Life Sciences 222 (2019) 29–35

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Sema6A-plexin-A2 axis stimulates RANKL-induced osteoclastogenesis through PLCγ-mediated NFATc1 activation

Jinliang Zhuang[a,b], Xun Li[c], Yi Zhang[a,b], Ruixin Shi[a], Ce Shi[b,d], Dongsheng Yu[a], Xingfu Bao[a], Min Hu[a,b,*]

[a] Department of Orthodontics, School and Hospital of Stomatology, Jilin University, Changchun, Jilin 130021, China
[b] Jilin Provincial Key Laboratory of Tooth Development and Bone Remodeling, China
[c] Department of Endodontics, School and Hospital of Stomatology, Jilin University, Changchun, Jilin 130021, China
[d] Department of Pathology, School and Hospital of Stomatology, Jilin University, Changchun, Jilin 130021, China

ARTICLE INFO

Keywords:
Sema6A
Plexin-A2
Osteoclastogenesis
PLCγ
NFATc1

ABSTRACT

Recently, several plexins and semaphorins have been associated with osteoclastogenesis, a vital process for bone remodeling. Plexin-A2 is implicated in bone homeostasis, however, whether it plays a role in osteoclastogenesis and the underlying mechanism remain unknown. We show that plexin-A2 expression is upregulated during RANKL-induced osteoclastogenesis. In addition, the soluble Sema6A fused with IgG1 Fc region (Fc-Sema6A) interacts with plexin-A2 from cell lysates of osteoclasts, suggesting that plexin-A2 acts as a receptor of Sema6A in osteoclasts. Moreover, Sema6A treatment stimulates RANKL-induced osteoclastogenesis, and this effect is abolished when plexin-A2 is neutralized, which illustrates an indispensable role of plexin-A2 in mediating Sema6A effect on osteoclastogenesis. Mechanistically, Sema6A-plexin-A2 axis enhances RANKL-induced activation of PLCγ as well as downstream target NFATc1, one master transcriptional factor of osteoclastogenesis. Lastly, inhibition of PLCγ by pharmacological inhibitor U73122 abrogates Sema6A-stimulated NFATc1 activation and RANKL-induced osteoclastogenesis, thus demonstrating that the PLCγ-mediated NFATc1 activation accounts for the promotive role of Sema6A-plexin-A2 axis in RANKL-induced osteoclastogenesis. Taken together, this study uncovers a novel role of Sema6A and plexin-A2 in osteoclastogenesis, and also offers them as possible therapeutic targets in the intervention of osteolytic diseases.

1. Introduction

A continuously fine-tuned dynamic remodeling between bone-resorption through osteoclasts and bone-formation through osteoblasts is critical for the maintenance of normal bone mass [1]. Excessive osteoclast activity leads to diseases with a low bone mass, and oppositely, disrupted osteoclastic bone resorption causes diseases with a high bone mass, such as osteopetrosis, periodontitis and osteoarthritis [2,3]. Therefore, agents including bisphosphonates that are able to modulate osteoclast activity have been developed for the treatment of bone diseases [4].

Osteoclasts are specialized multinucleated cells developed from bone marrow-derived monocyte/macrophage precursor cells [5]. The differentiation of osteoclasts is controlled by signaling pathways mediated by receptor activator of nuclear factor-kB ligand (RANKL), macrophage colony-stimulating factor (M-CSF), as well as immunoreceptor tyrosine-based activation motif (ITAM) [6]. RANKL induces osteoclast differentiation in the presence of M-CSF by activating the nuclear factor of activated T cells c1 (NFATc1), the master transcription factor for osteoclastogenesis, through three main pathways, including the tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Fos and phospholipase Cγ (PLCγ)-calcium pathways [7]. However, despite these knowledge, the molecular basis underlying osteoclastogenesis is not fully understood.

Plexins are type I transmembrane receptors originally known to transduce signals from semaphorins, which are axon-guidance molecules vital for neural development [8]. Beyond the nervous system, plexins also function as signal transducers to regulate a variety of biological activities, such as cell migration, immune response, tissue development, and neoplastic transformation [9]. More recently, several plexins and semaphorins have been connected to the regulation of differentiation of osteoclasts and osteoblasts, whereby influencing the bone remodeling, such as ephrinA2-EphA2 [10], ephrinB2-EphB4 [11], Sema4D-plexin-B1 [9]. Plexin-A2 is implicated in bone homeostasis

* Corresponding author at: Department of Orthodontics, School and Hospital of Stomatology, Jilin University, No.1500, Qinghua Road, Changchun City, China.
E-mail address: humjlu159@163.com (M. Hu).

https://doi.org/10.1016/j.lfs.2019.01.060
Received 22 November 2018; Received in revised form 16 January 2019; Accepted 16 January 2019
Available online 28 February 2019

[12] and osteoblast differentiation [13]. We asked whether plexin-A2 plays a functional role during osteoclastogenesis. In the present study, we found that plexin-A2 was upregulated during RANKL-induced osteoclastogenesis, and that through interacting with its ligand, Sema6A, plexin-A2 enhanced RANKL-induced osteoclastogenesis through PLCγ-mediated NFATc1 activation.

2. Materials and methods

2.1. Antibodies and reagents

Primary antibodies against plexin-A2 (MAB-5486) and PLCγ (NBP2-52533) were obtained from Novus Biologicals; V-ATPase d2 (sc-517,031), NFATc1 (sc-7294) and β-actin (sc-47,778) were obtained from Santa Cruz; Cdk5 (2506) and p-PLCγ (14008) were obtained from Cell Signaling. p-Cdk5 (SAB4301418) was obtained from Sigma-Aldrich. Neutralizing anti-plexin-A2 antibody (AF5486) and isotype IgG antibody (AB-108-C) and recombinant mouse Sema6A Fc chimera proteins (9017-S6-050) were obtained from R&D Systems. Secondary antibodies of goat anti-mouse IgG-HRP (sc-2005) and goat anti-rabbit IgG-HRP (sc-2004) were obtained from Santa Cruz. U73122 hydrate (U6756) was obtained from Sigma-Aldrich.

