Journal of Reproduction and Development, Vol. 64, No 5, 2018

—Original Article—

GDF9 and BMP15 induce development of antrum-like structures by bovine granulosa cells without oocytes

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Abstract. The role of oocytes in follicular antrum formation is not well understood. We examined the effect of oocyte-derived growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), on the formation of antrum-like structures by cultured bovine oocyte-granulosa cell complexes (OGCs). OGCs containing growing oocytes (105–115 μm in diameter) were collected from early antral follicles (1.2–1.8 mm) and used to prepare oocytectomized complexes (OXCs) and granulosa cell complexes (GCs). The mRNAs of *GDF9* and *BMP15* were expressed in the oocytes, but not in the granulosa cells. The complexes were cultured for five days with or without GDF9 and BMP15 either alone or in combination. The OGCs maintained their complex integrity and developed antrum-like structure, whereas OXCs and GCs neither maintained their integrity nor developed any antrum-like structure without growth factors. GDF9 or BMP15 alone increased the integrity of these complexes and induced antrum-like structures in OXCs and GCs. Moreover, the combination of GDF9 and BMP15 was more potent for both phenomena in all types of complexes. In OXCs and GCs cultured without GDF9 and BMP15 or with BMP15 alone, outgrowing granulosa cells differentiated into fibroblast-like cells. The combination of GDF9 and BMP15 suppressed the appearance of fibroblast-like cells in OXCs and GCs during incubation. Instead, the granulosa cells appeared rhomboid and pebble-like in shape, similar to those in OGCs cultured without supplementation of GDF9 and BMP15. These results suggest that oocytes maintain complex integrity by preventing granulosa cell differentiation and participate in follicular antrum formation via GDF9 and BMP15.

Key words: Antrum formation, Bone morphogenetic protein 15 (BMP15), Bovine granulosa cells, Growth differentiation factor 9 (GDF9), Oocyte

(**J. Reprod. Dev. 64:** 423–431, 2018)

Mammalian ovaries contain a vast number of primordial follicles. A small population of these follicles enters the growth phase and develops through the primary and secondary follicle stages. Follicular granulosa cells proliferate to develop a multilayered structure during the progression of follicular development. Later, a fluid-filled cavity called an antrum is formed inside the follicles as they reach the antral follicle stage. In antral follicles, granulosa cells separate to form cumulus granulosa cells which enclose the oocytes, and mural granulosa cells which form the inner layer of the follicle wall [1].

Bidirectional communication between oocytes and granulosa cells is required for ovarian function and fertility [2]. Granulosa cells supply nutrients, metabolites, and molecular signals to the oocytes, while oocytes promote the proliferation, differentiation and function of granulosa cells. The oocytes play a crucial role in the regulation of follicular development [3]. Oocytes are required for initial primordial follicle formation [4] and regulate the specific characteristics and functions between cumulus granulosa cells and mural granulosa cells in mice [5]. During oocyte maturation, oocytes secrete a cumulus expansion-enabling factor to induce cumulus expansion [6, 7]. In addition, it has been suggested that oocytes promote contact between granulosa cells and induce antrum formation in pigs [8].

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are two important oocyte-derived factors that regulate ovarian functions [9, 10]. In GDF9 null mice, folliculogenesis does not progress beyond the primary follicle stage and defective granulosa cell proliferation is observed in the follicles [11]. BMP15 null mice exhibit subfertility [12] and ewes carrying BMP15 mutations are infertile and exhibit arrest of follicular development at the primordial stage [13]. Immunization against GDF9 and BMP15 has been shown to reduce the follicular development and ovulation rate in cattle [14]. Under various culture conditions, GDF9 and BMP15 promote granulosa cell proliferation and function in mice [15], humans [16], cattle [17], and sheep [17]. Although it has been suggested that both GDF9 and BMP15 are important in follicular development, their role in follicular antrum formation has not been elucidated.

Many approaches have been considered to grow mammalian oocytes *in vitro*; some studies have reported that cultured follicles and oocyte-granulosa cell complexes (OGCs) developed antrum-like structures [18]. Other studies have reported the development of antrum-like structures in mice [19], humans [20], and pigs [21] by *in vitro* incubation of the preantral follicles. In addition, rat [22] and bovine [23] OGCs have been shown to form antrum-like structures in culture. Hirao *et al*. [24] developed a growth culture system in

Received: June 13, 2018
Accepted: June 30, 2018
Published online in J-STAGE: July 21, 2018

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which bovine OGCs containing oocytes less than 100 µm in diameter were cultured in medium supplemented with a high concentration of polyvinylpyrrolidone for 14 days. This system efficiently supported the growth of bovine small oocytes, and OGCs formed antrum-like structures during the incubation. However, it is not yet known whether oocytes or oocyte-derived factors induce the formation of antrum-like structures *in vitro*.

In the present study, we cultured bovine growing oocytes with surrounding cumulus granulosa cells collected from early antral follicles (1.2–1.8 mm in diameter) for five days. OGCs formed antrum-like structures, whereas the oocytectomized complexes (OXCs) and granulosa cell complexes (GCs) did not. We therefore investigated the effect of GDF9 and BMP15 on the formation of antrum-like structures. Surprisingly, our results showed that GDF9 and BMP15 induced the development of antrum-like structures by OXCs and GCs.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. GDF9 (Recombinant Mouse GDF-9; 739-G9) and BMP15 (Recombinant Human BMP-15; 5096-BM) were purchased from R&D Systems (Minneapolis, MN, USA).

