Effects of Wnt-β-Catenin Signaling and Sclerostin on the Phenotypes of Rat Pheochromocytoma PC12 Cells

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Abstract

Pheochromocytomas and paragangliomas (PPGLs) are classified into 3 major categories with distinct driver genes: pseudohypoxia, kinase signaling, and Wnt-altered subtypes. PPGLs in the Wnt-altered subtype are sporadic and tend to be aggressive with metastasis, where somatic gene fusions affecting mastermind-like 3 (*MAML3*) and somatic mutations in cold shock domain containing E1 (*CSDE1*) cause overactivation of Wnt-β-catenin signaling. However, the relation between Wnt-β-catenin signaling and the biological behavior of PPGLs remains unexplored. In rat pheochromocytoma PC12 cells, Wnt3a treatment enhanced cell proliferation and suppressed mRNA expression of tyrosine hydroxylase (*TH*), the rate-limiting enzyme of catecholamine biosynthesis, and dopamine secretion. We identified the expression of sclerostin in PC12 cells, which is known as an osteocyte-derived negative regulator for Wnt signaling-driven bone formation. Inhibition of endogenous Wnt pathway by XAV939 or sclerostin resulted in attenuated cell proliferation and increased *TH* expression. Furthermore, Wnt3a pretreatment suppressed bone morphogenetic protein (BMP)-induced Smad1/5/9 phosphorylation whereas BMPs enhanced sclerostin expression in PC12 cells. In the Wnt-altered subtype, the increased Wnt-β-catenin pathway may contribute the aggressive clinical behavior with reduced catecholamine production. Furthermore, upregulated expression of sclerostin by BMPs may explain the osteolytic metastatic lesions observed in metastatic PPGLs.

Key Words: Wnt-β-catenin signaling, sclerostin, catecholamine, PPGL, PC12

Abbreviations: BMP, bone morphogenetic protein; *CSDE1*, cold shock domain containing E1; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HS, horse serum; LRP5/6, low-density lipoprotein receptor-related proteins 5/6; *MAML3*, mastermind-like 3 gene; PCR, polymerase chain reaction; PPGL, pheochromocytoma/paraganglioma; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCGA, The Cancer Genome Atlas; *Th*, tyrosine hydroxylase.

Pheochromocytomas and paragangliomas (PPGLs) are neuroendocrine tumors that arise from neural crest–derived cells of the adrenal medulla and extra-adrenal paraganglia, respectively. According to the most recent edition of the World Health Organization (WHO) classification of endocrine tumors published in 2017, all PPGLs have metastatic potential [1]. However, patients with metastatic PPGL have limited treatment options and poor prognosis. PPGLs are also considered to have the highest degree of heritability of any human tumor type. More than one-third of PPGLs are associated with inherited cancer susceptibility syndromes. The Cancer Genome Atlas (TCGA) proposed that PPGLs can be classified into 3 main molecular subgroups linked to distinct driver genes: pseudohypoxia (*SDHA, SDHB, SDHC, SDHD, SDHAF2, FH, VHL, EPAS1*, and *EGLN1*), kinase signaling (*RET, NF1, TMEM127, MAX, HRAS, FGFR1*, and *MET*) and Wnt-altered (*CSDE1* or *MAML3*) [2]. The Wnt-altered subtype of PPGL is relatively newly recognized and includes somatic gene fusions affecting mastermind-like 3 (*MAML3*) and somatic mutations in cold shock domain containing E1 (*CSDE1*) [2]. *MAML3* fusion genes have been shown to lead

to overactivation of Wnt/Hedgehog signaling. A gain-of-function mutation in *CSDE1* has been reported to cause the overactivation of β-catenin, a target of Wnt signaling [2].

The Wnt-β-catenin signaling pathway plays important roles in cell proliferation and differentiation during embryogenesis and adult tissue homeostasis [3]. Hyperactivation of the Wnt signaling pathway has been demonstrated in diverse cancers, including colorectal, breast, lung, and hematopoietic malignancies [4]. Three different pathways are thought to be activated upon Wnt receptor activation: the canonical Wnt-β-catenin signaling pathway, the noncanonical planar cell polarity pathway, and the Wnt/Ca^{2+} pathway. In the canonical Wnt-β-catenin signaling, binding of Wnt to its cell surface receptor, frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), antagonizes the phosphorylation of β-catenin. Once β-catenin phosphorylation is reduced, it is no longer degraded, resulting in its accumulation in the cytoplasm. Stabilized β-catenin is translocated into the nucleus, where it binds to the transcription factors T-cell factor (Tcf) and lymphoid enhancer binding factor (Lef) and thereby stimulates the transcription of Wnt target genes including

Axin2 and *Lef1* [5]. The canonical Wnt signaling pathway involves Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt8, Wnt8b, and Wnt10a. Of the canonical Wnts, Wnt3a plays crucial roles in both proliferation and differentiation in several types of cancer cells and stem cells [6]. All reported PPGLs with Wnt-altered subtype were sporadic and *MAML3* fusion genes were proposed to be associated with metastatic disease and poor aggressive-disease-free survival [2]. However, the molecular mechanisms modulated by overactivated Wnt-β-catenin signaling that may contribute to the aggressive disease of the Wnt-altered PPGL subtype are unknown. In the present study, we investigated the role of the Wnt-β-catenin signaling pathway in PPGL and its related intracellular signaling pathways using rat pheochromocytoma PC12 cells.