2.2. In vitro osteoclast differentiation

Osteoclast differentiation was performed as previously described [14]. Briefly, the bone marrow cells were isolated from femurs and tibias of 8-week-old C57BL/6 mice and then cultured in a petri dish in αMEM supplemented with 20% FBS and 50 ng/ml M-CSF for 6days to generate bone marrow-derived macrophages (BMMs). For inducing osteoclast differentiation, BMMs were cultured in αMEM supplemented with 50 ng/ml RANKL and 50 ng/ml M-CSF for different days according to experimental purposes. The culture medium was changed with fresh medium every day. The C57BL/6 mice were housed in pathogen-free facilities. All the animal experimental procedures were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee of School and Hospital of Stomatology, Jilin University.

TRAP staining and multinucleated cell counting.

At day 3 after the induction of osteoclast differentiation, osteoclasts were fixed in 10% buffered formalin for 10 min and then visualized by TRAP staining using a commercial kit following the manufacturer's instructions (Cosmo Bio, PMC-AK04F-COS). TRAP-staining positive multinucleated cells containing at least three nuclei were counted as osteoclast cells. The number and area of TRAP-staining positive multinucleated cells were analyzed by Image J software [15].

2.3. Osteoclast cell treatment

BMMs were differentiated with the treatment of 50 ng/ml RANKL and 50 ng/ml M-CSF for 3 days, and then further treated with 0, 20 or 40 ng/ml Fc-Sema6A for 4 h in the presence or absence of 50 μg/ml or 100 μg/ml anti-plexin-A2, or 10 μM U73122. After treatment, cells were harvested for further biochemical analyses.

2.4. RNA extraction and qRT-PCR analysis

Total RNA was extracted from BMMs and osteoclast cells using the TRIzol reagent (ThermoFisher Scientific, 15596026). The complimentary cDNA was synthesized using the M-MLV Reverse Transcriptase Kit (Promega, M1701). Real-time qRT-PCR was conducted using the SYBR Premix Ex Taq Kit (TaKaRa, RR420A) and the Real-Time PCR Detection System (bio-rad, CFX96 Touc). Real-time PCR primers used in this study for amplifying mouse target genes are listed as follows: Plxna2 forward 5′-CCATCCTTTCCACCCAGAGT-3′, reverse 5′-CTTAGGAACCAGAGCCACCA-3′; Trap forward 5′-CAAAGAGATCGCCAGAACCG-3′, reverse 5′-GAGACGTTGCCAAGGTGATC-3′; Nfatc1 forward 5′-TTGAGCTGAGGAAAGGGGAG-3′, reverse 5′-TGACTGGGTAGCTGTCTGTG-3′; *C-Fos* forward 5′-TCCCCAAACTTCGACCATGA-3′, reverse 5′-AGTTGGCACTAGAGACGGAC-3′; Traf6 forward 5′-CAAACCAGAACTGCTTGCCT-3′, reverse 5′-GCATCAGTACTTCGTGGCTG-3′; Actb forward 5′-TCTTTGCAGCTCCTTCGTTG-3′, reverse 5′-TCCTTCTGACCCATTCCCAC-3′. The house-keeping gene β-Actin was used as an internal control. Data were analyzed with a threshold cycle (Ct), indicating the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold. The relative expression was calculated with the comparative ΔΔCt method using the formula $2^{-\Delta\Delta Ct}$ [16].

2.5. Immunoblotting

Total protein was extracted from BMMs and osteoclast cells using the RIPA lysis buffer (Beyotime, P0013B) containing one tablet of protease inhibitor cocktail (ThermoFisher Scientific, A32963). The protein concentration was quantified by the BCA method. Equal amount of proteins in each sample were separated by SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked for 1 h at room temperature in 5% non-fat dry milk diluted in TBS with 0.1% Tween 20 (TBST), and then incubated overnight with primary antibodies at 4 °C and subsequently 1 h with HRP-linked secondary antibodies at room temperature. After adequate wash with TBST, the protein bands on membranes were detected using ECL Substrates (ThermoFisher Scientific, 32106). The band intensity was analyzed with the aid of Image J software. β-Actin was used as a loading control for each sample. Quantification results of target proteins were normalized to that of β-Actin.

2.6. In vitro pull-down assay

In pull-down assays using Fc-Sema6A, osteoclast cells differentiated for 3 days were lysed with pull-down lysis buffer (10 mM HEPES pH 7.5; 100 mM NaCl, 2 mM EDTA and 0.5% NP-40) supplemented with one tablet of protease inhibitor cocktail for 20 min at 4 °C. The whole cell lysates were centrifuged at 12000 ×*g* for 10 min at 4 °C. The supernatants were incubated for 4 h at 4 °C with or without 100, 200, and 500 ng Fc-Sema6A. Pierce Protein A/G Agarose beads (ThermoFisher Scientific, 20421) were used to pull down Fc-Sema6A. The bound products were subjected to immunoblotting analysis with antibodies against plexin-A2 and Cdk5.

