Smad5 and DPC4 Are Key Molecules in Mediating BMP-2-induced Osteoblastic Differentiation of the Pluripotent Mesenchymal Precursor Cell Line C2C12*

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Since the bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily that induce the differentiation of mesenchymal precursor cells into the osteogenic cells, we identified the relevant signaling molecules responsible for mediating BMP-2 effects on mesenchymal precursor cells. BMP-2 induces osteoblastic differentiation of the pluripotent mesenchymal cell line C2C12 by increasing alkaline phosphatase activity and osteocalcin production. As recent studies have demonstrated that cytoplasmic Smad proteins are involved in TGF-β superfamily signaling, we plan to isolate the relevant Smad family members involved in osteoblastic differentiation. We identified human Smad5, which is highly homologous to Smad1. BMP-2 caused serine phosphorylation of Smad5 as well as Smad1. In contrast, TGF-β failed to cause serine phosphorylation of Smad1 and Smad5. We found Smad5 is directly activated by BMP type Ia or Ib receptors through physical association with these receptors. Following phosphorylation, Smad5 bound to DPC4, another Smad family member, and the complex was translocated to the nucleus. Overexpression of point-mutated Smad5 (G419S) or a C-terminal deletion mutant DPC4 (DPC4ΔC) blocked the induction of alkaline phosphatase activity, osteocalcin production, and Smad5-DPC4 signaling cascades upon BMP-2 treatment in C2C12 cells. These data suggest that activation of Smad5 and subsequent Smad5-DPC4 complex formation are key steps in the BMP signaling pathway, which mediates BMP-2-induced osteoblastic differentiation of the C2C12 mesenchymal cells.

The bone morphogenetic proteins (BMPs)1 are members of the transforming growth factor-β (TGF-β) superfamily which have been implicated in embryogenesis, organogenesis, and morphogenesis (1). Among BMPs, BMP-2 and BMP-4 have been shown to promote the development of bone and cartilage by inducing the differentiation of undifferentiated mesenchymal cells into the osteoblastic cells or cartilage cells, respectively (2). BMP-2 has been shown to induce ectopic bone or cartilage formation when implanted in muscular tissue in vivo (2, 3) and stimulate osteoblastic differentiation of the mesenchymal cells in vitro as assessed by the stimulation of calcification, alkaline phosphatase (ALP) expression, or osteocalcin production (4, 5). However, the molecular mechanisms responsible for the effects of BMP on differentiation of these cells toward bone and cartilage are poorly understood.

BMPs exert their diverse biological effects through two types of transmembrane receptors, BMP type I (BMPIR) and type II (BMPRII) receptors (6, 7), which possess intrinsic serine/threonine kinase activity (6, 7). BMPIR is further subclassified into BMP type IaR (also called ALK3) and IbR (also called ALK6) (6, 7), but their functional difference in BMP signaling is unknown at the present time. Upon binding to the type II receptors, BMPs induce heterodimerization between BMP type I and type II receptors, and transduce signals into the cytoplasm (6, 7). Recent studies have shown that cytoplasmic signaling molecules, including Mad (mother against dpp), the Xenopus homologue of Mad, Xmad1, Xmad2, and several human homologues of Mad, Smads, play critical roles in TGF-β superfamily signaling (7–9). To date, seven Smad family members that possess ligand selectivity have been identified (7, 8). For example, Smad1 has been implicated in BMP responses (10–12), whereas Smad3 (13, 14) and Smad4 (15, 16) are activated upon treatment with TGF-β. DPC4 (Smad4) was initially found to be a tumor suppressor in pancreatic cancers (17). DPC4 is not phosphorylated but forms a complex with Smad1, Smad2, and Smad3 upon BMP or TGF-β treatment (18, 19). Thus, DPC4 may have unique roles in TGF-β superfamily signaling. Interestingly, Smad6 (20) and Smad7 (20, 21) are found to possess unique structures compared with other Smad family members and both Smad6 and Smad7 inhibit TGF-β effects. Despite these data, it is not known whether these Smad family members are specific for these growth factors, or whether the growth factors may utilize different Smad family members for different biological effects. Furthermore, considering the multifunctional properties of the BMPs, it is possible that there are still unidentified Smad family members.

