Lrp4, a Novel Receptor for Dickkopf 1 and Sclerostin, Is Expressed by Osteoblasts and Regulates Bone Growth and Turnover In Vivo

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Abstract

Lrp4 is a multifunctional member of the low density lipoprotein-receptor gene family and a modulator of extracellular cell signaling pathways in development. For example, Lrp4 binds Wise, a secreted Wnt modulator and BMP antagonist. Lrp4 shares structural elements within the extracellular ligand binding domain with Lrp5 and Lrp6, two established Wnt receptors with important roles in osteogenesis. Sclerostin is a potent osteocyte secreted inhibitor of bone formation that directly binds Lrp5 and Lrp6 and modulates both BMP and Wnt signaling. The anti-osteogenic effect of sclerostin is thought to be mediated mainly by inhibition of Wnt signaling through Lrp5/6 within osteoblasts. Dickkopf1 (Dkk1) is another potent soluble Wnt inhibitor that binds to Lrp5 and Lrp6, can displace Lrp5-bound sclerostin and is itself regulated by BMPs. In a recent genome-wide association study of bone mineral density a significant modifier locus was detected near the SOST gene at 17q21, which encodes sclerostin. In addition, nonsynonymous SNPs in the LRP4 gene were suggestively associated with bone mineral density. Here we show that Lrp4 is expressed in bone and cultured osteoblasts and binds Dkk1 and sclerostin in vitro. MicroCT analysis of Lrp4 deficient mutant mice revealed shortened total femur length, reduced cortical femoral perimeter, and reduced total femur bone mineral content (BMC) and bone mineral density (BMD). Lumbar spine trabecular bone volume per total volume (BV/TV) was significantly reduced in the mutants and the serum and urinary bone turnover markers alkaline phosphatase, osteocalcin and deoxypyridinoline were increased. We conclude that Lrp4 is a novel osteoblast expressed Dkk1 and sclerostin receptor with a physiological role in the regulation of bone growth and turnover, which is likely mediated through its function as an integrator of Wnt and BMP signaling pathways.

Introduction

Osteoporosis and the inherently increased susceptibility to sustain fractures associated with this disease represent a major challenge in our aging western societies. Bone mineral density (BMD) is used as a reference to diagnose and to monitor osteoporosis and is the best predictor of fractures and a valuable tool for fracture risk assessment [1,2]. Although much progress has been made in the molecular understanding of bone metabolism in recent years, no therapy is yet available to cure osteoporosis [3]. Many of the current approaches to identify potential therapeutic targets are focused on the Wnt/β-catenin signaling pathway, which is of fundamental importance for osteogenesis [4–6]. In a recent genome-wide association study of BMD in individuals of European descent, a new locus was identified near the SOST gene at 17q21, which encodes for the osteocyte secreted protein sclerostin [7], a potent inhibitor of bone formation. Within the same study, nonsynonymous SNPs in the LRP4 gene at 11p11 were suggestively associated with bone density (BMD) [7], confirming previous findings of another study from the same group in which non-significant association of SNPs within or close to the LRP4 locus with BMD and fractures had been reported [8]. Sclerostin functions as a secreted antagonist of both the bone morphogenetic protein (BMP) [9,10] and Wnt signaling pathways [11,12] and Lrp4 has been proposed to function as an integrator of BMP and Wnt signaling [13]. However, whether sclerostin and Lrp4 bind physically to each other, or whether they form an indirect functional interaction has not been known.
Lrp4 is a member of the multifunctional low-density lipoprotein receptor (Ldlr) gene family [14–16]. Physiological functions for this ancient gene family include the endocytosis of a large number of macromolecules, including lipoproteins, proteases and protease inhibitors, as well as functions as direct signal transducers or modulators of several fundamental signal transduction pathways, including BMP, TGFβ, PDGF, reelin and canonical Wnt signaling.

Insight into the physiological functions of Lrp4 has been gained through naturally occurring or genetically engineered mutations in mice and cattle. Lrp4 is expressed in various organs [17–19], as well as in bone (this study). Mice bearing functional Lrp4 null mutations die perinatally due to a failure of forming neuromuscular junctions [20–22]. In addition, limb development is also abnormal [18,20]. Hypomorphic mutations of the Lrp4 gene are compatible with survival and present with a variable degree of skeletal abnormalities, in particular fusion of digits at the hind and fore limbs (polydactyly). By engineering a stop codon just upstream of the transmembrane domain of the murine Lrp4 gene, we have generated such a hypomorphic dysfunctional receptor (Lrp4 ECD), in which the lack of a membrane anchor prevents the efficient interaction of Lrp4 with its extracellular ligands. Animals carrying this mutation are viable but present with growth retardation, polydactyly and tooth developmental abnormalities [19,18]. A similar polydactyly phenotype has been observed also with other allelic mutations in the murine [23] and bovine [24] Lrp4 gene. It was recently shown that Lrp4 integrates BMP and Wnt signaling during tooth development by binding the BMP antagonist Wise [13]. The role of Lrp4 as an antagonist of canonical Wnt signaling pathway is thought to be mediated in part by a displacement of the homologous Lrp5/6 proteins in the co-receptor complex formed by frizzled proteins (fzd) with Lrp5/6, which is required to bind Wnt proteins and to transduce the Wnt signal to downstream elements of the canonical cascade.

