Review

Contributions to osteoclast biology from Japan

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(Contributed by Tatsuo SUDA, M.J.A.)

Abstract: Bone is a dynamic tissue, in which bone formation by osteoblasts and bone resorption by osteoclasts continue throughout life. In 1998, we molecularly cloned osteoclast differentiation factor (ODF), a long-thought factor responsible for osteoclast formation. This review article describes how Japanese scientists contributed to osteoclast biology before and after the discovery of ODF. This review article is based on the Louis V. Avioli Memorial Lecture of the American Society for Bone and Mineral Research (ASBMR) held in Honolulu in September, 2007.

Keywords: osteoclast formation and activation, M-CSF, ODF/RANKL, OCIF/OPG, DC-STAMP, NFATc1/NFAT2

Introduction

Bone is a dynamic tissue, in which bone formation and resorption continue throughout life. This process is called "bone remodeling". The bone tissue contains various types of cells. Among them, the bone-forming osteoblasts and the bone-resorbing osteoclasts are two major types of cells responsible for bone remodeling. Osteoblasts are believed to be derived from undifferentiated mesenchymal cells present in bone marrow, which further differentiate into osteocytes and are embedded in calcified bone.[1)] In contrast, osteoclasts are multinucleated giant cells present only in bone.[2)] It is believed that osteoclasts are recruited from hemopoietic cells of the monocyte-macrophage lineage. Osteoclast progenitors then proliferate and differentiate into mononuclear pre-osteoclasts and fuse each other to form multinucleated osteoclasts. In ages under 50, the rate of bone resorption and formation is well balanced, thus bone maintains a level of peak bone mass.[3)] However, in ages over 50, humans lose bone mass annually at a rate of 1%. Thus, humans with 80 years old, contain only 70% of the peak bone mass. In addition, most women lose additional 10% of bone mass between 50 and 60 years old after menopause. This is due to the fact that osteoclastic bone resorption exceeds osteoblastic bone formation owing to estrogen deficiency. In patients with osteoporosis and rheumatoid arthritis, bone resorption also exceeds bone formation, which results in serious bone loss and bone damage. In Japan, the number of patients with osteoporosis has been estimated to be over 10 million, and the number of annual hip fracture has exceeded 120,000 cases.[4)]

We have been working in osteoclast biology for the past two decades.[2)] Two years ago, I had an e-mail from Harald Jueppner, the Chairman of the 2007 Program Committee of the American Society for Bone and Mineral Research (ASBMR), who asked me to deliver a Louis Avioli Memorial Lecture held in Honolulu in September, 2007. The ASBMR is the biggest society for bone research in the world. It is a domestic society for American bone community, but more than 50% of the members of the ASBMR come from outside of the US. The late Louis Avioli is the founder of the ASBMR. Louis's dynamic nature has attracted us very much since we met him for the first time in 1974. His influence on our field over the past four decades resulted from his extraordinary leadership, intelligence, vision, energy and understanding of

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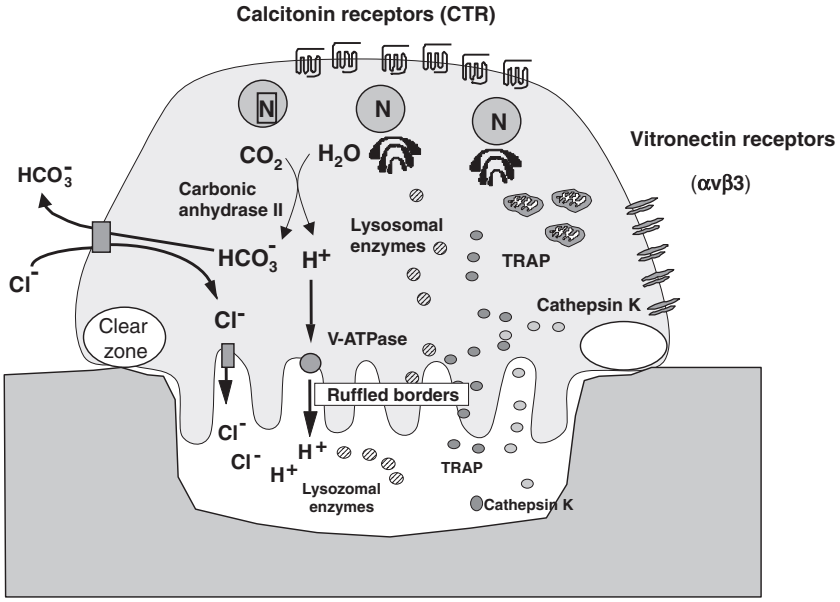


Fig. 1. Structure of osteoclasts.

human relations. Therefore, we thought this is undoubtedly a great honor not only for us but also for all active Japanese members of the ASBMR. During that time we worked in the field of osteoclast biology, we had an acquaintance with many friends not only from Japan, but also from all over the world. In the molecular cloning of the osteoclast differentiation factor (ODF), we collaborated or competed with many foreign research groups. In the modern research on osteoclast biology, Japanese scientists discovered a number of important findings. Thus, in our Avioli lecture, we wanted to introduce the contributions to osteoclast biology not only from our own work but also from major work by Japanese scientists. This review article is based on the Avioli Memorial Lecture of the ASBMR held in Honolulu in September, 2007.

1. Characteristic features of osteoclasts

Osteoclasts exhibit typical ultra-structural features such as the presence of a number of nuclei, abundant pleomorphic mitochondria, and a large number of vacuoles and many stacks of Golgi membranes.[5),6)] The most characteristic features of osteoclasts are the presence of ruffled borders and clear zones (Fig. 1). The ruffled border is a complex structure of deeply infolded finger-like plasma membranes adjacent to the bone surface. The ruffled border is surrounded by a clear zone, which is totally devoid of cellular organelles, except for actin filaments. It is generally accepted that the clear zone serves for the attachment of osteoclasts to bone surface and for separating the bone resorbing area beneath the ruffled border from the unresorbed area.

Osteoclasts have several specific marker enzymes like tartrate-resistant acid phosphatase (TRAP) and cathepsin K in lysosomes, and vacuolar type proton ATPase (v-ATPase) in ruffled borders.[5),6)] Osteoclasts have a number of calcitonin receptors on the basolateral membranes. It was essential to generate osteoclasts with these characteristic features *in vitro* to understand how osteoclast differentiation and function are controlled.

2. Osteoblasts intervene in the process of osteoclastic bone resorption

The idea that osteoblasts or osteoblastic stromal cells may intervene in the process of osteoclastic bone resorption was first reported by Gideon Rodan and Jack Martin in 1981.[7)] Their argument for such a mechanism was based on the observations that first, bone-resorbing hormones and cytokines such as parathyroid hormone (PTH), interleukin 1 (IL-1) and 1α,25-dihydroxyvitamin D_3 [1α,

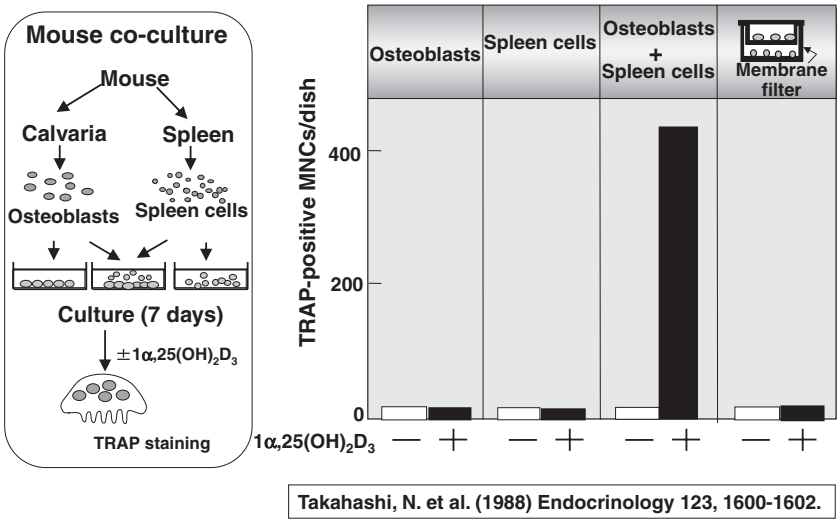


Fig. 2. Osteoclast formation in co-cultures of osteoblasts and spleen cells.

$25(OH)_2D_3$] have their receptors in osteoblasts but not in osteoclasts, and second, the relative potencies of these bone-resorbing factors in osteoblasts resemble those in inducing bone resorption. The same conclusion was reached independently by Tim Chambers,[8] who proposed that a factor called osteoclast activating factor (OAF) is produced by osteoblasts in response to bone-resorbing hormones and cytokines, which stimulates osteoclast activation.