In the present study, we first sought Smad family members transforming growth factor-β (TGF-β) superfamily signal-
that might be involved in BMP-induced osteoblastic differentiation of pluripotent mesenchymal precursor cells. We isolated several human Smad family members including Smad5. Because the functional roles of Smad5 in TGF-β superfamily signaling have not been fully characterized as yet, we focused our efforts on Smad5 and found that Smad5 was directly activated by BMP type Ia or Ib receptors upon BMP-2 stimulation and transduced BMP-2 signals to the nucleus by forming a complex with DPC4. Moreover, we demonstrate that Smad5 and DPC4 play a critical role in the induction of the osteoblastic differentiation of the pluripotent mesenchymal cells C2C12 by BMP-2.

MATERIALS AND METHODS

Cells and Antibodies—293 cells, L6 cells and C2C12 cells were cultured in DMEM containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). NuMuG cells were purchased from ATCC and cultured in DMEM containing 10% FBS and 10 μg/ml insulin (Sigma). Anti-HA polyclonal antibody, anti-phosphotyrosine monoclonal antibody, and anti-Flag monoclonal antibody were purchased from BAbCO, Transduction Laboratories, and IBI, respectively. Anti-phosphoserine and anti-phosphothreonine polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Abbott Park, IL).

cDNA Cloning—Human homologues of Mad cDNA were isolated using the PCR-nested cloning approach. Degenerate oligonucleotide primers for PCR were designed based on the conserved region of Drosophila Mad (22, 23) and Caenorhabditis elegans Sma-2 (24) proteins (forward primer; 5′-CA/C/CTAT/AT/GCTGGAA/G/AAGGGGT-3′ encoding HIGKGV; reverse primer; 5′-TGAG/GAT/CT/TAT/CTATCCAA/A (G/C)GNGCT-3′, encoding TPCWIEIH). After PCR amplification, predicted size PCR products were subcloned into TA cloning vector (A/G)CANGGNGT-3′ encoding HIGKGV; reverse primer; 5′-99-39).

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FIG. 1. DNA and amino acid sequence of human Smad5. Mad homology domain 1 (MH1) and Mad homology domain 2 (MH2) are shown as underlined and bold, respectively (GenBank™ accession number U73825).

with horseradish peroxidase coupled to protein A (KPL) or horseradish peroxidase-coupled anti-mouse IgG antibodies (Cappel), and enhanced by ECL detection kits (Amersham).

For 32P metabolic labeling, serum-starved cells were incubated with 0.5 mCi/ml [32P] orthophosphate for 15 min. The lysates of cells treated with or without 100 ng/ml BMP-2 or 10 ng/ml TGF-β for 15 min were immunoprecipitated with anti-Flag antibody and subjected to SDS-PAGE, followed by autoradiography.

Immunofluorescent Staining—The cells were serum-starved with DMEM containing 0.2% FBS for 16 h, and treated with 100 ng/ml BMP-2 for 40 min. The cells were washed three times with ice-cold PBS and fixed with 3.8% paraformaldehyde-PBS. The number of cells whose nucleus was stained by anti-Flag antibody was counted per hundred cells at four independent fields. The cells whose nucleus was stained by anti-Flag antibody was counted per hundred fields at four independent fields.

GST Fusion Proteins and in Vitro Binding Assay—Smad5 and DPC4 cDNA were subcloned into pGEX-2T (Pharmacia Biotech Inc.) frame. GST fusion proteins were expressed and purified by glutathione-agarose beads and were used as described.

