Lrp4 expression by adipocytes and osteoblasts differentially impacts sclerostin’s endocrine effects on body composition and glucose metabolism

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Running Title: Lrp4 enables sclerostin endocrine actions

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Abstract
Sclerostin exerts profound local control over bone acquisition and also mediates endocrine communication between fat and bone. In bone, sclerostin’s anti-osteoclastic activity is enhanced by low-density lipoprotein receptor-related protein-4 (Lrp4), which facilitates its interaction with the Lrp5 and Lrp6 Wnt co-receptors. To determine whether Lrp4 similarly affects sclerostin’s endocrine function, we examined body composition as well as glucose and fatty acid metabolism in mice rendered deficient for Lrp4 in the adipocyte (AdΔLrp4) or the osteoblast (ObΔLrp4). AdΔLrp4 mice exhibit a reduction in adipocyte hypertrophy and improved glucose and lipid homeostasis, marked by increased glucose and insulin tolerance and reduced serum fatty acids, and thereby mirror the effect of sclerostin-deficiency on whole-body metabolism. Indeed, epistasis studies place adipocyte-expressed Lrp4 and sclerostin in the same genetic cascade that regulates adipocyte function. Intriguingly, ObΔLrp4 mice, which exhibit dramatic increases in serum sclerostin, accumulate body fat and develop impairments in glucose tolerance and insulin sensitivity, despite the development of a high bone mass phenotype. These data indicated that the expression of Lrp4 by both the adipocyte and osteoblast is required for normal sclerostin endocrine function and that the impact of sclerostin deficiency on adipocyte physiology is distinct from the effect on osteoblast function.

The comorbidity of obesity and osteopenia/osteoporosis illustrates the coordination of adipose and bone metabolism via endocrine communication and by common regulatory mechanisms. Adipose-derived hormones, like leptin and adiponectin, affect bone mass accrual through both direct and indirect effects on the osteoblast (1,2) and recent advances have identified bone-specific factors that affect adipocyte hypertrophy and insulin-sensitivity (3-5). Moreover, the fate-specification of bi-potential progenitors, present in the bone marrow and adipose stromal vascular fraction, to the osteoblastic and adipocytic lineages are reciprocally regulated by Map kinase (6), bone morphogenetic (7) and Wnt/β-catenin signaling (8,9).

Sclerostin, a cysteine-knot glycoprotein, is a potent inhibitor of bone acquisition that antagonizes Wnt/β-catenin signaling (10,11). Secreted primarily by bone matrix-embedded osteocytes, sclerostin binds the first β-propeller of the Wnt co-receptors low density lipoprotein receptor-related protein-5 (Lrp5) and Lrp6 expressed by osteoblasts and their progenitors. This interaction impedes the recognition of Wnt1 class ligands by Lrp5/6 (12-
Lrp4 enables sclerostin endocrine actions

14) and thereby prevents the formation of the ternary Wnt:Frizzled:Lrp5/6 complex necessary for the initiation of β-catenin signals that drive osteoblast commitment and the attainment of a mature phenotype (15,16).

We recently demonstrated that in addition to its local actions in bone, sclerostin fulfills an endocrine function that regulates body composition and adipocyte metabolism. Sost−/− mice accumulate less body fat and exhibit an increase in insulin sensitivity, in association with increased markers of Wnt/β-catenin signaling activation in adipose tissue depots and alterations in the ratio of anabolic to catabolic metabolism in adipocytes (3).

Pharmacological inhibition of sclerostin activity with neutralizing antibodies produces an identical phenotype, while ectopic sclerostin overexpression stimulates adipocyte hypertrophy and impedes glucose homeostasis. These data accord with a growing number of studies that document a correlation between serum sclerostin levels and metabolic disease in humans. Serum sclerostin levels are increased in type 2 diabetics (17-19) and are positively associated with fat mass (20,21) and tissue insulin resistance (22,23).

Within the bone microenvironment, the antianabolic actions of sclerostin are facilitated by Lrp4, a member of the same protein family as Lrp5 and Lrp6 (24). Mice globally deficient for this receptor exhibit impairments in embryonic limb development and perinatal lethality, likely the result of Lrp4’s function in neuromuscular junction formation (25,26), but point mutations in the extracellular domain of human LRP4 (R1170W and W1186S) are associated with a bone overgrowth phenotype (27) reminiscent of that in Sclerosteosis and Van Buchem disease patients that lack normal sclerostin protein production (28-30). Direct interaction of Lrp4 and sclerostin was confirmed in a mass spectroscopy screen by Leupin et al (27), who also demonstrated that genetic knockdown of the receptor in vitro ablated sclerostin’s inhibitory effect on osteoblast differentiation. Likewise, targeted ablation of Lrp4 expression in the osteoblast lineage (31,32) or pharmacological inhibition of the Lrp4:sclerostin interaction in vivo (31) dramatically increases bone formation and bone mass.

