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# Effect of the interaction of metformin and bone morphogenetic proteins on ovarian steroidogenesis by human granulosa cells

Nahoko Iwata [a], Toru Hasegawa [b], Shiho Fujita [b], Satoko Nagao [a], Yasuhiro Nakano [a], Takahiro Nada [a], Yuki Nishiyama [c], Takeshi Hosoya [c], Fumio Otsuka [a, *]

[a] Department of General Medicine, Japan
[b] Department of Obstetrics and Gynecology, Japan
[c] Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama, 700-8558, Japan

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ABSTRACT

In the present study, we studied the effects of metformin and its interactions with the actions of bone morphogenetic proteins (BMPs) on ovarian steroidogenesis. It was revealed that metformin treatment enhanced progesterone production by human granulosa KGN cells and rat primary granulosa cells induced by forskolin and FSH, respectively. In human granulosa cells, it was found that metformin treatment suppressed phosphorylation of Smad1/5/9 activated by BMP-15 compared with that induced by other BMP ligands. Moreover, metformin treatment increased the expression of inhibitory Smad6, but not of that Smad7, in human granulosa cells, while metformin had no significant impact on the expression levels of BMP type-I and -II receptors. Thus, the mechanism by which metformin suppresses BMP-15-induced Smad1/5/9 phosphorylation is likely, at least in part, to be upregulation of inhibitory Smad6 expression in granulosa cells. The results suggest the existence of functional interaction between metformin and BMP signaling, in which metformin enhances progesterone production by down-regulating endogenous BMP-15 activity in granulosa cells.



## 1. Introduction

Metformin is an insulin-sensitizing agent and has been widely used for patients with type II diabetes. Metformin increases insulin sensitivity by decreasing glucose production in the liver and increasing glucose uptake in muscle. In addition to its anti-diabetic effects, metformin has been shown to exert beneficial effects on polycystic ovary syndrome (PCOS) and protective effects against cardiovascular diseases and cancers [1]. PCOS, which affects women of reproductive age, is characterized by the defect of ovulation and excess of androgens with manifesting menstrual irregularity, hirsutism and infertility [2]. Patients with PCOS also have increased risk factors for cardiovascular diseases just as shown in the patients with metabolic syndrome [3]. It has been considered that the existence of insulin resistance is highly involved in the pathogenesis of PCOS [4]. Therefore, clinical attention has been paid to the effects of metformin, which is linked to the improvement of insulin resistance, on changes of reproductive function in patients with PCOS.

On the other hand, it has been demonstrated that various growth factors expressed in the ovary play crucial and unique roles in integrating female reproduction in autocrine and/or paracrine fashion [5]. The growth factors, including bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), mutually interact with gonadotropin actions in the ovary, leading to normal process of folliculogenesis and the following ovulation. Among these, the BMP system in ovarian follicles can mainly regulate the activity of FSH receptor (FSHR) signaling in granulosa cells [5,6].

In the present study, the effect of metformin on ovarian

*Abbreviations:* ActRII, activin type-II receptor; ALK, activin receptor-like kinase; AMPK, AMP-activated protein kinase; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; FSH, follicle-stimulating hormone; FSK, forskolin; FSHR, FSH receptor; GCs, granulosa cells; GDF, growth and differentiation factor; IGF, insulin-like growth factor; 3βHSD, 3β-hydroxysteroid dehydrogenase; MAPK, mitogen-activated protein kinase; Met, Metformin; PCOS, polycystic ovary syndrome; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein.

* Corresponding author.
*E-mail address:* fumiotsu@md.okayama-u.ac.jp (F. Otsuka).

