Original Article

A regulatory role of androgen in ovarian steroidogenesis by rat granulosa cells.

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Abbreviations:

1 ALK, activin receptor-like kinase
2 ActRI, activin type-I receptor
3 ActRII, activin type-II receptor
4 BMP, bone morphogenetic protein
5 BMPRI, BMP type-I receptor
6 BMPRII, BMP type-II receptor
7 FSH, follicle-stimulating hormone
8 FSHR, FSH receptor
9 GDF, growth and differentiation factor
10 IGF-I, insulin-like growth factor-I
11 IGF-IR, IGF-I receptor
12 3βHSD, 3β-hydroxysteroid dehydrogenase
13 LH, luteinizing hormone
14 PCOS, polycystic ovary syndrome
15 P450arom, P450 aromatase
16 P450scc, P450 steroid side-chain cleavage enzyme
17 StAR, steroidogenic acute regulatory protein
18 TGF, transforming growth factor

Abstract

Excess androgen and insulin-like growth factor (IGF)-I in the ovarian

follicle has been suggested to be involved in the pathophysiology of polycystic

ovary syndrome (PCOS). Here we investigated the impact of androgen and

IGF-I on the regulatory mechanism of ovarian steroidogenesis using rat primary

granulosa cells. It was revealed that androgen treatment with

dihydrotestosterone (DHT) amplified progesterone synthesis in the presence of

FSH and IGF-I, whereas it had no significant effect on estrogen synthesis by rat

granulosa cells. In accordance with the effects of androgen on steroidogenesis,

DHT enhanced the expression of progesterogenic factors and enzymes,

including StAR, P450scc and 3βHSD, and cellular cAMP synthesis induced by

FSH and IGF-I. Of note, treatment with DHT and IGF-I suppressed Smad1/5/8

phosphorylation and transcription of the BMP target gene Id-1, suggesting that

androgen and IGF-I counteract BMP signaling that inhibits FSH-induced

progesterone synthesis in rat granulosa cells. DHT was revealed to suppress

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the expression of BMP-6 receptors, consisting of ALK-2, ALK-6 and ActRII, while it increased the expression of inhibitory Smads in rat granulosa cells.   In addition, IGF-I treatment upregulated androgen receptor (AR) expression and DHT treatment suppressed IGF-I receptor expression on rat granulosa cells. Collectively, the results indicate that androgen and IGF-I mutually interact and accelerate progesterone production, at least in part, by regulating endogenous BMP signaling in rat granulosa cells.   Cooperative effects of androgen and IGF-I counteract endogenous BMP-6 activity in rat granulosa cells, which is likely to be functionally linked to the steroidogenic property shown in the PCOS ovary.

Introduction

In patients with polycystic ovary syndrome (PCOS), endogenous insulin

levels are increased due to insulin resistance [1].   Hyperinsulinemia reinforces

local insulin-like growth factor (IGF)-I activity in the ovary and, as a result, this

induces an androgenic composition in theca cells [2].   Both increased IGF-I

activity and a high androgenic environment appear to be involved in the

ovulation disorder in PCOS patients [3].

The growing and maturating process of ovarian follicles occurs as a

consequence of functional interactions between gonadotropins and various

autocrine/paracrine factors in the ovary.   It has been shown that the ovarian

bone morphogenetic protein (BMP) system plays a critical role in the regulation

of follicular steroidogenesis in an autocrine/paracrine manner [4, 5].   BMPs

exert a common biological activity to suppress progesterone synthesis

stimulated by follicle-stimulating hormone (FSH) as a luteinizing inhibitor.   In

growing follicles, exquisite control of FSH responsiveness is necessary for the

physiological selection of dominant follicles and the successive ovulation.

BMPs are key regulatory molecules for the maintenance of FSH activity as well

as FSH sensitivity during the process of normal folliculogenesis [4, 6].

The BMP system molecules including BMP ligands and the receptors

exhibit a cell-specific expression pattern in ovarian follicles.   Among these, the

expression of BMP-6 is localized in oocytes and granulosa cells at Graafian

stages of rat healthy follicles [6].   BMP-6 has an inhibitory effect on FSH

receptor actions by suppressing adenylate cyclase, leading to cAMP reduction,

in rat granulosa cells [7, 8].   BMP-6 expressed in rat granulosa cells appears to

be reduced at the step of selecting dominant follicles [6], suggesting that BMP-6

physiologically conduces to the selection process.

