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Keywords: Bone morphogenetic protein, Gonadotrope, Gonadotropin-releasing hormone, Luteinizing hormone, and Somatostatin.

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Response to Reviewers: Dear Dr. Karl-Heinz Herzig, MD, PhD
Editor of Peptides

Ms. Ref. No.: PEPTIDES-D-15-00607-R1:
Title: “BMP-6 modulates somatostatin effects on luteinizing hormone production by gonadrotrope cells.” by Toma et al.

We would like to thank the referees and the editor for their careful review of the manuscript and constructive comments. We have responded to all of the comments from the reviewers. Our manuscript was revised on the basis of the editor’s and reviewer’s comments and critiques (changes indicated with underlining).

We would also like to thank you for allowing us to resubmit our revised manuscript. We hope that the revised manuscript is now acceptable for publication in Peptides.

Our responses to the referees’ specific comments are provided in the attachment file.

Sincerely yours,

Kishio Toma, M.D.
Fumio Otsuka, M.D., Ph.D.

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LH secretion. The authors have provided a simple yet detailed study of the negative impact of somatostatin receptor activation on GnRH regulation of LH. Of particular interest is the demonstration that BMP6 exaggerates somatostatin inhibition in gonadotropes and that this correlates with increased expression of SSTR5. Although the authors do not directly knock down SSTR5 activity, thereby demonstrating a direct link, the correlative evidence is well presented. It is understood that the capacity for redundant SSTR expression may complicate these studies. I have only one minor comment. The authors should present in their discussion the rationale regarding why SSTR5 receptor was not only minimally responsive at the protein level. This could potentially be due to rec3eptor turnover, but may also be a technical issue with regard to imaging technique.. Although they state that ImageJ was used for quantitation, they do not discuss how the image was obtained. Was this from a luminescent imaging or derived from film exposure? Film is generally inadequate for quantitative measurements and should be avoided. Although the image was modestly significant, the authors should state the imaging technique used clearly in the methods and figure legend for 1F.

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Thank you for your review.

Title:

BMP-6 modulates somatostatin effects on luteinizing hormone production by gonadrotrope cells.

Authors:

Kishio Toma and Fumio Otsuka, *et al.*

Highlight:

- BMP-6 enhanced inhibitory effects of somatostatin analogs on LH secretion by upregulating SSTR5.
- Somatostatin analogs suppressed GnRH-induced ERK phosphorylation in the presence of BMP-6.
- Somatostatin analogs impaired Smad signaling by regulating BMPR and inhibitory Smad expression.
- BMP-6 modulates responsiveness of somatostatin analogs on LH production by gonadotrope cells.

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BMP-6 modulates somatostatin effects on luteinizing hormone production by gonadrotrope cells.

Kishio Toma[1], Fumio Otsuka[2,3], Kohei Oguni[1,2], Tomohiro Terasaka[1,3], Motoshi Komatsubara[1], Naoko Tsukamoto-Yamauchi[1], Kenichi Inagaki[1] and Hirofumi Makino[3]

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Short title: Somatostatin and BMP-6 in LH regulation.

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Abstract

The effects of somatostatin analogs and roles of BMP-6 in the regulation of luteinizing hormone (LH) secretion were investigated using mouse gonadotrope LβT2 cells. LH mRNA expression and LH secretion induced by GnRH were suppressed by treatments with somatostatin analogs, including octreotide and pasireotide, in LβT2 cells. Of note, the inhibitory effects of somatostatin analogs on LH secretion were enhanced by the action of BMP-6. BMP-6 increased the expression levels of somatostatin receptor (SSTR)5, suggesting that BMP-6 upregulates SSTR activity that leads to reduction of GnRH-induced LH secretion. In addition, GnRH-induced phosphorylation of MAPKs including ERK, but not P38 or SAPK, was suppressed by pasireotide in the presence of BMP-6. Given that each inhibitor of ERK, JNK or P38 signaling suppressed GnRH-induced LH transcription, MAPKs are individually involved in the induction of LH production by LβT2 cells. Somatostatin analogs also impaired BMP-6-induced Smad1/5/8 phosphorylation by suppressing BMPRs and augmenting Smad6/7 expression. Collectively, the results indicate that somatostatin analogs have dual effects on the modulation of GnRH-induced MAPK signaling and BMP activity. The pituitary BMP system may play a regulatory role in GnRH-induced LH secretion by tuning the responsiveness to somatostatin analogs in gonadotrope cells.

Key words: Bone morphogenetic protein, Gonadotrope, Gonadotropin-releasing hormone, Luteinizing hormone, and Somatostatin.

Introduction

Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus and local factors that reside in the pituitary play key roles in the secretion of gonadotropins. Bone morphogenetic proteins (BMPs) are growth factors that belong to the transforming growth factor (TGF)-β superfamily. In addition to the promotive effects of BMPs on bone formation, various biological activities of BMPs in endocrine tissues including the ovary and pituitary have been uncovered [1-3].

BMPs have been shown to play important roles in initial development of the anterior pituitary [4]. Recent studies have revealed that BMPs also modulate the pathogenesis of differentiated pituitary lineages [5, 6]. We previously reported that pituitary BMPs are involved in the regulation of follicle-stimulating hormone (FSH) secretion by gonadotropes [7-9]. BMP-6 and GnRH mutually augmented FSH transcription, while GnRH-induced FSH transcription was impaired by BMP-7 in LβT2 gonadotrope cells [10]. However, the regulatory mechanism of luteinizing hormone (LH) production by BMPs has not yet been elucidated.

Somatostatin analogs are effective for controlling functioning pituitary adenomas such as growth hormone-, thyrotropin- and adrenocorticotropin-producing tumors, depending on the expression profiles of somatostatin receptors (SSTRs). However, little is known about the expression of SSTRs in gonadotropin-producing adenomas. Many pituitary adenomas classically diagnosed as nonfunctioning tumors have been shown to possess FSH

productivity [11, 12], implying that gonadotropinomas are originally derived from the same clone as that from which nonfunctioning tumors are derived [13, 14]. In nonfunctioning adenomas, a variable SSTR subtype expression profile, with SSTR2 and, secondarily, SSTR3 being the major subtypes has been reported [15], though the efficacy of somatostatin ligands remains unclear. It was recently reported that pituitary gonadotropinomas express different patterns of SSTR subtypes depending on the tumor condition and aggressiveness, leading to varied reactions to somatostatin analogs [16]. Gonadotropinomas undergoing early recurrence had lower expression levels of SSTR2/3 than the levels in those without a five-year recurrence. However, recurrent tumors had higher expression levels of SSTR1, 2, 3 and 5, suggesting higher sensitivity to somatostatin analogs to possibly counteract the recurrence [16].

In this study, we investigated the effects of somatostatin analogs and roles of BMP-6 in the regulation of LH secretion by gonadotrope LβT2 cells. It was revealed that somatostatin analogs have dual effects on the modulation of GnRH-induced MAPK signaling and BMP-6 activity. A functional link between the BMP system and GnRH-induced LH transcription was newly uncovered.

