Lrp4 in osteoblasts suppresses bone formation and promotes osteoclastogenesis and bone resorption

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Bone mass is maintained by balanced activity of osteoblasts and osteoclasts. Lrp4 (low-density lipoprotein receptor related protein 4) is a member of the LDL receptor family, whose mutations have been identified in patients with high–bone-mass disorders, such as sclerosteosis and van Buchem diseases. However, it remains unknown whether and how Lrp4 regulates bone-mass homeostasis in vivo. Here we provide evidence that Lrp4-null mutation or specific mutation in osteoblast-lineage cells increased cortical and trabecular bone mass, which was associated with elevated bone formation and impaired bone resorption. This phenotype was not observed in osteoclast-selective Lrp4 knockout mice. Mechanistic studies indicate that loss of Lrp4 function in osteoblast-lineage cells increased serum levels of sclerostin, a key factor for bone-mass homeostasis that interacts with Lrp4, but abolished the inhibition of Wnt/β-catenin signaling and osteostatic differentiation by sclerostin. Concomitantly, sclerostin induction of RANKL (receptor activator of nuclear kappa B ligand) was impaired, leading to a lower ratio of RANKL over OPG (osteoprotegerin) (a key factor for osteoclastogenesis). Taken together, these results support the view for Lrp4 as a receptor of sclerostin to inhibit Wnt/β-catenin signaling and bone formation and identify Lrp4 as a critical player in bone-mass homeostasis.

Lrp4 | sclerostin | osteoblasts | osteoclasts | β-catenin

Bone remodeling is a dynamic process essential for maintenance of skeletal integrity and bone homeostasis (1). Bone mass is tightly regulated by bone-forming osteoblasts (OBs) and bone-resorbing osteoclasts (OCs). OBs are differentiated from bone marrow stromal cells (BMSCs) or mesenchymal progenitor cells, whereas OCs are derived from hematopoietic bone marrow macrophages or myeloid monocytes (BMMs). The balance of bone formation and resorption is critical for maintenance of healthy bone mass. The imbalance of bone formation and resorption could result in high–bone-mass disorders such as sclerosteosis and van Buchem disease or bone loss such as osteoporosis.

The canonical Wnt/β-catenin signaling is critical to regulate bone-mass homeostasis (1, 2). Binding of Wnt ligands to a dual-receptor complex of frizzled and Lrp5/6 leads to accumulation of cytoplasmic β-catenin and translocation of β-catenin into the nucleus to regulate gene expression. This pathway is required for commitment of mesenchymal stem cells to the OB lineage, OB precursor cell proliferation and differentiation, and OC genesis and activation (1–3). Clinically, Lrp5 mutations are associated with the osteoporosis–pseudoglioma syndrome, a low–bone-mass disorder (4), as well as with high–bone-mass disorders (5, 6).

Lrp4 is a member of LDL family protein, containing a large extracellular region with multiple LDLs, EGF-like, and β-propeller repeats, a transmembrane domain, and a short C-terminal region. Lrp4 is a receptor of agrin (7, 8), critical for neuromuscular junction formation. Mice lacking Lrp4 (null allele) die at birth because of inability to breathe (9). Lrp4 is also highly related to Lrp5/6 and interacts with sclerostin, a key extracellular factor for bone remodeling (10–13). Mutations have been identified in Lrp4 and in sclerostin in patients with high–bone-mass disorders, such as sclerosteosis and van Buchem disease (10–16). However, how Lrp4 regulates bone-mass homeostasis remains unclear. Mice harboring a stop codon upstream of the transmembrane domain of Lrp4 exhibit increased bone formation and elevated bone resorption, but decreased bone mineral density (11). Because the extracellular domain of Lrp4 remains intact, it is unclear whether this mutant mouse model represents a gain or loss of Lrp4 function.

Here we provide evidence that Lrp4 in OB-lineage cells suppresses bone overgrowth. Both muscle-rescued Lrp4-null (mr-Lrp4mut) and OB-selective Lrp4 knockout mice (Lrp4Ocx-cko) showed increased cortical and trabecular bone mass, elevated bone formation, and impaired bone resorption. In contrast, these phenotypes were not observed in OC-selective Lrp4 knockout (Lrp4Ocn-cko) mice. Loss of Lrp4 function in OB-lineage cells abolished sclerostin inhibition of Wnt/β-catenin signaling and OB differentiation, despite the fact that serum sclerostin was increased. In addition, Lrp4 deficiency in OB-lineage cells impaired sclerostin induction of receptor activator of nuclear kappa B ligand (RANKL), thus reducing the ratio of RANKL over osteoprotegerin (OPG). These results suggest that Lrp4 in OB-lineage cells is necessary to prevent bone formation and to promote bone resorption, therefore maintaining adequate bone homeostasis. Loss of Lrp4 function in OB-lineage cells results in high bone mass, providing a pathophysiological mechanism of relevant high–bone-mass disorders.

