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Original Article

Effects of oocyte-derived growth factors on the growth of porcine oocytes and oocyte–cumulus cell complexes *in vitro*

Riho MORIKAWA[1)], Jibak LEE[1)] and Takashi MIYANO[1)]

[1)]*Laboratory of Developmental Biotechnology, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

Abstract. During oocyte growth and follicle development, oocytes closely communicate with cumulus cells. We examined the effects of oocyte-derived growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), on the growth and acquisition of meiotic competence of porcine oocytes collected from early antral follicles (1.2–1.5 mm). First, we confirmed that *GDF9* and *BMP15* mRNAs were expressed almost exclusively in the oocytes. Oocyte–cumulus cell complexes (OCCs) collected from early antral follicles were cultured in growth medium supplemented with 0–100 ng/ml of GDF9 or BMP15 for 5 days. GDF9 dose-dependently increased the OCC diameter, while BMP15 did not. GDF9 and BMP15 had no significant effects on oocyte growth ($P > 0.05$). When OCCs that had been cultured with 50 and 100 ng/ml BMP15 were subjected to a subsequent maturation culture, they expanded fully by gonadotropic stimulation and 49% and 61% of oocytes matured to metaphase II (MII), respectively. In contrast, GDF9 did not promote cumulus expansion, and < 10% of oocytes matured to MII. Based on the difference in cumulus expansion, we compared the expression of *luteinizing hormone/choriogonadotropin receptor* (*LHCGR*) and *follicle stimulating hormone receptor* (*FSHR*) mRNAs in cumulus cells. The level of *LHCGR* mRNA was increased in cumulus cells of the BMP15 group, although there were no significant differences in *FSHR* mRNA levels among the groups. These results suggest that GDF9 promotes the growth of OCCs and that BMP15 promotes *LHCGR* mRNA expression in cumulus cells during oocyte growth culture, which may contribute to cumulus expansion and oocyte maturation.

Key words: Bone morphogenetic protein 15 (BMP15), Cumulus expansion, Growth differentiation factor 9 (GDF9), Oocyte growth, Pig

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The basic functional unit of the ovary is the ovarian follicle. This follicle consists of an oocyte surrounded by cumulus cells, granulosa cells, basal lamina, and theca cells. Oocytes grow in follicles and acquire nuclear and cytoplasmic competence for meiotic maturation and fertilization. After gonadotropic surge, the cumulus expands and fully grown oocytes resume meiosis and mature to the second metaphase in antral follicles. It has been suggested that oocytes are the central regulators of follicle function and play critical roles in follicle development, ovulation, and thus female fertility [1]. Conversely, oocyte growth is strongly dependent on the surrounding somatic cells, which supply essential metabolic substrates and regulatory molecules [2].

Oocyte-secreted factors may form the basis of a complex bi-directional communication network between the oocyte and its surrounding somatic cells [1, 3, 4]. Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are members of the transforming growth factor-beta (TGF-β) family and have been identified as oocyte-secreted factors [1]. In GDF9-null mice, proliferation of granulosa cells is retarded in the follicles, and folliculogenesis does not progress beyond the primary follicle stage [5]. BMP15-null female mice exhibit subfertility [6], ewes carrying BMP15 mutations are infertile, and their follicular development is arrested at the primordial stage [7]. BMP15-knockdown gilts show abnormal ovarian follicular development and ovulation [8].

In culture experiments of oocytes with cumulus/granulosa cells, oocyte-secreted factors have been suggested to target these cells to regulate a broad range of functions, such as proliferation [9], glycolysis [10], luteinization [11], and cumulus expansion in mice [2]. The role of oocytes in cumulus expansion has been examined by physically removing oocytes from oocyte–cumulus/granulosa cell complexes [2, 12, 13], and the oocyte-derived cumulus expansion has been attributed to GDF9 [14].

The direct effects of exogenous GDF9 and BMP15 have been examined using cultured granulosa cells. GDF9 reportedly promoted the proliferation of mouse granulosa cells [15] and suppressed progesterone and estradiol production in rat granulosa cells [16] and apoptosis of rat cumulus cells [17]. Similarly, BMP15 promoted the proliferation of rat granulosa cells [18] and inhibited progesterone production in rat granulosa cells [18], in addition to inhibiting apoptosis of bovine cumulus cells [19]. GDF9 was reported to promote follicle development in organ-cultured rat ovaries [20] and cultured human ovarian cortical tissues [21]. Recently, we reported that GDF9 and BMP15 promoted the formation of antrum-like structures in bovine oocyte–granulosa cell complexes [22]. Studies in pigs have demonstrated that GDF9 promotes proliferation of mural granulosa cells [23] and GDF9 and BMP15 prevent cumulus cell apoptosis [24, 25].

In the final stage of oocyte growth in porcine antral follicles, oocytes become able to mature [26] and cumuli become able to expand [27]. Although GDF9 and BMP15 are thought to participate

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Correspondence: R Morikawa (e-mail: 172a217a@stu.kobe-u.ac.jp)

in the bidirectional communication network between oocytes and surrounding cumulus cells at this stage, the effects of exogenous GDF9 and BMP15 on the growth and acquisition of meiotic competence of oocytes have not been studied in pigs. In this study, we dissected early antral follicles (1.2–1.5 mm in diameter) containing growing oocytes from porcine ovaries. We confirmed the expression of *GDF9* and *BMP15* mRNAs in oocytes. Next, we cultured oocyte–cumulus cell complexes (OCCs) collected from the early antral follicles over 5 days to examine the effects of GDF9 and BMP15 on OCC and oocyte growth. The cultured OCCs were stimulated by gonadotropin in GDF9- and BMP15-free media to induce oocyte maturation and the maturational competence of the *in vitro* grown oocytes was compared. The results suggested that the presence of BMP15 during the oocyte growth culture period promoted cumulus expansion and oocyte maturation. Thus, we considered that the cumulus cells in OCCs cultured with BMP15 became more sensitive to gonadotropin. To investigate this hypothesis, we further compared the expression of the mRNAs of luteinizing hormone/choriogonadotropin receptor (LHCGR) and follicle stimulating hormone receptor (FSHR) in cumulus cells from cultured OCCs.

Materials and Methods

Chemicals

All chemicals were purchased from MilliporeSigma (St. Louis, MO, USA) unless otherwise noted.

