Neogenin, a Receptor for Bone Morphogenetic Proteins*

Received for publication, September 1, 2010, and in revised form, November 5, 2010. Published, JBC Papers in Press, December 13, 2010, DOI 10.1074/jbc.M110.180919

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Bone morphogenetic proteins (BMPs) regulate many mammalian physiologic and pathophysiologic processes. These proteins bind with the kinase receptors BMPR-I and BMPR-II, thereby activating Smad transcription factor. In this study, we demonstrate that neogenin, a receptor for netrins and proteins of the repulsive guidance molecule family, is a receptor for BMPs and modulates Smad signal transduction. Neogenin was found to bind directly with BMP-2, BMP-4, BMP-6, and BMP-7. Knockdown of neogenin in C2C12 cells resulted in the enhancement of the BMP-2-induced processes of osteoblastic differentiation and phosphorylation of Smad1, Smad5, and Smad8. Conversely, overexpression of neogenin in C2C12 cells suppressed these processes. Our results also indicated that BMP-induced activation of RhoA was mediated by neogenin. Inhibition of RhoA promoted BMP-2-induced processes of osteoblastic differentiation and phosphorylation of Smad1/5/8. However, treatment with Y-27632, an inhibitor of Rho-associated protein kinase, did not modulate BMP-induced phosphorylation of Smad1/5/8. Taken together, our findings suggest that neogenin negatively regulates the functions of BMP and that this effect of neogenin is mediated by the activation of RhoA.

Bone morphogenetic proteins (BMPs) are a unique family belonging to the transforming growth factor-β superfamily. BMPs regulate various processes associated with the differentiation, growth, and death of cells (1). BMPs bind to two different serine/threonine kinase receptors and mediate their signals through Smad-dependent and Smad-independent pathways (1). The biological activities of these proteins are regulated by intracellular signaling cascades triggered by various growth factors and cytokines. In particular, the functions of BMPs are known to be modulated by small GTPase Rho. Rho and one of its effectors, ROCK, participate in a variety of biological processes such as vascular contraction, tumor invas-

* This work was supported by a Grant-in-Aid for Young Scientists (S) from JSPS.

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‡ The abbreviations used are: BMP, bone morphogenetic protein; ROCK, Rho-associated protein kinase; RGM, repulsive guidance molecule; ALP, alkaline phosphatase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ECD, extracellular domain; BMPR, BMP receptor; VSV-G, vesicular stomatitis virus G protein.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were used in this study: mouse monoclonal antibodies to BMP-2 (R & D Systems, Minneapolis, MN), α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), BMP-4, RhoA, and His tag (Santa Cruz Biotechnology); rabbit polyclonal antibodies to neogenin (Santa Cruz Biotechnology), human IgG-Fc (MP Biomedicals, Irvine, CA), and vesicular stomatitis virus tag
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(Sigma-Aldrich); and rabbit monoclonal phospho-specific antibody to Smad1/5/8 (Cell Signaling Technology, Beverly, MA). Disuccinimidyl suberate was purchased from Thermo Fisher Scientific (Waltham, MA). 5’ adenosine monophosphate-activated protein kinase inhibitor compound C (Dormorphin) were obtained from Calbiochem. Y27632 was obtained from Biomol Research Laboratories (Plymouth, PA). The recombinant proteins rhBMP-2, rhBMP-4, rhBMP-6, rhBMP-7, and rmNeogenin-Fc were obtained from R & D Systems. We also used rhBMP-2 purified from Escherichia coli, which was prepared by Osteopharma Inc. in collaboration with the Department of Physiologic Chemistry II at the University of Würzburg. The mature domain of human BMP-2 was expressed in E. coli, isolated from inclusion bodies, renatured, and purified using a previously described method (12, 13).

Cell Culture and Transfection—HEK293T and C2C12 cells (both cell types from ATCC, Manassas, VA) were cultivated in DMEM (Invitrogen) supplemented with 10% (v/v) FBS and 100 mg/ml penicillin/streptomycin (Invitrogen). ST2 cells were cultivated in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FBS and 100 mg/ml penicillin/streptomycin (Invitrogen). These cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were assayed 48 h after transfection.

