—Mini Review—

The Key Signaling Cascades in Granulosa Cells during Follicular Development and Ovulation Process

Masayuki Shimada[1]* and Yasuhisa Yamashita[2]

[1]Laboratory of Reproductive Endocrinology, Graduate School of Biosphere Science, Hiroshima University, Hiroshima 739-8528, Japan
[2]Laboratory of Animal Physiology, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Hiroshima 727-0023, Japan

Abstract: *Follicular development and ovulation process are complex events in which a sequential process is started by two pituitary hormones, FSH and LH. Recent studies using microarray analysis have shown that numerous endocrine factors are expressed and specifically activate the signal transduction cascades to upregulate or downregulate the expression of target genes in granulosa cells and cumulus cells. Especially, the PI 3-kinase-AKT pathway during the follicular development stage and the ERK1/2 pathway after LH surge are essential for initiating the dramatic changes in follicular function. The proliferation of granulosa cells and cell survival are dependent on the FSH-induced PI 3-kinase-AKT pathway in a c-Src-EGFR dependent manner. On the other hand, the transient activation of ERK1/2 by LH surge is dependent on de-novo transcription and translation. EGF-like factors, especially amphiregulin, that are expressed in a cAMP-PKA-CREB dependent manner, act on EGFR expressed on granulosa cells and cumulus cells. The phosphorylated EGFR induces the RAS-cRAF-MEK-ERK1/2 pathway in both cell types. One of the ERK1/2 target molecules is C/EBP that is a member of transcription factors increasing the expression of genes involved in granulosa cell luteinization, cumulus expansion and oocyte maturation. These signaling cascades upregultaed by FSH or LH in granulosa cells play important roles in follicular development and ovulation process.*

Key words: *Signaling cascade, PI 3-kinase, ERK1/2, Follicular development, Ovulation*

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*To whom correspondence should be addressed.
e-mail: mashimad@hiroshima-u.ac.jp

Introduction

Follicle stimulating hormone (FSH) secreted from the pituitary gland acts on granulosa cells in follicles at the secondary and small antral stages to direct and ensure the development of preovulatory follicles [1]. In FSH-stimulated granulosa cells, *Ccnd2* mRNA is expressed and its translated product (Cyclin D2) binds to cyclin dependent kinase 4 (CDK4), inducing cell proliferation. FSH also increases estradiol 17β (E2) production that is converted from androgens via induction of the enzyme aromatase [1–3]. E2 acts on ESR2 (estrogen receptor beta; ERβ) that is expressed in granulosa cells, and enhances FSH-mediated granulosa cell proliferation and also differentiation (*Lhcgr* induction) [4–6]. Elevated serum E2 activates neuronal estrogen receptor alpha (ERS1) inducing GnRH synthesis/release, leading to the LH surge and the initiation of ovulation and luteinization [7–9]. *Esr2* [10] and *Esr1* mutant mice [11] are subfertile or infertile.

The surge of luteinizing hormone (LH) acts via its receptor on granulosa cells of preovulatory follicles to induce luteinization, cumulus cell-oocyte complex (COC) expansion, oocyte maturation and follicle rupture [1, 12]. During these dramatic changes, LH decreases *Cyp19* expression that encodes aromatase, but markedly induces the expression of genes (*Cyp11a1, Star*) regulating progesterone biosynthesis [1]. In progesterone receptor (*Pgr*) knockout (PRKO) mice, follicle rupture is completely suppressed [13, 14]. Prostaglandin E2 (PGE2) that is derived from arachidonic acid by the rate-limiting enzyme prostaglandin synthase 2 (PTGS2; also known as cyclooxygenase 2, COX-2) [15], is also increased by LH stimulation. In *Ptgs2* KO mice, or the PGE2 receptor

(EP2) null mice, ovulation is impaired, and oocytes resume meiosis and reach the metaphase II stage in both *in vitro* and *in vivo* [16, 17].

