

1 *Original article*

2
3 **Activities of bone morphogenetic proteins in prolactin regulation by**
4 **somatostatin analogs in rat pituitary GH3 cells**

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26 *Abbreviations:*

27 ActRI and ActRII, activin type I and type II receptor
28 ALK, activin receptor-like kinase
29 BMP, bone morphogenetic protein
30 BMPRI and BMPRII, BMP type I and type II receptor
31 BRC, bromocriptine
32 DA, dopamine agonists
33 D2R, dopamine D2 receptor
34 FSK, forskolin
35 OCT, octreotide
36 SSTR, somatostatin receptor
37 TGF- β , transforming growth factor- β

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Abstract

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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

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

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Introduction

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

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

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

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

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

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Materials and Methods

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Reagents and supplies

3 A 1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham F-12 medium
4 (DMEM/F12), penicillin-streptomycin solution, forskolin (FSK), and
5 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Corp. (St.
6 Louis, MO). Recombinant human BMP-4, BMP-6 and mouse Noggin were purchased
7 from R&D Systems (Minneapolis, MN). Bromocriptine mesylate (BRC), octreotide
8 acetate (OCT) and pasireotide, also known as SOM230 (SOM), were provided by
9 Novartis International Pharmaceutical Ltd. (Basel, Switzerland).
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Cell culture and cAMP measurement

12 Rat pituitary somato-lactotrope tumor GH3 cells were cultured in DMEM/F12 medium
13 supplemented with 10% fetal calf serum (FCS) and antibiotics in a 5% CO₂ atmosphere
14 at 37°C. GH3 cells (1×10^5 viable cells) were seeded in 24-well plates with
15 DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the
16 medium was changed to serum-free DMEM/F12 containing penicillin-streptomycin and
17 0.1 mM IBMX (a specific inhibitor of phosphodiesterase activity), and then the cells
18

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

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9 *Determination of prolactin levels*

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

1 and 13.4%, respectively.

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3 *RNA extraction and quantitative real-time PCR analysis*

4 After preculture, cells (3×10^5 viable cells) were treated with indicated concentrations
5 of FSK, BRC, OCT, SOM, BMP-4, BMP-6 and Noggin in serum-free DMEM/F12.

6 After 24-h culture, the medium was removed and total cellular RNA was extracted
7 using TRIzol® (Invitrogen Corp., Carlsbad, CA). Total RNA was quantified by
8 measuring the absorbance of the sample at 260 nm and was stored at -80°C until assay.

9 The extracted RNA (1 µg) was subjected to RT reaction using First-Strand cDNA
10 Synthesis System® (Invitrogen Corp.) with random hexamer (2 ng/µl), reverse
11 transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50
12 min and at 70°C for 10 min. For the quantification of indicated mRNA levels of PRL,
13 D2R, SSTRs, Id-1 and housekeeping gene ribosomal L19 (RPL19), real-time PCR was
14 performed using LightCycler-FastStart DNA Master SYBR Green I system® (Roche
15 Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60°C with 4 mM MgCl₂,
16 following the manufacturer's protocol. Accumulated levels of fluorescence for each
17 product were analyzed by the second derivative method after melting-curve analysis
18 (Roche Diagnostic Co.), and then, following assay validation by calculating each

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

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14 *Western immunoblot analysis*

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

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

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8 *Statistical analysis*

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

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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

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

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

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

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

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

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Discussion

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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. Secondly, 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

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

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

12 The BMP system including BMP-4 has been shown to play important roles in
13 initial development of the anterior pituitary involving lactotrope (Scully and Rosenfeld,
14 2002). Each of type I and type II receptor for BMPs exhibits serine/threonine kinase
15 activity, in which several preferential combinations of BMP ligands and receptors have
16 been recognized to date. Since ALK-6 is not expressed in GH3 cells, the receptor pair
17 of ALK-3 and BMPRII is likely to be the major functional complex for BMP-4 for
18 regulating PRL and cAMP production.