Lrp5/6 are established Wnt co-receptors with important roles in osteogenesis. Gain of function and loss of function mutations in the Lrp5 gene lead to a high bone mass and low bone mass phenotype, respectively, both in mice and humans (for review, see [25]). Mutations in the Lrp6 gene display a partially overlapping bone phenotype with the various Lrp5 mutations (for review, see [26]). Sclerostin is a potent osteocyte secreted inhibitor of bone formation that directly binds to Lrp5 and Lrp6 [12,27]. The powerful anti-osteogenic effect of sclerostin is thought to be mediated mainly by inhibition of Wnt-signaling through Lrp5/6 within osteoblasts by disrupting the Wnt induced frizzled/Lrp complex formation, although sclerostin was first found to inhibit not Wnt, but the action of murine and human BMPs in vitro [9,10]. Sclerostin is predominantly expressed in skeletal tissues [9,10]. Mutations in SOST cause the human disease sclerosteosis, which is characterized by massive bone overgrowth [28,29]. Van Buchem’s disease is a similar disorder with generalized hyperostosis and a 52-kb deletion downstream (35 kb) of the SOST gene that removes a SOST-specific regulatory element [30,31]. Consistent with a function of sclerostin as an inhibitor of bone formation, transgenic mice overexpressing human SOST, display a low bone mass phenotype [10,32] and Sost knockout mouse have higher bone mass with increased BMD and bone strength [33]. Interestingly, overexpression of human SOST in transgenic mice resulted in an additional phenotype with fused or missing digits of the fore and hind limbs, reminiscent of the phenotype of mice with dysfunctional Lrp4 [32].

Dickkopf1 (Dkk1) is another soluble inhibitor of Wnt/β-catenin signaling that binds to Lrp5 and Lrp6 [34–37]. Dkk1 is required for embryonic head and limb development. It also regulates postnatal bone accretion and maintenance of bone mass mainly by binding to Lrp5/6 in a process that involves the transmembrane proteins Kremen 1 or Kremen 2 [34,38], although at least some of the Wnt-inhibitory effects of Dkk1 mediated by Lrp5/6 seem to be independent of Kremen [39]. Dkk1 null mice die perinatally and show severe developmental phenotypes, including head and limb dysmorphogenesis [40]. A transgenic mouse mutant with reduced Dkk1 expression displays postnatal polydactyly, which can be partially rescued by the concomitantly reduced expression of Lrp5/6 [41]. Overexpression of Dkk1 in osteoblasts causes osteopenia [42] and Lrp5 mutants that cannot bind Dkk1 show increased bone mass both in mice and in humans [37,43]. Dkk1 binds to the first EGF-like domain of Lrp5/6, with which also sclerostin and Wnts interact [34,44]. It has been shown that Dkk1 can displace sclerostin from the Lrp5 sclerostin complex [45]. Moreover, the expression of Dkk1 has been reported to be regulated by BMPs [46].

The current study was prompted by the association of both the SOST and the LRP4 gene with BMD [7,8,47,48]. The established function of both Sost and Lrp4 in the modulation of BMP and Wnt signaling [13,49,50], the partially overlapping developmental phenotypes in genetically manipulated mice of the Sost, the Dkk1 and the Lrp4 genes, and our previous findings that Lrp4 binds Wise (a.k.a. Sostdc1) through its extracellular domain which is homologous to that of Lrp5/6 which interacts with sclerostin and Dkk1.

Here, we have used in vitro and in vivo analysis of wild type mice and of two Lrp4 mutant mouse strains, one fully deficient (null mutant, Lrp4/-; Dietrich et al., in preparation) and a functional hypomorph (Lrp4-ECD; [18]) to show that Lrp4 is an osteoblast expressed receptor for Dkk1 and sclerostin and regulates bone growth and bone turnover in vivo.

Materials and Methods

Ethics Statement

All animal work has been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, “The use of non-human primates in research,” the following statement to this effect has been included to document the details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates: This work has been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (IACUC). This committee functions in close cooperation with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The performance site for this work (UT Southwestern) is an AAALAC accredited institution.

