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The induction of RANKL molecule clustering could stimulate early osteoblast differentiation

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ABSTRACT

We recently found that the membrane-bound receptor activator of NF-κB ligand (RANKL) on osteoblasts works as a receptor to stimulate osteoblast differentiation, however, the reason why the RANKL-binding molecules stimulate osteoblast differentiation has not been well clarified. Since the induction of cell-surface receptor clustering is known to lead to cell activation, we hypothesized that the induction of membrane-RANKL clustering on osteoblasts might stimulate osteoblast differentiation. Immunoblotting showed that the amount of RANKL on the membrane was increased by the RANKL-binding peptide OP3-4, but not by osteoprotegerin (OPG), the other RANKL-binding molecule, in *Gfp-Rankl*-transfected ST2 cells. Observation under a high-speed atomic force microscope (HS-AFM) revealed that RANKL molecules have the ability to form clusters. The induction of membrane-RANKL-OPG-Fc complex clustering by the addition of IgM in *Gfp-Rankl*-transfected ST2 cells could enhance the expression of early markers of osteoblast differentiation to the same extent as OP3-4, while OPG-Fc alone could not. These results suggest that the clustering-formation of membrane-RANKL on osteoblasts could stimulate early osteoblast differentiation.

1. Introduction

Receptor activator of NF-κB ligand (RANKL) is known to activate osteoclast formation and bone resorption activity through RANKL and the receptor activator of NF-κB (RANK) interactions [1,2]. We recently showed that the RANKL expressed on osteoblasts works as a receptor of RANKL-binding molecules, which activate osteoblast differentiation. This stimulatory signal in osteoblasts was designated "RANKL-reverse signaling", since the ligand works as a receptor [3]. However, osteoprotegerin (OPG), which is known to be a RANKL-binding molecule [2,4], does not have the anabolic effects on bone formation since the deletion of OPG does not reduce bone formation [5]. Furthermore, OPG-Fc, which like OPG has been used as a RANKL-binding molecule [6–8], did not stimulate the RANKL-reverse signaling or osteoblast differentiation in ST2 cells (a murine

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osteoblastic cell line) [3]. Thus, it became known that not all the RANKL-binding molecules always enhance osteoblast differentiation.

RANKL-binding peptides, another type of RANKL-binding molecule, have been shown to stimulate osteoblast differentiation and bone formation [9–12]. Both W9 and OP3-4 are known as RANKL-binding peptides [9,13–16], and they are also known to be able to activate mammalian target of rapamycin complex 1 (mTORC1) activity in osteoblasts [12]. Since mTORC1 activation has been demonstrated to occur downstream of RANKL signaling in osteoblasts [3], both RANKL-binding peptides are suggested to activate RANKL-reverse signaling. Furthermore, the activation of osteoblast differentiation by RANKL-binding peptides is retarded when RANKL-deficient osteoblasts are used [3,9], suggesting that the RANKL-binding peptides stimulate osteoblast differentiation under a RANKL-dependent mechanism. However, how the RANKL-binding peptides enhance osteoblast differentiation has not been clarified.

On the other hand, the induction of membrane receptor clustering is known to enhance cellular activities, through the mechanisms such as the stimulation of proliferation and/or differentiation [17] of several types of hematopoietic lineage cells. For example, Kosmides et al. showed that the magnetically-induced clustering of membrane molecules stimulated T cell activation [18]. Furthermore, small molecules that induce the dimerization of thrombopoietin receptors activate thrombopoietin-specific signal transduction in platelet precursors [19]. The induction of granulocyte-colony stimulating factor (GCSF)-receptor clustering activates granulopoiesis in hematopoietic lineage cells [19]. Furthermore, the induction of erythropoietin-receptor-dimerization also enhances the production of erythrocytes [20]. Thus, we hypothesized that the difference in osteoblast differentiation between OP3-4 and OPG could be explained by the different molecular characteristics following the formation of RANKL molecule clusters.

2. Materials and Methods

2.1. Culture conditions and the alkaline phosphatase (ALP) assay

ST2 (a murine osteoblastic cell line, RIKEN, Ibaragi, Japan) cells, were seeded at a density of 1×10^4/well and cultured in an osteogenic medium as described elsewhere [3,12].