Materials and Methods

Cell Culture

The rat pheochromocytoma cell line PC12 was obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). PC12 cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10% horse serum (HS), penicillin, and streptomycin, which were purchased from Sigma-Aldrich (St. Louis, MO, USA). The culture medium was changed twice per week, and the cultures were passaged at 80% confluence.

Catecholamine Assays

PC12 cells were precultured in 12-well plates with DMEM containing 10% FCS and 10% HS for 24 hours. The medium was then changed to DMEM containing 1% FCS and 1% HS, and the cells were treated with the indicated concentrations of recombinant mouse Wnt3a (R&D, Minneapolis, MN, USA, 1324-WN), recombinant mouse sclerostin protein (R&D, 1589-ST), recombinant human bone morphogenetic protein (BMP)-2 protein (R&D, 355-BM), recombinant human BMP-4 protein (R&D, 314-BP), recombinant human BMP-7 protein (R&D, 354-BP) or XAV939 (Sigma-Aldrich, X3004), a tankyrase inhibitor. XAV939, which stabilizes Axin and antagonizes Wnt-β-catenin signaling [7], was dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium. The culture medium was collected after 24-hour culture, and the levels of catecholamines were determined using high-performance liquid chromatography (HPLC; BML, Inc., Saitama, Japan).

RNA Extraction, Quantitative Real-Time Polymerase Chain Reaction Analysis

PC12 cells were precultured in 12-well plates with DMEM containing 10% FCS and 10% HS for 24 hours. The medium was then replaced with DMEM containing 1% FCS and 1% HS, and the cells were treated with Wnt3a, XAV939, sclerostin, BMP2, BMP4, BMP7, or a combination of the reagents at the indicated concentrations. After culturing, the medium was removed and total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The extracted RNA (1 μg) was subjected to reverse transcription (RT) using a First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamers (2 ng/mL), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42 °C for 50 minutes and at 70 °C for 10 minutes. All primer sequences are listed in Table 1. Aliquots of the polymerase chain reaction (PCR) products were electrophoresed on 1.5% agarose gels and visualized using fluorescent nucleic acid staining assays (GelRed, purchased from Biotium, Fremont, CA, USA). For quantification of each target mRNA level, real-time PCR was performed using the QuantStudio real-time PCR system (Applied Biosystems, Waltham, MA, USA). The relative expression of each mRNA was determined using the ΔCt method, where ΔCt is the value obtained by subtracting the Ct value of ribosomal protein L19 (*RPL19*) mRNA from that of the target mRNA. The amount of target mRNA relative to *RPL19* mRNA was expressed as $2^{-(\Delta Ct)}$, and the results were expressed as the ratio of target mRNA to *RPL19* mRNA.

Western Blot Analysis

PC12 cells were pretreated with the indicated concentrations and periods of Wnt3a, sclerostin, XAV939, BMP2, BMP4, and BMP7 in DMEM. After culture, cells were solubilized and the cell lysates were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with anti-non-phospho (active) β-catenin (Ser33/37/Thr41) rabbit monoclonal antibodies (Cell Signaling Technology, Beverly, MA, USA, D13A1, RRID: AB_11127203), anti-β-catenin rabbit monoclonal antibodies (Cell Signaling Technology, D10A8, RRID: AB_11127855), anti-phospho-Smad 1/5/9 antibody (Cell Signaling Technology, #13820, RRID: AB_2493181), anti-SOST antibody (R&D, AF1589 RRID: AB_2195345) and anti-ACTIN antibody (Sigma-Aldrich, A2066, RRID: AB_476693). The integrated signal density of each protein band was analyzed using the LAS-4000 mini-instrument (FUJIFILM, Tokyo, Japan).

Cell Proliferation Assay

For cell proliferation assays, the CellTiter 96 AQueous One Solution Cell Proliferation Assay System (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. PC12 cells were plated in each well of a 96-well

Table 1. Primer sequences used for PCR analysis

gene	Forward primer (5'-3')	Reverse primer (5'-3')
Rpl19	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
Th	TCCCCTGGTTCCCAAGAAAAG	GTGCATTGAAACACGCGGAAG
Wnt3a	ACCGTCACAACAATGAGGC	GATAGTCCGTGGCATTTGC
Sost	GCCTCCTCAGGAACTAGAGAAC	TACTCGGACACGTCTTTGGTG
Lrp5	GCTGCTCCCATATCTGCATC	AGCCTTCCTCATCACTCTGG
Lrp6	GGATGAGCTGTCCTGTGGAG	TTCGCGTCACCATTGCATC
Axin2	GGCCTATCCAGCAAAACTC	CCTACGTGATAAGGATTAACCG
Lef1	CCTGAAATGCCCACCTTCTACC	CCACCCGTGATGGGATAAACAG

plate and the indicated concentrations of Wnt3a, sclerostin, XAV939, BMP2, BMP4, and BMP7 was added. After 24-hour incubation in a humidified 5% CO_2 atmosphere, PC12 cells were incubated with 20 μL of CellTiter 96 AQueous One Solution reagent per well. The absorbance at 490 nm was measured using a 96-well plate reader (Bio-Rad, Hercules, CA, USA, Model 680 XR).