Collection of OGCs

Oocytes with surrounding cumulus granulosa cells (OGCs) were collected from bovine ovaries as described previously [25] with some modifications. Briefly, bovine ovaries were obtained from a local slaughter house and transported to the laboratory. Ovaries were washed once with 0.2% (w/v) cetyltrimethylammonium bromide and then three times with Dulbecco's PBS containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA). Ovarian cortical slices were collected using a surgical blade (No. 10; Feather Safety Razor, Tokyo, Japan) and forceps. Small antral follicles (1.2–1.8 mm in diameter) were collected from ovarian cortical slices in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% PVA, 0.85 mg/mL sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate. The follicles were opened using a blade (No. 10) and forceps to collect OGCs.

Preparation of complexes

OGCs were used to prepare two types of complexes: OXCs and GCs. OXCs were prepared by removing the oocyte cytoplasm with the germinal vesicle (GV) from the OGCs using a microinjection apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan) attached to an inverted microscope (Olympus IX70 Multi-parameter Fluorescence Microscope; Olympus, Tokyo, Japan). Holding pipettes were prepared by pulling glass capillary tubes with a pipette puller (P-97/IVF; Sutter Instrument, Novato, CA, USA), followed by processing with a Microforge (MF-79; Narishige Scientific Instrument Lab.). Injection pipettes (MIC-CUST-30) were purchased from Origio Humagen (Charlottesville, VA, USA). The injection pipettes had an inner diameter of 11 µm at the needle tip. The oocyte cytoplasm along with the GV was suctioned by the negative pressure of the injection pipette after insertion into the oocyte. The resulting OXCs contained a zona pellucida and granulosa cells. The GCs were prepared by removing whole oocytes with the zona pellucida from OGCs using a small bore pipette.

RNA extraction and RT-PCR

Bovine denuded oocytes, GCs and OGCs were used for extraction of total RNA using an RNeasy Plus Micro Kit (Qiagen, Austin, TX, USA) according to the manufacturer's instructions and eluted with 14 µl of RNase-free water. At least 30 mechanically denuded oocytes, clumps of GCs separated from 30 denuded oocytes, and 30 OGCs were used for RNA extraction. Reverse transcription was performed using ReverTra Ace® qPCR RT Master Mix (Toyobo, Tsuruga, Japan) according to the manufacturer's instructions in a final volume of 20 µl. The RT-PCR reactions were carried out in a 25 µl reaction volume using PCR Master Mix (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Cycling conditions for the amplifications were 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 65°C for 1 min, and 72°C for one minute, followed by 72°C for 5 min; with a final hold at 4°C. The primers were designed according to known sequences in GenBank (accession numbers: bovine *β-ACTIN*, NM_173979.3; *GDF9*, NM_174681.2; and *BMP15*, NM_001031752.1). The primers were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The primer sequences and their expected PCR product sizes are shown in Table 1.

RT-PCR products were electrophoresed and visualized in 1% (w/v) agarose gel containing ethidium bromide. From the agarose gel, RT-PCR products were purified using a QIAquick gel extraction kit (Qiagen). Sequencing of gel-purified products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130 Genetic Analyzer (Applied Biosystems). We confirmed that the nucleotide sequences of the RT-PCR products were identical to the bovine cDNA sequences of *β-ACTIN*, *GDF9*, and *BMP15*.

In vitro culture of complexes

The collected complexes (OGCs, OXCs, and GCs) were cultured for five days in 96-well culture plates (BioCoat^{TM} Collagen I Cellware; Becton Dickinson Biosciences, San José, CA, USA) containing 200 µl of culture medium. One complex was placed into each well of a 96-well plate for individual incubation. The culture was carried out under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. The basic culture medium was Minimum Essential Medium α (GIBCO, Invitrogen, Paisley, Scotland, UK) supplemented with 4% (w/v) polyvinylpyrrolidone (molecular weight 360 000), 5% (v/v) fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), 4 mM hypoxanthine, 50 µg/ml ascorbic acid 2-glucoside (Hayashibara Biochemical Laboratories, Okayama, Japan), 55 µg/ml cysteine, 0.05 mM dexamethasone, 100 ng/ml 17β-estradiol, 10 ng/ml androstenedione, 1 mM sodium pyruvate, 2.2 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate [24, 25]. The culture medium was further supplemented with 100 ng/ml of GDF9, 10 ng/ml of BMP15, or a combination of 100 ng/ml of GDF9 and 10 ng/ml of BMP15. The combination of GDF9 and BMP15 was used because the heterodimers of these oocyte-derived factors are 10–3000 fold more bioactive than the homodimers [26]. The

Table 1. Primers used for RT-PCR and the expected size of their PCR products

Gene	Primer	Nucleotide sequence	Expected size (bp)
β-ACTIN	*β-ACTIN* -F	5′-AAGATCAAGATCATCGCGCCC- 3′	350
	β-ACTIN -R	5′-CTTTGGGAATGCTCGATCCAACC- 3′	
GDF9	*GDF9* -F	5′-CCATGGCGCTTCCCAACAAAT- 3′	416
	GDF9 -R	5′-CACTGATGGAAGGGTTCCTGCT- 3′	
BMP15	*BMP15* -F	5′-TCTCAGAGGCTCCTGGCACAT- 3′	489
	BMP15 -R	5′-TGACGAGCCCTCCTCAAGAGA- 3′	

F: forward primer and R: reverse primer. Both forward and reverse primer sequences for all genes of interest are given in the 5′ to 3′ direction.

concentrations of GDF9 and BMP15 were selected on the basis of their ED_{50} values and previous reports [17, 27]. The day of complex preparation was considered Day 0, and half (100 µl) of the culture medium was replaced with new medium at Day 3. The formation of antrum-like structures by complexes was examined at Day 1, Day 3 and Day 5 by identifying visible spaces surrounded by granulosa cells. Complexes showing cytoplasmic degeneration of oocytes (in OGCs), detachment of granulosa cells from the zona pellucida (in OGCs and OXCs), or loss of the aggregated structure of granulosa cells (in all complexes) were classified as disintegrated complexes; all others were considered complexes maintaining their integrity.

