Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and ovulation-related genes that impact oocyte maturation *in vivo* and *in vitro*

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Abstract

In this study, we collected follicular fluid, granulosa cells, and cumulus cells from antral follicles at specific time intervals following equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) treatment of gilts. The treatment with eCG increased the production of estrogen coordinately with up-regulated proliferation of granulosa and cumulus cells. eCG also induced the expression of *LHCGR* and *PGR* in cumulus cells and progesterone accumulation was detected in follicular fluid prior to the LH/hCG surge. Moreover, progesterone and progesterone receptor (*PGR*) were critical for FSH-induced *LHCGR* expression in cumulus cells in culture. The expression of *LHCGR* mRNA in cumulus cells was associated with the ability of LH to induce prostaglandin production, release of epidermal growth factor (EGF)-like factors, and a disintegrin and metalloprotease with thrombospondin-like repeats 1 expression, promoting cumulus cell oocyte complexes (COCs) expansion and oocyte maturation. Based on the unique expression and regulation of *PGR* and *LHCGR* in cumulus cells, we designed a novel porcine COCs culture system in which hormones were added sequentially to mimic changes observed *in vivo*. Specifically, COCs from small antral follicles were pre-cultured with FSH and estradiol for 10 h at which time progesterone was added for another 10 h. After 20 h, COCs were moved to fresh medium containing LH, EGF, and progesterone. The oocytes matured in this revised COC culture system exhibited greater developmental competence to blastocyst stage. From these results, we conclude that to achieve optimal COC expansion and oocyte maturation in culture the unique gene expression patterns in cumulus cells of each species need to be characterized and used to increase the effectiveness of hormone stimulation.

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Introduction

During the development of mammalian ovarian follicles, the pituitary gonadotropin follicle-stimulating hormone (FSH) is essential for granulosa cells' estrogen production (estradiol 17 β , E₂) and increased estrogen concentrations in follicular fluid. In follicles and more specifically in granulosa cells, estradiol acts via intracrine mechanisms involving estrogen receptor type β (ER β or ESR2) to enhance granulosa cell responses to FSH (Richards 1994, Tremblay *et al.* 1997). In ER β (*Esr2*) knockout mice, granulosa cells within developing follicles fail to differentiate and do not express genes critical for ovulation (Couse *et al.* 2005), suggesting that the estrogen–ER β

pathway plays an important role in the development of follicles that are competent to respond to the luteinizing hormone (LH) surge and ovulate. The temporal pattern of steroid hormone production within follicles changes dramatically following the LH surge and the induction of ovulation and luteinization (Ainsworth *et al.* 1980, Richards 1994). Genes controlling estradiol biosynthesis are turned off, whereas genes controlling the production of progesterone are elevated (Richards 1994). The LH surge also induces in preovulatory follicles the production of prostaglandins, principally PGE₂ (Zor & Lamprecht 1977, Hedin *et al.* 1987) and epidermal growth factor (EGF)-like growth factors that impact ovulation process, cumulus cell oocyte complex (COC) expansion, and

meiotic resumption of meiosis (Park *et al.* 2004, Hsieh *et al.* 2007). Using both progesterone receptor (*PGR*) null mice and prostaglandin synthase 2 (*PTGS2*) null mice, we recently showed that LH induction of the EGF-like growth factors, amphiregulin (*Areg*) and epiregulin (*Ereg*), was dependent on both *PGR*- and *PGE*₂-dependent signaling pathways (Shimada *et al.* 2006). Thus, in mice, FSH-induced production of estrogen stimulates follicular development to the preovulatory stage and LH surge induces secondary factors, such as progesterone, *PGE*₂, and EGF-like factors that mediate cumulus expansion and oocyte maturation.

In the pig ovary, numerous early antral follicles are present at the pro-estrous stage of the estrous cycle (Ainsworth *et al.* 1980). In response to equine chorionic gonadotropin (eCG) injection, the small antral follicles (more than 3 mm in diameter) enlarge to more than 10 mm diameter reaching a preovulatory stage of follicle development by 72 h post hormone stimulation. Ainsworth *et al.* (1980) reported that not only estrogen production but also progesterone accumulation were increased after eCG injections alone. Moreover, the functional roles of progesterone in the ovulation process of mice and pigs are different. Whereas *PGR* null mice (PRKO) exhibit impaired ovulation, the matured oocytes recovered from these follicles were developmentally competent after *in vitro* fertilization (Lydon *et al.* 1995). By contrast, in the pig, either a progesterone synthesis inhibitor or *PGR* antagonist potentially suppressed the cumulus cell functions (Shimada *et al.* 2004a, 2004b), the meiotic resumption (Yamashita *et al.* 2003), and the developmental competence of oocytes (Shimada *et al.* 2004b). Moreover, in pig follicles, the *PGR* is expressed in both cumulus and granulosa cells when cultured with FSH alone (Shimada *et al.* 2004c) or recovered from eCG-primed gilts (Slomaczynska *et al.* 2000). These observations suggest that, in the pig, progesterone as well as estrogen impacts functional changes in cumulus and granulosa cells that are critical for the acquisition of responses to the ovulatory LH signal. Although *PGE*₂ accumulates within follicular fluid and the level was significantly increased after gonadotropin surge (Hunter & Poyser 1985, Xie *et al.* 1990), little is known about what regulates the production of *PGE* in porcine follicles. Moreover, there is little information in the pig model about the time course changes in the expression genes encoding steroidogenic enzymes, the effects of steroid hormones on cumulus cell function, the regulated expression of the EGF-like growth factors or *PTGS2* mRNA by LH, or the role of the factors in cumulus cell-mediated regulation of oocyte maturation *in vivo* and *in vitro*.

Characterizing the mechanisms by which hormones control follicular development to the preovulatory stage and ovulation *in vivo* provides basic information for applying and improving hormonal treatments to stimulate successful maturation of porcine oocytes *in vitro*.