Recent genetic and molecular approaches have determined that FSH and LH activate multiple and specific intracellular signaling cascades that have an impact on follicular development and ovulation process [18–20]. Each cascade is transiently activated at a specific time, and some cascades act synergistically, while others act competitively as inducers of granulosa cell survival, proliferation, and differentiation of granulosa cells. Specifically, our recent studies indicate that the extra cellular regulated kinases 1/2 (ERK1/2; also known as MAPK3/1) are essential mediators by which LH dictates the dramatic changes in follicular cell fate during ovulation and luteinization [21]. The PI3K/PKB(AKT)/(FOXO) pathway is related to the survival of granulosa cells in preovulatory follicles [22–25]. Although the activation and roles of these signaling cascades have been clarified in experimental animals, especially mice over the past decade, there are few reports on their roles in human and domestic animals. Such information would contribute to our understanding of how to control ovarian stimulation in infertility care of humans and in the field of reproductive technology of domestic animals. This mini-review focuses on the signaling cascades in granulosa cells during follicular development and ovulation process in mice.

PI 3-kinase/PKB (AKT)/FOXO Pathway in the Follicular Development Stage

Gonzalez-Robayna *et al*. [22] showed that in granulosa cells FSH increases protein kinase B (PKB, also known as AKT) phosphorylation and activation in a way that is cAMP-dependent and phosphatidylinositol 3-kinase (PI 3-kinase)-dependent. It is known that cAMP binds to the regulatory subunit of protein kinase A (PKA) to release and activate the catalytic subunit of PKA in granulosa cells. However, cAMP also activates two small GTP binding proteins, RAP1 and RAS in FSH-stimulated granulosa cells. In the neuronal cells, EPAC activates RAP1 independently of PKA, and EPAC-mediated RAP1 activation leads to PKB phosphorylation [26]. Because EPAC1/2 is expressed in granulosa cells, Wayne *et al*. [27] investigated the different effects of specific cAMP analog on the activation of PKA, RAP1 and EPAC. Their results show that the PKA pathway does not induce the activation of the PI 3-kinase-AKT pathway, that EPAC1/2 mediates the phosphrorylation of AKT in cultured granulosa cells, and that FSH stimulation increases the activation level of RAS, small GTP binding protein, in granulosa cells during follicular development stage [27]. RAS regulates multiple signaling cascades, such as the c-RAF-MEK [MAP or extracellular regulated protein kinase (ERK)]-ERK1/2 pathway, and the c-Src tyrosine kinase pathway [24]. The ERK1/2 pathway is not fully activated in granulosa cells during the follicular development stage but is induced during the ovulation process in the following section. It is possible that FSH-activated PKA suppresses c-RAF to prevent ERK1/2 activation because the pathway plays important roles in both the suppression of cell proliferation and the induction of the luteinization of granulosa cells. Thus, in FSH-stimulated granulosa cells, c-Src is the prime target of the RAS signaling pathway, and the activated form of c-Src (phosphohrylated c-Src) is detected in developing follicles [27]. One of the substrates of c-Src is EGF receptor (EGFR), and the phosphorylation of EGFR is observed in FSH-stimulated granulosa cells in a ligand of EGFR independent manner. When granulosa cells were cultured with FSH, the phosphorylation of c-Src and EGFR was induced [27]. However, treatment with c-Src inhibitor, PP2 suppressed the FSH-function, whereas *de-novo* transcription inhibitor (α-amanitin) or protein synthesis inhibitor (cycloheximide) did not [27], suggesting that the phosphorylation of EGFR is independent of the synthesis of its specific ligands. The phosphorylation of EGFR by c-Src induces self-activation of tyrosine kinase, and then induces the binding of the regulatory subunit of PI 3-kinase. PI 3-kinase is a heterodimer of its regulatory subunit and catalytic subunit that phosphorylates lipid, phosphatidylinositol di-phosphates (PIP2) to phosphatidylinositol tri-phosphates (PIP3). PIP3 binds to the PH domain of PDK1, an upstream factor of AKT.

