Inhibiting Wnt Secretion Reduces High Bone Mass Caused by Sost Deficiency or Point Mutations in Lrp5

Bart Williams (✉ bart.williams@vai.org)  
Van Andel Institute  https://orcid.org/0000-0002-5261-5301

Cassandra Diegel  
Van Andel Research Institute

Gabrielle Foxa  
Van Andel Institute

Mitchell McDonald  
Van Andel Research Institute

Zachary Madaj  
Van Andel Institute

Ina Kramer  
Novartis Institutes for Biomedical Research

Charles Moes  
Novartis Institutes for Biomedical Research

Sabine Guth  
Novartis Institutes for Biomedical Research

Jun Liu  
Novartis Institutes for Biomedical Research

Jennifer Harris  
Novartis Institutes for Biomedical Research

Michaela Kneissel  
Novartis Institutes for Biomedical Research

Article

Keywords:

Posted Date: January 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2436798/v1
Abstract

Proper regulation of Wnt signaling is critical for normal bone development and homeostasis. Mutations in several Wnt signaling components, which increase the pathway's activity in the skeleton, cause high bone mass in human patients and mouse models. Increased bone mass is often accompanied by severe headaches from increased intracranial pressure, which can lead to fatality and loss of vision or hearing due to the entrapment of cranial nerves. In addition, progressive bossing of the forehead and mandibular overgrowth occur in almost all patients. Treatments that would provide symptomatic relief in these patients are limited. Porcupine-mediated palmitoylation is necessary for Wnt secretion and binding to the Frizzled receptor. Chemical inhibition of porcupine is a highly selective inhibitor of all Wnt signaling. We treated three different mouse models of high bone mass caused by aberrant Wnt signaling: homozygosity for loss-of-function in SOST, which models Sclerosteosis, and two strains of mice carrying different point mutations in LRP5 (equivalent to human G171V and A214V) with porcupine inhibitors for 5–6 weeks. Treatment significantly reduced both trabecular and cortical bone mass in all three models. This demonstrates that porcupine inhibition is potentially therapeutic for symptomatic relief in patients who suffer from these disorders and further establishes that the continued production of Wnts is necessary for sustaining high bone mass in these models.

Introduction

Families with inherited mutations in Wnt/β-catenin signaling have dramatic alterations in bone mass [1]. Mutations in humans in the low-density lipoprotein receptor-related protein 5 (Lrp5) lead to overactive Wnt/β-catenin signaling and enhanced bone formation, causing autosomally dominant high-bone-mass (HBM). In contrast, loss of Lrp5 causes Osteoporosis pseudoglioma, an autosomal recessive disorder that causes pediatric osteoporosis [2]. Gain of function missense mutations in LRP5 that encode for proteins incapable of binding the secreted inhibitors DKK1 and Sclerostin (SOST) cause HBM in an autosomal dominant manner [3, 4]. Subsequent studies reported that patients homozygous for loss-of-function mutations in the SOST gene developed extreme HBM, a disease known as Sclerosteosis [5, 6]. Another HBM disorder, van Buchem's Disease, was subsequently found to be caused by homozygosity for a 52-kb deletion that removes a SOST-specific regulatory element approximately 35 kb downstream of the SOST gene [7, 8]. These observations were the driving force in developing the anti-Sclerostin antibodies to treat osteoporosis, a process that culminated in the FDA approval of Romosozumab [9, 10].

While patients with Van Buchem disease or HBM associated with LRP5 mutations have a normal lifespan, they can suffer from neuralgia, headaches, deafness, and facial palsy [11]. Sclerosteosis patients have even more severe symptoms that can reduce lifespan. These include cranial vascular and neural foraminal narrowing and reduced intracranial volume, frequent seventh nerve palsy, progressive optic and cranial neuropathies, mixed hearing loss, brainstem compression, intracranial hypertension with increased elastance, and sudden, premature death [12–15]. Thus, it is critical to identify approaches that can reduce the pain and morbidity seen in these patients to improve their quality of life.
Wnt/β-catenin signaling is initiated at the plasma membrane via the coordinated action of a Frizzled (Fzd) receptor and an LRP5 or LRP6 co-receptor following Wnt ligand binding [16, 17]. Extracellular Wnt binding to its cognate receptor and co-receptor initiates intracellular Wnt signaling, often via β-catenin. Paracrine-acting SOST inhibits Wnt/β-catenin signaling by binding to LRP5/6 and Frizzled co-receptors [18–20]. Notably, Wnts must be acylated and glycosylated by porcupine (PORCN), an endoplasmic-reticulum resident O-acyltransferase, for their secretion [21–23]. Pharmacological inhibition of PORCN with PORCN inhibitors decreases growth of Wnt-driven mammary cancers in vivo [22–26], supporting their use in cancer clinical trials [27–29]. However, treatment with PORCN inhibitors or other broad Wnt signaling inhibitors significantly reduces bone mass in both mice and humans, necessitating careful administration of these agents to reduce on-target bone side effects [27, 29–31].

