Expression of two progesterone receptor isoforms in cumulus cells and their roles during meiotic resumption of porcine oocytes

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Abstract

The present study aimed to investigate progesterone receptor (PR) gene expression in cumulus cells and their roles during meiotic resumption of porcine oocytes. The amount of PR-A or PR-B mRNA was analyzed by RT-PCR using primer sets for the PR-B region or the PR-A/B common region. The level of PR-B mRNA in cumulus cells was up-regulated by FSH and LH during the first 8 h of cultivation but the level significantly decreased at 12 h. However, a high level of total PR mRNA was maintained up to a cultivation period of 20 h. The level of PR-B protein in cumulus cells reached its maximum at 4 to 12 h, whereas PR-A predominated in cumulus cells of cumulus–oocyte complexes (COCs) at 20 h. Accompanying the shift in expression of PR isoforms, progesterone production in cumulus cells was significantly increased, and both the proliferative activity of cumulus cells during a 10- to 20-h cultivation period and the level of connexin-43, a major component of the gap junction, in cumulus cells significantly decreased. When COCs were cultured with FSH and LH for 10 h and then further cultured with additional RU486, there was a significant suppression in the shift in PR isoforms and in progesterone production, a loss of proliferative activity, and a decrease in connexin-43 mRNA in cumulus cells. Moreover, treatment with RU486 after 10-h cultivation of COCs inhibited the meiotic resumption of oocytes and cumulus cell expansion. These results suggest that the induction of PR isoforms in cumulus cells and their binding to progesterone appear to impact on proliferation and differentiation in a time-dependent manner, and the shift from PR-B to PR-A may help mediate certain events.

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Introduction

During *in vitro* meiotic maturation of cumulus–oocyte complexes (COCs), it has been shown in human (Chian *et al.* 1999), rat (Zhang & Armstrong 1989), cattle (Armstrong *et al.* 1996) and pig (Xia *et al.* 1994, Coskun *et al.* 1995, Shimada & Terada 2002a) that progesterone is produced by cumulus cells, and that the level of progesterone is increased by stimulation with luteinizing hormone (LH), follicle-stimulating hormone (FSH) or forskolin. In our previous study (Yamashita *et al.* 2003), we showed that there was a significant positive correlation between the germinal vesicle breakdown (GVBD) rate of oocytes and the progesterone concentration in each well. When porcine COCs

were cultured with a P450 scc inhibitor, progesterone production was almost completely suppressed in cumulus cells, and a reduction of the GVBD rate was also observed (Shimada & Terada 2002a). Therefore, progesterone secreted by cumulus cells is clearly associated with meiotic maturation of porcine oocytes.

Many of the biological activities of progesterone are mediated by an intracellular receptor, a hormonally regulated DNA-binding protein that belongs to a superfamily of ligand-activated transcription factors (Beato *et al.* 1989). The progesterone receptors (PR) are expressed as two protein isoforms, PR-A and PR-B, produced from a single gene by transcription at two distinct promoters and by translation initiation at two

alternative AUG signals (Kraus *et al.* 1993, 1994). The distal and proximal promoters have putative binding sites for the estrogen receptor, called estrogen response element (ERE)-like regions (Kraus *et al.* 1993, 1994). In MCF-7 breast cancer cells, stimulation with estradiol-17 β induced the expression of the PR gene (Fazzari *et al.* 2001). However, in granulosa cells, expression of the PR gene is not observed by stimulation with estrogen; the cAMP-protein kinase A (PKA) pathway has been documented to directly activate the distal promoter activity of the PR gene in the cells (Clemens *et al.* 1998). Park and Mayo (1991) reported that PR mRNA was highly expressed in the granulosa cells of large follicles in the ovaries of animals treated with pregnant mare's serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG). In *in vitro* culture of rat granulosa cells, high concentrations of either FSH or LH stimulated PR gene expression (Natraj & Richards 1993). In pigs, Slomczynska *et al.* (2000) showed that in granulosa cells of small antral follicles, PRs were not detected; however, the treatment of small follicles with LH or FSH *in vitro* induced the expression of the PR protein. Although it is known that the expression of the PR in granulosa cells is regulated by the gonadotropin surge, there has until now been no description of the ratios of PR-A to PR-B mRNA in cumulus cells surrounding oocytes during *in vitro* meiotic maturation of COCs.

It has been reported that PR-A is a truncated form of PR-B that lacks the first 164 N-terminal amino acids, and that PR-A and PR-B are functionally distinct mediators of progesterone action in the uterine epithelium (Conneely *et al.* 2001). Using a PR-A-deficient mouse model, Mulac-Jericevic *et al.* (2000) showed that the binding of progesterone to PR-B resulted in a gain of proliferative activity in the uterus; however, uterine expression of PR-A isoforms was required to oppose progesterone-induced proliferation through the PR-B function. Stimulation of PR-A by progesterone has also been reported to promote the differentiation of cells (Shiozawa *et al.* 2001, De-Vivo *et al.* 2002). Schuetz *et al.* (1996) showed that during *in vivo* ovulation, the loss of proliferative activity appeared to be closely associated with LH regulation of cumulus cell functions. The functions of differentiated cumulus cells, such as progesterone production, hyaluronan synthesis, or the close of

gap junctional communication, were involved in the meiotic maturation of cumulus-enclosed oocytes (Tanghe *et al.* 2002). Judging from these reports, it is estimated that a high ratio of PR-A to PR-B expression plays an important role in cumulus cell differentiation; however, the functional differences between PR-A and PR-B in cumulus cells during meiotic resumption of porcine oocytes remain unclear.