Recent studies have demonstrated that the downstream signaling of PI 3-kinase/AKT induces the expression of genes involved in granulosa cell differentiation [28]. In a proliferating model of granulosa cells cultured with FSH, PI 3-kinase/AKT targets forkhead box O, subfamily 1 (FOXO1) [23, 24]. The phosphorylated form of FOXO1 translocates from the nucleus to the cytoplasm and is then degradated. Since FOXO1 suppresses *Ccnd2,* steroidogenic factor-1 (*Nr5a1*), inhibin-α (*Inha*) and aromatase cytochrome P-450 (*Cyp19a1*) expression [23], the PI3-kinase-AKT pathway is required for the induction of both granulosa cell proliferation and differentiation. Liu *et al*. [30] also showed the target genes of FOXO1 from the microarray analysis of granulosa cells infected with a constitutive

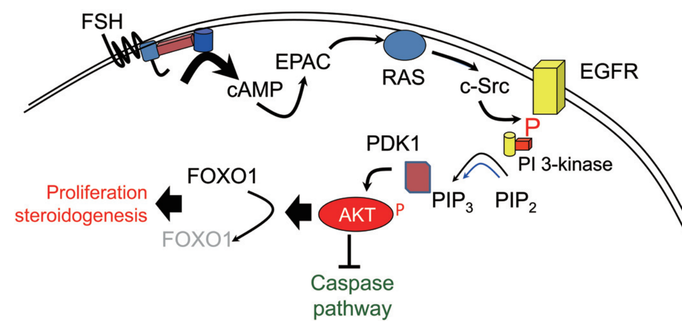

Fig. 1. The PI 3-kinase-AKT signaling pathway in FSH-stimulated granulosa cells.

active form of FOXO1. This data provides evidence that FOXO1 plays a key role in granulosa cells modulating lipid and sterol biosynthesis. Therefore, the PI 3-kinase-AKT activated by FSH stimulation in granulosa cells is required for cell proliferation and differentiation during the follicular development stage via the phosphorylation and degradation of FOXO1 dependent mechanisms (Fig. 1).

The PI 3-kinase-AKT pathway is well known as a cell survival factor in numerous kinds of cell types. Wang *et al*. [31] reported that the PI 3-kinase/AKT pathway activated the X-linked inhibitor of apoptosis (XIAP) expression, suppressing the induction of apoptosis in rat granulosa cells. The inhibitor of apoptosis (IAP) family includes X-linked IAP (XIAP or cIAP-3), human IAP-1 (HIAP-1 or cIAP-2), human IAP-2 (HIAP-2 or cIAP-1), neuronal apoptosis inhibitory protein, survivin, and livin [32-34]. Although the subcellular action of these anti-apoptotic proteins is not clear in all molecules, XIAP, HIAP-1, and HIAP-2 have been shown to be direct inhibitors of caspase-3 and caspase-7 [35, 36]. In the ovary, XIAP is upregulated by gonadotropin and it is necessary for follicular development in both *in vivo* and *in vitro* [31, 37]. The induction of XIAP in granulosa cells is dependent on NFκB activation and translocation to the cell nucleus. This process is mediated through the PI 3-kinase/AKT pathway and is IκB phosphorylation and degradation independent [31].

ERK1/2 Pathway in Granulosa Cells during the Ovulation Process

During the ovulation process, multiple signaling pathways are activated in granulosa cells and cumulus cells. Among them, the ERK1/2 pathway is a key signaling cascade in the change of the follicular development stage to the ovulation stage via the suppression of granulosa cell proliferation, the induction of final differentiation (luteinization) of granulosa cells and cumulus cell expansion. ERK1/2 is activated by the RAS-cRAF-MEK1 pathway in both cell types after LH surge [21, 38]. When COCs were cultured with the MEK1 inhibitor (PD98059 or U0126), cumulus expansion was blocked dramatically [39–41]. In granulosa cells, MEK inhibitors suppress the induction of *Cyp11a1* and *Star* expression [42], suggesting that the ERK1/2 pathway is required for luteinization of granulosa cells. To clarify the functional roles of ERK1/2 in more detail, Fan *et al*. [21] generated granulosa cell- and cumulus cell-specific ERK1/2 mutant mice using the Cre/LoxP technique. In ERK1/2 mutant mice in which both kinases are depleted in granulosa cells and cumulus cells, not only cumulus cell expansion and granulosa cell luteinization but also oocyte meiotic resumption are completely suppressed [21], suggesting that the ERK1/2 pathway in granulosa cells and cumulus cells works as a rate-limiting factor on the ovulation process.

