TACE-Mediated Shedding Supports a Role for Semaphorin 4D Expressed on Osteoclasts in Up-Regulation of RANKL-Induced Osteoclastogenesis

TACE-mediated shedding supports a role for Semaphorin 4D expressed on osteoclasts in the upregulation of RANKL-induced osteoclastogenesis

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Jae Young Kim, DDS

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Research Mentor:

Toshihisa Kawai, DDS, PhD

The Forsyth Institute

Harvard School of Dental Medicine

Boston, Massachusetts

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Abbreviations

Sema4D: Semaphorin 4D

TACE: TNF alpha convertase enzyme

MT1-MMP: membrane type 1-matrix metalloproteinase

mAb: Monoclonal antibody

RANK: Receptor activator of NF kappa B

RANKL: RANK ligand

MCSF: Macrophage colony-stimulating factor

IL: Interleukin

β-GP: beta glycerophosphate

ALP: Alkaline phosphatase

Abstract:

Background: This proposal targets a recently identified bone anabolic mechanism, Semaphorin 4D, an anti-coupling factor, which is elicited by bone cell-cell communication. During bone remodeling, osteoblast-activating molecules are released from demineralized bone matrix to replace the same amount of bone resorbed by osteoclasts. According to Howard et al., anabolic molecules released by activated osteoclasts are termed "coupling factors". Important to this study, osteoclasts were reported to produce a particular anti-coupling factor known as Semaphorin 4D (Sema 4D), which is purported to suppress osteoblastogenesis until bone resorption is accomplished. However, the possible effects of Sema4D on RANKL-mediated osteoclastogenesis are still unknown. According to Li Zhu et al., the extracellular domain of Sema4D is shed from the platelet surface by the metalloprotease TACE (TNFα-converting enzyme), also known as ADAM17. However, the expression and function of TACE on osteoclasts is virtually unknown. Since TACE mediates the shedding of Sema4D from the platelet surface, we reasoned that TACE expressed on osteoclasts could shed the membrane-bound form of Sema4D (mSema4D) to its soluble form, sSema4D, which, in turn, would further promote RANKL-induced osteoclastogenesis, while, at the same time, suppressing osteoblastogenesis. To test this theory, this study aimed to examine the possible impact of Sema4D on osteoclastogenesis using a novel anti-Sema4D monoclonal antibody (anti-Sema4D-mAb), along with assessing the expression and function of TACE on osteoclasts using TACE siRNA and anti-TACE-mAb.

Materials and Methods: The expression of Sema4D on osteoclast cells and its effects were examined *in vitro* by Western blot analysis and TRAP staining/Pit formation assay, respectively.

ALP assay was also performed to examine the effects of Sema4D on osteoblastogenesis by culturing osteoblast cells in a culture of osteoclast supernatant. TACE and MT1-MMP expressed on osteoclast cells were detected via Western blot assay, RT-PCR, immunostaining, and fluorescence microscopy. Differentiation of osteoclast cells was evaluated through TRAP staining and pit formation assay under RNA-mediated silencing of TACE and MT1-MMP in a loss-of-function study confirmed by Western blot and RT-PCR. Osteoblast cells were cultured with and without TACE siRNA- and MT1-MMP siRNA-treated osteoclast culture medium to examine the function of TACE and MT1-MMP on osteoblastogenesis through ALP assay. As proof-of-concept, the expression of mSema4D and sSema4D was detected by Western blot, PCR, immunostaining and fluorescence microscopy after treatment with anti-TACE-mAb and anti-MT1-MMP-mAb, followed by TRAP/pit formation assays and ALP assay on osteoclastogenesis and osteoblastogenesis, respectively.

Results: Sema4D was expressed on osteoclasts in both membrane and soluble forms. The level of osteoclastogenesis was significantly suppressed in the group with anti-Sema4D-mAb compared to nontreated control, while the recombinant Sema4D (rSema4D) group showed significantly higher expression of osteoclastogenesis. Mature osteoclasts with more than 10 nuclei per cell had greater pit formation activity ($p<0.01$). Furthermore, rSema4D and culture supernatant from RANKL-stimulated osteoclasts suppressed *in vitro* osteoblastogenesis, as determined by ALP assay. TACE and MT1-MMP were also expressed on osteoclast cells. When they lost their function by siRNA, osteoclastogenesis was inhibited, while osteoblastogenesis was enhanced. Similarly, anti-TACE-mAb and anti-MT1-MMP-mAb suppressed osteoclastogenesis and enhanced osteoblastogenesis, as determined by TRAP/pit formation and ALP assay, respectively.

Conclusion: Sema4D can elicit an anti-coupling effect on bone remodeling processes in two ways. Sema4D initially expressed on the surface of preosteoclasts can serve as a ligand to inhibit osteoblastogenesis via preosteoclast-preosteoblast interactions (Negishi-Koga et al. 2011). Then, after shedding from the preosteoclast surface, Sema4D can serve as a bioactive soluble molecule capable of interacting with receptor CD72 expressed on osteoclast precursor cells, as well as remote cells. To facilitate this, TACE and MT1-MMP play a crucial role in shedding the extracellular domain of Sema4D as an autocrine agent. Furthermore, among the known Sema4D receptors, CD72, but not Plexin B1/B2, expressed on the cell surface of osteoclasts functions as the receptor for sSema4D. These results suggest that TACE and MT1-MMP expressed on osteoclast cells shed mSema4D to sSema4D, which, in turn, further promotes RANKL-induced osteoclastogenesis and suppresses osteoblastogenesis. Plexin B2 functions as a receptor for Sema4D on osteoblasts, whereas Sema4D binds to its receptor CD72 expressed on osteoclasts to promote RANKL-mediated osteoclastogenesis.

Introduction

Throughout life, bone remodeling occurs within a temporary anatomic structure known as the "bone multicellular unit (BMU)" [1,2]. The BMU consists of a group of osteoclasts in the front, forming the cutting cone and a group of osteoblasts in the rear, forming the closing cone. The process of bone remodeling is divided into initiation, transition and formation. In the initiation phase, mechanical loading and microdamage are sensed by osteocytes, which stimulate the recruitment of osteoclast precursor cells. Then, in the transition phase, classical coupling factors stimulate migration of osteoprogenitor cells to the resorbed site and promote differentiation into osteoblast cells. Each of these complex processes requires tight control through some signaling.

During bone remodeling, osteoblast-activating molecules are released from the demineralized bone matrix to replace the same amount of bone resorbed by osteoclasts. Anabolic molecules, such as TGF-β and IGF-1, termed as "coupling factors", are released by activated osteoclasts [3]. In the initiation phase, however, osteoblast differentiation and formation within the BMU need to be suppressed until bone resorption is accomplished. A molecule known as Semaphorin4D (Sema4D) is produced by osteoclasts and is thought to suppress osteoblastogenesis as an anti-coupling factor, both in bone remodeling and in bone pathogenesis[4]. In addition to suppressing osteoblastogenesis, autocrine Sema4D can also promote RANKL-induced osteoclastogenesis in osteoclast precursor cells. This proposal aims to examine this recently identified bone anabolic molecule, Sema4D, as an anti-coupling factor, elicited by bone cell-cell communication.

Sema4D was originally found as an axonal growth cone guidance molecule in the central nervous system[5-7], a 150kDa transmembrane protein expressed on human normal lymphocytes.

To date, several roles of Sema4D have been identified. It plays relevant roles in the differentiation and activation of both neurological and immunological systems by inducing growth cone collapse during neuronal development. In epithelial and endothelial cells, Sema4D binds Plexin B1, thereby inducing chemorepulsive signals in neuronal cells, as well as angiogenesis[8,9]. Sema4D also binds Plexin B2 on keratinocytes to restore epithelial integrity[10]. In the immune system, however, Sema4D uses CD72 as a functional receptor and enhances the activation of B cells, dendritic cells, and monocytes by diminishing inhibitory signals from CD72[11].

Sema4D is known to express in two forms: membrane-bound form (mSema4D) and a secretory form (sSema4D). However, important to this study, Basile and colleagues reported that many human cancers overexpress Sema4D, which promotes neovascularization on endothelial cells[12]. In order to exert its proangiogenic functions, it was also reported that Sema4D needs to be processed and released from its membrane-bound form by membrane-type 1 metalloproteinases (MT1-MMP) to act in a paracrine manner on endothelial cells. This observation raises the importance of sSema4D when it acts both remotely and locally. In addition, Li Zhu et al. showed that mSema4D is shed by metalloprotease tumor necrosis factor-α-converting enzyme (TACE) from the platelet surface as thrombus formation proceeds. After shedding, Sema4D serves as a soluble bioactive molecule capable of interacting with receptors on endothelial cells and monocytes, as well as those on nearby platelets[11,13]. From these studies, shedding of mSema4D by TACE and MT1-MMP might affect the activity of other cells.

In the field of bone homeostasis, Negishi-Koga, et al. reported that osteoclasts produce the anti-coupling factor, Sema4D, and that Sema4D secreted by osteoclasts inhibits

osteoblastogenesis-mediated bone formation (*Nature Medicine*, 2011). It was found that the ligation of Sema4D to its receptor, Plexin B1 expressed on osteoblast cells, is critically involved in counter-regulation of osteoblast differentiation promoted by the coupling factor IGF-1. It was also demonstrated that Sema4D neutralizing monoclonal antibody (mAb) efficiently enhances bone formation by promoting osteoblast differentiation that occurs during homeostatic bone remodeling, as well as pathogenic bone remodeling in ovariectomy-induced osteoporosis.

While the role of Sema4D on osteoblast differentiation has been identified, the possible effects of autocrine Sema4D on osteoclastogenesis and the expression of receptors on osteoclast cells have not yet been identified. Furthermore, since Sema4D is expressed in membrane-bound form on osteoclasts, the mechanism underlying the secretion of soluble Sema4D by osteoclasts remains unclear. Previous results suggest that Sema4D, initially attached to the osteoclast, serves as a ligand for cell-cell interaction that promotes osteoclastogenesis and that following TACE/MT1-MMP-mediated shedding from the osteoclast cell surface, sSema4D interacts with receptors on osteoclast cells. We know, however, that mSema4D is physically limited because it can only act through cell-cell contact, while sSema4D diffuses in the cellular matrix without such local constraint. In the present study, we show that osteoclasts express Sema4D and its receptor, providing evidence that these molecules promote osteoclastogenesis through an autocrine mechanism. Following TACE/MT1-MMP shedding, we also show that the level of sSema4D increases during osteoclastogenesis. Taken together, results indicate that Sema4D and TACE may be candidate therapeutic targets and that the development of anti-Sema4D and anti-TACE monoclonal antibodies (mAbs) might generate an alternative approach to treat bony pathogenesis, but with fewer side effects or complications. Therefore, this study aims to examine the effects of anti-Sema4D and anti-TACE-mAbs on bone cells.

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Review of Literature:

1. Monoclonal Antibody

Antibodies bind specific target cells or protein with a longer half-life. The development of monoclonal antibodies has adopted this same concept. The advantage of mAb drugs is derived from their high specificity to the functional protein expressed on the cell surface or secreted extracellularly. The large molecular size of mAbs blocks the interactions between functional proteins and their cognate receptors, resulting in agonist or antagonist signaling in target cells. However, mAbs derived from mice possess many limitations, including immunogenicity, inefficient effector functions and half-lives that are typically less than 20 hours. Notably, these limitations have been addressed by molecular engineering technologies aimed at replacing the constant region of murine mAbs with that of human, while, at the same time, maximizing specific therapeutic actions [14]. These efforts have resulted in the development of novel artificial subtypes of mAbs, including chimeric, humanized and fully human mAbs[15]. Over the decades, mAbs have been accepted as an alternative approach for treating various diseases, in particular, cancer, inflammatory diseases and hematological disorders[16]. Antibodies, or IgG-based molecules, have advantages over small-molecule drugs in many respects, including tissue-specific targeting, serum half-life and effector functions via complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or drug conjugates[17]. Important to this study, an anti-Sema4D-mAb has recently been developed, and its potency to abrogate systemic bone resorption by enhancing osteoblastogenesis was proven using a mouse model of ovariectomy-induced osteoporosis[4]. However, no previous

studies have investigated the effect of anti-Sema4D-mAb inhibition on osteoclastogenesis and anti-TACE-mAb inhibition on mSema4D shedding from the surface of osteoclast cells.

2. Osteoclastogenesis

Osteoclasts play an important role in maintaining serum calcium levels and skeletal integrity for bone homeostasis. Osteoclasts originate from the monocyte/macrophage hematopoietic lineage and differentiate into multinucleated osteoclast cells responsible for bone resorption by fusion of mononuclear precursors.

