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## CROSS-TALK BETWEEN WNT AND BONE MORPHOGENETIC PROTEIN (BMP)- 2 SIGNALLING IN DIFFERENTIATION PATHWAY OF C2C12 MYOBLASTS \*

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Running Title: Cross-talk between Wnt3a and BMP-2

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Loss of function of the Wnt co-receptor, lipoprotein receptor-related protein 5, decreases bone formation, and a point mutation in this gene results in high bone mass, indicating the importance of this signalling pathway in bone formation. However, the exact mechanism is currently unknown. We examined a potential role for Wnt signalling and functional cross-talk of bone morphogenetic protein (BMP)-2 in osteoblast differentiation. To assess the contribution of Wnt, we generated C2C12 cells overexpressing Wnt3a or Wnt5a and treated these with BMP-2. We showed that expression of matrix extracellular phosphoglycoprotein was induced by BMP-2 in Wnt3a over-expressing C2C12 cells but not in Wnt5a over-expressing C2C12 cells. Overexpression of Wnt3a blocked BMP-2-induced inhibition of myotube formation in C2C12 cells when switched to low mitogen medium. In these cultures, expression of inhibitor of DNA binding/differentiation (Id) 1, a basic helix-loop-helix protein induced by BMP-2, decreased in stable Wnt3a, but not Wnt5a, expressing cells. This suppression is mediated by a GC rich region of the BMP-2 responsive element of the Id1 gene promoter and interaction between Smad1/4 and  $\beta$ -catenin is crucial for Wnt-mediated suppression of the BMP-2 response in C2C12 cells. Overexpression of the inhibitor of canonical Wnt signalling, Dickkopf, inhibits this suppression. In contrast, BMP-2 or Smad1/4 up-regulated Wnt3a or activated  $\beta$ -catenin-induced lymphoid enhancing factor 1/T cell factor-dependent

transcriptional activity. These findings identify functional cross-talk of Id1 expression between Wnt and BMP signalling and demonstrate a novel mechanism for Wnt regulation of the BMP-2 response, linking Id1 expression to Wnt/ $\beta$ -catenin signalling.

The Wnt secreted proteins are a family of glycosylated-lipid-modified proteins which are powerful regulators of embryonic development, cell differentiation, proliferation and migration. Signalling is initiated by Wnt ligand binding to two receptor molecules, the seven transmembrane-domain protein receptors of the Frizzled family and lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (1). Two types of Wnt proteins have been identified; one class of which is  $\beta$ -catenin-dependent canonical Wnts such as Wnt1, Wnt2, Wnt3 and Wnt3a. The other class is the so-called "noncanonical" Wnts such as Wnt4, Wnt5a, Wnt5b, Wnt6 and Wnt7a which is independent of or inhibits  $\beta$ -catenin signalling (2, 3). According to the current model of canonical Wnt action, in cells lacking Wnt signal, glycogen synthase kinase (GSK)-3 $\beta$  phosphorylates  $\beta$ -catenin, inducing rapid degradation of  $\beta$ -catenin via the ubiquitin/proteasome pathway.  $\beta$ -Catenin is also found cytoplasmically interacting with several molecules including the adenomatous polyposis tumor suppressor protein, lymphoid enhancer factor 1/T cell factor (Lef1/Tcf), subunits of protein phosphatase 2A and axin. Conventional Wnt signalling causes stabilization of  $\beta$ -catenin and its accumulation in a complex with the transcription factor Lef1/Tcf that regulates

expression of target genes such as c-myc and cyclin D1 (2, 3). Dickkopf (Dkk) is a secreted protein that acts as a Wnt pathway inhibitor by binding to and antagonizing LRP5/6 (4). Conversely, the so-called “noncanonical Wnt pathway” mediated by the Wnt5a subclass of Wnts triggers intracellular  $\text{Ca}^{2+}$  release to activate protein kinase C and  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II, and then activates TAK1 mitogen-activated protein kinase (MAPK) kinase kinase and NLK MAPK (5). NLK phosphorylates Lef1/Tcf and inhibits the interaction of the  $\beta$ -catenin-Lef1/Tcf complex with DNA (6). This distinct Wnt pathway converges in an antagonistic manner that is conserved between vertebrates and *C. elegans*.

Recently, Wnt signalling has been suggested to be involved in regulation of bone mass and bone formation. A loss of function mutation in LRP5 was found to associate with osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive disorder (7, 8). Moreover, a Gly171-to-Val substitution mutation in LRP5 results in a high-bone-mass phenotype (9, 10). These phenotypes associated with the loss of function or substitution mutations of LRP5 indicated that Wnt signalling might be involved in modulating regulation of bone mass and bone formation (11). During osteogenesis, pluripotent mesenchymal stem cells differentiate into preosteoblasts, which then differentiate into mature osteoblasts that deposit the necessary components to form bone matrix and subsequent mineralization. Upon differentiation into osteoblasts, the cells express differentiation-related phenotypes such as a high level of alkaline phosphatase (ALP), parathyroid hormone receptor, type I collagen, osteocalcin, matrix extracellular phosphoglycoprotein (MEPE) and bone sialoproteins (12). In cultured cells, Bain *et al.* described that stimulation of canonical Wnt signalling using constitutively active forms of  $\beta$ -catenin induced the activity of ALP which is one of the differentiation markers in osteoblastic cells (13). However, the exact mechanisms and signalling regulating osteoblastic differentiation or function and bone formation still remain to be elucidated.

MEPE was one of the bone matrix proteins identified from cDNA as a tumor-derived phosphaturic factor in oncogenic hypophosphatemic osteomalacia (14) and this cDNA coded for a matrix protein consisting of a serine/glycine-rich secreted peptide that contained numerous potential phosphorylation sites and one RGD motif (15). This protein is a member of the SIBLING (Small integrin-binding ligand, N-linked glycoprotein) family of matrix proteins, which includes MEPE, bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein, enamelin and osteopontin, expressed in bones and/or teeth (16). The tissue distribution of this mRNA is bone specific and highly expressed in osteoblasts and osteocytes (15). Furthermore, targeted disruption of the MEPE gene in mice results in increased bone formation and bone mass (17). Although MEPE is perhaps involved in the regulation of bone metabolism *in vivo*, regulation of the expression of this gene during osteoblastic differentiation has not yet been identified.

Bone morphogenetic protein (BMP)s, members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, regulate the proliferation, differentiation and apoptosis of various types of cells and organs not only in embryonic development but also in postnatal physiological function (18). At least 15 types of BMPs have been identified in humans. Among them, BMP-2 alone is sufficient to induce ectopic bone formation when it is implanted into the tissues of rodents. BMP-2 is reported to trigger osteoblast differentiation and to up-regulate the expression of most genes encoding osteoblastic phenotype-related proteins *in vitro* (18). Genetic disruptions of BMPs have resulted in various skeletal and extra-skeletal developmental abnormalities (19). Signalling by TGF- $\beta$  superfamily members including BMPs is initiated following their binding to two types (type I and II) of their respective serine/threonine kinase receptors. In the intracellular signalling of BMPs, eight Smad proteins have been identified to play critical roles in mammals (20). The receptor Smads (R-Smads), consisting of Smad1, Smad2, Smad3, Smad5 and Smad8, are directly phosphorylated by type I receptors, and then

form complexes with the Co-Smad, Smad4, and move into the nucleus, where they bind to the regulatory regions of the target genes and regulate their expression.

Inhibitor of DNA binding/differentiation (Id) 1, one of the basic helix-loop-helix proteins (HLHs), was identified as a gene for inhibition of myogenesis and as a typical early response gene in BMP treatment in various types of cells in mice and humans (21). We previously reported that the expression of Id1 was stimulated within 1 h after the addition of BMP-2 in C2C12 cells (22). Id family proteins such as Id1, Id2 and emc, which lack the basic DNA binding domain, are capable of forming heterodimers with other basic HLH family transcription factors, such as MyoD family proteins, that have been implicated in transcriptional regulation during differentiation of certain cell types including myoblasts. The HLH heterodimers formed with Id proteins inhibit transcriptional activity as Id proteins lack DNA-binding activity (23). Previously, we identified a 29-bp GC rich element as a BMP-2 responsive element in the 5' flanking region of the human Id1 gene and showed that complexes of Smad1 and Smad4 recognized this element in response to BMP-2 (24).

Here, we established stimulatory Wnt3a and inhibitory Wnt5a canonical Wnt-expressing pluripotent C2C12 cells to elucidate the responses of canonical Wnt signalling to modulation by BMP-2. In these cultures, expression of bone matrix protein, MEPE was induced by BMP-2 in Wnt3a over-expressing C2C12 (Wnt3a-C2C12) cells but not in either Wnt5a over-expressing C2C12 (Wnt5a-C2C12) cells or vehicle-transfected C2C12 cells. C2C12 cells form multinucleated myotubes when switched to low mitogen medium, and this can be inhibited by the addition of BMP-2. However overexpression of Wnt3a blocked this BMP-2-induced inhibition of myotube formation. We also show that Wnt3a down-regulates expression of the BMP-2-responsive gene, Id1, and this suppression is mediated by a 29-bp GC rich region of the BMP-2 responsive element of the Id1 gene promoter. Interaction between  $\beta$ -catenin and Smad1/4 is found to be crucial for

Wnt-mediated suppression of the BMP-2 response. In contrast, BMP-2 or Smad1/4 up-regulates  $\beta$ -catenin-induced Lef1/Tcf-dependent transcriptional activity. These findings reveal that Wnt signalling links BMP-target gene expression and illustrate the functional role of Wnt signalling in BMP-2-regulated mesenchymal cell differentiation.