For *in vitro* maturation of pig oocytes, COCs are typically recovered from small antral follicles (3–5 mm in diameter; Funahashi *et al.* 1994, 1997, Shimada *et al.* 2002, 2003) because oocytes recovered from COCs of follicles greater than 3 mm in diameter and cultured with FSH and LH can resume meiosis and progress to the metaphase II stage (Sun *et al.* 2004). However, *in vitro*-matured oocytes exhibit limited developmental competence as revealed by their inability to develop beyond the blastocyst stage. Additionally, cumulus cells of COCs recovered from small antral follicles exhibit limited responsiveness to LH (Shimada *et al.* 2003) and EGF (Prochazka *et al.* 2003), indicating that treatment with FSH is required for cumulus cells to acquire responses to LH and thereby mimic conditions *in vivo*. Thus, we hypothesized that cumulus cells of COCs recovered from small antral follicles exhibit minimal responses to LH due to their early stage of development and limited exposure to FSH and/or steroid hormones.

To define the *in vivo* and *in vitro* conditions that permit optimal maturation of pig oocytes, we collected follicular fluid, granulosa, and cumulus cells from follicles beginning at the small antral stage (3–5 mm) to preovulatory stage (more than 10 mm) following the treatment of swine with exogenous eCG. Additionally, the cumulus cells of COCs were recovered from the ovaries of eCG-primed swine at selected times after treatment with human chorionic gonadotropin (hCG). Using these samples, we documented that the temporal changes of steroid hormone production and the expression patterns of specific genes including those encoding the EGF-like factors were distinct compared with those observed in mouse models. On the basis of the unique porcine mechanisms *in vivo*, we altered the *in vitro* culture conditions for oocyte maturation (Fig. 1) and showed that if COCs are cultured first with FSH and steroid hormone and then cultured with LH and EGF-like factors, oocyte maturation, and blastocyst development are improved.

Figure 1 A schematic with regard to the novel culture system of porcine COCs when compared with two-step culture as described in our previous study (Shimada *et al.* 2003) or plain one-step culture.

Results

Experiment 1

Steroid hormone production

The mean concentrations of E_2 , progesterone, and testosterone in each follicular fluid sample obtained at various times after eCG/hCG treatment are presented in Table 1. The follicular concentration of testosterone was significantly higher in the small antral follicles (3–5 mm in diameter) than in larger follicles (> 5 mm diameter) with or without eCG treatment. By contrast, levels of E_2 were up-regulated significantly in middle-sized follicles (5–10 mm in diameter) and then further increased at 72 h after eCG injection. In these same samples, progesterone concentrations also increased significantly when compared with those in the follicles without hormone stimulation.

Changes in the expression of mRNAs encoding steroidogenic genes exhibited patterns of expression that coordinated with steroid production. High levels of aromatase (*CYP19A1*) mRNA was detected in the granulosa cells of middle-sized follicles and large antral follicles obtained at 72 h after eCG injection whereas *CYP19A1* mRNA levels decreased significantly following hCG stimulation (Fig. 2). Although the expression levels of P450scc (*CYP11A1*) mRNA in granulosa cells did not change during follicular development or ovulation, expression of 3 β HSD (*HSD3B1*) mRNA was up-regulated significantly after 72 h eCG priming; the level was maintained after hCG injection (Fig. 2).

The expression of *PTGS2*, EGF-like factors (*AREG* and *EREG*), and a specific protease (*TACE/ADAM17*) in granulosa cells

The expression of *PTGS2* mRNA in granulosa cells was low in small antral follicles and remained low even at 72 h after eCG injection. However, *PTGS2* mRNA increased rapidly and significantly at 12-h post-hCG stimulation and the high level was still detected at 24 h after hCG stimulation (Fig. 3). By contrast, induction of the EGF-like factor (*AREG* and *EREG*) mRNA was observed at 72 h after eCG injection, maintained at

12 h post hCG, and decreased after 24 h hCG stimulation. The specific protease, *TACE/ADAM17* mRNA, increased in granulosa cells only at 12 h post hCG (Fig. 3).

Gonadotropin receptor (*FSHR* and *LHCGR*) mRNA expression in granulosa cells and cumulus cells

The expression of *FSHR* mRNA in both granulosa cells and cumulus cells was low in small antral follicles (3–5 mm in diameter). The levels were significantly increased to 10 mm in size during the development of follicles. However, the injections of eCG significantly down-regulated *FSHR* mRNA levels in granulosa cells (Fig. 4).

Three splice variants of *LHCGR* mRNA, in addition to the largest transcript, were detected in both granulosa and cumulus cells. The intensity of large transcript in both cell types increased significantly after eCG priming. However, after hCG stimulation, the levels of *LHCGR* mRNA transcripts decreased dramatically in both cell types (Fig. 4).

The gene expressions involved in cumulus cell differentiation in vivo

The expression of the cell-cycle regulators, cyclin D2 (*CCND2*) and p27^{kip1} (*CDKN1B*), was significantly higher in cumulus cells obtained from middle-sized follicles (5–10 mm in diameter) than in cells from small antral follicles (3–5 mm in diameter) and decreased in response to eCG (Fig. 5). Low levels were maintained until 48 h after post hCG. By contrast, expression of *AREG* mRNA increased following eCG at 72 h and a further significant increase at 12-h post hCG. However, the increase in cumulus cells was only 5-fold when compared with 40-fold in granulosa cells (Fig. 3). In addition to *AREG* mRNA, the expression of other genes (*EGFR*, hyaluronan synthase 2 (*HAS2*), tumor necrosis factor α -induced protein 6 (*TNFAIP6*), *PTGS2*, and *PGR*) was increased dramatically in COCs obtained from eCG- (72 h) and hCG- (12 h) treated gilts (Fig. 5). Unlike these genes, significant increases in a disintegrin and metalloprotease with thrombospondin-like repeats 1 (*ADAMTS1*) and *ADAM17*

Table 1 Steroid hormone concentration (mean $\pm$ S.E.M., ng/ml) in follicular fluid of gilts treated with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to induce follicular growth and ovulation.

