Kruppel-like factor 4 attenuates osteoblast formation, function, and cross talk with osteoclasts

Jung Ha Kim,[1] Kabsun Kim,[1] Bang Ung Youn,[1] Jongwon Lee,[1] Inyoung Kim,[1] Hong-In Shin,[2] Haruhiko Akiyama,[3] Yongwon Choi,[4] and Nacksung Kim[1]

[1]Department of Pharmacology, Medical Research Center for Gene Regulation and BK21 plus, Chonnam National University Medical School, Gwangju 501-746, Korea
[2]Institute for Hard Tissue and Bio-Tooth Regeneration, Department of Oral Pathology, School of Dentistry, Kyungpook National University, Daegu 700-412, Korea
[3]Department of Orthopaedics, Kyoto University, Kyoto 606-8507, Japan
[4]Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

Osteoblasts not only control bone formation but also support osteoclast differentiation. Here we show the involvement of Kruppel-like factor 4 (KLF4) in the differentiation of osteoclasts and osteoblasts. KLF4 was down-regulated by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in osteoblasts. Overexpression of KLF4 in osteoblasts attenuated $1,25(OH)_2D_3$-induced osteoclast differentiation in co-culture of mouse bone marrow cells and osteoblasts through the down-regulation of receptor activator of nuclear factor κB ligand (RANKL) expression. Direct binding of KLF4 to the RANKL promoter repressed $1,25(OH)_2D_3$-induced RANKL expression by preventing vitamin D receptor from binding to the RANKL promoter region. In contrast, ectopic overexpression of KLF4 in osteoblasts attenuated osteoblast differentiation and mineralization. KLF4 interacted directly with Runx2 and inhibited the expression of its target genes. Moreover, mice with conditional knockout of KLF4 in osteoblasts showed markedly increased bone mass caused by enhanced bone formation despite increased osteoclast activity. Thus, our data suggest that KLF4 controls bone homeostasis by negatively regulating both osteoclast and osteoblast differentiation.

Introduction

Bone is a highly dynamic tissue that is continuously remodeled throughout the lifetime. Consequently, bone is maintained by a delicate balance between activities of osteoclasts and those of osteoblasts, which are responsible for bone resorption and formation, respectively. The balance between osteoclasts and osteoblasts determines bone mass. This balance can be disturbed by alteration of hormones including estrogen deficiency and chronic inflammation. The resulting imbalance can lead to bone diseases such as osteoporosis, periodontal disease, Paget's disease, and rheumatoid arthritis (Walsh et al., 2006).

Bone-resorbing osteoclasts are tartrate-resistant acid phosphate (TRAP)–positive multinuclear cells (MNCs) that differentiate from the monocyte/macrophage lineage upon stimulation by two essential cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF binds to its receptor, c-Fms, on the osteoclast precursor cells and induces the expression of RANK, which is the sole receptor on these cells for RANKL (Arai et al., 1999). In particular, RANKL can induce the complete differentiation, fusion of osteoclast precursor cells into mature osteoclasts, and survival of osteoclasts in the presence of low levels of M-CSF. Binding of RANKL to the RANK stimulates nuclear factor κB, JNK, p38, and extracellular signal-regulated kinase, which are important for osteoclast differentiation, and induces transcription factors such as c-fos and nuclear factor of activated T cells c1 (NFATc1), which are necessary for sufficient osteoclast differentiation (Teitelbaum, 2000; Khosla, 2001; Takayanagi et al., 2002; Boyle et al., 2003).

Bone-forming osteoblasts are mononuclear cells that differentiate from mesenchymal progenitors under the control of

Correspondence to Nacksung Kim: nacksung@jnu.ac.kr

Abbreviations used in this paper: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; BMM, bone marrow–derived macrophage-like cell; BSP, bone sialoprotein; ChIP, chromatin immunoprecipitation; CTx-I, C-terminal cross-linking telopeptide of type 1 collagen; KLF4, Kruppel-like factor 4; M-CSF, macrophage colony-stimulating factor; μCT, micro-computed tomography; MNC, multinuclear cell; NFATc1, nuclear factor of activated T cells c1; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphate; VDR, vitamin D receptor.

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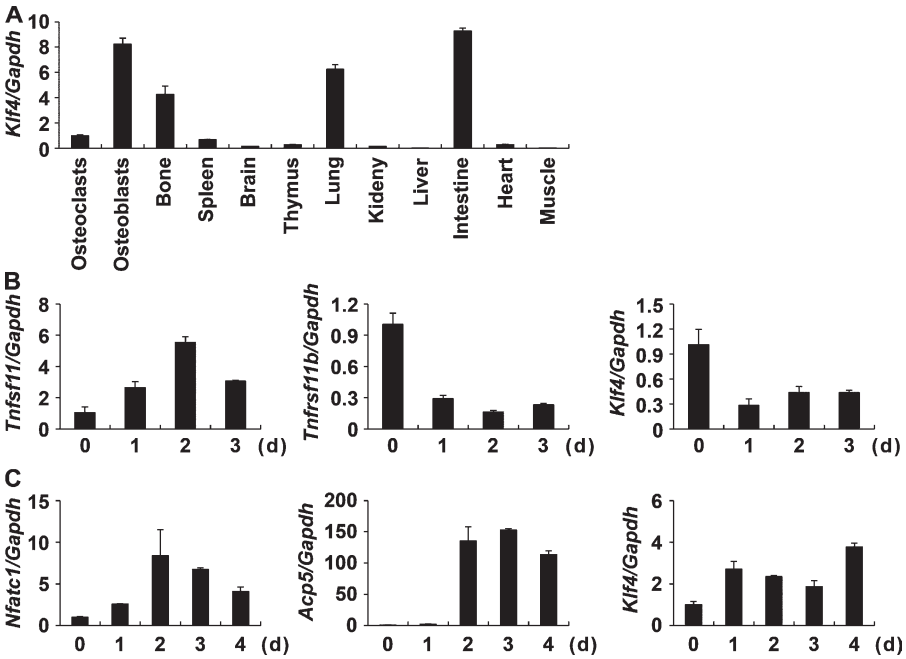

Figure 1. **Expression of KLF4 in osteoclasts and osteoblasts.** (A) Total RNA was isolated from bone, spleen, brain, thymus, lung, kidney, liver, intestine, heart, and muscle of mice. To obtain osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 3 d. To obtain osteoblasts, primary osteoblast precursor cells were cultured with osteogenic medium containing BMP2 (100 ng/ml), ascorbic acid (50 µg/ml), and β-glycerophosphate (100 mM) for 6 d. Quantitative real-time PCR was performed for the mRNA expression of *Klf4*. (B) Primary calvarial osteoblasts were cultured with $1,25(OH)_2D_3$ (10 nM) for the indicated times. mRNA expression of *Tnfsf11* (RANKL), *Tnfrsf11b* (OPG), and *Klf4* was measured by quantitative real-time PCR. (C) For osteoclast differentiation, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for the indicated times. mRNA levels of *Nfatc1*, *Acp5* (TRAP), and *Klf4* were assessed by quantitative real-time PCR. Data represent means ± SD of triplicate samples.

several transcriptional factors and signaling cascades. In particular, Runx2, a cell-specific member of the Runt family of transcription factors, is essential for osteoblast and hypertrophic chondrocyte differentiation (Komori, 2005, 2011). Runx2 induces the expression of target genes by binding to the Runx2 binding sites in the promoter region of genes such as AP, bone sialoprotein (BSP), and osteocalcin, which are key regulators of osteoblast differentiation and function (Komori, 2005, 2011). Moreover, Runx2-null mice show a deficiency of osteoblasts and bone formation, which suggests that Runx2 is a master transcription factor of osteogenesis (Komori et al., 1997).

In addition to stimulating bone formation, osteoblasts support osteoclast differentiation through up-regulation of RANKL expression upon stimulation by various factors, such as 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), prostaglandin E_2, glucocorticoids, TNF, and interleukin-1. In particular, $1,25(OH)_2D_3$ stimulates osteoclast differentiation in co-cultures of osteoclast precursors and osteoblasts (Hofbauer et al., 1999; Kitazawa et al., 1999; Suda et al., 1999; Lee et al., 2008). $1,25(OH)_2D_3$ is known to induce RANKL up-regulation primarily through actions initiated by its receptor, vitamin D receptor (VDR). Mice lacking VDR by gene targeting cannot produce RANKL in response to $1,25(OH)_2D_3$ and therefore cannot support $1,25(OH)_2D_3$-mediated osteoclastogenesis (Takeda et al., 1999). These results suggest that binding of the VDR–$1,25(OH)_2D_3$ complex to its responsive elements within the RANKL promoter is essential for the up-regulation of RANKL and $1,25(OH)_2D_3$-mediated osteoclast formation.

