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BMP-2 Modulates β-Catenin Signaling Through Stimulation of *Lrp5* Expression and Inhibition of β-TrCP Expression in Osteoblasts

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ABSTRACT

Canonical BMP and Wnt signaling pathways play critical roles in regulation of osteoblast function and bone formation. Recent studies demonstrate that BMP-2 acts synergistically with β-catenin to promote osteoblast differentiation. To determine the molecular mechanisms of the signaling cross-talk between canonical BMP and Wnt signaling pathways, we have used primary osteoblasts and osteoblast precursor cell lines 2T3 and MC3T3-E1 cells to investigate the effect of BMP-2 on β-catenin signaling. We found that BMP-2 stimulates *Lrp5* expression and inhibits the expression of β-TrCP, the F-box E3 ligase responsible for β-catenin degradation and subsequently increases β-catenin protein levels in osteoblasts. In vitro deletion of the β-catenin gene inhibits osteoblast proliferation and alters osteoblast differentiation and reduces the responsiveness of osteoblasts to the BMP-2 treatment. These findings suggest that BMP-2 may regulate osteoblast function in part through modulation of the β-catenin signaling. J. Cell. Biochem. 108: 896–905, 2009.

KEY WORDS: BMP-2; β-CATENIN; LRP5; β-TrCP; OSTEOBLAST DIFFERENTIATION

The Wnt family consists of a numbers of small, cysteine-rich, secreted glycoproteins involved in regulation of a variety of cellular activities and plays critical roles early during development controlling mesoderm induction, patterning, cell fate determination and morphogenesis [Huelsken and Birchmeier, 2001; Moon et al., 2002; Westendorf et al., 2004]. Wnt proteins trigger signaling pathways inside cells that proceed through several protein complexes. One protein in these pathways is β-catenin. The canonical Wnt signaling pathway affects cellular functions by regulating β-catenin expression and its subcellular localization. In the absence of Wnt, β-catenin levels are kept at a steady-state. Any β-catenin molecules that are not bound to cadherins are ubiquitinated and degraded by the 26S proteasome [Aberle et al., 1997]. A multi-protein complex containing kinases such as glycogen synthase kinase (GSK) 3β and casein kinase 1 (CK1) and scaffolding proteins such as Axis inhibition protein (Axin), Axin2 (also known as conductin), adenomatous polyposis coli (APC) and disheveled (Dsh) mediate the degradation of excess β-catenin by phosphorylating specific amino terminal residues and creating docking sites for F-box protein/E3 ligase complexes [Behrens et al., 1998; Jiang and Struhl, 1998; Westendorf et al., 2004]. Therefore, inhibition of β-catenin phosphorylation prevents its degradation and increases its cytoplasmic levels and nuclear translocation. Signaling from Wnt releases β-catenin from its binding protein,

Abbreviations used: BMP-2, bone morphogenetic protein 2; DKK1, Dickkopf-1; LRP5, low-density-lipoprotein-receptor related protein 5; Col-1, type I collagen; ALP, alkaline phosphatase; Wnt3a, Wingless/Int-3a; TCF, T-cell factor; LEF, lymphocyte enhancer binding factor; GSK, glycogen synthase kinase.

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allowing it to move to the nucleus, where it interacts with the TCF/LEF transcription factors, to activate expression of target genes [Staal and Clevers, 2000; Moon, 2004].

At the surface of cells, Wnts interact with two kinds of protein; Frizzled receptor and low-density-lipoprotein (LDL) receptor related protein 5 or 6 (LRP5/6). The intracellular parts of the receptors pass on the information, turning on the pathway through β-catenin inside the cell. LRP5 or 6 receptors have a second function. They bind to Dickkopf-1 (DKK1), a molecule that counteracts Wnt and blocks Wnt function. Binding of DKK1 to LRP5/6 might alter the conformation of LRP5/6 so that LRP5 or 6 can no longer interact with Wnt and Frizzled, thus halting the intracellular signaling. The intracellular tail of LRP5/6 binds Axin and causes dissociation of β-catenin from its protein complex and activates β-catenin signaling [Mao et al., 2001]. Most recently, mouse genetic evidence further indicates that LRP5 controls bone mass through a β-catenin independent mechanism. LRP5 blocks serotonin production in enterochromaffin cells of the duodenum through inhibition of the expression of Tph1, the rate-limiting biosynthetic enzyme for serotonin, a negative regulator of osteoblast proliferation [Yadav et al., 2008]. It seems that there are direct and indirect effects of LRP5 on bone cells.

Bone morphogenetic protein 2 (BMP-2) stimulates osteoblast differentiation and bone formation in vitro and in vivo [Wozney and Rosen, 1988; Chen et al., 1997, 1998; Zhao et al., 2002]. The downstream target genes of BMP-2 signaling and cross-interaction between BMP-2 and other signaling pathways in osteoblasts remain poorly understood. Recent studies demonstrate that BMP-2 has a synergic effect with β-catenin on osteoblast differentiation in vitro [Rawadi et al., 2003; Mbalaviele et al., 2005]. In the present studies, we investigated the mechanism by which BMP-2 modulates β-catenin signaling. We found that BMP-2 stimulates *Lrp5* expression and inhibits β-TrCP expression and subsequently enhances protein levels of the non-phosphorylated active form of β-catenin in osteoblasts. In vitro deletion of the *β-catenin* gene inhibits osteoblast proliferation and differentiation and partially inhibits the responsiveness of BMP-2 on osteoblast differentiation. Our findings provide new insights into cross-interaction between canonical BMP-2 and β-catenin signaling pathways in osteoblasts.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

2T3 and MC3T3-E1 osteoblast precursor cells were cultured in a minimal essential medium (α-MEM) and supplemented with 10% fetal calf serum (FCS). Primary osteoblasts were isolated from 3-day-old C57BL/6J wild-type (WT) mice or *β-catenin-loxP* mice and cultured in α-MEM supplemented with 10% FCS. Bone marrow stromal (BMS) cells were isolated from 2-month-old WT and *β-catenin-loxP* mice and cultured in α-MEM supplemented with 10% FCS. Empty vector (pcDNA3, 1 μg/well), mutant Smad1 (G418S) or β-TrCP expression plasmids were transfected into 2T3 cells in 6-well culture plates using Lipofectamine plus reagents (Life Technologies, Inc., Rockville, MD). Cells were treated with different concentrations of BMP-2 (0–200 ng/ml) for different period of time (0–24 h). Cell lysates were extracted and Western blot were performed.