Collection of OCCs

Porcine ovaries were collected at a local slaughterhouse and transported to the laboratory at room temperature (20–25°C). The ovaries were washed once with 0.2% (w/v) cetyltrimethylammonium bromide (FUJIFILM Wako Pure Chemical, Osaka, Japan) and three times with Dulbecco's phosphate buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA). The ovaries were sliced with a surgical blade (No. 21; Keisei Medical Industrial, Niigata, Japan), and the slices were pooled in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate. Early antral follicles (1.2–1.5 mm in diameter) were collected from the ovarian slices and pooled in HEPES-199. The follicles were opened using a surgical blade (No. 21) and OCCs containing growing oocytes were collected. OCCs containing oocytes that were partly or completely denuded and whose ooplasms were not homogenous were excluded from the experiment.

As an *in vivo* control, OCCs containing fully grown oocytes were collected from antral follicles (4.0–6.0 mm in diameter). The follicles were dissected using surgical blades (No. 11; Feather Safety Razor, Osaka, Japan) and opened to collect OCCs. OCCs with oocytes that were partly or completely denuded and whose ooplasms were not homogenous were excluded.

RNA extraction and cDNA synthesis

After washing in PBS-PVA, cumulus cells were removed from a group of 30 OCCs using a fine pipette to collect denuded oocytes. The 30 denuded oocytes, along with a group of 30 OCCs, were each transferred into a 1.5 ml microtube with a minimum volume of PBS-PVA and stored at –80°C until use. The cumulus cells collected from the 30 OCCs and mural granulosa cells derived from 30 follicles were each transferred into a 1.5 ml microtube with PBS-PVA. The microtubes were centrifuged at 10,000 rpm (9,100 × *g*) for 5 min. After discarding the supernatant, the cumulus cells and mural granulosa cells were washed three times in 300 µl PBS-PVA by centrifuging for 5 min each time. After washing, they were stored at –80°C with a minimum volume of PBS-PVA until use.

Total RNA was extracted from the collected OCCs, oocytes, cumulus cells, and mural granulosa cells using an RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR)

RT-PCR was performed using PCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. Primers for *GDF9*, *BMP15*, and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) for internal control were designed according to known sequences in GenBank (accession numbers: porcine *GDF9*, NM_001001909; *BMP15*, NM_001005155; and *GAPDH*, NM_001206359) and were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primer sequences are shown in Supplementary Table 1. The cycling conditions for the amplification were 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 1 min, followed by 72°C for 5 min with a final hold at 4°C. RT-PCR products were electrophoresed in 2% (w/v) agarose gels containing ethidium bromide at 120 V and 180 mA for 30 min and visualized by exposure to ultraviolet (UV) light.

A QIAquick gel extraction kit (QIAGEN) was used to purify RT-PCR products from the agarose gels according to the manufacturer's instructions. Sequencing of gel-purified products was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3130 Genetic Analyzer (Applied Biosystems). The nucleotide sequences of the RT-PCR products were checked against the porcine cDNA sequences of *GDF9*, *BMP15,* and *GAPDH*.

The qPCR reactions were performed using the THUNDERBIRD SYBR qPCR Mix (TOYOBO) according to the manufacturer's instructions. Primers for *GDF9*, *BMP15*, *LHCGR*, *FSHR*, *GAPDH*, and *actin beta (ACTB)* were designed according to known sequences in GenBank (accession numbers: porcine *GDF9*, NM_001001909; *BMP15*, NM_001005155; *FSHR*, XM_021085881.1; *GAPDH*, NM_001206359; and ACTB, XM_003124280) or a previous report (*LHCGR*, [28]). They were purchased from Thermo Fisher Scientific (Supplementary Table 1). *GAPDH* and *ACTB* were used as internal controls. Results are indicated only with *GAPDH* because those of *ACTB* were almost identical. The cycling conditions for amplification were 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 63°C (for *GDF9* and *BMP15*) or 61.4°C (for *LHCGR* and *FSHR*) for 30 sec. Melt curve analyses were carried out for all genes. The specificity of the PCR products was checked by a single peak.

In vitro growth culture of OCCs

Porcine OCCs collected from early antral follicles were cultured as previously reported [22] with some modifications. Briefly, OCCs were cultured individually for 5 days in 96-well culture plates (BioCoat Collagen I Cellware; Becton Dickinson, San Diego, CA, USA) containing 200 µl of growth medium in each well under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. The basic growth medium was Minimum Essential Medium α (GIBCO Invitrogen, Inchinnin, Scotland, UK) supplemented with 2.2 mg/ml sodium bicarbonate, 0.08 mg/ml kanamycin sulfate, 2% (w/v) polyvinylpyrrolidone (molecular weight 360,000), 55 µg/ml

L-cysteine, 0.05 μM dexamethasone, 4.0 mM hypoxanthine, 50 μg/ml ascorbic acid 2-glucoside (Hayashibara, Okayama, Japan), and 5% (v/v) fetal bovine serum (ICN Biomedicals, Solon, OH, USA). The medium was also supplemented with 10 μM 17β-estradiol and 0.01 IU/ml recombinant human follicle stimulating hormone (rhFSH; MSD, Tokyo, Japan). In the experimental groups, 10, 50, or 100 ng/ml of GDF9 (recombinant mouse GDF-9 protein: 739-G9; R&D Systems, Minneapolis, MN, USA) or BMP15 (recombinant human BMP-15 protein: 5096-BM; R&D Systems) were further added to the culture medium. Mouse GDF9 (Gly307-Arg441) and human BMP15 (Gln268-Arg392) are bioactive mature forms that share 88% and 80% amino acid sequence identity with porcine GDF9 and BMP15, respectively. In addition, their effects on cumulus/granulosa cells or OCCs have been reported in several mammalian species, including pigs [22, 29–33].