In this study, we examined the time-dependent changes in PR gene expression in cumulus cells of COCs. The amount of PR-A or PR-B mRNA was analyzed by RT-PCR using primer sets for the PR-B region or the PR-A/B common region. The protein levels of the two isoforms were detected by Western blotting analysis. To investigate the roles of PR in cumulus cells, COCs were cultured with RU486 or aminoglutethimide. The proliferative activity of cumulus cells, the amount of connexin-43 mRNA and progesterone production in cumulus cells, and the nuclear status of oocytes were analyzed.

Materials and methods

In vitro maturation of porcine COCs

Isolation of porcine COCs has been described previously (Shimada & Terada 2001). The COCs were cultured in the maturation medium supplemented with or without both 20 ng/ml highly purified porcine FSH (NIDDK, Torrance, CA, USA) and 500 ng/ml porcine LH (NIDDK) (FSH+LH). Some COCs were cultured with 100 ng/ml estradiol-17 β (Sigma Chemical Co., St Louis, MO, USA) or 5 μ M forskolin (Sigma). The maturation medium was modified NCSU37 (Petters & Reed 1991) supplemented with essential amino acids (Gibco BRL, Grand Island, NY, USA), non-essential amino acids (Gibco BRL), 7 mM taurine (Sigma) and 4 mM hypoxanthine (Sigma). Estradiol-17 β was dissolved in ethanol at 0.1 mg/ml, and was stored at 4 °C. Forskolin was dissolved in DMSO (Sigma) at 50 mM and stored at -20 °C. The final concentration of estradiol-17 β or forskolin was obtained by dilution with the basic medium. 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 $\times$) for evaluation of their

Table 1 Primers used for the determination of porcine PR, connexin-43 and beta-actin mRNA by RT-PCR

	Primer sequence	Predicted product size (bp)	Amplification cycles
mRNA			
PR-A/B	F: 5'-AGC CCA CAA TAC AGC TTC GAG-3' R: 5'-TTT CGA CCT CCA AGG ACC AT-3'	254	30
PR-B	F: 5'-ACT GAG CTG AAG GCA AAG GGT-3' R: 5'-GTC CTG TCC CTG GCA GGG C-3'	198	33
Connexin-43	F: 5'-TGTCTTCTTCAAGGGTGTTAAGG-3' R: 5'-CACTCGCTTGTTTGTGTAATTG-3'	230	26
Beta-actin	F: 5'-CTA CAA TGA GCT GCG TGT GG-3' R: 5'-TAG CTC TTC TCC AGG GAG GA-3'	450	26

F, forward; R, reverse

chromatin configuration. Some COCs were cultured for 44 h, and then the matured oocytes were used for *in vitro* fertilization and subsequent *in vitro* culture to the blastocyst stage, according to our previous study (Shimada *et al.* 2003).

RNA isolation

After cumulus cells were separated from 20 COCs, they were washed three times in PBS. Total RNA was extracted from cumulus cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the instruction manual, and dissolved in nuclease-free water. The final RNA concentrations were determined by absorbance using a spectrophotometer.

RT-PCR

RT-PCR was performed according to a coupled one-step procedure using the Access RT-PCR System (Promega) with some modifications (Shimada *et al.* 2003). Briefly, 10 ng total RNA were reverse transcribed at 48 °C for 45 min, denatured at 94 °C for 2 min, and amplified for 24, 30 or 33 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C (PR-B, connexin-43) or 56 °C (PR-A/B, β -actin) for 1 min, and extension at 68 °C for 1 min, with a final extension step of 7 min at 68 °C. The amplified products were analyzed by electrophoresis on 2% agarose gels. One primer set was directed at the sequence specific for the PR-B (i.e. within the 164 amino acids at the N-terminus) and therefore detected only mRNA transcripts encoding PR-B (Table 1, Fig. 1). The other primer set was directed to the section of the PR common to PR-A and PR-B

(PR-A/B) and therefore detected total PR mRNA (Table 1, Fig 1). The PR primers were based on the human sequence (GenBank accession no. NM00926). The primers used for amplification of connexin-43 were designed from the known cDNA sequence of porcine connexin-43 (GenBank accession no. X86026). β -Actin was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. The β -actin primers were based on the mouse sequence (GenBank accession no. NM009609).

DNA sequence

Direct sequence was carried out to identify RT-PCR products using a fluorescent dye terminator (DYEnamic ET Terminator Cycle Sequence Kit, Amersham Biosciences, Uppsala Sweden) and the ABI PRISM 377XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA). According to the instructions supplied with the sequencing kit,

Figure 1 Schematic diagram indicating positions of the oligonucleotide primers on the putative porcine PR gene. Although unknown, the genomic structure of the porcine PR is depicted in a similar fashion to the human PR gene (Gen Bank, accession number, NM 000926).

the cDNA fragments were mixed with four dye-labeled dideoxynucleotides as terminators, a thermally stable AmpliTaq DNA polymerase, and upstream or downstream primer, then set on the thermal cycler for the sequencing reaction. Extended products were analyzed on the DNA sequencer.