**Results**

**High-Bone-Mass Deficit in Muscle-Rescued Lrp4-Null Mice.** Lrp4mut is considered as a null allele because the mutation results in a stop codon at the amino acid 377 (Fig. SL4) (9). Lrp4mut mice die
immediately after birth, due to the absence of neuromuscular junction and hence inability to breathe (9, 17, 18). To investigate functions of Lrp4 in bone remodeling, we crossed Lrp4\textsuperscript{f/f} mice with HSA (human skeletal α-actin) promoter-driven Lrp4 transgenic mouse (HSA-Lrp4), where Lrp4 was expressed specifically in skeletal muscles (Fig. S1 A and B). Western blot analysis indicated that in muscle-rescued Lrp4\textsuperscript{mutt} (thus referred to as mr-Lrp4\textsuperscript{mutt}), Lrp4 was detected only in skeletal muscle, but not in other tissues (Fig. S1 C and D). In agreement, muscle expression of Lrp4 restores neuromuscular junction formation (19), but not digit development (Fig. S1 E and F). The syndactyly-like digit deficit was detected in mr-Lrp4\textsuperscript{mutt} mice (Fig. S1 E and F), as it was in Lrp4\textsuperscript{mutt} mice (9, 17, 18). However, the mr-Lrp4\textsuperscript{mutt} mice were viable and had relatively normal body size with a slightly reduced body weight (Fig. S1E). Intriguingly, the mutant long bones (e.g., tibia and femurs) appeared to be white/pale in color (Fig. S1F), that may be due to reduced bone marrow cells. Microcomputer tomographic (μCT) analysis of femurs from 3-mo-old mr-Lrp4\textsuperscript{mutt} mice showed decreased bone marrow cavity and increased cortical bone volumes/total volumes (Cb, BV/TV), and trabecular bone thickness (Tb.Th) were increased, respectively. Lrp4 level in BMSC was moderate, but increased significantly in OB, OC, and precursor cells (BMSC and BMM, respectively). Lrp4 level in BMSC was moderate, but increased in OBs; on the other hand, Lrp4 in BMMs was relatively high, but diminished in OCs (Fig. 2 A and B). To determine whether Lrp4 in OBs is crucial, we crossed floxed Lrp4 (Lrp4\textsuperscript{f/f}) mice with osteocalcin (Ocn)–Cre that expresses Cre selectively in mature macrophages (precursors of OCs) (Fig. S2A). As shown in Fig. 2, μCT analysis of 3-mo-old Lrp4\textsuperscript{Ocn-cko} mice showed increased trabecular and cortical bone volumes, compared with littermate controls (Lrp4\textsuperscript{f/f}). The trabecular thickness was also increased in Lrp4\textsuperscript{Ocn-cko} femurs (Fig. 2 C and D). These phenotypes appeared to be more severe in Lrp4\textsuperscript{Ocn-cko} mice at the age of 8 mo old (Fig. 2 E and F). Together, these results indicate that Lrp4 in OB-lineage cells is critical for decrease of bone mass. This notion was further supported by studies of Lrp4\textsuperscript{LysM-cko} mice, where Lrp4 was expressed specifically in macrophages (LysM)–Cre that expresses Cre selectively in mature macrophages (precursors of OCs) (Fig. S2C and D) (21). As expected, Lrp4 level was diminished in BMSCs in Lrp4\textsuperscript{LysM-cko} mice (Fig. S3 A and B). Compared with the controls, no significant change was observed
Increased Bone Formation in mr-Lrp4mitt and Lrp4Ocn-cko Mice. The bone-mass increase in both mr-Lrp4mitt and Lrp4Ocn-cko mice may result from enhanced bone formation and/or decreased bone resorption. To address this question, we asked whether bone formation is altered by loss of Lrp4 in OB-lineage cells. Neonatal mice were injected with two doses (separated by a 12-d interval) of calcein and alizarin red, both fluorescent markers of bone formation. Mineral apposition rate (MAR), bone formation rate (BFR), and mineral surface (MS) / bone surface (BS) were assessed in nondecalcified histological sections of femurs and tibia. As shown in Fig. 3A–D, the MAR, BFR, and MS/BS were all increased in 1-mo-old mr-Lrp4mitt mice, compared with littermate controls (Fig. 3A and D). These results demonstrate increased bone formation in mice lacking Lrp4 in OB-lineage cells. As with bone formation deficit, PYD reduction was detected in 1-mo-old mr-Lrp4mitt, but not Lrp4Ocn-cko mice (Fig. 4A and E). The reduction appears to be specific because it was not observed in OC-selective Lrp4 knockout (Lrp4Ocn-cko) mice (Fig. S4A). In fact, serum PYD was increased in Lrp4Ocn-cko mice (Fig. S4A). These results suggest an age-dependent inhibition of bone resorption by loss of Lrp4 in OB-lineage cells. Altered bone resorption could be due to a change in OC number and/or function. We thus asked if OC genesis is impaired in Lrp4 mutant mice. As shown in Fig. 4B and C, the number of tartrate-resistant acid phosphatase positive (TRAP+) OCs in femurs was lower in mr-Lrp4mitt mice than in littermate controls at the age of 1 mo old, suggesting that Lrp4 is necessary for proper control of OC genesis. Intriguingly, TRAP+ OCs were also lower in 3-mo-old, but not 1-mo-old, Lrp4Ocn-cko mice than in controls (Fig. 4D and E). In contrast, the number of TRAP+ OCs in Lrp4Ocn-cko mice was slightly greater than that of littermate controls (Fig. S4B–F). These observations suggest that OC genesis appears to be positively regulated by Lrp4 in OB-lineage cells, but negatively regulated by Lrp4 in OC-lineage cells.

