—Mini Review—

**Cumulus Cells are an Essential Mediator of Ovulation Stimuli from Granulosa Cells to Oocyte**

Masayuki Shimada¹* and JoAnne S. Richards²

¹Laboratory of Reproductive Endocrinology, Graduate School of Biosphere Science, Hiroshima University, Hiroshima 739-8528, Japan
²Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, 77030, USA

**Abstract:** In preovulatory follicles, oocytes are surrounded by numerous layers of cumulus cells in a known as the cumulus cell-oocyte complex (COC). After stimulation of ovulation by the LH surge, the morphology of COC is dramatically changed as a hyaluronan rich matrix accumulates within cumulus cell layer, and the oocyte resumes meiosis by progressing to the metaphase II. Although both changes induced by LH surge are essential for successful fertilization in vivo, the expression of LH receptors is not detected in the oocyte and is minimal (negligible) in cumulus cells compared with granulosa cells. However, cumulus cells express members of the EGF receptor family (ErbB family), prostaglandin receptors (EP2 and EP4) and cytokine family receptors that respond to specific ligands secreted by granulosa cells during the ovulation process. By these intermediary steps, the cumulus cells mediate LH signaling from granulosa cells to induce oocyte maturation. This minireview focuses on the role of cumulus cells in oocyte maturation at the physiological and molecular levels.

**Key words:** EGF like factor, Progesterone, PGE2, ERK1/2, PI 3-kinase

**Introduction**

Mammalian oocytes arrested at the diplotene stage of the first meiotic prophase are closely surrounded by somatic cells (designated pre-granulosa cells in primordial follicles and cumulus cells in growing follicles). During early stages of follicle growth, granulosa cells and cumulus cells transport energy sources and other factors into oocytes via numerous gap junctions, promoting oocyte growth to full development size [1–3]. After the preovulatory LH surge, the oocytes resume meiotic maturation, complete germinal vesicle breakdown (GVBD), and progress to metaphase II (MII) before ovulation. Because LH receptors (LHCR) are not present on the surface of oocytes and because the expression in cumulus cells is minimal compared with that in granulosa cells [4], the LH surge dramatically and directly changes the pattern of gene expression and protein synthesis in granulosa cells [5]. Factors induced and secreted rapidly from granulosa cells then act on cumulus cells to induce hyaluronan synthesis and its accumulation within cumulus cell layers during the ovulation process [6]. Concomitantly with these differentiation-dependent changes in cumulus cell function, meiotic nuclear maturation and cytoplasmic maturation are also induced, thereby supporting the formation of the pronucleus and development of the early embryo [7]. These data suggest that cumulus cells play a critical role in oocyte maturation, however there is less information about the mechanisms by which cumulus cells differentiate and how these changes in cumulus cells impact oocyte maturation at the molecular level. The specific gene expression profiles, protein synthesis patterns and other modifications in cumulus cells of COCs during the ovulation process are the focus of this review.

**The Function of EGF Like Factor on Cumulus Cells**

Because cumulus cells of mouse and rat possess few...
if any LH receptors, the molecular and biochemical mechanisms by which LH impacts COC expansion have remained unclear. However, earlier studies have indicated that factors other than LH can induce cumulus expansion and oocyte maturation in culture, including growth factors [8].

A possible role of EGF or EGF-like factors in the ovulation process was strengthened by recent observations showing that LH induces the expression of mRNAs encoding the EGF-like factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in mouse and rat preovulatory follicles [9, 10]. Furthermore, an EGF receptor (EGFR) tyrosine kinase inhibitor (AG1478) blocked LH-mediated COC expansion and oocyte maturation in explanted whole follicles in culture [9–11]. Additionally, mutant mice null for both Areg and homozygous for Egfr wa2 (Areg−/−Egfrwa2/wa2), exhibited significant reduction of EGFR phosphorylation in cumulus cells, impaired expansion of cumulus oocyte complexes and arrest of meiosis at the germinal vesicle (GV) stage in oocytes [12]. Thus, LH induction of EGF-like factors in granulosa cells plays a critical role in oocyte maturation via an EGFR-dependent mechanism operative in cumulus cells (Fig. 1).