For performing the ALP assay, we used 96-well plates. The culture conditions are described above. The culture was stimulated with 100 or 200 μM OP3-4 (YCEIEFCYLIR; the RANKL-binding peptide, which was designed based on the critical contact site on OPG for RANKL [15] (Atlantic Peptides, Lewisburg, PA, USA) (Fig. 1A)), 5 or 20 ng/ml OPG (Prospec-Tany Technogene, Rehovot, Israel). The conditioned medium was changed on day 3. ALP staining was performed on day 6 using a Lab Assay ALP (FUJIFILM Wako Pure Chemical Corp.) in accordance with the manufacturer's protocol. The ALP activity in each well was measured at 405 nm with a microplate reader (iMark, Bio-Rad, Hercules, CA, USA).

2.2. Cell surface RANKL detection

The *mouse Rankl* gene was subcloned into pEGFP-C1 vector. Plasmids encoded with green fluorescent protein (GFP) at the N-terminal were constructed by site-directed mutagenesis against pEGFP-based constructs [21].

For the preparations to detect the amount of surface GFP-RANKL, we used 12-well plates cultured for 72 h under the culture conditions as described in section 2.1., excluding ascorbic acid, β-glycerophosphate, and dexamethasone. After the first 24 h, *Gfp-mouse-Rankl* was transfected using Lipofectamine 2000 (a transfection reagent; Thermo Fisher Scientific, Waltham, MA, USA). ST2 cells were maintained in 100 or 200 μM OP3-4, 100 or 200 μM W9 (YCWSQYLCY; Atlantic Peptides) [13], or 20 ng/ml OPG for another 6 or 24 h. The cell surface proteins were biotinylated using EZ-Link sulfo-NHS-SS-biotin (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The cells were then lysed with a buffer composed of the following reagents; PBS, pH 7.4; 1.0% Triton X-100; protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail, Merck & Co., Rahway, NJ, USA, #5056489001). After lysing, the cells were centrifuged at 15,000×*g* for 10 min. The protein concentration of each supernatant was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific). Biotinylated protein in the supernatants was collected with Manga Bind Streptavidin Beads (Thermo Fisher Scientific). The blots were probed and proteins were detected with anti-GFP rabbit polyclonal (Thermo Fisher Scientific, #A-11122), anti-Na^+/K^+-ATPase, rabbit polyclonal (SantaCruz, Dallas, TX, USA, #sc-28800) and anti-GAPDH, rabbit monoclonal (Cell Signaling Technology, Danvers, MA, USA, #2118). Immunolabeled proteins were detected using anti-rabbit IgG, HRP-linked whole Ab donkey (GE Healthcare, Buckinghamshire, UK, #NA934) as corresponding secondary antibodies, and ECL prime reagents (GE Healthcare) were used for final detection.

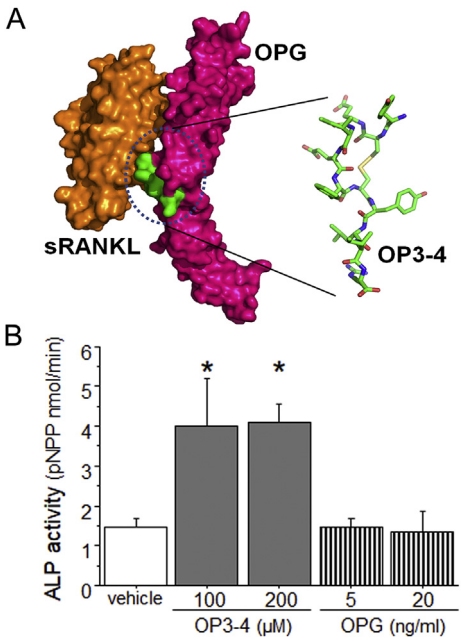

Fig. 1. OPG, a RANKL-binding molecule, did not enhance ALP activity, while OP3-4, a RANKL-binding peptide, stimulated it.
A. Scheme of OPG (purple)-RANKL (yellow) complex with putative location of OP3-4 binding (shown in green). OP3-4 was designed from the contact site of RANKL on OPG using structure-based approach.
B. OPG did not stimulate ALP activity. ST2 cells were cultured in osteogenic medium for 6 days in the presence of the indicated reagents. The ALP activity was measured as shown in 2.1. of the Materials and Methods section. Data are shown as the mean ± SD. *, $p < 0.01$ vs vehicle-control.