V-SVG-tagged human neogenin in pcDNA 3.1 was kindly gifted by Dr. Eric R. Fearon (14). The target sequence of the siRNA for mouse neogenin (Sigma-Aldrich) was described previously (11). The cells were incubated with the siRNAs for 24 h, and then the medium was replaced and incubated further for 24 h.

Ligand Binding Assay—Neogenin-transfected HEK 293T cells were starved overnight and incubated with or without 0.5 μg/ml of rhBMP-2 and rhBMP-4 in binding buffer (PBS containing 25 mM BSA) for 2 h. The cells were washed in the washing buffer (12.5 mM Hepes, 140 mM NaCl, 5 mM KCl) three times and then treated with 0.5% disuccinimidyl suberate. The cells were then incubated for 30 min on ice and then fixed for 30 min at room temperature with 4% paraformaldehyde. To detect binding of BMPs, the cells were stained with anti-BMP-2 or anti-BMP-4. The samples were examined under a fluorescence microscope (Olympus BX51, Tokyo, Japan) with 40× objectives.

Immunoprecipitation—The transfected HEK293T cells or C2C12 cells were serum-starved overnight by incubation in DMEM without FBS and stimulated with 1 μg/ml of rhBMP-2 or rhBMP-4 in binding buffer (PBS containing 25 mM BSA) for 2 h. After washing the cells in the washing buffer (12.5 mM Hepes, 140 mM NaCl, 5 mM KCl) three times, cell lysis was then carried out using a lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Hepes, pH 7.5, complete EDTA-free protease inhibitors (Roche Applied Science), and 1 mM phenylmethylsulfonyl fluoride), and immunoprecipitation was performed by treatment with 10 μg of the corresponding antibody and protein G-Sepharose beads for 3 h or overnight under centrifugation at 4 °C. After the protein precipitates were washed several times, the proteins were separated by SDS-PAGE followed by immunoblotting.

ELISA—ELISA was performed using 96-well microplates (Thermo Fisher Scientific) that were coated with 0.5 μg/ml of BSA or BMP-2, -4, -6, and -7. The wells were then incubated with rhBMP1RA-Fc (170 – 0 nm) or neogenin-Fc (170 – 0 nm) diluted in 1% BSA/PBS. Two hours after incubation, the plates were washed and diluted, and anti-IgG Fc antibody was added. HRP-conjugated secondary antibodies, the substrate reagent, and stop solution (R & D Systems) were used to detect protein binding. Absorbance was measured at 450 nm. For competition experiments using ELISA, the microplates were coated with BMP-2 (3 pm) or BSA. Then the wells were incubated with the solution that included 70 nm neogenin-Fc and BMP-2 (0 – 300 pm). The binding of neogenin-Fc to the coated BMP-2 was measured.

Affinity Precipitation of GTP-RhoA—After treatment with 100 ng/ml rhBMP-2 or control medium for 30 min, the cells were lysed in a solution containing 50 mM Tris, pH 7.5, 1% Nonidet P-40, 5% glycerol, 1 mM Na2VO4, 1 mM NaF, 150 mM NaCl, 30 mM MgCl2, 1 mM DTT, and 10 μg/ml each of leupeptin and aprotinin. The cell lysates were clarified by centrifugation (15,000 g) at 4 °C for 10 min, and the supernatants were incubated with 20 μg of Rho-binding domain of rotekin beads (15) at 4 °C for 45 min. The beads were washed four times with the lysis buffer and subjected to SDS-PAGE followed by immunoblotting with anti-RhoA antibody. The cell lysates were also immunoblotted for total RhoA. The levels of RhoA activation were calculated by comparing the band intensities of active RhoA bands with those of total RhoA in each lane using Multi Gauge software (Fuji Film Corporation, Tokyo, Japan). The values obtained were then divided by those of control, and the results were expressed as fold increases over the levels in the controls.