Hematoxylin and eosin staining of complexes

Next, the complexes were subjected to hematoxylin and eosin Y staining. The media were carefully removed from the 96-well plates after five days of incubation and the complexes were washed three times with PBS-PVA and fixed in 4% (w/v) paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 30 min. The complexes were then washed three additional times with PBS-PVA. Mayer's hematoxylin solution (Wako Pure Chemical Industries) was used to stain the complexes for 10 min, then washed out with tap water for 10 min. Finally, the complexes were stained with eosin Y (1%; Wako Pure Chemical Industries) for 2 min, and the eosin Y was washed out with tap water for one minute. A glycerol-water mixture (1:4) was used to mount the complexes.

Preparation of histological sections

To prepare the histological sections, the complexes were cultured on Millicell® inserts (Merck Millipore, Billerica, MA, USA) placed in petri dishes (Becton Dickinson and Company, Bedford, MA, USA) according to a method described previously [28]. After five days of incubation, the complexes were washed three times with PBS-PVA and fixed in 4% paraformaldehyde for 2 h. Ovarian follicles 1.2–1.8 mm in diameter were fixed for three hours as controls. The complexes and follicles were washed three times with PBS-PVA and dehydrated by a series of increasing concentrations of ethanol (50% for 30 min, followed by 70%, 80%, 90%, 100% and 100% for 20 min each). The complexes that maintained integrity were detached from the Millicell® inserts before the final dehydration by 100% ethanol. They were infiltrated and embedded in JB-4 resin (PolySciences, Niles, IL, USA). A rotary microtome (HM 335 E; MICROM International GmbH, Walldorf, Germany) was used to make 7–8 µm sections from the specimens. Mayer's hematoxylin and eosin Y were used to stain the sections as described above.

Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's HSD (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Values of $P < 0.05$ were considered statistically significant.

Results

Expression of GDF9 and BMP15 mRNAs in bovine oocytes and granulosa cells

The expression levels of *GDF9* and *BMP15* mRNAs in bovine oocytes and GCs were examined by RT-PCR (Fig. 1). Bovine *β-ACTIN* was used as an internal control. OGCs and oocytes (Oo) showed bands of PCR products for the *GDF9* and *BMP15* mRNAs at the expected size, whereas GCs did not show any bands.

Effect of GDF9 and BMP15 on the integrity of complexes

The typical morphologies of complexes during culture are shown

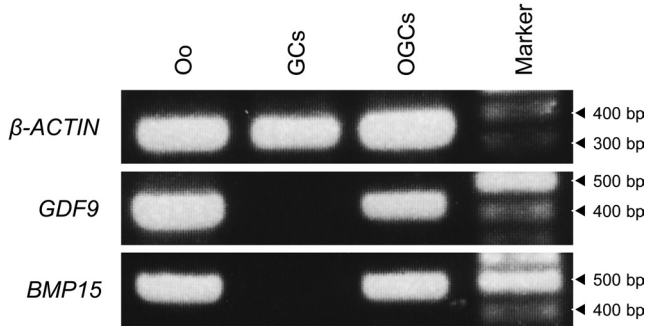

Fig. 1. Expression of *GDF9* and *BMP15* mRNAs in bovine oocytes and granulosa cell complexes. Bovine oocytes (Oo), granulosa cell complexes (GCs), and oocyte-granulosa cell complexes (OGCs) were used for extraction of total RNA. The cDNA was synthesized using total RNA as a template for RT-PCR. The RT-PCR product bands using primer sets specific to bovine *β-ACTIN, GDF9,* and *BMP15* are shown in the top, middle, and bottom panels, respectively. Bovine *β-ACTIN* was used as an internal control. The right lane represents the molecular mass marker. The expected sizes of the PCR products for *β-ACTIN, GDF9* and *BMP15* were 350 bp, 416 bp, and 489 bp, respectively.

