XAF1 is a promising target to regulate osteoclastogenesis

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Abstract

Over-activated osteoclast (OC) is a major cause of diseases related to bone loss. Both bone resorption inhibition and apoptosis induction of osteoclast are crucial in treating these diseases. However, the mechanisms that determine osteoclast function and lifespan are limited. Here, our findings were presented indicating that the newly characterized gene *X-linked inhibitor of apoptosis protein (XIAP)-associated factor 1 (Xaf1)* was an important interferon-stimulated gene for termination of osteoclastogenesis via apoptosis induction. We showed that Xaf1 ablation enhanced osteoclast generation in vitro. Xaf1 knockout increased osteoclast number and bone resorption, thereby exacerbating bone loss in both OVX and osteolysis models. Activation of XAF1 with BV6 (XIAP inhibitor) suppressed osteoclast formation. Mechanistically, Xaf1 deletion decreased osteoclast apoptosis via increasing interaction between XIAP and caspase-3/7. Collectively, our data illustrated an essential role of Xaf1 in the regulation of osteoclastogenesis in both osteoporosis and osteolysis models and highlighted its underlying mechanism.

Introduction

Osteoporosis is a common disease that causes fragility fractures as a result of a systemic deterioration of bone mass and microarchitecture. The medical and socioeconomic costs of osteoporosis in general, and postmenopausal osteoporosis in particular, will increase further as the population ages. Bone turnover is a strictly controlled process in the physiologic steady state where the rate of osteoclast-mediated bone resorption and osteoblast-mediated bone formation are in balance. Across a variety of osteoporosis types, decreased bone resorption provides a positive bone balance for osteoporosis treatment.

Mounting evidences indicate that bone is an immune regulatory organ and factors once thought to be immune system-related significantly affect bone health and disease. OCs are multinucleated cells differentiated from bone marrow-derived macrophages (BMMs), which are a subset of the innate immune system. Immune system B cells produce antibodies and RANKL to directly stimulate OC precursors and T cells suppress the generation of OCs to lessen bone loss. Interferons (IFN) have been reported to be essential for a variety of immunological responses, such as the stimulation of anticancer activity, which are also involved in OCgenesis. IFN-α inhibits the formation of OCs via preventing c-Fos expression and IFN-β exerts inhibitory effects on OCgenesis via JAK1/STAT3/c-Fos signaling, indicating IFN signaling is involved in OCs.

X-linked inhibitor of apoptosis (XIAP) associated factor 1 (Xaf1) was identified as a novel IFN-stimulated gene (ISG) by Leaman et al in 2002. Both IFN-α and IFN-β increased the levels of Xaf1 messenger RNA (mRNA) in WM9 cells. Meanwhile Xaf1 is also concerned with IFN-induced apoptosis. It acts as an antagonist of XIAP by restoring caspase activity that XIAP has inhibited. The co-expression of XAF1 and XIAP enhance nuclear translocation of XIAP and hinder XIAP-dependent caspase-3 inhibition, as
demonstrated by the incubation of recombinant XIAP with caspase-3 in the absence or presence of XAF1. Spontaneous apoptosis is required for OC longevity, which is associated with activation of the caspase downstream of the extrinsic and intrinsic complementary pathways. It’s of critical importance for us to understand osteoclast behavior during bone resorption and remodeling via OC apoptosis and its modulation. Previous study has shown that OC apoptosis occurs among patients with osteoporosis. Whether Xaf1 is involved in OC genesis and OC apoptosis has been unclear. Therefore, it is crucial to clarify the role and mechanism of Xaf1 in OCs.

Here, we investigated the effect of Xaf1 on OCs by global deletion and bone marrow chimera during steady-state conditions in mouse models of chronic (OVX–induced osteoporosis) and acute (Ti-particle-induced osteolysis) bone loss. Mechanistically, we demonstrated that Xaf1 functions as osteoprotective regulator that increased and preserved bone mass in response to both chronic and acute conditions. This bone-protective effect of Xaf1 involved in a decrease in the expression of osteoclastic genes and amplification of OC apoptosis, rather than depended on a direct effect on bone-formation osteoblasts. Xaf1 deletion led to a robust interaction between XIAP and caspase. These findings implied that Xaf1 may serve as an osteoprotective regulator to restore bone homeostasis.

