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Wnt/β-catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts

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ABSTRACT

The BMP and Wnt/β-catenin signaling pathways cooperatively regulate osteoblast differentiation and bone formation. Although BMP signaling regulates gene expression of the Wnt pathway, much less is known about whether Wnt signaling modulates BMP expression in osteoblasts. Given the presence of putative Tcf/Lef response elements that bind β-catenin/TCF transcription complex in the BMP2 promoter, we hypothesized that the Wnt/β-catenin pathway stimulates BMP2 expression in osteogenic cells. In this study, we showed that Wnt/β-catenin signaling is active in various osteoblast or osteoblast precursor cell lines, including MC3T3-E1, 2T3, C2C12, and C3H10T1/2 cells. Furthermore, crosstalk between the BMP and Wnt pathways affected BMP signaling activity, osteoblast differentiation, and bone formation, suggesting Wnt signaling is an upstream regulator of BMP signaling. Activation of Wnt signaling by Wnt3a or overexpression of β-catenin/TCF4 both stimulated BMP2 transcription at promoter and mRNA levels. In contrast, transcription of BMP2 in osteogenic cells was decreased by either blocking the Wnt pathway with DKK1 and sFRP4, or inhibiting β-catenin/TCF4 activity with FWD1/β-TrCP, ICAT, or ΔTCF4. Using a site-directed mutagenesis approach, we confirmed that Wnt/β-catenin transactivation of BMP2 transcription is directly mediated through the Tcf/Lef response elements in the BMP2 promoter. These results, which demonstrate that the Wnt/β-catenin signaling pathway is an upstream activator of BMP2 expression in osteoblasts, provide novel insights into the nature of functional cross talk integrating the BMP and Wnt/β-catenin pathways in osteoblastic differentiation and maintenance of skeletal homeostasis.

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Introduction

Bone morphogenetic protein 2 (BMP2) is a critical autocrine and paracrine growth factor that directs osteoblast differentiation and bone formation [1–4]. Signaling of BMP2 induces mesenchymal precursor cells to differentiate into mature osteoblasts by regulating signals that stimulate specific transcriptional programs required for bone formation [5,6]. Previous studies have shown that BMP2 expression is tightly associated with the status of osteoblast maturation [7,8], and is regulated in osteoblasts by other bone-related factors and signaling pathways including the TGF-β, hedgehog/Gli, PTH/CREB, estrogen receptor, NF-κB, PGE2, and microtubule signaling pathways [9–17]. Expression of BMP2 also is regulated by the BMP signaling pathway itself because BMP2 is an autoregulated protein [18–20]. These multiple mechanisms involved in regulation of BMP2 gene expression may provide potential therapeutic targets for skeletal diseases with bone loss. However, the major anabolic mechanisms, particularly at the transcriptional level, that control BMP2 expression for osteoblastic bone formation are still unknown.

Abbreviations: ALP, alkaline phosphatase; BGJ medium, Biggers, Gwatkin, and Judah medium; BMP, bone morphogenetic protein; β-TrCP, β-transducin repeat-containing protein; Col1a1, type I collagen 1a1; CTGF, connective-tissue growth factor; CREB, cAMP responsive element binding protein; DKK1, Dickkopf-related protein 1; FWD1, F-box/WD40-repeat protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3, glycogen synthase kinase 3; H&E, hematoxylin and eosin; ICAT, murine inhibitor of β-catenin and transcription factor; LRP5/6, low-density lipoprotein receptor-related protein 5/6; Lef, lymphoid enhancer binding factor; NF-κB, nuclear factor κB; OSE2, Runx2 binding elements; PGE2, prostaglandin E2; PTH, parathyroid hormone; RT-PCR, reverse transcription polymerase chain reaction; SBE, Smad binding element; sFRP4, secreted frizzled-related protein 4; sKremen, soluble form of the extracellular ligand-binding domain (amino acids 20–395) of Kremen-1; TCF (Tcf), T cell specific factor; TGF-β, transforming growth factor beta; TRE, Tcf/Lef response elements or transcription regulatory elements; Wnt, wingless integration site.