Production of Recombinant Proteins and Binding Assay

HEK293A cells were transfected with pCDNA3-AP, pCDNA3-RAP-AP, pCDNA3-Wise-AP, pCDNA3-RSpondin2-AP, pCDNA3-DKK1-AP, or pCDNA3-SOST-AP constructs using FuGENE 6 (Roche) in DMEM plus 0.2% BSA medium to produce conditioned media containing AP or AP-tagged recombinant proteins. After 48 h transfection, media were collected and the production levels of AP and AP-tagged proteins in the media were determined by AP activity assay using p-Nitrophenyl phosphate (Calbiochem) as a substrate. To investigate the binding of AP-tagged proteins to Lrp4 in a cell-free system, media containing of the Lrp4 ectodomain fused to the first EGF-like domain of Lrp5/6 was produced by transfection of HEK293A cells with pCDNA3-LRPecto-Fc in the constant region of Immunoglobulin G (Lrp4ecto-Fc) was produced by transfection of HEK293A cells with pCDNA3-LRPecto-Fc in...
IACUC. University of Texas Southwestern Medical Center at Dallas Committee for Use and Care of Laboratory Animals of the accordance with the protocols approved by the Institutional lumbar spine, and femur. All procedures were performed in prior to collection of blood followed by harvesting of the calvaria, After urine collection, the mice were euthanized with isoflurane experimental mice were fasted for 4 h prior to collection of urine. Teklad, Madison, WI) with access to water cycle and fed a standard rodent chow diet (Diet 7001, Harlan genetic drift and vertical inbreeding, mice from the entire intracellular domains of LRP4 [18]. To minimize the effects of truncation of the protein and the loss of the transmembrane and Lrp4ECD heterozygote pool of our colony on a 129SvEv x osteoblast differentiation was induced at 80% confluency in calvariae from 3–4 day old wildtype C57Bl/6 mice as described osteoblasts were obtained by sequential collagenase digestion of connection with scalpel and snap-frozen in liquid nitrogen for storage until the extraction of protein and RNA. Primary osteoblasts were obtained by sequential collagenase digestion of calvariae from 3–4 day old wildtype C57Bl/6 mice as described osteoblast differentiation was induced at 80% confluency in calvariae from 3–4 day old wildtype C57Bl/6 mice as described 250 ng/µl of the product with the expected length was confirmed with gel electrophoresis. The following primers were used: LRP4, 5′-TGTGCGAGACCGGAAATAGC (forward), 5′-GCGCTACCGGACATGT (reverse); 36B4, 5′-CACTGGTCTAAGACCCCGAGAAG (forward), 5′-GGTGCCCTGAGAGATTTTCG (reverse). µCT analysis of Bone The femoral and spinal bones of mouse were scanned at an isotropic voxel size of 27 µm (80 kV, 450 µA and 2000 ms integration time) using the cXplore Locus micro-CT scanner (GE Health Care). The scan was done transversely which generated 720 tomographic slices from a 33 mm sample depth. Three dimensional images were then reconstructed from these two-dimensional gray-scale image slices using the reconstruction utility (GE Medical System) and visualized using Microview Software (GE Medical System). Density values for soft tissue and bone were calibrated using a phantom (GE Health Care) containing air bubble, water and hydroxyapatite rod. The region of interest (ROI) for each animal tissue was defined based on skeletal landmarks from the gray-scale images. Bone analysis was conducted using Microview software. For femoral analysis, bone mineral density (BMD) and bone mineral content (BMC) were measured in cortical bones. For determination of cortical bone, an appropriate ROI (region of interest) was defined and the histogram was created on this selected region. The separation of cortical region was calculated from the grey scale values (upper threshold 4300 and lower threshold 900) on the histogram. BMD, BMC and trabecular structural measurement in vertebral bone was done by Microview using the threshold selected for trabecular bone (upper threshold 1400 and lower threshold 600). Data analysis and calculations were automatically performed by the software. All µCT analyses were done with samples in a randomly selected sequence by an independent blinded investigator who did not know the study hypothesis. Measurement of Bone Formation and Resorption Markers All serum and urine samples had been stored at minus 70°C immediately after the blood draw and were not unoxygenated prior to analysis. Serum levels of osteocalcin were measured using a two-site immunoradiometric assay (ImmunoLigand) according to the manufacturer’s instructions. Alkaline phosphatase activity in serum, was measured using the pNPP method. Briefly, 5 µl of serum were mixed with 50 µl of substrate solution containing 2 mg/ml of p-Nitrophenyl Phosphate (Sigma) in alkaline buffer solution (Sigma). After incubation at 37°C for 15 min, the reaction was stopped by adding 500 µl of 0.5M NaOH and the absorbance at 405 nm was measured. Alkaline phosphatase activity was determined by using p-nitrophenol solution (Sigma) as standard substrate.
Bone resorption was determined by measuring urinary levels of deoxypyridinoline (DPD) using MicroVue DPD (Quidel) according to the manufacturer’s instructions. The DPD values were normalized to the levels of urinary creatinine measured using the MicroVue Creatinine Assay Kit (Quidel).

