Original Research Paper

Title: Relationship between *in vitro* growth of bovine oocytes and steroidogenesis of granulosa cells cultured in medium supplemented with bone morphogenetic protein-4 and follicle stimulating hormone

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Abstract

Bone morphogenetic protein-4 (BMP-4) and FSH play important regulatory roles in follicular growth and steroidogenesis *in vivo*. The purpose of this study was to investigate the effects of BMP-4 and FSH on *in vitro* growth (IVG) and steroidogenesis of bovine oocyte-cumulus-granulosa complexes (OCGCs). We cultured OCGCs collected from early antral follicles (0.5-1 mm) in medium without BMP-4 and FSH for 4 days and investigated the appearance of OCGCs and their steroidogenesis. During the first 4 days of IVG, morphologically normal OCGCs produced more estradiol-17β (E_2), but less progesterone (P_4). Morphologically normal OCGCs were subjected to an additional culture in medium supplemented with BMP-4 (0, 10, and 50 ng/mL) and FSH (0 and 0.5 ng/mL) until day 12. We examined the viability and steroidogenesis of OCGCs after 8 and 12 days of culture. Oocyte growth, characteristics of granulosa cells, and the maturational competence of oocytes were also investigated. On day 8, the viability of OCGCs cultured without FSH was higher in the 10 ng/mL BMP-4 group than in the 50 ng/mL BMP-4 group ($P<0.05$). No significant difference was observed in the viability of groups cultured with FSH, regardless of the addition of BMP-4, and FSH improved the viability of 50 ng/mL BMP-4 group similar to 10 ng/mL BMP-4 group. The total number of granulosa cells was larger in 10 ng/mL BMP-4 group cultured with FSH than in 50 ng/mL BMP-4 group cultured with FSH on day 8 ($P<0.05$). E_2 production decreased from days 8 to 12, and P_4 production increased throughout IVG culture, regardless of the addition of BMP-4 and FSH ($P<0.05$). No significant differences in E_2

production were observed between groups from days 4 to 8, regardless of whether BMP-4 was added without FSH; however, E_2 production in the group cultured with 50 ng/mL BMP-4 was suppressed by FSH. BMP-4 suppressed E_2 production from days 8 to 12, regardless of whether FSH was added. The group cultured with 10 ng/mL BMP-4 without FSH showed the lowest P_4 production among all groups for all culture periods. OCGCs that produced mature oocytes tended to secrete more E_2 and less P_4 than OCGCs that produced immature oocytes. In conclusion, until day 8 of the IVG culture, P_4 production by OCGCs was suppressed by the addition of 10 ng/mL BMP-4 in the absence of FSH, without inhibiting E_2 production. These conditions appear to mimic growing follicles until day 8 and mimic degenerating follicles from days 8 to 12 of culture.

Keywords: BMP-4; Early antral follicle; FSH; *In vitro* growth; Maturational competence; Steroidogenesis.

1. Introduction

A large number of primordial follicles exist in mammalian ovaries, and granulosa cells multiply and oocytes become developmentally competent as they grow. However, most follicles degenerate during follicular growth, and only a small proportion of follicles develop sufficiently to undergo ovulation [1, 2]. If it is possible to develop a culture system that enables early stage oocytes or follicles to grow to the ovulatory stage, more embryos may be produced and an experimental model may also be established to investigate the mechanisms underlying follicular recruitment, selection, and ovulation. In mice, tissue culture of neonatal ovaries combined with *in vitro* growth (IVG) culture of oocyte-cumulus-granulosa complexes (OCGCs) allows pups to be produced from primordial follicles *in vitro* [3, 4]. However, in cattle, no studies have currently reported the production of calves from follicles at stages earlier than preantral follicles. Previous studies described bovine calves derived from IVG oocytes that originated from OCGCs in early antral follicles [5, 6, 7]; however, their developmental competence to transferable embryos was lower than the competence of *in vivo* matured oocytes [5-13].

The primary roles of follicular cells are to support the growth and maturation of oocytes, as well as the production of sex steroid hormones by granulosa cells. Therefore, in order to mimic *in vivo* follicular growth via an *in vitro* culture system, the growth of oocytes and production of sex steroid hormones by granulosa cells both need to be investigated. A previous study that combined histological observations with the measurement of sex steroid hormones in follicular fluid revealed

70 that estradiol-17β (E_2) concentrations in growing follicles increased as the follicles grew, with a peak
71 at estrus in cattle, and the degeneration of follicles led to increases in progesterone (P_4)
72 concentrations [14]. Furthermore, E_2 concentrations in dominant follicles increased concomitantly
73 with follicular development and the E_2/P_4 ratio also increased; however, subordinate follicles showed
74 low E_2/P_4 ratios [14, 15]. These findings indicate that a culture system of OCGCs that produces
75 more E_2 and less P_4 is needed to mimic *in vivo* dominant follicular development. In conventional
76 IVG, serum has typically been added to culture media to promote cell growth and survival [5, 6, 7].
77 However, granulosa cells cultured in media containing serum luteinize, compromise E_2 production,
78 and begin to produce P_4 [16, 17]. Previous studies attempted to culture OCGCs in serum-free
79 media [18, 19]; however, the oocytes derived from serum-free cultures had low maturational
80 competence and low fertilizability. Thus, a culture system for OCGCs that produces oocytes with
81 high developmental competence and inhibits the luteinization of granulosa cells under culture
82 conditions containing serum needs to be developed. As shown in our previous study, the addition
83 of bone morphogenetic protein-4 (BMP-4) to the growth medium inhibited the luteinization of
84 granulosa cells [20].

85 BMP-4 is produced by theca cells in bovine follicles, and its receptor is primarily
86 expressed in oocytes and granulosa cells [21]. An *in situ* hybridization analysis of rat ovaries
87 revealed that the expression level of BMP-4 increases during follicular growth [22]. Based on
88 previous studies of cultured granulosa cells without oocytes, BMP-4 promotes E_2 production by

89 inhibiting apoptosis [23] and promoting aromatase (P450arom) activity in cattle [21]. In addition,
90 P_4 production is inhibited by the suppression of steroidogenic acute regulatory protein (StAR) in
91 cattle [24] and sheep [25] and cholesterol side chain cleaving (P450scc) in sheep [25] at the
92 messenger RNA (mRNA) and protein levels. Moreover, BMP-4 promotes the FSH-mediated
93 activation of E_2 production, which is increased in the presence of oocytes [26]. On the other hand,
94 P_4 production was inhibited in a manner independent of the presence of oocytes in an *in vitro* study
95 of rat granulosa cells [26]. Therefore, the addition of BMP-4, which compensates for the lack of
96 theca cells, and FSH to the medium of IVG for bovine OCGCs may promote oocyte growth by
97 promoting E_2 production and inhibiting P_4 production. According to our recent report, the addition
98 of BMP-4 (10 ng/mL) to an IVG culture suppressed P_4 production and did not affect oocyte growth,
99 nuclear maturation, or fertilization, but impaired subsequent embryonic development and, at a higher
100 concentration (50 ng/mL), even compromised the viability of OCGCs by suppressing the
101 proliferation of granulosa cells [20]. In that study [20], we cultured OCGCs in medium that
102 contained high concentrations of E_2 (1 μg/mL) to increase the E_2/P_4 ratio similar to a dominant
103 follicle; therefore, we were unable to correctly investigate the effects of BMP-4 and FSH on the
104 steroidogenesis of granulosa cells. In the present study, we added BMP-4 and FSH to the growth
105 medium without the addition of E_2 and examined the production of sex steroid hormones from
106 individually cultured OCGCs. We also retrospectively analyzed the correlation between the
107 steroidogenesis of OCGCs during the IVG culture and the nuclear maturation of the corresponding

oocytes.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise.

