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Rictor is required for optimal bone accrual in response to anti-sclerostin therapy in the mouse

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Abstract

Wnt signaling has emerged as a major target pathway for the development of novel bone anabolic therapies. Neutralizing antibodies against the secreted Wnt antagonist sclerostin (Scl-Ab) increase bone mass in both animal models and humans. Because we have previously shown that Rictor-dependent mTORC2 activity contributes to Wnt signaling, we test here whether Rictor is required for Scl-Ab to promote bone anabolism. Mice with Rictor deleted in the early embryonic limb mesenchyme (Prx1-Cre;Rictor^{fl/f}, hereafter RiCKO) were subjected to Scl-Ab treatment for 5 weeks starting at 4 months of age. In vivo micro-computed tomography (μCT) analyses before the treatment showed that the RiCKO mice displayed normal trabecular, but less cortical bone mass than the littermate controls. After 5 weeks of treatment, Scl-Ab dose-dependently increased trabecular and cortical bone mass in both control and RiCKO mice, but the increase was significantly blunted in the latter. Dynamic histomorphometry revealed that the RiCKO mice formed less bone than the control in response to Scl-Ab. In addition, the RiCKO mice possessed fewer osteoclasts than normal under the basal condition and exhibited lesser suppression in osteoclast number by Scl-Ab. Consistent with the fewer osteoclasts in vivo, bone marrow stromal cells (BMSC) from the RiCKO mice expressed less Rankl but normal levels of Opg or M-CSF, and were less effective than the control cells in supporting osteoclastogenesis in vitro. The reliance of Rankl on Rictor appeared to be independent of Wnt-β-catenin or Wnt-mTORC2 signaling as Wnt3a had no effect on Rankl expression by BMSC from either control or RiCKO mice. Overall, Rictor in the limb mesenchymal lineage is required for the normal response to the anti-sclerostin therapy in both bone formation and resorption.

Keywords

Rictor; mTORC2; Wnt; Sost; Bone; Osteoblast; Osteoclast

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1. Introduction

Wnt signaling has emerged as a key regulator of bone development and homeostasis [1,2]. In particular, β -catenin, a critical effector for Wnt-induced gene transcription, is indispensable for osteoblast development in the mouse embryo [3–5]. Similarly, the Wnt co-receptors Lrp5 and Lrp6 are jointly required for both embryonic osteoblast formation and postnatal bone acquisition [6,7]. β -catenin also regulates osteoblast activity and life span in postnatal mice [8]. In addition, Wnt- β -catenin signaling in osteoblasts has been shown to suppress osteoclast differentiation through stimulation of Opg production [9,10]. Overall, mouse genetic studies have identified Wnt-Lrp5/6- β -catenin signaling as an important mechanism in regulating the skeleton.

Besides β -catenin, Wnt proteins also activate other intracellular signaling molecules. For example, Wnt has been shown to activate PKC δ through phosphatidylinositol signaling in osteoblast-lineage cells [11]. Multiple Wnt ligands have been reported to activate mTOR (mammalian target of rapamycin). For instance, mTORC1 (mTOR complex 1) was activated by overexpression of either Wnt 10b or Wnt 7b in bone [12, 13]. mTORC2 (mTOR complex 2) was also activated by Wnt7b and through Lrp5 signaling in bone [14]. The importance of mTORC1 or mTORC2 in bone was demonstrated by genetic deletion of either Raptor or Rictor, respectively, in the osteoblast lineage [13–16]. Most notably, mice with Rictor deleted in the limb mesenchymal cell lineage formed thinner bones and were less responsive to loading in forming new bone [15]. However, it is not known whether Rictor deletion alters the bone anabolic response to Wnt signaling in vivo.

Sclerostin, a secreted Wnt antagonist primarily from osteocytes, has become an important target for developing bone anabolic therapies. Sclerostin functions by binding to Lrp5 or Lrp6 to impede their interaction with Wnt ligands [17–19]. Sclerostin deficiency in humans causes high bone mass syndromes such as sclerosteosis [20] and Van Buchem disease [21]. Monoclonal antibodies against sclerostin (Scl-Ab) successfully increased bone mass not only in animals but also in patients enrolled in clinical trials [22–26]. However, it is not known what intracellular pathways are responsible for the bone anabolic effect of Scl-Ab.

