Bone 91 (2016) 53–63

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Targeted disruption of BMP signaling through type IA receptor (BMPR1A) in osteocyte suppresses SOST and RANKL, leading to dramatic increase in bone mass, bone mineral density and mechanical strength☆

Nobuhiro Kamiya [a,b,c,*,1], Lin Shuxian [d,e,1], Ryosuke Yamaguchi [a], Matthew Phipps [a], Olumide Aruwajoye [a], Naga Suresh Adapala [a,b], Hui Yuan [d], Harry K.W. Kim [a,b], Jian Q. Feng [d,**]

[a] Texas Scottish Rite Hospital for Children, Dallas, TX 75219, USA
[b] Orthopaedic Surgery, University of Texas Southwestern Medical Center, Dallas, TX 75390-8883, USA
[c] Sports Medicine, Tenri University, Tenri, Nara 632-0071, Japan
[d] Department of Biomedical Sciences, Texas A&M University College of Dentistry, Dallas, TX, USA
[e] Department of Prosthodontics, Ninth People's Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai 200011, China

ARTICLE INFO

Article history:
Received 24 February 2016
Revised 5 July 2016
Accepted 6 July 2016
Available online 8 July 2016

Keywords:
Osteocyte
BMPR1A
RANKL
SOST/sclerostin
Wnt/β-catenin
Proliferation

ABSTRACT

Recent studies suggest a critical role of osteocytes in controlling skeletal development and bone remodeling although the molecular mechanism is largely unknown. This study investigated BMP signaling in osteocytes by disrupting *Bmpr1a* under the *Dmp1*-promoter. The conditional knockout (cKO) mice displayed a striking osteosclerotic phenotype with increased trabecular bone volume, thickness, number, and mineral density as assessed by X-ray and micro-CT. The bone histomorphometry, H&E, and TRAP staining revealed a dramatic increase in trabecular and cortical bone masses but a sharp reduction in osteoclast number. Moreover, there was an increase in BrdU positive osteocytes (2–5-fold) and osteoid volume (~4-fold) but a decrease in the bone formation rate (~85%) in the cKO bones, indicating a defective mineralization. The SEM analysis revealed poorly formed osteocytes: a sharp increase in cell numbers, a great reduction in cell dendrites, and a remarkable change in the cell distribution pattern. Molecular studies demonstrated a significant decrease in the *Sost* mRNA levels in bone (>95%), and the SOST protein levels in serum (~85%) and bone matrices. There was a significant increase in the *β-catenin* (>3-fold) mRNA levels as well as its target genes *Tcf1* (>6-fold) and *Tcf3* (~2-fold) in the cKO bones. We also showed a significant decrease in the RANKL levels of serum proteins (~65%) and bone mRNA (~57%), and a significant increase in the *Opg* mRNA levels (>20-fold) together with a significant reduction in the *Rankl*/*Opg* ratio (>95%), which are responsible for a sharp reduction in the cKO osteoclasts. The values of mechanical strength were higher in cKO femora (i.e. max force, displacement, and work failure). These results suggest that loss of BMP signaling specifically in osteocytes dramatically increases bone mass presumably through simultaneous inhibition of RANKL and SOST, leading to osteoclast inhibition and Wnt activation together. Finally, a working hypothesis is proposed to explain how BMPR1A controls bone remodeling by inhibiting cell proliferation and stimulating differentiation. It is reported that RANKL and SOST are abundantly expressed by osteocytes. Thus, BMP signaling through BMPR1A plays important roles in osteocytes.

1. Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) gene superfamily [1]. BMPs signal through transmembrane serine/threonine kinase receptors such as BMP types I and II. Upon ligand binding, a highly conserved glycine- and serine-rich domain between the transmembrane and kinase domains in the type I receptor is phosphorylated and activated. Three type I receptors (BMPR1A, BMPR1B and ACVR1) relay the signal from the cytoplasm to the nucleus by phosphorylating downstream targets [2]. BMP2, BMP4, and their potent receptor BMPR1A are abundantly expressed in bone [3–5].

☆ This work was supported by the Intramural Research Program of Texas Scottish Rite Hospital for Children (N.K.) and the U.S. National Institutes of Health grants DE025014 and R56DE022789 (J.Q.F).