Our previous study demonstrates that a 4-week treatment of wild-type (WT) mice with PORCN inhibitors substantially reduced bone mass compared to vehicle-only treated animals [27]. We wanted to extend this work to test whether PORCN inhibitors could reverse the abnormal bone density observed in mice with HBM induced by either loss of function mutations in Sost or by point mutations in LRP5. To do so, we analyzed two models of human HBM disease, one evaluating mice with the Lrp5 human HBM missense mutations, Lrp5A214V and Lrp5G171V, knocked into the endogenous Lrp5 locus [32, 33], and the other with mice deficient in Sost [34] to model Sclerosteosis. Both mouse models exhibit higher bone strength and material properties, i.e. a HBM phenotype [34] [32, 33]. Mice were subjected to daily treatment with the potent and selective PORCN inhibitor, LGK974 (WNT974) or a related small-molecule inhibitor with further improved physicochemical properties, GNF-6231 [35], for 5–6 weeks.

In this study, we investigated whether porcupine inhibitor treatment was sufficient to restore bone mass in the HBM mice to WT levels to assess whether using PORCN inhibitors may constitute a potential treatment option to alleviate sclerosing symptoms in HBM patients with mutations in SOST or LRP5.

Results

Blocking Wnt secretion reduces bone mass and density in Sost KO mice

We previously demonstrated that female mice deficient in Sost show a more pronounced cancellous HBM phenotype than Sost-deficient male littermates [36]. To understand whether PORCN inhibition (PORCNi) can reduce HBM and bone architecture defects in Sost KO mice, we focused on female gender only. We treated adult homozygous Sost knockout (KO) and wild-type (WT) female mice twice a day orally with 0.3, 1, or 3 mg/kg of GNF-6231 or vehicle control for 6 weeks (Fig. 1a). Peripheral quantitative computed tomography (pQCT) was performed on both the distal femur metaphysis and proximal tibia metaphysis before treatment and at 5 weeks during treatment for longitudinal bone morphology analysis. All doses of PORCNi significantly decreased femoral and tibial total bone mineral content and density, trabecular BMD, and cross-sectional cortical thickness in a dose-dependent manner in WT and Sost KO mice as measured by pQCT (Fig. 1b-e). Even at the lowest inhibitor dose, normal late-stage long bone gain was
blocked in WT mice. Similarly, elevated bone gain in Sost KO animals was reduced at the lowest dose level, and bone loss was induced at the highest dose. Bone gain was similarly suppressed in the cancellous and cortical bone compartments (Fig. 1f-i). Ex vivo microstructural analyses of the femur after the study by high-resolution micro-CT (µCT) confirmed significant reductions in cancellous (from distal femur) and cortical (from femoral mid-shaft) bone morphometric indices. Dramatic changes in bone mineral density (BMD), bone/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and number (Tb.N) were detectable in a dose-dependent manner following PORCNi treatment (Fig. 2). While all treatments reduced the sclerosing bone gain in Sost KO female mice, the highest dose of 3 mg/kg was best at decreasing both cortical and cancellous bone parameters near to those seen in WT vehicle-treated mice (Fig. 2a-j). At this dose, a 66% reduction in trabecular BMD was observed in Sost KO mice (Fig. 2b) and a 68% decrease in bone volume over total tissue volume (BV/TV) (Fig. 2c) relative to vehicle-treated Sost KO mice. Tb.Th decreased by 13%, Tb.N reduced by 24%, while spacing between trabeculae, Tb.Sp, increased by 40% compared to Sost KO vehicle-treated controls (Fig. 2c-e). These parameters indicate a substantial reduction of the HBM phenotype seen in sclerostin-deficient bone. However, note that 6-week PORCNi treatment in the Sost KO mice was not sufficient to completely reverse the HBM phenotype already present at the pre-treatment baseline, compared to WT vehicle-treated controls.

Cortical bone morphometric indices were also significantly changed in a dose-dependent manner (Fig. 2g). In Sost KO females treated with 3 mg/kg of inhibitor, diaphyseal cortical tissue mineral density (TMD) showed no statistical significance compared to vehicle-treated Sost KO mice (Fig. 2h), while cortical bone area fraction (CAF) was decreased by 24%, and cross-sectional cortical thickness (Ct.Th) decreased by 15%. This indicates that PORCNi treatment reduces radial bone growth compared to vehicle-treated Sost KO mice without impacting cortical bone quality. Note that a similar change in cortical bone thickness also occurs in the WT mice following PORCNi treatment. Thus, PORCN inhibition was sufficient to normalize bone gain in Sost deficient mice to wild-type levels during the late-stage long bone growth period.