ALP Staining—C2C12 cells were plated onto 24-well plate at the density 1 × 105 cells/ml. At 48 h after plating, the culture media were replaced with DMEM (without FBS) containing 100 ng/ml rhBMP-2. Where indicated, 10 μM Y-27632 was added. Cultured for 3 days, the cells were fixed for 30 min with 4% formaldehyde/PBS at room temperature. After washing with PBS, nodules were stained with Western Blue stabilized substrate for ALP (Promega, Madison, WI). The results were expressed as fold increases over the levels in the controls.

Real Time RT-PCR—Approximately 800 ng of total RNA was used as a template to synthesize the first strand cDNA with specific reverse primers for the genes ALP and GAPDH, using reverse transcription. Subsequently, 2 μl of the cDNA mixture was used in a real time PCR (total volume, 20 μl) performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. In addition, the PCR products were purified and sent for sequence analysis using the 7300 real time PCR system (Applied Biosystems) to verify the amplification of the targeted genes. The optimal conditions were defined as follows: Step 1, 50 °C for 2 min; Step 2, 95 °C for 10 min; Step 3, 95 °C for 15 s and 60 °C for 1 min, repeated for 40 cycles; and Step 4, 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The relative mRNA expression of ALP was adjusted with that of GAPDH. The primer pairs used for PCR are as follows: GAPDH, up 5’-tgacgtgcgctgagagaa-3’, down 3’-agtgtgc-
ccaagatgcccttcag-5′; ALP, up 5′-cgagttctgccaaaacc-3′, down 3′-ctcatgtctccctgtctcaat-5′; and neogenin up 5′-ggaagggggaatgagacc-3′, down 3′-atgaggggaatgagacc-5′.

**Statistical Analysis**—The quantitative data are expressed as the means (S.D.) of at least three (indicated in the figure legends, when the number was more than three) independent experiments. Statistical analysis of these values was performed using one-way analysis of variance followed by Scheffe’s multiple comparison tests. *p* values of less than 0.05 were considered significant.

