The Novel Pig In Vitro Maturation System to Improve Developmental Competence of Oocytes Derived from Atretic Nonvascularized Follicles1

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ABSTRACT

The in vitro maturation (IVM) technique is beneficial for producing animal offspring, but the blastocyst rate is low after IVM. In this technique, cumulus-oocyte complexes (COCs) are collected from medium-size follicles. The follicles are ultimately selected as large dominant follicles or atretic follicles; therefore it is possible that the COCs collected using IVM are contaminated by follicles that will develop into large follicles and induce atresia. In the dominant follicles, estradiol-17β and progesterone induce the differentiation of follicular somatic cells that exhibit the ability to respond to ovulation during follicular development. Thus, we hypothesized that changes in the hormonal condition of healthy follicles are essential for oocyte maturation during IVM. In this study, we performed a comparative analysis of the steroid hormone concentrations in nonvascularized follicles (NVFs) and vascularized follicles (VFs). The estradiol-17β concentration increased in medium VFs, whereas the level was low in NVFs of the same size. The progesterone concentration increased with large follicular size in VFs, but the level remained low in follicles of any size among NVFs. To improve the oocyte quality derived from NVFs, NVF COCs were cultured with follicle-stimulating hormone (FSH) alone or FSH under the VF hormonal conditions. Cultivation under the VF hormonal conditions dramatically improved the proliferation and survival of cumulus cells, meiotic maturation of oocytes, cumulus expansion, and blastocyst rate following in vitro fertilization. Thus, the cultivation of NVF COCs under VF hormonal conditions improves the developmental potential to the blastocyst stage by VF oocytes.

cumulus cells, estradiol-17β, follicular development, follicular selection, in vitro maturation (IVM), oocyte, pig, progesterone

INTRODUCTION

An in vitro maturation (IVM) technique for the oocyte cumulus-oocyte complex (COC) has been developed and applied to produce offspring in mammals, including cattle [1, 2] and pigs [2–4], as well as in clinical infertility treatment [5]. The IVM technique generally uses follicle-stimulating hormone (FSH), growth factors such as endothelial growth factor (EGF) [6, 7], and steroid hormones [8, 9] to stimulate oocyte maturation. However, the rate of blastocyst formation following in vitro fertilization (IVF) is still low using IVM oocytes compared with that of in vivo-matured oocytes, especially in large livestock animals, such as pigs [10, 11]. One of the reasons for the difference in developmental competence by IVM oocytes from various animal species is the origin of oocytes. In pigs, the COCs are usually collected from medium-size antral follicles (>3 mm diameter) where the oocyte has the ability to induce oocyte maturation, whereas follicular somatic cells do not acquire the ability to respond to ovulation stimuli. Thus, a novel IVM technique is required based on information regarding how the oocytes mature during follicular development in vivo in each animal to generate in vitro-matured oocytes with high developmental competence.

Ovarian follicles make up a single oocyte surrounded by somatic cells, which are called granulosa cells. The development of primary follicles is induced by oocyte-secreted factors, such as GDF-9, to form secondary follicles where granulosa cells express FSH receptors [12]. The secretion of FSH from the pituitary gland in the brain induces further follicle development and the formation of an antrum in the follicles. Most of the small antral follicles undergo atretic degeneration, and only a few develop selectively into full-grown preovulatory follicles with the responsive ability to FSH [13, 14]. When FSH is injected into animals, most of the antral follicles develop into preovulatory follicles without atretic degeneration due to FSH-induced ant apoptotic functions [15]. In addition, the follicles collected from preovulatory follicles in FSH-primed mice are easily matured in vitro with a high fertilization likelihood and developmental competence. In contrast, in pigs, ovaries are collected from nonhormonally stimulated 5- to 7-month-old prepubertal giltst at the local slaughterhouse. Most of these ovaries contain only small or medium-size antral follicles measuring <6 mm in diameter [8–11], indicating that the follicular somatic cells do not acquire the sufficient capacity to support oocyte maturation with developmental competence to the blastocyst stage, and, more important, some of them are degenerating follicles. Therefore, understanding the mechanisms that allow follicular somatic cells to differentiate and support oocyte maturation during follicular development is required to improve the IVM technique in pigs.

In the small antral follicles, FSH mainly stimulates the proliferation of granulosa cells to form the antrum between the
granulosa and cumulus cell layers, which directly surround the oocyte. In the small antral follicles, the antrum is filled with follicular fluid that contains a high level of the androgen [16]. The androgen increases the expression of Fshr in granulosa cells, where FSH induces the expression of Cyp19a1, which encodes an aromatase involved in the production of estradiol-17β in mice [17–20], rats [21], cattle [22], and pigs [23]. The positive effects of estradiol-17β induce functional changes in granulosa cells and cumulus cells, which can enable ovulation following a surge in luteinizing hormone (LH) [24]. Indeed, in estrogen receptor β (ERβ, which is also known as NR3A) knockout mice, follicular development with the ability to respond to an LH surge (LH receptor expression) is not fully induced in the natural estrus cycle or even by exogenous FSH stimulation [25]. In contrast, the high level of estradiol-17β in the serum suppresses FSH secretion, which decreases the survival of granulosa cells, thereby leading to atretic degeneration of the follicles, and only a few follicles that express a high level of FSH receptor develop selectively into fully grown large antral follicles, where there is a concomitant high level of estradiol-17β [14]. After follicular development, a transient surge in the pituitary-gland-derived LH level induces Star, Hsd3b1, and Cyp11a1 mRNA expression in the granulosa cells of mice [26, 27], cattle [28], and pigs [29]. The expression of genes involved in progesterone production is induced during the latter stage of the follicular development process but mainly during the ovulation process. In progesterone receptor knockout mice, ovulation is defective, but follicular development is normal [30, 31]. Overall, these findings indicate that the sequential transition of the steroid hormonal environment in follicular fluid from estradiol-17β to progesterone is essential for follicular development and oocyte maturation. Thus, we hypothesized that the optimal and sequential supplementation of estradiol-17β and progesterone in the IVM medium will improve oocyte maturation with developmental competence.