Materials and Methods

Reagents and supplies

Dulbecco's modified Eagle's medium (DMEM) and GnRH human acetate salt were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-6 was purchased from R&D Systems, Inc. (Minneapolis, MN). The ERK inhibitor U0126 and P38 inhibitor SB203580 were from Promega Corp. (Madison, WI), and the SAPK/JNK inhibitor SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA). Octreotide acetate (OCT) and pasireotide, also known as SOM230 (SOM), were provided by Novartis International Pharmaceutical Ltd. (Basel, Switzerland). LβT2 cells and a plasmid of LHβ-Luc that contains 1800 bp of the rat LHβ promoter were provided by Drs. Pamela L. Mellon and Mark A. Lawson, University of California, San Diego.

Cell culture and LH assay

LβT2 cells were grown in DMEM containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2 in air. The cells were grown in 75-cm^2 flasks until they became confluent and were then transferred to 12-well plates. After preculture, the medium was replaced with fresh serum-free DMEM, and indicated combinations of GnRH, BMP-6, and somatostatin analogs were added to the culture medium. After 48-h culture, the conditioned medium was collected and LH concentrations were determined by a Rodent LH ELISA with the minimal detectable concentration of rodent LH being ~0.5ng/ml (Endocrine Technologies, Inc. Newark, CA).

Transient transfection and luciferase assay

After 24-h preculture, LβT2 cells (~60% confluent) were transiently transfected with 500 ng of the luciferase reporter plasmid LHβ-Luc and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 16 h. The cells were treated with GnRH and BMP-6 in fresh serum-free DMEM for 24 h and then washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-galactosidase (β-gal) activity of the cell lysate were measured by a luminometer. The data were shown as the ratio of luciferase to β-gal activity.

RNA extraction and quantitative real-time PCR analysis

LβT2 cells (3×10^5 viable cells/well) were cultured in 12-well plates with 10% FCS. The medium was then changed to serum-free DMEM, and the cells were subsequently treated with indicated concentrations of GnRH, BMP-6, octreotide and pasireotide alone or in combination with various inhibitors of intracellular MAPK signaling. After 24-h culture, total cellular RNAs were extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1 μg) was subjected to RT reaction using the First-Strand cDNA Synthesis System® (Invitrogen Corp.) at 42°C for 50 min and 70°C for 10 min. PCR primer pairs were selected from different exons of the corresponding genes as follows: GnRHR: 671-680 and 902-921 (NM_010323); LHβ: 138-157 and 348-

367 (NM_008497); SSTR1: 526-546 and 869-889 (from GenBank accession No. X62314); SSTR2: 240-260 and 559-579 (M93273); SSTR3: 501-521 and 782-802 (X63574); SSTR4: 400-420 and 781-801 (M96544); and SSTR5: 781-801 and 1031-1051 (L04535). Primer pairs for ALK-2, -3, ActRII, BMPRII, Smad6/7 and a house-keeping gene, ribosomal protein L19 (RPL19), were selected as reported previously [9, 17, 18]. For the quantification of GnRHR, LHβ, SSTR1-5, BMPRs, Smads and RPL19 mRNAs, real-time PCR was performed using the LightCycler-FastStart DNA master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan). Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis, and then, following assay validation by calculating amplification efficiency of each amplicon, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. 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 the target gene by the quantity of RPL19 in each sample.

Western immunoblot analysis

LβT2 cells (1.5×10^5 viable cells/well) were treated by 15- to 60-min stimulation or 24-h culture with BMP-6, octreotide, pasireotide and GnRH either alone or in the indicated combinations in serum-free DMEM. After the stimulation, the cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, 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 an anti-SSTR5 (H-54) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho- and anti-total-ERK1/2, P38 and SAPK/JNK antibodies, anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA), and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal densities were analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) or by NIH image J 1.34s. For evaluating SSTR5 expression and protein phosphorylation, ratios of the signal intensities of SSTR5 and phosphorylated protein / total protein or actin were calculated, respectively.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data were subjected to ANOVA or unpaired *t*-test to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If differences were detected by ANOVA, Fisher's protected least significant difference (PLSD) test was used to determine which means differed (StatView 5.0 software). *P* values < 0.05 were accepted as statistically significant.

Results

First, we examined the effects of GnRH, somatostatin analogs and BMP-6 on LH production by LβT2 cells. GnRHR, all of the receptor subtypes for SSTRs including SSTR1 to 5, and BMP receptors including ALK-2, -3 but not ALK-6, ActRII and BMPRII were shown to be expressed in LβT2 cells by RT-PCR [7]. As shown in **Fig. 1A**, the basal levels of LHβ mRNA were increased by GnRH (10 nM) but were not changed by somatostatin analogs, including octreotide and pasireotide (10 μM), or by BMP-6 (100 ng/ml). Activity of LHβ transcription was induced by treatment with GnRH (10 nM) but not by treatment with BMP-6 (100 ng/ml) (**Fig. 1B**).

As shown in **Fig. 1C**, LH production in the medium was also significantly increased by GnRH (10 nM) but not by BMP-6 (100 ng/ml). Interestingly, the levels of GnRH-induced secretion of LH were decreased in the presence of somatostatin analogs (10 μM) including octreotide, an agonist for SSTR2, or pasireotide, an agonist for SSTR1, 2, 3 and 5 (**Fig. 1C**). Of note, the suppressive effects of octreotide and pasireotide on GnRH-induced LH expression were enhanced in the presence of BMP-6 (100 ng/ml) (**Fig. 1D**). To determine the mechanism by which BMP-6 facilitates the suppressive effects of somatostatin analogs on GnRH-induced LH expression, the effects of BMP-6 on the expression of GnRHR and key receptors for somatostatin analogs were examined. BMP-6 (100 ng/ml) treatment had no significant effect on GnRHR mRNA expression (**Fig. 1E**). Of interest, BMP-6 (100 ng/ml) increased SSTR5

mRNA and protein (**Fig. 1E, F**) levels in LβT2cells, while BMP-6 had no significant effect on SSTR2 expression (**Fig. 1E**). The results suggested that upregulation of SSTR5 induced by BMP-6 might be involved in the reduction of GnRH-induced secretion of LH by somatostatin analogs.

Involvement of MAPK, which has been reported to be a key functional pathway in LβT2 cells [10, 19, 20], in the regulation of GnRH-induced LH production was then examined. The results of experiments using MAPK inhibitors including U0126, SB203580 and SP600125 (1 to 10 μM) showed that all of the MAPKs are, at least in part, involved in the GnRH (10 nM)-induced LH expression (**Fig. 2A**). As shown in **Fig. 2B**, GnRH (10 nM) treatment readily induced phosphorylation of MAPK pathways including ERK, P38 and SAPK, and co-treatment with BMP-6 (100 ng/ml) had no specific effect. In the presence of pasireotide (10 μM), GnRH-induced ERK, but not P38 or SAPK, phosphorylation was slightly reduced (**Fig. 2B**). BMP-6 treatment (100 ng/ml) enhanced the effects of pasireotide (10 μM), leading to a significant reduction of GnRH (10 nM)-induced ERK activation (**Fig. 2C**). The results thus suggested that the effects of SSTR5, possibly augmented by BMP-6, suppressed the ERK phosphorylation induced by GnRH.