Western blotting

Western blotting analysis was based on the procedures reported in our previous study (Shimada *et al.* 2001). After cultivation of COCs, cumulus cells were separated from the COCs. The cumulus cells were lysed in Laemmli sample buffer. After denaturing by boiling for 5 min, each protein sample (4 µl) was separated by SDS-PAGE on a 7.5% polyacrylamide gel (Amersham Biosciences), then transferred onto a PVDF membrane (Amersham Biosciences). The membrane was blocked by 5% (w/v) nonfat dry milk (Amersham Biosciences), then incubated with anti-PR rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) which allowed recognition of both PR-A and PR-B at 1:200 dilution overnight at 4 °C. After four washes in 0.1% (v/v) Tween 20 (Sigma)/PBS (T-PBS), the membranes were treated with horseradish-peroxidase-labeled anti-rabbit IgG (1:5000, Amersham Biosciences) for 1 h at room temperature. After four 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.

5-Bromo-2-deoxyuridine (BrdU) labeling for cell proliferation

The proliferative response of cumulus cells was measured with a BrdU proliferation kit (Roche Molecular Biochemicals, Basel, Switzerland). In short, the 10 COCs in each well were cultured with a 100 µM BrdU for a 10-h period. At the end of cultivation, cumulus cells were separated from COCs, then washed 3 times with PBS. They were suspended at 5×10^3 cells/well in the 96-well culture plates, and then the plates were dried for 1 h at 60 °C. FixDenat solution (200 µl) was added to each well, and the plates were incubated for 30 min at 25 °C. The FixDenat solution was removed and peroxidase-labeled anti-BrdU mono-

clonal antibody (100 µl/well) that had been diluted 100-fold with the antibody dilution solution was added, before the plates were incubated for 1 h at 25 °C. The peroxidase-labeled anti-BrdU monoclonal antibody solution was removed, and each well was washed three times with PBS (200 µl each). A tetramethylbenzidine solution (100 µl/well) was added, and the plates were incubated for 15 min. After adding 1 M H₂SO₄ (25 µl/well) to stop the enzymatic reaction, the absorbance at 450 nm was measured with a microplate reader.

Treatment of COCs with RU486

Some COCs were cultured with FSH, LH and 20 ng/ml progesterone, and/or 0.25, 2.5 or 25 µM RU486 (Sigma) for 10 h. Other COCs were pre-cultured with FSH and LH for 10 h and then further cultured with 0.25, 2.5 or 25 µM RU486 for an additional 10 h. The range of RU486 concentrations has been used to test progesterone receptor function in granulosa cells (Natraj & Richards 1993, Park *et al.* 2000, VandeVoort *et al.* 2000, Mussche & D'Herde 2001, Shao *et al.* 2003). RU486 was dissolved in ethanol at 25 mM, and was stored at 4 °C. Each final concentration was obtained by dilution in the maturation medium.

Treatment of COCs with aminoglutethimide

COCs were cultured for 20 h in maturation medium supplemented with 0.5 mM aminoglutethimide (AGT, Sigma). In our previous study (Shimada & Terada 2002a), progesterone production in cumulus cells of COCs during *in vitro* maturation was decreased by AGT in a dose-dependent fashion, and the maximum inhibitory effect was observed at 0.5 mM. AGT was dissolved in DMSO at 0.5 M, and was stored at -20 °C. Each final concentration was obtained by dilution in the maturation medium.

Statistical analysis

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

Figure 2 Comparison of partial cDNA sequences of porcine PR-B (A) and PR-A/B common region (B) with human PR (accession number, NM 000926) and mouse PR (accession number, NM 008829). Conserved nucleotides are indicated by a dash (-) and deletions by a colon (:).

Results

Regulation of PR gene expression in cumulus cells of COCs

Partial cDNAs were amplified by RT-PCR from porcine cumulus cells using primers that are sequence-specific for human PR-B (i.e. within the 164 amino acids at the N-terminus) and common to human PR-A and PR-B (PR-A/B). Each sequence was compared for homology with the human and mouse cDNA sequences. The similarity of the partial porcine PR-B region sequence was 83.3% to human PR-B and 82.8% to mouse PR-B (Fig. 2A). The porcine PR common region sequence showed a high degree of similarity to human PR (98.1%) and mouse PR (90.5%) (Fig. 2B). Sequence comparisons indicate that the partial porcine cDNAs amplified with human PR primers conformed to the porcine PR-B region and PR common region (PR-A/B) respectively.

After 4-h cultivation of COCs without stimulation, the total PR mRNA (PR-A/B) level in cumulus cells was very low (Fig. 3). This level was not significantly increased by stimulation with estradiol-17 β (Fig. 3). Cultivation with FSH for 4 h significantly increased the level of total PR-A/B mRNA and PR-B mRNA in cumulus cells of COCs (Fig. 3). Moreover, the higher levels of PR gene expression in cumulus cells that were induced

by FSH were comparable with those in cumulus cells stimulated by 5 μ M forskolin (Fig. 3) which elevated cAMP level in cumulus cells to the same

Figure 3 The expression of the PR gene in cumulus cells which were cultured for 4 h with estradiol-17 β , FSH or forskolin. COCs were cultured for 4 h without any hormones (control, C), with 100 ng/ml estradiol-17 β (E₂), with 20 ng/ml FSH (FSH) or with 5 μ M forskolin (For). * P < 0.01, significant effect of FSH on the level of PR-A/B; ** P < 0.01, significant effect of forskolin on the level of PR-A/B; # P < 0.01, significant effect of FSH on the level of PR-B; ## P < 0.01, significant effect of forskolin on the level of PR-B. Values are means $\pm$ S.E.M. of 3 replicates.

