*Original Article*

# Incretins modulate progesterone biosynthesis by regulating bone morphogenetic protein activity in rat granulosa cells.

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*Abbreviations:*

AC, adenylate cyclase
ALK, activin receptor-like kinase
ActRII, activin type-II receptor
BMP, bone morphogenetic protein
BMPRII, BMP type-II receptor
FSH, follicle-stimulating hormone
FSHR, FSH receptor
GDF, growth and differentiation factor
GIP, glucose-dependent insulinotropic polypeptide
GLP-1, glucagon-like peptide-1
IGF-I, insulin-like growth factor-I
3βHSD, 3β-hydroxysteroid dehydrogenase
LH, luteinizing hormone
PCOS, polycystic ovary syndrome
P450arom, P450 aromatase
P450scc, P450 steroid side-chain cleavage enzyme
StAR, steroidogenic acute regulatory protein
TGF, transforming growth factor

## Abstract

The effects of incretins on ovarian steroidogenesis have not been clarified. In this study, we investigated the effects of incretins, including GIP and GLP-1, on ovarian steroidogenesis using rat primary granulosa cells. Treatment with incretins significantly suppressed progesterone synthesis in the presence of FSH, and the effect of GIP was more potent than that of GLP-1. In contrast, incretins had no significant effect on estrogen synthesis by rat granulosa cells. In accordance with the effects of incretins on steroidogenesis, GIP and GLP-1 suppressed the expression of progesterogenic factors and enzymes, including StAR, P450scc, 3βHSD, but not P450arom, and cellular cAMP synthesis induced by FSH. In addition, incretins moderately increased FSHR mRNA expression in granulosa cells. Of note, treatment with GIP, but not treatment with GLP-1, augmented Smad1/5/8 phosphorylation and transcription of the BMP target gene Id-1 induced by BMP-6 stimulation, suggesting that GIP upregulates BMP receptor signaling that can inhibit

FSH-induced progesterone synthesis in rat granulosa cells. On the other hand, BMP-6 treatment suppressed the expression of GIP receptor but not that of GLP-1 receptor. Expression of the BMP type-I receptor ALK-3 was upregulated by treatment with GIP and GLP-1 and that of ALK-6 was also increased by GIP, while inhibitory Smad6 expression was impaired by GIP and GLP-1 in rat granulosa cells. Collectively, the results indicate that incretins, particularly GIP, impair FSH-induced progesterone production, at least in part, by upregulating BMP signaling in rat granulosa cells. The modulatory effects of incretins on endogenous BMP activity may be applicable to treatment of dysregulated steroidogenesis such as polycystic ovary syndrome.

## Introduction

Incretins, including glucose-dependent insulinotropic polypeptide (also called gastric inhibitory polypeptide; GIP) and glucagon-like peptide (GLP)-1, are peptide hormones secreted from the intestine. Incretins stimulate insulin secretion following oral glucose intake in a glucose-dependent manner [1-3]. The postprandial release of GIP and GLP-1 from the proximal and distal small intestine, respectively, accounts for more than 50% of the postprandial insulin response as incretin effects [1].

Since insulin resistance is considered to play an important role in the pathogenesis of polycystic ovary syndrome (PCOS) [4], attention has been paid to the altered pattern of incretin secretion and the insulinotropic activity of incretins in diabetic, obese [2, 5-7], and PCOS patients [8]. As for the reproductive impact of incretins, an *in vitro* study using the hypothalamic cell line GT1-7 demonstrated that GLP-1 enables stimulation of gonadotropin release, at least in part by activating gonadotropin-releasing hormone neurons, suggesting

a physiological role of GLP-1 as a new metabolic signal to the reproductive system [9]. An *in vivo* study also showed a reproductive effect of GLP-1 based on the knockout female phenotype of the GLP-1 receptor (GLP-1R) [10]. Female mice with a defect of GLP-1R showed a consistent delay in onset of the first ovarian cycle and reduction in the number of developing follicle, but they eventually became fertile and did not exhibit a critical impairment of reproductive capacity [10]. However, the pathophysiological role of incretins in ovarian steroidogenesis and the significance of the alteration in incretin levels in PCOS patients have yet to be elucidated.

