



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
Author(s)	Sakaguchi, Kenichiro; Huang, Weiping; Yang, Yinghua; Yanagawa, Yojiro; Nagano, Masashi
Citation	Theriogenology, 97, 113-123 https://doi.org/10.1016/j.theriogenology.2017.04.030
Issue Date	2017-04-23
Doc URL	http://hdl.handle.net/2115/69418
Rights	© 2017. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Sakaguchi et al HUSCAP.pdf



[Instructions for use](#)

1 Original Research Paper

2

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

6

7 Authors: Kenichiro Sakaguchi ¹, Weiping Huang^{1,2}, Yinghua Yang¹, Yojiro Yanagawa¹, Masashi
8 Nagano^{1*}

9 Author details

10 ¹Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of
11 Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

12 ²Anhui Provincial Laboratory of Animal Genetic Resources Protection and Breeding, College of
13 Animal Sciences and Technology, Anhui Agricultural University, Hefei, Anhui, China

14 E-mail addresses

15 Kenichiro Sakaguchi (ksaka@vetmed.hokudai.ac.jp); Weiping Huang (huangwp@ahau.edu.cn);

16 Yinghua Yang (young@vetmed.hokudai.ac.jp); Yojiro Yanagawa (yoji-y@vetmed.hokudai.ac.jp);

17 Masashi Nagano (mnaga@vetmed.hokudai.ac.jp);

18 *Corresponding author: Masashi Nagano

19

20 **Abstract**

21 Bone morphogenetic protein-4 (BMP-4) and FSH play important regulatory roles in
22 follicular growth and steroidogenesis *in vivo*. The purpose of this study was to investigate the
23 effects of BMP-4 and FSH on *in vitro* growth (IVG) and steroidogenesis of bovine
24 oocyte-cumulus-granulosa complexes (OCGCs). We cultured OCGCs collected from early antral
25 follicles (0.5-1 mm) in medium without BMP-4 and FSH for 4 days and investigated the appearance
26 of OCGCs and their steroidogenesis. During the first 4 days of IVG, morphologically normal
27 OCGCs produced more estradiol-17 β (E₂), but less progesterone (P₄). Morphologically normal
28 OCGCs were subjected to an additional culture in medium supplemented with BMP-4 (0, 10, and 50
29 ng/mL) and FSH (0 and 0.5 ng/mL) until day 12. We examined the viability and steroidogenesis of
30 OCGCs after 8 and 12 days of culture. Oocyte growth, characteristics of granulosa cells, and the
31 maturational competence of oocytes were also investigated. On day 8, the viability of OCGCs
32 cultured without FSH was higher in the 10 ng/mL BMP-4 group than in the 50 ng/mL BMP-4 group
33 (P<0.05). No significant difference was observed in the viability of groups cultured with FSH,
34 regardless of the addition of BMP-4, and FSH improved the viability of 50 ng/mL BMP-4 group
35 similar to 10 ng/mL BMP-4 group. The total number of granulosa cells was larger in 10 ng/mL
36 BMP-4 group cultured with FSH than in 50 ng/mL BMP-4 group cultured with FSH on day 8
37 (P<0.05). E₂ production decreased from days 8 to 12, and P₄ production increased throughout IVG
38 culture, regardless of the addition of BMP-4 and FSH (P<0.05). No significant differences in E₂

39 production were observed between groups from days 4 to 8, regardless of whether BMP-4 was added
40 without FSH; however, E₂ production in the group cultured with 50 ng/mL BMP-4 was suppressed
41 by FSH. BMP-4 suppressed E₂ production from days 8 to 12, regardless of whether FSH was
42 added. The group cultured with 10 ng/mL BMP-4 without FSH showed the lowest P₄ production
43 among all groups for all culture periods. OCGCs that produced mature oocytes tended to secrete
44 more E₂ and less P₄ than OCGCs that produced immature oocytes. In conclusion, until day 8 of the
45 IVG culture, P₄ production by OCGCs was suppressed by the addition of 10 ng/mL BMP-4 in the
46 absence of FSH, without inhibiting E₂ production. These conditions appear to mimic growing
47 follicles until day 8 and mimic degenerating follicles from days 8 to 12 of culture.

48

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

51 **1. Introduction**

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

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

70 that estradiol-17 β (E₂) 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 (P₄)
72 concentrations [14]. Furthermore, E₂ concentrations in dominant follicles increased concomitantly
73 with follicular development and the E₂/P₄ ratio also increased; however, subordinate follicles showed
74 low E₂/P₄ ratios [14, 15]. These findings indicate that a culture system of OCGCs that produces
75 more E₂ and less P₄ 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 E₂ production,
78 and begin to produce P₄ [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 E₂ production by

89 inhibiting apoptosis [23] and promoting aromatase (P450arom) activity in cattle [21]. In addition,
90 P₄ 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 E₂ production, which is increased in the presence of oocytes [26]. On the other hand,
94 P₄ 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 E₂ production and inhibiting P₄ production. According to our recent report, the addition
98 of BMP-4 (10 ng/mL) to an IVG culture suppressed P₄ 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 E₂ (1 µg/mL) to increase the E₂/P₄ 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 E₂ 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

108 oocytes.

