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Positive Feedback Loop Between Prostaglandin E2 and EGF-Like Factors Is Essential for Sustainable Activation of MAPK3/1 in Cumulus Cells During In Vitro Maturation of Porcine Cumulus Oocyte Complexes[1]

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ABSTRACT

During in vitro maturation of porcine cumulus-oocyte complexes (COCs), follicle-stimulating hormone (FSH) increases both prostaglandin E2 (PGE2) production and the expression levels of EGF-like factors. The ligands act on cumulus cells by the autocrine system due to their specific receptors, EP2, EP4, or EGF receptor. When each pathway is suppressed by inhibitors, complete cumulus expansion and oocyte maturation do not occur. In this study, we examined the relationship between both of these pathways in cumulus cells of porcine COCs. When COCs were cultured with FSH, *Fshr* mRNA expression was immediately decreased within 5 h, whereas *Ptger2*, *Ptger4,* and *Ptgs2* expression levels were significantly increased in cumulus cells in the culture containing FSH for 5 or 10 h. The PTGS2 inhibitor NS398 significantly suppressed not only PGE2 secretion at any culture time point but also *Areg*, *Ereg,* and *Tace/Adam17* expression in cumulus cells at 10 and 20 h but not at 1 or 5 h. During the early culture period, phosphorylation of MAPK3 and MAPK1 (MAPK3/1) was not affected by NS398; however, at 10 and 20 h, phosphorylation was suppressed by the drug. Furthermore, down-regulations of MAPK3/1 phosphorylation and expression of the target genes by NS398 was overcome by the addition of either PGE2 or EGF. FSH-induced cumulus expansion and meiotic progression to the MII stage were also suppressed by NS398, whereas these effects were also overcome by addition of either PGE2 or EGF. These results indicated that PGE2 is involved in the sustainable activation of MAPK3/1 in cumulus cells via the induction of EGF-like factor, which is required for cumulus expansion and meiotic maturation of porcine COCs.

cumulus cells, in vitro maturation, oocyte maturation, ovulation

INTRODUCTION

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are secreted from the pituitary gland under the control of follicular development and ovulation. Their receptors (FSHR and luteinizing hormone/choriogonadotropin receptor [LHCGR]) belong to a member of the seven-transmembrane domain G-protein-coupled receptor that activates adenyl cyclase to produce cAMP as a second messenger [1, 2]. In the cumulus-oocyte complex (COC), the cAMP-dependent pathway in cumulus cells induced oocyte maturation and cumulus expansion [3] concomitantly with up-regulation of the cumulus expansion-related *Has2* and *Tnfaip6* genes [4]. It has been shown that prostaglandin E2 (PGE2) also increases cAMP levels in cumulus cells during the ovulation process [5]. PGE2 synthesized from arachidonic acid by the rate-limiting enzyme PTGS2 acts on a family of four different G-protein-coupled receptors, EP1, EP2, EP3, and EP4 [6]. When receptors EP2 and EP4, encoded by the *Ptger2* and *Ptger4* genes, respectively, were activated, the intracellular cAMP level was dramatically and immediately increased within cytoplasm [7], and both types of receptors were dominantly expressed in cumulus and granulosa cells [8]. In *Ptgs2*- or *Ptger2*-deficient mice, cumulus expansion and meiotic maturation of oocyte are not successfully induced in vivo [9, 10], indicating that the increase in cAMP in cumulus cells by not only gonadotropins but also by PGE2 could play an important role in oocyte maturation process.

Recently, Park et al. [11] reported using a mouse model in which the EGF-like factors amphiregulin (AREG), epiregulin (EREG), and β-cellulin are expressed in granulosa cells and transmit the LH signal from granulosa cells to cumulus cells [11]. Levels of *Areg* and *Ereg* expression were directly regulated by the cAMP-PKA-CREB (PKA, protein kinase A; CREB, cAMP response element-binding protein) pathway in cumulus and granulosa cells [12, 13]. Using *Ptgs2* knockout mice, we revealed that *Areg* and *Ereg* mRNA expression levels were significantly lower than those in granulosa cells

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TABLE 1. List of primers used for RT-PCR, annealing temperature, and the amplified cycle number.

mRNA	Primer sequences	Annealing temperature (°C)	Amplification cycle
Actb	F:5′-CTA CAA TGA GCT GCG TGT GG-3′ R:5′-TAG CTC TTC TCC AGG GAG GA-3′	58	31
Fshr	F:5′-GCT AAT TGC AGC ACC AGA CA-3′ R:5′-TGT TGG TTC CCT TTT TCA CC-3′	62	29
Ptger2	F:5′-GGT GCT GGC TTC TTA TGC TC-3′ R:5′-CAG CGC TGG TAG AAG TAG GG-3′	64	27
Ptger4	F:5′-AAG CTG GGA CTC GTC TTT GA-3′ R:5′-GCT TTC ACC TTG TCC TGC TC-3′	64	29
Ptgs2	F:5′-CTG CCG TGT CGC TCT GCA CTG-3′ R:5′-TCA TAA CTC CAT ATG GCT TGA AC-3′	58	35
Egfr	F:5′-CAC CAA ATG AGC CTG GAC AA-3′ R:5′-ACA TGG CYG TCA GGA GCA GA-3′	60	33
Areg	F:5′-CAC CAG AAC AAA AAG GTT CTG TC-3′ R:5′-AAG TCC ATG AAG ACT CAC ACC AT-3′	58	35
Ereg	F:5′-AAG ACA ATC CAC GTG TGG CTC AAG-3′ R:5′-CGA TTT TTG TAC CAT CTG CAG AAA-3′	58	35
Tace/Adam17	F:5′-GAC ATG AAT GGC AAA TGT GAG AAA C-3′ R:5′-AGT CTG TGC TGG GGT CTT CCT GGA-3′	58	34
Has2	F:5′-GAA TTA CCC AGT CCT GGC TT-3′ R:5′-GGA TAA ACT GGT AGC CAA CA-3′	54	35
Tnfaip6	F:5′-TCA TAA CTC CAT ATG GCT TGA AC-3′ R:5′-TCT TCG TAC TCA TTT GGG AAG CC-3′	54	32

collected from heterozygous mice [14]. On the other hand, AREG induced *Ptgs2* expression in cumulus and granulosa cells [14], suggesting that the positive loop of EGF-like factors and PGE2 pathways is activated and regulated by cumulus cell function and oocyte maturation. Because cumulus cells have less LH receptor, it is possible that the positive feedback system is required for the activation of cAMP-dependent signaling pathway in cumulus cells during the ovulation process.

In the medium used for in vitro maturation of oocytes, FSH is usually added to directly increase cAMP level in cumulus cells of COCs in mice, cattle, and pigs [15–17]. Our previous study showed that the addition of FSH increased *Areg* and *Ereg* expression levels in cumulus cells of porcine COCs and that this induction was required for cumulus expansion, oocyte meiotic progression, and developmental competence of the oocyte [18]. The PKA inhibitor H89 significantly suppressed EGF-like factor expression in cumulus cells of cultured COCs [12]. Moreover, several studies have shown that the addition of dibutyl cAMP or phosphodiesterase inhibitor (IBMX) to maturation medium for the first half of culture time increases developmental competence of porcine oocyte of COCs under in vitro culture condition [18–20], indicating that the cAMP-dependent pathway in cumulus cells is important for oocyte maturation in vitro, possibly via an EGF-like factor-dependent manner. However, there is little direct evidence to show how the level of cAMP is regulated in cumulus cells and which culture condition physiologically mimics the in vivo ovulation process.