Kruppel-like factor 4 (KLF4), also known as gut-enriched KLF, is a zinc finger transcription factor. KLF4 is expressed in various tissues including the epithelium of intestine, testis, skin, and bone and plays an important role in various biological processes such as proliferation, differentiation, survival, and development (Evans and Liu, 2008). However, the role of KLF4 in bone homeostasis is poorly understood even though KLF4 is highly expressed in bone (Fig. 1 A). Herein, we found that KLF4 inhibited $1,25(OH)_2D_3$-mediated osteoclast formation and osteoblast differentiation. Moreover, osteoblast-specific KLF4 knockout mice showed increased bone mass even with the increasing osteoclast number, which suggests an important role of KLF4 in the bone homeostasis regulated by the cross talk between osteoclasts and osteoblasts.

Results

KLF4 is down-regulated by $1,25(OH)_2D_3$ in osteoblasts

$1,25(OH)_2D_3$ induces osteoclast differentiation indirectly through regulation of RANKL and osteoprotegerin (OPG) expression in osteoblasts. In this study, we used microarray analyses to examine gene expression profiles during $1,25(OH)_2D_3$-induced osteoclast differentiation in co-cultures of mouse bone marrow cells and osteoblasts and found that KLF4 was down-regulated during $1,25(OH)_2D_3$-induced osteoclast differentiation (Table S1). To confirm the down-regulation of KLF4, we examined the expression patterns of KLF4 by stimulation of osteoclastogenic factors in both osteoclasts and osteoblasts. Consistent with previous results (Lee et al., 2002; Balga et al., 2006), stimulation of $1,25(OH)_2D_3$ in primary calvarial osteoblasts increased RANKL (*Tnfsf11*) expression and decreased OPG (*Tnfsf11b*) expression. Expression of KLF4 was down-regulated by $1,25(OH)_2D_3$ stimulation in osteoblasts, similar to the microarray results (Fig. 1 A). When we cultured bone marrow–derived macrophage-like cells (BMMs), which are osteoclast precursor cells, in the presence of RANKL and M-CSF, however, the expression of KLF4 was increased during RANKL-induced osteoclast formation. Expression levels of NFATc1 and TRAP, important markers of osteoclastogenesis, increased as the cells differentiated (Fig. 1 B). These results suggest that KLF4 is down-regulated by $1,25(OH)_2D_3$ in osteoblasts and may play a role in $1,25(OH)_2D_3$-induced osteoclast differentiation.

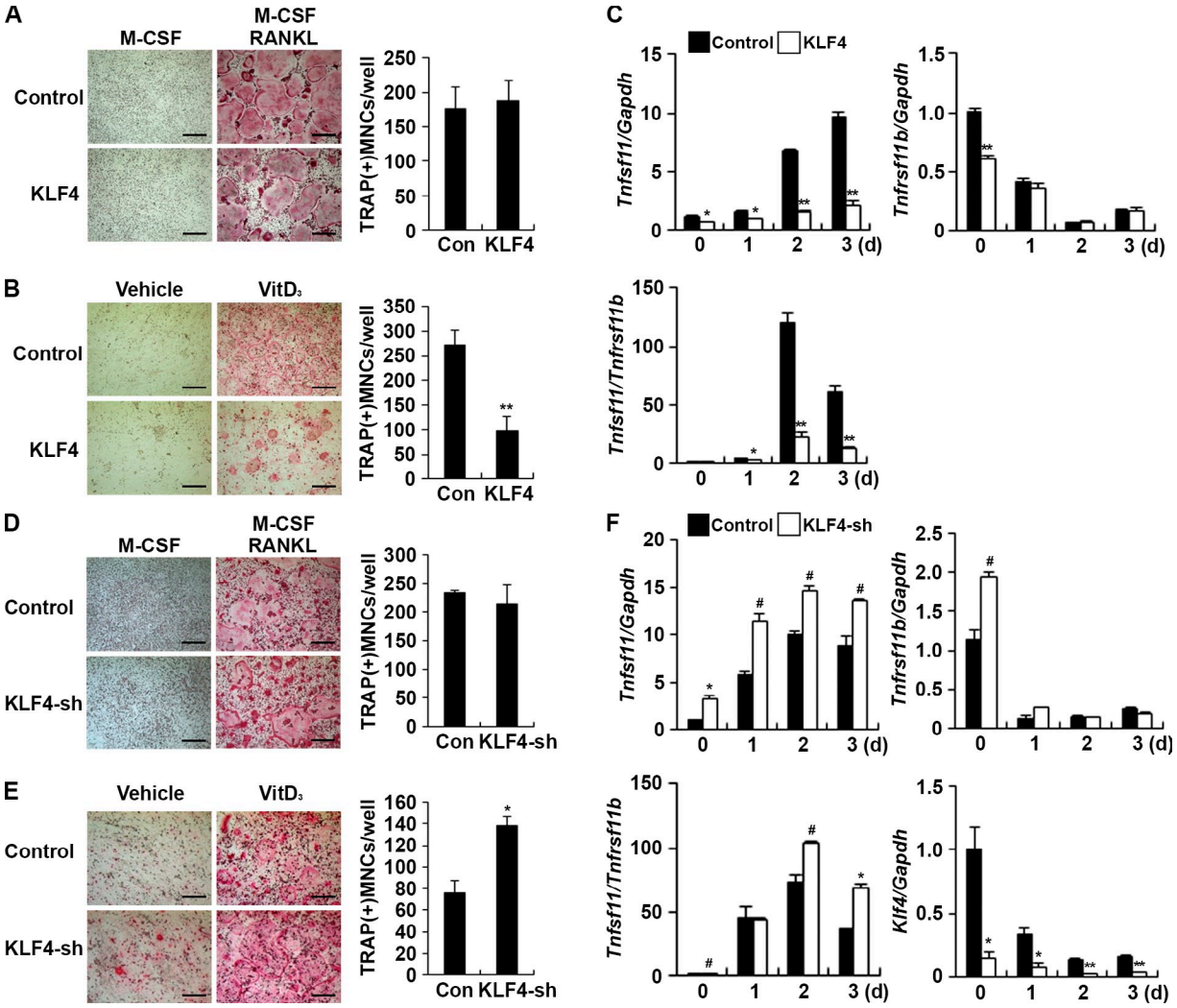

Figure 2. **Inhibition of $1,25(OH)_2D_3$-induced osteoclastogenesis by KLF4.** (A) BMMs were transduced with pMX-IRES-*EGFP* (control) or KLF4 retrovirus and cultured with M-CSF (30 ng/ml) in the absence or presence of RANKL (100 ng/ml) for 3 d. Cultured cells were stained for TRAP (left), and the number of TRAP-positive MNCs per well was counted (right). Bars, 200 μm. (B) Osteoblasts were transduced with pMX-IRES-*EGFP* (control) or KLF4 retrovirus and co-cultured with bone marrow cells in the absence or presence of $1,25(OH)_2D_3$ (10 nM) for 5 d. Cultured cells were stained for TRAP (left), and the number of TRAP-positive MNCs per well was counted (right). Bars, 200 μm. (C) Control or KLF4 transduced osteoblasts were cultured with $1,25(OH)_2D_3$ (10 nM) for the indicated times. mRNA expression of *Tnfsf11* (RANKL) and *Tnfrsf11b* (OPG) was assessed by quantitative real-time PCR. (D) BMMs were transduced with pSuper (control) or shKLF4 retrovirus and cultured with M-CSF (30 ng/ml) in the absence or presence of RANKL (100 ng/ml) for 3 d. Cultured cells were stained for TRAP (left), and the number of TRAP-positive MNCs per well was counted (right). Bars, 200 μm. (E) Osteoblasts were transduced with pSuper (control) or shKLF4 retrovirus and co-cultured with bone marrow cells in the absence or presence of $1,25(OH)_2D_3$ (10 nM) for 5 d. Cultured cells were stained for TRAP (left), and the number of TRAP-positive MNCs per well was counted (right). Bars, 200 μm. (F) Control or shKLF4 transduced osteoblasts were cultured with $1,25(OH)_2D_3$ (10 nM) for the indicated times. mRNA expression of *Tnfsf11* (RANKL), *Tnfrsf11b* (OPG), and *Klf4* was assessed by quantitative real-time PCR. Data represent means ± SD of triplicate samples. #, $P < 0.05$; *, $P < 0.01$; **, $P < 0.001$ versus control.

KLF4 negatively regulates $1,25(OH)_2D_3$-mediated osteoclastogenesis by inhibiting RANKL expression

Given our observation that KLF4 expression is regulated by $1,25(OH)_2D_3$, an osteoclastogenic factor, in osteoblasts and is detectable during osteoclast differentiation, we examined the effect of KLF4 on osteoclast differentiation. Bone marrow cells were cultured with M-CSF to generate BMMs, which were used as osteoclast precursor cells. KLF4 was overexpressed in the BMMs by retrovirus and the BMMs were cultured with RANKL and M-CSF. Overexpression of KLF4 in BMMs did not affect the formation of TRAP-positive MNCs mediated by RANKL when compared with overexpression of control vector (Fig. 2 A). Next, to examine the role of KLF4 in the osteoclastogenic activity of osteoblasts, we co-cultured KLF4-transduced osteoblasts with bone marrow cells in the presence of $1,25(OH)_2D_3$. Osteoclast differentiation induced by KLF4-transduced osteoblasts was significantly lower than that of osteoblasts transduced with control retrovirus (Fig. 2 B).

The ratio of RANKL and its natural decoy receptor OPG determines the rate of osteoclastogenesis and thereby directly influences the bone mass. Thus, to determine whether the ratio of RANKL/OPG expression in osteoblasts transduced with KLF4 was responsible for the inhibitory effect of KLF4 on osteoclast differentiation, we examined the effect of KLF4 on the expression of RANKL and OPG. KLF4 considerably inhibited RANKL

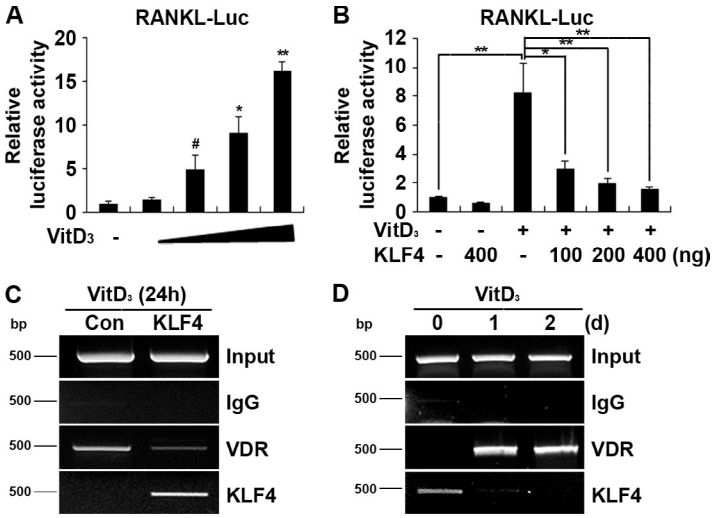

Figure 3. **Inhibition of $1,25(OH)_2D_3$-mediated RANKL expression by KLF4.** (A) UAMS-32 cells were transfected with a RANKL 0.6-kb promoter luciferase reporter and stimulated with increasing concentrations of $1,25(OH)_2D_3$. (B) UAMS-32 cells were cotransfected with a RANKL 0.6-kb promoter luciferase reporter and increasing amounts of KLF4 and then stimulated with $1,25(OH)_2D_3$ (10 nM). (A and B) Data represent means ± SD of triplicate samples. #, $P < 0.05$; *, $P < 0.01$; **, $P < 0.001$ versus control. (C) Osteoblasts were transduced with pMX-IRES-*EGFP* (control) or KLF4 retrovirus and stimulated with $1,25(OH)_2D_3$ (10 nM) for 24 h. After cross-linking, samples were immunoprecipitated with indicated antibody, respectively. The precipitated DNA was subjected to PCR with primers specific to the RANKL promoter region containing vitamin D responsive element binding sites. (D) Osteoblasts were cultured with $1,25(OH)_2D_3$ (10 nM) for the indicated times. Samples were immunoprecipitated with rabbit polyclonal anti-VDR or rabbit polyclonal anti-KLF4 antibody for ChIP analysis. PCR analysis was performed with primers containing the vitamin D responsive element binding sites of the RANKL promoter region.

expression without affecting the expression of OPG in osteoblasts treated with $1,25(OH)_2D_3$ (Fig. 2 C, top). Importantly, overexpression of KLF4 in osteoblasts significantly inhibited the RANKL/OPG ratio (Fig. 2 C, bottom).

To further confirm these results, we used shKLF4, which can silence KLF4 expression via RNA interference. Down-regulation of KLF4 by shKLF4 retrovirus in BMMs did not affect the formation of TRAP-positive MNCs mediated by RANKL when compared with control shRNA retrovirus (Fig. 2 D). In contrast, when shKLF4 retrovirus-infected osteoblasts were co-cultured with bone marrow cells in the presence of $1,25(OH)_2D_3$, osteoclast formation was significantly enhanced compared with control shRNA retrovirus-infected osteoblasts (Fig. 2 E). Also, shKLF4 induced the expression of RANKL without affecting the expression of OPG in osteoblasts treated with $1,25(OH)_2D_3$ and considerably induced the ratio of RANKL/OPG expression (Fig. 2 F). These results suggest that KLF4 plays a negative regulatory role in $1,25(OH)_2D_3$-induced osteoclast differentiation by inhibiting the production of RANKL in osteoblasts.

RANKL expression is stimulated by other factors in addition to $1,25(OH)_2D_3$ in osteoblasts. We therefore examined the effect of KLF4 on RANKL induction by various factors such as PTH, TNF, and interleukin-1. Overexpression of KLF4 slightly inhibited PTH- and IL-1–induced RANKL expression but not that induced by TNF (Fig. S1). These results suggest that KLF4 chiefly regulates the $1,25(OH)_2D_3$-mediated signaling pathway to inhibit RANKL production in osteoblasts.

KLF4 competes with VDR for binding to RANKL promoter

To further investigate the mechanism by which KLF4 regulates $1,25(OH)_2D_3$-induced RANKL expression, we performed transient transfection assays using RANKL promoter plasmid in UAMS-32 cells, a stromal/osteoblast cell line (O'Brien et al., 1999). RANKL promoter activity was significantly increased by $1,25(OH)_2D_3$ in a dose-dependent manner (Fig. 3 A). However, $1,25(OH)_2D_3$-induced RANKL promoter activity was strongly inhibited by KLF4 (Fig. 3 B). $1,25(OH)_2D_3$ is known to induce RANKL up-regulation through signals initiated by the VDR. Binding of VDR to the vitamin D responsive elements in the RANKL promoter region is required for sufficient induction of RANKL by $1,25(OH)_2D_3$ (Kitazawa and Kitazawa, 2002). Given our observation that KLF4 prevents $1,25(OH)_2D_3$-induced RANKL expression, we examined whether KLF4 could block binding of VDR to the RANKL promoter region by using a chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 3 C, KLF4 inhibited the direct binding of VDR to the RANKL promoter region. Next, we examined whether endogenous KLF4 binds to the RANKL promoter. Interestingly, ChIP analysis using a KLF4 antibody showed that KLF4 associated with the RANKL promoter before $1,25(OH)_2D_3$ treatment (Fig. 3 D). Upon $1,25(OH)_2D_3$ stimulation, however, binding of KLF4 to the RANKL promoter was diminished and recruitment of VDR to the RANKL promoter region was simultaneously increased (Fig. 3 D). Collectively, our results demonstrate that KLF4 competes with the VDR for binding to the RANKL promoter region and thereby attenuates $1,25(OH)_2D_3$–VDR signaling pathway–mediated RANKL expression.

KLF4 expression is increased during osteoblast differentiation

Because KLF4 was expressed in osteoblasts (Fig. 1 B), we examined expression patterns during osteoblast differentiation. When we cultured primary calvarial osteoblasts in the presence of osteogenic factors containing ascorbic acid, β-glycerophosphate, and BMP2, KLF4 expression was gradually increased during osteoblast differentiation. Expression levels of Runx2, AP, and BSP, well-known marker genes of osteoblast differentiation, increased as the cells differentiated (Fig. 4).

KLF4 negatively regulates osteoblast differentiation and bone formation

To investigate the role of KLF4 in osteoblast differentiation, osteoblasts were infected with control or KLF4 retrovirus and further cultured in the presence of osteogenic factors. AP activity and bone nodule formation were evaluated as markers of osteoblast differentiation and function. AP staining and activity were significantly inhibited in the KLF4-overexpressing osteoblasts

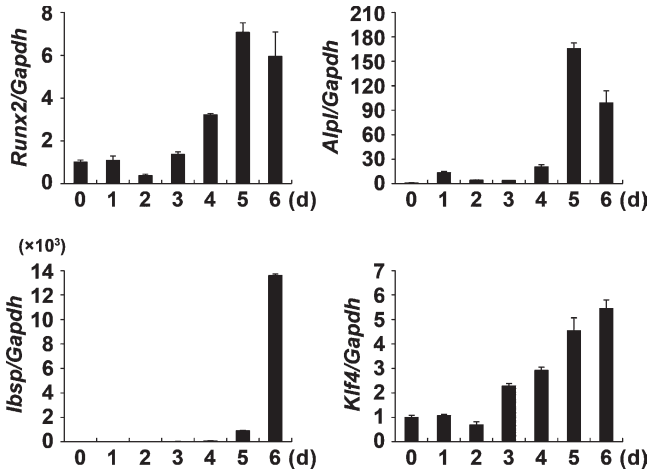

Figure 4. **Expression of KLF4 during osteoblast differentiation.** Osteoblasts were cultured with osteogenic medium containing BMP2 (100 ng/ml), ascorbic acid (50 µg/ml), and β-glycerophosphate (100 mM) for the indicated times. Quantitative real-time PCR was performed for the mRNA expression of *Runx2*, *Alpl* (AP), *Ibsp* (BSP), and *Klf4*. Data represent means ± SD of triplicate samples.

(Fig. 5 A). Also, similar with the AP results, overexpression of KLF4 strongly inhibited bone nodule formation and alizarin red activity under the osteogenic conditions (Fig. 5 B).

Next, we examined the effect of KLF4 on the expression of osteogenic marker genes. KLF4 slightly inhibited the expression of Runx2 and strongly inhibited the expression of AP and BSP induced by the osteogenic medium (Fig. 5 C). To further confirm these results, we used shKLF4 to silence KLF4 expression during osteoblast differentiation. When we cultured osteoblasts infected with pSuper control shRNA or shKLF4 retrovirus in the osteogenic medium, AP staining and activity were higher in osteoblasts infected with shKLF4 retrovirus than in osteoblasts infected with pSuper retrovirus (Fig. 5 D). Down-regulation of KLF4 expression in osteoblasts also increased bone nodule formation and alizarin red activity (Fig. 5 E). Moreover, shKLF4 retrovirus enhanced the expression of Runx2, AP, and BSP during osteoblast differentiation (Fig. 5 F). Collectively, these data suggest that KLF4 plays a role as a negative regulator in osteoblast differentiation and mineralization by regulation of osteoblast-specific genes.

KLF4 inhibits transcriptional activity of Runx2 through direct interaction

Runx2 is a key transcription factor that induces expression of osteogenic genes such as AP and BSP during osteoblast differentiation. Our results showed that although KLF4 slightly inhibited Runx2 expression, the expression of AP and BSP was greatly inhibited in osteoblasts transduced with KLF4. Therefore, we examined whether KLF4 could affect the transcriptional activity of Runx2. When a reporter plasmid containing the AP promoter region was cotransfected with Runx2 into 293T cells, relative luciferase activity was increased. However, luciferase activity induced by Runx2 was significantly decreased when KLF4 was cotransfected (Fig. 6 A). Also, BSP promoter-dependent luciferase activity was significantly suppressed by KLF4 (Fig. 6 B). These results showed that KLF4 could inhibit the transcriptional activity of Runx2. It has been reported that KLF4 can repress β-catenin–mediated gene expression via direct interaction with β-catenin (Zhang et al., 2006). Thus, we examined whether KLF4 could interact with Runx2 directly by using an immunoprecipitation assay. 293T cells were cotransfected with HA-tagged Runx2 and Flag-tagged KLF4. As shown in Fig. 6 C, KLF4 could interact with Runx2 directly. In addition, the association between endogenous Runx2 and KLF4 was found in primary osteoblasts (Fig. S2 A). Next, we investigated whether the interaction between KLF4 and Runx2 could modulate the DNA binding activity of Runx2 by using a biotinylated DNA affinity precipitation assay. 293T cells were cotransfected with HA-tagged Runx2 and Flag-tagged KLF4, and cell lysates were precipitated with a biotinylated OSE (Runx2 DNA-binding elements) probe. Precipitated samples were subjected to SDS-PAGE and Western blotting with anti-HA antibodies. As shown in Fig. 6 D, binding of Runx2 to the OSE probe was attenuated by KLF4. This inhibitory effect of KLF4 on Runx2 DNA binding activity was confirmed by ChIP analysis. When KLF4 was overexpressed in osteoblasts, binding of Runx2 to the AP or BSP promoter was diminished (Fig. S2 B). Collectively, these results clearly demonstrate that KLF4 can act as a negative regulator of Runx2 by inhibiting the transcriptional and DNA binding activity of Runx2 via a direct interaction.

KLF4 deficiency in osteoblasts results in increased bone mass in vivo

To examine the effect of KLF4 on bone homeostasis in vivo, we generated conditional KLF4 knockout mice by crossing floxed KLF4 ($KLF4^{fl/fl}$) mice with collagen type I promoter-driven Cre (*Col1α-Cre*) mice. KLF4 conditional knockout mice were viable and developed normally with no obvious phenotypical abnormality. We assessed the metaphyseal femoral region of the long bones of 8-wk-old KLF4 conditional knockout mice and their littermate control mice by using micro-computed tomography (µCT) imaging. As shown in Fig. 7 A, conditional deletion of KLF4 in osteoblasts increased bone mass compared with that in littermate control mice in vivo. µCT showed that bone volume, trabecular separation, and trabecular number were significantly different between KLF4 conditional knockout mice and littermate control mice, whereas trabecular thickness was not significantly different between the two groups (Fig. 7 B). Next, to examine the effect of osteoblast-dependent KLF4 deficiency on the formation of osteoclasts and osteoblasts in vivo, we conducted histomorphometric analyses of the proximal tibiae. The numbers of both osteoclasts and osteoblasts were significantly increased in KLF4 conditional knockout mice (Fig. 7 C). We next assessed levels of serum C-terminal cross-linking telopeptide of type 1 collagen (CTx-I) and the mineral apposition rate to confirm the effect of KLF4 deficiency on osteoclast function and bone formation in vivo, respectively. The levels of serum CTx-I were significantly increased in KLF4 conditional knockout mice compared with those of control littermates (Fig. 7 D). Also, double calcein labeling analysis of KLF4 conditional knockout mice revealed a significant increase in the mineral apposition rate (Fig. 7 E). Therefore, these findings revealed that KLF4 conditional knockout mice show increased bone mass even with increased osteoclast differentiation and function, and this phenotype is associated with increased osteoblast differentiation

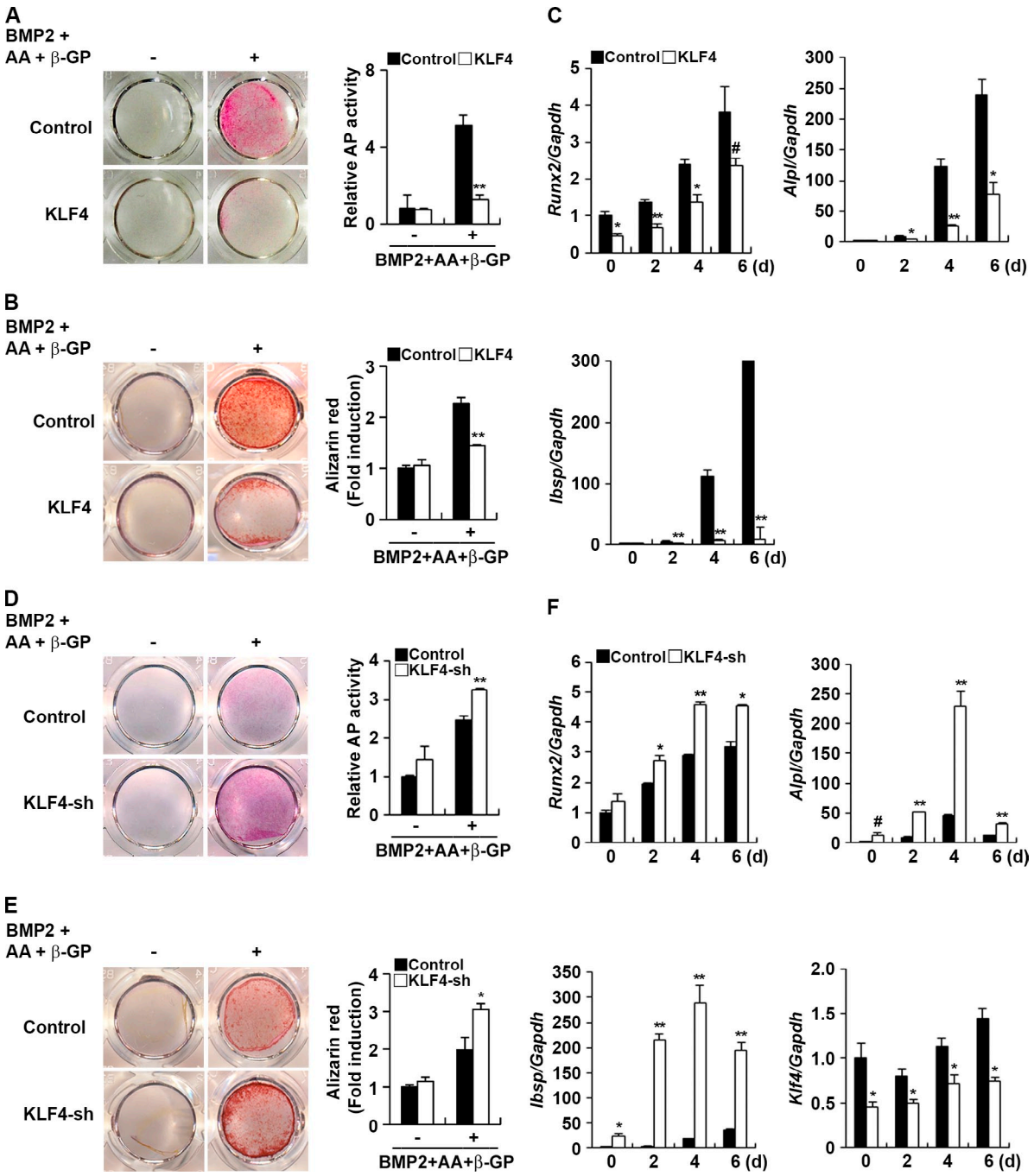

Figure 5. **Inhibition of osteoblast differentiation and mineralization by KLF4.** (A–C) Osteoblasts were transduced with pMX-IRES-*EGFP* (control) or KLF4 retrovirus and cultured with osteogenic medium. (A) Cells cultured for 4 d were fixed and stained for AP (left). AP activities were measured by densitometry at 405 nm (right). (B) Cells cultured for 9 d were fixed and stained for Alizarin red (left). Alizarin red staining activity was quantified by densitometry at 562 nm (right). (C) Transduced cells were cultured for the indicated times and quantitative real-time PCR analysis of mRNA expression of *Runx2*, *Alpl* (AP), and *Ibsp* (BSP) was performed. (D–F) Osteoblasts were transduced with pSuper (control) or shKLF4 retrovirus and cultured with osteogenic medium. (D) Cells cultured for 4 d were fixed and stained for AP (left). AP activities were measured by densitometry at 405 nm (right). (E) Cells cultured for 9 d were fixed and stained for Alizarin red (left). Alizarin red staining activity was quantified by densitometry at 562 nm (right). (F) Transduced cells were cultured for the indicated times and quantitative real-time PCR analysis of mRNA expression of *Runx2*, *Alpl* (AP), *Ibsp* (BSP), and *Klf4* was performed. Data represent means ± SD of triplicate samples. #, $P < 0.05$; *, $P < 0.01$; **, $P < 0.001$ versus control.