In Vitro Assay for Smad5—BMIPIb or TGF-βRII was immu-
FIG. 2. A, Smad5 is phosphorylated by BMP-2, but not by TGF-β. Wild-type (WT) (lanes 1, 2, 5, and 6) or mutant (G419S) (lanes 3 and 4) of Flag-epitope-tagged Smad5 was co-transfected with BMPⅢR and BMPⅢRI (lanes 1–4) or TGF-βRI and TGF-βRII (lanes 5 and 6) into 293 cells. Cells were metabolically labeled with 32P for 3 h, and stimulated with 100 ng/ml BMP-2 (lanes 2 and 4) or 10 ng/ml TGF-β (lane 6) for 15 min. The cell lysates were immunoprecipitated with anti-Flag antibody (IBI) and subjected to SDS-PAGE followed by autoradiography. Expression levels of Flag-Smad5 in the lysates from unlabeled cultures prepared in parallel were determined by Western blotting using anti-Flag antibody (bottom). Note slower migration in BMP-2-treated groups due to hyperphosphorylation in 293 cells.

B, Smad5 is serine-phosphorylated by BMP-2, but mutant Smad5 (G419S) is not. Wild-type (WT) (lanes 1, 2, and 5–8) or mutant (G419S) (lanes 3 and 4) Flag-Smad5 was co-transfected with BMPⅢR and BMPⅢRI into 293 cells. Cells were stimulated with 100 ng/ml BMP-2 (lanes 2, 4, 6, and 8) for 15 min, lysed, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-phosphoserine (α-pSerine, lanes 1–4), anti-phosphotyrosine (α-pTyr, lanes 5 and 6), or anti-phosphothreonine antibody (α-pThr, lanes 7 and 8). Expression levels of Flag-Smad5 in the lysates were determined by Western blotting using anti-Flag antibody (bottom).

C, Smad5 is phosphorylated by BMP-2 treatment, but not by TGF-β treatment in NMuMg cells. Flag-Smad1 (lanes 1–3), Flag-Smad5 (lanes 4–6), or Flag-Smad3 (lanes 7–9) was co-transfected with BMPⅢR and BMPⅢRII into NMuMg cells. Cells were stimulated with 100 ng/ml BMP-2 (lanes 3, 6, and 9) or 20 ng/ml TGF-β (lanes 2, 5, and 8) for 15 min, lysed, immunoprecipitated with anti-Flag antibody (IBI), and subjected to SDS-PAGE followed by autoradiography. Expression levels of Flag-Smad1, Flag-Smad5, and Flag-Smad3 in the lysates from unlabeled cultures prepared in parallel were determined by Western blotting using anti-Flag antibody (bottom). D, Smad5 is serine-phosphorylated by constitutively active BMPⅢaR (233D) and BMPⅢbR (203D), but not by constitutively active TGF-βRI (204D). 293 cells were transfected together.
**RESULTS**

**Isolation of Human Smad5**—To identify Smad-related genes, human Smad genes were isolated by cDNA cloning from 293 cells overexpressing HA-tagged BMPIbR or BMPIbR and BMPRII, and stimulated with wild-type (WT) BMP-2 (lanes 1 and 2) or mutant (G419S) BMP-2 (lanes 3 and 4) for 15 min. The cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-HA antibody. Expression levels of Flag-Smad5 or HA-BMPIbR in the lysates were determined by Western blotting using anti-Flag or anti-HA antibody. D, wild-type BMPIbR (lanes 1 and 2) or BMPIbR (lanes 3 and 4) was co-transfected into 293 cells with BMPRII. Cells were stimulated with 100 ng/ml BMP-2 (lanes 2 and 4) for 15 min, lysed, and incubated with GST-Smad5, and the complexes were determined by immunoblotting with anti-HA antibody. The difference in the position of two bands is due to different molecular sizes for BMP-2 and BMP-2. C, wild-type BMPIbR (lanes 1 and 2), GST-Smad5 (lanes 3 and 4), or GST-DPC4 (lanes 5 and 6), and the complexes were visualized by immunoblotting with anti-HA antibody. D, MH2 domain of Smad5 is required for binding to BMPIbR. 293 cells were transfected with HA-tagged BMPIbR and BMPRII, and stimulated with 100 ng/ml BMP-2 (lanes 2, 4, and 6) for 15 min. The cell lysates were incubated with GST (lanes 1 and 2), GST-Smad5 (lanes 3 and 4), or GST-DPC4 (lanes 5 and 6), and the complexes were visualized by immunoblotting with anti-HA antibody. E, GST-Smad5 associates with activated BMPIbR. The supernatants were collected, boiled in the presence of SDS-sample buffer at 95 °C for 5 min, and subjected onto SDS-PAGE, followed by autoradiography.

