-CSF (50 ng/ml) for 60-72 h. Cells were stimulated with MIG (100 ng/ml) for the indicated time. Total RNA was extracted from the treated cells. RNA was reverse transcribed and PCR amplified with *MIG* or *GAPDH* primers. PCR products were separated on a 1.2% agarose gel and were stained with ethidium bromide.

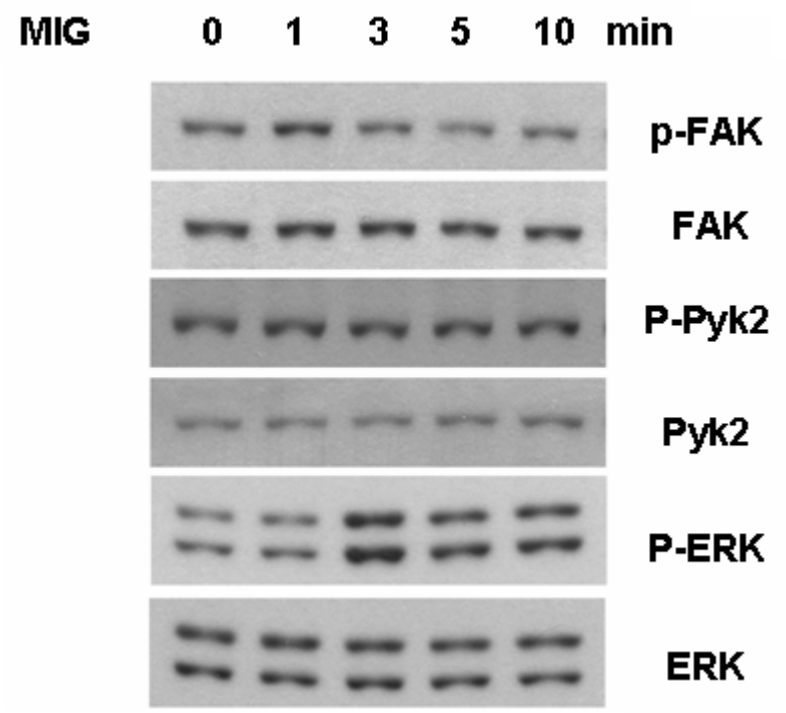


Figure 20. Activation of ERK and FAK by MIG on osteoclast precursors

Osteoclast precursors were stimulated with M-CSF (50 ng/ml) for 60 to 72 h. Cells were stimulated with MIG (100 ng/ml) for the indicated time. Cell lysates were prepared and subjected to Western blot analysis with anti-phospho-FAK, Pyk2, and ERK antibody. The same membrane was stripped and reprobed with anti-FAK, Pyk2, and ERK antibody.

III.4.7. MIG secreted by osteoclast precursors triggers cell migration

I next examined the effect of conditioned media derived from the osteoclast precursors stimulated by RANKL for 24 h on cell migration. In addition, because MIG was not expressed in STAT1-deficient mice, I used the conditioned media from STAT1-deficient cells treated with RANKL. As shown in Figure 23A, cell migration was more greatly increased by conditioned medium obtained from wild type cells stimulated by RANKL (lane 2) than it was by conditioned medium obtained from unstimulated cells (lane 1). The possibility that RANKL itself, which remained in the conditioned medium, might function as a chemotactant factor was excluded because directly adding RANKL to conditioned medium did not stimulate migration (lane 3). In contrast to the situation in wild type cells, cell migration in response to conditioned media from STAT1 deficient cells was not increased by RANKL treatment (lane 5). Adding MIG directly to the conditioned medium from STAT1-deficient cells restored the cell migration response (lane 6). Furthermore, adding a neutralizing antibody against MIG to the conditioned medium obtained from wild-type cells incubated with RANKL reduced the migration response (Figure 23B). The RANKL induction of MIG expression was abolished in the presence of SB203580 at both mRNA and protein levels (Figure 5). Including of the p38 inhibitor SB203580 during RANKL treatment to produce conditioned medium attenuated the extent of migration (Figure 24). These results demonstrate that MIG induced by RANKL in osteoclast precursors can

stimulate migration of osteoclasts.