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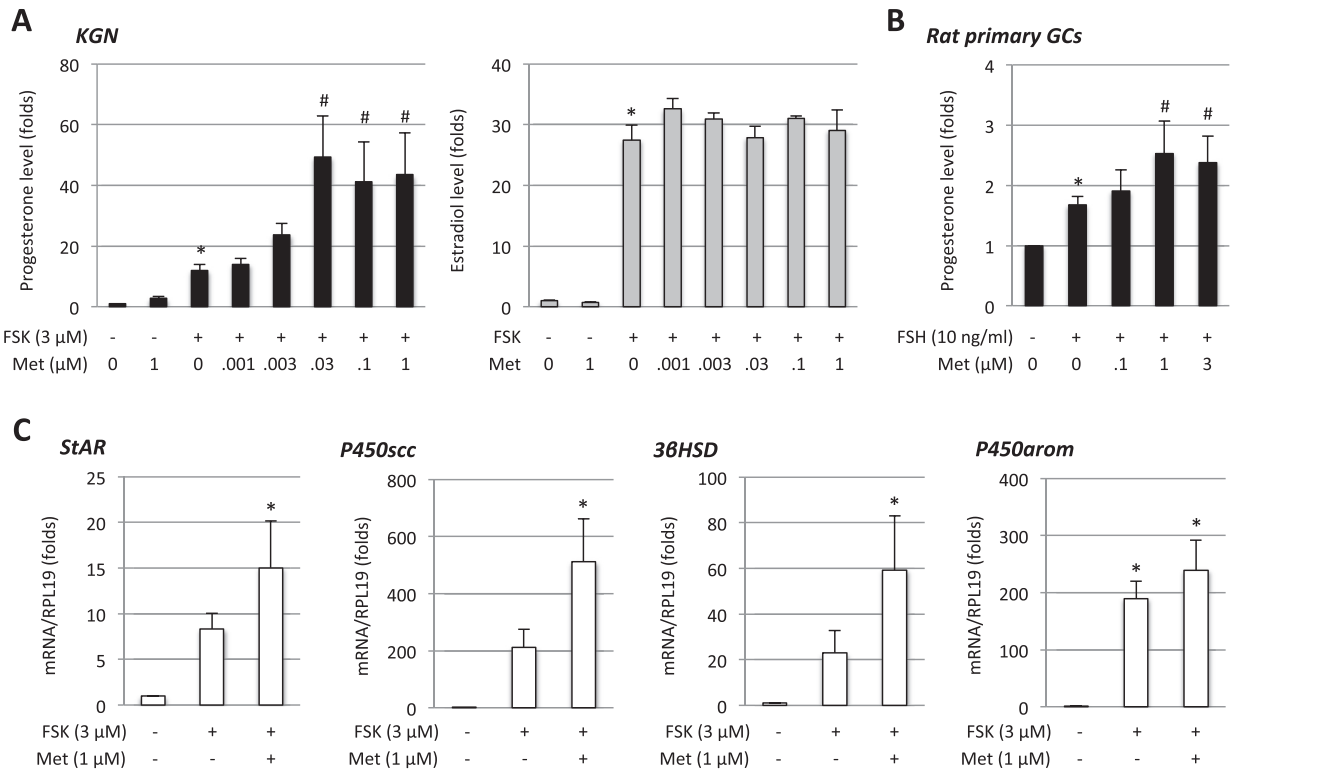


**Fig. 1. Effects of metformin on steroidogenesis by human and rat granulosa cells. A, B**) Conditioned media were collected from (**A**) human granulosa KGN cells and (**B**) rat primary granulosa cells (GCs) treated in a serum-free condition with the indicated concentrations of metformin either alone or in combination with FSK (3 μM) for 24 h or FSH (10 ng/ml) for 48 h, respectively, and the levels of progesterone and estradiol production were measured by CLIA and expressed as fold changes. **C**) Total cellular RNA was extracted from KGN cells treated with FSK (3 μM) either alone or in combination with metformin (1 μM) in a serum-free condition for 24 h, and mRNA expression levels of steroidgenetic genes including StAR, P450scc, 3βHSD and P450arom were determined by quantitative PCR. The expression levels of mRNA were normalized by RPL19 level and 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. $^{*}P < 0.05$ vs. control groups; and $\#P < 0.05$ vs. the groups treated with FSK or FSH alone.

steroidogenesis and the functional interaction between metformin and BMP activity were investigated by utilizing the KGN cell line and rat primary granulosa cells. Actually, it has been recognized that treatment of PCOS patients with metformin alleviates hyperandrogenism and restores the menstrual cycle and ovulatory process [7]. However, the mechanisms underlying the effects of metformin on reproductive function and steroidogenesis have yet to be clarified. Here, it was uncovered that metformin is directly and functionally involved in progesterone production by modulating the BMP system in granulosa cells.

