Signaling pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig

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Abstract

To define signaling pathways that drive FSH- and epidermal growth factor (EGF)-like peptide-induced cumulus expansion and oocyte meiotic resumption, *in vitro* cultured pig cumulus–oocyte complexes were treated with specific protein kinase inhibitors. We found that FSH-induced maturation of oocytes was blocked in germinal vesicle (GV) stage by protein kinase A (PKA), MAPK14, MAPK3/1, and EGF receptor (EGFR) tyrosine kinase inhibitors (H89, SB203580, U0126, and AG1478 respectively) whereas phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) inhibitor (LY294002) blocked maturation of oocytes in metaphase I (MI). Amphiregulin (AREG)-induced maturation of oocytes was efficiently blocked in GV by U0126, AG1478, and low concentrations of LY294002; H89, SB203580, and high concentrations of LY294002 allowed the oocytes to undergo breakdown of GV and blocked maturation in MI. Both FSH- and AREG-induced cumulus expansion was incompletely inhibited by H89 and completely inhibited by SB203580, U0126, AG1478, and LY294002. The inhibitors partially or completely inhibited expression of expansion-related genes (*HAS2*, *PTGS2*, and *TNFAIP6*) with two exceptions: H89 inhibited only *TNFAIP6* expression and LY294002 increased expression of *PTGS2*. The results of this study are consistent with the idea that PKA and MAPK14 pathways are essential for FSH-induced transactivation of the EGFR, and synthesis of EGF-like peptides in cumulus cells and MAPK3/1 is involved in regulation of transcriptional and posttranscriptional events in cumulus cells required for meiotic resumption and cumulus expansion. PI3K/AKT signaling is important for regulation of cumulus expansion, AREG-induced meiotic resumption, and oocyte MI/MII transition. The present data also indicate the existence of an FSH-activated and PKA-independent pathway involved in regulation of *HAS2* and *PTGS2* expression in cumulus cells.

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Introduction

The resumption of meiosis is initiated in ovarian follicles by the preovulatory peak of LH. In the mouse and rat, LH binds to its receptors on mural granulosa cells and stimulates expression of epidermal growth factor (EGF)-like peptides (Park *et al.* 2004, Ashkenazi *et al.* 2005). These peptides (amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC)) are produced as membrane-bound pro-peptides that require a proteolytic step to be released into the follicular environment (Ashkenazi *et al.* 2005). They then act directly on mural granulosa as well as cumulus cells and stimulate resumption of meiosis and expansion of the cumulus. In addition, a regulatory loop between the mural granulosa and the cumulus cells exists in the preovulatory follicles, which ensures production of AREG and EREG also in the cumulus cell compartment (Shimada *et al.* 2006). The EGF-like peptides are also produced in cumulus cells of *in vitro* cultured cumulus–oocyte complexes (COCs) upon stimulation by FSH and trigger resumption of meiosis (Downs & Chen 2008). The data published

by our and other laboratories strongly suggest that similar mechanisms work also in large animal species (Prochazka *et al.* 2000, 2003, 2011, Akaki *et al.* 2009, Yamashita *et al.* 2009). We have recently shown that FSH induces expression of AREG and EREG but not BTC in cultured pig COCs and that these peptides stimulate expansion of cumulus cells, maturation of oocyte, and acquisition of the oocyte developmental competence. However, we have also pointed out lower efficiency of EGF-like peptides compared with FSH, in inducing expression of expansion-related genes and their inability to stimulate protein kinase A (PKA) and production of progesterone in cultured COCs (Prochazka *et al.* 2011). These results raise a question about specific roles of gonadotropin- and EGF-like factor-stimulated signaling pathways in cumulus cells and about their significance for resumption of meiosis and cumulus expansion.

FSH/LH binding to their membrane receptors leads to a prompt activation of several signaling pathways. A well-characterized signaling cascade is the G-protein-mediated activation of adenylate cyclase and subsequent

production of cAMP that in turn activates PKA. The PKA phosphorylates many substrates within the cell including transcription factors SP1/SP3 and CREB that control transcription of specific genes in cumulus and granulosa cells (Gonzalez-Robayna *et al.* 2000). However, recent studies have shown that FSH signaling in granulosa and also in cumulus cells is more complex and some molecular events occur even independently of PKA activation through cAMP-activated exchange factor pathway (Wayne *et al.* 2007). The gonadotropin-induced production of EGF-like peptides leads to a transactivation of EGF receptor (EGFR; Conti *et al.* 2006) as these peptides bind to a classical EGFR (ERBB1), activate its intrinsic tyrosine kinase, and downstream signaling cascades, including MAPK3/1, MAPK14, and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathways (Shimada *et al.* 2006, Li *et al.* 2008, Hsieh *et al.* 2011). Therefore, gonadotropins activate a broad signaling network in cultured COCs, which results in essential changes in transcription profiling in the cumulus cells and consequently in series of posttranscriptional molecular events in the oocytes leading to the resumption of meiosis.

The aim of this work was to define specific roles of signaling pathways activated in cumulus cells by the FSH and EGF-like peptides in major events accompanying meiotic resumption in the pig. For this purpose, specific inhibitors of PKA, MAPK14, MAPK3/1, PI3K/AKT, and EGFR tyrosine kinase were employed and their effects on maturation of oocytes, expansion of cumulus cells, and expression of the expansion-related genes, namely hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxidase synthase 2 (*PTGS2*), and tumor necrosis factor α -induced protein 6 (*TNFAIP6*), were studied.

