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REVIEW ARTICLE

Roles of epidermal growth factor (EGF)-like factor in the ovulation process

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Abstract Luteinizing hormone (LH) surge stimulates preovulatory follicles to induce the ovulation process, including oocyte maturation, cumulus expansion, and granulosa cell luteinization. The matured oocytes surrounded by an expanded cumulus cell layer are released from follicles to the oviduct. However, LH receptors are dominantly expressed in granulosa cells, but less in cumulus cells and are not expressed in oocytes, indicating that the secondary factors expressed and secreted from LH-stimulated granulosa cells are required for the induction of the ovulation process. Prostaglandin and progesterone are well-known factors that are produced in granulosa cells and then stimulate in both granulosa and cumulus cells. The mutant mice of prostaglandin synthase (*Ptgs2*KO mice) or progesterone receptor (PRKO mice) revealed that the functions were essential to accomplish the ovulation process, but not to induce the ovulation process. To identify the factors initiating the transfer of the stimuli of LH surge from granulosa cells to cumulus cells, M. Conti's lab and our group performed microarray analysis of granulosa cells and identified the epidermal growth factor (EGF)-like factor, amphiregulin (AREG), epiregulin (EREG), and β-cellulin (BTC) that act on EGF receptor (EGFR) and then induce the ERK1/2 and Ca^{2+}-PLC pathways in cumulus cells. When each of the pathways was down-regulated using a pharmacological approach or gene targeting study, the induction of cumulus expansion and oocyte maturation were dramatically suppressed, indicating that both pathways are inducers of the ovulation process. However, an in vitro culture study also revealed that the EGFR-induced unphysiological activation of PKC in cumulus cells accelerated oocyte maturation with low cytostatic activity. Thus, the matured oocytes are not arrested at the metaphase II (MII) stage and then spontaneously form pronuclei. The expression of another type of EGF-like factor, neuregulin 1 (NRG1), that does not act on EGFR, but selectively binds to ErbB3 is observed in granulosa cells after the LH surge. NRG1 supports EGFR-induced ERK1/2 phosphorylation, but reduces PKC activity to physiological level in the cumulus cells, which delays the timing of meiotic maturation of oocytes to adjust the timing of ovulation. Thus, both types of EGF-like factor are rapidly induced by LH surge and then stimulate cumulus cells to control ERK1/2 and PKC pathways, which results in the release of matured oocytes with a fertilization competence.

Keywords Cumulus cells · Granulosa cells · Oocytes · Progesterone · Prostaglandin

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Introduction

Mammalian female germ cells form a cluster with their proliferation before birth [1, 2]; however, the number of germ cells dramatically decreases in mice due to the reduction of estrogen and progesterone levels [3–5]. These dramatic changes induce the meiosis of female germ cells, and the oocytes are arrested at the diplotene stage of the first meiotic prophase by the timing of ovulation [6, 7]. At this stage, the clusters of oocytes are dissolved to form primordial follicles so that one layer of somatic cells surrounds the oocyte [8–10]. The primordial follicles remain at this stage until the follicles initiate follicular growth to

form preovulatory follicles during each estrous cycle [11, 12]. During the early stages of follicle growth, follicular somatic cells are differentiated to granulosa cells and then are proliferated to develop the follicles to secondary follicles [13–15]. The granulosa cells of secondly follicles are stimulated by FSH secreted from the pituitary gland to proliferate further and form an antrum between the spaces of the granulosa cell layers (mural granulosa cells) interlining the follicular wall and granulosa cell-derived cumulus cells directly surrounding the oocyte [16–18]. The cumulus cells transport energy sources and other factors into oocytes via numerous gap junctions, thereby promoting oocyte growth to full development size [19–21]. The full grown oocytes have the ability to resume meiosis from prophase I to progress to the metaphase II (MII) stage when the oocytes are released from the follicles to in vitro culture medium or when the oocytes are ovulated with the stimulation of LH surge [22].

The size of oocytes reaches the maximum level at the middle size of antral follicles; however, the LH surge can not permit the initiation of oocyte maturation with a progressing ovulation process of middle-size antral follicles. LH is secreted from the pituitary gland in a similar manner to FSH, and the basal level of LH acts on theca cells of the follicular walls of the antral follicles [23–25]. The LH surge acts directly on the granulosa cells of preovulatory follicles, which are maximum-size antral follicles [26–28]. The ovulation stimuli determine the final differentiation of granulosa cells to form a corpus luteum, cumulus cells to form a hyaluronan-rich matrix and accumulate the matrix within a cumulus cell layer, called cumulus expansion, and oocytes to resume meiosis to the MII stage [29]. More importantly, although the functions and morphology of oocytes and cumulus cells are dramatically changed by the LH surge, both types of cells either don't express or express fewer LH receptors [30]. Thus, the factors induced and secreted rapidly from LH-stimulated granulosa cells act on cumulus cells and/or oocytes to induce cumulus expansion and oocyte maturation.

When cumulus oocyte complexes (COCs) were collected and cultured in vitro, the oocytes progressed to the MII stage with fertilization and developmental competence [31]. However, the denuded oocytes predicted low fertilization and developmental competence after maturation [32], suggesting that cumulus cells play a critical role in oocyte maturation. Therefore, the majority of secreted factors from LH-stimulated granulosa cells act on cumulus cells to induce cell differentiation for impacting not only cumulus expansion, but also the regulation of oocyte maturation. The secondary factors secreted from granulosa cells during the ovulation process to stimulate the cumulus cells of COCs are the focus of the current review.

Progesterone and prostaglandin E2 (PGE_2)

The LH surge stops cell proliferation of granulosa cells by decreasing *Ccnd2* expression [33, 34]. The molecular mechanism was reported by Richards et al. [35] to be involved in AP-1 transcription factors. These are regulated by c-jun or other members of mitogen-activated protein (MAP) kinase family, including ERK1/2 and then strongly induce marked functional (endocrine, biochemical, and molecular) changes in the preovulatory follicle [35]. Estrogen concentrations decline in follicular fluid as a consequence of the decreased expression of aromatase (*Cyp19a1*) in granulosa cells [36, 37]. Conversely, progesterone concentrations increase in the follicular fluid in association with the induction of P450scc (*Cyp11a1*) and StAR (*Star*) in granulosa cells [38–40]. These two genes are induced by the activation of cAMP response element binding protein (CREB), the CCAT enhancer binding protein (C/EBP) family, and AP-1 transcription factors in the cells during the ovulation process [41–43]. LH increases the cAMP level in granulosa cells, which activates PKA to phosphorylate CREB and E/PAC to activate the RAS-c-raf-MEK1–ERK1/2 pathway, which phosphorylate C/EBP family and the AP-1 transcription factor family [44–46]. The in vitro culture study of granulosa cells showed that the induction of cAMP by forskolin treatment rapidly activated the signaling pathway within a few minutes and expressed *Cyp11a1* and *Star* with an increasing level of progesterone in the medium [47, 48].

