Nephronectin Expression Is Up-Regulated by BMP-2

Tamaki Kurosawa,*,a Atsushi Yamada,a,b Dai Suzuki,a Naoko Morimura,c Yoshiyuki Sasagane,a Hiroyuki Itabe,b and Ryutaro Kamijo,a

Department of Biochemistry, School of Dentistry, Showa University; 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; Division of Biological Chemistry, Department of Molecular Biology, Showa University School of Pharmacy; 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; and Brain Science Laboratory, The Research Organization of Science and Technology, Ritsumeikan University; 1–1–1 Noji-higashi, Kusatsu, Shiga 525–8577, Japan.

Received November 25, 2015; accepted April 25, 2016

Nephronectin (Npnt), known to be a ligand of integrin αβ1, plays important roles in the development and function of various tissues, including those of the kidneys, liver, bones, and muscles. In previous studies, we showed that the expression of Npnt mRNA was regulated by various cytokines, including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and oncostatin M (OSM), and that over-expression of Npnt enhanced osteoblast differentiation. In this study, we found that bone morphogenic protein-2 (BMP-2), known as an osteogenesis inducing cytokine, strongly up-regulated the expression of Npnt mRNA in a murine skeletal muscle cell line (C2C12) via the BMP-SMAD signaling pathway.

Key words  nephronectin; bone morphogenetic protein-2; C2C12; SMAD signaling pathway

MATERIALS AND METHODS

Cell Cultures  C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) with l-glutamine and Phenol Red medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan; Cat. No. 044-29765), supplemented with 15% fetal bovine serum (FBS) (Life Technologies, U.S.A.; Cat. No. 10099-14) and 1% penicillin-streptomycin (Life Technologies; Cat. No. 15596-044). Quantitative real-time PCR was performed with a StepOneTM Real Time PCR System (Applied Biosystems, U.S.A.) using SYBR Green Fast PCR Master Mix (Applied Biosystems) with the following specific PCR primers.

Western Blotting  Cells were lysed using 10 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100, protease inhibitors, and phosphatase inhibitors. After boiling, total protein was separated by SDS–PAGE and transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies against Smad 4, 5, and 6, and GAPDH as a loading control. The specific bands were visualized by the ECL detection system (GE Healthcare, U.S.A.).

Note

© 2016 The Pharmaceutical Society of Japan
(pH 7.8) containing 1% Nonidet P-40, 0.15 m NaCl, and a protease inhibitor mixture containing ethylenediaminetetraacetic acid (EDTA) (Roche Applied Science, U.S.A.). Lysates were cleared by centrifugation at 13000×g for 10 min, then placed onto 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoresed following dilution with Sample Buffer Solution with Reducing Reagent (6x) for SDS-polyacrylamide gel electrophoresis (PAGE) (Nacalai Tesque, Kyoto, Japan; Cat. No. 09499-14). After transferring, polyvinylidene difluoride (PVDF) membranes were blocked with 0.1% Tween20-TBS containing 1% skim milk for 1 h and incubated with the primary antibody overnight at 4°C. Antibodies against the following proteins were used for Western blotting analysis: Smad1 (1/1000, Cell Signaling Technology, U.S.A.; #9743), Smad5 (1/625, Cell Signaling Technology; #12534), phospho-Smad1/5 (1/1000, Cell Signaling Technology; #9516), Smad4 (1/1000, Cell Signaling Technology; #9515), Npnt (1/500, R&D Systems, Inc. #AF4298), and actin (1/2000, SIGMA Life Science, U.S.A.; #A5060). Anti-rabbit immunoglobulin G (IgG), horseradish peroxidase linked whole antibody (1/2500, GE Healthcare, U.S.A.; Cat. No. NA934V), and peroxidase-conjugated anti-goat IgG (1/5000, Jackson Immunoresearch, U.S.A.; #705-035-147) were used as secondary antibodies. All antibodies were placed in TBS-0.1% Tween20 containing 1% skim milk. To visualize antigenic bands, peroxidase reactions were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare, U.S.A.; RPN2232).