During the follicular developmental stage, impairment of the blood vasculature in the theca interna is associated with the induction of follicular atresia [32–36]. Furthermore, it has been shown that the blood vessel extension induced by vascular EGF in the theca interna occurs in the dominant follicles in cattle but not in the atretic follicles [37–39]. Therefore, in this study, we performed a comparative analysis of estradiol-17β and progesterone production in nonvascularized follicles (NVFs) and vascularized follicles (VFs). We also tried to improve the maturation of oocytes derived from NVFs by treating them under steroid hormone conditions detected in the VFs.
Steroid Hormone Condition Affects In Vitro-Matured Oocyte Quality

Porcine ovaries were collected from 5- to 7-mo-old prepubertal gilts at a local slaughterhouse and transported within 1 h to the laboratory in 0.85% NaCl containing 1% penicillin-streptomycin mixed solution (Nakarai Tesque) at about 30°C. Follicles measuring 1–3 mm (small antral follicle), 4–7 mm (medium antral follicle), and >8 mm (large antral follicle) in diameter were cut with scissors. The dominant follicle had a vascular sheath on the follicular surface and turbidity due to follicular fluid [32, 33, 36]; therefore, the NVFs and VFVs were classified macroscopically by observing their status (Fig. 1A–D). Follicular fluids, granulosa cells, and COCs were collected from NVFs and VFVs by cutting with a surgical blade in a Petri dish and then stored in 1.5-ml tube. Each tube was centrifuged at 1000 rpm for 5 min, and the supernatant was stored at -80°C until the analysis of the estradiol-17β and progesterone concentrations. The precipitates containing granulosa cells and COCs were placed in a Petri dish, and COCs were collected with a Pasteur pipette using a stereomicroscope. The residual granulosa cells were used for total RNA isolation, and the COCs collected from the medium-size VFVs or NVFs were used for IVM.

In Vitro Culture of Porcine COCs

For IVM of COCs collected from medium-size VFVs or NVFs, oocytes having an evenly granulated cytoplasm with at least four layers of unexpanded cumulus cells were selected by microscopic examination and washed three times. Twenty COCs were cultured at 39°C in each well of a 96-well plate (Thermo Fisher Scientific) containing 100 µl of modified NCSU37 medium (maturation medium) without covering with mineral oil in a humidified incubator (95% air, 5% CO₂), as described in our previous studies [41, 42]. To avoid the evaporation of the maturation medium during IVM in the 96-well plate, we placed the water in all of the wells in which COCs were not cultured. The maturation medium was supplemented with 10% (v/v) FCS (Gibco BRL) and 7 mM of taurine (Sigma). COCs were cultured in each treatment group shown in Figure 2. For conventional IVM, the maturation medium was supplemented with 100 ng/mL of highly purified FSH (NIDDK). COCs were cultured for 24 h in the medium and then replaced with medium containing the same dose of FSH before a further 24-h culture (conventional system). In the NVF E2 P4 culture system that mimicked the steroid hormone conditions in the follicular fluid of NVFs, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH, 15 ng/mL of E2, and 35 ng/mL of P4 for 24 h. The 24-h-cultured COCs were washed twice with the maturation medium and then cultured in maturation medium supplemented with 100 ng/mL of FSH, 85 ng/mL of E2, and 5 ng/mL of P4 for another 24 h. In the VF E2 culture system, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for 24 h. The 24-h-cultured COCs were then cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for another 24 h. In the VF P4 culture system, COCs were cultured in fresh maturation medium supplemented with 100 ng/mL of FSH, 50 ng/mL of E2, and 50 ng/mL of P4 for the first 24 h. The COCs were then cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 150 ng/mL of P4 for another 24 h. To culture COCs under the conditions that mimicked the levels of E2 and P4 in the follicular fluid of VFVs, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH, 85 ng/mL of E2, and 35 ng/mL of P4 for another 24 h. The COCs were then washed twice with the maturation medium and cultured in maturation medium containing 100 ng/mL of FSH, 85 ng/mL of E2, and 150 ng/mL of P4 for another 24 h. After cultivation, the diameters of the COC expanded matrix.

Materials and Methods

Materials

Highly purified FSH was a kind gift from the National Hormone and Pituitary Program (National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK)), and it was used at 100 ng/mL. Estradiol-17β (E2) and progesterone (P4) were purchased from Sigma Chemical Company. They were dissolved in ethanol and stored at -20°C until use. The final concentration of ethanol in the medium was 0.1% (v/v), which did not affect the functions of cumulus cells during meiosis [40]. Fetal calf serum (FCS) was purchased from Invitrogen. Oligonucleotide poly-dT was obtained from GE Healthcare. Avian myeloblastosis virus reverse transcriptase and Taq DNA polymerase were obtained from Promega. Routine chemicals and reagents were purchased from Nakarai Tesque or Sigma.

In Vitro Fertilization

Oocytes from cultured COCs were used for IFS as described in our previous study [45]. Briefly, cumulus cell-free oocytes were washed three times with the fertilization medium, which comprised modified Tris-buffered medium (mTBM) supplemented with 0.1% (w/v) BSA (fraction V, A 7888; Sigma) and 1-mM caffeine (Sigma). After washing, 20 oocytes were placed in 50-µL drops of the fertilization medium, which was covered with mineral oil in a 35 × 10-mm petri dish cultured in a 5% CO₂ incubator (Thermo Fisher Scientific) containing 95% air, 5% CO₂, and 100% relative humidity. The dishes were kept in the incubator for approximately 30 min until adding the spermatozoa for fertilization. Fresh spermatozoa from a Duroc boar were purchased from Swinegenetics. These spermatozoa were washed by centrifugation at 700 × g for 5 min in washing