In this study, to answer the above questions, we examined the kinetic changes of *Fshr*, *Ptger2*, *Ptger4*, and *Ptgs2* mRNA expression; cAMP concentration in cumulus cells; and PGE2 production in cultured medium during in vitro maturation of porcine COCs. Because results showed that induction of the *Ptgs2* gene was not required for the increase in cAMP levels but was required to maintain a high level of cAMP in cumulus cells, we investigated the effects of the PTGS2 inhibitor NS398 on the EGF-like factor pathway in cumulus cells at several culture periods. We also tested the effect of NS398 on MAPK3 and MAPK1 (MAPK3/1) phosphorylation and expression of their target genes (*Has2* and *Tnfaip6*) in cumulus cells. Finally, cumulus expansion and meiotic maturation of porcine oocytes during in vitro maturation of porcine COCs were examined.

MATERIALS AND METHODS

Materials

Highly purified porcine FSH was a gift from the National Hormone and Pituitary Program (Rockville, MD). Fetal calf serum was obtained from Invitrogen (Carlsbad, CA). Oligonucleotide poly(dT) was purchased from Amersham Pharmacia Biotech (Newark, NJ). Avian myeloblastosis virus reverse transcriptase and *Taq* DNA polymerase were from Promega (Madison, WI). Routine chemicals and reagents were obtained from Nakarai Chemical Co. (Osaka, Japan).

Collection of Cumulus and Granulosa Cells from Preovulatory and Periovulatory Follicles of eCG- and hCG-Treated Sows

Landrace sows (12–14 mo old, weighing approximately 150 kg), used for breeding, were weaned at 28 days after farrowing. Estrus was induced by injection of 1000 IU of equine chorionic gonadotropin (eCG) at 24 h after weaning, followed 72 h later with 500 IU of human chorionic gonadotropin (hCG). Estrus detection was performed twice a day (0900 and 1600 h), beginning 2 days after eCG administration, by allowing females to have nose-to-nose contact with a mature boar and by applying back pressure. Treated gilts were slaughtered at 0, 6, 12, 24, or 48 h post-hCG injection, and preovulatory and periovulatory follicles of more than 10 mm in diameter were aspirated by syringe for collection of cumulus and granulosa cells. Animals were treated according to the Animal Care and Use Committee at Hiroshima University.

In Vitro Culture of Porcine COCs

Isolation of porcine COCs was carried out as described previously [21, 22]. Briefly, porcine ovaries were collected from 5- to 7-mo-old prepubertal gilts at a local slaughterhouse. COCs were collected from the surfaces of intact healthy antral follicles measuring 3–5 mm in diameter. Oocytes having evenly granulated cytoplasm with at least four layers of unexpanded cumulus cells were selected and washed three times with maturation medium. Twenty COCs were cultured for up to 40 h in 300 μl of maturation medium supplemented with 20 ng/ml highly purified porcine FSH (National Institute of Diabetes and Digestive and Kidney Disease, Torrance, CA) at 39°C in a humidified incubator (95% air, 5% CO_2). The maturation medium consisted of modified NCSU37

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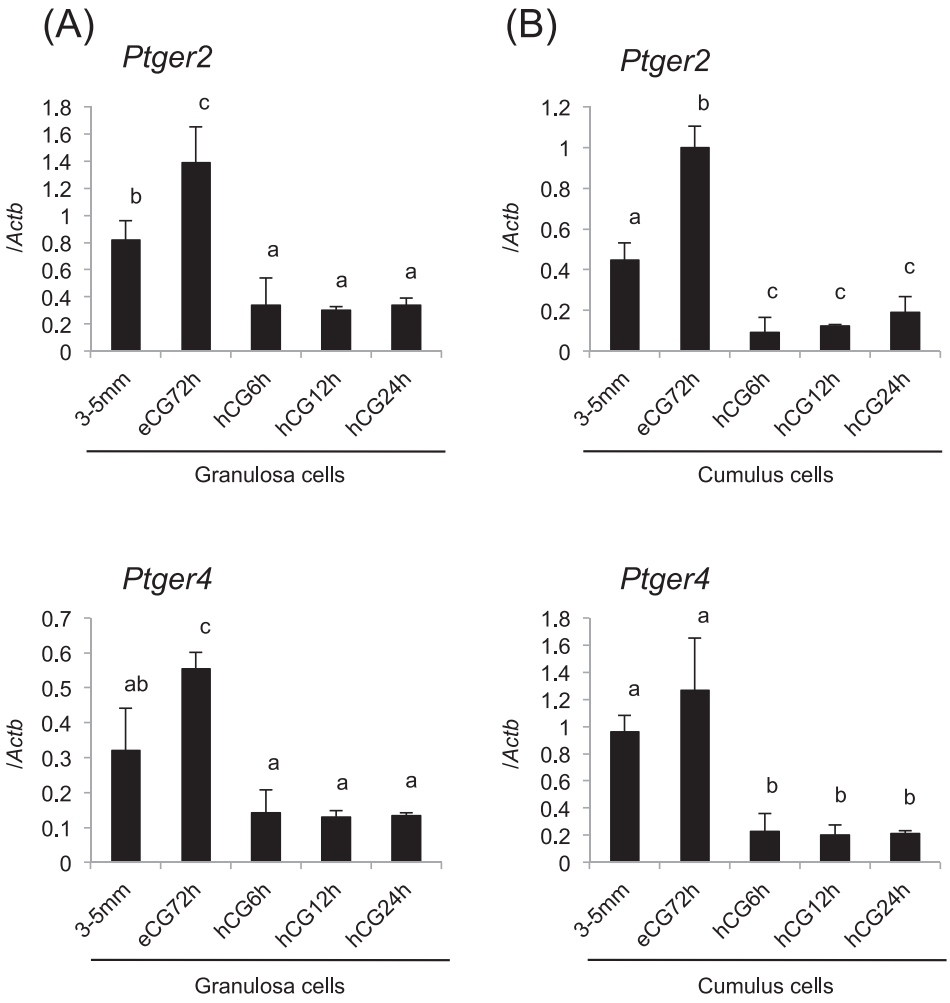

FIG. 1. Kinetic changes in *Ptger2* and *Ptger4* mRNA expression levels in granulosa cells (**A**) and cumulus cells (**B**) during follicular development and ovulation processes are shown. Granulosa cells and cumulus cells were collected from 3- to 5-mm follicles of gilts without eCG and hCG injections, from follicles of more than 10-mm diameter from gilts 72 h after eCG injection, or from follicles of 10 mm in diameter from gilts at 6, 12, or 24 h after hCG injection followed by eCG priming. Values are means ± SEM calculated on the basis of three animals. Bars without a common superscript are significantly different ($P < 0.05$).

[22] supplemented with 10% (v/v) fetal calf serum (Gibco BRL, Grand Island, NY) and 7 mM taurine (Sigma Chemical Co., St. Louis, MO). At selected time intervals, COCs were collected for RNA and protein isolation.

Assessment of cumulus expansion was based on the diameter of COCs, measured with an eyepiece micrometer, using a phase-contrast microscope (IMT2 model; Olympus, Tokyo, Japan) and a 10× objective as described previously [21, 22]. The diameter selected for measurement was defined as the greatest distance across the COC expanded matrix. Oocytes were fixed with an acetic acid-ethanol (1:3) solution for 48 h and stained with acetolacmoid before being examined with phase-contrast microscopy (at ×400 magnification) for evaluation of their chromatin configuration.