Requirement of Lrp4 in OB-Lineage Cells for Sclerostin to Inhibit OB Differentiation. Lrp4 interacts with multiple extracellular proteins, including sclerostin (SOST), Wise (a paralog of sclerostin) and DKK1, and agrin affect OB differentiation and, if yes, whether sclerostin, Wise, and DKK1 are negative regulators of Wnt/β-catenin signaling and/or bone formation (1, 13, 23, 24). To determine whether their effect requires Lrp4, we cultured BMSCs from mr-Lrp4mitt and control littermates and examined in vitro OB differentiation by ALP (alkaline phosphatase) staining and osteocalcin expression. No difference of ALP function/bone formation. We then determined whether sclerostin, DKK1, and agrin affect OB differentiation and, if yes, whether

Decreased Bone Resorption in Both mr-Lrp4mitt and Lrp4Ocn-cko Mice. We next determined whether Lrp4 deficiency alters bone resorption by measuring serum levels of deoxy-pyridinoline (PYD), a collagen cross-link molecule that provides valuable information on bone resorption. At the age of 3 mo old, serum PYD was significantly decreased in both mr-Lrp4mint and Lrp4Ocn-cko mice, compared with littermate controls (Fig. 4A). As with bone formation deficit, PYD reduction was detected in 1-mo-old mr-Lrp4mint, but not Lrp4Ocn-cko mice (Fig. 4A and E). The reduction appears to be specific because it was not observed in OC-selective Lrp4 knockout (Lrp4Ocn-cko) mice (Fig. S4A). In fact, serum PYD was increased in Lrp4Ocn-cko mice (Fig. S4A). These results suggest an age-dependent inhibition of bone resorption by loss of Lrp4 in OB-lineage cells. Altered bone resorption could be due to a change in OC number and/or function. We thus asked if OC genesis is impaired in Lrp4 mutant mice. As shown in Fig. 4B and C, the number of tartrate-resistant acid phosphatase positive (TRAP+) OCs in femurs was lower in mr-Lrp4mitt mice than in littermate controls at the age of 1 mo old, suggesting that Lrp4 is necessary for proper control of OC genesis. Intriguingly, TRAP+ OCs were also lower in 3-mo-old, but not 1-mo-old, Lrp4Ocn-cko mice than in controls (Fig. 4D and E). In contrast, the number of TRAP+ OCs in Lrp4Ocn-cko mice was slightly greater than that of littermate controls (Fig. S4B–F). These observations suggest that OC genesis appears to be positively regulated by Lrp4 in OB-lineage cells, but negatively regulated by Lrp4 in OC-lineage cells.

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the effect requires Lrp4. Condition media (CMs) containing sclerostin, DKK1, or agrin were collected from respectively transfected HEK293 cells (Fig. S6 A–C). Control and Lrp4 mutant BMSCs were induced for OB differentiation, in agreement with previous reports (1, 13, 23, 24). Interestingly, the inhibitory effect of sclerostin was diminished in mr-Lrp4mut and Lrp4Ocn-cko BMSCs (Fig. 5 B and C), suggesting that Lrp4 is necessary for sclerostin inhibition of OB differentiation. To further test this view, we examined osteocalcin expression in BMSCs treated with or without sclerostin. Indeed, sclerostin reduced osteocalcin expression in control, but not Lrp4 mutant, BMSCs (Fig. 5D). Together, these results indicate the requirement of Lrp4 for sclerostin inhibition of OB differentiation/function. Notice that Lrp4 is also necessary for Wnt inhibition of OB differentiation, but not for the regulation by DKK1 (Fig. 5 B and C), indicating the specificity of the effect. Moreover, the inhibitory effect of sclerostin and Wise was attenuated by Lrp4-extracellular domain (Lrp4–ECD), the polypeptide containing the entire Lrp4 extracellular domain that is able to interact with sclerostin, Wise, and agrin (7, 8, 11, 18, 22). This suggested that sclerostin/Wise inhibition is mediated by direct binding to the extracellular region of Lrp4, supporting Lrp4 as a receptor of sclerostin.

Necessity of Lrp4 in OB-Lineage Cells for Sclerostin to Suppress Wnt/β-Catenin Signaling. Sclerostin is believed to be an antagonist of Wnt/β-catenin signaling to inhibit OB differentiation/function (1). We asked whether Lrp4 in BMSCs is required for sclerostin to inhibit Wnt/β-catenin signaling. The level of β-catenin, a key indicator of Wnt canonical signaling, was examined in control and Lrp4-deficient BMSCs treated with or without sclerostin. A marked increase in β-catenin was detected in Lrp4-deficient BMSCs, compared with the WT controls (Fig. 6 A and B). In response to sclerostin, β-catenin was decreased in control, but not Lrp4 mutant, BMSCs (Fig. 6 A and B). Notice that transcripts of SOST (sclerostin) and Wnt5a were unchanged, and those of DKK1 (an inhibitor of Wnt signaling) were increased in Lrp4 mutant BMSCs (Fig. S7 A and B), suggesting that the β-catenin alteration is not due to the change in Wnt5a activation or DKK1 inactivation. These results thus support the view for Lrp4 as a receptor of sclerostin to negatively regulate Wnt/β-catenin signaling.