The expression of EGF-like factors is induced rapidly (within 1 hr) by the LH surge, and reaches a maximum level at 2 hr post-LH treatment [9]. Factors downstream of EGF receptor activation are also transiently activated in both granulosa cells and cumulus cells within 2 hr after LH [13, 14], however the expression level of genes involved in cumulus expansion, such as Has 2, Tnfai6 or Pttx3 are also increased within 2 hr and are maintained at high levels for at least 4 hr after the LH surge [15], suggesting that the cumulus cell expansion is achieved by additional factors. In our microarray database derived from analyses of gene expression profiles in cultured granulosa cells, AREG upregulates Ptgs2 and Il6 mRNA expression (Shimada et al., unpublished data). Ptgs2 encodes prostaglandin synthase 2, the rate-limiting enzyme in the synthesis of PGE2 [16, 17]. When mouse COCs are cultured with PGE2, cumulus expansion is observed [18]. In mice null for the prostaglandin E receptor 2 (Ptger2) or Ptgs2, the expression levels of Tnfai6 mRNA and protein in cumulus cells are significantly reduced compared to wild type mice [18] and the degree of cumulus expansion is impaired in both mutant mouse models [19, 20]. Thus, PGE2 is a secondary mediator of LH-EGF-like factor induction of cumulus expansion during the ovulation process (Fig. 1).

Recently, we have shown that the cytokine family, including IL-6, is expressed in granulosa cells and secreted by the Ca^2+–regulated exocytosis system [21]. The exocytosis process is dependent on the binding of...
SNAP 25 and synaptotagmin 1 (SYT1) that are also induced in granulosa cells during ovulation process [22]. The secreted IL-6 can activate the co-receptors, IL6R and GP130, that are expressed on cumulus cells and thereby induce cumulus expansion, and oocyte cytoplasmic maturation [23]. Because AREG induces Il6, Snap25 and Syt 1 gene expression in cultured granulosa cells, the cytokine pathway might be required for the progression of cumulus expansion and oocyte maturation.

Signal Transduction in Cumulus Cells during Ovulation Process

During the ovulation process, multiple signaling pathways are activated in cumulus cells and granulosa cells. The activated EGFR, in turn, activates the RAS-cRAF-MEK1-ERK1/2 pathway in cumulus cells [14, 24]. When COCs are cultured with the MEK1 inhibitor (PD98059 or U0126), cumulus expansion is blocked dramatically [25, 26]. In ERK1/2 mutant mice in which both kinases are depleted in granulosa cells and cumulus cells, not only cumulus cell expansion but also oocyte meiotic resumption are completely suppressed [14], suggesting that the ERK1/2 pathway in cumulus cells plays an important role in oocyte maturation. 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 [27, 28]. The promoter of the Tnfaip6 gene has an AP-1 site, and Fra2 and JunD present in nuclear extracts purified from bovine granulosa cells and bind the AP-1 site of Tnfaip6 promoter region [29]. Other ERK1/2 target transcription factors are the CCAAT enhancing binding proteins (C/EBPα/β) [30, 31] that are expressed during ovulation and C/EBPβ can mediate induction of selected genes [14]. In C/EBPβ null mice, Ptgs2 expression level was significantly lower than that in wild type mice [14, 32]. In Ptgs2 KO mice, Tnfaip6 expression was significantly decreased [18], suggesting that ERK1/2 regulates Tnfaip6 gene expression by both direct and indirect mechanisms via PGE2 production to induce cumulus cell expansion.

The cAMP-PKA pathway is also essential for the induction of ovulation and oocyte maturation. A cAMP analog that activated PKA strongly induced COC expansion [33], whereas a PKA inhibitor suppressed the cumulus cell differentiation and oocyte maturation in in vitro culture [34, 35]. In granulosa cells, the LH receptor (LHCGR) directly induced cAMP production via G protein α subunit type S (GαS)-induced adenylcyclase activation [36], whereas in cumulus cells the expression level of the receptor is much lower or even absent [4]. However, in cumulus cells, PGE2 rather than LH increases the level of cAMP [18, 37]. cAMP binds to the regulatory subunit of PKA and releases the catalytic (C) subunit [38], thereby allowing the free catalytically active C subunit to phosphorylate targeted proteins [39]. One such target is the cAMP response element binding protein (CREB). When CREB is phosphorylated by PKA, it binds to CRE sites of the promoter regions of specific genes [40] including the LH targets Areg, Ptgs2 and Tnfaip6 [41–43]. However, of these genes, only Areg and Ereg are induced by LH in ERK1/2 null mice indicating that ERK1/2 transcription factor targets are most potent in the control of Ptgs2 and Tnfaip6 [14].

The Role of Cumulus Cells in Oocyte Maturation

Although the ERK1/2 pathway in oocytes is not essential for the resumption of meiosis, the MEK inhibitor significantly suppresses this process [25, 46] presumably by blocking the activity or ERK1/2 in cumulus cell and granulosa cells. Moreover, EGF like factors can induce oocytes to resume meiosis and reach metaphase II stage, indicating that the activated
EGF receptor pathway in cumulus cells is required for the resumption of meiosis [9, 47]. Additionally, in Ptgs2 knockout mice, in which the expression of EGF like factors is reduced [10], oocyte meiotic resumption and progression to the MII stage are delayed as compared with those in wild type mice [37]. Collectively, these results indicate that the EGF like factor/EGFR/ERK1/2 pathway is critical for oocyte maturation. This conception was supported recently by the total block of oocyte maturation in mutant mice lacking ERK1/2 in somatic cumulus/granulosa cells but not in the oocyte [14].