The effects of somatostatin analogs on BMP signaling were also examined. As shown in **Fig. 3A**, BMP-6 (100 ng/ml) induced Smad1/5/8 phosphorylation, and its activity was reduced by co-treatment with GnRH (10 nM). Of note, treatments with somatostatin analogs (10 μM) significantly suppressed BMP-6 (100 ng/ml)-induced Smad1/5/8 phosphorylation, the effect

of pasireotide being more efficient than that of octreotide (**Fig. 3A**). To determine the mechanism by which somatostatin analogs suppress BMP-Smad signaling in LβT2 cells, the effects of somatostatin analogs on BMP receptor expression were assessed. As shown in **Fig. 3B**, somatostatin analogs (10 μM) reduced the mRNA expression of BMP receptors including ALK-2, -3, ActRII and BMPRII. The effects were more potent in the case of pasireotide than octreotide, being in accordance to the changes seen in Smad phosphorylation. The changes in inhibitory Smad6/7 expression were also examined. As shown in **Fig. 3C**, somatostatin analogs (10 μM) individually increased mRNA levels of Smad6/7 in the presence of BMP-6 (100 ng/ml). Thus, BMPR downregulation as well as increase in inhibitory Smads are likely to be involved in the suppressive effects of somatostatin analogs on BMP signaling in LβT2 cells.

Discussion

In the present study, the effects of somatostatin analogs and roles of BMP-6 in the regulation of LH secretion by gonadotrope LβT2 cells were uncovered. Somatostatin analogs have dual effects on modulation of GnRH-induced MAPK signaling and BMP-6 activity (**Fig. 4**). LH production induced by GnRH was suppressed by somatostatin analogs in LβT2 cells. Of note, the inhibitory effects of somatostatin analogs on LH secretion were enhanced by BMP-6 treatment. Since it was shown that BMP-6 increased the expression levels of SSTR5, the results suggested that BMP-6 upregulates SSTR actions, leading to reduction of GnRH-induced secretion of LH by pasireotide in LβT2 cells.

The effects of somatostatin are elicited by binding of five SSTRs being coupled with the G-protein, which are widely distributed in many endocrine tissues. The efficacy and potency of somatostatin analogs are functionally linked to the selectivity profile of SSTRs, in which the binding capability to SSTR2/5 is critical [21]. SSTR2/5 is intracellularly coupled to adenylate cyclase, leading to reduction of cellular cAMP synthesis [22]. SSTR actions are also mediated by calcium influx through a direct action on calcium channels [23] and an indirect action through potassium channels [24]. The effectiveness of a somatostatin analog is, in general, dependent on the expression of a sufficient amount of appropriate SSTRs.

Locally produced BMPs play critical roles in differentiation of pituitary gonadotrope cells [4]. As for their receptors in the pituitary, expression of

BMPRII, ActRII, ALK-2 and ALK-3 mRNAs in mice [7], ALK-6 mRNA in sheep, and ActRII and ActRIIB mRNAs in rats [1] has been reported. Regarding the function on gonadotropin production, it has been shown that BMP-6 and -7 stimulate FSH synthesis and secretion in the gonadotrope cell line LβT2 [25]. BMP-15 has also been shown to stimulate FSH biosynthesis and secretion by rat primary pituitary cells without regulating LH secretion [7]. Differential expression of a BMP-binding protein, follistatin, was also shown in human FSH-producing and nonfunctioning adenomas [8]. In nonfunctioning tumors, the expression of follistatin is abundant and thereby activation of activin/BMP signaling is insignificant, resulting in less production of FSH. On the other hand, in gonadotropinomas, the effect of follistatin is negligible and thereby activins can bind to their receptors and effectively transduce the signaling to stimulate FSH production. The intrapituitary regulation of activin/BMP may be involved in the functional transition of gonadotropin productivity.

The results of this study suggested that the pituitary BMP system plays a regulatory role in GnRH-induced LH secretion by modulating the responsiveness to somatostatin analogs in gonadotrope cells. GnRH-induced phosphorylation of ERK was impaired by a somatostatin analog in the presence of BMP-6. ERK, JNK and P38 are individually involved in the induction of LH secretion by GnRH, although FSH secretion was shown to be predominantly regulated by ERK signaling [10]. Somatostatin analogs also impaired Smad1/5/8 phosphorylation induced by BMP-6. Namely, a functional link between the BMP system and GnRH-induced LH transcription was uncovered.

As for the interrelationship between somatostatin effects and BMPs, we previously reported that somatostatin analogs, including octoreotide and pasireotide, upregulated BMP-Smad1/5/8 activity with increased expression of ALK-3/BMPRII but downregulated inhibitory Smad6/7 molecules in mouse corticotrope AtT20 cells [18, 26]. Endogenous BMP activity is thus involved in the mechanism by which somatostatin analogs suppress adrenocorticotropin production [18]. In addition, BMP-4 showed a significant inhibition of cell mitosis in the presence of pasireotide and octreotide [18]. These findings suggested that somatostatin analogs facilitate BMP signal transduction in corticotrope cells.

A functional link between the BMP system and SSTR expression was also seen in prolactin secretion by lactotrope cells [27]. BMP-4 and -6 act to increase prolactin release, while they reduced SSTR2 expression but increased SSTR5 expression in rat lactotrope GH3 cells [27, 28]. The effect of the SSTR5-preferring agonist pasireotide, which reduced prolactin secretion induced by forskolin, was facilitated in the presence of BMP-4 but blocked by a BMP-binding protein, noggin. In the presence of noggin, the SSTR2-preferring agonist octreotide exerted an inhibitory effect on prolactin release. These results suggested that endogenous BMPs also play regulatory roles in SSTR sensitivity of lactotorope cells [27].

In the present study, a functional interaction of BMP-6 and somatostatin analogs in GnRH-induced LH secretion by gonadrotrope cells was revealed. GnRH-induced LH production was suppressed by pasireotide and octreotide. The inhibitory effects of somatostatin analogs on LH secretion were enhanced

in the presence of BMP-6 by upregulating SSTR5 in gonadotrope cells. GnRH-induced phosphorylation of ERK was suppressed by somatostatin analogs and BMP-6, while somatostatin analogs impaired BMP-induced Smad1/5/8 activation. The pituitary BMP system may play a regulatory role in GnRH-induced LH secretion by modulating the responsiveness to somatostatin analogs in gonadotrope cells (**Fig. 4**).

Collectively, the results indicate that the pituitary BMP system may act as a functional modulator for somatostatin analogs in an autocrine/paracrine manner, leading to fine-tuning of the sensitivity in gonadotropes, as shown in lactotropes and corticotropes. Regulation of the endogenous BMP system, which is a functional determinant of SSTR sensitivity, may be a future strategy for medication of pituitary adenomas.

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Figure Legends:

Fig. 1. Effects of somatostatin analogs on LH expression and production by LβT2 cells. A) LβT2 cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH, BMP-6, octreotide (OCT) and pasireotide (SOM) in serum-free DMEM. After 24-h culture, total cellular RNAs were extracted and LHβ mRNA levels were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **B)** Cells were transiently transfected with LHβ-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24-h treatment with the indicated concentrations of GnRH and BMP-6, cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-galactosidase (β-gal) activity. **C)** Cells (3×10^5 cells/well) were treated with the indicated concentrations of BMP-6, somatostatin, OCT or SOM in the presence of GnRH in serum-free conditions. After 48-h culture, LH concentrations in the culture media were determined by EIA assays. **D, E)** Cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH, BMP-6, OCT and SOM in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of LHβ, GnRHR, SSTR2 and SSTR5 were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **F)** Cells (1.5×10^5 cells/well) were treated with the indicated BMP-6 concentration in serum-free conditions for 24 h. The cells were then lysed and subjected to immunoblot (IB) analysis using antibodies that detect SSTR5 and actin as an internal control.