Requirement of Lrp4 in OB-Lineage Cells for Sclerostin to Induce RANKL Expression. To understand how Lrp4 in OB-lineage cells regulates OC genesis and activation, we determined whether Lrp4 is necessary for expressing proteins crucial for OC genesis. They included macrophage colony-stimulating factor (M-CSF), which is critical for OC precursor cell proliferation and survival; RANKL, which is essential for OC genesis and activation; and OPG, an antagonist of RANKL (25, 26). M-CSF mRNA in Lrp4-deficient BMSCs was comparable to that of control cells (Fig. S7C). In contrast, RANKL was markedly reduced, but OPG was slightly increased, in mutant cells (Fig. S7C). This resulted in a significant decrease in the ratio of RANKL over OPG (Fig. S7D). Intriguingly, sclerostin increased RANKL, but reduced OPG, in control BMSCs, resulting in an elevation of the ratio of RANKL over OPG (Fig. 6 C–E). This sclerostin’s effect was also abolished in Lrp4-deficient BMSCs (Fig. 6 C–E), demonstrating the necessity of Lrp4 for sclerostin to induce RANKL/OPG expression.

Elevated Serum Sclerostin in Both mr-Lrp4mut and Lrp4Ocn-cko, but Not Lrp4Δsmc-cko, Mice. The requirement of Lrp4 for sclerostin to suppress β-catenin signaling and OB differentiation and to induce RANKL/OPG expression supports the view for Lrp4 in OB-lineage cells as a receptor of sclerostin. Sclerostin is a soluble protein in the blood/serum as well as in bone matrix (1). To determine whether Lrp4 regulates its distribution, we measured serum sclerostin by ELISA analysis. Serum sclerostin levels were markedly elevated in both mr-Lrp4mut and Lrp4Ocn-cko mice, compared with control littermates (Fig. 6 F and G). The increase was detected at neonatal age of Lrp4 mutant mice (Fig. 6F), and not due to the increased expression, because SOST transcripts in Lrp4 mutant BMSCs were comparable to those of controls (Fig. S7A). Sclerostin levels in CMs of Lrp4 mutant BMSCs were similar to those of controls (Fig. 6H), excluding altered secretion or uptake of sclerostin in Lrp4-deficient BMSCs. Finally, serum sclerostin levels in Lrp4Δsmc-cko mice were similar to those of controls, indicating the specific function of Lrp4 in OB-lineage cells. Together, these results suggest that Lrp4 in OB-lineage cells is critical to control serum sclerostin levels, probably by interacting and sequestering sclerostin into the bone matrix.

Discussion

We showed that Lrp4 loss in OB-lineage cells increased cortical and trabecular bone mass. The deficits were observed in Lrp4 null and OB-selective Lrp4 knockout mice, but not in OC-specific mutant mice. The increased bone mass in Lrp4 mutant mice was associated with elevated bone formation and impaired OC genesis and bone resorption. Loss of Lrp4 in OB-lineage cells abolished sclerostin inhibition of Wnt/β-catenin signaling and OB differentiation and sclerostin induction of the ratio of RANKL over OPG (a key factor for osteoclastogenesis). These results thus identify Lrp4 as a key player in bone-mass homeostasis and support a working model depicted in Fig. 6I. In this model, Lrp4 in OB-lineage cells suppresses bone formation and promotes bone resorption probably by sequestering sclerostin into the bone matrix and serving as a sclerostin receptor to negatively regulate Wnt/β-catenin signaling and promote RANKL-induced osteoclastogenesis.

The high–bone-mass phenotype in mr-Lrp4mut and Lrp4Ocn-cko, cko mice was remarkably similar to that of SOST mutant mice and/or animals treated with anti-sclerostin antibodies, supporting the view for Lrp4 as a receptor of sclerostin in vivo. The high–bone-mass phenotypes in Lrp4 mutant mice also resemble
clinical features of sclerosteosis and van Buchem disease (10, 14–16). Interestingly, mutations in both Lrp4 and SOST genes have been identified in patients with sclerosteosis and van Buchem diseases (10, 14–16). SOST’s gene product, sclerostin, binds to Lrp4 (10, 11). Lrp4 loss in OB-lineage cells impaired sclerostin inhibition of Wnt/β-catenin signaling and OB differentiation (Figs. 5 and 6) and increased serum levels of sclerostin (Fig. 6). These observations suggest that osteoblastic Lrp4 serves as a receptor for sclerostin, and impaired sclerostin–Lrp4 pathway in OB-lineage cells might be a pathophysiological mechanism for sclerosteosis and van Buchem diseases.