2.3. High-speed atomic force microscope (HS-AFM)

Observations were performed in tapping mode using a lab-made HS-AFM apparatus [22] and short cantilevers (BL-AC10DS, Olympus, Tokyo, Japan). For the observation of soluble RANKL (FUJIFILM Wako Pure Chemical Corp.) in the absence or presence of OP3-4, 100 μM RANKL dissolved in solution A (100 mM NaCl, 20 mM Tris-HCl pH 8.0) was deposited on a freshly cleaved mica surface. After 3 min of incubation, unattached RANKL was washed

out with solution A. For the observation of RANKL in the presence of OPG, a drop of 10 nM RANKL mixed with 10 nM OPG in solution A was deposited on mica. After 3 min of incubation, unattached RANKL and OPG was washed out with solution A. The HS-AFM imaging conditions were as follows: scan size and pixel size, 80×80 nm^2 (80×80 pixels), 100×100 nm^2 (100×100 pixels), or 50×50 nm^2 (80×80 pixels); imaging rate, 5.0–6.7 frames/sec (fps). Imaging was performed for RANKL alone in solution A, RANKL plus OP3-4 (100 μM) in solution A, and RANKL plus OPG in 50 mM Tris-HCl (pH 8.0).

2.4. mRNA quantification in the crosslink culture

We used 6-well plates and cultured ST2 cells for 5 days under the culture conditions described in section 2.1. and the cells were transfected as described in section 2.2. The stimulants in this assay were 0.09 nM OPG-Fc (BioLegend, San Diego, CA, USA), 0.45 nM mouse anti-human IgG Fc CH3 secondary antibody (IgM; LifeSpan BioSciences, Seattle, WA, USA), and OPG-Fc + IgM. To induce RANKL molecule clustering, we added IgM 1 h after adding OPG-Fc to the transfected cells. To check the marker-gene-expressions of early osteoblast differentiation, reverse transcription and a PCR were performed using cDNA from ST2 cells, with GoTaq PCR mix (Promega, San Luis Obispo, CA, USA). mRNA was quantified by a quantitative real-time PCR with SYBR GreenER qPCR SuperMix Universal (Life Technologies, Carlsbad, CA, USA) and an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). The software program and primers are described as follows;

5′-GAGTCAGATTACAGATCCCA-3′ and 5′-TGGCTCTTCTTACTGAGAGA-3′ for mouse *Runx2*, 5′-CCCCAAGATGTCTATAAGCC-3′ and 5′-CGCTCTAGCTCCTGACAGTT-3′ for mouse *Osterix*, 5′-CCCCAACCCTGGAAACAGAC-3′ and 5′-GGTCACGTTCAGTTGGTCAAAGG-3′ for mouse *Col1a1*, 5′-GGGCGTCTCCACAGTAACCG-3′ and 5′-ACTCCCACTGTGCCCTCGTT-3′ for mouse *Alp*, 5′-ATGTGTCCGTCGTGGATCTG-3′ and 5′-TGAAGTCGCAGGAGACAACC-3′ for mouse *Gapdh*.

2.5. Statistics

All of the data were expressed as the mean ± SD. A *t*-test with Bonferroni correction was used to analyze multiple comparisons between two groups. All statistical analyses were performed using the SPSS software program (ver. 22.0, IBM, Chicago, IL, USA).

3. Results

3.1. RANKL-binding molecules stimulated osteoblast differentiation

First, we performed an osteoblast differentiation assay to confirm the previous evidence showing that OP3-4 stimulates osteoblast differentiation but OPG does not. As shown in Fig. 1B, OP3-4, a RANKL-binding peptide, stimulated ALP activity, a marker of early osteoblast differentiation in the osteogenic culture condition, while OPG, which also binds to RANKL did not. The concentrations of OP3-4 and OPG used in Fig. 1B are known to enhance osteoblast differentiation and to inhibit osteoclastogenesis, respectively [12,23].

