A Dishevelled-1/Smad1 Interaction Couples WNT and Bone Morphogenetic Protein Signaling Pathways in Uncommitted Bone Marrow Stromal Cells*

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Morphogenetic Protein Signaling Pathways in Uncommitted Bone Marrow Stromal Cells

Genetic evidence from both humans and mice suggests that Wnt/β-catenin and bone morphogenetic protein (BMP) signaling pathways are essential for bone marrow mesenchymal stem cells to differentiate into osteoblasts. Here we describe a mechanism through which BMPs antagonize Wnt signaling and retard bone marrow mesenchymal stem cell proliferation. Treatment with Wnt3a, but not BMP-2, stimulated Lef1-mediated transcriptional activity, whereas co-stimulation with both Wnt3a and BMP-2 markedly reduced Wnt3a-induced reporter activity. Immunoprecipitation assays in 293T cells transfected with individual Smads and Wnt pathway components revealed a specific interaction between Dvl-1 and Smad1 that was dependent on the presence of Wnt3a or BMP-2. Under unstimulated conditions, Dvl-1 and Smad1 are co-immunoprecipitated and form a complex through the linker region of Smad1. Wnt3a treatment transiently disrupted the Dvl-1/Smad1 interaction coincident with nuclear accumulation of β-catenin. In contrast, when cells were exposed to both Wnt3a and BMP-2, there was an enhanced accumulation of the Dvl-1-Smad1 complex and a decreased nuclear accumulation of β-catenin. Expression of a mutant Smad1 protein, which cannot be phosphorylated in response to BMP, eliminated the inhibitory effect of BMP on Wnt-induced β-catenin accumulation and transcriptional activity. These results identify a potential mechanism whereby BMP-2 antagonizes Wnt signaling in osteoblast progenitors by promoting an interaction between Smad1 and Dvl-1 that restricts β-catenin activation.

The development of the vertebrate skeleton is controlled by the balanced actions of morphogens and growth factors. Prominent among these are the members of the Wnt and BMP families of signaling molecules (1–3). Wnts and their downstream signaling components are highly conserved signaling molecules that control a wide variety of processes in embryonic development (4–7). Wnts bind to cell surface receptors encoded by the Frizzled gene family, which when activated stimulate Dishevelled (Dvl), the most proximal cytosolic component known in this pathway. In the absence of Wnt, β-catenin is sequestered with Axin, adenomatous polyposis coli, and GSK-3β, is phosphorylated, and then degraded. In the presence of Wnt, Frizzleds bind and activate Dvl. Phosphorylated Dvl causes inhibition of GSK-3β. These events prevent degradation of β-catenin, which is translocated to the nucleus where it binds to the Lef/Tcf family of transcription factors and initiates the transcription of several target genes including cyclin D1, Myc, Tcf-1, and Xnr3 (8–10). In both vertebrates and invertebrates, conventional Wnt signal transduction results in tissue-specific cell fate decisions during embryogenesis and regulates cell proliferation in adult tissues (11, 12).

BMPs are multifunctional growth factors, which belong to the transforming growth factor β (TGF-β) superfamily. BMPs exert critical actions during skeletal development that are different from those influenced by Wnts (13–15). BMPs bind and activate a family of BMP/TGF-β receptors that possess intrinsic serine/threonine kinase activity that triggers phosphorylation of receptor-regulated Smads (R-Smads). Smad2 and Smad3 are phosphorylated by TGF-β and activin type I receptors, whereas Smad1, -5, and -8 act downstream of BMP type I receptors. Activated R-Smads by phosphorylation form heteromeric complexes with common-partner Smad and translocate to the nucleus where they regulate the transcription of target genes in cooperation with other transcription factors. Inhibitory Smads (Smad6 and -7) serve to oppose the action of R-Smads (16).

