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# Smad mediates BMP-2-induced upregulation of FGF-evoked PC12 cell differentiation

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**Abstract We previously reported that bone morphogenetic protein (BMP)-2 augments fibroblast growth factor (FGF)-induced neuronal differentiation of PC12 cells by selectively upregulating FGF receptor (FGFR)-1 expression. Here we describe the underlying mechanism. BMP-2 activated Smad proteins in PC12 cells. Overexpression of Smad7 or Smad1, inhibitory and receptor-regulated isoforms, respectively, suppressed or enhanced BMP-2-induced upregulation of FGFR-1 expression. Smad 7 also inhibited the FGF-induced PC12 differentiation. Our findings indicate that activation of a Smad signaling pathway is required for upregulation of FGFR-1 expression by BMP-2 and for the synergistic induction of PC12 differentiation by BMP-2 and FGF.**


*Key words:* Smad; Bone morphogenetic protein-2; Fibrobast growth factor; Fibrobast growth factor receptor type-1; PC12 cells

## 1. Introduction

All of the more than twenty known fibroblast growth factor isoforms (FGFs) are considered to transmit their signal by activating specific cell surface tyrosine kinase receptors [FGF receptors (FGFRs)] [1–3]. In each case, FGFR signaling follows dimerization of the receptor molecules brought about by ligand binding in cooperation with heparan sulfate proteoglycans [4–6]. Thus far, genes encoding four FGFRs (*FGFR-1–4*) expressed in respective subsets of neurons in both the peripheral and central nervous systems have been identified. Their expression patterns, along with those of agonist FGFs, suggest that FGFs exert specific effects on distinct neuronal cell types during the course of development [7–13].

Bone morphogenetic proteins (BMPs) are a subclass of the transforming growth factor (TGF)-β superfamily active in both the developing and adult nervous systems [14,15]. BMPs bind to and activate two different serine/threonine kinase receptors (BMPR-I and BMPR-II). Upon activation, BMP receptors recruit and phosphorylate several receptor-regulated Smad transcription factors (Smad1, Smad5 or Smad8), which in turn translocate into the nucleus to regulate gene expression [16,17]. In addition, a separate pathway involving the TGF-β-activated kinase 1 (TAK1) and p38 mitogen-activated protein kinase (MAPK) is reportedly activated by BMPs in some cells [18,19].

Cellular responsiveness to a particular growth factor is determined by a host of factors, including expression of its receptor, and is the key determinant of cell fate during development, differentiation and many other physiological and pathological processes. In that regard, polypeptide growth factors, including FGF and TGF-β, often act in concert to regulate biological events, and several growth factors have been shown to upregulate FGFR-1 expression. For example, an intracellularly localized, high-molecular-weight form of FGF-2 upregulates FGFR-1 expression in a pancreatic cell line, thereby potentiating signals evoked by endogenous FGF [20]; TGF-β1 upregulates FGFR-1 expression in lung fibroblasts and enhances their mitogenic response to FGF [21]; insulin-like growth factor (IGF)-1 upregulates FGFR-1 in rabbit vascular smooth muscle cells and increases FGF-2-induced mitogenesis [22]; and PDGF (platelet-derived growth factor)-bb upregulates FGFR-1 in murine brain endothelial cells and augments FGF-2-induced plasminogen activator activity [23]. In the presence of certain neurotrophic factors, PC12 cells, which originate from the rat adrenal medulla and thus neural crest, differentiate into sympathoadrenal neurons. One of those neurotrophic factors is FGF, and BMP-2 also reportedly exerts a neurotrophic effect on PC12 cells [24,25], though in our hands its activity was much weaker than previously reported. Instead, we found that when present in combination, BMP-2 and FGF act synergistically to induce PC12 cell differentiation, i.e. BMP-2 upregulated expression of FGFR-1, which in turn enhanced the effect of FGF on PC12 differentiation [26]. To better understand the mechanism by which BMP-2 upregulates FGFR-1 expression, in the present study we examined the signaling pathway by which BMP-2 acts.