3.2. RANKL-binding peptide increased the amount of membrane-RANKL, but OPG did not

Since we thought that a certain amount of membrane-RANKL is necessary to induce the clustering of RANKL molecules on the osteoblast membrane, we tried to find the difference in the amount of membrane-RANKL after stimulation of RANKL-binding molecules in *Gfp-Rankl*-transfected ST2 cells. As shown in Fig. 2, both OP3-4 and W9, another RANKL-binding peptide, increased the amount of membrane-GFP-RANKL, after 6 h of stimulation, while OPG did not. The expression level of Na^+/K^+-ATPase, a standard cell-surface-marker protein, appeared to be similar in all conditions. After 24 h of stimulation with both peptides, the amount of membrane-GFP-RANKL was still higher in comparison to that after stimulation with OPG. On the other hand, the amount of GFP-RANKL in the whole cell lysates was similar among all conditions (Fig. 2), suggesting that the RANKL localization shifted from the intracellular organelle to the cell membrane after the stimulation with the RANKL-binding peptides. On the other hand, confocal laser scanning microscope (CLSM) images obtained from the cells in the same experimental conditions as shown in Fig. 2 did not show apparent changes of localization of GFP-RANKL after 6 h of stimulations of the RANKL-binding peptides or OPG (Supplementary Fig. 1), suggesting that the portion of the RANKL protein, which shifted from the intracellular organelle to the plasma membrane, was not a major portion of the RANKL protein compared to the whole amount of RANKL protein in the cells even in the immunoblotting analyses, which showed the apparent increase of the membrane-RANKL by the RANKL-binding peptides.

3.3. HS-AFM revealed the ability of RANKL molecules to form clusters and highly flexible OPG clinging to RANKL

To reveal the molecular features of RANKL in clustering, we observed RANKL molecules on mica in solution, by HS-AFM, which allows for the direct imaging of protein molecules in dynamic action without disturbing their function [24]. As shown in Fig. 3A, linear cluster-like structures were observed, as well as monomers with a height of ~1.5 nm. While the clusters were sometimes disassembled into smaller parts or monomers, oligomers including trimers or monomers sometimes made contact with other clusters, resulting in the formation of larger clusters. This cluster formation of RANKL was not inhibited by the presence of OP3-4 (Fig. 3B). Note that the HS-AFM apparatus could not image the OP3-4 molecules due to their small size (11 amino acids) and/or very fast diffusion on mica. The characteristic behaviors of RANKL molecules in the presence or absence of OP3-4 are shown in Video S1A.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.12.093.

Next, we imaged RANKL in the presence of OPG. As shown in Fig. 3C and Video S1B, one OPG molecule with high flexibility clung to and moved around to one RANKL molecule. This intermolecular interaction would prevent RANKL molecules from forming large clusters.

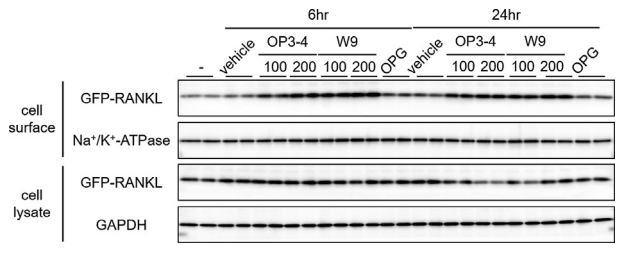

Fig. 2. The RANKL-binding peptides OP3-4 and W9 increased the membrane-RANKL, but OPG did not.

Gfp-mouse-Rankl was transfected to ST2 cells and the indicated reagents were added to the transfected cells 2 days after the transfection. The cells were lysed 6 h and 24 h after each condition, and biotinylated surface proteins were collected using streptavidin beads. Membrane-RANKL was detected using anti-GFP antibody. Na^+/K^+-ATPase was used as a representative cell surface protein. OP3-4 and W9: 100 or 200 μM, OPG: 20 ng/ml.