Results

1. XAF1 was increased in both osteoporotic patients and OVX mice

To explore the specific gene in OC genesis and osteoporosis process, RNA-seq was performed between OVX and sham mice. Notably, pathway enrichment analysis of these genes revealed significant enrichment for the OC differentiation, IFN signaling and apoptosis, which are essential for OC function and survival (Fig. 1A). Meanwhile, among all the differentially expressed genes (DEGs) in the femurs treated with OVX, IFN and apoptosis signaling were highly and significantly enriched (Fig. 1B). To further explore the common target for osteoporosis both in human and mice, data from GSE230665 was utilized to overlap with data from mice, demonstrating 489 DEGs in common (Fig. 1C). In the view of the significance of apoptosis and IFN, we next overlap DEGs with interferon-stimulated genes and apoptosis genes, XAF1, IFIT2 and IRF1 were screened out (Fig. 1D). The intimate connection between XAF1, IFIT2 and IRF1 expression and OC function prompted us to explore the pathological involvement of them in human osteoporosis. We obtained bone specimens from patients with osteoporosis (OP) and normal bone mineral density. The source of normal BMD bone specimens was lamina bone of young patients with lumbar disc herniation for laminectomy surgery. RT-PCR was performed, indicating that XAF1 increased with TRAP, CTSK and MMP9 in OP patients instead of IRF1 and IFIT2 (Fig. 1E, S1A). The expression of the XAF1 protein in the OP group was higher than that in the control group (Fig. 1F). These data suggested that XAF1 be closely associated with osteoporosis. Hence, Xaf1 global knockout mice was generated as displayed (Fig. 1G) and knockout efficiency was presented in Supplementary Fig. 1B-C.

2. Global deletion of Xaf1 promoted RANKL-induced osteoclastogenesis
To determine the influence of Xaf1 targeting OCs, BMMs were isolated from WT and Xaf1<sup>−/−</sup> mice and stimulated with RANKL for 7 d. Mature OCs were assessed by TRAP staining and bone resorption assay. Xaf1<sup>−/−</sup> BMMs formed more TRAP<sup>+</sup> multinucleated OCs (Fig. 2A-B) and exhibited increased bone resorption capacity than BMMs derived from WT mice, manifested as increased number and area of OCs as well as more resorption pits and trails on the bone slices (Fig. 2C-D). Consistent with results above, a striking size increase in actin ring structure was observed (Fig. 2E) and both the number of nuclei per osteoclast and actin rings per osteoclast were increased via phalloidin staining in Xaf1<sup>−/−</sup> OCs (Fig. 2F). Moreover, a significant increase in the OC-specific gene expression, such as Acp5, Oscar, Atp6v0d2, Ctsk, Dcstamp, and Mmp9 were observed in Xaf1<sup>−/−</sup> BMMs with RANKL stimulation (Fig. 2G). Subsequent western blot analyses of the key OC-specific makers NFATc1, c-Fos, MMP9, TRAP and cathepsin K (CTSK) showed that RANKL induced the expression of all these markers, and Xaf1 deletion augmented RANKL-induced expression of all markers compared with WT during OCgenesis (Fig. 2H).

ALP (Fig. S2A-B) and alizarin red staining (Fig. S2C-D) were used to determine osteogenic activity, which demonstrated no difference between WT and Xaf1<sup>−/−</sup> BMSCs. Likewise, gene expression levels of a series of osteoblast markers, including Runx2, Osterix, Ocn, and Osteopontin were indistinguishable between WT and Xaf1<sup>−/−</sup> BMSCs (Fig. S2E). In summary, depletion of XAF1 did not significantly affect osteoblast differentiation in vitro. Together, these results indicated that Xaf1 deletion selectively enhanced OC generation without affecting osteogenesis in vitro.

3. Xaf1 deletion exacerbated OVX-induced bone loss in vivo

After establishing the cellular effects of Xaf1 on OCs, we sought to define its role in vivo. OVX or sham surgeries were performed in 8-week old WT and Xaf1<sup>−/−</sup> mice. Reconstructed three-dimensional (3D) imaging was conducted using micro-computed tomography (μCT) (Fig.3A). OVX treated Xaf1<sup>−/−</sup> mice revealed decreased bone mass, BMD, BV/TV, Tb.N, as well as increased Tb.Sp compared with OVX treated WT mice (Fig.2B).

Complementing these findings, serum was analyzed for the concentrations of RANKL, which promoted OCgenesis, and its regulatory decoy receptor OPG. Xaf1 deletion significantly increased the RANKL/OPG ratio in blood serum (Fig. 3C). Elevated OC formation and bone resorption were often the underlying causes of low-bone mass osteoporotic phenotypes. In order to determine the number and activity of OCs, femur bone slices were stained with TRAP and H&E. As seen in Fig. 3D-E, femoral sections from Xaf1<sup>−/−</sup> mice had higher numbers of OCs than WT mice and exhibited low-bone mass osteoporotic phenotype in TRAP-stained and H&E-stained tissue samples, respectively. This observation revealed that Xaf1 may have an osteoprotective impact, which accounted for the decreased bone mass in Xaf1<sup>−/−</sup> mice.

4. Xaf1 deletion exacerbated Ti-particle induced osteolysis
Osteolysis was an inflammatory bone loss caused by excessive OC formation and activity. Given the effect of Xaf1 on noninflammatory osteoporosis, we considered if Xaf1 conferred additional protection during osteolysis. To induce osteolysis, Ti particles were injected onto the calvaria of WT and Xaf1−/− mice. μCT analysis revealed that the Xaf1−/− mice exhibited a significant decrease in osteolysis compared to WT mice (Fig. 4A). Morphological analysis revealed a significant reduction in BV/TV and an increase in the number of pores and the percentage of porosity from Xaf1−/− mice compared to WT mice (Fig. 4B). Serum levels of RANKL were upregulated from Xaf1−/− mice, while OPG levels were comparable, resulting in the increase of RANKL/OPG ratio, a parameter for assessing OCgenesis (Fig. 4C). Collectively, these results indicated that Xaf1 deletion exacerbated bone loss in Ti-particle-induced osteolysis.