In this study, we test the hypothesis that mTORC2 signaling mediates the bone anabolic effect of Scl-Ab. We show that mice with Rictor deleted in the mesenchymal lineage of the limb have a muted response in bone formation in response to Scl-Ab. We further show that Rictor deficiency suppresses osteoclastogenesis by reducing Rankl expression independent of Wnt- β -catenin or Wnt-mTORC2 signaling.

2. Materials and methods

2.1. Mouse strains and antibody injections

All mouse procedures were approved by Washington University Animal Studies Committee. Prx1-Cre mice (Jackson Laboratory, Bar Harbor, ME, USA), and *Rictor*^{flox/flox} (here after *Rictor*^{f/f}, kindly provided by Dr. Jeffrey Arbeit, Washington University in St. Louis) were as previously described [27,28]. Mice with the genotype of Prx1-Cre;*Rictor*^{f/f} (hereafter RiCKO) were produced as before [15]. Cohorts of RiCKO versus *Rictor*^{f/f} mice were

produced by crossing the RiCKO and the *Rictor^{fl/fl}* mice. Four-month-old sex-matched littermate pairs (*Rictor^{fl/fl}* versus RiCKO) were subjected to intraperitoneal injections of either vehicle (0.004% Tween) or a sclerostin monoclonal antibody (Scl-Ab; Amgen, USA) at 5 or 25 mg/kg [29]. The animals were injected on Tuesdays and Fridays for 5 consecutive weeks, and sacrificed on the third day after the final injection. Selected groups of mice were used for μ CT measurements, serum biochemistry, or histomorphometry as detailed below.

2.2. In vivo μ CT analyses

A total of nine male ($n = 5$) or female ($n = 4$) *Rictor^{fl/fl}* versus RiCKO sex-matched littermate pairs injected as described above were analyzed for bone mass changes with in vivo μ CT. The animals were first analyzed with in vivo μ CT before the injections with either vehicle (2 female pairs, 1 male pair), or the sclerostin antibody at 5 mg/kg (2 female pairs, 1 male pair) or 25 mg/kg (3 male pairs). The animals were again analyzed with in vivo μ CT at the end of treatment before harvest. In vivo micro-computed tomography (μ CT) was performed on the right tibia of each mouse (Scanco VivaCT40). The thresholds for quantification of trabecular and cortical bone parameters were set at 200/1000 and 250/1000, respectively. The voxel size was 10.5 μ m. Scanning and analyses were performed as reported previously [15,30]. Briefly, analyses of cortical bone parameters were performed on 50- μ CT slices (0.8 mm total) at the mid-point of the shaft of the tibia; trabecular parameters were assessed on 120- μ CT slices (1.6 mm total) immediately below the proximal growth plate of the tibia.

2.3. Serum biochemical markers

A total of 12 pairs of mice injected with vehicle (3 female pairs, 3 male pairs) or 25 mg/kg antibody (3 female pairs, 3 male pairs) as described above were used for serum biochemistry. Before harvest, the animals were fasted for 6 hours before serum collection [13]. N-terminal propeptide of procollagen type I (PINP) was evaluated by enzyme immunoassay (EIA) (Rat/Mouse PINP EIA; IDS; Fountain Hills, AZ, USA). Serum CTX-I assays were performed with the RatLaps ELISA kit (Immunodiagnostic Systems, Ltd.).

2.4. Bone histomorphometry

Tibias were collected from a subset of the mice for histomorphometry. H&E and TRAP staining on paraffin sections was performed according to the standard protocols. Static histomorphometry (osteoblast and osteoclast number) was performed with the Image J software (NIH, USA) for four male pairs for each treatment (vehicle versus 25 mg/kg antibody), with three medial sections from each mouse. For dynamic histomorphometry, three male pairs for each treatment were injected with calcein (10 mg/kg; Sigma-Aldrich; St. Louis, MO, USA) at 10 and 3 days before sacrifice and tibias were fixed in 70% ethanol and embedded in methyl-methacrylate for plastic sections. Dynamic histomorphometry was performed with the commercial software Bioquant Osteo II (Nashville, TN, USA).