## 2. Materials and methods

### 2.1. Reagents and supplies

1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham's F-12 medium (DMEM/F12), HEPES buffer, McCoy's 5A and Medium 199 were purchased from Invitrogen Corp. (Carlsbad, CA); 4-androstene-3,17-dione, diethylstilbestrol (DES), 3-isobutyl-1-methylxanthine (IBMX), ovine pituitary FSH, forskolin (FSK), and penicillin-streptomycin were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO); recombinant human BMP-2, -4, -6, -7, -9 and -15 were purchased from R&D Systems Inc. (Minneapolis, MN); and metformin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### 2.2. Cell preparations of KGN and rat granulosa cells

The KGN cells, originated from the human ovarian granulosa-like tumor cell line [8,9], were cultured in DMEM/F12 supplemented with 10%FCS and penicillin-streptomycin at 37 °C in an atmosphere of 5%$CO_2$. Primary culture of rat granulosa cells (GCs) was obtained from the ovaries of female Sprague-Dawley rats (Charles River, Wilmington, MA) exposed to DES-containing capsules (10 mg/tube) for 3 days by puncturing ovarian follicles with a 27-gauge needle and by filtering the cell suspension (BD Falcon, Bedford, MA) [10], and then the isolated granulosa cells were cultured in a serum-free McCoy's 5A medium supplemented with antibiotics at 37 °C with 5%$CO_2$ conditions. The protocol for animal experiments (OKU-2016065) was approved by Okayama University Institutional Animal Care and Use Committee.

### 2.3. Assays for estradiol and progesterone

KGN cells ($1 \times 10^5$ viable cells/ml) were cultured in serum-free DMEM/F12 with androstenedione (100 nM; a substrate for aromatase) in 12-well plates, and then treated with FSK (3 μM) either alone or in combination with the indicated concentrations of metformin for 24 h. Rat granulosa cells ($1 \times 10^5$ viable cells/0.2 ml) were cultured in serum-free McCoy's 5A with androstenedione (100 nM) in 96-well plates, and then treated with FSH (10 ng/ml) either alone or in combination with the indicated concentrations of metformin for 48 h. A range of metformin concentrations from 10 nM to 10 μM was screened, and by preliminary experiments, 1–3 μM of metformin was shown to elicit considerable changes in steroidogenesis. On the basis of results of our earlier *in vitro* experiments on the same culture conditions [11–15], each concentration of FSK (3 μM), FSH (10 ng/ml) and BMP ligands (100 ng/ml)

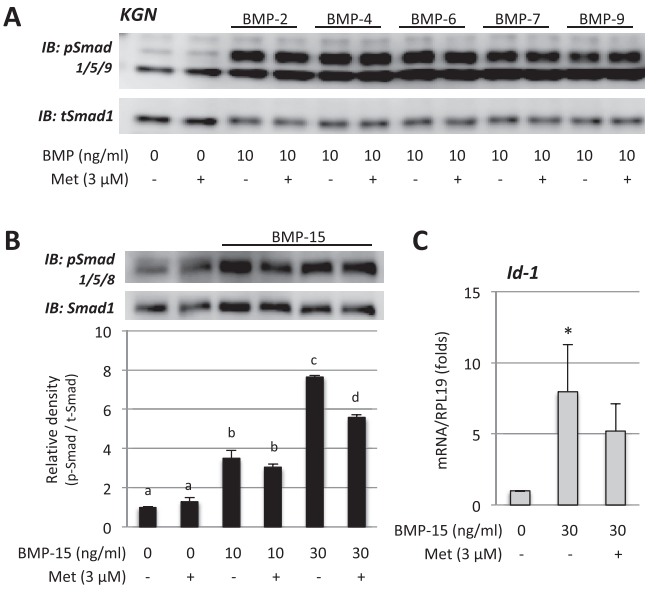


**Fig. 2. Effects of metformin on Smad signaling induced by BMPs in human granulosa cells. A**, **B**) KGN cells were cultured in serum-free DMEM/F12 with indicated concentrations of metformin for 24 h, and then stimulated with BMP-2, -4, -6, -7, -9, and -15 (10–30 ng/ml) for 60 min. The protein cell lysates were analyzed by SDS-PAGE/immunoblotting using anti-pSmad1/5/9 antibodies. The signal intensities of pSmad1/5/9 were normalized by those of total Smad1 in each sample and then expressed as fold changes. **C**) Total cellular RNA was extracted from KGN cells treated with BMP-15 (30 ng/ml) either alone or in combination with metformin (3 μM) in a serum-free condition for 24 h, and the mRNA level of Id-1 was determined by quantitative PCR. 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. Values with different superscript letters are significantly different at $P < 0.05$; and $^{*}P < 0.05$ vs. control groups.

were selected in the present study. The levels of estradiol and progesterone production in the culture media were analyzed using Architect kits for estradiol and progesterone (Cayman Chemical Co., Ann Arbor, MI, USA) by chemiluminescent immunoassay (CLIA). Steroid levels were undetectable (progesterone content of <10 pg/ml and estradiol content of <15 pg/ml) in each cell-free culture medium.