ADENOVIRUS INFECTION

An adenovirus vector carrying an EGFP gene driven by CMV promoter (Ad-GFP), and an adenovirus vector carrying the Cre recombinase gene of P1 phage driven by CMV promoter (Ad-Cre), were made by CAGT Vector Development Laboratory (Baylor College of Medicine, Houston, TX) (4×10^6 plaque formation unit, PFU). Primary osteoblasts were isolated from the calvaria of 3-day-old *β-catenin-loxP* mice and BMS cells were isolated from 2-month-old WT and *β-catenin-loxP* mice. Cells were infected with Ad-Cre and Ad-GFP (multiplicity of infection, MOI = 2). Twenty-four hours after infection, the medium was changed and cells were recovered after additional 24 h incubation. Cells were treated with different concentrations of BMP-2 as indicated in figures.

RNA ANALYSIS

Primary osteoblasts isolated from *β-catenin-loxP* mice were infected with Ad-Cre or Ad-GFP (control). Total RNA was extracted from these cells using RNAzol B solution (Tel-Test, Inc., Friendswood, TX). Expression of osteoblast marker genes was analyzed by real-time RT-PCR. DNAse I-treated total RNA was reverse transcribed-using oligo-(dT) and cDNA were amplified using primers specific for these marker genes.

WESTERN BLOT ANALYSIS

To determine effects of BMP-2 on expression of β-catenin in osteoblasts, 2T3 or MC3T3-E1 cells were treated with different concentrations of BMP-2 (50 and 100 ng/ml) or treated with 100 ng/ml of BMP-2 for different time periods (0–24 h) and cell lysates were extracted. Wnt3a (100 ng/ml) was used as a positive control. 2T3 or MC3T3-E1 cells were lysed on ice for 30 min in a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). Proteins were fractionated by SDS–PAGE, transferred to a nitrocellulose membrane and detected using the following antibodies: anti-β-catenin monoclonal antibody (clone E-5) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-active-β-catenin (clone 8E7) and anti-TCF4 (clone 6H5-3) monoclonal antibodies (Upstate Cell Signaling solutions, Lake Placid, NY), anti-TCF3 (clone 2G2e4) and anti-β-TrCP (clone 1B1D2) monoclonal antibodies (Zymed Laboratories, Inc., South San Francisco, CA). Immunostaining was detected using an enhanced chemiluminescence (ECL) system (Amersham, Backinghamshire, UK).

RESULTS

β-CATENIN AND ITS TRANSCRIPTION FACTORS Tcf/Lef ARE EXPRESSED IN BONE CELLS

To determine if β-catenin protein is expressed in osteoblasts in vivo, we have performed immunostaining using an anti-β-catenin

monoclonal antibody on histological sections of tibiae from 3-day-old (P3) C57BL/6J mice. Strong β-catainin staining was detected in osteoblasts on the trabecular bone surfaces in the primary spongiosa and endosteal surfaces of cortical bone (Fig. 1A–C). When β-catenin enters the nucleus, it interacts with TCF/LEF transcription factors to activate downstream target genes. Four TCF/LEF transcription factors have been identified: TCFs 1, 3, and 4 and LEF1. In the present studies, we analyzed *Tcf/Lef* expression in primary osteoblasts. Results showed that mRNA of these four transcription factors was expressed in primary osteoblasts isolated from calvariae of 3-day-old C57BL/6J mice. *Tcf3* and *Tcf4* are highly expressed and *Tcf1* and *Lef* are only weakly expressed in primary osteoblasts (Fig. 1D). The protein expression of TCF3 and TCF4 was detected by Western blotting in primary osteoblasts and osteoblastic cell lines. TCF3 was highly expressed in 2T3 and MC3T3-E1 cells and primary mouse and rat osteoblasts and weakly expressed in C2C12 cells. TCF4 was highly expressed in MC3T3-E1 cells and was weakly expressed in primary osteoblasts (Fig. 1E). Taken together, these results demonstrated that osteoblasts express β-catenin protein and its interacting transcription factors and indicate that β-catenin plays an important role in osteoblast functions.

BMP-2 MODULATES β-CATENIN SIGNALING IN OSTEOBLASTS

It has been reported that BMP-2 has a synergistic effect with β-catenin to promote chondrocyte and osteoblast differentiation [Fischer et al., 2002a,b; Bain et al., 2003; Rawadi et al., 2003; Mbalaviele et al., 2005; Winkler et al., 2005]. In the present studies, we examined the effect of BMP-2 on steady-state protein levels of β-catenin in osteoblasts. We determined the time-course effect of BMP-2 on β-catenin expression and found that BMP-2 increased the protein levels of the non-phosphorylated active form of β-catenin in 2T3 osteoblast precursor cells [Ghosh-Choudhury et al., 1996; Chen et al., 1998] and maximal effect was observed at 24-h time point (Fig. 2A). We then tested the effect of 50 and 100 ng/ml of BMP-2 on β-catenin expression and used Wnt3a as a positive control. We found both doses of BMP-2 had similar potency on β-catenin expression and Wnt3a, as a positive control, also increased β-catenin expression in 2T3 cells (Fig. 2B). To determine if