The results shown are representative of those obtained from three independent experiments. The integrated signal density of each protein band was digitally analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences), and the ratios of signal intensities of SSTR5/actin were expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A-C)** and unpaired t-test **(D-F)**. **P* < 0.05 and ***P* < 0.01 vs. control or between the indicated groups. n.s., not significant.

Fig. 2. Effects of somatostatin analogs on GnRH-induced MAPK signaling in LβT2 cells. A) LβT2 cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH and each inhibitor of intracellular MAPKs including U0126, SP203580 and SP600125 in serum-free DMEM for 24 h. Total cellular RNAs were extracted and the mRNA levels of LHβ were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **B)** Cells (1.5×10^5 cells/well) were pretreated with the indicated concentrations of BMP-6, OCT and SOM in serum-free conditions for 24 h, and then the indicated concentration of GnRH was added to the medium. After 15-min stimulation, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect phosphorylation of MAPKs. The results are representative of those obtained from three independent experiments. **C)** The relative integrated density of the ERK protein band was digitized, with the phosphorylated levels being normalized by the total levels, and then the results were expressed as fold changes. Results in all panels are shown as means ±

SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A, C)**. **P* < 0.05 vs. control and $^{\#}P$ < 0.05 vs. GnRH group.

Fig. 3. Effects of somatostatin analogs on BMP-6 signaling in LβT2 cells. **A)** LβT2 cells (1.5 × 10^5 cells/well) were pre-treated with the indicated concentrations of GnRH, OCT and SOM in serum-free DMEM for 24 h. After 1-h stimulation with BMP-6, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect pSmad1/5/8 and actin. The results are representative of those obtained from three independent experiments. The relative integrated density of the pSmad1/5/8 protein band was digitized, with the phosphorylated levels being normalized by actin levels, and then the results were expressed as fold changes. **B, C)** Cells (3 × 10^5 cells/well) were treated with the indicated concentrations of BMP-6, OCT and SOM in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of **(B)** ALK-2, -3, ActRII, BMPRII and **(C)** Smad6/7 were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A-C)**. For each result within a panel, the values with different superscript letters are significantly different at *P* < 0.05. **P* < 0.05 vs. control.

Fig. 4. Functional interaction of BMP-6 and somatostatin in GnRH-induced production of LH by gonadrotrope cells. LH production induced by GnRH was suppressed by somatostatin (SS) analogs, pasireotide and octreotide. The inhibitory effects of SS analogs on LH production were enhanced by BMP-6 action. BMP-6 increased SSTR5 expression, leading to reduction of GnRH-induced LH production by suppressing ERK. SS analogs also impaired BMP-induced Smad1/5/8 signaling via BMPR reduction and Smad6/7 expression. BMP-6 plays a regulatory role in GnRH-induced LH production by modulating the responsiveness to SS analogs.

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Disclosure Statement

FO receives speaker honoraria from Novo Nordisk. HM is a consultant for AbbVie, Astellas and Teijin, receives speaker honoraria from Astellas, Boehringer-ingelheim, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novartis, Pfizer, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novo Nordisk, Takeda, and Tanabe Mitsubishi.

Abbreviations

ActRII, activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; SSTR, somatostatin receptor; and TGF-β, transforming growth factor-β.

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BMP-6 modulates somatostatin effects on luteinizing hormone production by gonadrotrope cells.

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Abstract

The effects of somatostatin analogs and roles of BMP-6 in the regulation of luteinizing hormone (LH) secretion were investigated using mouse gonadotrope LβT2 cells. LH mRNA expression and LH secretion induced by GnRH were suppressed by treatments with somatostatin analogs, including octreotide and pasireotide, in LβT2 cells. Of note, the inhibitory effects of somatostatin analogs on LH secretion were enhanced by the action of BMP-6. BMP-6 increased the expression levels of somatostatin receptor (SSTR)5, suggesting that BMP-6 upregulates SSTR activity that leads to reduction of GnRH-induced LH secretion. In addition, GnRH-induced phosphorylation of MAPKs including ERK, but not P38 or SAPK, was suppressed by pasireotide in the presence of BMP-6. Given that each inhibitor of ERK, JNK or P38 signaling suppressed GnRH-induced LH transcription, MAPKs are individually involved in the induction of LH production by LβT2 cells. Somatostatin analogs also impaired BMP-6-induced Smad1/5/8 phosphorylation by suppressing BMPRs and augmenting Smad6/7 expression. Collectively, the results indicate that somatostatin analogs have dual effects on the modulation of GnRH-induced MAPK signaling and BMP activity. The pituitary BMP system may play a regulatory role in GnRH-induced LH secretion by tuning the responsiveness to somatostatin analogs in gonadotrope cells.

Key words: Bone morphogenetic protein, Gonadotrope, Gonadotropin-releasing hormone, Luteinizing hormone, and Somatostatin.

Introduction

Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus and local factors that reside in the pituitary play key roles in the secretion of gonadotropins. Bone morphogenetic proteins (BMPs) are growth factors that belong to the transforming growth factor (TGF)-β superfamily. In addition to the promotive effects of BMPs on bone formation, various biological activities of BMPs in endocrine tissues including the ovary and pituitary have been uncovered [1-3].

BMPs have been shown to play important roles in initial development of the anterior pituitary [4]. Recent studies have revealed that BMPs also modulate the pathogenesis of differentiated pituitary lineages [5, 6]. We previously reported that pituitary BMPs are involved in the regulation of follicle-stimulating hormone (FSH) secretion by gonadotropes [7-9]. BMP-6 and GnRH mutually augmented FSH transcription, while GnRH-induced FSH transcription was impaired by BMP-7 in LβT2 gonadotrope cells [10]. However, the regulatory mechanism of luteinizing hormone (LH) production by BMPs has not yet been elucidated.

Somatostatin analogs are effective for controlling functioning pituitary adenomas such as growth hormone-, thyrotropin- and adrenocorticotropin-producing tumors, depending on the expression profiles of somatostatin receptors (SSTRs). However, little is known about the expression of SSTRs in gonadotropin-producing adenomas. Many pituitary adenomas classically diagnosed as nonfunctioning tumors have been shown to possess FSH

productivity [11, 12], implying that gonadotropinomas are originally derived from the same clone as that from which nonfunctioning tumors are derived [13, 14]. In nonfunctioning adenomas, a variable SSTR subtype expression profile, with SSTR2 and, secondarily, SSTR3 being the major subtypes has been reported [15], though the efficacy of somatostatin ligands remains unclear. It was recently reported that pituitary gonadotropinomas express different patterns of SSTR subtypes depending on the tumor condition and aggressiveness, leading to varied reactions to somatostatin analogs [16]. Gonadotropinomas undergoing early recurrence had lower expression levels of SSTR2/3 than the levels in those without a five-year recurrence. However, recurrent tumors had higher expression levels of SSTR1, 2, 3 and 5, suggesting higher sensitivity to somatostatin analogs to possibly counteract the recurrence [16].

