Wnt4 signaling prevents skeletal aging and inflammation by inhibiting nuclear factor-κB

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Aging-related bone loss and osteoporosis affect millions of people worldwide. Chronic inflammation associated with aging promotes bone resorption and impairs bone formation. Here we show that Wnt4 attenuates bone loss in osteoporosis and skeletal aging mouse models by inhibiting nuclear factor-κB (NF-κB) via noncanonical Wnt signaling. Transgenic mice expressing Wnt4 from osteoblasts were significantly protected from bone loss and chronic inflammation induced by ovariectomy, tumor necrosis factor or natural aging. In addition to promoting bone formation, Wnt4 inhibited osteoclast formation and bone resorption. Mechanistically, Wnt4 inhibited NF-κB activation mediated by transforming growth factor-β–activated kinase-1 (Tak1) in macrophages and osteoclast precursors independently of β-catenin. Moreover, recombinant Wnt4 alleviated bone loss and inflammation by inhibiting NF-κB in vivo in mouse models of bone disease. Given its dual role in promoting bone formation and inhibiting bone resorption, our results suggest that Wnt4 signaling could be an attractive therapeutic target for treating osteoporosis and preventing skeletal aging.

Normal bone remodeling maintains constant bone mass by an orchestrated balance between the destruction of preexisting bone by osteoclasts and rebuilding it by osteoblasts1-2. Aging is associated with substantial changes to the skeletal system that are characterized by structural alterations including reduction in trabecular bone volume, density and strength3-4, as well as a shift in tissue microenvironment with increasing proinflammatory cytokine levels in bone marrow and the serum5-9. Advancing age is also a critical risk factor for osteoporosis, which is the most common metabolic bone disease and a leading cause of morbidity and mortality in our aging population10-11. In osteoporosis, bone homeostasis is dysregulated by hormonal deficiency and aging, leading to increased bone turnover with enhanced bone formation and even greater rates of bone resorption, resulting in a net bone loss. Skeletal aging also manifests through other scenarios of abnormal bone remodeling, such as reduced formation and accelerated resorption in inflammatory bone diseases and low bone turnover in physiological aging. Both bone formation and resorption are regulated on the local level by factors secreted by bone cells, as well as on the systemic level by hormones4,11-14.

Chronic inflammation has been found to be associated with osteoporosis and aging-related bone loss1,6,7. In general, the transcription factor NF-κB is activated during inflammatory processes15. Growing evidence suggests that NF-κB plays an important role in aging-related disorders, including aging-related bone loss and osteoporosis16-19. Inhibition of NF-κB has been shown to attenuate osteoporosis and arthritis20,21. We previously reported that NF-κB activation inhibits bone formation in estrogen deficiency–induced bone loss22. Thus, targeting NF-κB may allow both inhibition of bone resorption and promotion of bone formation.

The Wnt family proteins are key regulators in growth and development, stem cell self-renewal and cancer development23,24. Wnt signaling has also emerged as a critical player in bone homeostasis25,26. The 19 Wnt family proteins are divided into canonical and noncanonical ligands based on their dependence on transduction through β-catenin27-29. Although there have been a few studies elucidating the role of noncanonical Wnt signaling in osteoblast differentiation30-33, little is known regarding how this signaling pathway affects osteoclast formation. Signaling between Wnt5a and receptor tyrosine kinase–like orphan receptor-2 (Ror2) has been found to promote osteoclastogenesis by activating the Wnt–c-Jun terminal kinase (JNK) pathway32. Previously, we found that Wnt4, a prototypical ligand for the noncanonical Wnt pathway, is able to promote osteoblast differentiation of mesenchymal stem cells (MSCs)33. To further explore the therapeutic potential of Wnt4, we generated transgenic mice that express Wnt4 in osteoblasts. We confirmed that Wnt4 could enhance bone formation in vivo, but we also found that Wnt4 could inhibit osteoclast formation and inflammation in vivo, thus attenuating bone loss and osteoporosis. Together with our previously published findings, our

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results suggest that Wnt4 signaling may represent an attractive target to treat bone loss as it promotes osteoblast generation and inhibits osteoclast formation.

RESULTS

Wnt4 promotes bone formation in vivo

To explore whether Wnt4 promoted bone formation in vivo, we generated transgenic mice in which Wnt4 is driven by the mouse 2.3-kb type 1 collagen (Col2.3) promoter (OB-Wnt4 mice). The Col2.3 promoter contains a 2.3-kb DNA fragment upstream of the transcription start site of the Col1a1 gene and has been shown to drive gene expression specifically in differentiated osteoblasts. The fragments of the Wnt4 transgene were microinjected into C57BL/6 × SJL mouse oocytes, and the oocytes were surgically transferred to pseudopregnant C57BL/6 dams. Seven of the ten potential founders screened displayed strong expression of the transgene (Supplementary Fig. 1a), thus allowing establishment of two separate transgenic mouse lines (TG1 and TG7). Whereas hemagglutinin-tagged transgenic Wnt4 was undetectable in primary calvarial cells from wild-type (WT) C57BL/6 mice, Wnt4 in calvarial cells from TG1 and TG7 mouse lines was induced as the cells differentiated into osteoblasts in osteogenic medium (Fig. 1a). RT-PCR confirmed that Wnt4 transgene mRNA was expressed in bone tissue but not in brain, heart, kidney, liver, spleen or muscle using the TG7 line (Fig. 1b).