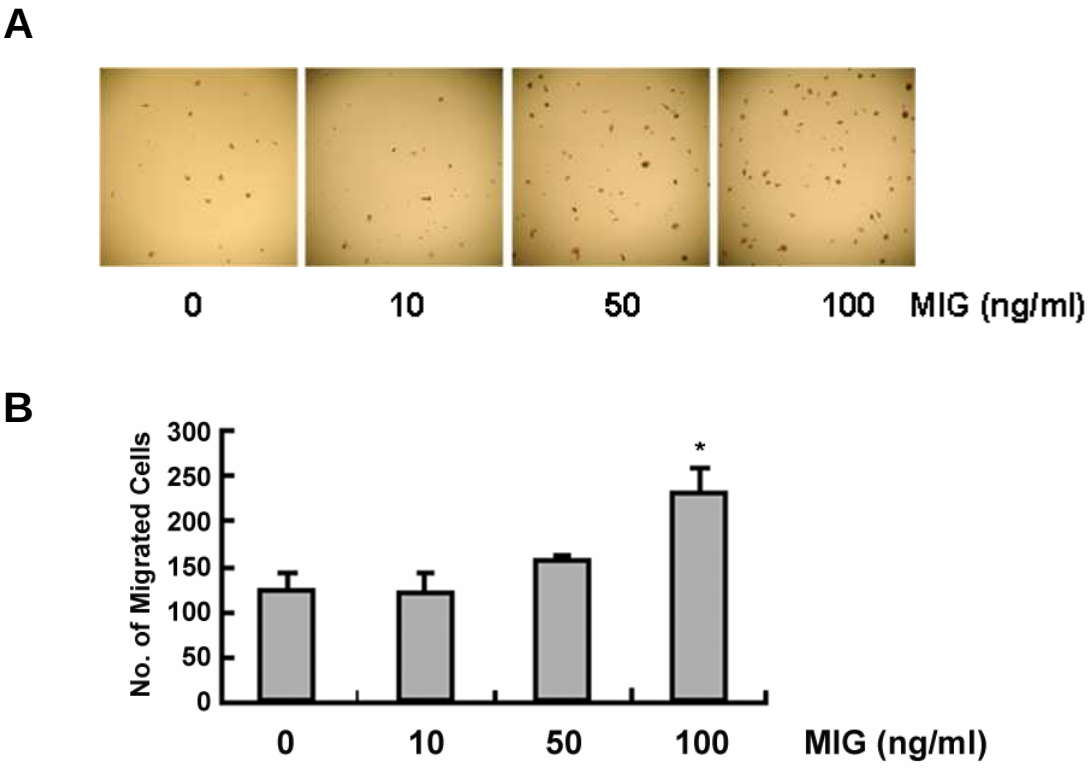


Figure 21. Effect of MIG on the migration of TRAP-positive osteoclasts

Osteoclast precursors were cultured in the presence M-CSF (50 ng/ml) plus RANKL (100 ng/ml) for 60 to 72 h. Cells were washed with PBS, suspended in serum-free α-MEM, and loaded to the upper well of transwell chambers. The lower well contained serum-free medium with the indicated concentration of MIG. After 6 to 8 h, cells migrated onto the lower well were fixed and stained with hematoxylin. *Significant difference from the medium control ($P < 0.01$).

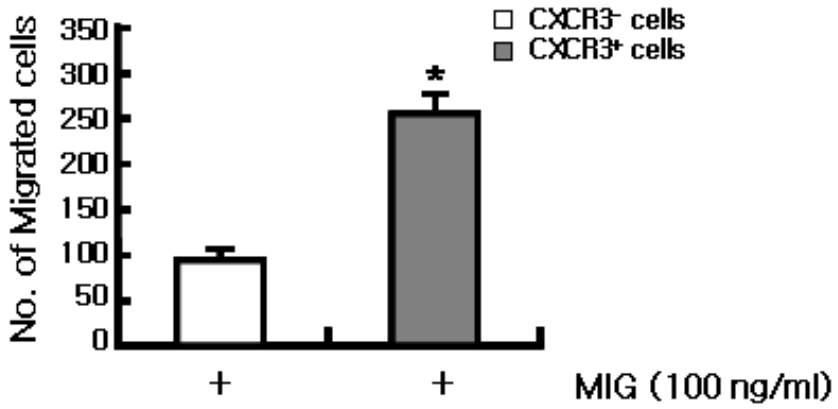


Figure 22. MIG induces the migration of CXCR3-expressing cells

$CXCR3^-$ cells were used as osteoclast precursors. $CXCR3^+$ cells were prepared from osteoclast precursors stimulated with M-CSF for 60 to 72 h. Cells were washed with PBS, suspended in serum-free α-MEM, and loaded to the upper well of transwell chambers. The lower well contained serum-free medium with MIG (100 ng/ml). After 6 to 8 h, cells migrated onto the lower well were fixed and stained with hematoxylin. *Significant difference from the medium control ($P < 0.01$).

