**Bone morphogenetic protein-2 enhances Wnt/β-catenin signaling-induced osteoprotegerin expression**

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**ABSTRACT**

Wnt/β-catenin signaling plays an important role in the developing skeletal system. Our previous studies demonstrated that Wnt/β-catenin signaling inhibits the ability of bone morphogenetic protein (BMP)-2 to suppress myotube formation in the multipotent mesenchymal cell line C2C12 and that this inhibition is mediated by Id1. In this study, we examined the role of intracellular signaling by Wnt/β-catenin and BMP-2 in regulating the expression of osteoprotegerin (OPG) and of the receptor activator of NFkB ligand (RANKL). *OPG* expression was induced by Wnt/β-catenin signaling in C2C12 cells and osteoblastic MC3T3-E1 cells. Silencing of glycogen synthase kinase-3β also increased *OPG* expression. In contrast, RANKL expression was suppressed by Wnt/β-catenin signaling. In a transfection assay, β-catenin induced the activity of a reporter gene, a 1.5 kilobase fragment of the 5'-flanking region of the *OPG* gene. Deletion and mutation analyses revealed that Wnt/β-catenin signaling regulates transcription of *OPG* via a promoter region containing two Wnt/β-catenin responsive sites. BMP-2 enhanced Wnt/β-catenin-dependent transcriptional activation of the *OPG* promoter. In response to BMP-2 stimulation, Smad 1 and 4 interacted with these Wnt/β-catenin responsive sites. These results show that the regulation of *OPG* expression is mediated through two transcription pathways that involve the OPG promoter.

## INTRODUCTION

Wnt proteins belong to a protein family that regulates embryonic development and cell differentiation, proliferation, and migration. Signaling is initiated by the binding of the Wnt ligand to receptor molecules of the Frizzled family and to lipoprotein receptor-related proteins (LRP) 5 and 6 (Nelson & Nusse 2004) (Clevers 2006). Two types of Wnt protein have been identified: β-catenin-dependent "canonical" Wnts, such as Wnt1 and Wnt3a; and the so-called "noncanonical" Wnts that are independent of or inhibit β-catenin signaling. According to the current model of Wnt/β-catenin (canonical Wnt) action, glycogen synthase kinase (GSK)-3β phosphorylates β-catenin and thereby induces rapid degradation of β-catenin in cells that lack Wnt signaling. Stabilized β-catenin interacts in the cytosol with several molecules, including lymphoid enhancer factor 1/T cell factor (Lef1/Tcf). A complex involving the transcription factor Lef1/Tcf and β-catenin regulates expression of several target genes (Yochum *et al.* 2007). Conversely, the "noncanonical" Wnt pathway, mediated by the Wnt5a subclass of Wnts, inhibits the "canonical" Wnt pathway. The pathway is regulated by Siah2, APC complex or Ror2, which promote GSK-3β independent β-catenin degradation or inhibit Lef1/Tcf –mediated transcriptional activation (Topol *et al.* 2003; Mikels & Nusse 2006).

The Wnt/β-catenin signaling pathway has been shown to be essential for the regulation and maintenance of bone mass (Westendorf *et al.* 2004), (Krishnan *et al.* 2006), (Baron & Rawadi 2007). Additionally, Day et al. demonstrated that mesenchymal progenitor cells differentiated into chondrocytes instead of osteoblasts in the absence of β-catenin during embryonic skeletogenesis, indicating that β-catenin is required to repress chondrogenesis (Day *et al.* 2005). In turn, bone morphogenetic protein (BMP)-2 was reported to induce ectopic bone formation and osteoblast differentiation (Canalis *et al.* 2003). In previous reports, we showed that a combination of BMP-2 and Wnt3a induced expression of matrix extracellular phosphoglycoprotein (MEPE), one of the bone matrix proteins, in C2C12 cells (Nakashima *et al.* 2005). In addition, Wnt3a was found to down-regulate expression of inhibitor of DNA binding/differentiation 1 (Id1), a

BMP-2-responsive gene that has been shown to inhibit myogenesis and that is a typical early response gene following BMP treatment of various cell types. Moreover, we found that BMP-2 up-regulated β-catenin-mediated Tcf-dependent transcription activity, indicating a possible mechanism through which the BMP-2 and Wnt/β-catenin intracellular signaling pathways cooperate to regulate expression of target genes (Nakashima *et al.* 2005).

The levels of bone mass are dependent on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are multinucleated cells derived from hematopoietic precursor cells**;** osteoclast precursor cells interact with osteoblasts to differentiate into mature osteoclasts. This interaction is mediated by the receptor activator of NFkB ligand (RANKL)**,** which is expressed on the osteoblast cell surface**,** and by the receptor activator of NFkB (RANK)**,** a cognate receptor expressed on hematopoietic precursor cells. Osteoprotegerin (OPG) is a secreted receptor of the tumor necrosis factor receptor family and lacks a transmembrane domain. OPG acts as a decoy receptor to block interaction between RANKL and RANK. OPG is a key osteoclastogenesis inhibitory factor that inhibits the formation and activity of osteoclasts in vitro and of bone resorption in vivo (Theoleyre *et al.* 2004). Until recently, little was known of the effects of Wnt/β-catenin signaling on the regulation of OPG/RANKL in osteoblastic cells and their progenitors.

In this report, we demonstrate that *OPG* is a target gene for both Wnt/β-catenin and BMP-2 signaling and that regulation of signaling is mediated by the two Lef1/Tcf binding sites of the *OPG* promoter.

## RESULTS

### *Regulation of OPG Expression by Wnt/β-catenin Signaling*

In a previous investigation of the role of Wnt/β-catenin signaling we established C2C12 cell lines that expressed either Wnt3a (Wnt3a-C2C12 cells), which stimulates Wnt/β-catenin signaling, or Wnt5a (Wnt5a-C2C12 cells), which stimulates non-canonical Wnt signaling. C2C12 is a multipotent cell line and is a well-characterized model system that has been reported to differentiate not only into myotubes but also into osteoblasts. The pattern of differentiation depends upon the specific culture conditions when the cells are incubated in the presence of BMPs (Katagiri *et al.* 1994). In our previous study, we found that BMP-2 in combination with Wnt3a induced MEPE mRNA expression (Nakashima *et al.* 2005). This suggests that Wnt/β-catenin signaling may be a potential stimulator of osteoblastic differentiation and function in bone tissue. Here we measured OPG levels in culture supernatants using ELISA. C2C12 and Wnt5a-C2C12 cells had undetectable levels of OPG, whereas a high level of OPG was detected in the conditioned media from Wnt3a-C2C12 cells (Fig. 1A).

In the Wnt/β-catenin signaling pathway, β-catenin is not phosphorylated by GSK3β but rather accumulates and is transported to the nucleus where it forms a transcription complex that regulates expression of target genes. To identify the β-catenin involved in OPG induction, an expression plasmid carrying an activating form of β-catenin that lacks sites of phosphorylation by GSK3β (β-cateninΔGSK) was transfected into the cells. After transfection of β-cateninΔGSK, *OPG* expression was induced to the same extent as that found 24 h after transfection with Wnt3a, and the enhancement lasted up to 48 h (Fig. 1B). The level of induced expression showed a dose-dependent relationship to the amount of plasmid transfected (Fig. 1C). These results indicate that OPG production in C2C12 cells is mediated by Wnt/β-catenin signaling.

It is known that OPG is constitutively expressed in osteoblasts. We therefore examined OPG induction by Wnt/β-catenin signaling in the murine calvaria-derived osteoblastic cell line, MC3T3-E1. The cells were transfected with either the Wnt3a expression plasmid or the Wnt5a

expression plasmid, and then selected to establish cell lines (Wnt3a-MC3T3E1 or Wnt5a-MC3T3E1 cells, respectively). As shown in Fig. 1D, while MC3T3-E1 cells did not produce any detectable Wnt3a expression, the RT-PCR analysis detected Wnt3a expression in Wnt3a-MC3T3E1 cells. The amount of OPG protein in Wnt3a-MC3T3E1 cells showed a 1.7-fold increase over that in MC3T3-E1 cells (Fig. 1D). Additionally, Wnt3a induced an increase in OPG expression in ST-2 cells (data not shown), indicating that Wnt/β-catenin signaling is effective for OPG induction in osteoblasts.

