Osteoblastic Lrp4 promotes osteoclastogenesis by regulating ATP release and adenosine-A<sub>2A</sub>R signaling

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Bone homeostasis depends on the functional balance of osteoblasts (OBs) and osteoclasts (OCs). Lrp4 is a transmembrane protein that is mutated in patients with high bone mass. Loss of Lrp4 in OB-lineage cells increases bone mass by elevating bone formation by OBs and reducing bone resorption by OCs. However, it is unclear how Lrp4 deficiency in OBs impairs osteoclastogenesis. Here, we provide evidence that loss of Lrp4 in the OB lineage stabilizes the prorenin receptor (PRR) and increases PRR/V-ATPase–driven ATP release, thereby enhancing the production of the ATP derivative adenosine. Both pharmacological and genetic inhibition of adenosine-A<sub>2A</sub>R receptor (A<sub>2A</sub>R) in culture and Lrp4 mutant mice diminishes the osteoclastogenic deficit and reduces trabecular bone mass. Furthermore, elevated adenosine-A<sub>2A</sub>R signaling reduces receptor activator of nuclear factor κB (RANK)–mediated osteoclastogenesis. Collectively, these results identify a mechanism by which osteoblastic Lrp4 controls osteoclastogenesis, reveal a cross talk between A<sub>2A</sub>R and RANK signaling in osteoclastogenesis, and uncover an unrecognized pathophysiological mechanism of high-bone-mass disorders.

Introduction

Bone homeostasis depends on the functional balance of osteoblasts (OBs) and osteoclasts (OCs). Each releases secretable factors that regulate the function of the other. For example, OB-released factors include receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG), cytokine interleukin-13, and EphB4 (Yasuda et al., 1998; Teitelbaum, 2000; Zhao et al., 2006; Zaiss et al., 2011), among which RANKL is the best studied and perhaps most important (Yasuda et al., 1998; Teitelbaum, 2000). RANKL is necessary for trabecular bone structures, because RANKL deletion in osteocytes reduces OC number on cancellous bone and increases bone volume (BV; Nakashima et al., 2011; Xiong et al., 2011). Via its receptor, receptor activator of nuclear factor κB (RANK), RANKL increases nuclear factor κB signaling and promotes the commitment of bone marrow macrophages/monocytes (BMMs) to OC progenitors, OC differentiation, and activation (Yasuda et al., 1998; Teitelbaum, 2000). In contrast, OPG acts as an antagonist of RANKL to inhibit OC genesis and function (Teitelbaum, 2000). The ratio of RANKL to OPG is thus a key factor in the control of OC genesis. This ratio is up-regulated by multiple factors, including sclerostin Lrp4 (low-density lipoprotein [LDL] receptor–related protein 4) pathway (Itoh et al., 2000; Ma et al., 2001; Huang et al., 2004; Silvestrini et al., 2008; Wijenayaka et al., 2011; Xiong et al., 2015) and down-regulated by factors such as the canonical Wnt–β-catenin signaling pathway (Huang et al., 2004; Fujita and Janz, 2007; Silvestrini et al., 2008; Takahashi et al., 2011; Folestad et al., 2015).

Lrp4 is a member of LDL family receptors containing a large extracellular region with multiple LDLa, EGF-like, and β-propeller repeats; a transmembrane domain; and a short C-terminal region (Nakayama et al., 1998; Tian et al., 1999; Herz and Bock, 2002; Suzuki, 2004; Wu et al., 2010; Shen et al., 2015). It is a receptor for agrin to mediate neuromuscular junction formation and stability (Kim et al., 2008; Zhang et al., 2008; Wu et al., 2012; Zong et al., 2012; Shen et al., 2014). It is also a receptor for sclerostin to negatively regulate bone homeostasis (Li et al., 2005; Semënov et al., 2005; Choi et al., 2009; Leupin et al., 2011; Xiong et al., 2015). Mutations in Lrp4 and sclerostin genes have been identified in patients with high bone mass, such as sclerosteosis and Van Buchem disease (Balemans et al., 2002; Loots et al., 2005; Semënov et al., 2005; Leupin et al., 2011). Deletion of Lrp4 or sclerostin gene in mice results in high-bone-
Results

Reduced OC differentiation of BMMs from osteoblastic Lrp4 mutant mice

To explore mechanisms of how osteoblastic Lrp4 knockout results in an OC genesis deficit, we first tested whether this deficit could be rescued by treatment with RANKL, a crucial factor necessary for OC genesis that is reduced in Lrp4-deficient OB-lineage cells (Xiong et al., 2015). BMMs were treated with exogenous RANKL to induce OC differentiation (Fig. 1 A). BMMs from control mice formed OC-like, tartrate-resistant acid phosphatase (TRAP)+ multinuclei cells (MNCs) at day 7 of treatment (Fig. 1 B). However, the number of TRAP+ MNCs was much lower in BMMs from hr-Lrp4mut (muscle-rescued Lrp4 null) mutant mice, even after treatment with 100 ng/ml RANKL, a concentration four times the sufficient dose of 25 ng/ml, for 7 d (Fig. 1, B and C), suggesting an OC differentiation deficit. Such a deficit was also observed in BMMs from OB-selective conditional knockout (Lrp4Ocn-cko) mice (or Lrp4f/f; osteocalcin [Ocn]–Cre; Fig. 1, D–G). The OC differentiation deficit was detected in BMMs from 3-mo-old mice (Fig. 1, H and I), indicating that they belong to macrophage/megakaryocyte cells. However, the percentage of RANK+ cells was caused by higher levels of ATP.

We next measured PPI, because it is known to inhibit OC differentiation (Nishikawa et al., 1996; Baron et al., 2011; Burr and Russell, 2011; Russell, 2011). Intriguingly, levels of PPI were much higher in CMs of Lrp4 mutant BMSCs and OBs than in those of controls (Fig. 2 C). PPI is derived from ATP metabolism (Russell, 2011), and both ATP and ATP’s hydrolytic product adenosine are implicated in the regulation of OC genesis (Mediero and Cronstein, 2013; Idzko et al., 2013; Chang et al., 2014; Xiong et al., 2015; Fijalkowski et al., 2016). As shown in Fig. 2 (A and B), the levels of both SOST and Wise were comparable in CMs of two genotypes of BMSCs, eliminating the possibility that SOST and Wise are involved in OC differentiation inhibition.