Figure 4 Time-dependent changes of PR expression in cumulus cells of COCs which were cultured with FSH and LH. (A) RT-PCR analysis of PR-A/B or PR-B mRNA in cumulus cells. The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). The respective values of PR-A/B and PR-B were normalized according to those of beta-actin to evaluate arbitrary units of the relative abundance of the targets. a-b, c-e Values with different letters are significantly different ($P < 0.05$). Values are means $\pm$ S.E.M. of 3 replicates. (B) Western blotting analysis of PR in cumulus cells. The intensity of the bands was analyzed using a Gel-Pro Analyzer. a-d, e-g Values with different letters are significantly different ($P < 0.05$). Values are means $\pm$ S.E.M. of 3 replicates.

extent as FSH (Racowsky 1985, Shimada & Terada 2002b).

Time-dependent changes of PR-A and PR-B expression in cumulus cells of COCs

To investigate the time-dependent changes in PR gene expression in cumulus cells, COCs were cultured with FSH and LH. Our data showed that the level of total PR mRNA (PR-A/B) was very low in cumulus cells just after collection from their follicles (Fig. 4A). The expression of PR-B in cumulus cells was up-regulated by FSH and LH during the first 8 h of cultivation, but the level had significantly decreased by 12 h of cultivation (Fig. 4A). Still, a high level of total PR mRNA was maintained up to the 20-h cultivation period (Fig. 4A). The result of Western blotting analysis of PR is shown in Fig. 4B. The level of PR-B protein in cumulus cells reached its maximum at 4–12 h. However, further cultivation for 8 h (a total 20-h

cultivation period) significantly decreased the level of PR-B protein in cumulus cells. The level of PR-A protein significantly increased in a time-dependent fashion when COCs were cultured with FSH and LH. The maximum level was reached at 20 h of cultivation. These results for mRNA and protein levels indicated that PR-B was initially expressed in cumulus cells, whereas the level of PR-B decreased and PR-A predominated in cumulus cells of COCs after 12 h of cultivation.

The roles of PR-A and PR-B in proliferation of cumulus cells

During the first 10-h cultivation period, the incorporation of BrdU in cumulus cells cultured with both FSH and LH was significantly lower than that in cumulus cells of COCs which were cultured without any hormones (Fig. 5A). A further decrease of proliferative activity was induced by the addition of 2.5 μ M RU486 to FSH+LH-containing

medium, but the difference was not significant. In the next experiment, we investigated the additional effects of 20 ng/ml progesterone in the FSH+LH-containing medium on the inhibitory function of the gonadotropins. When COCs were cultured with FSH, LH and 20 ng/ml progesterone for 10 h, the incorporation of BrdU in cumulus cells increased significantly compared with that of COCs without the additional progesterone (Fig. 5A). The positive effect of progesterone on cell proliferation was significantly suppressed by RU486 in a dose-dependent fashion (Fig. 5A). The effect was reached at 2.5 μ M. In cumulus cells of COCs which were cultured with progesterone and/or RU486, the level of PR-B mRNA was comparable to that of COCs cultured with only gonadotropin (Fig. 5B). The progesterone production by COCs could not be affected by treatment with RU486 (Fig. 5C). Thus, during the first 10-h cultivation period, the high level of PR-B in cumulus cells may have the potential to promote the proliferation of the cells.

We have already shown that progesterone production in cumulus cells is significantly increased after 10 h of cultivation (Shimada *et al.* 2002). In Fig. 6D, 29.8 ± 4.1 ng/ml progesterone were secreted by FSH- and LH-stimulated COCs. Progesterone has been shown to be a potent antagonist of proliferation of uterine epithelium when PR-A is expressed in the cells (Mulac-Jericevic *et al.* 2000). Since the relatively high ratios of PR-A to PR-B were observed in cumulus cells after 10-h cultivation of COCs, it was estimated that the proliferative activity of cumulus cells during the 10- to 20-h cultivation period would decrease due to the binding of progesterone to PR-A. In order to test this hypothesis, COCs were pre-cultured with FSH+LH for 10 h and then further cultured with FSH, LH and/or RU486 for 10 h. The proliferative activity in cumulus cells during the latter 10-h cultivation period was increased by RU486 in a dose-dependent fashion (Fig. 6A). The rate of proliferative activity in cumulus cells cultured with FSH+LH and either 2.5 or 25 μ M RU486 after 10 h of cultivation with FSH+LH was significantly higher than that in cumulus cells cultured with FSH+LH for 20 h (Fig. 6A). The addition of 25 μ M RU486 suppressed the decrease of PR-B mRNA and protein level in cumulus cells of COCs which were cultured with FSH and LH for 20 h (Fig. 6B and C). The level of

PR-B mRNA in cumulus cells treated with RU486 was significantly higher than that in cumulus cells of COCs which were cultured with FSH and LH for 20 h, whereas the level of total PR mRNA (PR-A/B) was not affected by the addition of RU486 (Fig. 6B). Moreover, the addition of RU486 to FSH+LH-containing medium after 10 h of cultivation significantly suppressed progesterone production by COCs (Fig. 6D). Thus, the further 10-h cultivation of COCs with RU486 after the 10-h cultivation with FSH and LH induced both a decrease in progesterone production and a suppression of the decrease in PR-B levels, which resulted in the failure of FSH- and LH-induced loss of proliferative activity in cumulus cells.