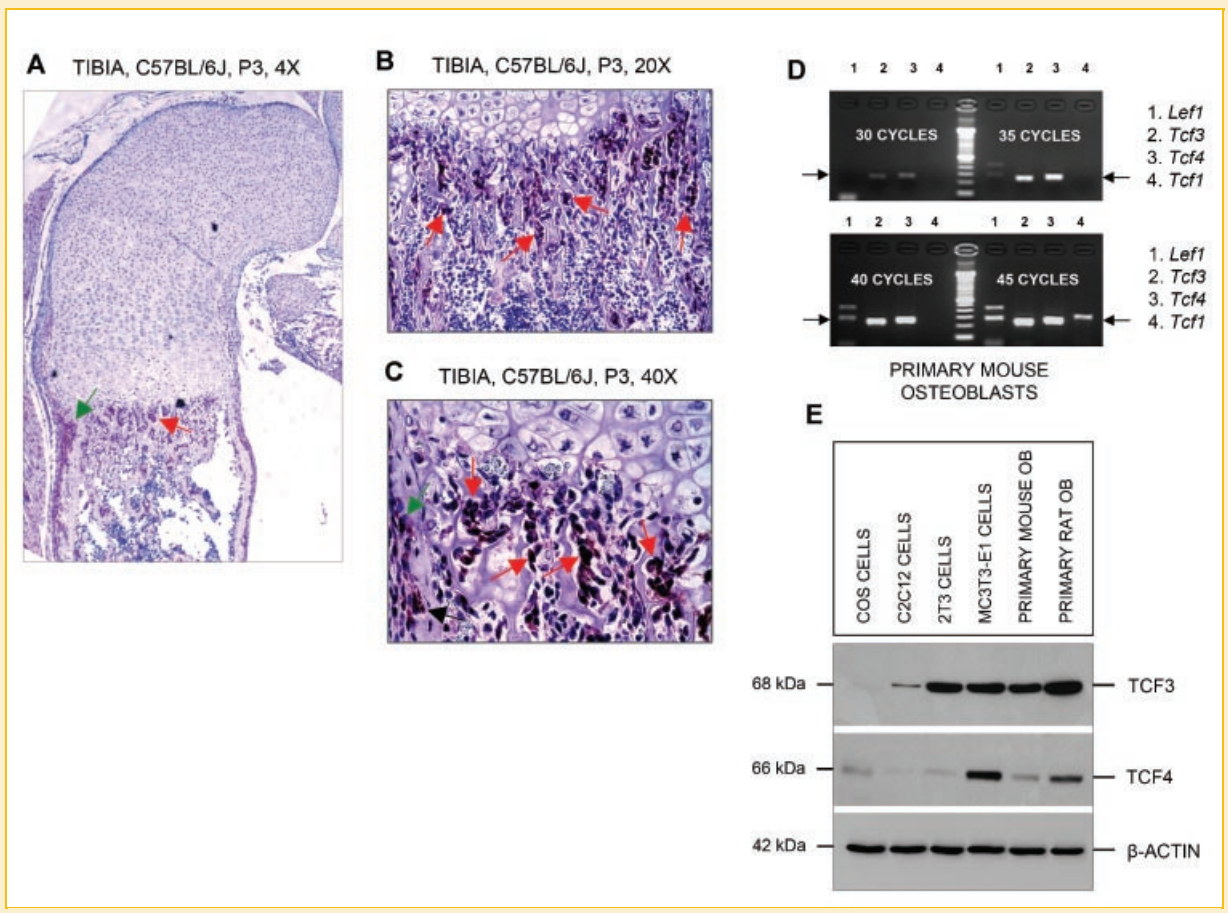

Fig. 1. β-Catenin signaling is active in bone cells. A–C: Expression of β-catenin protein was detected in osteoblasts on trabecular (red arrow) and cortical (green arrow) bones in tibiae of 3-day-old (P3) C57BL/6J mice by immunostaining using the anti-β-catenin monoclonal antibody (A, 4×; B, 20×; C, 40×). D: The mRNA expression of *Tcf* and *Lef* was detected in primary osteoblasts isolated from 3-day-old C57BL/6J mice by RT-PCR. *Tcf3* and *Tcf4* were highly expressed and *Tcf1* was only weakly expressed in osteoblasts. E: The protein expression of TCF3 and TCF4 in osteoblasts was detected by Western blot. TCF3 protein was highly expressed in 2T3 and MC3T3 cells and in primary osteoblasts and was weakly expressed in C2C12 cells. TCF4 is highly expressed in MC3T3 cells but weakly expressed in primary osteoblasts.

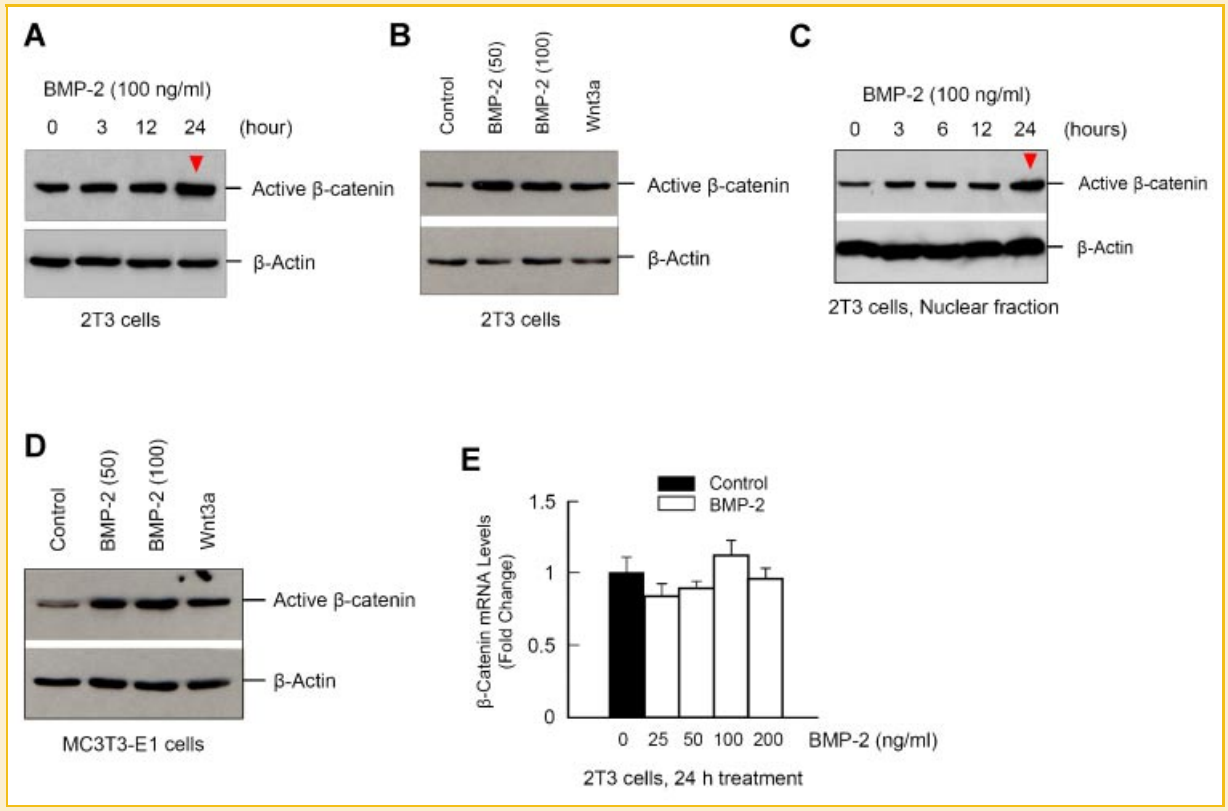

Fig. 2. BMP-2 enhances β-catenin protein levels in osteoblasts. A: 2T3 osteoblast precursor cells were treated with BMP-2 (100 ng/ml) for 3, 12, and 24 h. BMP-2 significantly increased the β-catenin protein level (non-phosphorylated active form) after 24 h treatment. B: 2T3 cells were treated with BMP-2 at 50 and 100 ng/ml concentrations for 24 h. Wnt3a (100 ng/ml) was used as a positive control. BMP-2 at both concentrations significantly increased β-catenin protein level (non-phosphorylated active form) after 24 h treatment. C: 2T3 cells were treated with BMP-2 for different periods of time (0–24 h) and nuclear protein was extracted. BMP-2 enhanced nuclear β-catenin protein level (active form) at 24 h time-point. D: MC3T3-E1 cells were treated with BMP-2 (50 and 100 ng/ml) and Wnt3a (100 ng/ml) for 24 h. Similar to 2T3 cells, BMP-2 significantly increased β-catenin protein levels in MC3T3-E1 cells. E: 2T3 cells were treated with different concentrations of BMP-2 (0–200 ng/ml) for 24 h and total RNA was extracted. BMP-2 had no significant effect on β-catenin mRNA expression.