**Isolation of Smad-related Genes**—Using PCR technique. We designed de-generated primers based on conserved region of Mad (22, 23) and Sma-2 (24). We isolated several human cDNA clones, which encode Smad-related genes including Smad3 (15, 16) and DPC4 (Smad4; Ref. 17) from a human 293 cell cDNA library. One of clones was approximately 90% homologous to the human Smad1 (10–12) and identical to dwarfin-C, which has been cloned previously and shown to mediate TGF-β superfamily signaling (28). A partial sequence of this clone was also reported as JV5–1 (29). According to the nomenclature for Smad family (30), we named the clone Smad5. Human Smad5 cDNA encodes 465 amino acids containing highly conserved MH1 and MH2 domains, which are separated by a proline-rich linker domain (Fig. 1). Smad5 is less homologous to other Smad family members, including Smad2 (13, 14) (55%), Smad3 (15, 16) (60%), and DPC4 (17) (31%) than Smad1.

**Specificity of Smad5 Responsiveness for BMP-2**—Since Smad5 shows high homology to Smad1 that is phosphorylated with wild-type (WT) (lanes 1, 2, 3, 4, 6, 9, and 10) or mutant (G419S) (lanes 3, 4, 7, and 8) Flag-Smad5 and wild-type BMP1aR (lanes 1 and 3), constitutively active BMP1aR (233D) (lanes 2 and 4), wild-type BMP1bR (lanes 5 and 7), constitutively active BMP1bR (203D) (lanes 6 and 8), wild-type BMP-2 (lane 9), or constitutively active TGF-βRII (204D) (lane 10). The cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-phosphoserine antibody. Expression levels of Flag-Smad5 and HA-BMP or TGF-β type I receptor in the lysates were determined by Western blotting using anti-Flag antibody (middle), or anti-HA antibody (bottom), respectively. E, GST-Smad5 is phosphorylated by BMP1bR, but not by TGF-βRII in vitro. GST-Smad5 (lanes 1 and 2) or GST-DPC4 (lanes 3 and 4) was incubated with BMP1bR (lanes 1 and 3) or TGF-βRII (lanes 2 and 4) immuno precipitates in the presence of MnCl2 and 1 μCi of [γ-32P]ATP at 28 °C for 20 min. The supernatants were determined by SDS-PAGE, followed by autoradiography.
by BMP-2 (10, 12), we examined whether BMP-2 also phosphorylates Smad5. To carry out the experiments, we tagged Flag-epitope (10, 11) to the N terminus of Smad5 to distinguish from other Smad proteins, and transiently expressed Flag-tagged Smad5 in 293 cells. As shown Fig. 2A, Smad5 was clearly phosphorylated by BMP-2 (lane 2). In contrast, TGF-β had no effect on the phosphorylation of Smad5 (Fig. 2A, lane 6). Western blotting analysis using antibodies to phosphoserine, phosphothreonine, and phosphotyrosine revealed that the serine residue(s) of Smad5 was phosphorylated (Fig. 2B, lane 2). Neither tyrosine nor threonine residues were phosphorylated (Fig. 2B, lanes 6 and 8). Point-mutated Smad5 in which the highly conserved 419 glycine residue was replaced by serine was not phosphorylated by BMP-2 (Fig. 2, lane 4 in A and B), as shown previously for Smad1 (10). Of note, Yingling et al. (28) have reported dwarfin-C, which is identical to Smad5, is phosphorylated by TGF-β, but not BMP-2 in NMuMG cells. Since their results are not consistent with our present data, we determined Smad5 phosphorylation in response to TGF-β or BMP-2 in NMuMG cells. As shown in Fig. 2C, BMP-2 caused Smad5 phosphorylation (lane 6), whereas TGF-β did not cause Smad5 phosphorylation in NMuMG cells (lane 5). Smad1 showed identical responsiveness to BMP-2 (lanes 2 and 3) to that of Smad5, as shown previously (10–12). On the other hand, we observed that Smad3 was not phosphorylated by BMP-2, but TGF-β clearly phosphorylated Smad3 in NMuMG cells (lanes 8 and 9).