Half (100 μl) of the culture medium was replaced with fresh medium on Day 3. At the start of culture (Day 0), Day 3 and Day 5, OCC morphologies were assessed and photographed. The OCC diameters were measured using the ImageJ software (NIH, Bethesda, MD, USA). Oocytes completely wrapped with cumulus cells were considered to be surviving. The formation of antrum-like structures was examined on Day 3 and Day 5 by identifying the visible spaces surrounded by cumulus cells. OCCs showing cytoplasmic degeneration of oocytes, detachment of cumulus cells from the zona pellucida, or loss of the aggregated structure of cumulus cells were classified as disintegrated complexes; all other complexes were considered to have maintained their integrity. *In vitro* grown OCCs were used for the measurement of oocyte diameters, nuclear stage analysis, *in vitro* maturation culture, and qPCR. For the measurement of oocyte diameters, the oocytes were denuded and the diameter excluding the zona pellucida was measured using an ocular micrometer (Olympus, Tokyo, Japan).

In vitro maturation culture of OCCs

After 5 days of growth culture, oocytes were assessed for their meiotic competence. *In vitro* grown OCCs were recovered and further cultured in maturation medium (0.5 ml) in 4-well multi dishes (Thermo Fisher Scientific) in an atmosphere of 5% CO_2 in air at 38.5°C for 48 h. The maturation medium was bicarbonate-buffered medium 199 supplemented with 2.2 mg/ml sodium bicarbonate, 0.08 mg/ml kanamycin sulfate, 0.1 mg/ml sodium pyruvate (Nacalai Tesque, Kyoto, Japan), 10% (v/v) fetal bovine serum, and 0.1 IU/ml human menopausal gonadotropin (hMG; ASKA Pharmaceutical, Tokyo, Japan). After 48 h of maturation culture, oocytes were denuded by gently pipetting with 0.1% (w/v) hyaluronidase.

In some experiments, oocytes after growth culture were denuded and used to induce spontaneous maturation to eliminate the effect of cumulus cells on oocyte maturation. In addition, some OCCs after growth culture were subjected to maturation culture with 10 ng/ml epidermal growth factor (recombinant human EGF: 236-EG; R&D systems) [34] instead of hMG.

OCCs containing growing oocytes before growth culture and OCCs containing *in vivo* grown oocytes were used as controls.

Analysis of nuclear stages of oocytes

Oocytes before and after growth culture and maturation culture were denuded mechanically, fixed in aceto-ethanol, and stained with 1% (w/v) aceto-orcein. The stained oocytes were then classified based on the morphologies of the chromatin and nuclear envelope [35, 36]. Oocytes containing the nuclear envelope were classified into filamentous chromatin (FC), stringy chromatin (SC), or germinal vesicle (GVI–IV) stages. Oocytes after germinal vesicle breakdown (GVBD) were classified as diakinesis (D), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII). Oocytes showing cytoplasmic or nuclear abnormalities were considered to be degenerated oocytes.

Statistical analysis

The frequencies of antrum formation and oocyte nuclear stage were compared using a chi-squared test. The mean diameters of the OCCs were subjected to a two-way analysis of variance (ANOVA) followed by a Tukey–Kramer test. The mean diameters of oocytes and the quantity of mRNAs were subjected to one-way ANOVA followed by the Tukey–Kramer test. Statistical significance was set at $P < 0.05$.

Results

Expression of GDF9 and BMP15 mRNAs in porcine follicles

Expression of *GDF9* and *BMP15* mRNAs in OCCs (Fig. 1A), oocytes (Oos; Fig. 1B), cumulus cells (CCs; Fig. 1C), and mural granulosa cells (MGCs; Fig. 1D) from early antral follicles (1.2–1.5 mm) was examined by RT-PCR (Fig. 1E). OCCs and Oos from both early antral follicles and antral follicles (4–6 mm) showed *GDF9* and *BMP15* PCR product bands of the expected sizes. In CCs and MGCs from both types of follicles, *GDF9* bands were faintly detected and *BMP15* bands were undetectable.

The qPCR results supported the expression of *GDF9* and *BMP15* in oocytes from both types of follicles (Figs. 1F and G). *GDF9* and *BMP15* mRNAs were mainly expressed in oocytes, whereas they were scarcely detected in CCs and MGCs. Oocytes from the early antral follicles expressed higher levels of *GDF9* and *BMP15* mRNAs. These levels decreased with follicle development, although the decrease was not statistically significant.

Effects of GDF9 and BMP15 on OCC growth

The typical morphologies of OCCs from early antral follicles during growth culture with GDF9 or BMP15 are shown in Fig. 2. Before culture (Day 0), the oocytes were covered with two to three layers of cumulus cells. During growth culture, the diameters of OCCs gradually increased and some of the OCCs formed antrum-like structures. The percentages of antrum formation were 10–30% in all groups. There were no obvious effects of GDF9 and BMP15. More than 85% of OCCs were completely covered with cumulus cells in all groups after culture.

Figures 3A and 3B show the changes in the diameter of OCCs during culture. Before culture (Day 0), the diameters of the OCCs were 170–180 μm. The diameters gradually increased in all groups, reaching more than 300 μm in the GDF9 group and 250 μm in the BMP15 group on Day 5. GDF9 induced a concentration-dependent increase in OCC diameter, whereas higher concentrations of BMP15 did not promote a further increase in OCC diameter. The mean diameter of OCCs cultured with 100 ng/ml GDF9 at Day 5 was 349.9 ± 9.0 μm (mean ± S.E.), which was significantly larger than the diameter in the control OCCs (302.0 ± 7.5 μm).

Effects of GDF9 and BMP15 on oocyte growth

After growth culture, more than 80% of oocytes showed normal morphology with no significant differences among the experimental groups. Figs. 3C and 3D show the diameters of the oocytes after growth culture. The diameters of cultured oocytes increased significantly compared to those before culture (approximately 110 μm). In all