BMP-2 increases the nuclear translocation of β-catenin, we examined the time-course effect of BMP-2 on nuclear β-catenin protein levels by extracting nuclear protein fractions from 2T3 cells. We found that BMP-2 (100 ng/ml) significantly increased the active form of nuclear β-catenin protein levels at the 24 h time point (Fig. 2C). Similar findings were also observed in pre-osteoblast MC3T3-E1 cells and BMP-2 as well as Wnt3a increased β-catenin protein levels in MC3T3-E1 cells (Fig. 2D). In contrast to its effect on β-catenin protein expression, BMP-2 had no significant effect on β-catenin mRNA expression (Fig. 2E). These results demonstrate that BMP-2 enhances the protein levels of non-phosphorylated active form of β-catenin in osteoblasts at different differentiation stages.

BMP-2 STIMULATES Lrp5 AND Wnt LIGAND EXPRESSION

We investigated the mechanism of BMP-2 on β-catenin expression and examined the effect of BMP-2 on *Lrp5* expression in osteoblasts. We treated 2T3 cells with 100 ng/ml of BMP-2 for 2, 24, or 48 h and examined *Lrp5* expression using Northern blot analysis. We found that BMP-2 stimulated the expression of *Lrp5* (two- to threefold increase) after 24 and 48 h treatment (Fig. 3A,B). The results suggest that the part of the effect of BMP-2 on β-catenin signaling may be due to activation of *Lrp5* in osteoblasts. The effect of BMP-2 on *Lrp5* expression was further confirmed by real-time PCR assay. To determine the signaling mechanism involved in BMP-2-induced Lrp5 expression, 2T3 cells were transfected with Smad6 or treated with SB 220025, a MAP kinase inhibitor, with or without BMP-2. BMP-2-induced *Lrp5* expression was completely inhibited by the MAP kinase inhibitor while expression of Smad6 had no effect on *Lrp5* expression induced by BMP-2 (Fig. 3C). To further determine the contribution of up regulation of *Lrp5* by BMP-2 in regulation of β-catenin expression, 2T3 cells were transfected with Lrp5 siRNA with or without BMP-2. Lrp5 siRNA significantly inhibited BMP-2-induced β-catenin expression (Fig. 3D), suggesting that regulation of *Lrp5* expression by BMP-2 is indeed involved in BMP-2 modulated β-catenin signaling. The knock-down efficiency of Lrp5 siRNA is over 70% (data not shown). We then analyzed if BMP-2-induced β-catenin expression is affected by DKK1, a LRP5 receptor antagonist, and found that addition of DKK1 significantly inhibited BMP-2-induced β-catenin expression (Fig. 3E). Since DKK1 interferes with Wnt ligands to interact with LRP5 receptor, we hypothesize that BMP-2 may also regulate Wnt ligand expression. We examined the effects of BMP-2 on the expression of *Wnt1*, *Wnt3a*, and *Wnt4* and found that BMP-2