2.7. Statistics

All data are expressed as mean ± standard deviation (s.d.). Pull-down and TRAP staining assays were conducted in three biological replicates, and for in vitro stimulation assays and other biochemical analyses, three technical replicates were performed in each independent experiment. Statistical analyses were conducted using one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Plexin-A2 expression is upregulated during RANKL-induced osteoclastogenesis

To explore whether plexin-A2 is involved in the regulation of osteoclastogenesis, we first compared its mRNA expression in untreated versus receptor activator of nuclear factor-κB ligand (RANKL)-treated bone marrow-derived macrophages (BMMs), which differentiate into osteoclasts in response to RANKL stimulation [17]. qRT-PCR analysis showed that plexin-A2 expression was increased during RANKL-induced osteoclastogenesis in a time-dependent manner as compared with untreated BMMs (Fig. 1A). The osteoclastogenesis was confirmed by the increased expression of tartrate-resistant acid phosphatase

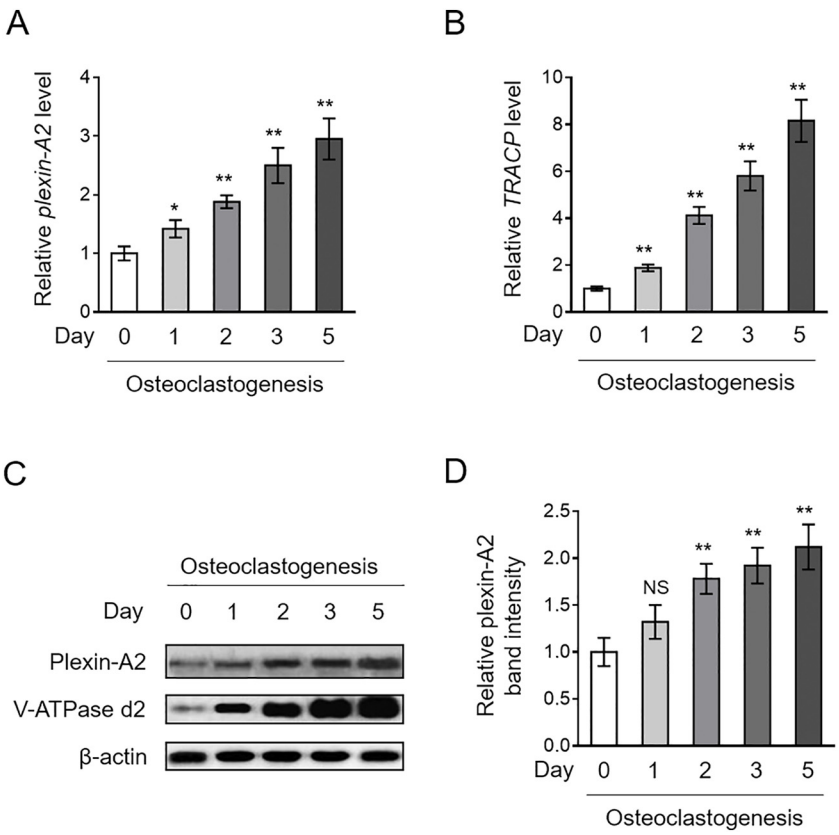

Fig. 1. Plexin-A2 is upregulated during RANKL-induced osteoclastogenesis.
(A–B) Bone marrow-derived macrophages (BMMs) were cultured with 50 ng/ml RANKL for different time periods as indicated for inducing osteoclastogenesis. The mRNA levels of plexin-A2 (A) and TRAP (B) were quantified by qRT-PCR analysis. β-Actin was used as an endogenous control. Results relative to 0 day group are shown. (C–D) BMMs were treated as in (A-B). The protein levels of Plexin-A2 and V-ATPase d2 were measured by immunoblotting analysis. β-Actin was used as a loading control. The representative images (C) and band intensity analysis of plexin-A2 (D) are shown. 50 ng/ml M-CSF was added into the culture medium throughout. Data are mean ± s.d. Data were compared using one-way ANOVA followed by Dunnett's test. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

(TRAP) (Fig. 1B), one specific marker gene for osteoclasts [18]. Next, immunoblotting analysis showed that consistent with its mRNA change, the protein level of plexin-A2 was also upregulated during osteoclastogenesis (Fig. 1C–D). As a positive control, another osteoclast specific marker V-ATPase d2 was concomitantly found to be induced during osteoclastogenesis (Fig. 1C) [19]. These results indicate that plexin-A2 expression is upregulated during osteoclastogenesis induced by RANKL stimulation.

3.2. Plexin-A2 is a receptor of Sema6A in osteoclasts

Plexin-A2 is a transmembrane receptor that transduces signals from semaphorins, leading to axon repulsion in the vertebrate nervous system [20]. In recent years, it has been demonstrated that plexin-A2 binds to ligand semaphorin-6A (Sema6A) in granule cells [21] and mossy fibers [22]. To determine whether plexin-A2 could be the receptor of Sema6A in osteoclasts, we performed a pull-down assay using the recombinant mouse Sema6A Fc chimera proteins (Fc-Sema6A) to check whether Sema6A could precipitate plexin-A2 protein present in cell lysates of osteoclasts. The results showed that Sema6A bound to plexin-A2, and more plexin-A2 was precipitated when the employed absolute amount of Fc-Sema6A was increased (ranging from 100 ng to 500 ng) (Fig. 2A). Previous studies have also shown that the cyclin-dependent kinase 5 (Cdk5) binds to plexin-A2 through an active Fyn-dependent manner [21,23]. We found that Cdk5 was concurrently precipitated with plexin-A2 by Fc-Sema6A, suggesting that the binding between Sema6A and plexin-A2 in osteoclasts may trigger a cellular signal transduction cascade. To test this hypothesis, the status of Cdk5 in osteoclasts was detected following Sema6A stimulation, as its phosphorylation was reported to be induced downstream of plexin-A2 [21]. Indeed, the phosphorylation of Cdk5 was increased by Fc-Sema6A treatment in a dose-dependent manner (Fig. 2B), which was abrogated when plexin-A2 was neutralized by plexin-A2-specific antibody (Fig. 2C). Together, these data suggest that plexin-A2 serves as a receptor of Sema6A in osteoclasts and their binding initiates the downstream signal transduction.