109

110 **2. Materials and Methods**

111 **2.1. Chemicals**

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

114

115 **2.2. Collection of OCGCs and the IVG culture**

116 Bovine ovaries obtained from a local abattoir were stored in plastic bags at 20°C and
117 transported to the laboratory within 6-10 h of their collection. After the ovaries were washed three
118 times with physiological saline, slices of ovarian cortex tissues (< 1 mm thickness) were prepared
119 using a surgical blade (no. 11) and stored in tissue culture medium 199 (TCM-199; Thermo Fisher
120 Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM
121 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and
122 50 µg/mL gentamicin sulfate (isolation medium, pH 7.4) at 37 °C, as described elsewhere [8].
123 Under a stereomicroscope, early antral follicles (0.5 to 1 mm in diameter) were dissected from sliced
124 ovarian tissues using a surgical blade (no. 20) and fine forceps in a 90-mm petri dish that had a
125 1-mm² scale on its bottom (FLAT Co., Ltd., Chiba, Japan). OCGCs were isolated from early antral
126 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 (A₄) as a precursor
131 for E₂ [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% CO₂. 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,

146 OCGCs were defined as abnormal.

147

148 **2.4. Evaluation of the characteristics of granulosa cells**

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

157

158 **2.5 Evaluation of nuclear status of oocyte after IVG and IVM**

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

165 for 15 min in the dark, as described elsewhere [28]. The nuclear status was then evaluated under an
166 inverted fluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) using a UV filter
167 (excitation 334–365 nm) to confirm meiotic arrest. Oocytes with a nuclear envelope were defined
168 as being in the germinal vesicle stage. Other oocytes were submitted to individual IVM as
169 previously described [29]. Oocytes surrounded by several layers of cumulus cells were washed
170 with IVM medium, which consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM
171 sodium pyruvate, 20 µg/mL FSH, 1 µg/mL E₂, 10% FCS, and 50 µg/mL gentamicin sulfate. IVM
172 of oocytes was performed in each well of micro-well plates filled with 6 mL of IVM medium at
173 39 °C under 5% CO₂ in air for 22 h. After IVM, oocytes were denuded from cumulus cells by
174 individually pipetting, photographed, and their diameters measured. Oocytes were mounted
175 individually on a slide glass and fixed with a mixture of acetic acid and ethanol (1:3) for 6 h. After
176 fixation, oocytes were stained with 1% (w/v) aceto-orcein and the status of their nuclei was
177 examined under a phase contrast microscope, as described elsewhere [30]. Oocytes that reached
178 metaphase II and had a polar body were defined as mature; oocytes with other nuclear statuses were
179 defined as immature.

180

181 **2.6. E₂ and P₄ assays**

182 Spent media (100 µL) from IVG cultures were assayed to determine the E₂ and P₄
183 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 Na₂HPO₄, 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 E₂ and P₄ 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 Na₂HPO₄, 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 H₂SO₄. All samples were assayed in triplicate. Assay
197 sensitivities were 7.1 pg/well for E₂ and 11.2 pg/well for P₄. The inter- and intra-assay
198 coefficients of variation were 15.8 and 4.0% for E₂ and 17.5 and 3.9% for P₄, respectively.

199

200 **2.7. Experimental design**

201 **2.7.1. Morphology and steroidogenic capacity of OGCs**

202 OGCs were cultured in growth medium without BMP-4 or FSH for 4 days to select

203 healthy OCGCs that exhibited steroidogenic activity similar to developing follicles *in vivo* (Fig. 2).
204 The correlation between the morphological normality of OCGCs and the production of steroid
205 hormones was evaluated by measuring the concentrations of E₂ and P₄ in spent media. OCGCs
206 defined as normal were subjected to subsequent experiments.

207 **2.7.2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa**
208 **cells, growth and maturation of oocytes, and steroidogenesis**

209 Normal OCGCs were randomly divided into six groups after 4 days of IVG culture to
210 evaluate the effects of BMP-4 and FSH on the functions of OCGCs. We cultured OCGCs for an
211 additional 8 days (a total of 12 days of IVG culture) in growth medium supplemented with different
212 concentrations of BMP-4 (0, 10, or 50 ng/mL; HZ-1045, Humanzyme, Chicago, IL, USA) and FSH
213 (0 or 0.5 ng/mL) (Fig. 2). The concentrations of BMP-4 and FSH were determined according to
214 previous studies in which granulosa cells were cultured *in vitro* [23, 25, 32]. In these studies, 10
215 and 50 ng/mL BMP-4 promoted cell viability [23] and suppressed P₄ production [25], whereas 0.5
216 ng/mL FSH enhanced the expression of the *P450arom* mRNA and E₂ production [32]. After 8 and
217 12 days of IVG culture, the viability of OCGCs was defined by the normality of their morphological
218 appearance (Fig. 1); *i.e.*, morphologically normal OCGCs were defined as having survived, whereas
219 morphologically abnormal OCGCs were defined as dead. In addition, antrum formation in the
220 granulosa cell layer was noted on day 12 of the IVG culture [6]. On day 12 of the IVG culture, 233
221 oocytes that were judged as having survived were subjected to IVM and nuclear maturation was

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

228 The concentrations of E₂ and P₄ in spent media collected on days 8 and 12 of the IVG
229 culture were measured to evaluate the steroidogenesis of OCGCs that survived for the 12 days of
230 IVG culture. Steroid hormone production during each period (days 4 to 8 and days 8 to 12) was
231 calculated using the following formula:

$$\begin{aligned} 232 \text{ Steroid hormone production (ng)} &= 0.2 \text{ (mL)} \times \text{Concentration at the end of the period (ng/mL)} \\ 233 &\quad - 0.1 \text{ (mL)} \times \text{Concentration at the start of the period (ng/mL)} \end{aligned}$$

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

237 OCGCs were divided based on whether an antrum had formed or not and whether OCGCs
238 produced mature or immature oocytes to evaluate the relationship between OCGC characteristics
239 and steroidogenesis. The relationships between these factors and steroidogenesis (the production of
240 E₂ and P₄ and the E₂/P₄ 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

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

261

262 **3. Results**

263 **3.1. Morphology and steroidogenic capacity of OCGCs**

264 Nine hundred eight OCGCs (8 to 60 OCGCs/replicate) were cultured in growth medium
265 without the addition of BMP-4 or FSH. After 4 days of IVG culture in the absence of both BMP-4
266 and FSH, 93.7% (851/908) of OCGCs had a normal appearance. E₂ and P₄ levels were then
267 measured in culture media collected from 475 OCGCs with a normal appearance and 10 OCGCs
268 with an abnormal appearance. As shown in Fig. 3, OCGCs with a normal appearance produced
269 more E₂ (P < 0.01) and less P₄ (P < 0.01) than abnormal OCGCs.