**Blocking Wnt secretion reverses HBM observed in LRP5 mutants**

We wanted to determine whether PORCNi and, by proxy, inhibition of Wnt ligand secretion can also reduce the HBM and bone architecture defects of Lrp5A214V and Lrp5G171V mice. Thus, we treated adult male and female heterozygous mutants, hereafter Lrp5 A/+ (A/+) and Lrp5 G/+ (G/+), and WT mice with 3 mg/kg LGK974 daily for 5 weeks (Fig. 3a). Because previous studies showed similar increases in bone mass in male and female mice, we included both in these studies. Mice heterozygous for these mutations and WT littermate controls were treated with vehicle or LGK974. Whole body areal dual-energy X-ray absorptiometry (DEXA) scans were collected before treatment and every 7 days thereafter (Fig. 3b-c) to analyze longitudinal BMD changes. After 5 weeks of treatment, femurs were collected, and µCT and histomorphometric analyses were performed. We quantified trabecular bone in the distal femur (Fig. 4) and cortical bone in the femoral midshaft (Fig. 5).
Longitudinal analysis of the whole-body areal BMD (aBMD) by DEXA gave us insight into the effects of LGK974 on overall bone architecture in both WT and Lrp5 mutant heterozygotes. Note that aBMD is significantly higher in vehicle-treated Lrp5 A/+ and Lrp5 G/+ male and female mice than their vehicle-treated WT littermates (Fig. 3b,c). aBMD significantly decreased in both female and male WT and A/+ treated animals starting 14 days after treatment (Fig. 3b), whereas Lrp5 G/+ animals responded differently to LGK974 treatment. In female Lrp5 G/+ mice, significant aBMD changes were first observed after 3 weeks of treatment (Fig. 3c). In contrast, male Lrp5 G/+ had minor but significant aBMD changes starting after the first week of treatment (Fig. 3c).

Bone ultrastructure and density is consistently and significantly elevated in vehicle-treated Lrp5 A/+ and Lrp5 G/+ mice for cancellous (Figs. 3, 4), and cortical bone (Fig. 5, 6) density parameters measured, in support of earlier work [32, 33]. As previously observed [33], average trabecular BMD was dramatically increased in vehicle-treated Lrp5 A/+ and Lrp5 G/+ mice compared to their vehicle-treated WT littermates (Fig. 4b). The cancellous bone framework was decreased in both sexes because of LGK974 treatment (Fig. 4a), although female mice had a more significant response to LGK974 treatment. Treatment with LGK974 significantly decreased BMD, very close to levels seen in WT vehicle-treated littermates in both female Lrp5 A/+ and Lrp5 G/+ mice (Fig. 4b). LGK974 treatment also significantly decreased BV/TV in both sexes of Lrp5 A/+ and Lrp5 G/+ mice compared to their vehicle-treated WT littermates (Fig. 4c). Moreover, Tb.Th and Tb.N in both LGK974 treated models decreased to levels closer to vehicle-treated WT mice (Fig. 4d,f). Similar to PORCNi treatment of Sost KO mice (Fig. 2), Tb.Sp increased in all conditions following LGK974 treatment (Fig. 4e). These parameters also show that treating Lrp5 HBM models with porcupine inhibitors can substantially reduce the HBM phenotype.

To test whether LGK974 treatment could normalize the altered bone architecture of Lrp5 mutant mice, we also quantified cortical bone density and structure (Fig. 5). The increase in cortical TMD, CAF, and bone cross-sectional cortical thickness (Ct.Th) observed in Lrp5 mutant variants was significantly reduced following LGK974 treatment (Fig. 5b-d). All these parameters demonstrate that cortical bone architecture became normalized compared to WT vehicle-treated littermates.

Like the Sost KO model, µCT analysis revealed that LGK974 successfully normalized the HBM phenotypes in Lrp5 A/+ and Lrp5 G/+ mice. When vehicle-treated WT mice were compared to LGK974-treated Lrp5 A/+ and Lrp5 G/+ mice, parameters for both cancellous and cortical bone were very similar. Thus, the HBM effects driven by Lrp5 A/+ and Lrp5 G/+ mutations were mitigated with the PORCNi to normalize bone volume and architecture.