### 2.4. Procedure of real-time RT-PCR

KGN cells ($1 \times 10^5$ cells/ml) were treated with FSK (3 μM) either alone or in combination with metformin (1 μM) in serum-free DMEM/F12 in 12-well plates. After 24-h culture, total RNA was extracted using TRI Reagent® (Cosmo Bio Co., Ltd., Tokyo, Japan) and the yields of RNA were measured by using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The primer sets were selected from different exons of the corresponding genes to eliminate PCR products arisen from chromosome DNA. Primer pairs for the genes of steroidogenic factors and enzymes, including steroidogenic acute regulatory protein (StAR), steroid side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), and aromatase (P450arom); BMP receptor (BMPR) signaling molecules, including activin type-II receptor (ActRIIA), BMP type-II receptor (BMPRII), activin receptor-like kinase (ALK)-2, −3 and −6, Id-1, and Smad6 and Smad7; and a housekeeping gene, ribosomal protein L19 (RPL19), were selected as we reported previously [9,11–15]. The extracted RNA (0.5–1 μg) was subjected to reverse transcription (RT) with ReverTra Ace® (TOYOBO CO., LTD., Osaka, Japan). Quantitative real-time PCR was performed using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) under the optimized annealing conditions and amplification efficiency [14]. The mRNA levels of target genes were analyzed by the Δ threshold cycle (Ct) method, in which ΔCt values were obtained by subtracting the Ct value of RPL19 from that of the target. The mRNA levels of target genes relative to RPL19 were expressed as $2^{-(\Delta Ct)}$, and then the data were expressed as the mRNA ratios of target to RPL19.

### 2.5. Western blots for smads

KGN cells ($1 \times 10^5$ cells/ml) were pretreated with metformin (3 μM) in serum-free DMEM/F12 for 24 h and subsequently stimulated with various BMP ligands (10–30 ng/ml) for 1 h. In another study, cells ($1 \times 10^5$ cells/ml) were treated with metformin (3 μM) in serum-free condition for 24–72 h. The cell lysates were then collected by solubilizing cells into RIPA lysis buffer (50 μl) (Upstate Biotechnology, Lake Placid, NY) containing 1 mM $Na_3VO_4$, 1 mM NaF, 2% SDS and 4% β-mercaptoethanol. The lysates were then subjected to SDS-PAGE/immunoblotting with antibodies against phospho-Smad1/5/9 (pSmad1/5/9), total-Smad1 (tSmad1; Cell Signaling Technology, Inc., Beverly, MA), Smad6 (Cell Signaling Technology, Inc., Beverly, MA), Smad7 (R&D Systems Inc.), and actin (Sigma-Aldrich Co. Ltd.). The integrated band intensities were analyzed to express numerical values by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) and the data were shown as the relative ratios of pSmad/tSmad or Smad/actin.

### 2.6. Statistics

Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples. Statistical analysis was performed by ANOVA with Fisher's protected least significant difference (PLSD) and the unpaired *t*-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were accepted as statistically significant.

## 3. Results

We first investigated the effects of metformin on the production of progesterone and estradiol by human KGN cells. To examine the effects of metformin on FSK-induced steroidogenesis in KGN cells, the conditioned media collected following 24-h culture under the presence or absence of metformin were tested for CLIA. As shown in Fig. 1A, levels of FSK (3 μM)-induced progesterone production were significantly enhanced by treatment with metformin (0.03–1 μM), whereas metformin did not change FSK-induced estradiol production. The same experiment was performed using primary rat granulosa cells to see whether similar results would be obtained. As shown in Fig. 1B, levels of FSH (10 ng/ml)-induced progesterone production were significantly augmented with more than 1 μM of metformin. Since metformin treatment upregulated the FSK-induced production of steroids, the mRNA levels of genes involving in steroidogenesis, StAR, P450scc, 3βHSD and P450arom, were examined by quantitative RT-PCR. As expected, metformin (1 μM) increased the mRNA expression of StAR, P450scc and 3βHSD, but not that of P450arom, induced by FSK (3 μM) (Fig. 1C).