270

271 **3.2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa cells, 272 growth and maturation of oocytes, and steroidogenesis**

273 Eight hundred fifty-one OCGCs were cultured in media containing BMP-4 (0, 10, or 50 ng/mL)
274 and FSH (0 or 0.5 ng/mL), and 718 OCGCs were cultured until day 12; the remaining 133 OCGCs
275 were cultured until day 8 to evaluate the characteristics of granulosa cells. The viabilities of
276 OCGCs on days 8 and 12 of IVG culture are shown in Table 1. After 8 days of culture, the group
277 cultured with 10 ng/mL BMP-4 in the absence of FSH showed higher viability than the group
278 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 P₄ production from days 4 to 8
318 (P < 0.05). Between days 8 and 12, the addition of BMP-4 decreased E₂ production, regardless of
319 whether FSH was added (P < 0.05). FSH decreased E₂ 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 P₄, 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 E₂ from days 4 to 8 (P < 0.01) and smaller amounts of P₄ 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 E₂ (P = 0.10)
328 and less P₄ (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 E₂ and P₄
330 production from days 8 to 12, the E₂/P₄ ratio varied markedly. E₂ and P₄ 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 E₂/P₄ ratios. No obvious
333 relationship was observed between the nuclear maturation of IVG oocytes and antrum formation in
334 the granulosa cell layer.

335

336 **4. Discussion**

337 In the present study, OCGCs that had a normal appearance 4 days after the initiation of the
338 IVG culture produced a large amount of E₂ and less P₄ during the initial period of the IVG culture.
339 During the *in vivo* development of a dominant follicle, the E₂ concentration increases as follicles
340 grow [14, 15]. Thus, we only used healthy OCGCs in subsequent experiments to evaluate the
341 effects of BMP-4 and FSH on steroidogenesis and oocyte maturation.

342 On day 8 of the IVG culture, the viability of the group cultured with 10 ng/mL BMP-4 was
343 higher than the group cultured with 50 ng/mL BMP-4 ($P < 0.05$) when OCGCs were cultured
344 without FSH. The low viability of OCGCs cultured with 50 ng/mL BMP-4 may stem from a
345 decrease in granulosa cell numbers, as we reported previously [20]. Actually, the lowest number of
346 granulosa cells was observed on day 8 in the group cultured with 50 ng/mL BMP-4 (approximately
347 41,000 cells/well) among all groups tested (approximately 47,000 cells/well) in the absence of FSH,
348 although the values were not significantly different. On the other hand, when 0.5 ng/mL FSH was
349 added to growth medium, the viability of OCGCs in the group cultured with 50 ng/mL BMP-4
350 improved on days 8 and 12. In the present study, we only examined OCGCs that had survived;
351 therefore, we could not find the increase of granulosa cell number. We speculate that the result may
352 be attributed to the increased proliferation of granulosa cells by the addition of 0.5 ng/mL FSH from
353 days 4 to 8 [34], and was particularly apparent in the group cultured with 10 ng/mL BMP-4 and FSH,
354 which showed the highest viability on day 12 (approximately 68,000 cells/well).

355 Between days 4 and 8 of the IVG culture, the addition of 10 ng/mL BMP-4 and 0.5 ng/mL
356 FSH did not affect E₂ production by OCGCs; however, this supplementation regime decreased P₄
357 production compared to cultures with 0.5 ng/mL FSH in the absence of BMP-4. On the other hand,
358 no significant differences were noted between the number of granulosa cells in both groups;
359 therefore, under the present culture conditions containing serum, 10 ng/mL BMP-4 may inhibit P₄
360 production by granulosa cells by suppressing StAR [24, 25] and P450_{scc} [25] at the mRNA and
361 protein levels. However, P450_{arom} may not be enhanced, because previous studies showed that
362 P450_{arom} activity is increased in granulosa cells cultured without serum [21, 34]. A study that
363 cultured granulosa cells for 6 days in the presence of serum showed that the expression of the
364 *P450arom* gene was suppressed, but not *StAR* and *P450scc* [17]. These findings indicate that the
365 luteinization of granulosa cells induced by serum counteracts the stimulatory effects of BMP-4 on E₂
366 production. Our results demonstrated that this supplementation regime permitted OCGCs to
367 partially mimic developing follicles, which secrete E₂ as follicles develop and the E₂/P₄ ratio
368 increases [15]. However, 0.5 ng/mL FSH increased P₄ production from days 4 to 8 and particularly
369 from days 8 to 12. Previous studies reported that *in vivo*-grown large luteal cells (38.4 μm in
370 diameter) originated from granulosa cells and *in vitro*-luteinized granulosa cells (38.4 μm) are larger
371 than granulosa cells in pre-ovulatory follicles (10.6 μm) [35, 36]. Under our culture conditions, the
372 mean diameter of granulosa cells on day 8 increased with the addition of FSH to medium containing
373 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 E₂ and less P₄ 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 E₂ and less P₄ 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 E₂. In the present study, we added A₄ to medium instead of
419 the E₂ 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 E₂ 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 cells 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 E₂ and
430 less P₄ are similar to follicles that are grown *in vivo*. However, E₂ production increased until day 8

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

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

440

441 **Acknowledgments**

442 This study was supported by Grants-in-Aid for Scientific Research from the Japan Society
443 for the Promotion of Science awarded to M. Nagano (nos. 25450441 and 16K08043).

444

445 **References**

- 446 [1] Aerts JMJ, Bols PEJ. Ovarian follicular dynamics: a review with emphasis on the bovine species.
447 Part I: Folliculogenesis and pre-antral follicle development. *Reprod Domest Anim* 2010;45:171–9.
448 doi: 10.1111/j.1439-0531.2008.01302.x.
- 449
- 450 [2] Aerts JMJ, Bols PEJ. Ovarian follicular dynamics. a review with emphasis on the bovine species.
451 Part II: Antral development, exogenous influence and future prospects. *Reprod Domest Anim*
452 2010;45:180–7. doi: 10.1111/j.1439-0531.2008.01298.x.

