Investigating Bone Morphogenetic Protein (BMP) Signaling in a Newly Established Human Cell Line Expressing BMP Receptor Type II

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Bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) cytokine superfamily, elicit various biological effects in different tissues (Hogan 1996). BMP receptor type II (BMPRII) contains a unique carboxyl-terminal region that interacts with multiple signaling molecules. However, expression of endogenous BMPRII is low in various mammalian cell lines, which hampers the analysis of BMP signaling. Therefore, we established a human cell line expressing BMPRII tagged with a Flag epitope (BMPRII-Flag) using the tetracycline-controlled Flp-In T-REx gene expression system. The BMPRII-Flag gene was introduced into the Flp-In T-REx 293 (FT293) cell line, a derivative of human 293 embryonic kidney fibroblasts. Then we analyzed the expression of key BMP target genes, inhibitors of DNA binding (Id) family members (Id1, Id2, and Id3) and the inhibitory Smads Smad6 and Smad7, in parental FT293 cells and an established cell line, FT293-BMPRII, by quantitative real-time PCR. Tetracycline treatment significantly increased the expression of BMPRII-Flag mRNA and protein in FT293-BMPRII cells, but induced no significant changes in expression of Id1, Id2, Id3, Smad6, or Smad7 mRNA. In contrast, treatment with a BMPRII ligand BMP2 induced the expression of Id1, Id2, Id3, and Smad6 in parental FT293 cells and FT293-BMPRII cells. Tetracycline-induced BMPRII-Flag expression significantly enhanced the induction of Id1, Id3, and Smad6 mRNA expression in FT293-BMPRII cells treated with BMP2. These findings provide evidence that although BMPRII has no obvious effect on the expression of representative BMP target genes, it differentially modulates the responsiveness of target genes to BMP2.

Keywords: bone morphogenetic protein; BMP receptor type II; stable cell line; tetracycline regulation; Flag-epitope tagging

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Bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) cytokine superfamily, elicit various biological effects in different tissues (Hogan 1996). They interact with two classes of transmembrane receptor serine-threonine protein kinases, termed type I and type II receptors (Wrana et al. 1994). Type II receptors phosphorylate and activate type I receptors, which in turn phosphorylate and activate the receptor-regulated Smad (R-Smad) proteins Smad1, Smad5, and Smad8 (Ishidou et al. 1995; Ebendal et al. 1998). The activated R-Smad proteins then translocate to the nucleus in association with the common mediator Smad4, where they act as transcription factors to regulate the expression of target genes, including members of the inhibitors of DNA binding (Id) family (Id1, Id2, and Id3) and the inhibitory Smads (I-Smad) Smad6 and Smad7 (Imamura et al. 1997; Miyazono and Miyazawa 2002). The Id proteins are a family of transcription factors that negatively regulate other transcription factors (Miyazono and Miyazawa 2002). They regulate cell growth and differentiation in embryonic and adult tissues (de
Candia et al. 2004). Smad6 and Smad7 inhibit BMP signaling by interfering with the activation of other Smads (Topper et al. 1997; Imamura et al. 1997). Their expression is induced by BMPs, which provides negative feedback control (Aframkh et al. 1998). In addition, the p38 mitogen-activated protein kinase (MAPK) signaling pathway, which controls cellular responses to cytokines and stress, is activated following the binding of BMPs to their receptors (Kozawa et al. 2002).

The BMP receptor type II (BMPRII) is a member of the type II receptor class, which binds to BMP2, BMP4, and BMP7, and is important for BMP signaling (Rosenzweig et al. 1995). In BMPRII homozygous null mice, development is arrested prior to gastrulation, an early phase in embryonic development (Beppu et al. 2000). Heterozygous mutations identified in the human BMPRII gene have been linked to pulmonary arterial hypertension (PAH), a disorder of the pulmonary arteries (Lane et al. 2000). This suggests that Id proteins play important roles in vascular tissue homeostasis.

