LGR4 is a receptor for RANKL and negatively regulates osteoclast differentiation and bone resorption

Jian Luo1,7, Zhengfeng Yang1,7, Yu Ma1, Zhiying Yue1, Hongyu Lin1, Guojun Qu1, Jiping Huang1, Wentao Dai2, Chenghai Li1, Chunbing Zheng1, Leqin Xu1, Huaqing Chen1, Jiqiu Wang3, Dali Li1, Stefan Siwko4, Josef M Penninger5, Guang Ning3, Jianru Xiao1,6 & Mingyao Liu1,4

Tumor necrosis factor (TNF) superfamily member 11 (TNFSF11, also known as RANKL) regulates multiple physiological or pathological functions, including osteoclast differentiation and osteoporosis. TNFRSF11A (also called RANK) is considered to be the sole receptor for RANKL. Herein we report that leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4, also called GPR48) is another receptor for RANKL. LGR4 competes with RANK to bind RANKL and suppresses canonical RANK signaling during osteoclast differentiation. RANKL binding to LGR4 activates the Goq and GSK3-β signaling pathway, an action that suppresses the expression and activity of nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATC1) during osteoclastogenesis. Both whole-body (Lgr4−/−) and monocyte conditional knockout mice of Lgr4 (Lgr4 CKO) exhibit osteoclast hyperactivation (including elevation of osteoclast number, surface area, and size) and increased bone erosion. The soluble LGR4 extracellular domain (ECD) binds RANKL and inhibits osteoclast differentiation in vivo. Moreover, LGR4-ECD therapeutically abrogated RANKL-induced bone loss in three mouse models of osteoporosis. Therefore, LGR4 acts as a second RANKL receptor that negatively regulates osteoclast differentiation and bone resorption.

Bone-mass regulation depends on the dynamic balance between bone formation and bone resorption, which are driven by osteoblast activation and osteoclast activation, respectively. RANKL is a central positive regulator of osteoclast differentiation, acting through its binding to TNFRSF11A to induce signaling through TNF-receptor-associated factor (TRAF) and nuclear factor (NF)-κB, which ultimately leads to the activation of NFATC1. Tnfsf11−/− mice exhibit osteoporosis as a result of a lack of osteoclasts1,2; defective T cell and B cell differentiation3; and a failure of mammary gland lobuloalveolar development during pregnancy3. RANKL has been implicated in breast carcinogenesis and bone metastasis4–7, diabetes8, and body-temperature regulation9. The balance between RANKL and its decoy receptor osteoprotegerin (OPG, also called TNFRSF11B) is considered to be a crucial determinant of bone resorption10. Denosumab, a human monoclonal antibody against RANKL, is an approved therapeutic for treating postmenopausal osteoporosis and giant cell tumor of bone11. LGR4, also known as GPR48, regulates multiple developmental pathways through either potential classical G-protein signaling12,13 or via the potentiation of Wnt signaling14,15. A nonsense mutation in LGR4 is associated with low bone mineral density (BMD) in humans16. However, the molecular mechanisms for this regulation are unknown. LGR4 belongs to the LGR family, in which another two members, thyroid-stimulating hormone receptor (TSHR) and follicle-stimulating hormone receptor (FSHR), regulate osteoclast differentiation and resorption17,18. Therefore, we speculated whether LGR4 could also regulate osteoclast differentiation. Tnfsf11−/− mice and Lgr4−/− mice present with similar sets of phenotypes, including disrupted immunity regulation19, mammalian development20, body-temperature modulation21,22, cancer metastasis4–23, and energy expenditure24,25. RANKL–RANK signaling regulates mammary gland lobuloalveolar progenitors, at least in part, through the LGR4 ligand R-spondin1 (RSPO1), which suggests the potential for crosstalk between RANKL–RANK and LGR4 signaling. Therefore, we hypothesized that RANKL and LGR4 act in the same pathways to regulate physiological functions. In this study, we reveal that LGR4 is another receptor for RANKL, and that it, via this ligand, acts to negatively regulate osteoclast differentiation and bone remodeling. These findings suggest that targeting LGR4 is a viable strategy for treating osteoporosis and other bone-resorption diseases.

RESULTS

LGR4 physically interacts with RANKL

To test our hypothesis that RANKL and LGR4 act in the same pathways, we employed five separate approaches to determine whether

1East China Normal University and Shanghai Changzheng Hospital Joint Research Center for Orthopedic Oncology, Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, China. 2Shanghai Key Laboratory of Orthopedic Oncology, Shanghai Academy of Science and Technology, Shanghai, China. 3Department of Endocrinology and Metabolism, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 4Department of Molecular and Cellular Medicine, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas, USA. 5IMBA, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria. 6Department of Orthopedic Oncology, Shanghai Changzheng Hospital, The Second Military Medical University, Shanghai, China. 7These authors contributed equally to this work. Correspondence should be addressed to J.L. (jluo@bio.ecnu.edu.cn), J.X. (jianru-xiao83@163.com) or M.L. (myliu@bio.ecnu.edu.cn or mliu@ibt.tamhsc.edu).

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RANKL directly interacts with human LGR4. First, by using a co-immunoprecipitation approach, we found that both the Flag-tagged extracellular domain (ECD) of LGR4 (amino acids (aa) 28–528; LGR4-ECD or N-terminal (NT)-LRR17) and NT-LRR8 (28–249 aa) of LGR4 physically interacted with RANKL (Fig. 1b,c and Supplementary Fig. 1a). Given that the NT domain and leucine-rich repeat domains (LRRs) 1–8 of LGR family proteins are essential for binding to R-spondins 1–4 (RSPOs)15,25, we made four Flag-tagged deletion constructs to examine the LGR4–RANKL interaction (Fig. 1a). ANT–Flag (58–528 aa) and ANT-LRR1–Flag (80–528 aa) of LGR4 interacted with RANKL but not with RSPO1 (Fig. 1d). The NT-LRR8–Flag of LGR4 (28–249 aa) was associated with RSPO1 but not with RANKL (Fig. 1e). However, the NT-LRR4–Flag of LGR4 (28–396 aa) was associated with RANKL (Fig. 1e). Therefore, different binding motifs are employed in the LGR4–RANKL and the LGR4–RSPO interactions.