Results

PKA inhibition

The FSH-induced activity of PKA was gradually inhibited in COCs by 5–20 μ M H89. The activity of PKA at the highest concentration of H89 (20 μ M) was indistinguishable from that found in control non-stimulated COCs (Fig. 1A, left panel). AREG did not stimulate activation of PKA in the COCs at all (Fig. 1A, right panel).

FSH and AREG equally stimulated maturation of the oocytes to metaphase II (MII; 78 vs 74% respectively). FSH-stimulated maturation of oocytes was almost completely blocked in germinal vesicle (GV) stage by H89 in concentrations of 5–15 μ M (Fig. 1B); higher concentrations caused irreversible damage and degeneration of the oocytes during the period of culture. In contrast, AREG-induced resumption of meiosis was not blocked by any of the used concentrations of H89 and most oocytes underwent breakdown of the GV (GVBD) or proceeded to MII; the completion of meiosis to MII was, however, gradually decreased by increasing

concentrations of H89 (Fig. 1B). The FSH- and AREG-induced expansion of cumulus cells was gradually but incompletely inhibited by H89 in concentrations of 5–20 μ M (Fig. 1C). Approximately 30% of COCs treated with 15 and 20 μ M H89 still exhibited expansion of outer layers of the cumulus (expansion 2). Both FSH- and AREG-induced expression of *HAS2* and *PTGS2* was not inhibited by H89 but the expression of *TNFAIP6* was significantly decreased by the drug (Fig. 1D).

MAPK14 inhibition

Both FSH- and AREG-induced activity of MAPK14 was reduced to a base level or completely abolished by SB203580 in concentrations higher than 10 μ M (Fig. 2A). The FSH- and AREG-induced maturation of oocytes was differently affected by the SB203580. The FSH-induced resumption of meiosis was efficiently blocked at GV by SB203580 in 80–100% of oocytes (Fig. 2B). In contrast, only 39.6–44.6% of the AREG-stimulated oocytes remained in GV after treatment with different concentrations of SB203580, which was not significantly different from the control group. Thus, the majority of AREG-stimulated oocytes resumed meiosis and underwent GVBD despite of the presence of the MAPK14 inhibitor, but the maturation was then blocked and few oocytes completed meiosis to MII stage (Fig. 2B). The expansion of cumulus cells was gradually inhibited by 5–20 μ M SB203580 regardless of whether it was induced by FSH or AREG (Fig. 2C). The expression of expansion-related genes was partially (*HAS2* and *PTGS2*) or completely (*TNFAIP6*) inhibited by the drug in FSH-stimulated COCs. In the AREG-stimulated COCs, the inhibition was complete for all the three genes (Fig. 2D).

MAPK3/1 inhibition

The FSH- and AREG-induced MAPK3/1 activity was abolished or attenuated to a base level by the use of U0126 over the range of assessed concentration (1–10 μ M; Fig. 3A). The U0126 had the same effect on both FSH- and AREG-induced maturation of oocytes. The resumption of meiosis was effectively blocked at GV stage by all concentrations of U0126 (1–10 μ M), and completion of meiosis to MII stage was significantly decreased in all groups (Fig. 3B). The degree of FSH- and AREG-induced cumulus expansion was gradually decreased with increasing concentrations of U0126; the AREG-stimulated COCs were significantly more inhibited than the FSH-stimulated ones (Fig. 3C). Corresponding with this observation, the U0126 partially inhibited the FSH-induced expression of the expansion-related genes, but the AREG-induced expression of the genes was completely inhibited (Fig. 3D).

Figure 1 Effect of PKA inhibition on meiotic resumption and cumulus cell expansion. (A) Effect of H89 on FSH-induced activation of PKA in cultured COCs (left panel) and assessment of PKA activity in AREG-stimulated COCs (right panel). The panels display migration of the phosphorylated (+) and non-phosphorylated (–) PKA-specific substrate in an electric field following the PKA assay. Negative (N) and positive (P) control assays were run without the cells or with 25 ng catalytic subunit of PKA respectively. C, control COCs cultured without stimulation; F, FSH. (B) Maturation of oocytes in medium with different concentrations of H89. Asterisks above the column indicate a significant difference from the corresponding control value found in oocytes cultured without H89; ** $P < 0.01$, and *** $P < 0.001$. Approximately 90 COCs were included in each group. (C) Degree of cumulus expansion in COCs cultured in medium with different concentrations of H89. Double asterisk above the value means significant difference from the corresponding value within the same concentration of H89; ** $P < 0.01$. (D) Effect of PKA inhibition on expression of expansion-related genes in cultured COCs. The relative abundance of specific gene mRNA was measured in COCs cultured in medium supplemented with 20 μM H89 for 4 h and is expressed in arbitrary units as fold strength increases in specific gene/*HPRT1* ratio over the level found in COCs cultured with FSH or AREG. Different superscripts above the columns indicate significant differences ($P < 0.05$).