Histological analysis was performed on calvarial sections to validate μCT results. H&E staining showed significant bone defects (Fig. 4D) and increased proportion of eroded surface in Xaf1−/− mice (Fig. 4E). TRAP staining confirmed that the activity of osteoclasts along the surface of trabecular bone was higher in Xaf1−/− mice than in WT mice (Fig. 4D). Osteoclast number per bone perimeter (N.Oc/B.pm) was larger in Xaf1−/− mice than in WT mice (Fig. 4F). Together, these results suggested that depletion of Xaf1 deteriorated osteolytic bone loss by aggravating OCgenesis in vivo.

5. Xaf1 deletion chimeras resulted in a low bone mass phenotype

In fact, global Xaf1 deletion led to several nonskeletal pathologies, making it challenging to distinguish between direct effects of Xaf1 deletion on bone and secondary effects on other organs. Then, bone marrow transplantation (BMT) from the WT and Xaf1−/− donors was performed to generate WT mice containing macrophages deficient in Xaf1 (Xaf1−/−→WT) and control mice (WT→WT) (Fig. 5A). The efficiency of BMT was verified using flow cytometry (Fig. S3A). Similar with global deletion, Xaf1−/− chimeric mice illustrated bone loss phenotype in OVX model (Fig. 4B). Morphometric analyses of trabecular including BV/TV, BS/TV, Tb. N and Tb.pf confirmed low bone mass in Xaf1−/− chimeric mice (Fig. 5C). H&E and TRAP staining showed decreased bone mass, trabecular area, and increased OC number in Xaf1−/− chimeric mice (Fig. 5D-E). Moreover, the elevated serum levels of RANKL and RANKL/OPG ratio in Xaf1−/− chimeric mice compared to WT chimeric mice further confirmed higher bone resorption activity (Fig. S3B). No difference in serum OPG content was found between Xaf1−/− chimeric mice and WT chimeric mice (Fig. S3B).

Meanwhile, severe bone loss in osteolysis model was observed in Xaf1−/− chimeric mice (Fig. 5F), confirmed by morphometric (Fig. 5G) and histological analysis (Fig. 5H), wherein an increase in the number and activity of OCs was observed (Fig. 5I). Serum levels of RANKL and RANKL/OPG ratio indicated a severe osteolysis phenotype (Fig. S3C). Both Xaf1 global depletion and Xaf1−/− chimera mice generation promoted osteoclastic bone resorption in the OVX and osteolysis model. Together, our findings demonstrated that Xaf1 deficient in macrophages demonstrated a low-bone mass phenotype due to elevated OC number and activation.
6. Xaf1 deletion inhibited the apoptosis of OCs

Previous studies had demonstrated that mature OCs undergo spontaneous apoptosis. However, multinucleated OCs derived from Xaf1<sup>−/−</sup> mice exhibited resistance to apoptosis due to the removal of cytokines compared with OCs derived from WT mice (Fig. 6A-B). These findings led us to examine induction of apoptosis in Xaf1<sup>−/−</sup> OCs. Therefore, we performed RT-PCR to determine the involvement of Xaf1 in OC apoptosis, which confirmed downregulation of pro-apoptotic Bax and upregulation of anti-apoptotic Bcl2 and Xiap in Xaf1<sup>−/−</sup> cells during OCgenesis (Fig. 6C). Meanwhile, inactivation of caspase-3/7, as indicated by cleaved caspase-3/7, was also observed in BMMs derived from Xaf1<sup>−/−</sup> mice throughout the differentiation process (Fig. 6D). When compared to the WT BMMs, Xaf1<sup>−/−</sup> BMMs had significantly fewer cells that were positive for DNA fragmentation (TUNEL<sup>+</sup>) after 3 days following RANKL stimulation, which suggested that Xaf1<sup>−/−</sup> BMMs suppressed spontaneous apoptosis during OCgenesis (Fig. 6E-F). Flow cytometry was performed to determine OC apoptosis, indicating a decreased percentage of OC apoptosis in Xaf1<sup>−/−</sup> BMMs treated with RANKL (Fig. 6G-H). These findings collectively suggested that Xaf1 deficiency causes excessive OC generation via inhibiting apoptosis.

7. BV6 (XIAP inhibitor) suppressed RANKL-induced OC formation

Xaf1 was identified as an XIAP inhibitor gene, which was verified by STRING analysis (Fig. 7A). RT-PCR (Fig. 7B) and western blot (Fig. 7C) analysis demonstrated that Xaf1 experienced a complementary change with XIAP during OCgenesis. BV6, a XIAP inhibitor, was used to determine if Xaf1 regulated OCgenesis via XIAP in vitro. CCK-8 assays were conducted to determine the viability of BMMs treated with different concentrations of BV6 for 24 and 48 hours, indicating that BV6 at concentrations up to 5 μM had no discernible effect on the viability of BMMs at all time points analyzed (Fig. S4A).