3.3. Sema6A-plexin-A2 axis stimulates RANKL-induced osteoclastogenesis

The upregulation of plexin-A2 during RANKL-induced osteoclastogenesis and its binding to Sema6A led us to analyze the functional role of Sema6A-plexin-A2 axis in this process. We supplemented medium containing RANKL with Fc-Sema6A in the presence or absence of plexin-A2-specific antibody. TRAP staining assay revealed that the RANKL-induced osteoclastogenesis was stimulated by Sema6A, as evidenced by the increased number of TRAP-positive multinucleated cells (Fig. 3A–B) as well as enlarged area of cytoplasmic compartment of differentiated osteoclasts (Fig. 3A and C). However these effects vanished when plexin-A2 was blocked by specific antibody (Fig. 3), therefore indicating that Sema6A signaling through plexin-A2 promotes RANKL-induced osteoclastogenesis.

3.4. Sema6A-plexin-A2 axis enhances RANKL-induced activation of PLCγ and NFATc1

RANKL-induced osteoclastogenesis relies on the activation of NFATc1, the master regulator for osteoclastogenesis [24], for which three main routes play an important role, including pathways of TRAF6, c-Fos and PLCγ [25,26]. To gain insight into the mechanism by which Sema6A-plexin-A2 axis exerts its effect during RANKL-induced osteoclastogenesis, we monitored their expression and activity alterations. qRT-PCR results showed that the expression of NFATc1 was induced when stimulated with Sema6A, whereas those of TRAF6 and c-Fos were not obviously affected (Fig. 4A). The promoted induction of NFATc1 by Sema6A was further validated by its increased protein expression, as detected by immunoblotting, and this alteration was abolished when further treated with plexin-A2-specific antibody (Fig. 4B). These results suggested that NFATc1 was induced by the stimulation of Sema6A-

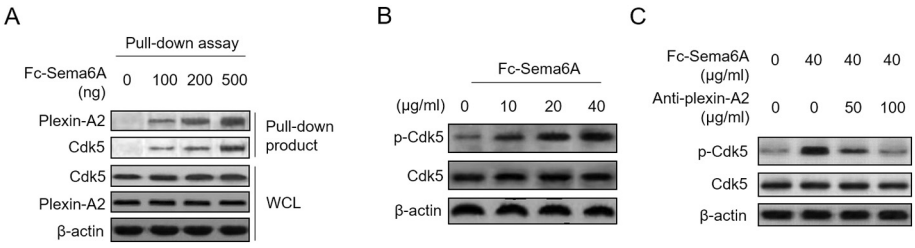

Fig. 2. Plexin-A2 functions as a receptor of Sema6A in osteoclasts.
(A) Cell lysates of osteoclasts were incubated with control IgG1 or increasing amount of Fc-Sema6A as indicated for 4 h. The whole cell lysates (WCL) and pull-down products were then analyzed by immunoblotting with antibodies to plexin-A2 and Cdk5. (B) BMMs were cultured with 50 ng/ml RANKL for 3 days, and further treated with increasing concentrations of Fc-Sema6A as indicated for 4 h. The protein expression of p-Cdk5 and Cdk5 was determined by immunoblotting. β-Actin was used as a loading control. (C) BMMs were cultured with 50 ng/ml RANKL for 3 days, and further treated with 40 ng/ml Fc-Sema6A for 4 h in the presence or absence of anti-plexin-A2. The protein expression of p-Cdk5 and Cdk5 was determined by immunoblotting. 50 ng/ml M-CSF was added into the culture medium throughout. β-Actin was used as a loading control. Experiments were conducted 3 times independently and representative images are shown.

plexin-A2 axis, however, during which TRAF6 and c-Fos pathways may be irrelevant. We next examined the status of PLCγ. As shown, the phosphorylation of PLCγ in osteoclasts was further increased by Sema6A, pointing to an exacerbated activation of this pathway (Fig. 4C). In addition, plexin-A2 blockade completely reversed this effect (Fig. 4C). Collectively, these findings suggest that Sema6A-plexin-A2 axis enhances RANKL-induced NFATc1 activation, which may be associated with activated PLCγ.

3.5. PLCγ inhibition abolishes Sema6A effect on RANKL-induced osteoclastogenesis

In order to clarify the contribution of PLCγ activation to the effect of Sema6A-plexin-A2 axis on NFATc1 activation and RANKL-induced osteoclastogenesis, we treated osteoclasts with U73122, a selective inhibitor of PLCγ [27]. The results showed that the promoted induction of NFATc1 by Sema6A addition in RANKL-induced osteoclastogenesis was totally recovered when PLCγ was inhibited by U73122 (Fig. 5A). In accordance with the change of NFATc1 activation, as shown by the number and area of TRAP-positive multinucleated cells, the

