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Original Article

Oocyte-derived growth factors promote development of antrum-like structures by porcine cumulus granulosa cells *in vitro*

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Abstract. Oocytes communicate with the surrounding somatic cells during follicular development. We examined the effects of two oocyte-derived growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), on the development of porcine oocyte–cumulus cell complexes (OCCs) *in vitro*. We collected OCCs from early antral follicles (1.2–1.5 mm) and prepared oocytectomized cumulus cell complexes (OXCs), which were then cultured in a growth medium supplemented with 0–100 ng/ml GDF9 and/or BMP15 for 7 days. In the medium without GDF9 or BMP15, OCCs developed during culture, and approximately 30% of them formed antrum-like structures. GDF9 promoted OCC development and structure formation in a dose-dependent manner. However, OXCs did not form antrum-like structures without growth factors. GDF9 promoted the development of OXCs, and 50 and 100 ng/ml GDF9 promoted the formation of the structures by 8% and 26%, respectively; however, BMP15 did not promote the formation of these structures. OXCs were then cultured with 100 ng/ml GDF9 and various concentrations of BMP15 to investigate their cooperative effects on the formation of antrum-like structures. BMP15 promoted the formation of antrum-like structures in a dose-dependent manner. In conclusion, GDF9 derived from oocytes is probably important for the formation of antrum-like structures in porcine OXCs, and BMP15 cooperates with GDF9 to form these structures.

Key words: Antrum formation, Bone morphogenetic protein 15 (BMP15), Cumulus cell, Growth differentiation factor 9 (GDF9), Pig

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In the first step of mammalian folliculogenesis, oocytes and flat granulosa cells form primordial follicles in the ovaries. Once these follicles begin to develop, the granulosa cells change shape and proliferate, and the follicles develop into primary and secondary follicular stages. As the granulosa cells proliferate and stratify, the follicles form fluid-filled cavities called the antrum among the granulosa cells, reaching the antral follicle stage. In the antral follicles, the granulosa cells differentiate into two cell types, i.e., cumulus (granulosa) cells surrounding the oocyte and mural granulosa cells lining the inner follicular wall. The cumulus cells supply metabolites and nutrients to the oocyte to support its growth, while the mural granulosa cells produce steroid hormones [1, 2]. Therefore, the expression of genes such as luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) differs between the two types of granulosa cells [3].

Follicular development is regulated by hormones synthesized at various levels in the hypothalamus–pituitary–ovarian axis. The hypothalamus secretes gonadotropin-releasing hormone, which stimulates the anterior pituitary gland to secrete two gonadotropins, FSH and LH. Ovaries stimulated by gonadotropins produce steroid hormones [4]. In follicles, FSH stimulates granulosa cell proliferation and androgen-to-estrogen aromatization, and the resulting estrogen stimulates granulosa cell proliferation [5]. Studies on follicle-stimulating hormone receptor (*Fshr*) knockout mice suggest that FSH is involved in follicular antrum formation [6].

Oocytes play an important role in follicular development. Oocytes secrete members of the transforming growth factor-beta (TGF-β) superfamily, including growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). GDF9 and BMP15 act on cumulus/granulosa cells in a paracrine manner, regulating follicle and oocyte growth [1, 7]. As the absence of these factors *in vivo* results in subfertility [8–11], GDF9 and BMP15 are thought to play critical roles in female fertility. In *in vitro* studies, GDF9 promoted the proliferation of mouse granulosa cells [7] and suppressed progesterone and estradiol production in rat granulosa cells [12]. Similarly, BMP15 promoted the proliferation of rat granulosa cells [13] and inhibited progesterone production in rat granulosa cells [13]. In addition, GDF9 promoted follicular development in organ-cultured rat ovaries [14] and in human ovarian epithelial tissues [15]. These reports have changed the concept that the process of follicular development leading to the ovulation of oocytes is regulated by gonadotropins in the hypothalamus–pituitary–ovarian axis and that the granulosa cells surrounding the oocytes unilaterally control oocyte growth. Oocytes have been proposed to play an important role in follicular development by controlling granulosa cell function *via* GDF9 and BMP15 [16].

The development of the follicular antrum begins with the formation of cavities filled with follicular fluids; however, the initial signal for antrum formation is not well understood [17]. During *in vitro* culture, porcine and bovine oocyte–granulosa cell complexes form antrum-like structures [18, 19]. A recent study found that GDF9 and BMP15 promoted the formation of antrum-like structures in bovine oocyte–granulosa cell complexes [20]. Studies in pigs have demonstrated that GDF9 promotes the proliferation of mural granulosa cells [21], and that GDF9 and BMP15 prevent cumulus

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cell apoptosis [22, 23]. In our previous study, we showed that GDF9 promoted the growth of porcine oocyte–cumulus cell complexes collected from early antral follicles, and some of the developing complexes formed antrum-like structures [24]. However, it has not been determined whether oocyte-derived growth factors are involved in antrum formation in pigs.