Results

Lrp4 Interacts with Dkk1 and Sclerostin In Vitro

Both Dkk1 and sclerostin modulate Wnt signaling by binding to EGF-like repeats of Lrp5 and Lrp6. The extracellular domains of Lrp5, Lrp6 and Lrp4 are highly conserved [18], raising the possibility that the spectrum of ligands that bind to Lrp5/6 and Lrp4 might overlap. In order to test for a physical interaction between Dkk1 and Lrp4 as well as sclerostin and Lrp4, we performed two types of in vitro binding assays. First, HEK293A cells transfected with Lrp4 were incubated with conditioned media containing the alkaline phosphatase (AP)-tagged putative Lrp4 ligands (Figure 1A). The 39kDa receptor-associated protein (RAP), a chaperone for Ldlr gene family members, is known to bind to Lrp4 [51]. We have previously shown that Wise interacts with Lrp4, while another modulator of the Wnt signalling pathway, R-spondin2 (RS2) does not [13]. Thus, RAP and Wise served as positive controls, RS2 and non-tagged AP as negative controls for these assays. AP-fusion proteins of controls and the putative ligands Dkk1 and sclerostin were pulled down with an antibody against AP and immunoblotted with an anti-Lrp4 antibody to detect co-precipitated receptor (Figure 1B). In a complementary, converse cell-free assay, the Lrp4-Fc fusion protein was immobilized on Protein A-agarose and incubated with the AP-tagged putative ligands and controls. Lrp4 bound ligands were then detected by immunoblotting against AP (Figure 1C). Both assays revealed that Lrp4 efficiently binds Dkk1 and sclerostin.

Lrp4 is Expressed in Osteoblasts

Sclerostin is a secreted protein expressed by osteocytes. Both sclerostin and Dkk1 bind to Lrp5/6, which play critical roles in the Wnt/β-catenin signal transduction in osteoblasts. The inhibition of bone formation by Dkk1 and sclerostin is thought to be mediated primarily through inhibition of wnt-signaling in osteoblasts. In light of our finding that Dkk1 and sclerostin bind to Lrp4 (Figure 1), we next analyzed whether Lrp4 is expressed in bone. Figure 2A-C and Table 1 demonstrate by quantitative RT-PCR and Western blot analysis that Lrp4 mRNA and protein are indeed expressed in calvaria and in the long bones of adult wild type mice. Murine embryonic brain (E10) from Lrp4 null mice and wild type littermates served as a negative and positive control for the RT-PCR and immunoblots, respectively (Figure 2A, B). Moreover, a polyclonal antibody derived against the recombinantly expressed Lrp4 ligand binding domain specifically recognizes Lrp4 in brain protein extracts (Figure 2B). In Lrp4 null animals, in which the promoter and exon 1 of Lrp4 have been deleted (Dietrich et al., in preparation) neither mRNA (Figure 2A, Table 1), nor Lrp4 protein was detectable (Figure 2B), whereas mRNAs and protein were present in wild type calvaria, femur (Figure 2A, B) and primary calvarial osteoblasts from newborn wild type mice that had undergone six days of in vitro differentiation with ascorbate and β-glycerolphosphate (Figure 2C). Taken together, these data demonstrate that Lrp4 is expressed by osteoblasts in murine bone.

Functional Lrp4 Deficiency Results in Impaired Skeletal Growth, Reduced Trabecular Bone Volume and Increased Bone Turnover