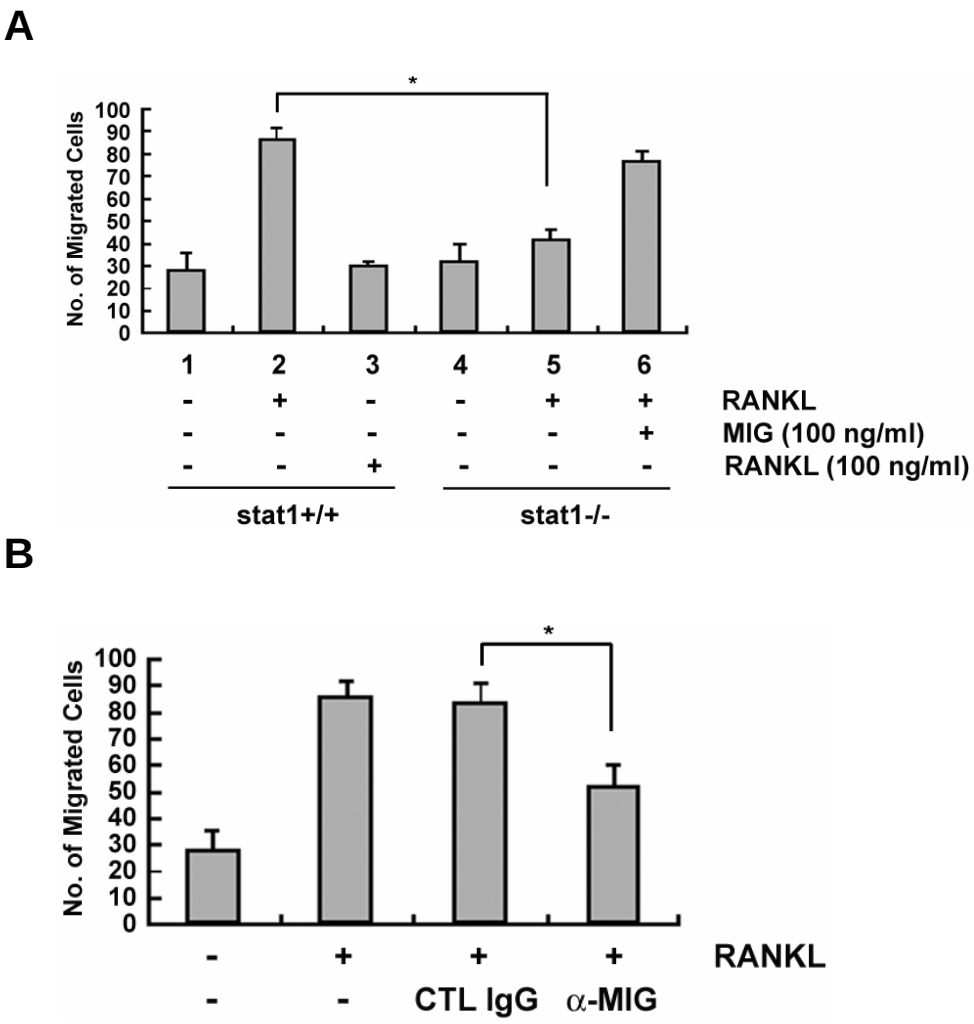


Figure 23. Osteoclast precursors derived conditioned medium induce the migration of osteoclast precursors

(A) Conditioned media were obtained from wild-type and STAT1-deficient osteoclast precursors incubated with RANKL for 24 to 30 h. Conditioned medium was added to the lower well, and osteoclasts were loaded to the upper well. After 6 to 8 h, cells that

had migrated to the lower well were counted. *Significant difference between the indicated groups ($P < 0.01$). (B) The lower well of transwell chambers contained conditioned medium from RANKL-treated cells and either a MIG neutralizing antibody or the control immunoglobulin G (IgG). Migrated cells were counted as described. *Significant difference between the indicated groups ($P < 0.01$).

