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Title: The IRF2BP2-KLF2 axis regulates osteoclast and osteoblast differentiation

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Running Title: Role of IRF2BP2-KLF2 axis in bone remodeling

Keywords: IRF2BP2; KLF2; NF-κB; osteoclast; osteoblast

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ABSTRACT

Kruppel-like factor 2 (KLF2) has been implicated in the regulation of cell proliferation, differentiation, and survival in a variety of cells. Recently, it has been reported that KLF2 regulates the p65-mediated transactivation of NF-κB. Although the NF-κB pathway plays an important role in the differentiation of osteoclasts and osteoblasts, the role of KLF2 in these bone cells has not yet been fully elucidated. In this study, we demonstrated that KLF2 regulates osteoclast and osteoblast differentiation. The overexpression of KLF2 in osteoclast precursor cells inhibited osteoclast differentiation by downregulating c-Fos, NFATc1, and TRAP expression, while KLF2 overexpression in osteoblasts enhanced osteoblast differentiation and function by upregulating Runx2, ALP, and BSP expression. Conversely, the downregulation of KLF2 with KLF2-specific siRNA increased osteoclast differentiation and inhibited osteoblast differentiation. Moreover, the overexpression of interferon regulatory protein 2-binding protein 2 (IRF2BP2), a regulator of KLF2, suppressed osteoclast differentiation and enhanced osteoblast differentiation and function. These effects were reversed by downregulating KLF2. Collectively, our data provide new insights and evidence to suggest that the IRF2BP2/KLF2 axis mediates osteoclast and osteoblast differentiation, thereby affecting bone homeostasis.

INTRODUCTION

Bone homeostasis is the process by which bone is continually renewed by bone-resorbing osteoclasts and bone-forming osteoblasts. The balance between osteoclasts and osteoblasts is crucial for maintaining bone and determining bone density. However, imbalances between these bone cells have been associated with various diseases, such as osteoporosis and rheumatoid arthritis. Therefore, it is important to identify the molecules that regulate both osteoclast and osteoblast differentiation to maintain bone homeostasis.

Osteoclasts differentiate in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), thus these factors are essential for osteoclastogenesis. RANKL stimulation activates components of the NF-κB and MAPK signaling pathways, such as ERK, JNK, and p38 MAPKs (1). RANKL also activates a variety of transcription factors important for osteoclast differentiation, among which nuclear factor of activated T cells (NFATc1) is the master regulator of this process (2). Activated NFATc1 is transported to the nucleus where it induces the expression of target genes involved in osteoclast differentiation, including tartrate-resistant acid phosphatase (TRAP) and osteoclast-associated receptor (OSCAR) (1, 3). In addition, c-Fos is an essential transcription factor for osteoclast differentiation (4). c-Fos regulates the induction of NFATc1 expression by RANKL, and the cooperation between c-Fos and NFATc1 activates genes involved in osteoclastogenesis (5).

Osteoblasts are mononuclear cells that differentiate from mesenchymal cells under the control of several transcription factors and signaling cascades (6). Bone morphogenetic protein 2 (BMP 2) plays an important role in the differentiation of undifferentiated mesenchymal cells into osteoblasts (7). Runt-related transcription factor 2 (Runx2) is a master regulator of osteoblast differentiation which induces the expression of several osteogenic genes, including alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin, which are key regulators of osteoblast differentiation and function (8).

NF-κB is a family of five transcription factors consisting of NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel, which are involved in the differentiation of osteoclasts and osteoblasts (9). RANKL-induced c-Fos expression and osteoclast differentiation is impaired in NF-κB double knockout precursor cells and is restored by c-Fos overexpression (10). In osteoblasts, it has been reported that the p65 subunit of NF-κB suppresses the DNA binding activity of Smad proteins during BMP2-induced osteoblast differentiation, resulting in the inhibition of bone formation by NF-κB (11). Furthermore, NF-κB suppression prevents osteoporotic bone loss while NF-κB RelB inhibits osteoblast differentiation and bone formation by regulating Runx2 (9, 12). Moreover, it is known that mammalian sterile 20-like kinase 2 (Mst2) regulates bone homeostasis by inhibiting osteoclast differentiation and enhancing osteoblast differentiation via the NF-κB pathway (13).