Previous studies have shown interactions between Wnt and BMP signaling pathways on a number of levels. In Xenopus, Smad4 and Lef1 cooperate to control the expression of the homeobox gene Xtwn in Spemann’s organizer (17). In mice, deficiency of one Smad4 allele synergizes with deficiency of adenomatous polyposis coli, a regulator of β-catenin degradation, to increase the number and invasiveness of intestinal tumors (18). These findings have been extended to the level of molecular interactions in Xenopus by the demonstration of Smad4-Lef1/Tcf and Smad4-β-catenin molecular complexes, and the cooperative interaction of Smad4 and Lef1/Tcf at the Xtwn promoter (17). Previous studies also suggested that Wnt and BMP signaling can alternatively synergize or antagonize the action of one another in uncommitted bone marrow stromal cells (BMSCs) (19–22). However, the molecular mechanisms responsible for these interactions remain unclear.

Here we report that BMP-2 attenuates Wnt signaling through the formation of specific complexes between Dvl-1 and Smad1. Exposure to Wnt3a induces disassembly of this complex and increased nuclear accumulation of β-catenin, whereas stimulation of cells with both ligands results in decreased nuclear β-catenin levels. Functional assays confirmed that Wnt3a-induced increases in Lef1 reporter activity were attenuated by co-addition of BMP. These results provide a potential mechanism whereby BMP-2 antagonizes Wnt3a-induced proliferation.
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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Wnt3a Conditioned Media (Wnt3a CM)—**Primary mouse BMSCs from 6-week-old male C57BL/6 mice were prepared and cultured as described previously (23). ST2 mouse BMSCs (24), 293T cells, and L cells overexpressing Wnt3a (ATCC) were grown in α-minimum Eagle’s medium or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) (Cellgro), and 1% glutamine/penicillin/streptomycin. All cells were grown in a 37 °C incubator in the presence of 5% CO₂. Wnt3a-containing conditioned media (CM) from L cells was collected from cultures grown to confluence, centrifuged at 1,000 rpm for 10 min, snap-frozen on dry ice, and stored at −80 °C until use. To stimulate cells with Wnt3a CM, cells were incubated in serum-free media for 1 h prior to the addition of the media over the time course of 0.5, 1, 3, and 5 h at 37 °C. Stimulated cells were quickly washed with ice-cold phosphate-buffered saline and lysed for use in immunoprecipitations and Western blotting.

**Co-immunoprecipitation Assay—**In the overexpression system, 293T cells were split and plated at 1 × 10⁶ cells per 100-mm dish and cultured with Dulbecco’s modified Eagle’s medium supplemented with 10% FBS for 24 h. Constructs encoding Myc-tagged Dvl-1 and HA-tagged Axin-1 (generously provided by Dr. Dianqing Wu, University of Connecticut Health Center) (25, 26) were co-transfected with constructs encoding FLAG-tagged Smads into 293T cells using Tfx-20 according to the manufacturer’s instructions (Promega) (27). The empty vector of pCDNA3 was a control plasmid. Thirty six hours after transfection, the cells were treated with either Wnt3a CM alone or together with 100 ng/ml BMP-2 (Sigma) over the same time course (0.5, 1, 3, and 5 h). The cells were then lysed in Triton X-100 buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 50 mM Tris buffer, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). For immunoprecipitations, 1 μg of anti-FLAG M2 monoclonal antibody (Sigma) was added into 1 ml (1 μg/ml) of cell lysate and rotated at 4 °C overnight. The protein complexes were immunoprecipitated by protein G- Sepharose beads (Amersham Biosciences), and the samples were loaded and run on a 10% SDS-polyacrylamide gel. The precipitates were transferred to a nitrocellulose membrane and immunoblotted with anti-HA or anti-Myc monoclonal antibodies (Upstate). For interference with BMP-2 signaling, ST2 cells were transfected with the Myc-tagged Dvl-1 construct together with a construct encoding FLAG-tagged Smad1¹⁵ mutant protein. The Smad1¹⁵ mutant contains a replacement of four amino acids in the COOH-terminal (SSVS into AAVA) in exon 7 that cannot be phosphorylated in response to BMP-2 (28). Forty eight hours after transfection, the cells were harvested, and immunoprecipitations were performed as described above. For endogenous immunoprecipitations, 1 μg of anti-Smad1 monoclonal antibody or purified mouse IgG1 (Santa Cruz Biotechnology) was added to 1 ml (1 μg/ml) of cell lysate and rotated at 4 °C overnight. The protein complexes were immunoprecipitated by protein G-Sepharose beads (29–31). The precipitates were loaded onto 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-Dvl-1 polyclonal antibody (Upstate).