***Knock-down of GSK-3β Increases OPG Production***

Since GSK-3β is known to mediate Wnt/β-catenin signaling, we examined the effect of knockdown of GSK-3β using either RNA interference or the long form of tRNA 3' processing endoribonuclease (tRNase ZL) utilizing gene silencing (Tamura *et al.* 2003) upon OPG induction. Knock-down of GSK-3β was confirmed by Western blot analysis (Fig. 2). We found that the level of GSK-3β protein decreased to approximately 10% (GSK-3βHP1, lane 6) or 22% (sgGSKL, lane 5) of the nontargeting control level (lane 4 or 3), confirming that the siRNA and sgRNA were effective in silencing the endogenous GSK-3β gene. Since suppression of the GSK-3β protein might lead to upregulation of β-catenin signaling, the level of OPG protein was examined. OPG levels dramatically increased after transfection with GSK-3βHP1 or sgGSKL (Fig. 2). These results indicate that OPG induction is GSK-3β dependent, and that the Wnt/β-catenin signaling pathway may be required for OPG induction.

***Wnt/β-catenin Induces OPG mRNA Expression and its Modulation by BMP-2***

In order to determine whether the induction of *OPG* occurs at the mRNA level, we performed a qRT-PCR analysis on total RNA isolated from C2C12, Wnt3a-C2C12, and Wnt5a-C2C12 cells. Neither C2C12 nor Wnt5a-C2C12 cells had a detectable level of *OPG* expression, but OPG mRNA was clearly present in Wnt3a-C2C12 cells (Fig. 3A). Osteoblasts

express at least two cytokines essential for osteoclast differentiation: RANKL and macrophage colony stimulating factor (M-CSF). However, RANKL expression was suppressed in Wnt3a-transfected cells, whereas expression of M-CSF was not changed after stable transfection with Wnt3a or Wnt5a (data not shown). Previously, we showed that BMP-2 could induce β-catenin-mediated Tcf-dependent transcription in C2C12 cells (Nakashima *et al.* 2005). We therefore investigated the effect of BMP-2 on the regulation of *OPG* and *RANKL* expression by Wnt/β-catenin signaling. We found that BMP-2 enhanced Wnt3a-mediated activation of OPG production, whereas RANKL expression was decreased in Wnt3a-C2C12 cells to a level comparable to that of C2C12 cells (Figs. 3A and B).

To confirm the role of BMP-2 in the regulation of *OPG* expression in Wnt3a-C2C12 cells, we analyzed the time and dose effects of culturing cells in BMP-2 on the level of *OPG*. In Wnt3a-C2C12 cells, the level of *OPG* mRNA began to rise after 24 h and remained constant from 24 h to 48 h (Fig. 3C). The level of *OPG* mRNA increased significantly after 50 ng/ml of BMP-2 and further increased with dose up to 500 ng/ml (Fig. 3D). As is shown in Fig. 3E, BMP-2 increased the level of OPG protein in the culture supernatant of Wnt3a-C2C12 cells. Our results show that Wnt/β-catenin signaling, in combination with BMP-2, regulates *OPG* expression.

***Activated β-catenin Induces OPG Promoter Activity***

To investigate the mechanisms by which Wnt/β-catenin signaling activates *OPG* transcription, we cloned an approximately 1.5 kilobase pair mouse genomic DNA fragment corresponding to the 5'-flanking promoter region (-1478/+37) of the *OPG* gene. The *OPG* promoter region was ligated into a luciferase reporter expression vector to examine its responsiveness to Wnt/β-catenin stimulation. Transient transfection of this construct (pOPG1.5-luc) into C2C12 cells with an activating form of β-catenin (β-cateninΔGSK) resulted in a significant increase in luciferase activity. In MC3T3-E1 cells, the activity was also increased by β-cateninΔGSK (data not shown). To identify the sequences in the 5'-flanking promoter region

that mediate β-catenin-dependent induction, we produced a series of deletion mutant constructs that contained sequential deletions of the 5'-flanking fragment placed upstream of the luciferase gene. We found that β-cateninΔGSK transfection enhanced the luciferase activities of four constructs in which the 5'-end was deleted up to nucleotide positions -982, -673, -522 and -395 (pOPG982-luc, pOPG673-luc, pOPG522-luc and pOPG395-luc, respectively). However, truncation of the 5' flanking sequence to -253 (pOPG253-luc) had no effect on induction (Fig. 4A). This deletion analysis implied the presence of transcriptional machinery that was sensitive to interference by β-catenin and that regulated transcriptional activity through interaction with the *OPG* gene promoter upstream from nucleotide position -253.

To identify the potential target sites on the *OPG* gene promoter that are regulated by β-catenin, we searched promoter sequences for potential Lef1/Tcf binding consensus sequences (Yochum *et al.* 2007). This search identified four putative Lef1/Tcf binding sites in the murine *OPG* promoter located at positions -998 to -993 (site 1, CTTTGAA), -539 to-534 (site 2, TACAAAG), -447 to -442 (site 3, CTTTGCA) and -358 to -353 (site 4, CTTTGGG). Sites 3 and 4 are imperfect matches to the consensus core Lef1/Tcf binding sequence 5'-CTTTGA/TA/T-3'. To determine whether these four sites contributed to the induction of *OPG* promoter activity by β-catenin, we introduced mutations into each by replacing four nucleotide sequences. The luciferase activity of construct pOPG1.5m1-luc was enhanced by β-cateninΔGSK in a similar fashion to that observed in pOPG1.5-luc, which lacks nucleotide substitutions. In contrast, the reporter constructs with mutations of sites 2, 3 and 4 (pOPG1.5m2-luc, pOPG1.5m3-luc and pOPG1.5m4-luc, respectively) showed a reduction of activity of approximately 20-50% of that of the control pOPG1.5-luc after induction by β-catenin (Fig. 4B), indicating that sites 2, 3 and 4 are potential responsive sites of an activator of transcription. To further investigate the functional significance of these sites for the activation of the *OPG* promoter, triple site mutations were introduced into pOPG1.5-luc. Simultaneous mutation of sites 1, 2 and 4 (pOPG1.5m124-luc) reduced the responsiveness to β-catenin, whereas simultaneous mutation of sites 1, 3 and 4

(pOPG1.5m134-luc) and sites 1, 2 and 3 (pOPG1.5m123-luc) did not significantly affect the response of the *OPG* promoter to β-catenin (Fig. 4B), indicating that sites 2 and 4 contribute to the inducible transcriptional activity of the *OPG* gene by Wnt/β-catenin signaling.

***Interaction of Nuclear Protein(s) with the Lef1/Tcf Motif of the OPG Promoter***

To characterize the binding activity of nuclear proteins that interact with sites 2 and 4 of the *OPG* promoter, EMSA was performed using an oligonucleotide containing the consensus Lef1/Tcf binding sequence (Tcf-Lef) as a probe. Wnt3a-C2C12 cells nuclear extracts formed a retarded band (Fig. 5A). As expected, the shifted band was not abolished when mutated Tec-Lef (Tcf-LefM) was used as the competitor. The binding of proteins could be competed out with unlabeled oligonucleotide (100-fold excess) containing the Tcf-Lef probe and the site 2 probe (Fig. 5A). However, binding activity was only slightly reduced when the site 4 oligonucleotide was used as the competitor. These findings suggest that the binding proteins of the consensus Lef1/Tcf binding sequence interact with site 2, and to a lesser degree with site 4, of the *OPG* promoter.

To further address whether the binding activity of sites 2 and 4 in the *OPG* promoter is regulated by Wnt/β-catenin signaling, we performed EMSA using nuclear extracts of C2C12 cells and Wnt-3a-C2C12 cells. The binding activity of site 2 or 4 in the nuclear extracts prepared from C2C12 cells was very low (Fig. 5B). In contrast, the oligonucleotide containing site 2 or 4 in the nuclear extracts prepared from Wnt3a-C2C12 cells had a higher binding activity (Fig. 5 B), indicating that the binding activity at sites 2 and 4 in nuclear extracts is regulated by Wnt/β-catenin signaling and is closely correlated to the promoter activity found using the reporter constructs in the site-directed and multiple mutation analyses (Fig. 4). No difference was observed between the binding activities of C2C12 and Wnt3a-C2C12 nuclear extracts when site 1 or 3 was used as the probe (data not shown). Binding to the site 2 probe was abolished when the site 4 probe was used as a competitor. Likewise, binding to site 4 probe was abolished when the

site 2 probe was used as a competitor, indicating that the binding complex interacts with both sites 2 and 4. Moreover, inclusion of an antibody against β-catenin or Tcf-1 in the incubation reaction reduced the formation of the complex in Wnt3a-C2C12 nuclear extracts when site 2 or 4 was used as the probe, confirming the presence of β-catenin and Tcf1 in the complex.