**Blocking Wnt ligand secretion reduces periosteal and endocortical bone formation in Lrp5 mutants**

To evaluate bone remodeling in our experiments, we employed dynamic histomorphometry using mineral apposition rate (MAR) and bone formation rate (BFR) to assess the cellular nature of the bone changes. Cortical bone was fluorochrome-labeled via calcein injections 10 days apart (at 17 days and 7 days before sacrifice; Fig. 3a). While no significant decrease in endocortical mineral apposition rate (Ec.MAR)
was observed in vehicle or LGK974-treated Lrp5 A/+ mice, Ec.MAR was significantly decreased in vehicle-treated Lrp5 G/+ mice compared to the vehicle-treated WT mice (Fig. 6b). In male Lrp5 G/+ mice, treatment with LGK974 increased Ec.MAR. Endocortical bone formation rate (Ec.BFR) trended upward in vehicle-treated male Lrp5 A/+ mice and was significantly elevated in Lrp5 G/+ mice compared to the WT vehicle group (Fig. 6c), suggesting increased bone remodeling in HBM males. This difference was not observed between vehicle-treated WT and mutant female mice (Fig. 6c). LGK974 treatment led to a significant decrease in Ec.BFR for male Lrp5 A/+ and Lrp5 G/+ mice in comparison to the vehicle-treated Lrp5 mutant mice (Fig. 6c). With our sample size we did observe a significant difference in female mice from either Lrp5 mutant treated with LGK974, in bone dynamics. The periosteal mineral apposition rate (Ps.MAR), in both A/+ and G/+ males treated with LGK974, was significantly decreased compared to vehicle-treated mutants (Fig. 6d). Additionally, male Lrp5 A/+ and wildtype littermate controls treated with LGK974 had a statistically significant decrease in periosteal bone formation (Ps.BFR) (Fig. 6e). The only significant difference in cortical dynamics for female mice following LGK974 treatment was observed in Lrp5 G/+ mice, where Ps.BFR was significantly reduced with LGK974 (Fig. 6e), while male mice were not significantly affected. In conclusion, endocortical and periosteal bone formation decreased when Lrp5 HBM mutants were treated with LGK974.

**Cellular bone changes are rescued following LGK974 treatment of Lrp5 mutant mice**

To investigate how PORCNi impacts the skeleton of Lrp5 mutant mice at the cellular level, we stained sections of cancellous bone with Goldner's Trichrome (Fig. 7a) and TRAP (Fig. 8). The trends for increased bone volume obtained by µCT were confirmed in the cancellous bone sections (Fig. 7b). BV/TV was significantly increased in both sexes of vehicle-treated Lrp5 A/+ and Lrp5 G/+ mice compared to their WT controls (Fig. 7b). Furthermore, Goldner's Trichrome staining showed changes in the number of adipocytes within females, specifically WT (Lrp5A214V littermates) and Lrp5 G/+ mice treated with LGK974 (Fig. 7c). Osteoid volume/bone volume (OV/BV) also significantly increased with the addition of LGK974 in WT mice from the Lrp5 A/+ and Lrp5 G/+ groups (Fig. 7d). Likewise, the same groups displayed a significant increase in osteoid surface/bone surface (OS/BS). A significant increase was observed in WT mice when treated with LGK974 (littermates of the Lrp5 A/+ mice: 72% change in females and 61% in males; littermates of Lrp5 G/+: 136% change in males only) (Fig. 7e). However, no significant differences were observed in either vehicle or LGK974 treated Lrp5 mutant mice (Fig. 7e). Significant increase in the number of osteoblasts/bone surface (N.Ob/BS) occurred between vehicle and LGK974 treated WT mice (Fig. 7f), in both females (by 93.5%) and males (by 57.6%). Within the Lrp5 G/+ group, treatment with LGK974 significantly increased N.Ob/BS in both WT and Lrp5 G/+ male mice (Fig. 7f).

We analyzed TRAP-stained sections of trabecular bone from male mice to investigate changes in osteoclast function in Lrp5 HBM mutants and LGK974-treated animals. Osteoclast surface/bone surface (Oc.S/BS) significantly decreased in vehicle-treated Lrp5 G/+ mice compared to vehicle-treated wild-type mice (Fig. 8b). When these Lrp5 G/+ mice were treated with LGK974, Oc.S/BS significantly increased compared to the vehicle-treated mutant mice (Fig. 8b). However, there was no significant change in
Oc.S/BS in vehicle or LGK974 treated $Lrp5$ A/+ mice. (Fig. 8b). The number of osteoclasts/bone surface (N.Oc/BS) decreased significantly in vehicle-treated $Lrp5$ G/+ mice (Fig. 8c). Additionally, when the $Lrp5$ G/+ mice were treated with LGK974, N.Oc/BS increased significantly (Fig. 8c). Again, there was no significant change in N.Oc/BS in vehicle or LGK974 treated, $Lrp5$ A/+ mice. (Fig. 8c).