## EXPERIMENTAL PROCEDURES

*Cell Cultures* – Cells of the mouse myoblast cell line C2C12 (Cell Systems, Kirkland, WA) were cultured in  $\alpha$ -MEM (Sigma, St.Louis, MO) containing 100  $\mu$ g /ml of kanamycin (Meiji, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Sigma) at 37°C in 100-mm cell culture dishes (Corning, Corning, NY) in a humidified atmosphere of 5% CO<sub>2</sub> in air.

*Establishment of stable transfected C2C12 cells* – Cells were plated 1 day before transfection in  $\alpha$ -MEM at a density of 1X10<sup>5</sup> cells per well (24-well plate). The cells were transfected with 1.0  $\mu$ g of Wnt3a-pUSEamp, Wnt5a-pUSEamp or empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two days after transfection, the medium was changed to  $\alpha$ -MEM containing neomycin/Geneticin (G418) (Promega, Madison, WI) at 0.1  $\mu$ g/ml. The cells were passaged, and the clones were selected in  $\alpha$ -MEM supplemented with G418 and 10% FBS. To rule out the possibility of clonal variation, we characterized at least three independent clones for each stable transfection. To examine the effects of Wnt overexpression on the action of BMP in endogenous differentiation, control C2C12 or stable transfected C2C12 cells were inoculated at 1.5 X 10<sup>6</sup>/cm<sup>2</sup> and cultured in  $\alpha$ -MEM containing 10% FBS (growth medium). On day 1 the medium was replaced with  $\alpha$ -MEM containing 2.5% FBS (low mitogen medium) and the cells were cultured with BMP-2 or vehicle for 3 to 9 days.

*Reagents* – Recombinant human BMP-2 (25) was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan).

*Reverse transcriptase-polymerase chain*

*reaction (RT-PCR)* – Total RNA was extracted from the cells at the indicated time points using Isogen (Nippongene, Toyama, Japan) and treated with RNase-free DNase (Qiagen, Valencia, CA) to remove any contaminating genomic DNA according to the manufacturer's protocol. Complementary DNA was synthesized with Omniscript reverse transcriptase (Qiagen) using an oligo(dT)<sub>15</sub> primer (1 μM) according to the manufacturer's instructions. Each PCR reaction was carried out in 50 μl of mixture containing 1 μl of cDNA, 5 μl of 10 x Qiagen PCR buffer, 10 μl of 5 x Q-Solution PCR buffer, 1 μl of 10 mM each deoxynucleotide triphosphate mix, 0.1 μM each of sense and antisense primers, and 0.5 μl of Taq DNA polymerase (Qiagen). Each reaction consisted of initial denaturation at 94°C for 1 min, followed by 3-step cycling: denaturation at 94°C for 15s, annealing at a temperature optimized for each primer pair for 30s and extension at 72°C for 1 min. After the requisite number of cycles (25 cycles), reactions underwent a final extension at 72°C for 7 min. The primer sequences used for PCR amplification were designed based on published cDNA sequences and were:

5'-ATTGAATTTGGAGGAATGGT-3' and 5'-CTTGAAGTACGTGTAACGTG-3' for Wnt3a (26), generating a 320 bp fragment; 5'-TCCTATGAGAGGGCGCACGCAT-3' and 5'-CAGCTTGCCCCGGCTGTTGA-3' for Wnt5a (27), generating a 230 bp fragment, 5'-GTGTCATCCAGTGGGAGTT-3' and 5'-TAATGTGTCGCCTGTCCAAA-3' for MEPE (14), generating a 235 bp fragment, 5'-CTGAGTCTGACAAAGCCTTC-3' and 5'-GCTGTGACATCCATACTTGC-3' for osteocalcin (28), generating a 312 bp fragment, 5'-TATGAGCGGACTGAGCTCAGCT-3' and 5'-CAGATGTGCACACTTGTCCAGG-3' for myogenin (29), generating a 942 bp fragment, 5'-CAATAAGCTTCGCGATAAGGAG-3' and 5'-AGGAAGCTTTTGTGTCGTTG-3' for muscle creatine kinase (MCK) (30), generating a 561 bp fragment, 5'-TCCTGCAGCATGTAATCGAC-3' and 5'-GAGAGGGTGAGGCTCTGTTG-3' for Id1 (31), generating a 355 bp fragment, and 5'-GGGTAAAGGGGA GCAAAGTCAGAT-3' and 5'-CTGGGGAAAGGAGGCACA

AAGAAG-3' for osterix (32), generating a 496 bp fragment. To control for the amount of RNA in different samples, expression of glucose-6-phosphate dehydrogenase (GAPDH) was used, amplified with primers: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' generating a 300 bp fragment (33). For subsequent use, amplified PCR products were subcloned into the pGEM<sup>®</sup>-T Vector (Promega) using the pGEM<sup>®</sup>-T Vector System II and the sequences of the subcloned cDNAs were checked with a DNA sequencer (Prism 310, Applied Biosystems, Foster city, CA). All the primers were synthesized by Hokkaido System Science (Sapporo, Japan). Amplification products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining followed by UV light illumination.

*ALP staining* – Confluent cells in 100 mm culture dishes were rinsed in phosphate-buffered saline; fixed in 100% methanol, rinsed with phosphate-buffered saline, and then overlaid with 5 ml of 0.15 mg/ml 5-bromo-4 chloro-3-indolyphosphate (Wako, Osaka, Japan) plus 0.3 mg/ml of nitro-blue tetrazolium (Wako) in 0.1 M Tris-HCl (pH 9.0), 0.01 N NaOH and 0.05 mM MgCl<sub>2</sub> followed by incubation at room temperature for 2 h.

*Histochemical Analysis*– To histochemically analyze expression of myosin heavy chain (MHC), the cells were fixed for 10 min at room temperature with 3.7% formaldehyde and then stained immunohistochemically using anti-MHC monoclonal antibody (MF-20; purchased from Developmental Studies Hybridoma Bank, Iowa City, IA) and a secondary antibody of biotinylated rabbit anti-mouse immunoglobulin as described (22). The reaction products of biotin-streptavidin were visualized using the AEC substrate kit (Histofine, Nichirei Co., Tokyo, Japan).

*Reporter Constructs* – Luciferase reporter plasmids of Id985WT-luc, Id985mutB-luc, IdWT4F-luc and IdmutB4F-luc were generated as described previously (24). In brief, the 1079-bp Id1 promoter fragment (-985 to +94) was subcloned to pGL3 Basic (Promega) (Id985WT-luc). Id985mutB-luc was generated by PCR using the specific primer of 5'-ttctcgagCATGGCGA

CCGCCCGCGCTTTGCCA-3' (lower case and underline indicate flanking sequences and mutations, respectively). To generate IdWT4F-luc and IdmutB4F-luc, the specific oligo DNAs of the 29-bp BMP responsive element with the wild-type or the mutB sequence flanked by six nucleotides were annealed, digested with XhoI and Sall and subcloned into the pGL3-Promoter (Promega). Topflash is a TCF reporter plasmid containing two sets (with the second set in the reverse orientation) of three copies of the Lef1/Tcf binding site (wild type) upstream of the thymidine kinase minimum promoter and luciferase open reading frame (Upstate Biotechnology, Lake Placid, NY). Fopflash is a TCF reporter plasmid containing two full and one incomplete copy of the Lef1/Tcf binding site (mutated) followed by three copies in the reverse orientation, upstream of the thymidine kinase minimum promoter and luciferase open reading frame (Upstate Biotechnology).

*Expression Plasmids* – Complementary DNA (cDNA) constructs including Hemagglutinin (HA)-tagged mouse full-length Wnt3a and Wnt5a cDNA in pUSEamp (Wnt3a-pUSEamp, Wnt5a-pUSEamp) under the control of the CMV promoter were purchased from Upstate Biotechnology. The mutated  $\beta$ -catenin expression plasmid of  $\beta$ -catenin  $\Delta$ GSK, in which amino acid residues 15-91 of the GSK phosphorylation site of human  $\beta$ -catenin cDNA are deleted, was kindly provided Dr. Ozawa, Kagoshima Univ., Kagoshima, Japan)(34). DKK1, DKK2, DKK3 and DKK4 expression vectors were prepared by subcloning from DKK1, DKK2, DKK3 and DKK4 cDNAs (kindly provided from Dr. Sean A. McCarthy, Univ. of Michigan, Ann Arbor, MI) into pUSEamp (Upstate Biotechnology)(35). Smad expression plasmids were generated into pFLAG-CMV-2 (Sigma-Aldrich) as previously described (24).