**Immunoblotting—**Cell lysates and immunoprecipitates were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed using an anti-β-catenin monoclonal antibody (BD Transduction Laboratories). The membranes were then incubated with horseradish peroxidase-linked secondary anti-mouse antibodies, and bound antibodies were visualized using the Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

**Immunofluorescence and Confocal Microscopy—**293T cells growing on glass coverslips placed in 6-well plates were co-transfected with a Myc-tagged Dvl-1 expression plasmid, a FLAG-tagged Smad1 expression plasmid, or an empty vector of pCDNA3. Thirty six hours after transfection, cells were treated with Wnt3a CM alone, 100 ng/ml BMP-2 alone, both Wnt3a CM and BMP-2, or vehicle over the same time course as above. After culturing and treatment, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 for 2 min, and incubated overnight at 4 °C with mouse anti-β-catenin monoclonal antibody (1:50; BD Transduction Laboratories). After washing, coverslips were incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes) for 1 h and mounted with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories) (32). Fluorescence localization was detected by confocal microscopy with a laser scanning microscope (Carl Zeiss LSM-510). Images were obtained with the same confocal settings for each set of experiments, and no autofluorescence was detected at these settings. Controls in which primary or secondary antibodies were omitted showed no staining. Images were processed using Adobe Photoshop software. The number of positive nuclei were counted in 15 randomly selected microscope fields (30 cells per field) per sample.

**Transfection and Luciferase Assay—**To identify β-catenin transcriptional activity, ST2 cells were split and plated at 5 × 10⁶ cells per 24-well plate and cultured with α-minimum Eagle’s medium supplemented with 10% FBS for 24 h. The cells were transfected with either 200 ng of empty vector pCDNA3 or 200 ng of Lef1 reporter construct (generously provided by Dr. Dianqing Wu, University of Connecticut Health Center), which is a reporter for measuring β-catenin transcriptional activity in a canonical Wnt signaling pathway (26). In some experiments, cells were also transfected with 100 ng of constructs encoding Myc-tagged Dvl-1, the FLAG-tagged Smad1¹⁵ mutant, and 200 ng of Lef1 reporter plasmid DNA. Twenty four hours after transfection, the aliquots of cells were treated with Wnt3a CM alone, 100 ng/ml BMP-2 alone, both Wnt3a CM and BMP-2, or vehicle. Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase™ assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was assayed and normalized against Renilla luciferase activity (31). Luciferase values shown in the figures are representative of transfection experiments performed in triplicate from at least three independent experiments.

**In Vitro Binding Assay—**The binding assay was modified and performed as outlined previously (33–35). Briefly, 293T cells were transfected with construct encoding Myc-tagged Dvl-1. Forty eight hours later, the cells were lysed in Triton X-100 buffer. Cell lysates were cleared by centrifugation at 16,000 × g for 15 min, and the supernatants were incubated with anti-Myc antibody at 4 °C overnight. The protein complexes were immunoprecipitated by protein G-Sepharose beads. After washing the beads four times with Triton X-100 buffer, 1 μg of GST-Smad1 full-length protein or each GST-Smad1 mutant protein (27) was added to the beads in 1 ml of binding buffer (Triton X-100 buffer). The mixture was incubated for 4 h at 4 °C. Complexes were washed four times with the binding buffer, and proteins were extracted by heating at 100 °C for 5 min in 20 μl of the sample buffer. The amount of GST-Smad1 full-length protein or each GST-Smad1 mutant protein present in the binding assay was determined by Western blotting using horseradish peroxidase-labeled rabbit anti-GST polyclonal antibody (GeneTex).
ATP-lite Assay—1 × 10^4 ST2 cells were seeded into 96-well plates and cultured overnight. Wnt3a CM, 100 ng/ml of BMP-2, both, or L cell CM as a control were added, and the cells were harvested 48 h later for the proliferation assay using the ATP-lite assay kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions (36, 37).