453

454 [3] Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol*
455 *Reprod* 1996;54:197–207.

456

457 [4] O'Brien MJ, Pendola JK., Eppig JJ. A revised protocol for in vitro development of mouse
458 oocytes from primordial follicles dramatically improves their developmental competence. *Biol*
459 *Reprod* 2003;68:1682–6. doi: 10.1095/biolreprod.102.013029.

460

461 [5] Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T. Development to live
462 young from bovine small oocytes after growth, maturation and fertilization in vitro. *Theriogenology*
463 1999;52:81–9.

464

465 [6] Hirao Y, Itoh T, Shimizu M, Iga K, Aoyagi K, Kobayashi M, Kacchi M, Hoshi H, Takenouchi N.
466 In vitro growth and development of bovine oocyte-granulosa cell complexes on the flat substratum:
467 effects of high polyvinylpyrrolidone concentration in culture medium. *Biol Reprod* 2004;70:83–91.
468 doi: 10.1095/biolreprod.103.021238.

469

470 [7] Huang W, Kang SS, Nagai K, Yanagawa Y, Takahashi Y, Nagano M. Mitochondrial activity
471 during pre-maturational culture in in vitro-grown bovine oocytes is related to maturational and
472 developmental competences. *Reprod Fertil Dev* 2016;28:349-56. doi: 10.1071/RD14023.

473

474 [8] Harada M, Miyano T, Matsumura K, Osaki S, Miyake M, Kato S. Bovine oocytes from early
475 antral follicles grow to meiotic competence in vitro: effect of FSH and hypoxanthine.
476 *Theriogenology* 1997;48:743–55.

477

478 [9] Alm H, Kątska-Książkiewicz L, Ryńska B, Tuchscherer A. Survival and meiotic competence of
479 bovine oocytes originating from early antral ovarian follicles. *Theriogenology* 2006;65:1422–34.
480 doi:10.1016/j.theriogenology. 2005.08.014.

481

482 [10] Hirao Y, Shimizu M, Iga K, Takenouchi N. Growth of bovine oocyte-granulosa cell complexes
483 cultured individually in microdrops of various sizes. *J Reprod Dev* 2009;55:88–93.

484

485 [11] Taketsuru H, Hirao Y, Takenouchi N, Iga K, Miyano T. Effect of androstenedione on the
486 growth and meiotic competence of bovine oocytes from early antral follicles. *Zygote*
487 2012;20:407–15. doi: 10.1017/S0967199411000268.

488

489 [12] Hirao Y, Shimizu M, Iga K, Takenouchi N. Optimization of oxygen concentration for growing

490 bovine oocytes in vitro: constant low and high oxygen concentrations compromise the yield of fully
491 grown oocytes. *J Reprod Dev* 2012;58:204-211.

492

493 [13] Sprícigo JFW, Diógenes MN, Leme LO, Guimarães AL, Muterlle CV, Silva BDM., Solà-Oriol
494 D, Pivato I, Silva LP, Dode MAN. Effects of different maturation systems on bovine oocyte quality,
495 plasma membrane phospholipid composition and resistance to vitrification and warming. *PLoS One*
496 2015;10:e0130164. doi: 10.1371/journal.pone.0130164.

497

498 [14] Kruip TA, Dieleman SJ. Steroid hormone concentrations in the fluid of bovine follicles relative
499 to size, quality and stage of the oestrus cycle. *Theriogenology* 1985;24:395–408.

500

501 [15] Beg MA, Bergfelt DR, Kot K, Wiltbank MC, Ginther OJ. Follicular-fluid factors and
502 granulosa-cell gene expression associated with follicle deviation in cattle. *Biol Reprod*
503 2001;64:432–41.

504

505 [16] Orly J, Sato G, Erickson GF. Serum suppresses the expression of hormonally induced functions
506 in cultured granulosa cells. *Cell* 1980;20:817–27. doi:10.1016/0092-8674(80)90328-1

507

508 [17] Kayani AR, Glistler C, Knight PG. Evidence for an inhibitory role of bone morphogenetic
509 protein(s) in the follicular-luteal transition in cattle. *Reproduction* 2009;137:67–78. doi:
510 10.1530/REP-08-0198.

511

512 [18] Senbon S, Miyano T. Bovine oocytes in early antral follicles grow in serum-free media: effect
513 of hypoxanthine on follicular morphology and oocyte growth. *Zygote* 2002;10:301–9. doi:
514 10.1017/S0967199402004033

515

516 [19] Senbon S, Fukumi Y, Hamawaki A, Yoshikawa M, Miyano T. Bovine oocytes grown in
517 serum-free medium acquire fertilization competence. *J Reprod Dev* 2004;50:541–7. doi:
518 10.1262/jrd.50.541

519

520 [20] Yang Y, Kanno C, Huang W, Kang SS, Yanagawa Y, Nagano M. Effect of bone morphogenetic
521 protein-4 on in vitro growth, steroidogenesis and subsequent developmental competence of the
522 oocyte-granulosa cell complex derived from bovine early antral follicles. *Reprod Biol Endocrinol*
523 2016;14:3. doi: 10.1186/s12958-016-0137-1.

524

525 [21] Glistler C, Kemp CF, Knight PG. Bone morphogenetic protein (BMP) ligands and receptors in
526 bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential

527 modulation of Smad-1 phosphorylation by follistatin. *Reproduction* 2004;127:239–54. doi:
528 10.1530/rep.1.00090.

529

530 [22] Erickson GF, Shimasaki S. The spatiotemporal expression pattern of the bone morphogenetic
531 protein family in rat ovary cell types during the estrous cycle. *Reprod Biol Endocrinol* 2003;1:9.
532

533 [23] Kayamori T, Kosaka N, Miyamoto A, Shimizu T. The differential pathways of bone
534 morphogenetic protein (BMP)-4 and -7 in the suppression of the bovine granulosa cell apoptosis.
535 *Mol Cell Biochem* 2009;323:161–8. doi: 10.1007/s11010-008-9976-1.

