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Regulatory role of BMP-9 in steroidogenesis by rat ovarian granulosa cells

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ABSTRACT

BMPs expressed in the ovary differentially regulate steroidogenesis by granulosa cells. BMP-9, a circulating BMP, is associated with cell proliferation, apoptosis and differentiation in various tissues. However, the effects of BMP-9 on ovarian function have yet to be elucidated. Here we investigated BMP-9 actions on steroidogenesis using rat primary granulosa cells. BMP-9 potently suppressed FSH-induced progesterone production, whereas it did not affect FSH-induced estradiol production by granulosa cells. The effects of BMP-9 on FSH-induced steroidogenesis were not influenced by the presence of oocytes. FSH-induced cAMP synthesis and FSH-induced mRNA expression of steroidogenic factors, including StAR, P450scc, 3βHSD2 and FSHR, were suppressed by treatment with BMP-9. BMP-9 mRNA expression was detected in granulosa cells but not in oocytes. BMP-9 readily activated Smad1/5/8 phosphorylation and Id-1 transcription in granulosa cells. Analysis using ALK inhibitors indicated that BMP-9 actions were mediated via type-I receptors other than ALK-2, -3 and -6. Furthermore, experiments using extracellular domains (ECDs) for BMP type-I and -II receptor constructs revealed that the effects of BMP-9 were reversed by ECDs for ALK-1 and BMPRII. Thus, the functional receptors for BMP-9 in granulosa cells were most likely to be the complex of ALK-1 and BMPRII. Collectively, the results of the present study showed that BMP-9 can affect luteinization and that there are two possible sources of BMP-9, serum and granulosa cells in the ovary.

1. Introduction

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor (TGF)-β superfamily, were originally identified as inducers for ectopic bone growth and cartilage formation. There has been substantial progress in elucidation of the multiple roles of BMPs ranging from roles in bone to other systemic actions [1]. BMPs regulate cell growth, differentiation, and apoptosis of various cell types, and they are crucial for tissue morphogenesis and differentiation.

Ovarian follicle growth and maturation occur as a result of elaborate interactions between pituitary gonadotropins and autocrine/paracrine growth factors produced within the ovary. Recent studies have established the concept that members of the TGF-β superfamily, including BMPs, growth differentiation factors (GDFs), activins and inhibins, play key roles as autocrine/paracrine factors in female fertility in mammals [2–4]. BMP ligands and receptors, including BMP-2, -3, -3b, -4, -6, -7 and -15, GDF-9, and BMP type-I and type-II receptors, are expressed in cell-specific patterns in ovarian cells that undergo dynamic changes during follicular development and corpora luteal formation [3].

Regulation of FSH responsiveness in granulosa cells is critical for the steps of follicular selection, dominant follicle formation and

Abbreviations: ALK, activin receptor-like kinase; ActRI, activin type-I receptor; ActRII, activin type-II receptor; BMP, bone morphogenetic protein; BMPRI, BMP type-I receptor; BMPRII, BMP type-II receptor; ECD, extracellular domain; FSH, follicle-stimulating hormone; FSHR, FSH receptor; GDF, growth differentiation factor; 3βHSD, 3β-hydroxysteroid dehydrogenase; IBMX, 3-isobutyl 1-methylxanthine; MAPK, mitogen-activated protein kinase; P450scc, P450 steroid side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TGF-β, transforming growth factor.

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subsequent ovulation in mammals. This process elicited by FSH receptor (FSHR) signaling is further modulated by many autocrine/paracrine factors [2]. BMPs play a key role in female fertility by regulating steroidogenesis and mitosis in granulosa cells. In particular, BMP ligands commonly suppress FSH-induced progesterone synthesis [3–6]. For instance, oocyte-derived BMP-15 attenuates FSH actions by suppressing FSHR expression in granulosa cells. Oocyte- and granulosa-derived BMP-6 reduces FSH-induced progesterone level by suppressing adenylate cyclase activity. BMP-2 from granulosa cells and BMP-4 and -7 from theca cells also suppress FSH-induced progesterone production. The finding that FSH-induced progesterone synthesis is inhibited by ovarian BMPs suggests a key role of BMPs as luteinization inhibitors.

BMP-9, also called GDF-2, is mainly expressed in the liver and shows hematopoietic, hepatogenic and osteo-chondrogenic activities [7]. BMP-9 also acts as a metabolic regulator for glucose, lipid and iron as well as a neuronal differentiation factor [8,9]. BMP-9 exists in human serum and plasma, in which the circulating concentration was found to be higher at the order of "ng/ml" compared with concentrations of other BMPs [10]. Recently, it was reported that BMP-9 is also associated with cancer cell proliferation, apoptosis and differentiation in the ovary, prostate, breast and thyroid tissues [11,12]. These findings indicate the possible involvement of BMP-9 in regulation of reproductive functions. However, the effects of BMP-9 on ovarian steroidogenesis and folliculogenesis have yet to be elucidated.