Inspired by those pioneering work, we established an efficient mouse co-culture system to recruit osteoclasts *in vitro* in 1988.[9] Osteoblasts were isolated from mouse calvaria, and cells isolated from splenic tissues were used as osteoclast progenitors. Spleen cells represent osteoclast progenitors, in other word "seeds", and osteoblasts represent supporting cells to provide a suitable microenvironment for osteoclast formation, in other words, "farm". They were either separately cultured or co-cultured in the presence of $1\alpha,25(OH)_2D_3$. Figure 2 shows the number of TRAP-positive multinucleated osteoclasts formed. When osteoblasts alone or spleen cells alone were cultured, no osteoclasts were formed even in the presence of $1\alpha,25(OH)_2D_3$. Multinucleated cells which satisfy almost all of the osteoclast phenotype were formed only when spleen cells and osteoblasts were co-cultured in the presence of $1\alpha,25(OH)_2D_3$. When osteoblasts and spleen cells were separately cultured in a trans-well through a membrane filter, no osteoclasts were formed, suggesting that cell-to-cell interaction between osteoblasts and spleen cells is important. Since this co-culture system was reproducible and efficient to recruit osteoclasts *in vitro*, most of the scientists working in osteoclast biology evaluated it highly useful.

The next question was to examine whether osteoblasts are also needed for activating osteoclast function (in other words, for inducing bone-resorbing activity of osteoclasts). To explore this issue, Eijiro Jimi[10] attempted to purify osteoclasts formed in our co-culture system. Since it was extremely difficult to detach osteoclasts formed on plastic dishes, he devised a new method, in which osteoblasts and bone marrow cells were co-cultured on collagen gel-coated dishes in the presence of $1\alpha,25(OH)_2D_3$ to generate osteoclasts. The co-culture was then treated with collagenase to recover most of the cells as a crude osteoclast preparation. The purity of osteoclasts in this preparation was only 5%. The crude osteoclast preparation was further subjected to a 30% Percoll solution and centrifuged to purify osteoclasts. The purity of osteoclasts increased up to 70%. The crude osteoclast preparation containing many osteoblasts did form resorption pits when they were cultured on dentine slices, whereas purified osteoclasts did not form resorption pits at all on the slices (Fig. 3). When osteoblasts were put onto purified osteoclasts, the pit-forming activity of the purified osteoclast preparation was strikingly recovered.[10]

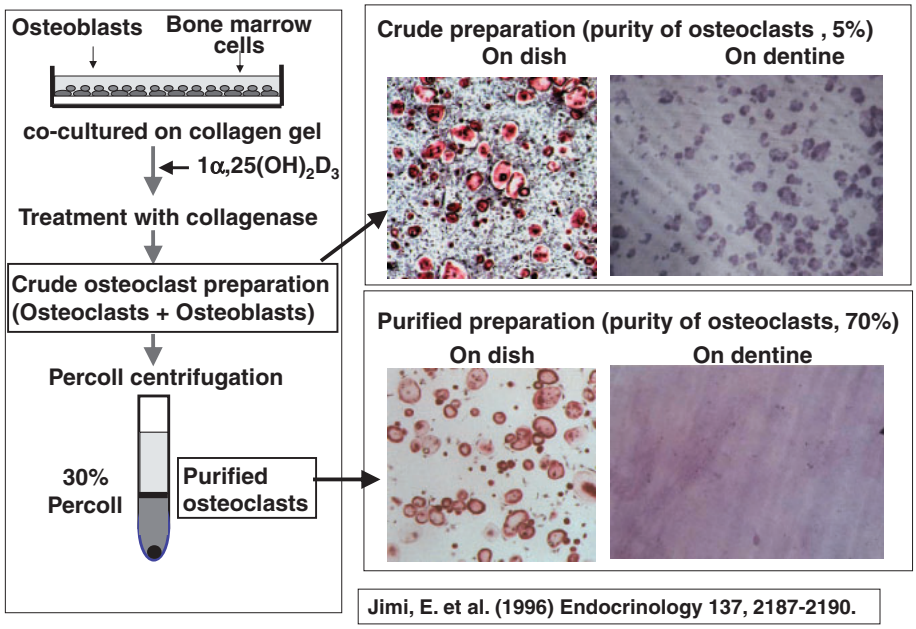


Fig. 3. Purification of osteoclasts formed in co-cultures.

From these experimental results, we concluded that not only osteoclast differentiation but also osteoclast activation requires the presence of osteoblasts. Osteoblasts appeared to perform those actions through cell-to-cell interaction with osteoclast progenitors or mature osteoclasts.

3. Role of M-CSF in osteoclastogenesis

The next important discovery in osteoclast biology was the requirement of M-CSF (macrophage-colony stimulating factor)/CSF-1 (colony stimulating factor 1) in osteoclastogenesis. Shinichi Hayashi and his associates were interested in osteopetrotic *op/op* mice. Since the osteopetrotic phenotype of *op/op* mutant mice was not cured by transplantation of normal bone marrow cells, the defect was considered to be present in local microenvironment in bone. Hisa Yoshida[11] found that there was an insertion of thymidine at bp 262 in the M-CSF gene of *op/op* mice, resulting in the formation of the stop codon TGA in the downstream (Fig. 4). This indicated that *op/op* mutant mice are unable to produce functionally active M-CSF.

Almost simultaneously, Rolf Felix and Herbie Fleisch[12] reported that administration of M-CSF into *op/op* mutant mice strikingly restored the osteopetrotic bone phenotype *in vivo*. The bone marrow cavity of *op/op* mutant mice was completely filled with bone mass without any appreciable osteoclasts, whereas the *op/op* mutant mice given M-CSF strikingly formed bone marrow cavity with appearance of a number of osteoclasts (Fig. 5). Thus, it is clear that M-CSF is essential for formation of bone marrow cavity. Hiroaki Kodama[13] also reported that osteoclast deficiency in *op/op* mice is cured by injections of M-CSF. These results suggested that osteoclast deficiency in the *op/op* mutant mouse is due to the failure of the functionally active M-CSF. The *op/op* mouse is now designated as the $Csf1^{op}/Csf1^{op}$ mouse.

4. Target cells of bone-resorbing hormones and cytokines

The next step in our study on osteoclast biology was to examine the target cells of osteotropic factors like PTH, $1\alpha,25(OH)_2D_3$ and IL-6 in osteoclastogenesis. Using vitamin D receptor (VDR) knockout mice, Shu Takeda[14] clearly showed that co-culture of VDR-deficient osteoblasts and wild type (WT) spleen cells failed to form osteoclasts in response to $1\alpha,25(OH)_2D_3$, whereas VDR-deficient spleen cells differentiated into osteoclasts in response to $1\alpha,25(OH)_2D_3$, when co-cultured with WT osteoblasts. Similarly, B.Y. Liu[15] showed that PTH receptor (PTHR)-deficient

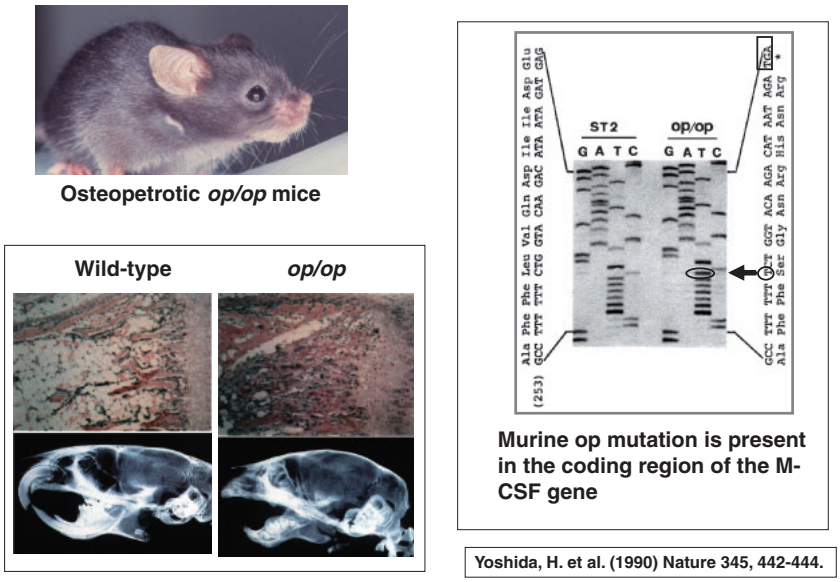


Fig. 4. *op/op* mice have a mutation in the cording region of the M-CSF gene. The radiographic features of *op/op* mice were provided by Dr. Shumpei Niida (National Center for Geriatrics and Gerontology, Aichi, Japan).