Time-dependent changes of connexin-43 gene expression in cumulus cells and the regulation by progesterone

Differentiated cumulus cells have been shown to be involved in meiotic resumption of cumulus-enclosed oocytes (Tanghe *et al.* 2002). One of the functions they perform is the closing of gap junctional communication between outer layers of cumulus cells (Larsen *et al.* 1986, 1987, Isobe *et al.* 1998). We have already reported that the connexin-43 protein, a major component of the gap junction, was up-regulated by cultivation with FSH and LH for 8 h, whereas the level significantly decreased after 12 h of cultivation and remained so for up to 28 h (Shimada *et al.* 2001). The present results showed that the level of connexin-43 gene expression was significantly increased and reached a maximum at 8 h (Fig. 7A). The sequence of the RT-PCR product was identified as part of the porcine connexin-43 cDNA (GenBank accession no. X86026), by using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990). However, further cultivation suppressed the expression of the connexin-43 gene, and this lower level was maintained for up to 20 h (Fig. 7A). The levels of connexin-43 mRNA at 16 and 20 h were significantly lower than those in cumulus cells of COCs that were cultured for 8 h (Fig. 7A). Thus, the disappearance of connexin-43 protein in cumulus cells of COCs could result from the suppression of connexin-43 gene expression, concomitantly with the shift in expression of PR isoforms.

Zhao *et al.* (1996) revealed that progesterone down-regulated the expression of the connexin-43 gene in human myometrial cells. In the next experiment, we investigated the effects of RU486 or aminoglutethimide (AGT) on connexin-43 gene expression in cumulus cells. When COCs were cultured for 8 h, there was no significant difference in the level of connexin-43 mRNA in cumulus cells among the treatment groups (Fig. 7B). The level of connexin-43 mRNA was decreased by 20-h cultivation with FSH+LH (Fig. 7A and C). However, 20-h cultivation of COCs with FSH+LH and 0.5 mM AGT significantly suppressed the decrease in connexin-43 levels in cumulus cells as compared with those in cumulus cells cultured without AGT (Fig. 7C). Moreover, the addition of RU486 to FSH+LH-containing medium after 10 h-cultivation with FSH+LH also induced the failure of disappearance of connexin-43 mRNA in cumulus cells (Fig. 7C).

Effects of RU486 or AGT on meiotic resumption of cumulus-enclosed oocytes

To investigate whether the binding of progesterone to PR in cumulus cells during a 10- to 20-h cultivation period induced the meiotic resumption of oocytes, COCs were cultured with RU486 after a 10-h cultivation with FSH and LH. As shown in Fig. 8A, the proportion of oocytes undergoing GVBD significantly decreased as the concentration of RU486 was increased in the medium with FSH+LH. When FSH+LH-stimulated progesterone production was suppressed by 0.5 mM AGT, a significantly lower GVBD rate than that in oocytes cultured without the drug was also observed (Fig. 8A).

It has been reported that the addition of RU486 to the *in vitro* culture medium from 0 to 24 h cannot suppress meiotic resumption of mouse oocytes (Batten *et al.* 1989). When COCs were cultured with FSH, LH and 25 μ M RU486 for 20 h, the proportion of oocytes exhibiting GVBD was comparable to that in oocytes cultured without the drug (Fig. 8B). However, a further 24-h cultivation with RU486 significantly reduced the proportion of oocytes that reached the metaphase of meiosis II (MII) stage (Fig. 8B). The matured oocytes which were cultured in the presence of FSH and LH supplemented with or without RU486 were used for *in vitro* fertilization. After 5 days cultivation of oocytes, $20 \pm 3.4\%$ of the matured oocytes without RU486 treatment developed to the blastocyst stage, whereas the rate was significantly decreased ($3.3 \pm 1.1\%$) by treatment with RU486. Additionally, the proliferative activity of cumulus cells which were cultured for 44 h with FSH, LH and 0.25 or 2.5 μ M RU486 was significantly lower than that in cumulus cells of COCs cultured without RU486 for 20 h (Fig. 8C). Thus, the oocytes matured without the stimulation of PR in cumulus cells have little developmental potential.

Expanded COCs were observed after 44-h cultivation with FSH and LH (Fig. 8Da). However, when COCs were cultured for 34 h with the addition of RU486 following 10-h cultivation in FSH+LH-containing medium, cumulus cells remained packed around the oocytes with little visible expansion (Fig. 8Db). On the other hand, 44 h of cultivation with FSH+LH and RU486 resulted in dispersion of cumulus cells from oocytes, but the majority of the cells became flattened on the culture dish with few remaining suspended in the expanded complex (Fig. 8Dc).