Chemicals

PTGS2 inhibitor (NS398; Sigma) and EGF receptor (EGFR) tyrosine kinase inhibitor (AG1478; Sigma) were dissolved in dimethylsulfoxide at 10 mM each and stored at −20°C. PGE2 (Sigma) was dissolved in ethanol at 500 ng/ml and stored at −20°C. EGF (Sigma) was dissolved in 1 mg/ml maturation medium and stored at −20°C. The final concentration of each compound (as describe above) was obtained by dilution (1:1000) with the maturation medium. The final concentrations of dimethylsulfoxide and ethanol were 0.1% (v/v), which did not affect the function of cumulus cells during meiotic resumption of porcine oocytes [23].

Measurement of cAMP Concentration in Cumulus Cells of COCs

Cumulus cells of cultured COCs were recovered and washed three times in PBS. Collected cumulus cells were stored at −80°C until used. cAMP concentration was determined by using a cAMP Complete EIA kit (Assay Designs, Ann Arbor, MI) according to the instruction manual.

Measurement of PGE2 Concentration in Cultured Medium

COCs were cultured for 20 h in maturation medium. The cultured medium was recovered and then centrifuged at 1500 rpm for 7 min. Supernatants were stored at −80°C until used for detection of the level of PGE2. PGE2 was detected by using a PGE2 high-sensitivity EIA kit (Assay Designs) according to the manufacturer's instructions.

RNA Isolation

After COCs were cultured, cumulus cells were recovered and washed three times in PBS. Total RNA was extracted from cumulus cells by using an RNeasy mini-kit (Qiagen Sciences, Valencia, CA) according to the instruction manual and dissolved in nuclease-free water. Final RNA concentrations were determined by absorbance using a spectrophotometer.

RT-PCR

RT-PCR analyses were performed as previously described [21, 22]. Briefly, total RNA was reverse-transcribed using 500 ng of poly(dT) and 0.25 U of avian myeloblastosis virus-reverse transcriptase at 42°C for 75 min and 95°C for 5 min. PCR conditions were as follows: cDNA was amplified by denaturation at 94°C for 30 sec, then primer annealing for 1 min, and extension at 68°C for 1 min, with a final extension step of 7 min at 68°C. The amplified cycle and annealing temperature are shown in Table 1. Amplified products were analyzed by electrophoresis on 2% agarose gels. The intensities of the objective bands were quantified by densitometric scanning using a Gel-Pro analyzer (Media Cybernetics, Inc., Bethesda, MD). Specific primer pairs were selected and analyzed as indicated in Table 1. Expression of the *Actb* gene was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction.

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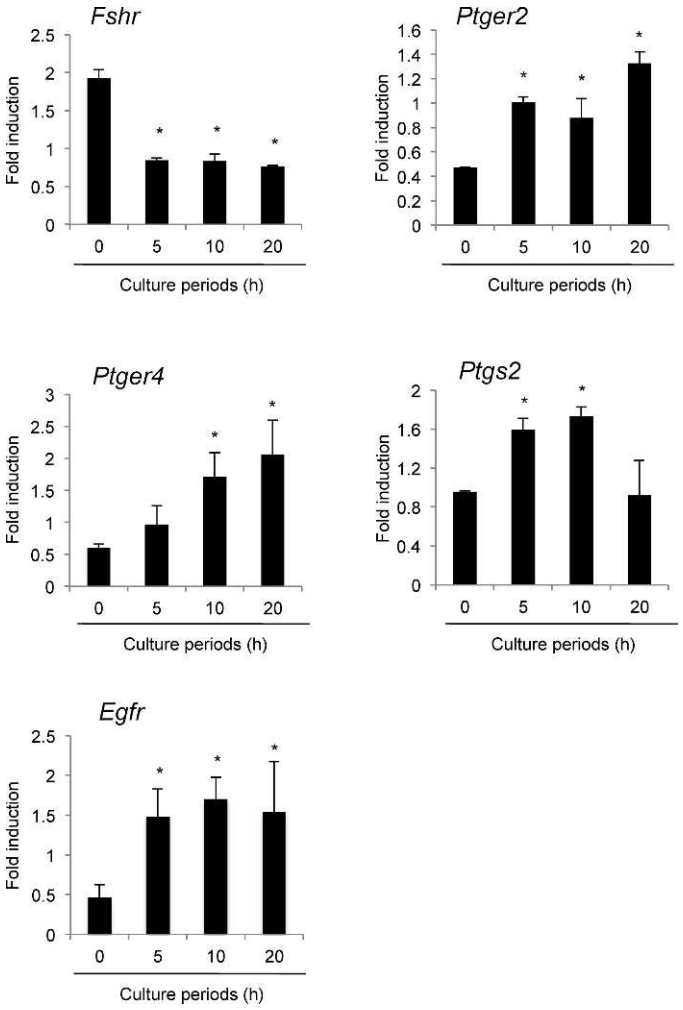

FIG. 2. Kinetic changes in *Fshr*, *Ptger2*, *Ptger4*, and *Ptgs2* mRNA expression levels are shown in cumulus cells of COCs cultured with FSH for 0, 5, 10, or 20 h. For reference, the 1-h COC value was set as 1, and data are presented as the fold-change in induction. Values are means ± SEM from three independent culture experiments. *, Indicates significant differences were observed compared with cumulus cells of COCs just collected from follicles ($P < 0.05$).