In this study, we investigated the effects of somatostatin analogs and roles of BMP-6 in the regulation of LH secretion by gonadotrope LβT2 cells. It was revealed that somatostatin analogs have dual effects on the modulation of GnRH-induced MAPK signaling and BMP-6 activity. A functional link between the BMP system and GnRH-induced LH transcription was newly uncovered.

Materials and Methods

Reagents and supplies

Dulbecco's modified Eagle's medium (DMEM) and GnRH human acetate salt were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-6 was purchased from R&D Systems, Inc. (Minneapolis, MN). The ERK inhibitor U0126 and P38 inhibitor SB203580 were from Promega Corp. (Madison, WI), and the SAPK/JNK inhibitor SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA). Octreotide acetate (OCT) and pasireotide, also known as SOM230 (SOM), were provided by Novartis International Pharmaceutical Ltd. (Basel, Switzerland). LβT2 cells and a plasmid of LHβ-Luc that contains 1800 bp of the rat LHβ promoter were provided by Drs. Pamela L. Mellon and Mark A. Lawson, University of California, San Diego.

Cell culture and LH assay

LβT2 cells were grown in DMEM containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2 in air. The cells were grown in 75-cm^2 flasks until they became confluent and were then transferred to 12-well plates. After preculture, the medium was replaced with fresh serum-free DMEM, and indicated combinations of GnRH, BMP-6, and somatostatin analogs were added to the culture medium. After 48-h culture, the conditioned medium was collected and LH concentrations were determined by a Rodent LH ELISA with the minimal detectable concentration of rodent LH being ~0.5ng/ml (Endocrine Technologies, Inc. Newark, CA).

Transient transfection and luciferase assay

After 24-h preculture, LβT2 cells (~60% confluent) were transiently transfected with 500 ng of the luciferase reporter plasmid LHβ-Luc and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 16 h. The cells were treated with GnRH and BMP-6 in fresh serum-free DMEM for 24 h and then washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-galactosidase (β-gal) activity of the cell lysate were measured by a luminometer. The data were shown as the ratio of luciferase to β-gal activity.

RNA extraction and quantitative real-time PCR analysis

LβT2 cells (3×10^5 viable cells/well) were cultured in 12-well plates with 10% FCS. The medium was then changed to serum-free DMEM, and the cells were subsequently treated with indicated concentrations of GnRH, BMP-6, octreotide and pasireotide alone or in combination with various inhibitors of intracellular MAPK signaling. After 24-h culture, total cellular RNAs were extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1 μg) was subjected to RT reaction using the First-Strand cDNA Synthesis System® (Invitrogen Corp.) at 42°C for 50 min and 70°C for 10 min. PCR primer pairs were selected from different exons of the corresponding genes as follows: GnRHR: 671-680 and 902-921 (NM_010323); LHβ: 138-157 and 348-

367 (NM_008497); SSTR1: 526-546 and 869-889 (from GenBank accession No. X62314); SSTR2: 240-260 and 559-579 (M93273); SSTR3: 501-521 and 782-802 (X63574); SSTR4: 400-420 and 781-801 (M96544); and SSTR5: 781-801 and 1031-1051 (L04535). Primer pairs for ALK-2, -3, ActRII, BMPRII, Smad6/7 and a house-keeping gene, ribosomal protein L19 (RPL19), were selected as reported previously [9, 17, 18]. For the quantification of GnRHR, LHβ, SSTR1-5, BMPRs, Smads and RPL19 mRNAs, real-time PCR was performed using the LightCycler-FastStart DNA master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan). Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis, and then, following assay validation by calculating amplification efficiency of each amplicon, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. 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 the target gene by the quantity of RPL19 in each sample.

Western immunoblot analysis

LβT2 cells (1.5×10^5 viable cells/well) were treated by 15- to 60-min stimulation or 24-h culture with BMP-6, octreotide, pasireotide and GnRH either alone or in the indicated combinations in serum-free DMEM. After the stimulation, the cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, 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 an anti-SSTR5 (H-54) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho- and anti-total-ERK1/2, P38 and SAPK/JNK antibodies, anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA), and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal densities were analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) or by NIH image J 1.34s. For evaluating SSTR5 expression and protein phosphorylation, ratios of the signal intensities of SSTR5 and phosphorylated protein / total protein or actin were calculated, respectively.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data were subjected to ANOVA or unpaired *t*-test to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If differences were detected by ANOVA, Fisher's protected least significant difference (PLSD) test was used to determine which means differed (StatView 5.0 software). *P* values < 0.05 were accepted as statistically significant.

Results

First, we examined the effects of GnRH, somatostatin analogs and BMP-6 on LH production by LβT2 cells. GnRHR, all of the receptor subtypes for SSTRs including SSTR1 to 5, and BMP receptors including ALK-2, -3 but not ALK-6, ActRII and BMPRII were shown to be expressed in LβT2 cells by RT-PCR [7]. As shown in **Fig. 1A**, the basal levels of LHβ mRNA were increased by GnRH (10 nM) but were not changed by somatostatin analogs, including octreotide and pasireotide (10 μM), or by BMP-6 (100 ng/ml). Activity of LHβ transcription was induced by treatment with GnRH (10 nM) but not by treatment with BMP-6 (100 ng/ml) (**Fig. 1B**).

As shown in **Fig. 1C**, LH production in the medium was also significantly increased by GnRH (10 nM) but not by BMP-6 (100 ng/ml). Interestingly, the levels of GnRH-induced secretion of LH were decreased in the presence of somatostatin analogs (10 μM) including octreotide, an agonist for SSTR2, or pasireotide, an agonist for SSTR1, 2, 3 and 5 (**Fig. 1C**). Of note, the suppressive effects of octreotide and pasireotide on GnRH-induced LH expression were enhanced in the presence of BMP-6 (100 ng/ml) (**Fig. 1D**). To determine the mechanism by which BMP-6 facilitates the suppressive effects of somatostatin analogs on GnRH-induced LH expression, the effects of BMP-6 on the expression of GnRHR and key receptors for somatostatin analogs were examined. BMP-6 (100 ng/ml) treatment had no significant effect on GnRHR mRNA expression (**Fig. 1E**). Of interest, BMP-6 (100 ng/ml) increased SSTR5

mRNA and protein (**Fig. 1E, F**) levels in LβT2cells, while BMP-6 had no significant effect on SSTR2 expression (**Fig. 1E**). The results suggested that upregulation of SSTR5 induced by BMP-6 might be involved in the reduction of GnRH-induced secretion of LH by somatostatin analogs.

Involvement of MAPK, which has been reported to be a key functional pathway in LβT2 cells [10, 19, 20], in the regulation of GnRH-induced LH production was then examined. The results of experiments using MAPK inhibitors including U0126, SB203580 and SP600125 (1 to 10 μM) showed that all of the MAPKs are, at least in part, involved in the GnRH (10 nM)-induced LH expression (**Fig. 2A**). As shown in **Fig. 2B**, GnRH (10 nM) treatment readily induced phosphorylation of MAPK pathways including ERK, P38 and SAPK, and co-treatment with BMP-6 (100 ng/ml) had no specific effect. In the presence of pasireotide (10 μM), GnRH-induced ERK, but not P38 or SAPK, phosphorylation was slightly reduced (**Fig. 2B**). BMP-6 treatment (100 ng/ml) enhanced the effects of pasireotide (10 μM), leading to a significant reduction of GnRH (10 nM)-induced ERK activation (**Fig. 2C**). The results thus suggested that the effects of SSTR5, possibly augmented by BMP-6, suppressed the ERK phosphorylation induced by GnRH.