Figure 5 Proliferative activity of cumulus cells of COCs during the first 10 h of cultivation. (A) COCs were cultured for 10 h in FSH+LH-containing medium with or without 20 ng/ml progesterone. Some COCs were cultured in 20 ng/ml progesterone-containing FSH+LH medium with RU486 (0 to 25 μ M). Data are expressed as percentage of the absorbance value at 450 nm in cumulus cells of COCs which were cultured for 10 h in the Free medium with 100 μ M BrdU. C (control), COCs were cultured for 10 h without any hormones. Free, COCs were cultured for 10 h with or without 2.5 μ M RU486. * $P < 0.01$, significant effect of FSH+LH on proliferative activity of cumulus cells; ** $P < 0.01$, significant effect of the addition of 20 ng/ml progesterone to FSH+LH-containing medium. ^{a-c}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 4 replicates. (B) RT-PCR analysis of PR-A/B or PR-B mRNA in cumulus cells when COCs were cultured for 10 h. The respective values of PR-A/B and PR-B were normalized according to those of beta-actin to evaluate arbitrary units of the relative abundance of the targets. * $P < 0.01$, significant effect of FSH+LH on the level of PR-A/B; ** $P < 0.01$, significant effect of FSH+LH on the level of PR-B. Values are means $\pm$ S.E.M. of 3 replicates. (C) The level of progesterone in the medium where COCs had been cultured for 10 h. * $P < 0.01$, significant effect of FSH+LH on the level of progesterone in the cultured medium. Values are means $\pm$ S.E.M. of 3 replicates.

Discussion

Promoters within the PR gene have putative binding sites for the estrogen receptor, known as estrogen response element (ERE)-like regions (Kraus *et al.* 1993, 1994). However, in granulosa cells, the expression of the PR gene was not apparently stimulated by estrogen; rather, the cAMP–PKA pathway has been documented to directly activate the distal promoter activity of the PR gene in these cells (Clemens *et al.* 1998). The present study showed that the expression of both PR-A and PR-B in cumulus cells was up-regulated by either FSH or forskolin in cumulus cells. It is well known that the level of cAMP in cumulus cells of COCs is elevated by the presence of FSH or forskolin (Racowsky 1985, Shimada & Terada 2002b). These results suggested that the expression of the PR gene was stimulated by the cAMP–PKA pathway in cumulus cells as well as in granulosa cells.

In rat gonadotropes or uterus where the expression of PR is up-regulated by estrogen, it has been reported that progesterone leads to a down-regulation of PR (Turgeon & Waring 2000, Fang *et al.* 2002). However, the inhibitory effect of progesterone on PR expression was not observed in granulosa cells (Iwai *et al.* 1991, Natraj & Richards 1993, Jo *et al.* 2002). In the present study, total PR mRNA level in cumulus cells of COCs which were cultured with FSH and LH was maintained at a

high level for up to 20 h of cultivation. Interestingly, we found that the level of PR-B decreased in cumulus cells from 12 to 20 h of cultivation, concomitantly with an increasing level of progesterone secreted by COCs. Natraj and Richards (1993) have also reported that PR-A mRNA appears to persist longer than PR-B mRNA in LH-stimulated rat granulosa cells. We examined the relationship between the decrease in PR-B levels and the high levels of secreted progesterone. The results showed that the reduction of PR-B was suppressed by the addition of RU486 following a 10-h cultivation with FSH and LH, but there was no effect on the total level of PR. Judging from these results, it is estimated that the pattern of PR expression, but not the amount of PR expression, is regulated by both the gonadotropin-induced cAMP–PKA pathway and the progesterone–PR pathway in cumulus cells during meiotic resumption of porcine oocytes.

When PR-B predominated in cumulus cells of COCs, stimulation with progesterone increased the proliferative activity of cumulus cells. The proliferative activity was decreased by FSH and LH from 10 to 20 h of cultivation. The decreases of both the proliferative activity and the level of PR-B in cumulus cells were restrained when COCs were cultured with the addition of RU486 following 10-h cultivation with FSH and LH. Since PR-A null mice have decreased ovulation rates (Mulac-Jericevic *et al.* 2000), and those that lack only the B form are not affected (Mulac-Jericevic *et al.* 2003), it