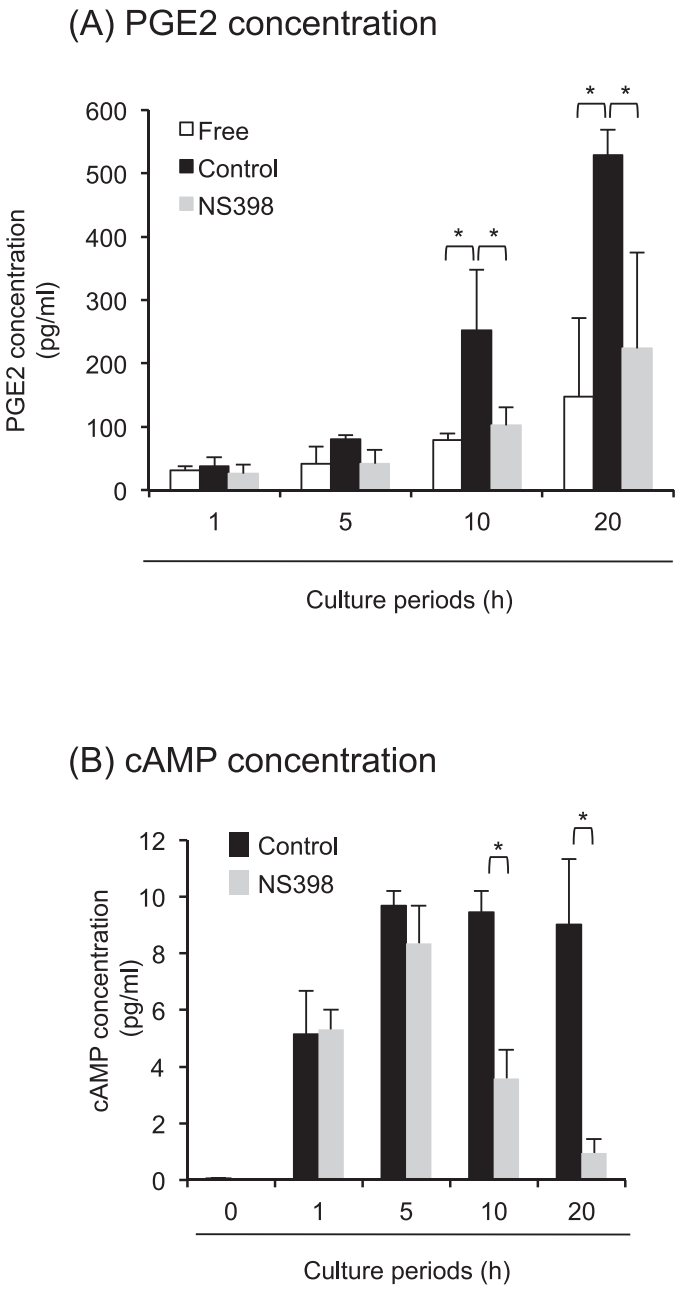

FIG. 3. Effect of NS398 on PGE2 concentration in cultured medium (**A**) and cAMP concentration in cumulus cells of COCs (**B**) are shown. **A**) COCs were cultured in FSH-containing medium with or without NS398 for 20 h. At the selected time points, cultured medium was collected and used for detection of PGE2 level by using the EIA method. **B**) COCs were cultured with FSH and NS398 for 0, 1, 5, 10, or 20 h. Cumulus cells were lysed, and cAMP levels were examined. Values are means ± SEM from three independent culture experiments. *, Indicates significant differences were observed compared with cumulus cells of COCs cultured with FSH ($P < 0.05$). Free, COCs were cultured without FSH; control, COCs were cultured with FSH; NS398, COCs were cultured with FSH and NS398.

Western Blot Analysis

Cumulus cells from cultured COCs were lysed in Laemmli sample buffer. After samples were denatured by boiling for 5 min, 10 μl of each sample containing equal amounts of protein (10 μg) was separated by SDS-PAGE on 10% polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes (GE Healthcare, Newark, NJ). Membranes were blocked with 5% (w/v) nonfat dry milk (GE Healthcare) in PBS. Primary antibodies were added in a solution of 2.5% (w/v) nonfat dry milk in 0.1% (v/v) Tween-20 (Sigma)-PBS (PBS-T) and incubated overnight at 4°C. A rabbit polyclonal antibody against human ADAM17 (Sigma) was used at a dilution of 1:2000. Anti-phospho-MAPK3/1, total MAPK3/1, and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and diluted 1:2000, 1:1000, and 1:10 000, respectively. After four washes in PBS-T, the membranes were incubated for 1 h with a 1:2000 dilution of goat anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG horseradish peroxidase-linked antibody (Cell Signaling Technology, Inc.) in 2.5% (w/v) nonfat dry milk in PBS-T at room temperature. After membranes were washed five times for 10 min each with PBS-T, peroxidase activity was visualized using the ECL Western blot detection system (GE Healthcare) according to the manufacturer's instructions.

Determination of TACE/ADAM17 Activity

Cumulus cells of cultured COCs were lysed in 25 mM of Tris buffer, pH 7.4, containing 1% (v/v) Triton X-100 (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), leupeptin (Sigma) at 10 μg/ml, and 3 mM aprotinin (Sigma). Lysed samples were centrifuged, and supernatants were stored at −80°C until analyzed. Twenty micrograms of protein extract was used to determine protease activity, using 4 mM fluorogenic peptide III (R&D Systems, Minneapolis, MN) as a substrate. TACE/ADAM17 cleaved this peptide sequence between Ala and Val, separating the fluorochrome from the quencher, thus allowing detection of fluorescence. The reaction was performed at 37°C, and the fluorescence intensity was determined by spectofluorometer (Shimazu, Kyoto, Japan) using

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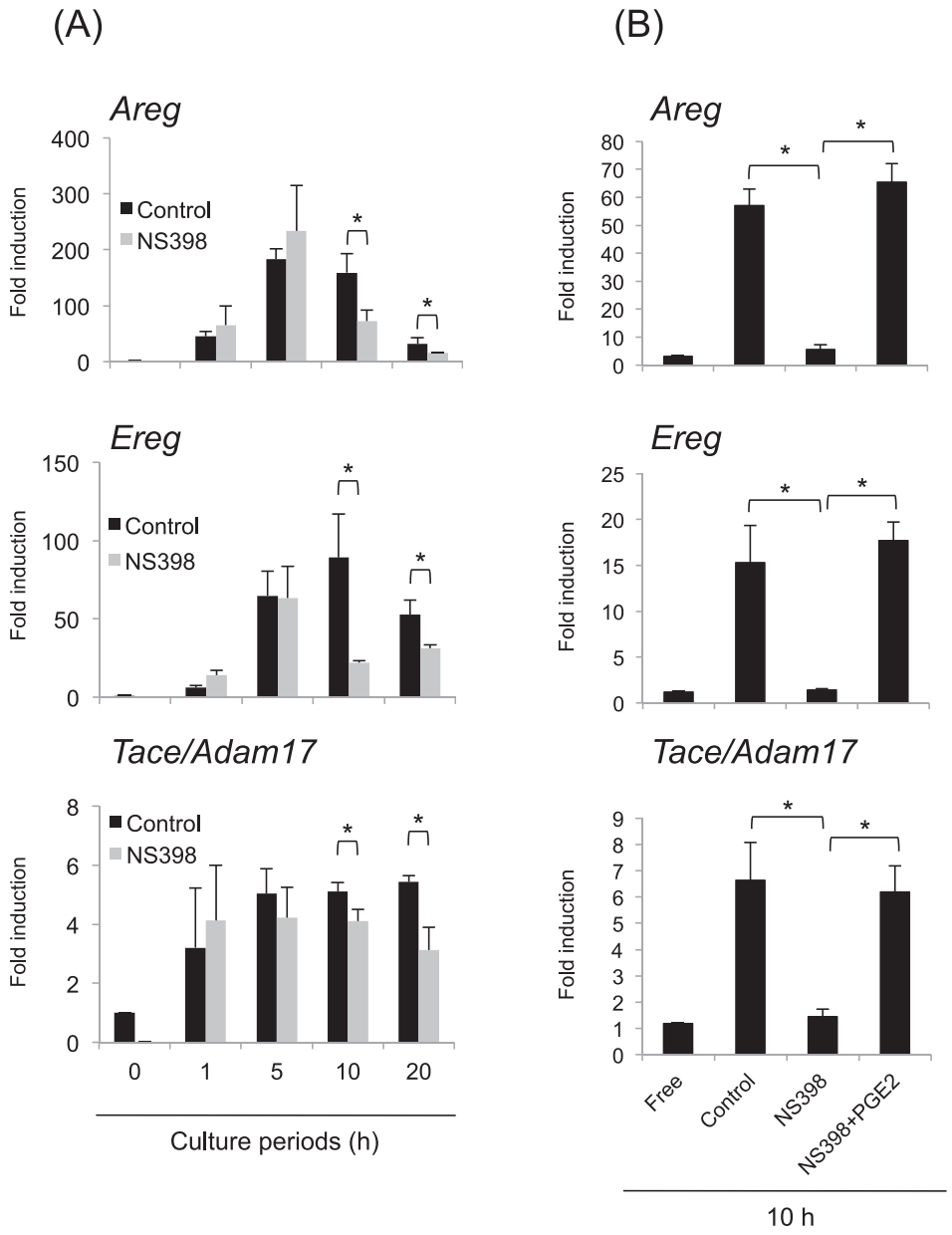

FIG. 4. Effect of NS398 on *Areg*, *Ereg*, or *Tace/Adam17* mRNA expression level in cumulus cells of COCs is shown. **A**) COCs were cultured with FSH for 0, 5, 10, or 20 h. For reference, the 0-h COC value was set as 1, and data are presented as the fold-change in induction. Values are means ± SEM from three independent culture experiments. *, Indicates significant differences were observed compared with cumulus cells of COCs cultured with FSH and NS398 ($P < 0.05$). Control, COCs were cultured with FSH; NS398, COCs were cultured with FSH and NS398. **B**) COCs were cultured for 10 h without any ligand or in drug-free medium, or in medium containing FSH (control), FSH plus NS398 (NS398), or FSH plus NS398 plus PGE2 (NS398+PGE2).

an excitation wavelength of 320 nm and an emission wavelength of 405 nm for 105 min.