Increased ATP, PPI, and adenosine in extracellular compartments of Lrp4-deficient OB-lineage cells

To identify the secretable factors that inhibit OC genesis, we first examined levels of SOST and Wise in CMs of WT and Lrp4-deficient BMSCs, because both SOST and Wise are believed to be Lrp4 ligands important for bone homeostasis and their serum levels are increased in Lrp4 mutant mice (Ahn et al., 2013; Chang et al., 2014; Xiong et al., 2015; Fijalkowski et al., 2016). As shown in Fig. 2 (A and B), the levels of both SOST and Wise were comparable in CMs of two genotypes of BMSCs, eliminating the possibility that SOST and Wise are involved in OC differentiation inhibition.

Next, we performed FACS analyses to determine whether the number of OC progenitor cells in BMMs from OB-Lrp4 mutant mice was reduced. BMMs isolated from 3-mo-old mice were sorted with antibodies against CD11b (a macrophage/megakaryocyte marker) and RANK (a receptor for RANKL, expressed in OC progenitor cells; Fig. 1 A). Approximately 99% of BMMs of all genotypes were positive for CD11b (CD11b+; Fig. 1, H and I), indicating that they belong to macrophage/megakaryocyte cells. However, the percentage of RANK+ cells was significantly lower in BMMs from OB-Lrp4 mutant mice than in those of control mice (Fig. 1, H and J), suggesting a problem with OC progenitor cell production. In accord, the RANK protein level was markedly lower in BMMs from Lrp4 mutant mice than in control mice (Fig. 1, K and L). The level of c-Fms, a receptor for macrophage colony-stimulating factor (M-CSF), was similar between control and mutant BMMs, indicating normal development of macrophages. These results suggest that OB-Lrp4 may promote the commitment of BMMs to RANK+ OC precursor cells in the bone marrow.

We then determined whether the conditioned medium (CM) of Lrp4-deficient OB-lineage cells contains an inhibitor of OC differentiation. We collected CMs from wild-type (WT) and OB-Lrp4 mutant bone marrow stromal cells (BMSCs); WT BMSCs were treated with the different CMs for 7 d in the presence of M-CSF and RANKL and subjected to TRAP staining for OC formation (Fig. 1 M). As shown in Fig. 1 (N–O), the number of TRAP+ MNCs was lower in BMMs treated with Lrp4mut-BMSC CM that in those treated with WT-BMSC CM. These results indicate that OB-Lrp4 mutant BMSCs regulate OC genesis via secretable factors (Fig. 1 P).

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platform to measure oxygen consumption rates (OCRs) and mitochondrial respiratory capacity in real time, we detected higher rates of both basal oxygen consumption and maximal respiratory capacity, which were induced by the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) in Lrp4-deficient OBs than in those of controls (Fig. 2, G and H). No difference in oxygen consumption was observed after treatment of cells with oligomycin, an inhibitor of ATP synthase (Fig. 2, G and H). These results thus indicate hyperfunctional mitochondria in Lrp4-deficient OBs, in line with the previous studies of hyper-OB function in Lrp4 mutant mice (Chang et al., 2014; Xiong et al., 2015).

Collectively, these observations support the notion that Lrp4 deficiency in OB-lineage cells increases both ATP synthesis and secretion, which then increases levels of PPi and adenosine (Fig. 2 I).

Figure 1. Reduced OC differentiation in BMMs from osteoblastic Lrp4 mutant mice or BMMs treated with CM of Lrp4-deficient OB-lineage cells. (A–G) TRAP staining analysis of cultured OCs derived from BMMs of different genotypes. (A) Experimental strategy. Cells were treated with 100 ng/ml RAN KL for 7 or 14 d as indicated. Representative images are shown in B, D, and F, and quantitative data of TRAP+ multinuclei cells (MNCs; more than three nuclei) per randomly selected visual field are shown in C, E, and G. Bars, 100 µm. Data represent mean ± SD from three different cultures; *, P < 0.05. (H) FACS analysis of primary BMMs. Bone marrow was isolated from the indicated mice and incubated overnight. BMMs were isolated from nonadherent cells by Ficoll-Hypaque gradient centrifugation and analyzed by FACS with antibodies against CD11b and RANK. (I and J) Quantitative data of FACS analysis. Data represent mean ± SD, n = 3; *, P < 0.05. (K and L) Western blot analysis of RANK and c-Fms expression in BMMs from indicated mice. Data represent mean ± SD, n = 3; *, P < 0.05. (M–O) TRAP staining of WT BMMs in the presence of CM of WT or Lrp4−/− BMSCs. (P) Experimental strategy. (N) Representative images. Bar, 100 µm. (O) Quantitative data of TRAP+ MNCs in N. Data represent mean ± SD, n = 3; *, P < 0.05. (P) Illustration of a working model. Lrp4-deficient OB-lineage cells release secretable factors to reduce OC differentiation.
Inhibition of adenosine-A2AR signaling diminishes the OC differentiation deficit

PPi is known to inhibit not only OC genesis but also bone mineralization (Nishikawa et al., 1996; Russell, 2011; Or-riss et al., 2013). However, previous studies, including ours, have demonstrated an increased bone mineralization in Lrp4 mutant mice (Leupin et al., 2011; Chang et al., 2014; Xiong et al., 2015). Serum levels of PPi were comparable between Lrp4 mutant mice and control mice (Fig. S1 D). These results suggest that PPi may play little to no role in Lrp4-deficient mice, although it was increased in cultured Lrp4-deficient OB-lineage cells.