The roles of progesterone in ovulation have been reported by numerous researchers using animal pharmacological models, in vitro culture studies and the knockout (KO) mice model. Mori et al. [49] injected anti-progesterone antiserum with hCG to superovulation rats, and the study revealed that ovulation was inhibited by the treatment [49]. The progesterone synthesis inhibitors, aminoglutethimide or epistane, also decreased the number of ovulated oocytes in superovulation rats [50, 51], suggesting that the LH-induced increase in progesterone is essential for ovulation. Progesterone binds to its receptor, PGR, and the activated receptor moves from the cytoplasm to the nucleus to induce target gene expression [52, 53]. In an in vitro culture study of pig COCs or rat preovulatory follicles, the addition of PGR antagonist, RU486, dramatically suppressed cumulus expansion and oocyte maturation [54–57]. Female mice null for *Pgr* (PRKO) failed to ovulate, even in response to exogenous hormone [58, 59]. Thus, LH induction of progesterone-dependent pathways in granulosa cells and cumulus cells changes gene expression patterns in both cells to mediate critical events during the ovulation process.

Detailed analysis of PRKO mice revealed that follicular development to preovulatory follicles was normal, and the gene expression patterns in cumulus cells and granulosa cells at 2 h after hCG injection were also similar to those in the wild type (WT) mice [60]. Oocyte maturation, cumulus expansion, and corpus luteum formation of KO mice were comparable to those of WT mice; however, matured oocytes were observed in the corpus luteum, but not in the oviduct [58]. The phenotype indicates that although progesterone is a factor secreted from granulosa cells during the ovulation process, its functions appear later during the ovulation process.

One of the other well-known factors secreted from granulosa cells during the ovulation process is prostaglandin (PG). In LH-stimulated granulosa cells, prostaglandin synthase (PTGS)-2, also known as COX-2, is expressed to produce dominantly PGE_2 [61–63]. Cumulus cells and granulosa cells have PGE receptors (EP2 and EP4), which increase cAMP level to activate PKA and other signaling pathways similar to those by LH [64, 65]. Thus, PGE_2 works as a second stimulator of granulosa cells and cumulus cells at a few hours after the LH surge or as a supporter to maintain cAMP level in both cell types during a few periods after the LH surge. In *Ptgs2* null mice and *Pger2* null mice, the ovulation was significantly suppressed and cumulus expansion was not normal due to decreasing gene expressions of *Has2* encoding hyaluronan (HA) synthase 2 and *Tnfaip6* encoding HA-binding protein compared with those in the WT mice [66, 67]. Moreover, the fertilization in vivo by natural mating and in vitro when the matured oocytes were collected from the oviduct was completely suppressed in both KO mice [67]. However, the target gene expressions were decreased at 8 h, but not at 4 h after the injection of hCG [60, 68], indicating that PGE_2 also worked later during the ovulation process similar to progesterone but not as secondary factors to initiate the ovulation process.

Ligands for epidermal growth factor (EGF) receptor (EGFR)

Because cumulus cells of the mouse and rat possess few, if any, LH receptors [30], the molecular mechanisms by which LH impacts cumulus cell differentiation to induce cumulus expansion and oocyte maturation have remained unclear. However, earlier studies have indicated that factors other than LH, progesterone or PGE_2 can induce cumulus expansion and oocyte maturation in a culture, including growth factors. The addition of epidermal growth factor (EGF) to in vitro culture medium phosphorylates EGF receptor (EGFR) in cumulus cells, but not in oocytes to activate the down-stream pathway, such as ERK1/2 [69]. The EGF–EGFR-activated ERK1/2 induces cumulus expansion and oocyte maturation of pigs [70, 71], mice [72], and cattle COCs [73, 74]. Cumulus cells also express insulin-like growth factor (IGF) receptors and insulin receptors, and these receptors are activated by exogenous IGF-1 and insulin that impacts cumulus cell expansion and oocyte maturation of COCs in pigs, cattle, and mice [75–77]. However, the expression level of IGF-1 is not changed, and the expression of EGF is not induced in granulosa cells during the ovulation process [78, 79], indicating that other types of ligands potentially activating EGFR and/or IGFR are expressed in granulosa cells just after the LH surge.

To confirm the aforementioned hypothesis, Conti and colleagues [80] attempted to perform microarray analysis using RNA recovered from granulosa cells before or after ovulation stimuli. Their observations showed that LH induced the expression of mRNAs encoding the EGF-like factor, amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) in mouse preovulatory follicles [80]. EGF-like factor consists of 13 families that are transmembrane proteins containing a signal sequence, a transmembrane domain and at least one EGF domain [81] (Fig. 1a). Some of these EGF-like factor function as a ligand to stimulate neighbor cells without releasing the ligand domain from the membrane bound form. However, most are shed by specific protease to release EGF domain to activate specific receptors expressed on not only neighbor cells, but also on separated cells by a paracrine system [82, 83] (Fig. 1c). Granulosa cell-expressed EGF-like factor, AREG, EREG, and BTC have a cysteine-rich domain in the extracellular domain that is a targeted sequence of tumor necrosis factor α-converting enzyme (TACE) [83]. TACE, which is also known as ADAM17, a disintegrin and metalloprotease family member, is expressed in the granulosa cells of the rat and pig ovary during the ovulation process [71, 84, 85]. The level of expression is dramatically increased in the cells after hCG injection with the induction of EGF-like factor [71, 86]. When the expression of TACE/ADAM17 was down-regulated by specific siRNA, the expression level of EGF-like factor was normal, whereas the phosphorylations of EGFR and ERK1/2 were dramatically decreased [71], suggesting that the secretion from granulosa cells to follicular fluid impacts the functional changes of granulosa cells themselves and cumulus cells to induce a successful ovulation process.

The expression of EGF-like factor, particularly AREG and EREG is rapidly induced within 2 h after hCG injection in mice granulosa cells [80] (Fig. 1b). An in vitro culture of mouse granulosa cells revealed that the cAMP dependent-PKA pathway directly activated the promoter activity of both genes. In the promoter region of *Areg*, cAMP-responsible element (CRE) is essential for the induction of its gene [87] (Fig. 2a). Phosphorylated CRE-binding protein (CREB) by PKA is recruited to the