BrdUrd Incorporation Assay—ST2 cells (8 × 10^4) and primary BMSCs were seeded into 6-well plates and cultured with α-minimum Eagle’s medium supplemented with 1% FBS overnight. Cells were then treated with Wnt3a CM, or 100 ng/ml BMP-2 individually or with both agents. After 36 h, BrdUrd (1 μM) was added to the medium, and cells were stained 48 h later using a BrdUrd assay kit (Pharmingen). Samples were analyzed by flow cytometry for fluorescence on a FACScan (38).

Statistical Analysis—Statistical significance was determined using the Student’s t test. A p value of less than 0.05 was considered significant.

RESULTS

BMP-2 Attenuates Wnt3a-stimulated Proliferation of BMSCs—Previous studies have shown that BMP-2 and Wnts participate in coordinating bone mesenchymal cell proliferation and differentiation (1, 22, 39, 40). In most cases, Wnt signaling through the canonical pathway promotes proliferation of pluripotent BMSCs and retards further differentiation, whereas BMP-2 signaling stimulates progression of the osteoblast lineage (1, 24, 40–42). This fact suggests the existence of a mechanism through which BMP-2 might restrict canonical Wnt signaling. To explore this idea further, we determined the effects of Wnt3a CM and BMP-2 alone or in combination on proliferation of ST2 or primary mouse BMSCs. As shown in Fig. 1, A and B, Wnt3a significantly

FIGURE 1. BMP-2 inhibits Wnt3a-induced proliferation. ST2 cells (A and B) and primary mouse BMSCs (C and D) were treated with either Wnt3a CM, BMP-2, both, or L cell CM as a negative control (NC). Forty eight hours later, proliferation assays were performed using the ATP-lite assay. BrdUrd labeling was performed as described under “Experimental Procedures.”

FIGURE 2. BMP-2 attenuates Wnt3a-induced β-catenin activation in ST2 cells. A, BMP-2 inhibits Wnt3a-induced β-catenin accumulation. ST2 cells were treated with either Wnt3a CM, BMP-2, both, or L cell CM as a negative control (NC) for 24 h. Total β-catenin levels were examined by Western blotting. B, BMP-2 suppresses β-catenin transcriptional activity. ST2 cells were transfected with Lef1 reporter plasmid DNA for 24 h and then treated with either Wnt3a CM, BMP-2, both, or L cell CM as a negative control (NC) for an additional 24 h. Luciferase activities were measured using the Dual-Luciferase™ assay kit. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Wnt-BMP Cross-talk in Bone Marrow Cells

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increased cell proliferation compared with controls, whereas the combination of Wnt3a and BMP-2 had an inhibitory effect on proliferation of ST2 cells compared with treatment with Wnt3a CM alone. Similar effects were seen in primary mouse BMSCs treated with Wnt3a and BMP-2 (Fig. 1, C and D).

**BMP-2 Inhibits Wnt3a-induced β-Catenin Accumulation and Activity**—To begin to investigate the mechanisms of the inhibitory action of BMP-2 on Wnt3a-induced proliferation, we determined the effect of BMP-2 treatment on Wnt3a-induced β-catenin accumulation and transcriptional activity in ST2 cells. Treatment of cells with Wnt3a CM alone increased β-catenin accumulation (Fig. 2A) and stimulated the activity of a Lef1 reporter construct (Fig. 2B) in ST2 cells. Addition of BMP-2 largely blocked Wnt3a-induced β-catenin accumulation and reporter activity indicating that BMP-2 inhibits Wnt actions by attenuating acute canonical signaling.

Dvl-1 Forms a Complex with Smad1—We reasoned that BMP might exert its inhibitory activity by promoting interactions between Smads and signaling molecules downstream of the Wnt receptors, Frizzled, and low density lipoprotein receptor-related proteins. To explore this hypothesis, we tested for interactions between Smad1 and proteins that control β-catenin phosphorylation using co-immunoprecipitation assays. Constructs encoding Myc-tagged Dvl-1 or HA-tagged Axin-1 and FLAG-tagged Smad1 were co-transfected into 293T cells, and extracts were subjected to immunoprecipitation with an anti-FLAG monoclonal antibody followed by immunoblotting with an anti-Myc or anti-HA monoclonal antibody (Fig. 3). Dvl-1 and Smad1 were consistently co-immunoprecipitated (Fig. 3A). By contrast, no specific interactions were observed between Smad1 and Axin (Fig. 3B) or β-catenin (data not shown).