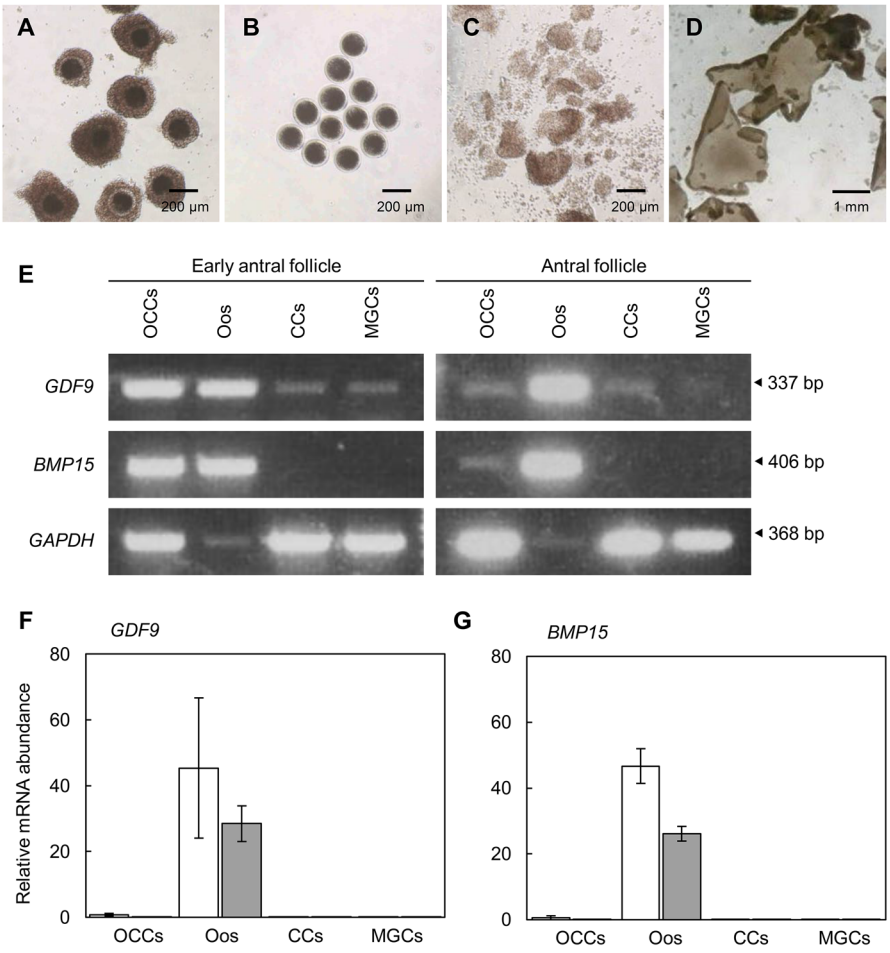

Fig. 1. Expression of *GDF9* and *BMP15* mRNAs in porcine oocyte–cumulus cell complexes (OCCs; A), oocytes (Oos; B), cumulus cells (CCs; C), and mural granulosa cells (MGCs; D) from early antral follicles (1.2–1.5 mm) and antral follicles (4.0–6.0 mm) was examined by RT-PCR (E) and qPCR (F and G). The RT-PCR product bands specific to porcine *GDF9*, *BMP15*, and *GAPDH* (internal control) mRNAs are shown in the top, middle, and bottom panels, respectively (E). Relative expression levels of *GDF9* (F) and *BMP15* (G) mRNAs are shown as the mean ± S.E. from more than three replicates. Porcine *GAPDH* was used as an internal control.

groups, the mean diameter of oocytes after culture was approximately 125 µm, which was similar to the mean diameters of *in vivo* fully grown oocytes (FO in Figs. 3C and D). There were no significant effects of GDF9 or BMP15 on oocyte growth.

The effects of GDF9 and BMP15 on the meiotic stage of oocytes were examined after growth culture (Supplementary Table 2). Most of the growing oocytes were at the FC or SC stage immediately after collection from early antral follicles. However, *in vivo* fully grown oocytes from antral follicles were at the GV (I–IV) stage. More than 70% of *in vitro* grown oocytes were at the GV stage and there were no significant effects of GDF9 and BMP15 on the meiotic stage of oocytes.

Effects of GDF9 and BMP15 on oocyte meiotic competence

To examine the meiotic competence of oocytes grown with GDF9 or BMP15, OCCs after growth culture were subjected to *in vitro* maturation culture. Figs. 4A–C show cumulus expansion after maturation culture. OCCs from antral follicles fully expanded (Fig. 4A, FO), while those from early antral follicles barely expanded (Fig. 4A, growing oocytes [GO]). OCCs cultured without growth factors swelled after maturation culture with cumulus cells loosely connected in the mass (Figs. 4B and C). OCCs that had been cultured with GDF9 showed swelling similar to that of the OCCs without growth factors (Fig. 4B). In contrast, OCCs cultured with 50 and 100 ng/ml BMP15 expanded fully after maturation culture in a manner similar to that of OCCs collected from antral follicles (Fig. 4C).

We further examined the meiotic stage of the oocytes after maturation culture. The GVBD rate of the growing oocytes from early antral follicles was low; none matured to MII, while almost all *in vivo* fully grown oocytes collected from antral follicles underwent GVBD and matured to MII (Table 1). Approximately 20% of *in vitro* grown oocytes in the control groups cultured without GDF9 and BMP15 underwent GVBD, and 15–16% matured to MII after maturation culture. Similarly, less than 10% of oocytes cultured with GDF9 (50 and 100 ng/ml) underwent GVBD and matured to MII. On the other hand, the percentage of oocytes that underwent GVBD and reached MII after culturing with BMP15 increased dose-dependently, and the MII rates in the 50 and 100 ng/ml BMP15 groups (49% and 61%, respectively) were significantly higher than those of the control oocytes (16%).

These data led us to speculate that the presence of high concentrations of BMP15 during the growth culture period promoted cumulus expansion after gonadotropic stimulation, ultimately resulting in oocyte maturation. Next, to eliminate the effect of cumulus cells