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Recent gain- and loss-of-function studies of LRP5/6, β-catenin, and many other molecules that participate in the Wnt signaling cascade identify the canonical Wnt signaling pathway as a crucial pathway in the skeleton controlling osteoblast differentiation and bone formation [21–28]. The evolutionarily conserved BMP and Wnt pathways are independent signaling mechanisms, by means of different ligands, receptors, and intracellular signal transducers. However, these two pathways control bone formation cooperatively. Activation of Wnt signaling induces differentiation of pluripotent mesenchymal cells into osteoblast progenitors that become osteoblasts, and maintains the precursor status of these osteoprogenitors. BMP signaling stimulates these cells to further differentiate into mature osteoblasts. After osteoprogenitors become osteoblasts, both pathways promote further differentiation, evidenced by increased alkaline phosphatase (ALP) activity and mineralization [29–32]. Functional integration of the BMP and Wnt signaling pathways mostly causes dependent and/or synergistic effects on osteoblast differentiation and bone formation [30,32,33].

Molecular studies have shown that functional communication between the BMP and Wnt pathways involves multiple mechanisms. Some extracellular proteins, such as sclerostin, CTGF, cerberus and sFRPs, are known to bind ligands/antagonists or/and receptors of both the BMP and Wnt pathways [34–40]. Intracellularly, Smads are found to form complexes with Wnt signaling molecules, such as Dishevelled-1, Axin, GSK3 and β-catenin. These complexes modulate phosphorylation and activity of Smads and β-catenin [41–46]. Perhaps the most compelling mechanism highlighting the cooperation between the BMP and Wnt pathways is the transcriptional regulation of their common target genes, which harbor both Smad and Tcf/Lef response elements (TREs). Smads can form a transcriptional complex with β-catenin/Tcf/Lef and co-activate transcription of many target genes through these binding elements in response to both BMP and Wnt signaling [47–52]. In our effort to identify potentially critical transcriptional mechanisms that control BMP2 expression in osteoblasts, we hypothesized that the Wnt signaling pathway is an upstream transactivator of BMP2 in osteoblasts. This takes into account that the BMP and Wnt signaling pathways tightly cooperate and regulate each other, and that multiple putative Tcf/Lef response elements (TREs) exist in the BMP2 promoter (vide infra). The purposes of the current study were to determine the Wnt/β-catenin signaling pathway activation effects on BMP2 gene expression and signaling in osteoblasts and to identify potential transcriptional mechanisms. Since both BMP and Wnt pathways are critical anabolic signaling pathways affecting bone formation, demonstration of Wnt regulation of BMP2 expression in osteoblasts will provide novel insights into the role of the functional communication between the BMP and Wnt signaling pathways that affects bone formation.