We further confirmed the specificity of Smad5 responsiveness to BMP-2 by overexpressing constitutively active BMPIR or TGF-β type I receptors (TGF-βRI). It has been demonstrated that point mutation of BMPIR at position 233 (glutamine to aspartic acid), BMPIR at 203 (glutamine to aspartic acid), or TGF-βRI at 204 (threonine to aspartic acid) renders these receptors constitutively active without their corresponding ligands and type II receptors (10). Smad5 was serine-phosphorylated in the presence of constitutively active mutant BMPIR (233D) or BMPIR (233D) without BMP-2 stimulation (Fig. 2D, lanes 2 and 6). In contrast, Smad5 phosphorylation did not occur in the presence of constitutively active mutant TGF-βRI (204D) (Fig. 2D, lane 10). Of note, the mutant Smad5 (G419S) in which glycine 419 was replaced by serine was not phosphorylated, even in the presence of constitutively active mutant BMPIR or BMPIR (Fig. 2D, lane 8). Wild-type BMPIR or BMPIR (lanes 1 and 5) failed to serine-phosphorylate Smad5 in the absence of BMP-2. Moreover, recombinant GST-Smad5 was also phosphorylated by BMPIRs, but not by TGF-βRI in vitro (Fig. 2E), whereas GST-DPC4 was not phosphorylated by BMPIR or TGF-βRI. These data clearly show that Smad5 is a downstream signaling molecule specific for BMP in two different cell models.

Association of Smad5 with BMPIR—To further determine the interaction of Smad5 with BMPIR, we next explored whether Smad5 physically associates with BMPIRs. We performed co-immunoprecipitation experiments in 293 cells that were co-transfected with HA-BMPIR and Flag-Smad5. As shown in Fig. 3A, Smad5 was co-immunoprecipitated with BMPIR in a BMP-2-dependent manner (lanes 1 and 2). Consistent with this result obtained in living cells, GST-Smad5 associated with BMP-2-stimulated BMPIR (Fig. 3B, lanes 1 and 2) or BMPIR (Fig. 3, lanes 3 and 4 in B and C). The

FIG. 4. A, BMP-2-activated Smad5 associates with DPC4. Wild-type (WT) (lanes 1, 2, 5, and 6) or mutant (G419S) (lanes 3 and 4) Flag-Smad5 was co-transfected into 293 cells with wild-type (WT) (lanes 1–4) or C-terminal deletion mutant (DPC4ΔC) (lanes 5 and 6) HA-tagged DPC4. Cells were stimulated with (lanes 2, 4, and 6) or without BMP-2 (lanes 1, 3, and 5) lysed, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA antibody. Expression of Flag-Smad5 or HA-DPC4 was determined by anti-Flag (middle) or HA (bottom) antibody, respectively. B, BMP-2-activated Smad5 associates with GST-DPC4 in vitro. Wild-type (WT) (lanes 1, 2, 5, and 6) or mutant (G419S) (lanes 3 and 4) Flag-Smad5 was transfected into 293 cells. Cells were stimulated with (lanes 2, 4, and 6) or without BMP-2 (lanes 1, 3, and 5) and incubated with GST-DPC4 (lanes 1–4) or GST-DPC4ΔC (lanes 5 and 6), and the complexes were visualized by immunoblotting with anti-Flag antibody.

