

# CCAAT/enhancer-binding protein beta (C/EBP $\beta$ ) is an important mediator of 1,25 dihydroxyvitamin D3 (1,25D3)-induced receptor activator of nuclear factor kappa-B ligand (RANKL) expression in osteoblasts

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**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/EBP $\beta$ ) has been proposed to function as a transcription factor and upregulate RANKL expression, but it is still uncertain how C/EBP $\beta$  is involved in 1,25D3-induced RANKL expression of osteoblasts. 1,25D3 stimulation increased the expression of RANKL and C/EBP $\beta$  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/EBP $\beta$ . In contrast, C/EBP $\beta$  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/EBP $\beta$  protein binding to the RANKL promoter. In conclusion, C/EBP $\beta$  is required for induction of RANKL by 1,25D3. [BMB Reports 2019; 52(6): 391-396]**

## INTRODUCTION

Bone constantly cycles through formation and absorption,

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<https://doi.org/10.5483/BMBRep.2019.52.6.166>

Received 20 July 2018, Revised 17 August 2018,  
Accepted 10 October 2018

**Keywords:** 1,25-dihydroxyvitamin D3 (1,25D3), C/EBP $\beta$ , Osteoblasts, RANKL

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- $\kappa$ B 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<sub>3</sub> 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/EBP $\beta$ ) 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/EBP $\beta$  activation by 1,25D3 and the functional role of C/EBP $\beta$  in osteoblasts are not fully understood.

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

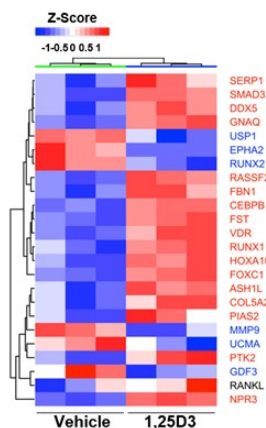
## RESULTS

### 1,25D3 significantly increases VDR and C/EBP $\beta$ 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/EBP $\beta$* , *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/EBP $\beta$*  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

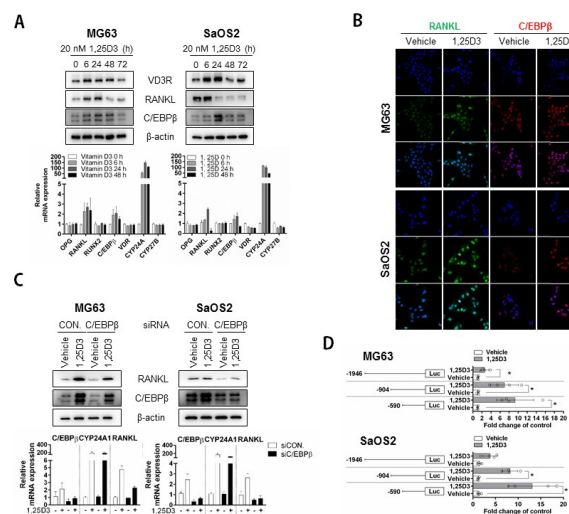


**Fig. 1.** 1,25D3 significantly increases VDR and C/EBP $\beta$  gene expression in murine osteoblasts. Using microarray datasets, we investigated gene expression changes in response to a treatment of  $10^{-7}$  M 1,25D3 for 24 h. In order to assign putative functional changes in bone development, 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.

viability or toxicity between 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/EBP $\beta$

As shown in Fig. 2A, increases in *CYP24A* mRNA and VD3R protein levels were affected by 1,25D3 treatment. In this



**Fig. 2.** 1,25D3 induces RANKL expression via regulation of C/EBP $\beta$ . (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/EBP $\beta$  ( $n = 3$ ). Scale bar is 50  $\mu$ m. (C) The cells were transfected with siRNA against C/EBP $\beta$  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  $\pm$  SD. P values indicate significant differences between two groups. \* $P < 0.05$ .

situation, we observed that expression of RANKL and C/EBP $\beta$  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/EBP $\beta$ , 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/EBP $\beta$  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/EBP $\beta$ .