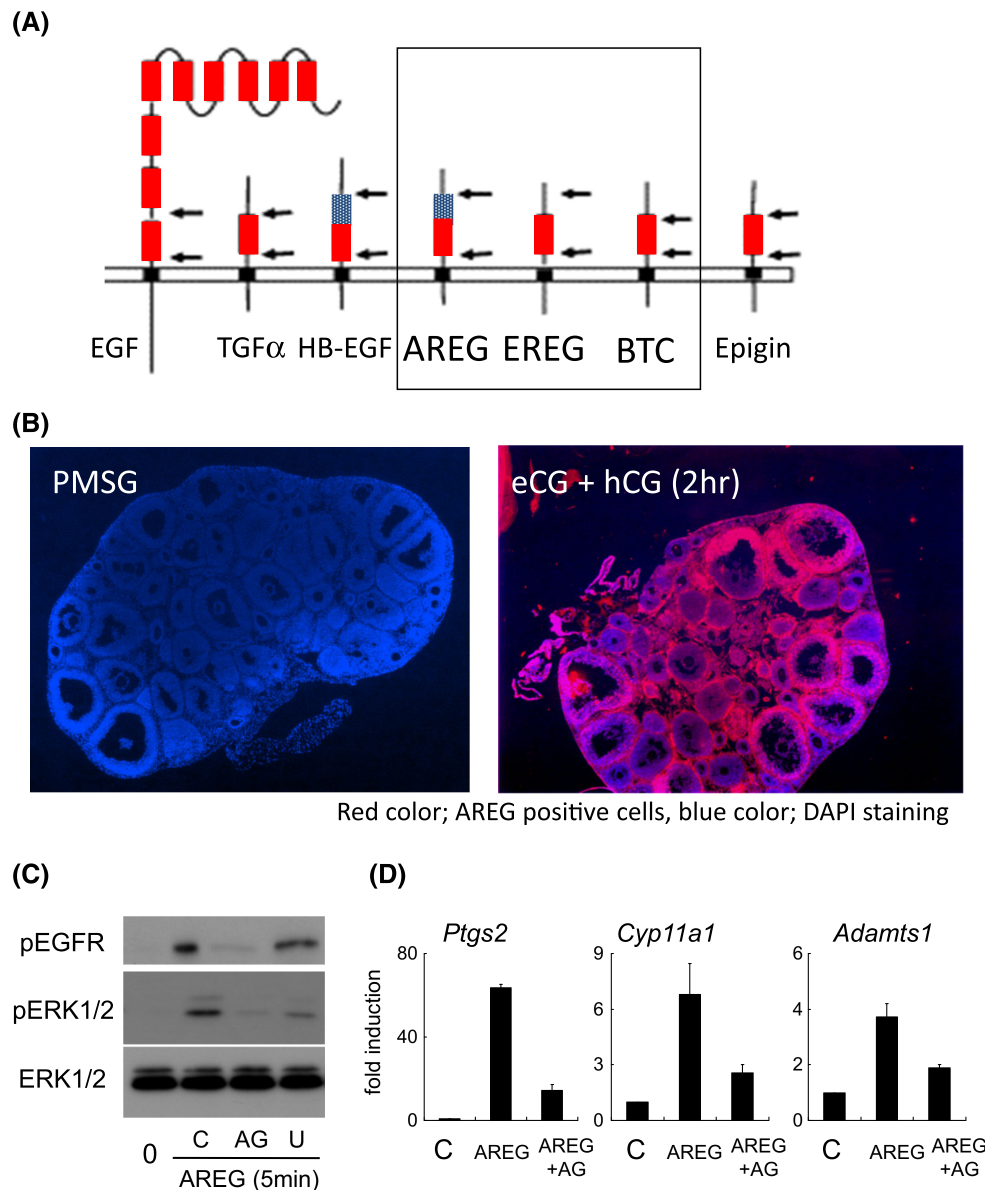

Fig. 1 The expression of EGF-like factor, AREG, EREG, BTC in granulosa cells during the ovulation process. **a** The typical members of EGF-like factor that contain a transmembrane domain, protease targeting sequences, heparin-binding domain and EGF domain, *red box* EGF domain, *blue box* heparin-binding domain, *black box* transmembrane domain, *black arrow* protease-targeting sequence. **b** The expression and localization of AREG in pre- or peri-ovulatory follicles before or after hCG injection in mice. The localization of AREG was detected by immunohistochemistry using anti-AREG antibody. AREG positive signals were visualized with Cy3 (*red color*) and nuclei were stained with DAPI (*blue color*). **c** The activation of EGFR and its downstream pathway by AREG in granulosa cells. Mouse granulosa cells recovered from eCG-stimulated mice were pre-cultured for overnight and then the cells were treated with AREG and either EGFR tyrosine kinase inhibitor (AG) or MEK1 inhibitor (U) for 5 min. C; Some of pre-cultured granulosa cells were further cultured with AREG alone for 5 min. 0; The granulosa cells were collected just after pre-cultivation. **d** The expression of genes well-known as ovulation markers in granulosa cells cultured with AREG. Mouse granulosa cells recovered from eCG-stimulated mice were pre-cultured for overnight and then the cells were treated with AREG alone (AREG) or AREG + EGFR tyrosine kinase inhibitor (AREG + AG) for 4 h. C; The granulosa cells were collected just after pre-cultivation

promoter region [88, 89], indicating that *Areg* is one of the rapid target genes of LH surge in granulosa cells. The promoter region of *Ereg* does not contain any CRE sites and is regulated by Sp1-binding sites [90]. The transcription factor Sp1 has numerous phosphorylated sites, some of which are phosphorylated by PKA to bind the cytosine- and guanine-rich repeats sequence, Sp1 site of the promoter region [91]. Thus, *Ereg* promoter in granulosa cells is also directly regulated by LH-induced signaling during the early phase ovulation process, estimating that AREG and EREG

