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Running Title: Role of C/EBPβ in 1,25D3-induced RANKL expression

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Abstract

Receptor activator of nuclear factor kappa B ligand (RANKL) expression in osteoblasts is regulated by 1,25-dihydroxyvitamin D3 (1,25D3). CCAAT/enhancer-binding protein beta (C/EBPβ) has been proposed to function as a transcription factor and upregulate RANKL expression, but it is still uncertain how C/EBPβ is involved in 1,25D3-induced RANKL expression of osteoblasts. 1,25D3 stimulation increased the expression of RANKL and C/EPBβ genes in osteoblasts and enhanced phosphorylation and stability of these proteins. Moreover, induction of RANKL expression by 1,25D3 in osteoblasts was downregulated upon knockdown of C/EBPβ. In contrast, C/EBPβ overexpression directly upregulated RANKL promoter activity and exhibited a synergistic effect on 1,25D3-induced RANKL expression. In particular, 1,25D3 treatment of osteoblasts increased C/EBPβ protein binding to the RANKL promoter. In conclusion, C/EBPβ is required for induction of RANKL by 1,25D3.

Keywords: 1,25-Dihydroxyvitamin D3 (1,25D3), RANKL, C/EBPβ, Osteoblasts

INTRODUCTION

Bone constantly cycles through formation and absorption, which is marked by coordinated activities between osteoclasts and osteoblasts (1). Osteoclasts, multinucleated cells derived from precursor monocyte lineages, play a critical role in bone resorption (2). Two molecules are necessary for mature osteoclasts: macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (3). RANKL expression in osteoblasts is critical for maintenance and coordination of the osteoblast-osteoclast process (4). Osteoblasts induce expression of RANKL through various cytokines and hormones. In particular, vitamin D3 is the key hormone that promotes osteoblastic activity and bone formation (5).

1,25-Dihydroxyvitamin D3 (1,25D3), a vitamin D_3 active metabolite, is known to induce expression of RANKL in osteoblasts and stromal cells through a vitamin D receptor (VDR) (6, 7). In RANKL regulation, vitamin D-responsive element (VDRE) and RUNX2 are responsible for the promoter located at the distal and proximal region, respectively (8-11). It is well known that 1,25D3 increases RANKL expression, but RUNX2 is more controversial since there are reports that it does not contribute to RANKL expression (12, 13). Thus, there may be other candidate genes that respond to 1,25D3, and their function can be regulated for RANKL expression.

1,25D3 induces CCAAT/enhancer-binding protein beta (C/EBPβ) via its binding site in the proximal region of RANKL (14, 15). Although the upregulation of RANKL by 1,25D3 has been intensively investigated, the relationship between the RANKL and C/EBPβ activation by 1,25D3 and the functional role of C/EBPβ in osteoblasts are not fully understood.

Here, we demonstrate that C/EBPβ, which is responsive to 1,25D3, potentiates RANKL expression. By overexpressing and knocking down C/EBPβ, we show that modulation of the gene directly contributes to activation and expression of the RANKL promoter.

RESULTS

1,25D3 significantly increases VDR and C/EBPβ gene expression in murine osteoblasts

High-throughput data with the accession number GSE51515 were downloaded from Gene Expression Omnibus (GEO) and analyzed. Using a publicly available microarray dataset, we investigated gene expression alterations after treatment with 10^{-7} M 1,25D3 for 24 hours. To assign a putative functional change in bone development, genes whose GO terms were related to bone development were selected. As a result, 122 genes were identified as bone development-related genes (Suppl. Fig. 1). Among them, 17 genes (*NPR3, COL5A2, DDX5, PTK2, FBN1, FST, GNAQ, HOXA10, FOXC1, PIAS2, VDR, RUNX1, C/EBPβ, SMAD3, SERP1, ASH1L,* and *RASSF2*) were upregulated when the POBs were treated with 1,25D3, and 6 genes (*GDF4, RUNX2, EPHA2, MMP9, UCMA,* and *USP1*) were downregulated (Fig. 1). *RANKL*, also known as *TNFSF11*, was upregulated, but it was not statistically significant (indicated by the red arrow in Suppl. Fig. 1). We noted that 1,25D3 resulted in increases in *C/EBPβ* and *RANKL* gene expression.

1,25D3 accelerates osteoblastic differentiation

To investigate cellular effects of 1,25D3 on osteoblasts, we evaluated cell viability and toxicity in both MG63 and SaOS2 osteoblasts that were treated with various concentrations of 1,25D3 for 3 days. There was no significant difference in cell viability or toxicity at 0 and 20 nM doses of 1,25D3, but these effect were observed at a higher dose of 50 nM (Suppl. Fig. 2A and B). In this situation, ALP activity was increased by 1,25D3 in a dose-dependent manner in MG63 osteoblasts but decreased with 20 nM 1,25D3 in SaOS2 osteoblasts (Suppl. Fig. 2C). Therefore, we selected concentrations of 10 and 20 nM for activation of osteoblast-related gene promoters. Luciferase activities of the known osteoblast-specific *ALP, OSE, OCN,* and *BSP* promoters were significantly elevated 2-3 fold by 20 nM 1,25D3 treatment (Suppl. Fig. 2D). Consistent with previous reports, addition of 1,25D3 under osteogenic stimuli resulted in

increased osteoblastic differentiation, as shown with ALP and Alizarin Red (ARS) staining (Suppl. Fig. 3). These data confirm that 1,25D3 has a potent effect on promoting osteoblast differentiation.

1,25D3 induces RANKL expression via regulation of C/EBPβ

As shown in Fig. 2A, increases in *CYP24A* mRNA and VD3R protein levels were affected by 1,25D3 treatment. In this situation, we observed that expression of RANKL and C/EBPβ was elevated in both MG63 and SaOS2 cells when treated with 1,25D3 in a time- and dose-dependent manners (Fig. 2A and Suppl. Fig. 4). ALP, OCN, C/EBPβ, and RANKL were increased in osteoblasts treated with 1,25D3 (Suppl. Fig. 5 and Fig. 2B). Intriguingly, upregulation of *RANKL* expression in response to 1,25D3 was attenuated by the *C/EBPβ* knockdown at the mRNA and protein levels (Fig. 2C). In addition, 1,25D3 treatment activated the *RANKL* promoter (approximately 2Kb) in both osteoblast cell lines (Suppl. Fig. 6), but showed relatively higher response in the promoter within 1Kb (Fig. 2D). Therefore, 1,25D3 induces *RANKL* expression by regulating *C/EBPβ*.

1,25D3 enhances phosphorylation and stability of the C/EBPβ protein

To further assess the regulation of the C/EBPβ protein by 1,25D3 in osteoblasts, we stimulated osteoblasts with various concentrations of 1,25D3 for 6 h and then analyzed phosphorylation of p38 and ERK protein, two 1,25D3 responsive kinases. 1,25D3 induced the phosphorylation of p38, ERK, and C/EBPβ proteins in a dose-dependent manner (Fig. 3A). We also used SB203580, a p38 inhibitor, and PD98059, an ERK inhibitor, to confirm an effect on the phosphorylated C/EBPβ protein. The two inhibitors prevented C/EBPβ-Thr235 phosphorylation through 1,25D3 without a change in VD3R protein (Fig. 3B). We next used the inhibitors cycloheximide (CHX) to inhibit protein synthesis and MG132 to inhibit the proteasome in order to observe changes at the protein level. VD3R, C/EBPβ, and RANKL proteins were reduced in the vehicle, whereas these proteins were only modestly reduced in

response to 1,25D3 (Fig. 3C). Moreover, 1,25D3-induced VD3R, C/EBPβ, and RANKL proteins were degraded by the proteasome pathway (Fig. 3D). These results suggest that 1,25D3 enhances the phosphorylation and stability of C/EBPβ protein.