This complex and fascinating process is mainly regulated by a large number of cytokines[18] and leads to initiation of bone remodeling. Macrophage colony-stimulating factor (M-CSF), a secreted cytokine, acts through its receptor, c-fms (colony-stimulating factor 1 receptor), on osteoclasts and triggers hematopoietic stem cells to differentiate into macrophages[19-21]. Subsequently, these macrophages are stimulated to express RANK, a member of the tumor necrosis family (TNF), also known as receptor activator of nuclear factor-ϰB, on the cell membranes to differentiate into osteoclast precursor cells[22,23]. RANK, which is expressed on osteoclast precursor cells, binds RANKL, the ligand of RANK, in order to differentiate into mature and active multinucleated osteoclast cells by activating nuclear factor- ϰB and nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1 (NFATc1). However, osteoprotegerin (OPG), which is produced by osteoblast cells and belongs to the TNF receptor superfamily, acts as a soluble decoy receptor to prevent RANKL from binding to RANK, thus inhibiting the formation of multinucleated osteoclasts[24,25]. Identification of the OPG/RANKL/RANK regulatory axis of osteoclastogenesis represents a

landmark discovery in bone biology[25]. Consequently, osteoclast differentiation is controlled by complex interactions among OPG, RANKL and RANK[25]. In addition to three known cytokines, many other cytokines affect osteoclastogenesis, such as IL-1[26], IL-6[27,28], IL-7[29,30], and TNF (Tumor necrosis factor) [31,32]. Upon some pathological challenge, physiological balance between osteoclasts and osteoblasts is interrupted, and levels of these cytokines both increase and decrease, leading to osteoclastogenesis, followed by bone resorption and bony pathogenesis.

3. Coupling and uncoupling factors in bone remodeling.

The first concept of sequential cellular activities in bone remodeling was advocated by Frost in the 1960s[33]. As previously noted, bone remodeling occurs in the bone multicellular unit, or BMU, throughout life [3], and cell-cell communication is crucial to preserve bone mass and integrity. This needs tight local control from cellular signals, also known as "coupling factors".

Four main classes of coupling factors interact in the BMU to promote osteoblastogenesis at the resorbed bone site. First, matrix-derived signals released during bone resorption, including TGF-β, BMP-2, PDGF, and IGF-1, are all growth factors deposited by osteoblasts during matrix production and then released by osteoclastic resorption on the bone surface. Second, factors, including cardiotrophin-1, sphiongosine-1-phosphate, Wnt10b, and BMP-6, are synthesized and secreted by mature osteoclasts to promote osteoblast precursor cell recruitment and differentiation and thereby promote bone formation in BMU. However, they are not produced exclusively by osteoclasts. Third, factors, such as EphrinB2, are expressed

on the osteoclast cell membrane. Fourth, osteoclasts cause topographical changes on the bone surface[34]. Consequently, coupling factors balance bone destructive and bone regenerative processes, ultimately reaching normal bone remodeling homeostasis [33].

However, osteoblastogenesis is sometimes halted. In the physiological phase, the bone anabolic mechanism needs to be blocked to allow bone resorption to run its course during the initiation phase of bone remodeling. Moreover, in the presence of inflammation or pathogenesis, bone resorption is promoted by suppressing osteoblastogenesis, as well as enhancing osteoclastogenesis. For this to occur, osteoclast cells secrete semaphorins, now known as uncoupling factors, but first found as an axonal growth cone guidance molecule, able to suppress osteoblastogenesis[35].

Important to this study, neutralizing the uncoupling factors secreted by osteoclast cells would enhance bone formation by abrogating the function of uncoupling. At the same time, we need to keep in mind that these coupling signals form a tight relationship between the activities of osteoclasts and osteoblasts in the context of normal and pathogenic bone remodeling.

4. Semaphorin 4D and its receptors Plexin B1, Plexin B2, and CD72

Semaphorins compose a large family of membrane-bound and soluble glycoproteins divided into 8 classes, depending on domain composition and membrane linkage. Semaphorins were first found in the central nervous system as axonal growth cone guidance molecules that play relevant roles in the activation and differentiation of immunological and neural systems by growth-cone collapse in neuronal development[6]. Among 8 classes of

semaphorins, Semaphorin 4D was found to be constitutively expressed on T cells, and its expression was upregulated along with T-cell activation[9,36]. At lower levels, Sema4D was also expressed in macrophages, B cells, NK cells, and neutrophils[37]. Sema4D consists of an NH_2-terminal signal peptide that is expressed on a cell surface as a monomer (150kDa) and a disulfide-linked homodimer (300kDa) as a dominant form[38]. Recent studies have shown that Sema4D can function in cell-cell communication between osteoclasts and osteoblasts during bone remodeling.

Sema4D has three functional receptors, namely Plexin B1, Plexin B2 and CD72[8,37,39]. Recent studies suggest that immune semaphorins play a critical role in many physiological and pathological processes. Furthermore, in the study field of immunology, ligation of Sema4D (CD100) expressed on T cells with its lower affinity receptor CD72, which is expressed on B cells, was reported to induce an activation signal in B cells[8,40]. This reported evidence indicates that Sema4D plays relevant roles in the differentiation and activation of neuro- and immunological systems. However, recent studies have also suggested that Sema4D is also involved in the cell-cell communication that occurs between osteoclasts and osteoblasts[35]. More specifically, osteoclast-derived Sema4D by its ligation with Plexin B1 expressed on osteoblasts inhibits IGF-1-induced osteoblastogenesis[4]. During bone remodeling, osteoblast-activating molecules, such as TGF-β and IGF-1, are released from demineralized bone matrix to replace the same amount of bone as that resorbed by RANKL-activated osteoclasts. The osteoblast-activating molecules released from bone matrix are called coupling factors that maintain physiological control between bone-destructing and bone-generating events (Howard et al. 1981)[3]. In view of this coupling system, Sema4D, which is produced by osteoclasts to counteract IGF-1-mediated osteoblastogenesis, can be

considered an "anti-coupling factor." Collectively, evidence shows that osteoclasts can suppress bone formation through the expression of Sema4D[41,42]. Our preliminary results demonstrated that Sema4D expression is elevated in the context of periodontitis and that upon its release at the site of periodontal lesion, it plays a key role in downregulating new bone formation. Furthermore, the proinflammatory factor TNF-α was found to upregulate Sema4D expression by osteoclasts. While the nature of inflammation induced at implant placement may be different from that of periodontitis, it is conceivable that insufficient osseointegration around the implant, as suggested in this proposal, may be derived from imbalanced coupling in the context of inflammation and that results, in part, from the production of Sema4D by osteoclasts.

5. Sema4D shedding enzymes TACE and MT1-MMP

Semaphorins have recently been implicated in a host of responses, including regulation of cell migration, immune response, tumor progression, and tissue organization during development[43-45]. In order to exert its function, Sema4D, a membrane bound protein, needs to be processed and released into a soluble form to act in a paracrine manner on endothelial cells. Sema4D is known to be expressed on the surface of cells as a homodimer, but its shedding into the surrounding environment through proteolytic cleavage has only recently been investigated[46]. The protease responsible for Sema4D cleavage is likely a matrix metalloproteinase (MMP), a group of zinc-dependent enzymes that hydrolyze numerous components of the extracellular matrix, because shedding could be partly inhibited by the MMP inhibitors EDTA and EGTA[46]. Upregulation of MMPs in cancer cells has been linked

to acquisition of an invasive phenotype, with cells acquiring the ability to digest extracellular matrix substrates, invade underlying tissue, and metastasize[47]. In this study, secreted Sema4D, acting through Plexin B1 on the surface of endothelial cells, enhances tumor growth and promotes angiogenesis.

MT1-MMP is a member of a family of metalloproteinase tethered to the cell membrane, and it confers peri-cellular proteolytic activity, but also participates in the processing of membrane-bound receptors and proteins[48]. MT1-MMP was not found to be expressed in non-tumorigenic epithelial cell lines, but its presence was found in several head and neck squamous carcinoma cell lines. MT1-MMP was required for processing and release of Sema4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis *in vitro* and tumor-induced angiogenesis *in vivo*. These results suggest that proteolytic cleavage of Sema4D may provide a molecular mechanism by which MT1-MMP controls tumor-induced angiogenesis.

In a similar manner, TACE (Tumor necrosis factor-alpha converting enzyme) plays an important role for cleavage of Sema4D. TNFα is a cytokine that induces protective inflammatory reaction and kills tumor cells, but it also causes severe damage when produced in excess, as in rheumatoid arthritis and septic shock. TACE was named after the function of cleaving the membrane-bound TNFα precursor to release soluble TNFα, suggesting that inhibition of TACE may be used to treat inflammation associated with arthritis. According to Zhu[13], mSema4D shedding is blocked by metalloprotease inhibitors and abolished in mouse platelets that lack the metalloprotease ADAM17 (TACE)[13]. TACE is also a Zn-dependent protease synthesized as an inactive precursor. Even though cytokines function in the membrane-bound form, cleavage of the prodomain renders solubilized cytokines active [49,50].

A catalytically inactive mutant form of TACE was observed in blood cells of TACE transplantation. In addition, while full-length Sema4D was present in platelets, cleavage did not occur. However, cleavage did occur when functional TACE was present. Therefore, TACE is necessary for regulated cleavage of Sema4D in mouse platelets.

Specific Aims

Specific Aim 1: To study the *in vitro* the effect of anti-Sema4D-mAb on osteoblasts and osteoclasts in relation to their differentiation and activation.

Hypothesis: Autocrine Sema4D can promote RANKL-induced osteoclastogenesis in osteoclast precursor cells.

Specific Aim 2: To investigate the *in vitro* effect of anti-TACE-mAb on osteoblastogenesis and osteoclastogenesis.

Hypothesis: TACE expressed on osteoclast cells sheds mSema4D to produce sSema4D which, in turn, further promotes RANKL-mediated osteoclastogenesis and suppresses osteoblastogenesis.

Materials and Methods

1. Mouse bone marrow cells culture and induction of osteoclastogenesis

Mononuclear cells were isolated from bone marrow of femur and tibia of 8- to 10-week-old male wild-type mice (C57BL/6j strain) by gradient density centrifugation using Histopaque 1083 (Sigma). The isolated mononuclear cells were cultured in alpha-modified Eagle's Medium (αMEM; Gibco®) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% gentamycin, 1% MEM Nonessential Amino Acid (NEAA) and 20ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA) (termed as complete αMEM) at 37°C with 100% humidity. More specifically, mononuclear cells were preincubated in 96-well plates at 1.0×10^5 cells/well in 200μl of complete αMEM in the presence of M-CSF (50ng/ml, Peprotech, Rocky Hill, NJ). After 3 days of preincubation to induce osteoclast precursors with M-CSF, 100μl of the mediums were replaced with fresh medium containing 50ng/ml M-CSF and 100ng/ml RANKL so that RANKL-mediated osteoclastogenesis would be induced from the osteoclast precursors. In some cultures, different concentrations of cytokines and monoclonal antibodies (10, 50μg/ml) were applied. On Day 3 after the addition of RANKL, 100μl of the medium (50% of medium) were replaced again, as previously described[51,52]. All animals were maintained in a specific-pathogen-free (SPF) environment at the Forsyth Institute Animal Facility, in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). All experimental protocols used in this study were approved by Forsyth's IACUC.

2. Western blot analysis

A previously published protocol for W-blot-based identification of proteins in osteoclasts[51,52] was used in this study. Proteins were isolated from cells and supernatant using a cell lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 10% Glycerol, 2.5mM Sodium-Pyrophosphate, 1.0mM β-Glycerophosphate, and Phosphatase Inhibitor Cocktail [Sigma]). Equal amount of protein sample (20μg) isolated from each condition was loaded into the well of the precast SDS-PAGE gel (NuPAGE 4-12% Bis-Tris, Invitrogen), along with molecular weight marker. The separated proteins in the SDS-PAGE gel were blotted to a nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA). After blocking with 5% non-fat milk, the NC membrane was incubated with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. The specific-antibody reaction was developed by ECL solution (Millipore, Billerica, MA) and then exposed to X-ray film (Kodak, Rochester, NY).

3. ELISA to detect sSema4D

Soluble Semaphorin 4D (sSmea4D) was detected by using a modified ELISA following the method published by Movila et al[53]. Anti-Sema4D-mAb (eBioscience) was captured on a 96-well ELISA plate overnight (Costar® Assay Plate, Corning, Kennebunk ME, USA) and washed three times with PBS with 0.05% Tween 20 (PBS-T) containing 0.5% skim milk. Culture supernatants were biotinylated (EZ-Link-Sulfo-NHS-Biotin, Thermo-Fisher) and then reacted with the above-noted immobilized anti-Sema4D-mAb coated on the ELISA plate. After washing the plate, horse radish peroxidase (HRPO)-conjugated-avidin was reacted to the sample-

derived biotin-labeled Sema4D. Biotin-labeled recombinant mouse sSema4D was used as a standard control for semi-quantification of sSema4D. Substrate solution containing 0.5 mg/ml o-phenylenediamine (OPD) was prepared in 0.1 M citrate-phosphate buffer, pH 5.0, with 0.03% hydrogen peroxide added to each well for 10min. To stop ELISA, 2 M of sulfuric acid was added to each well. Color change was read at OD 492 using a Gen5^{TM} plate reader (Biotek®, Winooski, VT, USA).