536

537 [24]. Yamashita H, Murayama C, Takasugi R, Miyamoto A, Shimizu T. BMP-4 suppresses
538 progesterone production by inhibiting histone H3 acetylation of StAR in bovine granulosa cells in
539 vitro. *Mol Cell Biochem* 2011;348:183–90. doi: 10.1007/s11010-010-0653-9.

540

541 [25] Pierre A, Pisselet C, Dupont J, Mandon-Pépin B, Monniaux D, Monget P, Fabre S. Molecular
542 basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine
543 granulosa cells. *J Mol Endocrinol* 2004;33:805–17. doi: 10.1677/jme.1.01545.

544

545 [26] Inagaki K, Otsuka F, Miyoshi T, Yamashita M, Takahashi M, Goto J, Suzuki J, Makino H.
546 p38-Mitogen-activated protein kinase stimulated steroidogenesis in granulosa cell-oocyte cocultures:
547 role of bone morphogenetic proteins 2 and 4. *Endocrinology* 2009;150:1921–30. doi:
548 10.1210/en.2008-0851.

549

550 [27] Huang W, Nagano M, Kang SS, Yanagawa Y, Takahashi Y. Effects of in vitro growth culture
551 duration and prematuration culture on maturational and developmental competences of bovine
552 oocytes derived from early antral follicles. *Theriogenology* 2013;80:793–9. doi:
553 10.1016/j.theriogenology.2013.07.004.

554

555 [28] Lodde V, Modina S, Galbusera C, Franciosi F, Luciano AM. Large-scale chromatin remodeling
556 in germinal vesicle bovine oocytes: Interplay with gap junction functionality and developmental
557 competence. *Mol Reprod Dev* 2007;74:740–9. doi: 10.1002/mrd.20639

558

559 [29] Nagano M, Kang SS, Koyama K, Huang W, Yanagawa Y, Takahashi Y. In vitro maturation
560 system for individual culture of bovine oocytes using micro-volume multi-well plate. *Jpn J Vet Res*
561 2013;61:149–54. doi:10.14943/jjvr.61.4.149.

562

563 [30] Nagano M, Katagiri S, Takahashi Y. Relationship between bovine oocyte morphology and in

564 vitro developmental potential. *Zygote* 2006;14:53–61. doi:10.1017/S0967199406003510.
565

566 [31] Yanagawa Y, Matsuura Y, Suzuki M, Saga S, Okuyama H, Fukui D, Bando G, Nagano M,
567 Katagiri S, Takahashi Y, Tsubota T. Accessory corpora lutea formation in pregnant Hokkaido sika
568 deer (*Cervus nippon yesoensis*) investigated by examination of ovarian dynamics and steroid
569 hormone concentrations. *J Reprod Dev* 2015;61:61–6. doi: 10.1262/jrd.2014-076.
570

571 [32] Scheetz D, Folger JK, Smith GW, Ireland JJ. Granulosa cells are refractory to FSH action in
572 individuals with a low antral follicle count. *Reprod Fertil Dev* 2012;24:327–36. doi:
573 10.1071/RD11020.
574

575 [33] Hirao Y, Naruse K, Kaneda M, Somfai T, Iga K, Shimizu M, Akagi S, Cao F, Kono T, Nagai T,
576 Takenouchi N. Production of fertile offspring from oocytes grown in vitro by nuclear transfer in
577 cattle. *Biol Reprod* 2013;89:57. doi: 10.1095/biolreprod.113.109439.
578

579 [34] Gutiérrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell
580 culture system: Induction and maintenance of estradiol production, response to follicle-stimulating
581 hormone, and morphological characteristics. *Biol Reprod* 1997;56:608–16.
582

583 [35] O’Shea JD, Rodgers RJ, D’Occhio MJ. Cellular composition of the cyclic corpus luteum of the
584 cow. *J Reprod Fertil* 1989;85:483–7. doi:10.1530/jrf.0.0850483.
585

586 [36] Meidan R, Girsh E, Blum O, Aberdam E. In vitro differentiation of bovine theca and granulosa
587 cells into small and large luteal-like cells: morphological and functional characteristics *Biol Reprod*.
588 1990;43:913–21. doi:10.1095/biolreprod43.6.913.
589

590 [37] Silva JM, Price CA. Effect of follicle-stimulating hormone on steroid secretion and messenger
591 ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in
592 bovine granulosa cells in vitro. *Biol Reprod* 2000;62:186–91.
593

594 [38] Sahmi M, Nicola ES, Silva JM, Price CA. Expression of 17beta- and 3beta-hydroxysteroid
595 dehydrogenases and steroidogenic acute regulatory protein in non-luteinizing bovine granulosa cells
596 in vitro. *Mol Cell Endocrinol* 2004;223:43–54. doi:10.1016/j.mce.2004.05.010.
597

598 [39] Portela M, Zamberlam G, Price CA. Cell plating density alters the ratio of estrogenic to
599 progestagenic enzyme gene expression in cultured granulosa cells. *Fertil Steril* 2010;93:2050–5.
600 doi: 10.1016/j.fertnstert.2009.01.151.

601

602 [40] Huang W, Kang SS, Yanagawa Y, Yang Y, Takahashi Y, Nagano M. Effects of in vitro-growth
603 culture duration on fertilizability of bovine growing oocytes and proliferation of cells surrounding
604 oocytes. *Jpn J Vet Res* 2014;62:135–41. doi: 10.14943/jjvr.62.3.135.

605

606 [41] Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and
607 transcriptional activity. *Mol Reprod Dev* 1995;42:437–42.

608

609 [42] Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Bovine oocyte diameter in relation
610 to developmental competence. *Theriogenology* 1997;48:769–74.
611 doi:10.1016/S0093-691X(97)00300-2.

612

613 [43] Arlotto T, Schwartz JL, First NL, Leibfried-Rutledge ML. Aspects of follicle and oocyte stage
614 that affect in vitro maturation and development of bovine oocytes. *Theriogenology* 1996;45:943–56.