*Transient cell transfection and assay for luciferase activity* – For the reporter assay, transfection of plasmid DNA into cells was performed using the cationic lipid reagent Lipofectamine 2000 (Invitrogen). In brief, cells were plated 24 h before transfection at a density of  $1 \times 10^5$  cells per well (24 well plate) and cultured in  $\alpha$ -MEM supplemented with 10%

FBS. The cells were transfected with reporter plasmid and expression plasmid as indicated in the figure legend. BMP-2 or vehicle was then added at the concentration indicated in the figure legend. The cells were subsequently cultured in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C for 24 h. The cells were then harvested and luciferase activities in the cell extracts were determined using a PicaGene<sup>®</sup> (Toyo Ink, Tokyo, Japan) and read using a Mini Lumat LB 9506 (Perkin Elmer). For each transfection, additional cells were transfected with 100 ng of the pTK $\beta$  plasmid (Invitrogen) for  $\beta$ -galactosidase assays to account for variation in transfection efficiency, and luciferase activity was normalized against  $\beta$ -galactosidase activity. All experiments were performed on triplicate samples and were independently repeated four to six times.

*Electrophoresis mobility shift assay (EMSA)* – EMSA was performed essentially as previously described (24). C2C12 cells were incubated for 1 h, with or without 300 ng/ml of BMP-2, and then nuclear extracts were prepared from the cells by the method of Muller et al. (1989) (36) with a protease inhibitor cocktail and phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany). Oligonucleotide probes were annealed by heating to 95°C for 5 min followed by cooling slowly to room temperature in the presence of 50 mM Tris-HCl (pH 8.0) and 10 mM MgCl<sub>2</sub>. Some sequences also contained cohesive Sall sites at the ends. The annealed probes were labelled by filling-in 5' protruding ends with [ $\alpha$ -<sup>32</sup>P]dCTP (New England Nuclear/Du Pont, Wilmington, DE) using the Klenow fragment (Pharmacia, Uppsala, Sweden) (37). The oligonucleotide sequences used as probes or competitors in EMSA were as follows: Wild-type BMP responsive element top strand, 5'-tcgacCATGGCGACCGCCCGCGCGGCGC CAGCCT-3'; wild-type BMP responsive element bottom strand, 5'-tcgacAGGCTGGCGCCGCGGGGCGGTC GCCATG-3'. Six micrograms of the nuclear extracts were incubated with a labeled BMP responsive element probe with the wild-type sequence. The reaction mixture was loaded onto a 5% polyacrylamide gel in 0.5 x TBE (44.5 mM Tris base, 44.5 mM boric acid and 1 mM

EDTA) and resolved by electrophoresis.

## RESULTS

*Establishment and characterization of Wnt3a or Wnt5a stably expressing C2C12 cells* – To examine a potential role for Wnt canonical signalling in mesenchymal cell differentiation and the functional contribution of BMP-2, we generated a pluripotent C2C12 cell line stably expressing Wnt3a or Wnt5a. C2C12 cells are a myoblastic cell line and are a well-characterized model system which have been reported to differentiate into not only myotubes but also osteoblasts depending upon the specific culture conditions when incubated in the presence of BMPs for 48-72 h (22). These culture conditions were previously shown to give rise to cells that expressed numerous mRNAs characteristic of osteoblasts including bone matrix proteins such as osteocalcin, osteopontin and transcription factors involved in osteoblastic differentiation such as Runx2 (22, 50). In first, several Wnt gene expressions in C2C12 cells were examined by RT-PCR analysis. Although Wnt4, Wnt5b and Wnt10b were expressed during day 1-5, Wnt1, Wnt2, Wnt2b, Wnt3a, Wnt5a, Wnt7a, Wnt8, Wnt8b, Wnt10a, Wnt11 and Wnt15 were not detected during all days of cultures (results not shown). Therefore, C2C12 cells were transfected with the Wnt3a expression plasmid for activating canonical Wnt signalling or Wnt5a expression plasmid for inhibiting canonical Wnt signalling, and then selected using G418 to establish cell lines. We denoted these cell lines Wnt3a-C2C12 cells and Wnt5a-C2C12 cells, respectively. While vehicle-transfected C2C12 cells did not produce any detectable Wnt3a or Wnt5a mRNA expression, Wnt3a-C2C12 cells or Wnt5a-C2C12 cells expressed high levels of Wnt3a or Wnt5a mRNA detected by RT-PCR analysis at day 2 in cultures (Fig. 1A).

To confirm activation or suppression of canonical Wnt signalling in these cells, we transfected with Topflash, the reporter plasmid that carries six tandem repeats of the Lef1/Tcf binding site. The promoter activity of Topflash is enhanced in Wnt3a-C2C12 cells. In contrast, Topflash activity is suppressed in Wnt5a-C2C12

cells (Fig. 1B). To determine whether Wnt canonical signalling might reflect osteoblastic phenotypic expression, we examined ALP activity, a defined marker of early osteoblast differentiation. Wnt3a-C2C12 cells had detectable ALP activity, while neither Wnt5a-C2C12 cells nor control C2C12 cells stained for ALP activity (Fig. 1C). However any staining was weak compared to that of the osteoblastic cell line MC3T3-E1 (Fig. 1C). To determine the formation of a mineralized matrix in these Wnt-expressing cells, the cells were continuously cultured until day 7, 14 and 28, and then assayed by von Kossa staining. Wnt3a mRNA was maintained at similar levels throughout the culture period, thus the ALP activity was induced in these cultures though calcium deposition was not detected (results not shown). Cells expressing Wnt3a exhibited a distinct morphological change compared with Wnt5a-C2C12 cells or vehicle-transfected C2C12 cells (Fig. 1D). The cells were more elongated and, upon visual inspection, appeared to be denser.

*Matrix extracellular phosphoglycoprotein (MEPE) and osteocalcin mRNA expressions and their regulation by BMP-2 and Wnt* – We next examined whether Wnt signalling combined with BMP-2 specifically modulates expression of bone matrix proteins in osteoblastic differentiation. Osteocalcin and MEPE transcripts were not detectable in Wnt3a-C2C12 cells, Wnt5a-C2C12 cells and vehicle-C2C12 cells, indicating that Wnt3a expression alone was not sufficient to induce all of the phenotypic markers for osteoblasts. Similar levels of osteocalcin mRNA expression were induced by BMP-2 treatment not only in vehicle-C2C12 cells but also in Wnt3a-C2C12 cells and Wnt5a-C2C12 cells, and Wnt3a did not change the level induced by BMP-2 (Fig. 1E). Interestingly, culturing the Wnt3a-C2C12 cells with BMP-2 dramatically induced MEPE expression as well (Fig. 1E). Time dependent changes in MEPE expression in response to the addition of BMP-2 revealed that expression was induced after 24 h then slightly decreased after 48 h (Fig. 1E). This induction was similar to that of the osteocalcin transcript except that osteocalcin expression was induced after 24 h and then reached maximal levels by 48 h after

BMP-2 treatment in Wnt3a-C2C12 cells as well as Wnt5a-C2C12 cells or vehicle-C2C12 cells. The levels of MEPE mRNA were increased by BMP-2 in a dose-dependent manner in Wnt3a-C2C12 cells (Fig. 1F). However, no induction of MEPE expression was seen in Wnt5a-C2C12 cells nor vehicle-C2C12 cells, indicating that Wnt3a and BMP-2 are required for the MEPE mRNA induction in C2C12 cells. Results shown indicate that the combination with canonical Wnt signalling induced by Wnt3a and BMP-2 positively regulates MEPE expression in osteoblastic differentiation.

*Effects of Wnt3a or Wnt5a overexpression on inhibition of myogenic terminal differentiation by BMP-2 in C2C12 cells* – Next, we examined the effects of Wnt overexpression on myogenic differentiation regulated by BMP-2 in the low mitogen culture medium. C2C12 cells generated numerous multinucleated myotubes at day 6 and the observed morphological transition from myoblasts to myotubes was also confirmed by immunohistochemical staining of MHC as a marker for mature muscle cells. By day 6 in the low mitogen medium, even Wnt3a- or Wnt5a-overexpressing C2C12 cells were also fused to form multinucleated myotubes (Fig. 2A). As previously shown, BMP-2 at 300 ng/ml inhibited the myotube formation in C2C12 cells and the cells remained as unfused mononuclear polygonal cells did not express MHC. As in the control C2C12 cells, neither myotubes nor MHC-positive cells were detected in Wnt5a-C2C12 cells treated with BMP-2. However, the MHC-positive multinucleated myotubes were observed in Wnt3a-C2C12 cells even in the presence of BMP-2 (Fig. 2A). The mRNA expressions for myogenic differentiation markers were examined by RT-PCR analysis. Each type of cells expressed similar levels of myogenin and MCK mRNAs at 3 d and these expressions increased in a time dependent manner, indicating that Wnt signaling did not modulate the endogenous myogenic differentiation in these cells (Fig. 2B). Treatment with BMP-2 completely suppressed the induction of these expressions in Wnt5a-C2C12 cells as previously shown in C2C12 cells (Fig. 2B and ref. 22). However, even in the presence of BMP-2 (300 ng/ml), both myogenin and MCK mRNA expressions

were detected at 3 d in Wnt3a-C2C12 cells and these expressions increased in a time-dependent manner until day 9 (Fig. 2B). These results indicate that Wnt canonical signalling suppresses the inhibitory effect of BMP-2 on the myogenic differentiation in C2C12 cells. They also suggest that Wnt canonical signalling modulates BMP-2 signalling during the myogenic cell differentiation.