FIG. 2. Schematic illustrating the conventional culture system, NVF E2 P4 culture system, VF E2 culture system, VF P4 culture system, and VF E2 culture system. Conventional culture system: COCs were cultured in maturation medium supplemented with 100 ng/mL of highly purified FSH (NIDDK) for 48 h. NVF culture system: COCs were cultured in maturation medium supplemented with 100 ng/mL of FSH, 15 ng/mL of E2, and 35 ng/mL of P4 for 24 h. The 24 h-cultured COCs were then cultured in maturation medium supplemented with 100 ng/mL of FSH, 85 ng/mL of E2, and 5 ng/mL of P4 for another 24 h. VF E2 culture system: COCs were cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for 24 h. The 24-h-cultured COCs were cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for another 24 h. VF P4 culture system: COCs were cultured in fresh maturation medium supplemented with 100 ng/mL of FSH, 50 ng/mL of E2, and 50 ng/mL of P4 for 24 h. The 24 h-cultured COCs were then cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 150 ng/mL of P4 for another 24 h. In the VF E2 P4 culture system, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for 24 h. The 24-h-cultured COCs were washed twice with the maturation medium and then cultured in maturation medium supplemented with 100 ng/mL of FSH, 85 ng/mL of E2, and 5 ng/mL of P4 for another 24 h. In the VF E2 culture system, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for 24 h. The 24-h-cultured COCs were then cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 85 ng/mL of E2 for another 24 h. In the VF P4 culture system, COCs were cultured in fresh maturation medium supplemented with 100 ng/mL of FSH, 50 ng/mL of E2, and 50 ng/mL of P4 for the first 24 h. The COCs were then cultured in fresh maturation medium supplemented with 100 ng/mL of FSH and 150 ng/mL of P4 for another 24 h. To culture COCs under the conditions that mimicked the levels of E2 and P4 in the follicular fluid of VFVs, COCs were first cultured in maturation medium supplemented with 100 ng/mL of FSH, 85 ng/mL of E2, and 35 ng/mL of P4 for another 24 h. The COCs were then washed twice with the maturation medium and cultured in maturation medium containing 100 ng/mL of FSH, 85 ng/mL of E2, and 150 ng/mL of P4 for another 24 h. After cultivation, the diameters of the COC expanded matrix.

Oocytes were separated and their nuclear status was assessed as described previously [41–44]. Separated cumulus cells were used for isolating total RNA and protein at selected intervals.

In Vitro Fertilization

Oocytes from cultured COCs were used for IFS as described in our previous study [45]. Briefly, cumulus cell-free oocytes were washed three times with the fertilization medium, which comprised modified Tris-buffered medium (mTBM) supplemented with 0.1% (w/v) BSA (fraction V, A 7888; Sigma) and 1-mM caffeine (Sigma). After washing, 20 oocytes were placed in 50-µL drops of the fertilization medium, which was covered with mineral oil in a 35 × 10-mm petri dish cultured in a 5% CO₂ incubator (Thermo Fisher Scientific) containing 95% air, 5% CO₂, and 100% relative humidity. The dishes were kept in the incubator for approximately 30 min until adding the spermatozoa for fertilization. Fresh spermatozoa from a Duroc boar were purchased from Swinegenetics. These spermatozoa were washed by centrifugation at 700 × g for 5 min in washing
medium, which comprised mTBM supplemented with 0.1% (w/v) BSA. The sperm pellet was resuspended and precultured for 90 min in preculture medium, that is, mTBM supplemented with 10% (v/v) FCS and 1 mM caffeine. The concentration of spermatozoa during precultivation was 2 × 10^6 cells/ml. The precultured spermatozoa were diluted to 2 × 10^6 cells/ml in the fertilization medium, and 50 μl of this sperm suspension were added to 50 μl of the fertilization medium containing oocytes (final concentration of sperm: 10^5 cells/ml). The oocytes were cocultured with spermatozoa at 39°C in an atmosphere of 5% CO2 in air for 6 h. The mTBS used for IVF was nearly identical to that used by Abeydeera and Day [46]. The pH of the mTBM was 8.8 immediately after preparation, that is, approximately 9.8–10.5; therefore, the mTBM was adjusted to a pH of 7.2–7.3 before use.

**In Vitro Production of Embryos**

After sperm-oocyte coinoculation, putative zygotes were cultured as described by Shimada et al. [47]. After coinoculation of the gametes for 6 h, the putative zygotes were washed three times and transferred to a culture in vitro (ICM) medium. The day of insemination was defined as Day 0. The ICM medium was NCSU37 medium containing 0.4% BSA (fraction V, A 8022; Sigma). In vitro-produced embryos were cultured in ICM medium supplemented with 0.1% (w/v) BSA. The concentration of spermatozoa during precultivation was 2 × 10^6 cells/ml. The precultured spermatozoa were diluted to 2 × 10^6 cells/ml in the fertilization medium, and 50 μl of this sperm suspension were added to 50 μl of the fertilization medium containing oocytes (final concentration of sperm: 10^5 cells/ml). The oocytes were cocultured with spermatozoa at 39°C in an atmosphere of 5% CO2 in air for 6 h. The mTBS used for IVF was nearly identical to that used by Abeydeera and Day [46]. The pH of the mTBM was basic immediately after preparation, that is, approximately 9.8–10.5; therefore, the final IVM medium was kept in an incubator (an atmosphere of 5% CO2 in air at 39°C) for 18–24 h to stabilize the pH to 7.2–7.3 before use.

**Quantification of E2 and P4 in Follicular Fluid**

Follicular fluid derived from VFs or NVFs was collected in plastic tubes. The concentration of total estradiol in the follicular fluid was determined using a specific Estradiol EIA Kit (Cayman Chemical Company) or Progesterone EIA Kit (Cayman Chemical Company), respectively, according to the manufacturer’s instructions. The estradiol EIA Kit and Progesterone EIA Kit were used to measure the concentrations of estradiol and progesterone in the follicular fluid. The estradiol level in the follicular fluid was determined using a specific Estradiol EIA Kit (Cayman Chemical Company) or Progesterone EIA Kit (Cayman Chemical Company), respectively, according to the manufacturer’s instructions.

**Morphological Analysis of VFs and NVFs**

For the morphological analysis, 4–7-mm-diameter sections of VFs and NVFs from porcine ovaries were fixed in 4% paraformaldehyde (Nakarai Tesque) for 24 h at 4°C. Subsequently, these tissues were washed with PBS and embedded in paraffin after dehydration. Paraffin tissue sections (5 μm) were stained with hematoxylin solution for 1 min and then stained with eosin for 30 min. After dehydration with alcohol and xylene, the mount was dropped on the slide and covered with a glass coverslip. Tissues were observed under a light microscope.