Activation of adenosine receptor A2AR has been shown to inhibit OC genesis (Mediero and Cronstein, 2013). We thus determined whether adenosine-A2AR signaling is involved in the OC genesis deficit by Lrp4 mutation. WT BMMs were treated with CMs of control and Lrp4 mutant BMSCs in the presence of SCH58261, an A2AR antagonist (Fig. 3 A). As shown in Fig. 3
(B and C), in vitro OC genesis (TRAP+ MNC formation) was impaired when treated with CM of Lrp4-deficient BMSCs; this effect was diminished by SCH58261. In contrast, A740003, an antagonist of the P2X7 receptor (P2X7R), showed little effect on the formation of TRAP+ MNCs (Fig. 3, B and C). These results suggest that inhibition of adenosine-A2AR signaling could diminish the OC genesis deficit.

To test this hypothesis in vivo, Lrp4 Ocn-cko mice at age postnatal day 70 were treated with SCH58261 (i.p., 2 mg/kg, daily) for 5 wk (Fig. 4 A), and their long bone samples were subjected to micro–computed tomography (µCT) analysis 5 wk later. As shown in Fig. 4 (B and C), SCH58261 reduced trabecular bone mass in Lrp4Ocn-cko mice compared with vehicle controls. This effect appeared to be selective, as SCH58261 did not affect cortical BV (Fig. 4, B and C) and had a lesser effect on control (Ocn-Cre) mice (Fig. 4, B and C). Although SCH58261 increased TRAP+ OCs and levels of serum pyridinoline (PYD) and calcium (markers of bone resorption; Fig. 4, D–G), it had no effect on serum Ocn levels (Fig. 4 H). In addition, although SCH58261 fully restored the number of TRAP+ cells in trabecular bone regions of Lrp4Ocn-cko mice (Fig. 4, D and E), its rescue effect on bone resorption (measured by serum levels of PYD and calcium) appeared to be partial (Fig. 4, F and G). These results indicate that inhibiting A2AR improves OC formation and activation in Lrp4 mutant mice. As a control, treatment of Lrp4Ocn-cko mice with A740003 (i.p., 2.75 mg/kg; Fig. S2, A–E) had no effect on bone resorption markers (TRAP+ OC and serum PYD; Fig. S2, F–H), in agreement with in vitro studies (Fig. 3, B and C). However, the treatment decreased trabecular bone mass (Fig. S2, B and C) and levels of serum Ocn (Fig. S2 I), a marker of bone formation. These results indicate that SCH58261 and A740003 have differential effects on OC-mediated bone resorption and OB-mediated bone formation in Lrp4 mutant mice and provide additional evidence for adenosine-A2AR signaling in OC formation and activation in Lrp4Ocn-cko mice.

To further explore A2AR’s function in the OC genesis deficit by Lrp4 mutation, we crossed Lrp4Ocn-cko mice with A2AR-null (A2AR-KO) mice. The resulting Lrp4Ocn-cko;A2AR-KO mice were subjected to µCT analysis and used for an in vitro OC genesis assay. Indeed, µCT analysis showed a reduced trabecular bone mass in Lrp4Ocn-cko mice compared with Lrp4Ocn-cko BMMs (Fig. 5, A and B), in agreement with the results of SCH58261 treatment. A marked reduction of trabecular bone mass was detected in A2AR-KO mice compared with WT controls (Fig. 5, A and B), in line with previous studies (Mediero et al., 2012, 2013). TRAP+ cells and bone resorption (serum PYD) were elevated in Lrp4Ocn-cko;A2AR-KO mice compared with Lrp4Ocn-cko mice (Fig. 5, C–F). In agreement, in vitro OC genesis assays showed that the number of TRAP+ MNCs was higher in Lrp4Ocn-cko;A2AR-KO BMMs than in Lrp4Ocn-cko BMMs (Fig. 6, A and B). These results suggest a negative role of A2AR in OC differentiation in vitro and in vivo. Intriguingly, whereas A2AR-KO had little effect on RANK L/OPG expression in BMMs (Fig. 6, C and D), Western blot and FACS analyses demonstrated that it restored RANK levels in BMMs from Lrp4 Ocn-cko mice (Fig. 6, E–H). These results suggest that A2AR may inhibit OC genesis by suppressing RANK signaling and/or the commitment of BMMs to RANK+ OC precursor cells in the bone marrow. This view was further supported by the finding that treatment of WT BMMs with high concentrations of 150 µM adenosine decreased RANK levels, and this decrease was prevented by SCH58261 (Fig. 6, I and J).

In aggregate, these results suggest that the impairment in OC genesis (including commitment of BMMs to RANK+ OC precursor cells, OC formation, and activation) in Lrp4 mutant mice is likely caused by increased adenosine-A2AR signaling.
Elevated V-ATPase–driven vesicular ATP loading and release in Lrp4-deficient OB-lineage cells

We next investigated the mechanisms by which osteoblastic Lrp4 regulates ATP release. First, we characterized the inhibitory effect of various chemicals on ATP release from Lrp4 mutant BMSCs. Because vesicular ATP release is thought to contribute to extracellular ATP levels in OBs (Orriss et al., 2009, 2013), we initially tested the effect of bafilomycin A1 (BafA1), an inhibitor of V-ATPase. At commonly used...
concentrations (e.g., 100 nM; Bowman et al., 1988; Zhang et al., 1994; Robinson et al., 2004), BafA1 inhibited ATP release from WT BMSCs, which may have complicated the interpretation of results from Lrp4 mutant BMSCs (Fig. 7 A). At 10 nM, however, BafA1 had no effect on ATP release from WT BMSCs (Fig. 7 A); in contrast, it abolished increased ATP release from Lrp4 mutant BMSCs (Fig. 7 A), suggesting an enhanced V-ATPase–dependent ATP release. The proton gradient is important for ATP loading into lysosomes (Coco et al., 2003; Tokunaga et al., 2010). To test the hypothesis further, Lrp4 mutant BMSCs were treated with chloroquine (a lysosomotropic agent that buffers pH in lysosomes; Chen et al., 2011). At a concentration of 5 µM, which had no effect on ATP release from WT BMSCs, chloroquine inhibited ATP release from Lrp4 mutant BMSCs (Fig. 7 B), further supporting the hypothesis. P2X7R has been implicated in the release of ATP release in an autocrine manner (King, 2007; Takai et al., 2014). However, treatment of Lrp4 mutant BMSCs with A740003 had no effect on ATP release (Fig. 7 C). Similar results were also observed in Lrp4 mutant OBs (Fig. S3 A). These results suggest that Lrp4 deficiency in OB-lineage cells may increase V-ATPase–dependent ATP release.