In this study, we explored the contribution of Lrp4 to sclerostin’s endocrine function by ablating its expression in adipose tissue and bone. Similar to its role in facilitating sclerostin function in bone, inhibiting Lrp4 function in adipocytes abolished sclerostin’s ability to enhance adipogenesis in vitro and resulted in a reduction in adipocyte hypertrophy and improved insulin sensitivity in vivo. Loss of Lrp4 function in osteoblasts leads to sclerostin overexpression and the opposite metabolic phenotype. Collectively, these data indicate that Lrp4 expressed by adipocytes and osteoblasts regulates normal sclerostin endocrine function by affecting its activity and expression, respectively.

Results

Lrp4 is required for sclerostin to enhance in vitro adipocyte differentiation

The ability of osteocyte-produced sclerostin to inhibit osteo-anabolic Wnt signaling is facilitated by its interaction with the Lrp4 receptor expressed by osteoblasts and their progenitors (27,32). Since Lrp4 is expressed in adipose tissue (Figure 1A and B) and by adipocytes induced to differentiation in vitro (Figure 1C), we predicted that Lrp4 also contributes to the metabolic actions of sclerostin. As a first step in testing this hypothesis, stromal vascular cells were isolated from the inguinal fat pads of mice containing Lrp4flox alleles and infected with adenovirus encoding Cre-recombinase to abolish Lrp4 expression (DLrp4, Figure 1D) or green fluorescent protein as a control. Cells were then cultured under adipogenic conditions in the presence of vehicle or recombinant mouse sclerostin (rSc1).

Consistent with our previous report (3), rSc1 treatment inhibited the expression of the Wnt target gene Axin2 (33) (Figure 1D) and enhanced the adipogenic differentiation of control cells as indexed by histochemical staining for accumulated lipids (Figure 1F) and qPCR analysis of Cebpa, Fabp4, Lpl and Pparg mRNA levels (Figure 1G). Dlp4 adipocytes exhibited increases in Axin2 mRNA (Figure 1D) and β-catenin protein levels (Figure 1E), an impairment in differentiation and were strikingly resistant to rSc1 treatment, which was unable to enhance lipid accumulation or the expression of adipocyte markers. A similar effect of Lrp4 loss of function was observed when radiolabeled tracers were used to assess adipocyte metabolism in vitro. Whereas rSc1 increased de novo fatty acid synthesis (Figure 1H), suppressed fatty acid oxidation (Figure 1J), and induced...
coordinate changes in anabolic and catabolic gene expression (Figure 1I and K) in control adipocytes. ΔLrp4 adipocytes were unaffected by rScl treatment. Importantly, loss of Lrp4 function did not affect the expression Dkk1, Sostdc1 and Agrin or the ability of Dkk1 to enhance adipocyte differentiation (Figure S1). Thus, Lrp4 is required for sclerostin to enhance adipocyte differentiation in vitro.

**AdΔLrp4 mice have small adipocytes and improved glucose metabolism**

To determine the role of Lrp4 in adipocytes in vivo, we crossed Lrp4<sup>fl</sup> mice with AdipoQ-Cre mice (34) to generate mice in which Lrp4 expression was ablated specifically in white and brown adipose (AdΔLrp4, Figure 1B and 2A). AdΔLrp4 mice were born at the expected Mendelian ratios (data not shown) and had normal body weight (Figure 2B), serum sclerostin levels (Figure 2C), and bone volume (Figure S2). In agreement with Lrp4’s role as a facilitator of Wnt signaling antagonism in bone, Axin2 expression (Figure 2D) and β-catenin protein levels (Figure 2E) were increased in the white adipose tissue of the mutant mice relative to controls.

We expected that the increase in Wnt signaling in white adipocytes of AdΔLrp4 mice would lead to the development of a lean phenotype similar to that observed in Sost<sup>−/−</sup> mice (3), but whole-body fat mass and the weights of individual fat pads in AdΔLrp4 were comparable to control littermates (Figure 2F-H). However, histological analysis of adipocyte morphology in both the gonadal and inguinal fat pads revealed significant reductions in adipocyte size in AdΔLrp4 mice relative to controls that led to an increase in adipocyte numbers per field (Figure 2I-L). The modest downregulation of genes associated with adipocyte differentiation (Figure 2M) together with alteration in genes involved in fatty acid synthesis and lipid catabolism (Figure 2N and O) suggest that the reduced adipocyte size is at least partially due to an inhibition of adipocyte hypertrophy and changes in the ratio of anabolic to catabolic metabolism. The maintenance of normal fat pad weights in the Lrp4 mutant mice is likely due to an increase in proliferation and the accumulation of small adipocytes as Ki67 staining in the stromal vascular fraction (Figure 2P), fat pad DNA content (Figure 2Q), and Ccnd1 expression were increased (Figure 2R), while the mRNA levels of cyclin-dependent kinase inhibitors were reduced in adipose tissue relative to controls. The expression of Dkk1 and Sostdc1 were not affected by the loss of Lrp4 function in white adipose (Figure S1) and the morphology of brown adipose tissue and liver were identical in the control and mutant mice (Figure S2).