Figure 6 The effects of the addition of RU486 following 10-h cultivation with FSH+LH on the proliferative activity of cumulus cells. (A) COCs were cultured for 10 h with FSH+LH, and then cultured for a further 10-h with 0, 2.5 or 25 µM RU486. Data are expressed as percentage of the absorbance value at 450 nm in cumulus cells of COCs which were cultured for 10 h with the addition of 100 µM BrdU following 10-h cultivation without any hormones. C (control), COCs were cultured for 10 h with the addition of 100 µM BrdU following 10-h cultivation without any hormones. Free, COCs were cultured for 10 h with the addition of 100 µM BrdU and/or 25 µM RU486 following 10-h cultivation without any hormones. FSH+LH, COCs were cultured for 10 h with the addition of 100 µM BrdU and RU486 (0 to 25 µM) following 10-h cultivation with FSH+LH. * $P < 0.01$, significant effects of FSH+LH on proliferative activity of cumulus cells. ^{a-c}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 4 replicates. (B) RT-PCR analysis of PR-A/B or PR-B mRNA in cumulus cells when COCs were cultured for 20 h. The respective values of PR-A/B and PR-B were normalized according to those of beta-actin to evaluate arbitrary units of the relative abundance of the targets. C (control), COCs were cultured without any hormone for 20 h. * $P < 0.01$, significant effect of FSH+LH on the level of PR-A/B; ** $P < 0.05$, significant effect of FSH+LH on the level of PR-B. ^{a,b}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 3 replicates. (C) Western blotting analysis of PR in cumulus cells. 10 h, COCs were cultured for 10 h with FSH+LH. * $P < 0.01$, significant effect of additional 10-h cultivation on the level of PR-B; ** $P < 0.01$, significant effect of additional RU486 following 10-h cultivation with FSH+LH on the level of PR-B. Values are means $\pm$ S.E.M. of 3 replicates. (D) The level of progesterone in the medium where COCs have been cultured for 20 h. Free, COCs were cultured for 20 h without any hormones. * $P < 0.01$, significant effect of FSH+LH on the level of progesterone in the cultured medium. ^{a,b}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 3 replicates.

Figure 7 Time-dependent changes of connexin-43 gene expression in cumulus cells of COCs and the regulation by progesterone. (A) RT-PCR analysis of connexin-43 mRNA in cumulus cells of COCs which were cultured with FSH+LH for up to 20 h. The respective values of connexin-43 were normalized according to those of beta-actin to evaluate arbitrary units of the relative abundance of the targets. ^{a-c}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 4 replicates. (B) The level of connexin-43 mRNA in cumulus cells of COCs cultured with FSH+LH for 8 h. FSH+LH+P, COCs were cultured for 8 h in FSH+LH-containing medium with 20 ng/ml progesterone and/or 25 μ M RU486. Values are means $\pm$ S.E.M. of 3 replicates. (C) The effects of AGT or RU486 on the level of connexin-43 mRNA in cumulus cells of COCs cultured for 20 h. AGT, COCs were cultured for 20 h with FSH+LH and 0.5 mM aminoglutethimide. RU486, COCs were cultured for 10 h with the addition of 25 μ M RU486 following 10-h cultivation with FSH+LH. * $P < 0.01$, significant effect of AGT on the level of connexin-43; ** $P < 0.01$, significant effect of additional RU486 following 10-h cultivation on the level of connexin-43. Values are means $\pm$ S.E.M. of 3 replicates.

may be supposed that the PR-A in cumulus cells is involved not only in the loss of proliferative activity but also in the induction of differentiation. A loss of proliferative activity and differentiation of cumulus cells was also required for meiotic resumption of cumulus-enclosed oocytes (Tanghe *et al.* 2002). It has been reported that a closing of gap junctional communication in cumulus cell layers is one of the important changes occurring in cumulus cells during meiotic resumption of oocytes (Larsen *et al.* 1986, 1987, Isobe *et al.* 1998).

The gap junctional channels are formed by hexameric structures consisting of connexin molecules (connexon) in numerous tissues (Grazul-Bilska *et al.* 1997). Porcine ovarian follicles have been reported to express five different connexin genes: connexin-26, connexin-30.3, connexin-32, connexin-43, and connexin-60 (Itahana *et al.* 1996, 1998). In our previous study (Shimada *et al.* 2001), a disruption of gap junctional communication in the outer layers of cumulus cells resulted from the disappearance of connexin-43 protein in those layers. Thus, the regulation of connexin-43 gene expression in cumulus cells plays an important role in meiotic resumption of cumulus-enclosed oocytes. The present study showed that the expression of connexin-43 gene was stimulated by cultivation with FSH and LH; however, the depletion of connexin-43 mRNA in cumulus cells of COCs was observed after 12 to 20 h of cultivation, concomitantly with increasing levels of PR-A expression. The down-regulation of connexin-43 gene expression was prevented by the addition of RU486 or aminoglutethimide. Giangrande *et al.* (1997) reported that in most cell lines human PR-A functions as a transcriptional repressor, whereas human PR-B functions as a transcriptional activator of progesterone-responsive genes. Moreover, when high levels of PR-B expression were detected in cumulus cells of COCs (8-h cultivation with FSH+LH), the addition of progesterone did not affect the level of connexin-43 mRNA compared with that in cumulus cells cultured with FSH and LH alone. Judging from these results, this is the first report to raise the possibility that a decrease in PR-B levels in cumulus cells is required for down-regulation of connexin-43 gene expression in cumulus cells during meiotic resumption of oocytes.

It has been reported that the *in vivo* administration of anti-progestin antiserum into

PMSG+hCG-treated female rats significantly decreases the incidence of meiotic resumption of oocytes within the large follicles (Mori *et al.* 1983). Osborn *et al.* (1986) also reported that when ovine follicles were cultured with FSH, LH and the P450 scc inhibitor AGT, the result was an almost complete inhibition of progesterone production and meiotic progression to the metaphase II stage in oocytes. Unexpectedly, the addition of progesterone receptor antagonist did not suppress meiotic resumption of mouse oocytes (Batten *et al.* 1989) and porcine oocytes (in the present study). The present study also showed that the predominant isoforms of PR expressed in cumulus cells shifted from PR-B to PR-A during the time course of COCs' cultivation with FSH and LH. Treatment with RU486 following a 10-h cultivation inhibited the meiotic resumption of porcine cumulus-enclosed oocytes, suggesting that the decrease in PR-B levels in cumulus cells was involved in meiotic resumption of porcine oocytes.