Kruppel-like factors (KLFs) are a subclass of zinc-finger transcription factors implicated in the regulation of cellular growth, differentiation, and inflammation (14). KLF2, originally known as lung KLF, plays an important regulatory role in aspects of hematopoietic cell biology such as cell quiescence, proliferation, differentiation, and survival (15). Furthermore, KLF2 negatively regulates monocyte activation and function by inhibiting pro-inflammatory gene expression and reducing NF-κB promoter activity (16). Additionally, in KLF2 hemizygous mice, bone marrow-derived monocytes show an enhanced ability to differentiate into osteoclasts (17). These findings suggest that KLF2 may inhibit RANKL-induced osteoclast differentiation; however, the mechanisms involved are unknown and the role of KLF2 in osteoblasts has not been elucidated.

Interferon regulatory factor 2-binding protein 2 (IRF2BP2) consists of an N-terminal C4 zinc finger and a C-terminal C3HC4 RING domain and acts as a co-repressor of IRF2 (18). IRF2BP2 acts as an activator of VEGFA expression by interacting with the

Vgll4/TEAD4/MEF2 complex, and as an activator of the function of the repressor (18-20). In addition, IRF2BP2 regulates macrophage inflammation and lipid homeostasis by increasing MEF2-dependent KLF2 expression (21). IRF2BP2 is expected to control the differentiation of bone cells through KLF2, but its roles in bone remodeling have not yet been revealed.

In this study, we investigated the roles of KLF2, which suppresses the transcription factor NF-κB, in osteoclast differentiation and osteoblast differentiation and function. KLF2 was shown to inhibit osteoclast differentiation, whilst enhancing osteoblast differentiation and function. In addition, IRF2BP2 suppressed osteoclast differentiation and promoted osteoblast differentiation. Therefore, our data suggest that the IRF2BP2/KLF2 axis is important for osteoclastogenesis and osteoblastogenesis.

RESULTS

KLF2 inhibits RANKL-induced osteoclast differentiation

To investigate the expression of KLF2 during osteoclast differentiation, BMMs were cultured with M-CSF and RANKL for 3 days. The expression of osteoclast marker genes such as c-Fos, NFATc1, and TRAP was significantly increased during osteoclast differentiation (Supplementary Figure 1A). KLF2 was expressed at a low level in BMMs on day 1, with KLF2 expression gradually increasing during RANKL-induced osteoclast differentiation (Supplementary Figure 1A).

To elucidate the role of KLF2 in osteoclast differentiation, we overexpressed KLF2 in BMMs by retroviral transduction. As shown in Figure 1A, RANKL strongly induced osteoclast differentiation in the control vector-infected BMMs, whereas the formation of TRAP-positive multinucleate cells (MNCs) was significantly decreased in BMMs compared to the control cells, when KLF2 was overexpressed. Next, we examined whether KLF2 overexpression affected the expression of osteoclast marker genes. Compared with the control cells, KLF2 overexpression inhibited the mRNA expression of osteoclast marker genes such as c-Fos, NFATc1, and TRAP (Figure 1B), and reduced the protein expression of c-Fos and NFATc1 (Figure 1C).

Since KLF2 overexpression inhibited RANKL-induced osteoclastogenesis, we investigated the physiological role of KLF2 in osteoclast differentiation using siRNA. KLF2 mRNA expression was significantly decreased in BMMs transfected with KLF2-specific siRNA (Figure 1D). KLF2 downregulation in BMMs enhanced osteoclast differentiation (Figure 1E) and increased the mRNA expression of osteoclast marker genes, such as c-Fos, NFATc1, and TRAP (Figure 1F) and the protein levels of c-Fos and NFATc1 (Figure 1G). These results indicate that KLF2 is a negative regulator of RANKL-induced osteoclast differentiation.

KLF2 positively regulates osteoblast differentiation and function

Next, we examined the expression of KLF2 in osteoblasts, another bone cell lineage. Primary osteoblasts were cultured in osteogenic medium (OGM) containing BMP2, ascorbic acid, and β-glycerophosphate. The expression of osteoblast marker genes such as Runx2, ALP, and BSP increased during osteoblast differentiation (Supplementary Figure 1B). KLF2 was expressed in osteoblast precursor cells and KLF2 expression gradually increased during osteoblastogenesis (Supplementary Figure 1B).

In order to investigate the role of KLF2 in osteoblast differentiation and function, primary osteoblast precursor cells were infected with control or KLF2 retroviruses and cultured in osteogenic conditions. ALP activity and bone nodule formation were measured to assess osteoblast differentiation and function. ALP activity was significantly increased in KLF2-overexpressing osteoblasts compared with the control (Figure 2A), whilst KLF2 overexpression significantly increased bone nodule formation and alizarin red activity under osteogenic conditions (Figure 2B). Next, we examined whether KLF2 affected the expression of osteoblast marker genes. KLF2 overexpression significantly increased the expression of Runx2, ALP, and BSP during osteoblast differentiation compared to the control (Figure 2C).

We also investigated the physiological role of KLF2 in osteoblast differentiation using KLF2-specific siRNA. When primary osteoblast precursor cells were transfected with KLF2 siRNA, KLF2 expression was significantly decreased (Figure 2D). The inhibition of KLF2 expression by KLF2-specific siRNA significantly reduced ALP activity (Figure 2E), bone nodule formation, and alizarin red activity (Figure 2F) compared to the control. During osteoblastogenesis, the expression of osteoblast marker genes was significantly decreased when KLF2 expression was reduced by siRNA (Figure 2G). These data demonstrate that KLF2 positively regulates osteoblast differentiation and function.

IRF2BP2, a KLF2 regulator, mediates osteoclast and osteoblast differentiation

It has been reported that IRF2BP2 is a novel regulator of KLF2 (21); therefore, we examined whether IRF2BP2 could regulate KLF2 expression in osteoclasts. IRF2BP2 overexpression significantly increased KLF2 expression in BMMs (Figure 3A), suggesting that IRF2BP2 acts upstream of KLF2 in osteoclasts. However, as the role of IRF2BP2 is unknown in osteoclasts, we examined whether IRF2BP2 affected osteoclast differentiation. IRF2BP2 overexpression significantly inhibited osteoclast differentiation in BMMs compared to the control (Figure 3B). During osteoclast differentiation, IRF2BP2 overexpression inhibited the mRNA expression of c-Fos, NFATc1, and TRAP (Figure 3C), and attenuated the protein expression of c-Fos and NFATc1 (Figure 3D). These data indicate that IRF2BP2 inhibits osteoclast differentiation in a manner similar to KLF2.

Since the role of IRF2BP2 in osteoblasts has not yet been reported, we investigated the effect of IRF2BP2 on osteoblast differentiation and function. Similar to its effect in osteoclasts, IRF2BP2 overexpression led to increased KLF2 expression in preosteoblasts (Figure 3E). Next, we examined the effect of IRF2BP2 on osteoblast differentiation and function. IRF2BP2 overexpression in osteoblasts significantly enhanced ALP activity (Figure 3F), nodule formation, and alizarin red activity (Figure 3G) compared to the control. Furthermore, IRF2BP2 overexpression significantly increased the expression of Runx2, ALP, and BSP compared to the control (Figure 3H). Thus, these data demonstrate that IRF2BP2 enhances osteoblast differentiation and function in a similar manner to KLF2. Collectively, our data suggest that IRF2BP2 may act upstream of KLF2 in bone cells to regulate the differentiation of these cells.

IRF2BP2 regulates osteoclast and osteoblast differentiation via KLF2

We hypothesized that IRF2BP2 might regulate the differentiation of osteoclasts and osteoblasts via KLF2. We first examined whether the reduction in osteoclast differentiation caused by IRF2BP2 overexpression could be restored by downregulating KLF2. The overexpression of IRF2BP2 in BMMs significantly reduced osteoclast differentiation (Figure 4A), and the inhibitory effect of IRF2BP2 was significantly reduced by downregulating KLF2 using siRNA (Figure 4A). The reduction in c-Fos expression by IRF2BP2 overexpression was reversed by downregulating KLF2 using siRNA (Figure 4B). These results indicate that IRF2BP2 regulates osteoclast differentiation via KLF2.

Next, we examined whether IRF2BP2 enhanced osteoblast differentiation and function via KLF2. IRF2BP2 overexpression significantly increased ALP activity (Figure 4C), nodule formation, and alizarin red activity (Figure 4D); however, these effects were significantly reduced by downregulating KLF2 using siRNA (Figure 4C-D). The increase in Runx2 expression caused by IRF2BP2 overexpression was reduced by downregulating KLF2 using siRNA (Figure 4E). These data indicate that IRF2BP2 mediates osteoblast differentiation and function via KLF2. Taken together, these results suggest that IRF2BP2 controls osteoclast and osteoblast differentiation via KLF2.

DISCUSSION

The bone marrow-derived monocytes of KLF2 hemizygous mice display elevated function and differentiation into mature osteoclasts compared to those of wild-type mice (17). KLF2 is recruited to P300/CBP-associated factor (PCAF) and inhibits the transcriptional activity of NF-κB, thereby reducing the expression of several inflammatory genes and cytokines (22, 23). It is known that NF-κB promotes osteoclast differentiation and inhibits osteoblast differentiation (24, 25). Previous reports have suggested that KLF2 negatively regulates osteoclast differentiation; however, the mechanisms by which KLF2 acts have not yet been elucidated and the role of KLF2 in osteoblasts remains unknown.

In this study, we report that KLF2 plays different roles in the differentiation of osteoclasts and osteoblasts. We showed that KLF2 overexpression in BMMs suppresses RANKL-induced osteoclast differentiation, whereas the downregulation of KLF2 using siRNA increased osteoclast formation. In contrast, KLF2 overexpression in osteoblast precursor cells increased osteoblast differentiation and function, while KLF2 downregulation decreased the formation and function of osteoblasts. These data suggest that KLF2 negatively regulates osteoclast differentiation and positively regulates osteoblast differentiation. Although KLF2 does not affect p65 or IκB kinase (IKK) expression and nuclear accumulation, or the phosphorylation and degradation of IκB, it has been reported that it interacts directly with PCAF, the coactivator of NF-κB, to suppress p65 transcriptional activity (16, 23). In fact, we observed that the overexpression of KLF2 in osteoclasts did not change the phosphorylation and degradation of IκB and that p65-mediated transcriptional activity was inhibited by KLF2 in 293T cells (data not shown). Therefore, these results suggest that KLF2 may mediate the differentiation of osteoclasts and osteoblasts by suppressing the activity of the NF-κB promoter.

KLF2 plays an important role in regulating the activity of various immune cells and inhibiting the activation of monocytes (26). KLF2 is highly expressed in monocytes, and its expression is reduced by LPS stimulation in THP-1 monocyte-like cells and is also decreased when these cells differentiate into macrophages (16). KLF2 inhibits pro-inflammatory gene expression and suppresses the function and phagocytic ability of monocytes (16, 17). KLF2 mRNA levels were the most abundant during the BMM stage; it decreased sharply following RANKL treatment. KLF2 seems to have a negative regulatory role in osteoclastogenesis, which was clearly evidenced by overexpression or knockdown experiments. Therefore, the high level of KLF2 in BMMs may have a role in maintaining these cells in the un-differentiated stages. Further studies are needed to elucidate the role of KLF2 in BMMs.

Chen *et al.* reported that KLF2 expression was found to be significantly reduced in IRF2BP2-deficient bone marrow-derived macrophages (21, 27). Using ChIP and reporter assays, they showed that IRF2BP2 binds to the promoter of KLF2, resulting in the upregulation of KLF2 expression (21). We found that the overexpression of IRF2BP2 in BMMs and preosteoblasts increased KLF2 expression, suggesting that IRF2BP2 acts upstream of KLF2 in bone cells. We also showed that IRF2BP2 overexpression inhibits osteoclast differentiation and promotes osteoblast differentiation and function in the same manner as KLF2. In addition, we observed that the IRF2BP2-mediated inhibition of osteoclast differentiation and c-Fos expression was restored by downregulating KLF2, and that the IRF2BP2-mediated enhancement of osteoblast differentiation and Runx2 expression was rescued by downregulating KLF2. Taken together, our results suggest that IRF2BP2 acts upstream of KLF2 in osteoclasts and osteoblasts, and that the IRF2BP2/KLF2 axis regulates bone homeostasis by regulating the transcriptional activity of NF-κB.

IRF2BP2 interacts with NFATc2 and inhibits the NFATc2-mediated transactivation of the IL-2 and IL-4 promoters and the TNF-α κ3 element (19). NFATc2 is an upstream regulator

of NFATc1, which affects osteoclast differentiation by binding the NFATc1 promoter (28, 29). Therefore, IRF2BP2 may directly modulate NFATc1 in a KLF2-independent manner to control osteoclast differentiation.

In conclusion, we demonstrated that KLF2 inhibits osteoclast differentiation by reducing c-Fos expression, whilst KLF2 promotes the differentiation and function of osteoblasts by increasing Runx2 expression. We also found that IRF2BP2 regulates KLF2 in bone cells. Therefore, our results suggest that the IRF2BP2/KLF2 signaling pathway plays an important role in the regulation of bone cells via the transcriptional activity of NF-κB, and that the IRF2BP2/KLF2/NF-κB axis could be a potential therapeutic target for various bone diseases.