When cumulus cells are stripped from the oocytes and then the denuded oocytes are cultured, oocyte meiosis progresses to the MII stage. However, the matured oocytes exhibit less fertilization and developmental competence than those in matured in intact COCs [7]. The data indicate that cumulus cells provide specific support for oocytes to acquire competence (cytoplasmic maturation). IL-6 may be one, but not the only factor needed to enhance cytoplasmic maturation [23].

In amphibians, sea urchins and fish, the follicle cell-secreted factors that directly act on oocyte to induce oocyte maturation have already been defined; however, the precise factors controlling mammalian oocyte maturation remain to be defined clearly. Bayskov et al. [48] reported that meiosis-activating sterols (FF-MAS) can induce meiotic resumption of mouse oocytes. FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol) was first purified from human follicular fluid and has been shown to be synthesized by follicular cells in response to LH (hCG) stimulation [49, 50]. This type of sterol is an intermediate in the cholesterol biosynthetic pathway where it is produced by 14 alpha-demethylase and is metabolized by delta 14-reductase [51, 52]. Because de-novo cholesterol synthesis and enzymes of the cholesterol biosynthetic pathway are induced and activated in granulosa cells and cumulus cells to produce progesterone [53–55], and because a receptor for FF-MAS has not been defined in the oocyte [56], the physiological function(s) of FF-MAS remain unclear. It has been reported that Leydig insulin-like 3 (INSL3) is expressed in theca cells after the LH surge and acts on LGR8, a G protein coupling receptor expressed in oocytes [57]. LGR8 activates Gxi that in turn can activate the PI 3-kinase pathway that is presumed to be involved in cAMP degradation via phosphodiesterase type III [57–59]. Thus, INSL3 can induce meiotic resumption of oocytes. However, the physiological significance of this is unclear because the meiotic resumption of oocytes is completely suppressed in mutant mice lacking ERK1/2 in granulosa/cumulus cells but not theca cells. Although in this context, INSL3 does not appear to be the necessary and sufficient factor, INSL3 expression in theca cells of ERK1/2 mutant granulosa cells and cumulus cells remains to be determined.

The Positive Effects of Cumulus Cell Expansion on Fertilization

Our microarray analyses revealed that pattern recognition receptors (PRRs) of the Toll-like receptor (TLR) family and related molecules are expressed in cumulus cells of ovulated COCs and exhibit functions similar to those in macrophages [60, 61]. Specifically, we showed that when COCs were cultured with the TLR4 ligand bacterial lipopolysaccharide (LPS), the expression of Il-6, Tnf alpha and Ptgs2 is induced [60]. However, the endogenous ligand for the TLR family and its physiological role in ovulated COCs is not known. Recently, Jiang et al. [62] reported that hyaluronan fragments, but not the high molecular weight hyaluronan polymers, can activate TLR2 and TLR4. Because cumulus cells produce a hyaluronan rich matrix during the ovulation process, and the matrix is broken down by sperm-secreted hyaluronidase during fertilization [6], this process might regulate TLRs in cumulus cells. In fact, high molecular weight hyaluronan did not stimulate cumulus cell TLR2 and TLR4, however, small hyaluronan fragments activated both receptors and increased mRNAs encoding specific cytokines/chemokines, Il6, Mip2 and Ccl5, ~7-fold, ~10-fold and ~15-fold, respectively [63]. Anti-TLR2 and anti-TLR4 monoclonal antibodies significantly suppressed hyaluronan fragment- and hyaluronidase-induced expression of these genes in cumulus cells of ovulated COCs [63]. Thus, TLR2/TLR4 on cumulus cells of ovulated COCs are functional and activated by hyaluronan fragments. Furthermore, during the fertilization process, CCL-5, G-CSF, IL-1b, IL-6, IL-9, IL-12 IL-16, MCP-1, MIP-1b, and TNFα are secreted from cumulus cells of COCs, and anti-TLR2 and anti-TLR4 antibodies significantly suppress the secretion of CCL-5, G-CSF, MCP-1, MIP-1b, and IL-6. Concomitantly, fertilization rates decrease to 32 % from 75 % in oocytes without antibodies. Moreover, Ccr1, Ccr2, Ccr3 and Ccr5 mRNAs that encode CCL5, MCP-1, and MIP-1b receptors are expressed in spermatozoa (Fig. 2). Sperm motility was enhanced by CCL5 suggesting that the chemokines secreted from cumulus cells of ovulated
COCs can induce sperm capacitation and subsequent oocyte penetration by mechanisms dependent on TLR2/4 activation (Fig. 2).