EGFR tyrosine kinase inhibition

The phosphorylation of EGFR was reduced to a base level by 0.5 μM AG1478 and by all higher concentrations. Such a pattern of inhibition was identical for both FSH- and AREG-induced phosphorylation (Fig. 4A).

The AG1478 exhibited the same pattern of action on both FSH- and AREG-induced maturation of oocytes. Corresponding with the sensitivity test, the concentration from 0.5 μM significantly inhibited resumption and completion of meiosis; 5 and 10 μM AG1478 blocked 80–100% of the treated oocytes at GV stage (Fig. 4B). The degree of cumulus expansion was gradually decreased in both groups treated with 0.5 μM AG1478 and higher concentrations of the drug. Nevertheless, the AREG-treated COCs displayed higher sensitivity to the EGFR tyrosine kinase inhibitor, as 5 and 10 μM completely inhibited their expansion whereas the mean cumulus expansion index (CEI) of FSH-treated COCs was

around 1.0 under the same conditions (Fig. 4C). AG1478 only partially decreased expression of *HAS2* and *TNFAIP6* in FSH-stimulated COCs whereas expression of *PTGS2* was completely inhibited and so was expression of all the three genes in the AREG-stimulated COCs (Fig. 4D).

PI3K/AKT inhibition

The FSH-induced AKT activity was gradually decreased by 5–50 μM LY29004. The concentration of 15 μM appeared to reduce the AKT activity to a base line and higher concentrations further reduced the activity to an undetectable level. The AREG-induced AKT activity was already undetectable after using 20 μM LY29004 (Fig. 5A).

The inhibitor did not block resumption of meiosis in the FSH-stimulated COCs; the great majority of oocytes underwent GVBD, but few of them reached MII stage

Figure 2 Effect of MAPK14 inhibition on meiotic resumption and cumulus cell expansion. (A) Effect of SB203580 on activation of MAPK14 in cultured COCs. The panels display immunoblotting of phosphorylated and total MAPK14 in FSH- and AREG-stimulated COCs cultured for 18 h in medium with different concentrations of SB203580. This experiment was repeated twice with similar results. C, control COCs cultured without stimulation; F, FSH; A, AREG. Position of molecular weight marker is on the right. (B) Maturation of oocytes in medium with different concentrations of SB203580. Asterisks above the column indicate a significant difference from the corresponding control value found in oocytes cultured without SB203580; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Approximately 90 COCs were included in each group. (C) Degree of cumulus expansion in COCs cultured in medium with different concentrations of SB203580. (D) Effect of MAPK14 inhibition on expression of expansion-related genes in cultured COCs. The relative abundance of specific gene mRNA was measured in COCs cultured in medium supplemented with 20 μ M SB203580 for 4 h and is expressed in arbitrary units as fold strength increases in specific gene/*HPRT1* ratio over the level found in COCs cultured with FSH or AREG. Different superscripts above the columns indicate significant differences ($P < 0.05$).

(Fig. 5B). To exclude a possibility that the drug only postponed maturation of oocytes, we extended the culture period to 48 h. The result was, however, the same – almost all oocytes passed GVBD and remained blocked at MI stage at the end of culture (data not shown). A paradoxical effect of LY29004 on AREG-induced maturation of oocytes was repeatedly observed in our experiments: low concentrations of LY294002 (5–10 μ M) efficiently blocked maturation of oocytes at GV stage whereas high concentrations of LY294002 (15–50 μ M) allowed majority of the oocytes to undergo GVBD and blocked maturation in MI (Fig. 5B). No differences were observed in effect of LY294002 on FSH- and AREG-induced expansion of cumulus cells. The tested concentrations of the drug gradually inhibited cumulus expansion; 20 μ M LY294002 caused almost complete inhibition of expansion in both groups (Fig. 5C). Higher concentrations of LY294002 (25 and 50 μ M) caused obvious degeneration of cumulus cells during 24 h of culture. The 24-h exposure of COCs to

5–20 μ M LY294002 did not lead to irreversible changes and the COCs underwent expansion after washing the drug whereas exposure of the COCs to 25 or 50 μ M LY294002 caused irreversible damage and the COCs lost a capability to undergo expansion after washing (data not shown).

The LY294002 affected FSH- and AREG-induced expression of expansion-related genes in the same manner: it significantly inhibited expression of *HAS2* and *TNFAIP6* and significantly increased expression of *PTGS2* (Fig. 5D).

Discussion

The preovulatory surge of gonadotropins causes activation of a broad cell-signaling network, which results in a dramatic change in gene expression and also posttranscriptional modifications in all compartments of the follicle. The signaling in granulosa and cumulus cell, also modified by signals from the oocyte, removes the