1,25D3-induced upregulation of C/EBPβ contributes to *RANKL* expression in osteoblasts

To extend on the above findings, we speculated that 1,25D3 might positively regulate changes in *RANKL* expression via C/EBPβ. Overexpression of *C/EBPβ* in combination with 1,25D3 stimulation exhibited a synergistic effect on *RANKL* expression in comparison to either 1,25D3 or *C/EBPβ* alone (Fig. 4A and B). *C/EBPβ* overexpression markedly induced *RANKL* promoter activity (Suppl. Fig. 7) and its expression was quantified by qRT-PCR (Suppl. Fig. 7, lower panel). *C/EBPβ* overexpression significantly also induced the proximal region (less than 1Kb) compared to a 2Kb promoter region of human *RANKL* gene (Fig. 4C). Interestingly, #1 of sites of three putative C/EBPβ binding sites on the proximal *RANKL* promoter was selectively enhanced in response to 1,25D3-induced C/EBPβ protein (Fig. 4D). Taken together, these findings indicate that 1,25D3-induced upregulation of C/EBPβ contributes to *RANKL* expression in osteoblasts.

DISCUSSION

In this study, we sought to identify the signaling pathways and transcription factors involved in stimulation of 1,25D3-induced C/EBPβ and RANKL gene expression. We observed that 1,25D3 results in p38- and ERK-dependent activation and phosphorylation of C/BEPβ in both MG63 and SaOS2 osteoblasts. Furthermore, C/EBPβ knockdown slightly reduced RANKL expression and attenuated 1,25D3-induced the gene level. In contrast, 1,25D3 and C/EBPβ overexpression had a synergistic effect on RANKL expression. Finally, we demonstrated that, in response to 1,25D3, C/EBPβ binds directly to the RANKL promoter in osteoblasts.

Vitamin D is an important regulator of bone mineralization and metabolism. 1,25D3 is the most active metabolite of vitamin D_3, with high affinity for nuclear VDR (16). Hydroxylation of vitamin D metabolites at D-24 hydroxylase (CYP24A1) is the first step in metabolite inactivation and excretion (17). Basal expression of CYP24A1 is usually low, but 1,25D3 strongly induces CYP24A1 gene expression (18). Consistent with previous results, we observed increases in VD3R protein and CYP24A1 mRNA levels in response to 1,25D3 (Fig. 2A and B).

It is well known that C/EBPβ is a critical factor during adipocyte and chondrocyte differentiation (19). C/EBPβ is an important indicator of differentiation and control of target gene transcription. Despite the fact that C/EBPβ is necessary for osteoblastic activity and bone formation via RUNX2 and ATF4 regulation (20), there are fewer reports on C/EBPβ function in osteoblasts than in other cell lineages. Our previous work has revealed that RUNX2 and C/EBPβ proteins in bone-derived cells cooperate and promote IL-23 expression in ankylosing spondylitis (AS) (21). In addition, inflammatory cytokines in AS patient sera stimulate RUNX2 and C/EBPβ proteins via osteogenic induction (22, 23). In this study, we propose that induction of the RANKL gene by 1,25D3 is mediated by C/EBPβ. We therefore believe that the function of the C/EBPβ gene is a crucial factor in osteoblasts and in bone research.

Molecular RANKL and OPG play key roles in regulating physiological and pathological bone homeostasis. In general, RANKL can be expressed in three different molecular forms consisting either of (1) a membrane-bound form, (2) a secreted form, or (3) an intercellular form. It has been reported that soluble RANKL is produced by B cells (24), neutrophils (25), activated T cells (26), synoviocytes (27), and osteoblasts (4). Relatively less is known about the molecular mechanism of RANKL in osteoblasts and its progenitor cells. In this study, 1,25D3 treatment in osteoblasts specifically induced upregulation of RANKL expression without changes in OPG mRNA (Fig. 2B). We tested secreted RANKL from the culture supernatant using an enzyme-linked immunosorbent assay (ELISA), and there was no detectable soluble RANKL in response to 1,25D3 (data not shown). There are two possible explanations for this. 1,25D3-dependent induction of RANKL in osteoblasts might produce either a transmembrane form to contact and interact with osteoclast precursors for osteoclast activation or intracellular form to accumulate in cells.

Previous studies have revealed many putative binding sites in the human RANKL promoter (8): C/EBPβ, VDRE, heat shock factor 2 (HSF2), cAMP-responsive element-binding protein (CREB), runt-related transcription factor 2 (RUNX2), and nuclear factor-erythroid-derived 2 (NF-E2). We analyzed whether either the C/EBPβ or RUNX2 gene was modulated in the presence of 1,25D3. Interestingly, the cells responded to 1,25D3 by increasing C/EBPβ expression and decreasing RUNX2 gene expression, as shown in the microarray data in Figure 1. We compared luciferase activity of the RANKL promoter to the expression of the RUNX2 and C/EBPβ genes. As expected, the RANKL promoter was dramatically induced in transduction of the C/EBPβ gene than RUNX2, indicating that C/EBPβ is an essential factor for RANKL expression (data not shown).

We provided evidence of a critical role for 1,25D3 in mediating the phosphorylation and stabilization of C/EBPβ, which facilitates RANKL gene upregulation, and contributes to our understanding of RANKL expression in osteoblasts.

MATERIALS AND METHODS

Cell lines, chemicals, and plasmids

Both MG63 and SaOS2 osteoblast cell lines were obtained from Heekyoung Chung (Department of Pathology, College of Medicine, Hanyang University) and Korean Cell Line Bank (Seoul, Korea). MG63 or SaOS2 was grown in high-glucose DMEM medium (Hyclone, SH30243.01) or RPMI1640 (Hyclone, SH30027.01) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10082-147) and 1× antibiotics (Gibco, 15140-122). 1,25D3 (Sigma-Aldrich, D1530) was dissolved in absolute ethanol. For *in vitro* drug treatment, 1,25D3 was prepared as a 20 μM stock solution and diluted in fresh medium at the indicated concentrations. SB203580 (559398) and PD98059 (513001) were purchased from Merck Millipore, and DMSO was used as the vehicle. Osteoblast-related promoters of alkaline phosphatase (ALP), osteocalcin (OCN), osteoblast-specific elements (OSE), and bone sialoprotein (BSP) were provided by Dr. Kwang Youl Lee (College of Pharmacy, Chonnam National University, Gwangju, Korea) (28). C/EBPβ and the empty vector were generously provided by Dr. Yung Jong Lee (Division of Rheumatology, Department of Internal Medicine, Seoul National University Bundang Hospital) (29). The RANKL promoter was generously provided by Dr. Sakamuri V. Reddy (Department of Pediatrics, Medical University of South Carolina) (30). siRNA was obtained from Genolution (Seoul, Korea). siRNA information is given in Supplementary Table 3.

Gene Expression Omnibus (GEO) analysis

Gene expression datasets were downloaded from the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) under the accession numbers GSE41954 and GSE51514 (31). MC3T3-E1 cells (pre-osteoblasts, POBs) were treated with vehicle or 10^{-7} M 1,25D3 for 24 hours. RNA was then isolated and applied to gene expression microarrays. The microarray experiments were conducted in biological triplicate. Differentially expressed genes were

assessed by a linear regression method using the R/Bioconductor limma package (32). An adjusted p-value<0.05 was considered statistically significant. All microarray analyses and visualization were conducted using R 3.4.1 (www.r-project.org).