2.2. Collection of OCGCs and the IVG culture

Bovine ovaries obtained from a local abattoir were stored in plastic bags at 20°C and transported to the laboratory within 6-10 h of their collection. After the ovaries were washed three times with physiological saline, slices of ovarian cortex tissues (< 1 mm thickness) were prepared using a surgical blade (no. 11) and stored in tissue culture medium 199 (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium, pH 7.4) at 37 °C, as described elsewhere [8]. Under a stereomicroscope, early antral follicles (0.5 to 1 mm in diameter) were dissected from sliced ovarian tissues using a surgical blade (no. 20) and fine forceps in a 90-mm petri dish that had a 1-mm^2 scale on its bottom (FLAT Co., Ltd., Chiba, Japan). OCGCs were isolated from early antral follicles using a pair of fine forceps and subjected to IVG as previously described [27]. The growth

127 medium was HEPES-buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 5% (v/v)
128 fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (MW
129 360,000), 50 μg/mL ascorbic acid 2-glucoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan),
130 55 μg/mL cysteine, 50 μg/mL gentamicin sulfate, and 10 ng/mL androstenedione (A_4) as a precursor
131 for E_2 [11]. OCGCs with oocytes surrounded by a cumulus investment and an attached mural
132 granulosa-cell layer (Fig. 1) were cultured individually in a 96-well culture plate (Primaria 353872,
133 Corning Life Sciences, Tewksbury, MA, USA) with 200 μL of growth medium at 39 °C for 12 days
134 in humidified air with 5% CO_2. At the onset of the IVG culture, OCGCs were photographed under
135 an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached CCD camera (Moticam
136 2000, Shimadzu Rika Corporation, Tokyo, Japan). The diameters of the oocytes were assessed
137 using software (Motic Images Plus 2.2s, Shimadzu). Every 4 days of the IVG culture, half (100 μL)
138 of the growth medium was replaced with the same amount of fresh medium. Spent media collected
139 at 4, 8, and 12 days of culture were stored at -30 °C until steroid hormone assays were conducted.
140
141 **2.3. Evaluation of OCGC morphology**
142 After 4 days of IVG culture, the morphological appearance of OCGCs was examined (Fig.
143 1). OCGCs with an evenly granulated ooplasm that were completely enclosed by several layers of
144 healthy cumulus and granulosa cells were defined as normal. When OCGCs had oocytes with an
145 abnormal appearance and/or oocytes were denuded by scattering cumulus and granulosa cells,

OCGCs were defined as abnormal.

2.4. Evaluation of the characteristics of granulosa cells

The total number, viability, and diameter of granulosa cells after growth culture from morphologically normal OCGCs on days 8 and 12 were assessed using an acridine orange/propidium iodide cell viability kit together with a cell counter (F23001 and L2000, respectively; Logos Biosystems, Gyunggi, Republic of Korea) as previously described [20]. The culture medium in the well of each viable OCGC was removed and replaced with 80 μL of Dulbecco's PBS without calcium and magnesium (DPBS) supplemented with 0.125% trypsin and 0.05% EDTA to prepare the granulosa cells for counting. After 10 min of trypsinization and pipetting several times, 20 μL of FCS were added to stop the digestion. Denuded oocyte was removed from the well and discarded.

2.5 Evaluation of nuclear status of oocyte after IVG and IVM

After 12 days of IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs by aspiration using a fine glass pipette. Immediately after IVG, some of the oocytes were denuded from cumulus cells by individually pipetting, and fixed in 10 μL of DPBS containing 60% (v/v) methanol in each well of micro-well plates (Mini Trays 163118, NUNC, Roskilde, Denmark) for 30 min. After fixation, oocytes were stained with 5 μg/mL Hoechst 33342 in 10 μL of DPBS in each well of the micro-well plate at 37 °C

for 15 min in the dark, as described elsewhere [28]. The nuclear status was then evaluated under an inverted fluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) using a UV filter (excitation 334–365 nm) to confirm meiotic arrest. Oocytes with a nuclear envelope were defined as being in the germinal vesicle stage. Other oocytes were submitted to individual IVM as previously described [29]. Oocytes surrounded by several layers of cumulus cells were washed with IVM medium, which consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 20 μg/mL FSH, 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate. IVM of oocytes was performed in each well of micro-well plates filled with 6 mL of IVM medium at 39 °C under 5% CO_2 in air for 22 h. After IVM, oocytes were denuded from cumulus cells by individually pipetting, photographed, and their diameters measured. Oocytes were mounted individually on a slide glass and fixed with a mixture of acetic acid and ethanol (1:3) for 6 h. After fixation, oocytes were stained with 1% (w/v) aceto-orcein and the status of their nuclei was examined under a phase contrast microscope, as described elsewhere [30]. Oocytes that reached metaphase II and had a polar body were defined as mature; oocytes with other nuclear statuses were defined as immature.

2.6. E_2 and P_4 assays

Spent media (100 μL) from IVG cultures were assayed to determine the E_2 and P_4 concentrations using a competitive double antibody enzyme immunoassay, as previously described

184 [31]. Samples were subjected to 2- to 2000-fold serial dilutions with assay buffer (145 mM NaCl,
185 40 mM Na_2HPO_4, and 0.1% BSA (w/v), pH 7.2). Diluted samples (20 μL) were incubated with
186 the primary antisera and HRP-labeled hormone (100 μL each) in the wells of a 96-well microplate
187 (Costar 3590, Corning, NY, USA) coated with the secondary antiserum for 16–18 h at 4 °C. The
188 primary antisera used for the E_2 and P_4 assays were anti-estradiol-17β-6-CMO-BSA (QF-121,
189 Teikoku Hormone Mfg. Co. Ltd., Kanagawa, Japan) and anti-progesterone-3-CMO-BSA
190 (7720-0504, Biogenesis, Poole, England), respectively. Goat anti-rabbit serum (270335,
191 Seikagaku, Tokyo, Japan) was used as the secondary antiserum. After washing all wells four
192 times with 300 μL of washing buffer (0.05% Tween 80), 150 μL of TMB solution (5 mM citric acid,
193 50 mM Na_2HPO_4, 500 mM UHP, 1 mM TMB, and 2% DMSO) were added to each well and
194 incubated at 37 °C for 40 min. The absorbance of the solution in the wells was measured at 450
195 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan) after stopping the
196 chromogenic reaction with 50 μL of 4 N H_2SO_4. All samples were assayed in triplicate. Assay
197 sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and intra-assay
198 coefficients of variation were 15.8 and 4.0% for E_2 and 17.5 and 3.9% for P_4, respectively.
199
200 **2.7. Experimental design**
201 **2.7.1. Morphology and steroidogenic capacity of OCGCs**
202 OCGCs were cultured in growth medium without BMP-4 or FSH for 4 days to select

healthy OCGCs that exhibited steroidogenic activity similar to developing follicles *in vivo* (Fig. 2). The correlation between the morphological normality of OCGCs and the production of steroid hormones was evaluated by measuring the concentrations of E_2 and P_4 in spent media. OCGCs defined as normal were subjected to subsequent experiments.