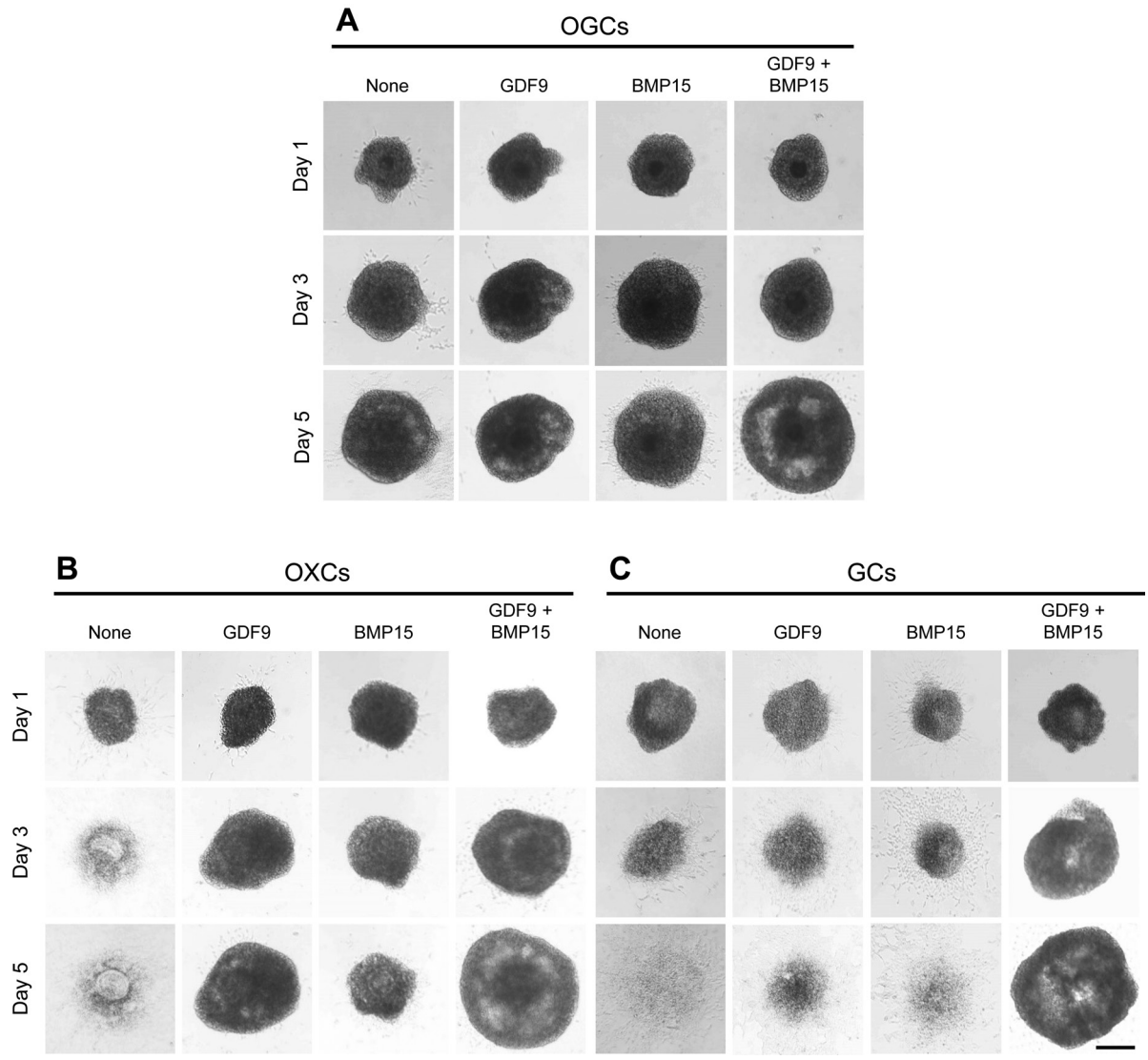

Fig. 2. Typical morphologies of complexes during culture. Bovine oocyte-granulosa cell complexes (OGCs; A), oocytectomized complexes (OXCs; B), and granulosa cell complexes (GCs; C) were cultured for 5 days with GDF9 (100 ng/ml), BMP15 (10 ng/ml), or a combination of GDF9 (100 ng/ml) and BMP15 (10 ng/ml). Complexes cultured without GDF9 or BMP15 are represented as "None". The top, middle, and bottom panels in each set of pictures (A, B and C) represents Day 1, 3, and 5 of incubation, respectively. The scale bar represents 200 μm.

in Fig. 2. In each complex, there were several layers of granulosa cells when they were initially placed in culture medium regardless of the presence of the oocyte or zona pellucida. During incubation, granulosa cells of OGCs adjacent to the culture plate were attached to it, and then proliferated to form multilayered rigid structures in some complexes (Fig. 2A). In other complexes, attached granulosa cells proliferated outward from the original complex. The combination of GDF9 and BMP15 promoted the development of the multilayered structure of OGCs (Fig. 2A). In OXCs and GCs, the initial aggregates composed of multilayered granulosa cells (Day 1 in Figs. 2B and 2C) lost their structure completely by Day 5 without supplementation of GDF9 and BMP15 (Day 5 in Figs. 2B and 2C). GDF9 or BMP15 alone maintained the multilayered structure in OXCs, but not in GCs. However, the combination of GDF9 and BMP15 promoted the development of a multilayered structure in both OXCs (Fig. 2B) and GCs (Fig. 2C). During incubation, some of the OGCs in all groups and some of the OXCs and GCs cultured with GDF9 and/or BMP15 formed visible antrum-like structures by developing spaces inside the multilayered granulosa cells (Fig. 2, Day 3). These antrum-like structures became more prominent by the end of incubation (Fig. 2, Day 5). However, no antrum-like structures were formed in OXCs (Fig. 2B) or GCs (Fig. 2C) without supplementation of GDF9 and BMP15.

The integrity of complexes is summarized in Fig. 3. In the presence of oocytes, 65% of OGCs maintained their integrity at Day 5 (Fig. 3A). Neither GDF9 nor BMP15 significantly increased the integrity