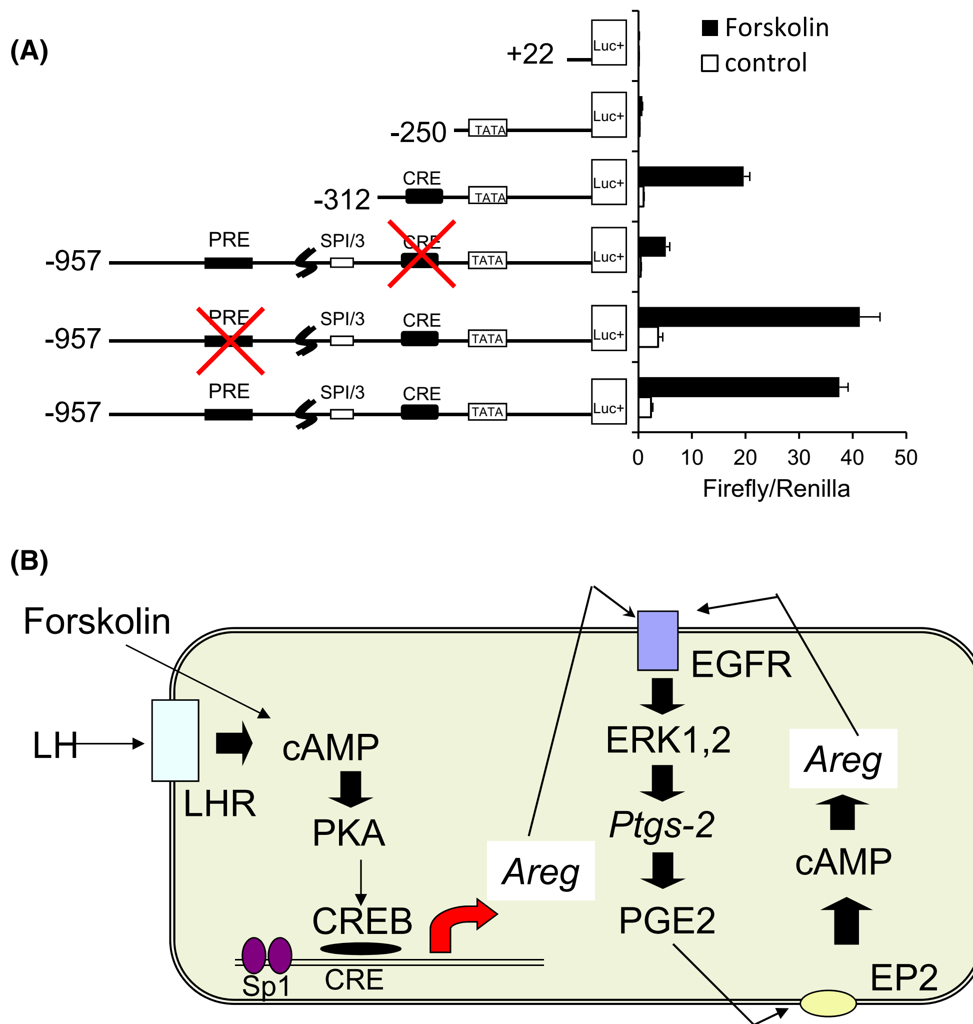

Fig. 2 The regulation of *Areg* expression in granulosa cells during the ovulation process. **a** The image of *Areg* promoter and the potential activity of *Areg* promoter in cultured granulosa cells. The putative *Areg* promoter has PRE, SP1/3 binding site, CRE and TATA box. Mouse granulosa cells were transiently transfected with the *Areg* promoter-luciferase constructs: −957, −312, −250, or +22 bp with or without mutations in PRE or CRE site, and stimulated with or without forskolin for 4 h. Firefly/Renilla; Firefly luciferase activities were normalized by *Renilla* luciferase activities. Values are mean ± SEM of three replicates. **b** A schematic diagram with regard to the induction and the maintenance of *Areg* expression in granulosa cells during the ovulation process. The induction of cAMP by LH or forskolin activates PKA–CREB pathway. The phosphorylated CREB moves to nuclei to bind CRE site of *Areg* promoter region. The induced AREG acts on its receptor, EGFR, which results in the activation of ERK1/2 pathway. Because one of the target genes of ERK1/2 pathway is *Ptgs2* encoding prostaglandin synthase 2, PGE_2 production is increased by AREG–EGFR dependent manner. PGE_2 acts on its receptor, EP2 to increase the level of cAMP, which induces *Areg* expression. Thus, the positive feedback loop in the expression of *Areg* is working to induce ovulation process

are secondary factors to transfer the signal of LH surge to cumulus cells to progress cumulus cell expansion and oocyte maturation.

Functions of EGF-like factor in the ovulation process

The addition of AREG or EREG to an in vitro culture medium of preovulatory follicles increased the expression levels of *Has2* and *Tnfaip6*, markers of cumulus expansion, and *Cyp11a1* and *Star*, markers of luteinization of granulosa cells [80, 92] (Fig. 1d). The gene expressions were also observed when COCs or granulosa cells were collected from preovulatory follicles and then cultured with AREG [60]. The inductions were rapidly induced within an hour by the addition of AREG, whereas forskolin or LH took more time to induce gene expression [60, 80, 93, 94]. Moreover, the inductions by the cAMP-producers were not detected by further treatment with EGFR tyrosine kinase inhibitor, AG1478 [60, 93]. In addition, mutant mice null for *Areg* and homozygous for *Egfr wa2* (*Areg*$^{-/-}$ *Egfr*$^{wa2/wa2}$) exhibited

significant decreases in cumulus expansion and oocyte maturation [95]. Thus, EGF-like factor secreted from granulosa cells are essential initiators to induce a successful ovulation process.

During the ovulation process, multiple signaling pathways are activated in cumulus cells and granulosa cells. EGF-like factor takes charge of the RAS-cRAF-MEK1–ERK1/2 pathway in cumulus cells and granulosa cells [94, 96]. When an EGFR tyrosine kinase inhibitor (AG1478) was added to a culture medium of preovulatory follicles, the induction of EGFR and ERK1/2 phosphorylation was blocked in LH-stimulated follicles [93]. The addition of AREG or EREG rapidly induced ERK1/2 phosphorylation within 5 min in cultured granulosa cells [60]. Because the release of the EGF domain from the membrane bound form of AREG and EREG is induced by TACE/ADAM17, we examined whether the inhibition of TACE/ADAM17 activity by a specific inhibitor or knockdown of the encoded gene by siRNA suppressed the luteinization of granulosa cells and expansion of cumulus cells. The study clearly revealed that the release of the EGF domain acted on EGFR in both granulosa cells and cumulus cells to activate the ERK1/2 pathway [71]. Moreover, when COCs were cultured with the MEK1 inhibitor (PD98059 or U0126), cumulus expansion was dramatically blocked [69, 97, 98]. 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 [94], suggesting that the EGF-like factor-activated ERK1/2 pathway in cumulus cells plays an important role in cumulus expansion and oocyte maturation.

Fan et al. [94] used granulosa cells recovered from an ovary 2 h after hCG injection of ERK1/2KO mice for microarray analysis to comprehensively understand the role of secondary factors during the ovulation process. Because genes encoding EGF-like factor, namely AREG, EREG, and BTC, are located at close sites of the same chromosome (chromosome 5) [99], it is very difficult to produce triple KO mice to clarify the role of secondary factors in the ovulation process. It has been known that their receptors are EGFR; however, EGF-like factor also acts on ErbB4 [81], which is other member of the ErbB family, to induce the Ras-ERK1/2 pathway. The expression of ErbB4 has been reported in the ovary during the ovulation process [100, 101], suggesting that ERK1/2 as the assembly point of EGFR- and ErbB4-downstream pathways are appropriate targets to understand the role of secondary factors in cumulus cells and granulosa cells during the ovulation process. Fan et al. [94] reported that 77 % of genes up-regulated by LH surge were decreased in ERK1/2KO mice compared with WT mice after hCG injection. Most of the genes that were already reported to be essential for the ovulation process were included in the down-regulated genes [94]. Some of the genes which decreased after the hCG injection, such as *Ccnd2* and *Cyp19a1*, remained detectable in ERK1/2KO mice. The mechanisms are explained by the ERK1/2-targeted transcription factors, AP-1 family, C/EBPα/β, and LRH1 (NR5a2).

Both Fra2 and JunD, present in nuclear extracts purified from bovine granulosa cells, bind to the AP-1 site of the *Tnfaip6* promoter region [35]. AP-1 also plays an important role in the arrest of the cell cycle due to the expression of cell cycle inhibitor, Kip1 [33, 102]. ERK1/2 also suppresses the expression of *Ccnd2* that is a key gene to induce the cell cycle to DNA replication in granulosa cells [94, 96]. The arrest of the cell cycle may play an important role in the differentiation of granulosa cells and cumulus cells during the ovulation process. Other ERK1/2 target transcription factors are C/EBPα/β that are expressed in granulosa cells and cumulus cells during ovulation and that can mediate the induction of selected genes [43, 103]. In C/EBPβ null mice, the number of oocytes ovulated was significantly decreased compared with that in WT mice. *Ptgs2* expression level was significantly lower than that of WT mice [104]. Thus, the EGF-like factor-induced EGFR–ERK1/2 pathway is mediated to PGE_2 production. The sequential production of secreted factors in granulosa cells mediates the ovulation process.