Unlike other type II receptors in the TGF-β superfamily, BMPRII has an unusually large carboxyl-terminal domain (cytoplasmic tail) with no enzymatic activity (Fig. 1A) (Nishihara et al. 2002). BMPRII mutants completely lacking this cytoplasmic tail domain can transduce BMP-Smad signals (Nishihara et al. 2002). However, recent studies suggest that the cytoplasmic tail functions as a mediator of both Smad and non-Smad BMP signaling (Foletta et al. 2003; Chan et al. 2007; Kudo et al. 2007).

Because native expression of BMPRII is quite low in various mammalian cell lines, it is difficult to study the functions and regulatory roles of endogenously expressed BMPRII. Therefore, we aimed to clarify the regulatory roles of BMPRII by developing a new model system. We generated a human cell line expressing human BMPRII using a tetracycline-controlled gene expression system, which enabled us to eliminate the potential influence of a genomic insertion site and gene dosage on gene expression (see Materials and Methods for details). A single copy of a tetracycline-inducible gene encoding Flag epitope-tagged human BMPRII (BMPRII-Flag) was introduced into the Flp-In T-REx 293 (FT293) parental cells to generate a stable cell line with a single integrated copy of the BMPRII-Flag expression vector (FT293-BMPRII), created through Flp recombinase-mediated DNA recombination at the FRT site. The FT293 cell line is a derivative of the human embryonic kidney 293 (HEK293) fibroblast cell line containing an FRT site. It stably expresses a bacterial transcription factor, Tet repressor protein (TetR). The expression of BMPRII-Flag in FT293-BMPRII cells is repressed by TetR in the absence of tetracycline. This repression is lifted when tetracycline is introduced.

Materials and Methods

Materials

Recombinant human BMP2 was obtained from Peprotech
(Rocky Hill, NJ, USA), the p38 MAPK-specific inhibitor SB203580 (Badger et al. 1996) was from Enzo Life Sciences (Farmingdale, NY, USA), and dorsomorphin, a specific inhibitor of type I BMP receptors (Yu et al. 2008; Hao et al. 2008) was from Calbiochem (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. A horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG antibody was purchased from Cell Signaling Technologies (Danvers, MA, USA).

**Plasmid construction and generation of stable cell lines**

A gene encoding human BMPRII with a Flag epitope fused to its C-terminus (BMPRII-Flag; Fig. 1A) was amplified by PCR and cloned into the inducible expression vector pcDNA5/FRT/TO-TOPO (Invitrogen). Flag epitope-tagging was employed to ease the detection of conditionally expressed BMPRII protein by Western blot (using an anti-Flag antibody), as well as to provide a useful means of purifying protein complexes containing conditionally expressed BMPRII (also using an anti-Flag antibody). A stable human cell line conditionally expressing tetracycline-regulated BMPRII-Flag was generated using the Flp-In T-REx system (Invitrogen)—a tetracycline-controlled gene expression system—as described by Hiratsuka et al. (2008). Briefly, a Flp recombinase expression vector, pOG44; Invitrogen) and the pcDNA5/FRT/TO-TOPO expression vector carrying BMPRII-Flag (which also contains a Flp recombination target [FRT] site, as well as a hygromycin resistance gene) were co-transfected into parental FT293 cells that carry a single FRT site and stably express the bacterial transcription factor Tet repressor protein (TetR), using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). The FRT site serves as both the recognition and cleavage site for the Flp, a site-specific yeast recombinase, and allows DNA recombination to occur immediately (O’Gorman et al. 1991). After co-transfection, Flp recombinase catalyzes a site-specific DNA recombination event between the FRT sites on the BMPRII-Flag expression vector and host cell DNA to generate a stable cell line carrying a single integrated copy of the BMPRII-Flag expression vector (Fig. 1B). Its use obviates the potential effects of a genomic insertion site and gene dosage on gene expression. TetR binds to the promoter region of the pcDNA5/FRT/TO-TOPO expression vector in the absence of tetracycline and represses transcription of the integrated gene. The binding of TetR by tetracycline renders the former unable to bind to the promoter, and hence transcription of the integrated gene is induced (Hillen and Berens 1994; Yao et al. 1998). To select for BMPRII-Flag-integrated cells, surviving cells were cultured in Eagle’s minimum essential medium (MEM; Wako Pure Chemical Industries, Osaka, Japan) containing the antibiotics blasticidin (30 μg/ml; Invitrogen), which allows selection of TetR stable transfectants in this system, and hygromycin (100 μg/ml; Invitrogen). A single copy of the cell line generated was tested in this study.