The effects of somatostatin analogs on BMP signaling were also examined. As shown in **Fig. 3A**, BMP-6 (100 ng/ml) induced Smad1/5/8 phosphorylation, and its activity was reduced by co-treatment with GnRH (10 nM). Of note, treatments with somatostatin analogs (10 μM) significantly suppressed BMP-6 (100 ng/ml)-induced Smad1/5/8 phosphorylation, the effect

of pasireotide being more efficient than that of octreotide (**Fig. 3A**). To determine the mechanism by which somatostatin analogs suppress BMP-Smad signaling in LβT2 cells, the effects of somatostatin analogs on BMP receptor expression were assessed. As shown in **Fig. 3B**, somatostatin analogs (10 μM) reduced the mRNA expression of BMP receptors including ALK-2, -3, ActRII and BMPRII. The effects were more potent in the case of pasireotide than octreotide, being in accordance to the changes seen in Smad phosphorylation. The changes in inhibitory Smad6/7 expression were also examined. As shown in **Fig. 3C**, somatostatin analogs (10 μM) individually increased mRNA levels of Smad6/7 in the presence of BMP-6 (100 ng/ml). Thus, BMPR downregulation as well as increase in inhibitory Smads are likely to be involved in the suppressive effects of somatostatin analogs on BMP signaling in LβT2 cells.

Discussion

In the present study, the effects of somatostatin analogs and roles of BMP-6 in the regulation of LH secretion by gonadotrope LβT2 cells were uncovered. Somatostatin analogs have dual effects on modulation of GnRH-induced MAPK signaling and BMP-6 activity (**Fig. 4**). LH production induced by GnRH was suppressed by somatostatin analogs in LβT2 cells. Of note, the inhibitory effects of somatostatin analogs on LH secretion were enhanced by BMP-6 treatment. Since it was shown that BMP-6 increased the expression levels of SSTR5, the results suggested that BMP-6 upregulates SSTR actions, leading to reduction of GnRH-induced secretion of LH by pasireotide in LβT2 cells.

The effects of somatostatin are elicited by binding of five SSTRs being coupled with the G-protein, which are widely distributed in many endocrine tissues. The efficacy and potency of somatostatin analogs are functionally linked to the selectivity profile of SSTRs, in which the binding capability to SSTR2/5 is critical [21]. SSTR2/5 is intracellularly coupled to adenylate cyclase, leading to reduction of cellular cAMP synthesis [22]. SSTR actions are also mediated by calcium influx through a direct action on calcium channels [23] and an indirect action through potassium channels [24]. The effectiveness of a somatostatin analog is, in general, dependent on the expression of a sufficient amount of appropriate SSTRs.

Locally produced BMPs play critical roles in differentiation of pituitary gonadotrope cells [4]. As for their receptors in the pituitary, expression of

BMPRII, ActRII, ALK-2 and ALK-3 mRNAs in mice [7], ALK-6 mRNA in sheep, and ActRII and ActRIIB mRNAs in rats [1] has been reported. Regarding the function on gonadotropin production, it has been shown that BMP-6 and -7 stimulate FSH synthesis and secretion in the gonadotrope cell line LβT2 [25]. BMP-15 has also been shown to stimulate FSH biosynthesis and secretion by rat primary pituitary cells without regulating LH secretion [7]. Differential expression of a BMP-binding protein, follistatin, was also shown in human FSH-producing and nonfunctioning adenomas [8]. In nonfunctioning tumors, the expression of follistatin is abundant and thereby activation of activin/BMP signaling is insignificant, resulting in less production of FSH. On the other hand, in gonadotropinomas, the effect of follistatin is negligible and thereby activins can bind to their receptors and effectively transduce the signaling to stimulate FSH production. The intrapituitary regulation of activin/BMP may be involved in the functional transition of gonadotropin productivity.

The results of this study suggested that the pituitary BMP system plays a regulatory role in GnRH-induced LH secretion by modulating the responsiveness to somatostatin analogs in gonadotrope cells. GnRH-induced phosphorylation of ERK was impaired by a somatostatin analog in the presence of BMP-6. ERK, JNK and P38 are individually involved in the induction of LH secretion by GnRH, although FSH secretion was shown to be predominantly regulated by ERK signaling [10]. Somatostatin analogs also impaired Smad1/5/8 phosphorylation induced by BMP-6. Namely, a functional link between the BMP system and GnRH-induced LH transcription was uncovered.

As for the interrelationship between somatostatin effects and BMPs, we previously reported that somatostatin analogs, including octoreotide and pasireotide, upregulated BMP-Smad1/5/8 activity with increased expression of ALK-3/BMPRII but downregulated inhibitory Smad6/7 molecules in mouse corticotrope AtT20 cells [18, 26]. Endogenous BMP activity is thus involved in the mechanism by which somatostatin analogs suppress adrenocorticotropin production [18]. In addition, BMP-4 showed a significant inhibition of cell mitosis in the presence of pasireotide and octreotide [18]. These findings suggested that somatostatin analogs facilitate BMP signal transduction in corticotrope cells.

A functional link between the BMP system and SSTR expression was also seen in prolactin secretion by lactotrope cells [27]. BMP-4 and -6 act to increase prolactin release, while they reduced SSTR2 expression but increased SSTR5 expression in rat lactotrope GH3 cells [27, 28]. The effect of the SSTR5-preferring agonist pasireotide, which reduced prolactin secretion induced by forskolin, was facilitated in the presence of BMP-4 but blocked by a BMP-binding protein, noggin. In the presence of noggin, the SSTR2-preferring agonist octreotide exerted an inhibitory effect on prolactin release. These results suggested that endogenous BMPs also play regulatory roles in SSTR sensitivity of lactotorope cells [27].

In the present study, a functional interaction of BMP-6 and somatostatin analogs in GnRH-induced LH secretion by gonadrotrope cells was revealed. GnRH-induced LH production was suppressed by pasireotide and octreotide. The inhibitory effects of somatostatin analogs on LH secretion were enhanced

in the presence of BMP-6 by upregulating SSTR5 in gonadotrope cells. GnRH-induced phosphorylation of ERK was suppressed by somatostatin analogs and BMP-6, while somatostatin analogs impaired BMP-induced Smad1/5/8 activation. The pituitary BMP system may play a regulatory role in GnRH-induced LH secretion by modulating the responsiveness to somatostatin analogs in gonadotrope cells (**Fig. 4**).

Collectively, the results indicate that the pituitary BMP system may act as a functional modulator for somatostatin analogs in an autocrine/paracrine manner, leading to fine-tuning of the sensitivity in gonadotropes, as shown in lactotropes and corticotropes. Regulation of the endogenous BMP system, which is a functional determinant of SSTR sensitivity, may be a future strategy for medication of pituitary adenomas.