4. Quantitative Polymerase chain reaction (qPCR)

RNA was isolated from cells using Trizol reagent (Invitrogen), following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 1μg of total RNA using iScript® cDNA synthesis kit (Bio-Rad Laboratories) by addition of reverse transcriptase (0.5μl) in a total 20μl of solution. qPCR was performed using the LightCycler® 480 system (Roche Applied Science) with LightCycler 480 Sybr 1 master mix (Roche) for 50 cycles. The typical PCR cycling procedures consisted of an initial denaturation step at 95°C for 5min, followed by 50 cycles of 1) denaturation at 95°C for 15 s, and 2) annealing/extension 59~60°C for 60 s. In order to confirm the specificity of designed primers, melting curve analysis was also performed using a single cycle of 95°C for 20s; 60°C for 20s; 40°C for 1s and 95°C prior to the final cooling. An amplified beta-actin gene run simultaneously in the same qPCR assay plate was used as an internal control[54].

5. Tartrate-resistant acid phosphatase (TRAP) staining

To determine the differentiation level of osteoclasts, TRAP staining was performed with the leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. In brief, cells were fixed with 5% formalin for 10 minutes. After subsequent washing with PBS, 100µl of citrate buffer (50mM, pH4.6) containing 10mM sodium Tartrate (Sigma-Aldrich, St. Louis, MO, USA) was applied to the osteoclastogenesis assay plates (96-well plates). The cells in the plate were incubated with a solution containing Naphthol AS-BI phosphoric acid and freshly diazotized fast garnet GBCA at 37°C for one hour. The substrate solution was aspirated and replaced with 200µl of water. TRAP-positive multinuclear cells having more than 3 nuclei are considered as mature differentiated osteoclasts and were counted microscopically.

6. Pit formation assay

Bone marrow cells (3 x 10^5 cells/well) were plated on a Corning® Osteo Assay Surface 96-well plate (Corning Incorporated, Life Science, MA, USA) and preincubated with M-CSF (50 ng/ml) in a complete ✔MEM. After three days of preincubation with M-CSF, RANKL (100ng/ml), M-CSF (50 ng/ml) and specific antibodies were added to each group by exchanging 50% of culture medium (100µl). At Day-10, supernatant in the well was aspirated, and the wells were washed with 10% sodium hypochlorite solution to remove existing adherent cells. Resorbed area on the plate was observed microscopically. ImageJ was used to measure pit areas on the well.

7. ALP assay for detection of osteoblastogenesis

The MC3T3-E1 osteoblast cell line (subclone 4; ATCC, Manassas, VA, USA) was cultured on type-I rat tail collagen-coated tissue culture plates in complete α-MEM. To induce osteogenic differentiation, 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate were added to the culture media. The cells were incubated to differentiate into mature osteoblasts for 7-14 days, changing the media every three days. To monitor the progression of osteoblast differentiation, the alkaline phosphatase (ALP) produced by osteoblasts was monitored using a ALP detection kit (Sigma). Briefly, after aspirating medium from the osteoblast (MC-3T3) culture, cells were incubated with 4% paraformaldehyde (PFA) for 10min to fix the cells. After washing the plates with PBS 2 times, NBT/BCIP solution was applied to the wells and incubated for 10-15 min at 37°C, followed by readout of color intensity developed in each well using a Gen5 plate reader (OD 405).

8. Immunostaining and fluorescence microscopy

Osteoblasts or osteoclasts cultured on a glass coverslip were fixed in ice-cold acetone at -20°C for 10min and blocked with 5% bovine serum albumin (BSA) for 1 hour in a humid chamber at room temperature. FITC-conjugated anti-mouse Sema4D monoclonal antibody (mAb) and Hoeschst 33342 (Thermo Fisher Scientific) was used to stain murine Sema4D and nuclei, respectively. For the staining of Plexin B2, CD72, MT1-MMP and TACE, fluorescent-conjugated mAbs, i.e., phycoerythrin (PE)-xPlexin B2-mAb (BioLegend), PE-CD72-mAb (LSBio), Alexa647-xMT1-MMP-mAb (R&D Systems) and Alexa647-xTACE-mAb (generated

in Kawai lab) were used. For the control, fluorescent-conjugated isotype-matched mAb was used. An inverted Zeiss LSM 780 confocal microscope with GAsP 34 channel detector was used to observe histological sections. Images were obtained with a Plan-Apochromat 40 x 1.40 NA oil immersion objective lens. Each scan was performed using identical laser power, gain, and offset values. Z-stack images were acquired at fixed intervals with a Z-depth of 4.0μm. Bi-dimensional reconstruction was performed using the Zeiss LSM image browser and then saved as TIFF files. Final images were prepared for publication by using Adobe Photoshop CS, version 6.0.

9. RNA silencing

Mononuclear cells were collected from mouse spleen using Histopaque 1083 and seeded to a 24-well plate at a density of 5 x 10^5 cell/well. At Day-3, transfection of siRNA for TACE (Santa Cruz), MT-MMP1 (Qiagen), or control scrambled siRNA (Santa Cruz) was performed following the published protocol [55]. More specifically, anti-TACE siRNA and anti-MT-MMP1 siRNA comprise the pool of 3 target-specific 20~25 nt siRNAs designed to knock down gene expression. Twenty-four hours after transfection of siRNA, mRNA and protein expressions of TACE and MT1-MMP were monitored to ensure effective silencing of target mRNA by real-time qPCR and Western blotting. The group receiving scrambled siRNA was used as negative control. Twenty-four hours after transfection, mononuclear cells were further stimulated with M-CSF and RANKL for 7 days with exchange of 50% of culture medium every 3 days.

Statistical analysis

The Student's *t*-test was used to compare means between the control and experimental groups. For all analyses where comparisons are made between more than two groups, an analysis of variance (ANOVA) was used, such as Student-Newman-Keuls multiple comparisons test. When significant differences were found by ANOVA, Bonferroni's method of multiple comparisons was used to determine which groups were significantly different from each other. Statistical significance level is set at $P < 0.05$. We assumed $P < 0.01$ and at least 80% effect in the power calculations.

Results

Chapter 1

Expression of Semaphorin 4D in membrane-bound form and soluble form by osteoclasts and effects of osteoclast-derived Semaphorin 4D on osteoclastogenesis and osteoblastogenesis

Expression of mSema4D and sSema4D is induced by RANKL-stimulated osteoclasts.

To verify the expression of Sema4D by osteoclast cells, cell lysate and culture supernatant collected from RANKL-stimulated osteoclast precursors derived from mouse bone marrow were subjected to W-blot to detect the membrane-bound and soluble forms of Sema4D based on different molecular weight (MW). In the W-blot assay, cell lysates treated with and without the reducing agent 2-Mercaptoethanol (2ME) showed positive bands at different molecular sizes, i.e., 150kDa under reducing conditions and 240kDa under nonreducing conditions. These two different MW bands appeared to reflect the monomer and homodimer of Sema4D expressed on osteoclasts in membrane-bound form. This finding corresponds to the report that mSema4D on lymphocytes[7] is expressed as a disulfide-linked homodimer. In supernatant, soluble Sema4D was detected in 3 different sizes (55, 65 and 90 kDa). The band sizes at 65kDa and 55kDa appeared to be monomers of sSema4D, while the 90kDa band appeared to be the dimer of sSema4D (Figure 1).

Recombinant Sema4D (rSema4D) promotes osteoclastogenesis, while mAb-mediated neutralization of Sema4D can suppress osteoclastogenesis

Although Negishi-Koga et al. reported that Sema4D can suppress osteoblastogenesis[4,56], it remains unclear whether Sema4D produced by osteoclasts can affect osteoclastogenesis. In order to evaluate the effects of osteoclast-derived Sema4D on osteoclasts in an osteoclastogenesis assay, two different concentrations of anti-Sema4D neutralizing-mAb (50μg/ml, 100μg/ml, IgG1)[53] were used, and the effects, both without anti-Sema4D-mAb and with control isotype-matched mAb (IgG1), were compared in a culture of mouse bone marrow cells (BMC)

(n=8/group)(Figure 2). As determined by TRAP staining, mature multinuclear osteoclasts were induced in the mouse BMC by stimulation with M-CSF and RANKL. Each experimental group was compared with the control IgG1-added group. To evaluate the effect of anti-Sema4D-mAb and rSema4D on osteoclastogenesis, osteoclasts were classified into 1) small TRAP+ osteoclast cells defined as having fewer than 10, but more than 3, nuclei and 2) large TRAP+ osteoclast cells defined as having more than 10 nuclei. The group treated with anti-Sema4D-mAb at both 50μg/ml and 100μg/ml showed significantly decreased emergence of both large and small TRAP+ osteoclasts ($p<0.01$). However, in the groups that received rSema4D, a significantly increased number of large osteoclasts were observed, along with a reduced number of small osteoclasts ($p<0.01$). On the other hand, the pit formation assay revealed that addition of rSema4D to the M-CSF/RANKL-stimulated BMC increased the ability of osteoclasts to form resorption pits, while antibody-mediated neutralization of Sema4D suppressed it. These results indicated that Sema4D produced by M-CSF/RANKL-stimulated bone marrow cells up-regulates osteoclastogenesis. Especially, the emergence of large TARP+ osteoclasts was increased by the effects of Sema4D, suggesting that rSema4D might promote the cell fusion event that increases their activity to resorb bone. Pit formation assay confirmed the induction of functionally active TRAP+ osteoclasts in the culture. It also showed that Sema4D promotes bone-resorption activity by osteoclasts (Figure 2 and 3).

Effects of supernatant harvested from the anti-Sema4D-mAb-treated RANKL-induced osteoclasts on the osteoblastogenesis of MC3T3-E1 cells

Osteoblast cells (MC-3T3) were stimulated with the osteogenesis factors ascorbic acid and β-glycerophosphate in the presence or absence of supernatant harvested from BMC incubated with RANKL for 48 hours (Figure 4). After a 7-day culture, ALP activity in MC-3T3 cells was measured. Addition of rSema4D to MC-3T3 cells cultured in medium containing the supernatant of RANKL-stimulated osteoclastogenesis further suppressed ALP production. On the other hand, the addition of anti-Sema4D-mAb to MC-3T3 cells cultured with the supernatant of RANKL-stimulated osteoclastogenesis upregulated ALP activity. These results indicated that Sema4D secreted by osteoclasts plays a crucial role in suppressing osteoblastogenesis.

Osteoclast precursor cells express CD72 and RANKL-induced osteoclastogenesis can be suppressed by neutralizing CD72 receptor

Three receptors, Plexin B1, Plexin B2 and CD72, are reported to serve as receptors for Sema4D[57]. PlexinB1 was originally found in epithelial and endothelial cells to promote angiogenesis[36], Plexin B2 was seen in keratinocytes for restoration of epithelial integrity[10], and CD72 was found to be a negative regulator of B cell activation[58] in lymphoid tissues. The down-regulatory function of CD72 is mediated by tyrosine phosphatase SHP-1, which is recruited to the ITIMs (immunoreceptor tyrosine-based inhibitory motifs) of CD72. Therefore, when recruitment of tyrosine phosphatase is blocked by sSema4D or agonistic CD72-specific mAbs, B cell activation is accelerated. According to Negishi-Koga, Plexin B1 was expressed on osteoblast cells to control their differentiation, whereas few studies have investigated the expression pattern

of Sema4D receptors expressed on osteoclast cells. In the present study, according to the qPCR-based profiling of genes encoding the three known Sema4D receptors, as noted above, RANKL-stimulated osteoclasts induced remarkable elevation of CD72, but little, or no, expression of Plexin-B2 or Plexin-B1, respectively (Figure 5A). The expression of CD72 protein was also identified on osteoclasts by fluorescent confocal microscopy (Figure 5C). Anti-CD72 neutralizing mAb inhibited *in vitro* RANKL-stimulated osteoclastogenesis in the TRAP staining assay (Figure 5B). In sum, we, for the first time, discovered that the Sema4D receptor CD72 is expressed on osteoclasts and that a Sema4D/CD72 axis is engaged in the upregulation of RANKL-induced osteoclastogenesis (Figure 5).

Chapter 2

Functional roles of TACE and MT1-MMP expressed on osteoclasts in shedding Sema4D

Expression of the sheddases TACE and MT1-MMP is induced in osteoclast precursors by stimulation with RANKL in conjunction with elevated Sema4D expression

RT-PCR was performed to monitor mRNA expression profiles for mSema4D, TACE, and MT1-MMP in RANKL-induced osteoclasts at different time points (0, 6, 12, 24, 48, and 72 hours). Expression of TACE mRNA increased from 6 hours and peaked at 72 hours, while the expression of MT1-MMP mRNA peaked at 48 hours (Figure 6A). Furthermore, according to fluorescent immunohistochemistry, colocalization of TACE and Sema4D on the cell surface of osteoclasts was detected, whereas MT1-MMP expression did not overlap with Sema4D expressed on osteoclasts (Figure 6B).

Detection of TACE enzymatic activity produced in RANKL-stimulated osteoclasts

The amount of TACE protein expression by osteoclasts increased and reached maximum level after stimulation with RANKL for 72 hours, as detected by Western blot assay in Figure 7. These data coincide with the expression of TACE mRNA, as shown in Figure 6.