The expression of *Areg* and *Ereg* was not affected in *Ptgs2*KO mice at 2 h after hCG injection, whereas the inductions were significantly decreased at the 4 or 8 h point after hCG injection [60]. The delayed suppressions are explained by the level of cAMP in granulosa cells. At the early time point, cAMP level is induced by LH, whereas the LH receptor is immediately degraded after the binding of ligand to its receptor [44, 105] (Fig. 2b). However, the cAMP level remains high at the 4 h point after the hCG injection because PGE_2 produces cAMP in the cells [106] (Fig. 2b). The PGE_2-increased cAMP activates PKA–CREB or PKA–Sp1 pathways to maintain the expression of *Areg* or *Ereg*, respectively [60, 87] (Fig. 2b). The positive feedback loop in the expression of EGF-like factor is also shaped by the progesterone-PGR pathway. The AP-1 transcription factors induce the expression of *Cyp11a1* and *Star*, which increases progesterone production in granulosa cells with the induction of *Pgr* [35, 107]. Although the promoter regions of *Areg*, *Ereg*, and *Btc* do not contain the functional PGR responsible element (PGRE), the overexpression of *Pgr* dramatically increases the promoter activities of those genes [60]. In PRKO mice, the expressions of *Areg*, *Ereg*, and *Btc* are similar to those in WT mice at 2 or 4 h after the hCG injection, whereas at the 8 h point, the expression levels were completely decreased in KO mice, but remained detected in WT mice [60, 108].

Thus, the progesterone-PGR pathway does not induce the expression of EGF-like factor; however, the pathway supports PKA-induced their expressions. This information supports our understanding of the essential roles of the EGF-like factor, AREG, EREG, and BTC to not only initiate but also accomplish the ovulation process.

During the follicular development process, granulosa cells and cumulus cells dominantly produce estrogen, but less progesterone. The estrogen that impacts the cell proliferation of cumulus cells and granulosa cells is converted from theca cell-produced androgen via an aromatase-dependent manner [109, 110]. The key enzyme aromatase is encoded by *Cyp19a1* that is expressed by FSH stimulation [16]. FSH receptor (FSHR) is a member of the G protein coupling receptors and activates adenylyl cyclase, which converts ATP to cAMP similar to LHR [111]. Important questions include the reasons for FSH increasing *Cyp19a1* expression to produce estrogen and LH decreasing *Cyp19a1* expression to induce the ovulation process. The answer to these questions is that during the follicular development process, both CREB activated by the cAMP–PKA pathway and LRH1 (NR5a2) bind to the promoter region of *Cyp19a1* to increase the gene expression [112, 113]. After the LH surge, CREB remains activated by LHR-produced cAMP and is located in the nucleus, whereas LRH1 is dropped from the nucleus to the cytoplasm due to the phosphorylation by ERK1/2 [87, 114]. The granulosa cell specific *Nr5a2* KO mice revealed that the morphology of follicular development appeared normal; however, the ability responded to LH surge was completely lost, indicating that ERK1/2 changed the phase from follicular development to the ovulation process [115]. Thus, EGF-like factor, AREG, EREG, and BTC are invariable factors to stop follicular growth, initiate, and accomplish the ovulation process.

Neuregulin1: another type of EGF-like factor

When mouse COCs were cultured with AREG alone, cumulus expansion and oocyte maturation were induced in a similar manner to those of COCs in vivo during the ovulation process. However, the progression of oocyte maturation of COCs was accelerated to approximately 2 h to resume meiosis and emit the first polar body [80, 101]. The matured oocytes have a limited fertilization competence and a low developmental ability to the blastocyst stage after in vitro fertilization [101, 116]. In pig COCs collected from 3 to 5 mm of antral follicles, EGFRs were expressed in cumulus cells, whereas the addition of AREG did not induce cumulus expansion and oocyte maturation [86]. Moreover, the phosphorylation of ERK1/2 in cumulus cells of COCs or cultured granulosa cells was temporally upregulated by the addition of AREG within 15 min, but was transiently downregulated to basal level at the 30-min point after stimulation [101] (Fig. 3A). In vivo, the phosphorylation of ERK1/2 was markedly increased and was maintained by at least 4 h after hCG injection in both cumulus cells and granulosa cells [94] (Fig. 3a), leading to the hypothesis that other secondary factors would be involved in the differentiation of EGF-like factor-stimulated cumulus cells and granulosa cells (Fig. 3b).

Most of the EGF-like factor, including AREG, EREG, and BTC bind to EGFR (also known as ErbB1) and then form a dimer to activate EGFR-containing tyrosine kinase [81, 117]. ErbB1 also forms a dimer with ErbB2 that contains a tyrosine kinase domain, but lacks a ligand-binding domain and is expressed in both granulosa cells and cumulus cells in preovulatory follicles [81, 101]. Other members of the ErbB family, ErbB3 and ErbB4, are also detected in both cell types, whereas the expression level of ErbB4 is markedly decreased after hCG injection [100, 101]. We performed an immunoprecipitation study to elucidate the activation status of the ErbB family in follicles during the ovulation process, and the data showed that not only EGFR, but also ErbB2 and ErbB3 were activated by the LH surge [101]. ErbB3 has a ligand-binding domain, but not a tyrosine kinase domain, and binding with ErbB2 is essential for the induction of signaling [117, 118]. Strikingly, ErbB3 formed the heterodimer with ErbB2 with the phosphorylation of both during the ovulation process [101], indicating that the ligands for ErbB3 are expressed and secreted during the ovulation process.

To identify the ligands for ErbB3 expressed in granulosa cells after ovulation stimuli, we searched the open access microarray databases of granulosa cells. However, we did not identify any known ErbB3 ligands encoding genes that were upregulated in granulosa cells after hCG injection. Thus, we constructed each primer set to recognize the ErbB3 ligand encoded genes, *Nrg1*, *Nrg2*, and *Nrg4* and then examined their expression levels in granulosa cells during the ovulation process in rats and mice. The real-time PCR studies revealed that the induction of *Nrg1* was markedly and rapidly observed in granulosa cells within 4 h after hCG injection [101]. *Nrg1* encodes neuregulin 1 (NRG1) that belongs to EGF-like factor family and has a high affinity to bind to ErbB3, but not to EGFR, and *Nrg1* has multiple transcription initiation sites [119, 120] (Fig. 4a). In granulosa cells during the ovulation process, type III *Nrg1* that lacks exon 1, but contains a ligand-binding domain (EGF-like domain) and transmembrane domain is selectively induced in granulosa cells [101] (Fig. 4b). The Affymetrix Mouse Genome 430 2.0 Array has been commonly used for genome-wide analysis of the functions of granulosa cells. It has a probe to recognize the exon 1 region that only recognizes type I *Nrg1*, but does