Note that in addition to high-bone-mass deficit, young adult mr-Lrp4mitt mice display additional phenotypes that were not observed in Lrp4Ocn-cko or Lrp4Lyse-cko mice. For example, the digit development was impaired in mr-Lrp4mitt, but not Lrp4Ocn-cko or Lrp1LysM-cko, mice (Figs. S1 and S2), suggesting that Lrp4 loss in other cell types (e.g., chondrocytes) may contribute to this deficit. Interestingly, the digit deficit resembles the hand defect for sclerosteosis patients as well as for patients with Cenani-Lenz syndrome (27–29). Lrp4 mutations are also identified in patients with Cenani-Lenz syndrome, in addition to bone-related diseases (27–29). A similar digit defect is also reported in SOST−/− mice (30). These observations thus suggest that the sclerostin-Lrp4 pathway plays an important role not only in adult bone-mass homeostasis, but also in limb development.

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**Fig. 5.** Impairment of sclerostin and Wise suppression of OB differentiation in Lrp4-deficient BMSCs. (A) Schematic of Lrp4 structure and its potential ligands. (B and C) Sclerostin and Wise, but not DKK1, reduced ALP activity in an Lrp4-dependent manner. The control, sclerostin, DKK1, and agrin condition were mixed with the OB differentiation medium (DM) at a ratio of 1:1 and incubated with control and Lrp4 mutant BMSCs to induce OB differentiation for 1 wk. The Wise recombinant protein (100 ng/mL) was added. OB-like cells were subjected to ALP-staining analysis. Representative images from three different cultures are presented in C. Data were normalized by WT controls. *P < 0.05. (D) Sclerostin reduced osteocalcin expression in an Lrp4-dependent manner. BMSCs were treated with control CM or sclerostin CM or Lrp4 CM for 24 h. The mRNAs were subjected to qRT-PCR analyses. The values were normalized to β-actin and WT controls. Means ± SD from three different experiments were presented. Data were normalized to their controls. Note that the control values of serum sclerostin in HSA-Lrp4 mice (1 mo old), Lrp4LysM (1 mo old), and LysMCre (3 mo old) were 750, 700, and 350 pg/mL, respectively. *P < 0.05, significant difference. (E-G) Increase of sclerostin/Wise in Lrp4−/− OB-lineage cells. BMSCs were treated with control CM or sclerostin CM for 24 h. The mRNAs were subjected to real-time PCR analyses. The values were normalized to β-actin and WT controls. Means ± SD from three different experiments are presented. **P < 0.01, significant difference. (H) A comparable level of CM-sclerostin between control WT and Lrp4 mutant BMSCs. In F–H, the serum and CM-sclerostin were measured by ELISA analysis (SI Materials and Methods). Means ± SD from three different animals per genotype or three different cultures are presented. Data were normalized to their controls.

**Fig. 6.** Impairment of sclerostin reduction of β-catenin and induction of RANKL/OPG in Lrp4-deficient BMSCs and increase of serum sclerostin in both mr-Lrp4mitt and Lrp4Ocn-cko, but not Lrp4Lyse-cko, mice. (A and B) Increase of β-catenin level and impairment of sclerostin reduction of β-catenin in Lrp4-deficient BMSCs. BMSCs were treated with control CM or sclerostin CM for 12 h. Cell lysates were subjected to Western blot analyses using indicated antibodies. Quantification analysis by ImageJ software is shown in B. The values of mean ± SD from three different experiments were presented. Data were normalized by β-actin loading and WT controls. *P < 0.05, significant difference. (C–E) Failure of sclerostin induction of RANKL/OPG in Lrp4Ocn-deficient BMSCs. BMSCs were treated with control CM or sclerostin CM for 24 h. The mRNAs were subjected to real-time PCR analyses. The values were normalized to β-actin and WT controls. Means ± SD from three different experiments are presented. **P < 0.01, significant difference. (F and G) Increased serum levels of sclerostin in both mr-Lrp4mitt and Lrp4Ocn-cko, but not Lrp4Lyse-cko, mice. (H) A comparable level of CM-sclerostin between control WT and Lrp4 mutant BMSCs. In F–H, the serum and CM-sclerostin were measured by ELISA analysis (SI Materials and Methods). Means ± SD from three different animals per genotype or three different cultures are presented. Data were normalized to their controls. Note that the control values of serum sclerostin in HSA–Lrp4 mice (1 mo old), Lrp4LysM (1 mo old), and LysMCre (3 mo old) were 750, 700, and 350 pg/mL, respectively. ***P < 0.01, significant difference. (I) Illustration of a working model for Lrp4 in OB-lineage cells to serve as a receptor of sclerostin, sequestering sclerostin into bone matrix, suppressing Wnt/β-catenin signaling and bone formation, and promoting RANKL-induced OC genesis and bone resorption, thus maintaining bone-mass homeostasis.

How does Lrp4 in OB-lineage cells regulate bone-mass homeostasis? High bone mass is temporally associated with increased bone formation and decreased bone resorption in both mr-Lrp4mitt and Lrp4Ocn-cko mutant mice, suggesting that Lrp4 in OB-lineage cells acts as an inhibitor of osteoblastic function, but a stimulator of osteoclastic activation, thus promoting homeostasis of bone mass. This view is supported by in vitro BMSC-OB culture studies. Lrp4 in OB-lineage cells is required...
Sclerostin inhibition of OB differentiation and function (Fig. 5), as well as sclerostin induction of RANKL/OPG (Fig. 6). Thus, the increased bone formation in Lrp4 mutant mice may result from loss of sclerostin’s inhibitory effect.