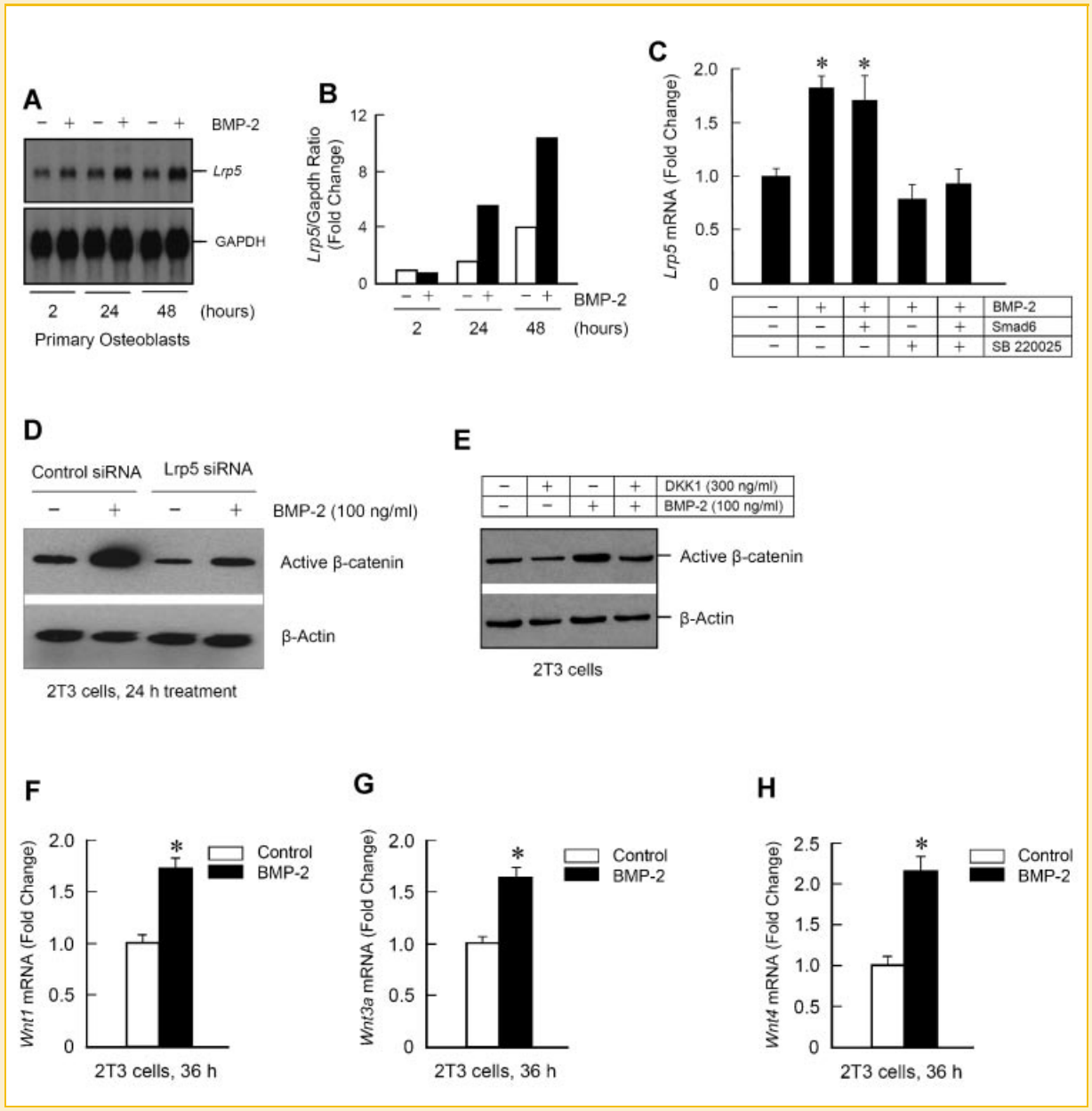

Fig. 3. BMP-2 stimulates *Lrp5* expression and inhibits β-TrCP expression. A,B: 2T3 cells were treated with BMP-2 (100 ng/ml) for 2, 24, and 48 h. BMP-2 stimulated *Lrp5* expression at 24 and 48 h cultures, detected by Northern blotting. C: 2T3 cells were treated with BMP-2 (100 ng/ml) with or without addition of the MAP kinase inhibitor SB 220025 (1 μM) or transfection of Smad6 for 24 h. *Lrp5* mRNA expression was measured by real-time PCR assay. Treatment with BMP-2 significantly increased *Lrp5* mRNA expression which was inhibited by addition of MAP kinase inhibitor. Transfection of Smad6 had no effect on BMP-2-induced *Lrp5* expression. D: 2T3 cells were treated with BMP-2 (100 ng/ml) for 24 h with or without transfection of Lrp5 siRNA. β-catenin expression (active form) was measured by Western blotting. BMP-2 significantly increased β-catenin expression and transfection of Lrp5 siRNA significantly inhibited BMP-2-induced up regulation of β-catenin protein levels. E: 2T3 cells were treated with DKK1 (300 ng/ml) with or without BMP-2 (100 ng/ml). The BMP-2-induced β-catenin expression (active form) was significantly inhibited by addition of DKK1. F–H: 2T3 cells were treated with BMP-2 (100 ng/ml) for 24 h and mRNA expression of canonical Wnt ligands Wnt1, Wnt3a, and Wnt4 was examined by real-time PCR. BMP-2 significantly up regulated the expression of Wnt1, Wnt3a, and Wnt4 in 2T3 cells.

significantly enhanced mRNA expression of these canonical Wnt signaling ligands in 2T3 cells (Fig. 3F–H).

BMP-2 INHIBITS β-TrCP EXPRESSION

The degradation of β-catenin is mediated by the F-box protein complex, $SCF^{\beta\text{-TrCP}}$ and β-TrCP is an E3 ubiquitin ligase in this protein complex. It is known that β-TrCP induces β-catenin degradation in other cell types [Hart et al., 1999; Wu et al., 2003a]. To confirm if β-TrCP mediates β-catenin degradation in osteoblasts, we transfected β-TrCP into 2T3 cells and found that β-TrCP significantly reduced steady-state protein levels of β-catenin in 2T3 cells (Fig. 4A). We then examined the effect of BMP-2 on β-TrCP