Follicle stage	Testosterone	Estradiol 17 β	Progesterone
3–5 mm	85.1 $\pm$ 12.6 ^a	28.5 $\pm$ 5.2 ^d	11.6 $\pm$ 2.1 ^g
5–10 mm	42.6 $\pm$ 9.3 ^b	89.4 $\pm$ 11.3 ^e	55.6 $\pm$ 6.7 ^h
eCG 72 h	15.4 $\pm$ 5.1 ^c	195.6 $\pm$ 21.2 ^f	116.2 $\pm$ 10.4 ⁱ
hCG 24 h	20.6 $\pm$ 3.8 ^c	52.6 $\pm$ 7.4 ^e	211.7 $\pm$ 33.7 ^j

3–5 mm: the small antral follicles (3–5 mm in diameter) were collected from gilts during pro-estrus stage (at day 11 of the estrus cycle). 5–10 mm: the middle size antral follicles were collected from gilts during pro-estrus stage (at day 11 of the estrus cycle). eCG 72 h: the follicular fluids were recovered from the follicles (more than 10 mm in diameter) of gilts 72 h after eCG injection. hCG 24 h: hCG was injected 72 h after eCG injection treatment. After 24-h hCG injection, the follicular fluid was collected from the follicles (more than 10 mm in diameter). Mean $\pm$ S.E.M. values were calculated on the basis of three or four animals sampled using individual animal means in those cases where more than two samples were collected from same animal. ^{a–c}, ^{d–f}, ^{g–j} Columns with no common superscripts are significantly different between groups ($P < 0.05$).

Figure 2 The kinetic changes of gene expression encoded the enzyme for steroid hormone production, *CYP19A1* (aromatase), *CYP11A1* (p450_{scc}), and *HSD3B1*, in granulosa cells during follicular development and ovulation process. Granulosa cells were collected from 3–5 or 5–10 mm follicles of gilts without eCG and hCG injection, from more than 10 mm in diameter follicles of gilts 72 h after eCG injection, or from more than 10 mm in diameter follicles of gilts 6, 12, or 24 h after hCG injection followed by eCG priming. For reference, the 3–5 mm value was set as 1 and the data were presented as the fold increase. Values are mean $\pm$ S.E.M. calculated on the basis of three animals. Bars without common superscript were significantly different ($P < 0.05$).

mRNAs were only detected at 12 and 24 h in *ADAM17* and 24 and 48 h in *ADAMTS1*; and the level of *ADAMTS1* mRNA remained significantly higher in cumulus cells of ovulated COCs 48 h after hCG injection (Fig. 5).

Experiment 2

A low dose of FSH priming enhances the responsiveness of COCs in culture to LH

To improve the responsiveness of cumulus cells of COCs recovered from early antral follicles to the level observed in COCs from preovulatory follicles, we cultured COCs recovered from small follicles with 0, 0.2, 2.0, or 20 ng/ml FSH for 20 h (in the presence of 3-isobutyl-1-methylxanthine (IBMX) to prevent spontaneous resumption of meiosis). We show that 2.0 ng/ml FSH dramatically increased the expression of *LHCGR* mRNA in cumulus cells (Fig. 6A). When COCs were cultured with FSH and E_2 to mimic *in vivo* expression (Fig. 2), the

Figure 3 The expressions of *PTGS2*, *AREG*, *EREG*, and *TACE/ADAM17* are regulated by gonadotropins in granulosa cells during follicular development and ovulation process. Each granulosa cell sample was collected as described in the legend of Fig. 2. For reference, the 3–5 mm value was set as 1 and the data were presented as the fold increase. Values are mean $\pm$ S.E.M. calculated on the basis of three animals. Bars without common superscript were significantly different ($P < 0.05$).

proliferative activity of cumulus cells (incorporation level of BrdU) was increased significantly compared with that in COCs with FSH alone, whereas the expression level of *LHCGR* mRNA was suppressed (Fig. 6B and C). To further mimic the changes in endogenous steroid production (Fig. 2; Table 1), progesterone was added to the FSH- and E_2 -containing medium after 10 h of COC culture. The presence of progesterone significantly suppressed the proliferative activity of cumulus cells when compared with that in COCs cultured with FSH and E_2 ; however, the level was still significantly higher than that in COCs cultured with FSH alone (Fig. 6B). The higher level of *CCND2* mRNA induced by FSH and E_2 was significantly suppressed by the addition of progesterone to the medium (Fig. 6D). The level of *PGR* mRNA was not affected by the

Figure 4 The changes of *FSHR* and *LHCGR* mRNA expression in granulosa and cumulus cells during follicular development and ovulation process. The levels of *FSHR* mRNA were analyzed by real-time RT-PCR. For reference, the 3–5 mm value was set as 1 and the data were presented as the fold increase. Values are mean $\pm$ S.E.M. calculated on the basis of three animals. Bars without common superscript were significantly different ($P < 0.05$). The expressions of *LHCGR* mRNA in cumulus cells of COCs and granulosa cells were detected by RT-PCR. The primer set was used for amplification of four porcine *LHCGR* mRNA isoforms. This primer pair predicts 185, 411, 855, and 1121 bp fragments. β -actin was used as a control for reaction efficiency and variations in concentration of mRNA in the original RT reaction. Results in each panel are representative of three separate experiments.

treatment with progesterone (Fig. 6D). Moreover, the addition of progesterone overcame the negative effects of E_2 on the expression of *LHCGR* mRNA (Fig. 6C).