Second, by using surface plasmon resonance (SPR) analysis, we found that the NT-LRR17–Flag and NT-LRR14 (28–396 aa) of LGR4–Flag and RANKL or RSPO1 association in HEK293T cells (e) Co-IP analysis of NT-LRR17–Flag and NT-LRR14–Flag, and NT-LRR8–Flag of LGR4 association with RANKL or RSPO1 in HEK293T cells. (f–h) SPR binding-affinity measurement of RANKL and three LGR4-ECD deletion mutants. RANKL and NT-LRR17 (LGR4-ECD) binding affinity is 52.2 nM (Fig. 1f). The binding affinity between NT-LRR14 and RANKL is 36.3 nM (kinetic analysis) or 1.527 µM (affinity analysis) (g). No binding between NT-LRR8 and RANKL was detected (h). (i) Immunofluorescence of LGR4 (green) and RANKL (red) in HEK293T cells. RANKL co-localization with RANK, and RSPO1 co-localization with LGR4, were performed as positive controls. Scale bars, 10 µm. Representative images of three fields of view per experiment are shown. Images are representative of more than ten experiments (b), three experiments (c,d), and more than three experiments (e,i) with biological replicate. Each co-IP was performed once per experiment and blotted separately each time.

Fourth, we found that RANKL co-localized with LGR4 on the plasma membrane of human embryonic kidney (HEK) 293T cells expressing LGR4, but did not do so in cells lacking LGR4 (Fig. 1i). Finally, we quantified RANKL binding to LGR4 in HEK293T cells. RANKL binding to cells that overexpressed LGR4 (143.54%) was higher than that to control HEK293T cells (100%) (Supplementary Fig. 2b). Conversely, HEK293T cells with knockdown of endogenous LGR4 had lower RANKL binding (60.37%) than did control cells (100%) (Supplementary Fig. 2c). Furthermore, LGR4-ECD protein inhibited RANKL binding to LGR4-positive HEK293T cells in a dose-dependent manner (Supplementary Fig. 2d). We also examined possible RANK–LGR4 interactions. RANK did not associate with LGR4, either with or without RANKL stimulation (Supplementary Fig. 2e,f). Collectively, our data suggest that RANKL directly binds LGR4.

**Figure 1 LGR4 interacts with RANKL.** (a) Schematic diagram of the mutation and deletion strategy for LGR4-ECD. (b) Co-immunoprecipitation (IP) analysis of LGR4-ECD and RANKL association in HEK293T cells transfected to express indicated proteins. Immunoblot (IB) probed using indicated antibodies. RSPO1 association with LGR4-ECD served as a positive control. Scale bars, 10 µm. Representative images of three fields of view per experiment are shown. Images are representative of more than ten experiments (b), three experiments (c,d), and more than three experiments (e,i) with biological replicate. Each co-IP was performed once per experiment and blotted separately each time.

LGR4 activates Gαq-Ca²⁺ signaling in response to RANKL
LGR4 is predicted to be a GPCR on the basis of its structural homology to rhodopsin-type GPCRs. However, previously reported LGR4 ligands—RSPOs and Norrie disease (NDP, also known as norrin)—fail to induce G-protein signaling14,15,26. Therefore, we investigated whether the binding of RANKL to LGR4 activated heterotrimeric G-protein signaling. RANKL dose-dependently stimulated serum response element (SRE)-luciferase reporter–gene expression in an
LGR4-dependent manner (Fig. 2a), without affecting cAMP-response element (CRE)-, nuclear factor of activated T cell response element (NFAT)-, or serum response factor–response element (SRF-RE)-luciferase reporter gene expression, and without altering the production of cAMP (Supplementary Fig. 3a,b). Moreover, siRNA-mediated knockdown of guanine-nucleotide binding protein (G protein), q polypeptide (encoded by GNAQ) blocked RANKL-induced SRE-luciferase reporter gene expression, whereas treatment with the G-protein subunit alpha i (encoded by GNAI) inhibitor PTX had little effect on RANKL-induced SRE-driven reporter expression (Fig. 2b,c). Similarly, transfection of either the regulator of G-protein signaling 2 (RGS2) or the C-terminal domain of Goq (Goq-CT), both of which selectively block Goq activation27, inhibited RANKL-induced reporter–gene expression (Fig. 2d). RANKL also stimulated SRE-luciferase reporter–gene expression in a dose-dependent manner through endogenous LGR4 in HEK293T cells (Fig. 2e). We also found that siRNA-mediated LGR4 knockdown blocked the induction of SRE-luciferase by RANKL, whereas co-transfection with an LGR4-expression plasmid restored RANKL-induced SRE-promoter activation (Fig. 2f).

Because Goq activation leads to intracellular calcium release28, we subsequently used calcium imaging to examine whether RANKL induces intracellular calcium release through LGR4. RANKL markedly stimulated calcium release in LGR4-overexpressing cells in a concentration-dependent manner, in contrast to the mild calcium release induced in control cells (Fig. 2g). Similar results were obtained with a fluorescence-imaging plate reader (FLIPR) calcium assay (Supplementary Fig. 3c). Consistently with our luciferase-assay results, all three Goq blockers—RGS2, Goq-CT, and siRNA of GNAQ—almost completely blocked RANKL-induced calcium release (Fig. 2h).

Furthermore, RSPO1 dose-dependently suppressed RANKL-LGR4-triggered calcium release (Fig. 2i). Therefore, our results indicate that RANKL triggers LGR4-mediated signaling via Goq.