To investigate the physiological significance of the expression of Lrp4 by osteoblasts, we performed micro computer tomography (μCT) of the femur and lumbar spine vertebrae of Lrp4ECID mutant mice and wild type littermates. We also measured several biochemical markers that are indicative of bone turnover. Ten week old male animals were analyzed (n = 6 per genotype for μCT, n = 14 per genotype for the determination of turnover markers). Fasting blood and urine samples were obtained before sacrifice according to a standardized time schedule to rule out any potentially confounding circadian alterations in the concentrations of the osteoblast activity markers alkaline phosphatase (ALP) and osteocalcin (OCN) as well as the urinary collagen breakdown product desoxypyridinoline (DPD) as a marker of osteoclast activity. Body weight of the animals was determined upon sacrifice. Ten week old wildtype mice had a significantly higher total body weight (25.7 g +/- 1.3 g) than their Lrp4ECID littermates (20.3 g +/- 1.4 g) (Figure 3B), consistent with our earlier findings [18]. Polysyndactyly of fore limbs and hind limbs and abnormal tooth development are fully penetrant [18]. MicroCT 3D and 2D reconstruction of the
femur did not reveal striking differences in the overall morphology of the bones (Figure 3A), but showed that the reduction of total body weight was accompanied by a significant reduction of femur length (13.5 +/− 0.3 mm vs 12.5 +/− 0.2 mm p = 0.0001) and a clear, albeit statistically non-significant trend towards a reduction in the combined height of lumbar vertebrae L3 and L4 (6.36 +/− 0.36 mm vs 5.78 +/− 0.58) of Lrp4ECD mutants as compared to controls (Figure 3B and 4B). Furthermore, the inner and outer cortical perimeter of the femur mid-diaphyses was significantly smaller in Lrp4ECD mutants (Figure 3B, p < 0.0001 and p = 0.002).

These findings indicate that at least part of the reduction of the total body weight was due to impaired skeletal growth. This is consistent with our previous report of a role of Lrp4 as a modulator of the BMP and Wnt signaling cascades in limb and tooth development, since both pathways also play a role in the enchondral ossification process in the growth plate that controls limb length growth. Total femur bone mineral content (BMC) and bone mineral density (BMD) were also significantly reduced in Lrp4ECD mutants (BMC 11.1 +/− 1.3 mg versus 9.0 +/− 1.5 mg, p < 0.05 and BMD 641.3 +/− 16.7 mg/cc versus 602.3 +/− 21.4 mg/cc, p < 0.01), while cortical thickness, cortical BMC and cortical BMD were unaffected (Figure 3B), suggesting that the difference in total femur BMD/BMC was due to differences in the trabecular compartment of the femur. For the assessment of trabecular bone structure and density in mice, the lumbar spine is better suited than the distal femur or the proximal tibia. We therefore performed μCT on the lumbar vertebrae L3 and L4. Again, 3D and 2D reconstructions did not unveil gross abnormalities of lumbar spine morphology (Figure 4A), but trabecular bone analyses revealed a significantly reduced bone volume per total volume (BV/TV, 18.9 +/− 2.5%) in Lrp4 ECD mutants as compared to wild type littermates, accompanied by the according changes in trabecular separation and trabecular number (Figure 4B). Trabecular thickness was not significantly affected (Figure 4B). Taken together, μCT analyses of the femur and lumbar spine indicate impaired bone growth in Lrp4ECD mutant mice, with smaller bones of otherwise mostly normal morphology and suggest a concomitant relative increase of bone resorption over bone formation, resulting in a net loss of trabecular bone.

To further explore the underlying cause of this phenotype, we measured the osteoblast activity markers osteocalcin (OCN, Figure 5A) and alkaline phosphatase (ALP, Figure 5B) in serum and urinary deoxypyridinoline (DPD) as an osteoclast activity marker (Figure 5C). All parameters were significantly elevated in the Lrp4ECD mutants (OCN: 15.1 +/− 1.9 ng/ml vs 22.0 +/− 3.9 ng/ml; ALP: 230.2 +/− 22.3 units/ml vs 365.5 +/− 41.1 units/ml; DPD/creatinine: 9.3 +/− 1.9 nmol/mmol vs 11.4 +/− 2.4 nmol/mmol), indicating increased bone turnover in comparison to wild type control animals.

**Discussion**

Here we report that Lrp4 binds the two secreted Wnt modulators Dkk1 and sclerostin, that Lrp4 is expressed by murine
bone, specifically by osteoblasts, and that mice carrying a functionally hypomorphic Lrp4 mutation have impaired bone growth and increased bone turnover.