To demonstrate how BV6 stimulates OCgenesis, we treated BMMs with BV6 at non-toxic concentrations for 7 days. However, a dose-dependent reduction in the number and size of OCs was observed in the BV6-treated groups (Fig. 7D), which was validated by number and area quantification of TRAP<sup>+</sup> OCs (Fig. 7E). Simultaneously, RT-PCR analyses showed that BV6 treatments dose-dependently induced the transcription of OC-specific genes in BMMs, including Oscar, Dcstamp, Ctsk, Atp6v0d2, Mmp9, and Acp5 (Fig. 7F). By using total cellular proteins extracted from BMMs stimulated with BV6, we observed the dose-dependent inhibition of NFATc1, c-Fos, MMP9, CTSK and TRAP (Fig. 7G). Thus, BV6 suppressed RANKL-induced OCgenesis and inhibited OC-specific gene and protein expression in a dose-dependent manner, implying XIAP functioned as XAF1 downstream signaling to mediate OC generation and function.

8. XAF1 interacted with XIAP to promote the caspase-3 activity

After establishing the cellular effects of Xaf1, we aimed to elucidate the underlying molecular mechanism. It’s been known that XIAP directly interacted with caspase and inhibits cell apoptosis. Based on these results, we hypothesized that XAF1 antagonize interaction between XIAP and caspase, thereby
promoting OC apoptosis. Co-immunoprecipitation assay was performed to validate the endogenous interaction between XIAP and caspase3/7 (Fig. 8A, B). Furthermore, in situ proximity ligation assay (PLA) revealed a direct interaction between endogenous XIAP and caspase3 in BMMs (Fig. 8C), which confirmed by increased number of XIAP-caspase3 interaction complex (Fig. 8D). Moreover, caspase3 activity was decreased in Xaf1−/− BMMs treated with RANKL compared to WT BMMs (Fig. 8E). These results were consistent with the effect of Xaf1 in antagonizing XIAP-caspase interaction.

Discussion

In this study, XAF1 was identified as a novel bone homeostasis regulator, which suppressed RANKL-induced OC genesis. Global or bone marrow chimera depletion of XAF1 increased the formation of OCs and led to a low-bone mass phenotype under chronic and acute conditions. Mechanistically, biochemical investigations revealed that XIAP functioned as a XAF1 interaction partner and XAF1 antagonized interaction between XIAP and caspase to mediate OC apoptosis.

XAF1 is a zinc-finger, pro-apoptotic protein that is originally identified in a yeast two-hybrid screen utilizing XIAP as the bait. Because of abnormal promoter methylation and gene silencing, XAF1 expression is commonly inactivated in human malignancies, implicating XAF1 in tumor suppression. XAF1 is involved in pancreatic, breast, and prostate cancer by inhibiting cell apoptosis. However, the role of XAF1 in osteoporosis and bone remodeling has been unknown. Here, we determined that XAF1 was present in OCs and played an essential role in OC genesis and bone resorption in vitro and in vivo. Our Xaf1 global deletion and bone marrow chimera animals showed osteoporotic and osteolytic low-bone mass phenotypes. Meanwhile, our study was the first to document that increased OC generation and bone resorption in vivo was the source of the osteoporotic phenotype brought on by the loss of Xaf1. Based on its significant role in shortening the lifespan of OCs, XAF1, as a protective molecule, played a critical role in the maintenance of bone homeostasis.

Apoptosis is related with several diseases, including cancer, neurodegenerative, and autoimmune diseases, such as rheumatoid arthritis (RA). Studies have shown that the decrease in cell apoptosis could lead to the accumulation of inflammatory cells and OCs in the synovial joints. Apoptosis induction inhibits synovial inflammation and bone erosion, demonstrating potential therapeutic effects in RA patients and mice. Moreover, attenuation in the apoptosis of OC causes bone loss in postmenopausal women with OP since estrogen deficiency decreases pro-apoptotic ligand FasL expression.

Furthermore, bisphosphonates are effective anti-resorptive drugs for treating patients with OP and promoting the apoptosis of OCs. These results indicate a close correlation between OC lifespan and the pathogenesis of bone metabolic diseases. Hence, targeting osteoclastic apoptosis in affected patients could be helpful for halting bone loss.

Inhibitor of apoptosis proteins (IAP) are the only endogenous proteins regulating the initiation and effector activity of cysteine proteases, which are associated with apoptosis. IAPs, including XIAP,
inhibits the activation of caspases and intrinsically regulates the caspase signaling pathway. XAF1, identified as XIAP inhibitor, induces nuclear translocation and antagonizes anti-trypsin activity of XIAP, which was consistent with our results, indicating that XAF1 and XIAP expression exhibited contrast changes under RANKL stimulation. Combining anti-tumor function with our findings, Xaf1 may be under strict transcriptional regulation to prevent abnormal cellular behavior and Xaf1 deletion prolonged OC survival by exerting anti-apoptotic effects. Notably, XAF1 is a crucial regulator of OC-mediated bone homeostasis through apoptosis.