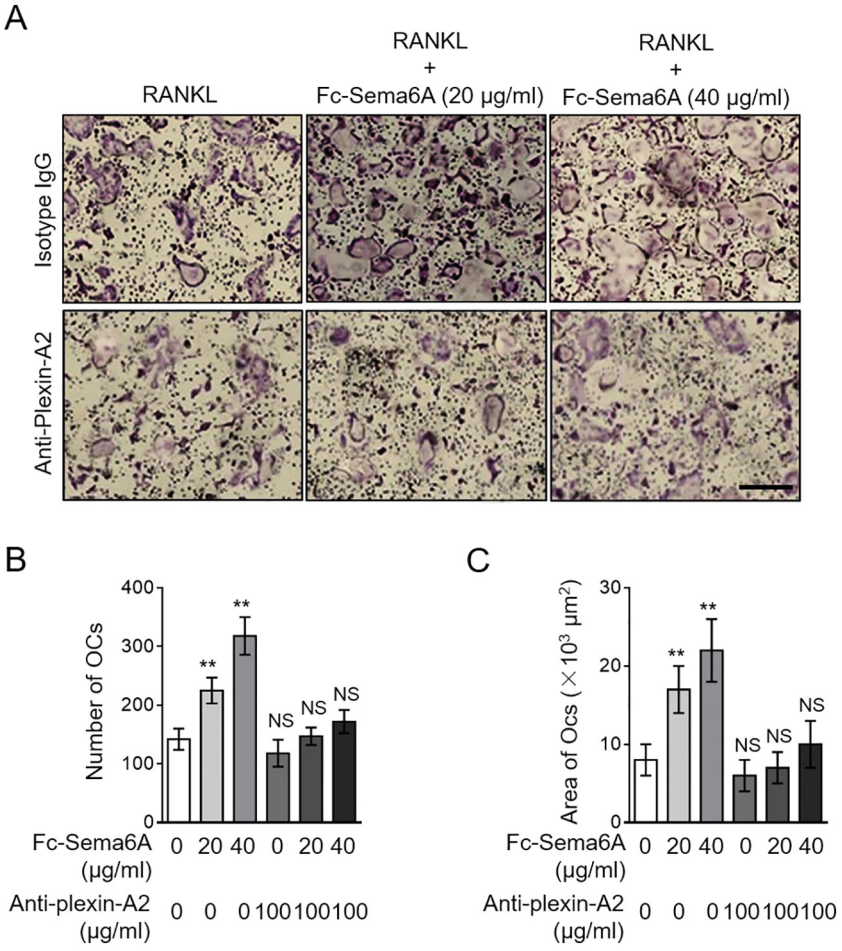

Fig. 3. Activation of Sema6A-plexin-A2 axis promotes RANKL-induced osteoclastogenesis.
(A) BMMs were cultured with 50 ng/ml RANKL for 3 days with or without addition of Fc-Sema6A or anti-plexin-A2 as indicated. Representative images of TRAP staining are shown. Scale bar, 100 μm. 50 ng/ml M-CSF was added into the culture medium throughout. (B–C) The number (B) and area (C) of TRAP-positive multinucleated cells shown in (A) were counted and analyzed. Data are mean ± s.d. Data were compared using one-way ANOVA followed by Dunnett's test. **, $P < 0.01$; NS, not significant.

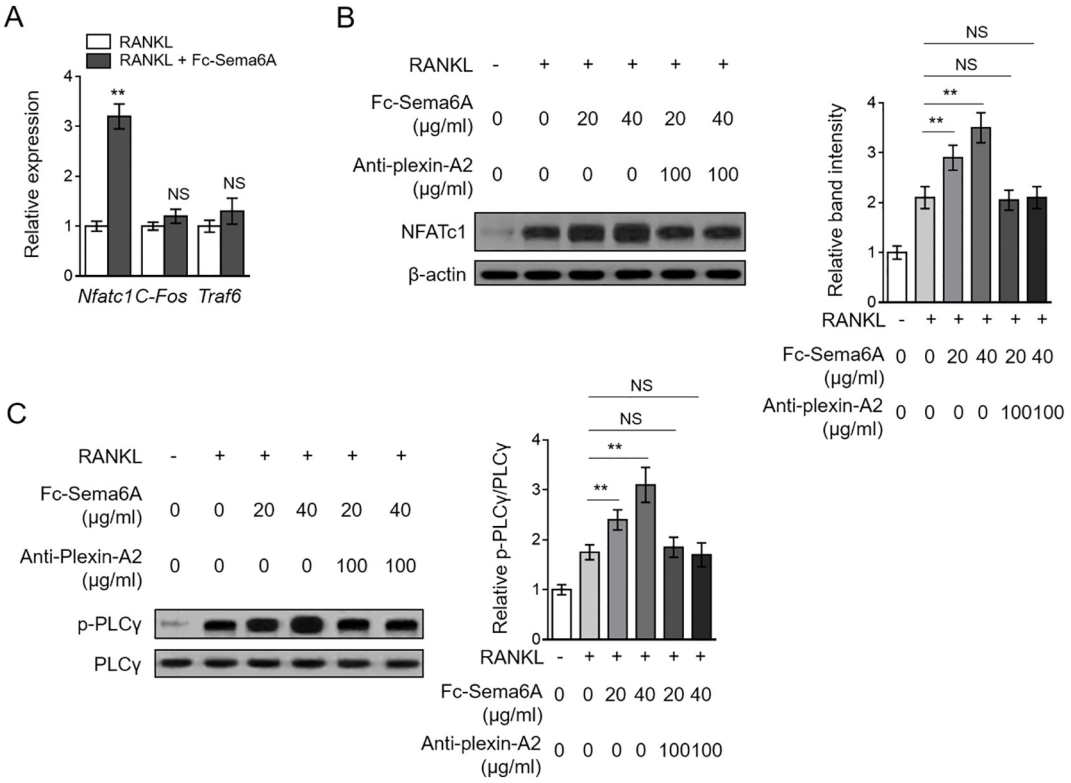

Fig. 4. Sema6A-Plexin-A2 axis stimulates RANKL-induced activation of PLCγ and NFATc1.
(A) BMMs were cultured with 50 ng/ml RANKL for 3 days with or without addition of Fc-Sema6A. The mRNA levels of NFATc1, C-FOS and TRAF6 were quantified by qRT-PCR analysis. β-Actin was used as an endogenous control. Results relative to RANKL alone group are shown. Data are mean ± s.d. Student *t*-test, **, $P < 0.01$; NS, not significant. (B–C) BMMs were cultured with 50 ng/ml RANKL for 3 days with or without addition of Fc-Sema6A or anti-Plexin-A2 as indicated. 50 ng/ml M-CSF was added into the culture medium throughout. The protein expression of NFATc1 (B) and p-PLCγ (C) was measured by immunoblotting. β-Actin or PLCγ was used as a loading control. The quantification of band intensity is shown at right. Data are mean ± s.d. Data were compared using one-way ANOVA followed by Dunnett's test. **, $P < 0.01$; NS, not significant.

