Original article

Activities of bone morphogenetic proteins in prolactin regulation by somatostatin analogs in rat pituitary GH3 cells

Naoko Tsukamoto, Fumio Otsuka†, Tomoko Miyoshi, Kenichi Inagaki, Eri Nakamura, Jiro Suzuki, Toshio Ogura, Yasumasa Iwasaki* and Hirofumi Makino

Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan; and *Department of Endocrinology, Metabolism, and Nephrology, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku 783-8505, Japan

Running title: PRL regulation by somatostatin and BMP

Key words: Bone morphogenetic protein, Bromocriptine, GH3, Octreotide, Pasireotide and Prolactin

Disclosure Statement: The authors have nothing to disclose.

†*Corresponding Author:* Fumio Otsuka, M.D., Ph.D.
Endocrine Center of Okayama University Hospital,
2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan.
Phone: +81-86-235-7235, Fax: +81-86-222-5214
E-mail: fumiotsu@md.okayama-u.ac.jp

Abbreviations:

ActRI and ActRII, activin type I and type II receptor
ALK, activin receptor-like kinase
BMP, bone morphogenetic protein
BMPRI and BMPRII, BMP type I and type II receptor
BRC, bromocriptine
DA, dopamine agonists
D2R, dopamine D2 receptor
FSK, forskolin
OCT, octreotide
SSTR, somatostatin receptor
TGF-β, transforming growth factor-β

Total word count: 3,287 words *Figure number:* 5 figures

Abstract

Involvement of the pituitary BMP system in the modulation of prolactin (PRL) secretion regulated by somatostatin analogs, including octreotide (OCT) and pasireotide (SOM230), and a dopamine agonist, bromocriptine (BRC), were examined in GH3 cells. GH3 cells are rat pituitary somato-lactotrope tumor cells that express somatostatin receptors (SSTRs) and BMP system molecules including BMP-4 and -6. Treatment with BMP-4 and -6 increased PRL and cAMP secretion by GH3 cells. The BMP-4 effects were neutralized by adding a BMP-binding protein Noggin. These findings suggest the activity of endogenous BMPs in augmenting PRL secretion by GH3 cells. BRC and SOM230 reduced PRL secretion, but OCT failed to reduce the PRL level. In GH3 cells activated by forskolin, BRC suppressed forskolin-induced PRL secretion with reduction in cAMP levels. OCT did not affect forskolin-induced PRL level, while SOM230 reduced PRL secretion and PRL mRNA expression induced by forskolin. BMP-4 treatment enhanced the reducing effect of SOM230 on forskolin-induced PRL level while BMP-4 did not affect the effects of OCT or BRC. Noggin treatment had no significant effect on the BRC actions reducing PRL levels by GH3 cells. However, in the presence of Noggin, OCT elicited an inhibitory effect on forskolin-induced PRL secretion and PRL mRNA expression, whereas the SOM230

effect on PRL reduction was in turn impaired. It was further found that BMP-4 and -6 suppressed SSTR-2 but increased SSTR-5 mRNA expression of GH3 cells. These findings indicate that Noggin rescues SSTR-2 but downregulates SSTR-5 by neutralizing endogenous BMP actions, leading to an increase in OCT sensitivity and a decrease in SOM230 sensitivity of GH3 cells. In addition, BMP signaling was facilitated in GH3 cells treated with forskolin. Collectively, these findings suggest that BMPs elicit differential actions in the regulation of PRL release dependent on cellular cAMP-PKA activity. BMPs may play a key role in the modulation of SSTR sensitivity of somato-lactotrope cells in an autocrine/paracrine manner.

Introduction

BMPs, which belong to the TGF-β superfamily, were originally identified as active components in bone extracts that are capable of inducing bone formation at ectopic sites. A variety of physiological BMP actions in many endocrine tissues, including the ovary, pituitary, thyroid and adrenal, have been discovered (Shimasaki et al., 2004; Otsuka, 2010). There is also increasing evidence that locally produced BMPs play key roles in differentiation of the pituitary. The BMP system is known to play important roles in initial development of the anterior pituitary (Scully and Rosenfeld, 2002). BMP-4 is required during the first stage of pituitary organogenesis for the proliferation of Rathke's pouch, which gives rise to Pit-1 lineage cells including lactotrope cells. During the subsequent stages of pituitary organogenesis, inhibition of BMP-2 by fibroblast growth factor (FGF)-8 leads to differentiation of corticotrope cells (Kioussi et al., 1999; Dasen and Rosenfeld, 2001). BMP-4 not only governs the pituitary organogenesis but also plays a key role in the pathogenesis of differentiated pituitary lineages (Giacomini et al., 2006; Labeur et al., 2010; Tsukamoto et al., 2010).

Dopamine agonists (DA) are the clinical treatment of choice for prolactin (PRL)-secreting pituitary adenomas (Casanueva et al., 2006). They control PRL

secretion and cell proliferation by interacting with the dopamine D2 receptor (D2R), which subsequently activates various transduction pathways (Missale et al., 1998). D2R agonists are efficient in the majority of cases; however, some patients with prolactinomas fail to obtain PRL normalization and reduction in tumor size even with the most potent dopamine agonist, cabergoline (Molitch, 2005; Gillam et al., 2006; Hofland et al., 2010). These prolactinomas, resistant to DA, are usually large and/or invasive, and surgery therefore cannot be a complete curative treatment. In such tumors poorly or partially responsive to DA, an alternative medical treatment is needed. Experimental data have demonstrated that different somatostatin receptor (SSTR) subtypes are expressed at various levels in prolactinomas, SSTR-5 being the most important in the regulation of PRL secretion (Shimon et al., 1997; Jaquet et al., 1999). A partial synergistic effect between D2R and SSTR-5 in suppressing PRL secretion has also been reported (Jaquet et al., 1999).