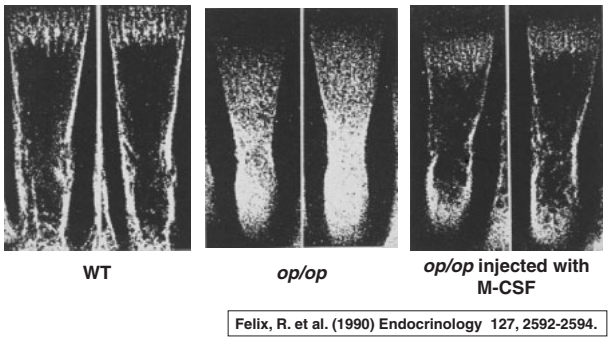
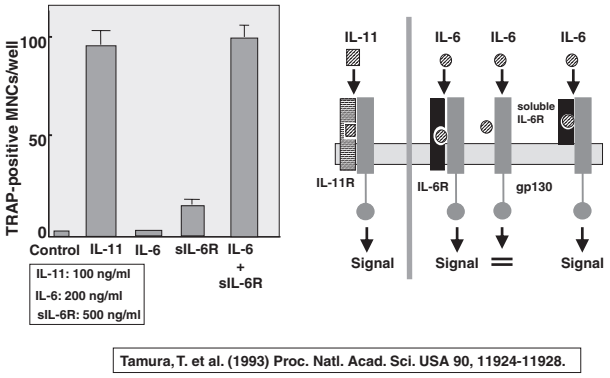


Fig. 5. Administration of M-CSF into *op/op* mice restores osteopetrotic bone phenotype.

spleen cells differentiated into osteoclasts in response to PTH, when co-cultured with WT osteoblasts. These results suggested that the target cells of $1\alpha,25(OH)_2D_3$ and PTH in osteoclastogenesis are osteoblasts, but not osteoclast progenitors.

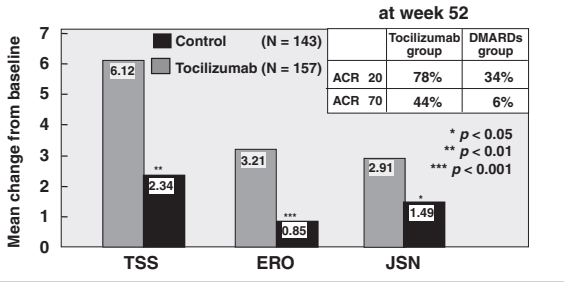
In 1992, Bob Jilka and Stavros Manolagas[16] reported that IL-6 is involved in increased osteoclast development after estrogen loss. Their finding was important, but the role of IL-6 in osteoclastogenesis was rather complicated. IL-6 exerts its biological activity via a cell surface receptor, which consists of two components; membrane-bound IL-6 receptor and its signal transducing 130 Kd glycoprotein (gp130).[17] When IL-6 receptor is occupied by IL-6, the ligand-receptor complex binds gp130, and IL-6 signals are transduced. IL-11 also belongs to the gp130 super-family. Tatsuya Tamura[18] reported that IL-11 formed osteoclasts in co-cultures of WT osteoclasts and bone marrow cells, but IL-6 did not. IL-6 induced osteoclast formation only in the presence of soluble IL-6 receptors, which lack trans-membrane and cytoplasmic domains of the receptors. Soluble IL-6 receptors are found in the serum of healthy humans with a level of 40 to 50 ng/ml. This indicated that the lack of membrane-bound IL-6 receptors in either osteoblasts or bone marrow cells was critical for osteoclast formation.

Using IL-6 receptor overexpressing mice, Nobi Udagawa[19] clearly showed that the presence of the membrane-bound IL-6 receptor in osteoblasts is critical for osteoclastogenesis induced by IL-6. Since WT osteoblasts do not appear to have enough numbers of membrane-bound IL-6 receptors, soluble IL-6 receptor could be substituted for the membrane-bound IL-6 receptors in osteoblasts (Fig. 6). Indeed, soluble IL-6 receptors in the synovial fluids are important for osteoclast formation induced by IL-6. Shigeru Kotake[20] found that the levels of both soluble IL-6 receptors as well as IL-6 in the synovial fluids are much higher in patients with rheumatoid arthritis (RA) than those in osteoarthritis (OA) patients. Multinucleated



Tamura, T. et al. (1993) Proc. Natl. Acad. Sci. USA 90, 11924-11928.

Fig. 6. Effects of mouse IL-6 and soluble IL-6 receptor (sIL-6R) on osteoclast formation in co-cultures of mouse osteoblastic cells and bone marrow cells.



TSS: Mean total Sharp score; ERO: Erosion score; JSN: Joint space narrowing score

Tocilizumab monotherapy shows superiority of blocking IL-6 signals to conventional DMARDs in inhibiting radiographic progression.

Nishimoto, N. et al. (2007) Ann. Rheum. Dis. 66, 1162 - 1167.

Fig. 7. The Samurai trial: Clinical results.

cells found in the synovial tissues of RA patients expressed osteoclast-specific markers such as TRAP, carbonic anhydrase II, vacuolar type proton ATPase, and vitronectin receptors.[20] These results indicated that soluble IL-6 receptors are involved in bone and joint destruction in RA patients.

Based on these experimental findings, Norihiro Nishimoto and Tadamitsu Kishimoto[21] prepared a humanized anti-IL-6 receptor monoclonal antibody (tocilizumab) and evaluated its ability to inhibit progression of structural joint damage in active RA patients. This clinical trial was called SAMURAI trial (Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor). In this clinical trial, 300 active RA patients were randomly allocated to receive either tocilizumab at 8 mg/kg *iv* every 4 weeks or conventional DMARDs including methotorexisate (MTX) for 52 weeks. No anti-TNF reagents were used in either group. At week 52, patients in the tocilizumab group showed significantly less radiographic progression, as measured by changes in TSS (total Sharp score), ERO (erosion score), and JSN (joint space narrowing score) (Fig. 7). The percentages of patients who achieved ACR20 were 78% in the tocilizumab group and 34% in the DMARDs group, and those who achieved ACR70 were 44% in the tocilizumab group and only 6% in the DMARDs group (Fig. 7).[21] The tocilizumab monotherapy was generally tolerated. This is the first trial showing the superiority of blocking IL-6 signals with tocilizumab to conventional DMARDs therapy in inhibiting radiographic progression. The tocilizumab therapy for RA patients has now received permission from the Ministry of Health, Welfare, and Labor, which is expected to be useful for the treatment of bone and joint destruction of RA patients worldwide.

Figure 8 summarizes our working hypothesis on the possible involvement of osteoblasts in osteoclast formation induced by several bone-resorbing factors.[22] It is important that the target cells of bone-resorbing hormones and cytokines are osteoblasts, but not osteoclast progenitors. The bone-resorbing factors were classified into three categories in terms of their signal transduction pathways. $1\alpha,25(OH)_2D_3$ induced osteoclast formation via vitamin D receptor (VDR) present in the nuclei. PTH, PGE_2 and IL-1 induced osteoclast formation via a protein kinase A system. The third group included IL-6, IL-11, LIF and oncostatin M, all of which transduced their signals via gp130. These 3 diverse signals appeared to stimulate osteoclast formation independently, since osteoclasts were present in either VDR-deficient, PTHR-deficient or gp 130-deficient mice. In other words, there is redundancy in bone-resorbing factors to recruit osteoclasts. We proposed that osteoclast differentiation factor (ODF) is commonly induced on the plasma membrane of osteoblasts in response to these bone-resorbing factors.[22] ODF appeared identical to SOFA (stromal osteoclast forming activity) proposed by Tim Chambers.[23] Osteoclast precursors having ODF receptor recognize ODF in osteoblasts through cell-to-cell interaction and differentiate into osteoclasts. M-CSF produced by osteoblasts also appeared to play an important role in the proliferation and differentiation of osteoclast progenitors.

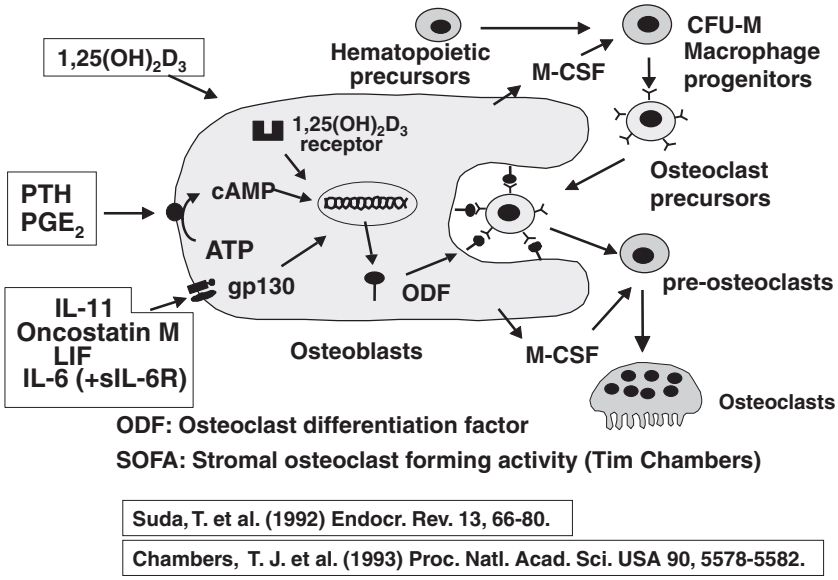


Fig. 8. Roles of osteoblasts in osteoclast formation.

