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FGF2 crosstalk with Wnt signaling in mediating the anabolic action of PTH on bone formation

Liping Xiao, Yurong Fei[1], Marja M. Hurley*

Department of Medicine, UConn Health, Farmington, CT 06030, USA

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ABSTRACT

The mechanisms of the anabolic effect of parathyroid hormone (PTH) in bone are not fully defined. The bone anabolic effects of PTH require fibroblast growth factor 2 (FGF2) as well as Wnt signaling and FGF2 modulates Wnt signaling in osteoblasts. In vivo PTH administration differentially modulated Wnt signaling in bones of wild type (WT) and in mice that Fgf2 was knocked out (*Fgf2KO*). PTH increased Wnt10b mRNA and protein in WT but not in KO mice. Wnt antagonist SOST mRNA and protein was significantly higher in KO group. However, PTH decreased Sost mRNA significantly in WT as well as in *Fgf2KO* mice, but to a lesser extent in *Fgf2KO*. Dickhopf 2 (DKK2) is critical for osteoblast mineralization. PTH increased *Dkk2* mRNA in WT mice but the response was impaired in *Fgf2KO* mice. PTH significantly increased Lrp5 mRNA and phosphorylation of Lrp6 in WT but the increase was markedly attenuated in *Fgf2KO* mice. PTH increased β-catenin expression and Wnt/β-catenin transcriptional activity significantly in WT but not in *Fgf2KO* mice. These data suggest that the impaired bone anabolic response to PTH in *Fgf2KO* mice is partially mediated by attenuated Wnt signaling.

1. Introduction

Osteoporosis is a disease characterized by low bone mass and a deterioration in the micro-architecture of bone tissue, which leads to bone fractures (Holroyd et al., 2008). In year 2025 an estimated 44 million Americans are threatened by osteoporosis and the cost for osteoporosis-related fractures is predicted to be $25.3 billion (NOF, 2013). Therefore, osteoporosis is an enormous health and economic problem. Parathyroid hormone (PTH) is currently the only anabolic agent for treatment of osteoporosis in the U.S. Since 2002, when the FDA approved intermittent PTH administration, great progress has been made in understanding how intermittent PTH treatment mediates its anabolic bone response. However, the detailed mechanisms of PTH actions are not fully defined.

We previously showed that maximal bone anabolic effects of PTH require fibroblast growth factor 2 (FGF2). PTH induced FGF2 and FGF receptor mRNA expression in osteoblast cells (Hurley et al., 1999). In addition, PTH treatment increased serum FGF2 in osteoporotic subjects together with enhanced bone formation (Hurley et al., 2005). However, the anabolic response of PTH on bone formation in mice was impaired in the absence of endogenous FGF2 (Hurley et al., 2006) (Fei et al., 2011b). These data suggest that endogenous FGF2 is required for maximal bone anabolic response of PTH.

FGF2 is one member of the FGF family. It is expressed in osteoblasts and stored in the extracellular matrix (Ornitz and Marie, 2015). FGF2 stimulates osteoblast precursor proliferation (Fei and Hurley, 2012; Hurley et al., 2002). Although continuous administration of FGF2 decreases osteoblast differentiation markers, intermittent FGF2 treatment stimulates osteoblast differentiation and bone formation in vitro and in vivo (Hurley et al., 2002; Montero et al., 2000). *Fgf2KO* mice further reveal the importance of FGF2 in bone. There is markedly reduced plate-like trabecular structures and loss of connecting rods of trabecular bone in the absence of endogenous FGF2 (Montero et al., 2000). Interestingly, we observed that FGF2 expression decreased in osteoblasts from aged subjects compared to cells from young subjects (Hurley et al., 2016). Clinical trials demonstrate that local application of FGF2 stimulates periodontal regeneration (Kitamura et al., 2011) and accelerates healing of tibial shaft fractures (Kawaguchi et al., 2010). These data support that FGF2 positively regulates osteoblast differentiation and bone formation.