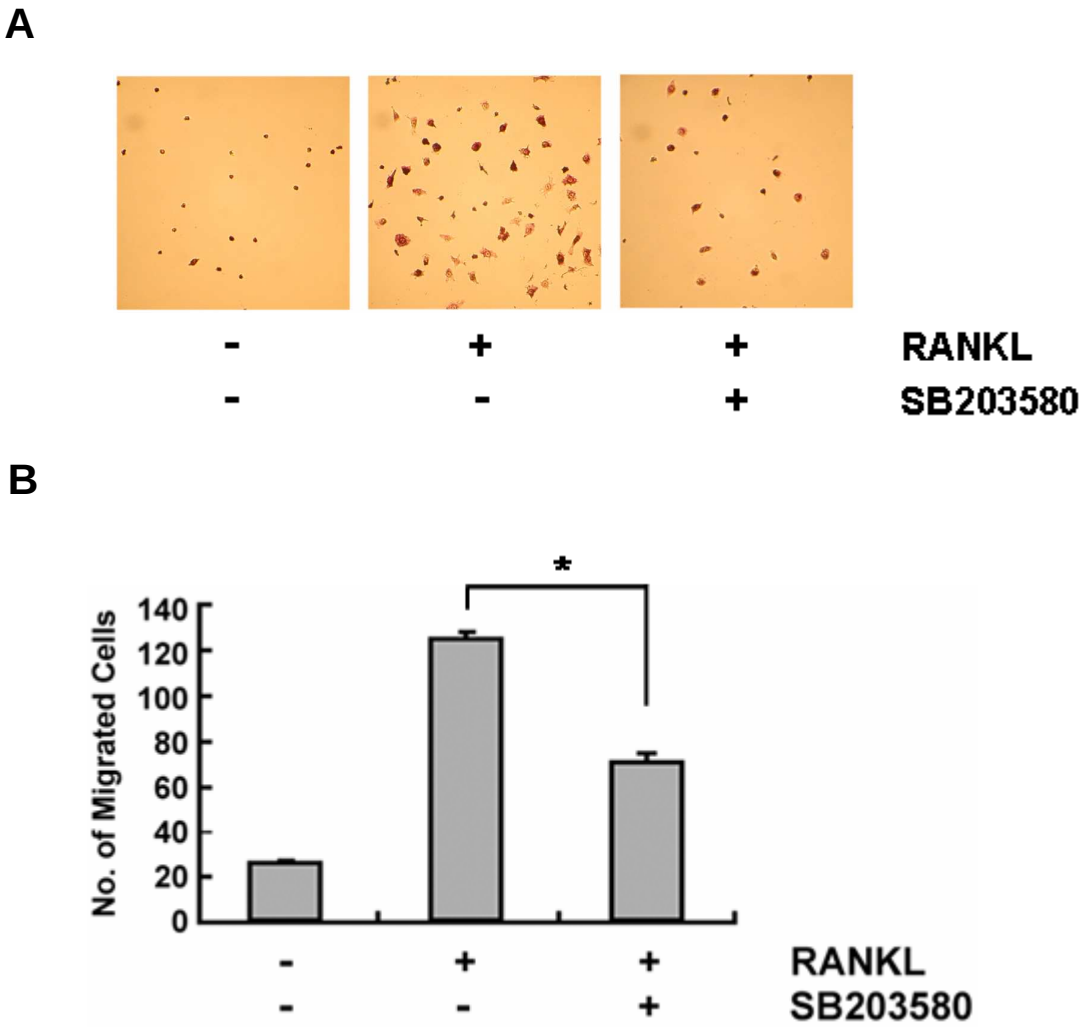


Figure 24. MIG triggers the migration of TRAP-positive osteoclasts

Conditioned media were obtained from osteoclast precursors treated for 24 h with RANKL (100 ng/ml) in the presence or absence of SB203580 (20 μM). Osteoclasts were added to the upper well and were allowed to migrate toward the conditioned medium added to the lower well. Migrated cells were scored after 8 h incubation.

*Significant difference between the indicated groups ($P < 0.01$).

IV. DISCUSSION

Bone is composed of organic component consisting of collagen which gives bone its flexibility, and a mineral component that includes calcium and phosphate salts. Also, bone is a dynamic tissue that is constantly remodeled, *i.e.* degraded and renewed. These two processes are accomplished by two main types of bone cells: bone-forming osteoblasts, of mesenchymal origin, and bone-resorbing osteoclasts, of hematopoietic origin (Theill *et al*., 2002). Understanding the generation and activation of these two cell types will help to unravel many processes involved in bone metabolism and remodeling. Osteoclasts are bone-resorptive multinucleated giant cells and they derive from M-CSF-dependent osteoclast precursors.

The key factors for osteoclastogenesis are the TNF receptor family member RANK, its lignad (RANKL), and the RANKL decoy receptor, OPG (Teitelbaum *et al*., 2003). RANKL and OPG, which are both produced by osteoblasts at differentiation stages of osteoclasts, account for some of the signals in osteoblast-osteoclast communication. The interaction of RANKL with RANK leads to the recruitment of members of the TNF receptor associated factor (TRAF) 2, 5, and 6 adapter proteins (Galibert *et al*., 1998) and the activation of the MAPKs (p38, JNK, and ERK) and NF-κB signaling pathways (Lee *et al*., 2003). These pathways play distinct roles in osteoclast differentiation, function and survival. NF-κB and JNK (leading to AP-1 activation) pathways are essential for osteoclast differentiation (Franzoso *et al*., 1997;

Grigoriadis *et al*., 1994). Moreover, NF-κB also plays a critical role in osteoclastic bone resorption (Gordon *et al*., 2005) and survival, while NFATc1 has been recently shown to be a critical transcription factor for osteoclast differentiation (Ishida *et al*., 2002; Takayanagi *et al*., 2002). Nevertheless, the RANK-initiated intracellular signaling pathways have not been completely elucidated. Thus, present efforts should be focused on investigating signaling mechanism by the RANKL. To this end, it will be important to further elucidate predicted downstream signaling molecules, to identify new signaling molecules.