5. Discovery of OPG and OCIF

In the course of searching a new member of TNF receptor super-family, Bill Simonet of Amgen[24] successfully isolated a novel secretory member of the TNF receptor super-family. Almost all of the members belonging to this super-family are trans-membrane proteins that elicit signal transduction in a wide variety of cells. However, the novel member isolated by Simonet lacked any apparent cell-association domain, indicating that it likely acts in the extra-cellular milieu (Fig. 9). This secretory protein contained four cysteine-rich domains and two death domain homologous regions, which mediate apoptotic signals (Fig. 9). Transgenic mice expressing this secretory protein exhibited a generalized increase in bone density like in osteopetrosis associated with a decreased number of osteoclasts.[24] This protein was named osteoprotegerin (OPG) to protect bone.

Eisuke Tsuda of Snow Brand Milk Products[25] independently and simultaneously isolated a novel protein termed osteoclastogenesis inhibitory factor (OCIF) as a heparin-binding glycoprotein from conditioned media of human embryonic lung fibroblasts (IMR-90). The molecular weight of this novel protein was 60 kDa for a monomer and 120 kDa for a homodimer. The cDNA sequence of OCIF was identical to that of OPG.

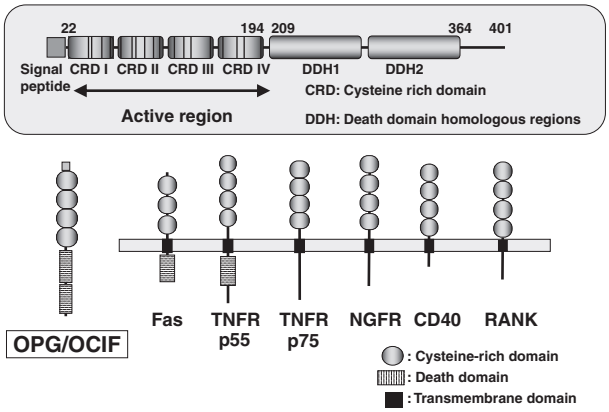


Fig. 9. Structure of OPG/OCIF.

$1\alpha,25(OH)_2D_3$ strikingly increased the specific binding sites for OPG/OCIF in stromal ST2 cells derived from mouse bone marrow.[26] Scatchard analysis indicated the presence of a single class of high affinity binding sites for OPG/OCIF in response to $1\alpha,25(OH)_2D_3$. These observations suggested that a ligand specifically bound to OPG/OCIF is present in ST2 cells. We hypothesized that ODF could be a common ligand for both ODF receptor and OPG/OCIF. ODF receptor must be located on the plasma membrane of osteoclast progenitors, whereas OPG/OCIF should be a secretory protein of the TNF receptor super-

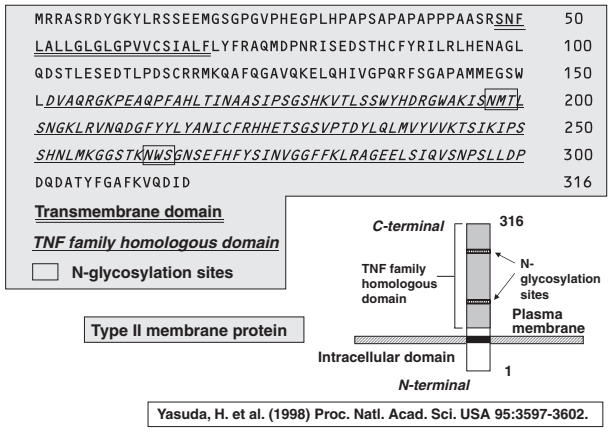


Fig. 10. Isolation of ODF as an OCIF-binding protein.

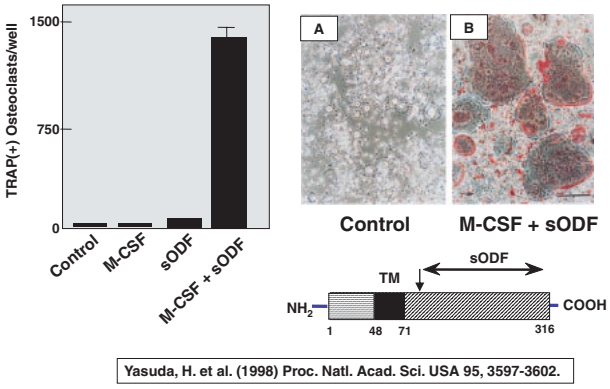


Fig. 11. Soluble ODF together with M-CSF induces osteoclasts in spleen cell cultures even in the absence of osteoblasts.

family. In other words, OPG/OCIF could act as a decoy receptor for ODF to inhibit osteoclast formation.

6. Molecular cloning of ODF

By screening a cDNA expression library of ST2 cells treated with $1\alpha,25(OH)_2D_3$ and dexamethasone, Hisataka Yasuda and Kanji Higashio[27] finally cloned a single positive clone of ODF, which contained a 1.65 Kb insert with an open reading frame encoding 316 amino acid residues (Fig. 10). Hydropathy analysis showed that ODF had no signal sequence, but had an internal hydrophobic domain consisting of 24 amino acid residues, which presumably represents a trans-membrane domain. A homology search of the Gen Bank sequence database revealed that extra-cellular C-terminal 165 amino acid residues had a significant homology to the TNF ligand super-family. This structure was typical of a type II trans-membrane protein: its C-terminal sequence exposed outside the cell and the N-terminal sequence intra-cellularly located (Fig. 10).

To examine whether ODF is indeed a long-sought factor for osteoclastogenesis, Hisataka Yasuda[27] produced a soluble form of ODF [$ODF^{76\text{-}316}$], and examined its effects on osteoclast formation in the absence of osteoblasts. When mouse spleen cells were cultured with M-CSF and soluble ODF in the absence of osteoblasts, numerous TRAP-positive osteoclasts were formed (Fig. 11). Adding M-CSF alone or soluble ODF alone failed to form osteoclasts. Like in co-cultures with osteoblasts, many TRAP-positive multinucleated cells were formed in pure spleen cell cultures in response to soluble ODF and M-CSF. This indicates that the combination of M-CSF and ODF can be substituted for osteoblasts in recruiting osteoclasts.

Our paper on the molecular cloning of ODF appeared in the 1998 March issue of the PNAS, USA,[27] and it was ranked as the 32nd of the Red Hot Papers in that year, which were selected and ranked in order by the citation numbers within the same year of publication. David Lacy of Amgen[28] reported the molecular cloning of OPG ligand (OPGL) in the April issue of the Cell. ODF and OPGL were the same molecule, which were also identical to TNF-related activation-induced cytokine (TRANCE) reported by Yangwon Choi of Rockefeller University[29] and receptor activator of NF-kB ligand (RANKL) reported by DM Anderson of Immunex (Fig. 12).[30] The ad hoc committee of the ASBMR chaired by Larry Riggs recommended the use of RANK, OPG and RANKL as the receptor, the decoy receptor and the ligand, respectively.[31]

Figure 13 summarizes the molecular mechanisms of osteoclast differentiation and activation. All bone-resorbing factors like $1\alpha,25(OH)_2D_3$, PTH and IL-11 act on osteoblasts to induce the membrane-associated factor called RANKL, which recognizes RANK present in osteoclast progenitors and osteoclasts by a mechanism involving cell-to-cell interaction. M-CSF is also an essential factor for osteoclast proliferation and differentiation, which is produced by osteoblasts in bone tissue.

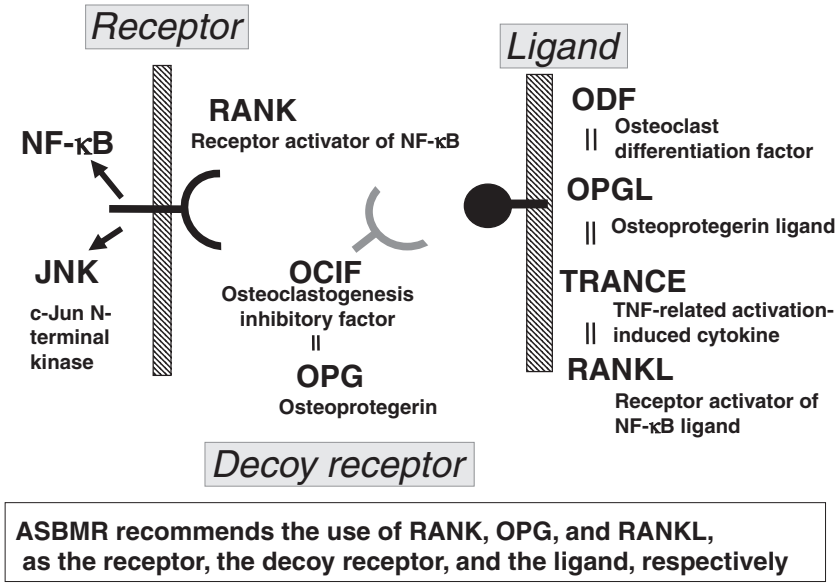


Fig. 12. RANKL-RANK interaction in osteoclastogenesis.