Lgr4 loss decreases bone mass and enhances osteoclast activity
RANKL is recognized as the key factor in osteoclastogenesis3. A nonsense mutation in Lgr4 is correlated with low BMD in humans15. We therefore tested whether the RANKL–Lgr4 interaction affects osteoclast differentiation and function in mice. Several LGR family members participate in bone remodeling13,16–18; only the expression of Lgr4, however, was dramatically induced during RANKL-driven osteoclast differentiation (Supplementary Fig. 4a). We confirmed LGR4 expression in osteoclasts by LacZ and TRAP co-staining bone sections from Lgr4+/− mice, which have the β-gal transcript knocked into the Lgr4 locus15 (Supplementary Fig. 4b). Furthermore, Lgr4 is a transcriptional target of RANKL–NFATC1 signaling during osteoclastogenesis (Supplementary Fig. 4c-g), which suggests that LGR4 could be a novel regulator during osteoclastogenesis.

To understand the functions of Lgr4 in vivo, we examined the bone phenotypes of mice deficient in Lgr4 (Lgr4−/− and Lgr4 CKO). Both mouse models exhibited low BMD (Fig. 3a–d and Supplementary Fig. 5a,b), which is consistent with the human phenotype15. Moreover, bone loss was exacerbated as the mice aged from 8 to 15 weeks old (data not shown). In a manner consistent with our observations of an increase in bone loss, we found greater numbers of TRAP-positive osteoclasts and larger osteoclast size in the femoral bones and calvaria of Lgr4−/− and Lgr4 CKO mice, as compared to those in wild-type mice, at all ages analyzed (Fig. 3e–h), which suggests that osteoclasts in Lgr4-deficient mice are hyperactivated. Bone-morphometric analysis revealed that osteoclast surface, osteoclast number, osteoclast size, and eroded surface were all markedly higher in Lgr4−/− and Lgr4 CKO mice than in control mice (Fig. 3f and Supplementary Table 1). Moreover, the serum-bone resorption (osteoclast) marker acid phosphatase 5, tartrate resistant (TRAP5b) was significantly higher in Lgr4 CKO mice than in control wild-type mice (Fig. 3i: P < 0.01), whereas no
**Lgr4 loss enhances the formation and blocks apoptosis of osteoclasts**

We next examined LGR4 in osteoclast differentiation in vitro. RANKL treatment resulted in notably greater osteoclast number and size in bone marrow monocytes (BMMs) from Lgr4 CKO mice (Fig. 4a), in Lgr4−/− BMMs (Supplementary Fig. 6a), and in Lgr4−/− knockdown pre-osteoclast RAW264.7 cells (Supplementary Fig. 6b) than in cells from control mice. Conversely, ectopic Lgr4 expression in RAW264.7 cells resulted in a lower number of and smaller-sized osteoclasts than those in vector-treated control cells (Supplementary Fig. 6c). The loss of Lgr4 also rendered BMMs more responsive than control BMMs to doses of RANKL lower than the standard dose (Fig. 4a).

Moreover, Lgr4 loss accelerated BMM differentiation, especially in later stages (Fig. 4b), probably owing to increased Lgr4 expression levels during BMM differentiation (Supplementary Fig. 4a,b). Lgr4 loss in osteoclasts deregulated bone resorption, with Lgr4−/− BMMs generating more pits with greater pit depth, perimeter, and area than those in BMMs from wild-type mice (Fig. 4c–e; P < 0.01). Osteoclast marker gene profiling in wild-type (WT) and Lgr4−/− osteoclasts also indicated that osteoclast formation (as determined by the expression of Nfatc1; of acid phosphatase 5, tartrate resistant (Acp5); and of Rous sarcoma oncogene (Src)) and resorption (as determined by the expression of calcinon receptor (Calcr) and cathepsin K (Ctksk)) were enhanced in Lgr4−/− BMMs (Supplementary Fig. 6d).

Because RANKL is an important survival factor for osteoclasts, we determined whether LGR4 also influences osteoclast survival in vitro. The number of dead cells in 8-day cultures of BMMs from both Lgr4−/− and Lgr4 CKO mice (i.e., mature osteoclasts) was markedly lower than in those from control osteoclasts (Fig. 4f,g). Lgr4 knockdown in RAW264.7 cells resulted in substantially fewer dead osteoclasts than did treatment with a control siRNA (Fig. 4h). Similar results were obtained in vivo via TUNEL staining of TRAP-positive osteoclasts (Fig. 4i), which suggests that LGR4 regulates osteoclast survival. Therefore, LGR4 inhibits RANKL-induced osteoclast differentiation, survival, and function in vivo and in vitro.

**LGR4 affects the canonical RANK-signaling pathway**

To investigate how LGR4 functions in osteoclastogenesis, we first examined whether RSPOs or norrin affect osteoclastogenesis. None of the reported LGR4 ligands had any effect on the osteoclast differentiation of BMMs or RAW264.7 cells (Fig. 5a,b and Supplementary Fig. 7a,b), which is consistent with a previous report. Moreover, neither RSPO nor norrin treatment affected osteoclastogenesis of Lgr4−/− BMMs (Fig. 5a,b). Furthermore, there was no significant difference in BMM differentiation between R-spondin 4 (Rspo4)−/−, Rspo4fl/fl, and Rspo4−/− mice (Fig. 5c). Taken together, our data suggested that Lgr4-deficiency-induced osteoclastogenesis is independent of RSPOs and norrin.

Next, we examined whether LGR4 affects the canonical RANKL–RANK signaling pathway. The LGR4–ECD dose-dependently inhibited RANKL binding to RANK (Fig. 5d), which suggests that LGR4 competes with RANK to interact with RANKL. Consequently,
LGR4 attenuated RANKL-induced association of RANK with its key downstream signaling molecule TRAF6 in a dose-dependent manner (Fig. 5c). Furthermore, LGR4 abrogated RANKL-induced NF-κB signaling by decreasing the phosphorylation of NF-κB or p65 and by inhibiting the degradation of NFκB inhibitor-α (IkBα, also called NFκBIA) (Fig. 5f and Supplementary Fig. 7c).