For these reasons, I thought it would be important to define the gene expression profile associated with RANKL-dependent osteoclastogenesis. cDNA microarray analysis of RANKL-induced signaling in osteoclast precursors has already begun to shed light on global gene changes occurring during osteoclastogenesis. Therefore, to investigate the effect of RANKL on the gene expression during osteoclastogenesis, cDNA microarray were used to analyze gene expression in mRNA derived from RANKL-treated precursors at stage of osteoclast differentiation, and the results were validated by RT-PCR and Western blotting analyses. One of several genes up-regulated by RANKL was *MIG*, originally isolated as a macrophage product with inflammatory and chemotactic properties (Faber, 1990). The differential expression of *MIG* mRNA was confirmed by RT-PCR (Figures 3 and 4) and Western blotting analyses (Figure 6). Moreover, RANKL induced an increase in *MIG* mRNA expression similar to that observed in the absence of M-CSF (Figure 4).

MIG was identified by differential screening of a cDNA library prepared from the RAW 264.7 macrophage cell line that had been treated with lymphokine-rich conditioned medium from concanavalin A-stimulated spleen cells (Faber, 1990). MIG has been known to be induced in macrophages almost exclusively by IFN-γ (Liao *et al*., 1995). Also, Ohmori *et al* reported that STAT1 is critical for IFN-γ-induced MIG expression (Ohmori *et al*., 1995). Therefore, RANKL could have increased MIG expression indirectly by inducing another gene, such as IFN-γ, that could up-regulate MIG. To determine whether new protein synthesis was involved in RANKL-induced *MIG* mRNA expression, osteoclast precursors were incubated with cyclohexamide before RANKL treatment. After treatment with cyclohexamide, RANKL induced an increase in *MIG* mRNA expression similar to that observed in the absence of cyclohexamide (Figure 5). These results indicate that RANKL can directly induce MIG gene expression in osteoclast precursors. It is the first to show that RANKL can induce MIG gene expression.

I found that NF-κB and p38 inhibitors could suppress the expression of MIG stimulated by RANKL (Figure 7). This result indicated that the expression of MIG was controlled by NF-κB and p38 MAP kinase signaling pathways. Horton *et al*. reported that MIG proximal promoter has two potential NF-κB binding sites and NF-κB plays an important role in the induction of *MIG* by IFN-γ (Horton *et al*., 2002). Also, γ-RE, which is a unique STAT1 binding site, is present in the MIG promoter (Horton *et al*., 1998). LPS, UV irradiation, and TNF-α induce the serine 727 phosphorylation of

STAT1 through the p38 MAP kinase pathway (Kovarik *et al*., 1999). Therefore, I hypothesized that p38 may mediate the *MIG* induction by RANKL via modulation of STAT1. In support of my hypothesis, RANKL stimulated the serine 727 phosphorylation of STAT1 in a p38-dependent manner (Figure 11). This is analogous to interferon-γ and IL-13-stimulated gene induction, for which p38 was required for STAT1 serine phosphorylation and transcriptional activation (Goh *et al*., 1999; Xu *et al*., 2003). The lack of MIG induction by RANKL in osteoclast precursors from STAT1-deficient mice further supports the requirement of STAT1 in this gene transcription (Figure 12).

STATs were activated by IFNs and appear to play a key role in the IFNs signaling (Bromberg *et al*., 1996). IFNs bind to a common receptor, and activate the receptor-associated Janus kinases (Jaks). Activation of the Jaks and phosphorylation of tyrosine residues by Jaks on the cytoplasmic domain of the receptors results in phosphorylation of STAT proteins. To function as transcription factors, STAT proteins should translocate to the nucleus. Tyrosine phosphorylation and subsequent dimerization through interaction with SH2 domain allows STATs to enter the nucleus and bind DNA (Darnell, 1997). While tyrosine phosphorylation provides the prerequisite for these transcription factors, the second phosphorylation on serine renders STATs transcriptionally active (Decker *et al*., 2000).

In the current study, serine 727 phosphorylation of STAT1 was detected 10 min after RANKL stimulation in osteoclast precursors, reached a maximum within 20

min. However stimulation of tyrosine 701 phosphorylation of STAT1 by RANKL was not observed (Figure 8). The puzzling question then arises how STAT1 mediates RANKL induction of *MIG* transcription without the tyrosine phosphorylation required for nuclear localization. One possible answer may derive from the weak but consistently observed basal level of tyrosine phosphorylation (Figure 8), which might have been sufficient to translocate some STAT1 proteins. In confocal microscopic analyses of immunostained osteoclast precursors, significant amount of STAT1 was observed in the nucleus and the nuclear localization was not increased by RANKL (Figure 10). The tyrosine phosphorylation of STAT1 observed in osteoclast precursors might be a consequence of M-CSF treatment applied to bone marrow cells for 3 days to obtain the osteoclast precursors. In fact, M-CSF caused rapid STAT1 tyrosine phosphorylation and increased nuclear localization of STAT1 in osteoclast precursors (Figures 9 and 10). Moreover, the serine 727 phosphorylation of STAT1 is necessary for maximal transcriptional activity (Decker *et al*., 2000).