How does Lrp4 in OB-lineage cells promote osteoclastic function in a cell nonautonomous manner? Several mechanisms may underlie this event. First, the reduced RANKL/OPG (Fig. 6) may impair OC genesis and bone resorption in mR-Lrp4 and Lrp4Δcko mice because the ratio of RANKL over matrix and change sclerostin conformation to expose its central domain. Sclerostin may result in local enrichment of sclerostin in bone matrix and promote OC genesis by inhibiting Wnt/β-catenin signaling (Fig. 6).

It remains unclear exactly how Lrp4 mediates sclerostin inhibition of Wnt/β-catenin signaling and bone formation. Sclerostin, Wise, and DKK1 are soluble factors that inhibit Wnt signaling by binding LRP5/6 (12, 13, 31). Why do sclerostin, Wise, but not DKK1, inhibit OB in an Lrp4-dependent manner? It is noteworthy that the central core region of sclerostin is critical for binding to the first β-propeller domain of LRP5/6, not for binding to Lrp4 (32). The interaction of sclerostin with Lrp4 is impaired by two sclerosteosis-associated mutations, R1170W and W1186S (10), suggesting that the third β-propeller domain of Lrp4 may interact with sclerostin. Moreover, serum levels of sclerostin are markedly increased in both mR-Lrp4 and Lrp4Δcko mice (Fig. 6, F and E). In light of these observations, we speculate that Lrp4 interaction with sclerostin may result in local enrichment of sclerostin in bone matrix and change sclerostin conformation to expose its central core binding site for LRP5/6 (Fig. 6). However, future work will be needed to investigate which domain or motif in Lrp4 interacts with sclerostin/Wise and how the interaction is regulated. Such knowledge may help to explain the complexity of Lrp4 behavior in maintaining bone-mass homeostasis.

Materials and Methods

Animals. All experimental procedures were approved by the Animal Subjects Committee at the Georgia Health Sciences University, according to US National Institutes of Health guidelines. Generation of Lrp4DΔm and Lrp4-flox mice were described previously (9, 18). Additional information is provided in SI Materials and Methods.

Measurements of Serum Levels of Osteocalcin, PYD, and Sclerostin. Mouse serum samples were collected and subjected to ELISA analysis. Additional information is provided in SI Materials and Methods.

RNA Isolation and Real-Time PCR. Total RNA was isolated from BMSCs by TRIzol extraction (Invitrogen). Additional information is provided in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Animals. Lrp4<sup>f/f</sup> mice, which were generated by using N-ethyl-N-nitrosourea (ENU) (1), were obtained from L. A. Niswander (Howard Hughes Medical Institute, University of Colorado). They were crossed with human skeletal α-actin (HSA) promoter-driven Cre (HSA–Cre) transgenic mice to generate muscle-rescued Lrp4<sup>null</sup> mice (2). Lrp4<sup>flox</sup> mice, which were generated by using Cre-recombinase enzyme were back-crossed into C57BL/6J mice. Mice were housed in a room with a 12 h light/dark cycle with ad libitum access to water and standard rodent chow diet (Harlan Teklad S-2335). Control littermates were processed in parallel for each experiment.

Microcomputed Tomography. The microcomputed tomography (μCT) imaging analysis was carried out as described previously (3, 4). In brief, microarchitectural of the distal trabecular bone and midshaft cortical bone of the femur were measured by Skyscan μCT 40 (Skyscan Medical AG, Brüttisellen, Switzerland). Bones were placed vertically in 12-mm-diameter scanning holders and scanned at the following settings: 12-μm resolution, 55-kVp energy, 145-μA intensity, and an integration time of 200 ms.

Bone Histomorphometric Analysis. Bone histomorphometric analyses were carried out as previously described (3, 4). Briefly, mouse tibia and femurs were fixed in 4% paraformaldehyde solution, decalcified in 10% (vol/vol) EDTA, embedded in paraffin, sectioned, and subjected for H & E, Goldner Trichrome stain, and TRAP-staining analyses, which were counterstained by fast green. Bone histomorphometric perimeters were determined by measuring the areas situated at least 0.5 mm from the growth plate, excluding the primary spongiosa and trabeculae connected to the cortical bone.

Measurements of Serum Levels of Osteocalcin, PYD, And Sclerostin. Mouse serum samples were collected and subjected to ELISA analysis of osteocalcin and sclerostin and RIA analysis of PYD as described previously (3, 4). Mouse osteocalcin Eliisa kit (Biomedical Technologies, Inc.), METRA Serum PYD RIA kit (QUIDEL Corporation), and mouse/rat SOST (sclerostin) Eliisa kit (R&D Systems) were used. All of the samples were measured in duplicate, and values were subjected to statistical analysis.

For mouse serum levels of osteocalcin test, it was measured at a 1:5 dilutions of collected serum samples. Serum samples (25-μL diluted samples) were incubated at 4 °C overnight with 100 μL of biotin–mouse osteocalcin antibody. After three washes, 100 μL of HRP–Strep A were added and incubated in the dark at room temperature for 30 min. One hundred microliters of tetramethylbenzidine– Perox were then added after three washes to incubate for 15 min at room temperature. The reaction was stopped, and the OD was measured at 450 nm within 30 min of stopping reaction and then converted to osteocalcin concentrations using a standard curve.