To assess whether transcription factors bind in vivo to the site 2 and 4 regions of the *OPG* promoter, a ChIP assay was used to identify protein-DNA complexes. Immunoprecipitation was performed using β-catenin, Tcf-1, or Tcf-4 antibody. PCR amplification using primers for sites 2 and 4 (P2 and P4, respectively) on chromatin cross-linked to the immunoprecipitates showed that complexes with β-catenin and Tcf-1 were bound to the site 2 and 4 regions of endogenous *OPG* promoter in Wnt3a-C2C12 cells (Fig. 5C). Tcf-4 did not bind these *OPG* promoter regions. However, no amplification product was obtained after immunoprecipitation with Tcf-1 antibodies in C2C12 cells (data not shown). Primers for the osteocalcin promoter and an anti-HA antibody were used as a negative control in the assay. The results of the ChIP analysis are consistent with those described above for inducible transcription activity and the EMSA assay. Our results provide evidence that Wnt/β-catenin signaling regulates transcription of *OPG* via a promoter region that includes sites 2 and 4.

***Smad Interactions Modulate Wnt/β-catenin and BMP-2 Signaling***

The data illustrated in Fig. 3 indicate that *OPG* expression is positively regulated by both Wnt/β-catenin and BMP-2. Therefore, we investigated whether the transcriptional activation of the *OPG* promoter is regulated by BMP-2. However, the luciferase activities of pOPG1.5-luc was not increased by addition of BMP-2, nor were the promoter activities enhanced by BMP-2 in cells transfected with β-cateninΔGSK (Fig. 6A). These results indicate that BMP-2 did not directly enhance the activity of the 1.5 kb *OPG* promoter but rather enhanced Wnt/β-catenin-dependent transcriptional activation of the *OPG* promoter. To identify which of

the β-catenin responsive sites are essential for BMP-2-mediated induction, we examined the effect of mutations within the *OPG* promoter. The reporter constructs pOPG1.5m2-luc and pOPG1.5m4-luc showed a reduced induction of transcriptional activity not only after β-cateninΔGSK transfection but also after the addition of BMP-2 (Fig. 6A). In contrast, the luciferase activity of construct pOPG1.5m1-luc was increased by both BMP-2 and β-catenin, in a similar fashion to that observed in pOPG1.5-luc, showing that sites 2 and 4, but not site 1, contributed to the inducible transcriptional activity of the *OPG* gene for both Wnt/β-catenin and BMP-2 signaling.

As an alternative to adding BMP-2, we transfected an activated form of Smad1 to determine whether Smad1 and Wnt/β-catenin signaling molecules could interact with sites 2 and 4 to induce the *OPG* promoter. Luciferase activity was significantly increased by co-transfection of both β-cateninΔGSK and Smad1 with site 2 (pOPG1.5m134-luc) or site 4 (pOPGm123-luc) of the *OPG* promoter (Fig. 6B). However, Smad3 did not enhance luciferase activity in the presence of β-cateninΔGSK (data not shown). The constitutively active mutated form of p300/CBP also caused a further increase in the induced promoter activity at site 2 in combination with Smad1 and β-cateninΔGSK (Fig. 6B). These results indicate that interaction between β-catenin/Tcf-1 and Smad1 modulates the Wnt/β-catenin response to BMP-2 and that association of p300/CBP could result in induction of *OPG* expression via site 2 of the *OPG* promoter. To assess whether Smad also bound in vivo to its target site in the *OPG* promoter, we performed a ChIP assay. Chromatin was prepared from Wnt3a-C2C12 cells treated with BMP-2 and immunoprecipitated with an antibody specific for either Smad1 or Smad4. PCR amplification using the primers for site 2 (P2) or 4 (P4) of the *OPG* promoter on chromatin cross-linked to the immunoprecipitates showed that complexes with Smad1 and Smad4 were bound to the endogenous site 2 and 4 regions of *OPG* promoter. This result demonstrates that BMP-2 stimulation led to the complex recruitment of Smad1 and Smad4 at both sites 2 and 4 (Fig.6C), indicating that β-catenin responsive sites in the *OPG* promoter might be induced by BMP-2 signaling. Moreover, we carried out IP assays in

Wnt3a-C2C12 cells, following treatment with BMP-2, to determine endogenous molecular complexes of Smads and β-catenin. Smads were immunoprecipitated and co-precipitated β-catenin was detected by Western blotting. We observed endogenous molecular complexes of β-catenin and Smad1, but not Smad4 in the Wnt3a-C2C12 cells following treatment with BMP-2 (Fig. 6D). These data show that Smads and β-catenin can interact physically in a BMP-2-dependent manner.

**DISCUSSION**

In this study, we used an over-expression procedure to show that Wnt/β-catenin signaling induces *OPG* expression. C2C12 cells were used in the present investigation; these cells differentiate into multinucleated myotubes. Previous studies showed that BMP-2 not only inhibits the myogenic differentiation of C2C12 cells but also converts their differentiation pathway into that of osteoblasts (Katagiri *et al.* 1994). C2C12 cells can also support the differentiation of spleen cells into multinucleated osteoclast-like cells in the presence of 1α,25-dihydroxyvitamin D3 and BMP-2 (Otsuka *et al.* 2003). In the present study, C2C12 cells were found to show Wnt3a induced expression of *OPG*.

Wnt/β-catenin was shown here to activate the *OPG* promoter. The osteoblast-specific transcription factor Runx2 is known to associate with the Runx2 binding element of the *OPG* promoter (Meyers *et al.* 1993) and to contribute to the expression of OPG (Thirunavukkarasu *et al.* 2000). Runx2 is expressed in MC3T3-E1 cells but not in C2C12 cells (Nakashima & Tamura 2006). The amount of OPG protein in MC3T3-E1 cells was reduced by transfection of Runx2 siRNA (data not shown), implying that basal OPG expression in osteoblasts depended upon Runx2. Upon activation by a Wnt/β-catenin signal, the HMG-box protein Lef1/Tcf can form a complex with β-catenin. The complex converts into a transcriptional activator of target genes. A number of target genes have been identified that respond to Wnt/β-catenin signaling and that have a Lef1/Tcf binding motif (Yochum *et al.* 2007). Previously, several gene promoters, including

Axin2, were found to contain multiple Lef1/Tcf binding sites and to contribute to the regulation of transcription (Jho *et al.* 2002). In this report, extensive deletion and mutation analyses allowed us to delimit two functional Lef1/Tcf binding sites in the *OPG* promoter that may provide a mechanism for the direct activation of *OPG* transcription by Wnt/β-catenin signaling. Similar to our studies, Glass DA et al. also demonstrated that β-catenin, along with Tcf, regulates the *OPG* expression in osteoblasts (Glass et.al, 2005). However, in their reports, only one *OPG* promoter site (we have identified two sites) was identified as the regulatory region of *OPG* expression by carrying out site-directed mutation assay using ROS 17/2.8 cells and transfection assay using COS cells. This discrepancy may be due to the differences in the experimental systems including the cell lines. Although site 2 was consistent with the consensus Lef1/Tcf binding motif, the site 4 sequence was not a perfect match. The results from our EMSA competition assay using a Tcf-Lef probe indicate that the binding affinity of site 4 might lower than that of site 2. A ChIP assay indicated that a complex including β-catenin and Tcf-1 interacted with sites 2 and 4 of the endogenous *OPG* promoter by activation of Wnt/β-catenin signaling. These results support the idea that an interaction of the transcriptional complex formed by the Lef1/Tcf site binding protein(s) with sites 2 and 4 of the *OPG* promoter is involved in induced transcription of the *OPG* gene by Wnt/β-catenin signaling. Thus, it appears that the *OPG* gene is a direct target of the Wnt/β-catenin pathway.