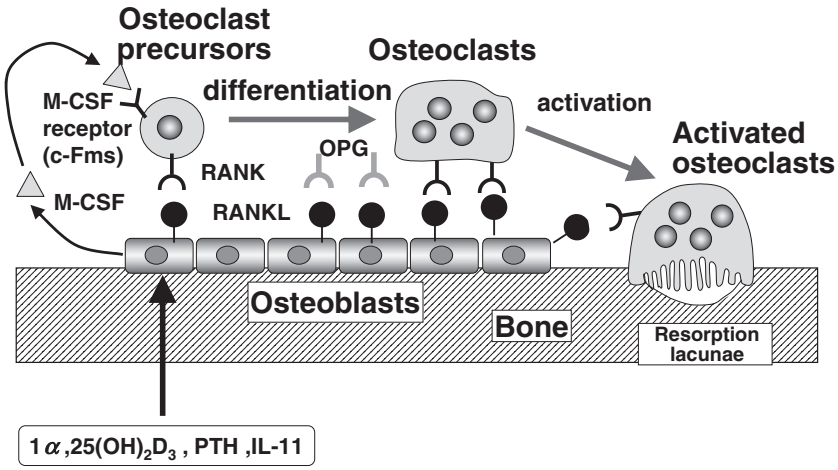


Fig. 13. Molecular mechanism of osteoclast differentiation and activation.

Osteoclast precursors differentiate into osteoclasts, then activated, both mechanisms are controlled by the same molecule RANKL. When OPG covers RANKL, osteoclast precursors and osteoclasts are unable to bind RANKL, thus OPG acts as a decoy receptor in the RANKL/RANK interaction.

The molecular mechanism of osteoclast formation and activation proposed by *in vitro* studies has now been confirmed by a number of *in vivo* studies using engineered gene-mutated transgenic and knockout mice of the RANKL-RANK-OPG system. Table 1 summarizes that OPG knockout mice induced osteoporotic phenotype with many osteoclasts.[32] Soluble RANKL (sRANKL) transgenic mice as well as administration of recombinant sRANKL also exhibited osteoporosis.[33] In contrast, OPG transgenic mice as well as administration of OPG induced osteopetrosis with no osteoclasts. Knockout mice of RANK or RANKL induced osteopetrosis as well. These results indicate that the proposed mechanism of osteoclast differentiation and activation occurs *in vivo* as well.

Table 1. Bone resorption and skeletal phenotype of gene-mutated mice of RANKL-RANK signals

	Bone resorption	Phenotype
OPG KO[32), 52)] sRANKL administration[53)] sRANKL TG[33)]	Up ↑	Osteoporosis
OPG TG/OPG administration[24)] RANKL KO[54)] RANK KO[55)] sRANK administration[56)] TRAF6 KO[36), 37)] c-src KO[57)] c-fos KO[58), 59)] NF-κB (p50/p52) KO[60), 61)] NFATc1 KO[42), 44)]	Down ↓	Osteopetrosis

7. DC-STAMP is involved in cell-to-cell fusion in osteoclastogenesis

Osteoclasts are multinucleated cells. Very recently, two Japanese research groups discovered DC-STAMP (a dendritic cell specific trans-membrane protein) as an essential factor involved in cell fusion of mononuclear pre-osteoclasts. Using the osteoclastogenic RAW cell line, Toshio Kukita of Kyushu University[34)] identified an interesting protein with a seven trans-membrane domain, called DC-STAMP. Mitsuru Yagi and Toshio Suda of Keio University[35)] generated DC-STAMP knockout mice. Figure 14 shows TRAP staining of tibia of 8-week-old wild type (WT) and DC-STAMP-deficient mice. To our surprise, no multinucleated osteoclasts were detected in DC-STAMP-deficient mice, though many TRAP-positive mononuclear cells were found. Osteoclast progenitors from DC-STAMP-deficient mice differentiated into TRAP-positive mononuclear cells but did not form multinucleated cells *in vitro*.[35)] These results confirm that DC-STAMP is essential for inducing cell-to-cell fusion of mononuclear pre-osteoclasts.

8. Involvement of TRAF6 in RANKL-induced osteoclast formation

The next mile-stone in the research on osteoclast biology was the discovery of involvement of TRAF6 (TNF receptor-associated factor 6) in RANKL-induced osteoclast formation. Asuka Naito and Jun-ichiro Inoue of the University of Tokyo,[36)] and Mark Lomaga of Amgen at the University of Toronto[37)] contributed a lot to our understanding of the role of TRAF6 in osteoclastogenesis. TRAF6 is involved in signaling pathways of not only RANK, but also CD40, IL1 receptor (IL-1R), and toll-like receptor (TLR4), the receptor for LPS (Fig. 15). TRAF6 signals activate both NF-κB and AP1. Mark Lomaga in Toronto and Asuka Naito in Tokyo independently generated TRAF6-deficient mice, which showed severe osteopetrotic phenotype with defects in tooth eruption due to impaired bone resorption.[36),37)] Histological features of TRAF6-deficient mice produced by the Tokyo group showed severe osteopetrosis with fewer numbers of osteoclasts (Fig. 15). These results suggest that TRAF6 plays an important role not only in osteoclast function but also in osteoclast formation.

To understand the molecular mechanism of TRAF6-mediated osteoclastogenesis, Jin Gohda[38)] examined RANKL-induced osteoclast formation in splenocyte cultures prepared from TRAF6-deficient mice. Osteoclasts were formed in wild type splenocyte cultures, but no osteoclasts were formed in TRAF6-deficient splenocyte cultures. Transfection of TRAF6 into splenocytes induced osteoclast formation, but TRAF2 transfection did not.[38)] These results suggest that the RANK-TRAF6 pathway leads to osteoclast differentiation and function.

9. Discovery of NFATc1 (also called NFAT2) as an essential transcription factor in RANKL-induced osteoclastogenesis

The next breakthrough in osteoclast research was the identification of NFATc1, also called NFAT2, a transcription factor, essentially involved in RANKL-induced osteoclastogenesis. NFAT was originally identified as a transcription factor responsible for inducing IL-2 after T-cell activation. Japanese scientists including Tatsuo Takeya and Hiro Takayanagi played major roles on this matter as well.

Norihiro Ishida of Takeya's group[39)] selected 635 genes that showed marked changes in expression after RANKL stimulation in RAW 264 cells. They were classified into 6 groups: up-regulated genes and down-regulated genes at early, middle, and late stages after RANKL stimulation (Fig. 16). Norihiro Ishida focused on a group of up-regulated early genes, and identified NFAT2 as an important

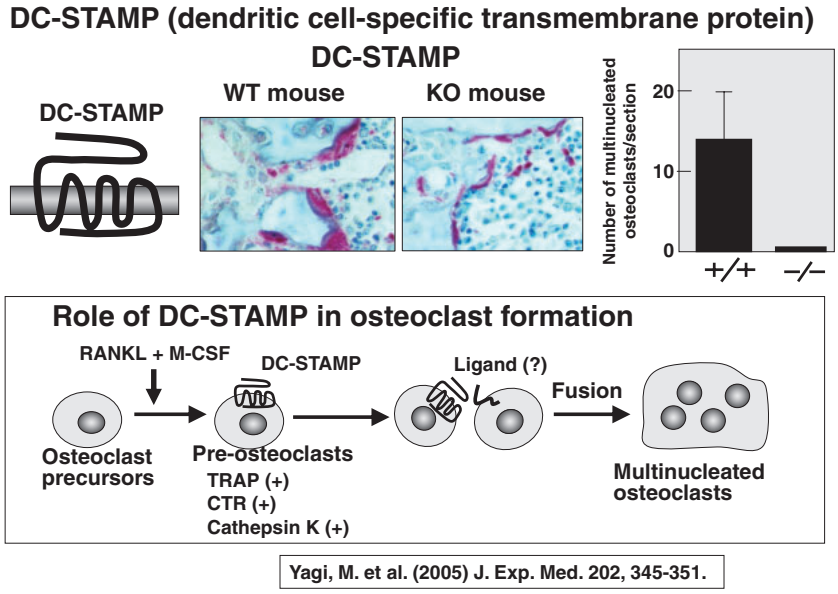


Fig. 14. DC-STAMP is involved in fusion of pre-osteoclasts.