Further analysis of AdΔLrp4 mice revealed an improvement in glucose metabolism. While random fed blood glucose levels were normal (Figure 3A), the mutant mice exhibited a significant reduction in serum insulin (Figure 3B), which is usually indicative of an increase in insulin sensitivity. In line with this idea, the mutants performed better in glucose tolerance tests (Figure 3C and D) and insulin tolerance tests (Figure 3E and F). The increase in insulin sensitivity is likely to be driven, at least in part, by changes in adipose tissue sensitivity as insulin-stimulated Akt phosphorylation was increased in the inguinal fat pad of the mutants (Figure 3G and H), and serum free fatty acid levels were reduced (Figure 3J) without a change in serum triglyceride levels (Figure 3I). Serum levels of leptin, adiponectin and osteocalcin were similar in the control and mutant mice (Supplementary Table I). The metabolic phenotype of female AdΔLrp4 mice mirrored that of male mutants (Figure S3). Therefore, adipocyte-specific Lrp4 mutants partially phenocopy the metabolic effects of sclerostin deficiency (3) and raise the possibility that Lrp4 mediates at least some of sclerostin’s endocrine function in white adipose tissue.

**Epistasis reveals a genetic interaction between adipocyte-expressed Lrp4 and sclerostin**

As a genetic test of the interaction between sclerostin and Lrp4 in adipocytes, we performed an epistasis study by crossing AdΔLrp4 mice with Sost<sup>+</sup>-/- mice to generate cohorts of compound heterozygous mice lacking one allele of the Sost gene globally and one allele of Lrp4 in adipocytes (AdΔLrp4<sup>+/−</sup>; Sost<sup>−/+</sup>) as well as the appropriate heterozygous controls (Sost<sup>−/+</sup> and AdΔLrp4<sup>+/−</sup>, See Experimental Procedures for breeding strategy). In support of the notion that sclerostin and adipocyte-expressed Lrp4 work in concert to regulate adipose tissue metabolism, the compound heterozygous
Lrp4 enables sclerostin endocrine actions

Mice exhibited an increase in Axin2 expression (Figure 4A) even though serum sclerostin levels were reduced by only 30% reduction (Figure 4B). More importantly, the compound mutants developed reductions in both whole-body fat mass and the weights of the gonadal and inguinal fat pads, while body weight remained normal (Figure 4C-F). Within white adipose tissue depots, adipocyte size (Figure 4G and H) was reduced in compound heterozygotes relative to controls and was accompanied by changes in metabolic gene expression that were similar to the genetic ablation of both Lrp4 alleles in adipocytes (Figure 4I). An increase in the abundance of multi-locular adipocytes was also evident in the compound heterozygotes, raising the possibility of an increase in the browning of this tissue. In all cases, Sost±/− and AdΔLrp4/+ mice were similar to controls.

The compound heterozygous mice also mirrored the improvements in glucose metabolism evident here in AdΔLrp4 mice (Figure 3) and previously in Sost−/− mice (3). Random fed blood glucose levels were comparable to controls, but serum insulin levels were significantly reduced in the compound heterozygotes (Figure 4J and K). Similarly, glucose tolerance (Figure 4L and M) and insulin tolerance (Figure 4N and O) were increased in the compound heterozygous mice, which also had significantly lower serum free fatty acid levels (Figure 4P and Q). Sost−/− and AdΔLrp4/+ were indistinguishable from controls in all of these measures. When taken together, these studies provide strong genetic evidence that Lrp4 facilitates sclerostin’s function in fat in a manner analogous to that in bone.

Lrp4-deficiency in adipocytes alters Bmp signaling

Since alterations in sclerostin function in adipocytes produce coordinate changes in Bmp signaling and the administration of noggin inhibits the effect of rScI on adipocyte differentiation in vitro (3), we predicted that the loss of Lrp4 expression would decrease the activation of this adipogenic pathway (30). Consistent with our hypothesis, Bmp4 mRNA expression levels and the phosphorylation of Smad1/5/9 were reduced in the inguinal fat pads of AdΔLrp4 mice (Figure 5A and B). Likewise, while rScI increased the expression of Bmp4 in in vitro cultures of control adipocytes, expression was reduced in ΔLrp4 cells and unresponsive to exogenous sclerostin (Figure 5C).