Wnt and BMPs are expressed in many overlapping tissues and dual regulation by Wnt and BMPs appear to be frequent in mammalian development. In the intracellular BMP-2 signaling pathway, Smad1 forms a complex with Smad4 that moves into the nucleus, where it binds to the Smad binding element (SBE) of target genes, thereby regulating their expression (Derynck *et al.* 1998). Expression of the homeobox gene, Emx2, has been shown to be regulated by both Smad and Lef1/Tcf binding sites in a synergistic fashion (Theil *et al.* 2002). In addition, both Smad and Lef1/Tcf binding sites are present in the promoter of *Xtwn* and are required for the synergistic activation of the gene (Labbé *et al.* 2000). Here, we showed that two Lef1/Tcf binding

sites in the *OPG* promoter are responsible for Wnt/β-catenin signaling, and that OPG expression could be induced by BMP-2. Two Hoxc-8 binding sites have been shown to mediate the activities of a human OPG promoter construct and of endogenous OPG gene expression in response to BMP-2 stimulation (Wan *et al.* 2001). However, the *OPG* promoter construct used our study (pOPG1.5-luc) did not contain any SBE sequence or Hoxc-8 binding sites and was not activated by BMP-2, suggesting that Smad could not bind *OPG* promoter directly. This discrepancy might reflect difference in the experimental systems. In this report, BMP-2 enhanced Wnt/β-catenin-dependent transcriptional activation of the *OPG* promoter, and mutation analyses of the promoter demonstrated that the two Lef1/Tcf site binding sites in the *OPG* promoter responded to BMP-2 stimulation. This observation was supported by the Smad1/4 transfection studies. Importantly, we previously observed that another Lef1/Tcf regulating element, a Top-flash reporter that did not contain the Smad binding site, was up-regulated by BMP-2. Inducible activity was also observed after transfection with Smad1/4, instead of the addition of BMP-2, in cells transfected with activated β-catenin (Nakashima *et al.* 2005). There are several reports that TGF-β signaling results in the C-Smads, Smad4 and Smad3, directly interacting with β-catenin/Lef1 and in the transcriptional activation of Lef1/Tcf-responsive promoters (Labbé *et al.* 2000; Theil *et al.* 2002; Hu & Rosenblum 2005). Our present results suggest that the *OPG* gene promoter functionally interacts with β-catenin/Tcf-1 in cooperation with Smads, and that these complexes then regulate graded expression of *OPG*. Thus, we proposed a model in which Smad regulation of Lef1/Tcf target elements depends on the physical association of the transcription factors and the formation of a complex. We also found that p300/CBP stimulates both Wnt/β-catenin and BMP-2 induced activation of site 2 of the *OPG* promoter. In agreement with our results, Takizawa *et al.* (2003) reported that BMP-2 enhances the transcriptional activation of the Hes-5 gene promoter by recruitment of the co-activator p300/CBP that interacts with β-catenin and Smad1 (Takizawa *et al.* 2003). Here, we show that one possible mechanism by

which these transcription factors influence *OPG* gene expression is by response to either the Wnt/β-catenin or BMP-2 pathways via the recruitment of a co-activator such as p300/CBP. We have previously reported that a combination of Wnt3a and BMP-2 induced the expression of MEPE in the C2C12 cells (Nakashima *et al.* 2005), suggesting that the promoter of MEPE gene may interact with β-catenin/Tcf/Lef1 in co-operation with Smads, and subsequently induce the expression of the gene. No induction of MEPE expression was observed in C2C12 cells overexpressing Wnt3a (Nakashima *et al.* 2005) and showing active β-catenin transfection (our unpublished data). We searched for consensus sequences for Lef1/Tcf binding sites at the 5′ proximal region of the murine MEPE promoter using search software. We could not identify putative Lef1/Tcf binding sites in the MEPE gene promoter. In conclusion, MEPE induction by Wnt3a and BMP-2, involving interaction between β-catenin/Tcf1 and Smads, may not simply mediate the transcriptional activation of the MEPE promoter. Further investigation is required to understand if a combination of Wnt3a and BMP-2 can induce the MEPE expression in C2C12 cells.

The differentiation and activation of osteoclasts are tightly regulated by osteoblasts. OPG is a potent inhibitor of the osteoclast-mediated bone resorption induced by osteoblasts (Bucay *et al.* 1998). Many bone-remodeling regulatory agents such as tumor necrosis factor and interleukin-1 have been implicated as inducers of *OPG* expression in osteoblasts (Theoleyre *et al.* 2004). *OPG* expression is down-regulated by agents that stimulate osteoclast formation, such as 1α25-dihydroxyvitamin D3, parathyroid hormone and prostaglandin E2. Our analyses showed that BMP-2 also enhanced *OPG* expression, and indicated that BMP-2 plays a role in modulating the *OPG* expression induced by Wnt/β-catenin signaling. Thus, although Wnt/β-catenin signaling may be sufficient to induce *OPG* expression, our findings indicate that a combination of Wnt/β-catenin and BMP-2 signaling may be necessary for maximum *OPG* expression in

osteoblasts. This combined effect of both signaling pathways might therefore be required to achieve the most effective inhibition of osteoclast-mediated bone resorption. Spencer et al. (Spencer *et al.* 2006) reported that activation of Wnt/β-catenin signaling down-regulated *RANKL* expression in osteoblasts. Our analyses showed that Wnt3a enhanced expression of *OPG* but reduced that of RANKL. This change in the expression of RANKL may also contribute to Wnt-mediated regulation of osteoclastogenesis in bone tissue. It is thus also possible that Wnt/β-catenin signaling in osteoblasts plays an indirect role in the regulation of osteoclast formation and differentiation via OPG and RANKL. Overall, our results suggest that net changes in bone mass induced by Wnt/β-catenin signaling may result from changes in the balance between bone formation and bone resorption through regulation of osteoclastic formation and activity.

In conclusion, we have shown that *OPG* is a target gene of Wnt/β-catenin. We have also provided evidence to support an indirect role for osteoblastic Wnt/β-catenin signaling in the regulation of osteoclast differentiation and formation via OPG and RANKL. This study has also helped to elucidate the molecular mechanisms of the Wnt/β-catenin and BMP-2 signaling pathways and the ways in which these pathways interact cooperatively to regulate the level of expression of OPG. The physiological function of Wnt/β-catenin in bone may be mediated at several levels by cross-talk with co-regulatory signaling pathways that also target Wnt/β-catenin target genes. It is likely that Wnt/β-catenin mimetic drugs may be of value for future therapies to improve bone mass in patients with bone disorders.

**Experimental Procedures**

*Cell cultures*-The mouse myoblast cell line C2C12 and the mouse calvarial osteoblast cell line MC3T3-E1 were obtained as described previously (Nakashima *et al.* 2005). Wnt3a-C2C12 cells and Wnt5a-C2C12 cells were established as described previously (Nakashima *et al.* 2005). Cells were cultured in α-MEM supplemented with 10% fetal bovine serum at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$ in air.

*Expression plasmids* – The plasmid constructs Wnt3a-pUSEamp, Wnt5a-pUSEamp, Tcf-1-pUSEamp and p300/CBP-pUSEamp were purchased from Upstate Biotechnology (Charlottesville, VA). The mutated β-catenin expression plasmid, β-cateninΔGSK, in which amino acid residues of the GSK phosphorylation site of β-catenin cDNA are deleted, was used (Obama & Ozawa 1997). Smad1 expression plasmids were generated using pFLAG-CMV-2 (Katagiri *et al.* 2002).

*Establishment of stable transfected cells* - MC3T3-E1 cells were transfected with 1.0 μg of Wnt3a-pUSEamp orWnt5a-pUSEamp as described previously (Nakashima *et al.* 2005).

*Quantification of OPG by enzyme-linked immunosorbent assay (ELISA)* - The level of OPG protein was quantified using the mouse OPG Immunoassay (Quantikine® HS; R&D Systems, Minneapolis, MN).

*Transfection of small interfering RNA (siRNA) and small guide RNA (sgRNA) expression plasmids* - The GSK-3β hairpin siRNA expression plasmid, U6-GSK-3βHP1, was kindly provided by Dr. D.L. Turner (Univ. Michigan, Ann Arbor, MI) (Yu *et al.* 2003). The sgRNA expression plasmids for silencing *GSK-3β* genes (pRNA Tin-H1.2/Neo-sgGSKL) were

constructed as described previously (Tamura *et al.* 2003; Nakashima *et al.* 2007). pU6-GSK-3αHP1 (Yu *et al.* 2003) or pRNATin-H1.2/Neo-Luc (Nakashima *et al.* 2007) was used as a non-targeting control. Cells were harvested 48 h after transfection, and culture supernatants were collected and the levels of OPG protein quantified by ELISA. Whole-cell extracts were subjected to Western blot analysis.

*Quantitation of gene expression by reverse transcription-polymerase chain reaction (RT-PCR)* - Quantitative RT-PCR (qRT-PCR) was performed using assay-on-demand TaqMan probes (Applied Biosystems, Foster city, CA) and StepOne® real time PCR system. The relative level of gene expression was quantified using the comparative $C_T$ method with glyceraldehyde-3-phosphate dehydrogenase as the endogenous control. RT-PCR was performed as previously described (Nakashima *et al.* 2005).