Next, we examined TACE enzymatic activity using a commercially available TACE enzyme activity detection kit (InnoZyme™ TACE Activity Kit, EMD Millipore) and monitored the magnitude of TACE activity present in RANKL-stimulated osteoclast precursors. TACE enzymatic activity was significantly up-regulated in the osteoclast precursors in response to stimulation with RANKL and reached peak level at 48 hours ($p < 0.01$) (Figure 7B).

Chapter 3

The role of TACE and MT1-MMP in the production of sSema4D

Effects of RNAi-mediated silencing of TACE mRNA and MT1-MMP mRNA on the shedding of mSema4D expressed on RANKL-stimulated osteoclasts

To evaluate the role of TACE and MT1-MMP in the shedding of mSema4D expressed on RANKL-stimulated osteoclasts, RNAi-based loss-of-function assay was performed (Figure 8A, 8B). In response to the treatment with siRNA for TACE and MT1-MMP, RANKL-stimulated osteoclasts showed significantly diminished expression of both TACE mRNA and MT1-MMP mRNA compared to RANKL-stimulated osteoclasts that received control scrambled siRNA. According to W-blot analysis, TACE and MT1-MMP proteins expressed on osteoclasts stimulated with RANKL for 48 hours were significantly reduced by the treatment with siRNA for TACE and MT1-MMP, respectively, compared to the treatment with control siRNA (Figure 8C). These results from qPCR and W-blot analyses indicated that RNAi for TACE, as well as that for MT1-MMP, could sufficiently suppress the expression of TACE and MT1-MMP. Important to this study, silencing of both TACE and MT1-MMP resulted in suppressing the secretion of sSema4D by RANKL-stimulated osteoclasts to the culture supernatant (Figure 9A). Furthermore, the level of sSema4D suppression was more pronounced in TACE siRNA compared with MT-MMP1 siRNA (Figure 9A). However, in Figure 9B, neither TACE nor MT1-MMP siRNA affected the expression of mRNA of Sema4D by RANKL-stimulated osteoclasts, suggesting that silencing and resultant loss-of-function did not affect the expression of Sema4D mRNA on osteoclasts. These results indicated that TACE and MT1-MMP are engaged in the shedding of mSema4D to produce sSema4D and that the shedding effect was greater by TACE enzymatic activity compared to MT1-MMP.

RNAi-mediated silencing of TACE mRNA and MT1-MMP mRNA suppressed the emergence of RANKL-induced TRAP+ osteoclasts

We next examined the effect of TACE and MT1-MMP siRNA on osteoclastogenesis induced in RANKL-stimulated BMC by TRAP staining and pit formation assay. TRAP-positive multinuclear cells containing more than 3 nuclei were counted as mature osteoclasts under the microscope. In contrast to the control group that received M-CSF alone, addition of RANKL to M-CSF-primed BMC efficiently induced the production of TRAP-positive multinucleated cells. However, when M-CSF-primed BMC was treated with anti-TACE-siRNA or anti-MT1-MMP-siRNA prior to stimulation with RANKL, the number of TRAP-positive multinucleated cells was significantly diminished (Figure 10). To determine whether such RNAi-mediated suppression of TACE and MT1-MMP affected the resorptive activity of osteoclasts, RNAi-treated BMC were cultured in Osteo Assay Surface of 96-well plates. When compared to RANKL-stimulated BMC that received control siRNA, a significantly diminished area of resorption pits was observed in the RANKL-stimulated BMC treated with anti-TACE-siRNA or anti-MT1-MMP-siRNA (Figure 10). In sum, these results indicated that shedding of mSema4D expressed on osteoclasts by TACE and MT1-MMP is responsible for the production of sSema4D which, in turn, further promotes RANKL-mediated osteoclastogenesis.

Effects of supernatant harvested from anti-TACE-siRNA- and anti-MT1-MMP- siRNA-treated RANKL-stimulated osteoclasts on osteoblastogenesis from MC-3T3 cells

Negishi-Koga et al. showed that supernatant from osteoclast culture does not suppress osteoblastogenesis, whereas soluble Sema4D-Fc protein could[4]. In contrast to this controversial

finding, our results clearly showed that supernatant from osteoclast culture suppressed osteoblastogenesis (Figure 11). This discrepancy could be explained by the higher numbers of osteoclasts in the culture or higher level of osteoclastogenesis induced by RANKL in our study compared to the experimental study design of Negishi-Koga et al. For this reason, it is questioned whether sSema4D cleaved from osteoclasts by the sheddases TACE and MT1-MMP, as discussed above, can suppress osteoblastogenesis after cleavage. To address this question, culture supernatant collected from RANKL-stimulated osteoclasts pretreated with or without anti-TACE-siRNA and anti-MT1-MMP-siRNA was applied to Vitamin C/β-GP-activated MC-3T3 cells and cultured for 7 days (Figure 11). Significantly elevated ALP activity was observed in the Vitamin C/β-GP- activated MC-3T3 cells incubated with anti-TACE-siRNA-treated osteoclast supernatant, compared to the other groups, suggesting that anti-TACE-siRNA-mediated sSema4D suppression resulted in canceling the anti-osteoblastogenesis activity present in the supernatant of osteoclast culture. In other words, the results supported that TACE-mediated production of sSema4D from osteoclasts could, in fact, suppress osteoblastogenesis.

Chapter 4

Effects of anti-TACE-mAb on osteoclastogenesis and osteoblastogenesis as proof-of-concept

Anti-TACE-mAb prevents the shedding of mSema4D expressed on osteoclasts.

The expression level and patterns of mSema4D on RANKL-stimulated OC precursors were monitored through immunofluorescence microscopy (Figure 12). The addition of anti-TACE-mAb remarkably increased the level of mSema4D expression on the RANKL-stimulated osteoclast precursors in the same experimental condition.

Anti-TACE-mAb applied to the culture of RANKL-stimulated mouse bone marrow osteoclast precursors suppressed the expression of sSema4D by suppressing the TACE enzymatic activity that leads to cleavage. In the same way, expression of mSema4D on the cell surface was promoted. As shown in Figure 13, Sema4D proteins were collected from cell lysate and supernatant to evaluate the concentration of mSema4D and sSema4D, respectively. Western blot assay revealed that the expression of mSema4D increased up to 72 hours in the group with anti-TACE-mAb, while the expression of sSema4D decreased in the same group. This result suggested that the anti-TACE-mAb, which was established by our group, can neutralize the shedding effects of TACE on mSema4D (Figure 13). The intensity of Western blot bands in Figure 13 was analyzed using densitometry, and quantitated levels of mSema4D and sSema4D are expressed in the histograms (Figure 14).

Neutralizing antibody of TACE suppressed the emergence of RANKL-induced TRAP+ osteoclasts and pit formation.

As proof-of-concept of silencing, neutralizing monoclonal antibodies were used to evaluate the effect of TACE on RANKL-induced osteoclastogenesis. Two different concentrations of anti-TACE-mAb (10μg/ml and 50μg/ml) were added to BMCs stimulated by

M-CSF on Day 3 and cultured for 10 days. TRAP-positive multinuclear cells containing more than three nuclei were considered as mature osteoclasts and were counted microscopically. Stimulation with RANKL and M-CSF showed significantly elevated TRAP staining compared to the group that received M-CSF alone. The RANKL/M-CSF-stimulated groups treated with control mAb (10μg/ml and 50μg/ml) did not show any effect on osteoclastogenesis compared to the one stimulated with RANKL/M-CSF. However, when 50μg/ml of neutralizing monoclonal antibody of TACE were added, the number of TRAP-positive multinucleated cells was significantly diminished (Figure 15), while no significant change was observed in the group that received 10μg/ml of anti-TACE-mAb. To determine if this suppression affected resorption activity, mouse bone marrow-derived mononuclear cells were cultured on Corning Osteo Assay multiple-well plates. Pit formation activity by osteoclasts was proportional to that of emergent TRAP+ multinuclear osteoclasts.

Effects of supernatant harvested from the anti-TACE-mAb-treated RANKL-stimulated osteoclasts on the osteoblastogenesis of MC-3T3 cells

Addition of culture supernatant harvested from RANKL/M-CSF-stimulated osteoclasts in the presence of control IgG1 (Day-1, 2 and 3) to Vitamin C/β-GP- activated MC-3T3 cells suppressed the expression of ALP by MC-3T3 (Figure 16). However, culture supernatant collected from the anti-TACE-mAb (50μg/ml) -treated RANKL-stimulated osteoclasts (Day-1, 2 and 3) showed higher ALP activity in Vitamin C/β-GP-activated MC-3T3 cells compared to the control IgG1 group. These results indicated that soluble Sema4D produced by TACE could functionally suppress osteoblastogenesis.

Discussion

This study revealed that Sema4D can mediate an anti-coupling effect within the confines of the BMS, composed of osteoclasts and osteoblasts, in two different ways. Sema4D, which is initially expressed on the osteoclast surface can serve as a ligand for Plexin B1 expressed on osteoblasts, induces the suppression of osteoblastogenesis. However, after TACE shedding and cleavage to its soluble form, sSema4D can serve as a bioactive molecule capable of interacting with receptors on osteoclast precursors and osteoblasts, as well as on remotely located cells. Anti-Sema4D-mAb was demonstrated to effectively inhibit RANKL-induced osteoclastogenesis by binding CD72 receptor expressed on osteoclast precursor cells. Furthermore, by interfering with its enzymatic activity, anti-TACE-mAb treatment led to a decrease in the production of cleaved Sema4D and, hence, attenuated RANKL-induced osteoclastogenesis, while, at the same time, promoting osteoblastogenesis. Compared to other *in vitro* study models that used RAW264.7 cells, all experiments were performed with BMC cells collected from wild-type mouse femur which reflects the physiological context of bone tissue. Although this study is limited by the absence of *in vivo* studies using animal models, the findings, for the first time, revealed that sSema4D, produced by osteoclasts, contributes to pathogenic bone destruction by promoting osteoclastogenesis, while simultaneously suppressing osteoblastogenesis.

Sema4D is known to have three receptors: Plexin B1 on epithelial and endothelial cells to enhance angiogenesis at the tumor site[36], Plexin B2 on keratinocytes to restore epithelial integrity[10], and CD72 on B and T lymphocytes to control immune cell differentiation[40]. Of these three, the strongest expression of CD72 was shown via PCR, which confirmed its expression through fluorescent immunostaining. CD72 is known as the immunoreceptor for tyrosine-based

inhibitory motifs (ITIMs) in its cytoplasmic domain, and it functions as a negative regulator of B cells[58]. The regulatory function of CD72 is mediated by the tyrosine phosphatase SHP-1, which is recruited to the phosphorylated ITIMs of CD72. Evidence has shown that Sema4D enhances B cell activation by turning off the inhibitory signals of CD72. Sema4D ligation inhibits physical association of CD72 with the B cell receptor (BCR) complex[59]. In our study, although anti-CD72-mAb suppressed RANKL-induced osteoclastogenesis, it is unclear whether SHP-1 is engaged in the cell signal elicited by Sema4D/CD72 ligation. Therefore, further experiments to investigate the molecular mechanism are required.

Semaphorins were first found in the early 1990s as an axonal guidance molecule by inducing growth cone collapse during neuronal development. Since then, about 20 types of semaphorins have been identified[5]. By their structure and amino acid sequences, semaphorins are divided into eight classes. Class I and II are found in invertebrates, and Classes III-VII are expressed in vertebrates[60]. Class I and IV-VII are found to be membrane-associated. Among all those classes of semaphorins, Sema4D belongs to class IV, which is comprised of an immunoglobulin (Ig)-like domain, followed by a Sema domain and then by transmembrane and cytoplasmic domains with tyrosine and serine phosphorylation tails. The 150kDa monomeric and 300kDa disulfide-linked homodimeric forms of membrane-bound Sema4D[38] are expressed and regulate immune response [37]. Important to this study, according to Negishi-Koga et al. (2011), osteoclasts control osteoblast differentiation through Sema4D's effect on osteoblast differentiation, motility and guidance of bone cell positioning. Sema4D binds its PlexinB1 receptor expressed on osteoblast cells and inhibits the IGF-1-elicited pathway, an essential signal for osteoblastogenesis. However, the effect of Sema4D on osteoclastogenesis remains to be elucidated. Basing our work on the study of Negishi-Koga, we extended the concept of

uncoupling factors by applying the novel function of Sema4D to promote osteoclastogenesis, which causes imbalance between bone resorption and formation. Therefore, when anti-Sema4D-mAb was added to the culture medium, osteoclastogenesis was significantly suppressed, suggesting that Sema4D plays a key role in osteoclastogenesis. However, when recombinant Sema4D (rSema4D) was added to the culture medium, a higher level of osteoclastogenesis resulted in comparison to the control group that did not receive rSema4D. Furthermore, anti-TACE-mAb suppressed *in vitro* RANKL-induced osteoclastogenesis, while inhibiting the generation of soluble Sema4D from RANKL-stimulated osteoclast precursors. Such results revealed that sSema4D, but not mSema4D, produced by osteoclast precursors, is responsible for the upregulation of RANKL-induced osteoclastogenesis which delineated the distinct role of sSema4D produced by osteoclast precursors in upregulating osteoclastogenesis in an autocrine manner.