2.5. Frozen sections and immunohistochemistry

Bones were incubated overnight at room temperature in 4% (wt/vol) paraformaldehyde followed by 3 days of decalcification in 14% (wt/vol) EDTA, pH 7.4. Bones were then rinsed, equilibrated in 20% (wt/vol) sucrose, embedded in optimum cutting temperature

(OCT) compound (Tissue-Tek), and frozen in liquid nitrogen. Sections at 10 μ m in thickness were cut using the Cryo-Jane Tape-Transfer system (Leica). Sections were rinsed, incubated briefly in 0.1% Triton X-100, and blocked with 5% (vol/vol) normal serum, followed by overnight incubation in osteocalcin antibody (1:50; Santa Cruz sc-30045) at 4 °C. Following secondary detection at room temperature, sections were rinsed and mounted with Vectashield containing DAPI (Vector Laboratories). The osteocalcin positive area normalized to bone surface was determined with Image J on three male pairs for each treatment, with three medial sections for each animal.

2.6. BMSC culture and in vitro osteoclastogenesis

Mouse bone marrow cells (BMSC) were isolated from tibiae and femurs of 4-month-old mice as described previously [11]. Briefly, bone marrow cells were seeded on 60 mm tissue culture dishes in α -MEM (Gibco, USA) containing 10% FBS. After 72 h, the non-adherent cells were removed. On the seventh day, the cells were trypsinized for subsequent experiments.

Primary bone marrow monocytes (BMM) were prepared as described previously [31]. Briefly, bone marrow was extracted from bilateral femurs and tibiae of 4-month-old *Rictor^{fl/fl}* mice and cultured on petri dishes in α -MEM (Gibco, USA) containing 10% FBS and 1:10 CMG (conditioned medium containing recombinant M-CSF) [32,33]. Cells were cultured at 37 °C in 5% CO₂ for 3 days and then washed with PBS, followed by dissociation with 1 $\times$ trypsin/EDTA (Invitrogen) in PBS for co-culture with BMSC as described above. 3×10^4 BMM and 4×10^4 BMSC were co-cultured in 500 μ l of α -MEM containing 10% FBS and 1 ng/ml vitamin D in 48-well tissue culture plates for 7 days. The medium was changed every 3 days. After co-culture for 7 days, cells were treated with collagenase, and the remaining cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity with a commercial kit (387-A, Sigma). The experiment was repeated three times, each with BMSC from one pair of *Rictor^{fl/fl}* versus *RiCKO* male littermates. Representative data from one pair are presented.

2.7. Wnt3a treatment and qPCR analyses of cell cultures

Recombinant mouse Wnt3a (R&D systems) was used at 100 ng/ml. As a vehicle control for Wnt3a, PBS with 0.1% CHAPS and 0.1 mM EDTA was used [34]. Cells were harvested 72 h later for qPCR. Total RNA was extracted from cells with RNAeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA was used for reverse-transcription with iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). qPCR was performed with SYBR green Supermix (Bio-Rad). Expression levels were normalized first to β -actin, and then to control samples with the 2^{-Ct} method. The primers used are listed in Table 3. The experiment was repeated three times, each with BMSC prepared from one pair of *Rictor^{fl/fl}* versus *RiCKO* male littermates. Representative data from one pair are presented.

2.8. Statistics

All quantitative data are presented as mean $\pm$ SEM with a minimum of three animals. Statistical analyses were performed with either Student's t-test, or two-way factorial

ANOVA (<http://vassarstats.net>) as indicated. Statistical significance was determined by a p value < 0.05 .

