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Regulatory role of kit ligand–c-kit interaction and oocyte factors in steroidogenesis by rat granulosa cells

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ABSTRACT

Although kit ligand (KL)–c-kit interaction is known to be critical for oogenesis and folliculogenesis, its role in ovarian steroidogenesis has yet to be elucidated. We studied the impact of KL–c-kit interaction in regulation of steroidogenesis using rat oocyte/granulosa cell co-culture. In the presence of oocytes, soluble KL suppressed FSH-induced estradiol production and aromatase mRNA expression without affecting FSH-induced progesterone production. The KL effect on steroidogenesis was interrupted by an anti-c-kit neutralizing antibody, suggesting that KL–c-kit interaction is involved in suppression of estrogen by granulosa cells through oocyte c-kit action. The cAMP-PKA pathway activity was not directly involved in the estrogen regulation by KL–c-kit action. It was of note that KL treatment increased the expression levels of oocyte-derived FGF-8, GDF-9 and BMP-6, while it reduced the expression levels of oocyte-derived BMP-15 in the oocyte-granulosa cell co-culture. Given the findings that FGF-8, but not GDF-9, BMP-6 or -15, suppressed FSH-induced estrogen production by granulosa cells, oocyte-derived FGF-8 is linked to suppression of FSH-induced estrogen production through the KL–c-kit interaction. Furthermore, the suppression of FSH-induced estrogen production by KL in the co-culture was reversed by a FGF receptor kinase inhibitor and the effect of the inhibitor was enhanced in combination with extracellular-domain protein of BMPRII, which interferes with BMP-15 and GDF-9 activities. Thus, the actions of endogenous oocyte factors including FGF-8 and BMP-15/GDF-9 were involved in the KL activity that inhibited FSH-induced estradiol production. Collectively, the results indicate that KL–c-kit interaction plays a role in estrogenic regulation through oocyte-granulosa cell communication.

1. Introduction

In the ovary, follicle growth and maturation are precisely regulated by interactions between pituitary gonadotropins and various autocrine/paracrine factors derived from follicular cells. Much attention has been paid to oocyte-derived growth factors, based on the concept of bidirectional communication between oocytes and surrounding somatic cells that is critical for normal follicular development (Eppig, 2001; Gilchrist et al., 2006).

In this process, local follicular factors, including several members of the transforming growth factor (TGF)-β superfamily such as bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins and inhibins, play key roles as autocrine/paracrine factors in female fertility in mammals by regulating steroidogenesis as well as mitogenesis in granulosa cells (Findlay et al., 2002; Shimasaki et al., 2003, 2004). The discovery that insufficiencies of two oocyte-specific growth factors, GDF-9 and BMP-15, cause female infertility due to defects of follicular development was a breakthrough in this field (Dong et al., 1996; Galloway et al., 2000). Expression of BMP system components, including ligands, receptors and intracellular signal transduction factors, in growing preantral follicles was revealed (Drummond et al., 2003; Erickson and Shimasaki, 2003), and bioassays have clarified many important roles of the BMP system in regulation of ovarian functions (Shimasaki et al., 2004; Otsuka and Inagaki, 2011a, Otsuka et al., 2011b). In addition, we recently reported roles of another oocyte-derived factor, FGF-8, in ovarian steroidogenesis, in which FGF-8 suppresses FSH-induced estrogen production and amplifies

Abbreviations: ActRII, activin type II receptor; ALK, activin receptor-like kinase; Arom, aromatase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; DES, diethylstilbestrol; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; FSK, forskolin; GDF, growth differentiation factor; IBMX, 3-isobutyl-1-methylxanthine; KL, kit ligand; PKA, protein kinase A; RPL19, ribosomal protein L19; StAR, steroidogenic acute regulatory protein.

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BMP signaling through oocyte-granulosa cell communication (Miyoshi et al., 2010).

Besides the communication mediated by oocyte growth factors, the physiological importance of granulosa-to-oocyte signaling was also recognized in naturally occurring mutations at the *W* (white spotting) and *Sl* (steel) loci in mice that resulted in developmental abnormalities in oogenesis and folliculogenesis leading to infertility (Driancourt et al., 2000; Hutt et al., 2006). Genetic approaches have revealed that the *W* locus encodes a member of the tyrosine kinase receptor family termed *c-kit* or *Kit* proto-oncogene. The *Sl* locus encodes a ligand for c-kit receptor termed Kit Ligand (KL; also called *Sl* factor, stem cell factor or mast cell growth factor). KL receptor, *c-kit* mRNA and c-kit protein are localized to oocytes at all stages of follicular development during postnatal ovarian development, while *KL* mRNA expression is localized to granulosa cells in the follicles (Driancourt et al., 2000). Thus, it is possible that KL action contributes to the communication from granulosa cells to oocytes via c-kit signaling (Hutt et al., 2006; Thomas and Vanderhyden, 2006).