Studies initially focus on the capacity of XIAP to bind to and inhibit caspases that control apoptotic cell death. Proapoptotic caspases are proteases that break down cells, thus they need to be carefully managed to avoid either uncontrolled cell death or the unintended survival of damaged or cancerous cells. A linker region between BIR domains 1 and 2 is where XIAP interacts to caspases 3 and 7 in apoptotic signaling. Our discovery that XAF1 mediated the activity of caspase3 and the interaction between XIAP and caspase3/7 may offer new insights into how OCgenesis could be precisely adjusted and controlled during pathological osteoporosis, which shifts the balance from normalcy towards apoptosis. Besides, it’s interesting to note that the lengthy 5’ and 3’ UTRs on the XIAP transcript may allow for independent regulation of XIAP protein levels from transcriptional regulation of the mRNA. The human XIAP 5’ UTR has an internal ribosome entry site (IRES) region that enables XIAP production in stressed cells even if cap-dependent translation is normally blocked. As a result, under the same stress circumstances, XIAP protein may be preferentially up-regulated in comparison to other cellular proteins, which supported for the effect of BV6 on OCgenesis in our study.

The Bcl2 family is involved in OC apoptosis and affects the survival of osteocytes and osteoblasts. Therefore, reducing the threshold of OC apoptosis could aid in developing new targeted therapies for patients with bone loss. Our results showed that XAF1 could affect the production and functions of OCs, but did not significantly affect osteoblasts (Figure S2). XAF1 induces intrinsic cell apoptosis by promoting Bax expression and inhibiting Bcl2 expression mediated by p53 and cAMP response element-binding protein, respectively, which in accordance with our results, demonstrating an increase in the mRNA expression levels of several apoptosis-related genes in OCs derived from Xaf1 deletion mice. Therefore, XAF1 plays an essential role in OC apoptosis.

In fact, multiple XAF1 transcripts, including full-length (XAF1A) and short truncated (XAF1B-E), are expressed via alternative splicing in normal human tissues. Recent study has shown the crosstalk between bone and other organs. Hence, the effect of XAF1 activation in the liver or other organs on bone health cannot be ignored. Based on this, bone marrow transplantation was performed to construct chimeric mice to mimic deletion of Xaf1 expression in macrophages. If permitted, bisphosphonates or denosumab can be utilized to rescue low-bone mass phenotype, validating the bone-dependent mechanism. However, inconsistent with our findings of RNA-seq, Xaf1 played a protective role in OCgenesis. From our perspective, inflammatory microenvironment of osteoporosis led to the increase of Xaf1 transcript factor, such as Irf1 and Stat1, enhancing Xaf1 expression. Meanwhile, XAF1...
functioned as an osteoprotective factor and its up-regulation prevented over-activated OCgenesis and excessive bone loss\textsuperscript{43–45}, which was indicated by deteriorative osteopenia phenotype in \textit{Xaf1}\textsuperscript{−/−} mice treated with OVX and Ti-particle.

In conclusion, our study demonstrated that \textit{Xaf1} promoted OC apoptosis via the XIAP-Caspase-3/7 signaling pathway, thereby inhibiting OC formation and function. To the best of our knowledge, our study is the first to demonstrate that \textit{Xaf1} could enhance OCgenesis by inhibiting cell apoptosis. Our results could shed light on the role of \textit{Xaf1} in bone homeostasis and targeting \textit{Xaf1} may be beneficial as a therapeutic approach.

\textbf{Materials and Methods}

\textbf{Study approval}

Human studies were approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. All participants have signed informed consent. All \textit{in vivo} animal experimentations and protocols were approved by the Institutional Animal Care and Use Committee of Soochow University.

\textbf{Mice}

\textit{Xaf1}\textsuperscript{−/−} mice with a C57BL/6N genetic background were generated by Cyagen Biosciences (Suzhou, China). In brief, a 4.4 kb chromosomal deletion on \textit{Xaf1} (Gene ID: 327959) was generated by single guide RNAs (sgRNAs) 5′-TACAAGTTAGCTAGGGCTGTTGG-3′ and 5′-TTCCGCTGTTCCAACGTGGTTGG-3′. Primers for genotyping mice identification were 5′-GATGGAATGGGTTGGCAGCGTTC-3′ (F1), 5′-CTCCTTGACACTCATGGGATTG-3′ (R1), and 5′-GTACCAGGGCAA CAGGCAACTTTG-3′ (R2). PCR product sizes were 635 bp (F1 + R1, WT mice) and 910 bp (F1 + R2, \textit{Xaf1}\textsuperscript{−/−} mice). WT C57BL/6N mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). All the mice were kept in the specific pathogen-free (SPF) environment at Suzhou Institute of Systems Medicine (ISM) under a controlled temperature (25°C) and a 12h day-night cycle. The Animal Service Center of ISM authorized all animal research in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. (ISM-IACUC-0011-R). For OVX model, 8-week-old female C57BL/6J mice were anesthetized and underwent bilateral ovariectomy or sham operation. After 8 weeks, blood samples, femora and tibias were harvested for further study. For osteolysis model, 8-week-old male C57BL/6J mice were anesthetized and implanted Ti particle (3mg/kg) in calvaria. After 2 weeks, blood samples and crania were harvested for further analysis.