*Wnt3a modulates BMP-2 induced Id1 mRNA expression in C2C12 cells* – It has already been shown that BMP-2 transiently induces Id1 mRNA expression in C2C12 cells. To investigate a potential role for canonical Wnt and the functional relationship of BMP-2, we first examined whether Wnt signalling specifically modulates BMP-2-induced Id1 mRNA expression. In Wnt-transfected cells, we added 300 ng/ml of BMP-2 for 24 h and then analysed gene expression by RT-PCR. The Id1 mRNA level induced by BMP-2 decreased in Wnt3a-C2C12 cells compared to vehicle-transfected C2C12 cells (Fig.3A). In contrast, no significant change of Id1 mRNA level was observed in Wnt5a-C2C12 cells with the addition of BMP-2 (Fig.3A). However, the mRNA level of osterix, a transcription factor essential for osteoblast differentiation, induced by BMP-2 was not changed in Wnt3a-C2C12 cells or Wnt5a-C2C12 cells (Fig. 3A).

To investigate the molecular mechanism underlying modulation of the BMP-2 response of the Id1 gene by canonical Wnt signalling, we analysed promoter activity in the 5'-flanking region of the Id1 gene. First, we used a luciferase reporter plasmid, Id985WT-luc, which carries a 985 bp fragment of the 5' flanking region of the Id1 gene (24). As shown in Figure 3B, BMP-2 stimulates the promoter activity of Id985WT-luc in C2C12 cells in agreement with our previous findings (24). By transfection of Wnt3a into C2C12 cells, promoter activity of Id985WT-luc induced by BMP-2 was reduced compared with that of vehicle-transfected cells (Fig. 3B). In contrast, Wnt5a increased Id985WT-luc induced by BMP-2, indicating that Wnt canonical signalling regulates Id1 promoter activity in response to BMP-2 (Fig. 3B). As the 29-bp GC rich region between -985 and -957 was identified as the BMP-2 responsive element of



the human *Id1* gene, we used *Id985mutB-luc* with a mutation in the BMP-2 responsive element sequence to investigate whether canonical Wnt signalling regulates the response element. Neither Wnt3a nor Wnt5a regulates the transcriptional activity of *Id985mutB-luc* (Fig. 3B), suggesting that this element involves not only the BMP-2 response but also a canonical Wnt response in regulation of the *Id1* gene.

*The BMP responsive element of the Id1 gene involves canonical Wnt signalling-dependent suppression* – As the 29-bp GC-rich region between -985 and -957 was identified as the BMP-2 responsive element of the human *Id1* gene, we used four copies of this 29-bp fragment with the wild-type or mutated sequence in tandem with the forward sequence (*IdWT4F-luc*) in front of the SV 40 promoter (Fig.3C). Wnt3a suppressed promoter activity of *IdWT4F-luc* induced by the addition of BMP-2 compared with that of vehicle-transfected cells, whereas Wnt5a enhanced the luciferase level induced by BMP-2 (Fig. 3C).

In the absence of conventional Wnt signalling,  $\beta$ -catenin is phosphorylated by GSK3 $\beta$ , and then degraded by proteasomes (4). When conventional Wnt signalling does not cause phosphorylation of  $\beta$ -catenin by GSK3 $\beta$  and its accumulation and transport to the nucleus, then it forms a complex with Lef1/Tcf that regulates target gene expression. An activating form of  $\beta$ -catenin which lacks sites of phosphorylation by GSK3 $\beta$  ( $\beta$ -catenin  $\Delta$ GSK) also suppressed *IdWT4F-luc* activity induced by BMP-2 (Fig. 4A). In contrast, the promoter activity of *IdmutB4F-luc*, which contains a 3-bp mutation introduced into the 29-bp GC rich region, was not affected by BMP-2 and/or Wnt3a or by Wnt5a or the constitutively activating form of  $\beta$ -catenin, indicating that this BMP-2 responsive element of the *Id1* gene also contains a canonical Wnt modulating region (Fig.4A).

DKK is an endogenous Wnt antagonist. Transfection of DKK1, 2, 3 and 4 rescued the Wnt3a suppressed promoter activity of *IdWT4F-luc* on the BMP-2 responsive promoter activity (Fig. 4B). In contrast, DKK1 has no effect on suppression by  $\beta$ -catenin  $\Delta$ GSK by BMP-2 induced *IdWT4F-luc* activity

(Fig. 4B), indicating an extracellular interaction between the Wnt receptor, Wnt3a and DKK.

*Interaction of Smad and  $\beta$ -catenin modulates the BMP-2 response by canonical Wnt signalling through the BMP responsive element of the Id1 gene* – Previously we showed that transcription factors including Smad1 and Smad4 bound to the 29-bp BMP responsive element using EMSA and supershift assay. Co-transfection of both Smad1 and Smad4 cDNA also up-regulated basal promoter activity of *IdWT4F-luc* in C2C12 cells as well as that induced by the addition of BMP-2 (24). In this induction, the constitutively active mutated form of  $\beta$ -catenin,  $\beta$ -catenin  $\Delta$ GSK, also inhibited the promoter activity of *IdWT4F-luc* induced by Smad1 and 4 (Fig. 5A), indicating that interaction between Smads and canonical Wnt signalling molecules such as  $\beta$ -catenin could associate in the suppression of the BMP-2 responsive element in the *Id1* gene. To investigate DNA binding activity in the nucleus, we prepared nuclear extracts from C2C12 cells, Wnt3a-C2C12 cells and Wnt5a-C2C12 cells within 1 h after addition of BMP-2 and performed EMSA using the 29-bp BMP responsive element as a probe. As shown as Fig. 5B, binding activity of the 29-bp probe in nuclear extracts prepared from control C2C12 cells was very low, while the binding activity in nuclear extracts prepared from C2C12 cells treated with BMP-2 was significantly increased. In contrast, binding activity was decreased in BMP-2-treated C2C12-Wnt3a cells (Fig. 5B). The mobility of the DNA-protein complexes from nuclear extracts of BMP-2-treated C2C12-Wnt5a cells was increased to that observed in C2C12 nuclear extracts (data not shown). These findings suggest that the binding activity of the BMP responsive element in nuclear extracts is also regulated by canonical Wnt signalling in C2C12 cells and its binding activity is closely correlated to the promoter activity and mRNA expression of the *Id1* gene.

*BMP-2 upregulates transcriptional activity of canonical Wnt signalling* – In turn, to examine further the relationship between Wnt signalling and BMP-2, we performed a reporter assay for promoter activity using Topflash, which carries six tandem repeats of the Wnt response element, to elucidate whether BMP-2 regulates the

canonical Wnt response. When BMP-2 was added to the medium for 24 h, the promoter activity of Topflash was not altered in C2C12 cells with the addition of BMP-2 (Fig. 6A). As previously shown, transfection of a canonical Wnt signalling molecule such as Wnt3a or  $\beta$ -catenin  $\Delta$ GSK induced promoter activity of Topflash. To our surprise, the addition of BMP-2 to transfected cells with activated canonical Wnt signalling involving Wnt3a or  $\beta$ -catenin  $\Delta$ GSK caused a further dramatic increase in Topflash activity and these inductions were dose-dependent (Fig. 6A). No inducible activity was detected with BMP-2 in cells transfected with Wnt5a (Fig. 6A). Inducible activity was also observed with transfection of Smad1/4 instead of addition of BMP-2 in cells transfected with either Wnt3a or  $\beta$ -catenin  $\Delta$ GSK (Fig. 6B). Therefore, BMP-2 signalling could increase the canonical Wnt response in this reporter system in these cells.

## DISCUSSION

C2C12 myoblasts differentiate into multinucleated myotubes when the concentration of FBS in culture medium is reduced from 10% to 2.5%. Our previous studies have shown that BMP-2 inhibits the myogenic differentiation of C2C12 cells into mature muscle cells but also converts their differentiation pathway into that of osteoblasts (22). We show that signaling induced by both canonical Wnt and BMP-2 are needed to induce the expression of late osteoblastic differentiation marker genes such as MEPE. Recent studies showed that antibodies to MEPE specifically immunostained highly differentiated osteoblasts in adult bone tissues and MEPE mRNA expression in cultured cells was greatly increased when cells were maintained at confluence in the presence of dexamethasone to stimulate differentiation along the osteoblastic lineage (15). Therefore, this body of evidence indicates that MEPE is a specific marker of the osteoblast. Previously we reported that MEPE expression was down-regulated by FGF2 in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells incubated with

dexamethasone,  $\beta$ -glycerophosphate and ascorbic acid (39). In our present study, not only BMP-2 alone but also BMP-2 in combination with Wnt3a was found to have inductive effects on MEPE mRNA expression, which opens up the possibility that such canonical Wnt signaling may be a potential stimulator of osteoblastic differentiation and function in bone tissue. Here we also found that Wnt canonical signalling induced by Wnt3a-overexpression suppressed the inhibitory effects of BMP-2 on the myogenic differentiation in C2C12 cells, indicating that the Wnt canonical signalling is capable to regulate the BMP-2 signalling. Therefore, a functional cross-talk between Wnt and BMP-2 signalling was further examined in this study.