2.7.2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa cells, growth and maturation of oocytes, and steroidogenesis

Normal OCGCs were randomly divided into six groups after 4 days of IVG culture to evaluate the effects of BMP-4 and FSH on the functions of OCGCs. We cultured OCGCs for an additional 8 days (a total of 12 days of IVG culture) in growth medium supplemented with different concentrations of BMP-4 (0, 10, or 50 ng/mL; HZ-1045, Humanzyme, Chicago, IL, USA) and FSH (0 or 0.5 ng/mL) (Fig. 2). The concentrations of BMP-4 and FSH were determined according to previous studies in which granulosa cells were cultured *in vitro* [23, 25, 32]. In these studies, 10 and 50 ng/mL BMP-4 promoted cell viability [23] and suppressed P_4 production [25], whereas 0.5 ng/mL FSH enhanced the expression of the *P450arom* mRNA and E_2 production [32]. After 8 and 12 days of IVG culture, the viability of OCGCs was defined by the normality of their morphological appearance (Fig. 1); *i.e.*, morphologically normal OCGCs were defined as having survived, whereas morphologically abnormal OCGCs were defined as dead. In addition, antrum formation in the granulosa cell layer was noted on day 12 of the IVG culture [6]. On day 12 of the IVG culture, 233 oocytes that were judged as having survived were subjected to IVM and nuclear maturation was

examined (5 to 20 oocytes/replicate). However, 12 oocytes were accidently collapsed during the pipetting procedures for denudation from cumulus cells. Other oocytes that were not subjected to IVM were used to evaluate meiotic arrest immediately after the IVG culture. *In vivo*-derived oocytes collected from antral follicles of 2-8 mm in diameter were also subjected to IVM and served as *in vivo* controls. Oocyte growth was evaluated as the difference in oocyte volume calculated based on diameters [33] before the IVG culture and after IVM.

The concentrations of E_2 and P_4 in spent media collected on days 8 and 12 of the IVG culture were measured to evaluate the steroidogenesis of OCGCs that survived for the 12 days of IVG culture. Steroid hormone production during each period (days 4 to 8 and days 8 to 12) was calculated using the following formula:

Steroid hormone production (ng) = 0.2 (mL) × Concentration at the end of the period (ng/mL)

− 0.1 (mL) × Concentration at the start of the period (ng/mL)

Three hundred forty-eight OCGCs were cultured to evaluate the characteristics of the granulosa cells, and the total number, viability, and diameter of granulosa cells from OCGCs that had survived on day 8 (90 OCGCs) and day 12 (93 OCGCs) were assessed.

OCGCs were divided based on whether an antrum had formed or not and whether OCGCs produced mature or immature oocytes to evaluate the relationship between OCGC characteristics and steroidogenesis. The relationships between these factors and steroidogenesis (the production of E_2 and P_4 and the E_2/P_4 ratio) were then retrospectively examined.

241

242 **2.8. Statistical analysis**

243 All statistical analyses were performed using software (JMP version 10, SAS Institute,
244 Cary, NC, USA and StatView 4.51, Abacus Concepts, Inc., Calabasas, CA, USA). Differences in
245 steroid hormone production from OCGCs which had normal or abnormal appearance were analyzed
246 using Student's *t*-test. The effects of BMP-4 and FSH on the viability of OCGCs and the nuclear
247 maturation of IVG oocytes were analyzed using a chi-square test. The differences in oocyte
248 volumes between experimental groups cultured with the same FSH concentrations were analyzed
249 using Tukey-Kramer's HSD test. Volumes of oocytes cultured with the same BMP-4
250 concentrations were analyzed using Student's *t*-test. Oocyte volumes measured before and after
251 IVG were compared using Dunnett's test. The volume before IVG served as a control. The
252 differences in the production of steroid hormones and the characteristics of granulosa cells between
253 experimental groups within the same culture period were compared using a one-way ANOVA
254 followed by Tukey-Kramer's HSD test. The differences in steroid production and the
255 characteristics of granulosa cells between days 4 to 8 and days 8 to 12 of culture were analyzed
256 using Student's *t*-test. In the retrospective analysis, the relationship between antrum formation and
257 steroid production was analyzed using Student's *t*-test. The characteristics of OCGCs (oocyte
258 volume and steroid production) between OCGCs that produced mature and immature oocytes were
259 compared using Student's *t*-test. The difference in antrum formation between OCGCs that

produced mature and immature oocytes was analyzed using a chi-square test.

3. Results

3.1. Morphology and steroidogenic capacity of OCGCs

Nine hundred eight OCGCs (8 to 60 OCGCs/replicate) were cultured in growth medium without the addition of BMP-4 or FSH. After 4 days of IVG culture in the absence of both BMP-4 and FSH, 93.7% (851/908) of OCGCs had a normal appearance. E_2 and P_4 levels were then measured in culture media collected from 475 OCGCs with a normal appearance and 10 OCGCs with an abnormal appearance. As shown in Fig. 3, OCGCs with a normal appearance produced more E_2 ($P < 0.01$) and less P_4 ($P < 0.01$) than abnormal OCGCs.

3.2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa cells, growth and maturation of oocytes, and steroidogenesis

Eight hundred fifty-one OCGCs were cultured in media containing BMP-4 (0, 10, or 50 ng/mL) and FSH (0 or 0.5 ng/mL), and 718 OCGCs were cultured until day 12; the remaining 133 OCGCs were cultured until day 8 to evaluate the characteristics of granulosa cells. The viabilities of OCGCs on days 8 and 12 of IVG culture are shown in Table 1. After 8 days of culture, the group cultured with 10 ng/mL BMP-4 in the absence of FSH showed higher viability than the group cultured with 50 ng/mL BMP-4 in the absence of FSH ($P < 0.05$). In the presence of 50 ng/mL

279 BMP-4, the group cultured with 0.5 ng/mL FSH showed higher viability than the group cultured
280 without FSH on days 8 ($P < 0.05$). The viability of the group cultured with 10 ng/mL BMP-4 and
281 0.5 ng/mL FSH was similar between days 8 and 12; however, the viability of other groups decreased
282 from days 8 to 12. As shown in Fig. 4, on day 8, the group cultured with 10 ng/mL BMP-4 and 0.5
283 ng/mL FSH showed the highest total number of granulosa cells (approximately 68,000 cells), which
284 was larger than that in the group cultured with 50 ng/mL BMP-4 and 0.5 ng/mL FSH (approximately
285 42,500 cells, $P < 0.05$). Also, the number was slightly larger than the group cultured with 10 ng/mL
286 BMP-4 in the absence of FSH (approximately 47,000 cells, $P = 0.06$). On day 12, no significant
287 differences were observed in the total number of granulosa cells between the experimental groups.
288 The total numbers of granulosa cells observed on days 8 and 12 were similar in the same
289 experimental groups. The viability of granulosa cells on day 8 was approximately 100% in all
290 experimental groups. On day 12, viability was greater than 90% in all groups and small differences
291 were observed between the experimental groups. The highest viability was observed for cells
292 cultured with 50 ng/mL BMP-4 in the absence of FSH (98.9%), and the lowest viability was
293 observed for cells cultured with 50 ng/mL BMP-4 and FSH (92.5%). The addition of FSH to the
294 groups cultured with 10 ng/mL BMP-4 (0 ng/mL FSH, 11.5 μm; 0.5 ng/mL FSH, 12.4 μm; $P < 0.01$)
295 and 50 ng/mL BMP-4 (0 ng/mL FSH, 11.1 μm; 0.5 ng/mL FSH, 12.0 μm; $P = 0.05$) increased the
296 diameters of granulosa cells measured on day 8; however, no significant differences were observed
297 between experimental groups on day 12.