Next, we investigated whether Wnt4 could enhance bone formation in vivo using the TG7 line. Of note, both lines of OB-Wnt4 mice had a phenotypically normal skeleton at birth (data not shown). Microcomputed tomography (µCT) analysis of the secondary spongiosa of the distal femur metaphysis revealed that the bone mineral density (BMD) of OB-Wnt4 mice was significantly higher compared to WT littermates at 1, 2 and 3 months of age (Fig. 1c,d). Similarly, the bone volume/tissue volume ratio (BV/TV) was significantly higher in OB-Wnt4 mice compared to WT mice (Fig. 1e), consistent with the greater amount of trabecular bones shown in H&E staining (Fig. 1f). Histomorphometric analysis revealed mildly higher osteoblast counts in 3-month-old OB-Wnt4 mice compared to WT mice (Fig. 1g). To further confirm whether the increased BMD was due to enhanced osteoblast function, we performed dynamic histomorphometric analysis over a 7-d period using calcein labeling and found that the bone formation rate (BFR) in 3-month-old OB-Wnt4 mice was significantly higher compared to WT mice (Fig. 1h). Similarly, characterization of the TG1 mouse line also confirmed that Wnt4 enhanced bone formation in vivo, ruling out variations due to mouse strains (Supplementary Fig. 1b,c).

To examine whether Wnt4 enhanced osteoblastic activity in a cell-autonomous manner, we isolated bone marrow MSCs from femurs of OB-Wnt4 mice and WT mice. Primary MSCs from OB-Wnt4 mice demonstrated enhanced osteogenic potential, as evidenced by the increased BMD and BV/TV (Fig. 1i). To determine whether Wnt4 enhanced bone formation in vivo, we generated transgenic mice in which Wnt4 is driven by the mouse 2.3-kb type 1 collagen (Col2.3) promoter (OB-Wnt4 mice). The Col2.3 promoter contains a 2.3-kb DNA fragment upstream of the transcription start site of the Col1a1 gene and has been shown to drive gene expression specifically in differentiated osteoblasts. The fragments of the Wnt4 transgene were microinjected into C57BL/6 × SJL mouse oocytes, and the oocytes were surgically transferred to pseudopregnant C57BL/6 dams. Seven of the ten potential founders screened displayed strong expression of the transgene (Supplementary Fig. 1a), thus allowing establishment of two separate transgenic mouse lines (TG1 and TG7). Whereas hemagglutinin-tagged transgenic Wnt4 was undetectable in primary calvarial cells from wild-type (WT) C57BL/6 mice, Wnt4 in calvarial cells from TG1 and TG7 mouse lines was induced as the cells differentiated into osteoblasts in osteogenic medium (Fig. 1a). RT-PCR confirmed that Wnt4 transgene mRNA was expressed in bone tissue but not in brain, heart, kidney, liver, spleen or muscle using the TG7 line (Fig. 1b).

Figure 1 Wnt4 promotes postnatal bone formation in vivo. (a) Western blot showing Wnt4 expression in primary calvarial cells extracted from WT and OB-Wnt4 (Wnt4) mice following osteogenic induction. HA, hemagglutinin. (b) RT-PCR analysis of Wnt4 mRNA expression in various tissues and organs. (c–e) µCT reconstruction (c), BMD (d) and BV/TV (e) of metaphyseal regions of distal femurs from 1-, 2- and 3-month-old WT and Wnt4 mice. Scale bars, 200 µm; n = 12 per group. (f) H&E staining of femur sections from 1-, 2- and 3-month-old WT (n = 8 per group) and Wnt4 (n = 10 per group) mice. Scale bars, 300 µm. (g) Histomorphometric analysis of osteoblast counts in 3-month-old Wnt4 (n = 10) versus WT (n = 8) mice. Ob.S, osteoblast surface; Ob.N, osteoblast number; BS, bone surface. (h) BFR and MAR measurements from dual-fluorescent calcein labeling of 3-month-old Wnt4 (n = 10) versus WT (n = 8) mice. (i) Alkaline phosphatase staining of femur bone marrow MSCs from Wnt4 versus WT mice after osteogenic induction. Data are presented as mean ± s.d., *P < 0.05, unpaired two-tailed Student’s t-test.
Figure 2 Wnt4 attenuates osteoporosis induced by OVX.
(a,b) μCT reconstruction (a) of metaphyses of distal femurs, as well as BMD and BV/TV (b), in WT versus OB-Wnt4 (Wnt4) mice at 2 months after OVX. Scale bars, 200 μm. (c) BFR measurement of calcine dual labeling in WT versus Wnt4 mice 2 months after OVX or sham operation. (d,e) Morphometric analysis of osteoblast (d) and osteoclast (e) counts in WT versus Wnt4 mice after OVX or sham operation. (f) TRAP staining of femur sections from WT and Wnt4 mice after OVX or sham operation. Scale bars, 30 μm. (g-i) ELISA of serum concentrations of osteocalcin (Ocn) (g), Trap5b (h) and Il-6 and Tnf (i) in WT versus Wnt4 mice after OVX or sham operation. (j) Immunostaining and quantification of active p65 in trabecular bone cells and surrounding bone marrow cells in WT and Wnt4 mice after OVX or sham operation. Scale bars, 30 μm. IOD, integral optical density. For b-e and g-j, n = 8 for sham groups and n = 12 for OVX groups. Data are presented as mean ± s.d., *P < 0.05, **P < 0.01, unpaired two-tailed Student’s t-test.

by alkaline phosphatase and Alizarin red staining, when cells were induced by osteogenic medium (Fig. 1j). Furthermore, real-time RT-PCR showed greater mRNA expression of the master osteogenic transcription factors Runx2 and Sp7 (Supplementary Fig. 1d,e), as well as the mineralization markers Ibsp and Bglap (Supplementary Fig. 1f.g), in differentiated osteoblasts from OB-Wnt4 mice compared with WT mice.