3. Results

3.1. Loss of Rictor diminishes the effect of Scl-Ab on bone mass in adult mice

We have previously reported that deletion of Rictor with Prx1-Cre in the mouse (Prx1-Cre; Rictor^{f/f}, hereafter RiCKO) results in less cortical bone and a subdued response to mechanical loading [15]. Because Rictor-mediated mTORC2 participates in Wnt signaling, we seek to understand whether Rictor deletion affects the bone anabolic response to the anti-sclerostin therapy [14]. To this end, we decided to treat skeletally mature mice (4 months old) with Scl-Ab, a monoclonal antibody previously shown to promote bone formation [22]. We first established the basal bone phenotype with in vivo μ CT before the antibody treatment. Consistent with our previous report with 6-week-old mice, the 4-month-old RiCKO mice showed normal trabecular bone parameters (Figs. 1A–D) [15]. On the other hand, the mutant mice exhibited a significant decrease in cortical bone area (Ct. Ar), total diaphyseal cross-sectional area (Tt. Ar), and cortical bone thickness (Ct. Th) when compared to the sex-matched Rictor^{f/f} littermates, even though the percentage of cortical bone area over total area (Ct. Ar/Tt. Ar) was not changed (Figs. 1E–I). Thus, deletion of Rictor in the embryonic limb mesenchyme reduces cortical but not trabecular bone mass in the long bones of adult mice.

We then treated the 4-month-old, sex-matched Rictor^{f/f}, and RiCKO littermates with either vehicle or Scl-Ab at two different doses (5 or 25 mg per kg of body weight) for 5 weeks. The mice were analyzed with in vivo μ CT at the end of the treatment. As expected, Scl-Ab, compared to the vehicle, dose-dependently increased both trabecular and cortical bone mass in the Rictor^{f/f} control mice (Figs. 2A, B, left panels). Scl-Ab also elicited a dose-dependent increase in bone mass in the RiCKO mice, but the final bone mass with either dose appeared to be lower than that achieved in the control mice by the same dose (Figs. 2A, B, right panels). To evaluate the potential quantitative differences between the genotypes in response to the antibody, we performed longitudinal analyses with the in vivo μ CT data before and after the treatment. Vehicle treatment did not affect trabecular bone mass (BV/TV), trabeculae number (Tb. N*) or thickness (Tb. Th*) in either Rictor^{f/f} or RiCKO mice (Table 1). On the other hand, Scl-Ab at 5 or 25 mg/kg caused both mice to increase trabecular bone volume and trabeculae thickness in a dose-dependent manner. However, the response of those parameters to either dose was significantly diminished in the RiCKO mice compared to the Rictor^{f/f} littermates (Table 1). Trabeculae number was generally less affected by Scl-Ab, with only 25 mg/kg showing a significant change in the Rictor^{f/f} mice. In the cortical bone, the vehicle treatment had no effect on any of the parameter, but Scl-Ab at either dose similarly increased the cortical thickness (Ct. Th*) and the cortical bone area (Ct. Ar), and at the higher dose also increased the total cross-sectional area (Tt. Ar) (Table 2). Again, the increase here was less pronounced in the RiCKO mice than that in the Rictor^{f/f} littermates. Thus, the effect of anti-sclerostin therapy on both trabecular and cortical bone mass is suppressed in the Rictor-deficient mice.

3.2. Scl-Ab induces less bone formation in Rictor-deficient mice

To determine the cellular basis for the lesser response to Scl-Ab in RiCKO mice, we measured serum levels of PINP, a common marker for bone formation activity, at the end of the antibody or vehicle treatment. Because the two different dosages elicited qualitatively the same effect, we focused further analyses on 25 mg/kg Scl-Ab that produced a more robust outcome. The serum level of PINP was lower in the RiCKO mice than the littermate controls after 5 weeks of vehicle treatment, consistent with a lower bone formation rate in the mutant animals under basal conditions (Fig. 3A, solid bars). The Scl-Ab treatment increased PINP levels in both Rictor^{f/f} and RiCKO mice, but the effect was significantly greater in the former than the latter ($p = 0.03$, interaction p value, ANOVA) (Fig. 3A, open bars). Histomorphometry detected a significant increase in osteoblast number normalized to bone surface in the Rictor^{f/f} but not the RiCKO mice after Scl-Ab treatment (Fig. 3B). Similarly, immunostaining of trabecular bone sections with an osteocalcin (OCN) antibody revealed that Scl-Ab increased the percentage of bone surface covered by OCN⁺ osteoblasts in the Rictor^{f/f} but not the RiCKO mice (Figs. 3C, D). Therefore, Rictor deficiency compromises the increase of osteoblast number by the anti-sclerostin therapy.