*Immunoprecipitation*- Cells were lysed in lysis buffer containing protease inhibitors and phosphatase inhibitors, and clarified by centrifugation. Supernatants were incubated with protein A-Sepharose that had been conjugated to the following antibodies: anti-Smad1 (A-4, Santa Cruz), anti-Smad4 (B-8, Santa Cruz), anti-β-catenin (Upstate) and anti-hemagglutinin (HA) tag antibody (Roche). Immune complexes were boiled in SDS sample buffer containing 10 mM dithiothreitol.

*Western blots* - Whole cell extracts or the immune complexes were separated, transferred to a PVDF membrane, and probed with the following antibodies: anti-β-catenin (Upstate) and anti-GSK3β (BD Bioscience, Palo Alto, CA) using the ECL detection system.

*Reagents* - Recombinant human BMP-2 was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan).

*Reporter constructs*- Luciferase reporter plasmids for the *OPG* promoter were generated as follows. The 1515-bp *OPG* promoter fragment (-1478 to +37) was isolated from mouse genomic DNA by PCR and subcloned into the pGL4.12 vector (Promega) to generate the luciferase reporter plasmid (pOPG1.5-luc). A deletion series of the reporter constructs were made by PCR amplification. The constructs with single and triple site mutations were generated by replacing the putative Lef1/Tcf binding sites (sites 1, 2, 3 and 4) in pOPG1.5-luc. Site-directed mutagenesis was performed by the overlap extension technique using PCR. The following mismatched oligonucleotides were used: site 1, 5'-TGAACTACGGCTAACTCATGA-3'; site 2, 5'-CTCCAGGTATGCGGAATTTAT-3'; site 3, 5'-CCAGGACACGGCACATGTTAA-3'; and site 4, 5'-TACAGAATCCTCAGGAAGGAG-3'. The nucleotide sequences of each mutated promoter region were verified by sequencing.

*Transient cell transfection and assay for luciferase activity* -The reporter assay was performed as described previously (Nakashima *et al.* 2005).

*Electrophoresis mobility shift assay (EMSA)* - EMSA was performed essentially as described previously (Nakashima *et al.* 2005). For competition experiments, 100-fold molar excess of unlabeled double stranded competitor oligonucleotides were incubated with the nuclear extracts for 15 min prior to the addition of the probe. In antibody interference experiments, 2 μg of an anti-β-catenin (Upstate) or anti-Tcf-1 (H-118, Santa Cruz Biotechnologies, Heidelberg) polyclonal antibody was incubated with the nuclear extracts for 15 min prior to the addition of the probe. The following oligonucleotide sequences were used as probes for EMSA: Tcf-Lef probe, 5'-CCCTTTGATCTTACC-3'; Tcf-Lef mutation (Tcf-LefM) probe, 5'-CCCTTTGGCCTTACC-3'; site 2 probe, 5'-AAATTCTTTGTACCTGGA-3'; site 3 probe, 5'-GGACACTTTGCATGTTAA-3'; site 4 probe, 5'-AGAATCTTTGGGAAGGAG-3'.

*Chromatin immunoprecipitation (ChIP) Assay*- A ChIP assay was performed using a kit from Upstate. Chromatin solutions were incubated overnight at 4 °C with rotation, and with 5 µg of one of the following antibody solutions: anti-β-catenin (Upstate), anti-Tcf-1 (H-118, Santa Cruz), anti-Tcf-4 (H-125, Santa Cruz), anti-Smad1 (A-4, Santa Cruz), anti-Smad4 (B-8, Santa Cruz) or control anti-HA tag antibody (Roche). The putative Lef1/Tcf reorganization sequences (sites 2 or 4) of the *OPG* gene were investigated using the PCR primers P2 or P4. PCRs using osteocalcin promoter primers (POc) served as negative controls. Primer sequences are available upon request.

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**FIGURE LEGENDS**

Figure 1. **Regulation of osteoprotegerin (OPG) production by Wnt/β-catenin signaling.**

**A.** C2C12, Wnt3a-C2C12 or Wnt5a-C2C12 cells were plated at $1x10^5$ cells/$cm^2$ and cultured for 48 h. The level of OPG protein in the culture supernatant was determined by an enzyme-linked immunosorbent assay (ELISA). **B and C.** C2C12 cells were plated at $1x10^5$ cells/$cm^2$ and cultured for 24 h. The cells were then transiently transfected with 100 ng/ml (B) or the indicated dose (C) of one of the following: an expression plasmid carrying β-cateninΔGSK, the active form of β-catenin that lacks the site of phosphorylation by glycogen synthase kinase (GSK)-3β**;** Wnt3a**;** or, a vehicle expression plasmid (pcDNA3). After the cells were further cultured for the indicated times (B) or for 48 h (C), the level of OPG in the culture supernatant was determined by ELISA. **D.** Establishment and characterization of MC3T3-E1 cells showing stable expression of Wnt3a. MC3T3-E1 cells were transfected with Wnt3a or Wnt5a expression plasmid, and then transfected cell clones (Wnt3a-MC3T3E1 or Wnt5a-MC3T3E1 cells, respectively) were selected. Expression of Wnt3a mRNA in cells was analyzed by RT-PCR (D, upper). The concentration of OPG protein in the culture supernatant was determined by ELISA (D, lower). Data are means ± S.D. Each assay represents a separate experiment performed in triplicate.

Figure 2. **Up-regulation of OPG production by gene silencing of murine *GSK*-3β.**

C2C12 cells were transiently transfected in 100 mm dishes with 6 μg of vector: lane 1 with pU6-pro (empty vector); lane 2 with pRNA Tin-H1.2/Neo (empty vector); lane 3 with pU6-GSK-3αHP1 (nontargeting control); lane 4 with psgLuc1 (nontargeting control); lane 5 with pRNA Tin-H1.2/Neo-sgGSKL (GSK3β sg RNA expression plasmid); and lane 6 with pU6-GSK-3beta HP1 (GSK3β siRNA expression plasmid) (18). After the cells were cultured for a further 48 h, the levels of GSK3β protein in the cells were determined by Western blot analysis using a GSK-3β antibody (upper panel). The level of OPG protein in the culture supernatants were determined by ELISA (lower panel). Data are means ± S.D. Each assay represents a separate

experiment performed in triplicate.

Figure 3. **Regulation of expression of *OPG* and receptor activation of NF-κB ligand (*RANKL)* by Wnt/β-catenin signaling and bone morphogenetic protein (BMP)-2.**

**A and B.** C2C12, Wnt3a-C2C12 and Wnt5a-C2C12 cells were plated at $1x10^5$ cells/cm² and cultured for 24 h. BMP-2 (300 ng/ml)(+) or vehicle (-) was added, after which the cells were cultured for a further 24 h. Total RNA was extracted from the cells and the mRNA levels of *OPG* (A) and *RANKL* (B) were determined by qRT-PCR. **C and D.** Time and dose dependent induction of *OPG* mRNA expression after addition of BMP-2 to Wnt3a-C2C12 cell cultures. Wnt3a-C2C12 cells were plated at $1x10^5$ cells/cm² and cultured. After 24 h, 300 ng/ml (C) or the indicated concentrations (D) of BMP-2 were added, after which the cells were cultured further for the indicated times (C) or for 24 h (D). Total cellular RNA was extracted and then qRT-PCR was performed to estimate the level of *OPG* mRNA expression. **E.** Wnt3a-C2C12 cells were plated at $1x10^5$ cells/cm$^2$ and cultured for 24 h. Then, the indicated dose of BMP-2 was added. After the cells had been cultured for a further 48 h, OPG levels in the culture supernatant were determined by ELISA. OPG levels are represented as the relative increase over no addition of BMP-2. Data are means ± S.D. Each assay represents a separate experiment performed in triplicate.