## 2. Materials and methods

### 2.1. Materials

PC12 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Recombinant human BMP-2 was kindly provided by Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba, Japan). Heparin was from Sigma (St. Louis, MO, USA). Anti-Smad1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Smad1 antibody was from Upstate Biotechnology (Lake Placid, NY, USA).

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*Abbreviations:* BMP, bone morphogenetic protein; FGF, fibroblast growth factor; FGFR, FGF receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; RT-PCR, reverse-transcription-polymerase chain reaction; TGF-β, transforming growth factor-β

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Anti-p38 and anti-phospho-p38 antibodies were from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology, respectively. The pGL3ti(SBE)4-luciferase reporter plasmid was a generous gift from Dr. W. Kruijer (Groningen Biomolecular Sciences and Biotechnology Institute, The Netherlands) [27]. Smad7 [28] and Smad1 [29] expression vectors were generous gifts from Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Sweden) and Dr. J.L. Wrana (University of Toronto, Canada), respectively.

### 2.2. Cell culture and induction of differentiation

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 10% horse serum. To assess their differentiation, the cells were first plated to a density of $5\times10^3$cells/well on collagen type IV-coated, 24-well culture plates (Becton Dickinson) for 24 h and then starved for 12 h in serum-free medium (DMEM containing 2 mg/ml bovine serum albumin, 1 μg/ml insulin, 2 μg/ml transferrin, 30 nM $Na_2SeO_3$, 20 nM progesterone, and 10 mM HEPES, pH 7.4). Thereafter, the cells were cultured in the serum-free medium with or without BMP-2. On day 2, the medium was replaced with fresh serum-free medium, and the culture was continued an additional day. On day 3, FGF was added to the medium, with or without BMP-2, and the culture was continued for the indicated times (0–6 days). Cell differentiation was evaluated by examining the cells under a phase-contrast microscope. Randomly selected fields containing approximately 100 cells each were photographed, and the numbers of undifferentiated and differentiated cells counted. The experimental criterion for distinguishing differentiated from undifferentiated cells was neurite outgrowth: cells having neurites greater in length than two cell body diameters were considered differentiated.

### 2.3. Transfection

We used the pGL3Ti(SBE)4-luciferase reporter plasmid, which contains four Smad-binding elements (SBE) from the *JunB* promoter [27], to monitor the inhibitory effect of Smad7. For transient transfection of pGL3ti(SBE)4-luciferase and β-galactosidase expression plasmid, cells were first plated to a subconfluent density on 24-well culture plates. The next day, they were transiently transfected with the plasmids using Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instruction, after which they were stimulated with 50 ng/ml BMP-2 for 48 h, lysed, and the luciferase activity in the lysate was measured using a Luciferase Assay System (Promega). As an internal control, luciferase activity was normalized to β-galactosidase activity measured using a Beta-Galactosidase Enzyme Assay System (Promega). Data are expressed as the means ± S.D. of three independent determinations.

### 2.4. Quantitation of mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)/Southern blot analysis

Semi-quantitative RT-PCR was carried out essentially as described previously (13, 27). Briefly, total RNA was isolated using Isogen (Nippon Gene), after which a 1-μg sample was reverse transcribed using Superscript II according to manufacturer's instructions. The specific primers used for amplification were 5′-ttc tgg gct gtg ctg gtc ac-3′ (sense) and 5′-gcg aac ctt gta gcc tcc aa-3′ (antisense) for FGFR-1, and 5′-ttc att gac ctc aac tac atg-3′ (sense) and 5′-gtg gca gtg atg gca tgg ac-3′ (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In a preliminary experiment, the expression levels of the targeted mRNAs in PC12 cells were assessed, and conditions limiting amplification to within the linear exponential range were determined (31 cycles of 1 min at 94°C, 2 min at 65°C and 1 min at 72°C for FGFR-1, and 18 cycles of 1 min at 94°C, 2 min at 65°C and 1 min at 72°C for GAPDH). Following PCR, aliquots of the product were run on 1.0% agarose gels, after which the resultant DNAs were denatured with 0.5 N NaOH and transferred onto Hybond-N+membranes. The filters were then hybridized with the corresponding DNA probe labeled with digoxygenin-conjugated dUTP using the random priming method. The intensity of each band on the image was measured, and the data were processed using NIH Image version 1.61 image processing software.