Figure 5. Diminished high-bone-mass phenotype in Lrp4Ocn-cko mice by A2AR knockout. The femurs and sera samples collected from 3-mo-old control (Ocn-Cre), A2AR-KO, Lrp4Ocn-cko, and Lrp4Ocn-cko;A2AR-KO mice were subjected to µCT and ELISA/RIA analyses. (A and B) µCT analysis. Five different male mice of each genotype per group were examined blindly. Representative 3D images are shown in A. Quantification analyses of trabecular bone (TB) volumes over total volumes (BV/TV), TB thickness (TB. Th), TB space (Tb. Sp), cortical bone (CB) BV/TV, CB cavity, and femur length by direct model of µCT analysis are presented in B. Data were analyzed by two-way ANOVA; *, P < 0.05. (C and D) TRAP staining analysis. Representative images are shown in C. Bar, 50 µm. Quantification analysis (mean ± SD, n = 5 femur samples for each group) are shown in D. (E–G) RIA analyses of serum PYD and Ocn, and colorimetric analysis of serum calcium levels. Values are presented as mean ± SD (n = 5). *, P < 0.05.
Second, we measured the activity of V-ATPase in WT and Lrp4 mutant BMSCs using in vitro ATP hydrolysis assay. As shown in Fig. 7 D, V-ATPase was more active in Lrp4-deficient cells than in controls. Third, we measured the pH in lysosomes, because V-ATPase is a proton pump that is essential for the vesicular proton gradient and acidification (Bankston and Guidotti, 1996; Rudnick, 2008; Wang and Hiesinger, 2013), and the proton gradient controls vesicular ATP loading (Coco et al., 2003; Tokunaga et al., 2010). As shown in Fig. 7 (E and F), vesicular fluorescence of LysoSensor (a pH sensor) was higher in Lrp4-deficient BMSCs than in controls, indicating increased vesicular acidification and providing additional support for elevated V-ATPase activity in Lrp4-deficient BMSCs. Using quinacrine and LysoTracker, which label vesicular ATP and lysosomal vesicles, respectively (Cao et al., 2014; Huang et al., 2014), we compared vesicular ATP levels between control and
Figure 7. Elevated v-ATPase–driven vesicular ATP loading and release in Lrp4-deficient OB-lineage cells. (A–C) Primary cultured WT and Lrp4-deficient BMSCs were treated with sham (PBS), BafA1, chloroquine, or A740003 at the indicated dose. After 2-h treatment, ATP levels in the culture medium were measured using a bioluminescence detection kit. Mean ± SD values from three different experiments are shown. *, P < 0.05. (D) Increased V-ATPase activity in Lrp4-deficient BMSCs. Mean ± SD values from experiments using three different preparations are shown. *, P < 0.05; [E and F] Increased vesicular acidification in Lrp4-deficient BMSCs. The green fluorescence intensity of LysoSensor was measured by ImageJ (mean ± SD; n = 20). Bar, 10 µm. *, P < 0.05. (G and H) Increased ATP loading in Lrp4-deficient BMSCs. ATP-containing vesicles were labeled with quinacrine (green channel), and lysosomes were stained with LysoTracker DND-99 (red channel). Bar, 10 µm. Quantification analysis is shown in H. Mean ± SD, n = 20; **, P < 0.001. (I and J) Primary cultured WT and Lrp4-deficient BMSCs were treated with 5 nM bafilomycin A1 (BafA1) for 1 h. Quinacrine and LysoTracker DND-99 were added. Fluorescence microscopy was undertaken to monitor quinacrine and LysoTracker staining. Bar, 10 µm. Quantification analysis is shown in J. Mean ± SD, n = 20; *, P < 0.05. (K) Illustration of a working model in which Lrp4 deficiency in OB-lineage cells increases V-ATPase activity, consequently enhancing vesicular ATP loading and release.
Lrp4-deficient BMSCs. The quinacrine signal was largely co-localized with LysoTracker in both control and Lrp4-deficient BMSCs (Fig. 7 G), in line with studies of ATP in lysosomal vesicles (Cao et al., 2014; Huang et al., 2014). More quinacrine-labeled ATP vesicles were detected in Lrp4-deficient BMSCs than in control cells (Fig. 7, G and H), indicating elevated vesicular ATP loading. Similar results were also detected in Lrp4-deficient OBs (Fig. S3, B–D). Moreover, both quinacrine-ATP and LysoTracker fluorescence signals were V-ATPase activity dependent, as treatment with 5 nM BafA1 largely reduced their fluorescence (Fig. 7, I and J). Together, these results support a working model (Fig. 7 K) in which Lrp4 deficiency in OB-lineage cells increases V-ATPase activity and thus enhances vesicular ATP loading and release.