**Western Blot Analysis**

Cumulus cells were denuded from the cultured COCs to analyze the phosphorylated ERK1/2 level in cumulus cells from NVF COCs cultured in the conventional system or the VF culture system. Protein samples from cumulus cells were prepared by homogenization in whole cell extract buffer and then diluted using 2X sodium dodecyl sulfate (SDS) sample buffer (Nakalai Tesque). Extracts (5 μg of protein) were resolved by SDS polyacrylamide gels (10%) electrophoresis and transferred to PVDF membranes (GE Healthcare). The membranes were blocked in Tris-buffered saline and Tween 20 (TBST; 10 mM Tris [pH 7.5], 150 mM NaCl, and 0.05% [v/v] Tween 20), containing 5% (w/v) nonfat Carnation instant milk (Nestlé Co.) at room temperature. Blots were then incubated overnight at 4°C with the appropriate primary antibodies. The membranes were then washed with TBST and incubated with secondary antibodies conjugated to horse radish peroxidase. The signals were detected using an enhanced chemiluminescence detection system (Bio-Rad Laboratories). The specific primers and annealing temperature were selected as indicated in Table 1. The results were first normalized against the expression levels of a housekeeping gene, 18S. To avoid false-positive signals, dissociation-curve analysis was performed at the end of amplification, and the PCR products were subjected to agarose gel electrophoresis to confirm their sizes.

**RNA Extraction and Quantitative RT-PCR Analysis**

Total RNA isolation was performed as described in our previous study [41]. Briefly, granulosa cells and cumulus cells were washed three times with PBS. Total RNA was extracted from cells using an RNasy Mini Kit (QIAGEN Sciences) according to the instruction manual and dissolved in nucleic-acid-free water. The final RNA concentration (10 ng/μl) was determined based on the absorbance measured using a microspectrophotometer (Thermo Fisher Scientific).

Reverse transcription was performed as previously described [42, 49]. Briefly, total RNA was reverse transcribed using 500 ng of poly-dT (Promega) and 0.25 U of avian myeloblastosis virus reverse transcriptase (Promega) for 75 min at 42°C and for 5 min at 95°C.

Real-time PCR analysis was performed as previously described [42]. Briefly, cDNA and primers were added to the KAPA SYBER Fast Universal qPCR Kit (Kapa Biosystems) to obtain a total reaction volume of 15 μl. The PCR reactions were then performed using the MiniOpticon Real-Time PCR Detection System (Bio-Rad). The cycle conditions were set according to the following parameters: 30 sec at 95°C, followed by 40 cycles each for 5 sec at 95°C and 45 sec. The specific primers and annealing temperature were selected and analyzed as indicated in Table 1. The results were first normalized against the expression levels of a housekeeping gene, 18S. To avoid false-positive signals, dissociation-curve analysis was performed at the end of amplification, and the PCR products were subjected to agarose gel electrophoresis to confirm their sizes.

**Table 1. List of primers used for quantitative RT-PCR and the annealing temperatures.**

<table>
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<tr>
<th>mRNA</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Accession no.</th>
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<td>58</td>
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<tr>
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<td>86</td>
<td>AF025377.1</td>
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<tr>
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<td>195</td>
<td>60</td>
<td>NM_214429.1</td>
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<tr>
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<td>179</td>
<td>56</td>
<td>NM_213755.2</td>
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<tr>
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<td>192</td>
<td>64</td>
<td>NM_214247.1</td>
<td></td>
</tr>
<tr>
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<td>245</td>
<td>60</td>
<td>NM_00100409.1</td>
<td></td>
</tr>
<tr>
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<td>NM_214088.1</td>
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<tr>
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<td>60</td>
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*F, forward; R, reverse.*
incubated overnight with p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (total ERK1/2; tERK) (1:1000 dilution; product no. 4695; Cell Signaling) or phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb (phosphorylated ERK1/2; pERK1/2) (1:1000 dilution; product no. 9106; Cell Signaling) at 4°C. After washing four times in TBST, the membranes were incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (1:5000 dilution; product no. 7074; Cell Signaling) or goat anti-mouse IgG HRP-linked antibody (1:3000 dilution; product no. 7076; Cell Signaling) at room temperature. After five washes for 10 min each with TBST, enhanced chemiluminescence (ECL) detection was performed using the ECL prime Western blotting detection system (GE Healthcare), according to the manufacturer’s instructions, and the blots were exposed as appropriate to Fuji X-ray films (Fujifilm). The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics, Inc.).

Immunofluorescence of Proliferating Cell Nuclear Antigen-Positive Cumulus Cells

To detect proliferating cell nuclear antigen (PCNA)-positive cumulus cells, COCs were placed on a glass slide, and the glass slide was then air-dried at 37°C for 2 h. The COCs on the glass slide were blocked using 4% paraformaldehyde (Nakalai Tesque) at room temperature for 15 min. The fixed COCs were permeabilized in PBS with 0.3% (v/v) Triton-X100 for 15 min before washing three times for 5 min in PBS. The COCs were blocked in PBS containing 5% (w/v) BSA at room temperature for 2 h and then incubated for 4 h with anti-PCNA (PC10) mouse monoclonal antibody (1:100 dilution; product no. 2586; Cell Signaling). After incubation, the COCs were washed three times in PBS and then incubated for 2 h with an anti-mouse IgG F(ab')2 fragment Cy3 antibody (1:100 dilution; product no. C2181; Sigma). After washing the COCs three times in PBS, they were mounted with a cover glass on a slide glass using Vectashield with DAPI (Vector Laboratories). The fluorescence of PCNA-positive cells was detected by confocal fluorescence microscopy using an excitation filter at 550 nm (Olympus FV10i; Olympus). The percentage of...
FIG. 4. Characteristics of follicular development in VFs and NVFs. The expression of genes encoding the FSH receptor (Fshr; A); enzymes for steroid hormone production, Cyp19a1 (B), Star (C), Cyp11a1 (D), and Hsd3b1 (E), in granulosa cells; and the concentrations of estradiol-17β (F) and progesterone (G) in follicular fluid derived from follicles with diameters of 1–3, 4–7, or >8 mm. The expression of genes encoding the FSH receptor (Fshr; A) and enzymes for steroid hormone production, Cyp19a1 (B), Star (C), Cyp11a1 (D), and Hsd3b1 (E), were analyzed by quantitative RT-PCR. A–E) Granulosa cells were collected from VFs and NVFs with diameters of 1–3, 4–7, or >8 mm. Expression of Fshr (A) and the enzymes for steroid hormone production,
PCNA-positive signals was calculated among the cumulus cells relative to the total number of cumulus cells.