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Figure Legends:

Fig. 1. Effects of somatostatin analogs on LH expression and production by LβT2 cells. A) LβT2 cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH, BMP-6, octreotide (OCT) and pasireotide (SOM) in serum-free DMEM. After 24-h culture, total cellular RNAs were extracted and LHβ mRNA levels were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **B)** Cells were transiently transfected with LHβ-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24-h treatment with the indicated concentrations of GnRH and BMP-6, cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-galactosidase (β-gal) activity. **C)** Cells (3×10^5 cells/well) were treated with the indicated concentrations of BMP-6, somatostatin, OCT or SOM in the presence of GnRH in serum-free conditions. After 48-h culture, LH concentrations in the culture media were determined by EIA assays. **D, E)** Cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH, BMP-6, OCT and SOM in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of LHβ, GnRHR, SSTR2 and SSTR5 were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **F)** Cells (1.5×10^5 cells/well) were treated with the indicated BMP-6 concentration in serum-free conditions for 24 h. The cells were then lysed and subjected to immunoblot (IB) analysis using antibodies that detect SSTR5 and actin as an internal control.

The results shown are representative of those obtained from three independent experiments. The integrated signal density of each protein band was digitally analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences), and the ratios of signal intensities of SSTR5/actin were expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A-C)** and unpaired t-test **(D-F)**. **P* < 0.05 and ***P* < 0.01 vs. control or between the indicated groups. n.s., not significant.

Fig. 2. Effects of somatostatin analogs on GnRH-induced MAPK signaling in LβT2 cells. A) LβT2 cells (3×10^5 cells/well) were treated with the indicated concentrations of GnRH and each inhibitor of intracellular MAPKs including U0126, SP203580 and SP600125 in serum-free DMEM for 24 h. Total cellular RNAs were extracted and the mRNA levels of LHβ were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. **B)** Cells (1.5×10^5 cells/well) were pretreated with the indicated concentrations of BMP-6, OCT and SOM in serum-free conditions for 24 h, and then the indicated concentration of GnRH was added to the medium. After 15-min stimulation, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect phosphorylation of MAPKs. The results are representative of those obtained from three independent experiments. **C)** The relative integrated density of the ERK protein band was digitized, with the phosphorylated levels being normalized by the total levels, and then the results were expressed as fold changes. Results in all panels are shown as means ±

SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A, C)**. *P < 0.05 vs. control and $^{\#}P$ < 0.05 vs. GnRH group.

Fig. 3. Effects of somatostatin analogs on BMP-6 signaling in LβT2 cells. **A)** LβT2 cells (1.5×10^5 cells/well) were pre-treated with the indicated concentrations of GnRH, OCT and SOM in serum-free DMEM for 24 h. After 1-h stimulation with BMP-6, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect pSmad1/5/8 and actin. The results are representative of those obtained from three independent experiments. The relative integrated density of the pSmad1/5/8 protein band was digitized, with the phosphorylated levels being normalized by actin levels, and then the results were expressed as fold changes. **B, C)** Cells (3×10^5 cells/well) were treated with the indicated concentrations of BMP-6, OCT and SOM in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of **(B)** ALK-2, -3, ActRII, BMPRII and **(C)** Smad6/7 were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA **(A-C)**. For each result within a panel, the values with different superscript letters are significantly different at P < 0.05. *P < 0.05 vs. control.

Fig. 4. Functional interaction of BMP-6 and somatostatin in GnRH-induced production of LH by gonadrotrope cells. LH production induced by GnRH was suppressed by somatostatin (SS) analogs, pasireotide and octreotide. The inhibitory effects of SS analogs on LH production were enhanced by BMP-6 action. BMP-6 increased SSTR5 expression, leading to reduction of GnRH-induced LH production by suppressing ERK. SS analogs also impaired BMP-induced Smad1/5/8 signaling via BMPR reduction and Smad6/7 expression. BMP-6 plays a regulatory role in GnRH-induced LH production by modulating the responsiveness to SS analogs.

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Disclosure Statement

FO receives speaker honoraria from Novo Nordisk. HM is a consultant for AbbVie, Astellas and Teijin, receives speaker honoraria from Astellas, Boehringer-ingelheim, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novartis, Pfizer, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novo Nordisk, Takeda, and Tanabe Mitsubishi.

Abbreviations

ActRII, activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; SSTR, somatostatin receptor; and TGF-β, transforming growth factor-β.