and activation. Given our observation that knockdown of KLF4 by shRNA in osteoblasts increases $1,25(OH)_2D_3$-induced RANKL expression and osteoblast differentiation and activity, we conducted a final experiment to confirm these results. We prepared primary osteoblast precursor cells from calvaria of KLF4 conditional knockout mice or littermate control mice and cultured these cells with $1,25(OH)_2D_3$ or osteogenic medium. Unexpectedly, $1,25(OH)_2D_3$-mediated RANKL expression as well as KLF4 expression were comparable in KLF4 conditional knockout and control osteoblasts (Fig. 7 F). However, when primary osteoblast precursor cells were cultured with osteogenic medium to induce osteoblast differentiation, the expression of

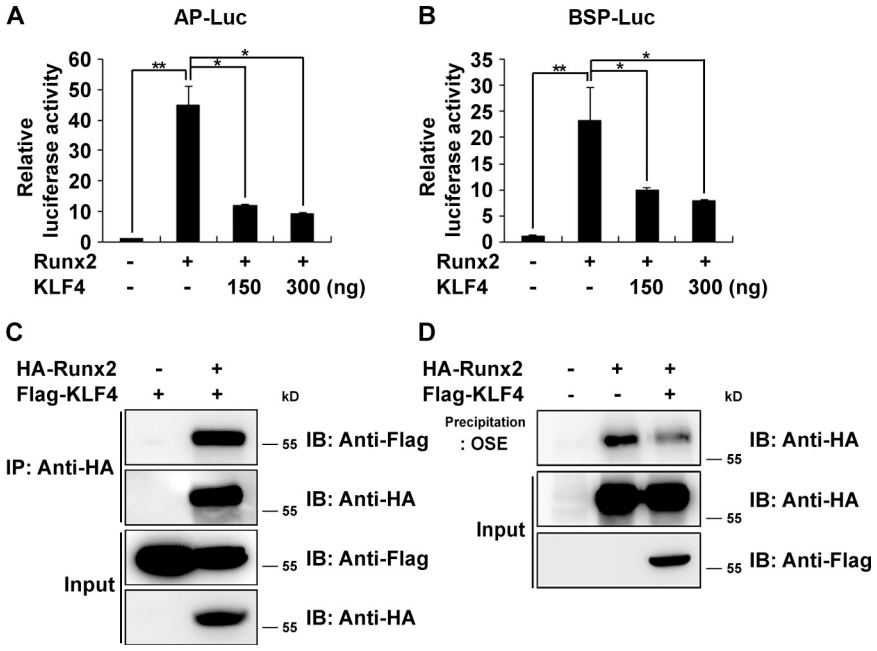

Figure 6. **Inhibition of Runx2 transcriptional activity by KLF4.** (A) An AP promoter luciferase reporter was cotransfected with Runx2 and increasing amounts of KLF4 into 293T cells. (B) A BSP promoter luciferase reporter was cotransfected with Runx2 and increasing amounts of KLF4 into 293T cells. (A and B) Cells were lysed and luciferase activities were determined. Data represent means ± SD of triplicate samples. *, $P < 0.01$; **, $P < 0.001$ versus control. (C) 293T cells were transfected with Flag-KLF4 and HA-Runx2. 2 d after transfection, cell lysates were immunoprecipitated (IP) with mouse monoclonal anti-HA antibodies, followed by immunoblot (IB) with the indicated antibodies. (D) 293T cells were transfected with Flag-KLF4 and HA-Runx2. 2 d after transfection, cell lysates were precipitated with biotinylated OSE probe and streptavidin-coated agarose beads. Precipitated samples were subjected to SDS-PAGE and followed by immunoblot with the indicated antibodies.

KLF4 was efficiently reduced and the expression of osteogenic marker genes including Runx2, AP, and BSP was strongly enhanced in KLF4 conditional knockout osteoblasts (Fig. 7 G). Our results using shKLF4 retrovirus or KLF4 conditional knockout mice–derived osteoblasts revealed that expression of RANKL and osteogenic marker genes is increased only when KLF4 expression is efficiently reduced in osteoblasts. Thus, collectively, these results confirmed that the expression level of KLF4 can regulate the function of osteoblasts as a supporter of osteoclasts or a regulator of bone formation. In the cells isolated from KLF4 conditional knockout mice, we observed a significant reduction in KLF4 expression during osteoblastogenesis (Fig. 7 G) even though KLF4 expression was not reduced in preosteoblasts (Fig. 7 F). This finding might have been a result of insufficient collagen expression in preosteoblasts for inducing Cre expression. Indeed, when we examined the expression level of KLF4 and RANKL in vivo, we observed increased RANKL expression and decreased KLF4 expression in long bone of KLF4 conditional knockout mice (Fig. S3). Collectively, these results suggest that KLF4 plays an important role in bone remodeling through regulation of two distinct roles of osteoblasts in vivo.

Discussion

In the present study, we identified KLF4 as a transcriptional repressor during $1,25(OH)_2D_3$-mediated osteoclastogenesis. Our data showed that KLF4 expression was decreased by $1,25(OH)_2D_3$ in osteoblasts. Additionally, our results from overexpression or down-regulation of KLF4 using retrovirus and shRNA, respectively, demonstrated that KLF4 affects $1,25(OH)_2D_3$-induced RANKL expression and thereby can indirectly regulate osteoclast differentiation. Unexpectedly, however, we did not observe an inhibitory effect of KLF4 on RANKL expression in KLF4 conditional knockout mice–derived osteoblasts owing to weak down-regulation of KLF4 expression. After collagen type I-Cre–mediated KLF4 deletion, KLF4 expression levels were weakly reduced in preosteoblasts but were significantly reduced in mature osteoblasts. This differential reduction of KLF4 expression suggests that collagen type I-Cre–mediated KLF4 deletion in bone occurs more efficiently in mature osteoblasts and is dependent on the stage of osteoblastic differentiation. Thomas et al. (2001) revealed that the basal mRNA level of RANKL is not changed during osteoblast differentiation and that RANKL expression is significantly enhanced by $1,25(OH)_2D_3$ treatment regardless of the stage of osteoblastic differentiation. These results suggest that $1,25(OH)_2D_3$ can stimulate RANKL expression not only in mature osteoblasts but also in preosteoblasts. Therefore, it seems that collagen type I-Cre–mediated KLF4 deletion in bone leads to down-regulation of KLF4 in mature osteoblasts rather than in preosteoblasts and that this down-regulation results in an increase in RANKL expression and osteoclast formation in vivo. Indeed, we observed decreased KLF4 expression and increased RANKL expression in long bone of KLF4 conditional knockout mice.