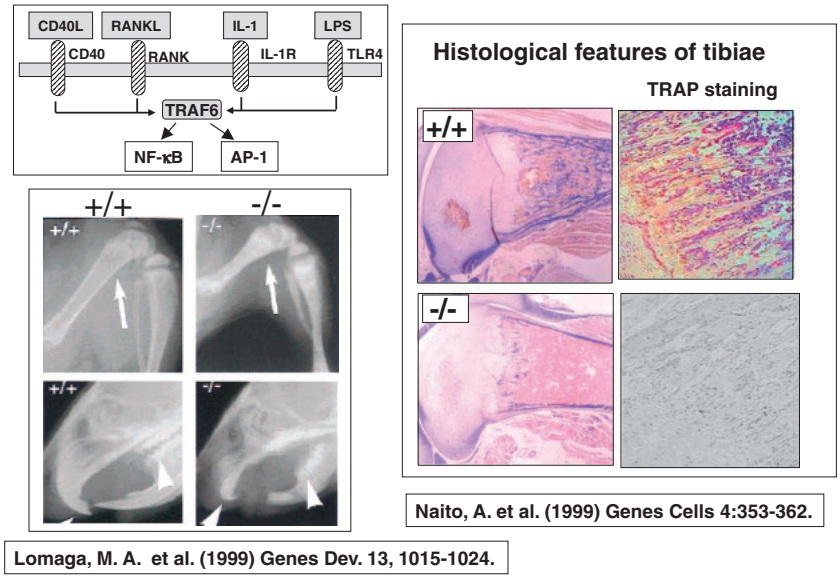


Fig. 15. Severe osteopetrosis in TRAF6-deficient mice.

transcription factor for osteoclast differentiation. Down-regulation by siRNA of NFAT2 mRNA in RAW264 cells (clone KD1 and KD14) suppressed the differentiation of RAW cells into osteoclasts (Fig. 16).[40] These results suggest that NFAT2 plays a key role in osteoclastogenesis.

Hiro Takayanagi[41] independently identified NFATc1 as an essential transcription factor for osteoclastogenesis. NFATc1 was the same molecule as NFAT2, which Takeya identified as an early upregulated gene after RANKL stimulation.[39] Hiro Takayanagi investigated the role of NFATc1 in osteoclastogenesis, using NFATc1-deficient ES cells. Heterogeneously NFATc1-deficient ES cells underwent differentiation into TRAP-positive osteoclasts, but ES cells totally lacking NFATc1 was

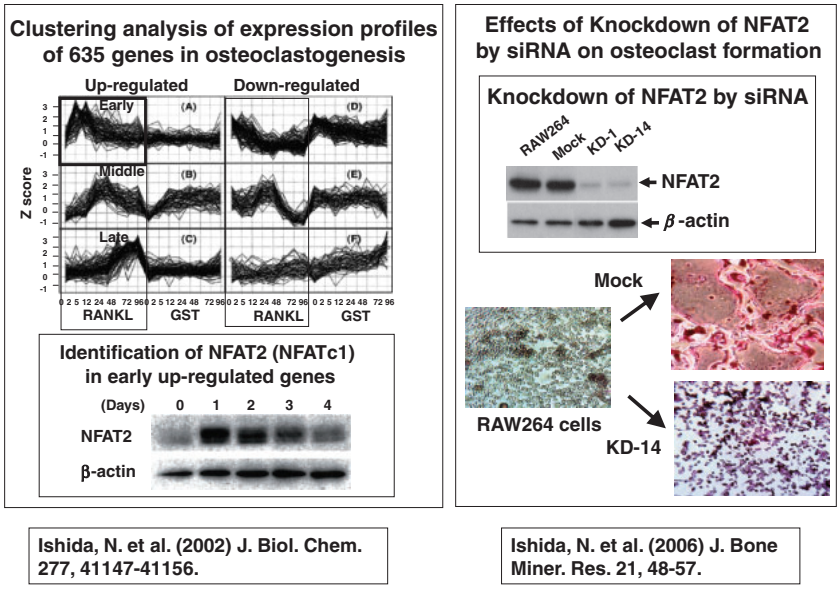


Fig. 16. Identification of NFAT2 in early up-regulated genes during oteoclastogenesis.
(The upper right panel adapted from J. Bone Miner. Res. 2006; 21:48–57 with permission of the American Society for Born and Mineral Research.)

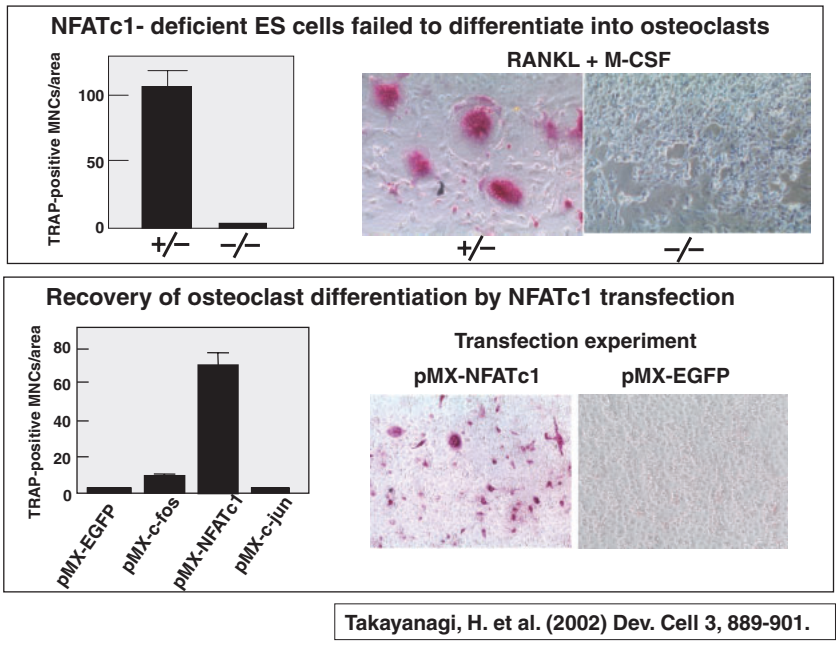


Fig. 17. NFATc1 is essential for differentiation of ES cells into osteoclasts.

defective in osteoclast formation (Fig. 17). Osteoclast differentiation of NFATc1-deficient ES cells was recovered specifically by NFATc1 transfection (Fig. 17). Neither c-Fos nor c-Jun transfection failed to generate osteoclast differentiation of NFATc1-deficient ES cells. These results suggest that NFATc1 (NFAT2) plays a key role in osteoclastogenesis. However, *in vivo* analysis of the role

c-Fos target genes	Fold increase in osteoclastogenic culture	
	Normal	c-Fos-deficient
Acp5 (TRAP)	3836	462
Ctsk (cathepsin K)	2862	61
Car2 (cabonic anhydrase 2)	971	14
Mmp9 (matrix metalloproteinase 9)	620	80
Nfatc1	752	absent

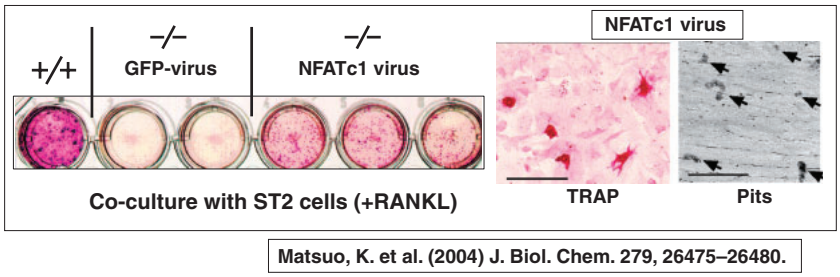


Fig. 18. NFATc1 is down-regulated in c-Fos-deficient osteoclast precursors.

of NFATc1 was hampered at that time, because NFATc1-deficient embryos died of defects in heart valve development. Asagiri *et al.*[42] provided genetic evidence for the first time that NFATc1 is essential for osteoclast differentiation *in vivo* using adoptive transfer of NFATc1-deficient hematopoietic stem cells to c-Fos-deficient mice, which lack osteoclast differentiation, originally reported by Agi Grigoriadis in 1994.[43] When hematopoietic stem cells obtained from WT mice were injected into c-Fos-deficient newborn mice, osteopetrosis was markedly rescued. In contrast, when NFATc1-deficient hematopoietic stem cells were transferred to c-Fos-deficient mice, they still exhibited severe osteopetrosis, and bone marrow cavities remained occupied with unresorbed bone.[42] Winslow *et al.*[44] generated viable NFATc1-deficient mice by expression of an NFATc1 transgene under the control of the endothelial-specific Ti-2 promoter in NFATc1-deficient embryos (Tie-2-NFATc1$^+$ embryos). Tie-2-NFATc1$^+$ mice appeared normal at birth, but gained much weight slower than their littermates and died before adulthood. These mice showed greatly reduced osteoclastogenesis as revealed by the failure of tooth eruption, osteopetrosis, and very few small TRAP-positive cells.[44] These results confirm that NFATc1 is indispensable for osteoclast formation *in vivo* as well.