Figure **4.** **Activation of OPG promoter by β-catenin and effect of mutations in putative Lef1/Tcf binding sites.**

**A.** Transcriptional activity of the 5' region of the *OPG* gene promoter in luciferase reporter constructs. C2C12 cells were transiently co-transfected in 24-well plates with 0.1 μg of a reporter plasmid carrying the full length (-1478/+37) *OPG* promoter (pOPG1.5-luc), or carrying a construct in which the 5'-end was deleted up to nucleotide position -982, -673, -522, -395 or -253 (pOPG982-luc, pOPG673-luc, pOPG522-luc, pOPG395-luc or pOPG253-luc, respectively), and 0.1 μg of pβ-cateninΔGSK (+) or vehicle expression plasmid (pcDNA3) (-). After the cells were

cultured for a further 48 h, luciferase activity was determined. Luciferase activity is shown as the relative reduction of pOPG1.5-luc compared to that of pcDNA3 transfection. Putative Lef1/Tcf binding sites 1-4 are indicated by ovals. **B.** Effects of mutations in the putative Lef1/Tcf binding sites on luciferase activity. Substitution mutations were introduced into Lef1/Tcf binding sites in pOPG1.5-luc to generate pOPG1.5m1-luc, pOPG1.5m2-luc, pOPG1.5m3-luc, pOPG1.5m4-luc, pOPG1.5m134-luc, pOPG1.5m124-luc and pOPG1.5m123-luc. Sites 1-4 are indicated by ovals. These constructs and 0.1 μg of pβ-cateninΔGSK or vehicle expression plasmid (pcDNA3) were transfected into C2C12 cells, and cell extracts were prepared 48 h after transfection and processed for luciferase assays. Normalized luciferase activity is represented as the relative increase over empty vector with each construct. Data are means ± S.D. Each assay represents a separate experiment performed in triplicate.

Figure 5. **Electrophoresis mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay demonstrates β-catenin and Tcf-1 binding to the OPG promoter.**

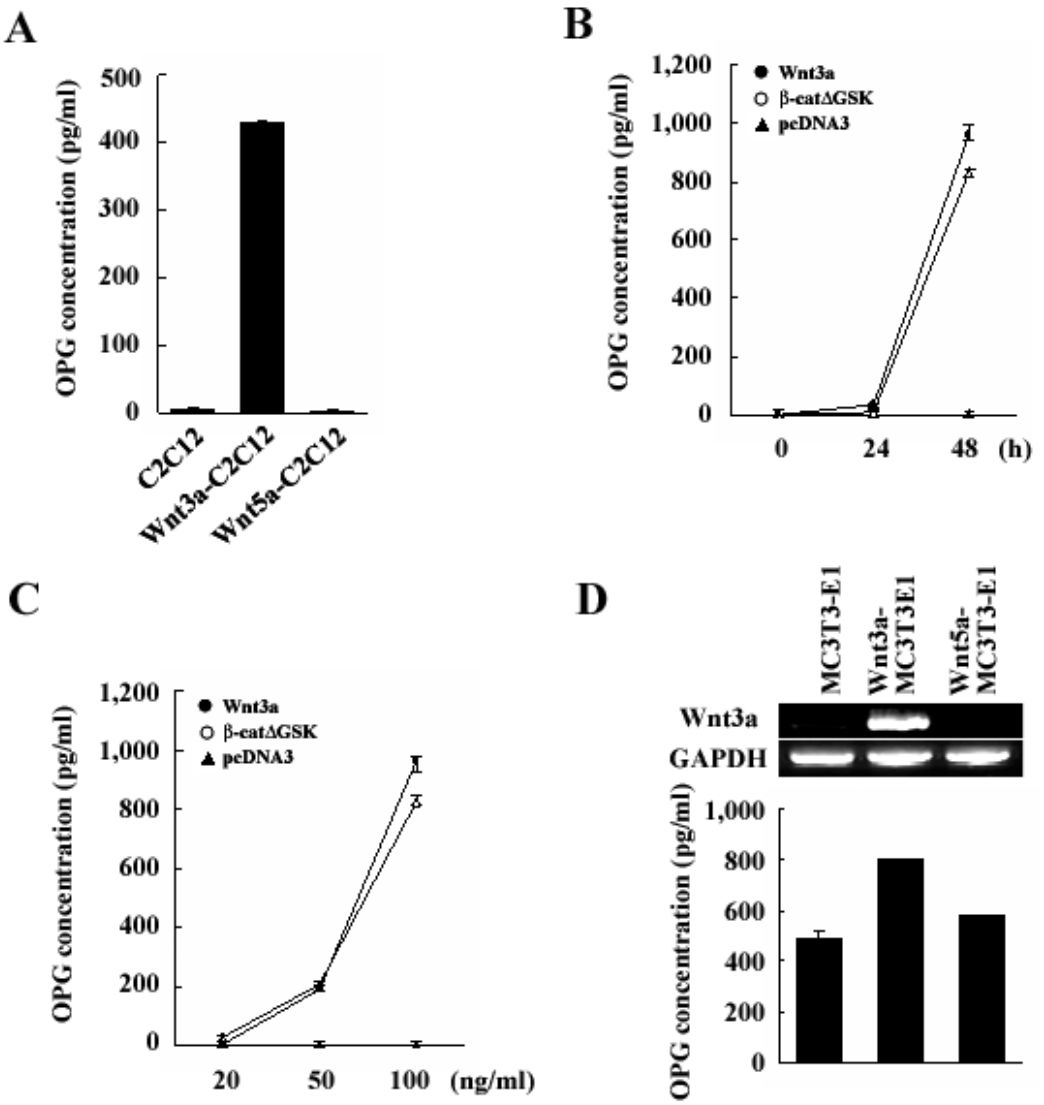
**A and B.** Nuclear extracts prepared from C2C12 cells (lane 1 in **A**, and lanes 1 and 8 in **B**) or Wnt3a-C2C12 cells (lanes 2-7 in **A,** and lanes 2-7, and 9-13 in **B**) were incubated with a radiolabeled probe for the Tcf-Lef consensus sequence (**A**), or a probe for site 2 or site 4 (**B**) for 20 min at room temperature. For competition experiments, **a** 100-fold molar excess of the indicated unlabeled double stranded competitor DNA was incubated with the nuclear extracts for 15 min prior to the addition of the probe (lanes 3-7 in **A**, and lanes 3, 4, 10 and 11 in **B**). For antibody experiments, the indicated antibody was incubated with the nuclear extracts for 15 min prior to the addition of the probe (lanes 5-7, 11 and 12 in **B**). Protein-DNA complexes were resolved by EMSA. The arrow indicates the protein-DNA complex. **C.** Protein/DNA complexes from Wnt3a-C2C12 cells were precipitated without antibody (input), or with a β-catenin antibody, Tcf-1 antibody, Tcf-4 antibody or nonspecific HA antibody. PCR amplification was performed using primers for *OPG* promoter site 2 (P2) or site 4 (P4). PCR using input DNA was used as the

positive control. PCR using osteocalcin promoter primers (POc) was used as the negative control.

Figure 6. **BMP-2 enhances Wnt/β-catenin mediated transcriptional activation of OPG gene promoter.**

**A and B.** C2C12 cells were transiently co-transfected in 24-well plates with 0.1 μg of pOPG1.5-luc, pOPG1.5m134 or pOPG1.5m123 as a reporter plasmid and 0.1 μg of pβ-cateninΔGSK, Smad1, Tcf-1, p300/CBP or vehicle expression plasmid (pcDNA3) as indicated. BMP-2 (300 ng/ml) (+) or vehicle (-) was added, after which cells were cultured for a further 48 h. Luciferase activities were then determined. Normalized luciferase activity is represented as the relative increase over p253OPG-luc with pcDNA3. Data are means ± S.D (n=5). **C**. ChIP assay. Wnt3a-C2C12 cells were plated and cultured for 24 h. BMP-2 (300 ng/ml) was added, after which cells were cultured for a further 48 h. The protein/DNA complexes were precipitated without antibody (input), or with a Smad1 antibody, Smad4 antibody or nonspecific HA antibody. PCR amplification was performed using the P2 or P4 primers. PCR using input DNA was used as the positive control. PCR using the POc primers was used as the negative control. **D.** Detection of molecular complexs by BMP-2 treatment. Wnt3a-C2C12 cells were plated and cultured for 24 h. BMP-2 (300 ng/ml) or vehicle was added, after which cells were cultured for a further 48 h. Cell lysate were subjects to immunoprecipitation with anti-Smad antibodies, anti-β-catenin antibody (positive control) or anti-HA antibody (negative control) and were analyzed by immunoblotting with anti-β-catenin antibodies.