In mice, the EGFR-RAS pathway is a key signaling pathway in the induction of the phosphorylation of ERK1/2 in cumulus cells and granulosa cells [38, 43, 44]. EGFR (ErbB1) is one member of the EGF receptor super family that is expressed in granulosa cells and cumulus cells but not in oocytes, however the receptor signaling pathway is known to impact oocyte maturation in LH-stimulated preovulatory follicle cultures [45, 46]. As ligands for EGFR, the EGF-like factors, amphiregulin (*Areg*), betacellulin (*Btc*) and epiregulin (*Ereg*) are transiently expressed after LH stimulation in granulosa cells and act on both granulosa cells and cumulus cells [43, 45, 47]. Especially, *Areg* is rapidly and dominantly expressed within 1 hr of LH surge and the expression is maintained up to 4 hr after LH stimulation [43, 48]. In mouse *Areg* gene promoter region, a putative cAMP responsible element (CRE) site is observed [48, 49]. The mutation of this region decreases the promoter activity in

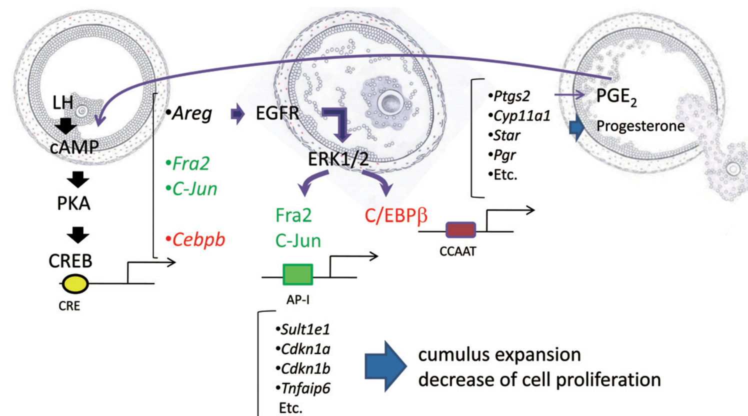

Fig. 2. ERK1/2 mediates a key signaling cascade to induce successful ovulation.

the luciferase promoter assay using primary culture of mouse granulosa cells, and CRE sequence binds to phosphorylated CREB (CRE binding protein) after LH stimulation [48]. Thus, the expression of *Areg* is directly regulated by the cAMP-PKA-CREB cascade in granulosa cells and cumulus cells during the ovulation process (Fig. 2). However, since the expression level of *Lhcgr* is quickly decreased after LH stimulation in granulosa cells and the expression level is very low level in cumulus cells [1], an other factor is required to maintain the level of cAMP in granulosa cells or increase the level in cumulus cells. G protein–coupled receptor subtypes EP2 (PTGER2) or EP4 (PTGER4) are activated and produce cAMP in both granulosa cells and cumulus cells during ovulation process [50]. EP2 and EP4 are receptors of prostaglandin E2 (PGE2) that is converted from arachidonic acid by the rate-limiting enzyme PTGS2 [15, 51, 52]. We documented that *Areg* and *Ereg* expression levels are markedly reduced in COCs and granulosa cells of *Ptgs2* null mice [43]. Thus, the initial induction of EGF-like factor expression is directly initiated by LH via the cAMP-PKA-CREB pathway, and the expression is maintained in a PGE2 production-dependent manner in order to elicit the maximum activation of ERK1/2 signaling cascade (Fig. 2).