exacerbation of RANKL-induced osteoclastogenesis by Sema6A stimulation was absent when further treated with U73122 (Fig. 5B-C). In sum, these lines of evidence suggest that the stimulation of RANKL-induced osteoclastogenesis by Sema6A-plexin-A2 axis depends on PLCγ pathway-mediated NFATc1 activation.

4. Discussion

The orchestrated regulation of bone remodeling through the participation of osteoclasts and osteoblasts is an important aspect encountered in the treatment of several bone and joint associated diseases, such as osteoporosis, rheumatoid arthritis, periodontitis and bone tumor [28,29]. The pathological bone resorption by osteoclasts could lead to excessive bone loss and several bone disorders, which renders osteoclasts as the key targets for the development of effective therapeutics [30]. In truth, most of the currently available treatments for intervening bone loss are based on disrupting osteoclast differentiation so as to inhibit bone resorption, for example bisphosphonate [31]. However, obvious side-effects were reported, such as osteonecrosis and femoral insufficiency fractures [32]. Therefore, searching novel regulators that control osteoclast differentiation may be of clinical significance in conceiving novel therapeutic strategy. In the present study, we show that plexin-A2, a transmembrane receptor previously known to mediate repulsive axon guidance in the nervous system [33,34],

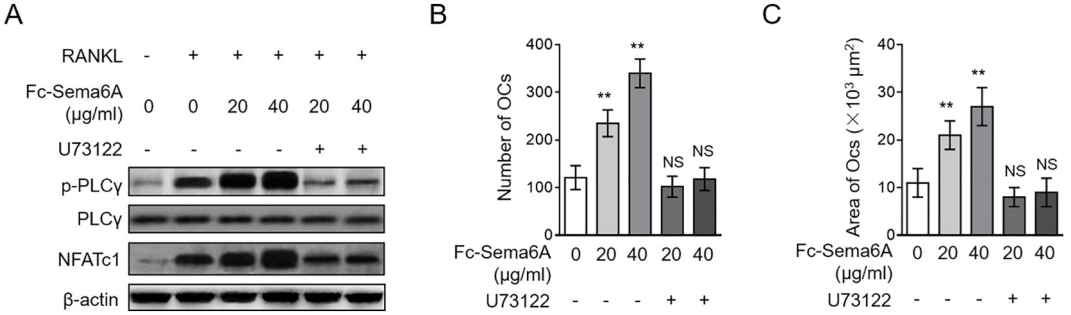

Fig. 5. Inhibition of PLCγ activity eliminates promotive effect of Sema6A on RANKL-induced osteoclastogenesis
(A) BMMs were cultured with 50 ng/ml RANKL for 3 days with or without addition of Fc-Sema6A or 10 μM U73122 as indicated. The protein levels of NFATc1, p-PLCγ, PLCγ were analyzed by immunoblotting. β-Actin was used as a loading control. (B–C) BMMs were treated as in (A). The number (B) and area (C) of TRAP-positive multinucleated cells differentiated by RANKL stimulation were counted, analyzed and presented. Data are mean ± s.d. Data were compared using one-way ANOVA followed by Dunnett's test. **, $P < 0.01$; NS, not significant.

serves as a receptor of Sema6A in osteoclasts, and that functionally, its signal transduction triggered by Sema6A stimulation promotes RANKL-induced osteoclastogenesis. Further mechanistic evidence suggests that this effect of Sema6A-plexin-A2 axis is dependent on PLCγ-induced activation of NFATc1, thus providing a molecular basis for targeting Sema6A-plexin-A2 axis as a potential therapeutic approach in the intervention of osteoclast-related bone diseases.

The expression of plexin-A2 was found to be upregulated during the course of RANKL-induced osteoclastogenesis at both transcript and protein levels, which implies a transcriptional activation of plexin-A2 gene when osteoclast differentiation occurs. Notably, this upregulation of plexin-A2 alone appears dispensable for osteoclast differentiation, since in BMMs untreated with Sema6A, plexin-A2 blockade using specific antibody did not obviously affect RANKL-induced formation of osteoclasts (Fig. 3A–C). Thus, it is very likely that the functional performance of upregulated plexin-A2 expressed by osteoclasts during differentiation is determined by the bioavailability of Sema6A and their interaction. Previous studies have shown that plexin-A2 interacts with Sema6A in sympathetic and sensory neurons, hippocampus and cerebellar granule cells [21,22]. We for the first time show that except for nervous system, plexin-A2 expression is detected in osteoclasts and is increased during their differentiation, and moreover, plexin-A2 could serve as a receptor of Sema6A, as demonstrated by pull-down assay in an in vitro system. These results indicate that the presence of plexin-A2 and Sema6A and their interaction are not limited to the nervous system. Further studies are required to elucidate the mechanisms by which plexin-A2 expression is regulated during osteoclast differentiation, and to examine whether the interaction between plexin-A2 and Sema6A also exists in other types of tissues and cells.