To determine the specificity of the Smad1 association with Dvl-1, we assessed complex formation of Myc-tagged Dvl-1 with various other FLAG-tagged BMP and TGF-β-specific R-Smads following transfection into 293T cells. As shown in Fig. 4A, no specific complexes were detected with TGF-β specific R-Smad2 or -3, whereas BMP-specific R-Smad8 showed a weak interaction with Dvl-1. To investigate whether endogenous Dvl-1 and Smad1 formed a complex in BMSCs, extracts were prepared from both ST2 cells and primary BMSC, and immunoprecipitated with an anti-Smad1 antibody followed by immunoblotting with the anti-Dvl-1 antibody. Under these conditions, endogenous Dvl-1 and Smad1 were co-immunoprecipitated (Fig. 4, B and C).

Dvl-1 Directly Binds Smad1—Smad1 consists of three distinct domains as follows: two highly conserved NH2- and COOH-terminal domains, referred to as MH1 and MH2 (mad homology 1 and 2) respectively, and a more divergent intervening linker region (27, 28). To identify the region in Smad1 responsible for binding to Dvl-1, we created individual GST-Smad1 mutant fusion constructs encoding different functional domains of Smad1, and we compared their binding to Dvl-1 using an in vitro binding assay (Fig. 5). As shown in Fig. 5, Myc-tagged Dvl-1 purified from transfected 293T cells bound to the full-length Smad1 protein and to the linker region. Smad1 MH1 and MH2 mutants lacking the linker region bound minimally or not at all.

**Wnt Stimulation Disrupts the Dvl-1-Smad1 Complex and Promotes Nuclear Accumulation of β-Catenin**—Binding of Wnt to its cognate receptors Frizzled/LRP5/6 induces phosphorylation of Dvl which then inhibits the activity of GSK-3β and stabilizes β-catenin (43). To determine the effect of activation of the Wnt signaling pathway on formation of the Dvl-1-Smad1 complex, we treated 293T cells expressing Myc-
tagged Dvl-1 and FLAG-tagged Smad1 with Wnt3a CM. Consistent with the results described above, under basal conditions, Dvl-1 and Smad1 were co-immunoprecipitated. Exposure of cells to Wnt3a CM caused an acute and rapid disruption of the Dvl-1/Smad1 interaction with reassembly occurring within 5 h (Fig. 6A). These changes coincided temporally with accumulation of total cellular β-catenin as deter-
FIGURE 7. Combined effects of BMP-2 and Wnt3a on the Dvl-1/Smad1 interaction and β-catenin accumulation in 293T cells. Constructs encoding Myc-tagged Dvl-1 and FLAG-tagged Smad1 were co-transfected into 293T cells. Thirty six hours after transfection, cells were treated with both Wnt3a CM and BMP-2 for 0, 0.5, 1, 3, and 5 h. A, cell lysates were immunoprecipitated (IP) using an anti-FLAG M2 monoclonal antibody. The immunocomplex was examined by Western blotting using an anti-Myc monoclonal antibody. B, total β-catenin levels in the same lysates were determined by Western blotting with a β-catenin monoclonal antibody. C, the percentage of β-catenin-positive nuclei was quantitated using a fluorescence microscope as described under “Experimental Procedures.” Data from three separate experiments is presented as mean ± S.E. D, nuclear accumulation of β-catenin was assessed by confocal microscopy.

minded by immunoblotting (Fig. 6B) and increased β-catenin in the nucleus assessed by staining with anti-β-catenin antibody (Fig. 6, C and D). These data indicate that activation of Wnt signaling by Wnt3a disrupts the Dvl-1-Smad1 complex allowing accumulation and nuclear translocation of β-catenin. By contrast, when cells were treated with both Wnt3a and BMP-2, there was an enhanced Dvl-1-Smad1 complex (Fig. 7A) over the first 3 h. This interaction was accompanied by decreased accumulation of β-catenin in the total cell extract and a decrease in nuclear accumulation as determined by confocal microscopy. At 5 h, levels of the Dvl-1-Smad1 complex had declined. This observation was associated with increased accumulation of β-catenin in whole cell extracts (Fig. 7B) and its accumulation in the nucleus (Fig. 7, C and D).