Luciferase assay

Cells were co-transfected with each promoter and renilla using Lipo3000 (Thermo Fisher, L3000008) and then stimulated as indicated. The luciferase assay was assessed according to the manufacturer's protocol (Promega, E1500) and measured by a Panomics Luminometer (Gentaur, Kampenhot, Belgium).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays (Millipore, 17-295) were performed as previously described (33). In brief, the cells stimulated in 10 cm dishes were fixed with a final concentration of 1% formaldehyde, sonicated in 1× PBS with protease inhibitor, and then immunoprecipitated with protein A agarose beads conjugated to the C/EBPβ antibody. DNA purification was performed using the phenol/chloroform/isoamyl alcohol (Sigma-Aldrich, P3803) method. DNA was precipitated using 3 M sodium acetate and eluted with DEPC water. Approximately 5-10 ng of eluted DNA was used for quantitative RT-PCR. The quantification and calculation of ChIP-qPCR have been previously described (34). The primers used in the ChIP assays are provided in Table 2 of the Supplementary data (14).

Other methods

Immunoblotting, qRT-PCR, immunofluorescence, measurement of cell viability and toxicity, and other procedures are described in the Supplementary Data.

Statistical analysis

All experiments were carried out at least three times, and data consistency was observed in repeated experiments. Differences between groups were analyzed by the Mann-Whitney U test. GraphPad Prism5.0 was used for statistical analysis and to present reported data. $p<0.05$ was

considered statistically significant.

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CONFLICTS OF INTEREST: The authors have no conflicts of interest to declare.

FIGURE LEGENDS

Figure 1. 1,25D3 significantly increases VDR and C/EBPβ gene expression in murine osteoblasts. Using microarray datasets, we investigated gene expression changes in response to a treatment of 10^{-7} M 1,25D3 for 24 h. In order to assign putative functional changes in bone development, genes whose GO terms were related to bone development were selected. In total, 122 genes were identified as bone development-related. A heat map of bone development-related genes in POB cells treated with vehicle or 1,25D3. Blue, low expression; red, high expression. Among them, 24 genes with RANKL (marked in black) were differentially expressed.

Figure 2. 1,25D3 induces RANKL expression via regulation of C/EBPβ. (A)Both MG63 and SaOS2 cells were treated with 1,25D3 as indicated, followed by immunoblotting (upper panel) and qRT-PCR (lower panel) (n=5). (B) The cells were stimulated with 20 nM 1,25D3 for 24 h, followed by immunofluorescence using RANKL and C/EBPβ (n=3). Scale bar is 50 μm. (C) The cells were transfected with siRNA against C/EBPβ and control (CON) using Lipo3000, incubated for 48 h, and stimulated with 20 nM 1,25D3 for 24 h. The stimulated cells were subjected to immunoblotting (upper panel) and qRT-PCR (lower panel) (n=5). (D) The cells were transfected with indicated deletion mutants of RANKL promoter, incubated for 48 h, treated with 1,25D3 for 24 h, and then analyzed with luciferase assay (n=4). The Mann-Whitney U test was performed to determine statistical significance. Data are presented as mean ± SD. *P* values indicate significant differences between two groups. *$p<0.05$;

Figure 3. 1,25D3 enhances C/EBPβ protein phosphorylation and stability. (A) Both MG63and SaOS2 cells were stimulated with 1,25D3 as indicated for 6 h. (B) The cells were pre-treated with SB203580 or PD98059 for 5 min and stimulated with 20 nM 1,25D3 for 6 h. (C) The cells were pre-treated with vehicle-alone (Ethanol) or 20 nM 1,25D3 for 1 day and harvested after stimulation with 20 μg/mL cyclohexamide (CHX) at the indicated times. (D)

The cells were pre-treated with MG132 for 5 min and stimulated with 20 nM 1,25D3 for 6 h. All samples were subjected to immunoblotting. All experiments were carried out at least four times, and data consistency was observed between experiments. Representative images are shown.

Figure 4. 1,25D3-induced upregulation of C/EBPβ contributes to *RANKL* expression in osteoblasts. Both MG63 and SaOS2 cells were transduced with *C/EBPβ* (2.5 μg) or empty vector for 48 h, treated with 1,25D3 for 24 h, and then analyzed by (A) immunoblotting (n=5) or (B) qRT-PCR (n=5). (C) Indicated deletion mutants of RANKL promoter was transiently co-transfected with C/EBPβ (2.5 μg) or empty vector in Both MG63 and SaOS2 cells. The transfected cells were incubated for 48 h and then analyzed using a luciferase assay (n=4). (D) Cells were stimulated with 20 nM 1,25D3 for 24 h and then analyzed with a chromatin immunoprecipitation (ChIP) assay using the C/EBPβ antibody (MG63, n=4; SaOS2, n=4). The Mann-Whitney U test was performed to determine statistical significance. Data are presented as mean ± SD. *P* values indicate significant differences between two groups. *$p<0.05$

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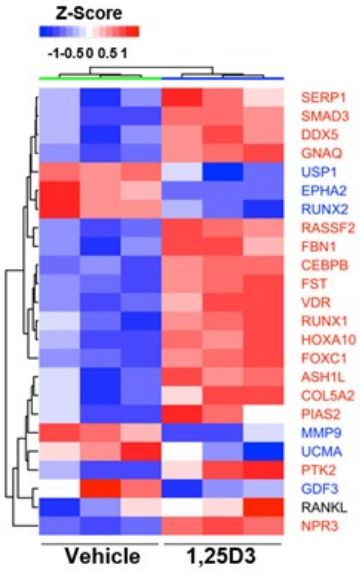


Fig. 1.