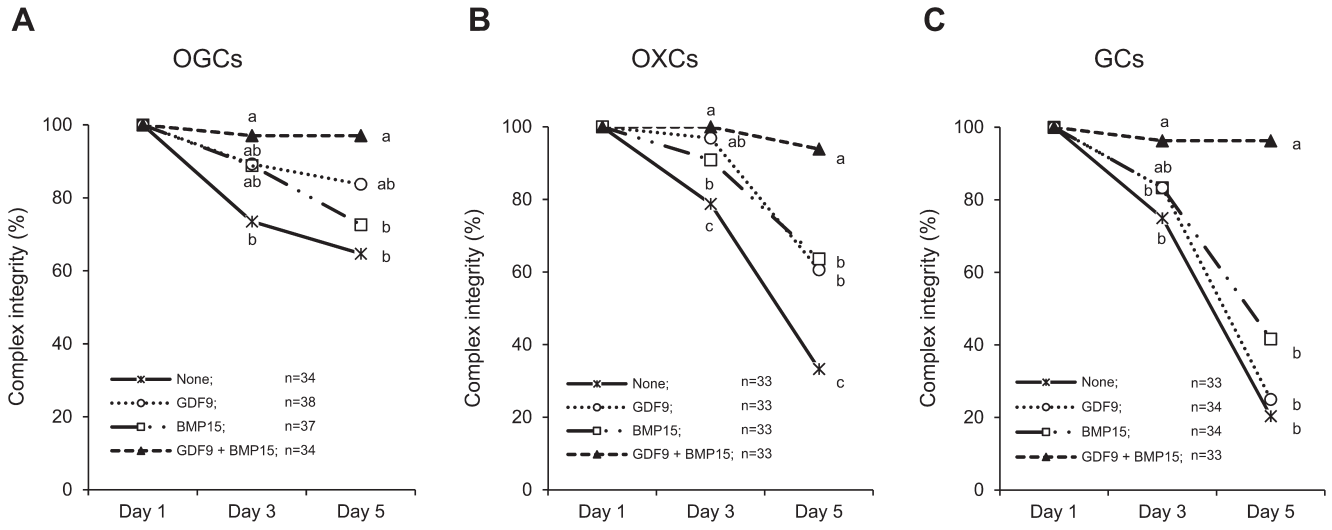

Fig. 3. Integrity of complexes (A: OGCs; B: OXCs; and C: GCs) during *in vitro* incubation with GDF9 and BMP15. Complexes showing degenerative signs, such as cytoplasmic degeneration of oocytes, detachment of granulosa cells from the zona pellucida, or loss of the aggregated structure of granulosa cells, were classified as disintegrated complexes. The numbers of complexes (n) used in each group are shown in each graph (A, B, and C). Different types of lines indicate GDF9 (100 ng/ml) and BMP15 (10 ng/ml) either alone or in a combination. Data are shown as average percentages from at least three replicated cultures. The letters "a–c" denote significantly different values ($P < 0.05$).

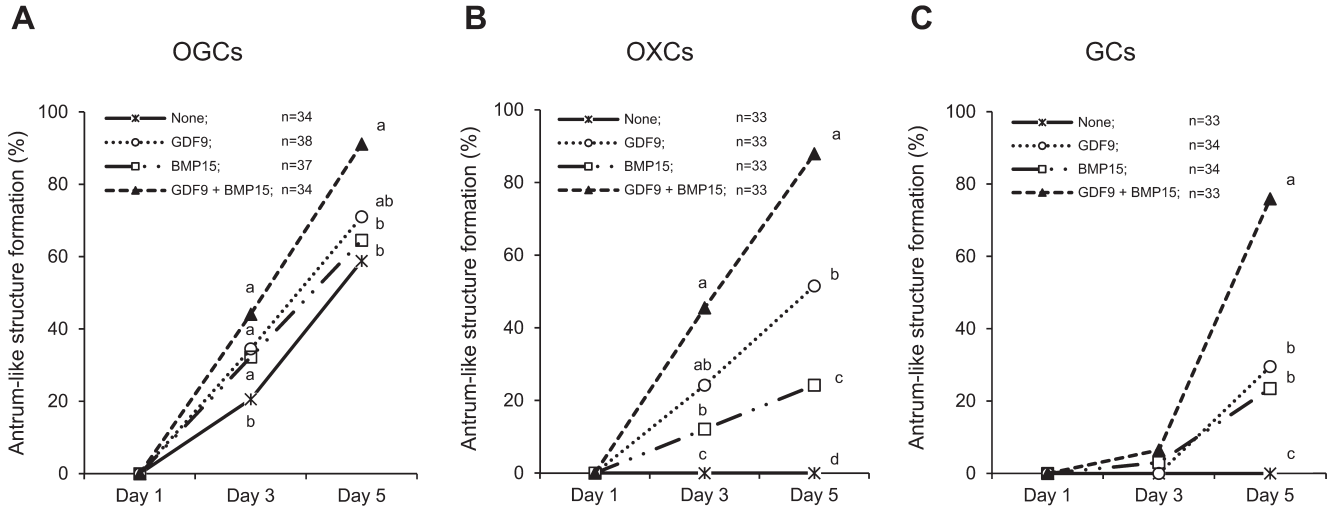

Fig. 4. Formation of antrum-like structures by complexes (A: OGCs; B: OXCs; and C: GCs) during *in vitro* incubation with GDF9 and BMP15. Formation of antrum-like structures was confirmed by examining spaces formed inside the granulosa cell layers on Day 1, Day 3 and Day 5 of the culture period. The numbers of complexes (n) used in each group are shown in each graph (A, B, and C). Different types of lines indicate GDF9 (100 ng/ml) and BMP15 (10 ng/ml) either alone or in a combination of both. Data are shown as average percentages from at least three replicated cultures. The letters "a–d" denote significantly different values ($P < 0.05$).

of OGCs when administered singly, but the combination of GDF9 and BMP15 increased the integrity of OGCs. The integrity of OXCs and GCs, both of which lacked oocytes, decreased markedly at Day 5 (Figs. 3B and 3C). However, either GDF9 or BMP15 alone maintained the integrity of OXCs, while the two in combination increased OXC integrity more than either growth factor administered singly (Fig. 3B). In GCs, neither GDF9 nor BMP15 alone increased integrity, but the combination of both increased the integrity significantly (Fig. 3C).