Here we studied a unique activity of the pituitary BMP system in PRL regulation by somatostatin analogs using rat somato-lactotropinoma GH3 cells. GH3 cells express somatostatin receptors including SSTR-1, -2, -3, -4 and -5 as well as dopamine D2R (Johnston et al., 1991; Yang et al., 2005; Miyoshi et al., 2008). In addition, we earlier reported that both GH3 cells and rat whole pituitaries express

BMP/activin type I receptors (ALK-2, -3 and -4), type II receptors (ActRII, ActRIIB and BMPRII) and Smads (Smad1, 2, 3, 4, 5, 6, 7 and 8) (Miyoshi et al., 2008). The whole pituitary tissues also express BMP/activin ligands, including BMP-2, -4, -6, -7, activinβA/βB, and inhibinα subunits. The predominant BMP ligands endogenously expressed in GH3 cells are reported to be BMP-4 and BMP-6 (Miyoshi et al., 2008). Based on the present findings on GH3 cells expressing both machineries for BMP system and SSTR signaling, we here propose that BMPs may play a key role in the modulation of SSTR sensitivity of pituitary tumor cells in an autocrine/paracrine manner.

Materials and Methods

Reagents and supplies

A 1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham F-12 medium (DMEM/F12), penicillin-streptomycin solution, forskolin (FSK), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human BMP-4, BMP-6 and mouse Noggin were purchased from R&D Systems (Minneapolis, MN). Bromocriptine mesylate (BRC), octreotide acetate (OCT) and pasireotide, also known as SOM230 (SOM), were provided by by Novartis International Pharmaceutical Ltd. (Basel, Switzerland).

Cell culture and cAMP measurement

Rat pituitary somato-lactotrope tumor GH3 cells were cultured in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a 5% CO_2 atmosphere at 37°C. GH3 cells (1×10^5 viable cells) were seeded in 24-well plates with DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12 containing penicillin-streptomycin and 0.1 mM IBMX (a specific inhibitor of phosphodiesterase activity), and then the cells

were treated with indicated concentrations and combinations of FSK, BRC, OCT, SOM, BMP-4, BMP-6 and Noggin. After 24-h culture, the medium removed from the cells was centrifuged. The supernatant of the culture media was collected and stored at -80°C until assay. After acetylation of each sample, the extracellular contents of cAMP were determined by an enzyme immunoassay with assay sensitivity of 0.039 nM (Assay Designs, Inc., Ann Arbor, MI). The intra- and inter-assay coefficients are 6.8% and 7.9%, respectively.

Determination of prolactin levels

GH3 cells (1×10^5 viable cells) were cultured in 24-well plates with DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12, and then the cells were treated with indicated concentrations and combinations of FSK, BRC, OCT, SOM, BMP-4, BMP-6 and Noggin. After 24-h culture, the medium removed from the cells was centrifuged. The supernatant of the culture media was collected and stored at -80°C until assay. The levels of PRL in the cultured media were determined by a rat-specific enzyme immunoassay with assay sensitivity of 1 pg/ml (Duhau et al., 1991) (SPI-BIO, Montigny-le-Bretonneux, France). The intra- and inter-assay coefficients are 10.6%

and 13.4%, respectively.

RNA extraction and quantitative real-time PCR analysis

After preculture, cells (3×10^5 viable cells) were treated with indicated concentrations of FSK, BRC, OCT, SOM, BMP-4, BMP-6 and Noggin in serum-free DMEM/F12. After 24-h culture, the medium was removed and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). Total RNA was quantified by measuring the absorbance of the sample at 260 nm and was stored at -80°C until assay. The extracted RNA (1 μg) was subjected to RT reaction using First-Strand cDNA Synthesis System® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and at 70°C for 10 min. For the quantification of indicated mRNA levels of PRL, D2R, SSTRs, Id-1 and housekeeping gene ribosomal L19 (RPL19), real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60°C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis (Roche Diagnostic Co.), and then, following assay validation by calculating each

amplification efficiency, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes as follows: PRL: 271-291 and 471-491 (NM_012629); D2R: 542-562 and 851-871 (from GenBank accession No. X56065); SSTR-2: 240-260 and 559-579 (M93273); SSTR-5: 98-118 and 368-388 (L04535); and Id-1, 225-247 and 364-384 (NM_010495). For each transcript, all treatment groups were quantified simultaneously in a single LightCycler run. To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of target gene by the quantity of RPL19 in each sample. The raw data of each target mRNA level (/RPL19) were statistically analyzed as indicated, and then shown as fold changes in the figures.

Western immunoblot analysis

Cells (3×10^5 viable cells) were cultured in 12-well plates in DMEM/F12 containing penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12 and cultured for 24 h, and then the cells were treated with indicated concentrations and combinations of OCT, SOM and BMP-4. After 1-h culture, cells

were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc.) and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The relative integrated density of each protein band was digitized by NIH image J 1.34s.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data of prolactin and cAMP levels, real-time PCR analysis and immunoblots densities were subjected to ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test, when appropriate, to determine differences. *P* values < 0.05 were accepted as statistically significant.

Results

We first examined the effects of BMPs on PRL secretion by GH3 cells for 24 h. As shown in **Fig. 1A**, BMP-4 and BMP-6 stimulated PRL production. In accordance with PRL release, cAMP synthesis was also increased by treatment with BMP-4 and BMP-6. The BMP-4 effects were neutralized by treatment with 30 ng/ml of a BMP-binding protein, Noggin. Subsequently, we examined the effects of somatostatin analogs, including octreotide (OCT) and pasireotide (SOM230), and a dopamine agonist, bromocriptine (BRC), on PRL and cAMP levels by GH3 cells. OCT had no effects on PRL and cAMP release, while SOM and BRC suppressed PRL and cAMP levels (**Fig. 1B**). To clarify the effects of OCT, SOM and BRC on PRL secretion by GH3 cells, GH3 cells were cultured in the presence of forskolin (FSK). FSK significantly activated PRL secretion with increasing cAMP level (**Fig. 1C**). OCT had no effect on FSK-induced PRL or cAMP level, whereas SOM showed a dose-responsive decrease of PRL as well as cAMP level (**Fig. 1C**). BRC showed more efficacious suppression of FSK-induced PRL secretion as well as cAMP level.

Next, BMP-4 actions on PRL secretion regulated by OCT, BRC and SOM were examined (**Fig. 2A**). BMP-4 did not affect PRL secretion in GH3 cells treated

with OCT or BRC regardless of the effects of FSK. Notably, BMP-4 treatment significantly potentiated the SOM effects reducing PRL secretion stimulated by FSK. To examine the involvement of endogenous BMP actions in PRL release by GH3 cells, cells were treated with Noggin (30 ng/ml). As shown in **Fig. 2B**, in the presence of Noggin, OCT treatment suppressed PRL and cAMP levels, while the actions of SOM reducing PRL and cAMP levels were reversed by Noggin. BRC actions were not affected by Noggin treatment. As shown in **Fig. 2C**, these Noggin effects were also enhanced in GH3 cells treated with FSK. That is, in the presence of Noggin, OCT suppressed PRL secretion, but the effects of SOM were reversed. BRC effects were not affected by addition of Noggin. These findings suggest that the endogenous BMP system is active to modulate OCT and SOM actions for regulating PRL secretion in GH3 cells.