MATERIALS AND METHODS

See supplementary information for Material and Methods. Kindly refer to the supplementary information section for the Material and Methods used in this study.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. KLF2 inhibits RANKL-induced osteoclast differentiation. (A) BMMs were transduced with pMX-IRES-EGFP (control) or KLF2 retroviruses and cultured in the presence of M-CSF and various RANKL concentrations for 3 days. Cultured cells were stained for TRAP (left panel). The number of TRAP-positive multinucleate cells (MNCs) per well was counted (right panel). (B-C) BMMs were transduced with either pMX-IRES-EGFP (control) or KLF2 retroviruses and cultured in the presence of M-CSF and RANKL for the indicated length of time. (B) The mRNA levels of c-Fos, NFATc1, TRAP, and KLF2 were assessed by real-time PCR. (C) Cell lysates were harvested from cultured cells and immunoblotted with the indicated antibodies. (D-G) BMMs were transfected with either control or KLF2 siRNAs. (D) The mRNA level of KLF2 was assessed by real-time PCR. (E) Transfected BMMs were cultured in the presence of M-CSF and various RANKL concentrations for 3 days. Cultured cells were then stained for TRAP (left panel). The number of TRAP-positive MNCs per well was counted (right panel). (F-G) Transfected BMMs were cultured in the presence of M-CSF and RANKL for the indicated length of time. (F) The mRNA levels of c-Fos, NFATc1, and TRAP were assessed by real-time PCR. (G) Cell lysates were harvested from cultured cells and immunoblotted with the indicated antibodies. Data represent the mean ± standard deviation (SD) of triplicate samples. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the control.

Figure 2. KLF2 enhances osteoblast differentiation and function. (A-C) Osteoblasts were transduced with either pMX-IRES-EGFP (control) or KLF2 retroviruses and cultured in an osteogenic medium. (A) Cells cultured for 3 days were subjected to an alkaline phosphatase (ALP) activity assay. ALP activity was measured as the change in the absorbance at 405 nm. (B) Cells cultured for 9 days were fixed and stained for alizarin red (left panel), which was quantified by densitometry at 562 nm (right panel). (C) The mRNA levels of Runx2, ALP, BSP,

and KLF2 were assessed by real-time PCR. (D-G) Osteoblasts were transfected with either control or KLF2 siRNAs and cultured in an osteogenic medium. (D) The mRNA level of KLF2 was assessed by real-time PCR. (E) Cells cultured for 3 days were subjected to an ALP activity assay. ALP activity was measured as the change in the absorbance at 405 nm. (F) Cells cultured for 9 days were fixed and stained for alizarin red (left panel), which was quantified by densitometry at 562 nm (right panel). (G) Transfected cells were cultured for the indicated length of time and the mRNA expression of Runx2, ALP, and BSP was assessed by real-time PCR analysis. Data represent the mean ± standard deviation (SD) of triplicate samples. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the control.

Figure 3. Interferon regulatory factor 2-binding protein 2 (IRF2BP2) regulates the differentiation of osteoclasts and osteoblasts. (A-D) Bone marrow-derived macrophage cells (BMMs) were transduced with either pMX-IRES-EGFP (control) or IRF2BP2 retroviruses. (A) The mRNA level of KLF2 was assessed by real-time PCR. (B) Transduced BMMs were cultured in the presence of M-CSF and various RANKL concentrations for 3 days. Cultured cells were stained for TRAP (left panel). The number of TRAP-positive multinucleate cells (MNCs) per well was counted (right panel). (C-D) Transduced BMMs were cultured in the presence of M-CSF and RANKL for the indicated length of time. (C) Transduced cells were cultured for the indicated length of time and the mRNA expression of c-Fos, NFATc1, and TRAP was assessed by real-time PCR. (D) Cell lysates were harvested from cultured cells and immunoblotted with the indicated antibodies. (E-H) Osteoblasts were transduced with either pMX-IRES-EGFP (control) or IRF2BP2 retroviruses. (E) The mRNA level of KLF2 was assessed by real-time PCR. (F-H) Transduced osteoblasts were cultured in an osteogenic medium. (F) Cells cultured for 3 days were fixed and subjected to an alkaline phosphatase (ALP) activity assay. ALP activity was measured by densitometry at 405 nm. (G) Cells cultured

for 9 days were fixed and stained for alizarin red (left panel). Alizarin red staining activity was quantified by densitometry at 562 nm (right panel). (H) Transduced cells were cultured for the indicated length of time and the mRNA expression of Runx2, ALP, and BSP was assessed ~~time~~ by real-time PCR. Data represent the mean ± standard deviation (SD) of triplicate samples. *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control.