Figure 3 Effect of MAPK3/1 inhibition on meiotic resumption and cumulus cell expansion. (A) Effect of U0126 on activation of MAPK3/1 in cultured COCs. The panels display immunoblotting of phosphorylated and total MAPK3/1 in FSH- and AREG-stimulated COCs cultured for 3 or 1 h respectively in medium with different concentrations of U0126. This experiment was repeated twice with similar results. C, control COCs cultured without stimulation; F, FSH; A, AREG. Position of molecular weight marker is on the right. (B) Maturation of oocytes in medium with different concentrations of U0126. Asterisks above the column indicate a significant difference from the corresponding control value found in oocytes cultured without U0126; * P <0.05, ** P <0.01, and *** P <0.001. Approximately 90 COCs were included in each group. (C) Degree of cumulus expansion in COCs cultured in medium with different concentrations of U0126. Asterisks above the value mean significant difference from the corresponding value within the same concentration of U0126; * P <0.05 and ** P <0.01. (D) Effect of MAPK3/1 inhibition on expression of expansion-related genes in cultured COCs. The relative abundance of specific gene mRNA was measured in COCs cultured in medium supplemented with 10 μ M U0126 for 4 h and is expressed in arbitrary units as fold strength increases in specific gene/*HPRT1* ratio over the level found in COCs cultured with FSH or AREG. Different superscripts above the columns indicate significant differences (P <0.05).

inhibitory influence of the follicle on the oocyte cell cycle and meiosis can resume (Sun *et al.* 2009, Conti *et al.* 2012).

The present results show a critical importance of PKA in regulation of FSH-induced meiotic resumption in pig oocytes. In the mouse, an increase in cAMP concentration and PKA activity is a prerequisite for gonadotropin-induced activation of MAPK3/1 in cumulus cells and induction of meiotic resumption (Su *et al.* 2002). Next, the cAMP/PKA signaling is crucial for production of EGF-like factors and transactivation of the EGFR in mouse follicles (Conti *et al.* 2006) and also for expression of AREG and EREG in cultured human ovarian cells (Freimann *et al.* 2004, Ben-Ami *et al.* 2006). Thus, it appears that a principal role of PKA toward resumption of meiosis in the preovulatory mammalian follicle is propagation of the gonadotropin signals in mural

granulosa cells leading to transition of the signals to cumulus cell and reentry of the oocyte into the meiotic cell cycle. The results of our study support this idea as inhibition of PKA blocked only FSH-stimulated resumption of meiosis but was not able to block the resumption stimulated by AREG. Thus, the PKA appears to work in pig COCs upstream of AREG production.

Under *in vitro* conditions, FSH induces expansion of cumulus cells in all studied mammalian species. The FSH-induced expansion is preceded by an increase in cAMP concentration in cumulus cells and increased expression of *HAS2* (Salustri 2000). The expansion can also be induced by forskolin, a direct activator of adenylate cyclase, or 8-Br-cAMP, a membrane permeable analog of cAMP (Su *et al.* 2002). All these data suggest that cAMP signaling is involved in physiological mechanisms regulating cumulus cell expansion.

Figure 4 Effect of EGFR tyrosine kinase inhibition on meiotic resumption and cumulus cell expansion. (A) Effect of AG1478 on phosphorylation of the EGFR in cultured COCs. The panels display phosphorylation of the EGFR on tyrosine residues in FSH- and AREG-stimulated COCs cultured for 15 min in medium with different concentrations of AG1478. This experiment was repeated twice with similar results. C, control COCs cultured without stimulation; F, FSH; A, AREG. Position of molecular weight marker is on the right. (B) Maturation of oocytes in medium with different concentrations of AG1478. Asterisks above the column indicate a significant difference from the corresponding control value found in oocytes cultured without AG1478; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Approximately 90 COCs were included in each group. (C) Degree of cumulus expansion in COCs cultured in medium with different concentrations of AG1478. Asterisk above the value means significant difference from the corresponding value within the same concentration of U0126; * $P < 0.05$ and ** $P < 0.01$. (D) Effect of EGFR tyrosine kinase inhibition on expression of expansion-related genes in cultured COCs. The relative abundance of specific gene mRNA was measured in COCs cultured in medium supplemented with 10 μ M AG1478 for 4 h and is expressed in arbitrary units as fold strength increases in specific gene/*HPRT1* ratio over the level found in COCs cultured with FSH or AREG. Different superscripts above the columns indicate significant differences ($P < 0.05$).

However, recent data suggest that PKA is not the only transmitter of the cAMP signals in mammalian cell. The PKA-independent cAMP signaling has recently been reported in rat granulosa cells (Gonzalez-Robayna *et al.* 2000, Wayne *et al.* 2007). The data of our study indicate that PKA is not the principal kinase regulating FSH-induced expansion of cumulus cells in the pig. Inhibition of PKA did not decrease FSH-stimulated expression of *HAS2*, the key enzyme regulating synthesis of hyaluronic acid, but significantly decreased expression of *TNFAIP6*, which is involved in stabilization of the hyaluronic acid in the matrix (Salustri 2000, Fulop *et al.* 2003, Nagyova *et al.* 2009). The PKA inhibition may thus adversely affect cumulus expansion by compromising the stability of the matrix rather than limiting its production by the cumulus cells. The AREG-induced expression of the *HAS2* and *PTGS2* was not affected by PKA inhibition at all, which is in correspondence with our finding that the EGF-like factors do not activate PKA (Prochazka *et al.* 2011;

this study), but surprisingly reduced expression of *TNFAIP6*. The inhibition of *TNFAIP6* was conceivably the reason why H89 inhibited degree of expansion not only in the FSH-stimulated COCs but also in the AREG-stimulated COCs.