To ensure that regulation of Bmp4 was indeed downstream of Lrp4 in the control of adipogenesis, we next treated differentiating control and AdΔLrp4 adipocytes with recombinant Bmp4. Bmp4 treatment enhanced in vitro adipogenesis in control cultures which was marked by an increase in oil red o staining and an increase in adipogenic gene expression (Figure 5D and E). Importantly, ΔLrp4 cultures were fully responsive to Bmp4 treatment, which was able to rescue the defect in adipogenesis associated with Lrp4 loss of function. Similarly, Bmp4 increased fatty acid synthesis (Figure 5F) and affected the expression of metabolic genes in both control and ΔLrp4 cultures (Figure 5G and H) and in both cases reversed the changes induced by Lrp4 deficiency. Therefore, alterations in Bmp4 expression and activity similar to those evident in Sost−/− mice (3) appear to underlie the differentiation and metabolic defects associated with Lrp4 deficiency in adipocytes.

ObΔLrp4 mice over-produce sclerostin and accumulate adipose tissue

While the loss of Lrp4 function in the osteoblast lineage increases bone mass, it also leads to the over-expression of Sost via an unknown compensatory mechanism (31,32). We questioned whether this de-regulation of sclerostin expression would in turn lead to alterations in body composition and metabolism by generating cohorts of male osteoblast-/osteocyte-specific Lrp4 mutants (Lrp4lox, Ocn-Cre, ObΔLrp4) and control littermates.

As previously reported (31,32), the ablation of Lrp4 expression in osteoblasts and osteocytes (Figure 6A), increased bone volume (Figure 6C and D) and induced a more that 4-fold increase in serum sclerostin levels (Figure 6B). Analysis of Sost mRNA levels in the femur indicated that the increase in serum abundance is at least partially due to increased expression (Figure 6A). The ObΔLrp4 mice maintained normal body weight (Figure 6E), but consistent with the idea that sclerostin favors adipose tissue accumulation via an endocrine mechanism the mutants exhibited significant increases in whole body fat mass (Figure 6F) and the weights of white adipose tissue depots (Figure 6H) and a reduction in lean tissue mass (Figure 6G).
Additionally, the osteoblast-specific mutants exhibited a significant increase in adipocyte hypertrophy in the gonadal and inguinal fat pads (Figure 6I and J) coincident with a reduction in Wnt target gene expression and increased expression of genes involved in de novo fatty acid synthesis (Figure 6K).

Glucose and lipid metabolism were also negatively affected by Lrp4 deficiency in osteoblasts and osteocytes and the resulting overproduction of sclerostin. Random fed ObΔLrp4 mice were hyperglycemic (Figure 6L) and hyperinsulinemic (Figure 6M) when compared to control littermates and performed poorly in standard glucose tolerance (Figure 6N and O) and insulin tolerance tests (Figure 6P and Q). Serum triglyceride levels (Figure 6R and S) were also elevated in the osteoblast-/osteocyte-specific mutants suggesting the development of a dyslipidemia but serum leptin, adiponectin and under-carboxylated osteocalcin were similar in the control and mutant mice (Supplementary Table I).

Lrp4 is well-expressed by white adipocytes, with mRNA levels commensurate with those found in bone, and it appears to function in a manner analogous to that described in osteoblasts. Our in vitro studies demonstrated that ablating Lrp4 expression produces a defect in the adipogenic differentiation of stromal vascular cells (which could be due to the presence of sclerostin in the serum used during in vitro culture) and renders adipocytes resistant to the effects of recombinant sclerostin on both adipogenesis and the ratio of catabolic to anabolic metabolism. Importantly, the adipogenic effects of Dkk1, which may also interact with Lrp4 (39), remained intact and the mRNA levels of other interacting proteins remained largely normal. The metabolic phenotype of mice lacking Lrp4 in adipocytes partially mirrors that of Sost-/- mice (3). Both mutants exhibit an increase in markers of Wnt signaling in white adipose tissue, reductions in adipocyte size, and improvements in insulin sensitivity that was at least partially driven by an increase in the sensitivity of the adipocyte. The development of these phenotypes also appears to be secondary to modifications in the same pathway as both AdΔLrp4 and Sost-/- mice exhibited reductions in Bmp4 expression and Smad phosphorylation, which has previously been reported to induce adipocyte hypertrophy by stimulating fatty acid synthesis (35). Further, our genetic epistasis experiment places Lrp4 and sclerostin in the same genetic pathway that regulates adipocyte physiology.