Statistical Analysis

Data are presented as the means ± SE of data from at least 3 separate experiments, each performed in triplicate. Differences between groups were analyzed for statistical significance using ANOVA with post hoc unpaired *t* test, when appropriate, to determine differences. Two-tailed *P* values less than 0.05 were regarded as statistically significant.

Results

Effect of Wnt-β-Catenin Signaling on Cell Proliferation and Catecholamine Synthesis

To demonstrate the existence of the Wnt-β-catenin signaling system in PC12 cells, the mRNA expression of key molecules involved in the canonical Wnt signaling was examined by reverse transcriptase PCR analysis (Fig. 1A). Wnt3a, LRP5/6, Axin2, and Lef1 were expressed, that agrees with previous reports [8]. The expression of tyrosine hydroxylase (*Th*), the rate-limiting step in catecholamine biosynthesis, was also confirmed. Notably, the mRNA (Fig. 1A) and protein (Fig. 1B) expression of sclerostin, a negative regulator in of the canonical Wnt pathway, was also detected by PCR and Western blotting, respectively in PC12 cells. Sclerostin is expressed mainly in osteocytes and suppresses osteoblast differentiation by binding to LRP5/6 and inhibiting the canonical Wnt signaling pathway [9]. To examine whether this Wnt-β-catenin system operates functionally, the effect of exogenous Wnt3a on β-catenin expression was evaluated with Western blots. As shown in Fig. 1C, Wnt3a increased total β-catenin, as previously reported [10], and nonphosphorylated β-catenin, the stabilized and active form of β-catenin (Fig. 1C). This Wnt3a-induced nonphosphorylated and total β-catenin expression was attenuated by pretreatment with sclerostin (Fig. 1C). Messenger RNA expression of Axin2 and Lef1, well-known canonical Wnt target genes, was also confirmed to be upregulated by treatment with Wnt3a (Fig. 1D).
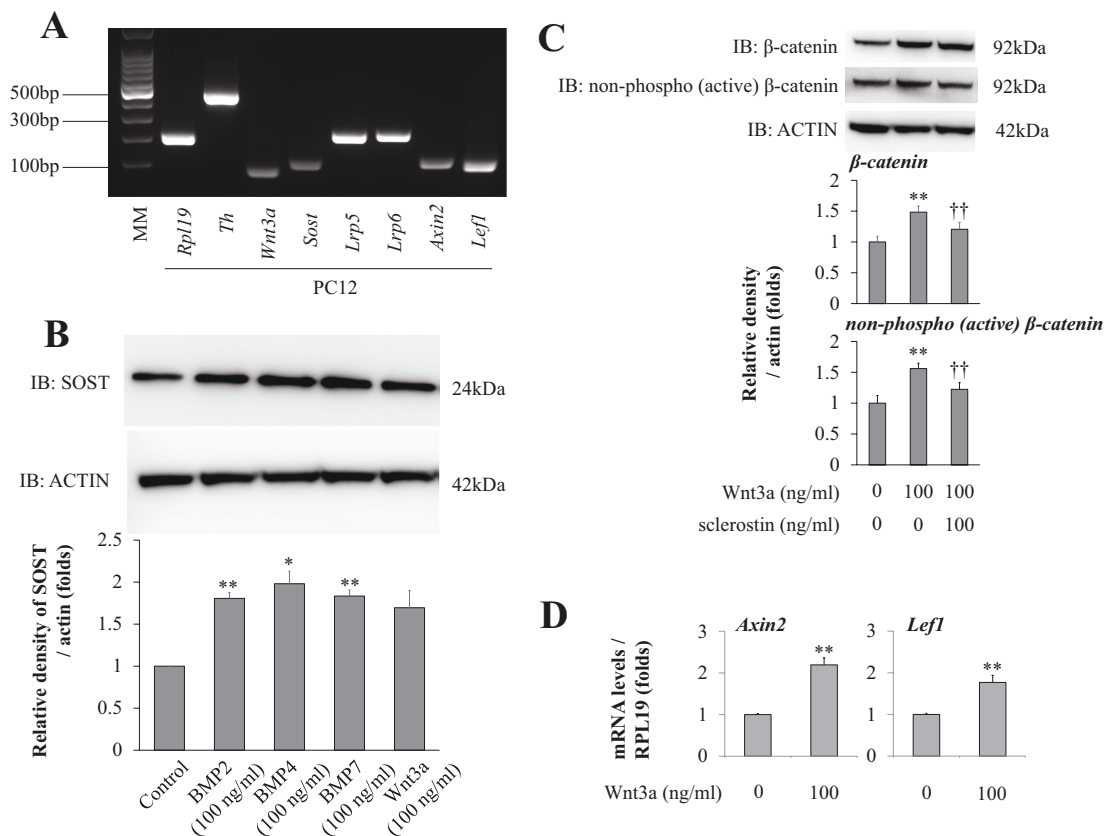