The physiological process of ovarian folliculogenesis occurs as a consequence of functional interactions between gonadotropins and various autocrine/paracrine factors in the ovary. It has been shown that the bone morphogenetic protein (BMP) system in the ovary plays a critical role in the regulation of ovarian steroidogenesis in an autocrine/paracrine manner [11, 12]. BMP system molecules consisting of BMP ligands and receptors are expressed in ovarian follicles in a cell-specific pattern. The expression of BMP-6 is

localized in oocytes as well as granulosa cells in Graafian-staged healthy follicles [13]. It has been shown that BMP-6 in granulosa cells elicits an inhibitory effect on follicle-stimulating hormone (FSH) receptor (FSHR)-dependent activities by suppressing adenylate cyclase (AC), leading to cAMP reduction [14, 15]. BMP-6 expressed in rat granulosa cells appears to be reduced when dominant follicles are selected [13], suggesting that BMP-6, in particular, contributes to the selection process of folliculogenesis. From clinical points of view regarding the pathophysiology of human PCOS, it is intriguing that the expression of BMP-6 was upregulated in human granulosa cells isolated from PCOS ovaries based on the histological and molecular workup [16-18].

In the present study, we used a primary culture of rat granulosa cells to investigate the functional roles of incretins in ovarian steroidogenesis induced by FSH with focus on the luteinizing inhibitor BMP-6. The findings can be linked to a therapeutic strategy for dysregulation of ovarian steroidogenesis such as PCOS.

## Materials and Methods

*Reagents and supplies*

Medium 199, McCoy's 5A medium and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Bovine serum albumin (BSA), diethylstilbestrol (DES), Glucagon-Like Peptide 1 (GLP-1) human and Gastric Inhibitory Polypeptide (GIP) human, 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), ovine pituitary FSH, and penicillin-streptomycin solution were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO), and recombinant human BMP-6 was from R&D Systems Inc. (Minneapolis, MN).

*Primary culture of rat ovarian granulosa cells*

Sprague-Dawley (SD) rats were purchased from Charles River (Wilmington, MA). Silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats to increase the number of granulosa cells. After 3 to 4 days of

exposure to DES, ovarian follicles were punctured with a 27-gauge needle. Granulosa cells were counted and separated from oocytes by filtering the oocyte/granulosa cell suspension through a 100-μm nylon mesh and then through a 40-μm nylon mesh (BD Falcon, Bedford, MA), which allowed granulosa cells but not oocytes to pass through [19, 20]. The isolated granulosa cells were then cultured in a serum-free McCoy's 5A medium supplemented with penicillin-streptomycin at 37°C in an atmosphere of 5% $CO_2$. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

*Determination of estradiol, progesterone and cAMP levels*

Rat granulosa cells ($1 \times 10^5$ viable cells in 200 μl) were cultured, as described above, in 96-well plates with 100 nM of androstenedione, a substrate for aromatase. FSH (30 ng/ml) was added to the culture medium either alone or in combination with indicated concentrations of GIP or GLP-1. After 48-h culture, the culture media were collected and stored at -30°C until assay. The

concentrations of estradiol and progesterone in the culture media were examined by a chemiluminescent immunoassay (CLIA) using Architect estradiol and progesterone kits (Cayman Chemical Co., Ann Arbor, MI, USA). Steroid contents were undetectable (progesterone <10 pg/ml and estradiol <15 pg/ml) in a cell-free medium. To evaluate cellular cAMP synthesis, rat granulosa cells (1 × $10^5$ viable cells in 200 µl) were cultured, as described above, in 96-well plates with 0.1 mM of IBMX (specific inhibitor of phosphodiesterase activity). After 48-h culture with indicated treatments, the conditioned medium was collected and stored at -30°C until assay. The concentrations of extracellular cAMP were measured by an enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Inc., NY, USA) after acetylation of each sample with an assay sensitivity of 0.039 pmol/ml.