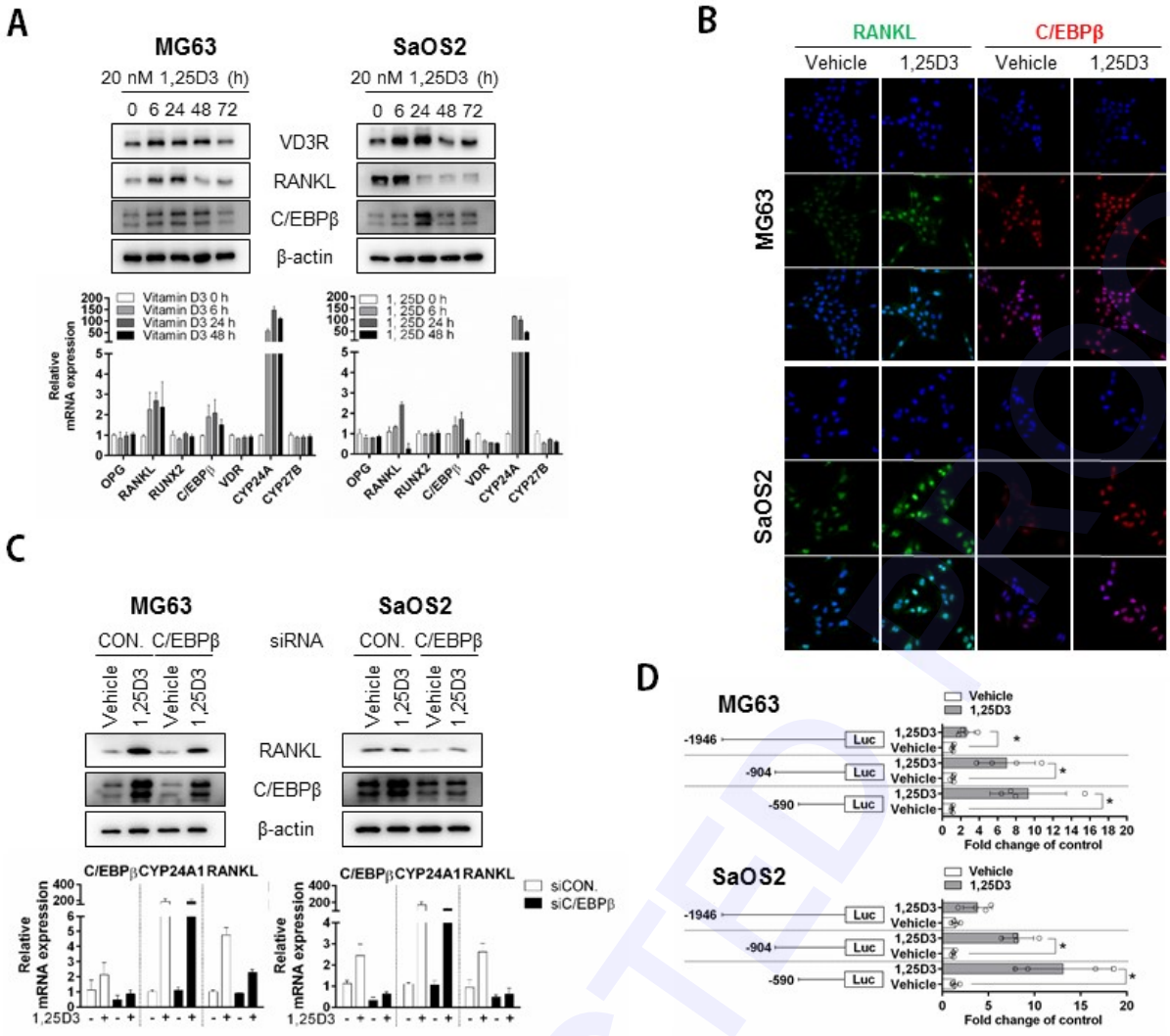


Fig. 2.

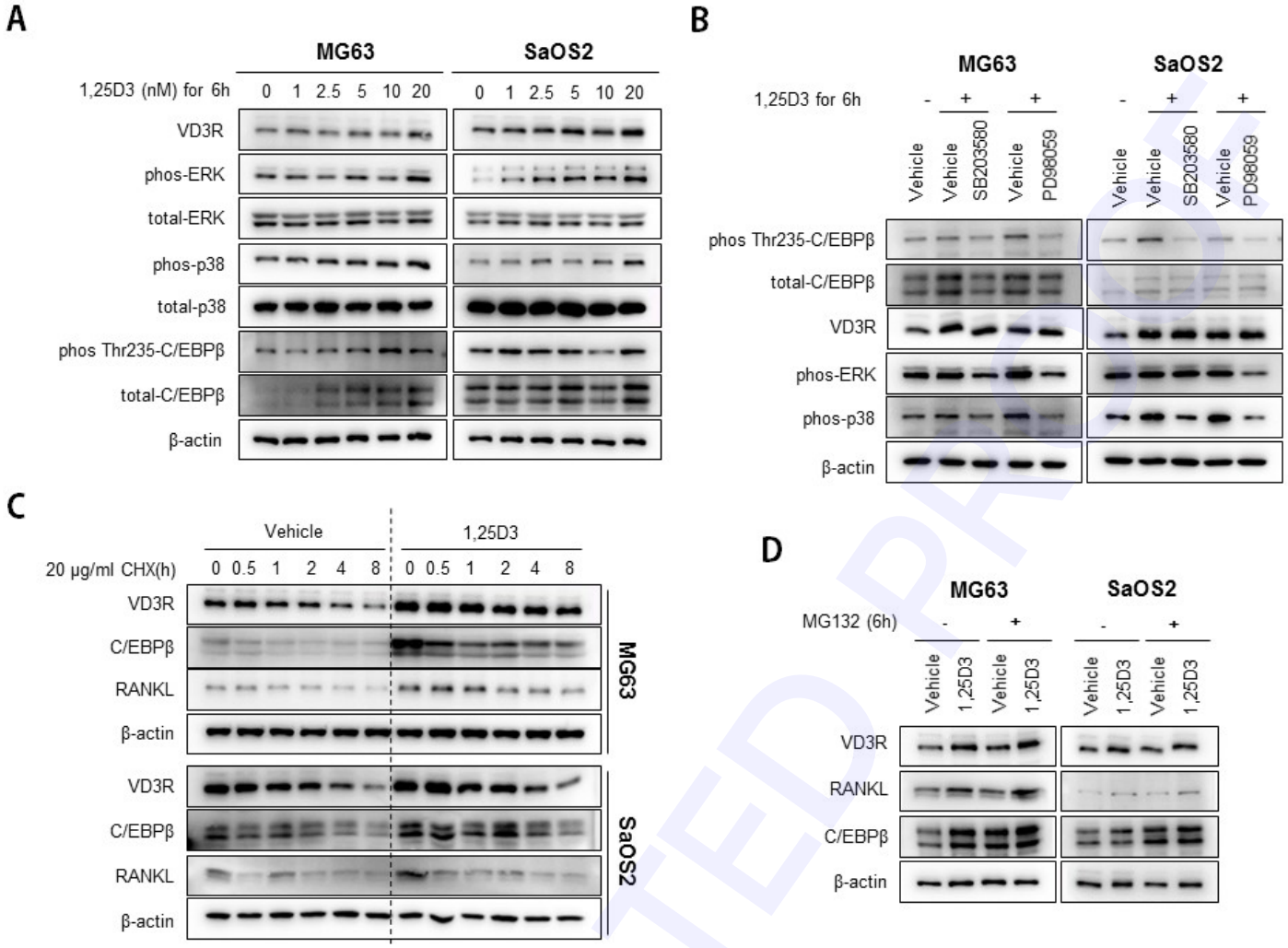


Fig. 3.