**RESULTS**

**BMPs Bind to Neogenin**—First, we sought to examine whether neogenin is associated with BMPs by using cell-based binding assays. HEK293T cells were transfected with neogenin or control plasmid, and the binding of rhBMP-2 (**left panels**) and rhBMP-4 (**right panels**) to the cells was assessed by immunocytochemistry. Scale bar, 100 μm. The relative luminance levels indicating the binding of rhBMP-2 or rhBMP-4 to the cells are shown in the right graph (**n = 3**). *, *p* < 0.01. **B**. Co-immunoprecipitation of neogenin with rhBMP-2 and rhBMP-4 using lysates prepared from the transfected HEK293T cells. The cells were transfected with the control plasmid or V-SVG-tagged neogenin and incubated with rhBMP-2 (**upper panels**) or rhBMP-4 (**lower panels**). In the V-SVG immunoprecipitates, the anti-BMP2 or anti-BMP4 antibody revealed the presence of the corresponding protein. **C**. BMP-2 was co-immunoprecipitated with neogenin in lysates from P7 mouse cerebral cortex. **D**. BMP-2 was co-immunoprecipitated with neogenin in lysates from P7 mouse cerebral cortex. The cells were incubated with rhBMP-2. In the anti-neogenin or anti-BMPR1a antibody immunoprecipitates, the anti-BMP2 antibody revealed the presence of the corresponding protein. **E**. BMP-2 was co-immunoprecipitated with neogenin in lysates from P7 mouse cerebral cortex. Western blot of His immunoprecipitates with the anti-BMP2 or anti-BMP4 antibody revealed the presence of the corresponding protein. cont, control; IP, immunoprecipitation. VSVG, vesicular stomatitis virus G protein.
the transfection, the cells were incubated with 100 ng/ml recombinant BMP-2 (rhBMP-2) or rhBMP-4 for 4 h, washed, and immunostained with anti-BMP-2 or anti-BMP-4 antibody, respectively. BMP-2 and BMP-4 were found to bind to cells expressing neogenin, but not those transfected with the control plasmid (Fig. 1A). These results suggest that BMP-2 and BMP-4 bind to neogenin. As the next step, we performed co-immunoprecipitations for both BMP-2 and BMP-4. HEK293T cells were transfected with either V-SVG-tagged neogenin or a control plasmid, cultured for 48 h, and incubated with 100 ng/ml rhBMP-2 or rhBMP-4 for 2 h. The cell extracts were then immunoprecipitated using the anti-V-SVG antibody (Fig. 1B). In the neogenin-V-SVG immunoprecipitates, the anti-BMP-2 and BMP-2 antibodies revealed the presence of BMP-2 and BMP-4, respectively, but not in the precipitates obtained from mock-transfected cells (Fig. 1B). We next assessed the interaction of BMP-2 and neogenin in the cells expressing these proteins endogenously. Co-immunoprecipitation experiments revealed that BMP-2 was co-immunoprecipitated with neogenin as well as BMPR-1A in C2C12 cells (Fig. 1C). An association of these molecules could also be observed in lysates from postnatal day 7 mouse cerebral cortex (Fig. 1D). Further, we assessed the direct interaction of recombinant His-tagged neogenin-extracellular domain (ECD) with rhBMP-2 or rhBMP-4. After immunoprecipitation with the anti-His antibody, the proteins were detected by immunoblotting for the BMPs (Fig. 1E). The results of the analysis showed that rhBMP-2 and rhBMP-4 bind directly with neogenin ECD. We investigated whether neogenin ECD interacted directly with rhBMP-2, rhBMP-4, rhBMP-6, and rhBMP-7 by performing an ELISA. Fc-tagged recombinant neogenin ECD (170–0 nm), Fc-tagged recombinant BMP receptor type 1A (170–0 nm), Fc-tagged recombinant p75 (170–0 nm; p75 is a neurotrophin receptor belonging to the tumor necrosis factor receptor superfamily), or bovine serum albumin (BSA) was added to plastic wells coated with 0.1 mg/ml of one of the abovementioned rh-BMPs. Although binding of Fc-neogenin ECD and Fc-BMP receptor type 1A with rhBMP-2 was detected, the interaction of Fc-tagged p75 ECD (170–0 nm) with BMP-2 was not significantly higher than the baseline level (Fig. 2A), indicating specific binding of rhBMP-2 with neogenin ECD. The data obtained by Scatchard plot analyses indicated that binding of BMP-2, BMP-4, BMP-6, and BMP-7 occurred at sites with the dissociation constants ($K_d$) of 25, 50, 50, and 38 nM, respectively (Fig. 2B). We next established an ELISA-based competition assay to further assess the interaction of neogenin with BMP-2. The microwells were coated with BMP-2 (3 pM) or BSA and were incubated with BMP-2 (0–300 pM) and 70 nM neogenin-Fc. The binding of neogenin-Fc to the coated BMP-2 was suppressed when 30 or 300 pM of BMP-2 was included in the solution (Fig. 2C). Hence, interaction of coated BMP-2 with neogenin was inhibited by excess BMP-2. These findings indicate that neogenin ECD directly binds to BMPs and thereby suggest that neogenin is a receptor for BMPs.