We next performed dynamic histomorphometry to assess the response in osteoblast activity to Scl-Ab treatment. With vehicle treatment, the trabecular bone of RiCKO mice exhibited fewer mineralizing bone surfaces (MS/BS) but a relatively normal mineral apposition rate (MAR), resulting in a lower bone formation rate (BFR/BS) than the Rictor^{f/f} littermates (Figs. 4A, B, E). Scl-Ab treatment increased all three parameters in both Rictor^{f/f} and RiCKO mice, but the extent of increase in MAR and BFR/BS was significantly less in the latter ($p < 0.05$, interaction p value, ANOVA) (Fig. 4E). In the cortical bone, with vehicle treatment, the RiCKO mice showed normal MS/BS but a lower MAR and BFR/BS than the Rictor^{f/f} control at the endosteal surface; neither mice had any appreciable amount of calcein labeling at the periosteal surface (Fig. 4F). The Scl-Ab treatment increased all three parameters at the endosteal surface in the Rictor^{f/f} mice but did not affect MAR in the RiCKO mice. Moreover, the increase in MS/BS and BFR/BS at the endosteal surface was subdued in the RiCKO mice compared to the Rictor^{f/f} littermates ($p < 0.05$, interaction p value, ANOVA) (Fig. 4G). Scl-Ab increased all three parameters at the periosteal surface in both animals but again to a lesser extent in the RiCKO mice. Thus, loss of Rictor compromises the increase in osteoblast activity in both trabecular and cortical bone in response to anti-sclerostin.

3.3. Rictor deficiency reduces basal bone resorption and blunts further suppression by anti-sclerostin therapy

We next examined the effect of Rictor deletion on bone resorption. With vehicle treatment, the RiCKO mice exhibited a lower level of CTX-I in the serum than the Rictor^{f/f} littermates, indicating reduced bone resorption under basal conditions (Fig. 5A, solid bars). Scl-Ab notably reduced the serum CTX-I level in the Rictor^{f/f} but not the RiCKO animals (Fig. 5A, open bars). Consistent with the lower CTX-I levels, TRAP staining on bone sections revealed a lower number of TRAP⁺ osteoclasts normalized to bone surface (N. Oc/B. Pm) in the RiCKO mice treated with vehicle (Fig. 5B, solid bars). Moreover, Scl-Ab decreased osteoclast number to a greater extent in the Rictor^{f/f} than the RiCKO mice ($p < 0.0001$,

interaction *p* value, ANOVA) (Fig. 5B, open bars). As a result, although the RiCKO mice exhibited a lower CTX-I level and fewer osteoclasts following vehicle treatment, both parameters became essentially equal between the RiCKO and the Rictor^{f/f} mice after the Scl-Ab treatment. Overall, Rictor deficiency in the mesenchymal lineage reduces the basal level of bone resorption and also blunts the suppressive effect of Scl-Ab on this activity.

As Rictor deletion in the RiCKO mice was specific to the mesenchymal cell lineage, the observed effect on osteoclasts was expected to be indirect. To demonstrate this directly, we performed co-culture experiments to assess the ability of bone marrow stromal cells (BMSC) from 4-month-old RiCKO or Rictor^{f/f} mice in supporting osteoclastogenesis. As indicated by the number of TRAP⁺ cells, BMSC from the RiCKO mice were notably deficient in supporting osteoclast differentiation in vitro (Figs. 6A, B). To examine the molecular basis for such deficiency, we assessed the expression levels of several known osteoclastogenic factors including Rankl, Opg, and M-CSF in BMSC cultures. Whereas Opg and M-CSF levels were similar between the RiCKO and the Rictor^{f/f} samples, Rankl was significantly lower in the RiCKO cells (Fig. 6C). Thus, reduction of Rankl expression by the mesenchymal lineage cells may be a major mechanism for the decrease in osteoclast number in the RiCKO mice.