Canonical Wnt-signaling is of crucial importance for osteogenesis and has probably been the most intensely studied signaling pathway in bone in recent years. Lrp5 and Lrp6 have been recognized as the main osteoblast expressed co-receptors of the frizzled family of proteins, which together form a receptor complex at the cell surface that binds the Wnt signaling proteins and thereby initiates the canonical signaling cascade. The finding that Lrp4 is also expressed by osteoblasts in bone has important implications for the understanding of the complexity of the

Figure 3. Lrp4ECD results in impaired bone growth. Panel A, Representative μCT images of WT and Lrp4ECD mouse femurs. Panel B, Total body weight of ten week old male mice was determined prior to sacrifice. Spinal column and femurs were dissected out, fixed in 4% paraformaldehyde and scanned. Calculations for the indicated parameters were performed using Microview software from GE Medical System. Results represent the average±S.E. of values from six male mice. doi:10.1371/journal.pone.0007930.g003
regulation of the canonical Wnt signaling pathway in bone. Sclerostin and Dkk1 are inhibitory modulators of the Wnt/β-catenin pathway and it has been generally assumed that these molecules exert their antagonistic effect mainly through direct binding to Lrp5/6, thereby blocking binding sites on these receptors for the pathway activating Wnts. Here we have shown that Lrp4 efficiently binds both sclerostin and Dkk1, suggesting that at the osteoblast surface, Lrp4 could either compete with

Figure 4. Lumbar spine trabecular bone is reduced in Lrp4ECD mice. Panel A, Representative μCT images of wild type and LRP4ECD mouse lumbar vertebra L3 and L4. Panel B, Lumbar vertebrae were fixed, scanned, and analyzed for the indicated parameters. Results represent the average ± S.E. of values from six male mice.

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Lrp5/6 for the interaction with sclerostin and Dkk1 or cooperate with the Lrp5/6/frizzled co-receptor complex by binding and presenting these ligands to Lrp5/6. LRP4, but not Lrp5/6, contains an NPXY endocytosis motif in its cytoplasmic domain. However, it is not known whether Lrp4 contributes to the endocytosis of extracellular molecules to a significant extent. The exact nature of both the cellular consequences of sclerostin and Dkk1 binding to Lrp4 as well as the impact that this process may have on the signaling function of Lrp5/6 in bone and other tissues will have to be determined by future studies. It is likely that mouse genetics with double and triple knockout combinations of receptor and ligand genes will be needed to fully understand these interactions.

How can we explain the bone phenotype of Lrp4ECD mice? We have previously shown that Lrp4 integrates BMP and Wnt signals in tooth development in mice [13] by binding Wise, a secreted BMP antagonist that also binds to Lrp6 and thereby inhibits Wnt signaling. In analogy to Wise, sclerostin and Dkk1 are two other Lrp5/6 ligands that we have now shown to also interact with Lrp4. One possible mechanism is that Lrp4 simply acts as a sink and competes with Lrp5/6 for the binding of these Wnt antagonists, which then are no longer available for suppression of the signal through the Lrp5/6 axis. When Lrp4 is dysfunctional as in the Lrp4ECD mutant strain that we used here, the availability of extracellular Wnt antagonists increases, resulting in a net inhibitory effect on canonical Wnt/β-catenin signaling in the cell, which is well known to decrease BMD and thus would provide an explanation for the reduced lumbar spine trabecular BV/TV in the Lrp4ECD mutant mice. Furthermore, both Wnt signaling and BMP signaling are involved in the growth plate organization and enchondral ossification process [53–56]. Moreover, Lrp4 can by itself inhibit Wnt signaling, presumably by competing for Lrp5/6 in the Wnt/Fz complex [18]. Since sclerostin can inhibit BMPs, and Dkk1 is itself regulated by BMPs, it is likely that the failure to properly integrate BMP and Wnt signaling pathways in the absence of a normal, functional Lrp4 is responsible for the reduced limb length growth in the Lrp4ECD mutants. Such a mechanism would be analogous to the role of LRP1 in the integration of PDGF and TGFβ signals in the vascular wall, where loss of LRPI expression in smooth muscle cells results in the simultaneous deregulation of PDGF as well as TGFβ signalling with medial hypertrophy, elastolysis and fibrosis [57–59].

The elevated concentration of biochemical bone turnover markers, serum osteocalcin, alkaline phosphatase and urinary DPD in Lrp4ECD animals indicate that both osteoblast and osteoclast activities are significantly increased in these mice. This could be either explained by an altered coupling of osteoblast and osteoclast activity due to osteoblast-specific changes in Wnt and BMP signaling, or by altered osteoclast function independent of osteoblasts. We failed to detect Lrp4 expression in primary osteoclast cultures from wildtype mice (data not shown), which would favour a mechanism in which altered coupling leads to increased osteoclast activity in this mouse model, possibly by a mechanism similar to the one observed in mutant mice with osteoblast-specific inactivation of β-catenin which results in increased bone resorption [60]. In addition to its role as a bone formation marker, the osteoblast-specific protein osteocalcin has been shown to regulate glucose metabolism and fat mass in mice [61]. Whether the elevated concentrations of osteocalcin in Lrp4ECD mice are also associated with an altered glucose metabolism will have to be determined.