From the standpoint of the pathophysiology of human PCOS,

examination of ovary tissues revealed that growth and differentiation factor

(GDF)-9 mRNA expression, compared to BMP-15, was delayed and decreased

in growing follicles from PCOS ovaries [9].   Moreover, it is of note that the

expression of BMP-6 is highly increased in human granulosa cells isolated from

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PCOS ovaries [10, 11].

In the present study, we investigated the functional roles of androgen and IGF-I in ovarian steroidogenesis by focusing on BMP-6, which suppresses progesterone biosynthesis, using primary culture of rat granulosa cells. It was clarified that androgen excess and IGF-I action interact mutually and accelerate steroidogenesis by regulating endogenous BMP signaling in rat granulosa cells.

Materials and Methods

Reagents and supplies

McCoy's 5A medium, Medium 199 and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Bovine serum albumin (BSA), diethylstilbestrol (DES), dihydrotestosterone (DHT), 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), insulin-like growth factor-I (IGF-I), ovine pituitary FSH, and penicillin-streptomycin solution were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-6 was purchased from R&D Systems Inc. (Minneapolis, MN).

Primary culture of rat ovarian granulosa cells

Female Sprague-Dawley (SD) rats were purchased from Charles River (Wilmington, MA). To increase the number of granulosa cells, silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats. After 3 days of exposure to DES, ovarian follicles were punctured with a 27-gauge needle. Granulosa cells were counted and separated from oocytes by filtering

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the oocyte/granulosa cell suspension through 100- and 40-µm nylon meshes (BD Falcon, Bedford, MA) that allowed granulosa cells but not oocytes to pass through [12, 13].   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 (1×10^5 viable cells in 200 µl) were cultured, as described above, in 96-well plates with 100 nM of androstenedione, a substrate for aromatase.   FSH (10 ng/ml) was added to the culture medium either alone or in combination with indicated concentrations of IGF-I and DHT.   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 medium 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 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 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.   Cells were treated with FSH (10 ng/ml) either alone or in combination with indicated concentrations of IGF-I and DHT.   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 stored at -80°C until assay.   Primer pairs for activin receptor-like

kinase (ALK)-2, ALK-3 and ALK-6, activin type-II receptor (ActRII), BMP type-II

receptor (BMPRII), Smad6 and 7, and ribosomal protein L19 (RPL19) were

selected as reported previously [14, 15].   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:

P450scc, 147-167 and 636-655 (from GenBank accession #J05156);

steroidogenic acute regulatory protein (StAR), 395-415 and 703-723

(AB001349); rat 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);   IGF-I receptor (IGF-IR), 1266-1287 and

1501-1522 (NM_052807); androgen receptor (AR), 2521-2543 and 2711-2734

(NM_013476); and RPL19, 401-421 and 575-595 (J02650).   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 (2.5×10^5 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 IGF-I and/or DHT, cells were treated with BMP-6 for

60 min.   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 subjected to

SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8 (pSmad1/5/8) 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 target proteins / 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 unpaired *t*-test, when appropriate (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA).   If differences were detected by ANOVA, Fisher's protected least significant difference (PLSD) test and Tukey-Kramer's post hoc test were used to determine which means differed (StatView 5.0 software). P values <0.05 were accepted as statistically significant.

Results

We first investigated the combined effects of androgen and IGF-I on regulation of ovarian steroidogenesis using rat primary granulosa cells isolated from immature female rat ovaries. In order to exclude the effect of conversion of testosterone to estradiol [16], we utilized DHT, instead of testosterone, as an androgen for assessment of the appropriate AR action.

As shown in **Fig. 1**, individual treatment with IGF-I (100 ng/ml) or DHT (100 nM) did not affect basal levels of estradiol (**Fig. 1A**) and progesterone (**Fig. 1B**) production by rat granulosa cells for 48-h culture. Treatment with IGF-I (0 to 300 ng/ml) increased FSH (10 ng/ml)-induced estradiol production (**Fig. 1A**) as well as progesterone production (**Fig. 1B**) by granulosa cells in a concentration-responsive manner. Treatment with DHT (100 nM) only marginally increased estradiol production in the presence of FSH plus IGF-I (0 to 300 ng/ml) (**Fig. 1A**). On the other hand, it was of note that DHT (100 nM) treatment significantly enhanced FSH-induced progesterone production in the

presence of IGF-I (0 to 300 ng/ml) (**Fig. 1B**).

To confirm the effects of androgen and IGF-I on steroidogenesis, we examined the mRNA levels of steroidogenic enzymes including StAR, P450scc, 3βHSD and P450arom by quantitative real-time PCR.   We previously reported that treatment with IGF-I enhances FSH-induced expression of StAR, P450scc, 3βHSD and P450arom in rat granulosa cells [17].   In the present study, we focused on the effects of androgen on steroidogenic enzyme expression induced by FSH and IGF-I.   As shown in **Fig. 2**, co-treatment with DHT (100 nM) further enhanced the effects of IGF-I (100 ng/ml) on expression of StAR, P450scc and 3βHSD mRNAs induced by FSH (10 ng/ml) in rat granulosa cells for 48 h (**Fig. 2A**).   On the other hand, in accordance with the results for estradiol production (**Fig. 1A**), the mRNA levels of P450arom were not significantly altered under the condition of the same treatment with DHT and IGF-I (**Fig. 2A**).

To determine the mechanism by which androgen and IGF-I regulate FSH-induced steroidogenesis by granulosa cells, cAMP synthesis in the conditioned medium of rat granulosa cell culture was examined.   FSH (10

ng/ml)-induced cAMP synthesis was not significantly increased by only IGF-I

(100 ng/ml) treatment, whereas co-treatment with DHT (100 nM) significantly

enhanced the cAMP synthesis induced by FSH and IGF-I by rat granulosa cells

for 48-h culture (**Fig. 2B**), being in accordance with the changes in progesterone

production induced by DHT (**Fig. 1B**).   To determine the mutual interaction

between the effects of androgen and IGF-I in granulosa cells, the expression

levels of IGF-IR and AR were examined by quantitative PCR.   As shown in **Fig.**

2C, the expression level of AR mRNA was upregulated by IGF-I (100 ng/ml)

treatment; on the other hand, the expression of IGF-IR was in turn suppressed

by DHT (100 nM) treatment for 48-h culture in rat granulosa cells.

We next examined the interaction of androgen, IGF-I and

BMP-receptor signaling in rat granulosa cells.   As shown in **Fig. 3A**, stimulation

with BMP-6 (100 ng/ml) for 1 h readily induced Smad1/5/8 phosphorylation,

which was significantly suppressed by IGF-I (100 ng/ml), DHT (100 nM) or their

combined treatment.   To clarify the changes in BMP-receptor signal

transduction caused by IGF-I and DHT, we also assessed mRNA level of the

BMP target gene Id-1.   As shown in **Fig. 3B**, IGF-I (100 ng/ml) and DHT (100

nM) had no effect on the basal Id-1 mRNA level; however, the Id-1 induction

caused by BMP-6 (100 ng/ml) was clearly suppressed by treatment with IGF-I,

DHT or their combination for 24-h culture of rat granulosa cells.

To determine the mechanism by which androgen suppresses

BMP-Smad signaling in granulosa cells, we further evaluated the changes in

BMP-receptor expression caused by androgen and IGF-I treatment.   As shown

in **Fig. 3C**, the expression levels of type-I (ALK-2 and ALK-6) and type-II (ActRII)

receptors for BMP-6 were reduced by DHT (100 nM) treatment for 48-h culture,

in which ALK-6 and ActRII were also decreased by IGF-I (100 ng/ml) treatment.

The expression level of another type-II receptor, BMPRII, was not significantly

changed by DHT treatment, although the effect was different from that induced

by IGF-I.   We also assessed changes in the expression of inhibitory Smads

including Smad6 and Smad7.   As shown in **Fig. 3C**, the expression levels of

Smad6 and Smad7 mRNA were significantly increased by IGF-I (100 ng/ml) and

DHT (100 nM), respectively, in rat granulosa cells for 48 h.