Experiment 3

The novel culture system of COCs dramatically improved cumulus cell function and oocyte maturation

To determine the functional activity of COCs, they were cultured using a novel culture system, two-step system, or plain one-step system. The diagram of culture conditions is in Fig. 1. The results showed that during the first 20 h of culture in the presence of IBMX, resumption of meiosis at the germinal vesicle stage was completely suppressed (Fig. 7A). Following the 20-h culture period, the COCs were moved to fresh medium containing LH and/or EGF+progesterone. Within 5 h of LH and/or EGF+progesterone stimulation, oocytes resumed meiosis concomitantly with the expansion of the COC matrix diameter. The stimulations also

Figure 5 The kinetic changes of gene expressions, *CCND2*, *CDKN1B*, *AREG*, *EGFR*, *HAS2*, *TNFAIP6*, *PTGS2*, *PGR*, *ADAMTS1*, and *TACE/ADAM17*, in cumulus cells during follicular development and ovulation process *in vivo*. Cumulus cells of COCs were collected as described in the legend of Fig. 2. For reference, the 3–5 mm value was set as 1 and the data were presented as the fold increase. Values are mean $\pm$ S.E.M. calculated on the basis of three animals. Bars without common superscript were significantly different ($P < 0.05$).

significantly increased the expression of *PTGS2* mRNA at 5 h, which was maintained until 10 h of culture with LH (Fig. 7C). Moreover, *PTGS2* mRNA in cumulus cells was significantly higher in this novel system when compared with that in COCs cultured in the two-step condition (Fig. 7C). Meiotic progression to the MII stage was synchronized and more than 80% of oocytes reached this stage at 24 h culture with LH, EGF, and progesterone (total 44 h). At that time, the accumulation of *ADAMTS1* within COCs cultured in the novel system was much higher than that in COCs in the plain one-step or two-step culture system (Fig. 7D). Additionally, the morphology of COCs was different between culture systems (Fig. 7E). After 20 h culture, the diameter of COCs in the novel culture system was larger than that in

Figure 6 The effects of estradiol 17 β and/or progesterone on proliferative activity and the expression of *LHCGR* in cumulus cells of COCs which were cultured with FSH and IBMX for 20 h. (A) The dose effects of FSH on the expression of *LHCGR* in cumulus cells of COCs cultured for 20 h in the presence of 0.5 mM of IBMX. β -actin was used as a control. Results in each panel are representative of three separate experiments. (B) The proliferative activity of cumulus cells of COCs during the 20-h culture was analyzed by BrdU incorporation assay. COCs were cultured for 20 h with FSH, FSH + 20 ng/ml progesterone (P4), FSH + 100 ng/ml estradiol 17 β (E₂), or FSH + E₂ and additional P4 at 10-h culture period in the presence of 100 μ M of BrdU and 0.5 mM of IBMX. Data are expressed as the percentage of the absorbance value at 450 nm in cumulus cells of COCs which were cultured for 20 h with FSH alone. Values are mean $\pm$ S.E.M. of three independent culture experiments. (C) The effects of estradiol 17 β (E₂) or progesterone (P4) on the expression of *LHCGR* in cumulus cells of COCs cultured with FSH and IBMX for 20 h. Cont, without additional progesterone. (D) The additional effects of progesterone (P4) on the expression of *CCND2* and *PGR* mRNA in cumulus cells of COCs cultured with FSH, E₂, and IBMX for 20 h. For reference, the FSH+E₂ value was set as 100 and the data were presented as the relative expression. Values are mean $\pm$ S.E.M. of three independent culture experiments.

the plain one-step system or two-step culture system, whereas the COC was not expanded. After 44 h, the full expansion of the COCs was observed in the novel culture system as shown in Fig. 7E. The relative amount of the apoptosis marker, cleaved caspase-3, was lower in cumulus cells of COCs cultured under the novel culture system when compared with COCs cultured in the plain one-step method (Fig. 7F). Finally, to investigate the developmental competence of *in vitro* matured oocytes, the oocytes matured in the plain one-step system, the two-step system, and the novel culture system were used for *in vitro* fertilization and culture to blastocyst stage. The results showed in Fig. 7G that the maturation rate and penetration rate were not affected by the culture

system; however, the development of the fertilized oocytes to the blastocyst stage was significantly enhanced by the novel culture system.

Discussion

Follicular development in mammals is dependent on many growth regulatory factors. Principally, among these are the gonadotropic hormones, FSH and LH, and the steroid hormones, estradiol and progesterone and the regulated expression of the receptors that bind to and mediate the action of these hormones. The studies herein document specific sequential changes *in vivo* in the expression of genes controlling the production of estradiol (*CYP19A1*) and progesterone (*HSD3B1*) as well as the induced expression of receptors for LH (*LHCGR*) and progesterone (*PGR*) and their target genes that impact the function of granulosa and cumulus cells during COC expansion in a porcine model. To mimic the changes in hormones observed in eCG- and hCG-treated gilts *in vivo*, immature COCs from early stage follicles were cultured with a specific temporal sequence of FSH, estradiol, progesterone, LH, and EGF-like factors. We provide solid evidence that the events *in vivo* as applied to COCs in culture are critical for optimal increases in cumulus cell proliferation (FSH and estradiol), cumulus cell differentiation (FSH, estradiol followed by progesterone at 10 h), and COC expansion and oocyte maturation (LH + EGF + progesterone added at 20–24 h). Whereas FSH and estradiol do have an impact on the level of expression of *CCND2* mRNA, progesterone acts synergistically with FSH and estradiol to reduce proliferation and induce expression of receptors for LH (*LHCGR*). Up-regulated expression of *LHCGR* and *EGFR* permits LH with *AREG* to induce expression of genes involved in COC expansion, *TNFAIP6* and *PTGS2*.