As Rictor-mediated mTORC2 participates in Wnt signaling in osteoblast-lineage cells, we next tested whether Rictor normally functions downstream of Wnt to stimulate Rankl expression. In particular, because we have previously shown that Wnt3a activates mTORC2, we explored the potential role of Wnt3a in this regulation. However, Wnt3a had no effect on Rankl expression by BMSC from either RiCKO or Rictor^{f/f} mice (Fig. 6D). On the other hand, Wnt3a modestly stimulated the expression of Opg in both RiCKO and Rictor^{f/f} cells, as expected from the previous finding of Opg as a β -catenin target [9]. Therefore, Rictor appears to support Rankl expression in the mesenchymal lineage cells independent of Wnt signaling mediated by either mTORC2 or β -catenin.

4. Discussion

We have investigated the role of Rictor in mediating the bone-enhancing effect of the anti-sclerostin therapy. In mice with Rictor deleted in the mesenchymal cell lineage of the limbs, we show that the impact of Scl-Ab on bone mass of the long bones was greatly compromised though not completely eliminated. In particular, loss of Rictor markedly suppressed the increase in both osteoblast number and function in response to Scl-Ab. Therefore, the sclerostin antibody increases bone mass partly through a Rictor-dependent mechanism.

The current prevailing model posits that anti-sclerostin stimulates bone formation through activation of Wnt signaling. Multiple Wnt ligands have been implicated in the regulation of bone accrual. For instance, deletion or overexpression of Wnt10b leads to osteopenia or high bone mass respectively in the mouse [35,36]. Mutations in Wnt1 have been linked with early-onset osteoporosis and osteogenesis imperfecta in human patients [37–40]. In addition, deletion of Wnt7b delays embryonic bone formation whereas overexpression of Wnt7b markedly increases bone mass in the mouse [11,41]. Thus, anti-sclerostin may stimulate

bone formation through the activity of multiple Wnt ligands but the precise identity of such ligands remains to be determined. The intracellular signaling pathways responsible for the bone anabolic function of anti-sclerostin are also not fully understood. Although β -catenin is critical for both embryonic and postnatal bone formation in the mouse, its role in the anti-sclerostin therapy cannot be readily tested due to the severe phenotypes caused by β -catenin deletion [3–5,42,43]. Here, by taking advantage of the RiCKO mice, we demonstrate that the full bone anabolic function of Scl-Ab requires Rictor, leading support to a model wherein anti-sclerostin promotes bone formation in part through Wnt-mTORC2 signaling. To our knowledge, this is the first study linking the bone anabolic function of anti-sclerostin with a specific intracellular signaling pathway downstream of Wnt. Moreover, since we have previously shown that Rictor contributes to loading-induced bone formation, Rictor-dependent mTORC2 signaling may serve as a common nexus for mediating bone anabolism in response to both mechanical and biochemical signals [15].

Besides promoting bone formation, Scl-Ab also markedly suppresses bone resorption. Therefore, both modes of action may contribute to the overall increase in bone mass following the anti-sclerostin therapy. Mechanistically, we found that Wnt3a stimulated Opg expression in BMSC without affecting either Rankl or M-CSF, raising that possibility that Scl-Ab may suppress osteoclastogenesis by activating Wnt- β -catenin signaling and Opg production in the bone marrow environment in vivo. Furthermore, Wnt3a induced Opg levels similarly in BMSC with or without Rictor deletion, indicating that Rictor/mTORC2 does not play a significant role in the β -catenin-mediated regulation of Opg. We have also discovered that Rictor positively regulates Rankl expression by BMSC either directly or indirectly, but apparently independent of Wnt- β -catenin or Wnt-mTORC2 signaling. This finding predicts a depressed level of Rankl in the bone marrow environment of the RiCKO mice. A critically low Rankl level can explain not only the reduced osteoclast number in the RiCKO mice under basal conditions, but also the muted response to Scl-Ab despite the expected increase of Opg in those mice. Overall, the data support the model that Rictor in the mesenchymal cell lineage supports normal osteoclastogenesis through Rankl, but do not mediate the suppression of bone resorption by the anti-sclerostin therapy, which is likely through Opg induction.