**In Vitro Maturation (IVM) of COC**

During the ovulation process, cumulus cells were stimulated by several kinds of granulosa cells-secreted factors, such as the EGF like factor, PGE2 and cytokines as described above. Some of these factors are expressed dominantly in granulosa cells, others are only expressed in granulosa cells but not in cumulus cells [61, 64]. Thus, it is possible that the supplementation of these factors to the maturation medium of cultured COCs will enhance oocyte maturation in vitro. Indeed, when pig COCs are cultured with EGF, the developmental rate of fertilized oocytes to the blastocyst stage is significantly higher than that in oocytes matured without EGF [65]. However, the positive effect of EGF is only observed when the COCs are pre-cultured with FSH [66]. Generally, porcine COCs are collected from small antral follicles (3–5 mm in diameter) for use in IVM, and the cumulus cells of these small follicles do not express a sufficient level of EGF receptor to respond efficiently to EGF [65]. Rather, during follicular development, the responsiveness of cumulus cells to EGF is enhanced by FSH stimulation [65], because when porcine COCs are recovered from medium- or large-sized follicles (more than 5 mm in diameter), EGF alone induces cumulus expansion [66]. More interestingly, the ERK1/2-targeted transcriptional factor, C/EBPβ is only expressed during the ovulation process but not follicular development stage [14]. Thus, the cAMP signaling pathway may be required to increase expression of specific transcription factors in cumulus cells in order to allow FSH pre-cultured COCs to respond fully to EGF or other factors enhancing oocyte competence and blastocyst development.

**The Future of Human Infertility Care Derived from Basic Research of Cumulus Cell Functions**

The positive roles of cumulus cells in oocyte maturation in vitro have been well known for the past four decades. Recent microarray studies have identified novel activators secreted from granulosa cells that induce the differentiation of cumulus cells. Additionally, gene targeting technology has shown clearly which signaling pathways are required in cumulus cells for oocyte maturation. The new information base should provide new methods for
improved in vitro oocyte maturation. We have already reported a new system of porcine COC maturation [65] which we briefly describe below.

1. COCs are recovered from small antral follicles (3–5 mm in diameter).
2. The recovered COCs are pre-cultured with 2 ng/ml FSH and 100 ng/ml estradiol 17β for 10 hr to induce cell proliferation.
3. At 10 hr, 20 ng/ml of progesterone is added to the FSH- and estradiol-containing medium to suppress cell proliferation and induce Lhcgr mRNA expression.
4. After 20 hr of culture, COCs are moved to fresh medium with 1 µg/ml LH, 1 ng/ml EGF and 100 ng/ml progesterone for an additional 24 hr.

Using this modified culture system based on in vivo changes in hormones and growth factor production, the matured porcine COCs exhibit full expansion, the cumulus cells remain healthy (low number of apoptotic cells) and when oocytes obtained from these COCs are used for in vitro fertilization, developmental competence to the blastocyst stage is significantly improved as compared with the conventional FSH+LH culture system.

In human COC maturation, the basic information about the changes of hormonal condition within follicles and the expression of genes involved in cumulus cell differentiation is limited. However, modification of human IVM conditions is required to get matured oocytes that have high developmental competence. The porcine culture system contributes to the development of an improved human IVM system.

Basic information about cumulus cells is also beneficial for judging whether a matured oocyte has developmental competence or not. We have shown that the high expression of Sult1e1 in cumulus cells of matured human COCs collected from ovarian stimulation cycles can be used as a predictive measure of oocyte developmental competence to the blastocyst stage (Tabata et al., unpublished data), other groups have reported significant correlations between oocyte developmental competence and the gene expression levels of Gremlin, Pgr, and Cx43 in cumulus cells recovered from matured human oocytes [67–69]. However, the key factors transported or secreted by cumulus cells that control oocyte nuclear and cytoplasmic maturation remain unclear. Further study to clarify the unknown factors is required for in vitro maturation of oocytes.

Acknowledgement

Dr. Heng-Yu Fan, Dr. Zhilin Liu, Dr. Yasuhisa Yamashita, and Dr. Tetsuji Okazaki have provided outstanding assistance and good suggestions. This work was supported in part, by Grant-in-Aid for Scientific Research (No. 18688016, No. 21688019, No. 21028014, No. 2148032) from the Japan Society for the Promotion of Science (JSPS) (to M.S.), and National Institute of Health (NIH)-HD-16229 and HD-07495 (Project II, Specialized Cooperative Program in Reproductive Research, SCPRR) (to J.S.R).

References

12) Hsieh, M., Lee, D., Panigone, S., Horner, K., Chen, R.,


61) Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Shimada, M., Wayne, C.M., Ochsner, S.A., White, L. and Richards,