**Cell culture**

Parental FT293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries) containing 10% horse serum (Invitrogen) and 30 μg/ml blasticidin. FT293-BMPRII cells were grown in DMEM containing 10% horse serum, 30 μg/ml blasticidin, and 100 μg/ml hygromycin. The expression of BMPRII-Flag was induced by treating cells seeded to 12-well plates with tetracycline (1 or 10 μg/ml) for 3, 12, or 24 h.

**Quantitative real-time PCR analysis**

Total RNA was extracted from cultured cells using an RNA purification kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). Total RNA was reverse-transcribed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer’s instructions. The resulting first-strand cDNA samples were diluted and used as a template in subsequent quantitative real-time PCR (QRT-PCR) analyses. These analyses were performed using a Thunderbird SYBR qPCR Mix (Toyobo) in conjunction with a Thermal Cycler Dice Real Time System (TP800; Takara Bio, Otsu, Japan) and Dice Real Time System software (version 2.10B; Takara Bio) according to the manufacturer’s instructions. Forty QRT-PCR cycles were performed. Then expression of the different target genes in each sample was quantified in separate tubes with the indicated primers (Table 1). All expression data were normalized to the levels of the internal control β-actin.

**Western blotting**

Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [w/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] sodium dodecyl sulfate [SDS], 0.2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and a cocktail of protease inhibitors [Roche Diagnostics]). The resulting cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatants were used as cell extracts. Extract protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of the protein extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrobotted onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare, Piscataway, NJ, USA). The membranes were blocked with 1% (w/v) blocking reagent (Roche Diagnostics Corp.) in Tris-buffered saline containing Tween 20 (TBST: 20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 0.1% [w/v] Tween 20) for 2 h at room temperature. After overnight incubation with anti-Flag antibody (diluted 1:2,500) or anti-β-actin antibody (1:500), the membranes were washed four times with TBST over a 20-min period and incubated with the appropriate secondary antibody conjugated with HRP (1:1,000) for 30 min. Following four washes with TBST, signals were visualized using the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection System (GE Healthcare). Protein expression was analyzed using ImageJ software (from the National Institutes of Health [NIH], http://rsb.info.nih.gov/ij/).

**Statistical analysis**

Data are presented as the mean ± the standard error of the mean (SEM). Significant differences between groups were identified using two-tailed Student’s t-tests or one-way analysis of variance (ANOVA) followed by Scheffé testing as appropriate. P values < 0.05 were considered statistically significant.

**Results**

**Tetracycline upregulates the expression of BMPRII-Flag mRNA in the FT293-BMPRII cell line**

To investigate whether the generated cell line (FT293-BMPRII) carries BMPRII-Flag and expresses BMPRII-Flag mRNA in response to tetracycline treatment, total RNA was harvested from FT293-BMPRII cells and control parental
FT293 cells that had been treated with 1 μg/ml tetracycline for 0, 3, or 24 h. Subsequently, QRT-PCR analysis was performed using a primer pair that recognizes both endogenous BMPRII mRNA and exogenous BMPRII-Flag mRNA (Table 1). Fig. 2A shows that tetracycline treatment had no marked effect on the combined expression of endogenous and exogenous BMPRII mRNA in FT293 cells. In contrast, overall expression of BMPRII mRNA was significantly up-regulated in FT293-BMPRII cells treated with tetracycline for 3 or 24 h.