10. Relationship between NFATc1 (NFAT2) with other transcription factors in RANKL-induced osteoclastogenesis

Investigation of the relationship between NFATc1 and other transcription factors like c-Fos and c-Jun was another important issue in osteoclast biology. AP-1 refers to a family of dimeric transcription factors composed of Fos and Jun. Koichi Matsuo and Toshi Yoneda also contributed a lot to our understanding of the relationship between NFATc1 and other transcription factors.

Koichi Matsuo of Keio University was interested in the osteopetrotic phenotype of c-Fos-knockout mice, which lack osteoclast differentiation. Using microarray analysis, Koichi[45] clearly showed that NFATc1 was down-regulated in c-Fos-deficient osteoclast precursors, like other osteoclast-specific genes encoding TRAP, cathepsin K, carbonic anhydrase 2, and MMP9 (Fig. 18). Moreover, TRAP staining as well as pit-forming activity of c-Fos-deficient splenocytes was rescued by transfection of NFATc1 virus, but not by GFP virus in the presence of RANKL (Fig. 18). These results clearly indicate that the lack of NFATc1 expression in c-Fos-deficient osteoclast precursors is the cause of the block of osteoclast differentiation.

To investigate the role of c-Jun in osteoclastogenesis, Fumiyo Ikeda of Toshi Yoneda's laboratory

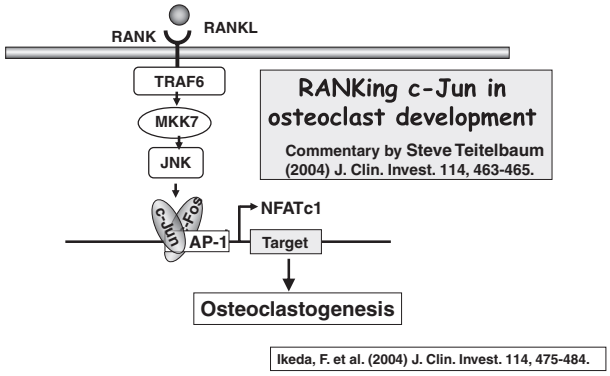


Fig. 19. Activation of c-Jun is essential for RANKL-induced osteoclastogenesis.

at Osaka University[46] generated transgenic mice expressing dominant negative c-Jun in the osteoclast lineage cells. They clearly showed that the transgenic mice of dominant negative c-Jun exhibited severe osteopetrosis due to impaired osteoclastogenesis.[46] The tooth eruption was missing in the transgenic mice, despite of the formation of tooth germs. The transgenic mice also showed increased radiodensity of long bones. TRAP staining of long bones showed the absolute lack of TRAP-positive osteoclasts in dominant negative c-Jun transgenic mice.

Taken together, these results indicate that the RANKL-RANK signals are transported into TRAF6, MKK7, JNK, AP1 and NFATc1, in that order. Steve Teitelbaum[47] has named this signalling cascade "RANKing c-Jun in osteoclast development" in the commentary in the same issue of the J. Clin. Invest. (Fig. 19).

11. Establishment of a new research field: Osteoimmunology

One of the most exciting findings in osteoclast biology for the past few years would be the establishment of a new research field called "Osteoimmunology." The crosstalk between the immune and bone systems has long been appreciated, but only recent work on bone destruction associated with inflammation as well as bone phenotype found in knockout mice of immune-related molecules has attracted attention to the interdisciplinary field called "Osteoimmunology".[48]

Takako Koga of Takayanagi's laboratory[49] found that mice lacking immune receptor tyrosine-based activation motif (ITAM)-harbouring adaptors, Fc receptor common gamma (FcR gamma) subunit and DNAX-activating protein 12 (DAP12) exhibit severe osteopetrosis owing to impaired osteoclast differentiation. Double knockout mice of FcR gamma and DAP12 developed much severer osteopetrosis (Fig. 20). This indicates that, in addition to RANKL- and M-CSF-induced signals, ITAM signals are also essential for osteoclast formation. It is known that FcR gamma and DAP12 are associated with multiple immunoreceptors and activate calcium signaling through phospholipase C gamma. Takako Koga[49] proved that FcR gamma and DAP12 activate calcium signaling through PLC gamma in osteoclast progenitors as well.

Figure 21 schematically describes an up-to-date model of signal transduction pathways in osteoclastogenesis.[50] There are three major signal transduction pathways between osteoblasts and osteoclast progenitors. The first one is the M-CSF signal, which is transduced through tyrosine kinase. The second one is the most important RANKL signal, which is transduced through RANK and TRAF6 in osteoclast progenitors. The induction of NFATc1 is dependent on NF-kB and AP1. The third essential signal is transduced through immunoglobulin-like receptors such as osteoclast-associated receptor (OSCAR) and triggering receptor 2 expressed on myeloid cells (TREM2) with ITAM-harboring adaptors, DAP12, and FcR gamma. The ITAM signal acts as a co-stimulatory signal for RANKL, and activates calcium signaling in osteoclast precursors. Recent studies have shown that the CAMK/CREB pathways play important roles in osteoclast differentiation and function.[51]

Summary and Acknowledgments

Finally, we would like to summarize the major contributions to osteoclast biology from Japan by thanking all of the Japanese scientists who allowed us to introduce their excellent scientific achievements.

1. Establishment of the mouse co-culture system to examine osteoclast differentiation brought about a new era to modern osteoclast biology research. This system was devised by Takuhiko Akatsu and Nobi Udagawa and ourselves (Showa University), based on the pioneering work by Gideon Rodan (Merck

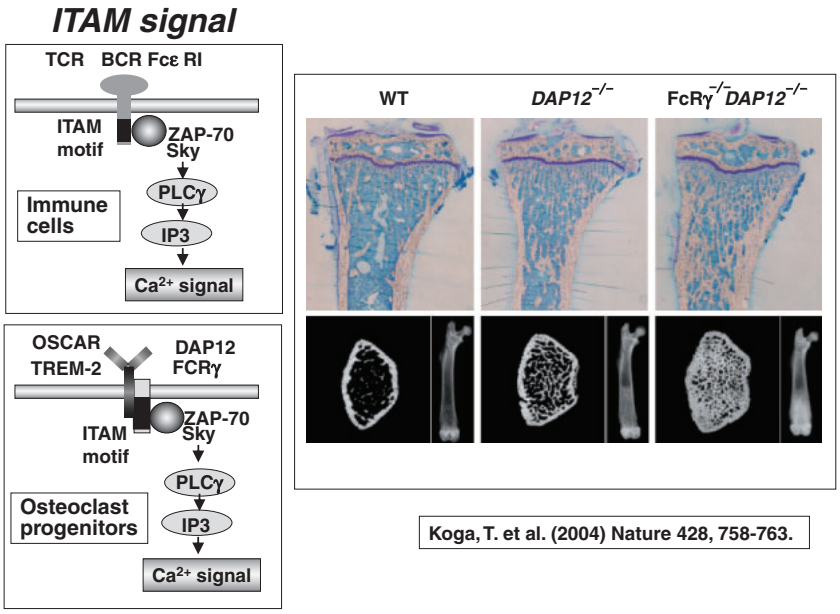


Fig. 20. Severe osteopetrosis in mice lacking both DAP12 and FcRγ.

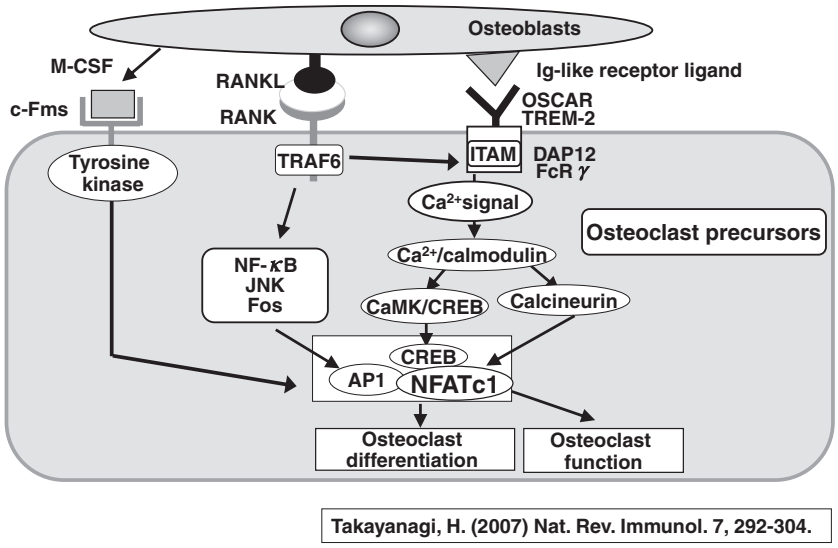


Fig. 21. Signaling pathways required for osteoclast differentiation.