KLF4 can act as a transcriptional repressor via simple competition with an activator for binding to a target DNA sequence (Zhang et al., 1998; Kanai et al., 2006). In this study, we showed that KLF4 directly binds to the RANKL promoter region, displacing the VDR from the promoter and resulting in repression of RANKL expression. KLF4 contains three tandem C2H2 zinc finger motifs at its C-terminal end that bind to CC-GT–rich or CACCC elements on target genes (Yet et al., 1998). Using a software suite to search for transcription factor binding sites, we found that an ~650–base pair region upstream from the transcription start site of the RANKL promoter contains several putative KLF4 binding sites that are located adjacently in the VDR binding site. Because the binding sites for VDR and KLF4 exist in close proximity to each other within the RANKL promoter, the binding of KLF4 on the RANKL promoter may interrupt

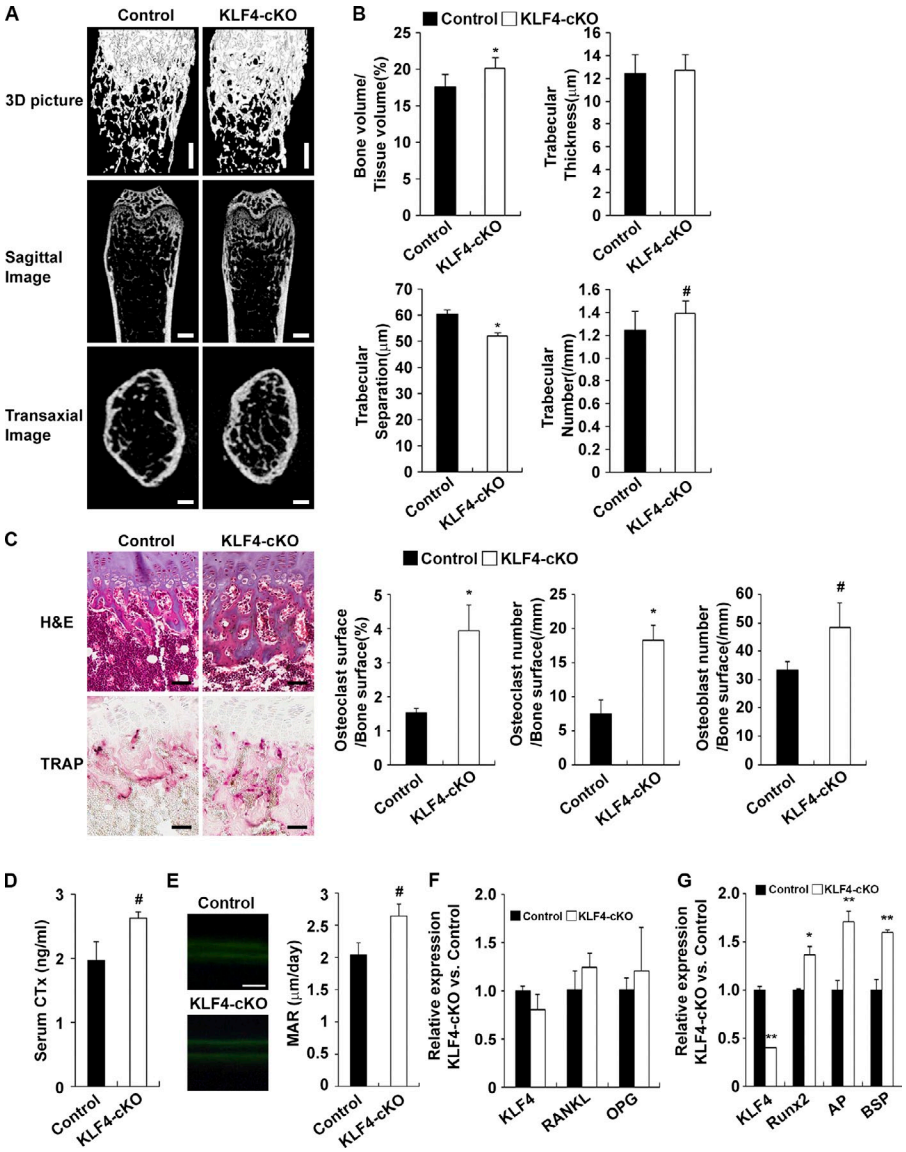

Figure 7. **Increase of bone mass in vivo by osteoblast-specific *Klf4* deficiency.** (A) Representative three- and two-dimensional images of femurs in control or KLF4 conditional knockout mice. Bars, 500 µm. (B) Bone volume per tissue volume, trabecular bone thickness, trabecular separation, and trabecular number were assessed from the µCT measurements (n = 10). (C) The hematoxylin/eosin (H&E) and TRAP staining of histological section of proximal tibiae (left). Bars, 50 µm. Osteoclast surface per bone surface, osteoclast number per bone surface, and osteoblast number per bone surfaced were assessed (right; n = 4). (D) Serum concentration of CTx-I in control or KLF4 conditional knockout mice (n = 3). (E) Representative images of calcein double labeling (×100; left). The mineral apposition rate (MAR) was assessed (right; n = 3). Bar, 50 µm. (F and G) Osteoblasts were isolated from control or KLF4 conditional knockout mice. (F) Control or KLF4 conditional knockout mice-derived osteoblasts were cultured with 1,25$(OH)_2D_3$ (10 nM) for 2 d. mRNA expression of *Tnfsf11* (RANKL), *Tnfrsf11b* (OPG), and *Klf4* was assessed by quantitative real-time PCR. (G) Control or KLF4 conditional knockout mice-derived osteoblasts were cultured with osteogenic medium for 5 d. mRNA expression of *Runx2*, *Alpl* (AP), *Ibsp* (BSP), and *Klf4* was assessed by quantitative real-time PCR. Data represent means ± SD. #, $P < 0.05$; *, $P < 0.01$; **, $P < 0.001$ versus control.

recruitment of the VDR to the promoter. Therefore, down-regulation of KLF4 by 1,25$(OH)_2D_3$ is required for sufficient RANKL expression during osteoblast-mediated osteoclast differentiation in co-culture of osteoblasts with bone marrow cells.

A previous study reported that KLF4 inhibits osteoblast differentiation (Michikami et al., 2012). However, the precise mechanism of this inhibition was unclear. In this study, we found that KLF4 physically associated with Runx2 to inhibit the DNA binding and transcriptional activity of Runx2. Repression of Runx2 transcriptional activity by KLF4 resulted in reduction of AP and BSP expression and inhibition of osteoblast differentiation and function. It has been well documented that Runx2 is a critical transcription factor in osteoblast differentiation and skeletal development (Ducy et al., 1999). Overexpression of Runx2 in osteoblasts induces several osteoblast-related genes such as AP, BSP, and osteocalcin. Also, deletion of Runx2 in mice causes an arrest of osteoblast differentiation, which suggests that Runx2 acts as a key modulator of osteoblastogenesis. Many genes have been identified to regulate Runx2 expression and its activity through transcriptional and posttranslational mechanisms. In particular, MEF, Nrf2, YAP, and p53 can interact with Runx2 and thereby inhibit its transcriptional activity (Zaidi et al., 2004; Hinoi et al., 2006; Lengner et al., 2006; Kim et al., 2007). Herein, we suggest that in addition to the genes mentioned here, KLF4 acts as a negative regulator of Runx2.

Although the inhibitory effect was very low, overexpression of KLF4 attenuated Runx2 expression as well as AP and BSP. Because Runx2 can induce its expression by binding to its own promoter, KLF4 in part can attenuate Runx2 expression through the inhibition of Runx2 transcriptional activity. Thus, up-regulation of KLF4 during osteoblastogenesis may fine-tune osteoblast differentiation via a negative feedback mechanism.

It has been reported that Runx2 plays a negative role in osteoblast differentiation during the late stage (Liu et al., 2001; Maruyama et al., 2007). Transgenic mice overexpressing Runx2 in both the early and the late stages of osteoblast differentiation exhibited an osteopenic phenotype because the fully differentiated osteoblasts were reduced in the transgenic mice (Liu et al., 2001). Also, these transgenic mice showed that the expression of AP and osteocalcin was decreased but the expression of

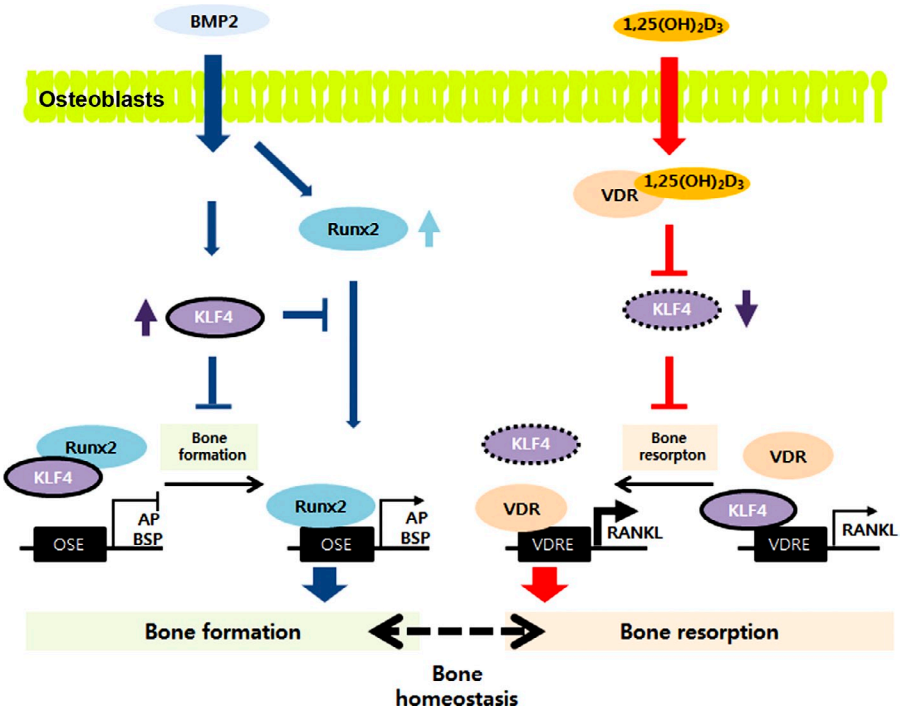

Figure 8. **Proposed model for role of KFL4 in bone homeostasis.** In osteoblasts, KLF4 expression is attenuated and enhanced by $1,25(OH)_2D_3$ and BMP2, respectively. KLF4 attenuates RANKL expression via binding competition with VDR to the RANKL promoter region. Thus, $1,25(OH)_2D_3$ could induce RANKL expression by down-regulation of KLF4 in osteoblasts. In contrast, BMP2 induces the expression of KLF4 during osteogenesis. KLF4 inhibits Runx2-mediated expression of AP and BSP through direct interaction with Runx2, thus providing a negative feedback in regulating bone formation. Reciprocal regulation of KLF4 expression by various factors can control normal bone homeostasis through the regulation of bone formation and osteoblast-mediated bone resorption.

osteopontin and BSP was increased. Interestingly, previous results (Michikami et al., 2012) and our results (Fig. S4) showed that overexpression of KLF4 dramatically enhances the expression of osteocalcin, which is restricted to mature osteoblasts. These results indicated that KLF4 acts as a transcriptional repressor of Runx2 in both the early and the late stages of osteoblast differentiation. Thus, up-regulation of KLF4 by osteogenic factors may be required for prevention of a negative role of Runx2 in mature osteoblasts, and adequate bone formation can be controlled by the delicate balance of actions between KLF4 and Runx2.