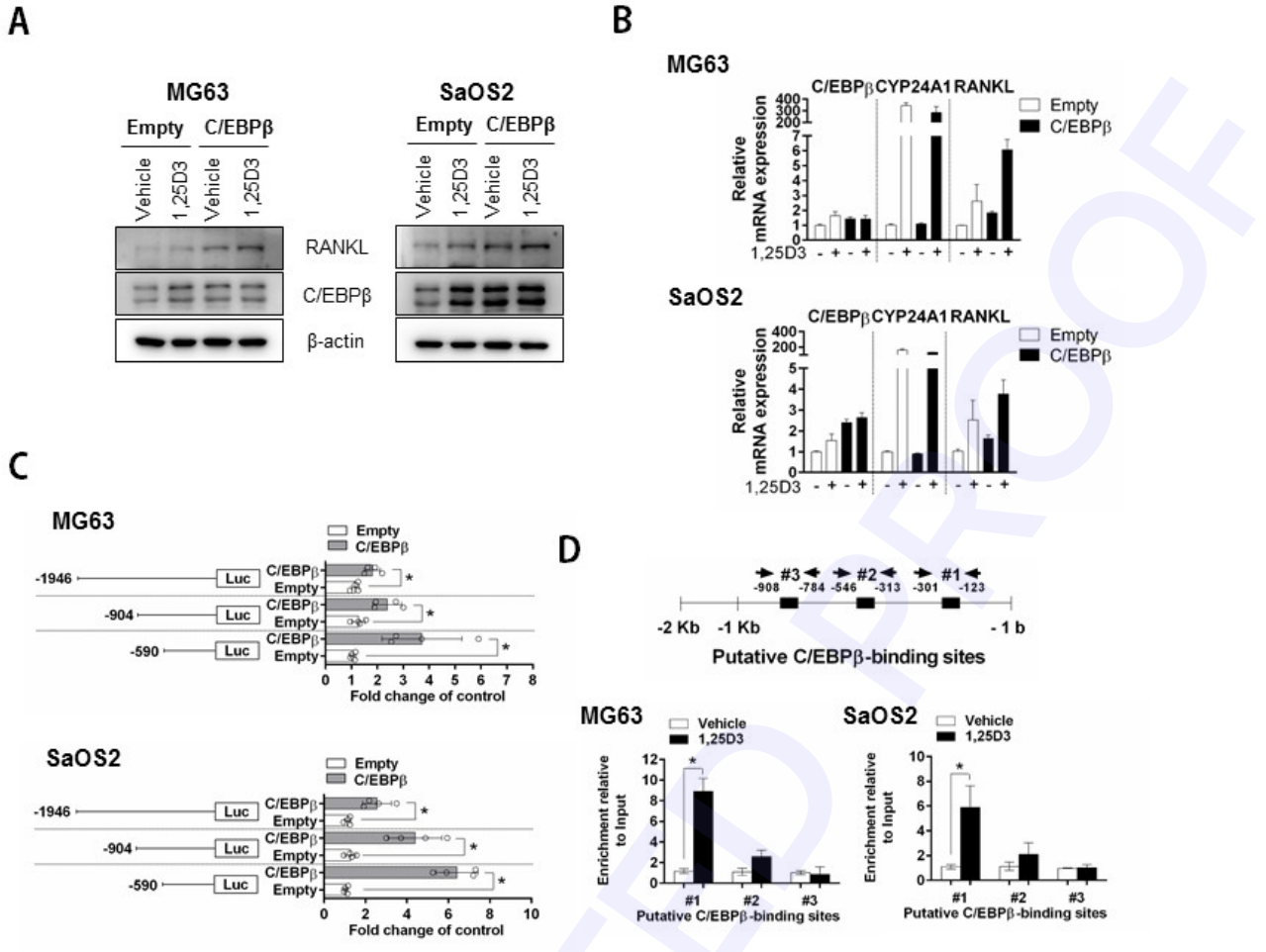
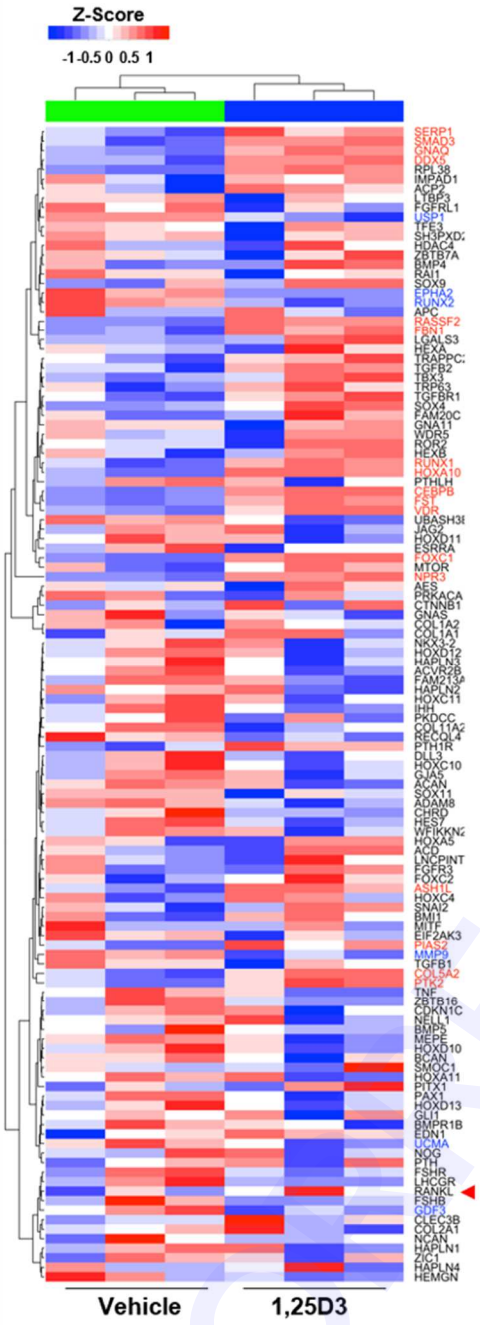


Fig. 4.

Supplementary Figure. 1



Symbol	Gene ID	RNA ID	Average Expression	logFC	Up/Down	Adjusted P value
NPR3	18162	NM_001039181	10.37	4.44	Up	8.07E-07
COL5A2	12832	NM_007737	9.29	1.22	Up	6.96E-03
DDX5	13207	NM_007840	12.75	0.54	Up	3.66E-02
PTK2	14083	NM_007982	8.57	1.16	Up	7.30E-03
FBN1	14118	NM_007993	13.58	0.89	Up	4.82E-03
FST	14313	NM_008046	11.94	1.50	Up	3.72E-04
GNAQ	14682	NM_008139	13.00	0.86	Up	4.37E-03
HOXA10	15395	NM_008263	11.57	0.90	Up	4.09E-03
FOXC1	17300	NM_008592	10.74	1.22	Up	5.39E-04
PIAS2	17344	NM_008602	10.02	0.64	Up	4.54E-02
VDR	22337	NM_009504	11.69	1.57	Up	1.05E-03
RUNX1	12394	NM_009821	11.69	0.66	Up	2.04E-02
CEBPB	12608	NM_009883	11.99	1.68	Up	1.27E-04
SMAD3	17127	NM_016769	12.86	0.73	Up	9.94E-03
SERP1	28146	NM_030685	12.92	0.58	Up	4.08E-02
ASH1L	192195	NM_138679	9.57	0.73	Up	1.52E-02
RASSF2	215653	NM_175445	14.05	1.04	Up	1.02E-03
RANKL	21943	NM_011613	7.14	0.21	Up	3.56E-01
GDF3	14562	NM_008108	7.44	-0.69	Down	4.04E-02
RUNX2	12393	NM_009820	12.23	-0.49	Down	4.70E-02
EPHA2	13836	NM_010139	12.40	-0.57	Down	2.95E-02
MMP9	17395	NM_013599	10.15	-0.94	Down	8.49E-03
UCMA	68527	NM_026754	8.73	-0.71	Down	4.83E-02
USP1	230484	NM_146144	13.10	-0.66	Down	2.79E-02

Figure S1. Differentially expression gene by treatment of 1,25D3. In total, 122 genes were identified as bone development-related. A heat map of bone development-related genes in POB cells treated with vehicle (V) or 1,25D3 (left panel). Among them, differentially expressed 23 genes were shown (right panel). Blue, low expression; red, high expression. Red arrow indicates Tnfsf11, also known as RANKL.