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

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

1 further mediated by Ca^{++} influx through a direct action on Ca^{++} channels (Chen et al.,
2 1997) and/or indirectly through activating K^{+} channels (Takano et al., 1997). The
3 success of *in vivo* peptide-targeted therapy is highly dependent on the presence and on
4 the localization in the tumor of a sufficient amount of the appropriate receptor. In this
5 regard, we recently reported the involvement of BMP system in corticotrope cells
6 (Tsukamoto et al., 2010). In that study, somatostatin analogs upregulated BMP-Smad
7 signaling by augmenting BMPRII/ALK-3 expression as well as by reducing the
8 expression of inhibitory Smad6/7 in corticotrope AtT20 cells. However, the effects of
9 SOM on BMP-4 signaling were significantly higher than the effects of OCT in AtT20
10 cells, indicating the differences of SSTR affinities between two somatostatin analogs
11 (Tsukamoto et al., 2010). Similar to our current study on lactotrope cells, the
12 interaction of BMP-Smad pathway and CRH receptor signaling was also functionally
13 involved in controlling ACTH production from corticotrope cells in an
14 autocrine/paracrine manner.

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

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

8 In clinical trials testing somatostatin analogs, the effects of cortistatin, a
9 neuropeptide that has high homology with somatostatin and binds with high affinity to
10 all SSTRs, on PRL suppression in prolactinoma patients were reported (Grottoli et al.,
11 2006). BIM-23206, a SSTR-5-selective agonist (Fusco et al., 2008), was also reported
12 to suppress PRL release in patients with DA-sensitive prolactinomas similar to
13 cabergoline. Another study on PRL-secreting adenomas also revealed significant
14 effects of cortistatin and a SSTR-5 agonist on PRL reduction in most of the
15 prolactinomas examined, whereas PRL inhibition by the SSTR-2 agonist was observed
16 in only one case of prolactinoma (Rubinfeld et al., 2006). These results support the
17 notion that SSTR-5 primarily regulates PRL suppression by somatostatin in
18 prolactinoma cells (Shimon et al., 1997). In addition, the existence of novel mRNA

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

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

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References

- 1
2
3 Casanueva FF, Molitch ME, Schlechte JA, Abs R, Bonert V, Bronstein MD, Brue T,
4 Cappabianca P, Colao A, Fahlbusch R, Fideleff H, Hadani M, Kelly P,
5 Kleinberg D, Laws E, Marek J, Scanlon M, Sobrinho LG, Wass JA, Giustina A
6 (2006) Guidelines of the Pituitary Society for the diagnosis and management of
7 prolactinomas. *Clin Endocrinol (Oxf)* 65:265-273.
- 8 Chen ZP, Xu S, Lightman SL, Hall L, Levy A (1997) Intracellular calcium ion
9 responses to somatostatin in cells from human somatotroph adenomas. *Clin*
10 *Endocrinol (Oxf)* 46:45-53.
- 11 Dasen JS, Rosenfeld MG (2001) Signaling and transcriptional mechanisms in pituitary
12 development. *Annu Rev Neurosci* 24:327-355.
- 13 Duhau L, Grassi J, Grouselle D, Enjalbert A, Grognet JM (1991) An enzyme
14 immunoassay for rat prolactin: application to the determination of plasma levels.
15 *J Immunoassay* 12:233-250.
- 16 Duran-Prado M, Gahete MD, Martinez-Fuentes AJ, Luque RM, Quintero A, Webb SM,
17 Benito-Lopez P, Leal A, Schulz S, Gracia-Navarro F, Malagon MM, Castano JP
18 (2009) Identification and characterization of two novel truncated but functional
19 isoforms of the somatostatin receptor subtype 5 differentially present in pituitary
20 tumors. *J Clin Endocrinol Metab* 94:2634-2643.
- 21 Fusco A, Gunz G, Jaquet P, Dufour H, Germanetti AL, Culler MD, Barlier A, Saveanu
22 A (2008) Somatostatinergic ligands in dopamine-sensitive and -resistant
23 prolactinomas. *Eur J Endocrinol* 158:595-603.
- 24 Giacomini D, Paez-Pereda M, Theodoropoulou M, Gerez J, Nagashima AC, Chervin A,
25 Berner S, Labeur M, Refojo D, Renner U, Stalla GK, Arzt E (2006) Bone
26 morphogenetic protein-4 control of pituitary pathophysiology. *Front Horm Res*
27 35:22-31.
- 28 Gillam MP, Molitch ME, Lombardi G, Colao A (2006) Advances in the treatment of
29 prolactinomas. *Endocr Rev* 27:485-534.
- 30 Grottoli S, Gasco V, Broglio F, Baldelli R, Ragazzoni F, Gallenca F, Mainolfi A,
31 Prodam F, Muccioli G, Ghigo E (2006) Cortistatin-17 and somatostatin-14
32 display the same effects on growth hormone, prolactin, and insulin secretion in
33 patients with acromegaly or prolactinoma. *J Clin Endocrinol Metab*
34 91:1595-1599.
- 35 Hofland LJ, Felders RA, de Herder WW, Lamberts SW (2010) Pituitary tumours: the