In addition to the higher level of osteoclastogenesis in the rSema4D group, multinucleated osteoclasts (i.e., mature osteoclasts with more than 10 nuclei) also appeared. In the pit formation assay, as shown in Figure 3, we found that these multinucleated osteoclasts showed larger pit areas than osteoclasts having more than 3, but less than 10, nuclei, suggesting that the bigger multinucleated osteoclasts are functionally more potent in bone resorption than small osteoclasts. While it is plausible that mSema4D is engaged in activating osteoclast precursor cells during the cell-cell contact-dependent osteoclast fusion process, we have shown that sSema4D, rather than mSema4D, functions as a bioactive molecule to increase the size of osteoclasts and, hence, induce stronger bone-resorbing activity.

In this study, we emphasized the importance of soluble Sema4D in promoting osteoclastogenesis. Wang et al. discovered that sSema4D is released by a still unidentified

metalloproteinase[61]. Zhu et al. found that the extracellular domain of Sema4D is shed from the platelet surface by the metalloproteinase TACE, which is also known as ADAM17 [13]. Using qPCR and Western blot, we showed that osteoclasts also express TACE and MT1-MMP on their cell surface. As confirmed by InnoZyme™ , TACE expression reached a peak at 72 hours, while its functional activity was highest at 48 hours. Unfortunately, neither global gene knockout strain nor osteoclast-specific conditional knockout mouse strain is available for TACE or MT1-MMP. Therefore, in order to evaluate loss-of-function, we conducted RNAi-based gene silencing to evaluate the role of TACE and MT1-MMP in the shedding of mSema4D expressed on osteoclasts. The silenced TACE group showed significantly less expression of sSema4D than control. Very importantly, such RNAi-based silencing of TACE or MT1-MMP did not affect the expression of Sema4D mRNA, as revealed by qPCR. Consequently, we could confirm that the different amount of sSema4D expression exclusively resulted from TACE and MT1-MMP loss-of-function, but not from cell damage via siRNA transfection reagent or stimulation of RNA-binding receptor with the siRNA tested. As expected, the silenced TACE and MT1-MMP groups suppressed osteoclastogenesis in conjunction with diminished production of sSema4D from osteoclast precursors. Furthermore, cultured MC-3T3 osteoblast cells that received supernatant of osteoclast culture showed less alkaline phosphatase activity, which was abrogated by addition of anti-TACE-mAb into the osteoclast culture prior to the addition to MC-3T3 cells (Figure 16). Interestingly, as shown in Figure 1, the mSema4D shedding activities by TACE and MT1-MMP could be distinguished by two different sizes of soluble bands (55kDa, and 65kDa), suggesting that the different cleavage sites of mSema4D extracellular domain are targeted by TACE and MT1-MMP, respectively, thus requiring further study.

In Figure 9 and 10, osteoclastogenesis and osteoclast resorption were almost completely suppressed in each sheddase-silenced group, while sSema4D was still present from the unsilenced enzyme, albeit in decreased amount. We also found that protein expression of TACE was decreased in the MT1-MMP silenced group, even though MT1-MMP protein expression was not significantly changed in the TACE-silenced group. Given the limitation of this study relying on the *in vitro* assay without confirmation of the finding in the in vivo context, possible explanations are that 1) TACE and MT1-MMP could be permissive to shed mSema4D, rather than acting independently, 2) TACE and MT1-MMP may also function on other cytokines, such as IL-1, -6, , and TNFα that are known to promote the RANKL-induced osteoclastogenesis, , and 3) autocrine sSema4D that resulted from shedding by TACE and MT1-MMP appeared to be required to facilitate the cell fusion between small osteoclast precursors during the osteoclastogenesis.

<u>Function of TACE and MT1-MMP could be permissive to generate sSema4D, rather than acting independently</u>

MMPs, including MT1-MMP, are enzymes known to be involved in shedding of a number of membrane bound molecules, including adhesion molecules and cytokines, thereby affecting migration, proliferation, and apoptosis of cells [11]. According to Sternlicht et al.[62], MT1-MMP plays a role in activating MMP2[63-65] by cleaving MMP2 from the cell surface. TACE is also expressed on the cell surface in an inactive form[67]. TACE is activated through cleavage of its extracellular, not cytoplasmic, domain by other molecules. Furthermore, the expression of TACE was reduced in the osteoclast precursors that received MT1-MMP siRNA (Figure 8). Therefore, since MT1-MMP can activate the catalytic activities of MMP2 and MMP9, it is

plausible that MT1-MMP may act as an activator of TACE. Another possibility is that most sSema4D molecules are homodimeric since the shedding process requires homodimerization[62]. Interestingly, in Figure 1, two different band sizes (55kD and 65 kD) were detected in the W-blot of 2-ME-treated supernatant from RANKL-stimulated osteoclasts, suggesting that MT1-MMP and TACE acted on different cleavage sites in mSema4D, resulting in the production of two different soluble forms. It is unclear if both forms of sSema4D can bind with Plexin B1/2 and CD72 and elicit the activation of cells. More in-depth investigation on the function and structure relationship of cleaved sSema4D is required.

In contrast to chemical-based drugs, monoclonal antibody drugs show more specificity and fewer side effects. Over the decades, mAbs have been accepted as an alternative approach to treating various diseases, in particular, cancer, inflammatory diseases and hematological disorders. These mAbs, or IgG-based molecules, have advantages over small-molecule drugs in many aspects, including tissue-specific targeting, serum half-life and effector functions via complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or the capacity for developing drug conjugates. It is true that an anti-Sema4D-mAb and anti-TACE-mAb have recently been developed by other groups and that the therapeutic effects of those mAbs were reported in animal models of several different diseases. However, no previous studies have investigated the effect of Sema4D on RANKL-induced osteoclastogenesis. Thus, the results in this study may lay the groundwork for the development of a novel therapeutic target against bone loss in bone disease. In this study, it was shown that anti-Sema4D-mAb could significantly decrease the level of osteoclastogenesis. This revelation promises a future therapeutic approach in bony pathogenesis, such as osteoporosis and periodontitis, by both suppressing osteoclastogenesis and promoting osteoblastogenesis.

It was also shown that TACE and MT1-MMP play a crucial role in RANKL-induced osteoclastogenesis and that sSema4D is a key driver of RANKL-induced osteoclastogenesis. Therefore, we propose the following model to explain the findings in this study. Upon activation of osteoclasts in response to RANKL, mSema4D on the surface of osteoclast cells interacts with PlexinB2 on nearby osteoblasts, suppressing formation of mature osteoblasts. As this continues, TACE-dependent cleavage of Sema4D releases a soluble fragment of Sema4D, further promoting RANKL-induced osteoclastogenesis.

For the first time, this study demonstrated that anti-Sema4D-mAb could suppress RANKL-induced osteoclastogenesis and promote osteoblastogenesis. Anti-TACE-mAb could also suppress RANKL-induced osteoclastogenesis by blocking cleavage of Sema4D on the osteoclast cell surface and inhibiting cleavage of sSema4D. Therefore, anti-Sema4D-mAb and anti-TACE-mAb could be developed as potential lead candidates for a therapeutic approach that targets osteoclast-mediated bony pathogenesis with minimal, or no, side effects.

Conclusions

- Membrane-bound Sema4D and sSema4D were detected in cell lysate and supernatant from Western blot.
- Anti-Sema4D-mAb suppressed osteoclastogenesis and promoted osteoblastogenesis. Conversely, rSema4D promoted osteoclastogenesis and suppressed osteoblastogenesis.
- CD72, the ligand of Sema4D, has higher expression on osteoclasts compared with Plexin B1 or Plexin B2.
- Expression of TACE and MT1-MMP sheddases is induced in osteoclast precursors by stimulation with RANKL in conjunction with elevated Sema4D expression.
- TACE enzymatic activity was significantly upregulated by RANKL stimulation which reached peak level at 48 h. The amount of TACE protein also increased and reached maximum level after 72-hour stimulation.
- TACE and MT1-MMP siRNAs suppressed the secretion of sSema4D to culture supernatant, while promoting the level of mSema4D on osteoclasts.
- Neither TACE nor MT1-MMP siRNAs affected the expression of Sema4D mRNA by RANKL-stimulated osteoclasts.
- Anti-TACE-mAb prevented the shedding of mSema4D expressed on osteoclasts.
- Anti-TACE-mAb suppressed RANKL-induced osteoclastogenesis by inhibiting cleavage of sSema4D.

- Osteoblastogenesis of MC-3T3 cells was promoted by the addition of supernatant from anti-TACE-mAb-treated osteoclast culture, suggesting that the shedding effects of TACE to cleave off mSema4D expressed on osteoclasts is responsible for the production of functionally active sSema4D that can inhibit osteoblastogenesis.

Future Directions

The physiological consequences of sSema4D by TACE shedding on osteoclastogenesis and osteoblastogenesis, as revealed in the present study, will open up a new field of study in oral biology and, especially, bone biology. Further research is required to investigate the regulatory pathways and confirm the function of Sema4D and TACE in periodontal inflammation.

Figures and Tables

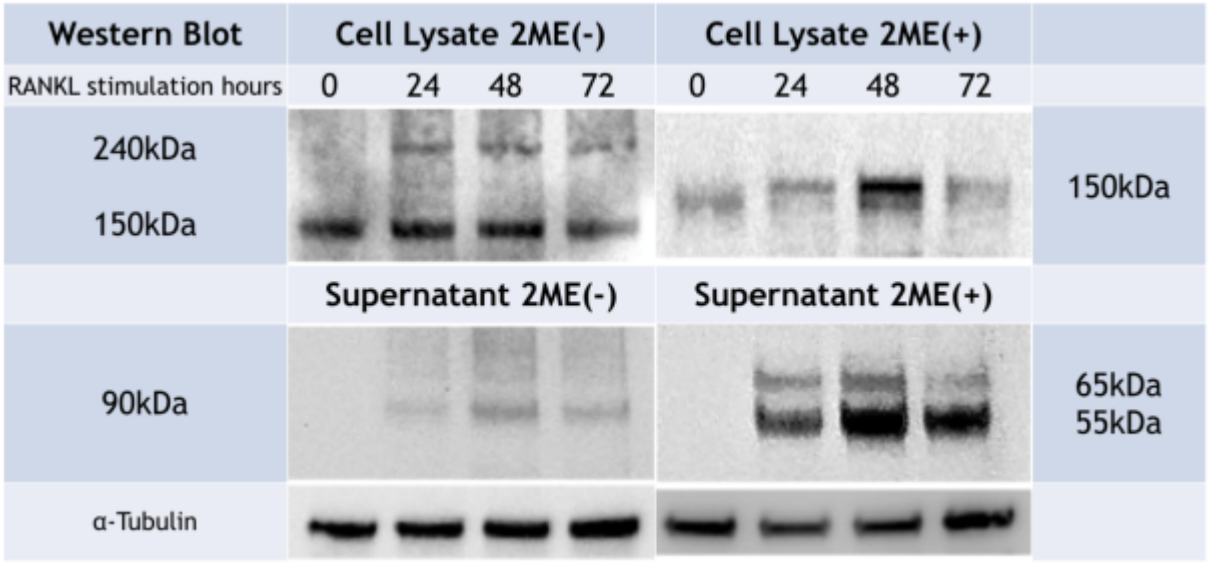
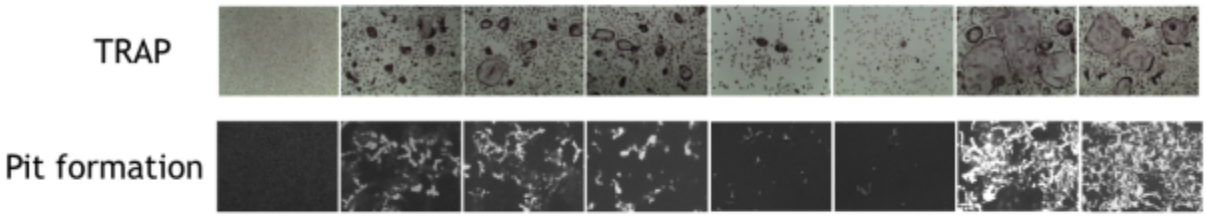


Figure 1. The expression of membrane-bound Sema4D (mSema4D), as well as soluble Sema4D (sSema4D), is induced by RANKL-stimulated osteoclasts. According to the Western blot analysis using anti-Sema4D antibody, mSema4D (240kD and 150kD) and sSema4D (65kD and 50kD) were detected in the cell lysates of RANKL-stimulated osteoclasts (mouse bone marrow cells (BMC)).