Effect of GDF9 and BMP15 on the formation of antrum-like structures by complexes

The formation of antrum-like structures by the complexes is shown in Figs. 4 and 5. OGCs developed antrum-like structures after incubation without supplementation of GDF9 and BMP15 (Fig. 4A), whereas OXCs and GCs did not form antrum-like structures without growth factors (Figs. 4B and 4C). GDF9 and BMP15 alone induced formation of antrum-like structures in OXCs and GCs, but

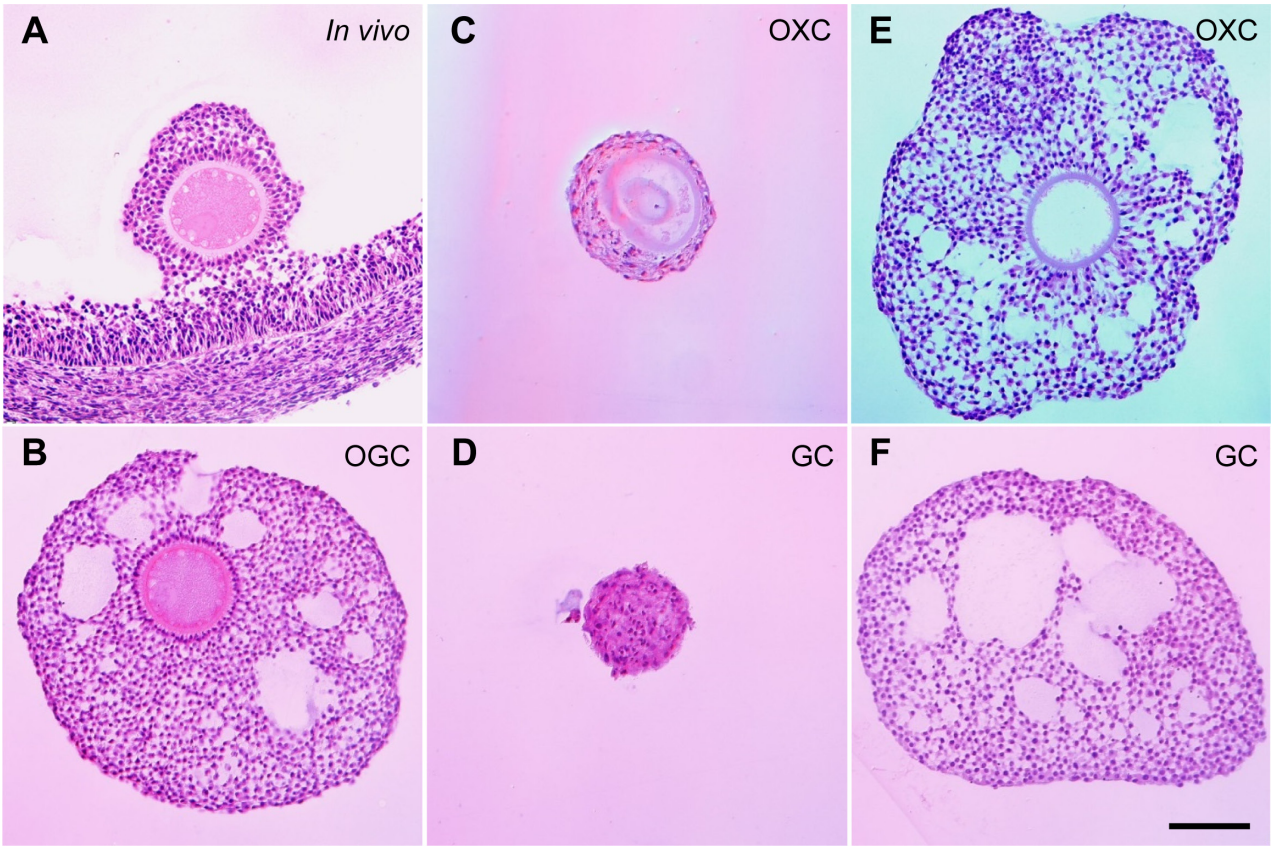

Fig. 5. Representative images of histological sections of complexes (B: OGCs; C and E: OXCs; and D and F: GCs) after 5 days of culture. There is an antrum inside the *in vivo*-grown bovine early antral follicle (1.2–1.8 mm in diameter) (A). OGCs developed antrum-like structures *in vitro* (B). OXCs (C) and GCs (D) did not develop antrum-like structures *in vitro*. However, OXCs (E) and GCs (F) developed antrum-like structures when they were cultured with GDF9 and BMP15. The scale bar represents 100 µm.

the combination of GDF9 and BMP15 achieved even more potent promotion of the development of antrum-like structures in all types of complexes (Fig. 4).

Representative histological images of antrum-like structures in the complexes are shown in Fig. 5. The *in vivo* grown follicles (1.2–1.8 mm) used as controls contained large antra (Fig. 5A). Cultured OGCs formed multiple spaces (antrum-like structures) between the oocytes and the peripheries of the complexes (Fig. 5B). Upon completion of incubation, OXCs contained the zona pellucida surrounded by granulosa cells, although no antrum-like structures were formed inside the complexes (Fig. 5C). Similarly, GCs did not develop any antrum-like structures without growth factors (Fig. 5D). However, GDF9 and BMP15 induced OXCs (Fig. 5E) and GCs (Fig. 5F) to form multiple antrum-like structures inside the complexes, which were similar to those in the OGCs grown without growth factors (Fig. 5B).