Chemokines are a family of molecules that regulate the chemotaxis of leukocytes in tissues. Chemokines have other roles, including promoting mitosis and the modulation of apoptosis, survival, and angiogenesis (Gerad *et al*., 2001). The chemokine family now consists of over 40 members that are subdivided into groups based on the motifs of their first two N-terminus cysteine residues. In particular, MIG is a member of a CXC chemokine superfamily. This secreted low molecular weight protein has been implicated in the directed migration, adhesion and, activation of

macrophages, T cells, and B cells that express CXCR3, the receptor for MIG (Faber, 1997; Moser *et al*., 2001; Rabin *et al*., 2003). CXCR3 is the only known receptor for MIG. MIG shares CXCR3 with IP-10 (Liao *et al*., 1995) and I-TAC (Cole *et al*., 1998), and CXCR3 is functionally expressed on subsets of human peripheral blood T, B, and NK cells (Qin *et al*., 1998). MIG and CXCR3 are produced by different cell types in response to IFN-γ (Gasperini *et al*., 1999). I hypothesized that the RANKL-induced expression of MIG in osteoclast precursors might trigger the adhesion and migration of CXCR3-expressing cells. To gain evidence supporting my hypothesis, I first examined whether osteoclast precursors express CXCR3 along the process of osteoclastogenesis and found that M-CSF treatment induced *CXCR3* gene transcription and surface expression (Figures 13 and 14). Granulocyte macrophage-colony stimulating factor (GM-CSF) has been reported to induce CXCR3 on $CD34^+$ hematopoietic progenitors from human cord blood, which led to chemotactic and adhesive responses to MIG and IP-10, another CXCR3 ligand (Jinquan *et al*., 2000). I examined the ability of MIG to induce adhesion and migration in M-CSF-stimulated osteoclast precursors and found that they induced significant adhesion and chemotactic migration in the stimulated cells (Figures 15 and 20). I report for the first time that M-CSF can induce the expression of CXCR3 in bone marrow-derived hematopoietic progenitors during osteoclastogenesis. I next investigated whether the CXCR3-expressing osteoclast precursors and differentiating osteoclasts show adhesion and migration stimulated by MIG. MIG or the conditioned medium from RANKL-treated osteoclast precursors

stimulated the migration and adhesion of osteoclast precursors and differentiating osteoclasts that were induced to express CXCR3 by M-CSF treatment (Figures 20 and 22).

Integrins are heterodimeric receptors that are involved in cell-cell and cell-matrix interactions. Integrins play a role both in adhesion and in transducing signals involved in a variety of cellular functions. It has been reported that fibronectin and vitronectin are a ligand of the integrins $\alpha4\beta1$, $\alpha5\beta1$, and αv and mediate the adhesion of bone marrow macrophages and osteoclasts, respectively, through these integrins (Duong *et al*., 1999). I found that MIG induced the expression of integrin $\alpha4$, $\alpha5$, and αv subunits in osteoclast precursors. These results suggest that up-regulation of MIG-induced integrins ($\alpha4$, $\alpha5$, and αv) plays a crucial role in the adhesion of M-CSF-stimulated osteoclast precursors. Also, one of the kinases involved in adhesion signaling is the focal adhesion kinase FAK. Phosphorylation of FAK has been observed in a variety of cell types (Hanks, *et al*, 1992; Hamawy *et al*., 1993). The FAK molecule is part of a general signaling pathway for adhesion signaling. MIG can induce the phosphorylation of FAK (Figure 20). These finding is in agreement with previous reports that FAK participates in adhesion signaling (Astier *et al*., 1997).

The induction of MIG by RANKL and that of CXCR3 by M-CSF during differentiation of osteoclasts from bone marrow cells suggest that this chemokine ligand-receptor pair may function in an autocrine or paracrine mode. As the time course of the ligand and receptor expression shows more than 24 h gap, it is likely that

MIG-CXCR3 work in a paracrine rather than an autocrine way between different stages of cells during osteoclastogenesis. In *in vitro* culture of bone marrow-derived cells for osteoclastogenesis, interactions between cells already fused and cells mononuclear (i.e. between cells more progressed and cells at an earlier stage) are frequently observed. Another physiological importance of CXCR3 expression in osteoclast lineage cells may lie on the possibility that MIG secreted by IFN-γ in the inflammatory sites may recruit osteoclast precursors and differentiating osteoclasts, which would results in bone resorption.