Disruption of the Dvl-1-Smad1 Complex Relieves BMP-2 Inhibition of Wnt3a-induced β-Catenin Accumulation and Activity—To investigate more directly the importance of the Smad1-Dvl-1 interaction to BMP-2 inhibition of Wnt actions, we determined the effect of introduction of a mutant Smad1 molecule that cannot be phosphorylated by BMP-2 (Fig. 8A). A construct encoding FLAG-tagged Smad1* mutant, co-expressed with Myc-tagged Dvl-1, failed to yield a Smad1-Dvl-1 complex in ST2 cells (Fig. 8B). Furthermore, in ST2 cells expressing the FLAG-tagged Smad1* and Myc-tagged Dvl-1, BMP-2 treatment failed to block Wnt3a-induced β-catenin accumulation (Fig. 8C) and Lef1 reporter activity (Fig. 8D). These data indicate that Smad1 phosphorylation by BMP-2 is required for BMP-2 inhibition of Wnt actions in ST2 cells.

DISCUSSION

In this study, we characterized a mechanism that couples Wnt and BMP signaling pathways in uncommitted BMSCs. Previous studies have shown that Wnt signaling promotes mesenchymal stem cell renewal by elevating β-catenin, which activates genes responsible for proliferation (43–45). By contrast, BMP-2/4 signaling is believed to promote stem cell differentiation and to stabilize lineage progression during mesenchymal stem cell development (21, 22, 46). This type of relationship suggested that Wnt and BMP signaling pathways might be functionally coupled to allow the effects of each factor to be coordinated during mesenchymal stem cell development. In accord with this concept, we found that BMP-2 inhibited Wnt3a-mediated proliferation in BMSCs, which has been reported previously in different mesenchymal stromal cells. For example, inhibition of BMP-2/4 signaling in the intestine by overexpressing the BMP antagonist Noggin results in increased polyp formation in association with increased β-catenin expression (44). This observation suggests that BMP/Smad signals normally antagonize the effect of Wnt on intestinal stem cell growth. Similarly, BMP-2 signaling in neural crest stem cells suppresses β-catenin-dependent sensory neurogenesis (47). In addition, in C3H10T1/2 cells, BMP-2 partially reverses Wnt3a-induced inhibition of osteocalcin and osteopontin gene expression (22). Taken together, these observations support a general paradigm in which BMP signaling serves to attenuate Wnt-mediated signals required for proliferation and self-renewal in a number of different cell types.

Previous studies have demonstrated interactions between signaling components of the canonical Wnt pathway and Smads on a number of levels. For example, Axin, the scaffold protein that regulates β-catenin phosphorylation, binds to Smad3 and facilitates TGF-β signaling (48). In addition, Dvl-1 has been identified in complexes with Smad3 in yeast two-hybrid assays (49). Other mechanisms may exist to couple Wnt and BMP pathways at the transcriptional level. As already mentioned, in
**Xenopus**, BMP and Wnt synergistically activate the Wnt target gene Xtwn through the formation of a complex between Smad4 and Lef/Tcf (17). The mechanisms described in this study are distinct from the ones mentioned above, but clearly additional interactions could be operative in the BMSCs that would participate in the interaction between the Wnt and BMP signaling pathways.