M-CSF	+	+	+	+	+	+	+	+
RANKL	-	+	+	+	+	+	+	+
Cont-IgG_1	-	-	50ug/ml	100ug/ml	-	-	-	-
xSema4D	-	-	-	-	50ug/ml	100ug/ml	-	-
rSema4D	-	-	-	-	-	-	50ug/ml	100ug/ml

Figure 2. Recombinant Sema4D promotes osteoclastogenesis, while antibody-mediated neutralization of Sema4D can suppress osteoclastogenesis. As determined by TRAP-staining, osteoclastogenesis from mouse BMC was induced by stimulation with M-CSF and RANKL. Addition of rSema4D to M-CSF/RANKL-stimulated BMC promoted osteoclastogenesis, while antibody-mediated neutralization of Sema4D suppressed it.

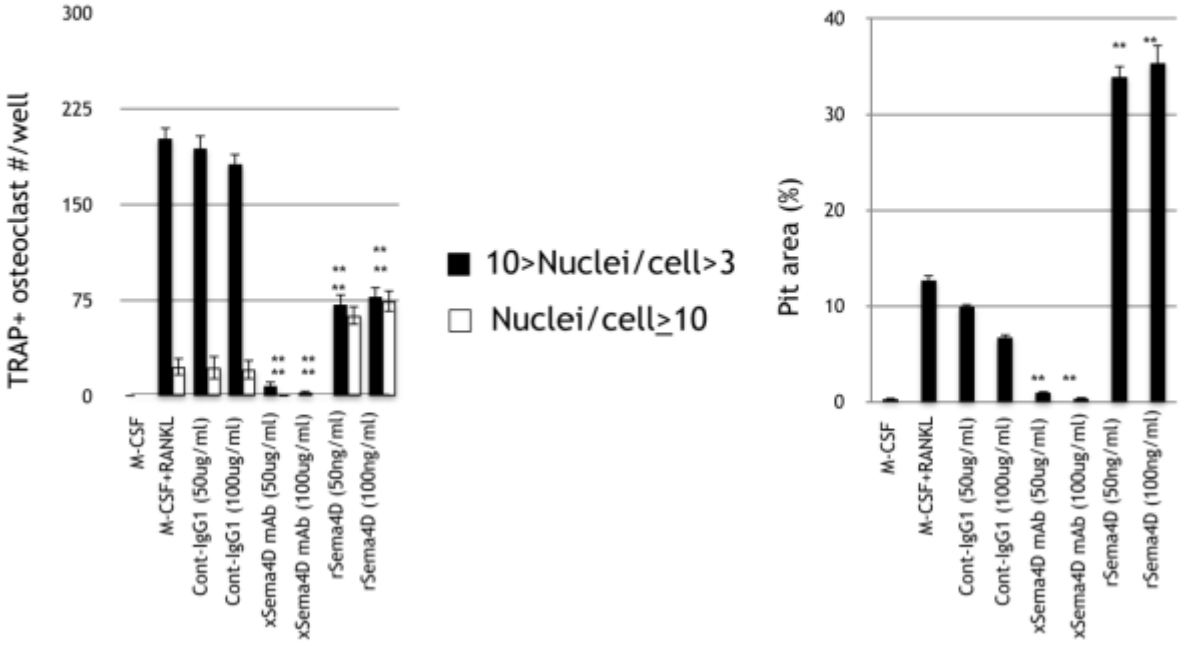


Figure 3. Recombinant Sema4D promotes osteoclastogenesis, while antibody-mediated neutralization of Sema4D can suppress it. Incubation of bone marrow cells with recombinant sSema4D resulted in the emergence of large TRAP+ osteoclasts (nuclei ≥ 10/cell) by TRAP staining and more pits by pit formation assay. However, recombinant sSema4D rather suppressed the number of small TRAP+ osteoclasts (10>nuclei≥3). Those results indicated that sSema4D prmote the osteoclast cell fusion which convert small osteoclasts to large osteoclasts by coalition. On the other hand, xSema4D-mAb suppressed emergency of both large and small TRAP+ osteoclasts.

	αMEM		Osteoclast culture medium (48hours)		
VitC + β-GP	—	+	+	+	+
xSema4DmAb	—	—	—	50ug/ml	—
rSema4D	—	—	—	—	50ug/ml

Figure 4. Effects of supernatant harvested from the anti-Sema4D-mAb- and rSema4D-treated, RANKL-stimulated osteoclasts on the osteoblastogenesis of MC-3T3 cells. Culture supernatant collected from the anti-Sema4D-mAb-treated, RANKL-stimulated osteoclasts showed significantly elevated ALP activity in Vitamin C/β-GP-activated MC-3T3 cells, compared to the other groups, suggesting that anti-Sema4D-mAb-mediated sSema4D suppression resulted in canceling the anti-osteoblastogenesis activity present in the supernatant of osteoclast culture.

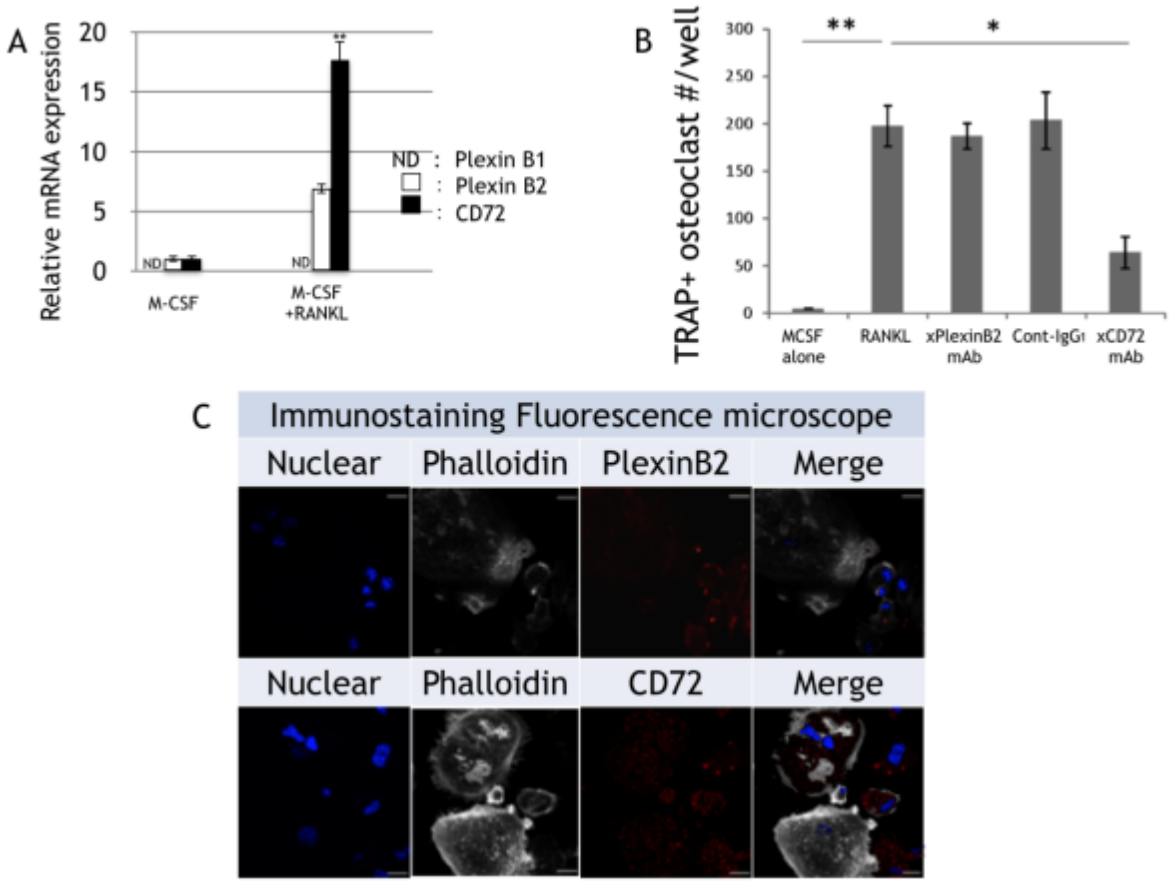


Figure 5. Osteoclasts express CD72, a receptor for Semaphorin 4D (Sema4D).

Among the three known receptors for Sema4D, including, CD72, PlexinB1 and PlexinB2, predominant expression of CD72 was detected by PCR (A) and immunofluorescent staining of cultured osteoclasts from mouse (C57BL/6) BMC (C). CD72 was expressed on the same osteoclasts. Anti-CD72-mAb could inhibit *in vitro* RANKL-induced osteoclastogenesis (B), suggesting that CD72 is associated with the upregulation of RANKL-induced osteoclastogenesis.

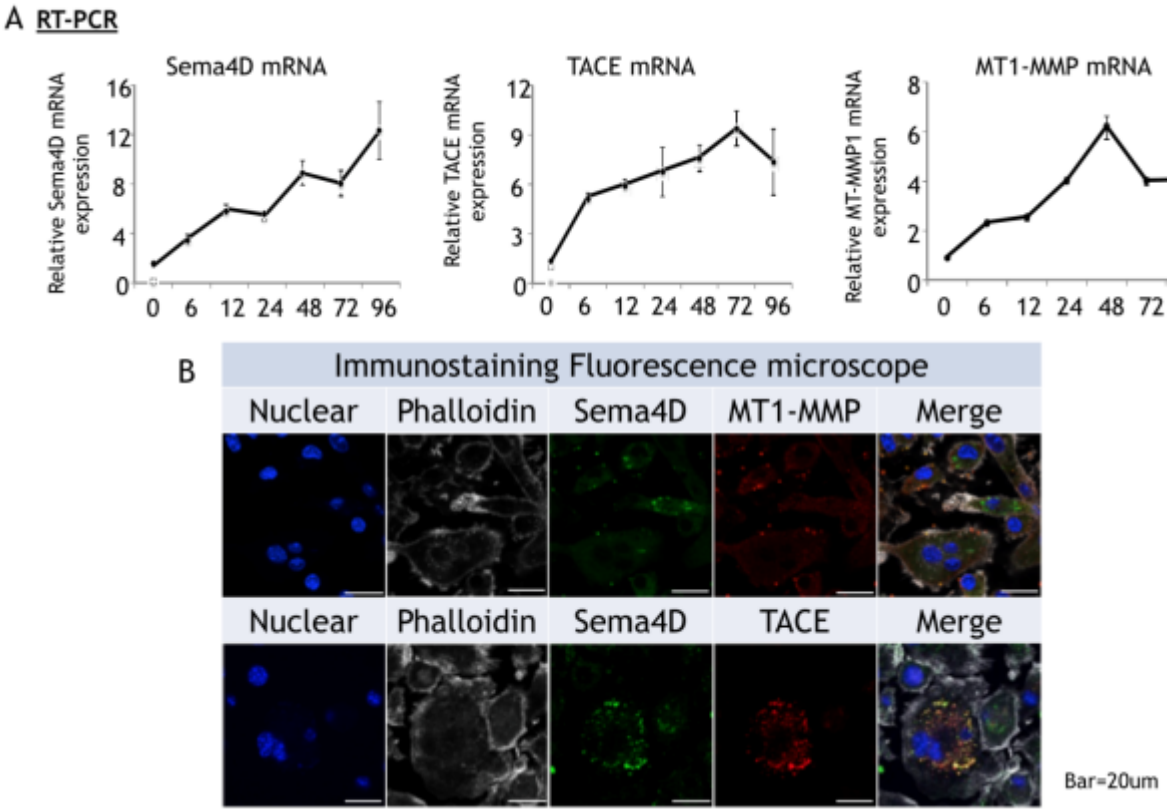


Figure 6. The expression of TACE and MT1-MMP, two sheddase enzymes, is induced in osteoclast precursors by stimulation with RANKL in conjunction with elevated Sema4D expression (A: RT-PCR; B: fluorescent immunohistochemistry). According to fluorescent immunohistochemistry, colocalization of TACE and Sema4D, but not MT1-MMP and Sema4D, was detected (B).