Figure 4. IRF2BP2 regulates osteoclastogenesis and osteoblastogenesis via KLF2. (A-B) Bone marrow-derived macrophage cells (BMMs) were transfected with control or KLF2 siRNAs. Transfected BMMs were transduced with pMX-IRES-EGFP (control) or IRF2BP2 retroviruses and cultured with M-CSF and RANKL. (A) Cultured cells were fixed and stained for TRAP (left panel). The number of TRAP-positive multinucleate cells (MNCs) per well was counted (right panel). (B) c-Fos mRNA expression was assessed by real-time PCR. (C-E) Osteoblasts were transfected with control or KLF2 siRNAs, then transduced with pMX-IRES-EGFP (control) or IRF2BP2 retroviruses and cultured in an osteogenic medium. Cells cultured for 3 days were fixed and subjected to an alkaline phosphatase (ALP) activity assay. ALP activity was measured by densitometry at 405 nm. (D) Cells cultured for 9 days were fixed and stained for alizarin red (left panel). Alizarin red staining activity was quantified by densitometry at 562 nm (right panel). (E) Runx2 mRNA expression was assessed by real-time PCR. Data represent the mean ± standard deviation (SD) of triplicate samples. *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control.

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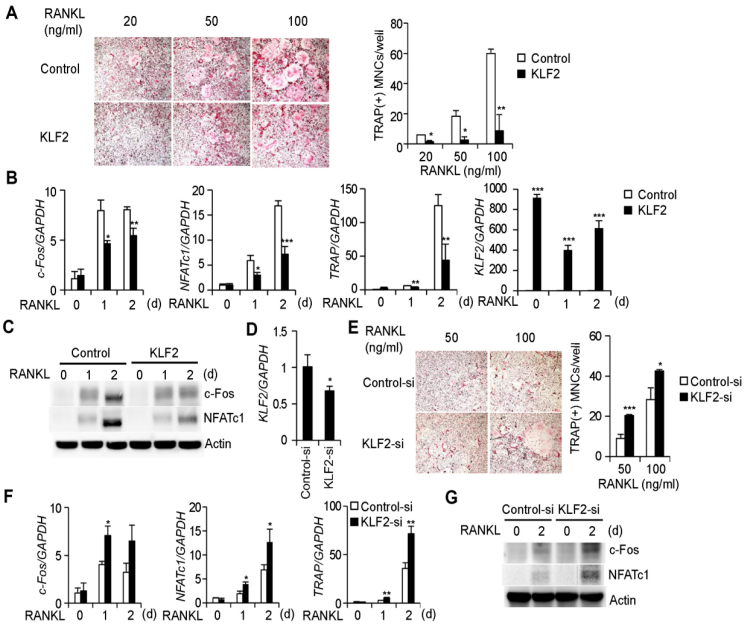

Figure 1

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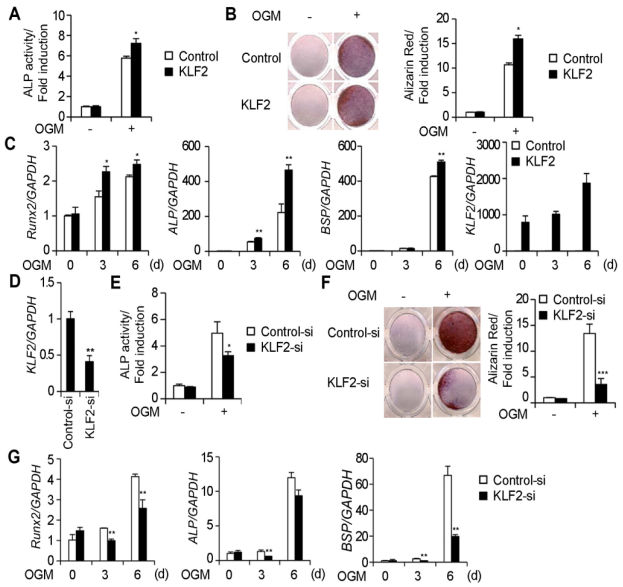