Supplementary Figure. 2

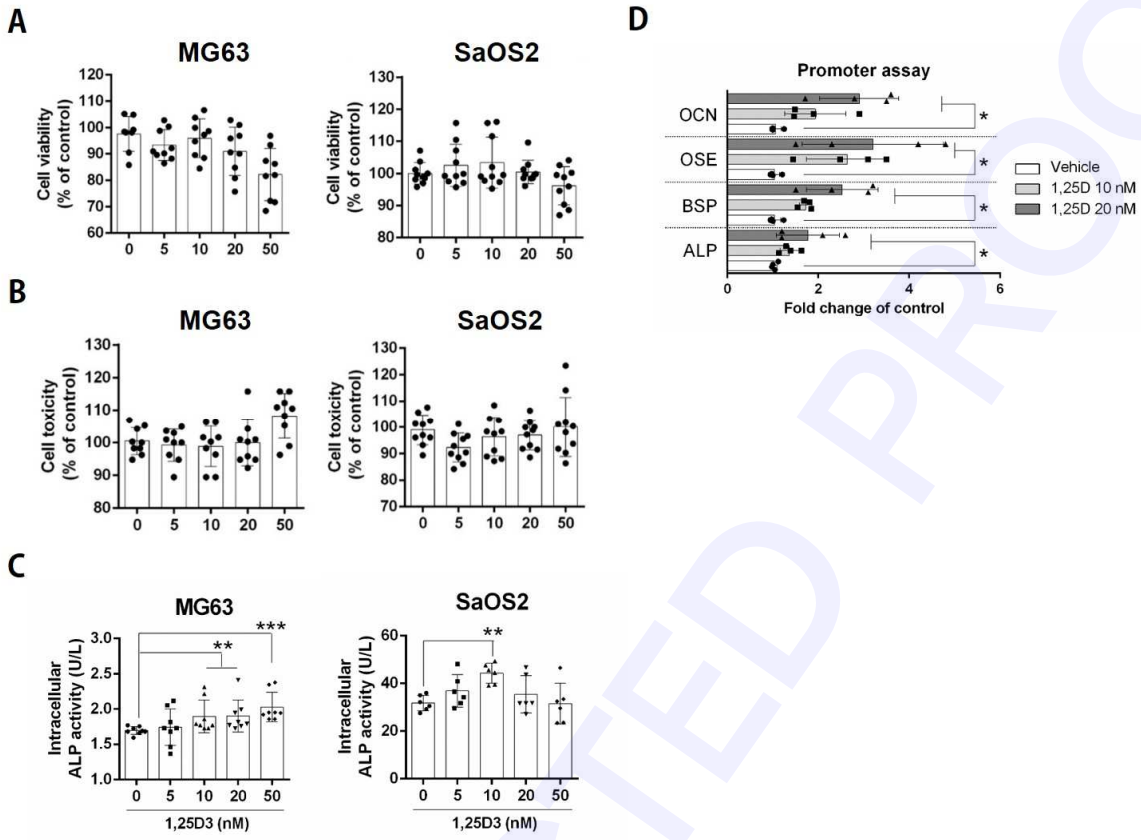


Figure S2. Effect of 1,25D3 on cell viability, toxicity, and osteoblastic activity. Both MG63 and SaOS2 osteoblasts were treated with various concentration of 1,25D3 for 3 days to measure (A) cell viability and (B) cell toxicity (MG63;n=9 and SaOS2;n=10), and (C) ALP activity (n=8). (D) 293T cells were transfected with osteoblast specific 4 promoters (ALP, BSP, OSE, and OCN), followed by incubation for 48h, treatment with 1,25D3 for 24h, analysis with Luciferase assay (n=4). The Mann-Whitney U test was performed to determine statistical significance. Data are presented as means ± SDs. *P* values indicate significant differences between the two groups. **p*<0.05; ***p* <0.01.

Supplementary Figure 3

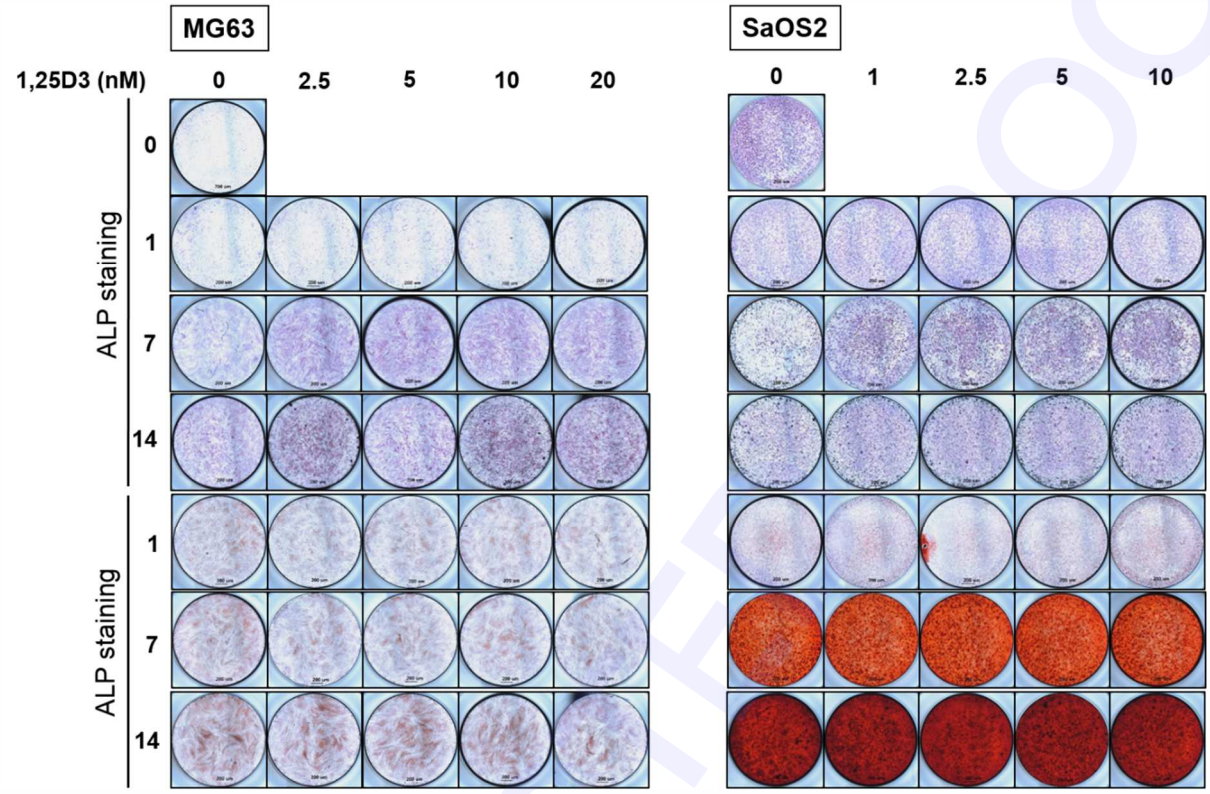


Figure S3. Effect of 1,25D3 on osteogenic differentiation in both MG63 and SaOS2. Both MG63 and SaOS2 (1E4 cells) were seeded on 96 wells plate in growth medium. Next day, the medium changed with osteogenic medium (ascorbic acid, beta-glycerolphosphate, and dexamethasone). Osteogenic medium was changed every 3 days. As indicated day, the cells were subjected to ALP and ARS staining. Independent experiments were performed four times. Representative images are presented, scale bar 200μm.

Supplementary Figure 4

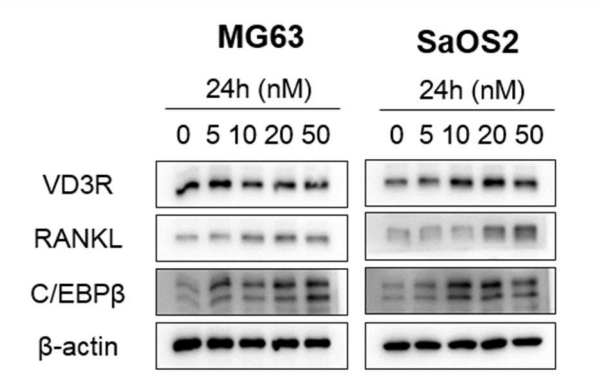


Figure S4. Analysis of 1,25D3-induced protein level in both MG63 and SaOS2. The both MG63and SaOS2 cells were stimulated with 1,25D3 for 24h as indicated dose, followed by immunoblotting (n=5).