Id1 is one of a group of negative regulatory proteins for muscle differentiation and its mRNA and protein levels are down-regulated upon initiation of myogenic differentiation. Id1 is the most significantly up-regulated gene upon BMP-2, BMP-6, or BMP-9 stimulation in various types of human and mouse cells (20). Previously, we have shown that the GC rich element between -985 bp and -957 bp of the human Id1 gene was the response element for BMP-2 (24). This 29-bp element showed 100% homology between the human and mouse Id1 gene, indicating that this element has a critical role in the expression of Id1 in mammals. Korchynskyi and ten Dijke also reported that BMPs, but not TGF- $\beta$ , rapidly activate the mouse Id1 promoter via two regions, one of which contains CGCC sequence elements flanked by CAGC motifs and the other of which harbors two Smad binding elements (40). Recently, BMP-dependent activation of Msx2 was shown to be mediated via binding of Smad1 and Smad4 at Smad binding elements of the Msx2 gene in murine embryonic stem cells (41). In this study, we show that down-regulation of Id1 gene promoter activity by canonical Wnt signalling is mediated via the Smad binding element of the Id1 gene promoter. Thus  $\beta$ -catenin could bind Smad1/4, leading to reduced DNA binding activity and transcriptional activation by complexes of transcription factors including Smad1/4. We hypothesized that  $\beta$ -catenin might interact with Smad and repress transcription by masking the

DNA binding domain of Smad, leading to inhibition of the BMP-2 target gene, Id1. Several lines of evidence suggest that the transcriptional activator p300/CBP is required for Smad-dependent transactivation of target genes and Wnt and BMP signalling could occur via enhanced accumulation of p300/CBP on the response element of the promoter. Ghosh *et al.* demonstrated that p53 also represses TGF- $\beta$  stimulation of promoter activity driven by minimal Smad-binding elements and that overexpression of the transcriptional coactivator p300 rescued TGF- $\beta$  stimulation of collagen  $\alpha$ 2(I) promoter activity in fibroblasts over-expressing p53, suggesting that ligand-dependent interaction of Smad3 with p300 may be one of the targets of p53-mediated inhibition of TGF- $\beta$  responses (42). Thus, it is possible that the transcriptional complexes decrease the affinity or the levels of coactivator molecules by binding of  $\beta$ -catenin to Smad1/4 in the Smad binding element of the Id1 gene promoter.

The BMP and Wnt signalling pathways have been extensively studied in the regulation of early embryonic development and in the control of cell differentiation and proliferation in adult tissues (2). The Wnt signalling pathway acts as a dorsal modifier and the BMP signalling pathway as a mesoendoderm inducer (43). Genetic interaction between these highly conserved and ubiquitous signalling pathways has been observed in multiple settings in fruit flies, amphibians, zebrafish, and mammals. Several studies have reported that these two signalling pathways link to biological responses via the formation of a nuclear transcription factor complex that acts on target genes, resulting in Wnt signalling repression of BMP-induced activation. For example, overexpression of Dkk1 enhances BMP4-triggered apoptosis in vertebrate limb development, indicating that Wnt signalling inhibits apoptosis induced by BMP signalling (44). In embryonic stem cells, Wnt signalling also inhibits neural differentiation and this differentiation can be partially restored by the addition of the BMP antagonist noggin, suggesting that Wnt signalling inhibits BMP signalling (45). Our observations provide evidence that Wnt may

negatively regulate BMP signalling not only in apoptosis in limb development or neural differentiation but also during differentiation of mesenchymal cells. Another report indicated that the Wnt signalling pathway indeed directly represses transcription of some genes including the osteocalcin gene mediated by Runx2 (46). Our results confirm the cooperative interaction between Smad1/4 and  $\beta$ -catenin in gene expression during mesenchymal cell differentiation.

In our data, BMP-2 or Smad1/4 enhance the response of the Lef1/Tcf reporter. Several reports demonstrated that TGF- $\beta$  signalling results in the C-Smads, Smad4 and Smad3, directly interacting with  $\beta$ -catenin/Lef-1 and the transcriptional activation of Lef1-responsive promoters (47, 48). Takizawa *et al.* reported that BMP-2 enhances Notch-induced transcriptional activation of the Hes-5 gene via the RBP-Jkappa binding sequence in the Hes-5 gene promoter by recruitment of co-activator p300/CBP interacting with  $\beta$ -catenin and Smad1 (49). Our present results suggest the possibility that promoter of the MEPE gene interacts with  $\beta$ -catenin/Tcf/Lef1 in cooperation with Smads, and then these complexes induce expression of this gene. It is therefore a possible mechanism that Smad1/4 could form a complex with  $\beta$ -catenin/Tcf1 in response to either the Wnt/ $\beta$ -catenin or BMP signalling pathway via the recruitment of a co-activator such as p300/CBP.

Wnts have been shown to play important roles in the regulation of many aspects of development, which may include chondrogenesis and osteogenesis (11). Since Wnt3a plays a key role in the development of the axial and appendicular skeleton and appears to regulate stem cell proliferation and differentiation, we sought to explore its effect on cells cultured under osteogenic conditions. Intriguingly, the synergism of expression of Wnt3a or Wnt5a with BMP-2 on gene transcription occurred without altering expression of Runx2 (data not shown), suggesting that canonical Wnt's actions are independent or downstream of this osteoblast-specific transcription factor (50). This is consistent with recent observations in

early events during skeletal development such as limb patterning. The phenotypic abnormalities observed in Wnt-deficient mice occur in the context of normal expression of a gene controlling osteogenesis, Runx2 (2), indicating a role for a Runx2-independent pathway in control of osteoblast proliferation and function. From our observations, BMP-2 did not regulate Topflash activity in C2C12 cells, indicating that it is unlikely that BMP-2 regulates GSK-3 $\beta$  enzymatic activity or increases  $\beta$ -catenin nuclearization or interaction with Lef1/Tcf in a transcription complex, at least in these cells. It has been reported that BMP-2 activates not only Smad proteins but also MAPK and PI3K to induce the respective intracellular signals (51). We cannot rule out the possibility that MAPK and/or PI3k are involved in the effects of BMP-2, but the contribution of these signalling pathways would be minor, at least in the expression of Id1 in C2C12 cells.

In osteogenic differentiation, activating  $\beta$ -catenin induces ALP expression, one of the early markers of osteoblastic differentiation, in the pluripotent mesenchymal progenitor cells C3H10T1/2, and it induces mineralization of the osteoblast-like cells MC3T3-E1 and participates in BMP-2 mediated signal transduction (13). We also observed ALP activity in Wnt3a-C2C12 cells but not Wnt5a-C2C12 cells. Recently, Mbalaviele *et al.* showed that BMP-2 and  $\beta$ -catenin synergize to promote osteoblast differentiation such as stimulation of ALP activity during osteogenic differentiation of multipotent embryonic cell lines, and new bone formation (52). Rawadi *et al.* proposed that BMP-2 induction of ALP in these cells partially relies on the Wnt expression cascade and that  $\beta$ -catenin might regulate osteoblastic differentiation via multiple mechanisms (53). However, in our experiments using C2C12 cells, BMP-2 did not induce canonical Wnt expression and Tcf/Lef1 dependent transcriptional activation. It could be ruled out that BMP-2 regulates canonical Wnt expression leading to direct regulation of  $\beta$ -catenin nuclearization or interaction with Tcf/Lef1 in a transcription complex, at least in C2C12 cells. Our results indicate that BMP-2-induced osteoblastic differentiation may be regulated by canonical Wnt signalling and

that  $\beta$ -catenin can also participate in non-BMP-2-dependent osteoblastic differentiation processes. Constitutive overexpression of the Id gene inhibited osteoblast differentiation initiated by BMP-9 and this BMP-9-regulated Id1 expression is Smad4-dependent (54, 55). Taken together, our findings suggest that canonical Wnt signalling may enhance osteoblastic differentiation via down-regulation of the Id1 HLH protein. Moreover, Id1 expression must be down-regulated during the terminal differentiation of committed osteoblasts, suggesting that a balanced regulation of Id1 expression may be critical to BMP-induced osteoblast lineage-specific differentiation of mesenchymal progenitor cells.