\textbf{Antibodies and other reagents}

Rabbit primary antibodies against TRAP (catalog ab2391), MMP9 (catalog ab38898), c-Fos (catalog #2250), NFATc1 (catalog ab25916), GAPDH (catalog #2118), CTSK (catalog #19027), XAF1 (catalog ab17204), XIAP (catalog #2042), Cleaved Caspase3 (catalog #9664), Caspase3 (catalog #14220), Cleaved Caspase7 (catalog #8438) and Caspase7 (catalog #12827) were purchased from Abcam and
Cell Signaling Technology. Mouse primary antibodies against XIAP (catalog sc-55550), Caspase3 (catalog sc-56053) were purchased from Santa Cruz. HRP-linked secondary antibodies specific for rabbit IgG and mouse IgG were also from Cell Signaling Technology. XIAP antagonist BV6 was purchased from MedChemExpress.

**Osteoclastogenesis assay**

Bone marrow cells from hindlimbs (tibia and femur) were cleansed and red blood cell lysis buffer was used to remove erythrocytes. Attached bone marrow cells were cultured in minimal essential medium (α-MEM, Gibco) with 10% FBS, macrophage colony-stimulating factor (MCSF, R&D system), and RANKL (R&D system) in 12-well plates after 16h. Every two days, culture media was replaced, and cells were observed for the emergence of large-size cells. Cells were stained for tartrate resistant acid phosphatase (TRAP) (Sigma-Aldrich) after 5 to 8 days of culture, and the quantity of osteoclasts was determined under microscope. As for bone resorption assay, mature osteoclasts went through ultrasonication after incubation in osteo assay surface (Corning) and resorption pits were observed with light microscope.

**Osteogenesis assay**

Bone marrow cells from hindlimbs (tibia and femur) were cleansed and red blood cell lysis buffer was used to remove erythrocytes. Cell suspensions were incubated with Dulbecco’s modified Eagle’s medium (DMEM) consisting of 50 µM ascorbic acid sodium salt, 100 nM dexamethasone, and 10 mM beta-glycerophosphate (Sigma). ALP assay was performed after 7 days, and Alizarin red S staining was performed after 21 days as per manufacturer’s protocol.

**Phalloidin staining**

BMMs seeded on glass coverslips were stimulated with MCSF and RANKL for 5–8 days. Cells were washed briefly with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and blocked with 5% goat serum. Mature osteoclasts were detected by phalloidin staining (Beyotime Biotechnology) in accordance with instructions. Fluorescent signal was observed and photographed using the Leica TCS SP6 fluorescent microscope.

**Real time PCR**

Total RNA was isolated from BMMs in the presence of RANKL using TRizol reagent (ThermoFisher Scientific) according to the manufacturer’s directions. Synthesis of cDNA was performed using the PrimeScript RT Master Mix (Takara). TB Green Premix Ex Taq (Tli RNaseH Plus) was used for RT-PCR amplification on a Roche LightCycler 480 II system. RT-PCR primer sequences for target genes were listed in Table S1.

**Western blot and immunoprecipitation (IP)**

Proteins were extracted from BMMs in the presence of MCSF and RANKL for specific days. For western blot analysis, proteins were extracted from BMMs using RIPA lysis buffer (Beyotime Biotechnology) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor PhosSTOP (Roche)
according to the manufacturer's instructions. Protein concentrations of the extracts were determined by bicinchoninic acid (BCA) assay (Beyotime Biotechnology) and adjusted with lysis buffer for equalization. Equal portions of protein extracts were loaded to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and then blotted with the chemiluminescence horseradish peroxidase (HRP) substrate (Millipore). For IP, BMM protein extractions were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), followed with protein concentration quantification. A total of 500 µg protein lysates was incubated overnight with Protein A-agarose beads (Sigma-Aldrich) plus appropriate antibodies. Fusion proteins were eluted from the agarose and performed with western blotting.

**Micro-computed tomography (µCT) analysis**

Femurs and calvariae were scanned with a SkyScan 1176 scanner (Bruker, Aartselaar, Belgium) and raw data was obtained, followed by reconstruction and reorientation. For quantitative analysis, region of interest (ROI) was selected among specific µCT slices and CTAn software (SkyScan, Aartselaar, Belgium) was utilized to generate three-dimensional (3D) images. 3D images were visualized using Mimics v10.01 software (Materialise, Leuven, Belgium).

**Bone histology**

Femur and calvaria specimens were harvested from mice operated with OVX or osteolysis and fixed in 4% paraformaldehyde for 24 hours at 4°C. After demineralization in EDTA (10%) for 20 days, specimens were dehydrated in graded concentrations of ethanol and embedded in paraffin. The serial sections (5 µm) were obtained with a microtome for H&E and TRAP staining.