Figure 1. Expression of the molecules related to Wnt-β-catenin signaling in PC12 cells. (A) Total cellular RNAs were extracted from PC12 cells. The expression of *Th*, *Wnt3a*, *Sost*, *Lrp5*, and *Lrp6* were detected by RT-PCR analysis. MM indicates molecular weight marker. (B) Protein expression of sclerostin in PC12 cells with or without treatment of BMPs and Wnt3a. PC12 cells were treated with indicated concentration of BMP2, BMP4, BMP7, and Wnt3a for 24 hours. The cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-SOST and anti-ACTIN antibodies. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. (C) PC12 cells were treated with indicated concentration of Wnt3a for 8 hours with or without pretreatment with sclerostin for 24 hours. The cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-non-phospho-β-catenin, β-catenin, and anti-ACTIN antibodies. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. (D) PC12 cells were treated with indicated concentration of Wnt3a for 8 hours. Total cellular RNAs were extracted, and mRNA levels were analyzed by quantitative PCR. The expression levels of target mRNA were standardized by Rpl19 level in respective sample, and then levels of mRNA of genes were expressed as fold changes. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with control, ††$P < 0.01$ compared with Wnt3a treatment alone as determined by unpaired *t* test.

These results demonstrate the existence of a functional Wnt-β-catenin signaling system including sclerostin in PC12 cells.

To investigate the influence of enhanced Wnt-β-catenin signaling on tumor growth in PPGL, we evaluated the proliferation of PC12 cells treated with various concentrations of Wnt3a by measuring viable cell viability using CellTiter 96. Previous studies have reported that Wnt3a has protective effects against apoptosis induced by neurocytotoxic substances, including ferrous sulfate and β-amyloid peptide, in PC12 cells [10, 11]. In the present study, exogenous Wnt3a treatment significantly enhanced cell proliferation (Fig. 2). Inhibition of the endogenous canonical Wnt pathway by XAV939 decreased viable cell number. A high concentration of sclerostin at 1000 ng/mL also reduced the cell number (Fig. 2).

To examine the influence of enhanced Wnt-β-catenin signaling on catecholamine synthesis and secretion of PPGL cells, we assessed Wnt3a-induced changes in *Th* mRNA expression (Fig. 3A) and dopamine secretion (Fig. 3B) in PC12 cells. The relative expression of *Th* standardized by *Rpl19* was examined using real-time PCR analysis. Interestingly, the expression levels of *Th* and dopamine in the conditioned medium were reduced by exogenous Wnt3a treatment. *Th* expression was increased by adding XAV939 or sclerostin and the secreted dopamine level was also increased by XAV939. Sclerostin did not significantly affect dopamine levels, although there was a trend toward a gradual increase in dopamine secretion. These results indicate that the activation of Wnt-β-catenin signaling suppresses catecholamine synthesis in PC12 cells.

Interaction of Wnt β-Catenin Signaling and BMP Signaling

To explore more detailed mechanisms by which the activation of Wnt-β-catenin signaling enhanced cell proliferation and reduced catecholamine synthesis in PPGL cells, we examined the interaction of Wnt and BMP signaling in PC12 cells. BMPs have been reported to regulate cell growth, apoptosis, migration, and invasion in many cancers, including breast cancer, hepatocellular carcinoma, gastric cancer, lung cancer, and prostate cancer [12, 13]. However, the function of BMPs on PPGL growth has been scarcely explored, and thus we examined the effects of BMPs on the proliferation of PPGL cells using PC12 cells. Intracellular BMP systems including BMP ligands such as BMP2, BMP4, BMP7, their type I and II receptors, and Smads, have been reported to be expressed in PC12 cells

[14]. In the present study, we added BMP2, BMP4, or BMP7 to the cell culture medium of PC12 cells, and changes in viable cell number were evaluated using CellTiter 96. As shown in Fig. 4A, BMPs enhanced cell proliferation. Subsequently, we assessed the interaction of the BMP and Wnt signaling on cell proliferation of PC12 cells. Cells were cultured with BMP2, BMP4, and BMP7 in combination with Wnt3a, and proliferation assays were performed. Wnt3a failed to enhance the BMPs-induced increase in the viable cell number (Fig. 4B), although Wnt3a alone was shown to stimulate proliferation. As Wnt-β-catenin activation exerted no additive effect on BMP-induced cell proliferation, we examined the effect of Wnt3a on BMP-induced receptor Smads phosphorylation, which is activated by BMP receptor kinase and mediates the signals of BMPs (Fig. 5). Immunoblotting analysis showed that pretreatment with Wnt3a reduced BMP2, BMP4, and BMP7-induced Smad1/5/9 phosphorylation. Subsequently, we assessed the effect of BMPs on the expression of key molecules in the Wnt pathway. As shown in Fig. 6, BMP2 and BMP4 reduced endogenous Wnt3a mRNA expression, and BMP2 diminished Lrp5/6 expression while BMPs alone did not affect the Wnt-β-catenin target genes, such as Axin2 and Lef1. Thus, BMPs alone are not likely to directly suppress the steady-state Wnt-β-catenin pathway. However, BMP2, BMP4, and BMP7 markedly upregulated *SOST* mRNA expression. Furthermore, BMPs increased sclerostin protein expression. Wnt3a also increased it, although this was not significant (Fig. 1B). These data indicate the possibility that BMPs could suppress activated Wnt-β-catenin signaling through sclerostin upregulated by BMPs. Taken together, these results suggest that BMP and Wnt signaling suppress each other's pathways rather than act cooperatively, although both stimulate the proliferation of PC12 cells.