Figure

Revised Fig. 1

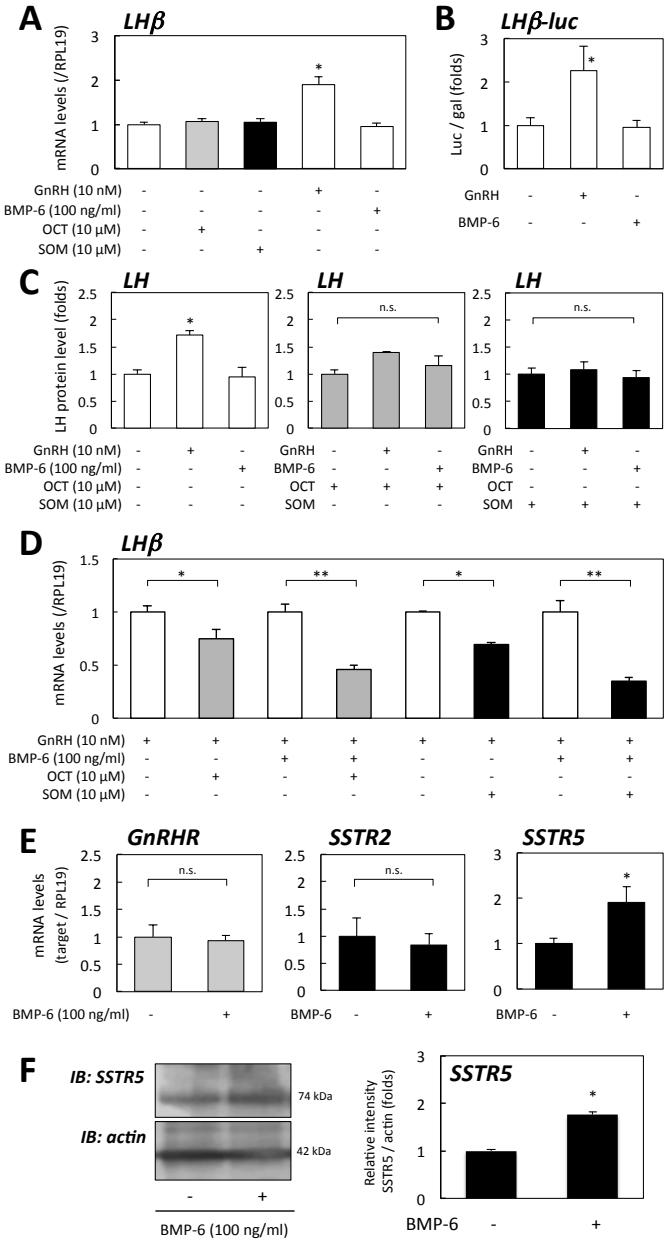


Fig. 2

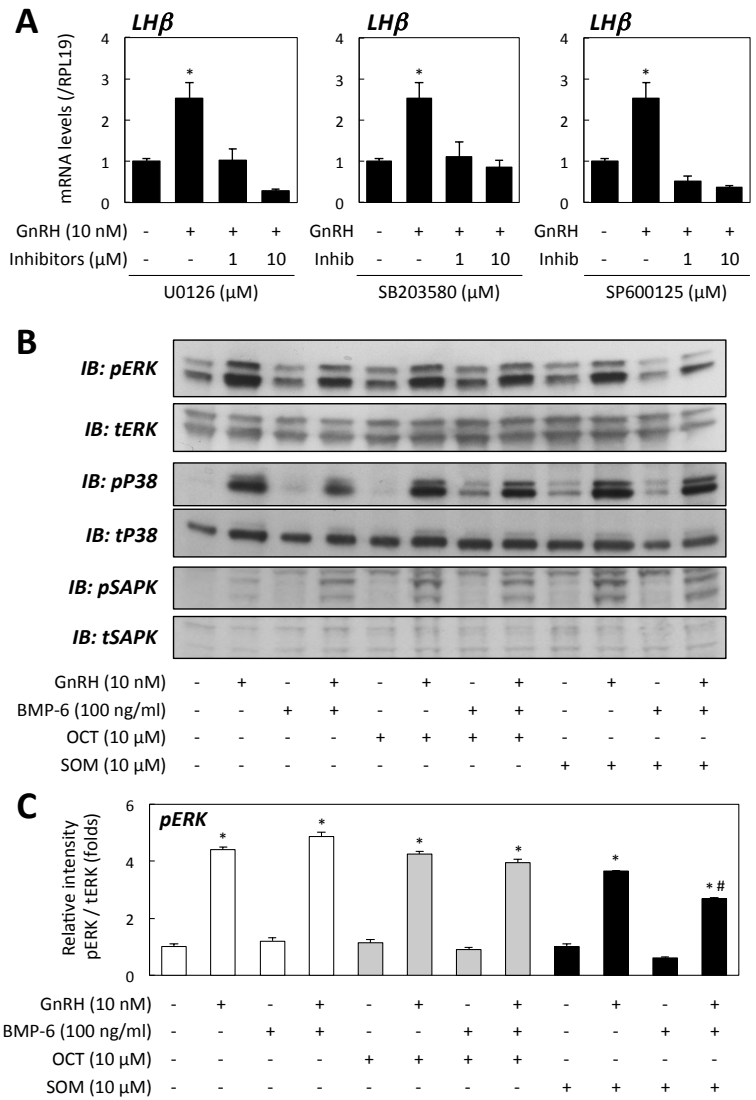

A
LHβ
LHβ
LHβ
mRNA levels (/RPL19)
GnRH (10 nM)
Inhibitors (μM)
Inhib
U0126 (μM)
SB203580 (μM)
SP600125 (μM)
B
IB: pERK
IB: tERK
IB: pP38
IB: tP38
IB: pSAPK
IB: tSAPK
GnRH (10 nM)
BMP-6 (100 ng/ml)
OCT (10 μM)
SOM (10 μM)
C
pERK
Relative intensity pERK / tERK (folds)
GnRH (10 nM)
BMP-6 (100 ng/ml)
OCT (10 μM)
SOM (10 μM)

Fig. 3

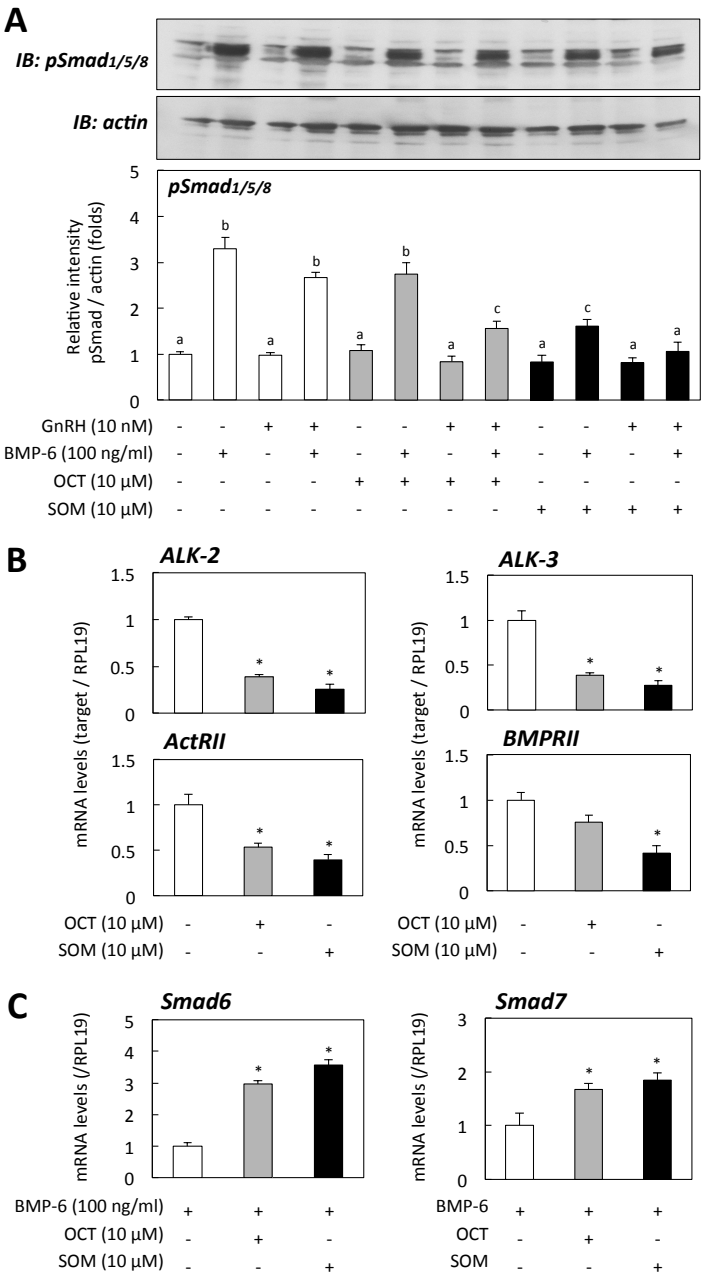
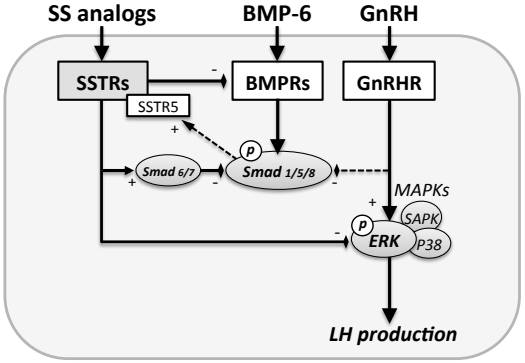

A
IB: pSmad1/5/8
IB: actin
pSmad1/5/8
Relative intensity pSmad / actin (folds)
GnRH (10 nM)
BMP-6 (100 ng/ml)
OCT (10 μM)
SOM (10 μM)
B
ALK-2
ALK-3
ActRII
BMPRII
mRNA levels (target / RPL19)
OCT (10 μM)
SOM (10 μM)
C
Smad6
Smad7
mRNA levels (/RPL19)
BMP-6 (100 ng/ml)
BMP-6
OCT (10 μM)
OCT
SOM (10 μM)
SOM

Fig. 4


SS analogs
BMP-6
GnRH
SSTRs
BMPRs
GnRHR
SSTR5
Smad 6/7
Smad 1/5/8
MAPKs
SAPK
ERK
P38
LH production