The BMP system that resides in the ovary plays an inhibitory role in FSH-included progesterone production during folliculogenesis. Therefore, involvement of BMP actions were investigated by SDS-PAGE/immunoblotting using protein lysates derived from BMP-treated KGN cells. As shown in Fig. 2A, the phosphorylation of Smad1/5/9 induced by BMP-2, -4, -6, -7 or -9 (10 ng/ml) was not affected by treatment with metformin (3 μM). However, the phosphorylation of Smad1/5/9 induced by BMP-15 (30 ng/ml) was suppressed by metformin (3 μM) (Fig. 2B). In addition, the mRNA

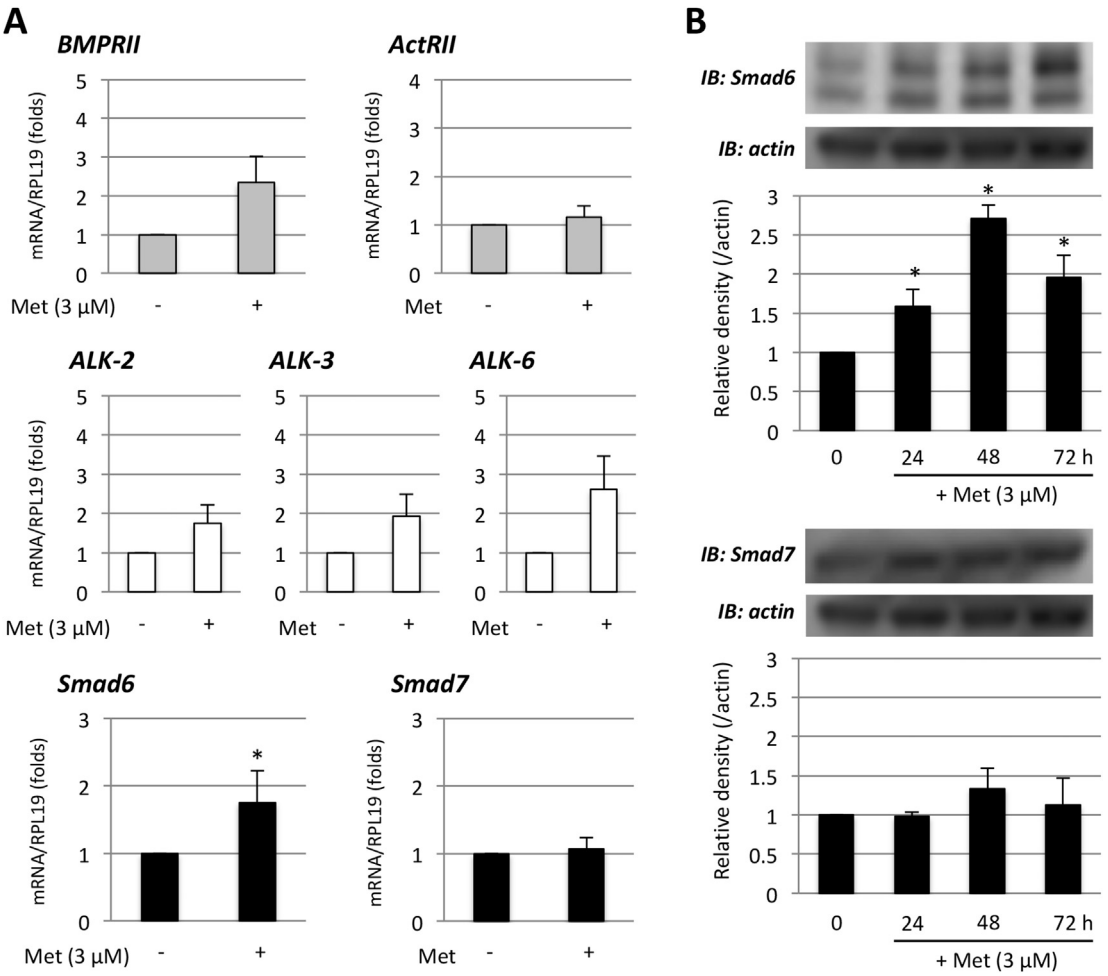


**Fig. 3. Effects of metformin on the expression of genes involved in BMP signaling. A**) Total cellular RNA was extracted from KGN cells treated with or not treated with metformin (3 μM) in a serum-free condition for 24 h, and mRNA expression levels of receptor genes, BMPRII, ActRII, ALK-2, -3 and -6, and inhibitory Smad6 and Smad7, were determined by quantitative PCR. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. **B**) The protein cell lysates were collected after the treatment with metformin (3 μM) for 24 h–72 h in a serum-free condition and analyzed by Western blot using anti-Smad6 and anti-Smad7 antibodies. The signal intensities of Smad6/7 were normalized by those of anti-actin antibody in each sample and then expressed as fold changes. The results were analyzed by the unpaired *t*-test (**A**) and ANOVA (**B**). Values with different superscript letters are significantly different at $P < 0.05$; and $^{*}P < 0.05$ vs. control groups.

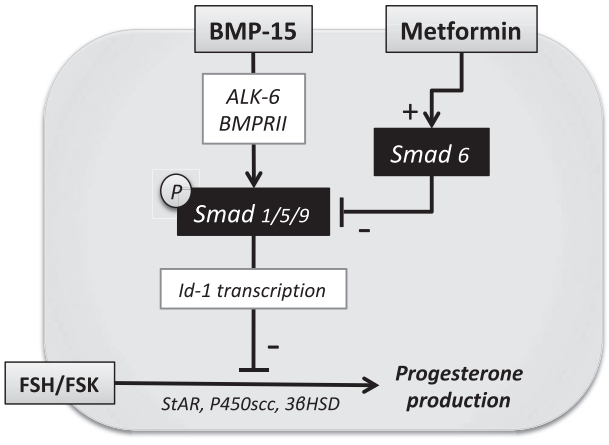