During bone repair process in skeletal fractures, it is reported that expression certain Wnt gene members and their target genes are expressed (56). The healing of skeletal fractures is essentially a replay of bone development, involving the closely regulated processes of osteogenesis. In preliminary, we attempted to deliver plasmid DNA containing Wnt3a gene to the repair process of fractured bone by electroporation-transfer in rat.  $\mu$ CT analysis at 14 days after fracture exhibited enlarged callus volume with Wnt3a expression, suggesting that Wnt3a may promote the cell differentiation in the callus and the repair of fractured bone (Negishi, A. *et al.* manuscripts in preparation). Several Wnt have recently been shown to be targets of sonic hedgehog (4). Since it was previously shown that hedgehog were expressed in the developing skeletal system, and thus hold important roles as regulators of skeletogenesis (12). The hedgehog pathway was also shown to be linked to the Wnt and BMP signaling pathways during skeletal development (12). Taken together, our results of the interaction of multiple signaling pathways may play a pivotal role in the successful mammalian bone regeneration, skeletal development, and wound repair.

In conclusion, our results reveal the molecular mechanisms of how BMP-2 and Wnt signalling pathways cooperate to regulate graded expression of Id1. Moreover, we propose that the results of this study suggest that canonical Wnt signalling has putative

physiological significance for BMP-2 regulated cell differentiation from mesenchymal progenitor cells. The physiological and functional interactions between  $\beta$ -catenin and Smad1/4 and the cooperation of two pathways

permitting tight regulation of this regulatory process may link the two pathways and contribute to the specification of cell fates and bone formation.

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## FOOTNOTES

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<sup>1</sup>The abbreviations used are: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; EMSA, electrophoresis mobility shift assay; GSK, glycogen synthase kinase; HLH, helix-loop-helix; Lef, lymphoid enhancer factor; LRP, low-density-lipoprotein related protein; Luc, luciferase; MCK, muscle creatine kinase; MEPE: matrix extracellular phosphoglycoprotein; MHC, myosin heavy chain; RT-PCR, reverse transcriptase chain reaction; Tcf, T-cell factor.

## FIGURE LEGENDS

**Fig. 1. Establishment and characterization of Wnt3a or Wnt5a stable expressing C2C12 cells.** C2C12 cells were transfected with 1.0  $\mu\text{g}$  of Wnt3a-pUSEamp, Wnt5a-pUSEamp or empty vector and then transfected cell clones (Wnt3a-C2C12, Wnt5a-C2C12 or C2C12 cells) were selected as described in Experimental Procedures. A. Expression of Wnt3a or Wnt5a mRNA in transfected C2C12 cells. Wnt3a-C2C12, Wnt5a-C2C12 or C2C12 cells were plated at  $1 \times 10^5$  cells/cm<sup>2</sup> in 100 mm cell culture dishes. After 2 days, total cellular RNA was extracted and then RT-PCR was conducted to estimate the level of Wnt3a or Wnt5a mRNA expression. Equal loading of RNA samples was checked by amplification of GAPDH cDNA. B. Transcriptional activity of Topflash reporter. Cells were transiently transfected in 24-well plates with 0.1  $\mu\text{g}$  of Topflash, after which cells were cultured for a further 24 h. Luciferase activity was determined as described in Experimental Procedures. Normalized luciferase activity is represented as fold induction over C2C12 cells. C. Alkaline phosphatase (ALP) enzyme activity. The cells were cultured until confluent and then stained for ALP activity as described in Experimental Procedures. D. Cell morphology viewed by phase contrast microscopy. Magnification: X100. E and F. Time and dose dependent induction of matrix extracellular phosphoglycoprotein (MEPE) and osteocalcin mRNA expression with the addition of BMP-2 in Wnt3a or Wnt5a stable expressing C2C12 cells. Wnt3a-C2C12, Wnt5a-C2C12 or C2C12 cells were plated at  $1 \times 10^5$  cells/cm<sup>2</sup> in 100 mm cell culture dishes. After 24h, the medium was changed and 300 ng/ml (E) or indicated concentrations (F) of BMP-2 was added, after which cells were cultured for a further indicated time (E) or 24 h (F). Total cellular RNA was extracted and then RT-PCR was performed to estimate the level of MEPE or osteocalcin mRNA expression as described in Experimental Procedures. The data represent one of three experiments with similar results.

**Fig. 2. The effects of Wnt3a or Wnt5a overexpression on inhibition of myogenic terminal differentiation by BMP-2 in C2C12 cells.** A. immunohistochemical analysis for myosin heavy chain (NHC) expression. The cells were cultured in low mitogen medium without (a, c and e) or with (b, d and f) 300 ng/ml of BMP-2. After 6 days, the cells were fixed and stained

immunohistochemically for MHC with a specific antibody. a and b, C2C12 cells; c and d, Wnt3a-C2C12 cells; e and f, Wnt5a-C2C12 cells. Magnification: X100. B. mRNA expression of proteins associated with terminal myogenic differentiation. The C2C12, Wnt3a-C2C12 or Wnt5a-C2C12 cells were inoculated at  $1.5 \times 10^6/\text{cm}^2$  and cultured in growth medium, then the medium was replaced on day 1 with low mitogen medium containing 300 ng/ml of BMP-2 (+) or vehicle (-). After the indicated days, total cellular RNA was extracted and RT-PCR was conducted to estimate myogenin and muscle creatine kinase (MCK) mRNA expression in these cells. RT-PCR for GAPDH was performed with the same samples as a control for the amount of reverse-transcribed cDNA present in the samples.

**Fig. 3. Wnt3a represses BMP-2-induced Id1 mRNA expression and the BMP responsive element of the Id1 gene is involved in Wnt canonical signalling-dependent suppression.**

A. RT-PCR analysis of Id1 mRNA expression. C2C12 cells, Wnt3a-C2C12 cells and Wnt5a-C2C12 cells were stimulated with 300 ng/ml of BMP-2 (+) or vehicle (-) for 24 h and then RT-PCR analysis was performed using Id1 primers or osterix primers as described in Experimental Procedures. Equal loading of RNA samples was checked by amplification of GAPDH cDNA. B. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu\text{g}$  of Id985WT-luc or Id985mutB-luc as a reporter plasmid, 0.1  $\mu\text{g}$  of Wnt3a, Wnt5a or vehicle expression plasmid as indicated and pTK $\beta$ . Then, 300 ng/ml of BMP-2 (+) or vehicle (-) was added, after which cells were cultured for a further 24 h. Luciferase activity was determined as described in Experimental Procedures. Normalized luciferase activity is represented as fold induction over Id985WT-luc with vehicle. Data are means  $\pm$  s.d. (n=5). C. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu\text{g}$  of IdWT4F-luc or IdmutB4F-luc and 0.2  $\mu\text{g}$  of Wnt3a, Wnt5a, or vehicle expression plasmid as indicated. Then, 300 ng/ml of BMP-2 (+) or vehicle (-) was added, after which cells were cultured for a further 24 h. Normalized luciferase activity is represented as fold induction over IdWT4F-luc with vehicle. Data are means  $\pm$  s.d. (n=5).

**Fig. 4. The constitutively active form of  $\beta$ -catenin represses transcriptional activity of the BMP responsive element induced by BMP-2 and overexpression of Dkk reverses Wnt3a-mediated suppression in the BMP responsive element of the Id1 gene.**

A. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu\text{g}$  of IdWT4F-luc and 0.2  $\mu\text{g}$  of  $\beta$ -catenin  $\Delta$ GSK,  $\beta$ -catenin, or vehicle expression plasmid as indicated. Then, 300 ng/ml of BMP-2 (+) or vehicle (-) was added, after which cells were cultured for a further 24 h. Normalized luciferase activity is represented as fold induction over IdWT4F-luc with vehicle. Data are means  $\pm$  s.d. (n=5). B. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu\text{g}$  of IdWT4F-luc, 0.2  $\mu\text{g}$  of Wnt3a,  $\beta$ -catenin  $\Delta$ GSK or vehicle expression plasmid and 0.2  $\mu\text{g}$  of DKK1, DKK2, DKK3, DKK4 or vehicle expression plasmid as indicated. Then, 300 ng/ml of BMP-2 (+) was added, after which cells were cultured for a further 24 h. Normalized luciferase activity is represented as fold induction over IdWT4F-luc with BMP-2. Data are means  $\pm$  s.d. (n=5).