These data in the pig model provide several similarities but also distinct differences from the patterns of regulation in rat and mouse models. Major similarities are observed in factors controlling proliferation. FSH and estradiol have been shown to act synergistically to enhance granulosa cell proliferation in a rat model (Rao *et al.* 1978, Robker & Richards 1998). The cell-cycle regulatory molecule cyclin D2 (*CCND2*) was expressed in granulosa cells during follicular development stage, and the *CCND2* knockout mice showed limited granulosa cell and cumulus cell proliferation and anovulatory phenotypes (Sicinski *et al.* 1996). Moreover, in the *in vitro* culture system of human COCs, the number of cumulus cells in COCs before culture was positively correlated ($P < 0.05$) with the developmental competence of oocytes after *in vitro* maturation (Sato *et al.* 2007), suggesting that a sufficient number of cumulus cells is required to support oocyte maturation.

The major differences reside in the temporal appearance, cell-specific expression, and functional roles of *PGR* and *LHCGR*. In pig follicles, *PGR* is expressed not

only in granulosa cells (Slomaczynska *et al.* 2000) but also in cumulus cells (Fig. 5) and is observed in these cells of antral follicles prior to the ovulatory hormone surge. Moreover, data herein document that progesterone acts coordinately after FSH and estradiol to induce the expression of full-length *LHCGR* mRNA but not the short form in cumulus cells of cultured COCs. It has been reported that LH receptor protein translated from the full-length *LHCGR* mRNA had biological activity (Loosfelt *et al.* 1989). In our previous study (Shimada *et al.* 2003), the full-length form of *LHCGR* mRNA in cumulus cells of porcine COCs was significantly increased by 20 h of culture with FSH. The addition of FSH also significantly increased the binding level of biotinylated hCG to the COCs (Shimada *et al.* 2003). Three other splicing variants that lack the putative transmembrane domain negatively regulate the function of the receptor (Nakamura *et al.* 2004), suggesting that porcine cumulus cells have functional LH receptor. Meanwhile, a low level of *LHCGR* mRNA expression was detected in cumulus cells of mouse COCs, since oocyte-secreted factors strongly suppressed the expression (Elvin *et al.* 1999). Thus, it is a possibility that porcine cumulus cells would have a unique role of progesterone–PGR pathway in the induction of functional LH receptor expression.

The addition of progesterone to these same COC cultures (i.e., 10 h after the combined FSH and estradiol treatment) reduced cumulus cell proliferation. These observations extend our previous study, which documented that the cumulus cell proliferation was down-regulated by the culturing COCs in the plain one-step culture system, i.e., with FSH and LH combined, and the reduction reversed by the PGR antagonist, RU486 (Okazaki *et al.* 2003, Shimada *et al.* 2004b). Moreover, eCG-induced increases in follicular levels of progesterone *in vivo* were associated with reduced levels of *CCND2* mRNA indicating that eCG and progesterone impact cell proliferation in large antral porcine follicles. Similarly, anti-proliferative effects of progesterone have been observed in other tissue models (Lydon *et al.* 1995, Wang *et al.* 2007). These results indicate that the increase of progesterone and the expression of *PGR* before LH surge are essential for the reduction of proliferative activity and induction of *LHCGR* and the ability of pig cumulus cells to respond directly to LH.

In addition to the expression and critical role of the PGR pathway in eCG-primed porcine follicles and COCs, mRNAs encoding the EGF-like factors AREG and EREG, were increased by eCG in granulosa cells and to a lesser extent in cumulus cells of follicles prior to the LH/hCG surge. Moreover, the EGF receptor is increased in eCG- and hCG-primed COCs. This eCG-response pattern also differs from that in mouse models where *AREG* and *EREG* mRNAs are expressed highly only after hCG stimulation (Park *et al.* 2004, Shimada *et al.* 2006). Additionally, Hsieh *et al.* (2007) showed that in *Areg* or

Ereg null mice, or in mice null for *Areg* and hypomorphic for EGF receptor, follicular development and *LHCGR* expression after eCG priming were not altered markedly. However, in mice null for *Areg* and hypomorphic for EGF receptor, the ovulation process was dramatically impaired with notable defects observed in oocyte maturation, cumulus cell expansion, granulosa cell luteinization, and follicle wall rupture. Because AREG and EREG are synthesized as transmembrane precursors, they are activated when cleaved at one or more sites in the extracellular domain by proteases such as TACE/ADAM17, thereby releasing the EGF peptide (Lee *et al.* 2003, Sahin *et al.* 2004). Our previous studies have shown the expression of TACE/ADAM17 in cumulus cells of porcine COCs cultured with FSH and LH, and that the protease activity was required for the activation of EGF receptor downstream pathway (Yamashita *et al.* 2007). Herein we document that *TACE/ADAM17* mRNA is expressed in granulosa cells recovered from the middle-sized and large antral follicles but the level was increased further by hCG. Additionally, the expression of EGF receptor (*EGFR*) mRNA was also up-regulated by hCG injection. These results suggest that the release of active EGF peptides from granulosa cells would reach maximal levels and stimulate the receptors localized on cumulus cells only after hCG stimulation.