### 2.5. Immunoblotting

Growth factor-stimulated PC12 cells were collected; washed twice with 1 ml of ice-cold phosphate-buffered saline; lysed for 10 min on ice in 100 μl of lysis buffer (20 mM Tris–HCl (pH7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, and 1 μg/ml leupeptin); and then centrifuged to remove the cell debris. The protein concentration in the supernatant was determined using a Bio-Rad protein assay, after which equal amounts of protein (40 μg for phospho-Smad1 and phospho-p38, 10 μg for Smad1 and p38) were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrophoretically transferred to PVDF transfer membranes (Immobilon-P; Millipore), and probed with the appropriate antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

## 3. Results

### 3.1. Effects of BMP-2 on neuronal differentiation and FGFR-1 expression in PC12 cells

We found that BMP-2 (50 ng/ml) did not induce significant differentiation of PC12 cells during the 6 days (144 h) of culture (Fig. 1A), which confirmed our earlier finding that, by itself, BMP-2 has only a very weak neurotrophic effect on PC12 cells [26]. In the same earlier study, we also found that BMP-2 significantly upregulated expression of FGFR-1 mRNA in PC12 cells [26]. Here we examined the time course of that effect more precisely (Fig. 1B). PC12 cells were treated with BMP-2 for the indicated period, and expression of FGFR-1 mRNA was evaluated using semi-quantitative RT-PCR/Southern blot analysis as previously described [13,30]. As shown, the level of FGFR-1 mRNA began to increase after 18 h of incubation with BMP-2, rising sharply for the

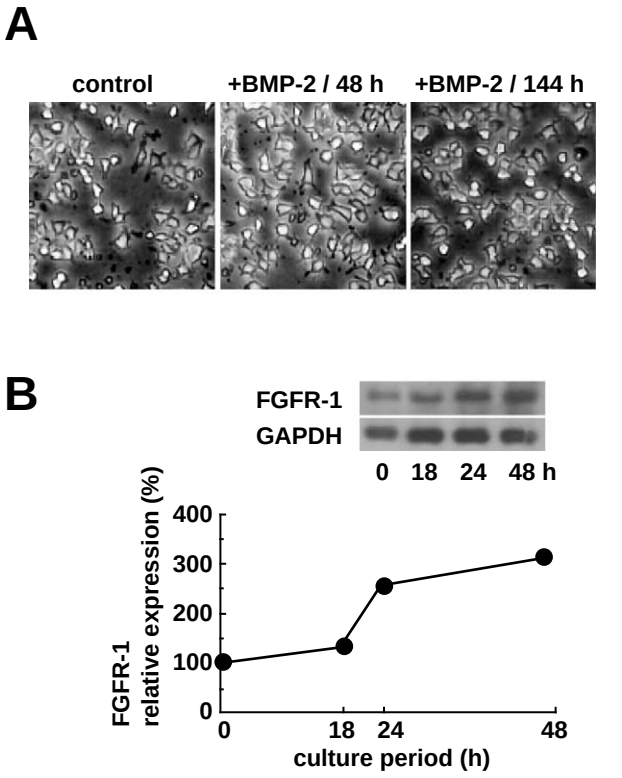



Fig. 1. BMP-2 upregulates FGFR-1 expression in PC12 cells but does not induce differentiation. A: Phase contrast micrographs showing untreated PC12 cells (control) and cells cultured for 48 h or 144 h with BMP-2 (50 ng/ml). B: Upregulation of FGFR-1 mRNA expression by BMP-2. PC12 cells were treated with BMP-2 (50 ng/ml) for the indicated times, after which expression of FGFR-1 mRNA was analyzed by semi-quantitative RT-PCR/Southern blot analysis. The signal intensities were quantified and normalized to the respective GAPDH signals. The initial expression (0 time) was assigned a value of 100%.