298 The mean volumes of oocytes in all groups before the IVG culture were similar; therefore, the
299 mean values of all groups were used as controls prior to the IVG culture. As shown in Fig. 5, the
300 mean volumes of oocytes in all groups were larger after IVG culture than before IVG ($P < 0.05$). In
301 addition, the mean volumes of oocytes were similar in the groups cultured with 0 and 10 ng/mL
302 BMP-4 regardless of whether FSH was added following IVM. However, in the groups cultured
303 with 50 ng/mL BMP-4, oocyte volumes were larger in the group cultured without FSH than in the
304 group cultured in the presence of FSH ($P < 0.05$).
305 Two hundred thirty-three oocytes were studied to evaluate effects of BMP-4 and FSH on
306 nuclear maturation. Immediately after IVG, before IVM culture, all oocytes were arrested at the
307 stage of the germinal vesicle. The results are summarized in Table 2. After the IVM culture, only
308 oocytes derived from OCGCs cultured with 50 ng/mL BMP-4 in the absence of FSH showed a
309 maturation rate similar to oocytes grown *in vivo*, whereas oocytes derived from other cultures had
310 lower rates of nuclear maturation compared to the *in vivo* control ($P < 0.05$).
311 E_2 and P_4 production were evaluated in 364 cultures with BMP-4 (0, 10, or 50 ng/mL) and
312 FSH (0 or 0.5 ng/mL), and results are shown in Fig. 6. E_2 was produced at a lower level from days
313 8 to 12 than from days 4 to 8, whereas P_4 production increased in all groups during culture ($P < 0.05$).
314 From days 4 to 8, E_2 production did not significantly differ between the groups cultured without
315 FSH, regardless of the presence or absence of BMP-4. In the presence of FSH, production
316 decreased in the group cultured with 50 ng/mL BMP-4 ($P < 0.01$). Additionally, the addition of

317 FSH to the groups cultured with 0 and 10 ng/mL of BMP-4 increased P_4 production from days 4 to 8
318 ($P < 0.05$). Between days 8 and 12, the addition of BMP-4 decreased E_2 production, regardless of
319 whether FSH was added ($P < 0.05$). FSH decreased E_2 production in the group cultured with 50
320 ng/mL BMP-4 ($P < 0.01$). The group cultured with 10 ng/mL BMP-4 in the absence of FSH
321 exhibited the lowest production of P_4, regardless of the culture period.

322 After 12 days of culture, 364 OCGCs were evaluated for antrum formation, which was
323 observed in 96 OCGCs. As shown in Table 3, OCGCs with antra on day 12 produced larger
324 amounts of E_2 from days 4 to 8 ($P < 0.01$) and smaller amounts of P_4 in both periods than OCGCs
325 without an antrum ($P < 0.01$). As shown in Table 4, oocytes that achieved nuclear maturation had
326 larger diameters than oocytes without nuclear maturation ($P < 0.01$) after the IVM culture. In
327 addition, OCGCs that produced mature oocytes generated slightly larger amounts of E_2 ($P = 0.10$)
328 and less P_4 ($P = 0.09$) between days 4 and 8 than OCGCs that produced immature oocytes.
329 Although no significant differences were observed in the mean values obtained for E_2 and P_4
330 production from days 8 to 12, the E_2/P_4 ratio varied markedly. E_2 and P_4 production varied in
331 OCGCs that generated mature oocytes throughout the duration of the IVG culture. Some of the
332 oocytes that achieved nuclear maturation showed markedly high E_2/P_4 ratios. No obvious
333 relationship was observed between the nuclear maturation of IVG oocytes and antrum formation in
334 the granulosa cell layer.
335

4. Discussion

In the present study, OCGCs that had a normal appearance 4 days after the initiation of the IVG culture produced a large amount of E_2 and less P_4 during the initial period of the IVG culture. During the *in vivo* development of a dominant follicle, the E_2 concentration increases as follicles grow [14, 15]. Thus, we only used healthy OCGCs in subsequent experiments to evaluate the effects of BMP-4 and FSH on steroidogenesis and oocyte maturation.

On day 8 of the IVG culture, the viability of the group cultured with 10 ng/mL BMP-4 was higher than the group cultured with 50 ng/mL BMP-4 ($P < 0.05$) when OCGCs were cultured without FSH. The low viability of OCGCs cultured with 50 ng/mL BMP-4 may stem from a decrease in granulosa cell numbers, as we reported previously [20]. Actually, the lowest number of granulosa cells was observed on day 8 in the group cultured with 50 ng/mL BMP-4 (approximately 41,000 cells/well) among all groups tested (approximately 47,000 cells/well) in the absence of FSH, although the values were not significantly different. On the other hand, when 0.5 ng/mL FSH was added to growth medium, the viability of OCGCs in the group cultured with 50 ng/mL BMP-4 improved on days 8 and 12. In the present study, we only examined OCGCs that had survived; therefore, we could not find the increase of granulosa cell number. We speculate that the result may be attributed to the increased proliferation of granulosa cells by the addition of 0.5 ng/mL FSH from days 4 to 8 [34], and was particularly apparent in the group cultured with 10 ng/mL BMP-4 and FSH, which showed the highest viability on day 12 (approximately 68,000 cells/well).

Between days 4 and 8 of the IVG culture, the addition of 10 ng/mL BMP-4 and 0.5 ng/mL FSH did not affect E_2 production by OCGCs; however, this supplementation regime decreased P_4 production compared to cultures with 0.5 ng/mL FSH in the absence of BMP-4. On the other hand, no significant differences were noted between the number of granulosa cells in both groups; therefore, under the present culture conditions containing serum, 10 ng/mL BMP-4 may inhibit P_4 production by granulosa cells by suppressing StAR [24, 25] and P450scc [25] at the mRNA and protein levels. However, P450arom may not be enhanced, because previous studies showed that P450arom activity is increased in granulosa cells cultured without serum [21, 34]. A study that cultured granulosa cells for 6 days in the presence of serum showed that the expression of the *P450arom* gene was suppressed, but not *StAR* and *P450scc* [17]. These findings indicate that the luteinization of granulosa cells induced by serum counteracts the stimulatory effects of BMP-4 on E_2 production. Our results demonstrated that this supplementation regime permitted OCGCs to partially mimic developing follicles, which secrete E_2 as follicles develop and the E_2/P_4 ratio increases [15]. However, 0.5 ng/mL FSH increased P_4 production from days 4 to 8 and particularly from days 8 to 12. Previous studies reported that *in vivo*-grown large luteal cells (38.4 μm in diameter) originated from granulosa cells and *in vitro*-luteinized granulosa cells (38.4 μm) are larger than granulosa cells in pre-ovulatory follicles (10.6 μm) [35, 36]. Under our culture conditions, the mean diameter of granulosa cells on day 8 increased with the addition of FSH to medium containing 10 ng/mL BMP-4 (0 ng/mL FSH, 11.5 μm vs. 0.5 ng/mL FSH, 12.4 μm; $P < 0.01$) or 50 ng/mL

374 BMP-4 (0 ng/mL FSH, 11.1 μm vs. 0.5 ng/mL FSH, 12.0 μm; P = 0.05). FSH appears to have
375 enhanced the luteinization of granulosa cells under the present IVG conditions, as has been reported
376 previously [37]. On day 12 of the IVG culture, no significant differences were observed in the
377 diameters of granulosa cells between experimental groups; however, the group cultured with 50
378 ng/mL BMP-4 in the absence of FSH showed a larger diameter (12.2 μm) than the same group on
379 day 8 of the IVG culture (11.1 μm, $P < 0.05$). These results indicate that the anti-luteinizing effects
380 of BMP-4 were lost when the culture period was extended.