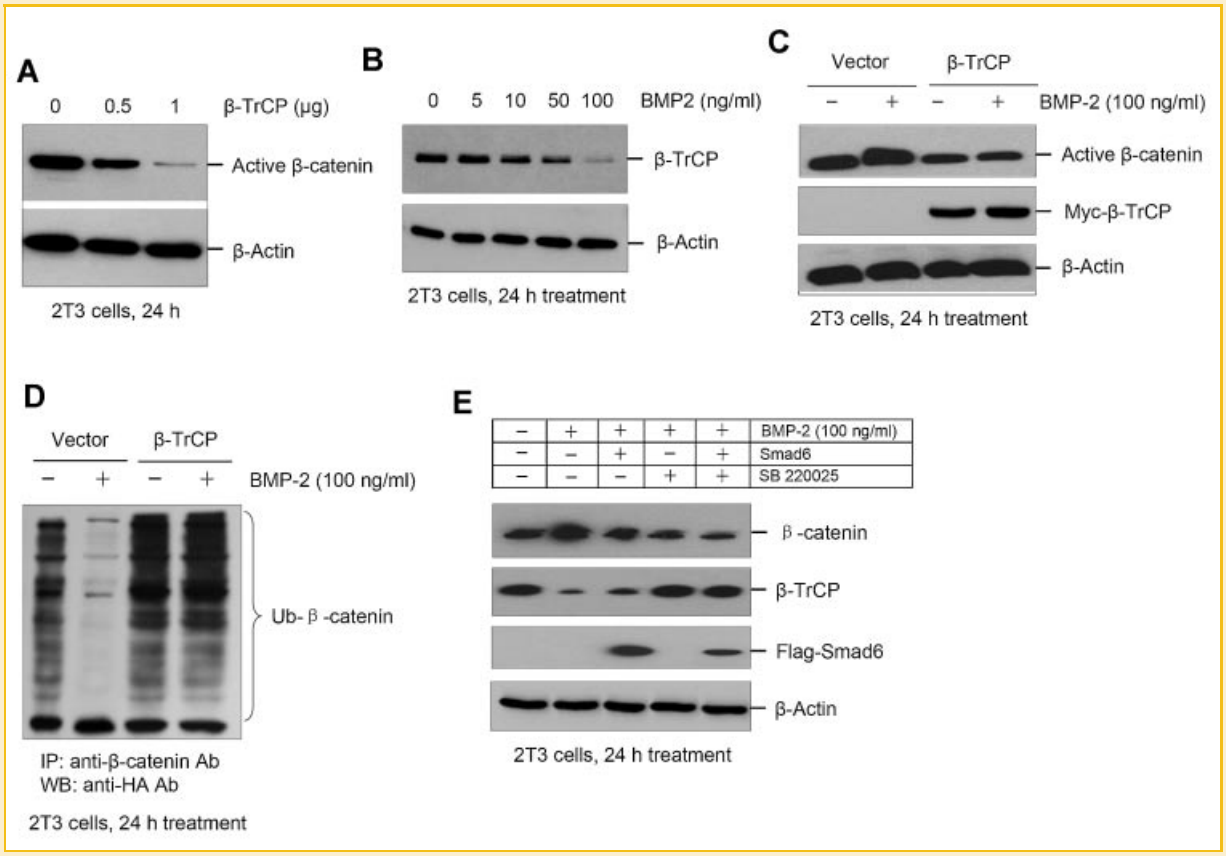

Fig. 4. BMP-2 inhibits β-TrCP expression. A: 2T3 cells were plated into six-well culture plates and transfected with 0.5 and 1 μg of β-TrCP expression plasmid. Western blot analysis showed that β-TrCP induced β-catenin degradation in 2T3 cells. B: 2T3 cells were treated with different concentrations of BMP-2 (0–100 ng/ml) for 24 h. Western blotting result demonstrated that BMP-2 inhibited β-TrCP expression in a dose-dependent manner in 2T3 cells. C: 2T3 cells were treated with BMP-2 (100 ng/ml) and transfected with β-TrCP or empty vector. β-catenin protein levels were examined by western blotting 24 h after BMP-2 treatment. BMP-2 significantly up regulated β-catenin protein levels which was completely reversed by the transfection of β-TrCP. D: 2T3 cells were transfected with β-TrCP or empty vector and HA-Ub and treated with BMP-2 (100 ng/ml) for 24 h. Immunoprecipitation (IP) was performed using the anti-β-catenin antibody followed by Western blotting using the anti-HA antibody. Treatment with BMP-2 significantly inhibited β-catenin ubiquitination. Transfection of β-TrCP completely reversed the inhibitory effect of BMP-2 on β-catenin ubiquitination. E: 2T3 cells were treated with BMP-2 (100 ng/ml) for 24 h with or without addition of MAP kinase inhibitor SB 220025 or transfection of Smad6. β-TrCP and β-catenin protein levels were examined by Western blotting. BMP-2 down regulated β-TrCP protein levels and up regulated β-catenin protein levels. Addition of MAP kinase inhibitor significantly reversed BMP-2-regulated β-TrCP and β-catenin protein levels. In contrast, transfection of Smad6 had minor effect on BMP-2 regulated β-TrCP and β-catenin protein levels.

expression and found that BMP-2 inhibited β-TrCP expression in a dose-dependent manner and high dose of BMP-2 almost completely abolished β-TrCP expression in 2T3 cells (Fig. 4B). To further determine the contribution of the inhibitory effect of BMP-2 on β-TrCP expression in BMP-2-regulated β-catenin expression, we transfected β-TrCP with or without BMP-2 treatment in 2T3 cells and found that BMP-2-induced up regulation of β-catenin protein levels was completely reversed by expression of β-TrCP in these cells (Fig. 4C). In addition to its inhibitory effect on β-TrCP expression, we also found that BMP-2 significantly inhibited β-catenin ubiquitination. Expression of β-TrCP completely reversed the inhibitory effect of BMP-2 on β-catenin ubiquitination (Fig. 4D), indicating that inhibition of β-TrCP expression and β-catenin ubiquitination is critical for BMP-2 modulated β-catenin protein levels in osteoblasts. To further determine the signaling mechanism involved in BMP-2-inhibited β-TrCP expression, 2T3 cells were transfected with Smad6 or treated with SB 220025 with or without BMP-2. Addition of SB 220025 significantly reversed the inhibitory effect of BMP-2 on β-TrCP expression and the stimulatory effect of BMP-2 on β-catenin expression while transfection with Smad6 had only minor effects on BMP-2-regulated β-TrCP or β-catenin expression (Fig. 4E). These findings indicate that MAP kinase signaling is involved in BMP-2-regulated β-TrCP expression leading to stabilization of the β-catenin protein in osteoblasts.

IN VITRO DELETION OF THE β-CATENIN GENE IN OSTEOBLASTS

To further determine the role of β-catenin in osteoblast function, we deleted *β-catenin* expression in vitro using primary osteoblasts isolated from *β-catenin*$^{fx/fx}$ mice (Fig. 5A) (obtained from Jackson Laboratories) and infected these cells with adenovirus expressing Cre recombinase (Ad-Cre). Ad-GFP was also infected into these cells as a control and the infection efficiency for Ad-GFP is over 80% (Fig. 5B). Deletion of the *β-catenin* gene in osteoblasts infected with Ad-Cre was determined by genotyping using primers as described previously [Brault et al., 2001] (Fig. 5C) and confirmed by western blot analysis. Significant reduction in the expression of β-catenin

protein was found in osteoblasts isolated from *β-catenin*$^{fx/fx}$ mice infected with Ad-Cre (Fig. 5D). A significant reduction in osteoblast proliferation has been observed in *Lrp5* KO mice [Kato et al., 2002]. In the present studies, we examined osteoblast proliferation by measuring the number of cells actively synthesizing DNA by 5-bromo-2′-deoxy-uridine (BrdU) incorporation in *β-catenin*-deleted osteoblasts and control cells. A significant reduction in BrdU-positive cells was detected in *β-catenin*-deleted osteoblasts compared with control cells (40% reduction) (Fig. 5E,F). It has been reported that β-catenin directly regulates cyclin D1 gene transcription in tumor cells [Tetsu and McCormick, 1999; Shtutman et al., 1999]. In the present studies, we found that cyclin D1 expression was significantly reduced in *β-catenin*-deficient osteoblasts (Fig. 5G). In contrast, Wnt3a significantly increased cyclin D1 expression in WT osteoblasts (Fig. 5H). We then examined changes in alkaline phosphatase (ALP) activity and expression of osteoblast marker gene *type I collagen* (*Col-1*) and found that ALP activity and expression of *type I collagen* (*Col-1*) were significantly decreased in *β-catenin*-deficient osteoblasts even in the presence of BMP-2 (Fig. 5I,J). To determine the effect of β-catenin on osteoblast terminal differentiation, we analyzed mineralized bone matrix formation and found that deletion of the *β-catenin* gene in osteoblasts significantly inhibited BMP-2-induced bone nodule formation in long-term cultures (Fig. 5K). These results suggest that BMP-2 may regulate osteoblast function partially through regulation of β-catenin.