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Fig. 1. Rictor deletion reduces cortical bone mass in 4-month-old mice

(A) Representative images of μ CT 3D reconstruction of trabecular bone in proximal tibiae of control (Rictor^{f/f}) versus mutant (RiCKO) male littermates. (B–D) Trabecular bone parameters in proximal tibiae by μ CT. (E) Representative images of μ CT 3D reconstruction of cortical bone at mid-point of tibia shaft of male littermates. (F–I) Cortical bone parameters at mid-point of tibia shaft by μ CT. Scale bar = 100 μ m. All bar graphs show mean $\pm$ SEM, * p < 0.05.

Fig. 2. Loss of Rictor diminishes the effect of Scl-Ab on bone mass in adult mice
 (A) Representative images of μ CT 3D reconstruction of trabecular bone in proximal tibiae of male littermates. (B) Representative images of μ CT 3D reconstruction of cortical bone at mid-point of the tibia shaft of male mice. Scale bar = 100 μ m.

Fig. 3. Scl-Ab induces less bone formation in Rictor-deficient mice

(A) Serum PINP levels. (B) Number of osteoblasts normalized to trabecular bone perimeter on tibia sections. (C) Representative images of immunofluorescence staining on longitudinal tibia sections. Red, osteocalcin; blue, DAPI. (D) Osteocalcin-positive region normalized to trabecular bone surface on tibia sections. Scale bar = 50 μ m. All bar graphs show mean $\pm$ SEM, $p < 0.05$.

Fig. 4. Rictor deficiency reduces osteoblast activity in response to Scl-Ab
 (A–D) Representative images of calcein double labeling in trabecular bone of primary ossification center in tibiae of Rictor^{f/f} (A, C) or RiCKO littermates (B, D) treated with vehicle (A, B) or Scl-Ab (25 mg/kg, C, D). (E) Bone formation parameters from the primary ossification center. (F) Representative images of calcein double labeling at the tibia shaft. (G) Bone formation parameters from cortical bone. Mice (f/f = Rictor^{f/f}, cko = RiCKO) treated with either vehicle (–) or 25 mg/kg Scl-Ab (+). Scale bar = 50 μ m. Es: endosteal surface; Ps: periosteal surface. All bar graphs show mean $\pm$ SEM, *p < 0.05.

Fig. 5. Rictor deficiency reduces bone resorption and the suppression by anti-sclerostin
 (A) Serum CTX-1 levels. (B) Osteoclast number normalized to trabecular bone surface in primary ossification center of proximal tibia. All bar graphs show mean $\pm$ SEM, * $p < 0.05$.

Fig. 6. Rictor deletion suppresses osteoclastogenesis through decrease of Rankl

(A) Representative TRAP staining of osteoclasts differentiated from macrophages co-cultured with BMSCs. (B) Quantification of TRAP+ cells. (C) qPCR analyses of gene expression in BMSC. (D) qPCR analyses of gene expression in BMSC after treatment with vehicle or Wnt3a for 72 h. Genotype: f/f = *Rictor^{fl/fl}*; cko = *RiCKO*. Scale bar = 50 μ m. All experiments performed with three pairs of male *Rictor^{fl/fl}* versus *RiCKO* littermates. All bar graphs show mean $\pm$ SEM, *p < 0.01.

In vivo μ CT analyses of trabecular bone in the tibia.