Bone anabolic response of PTH also requires the Wnt signaling pathway (Fei and Hurley, 2012; Jilka, 2007). Wnt signaling can occur either through the non-canonical pathway or the canonical Wnt/β-catenin signaling. Non-canonical pathway includes the Wnt/calcium pathway and the Wnt/planar cell polarity pathway (Piters et al., 2008). The canonical Wnt/β-catenin pathway is well studied in bone. To

* Corresponding author at: Department of Medicine, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030, USA.
E-mail address: hurley@uchc.edu (M.M. Hurley).
[1] Current address: Springer Nature, New York, NY, 10013.

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Table 1
Primers used for quantitative real time-PCR.

Gene	Forward	Reverse
Gapdh	5′-CAGTGCCAGCCTCGTCCCGTAGA-3′	5′-CTGCAAATGGCAGCCCTGGTGAC-3′
β-Catenin	5′-TGCTGAAGGTGCTGTCTGTC-3′	5′-GCTGCACTAGAGTCCCAAGG-3′
Dkk1	5′-GGGAGTTCTCTATGAGGGCG-3′	5′-AAGGGTAGGGCTGGTAGTTG-3′
Dkk2	5′-CTGGGATGGCAGAATCTAGG-3′	5′-AATCCAGGTTTCCATCATGC-3′
Lrp5	5′-ACCCGCTGGACAAGTTCATC-3′	5′-TCTGGGCTCAGGCTTTGG-3′
Lrp6	5′-GGTGTCAAAGAAGCCTCTGC-3′	5′-ACCTCAATGCGATTTGTTCC-3′
Sost	5′-GGAATGATGCCACAGAGGTCAT-3′	5′-CCCGGTTCATGGTCTGGTT-3′
Wnt3a	5′-CTCCTCTCGGATACCTCTTAGTG-3′	5′-CCAAGGACCACCAGATCGG-3′
Wnt10b	5′-TTCTCTCGGGATTTCTTGGATTC-3′	5′-TGCACTTCCGCTTCAGGTTTTC-3′

initiate signaling, ligand Wnt bind to receptor Lrp5/Lrp6 and Frizzled. This binding will block the destruction complex including kinase glycogen synthase kinase 3β (GSK3β), therefore, β-catenin will be stabilized and accumulate in the nucleus, where it binds to transcription factor T cell specific transcription factor and activate downstream target genes. Wnt signaling is regulated by Wnt antagonists, such as sclerostin (the gene coding sclerostin is Sost) and dickkopfs (DKKs) (Kamiya et al., 2008; Kawano and Kypta, 2003).

Extensive studies support that PTH treatment regulates genes of the Wnt signaling family (Kulkarni et al., 2005; Li et al., 2007; Onyia et al., 2005; Qin et al., 2003). We previously reported that FGF2 stimulation of osteoblast differentiation is partially through modulation of the Wnt/β-catenin signaling pathway (Fei et al., 2011a). We hypothesize that the impaired bone anabolic response of PTH in *Fgf2KO* mice is mediated by attenuated Wnt signaling. In the present study, we examined acute effects of PTH treatment (single injection, 20 μg/kg body weight) on Wnt signaling. We observed attenuated Wnt/β-catenin signaling in bones of *Fgf2KO* mice, which may contribute to the impaired bone anabolic response to PTH.

2. Materials and methods

2.1. Generation of 3.6Col1GFPsaph$^{Tg/Tg}$; Fgf2$^{-/-}$ mice

To generate mice in which osteoblast lineage cells are labeled with green fluorescent and Fgf2 gene is globally knocked out, 3.6Col1GFPsaph$^{Tg/Tg}$ mice on a FVB/N genetic background previously made in our lab (Xiao et al., 2009) was crossed with *Fgf2* null mice on a black swiss 129Sv genetic background (Zhou et al., 1998). 3.6Col1GFPsaph$^{Tg/Tg}$; Fgf2$^{+/-}$ × 3.6Col1GFPsaph$^{Tg/Tg}$; Fgf2$^{+/-}$ breeding pairs were maintained in the transgenic facility in the Center for Comparative Medicine at the UConn Health to generate 3.6Col1GFPsaph$^{Tg/Tg}$; Fgf2$^{+/+}$ (WT) and 3.6Col1GFPsaph$^{Tg/Tg}$; Fgf2$^{-/-}$ (*Fgf2KO*) mice used in this study. Three months-old WT and *Fgf2KO* littermate female mice were used in the present study unless otherwise specified. These mice are on a mixed black swiss 129Sv/FVB/N genetic background and osteoblast lineage cells are labeled with green fluorescence sapphire. Mice genotype was performed using primers as previously described (Montero et al., 2000). Mice were sacrificed by CO_2 narcosis and cervical dislocation. The UConn Health Institutional Animal Care and Use Committee approved all animal protocols.