**Small Interfering RNA (siRNA) Knockdown of Gene Expression** For siRNA transfection, Lipofectamine RNAiMAX Reagent (Invitrogen, U.S.A.; #13778-150) was used. Both of Smad4 siRNA (Cell Signaling Technology; #12791) and StealthTM RNAi Negative Control Medium GC Duplex #2 (Invitrogen; PN12935112) were diluted by adding Opti-MEM (Gibco, U.S.A.; REF 31985-070) in different tubes. Duplex #2 (Invitrogen; PN12935112) was used for a negative control siRNA. 14) Luminosity was detected using a Dual-Luciferase Reporter Assay System (Promega, U.S.A.; E1910) after 24 h of incubation with or without 300 ng/mL of BMP-2. The relative firefly luciferase reaction was normalized to the luminescent reactions of *Renilla* luciferase from the TK vector. A pGL4 [luc2/Neo] vector was used as the negative control vector.

**Myosin Heavy Chain (MHC) and Alkaline Phosphatase (ALP) Staining** To examine C2C12 cell differentiation, we checked expression of MHC and activity of ALP. First, to analyze the expression of MHC, cells were fixed with 10% formaldehyde for 10 min at room temperature, then treated with an acetone–ethanol mixture (50:50, v/v) for 1 min and incubated with a mouse anti-MHC monoclonal antibody (MF-20; Developmental Studies Hybridoma Bank, Iowa, IA, U.S.A.) at room temperature for 1 h. After washing with PBS, cells were incubated for 30 min with a secondary antibody (#424134; Nichirei, Tokyo, Japan). To determine ALP activity, after washing with PBS, cells were exposed to an ALP activity solution containing 0.1 mg/mL of naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide (Wako Pure Chemical Industries, Ltd.), 2 mM MgCl₂, and 0.6 mg/mL of fast blue BB salt (Sigma) in 0.1 mM Tris–HCl (pH 8.5) for 30 min at room temperature. After washing with PBS, MHC was visualized using an AEC substrate kit (#415184, Nichirei).

**Statistical Analysis** All results are expressed as the
mean ± standard deviation (S.D.). Statistical analysis results shown in Figs. 1, 2A, and 3A, and Supplementary Fig. 2B were obtained using one-way ANOVA, while those show in Figs. 2B, 4A, and B were obtained using a two-tailed Student's t-test. p Values <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

C2C12 cells which are one of the mouse myoblast cell lines are used as a model for osteoblast differentiation induced by BMP-2. Analyses of ALP activity and MHC immunohistochemistry are revealed that C2C12 cells were differentiated into osteoblasts or myotubes using low FBS (0.5%) containing medium with or without BMP-2 (Fig. 1A). To examine their effect on Npnt mRNA expression, we exposed C2C12 cells to various BMPs for 48 h, then harvested after 24, 36, 48, or 72 h. Real-time PCR analyses were performed using cDNA from each sample to examine the mRNA levels of Npnt and Gapdh. Values are shown as the mean ± S.D. of 3 samples folded over the group taken at 0 h. **p <0.01, relative to level in cells with no treatment at each time point (Student's t-test).

Fig. 2. BMP-2 Up-Regulated the Level of Npnt mRNA Expression in Both Dose- and Time-Dependent Manners

(A) Dose-dependent effects of BMP-2 on expression of Npnt mRNA in C2C12 cells after treatment with 0, 3, 30, 300, or 1000 ng/mL for 48 h. At higher concentrations, Npnt mRNA was remarkably up-regulated by BMP-2. Values are shown as the mean ± S.D. of 3 samples folded over the group without treatment. **p <0.01, relative to level in cells without treatment (ANOVA). (B) Time-dependent effects of BMP-2 on Npnt mRNA expression in C2C12 cells. After a 24-h pre-culture, cells were treated with 300 ng/mL of BMP-2, then harvested after 24, 36, 48, or 72 h. Real-time PCR analyses were performed using cDNA from each sample to examine the mRNA levels of Npnt and Gapdh. Values are shown as the mean ± S.D. of 3 samples folded over the group taken at 0 h. **p <0.01, relative to level in cells with no treatment at each time point (Student's t-test).