In summary, I have shown that RANKL directly induces MIG expression during osteoclastogenesis. The RANKL induction of MIG requires STAT1 serine phosphorylation, which is stimulated through p38 MAPK. I have also found that differentiating osteoclasts express the MIG receptor CXCR3 on the surface and migrate toward MIG.

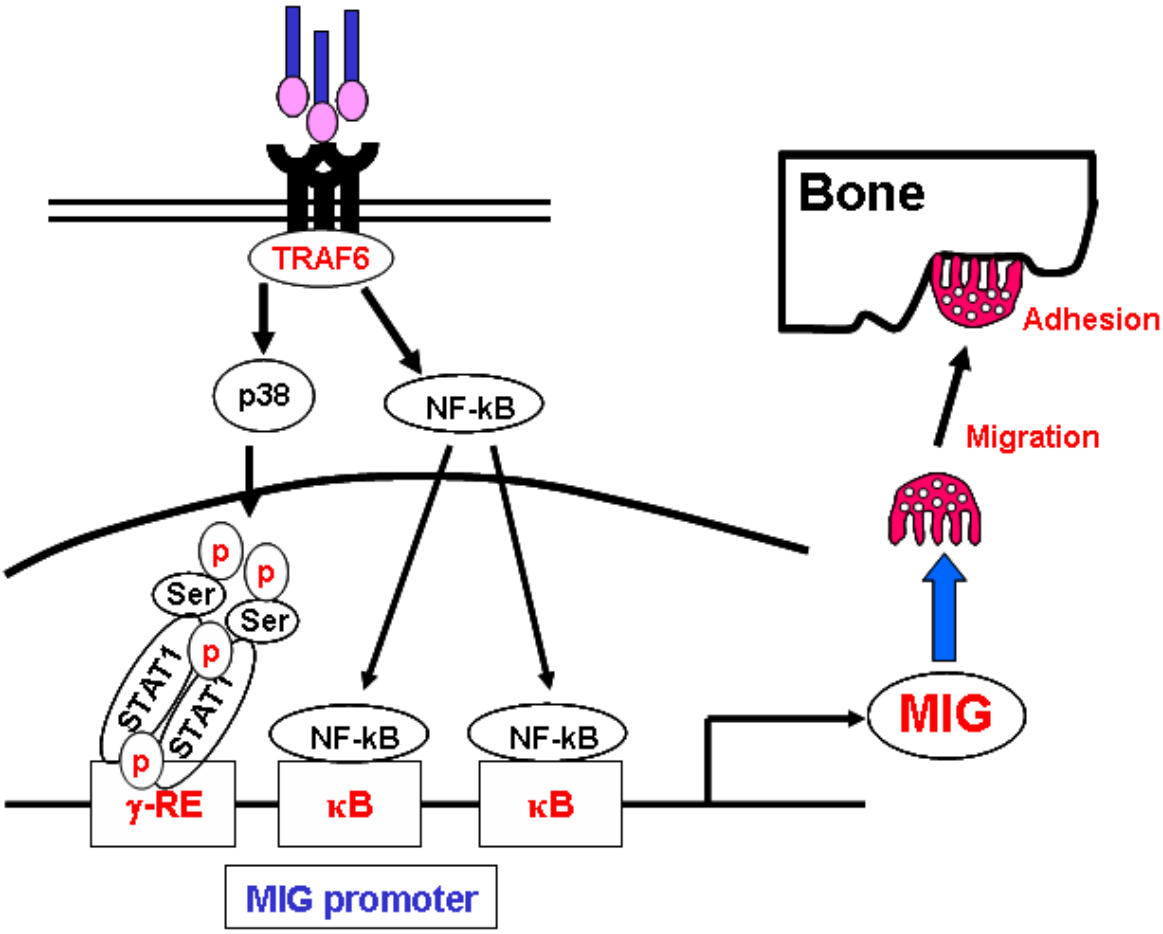


Figure 25. Model of osteoclast migration and adhesion induced by MIG

MIG, a CXC chemokine greatly expressed by osteoclast precursors, may be a key signal for the migration of $CXCR3^{+}$ cells, such as osteoclast precursors and osteoclasts into bone.

V. 적요

파골세포의 부착 및 이동과 RANKL에 의해 유도되는 Monokine induced by interferon-gamma (MIG)의 연관성에 대한 연구

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골(骨)은 일생 동안 끊임없이 생성과 소멸을 반복하는데 골 밀도는 인체 내의 내분비 호르몬 (hormone)과 국지적인 염증 유발인자 (Inflammatory cytokine)에 의해 조절된다고 보고 되었다. 골 밀도를 조절하는 중요한 두 가지 세포 중에 조골세포 (Osteoblast)는 골기질의 합성으로 뼈를 형성시키고 반면 파골세포 (Osteoclast)는 조골세포에 의해 만들어진 골 기질을 흡수하는 역할을 한다. 그러나 과도하게 파골세포의 활성이 높아지면 골 밀도가 감소하게 되어 골다공증 및 류마티스 관절염과 같은 골 질환이 유발된다.