In the present study, we used *in vitro* culture systems to determine the effects of exogenous GDF9 and BMP15 on the formation of antrum-like structures by porcine cumulus cells. We dissected early antral follicles (1.2–1.5 mm in diameter) containing growing oocytes from porcine ovaries. Then, we collected oocyte–cumulus cell complexes (OCCs) from the follicles and prepared oocytectomized cumulus cell complexes (OXCs) to exclude the effects of oocyte-secreted factors. These two complexes were cultured for 7 days to examine the effects of GDF9 and BMP15 on the formation of antrum-like structures. The findings of this study suggest that GDF9 derived from oocytes is important for the formation of antrum-like structures in porcine OXCs and that BMP15 cooperates with GDF9 to form these structures.

Materials and Methods

Chemicals and media

All chemicals were purchased from Millipore Sigma (St. Louis, MO, USA) unless otherwise noted. To collect and handle ovarian follicles and OCCs, 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) polyvinyl alcohol (PVA), 0.85 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate was used. The basic growth medium was Minimum Essential Medium α (GIBCO Invitrogen, Paisley, 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), 10 µM 17β-estradiol, 0.01 IU/ml Gonalef 75 (recombinant human follicle-stimulating hormone; Merck Biopharma Japan, Tokyo, Japan), and 5% (v/v) fetal bovine serum (ICN Biomedicals, Costa Mesa, CA, USA) as reported previously [24].

Collection of OCCs and OXCs

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at room temperature. 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) PVA (PBS-PVA). The ovaries were sliced with a surgical blade (No. 21; Keisei Medical Industrial, Niigata, Japan), and pooled in HEPES-199. 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 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 and were excluded from the experiment.

Some OCCs were used to prepare OXCs. The oocytes were crushed, and the ooplasm was removed from the OCCs by aspirating the oocytes using a fine glass pipette with an inner diameter similar to the oocyte diameter (without zona pellucida). The resulting OXCs contained the zona pellucida and cumulus cells. The OXCs were washed three times with PBS-PVA.

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 the OCCs. 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 and were excluded from the experiment.

In vitro growth culture

Porcine OCCs and OXCs were cultured as previously reported [24], with some modifications. Briefly, OCCs and OXCs were cultured individually for 7 days in 96-well culture plates (Nunclon Sphera low-attachment surface: 174927 for growth culture; 174925 for quantitative PCR (qPCR); Thermo Fisher Scientific, Waltham, MA, 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 growth medium was supplemented with 0–100 ng/ml GDF9 (recombinant mouse GDF-9 protein: 739-G9; R&D Systems, Minneapolis, MN, USA) and/or BMP15 (recombinant human BMP-15 protein: 5096-BM; R&D Systems).

Half (100 µl) of the culture medium was replaced with fresh medium on Day 3 and Day 5. The morphologies of OCCs and OXCs were assessed and photographed at the start of culture (Day 0) and on Days 3 (72 h), 5 (120 h), and 7 (168 h). The complex diameters were measured using ImageJ software (NIH, Bethesda, MD, USA). The lengths in two right-angled directions passing through the center of the oocyte or zona pellucida in each complex were measured, and the average value was taken as the diameter of the complex. OCCs showing cytoplasmic degeneration of oocytes, OCCs and OXCs showing detachment of cumulus cells from the zona pellucida, or OCCs and OXCs showing loss of the aggregated structure of cumulus cells were classified as disintegrated complexes (Supplementary Fig. 1). All other complexes were considered to have maintained their integrity. The formation of antrum-like structures was examined for all complexes on Days 3, 5, and 7 by identifying the visible spaces surrounded by cumulus cells. The cultured complexes were used for histological assessment and qPCR.

Preparation of histological sections

Histological sections of OCCs and OXCs were prepared in accordance with a previously described method [20] with some modifications. Briefly, the complexes after 7 days of incubation were washed three times with PBS-PVA and fixed with 4% (w/v) paraformaldehyde (FUJIFILM Wako Pure Chemical) for 60 min. The complexes were washed three times with PBS-PVA and detached from the 96-well plate. They were dehydrated using a graded ethanol series (50% for 60 min followed by 70%, 80%, 90%, 100%, and 100%, for 30 min each), and then embedded in JB-4 resin (PolySciences, Niles, IL, USA). A rotary microtome (HM 335 E; MICROM International Walldorf, Germany) was used to prepare 5−8 µm sections from the specimens. The sections were stained with Mayer's hematoxylin (Wako Pure Chemical Industries) and 1% (w/v) eosin Y (Wako Pure Chemical Industries), and mounted using Eukitt mounting medium (O. Kindler, Freiburg, Germany) to observe the structures.

qPCR

qPCR was performed as previously reported [24] with some modifications. Briefly, after culture, 15 OXCs from each experimental group were washed with PBS-PVA, transferred into a 1.5 ml microtube with a minimum volume of PBS-PVA, and stored at –80°C until use.

As controls, we used cumulus cells collected from OCCs in early antral follicles, cumulus cells and mural granulosa cells collected from antral follicles, and cumulus cells of OCCs cultured for 7 days. The cumulus cells were removed from a group of 15 OCCs using a fine pipette and transferred into a 1.5 ml microtube with PBS-PVA. Mural granulosa cells collected from 15 antral follicles were transferred into a 1.5 ml microtube with PBS-PVA. The microtubes were centrifuged at 3,000 rpm (800 × *g*) for 3 min. After discarding the supernatant, the cumulus and mural granulosa cells were washed three times in 200 µl PBS-PVA by centrifugation for 3 min each time. After washing, the samples were stored at –80°C with a minimum volume of PBS-PVA until use.

