The Release of EGF Domain from EGF-like Factors by a Specific Cleavage Enzyme Activates the EGFR-MAPK3/1 Pathway in Both Granulosa Cells and Cumulus Cells During the Ovulation Process

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Abstract. In mammalian preovulatory follicles, LH stimulation induces the ovulation process, including follicular wall rupture, granulosa cell luteinization, cumulus cell expansion and meiotic maturation of the oocyte. The receptor for LH (LHCGR) is expressed mostly in granulosa cells of preovulatory follicles, and is rarely expressed in cumulus cells or oocytes. The expression level in granulosa cells dramatically decreases after ovulation stimuli. Thus, a potent factor(s) secreted by granulosa cells is required to stimulate not only granulosa cells via an autocrine manner but also cumulus cells and/or oocytes via a paracrine pathway. Recent reports showed that granulosa cells and cumulus cells express EGF-like factors that activate the EGF receptor (EGFR)-mitogen-activated protein kinase3/1 (MAPK3/1) (also known as extracellular signal-regulated kinase1/2 (ERK1/2)) pathway in both cell types. EGF-like factors are composed of a signal sequence, transmembrane domain and EGF domain, suggesting that release of the EGF domain by a specific enzyme is essential for interaction with the EGFR to induce the ovulation process. In our studies, TACE/ADAM17, which is known to be a proteolytic enzyme of EGF-like factors in many types of tissue, was found to be expressed in FSH/LH-stimulated granulosa cells and cumulus cells together with activation of the EGFR-MAPK3/1 pathway. When TACE/ADAM17 activity was decreased by a specific inhibitor or siRNA technique, granulosa cell luteinization, cumulus expansion and oocyte maturation were suppressed in an *in vitro* culture. Thus, TACE/ADAM17 is one of the key genes expressed in both granulosa cells and cumulus cells for induction of the ovulation process.

Key words: cAMP, EGF like factors, PGE2, Progesterone, Ovulation

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The surge of LH from the pituitary gland stimulates preovulatory follicles, and the stimulus induces a series of ovulation processes: follicular wall rupture, granulosa cell luteinization, cumulus expansion and meiotic maturation of the oocyte. Because the receptor for LH (LHCGR) is dominantly expressed in granulosa cells and is dramatically broken-down after an LH surge [1], a potential factor(s) secreted from granulosa cells to interact with granulosa cells, cumulus cells or oocytes must be required to induce these events [2].

Recently, Conti and coworkers conducted global mRNA expression profiling of mouse granulosa cells in response to PMSG and subsequent hCG stimuli. The results showed that the expression levels of EGF-like factors, amphiregulin (AREG), epiregulin (EREG) and β -cellulin (BTC) were significantly upregulated by hCG in granulosa cells, and addition of these factors to maturation medium enhanced cumulus cell expansion and meiotic maturation of follicle-enclosed mouse oocytes [3]. EGF-like factors act on EGFR to activate the Ras-MAPK3/1 pathway in granulosa cells and cumulus cells. Granulosa cell-specific and cumulus cell-specific mutation of the *Erk1/2* gene

impairs oocyte maturation, cumulus expansion and granulosa cell luteinization [4, 5], indicating that EGF-like factors are one of the key factors likely to work as autocrine factors in granulosa cells, and transmit the LH-dependent signal from granulosa cells to cumulus cells and the oocyte.

EGF-like factors are transmembrane proteins comprising a signal sequence, a transmembrane domain and at least one EGF domain. To activate cognate receptors by EGF-like factors working as a ligand, metalloprotease activity is critical for release of the EGF domain. Importantly, Sahin *et al.* [8] showed that tumor necrosis factor α -converting enzyme (TACE) is the main sheddase of AREG and EREG in mouse embryonic cells. TACE, which is also known as ADAM17, is a member of the ADAM (a disintegrin and metalloprotease) family of proteases. Expression of TACE/ADAM17 has been observed in many tissues, including the heart, brain, lung, liver, muscle, kidney and testis. Although mechanisms of EGF-like factor expression during the ovulation process have been reported [6, 7], until recently there was little information about how to specifically activate these factors as ligands, especially regarding the expression and functional roles of TACE/ADAM17. This mini-review focuses on mechanisms underlying the expression and activation of TACE/ADAM17 and its physiological roles in granulosa cells and cumulus cells during the oocyte maturation process.