Discussion

Seminal work done over twenty years ago established the critical role of Wnt signaling in bone homeostasis. Homozygosity for loss-of-function mutations in LRP5 was identified as the underlying cause of Osteoporosis pseudoglioma [35], a syndrome characterized partly by early-onset, severe osteoporosis. Almost concurrently, patients carrying autosomal dominant point mutations in LRP5 were shown to develop high bone mass [3, 4]. These point mutants resulted in the production of an altered form of LRP5 that could no longer be bound and inhibited by negative regulators of the pathway such as Dickkopf-1 and Sclerostin [4, 18, 19, 36, 37]. Subsequent work showed that patients either completely lacking Sost expression (Sclerosteosis [6, 38]) or having severely diminished SOST expression due to loss of a regulatory enhancer sequence that acted on the SOST promoter (Van Buchem's disease [39]) also developed extremely high bone mass. These observations were the basis for the biotechnology industry investing in developing therapeutic antibodies that blocked SOST activity, which ultimately resulted in the successful development of an FDA-approved therapeutic, Romosozumab, to treat severe osteoporosis [40–42]. While this may benefit thousands of patients with osteoporosis in the future, the patients with the high bone mass disorders (and those found to have analogous mutations in LRP6 [43, 44] to those found in patients with LRP5 point mutations) continue to suffer from the secondary effects of bone overgrowth, such as increased intracranial pressure, loss of vision and hearing, and progressive overgrowth of other craniofacial structures. We sought to test whether selectively inhibiting Wnt signaling could lessen these symptoms by using well-validated mouse models of these HBM disorders.

Porcupine is a membrane-bound O-acyl transferase with highly specific activity directed at adding a palmitoleic acid hydrocarbon chain at a conserved serine residue in all Wnts [21, 45, 46]. Given the increased Wnt signaling activity associated with many human tumors [47, 48], Porcupine inhibitors were developed as potential treatments for cancer patients. While these showed significant activity against tumor growth, Phase 1 clinical trials with Porcupine inhibitors were paused due to increased risks of bone fracture associated with decreased bone mass [27, 28, 49]. Consistent with this observation, genetically-engineered mouse models with osteoblast-specific inactivating mutations in Porcupine or the dedicated Wnt chaperone, GPR177/Wntless, have low bone mass [50–52]. We previously showed that rapid bone loss was caused by treating wild-type mice with Porcupine inhibitors [27]. Our current work extends this concept further showing bone loss in high-bone-mass disorders caused by mutations in components of the Wnt signaling pathway.

Our work indicates that the effects of $Sost$ and $Lrp5$ mutations in causing HBM can be normalized by blocking Wnt ligand secretion. This is consistent with previous work showing that HBM-associated variants were not intrinsically more active than wild-type variants but instead were no longer inhibited by
endogenous Wnt inhibitors [4, 19, 36, 37]. This further supports the notion that maintenance of HBM associated with Lrp5A214V and Lrp5G171V mutations and loss of function of Sost is dependent on the continued presence of Wnt ligands, validating our earlier in vitro [36] and in vivo observations [27].

Patients with Sclerosteosis, van Buchem Disease, or HBM associated with LRP5 mutations often suffer from neuropathies and other sequelae due to nerve impingements caused by increased bone mass [11–15]. We show here that when treated with Wnt secretion inhibitors, genetic mouse models that replicate human HBM have bone mass reduced to more normal levels, therefore establishing this approach as a potential strategy for treating HBM patients to reduce neuropathy. This concept is supported by the observed loss in bone mass associated with clinical use of PORCN inhibitors [27]. Our work shows that a 4- to 6-week treatment regimen is sufficient to reduce bone mass. We expect that this same treatment length, or potentially even shorter treatments, with PORCN inhibitors will be sufficient to provide symptomatic relief of neuropathies. This work serves as the conceptual foundation to conduct longitudinal studies on treatment frequency needed to mitigate HBM-related neuropathies.

Materials And Methods

Animals

Mice with the Lrp5 conditional knock-in HBM alleles (p.G171V and p.A214V), were previously described [32, 33]. Animals used in this study were initially crossed to a ubiquitous Cre (CMV-Cre) transgenic mouse to excise the neomycin-resistant-cassette flanked by loxP sites and then backcrossed to C57BL/6J animals to breed out the Cre transgene. We used wildtype (+/+) and heterozygous Lrp5-A214V (A/+) or Lrp5-G171V (G/+) mice, as previously described [36]. Female and male 3-month-old +/+ (wild-type, WT) and heterozygous Lrp5-A214V (A/+) or Lrp5-G171V (G/+) mice were treated daily with either 3 mg/kg LGK974 (Novartis) or with vehicle for 5 weeks by oral gavage at a dosing volume of 10 µL/g animal body weight. LGK974 is poorly soluble and is therefore administered as a suspension in 0.5% methylcellulose / 0.5% Tween 80 [23, 53]. Lrp5 HBM mice were maintained in accordance with institutional animal care and use guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the Van Andel Institute Mice were housed in Thoren Maxi-Miser IVC caging systems with a 12-h/12-h light/dark cycle and fed a breeder rodent diet containing 23% protein and 24% fat with an energy content of 19.3 MJ/kg (5021, LabDiet St. Louis MO) with food and water provided ad libitum.