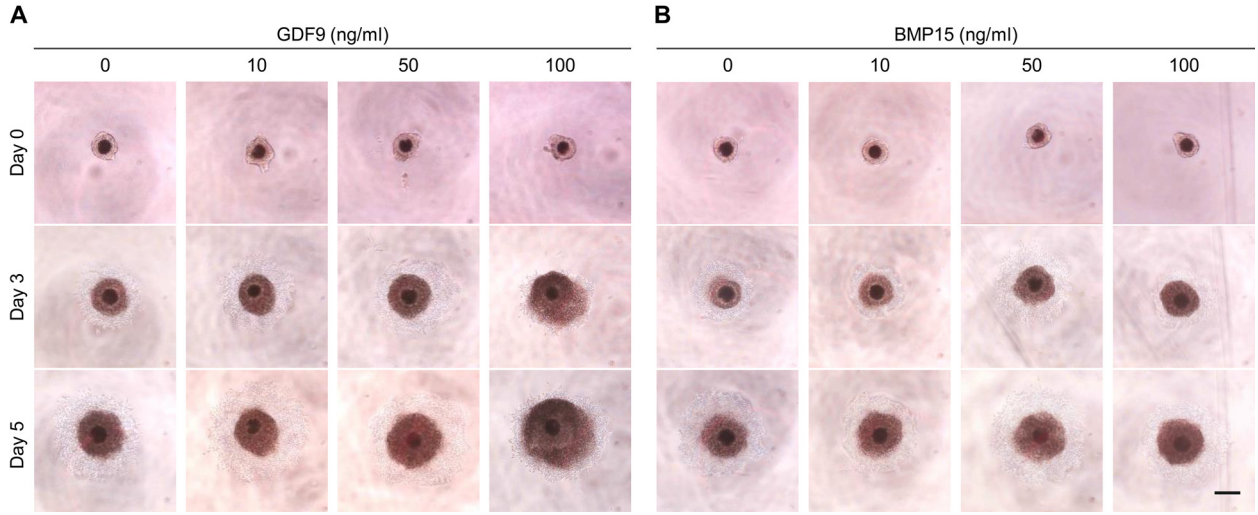

Fig. 2. Typical morphologies of porcine oocyte–cumulus cell complexes (OCCs) cultured with GDF9 (A) and BMP15 (B) during growth culture. The top, middle, and bottom panels in each set of pictures show results from Day 0, 3, and 5 of incubation, respectively. The different concentrations (ng/ml) of growth factors are shown in increasing order from left to right. The scale bar represents 200 μm.

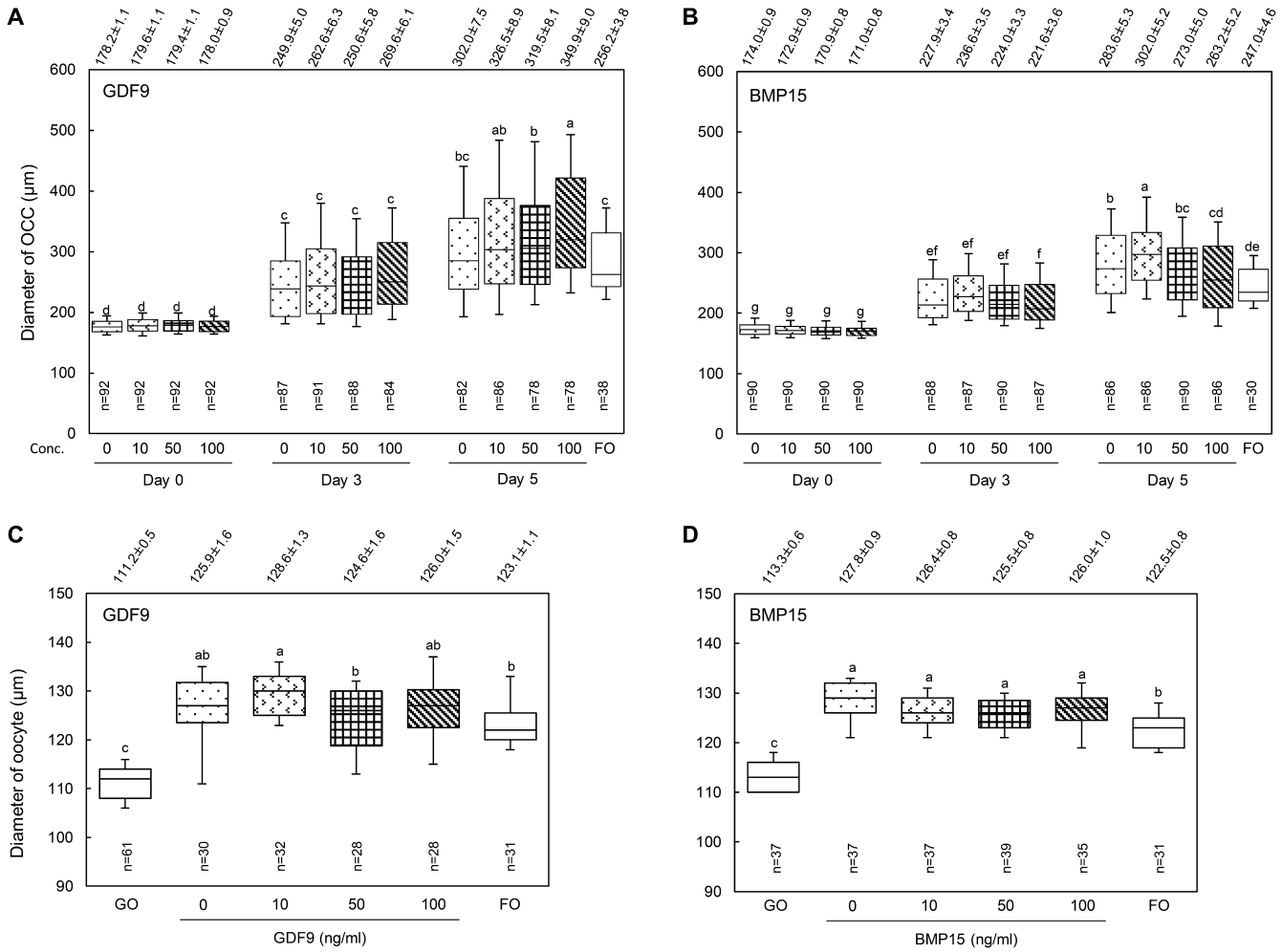

Fig. 3. Effects of GDF9 and BMP15 on the diameters of porcine oocyte–cumulus cell complexes (OCCs) during growth culture (A and B), and the diameters of oocytes after growth culture (C and D). OCCs were cultured in the growth medium supplemented with GDF9 (A and C) and BMP15 (B and D). The diameters of OCCs excluding disintegrated complexes were measured at Day 0, 3, and 5. The diameters of OCCs including fully grown oocytes collected from antral follicles (4.0–6.0 mm) were measured as *in vivo* controls (FO) in (A) and (B). The diameters of growing oocytes (GO) from early antral follicles (1.2–1.5 mm) and FO were measured as *in vivo* controls in (C) and (D). GDF9 and BMP15 concentrations are shown in ascending order under each set of boxes. The numbers of OCCs or oocytes (n) used for each experiment are shown at the bottom of each box. The mean diameter ± S.E. is shown at the top of each box. Boxes with different letters (a–g) in each figure are significantly different (two-way ANOVA for OCC diameters, and one-way ANOVA for oocyte diameters; $P < 0.05$). The experiments were replicated more than three times.