RANK–LGR4–Gαq–GSK3-β–NFATC1 pathway blocks osteoclastogenesis

We next determined whether LGR4–Gαq signaling regulates osteoclastogenesis. Knockdown or overexpression of GNAQ, similarly to changes in Lgr4 expression, showed that Gαq negatively regulated osteoclast differentiation (data not shown). Moreover, overexpression of constitutively active Gαq (Gαq(CA)) prevented Lgr4-knockdown-induced osteoclast differentiation (Fig. 5g). Gαq signaling also negatively regulated the expression of NFATC1, the key transcription factor in osteoclastogenesis, and its downstream target genes (Supplementary Fig. 7d and data not shown). Moreover, Gαq inhibited RANKL-induced NFATC1 nuclear translocation (Supplementary Fig. 7d), most probably via the inhibition of glycogen synthase kinase 3-β (GSK3-β) serine 9 (Ser9) phosphorylation (Fig. 5h). The overexpression of either RGS2 or GNAQ-CT restored GSK3-β Ser9 phosphorylation (Supplementary Fig. 7e). In addition, RANKL-induced GSK3-β Ser9 phosphorylation occurred in Rank knockout cells (Supplementary Fig. 7f), which suggests that RANKL-induced GSK3-β Ser9 phosphorylation is independent of RANK expression.

To confirm the relevance of this pathway in osteoclastogenesis, we investigated whether knockdown of GSK3-β can normalize osteoclast differentiation suppressed by GNAQ overexpression. We observed that Gsk3-β knockdown by siRNA rescued the inhibition of osteoclast differentiation in Gαq(CA)-overexpressing cells (Fig. 5i). These data show that LGR4–Gαq signaling affects RANKL-induced osteoclastogenesis.
Soluble LGR4-ECD protein ameliorates bone loss

RANKL blockade has been effective in treating multiple diseases that result in bone loss, including osteoporosis\(^1\). Therefore, we examined whether soluble LGR4-ECD protein, which contains the RANKL interaction domain, could ameliorate osteoporosis. We validated that LGR4-ECD suppressed RSPO1-induced top- and fop-FLASH luciferase-reporter expression in a concentration-dependent manner (data not shown). We then used four models of osteoclast differentiation of RAW264.7 pre-osteoclast cells treated as indicated. Representative images (\(n = 3\) images taken in total, one image each from one well, each with triplicate repeated wells) (a) and osteoclast quantitation (b). \(n = 3\) per group. Scale bars, 500 µm. (c) TRAP staining of BMMs from Lgr4\(^{-/-}\) and Lgr4 CKO mice treated with the indicated stimulation. Representative images (\(n = 3\) images taken in total, one image each from one well, each with triplicate repeat wells) (left) and osteoclast quantitation (right). \(n = 3\) images taken in total, one image each from one well, each with four repeated wells) of osteoclast formation in RAW264.7 pre-osteoclast cells treated as indicated. Representative images (\(n = 3\) images taken in total, one image each from one well, each with four repeated wells) of osteoclast differentiation of RAW264.7 pre-osteoclast cells treated as indicated. \(n = 4\) per group. Scale bars, 200 µm. (d) Western blot of GSK3-β and p65 phosphorylation in RAW264.7 cells at different amounts of LGR4. Images representative of two experiments (biological replicate). (e) Representative images (\(n = 4\) images taken in total, one image each from one well, each with four repeated wells) of osteoclast formation in RAW264.7 pre-osteoclast cells treated as indicated. \(n = 4\) per group. Scale bars, 200 µm. (f) Western blot of GSK3-β phosphoSer9 in HEK293T cells transfected as indicated. Images representative of two experiments (biological replicate) and one experiment (technical replicate). (g) Representative TRAP staining images (\(n = 4\) images taken in total, one image each from one well, each with four repeated wells) of osteoclast differentiation of RAW264.7 pre-osteoclast cells treated as indicated. \(n = 4\) per group. Scale bars, 200 µm.

For a–c,g,i, error bars are mean ± s.d.; \(*P < 0.05\); \(**P < 0.01\); \(***P < 0.001\); n.s., not significant, unpaired two-tailed Student’s \(t\) test.

DISCUSSION

We here show that LGR4 is a novel RANKL receptor that competes with RANK for RANKL binding in osteoclasts. LGR4 inhibits RANKL-induced osteoclast differentiation by blocking RANK–TRAF6 signaling, as well as through Gq-mediated inhibition of NFATC1. Furthermore, LGR4 is a downstream target of RANKL–RANK signaling, which suggests that LGR4 functions in a negative feedback loop.
loop to limit RANKL osteoclastogenesis and to reduce the numbers of osteoclasts in vivo. The injection of soluble LGR4-ECD inhibited osteoclast differentiation in vitro and osteoporosis in three mouse models, which suggests that this may be a viable strategy for the treatment of osteoporosis and other bone-resorption diseases in humans.

RANKL regulates osteoclast survival by reducing the expression of the death receptor Fas in mature osteoclasts. However, mature osteoclasts ultimately undergo apoptosis in a RANKL-containing environment, which suggests the existence of a RANKL-induced signaling pathway that limits the survival of mature osteoclasts. Our data implicate LGR4 as a crucial component of a negative-feedback mechanism to limit osteoclast function in vivo. LGR4 expression is induced by RANKL–NFATC1 signaling during osteoclast differentiation. In mature osteoclasts, the expression level of Lgr4 is especially elevated, which indicates that LGR4 inhibition of RANKL–RANK signaling, increased Fas expression, and apoptosis induction (Supplementary Fig. 8h). This mechanism could explain why mature osteoclasts still undergo apoptosis with RANKL present, and why the loss of Lgr4 prolongs osteoclast survival.