**Neogenin Negatively Regulates the BMP-2-induced Osteoblastic Differentiation of C2C12 Cells**—Because BMPs bind directly with neogenin, we attempted to explore the functional implications of neogenin as a BMP receptor. For this purpose, we performed a cell-based assay using mesenchymal C2C12 cells, which differentiate into mature osteoblasts upon BMP stimulation. We examined neogenin expression in these cells.
cells by using real time RT-PCR. When the cultured cells were incubated with rhBMP-2 (100 ng/ml) for 3 days, mRNA expression of neogenin in C2C12 cells and ST2 cells, as well as in ST2 cells, which also differentiate into mature osteoblasts upon BMP stimulation (Fig. 3A). Time course experiments revealed that neogenin mRNA in C2C12 cells increased gradually for up to 5 days after stimulation with rhBMP-2 (100 ng/ml). After the cells were cultured for 3 more days, they were fixed and stained for ALP, and the activity was measured. The graph shows fold increase as compared with the levels in the controls. G, the BMP-induced ALP mRNA expression was quantitatively analyzed by real time RT-PCR. The expression of ALP mRNA was normalized to that of GAPDH. H, the effect of neogenin overexpression on the ALP mRNA expression. C2C12 cells transfected with neogenin or control vector were cultured for 48 h. The cells were cultured for an additional 3 days with rhBMP-2 (100 ng/ml) and subjected to real time RT-PCR analysis. I, neogenin knockdown did not affect the cell number. The C2C12 cells transfected with neogenin siRNA or control siRNA were cultured for 48 h and treated with rhBMP-2 (100 ng/ml) for 3 days. Cell viability was assessed by MTT assay and was expressed as an absorbance at 570 nm. For A–C, F, H, and I, the graphs represent data obtained from three independent experiments. *, p < 0.01; **, p < 0.05. For G, n = 5. *, p < 0.01; **, p < 0.05.

FIGURE 3. Neogenin negatively regulates osteoblastic differentiation of the C2C12 cells induced by BMP-2. A, levels of mRNA expression of neogenin in C2C12 cells and ST2 cells, as determined by real time RT-PCR. B, the time course of the mRNA expression of neogenin in rhBMP-2-stimulated C2C12 cells. C, the effect of neogenin siRNA on the expression of neogenin mRNA in C2C12 cells. C2C12 cells transfected with neogenin siRNA or control siRNA were cultured for 48 h, and the levels of expression of neogenin mRNA were examined and normalized to that of GAPDH. D, the effect of neogenin siRNA on the expression of neogenin in C2C12 cells. Western blots for neogenin and α-tubulin are shown. E, no off-target effect of neogenin siRNA was seen on the expression of DCC, UNC5B, and α-tubulin in C2C12 cells. C2C12 cells transfected with neogenin siRNA or control siRNA were cultured for 48 h and subjected to Western blot analysis. F, the BMP-induced ALP expression was enhanced by neogenin knockdown. C2C12 cells transfected with neogenin siRNA or control siRNA were cultured for 48 h and then treated with rhBMP-2 (100 ng/ml). After the cells were cultured for 3 more days, they were fixed and stained for ALP, and the activity was measured. The graph shows fold increase as compared with the levels in the controls. G, the BMP-induced ALP mRNA expression was quantitatively analyzed by real time RT-PCR. The expression of ALP mRNA was normalized to that of GAPDH. H, the effect of neogenin overexpression on the ALP mRNA expression. C2C12 cells transfected with neogenin or control vector were cultured for 48 h. The cells were cultured for an additional 3 days with rhBMP-2 (100 ng/ml) and subjected to real time RT-PCR analysis. I, neogenin knockdown did not affect the cell number. The C2C12 cells transfected with neogenin siRNA or control siRNA were cultured for 48 h and treated with rhBMP-2 (100 ng/ml) for 3 days. Cell viability was assessed by MTT assay and was expressed as an absorbance at 570 nm. For A–C, F, H, and I, the graphs represent data obtained from three independent experiments. *, p < 0.01; **, p < 0.05. For G, n = 5. *, p < 0.01; **, p < 0.05.
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sis in some but not all of the cell types (17, 18). To test the validity of this possibility, we performed the MTT assay to assess the cell viability. The results of the assay indicated that the transfection of neogenin siRNA with C2C12 cells did not affect the cell viability, irrespective of the presence or absence of rhBMP-2 (Fig. 3I). These results demonstrate that neogenin suppresses BMP-2-induced osteoblastic differentiation of C2C12 cells.