In cultured granulosa cells, the phosphorylation of ERK1/2 is observed within 5 min of AREG stimulation, however the phosphorylation is transient and is not detected after 30 min [43, 53]. Moreover, the expression of ERK1/2 target genes, such as *Tnfaip6,* is significantly lower in both granulosa cells and cumulus cells cultured with AREG than in follicles cultured with LH [53]. Comparative *in vivo* and *in vitro* studies indicate that LH not only induces the expression of known EGF-like factors but also regulates other growth factor-receptor system(s) for successful ovulation, COC expansion and the resumption of meiosis. In fact, not only ERBB1 but also ERBB2 and ERBB3 are phosphorylated in granulosa cells during the ovulation process, suggesting that these receptors are activated by a specific ligand(s) [53]. ERBB2 has no ligand binding site whereas ERBB3 has a ligand binding site but lacks receptor tyrosine kinase activity, and therefore must heterodimerize mainly with ERBB2, to form an active receptor complex [54, 55]. Of the known members of the ERBB3 ligand family, we detected strong induction of *Nrg1* mRNA in granulosa cells but not in cumulus cells within 2–4 hr of hCG injection [53]. The *Nrg1* promoter region has three putative CCAAT enhancing binding protein (C/EBP) binding sites and a CRE site, and the most distal C/EBP binding site appears to play a critical role in the increase of promoter activity in granulosa cells. Since the C/EBP family is phosphorylated by ERK1/2 leading to increase transcriptional activity, *Nrg1* expression is dependent on ERK1/2. Interestingly, when granulosa cells or COCs were co-cultured with NRG1 and AREG, the phosphorylation of ERK1/2 was enhanced whereas NRG1 alone did not induce the phosphorylation. Thus, NRG1 is a positive feedback factor for extending activation of ERK1/2 phosphorylation, which is potentially required to induce the differentiation of both granulosa cells and cumulus cells, and oocyte maturation with a high developmental competence.

The activation of ERK1/2 can regulate numerous genes via the phosphorylation and activation of key transcription factors, such as members of the AP-1 family (Fra2 and C-Jun) that are present in granulosa

cells [56, 57]. The promoter of the *Tnfaip6* gene has an AP-1 site and Fra2 and JunD, present in nuclear extracts purified from bovine granulosa cells, bind to the AP-1 site of the *Tnfaip6* promoter region [58]. Other ERK1/2 target transcription factors are C/EBPα/β that are expressed during the ovulation process [21]. The conditional knockout mice of *Cebpb* and/or *Cebpa* in granulosa cells show similar phenotypes of ERK1/2KO mice [59]. C/EBPα/β protein is increased rapidly by hCG in granulosa cells *in vivo*, and in cultured cell lines [21]. When undifferentiated granulosa cells were infected with an adenoviral vector encoding C/EBPβ, C/EBPβ was expressed and phosphorylated in response to AREG, which increased the expression of target genes (*Ptgs2*, *Tnfaip6*, *Pgr*, and *Star*) [21]. Thus, C/EBPα/β is a downstream effector of ERK1/2 in granulosa cells during ovulation and luteinization (Fig. 2).

Conclusion

It is well known that both FSH and LH increase the level of cAMP in granulosa cells and cumulus cells to upregulate PKA dependent mechanisms [18, 30, 55]. However, using the same downstream pathway of LH and FSH receptors, the cell responses are completely different. Recent studies have shown that a specific signaling cascade is activated at selected times. One of the key factors during follicular development stage is the PI 3-kinase-AKT pathway that induces cell proliferation and prevents apoptosis. However, if the pathway is not activated during this stage, the gene expression patterns change, resulting in low numbers of granulosa cells expressing low levels of LH receptor [30]. During the ovulation process, the cAMP-PKA pathway increases the expression level of EGF-like factors that impact on the ERK1/2 signaling cascade [41, 48, 55]. ERK1/2 regulates the target transcription factors to increase the expression of genes involved in luteinization and cumulus expansion, or decrease the expression of genes that are required for follicular development [21, 40, 43, 45, 46]. The basic information about granulosa cells and cumulus cells at the molecular level is beneficial for our understanding of ovarian stimulation and for *in vitro* culture techniques in human infertility care and in the reproductive technology of animals.

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