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Expression of TACE/ADAM17 in Granulosa and Cumulus Cells

TACE/ADAM17 belongs to the ADAM family of proteins, which currently comprises more than 30 members. About 50% of ADAM proteins are expressed predominantly in the testis and play a role in spermatogenesis and fertilization. The others are involved in neurogenesis, myogenesis, and osteogenesis, and in the regulation of immune responses [9–11]. Unlike other types of ADAM protein, *Tace/Adam17* mRNA is ubiquitously expressed in numerous tissues [12]; however, a high level of expression is observed in cancer cells, where it is involved in activation of the EGFR-MAPK3/1 pathway to induce cell proliferation and migration. As in cancer cells, the EGFR-MAPK3/1 pathway is transiently and dramatically activated in granulosa cells and cumulus cells during the ovulation process due to the expression of EGF-like factors. From the above information, we hypothesized that TACE/ADAM17 might be expressed in granulosa cells and cumulus cells, where it might work as a shedding enzyme on EGF-like factors during the ovulation process.

Using mRNA recovered from granulosa cells or cumulus cells of antral follicles, preovulatory follicles, and periovulatory follicles of eCG/hCG-primed swine, we investigated the temporal changes in *Tace/Adam17* expression. The results revealed that administration of eCG to induce follicle development to the preovulatory stage did not increase the level of *Tace/Adam17* mRNA in either granulosa or cumulus cells, whereas mRNA expression was significantly induced by subsequent hCG stimuli [13].

To clarify how *Tace/Adam17* is expressed during the ovulation process, *in vitro* culture studies of granulosa cells and cumulus-oocyte complexes (COCs) were carried out. Treatment with FSH and/or LH also increased the expression level of *Tace/Adam17* mRNA in both cell types, concomitantly with *Areg* and *Ereg* mRNA expression. Western blotting analysis also showed upregulation of the protein by FSH and LH in cumulus cells of COCs [14]. Furthermore, expression of *Tace/Adam17* was significantly suppressed by inhibitors of the PKA, p38MAPK (MAPK14) or MAPK3/1 pathway [15]. Its proximal promoter contains multiple AP2 and Sp1 transcription factor binding sites and includes a GC box and a CCAAT box; thus the region including both boxes has potential promoter activity [16]. C/EBP α and C/EBP β , which directly bind to a CCAAT box in promoter regions, are well-known targets of MAPK3/1; however, they are also activated by both PKA and p38MAPK. Following LH stimuli, PKA and p38MAPK are rapidly activated by a nongenomic pathway within 1 h, and then phosphorylation of MAPK3/1 is induced by an EGF-like factor expressed in mouse granulosa cells [17]. Thus, TACE/ADAM17 expressed by PKA- and p38MAPK-dependent mechanisms releases the EGF domain from EGF-like factors, which then increase the phosphorylation level of MAPK3/1. The MAPK3/1-C/EBP pathway further increases the expression level of TACE/ADAM17 to maintain the activation status of the signaling pathway (Fig. 1). This positive feedback system is required to induce a successful ovulation process as follows.

Fig. 1. The mechanism of TACE/ADAM17 expression in granulosa cells and cumulus cells. FSH/LH stimuli activates the PKA- and p38MAPK-C/EBP pathway, and then induces *Tace/Adam17* mRNA expression in granulosa cells and cumulus cells. The EGF-like factors released by TACE/ADAM17 activate the EGFR-MAPK3/1-C/EBP pathway, which results in the increase in *Tace/Adam17* mRNA level in granulosa cells and cumulus cells during the ovulation process.