381 A previous study that cultured granulosa cells for 6 days in serum-free media demonstrated
382 that E_2 and P_4 production increased as the culture period was extended [38]. According to our
383 results, the production of E_2 from cultured OCGCs was maintained at least until day 8, even when
384 the medium contained serum. However, granulosa cells are apparently unable to stably produce E_2
385 for the 12 days of IVG culture. High densities of granulosa cells have been shown to inhibit E_2
386 secretion and *P450arom* expression and also to increase P_4 secretion and the levels of mRNA
387 encoding progestogenic enzymes, such as *StAR* and *P450scc* [39]. We previously reported that the
388 number of granulosa cells peaked on day 12 of the IVG culture and then decreased [40]. These
389 findings indicate that luteinization progresses as the density of granulosa cells increases.

390 OCGCs cultured with 50 ng/mL BMP-4 in the absence of FSH showed the lowest
391 viability; however, the surviving oocytes derived from these OCGCs had the largest volumes and
392 highest levels of meiotic competence. The meiotic competence of bovine oocytes grown *in vivo*

393 increases as oocytes [41, 42] and follicles [43] grow. The administration of exogenous BMP-4
394 during IVG (50 ng/mL) [20] and IVM (100 ng/mL) [44, 45] did not affect oocyte nuclear maturation
395 in previous studies. Thus, BMP-4 does not directly promote oocyte nuclear maturation. However,
396 OCGCs, which can produce oocytes with higher maturational competences, may survive in the
397 presence of higher concentrations of BMP-4 in the absence of FSH, and the highest viability of
398 granulosa cells was also observed on day 12 (98.9%). Based on these results, we speculate that
399 OCGCs producing oocytes with high intrinsic developmental competence may survive in the
400 presence of high concentrations of BMP-4. In further studies, the relationship between oocyte
401 competence and the function of granulosa cells needs to be examined.

402 In addition, FSH did not promote the nuclear maturation of oocytes in the present study.
403 A previous study that cultured bovine OCGCs for 14 days with a higher concentration of FSH (3.5
404 mg/mL) showed that no oocytes progressed to the metaphase II stage and 73.3% of oocytes
405 degenerated after IVM [46]. According to another study, the addition of 10 μg/mL FSH improved
406 cumulus expansion, whereas the proportion of oocytes at metaphase II after IVM did not
407 significantly differ from cultures that did not include FSH [47]. These findings and the present
408 results suggest that FSH does not affect the maturational competence of bovine oocytes, although
409 FSH improves granulosa cell proliferation [34] and cumulus expansion [47].

410 In the present study, OCGCs that formed antra produced more E_2 and less P_4 than OCGCs
411 without an antrum. As reported in the study by Endo *et al*. [48], OCGCs that formed antra

412 exhibited similar levels of gene expression to healthy follicles that grew *in vivo*. Thus, antrum
413 formation in the granulosa cell layer is related to the steroidogenesis of OCGCs. On the other hand,
414 we were unable to detect a relationship between oocyte maturation and antrum formation in the
415 granulosa cell layer, as has been described in a previous study [48]; however, OCGCs that produced
416 mature oocytes produced slightly more E_2 and less P_4 during the IVG culture than OCGCs that
417 produced immature oocytes. Furthermore, some of the OCGCs that produced mature oocytes
418 secreted extremely large quantities of E_2. In the present study, we added A_4 to medium instead of
419 the E_2 used in the previous study [20], and did not observe any effects of BMP-4 on nuclear
420 maturation. These results suggest that the ability to produce E_2 by granulosa cells has an important
421 effect on the acquisition of oocyte ability. In future studies, we need to investigate the relationships
422 between the maturational competence of oocytes, the steroidogenesis of granulosa calls and antrum
423 formation, and the expression dynamics of growth factors and their receptors, such as BMP-4, in
424 more detail.
425
426 **5. Conclusions**
427 Based on the results of the present study, BMP-4 inhibits the luteinization of granulosa
428 cells and FSH enhances the proliferation of granulosa cells, viability of OCGCs and the luteinization
429 of granulosa cells. Moreover, cultured OCGCs with antra that produce a large amount of E_2 and
430 less P_4 are similar to follicles that are grown *in vivo*. However, E_2 production increased until day 8

of the culture and then decreased. In conclusion, an IVG culture with 10 ng/mL BMP-4 in the absence of FSH partially mimics *in vivo* steroidogenesis and the development of growing follicles until day 8 of culture. The same conditions also mimic the steroidogenesis of degenerating follicles from days 8 to 12 of the culture, particularly in the presence of FSH.

The addition of serum to the growth medium may have enhanced the luteinization of granulosa cells in the present study; however, oocytes derived from serum-free cultures had low maturational competence and low fertilizability in previous studies [18, 19]. In further studies, we should develop an IVG system that does not use serum but enhances oocyte competence, or a system that inhibits the luteinization of granulosa cells even when the growth medium contains serum.

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638 **Figure Legends**

639 Fig. 1. Oocyte-cumulus-granulosa complexes (OCGCs) before and after day 4 of the growth culture.

640 The left panel shows an OCGC before the growth culture. OCGCs with oocytes surrounded by a

641 cumulus investment and an attached mural granulosa cell layer were subjected to the growth culture.

642 The white arrow head indicates the cumulus investment. The black arrow head indicates the mural

643 granulosa cell layer. The central panel shows an OCGC with an evenly granulated ooplasm

644 enclosed by several layers of healthy granulosa cells. The right panel shows an OCGC that has an

645 abnormal appearance with a degenerated oocyte. Scale bars indicate 100 μm.

646

647 Fig. 2. Schematic of experimental design

648 Firstly, oocyte-granulosa cell complexes (OCGCs) were cultured in growth medium without bone

649 morphogenetic protein-4 (BMP-4) and FSH for 4 days. Morphologically normal OCGCs were

650 cultured for an additional 8 days in growth medium supplemented with different concentrations of

651 BMP-4 (0, 10, or 50 ng/mL) and FSH (0 or 0.5 ng/mL). On day 12 of the IVG culture, oocytes

652 judged as having survived were subjected to IVM. The assessments described in the schematic

653 were conducted at days 0, 4, 8, and 12 and after IVM.

654

655 Fig. 3. Steroidogenesis of oocyte-cumulus-granulosa-complexes (OCGCs) on day 4 of the growth

656 culture.

657 ** Asterisks indicate significant differences between normal and abnormal OCGCs ($P < 0.01$).

658 Numbers in parentheses indicate the number of OCGCs evaluated.

659 Error bars indicate the SEM.

660

661 Fig. 4. Effects of bone morphogenetic protein-4 (BMP-4) and FSH on the number, viability, and

662 diameter of granulosa cells at different culture periods.

663 a,b Different letters indicate significant differences between OCGCs cultured without FSH in the

664 same culture period ($P < 0.05$).

665 x,y Different letters indicate significant differences between OCGCs cultured with FSH in the same

666 culture period ($P < 0.05$).

667 * Asterisks indicate significant differences between OCGCs cultured with the same BMP-4

668 concentration in the same culture period ($P < 0.05$).

669 Error bars indicate the SEM.

670

671 Fig. 5. Effects of bone morphogenetic protein-4 (BMP-4) and FSH on the oocyte volume after the

672 growth culture.

673 Lines on the boxes delineate the 25th, 50th, and 75th percentiles, whereas the whiskers depict the

674 10th and 90th percentiles.

675 Values above boxes indicate the mean diameters (μm) of oocytes.

676 Values under boxes indicate the numbers of oocytes examined and the number of replicates are

677 shown in parentheses.

678 * An asterisk indicates a significant difference between groups cultured with and without FSH in the

679 presence of the same BMP-4 concentration ($P < 0.05$).

680 † A dagger indicates a significant difference between the values measured before and after IVG in all

681 groups ($P < 0.05$).

682

683 Fig. 6. Effects of bone morphogenetic protein-4 (BMP-4) and FSH on steroidogenesis in

684 oocyte-cumulus-granulosa complexes (OCGCs) at different culture periods.