*Granulosa cellular RNA extraction and quantitative RT-PCR*

Rat granulosa cells (5 × $10^5$ viable cells in 1 ml) were cultured in 12-well plates with serum-free McCoy's 5A. The cells were treated with FSH (30 ng/ml) either

alone or in combination with indicated concentrations of GIP and GLP-1. After 48-h culture, total cellular RNA was extracted using TRIzol® (Invitrogen Corp.). Total RNA amount was quantified by measuring the absorbance of the sample at 260 nm, and the RNA was stored at -80°C until assay. Primer pairs for activin receptor-like kinase (ALK)-2, ALK-3, activin type-II receptor (ActRII), Smad6 and 7, and ribosomal protein L19 (RPL19) were selected as reported previously [21, 22]. For all of the genes, the primer pairs were selected from different exons of the corresponding genes to distinguish PCR products that might arise from chromosome DNA contaminants as follows: FSHR, 720-739 and 913-932 (from GenBank accession #NM_199237); P450scc, 147-167 and 636-655 (J05156); steroidogenic acute regulatory protein (StAR), 395-415 and 703-723 (AB001349); type-I 3β-hydroxysteroid dehydrogenase (3βHSD), 336-355 and 841-860 (M38178); P450arom, 1180-1200 and 1461-1481 (M33986); Id-1, 225-247 and 364-384 (NM_010495); GIPR, 333-352 and 479-498 (NM_012728); GLP-1R, 749-768 and 980-999 (NM_012714); ALK-6, 227-246 and 450-469 (NM_001024259); and BMP type-II receptor (BMPRII),

1785-1804 and 1942-1961 (NM_080407). The extracted RNA (1 μg) was subjected to reverse transcription using a First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and at 70°C for 10 min. To quantify the level of target gene mRNA, real-time PCR was performed using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) after optimization of annealing conditions. The relative mRNA expression was calculated by the Δ threshold cycle (Ct) method, in which ΔCt is the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RPL19 mRNA was expressed as $2^{-(\Delta Ct)}$. The data are expressed as the ratio of target mRNA to RPL19 mRNA.

*Western immunoblotting analysis*

Rat granulosa cells ($2.5 \times 10^5$ viable cells in 500 μl) were cultured in 24-well plates in serum-free McCoy's 5A medium. After preculture either alone or with

indicated concentrations of GIP or GLP-1, the cells were treated with BMP-6 for 60 min. The cells were solubilized in 50 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 subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8(9) (pSmad1/5/8(9)) antibody (Cell Signaling Technology, Inc., Beverly, MA) and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). To evaluate the target protein levels, the ratios of the signal intensities of pSmad / actin were calculated.

*Statistical analysis*

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were statistically analyzed using ANOVA or the unpaired *t*-test when appropriate (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If

differences were detected by ANOVA, Tukey-Kramer's post hoc test was used to determine which means differed (StatView 5.0 software). *P* values <0.05 were accepted as statistically significant.

## Results

First of all, we examined the expression of incretin receptors in the rat ovary. As shown in **Fig. 1A**, the expression of GIPR and GLP-1R was detected by RT-PCR in the rat ovary as well as in rat pancreas tissues. The effects of incretins, including GIP and GLP-1, on FSH-induced ovarian steroidogenesis were examined by using rat primary granulosa cell culture for 48 h. Treatment with GIP or GLP-1 (100 nM) alone had no significant effect on basal progesterone (**Fig. 1B**) and estradiol (**Fig. 1C**) synthesis. Of note, treatment with incretins (30 to 300 nM) significantly suppressed progesterone synthesis induced by FSH (30 ng/ml). The effect of GIP (300 nM) was more potent than that of GLP-1 (300 nM); treatment with GIP and GLP-1 resulted in reductions of 35% and 19%, respectively, compared with FSH-induced progesterone levels. In contrast, GIP or GLP-1 (30 to 300 nM) had no significant effect on basal as well as FSH-induced estrogen synthesis by rat granulosa cells. To clarify the mechanism by which incretins suppress FSH (30 ng/ml)-induced

steroidogenesis by granulosa cells, the change in cAMP synthesis was determined by EIA. As shown in **Fig. 2A**, FSH-induced cAMP production was suppressed by treatment with GIP (100-300 nM) and GLP-1 (30-100 nM) for 48 h. FSHR mRNA expression in granulosa cells was moderately but significantly augmented by either GIP or GLP-1 (300 nM) for 48-h culture. In accordance with the effects of incretins on steroid biosynthesis, GIP and GLP-1 (300 nM) were found to reduce mRNA levels of progesterogenic factors and enzymes including StAR, P450scc and 3βHSD stimulated by FSH (30 ng/ml), although GIP or GLP-1 alone had no specific effect on the basal mRNA levels of progesterogenic factors and enzymes (**Fig. 2C**). On the other hand, the level of FSH (30 ng/ml)-induced P450arom mRNA was not altered by treatment with GIP or GLP-1 (300 nM) (**Fig. 2C**).