**TUNEL assay**

BMMs were stimulated with RANKL for 3 days and harvested after fixation, rinsing and permeabilization. Apoptosis was detected using a TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay, which specifically labeled the 3'-hydroxyl terminal of DNA strand breaks, according to the manufacturer's instructions.

**Flow cytometry**

BMMs were pretreated with RANKL for 3 days and prepared as a single cell suspension for staining. For analysis of apoptosis, cell suspension was performed by Annexin V-FITC and PI apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Data were obtained by a flow cytometer (LSR Fortessa, BD Biosciences) and analyzed by FlowJo™ (BD Biosciences).

**In Situ Proximity Ligation Assay (In Situ PLA)**

Duolink in situ PLA (Duolink Detection kit) was used to detect interactions between XIAP and caspase3. Briefly, BMMs plated on glass coverslips in the presence of RANKL (50 ng/mL) for 3 days were fixed using 4% formaldehyde. The fixed cells were incubated with mouse anti-caspase3 and rabbit anti-XIAP primary antibodies. Each of the primary antibodies was given oligonucleotide-labeled secondary
antibodies (PLA probes) by the Duolink system, which combined with a DNA amplification-based reporter system to generate a signal only when the two primary antibodies were sufficiently near to one another. The signal from each detected pair of primary antibodies was visualized as a spot according to the manufacturer’s instructions. Slides were evaluated using a LEICA TCA SP8 confocal microscope.

**Bone marrow transplantation**

8-week-old C57BL/6J mice were selected as donors and recipients. Before bone marrow transplantation, CD45.1 positive receptor mice were irradiated at a high dose (800 rad, 8 Gy) for endogenous hematopoietic cell ablation. WT/\(Xaf1^{-/-}\) mice with CD45.2 were selected as donors for BMMs (extraction described as above). Bone marrow single cell suspension were injected through the tail vein into the receptor. Recombinant bone marrow chimera mice were kept in a SPF environment for further study.

**RNA-seq analysis**

Total RNA was extracted from mice bone specimens and then subjected to cDNA synthesis by reverse transcription, followed by synthesis of biotinylated cRNA through *in vitro* transcription. Qubit 2.0 Fluorometer (Thermo Fisher Scientific) was used to detect the concentration of RNA samples. According to the instructions of the Agent 2100 bioanalyzer, samples were added to evaluate the integrity of the RNA. Ribosomal RNA Removal Kit (TruSeq Stranded mRNA Sample Prep Kit, Illumina) was used to construct the database and Illumina HiSeq*10* high-throughput analyzer was performed for RNA-Seq sequence. Adapter sequence was removed from raw data by CLC Genomics Workbench 12 (Qiagen), and all the samples were standardized by mouse mm10 reference genome for gene expression (RPKM, Reads Per Kilobase per Million mapped reads). CLC was also used to detect the difference in gene expression based on the p-value and fold change (p-value < 0.05, FC ≥ 1.5). Finally, these differentially expressed genes were analyzed by Gene Ontology or KEGG pathway functions.

**Statistical analysis**

Statistical analyses were performed using an unpaired two-tailed Student’s t-test in GraphPad Prism 8 software. All values are expressed as the mean ± SEM. Results are presented as representative examples of more than three independent experiments. Statistical tests were justified as appropriate for the results presented and the data met the assumptions of the tests.

**Declarations**

**Declaration of interests**

The authors declare no competing interests

**Author Contribution**
X.Z., F.M. and Q.D. conceived the idea for the study and designed the experiments. M.Z. and Y.H. performed all the experiments. L.S., X.G., Y.N., J.B., Q.D. and B.T. provided the reagents and suggestions. X.Z. and M.Z. analyzed the data and wrote the manuscript.

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Figures
**Figure 1**

*XAF1 was increased in both osteoporotic patients and OVX mice.* (A) Pathway enrichment analysis for genes expressed differentially between OVX and sham femurs. (B) GSEA analysis of IFN and apoptosis signaling for mice femurs. (C) Schematic diagram of overlap DEGs between mice and human. (D) Schematic diagram of overlap genes among DEGs, ISGs and apoptosis genes. (E) Real time PCR analysis of *XAF1, CTSK* and *MMP9* gene expression in bone specimens from lumbar disc herniation (LDH, control group, patients with normal BMD) and osteoporosis (OP, patients with low BMD) patients. (F) Western blot analysis of XAF1 protein in bone specimens from Ctrl and OP groups. (G) Schematic diagram of Xaf1 global deletion mice.
Figure 2

**Xaf1 deletion promoted RANKL-induced osteoclastogenesis.** (A) TRAP staining of osteoclasts. Scale bar, 50μm. (B) Quantification of the number and average size (area) of TRAP+ osteoclasts. (C) Bone resorption assay of osteoclasts. Scale bar, 50 μm. (D) Quantification of the (area) of resorption pits. (E) Phalloidin staining of osteoclasts. Red, actin ring; blue, DAPI. Scale bar, 50μm. (F) Real-time qPCR analysis of Acp5, Oscar, Atp6v0d2, Ctsk, Dcstamp and Mmp9 in BMMs stimulated with RANKL for 3d. (H) Western blot analysis of NFATc1, c-Fos, TRAP and MMP9 levels upon RANKL stimulation for 3d in BMMs. All bar graphs showed mean ± SD. n=3/group. Comparisons were conducted using Student’s t test.
Figure 3