We expected AdΔLrp4 mice would exhibit the decrease in adipose tissue mass evident in Sost-/- mice, but assessment of body composition by qNMR analysis and at necropsy revealed that fat mass is equivalent in the Lrp4 mutants and their control littermates. Our histological studies suggest that the mutants are able to maintain normal fat mass by accumulating small adipocytes likely through a compensatory increase in proliferation in the stromal vascular compartment and potentially the subsequent commitment to the adipocyte lineage. Additional studies including lineage tracing will be required to more fully evaluate the impact of sclerostin and Lrp4 on the proliferation and differentiation of cells within this compartment. Since Lrp4 expression increases during the differentiation process, these findings clearly indicate that Lrp4 facilitates the effects of sclerostin on adipocyte hypertrophy. However, we
cannot rule out a possible role for sclerostin and Lrp4 in adipocyte specification since the AdipoQ-Cre transgene we utilized primarily drives recombination in mature adipocytes and does not impact the stromal vascular compartment that contains progenitor cells (40,41). Examining the interaction of sclerostin and Lrp4 at this earlier stage of differentiation will require additional studies using stage-specific Cre lines and lineage tracing studies.

A surprising finding in these studies is the lack of an effect of the genetic ablation of Lrp4 in adipocytes on serum sclerostin levels. Most endocrine interactions are associated with feedback loops, wherein the level of signaling in the target tissue influences the production and release of the endocrine factor. According to this paradigm, loss of Lrp4 function in adipocytes would be expected to increase sclerostin production and perhaps indirectly impact skeletal homeostasis. Our data indicates that in mice fed a chow diet signals downstream of the sclerostin:Lrp4 interaction do not signal back to bone. It remains to be seen whether signaling from adipocytes regulates sclerostin production under obesogenic conditions, but the recent observation that PPARγ contributes to the regulation of sclerostin expression in osteocytes could represent a potential mechanism (42).

The increase in sclerostin serum levels following the genetic ablation of Lrp4 in the osteoblast lineage is consistent with previous studies (31,32), which suggested a sclerostin chaperone action for Lrp4 that retains the protein within the bone microenvironment because an increase in expression was not observed. Our studies suggest the sclerostin:Lrp4 interaction may also induce feedback inhibition of sclerostin expression in the osteocyte as Sost mRNA levels were increased in the femoral bone of the osteoblast-specific mutants. The discrepancy between our findings and those of Chang et al (31) could be due to the age or genetic background of the mice. Nonetheless, loss of Lrp4 function in the osteoblast is sufficient to alter body composition and phenocopy the effects of AAV-mediated Sost overproduction (3), which include the accumulation of white adipose tissue and the development of impaired glucose homeostasis. Thus, the expression of Lrp4 by two cell types, the osteoblast and the adipocyte, is required for normal sclerostin endocrine actions.

The phenotypes of these mutant mice are also informative with regard to the interactions of sclerostin’s effects on metabolism and bone mass. A number of recent studies, including some from our own laboratory, indicate that Wnt signaling regulates the intermediary metabolism of the osteoblast and thereby influences metabolic homeostasis (43-46). The coexistence of a high bone mass phenotype due to increased Wnt signaling and increased fat mass in ObΔLrp4 mice indicates that the effect of sclerostin on adipose tissue mass and metabolism is distinct from its effect on bone metabolism.

In summary, these new studies elaborate on the mechanisms by which sclerostin influences whole body metabolism and the mechanisms of cross-talk between adipose tissue and bone. We expect they will have important implications for the use of sclerostin neutralizing therapeutics that are intended to treat osteopenia/osteoporosis but may also be useful in the treatment of obesity and metabolic disease.

**Experimental Procedures**

**Mouse models** - The Institutional Animal Care and Use Committee of the Johns Hopkins University approved all procedures involving mice. Lrp4^floX/floX mice, in which exon 1 of the Lrp4 gene is flanked by loxP sites, were described previously (26,32). To generate adipocyte- and osteoblast-specific mutants, mice were crossed with AdipoQ-Cre mice (34) and Ocn-Cre mice (47), respectively. The resulting Lrp4^floX/+; Cre^-/- progeny were then backcrossed to Lrp4^floX/floX mice to generate the tissue specific knockouts. Breeding pairs of Lrp4^floX/floX; Cre^-/- and Lrp4^floX/floX mice were used to generated control (Lrp4^floX/floX mice) and knockout littermates for all studies. Sost^-/- mice (Sost<sup>m1(KOMP)Vlcg</sup>) were originally obtained from the KOMP repository and reconstituted as previously described (3). For genetic epistasis studies, Lrp4^floX/floX; AdipoQ-Cre^-/- mice were cross-bred with Sost^-/- mice to generate littermates with the four required genotypes: Sost<sup>+</sup>; Lrp4<sup>flox</sup>/flox (referred to as control), Sost<sup>+</sup>; Lrp4<sup>flox</sup>/flox (referred to as AdΔLrp4/+), Sost^-/-; Lrp4<sup>flox</sup>/flox (referred to as Sost^-/-), and Sost^-/-; Lrp4<sup>flox</sup>/flox (referred to as AdΔLrp4/+; Sost^-/-).
All mice were maintained on a C57BL/6 background. Mice were housed on ventilated racks on a 14 hr light/10 hr dark cycle and fed ad libitum with a standard chow diet (Extruded Global Rodent Diet, Harlan Laboratories).