615

616 [44] Fatehi AN, Van den Hurk R, Colenbrander B, Daemen AJ, Van Tol HT, Monteiro RM, Roelen
617 BA, Bevers MM. Expression of bone morphogenetic protein2 (BMP2), BMP4 and BMP receptors in
618 the bovine ovary but absence of effects of BMP2 and BMP4 during IVM on bovine. *Theriogenology*
619 2005;63:872–89. doi:10.1016/j.theriogenology.2004.05.013.

620

621 [45] La Rosa I, Camargo LSA, Pereira MM, Fernandez-Martin R, Paz DA, Salamone DF. Effects of
622 bone morphogenic protein 4 (BMP4) and its inhibitor, Noggin, on in vitro maturation and culture of
623 bovine preimplantation embryos. *Reprod Biol Endocrinol* 2011;9:18. doi: 10.1186/1477-7827-9-18.

624

625 [46] Kańska-Książkiewicz L, Alm H, Torner H, Heleil B, Tuchscherer A, Ryńska B. Mitochondrial
626 aggregation patterns and activity in in vitro cultured bovine oocytes recovered from early antral
627 ovarian follicles. *Theriogenology* 2011;75:662–70.

628

629 [47] Harper KM, Brackett BG. Bovine blastocyst development after in vitro maturation in a defined
630 medium with epidermal growth factor and low concentrations of gonadotropins. *Biol Reprod*
631 1993;48:409–16.

632

633 [48] Endo M, Kawahara-Miki R, Cao F, Kimura K, Kuwayama T, Monji Y, Iwata H. Estradiol
634 supports in vitro development of bovine early antral follicles. *Reproduction* 2013;145:85–96. doi:
635 10.1530/REP-12-0319.

636

637

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.