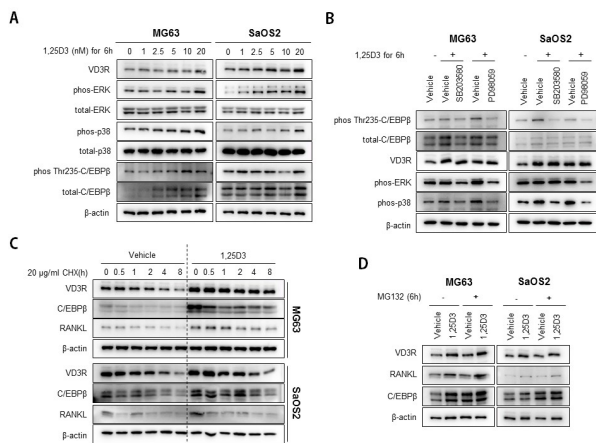
### 1,25D3 enhances phosphorylation and stability of the C/EBP $\beta$ protein

To further assess the regulation of the C/EBP $\beta$  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/EBP $\beta$  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/EBP $\beta$  protein. The two inhibitors prevented C/EBP $\beta$ -Thr235 phosphorylation through 1,25D3 without a

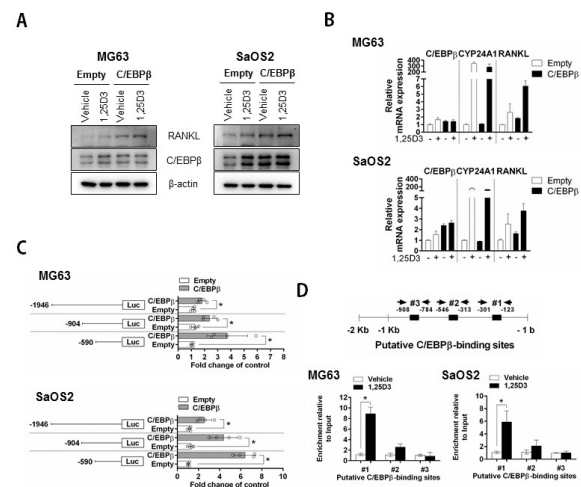
change in VD3R protein (Fig. 3B). We next used the inhibitors cycloheximide (CHX) to inhibit protein synthesis and MG132 to inhibit the proteasome in order to observe changes at the protein level. VD3R, C/EBP $\beta$ , and RANKL proteins were reduced in the vehicle, whereas these proteins were only modestly reduced in response to 1,25D3 (Fig. 3C). Moreover, 1,25D3-induced VD3R, C/EBP $\beta$ , and RANKL proteins were degraded by the proteasome pathway (Fig. 3D). These results suggest that 1,25D3 enhances the phosphorylation and stability of C/EBP $\beta$  protein.

### 1,25D3-induced upregulation of C/EBP $\beta$ 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/EBP $\beta$ . Overexpression of C/EBP $\beta$  in combination with 1,25D3 stimulation exhibited a synergistic effect on RANKL expression in comparison to either 1,25D3 or C/EBP $\beta$  alone (Fig. 4A and B). C/EBP $\beta$  overexpression markedly induced RANKL promoter activity (Suppl. Fig. 7) and its expression was quantified by qRT-PCR (Suppl. Fig. 7, lower panel). C/EBP $\beta$  overexpression significantly also induced the proximal region



**Fig. 3.** 1,25D3 enhances C/EBP $\beta$  protein phosphorylation and stability. (A) Both MG63 and 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 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  $\mu$ g/ml cycloheximide (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.