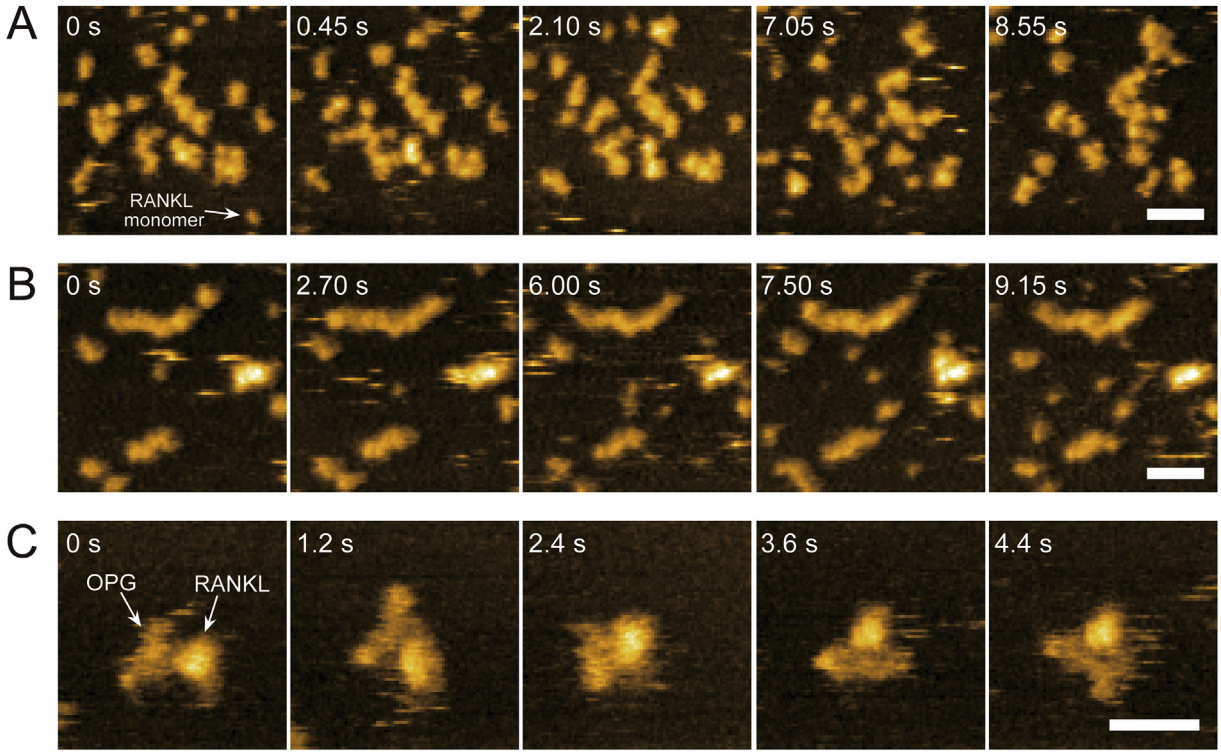

Fig. 3. HS-AFM imaging revealed the formation of clusters of RANKL molecules and highly flexible OPG clinging to RANKL.
Representative HS-AFM images showing molecular interaction among RANKL molecules in the absence (A) and presence (B) of 100 μM OP3-4. (C) HS-AFM images showing molecular interaction between RANKL and OPG. Scale bars, 20 nm. The detailed experimental conditions are shown in the Materials and Methods section. See also Videos S1A and B.

3.4. Cross-linker of the OPG-Fc-RANKL complex stimulated early osteoblast differentiation

The mRNA expression of osteoblast differentiation markers was investigated when IgM was used as a cross-linker of Fc-fusion protein. First we found the enhanced expression of markers of early osteoblast differentiation (*Runx2*, *Osterix*, *Col1a1* and *Alkaline phosphatase*), in the OP3-4-treated *Gfp-Rankl*-transfected ST2 cells (Fig. 4A). These osteoblast differentiation markers were significantly enhanced when IgM was added to the OPG-Fc culture, while they were not enhanced in the presence of OPG-Fc or IgM alone (Fig. 4A). Since the amount of membrane-RANKL might be a key to stimulating osteoblast differentiation, we also performed immunoblotting to clarify the difference in the amount of membrane-RANKL after using IgM, a cross linker of OPG-Fc fusion protein (Fig. 4B). As we expected, the amount of membrane-RANKL protein appeared to show a similar tendency to the markers of osteoblast differentiation (Fig. 4B).