MAPK14 was first characterized as a kinase responding to different environmental stress stimuli and inflammatory cytokines and regulating cell proliferation, differentiation, motility, and apoptosis. In addition, recent experiments disclosed an involvement of this kinase in transduction of gonadotropin-induced signals in somatic follicular cells (Villa-Diaz & Miyano 2004, Diaz *et al.* 2006, Shimada *et al.* 2006). The results of our study revealed high sensitivity of the FSH-stimulated meiotic resumption and low sensitivity of the AREG-stimulated meiotic resumption to the MAPK14 inhibitor, indicating an important role of MAPK14 upstream of AREG production. Indeed, MAPK14 has been reported to be involved in FSH-stimulated initial production of

Figure 5 Effect of PI3K/AKT inhibition on meiotic resumption and cumulus cell expansion. (A) Effect of LY294002 on activation of AKT in cultured COCs. The panels display immunoblotting of phosphorylated and total AKT in FSH- and AREG-stimulated COCs cultured for 1 h in medium with different concentrations of LY294002. This experiment was repeated twice with similar results. C, control COCs cultured without stimulation; F, FSH; A, AREG. Position of molecular weight marker is on the right. (B) Maturation of oocytes in medium with different concentrations of LY294002. Asterisks above the column indicate a significant difference from the corresponding control value found in oocytes cultured without LY294002; ** $P < 0.01$ and *** $P < 0.001$. Approximately 90 COCs were included in each group. (C) Degree of cumulus expansion in COCs cultured in medium with different concentrations of LY294002. (D) Effect of PI3K/AKT inhibition on expression of expansion-related genes in cultured COCs. The relative abundance of specific gene mRNA was measured in COCs cultured in medium supplemented with 25 μ M LY294002 for 4 h and is expressed in arbitrary units as fold strength increases in specific gene/*HPRT1* ratio over the level found in COCs cultured with FSH or AREG. Different superscripts above the columns indicate significant differences ($P < 0.05$).

Areg in cultured mouse COCs that precedes activation of EGFR and MAPK3/1-dependent induction of the expansion-related genes (Shimada *et al.* 2006). We may thus speculate that similar FSH-induced and MAPK14-dependent production of AREG also occurs in pig COCs and that the initial and rapid production of AREG plays an important role in induction of meiotic resumption. A possible target of such signaling pathway might be the regulation of gap junctions closure within the cumulus compartment that occurs soon after gonadotropin stimulation, plays a crucial role in meiotic resumption, and requires participation of EGF-like factors (Sela-Abramovich *et al.* 2005, Norris *et al.* 2008, Vaccari *et al.* 2009). Moreover, our results indicate that MAPK14 may also be required for completion of meiosis. In agreement with this observation, MAPK14 was found active in pig oocytes during MI/II transition (Villa-Diaz & Miyano 2004).

MAPK14 was reported to play an important role in a paracrine- and autocrine-positive feedback loop between mouse granulosa and cumulus cells, in which prostaglandin E2 (PGE2), through PGE2 receptor and MAPK14, enhances expression of EGF-like factors that stimulate expression of the expansion-related genes, inclusive of *PTGS2*, a major regulator of PGE2 synthesis (Shimada *et al.* 2006). Therefore, through this mechanism, the MAPK14 is involved in regulation of mouse cumulus expansion. Such a MAPK14-dependent regulatory loop seems to also work in the pig as indicated by results presented in this study and also in other studies reporting inhibition of pig cumulus expansion by SB203580 (Yamashita *et al.* 2009, Gilchrist & Ritter 2011). In the mouse, FSH- but not EGF-induced cumulus expansion and elevation of expansion-related transcripts (*Has2* and *Ptgs2*) required MAPK14 activity (Diaz *et al.* 2006). We have observed similar differences

between FSH and AREG stimulation in relation to meiotic resumption but the expansion-related events were suppressed by MAPK14 inhibitor irrespective of the mode of stimulation in this study. The reason of this discrepancy is not clear, but it is not associated with a different role of oocyte-derived factors in regulation of cumulus expansion in the mouse and pig (Prochazka *et al.* 1991, Vanderhyden 1993), as these factors act in mouse cumulus cells downstream of MAPK14 activation (Diaz *et al.* 2006). Interestingly, mice with granulosa cell-specific knockout of the MAPK14 α isoform displayed an aberrant cumulus expansion *in vitro* whereas *in vivo* expansion of cumulus and ovulation occurs normally (Liu *et al.* 2010). The significance of MAPK14 signaling for *in vivo* oocyte maturation and ovulation is not known in other species and should be studied.