We previously found that Lgr4 affects bone development by regulating osteoblast differentiation, potentially affecting bone mass via osteoblast regulation. However, using monocyte-specific Lgr4-knockout mice, our data here show that Lgr4 CKO mice had similar phenotypes to global Lgr4 knockout mice, including strikingly decreased bone mass, and sharply increased osteoclast differentiation and bone resorption, as compared to control mice, in vivo and in vitro. Although our results here do not preclude the contribution of osteoblast dysregulation to the Lgr4 CKO decreased bone mass phenotype, they do suggest that osteoclast Lgr4-deficiency predominates in driving the low bone mass seen in mice and humans with an Lgr4 mutation. The truncated OPG protein (OPG-Fc),
METHODS

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

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AUTHOR CONTRIBUTIONS

J.L. and Z. Yang generated the initial idea, proposed the hypothesis, and designed the study. Z.Y. conducted the key experiments. J.L. and M.L. supervised the study and performed the data analysis, interpreted results and wrote the manuscript. Y.M., Z. Yue, H.L., G.Q., J.H., C.L., and C.Z. performed the experiments. W.D. performed the docking and molecular modeling. L.X. and J.X. prepared and analyzed human samples. H.C., J.W., D.L., S.S., J.M.P., L.C., and Z. Yang performed the data analysis, interpreted results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Immunoprecipitation analysis. For immunoprecipitation analysis, RAW264.7 cells were transfected with vector or LGR4 plasmids. After 48 h, cell lysates were prepared with RIPA buffer (1% Nonidet P-40 in 150 mM NaCl, 50 mM Tris-HCl, 0.25% sodium deoxycholate, 2 mM PMSE, pH 7.4) with complete protease-inhibitor cocktail (Roche Applied Science, 0469312401). The supernatant was incubated with RANK (Santa Cruz, sc-9072, 1:50) or TRAF6 (Abcam, ab33915, 1:50) antibodies at 4 °C overnight, which was followed by protein A/G bead incubation for another 3 h at 4 °C. Immune complexes were washed three times with phosphate-buffered saline (PBS) and subjected to western blot analysis using specific antibodies for TRAF6 (Abcam, ab33915, 1:1000) and RANK (Santa Cruz, sc-9072, 1:500). To detect the association of RANKL with LGR4–ECD, RANKL with NT-LRR14 and RANKL with NT-LRR8, HEK293T cells were transfected with indicated plasmids, and cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer. The supernatant was incubated with 500 ng RANKL or 200 ng RSPO1–His at 4 °C overnight, and this was followed by incubation with Flag–M2 beads (Sigma–Aldrich, A2220, 5 µl per sample) for another 3 h at 4 °C. Immune complexes were then subjected to western blot using specific antibodies for Flag (Sigma–Aldrich, F7425, 1:2,000), His (Abmart, M30111, 1:5000), or RANKL (IMAGEGEX, IMG-185A, 1:2,000). To perform the RSPO1 competition experiment, HEK293T cells were transfected with LGR4–ECD plasmids, and cell lysates were prepared with RIPA buffer. The supernatant was incubated with RSPO1–His in 200 ng/ml, 500 ng/ml and 1,000 ng/ml at 4 °C for 12 h, respectively. Then 500 ng RANKL was added, and the complex was followed by incubation with Flag–M2 beads (Sigma–Aldrich, A2220, 5 µl per sample) for another 3 h at 4 °C. Immune complexes were then subjected to western blot using specific antibodies for Flag (Sigma–Aldrich, F7425, 1:2,000), His (Abmart, M30111, 1:5000), or RANKL (IMAGEGEX, IMG-185A, 1:2,000).

Protein expression and purification. cDNA of human LGR4–ECD (amino acids 25–528) and ΔNT–LRR1 (80–528 aa) were subcloned into the PET28a+ vector at the BamHI and EcoRI restriction sites. PT28a+-ECD and PT28a+-ΔNT&LRR1 proteins were expressed in Escherichia coli (Rosetta DE3), His-ECD and His-ΔNT&LRR1 were purified from E. coli lysates under native conditions and purified with the nickel–nitrilotriacetic acid (Ni–NTA) system. Briefly, E. coli transformed with PT28a+-ECD and PT28a+-ΔNT&LRR1 were treated with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 25 °C; the cell pellets were then lysis in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) for 10 min on ice, treated with 1 mg/ml lysozyme (Beyotime, China, ST206), and processed through multiple freeze–thaw cycles in liquid nitrogen. The lysate was then sonicated, centrifuged, and subjected to Ni–NTA purification. The beads were washed with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) four times, and eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). For expression of NT-LRR14 and NT-LRR8 protein, cDNAs of human LGR4 NT-LRR14 (28–396 aa) and LGR4 NT-LRR8 (28–249 aa) were subcloned into the pcDNA4T0 vector at the BamHI and EcoRI restriction sites with the CD8 sequence as a signal peptide. pcDNA4T0–NT-LRR14 and pcDNA4T0–NT-LRR8 were transfected and expressed in HEK293T cells. His-tagged NT-LRR14 and NT-LRR8 proteins were purified from HEK293T cell culture medium with the Ni–NTA system.

Surface plasmon resonance. SPR was determined using a Biacore X-100 plus instrument (GE). RANKL peptides were immobilized on the sensor chip (CM5) according to the manufacturer’s recommendations. The kinetics and affinity assay were examined at 25 °C at a flow rate of 30 µl/min using PBS buffer. Diluted ECD peptides, NT-LRR14 peptides, NT-LRR8 peptides and OPG–Fc peptides were injected at 0 °C and placed into the race tray before injection. The Kd values were calculated with the kinetic and affinity analysis option of Biacore X–100 plus evaluation software. Competition analysis was performed according to the software program “manual run”. The LGR4–ECD and RANKL protein interaction was analyzed by regeneration with pH 2.0 Gly-HCl buffer, OPG–Fc protein was loaded as analyte to make sure that the sensor chip was fully intact, and then LGR4–ECD protein was reloaded to compete with the OPG–RANKL interaction.

Docking and molecular modeling. We extracted single-chain truncated structures of RANKL and LGR4 from proteins 1QQA and 4KT1 (PDB ID), respectively. The crystal water in these proteins was removed in the extraction process. The 82–411 aa region of LGR4–ECD and the 161–316 aa region of RANKL were selected as candidate interfaces, and a truncation of RANKL (161–316 aa) and LGR4–ECD (25–528 aa) were docked into a complex with ZDock v3.0.2 software. The top ten complex models were selected as candidates. We selected the highest-confidence model from the ten candidate complexes using METop program (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). The complex model of RANKL–LGR4–ECD was shown by PyMol.