FIG. 5. Smad5 and DPC4 translocate to the nucleus upon stimulation with BMP-2. Wild-type Flag-Smad5 (A and B), mutant Flag-Smad5 (G419S) (C and D), wild-type Flag-DPC4 (E and F), or mutant Flag-DPC4(DPC4ΔC) (G and H) was co-transfected with BMPIR and BMPRII into 293 cells. Cells were stimulated with (B, D, F, and H) or without (A, C, E, and G) 100 ng/ml BMP-2 for 40 min and immunostained with anti-Flag antibody, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. Fluorescence was detected under a fluorescence microscope (Zeiss). Pictures show representative staining with anti-Flag antibody. Nuclear translocation of wild-type Flag-Smad5, mutant-Flag-Smad5 (G419S), wild-type DPC4, or mutant-DPC4 (DPC4ΔC) was observed in 85 ± 5%, 5 ± 0.6%, 78 ± 4%, or 3 ± 0.6% of cells, respectively.
difference in the position of the bands of BMPIaR and BMPIbR observed in Fig. 3B is due to the difference of molecular size of BMPIaR and BMPIbR. GST alone (Fig. 3C, lanes 1 and 2) or GST-DPC4 (lanes 5 and 6) was unable to bind to BMPIR. We also found that GST-Smad5 was phosphorylated by BMPIRs in vitro (Fig. 2E). These findings suggest that activated BMPIRs
directly phosphorylate Smad5 through physical association. Interestingly, the mutant Smad5 (G419S) that is not phosphorylated by BMP-2 was also able to bind to activated BMPbR (Fig. 3A, lane 4). The result suggests that the mutant Smad5 (G419S) may compete with the intact Smad5 for the binding to BMPIRs regardless the state of phosphorylation. We also found that GST-Smad5 lacking the MH2 domain was unable to bind to activated BMPbR (Fig. 3D, lanes 5 and 6), whereas MH1 domain-deleted GST-Smad5 still retained binding capacity (Fig. 3D, lanes 3 and 4). These data indicate that the MH2 domain is responsible for the physical association of Smad5 to BMPbR.

**Association of Activated Smad5 with DPC4—**Smad1 that has high homology with Smad5 is shown to form a heterocomplex with DPC4 (18, 19). To unravel further downstream of Smad5, we determined the interaction of Smad5 with DPC4 by co-immunoprecipitation and an *in vitro* binding assay using GST-DPC4 (25). As shown in Fig. 4A, Smad5 associated with DPC4 in a BMP-2-dependent manner (lane 2). In contrast, the mutant Smad5 (G419S) that is not phosphorylated by BMP-2 failed to bind to DPC4 (Fig. 4A, lane 4). Of note, BMP-2-activated Smad5 failed to associate with the C-terminal deletion mutant DPC4 (DPC4ΔC) (Fig. 4A, lane 6). Consistent with these results obtained in co-immunoprecipitation experiments, BMP-2-activated Smad5 also associated with GST-DPC4 (Fig. 4B, lane 2), but not with GST-DPC4ΔC (lane 6) *in vitro*. Furthermore, mutant Smad5 (G419S) was not able to bind to GST-DPC4 (lane 4). These results demonstrate that the phosphorylated Smad5 forms heterocomplex with DPC4 and suggest that the phosphorylation of Smad5 and the presence of the C terminus of DPC4 are required for heterocomplex formation between Smad5 and DPC4. Since DPC4 did not physically associate with BMPIRs (Fig. 3C, lane 6), DPC4 most likely serves as a downstream molecule of Smad5 in the BMP-2 signaling pathway.

