Bone 85 (2016) 1–8

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

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

Weiwei Sun [a,d], Yu Shi [a], Wen-Chih Lee [a], Seung-Yon Lee [a], Fanxin Long [a,b,c,*]

[a] Department of Orthopaedic Surgery, Washington University School of Medicine, St. Louis, MO 63110, USA
[b] Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA
[c] Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
[d] Department of Anatomy, Histology and Embryology, Nanjing Medical University, Nanjing, China

ARTICLE INFO

Article history:
Received 2 September 2015
Revised 23 December 2015
Accepted 13 January 2016
Available online 15 January 2016

Keywords:
Rictor
mTORC2
Wnt
Sost
Bone
Osteoblast
Osteoclast

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$^{f/f}$, 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.

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 PKCδ 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

* Corresponding author at: Department of Orthopaedic Surgery, Washington University School of Medicine, St. Louis, MO 63110, USA.
E-mail address: flong@wustl.edu (F. Long).

http://dx.doi.org/10.1016/j.bone.2016.01.013
8756-3282/

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$^{f/f}$ mice. Four-month-old sex-matched littermate pairs (*Rictor*$^{f/f}$ 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 μCT measurements, serum biochemistry, or histomorphometry as detailed below.

2.2. In vivo μCT analyses

A total of nine male (n = 5) or female (n = 4) *Rictor*$^{f/f}$ versus RiCKO sex-matched littermate pairs injected as described above were analyzed for bone mass changes with in vivo μCT. The animals were first analyzed with in vivo μCT 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 μCT at the end of treatment before harvest. In vivo micro–computed tomography (μCT) 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 μm. Scanning and analyses were performed as reported previously [15,30]. Briefly, analyses of cortical bone parameters were performed on 50-μCT slices (0.8 mm total) at the mid-point of the shaft of the tibia; trabecular parameters were assessed on 120-μCT 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 houses before serum collection [13]. N-terminal propeptide of procollagen type I (P1NP) 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 μm 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 tibias of 4-month-old *Rictor*$^{f/f}$ 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% CO_2 for 3 days and then washed with PBS, followed by dissociation with 1× 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 μl 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*$^{f/f}$ 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 $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table 3. The experiment was repeated three times, each with BMSC prepared from one pair of *Rictor*$^{f/f}$ versus *RiCKO* male littermates. Representative data from one pair are presented.