PRR in ATP release from Lrp4-deficient OB-lineage cells

PRR, also known as ATPase H+-transporting lysosomal accessory protein 2 (ATP6AP2), is an accessory subunit of the V-ATPase (Crucciat et al., 2010; Ichihara, 2012; Rousselle et al., 2014; Trepiccione et al., 2016). To understand how osteoblastic Lrp4 regulates V-ATPase, we compared their levels in control and Lrp4-deficient BMSCs. Remarkably, levels of both PRR and V-ATPase A1 were higher in Lrp4-deficient BMSCs than in those of controls (Fig. 8, A and B). The PRR increase was also detected in Lrp4-deficient OBs (not depicted) and MC3T3 cells (an OB cell line), whose Lrp4 expression was suppressed by miRNA (Fig. 8, C and D). The increase in PRR or V-ATPase A1 levels was not caused by altered transcription, as their mRNA levels were similar in mutant and control cells (Fig. S4, A and B), implicating a posttranscriptional regulation. Indeed, PRR appeared to be more stable in Lrp4-deficient BMSCs, with a half-life of ~4 h, twice longer than that of control cells (Fig. 8, E and F). We further examined PRR levels in MC3T3 cells overexpressing Lrp4. The PRR fluorescence signal was lower in cells expressing exogenous Lrp4 than in untransfected cells (Fig. 8, C and D). These results suggest that Lrp4 in OB-lineage cells is necessary and sufficient for PRR down-regulation or degradation.

To determine if increased PRR levels enhance ATP loading and release, MC3T3 cells were transfected with PRR and mCherry. In MC3T3 cells that were positive for exogenous PRR, there was stronger signal of quinacrine staining, compared with untransfected cells or cells transfected with control vector (Fig. 9, A and B), suggesting that a high level of PRR is associated with vesicular ATP loading. In accord, acidification was increased in these cells (Fig. 9, A and C). Next, we infected MC3T3 cells with lentiviral particles encoding shRNA-PRR, which was able to reduce PRR level in infected cells (Fig. 9, A and D). Reducing PRR levels in MC3T3 cells reduced quinacrine staining when compared with control cells (Fig. 9, D and E), indicating compromised vesicular ATP loading. The shRNA-PRR viral infection of Lrp4-deficient BMSCs also reduced ATP level in the CM (Fig. 9, F and G), in D. (E and F) Time-course analysis of PRR protein levels after cycloheximide (CHX) treatment. Primary cultured BMSCs from 3-mo-old control and Lrp4<sup>Δ<sup>0<sub>cKO</sub></sup></sup> mice were cultured with 50 µg/ml CHX for the indicated time. PRR protein levels were analyzed by Western blotting. Representative blots are shown in E, and quantification analysis (mean ± SD from three separate experiments; *, P < 0.03) is presented in F.
and the CM was less effective in causing OC genesis deficits (Fig. 9, H–J). Collectively, these results demonstrate that PRR is necessary and sufficient for vesicular ATP loading and release, revealing a mechanism of elevated ATP release in Lrp4-deficient OB-lineage cells.

**Critical role of the interaction between Lrp4 and PRR in Lrp4 down-regulation of PRR and V-ATPase activity**

To understand how Lrp4 down-regulates PRR, we first determined whether the two proteins form a complex. HEK293 cells were transfected with Flag-Lrp4 and V5-PRR, and cell lysates were subjected to precipitation with anti-Flag antibody. PRR was detected in precipitates by anti-Flag antibody, but not those precipitated by a nonspecific IgG, suggesting PPR and Lrp4 may form a complex (Fig. S5). To map the domain in Lrp4 to interact with PPR, HEK293 cells were transfected with V5-PRR and a series of Lrp4 deletion mutants (Fig. 10, A and B). As with full-length Lrp4, its extracellular domain was required and sufficient to communoprecipitate with V5-PRR (Fig. 10 B). The interaction was not altered by deleting LDLR (LDLR-A) or the β1 propeller domain, suggesting that these regions are not necessary for interaction. However, deleting β1234 (LDLR-B) or the β12 propeller domains abolished Lrp4’s interaction with PRR (Fig. 10 B). These results indicate a role of the β12 propeller domains in forming a complex with PRR.

It is of interest to note that syndactylly is a rare condition in which bones are misshapen due to abnormal PRR and V-ATPase activity

**Discussion**

In this paper, we investigated the molecular mechanism by which osteoblastic Lrp4 regulates OC genesis and function. We found that Lrp4 deficiency in OB-lineage cells increased the PRR/V-ATPase activity and vesicular ATP loading, releasing elevated levels of PPI and adenosine in the extracellular compartment. Second, both pharmacological blocking and genetic ablation A2AR diminished the OC genesis deficit in Lrp4 mutant mice, demonstrating a critical role for adenosine-A2AR signaling in the inhibition of OC genesis. Third, Lrp4 interacted with PRR and was necessary for PRR degradation. These results support a working model depicted in Fig. 10 H, where Lrp4 in OB-lineage cells promotes OC genesis and bone resorption by controlling PRR/V-ATPase-driven vesicular ATP release and thus maintaining extracellular levels of ATP and its derivative, adenosine.

When Lrp4 is mutated in OB-lineage cells, the PRR level is increased, which enhances vesicular ATP loading and release and levels of PPI and adenosine in the extracellular compartment. We propose that adenosine-A2AR signaling may underlie impaired OC genesis via osteoblastic Lrp4 deficiency for the following reasons. First, inhibition of A2AR signaling by its antagonist, SCH58261, but not A740003, an antagonist of ATP-P2X7R, was capable of restoring OC genesis and bone resorption in Lrp4 mutant mice (Figs. 3, 4, and S2). Second, knocking out A2AR in Lrp4 mutant mice diminished the OC genesis deficits and restored RANK levels and RANK-mediated OC differentiation (Figs. 5 and 6). Third, the idea that adenosine-A2AR signaling suppresses OC genesis and function is also in line with the previous findings that activation of A2AR by agonist CGS21680 inhibits OC differentiation and function in culture and in vivo (Mediero et al., 2012, 2013), inhibiting A3AR enhances RAN KL-induced OC genesis (Mediero and Cronstein, 2013), and A2AR mutant mice exhibit marked reduction in bone density, with increased number and function of TRAP+ OCs (Mediero et al., 2012, 2013). Fourth, although the level of PPI, an inhibitor of OC genesis, was increased in Lrp4-deficient OB-lineage cells, the presence of abundant tissue-nonspecific alkaline phosphatase or alkaline phosphatase in bone tissue (Orimo, 2010; Narisawa et al., 2013) may prevent its accumulation and inhibitory effect in Lrp4 mutant mice. In line with this view, serum levels of PPI are unchanged in Lrp4 mutant mice (Fig. S1 D), and PPI’s inhibitory effect on bone mineralization (Nishikawa et al., 1996; Baran et al., 2011; Burr and Russell, 2011; Russell, 2011) is undetectable in Lrp4 mutant mice (Leupin et al., 2011; Chang et al., 2014; Xiong et al., 2015). Finally, other adenosine receptors, such as A1R, A3R, and A2R, are unlikely to inhibit OC genesis and function (Mediero and Cronstein, 2013). In contrast, activation of A1R increases OC genesis and function (Mediero and Cronstein, 2013).