**TUNEL Assay**

Apoptotic COC cells were visualized in a chamber slide using the TUNEL method (in Situ Cell Death Detection Kit, Roche Diagnostics GmbH), according to the manufacturer’s instructions. Briefly, COCs were placed on slides, which were then dried at 37°C for 2 h. The COCs were permeabilized in PBS with 0.1% Triton-X for 10 min before washing twice for 10 min in PBS containing 3 mg/ml PVP. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 37°C. After incubation, the COCs were washed three times in PBS with 3 mg/ml PVP for 5 min each before mounting with a cover glass on a slide glass using Vectashield with DAPI (Vector Laboratories). The fluorescence of the fragmented DNA ends was detected by confocal fluorescence microscopy using an excitation filter at 495 nm (Olympus FV10i). The number of TUNEL-positive signals was counted among the cumulus cells per COC.

**Statistical Analyses**

All of the results were expressed as means ± SEM. To compare gene expression in granulosa cells derived from VFs and NVFs, the data were analyzed using t-tests (Statview; Abacus Concepts). To compare the developmental competence to the blastocyst stage, the data were analyzed using chi-square tests (Statview). Other data were subjected to one-way analysis of variance followed by t-tests (Statview). All of the data that were expressed as percentages were arcsine transformed before analysis; P < 0.05 was considered significantly different.

**RESULTS**

**Morphological Differences Between VFs and NVFs in the Porcine Ovary**

In the VFs, the granulosa cells adhered tightly to each other, and most of the recovered COCs had at least four layers of intact cumulus cells (Fig. 3, A and B). In the NVFs, the granulosa cells were dispersed, and cells were detached from the basal membrane (Fig. 3C). However, some of the COCs recovered from NVFs retained a sufficient proportion of cumulus cells (Fig. 3D). Morphological differences were not observed by microscopy in the COCs from VFs and NVFs (Fig. 3, B and D).

When COCs derived from VFs or NVFs were cultured with FSH alone for 24 or 48 h, the GVBD and MII rates for oocytes derived from NVFs were significantly lower than those for oocytes derived from VFs (GVBD rate: 85.5 ± 1.5 vs. 58.5 ± 8.6; MII rate: 60.1 ± 4.6 vs. 23.9 ± 10.9) (Fig. 3, E and F).

**Characteristics of Follicular Development in VFs and NVFs**

In VFs, the Fshr mRNA levels in granulosa cells from follicles with diameters of 1–3 mm, 4–7 mm, or >8 mm were higher than those in each of the NVF sizes. There was a significant difference in the VFs with diameters of 1–3 mm compared with the NVFs of the same size (Fig. 4A). Cyp19a1 expression was induced significantly in granulosa cells from VFs with diameters of 4–7 mm compared with that in granulosa cells recovered from VFs with diameters of 1–3 mm or >8 mm. The expression level of Cyp19a1 in granulosa cells derived from NVFs with diameters of 4–7 mm was significantly lower than that in those from VFs of the same size. The difference was not significant, but the Cyp19a1 mRNA expression level in granulosa cells from NVFs with diameters of >8 mm was higher compared with that in granulosa cells from VFs with diameters of >8 mm (Fig. 4B).

In VFs and NVFs, Star and Hsd3b1 mRNA were constitutively expressed in granulosa cells at all follicular sizes (Fig. 4, C and E). The Cyp11a1 mRNA expression level was also high compared with that in granulosa cells derived from VFs and NVFs up to 4–7 mm. The expression level was high in VFs with diameters of >8 mm, whereas the expression level in granulosa cells derived from NVFs with diameters of >8 mm was significantly lower compared with that in granulosa cells derived from VFs (Fig. 4D).

The estradiol-17β level was 22.0 ± 7.0 ng/ml in VFs with diameters of 1–3 mm. The level was significantly higher at 4–7 mm (84.3 ± 18.6 ng/ml) and even higher at >8 mm (88.7 ± 38.6 ng/ml) (Fig. 4F). The estradiol-17β concentrations were low in the follicular fluid from NVFs with diameters of 1–3 mm and 4–7 mm (1–3 mm: 16.4 ± 1.7 ng/ml; 4–7 mm: 15.9 ± 1.8 ng/ml). However, the concentration of estradiol-17β was higher in the NVFs with diameters of >8 mm, and the E2 concentration was similar to that in VFs with diameters of >8 mm (Fig. 4F).

The progesterone concentration was 13.4 ± 10.6 ng/ml in VFs with diameters of 1–3 mm. The level increased significantly after the induction of follicular development (48.8 ± 15.6 ng/ml in VFs = 4–7 mm; 137 ± 16.9 ng/ml in VFs >8 mm). The production of progesterone did not differ in NVFs with diameters of 1–3 mm or 4–7 mm compared with the VFs of the same size. In NVFs with diameters >8 mm, the induction of progesterone production was not detected, and the level was significantly lower than that in follicular fluid recovered from VFs with diameters >8 mm (Fig. 4G).