- 1 sst/D(2) receptors as molecular targets. *Mol Cell Endocrinol*:in press.
- 2 Hofland LJ, van der Hoek J, van Koetsveld PM, de Herder WW, Waaijers M,
3 Sprij-Mooij D, Bruns C, Weckbecker G, Feelders R, van der Lely AJ, Beckers A,
4 Lamberts SW (2004) The novel somatostatin analog SOM230 is a potent
5 inhibitor of hormone release by growth hormone- and prolactin-secreting
6 pituitary adenomas in vitro. *J Clin Endocrinol Metab* 89:1577-1585.
- 7 Jaquet P, Ouafik L, Saveanu A, Gunz G, Fina F, Dufour H, Culler MD, Moreau JP,
8 Enjalbert A (1999) Quantitative and functional expression of somatostatin
9 receptor subtypes in human prolactinomas. *J Clin Endocrinol Metab*
10 84:3268-3276.
- 11 Johnston JM, Wood DF, Bolaji EA, Johnston DG (1991) The dopamine D2 receptor is
12 expressed in GH3 cells. *J Mol Endocrinol* 7:131-136.
- 13 Kioussi C, Carriere C, Rosenfeld MG (1999) A model for the development of the
14 hypothalamic-pituitary axis: transcribing the hypophysis. *Mech Dev* 81:23-35.
- 15 Labeur M, Paez-Pereda M, Haedo M, Arzt E, Stalla GK (2010) Pituitary tumors: Cell
16 type-specific roles for BMP-4. *Mol Cell Endocrinol*:in press.
- 17 Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors:
18 from structure to function. *Physiol Rev* 78:189-225.
- 19 Miyoshi T, Otsuka F, Otani H, Inagaki K, Goto J, Yamashita M, Ogura T, Iwasaki Y,
20 Makino H (2008) Involvement of bone morphogenetic protein-4 in GH
21 regulation by octreotide and bromocriptine in rat pituitary GH3 cells. *J*
22 *Endocrinol* 197:159-169.
- 23 Molitch ME (2005) Pharmacologic resistance in prolactinoma patients. *Pituitary*
24 8:43-52.
- 25 Otsuka F (2010) Multiple endocrine regulation by bone morphogenetic protein system.
26 *Endocr J* 57:3-14.
- 27 Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y,
28 Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK,
29 Arzt E (2003) Involvement of bone morphogenetic protein 4 (BMP-4) in
30 pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk.
31 *Proc Natl Acad Sci U S A* 100:1034-1039.
- 32 Reisine T, Bell GI (1995) Molecular biology of somatostatin receptors. *Endocr Rev*
33 16:427-442.
- 34 Rubinfeld H, Hadani M, Barkai G, Taylor JE, Culler MD, Shimon I (2006) Cortistatin
35 inhibits growth hormone release from human fetal and adenoma pituitary cells
36 and prolactin secretion from cultured prolactinomas. *J Clin Endocrinol Metab*