We also investigated the effects of BMP-4 and Noggin on PRL mRNA levels in GH3 cells treated with FSK (**Fig. 3**). FSK significantly enhanced mRNA expression of PRL, which was not directly influenced by treatment with Noggin or BMP-4. BRC significantly suppressed the PRL mRNA levels in FSK-treated GH3 cells. The BRC effects on PRL mRNA levels were not affected by BMP-4 or Noggin. Although OCT alone did not affect PRL expression, PRL mRNA levels were

significantly reduced by OCT in cells treated with Noggin but not with BMP-4. On the other hand, PRL mRNA levels were reduced by SOM alone, which was reversed by adding Noggin. In addition, the reduction of PRL expression by SOM treatment was further suppressed by BMP-4 (**Fig. 3**).

To investigate the mechanism by which neutralization of endogenous BMPs influenced the effects of somatostatin analogs on PRL secretion, expression of the key receptors D2R, SSTR-2 and SSTR-5 was evaluated by real-time PCR analysis. As shown in **Fig. 4A**, D2R expression was not affected by BMP-4 and BMP-6 regardless of the presence of FSK. However, SSTR-2 mRNA expression was downregulated by addition of BMP-4 and BMP-6, whereas SSTR-5 expression was upregulated by BMP-4 and BMP-6. These changes were prominent when cells were activated with FSK. It was thus thought that pretreatment with Noggin maintains SSTR-2 level but decreases SSTR-5 expression. Therefore, endogenous BMP actions may be involved in enhancing SOM effects but impairing OCT effects on the reduction of PRL level. In addition, Id-1 mRNA levels induced by BMP-4 and -6, indicating BMP signaling activity, were also found to be enhanced by FSK treatment (**Fig. 4B**). The phosphorylation of Smad1/5/8 signaling induced by BMP-4 and -6 was not directly affected by treatment with SOM or OCT (**Fig. 4C**), although BMP-4 and -6 changed

SSTR-2 and -5 expression differentially (**Fig. 4A**).

Discussion

In the present study, a unique action of the pituitary BMP system in the modulation of PRL secretion regulated by somatostatin analogs was uncovered in GH3 cells expressing SSTRs and BMP system (**Fig. 5**). Firstly, endogenous BMP actions are involved in augmenting PRL secretion, since BMP-4 and -6 increased PRL and cAMP levels and the effects were neutralized by a BMP-binding protein Noggin. Secondarily, BMPs modulate SSTR sensitivity of GH3 cells in an autocrine/paracrine manner. Namely, BMP-4 and -6 reduced SSTR-2 expression but increased SSTR-5 expression. The effect of SOM230 (a SSTR-5-preferring agonist) that reduced PRL secretion induced by FSK was facilitated by adding BMP-4 and in turn blocked by Noggin. On the contrary, in the presence of Noggin, OCT (a SSTR-2-preferring agonist) rather exerted an inhibitory effect on the PRL release.

In the presence of BMP-4 actions, SSTR-2 expression could be downregulated, and therefore, OCT effects on PRL release remained latent regardless of the presence or absence of FSK. This phenomenon was in contrast to the effects of BRC and SOM on the PRL release, since D2R was not affected by BMP-4 and SSTR-5 was rather upregulated by BMP-4. Noggin by itself had no specific effect on cAMP

level; however, in the presence of Noggin, OCT effects on cAMP and PRL reduction became apparent because Noggin could neutralize the activities of endogenous BMPs which suppress SSTR-2 expression. It is thus suggested that the blockage of endogenous BMP actions by Noggin rescues SSTR-2 signaling but downregulates SSTR-5, leading to an increase in OCT sensitivity of GH3 cells.

In addition, BMP-Smad signaling shown as Id-1 transcription was facilitated by stimulation with FSK in GH3 cells. Certainly, BMP-4 action on increasing SSTR-5 expression was apparent when cells were treated with FSK, and the BMP-4 effect on potentiating the SOM actions reducing PRL secretion was significant when cells were stimulated by FSK. These findings also suggest that BMPs elicit differential actions in the regulation of PRL level dependent on cellular cAMP-PKA activity.

The BMP system including BMP-4 has been shown to play important roles in initial development of the anterior pituitary involving lactotrope (Scully and Rosenfeld, 2002). Each of type I and type II receptor for BMPs exhibits serine/threonine kinase activity, in which several preferential combinations of BMP ligands and receptors have been recognized to date. Since ALK-6 is not expressed in GH3 cells, the receptor pair of ALK-3 and BMPRII is likely to be the major functional complex for BMP-4 for regulating PRL and cAMP production.

Overexpression of the BMP-binding protein Noggin or dominant-negative ALK-3 in the anterior pituitary leads to arrest of the development of the Pit-1-expressing lineage (Scully and Rosenfeld, 2002). BMP-4 is overexpressed in lactotrope adenomas derived from D2R-null mice as well as human prolactinomas (Paez-Pereda et al., 2003). Moreover, Noggin expression is conversely downregulated in prolactinomas from D2R null mice (Paez-Pereda et al., 2003), suggesting that BMP-4 promotes cell proliferation in lactotoropes in conjunction with Smad-estrogen receptor interaction. In the present study, we discovered an interrelationship between BMP effects and SSTR expression for PRL secretion. BMPs act to increase PRL release at least in part via a cellular cAMP-PKA pathway. Hence, it is possible that the endogenous BMP system plays a key role in the modulation of SSTR sensitivity of lactotorope tumor cells in an autocrine/paracrine manner.