**Fig. 4. Potential interaction of metformin and BMP-receptor signaling in modulation of progesterone production by granulosa cells.** In human granulosa KGN cells, metformin was found to enhance progesterone production induced by FSH or FSK. Metformin was also found to suppress Smad1/5/9 phosphorylation induced by BMP-15 through upregulation of inhibitory Smad6 in human KGN granulosa cells.

expression of Id-1, a target gene of BMP signaling, was studied by quantitative PCR. Fig. 2C shows that treatment with BMP-15 (30 ng/ml) upregulated the expression of Id-1 mRNA, which was significantly suppressed by treatment with metformin (3 μM) as shown by Western blots.

To determine the mechanism by which metformin affects BMP signaling in KGN cells, the effects of metformin on the expression of genes involved in BMP-15 signaling were examined by quantitative PCR. As shown in Fig. 3A, treatment with metformin (3 μM) did not significantly affect mRNA expression of the type-I or -II receptor subunits in KGN cells, though slight increases in the expression levels of BMPRII and ALK-2, -3 and -6 were observed. Of note, among the inhibitory Smads, the mRNA expression of Smad6, but not that of Smad7, was significantly upregulated by treatment with metformin (3 μM). To determine whether this was also detectable at the protein levels of inhibitory Smads, Western blotting was performed for Smad6 and Smad7. As shown in Fig. 3B, treatment with metformin (3 μM) significantly increased the protein expression level of Smad6, but not that of Smad7, for 24–72 h. Thus, the results suggested that metformin acts to upregulate Smad6 expression that regulates BMP-15 activity, leading to the enhancement of progesterone production induced by granulosa cells induced by FSK or FSH (Fig. 4).