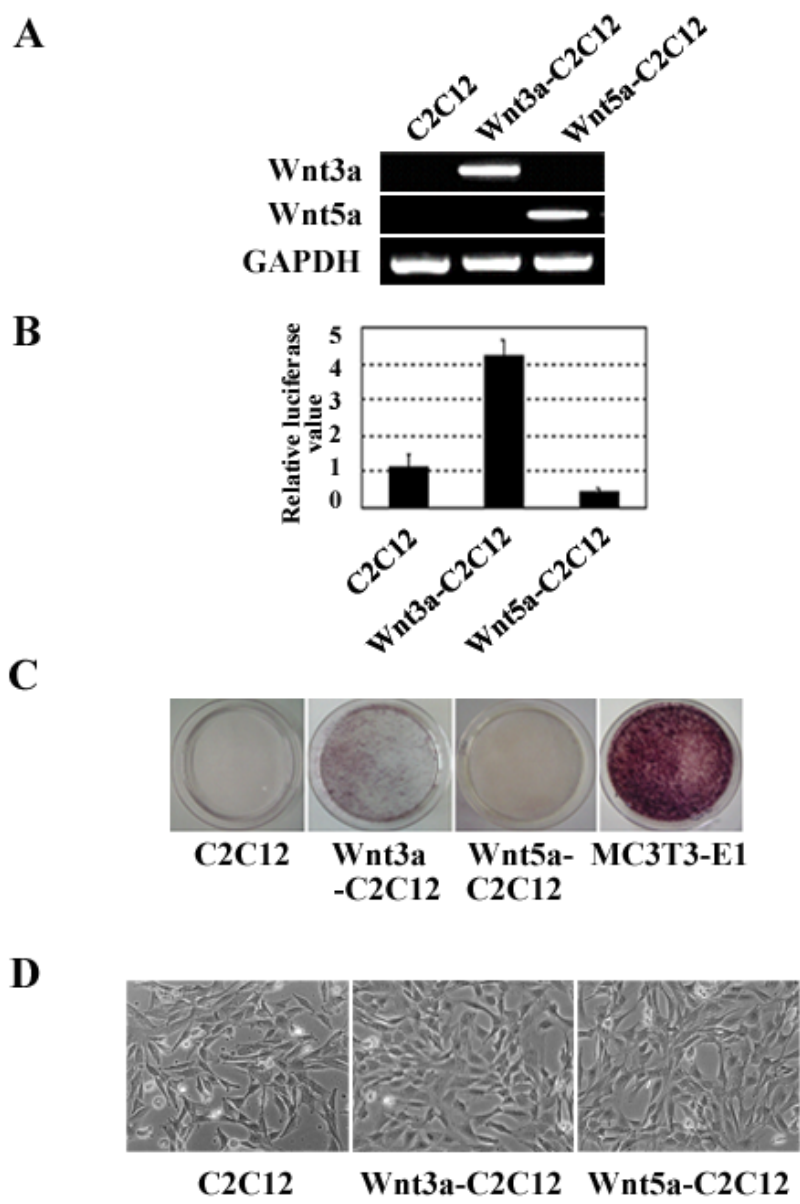
**Fig. 5. Canonical Wnt signalling suppresses Smad-induced transcriptional activities and binding activity of the GC-rich 29bp BMP responsive element of the Id-1 gene.**

A. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu\text{g}$  of IdWT 4F-luc, Smad1 and Smad4 expression plasmid, and 0.2  $\mu\text{g}$  of  $\beta$ -catenin  $\Delta$ GSK or vehicle expression plasmid as indicated. Cells were then cultured for a further 24 h. Normalized luciferase activity is represented as fold induction over IdWT4F-luc with vehicle. Data are means  $\pm$  s.d. (n=3). B. Electrophoresis mobility shift assay of the GC-rich 29bp binding activity in C2C12 cells, C2C12-Wnt3a cells and C2C12-Wnt5a cells. Nuclear Extracts were prepared from cells treated

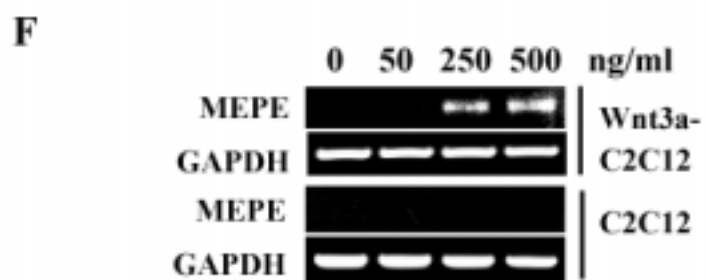
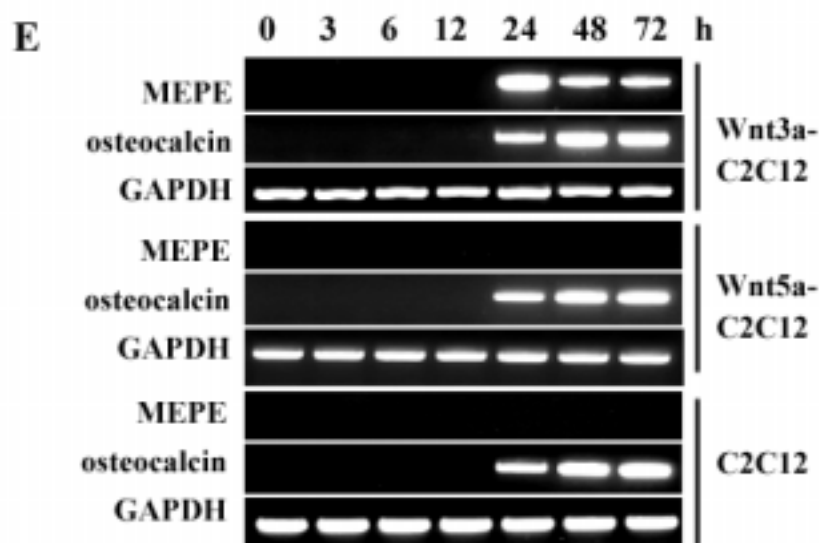


with and without BMP-2. EMSA was performed using the BMP responsive element as a probe as described in Experimental Procedures.

**Fig. 6. BMP-2 enhances Wnt3a or  $\beta$ -catenin mediated transcriptional activation of Lef1/Tcf reporter in C2C12 cells.** A. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu$ g of Topflash and expression plasmid of Wnt3a or  $\beta$ -catenin  $\Delta$ GSK as indicated. Then 300 ng/ml of BMP-2 was added, after which cells were cultured for a further 24 h. B. C2C12 cells were transiently co-transfected in 24-well plates with 0.1  $\mu$ g of Topflash, expression plasmid of Wnt3a,  $\beta$ -catenin  $\Delta$ GSK or vehicle, and Smad1 and Smad4 expression plasmid as indicated, then cells were cultured for a further 24 h. Normalized luciferase activity is represented as fold induction over Top-Flash with vehicle. Data are means  $\pm$  s.d. (n=5).