Somatostatin acts by binding five subtypes of G protein–coupled receptors that are widely distributed in many endocrine and nonendocrine tissues. The efficacy of somatostatin analogs is linked to the SSTR selectivity profile, in which binding capability to SSTR-2 and SSTR-5 appears critical (Shimon et al., 1997). SSTR-2 and SSTR-5 are negatively coupled to adenylyl cyclase, the activation of which results in a reduction of intracellular cAMP levels (Reisine and Bell, 1995). The SSTR effects are

further mediated by Ca^{++} influx through a direct action on Ca^{++} channels (Chen et al., 1997) and/or indirectly through activating K^{+} channels (Takano et al., 1997). The success of *in vivo* peptide-targeted therapy is highly dependent on the presence and on the localization in the tumor of a sufficient amount of the appropriate receptor. In this regard, we recently reported the involvement of BMP system in corticotrope cells (Tsukamoto et al., 2010). In that study, somatostatin analogs upregulated BMP-Smad signaling by augmenting BMPRII/ALK-3 expression as well as by reducing the expression of inhibitory Smad6/7 in corticotrope AtT20 cells. However, the effects of SOM on BMP-4 signaling were significantly higher than the effects of OCT in AtT20 cells, indicating the differences of SSTR affinities between two somatostatin analogs (Tsukamoto et al., 2010). Similar to our current study on lactotrope cells, the interaction of BMP-Smad pathway and CRH receptor signaling was also functionally involved in controlling ACTH production from corticotrope cells in an autocrine/paracrine manner.

Lactotrope adenomas are the most frequent pituitary tumors. The main pharmacologic treatment of PRL-secreting adenomas is dopamine agonists (DA). However, treatment is ineffective in 10% to 15% of treated patients, even with the most potent cabergoline (Molitch, 2005; Gillam et al., 2006). Although D2R is expressed in

nearly all prolactinomas and is the target for much current therapy, some patients are resistant to DA. SSTR is known to be expressed in prolactinomas, the majority of which express SSTR-5 but not SSTR-2 (Jaquet et al., 1999). This expression pattern indicates that established somatostatin analogs such as octreotide (OCT) and lanreotide, which bind primarily to SSTR-2, are much less effective for suppressing PRL secretion from prolactinomas compared with pasireotide (SOM), having a 40-fold greater binding affinity to SSTR-5 (Hofland et al., 2004).

In clinical trials testing somatostatin analogs, the effects of cortistatin, a neuropeptide that has high homology with somatostatin and binds with high affinity to all SSTRs, on PRL suppression in prolactinoma patients were reported (Grottoli et al., 2006). BIM-23206, a SSTR-5-selective agonist (Fusco et al., 2008), was also reported to suppress PRL release in patients with DA-sensitive prolactinomas similar to cabergoline. Another study on PRL-secreting adenomas also revealed significant effects of cortistatin and a SSTR-5 agonist on PRL reduction in most of the prolactinomas examined, whereas PRL inhibition by the SSTR-2 agonist was observed in only one case of prolactinoma (Rubinfeld et al., 2006). These results support the notion that SSTR-5 primarily regulates PRL suppression by somatostatin in prolactinoma cells (Shimon et al., 1997). In addition, the existence of novel mRNA

splice variants of the human SSTR-5 gene, having five and four transmembrane domains, was reported in different human tissues, including a prolactinoma (Duran-Prado et al., 2009). Since these novel SSTR-5 variants have been found to show different patterns of cellular localization than that of the original isoform of SSTR-5, the altered expression may also be involved in pathophysiological sensitivity of SSTRs in prolactinomas.

Collectively, this functional link between BMP-Smad signaling and SSTR actions may be involved in the individual tolerance to somatostatin analogs for controlling PRL secretion from prolactinomas (**Fig. 5**). Somatostatin analogs with modified receptor subtype affinities with prolonged binding capacity would have the potential to be developed as new treatment modalities for functioning pituitary adenomas. Otherwise, the establishment of regulatory methods of the endogenous BMP/Noggin system, which is a functional determinant for SSTR sensitivity, may be a future strategy for the treatment of DA-resistant prolactinomas. Further study is needed to elucidate the detailed molecular mechanism by which BMPs differentially regulate SSTR expression by human prolactinomas.

Acknowledgements

We thank Dr. R. Kelly Moore for helpful discussion and critical reading of the manuscript. We also thank Novartis for providing bromocriptine, octreotide and pasireotide. This work was supported in part by Grants-in-Aid for Scientific Research, WESCO Scientific Promotion Foundation, Ryobi Teiten Memory Foundation and Takeda Science Foundation.

References

Casanueva FF, Molitch ME, Schlechte JA, Abs R, Bonert V, Bronstein MD, Brue T, Cappabianca P, Colao A, Fahlbusch R, Fideleff H, Hadani M, Kelly P, Kleinberg D, Laws E, Marek J, Scanlon M, Sobrinho LG, Wass JA, Giustina A (2006) Guidelines of the Pituitary Society for the diagnosis and management of prolactinomas. Clin Endocrinol (Oxf) 65:265-273.

Chen ZP, Xu S, Lightman SL, Hall L, Levy A (1997) Intracellular calcium ion responses to somatostatin in cells from human somatotroph adenomas. Clin Endocrinol (Oxf) 46:45-53.

Dasen JS, Rosenfeld MG (2001) Signaling and transcriptional mechanisms in pituitary development. Annu Rev Neurosci 24:327-355.

Duhau L, Grassi J, Grouselle D, Enjalbert A, Grognet JM (1991) An enzyme immunoassay for rat prolactin: application to the determination of plasma levels. J Immunoassay 12:233-250.

Duran-Prado M, Gahete MD, Martinez-Fuentes AJ, Luque RM, Quintero A, Webb SM, Benito-Lopez P, Leal A, Schulz S, Gracia-Navarro F, Malagon MM, Castano JP (2009) Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors. J Clin Endocrinol Metab 94:2634-2643.

Fusco A, Gunz G, Jaquet P, Dufour H, Germanetti AL, Culler MD, Barlier A, Saveanu A (2008) Somatostatinergic ligands in dopamine-sensitive and -resistant prolactinomas. Eur J Endocrinol 158:595-603.

Giacomini D, Paez-Pereda M, Theodoropoulou M, Gerez J, Nagashima AC, Chervin A, Berner S, Labeur M, Refojo D, Renner U, Stalla GK, Arzt E (2006) Bone morphogenetic protein-4 control of pituitary pathophysiology. Front Horm Res 35:22-31.