685 In all experimental groups, E_2 was produced at a lower level between days 8 and 12 than between

686 days 4 and 8 ($P < 0.05$); P_4 was produced at a higher level between days 8 and 12 than between days

687 4 and 8 ($P < 0.05$).

688 ^{a,b} Different letters indicate significant differences between OCGCs cultured without FSH in the

689 same culture period ($P < 0.05$).

690 ^{x,y} Different letters indicate significant differences between OCGCs cultured with FSH in the same

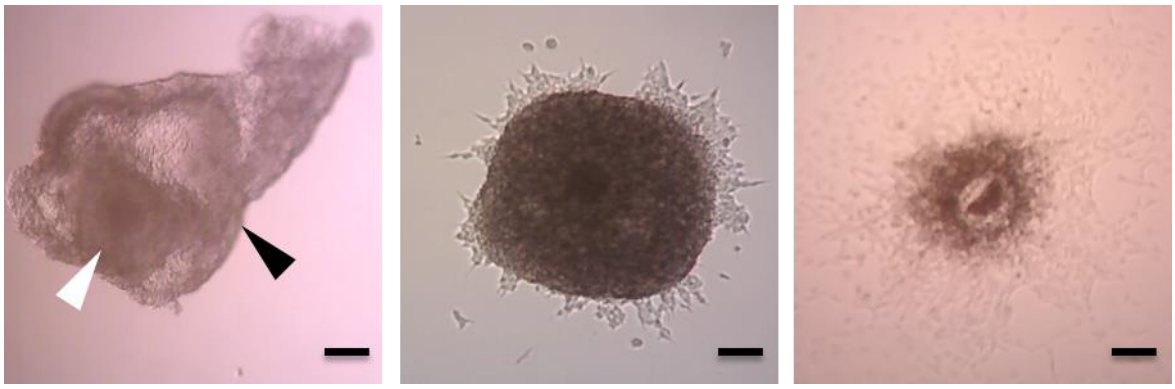
691 culture period ($P < 0.05$).

692 *,** Asterisks indicate significant differences between OCGCs cultured with the same BMP-4

693 concentration in the same culture period (*$P < 0.05$; **$P < 0.01$).

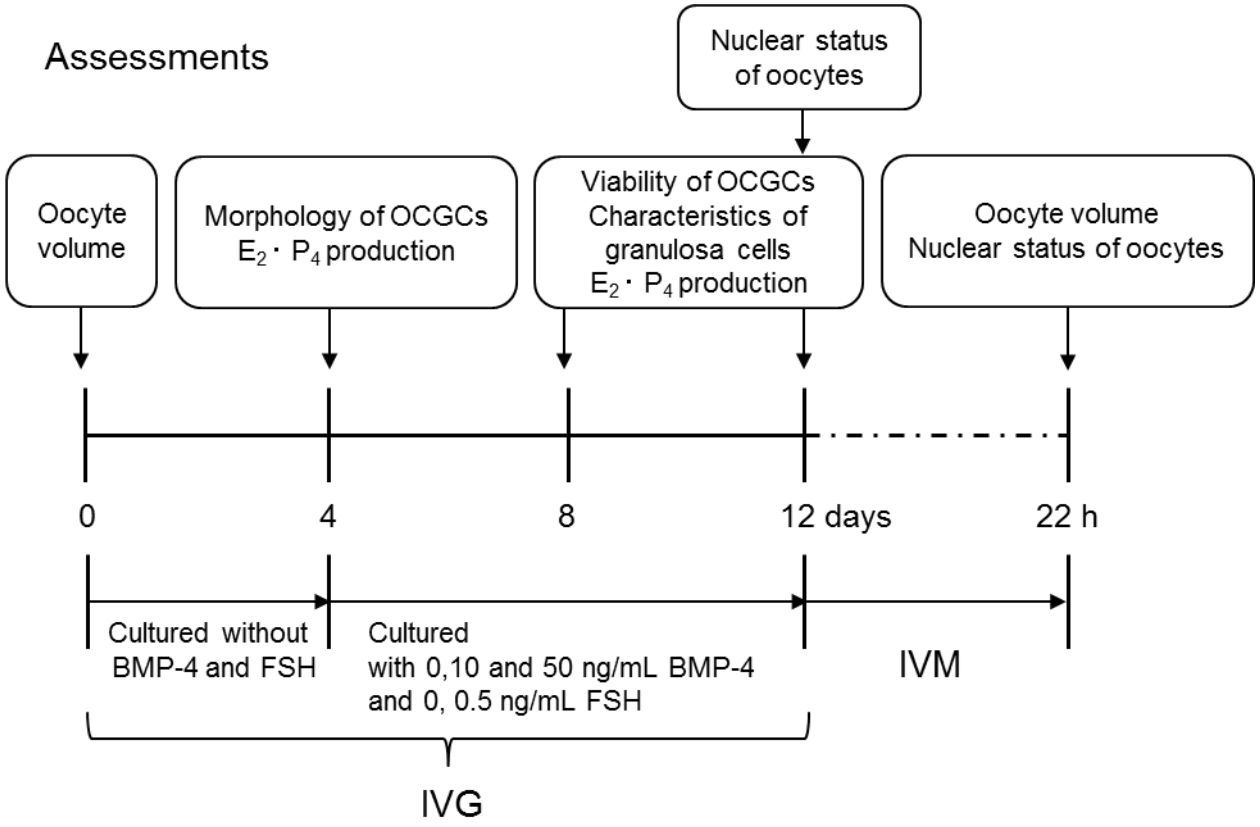
694 Error bars indicate the SEM.