Wnt4 prevents estrogen deficiency–induced bone loss
To mimic the molecular pathogenesis of osteoporotic bone loss, mouse ovariectomy (OVX) has been widely used as an animal model of this condition. We performed OVX or sham operation on 3-month-old WT and OB-Wnt4 mice, followed by μCT analysis of femurs from these mice. We found noticeable trabecular bone loss in WT mice compared to sham controls 2 months after OVX. In contrast, bone loss was markedly lower in OB-Wnt4 mice after OVX compared to WT mice (Fig. 2a). Quantitative measurements showed that whereas 47% of BMD and 48% of BV/TV were lost in WT mice after OVX, only 27% of BMD and 24% of BV/TV were lost in OB-Wnt4 mice (Fig. 2b). Following OVX, BFR was higher in WT mice to compensate for the accelerated bone resorption, whereas in OB-Wnt4 mice, osteoblastic activity was further enhanced (Fig. 2c). Similarly, toluidine blue staining revealed significantly higher osteoblast number and osteoblast surface in OB-Wnt4 as compared to WT mice in both OVX and sham groups (Fig. 2d). In contrast, we observed lower osteoclast number and surface in OB-Wnt4 mice compared to WT mice in both OVX and sham groups (Fig. 2e,f). We also performed ELISA to assess the serum markers of bone turnover. The serum concentrations of osteocalcin, a marker of bone formation, were significantly higher in OB-Wnt4 mice compared to WT mice after OVX (Fig. 2g). In contrast, OVX induced higher serum concentrations of Trap5b, a marker of bone resorption, in WT but not in OB-Wnt4 mice (Fig. 2h).

Studies have implicated proinflammatory cytokines, including tumor necrosis factor (Tnf) and interleukin-6 (Il-6), as important mediators of accelerated bone loss in osteoporosis. Consistent with this, OVX induced an elevation in serum concentrations of Tnf and Il-6 in WT mice, but such induction was suppressed in OB-Wnt4 mice (Fig. 2i). Immunostaining of activated p65 in femur sections revealed enhanced NF-κB activity in osteoclasts and bone marrow cells surrounding trabecular bones following OVX. In contrast, the NF-κB activation by OVX was significantly less pronounced in OB-Wnt4 mice (Fig. 2j). To further confirm the inhibition of NF-κB by Wnt4 in vivo, we immunostained NF-κB–dependent targets, including Tnf, cyclooxygenase-2 (Cox-2) and matrix metallopeptidase-9 (Mmp9). Consistent with activated p65 staining, we found that Wnt4 also potently reduced the expression of Tnf, Cox-2 and Mmp9 induced by OVX in vivo (Supplementary Fig. 2a–c).

Wnt4 inhibits TNF-induced inflammatory bone loss
TNF potently induces inflammation by activating NF-κB. Transgenic mice overexpressing human TNF (TNFtg) develop systemic bone loss and osteoporosis in addition to erosive arthritis due to a higher degree of osteoclastogenesis and inhibition of bone formation. To further determine whether Wnt4 could directly inhibit inflammation–associated bone loss, we bred TNFtg mice with OB-Wnt4 mice (TNFtg/OB-Wnt4 mice). There was severe paw and joint swelling, often associated with joint deviation, in 1-year-old TNFtg mice. We also performed μCT and histological analyses, which revealed extensive joint cartilage degeneration, in 1-year-old TNFtg mice.
Wnt4 prevents skeletal aging and bone loss

Aging creates a proinflammatory environment with elevated levels of cytokines that contribute to various chronic diseases including osteoporosis and osteopenia. We further examined whether Wnt4 could protect against natural aging–mediated bone loss or destruction and bone erosions due to invasion of inflammatory cells (Supplementary Fig. 3a–e). In contrast, there was significantly less joint swelling, bone erosion and inflammation in TNFtg/OB-Wnt4 mice than in TNFtg mice of comparable age (Supplementary Fig. 3a–e).

Consistent with previous studies, μCT analysis revealed systemic bone loss suffered by 1-year-old TNFtg mice compared with WT mice. However, bone loss in femurs from 1-year-old TNFtg/OB-Wnt4 mice was markedly mitigated (Fig. 3a). Quantitative measurements revealed that whereas 31% of BMD and 68% of BV/TV were lost in TNFtg mice compared to WT mice, only 18% of BMD and 28% of BV/TV were lost in TNFtg/OB-Wnt4 mice (Fig. 3b). As the reduced bone loss could be due to either higher bone formation or lower bone resorption (or both), we examined the effect of Wnt4 on both components of bone homeostasis. The lower degree of BFR and mineral apposition rate (MAR) in TNFtg mice compared to WT mice were alleviated in TNFtg/OB-Wnt4 mice (Fig. 3c). Consistent with this finding, histomorphometric analysis also showed 24% greater osteoblast counts in TNFtg/OB-Wnt4 mice than in TNFtg mice (Fig. 3d).