Fig. 3. ALK2/3 Inhibitor Blocked Up-Regulation of Npnt mRNA Expression

(A) The ALK2/3 inhibitor negated the up-regulation of Npnt mRNA expression by BMP-2. To block BMP-2 up-regulation, cells were treated with 300 ng/mL of BMP-2 and 300 nM of an ALK2/3 inhibitor for 48 h. Real-time PCR was performed using cDNA derived from total cellular RNA from each sample to determine the expression levels of Npnt and Gapdh mRNAs. Values are shown as the mean ± S.D. of 3 samples folded over the group without treatment. **p <0.01, relative to level in cells without treatment (ANOVA). (B) ALK2/3 inhibitor blocked BMP-2-induced phosphorylation of Smad1 and Smad5 in C2C12 cells. After pre-treatment with 300 nM of the ALK2/3 inhibitor for 1 h, cells were exposed to medium containing 300 ng/mL of BMP-2 and 300 nM of the inhibitor, or to control medium without the inhibitor for 15 min. All proteins were extracted and used to determine the levels of phosphorylated and total Smad1 and Smad5.

hand, no increase was seen with BMP-3 treatment. It has been reported that, BMP-2, -4, -6, and -7 bind to the type I receptor ALK2/3 based on their affinity for BMP receptors, while BMP-3 is known as an agonist of activin receptor type 2b (Acvr2b). Moreover, BMP-3 has only a 50% identical amino acid sequence with other osteogenic BMPs and, in some cases, suppresses BMP functions. In consideration of these inherent differences, we believe that the present results are in agreement with previous reports. The expression level of Npnt protein was also increased in C2C12 cells after 48 h of incubation with BMP-2 (Fig. 1C).

In order to confirm that the observed up-regulation of Npnt mRNA expression was caused by the BMPs, we exposed C2C12 cells to various doses of BMP-2, the most widely used BMP to drive osteoblast differentiation, for different periods of time, and found that the expression was up-regulated in both dose- and time-dependent manners (Figs. 2A, B).

Based on those findings, we next utilized an ALK2/3 inhibitor (LDN193189) to determine the mechanism by which BMP-2 regulates the expression of Npnt mRNA in C2C12 cells (Fig. 3A). Since exposure to the ALK inhibitor canceled
up-regulation by BMP-2, we speculated that the up-regulation of Npnt mRNA expression occurred via activation of the BMP receptor. To examine the effect of the ALK2/3 inhibitor, we performed Western blotting and detected phosphorylated Smad1 and Smad5, which were located in the downstream signaling pathway triggered by ALK2/3 receptors. As shown in Fig. 3B, the ALK2/3 inhibitor completely blocked phosphorylation of Smad1 and Smad5. Furthermore, we investigated whether the SMAD signaling pathway is activated on BMP-2 induced up-regulation of Npnt mRNA expression using siRNA of Smad4, a SMAD signaling factor that forms a complex with phosphorylated Smad1/5 and transmits BMP signals into the nucleus. Western blotting analysis showed that cells transfected with Smad4 siRNA had lower levels of Smad4 protein as compared to those transfected with control siRNA (Fig. 4A). Moreover, we transfected a construct containing a Smad4-responsive element (id1.0-luc vector) into Smad4 knockdown C2C12 cells and performed luciferase assay analysis (Fig. 4B). C2C12 cells transfected with the id1.0-luc vector demonstrated a high level of luciferase activity after BMP-2 treatment (control), while Smad4 knockdown cells showed only one-third of the activity of the control. Smad4 knockdown of C2C12 cells also resulted in a weaker up-regulation of Npnt mRNA expression by BMP-2 as compared to the control siRNA-transfected cells (Figs. 4C, D). Based on these results, we concluded that BMP-2 up-regulates Npnt mRNA expression in C2C12 cells via the BMP-SMAD signaling pathway.

Our finding that up-regulation of Npnt by BMP-2 requires Smad4 implies the presence of a mechanism that regulates the expression of Npnt mRNA. Other recent studies have also indicated a competitive relationship between Smad2 and Smad3 or Smad4 involved in regulation of the expressions of
some genes, such as hepatocyte growth factor (HGF). As for the expression of Npnt, Tsukasaki et al. reported its suppression by Smad2, while the present findings demonstrated its activation by Smad4. Together, these observations led us to speculate that the Npnt expression is also regulated by a competition between Smad2 and Smad4, though additional experiments are needed to more fully reveal the mechanism details.

Acknowledgments The authors express their gratefulness to Dr. Kentaro Yoshimura for the valuable discussion. We also thank Dr. Satomi Nimura for guidance regarding the use of statistics software produced by SSRI for one-way ANOVA. This work was supported in part by the Project to Establish Strategic Research Center for Innovative Dentistry by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