Fig. 1



703 Fig. 2

704

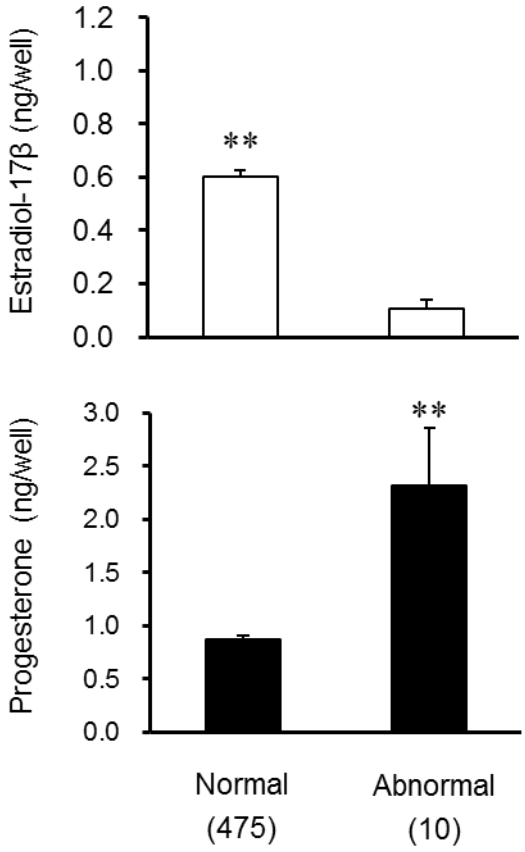


705

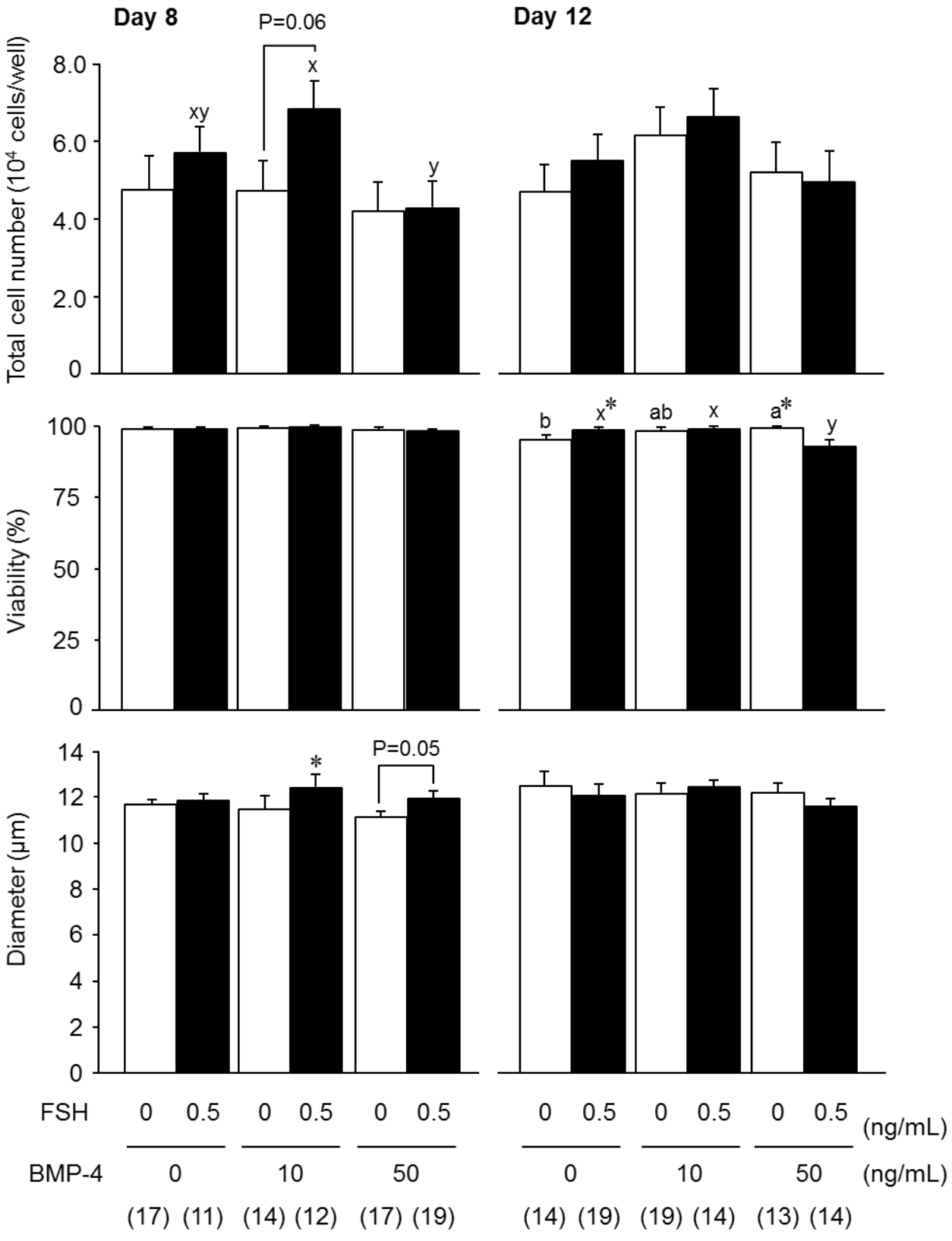
706

707

Fig. 3

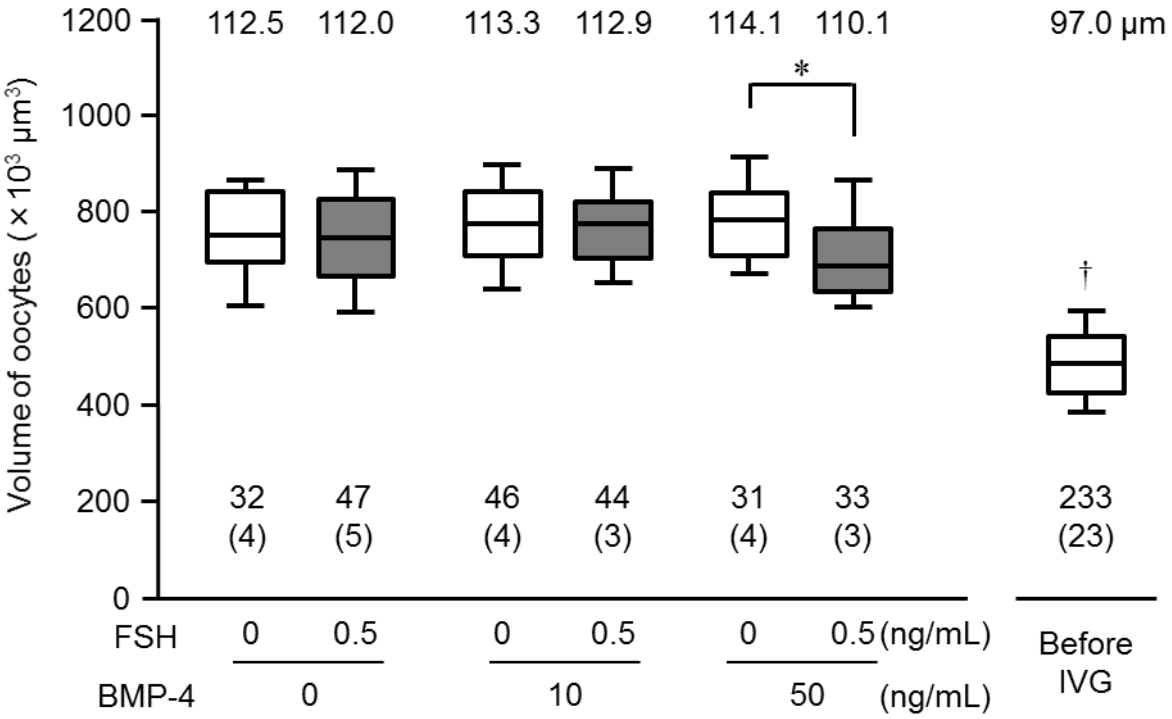

Estradiol-17β (ng/well)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Progesterone (ng/well)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Normal
(475)
Abnormal
(10)

712 Fig. 4


Day 8
P=0.06
Day 12
Total cell number (10^4 cells/well)
8.0
6.0
4.0
2.0
0
xy
x
y
Viability (%)
100
75
50
25
0
b
x*
ab
x
a*
y
Diameter (μm)
14
12
10
8
6
4
2
0
*
P=0.05
FSH 0 0.5 0 0.5 0 0.5 0 0.5 0 0.5 0 0.5 (ng/mL)
BMP-4 0 10 50 0 10 50 (ng/mL)
(17) (11) (14) (12) (17) (19) (14) (19) (19) (14) (13) (14)