**Translocation of Smad5 and DPC4 into Nucleus by BMP-2**—We next examined whether Smad5-DPC4 complex translocates into the nucleus, as shown in the case of Smad1 and Smad2 (11, 31). Immunofluorescent staining demonstrated Flag-Smad5 and Flag-DPC4 clearly translocated and accumulated in the nucleus as early as 40 min after BMP-2 treatment (Fig. 5, A, B, E, and F). The mutant Smad5 (G419S) or mutant DPC4 (DPC4ΔC) did not show nuclear translocation by BMP-2 treatment (Fig. 5, C, D, G, and H). These results suggest that phosphorylated Smad5, following complex formation with DPC4, translocates to the nucleus and might function as a transcription-regulating factor, as is the case of Smad2 (31).

**Role of Smad5 and DPC4 in Osteoblastic Differentiation of C2C12 Cells**—Since BMP-2 plays an important role in osteogenesis by regulating the differentiation of the undifferentiated mesenchymal cells *in vivo* (2, 3, 32) and *in vitro* (4, 5, 27), we next explored the biological role of Smad5 in a pluripotent mesenchymal cell line C2C12, which shows osteoblastic differentiation in the presence of BMP-2 (27). BMP-2 induced serine phosphorylation of Flag-Smad5 that was translocated in C2C12 cells (Fig. 6A). In conjunction with this, BMP-2 also induced a marked increase in ALP activity (Fig. 6B) and production of osteocalcin (Fig. 6C) in C2C12 cells as reported previously (27). ALP and osteocalcin are widely recognized phenotypic markers of cells of osteoblast lineage (27). Importantly, overexpression of the mutant Smad5 (G419S) in C2C12 cells strongly inhibited BMP-2-induced ALP activity (Fig. 6B) and osteocalcin production (Fig. 6C). In addition, co-transfection of the mutant Flag-Smad5 (G419S) with wild-type Flag-Smad5 into C2C12 cells abolished BMP-2-induced serine phosphorylation of wild-type Flag-Smad5, showing dominant negative effects of the mutant Smad5 (G419S) on Smad5 activation (Fig. 6D). This dominant-negative effect is probably due to a competition for the binding to BMPIR between wild type and mutant Smad5 (Fig. 3A). The data demonstrate an inhibition of Smad5 phosphorylation by dominant-negative mutant Smad5 is associated with an inhibition of BMP-2-induced osteoblastic differentiation of C2C12 cells and suggest that the phosphorylation of Smad5 is necessary for the osteoblastic differentiation of C2C12 cells induced by BMP-2.

In the same set of experiments, dominant-negative Smad1 (G419S) inhibited the osteoblastic differentiation of C2C12 cells induced by BMP-2 (Fig. 6, B and C), whereas dominant-negative Smad3 (G379S) showed no effects. The mutant Smad5 (G419S) blocked BMP-2-induced phosphorylation of Smad1, but did not affect the phosphorylation of Smad3 induced by TGF-β (Fig. 6D).

We also determined the biological role of DPC4 in the osteoblastic differentiation of C2C12 cells using Flag-DPC4ΔC, which is unable to associate with the phosphorylated Smad5 (Fig. 4, A and B). DPC4ΔC profoundly decreased BMP-2-induced ALP activity and osteocalcin production in C2C12 cells (Fig. 6, B and C). The dominant-negative effects of DPC4ΔC have been reported previously (15, 18). We found that DPC4ΔC did not affect the phosphorylation of Smad5 but instead specifically blocked the association of Smad5 with intact DPC4 (Fig. 6E). Thus, the results suggest that the heterocomplex formation of phosphorylated Smad5 with DPC4 is also essential for the osteoblastic differentiation of C2C12 cells.