Analysis of histology of PR null mice has revealed normal development of intraovarian follicles to the tertiary follicular stage, and the follicles contain a mature oocyte that is fully functional when isolated and fertilized *in vitro* (Lydon *et al.* 1995). Despite the failure of ovulation to occur in PR null mice, cumulus cell expansion proceeds normally (Robker *et al.* 2000). The cultivation of porcine COCs with RU486 from 0 to 44 h had less of an effect on the induction of meiotic resumption and slightly affected the progression to the MII stage; however, the matured oocytes had little developmental competence to proceed to the blastocyst stage. Additionally, cumulus cells became flattened on the culture dish with few remaining suspended in the expanded complex. The present results indicate that the PR-dependent pathway in cumulus cells is essential for normal *in vitro* maturation of porcine oocytes, but in mouse the oocytes may not be dependent on this pathway. The differences of PR expression among the species were also observed during follicular development in ovaries. In rodent and primate ovaries, follicles that developed beyond the primary stage no longer expressed PR in granulosa cells until after the surge of LH, and the LH surge induced a rapid increase in expression of PR in granulosa cells (Hild-Petito *et al.* 1988, Park & Mayo 1991, Shao *et al.* 2003). In pig, early antral follicles contained PR in granulosa cells, and the

stimulation of FSH increased the level in granulosa cells (Slomczynska *et al.* 2000). In dog, Vermeirsch *et al.* (2001) reported that the amount of PR in granulosa cells increased with the stage of follicle development, the level being significantly higher in the secondary and tertiary follicles compared with the primordial and primary follicles. Yoshimura *et al.* (1995) showed in chicken ovary that both PR-A and PR-B were expressed in small antral follicles but only PR-A was expressed in large antral follicles. These reports, including our own study, indicate that the role of PR in follicle cells should be investigated more thoroughly in each species to understand the mechanisms of follicular development and *in vitro* maturation of oocytes.

In summary, PR-B was predominantly expressed in cumulus cells of COCs cultured with FSH and LH. However, the level of PR-B mRNA dropped significantly at 12 h, whereas a high level of total PR mRNA was maintained up to 20 h. Accompanying the decrease of PR-B levels in cumulus cells, the loss of proliferative activity, down-regulation of connexin-43 gene expression and progesterone production in cumulus cells were also observed. The addition of RU486 after 10-h cultivation of COCs with FSH and LH suppressed the functional changes of cumulus cells and the meiotic resumption of oocytes. These results suggest that the induction of PR isoforms in cumulus cells and their binding to progesterone appear to have an impact on proliferation and differentiation in a time-dependent manner, and the shift from PR-B to PR-A may help mediate certain events. The differentiation of cumulus cells mediated by a

PR-dependent pathway is essential for meiotic resumption of porcine oocytes *in vitro*.

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Figure 8 The effects of RU486 on the oocyte maturation *in vitro*. (A) The proportion of oocytes exhibiting GVBD when COCs were cultured for 10 h with additional RU486 (0 to 25 μ M) after 10-h cultivation with FSH+LH. RU486, COCs were cultured for 10 h with FSH+LH, and further cultured for 10-h with 0, 0.25, 2.5 or 25 μ M RU486. AGT, COCs were cultured for 20 h with FSH+LH and 0.5 mM aminoglutethimide. *The additional effect of 0.5 mM AGT to FSH+LH-containing medium is significant ($P < 0.01$). ^{a-d}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 4 replicates. (B) COCs were cultured for 20 or 44 h with FSH+LH and/or 25 μ M RU486. The proportion of oocytes exhibiting GVBD or that reached metaphase II (MII) stage was checked after 20 h or 44 h of cultivation respectively. The developmental competence of matured oocytes to progress to the blastocyst stage was checked at 5 days after *in vitro* fertilization. * $P < 0.05$, significant effect of RU486 on the number of oocytes that reached the MII stage; ** $P < 0.01$, significant effect of RU486 on the developmental competence to progress to the blastocyst stage after *in vitro* fertilization. Values are means $\pm$ S.E.M. of 3 replicates. (C) Proliferative activity of cumulus cells of COCs cultured for 44 h with FSH+LH and RU486 (0 to 25 μ M). Data are expressed as percentage of the absorbance value at 450 nm in cumulus cells of COCs which were cultured for 44 h in the Free medium with 100 μ M BrdU. C (control), COCs were cultured for 44 h with 100 μ M BrdU. * $P < 0.01$, significant effect of FSH+LH on proliferative activity of cumulus cells. ^{a-c}Different superscripts are significantly different at the $P < 0.05$ level. Values are means $\pm$ S.E.M. of 3 replicates. (D) The expanded COCs were observed after 44-h cultivation with FSH+LH. (a) COCs were cultured for 44 h with FSH+LH. (b) COCs were cultured for 34 h with additional 25 μ M RU486 after 10-h cultivation with FSH+LH. (c) COCs were cultured for 44 h with FSH+LH and 25 μ M RU486.

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