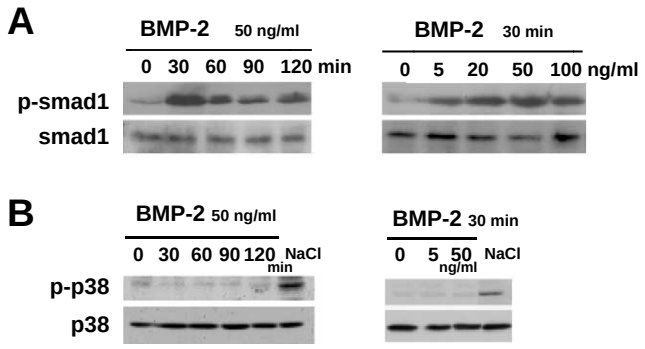



Fig. 2. BMP-2 activates Smad1 but not p38/MAPK in PC12 cells. A: Activation of Smad1 by BMP-2. Serum-starved PC12 cells were treated with BMP-2 (50 ng/ml) for the indicated times (left panels) or with the indicated concentrations for 30 min (right panels). The cell proteins were resolved by SDS–PAGE, transferred to a membrane, and probed with an anti-phospho-Smad1 (upper panels) or anti-Smad1 (lower panels) antibody. B: Absence of p38/MAPK activation in BMP-2-treated cells. Proteins isolated from BMP-2-treated cells were processed as in A and probed with anti-phospho-p38/MAPK (upper panels) or anti-p38/MAPK (lower panels) antibody. As a positive control for p38 activation, a separate culture was osmotically stressed for 15 min in 200 mM NaCl.

first 6 h and then more slowly for an additional 24 h thereafter. Stimulation of the PC12 cells with BMP-2 for periods shorter than 18 h did not affect FGFR-1 expression (data not shown).

### 3.2. *BMP-2 activates Smad signaling*

There are two pathways along which BMP-2 signaling is transduced, one via receptor-regulated Smads [16,17] and the other via p38/MAPK [31,32]. The first is a well-characterized signal transduction pathway for TGF-β family proteins; in fact, we previously showed that BMP-2 induced phosphorylation of Smad1 in PC12 cells [26]. On the other hand, Yanagisawa et al. [33] showed that introduction of a kinase-negative form of TAK1, an upstream kinase that may phosphorylate p38/MAPK, inhibited differentiation of PC12 cells stimulated with BMP-2. Both of these signaling pathways can be activated by the binding of BMP-2 to its cell surface receptors and their subsequent translocation to the nucleus. We therefore investigated whether one or both were responsible for the upregulation of FGFR-1 expression by BMP-2. PC12 cells were treated with selected concentrations of BMP-2 for the indicated periods of time, and the phosphorylated forms of Smad1 and p38/MAPK were analyzed by immunoblotting using specific antibodies (Fig. 2). BMP-2 evoked concentration-dependent phosphorylation of Smad1 that reached a maximum within 30 min and then persisted throughout the 120-min period of BMP-2 stimulation (Fig. 2A). By contrast, BMP-2 had no effect on p38/MAPK phosphorylation at any time or at any concentration (Fig. 2B), although phosphorylation was clearly induced by osmotic stress (Fig. 2B, NaCl), as was reported previously [34].