Figure 2

Fig. 2. Figure 2

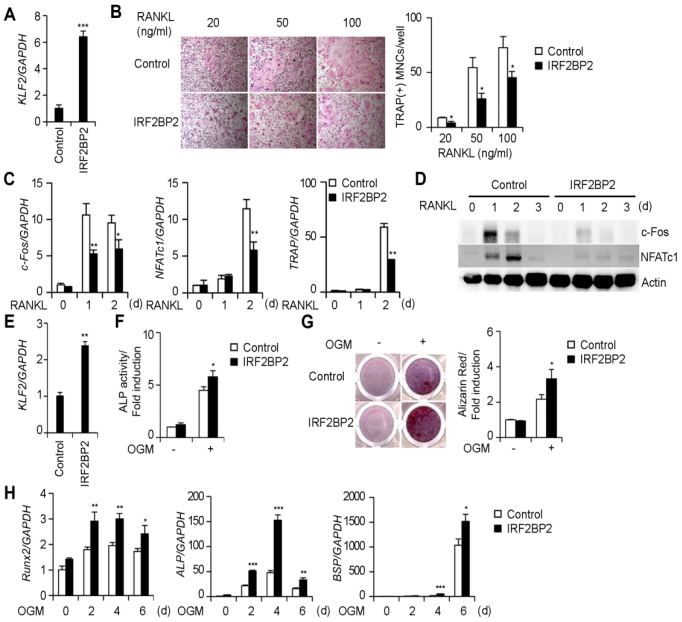


Figure 3

Fig. 3. Figure 3

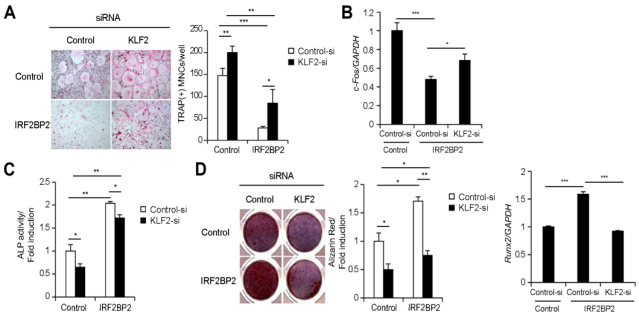
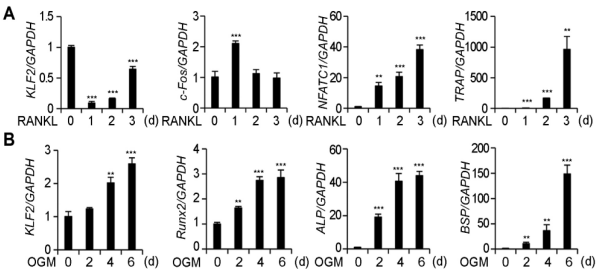


Figure 4

Fig. 4. Figure 4



Supplementary Figure 1

Sup. 1.

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

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Osteoclast differentiation

Bone marrow cells were isolated from the tibiae and femurs of 6-week-old ICR mice by flushing the bone marrow with α-MEM. Bone marrow cells were cultured in α-MEM containing 10 % fetal bovine serum (FBS) in the presence of macrophage colony-stimulating factor (M-CSF; 30 ng/ml) for 3 days. The adherent bone marrow-derived macrophages (BMMs) used as osteoclast precursors were cultured with M-CSF (30 ng/ml) and RANKL (20-100 ng/ml) for 3 days. Cultured cells were fixed with 10 % formalin and stained for TRAP solution. TRAP-positive cells with more than three nuclei were counted as osteoclasts.

Osteoblast differentiation

Primary osteoblast precursor cells were isolated from the calvarial bone of neonatal mice by successive enzymatic digestion with α-MEM containing 0.1 % collagenase (Thermo Fisher Scientific, Waltham, MA, USA) and 0.2 % dispase II (Roche Diagnostics, Rotkreuz, Switzerland). After removing the enzymes, the collected cells were cultured in osteogenic medium containing BMP2 (100 ng/ml), ascorbic acid (50 ng/ml), and β-glycerophosphate (100 mM). To assess osteoblast differentiation, osteoblast precursor cells cultured for 3 days were subjected to an ALP assay. Briefly, cells were lysed with osteoblast lysis buffer (50 mM Tris-HCl [pH 7.4], 1 % Triton X-100, 150 mM NaCl, and 1 mM EDTA) and the lysates were incubated with p-nitrophenyl phosphate substrate (MilliporeSigma, Burlington, MA, USA). ALP activity was measured as the change in absorbance at 405 nm using a spectrophotometer.