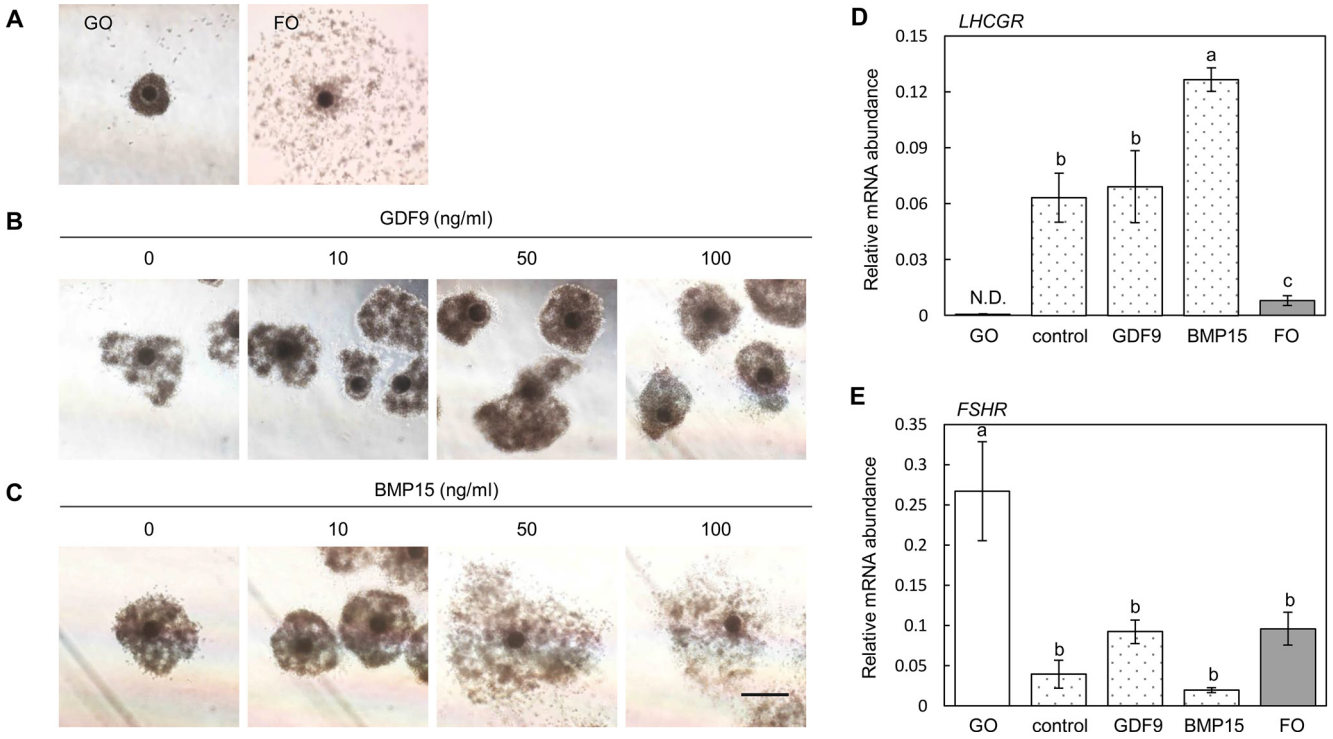

Fig. 4. Typical morphologies of the cumulus expansion of oocyte–cumulus cell complexes (OCCs, A–C). After growth culture for 5 days with 0, 10, 50, or 100 ng/ml GDF9 (B) or BMP15 (C), OCCs were futher cultured for maturation without GDF9 and BMP15. OCCs collected from early antral follicles (1.2–1.5 mm; A, GO) and antral follicles (4.0–6.0 mm; A, FO) were cultured in the maturation medium as *in vivo* controls. The scale bar represents 500 µm. Relative expression levels of *LHCGR* (D) and *FSHR* (E) mRNAs in porcine cumulus cells after 5 days of growth culture with GDF9 (100 ng/ml) or BMP15 (100 ng/ml). Cumulus cells from early antral follicles (GO: 1.2–1.5 mm; open bar) and antral follicles (FO: 4.0–6.0 mm; grey bar) were used as *in vivo* controls. Porcine *GAPDH* was used as an internal control. Values with different letters (a–c) are significantly different (one-way ANOVA; $P < 0.05$). As *LHCGR* mRNA of GO was not detected in several analyses (shown as N.D.), it was excluded from the statistical analysis. Data are shown as the mean ± S.E. from three replicates.

on oocyte maturation, OCCs were cultured with GDF9 or BMP15 for 5 days, after which the oocytes were denuded from cumulus cells and subjected to maturation culture (Table 2). We used 100 ng/ml of GDF9 and BMP15 for this experiment, because 100 ng/ml GDF9 yielded the greatest increase in OCC diameter and 100 ng/ml BMP15 showed the greatest promotion of oocyte maturation. After maturation culture of denuded oocytes, approximately half of the oocytes underwent GVBD and more than 40% of oocytes matured to MII in the control (41%) and GDF9 groups (45%). These percentages were not significantly different from those in the BMP15 group (70%). By removing the cumulus cells, the oocyte maturation rates of the control and GDF9 groups were improved compared to the rates in Table 1.

It has been reported that the EGF network transmits the LH signal from the somatic cells of the follicle to the oocyte to promote oocyte meiotic resumption [37, 38]. Thus, we also examined the effects of GDF9 and BMP15 on EGF-stimulated maturation of *in vitro* grown oocytes (Table 2). There were no significant differences in both GVBD and MII rates among the control, GDF9, and BMP15 groups. These results indicated that the oocytes in any group acquired meiotic competence after growth culture. However, the cumulus cells of the control and GDF9 groups did not acquire sufficient ability to respond to gonadotropic stimulation for oocyte maturation.