Analysis of Smad1 constructs encoding different regions of the molecule using *in vitro* binding assays revealed that Dvl-1 bound preferen-

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**FIGURE 8.** Disruption of the Dvl-1-Smad1 complex relieves BMP-2 inhibition of Wnt3a-induced β-catenin accumulation and activity. A, diagram illustrating the Smad1*Δ* mutant construct (28). B, ST2 cells were transfected with the constructs encoding Myc-tagged Dvl-1 and FLAG-tagged Smad1*Δ* mutant or FLAG-tagged Smad1 (as a positive control). Forty eight hours later, cell lysates were immunoprecipitated (IP) using an anti-FLAG M2 monoclonal antibody. The immunocomplex was examined by Western blotting using an anti-Myc monoclonal antibody. C, ST2 cells were transfected with constructs encoding Myc-tagged Dvl-1 and the FLAG-tagged Smad1*Δ* mutant. Twenty four hours later, the cells were treated with either Wnt3a CM, BMP-2, both, or L cell CM as a negative control (NC) for an additional 24 h. Total β-catenin levels were examined by Western blotting. D, ST2 cells were transfected with the constructs encoding Myc-tagged Dvl-1, FLAG-tagged Smad1*Δ* mutant, and Lef1 reporter for 24 h and then treated with either Wnt3a CM, BMP-2, both, or L cell CM as a negative control (NC) for an additional 24 h. Luciferase activities were measured using the Dual-Luciferase™ assay kit as described under “Experimental Procedures.” GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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**FIGURE 9.** Proposed model for Wnt3a and BMP-2 cross-talk in BMSCs. *Unstimulated*, under basal conditions, Dvl-1 interacts with Smad1; β-catenin is phosphorylated by GSK-3β and then degraded by the proteasome. *Wnt3a*, in the presence of Wnt3a, Dvl-1 is activated, separates from Smad1, and inhibits GSK-3β. β-Catenin accumulates in the cytoplasm, translocates to the nucleus, and activates transcription of Lef/Tcf target genes. *Wnt3a and BMP-2*, in the presence of both Wnt3a and BMP-2, Dvl-1 and Smad1 are activated and form a complex that inhibits Wnt signaling through the canonical pathway. Fz, Frizzled; LRP, low density lipoprotein receptor proteins.
entially to mutants containing MH1 and linker domains. Smad1 consists of three distinct domains as follows: two highly conserved NH2- and COOH-terminal domains, referred to as MH1 and MH2, respectively, and a more divergent intervening linker region. In the inactive state, MH1 and MH2 bind to one another, mutually inhibiting the function of each domain. In the active state, the phosphorylation of Smad1 opens up this structure to allow association with Smad4 or other DNA-binding proteins via the MH2 domain (50–52). Previous studies have shown that cross-talk between Smad1 and the mitogen-activated protein kinase signaling pathways is conferred by the linker region (28). In addition, the MH1 and the linker domains are required for interaction between Smad1 and Hoxc-8 (27). Our demonstration that the phosphorylation-defective Smad1c mutant protein failed to form a complex with Dvl-1 and relieved BMP-2 inhibition of Wnt3a-induced β-catenin-mediated transcriptional activity suggests this region is also critical for cross-talk between BMP and Wnt signaling pathways.

The close temporal association between the Dvl-1-Smad1 complex formation and the changes in nuclear accumulation of β-catenin suggest a model in which BMP-2 would restrict in time the activity generated by canonical Wnt signaling (Fig. 9). Thus, under basal conditions, Dvl-1 and Smad1 form a complex that is acutely disrupted by addition of Wnt3a. It is known that binding of Wnt to Frizzled/LRP5/6 induces activation of Dvl by phosphorylation, which then inhibits the activity of GSK-3β and stabilizes β-catenin (43, 53). In the presence of Wnt3a, Dvl-1 is activated and would separate from Smad1. Activated Dvl-1 can then inhibit GSK-3β. The β-catenin accumulates in the cytoplasm and then translocates to the nucleus, combining with transcription factors Lef/Tcf to activate target genes. Consistent with this model, we showed that exposure of cells to Wnt3a CM caused an acute and rapid disruption of the Dvl-1-Smad1 interaction, and these changes coincided temporally with accumulation of total cellular β-catenin and increased β-catenin in the nucleus. These effects are transient, however, such that reassembly of the Dvl-1-Smad1 complex may serve to restrict further signal flow through the β-catenin. Such a model would enable BMP-2 to temporally release cells from Wnt-induced proliferative effects while allowing BMP-2 to exert effects on cell differentiation.

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