**DISCUSSION**

The bone- and cartilage-inducing effects of the BMPs have been studied extensively (2,–5, 27, 32). Recent studies have markedly increased our understanding of BMP signal transduction pathways at molecular levels through identification of BMP-activated cytoplasmic signaling molecules including Smads and DPC4 (7, 8). Nevertheless, the precise role of these signaling molecules in the bone-inducing effects of BMPs is not defined to date. In this study, we isolated human Smad5 and found that Smad5 was involved in BMP-2 signaling cascades, which mediate the bone-inducing effects of BMP-2. Smad5 was directly serine-phosphorylated by BMPIR through a physical interaction. The activated Smad5 subsequently formed a complex with DPC4, and this complex was then translocated to the nucleus. Overexpression of mutant Smad5 (G419S), which inhibits the phosphorylation of intact Smad5, blocked the BMP-2-induced osteoblastic differentiation of C2C12 cells. Furthermore, suppression of the complex formation of the Smad5 with DPC4 by overexpressing DPC4ΔC also blocked BMP-2-induced osteoblastic differentiation of C2C12 cells. Thus, interruption of BMP-2 signaling cascades by inhibiting Smad5 activation or interfering with the association between Smad5 and DPC4 abolished the osteogenic effects of BMP-2 on C2C12 cells. These results strongly suggest that both activation of Smad5 and following heterocomplex formation with DPC4 are critical to the BMP-2 signaling, which mediates the induction of the osteoblastic differentiation of the pluripotent mesenchymal cells C2C12. In contrast, TGF-β did not induce the phosphorylation of Smad5 in living cells and *in vitro*, constitutively active TGF-βRI failed to phosphorylate Smad5, Smad5 was unable to bind to the activated TGF-βRI, and a previous study has shown that TGF-β fails to promote osteoblastic differentiation of C2C12 cells (27). Collectively, these results suggest that Smad5, in addition to Smad1, is an intracellular molecule specifically involved in the BMP signaling. Thus, Smad5 is a new cytoplasmic signaling molecule of human Smad family members that mediates the osteogenic effects of BMP-2.

In conflict with our data, an earlier study has reported that
mouse homologues for Smad5 and Smad1, dwarfin-C and dwarfin-A, respectively, are phosphorylated by TGF-β, but not by BMP-2 (28). To examine whether this apparent discrepancy between that study and ours is due to a difference in experimental models, we performed identical experiments to those described here in NMuMg cells that were used in the previous study. In our hands, Smad5 was selectively activated by BMP-2 and TGF-β did not activate Smad5 in NMuMg cells. It is possible that Smad1 and Smad5 antibodies used in the previous study might recognize other Smads including Smad2 and Smad3 that are responsive to TGF-β (13–16), since homology between these Smad members is high (7, 8). Indeed, the authors raised the same possibility in the report. Furthermore, we experienced that the antibody we generated against GST-Smad5 recognized other Smads including Smad1, Smad3, and DPC4. However, it still remains possible that endogenous Smad5 behaves in different manners from that of transfected exogenous Smad5 or Smad1. Resolution of this issue awaits generation of specific antibodies for Smad5.

Finally, our results suggest that there are no distinctive functional differences between Smad1 and Smad5 in BMP-2 signal transduction and BMP-2-induced osteoblastic differentiation in C2C12 cells. Dominant-negative Smad1, like dominant-negative Smad5, inhibited BMP-2-induced C2C12 differentiation into osteoblasts. Dominant-negative Smad5 interfered with Smad1 phosphorylation by BMP-2 stimulation. Whether Smad5 characterized herein has specific roles that are distinguishable from those of Smad1 in BMP-2 signaling that mediates the biological effects of BMP-2 is an important issue. However, this is beyond the scope of the present study. Antibodies that specifically recognize Smad5 and development of additional experimental models to C2C12 cells may clarify this point.

In conclusion, we demonstrate an important role of Smad5 and DPC4 in the BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal stem cell C2C12.

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