### 3.3. *Smad signaling mediates BMP-2-induced upregulation of FGFR-1 expression*

By inhibiting phosphorylation of receptor-regulated Smads, Smad7 selectively inhibits signaling by several TGF-β family polypeptides, including BMP-2 [26]. We therefore overexpressed Smad7 in PC12 cells to test whether Smad signaling is necessary for BMP-2-induced upregulation of *FGFR-1* expression. To monitor the inhibitory effect of Smad7, we used the pGL3Ti(SBE)4-luciferase reporter plasmid, which contains four SBE from the *JunB* promoter [27]. The cells were transfected with pGL3Ti(SBE)4-luciferase plasmid, with or without the Smad7 expression vector, then incubated with or without BMP-2 for 2 days, after which they were solubilized, and the luciferase activity was detected. As shown in Fig. 3A, treating cells with BMP-2 clearly enhanced SBE activity, and the effect was completely blocked by overexpression of Smad7. Moreover, transfecting cells with Smad7 also completely blocked the BMP-2-induced upregulation of *FGFR-1* expression (Fig. 3B).

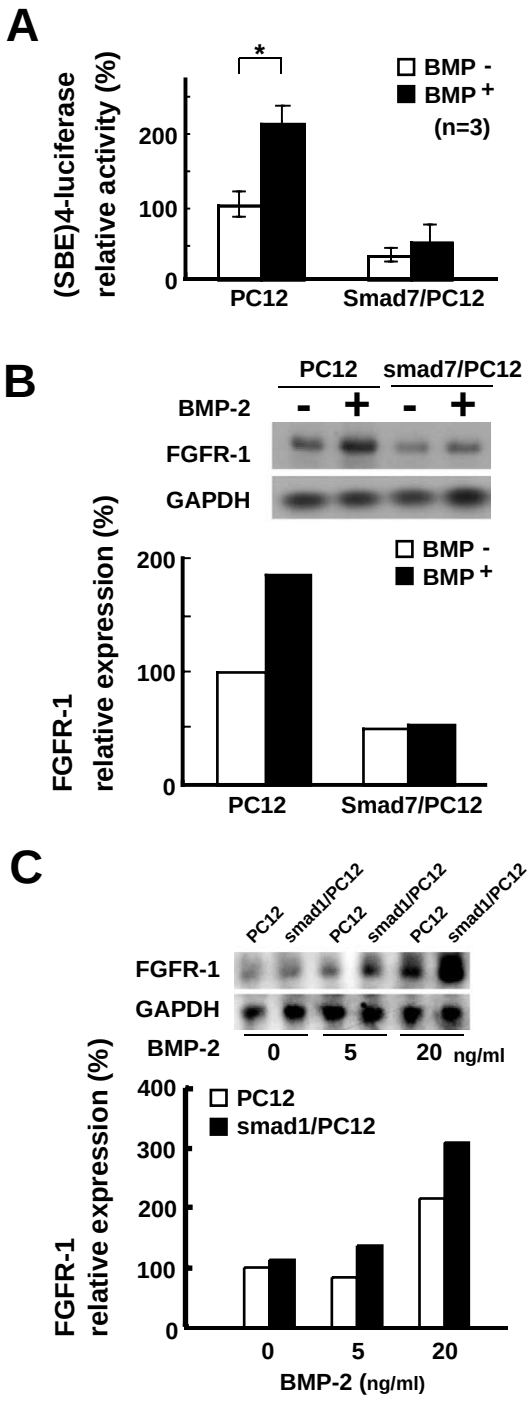
To directly determine whether augmentation of Smad signaling results in the augmentation of BMP-2-induced upregulation of FGFR-1, we transfected PC12 cells with an expression vector encoding Smad1, a receptor-regulated isoform of Smad that transduces BMP-2 signaling in PC12 cells (Fig. 2A). After confirming the overexpression of Smad1 protein by immunoblotting (data not shown), the transfectants were stimulated with various concentrations of BMP-2, and the level of FGFR-1 mRNA expression was evaluated with semi-quantitative RT-RCR/Southern blot analysis [13,30]. As shown in Fig. 3C, BMP-2 induced upregulation of FGFR-1 expression more strongly in cells overexpressing Smad1 than in the mock transfectants, most notably at 20 ng/ml. Thus, the effects of overexpressing an inhibitory and a receptor-regulated Smad both confirmed that Smad signaling mediates BMP-2-induced upregulation of FGFR-1 expression.