Discussion

The Wnt-altered subtype of PPGL was proposed by Fishbein et al in 2017, more recently than identification of the other 2 subtypes, pseudohypoxic and kinase signaling [2]. This subtype of PPGLs is sporadic and tends to be clinically aggressive and metastatic. Wnt-related gene alterations, including *MAML3* fusions and *CSDE1* mutations, lead to overactivation of β-catenin. In this study, we examined the molecular mechanisms modulated by overactivated Wnt-β-catenin signaling,
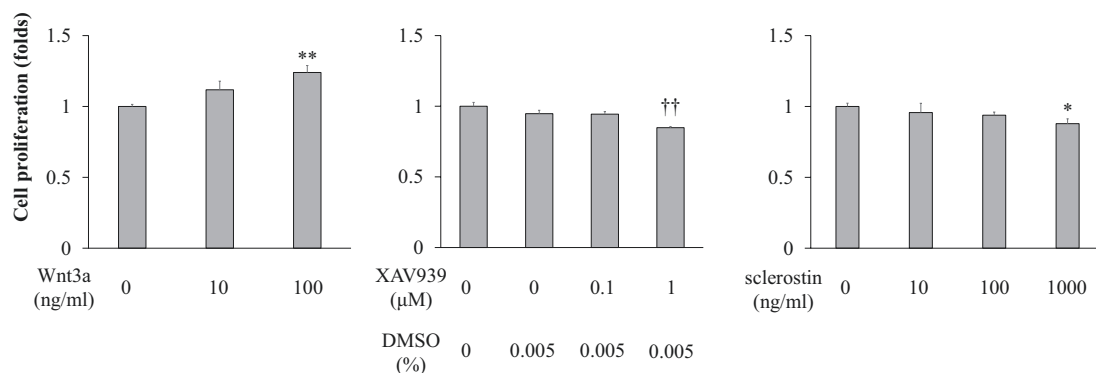
Figure 2. Effect of activated Wnt-β-catenin signaling on cell proliferation. Cells were seeded into 96-well culture plates at a density of 3000 cells/well, then incubated for 24 hours at 37 °C in a CO_2 incubator with indicated concentration of Wnt3a, XAV939, and sclerostin. Viable cells were measured by CellTiter 96. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with control, ††$P < 0.01$ compared with DMSO treatment alone as determined by unpaired *t* test.
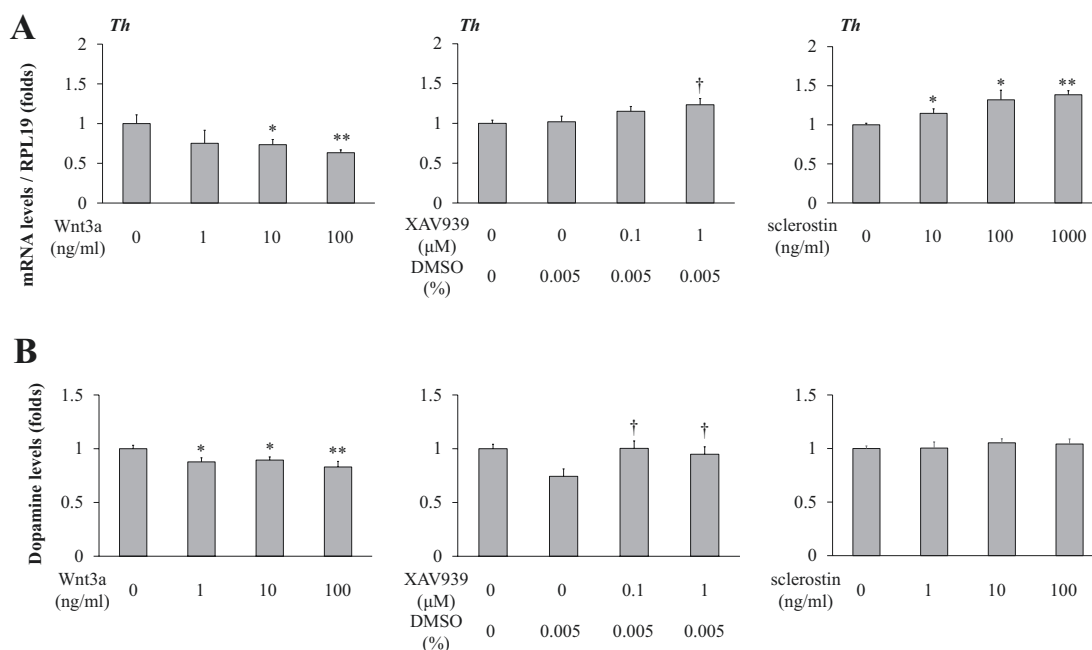
Figure 3. Effect of activated Wnt-β-catenin signaling on catecholamine synthesis. PC12 cells were cultured in DMEM containing 1% FCS and 1% HS, and then the cells were treated with the indicated concentrations of Wnt3a, XAV939, and sclerostin. (A) Total cellular RNAs were extracted after 24-hour culture, and mRNA levels of *Th* and *Rpl19* were analyzed by quantitative PCR. The expression levels of *Th* were standardized by *Rpl19* level in each sample, and levels of mRNA of target genes were expressed as fold changes. (B) The culture media were collected after 24-hour culture, and dopamine levels were measured by HPLC and expressed as fold changes standardized by each control level. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with control, †$P < 0.05$ compared with DMSO treatment alone as determined by unpaired t test.
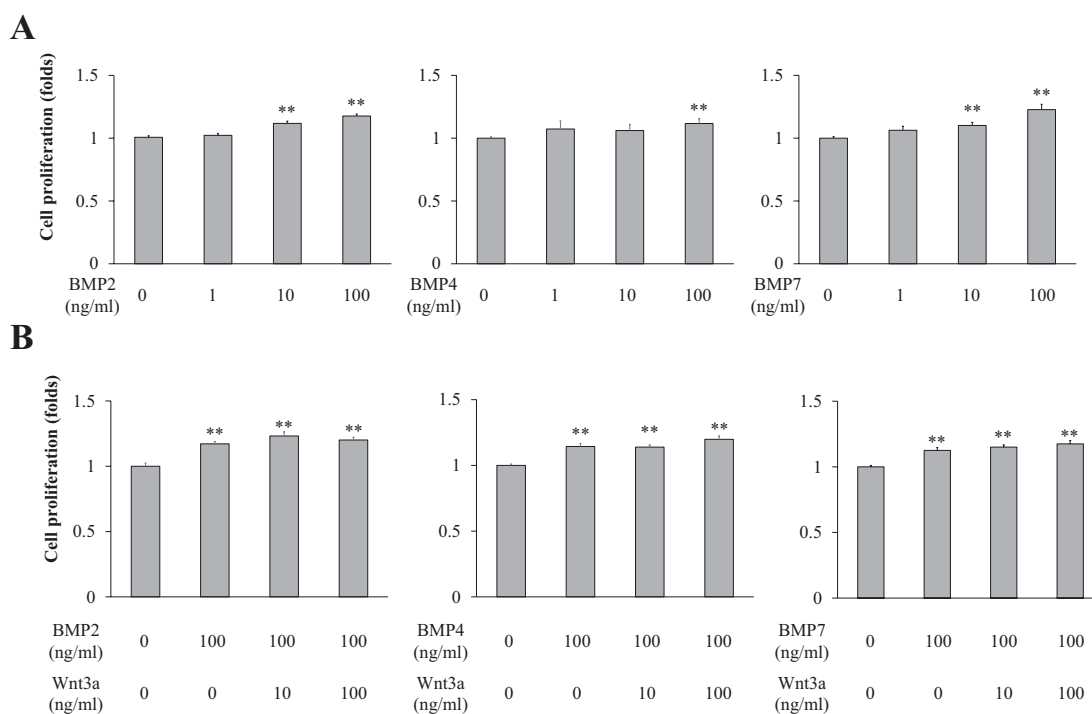