Gillam MP, Molitch ME, Lombardi G, Colao A (2006) Advances in the treatment of prolactinomas. Endocr Rev 27:485-534.

Grottoli S, Gasco V, Broglio F, Baldelli R, Ragazzoni F, Gallenca F, Mainolfi A, Prodam F, Muccioli G, Ghigo E (2006) Cortistatin-17 and somatostatin-14 display the same effects on growth hormone, prolactin, and insulin secretion in patients with acromegaly or prolactinoma. J Clin Endocrinol Metab 91:1595-1599.

Hofland LJ, Feelders RA, de Herder WW, Lamberts SW (2010) Pituitary tumours: the

sst/D(2) receptors as molecular targets. Mol Cell Endocrinol:in press.

Hofland LJ, van der Hoek J, van Koetsveld PM, de Herder WW, Waaijers M, Sprij-Mooij D, Bruns C, Weckbecker G, Feelders R, van der Lely AJ, Beckers A, Lamberts SW (2004) The novel somatostatin analog SOM230 is a potent inhibitor of hormone release by growth hormone- and prolactin-secreting pituitary adenomas in vitro. J Clin Endocrinol Metab 89:1577-1585.

Jaquet P, Ouafik L, Saveanu A, Gunz G, Fina F, Dufour H, Culler MD, Moreau JP, Enjalbert A (1999) Quantitative and functional expression of somatostatin receptor subtypes in human prolactinomas. J Clin Endocrinol Metab 84:3268-3276.

Johnston JM, Wood DF, Bolaji EA, Johnston DG (1991) The dopamine D2 receptor is expressed in GH3 cells. J Mol Endocrinol 7:131-136.

Kioussi C, Carriere C, Rosenfeld MG (1999) A model for the development of the hypothalamic-pituitary axis: transcribing the hypophysis. Mech Dev 81:23-35.

Labeur M, Paez-Pereda M, Haedo M, Arzt E, Stalla GK (2010) Pituitary tumors: Cell type-specific roles for BMP-4. Mol Cell Endocrinol:in press.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189-225.

Miyoshi T, Otsuka F, Otani H, Inagaki K, Goto J, Yamashita M, Ogura T, Iwasaki Y, Makino H (2008) Involvement of bone morphogenetic protein-4 in GH regulation by octreotide and bromocriptine in rat pituitary GH3 cells. J Endocrinol 197:159-169.

Molitch ME (2005) Pharmacologic resistance in prolactinoma patients. Pituitary 8:43-52.

Otsuka F (2010) Multiple endocrine regulation by bone morphogenetic protein system. Endocr J 57:3-14.

Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK, Arzt E (2003) Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. Proc Natl Acad Sci U S A 100:1034-1039.

Reisine T, Bell GI (1995) Molecular biology of somatostatin receptors. Endocr Rev 16:427-442.

Rubinfeld H, Hadani M, Barkai G, Taylor JE, Culler MD, Shimon I (2006) Cortistatin inhibits growth hormone release from human fetal and adenoma pituitary cells and prolactin secretion from cultured prolactinomas. J Clin Endocrinol Metab

91:2257-2263.

Scully KM, Rosenfeld MG (2002) Pituitary development: regulatory codes in mammalian organogenesis. Science 295:2231-2235.

Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004) The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101.

Shimon I, Yan X, Taylor JE, Weiss MH, Culler MD, Melmed S (1997) Somatostatin receptor (SSTR) subtype-selective analogues differentially suppress in vitro growth hormone and prolactin in human pituitary adenomas. Novel potential therapy for functional pituitary tumors. J Clin Invest 100:2386-2392.

Takano K, Yasufuku-Takano J, Teramoto A, Fujita T (1997) Gi3 mediates somatostatin-induced activation of an inwardly rectifying K+ current in human growth hormone-secreting adenoma cells. Endocrinology 138:2405-2409.

Tsukamoto N, Otsuka F, Miyoshi T, Yamanaka R, Inagaki K, Yamashita M, Otani H, Takeda M, Suzuki J, Ogura T, Iwasaki Y, Makino H (2010) Effects of bone morphogenetic protein (BMP) on adrenocorticotropin production by pituitary corticotrope cells: involvement of up-regulation of BMP receptor signaling by somatostatin analogs. Endocrinology 151:1129-1141.

Yang SK, Parkington HC, Blake AD, Keating DJ, Chen C (2005) Somatostatin increases voltage-gated K+ currents in GH3 cells through activation of multiple somatostatin receptors. Endocrinology 146:4975-4984.

Figure Legends

Fig. 1. Effects of BMPs and somatostatin analogs on prolactin secretion. **A)** GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were then treated with BMP-4 (10-100 ng/ml), BMP-6 (10-100 ng/ml) and Noggin (10-100 ng/ml). After 24-h culture, the supernatants of culture media were collected, and prolactin (PRL) and cAMP levels were determined by specific enzyme immunoassays. For measurement of cAMP levels, cells were cultured with serum-free medium containing 0.1 mM of IBMX. **B)** and **C)** GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. Cells were treated with octreotide (OCT; 0.1-10 μM), bromocriptine (BRC; 1-100 μM) or pasireotide (SOM; 0.1-30 μM) in the absence (**B**) or presence (**C**) of forskolin (FSK; 1 μM). After 24-h culture, the culture media were collected, and PRL and cAMP levels were determined. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, $P < 0.05$ vs. control group in each panel; and the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 2. Effects of BMP-4 and Noggin on prolactin secretion regulated by somatostatin analogs in GH3 cells. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were treated with octreotide (OCT; 1-10 μM), bromocriptine (BRC; 1-10 μM) and pasireotide (SOM; 1-10 μM) in combination with (**A**) BMP-4 (100 ng/ml) and (**B, C**) Noggin (30 ng/ml) in the absence or presence of forskolin (FSK; 1 μM). After 24-h treatment, prolactin (PRL) and cAMP levels in culture media were determined by enzyme immunoassays. For measurement of cAMP levels, cells were cultured with serum-free medium containing 0.1 mM of IBMX. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, $P < 0.05$ vs. control group in each panel; and the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 3. Effects of BMP-4 and Noggin on prolactin mRNA expression regulated by somatostatin analogs in GH3 cells. GH3 cells (3×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were treated with octreotide (OCT; 10 μM), bromocriptine (BRC; 10 μM) and pasireotide (SOM; 10 μM) in the absence or presence