**Additional Effects of the Physiological E2 and P4 Levels Detected in VF Follicular Fluid or NVF Follicular Fluid on the Maturation of COCs Derived from VFs**

To evaluate the additional effects of the physiological E2 and P4 levels detected in VF follicular fluid or NVF follicular fluid on oocyte maturation, COCs derived from VFs (VF COCs) were cultured in a conventional culture system, VF E2 P4 culture system, or NVF E2 P4 culture system for 48 h. When VF COCs were cultured in the conventional culture system, the cumulus cells were fully expanded. The VF E2 P4 condition also fully induced cumulus expansion, whereas the NVF E2 P4 condition suppressed cumulus expansion compared with VF COCs cultured in the conventional culture system or the VF E2 P4 culture system (Fig. 5A). The diameter reached almost 400 mm during the culture of VF COCs in the conventional condition or the VF E2 P4 condition, whereas the diameter was significantly smaller after culturing VF COCs in the NVF E2 P4 culture system (Fig. 5B). The GVBD rate was suppressed significantly by culturing in the NVF E2 P4 culture system compared with that of VF COCs cultured in the conventional system or the NVF E2 P4 culture system (Fig. 5C) (conventional culture system: 77.9 ± 3.7%; VF E2 P4 culture system: 76.1 ± 1.5%; NVF E2 P4 culture system: 50.7 ± 6.1%). Furthermore, the NVF E2 P4 condition significantly suppressed GVBD.

Cyp19a1 (B), Star (C), Cyp1l1 (D), and Hsd3b1 (E), were analyzed by quantitative RT-PCR. Values represent the mean ± SEM based on at least three independent culture experiments. F, G, I, J: Follicular fluid was recovered from VFs and NVFs with diameters of 1–3, 4–7, or >8 mm. The concentrations of estradiol-17β (F) and progesterone (G) were analyzed by ELISA. Values represent the mean ± SEM based on at least three independent culture experiments. a–c: Significant differences were observed among VFs with diameters of 1–3 mm, 4–7 mm, and >8 mm (P < 0.05). *Statistical analyses were performed using t-tests, and significant differences were observed between VFs and NVFs (P < 0.05).
FIG. 5. Additional effects of the physiological E2 and P4 levels detected in VF or NVF follicular fluid on the maturation of COCs derived from VFs. 

A) Morphology of VF COCs cultured for 48 h in the conventional culture system, VF E2 P4 culture system, and NVF E2 P4 culture system. 

B) Diameters of VF COCs cultured for 48 h in the conventional culture system, VF E2 P4 culture system, and NVF E2 P4 culture system. After 48 h of cultivation, the diameters of COCs were examined using an eyepiece micrometer and phase-contrast microscopy. 

C, D) GVBD rate (C) and MII rate (D) of VF COCs cultured for 48 h in the conventional culture system, VF E2 P4 culture system, and NVF E2 P4 culture system. VF COCs were collected from VF follicles cultured for 24 h (C) or 48 h (D) in the conventional culture system, VF E2 P4 culture system, and NVF E2 P4 culture system. Values represent the mean ± SEM based on at least three independent culture experiments. *Significant differences were observed (P < 0.05).
FIG. 6. Additional effects of the physiological E2 and P4 levels detected in VF follicular fluid on oocyte maturation of COCs derived from NVFs. A) Morphology of NVF COCs after 48 h of culture in the conventional culture system, VF E2 culture system, VF P4 culture system, or VF E2 P4 culture system. B) Diameter of NVF COCs after 48 h of culture in the conventional culture system, VF E2 culture system, VF P4 culture system, or VF E2 P4 culture system. After 48 h of culture, the diameter of COC was examined by an eyepiece micrometer and phase-contrast microscopy. C, D) GVBD rate (C) or MII rate (D) of NVF COCs after 48 h of culture in the conventional culture system, VF E2 culture system, VF P4 culture system, or VF E2 P4 culture system. COCs collected from NVFs were cultured for 24 h (C) or 48 h (D) in the conventional culture system, VF E2 culture system, VF P4 culture system, or VF E2 P4 culture system. Values are mean ± SEM of at least three independent culture experiments. *Significant differences were observed ($p < 0.05$).
FIG. 7. Additional effects of the physiological E2 and P4 levels detected in VF follicular fluid on cumulus cell proliferation of COCs derived NVFs. A, B) Immunofluorescence of PCNA-positive cumulus cells (Cy3) from NVF COCs. NVF COCs were cultured in the conventional culture system and VF E2 P4 culture system (B) for 24 h. PCNA-positive cumulus cells were detected after cultivation. ×100: COCs were observed at ×100 magnification. ×600: Cumulus cells were observed at ×600 magnification. DAPI staining was used for DNA detection. C) Quantification of A and B. The numbers of PCNA-positive cumulus cells relative to total cumulus cells from NVF COCs cultured in the conventional culture system and VF E2 P4 culture system. NVF COCs...
suppressed progression to the MII stage (Fig. 5D) (conventional culture system: 60.1 ± 3.7%; VF E2 P4 culture system: 60.1 ± 4.6%; NVF E2 P4 culture system: 23.9 ± 10.9%).

**Additional Effects of the Physiological E2 and P4 Levels Detected in VF Follicular Fluid on Oocyte Maturation of COCs Derived from NVFs**

To investigate the additional effects of the physiological steroid hormone conditions detected in VF follicular fluid on cumulus expansion and the meiotic maturation of NVF COCs, NVF COCs were cultured in the conventional culture system, VF E2 culture system, VF P4 culture system, or VF E2 P4 culture system for 24 or 48 h. The induction of full cumulus expansion was not observed in NVF COCs cultured with FSH alone (conventional culture system), FSH and E2 (VF E2 culture system), or FSH and P4 (VF P4 culture system). However, the VF E2 P4 culture system dramatically expanded the cumulus cell layers in NVF COCs (Fig. 6A). When COCs were cultured in the conventional culture system, the diameter of the NVF COCs was 207.2 ± 12.8 μm. Cultivation in the VF E2 culture system or the VF P4 culture system did not significantly increase the diameter of the COCs (VF E2 culture system: 202.5 ± 18.2 μm; VF P4 culture system: 222.8 ± 17.7 μm). However, when NVF COCs were cultured in the VF P4 culture system, the diameter was significantly increased (377.8 ± 24.6 μm) compared with that of NVF COCs cultured in the conventional culture system, VF E2 culture system, or VF P4 culture system (Fig. 6B).