BV: bone volume; TV: total volume; Tb, N*: trabeculae number; Tb, Th*: trabeculae thickness; data obtained from 120 of 16 mm slices immediately below growth plate, n = 3 for each group. Change (%): negative and positive values denote decrease and increase caused by treatment, respectively. *p* value denotes significant difference in extent of change between the genotypes (interaction p, ANOVA).

* Statistically significant changes before and after treatment for the same genotype ($p < 0.05$, Student's *t*-test).

Table 2

In vivo μ CT analyses of cortical bone in the tibia.

	Ct. Ar					
	Before treatment			After treatment		
	RiCTOR ^{eff}	RiCKO		RiCTOR ^{eff}	RiCKO	<i>p</i> value
Tween	0.61 $\pm$ 0.05	0.46 $\pm$ 0.03		0.63 $\pm$ 0.05	0.47 $\pm$ 0.03	5 $\pm$ 4 2 $\pm$ 1
Scl-Ab (5 mg/kg)	0.54 $\pm$ 0.04	0.44 $\pm$ 0.04		0.68 $\pm$ 0.02	0.49 $\pm$ 0.04	27 $\pm$ 6* 13 $\pm$ 3* 0.040
Scl-Ab (25 mg/kg)	0.58 $\pm$ 0.03	0.42 $\pm$ 0.03		0.73 $\pm$ 0.05	0.5 $\pm$ 0.04	29 $\pm$ 4* 18 $\pm$ 3* 0.007
<i>Tt. Ar</i>						
Tween	1.04 $\pm$ 0.01	0.82 $\pm$ 0.09		1.05 $\pm$ 0.01	0.86 $\pm$ 0.05	1 $\pm$ 1 5 $\pm$ 5
Scl-Ab (5 mg/kg)	0.97 $\pm$ 0.08	0.72 $\pm$ 0.05		1.02 $\pm$ 0.06	0.74 $\pm$ 0.1	6 $\pm$ 3 3 $\pm$ 1
Scl-Ab (25 mg/kg)	0.96 $\pm$ 0.1	0.75 $\pm$ 0.1		1.19 $\pm$ 0.1	0.87 $\pm$ 0.12	23 $\pm$ 1* 15 $\pm$ 0.4* 0.009
<i>Ct. Th</i> [*]						
Tween	0.19 $\pm$ 0.008	0.16 $\pm$ 0.009		0.19 $\pm$ 0.006	0.16 $\pm$ 0.01	-3 $\pm$ 3 -1 $\pm$ 1
Scl-Ab (5 mg/kg)	0.17 $\pm$ 0.009	0.14 $\pm$ 0.003		0.22 $\pm$ 0.01	0.17 $\pm$ 0.003	27 $\pm$ 2* 16 $\pm$ 3* 0.010
Scl-Ab (25 mg/kg)	0.19 $\pm$ 0.01	0.15 $\pm$ 0.01		0.25 $\pm$ 0.003	0.19 $\pm$ 0.01	33 $\pm$ 3* 25 $\pm$ 2* 0.020

Ct. Ar: cortical bone area; Tt. Ar: total cross-sectional area; Ct. Th^{*}: cortical thickness; data obtained from 50 of 16 mm slices at the mid-point of tibia shaft, $n = 3$ for each group. Change (%): negative and positive values denote decrease and increase caused by treatment, respectively. *p* value denotes significant difference in extent of change between the genotypes (interaction *p*, ANOVA).

* Statistically significant changes before and after treatment for same genotype ($p < 0.05$, Student's *t*-test).

Table 3

DNA sequence of qPCR primers.

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
<i>RANKL</i>	GGTCGGGCAATTCTGAATT	GGGGAATTACAAAGTGCACCAG
<i>OPG</i>	TGGAGATCGAATTCTGCTTG	TCAAGTGCTTGAGGGCATACT
<i>M-CSF</i>	CCCATATTGCGACACCGAA	AAGCAGTAACTGAGCAACGGG
<i>Rictor</i>	GAATACGAGGGCGGAATGAC	GGCCCAGCTTTCTCATATTTG