4. Discussion

In this study, we attempted to reveal reason why RANKL-binding peptides simulate stimulate osteoblast differentiation, but OPG does not. Both OPG and OP3-4 are RANKL-binding molecules, but the affinity of OPG to RANKL is much higher than that of OP3-4 [1,15,23], suggesting that we cannot explain the difference of both molecules on osteoblast differentiation by comparing the difference in affinity to RANKL. We mainly showed the difference that both molecules made on the amount of RANKL on the osteoblast membrane at 6 and 24 h after stimulation. Immunoblotting showed that OP3-4 enhanced the amount of membrane-RANKL on the cell surface, but that OPG did not (Fig. 2). Based on the HS-AFM data showing that RANKL molecules have an ability to cluster together (Fig. 3A), it is considered that the higher amount of RANKL molecules on the cell membrane induced by OP3-4 means that more clusters can form on the cell membrane at a higher rate in comparison to when a smaller amount of RANKL is induced by OPG. The osteoblast differentiation stimulated upon OPG-Fc crosslinking by IgM is possibly accounted for by the increased RANKL clustering on the cell membrane through the crosslinked OPG (Fig. 4A). Although the signaling to increase the amount of RANKL by the induction of the crosslinking is not clarified yet, the accumulation of RANKL on the cell membrane might be taken place in the similar way as shown in the previous study using the RANK-coated beads [21,25]. On the other hand, a single OPG molecule was observed to cling to a RANKL molecule (Fig. 3C). This sticky intermolecular association is very likely to prevent RANKL clustering, which could be a key to clarify why OPG has no stimulatory effect on osteoblast differentiation.

As mentioned earlier, we have found that RANKL-reverse signaling stimulated osteoblast differentiation [3]. In this study, OP3-4 probably worked like an agonist, binding to the membrane-RANKL to enhance osteoblast differentiation (Figs. 1B and 4A). The amount of membrane-RANKL after 6 h of OP3-4 stimulation was higher in comparison to the vehicle-control (Fig. 2), suggesting that some unknown signaling might be initiated to the osteoblastic cells by OP3-4. The induction of RANKL clustering was probably took place on the osteoblastic cell membrane. Kariya et al. showed that RANKL was predominantly expressed in the intracellular compartments of ST2 with an almost undetectable level of RANKL in the plasma membrane [21], suggesting almost no endogenous RANKL protein was expressed on the ST2 plasma membrane in this study, either. Both the accumulation of RANKL protein and the formation of RANKL clusters might be an induction-switch of both the enhancement of osteoblast differentiation and the accumulation of RANKL molecules on the cell membrane. Further studies are