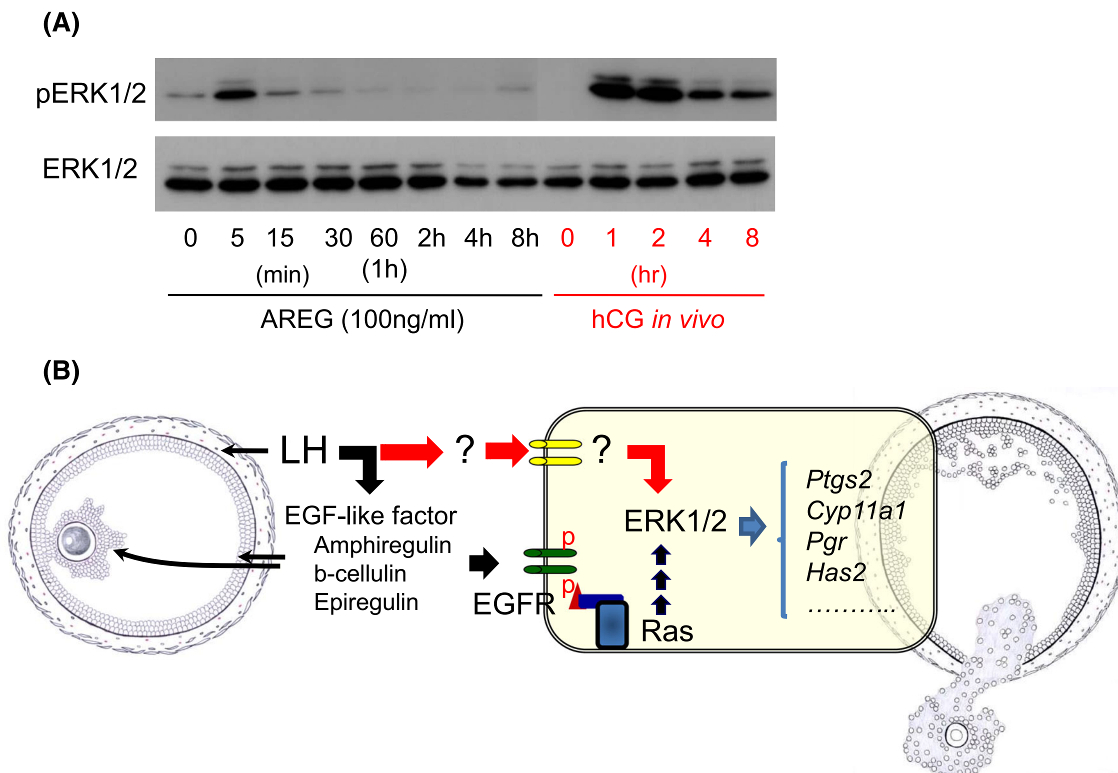

Fig. 3 Further unknown secondary factors are required for the induction of the successful ovulation process. **a** The induction of ERK1/2 phosphorylation in AREG-stimulated granulosa cells is much lower than that in granulosa cells of hCG-injected mice. Mouse granulosa cells recovered from eCG-stimulated mice were pre-cultured for overnight and then the cells were treated with AREG up to 8 h. Other granulosa cells were collected from periovulatory follicles at 0, 1, 2, 4, and 8 h after hCG injection. These granulosa cells were used for the detection of phosphorylated status of ERK1/2 by western blotting. **b** Schematic showing the mechanisms how to induce the maximum level of ERK1/2 phosphorylation in granulosa cells during ovulation process

not have any probes for recognizing the common region of *Nrg1*. Therefore, the researchers who performed the microarray analysis of mouse granulosa cells missed the expression of type III *Nrg1*.

Type III NRG1, which contains a signal sequence, two transmembrane domains, two protease targeting sequences, and a ligand domain forms a loop structure to present the ligand domain in the extracellular domain [121, 122] (Fig. 4c). One of the protease targeting sequences is shed by ADAM19, also expressed in granulosa cells during the ovulation process [101, 123]. MMP4 that has been known to be expressed and activated in follicles during the ovulation process recognizes the C terminal sequence of NRG1 [101, 124, 125]. Both of these proteases are activated by hCG injection with the induction of type III NRG1, indicating that the ligand domain of NRG1 is released to the follicular fluid of periovulatory follicles (Fig. 4c). In fact, the positive signals for antiserum to the ligand domain of NRG1 were detected on the surface of granulosa cells and cumulus cells and in the antrum of follicles at 4 h after hCG injection [126]. On the other hand, the signals for the anti-C terminal region of NRG1 IgG were only observed on the surface of granulosa cells because this region is located in the cytoplasm [126]. These data show that NRG1 is expressed and secreted from granulosa cells and then acts on both granulosa cells and cumulus cells during the ovulation process.

When mouse granulosa cells were cultured with NRG1, the phosphorylation of ERK1/2 was not induced, and the expression of genes that were observed in granulosa cells during the ovulation process was not increased, similar to those in granulosa cells cultured without any ligands [101]. However, the addition of NRG1 to AREG-containing medium induced the maximum level of ERK1/2 phosphorylation, and the induction was maintained up to 1 h, whereas AREG alone transiently phosphorylated ERK1/2 at the 15-min point [101]. In addition, the expression levels of genes involved in the luteinization of granulosa cells were significantly higher in the AREG+NRG1 treatment group than in the AREG alone group [101]. In COCs, the addition of NRG1 increased AREG-induced ERK1/2 phosphorylation in the cumulus cells, and the oocyte maturation with developmental competence was also improved by the addition of NRG1 to AREG-containing