For measurement of PYD, serum samples (25 μL) were incubated with PYD antibody solution (75 μL) overnight at 4 °C in the dark. After three washes, samples were incubated with enzyme conjugates for 60 min at room temperature and followed by three washes and incubation with substrate solution (150 μL) at room temperature for 40 min. The reaction was stopped, and the OD measured at 405 nm was converted to PYD concentrations using a standard curve.

For mouse serum levels of sclerostin, serum samples were measured at a twofold dilution and incubated for 3 h at room temperature with mouse/rat sclerostin antibody. After five washes, 100 μL conjugate were added and incubated for 1 h at room temperature and followed by five washes and incubation with 100 μL substrate solution for 30 min at room temperature in the dark. The reaction was stopped, and the OD was measured at 450 nm. Five hundred forty nanometers was used for wavelength correction and then converted to sclerostin concentrations using a standard curve.

Dynamic Bone Histomorphometry to Measure the Rate of Bone Formation In Vivo. Briefly, mice (2 wk old) were injected (intraperitoneally) with fluorochrome-labeled calcein green (10 mg/kg, Sigma–Aldrich) and then with Alizarin Red S (50 mg/kg, Sigma–Aldrich) (12 d interval). The mice were killed 2 d after the second injection. The left tibia and femurs were fixed in 70% (vol/vol) ethanol overnight, embedded in methyl methacrylate, and sectioned at 7–10 μm. Images were obtained using a 25× objective fluorescence microscope (laser scanning microscope 510; Carl Zeiss). The mineral apposition rate (MAR) in μm/d and bone formation rate (BFR) [BFR = MAR × MS (mineral surface)] were calculated from fluorochrome double-labeled at the periosteal and endocortical surfaces.

Plasmids and Transient Transfection. The SOST cDNA was cloned into pcDNA3.1 plasmid. Both DKK1 and agrin expression plasmids were generated by insert DKK1/agrin into pFlag–CMV1 downstream of a signal peptide. The Flag–Lrp4 and Flag–ecto-Lrp4 (Lrp4–ECD) plasmids were generated by insertions of the full-length Lrp4 and ecto-Lrp4 cDNA into the pFlag–CMV1 vector, which has an artificial signal peptide and a Flag epitope. The authenticity of all constructs was verified by DNA sequencing.

HEK293 cells were maintained in Dulbecco-modified Eagle medium supplemented with 10% FCS and 100 units/mL of penicillin G and streptomycin (Gibco). Calcium phosphate method was used for transfection of HEK293 cells. Forty-eight hours following transfection, cells were lysed in modified RIPA immunoprecipitation assay buffer (50 mM Tris·HCl, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.25% sodium deoxycholate, proteinase inhibitors). Lysates and medium were subjected to immunoblotting analyses.

In Vitro OB Culture and Treatments with Lrp4 Ligands: Sclerostin, DKK1, Agrin, Wise, and Lrp4–ECD. Whole bone marrow cells were isolated from long bones of 8-wk-old WT and Lrp4 mutant mice and plated on 100-mm tissue culture plates in DMEM containing 10% (vol/vol) FBS, 1% penicillin/streptomycin (PS)/. After 7 d, passing cells by trypsin digestion, 1 × 10<sup>5</sup/cm<sup>2</sup> were plated for osteogenesis differentiation in the presence of osteogenic medium [DMEM containing 10% (vol/vol) FBS, 1% PS/S, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid–2-phosphate]. Medium contained control (pcDNA3.1), sclerostin, Flag–DKK1, Flag–agrin, or mixed Lrp4–ECD was added to the differentiation medium (DM) (at a ratio of 1:1) at every medium change. Recombinant Wise protein (Abcam) was added to the DM at a
concentration of 100 ng/mL 7 d after incubation, ALP-staining (Sigma) and quantification analyses were performed.

RNA Isolation and Real-Time PCR. Total RNA was isolated from BMSCs by TRIzol extraction (Invitrogen). Quantitative PCR was performed with a Quantitect SYBR Green PCR Kit (Bio-Rad), according to the manufacturer’s instructions, and with a Real-Time PCR System with analytical software (Opticon Monitor 3). The following primers were used: M-CSF, 5′-TTGGCTTGGGATGATTCTCAG-3′ and 5′-GCCCTGGGTCTGTCAGTCTC-3′; RANKL, 5′-ATCCCATCGGGTTCCCATAA-3′ and 5′-TCCGT TGCTTAACGTCATGTTAG-3′; OP G, 5′-GGCCTGACGTGCTTCCAAC-3′ and 5′-CATGGATGCACAGATTCC-3′; Wnt5a, 5′-CAACTGCGAAGCCTTTCTCAA-3′ and 5′-CATCTCGATGCACCACT-3′; SOST, 5′-AGCCTCAGAATGGATGCCAC-3′ and 5′-CTTTGCGCTCATAGGGATGT-3′; and DKK1, 5′-CTCATCAATTCCAACCGATACA-3′ and 5′-GCCCTCATAGAAGCTCCCG-3′. β-actin primers (5′-AGGTATCATCTATGGCAACGA-3′ and 5′-CATGGATGCACAGATTCC-3′) were used for normalization.