To determine whether the integrated BMPRII-Flag gene was responsible for the increased expression of BMPRII mRNA in tetracycline-treated FT293-BMPRII cells, we performed QRT-PCR analysis using a specific primer pair designed to recognize only mRNA transcribed from the exogenous BMPRII-Flag gene (Table 1). While BMPRII-Flag mRNA was not expressed in parental FT293 cells, its expression, like that of overall BMPRII, was upregulated in the FT293-BMPRII cells treated with tetracycline for 3 or 24 h (Fig. 2B). These results indicate that the FT293-BMPRII cell line, but not parental FT293 cells, carries the tetracycline-inducible BMPRII-Flag gene.

We also demonstrated that tetracycline-dependent induction of BMPRII-Flag expression in FT293-BMPRII cells did not change significantly after co-treatment with either the type I BMP receptor inhibitor dorsomorphin or the p38 MAPK-specific inhibitor SB203580 (Fig. 2C). This indicates that each inhibitor may be used in combination with this cell line to investigate BMP- Smad and BMP-p38 MAPK signaling, respectively. The concentrations of SB203580 and dorsomorphin used in this study (0.5 and 2 μM, respectively) were previously reported to be sufficient to inhibit p38 MAPK and Smad activation, respectively (Peregrin et al., 2006; Hao et al. 2008).

### Tetracycline increases BMPRII-Flag protein expression in FT293-BMPRII cells

Next we examined the tetracycline-dependent expression of BMPRII-Flag protein. We harvested FT293-BMPRII cells treated with tetracycline (1 or 10 μg/ml) for 24 h and untreated control FT293-BMPRII cells and analyzed the resulting lysates by Western blotting, performed using an anti-Flag antibody. The expression of BMPRII-Flag protein was upregulated by both concentrations of tetracycline tested (Fig. 3A). We also evaluated the effects of exposing FT293-BMPRII cells to 1 μg/ml tetracycline for different periods of time (0, 3, 12, or 24 h). We found that BMPRII-Flag protein levels increased time-dependently in tetracycline-treated cells (Fig. 3B).

### Tetracycline-induced BMPRII-Flag expression in FT293-BMPRII cells has no effect on the expression of representative BMP target genes

To investigate whether the tetracycline-dependent induction of BMPRII-Flag expression in FT293-BMPRII cells had any effect on the expression of representative BMP target genes (Id1, Id2, Id3, Smad6, and Smad7), we used QRT-PCR to measure their expression, as well as that of BMPRII-Flag (as a control), in untreated FT293-BMPRII cells and FT293-BMPRII cells treated with 1 μg/ml tetracycline for 24 h. We also evaluated the effect of applying this same treatment to parental FT293 cells, which do not express BMPRII-Flag when treated with tetracycline. We found that treatment with tetracycline had no significant effect on the expression of the five target genes tested in either parental FT293 cells (Fig. 4A) or FT293-BMPRII cells (in which it induces the expression of BMPRII-Flag) (Fig. 4B).

### Tetracycline-induced BMPRII-Flag expression in FT293-BMPRII cells enhances the induction of several representative BMP target genes by BMP2