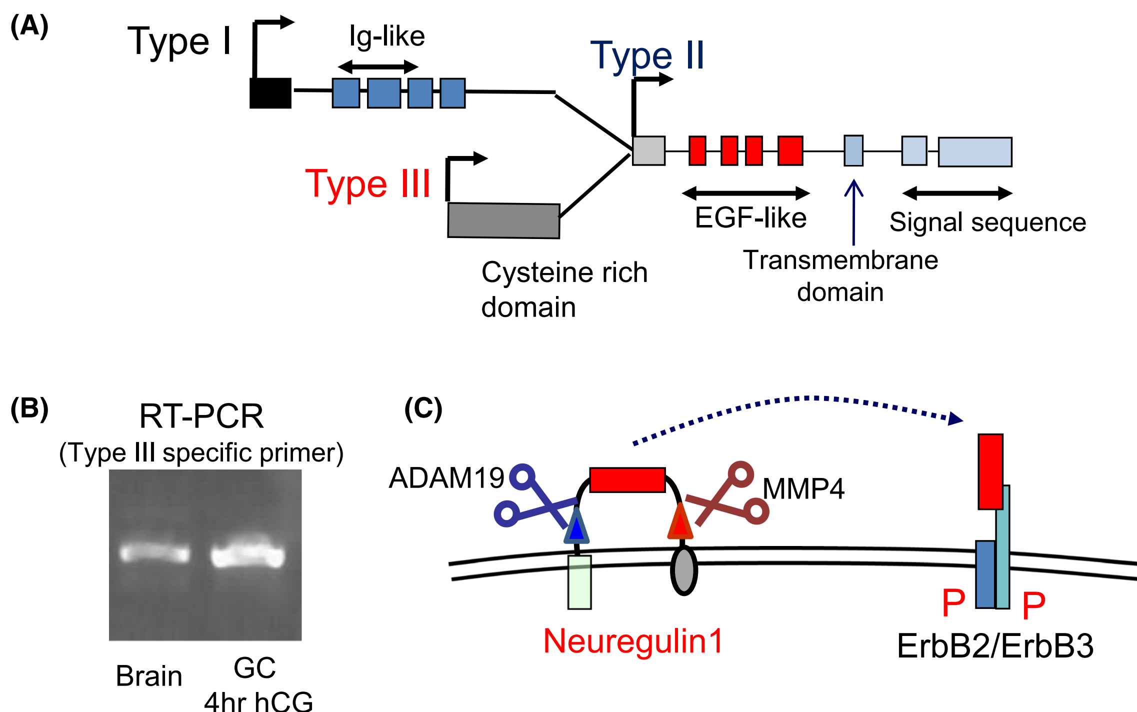

Fig. 4 The expression of *Nrg1* in granulosa cells during the ovulation process. **a** The gene construction of *Nrg1*. *Nrg1* has three different transcription start sites. Type I *Nrg1* is expressed from exon1, whereas type I *Nrg1* mRNA lucks the exon encoding cysteine rich domain via the specific splicing dependent manner. Type II *Nrg1* lucks the exons encoding Ig-like domain and cysteine rich domain. Type III *Nrg1* lucks Ig-like domain, but has a cysteine-rich domain. All of types of *Nrg1* mRNA contain exons encoding EGF-like domain, transmembrane domain, and signal sequence. **b** Type III *Nrg1* is only induced in granulosa cells during the ovulation process. The specific primer sets to recognize each type of *Nrg1* were used for RT-PCR study. The data revealed that type III *Nrg1* was expressed in mouse granulosa cells at 4 h after hCG injection. mRNA recovered from brain was used as positive control. **c** The construction of NRG1 and the image how to activate its receptor. Type III NRG1 that contains a signal sequence (*black box*), two transmembrane domains (*green box and purple circle*) and two protease targeting sequences (*blue and red triangles*) and a ligand domain (*red box*) forms a loop structure to present the ligand domain in the extracellular domain. Both proteases, ADAM19 (*blue scissors*) and MMP4 (*brown scissors*), are activated by hCG injection, which results in the release of the ligand domain of NRG1 to activate ErbB2/ErbB3 heterodimer

medium, indicating that NRG1 supported the EGF-like factor-induced ovulation process [101].

Reproductive phenotype of *Nrg1* null mice

Because the expression of *Nrg1* is observed in the brain and heart during embryogenesis, the gene mutation of *Nrg1* induces embryo lethality due to heart hypoplasia [127]. Thus, to overcome this severe limitation, we attempted to develop ovarian-specific *Nrg1* mutant mice by the mating of granulosa cell-specific Cre mice (*Cyp19a1Cre* mice) with *Nrg1*$^{flox/flox}$ mice [126]. A mating test with WT male mice was performed to assess the fertility in the mutant female mice. The number of pups per delivery was significantly decreased to approximately 20 % in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice compared with WT female mice. Although the number of oocytes ovulated by superovulation hormone treatments was more than 50 in both genotypes, the meiotic progression was accelerated to approximately 2 h in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice. The early matured oocytes were arrested at the MII stage by the 16 h point after hCG injection (just after ovulation), whereas most of them spontaneously formed pronuclei without fertilization at 20 h after hCG injection. In other words, the fertilization normally occurred just after ovulation but was abnormal by a few hours after ovulation in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice. When the mating did not occur just after hCG injection, the fertilization was not normally induced, and in most of the sperm-entered eggs, three pronuclei were observed with a low developmental competence to the blastocyst stage.

To elucidate the relationship between abnormal meiotic progression and abnormal fertilization in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice in more detail, particularly why the number of pups per delivery was decreased by only 20 %, but not completely lost, we focused on the timing of fertilization in each oocyte. Dr. Okabe's group reported that each ovulated oocyte is fertilized sequentially in vivo, but not concurrently [128], estimating that the latter timing

of fertilization in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice is outside of the fertilization window. To validate this hypothesis, the mice were assessed for the plug formation every 1 h after hCG injection to determine the timing of mating [126]. The successful fertilization rate was linearly decreased with the time of mating (plug formation) in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice but not in WT mice. Thus, the reason for a decreasing number of pups per delivery in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice is caused by the limiting the duration of oocyte fertilization competence at the MII stage due to the acceleration of meiotic resumption.

Because ErbB2/3 is not localized on the surface of oocytes, but on cumulus cells and granulosa cells [101], and because the maximum level of NRG1 protein is detected at 2 h after hCG injection, the NRG1-induced signaling pathway and expression of genes at the 2 h point in granulosa cells and cumulus cells may impact on the timing of oocyte meiotic resumption. Microarray analysis was performed for the comprehensive understanding of alternations in both cells in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice (Fig. 5). The clustering analysis of microarray data of granulosa cells in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice showed that the expression pattern of genes induced by hCG injection was similar in WT mice to that in the mutant mice (Fig. 5). More than twofold induction was observed in 1230 genes by hCG than that before hCG injection. In the mutant mice, 221 of upregulated genes at 2 h after hCG injection were further increased to more than twofold than those in WT mice.

Most of upregulated genes (88 %) have putative CREB response elements (CRE sites) and/or Sp1 binding sites (Fig. 5). It has been known that CREB and Sp1 have multiple phosphorylation sites, which are targeted by PKA, PKC, etc. [88, 91, 129, 130]. Moreover, the reporter assay study revealed that *Pgr* promoter containing Sp1-binding sites was activated in cultured granulosa cells by forskolin, cAMP inducer [54], and that the induction was further increased by additional PMA that was a PKC activator [131]. The promoter region of *Snap25*, including the further upregulated genes in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice also contains Sp1-binding sites, and its activity is regulated by both the PKA and PKC pathways [108], thereby estimating that the further induction of genes in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* mice is caused by alternation of PKA or PKC activity. Strikingly, PKC activity similar to PKA activity was significantly increased by hCG injection compared with that in granulosa cells before hCG injection. The PKC, but not PKA, activity was further increased in the mutant mice compared with that in WT mice.

An important question relates to the relationship between erratic PKC activity in cumulus cells and the acceleration of meiotic resumption in oocytes. The first step of oocyte meiotic resumption is known to be the closure of gap junctional communication between the cumulus cells and oocyte [132–136]. The closure is induced by the phosphorylation of connexin-43, which results in the decrease of the cGMP transfer level from the cumulus cell to oocyte [137–139]. The decreased level of cGMP in the oocyte activates phosphodiesterase 3A to decrease the level of cAMP [140–143]. The phosphorylation of connexin-43 is induced by ERK1/2 in cumulus cells [144]; however, connexin-43 contains the phosphorylated site (Ser 368) by PKC [145]. Although the PKC-induced phosphorylation of connexin-43 was at a weak level in LH-stimulated follicles [144], we hypothesized that the unphysiological phosphorylation would induce the acceleration of meiotic resumption in *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* female mice. The antibody that recognized only PKC-phosphorylated connexin-43 was used for detection in the study [126]. The band shift of the positive signal of total

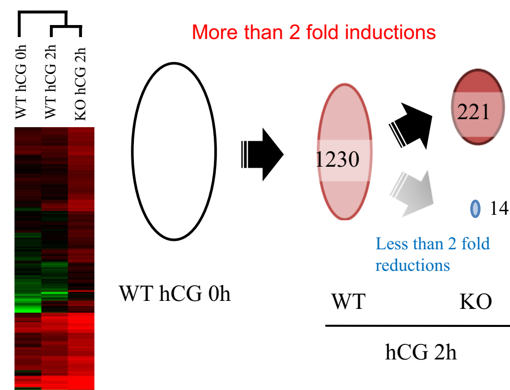

Table. Putative transcription factor binding sites in genes that were up-regulated in *Nrg1*KO mice

Transcription factor	(%)	Gene name
CREB	49%	*Areg,Ptgs2,Pgr* etc.
SP-1	76%	*Ereg,Btc,Ptx3* etc.
CREB or SP-1	**88%**	
CREB+SP-1	37%	*Snap25, Has2* etc.