Immunofluorescence. For immunofluorescence, HEK293T cells were seeded in a 24-well plate on 0.1% gelatin–treated glass coverslips. All of the cells were transfected with siRNA for RANK (5′-CCAGAAGAUUGGUACCAUU-3′, 5′-UUGGUAAGCACAUUUCCUGGUU-3′). Each well was transfected with 0.5 µg of empty vector, human LGR4, or mouse RANK plasmid, respectively. Cells were subsequently incubated with either RANKL or RSPO1 at 37 °C for 20 min. After being washed twice with PBS, the cells were fixed in 4% paraformaldehyde, and incubated with anti-RANKL (IMAGEGEX, IMG-185A, 1:50) or anti-His antibody (Abmart, M30111, 1:1000) at 37 °C for 1 h. The images were obtained by laser-scanning confocal microscopy (Leica).

Flow-cytometric analysis. HEK293T cells were transfected with control siRNA or RANKL siRNA and vector or LGR4 plasmids, incubated with 500 ng/ml of recombinant RANKL at 37 °C for 45 min, and then fixed with 4% paraformaldehyde. Cells were then incubated with 1 µg RANKL antibodies (IMAGEGEX, IMG-185A) or mouse IgG at 4 °C for 1 h. After washing, the cells were incubated with Alexa Fluor 488–labeled goat anti-mouse IgG (Invitrogen, a1001, 1:1000) at 4 °C for 1 h, and then subjected to fluorescence-activated cell sorting (FACS) analysis (FACS caliber, BD) after washing.

Reporter-gene assay. To identify the possible G proteins that RANKL may activate, we used a luciferase reporter–gene system, as previously reported. The experiments were conducted in triplicate. Briefly, HEK293T cells were co-transfected with vector, luciferase and rennilla or LGR4, luciferase and rennilla plasmids as indicated. After seeding into 24-well plates, in some experiments, the cells were transfected with siRNA for RANK (5′-CCA GAAGAUUGGUACCAUU-3′, 5′-UUGGUAAGCACAUUUCCUGGUU-3′) or siRNA for LGR4 (ref. 17) (5′-GAAGGAACUGUGCUACCAUU-3′, 5′-UUGGACACAGAUUUCCUGGUU-3′) by Lipofectamine 2000 (Invitrogen, l6668019). Then, cells were serum-starved with 1% FBS for 4 h, and incubated with indicated stimulators for 24 h. The luciferase assay was performed according to the manufacturer’s protocol (Promega, E1960). To examine the bioactivity of LGR4–ECD protein, we used the TOP–FLASH system, as previously reported. Briefly, 50 ng/ml RSPO1 and LGR4–ECD protein at the indicated concentrations were incubated for 12 h at 4 °C in DMEM culture containing 1% FBS. HEK293T cells were co-transfected with TOP–FLASH and rennilla or POP–FLASH and rennilla plasmids. After seeding into 24-well plates, cells were stimulated with RSPO1–LGR4–ECD mixture as indicated for 24 h. The luciferase assay was performed according to the manufacturer’s protocol (Promega, E1960).

Enzyme-linked immunosorbent assay (ELISA). For CAMP ELISA, HEK293T cells were transfected with vector or LGR4 plasmids for 24 h. Cells were then pretreated with 250 nM 3-isobutyl-1-methylxanthine (IBMX) for 30 min, and incubated with RANKL at the indicated concentration for 24 h. cAMP production was examined according to the manufacturer’s protocol (R&D, KGE02B) with samples pretreated at 95 °C for 15 s. For TRAP5b, PINP and osteocalcin ELISA, 8-week-old mice were euthanized for serum collection. Serum samples were then sent to USCN Life Science, Inc. (Wuhan, China) for analysis.

Calcium imaging and FLIPR calcium assay. We performed calcium imaging for the detection of intracellular calcium release, as previously described.
For transient calcium mobilization, HEK293T cells were transiently transfected with plasmids using the calcium-phosphate method and following the standard protocol. 24 h after transfection, cells were transfected with 2 μM fura-2 AM (Molecular Probes, F2121). The basal 340/380 fluorescence signal of the cells in the field of view was monitored for 30 s, and then the cells were stimulated with 100 ng/ml or 200 ng/ml RANKL. After waiting for 20 s, transient calcium release was detected and imaged by LAMBDA DG-4 (Novato, CA, USA). For the RSP01 competition assay, HEK293T cells were transiently transfected with Rank siRNA (5′-CCAGAAGAUUGCCCIACCACUU-3′, 5′-UGGGUAGACAUCCUUCCGUG-3′) using Lipofectamine 2000 (Invitrogen, 11668019), and with LGR4 plasmid using the calcium-phosphate method and following the standard protocol. 24 h after transfection, cells were pretreated with RSP01 at 50 ng/ml, 100 ng/ml and 200 ng/ml, respectively, for 12 h. Cells were then loaded with 2 μM fura-2 AM (Molecular Probes, F2121). The basal 340/380 fluorescence signal of the cells in the field of view was monitored for 30 s, and then the cells were stimulated with 200 ng/ml RANKL. After waiting for 20 s, transient calcium release was detected and imaged by LAMBDA DG-4 (Novato, CA, USA). To examine the calcium response, we performed a FLIPR calcium assay. Briefly, HEK293T cells were transiently transfected with plasmids using the calcium-phosphate method and following the standard protocol. 24 h after transfection, cells were seeded in a 96-well plate at 8 × 10^4 cells per well, and then cultured with DMEM containing 10% FBS and 2 mM CaCl_2 for 12 h. Cells were washed with Hank’s balanced salt solution (HBSS) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and loaded with calcium-indicator dye from the FLIPR Calcium 5 Assay Kit (Molecular Devices, Sunnyvale, CA, USA), and incubated for 60 min at 37 °C. Measurements were performed via a FlexStation3 (Molecular Devices) set at 26 °C. Calcium signals (excitation at 485 nm and emission at 525 nm) were recorded for 2 min at 1.52 s intervals. The response of each well was calculated as (maximum fluorescence value – minimum fluorescence value)/ minimum fluorescence value.