Total RNA was extracted from the collected cumulus and mural granulosa cells using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), according to the manufacturer's instructions. qPCRs were performed using the Thunderbird SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions. Primers for *LHCGR*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and actin beta (*ACTB*) were designed according to the corresponding sequences either hosted on GenBank (accession numbers: porcine *GAPDH*, NM_001206359; ACTB, XM_003124280) or reported in a previous study (*LHCGR* [25]). Primers were purchased from Thermo Fisher Scientific (Supplementary Table 1). *GAPDH* and *ACTB* were used as internal controls. The results are indicated only with *GAPDH* because those of *ACTB* were almost identical to those of *GAPDH*. The cycling conditions for amplification were 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 63.3°C for 30 sec. Melt curve analyses were performed for all genes. The specificity of the PCR products was determined based on the presence of a single peak.

Statistical analysis

The integrity and frequency of antrum formation in OCCs and OXCs were analyzed using a chi-square test. The mean diameters of the complexes were subjected to two-way analysis of variance (ANOVA), followed by a Tukey–Kramer test. Moreover, the diameters of the complexes on Day 7 were compared with those of the OCCs from antral follicles using a *t*-test. The mRNA levels were analyzed using one-way ANOVA followed by the Tukey–Kramer test. Significance was set at $P < 0.05$.

Results

Effects of GDF9 and BMP15 on OCC development

Typical morphologies of OCCs during growth culture with GDF9 or BMP15 is shown in Figs. 1A and 1B. The oocytes were wrapped with 2–3 layers of cumulus cells before culture (Day 0). The diameters of the complexes gradually increased, and some OCCs formed antrum-like structures during growth culture. After culture, cumulus cells were maintained to surround the oocytes so as to avoid their migration to the bottom of the wells where they formed spherical or dome-shaped structures. At the end of the culture period (Day 7), more than 80% of oocytes in each group were completely wrapped with cumulus cells (Supplementary Fig. 2).

We examined the changes in the diameters of the OCCs during culture (Supplementary Fig. 3). The diameters were 170–180 µm before culture (Day 0) and gradually increased in all groups, reaching approximately 350 µm in the control group on Day 7. On Day 7, GDF9 induced a concentration-dependent increase in OCC diameter, whereas BMP15 did not promote a further increase in OCC diameter (Figs. 1C and 1D). The mean diameter of OCCs cultured with 100 ng/ml GDF9 on Day 7 was 453.8 ± 11.4 µm (mean ± S.E.), which was significantly larger than that of the control OCCs (346.4 ± 11.8 µm).

Figures 1E and 1F show the percentage of OCCs that formed antrum-like structures during culture. The first structures began to form after Day 3, and the percentage of OCCs increased to 26–30% in the control group on Day 7. In the GDF9 group, the percentage of OCCs forming antrum-like structures increased dose-dependently, and in the 100 ng/ml GDF9 group, the percentage reached 80%, which was significantly higher than the control value of 26%. In contrast, BMP15 did not promote the formation of antrum-like structures (0 ng/ml: 30% *vs*. 100 ng/ml: 27%).

Effects of GDF9 and BMP15 on OXC development

Typical morphologies of OXCs during growth culture with GDF9 or BMP15 are shown in Figs. 2A and 2B. The zona pellucida was wrapped with 2–3 layers of cumulus cells before culturing (Day 0). In OCCs as the positive control, the diameter increased during culture, and spherical or dome-shaped structures developed, each containing an oocyte. However, the diameters of the OXCs changed little during growth culture in both the control and BMP15 groups. In the GDF9 group, the diameter of the complexes gradually increased during culture. After culturing, cumulus cells of OXCs enclosed the zona pellucida without migrating to the bottom of the wells and formed spherical structures. On Day 7, more than 70% of the OXCs in each group maintained structures close to the zona pellucida (Supplementary Fig. 4).

We examined the changes in the diameters of OXCs during culture (Supplementary Fig. 5). In OCCs used as the positive control, the diameter gradually increased from 170–180 µm on Day 0 to 300 µm on Day 7. On the other hand, in OXCs without GDF9 or BMP15, the diameters were approximately 200 µm on Day 7, which was significantly larger than that on Day 0. GDF9 increased the OXC diameter in a concentration-dependent manner, whereas the effect of BMP15 was minimal (Figs. 2C and 2D). The mean diameter of OXCs cultured with 100 ng/ml GDF9 on Day 7 was 318.5 ± 11.9 µm, which was significantly smaller than the diameter of OCCs (377.8 ± 9.8 µm).

Figures 2E and 2F show the percentages of OXCs that formed antrum-like structures during culture. As the positive control, OCCs started to form antrum-like structures after Day 5, and the percentage increased to 35–43% on Day 7. In contrast, OXCs in the control and BMP15 groups did not form any antrum-like structures. In the GDF9 groups, OXCs cultured with 50 and 100 ng/ml GDF9 formed antrum-like structures (8% and 26%, respectively).