Statistical Analysis

Statistical analyses of all data from three or four replicates for comparison were carried out by one-way ANOVA followed by Duncan's multiple-range test (Statview software; Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arcsine transformation before analysis.

RESULTS

Kinetic Changes in Ptger2 *and* Ptger4 *Expression in Cumulus Cells and Granulosa Cells of Ovulating Follicles*

We previously showed in a pig in vivo model that cumulus expansion and ovulation were usually detected at approximately 20 and 40 h, respectively, after hCG injection [15]. However, EGF-like factors, the *Ptgs2* and MAPK3/1 target genes (*Has2* and *Tnfaip6*), were induced by eCG but not by hCG in cumulus cells. In this study, we investigated whether levels of *Ptger2* and *Ptger4* mRNA expression were also induced by eCG, or not.

Expression levels of *Ptger2* and *Ptger4* mRNA in cumulus and granulosa cells were relatively low in small antral follicles (3–5 mm; immature), whereas these expression levels were rapidly and significantly increased at 72 h after eCG injection (Fig. 1, A and B). However, these high expression levels in cumulus and granulosa cells were significantly decreased at 6 h post-hCG stimulation, and the low levels were still detected 24 h after hCG stimulation (Fig. 1, A and B).

Kinetic Changes in Fshr, Ptger2, Ptger4, Ptgs2, *and* Egfr *Expression in Cumulus Cells of COCs Cultured with FSH In Vitro*

When COCs were cultured with FSH for up to 20 h, *Fshr* mRNA expression was immediately and significantly decreased within 5 h compared to that in cumulus cells of COCs just after collection (Fig. 2). In the PGE2 receptors, *Ptger2* and

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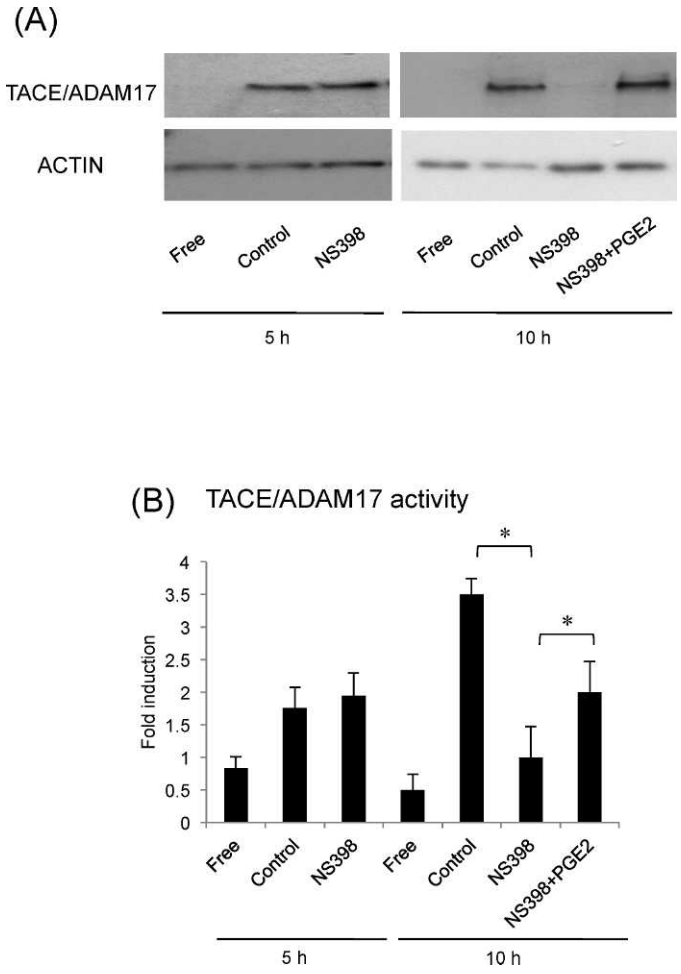

FIG. 5. Effect of NS398 on TACE/ADAM17 protein level (**A**) and TACE/ADAM17 protease activity (**B**) in cumulus cells of COCs are shown. COCs were cultured with FSH and NS398 for 5 or 10 h. Some COCs were cultured with FSH and NS398 for 10 h. Values are means ± SEM from three independent culture experiments. *, Indicates significant differences were observed compared with cumulus cells of COCs cultured with FSH and NS398 ($P < 0.05$). Free, COCs were cultured without FSH; control, COCs were cultured with FSH. NS398, COCs were cultured with FSH and NS398; NS398+PGE2, COCs were cultured with FSH, NS398, and PGE2.

Ptger4 expression levels were significantly increased during culture with the induction of *Ptgs2* mRNA (Fig. 2). The maximum *Ptgs2* mRNA level was reached at the 10-h culture point and then declined to a level similar to that detected in cumulus cells of COCs just after collection (Fig. 2). *Egfr* mRNA expression was increased by cultivation with FSH for 5 h and maintained high expression level at 20 h (Fig. 2).

Effects of NS398 on Secretion of PGE2 and cAMP Concentration in Cumulus Cells of COCs Cultured with FSH

PGE2 levels in the medium in which COCs were cultured without FSH (FSH-free medium) for 1, 5, 10, or 20 h were 31.9 ± 5.3 pg/ml, 41.8 ± 27.1 pg/ml, 78.5 ± 9.2 pg/ml, or 147.5 ± 123.6 pg/ml, respectively. The levels were significantly increased by FSH stimuli at 10 h (253.0 ± 95.7 pg/ml) or 20 h (529.0 ± 40.5 pg/ml) (Fig. 3A). These inductions were significantly suppressed by NS398 to the levels detected in FSH-containing maturation medium cultured for 10 or 20 h (Fig. 3A).

When COCs were cultured with FSH for 0, 1, 5, 10, or 20 h, cAMP concentration in cumulus cells was significantly increased within a 1-h culture point and then reached the maximum level (9.7 ± 0.5 pmol/ml) at 5 h (Fig. 3B). At the 5-h culture period, the negative effect of NS398 on cAMP level was not observed in cumulus cells of COCs compared to the cAMP level observed in cumulus cells cultured with FSH only (control). However, at the 10-h culture period, the level of cAMP in the NS398-treated group was significantly decreased compared to that in the control group (3.6 ± 1.0 vs. 9.4 ± 0.8 pmol/ml, respectively), and a significantly decreased level was also detected in the inhibitor-treated group at the 20-h culture period (Fig. 3B).

Effect of NS398 on Areg, Ereg, *or* Tace/Adam17 *Expression in Cumulus Cells of COCs Cultured with FSH*

In our previous study, we showed that *Ptgs2* mRNA was up-regulated after eCG injection and that the induction was synchronous with expression levels of *Areg*, *Ereg*, and *Tace/Adam17* mRNA in vivo in pig [18]. To investigate the physiological role of PGE2 in gene expression during in vitro maturation of porcine COCs, COCs were cultured with FSH and the inhibitor NS398 for 0, 1, 5, 10, or 20 h.