* Correspondence to: N. Kamiya, Sports Medicine, Tenri University, 80, Tainosho, Tenri, Nara 632-0071, Japan.
** Correspondence to: J. Q. Feng, Texas A&M College of Dentistry, 3302 Gaston Avenue, Sciences Building, Room 228A, Dallas, TX 75246, USA.
E-mail addresses: nkamiya1@sta.tenri-u.ac.jp (N. Kamiya), Jfeng@bcd.tamhsc.edu (J.Q. Feng).

[1] First author: Equally contributed to this study as a first author.

http://dx.doi.org/10.1016/j.bone.2016.07.002
8756-3282/

Osteoblasts are essential for bone development and metabolism because they secrete bone matrix proteins and regulate skeletal mineralization. For many decades, BMPs and their related signaling pathway have been known to play important roles in these osteoblast functions as studied mainly in vitro [2]. In vivo, the conventional knockout mouse lines for BMP2, BMP4, and BMPR1A are all embryonically lethal [6–8]; thus, to elucidate the role of BMP signaling specifically in bone, conditional knockout mouse or transgenic mouse lines have been generated by targeting the osteoblasts. The overexpression of BMP4 in the osteoblasts reduced the bone mass due to increased osteoclastogenesis [9]. The disruption of BMPR1A in osteoblasts increased bone mass and reduced the osteoclastogenesis with a reduction in RANKL [9–12]. These findings suggest that the BMP signal in osteoblasts plays an orthotopic, unexpected (i.e., endogenous) role because it activates bone resorption and reduces bone mass in vivo.

It is generally believed that some osteoblasts become bone-lining cells or undergo apoptosis, and others are passively embedded in the bone matrix and transformed into osteocytes as the surrounding mineral accumulates [13–15]. While it was recently demonstrated that the osteocytes, which compose 90% to 95% of all bone cells in adult bone, are crucial for bone biology because of their functions of regulating mineral metabolism and bioreacting to mechanical loading [16], the role of BMP signaling in osteocytes is largely unknown.

Furthermore, it has been widely believed that osteoblasts control osteoclast formation and maturation by mediating the ratio of RANKL/OPG during bone remodeling. Recently, two groups independently demonstrated that osteocytes, instead of osteoblasts, are the essential sources of the RANKL by controlling osteoclastogenesis and bone remodeling [17,18]. As a molecular mechanism by which osteocytes regulate osteoclasts, a recent study showed that activation of Wnt/β-catenin signaling conditionally in osteocytes gained bone mass concomitant with enhanced bone catabolism thorough RANKL [19]. In addition, SOST/Sclerostin, which is highly expressed in osteocytes as a potent inhibitor of bone anabolic signal Wnt/β-catenin, plays a critical role in the regulation of bone mass and mechanical loading [20–22]. Interestingly, we and other groups demonstrated that these two key molecules in osteocytes (i.e., RANKL and SOST) are downstream targets of BMP signaling [23–25].

The purpose of this study was to investigate the role of BMP signaling in osteocytes in vivo and to elucidate its key molecules SOST and RANKL. For this purpose to understand physiological impacts on bone phenotype, we generated conditional knockout (cKO) mice with osteocyte-specific deletion of BMPR1A under *DMP1* promoter. Here we found dramatically sclerotic bone phenotype with SOST and RANKL suppression in the mutant mice, where bone mineral density (BMD) and mechanical strength were increased.

2. Materials and methods

2.1. Generation of osteocyte-specific BMPR1A-deficient mice

The animal protocols for this study were approved by the local IACUC (Institutional Animal Care and Use Committee) at the University of Texas, Southwestern Medical Center. In order to delete the *Bmpr1a* gene specifically in osteocytes, we used a transgenic mouse line expressing Cre recombinase under the control of the *Dmp1* promoter (i.e. *Dmp1-Cre* mice) [26]. We bred the *Dmp1-Cre* mice with *Bmpr1a* floxed (*Bmpr1afx/fx*) mice [27] to generate experimental *Dmp1Cre:Bmpr1afx/fx* mice and control *Bmpr1afx/fx* mice (hereafter, cKO and control mice, respectively). The Cre activity of the *Dmp1-Cre* mice was identified as osteocytes as we previously reported [26]. There was no phenotypical difference by gender in the cKO group and both male and female were used in the entire experiments. The cKO mice were delivered normally and appeared to be healthy until the endpoint of the study protocol (i.e. 16 weeks of age).