Culture of primary adipocytes - Primary adipocyte precursors were harvested from the inguinal fat pads by collagenase digestion as previously described (48) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Sigma-Aldrich). Lrp4 gene deletion in vitro was induced by infection with adenovirus directing the expression of Cre-recombinase (MOI 100, Vector Biolabs). An adenovirus directing the expression of green fluorescent protein was used as a control. Upon reaching confluence, cells were induced to differentiate by treatment with 0.5 mM 3-isobutyl-1-methylxanthin (IBMX), 1 µM dexamethasone, and 167 nM insulin for 2 days, followed by continued culture in 167 nM insulin, which was changed every two days thereafter until analysis. Recombinant mouse sclerostin (R&D Systems) and Bmp4 (PeproTech) were replaced with each media change. Oil red o staining was carried out according to standard technique.

Fatty acid metabolism - Fatty acid oxidation by cultured adipocytes was measured in flasks with stoppers equipped with center wells as previously described (49). Reactions were incubated at 37°C in media containing 0.5 mM L-carnitine, 0.2% BSA and 14C-oleate (PerkinElmer). Expired 14CO2 was captured and counted by the addition of 1N perchloric acid to the reaction mixture and 1M NaOH to the center well containing Whatman filter paper. To measured de novo lipogenesis, cellular lipids were collected by the Folch method 3 hours after administration of 0.1µCi 3H-acetate.

Gene expression studies - Total RNA was extracted using TRizol (Life Technologies). For adipose tissue, samples were centrifuged prior to RNA purification to remove excess lipid. Bone samples were washed free of marrow before RNA isolation. Reverse transcriptase reactions were carried out using 1 µg of RNA and the iScript cDNA Synthesis system (Bio-Rad). Real-time qPCR was carried out using iQ Sybr Green Supermix (Bio-Rad) and primer sequences obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.htm). Reactions were normalized to endogenous 18S reference transcripts. Antibodies for Western blotting were obtained from Cell Signal Technologies: phospho-Akt (cat no. 4060), Akt (cat no. 4685), phospho-Smad1/5/9 (cat no. 13820), Smad1 (cat no. 9743), or Invitrogen: Lrp4 (cat no PA5-68218), Lrp5 (36-5400), Lrp6 (cat no. PA5-67902).

Metabolic phenotyping and bioassays - Whole-body fat and lean mass were assessed by qNMR (Echo MRI). Plasma triglycerides and free fatty acids were measured colorimetrically (Sigma) in plasma collected 3 hours after the initiation of the light cycle. Serum sclerostin and insulin were measured in plasma by ELISA (Alpco). Glucose levels were measured using a Bayer Contour handheld glucose monitor. For glucose tolerance testing, glucose (2 g/kg BW) was injected IP after a 6 hour fast. For insulin tolerance testing, mice were fasted for 4 hr and then injected IP with insulin (0.25 U/kg BW). Insulin signaling in WAT was assessed by injection of insulin (1 U/kg BW) into the portal vein before excising tissue and snap freezing for immunoblot analysis. Tissue for histological analysis were collected at necropsy, weighed, and then fixed in 4% paraformaldehyde before embedding and sectioning. Adipocyte size was assessed using ImageJ. DNA was isolated from WAT using the Quick-DNA Universal kit (Zymo Research) following proteinase K digestion. Ki67 (Abcam, Ab15580) staining was performed on frozen adipose tissue sections according to standard immune-histochemical techniques.

Imaging - High-resolution images of the mouse femur were acquired using a desktop microtomographic imaging system (Skyscan 1275, Bruker) in accordance with the recommendations of the American Society for Bone and Mineral Research (ASBMR) (50). Bones were scanned at 65 keV and 153 µA using a 1.0 mm aluminum filter with an isotropic voxel size of 10 µm. Trabecular bone parameters were assessed in the 500 µm proximal to the growth plate and extending for 2 mm (200 CT slices).