Effect of GDF9 and BMP15 on granulosa cell morphology

OGCs, OXCs and GCs were stained with hematoxylin and eosin after five days of incubation to examine the morphology of granulosa cells. Because the masses of granulosa cells in the original complexes were heavily stained, we could not observe the shape of individual granulosa cells. However, granulosa cells outgrowing from the complexes showed distinctive shapes according to the type of complex and/or treatment. Granulosa cells adjacent to the OGC were pebble-like in shape (Fig. 6A), whereas cells more distant from the original complex exhibited spreading into fibroblast-like shapes with large nuclei and flat cytoplasm (Fig. 6B). Between these two types of cells was another cell-type with a rhomboid shape and long cellular projections (Fig. 6C). Outgrowing granulosa cells from OGCs showed these three types of cells without growth factors (Fig. 7A). In the absence of oocytes and exogenous growth factors in OXCs and GCs, granulosa cells exhibited only fibroblast-like shape and spread extensively from the complexes (Figs. 7B and 7C). OXCs and GCs cultured with GDF9 showed both fibroblast-like and rhomboid-shaped cells, although the complexes cultured with BMP15 alone showed only fibroblast-like cells. However, the combination of GDF9 and BMP15 reduced fibroblast-like cells, while rhomboid-shaped and pebble-like cells were observed in all types of complexes.

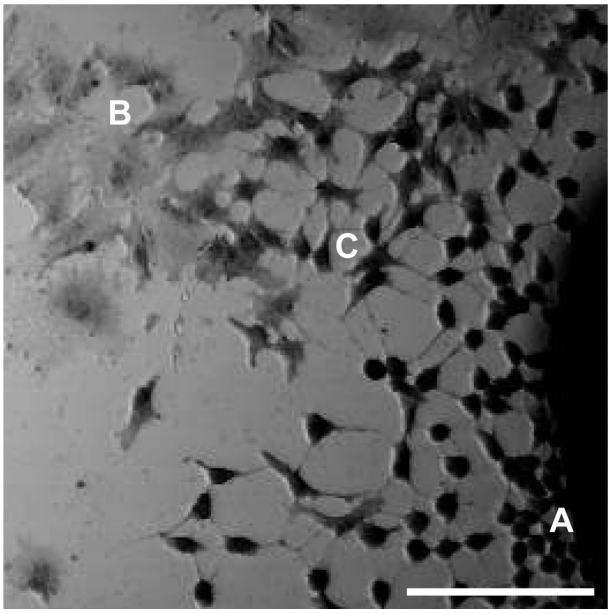

Fig. 6. A representative image of distinctive shapes of outgrowing granulosa cells from OGCs cultured with GDF9. Outgrowing granulosa cells exhibited pebble-like shape (A), fibroblast-like shape (B), or rhomboid shape with long cell projections (C). The scale bar represents 100 µm.

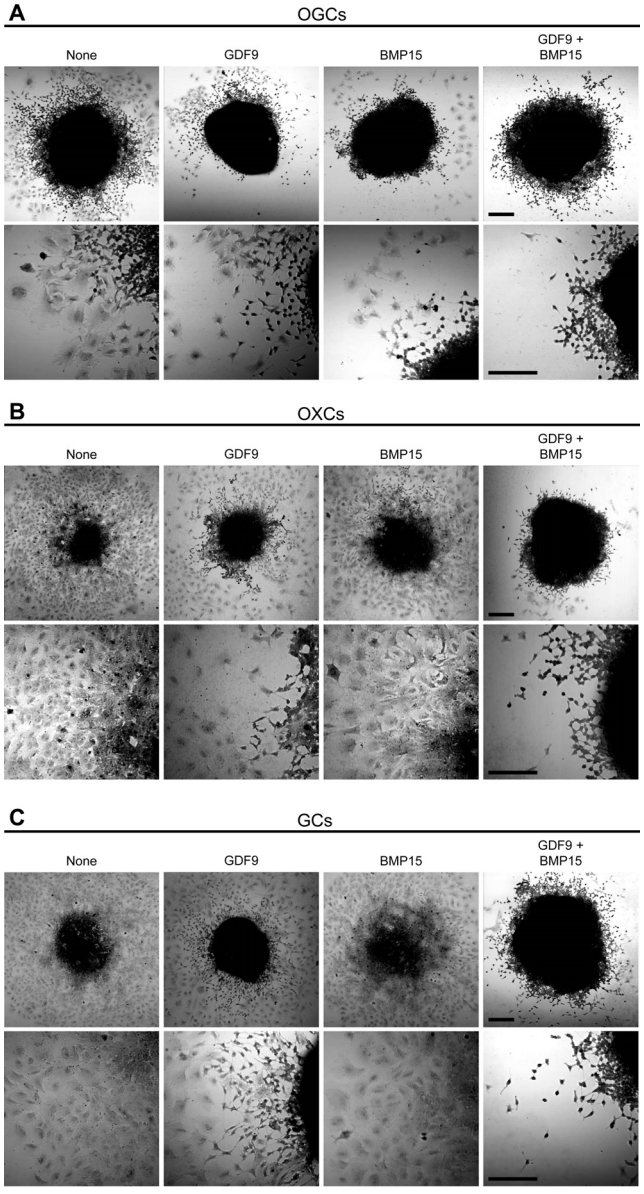

Fig. 7. Typical morphologies of outgrowing granulosa cells from complexes (A: OGCs; B: OXCs; and C: GCs) after 5 days of incubation. The complexes were cultured with GDF9 (100 ng/ml), BMP15 (10 ng/ml), or a combination of GDF9 and BMP15. Complexes cultured without GDF9 or BMP15 are represented as "None". The scale bars represent 200 µm.