Neogenin Suppresses BMP-2-induced Phosphorylation of Smad1/5/8—Because neogenin negatively regulates rhBMP-2-induced osteoblastic differentiation of C2C12 cells, we explored the molecular mechanism underlying the inhibition of BMP signal transduction by neogenin. We sought to ascertain the roles of major signaling pathways involving BMP and the BMP receptors (BMPR) Smads in the negative regulation of osteoblastic differentiation. Smad signals were analyzed by monitoring the phosphorylation levels of a set of receptor-activated Smads (Smad1, Smad5, and Smad8; Smad1/5/8). We treated C2C12 cells with rhBMP-2 (100 ng/ml) for 30 min and analyzed the phosphorylation state of the receptor proteins Smad 1/5/8 by using antibodies that specifically recognize phosphorylated Smad 1/5/8. Treatment of these cells with rhBMP-2 resulted in an increase in the extent of the phosphorylation of Smad 1/5/8; however, the extent of Smad 1/5/8 phosphorylation in the control siRNA-transfected cells was less than that in the neogenin siRNA-transfected cells (Fig. 4A). We also assessed the expression of Id1, a downstream transcriptional target of Smad 1/5/8 (19). The results of the analysis revealed that the Id1 protein level was elevated 30 min after the administration of rhBMP-2 in both the cells transfected with control siRNA and those transfected with neogenin siRNA and that the elevation in the latter was greater than that in the former (Fig. 4B). Conversely, overexpression of neogenin-V-SVG suppressed the increase in the rhBMP-2-induced phosphorylation of Smad 1/5/8 (Fig. 4C). The increase in Id1 induced by rhBMP-2 was also suppressed by the overexpression of V-SVG-tagged neogenin (Fig. 4D). These findings indicate that in C2C12 cells, neogenin negatively regulates Smad 1/5/8 phosphorylation downstream of BMP-BMPR.

BMP-2 Binding to Neogenin Leads to Activation of RhoA—Previous studies have shown that the binding of RGMa to neogenin results in the activation of RhoA (10). Leukemia-associated Rho guanine nucleotide exchange factor is associated with the receptor complex for RGMa and is involved in activation of RhoA (11). These findings prompted us to examine whether the regulation of RhoA activity by BMP was mediated by neogenin. We measured the activity of RhoA by a pull-down assay using the GST-fused Rho-binding domain of rhodokin beads. C2C12 cells were treated with rhBMP-2 (100 ng/ml) for 30 min. The level of active GTP-bound RhoA was increased by rhBMP-2 treatment (Fig. 5A). To determine whether neogenin mediates BMP-induced RhoA activation, we performed a knockdown experiment for endogenous neogenin. Activation of RhoA induced by rhBMP-2 was completely abolished in the C2C12 cells transfected with the neogenin siRNA (Fig. 5A). This result demonstrates that rhBMP-2 activated RhoA by a neogenin-dependent mecha-

![Graph A](image1)