As it has been shown that osteoclastogenesis and bone resorption were enhanced in TNFtg mice, we next examined the effect of Wnt4 on accelerated bone resorption in TNFtg mice. Both histomorphometric analysis and tartrate-resistant acid phosphatase (TRAP) staining revealed that whereas osteoclast activity was higher in TNFtg mice compared to WT controls, it was significantly lower in TNFtg/OB-Wnt4 mice (Fig. 3e,f). Consistent with this, the serum concentrations of osteocalcin were significantly lower in TNFtg mice than in TNFtg/OB-Wnt4 mice (Fig. 3g). On the other hand, the serum concentrations of Trap5b were significantly higher in TNFtg mice than in TNFtg/OB-Wnt4 mice (Fig. 3h). We observed that the serum Il-6 concentration in TNFtg/OB-Wnt4 mice was only 55% of that in TNFtg mice (Fig. 3i). As TNF is a potent activator of NF-κB that is associated with osteoporosis and skeletal aging, we next examined whether Wnt4 inhibited TNF-induced NF-κB activation in the TNFtg/OB-Wnt4 mice. Immunostaining of active p65 revealed markedly enhanced NF-κB activity in the proximity of trabecular bones in TNFtg mice, whereas NF-κB staining was significantly reduced in TNFtg/OB-Wnt4 mice (Fig. 3j). Moreover, Wnt4 also inhibited the expression of Cox-2 and Mmp9 in osteoclasts and bone marrow cells induced by TNF in vivo (Supplementary Fig. 3f,g).

Wnt4 prevents skeletal aging and bone loss

Aging creates a proinflammatory environment with elevated levels of cytokines that contribute to various chronic diseases including osteoporosis and osteopenia. We further examined whether Wnt4 could protect against natural aging–mediated bone loss or
Figure 4 Wnt4 attenuates skeletal aging by inhibiting NF-κB. (a–c) μCT reconstruction (a), BMD and BV/TV (b) and H&E staining (c) of distal femoral metaphyseal regions from 6-, 18- and 24-month-old WT and OB-Wnt4 (Wnt4) mice. Scale bars, 200 μm (a) and 300 μm (c). (d) Morphometric analysis of osteoblast counts in distal femoral metaphyses from 3-, 18- and 24-month-old WT and Wnt4 mice. (e) ELISA of Ocn concentrations in serum from 3-, 18- and 24-month-old WT and Wnt4 mice. (f) Morphometric analysis of osteoclast counts in distal femoral metaphyses from 3-, 18- and 24-month-old WT and Wnt4 mice. (g,i) Immunostaining with antibody to active p65 (red arrowheads) (i) and quantification of NF-κB activity surrounding the trabecular bones from 24-month-old WT and Wnt4 mice (j). Scale bars, 25 μm. For b, d–h and j, n = 12 mice per group. Data are presented as mean ± s.d., *P < 0.05, **P < 0.01, unpaired two-tailed Student’s t-test.

Wnt4 inhibits Tak1–NF-κB signaling

Our in vivo results suggest that Wnt4 secreted by osteoblasts may inhibit osteoclast formation and bone resorption in a paracrine fashion. To confirm our hypothesis, we examined whether Wnt4 could directly inhibit osteoclast differentiation using recombinant Wnt4 (rWnt4) protein. As evidenced by TRAP staining, rWnt4 protein significantly inhibited osteoclast differentiation of primary bone marrow macrophages induced by receptor activator of NF-κB ligand (Rankl; Supplementary Fig. 5a). Similarly, the osteoclast–like differentiation of RAW264.7 cells induced by Rankl was also attenuated by Wnt4 (Supplementary Fig. 5b). Real-time RT-PCR confirmed that rWnt4 inhibited the expression of osteoclast marker genes, including Acp5, Mmp9 and Ctsk, induced by Rankl in bone marrow macrophages and RAW264.7 cells (Supplementary Fig. 5c,d). As Wnt4 inhibited the expression of NF-κB target genes in vivo, we also examined whether rWnt4 inhibited the expression of NF-κB target genes induced by Rankl. Real-time RT-PCR revealed that rWnt4 potently inhibited induction of the NF-κB–dependent genes Il6 and Birc3 by Rankl in bone marrow macrophages (Supplementary Fig. 5e) and in RAW264.7 cells (Supplementary Fig. 5f). Consistent with our findings from immunostaining in vivo, rWnt4 also significantly
suppressed the NF-κB–dependent genes *Tnf* and *PtgS2* in bone marrow macrophages (Supplementary Fig. 5g).

To further elucidate the molecular mechanism by which Wnt4 inhibited NF-κB and osteoclastogenesis, we examined each key step of NF-κB activation induced by Rankl. Activation of the Rank receptor leads to association of its cytoplasmic domain with Tnf receptor–associated factor-6 (TrAF6), which is essential in osteoclast differentiation.47,48 TrAF6 forms a complex with Tak1 and Tak1 binding protein-2 (Tab2), leading to phosphorylation and activation of Tak1 (ref. 49). In canonical NF-κB signaling, Tak1 then phosphorylates IκB kinase (IKK) complex and thereby initiates degradation of IκBα, followed by phosphorylation and nuclear translocation of p65 to activate downstream target genes.49 Western blot analysis revealed that rWnt4 potently inhibited Tak1 phosphorylation, as well as the subsequent phosphorylation of p65 and the phosphorylation and degradation of IκBα induced by Rankl (Fig. 5a). Furthermore, rWnt4 also suppressed Rankl-induced nuclear translocation of p65 (Fig. 5b). Moreover, rWnt4 inhibited NF-κB–dependent transcription, as determined by the NF-κB–dependent luciferase reporter assay (Fig. 5c).