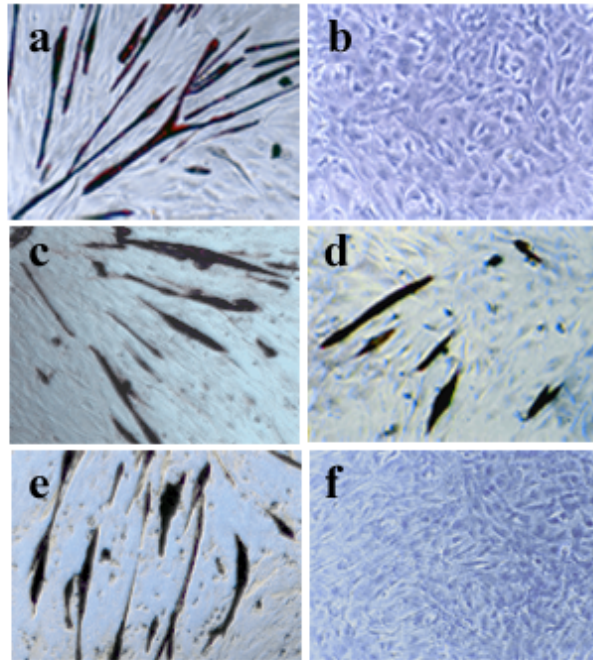


**Fig. 1**

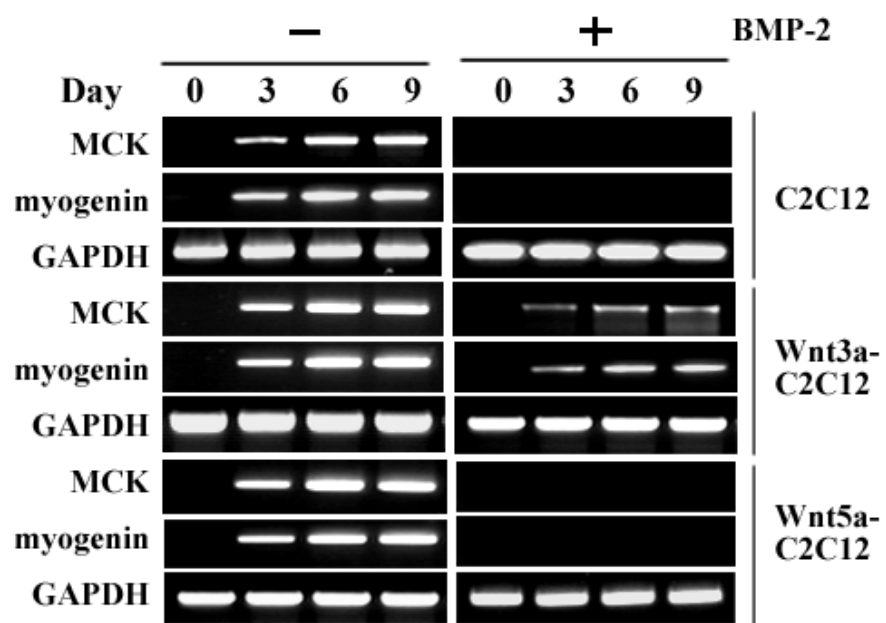


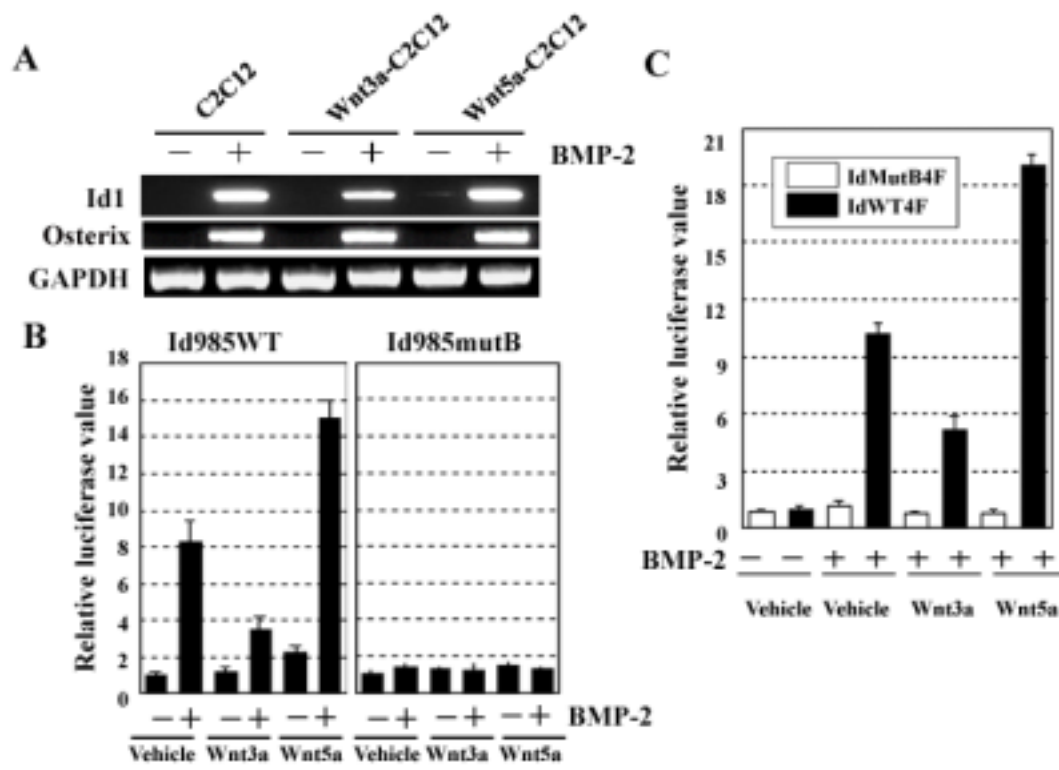
**Fig. 1**

**A**

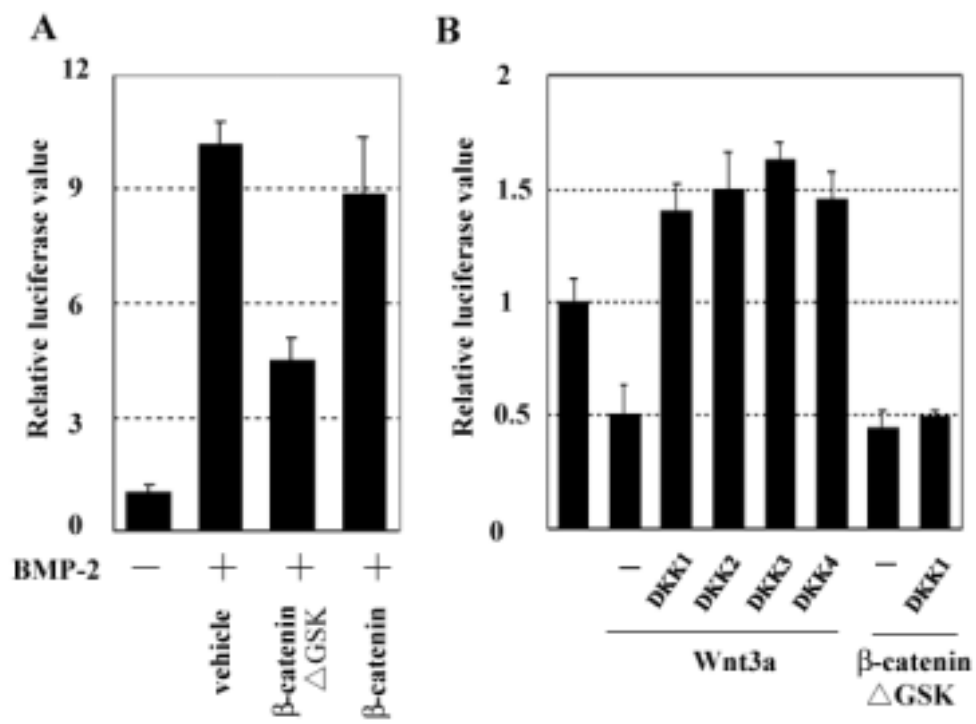


**Fig. 2**

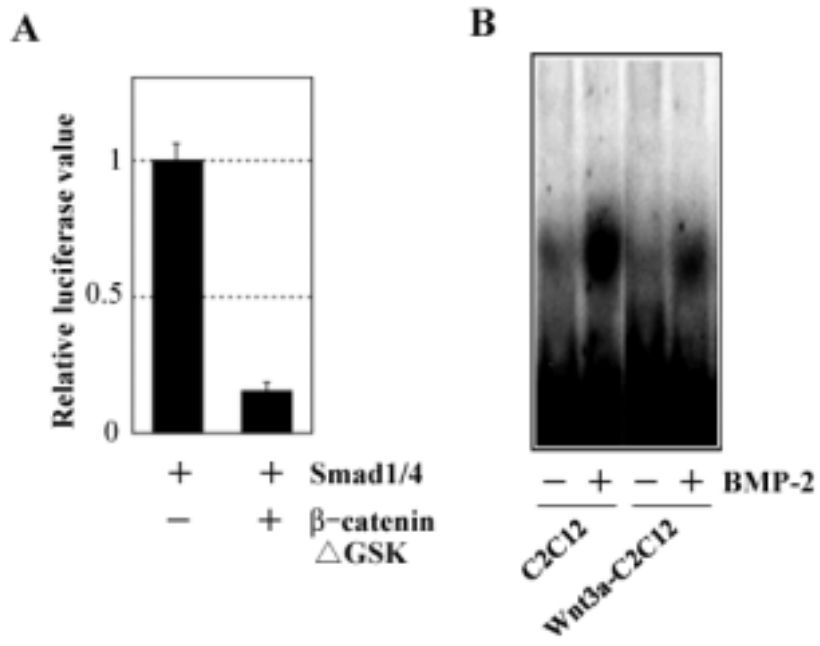
**B****Fig. 2**



**Fig. 3**

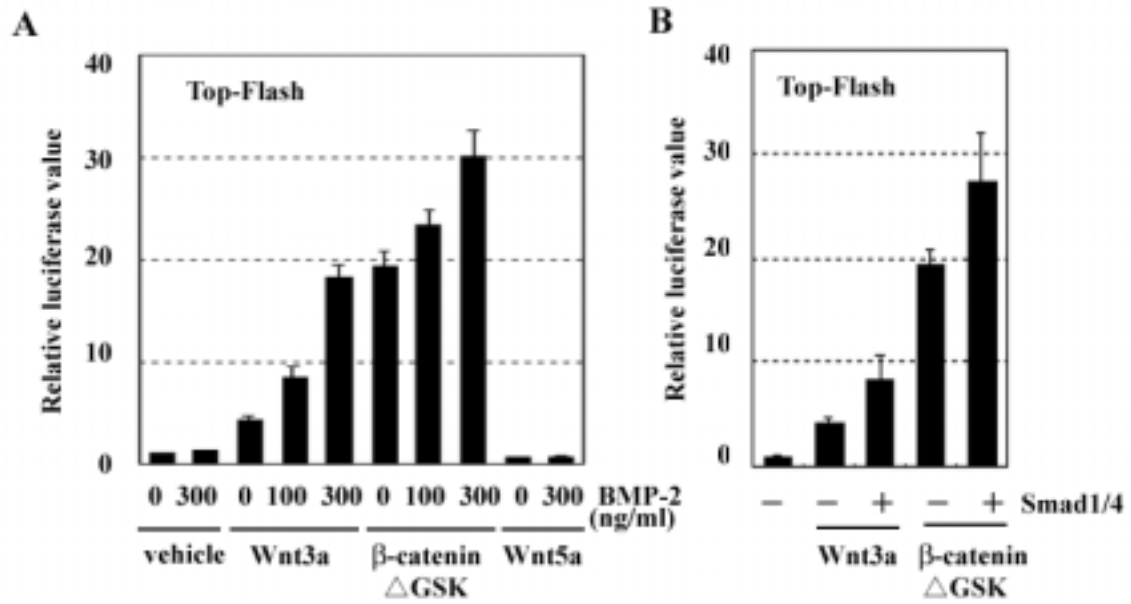


**Fig. 4**



**Fig. 5**





**Fig. 6**