The transactivation of the EGFR and activation of the MAPK3/1 pathway in follicular somatic cells are considered to be important steps in gonadotropin-induced cumulus expansion and oocyte maturation (Su *et al.* 2002, Park *et al.* 2004, Conti *et al.* 2006, Shimada *et al.* 2006, Fan *et al.* 2009). In rat follicles, MAPK3/1 mediates the LH-induced breakdown of cell-to-cell communication, possibly by phosphorylation of connexin 43 (Sela-Abramovich *et al.* 2005). The drop in the intercellular communication is necessary for meiosis reinitiation and is supposed to limit transfer of meiosis blocking substances to the oocyte. In the mouse, activation of EGFR is involved in LH-induced decrease in cGMP transport from granulosa and cumulus cells to the oocyte and both EGFR and MAPK3/1 activities participate in gap junction closure (Vaccari *et al.* 2009, Norris *et al.* 2009, 2010, Hsieh *et al.* 2011). These mechanisms contribute to relieve meiotic block as cGMP works in oocytes as a natural inhibitor of phosphodiesterase 3A, maintaining high intracellular level of cAMP and meiotic arrest of the oocyte (Masciarelli *et al.* 2004). In the pig, activation of MAPK3/1 in cumulus cells is also a prerequisite for gonadotropin-stimulated resumption of meiosis (Meinecke & Krschek 2003, Liang *et al.* 2005, Li *et al.* 2008). In this study, we show that both FSH- and AREG-induced resumption of meiosis in pig oocytes is dependent on EGFR and MAPK3/1 activity, which supports the idea that also in pig COCs, FSH drives resumption of meiosis by transactivation of the EGFR and activation of MAPK3/1 in the cumulus cells.

EGFR tyrosine kinase and MAPK3/1 activities are crucial for expansion of cumulus cells in different species including the pig (Prochazka *et al.* 2003, Chen *et al.* 2008, Nagyova *et al.* 2011). Nevertheless, the data of this study indicate that EGFR/MAPK3/1 pathway is not the only pathway controlling gonadotropin-induced expression of the expansion-related genes, at least in the *in vitro* system. First, FSH induced much greater increase in *HAS2*, *PTGS2*, and *TNFAIP6* expression in the cultured COCs than AREG (see also

Prochazka *et al.* (2011)). Secondly, AG1478 and U0126 completely inhibited the AREG-induced expression of the expansion-related genes on a level comparable with the control non-stimulated COCs, but the FSH-stimulated expression of the genes was only partially decreased by these inhibitors. Thirdly, the FSH-stimulated COCs were less sensitive to AG1478 than the AREG-stimulated COCs regarding the degree of expansion assessed by CEI. The complete inhibition of EGF-stimulated cumulus expansion and only partial decrease of FSH-stimulated expansion by AG1478 were reported previously in the pig (Chen *et al.* 2008).

The involvement of PI3K/AKT signaling in meiotic resumption and cumulus cells expansion has been extensively studied in the pig. An incentive for these studies was a finding that insulin-like growth factor 1, a known activator of PI3K/AKT signaling, is the component of serum that enables expansion of pig cumulus cells *in vitro* in response to FSH (Singh & Armstrong 1997). Further studies have revealed that PI3K/AKT signaling is involved in regulation of connexin 43 phosphorylation (Shimada *et al.* 2001) and in promotion of FSH-stimulated synthesis and retention of hyaluronic acid in pig COCs (Nagyova *et al.* 1999, Nemcova *et al.* 2007). Inhibition of PI3K/AKT signaling in matured pig COCs by LY294002 showed interesting differences between FSH- and AREG-induced maturation in our study. FSH-stimulated oocytes were arrested at MI stage whereas AREG-stimulated oocytes were blocked in GV by low concentrations of the inhibitor whereas high concentrations of the inhibitor led to resumption of meiosis and interruption of oocyte maturation at MI stage. Thus, FSH-induced resumption of meiosis does not seem to be regulated by PI3K/AKT, but this pathway appears important for progression of meiosis to MI/MII stage. This observation is in agreement with previous studies in the pig (Shimada *et al.* 1988, Kalous *et al.* 2009). The mechanism of the interruption of meiosis may consist in increased activity of glycogen synthase kinase 3 in centrosomal region, which leads to formation of aberrant MI spindles (Kalous *et al.* 2009). The AREG-stimulated resumption of oocyte maturation, however, seems to be dependent on PI3K/AKT activity in cumulus cells as shown in our study and also in another study carried out on the pig model (Li *et al.* 2008). The failure of high concentrations of LY29004 to block AREG-induced meiotic resumption observed in our study can be explained by a damage of the surrounding cumulus cells that are undoubtedly involved in that inhibitory mechanism. The damage of cumulus cells was displayed in our experiments by a lack to undergo expansion after washing and it was also documented by high incidence of apoptotic cells in cumuli treated by high doses of LY294002 (Shimada *et al.* 2003).

PI3K/AKT activity is required for gonadotropin-induced expansion of pig cumulus cells *in vitro* (Shimada *et al.* 1988, 2001, Nemcova *et al.* 2007).

The data of this study show that AREG-induced expansion is also sensitive to PI3K/AKT inhibition. No differences between FSH and AREG were found regarding the degree of expansion expressed by CEI and also the effect on expression of expansion-related genes was very similar. Interestingly, the expression of *PTGS2* was increased in both groups by the LY294002 whereas the expression of the other genes was decreased. The mechanism of the stimulatory effect of LY294002 on *PTGS2* expression is not known and should be studied in further experiments.

The results of this study are summarized in Fig. 6 and document that FSH-stimulated, but not the AREG-stimulated, resumption of meiosis depends on the PKA and MAPK14 activities; both modes of stimulation require activation of EGFR and MAPK3/1. Taken together, these results are consistent with the idea that PKA and MAPK14 pathways are essential for FSH-induced transactivation of the EGFR and synthesis of EGF-like peptides. On the other hand, EGFR tyrosine kinase and MAPK3/1 activities in cumulus cells are involved in regulation of transcriptional and posttranscriptional events required for resumption of oocyte meiosis. PI3K/AKT signaling is important for regulation of MI/MII transition and AREG-induced GVBD. Both FSH- and AREG-induced expression of the expansion-related genes was dependent on MAPK14 and MAPK3/1, and with exception for *PTGS2* also on PI3K/AKT activity.