Because Tak1 also forms a complex with Nemo-like kinase (Nlk) and Tab2 in noncanonical Wnt signaling,27,50 we hypothesized that rWnt4 stimulation may interfere with the formation of the TrAF6-Tak1-Tab2 complex induced by Rankl. Immunoprecipitation using antibodies specific to TrAF6 revealed that Rankl induced the formation of the TrAF6-Tak1-Tab2 complex (Fig. 5d). However, addition of rWnt4 drastically inhibited the formation of the TrAF6-Tak1-Tab2 complex (Fig. 5d). In contrast, we observed that rWnt4 stimulation induced the formation of the Tak1-Tab2-Nlk complex, and the addition of Rankl partially reduced the formation of the Tak1-Tab2-Nlk complex (Fig. 5d). As TrAF6-Tak1 signaling also activates p38 mitogen-activated protein kinase, Jnk and extracellular signal–regulated kinase (Erk), we examined whether rWnt4 inhibited the activation of p38, Jnk and Erk induced by Rankl. We found that rWnt4 partially inhibited the phosphorylation of Erk, p38 and Jnk induced by Rankl (Supplementary Fig. 5h).

As the nuclear factor of associated T cells-1 (Nfatc1) is the key transcription factor for osteoclastogenesis,51 we examined the effect of rWnt4 treatment on its expression following Rankl stimulation in bone marrow macrophages. We found that the induction of Nfatc1 by Rankl was repressed by rWnt4 (Fig. 5e). Previously, it has been shown that activation of NF-κB induces expression of *Nfatc1*, which in turn activates osteoclast differentiation.52 Both NF-κB and NFAT consensus binding sites exist at the *Nfatc1* promoter. Upon induction by Rankl, p65 is recruited to the *Nfatc1* promoter to activate its transcription, and subsequently, the newly generated Nfatc1 can autoamplify itself.52 Chromatin immunoprecipitation (ChIP) assays revealed that rWnt4 significantly suppressed Rankl-induced p65 binding to the *Nfatc1* promoter (Fig. 5f). Consequently, rWnt4 also potently reduced Nfatc1 binding at its own promoter induced by Rankl (Fig. 5g).

We previously found that Wnt4 activates noncanonical Wnt signaling in MSCs,53 but Wnt4 might also stimulate canonical Wnt signaling by stabilizing β-catenin. To rule out this possibility, we examined whether rWnt4 protein increased the levels of cytosolic and nuclear β-catenin in bone marrow macrophages. Subcellular fractionation revealed that whereas rWnt3a increased the levels of cytosolic and nuclear β-catenin, rWnt4 did not induce the accumulation of β-catenin (Fig. 5h). Moreover, we also examined whether rWnt4 induced β-catenin–dependent transcription using a TOPFlash luciferase reporter. rWnt3a, but not rWnt4, significantly activated the luciferase reporter in bone marrow macrophages (Fig. 5i). In addition, two
Figure 6 rWnt4 proteins attenuate established bone loss by inhibiting NF-κB. (a–c) μCT reconstruction (a), BMD and BV/TV (b) and H&E staining (c) of distal femoral metaphyseal regions from mice after sham operation, OVX and OVX with rWnt4 injection. Scale bars, 200 µm (a) and 300 µm (c). (d,e) Morphometric analysis of osteoblast (d) and osteoclast (e) counts in distal femoral metaphyses from mice after sham operation, OVX and OVX with rWnt4 injection. (f) TRAP staining showing osteoclasts surrounding trabecular bones in mice after sham operation, OVX and OVX with rWnt4 injection. Scale bars, 30 µm. (g,h) ELISA of Trap5b (g) and Ocn (h) concentrations in serum from mice after sham operation, OVX and OVX with rWnt4 injection. (i) Immunostaining with antibody to active p65 and quantification of NF-κB activity surrounding the trabecular bones from mice after sham operation, OVX and OVX with rWnt4 injection. Scale bars, 200 µm. (j) ELISA of II-6 and Tnf concentrations in serum from mice after sham operation, OVX + PBS and OVX + rWnt4 injection. For all panels with error bars, n = 8 mice for sham group; n = 12 mice per group for mice receiving OVX with PBS or with rWnt4 injection. Data are presented as mean ± s.d., *P < 0.05, **P < 0.01, unpaired two-tailed Student’s t-test.

rWnt4 inhibits OVX-induced bone loss

To explore whether Wnt4 can be used clinically, we first tested whether rWnt4 prevents bone loss by inhibiting NF-κB in an OVX mouse model. We ovariectomized 3-month-old mice and immediately began intraperitoneal administration of rWnt4 once a day for 3 weeks. We then performed μCT analysis, which showed that whereas mice that underwent OVX suffered marked loss in trabecular BMD and BV/TV 1 month after OVX, mice injected with rWnt4 had significantly less bone loss (Supplementary Fig. 6a–c). Histological analysis also confirmed that rWnt4 significantly inhibited trabecular bone loss induced by OVX (Supplementary Fig. 6e–g). Moreover, rWnt4 also reduced serum Trap5b levels (Supplementary Fig. 6h). Immunostaining showed that rWnt4 inhibited NF-κB activity in osteoclasts and adjacent inflammatory cells upon OVX (Supplementary Fig. 6i). Consistent with this, we found that serum levels of II-6 and Tnf induced by OVX were significantly reduced by rWnt4 (Supplementary Fig. 6j).

To further evaluate the therapeutic value of rWnt4, we examined whether rWnt4 could reverse established bone loss in mice induced by OVX. We first performed OVX on 3-month-old mice and waited for 1 month to establish bone loss. We then administered rWnt4 or the vehicle control (PBS) to mice for 1 month. We performed μCT analysis and found that rWnt4 treatment in the OVX group was associated with significantly higher degrees of BMD and BV/TV compared to PBS-treated OVX mice (Fig. 6a,b). Histological staining confirmed that rWnt4 treatment was associated with lower trabecular bone loss induced by OVX compared to PBS-treated OVX mice (Fig. 6c). Histomorphometric analysis also showed that the rWnt4-treated OVX mice had significantly higher osteoblast counts and lower osteoclast numbers compared to PBS-treated OVX mice (Fig. 6d–f). Consistent with these results, rWnt4 treatment of OVX mice was associated with lower serum Trap5b concentrations and higher serum osteocalcin concentrations versus those treated with PBS (Fig. 6g,h). Immunostaining revealed that rWnt4 potently inhibited NF-κB activity in osteoclasts and adjacent bone marrow cells (Fig. 6i), as well as the expression of Tnf, Cox-2 and Mmp9 (Supplementary Fig. 7). We found that rWnt4 treatment of OVX mice was also significantly associated with lower serum concentrations of II-6 and Tnf than those treated with PBS (Fig. 6j).