- 1 91:2257-2263.
- 2 Scully KM, Rosenfeld MG (2002) Pituitary development: regulatory codes in
3 mammalian organogenesis. *Science* 295:2231-2235.
- 4 Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004) The bone morphogenetic
5 protein system in mammalian reproduction. *Endocr Rev* 25:72-101.
- 6 Shimon I, Yan X, Taylor JE, Weiss MH, Culler MD, Melmed S (1997) Somatostatin
7 receptor (SSTR) subtype-selective analogues differentially suppress in vitro
8 growth hormone and prolactin in human pituitary adenomas. Novel potential
9 therapy for functional pituitary tumors. *J Clin Invest* 100:2386-2392.
- 10 Takano K, Yasufuku-Takano J, Teramoto A, Fujita T (1997) Gi3 mediates
11 somatostatin-induced activation of an inwardly rectifying K⁺ current in human
12 growth hormone-secreting adenoma cells. *Endocrinology* 138:2405-2409.
- 13 Tsukamoto N, Otsuka F, Miyoshi T, Yamanaka R, Inagaki K, Yamashita M, Otani H,
14 Takeda M, Suzuki J, Ogura T, Iwasaki Y, Makino H (2010) Effects of bone
15 morphogenetic protein (BMP) on adrenocorticotropin production by pituitary
16 corticotrope cells: involvement of up-regulation of BMP receptor signaling by
17 somatostatin analogs. *Endocrinology* 151:1129-1141.
- 18 Yang SK, Parkington HC, Blake AD, Keating DJ, Chen C (2005) Somatostatin
19 increases voltage-gated K⁺ currents in GH3 cells through activation of multiple
20 somatostatin receptors. *Endocrinology* 146:4975-4984.

21
22

1 **Figure Legends**

2

3 **Fig. 1. Effects of BMPs and somatostatin analogs on prolactin secretion.** A) GH3

4 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were

5 then treated with BMP-4 (10-100 ng/ml), BMP-6 (10-100 ng/ml) and Noggin (10-100

6 ng/ml). After 24-h culture, the supernatants of culture media were collected, and

7 prolactin (PRL) and cAMP levels were determined by specific enzyme immunoassays.

8 For measurement of cAMP levels, cells were cultured with serum-free medium

9 containing 0.1 mM of IBMX. B) and C) GH3 cells (1×10^5 viable cells) were

10 precultured in serum-free DMEM/F12. Cells were treated with octreotide (OCT;

11 0.1-10 μ M), bromocriptine (BRC; 1-100 μ M) or pasireotide (SOM; 0.1-30 μ M) in the

12 absence (B) or presence (C) of forskolin (FSK; 1 μ M). After 24-h culture, the culture

13 media were collected, and PRL and cAMP levels were determined. Results in all

14 panels are shown as mean \pm SEM of data from at least three separate experiments, each

15 performed with triplicate samples. The results were analyzed by ANOVA with

16 Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, $P <$

17 0.05 vs. control group in each panel; and the values with different superscript letters are

18 significantly different at $P < 0.05$.

19

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

14

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

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

10

11 **Fig. 4. Effects of BMPs on somatostatin receptors (SSTR) expression and effects**
12 **of forskolin and somatostatin analogs on BMP receptor signaling in GH3 cells.**

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

1 **B)** After preculture, cells were treated with BMP-4 and BMP-6 (100 ng/ml) in the
2 absence or presence of forskolin (FSK; 1 μ M). After 24-h culture, total cellular RNA
3 was extracted and subjected to RT-PCR reaction. The mRNA expression levels of
4 Id-1 were quantified by real-time PCR analysis. The expression levels of target genes
5 were standardized by RPL19 level in each sample. **C)** Cells (3×10^5 viable cells) were
6 precultured in serum-free DMEM/F12. After pretreatment with octreotide (OCT; 10
7 μ M) and pasireotide (SOM; 10 μ M) for 1 h, the cells were treated with BMP-4 and
8 BMP-6 (100 ng/ml) for 60 min. The cell lysates were then subjected to SDS-PAGE
9 immunoblotting (IB) analysis using anti-phospho-Smad1/5/8 antibody and anti-actin
10 antibody. The relative integrated density of each protein band was digitized and
11 pSmad1/5/8 levels were normalized by actin level in each sample. Results in all
12 panels are shown as mean \pm SEM of data from at least three separate experiments, each
13 performed with triplicate samples. The results were analyzed by ANOVA with
14 Tukey-Kramer's post hoc test or unpaired *t*-test. For each result within a panel, *, *P* <
15 0.05 vs. control group in each panel; and the values with different superscript letters are
16 significantly different at *P* < 0.05.