### 3.4. *Inhibition of Smad signaling suppresses the BMP-2-mediated enhancement of FGF-induced PC12 cell differentiation*

Finally, to confirm that BMP-2 augments FGF-induced PC12 cell differentiation through upregulation of FGFR-1 expression, we examined the effect of Smad7 overexpression on the synergistic relation between FGF and BMP-2. PC12 cells transfected with Smad7 expression vector were exposed to BMP-2 (50 ng/ml) for 3 days and then to FGF-1 (5 ng/ml) for an additional 3 days (Fig. 4). While $>30\%$ of mock transfectants differentiated within the 6 days, significantly fewer Smad7 transfectants exhibited FGF-induced differentiation (Fig. 4A,B). Apparently, BMP-2-induced Smad signaling is responsible for the augmentation of FGF-evoked PC12 cell differentiation.

## 4. Discussion

To better understand the molecular mechanism by which BMP-2 augments expression of *FGFR-1* and promotes FGF-dependent neuronal differentiation of PC12 cells, we examined two possible signaling pathways for BMP-2 activity, one Smad-dependent and the other p38/MAPK-dependent. We found that Smad signaling was clearly activated by BMP-2 and that overexpression of Smad7, an inhibitory Smad isoform that blocks phosphorylation of receptor-regulated Smads, abolished BMP-2′s ability to upregulate FGFR-1 expression and augment FGF-dependent neuronal differentiation. Furthermore, overexpression of Smad1, a receptor-regulated Smad that transduces BMP-2 signaling, enhanced BMP-2-induced upregulation of FGFR-1 expression. Thus, BMP-2-




induced upregulation of FGFR-1 expression is apparently mediated via a Smad signaling pathway.

By contrast, activation of p38/MAPK by BMP-2 was not detected. Our finding that BMP-2 does not significantly activate p38/MAPK or induce neurite outgrowth from PC12 cells differs from earlier studies that suggest activation of a p38/MAPK pathway by BMP-2 is sufficient for neuronal differentiation of PC12 cells [18,19]. This is despite the fact that we examined separate PC12 cell stocks obtained independently from four depositories and laboratories. Moreover, our in vitro kinase assays using ATF-2 as a substrate for p38/MAPK confirmed that treating PC12 cells with BMP-2 (50 ng/ml) did not activate p38/MAPK, even though it was clearly

Fig. 3. Smad signaling mediates BMP-2-induced upregulation of FGFR-1 expression. A: Inhibition of Smad signaling by Smad7. Smad signaling was measured as a function of SBE activity. PC12 cells cotransfected with pGLti(SBE)4 reporter plasmid, β-galactosidase expression plasmid, and Smad7 expression plasmid or a control plasmid (pcDNA3) were treated with BMP-2 or left untreated. Luciferase activity normalized to β-galactosidase activity is shown. The data are represented as means ± S.D. ($n = 3$) and were analyzed by 2-way analysis of variance (ANOVA) using DA Stats software; $^*P < 0.05$. B: Inhibition of BMP-2-induced FGFR-1 upregulation by Smad7. PC12 cells transfected with Smad7 or control plasmid were cultured for 2 days in the presence or absence of BMP-2 (50 ng/ml). Expression of FGFR-1 mRNA was analyzed as in Fig. 1. The expression level in control transfectants without BMP-2 was assigned a value of 100%. C: Promotion of BMP-2-induced FGFR-1 upregulation by Smad1. PC12 cells transfected with Smad1 or control plasmid were cultured for 2 days with BMP-2 at the indicated concentrations. The data were processed as in B. Three independent experiments yielded essentially the same results.
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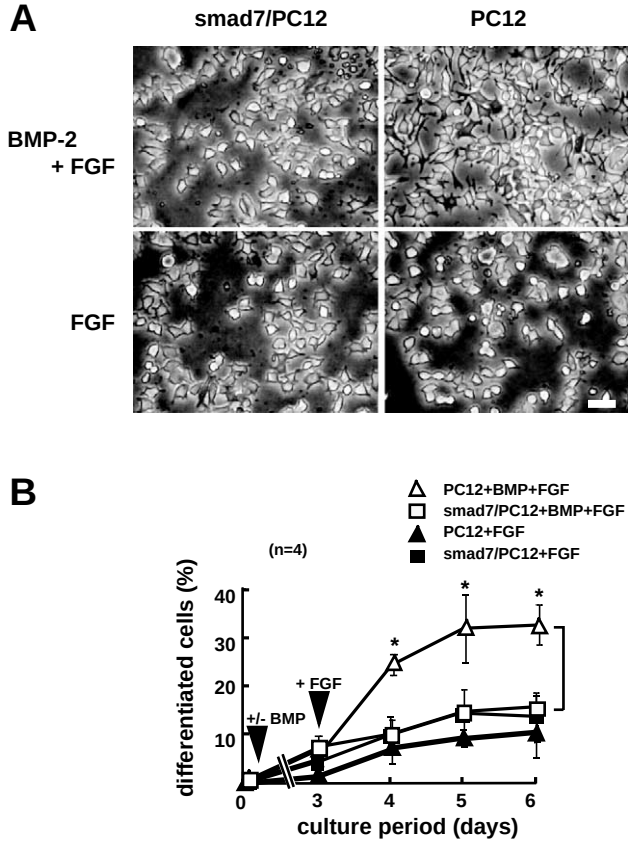