Effects of combining GDF9 and BMP15 on OXC development

Typical morphologies of OXCs during growth culture with 100 ng/ml GDF9 and various concentrations of BMP15 are shown in Fig. 3A. The diameters of the complexes gradually increased, and some formed antrum-like structures during growth culture. After culture, cumulus cells were maintained to surround the oocytes and formed spherical or dome-shaped structures, whereas in the high-concentration BMP15 groups, the complexes had lumpy surfaces. At the end of culture (Day 7), more than 75% of OXCs in each group maintained structures that enclosed the zona pellucida (Supplementary Fig. 6).

The diameter of the OXCs was approximately 180 µm before culture (Day 0, Supplementary Fig. 7). The diameter gradually increased in all groups, reaching approximately 350 µm in the control group on Day 7. BMP15 increased the OXC diameter in a concentration-dependent manner, and the mean diameter in the 100

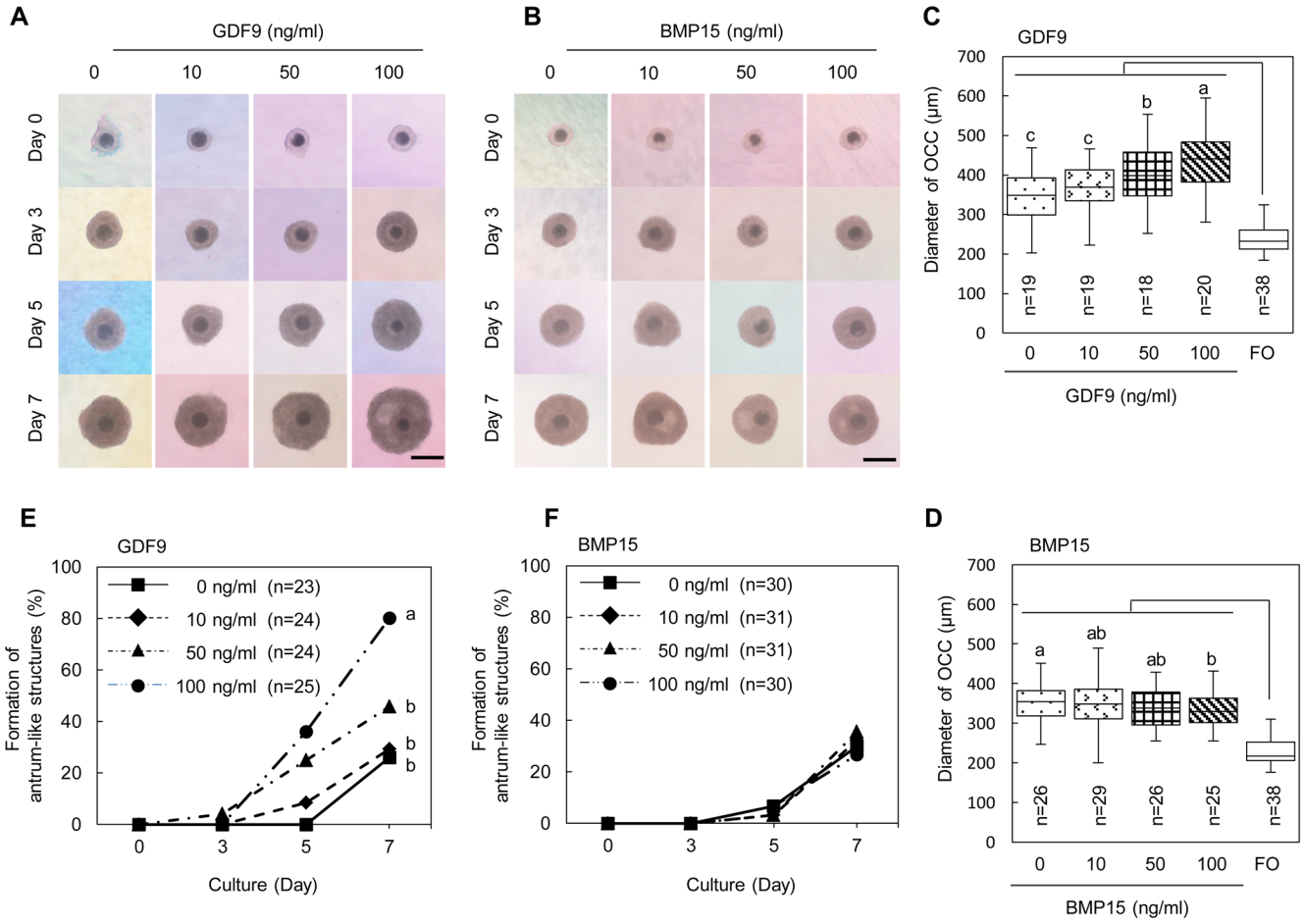

Fig. 1. Effects of GDF9 and BMP15 on porcine OCC development. Porcine oocyte–cumulus cell complexes (OCCs) were cultured in a growth medium supplemented with GDF9 or BMP15 for 7 days. (A and B) Typical morphologies of porcine OCCs cultured for 0, 3, 5, and 7 days are shown. Scale bars = 200 μm. (C and D) The diameters of OCCs excluding disintegrated complexes, were measured on Days 0, 3, 5, and 7 (Supplementary Fig. 3). The results on Day 7 are shown. The diameters of OCCs, including fully grown oocytes collected from antral follicles (4.0–6.0 mm), were measured as *in vivo* controls (FO). The GDF9 and BMP15 concentrations are shown in ascending order under the respective sets of boxes, and the numbers of OCCs (n) used for each experiment are shown at the bottom of each box. Boxes with different letters (a–c) in each figure are significantly different (two-way ANOVA, $P < 0.05$). The diameters of the complexes on Day 7 were compared with those of OCCs from antral follicles by *t*-test (square brackets: $P < 0.05$). (E and F) The percentage of OCCs that formed antrum-like structures was examined. Values with different letters (a, b) are significantly different (chi-square test; $P < 0.05$). Experiments were replicated more than three times.