Figure 4. (A) Effect of BMP2, BMP4, and BMP7 on cell proliferation. Cells were seeded into 96-well culture plates at a density of 3000 cells/well, then incubated for 24 hours at 37 °C in a CO_2 incubator with indicated concentration of BMP2, BMP4, and BMP7. Viable cells were measured by CellTiter 96. (B) Effect of Wnt3a on cell proliferation induced by BMP2, BMP4, and BMP7. Cells were seeded into 96-well culture plates at a density of 3000 cells/well, then incubated for 24 hours at 37 °C in a CO_2 incubator with indicated concentration of BMP2, BMP4, and BMP7 with or without indicated concentrations of Wnt3a. Viable cells were measured by CellTiter 96. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. **$P < 0.01$ compared with control as determined by unpaired t test.
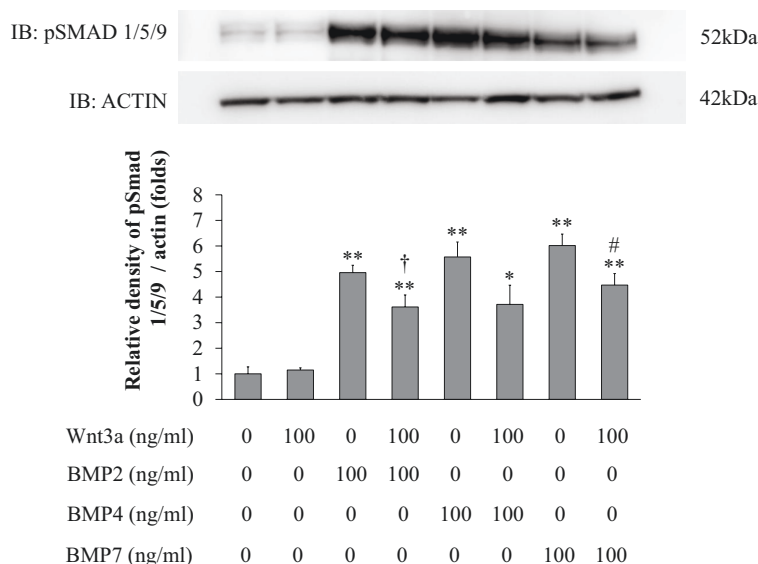
Figure 5. Effect of Wnt3a on BMP-induced phosphorylation of Smad1/5/9. After preculture with Wnt3a for 24 hours, cells were stimulated with BMP2, BMP4, and BMP7 for 1 hour. The cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-pSMAD1/5/9 and anti-ACTIN antibodies. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with control, †$P < 0.05$ compared with BMP2 treatment alone, #$P < 0.05$ compared with BMP7 treatment alone as determined by unpaired t test.

which may contribute to aggressive disease. We found that activation of Wnt-β-catenin enhanced cell proliferation whereas it slightly reduced catecholamine synthesis in PC12 cells, sclerostin was expressed in PC12, and the expression levels were upregulated by BMPs. Sclerostin, a negative regulator of Wnt signaling, plays an important role in malignant diseases with bone involvement [15-18].