Interestingly, the FSH-induced expression of the *HAS2* and *PTGS2*, but not that of *TNFAIP6*, was driven by a PKA-independent pathway that should be defined in future experiments.

Materials and Methods

Isolation and culture of COCs

Ovaries of slaughtered gilts were collected at a local abattoir and transported to the laboratory in a thermos at 37 °C. The contents of the medium size follicles (3–6 mm in diameter) was aspirated by a syringe connected with a 20 G needle, pooled in a Petri dish, and washed twice with PBS. COCs were picked out from the diluted aspirate and washed in the PBS. Only COCs surrounded by compact multi-layered cumulus were selected for experiments. About 30 COCs were cultured in 0.5 ml medium M-199 (Gibco, Life Technologies) supplemented with 0.91 mM sodium pyruvate, 0.57 mM cysteine, 2.3 mM HEPES, antibiotics, and 5% FCS (Sigma). The COCs were cultured in four-well dishes (Nunc, Roskilde, Denmark) at 38.5 °C in a humidified atmosphere of 5% CO₂ in the air. The maturation of oocytes and expansion of cumulus cells were stimulated by the addition of ovine pituitary FSH (1 IU/ml; #F8174; Sigma) or recombinant human AREG (100 ng/ml; #A7080; Sigma) into the culture medium. The FSH and AREG were dissolved in PBS according to the manufacturer's instructions and working aliquots were stored at –20 °C for up to 3 months. For inhibition of PKA, MAPK14, MAPK3/1, EGFR tyrosine kinase, and PI3K/AKT, the COCs were first exposed for 1 h to different

Figure 6 Scheme of signaling pathways activated in pig cumulus cells by FSH and AREG under *in vitro* conditions. The scheme summarizes data of this study in context of our current knowledge on regulation of meiotic resumption and cumulus cell expansion. The signaling pathway drawn in dotted line was activated only by exogenous AREG. The regulatory loop between EGFR and MAPK14 appears important only for cumulus expansion and might represent part of the FSH-stimulated but PKA-independent mechanism regulating expression of the expansion-related genes; the second part of this mechanism seems to be independent of both PKA and EGFR activity. Question marks indicate unidentified links.

concentrations of H89 (Sigma), SB203580 (Merck Chemicals Ltd.), U0126 (Merck Chemicals Ltd.), AG1478 (Sigma), or LY294002 (Sigma) respectively and then FSH or AREG was added. The inhibitors were dissolved in dimethyl sulfoxide (DMSO) and 10 or 100 mM stocks were stored frozen at -20°C for a maximum period of 3 months. The maximum final concentration of DMSO in culture medium was 0.2% (v/v).

Assessment of oocyte maturation and cumulus cell expansion

To assess the nuclear maturation, oocytes were cultured for 42 h and then removed of cumulus cells by vortexing, mounted on slides, and fixed in acetic ethanol for 48 h. Oocytes were then stained with 1% orcein and observed with a light microscope. Oocytes were scored for GV, GVBD (comprising mostly oocytes at MI stage and few oocytes at late diakinesis, anaphase I or telophase I), and for MII stage. The degree of cumulus expansion was assessed at 24 h after the onset of culture using a subjective scoring method (Vanderhyden *et al.* 1993). Briefly, no response is scored as 0, minimum observable response, the cells in outermost layer of the cumulus become round and glistening as 1, the expansion of outer COCs layers as 2, the expansion of all COCs layers except corona radiata as 3, and the expansion of all COCs layers as 4. Afterward, a CEI (0–4) was calculated as an average degree of expansion in the experimental group of COCs.

Real-time RT-PCR

Based on our previous results (Prochazka *et al.* 2011), the expression of the expansion-related genes was assessed at 4 h after the onset of COC stimulation. Total RNA from 30 COCs was extracted with the use of an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was eluted in 50 μl RNase-free water and stored at -80°C . The concentration of total RNA in samples was measured by a spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

The relative abundance of *HAS2*, *TNFAIP6*, and *PTGS2* mRNA in pig COCs was assessed by a real-time RT-PCR using a One-Step RT-PCR Kit (Qiagen) and primers designated for the specific sequences (Table 1). The total RNA of the samples was reverse transcribed and subsequently amplified in a reaction mixture (total volume 25 μl) containing 5 μl 5 $\times$ reaction buffer, 1 μl dNTP mix (10 mM stock of each), 0.5 μl of both reverse

and forward primers (0.02 mM stock), 0.15 μl RNasine (20 U/ μl stock; Promega), 1 μl enzyme mix, and 4 μl RNA. In addition, 0.5 μl SYBR Green I of 1:1000 $\times$ stock solution (Molecular Probes, Eugene, OR, USA) was added in each reaction. The amplification was performed on the RotorGene 3000 cyclor (Corbett Research, Sydney, Australia). The reaction conditions were as follows: cDNA synthesis at 50°C for 30 min, pre-denaturation at 95°C for 15 min, followed by various numbers of PCR cycles consisting of denaturation (95°C for 15 s), annealing (20 s) at temperature specific for each set of primers (Table 1), extension (72°C for 20 s), and final extension at 72°C for 5 min. Fluorescence data were acquired during an additional step at $\sim 3^{\circ}\text{C}$ below the product's melting temperature. After the cycling, the melting curve was generated to verify the amplification of one specific target (one peak at a specific melting temperature demonstrates the specificity). In addition, gel electrophoresis and ethidium bromide staining assessed the specificity of RT-PCR products.