of BMP-4 (100 ng/ml) and Noggin (30 ng/ml) and forskolin (FSK; 1 μM). After 24-h culture, total cellular RNA was extracted and subjected to RT-PCR reaction. The mRNA expression levels of prolactin (PRL) were quantified by real-time PCR analysis. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, $P < 0.05$ vs. control group in each panel; and the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 4. Effects of BMPs on somatostatin receptors (SSTR) expression and effects of forskolin and somatostatin analogs on BMP receptor signaling in GH3 cells. **A)** GH3 cells (3×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were treated with BMP-4 and BMP-6 (100 ng/ml) in the absence or presence of forskolin (FSK; 1 μM). After 24-h culture, total cellular RNA was extracted and subjected to RT-PCR reaction. The mRNA expression levels of dopamine type-2 receptor (D2R), SSTR-2 and SSTR-5 were quantified by real-time PCR analysis. The expression levels of target genes were standardized by RPL19 level in each sample.

B) After preculture, cells were treated with BMP-4 and BMP-6 (100 ng/ml) in the absence or presence of forskolin (FSK; 1 μM). After 24-h culture, total cellular RNA was extracted and subjected to RT-PCR reaction. The mRNA expression levels of Id-1 were quantified by real-time PCR analysis. The expression levels of target genes were standardized by RPL19 level in each sample. **C)** Cells (3×10^5 viable cells) were precultured in serum-free DMEM/F12. After pretreatment with octreotide (OCT; 10 μM) and pasireotide (SOM; 10 μM) for 1 h, the cells were treated with BMP-4 and BMP-6 (100 ng/ml) for 60 min. The cell lysates were then subjected to SDS-PAGE immunoblotting (IB) analysis using anti-phosho-Smad1/5/8 antibody and anti-actin antibody. The relative integrated density of each protein band was digitized and pSmad1/5/8 levels were normalized by actin level in each sample. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, $P < 0.05$ vs. control group in each panel; and the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 5. A possible interaction of BMP system and prolactin secretion in

lactotrope cells. BMPs increased prolactin (PRL) secretion with increasing cAMP level by GH3 cells via proper BMP receptors (BMPRs), and the effects were neutralized by a BMP-binding protein Noggin. BMPs reduced SSTR-2 expression but increased SSTR-5 expression in GH3 cells. The effect of SOM230, which reduced PRL secretion induced by forskolin (FSK), was facilitated by BMP treatment but in turn blocked by adding Noggin. On the contrary, in the presence of Noggin, OCT exerted an inhibitory effect on the PRL secretion. BRC effects, which suppressed PRL and cAMP levels via dopamine D2 receptor (D2R), were not affected by BMP or Noggin treatment. BMP signaling was also facilitated by FSK stimulation. Thus, BMPs may play a key role in the modulation of SSTR sensitivity of lactotrope cells in an autocrine/paracrine manner.