Discussion

The mechanisms regulating follicular antrum formation have received scant attention [29, 30] and the role of oocytes in particular has not been sufficiently elucidated. One study suggested that oocytes are required for antrum formation in pigs [8]. In that study, oocyte-cumulus-granulosa cell complexes (OCGs) from pig early antral follicles formed antrum-like structures when cultured in collagen gels for eight days. When the oocytes surrounded by cumulus cells in OCGs were replaced by denuded oocytes (O/G complexes) or Sephadex G-25 beads (B/G complexes), the O/G complexes formed antrum-like structures, whereas the B/G complexes did not. Hirao *et al*. developed a culture system for growing OGCs collected from bovine early antral follicles (0.4–0.7 mm) in 4% polyvinylpyrrolidone for 14 days; the complexes developed antrum-like structures in this culture system [24]. In our recent report using similar culture conditions, the inhibition of oocyte-specific PDE3A promoted the formation of antrum-like structures by bovine oocyte-cumulus complexes [31], which further indicated the engagement of oocytes in the process of antrum formation.

The present study showed that *GDF9* and *BMP15* mRNAs from bovine early antral follicles were expressed only in oocytes, but not in granulosa cells. The oocyte-specific expression of these mRNAs has been reported previously in several mammalian species, including mice [9], cattle [32], sheep [32], and humans [33]. GDF9 and BMP15 have been shown to regulate several follicular events [34]. GDF9-deficient female mice form primordial and primary follicles, but they exhibit a block in follicular development beyond the primary follicle stage, leading to infertility [11]. Buccione *et al*. found that oocytes were required for cumulus expansion during oocyte maturation through the secretion of a putative cumulus expansion-enabling factor [6]. Later, oocyte-derived GDF9 and BMP15 were identified as the cumulus expansion-enabling factors in mice [12, 35]. We have shown here that bovine growing oocytes participate in the formation of antrum-like structures and that GDF9 and BMP15 are able to substitute for this oocyte role. Antrum-like structures were formed in OGCs due to the presence of oocytes while GDF9 and

BMP15 also promoted the formation of antrum-like structures in OGCs. Moreover, OXCs and GCs, both of which lack oocytes, did not form antrum-like structures, but GDF9- and BMP15-induced formation of antrum-like structures was observed in these complexes. These results provide clear evidence that oocyte-derived factors are required for the formation of antrum-like structures in bovine OGCs and suggest that oocytes participate in follicular antrum formation via GDF9 and BMP15.

The OGCs used in this study contained oocytes and surrounding cumulus granulosa cells. We cannot rule out the possibility that the complexes are the mixture of cumulus granulosa cells and some parietal granulosa cells. The complexes might also contain undifferentiated granulosa cells. The ability of parietal granulosa cells to form an antrum structure has been examined in the pig [8]. In the experiment, parietal granulosa cells formed an antrum-like structure in the presence of denuded oocytes [8]. The role of oocytes in the proliferation of (i) undifferentiated granulosa cells from preantral follicles and (ii) more differentiated mural granulosa cells and cumulus granulosa cells from antral follicles was studied in mice [36]. Oocyte-secreted factors promoted proliferation of undifferentiated granulosa cells and differentiated mural granulosa cells but did not promote the proliferation of terminally differentiated cumulus cells [36]. These observations indicated that oocyte-secreted factors act on granulosa cells only at specific stages of their differentiation [36]. In the present study, we demonstrated that GDF9 and BMP15 might act on undifferentiated granulosa cells or parietal granulosa cells in the complexes to make multilayered structures and promote the formation of antrum-like structures.

Outgrowing granulosa cells from bovine OGCs appeared fibroblast-like, rhomboid, and pebble-like in culture. Granulosa cells from OXCs and GCs cultured without growth factors or with BMP15 alone showed only a fibroblast-like shape. Cultured rat granulosa cells contiguous to oocytes maintained their granulosa-like appearance, while those farthest away from oocytes showed a fibroblast-like shape [37]. Rat granulosa cells cultured without oocytes differentiated into a fibroblast-like shape [37]. In the present study, the bovine granulosa cells were thought to differentiate into fibroblast-like cells without oocytes. When OXCs and GCs were cultured with GDF9, the granulosa cells appeared fibroblast-like and rhomboid-shaped. The culture of these complexes with GDF9 and BMP15 reduced the fibroblast-like cells, similar to the OGCs cultured without GDF9 and BMP15. Moreover, the rhomboid cells were connected to each other by long, thin cytoplasmic projections resembling filopodia. A previous morphodynamics study of oocyte-follicle cell association during the development of human ovarian follicles revealed rhomboid-shape cumulus cells with microvilli and cilia in the antral follicles [38]. A recent study indicated that GDF9 from mouse growing oocytes induced granulosa cells to generate specialized filopodia, which penetrated the oocytes and provided a foundation for oocyte-granulosa cell communication [39]. Together with these reports, our present results suggest that GDF9 might promote the generation of filopodia in outgrowing granulosa cells, changing the morphology of granulosa cells to a rhomboid shape. OXCs and GCs did not maintain complex integrity during the 5-day incubation, although addition of GDF9 and BMP15 maintained the integrity of these complexes. The connection between granulosa cells whose growth was induced by GDF9 and BMP15 seems to contribute to the maintenance of complex integrity.

In summary, we have shown that GDF9 and BMP15 are required for the formation of antrum-like structures by bovine granulosa cells, thereby suggesting that oocytes participate in follicular antrum formation through these oocyte-derived factors.

Acknowledgement

We thank Dr Yuji Hirao at the NARO Institute of Livestock and Grassland Science, Tsukuba, Japan for his valuable suggestions. We are grateful to the staff of Kobe-Branch, Animal Biotechnology Center, Livestock Improvement Association of Japan; for the provision of bovine ovaries. This work was supported in part by a Japan Society for the Promotion of Science KAKENHI grant (17K08137) to TM.

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