When COCs were cultured with FSH and NS398 for 0, 1, or 5 h, NS398 treatment did not affect FSH-induced *Areg*, *Ereg,* and *Tace/Adam17* expression levels (Fig. 4A). However, at 10 or 20 h, NS398 significantly suppressed the expression levels of these genes in cumulus cells compared with those in cumulus cells of COCs cultured without the drug. Furthermore, the down-regulated levels caused by NS398 were overcome by the addition of PGE2 to both FSH- and NS398-containing medium (Fig. 4B).

The TACE/ADAM17 protein level and its protease activity were increased after 5 h of culture with FSH (Fig. 5, A and B). At that time point, the negative effects of NS398 on the protein level and its activity were not detected. However, after cells were cultured for 10 h with FSH, the addition of NS398 led to a decrease in protein level and activity compared with those in cumulus cells cultured without the inhibitor (Fig. 5, A and B).

Effect of NS398 on MAPK3/1 Phosphorylation in Cumulus Cells

When COCs were cultured with FSH for 1, 5, 10, or 20 h, the phosphorylation status of MAPK3/1 was sustainably detected in cumulus cells at any of the time points (Fig. 6A). At the early time points (1 or 5 h), NS398 had no effect on the phosphorylation of MAPK3/1 (Fig. 6A), whereas, at 10 or 20 h, the intensity was markedly decreased by NS398 (Fig. 6A). At the 10-h culture point of COCs, the negative effects of NS398 on MAPK3/1 phosphorylation were overcome by the addition of either PGE2 or EGF (Fig. 6B). In COCs treated with the inhibitor AG1478, neither PGE2 nor EGF induced phosphorylation of MAPK3/1 in cumulus cells of COCs (Fig. 6B).

Effect of NS398 on Has2 *and* Tnfaip6 *mRNA Expression in Cumulus Cells of COCs*

To investigate the effects of NS398 on *Has2* or *Tnfaip6* mRNA expression in cumulus cells, COCs were cultured in FSH-containing medium with or without NS398 for 5–20 h (Fig. 7, A and B). The expression of *Has2* mRNA was significantly increased in response to FSH in cumulus cells of COCs cultured for 10 or 20 h. At the 5-h point, the

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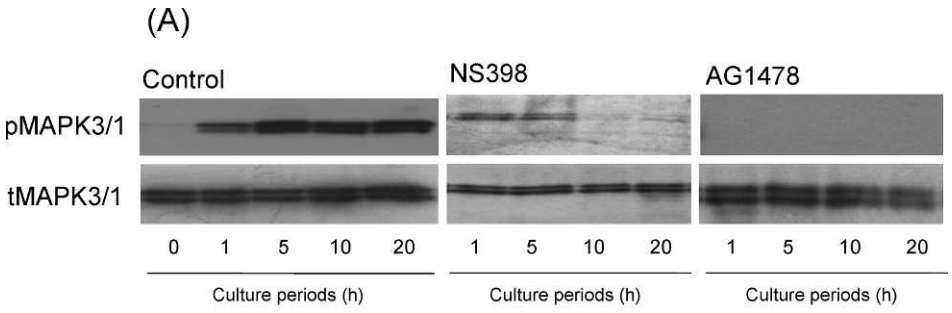

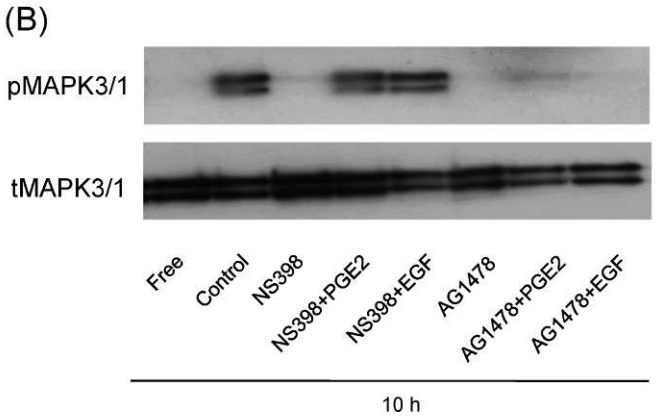

FIG. 6. Effect of NS398 or AG1478 on MAPK3/1 phosphorylation in cumulus cells of COCs is shown. **A**) COCs were cultured with FSH and NS398 or AG1478 for 1, 5, 10, or 20 h. **B**) COCs were cultured with FSH and NS398 or AG1478 for 10 h. PGE2 or EGF was used to test recovery from these inhibitors of phosphorylation of MAPK3/1 in cumulus cells. MAPK3/1 phosphorylation in cumulus cells was analyzed by Western blotting. Free, COCs were cultured without FSH; control, COCs were cultured with FSH; NS398, COCs were cultured with FSH and NS398; NS398+PGE2, COCs were cultured with FSH, NS398, and PGE2; NS398+EGF, COCs were cultured with FSH, NS398, and EGF.

pharmacological effect of NS398 in cumulus cells was not observed (Fig. 7A), whereas *Has2* expression in cumulus cells cultured for 20 h was significantly suppressed by NS398 compared to that in control cells (Fig. 7B). At this culture point, the inhibitory effect on *Has2* expression was overcome by the addition of either PGE2 or EGF. *Tnfaip6* mRNA expression was also increased in response to FSH stimulation at 5 or 20 h of culture (Fig. 7A). At 5 h, a negative effect of NS398 was not observed (Fig. 7B), whereas at the 20-h point, FSH-induced gene expression was down-regulated by the drug (Fig. 7B). The negative effect of NS398 was overcome by the addition of either PGE2 or EGF to the maturation medium.

Effect of NS398 on Induction of Cumulus Expansion of COCs During In Vitro Maturation

To examine the effects of NS398 on cumulus expansion, COCs were cultured for 0, 10, 20, 30, and 40 h. In the cell group without FSH and NS398 (FSH-free medium), the diameter of COCs was not dramatically changed by the culture up to 40 h (Fig. 8A). In response to FSH stimulation, the diameter of COCs was significantly increased from 20 h to 40 h. The increase in COCs diameter by FSH stimulation was significantly suppressed by NS398 at 30 and 40 h (Fig. 8A). Furthermore, the negative effect induced by NS398 on COCs diameter was overcome by the addition of either PGE2 or EGF (Fig. 8B).

Effect of NS398 on Meiotic Progression of Oocyte During In Vitro Maturation of COCs

Figure 9, A and B, shows the effect of NS398 on the meiotic progression of the oocyte (germinal vesicle breakdown [GVBD] rate and MII rate). When COCs were cultured without FSH for 20 h (FSH-free medium), the GVBD rate was less than 40%. Treatment with only NS398 did not induce spontaneous meiotic maturation of oocytes (Fig. 9A). FSH significantly elevated the GVBD rate of oocytes compared with oocytes cultured without FSH (65.21 ± 2.99%). The addition of NS398 to FSH-containing medium did not affect the rate of oocyte maturation (65.21 ± 2.99% vs. 56.42 ± 9.11%) (Fig. 9A). When COCs were cultured without FSH for 40 h, the rate at which oocytes reached MII stage (MII rate) was less than 40%. FSH significantly increased the MII rate compared with that of oocytes cultured without FSH (71.4 ± 5.27%). However, the induction was significantly decreased by NS398 (58.95 ± 4.47%). The lower MII rate was significantly increased by the addition of either PGE2 (73.42 ± 8.5%) or EGF (74.63 ± 6.76%) to the maturation medium (Fig. 9B).