The induction and activation of the EGF-like factors combined with prostaglandin production have been shown to be essential for cumulus cell oocyte complex expansion and oocyte maturation in mouse models (Ochsner *et al.* 2003, Shimada *et al.* 2006). Expansion is mediated by the production of an extracellular matrix comprised a hyaluronan backbone, stabilized by various hyaluronan-binding proteins, including the serum-derived factor, inter- α -trypsin inhibitor as well as TNFAIP6 and PTX3 expressed by cumulus cells (Chen *et al.* 1992, Camaioni *et al.* 1993, Fülöp *et al.* 1997, Salustri *et al.* 2004). Mice null for *Ambp*, *Tnfaip6*, and *Ptx3* exhibit impaired expansion and ovulation (Sato *et al.* 2001, Zhuo *et al.* 2001, Varani *et al.* 2002, Fülöp *et al.* 2003, Salustri *et al.* 2004). *Adamts1*, a target of progesterone and PGR action in mouse models (Robker *et al.* 2000) also impacts matrix formation and stability, presumably due to its ability to cleave the hyaluronan-binding proteoglycan, versican (*Csg2*) (Russell *et al.* 2003). In the porcine model, we reported previously that *ADAMTS1* mRNA expression required the expression and activation of cumulus cell PGR, and was critical for expansion of porcine COCs (Shimada *et al.* 2004a). Herein, we document that *ADAMTS1* and *PTGS2* mRNAs are induced only after hCG stimulation *in vivo* whereas *HAS2*, *TNFAIP6*, and *PGR* are increased in eCG-primed follicles in which COCs are not expanded. In summary, changes in follicular (granulosa and cumulus) cell function in the porcine system show many similarities but also important specific differences to the mouse models. These differences are functionally

important for appropriate COC expansion and oocyte maturation. Thus, COC culture conditions for the pig differ from the mouse, indicating that culture conditions for other species may also need to be carefully adjusted to changes that occur *in vivo*.

The *in vivo* data revealed that COCs recovered from small antral follicles are not fully differentiated and do not contain a sufficient number of cumulus cells to support oocyte maturation. However, when the COCs recovered from early antral follicles primed with FSH and estradiol, followed by progesterone in culture, they exhibited enhanced responsiveness to LH and EGF-like factors. Thus, an improved COC culture system for *in vitro* maturation of porcine oocytes is proposed.

1. COCs are recovered from small antral follicles (3–5 mm in diameter).
2. The COCs are pre-cultured with 2 ng/ml FSH and 100 ng/ml E₂ for 10 h to induce cell proliferation.
3. At 10 h, 20 ng/ml progesterone is added to the FSH- and estradiol-containing medium to suppress cell proliferation and induce *LHCGR* mRNA expression.
4. After 20 h culture, COCs are moved to fresh medium with 1 µg/ml LH, 1 ng/ml EGF, and 100 ng/ml progesterone for an additional 24 h.

Using this modified culture system based on *in vivo* changes in hormones and growth factor production, the matured porcine COCs exhibited full expansion, the cumulus cells remained healthy (low number of apoptotic cells) and when oocytes obtained from these COCs were used for *in vitro* fertilization, the developmental competence to blastocyst stage was significantly improved when compared with that using the plain one-step FSH+LH culture system, our two-step culture system as described in Shimada *et al.* (2002, 2003), or other two-step culture systems (Funahashi *et al.* 1997, Kikuchi *et al.* 1999). In conclusion, we have documented that the study of functional changes of porcine cumulus cells before and after LH stimulation *in vivo* permitted us to establish a better *in vitro* maturation system. We propose that because each species has a unique pattern of gene expression in cumulus cells, it is

important to establish that there is disparity in molecular profiles among the species and they need to be assessed in developing optimal culture condition of COCs.

Materials and Methods

Materials

eCG (Veterinary Peamex) was purchased from Sankyo (Tokyo, Japan). hCG was purchased from Asuka Seiyaku (Tokyo, Japan). Highly purified porcine FSH and porcine LH were a gift from the National Hormone and Pituitary Program (Rockville, MD, USA). E₂, progesterone, and IBMX were from Sigma Chemical Co. BSA (Fraction V; Sigma A7888) and BSA (Sigma 8022) were added to the *in vitro* fertilization medium or *in vitro* embryo culture medium respectively. Fetal calf serum (FCS) was obtained from Life Technologies Inc. Oligonucleotide poly-(dT) was purchased from GE Pharmacia Biotech (Newark, NJ, USA), and avian myeloblastosis virus reverse transcriptase was from Promega. Routine chemicals and reagents were obtained from Nakarai Chemical Co. (Osaka, Japan) or Sigma.

General procedure

Isolation of porcine COCs from small antral follicles was performed as described previously (Shimada & Terada 2001). The COCs were cultured for a total of 44 h as described below. After culture, COCs were visualized using phase-contrast microscope (Olympus IMT2, Olympus, Tokyo, Japan) and ×10 objective. The oocytes were fixed with acetic acid/ethanol (1:3) for 48 h and stained with aceto-lacmoid before examination under a phase-contrast microscope (400×) for evaluation of their chromatin configuration. Some oocytes were used for *in vitro* fertilization according to our previous study (Shimada *et al.* 2003). Visualization of apoptotic cells (cleaved caspase-3 positive cells) in COCs was detected by immunofluorescence using anti-cleaved caspase-3 antibody (Cell Signaling Technologies, Danvers, MA, USA) under a fluorescence microscope (Nikon DS-Fi1; Nikon, Tokyo, Japan) (Shimada *et al.* 2001). The proliferative activity of cumulus cells was measured with a BrdU proliferation kit (Roche Molecular Biochemicals) as described in Okazaki *et al.* (2003). The follicular fluid was used for the analyses of testosterone, E₂ and progesterone levels according to our previous study (Shimada & Terada 2002, Shimada *et al.* 2002).