*Xaf1* deletion exacerbated OVX-induced bone loss in vivo. (A) Representative 3D reconstructed μCT images of femurs. (B) Quantitative morphometric assessment of μCT bone parameters, including BMD, trabecular BV/TV, BS/TV, Tb. N (mm$^{-1}$) and Tb. Sp (mm). (C) ELISA assessment of serum RANKL, OPG levels and RANKL/OPG ratio. (D) Representative images of H&E and TRAP staining of femoral sections. Scale bar, 200μm. (E) Quantification of Tb. N and trabecular area in H&E and TRAP$^+$ OC in TRAP staining. All bar graphs showed mean ± SD. Sham, n=4; OVX, n= 6. Comparisons were conducted using Student’s t test.
Figure 4

*Xaf1* deletion exacerbated Ti-particle induced osteolysis. (A) Representative 3D reconstructed μCT images of calvariae. (B) Quantitative morphometric assessment of μCT bone parameters. (C) ELISA assessment of serum RANKL, OPG levels, and RANKL/OPG ratio. (D) Representative images of H&E and TRAP staining of calvarial sections. (E) Quantitative histomorphometric assessment of the eroded surface in H&E staining sections. Scale bar, 200μm. (F) Quantitative histomorphometric assessment of TRAP+ osteoclast number per bone perimeter. All bar graphs showed mean ± SD. Sham, n=4; Ti, n=8. Comparisons were conducted using Student’s t test.
Figure 5

**Xaf1 deletion chimeras resulted in a low bone mass phenotype.** (A) Schematic diagram of Xaf1 chimera mice generation by bone marrow transplantation. (B) Representative 3D reconstructed μCT images of femurs. (C) Quantitative morphometric assessment of μCT bone parameters. (D) Representative images of H&E and TRAP staining of femur sections. (E) Quantification of trabecular area and TRAP$^+$ osteoclast number on H&E and TRAP stained femur sections, respectively. Scale bar, 200μm. (F) Representative 3D reconstructed μCT images of calvariae. (G) Quantitative morphometric assessment of μCT bone parameters. (H) Representative images of H&E and TRAP staining of calvaria sections. Scale bar, 200μm. (I) Quantification of eroded surface and TRAP$^+$ osteoclast number on H&E and TRAP stained calvaria
sections, respectively. All bar graphs showed mean ± SD. Sham, n=4; OVX, n=8; Ti, n=6. Comparisons were conducted using Student’s t test.

Figure 6

*Xaf1* deletion inhibited the apoptosis of OCs. (A) TRAP staining at 24, 48, 72h by removing cytokines. Scale bar, 50μm. (B) Quantification of OC survival rate. (C) Real time PCR analysis of apoptotic gene expression including *Bax*, *Bcl2* and *Xiap* in BMMs stimulated with RANKL for 3d. (D) Western blot analysis of cleaved caspase-3/7 in BMMs stimulated with RANKL for 0, 15, 30, 60min. (E) TUNEL staining of OCs treated with RANKL for 3d. Green, TUNEL; blue, DAPI. Scale bar, 50μm. (F) Quantification...
of the TUNEL+ BMMs. (G) Flow cytometry analysis of OC apoptosis treated with RANKL for 3d. (H) Quantification of OC apoptosis rate. All bar graphs showed mean ± SD. n=3. Comparisons were conducted using Student’s t test.

Figure 7

**BV6 (XIAP inhibitor) suppressed RANKL-induced OC formation.** (A) STRING analysis of XAF1 and XIAP interaction. (B) Real time PCR analysis of *Xaf1* and *Xiap* gene expressions in BMMs treated with RANKL at 1, 2, 3d. (C) Western blot analysis of XAF1 and XIAP protein expressions in BMMs treated with RANKL at the indicated time. (D) TRAP staining of osteoclasts treated with BV6 after 7d. Scale bar, 50 μm. (E) Quantification of OC number and average size (area). (F) Real time PCR analysis of *Oscar, Dcstamp, Ctsk, Atp6v0d2, Mmp9,* and *Acp5* expression in BMMs treated with BV6 for 3d. (G) Western blotting analysis of OC-specific proteins in BMMs treated with BV6 for 3d. All bar graphs showed mean ± SD. n=3/group. Comparisons were conducted using Student’s t test.
**Figure 8**

**XAF1 interacted with XIAP to promote caspase activity.** (A-B) Coimmunoprecipitation analysis of XIAP and caspase3/7 protein interactions. (C) *In situ* PLA of interaction between endogenous XIAP and caspase3 in BMMs treated with RANKL for 3d. Red, positive interaction complexes; blue, DAPI. Scale bar, 50μm (D) Quantification of positive interaction complexes. (E) Caspase-3 activity assay of BMMs treated with RANKL for 3d. All bar graphs showed mean ± SD. n=3. Comparisons were conducted using Student’s t test.

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