Discussion

In the present study, we hypothesized that a high androgenic environment and increased IGF-I activity are mutually involved in the dysregulation of steroidogenesis in PCOS, leading to ovulation disorders. By using rat primary granulosa cell culture, it was revealed that the combined effect of androgen and IGF-I promoted FSH-induced progesterone production, compared with estradiol production, by suppressing endogenous BMP signaling (**Fig. 4**).

The ovarian IGF-I system plays key roles in normal folliculogenesis and also in the degenerative process of follicular atresia [18, 19], in which IGF-I is involved in the enhancement of FSH sensitivity by upregulating FSH receptor (FSHR) expression on granulosa cells. The significance of IGF-I activity in the development of PCOS pathology and enhanced IGF-IR expression during early preantral development has been reported [2]. On the other hand, roles of androgen and its receptor AR in the pathophysiology of PCOS have also been recognized [20]. AR is expressed in oocytes, granulosa cells and theca cells of

preantral follicles in the rat ovary, in which the expression pattern of AR is different depending on the development stage of follicles [20].   In the present study, it was found that IGF-I treatment upregulated the expression of AR mRNA, while androgen treatment in turn suppressed the expression of IGF-IR mRNA in rat granulosa cells.   An androgen excess is clinically related to insulin resistance and/or metabolic syndrome in women [21].   In rodents, long-term treatment with DHT has been demonstrated to cause various reproductive and metabolic features of PCOS.   For instance, DHT-treated rodents exhibited reduced ovulation with polycystic changes of the ovaries encompassing atretic follicles, thickened theca and thinned granulosa cells [22-24], suggesting a direct effect of AR actions on the development of reproductive and metabolic features of PCOS but with species-dependent differences.

Relationships between androgen and ovarian growth factors have been reported for IGFs and transforming growth factor (TGF) β superfamily members. In addition to the synergistic effects of androgen and FSH, androgen enhances the proliferation of granulosa cells induced by IGF-I alone or GDF-9 in the

presence of IGF-I [25, 26]. Direct AR effects also enhance the differentiation of rat granulosa cells, with testosterone acting via the AR to increase the expression of P450arom and P450scc [27]. The expression levels of factors for the oocyte–granulosa cell regulatory loop, such as Kit ligand, BMP-15 and GDF-9, are reduced in AR-deficient mouse ovaries at the preovulatory stage [28]. Pathophysiological involvement of BMP system molecules in PCOS has also been reported [9, 29, 30]. Of interest, female GDF-9-knockout mice showed PCO-like morphological features of the ovaries containing ovarian follicular cysts lined by several layers of flattened granulosa cells [31]. Examination of ovarian tissues derived from PCOS patients clarified that GDF-9 expression was significantly delayed and weakened during the differentiation phase [9]. Therefore, it is presumed that ovarian growth factors such as GDFs and BMPs are directly linked to the functional and morphological changes shown in PCOS.

We previously reported that the ovarian growth hormone (GH)/IGF-I system acts to modulate steroidogenesis in rat granulosa cells [17]. IGF-I impaired BMP-Smad1/5/8 signaling via downregulation of the expression of

BMP receptors [17], while BMPs also counteracted GH receptor, IGF-I and IGF-IR expression in rat granulosa cells. The mutual interaction between GH/IGF-I and BMP activities may be critical for the regulation of follicular steroidogenesis. However, the impact of androgen, in relation to IGF-I activity, on ovarian steroidogenesis and its mechanism remain uncertain. In the present study, we further demonstrated that androgen and IGF-I interaction reinforced progesterone production induced by FSH in rat granulosa cells. In addition, IGF-I enhanced the expression of AR, while androgen suppressed the expression of IGF-IR, suggesting the presence of a negative feedback loop within rat granulosa cells.

Clinical investigations have shown that the expression of BMP-6 mRNA was significantly increased in human granulosa cells isolated from PCOS patients in comparison with that in granulosa cells from control patients who underwent *in vitro* fertilization [10, 11]. Since BMP-6 expression is abundant in granulosa cells of healthy growing follicles but not in atretic follicles in the human ovary [32], the overexpression of BMP-6 in granuslosa cells may indicate the

existence of growth-disturbed follicles in PCOS ovaries.   Alternatively, the

upregulation of BMP-6 in granulosa cells isolated from PCOS patients may

reflect the existence of a feedback mechanism caused by suppression of

BMP-Smad signaling under the influence of excess androgen and IGF-I.