In the conventional system, the GVBD rate and MII rates were low (GVBD rate: 46.7 ± 4.7%; MII rate: 16.7 ± 6.4%). The cultivation of NVF COCs in the VF E2 culture system or the VF P4 culture system for 24 h did not increase the GVBD and MII rates (GVBD rate: VF E2 culture system: 11.6 ± 4.6%, VF P4 culture system: 23.2 ± 0.7%; MII rate: VF E culture system: 21.4 ± 0.5%, VF P4 culture system: 11.7 ± 3.7%), but the GVBD and MII rates were significantly increased by the VF E2 P4 culture system (GVBD rate: 73.3 ± 6.8%; MII rate: 62.1 ± 5.1%) (Figure 6C and D).

**Additional Effects of the Physiological E2 and P4 Levels Detected in VF Follicular Fluid on the Proliferation of Cumulus Cells of NVF COCs**

To examine whether the physiological E2 and P4 levels detected in VF follicular fluid affected the proliferation of cumulus cells among NVF COCs, COCs were cultured for 24 h in the conventional culture system or the VF E2 P4 culture system and then stained with anti-PCNA antibody. There were fewer PCNA-positive cumulus cells among the COCs cultured in the conventional culture system (Fig. 7A), whereas more positive cells were observed after cultivation in the VF E2 P4 condition (Fig. 7B). The percentage of PCNA-positive cells relative to the total number of COCs was about 4% in the conventional culture system, but the percentage increased significantly to about 15% using the VF E2 P4 culture system (Fig. 7C). Furthermore, the expression of Ccnd2 in cumulus cells among NVF COCs was increased significantly by cultivation in the VF E2 P4 culture system compared with that using the conventional culture system (Fig. 7D).

**Additional Effects of the Physiological E2 and P4 Levels Detected in VF Follicular Fluid on the Expression of EGF-Like Factor, Phosphorylation of ERK1/2, and Expression of Genes Involved in Cumulus Expansion in Cumulus Cells of COCs Derived from NVFs**

Our previous studies showed that the EGF-like factor (amphiregulin and epiregulin) EGFR-ERK1/2 pathway in cumulus cells affects oocyte maturation in porcine COCs [41, 42]; therefore, we also examined the effects of the physiological E2 and P4 levels detected in VF follicular fluid on the expression of genes encoding EGF-like factor and the phosphorylation of ERK1/2 in cumulus cells from NVF COCs. Areg expression was induced significantly in cumulus cells from NVF COCs cultured in the VF E2 P4 culture system compared with that in cumulus cells from the COCs cultured in the conventional culture system (Fig. 8A). Significant differences were not found in the Areg mRNA levels in cumulus cells from NVF COCs cultured in the VF E2 P4 culture system compared with those of NVF COCs cultured in the conventional culture system (Fig. 8B). The phosphorylation of ERK1/2 in cultured cumulus cells was induced by cultivation in the VF E2 P4 hormonal condition compared with that in the conventional condition (Fig. 8C). Furthermore, the expression levels of genes such as Has2, Tnfapi6, and Ptx3 were significantly upregulated in cumulus cells from COCs cultured in the VF E2 P4 culture system compared with those in the conventional culture system (Fig. 8D–F).

In VF COCs just after collection from their follicle, the positive signals of apoptosis in cumulus cells were less detected. Few apoptotic signals were observed after the COCs were cultured in the conventional culture system or the VF E2 P4 culture system. However, some positive cells were detected in VF COCs cultured in the NFV E2 P4 culture system (Fig. 9A). The percentage of TUNEL-positive cells relative to the total number of cumulus cells from COCs was low in the conventional culture system or the VF E2 P4 culture system (Fig. 9B), but the percentage was significantly higher in VF COCs cultured in the NVF E2 P4 culture system (Fig. 9C). In contrast, in the NFVs, the cumulus cells immediately exhibited positive signals that indicated apoptosis after their recovery from the follicles. Positive signals of apoptosis were also detected after the cultivation of NVF COCs for 48 h in the conventional culture system or the NVF E2 P4 culture system. However, after the cultivation of NVF COCs in the VF E2 P4 culture system, the positive signals that indicated apoptosis in cumulus cells were abolished, and most of the cumulus cells were viable (Fig. 9B). The number of positive signal cells per COC was almost 50 when NVF COCs were cultured in the conventional culture system or the NVF E2 P4 culture system, but the intensity of the signal was reduced significantly by cultivation in the VF E2 P4 culture system (Fig. 9D).
FIG. 8. Additional effects of the physiological E2 and P4 levels detected in VF follicular fluid on markers for cumulus expansion in COCs derived from NVFs. A, B) RT-PCR analysis of Areg (A) and Ereg (B) mRNA in cumulus cells. NVF COCs were cultured in the conventional culture system and VF culture system for 3 h. The expression of Areg and Ereg was analyzed by quantitative RT-PCR after cultivation. Values represent the mean ± SEM based on at least three independent culture experiments. *Significant differences were observed (P < 0.05). C) Western blotting analysis of pERK1/2 or tERK1/2 in cumulus cells. NVF COCs were cultured in the conventional culture system and VF E2 P4 culture system for 24 h; pERK1/2 and tERK were detected by Western blot.
Additional Effects of the Physiological E2 and P4 Levels in VF Follicular Fluid on the Developmental Competence of Oocytes of COCs Derived from VFs or NVFs

After insemination, about half (45.5 ± 10.2%) of the oocytes from VF COCs cultured in the conventional culture system reached the blastocyst stage. The high rate of blastocyst formation was not affected significantly by culture in the VF E2 P4 culture system (42.0 ± 4.5%). The blastocyst formation rate (6.97 ± 4.9%) was significantly lower in NVF COCs compared with that in VF COCs when cultured in the conventional culture system. However, the rate was significantly higher after culture in the VF E2 P4 culture system, where the percentage was similar to that in VF COCs cultured in the conventional culture system (Fig. 10).