Statistical Analysis - All results are expressed as mean ± SEM. Statistical analyses were performed using unpaired, two-tailed Student’s t or ANOVA.
Lrp4 enables sclerostin endocrine actions

tests followed by post hoc tests. A p-value less than 0.05 was considered significant. In all figures, * p ≤ 0.05.

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Lrp4 enables sclerostin endocrine actions

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**Footnotes**
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The abbreviations used are: Lrp4, low-density lipoprotein receptor-related protein-4; Lrp5, low-density lipoprotein receptor-related protein-5; Lrp6, low-density lipoprotein receptor-related protein-6, rScl, recombinant mouse sclerostin; qPCR, Quantitative Polymerase Chain Reaction;

**Figure Legends**

**Figure 1. Lrp4 mediates the adipogenic effects of sclerostin in vitro.** (A) qPCR analysis of Lrp4 expression in femur, gWAT, iWAT and BAT. (B) Immunohistochemical detection of Lrp4 expression in inguinal adipose tissue of control and AdΔLrp4 mice. (C) Western blot analysis of Lrp4, Lrp5, and Lrp6 expression during the in vitro adipogenic differentiation of stromal vascular cells isolated from iWAT. (D) qPCR analysis of Lrp4 and Axin2 mRNA levels in control and ΔLrp4 adipocyte cultures treated with vehicle or recombinant mouse sclerostin (rScl) for 7 days. (E) Western blot analysis of β-catenin protein...
Lrp4 enables sclerostin endocrine actions

levels in control and ΔLrp4 adipocytes. (F) Oil red O staining of primary adipocyte cultures treated with vehicle or recombinant mouse sclerostin. 20X magnification. Relative absorbance was calculated after stain extraction. (G) qPCR analysis of markers of adipocyte differentiation. (H) Relative de novo lipogenesis measured by the incorporation of \(^3\)H-acetate into cellular lipids. (I) qPCR analysis of enzymes involved in fatty acid synthesis. (J) Relative oleate oxidation measured by the conversion of \(^1\)4C-oleic acid to \(^1\)4CO\(_2\). (K) qPCR analysis of Cpt1a and Ppargc1a mRNA levels. In vitro studies were repeated in at least 2 independent experiments (n = 6-9 replicates). All data are represented as mean ± the SEM. *, p <0.05 vs Con unless otherwise indicated. Scale bar is 200µm.

Figure 2. Loss of Lrp4 function alters adipocyte physiology. (A) qPCR analysis of Lrp4 mRNA levels in gonadal, inguinal and intrascapular brown adipose tissue (n = 6 mice/group). (B) Body weight was assessed weekly (n = 10 mice/group). (C) Serum sclerostin levels in 16 week old male control and AdΔLrp4 mice (n = 8 mice/group). (D) qPCR analysis of Axin2 expression in the fat pads of control and AdΔLrp4 mice (n = 6 mice/group). (E) Western blot analysis of B-catenin protein levels in the inguinal fat pad of control and AdΔLrp4 mice. (F-G) qNMR analysis of fat mass and lean mass (n = 8 mice). (H) Adipose depot weights of 16 week old male control and AdΔLrp4 mice (n = 6-7 mice). (I) Representative histological sections of the gonadal and inguinal fat pads. 10X magnification. (J) Quantification of adipocyte number per 10X magnified field (n = 6-7 mice). (K-L) Frequency distribution of adipocyte size in the gonadal and inguinal fat pads (n = 6-7 mice). (M-O) qPCR analysis of genes associated with adipocyte differentiation, de novo fatty acid synthesis, fatty acid catabolism and adipose tissue browning. (P) Ki67 immunostaining in the inguinal fat pads of control and AdΔLrp4 mice. Merged FITC and bright field images are shown. (Q) Relative DNA content in the gonadal and inguinal fat pads (n = 4-5 mice). (R) qPCR analysis of cell cycle regulator expression in the inguinal fat pad of control and AdΔLrp4 mice (n = 6 mice/group). All data are represented as mean ± the SEM. *, p <0.05. Scale bar is 200µm.

Figure 3. Loss of Lrp4 function in adipocytes affects glucose and lipid metabolism. (A) Random fed glucose levels in 16 week old male control and AdΔLrp4 mice (n = 10 mice/group). (B) Random fed insulin levels (n = 8 mice/group). (C-D) Glucose tolerance testing (GTT) and area under the curve analysis (n = 8-9 mice/group). (E-F) Insulin tolerance testing (ITT) and area under the curve analysis (n = 7-8 mice/group). (G-H) Western blot analysis and quantification of Akt phosphorylation in the inguinal adipose tissue before and after insulin stimulation (n = 6 mice/group). (I-J) Serum triglyceride and free fatty acid levels in random fed control and AdΔLrp4 mice (n = 10 mice/group). All data are represented as mean ± the SEM. *, p <0.05.