Function of TACE/ADAM17 in Granulosa and Cumulus Cells

The metalloproteinase domain of TACE/ADAM17 contains the zinc-binding consensus motif (HWLGHNFGAEHD) involved in coordinating zinc with histidine residues and creating the active site of the enzyme [12, 18]. In many tissues and cell types, TACE/ADAM17 has been reported to have putative substrates, including transforming growth factor- α (TGF α), heparin-binding EGF (HB-EGF), Notch, AREG and EREG [8, 19]. In the mouse ovary, the expression level of TGF α and HB-EGF is low and does not change during follicular development and ovulation [20], indicating that these factors are not the potential target proteins of TACE/ADAM17. Johnson *et al.* [21] reported that *Notch* mRNA was detected in response to PMSG stimuli in growing follicles, although the functional role of Notch in the oocyte maturation process has not yet been demonstrated.

It has been shown that *Areg* and *Ereg* are expressed in granulosa and cumulus cells during the ovulation process [3, 17], and their induction is observed concomitantly with the increase in *Tace/Adam17* mRNA level [14, 15], suggesting that AREG and EREG might be physiological substrates of TACE/ADAM17 in periovulatory follicles. When porcine COCs were cultured for up to 40 h, the enzyme activity of TACE/ADAM17 was upregulated by gonadotropins as compared with cumulus cells of COCs before culture [14]. Phosphorylation of MAPK3/1 was induced by the addition of EGF or gonadotropins (FSH+LH) to cumulus cells of COCs. This phosphorylation of MAPK3/1 was completely downregulated by

addition of the TACE/ADAM17 selective inhibitor, TAPI-2, when porcine COCs or granulosa cells were cultured with FSH and LH [14]. However, these inhibitory effects were not observed in COCs or granulosa cells with EGF-like factors. In addition, we showed that LH-induced phosphorylation of MAPK3/1 was suppressed by transfection with *Tace/Adam17* siRNA in rat granulosa cells [14]. Thus, TACE/ADAM17 expressed in granulosa cells and cumulus cells facilitates release of the EGF domain of EGF-like factors, which then activates the EGFR-MAPK3/1 pathway in cumulus cells and granulosa cells during the ovulation process.

Positive Feedback Loop of EGF-like Factors, TACE/ADAM17 and PGE2 in Cumulus Cells of Porcine COCs

Prostaglandin E2 (PGE2) is synthesized and accumulates within the follicular fluid of periovulatory follicles. Mice deficient in *Ptgs2* (encoding cyclooxygenase-2; COX-2) or *Pger2* (encoding PGE2 receptor 2, PGER2) show a decreased number of ovulated oocytes and complete loss of fertilization ability [22]. Importantly, our previous study showed the downregulation of EGF-like factor expression in *Ptgs2*-deficient mice during administration of hCG. Interestingly, the expression of *Ptgs2* was upregulated by EGF-like factors [17], strongly indicating the existence of a positive feedback system between EGF-like factors and PGE2 during the ovulation process.

In an *in vitro* culture system of porcine COCs, expression of *Ptgs2* and *Ptger2* was induced by FSH concomitant with *Areg*, *Ereg* and *Tace/Adam17* expression [23]. The expression of *Areg*, *Ereg* and *Tace/Adam17* was significantly downregulated by the PTGS2 inhibitor, NS398, in cumulus cells of COCs cultured for 10 h, but not in those cultured for 5 h. During *in vitro* maturation of porcine COCs, the cAMP level was dramatically increased within 5 h, and this level was then maintained for up to 40 h [24]. However, the level of *Fshr* mRNA that encoded the FSH receptor was rapidly and significantly decreased in cumulus cells of COCs cultured with FSH alone [23], suggesting that other stimulators that increase the cAMP level in cumulus cells must be secreted from cumulus cells. Strikingly, the cAMP level was not changed by NS398 at 5 h, whereas it was significantly decreased at 10 h. Furthermore, NS398 dramatically suppressed MAPK3/1 after 10 h of culture but not after 5 h of culture. The addition of PGE2 alone induced cumulus expansion and/or oocyte meiotic maturation not only in pigs but also in mice [25, 26], indicating that PGE2 is the second factor that acts on cumulus cells to maintain the high level of cAMP. The results demonstrate that the initial expression of EGF-like factors and TACE/ADAM17 is induced in a gonadotropin-dependent manner, whereas sustainable expression of EGF-like factors and TACE/ADAM17 is dependent on the PGE2-PGER2 pathway [23] (Fig. 2).