DISCUSSION

Using three different but complementary animal models, we demonstrated that Wnt4 could reduce OVX-, inflammation- and aging-related bone loss by inhibiting NF-κB, revealing a previously uncharacterized cross-talk between noncanonical Wnt signaling and NF-κB. Gain- or loss-of-function mutations of Wnt signaling components have been identified in a variety of human bone disorders16,53,54. Recently, Wnt5a has been found to enhance osteoclast formation and bone resorption by activating the noncanonical JNK signaling pathway. Wnt5a enhanced osteoclastogenesis induced by Rankl through the Ror2 receptor12, suggesting that targeting Wnt5a may prevent bone erosion in arthritis. However, Wnt5a-haploinsufficient mice had a bone-loss phenotype with increased adipogenesis in bone marrow50. Thus, Wnt5a might not be an ideal therapeutic agent for arthritis and
metabolic bone loss. On the contrary, we found that Wnt4 inhibited osteoclastogenesis and bone resorption in vitro and in vivo by inhibiting NF-κB while promoting bone formation, thereby holding more promise as a potential therapeutic agent for preventing skeletal aging, osteoporosis and arthritis compared to Wnt5a.

Various Wnt ligands can elicit different responses depending on their receptors and cell contexts. Wnt5a acts via Ror2 to enhance the expression of Rank in osteoclast precursors by stimulating activator protein-1 and promotes Rankl-induced osteoclast formation[32]. Notably, we found that Wnt4 suppresses Tak1 activation induced by Rankl, resulting in the inhibition of IKK-NF-κB signaling activation in macrophages and osteoclast precursors. Although Tak1 plays a role in noncanonical Wnt signaling by interacting with Nlk[50], it also modulates canonical Wnt signaling[55,56]. The definitive role of Tak1 in both canonical and noncanonical signaling might depend on cell context and individual Wnt ligands. Our results suggest that Wnt4 might activate its receptors to promote Tak1-mediated noncanonical Wnt signaling in osteoclasts and subsequently sequester Tak1 from effectively binding with Traf6 to induce the NF-κB signaling cascade. Although we showed that Wnt4 promoted Tak1 binding to Nlk, it is possible that Wnt4 might also promote the interaction between Tak1 and the Wnt signaling components, as it has been reported that Ror2 interacts with Tak1 (ref. 55).

Most drugs currently used for osteoporosis are inhibitors of bone resorption, but they cannot restore the substantial bone loss that has already occurred at the time of diagnosis. Therefore, a better treatment module for osteoporosis would not only block bone catabolism but also promote bone anabolism while controlling local inflammation[6–8,11]. Multiple Wnt proteins, including Wnt4, have been detected in bone tissues or bone marrow[54,57,58]. Although the inhibition of aging-associated bone loss and inflammation is mainly based on transgenic overexpression of Wnt4, multiple noncanonical Wnt ligands, including Wnt4, Wnt6, Wnt11 and Wnt16, are expressed in osteoprogenitors[57,58]. They may collectively protect against aging-associated bone loss and inflammation. Notably, we show that rWnt4 proteins effectively inhibit OVX-induced bone loss by inhibiting NF-κB. Although canonical Wnt proteins have potential therapeutic value for treating osteoporosis by promoting bone formation, the constitutive activation of β-catenin might also increase the risk for cancer development that is associated with aging[23,24]. As Wnt4 does not activate β-catenin in either osteoblasts or osteoclasts, and by inhibiting NF-κB, our results suggest that rWnt4 may be a better, and perhaps safer, therapeutic agent for preventing skeletal aging-related bone loss and treating inflammatory bone diseases.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.Y., J.C., Y.L., J.L. and K.K. performed the experiments. K.A.-H., D.T.G., N.-H.P. and C.-Y.W. designed experiments and analyzed data. B.Y. and C.-Y.W. wrote the manuscript.

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We subcloned the mouse Wnt4 gene into pGL647, flanked by the Col2.3 promoter. The fragments of the Wnt4 transgene were purified and microinjected into C57BL/6 × SJL mouse oocytes (Charles River Laboratory), and the oocytes were surgically transferred to pseudopregnant C57BL/6 dams by the University of Michigan Transgenic Animal Model Core. We screened the founders by PCR using mouse tail genomic DNA and confirmed them by Southern blot analysis. We bred two transgenic founder mice with C57BL/6 mice for six generations to obtain a defined genetic background. TNPtg mice expressing hemizygous human TNF were purchased from Taconic Farms (#1006; B6.Cg(SJL)-Tg(TNF) N21+; Oxnard, California). WT C57BL/6 mice for Wnt4 injection were purchased from Jackson Laboratory (Bar Harbor, Maine). In all experiments, female transgenic mice and female WT littermates as controls were used. We established a sample size of at least 8 mice per group in OVX and aging experiments based on our previous experience.22 We used a sample size of at least 6 mice per group in TNPtg/Wnt4 experiments. The animals were randomly assigned to procedure groups including sham, OVX and rWnt4 injection. However, not all animal experiments were conducted in a completely blinded fashion. We overcameotized 3-month-old transgenic and WT mice to induce osteoporosis. Two months after operation, we euthanized the mice and gathered their femurs for histological and μCT analysis. We collected blood samples and isolated serum for serology. Serum ELISAs were performed with a mouse Trap5b assay kit (SBA Sciences), an Ocn ELISA kit (Biomedical Technologies), Il-6 and Tnf Quantikine ELISA kits (R&D Systems). All mouse protocols were approved by The University Committee on Use and Care of Animals at the University of Michigan, the Animal Research Committee at the University of California, Los Angeles, or both.