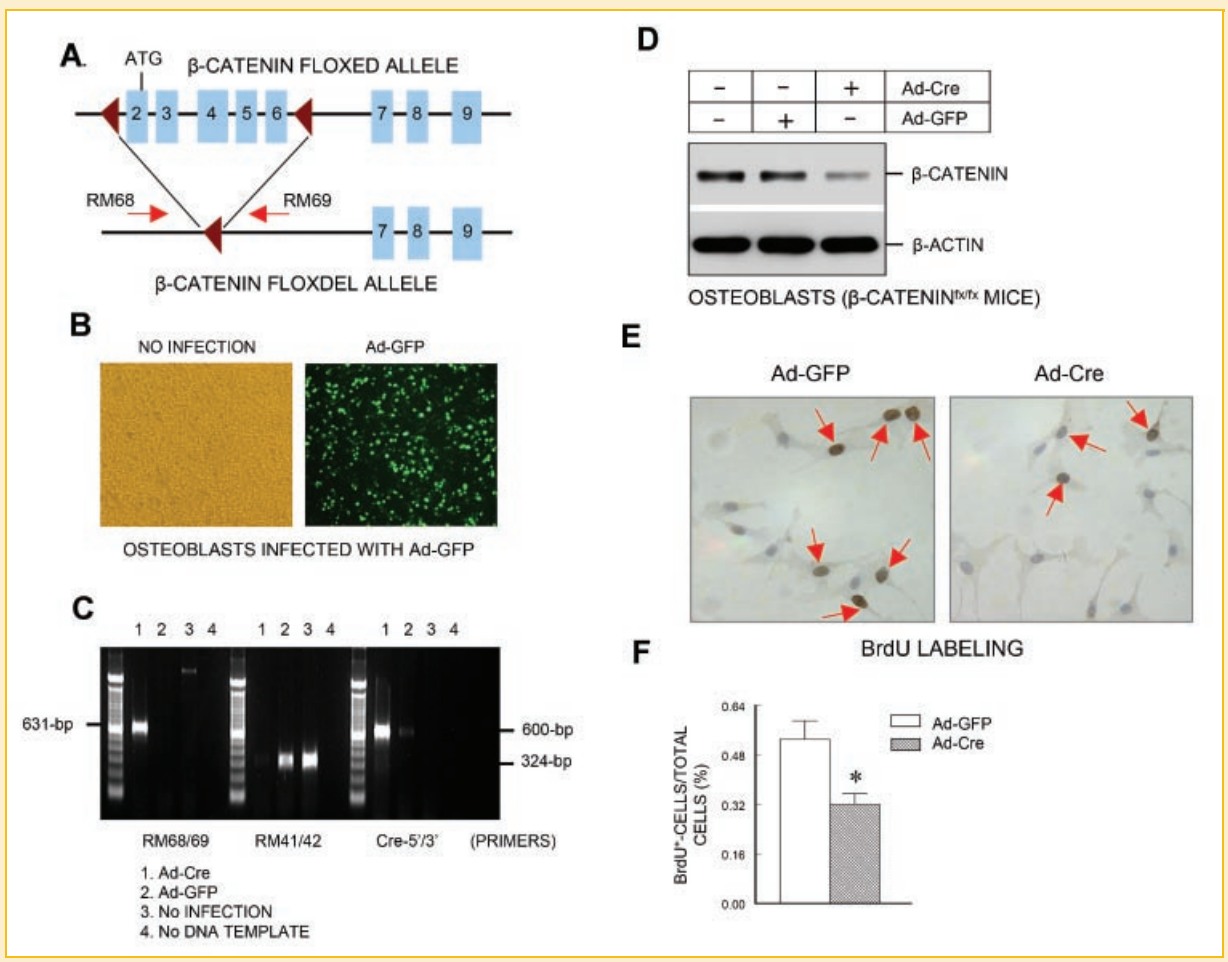

Fig. 5. Deletion of the β-catenin gene in osteoblasts impairs osteoblast proliferation and differentiation. Primary osteoblasts were isolated from 3-day-old *β-catenin*$^{fx/fx}$ mice and infected with same amounts of Ad-Cre or Ad-GFP (control). A: Genomic structure of floxed and deleted β-catenin gene. B: Over 80% infection efficiency was achieved when Ad-GFP was infected into primary mouse osteoblasts. C: The genotyping showed that Cre-mediated recombination (631-bp PCR product) was only observed in the cells infected with Ad-Cre (lane 1, far left). The PCR product of floxed β-catenin gene is shown in lane 2 and 3 (324-bp, middle panel). D: Western blotting result showed that β-catenin protein level was significantly reduced in osteoblasts infected with Ad-Cre. E,F: Osteoblast proliferation detected by BrdU labeling was decreased 40% in *β-catenin*-deleted osteoblasts. G: The expression of cell cycle protein cyclin D1 was reduced in *β-catenin*-deleted osteoblasts. H: Treatment with Wnt3a (100 ng/ml) increased cyclin D1 expression in WT primary mouse osteoblasts. I,J: ALP activity and the expression of *type I collagen* (*Col-1*) were determined. The ALP activity (I) and the expression of *Col-1* (J) were significantly increased by the treatment with BMP-2 (100 ng/ml) which was inhibited by deletion of the *β-catenin* gene in osteoblasts. K: The formation of mineralized bone matrix was further analyzed. The BMP-2 (100 ng/ml)-induced mineralized bone matrix formation was significantly inhibited in *β-catenin*-deleted osteoblasts in long-term culture, demonstrated by von Kossa staining. $^{*}P < 0.05$, compared to the Ad-GFP control group ($n = 3$), unpaired Student *t*-test.