**Figure 1**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Wnt3a-C2C12
Wnt5a-C2C12
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OPG concentration (pg/ml)
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800
600
400
200
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peDNA3
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24
48
(h)
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(ng/ml)
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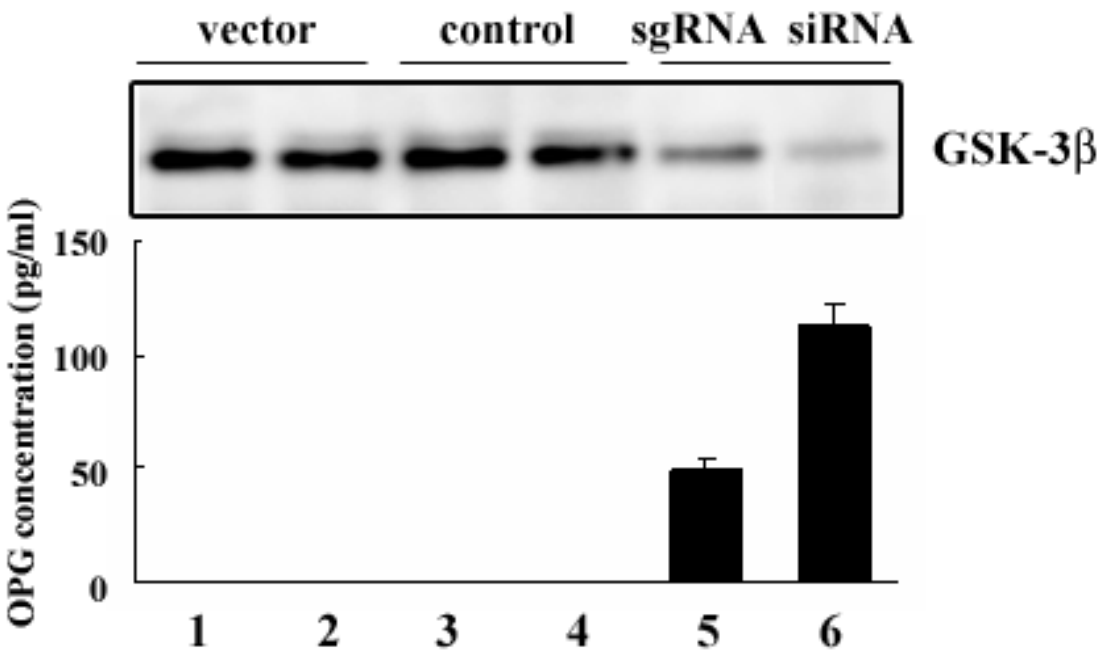
**Figure 2**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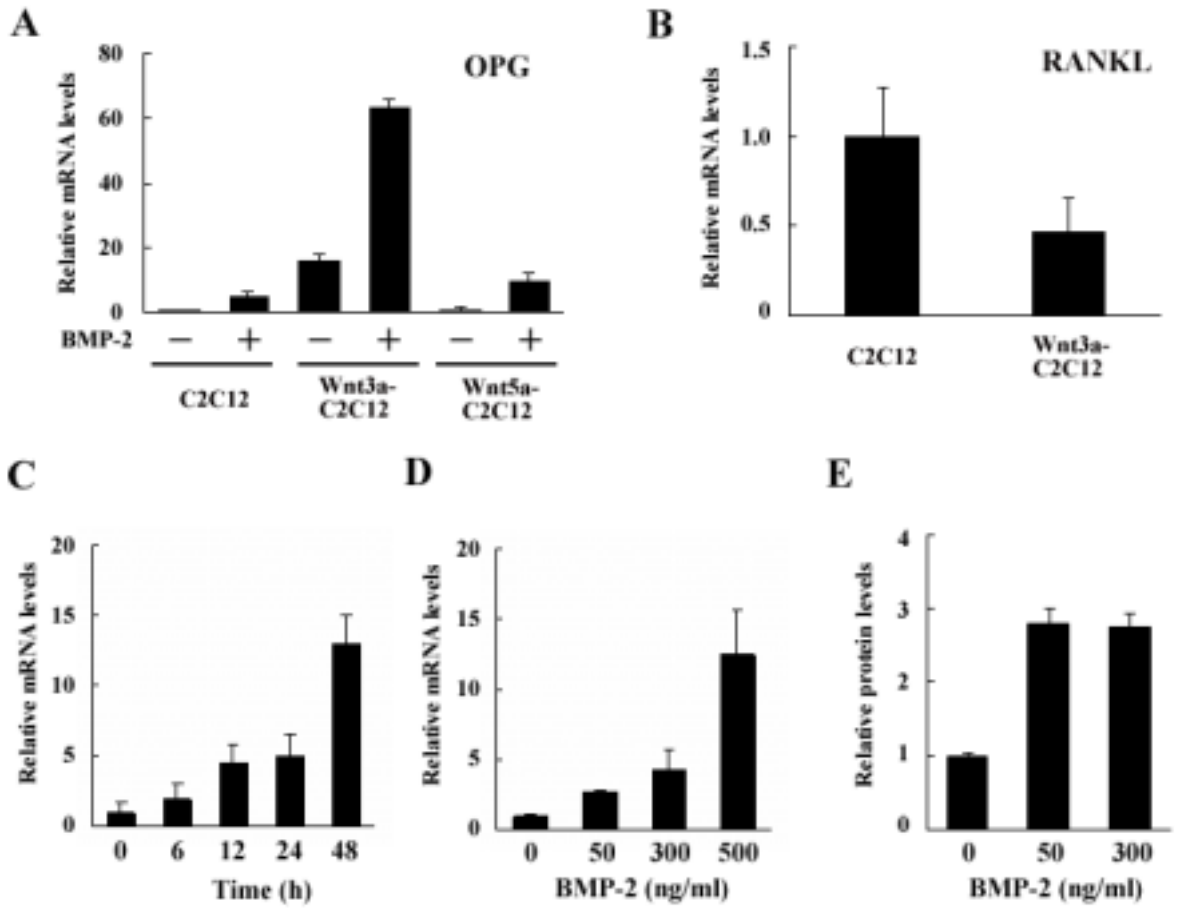
2
3
4
5
6