Female three-month-old wild-type C57BL/6J (Charles River Laboratories Germany) and Sost deficient (Sost KO) mice [34] were treated twice daily for six weeks with either vehicle (0.5% methylcellulose / 0.5% Tween 80 in water) or GNF-6231 suspension at 0.3, 1 or 3 mg/kg by oral gavage at a dosing volume of 10 µL/g animal body weight. Mice were housed at 22°C with a 12-h/12-h light/dark cycle and were fed a standard rodent diet containing 18.2% protein and 3.0% fat with an energy content of 15.8 MJ/kg (3890, Provimi Kliba SA) with food and water provided ad libitum. Procedures with Sost mice conformed to Swiss federal law for animal protection controlled by the Basel-Stadt Cantonal Veterinary Office, Switzerland.
For dynamic histomorphometry analysis of Lrp5 HBM models, animals were administered the fluorescent dye calcein (10 mg/kg split equally between i.p. and sub.q.; MilliporeSigma) 17 and 7 d before euthanasia. All animals were euthanized at 17-wk-old, following five weeks of treatment. Femurs were isolated and fixed in 10% neutral-buffered formalin (NBF) at room temperature for 48 h then changed to 70% ethanol before analysis and histological processing.

Table 1 details the number of specimens analyzed for each application.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Vehicle Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LRP5&lt;sup&gt;1214V&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>A/+ female</td>
</tr>
<tr>
<td></td>
<td>LRP5&lt;sup&gt;1214V&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>A/+ male</td>
</tr>
<tr>
<td></td>
<td>LRP5&lt;sup&gt;517V&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G/+ female</td>
</tr>
<tr>
<td></td>
<td>LRP5&lt;sup&gt;517V&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G/+male</td>
</tr>
<tr>
<td>DEXA and µCT</td>
<td>6</td>
</tr>
<tr>
<td>Dynamic histomorphometry</td>
<td>5</td>
</tr>
<tr>
<td>Static histomorphometry</td>
<td>5</td>
</tr>
<tr>
<td>TRAP</td>
<td>5</td>
</tr>
<tr>
<td>DEXA and µCT</td>
<td>5</td>
</tr>
<tr>
<td>Dynamic histomorphometry</td>
<td>4</td>
</tr>
<tr>
<td>Static histomorphometry</td>
<td>5</td>
</tr>
<tr>
<td>TRAP</td>
<td>5</td>
</tr>
<tr>
<td>LGK974 Treatment</td>
<td>5</td>
</tr>
<tr>
<td>DEXA and µCT</td>
<td>6</td>
</tr>
<tr>
<td>Dynamic histomorphometry</td>
<td>4</td>
</tr>
<tr>
<td>Static histomorphometry</td>
<td>5</td>
</tr>
<tr>
<td>TRAP</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Vehicle</th>
<th>Wildtype</th>
<th>Sos&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.3 mg/kg</td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>µCT</td>
<td>10</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>pQCT</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

**DEXA**

We performed whole body dual-energy X-ray absorptiometry (DEXA) to measure areal bone mineral density (aBMD; gm cm<sup>−2</sup>) and bone mineral content (BMC; gm) for the postcranial skeleton. A PIXImus II bone densitometer (GE Lunar) was used for analysis. aBMD values were collected at 0, 7,14, 21, 28, and 35 days into treatment.

**Microcomputed Tomography (µct)**
The *Lrp5* HBM models were analyzed using a SkyScan 1172 µCT system (Bruker MicroCT: Kontich, Belgium). Femora were scanned using an X-ray voltage of 60 kV, current of 167 µA, and 0.5 mm aluminum filter. Images were obtained with 2000 x 1200 pixel resolution and 7.98 µm pixel size. Femoral images were reconstructed using NRecon 1.7.4.6 (Bruker MicroCT). The mineralized tissue was oriented, and a volume of interest (VOI) was defined using DataViewer 1.5.6.3 (Bruker MicroCT). Regions of interest (ROI) were defined for cortical and trabecular bone using CTAn 1.18.8.0 (Bruker MicroCT). A trabecular ROI was drawn in the distal epiphysis for each femur, beginning 0.25 mm proximal to the growth plate and 2.5 mm in height. To define the position of each cortical ROI, the distal end of the region was set to be 45% of the femur length. The ROI was 0.8 mm in height toward the proximal end of the bone, within the midshaft. Trabecular 3D analysis was performed to quantify bone mineral density (BMD), bone volume/tissue volume (BV/TV), bone surface/bone volume, trabecular thickness (Tb.Th), trabecular separation, (Tb.Sp), and trabecular number (Tb.N). Cortical 2D analysis was performed to quantify tissue mineral density (TMD), tissue area, bone area, cortical area fraction (bone area/tissue area, CAF), cross-sectional thickness, and bone perimeter.