Fig. 5 The mutation of *Nrg1* increases the expression level of genes that have putative CRE or Sp1-binding sites in their promoter regions. *Image* hierarchal clustering analyses were performed on microarray data obtained from granulosa cells and cumulus cells from preovulatory or periovulatory follicles of gc*Nrg1*KO (KO) and WT mice at 0 or 2 h after hCG. Schematic shows the upregulation and downregulation of genes in granulosa/cumulus cells of gc*Nrg1*KO mice. One thousand two hundred and thirty genes were upregulated by hCG, and an additional 221 genes were more highly expressed in cells of the gc*Nrg1*KO mice as compared with those in WT mice at 2 h. *Table* Putative transcription factor binding sites in genes that were upregulated in granulosa cells of *Nrg1*$^{flox/flox}$;*Cyp19a1Cre* mice at 2 h after hCG injection

connexin-43 was detected in both WT and the mutant mice. However, the phosphorylation level of connexin-43 at Ser 368 was much higher at 2 and 4 h after hCG injection in the mutant mice. In an in vitro culture, with the addition of NRG1 to AREG-containing medium, NRG1 decreased AREG-induced Ca^{2+} level and PKC activity in cumulus cells of COCs, which delayed the timing of meiotic resumption and progression to the MII stage, with a similar timing to those in vivo during the ovulation process in WT mice [126]. Taken together, NRG1 modulates EGF-like factor-induced ovulation processes that include not only luteinization and cumulus expansion, but also the timing of the meiotic progression of oocytes via the regulation of PKC activity (Fig. 6).

PKC consists of more than ten isoforms classified to three groups: (1) conventional PKC, (2) novel PKC, and (3) atypical PKC [146]. The increase of Ca^{2+} and diacylglycerol (DG) level induces conventional PKC activity, whereas Ca^{2+} is not required for the induction of novel PKC activity [147, 148]. In granulosa cells, phorbol 12-myristate 13-acetate (PMA) treatment increases the promoter activity of genes that are expressed in a significantly higher level in *Nrg1*$^{flox/flox}$*;Cyp19a1Cre* female mice [108, 131, 149]. Because PMA binds to the DG-binding site of PKC [150], there is a possibility that novel and/or conventional PKC are abnormally activated in granulosa cells of *Nrg1*$^{flox/flox}$*;Cyp19a1Cre* female mice and are regulated to a physiological level in WT mice. DG is converted with inositol-tri-phosphate (IP_3) from phosphatidylinositol-di-phosphate (PIP_2) in a phospholipase C (PLC)-dependent manner [151, 152]. IP_3 binds to its receptor localized on ER to release Ca^{2+} [153], and the increased level of Ca^{2+} in cumulus cells is directly induced by a EGFR-dependent pathway [154, 155], suggesting that γPLC is activated by the binding to tyrosine phosphorylated EGFR to increase levels of both DG and Ca^{2+}. When mouse COCs were cultured with additional NRG1 to AREG-containing medium, both PKC activity and Ca^{2+} level were significantly decreased compared with those in COCs cultured with AREG alone [126]. Thus, NRG1 downregulates AREG–EGFR-induced PLC activity in cumulus cells and granulosa cells during the ovulation process. ErbB2/3 heterodimer that is the NRG1 receptor contains a γPLC-binding tyrosine-phosphorylated site [81], whereas NRG1 has a high affinity for the induction of the PI 3-kinase-AKT pathway in granulosa cells and cumulus cells [101]. One possibility is that because PLC and PI 3-kinase use the same substrate PIP_2 to convert IP_3 and DG or phosphatidylinositol-tri-phosphate (PIP_3), respectively, the production of IP_3 and DG is competitively suppressed by the activation of PI 3-kinase [156].

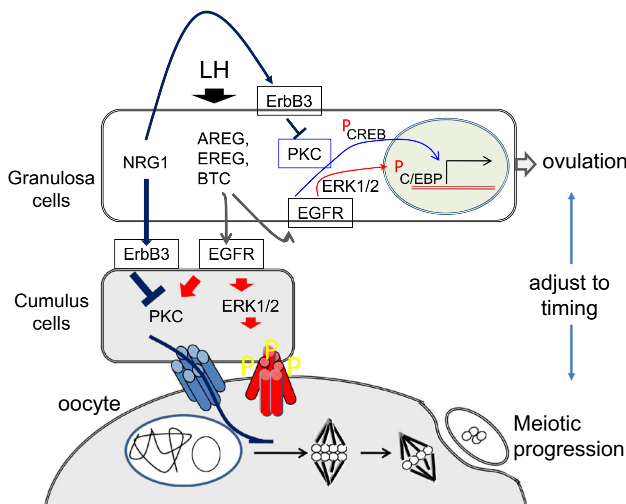

Fig. 6 Schematic showing the roles of EGFR ligands and NRG1 in the regulation of timing of ovulation and oocyte maturation. LH induces the expression of EGF-like factor, AREG, EREG, and BTC in granulosa cells. These factors act on EGFR expressed in both granulosa cells and cumulus cells to stimulate both the ERK1/2 pathway and PKC-dependent signaling pathway. Both pathways activate gene expressions involved in the ovulation process in granulosa cells. However, the erratic activation of PKC accelerates oocyte meiotic progression due to the early closure of gap junctional communication between cumulus cells and oocyte. LH-induced NRG1 that selectively acts on ErbB3/2 heterodimer decreases PKC activity to a physiological level. The controlling functions by NRG1 adjust the timing of oocyte maturation to the timing of ovulation

Conclusion

LH surge acts on granulosa cells to induce two types of EGF-like factor to transfer the stimuli from granulosa cells to cumulus cells, which impacts on cumulus expansion and oocyte maturation. One of these is a conventional EGF-like factor, AREG, EREG, and BTC that act on EGFR expressed in both granulosa cells and cumulus cells to stimulate both the ERK1/2 pathway and PKC-dependent signaling pathway. Both pathways are inducers to gene expressions involved in the ovulation process; however, the erratic activation of PKC results in the delicate progression of ovulation being placed out of order. In particular, the timing of oocyte meiotic progression is accelerated by the EGFR-induced Ca^{2+}-PKC pathway in cumulus cells. The fringe member of EGF-like factor, NRG1 that selectively acts on ErbB3/2 heterodimer supports EGFR-induced ERK1/2 activation, but suppresses the increase of Ca^{2+} level in cumulus cells, which results in the decrease of PKC activity to a physiological level. Thus, AREG, EREG, and BTC operate as inducers; however, NRG1 plays a modulating role in periovulatory follicles to adjust the timing of oocyte maturation to follicle rupture. The controlling functions by NRG1 are essential for increasing the probability to induce successful fertilization after

ovulation. Therefore, both types of EGF-like factor are notable players that transfer the stimuli of LH surge to cumulus cells and oocyte from granulosa cells to stop the follicular growth stage and then not only initiate but also accomplish the ovulation process.

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Compliance with ethical standards

Conflict of interest Masayuki Shimada, Takashi Umehara and Yumi Hoshino declare that they have no conflict of interest.

Human studies This article does not contain any studies with human subjects performed by any of the authors.

Animal studies All institutional and national guidelines for the care of use of laboratory animals were followed.

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