Cell culture and viral infection. We grew cells in a humidified 5% CO₂ incubator at 37 °C in alpha modified Eagle’s medium supplemented with 15% FBS (FBS; Invitrogen, California, USA). Viral packaging was prepared as described previously.55 For viral infection, we plated cells overnight and then infected them with lentiviruses or retroviruses in the presence of polybrene (6 μg ml⁻¹, Sigma-Aldrich, USA) for 6 h. We then selected the cells with puromycin for 3 d. Resistant clones were pooled and knockdown or overexpression was confirmed via western blot analysis. For culturing of RAW264.7 cells, we used Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. For primary bone marrow macrophages, we extracted bone marrow cells from mouse femurs and treated them with 100 ng ml⁻¹ mouse macrophage colony-stimulating factor (M-CSf; R&D Systems) for 2 d. This allowed the induction to form osteoclast precursors used in the experiments. For induction of osteoclastogenesis, we treated the osteoclast precursors with 100 ng ml⁻¹ mouse Rankl (R&D systems) for up to 3 d. In all in vitro experiments involving Wnt3a and Wnt4 recombinant proteins (R&D systems) and Rankl, we used 100 ng ml⁻¹.

Western blot analysis. We lysed cells in RIPA buffer (10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). We then separated lysates on a 10% SDS polyacrylamide gel and transferred to membranes by a semidry transfer apparatus (Bio-Rad). We blocked membranes with 5% milk for 1 h and then incubated with primary antibodies overnight. After rinsing, we incubated the immunocomplexes with horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI) and visualized the membranes with the Western blotting kit (Biomedical Technologies). For detection of γH2AX, we incubated the immunocomplexes with horseradish peroxidase–conjugated anti-γH2AX mouse IgG (Abcam) and visualized the membranes with Western blotting kit (Biomedical Technologies). We quantified the band intensities using ImageJ software.

ONLINE METHODS

Generation of transgenic mice and experimental animals. We used the plasmid PGL647, which contained the Col2.3 promoter, to specifically drive osteoblast-specific gene expression in vivo. We subcloned the mouse Wnt4 gene into pGL647, flanked by the Col2.3 promoter. The fragments of the Wnt4 transgene were purified and microinjected into C57BL/6 × SJL mouse oocytes (Charles River Laboratory), and the oocytes were surgically transferred to pseudopregnant C57BL/6 dams by the University of Michigan Transgenic Animal Model Core. We screened the founders by PCR using mouse tail genomic DNA and confirmed them by Southern blot analysis. We bred two transgenic founder mice with C57BL/6 mice for six generations to obtain a defined genetic background. TNPtg mice expressing hemizygous human TNF were purchased from Taconic Farms (#1006; B6.Cg(SJL)-Tg(TNF) N21+; Oxnard, California). WT C57BL/6 mice for Wnt4 injection were purchased from Jackson Laboratory (Bar Harbor, Maine). In all experiments, female transgenic mice and female WT littermates as controls were used. We established a sample size of at least 8 mice per group in OVX and aging experiments based on our previous experience.22 We used a sample size of at least 6 mice per group in TNPtg/Wnt4 experiments. The animals were randomly assigned to procedure groups including sham, OVX and rWnt4 injection. However, not all animal experiments were conducted in a completely blinded fashion. We overcameotized 3-month-old transgenic and WT mice to induce osteoporosis. Two months after operation, we euthanized the mice and gathered their femurs for histological and μCT analysis. We collected blood samples and isolated serum for serology. Serum ELISAs were performed with a mouse Trap5b assay kit (SBA Sciences), an Ocn ELISA kit (Biomedical Technologies), Il-6 and Tnf Quantikine ELISA kits (R&D Systems). All mouse protocols were approved by The University Committee on Use and Care of Animals at the University of Michigan, the Animal Research Committee at the University of California, Los Angeles, or both.

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We performed ChIP assays using a ChIP assay kit (Upstate, USA) following the manufacturer’s recommendation. Briefly, we incubated cells with a dimethyl 3′,5′-dithiobispropionimidate·HCl (Pierce) solution (5 mM) for 10 min at room temperature, followed by formaldehyde treatment for 15 min in a 37 °C water bath. For each ChIP reaction, we used 2 × 10⁶ cells. We then quantified resulting precipitated DNA samples with real-time PCR and expressed data as the percentage of input DNA. Antibodies for ChIP assays were purchased from the following commercial sources: polyclonal anti-p65 (Millipore); polyclonal anti-NFATc1 (Santa Cruz). The primers for Nfatc1 are: forward, 5′-CTGTGTTCCCATGTCCTC-3′; reverse, 5′-GGCCTCGAGTGTGTCTTT-3′. 9 kb downstream for Nfatc1 are: forward, 5′-CTGGCACCAAGTTGAGAGA-3′; reverse, 5′-GATG GCTTACCTGACAGA-3′.