In the present study, we investigated regulatory mechanism of BMP-9 for steroidogenesis by rat primary granulosa cells. BMP-9 might be an endocrine regulator of progesterone and luteinization in the process of folliculogenesis.

2. Materials and methods

2.1. Reagents and supplies

Female Sprague-Dawley (SD) rats were purchased from Charles-River (Wilmington, MA). Medium 199, McCoy's 5A medium and HEPES buffer were purchased from Invitrogen Corp. (Carlsbad, CA). Diethylstilbestrol (DES), ovine pituitary FSH, 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin, and penicillin–streptomycin solution were from Sigma–Aldrich Co., Ltd. (St. Louis, MO). Recombinant human BMP-4, -9, noggin and extracellular domains (ECDs) that lack trans-membrane and intra-cellular domains of human ALK-1, -2, -3, -6, ActRIIA, ActRIIB and BMPRII [13,14] were purchased from R&D Systems, Inc. (Minneapolis, MN).

2.2. Primary culture of granulosa cells and oocytes

Silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats to increase the number of granulosa cells. After 4 days of DES exposure, ovarian follicles were punctured with a double-thickness needle of 27 gauge, and the isolated mixture of granulosa cells and oocytes was cultured in serum-free McCoy's 5A medium supplemented with penicillin–streptomycin at 37 °C in an atmosphere of 5% CO_2. Granulosa cell and oocyte numbers were counted in an oocyte/granulosa cell suspension that was filtered by cell strainers (100-μm nylon mesh; BD Falcon, Bedford, MA) to eliminate cell aggregation. For indicated experiments, granulosa cells were separated from oocytes by filtering the oocyte/granulosa cell suspension through an additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through as previously reported [15]. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

2.3. Measurements of estradiol, progesterone and cAMP

Rat granulosa cells (1×10^5 viable cells in 200 μl) with or without oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free McCoy's 5A medium containing 100 nM of androstenedione, a substrate for aromatase. Cells were cultured either alone or in combination with indicated concentrations of FSH and BMP-9 in the presence of ALK inhibitors and BMP-receptor ECDs for 48 h. The levels of estradiol and progesterone in the media were determined by a chemiluminescent immunoassay using Architect estradiol and progesterone kits (Abbott Co., Ltd., Tokyo, Japan). Steroid contents were undetectable (progesterone <0.1 ng/ml and estradiol <8 pg/ml) in cell-free medium. To assess cellular cAMP synthesis, rat granulosa cells (1×10^5 viable cells in 200 μl) without oocytes were cultured in 96-well plates with serum-free McCoy's 5A medium containing 0.1 mM of IBMX (a phosphodiesterase inhibitor) for 48 h. The extracellular contents of cAMP were determined by an enzyme immunoassay (Assay Designs, Ann Arbor, MI) after acetylation of each sample with assay sensitivity of 0.039 nM.

2.4. Cellular RNA extraction, RT and quantitative real-time PCR

Isolated granulosa cells and oocytes were cultured in 12-well plates in serum-free conditions. Rat granulosa cells (5×10^5 viable cells in 1 ml) without oocytes were cultured either alone or in combination with indicated concentrations of FSH and BMP-9 in serum-free McCoy's 5A medium. After 48-h culture, the medium was removed and total cellular RNA was extracted using TRIzol® (Invitrogen Corp.) and quantified by measuring the absorbance of the sample at 260 nm. Primer pairs for steroidogenic acute regulatory protein (StAR), P450 steroid side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), FSHR, BMPRII and ribosomal protein L19 (RPL19) were selected as we reported previously [16]. PCR primer pairs for other target genes were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants as follows: BMP-9, 310–330 and 581–602 (from GenBank accession #NM_001106096), and ALK-1, 1499–1519 and 1949–1972 (NM_022441). The extracted RNA (1 μg) was subjected to an RT reaction 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. For the quantification of mRNA levels of each target gene, real-time PCR was performed using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) under optimized annealing conditions at 60–62 °C. The relative expression of each mRNA was calculated by the Δ threshold cycle (Ct) method, in which ΔCt was 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 Ct value of RPL19 mRNA did not significantly change with treatment in any study, and the results were expressed as the ratio of target mRNA to RPL19 mRNA.

2.5. Western immunoblot analysis

Rat granulosa cells (5×10^5 viable cells in 1 ml) without oocytes were cultured in 12-well plates in serum-free McCoy's 5A medium. After preculture with noggin (30 ng/ml) for 24 h to exclude endogenous effects of BMPs, cells were treated with BMP-4 and BMP-9 for 60 min. Cells were solubilized by a sonicator 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.