Activation of the Wnt-β-catenin pathway promoted cell proliferation and blockade of the endogenous Wnt-β-catenin pathway by sclerostin as well as XAV939 reduced cell proliferation. However, tyrosine hydroxylase mRNA expression and dopamine secretion were decreased by enhanced Wnt-β-catenin signaling. To the best of our knowledge, the catecholamine synthesis potential of Wnt-altered PPGL has not been evaluated till date. In contrast, Rao et al investigated the relationship between genotype-specific differences in catecholamine content in PPGL tumor tissues, comparing pseudohypoxic subtype with mutations of *SDHA, SDHB, SDHC, SDHD,* and *SDHAF2* (*SDHx*) to kinase signaling subtype with mutations of *RET* [19]. In their study, the total catecholamine content per tumor tissue weight was higher in PPGLs with *RET* mutations than in tumors with *SDHx* mutations. This difference was speculated to be partially due to increased tyrosine hydroxylase activity in tumors carrying gain-of-function mutations in *RET* [19]. On the contrary, tyrosine hydroxylase expression levels in the activated Wnt-β-catenin condition, as a model of the Wnt-altered subtype, were decreased in the present study. The effects of Wnt-β-catenin signaling on cell proliferation and catecholamine synthesis were similar to those of BMP-Smad signaling. BMPs, another type of growth factor that plays pro- or anti-oncogenic roles, suppress catecholamine synthesis [14]. In the present study, they promoted proliferation of PC12 cells as shown in Fig. 4. Our observation agreed with a previous study in which transfection of a BMP7 plasmid into PC12 cells promoted cell proliferation, as assessed by tetrazolium salt assay [20]. However, Wnt signaling inhibited BMP-induced Smad phosphorylation and BMPs enhanced sclerostin expression. Therefore, these 2 signaling pathways did not exert any additive or synergistic effects.