**Fig. 4.** 1,25D3-induced upregulation of C/EBP $\beta$  contributes to RANKL expression in osteoblasts. Both MG63 and SaOS2 cells were transfected with C/EBP $\beta$  (2.5  $\mu$ g) 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/EBP $\beta$  (2.5  $\mu$ g) 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/EBP $\beta$  antibody (MG63, n = 4; SaOS2, n = 4). The Mann-Whitney U test was performed to determine statistical significance. Data are presented as mean  $\pm$  SD. P values indicate significant differences between two groups. \*P < 0.05.

(less than 1Kb) compared to a 2Kb promoter region of human RANKL gene (Fig. 4C). Interestingly, #1 of sites of three putative C/EBP $\beta$  binding sites on the proximal RANKL promoter was selectively enhanced in response to 1,25D3-induced C/EBP $\beta$  protein (Fig. 4D). Taken together, these findings indicate that 1,25D3-induced upregulation of C/EBP $\beta$  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/EBP $\beta$  and RANKL gene expression. We observed that 1,25D3 results in p38- and ERK-dependent activation and phosphorylation of C/EBP $\beta$  in both MG63 and SaOS2 osteoblasts. Furthermore, C/EBP $\beta$  knockdown slightly reduced RANKL expression and attenuated 1,25D3-induced the gene level. In contrast, 1,25D3 and C/EBP $\beta$  overexpression had a synergistic effect on RANKL expression. Finally, we demonstrated that, in response to 1,25D3, C/EBP $\beta$  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<sub>3</sub>, 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/EBP $\beta$  is a critical factor during adipocyte and chondrocyte differentiation (19). C/EBP $\beta$  is an important indicator of differentiation and control of target gene transcription. Despite the fact that C/EBP $\beta$  is necessary for osteoblastic activity and bone formation via RUNX2 and ATF4 regulation (20), there are fewer reports on C/EBP $\beta$  function in osteoblasts than in other cell lineages. Our previous work has revealed that RUNX2 and C/EBP $\beta$  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/EBP $\beta$  proteins via osteogenic induction (22, 23). In this study, we propose that induction of the RANKL gene by 1,25D3 is mediated by C/EBP $\beta$ . We therefore believe that the function of the C/EBP $\beta$  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/EBP $\beta$ , 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/EBP $\beta$  or RUNX2 gene was modulated in the presence of 1,25D3. Interestingly, the cells responded to 1,25D3 by increasing C/EBP $\beta$  expression and decreasing RUNX2 gene expression, as shown in the microarray data in Fig. 1. We compared luciferase activity of the RANKL promoter to the expression of the RUNX2 and C/EBP $\beta$  genes. As expected, the RANKL promoter was dramatically induced in transduction of the C/EBP $\beta$  gene than RUNX2, indicating that C/EBP $\beta$  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/EBP $\beta$ , 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 and SaOS2 were grown in high-glucose DMEM medium (Hyclone, SH30243.01) and RPMI1640 (Hyclone, SH30027.01) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10082-147) and 1 $\times$  antibiotics (Gibco, 15140-122). 1,25D3 (Sigma-Aldrich, D1530) was dissolved in absolute ethanol, respectively. For *in vitro* drug treatment, 1,25D3 was prepared as a 20  $\mu$ M 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/EBP $\beta$  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](http://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, Kampenhout, 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  $1\times$  PBS with protease inhibitor, and then immunoprecipitated with protein A agarose beads conjugated to the C/EBP $\beta$  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.

## ACKNOWLEDGEMENTS

We thank Dr. Sung Eun Wang (Hanyang Biomedical Research Institute, Hanyang University) for technical assistance regarding the ChIP assay. Immunofluorescence images were analyzed by a confocal microscope at Hanyang LINC Analytical Equipment Center (Seoul).

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, & Future (NRF-2016R1A2B4008606) and the Ministry of Education (2017R1A6A3A11034394). The study was also supported by a Korea Health Technology R&D grant through the Korea Health Industry Development Institute (KHIDI), which is funded by the Ministry of Health & Welfare, Republic of Korea (HI17C0888).

## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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