Effects of GDF9 and BMP15 on the expression of LHCGR and FSH mRNAs

We considered that the cumulus cells of the BMP15 group responded more sensitively to gonadotropin than the cumulus cells of the other groups. To investigate this, we collected cumulus cells from OCCs cultured with GDF9 and BMP15 for 5 days and subjected them to qPCR to compare their expression of *LHCGR* and *FSHR* mRNAs (Figs. 4D and E). The expression of *LHCGR* mRNA increased with follicle development (from GO to FO in Fig. 4D), while the expression of *FSHR* mRNA decreased (Fig. 4E). For the *LHCGR* mRNA, cumulus cells from the control, GDF9, and BMP15 groups expressed higher levels of *LHCGR* mRNA than cumulus cells from the early antral and antral follicles, while the BMP15 group showed the highest level of expression. The expression level of *FSHR* mRNA was higher in cumulus cells from the GDF9 group than in those from the control and BMP15 groups; however, these differences were not significant.

Discussion

Porcine *GDF9* mRNA was expressed mainly in oocytes in early antral follicles and faintly in cumulus cells and mural granulosa cells, whereas *BMP15* mRNA was expressed specifically in oocytes. Oocyte-specific expression of *GDF9* mRNA has been reported in mouse [39–42], rat [43], cow [22, 44], sheep [44], and human [45] follicles. In pigs, *GDF9* mRNA is produced by oocytes and also by cumulus cells and mural granulosa cells [46]. *BMP15* mRNA expression was also shown to be oocyte-specific in mouse [41, 47], rat [44], human [45], and bovine [22] follicles. One study reported that the expression of *GDF9* mRNA in porcine granulosa cells was

Table 1. Nuclear stages of porcine oocytes after *in vitro* maturation culture with hMG

Follicle diameter (mm)	Growth culture (day)	Treatment	Conc. (ng/ml)	No. oocytes examined	No. (%) oocytes in GV stage *			No. (%) oocytes after GVBD *				No. (%) degenerated oocytes
					Total GV	FC–SC	GVI–IV	Total GVBD	D–MI	AI–TI	MII	
1.2–1.5	0	–	–	30	21 (70) ^{ab}	16 (53) ^{a}	5 (17) ^{a}	7 (23) ^{a}	7 (23) ^{a}	0 (0)	0 (0)	2 (7)
	5	GDF9	0	52	27 (52) ^{a}	1 (2) ^{b}	26 (50) ^{b}	11 (21) ^{a}	3 (6) ^{b}	0 (0)	8 (15) ^{a}	14 (27)
	5		10	54	33 (61) ^{ab}	0 (0)	33 (61) ^{bc}	12 (22) ^{a}	6 (11) ^{ab}	2 (4)	4 (7) ^{ab}	9 (17)
	5		50	50	37 (74) ^{b}	0 (0)	37 (74) ^{c}	4 (8) ^{a}	3 (6) ^{ab}	0 (0)	1 (2) ^{b}	9 (18)
	5		100	50	37 (74) ^{b}	2 (4) ^{b}	35 (70) ^{c}	4 (8) ^{a}	1 (2) ^{b}	0 (0)	3 (6) ^{ab}	9 (18)
4.0–6.0	0	–	–	30	0 (0)	0 (0)	0 (0)	28 (93) ^{b}	0 (0)	0 (0)	28 (93) ^{c}	2 (7)
1.2–1.5	0	–	–	29	18 (62) ^{A}	7 (24) ^{A}	11 (38) ^{A}	6 (21) ^{A}	6 (21) ^{A}	0 (0)	0 (0)	5 (17) ^{AB}
	5	BMP15	0	49	34 (69) ^{A}	0 (0)	34 (71) ^{B}	10 (20) ^{A}	2 (4) ^{AB}	0 (0)	8 (16) ^{A}	5 (10) ^{AB}
	5		10	49	34 (69) ^{A}	1 (2) ^{B}	33 (67) ^{B}	12 (24) ^{A}	7 (14) ^{AB}	0 (0)	5 (10) ^{A}	3 (6) ^{A}
	5		50	51	13 (25) ^{B}	0 (0)	13 (25) ^{A}	32 (63) ^{B}	7 (14) ^{AB}	0 (0)	25 (49) ^{B}	6 (12) ^{AB}
	5		100	51	6 (12) ^{B}	2 (4) ^{B}	4 (8) ^{C}	32 (63) ^{B}	1 (2) ^{B}	0 (0)	31 (61) ^{B}	13 (25) ^{B}
4.0–6.0	0	–	–	30	0 (0)	0 (0)	0 (0)	29 (97) ^{C}	0 (0)	0 (0)	29 (97) ^{C}	1 (3) ^{A}

* Stages of meiotic division: FC, filamentous chromatin stage; SC, stringy chromatin stage; GVI–IV, germinal vesicle stage (I–IV); GVBD, germinal vesicle breakdown; D, diakinesis; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II. The oocytes were classified on the basis of the morphology of the chromatin and nuclear envelope as described previously [35, 36]. $^{a–c), A–C)}$ Values with different letters are significantly different (χ^2; $P < 0.05$). The experiments were replicated more than three times.

Table 2. Nuclear stages of porcine oocytes after *in vitro* maturation culture by the denudation of oocytes, and with EGF

Follicle diameter (mm)	Growth culture			Maturation culture	No. oocytes examined	No. (%) oocytes in GV stage *			No. (%) oocytes after GVBD *				No. (%) degenerated oocytes
	Day	Treatment	Conc. (ng/ml)			Total GV	FC–SC	GVI–IV	Total GVBD	D–MI	AI–TI	MII	
1.2–1.5	0	–	–	denudation	30	6 (20)	5 (17)	1 (3)	17 (57) ^{a}	14 (47) ^{a}	3 (10)	0 (0)	7 (23) ^{ab}
	5	–	0	denudation	22	5 (23)	0 (0)	5 (23)	12 (55) ^{a}	3 (14) ^{b}	0 (0)	9 (41) ^{a}	5 (23) ^{ab}
	5	GDF9	100	denudation	22	5 (22)	0 (0)	5 (23)	11 (50) ^{a}	1 (5) ^{b}	0 (0)	10 (45) ^{a}	6 (27) ^{a}
	5	BMP15	100	denudation	23	4 (17)	0 (0)	4 (17)	17 (74) ^{ab}	1 (4) ^{b}	0 (0)	16 (70) ^{ab}	2 (9) ^{ab}
4.0–6.0	0	–	–	denudation	30	2 (7)	0 (0)	2 (7)	27 (90) ^{b}	0 (0)	0 (0)	27 (90) ^{b}	1 (3) ^{b}
1.2–1.5	0	–	–	EGF	30	20 (67) ^{A}	0 (0)	20 (67) ^{A}	8 (27) ^{A}	7 (23)	0 (0)	1 (3) ^{A}	2 (7) ^{A}
	5	–	0	EGF	26	3 (12) ^{B}	0 (0)	3 (12) ^{B}	15 (58) ^{B}	0 (0)	1 (4)	14 (54) ^{B}	8 (31) ^{B}
	5	GDF9	100	EGF	26	8 (31) ^{B}	0 (0)	8 (31) ^{B}	12 (46) ^{AB}	1 (4)	1 (4)	10 (39) ^{B}	6 (23) ^{AB}
	5	BMP15	100	EGF	24	7 (29) ^{B}	0 (0)	7 (29) ^{B}	12 (50) ^{AB}	1 (4)	0 (0)	11 (46) ^{B}	5 (20) ^{AB}
4.0–6.0	0	–	–	EGF	30	7 (23) ^{B}	0 (0)	7 (23) ^{B}	21 (70) ^{B}	5 (17)	1 (3)	15 (50) ^{B}	2 (7) ^{A}