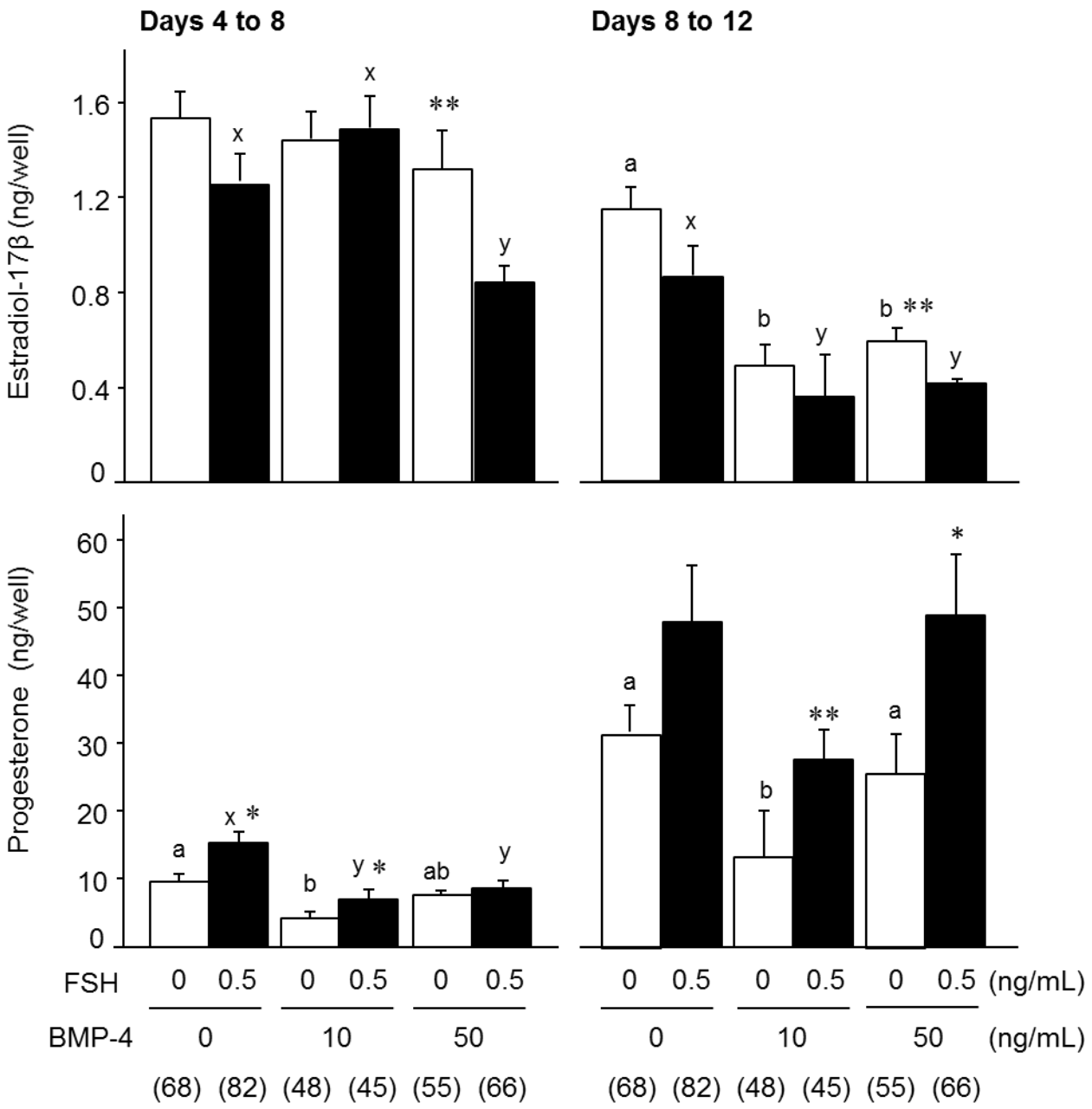
713

Fig. 5


Volume of oocytes (× 10³ μm³)
1200
1000
800
600
400
200
0
112.5
112.0
113.3
112.9
114.1
110.1
97.0 μm
*
†
32
(4)
47
(5)
46
(4)
44
(3)
31
(4)
33
(3)
233
(23)
FSH
0
0.5
0
0.5
0
0.5
(ng/mL)
BMP-4
0
10
50
(ng/mL)
Before IVG

719 Fig. 6

720



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722

723 **Tables**

724 Table 1. Effects of bone morphogenetic protein-4 (BMP-4) and FSH on the viability of
725 oocyte-cumulus-granulosa complexes (OCGCs) during the growth culture.

Experimental groups		Day 8		Day 12	
BMP-4 (ng/mL)	FSH (ng/mL)	No. of oocytes (replicates)	% of viable OCGCs	No. of oocytes (replicates)	% of viable OCGCs
0	0	158 (14)	72.1^{ab}	132 (11)	62.1^{ab}
	0.5	172 (16)	76.8^{abx}	154 (13)	65.6^{ay}
10	0	120 (10)	80.8^{ax}	100 (7)	67.0^{ay}
	0.5	103 (8)	80.6^{ab}	83 (5)	71.1^{a}
50	0	153 (10)	69.9^{bx}	127 (7)	53.5^{by}
	0.5	145 (10)	80.0^{ax}	122 (7)	65.6^{aby}
Total		851* (68)	76.3	718 (50)	63.6

726 ^{a,b} Different superscripts indicate differences between groups in the same culture period ($P < 0.05$).
727 ^{x,y} Different superscripts indicate significant differences between days 8 and 12 in the same group (P
728 < 0.05).
729 * One hundred and thirty-three OCGCs were used to evaluate the granulosa cell characteristics on
730 day 8.
731
732
733 Table 2. Effects of bone morphogenetic protein-4 (BMP-4) and FSH on the nuclear maturation of
734 oocytes after the maturational culture.

Experimental groups		No. of oocytes (no. of replicates)	% of nuclear maturation
BMP-4 (ng/mL)	FSH (ng/mL)		
0	0	32 (4)	78.1^{b}
	0.5	47 (5)	72.3^{b}
10	0	46 (4)	60.9^{b}
	0.5	44 (3)	66.7^{b}
50	0	31 (4)	80.6^{ab}
	0.5	33 (3)	75.8^{b}
Oocytes grown *in vivo*		79 (7)	92.4^{a}

735 ^{a,b} Different superscripts indicate significant differences within a column ($P < 0.05$).
736

Table 3. Relationship between antrum formation in the granulosa cell layer on day 12 and steroidogenesis in oocyte-cumulus-granulosa complexes (OCGCs) in different culture periods.

Antrum formation on day 12 (n)	E_2 (ng/well)		P_4 (ng/well)	
	Days 4 to 8	Days 8 to 12	Days 4 to 8	Days 8 to 12
Yes (96)	1.5 ± 0.9^{a}	0.8 ± 1.0	5.8 ± 6.4^{b}	13.9 ± 18.4^{b}
No (268)	1.2 ± 1.0^{b}	0.7 ± 0.8	10.9 ± 12.4^{a}	42.3 ± 58.4^{a}

Values are presented as means ± SD.

^{a,b} Different superscripts indicate significant differences within a column ($P < 0.01$).

Table 4. Relationship between the nuclear maturation of oocytes, oocyte growth, steroidogenesis, and antrum formation by oocyte-cumulus-granulosa complexes (OCGCs).

Nuclear Status* (n)	Diameter of oocytes (µm)		E_2 (ng/well)		P_4 (ng/well)		E_2/P_4 ratio (range)		% of antrum formation (n)
	before IVG	after IVM	Days 4 to 8	Days 8 to 12	Days 4 to 8	Days 8 to 12	Day 8	Day 12	
Mature (167)	97.3 ± 5.0	113.5 ± 4.9[a]	1.2 ± 0.8	0.5 ± 0.7	9.0 ± 10.7	32.3 ± 57.0	0.92 ± 3.31 (0.005-40.7)	0.37 ± 1.70 (0.0004-21.6)	27.5 (46)
Immature (66)	96.3 ± 5.1	110.0 ± 5.6[b]	1.0 ± 0.9	0.4 ± 0.7	12.1 ± 16.6	34.6 ± 48.5	0.35 ± 0.67 (0.005-5.1)	0.08 ± 0.11 (0.005-0.039)	19.7 (13)

Values are presented as means ± SD.

* Oocytes at metaphase II were defined as mature.

[a,b] Different superscripts indicate significant differences within a column ($P < 0.01$).