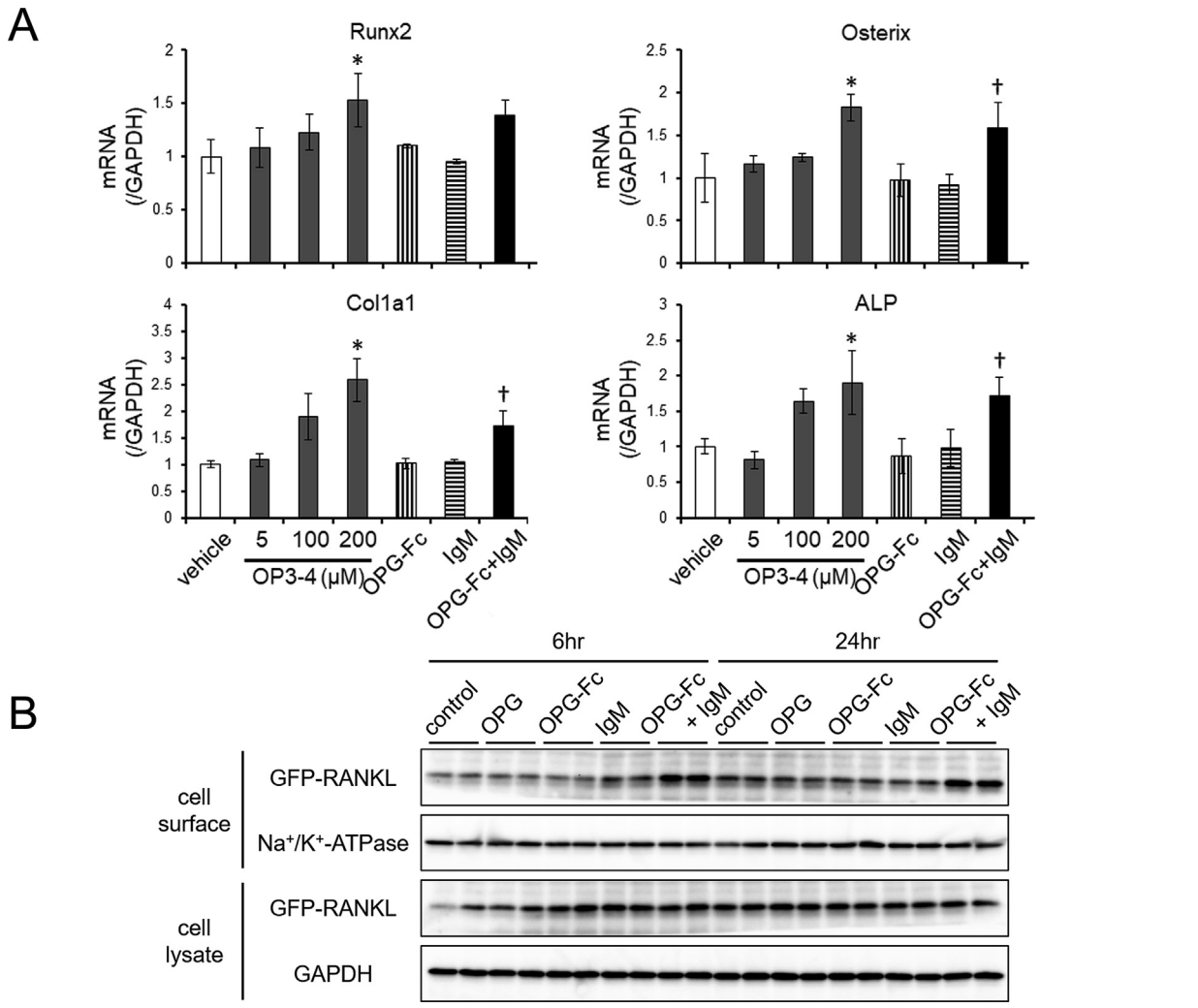

Fig. 4. OPG-Fc increased markers of early osteoblast differentiation when RANKL molecules were oligomerized by crosslinking OPG-Fc
A. Comparison of the mRNA expression levels of early osteoblast differentiation markers in ST2 cells after 5 days of stimulation with OP3-4, OPG-Fc, IgM, or OPG-Fc + IgM. Data are shown as the means ± SD. *, $p < 0.05$ vs vehicle-control. †, $0.05 < p < 0.1$ vs vehicle-control.
B. Comparison of the RANKL expression on ST2 cells stimulated with OPG, OPG-Fc, IgM, or OPG-Fc + IgM.

needed to clarify the details of this stimulatory signaling and whether the signaling is the same as the reverse signaling as shown in our previous study [3,12].

In conclusion, we showed that after OP3-4 stimulation, the amount of RANKL on the plasma membrane of *Gfp-Rankl*-transfected ST2 cells became higher in comparison to after OPG stimulation. HS-AFM revealed the characteristic ability of RANKL molecules to form clusters, and highly flexible OPG clinging to RANKL prevented productive clustering. Since the induction of the clustering of RANKL molecules on the plasma membrane stimulated early osteoblast differentiation, the induction of RANKL-clustering on the membrane could be a molecular switch that induces osteoblast differentiation.

Conflicts of interest

The authors declare no conflicts of interest in association with the present study.

Author contributions

E.S. designed the study, performed experiments, analyzed data and wrote the manuscript. D.N. performed HS-AFM analyses. Y.I. performed immunoblot analyses. M.N. and T.F. obtained the CLSM images. M.K. supervised for cell culture. Y.T. taught the skills for cell culture. M.I. assisted statistical analysis. M.O. advised and edited the manuscript. R.M., T.Y., M.H. and H.S. provided critical advices to the manuscript. T.A. supervised the AFM experiments and edited the manuscript. K.A. designed and supervised the study and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.093.

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