The treatment of Fc-Sema6A in osteoclasts induces the phosphorylation of Cdk5, suggesting that the binding of Sema6A with plexin-A2 triggers its activation and engenders bioactivity, as was previously found following the binding of Sema3A with plexin-A2 [23]. This similarity hints that plexin-A2 binding with either Sema6A or Sema3A shares a common signal transduction when activated. Cdk5 activation has been implicated in the regulation of neuronal migration as well as actin polymerization [35,36]. Whether Sema6A-induced plexin-A2 activation elicits certain morphological changes in osteoclasts needs to be addressed in the future.

We next found that Fc-Sema6A treatment stimulated RANKL-induced osteoclastogenesis through PLCγ-mediated activation of NFATc1, and this effect was abrogated when plexin-A2 was neutralized, hence emphasizing the important role of plexin-A2 in mediating the effect of Sema6A on osteoclastogenesis. The intracellular calcium oscillation downstream of PLCγ pathway activation is important for the activation of NFATc1 [7]. Noteworthily, NFATc1 is reportedly activated by both calcium oscillation-dependent and -independent pathways [37]. How Sema6A-plexin-A2 axis induces PLCγ activation and by what mechanism of action PLCγ induces subsequent NFATc1 activation remain obscure, and more intensive studies are warranted to clarify these issues. Moreover, it should be noted that PLC inhibitor U73122 has some side-effects which could possibly intervene our results. For instance, U73122 has been shown to inhibit RANKL-induced Ca^{2+} oscillations in BMMs [38]. It would be interesting to test whether Ca^{2+} oscillations contribute to the effect of Sema6A-plexin-A2 axis on RANKL-induced osteoclastogenesis. Other possible interfering factors which may not be easily excluded are the duration of U73122 stability and its modulatory role in the transient receptor potential channels [39]. Clarifying these issues may help us to better understand how exactly U73122 affects RANKL-induced osteoclastogenesis at a molecular level.

In recent years, semaphorins and their receptors have been shown to play a variety of roles in bone development, homeostasis and diseases [40]. Interestingly, plexin-A2 was reported to mediate osteoblast differentiation via regulating BMP2 signaling, although the ligand was not identified [41]. Nonetheless, together with our findings, we suspect that plexin-A2 may play a crucial role in bone remodeling, as it participates in the regulation of differentiation of both osteoclasts and osteoblasts. Further, the clinical relevance of plexin-A2 and Sema6A to bone and joint diseases associated with dysregulation of osteoclasts and osteoblasts merits deeper investigations, which may shed light on their contribution to disease physiopathology and help to discover new therapeutic approaches.

Disclosure of conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.060.

References

[1] G. Karsenty, E.F. Wagner, Reaching a genetic and molecular understanding of skeletal development, Dev. Cell 2 (2002) 389–406.
[2] R. Kitazawa, R. Haraguchi, M. Fukushima, S. Kitazawa, Pathologic conditions of hard tissue: role of osteoclasts in osteolytic lesion, Histochem. Cell Biol. 149 (2018) 405–415.
[3] J. Lorenzo, The many ways of osteoclast activation, J. Clin. Invest. 127 (2017) 2530–2532.
[4] J.B. Lian, G.S. Stein, A. Javed, A.J. van Wijnen, J.L. Stein, M. Montecino, et al., Networks and hubs for the transcriptional control of osteoblastogenesis, Rev. Endocr. Metab. Disord. 7 (2006) 1–16.
[5] M. Asagiri, H. Takayanagi, The molecular understanding of osteoclast differentiation, Bone 40 (2007) 251–264.
[6] M. Shinohara, T. Koga, K. Okamoto, S. Sakaguchi, K. Arai, H. Yasuda, et al., Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals, Cell 132 (2008) 794–806.
[7] L.H. Parker, M. Schmidt, S.W. Jin, A.M. Gray, D. Beis, T. Pham, et al., The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation, Nature 428 (2004) 754–758.
[8] Y. Zhou, R.A. Gunput, R.J. Pasterkamp, Semaphorin signaling: progress made and promises ahead, Trends Biochem. Sci. 33 (2008) 161–170.
[9] T. Negishi-Koga, M. Shinohara, N. Komatsu, H. Bito, T. Kodama, R.H. Friedel, et al., Suppression of bone formation by osteoclastic expression of semaphorin 4D, Nat. Med. 17 (2011) 1473–1480.
[10] N. Irie, Y. Takada, Y. Watanabe, Y. Matsuzaki, C. Naruse, M. Asano, et al., Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis, J. Biol. Chem. 284 (2009) 14637–14644.
[11] C. Zhao, N. Irie, Y. Takada, K. Shimoda, T. Miyamoto, T. Nishiwaki, et al., Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis, Cell Metab. 4 (2006) 111–121.
[12] J.Y. Hwang, J.Y. Lee, M.H. Park, K.S. Kim, K.K. Kim, H.J. Ryu, et al., Association of PLXNA2 polymorphisms with vertebral fracture risk and bone mineral density in postmenopausal Korean population, 17 (2006), pp. 1592–1601.
[13] Oh JE, Kim HJ, Kim WS, Lee ZH, Ryoo HM, Hwang SJ, et al. PlexinA2 Mediates Osteoblast Differentiation Via Regulation of Runx22012a.
[14] U. Harre, S.C. Lang, R. Pfeifle, Y. Rombouts, S. Fruhbeisser, K. Amara, et al., Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss, Nat. Commun. 6 (2015) 6651.
[15] Abràmoff MD, Magalhães PJ, Ram SJJBi. Image Processing With ImageJ. 2004;vol. 11:36–42.
[16] Y. Li, K. Wang, L. Chen, X. Zhu, J.J.M.M.B. Zhou, Quantification of mRNA Levels Using Real-Time Polymerase Chain Reaction (PCR), vol. 1406, (2016), pp. 73–79.
[17] I. Song, J.H. Kim, K. Kim, H.M. Jin, B.U. Youn, N. Kim, Regulatory mechanism of NFATc1 in RANKL-induced osteoclast activation, FEBS Lett. 583 (2009) 2435–2440.
[18] Luchin A, Purdom G, Murphy K, Clark MY, Angel N, Cassady AI, et al. The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. Journal of Bone and Mineral Research: the official journal of the American Society for Bone and Mineral Research. 2000;15:451–60.
[19] Feng H, Cheng T, Steer JH, Joyce DA, Pavlos NJ, Leong C, et al. Myocyte enhancer factor 2 and microphthalmia-associated transcription factor cooperate with NFATc1 to transactivate the V-ATPase d2 promoter during RANKL-induced osteoclastogenesis. J. Biol. Chem.. 2009;284:14667–76.
[20] Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, et al. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell. 1999;99:71–80.
[21] Renaud J, Kerjan G, Sumita I, Zagar Y, Georget V, Kim D, et al. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nat. Neurosci.. 2008;11:440–9.
[22] Suto F, Tsuboi M, Kamiya H, Mizuno H, Kiyama Y, Komai S, et al. Interactions between plexin-A2, plexin-A4, and semaphorin 6A control lamina-restricted projection of hippocampal mossy fibers. Neuron. 2007;53:535–47.