**RT-qPCR analysis.** For RT-qPCR analysis, total cellular RNA was extracted from cells using TRIzol reagent (Takara, 9109). PCR primers for Nfatc1, Rous sarcoma oncogene (Src, also called c-Src), cathepsin K, Trap, calcitonin receptor (Calcrl, also called Ctr) Ctr, Rank, Gapdh, Fshr, Ucgcrg, Tgr, Lgr4, Lgr6, relaxin/insulin-like family peptide receptor 1 (Rxfp1, also called Lgr7) and Rxfp2 (also called Lgr8) are provided in **Supplementary Table 2**.

**β-galactosidase (LacZ) and TRAP double-staining.** 10-day-old Lgr4^{−/−} mice were euthanized for femur bone isolation. The bones were washed with ice-cold LacZ fixation buffer (2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 in PBS) twice (20 min each time). After being incubated in LacZ staining buffer (5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II) tributyrate, 1 mg/ml X-gal in LacZ wash buffer) for 36 h at room temperature, the bone underwent decalcification with 0.5 M EDTA for 1 d followed by paraffin embedding, histological sectioning and TRAP staining. The same slides were captured before and after TRAP staining. The LacZ–TRAP double-staining regions were used for analysis.

**Chromatin immunoprecipitation assay.** RAW264.7 cells were treated with or without 20 ng/ml RANKL for 24 h, followed by ChIP analysis as previously reported11, with only minor modifications. Briefly, cells were sonicated on ice with six cycles of Biorupter (Diagenode). The supernatant was incubated with 2 μg IgG or Nfatc1 antibody (Santa Cruz, sc-7294) at 4 °C for 4 h. Genomic DNA in immune complexes was extracted and prepared for PCR reactions. The primer sequences are provided in **Supplementary Table 2**.

**Cell culture.** For osteoclast differentiation analyses in vitro, we isolated bone marrow macrophages (BMMs) from 4-week-old WT and Lgr4^{−/−} or Lgr4 CR2 mouse femur and tibia bones, as previously described9. The differentiation experiments were conducted in triplicate. BMMs were seeded into 24-well plates at a concentration of 1.5 × 10^6 cells per well. Cells were stimulated with 100 ng/ml RANKL (R&D, 462-TEC) and 10 ng/ml M-CSF (R&D, 416-ML) for 6 d, or for 8 d to assess osteoclast survival. Osteoclasts were fixed and stained using the TRAP staining kit (Sigma-Aldrich, 387A-1KT). For the osteoclast survival assay, osteoclast ghosts were quantitated as dead osteoclasts. RAW264.7 cells were transfected with Lgr4 siRNA (5′-GCAUCAUGCAAAAUATT−3′, 5′-UUAAUGUUAUAAAGAAGG−3′) or human LGR4 plasmids using FugeneHD transfection reagent, as previously reported42. Cells were then treated with RANKL for 3.5 d. For the pit-formation assay, mature osteoclasts were isolated, as previously described43. Pits (n = 50) were stained with toluidine blue, and pit perimeter, area and depth were examined by laser-scanning confocal microscopy, as previously described43. To assay osteoclast apoptosis, we performed TUNEL staining. Briefly, after RAW264.7 cells were transfected with siRNA using FugeneHD transfection reagent according to the manufacturer’s instructions, the cells were seeded into 96-well plates and stimulated with 20 ng/ml RANKL for 3 d. TUNEL (Promega, G7130) staining was performed according to manufacturer’s instructions. RAW264.7 and HEK293T cells were purchased from the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank. The cell lines were authenticated and mycoplasma-tested by the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank using PCR analysis.

**Nuclear and cytoplasm extraction.** RAW264.7 cells were transfected with Gqα–Gqβ–GSK3-β using Fugene HD (Roche Applied Science, 04709705001), and stimulated with 20 ng/ml RANKL for indicated times. Cells were then washed with cold PBS and resuspended in cell lysis buffer for 15 min, 3% NP-40 was added with vortexing. Samples were then rapidly centrifuged for 1 min at 14,000 rpm at 4 °C. The supernatant was the cytoplasmic extract (CE), and the pellet was the nuclear extract (NE). Both the CE and NE were lyzed with SDS-loading buffer and subjected to western blot using specific antibodies for Nfatc1 (Santa Cruz, sc-7294, 1:1,000), actin (Sigma-Aldrich, A5441, 1:5,000), Histone3 (Cell Signaling Technologies, 9715L, 1:1,000) and Gapdh (Santa Cruz, sc-393, 1:1,000).

**Western blot.** For NF-κB signaling analysis, RAW264.7 cells were transfected with or without 0.1, 0.2 μg LGR4 plasmids in 24-well plates. After 48 h, cells were stimulated with 100 ng/ml RANKL for 20 min, lysed in 1× SDS loading buffer and subjected to western blot using specific antibodies for phospho-p65 (Cell Signaling Technologies, 3033, 1:1,000), p65 (Cell Signaling Technologies, 3034, 1:1,000), IkBz (Cell Signaling Technologies, 4814, 1:1,000) and actin (Sigma-Aldrich, A5441, 1:5,000). For LGR4–Gqα–Gqβ–GSK3-β signaling, HEK293T cells were transfected either with LGR4–Flag or Gqα–Gqβ–Flag plasmid. Cells were then stimulated or without with 100 ng/ml RANKL for 5, 15 and 30 min, lysed in 1× SDS loading buffer and subjected to western blot using specific antibodies for phospho-GSK3-β (Cell Signaling Technologies, 9336, 1:1,000), GSK3-β (Cell Signaling Technologies, 9315, 1:1,000), Flag (Sigma-Aldrich, F7425, 1:5,000), Gqα (Santa Cruz, sc-393, 1:1,000) and GAPDH (Abmart, M20060F, 1:5,000).