ng/ml GDF9 + 100 ng/ml BMP15 group on Day 7 was 444.1 ± 16.9 μm, which was significantly larger than that in the control OCCs (344.8 ± 12.9 μm, Fig. 3B).

Figure 3C shows the percentage of OXCs that formed antrum-like structures during culture. OXCs and OCCs began to form antrum-like structures after Day 3, and the percentage increased to 14% in the OCC group on Day 7. BMP15 promoted the formation of antrum-like structures in a dose-dependent manner, and in the 100 ng/ml GDF9 + 100 ng/ml BMP15 group, the percentage reached 58%, which was significantly higher than the 16% increase in the 100 ng/ml GDF9 + 0 ng/ml BMP15 group.

Figure 4 shows representative images of histological sections of the complexes. In the OCCs, cumulus cells surrounded the oocyte. In the OXCs, the cytoplasm of the oocyte was not observed, and the zona pellucida was observed in the center of each complex consisting of cumulus cells. Inside the complexes, antrum-like structures were sometimes observed among cumulus cells. In the OXCs cultured with GDF9 alone, cumulus cells showed a pebble-like morphology, and the distance between cells was short. On the other hand, in the OXCs cultured with combinations of GDF9 and BMP15, cumulus cells were separated from each other and formed elongated thin protrusions like filopodia. In addition, depending on the concentration of BMP15, larger antrum-like structures were observed.

The expression levels of *LHCGR* mRNA are shown in Supplementary Fig. 8. The cumulus cells collected from early antral follicles (EACC) and antral follicles (ACC) hardly expressed *LHCGR* mRNA, whereas the mural granulosa cells collected from antral follicles (AMGC) expressed considerable levels of mRNA. All groups of cultured complexes expressed higher levels of *LHCGR* mRNA than EACC. The OXCs cultured without growth factors expressed higher levels of *LHCGR* mRNA than those cultured with oocytes (OCC) or GDF9. Cumulus cells of OXCs cultured with BMP15 expressed a higher level of *LHCGR* mRNA than those of the OCC, GDF9, and GDF9 + BMP15 groups; however, the mRNA levels did not significantly differ among the groups.

Discussion

GDF9 promoted porcine OCC development to produce antrum-like structures in a concentration-dependent manner. In contrast, BMP15