Fig. 1

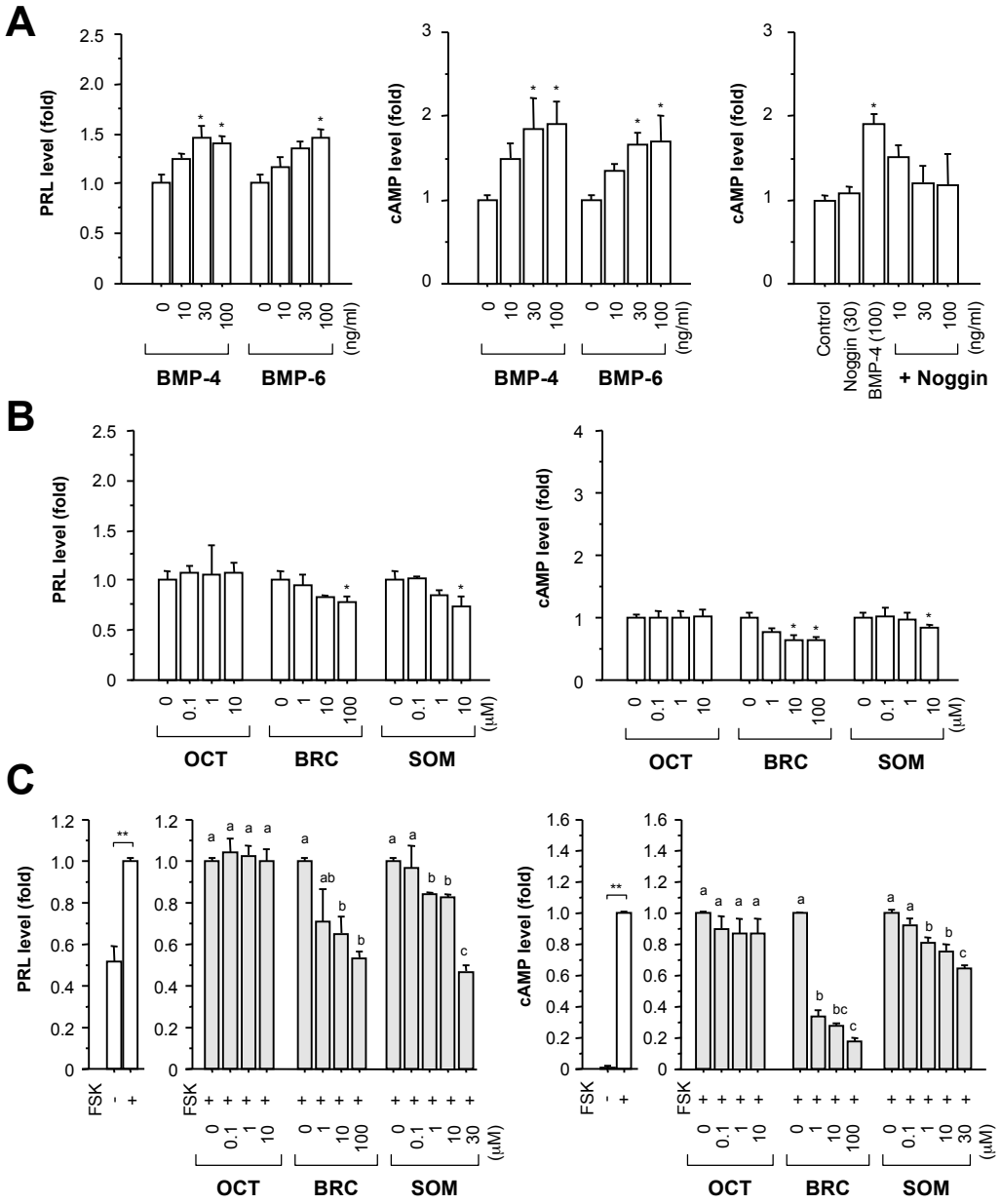

A
PRL level (fold)
cAMP level (fold)
cAMP level (fold)
0 10 30 100 (ng/ml)
BMP-4 BMP-6
Control
Noggin (30)
BMP-4 (100)
10 30 100 (ng/ml)
+ Noggin
B
PRL level (fold)
cAMP level (fold)
0 0.1 1 10 (μM)
0 1 10 100 (μM)
OCT BRC SOM
C
PRL level (fold)
cAMP level (fold)
FSK
0 0.1 1 10 (μM)
0 1 10 100 (μM)
0 1 10 30 (μM)
OCT BRC SOM

Fig. 2

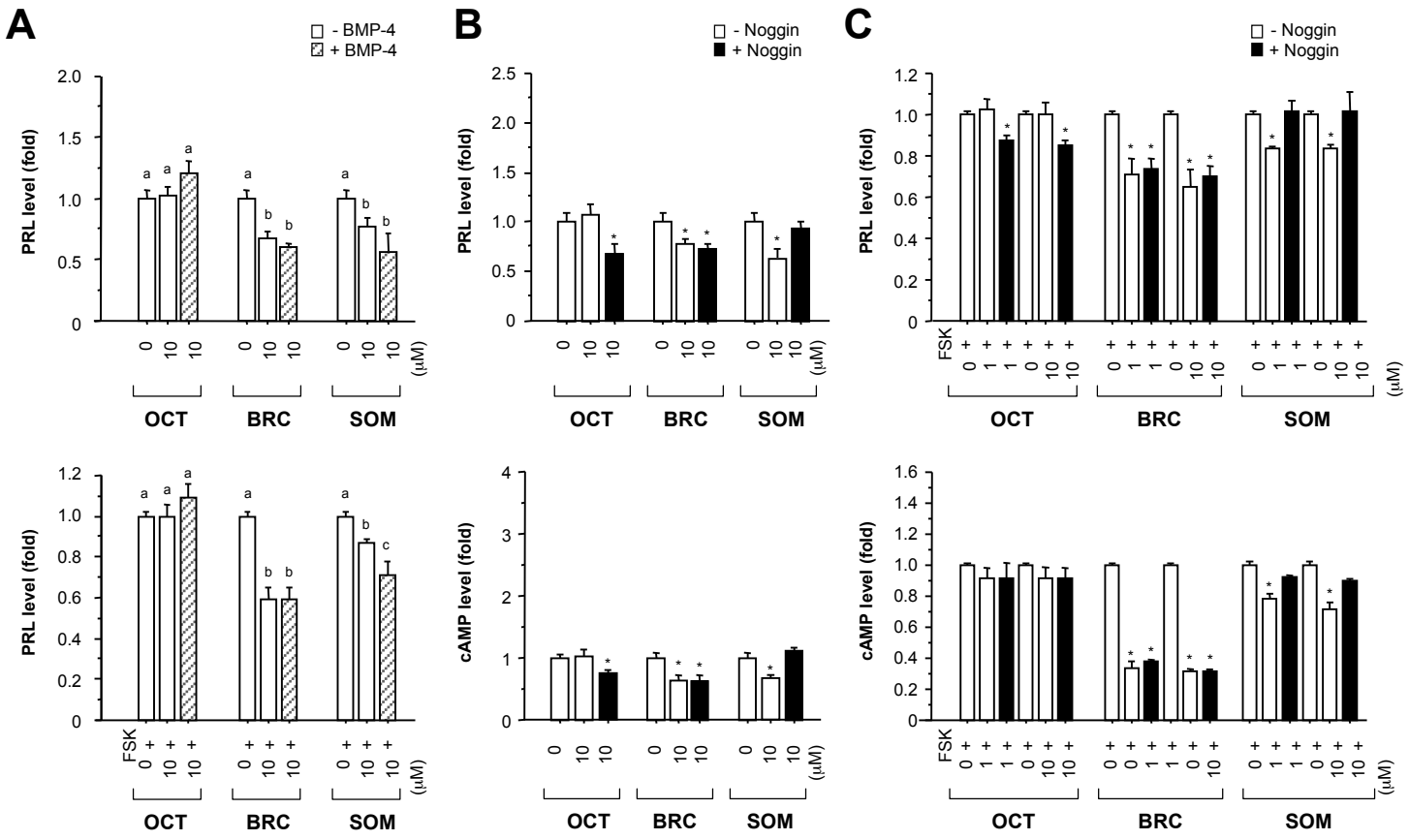

A
- BMP-4
+ BMP-4
PRL level (fold)
cAMP level (fold)
FSK
(μM)
OCT
BRC
SOM
B
- Noggin
+ Noggin
C
- Noggin
+ Noggin