**FIGURE 4.** *Neogenin suppresses BMP-induced Smad1/5/8 phosphorylation.* A, neogenin knockdown enhanced BMP-induced Smad1/5/8 phosphorylation. C2C12 cells were transfected with neogenin siRNA or control siRNA and were treated with rhBMP-2 (100 ng/ml) for 30 min. Representative Western blots for phosphorylated Smad1/5/8 (top panel), Smad1 (middle panel), and α-tubulin (bottom panel) are shown. The relative levels of the phosphorylated Smad1/5/8 normalized by Smad1 levels are shown in the graph. B, neogenin knockdown enhanced BMP-induced Id-1 expression. Representative Western blots for Id-1 (upper panel) and α-tubulin (lower panel) are shown. The relative levels of Id-1 are shown in the graph. C, neogenin suppressed BMP-induced Smad1/5/8 phosphorylation. C2C12 cells were transfected with neogenin or control vector, and 48 h later, they were treated with rhBMP-2 (100 ng/ml) for 30 min. The relative levels of the phosphorylated Smad1/5/8 are shown in the graph. D, neogenin suppressed BMP-induced Id-1 expression. The relative levels of Id-1 are shown in the graph. A–D, the graphs represent the data from three independent experiments. *, p < 0.01; **, p < 0.05.
FIGURE 5. BMP-2-induced activation of RhoA is dependent on neogenin and suppresses Smad1/5/8 phosphorylation. A, BMP-2 activates RhoA in C2C12 cells via neogenin. C2C12 cells were transfected with neogenin siRNA or control siRNA. Transfected cells were treated with or without rhBMP-2 (100 ng/ml) for 30 min and subjected to rhotekin pull-down assays to detect the active form of RhoA (upper panels). Whole cell lysates were also immunoblotted with anti-RhoA antibody (lower panels). B and C, the cells were pretreated with dorsomorphin and then were treated with rhBMP-2 for 30 min. Dorsomorphin treatment inhibited BMP-2-induced phosphorylation of Smad1/5/8 (B) but did not modulate the effect of BMP-2 on the RhoA activity (C). Representative blots are shown from four (B) or five (C) independent experiments. The relative RhoA activities are shown in the graphs. D, the BMP-2-induced ALP induction was enhanced by the ROCK inhibitor, Y-27632. The cells were pretreated with or without Y-27632 for 30 min and then were treated with rhBMP-2 for 3 days, followed by immunostaining for ALP. E, the ALP mRNA expression was analyzed by real time RT-PCR. The expression of ALP mRNA was normalized by that of GAPDH (n = 3). F, treatment with Y-27632 did not affect the BMP-2-induced phosphorylation of Smad1/5/8. The cells were pretreated with or without Y-27632 for 30 min and then were treated with rhBMP-2 for 30 min. Representative Western blots for phosphorylated Smad1/5/8 (top panel), Smad1 (middle panel), and α-tubulin (bottom panel) are shown. The relative level of phosphorylated Smad1/5/8 is shown in the graph (n = 7). G, inhibition of RhoA enhanced BMP-2-induced phosphorylation of Smad1/5/8. C2C12 cells transfected with a dominant negative form of RhoA (RhoDN) or control plasmid were cultured for 24 h. The cells were then treated with rhBMP-2 for 30 min. The relative level of phosphorylated Smad1/5/8 is shown in the graph. For A, C, E, F, and G, *, p < 0.01; **, p < 0.05.
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RhoA activation induced by rhBMP-2 is independent of BMPR.

**RhoA Negatively Regulates BMP-induced Smad Phosphorylation**—To examine whether the activation of RhoA occurring as a result of the binding of BMP to neogenin plays a role in suppressing the effects of BMP, we performed further experiments using Y-27632, an inhibitor of ROCK. We examined whether blockage of ROCK affected the BMP-2-induced increase in ALP activity. The C2C12 cells were cultured with rhBMP-2 (100 ng/ml) in the presence or absence of Y-27632 for 3 days and then subjected to ALP staining. The number of the ALP-positive cells in the Y-27632-treated C2C12 cells was markedly higher than that in the cells not treated with Y-27632 (Fig. 5D). In line with this finding, the increase in the BMP-2-induced ALP mRNA expression at 3 days was significantly higher in the cells treated with Y-27632 than in the cells not treated with Y-27632 (Fig. 5E). These results demonstrate that the inhibition of ROCK promoted BMP-2-induced osteoblastic differentiation of C2C12 cells. Thus, our findings support the notion that the RhoA/ROCK signals negatively regulate BMP-2-induced osteoblastic differentiation. Next, we tested whether the negative regulation of Smad signals by neogenin was also dependent on RhoA/ROCK. Although the stimulation of the cells with rhBMP-2 (100 ng/ml) increased the phosphorylation of Smad1/5/8, this increase was not significantly altered by the presence of Y27632 (Fig. 5F). However, C2C12 cells transfected with the dominant negative mutant of RhoA (RhoA17N) showed a significantly higher level of rhBMP-2-induced phosphorylation of Smad1/5/8 than C2C12 cells transfected with the mock plasmid (Fig. 5G). These results demonstrate that RhoA negatively regulates the BMP-2-induced phosphorylation of Smad1/5/8 in C2C12 cells and that this effect is not mediated by ROCK.