Femora from *Sost* knockout mice were analyzed by high-resolution *ex vivo* mCT using a vivaCT 40 instrument (Scanco Medical AG) at an isotropic nominal resolution of 6 µm. A Gaussian filter (σ = 0.7, support of one voxel) was used in all analyses to suppress noise and a segmentation threshold of 275 was applied.

### Peripheral Quantitative Computed Tomography (Pqct)

Treatment efficacy was assessed longitudinally by *in vivo* pQCT under isoflurane inhalation using an adapted Stratec-Norland XCT-2000 instrument fitted with an Oxford (Oxford, UK) 50-mmX-ray tube (GTA6505M/LA) and a 0.5-mm-diameter collimator (voxel size: 0.07 mm x 0.07 mm x 0.4 mm).

### Static And Dynamic Histomorphometry

Detailed methods for static and dynamic histomorphometry tissue preparation and analysis are found in our published protocol for skeletal tissue phenotyping [54].

Briefly, fixed fluorochrome-labeled femurs were dehydrated in graded ethanol and cleared using xylene. Samples were infiltrated and embedded in plastic using 85–100% MMA and 15% dibutyl phthalate until polymerization. Femurs were sectioned coronally, deplasticized, Goldner's Trichrome stained, and coverslipped for static histomorphometry.

Additional femurs were fixed and decalcified in 10% EDTA for 14 d, embedded in paraffin, and sectioned sagittally. Sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP kit 387A; Millipore Sigma, St. Louis, MO, USA). Indices measured for Goldner's Trichrome stained slides included bone volume/tissue volume (BV/TV), adipocyte number/tissue volume (Ad.V/TV), osteoid volume/bone volume (OV/BV), osteoid surface/bone surface (OS/BS), and the number of
osteoblasts/bone surface (N.Ob/BS). Parameters measured for TRAP-stained slides included osteoclast surface/bone surface and the number of osteoclasts/bone surface. All histomorphometric analysis was performed using BIOQUANT OSTEO software (v19.2.60; BIOQUANT Image Analysis Corporation, Nashville, USA).

For dynamic histomorphometry, femoral midshafts were cross-sectioned and coverslipped. Endocortical mineral apposition rate (MAR), endocortical bone formation rate (BFR), periosteal MAR, and periosteal BFR were measured for each sample.

**Statistical Analyses**

All analyses except DEXA were performed using the same methods. All percentage data, including BV/TV (µCT), CAF, BV/TV (histomorphometry), OV/BV, OS/BS, and Oc. S/BS were analyzed via beta regression. All other outcomes were analyzed via robust linear regression with natural log (y + 0.01) transformation to improve the models’ fit. All analyses and outcomes were Benjamini-Hochberg false discovery rate (FDR) adjusted to account for multiple testing. To determine if the two groups were similar, an equivalence test was performed such that any non-significant difference with a 95% confidence interval entirely within the range of 0.9–1.1 (fold-change [FC]) is considered equivalent. For any differences we could confidently say are smaller than 10%, we can conclude that there is sufficient evidence to support a hypothesis that there is no difference between the groups.

DEXA data were analyzed using linear mixed-effects models with random slope and intercept for each animal. FDR-adjusted linear contrasts were used to test specific hypotheses. Analyses were stratified by sex. For all models, a 3-way interaction between days, genotype, and treatment was initially fit. All two-way interactions were also tested and dropped if p-values exceeded 0.15 and saved for the interaction between days and treatment. This allowed for testing of a treatment effect in all comparisons.

**Declarations**

**Author Contributions**

C.R.D. contributed to the conceptualization of experiments, validation, investigations, data visualization, and writing. G.E.F. performed investigations, methodology, formally analyzed and visualized data, and wrote the original draft. C.M. and M.J.M performed investigations and formally analyzed data. Z.B.M. formally analyzed data. B.O.W., J.L., I.K., M.K., S.G., and S.L.H. contributed to conceptualization, methodology, supervision, funding acquisition, and writing.

**Acknowledgements**

We thank other members of the Williams lab for technical assistance and manuscript editing. We thank members of the VAI Vivarium and Transgenics Core for their exceptional care of the animals in this study and the VAI Pathology and Biorepository core for assistance. We thank Dr. Sonya Craig for editorial
assistance. Treatment schematics were created using BioRender. We also thank C. Kaffka for experimental contributions and excellent technical assistance.

**Conflicts of interest**

BOW is a stockholder and member of the Scientific Advisory Board for Surrozen. The Williams Laboratory has also received recent support from Janssen Pharmaceuticals for work unrelated to the studies reported here. CM, JL, IK, MK, and SG are employees and shareholders of Novartis.

**Data Availability**

The data analyzed to support the findings in this study are available from the corresponding author upon request.