Figure 7 The novel culture system of porcine COCs dramatically improved cumulus cell function and oocyte maturation. (A and B) Time-dependent changes of nuclear status in oocytes of COCs cultured in (A) novel culture system or (B) plain one-step culture system. GV, germinal vesicle stage; MI, metaphase of meiosis I stage; AT-I, anaphase or telophase of meiosis I stage; MII, metaphase of meiosis II stage. (C) The expressions of *PTGS2* mRNA in cumulus cells of COCs cultured by novel culture system, two-step culture system, or plain one-step culture system. The expressions were detected by real-time RT-PCR. For reference, the 0-h COC value was set as 1 and the data were presented as the fold increase. Values are mean ± S.E.M. of three independent culture experiments. Significantly lower levels were observed in COCs cultured by plain one-step culture system: **P* < 0.01. (D) Western blotting analysis of ADAMTS1 in cumulus cells of COCs. Pre, the 110 kDa molecular size is ADAMTS1 precursor; MP, 85 kDa prodomain truncated form of ADAMTS1. (E) Cumulus expansion of COCs cultured for 20 or 44 h. The expansion of COCs was observed using phase-contrast microscopy (×10). (F) The apoptosis of cumulus cells of COCs cultured for 44 h by plain one-step culture or novel culture system. The positive cells by anti-cleaved caspase-3 were defined as apoptotic cells. (G) Effects on the penetration and development to blastocyst stage following IVF of COCs matured by plain one-step, two-step, or novel culture systems. Values are mean ± S.E.M. of three independent culture experiments. Bars without common superscripts were significantly different (*P* < 0.05).

In vivo maturing COCs

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

Culture of COCs

Oocytes having evenly granulated cytoplasm with at least four layers of unexpanded cumulus oophorus cells were selected and washed thrice with maturation medium. The maturation medium was modified using NCSU37 (Petters & Reed 1991) containing 10% (v/v) FCS and 7 mM taurine (Sigma). Twenty COCs were cultured in 500 µl culture medium per well at 39 °C in a humidified atmosphere of 5% CO₂ in air using Nunc 4-well multi-dishes (Nunc, Roskilde, Denmark). Some COCs were cultured with 20 ng/ml FSH and/or 1 µg/ml LH (plain one-step culture system, Okazaki *et al.* 2003). In other experiments, we used a two-step culture system as described by Shimada *et al.* (2003). Briefly, during the first step, COCs were cultured with or without 2.0 ng/ml FSH in the presence of 0.5 mM IBMX for 20 h. The cultured COCs were further cultured with 1 µg/ml LH. To investigate the additional effects of estrogen, COCs were cultured with IBMX, FSH, and 100 ng/ml E₂ during the first step culture. Other COCs were cultured using a novel culture system. The COCs were cultured with IBMX, FSH, and E₂, and 10 ng/ml of progesterone added to the medium at the 10-h point. The 20-h cultured COCs were washed twice by maturation medium and then were further cultured with 1 µg/ml LH, 1 ng/ml EGF, and 100 ng/ml progesterone for 24 h (novel culture system). The culture systems are shown in Fig. 1.

RT-PCR analysis

Total RNA was obtained from COCs or granulosa cells using the RNeasy mini kit (Qiagen sciences) according to the manufacturer's instructions, and semiquantitative RT-PCR analyses were performed as previously described (Shimada *et al.* 2004a). Briefly, total RNA was reverse transcribed using 500 ng poly-dT and 0.25 U avian myeloblastosis virus reverse transcriptase at 42 °C for 75 min and 95 °C for 5 min. LH receptor protein translated from the full-length *LHCGR* mRNA has physiological functions (Loosfelt *et al.* 1989). The other three splicing variants lack the putative transmembrane domain; however, they negatively regulate function of the receptor (Nakamura *et al.* 2004). Additionally, we detected the four types of *LHCGR* mRNA in cumulus cells of porcine COCs (Shimada *et al.* 2003). From these reports, we adopted a regular PCR method to detect

LHCGR mRNA because the four splicing variant could not be detected by real-time PCR. Oligonucleotide primers used for the amplification of *LHCGR* were designed from known cDNA sequences of four porcine *LHCGR* isoforms (Loosfelt *et al.* 1989). The upstream primer (5'-CCAATCTCCTAGATGCCA-CATTGAC-3') is identical to nucleotides 861–885 of the porcine cDNA, while the downstream primer (5'-GCTCAG-CAACAGAAAGAAATCCC-3') represents the reverse complement of nucleotides 1959–1981. This primer pair predicts 185, 411, 855, and 1121 bp fragments. β-Actin was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. Using the specific primer pairs, the cDNA products were amplified for 24 cycles (β-actin) or 32 cycles (*LHCGR*). The RT-PCRs were run with linear range. The amplified products were analyzed by 2% agarose gel electrophoresis. The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA).

Real-time RT-PCR analyses

cDNA and primers were added to 15 µl total reaction volume provided in the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The PCRs were then performed using the iCycler thermocycler (Bio-Rad Laboratories, Inc). Conditions were set to the following parameters: 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at the annealing temperature. Each annealing temperature and specific primer pairs were selected and analyzed as indicated in Table 2.

Western blot analysis

Intact COCs were lysed in Laemmli sample buffer and protein extracts stored at –80 °C until use. Protein concentrations were determined by a Lowry assay using a protein assay kit (Bio-Rad), according to the manufacturer's procedure. After denaturing by boiling for 5 min, 10 µl of each protein sample (10 µg) was separated by SDS-PAGE on 12.5% polyacrylamide gel, then transferred to PVDF membrane (Amersham Biosciences). The membrane was blocked with 5% (w/v) nonfat dry milk (Amersham Biosciences) in 0.1% (v/v) Tween 20 (Sigma)/PBS (T-PBS). A rabbit polyclonal antibody against the anti-C terminal of human ADAMTS1 (Sigma) were added to 2.5% (w/v) nonfat dry milk in PBS-T at a dilution of 1:2000, and incubated overnight at 4 °C. After four washes in T-PBS, the membranes were incubated for 1.5 h with a 1:2000 dilution of goat anti-rabbit IgG horseradish-peroxidase-labeled antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) in 2.5% (w/v) nonfat dry milk in PBS-T at room temperature. After five washes of 10 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (Amersham Biosciences), according to the manufacturer's instructions. The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics).

Table 2 List of primers employed for RT-PCR and the expected size of the amplified product.