Collectively, the results indicate that androgen enhances IGF-I-induced

progesterone production by counteracting endogenous BMP-6 actions in

granulosa cells (**Fig. 4**), which might be functionally linked to the altered

steroidogenic property shown in the PCOS ovary.   In this regard, we earlier

reported the activity of melatonin, which has been shown to be effective for the

infertility of PCOS [33], on ovarian steroidogenesis [34].   In that study, it was

found that melatonin plays an antagonistic role in BMP-6 actions in rat granulosa

cells.   Since BMP-6 has the property of a luteinization inhibitor in growing

follicles, the results indicated that melatonin maintains progesterone synthesis

and luteinization by inhibiting BMP-6 activity in rat granulosa cells.   Such

inhibitory effects of melatonin or androgen on BMP-6 activity could be effective

for compensating the impaired progesterone production and the arrest of

follicular development observed in PCOS cases. Based on the present findings, it is suggested that balanced intensities between androgen/IGF-I and the BMP system are physiologically critical for fine-tuning ovarian steroidogenesis in growing follicles (**Fig. 4**).

Acknowledgments

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Figure legends:

Fig. 1. Effects of androgen and IGF-I on FSH-induced steroidogenesis by rat granulosa cells. <mark>Granulosa cells were cultured in a serum-free condition with FSH either alone or in combination with IGF-I and/or DHT. After 48-h culture, the levels of estradiol (**A**) and progesterone (**B**) in the medium were determined by CLIA.</mark> Results in all panels are shown as means ± SEM. The results were analyzed by ANOVA. Values with different superscript letters are significantly different at $P < 0.05$.

Fig. 2. Effects of androgen and IGF-I on steroidogenic enzyme expression, cAMP synthesis, and expression of AR and IGF-IR in rat granulosa cells.

A) <mark>Total cellular RNA was extracted from granulosa cells treated with FSH, IGF-I and DHT for 48 h in a serum-free condition, and StAR, P450scc, 3βHSD and P450arom mRNA levels were determined by quantitative PCR.</mark> **B**) <mark>Granulosa cells were cultured in a serum-free medium containing IBMX with FSH either alone or in combination with IGF-I and/or DHT.</mark> After 48-h culture, the levels of

cAMP in the medium were determined by ELISA.   **C**) <mark>Total cellular RNA was extracted from granulosa cells treated with IGF-I or DHT for 48 h, and AR and IGF-IR mRNA levels were determined by quantitative PCR.   The expression levels of target gene mRNA were standardized by RPL19 level and expressed as fold changes (**A**, **C**).</mark>   Results in all panels are shown as means ± SEM. The results were analyzed by the unpaired *t*-test (**A**, **C**), or ANOVA (**B**).   *$P <$ 0.05 and **$P <$ 0.01 vs. control group and between the indicated groups; The values with different superscript letter are significantly different at $P <$ 0.05; n.s., not significant.

Fig. 3. Effects of androgen and IGF-I on BMP-receptor signaling and expression of BMP receptors in rat granulosa cells.   **A**) After preculture in a serum-free condition with IGF-I and DHT 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.   <mark>The signal intensities of pSmad1/5/8 were standardized by actin</mark>

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 IGF-I and DHT for 24 h, and Id-1 mRNA levels were determined by quantitative PCR. **C**) Total cellular RNA was extracted from granulosa cells treated with IGF-I or DHT for 24 h, and BMPRs and Smad6/7 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. Values with different superscript letters are significantly different at $P < 0.05$. *$P < 0.05$ vs. control group or between the indicted groups.

Fig. 4. Possible mechanism by which androgen and IGF-I regulate ovarian steroidogenesis in rat granulosa cells. DHT promotes FSH-induced progesterone production in the presence of IGF-I action by inhibiting the BMP system in cooperation with each other. IGF-I and DHT suppress Smad1/5/8

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signaling by downregulating ALK-2, -6 and ActRII, and upregulating Smad6/7.

IGF-I upregulates AR expression, while DHT downregulates IGF-I receptor expression. The combined effects of androgen and IGF-I counteract endogenous BMP-6 activity in rat granulosa cells.