**Figure 3**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+
−
+
−
+
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Wnt3a-C2C12
Wnt5a-C2C12
B
RANKL
Relative mRNA levels
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1.0
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Wnt3a-C2C12
C
Relative mRNA levels
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5
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6
12
24
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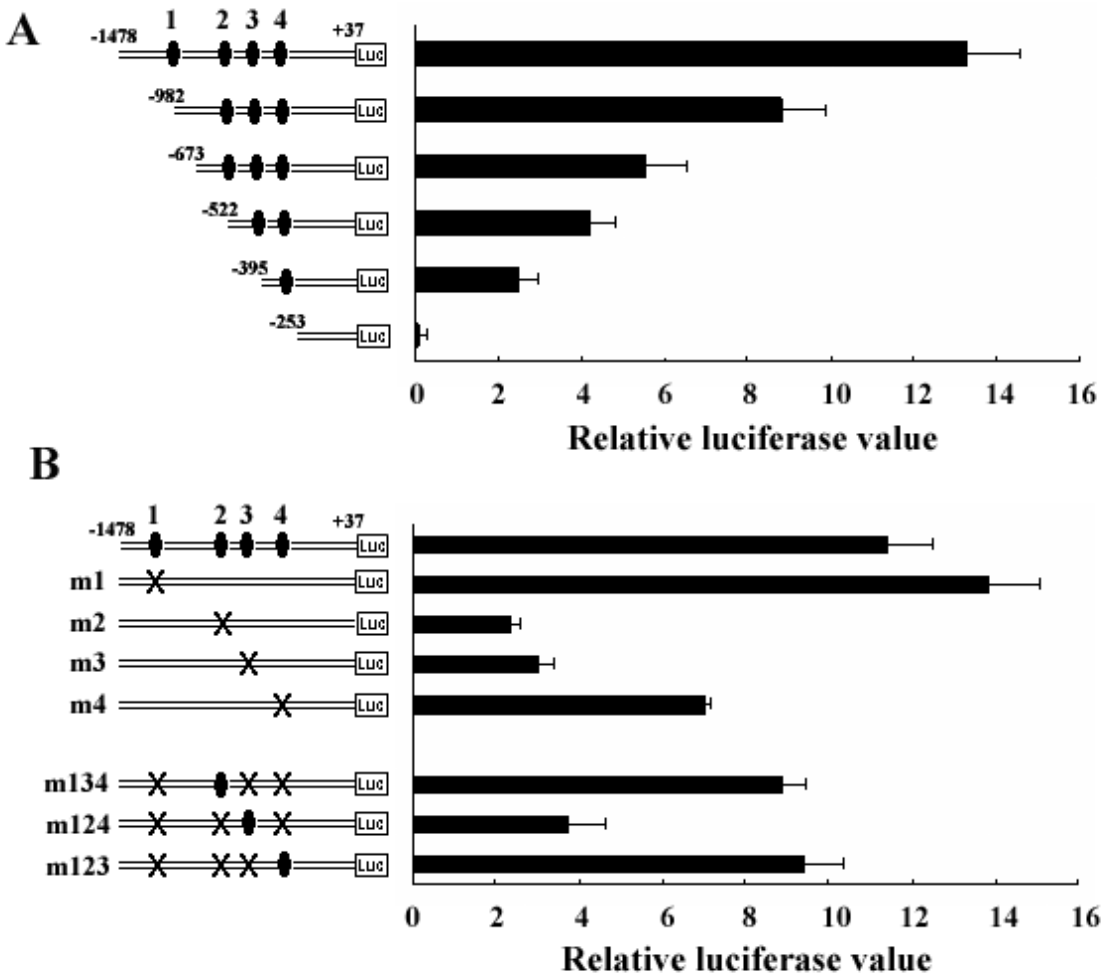
**Figure 4**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m2
m3
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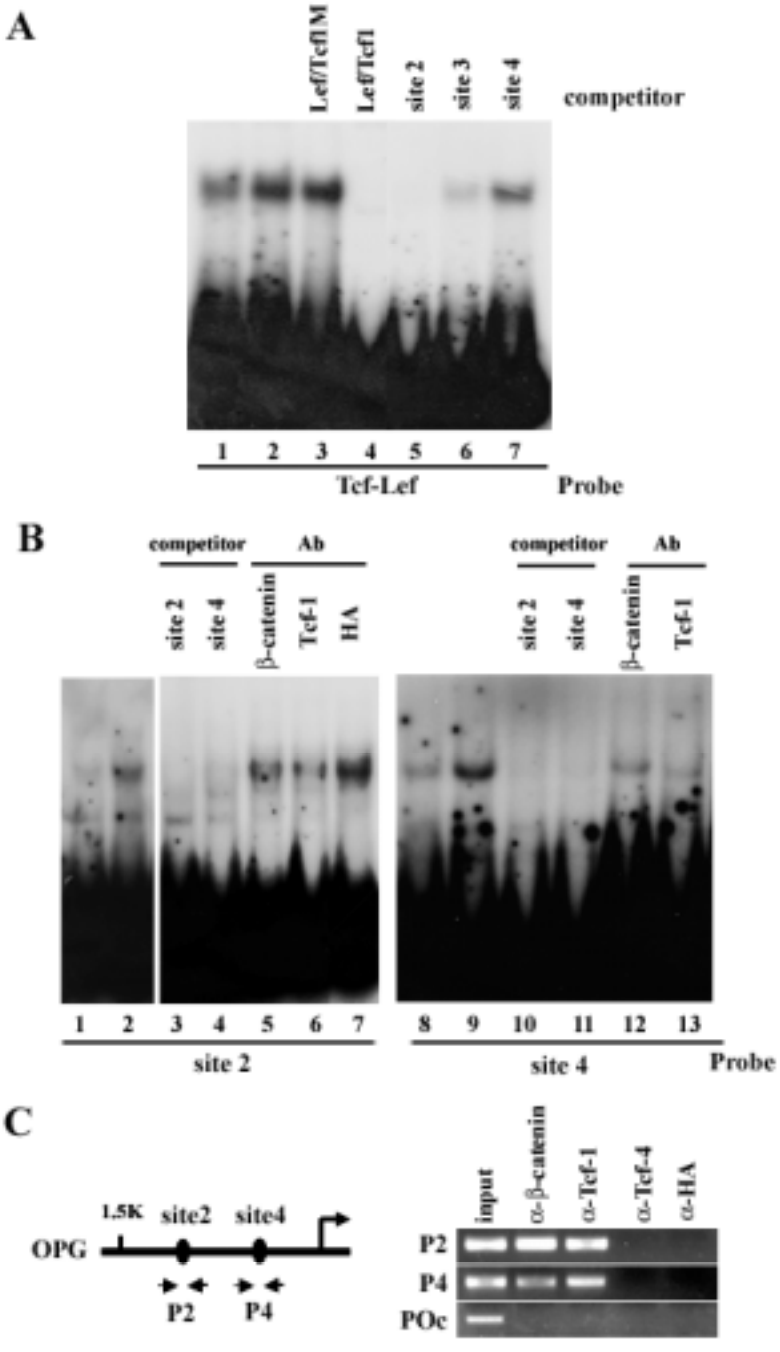
m123

**Figure 5**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site 2
site 4
β-catenin
Tcf-1
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Ab
site 2
site 4
β-catenin
Tcf-1
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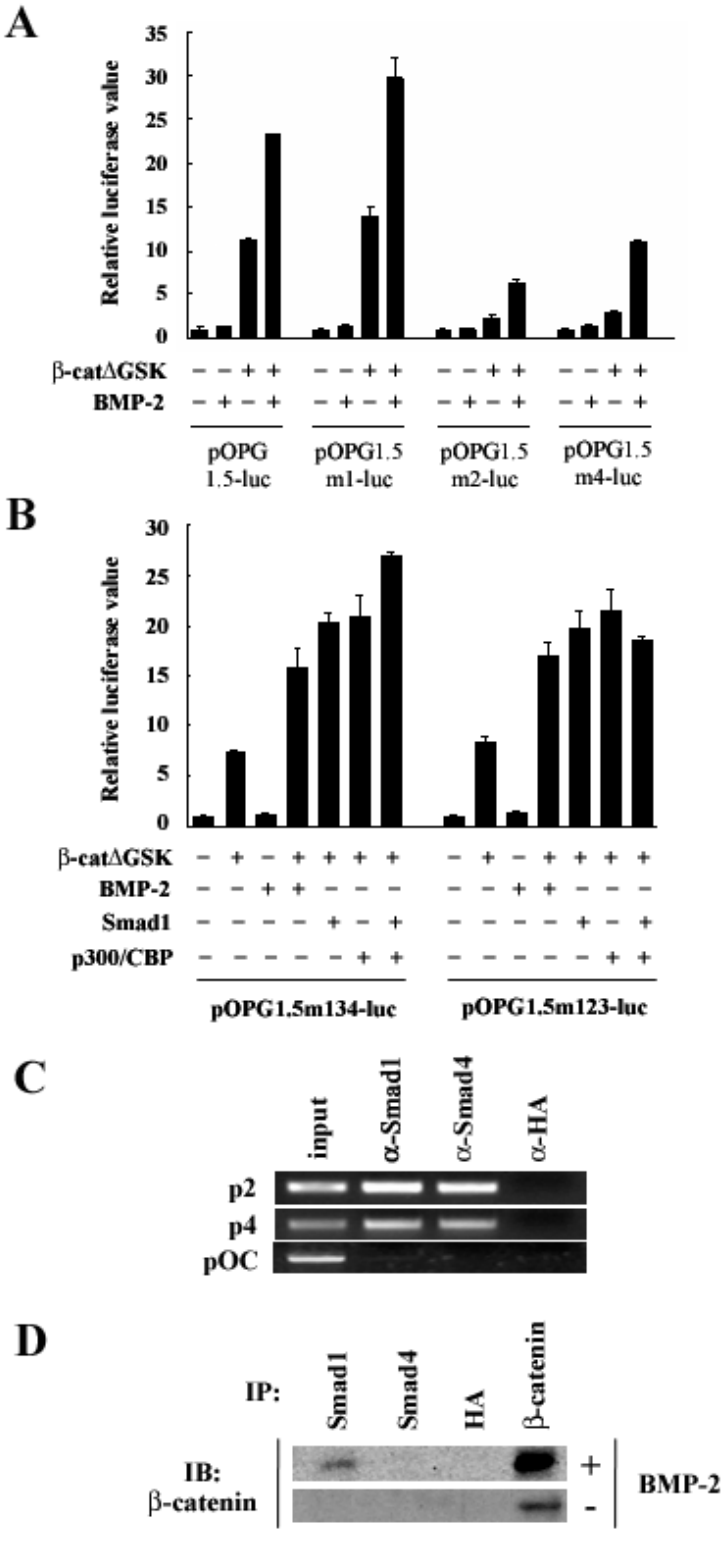
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pOPG1.5 m2-luc
pOPG1.5 m4-luc
B
Relative luciferase value
30
25
20
15
10
5
0
β-catΔGSK
BMP-2
Smad1
p300/CBP
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β-catenin
+
-
BMP-2