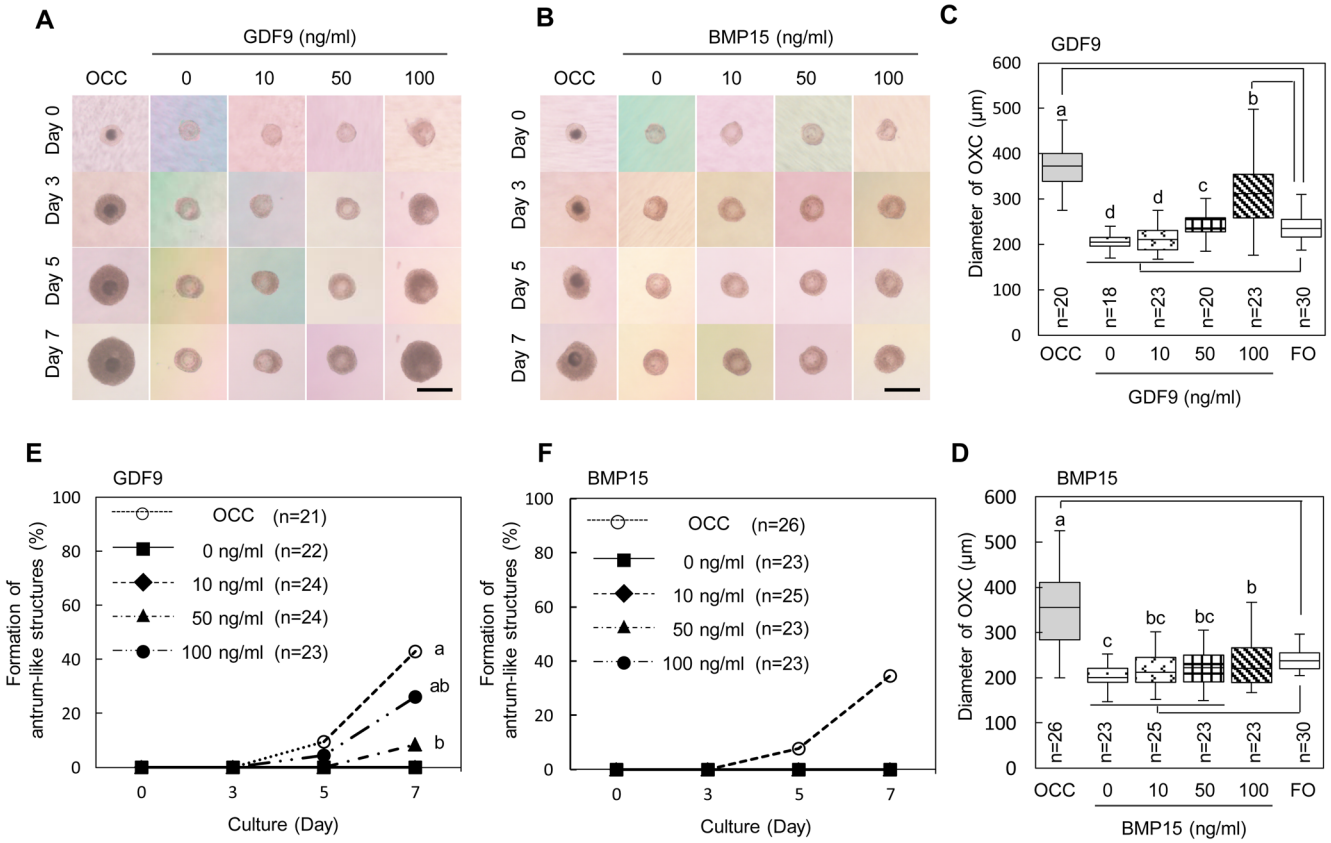

Fig. 2. Effects of GDF9 and BMP15 on porcine OXC development. Porcine oocytectomized cumulus cell complexes (OXCs) were cultured in a growth medium supplemented with GDF9 or BMP15 for 7 days. Oocyte–cumulus cell complexes (OCCs) were cultured without growth factors as the control. (A and B) Typical morphologies of porcine OXCs cultured for 0, 3, 5, and 7 days are shown. Scale bars = 200 μm. (C and D) The diameters of OXCs excluding disintegrated complexes were measured on Days 0, 3, 5, and 7 (Supplementary Fig. 5). The results on Day 7 are shown. The diameters of OCCs, including fully grown oocytes collected from antral follicles (4.0–6.0 mm), were measured as *in vivo* controls (FO). The GDF9 and BMP15 concentrations are shown in ascending order under the respective sets of boxes, and the numbers of OXCs (n) used for each experiment are shown at the bottom of each box. Boxes with different letters (a–d) in each figure are significantly different (two-way ANOVA, $P < 0.05$). The diameters of the complexes on Day 7 were compared with those of OCCs from antral follicles by *t*-test (square brackets: $P < 0.05$). (E and F) The percentage of OXCs that formed antrum-like structures was examined. Values with different letters (a, b) are significantly different (chi-square test; $P < 0.05$). Experiments were replicated more than three times.

did not significantly increase the OCC diameter or the formation of antrum-like structures. These results were mostly similar to the results obtained in our previous report [24], which suggested that GDF9 promoted the proliferation of cumulus cells to increase complex diameter, while BMP15 had little or no effect on OCC development. GDF9 promotes the proliferation of cultured granulosa cells in various species [7, 21]. As the number of OCCs that formed antrum-like structures increased as the OCC diameter increased, our findings suggest that GDF9 induces OCC development by promoting the proliferation of cumulus cells, which might be a prerequisite for the formation of antrum-like structures.

In the OCC growth culture used in the present study, we assumed that the oocytes secreted GDF9 and BMP15. Therefore, we cultured OXCs under the same conditions to exclude the effects of the oocyte-secreted factors. This enabled us to clarify the effects of GDF9 and BMP15 on the formation of the antrum-like structures in the complexes. As the positive control, OCCs developed and formed antrum-like structures after culture. In contrast, OXCs cultured without GDF9 or BMP15 developed slightly but did not form any antrum-like structures. This result indicates that oocytes induced the development of complexes and formation of antrum-like structures. BMP15 slightly promoted the development of OXCs, but did not induce the formation of any antrum-like structures. Although GDF9 promoted the development of OXCs and formation of antrum-like structures, the effect was weaker than that of the oocytes in the OCCs (Fig. 1E and Fig. 2E). Alam *et al.* who reported similar results using bovine oocyte–granulosa cell complexes and OXCs [20], suggested that bovine oocytes participate in antrum formation by the complexes and that both GDF9 and BMP15 induce the formation of antrum-like structures. This report is consistent with the results of the present study, except for the effects of BMP15, which suggests that oocytes and oocyte-derived GDF9 may promote the formation of antrum-like structures by cumulus/granulosa cells in mammals. However, the effects of BMP15 differed between pigs and cows. In pigs, OXCs cultured with BMP15 do not grow large enough to form antrum-like structures. Moreover, the percentage of antrum-like structures formed by porcine OXCs cultured with GDF9 was lower than that formed by OCCs. Thus, it is reasonable to speculate that other oocyte-derived factors, including BMP15, may cooperate with GDF9 and participate in the formation of the antrum-like structures.