To assess their function, osteoblasts cultured for 9 days were fixed with 70 % ethanol and stained with 40 mM alizarin-red (pH 4.2). Nonspecific staining was removed with PBS, stained alizarin-red was dissolved with 10 % cetylpyridinium chloride (MilliporeSigma), and the absorbance of the extracted solution was measured at 562 nm.

Retroviral gene transduction

Retroviral vectors were transfected into the packaging cell line (Plat E) using FuGENE 6 (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Viral supernatants were collected from the culture medium 48 h after transfection. BMMs or osteoblasts were incubated with the viral supernatants for 6 h in the presence of polybrene (10 μg/ml) (MilliporeSigma).

Small interfering RNA transfection

The small interfering RNA (siRNA) sequences used to knock down KLF2: a nonspecific control siRNA (5'-CCU GGC GCC UUC GGU CUU UUU-3') and mouse KLF2-specific siRNA (5'-GCA CGG AUG AGG ACC UAA A-3'). siRNAs were transfected into BMMs and osteoblasts using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitative real-time PCR analysis

Total RNA was extracted from the cultured cells using Qiazol lysis reagent (Qiagen, Venlo, Germany) according to the manufacturer's instructions, and 2 μg RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR analysis was performed in triplicate using a Rotor-Gene Q with SYBR Green (Qiagen). The following sequences were used to assess mRNA expression: c-Fos, 5′-ATG GGC

TCT CCT GTC AAC ACA-3′ and 5′-TGG CAA TCT CAG TCT GCA ACG CAG-3′; NFATc1, 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′ and 5′-CGG CTG CCT TCC GTC TC ATAG-3′; TRAP, 5′-CTG GAG TGC ACG ATG CCA GCG ACA-3′ and 5′-TCC GTG CTC GGC GAT GGA CCA GA-3′; GAPDH, 5′-TGA CCA CAG TCC ATG CCATCA CTG-3′ and 5′-CAG GAG ACA ACC TGG TCC TCA GTG-3′; Runx2, 5′-CCC AGC CAC CTT TAC CTA CA-3′ and 5′-CAG CGT CAA CAC CAT CAT TC-3′; ALP, 5′-CAA GGA TAT CGA CGT GAT CAT G-3′ and 5′-GTC AGT CAG GTT GTT CCG ATT C-3′; BSP, 5′-GGA AGA GGA GAC TTC AAA CGA AG-3′ and 5′-CAT CCA CTT CTG CTT CTT CGT TC-3′; KLF2, 5′-CTG GCG CCT TCG GTC TTT TC-3′ and 5′-CGC ATC CTT CCC AGT TGC AA -3′.

Western blotting analysis

Cultured cells were lysed in extraction buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40, 1 mM PMSF, and a protease inhibitor mixture). Equal amounts of protein were subjected to SDS-PAGE and transferred electrophoretically onto a polyvinylidene difluoride membrane (MilliporeSigma). Membranes were incubated with antibodies against actin (MilliporeSigma), c-Fos, and NFATc1 (Santa Cruz Biotechnology, Dallas, TX, USA). Immune complexes were detected using ECL (MilliporeSigma) and analyzed with a LAS3000 luminescent image analyzer (GE Healthcare, Chicago, IL, USA).

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t* test. All data are presented as the mean ± SD. *P* values < 0.05 were considered statistically significant.

Supplementaet Figure 1. Expression profile of KLF2 in ostoeclastogenesis and Osteoblastogenesis. (A) Bone marrow-derived macrophage cells (BMMs) were cultured with M-CSF and RANKL for the indicated length of time. The mRNA levels of KLF2, c-Fos, NFATc1, and TRAP were assessed by real-time PCR. (B) Osteoblasts were cultured with osteogenic medium (OGM) containing BMP2 (100 ng/ml), ascorbic acid (50 μg/ml), and β-glycerophosphate (100 mM) for the indicated length of time. The mRNA expression of KLF2, Runx2, ALP, and BSP was assessed by real-time PCR. (B-D) Osteoblasts were transduced with either pMX-IRES-EGFP (control) or KLF2 retroviruses and cultured in an osteogenic medium.