In addition to adenosine-A2AR signaling, the mechanisms of OC genesis deficits in Lrp4 mutant mice may be complex. We previously detected a reduced RANKL/OPG ratio in mr-Lrp4mut and Lrp4Δkina-cko mutant mice, which may also impair OC genesis and bone resorption (Xiong et al., 2015). The results presented in Fig. 1 (F and G) suggest a delayed RAN KL-induced in vitro OC differentiation in BMMs from Lrp4 mutant mice, which do not exclude RANKL/OPG’s contribution in vivo. In fact, the partial rescue of the bone resorption in Lrp4 mutant mice by inhibition of A2AR (Figs. 4 F and 5 E) suggest that additional factors are involved in this event. Furthermore, increased DKK1 in Lrp4 mutant mice may also inhibit OC genesis by inhibiting Wnt5a-induced OC genesis (Baron and Kneissel, 2013). These alternative mechanisms warrant additional investigation.

Intriguingly, blocking P2XR (by A740003), a primary receptor of ATP, diminished overgrowth of bone mass in Lrp4 mutant mice (Fig. S2), suggesting a potential involvement of ATP activation of P2XR in eventual phenotype manifestation. However, A740003 did not increase the number of TRAP+ cells or elevate bone resorption in Lrp4Δkina-cko mice (Fig. S2, F–H). Instead, serum Ocn levels were reduced in A740003-treated mice (Fig. S2 I). These observations suggest that the ATP-P2XR pathway may promote OB-mediated bone formation instead of OC-mediated bone resorption.

PRR appears to be a multifunctional protein. It was initially identified as a PRR critical for renin signaling and function (Nguyen, 2011). It is also an important component of V-ATPase
Figure 9. PRR is critical for ATP release from Lrp4-deficient OB-lineage cells. (A–C) Increased ATP loading and vesicular acidification in MC3T3 cells expressing V5-PRR. ATP-containing vesicles were labeled with quinacrine. The green fluorescent intensity of LysoSensor was measured by Image J (mean ± SD; n = 20). Quantification analysis is shown in B and C. Bar, 10 µm. *, P < 0.05. (D and E) Decreased ATP loading in MC3T3 cells suppressing PRR. Cells were costained with LysoTracker DND-99 and quinacrine. White bar, 10 µm; and yellow bar, 5 µm. Quantification analysis is shown in E. Mean ± SD, n = 20; *, P < 0.05. (F) Western blot analysis of Lrp4 and PRR expression in control and PRR-KD BMSCs. BMSCs derived from 1-mo-old control and mrlrpp4 mice were infected with control and shRRPRR lentiviruses. The cell lysates were subjected to the Western blot analysis using indicated antibodies. (G) Medium ATP levels in PRR-KD BMSCs. The values of mean ± SD from three different experiments were presented. *, P < 0.05. (H) Experimental strategy. TRAP staining of WT BMMs that were treated with CM of control or PRR-KD BMSCs. (I and J) TRAP staining analysis of OC cultures that were treated with CM plus RANKL for 7 d. Representative images are shown in I. Bar, 150 µm. Quantitative analysis of TRAP+ multinuclei cells (MNCs) per field is presented in J. Mean ± SD values from three different cultures are shown. *, P < 0.05.
Figure 10. Critical role of Lrp4’s interaction with PRR in Lrp4’s down-regulation of PRR and V-ATPase activity. (A) Illustration of various deletion mutants of Lrp4. (B and C) Coimmunoprecipitation analysis of Lrp4 and its mutant with PRR. HEK293T cells were transfected with the indicated plasmids. 48 h after transfection, ∼500 µg cell lysates was immunoprecipitated by anti-Flag M2-agarose. The resulting lysates were subjected to Western blot analysis using indicated antibodies. Approximately 50 µg cell lysates was used as an input. Data presented are representative of three independent experiments. (D and F) Effects of Lrp4 and its mutant on PRR levels in MC3T3 cells. MC3T3 cells transfected with mCherry plus Flag-Lrp4, Flag-Lrp4-dβ12, Flag-Lrp4-G907R, or Flag-Lrp4-L953P were fixed and immunostained with anti–PRR antibody. Representative images are shown in D. Transfected cells are marked with white line. Bar, 10 µm. Quantification analysis of data from D is shown in F; mean ± SD; n = 20. * P < 0.05. (E and G) Effects of Lrp4 and its mutant on ATP loading in MC3T3 cells. Bar, 10 µm. Quantification analysis is shown in G; mean ± SD; n = 20. * P < 0.05. (H) Illustration of a working model. Lrp4 deficiency in OB-lineage cells increases PRR-associated V-ATPase activity in lysosomes, thus promoting vesicle H+ gradient and acidification, accelerates vesicular ATP release, and elevates extracellular adenosine production, which inhibits OC genesis.