Statistical Analysis. All data were expressed as mean ± SD. The significance level was set at P < 0.05. Student t test was used for statistical analysis.


Fig. S1. Generation of muscle-rescued Lrp4-null (mr-Lrp4mits) mice. (A) Illustration of the LRPA protein structure and the stop codon in Lrp4mits mice. (B) Western blot analysis of Lrp4 (flag tagged) transgene expression in various tissue homogenates from HSA–Lrp4 mice. (C and D) Western blot analyses of Lrp4 expression in indicated tissue/cell lysates from control and mr-Lrp4mits mice. (E) Images of control and mr-Lrp4mits mice at the age of 3 mo old. (F) Digit defect and pale long bone in 3-mo-old mr-Lrp4mits mice.

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Fig. S2. Generation of OB- and OC-selective Lrp4 knockout mice. (A and C) Illustrations of breeding strategies for generation of Lrp4\textsuperscript{Ocn-cko} (A) and Lrp4\textsuperscript{LysM-cko} (C) mice. (B and D) Images, digits, and body weights of control vs. Lrp4\textsuperscript{Ocn-cko} (B) or vs. Lrp4\textsuperscript{LysM-cko} (D) mutant mice.

Fig. S3. Reduced trabecular, but not cortical, bone volumes in young adult Lrp4\textsuperscript{LysM-cko} mice. (A and B) Western blot analysis of LRP4 expression in BMSCs, OBs, BMMs, and OCs derived from 2-mo-old Lrp4\textsuperscript{f/f} (control) and Lrp4\textsuperscript{LysM-cko} mice. Representative blots are shown in A. Quantification analysis (mean ± SEM, n = 3) is presented in B. (C and D) The μCT analysis of femurs from ∼3-mo-old control (Lrp4\textsuperscript{f/f}) and Lrp4\textsuperscript{LysM-cko} littermates. Three different male mice of each genotype were examined blindly. Representative images are shown in C. Quantification analyses (mean ± SD, n = 3) are presented in D. *P < 0.05, significant difference from the control littermates.
Fig. S4. Increased serum PYD and TRAP⁺ OCs in Lrp4<sup>LysM</sup>-cko mice. (A) Measurements of serum levels of PYD in ~3-mo-old control and Lrp4<sup>LysM</sup>-cko mice by RIA analyses. The values of mean ± SD from three different males per genotype are shown. *P < 0.05, significant difference from the control. (B and C) TRAP-staining analysis of femur sections from ~3-mo-old control and Lrp4<sup>LysM</sup>-cko littermates (three mice per age group per genotype, both males and females). Representative images are shown in B. (Scale bar, 100 μm.) The quantitative analysis of TRAP⁺ cells per unit bone surface (BS) in trabecular bones is presented as values of mean ± SD from three different animals in C. *P < 0.05, significant difference from the control. (D–F) TRAP-staining analysis of cultured OCs derived from BMMs of 1.5-mo-old control and Lrp4<sup>LysM</sup>-cko mice. Representative images of the cultures treated with RANKL for 4 d (day 4) are shown in D. (Scale bar, 150 μm.) The quantitative analyses of TRAP⁺ multinuclei cells (MNCs) over total cells at days 4 and 7 of RANKL treatments are presented in E and F, respectively. The values of mean ± SD from three different cultures are shown. *P < 0.05, significant difference from the control.
Fig. S5. Normal ALP activity, but increased osteocalcin expression, in Lrp4-deficient BMSCs. (A–D) Normal in vitro OB differentiation of BMSCs derived from control and indicated Lrp4 mutant mice (2 mo old). Representative images of ALP-staining analysis of OBs (day 14 OB culture of BMSCs isolated from 2-mo-old control and indicated Lrp4 mutant femur bone marrows) are shown in A and C. Quantitative analyses of the average ALP activities (mean ± SD from three different cultures) are presented in B and D. (Scale bars, 80 μm.) (E and F) Increased expression of osteocalcin (F), but not collagen X (E), in mr-Lrp4<sup>mitt</sup> BMSC culture by real-time PCR analysis. The values of mean ± SD from three different cultures are presented and normalized by their controls. *P < 0.05, significant difference.

Fig. S6. Expression of sclerostin, DKK1, agrin, and Lrp4–ECD. HEK293 cells were transfected with indicated plasmids. The lysates and condition medium were subjected to Western blot analyses using indicated antibodies. Representative blots are shown in A–D.

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Fig. S7. Increased DKK1 and OPG, but decreased RANKL, in Lrp4-deficient BMSCs. (A and C) Real-time PCR analysis of the mRNA levels of Wnt5a, DKK1, M-CSF, RANKL, and OPG. The values are normalized to β-actin and controls (HSA–Lrp4 as control mice). Ratios of Wnt5a/DKK1 and RANKL/OPG are shown in B and D, respectively. Means ± SD values from three different experiments are presented. *P < 0.05, significant difference.