Gene	Forward primer	Reverse Pprimer	Size (bp)	Annealing temperature
β -Actin	5'-CTA CAA TGA GCT GCG TGT GG-3'	5'-TAG CTC TTC TCC AGG GAG GA-3'	450	58
ADAMTS1	5'-GGA AGC ATA AGA AAG AAG CGA TTT-3'	5'-TGG GCT GTG GTG AAG GCA GCT TGT-3'	467	58
AREG	5'-CAC CAG AAC AAA AAG GTT CTG TC-3'	5'-AAG TCC ATG AAG ACT CAC ACC AT-3'	409	58
CCND2	5'-CAA GTG CGT GCA GAA GGA TA-3'	5'-TGG CGA ACT TGA AGT CAG TG-3'	455	60
CDKN1B	5'-CTG GCT GCA AAT CAT TGC TA-3'	5'-GCC AGA TTT TTG CCC AGT TA-3'	207	60
CYP11A1	5'-GCT AAT TGC AGC ACC AGA CA-3'	5'-TGT TGG TTC CCT TTT TCA CC-3'	501	62
CYP19A1	5'-GAT GGA GCT GCC TTG TGA CAG GA-3'	5'-AGG GTA CCA CCG TTC TGA AGA GC-3'	272	60
EGFR	5'-CAC CAA ATG AGC CTG GAC AA-3'	5'-ACA TGG CTG TCA GGA GCA GA-3'	237	60
EREG	5'-AAG ACA ATC CAC GTG TGG CTC AAG-3'	5'-CGA TTT TTG TAC CAT CTG CAG AAA-3'	617	58
FSHR	5'-GAT TTG GGG ACC TGG AGA AA-3'	5'-TTT GGG CAG ATT GGA GAA CA-3'	86	60
HAS2	5'-CTC CTG GGT GGT GTG ATT TT-3'	5'-TCTCCTGGGTGGCATTATC-3'	305	58
HSD3B1	5'-TTC CGA AAG TGA GGT CCC ATT TA-3'	5'-TGA GCA TGG GGA CAC TAG TGT GG-3'	531	62
LHCGR	5'-CCA ATC TCC TAG ATG CCA CAT TGA C-3'	5'-GCT CAG CAA CAG AAA GAA ATC CC-3'	1121, 855, 411, 185	60
PGR	5'-AGC CCA CAA TAC AGC TTC GAG-3'	5'-TTT CGA CCT CCA AGG ACC AT-3'	254	60
PTGS2	5'-GGC TGC GGG AAC ATA ATA GA-3'	5'-GCA GCT CTG GGT CAA ACT TC-3'	183	58
TNFAIP6	5'-TCA TAA CTC CAT ATG GCT TGA AC-3'	5'-TCT TCG TAC TCA TTT GGG AAG CC-3'	396	54
TACE/ADAM17	5'-GAC ATG AAT GGC AAA TGT GAG AAA C-3'	5'-AGT CTG TGC TGG GGT CTT CCT GGA-3'	435	58

Statistical analysis

Data were tested for homogeneity of error variance by Bartlett's test before statistical analysis. Since heterogeneity of variance was not detected in this study, statistical analyses of all data from three or four replicates for comparison were carried out by analysis of one-way ANOVA followed by Duncan's multiple ranges test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data were subjected to arcsine transformation before statistical analysis. Values were determined to be significant when $P < 0.05$.

Experimental designs

Experiment 1 was conducted to examine the time-dependent changes of follicular cell function during hormonally stimulated follicular development from the early antral stage to the preovulatory stage and during the ovulation process. Follicular fluid was used for analyses of the level of testosterone, E_2 and progesterone. Total RNA was extracted from granulosa cells to analyze the expression of the genes encoding steroidogenic enzymes (aromatase (*CYP19*), P450scc (*CYP11A1*), and 3β HSD (*HSD3B1*)) and prostaglandin E biosynthesis (*PTGS2*), the EGF-like factors (*AREG* and *EREG*), a specific protease (*TACE/ADAM17*), and the gonadotropin receptors (*FSHR* and *LHCGR*). COCs were also collected from each follicle and used to analyze expression of selected genes involved in gonadotropin and progesterone reception (*FSHR*, *LHCGR*, and *PGR*), cumulus cell proliferation (cyclin D2; *CCND2* and p21kip1, *CDKN1B*) and differentiation (*ADAMTS1*, *HAS2*, *TNFAIP6*, and *PTGS2*).

In experiment 2, we examined the effects of E_2 and/or progesterone when added to the FSH-containing medium on cumulus cell proliferation and responses to LH *in vitro*. The gene expression pattern in cumulus cells of COCs cultured with FSH and LH as described in our previous study (Yamashita *et al.* 2007) was markedly different from that observed *in vivo*. Because we observed in the results of experiment 1, that testosterone was dominantly accumulated in small antral

follicles whereas E_2 was increased rapidly during follicular development, and that increased production of progesterone occurred later within preovulatory follicles, steroids were added to the culture medium to mimic these changes. Specifically, COCs recovered from small antral follicles were cultured with FSH and E_2 , and progesterone was added to the medium at 10 h of culture point. Total RNA was recovered from the COCs at 10 or 20 h of culture, and then used for RT-PCR analysis. The proliferative activity of cumulus cells were examined using BrdU proliferation kit. All the media contained 0.5 mM of IBMX to block spontaneous resumption of meiosis in the oocytes.

Experiment 3 was performed to investigate the functional activities of COCs cultured using the novel culture system as described in Fig. 1. Total RNA was recovered from the COCs at each culture point, and then used for RT-PCR analysis. Protein samples were used for Western blotting to detect the accumulation of ADAMTS1 within COC. Expansion of the COC and apoptosis of cumulus cells within the COCs were analyzed after 44 h. Some COCs cultured for 44 h were used for *in vitro* fertilization, and the gametes were cultured *in vitro* to develop to blastocyst stage.

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