* See footnotes in Table 1. In the upper set of the table, oocytes after growth culture were denuded to induce maturation. In the lower set of the table, OCCs after growth culture were subjected to maturation culture with EGF. $^{a, b), A, B)}$ Values with different letters are significantly different (χ^2; $P < 0.05$). The experiments were replicated more than three times.

less than 1/30 that of *BMP15* mRNA [48]. The results of the present study are in good agreement with these reports. The present results suggest that, in pigs, *GDF9* mRNA is produced mainly in oocytes, whereas *BMP15* mRNA is produced exclusively in oocytes.

In the present study, the expression levels of *GDF9* and *BMP15* mRNAs did not change significantly during antral follicle development. In previous reports, the mRNA levels of *GDF9* were higher in bovine oocytes in antral follicles than in oocytes in secondary follicles [49]. Furthermore, *GDF9* and *BMP15* mRNAs were expressed constantly in bovine oocytes throughout the antral follicle stage [50]. The present results indicate that *GDF9* and *BMP15* mRNAs are also expressed constantly in oocytes throughout the development of antral follicles in pigs.

Under our culture conditions, the diameter of OCCs increased in all groups after the growth culture. GDF9 increased the OCC diameter in a dose-dependent manner, whereas the effects of BMP15 on the diameter were not dose-dependent. Studies in different species have shown that GDF9 promotes the proliferation of cultured granulosa cells in mice [15], rats [51], cows [52], and pigs [23]. The diameters of secondary follicles cultured with GDF9 were increased in mice [53] and rats [51]. Our findings suggest that GDF9 probably promotes cumulus cell proliferation and increases the diameter of porcine OCCs.

Studies on the effects of GDF9 and BMP15 on oocyte growth are limited. In the present study, GDF9 and BMP15 had no significant effect on either oocyte growth or meiotic arrest after growth culture. However, when OCCs cultured with GDF9 for 5 days were subjected to a subsequent maturation culture, gonadotropin did not induce oocyte maturation, similar to the findings in control OCCs. In contrast, oocytes cultured with higher concentrations of BMP15 matured to MII. Moreover, OCCs cultured with GDF9 expanded loosely, whereas those with high concentrations of BMP15 expanded fully in the manner of *in vivo* grown OCCs. When the oocytes were denuded after growth culture and subjected to maturation culture, there were no significant differences in maturation rates among the

experimental groups.

BMP15 treatment during the growth culture period promoted cumulus expansion during the subsequent maturation culture, whereas GDF9 did not. This difference may be related to the different oocyte maturation rates. We added hMG, which has both FSH and LH activities [54], to the maturation culture medium. It has been suggested that LH induces the production of EGF-like peptides in the granulosa cells and that the peptides bind to the EGF receptor in the cumulus cells and activate the signaling pathways leading to cumulus expansion and oocyte meiotic resumption [37, 38]. Therefore, we conducted two experiments. In one, OCCs after growth culture were matured with EGF instead of hMG. In the second, expression of *LHCGR* and *FSHR* mRNAs in cumulus cells of OCCs that had been cultured with GDF9 or BMP15 for 5 days was compared. When OCCs after growth culture were stimulated by EGF, there were no significant differences in oocyte GVBD and MII rates among the experimental groups. These results suggest that the cumulus cells gained the ability to respond to EGF after growth culture regardless of the supplementation of GDF9 or BMP15. However, the *LHCGR* mRNA level was significantly increased by BMP15, although there were no significant differences in *FSHR* mRNA levels among the culture groups.

The expression of *LHCGR* mRNA in cumulus cells from early antral follicles before growth culture was quite low, whereas the expression of *LHCGR* mRNA in cumulus cells from antral follicles was substantial. In several species, including pigs, *LHCGR* mRNA has been reported to be absent in cumulus cells, or to be present at much lower levels than in mural granulosa cells [55–58]. In contrast, upregulation of *LHCGR* mRNA in cumulus cells was observed in all culture groups in the present study. This upregulation was probably because we supplemented the growth medium with FSH, which is essential for the growth of porcine oocytes [59]. It has been reported that FSH promotes differentiation of granulosa cells and upregulates *LHCGR* mRNA in mouse [60, 61] and porcine [62, 63] OCCs. The addition of BMP15 during growth culture further enhanced *LHCGR* mRNA expression in cumulus cells in the present study. As *LHCGR* is expressed in mural granulosa cells in the follicle [58], it is possible that the outer layer of cumulus cells of OCCs after growth culture with BMP15 could differentiate into mural granulosa cell-like cells. Considering that OCCs from the GDF9 and control groups did not show a full expansion and the oocytes did not mature to MII, the *in vitro* cultured OCCs may require a higher level of *LHCGR* mRNA to achieve cumulus expansion in response to LH.

These results suggest that GDF9 is produced in oocytes and promotes the growth of OCCs and that BMP15 produced by oocytes promotes the expression of *LHCGR* mRNA in cumulus cells during oocyte growth culture, which may contribute to cumulus expansion and oocyte maturation.

Conflict of interests: The authors declare that there are no conflicts of interest related to this manuscript.

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