695
696
697
698
699
700

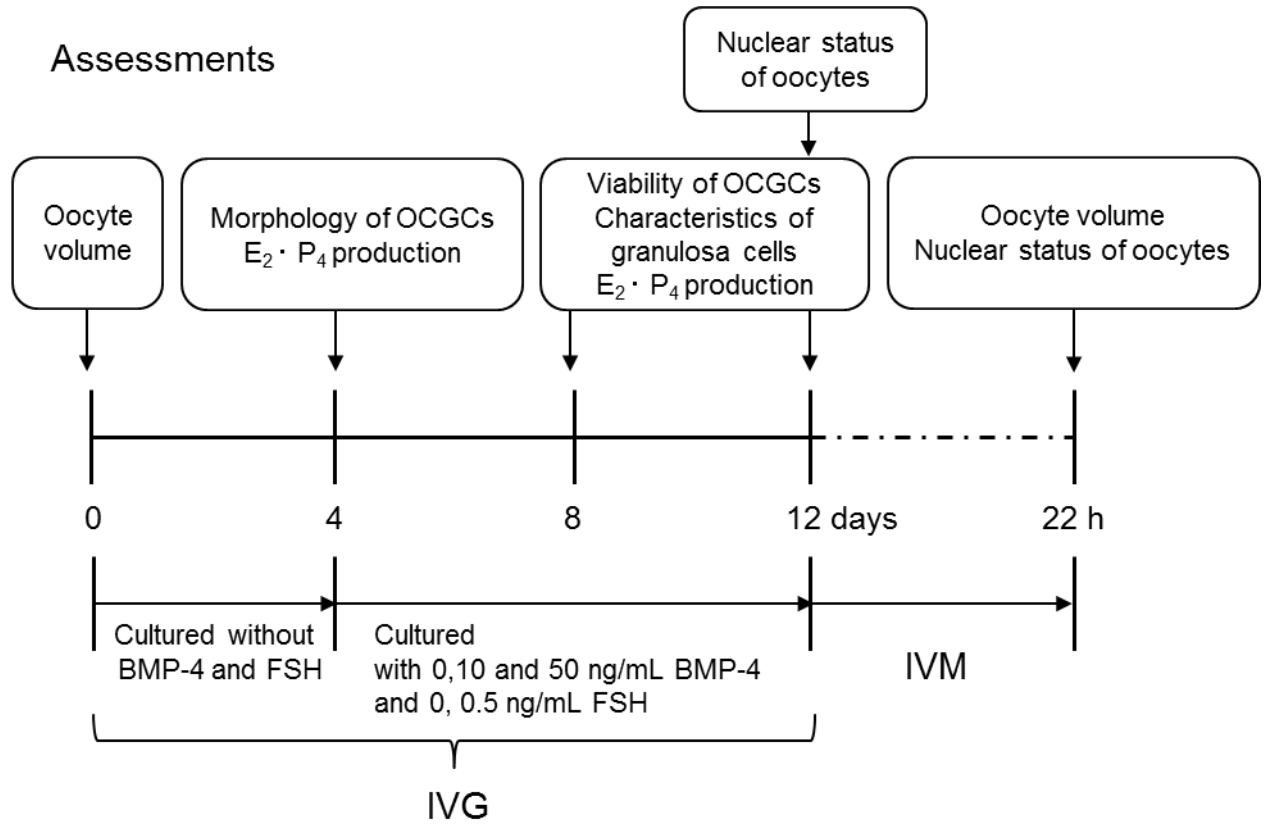
Fig. 1



701
702

703 Fig. 2

704



705

706

707

708 Fig. 3

709

710

711

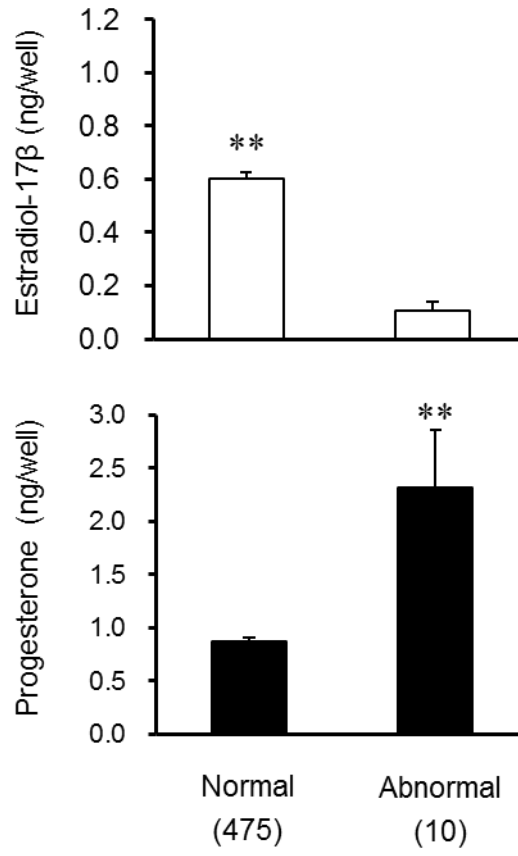
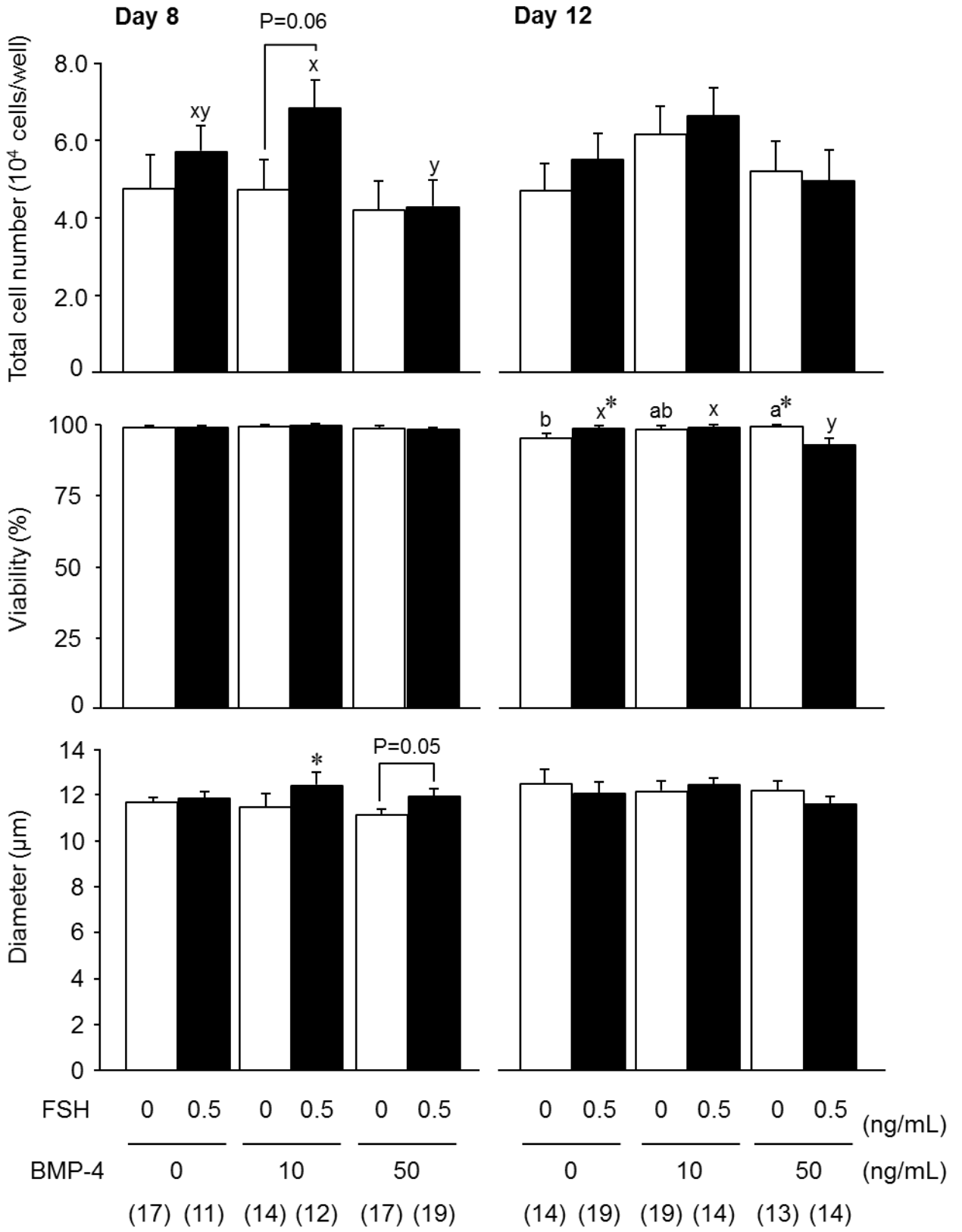
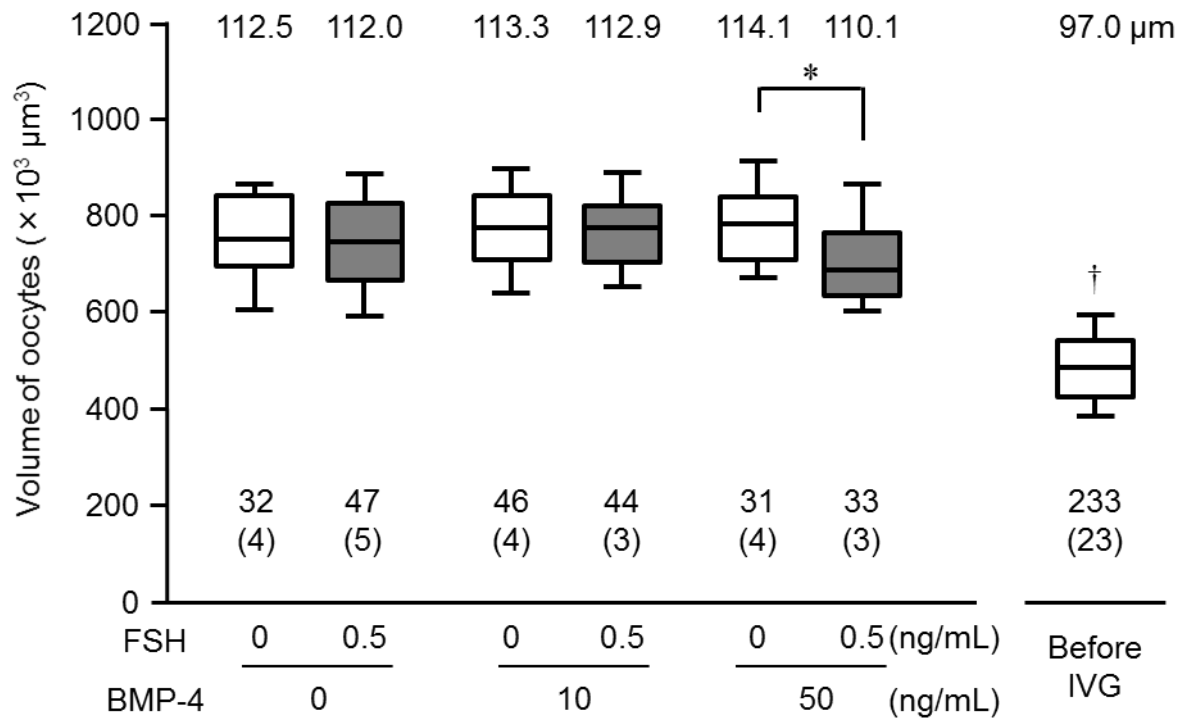