Osteoblastic Lrp4 regulates osteoclastogenesis • Xiong et al. 773
Materials and methods

Reagents and animals

Rabbit polyclonal antibodies, including PRR (HPA003156; Sigma-Aldrich), V-ATPase A1 (sc-28801; Santa Cruz Biotechnology, Inc.), pErk1/2 (4370S; Cell Signaling Technology), pAkt (4060P; Cell Signaling Technology), c-Fms (M-CSF receptor antibody; #3152; Cell Signaling Technology), adenosine receptor A2a (ab3461; Abcam), and pErk1/2 (4370S; Cell Signaling Technology), adenosine receptor A2a (ab3461; Abcam), and

**Plasmids and lentiviruses**

Original Lrp4 constructs were gifts from T. Suzuki (Shinshu University, Matsumoto, Japan). To generate Flag-Lrp4 and Flag-Lrp4 mutants, we amplified full-length Lrp4 and mutant Lrp4 cDNA by PCR from the original Lrp4 construct and subcloned the cDNA into pFlag-CMV1 downstream of an artificial signal peptide sequence and a Flag epitope (Wu et al., 2012). G907R (CGA to CGA) and L953P (CTA to CCA) point mutations were made from the Flag-Lrp4 construct using the Phusion High-Fidelity PCR kit (E0535L; New England Biolabs, Inc.) and Restriction Endonucleases DpnI (R0176L; New England Biolabs, Inc.). Lrp4-mRNA construct miLrp4-1062 was generated using the BLOCK-it Pol II miR RNAi expression vector kit (K4936-00; Invitrogen), which has been previously described and verified to be most potent in inhibiting Lrp4 expression (Zhang et al., 2008). V5-PRR plasmid was purchased from DNAUS (ATP6AP2 in pLX304, HeC0046844). Renin receptor shRNA lentiviral particles (shR-PRR) were obtained from Santa Cruz Biotechnology, Inc. (sc-62935-V). The authenticity of all constructs was verified by DNA sequencing.

**In vitro OB/OC lineage cell culture**

Whole bone marrow cells were flushed from long bones of WT and Lrp4-deficient mice and plated on 100-mm culture plates in DMEM containing 1% penicillin/streptomycin (P/S) and 10% FBS for 2 d. For OB-lineage culture, plates with adherent cells were replaced with fresh culture medium every 3 d. After 7 d of passaging by trypsin digestion, 1 × 10^6/cm^2 BMMs were plated for experiments. For OC lineage culture, nonadherent cells were harvested and subjected to Ficoll-Hypaque gradient centrifugation for purification of BMMs. Cells were plated on 100-mm culture dishes in α-MEM containing 10% FBS, 1% P/S, and 10 ng/ml recombinant M-CSF.

For osteoclastogenesis, 5 × 10^5 BMMs were incubated with OC differentiation medium containing 10 ng/ml recombinant M-CSF and 100 ng/ml recombinant RANKL. Mature OCs began to form at day 4 to 5 after RANKL treatment. The cells were then subjected to TRAP staining to confirm their OC identity.

For CM treatment, BMSCs were plated on 100-mm tissue culture plates in α-MEM containing 1% FBS and 1% P/S. BMMs derived from WT mice were placed onto presterilized glass coverslips on 12-well plates. CM from OB-lineage cells plates plus 10 ng/ml recombinant M-CSF and 100 ng/ml recombinant RANKL were added to 12-well plates containing BMM every day. After 7 d, cells were subjected to TRAP staining.

Primary OB cultures were prepared from long bones of 1-mo-old WT/mr-Lrp4null or 3-mo-old Ocn-Cre/Lrp4null mice. In brief, small bone pieces were incubated in collagenase solution to remove all remaining soft tissue and adhering cells and then transferred to 60-mm culture dishes containing DMEM medium supplemented with 10% FBS, 1% P/S, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate. Culture medium was replaced three times per week. Bone cells started to migrate from the bone chips after 3–5 d. After 2 wk, the monolayer was trypsinized by incubating the cells with trypsin solution.

**Cell lines and transfection**

MC3T3-E1 or HEK293 cells were maintained in DMEM supplemented with 10% FCS and 1% P/S. For transient transfection, MC3T3-E1 cells were plated at a density of 10^5 cells per 10-cm culture dish and allowed to grow for 12 h before transfection using a Lipofectamine 3000 Transfection kit (L3000; Invitrogen). 48 h after transfection, cells were
subjected to immunostaining analysis. HEK293 cells were transfected by polyethylenimine (PEI), as described previously (Xia et al., 2013; Xiong et al., 2015). In brief, 12 µg DNA mixture was prepared in serum-free DMEM. 6 µl PEI (based on a 3:1 ratio of PEI/total DNA) was added to the diluted DNA and mixed immediately by pipetting. After incubation for 20 min at room temperature, the DNA/PEI mixture was added to cells, and 48 h later, transfected cells were subjected to Western blot or coimmunoprecipitation assay.

The PRR-KD cell line or PRR-KD BMSCs were obtained by infection of MC3T3-E1 cells or BMSCs with lentiviral particles encoding scramble control or shRNA-PRR, respectively. In brief, cells were infected with the lentiviral particles for 1 d in 2 µg/ml polybrene medium. At day 3, the culture medium was removed and replaced with complete medium (without polybrene). After 5–6 d, stable clones expressing the shRNA were selected via 5 µg/ml puromycin dihydrochloride, which induces death of untransduced cells.

FACS analysis
FACS was performed as described previously (Xia et al., 2013). In brief, BMMs derived from 3-mo-old OcnCre, Lrp4Ocn-cko, A2AR-KO, and Lrp4Ocn-cko;A2AR-KO mice were resuspended in 2% acetic acid in ddH2O) was then added and allowed in incubate for 15 min at room temperature. Fluorescent quantification was performed using Zen software (ZEISS) by sequential excitation at 488 nm for quinacrine and 543 nm for LysoTracker Red. Images were acquired using Zen software according to the manufacturer’s instructions (ZEISS).