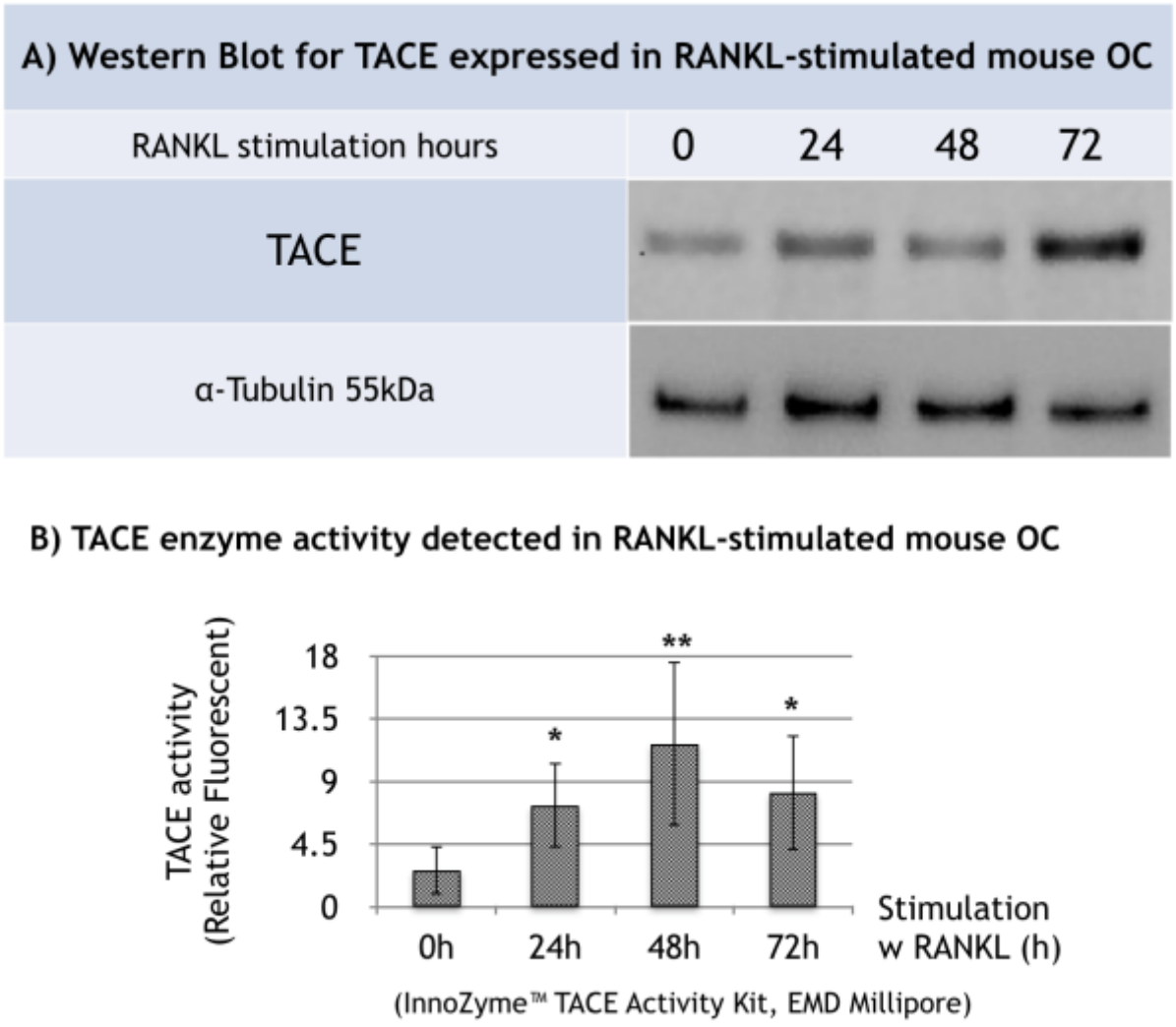


Figure 7. Detection of TACE enzymatic activity produced in RANKL-stimulated osteoclasts and the expression patterns of TACE protein in osteoclasts. Using a commercially available TACE enzyme activity detection kit (InnoZyme™ TACE Activity Kit, EMD Millipore), TACE activity in RANKL-stimulated osteoclast precursors was monitored. This activity was significantly upregulated by RANKL stimulation, which reached a peak at 48 hours (B). The amount of TACE protein also increased and reached maximum level after 72 hours of stimulation (A).

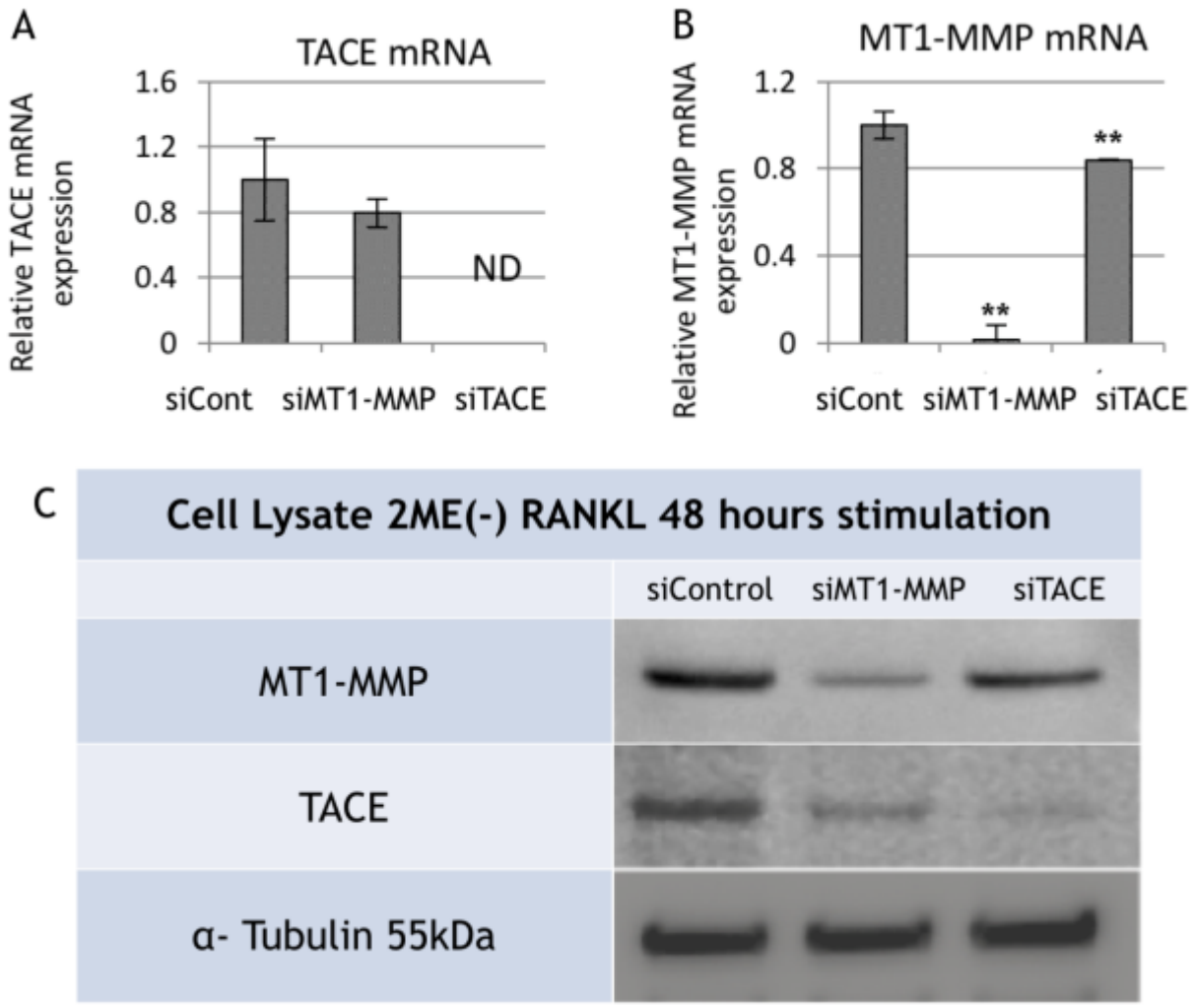


Figure 8. The effectiveness of TACE and MT1-MMP siRNA, as determined by PCR (A) and Western blot analysis (B).

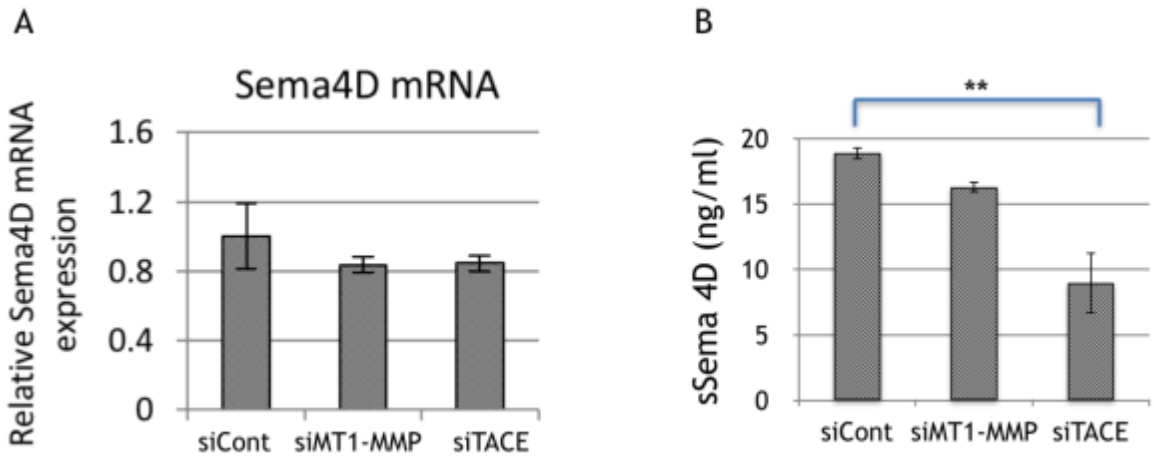


Figure 9. Effects of RNAi-mediated silencing of TACE mRNA and MT1-MMP mRNA on sSema4d expression by RANKL-stimulated osteoclasts. Secretion of sSema4D decreased significantly by TACE siRNA ($p < 0.01$). (B) However, neither silencing group affects the expression of Sema4D by the RANKL-stimulated osteoclasts (A).

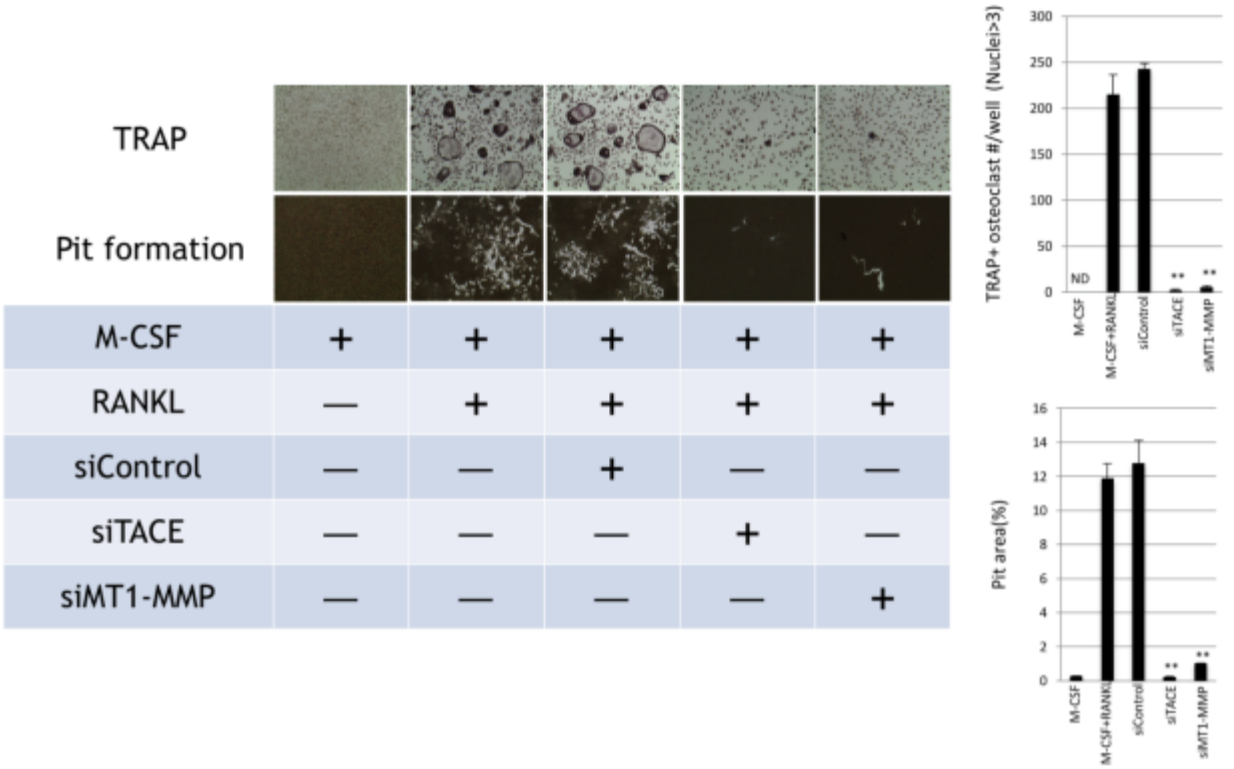
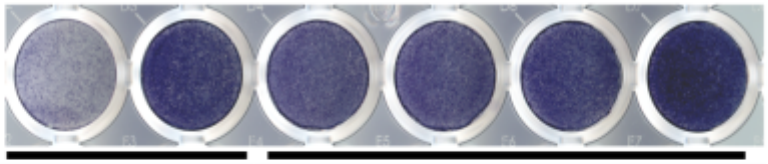


Figure 10. RNAi-mediated silencing of TACE and MT1-MMP mRNA suppressed the emergence of RANKL-induced TRAP+ osteoclasts. Pit formation by osteoclasts was proportional to the increase in the number of TRAP+ osteoclasts (magnification 40x).