Next we investigated the effects of tetracycline-dependent induction of BMPRII-Flag on the expression of representative BMP target genes in FT293-BMPRII cells treated with BMP2 (100 ng/ml). The effect of tetracycline on the expression of these same target genes in BMP2-treated parental FT293 cells was also investigated as a control.
Fig. 2. Tetracycline-induced BMPRII-Flag mRNA expression in established FT293-BMPRII cells. A: Relative expression of BMPRII mRNA. Quantitative real-time PCR (QRT-PCR) was performed using primers that recognize both endogenous BMPRII mRNA and exogenous BMPRII-Flag mRNA (Table 1). B: Relative expression of BMPRII-Flag mRNA. QRT-PCR was performed using primers specific only for BMPRII-Flag mRNA (the reverse primer recognizes a part of the sequence that codes the Flag-tag peptide [Table 1]). N.D., not detectable. A and B: Parental FT293 cells or the FT293-BMPRII cells were treated for 0, 3, or 24 h with 1 μg/ml tetracycline. *P < 0.05, **P < 0.01 vs. 0 h control. C: Effects of inhibitors of the BMP type I receptor (dorsomorphin) and p38 (SB203580) on tetracycline-induced BMPRII-Flag mRNA expression. FT293-BMPRII cells were treated/not treated for 24 h with 1 μg/ml tetracycline in the presence or absence of dorsomorphin (2 μM) or SB203580 (0.5 μM). **P < 0.01 vs. untreated cells. n.s., not significant. A, B, and C: Target mRNA expression was normalized to that of the internal control β-actin. Results are presented as fold changes relative to a 0 h control (A and B) or untreated cells (C). Data represent the mean ± SEM of three (A and B) or four (C) replicates.
Total RNA was isolated from FT293-BMPRII or FT293 cells treated with BMP2 for 3 h and pretreated/not pretreated with tetracycline for 24 h. By QRT-PCR, we evaluated the effects of BMP2 on the expression of the BMP target genes \textit{Id1}, \textit{Id2}, \textit{Id3}, \textit{Smad6}, and \textit{Smad7}. We also examined the effects of BMP2 on BMPRII-Flag mRNA expression in FT293-BMPRII cells.

In parental FT293 cells and FT293-BMPRII cells, treatment with BMP2 alone significantly increased the expression of \textit{Id1}, \textit{Id2}, \textit{Id3}, and \textit{Smad6} mRNAs, but not \textit{Smad7} mRNA (Fig. 5A and 5B). BMP2 also did not alter the expression of BMPRII-Flag mRNA. Furthermore, tetracycline pretreatment enhanced BMP2-mediated induction of \textit{Id1}, \textit{Id3}, and \textit{Smad6} mRNA expression in FT293-BMPRII cells (Fig. 5B). In contrast, tetracycline did not significantly influence BMP2-mediated induction of \textit{Id1},
Id3, or Smad6 in parental FT293 cells (Fig. 5A).

**Discussion**

To investigate BMPRII-mediated BMP signaling, we used a tetracycline-controlled gene expression system to establish the FT293-BMPRII cell line, which expresses BMPRII-Flag mRNA and protein (Figs. 2 and 3). This system enables the targeted insertion of exogenous genes in a site-specific manner in all transfected cells. Thus, it eliminates the potential influence of the genomic insertion site and ensures homogeneous levels of exogenous gene expression (Thomas et al. 2004; Fujimoto et al. 2006). Next, we assessed the effects of tetracycline treatment on BMP target gene expression in parental FT293 cells and FT293-BMPRII cells. We found, as shown in Figure 4A, that tetracycline itself does not disrupt the expression of representative BMP target genes in FT293 cells. Furthermore, tetracycline-dependent induction of BMPRII expression did not significantly influence the expression of these genes (Fig. 4B). Our findings are important for future studies involving the FT293-BMPRII cell line, because they show that this cell line can be used to investigate BMP2-dependent cellular responses. Had the tetracycline-dependent induction of BMPRII expression activated BMP-mediated signaling in the absence of a BMP ligand, it would have been difficult to analyze the BMP-dependent responses of tetracycline-pretreated FT293-BMPRII cells, as well as changes in the molecules interacting with BMPRII's cytoplasmic tail domain following BMP stimulation.

Significant upregulation of Id1, Id2, Id3, and Smad6 mRNA expression was detected in non-tetracycline-pretreated FT293 and FT293-BMPRII cells exposed to BMP2 for 3 h (Fig. 5B). However, BMP treatment did not alter Smad7 mRNA expression in FT293-BMPRII cells (Fig. 5B). These results might reflect a property of the parental FT293 cell line, because BMP2 similarly failed to influence Smad7 mRNA expression in FT293 cells (Fig. 5A).