Research Laboratories, West Point, PA.) and Jack Martin (St. Vincent's Institute of Medical Research, Melbourne), and also by Tim Chambers (St. George's Hospital Medical School, London). We never forget the great contributions of Eijiro Jimi (Showa University), who proved the essential roles of osteoblasts in activating osteoclast function as well.

2. The next big contribution from Japan was the establishment of requirement of M-CSF in osteoclast differentiation. Hisa Yoshida, Shin-ichi Hayashi (Kumamoto University), Hiro Kodama (Ohu University), and Sakae Tanaka (University of Tokyo) established the role of M-CSF in osteoclastogenesis. Nobi Udagawa also showed that even mature macrophages can differentiate into osteoclasts. Their contributions confirmed that the origin

of osteoclasts is indeed haemopoietic cells of the monocyte-macrophage lineage.

3. The next step in the understanding of the mechanism of osteoclast formation was the proposal of osteoclast differentiation factor "ODF" by Jack Martin and ourselves (Showa University). Tim Chambers also proposed the presence of stromal osteoclast forming activity "SOFA" in osteoblasts. We believe that the proposal of ODF and SOFA made a driving force to clone the RANK ligand.
4. The discovery of the identification of osteoblasts as the target cells of vitamin D is due to the excellent work by Shu Takeda, Toshio Matsumoto and Shige-aki Kato (University of Tokyo).
5. Isolation of OCIF and molecular cloning of ODF by Snow Brand Milk Products chaired by Kanji Higashio was one of the highlights in modern osteoclast biology. His excellent research group consists of Eisuke Tsuda, Hisataka Yasuda, Nobuyuki Shima, and many other brilliant collaborators.
6. The pioneering work of Bob Jilka and Stavros Manolagas (University of Arkansas for Medical Science) that IL-6 is involved in osteoclastic bone resorption was further extended by Tatsuya Tamura and Shigeru Kotake (Showa University). Based on these basic findings, Norihiro Nishimoto and Tadamitsu Kishimoto (Osaka University) prepared a humanized anti-IL-6 receptor monoclonal antibody called "Tocilizumab" and evaluated its ability to inhibit progression of structural joint damage in active RA patients. This clinical study called the SAMURAI trial has recently established a new therapeutic strategy for RA patients.
7. Two research groups have successfully identified DC-STAMP (dendritic cell specific transmembrane protein) as a key molecule for cell fusion of mononuclear pre-osteoclasts. Toshio Kukita, Akiko Kukita, and Naohisa Wada (Kyushu University), and Mitsuru Yagi, Takeshi Miyamoto and Toshio Suda (Keio University) contributed to this exciting discovery.
8. The discovery of the importance of TRAP6 signals for osteoclastogenesis by Jun-ichiro Inoue, Asuka Naito, and Jin Gohda (Institute for Medical Sciences, University of Tokyo) contributed a lot to establish the RANK-mediated signaling pathway.
9. NFATc1 (also called NFAT2), a key transcription factor for osteoclastogenesis, was independently discovered by two research groups in Japan. Tatsuo Takeya, Norihiro Ishida (Nara Institute of Science and Technology), and Hiro Takayanagi, and Masataka Asagiri (Tokyo Medical and Dental University) were involved in the establishment of this key transcription factor for osteoclastogenesis. Koichi Matsuo (Keio University) discovered a close relation between c-Fos and NFATc1 in osteoclastogenesis.
10. Discovery of the ITAM motif-mediated costimulatory signals in the RANKL-induced osteoclastogenesis by Takako Koga, Kajiro Sato, and Hiro Takayanagi (Tokyo Medical and Dental University) was another breakthrough in understanding the signal transduction pathways in osteoclastogenesis. This discovery brought about the establishment of a new research field called "Osteoimmunology", which was originally proposed by Yangwon Choi and Masamichi Takami (Rockefeller University).
11. Toshi Yoneda and his colleagues (Osaka University) also greatly contributed to osteoclast biology. Fumiyo Ikeda, Riko Nishimura of Toshi's laboratory (Osaka University) established essential roles of c-Jun signals in RANKL-induced osteoclastogenesis.

We would like to congratulate all of these great contributions to osteoclast biology by Japanese scientists. We also deeply appreciate their encouragement and friendship to allow us to include their exciting work in this review article.

Abbreviations

1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3
DMARDs, disease-modifying anti-rheumatic drugs
ERO, erosion score
FcRγ, Fc receptor common γ
CRD, cysteine-rich domain
CTR, calcitonin receptor
DC-STAMP, dendritic cell specific trans-membrane protein
DDH, death domain homologous region

DAP12, DNAX-activating protein 12
gp130, 130 kDa glycoprotein
OAF, osteoclast activating factor
OCIF, osteoclastogenesis inhibitory factor
ODF, osteoclast differentiation factor
OPG, osteoprotegerin
OPGL, OPG ligand
OSCAR, osteoclast-associated receptor
IL-6, interleukin 6
IL-6R, IL-6 receptor
sIL-6R, soluble IL-6 receptor
IL-11, interleukin 11
IL-11R, IL-11 receptor
ITAM, immune receptor tyrosine-based activation motif
JSN, joint space narrowing score
M-CSF, macrophage colony stimulating factor
MNC, multinucleated cell
MMP9, matrix metalloproteinase 9
MKK7, mitogen-activated protein kinase kinase 7
MTX, methotorexisate
NFAT, nuclear factor for activating T-cells
OSCAR, osteoclast-associated receptor
RA, rheumatoid arthritis
SAMURAI, Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor
RANKL, receptor activator of NF-κB ligand
sRANKL, soluble RANKL
SOFA, stromal osteoclast forming activity
TLR4, toll-like receptor 4
TNF, tumor necrosis factor
TNFR, TNF receptor
TRAF6, TNF receptor-associated factor 6
TRANCE, TNF-related activation-induced cytokine
TRAP, tartrate-resistant acid phosphatase
TREM, triggering receptor expressed on myeloid cells
TSS, total sharp score
v-ATPase, vacuolar type proton ATPase
VDR, vitamin D receptor

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Profile

Tatsuo Suda was born in Tokyo, Japan, in 1935. After graduated from the School of Dentistry, Tokyo Medical and Dental University in 1960, he started his research career on bone biology as a graduate student of the alma mater, inspired by the monograph entiteled "The Chemical Dynamics of Bone Mineral" written by William F. and Margaret W. Neuman. He was a postdoctoral fellow from 1968 to 1971 at the University of Wisconsin, where he was involved in the isolation and identification of vitamin D metabolites including 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 under the guidance of Professor Hector. F. DeLuca. After he came back to Tokyo, he developed 1α-hydroxyvitamin D_3 [1$\alpha$$(OH)D_3$] as a synthetic analog of 1α,25$(OH)_2D_3$ for the treatment of renal osteodystrophy and osteoporosis in collaboration with Yasuho Nishii of Chugai. In Japan, over one million patients with osteoporosis have been taking 1$\alpha$$(OH)D_3$ every day since 1983. In collaraboration with Naoyuki Takahashi and many other colleagues at Showa University, he proposed a factor responsible for osteoclast differentiation called osteoclast differentiation factor (ODF) induced by 1α,25$(OH)_2D_3$ in 1992. ODF was finally molecularly cloned in 1998 in collaboration with Hisataka Yasuda and Kanji Higashio of Snow Brand Milk Products. He received numerous awards and recognition for his basic research including the William F. Neuman Award from the American Society for Bone and Mineral Research (ASBMR) (1997), the Purple Ribbon Medal (1998), the Asahi Prize (2000), and the Japan Academy Prize (2001). He was elected as a member of the Japan Academy in 2007.

Profile

Naoyuki Takahashi was born in Kanagawa, Japan, in 1952. After finished the master course of the Graduate School of Iwate University (specialized in Agricultural Chemistry), he joined the Department of Biochemistry (Professor: Tatsuo Suda) of Showa University Dental School as a research associate in 1978. In 1984, he took Ph.D. degree from Tokyo Medical and Dental University for the study on the role of vitamin D in bone metabolism in Japanese quail under the guidance of Professor Suda. Nao joined the bone research group in the University of Texas Health Science Center at San Antonio in 1985 as a postdoctoral fellow. He started his life work on osteoclast biology there under the guidance of Professor G. R. Mundy and G. D. Roodman. After Nao returned to Showa University, he organized his research group on osteoclast biology in 1987. In 1988, he established a co-culture system of osteoblasts and hematopoietic cells to study osteoclast formation, and proposed an important concept that osteoblasts express osteoclast differentiation factor (ODF) in response to several bone resorbing factors. The concept was finally proved by the molecular cloning of ODF (known as RANKL) in collaboration with Drs. Hisataka Yasuda and Kanji Higashio of Snow Brand Milk Products. Nao moved to Matsumoto Dental University Institute for Oral Science as Professor of Hard Tissue Research Division in 2001. Since then, he is a leader of the hard tissue research group in Matsumoto Dental University.