2.2. Radiography and micro-CT analysis

Radiographic images of hindlimbs, spines, and rib cages at 12 weeks of age were obtained using a Faxitron X-ray system (Faxitron). For the micro-CT scans (SkyScan 1172 Micro-CT, Bruker MicroCT, Belgium), each undecalcified specimen (i.e., spine, femora) at 12 weeks of age was wrapped with gauze soaked in 70% ethanol and scanned at a 50 kV and 200 μA setting and a 0.5 mm aluminum filter. The resolution was set at 26.6 μm per pixel, and each rotation step was 0.70 degrees over a range of 180 degrees. After reconstruction with NRecon Reconstruction software (version 1.6.8.), all the serial section grayscale images were set at a threshold to identify the bone that matched the original grayscale X-ray and to remove any soft tissue contribution. An analysis was performed using the manufacturer's software to obtain the trabecular bone parameters (i.e., bone volume, thickness, number, separation, bone mineral density). The binarized 3-dimensional images were visualized with CTVox (version 2.4). It should be noted that cortical bone parameters could not be measured because the borderline between the trabecular and cortical bone was not clear in the cKO femora and spines.

2.3. Histology, immunostaining, and bone histomorphometry

Immediately after sacrifice, the bones were harvested and fixed with 10% formalin for 5 days, followed by decalcification with 10% EDTA for 5 days. The bones were embedded in paraffin, and 3 μm sections were prepared. Toluidine blue and hematoxylin and eosin (H&E) stains were performed following a standard protocol as previously described [11,23]. To detect the osteoclasts, TRAP staining was performed as previously reported [11].

For immunostaining, the femora were immediately dissected from the mice and fixed with freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 2 days, then stored in 0.5% paraformaldehyde. The bones were embedded in paraffin and 4.5 μm sections were prepared. Immunostaining was performed using an anti-sclerostin polyclonal antibody (1:100, AF1589, R&D Systems, Minneapolis, MN, USA), anti-Bmpr1a polyclonal antibody (1:100, AP2004B, Abgent, San Diego, CA, USA), and anti-beta-catenin active form monoclonal antibody (1:200 EMD Millipore, Billerica, MA, USA), followed by secondary antibodies. Antibody binding was visualized with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) before briefly counterstaining with hematoxylin (Invitrogen). Negative controls without primary antibodies were used.

For the BrdU staining, mice were injected twice with BrdU at 2 weeks of age (5-bromo-2-deoxyuridine, 100 μg/g mouse, intraperitoneal; Sigma-Aldrich). The first injection was made 24 h before mouse sacrifice, and the second was made 2 h prior to sacrifice. The femora (control: n = 4, cKO: n = 4) were fixed with paraformaldehyde for two days, decalcified with EDTA, and embedded by paraffin. Sections (4.5 μm thick) were dewaxed, rehydrated for BrdU staining; (BrdU staining kit, Invitrogen), dehydrated and mounted after staining and finally counted and counted after staining.

For standard bone histomorphometry as well as osteocyte-morphometry, the adult mice at 12 weeks of age received tetracycline (20 mg/Kg; Sigma-Aldrich) 5 days before death and calcein (10 mg/kg; Sigma-Aldrich) intraperitoneally 2 days before death. The femora (control: n = 3, cKO: n = 3) were fixed in 70% ethanol and embedded in methyl-methacrylate (Wako Chemicals, Kanagawa, Japan) without decalcification. Serial sections were cut and stained with Villanueva bone stain for bone histomorphometry. The histomorphometric analysis was performed at the Ito Bone Science Institute (Niigata, Japan). The cancellous bone was measured in the secondary spongiosa located at 250 μm from the epiphyseal growth plate (i.e. distal femora) and 125 μm from the endocortical surface using a semiautomatic image analysis system (System Supply, Nagano, Japan) and a fluorescent microscope (OLYMPUS BX-51, Japan).