The relative concentration of templates in different samples was determined using comparative analysis software (Corbett Research). The results for individual target messages were normalized according to the relative concentration of the internal standard, the *HPRT1* (*HPRT*).

PKA activity assay

PKA activity was assessed by a PepTag Assay for Non-Radioactive Detection of cAMP-Dependent Protein Kinase kit (Promega) according to the manufacturer's instructions. Unless otherwise indicated, 50 COCs were cultured for 1 h and then stored frozen at -70°C in a minimum volume of PBS until use in the assay. The samples were lysed in 10 μl cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and mixed in a test tube with 5 μl PKA reaction buffer (100 mM Tris, pH 7.4, 50 mM MgCl_2 , and 5 mM ATP), 5 μl PKA-specific fluorescent peptide substrate (Kemptide, 0.4 $\mu\text{g}/\mu\text{l}$ stock), and 5 μl deionized water. Negative control assay was run without the cell sample; in positive control assays, the sample was substituted with 25 ng catalytic subunit of PKA (diluted in 5 μl 350 mM K_3PO_4 with 0.1 mM dithiothreitol) and 5 μl PKA activator solution (5 μM cAMP in water). The reaction mixture was incubated at room temperature for 30 min and then stopped by placing the test tubes in boiling water for 10 min. The samples were loaded into the wells in 0.8% agarose gel and run at 100 V for 15 min. The phosphorylated substrate migrated toward the positive electrode while the non-phosphorylated substrate migrated toward the negative

Table 1 Primers used for real-time RT-PCR.

Gene transcript	Primers	Amplicon length (bp)	T _{an} ($^{\circ}\text{C}$)	GenBank accession number
<i>HAS2</i>	F: 5'-GAA GTC ATG GGC AGG GAC AAT TC-3' R: 5'-TGG CAG GCC CTT TCT ATG TTA-3'	407	54	NM_214053
<i>PTGS2</i>	F: 5'-TCG ACC AGA GCA GAG AGA TGA GAT-3' R: 5'-ACC ATA GAG CGC TTC TAA CTC TGC-3'	260	55	NM_214321
<i>TNFAIP6</i>	F: 5'-TCA TAA CTC CAT ATG GCT TGA AC-3' R: 5'-TCT TCG TAC TCA TTT GGG AAG CC-3'	396	54	Yamashita <i>et al.</i> (2009)
<i>HPRT1</i>	F: 5'-CCA GTC AAC GGG CGA TAT AA-3' R: 5'-CTT GAC CAA GGA AAG CAA GG-3'	129	55	NM_001032376

T_{an}, annealing temperature.

electrode. The gel was photographed in u.v. light and the images were assessed for the proportions of phosphorylated and non-phosphorylated substrate.

Immunoblotting

Groups of 25–50 COCs were lysed in 15 µl Laemmli sample buffer for SDS–PAGE, heated at 100 °C for 3 min, and stored at –80 °C. The proteins of the samples were separated on 7.5% polyacrylamide gel and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). The MAPK14 activity was assessed as described by Villa-Diaz & Miyano (2004). For the other protein detections, membranes were blocked in 5% low-fat dry milk or 10% FCS (PY20) in Tris-buffered saline (TBS) with 0.5% of Tween 20 for 2 h at room temperature and then incubated with primary antibody diluted 1:1000 in 5% BSA in TBS–Tween at 4 °C overnight. The following primary antibodies were used: phospho-AKT (Ser 473), AKT – both from Cell Signaling Technology, p-ERK, ERK (detecting MAPK3/1) – both from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-p38 MAPK, p38 MAPK (detecting MAPK14) – both from Cell Signaling, and anti-phosphotyrosine antibody PY20 from BioLegend (San Diego, CA, USA). The secondary antibody (anti-mouse or anti-rabbit IgG conjugated with HRP; GE Healthcare, Little Chalfont, UK) was diluted 1:5000 in 2% BSA in TBS–Tween. The membranes were incubated with the secondary antibody for 1 h at room temperature and washed intensively in TBS–Tween. The immune reaction was detected by ECL (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Following detection, the antibodies were stripped by incubation of the membrane in 25 mM Tris with 2% β-mercaptoethanol and 0.2% SDS at 60 °C for 20 min and reprobed with the next primary antibody.

Statistical analysis

Unless otherwise indicated, each experiment was performed in at least three replicates. Each replicate was performed on different day on a single batch of COCs that were randomly allocated to the treatments. The differences in percentages of maturing oocytes and the quantifications of RT-PCR results were compared by the ANOVA followed by Tukey's posttest. The differences of CEI between FSH- and AREG-stimulated COCs were performed by χ^2 analysis. Error bars indicate the S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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