RANKL는 파골세포의 분화와 활성에 없어서는 안될 중요한 인자로 RANKL에 의한 파골세포의 분화는 다양한 유전자 (gene)의 발현에 의해 유도된다. 따라서 RANKL에 의해 유도되는 유전자를 규명하는 것은

파골세포의 분화와 활성을 유도하는 기작 (mechanism)을 알 수 있는 중요한 일이라 할 수 있다.

파골세포의 전구세포 (Osteoclast precursor)를 RANKL로 자극하여 파골세포의 분화를 유도하였고, cDNA microarray 기법을 사용하여 파골세포의 분화 과정에서 유도되는 유전자를 확인 하였다. 그 결과 RANKL에 의해 유도되는 유전자 중에서 MIG (Monokine induced by interferon-γ)라 불리는 chemokine을 확인 하였다. MIG는 혈관내피 세포 (Endothelial), 섬유아 세포 (Fibroblast), 대식세포 (Macrophage)등에서 발현된다고 보고되었으며 여러 조직 (간, 신장, 심장 등)에서도 MIG가 분비된다고 보고되었다. 위의 세포와 조직에서 분비된 MIG는 면역 계통 세포의 활성에 중요하게 작용하며 다른 조직 (Tissue)으로의 이동과 부착에 관련되어있다고 보고되었다.

먼저 파골세포의 전구세포에 RANKL를 자극하여 MIG 발현 정도를 확인하였다. MIG는 RANKL의 자극에 의해 24 시간 까지 발현이 증가되었고 40-50 ng/ml 농도로 분비되었다. 다음으로 RANKL에 의해 유도되는 MIG가 어떤 기작을 통해 유도되는지 확인하기 위해 신호 전달 억제제 (Signaling inhibitor)를 사용하여 실험하였다. 그 결과 전사 유도인자 (Transcription factor)인 NF-kB와 신호 단백질인 p38 MAPK에 의존적으로 전사 유도인자 STAT1이 MIG 유도에 관련되어 있다는 것을 확인하였다. 그 결과 RANKL가 파골세포의 분화 과정에서 전사 유도인자인 NF-kB와 STAT1에 의해서 MIG 발현을 조절한다는 것을 확인하였다.

MIG는 수용체 (Receptor)인 CXCR3를 자극하여 CXCR3를 발현하는 세포의 활성을 조절한다고 보고되었다. 따라서 파골세포의 전구세포가 CXCR3를 발현하는지 확인 하였고 M-CSF에 의해서 파골세포의 전구세포뿐만 아니라 파골세포 에서도 CXCR3가 발현된다는 것을 확인하였다. 또한 MIG의 수용체를 가지고 있는 파골세포의 전구세포에 MIG로 자극하여 파골세포의 분화와 활성에 어떤 효과를 나타내는지 확인 하였다. MIG는 RANKL에 의해 많은 양이 분비 되었지만 파골세포의 분화와 활성에는 차이를 보이지 않았다.

골수에서 유래된 파골세포와 전구세포는 뼈 조직으로 이동하여 뼈를 흡수하게 된다. 그러나 골수에서 유래된 파골세포와 전구세포가 어떻게 뼈 조직으로 이동하는지는 자세히 알려지지 않았다. 그래서 MIG가 파골세포의 이동과 기질 (Fibronectin, Vitronectin)로의 부착을 촉진 하는지 확인 하였다. 그 결과 MIG는 파골세포와 전구세포의 이동과 부착을 농도에 의존적으로 증가시키는 것을 확인하였으며 파골세포의 전구세포에서 분비된 MIG가 다른 파골세포와 전구세포의 이동을 유도한다는 것 역시 확인 하였다. 이 결과로 MIG가 파골세포의 이동과 기질로의 부착을 유도함으로써 파골세포가 뼈 조직으로 이동하는데 중요한 역할을 할 것이라고 제안한다.

결론으로 파골세포에 중요한 인자인 RANKL가 파골세포의 분화 과정 동안에 chemokine의 일종인 MIG의 분비를 촉진하였고 이런 과정은 전사 유도인자인 NF-kB와 STAT1에 의해 조절되었다. 또한 파골세포의

전구세포 에서 분비된 MIG는 다른 파골세포와 전구세포의 이동과 기질로의 부착을 촉진 시켰다. 이런 결과로 MIG는 생체 내에서 파골세포가 뼈로 이동하는데 있어서 중요한 인자로 생각된다.

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