The current study cannot distinguish whether the observed bone phenotype of the Lrp4ECD mutant is due to an impaired interaction of osteoblast expressed Lrp4 with Dkk1, with sclerostin, or most likely with both proteins. Future studies will have to address to what degree the phenotype is synthetic, e.g. by analyzing mice with osteoblast-specific inactivation of Lrp4 in combination with mutations in Dkk1 and sclerostin.
In conclusion, the expression of Lrp4 by osteblasts described here adds another player to the long list of established factors that modulate canonical Wnt signaling in bone. By demonstrating that in addition to Wnt, Lrp4 is able to interact with two additional important modulators of Wnt and BMP signaling, our perspective of the mechanisms of the integration of BMP and Wnt signaling pathways on the osteoblast surface has expanded further. Extensive further studies are clearly necessary to fully understand the mechanisms by which Lrp4 deficiency leads to impaired bone growth, increased bone turnover and polyostely in these mutant mice. Nevertheless the recently described association of both the SOST and LRP4 genes with BMD in humans, together with our findings suggest that LRP4 plays a physiologically important role in the skeletal development and bone metabolism not only in rodents, but in humans as well.

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Author Contributions

Conceived and designed the experiments: JH AN. Performed the experiments: HYC MD AN. Analyzed the data: HYC MD JH AN. Wrote the paper: JH AN.

References

1. McClung MR (2005) The relationship between bone mineral density and fracture risk. Curr Osteoporos Rep 3: 37–45.
2. Kanis JA, Oden A, Johansson H, De Laet C, et al. (2007) The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. Osteoporos Int 18: 1033–1046.
3. Deal C (2009) Potential new drug targets for osteoporosis. Nat Clin Pract Rheumatol 5: 127–137.
4. Hoepner LH, Secretto EJ, Westerendt JD (2009) Wnt signaling as a therapeutic target for bone diseases. Expert Opin Ther Targets 13: 483–496.
5. Baron R, Rawadi G (2007) Targeting the Wnt/beta-catenin pathway to regulate bone formation in the adult skeleton. Endocrinology 148: 2935–2943.
6. Piers E, Boudin E, Van Hul W (2008) Wnt signaling: a win for bone. Arch Biochem Biophys 473: 112–116.
7. Styrrkardottir U, Haldorsdottir BV, Grettardottir S, Guðbjartsson DF, Walters GB, et al. (2009) New sequence variants associated with bone mineral density. Nat Genet 41: 15–17.
8. Styrrkardottir U, Haldorsdottir BV, Grettardottir S, Guðbjartsson DF, Walters GB, et al. (2009) Multiple genetic loci for bone mineral density and fractures. N Engl J Med 356: 2355–2366.
9. Kong X, Laursen SK, Imanishi M, Usui H, Konishi M, et al. (2003) Sclerostin is a novel secreted osteostat-derived bone morphogenetic protein antagonist with unique ligand specificity. J Biol Chem 278: 24113–24117.
10. Winkler DG, Sutherland MK, Georgiann JC, Yu C, Hayes T, et al. (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J 22: 6276–6277.
11. Winkler DG, Sutherland MS, Ojala E, Turcott E, Georgiann JC, et al. (2005) Sclerostin inhibition of Wnt-3a-induced C3H10T1/2 cell differentiation is indirect and mediated by bone morphogenetic proteins. J Biol Chem 280: 24981–24982.
12. Li X, Zhang Y, Kang H, Liu W, Liu P, et al. (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J Biol Chem 280: 19833–19837.
13. Okhaza A, Johnson EB, Ota MS, Choi HY, Pontavrettes T, et al. (2006) Lrp4 modulates extracellular integration of cell signaling pathways in development. PLoS One 3: e4092.
14. Willnow TE, Nykjaer A, Herz J (1999) Lipoprotein receptors: new roles for ancient proteins. Nat Cell Biol 1: E157–E162.
15. Wang K, Zhang Y, Li X, Chen L, Wang H, et al. (2008) Characterization of the bone strength. J Bone Miner Res 23: 860–869.
16. Mao B, Wu W, Li Y, Hoppe D, Stannaker P, et al. (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411: 321–325.
17. Balensiefer MM, Tatro JS, Merkens KM, Tatro LM, et al. (2009) Novel deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. Genome Res 19: 184–195.
18. Ai M, Holmen SL, Van Hul W, Williams BO, Warman ML (2005) Reduced bone mass-associated missense mutations in LRP5 affect canonical Wnt signaling. J Biol Chem 280: 659–666.
19. Tao Y, Liu H, Zhao B, Zhang J, et al. (2007) BMP5 and Lrp6 signaling in bone. J Bone Miner Res 24: 171–178.
20. Williams BO, Inouga KL (2009) Where Wnts went: the exploring field of Lrp5 and Lrp6 signaling in bone. J Bone Miner Res 24: 171–178.
21. Ellis DL, Viviano B, McCarthy J, Rey JP, Inazaki N, et al. (2006) Bone density linder, Sclerostin, directly interacts with LRP5 but not LRP6/G17IV to modulate Wnt activity. J Bone Miner Res 21: 1738–1749.
22. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, et al. (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10: 537–543.
23. Balemans W, Van Den EJ, Freire-Pawlos A, Dikkers FJ, Willems PJ, et al. (1999) Localization of the gene for sclerosteosis to the van Buchem disease-gene region on chromosome 17q12-q21. Am J Hum Genet 64: 1661–1669.
24. Balemans W, Patel N, Ebeling M, Van Hul E, Watts Y, et al. (2002) Identification of a 52 kb deletion downstream of the SOST gene in patients with Van Buchem disease. J Med Genet 39: 91–97.
25. Ntini GG, Varelas E, Goulioudis E, Ntini C, et al. (2002) Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. Genome Res 12: 1945–1953.
26. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, et al. (2008) Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone mass strength. J Bone Miner Res 23: 860–869.
27. MacDonald BT, Adamska M, Meisler MH (2004) Hypomorphic expression of the CTs, and to Walter Tauscher, Priscilla Rodriguez, Isaac Rocha, Wen-Ling Niu and Huichuan Reyna for excellent technical assistance.