Fig. 3

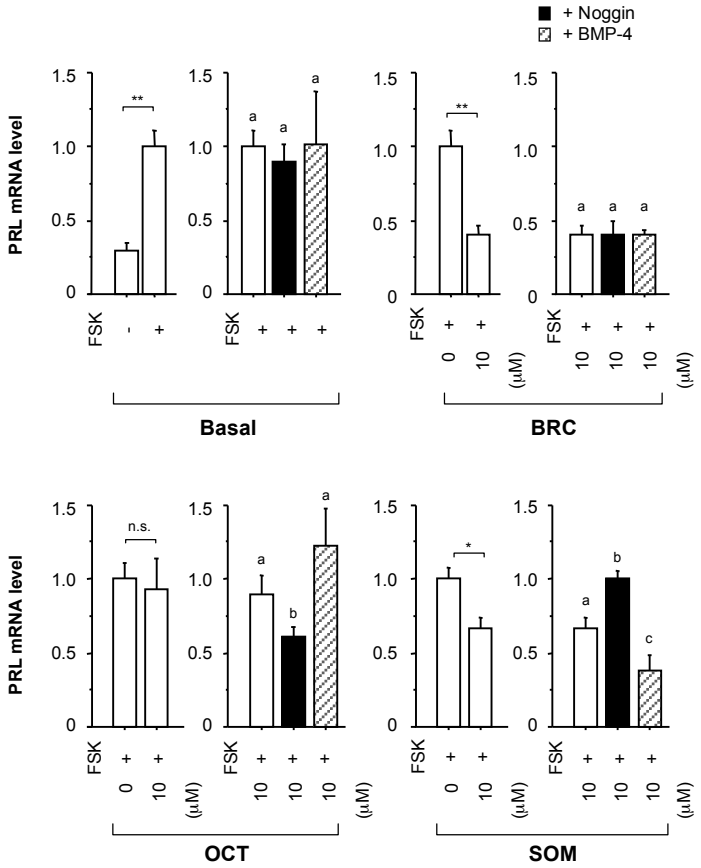

+ Noggin
+ BMP-4
PRL mRNA level
1.5
1.0
0.5
0
**
n.s.
*
a
b
c
FSK
-
+
0
10
(μM)
Basal
BRC
OCT
SOM

Fig. 4

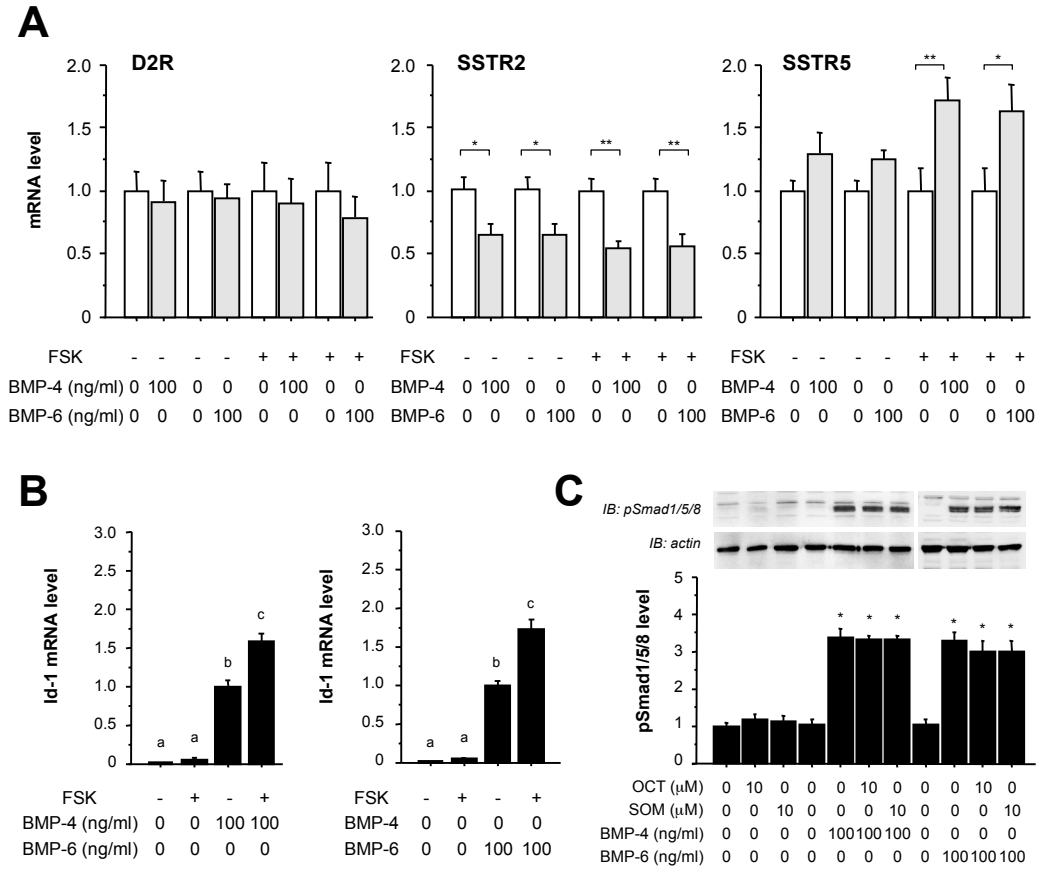
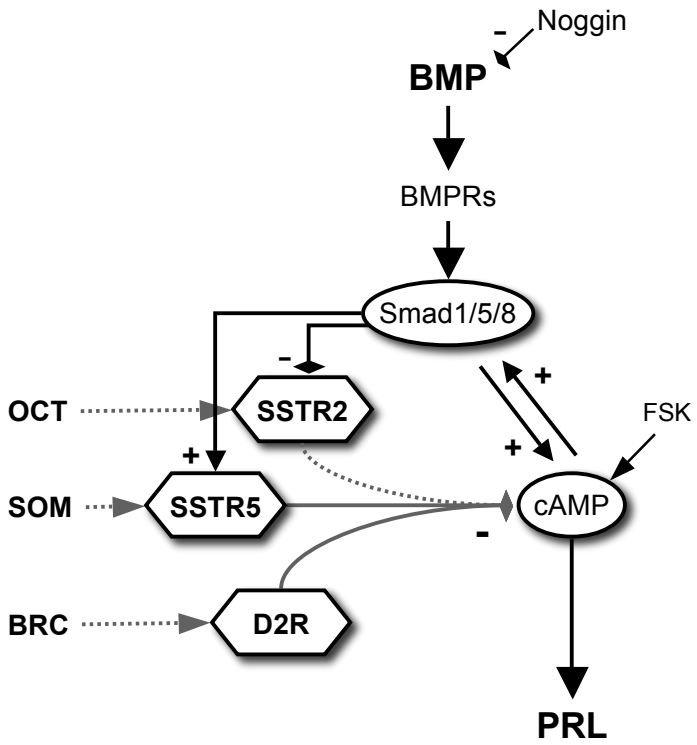

A
D2R
SSTR2
SSTR5
mRNA level
FSK
BMP-4 (ng/ml)
BMP-6 (ng/ml)
B
Id-1 mRNA level
C
IB: pSmad1/5/8
IB: actin
pSmad1/5/8 level
OCT (μM)
SOM (μM)

Fig. 5


Noggin
-
BMP
BMPRs
Smad1/5/8
-
+
OCT
SSTR2
FSK
+
+
SOM
SSTR5
cAMP
-
BRC
D2R
PRL