Previously, Michikami et al. (2012) reported that when KLF4 is overexpressed in osteoblasts in vivo, the formation of calvarial bones is severely impeded. However, because they analyzed the bone phenotype of transgenic embryos at E18.5 to overcome the possible embryonic or perinatal lethality, the previous study was limited for fully understanding the role of KLF4 in bone homeostasis. Herein, we further studied the consequences of osteoblast-dependent KLF4 deficiency on bone phenotype regulation in vivo. Osteoblast-specific deletion of KLF4 in mice resulted in increased bone mass owing to increased osteoblast differentiation and activation even with the increased osteoclast differentiation and function. These findings indicate that the increased osteoblast differentiation and activation in KLF4 deficiency are more prominent than the enhanced osteoclast differentiation and function with regard to the overall extent of bone homeostasis in vivo. Thus, the present study along with the previous results collectively support that KLF4 is a potent negative regulator of osteoblast differentiation and function in vitro and in vivo. In addition, the present study provides a novel finding that KLF4, with respect to osteoclasts, is a negative regulator that indirectly acts through regulation of RANKL in osteoblasts.

Indeed, it is well known that KLF4 is a repressor of the Wnt–β-catenin signaling pathway in colorectal cancer (Zhang et al., 2006; Evans et al., 2010). Also, with regard to osteoclasts, it has been well elucidated that the expression of RANKL is increased and the level of OPG is decreased in osteoblasts lacking β-catenin (Glass et al., 2005; Kubota et al., 2009). However, our findings reveled that overexpression of KLF4 significantly decreased RANKL expression induced by stimulation with $1,25(OH)_2D_3$ without affecting OPG expression. Therefore, these results indicate that KLF4 acts primarily as a negative regulator of the $1,25(OH)_2D_3$-VDR signaling pathway rather than as an inhibitory regulator of β-catenin to modulate osteoblast-mediated osteoclast differentiation.

Recently, it was reported that postnatal deletion of β-catenin in the osteoblastic lineage causes a striking reduction in bone mass and a remarkable increase in bone marrow adiposity (Song et al., 2012). These results provide direct evidence that β-catenin, a key component of the Wnt pathway, is important for bone acquisition in vivo. In fact, the bone phenotype of mice lacking β-catenin in osteoblasts is very similar to that of transgenic mice expressing KLF4 in osteoblasts (Michikami et al., 2012; Song et al., 2012). Although KLF4 regulates RANKL expression in osteoblasts through Wnt–β-catenin–independent signaling pathways, we cannot exclude the possibility that KLF4 regulates osteoblast differentiation and function through two distinct mechanisms: Wnt–β-catenin–independent and –dependent signaling pathways. Thus, further study will be required to elucidate the precise molecular mechanisms that govern the function of KLF4 in osteoblasts.

To summarize, we have proposed a model for the role of KLF4 in bone remodeling (Fig. 8). Advanced bone formation might be controlled by transcriptional regulation of KLF4 through two distinct mechanisms in osteoblasts: down-regulation

by $1,25(OH)_2D_3$ and up-regulation by osteogenic factors. Down-regulation of KLF4 by $1,25(OH)_2D_3$ facilitates recruitment of VDR to the RANKL promoter by displacing KLF4 from the promoter, which then induces RANKL expression. Also, up-regulation of KLF4 by osteogenic factors provides negative feedback for the Runx2-mediated signaling pathway. Direct interaction of KLF4 with Runx2 can reduce excessive transcriptional activity of Runx2 and result in inhibition of bone formation. In this study, we showed that osteoblast-specific inhibition of KLF4 in vivo resulted in an overall increase in bone mass. Collectively, our results suggest that KLF4 may contribute to maintenance of normal bone homeostasis, using different repression mechanisms depending on the target gene in osteoblasts.

Materials and methods

Reagents

Recombinant human sRANKL was purified from insect cells and recombinant human M-CSF was a gift from D. Fremont (Washington University, St. Louis, MO). Recombinant human BMP2 was purchased from Cowellmedi. Alizarin red, β-glycerophosphate, AP assay kits, and vitamin D_3 were purchased from Sigma-Aldrich. Ascorbic acid was purchased from Junsei Chemical. The luciferase assay kits and ChIP assay kits were obtained from Promega and EMD Millipore, respectively.

Plasmids

For knockdown of KLF4, the following oligonucleotides targeting mouse *Klf4* sequences (in capital letters) were annealed and inserted to *pSuper-retro-puro*: 5′-gatccccAGACCAGATGCAGTCACAAttcaagagaTTGTGACTGCATCTG-GTCTttttc-3′ and 5′-tcgagaaaaaAGACCAGATGCAGTCACAAtctcttgaaTT-GTGACTGCATCTGGTCTggg-3′. The promoter regions of RANKL (0.6 kb) were amplified by PCR by using mouse genomic DNA. The amplified PCR fragments were subsequently cloned into the pGL2 basic luciferase vector (Promega). AP-Luc and BSP-Luc reporter plasmids were provided by J.T. Koh (Chonnam National University, Gwangju, Korea).

Animals

$KLF4^{fl/fl}$ mice with a floxed exon 2 and 3 of the *Klf4* gene were obtained from Mutant Mouse Regional Resource Centers. To generate KLF4 conditional knockout mice ($KLF4^{fl/fl}$;*Col1α-Cre*), $KLF4^{fl/fl}$ mice were crossed with *Col1α-Cre* mice harboring the *Col1a1-Cre* transgene with *Cre* recombinase, which is active in osteoblasts under the control of the 2.3-kb collagen type 1 α promoter (Baek et al., 2009). Heterozygous floxed KLF4 offspring with *Col1α-Cre* ($KLF4^{fl/+}$;*Col1α-Cre*) were backcrossed to $KLF4^{fl/fl}$ mice. Homozygous floxed KLF4 littermates with *Col1α-Cre* ($KLF4^{fl/fl}$;*Col1α-Cre*) and homozygous floxed KLF4 littermates lacking *Col1α-Cre* ($KLF4^{fl/fl}$) were used as KLF4 conditional knockout mice and control mice, respectively. All mice were maintained and procedures were performed as per guidelines by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. The Ethics Committee of Chonnam National University Hospital approved our experimental protocols.

Cell cultures

To generate osteoclasts, mouse bone marrow cells were isolated from tibiae and femurs of 6-wk-old mice by flushing the bone marrow with α-MEM. Cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) in the presence of 30 ng/ml M-CSF for 3 d. Subsequently, nonadherent cells were removed and adherent BMMs were further cultured with 30 ng/ml M-CSF and 100 ng/ml RANKL for 3 d.

For osteoblast-mediated osteoclast generation, bone marrow cells were cultured with calvarial osteoblast precursors in the presence of $1,25(OH)_2D_3$ (10 nM) for 5 d and the culture medium was replaced every 3 d. Then, cells were fixed and stained for TRAP. TRAP-positive MNCs (>3 nuclei/cell) were determined as osteoclasts and counted. Cells were imaged with a ProgRes CFscan (Jenoptik) camera attached to a DMIRB microscope (Leica) using a 10× objective (NA 0.25) at room temperature. Images were captured using a ProgRes Capture Pro (Jenoptik).

Primary osteoblast precursor cells were isolated from the calvarial bone from newborn mice by enzymatic digestion with α-MEM containing 0.1% collagenase (Invitrogen) and 0.2% dispase II (Roche). In short, enzymes were removed and cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Osteoblast differentiation was promoted with osteogenic medium containing 100 ng/ml BMP2, 50 µg/ml ascorbic acid, and 100 mM β-glycerophosphate for 4 to 9 d and culture medium was replaced every 3 d. For the AP activity assay, cells were either stained with AP or lysed at day 4 of culture with osteoblast lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA). Then, cell lysates were incubated with p-nitrophenyl phosphate (Sigma-Aldrich) and AP activity was measured by using a spectrophotometer at 405 nm. For the mineralization assay, cells cultured for 9 d were fixed with 70% ethanol and stained with 40 mM alizarin red, pH 4.2, at room temperature. Subsequently, nonspecific stains were removed by washing with PBS and the plates were scanned using a CanoScan 4400F (Canon USA, Inc.). For quantification, stained alizarin red was dissolved with 10% cetylpyridinium (Sigma-Aldrich) for 15 min at room temperature. Then, the extracted solution was measured by using a spectrophotometer at 562 nm.