## 4. Discussion

In the present study, it was revealed that metformin enhanced progesterone synthesis by granulosa cells induced by FSK or FSH, whereas metformin had no effect on estradiol levels. The effects of metformin are linked to the activation of hepatic and muscular AMP-activated protein kinase (AMPK), leading to suppression of fatty acid synthesis in the liver and increase of glucose uptake in muscle [16,17]. It has been reported that a relatively low concentration of metformin (100 nM) attenuates basal and insulin-stimulated aromatase expression via the mitogen-activated protein kinase (MAPK) pathway in human luteinized granulosa cells [18] and that it also inhibits FSHR activity in human granulosa KGN cells [19]. A high concentration (10 mM) of metformin has been reported to reduce both estrogen and progesterone production via an AMPK-dependent pathway in rat granulosa cells [20] and bovine granulosa cells [21,22]. The effects of metformin on ovarian steroidogenesis seem to be concentration-dependent. In the present study, we utilized a medium range of metformin concentrations (1–3 μM) that augmented FSK-induced progesterone synthesis, but not estradiol level, by human KGN granulosa cells. It was of interest that metformin treatment suppressed phosphorylation of Smad1/5/9 activated by BMP-15 compared with that induced by BMP-2, -4, -6, -7 and -9. Moreover, metformin treatment enhanced the expression of inhibitory Smad6 in KGN cells. Thus, the mechanism by which metformin suppresses BMP-15-induced Smad1/5/9 phosphorylation is likely, at least in part, to be upregulation of inhibitory Smad6 expression in granulosa cells.

It is thought that the BMPs also have roles in the pathogenesis of PCOS, although the approaches to BMP-15 and GDF-9 genes have not yet revealed significant relevance to the etiology of PCOS [23,24]. The results of the present study indicated that metformin enhanced progesterone production by suppressing intracellular BMP-15 signaling; however, the endogenous levels of BMP-15 and GDF-9 expression do not seem to be stable in ovaries derived from patients with PCOS. Analysis of the expression of these factors in PCOS provided some interesting results. For instance, it was reported that GDF-9 transcription was delayed and decreased in the growing and differentiating stages of human PCOS ovaries [25]. The expression levels of GDF-9 and BMP-15 per oocytes were shown by single-cell expression analysis to be higher in PCOS patients [26]. Another study showed distinct changes in the expression levels of BMP-15 and GDF-9: GDF-9 expression level was lower and BMP-15 expression level was higher in granulosa cells from FSH-stimulated PCOS ovaries [27]. It was also shown that GDF-9 expression levels in cumulus cells were lower in PCOS patients [28] and that the dynamic changes in the expression pattern of GDF-9 and BMP-15 disappeared in oocytes from patients with PCOS [29]. Another study also showed that the expression levels of GDF-9 and BMP-15 were impaired and delayed in the early stage of follicular development of PCOS tissues [30].

Regarding expressional changes of BMP-15 and GDF-9 in oocytes of PCOS patients treated with metformin, it was reported that the level of BMP-15 protein in oocytes were not changed but that the expression level of GDF-9 in oocytes was significantly increased by metformin treatment [31]. Besides the actions of metformin, we previously reported that melatonin [32], androgen [11], insulin-like growth factor (IGF)-I [12] are key factors that enable to induce Smad6 and/or Smad7 expression. On the contrary, prolactin, somatostatins and incretins were suggested to be suppressors for Smad6/7 in granulosa cells [33]. Thus, a modulatory effect on BMPR signaling mediated by Smad6/7 seems to be crucial for integrating ovarian steroidogenesis via the endogenous BMP activity in granulosa cells.

The specificity of the interaction between metformin and BMP-15 action, compared with the other BMP ligands, was not clarified in our study. Changes in the expressional pattern of BMP type-I and -II receptors in KGN cells might be involved in this specificity [9]. Namely, the impact of Smad6 upregulation on Smad1/5/9 signaling may differ among the BMP ligands depending on the abundance of BMPR subunits expressed. The upregulation of BMPRII and/or ALK-2, -3, and -6, though the changes were insignificant, might have counter-regulated the enhanced expression of inhibitory Smad6, resulting in neutralization of these mutual effects on the intensity of BMPR signaling. In addition, the regulatory mechanism of BMPs in KGN steroidogenesis was somewhat differed from the data obtained in primary granulosa cells, which may reflect the characteristics of KGN cells derived from neoplastic granulosa cells [9]. Further studies are necessary to conclude the detailed molecular mechanism of metformin and its intracellular actions related to BMPR signaling.

Collectively, the results showed that metformin augments progesterone synthesis, at least in part, by downregulating BMP-15 signaling in granulosa cells (Fig. 4). The mechanism by which metformin impairs BMP-15-induced Smad1/5/9 phosphorylation is likely, at least in part, to be upregulation of inhibitory Smad6 in granulosa cells. The findings suggest the possibility that metformin is applicable for maintenance of steroidogenesis in PCOS ovaries via modulating endogenous BMP activity.

## Disclosure statement

The authors have nothing to disclose.

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## Transparency document

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