The functional interaction of granulosa-derived KL and oocyte c-kit is indispensable for normal fertility. Previous studies have shown that injection of anti-c-kit antibodies into the ovaries of immature mice causes a disruption of granulosa proliferation in early antral follicles (Yoshida et al., 1997). Interestingly, BMP-15 and KL are concomitantly expressed in the early stages of follicular development and they appear to be involved in granulosa cell mitosis (Otsuka and Shimasaki, 2002) by forming a negative feedback loop between the oocyte and surrounding granulosa cells. The combination of increased KL expression and subsequent reduction of BMP-15 expression leads to effective and balanced induction of granulosa cell proliferation (Otsuka and Shimasaki, 2002).

Hence, oocytes play an organizing role in cell-to-cell communication by secreting soluble growth factors that act on neighboring follicular cells. It has been reported that oocyte-derived BMP-15 stimulates KL expression in rat granulosa cells, leading to stimulation of granulosa cell proliferation (Otsuka and Shimasaki, 2002), while GDF-9 suppresses KL expression in mouse granulosa cells (Joyce et al., 2000). Therefore, it is hypothesized that KL–c-kit interaction plays a regulatory role not only in the early process of mitogenesis but also in steroidogenesis by granulosa cells, which is further controlled by oocyte factors. However, the significance of KL–c-kit interaction in regulation of ovarian steroidogenesis has yet to be elucidated. In the present study, we investigated the biological significance of KL–c-kit interaction in steroidogenesis by granulosa cells with focus on the oocyte-granulosa communication network.

2. Materials and methods

2.1. Reagents and supplies

Female Sprague–Dawley (SD) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Medium 199, McCoy's 5A medium and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Diethylstilbestrol (DES), ovine pituitary FSH, forskolin (FSK), N^6,O^2-dibutyryl adenosine-3',5'-cyclic monophosphate monosodium salt (BtcAMP), 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-6, BMP-15 and GDF-9 and mouse FGF-8b were purchased from R&D Systems (Minneapolis, MN). The extracellular domain (ECD) that lacks transmembrane and intracellular domains of human BMPRII (BMPRII-ECD) (Moore et al., 2003; Inagaki et al., 2006; Takeda et al., 2012) was purchased from R&D Systems. Recombinant soluble KL (amino-terminal 165 amino acid-chain of mouse KL produced by *Escherichia coli*), anti-c-kit blocking antibody (IgG fraction of goat polyclonal antibody immunized with purified extracellular domain of human c-kit) (Otsuka and Shimasaki, 2002) and a control goat antibody were from R&D Systems. The FGF receptor kinase inhibitor SU5402 was purchased from Calbiochem (Gibbstown, NJ).

2.2. Primary culture of granulosa cells and co-culture with oocytes

Female 22-day-old SD rats were implanted with silastic capsules containing 10 mg of DES to increase granulosa cell number. After 4 days of DES exposure, the ovarian follicles were punctured with a 28-gauge needle, and isolated granulosa cells or a mixture of oocyte/granulosa cells were cultured in serum-free McCoy's 5A medium supplemented with penicillin–streptomycin at 37 °C in an atmosphere of 5% CO_2 as previously reported (Erickson, 1983). In indicated experiments, a mixture of oocyte/granulosa cells isolated from DES-untreated 25-day-old rat ovaries was cultured in a serum-free condition. Granulosa cell and oocyte numbers were counted in the 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 (Otsuka and Shimasaki, 2002). The purified granulosa cells were cultured in serum-free McCoy's 5A medium as described above. In the experiments that included oocytes in the cultures, oocytes were used at a ratio of 1:5000 (oocytes:granulosa cells), based on our previous observation of the numbers of oocytes and granulosa cells extracted from DES-treated immature rats. 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 P450 aromatase. FSH (30 ng/ml equivalent to 1.5 mIU/ml), FSK (10 μM) or BtcAMP (1 mM) was added to the culture medium either alone or in combination with indicated concentrations of KL, anti-c-kit or control antibodies, BMPs, GDF-9, FGF-8, SU5402 and BMPRII-ECD as we have previously reported (Miyoshi et al., 2007, 2010). After 48-h culture, the culture media were collected and stored at −80 °C until assay. The levels of estradiol and progesterone in the media were determined by a chemiluminescent immunoassay (CLIA) 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) with oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free McCoy's 5A medium containing 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 −80 °C until assay. The extracellular contents of cAMP were determined by an enzyme immunoassay (EIA; Assay Designs, Ann Arbor, MI) after acetylation of each sample with assay sensitivity of 0.039 nM. Intra- and inter-assay coefficients for progesterone, estradiol and cAMP measurements are 2.4% and 2.9%, 2.3% and 2.5%, and 6.8% and 7.9%, respectively.

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

Rat granulosa cells (5×10^5 viable cells in 1 ml) with oocytes (100 oocytes/ml) were cultured in 12-well plates with serum-free