To investigate the effects of the cooperation between GDF9 and BMP15 on the formation of antrum-like structures, OXCs were cultured with 100 ng/ml GDF9 and various concentrations of BMP15. The results showed that BMP15 promoted the formation

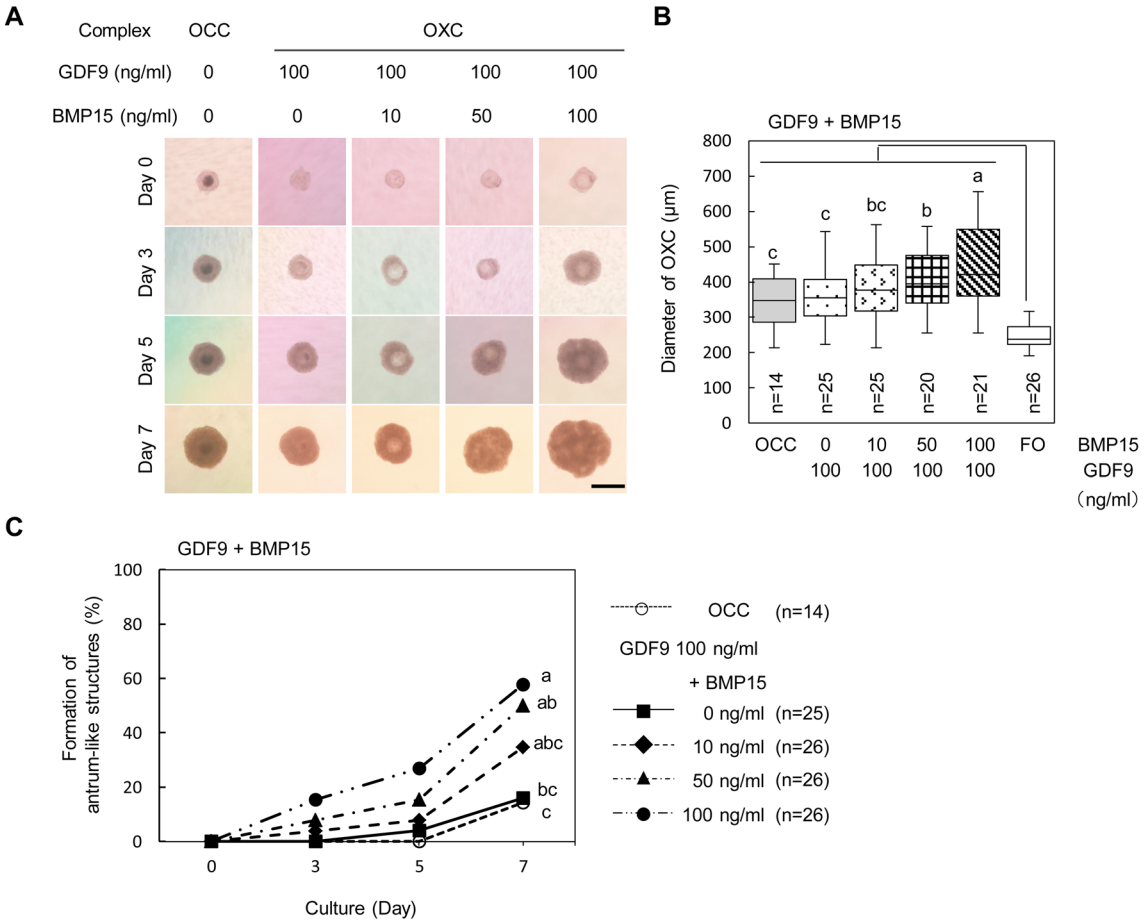

Fig. 3. Effects of combinations of GDF9 and BMP15 on porcine OXC development. Porcine oocytectomized cumulus cell complexes (OXCs) were cultured in a growth medium supplemented with 100 ng/ml GDF9 and various concentrations of BMP15 for 7 days. Oocyte–cumulus cell complexes (OCCs) were cultured without growth factors as the control. (A) Typical morphologies of porcine OXCs cultured for 0, 3, 5, and 7 days are shown. Scale bar = 200 μm. (B) The diameters of OXCs excluding disintegrated complexes were measured on Days 0, 3, 5, and 7 (Supplementary Fig. 7). The results on Day 7 are shown. The diameters of OCCs, including fully grown oocytes collected from antral follicles (4.0–6.0 mm), were measured as *in vivo* controls (FO). The GDF9 and BMP15 concentrations are shown in ascending order under the boxes, and the numbers of OXCs (n) used for each experiment are shown at the bottom of each box. Boxes with different letters (a–c) are significantly different (two-way ANOVA, $P < 0.05$). The diameters of the complexes on Day 7 were compared with those of OCCs from antral follicles by *t*-test (square bracket: $P < 0.05$). (C) The percentage of OXCs that formed antrum-like structures was examined. Values with different letters (a–c) are significantly different (chi-square test; $P < 0.05$). Experiments were replicated more than three times.