In this study, we showed that sclerostin was expressed at the mRNA and protein levels and that the expression levels were increased by treatment with exogenous BMPs in PC12 cells. To the best of our knowledge, this is the first study to focus the sclerostin expression in PPGL tumors or cell lines. However, according to the expression profile data of 178 PPGL tumors from 173 patients in the TCGA transcriptional study [2] obtained from the publicly available cBioPortal platform (https://www.cbioportal.org/), 72 PPGL tumors, including 5 tumors with *MAML3* fusion genes and a tumor with a *CSDE1* somatic mutation, presented various levels of *SOST* mRNA expression. Sclerostin is prominently produced by osteocytes and functions as a key regulator of normal bone remodeling, which inhibits bone formation by inhibiting Wnt signaling [21]. Furthermore, sclerostin is expressed in certain pathological conditions, including malignant diseases with bone involvement [15-18]. Sclerostin mRNA and protein expression has been reported in multiple myeloma cell lines (H929, RPMI-8226, U266, and Karpas909) and myeloma cells derived from patients with multiple myeloma [16]. The expression levels are higher in myeloma cells isolated from multiple myeloma patients with osteolytic disease than in those without bone disease [15]. The study with co-culture of human myeloma cell lines and bone marrow stromal cells differentiated into osteoblasts by Colucci et al shows that sclerostin produced by myeloma cells can suppress bone formation in the osteolytic disease of multiple myeloma [16]. Similarly, sclerostin, which is produced by cancer cells, is involved in breast cancer bone metastasis, which is mostly osteolytic and is observed in almost 70% of breast cancer patients [17, 18]. In a study by Zhu et al, sclerostin was overexpressed in tumor tissue from breast cancer patients with bone metastasis and in breast cancer cell lines. In addition, sclerostin-neutralizing antibody suppressed the migration and invasion of breast cancer cell lines and prevented osteolytic lesions resulting from tumor metastasis in a xenograft model [18]. Bone has been reported to be the most common site of distant metastasis of PPGL, accounting for approximately 70% [22, 23]. Our results and previous reports regarding the effect of sclerostin on bone lesions in multiple
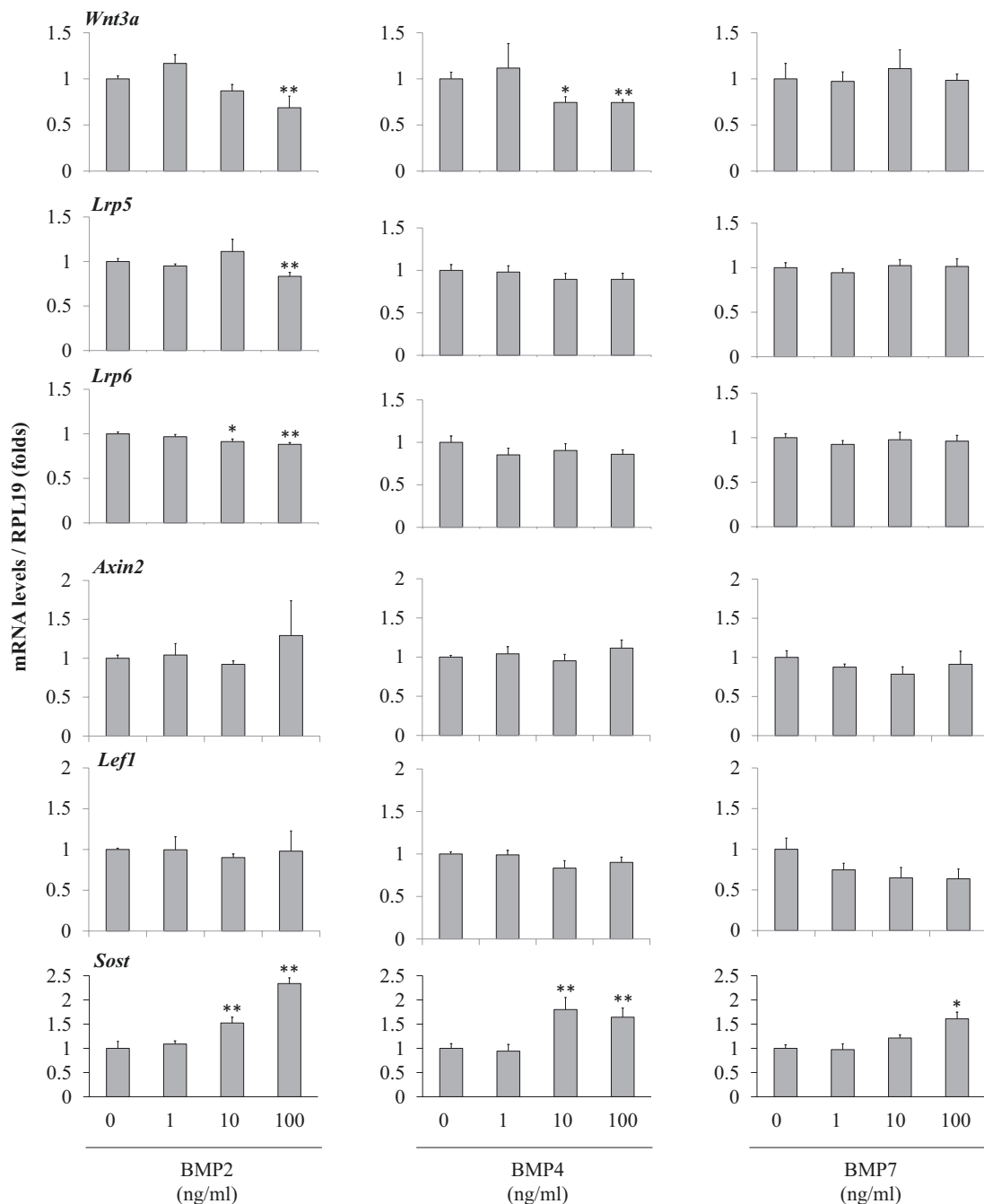
Figure 6. Effect of BMP on Wnt-β-catenin signaling. Total cellular RNAs were extracted after 24-hour culture, and mRNA levels were analyzed by quantitative PCR. The expression levels of target mRNA were standardized by *Rpl19* level in respective sample, and then levels of mRNA of target genes were expressed as fold changes. Data are shown as mean ± SE from at least 3 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with control as determined by unpaired t test.

myeloma and breast cancer suggest that sclerostin derived from PPGL cells may be involved in bone metastasis by suppressing osteoblast differentiation and that BMPs produced by osteoblasts may induce PPGLs sclerostin production.

However, this study contains some limitations. All of the presented data were obtained from the experiments using a single rodent cell line, PC12. Stimulation by the treatment with Wnt3a was used to evaluate the effects of activated Wnt-β-catenin signaling on cell proliferation and catecholamine synthesis in the PPGL cells instead of the gene transfection and mutagenesis of the *MAML3* fusions and the *CSDE1* mutations reported in the study by Fishbein et al [2]. In addition,

PC12 cells carry a *MAX* gene mutation [24] belonging to kinase signaling subtype. This could influence the results of this study, although there is no evidence that suggests any intracellular signaling link between kinase signaling subtype and Wnt-altered subtype. Furthermore, although sclerostin is expressed in PPGL cells of some clinical cases, the difference in sclerostin expression levels between primary tumors and bone metastatic lesions remains to be evaluated to clarify the effect of sclerostin in the bone metastasis of PPGL. Co-culture studies of PPGL cells and osteoblasts are also needed to examine the presence of the interaction between these cells, which could be mediated by BMPs and sclerostin.

In conclusion, we have shown that Wnt-β-catenin systems including sclerostin in PC12 cells can modulate cell proliferation and catecholamine synthesis. Further studies are needed to clarify the detailed mechanisms by which genetic changes in the Wnt-altered PPGL subtype cause a clinically aggressive phenotype.

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Disclosures

The authors have nothing to disclose.

Data Availability

All datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

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