2.2. PTH injection

Three independent experiments were performed using three-month old female mice. Mice were weighed and injected subcutaneously with vehicle (Veh, 0.001 M HCl in PBS with 1 mg/ml BSA) or 20 μg/kg body weight human PTH (1–34) (Bachem, Torrance, CA, USA). Eight hours after injection, mice were sacrificed by CO_2 narcosis and cervical dislocation. Tibiae were dissected, epiphyses were removed and the remaining bones were snap frozen in liquid nitrogen for RNA or protein extraction.

2.3. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from whole tibia (including bone and bone marrow) utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol. Three microgram RNA was reverse transcribed with a commercial kit (Clontech, CA, USA). Quantitative real-time PCR (qPCR) was carried out using the QuantiTect™ SYBR Green PCR kit by a MyiQ™ instrument (Bio-Rad, Laboratories Inc. Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an internal control for each sample. Relative mRNA expression was calculated using a formula reported previously (Pfaffl, 2001). mRNA was normalized to Gapdh mRNA level and expressed as the fold-change relative to the first sample for each experimental group. The mouse specific primers used are shown in Table 1.

2.4. Protein extraction and western blot

Whole tibiae (including bone and bone marrow) were broken down using polytron homogenizer and protein extracts were harvested in RIPA buffer (Cell Signaling, MA, USA). Protein concentrations were measured with BCA protein assay reagent (Pierce, IL, USA). Equal amounts of protein were fractioned on SDS-PAGE gel and transferred onto a PVDF membrane (Bio-Rad, CA, USA). Membranes were blocked for 1 h with 5% non-fat dry milk, and then incubated overnight at 4 °C with antibody against Wnt10b (4μg/ml, catalog number: ab70816, Abcam, MA, USA), pLRP6 antibody (1:1000, catalog number: 2568s, Cell Signaling Technology, MA, USA), LRP6 (1:500, catalog number: sc-15399, Santa Cruz, CA, USA), inactive GSK3β (1:1000, catalog number: 9336, Cell Signaling Technology, MA, USA), total GSK3β (1:1000, catalog number: 9315, Cell Signaling Technology, MA, USA), SOST (1:5000, AF1589, R&D System, MN, USA), active β-catenin (1:1000, catalog number: 8841, Cell Signaling Technology, MA, USA), total β-catenin (1:500, sc-7199, Santa Cruz, CA, USA), or Actin (1:10000, sc-47778, Santa Cruz, CA, USA). Membranes were incubated with anti-rabbit secondary antibody (1:10000, catalog number: 7074s, Cell Signaling Technology, MA, USA) or anti-goat secondary antibody (1:10000, catalog number: sc-2020, Santa Cruz, CA, USA) at room temperature for 1 h. Blots were developed with Super Signal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). Western blot bands were analyzed by NIH Image (version 1.61; NIH, Bethesda, Maryland, USA).

2.5. Immunofluorescence staining

For histological analysis, femurs were immediately fixed in 4% PFA at 4 °C for 48 h. Each sample was embedded in Shandon Cryomatrix (Thermo Electron Corporation, PA, USA). Frozen samples were cut into 6-μm sections on Cryofilm type IIC (Choung et al., 2001). The sections were washed in 1× PBS/1%FBS and permeabilized with 0.25% Triton X-100 in 1× PBS/1%FBS for 10 min. After rinsing with 1× PBS/1% FBS, the sections were stained with anti-SOST antibody (1:40, catalog