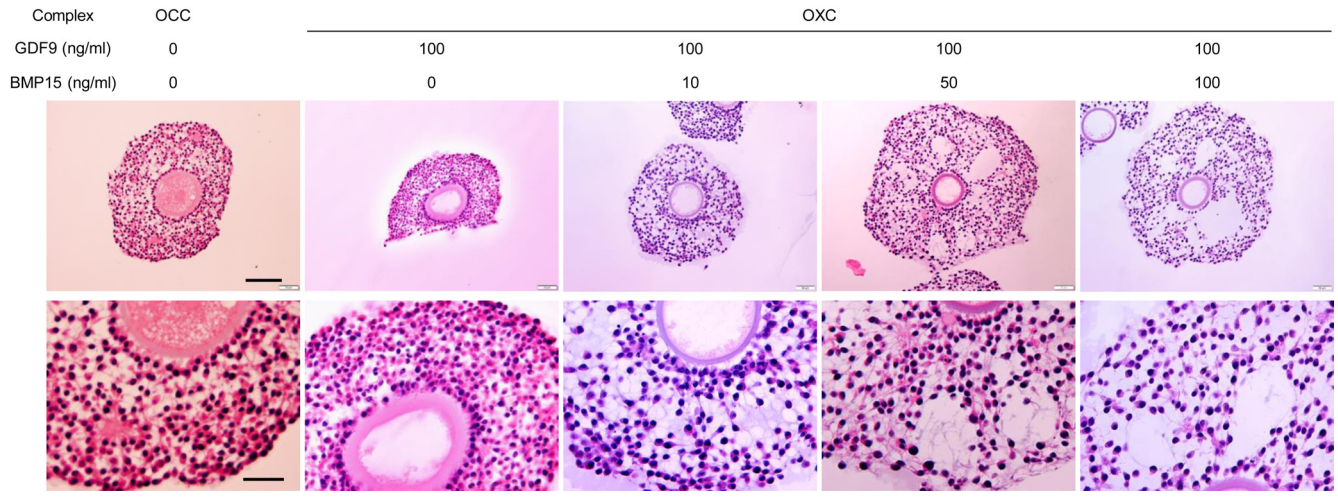

Fig. 4. Representative images of histological sections of porcine OXCs cultured with GDF9 and BMP15. Porcine oocytectomized cumulus cell complexes (OXCs) were cultured in a growth medium supplemented with combinations of 100 ng/ml GDF9 and 0 ng/ml (n = 3), 10 ng/ml (n = 7), 50 ng/ml (n = 8), or 100 ng/ml (n = 5) BMP15 for 7 days. Oocyte–cumulus cell complexes (OCCs, n = 6) were cultured as controls without growth factors. Scale bars = 100 μm (top) and 40 μm (lower).

of antrum-like structures in a dose-dependent manner, suggesting that BMP15 strengthened the effect of GDF9. Mottershead *et al.* [26] suggested that GDF9 and BMP15 formed a heterodimer, called cumulin and that cumulin was more effective for the proliferation of granulosa cells than GDF9 or BMP15 alone. In this study, we used recombinant mouse GDF9 and human BMP15. Although it is not certain whether they formed cumulin during culture, our results clearly showed that the combination of GDF9 and BMP15 had a more potent effect on the development of OXCs than GDF9 alone. Alam *et al.* [20] reported that almost all bovine OXCs cultured with GDF9 and BMP15 formed antrum-like structures. In our study, 60% of porcine OXCs cultured with 100 ng/ml GDF9 and 100 ng/ml BMP15 formed antrum-like structures. Therefore, both studies consistently suggest that the combination of GDF9 and BMP15 promotes the formation of antrum-like structures in pigs and cows.

Granulosa cells differentiate into cumulus and mural granulosa cells when follicles form an antrum *in vivo*, and mural granulosa cells express high levels of *LHCGR* mRNA [27–30]. We hypothesized that the cultured cumulus cells proliferated and that some of them may have differentiated into mural granulosa cells. Therefore, we examined the histological sections of cultured OXCs and performed qPCR to check the mRNA levels of *LHCGR*. In the OXCs cultured with GDF9, the cumulus cells showed a pebble-like morphology, whereas, in the OXCs cultured with GDF9 and BMP15, the cumulus cells exhibited elongated processes that seemed to be filopodia. In mice and cows, GDF9 induces granulosa cells to form filopodia [20, 31]. However, in pigs, GDF9 and BMP15 collaborate to promote the formation of filopodia, which increases the distance between cumulus cells to form spaces that probably develop into antrum-like structures. qPCR revealed that the expression levels of *LHCGR* mRNA were quite low in the cumulus cells before culture, whereas the levels were increased in the control (without growth factors) and BMP15 groups during culture. In contrast, these levels were suppressed in the OCC, GDF9, and GDF9 + BMP15 groups, which formed antrum-like structures. These results suggest that oocyte-derived GDF9 reduces *LHCGR* mRNA expression in cumulus cells, which may be important for the formation of antrum-like structures by these cells.

In conclusion, GDF9 derived from oocytes is probably important for the formation of antrum-like structures in porcine OXCs, and BMP15 cooperates with GDF9 to form these structures.

Conflict of interests: The authors declare that there is no conflict in relation to this manuscript.

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