DISCUSSION

In the dominant follicle, vascular formation is induced in the follicular theca interna, whereas vascularization on the surface of the follicular walls is not observed in atretic follicles. In addition, cellular debris from granulosa cells is present in the follicular fluid of atretic follicles [32, 33, 36]. In this study, we classified the dominant follicles as VFs and atretic follicles as NVFs according to these criteria. In VFs, the granulosa cells adhered tightly to the follicular basement membrane. In NVFs, the granulosa cells were separated, and each of the cells floated in the follicular antrum. When COCs were collected from NVFs, some of the cumulus cells were stained using the TUNEL method. The oocyte maturation competence was low in NVFs due to the low quality of the follicular environment, including the steroid hormone conditions and cumulus cell functions. However, the morphology of COCs observed by stereomicroscope was not different between VFs and NVFs, indicating that one of the reasons why oocytes matured in vitro presented the low developmental competence would be explained by the contamination of the low quality of COCs derived from NVFs.

Previous studies have shown that the upregulation of Cyp19a1 depends on stimulation by FSH during follicular development [22, 50–52]. Tilly et al. [53] showed that apoptosis by atretic follicles is associated with decreased Fshr and Cyp19a1 mRNA levels in porcine granulosa cells. In this study, we also investigated whether the Fshr mRNA expression level was increased in cumulus cells after the cultivation of NVF COCs in the VF E2 P4 condition. In NVF COCs, apoptotic cumulus cells were detected before culture, but cultivation in the VF E2 P4 condition dramatically decreased the percentage of apoptotic cumulus cells. Under these culture conditions, the expression level of Ccnd2 required for the proliferation of follicular somatic cells in vivo [54] was increased significantly in the cumulus cells from NVF COCs. The proliferation maker, PCNA, was also detected in about 15% of the cumulus cells, and the percentage was significantly higher than that in cumulus cells from NVF COCs cultured in the conventional system. Given these results, it is possible that survival and proliferation are induced by the physiological levels of E2 and P4 detected in VFs. In our previous studies, the number of cumulus cells in porcine COCs was increased by FSH and E2 [55]. The addition of FSH induced cell survival by cumulus cells from porcine COCs due to the activation of the PI 3-kinase-Akt pathway [56]. The results of the present study demonstrate that the physiological levels of E2 and P4 detected in VFs increased the expression level of Fshr in cumulus cells from NVF COCs (Supplemental Figure S1; Supplemental Data are available online at www.biolreprod.org), so we hypothesize that in NVFs, the low expression level of FSHR, due to the low levels of E2 and P4, would induce atresia. Thus, if NVF COCs are used for IVM, the COCs should be treated with the physiological levels of E2 and P4 detected in VFs to induce FSHR expression, thereby enhancing cumulus cell proliferation and functions and improving the oocyte developmental competence after culture. Indeed, the cultivation of NVF oocytes in the VF E2 P4 condition increased the blastocyst stage embryo rate after IVF to a level similar to that of the mature oocytes from VF COCs.

In null mice for Pr, which encodes the progesterone receptor, ovulation is suppressed completely [31]. However, the matured oocytes collected from periovulatory follicles develop to the blastocyst stage following IVF, thereby indicating that progesterone is required only for the rupture of the follicle walls in mice during the ovulation process. However, the present study using a pig model showed that the progesterone level in follicular fluid increased during the follicular development process. A high level of progesterone induces Lhcgr expression in cumulus cells, but it is also required for the induction of cumulus expansion in porcine COCs [45, 57]. In NVFs, the expression level of Cyp11a1 in granulosa cells with diameters >8 mm and the progesterone concentration in follicular fluid were not increased in follicles growing in NVFs. Therefore, we estimated that the low level of progesterone would cause the low functions of cumulus cells of NVF COCs.

In VF COCs, cultivation in the conventional culture system and the VF E2 P4 culture system induced full cumulus expansion and oocyte maturation, thereby indicating that COCs derived from VFs have the potential to undergo oocyte maturation (Supplemental Figure S2). In NVF COCs, cultivation in the conventional culture system (with FSH alone) did not induce cumulus expansion and oocyte maturation. Furthermore, the cultivation of NVF COCs with E2 or P4 alone did not overcome the low levels of cumulus expansion and oocyte meiotic maturation. However, these indexes were improved in NVF COCs and oocytes by cultivation in the VF E2 P4 culture system, which contained a moderate level of E2 and a high dose of progesterone, so we conclude that the balance between E2 and P4 is important for allowing the cumulus cells from COCs to induce oocyte maturation during follicular development. Thus, the optimal steroid hormonal supplementation with estradiol-17β and progesterone is essential for improving the oocyte maturation ability and developmental competence, even when culturing oocytes derived from NVFs.

In conclusion, the level of estradiol-17β was increased, followed by the induction of progesterone in VF antral follicles. However, the sequential induction of steroid hormones condition was not observed in NVF follicles. This abnormal condition decreased cell survivability and suppressed analysis after cultivation. The intensity of the bands was analyzed using a Gel-Pro Analyzer. Values represent the mean ± SEM based on at least three independent culture experiments. *Significant differences were observed (P < 0.05).
FIG. 9. Additional effects of the physiological E2 and P4 levels detected in VF follicular fluid on the apoptosis of cumulus cells of COCs derived from VFs or NVFs. **A, B** Apoptosis of cumulus cells of COCs derived from VFs (A) and NVFs (B) cultured in the conventional culture system, NVF E2 P4 culture system, and VF E2 P4 culture system, which were analyzed using the TUNEL method. TUNEL-positive staining (FITC) was used to detect cumulus cells apoptosis, and DAPI staining was used for DNA detection. Before culture: COCs were collected from VFs or NVFs and then analyzed by TUNEL method. **C** Quantification of A showing the number of TUNEL-positive cells for cumulus cells of COCs derived from VFs. **D** Quantification of B showing the number of TUNEL-positive cells for cumulus cells of COCs derived from NVFs. Values represent the mean ± SEM based on at least three independent culture experiments. *Significant differences were observed (P < 0.05).
proliferation ability in cumulus cells of COCs in NVF follicles, thereby resulting in the low maturation competence of oocytes after IVC. Based on these results, we developed a novel culture system (VF E2 P4) that mimics the steroid hormone condition in VF antral follicles. Culture in the VF E2 P4 condition improved the functions of the cumulus cell layer to induce oocyte maturation with a developmental competence. Overall, our results indicate that the VF E2 P4 culture system is a beneficial tool for improving the developmental competence of cultured oocytes derived from NVFs.

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REFERENCES