Supplementary Figure 5

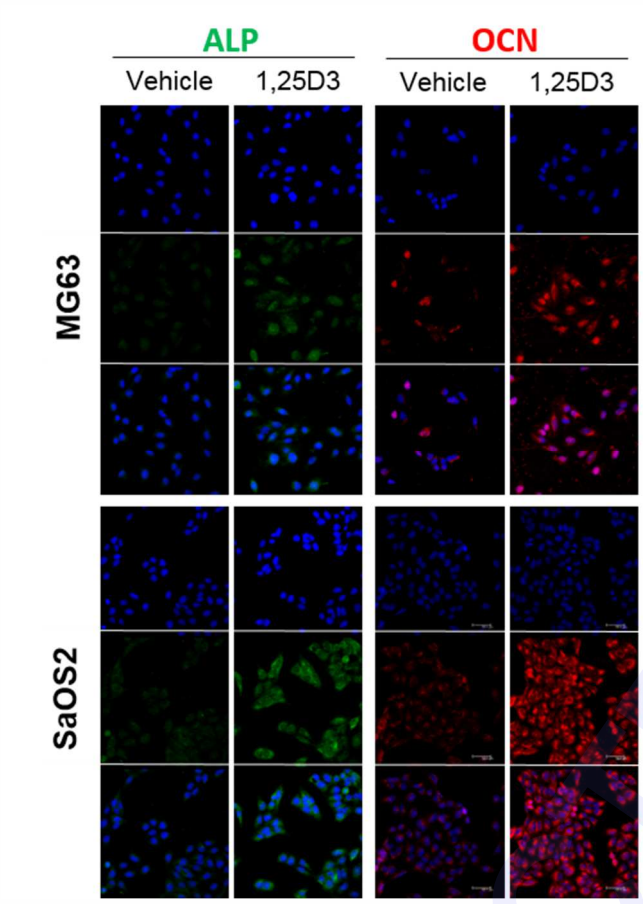


Figure S5. Analysis of 1,25D3-induced protein level in both MG63 and SaOS2. The both MG63 and SaOS2 cells were stimulated with 20 nM 1,25D3 for 24 h, followed by immunofluorescence using ALP and OCN (n=3). Scale bar is 50 μm.

Supplementary Figure 6

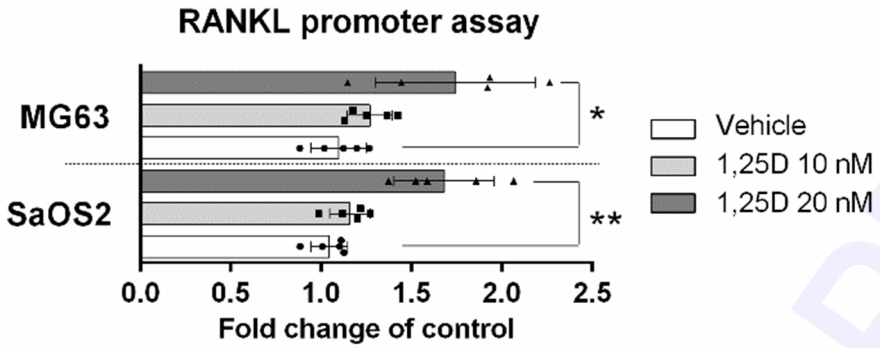


Figure S6. Analysis of 1,25D3-induced RANKL promoter activity in both MG63 and SaOS2. The both MG63 and SaOS2 cells were transfected with RANKL promoter (5 μg), incubated for 48 h, treated with 1,25D3 for 24 h, and then analyzed with luciferase assay (n=5). The Mann-Whitney U test was performed to determine statistical significance. Data are presented as mean ± SD. *P* values indicate significant differences between two groups. **p*<0.05; ***p*<0.01.

Supplementary Figure 7

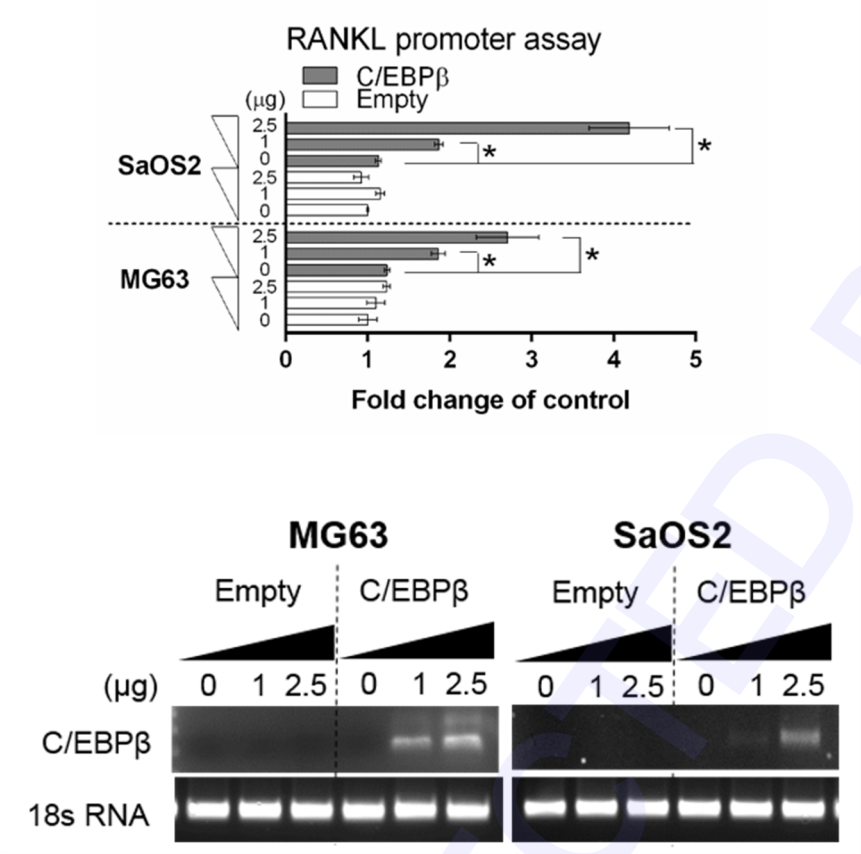


Figure S7. Analysis of C/EBPβ-mediated RANKL promoter activity in both MG63 and SaOS2. Both MG63 and SaOS2 cells were co-transfected with RANKL promoter (5 μg) and C/EBPβ or empty vector as indicated for 48 h and then analyzed using a luciferase assay (n=4). The lower panel (C) shows transfected C/EBPβ gene expression using RT-PCR. The Mann-Whitney U test was performed to determine statistical significance. Data are presented as mean ± SD. *P* values indicate significant differences between two groups. *$p<0.05$.

Table 1. Primary antibodies used in Immunoblotting (IB), Immunostaining (IF), and Chromatin immunoprecipitation (ChIP).