Terminal Regulation of TACE/ADAM17 Expression by the PGR Pathway in Cumulus Cells of Porcine COCs

Progesterone is synthesized and secreted during the ovulation process and formation process of the corpus luteum. Mice deficient in *Nr3c3* (encoding the progesterone receptor, PGR) fail to ovulate [27, 28]; however, histological analysis of the ovaries of these mice revealed that cumulus expansion is normally induced after

hCG injection. On the other hand, our previous studies showed that pharmacological inhibition of PGR or progesterone production during *in vitro* maturation of porcine COCs dramatically suppresses both cumulus expansion and oocyte maturation [29–32]. In addition, when one COC was cultured in each well, oocyte meiotic resumption was delayed as compared with wells where 20 COCs were cultured. The level of progesterone in the medium was raised by increasing the number of COCs in each well [33], suggesting that the progesterone-PGR pathway is required to induce cumulus expansion and oocyte maturation in pig COCs, at least in *in vitro* culture.

To clarify the roles of the progesterone-PGR pathway in more detail, we focused on the EGFR-MAPK3/1 pathway. Addition of the PGR antagonist, RU486 did not affect FSH-induced *Areg* and *Ereg* expression at any cultivation time point. RU486 also did not affect *Tace/Adam17* mRNA expression in cumulus cells of COCs cultured up to 20 h; at 30 or 40 h, however, RU486 significantly suppressed *Tace/Adam17* mRNA expression. Although the phosphorylation level of MAPK3/1 and expression of its target genes (*Has2* and *Tnfrsf6*) were not affected by RU486 up to 20 h, these levels were dramatically suppressed by RU486 at 40 h. Furthermore, cumulus expansion and the percentage of oocytes reaching the MII stage were also suppressed by RU486. Thus, the results indicated that the progesterone-PGR pathway regulates the expression level of *Tace/Adam17* during late cumulus expansion but not the levels of EGF-like factors, which are essential for the terminal secretion of EGF-like factors during cumulus expansion and oocyte meiotic maturation in pigs [34] (Fig. 2). However, because the promoter region of the *Tace/Adam17* gene does not contain the progesterone responsive element (PRE) [16], further study is required to understand how terminal *Tace/Adam17* gene expression is regulated by the progesterone-PGR pathway during this phase.

Conclusion

EGF-like factors are the most important and potent factors that enhance oocyte maturation and ovulation. Our studies have focused on the mechanisms that activate EGF-like factors. We have shown that the high expression of *Tace/Adam17* in granulosa and cumulus cells of porcine COCs is induced in a PGE2-dependent manner. The sequential induction of EGF-like factors enhances activation of MAPK3/1 in cumulus cells. Furthermore, we revealed that the progesterone-PGR pathway is required for maintenance of *Tace/Adam17* mRNA gene expression, but not for maintenance of the gene expression of EGF-like factors, in order to sustain MAPK3/1 activation in late cumulus expansion. In summary, activation of the EGFR-MAPK3/1 pathway via a TACE/ADAM17-released EGF domain from an EGF-like factor induces cumulus expansion and oocyte maturation in porcine COCs.

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Fig. 2. The expressions of TACE/ADAM17 and EGF-like factors in granulosa cells and cumulus cells during the ovulation process. FSH/LH stimuli induce EGF-like factor and TACE/ADAM17 expressions via a cAMP-dependent pathway in granulosa cells and cumulus cells. The EGF-like factors released by TACE/ADAM17 activate the EGFR-MAPK3/1 pathway, which enhances the expression of *Ptg2* mRNA in granulosa cells and cumulus cells. The produced PGE2 acts on PTGER2/4 and induces the maximum level of EGF-like factors and TACE/ADAM17 expressions in both types of cells, which results in the induction of sustainable activation of MAPK3/1 during the ovulation process. The sustainable activation of MAPK3/1 induces cumulus expansion, oocyte meiotic maturation and luteinization of granulosa cells during the ovulation process.

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