Retroviral gene transduction

To obtain retroviral supernatant, retroviral vectors were transfected into the packaging cell line, Plat E, by using FuGENE 6 (Roche) according to the manufacturer's instructions. Retroviral supernatant was collected from culture media 48 h after transfection. For retroviral infection, BMMs or osteoblast precursors were incubated with viral supernatant for 8 h in the presence of polybrene (10 µg/ml).

Western blotting and immunoprecipitation

After 293T cells were transfected with Flag-KLF4 and HA-Runx2 for 48 h, the cells were washed with chilled PBS and lysed in extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, PMSF, and protease inhibitors). Cell lysates were immunoprecipitated with mouse monoclonal anti-HA antibody (Roche). Cell lysates or immunoprecipitated samples were subsequently separated by SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were blocked with TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk and probed with HRP-conjugated antibodies including mouse monoclonal anti–Flag-HRP (Sigma-Aldrich) and mouse monoclonal anti–HA-HRP (Sigma-Aldrich). Signals were detected with enhanced chemiluminescence and analyzed by use of a luminescent image analyzer (LAS3000; Fujifilm).

ChIP assays

ChIP assays were performed with a ChIP kit (EMD Millipore) according to the manufacturer's instructions. In brief, cultured cells were cross-linked with 1% formaldehyde and resuspended in SDS lysis buffer. Lysates were sonicated to shear DNA into small fragments of a mean length between 200 and 1,000 base pairs. Sonicated cell supernatant was diluted 10-fold in ChIP dilution buffer and incubated with the antibodies indicated in the figure legends (rabbit polyclonal anti-VDR, rabbit polyclonal anti-KLF4, and rabbit polyclonal anti-Runx2 [Santa Cruz Biotechnology, Inc.]) or normal rabbit IgG (Santa Cruz Biotechnology, Inc.) at 4°C overnight. Immune complexes were precipitated with protein A–agarose and eluted in fresh elution buffer after washing nonspecific binding. After cross-link reversal, DNA samples were recovered by phenol/chloroform extraction and ethanol precipitation. Recovered DNA samples were subjected to PCR amplification with forward primer (5′-ATGGCCCAGACCCAGTGTCTGGTG-3′) and reverse primer (5′-GCACAATGAGGCGAGAGCAGTGTG-3′) to identify the vitamin D responsive element binding site on the RANKL promoter region. The primers used for the AP promoter were 5′-GGCTGGGACAGACAGA-ATGT-3′ (forward) and 5′-CTTTGTCCCTCGATGGTTGT-3′ (reverse) and those for the BSP promoter were 5′-GCCTCAGTTGAATAAACATGAAA-3′ (forward) and 5′-TCCTCACCCTTCAATTAAATCCCACAA-3′ (reverse).

μCT analysis

The femurs were collected from 8-wk-old mice, cleaned free of soft tissues, and fixed in 70% ethanol. μCT images of distal femurs were taken by using the high-resolution Skyscan 1172 system (Bruker) with the x-ray source at 50 kV and 201 mA with a 0.5-mm aluminum filter. Images were captured with a pixel size of 11 mm, every 0.7°, over an angular range of 180°. Raw images were reconstructed into serial cross section images with identical thresholds for all samples (0 to 6,000 in Hounsfield units) by use of image reconstruction software (CTAn; Bruker). A total of 300 steps of trabecular

bones of proximal femurs starting from 80 steps away from the epiphyseal plate were manually designated as a region of interest, and femoral morphometric parameters were determined by using data analysis software (CTAn). Trabecular morphometry was characterized by measuring the bone volume over tissue volume, trabecular thickness, trabecular number, and trabecular separation. For three-dimensional visualization, pixels between thresholds of 90 and 165 were regarded as bones and processed by use of model visualization software (CTAn).

Double calcein labeling

Double calcein labeling was performed by intraperitoneal injection of mice with 30 mg/kg body weight of calcein (Sigma-Aldrich) on days 10 and 3 before sacrifice. Bones were harvested and fixed in 4% paraformaldehyde for 2 d at 4°C. Fixed bones were embedded in methyl methacrylate and then sectioned. Dynamic histomorphometric analyses were conducted using the Bioquant program (Bio-Quant Inc.).

Serum CTx-I measurement

Blood samples were collected retro-orbitally under anesthesia immediately before sacrifice. Serum CTx-I was measured by using an ELISA kit (Novateinbio) according to the manufacturer's instructions.

Bone histomorphometric analysis

Tibiae collected from 8-wk-old mice were fixed in 4% paraformaldehyde and decalcified in 5.5% EDTA buffer for 2 wk at 4°C. Samples were gradually dehydrated and embedded in paraffin and cut into 4-µm-thick longitudinal sections. After being deparaffinized with xylene, the sections were stained with hematoxylin and eosin or TRAP to count osteoblasts and osteoclasts, respectively.

Quantitative real-time PCR analysis

Cells were lysed in Qiazol (QIAGEN) and total RNA was isolated according to the manufacturer's instructions. RNA in amounts of 2 µg was reverse transcribed into cDNA by use of Superscript II Reverse transcription (Invitrogen). Quantitative real-time PCR analysis was performed in triplicate with a Rotor-Gene Q (QIAGEN) with SYBR Green (QIAGEN). The thermal cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Amounts of mRNAs were normalized to an endogenous housekeeping gene, *Gapdh*. The relative quantitation value for each target gene compared with the calibrator for that target was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to *Gapdh*). The relative expression levels of samples were presented by semi-log plot. The primer sequences were as follows: Runx2, 5′-CCCAGCCACCTTTACCTACA-3′ and 5′-CAGCGTCAACACCATCATTC-3′; AP, 5′-CAAGGATATCGACGTGATCATG-3′ and 5′-GTCAGTCAGGTTGTTCCGATTC-3′; BSP, 5′-GGAAGAGGAGACTTCAAACGAAG-3′ and 5′-CATCCACTTCTGCTTCTTCGTTC-3′; KLF4, 5′-ACTACCCCTACACTGAGTCCCGAG-3′ and 5′-TAGTGCCTGGTCAGTTCATCGGAG-3′; NFATc1, 5′-CTCGAAAGACAGCACTGGAGCAT-3′ and 5′-CGGCTGCCTTCCGTCTCATAG-3′; TRAP, 5′-CTGGAGTGCACGATGCCAGCGACA-3′ and 5′-TCCGTGCTCGGCGATGGACCAGA-3′; RANKL, 5′-CCTGAGACTCCATGAAAACGC-3′ and 5′-TCGCTGGGCCACATCCAACCATGA-3′; OPG, 5′-CGGCGTGGTGCAAGCTGGAAC-3′ and 5′-CCTCTTCACACAGGGTGACATC-3′; GAPDH, 5′-TGACCACAGTCCATGCCATCACTG-3′ and 5′-CAGGAGACAACCTGGTCCTCAGTG-3′.

Luciferase assay

UAMS-32 cells or 293T cells were plated on 24-well plates (2×10^4 cells/well) 1 d before transfection. Cells were cotransfected with reporter plasmid and various expression vectors as indicated in the figure legends. 24 h after transfection, UAMS-32 cells were stimulated with $1,25(OH)_2D_3$ (10 nM) for 24 h. After 48 h of transfection, cells were lysed in passive lysis buffer (Promega). Luciferase activity was measured by using a dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

Biotinylated DNA affinity precipitation assay

293T cells were transfected with Flag-KLF4 and HA-Runx2. After transfection for 48 h, the cells were washed with chilled PBS and lysed in extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, PMSF, and protease inhibitors). Cell lysates were precipitated with biotinylated OSE probe and streptavidin-coated agarose beads. Precipitated samples were subjected to SDS-PAGE and immunoblotted with antibodies as indicated in the figures (mouse monoclonal anti–Flag-HRP and mouse monoclonal anti–HA-HRP).

Statistical analysis

Statistical analyses were performed using unpaired Student's *t* tests. $P < 0.05$ was considered statistically significant, and all data are presented as the means ± SD.

Online supplemental material

Fig. S1 shows the effect of KLF4 on RANKL expression by various stimulators. Fig. S2 shows that KLF4 inhibits the DNA binding activity of Runx2 through direct interaction with Runx2 in osteoblasts. Fig. S3 shows that down-regulation of KLF4 in long bone increases RANKL expression. Fig. S4 shows the negative role of KF4 in osteocalcin expression during osteoblast differentiation. Table S1 shows the microarray data at day 4 of co-culture of mouse bone marrow cells and osteoblasts treated with $1,25(OH)_2D_3$ versus without $1,25(OH)_2D_3$. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201308102/DC1.

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The authors declare no competing financial interests.

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