Antigen	Manufacturer	Species, type	Catalog number	Dilution
Alkaline Phosphatase (ALP)	Santa Cruz / TX, USA	Mouse monoclonal	Sc-365765	1:100 (IF)
Osteocalcin (OCN)	Abcam / Cambridge, UK	Mouse monoclonal	Ab13420	1:100 (IF)
Osteocalcin (OCN)	Santa Cruz / TX, USA	Rabbit polyclonal	Sc-30045	1:100 (IF)
Vitamin D3 Receptor (VD3R)	Cell signaling / MA, USA	Rabbit monoclonal	12550	1:100 (IF) 1:1,000 (IB)
RANKL	Cell signaling / MA, USA	Rabbit polyclonal	4816	1:1,000 (IB)
RANKL	Santa Cruz / TX, USA	Mouse monoclonal	Sc-377079	1:100 (IF)
Phos-C/EBPβ(Thr235)	Cell signaling / MA, USA	Rabbit monoclonal	3084	1:1,000 (IB)
C/EBPβ	Santa Cruz / TX, USA	Rabbit polyclonal	Sc-150	1:1,000 (IB) 5 µg (ChIP)
Phos-ERK	Cell signaling / MA, USA	Rabbit polyclonal	9101S	1:1,000 (IB)
Total-ERK	Cell signaling / MA, USA	Rabbit polyclonal	9102S	1:1,000 (IB)
Phos-p38	Cell signaling / MA, USA	Rabbit polyclonal	9215S	1:1,000 (IB)
Total-p38	Cell signaling / MA, USA	Rabbit polyclonal	9212	1:1,000 (IB)
β-actin	Cell signaling / MA, USA	Rabbit polyclonal	4970	1:10,000 (IB)
IgG	Milipore/ CA, USA	Rabbit monoclonal	pp64	5 µg (ChIP)

Table 2. Primer Sequences for qPCR

Gene	5' ------ Forward ------ 3'	5'------ Reverse ------ 3'
18s RNA	GTAACCCGTTGAACCCCATTC	CCATCCAATCGGTAGTAGCG
OSTEOPROTEGERIN (OPG)	CCTGGCACCAAAGTAAACGC	GCACGCTGTTTTCACAGAGG
RANKL	GCGTCGGCCTGTTCTTCTAT	TGACTCTCCAGAGTTGTGTCT
RUNX2	ACGAGCTGAACAGGAACAACGT	CACCAGCAAGAAGAAGCCTTTG
C/EBPβ	AGGGCATCCTCTCCACAAAA	CGCTGTTTGTGTTTGGCTTG
VDR	TGGAGACTTTGACCGGAACG	GGGCAGGTGAATAGTGCCTT
CYP24A	ACGTTGGCTTCAGGAGAAGG	TTGTCTGTGGCCTGGATGTC
CYP27B	GAGTTGCTATTGGCGGGAGT	AGAACAGTGGCTGAGGGGTA
GAPDH	CAAGATCATCAGCAATGCC	CTGTGGTCATGAGTCCTTCC
GAPDH for CHIP	tgcaacaccaactgcttagc	ggcatggactgtggtcatgag
RANKL#1 for CHIP	cctggtgggaggtaattgaa	aatcatggtgaaaggcaagg
RANKL#2 for CHIP	ccagccacatggaactgtaa	gtgctccacaggctcctaag
RANKL#3 for CHIP	ctcagacaaggggggaaatga	atttggggtcagcaaaaatg

Table 3. siRNA Sequences

siRNA	5' ------ Sense ------ 3'	5'------ Antisense ------ 3'
siControl	CCUCGUGCCGUUCCAUCAGGUAGUU	CUACCUGAUGGAACGGCACGAGGUU
siC/EBPβ	ACAACAUCGCCGUGCGCAAUU	UUGCGCACGGCGAUGUUGUUU

Supplementary methods

Measurements of cell viability and toxicity

EZ-CYTOX (EZ-1000, Dogen, Seoul, Korea) and EZ-LDH (DG-LDH1000, Dogen, Seoul, Korea) were used to quantify relative cells viability and toxicity, respectively. Assays were performed according to the manufacturer's instructions.

Gene transfer

Osteoblasts were transfected with siRNA or plasmids using Lipo3000 (Thermo Fisher, 32106) and incubated for 48 h. The cells were then stimulated for 24 h as indicated and subjected to qRT-PCR to evaluate mRNA levels and immunoblotting to evaluate protein levels.

qRT-PCR and immunoblotting

RNA and proteins were extracted from the stimulated cells with Nucleozol and 1× RIPA buffer, respectively. Complementary DNA was generated from 1 μg of total RNA with reverse transcriptase (Thermo Scientific, #EP0442). The cells were lysed with 1× RIPA buffer which included phosphatase (Cell signal, 5870S) and protease (Calbiochem, #535140) inhibitors and quantified with a Bradford assay. A total of 20~50 μg of protein were subjected to immunoblotting. Primary antibodies and primers information are given in Supple. Table 1 and Table 2, respectively.

Immunofluorescence

Cells grown on cover slips were fixed with ice-cold absolute methanol for 10 min. Cells were permeabilized and blocked with 0.3% Triton X-100 and 3% FBS in 1× PBS for 1 h at room temperature and then incubated with the appropriate primary antibodies in 0.1% Triton X-100 and 3% BSA in 1× PBS overnight at 4°C. Antibody binding was visualized using secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, A-11001) or Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, 111-165-144). Nuclei were counterstained with DAPI (VECTOR, H-1200). Immunofluorescence images were

analyzed by confocal microscopy (Leica Microsystems, Wetzlar, Germany). The primary antibodies that were used are listed in Table 1.

Constructions for deletion mutants of human *RANKL* promoter

Human *RANKL* promoter was cloned by PCR from pGL2-Basic-RANKLpromoter. The constructs were obtained using forward primers (containing a SacI-site): 5'-TCCGAGCTCATTCAAGACCTGCCTCGCTC-3' for *RANKL*(-)590, and 5'-TCCGAGCTCTCATGGGTGTTTAAATTGGTCAGC-3' for *RANKL*(-)904. The reverse primer (containing a XhoI-site) was commonly used 5'-CCGCTCGAGGTCCTGGGGCGCGC-3'. PCR mixtures contained 1㎕ of pGL2-Basic-hRANKLpromoter, 5 ㎕ of 10 x PCR buffer, 5 ㎕ of dNTP mix (2.5 mM), 5 % DMSO, 1 ㎕ of forward primer (10 pM), 1 ㎕ of reverse primer (10 pM), 1 ㎕ of Taq polymerase (10 units/㎕ Pyrobest, Takara bio, Ohtsu, Japan) adjusted to 50 ㎕ with distilled water. The PCR amplification procedure was consisted of denaturation for 5 min at 95℃ followed by 30 cycles of 30 s at 95 ℃, 30 s at 60 ℃, and 90 s at 72℃, with final extension for 7 min at 72℃. The PCR product was loaded and run by electrophoresis on 1.2 % agarose gel, and then visualized using a benchtop 3UV™ transilluminator (UVP, Upland, CA, USA). The DNA band of expected size was excised and purified using a FavorPREP™ gel/PCR purification mini kit (Favorgen biotech Corp., Taiwan), and digested with restriction enzymes (XhoI and SacI; New England Biolabs, Beverly, MA, USA) at 37℃. The digested sample was ligased into pGL3-Basic Luciferase Reporter Vector (Promega, Madison, WI, USA). The cloned mutants of human *RANKL* promoter were confirmed by sequencing (Cosmo Genetech, Seoul, Korea) and alignment with the NCBI human RANKL gene, promoter region sequence (NCBI AF544022.1)

using the Genomatix pairwise comparison program (http://www.genomatix.de/cgi-bin/dialign/dialign.pl).