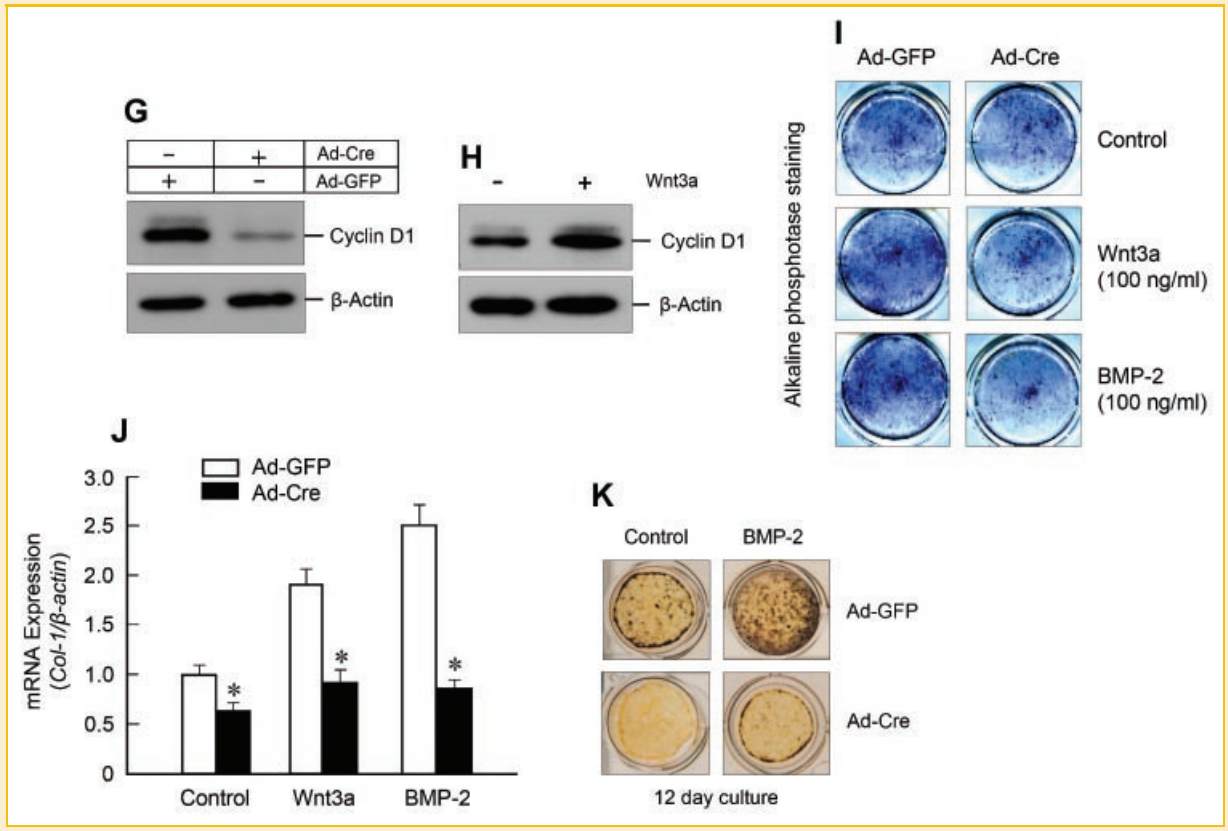

Fig. 5. (*Continued*)

DISCUSSION

Cumulative evidences demonstrate that BMP-2 stimulates bone formation in vivo [Chen et al., 1997; Yoshida et al., 2000; Zhao et al., 2002; Devlin et al., 2003; Winkler et al., 2003; Wu et al., 2003b] but the downstream signaling pathways activated by BMP-2 in osteoblasts remain to be further defined. Recent studies suggest that LRP5 and β-catenin signaling plays a critical role in bone formation in vivo [Gong et al., 2001; Kato et al., 2002; Days et al., 2005; Hill et al., 2005]. Several in vitro studies have shown that BMP-2 has a synergistic effect with β-catenin on chondrocyte and osteoblast differentiation [Fischer et al., 2002a,b; Rawadi et al., 2003; Mbalaviele et al., 2005] but the detailed molecular mechanisms remain unclear. In the present studies, we provide new evidence that BMP-2 enhances protein levels of the non-phosphorylated active form of β-catenin and increases nuclear β-catenin levels. In contrast, BMP-2 had no effect on β-catenin mRNA expression. BMP-2 activates β-catenin signaling by targeting several critical molecules in the β-catenin signaling pathways. BMP-2 stimulates mRNA expression of canonical Wnt ligands Wnt1, Wnt3a and Wnt4 and *Lpr5* mRNA expression in osteoblasts. Consistent with our findings, it has been reported that BMP-2 also up-regulates *Lrp5* expression in ST-2 cells [Gong et al., 2001]. BMP-2 also inhibits the protein expression of β-TrCP, the critical emzyme which mediates the degradation of phosphorylated β-catenin protein. These effects of BMP-2 lead to the prevention of β-catenin degradation and the induction of β-catenin nuclear translocation in osteoblasts (Fig. 6). We have further demonstrated that BMP-2-regulated *Lrp5* and β-TrCP expression is mediated by the MAP kinase pathway.

In the present studies, we have also examined profile of cells expressing β-catenin and its interacting transcription factors. Osteoblasts in trabecular and cortical bone express high levels of β-catenin protein and TCF3 and TCF4 are the two major transcription factors expressed in osteoblasts. We examined β-catenin function by deleting the *β-catenin* gene in osteoblasts by isolating primary osteoblasts from $\beta\text{-catenin}^{fx/fx}$ mice and infecting them with Ad-Cre. Our results demonstrate that β-catenin plays an important role in osteoblast proliferation and differentiation and BMP-2 responsiveness in osteoblasts.

Conditional deletion of the *β-catenin* and *Bmpr-1a* genes by *Brn4-Cre* (Cre activity is restricted to limb ectoderm) shows the interaction between canonical Wnt and BMP signaling pathways during limb development [Soshnikova et al., 2003]. The β-catenin acts downstream of the BMP receptor IA in apical ectodermal ridge (AER) induction and β-catenin controls *Bmp4* expression in the ectoderm as well, which is responsible for the formation of positive feedback loop. In addition, β-catenin acts upstream or in parallel to the BMP receptor IA during dorsal-ventral patterning [Soshnikova et al., 2003]. Our recent data also suggest that such positive feedback loop also exists in bone cells and activation of β-catenin signaling

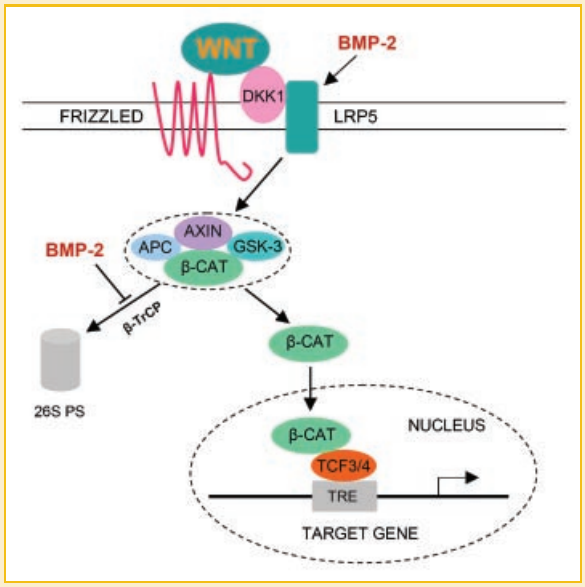

Fig. 6. BMP-2 acts at multiple steps of β-catenin signaling to modulate β-catenin levels. Our current findings suggest that BMP-2 may regulate β-catenin protein levels through stimulating *Lrp5* expression and inhibiting the expression of E3 ubiquitin ligase β-TrCP in osteoblasts.

enhances *Bmp2* and *Bmp4* expression and activates BMP signaling in chondrocytes [Chen et al., 2008; Zhu et al., 2009]. Although our in vitro studies demonstrate that BMP-2 activates osteoblast differentiation partially through activation of β-catenin signaling, further in vivo studies need to be implemented using different animal models to further determine the in vivo cross-interaction between these two signaling pathways.

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