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Conceived and designed the experiments: JH AN. Performed the experiments: HYC MD AN. Analyzed the data: HYC MD JH AN. Wrote the paper: JH AN.
46. Grotewold L, Ruther U (2002) The Wnt antagonist Dickkopf-1 is regulated by Bmp signaling and c-Jun and modulates programmed cell death. EMBO J 21: 966–975.
47. Uitterlinden AG, Arb PP, Parper BW, Charmley P, Proll S, et al. (2004) Polymorphisms in the sclerosteosis/van Buchem disease gene (SOST) region are associated with bone-mineral density in elderly whites. Am J Hum Genet 75: 1032–1045.
48. Sims AM, Shephard N, Carter K, Duan T, Dowling A, et al. (2008) Genetic analyses in a sample of individuals with high or low BMD shows association with multiple Wnt pathway genes. J Bone Miner Res 23: 499–506.
49. Kamiya N, Ye L, Kobayashi T, Mochida Y, Yamauchi M, et al. (2008) BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. J Bone Miner Res 23: 3901–3911.
50. van Bezooijen RL, Svensson JP, Eefting D, Visser A, van der HG, et al. (2007) Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. J Bone Miner Res 22: 19–28.
51. Willnow TE, Sheng Z, Ishibashi S, Herz J (1994) Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. Science 264: 1471–1474.
52. Duch P, Amling M, Takada S, Priemel M, Schilling AF, et al. (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 100: 197–207.
53. Chen M, Zhu M, Awad H, Li TF, Sheu TJ, et al. (2008) Inhibition of beta-catenin signaling causes defects in postnatal cartilage development. J Cell Sci 121: 1453–1465.
54. Nagayama M, Iwamoto M, Hargett A, Kamiya N, Tamamura Y, et al. (2008) Wnt/beta-catenin signaling regulates cranial base development and growth. J Dent Res 87: 244–249.
55. Kobayashi T, Lyons KM, McMahon AP, Kronenberg HM (2005) BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. Proc Natl Acad Sci U S A 102: 18023–18027.
56. Perry MJ, McDougall KE, Hou SC, Tobias JH (2008) Impaired growth plate function in bmpr-6 null mice. Bone 42: 216–225.
57. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J (2003) LRP1 role in vascular wall integrity and protection from atherosclerosis. Science 300: 329–332.
58. Boucher P, Li WP, Matz RL, Takayama Y, Auwerx J, et al. (2007) LRP1 functions as an atheroprotective integrator of TGFbeta and PDGF signals in the vascular wall: implications for Marfan syndrome. PLoS ONE 2: e448.
59. Zhou L, Takayama Y, Boucher P, Tallquist MD, Herz J (2009) LRP1 regulates architecture of the vascular wall by controlling PDGFβ-receptor-dependent phosphatidylinositol 3-kinase activation. PLoS ONE 4: e6922.
60. Glass DA, Iaakl K, Ahn JD, Starback M, Patel MS, et al. (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8: 751–764.
61. Lee NK, Sowa H, Himori E, Ferron M, Ahn JD, et al. (2007) Endocrine regulation of energy metabolism by the skeleton. Cell 130: 456–469.