Fig. 4. Smad7 inhibits synergistic effect of BMP-2 on FGF-induced differentiation of PC12 cells. A: Phase contrast micrographs of PC12 cells transfected with a Smad7 expression plasmid (Smad7/PC12) or a control plasmid (PC12). Cells were cultured for 12 h, replated, and then cultured for the indicated time in the presence or absence of 50 ng/ml BMP-2. The cultures received FGF-1 (5 ng/ml) plus heparin (5 μg/ml) on day 3 and continued until day 6. The micrographs were obtained on day 5, and the state of their differentiation was determined. Bar, 50 μm. B: Cultures were conducted as in A, and neuronal differentiation was evaluated on the indicated days; more than 650 cells from four separate cultures were observed for each condition. The data were analyzed by 2-way ANOVA using DA Stats software; $^*P < 0.01$ vs. Smad7/PC12+BMP+FGF (open squares).

activated by osmotic stress (Hayashi, unpublished observation). We reasoned, therefore, that the observed lack of p38/MAPK activation by BMP-2 in our experiment might be due to lower expression of TAK1, a putative upstream kinase for p38/MAPK. To address this question, we established PC12 transfectants overexpressing TAK1 and analyzed their differentiation. However, even in these cells, phosphorylation of p38/MAPK was not detected after BMP-2 treatment (Hayashi, unpublished observation). It appears that, by itself, TAK1 overexpression is not sufficient to activate p38/MAPK in BMP-2-stimulated PC12 cells, and that upregulation of FGFR-1 expression by BMP-2 is independent of the p38/MAPK pathway. Still, the reason for the lack of p38/MAPK activation in our experiment remains unclear; perhaps an upstream regulator of p38/MAPK other than TAK1 is involved; or the level of p38/MAPK phosphorylation is too low to be detected.

The transcriptional regulators and regulatory elements that directly affect expression of *FGFR-1* are presently unknown; however, the finding that upregulation of FGFR-1 mRNA expression only occurs when cells are exposed to BMP-2 for longer than 18 h (Fig. 1A) suggests the effect is indirect. With respect to the regulatory elements, previous studies of the regulation of *FGFR-1* expression concluded that the fact that the gene lacks TATA or CCAAT elements in its promoter region, and that the 5′-flanking region from position −62 to −42 lacks any consensus sequence plays a pivotal role in its expression [35]. On the other hand, two Sp1-binding elements located well upstream of the transcription start site were determined to regulate *FGFR-1* expression in myoblasts [36]. Characterization of the regulatory elements governing *FGFR-1* expression induced by BMP-2 awaits further study.

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