Since incretins specifically suppressed FSH-induced progesterone production without altering estradiol level, the involvement of BMP activity was implicated in the regulatory mechanism of the actions of incretins on steroidogenesis. We therefore examined the effects of incretins on

BMP-receptor signaling including Smad1/5/8 phosphorylation and BMP target gene Id-1 transcription. As shown in **Fig. 3A**, BMP-6 (30 ng/ml) treatment for 1 h readily stimulated Smad1/5/8 phosphorylation and, of note, 24-h co-treatment with GIP (300 nM) significantly enhanced the Smad1/5/8 phosphorylation induced by BMP-6 (30 ng/ml) stimulation. An enhancing effect of BMP-6-induced Smad1/5/8 activity was not observed in granulosa cells treated with GLP-1 (300 nM) for 24 h (**Fig. 3A**). In accordance with the effects on Smad activation, Id-1 mRNA induction by BMP-6 (30 ng/ml) was also enhanced by co-treatment with GIP but not by co-treatment with GLP-1 (300 nM) (**Fig. 3B**), suggesting that GIP upregulates BMP-receptor signaling that selectively inhibits FSH-induced progesterone synthesis [11], leading to effective reduction of the levels of progesterone produced by granulosa cells. On the other hand, as shown in **Fig. 3C**, BMP-6 (30 ng/ml) treatment for 24 h significantly suppressed the mRNA expression of GIPR but not that of GLP-1R, implying the presence of a feedback loop between the activities of GIP and BMP-6.

In order to assess the influence of incretins on BMP-receptor activity in

granulosa cells, mRNA levels of BMP type-I and type-II receptors and inhibitory Smads were evaluated. As shown in **Fig. 4A**, treatments with GIP and GLP-1 (300 nM) for 48 h increased ALK-3 mRNA expression among the type-I receptors (ALK-2, -3 and -6), and GIP treatment also increase ALK-6 mRNA, whereas they did not affect the expression of type-II receptors (BMPRII and ActRII). It was also found that treatment with GIP and GLP-1 (300 nM) for 48 h conversely reduced the mRNA level of inhibitory Smad6, but not that of Smad7, in rat granulosa cells (**Fig. 4B**).

## Discussion

In the present study, we examined the roles of incretins in ovarian steroidogenesis using rat primary granulosa cells. The effects of incretins on FSH response and/or FSH-induced estrogen and progesterone synthesis have remained unknown. It was found that the receptors for GIP and GLP-1 were both expressed in the rat ovary and that the suppressive effect of GIP on FSH-induced progesterone synthesis was more potent than that of GLP-1. The mechanism by which GIP reduces progesterone production involved the upregulation of BMP-receptor signaling with an increase in ALK-3 and ALK-6 expression and a decrease in Smad6 expression in granulosa cells (**Fig. 5**). A functional interaction between the receptor signaling of BMP, GIP and FSH was uncovered; it was found that BMP downregulates GIPR expression and that GIP upregulates BMP and FSH receptor signaling. Thus, GIP modulates FSH-induced ovarian steroidogenesis by regulating BMP-receptor sensitivity in rat granulosa cells (**Fig. 5**).

Pathophysiological roles of BMP family molecules in PCOS have been suggested [23-25]. For instance, the PCOS phenotype with single unilateral or bilateral ovarian cysts lined by several layers of flattened granulosa cells was observed in female GDF-9-knockout mouse ovaries [26]. Also, GDF-9 mRNA expression was delayed and reduced during the growth and differentiation phase of human PCOS ovaries [23]. As for the pathophysiological role of BMP-6 in the development of PCOS, BMP-6 expression was found to be upregulated in human granulosa cells isolated from PCOS cases in comparison with the expression in normal granulosa cells derived from patients who underwent *in vitro* fertilization [16, 17]. In the present study, it was found that treatment with GIP augmented BMP-6-induced Smad1/5/8 signaling in rat granulosa cells via increases in the expression of type-I receptors and concomitant decrease in the expression of inhibitory Smad6. Thus, incretins seem to impair FSH-induced progesterone production, at least in part, by upregulating BMP-receptor signaling in rat granulosa cells.