17

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

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

12

Fig. 1

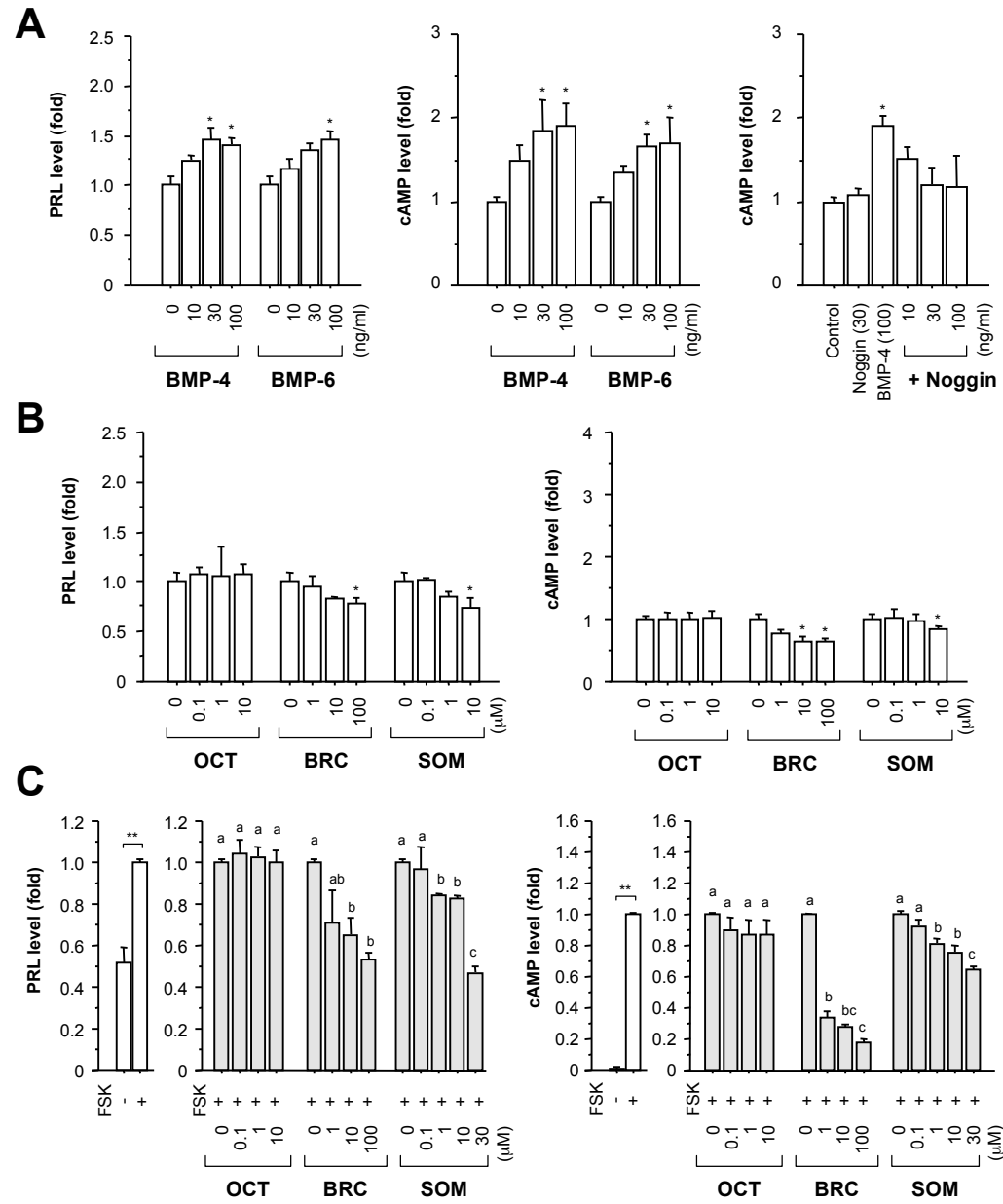


Fig. 2

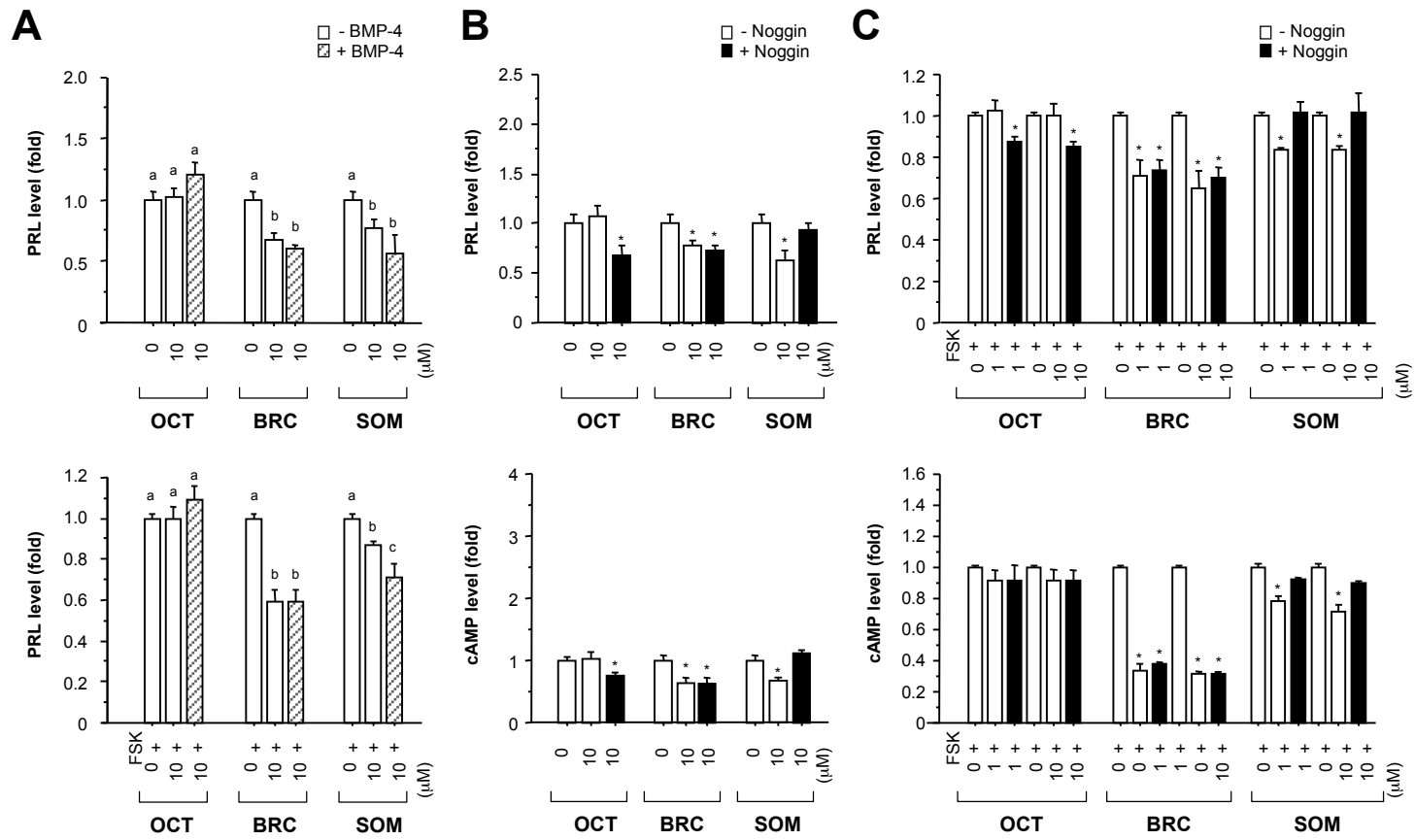


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