V-ATPase activity assay
Each sample (20 µg protein) was added to a solution containing 2 mM ATP, 3 mM MgSO4, 25 mM Tris-SO4, pH 8.0, and 5 mM NaN3. The samples (with or without 1 µM BafA1 to test V-ATPase specific activity) were incubated at 37°C for 20 min, and the reaction was stopped by the addition of 150 µl solution A (12% SDS). Inorganic phosphate was measured using a modified Chifflet’s assay (Chifflet et al., 1988; González-Romo et al., 1992). Different concentrations of K2HPO4 (ranging from 0 to 200 µM) were used to generate a standard curve. Solution B (12% acetic acid in 1 N HCl and 200 µM EDTA) and solution C (2% ammonium molybdate in 1 N HCl) were combined 1:1 at the time of the experiment (named solution D) and added to each of the samples (300 µl per group). After 3 min, solution E (2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid in ddH2O) was then added and incubated for 20 min at room temperature. Absorbance was read using a spectrophotometer at 850 nM. The color developed was stable up to 5 h. V-ATPase activity was calculated as BafA1-inhibited ATP hydrolysis.

µCT
The µCT analyses were performed as described previously (Xia et al., 2013; Xiong et al., 2015). Excised femurs from mice were scanned using the Scanco µCT40 desktop cone-beam micro-CT scanner (Scanco Medical) using µCT Tomography v5.44. Scans were automatically reconstructed into 2D slices, and all slices were analyzed using the µCT Evaluation Program (v6.5-2; Scanco Medical). The femur was placed inverted in a 12-mm-diameter scanning holder and scanned at the following settings: 12 µm resolution, 55 kVp, and 145 µA with
an integration time of 200 ms. For the cortical analysis, the bone was scanned at the midshaft of the bone for a scan of 25 slices. The region of interest was drawn on every slice and fitted to the outside of the cortical bone to include all the bone and marrow. The threshold for cortical bone was set at 329. 3D reconstruction (µCT Ray v3.8) was performed using all outlined slices. Data were obtained on BV, total volume (TV), BV/TV, bone density, and cortical thickness. For the trabecular bone, the scan was started at the growth plate and consisted of 211 slices. The region of interest was outlined starting below the growth plate (for the femurs from 1-mo-old mice) and where the condyles ended (for the femurs from older mice). 100 slices were outlined from this point, on the outside of the cortical bone, enclosing only the trabecular bone and marrow. Trabecular bone was thresholded at 245 and the 3D analysis performed on the 100 slices. Data were obtained on BV, density, TV, trabecular number, thickness, and separation.

**Bone histomorphometric analysis**

Bone histomorphometric analyses were performed as previously described (Xia et al., 2013; Xiong et al., 2015). In brief, mouse tibia and femurs were fixed overnight in 10% formalin, decalcified in 14% EDTA, embedded in paraffin, sectioned, and subjected to hematoyxin and eosin and TRAP staining. Morphometric parameters were determined by measuring the areas situated at least 0.5 mm from the growth plate, excluding the primary sponiosa and trabeculae connected to the cortical bone.

**Measurements of serum levels of Ocn, PYD, and calcium**

Blood samples were collected, allowed to clot for 30 min, and centrifuged for 10 min at 3,000 rpm. Serum was frozen at −80°C until use. Ocn, PYD, and calcium were measured in duplicate with an Ocn ELISA kit (Biomedical Technologies, Inc.), a METRA serum PYD RIA kit (Quidel Corporation), and a calcium detection kit (Abcam), respectively, as described previously (Xia et al., 2013; Xiong et al., 2015). Concentrations were obtained by comparing readings against standard curves.

**RNA isolation and real-time PCR**

Total RNA was isolated by TRizol extraction (Invitrogen). Quantitative PCR was performed using a Quantitect SYBR Green PCR kit (Bio-Rad Laboratories) with a real-time PCR System (Opticon Monitor 3). The following primers were used: CD39, 5′-AGGCTTAAACCAAGGATA CGA-3′ and 5′-AGGGGACGTGCTTGTTGCT-3′; CD73, 5′-TCCGCA AGGAAGACCCA-3′ and 5′-GTGCCCATGACCTGACCC-3′; PRR, 5′-CAGGCCTACTCCTCACCC-3′ and 5′-ACACCCGATTTG CTTTCC-3′; V-ATPase, 5′-ACTAAGCAAAGAGAGAGGGAG-3′ and 5′-ACCCGACAGCTCAAAACA-3′; RANKL, 5′-ATCCATCG GTTCCCATAA-3′ and 5′-TCCGTGGCTTTAAGTCTGATG-3′; OPG, 5′-GGCCTGATGATGCCCTCA-3′ and 5′-GTCGAG GAACCTGATGCTTCC-3′; β-actin primers (5′-AGGTCTACACTA TTGGGAAAG-3′ and 5′-CATGATGGCAGCAGATTCC-3′) were used for normalization.

**Statistical analysis**

All data are expressed as mean ± SD. For in vivo studies, five or six mice per genotype per assay were used. For in vitro cell biological and biochemical studies, each experiment was repeated three times. 10–50 cells were quantified for immunostaining analyses. Data were analyzed by Student’s t test, two-way analysis of variance (ANOVA), and post-hoc test (GraphPad Software Prism 5). The significance level was set at P < 0.05.

**Online supplemental material**

Fig. S1 shows that ATP levels were increased in CMs of Lrp4-deficient BMSCs. Fig. S2 demonstrates trabecular bone loss in Lrp4−/−cko mice by A740003, an antagonist of P2X-R. Fig. S3 shows elevated v-ATPase–driven vesicular ATP loading and release in Lrp4-deficient OBs. Fig. S4 shows unchanged expression levels of PRR in Lrp4-deficient BMSCs and MC3T3 cells. Fig. S5 demonstrates the interaction of Lrp4 with PRR.

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**Author contributions:** W.-C. Xiong and L. Xiong designed research. L. Xiong performed the experiments shown in Figs. 1–10, and J.-U. Jung performed the experiments shown in Fig. 1. H.-H. Guo and J.-X. Pan assisted in primary cell cultures and bone histomorphometric analysis. X.-D. Sun assisted in ATP measurements. W.-C. Xiong, L. Xiong, and L. Mei analyzed data. W.-C. Xiong, L. Mei, and L. Xiong wrote the paper.

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Osteoblastic LRP4 regulates osteoclastogenesis • Xiong et al. 777
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