DISCUSSION

The LH surge from the pituitary gland acts strongly on granulosa cells of preovulatory follicles to induce the ovulation process, including cumulus expansion and oocyte maturation. However, stimuli effects were transient because down-regulation of the receptor was observed at both the post-transcriptional and transcriptional levels [18]. Additionally, cumulus cells have less LH receptor, and oocytes do not have the receptor [24]. Thus, the mediators secreted from granulosa cells by LH stimuli are required for the induction of the ovulation process. It is well known that PGE2 is one of the mediators secreted from granulosa cells and that it then acts on both granulosa and cumulus cells [9, 10]. The other mediators are EGF-like factors that are also produced in granulosa cells, and the specific receptor is expressed in both cumulus and

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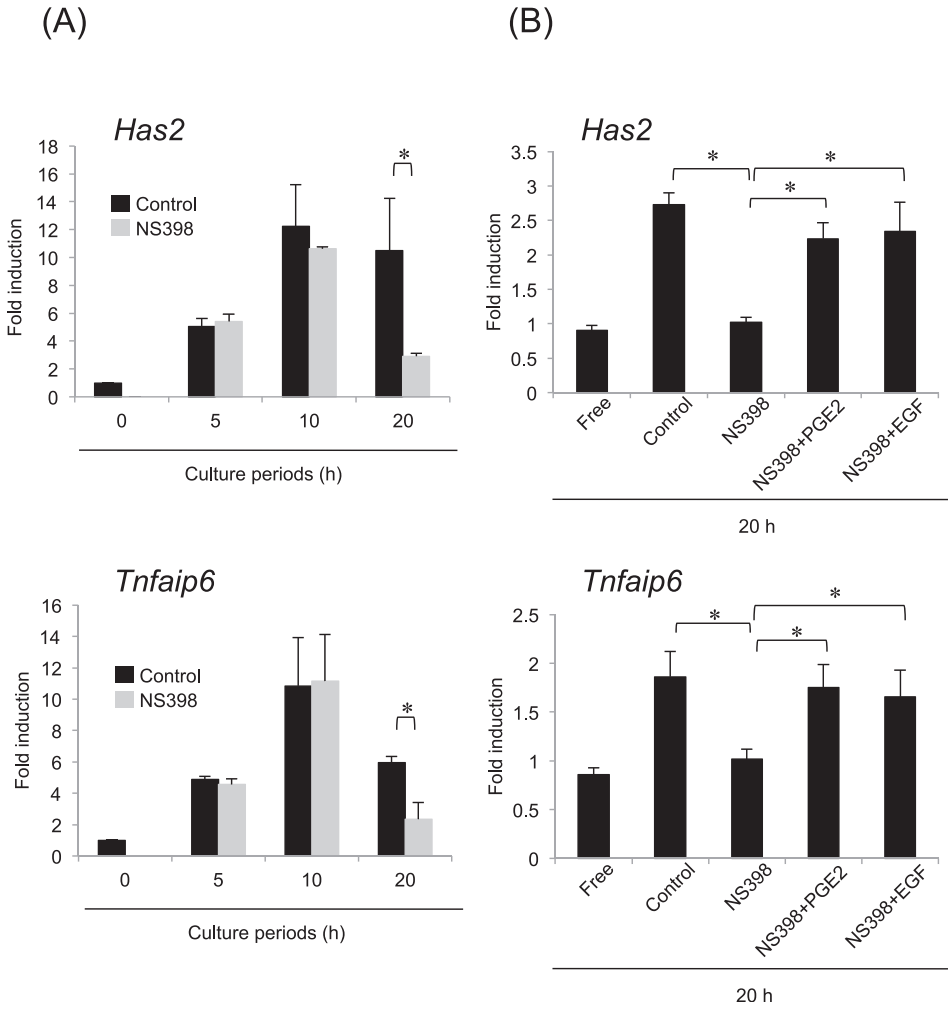

FIG. 7. Effect of NS398 on *Has2* and *Tnfaip6* mRNA expression is shown in cumulus cells of COCs. **A**) COCs were cultured with FSH and NS398 for 0, 5, 10, or 20 h. **B**) COCs were cultured with FSH and NS398 for 20 h. For reference, the 0-h COC value was set as 1, and data are presented as the fold-change in strength. Values are means ± SEM from three independent culture experiments. *, Indicates significant differences were observed compared with cumulus cells of COCs cultured with FSH and NS398 ($P < 0.05$). Free, COCs were cultured without FSH; control, COCs were cultured with FSH; NS398, COCs were cultured with FSH and NS398; NS398+PGE2, COCs were cultured with FSH, NS398, and PGE2; NS398+EGF, COCs were cultured with FSH, NS398, and EGF.

granulosa cells [11, 12, 25]. The mutant mice model of the PGE2 pathway and EGF-like factors pathway showed that both pathways are essential for the oocyte maturation process.

In in vitro maturation of mammalian oocytes, including those of pig, COCs were recovered from 3- to 5-mm antral follicles but not from preovulatory follicles. In cumulus cells of porcine COCs, the *Lhcgr* expression level is much lower than that in granulosa cells of preovulatory follicles [18]. Thus, the addition of LH to maturation medium did not enhance cumulus expansion, progesterone production, and oocyte maturation

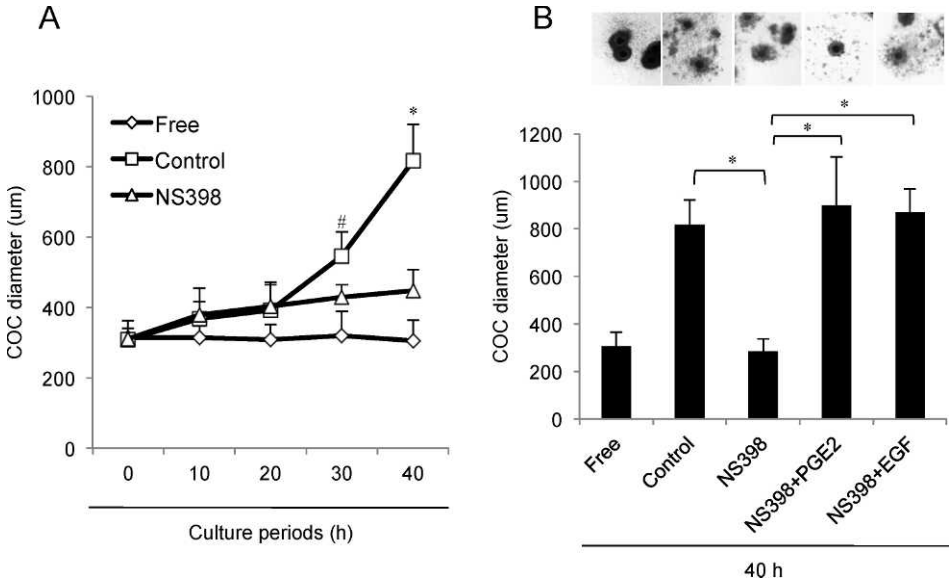

FIG. 8. Effect of NS398 on the COC diameter during in vitro maturation of porcine COCs is shown. **A**) COCs were cultured with FSH and NS398 for 0, 5, 10, 20, 30, or 40 h. **B**) COCs were cultured with or without NS398 in FSH-containing medium. PGE2 or EGF was used to test for recovery from inhibitor effects on the diameter of COCs cultured for 40 h. Values are means ± SEM from three replicates. #, Indicates significant differences were observed compared with cumulus cells of COCs cultured with FSH and NS398 for 30 h ($P < 0.05$). *, Indicates significant differences were observed compared with COCs cultured with FSH and NS398 for 40 h ($P < 0.05$). Free, COCs were cultured without FSH; control, COCs were cultured with FSH; NS398, COCs were cultured with FSH and NS398; NS398+PGE2, COCs were cultured with FSH, NS398, and PGE2; NS398+EGF, COCs were cultured with FSH, NS398, and EGF.