Androgen's action is likely to be another key factor contributing to the

increased secretion of incretins in PCOS patients [27]. The clinical interrelationship between GIP and androgen in PCOS patients suggests that both the actions of androgen and incretins are functionally involved in the pathophysiology of insulin resistance and reproductive dysfunction in PCOS [27]. In this regard, we recently demonstrated that the interaction of androgen and IGF-I accelerates progesterone synthesis induced by FSH in rat granulosa cells [28]. In the present study, both GIP and GLP-1 were found to suppress FSH-induced production of progesterone, but not that of estradiol, and the effect of GIP for reducing FSH-induced progesterone level was found to be more potent. GIP activates BMP-6-induced Smad1/5/8 signaling by upregulating BMP type-I receptor expression and by downregulating inhibitory Smad6 expression in granulosa cells. GIP also increases FSHR expression, while BMP-6 suppresses GIPR expression. GIP plays regulatory roles in progesterone biosynthesis by facilitating BMP-6 action that enables suppression of FSH-induced progesterone production by suppressing cAMP synthesis through inhibition of AC in granulosa cells [14]. In pancreatic tissues and cells,

incretins have been shown to increase intracellular levels of cAMP and calcium [29, 30], and calcium influx was attributed to the activation of voltage-dependent calcium channels [29]. However, in the present study, GIP or GLP-1 did not show any stimulatory effect on cAMP synthesis. They in fact suppressed FSH-induced cAMP synthesis by granulosa cells, suggesting tissue-dependent differences in the actions of incretins and/or their receptor signaling on endocrine modulation.

A disturbance in the secretion of incretins has been observed in PCOS with impaired glucose regulation. Decreased levels of late-phase GLP-1 after an oral glucose test but increased levels of total GIP were observed in European PCOS patients [31]. In another study, it was found that GIP levels were lower in obese women with PCOS than in lean women with PCOS [32]. It has also been reported that obese women with PCOS showed lower GIP levels in response to oral glucose intake but that the effect of an incretin was rather augmented in obese women with PCOS, suggesting that an increased insulinotropic effect could counteract the blunted GIP response [33]. A recent

study in which a comparison was made with gestational diabetic patients also showed decreased secretion of incretins in lean women with PCOS [34]. On the other hand, a study on Asian cases showed that PCOS patients have elevated glucose-induced responses of GIP [35] and GLP-1 [36] Hence, the results regarding incretin levels in PCOS patients have not been uniform; however, it is possible that the alteration of incretin levels is, at least in part, involved in the pathophysiology and/or disease activity of PCOS.

Considering the finding that GIPR and GLP-1R were expressed in the ovary, there is a possibility that incretins have a direct impact on reproductive function and steroidogenesis. In view of the therapeutic aspect for PCOS, it has been shown that GLP-1R agonists are effective for weight loss and adiposity reduction in patients with PCOS [37, 38]. Combined therapy with GLP-1R agonists and metformin was also reported to be more effective for reproductive functions such as menstrual cycle and hormonal and metabolic derangements than either GLP-1R agonist or metformin alone [39]. We previously reported the effects of melatonin and somatostatin analogs, which have been shown to

be effective for infertility in women with PCOS [40, 41], on the regulation of ovarian steroidogenesis [42, 43]. In contrast to the actions of incretins and somatostatin analogs, melatonin sustained progesterone synthesis and luteinization by inhibiting BMP-6 activity in rat granulosa cells. These agents including incretins are potent effectors for controlling progesterone level and follicular development, in which endogenous activity of the ovarian BMP system might be physiologically critical for fine-tuning ovarian steroidogenesis in growing follicles.

Collectively, the results suggest that incretins suppress FSH-induced progesterone biosynthesis, at least in part, by upregulating BMP-receptor signaling in rat granulosa cells. Modulation of endogenous BMP activity by incretins may be applicable to treatment of dysregulated steroidogenesis as seen in PCOS ovaries.