Figure 4. Sclerostin and adipocyte-expressed Lrp4 are in the same genetic cascade. (A) qPCR analysis of Lrp4 and Axin2 mRNA levels in the inguinal fat pad of 16 week old control, Sost\(^{+/-}\), AdΔLrp4/+, and AdΔLrp4/++; Sost\(^{+/-}\) mice (n = 5-6 mice/group). (B) Serum sclerostin levels (n = 7-10 mice/group). (C) Body weights at 16 weeks of age (n = 7-10 mice/group). (D-E) qNMR analysis of fat mass and lean mass (n = 7-10 mice/group). (F) Adipose depot weights of 16 week old male mice (n = 7-10 mice/group). (G-H) Representative histological sections (10X magnification) and frequency distribution of adipocyte size in the inguinal fat pad (n = 7-10 mice/group). (I) qPCR analysis of genes involved in de novo fatty acid synthesis and fatty acid catabolism (n = 5-6 mice/group). (J) Random fed glucose levels (n = 7-10 mice/group). (K) Random fed insulin levels (n = 7-10 mice/group). (L-M) Glucose tolerance testing (GTT) and area under the curve analysis (n = 7-10 mice/group). (N-O) Insulin tolerance testing (ITT) and area under the curve analysis (n = 7-10 mice/group). (P-Q) Serum triglyceride and free fatty acid levels in random fed control and AdΔLrp4 mice (n = 7-10 mice/group). All data are represented as mean ± the SEM. *, p <0.05. Scale bar is 200µm.
Figure 5. Bmp signaling is reduced in Lrp4 deficient adipocytes. (A) qPCR analysis of Bmp4 mRNA levels in iWAT of 16 week old control and AdΔLrp4 mice (n = 6 mice/group). (B) Immunoblot analysis of phosphorylated Smad1/5/9 levels in iWAT (results are representative of blots for n = 6 mice/group). (C) qPCR analysis of Bmp4 mRNA levels in control and ΔLrp4 adipocyte cultures treated with vehicle or rScl for 7 days. (D) Oil red O staining of primary adipocyte cultures treated with vehicle or recombinant Bmp4. 20X magnification. Relative absorbance was calculated after stain extraction. (E) qPCR analysis of markers of adipocyte differentiation. (F) Relative de novo lipogenesis measured by the incorporation of $^3$H-acetate into cellular lipids. (G) qPCR analysis of enzymes involved in fatty acid synthesis. (H) qPCR analysis of Cpt1a and Ppargc1a mRNA levels. In vitro studies were repeated in at least 2 independent experiments (n = 6-12 replicates). All data are represented as mean ± the SEM. *, p <0.05. Scale bar is 200µm.

Figure 6. Loss of Lrp4 function in the osteoblast increase sclerostin expression and fat mass. (A) qPCR analysis of Lrp4 and Sost mRNA levels in the femur of 12 week old control and ObΔLrp4 mice (n = 8 mice/group). (B) Serum sclerostin levels (n = 8 mice/group). (C-D) Representative microCT images and quantification of trabecular bone volume per tissue volume (BV/TV) in the distal femur (n = 5 mice/group). Scale bar is 2mm. (E) Body weight was assessed weekly (n = 10-11 mice/group). (F-G) qNMR analysis of fat mass and lean mass (n = 9-13 mice/group). (H) Adipose depot weights at 12 weeks of age (n = 11-12 mice/group). (I) Representative histological sections of the gonadal and inguinal fat pads. 10X magnification. Scale bar is 200µm. (J) Frequency distribution of adipocyte size in the inguinal fat pad (n = 5 mice). (K) qPCR analysis of gene expression in the inguinal fat pad (n = 6 mice/group). (L) Random fed glucose levels (n = 12-13 mice/group). (M) Random fed insulin levels (n = 8 mice/group). (N-O) Glucose tolerance testing (GTT) and area under the curve analysis (n = 12-13 mice/group). (P-Q) Insulin tolerance testing (ITT) and area under the curve analysis (n = 9-10 mice/group). (R-S) Serum triglyceride and free fatty acid levels in random fed control and AdΔLrp4 mice (n = 8 mice/group). All data are represented as mean ± the SEM. *, p <0.05.
Figure 1
Figure 2
Figure 3.
Figure 4.
Figure 5.
Figure 6
Lrp4 expression by adipocytes and osteoblasts differentially impacts sclerostin's endocrine effects on body composition and glucose metabolism
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