**DISCUSSION**

Our study revealed that neogenin was a receptor of BMPs. BMP-2 binding to neogenin activated RhoA (Fig. 6). The molecular mechanism underlying the neogenin-mediated activation of RhoA by BMPs may be similar to the mechanism underlying the activation of RhoA by RGM. We have previously shown that RGMa binds to neogenin to activate RhoA, which induces inhibitory or repulsive signals that eventually lead to the collapse of the neuronal growth cone. Unc5B, a member of the netrin receptor family, interacts with neogenin to act as a co-receptor for RGMa (11). Although RGMa does not directly bind to Unc5B, leukemia-associated Rho guanine nucleotide exchange factor associates with Unc5B to transduce the RhoA signals. Focal adhesion kinase is additionally involved in RGMa-induced tyrosine phosphorylation of leukemia-associated Rho guanine nucleotide exchange factor as well as RhoA activation. Thus, neogenin acts as a binding partner of the ligands, and Unc5B plays a role in transducing the RhoA signal. Therefore, neogenin may form a receptor complex with Unc5B for signal transduction between BMPs to RhoA.

Previous studies have suggested that RhoA plays a role in BMP signal transduction. We have previously shown that continuous infusion of Y-27632 in mice enhanced ectopic bone formation induced by rhBMP-2 impregnated into an atelocollagen carrier without affecting the systemic bone metabolism (2). Treatment with Y-27632 also enhanced the BMP-2-induced osteoblastic differentiation of cultured murine neonatal calvarial cells and ST2 cells. These effects were associated with the increased expression of the gene encoding BMP-4. Expression of a dominant negative mutant of ROCK in ST2 cells promoted osteoblastic differentiation, whereas a constitutively active mutant of ROCK attenuated osteoblastic differentiation, and a ROCK inhibitor reversed this phenotype. Thus, it is suggested that ROCK negatively regulates osteogenesis by suppressing BMP-4 expression. Our data show that the administration of Y-27632 up-regulated the rhBMP-2-induced osteoblastic differentiation of the C2C12 cells, although Y-27632 itself had no effect. Because the inhibition of ROCK had no effect on rhBMP-2-induced Smad1/5/8 phosphorylation, we can consider that ROCK inhibits the signal transduction downstream of Smads. Thus, our data are consistent with the notion that inhibition of ROCK facilitates osteogenic differentiation by inducing BMP-4. However, the binding of BMP to neogenin negatively regulated Smad1/5/8 phosphorylation, and this regulation was dependent on RhoA activity. Thus, RhoA may directly or indirectly suppress Smad1/5/8 phosphorylation.

It appears that the cells have two receptor systems; one is to activate and the other is to inhibit the BMP signal transduction. The sensitivity of the cells to BMP may be regulated by the relative expression levels of these receptors. Therefore, future studies should elucidate the regulatory mechanism of expression of these receptors.
Intriguingly, RGMs, ligands for neogenin (3), are BMP co-receptors and enhance the effects of BMP (20). Thus, RGMs positively regulate the functions of BMP by binding to BMPs and BMP receptors. Promotion of the functions of BMP by RGMs appears to depend on the formation of a complex of BMPs, BMP receptors, and RGMs in the extracellular domains, because RGMs are glycosylphosphatidyl inositol-anchored proteins and are therefore incapable of transducing intracellular signals by themselves. Instead, neogenin negatively regulates the functions of BMP by activating the intracellular signaling molecule RhoA. Although the results of ELISA in this study show that BMPs directly bound with neogenin, RGMs may affect the BMP-neogenin signal transduction by binding to neogenin. The cross-talk between these molecules should be investigated in future studies.

Recently, it was reported that mice lacking neogenin reveal impaired digit/limb development and endochondral ossification (21), suggesting that neogenin mediates the signal of BMPs. Our present study provides evidence to suggest that neogenin, in addition to the ROCK inhibitors, may be a promising molecular target for promoting osteogenesis.

Acknowledgments—We thank Prof. E. Fearon for providing the vesicular stomatitis virus-tagged neogenin construct. rhBMP-2 purified from E. coli was provided by Osteopharma Inc.

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