Fig. 4



714
715
716

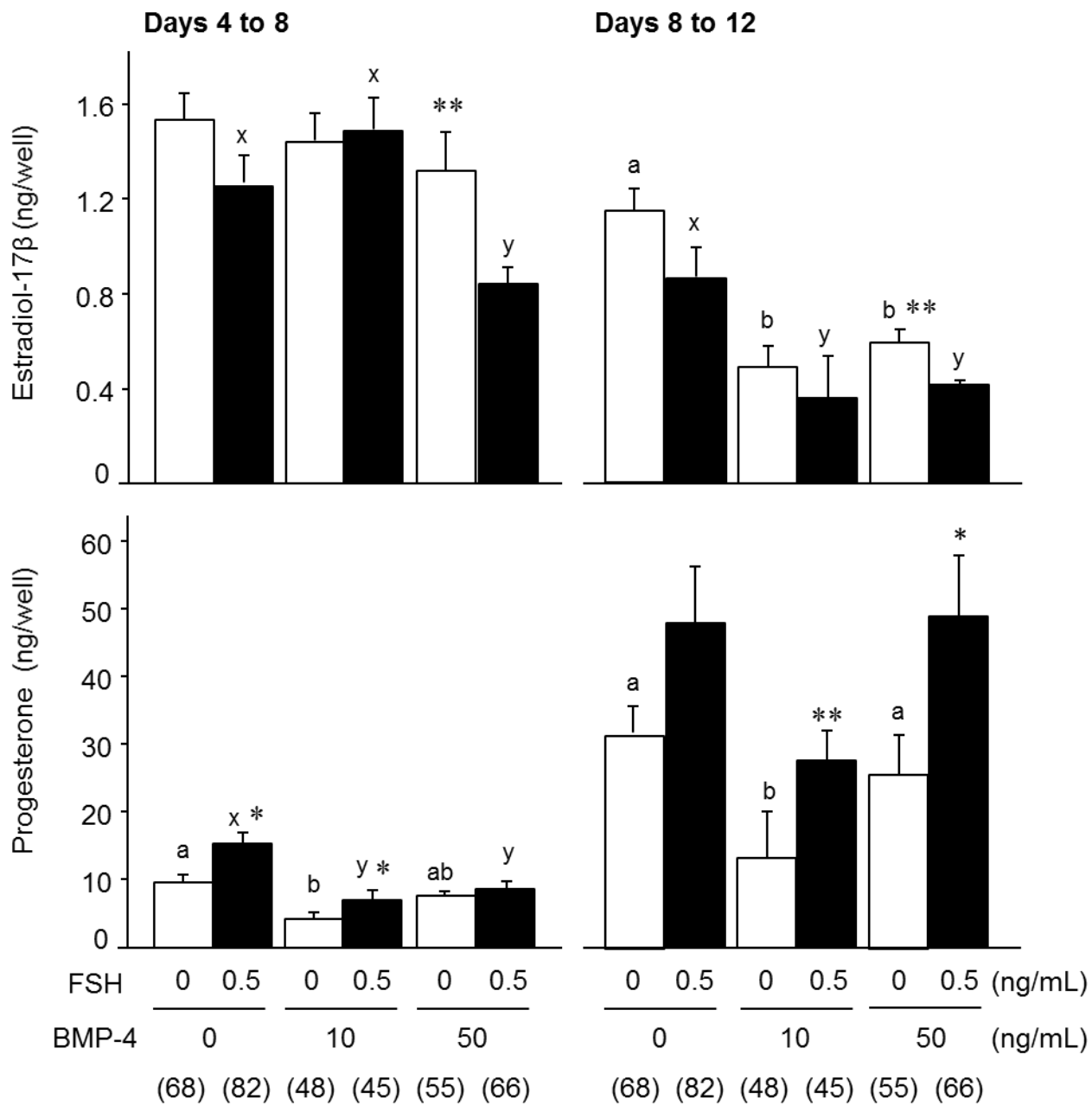
Fig. 5



717
718

719 Fig. 6

720



721

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 <i>in vivo</i>		79 (7)	92.4 ^a

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

736

737

738

739 Table 3. Relationship between antrum formation in the granulosa cell layer on day 12 and
740 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

741 Values are presented as means ± SD.

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

743 Table 4. Relationship between the nuclear maturation of oocytes, oocyte growth, steroidogenesis, and antrum formation by oocyte-cumulus-granulosa complexes
 744 (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)

745 Values are presented as means ± SD.

746 * Oocytes at metaphase II were defined as mature.

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

748

749

750