O VX, bone histomorphometry and scoring of arthritic joint swelling. We performed OVX or sham operation on 3-month-old female WT and OB-Wnt4 mice under isofluorane anesthesia. For the preventive model, rWnt4 proteins (8 µg kg⁻¹) were intraperitoneally injected daily for 3 weeks immediately after the surgery. For dual labeling, mice received intraperitoneal injection of calcine (0.5 mg per mouse, Sigma-Aldrich) 10 and 3 d before euthanasia. Mice were euthanized 4 days after O VX for the therapeutic model, we first performed OVX on 3-month-old mice and waited for 1 month to establish bone loss. Mice received intraperitoneal injection of rWnt4 (20 µg kg⁻¹) or vehicle control daily for 1 month before collection of bone samples. Eight to twelve mice were used in each group.

Following euthanasia, we fixed right femurs in 70% ethanol for 48 h and embedded in methyl methacrylate. 8-µm longitudinal sections were either stained with toluidine blue for osteoblast count or examined under fluorescent microscope to evaluate BFR and MAR as described previously.⁵² We fixed left femurs in 10% formaldehyde and embedded them in paraffin for preparation in 5-µm-thick sections. We analyzed osteoclast parameters after TRAP staining as described. For all brightfield and fluorescent microscopy analysis, we used Olympus-IX51 inverted microscope with SPOT advanced 4.0 and CellSens software. We scored the swelling of hindpaws on 1-year-old TNFtg and TNFtg/OB-Wnt4 mice on a scale of 0 to 3, as previously described⁴⁶,⁶¹; 1 = mild arthritis (mild swelling of joint and paw); 2 = moderate arthritis (severe swelling and joint deviation); and 3 = severe arthritis (ankylosis detected upon flexion). We used histological sections of hindpaw and ankle joints to examine the tibiotaral and interdigital joints and performed μCT imaging to further evaluate bone erosion and destruction of joint space associated with arthritis. Immunostaining and μCT analysis of mice. We extracted femurs from euthanized mice and fixed them in 10% neutral buffered formalin for 24 h. For μCT scanning, the specimens were fitted in a cylindrical sample holder (20.5 mm in diameter) with the long axis of the femur perpendicular to the X-ray source. We used a Scanco μCT40 scanner (Scanco Medical) set to 55 kVp and 70 µA. The bone volume (mm³) over tissue volume and bone mineral density in the region of interest were measured directly with μCT Evaluation Program V4.4A (Scanco Medical). We defined the regions of interest as the areas between 0.3 mm and 0.4 mm proximal to the growth plate in the distal femurs in order to include the secondary trabecular spongiosa. A threshold of 250 was used for evaluation of all scans.²² For visualization, we imported the segmented data and reconstructed them as a three-dimensional image displayed in μCT Ray V3.0 (Scanco Medical).

After scanning, we decalcified the specimens and sectioned them for staining as previously described. Antibodies used included rabbit polyclonal anti-NLS-p65 (600–401-271; 1:200; Rockland), rabbit polyclonal anti-Mmp9 (38898; 1:500; Abcam), rabbit polyclonal anti-Tnf (34674, 1:200; Abcam), and rabbit polyclonal anti-Cox2 (15191, 1:400, Abcam). For quantification of p65 positive staining, we selected at least ten images from each section per femur, measured the integral optical density (IOD) of nuclear-stained p65 using the Image Pro Plus 6.0 software (MediaCybernetics). We normalized the IOD by staining area and presented the data as reported previously.²²

Statistical analyses. Numerical data and histograms were expressed as the mean ± s.d. Two-tailed Student’s t-test was performed between two groups and a difference was considered statistically significant with P < 0.05.

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Addendum: Wnt4 signaling prevents skeletal aging and inflammation by inhibiting nuclear factor-κB

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In the published article, we, the authors, interpreted the data in Figure 4a–e to mean that transgenic expression of Wnt4 in osteoblasts of mice protects them from age-related bone loss. However, after publication it has been pointed out to us that, at all the ages examined, the transgenic mice had greater trabecular bone mass than control mice. Thus, we have re-examined the data in Figure 4b using statistical tests to examine the relative rate of change of bone mass and bone volume/total volume over the four age groups presented, and we find there is no statistical difference for the rate of these parameters between the transgenic and control groups. Thus, our conclusions with respect to this aspect of the study were incorrect, and further we conclude that we placed an improper emphasis on these findings in the title of the paper.

We have also reanalyzed the data in the rest of the paper using more proper statistical tests in several instances. In particular, the standard deviations in Figures 1g, 1h, 2c–e, 3c–e, 4d, 4f, 6d, 6e and Supplementary Figure 6g were reported inappropriately. We used values from each histology image (3–6 images per mouse) as individual data points, instead of the mean values for each mouse, leading to an increased standard deviation. Furthermore, for morphometric and serum analysis in Figures 2, 3, 4h, 6 and Supplementary Figure 6, one-way analysis of variance with Tukey’s post hoc test should have been used to account for multiple comparisons and adjustments for type I errors. Upon reanalysis, the comparison of osteoclast numbers/bone surface between the wild-type (WT) sham group and the Ob-Wnt4 sham group in Figure 2e, and the comparison of osteoclast surface/bone surface between the WT and Wnt4 groups in Figure 3e lost statistical significance, as was stated in the article.

Although our conclusion about the effect of transgenic expression of Wnt4 in osteoblasts on skeletal aging appears to be incorrect, the above changes regarding our statistical analyses do not alter the conclusions drawn in the manuscript with respect to the effect of Wnt4 transgenic expression on bone mass compared to non-transgenic mice at static time points, the effect of recombinant Wnt4 on bone loss in the ovariectomy model nor on the molecular mechanisms for these effects. Nonetheless, we apologize for any confusion these original analyses or conclusions may have caused.