[23] Sasaki Y, Cheng C, Uchida Y, Nakajima O, Ohshima T, Yagi T, et al. Fyn and Cdk5 mediate semaphorin-3A signaling, which is involved in regulation of dendrite orientation in cerebral cortex. Neuron. 2002;35:907–20.
[24] Q. Zhao, X. Wang, Y. Liu, A. He, R. Jia, NFATc1: functions in osteoclasts, Int. J. Biochem. Cell Biol. 42 (2010) 576–579.
[25] Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, et al. Tyrosine Kinases Btk and Tec Regulate Osteoclast Differentiation by Linking RANK and ITAM Signals. 2008b;vol. 132:794–806.
[26] Zhao Q, Wang X, Liu Y, He A, Jia RJTijob, Biology c. NFATc1: Functions in Osteoclasts. 2010b;vol. 42:576–9.
[27] Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ, et al. Selective Inhibition of Receptor-coupled Phospholipase C-dependent Processes in Human Platelets and Polymorphonuclear Neutrophils. 1990;vol. 255:756–68.
[28] W. Xiao, S. Li, S. Pacios, Y. Wang, D.T. Graves, Bone remodeling under pathological conditions, Frontiers of oral biology 18 (2016) 17–27.
[29] W. Xiao, Y. Wang, S. Pacios, S. Li, D.T. Graves, Cellular and molecular aspects of bone remodeling, Frontiers of oral biology 18 (2016) 9–16.
[30] B.F. Boyce, E. Rosenberg, A.E. de Papp, L.T. Duong, The osteoclast, bone remodelling and treatment of metabolic bone disease, Eur. J. Clin. Investig. 42 (2012) 1332–1341.
[31] M.J. Rogers, J.C. Crockett, F.P. Coxon, J. Monkkonen, Biochemical and molecular mechanisms of action of bisphosphonates, Bone 49 (2011) 34–41.
[32] J.D. Isaacs, L. Shidiak, I.A. Harris, Z.L. Szomor, Femoral insufficiency fractures associated with prolonged bisphosphonate therapy, Clin. Orthop. Relat. Res. 468 (2010) 3384–3392.
[33] Maier V, Jolicoeur C, Rayburn H, Takegahara N, Kumanogoh A, Kikutani H, et al. Semaphorin 4C and 4G are ligands of Plexin-B2 required in cerebellar development. Mol. Cell. Neurosci.. 2011;46:419–31.
[34] H. Tawarayama, Y. Yoshida, F. Suto, K.J. Mitchell, H. Fujisawa, Roles of semaphorin-6B and plexin-A2 in lamina-restricted projection of hippocampal mossy fibers, J. Neurosci. 30 (2010) 7049–7060.
[35] T. Kawauchi, K. Chihama, Y. Nabeshima, M. Hoshino, Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration, Nat. Cell Biol. 8 (2006) 17–26.
[36] Z. Xie, B.A. Samuels, L.H. Tsai, Cyclin-dependent kinase 5 permits efficient cytoskeletal remodeling–a hypothesis on neuronal migration, Cereb. Cortex 16 (Suppl. 1) (2006) i64–i68.
[37] Y. Kuroda, C. Hisatsune, T. Nakamura, K. Matsuo, K. Mikoshiba, Osteoblasts induce Ca2+ oscillation-independent NFATc1 activation during osteoclastogenesis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 8643–8648.
[38] Seuk KM, Yu-Mi Y, Aran S, Shun TY, Syng-Ill L, Won KS, et al. RANKL-Mediated Reactive Oxygen Species Pathway that Induces Long Lasting Ca2+ Oscillations Essential for Osteoclastogenesis. 2010;vol. 285:6913–21.
[39] Leitner MG, Michel N, Behrendt M, Dierich M, Dembla S, Wilke BU, et al. Direct Modulation of TRPM4 and TRPM3 Channels by the Phospholipase C Inhibitor U73122. 2016;vol. 173:2555–69.
[40] S. Kang, A. Kumanogoh, Semaphorins in bone development, homeostasis, and disease, Semin. Cell Dev. Biol. 24 (2013) 163–171.
[41] Oh JE, Kim HJ, Kim WS, Lee ZH, Ryoo HM, Hwang SJ, et al. PlexinA2 mediates osteoblast differentiation via regulation of Runx2. Journal of Bone and Mineral Research: the official journal of the American Society for Bone and Mineral Research. 2012b;27:552–62.