**References**


**Figures**
Figure 1

**Sost treatment schematic and in vivo femoral and tibial pQCT measurements.** (a) Schematic diagram of the experimental design. Longitudinal pQCT measurements of distal femur and proximal tibia metaphysis in female wild-type (WT) and SostKO mice, treated with vehicle or GNF-6231 daily for 40 days. The parameters measured include (b, c) total cross-sectional BMC, (d, e) total cross-sectional BMD, (f, g) cancellous BMD, and (h, i) cross-sectional cortical thickness. For all graphs, means of each group
are indicated by the shape, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 6 animals per genotype, per condition were analyzed (see Table 1).

Figure 2

Cancellous and cortical bone analysis of LGK974 treated Sost KO mice via microCT. Representative images of cancellous bone (a) in distal femur and cortical bone (g) in the midshaft of the femur, demonstrating the effects of GNF-6231 treatment on wild-type (WT) and Sost KO mice. The trabecular parameters measured include (b) bone mineral density (BMD), (c) bone volume/tissue volume (BV/TV), (d) trabecular thickness (Tb.Th), (e) trabecular separation (Tb.Sp), and (f) trabecular number (Tb.N). The cortical parameters measured include (h) tissue mineral density (TMD), (i) cortical area fraction (CAF), and (j) cross-sectional thickness (Ct.Th). For all graphs, means of each group are indicated by the middle
line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 5 animals per genotype, per condition were analyzed (Table 1).

Figure 3

Treatment schematic and whole-body areal bone mineral density measured by DEXA. (a) Schematic diagram of the experimental design. (b) Longitudinal whole body areal bone mineral density (aBMD)
measured by DEXA of \( Lrp5^{A214V} \) littermate wild-type (WT) and \( A/+ \) females and males respectively, treated with vehicle or LGK974 daily for 35 days. (c) Longitudinal whole body areal bone mineral density (aBMD) measured by DEXA of \( Lrp5^{G171V} \) littermate wild type and \( G/+ \) females and males respectively, treated with vehicle or LGK974 daily for 35 days. For all graphs, means of each group are indicated by the shape, upper and lower lines represent standard deviations, and \(* = p < 0.05\). A minimum of 5 animals per sex, per genotype, per condition were analyzed (see Table 1).

Figure 4
Cancellous bone analysis of LGK974 treated mice via microCT. (a) Representative images of trabecular bone in distal femurs, demonstrating the effects of LGK974 treatment on wildtype and Lrp5 mutant mice. The parameters measured included (b) bone mineral density (BMD), (c) bone volume/tissue volume, (d) trabecular thickness (Tb.Th), (e) trabecular separation (Tb.Sp), and (f) trabecular number (Tb.N). For all graphs, means of each group are indicated by the middle line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 5 animals per sex, per genotype, per condition were analyzed (Table 1).

Figure 5

Cortical bone analysis of LGK974 treated mice via microCT. (a) Representative images of cortical bone in the midshaft of femurs, demonstrating the effects of LGK974 treatment on wildtype and Lrp5 mutant mice. Trabecular bone parameters of treated Lrp5 A214V mice, Lrp5 G171V mice, and their respective controls were measured. These parameters included (b) tissue mineral density (TMD), (c) cortical area fraction (CAF), and (d) cross-sectional thickness (Ct.Th). For all graphs, means of each group are indicated by the middle line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 5 animals per sex, per genotype, per condition were analyzed (Table 1).
Figure 6

Dynamic histomorphometry of femoral cortical bone. (a) Representative images of cortical cross-sections from fluorochrome-labeled femurs. (b) Endocortical mineral apposition rate (Ec.MAR), (c) endocortical bone formation rate (Ec.BFR), (d) periosteal mineral apposition rate (Ps.MAR), and (e) periosteal bone formation rate (Ps.BFR). For all graphs, means of each group are indicated by the middle line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 3 animals per sex, per genotype, per condition were analyzed (Table 1).
Figure 7

Static histomorphometry of femoral trabecular bone. (a) Representative images of Goldner’s Trichrome stained trabecular bone in the distal femurs of mice. (b) bone volume/tissue volume (BV/TV), (c) adipocyte number/tissue volume (Ad.N/TV), (d) osteoid volume/bone volume (OV/BV), (e) osteoid surface/bone surface (OS/BS), (f) osteoid width (O.Wi), and (g) number of osteoblasts/bone surface (N.Ob/BS). For all graphs, means of each group are indicated by the middle line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 3 animals per sex, per genotype, per condition were analyzed (Table 1).
Figure 8

Osteoclast quantification in distal femurs of mice. (a) Representative images of TRAP stained femoral sagittal sections, (b) osteoclast surface/bone surface (Oc.S/BS), (c) number of osteoclasts/bone surface (N.Oc/BS). For all graphs, means of each group are indicated by the middle line, upper and lower lines represent standard deviations, and * = p < 0.05. A minimum of 2 animals per genotype and per condition were analyzed.