**Primary cultures of human peripheral blood mononuclear cells (PBMC) and bone giant cell tumor (GCTB) cells.** The use of all patient-derived tumor specimens was approved by the Institutional Review Board and the research ethics committee of Shanghai Changzheng Hospital under reference of 2010/081, which appeared in the proceedings of the meeting of the Ethics Committee on 18 November 2010. Informed consent was obtained from all tissue donors. The GCTB cells were isolated from tumor samples derived from tumor resections in Shanghai Changzheng Hospital, which were cultured in 37 °C with 5% CO2. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll gradient centrifugation (provided by the Shanghai Blood Center). The culture medium consisted of α-MEM supplemented with 10% FBS (FBS). For osteoclastogenesis, 5 × 10^5 PBMCs were seeded in a 96-well plate with 20 ng/ml human CSF1 (Sino Biological, Inc, 11792-H08Y). After 36 h, cells were stimulated with 30 ng/ml human RANKL (R&D, 6449-TEC) and 20 ng/ml human CSF1 for 8–9 d. Medium was changed every 2 d. Osteoclasts were fixed and stained using the TRAP-staining kit (Sigma-Aldrich, 387A-1KT).

**Mice.** Generation of Lgr4^{−/−} mice was previously described12,31. Tnfαfl/fl^{−/−} mice (strain 129S1/Sv) were purchased from the Shanghai Research Center For
Model Organisms. Rspo1−/− mice (strain FVB) were generated from the Animal Center of East China Normal University. The generation for Lgr5floxed mice (strain C57/BL/6) is described in reference 9. LysM-Cre mice (strain C57BL/6) were described in reference 44. Both male and female mice were used in all experiments, except only male mice were used for LGR4-ECD treatment experiments and only female mice for the ovariectomy model. All of the mice were randomly assigned to groups. Maintenance, use and treatment of all animals were in accordance with accepted standards of the Ethics Committee at ECNU.

Micro-CT analyses. 3D micro-CT analyses and osteoclast morphometric analyses were performed as previously described 13. For micro-CT, we scanned the bone using in vivo X-ray microtomography (Skyscan 1076, Bruker microCT) at a pixel size of 18 µm, and analyzed the results according to the manufacturer’s instructions. Region-of-interest (ROI) was defined from 0.215 mm (12 image slices) to 1.72 mm (106 image slices), where the growth plate slice was defined as 0 mm. Contrast was defined from 68–255; 3D analysis, BMD and 3D models were analyzed using CTAn software (Bruker microCT). 3D models were adjusted in CTVol software (Bruker microCT). 3D models were defined as 0 mm. Contrast was defined from 68–255; 3D model were analyzed using CTAn software (Bruker microCT). For osteoclast morphometric analyses, 8-week-old, 16-week-old, and 24-week-old mouse femur bones and calvaria bones were isolated and fixed in 4% formaldehyde for 24 h. After decalcification with 0.5 M EDTA for 1–2 weeks, histological sectioning and TRAP staining for osteoclasts was performed. Osteoclast numbers, osteoclast surface area and eroded surface area were assessed by the OsteoMeasure Analysis System (Osteometrics, Atlanta, GA, USA), according to standard criteria.

Treatment with recombinant LGR4-ECD protein in vivo. For animal studies in vivo, mice were randomized for weight. For the ovariectomy-induced bone loss model, we sham-operated or ovariec-tomized 3-month-old C57BL/6 mice to induce osteoporosis. Ovariectomized mice were randomly divided into two groups (vehicle (PBS) versus recombinant LGR4-ECD protein, n = 8 per group). To analyze the therapeutic effect of recombinant LGR4-ECD protein, we injected LGR4-ECD protein (1 mg/kg/day) or vehicle into the tail vein after waiting 1 month beyond surgery for peak bone loss. After 5 weeks of treatment, the femurs and the L3 lumbar were isolated for micro-CT and histomorphometric analysis. For the RANKL-injection bone-resorption mouse model, the control protein, LGR4-ECD protein, RANKL–control protein, or RANKL–LGR4-ECD protein were injected into the calvaria of 6-week-old C57BL/6 male mice (n = 6) every day for 2 weeks. The concentration of control protein, LGR4-ECD protein, and RANKL protein was 1 mg/kg. At 15 d after the first injection, the mice were euthanized, and calvaria were collected. Calvaria were fixed in 4% formaldehyde, permeabilized by 0.1% Triton X-100 for 1 h, and stained for TRAP activity with a TRAP kit (Sigma-Aldrich, 387A-1KT). 3D micro-CT analyses were performed according to a standard protocol. BMD and bone volume were analyzed by CT-analysis software (CTAn, Bruker microCT, Kontich, Belgium) and images were reconstituted by CT-volume software (CTvol, skyscan, CTAn, Bruker microCT, Kontich, Belgium). Similarly, 5-month-old 129 male Tnfrsf11b−/− mice (n = 6 per group) were injected with control protein and LGR4-ECD protein into the calvaria or tibia bone at a concentration of 1 mg/kg every day for 2 weeks. For the tibia bone assay, PBS was injected into the left leg, and LGR4-ECD protein was injected into the right leg in the same mouse (n = 11 per group). Investigators were not blinded with respect to which protein was injected.

Statistical analyses. Data are represented as mean ± s.d. for absolute values, as indicated in the vertical axis legend of the figures. The statistical significance of differential findings between experiments and controls was calculated by Excel 2007 (Microsoft Corp., Redmond, WA) using the two-tailed homoscedastic Student’s t test. Significance was considered to be P < 0.05. Results are representative examples of more than two independent experiments. Data distribution was previously tested with the Kolmogorov–Smirnov test. Animal-experiment sample size was selected on the basis of power calculations seeking 80% power to detect a difference of 30% between groups with α = 0.05. No animals were excluded. Investigators were not blinded during animal experiments.