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**Figure legends:**

**Fig. 1. Expression of incretin receptors and effects of incretins on FSH-induced steroidogenesis by rat granulosa cells.** **A**) Total cellular RNA was extracted from rat ovary and pancreas tissues, and the expression of GIPR, GLP-1R and RPL19 was examined by RT-PCR. **B**, **C**) Granulosa cells were cultured in a serum-free condition with FSH either alone or in combination with GIP or GLP-1. After 48-h culture, the levels of progesteone (**B**) and estradiol (**C**) in the medium were determined by CLIA. Results in all panels are shown as means ± SEM. The results were analyzed by ANOVA and by the unpaired *t*-test (**B**, **C**). **P* < 0.05 vs. control group or between the indicted groups; #*P* < 0.05 vs. FSH-treated groups. MM indicates molecular weight marker.

**Fig. 2. Effects of incretins on synthesis of cAMP and expression of FSH receptor and steroidogenic enzymes in rat granulosa cells.** **A**) Granulosa cells were cultured in a serum-free medium containing IBMX with FSH either alone or in combination with GIP or GLP-1. After 48-h culture, the levels of

cAMP in the medium were determined by ELISA. **B**) Total cellular RNA was extracted from granulosa cells treated with GIP or GLP-1 for 48 h, and FSHR mRNA levels were determined by quantitative PCR. **C**) Total cellular RNA was extracted from granulosa cells treated with FSH, GIP and GLP-1 for 48 h in a serum-free condition, and StAR, P450scc, 3βHSD and P450arom mRNA levels were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level and expressed as fold changes (**B**, **C**). Results in all panels are shown as means ± SEM. The results were analyzed by ANOVA (**A**, **C**) and the unpaired *t*-test (**B**). Values with different superscript letters are significantly different at $P < 0.05$; $^{*}P < 0.05$ vs. control group; n.s., not significant.

**Fig. 3. Effects of incretins on BMP-receptor signaling and effects of BMP-6 on incretin receptor expression in rat granulosa cells.** **A**) After preculture in a serum-free condition with incretins for 24 h, granulosa cells were stimulated with BMP-6 for 60 min. Cells were lysed and subjected to

SDS-PAGE/immunoblotting analysis using anti-pSmad1/5/8 and anti-actin antibodies. The signal intensities of pSmad1/5/8 were standardized by actin signal intensities in each sample and then expressed as fold changes. **B**) Total cellular RNA was extracted from granulosa cells treated with BMP-6 either alone or in combination with incretins for 24 h, and Id-1 mRNA levels were determined by quantitative PCR. **C**) Total cellular RNA was extracted from granulosa cells treated with BMP-6 for 24 h, and GIPR and GLP-1R mRNAs were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level and expressed as fold changes (**B**, **C**). Results in all panels are shown as means ± SEM. The results were analyzed by ANOVA (**A**, **B**) and by the unpaired *t*-test (**C**). Values with different superscript letters are significantly different at $P < 0.05$. *$P < 0.05$ vs. control group.

**Fig. 4. Effects of incretins on the expression of BMP receptors in rat granulosa cells.** Total cellular RNA was extracted from granulosa cells treated with GIP or GLP-1 for 48 h, and mRNA levels of BMP type-I (**A**) and

type-II (**B**) receptors and Smad6/7 (**C**) were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level and expressed as fold changes. Results in all panels are shown as means ± SEM. The results were analyzed by the unpaired *t*-test. **P* < 0.05 vs. control group.

**Fig. 5. Possible mechanism by which incretins modulate ovarian steroidogenesis in rat granulosa cells.** Both GIP and GLP-1 suppress FSH-induced progesterone production but not estradiol production, and the effect of GIP for reducing FSH-induced progesterone level is more potent. GIP activates BMP-6-induced Smad1/5/8 signaling by upregulating BMP type-I receptor expression and by downregulating inhibitory Smad6 expression in granulosa cells. GIP increases FSHR expression, while BMP-6 suppresses GIPR expression. GIP plays a regulatory role in progesterone synthesis by activating BMP-6 signaling that enables suppression of FSH-induced progesterone production by suppressing cAMP synthesis through inhibition of

adenylate cyclase (AC) in granulosa cells.