	αMEM		Osteoclast culture medium (48hours)			
VitC + β-GP	—	+	+	+	+	+
siControl	—	—	—	+	—	—
siMT-MMT1	—	—	—	—	+	—
siTACE	—	—	—	—	—	+

Figure 11. Effects of supernatant harvested from TACE/MT-MMP1 siRNA-treated, RANKL-stimulated osteoclasts on the osteoblastogenesis of MC-3T3 cells. Osteoblast cells were cultured with the supernatant collected from TACE siRNA-treated, RANKL-stimulated osteoclasts. ALP assay showed significantly elevated ALP activity in the TACE siRNA group with Vitamin C/β-GP-activated MC-3T3 cells compared to the other groups.

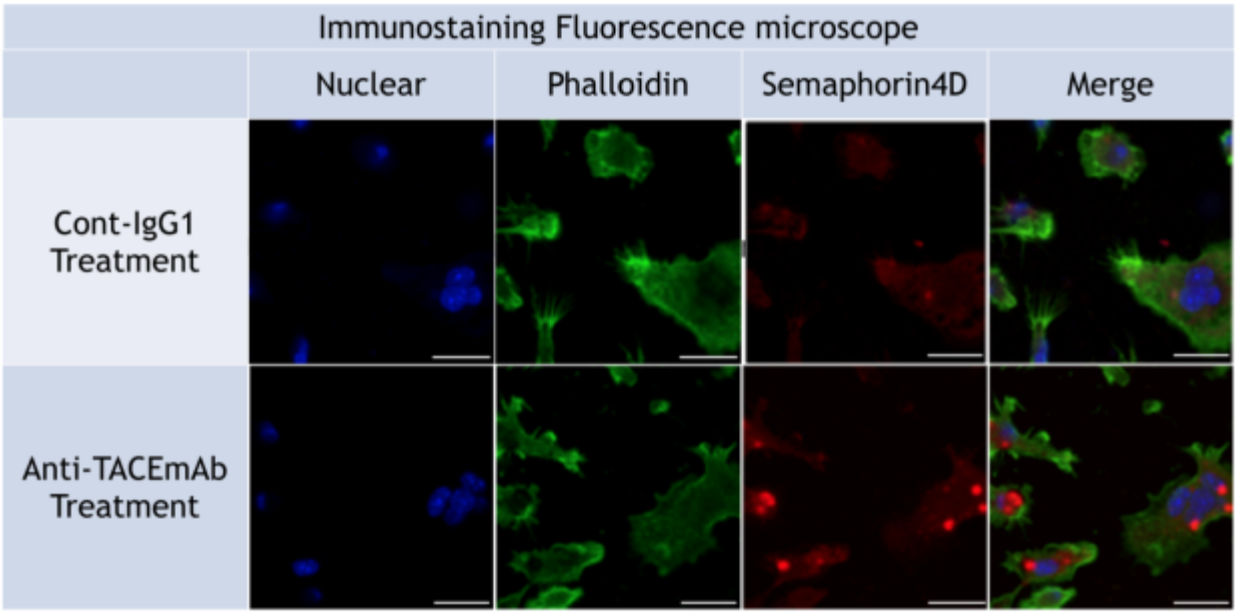


Figure 12. Anti-TACE-mAb prevents the shedding of mSema4D expressed on osteoclasts. The presence of Sema4D on osteoclasts when treated with anti-TACE-mAb, as viewed through immunofluorescence microscopy.

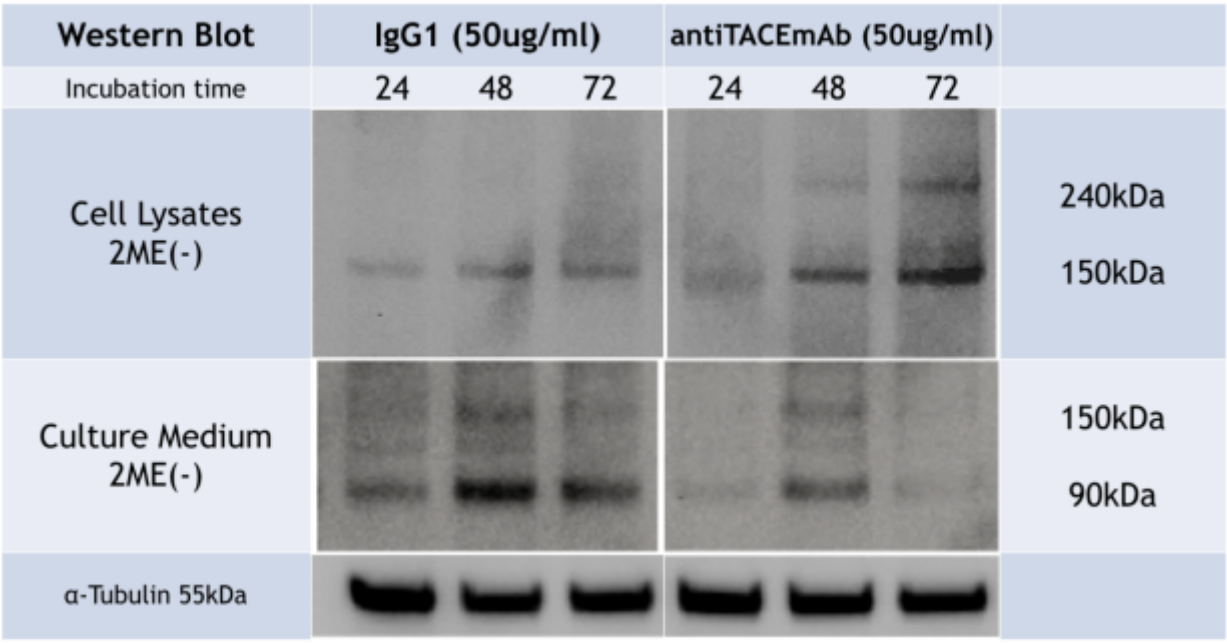


Figure 13. Sema4D was recognized in 240kDa and 150kDa as membrane bound form in monomeric and dimeric forms in cell lysate and 150kDa and 90kDa as soluble form in culture medium. mSema4D expression increased up to 72 hours, while sSema4D expression decreased in culture medium.

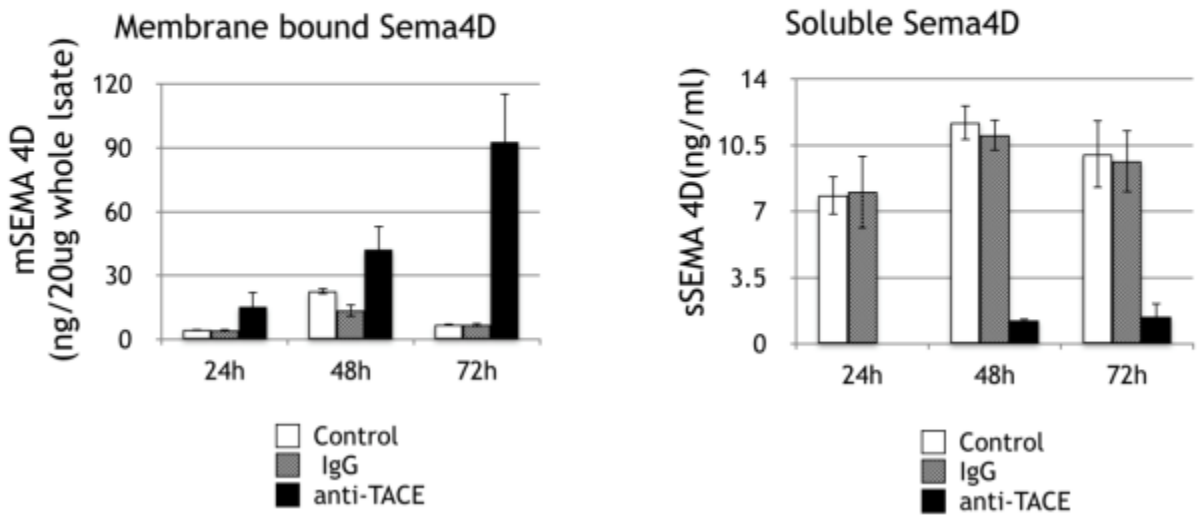


Figure 14. Intensity of Western blot bands in Figure13 was analyzed using densitometry, and quantitated levels of mSema4D and sSema4D are expressed in the histograms.

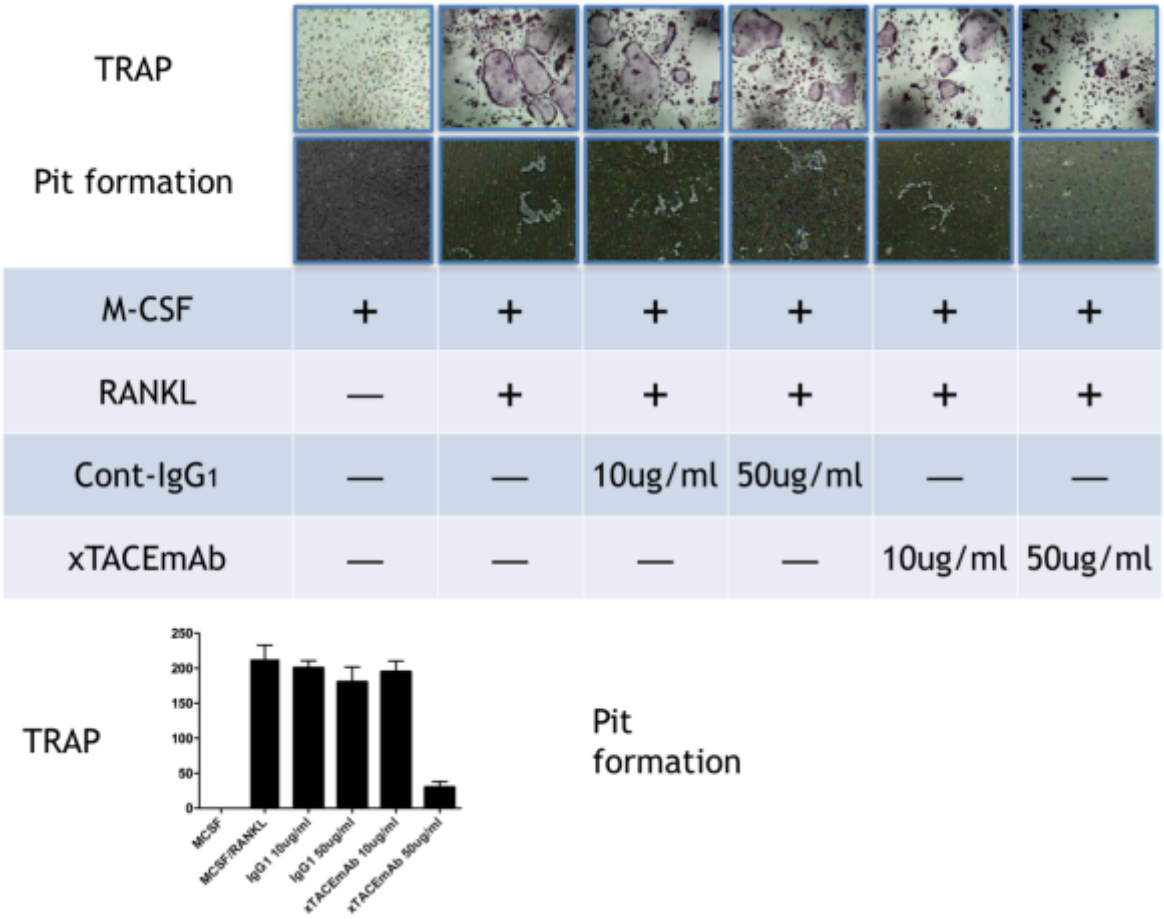
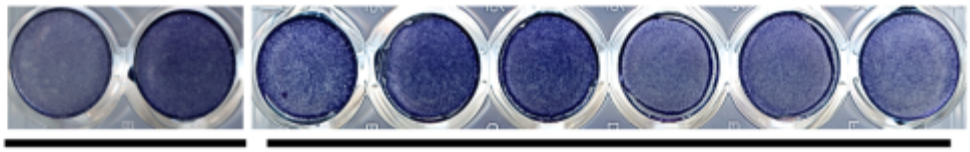


Figure 15. Effect of anti-TACE-mAb on osteoclastogenesis in mouse BMC. As determined by TRAP staining, osteoclastogenesis from mouse BMC was induced by stimulation with M-CSF (50ng/ml) and RANKL (50ng/ml). Addition of anti-TACE-mAb to M-CSF/RANKL-stimulated BMC suppressed osteoclastogenesis. Pit formation assay supported that TRAP+ osteoclasts induced in the culture are functionally active.



	αMEM		Osteoclast culture medium					
VitC + β-GP	—	+	+	+	+	+	+	+
Ab treatment	—	—	xTACE	xTACE	xTACE	Cont-IgG1	Cont-IgG1	Cont-IgG1
OC supernatant	—	—	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs

Figure 16. Effects of supernatant harvested from the anti-TACE-mAb-treated (50μg/ml), RANKL-stimulated osteoclasts on the osteoblastogenesis of MC-3T3 cells were tested. Results showed elevated ALP activity in Vitamin C/β-GP- activated MC-3T3 cells, compared to the other groups.

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