Of the genes whose expression in FT293-BMPRII cells was altered by treatment with BMP2 alone (Id1, Id2, Id3, and Smad6), tetracycline-mediated induction of BMPRII-Flag expression prior to BMP2 treatment significantly enhanced BMP2-mediated induction of Id1, Id3, and Smad6 expression (Fig. 5B). These tetracycline-mediated increases in the BMP2-induced gene expression were not observed in tetracycline-pretreated FT293 cells (Fig. 5A). Collectively, these results suggest that the observed effects on mRNA expression stemmed from the tetracycline-dependent induction of BMPRII-Flag expression and not from an adverse reaction to tetracycline itself. It should be noted that pretreatment with tetracycline did not alter the expression of Id2 mRNA in BMP2-treated FT293-BMPRII cells, despite the tetracycline-dependent induction of BMPRII-Flag expression (Fig. 5B), suggesting that the mechanism behind its regulation is different than that of Id1, Id3, and Smad6.
mRNA.

The Id protein family members play distinct and pivotal roles in physiological and pathological biology. Id1 and Id3 display extensive sequence homology and similar patterns of expression during embryogenesis and in adult tissues. In contrast, Id2 plays a critical role in breast differentiation (de Candia et al. 2004). The I-Smads (Smad6 and Smad7) similarly produce different inhibitory effects in intracellular signaling. Smad7, but not Smad6, inhibits signal transduction activated by activin A, a member of the TGF-β super family (Ishisaki et al. 1999). Therefore, the differential effects of induced BMPRII expression on the BMP2-mediated expression of BMP target genes observed in this study might reflect BMPRII-mediated BMP signaling.

Tetracycline is a small lipophilic compound that easily enters eukaryotic cells by passive diffusion. Tetracycline-controlled gene expression systems are extremely powerful tools for producing modifiable expression of exogenous genes (Kitamura 1998), and they have applications in many areas of biology and medicine (Shockett and Schatz 1996). Because the expression of endogenous BMPRII protein in various mammalian cell types is too low to allow analysis of BMPRII function, we believe that the FT293-BMPRII cell line, which is the presence of tetracycline expresses Flag epitope-tagged BMPRII from a single copy of the BMPRII-Flag gene, will facilitate numerous key analyses in the future, including the following: (i) global identification of the interacting partners of BMPRII-Flag under various cellular conditions through immunoprecipitation with an anti-Flag antibody and proteomic analysis, including two-dimensional electrophoresis; (ii) evaluation, using an anti-Flag antibody, of the mechanisms controlling the degradation of BMPRII, which is enhanced by Dullard, an enzyme that functions as an inhibitor of BMP signaling (Satow et al. 2000); and (iii) a detailed investigation of the differences in phenotype between wild-type BMPRII and BMPRII carrying mutations in its cytoplasmic tail and/or other regions, which might be achieved through the generation of a mutant BMPRII-inducible cell line using the methods described in this report.

Failure to properly control BMPRII expression affects normal body development and the pathogenesis of several diseases, including PAH (Beppu et al. 2000; Lane et al. 2000; Atkinson et al. 2002). Therefore, investigations into the relationship between BMPRII expression and proteins involved in BMP signaling using the FT293-BMPRII cell line might be extremely valuable for studying the physiology of BMPRII-mediated cell signaling, as well as the disorders with which it is associated.

In conclusion, using an established stable cell line (FT293-BMPRII) that expresses BMPRII-Flag in a tetracycline-dependent manner, we showed that the induction of BMPRII-Flag itself has no obvious effect on the expression of representative BMP target genes. Our results also show that the induction of BMPRII-Flag protein expression through pretreatment with tetracycline enhances BMP2-induced expression of the Id1, Id3, and Smad6 genes, but not Id2, in FT293-BMPRII cells. This report is the first to show that the induction of exogenous BMPRII differentially affects the responses of these genes to BMP2 stimulation.

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