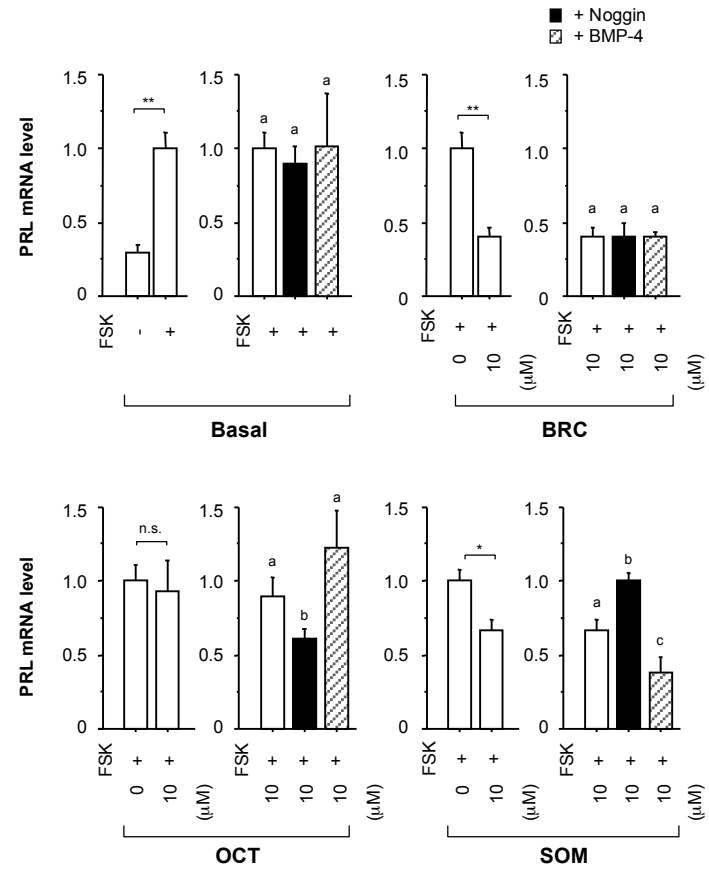


Fig. 4

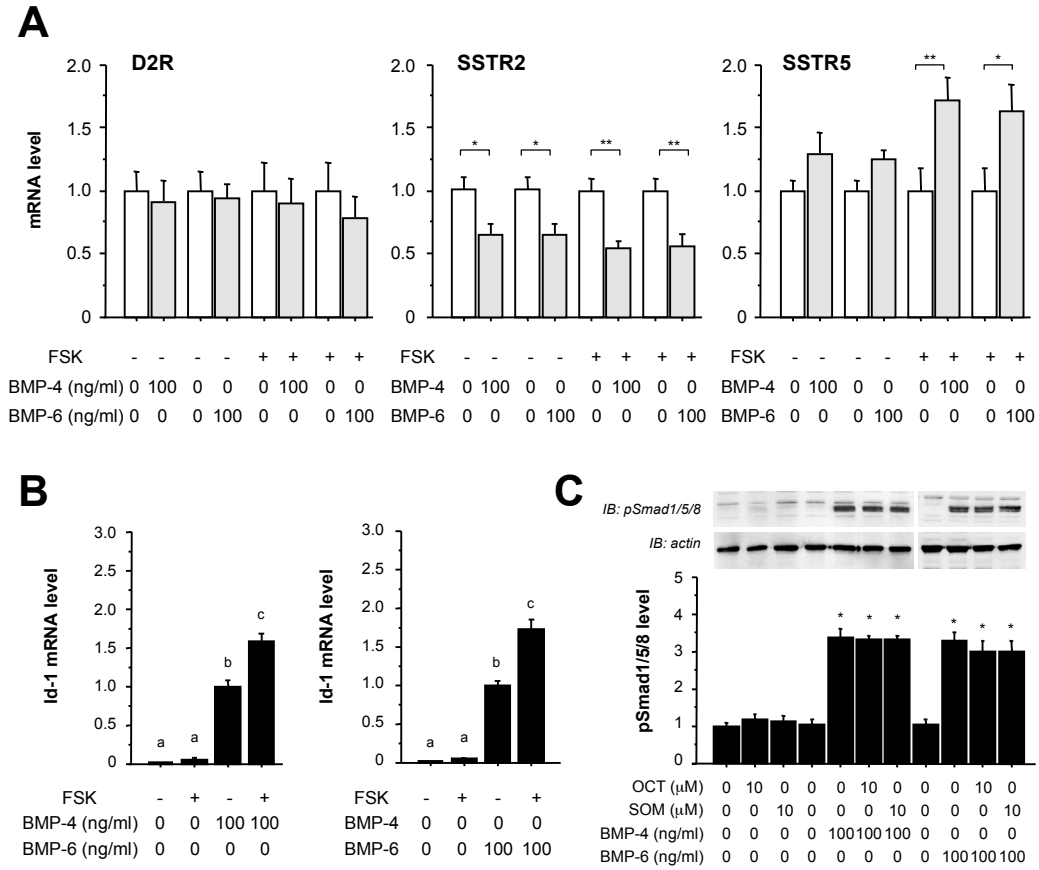


Fig. 5