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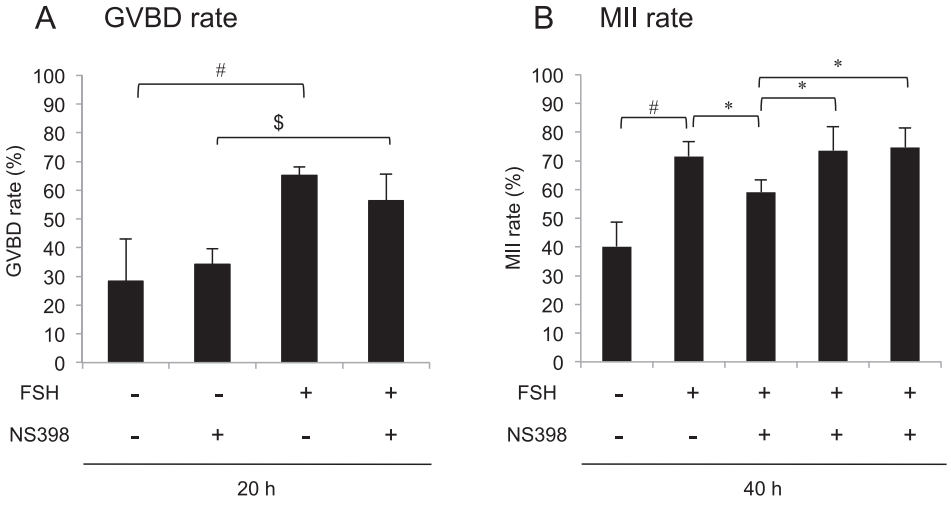

FIG. 9. Effect of NS398 on the proportion of oocytes in which GVBD was induced (**A**) and the proportion that reached MII stage (**B**) is shown. COCs were cultured with or without FSH and NS398 for 20 h (**A**) or 40 h (**B**). PGE2 or EGF was used to test recovery from the inhibitor effects by the proportion of oocytes exhibiting GVBD (**A**) versus those that reached MII stage. Values are means ± SEM from three replicates. $^{\#}$, Indicates significant differences were observed compared with oocytes of COCs cultured without FSH. $^{\$}$, Indicates significant differences were observed compared with oocytes of COCs cultured with NS398. *, Indicates significant differences were observed compared with oocytes of COCs cultured with FSH and NS398 for 40 h. FSH, COCs were cultured with FSH for 20 or 40 h; NS398, COCs were cultured with NS398 for 20 or 40 h.

[17, 26]. On the other hand, because the *Fshr* gene is highly expressed in cumulus cells [18], FSH is usually added to the in vitro oocyte maturation medium. In this study, FSH increased cAMP levels in cumulus cells, and the cAMP-dependent pathway induced cumulus expansion and oocyte maturation. The time schedule in in vitro mimics that in the in vivo model, in which cumulus expansion started 24 h after hCG injection, and the first polar body was detected 40 h after hCG injection [18]. However, the FSH receptor (*Fshr* expression) level was markedly decreased within 5 h in cumulus cells of COCs in vitro. Interestingly, down-regulation of the *Fshr* mRNA level was also observed in cumulus cells within 6 h after hCG injection in pig [18]. Moreover, the circular level of FSH is elevated concomitantly with LH surge [27]. Therefore, it is possible that because FSH acts on cumulus cells but stimuli are transient, the second factor to act on the cumulus cell itself is essential to maintain cAMP production during in vitro maturation of the oocyte.

In mouse ovary, LH stimuli increase expression of EGF-like factors in granulosa and cumulus cells in a cAMP-CREB-dependent manner [13]. Because the *Mapk3/1* genes of ovarian-specific knockout mice, which are activated by EGF-like peptide via EGFR, do not induce COC expansion or meiotic maturation and ovulation, the EGF-like factor MAPK3/1 pathway is essential for the ovulation process [28]. EGF-like factor expression is induced within 4 h after injection of hCG in mouse granulosa and cumulus cells, whereas in pig, the expressions are maintained for up to 12 h after hCG injection [18]. These results suggested that the expression levels were regulated not only by the LH surge but also by other secondary factors expressed by the LH surge. In the previous and present studies, we showed that *Fshr* mRNA was down-regulated, whereas *Ptgs2*, *Ptger2*, and *Ptger4* mRNA were increased after eCG injection in cumulus cells in vivo [18]. In vivo study also showed that expression levels of the *Has2* and *Tnfaip6* cumulus expansion-related genes were increased in response to eCG [18]. These expression patterns were coincident with reduction of *Fshr* mRNA expression and *Ptger2*, *Ptger4*, and *Ptgs2* up-regulation in vivo. In vitro study also showed similar results, that is, FSH induced *Ptger2*, *Ptger4*, and *Ptgs2* expression and cumulus expansion-related genes in cumulus cells of COCs. Furthermore, addition of NS398 to the maturation medium inhibited expression of EGF-like factors and cumulus expansion-related genes, phosphorylation of MAPK3/1, and cumulus expansion and oocyte maturation. Because the in vivo study using the mouse model showed that administration of a PTGS2 inhibitor impaired cumulus expansion and ovulation [5], we estimated in pig that PGE2 worked as the second messenger to maintain the expression of EGF-like factors, which induces physiological maturation process in vivo and in vitro.

During in vitro maturation of porcine COCs, we considered that the addition of PGE2 to maturation medium improved the in vitro culture condition in pig. However, we usually collected COCs from prepubertal gilt so that cumulus cells of the COC had not yet formed PGE2 receptors. Therefore, following supplementation of PGE2, after 10 h of cultivation, with FSH to await the formation of PGE2 receptors effectively and physiologically induced adequate oocyte maturation under in vitro conditions in pig. Furthermore, we previously showed that the supplemental number of COCs in maturation medium is critical for meiotic maturation of porcine oocytes because of alternation of concentration of maturation-inducing factors [28]. The result suggested that a high concentration of PGE2 in maturation medium is essential for the maturation process. In this study, 500 pg/ml PGE2 was added to maturation medium; however, we could not determine whether the concentration was sufficient to the maturation condition of oocytes or not. Thus, further study is required to answer the above-outlined questions.

In conclusion, we showed that levels of EGF-like factors and TACE/ADAM17 expression in cumulus cells were first induced in an FSH-dependent manner within 5 h of cultivation. Secreted EGF-like factors by TACE/ADAM17 enhanced activation of EGFR downsteam signaling pathway in cumulus cells, which enhanced induction of *Ptgs2* expression. Produced PGE2 maintained EGF-like factor mRNA expression levels during in vitro maturation of porcine COCs. The sequential induction of expression of EGF-like factors enhanced sustainable activation of MAPK3/1 in cumulus cells, which resulted in induction of cumulus expansion and oocyte maturation during in vitro maturation of porcine COCs.

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