Material and methods

DNA constructs and recombinant proteins

The following expression plasmids were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD): pCI-neo-β-catenin for human β-catenin; pCI-neo-β-catenin(Δ45) for stabilized β-catenin form in which Ser^{45} is deleted; pCI-neo-β-catenin(S33Y) for stabilized β-catenin form in which Ser^{33} is replaced by Tyr^{33} [53]; pcDNA-Myc-TCF4 for human TCF4; pcDNA-Myc-ΔTCF4 for the dominant negative mutant form of TCF4 (ΔTCF4) that lacks amino acids 1–30; and the Wnt signaling reporter TOPFLASH (pGL3-OT) [54]. Murine inhibitor of β-catenin and TCF (ICAT) was expressed in a vector as pcDNA3.1-Flag-ICAT [55]. Expression plasmids pcDNA3-Flag-FWD1 for F-box/WD40-repeat protein 1 (FWD1), the mouse ortholog of β-transducin repeat-containing protein (β-TrCP), and pcDNA3-Flag-FWD1ΔF-dominant negative form of FWD1 in which the F-box is deleted [56] were gifts from Drs. Kei-ichi Nakayama and Hatakeyama (Kyushu University, Japan). Osteoblast-specific multiple-signaling reporter, 9x6-OC-Luc, was constructed by linking a sequence containing 9 copies of Tcf/Lef response elements (TRE), 9 copies of Smad binding elements (SBE), and 6 copies of Runx2 binding elements (OSE2) upstream of a basal mouse osteocalcin promoter and linked to a luciferase reporter in pGL3 vector (Promega, Madison, WI). The BMP2 promoter reporter −2712/+165-Luc, made by linking mouse BMP2 promoter sequence −2712/+165 to a cDNA for firefly luciferase in pGL3 vector, has been previously described [6,13,14,57]. The BMP signaling reporter, 12SBE-Luc that we previously described [13,17,57,58], was constructed by connecting 12 copies of BMP-specific SBEs upstream of a basal mouse osteocalcin promoter and firefly luciferase coding sequence in pGL3 vector. All recombinant murine proteins (Wnt3a, BMP2, noggin, DKK1, serum frizzled-related protein 4 [sFRP4], and a genetically engineered soluble form of the extracellular ligand-binding domain [amino acids 20–395] of Kremen-1 [sKremen]) were purchased commercially (R&D Systems, Minneapolis, MN).

Cell culture and transfection

Osteoblast and osteoblast precursor MC3T3-E1 and 2T3 cells [6,13,14,57] were cultured in α-minimal essential medium (α-MEM). Pluripotent mesenchymal C3H10T1/2 cells were cultured in RPMI medium 1640. Myoblastic C2C12 cells were cultured in Dulbecco's modified Eagle medium (DMEM). Cells were cultured at 37 °C in a humidified 5% CO_2 incubator. The medium was supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% L-glutamine. Cells at 70–80% confluence were transfected with luciferase reporters and/or expression plasmids using LipoFectamine Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Bone organ culture assay

Neonatal mouse calvarial cultures were performed as previously described [11,59,60]. Briefly, calvariae from 4-day old Institute for Cancer Research (ICR) Swiss pups were dissected and cut in half with the excised hemi-calvariae explanted on metal grids (on the surface) in 1 mL BGJ medium with Fitton-Jackson modification BGJ medium (Sigma) containing 0.1% BSA with glutamine. The bones were incubated at 37 °C in a 5% humidified incubator for 24 h, transferred to wells containing 1 mL of medium with test compounds, and then further incubated under the above conditions for 72 h. The bones were then removed, fixed in 10% buffered formalin for 24 h, decalcified in 14% EDTA overnight, and embedded in paraffin. Sections (7 μm thick) were then cut and stained with hematoxylin and eosin (H&E) to facilitate assessment of new bone formation and osteoblast proliferation (cellularity of sections) as detailed previously (60).

Western blot

Twenty-four hours after incubation of MC3T3-E1, 2T3, C3H10T1/2, and C2C12 cells with Wnt3a at 40 ng/mL or vehicle, or 36 h after transfection of C2C12 cells with the β-catenin expression vector, cells were lysed with RIPA cell lysate buffer. Cell lysates mixed with sample buffer were loaded on SDS-PAGE gels (Mini-PROTEIN II Ready gels, Bio-Rad, Hercules, CA). Proteins were transblotted onto a PVDF membrane (Bio-Rad) in a transblotting buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8.0) at 4 °C and 100 V for 1 h. The membrane was blocked with 5% dry milk in TBS-T for 1 h at room temperature. Then the membrane was incubated with either a rabbit monoclonal anti-β-catenin antibody (Abcam, ab32572, Cambridge, MA) or a mouse monoclonal anti-β-actin antibody (Abcam, ab8226) in TBS-T for 2 h at room temperature. Incubation with horseradish peroxidase-conjugated anti-goat IgG antibody (Amersham Biosciences, Piscataway, NJ) was performed at room temperature for 1 h. After washes, immunoblots were detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences).