In Vitro Growth of Bovine Oocytes Cultured with Steroid Hormones

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博士論文

In Vitro Growth of Bovine Oocytes Cultured with Steroid Hormones

ウシ卵母細胞の体外発育に及ぼすステロイドホルモンの影響

平成 **28** 年 **1** 月

神戸大学大学院農学研究科

牧田　美穂

Index

Abbreviations

A_4: androstenedione

AI-TI: anaphase I and telophase I

AR: androgen receptor

BSA: bovine serum albumin

DAPI: 4',6-diamidino-2-phenylidone

DHT: dihydrotestosterone

E_2: 17β-estradiol

FBS: fetal bovine serum

FC: filamentous chromatin

FSH: follicle stimulating hormone

GDF-9: growth differentiation factor-9

GV: germinal vesicle I–IV

GVBD: germinal vesicle breakdown

HEPES-199: HEPES-buffered medium 199

hMG: human menopausal gonadotropin

IGF: insulin-like growth factor

IgG: immunoglobulin G

LH: luteinizing hormone

MI: metaphase I

MII: metaphase II

OGC: oocyte–granulosa cell complex

OHF: hydroxyflutamide

PBS: phosphate-buffered saline

PFA: paraformaldehyde

Phalloidin-FITC: fluorescein isothiocyanate-labeled phalloidin

PN: pronucleus

PVA: polyvinyl alcohol

SC: stringy chromatin

Suc: sucrose

T: testosterone

TZP: transzonal projection

α-MEM: Minimum Essential Medium alpha medium

CHAPTER I

General introduction

Mammalian oogenesis starts during embryogenesis. Primordial germ cells arise at the base of the incipient allantois before genital ridge formation. Then, they migrate to hindgut and further migrate to colonize the genital ridge (Saitou and Yamaji, 2012). Primordial germ cells are then called oogonia, and they undergo several times of mitosis and form clusters known as germ cell nests (Tam and Snow, 1981; Pepling and Spradling, 1998). Within the nests, oogonia start to undergo meiosis, then are called oocytes, and arrest meiosis I at diplotene stage (Pepling and Spradling 1998). When ovarian somatic cells, called pre-granulosa cells, invade into the nests, germ cell nests break down and pre-granulosa cells surround individual oocytes to form primordial follicles (Mayo *et al.*, 2007). In the ovary, the primordial follicle is the earliest recognizable follicle and is comprised of a non-growing oocyte and a single layer of flattened granulosa cells (pre-granulosa cells). When oocytes start to grow, surrounding granulosa cells become cubic (Mhawi *et al.*, 1991); these represent primary follicles (Fig. 1). After granulosa cells proliferate and become multilayered, and theca cell layers appear, the follicles are called secondary follicles. As granulosa cells proliferate and the follicle development proceeds, an antral cavity is formed in granulosa cell layers and the follicles become antral

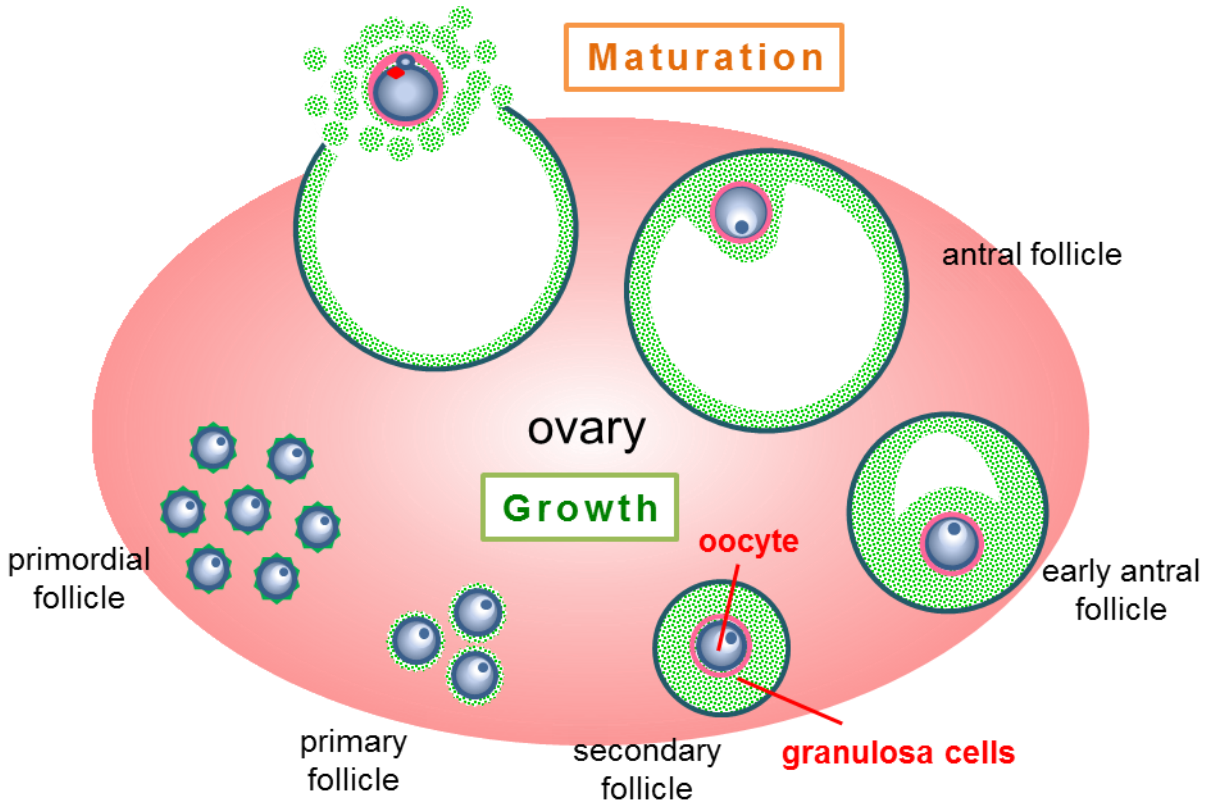

Figure 1 Oocyte growth and maturation in the ovary. When primordial follicles start to develop, they become primary follicles that have a single layer of cubic granulosa cells, then secondary follicles that have multilayered granulosa cells, and antral follicles that have an antral cavity. During follicle development, oocytes grow in the follicles. Only fully grown oocytes can be matured and ovulated.

follicles. During the follicle development, oocytes grow to their final size and acquire meiotic competence. After reaching the final size, the oocytes are released from meiotic arrest upon the luteinizing hormone (LH) surge and then mature to metaphase II to be ovulated. The matured and ovulated oocytes (eggs) are competent to be fertilized with spermatozoa.

Mammalian ovary contains a huge number of oocytes. A small population of oocytes enter the growth phase and an even smaller number of these complete their growth, mature and are ovulated (Baker, 1982). Most of the oocytes in the ovary are lost either before the initiation of their growth or at various stages of their growth. To utilize these oocytes, many technologies have been developed. Since the late 1950s when mammalian oocytes were fertilized *in vitro* for the first time (Chang, 1959), successful *in vitro* fertilization was reported in several mammals in the 1960s and 1970s. Although *in vitro* maturation of mammalian oocytes was first reported in 1935 (Pincus and Enzmann, 1935), the production of offspring from *in vitro* matured oocytes was first reported as late as 1970s. Since then, *in vitro* maturation has been succeeded in variety of species (Picton, 2002; Banwell and Thompson, 2008), and live offspring have been produced by *in vitro* maturation and fertilization of fully grown oocytes in several mammalian species (Mouse: Minato and Toyoda, 1983, Cow: Hanada *et al.*, 1986, Pig: Mattioli *et al.*, 1989, Rat: Vanderhyden and Armstrong, 1989) including the human (Cha *et al.*, 1991).

More recently, many efforts have been made to develop *in vitro* growth culture systems

of mammalian oocytes. Growth culture systems should satisfy at least the following three conditions: (i) oocytes remain healthy and functional so that they can execute intrinsic programs that direct their growth and development; (ii) granulosa cells that are adjacent to oocytes proliferate efficiently to prevent oocytes from becoming denuded; and (iii) granulosa cells maintain (and develop) appropriate associations with oocytes during the culture period (Hirao, 2011). In 1996, *in vitro* organ culture of neonatal mice ovaries and subsequent oocyte–granulosa cell complex culture succeeded in the production of offspring by the following *in vitro* fertilization and embryo transfer of matured oocytes (Eppig and O'Brien, 1996). Research in rodents has demonstrated the ability of *in vitro* growth culture to produce fertilizable oocytes; however, it was difficult to replicate these systems for murine oocytes in domestic animals whose follicles develop to the much larger size taking a longer period than murine ones. In the domestic species, Hirao *et al.* (1994) first reported the successful *in vitro* growth culture that supported the growth of pig oocytes from preantral follicles to the final size. In addition, offspring have been derived from bovine oocytes of early antral follicles after growth, maturation and fertilization *in vitro*, but with only a few reports (Yamamoto *et al.*, 1999; Hirao *et al.*, 2004). Thus, the reliable method for *in vitro* growth of oocytes in domestic animals remains to be developed.

Steroid hormones have crucial roles in follicle development and oocyte growth. Several

studies using knockout mice have determined that both estrogens (Krege *et al.*, 1998; Dupont *et al.*, 2000; Hewitt and Korach, 2003) and androgens (Hu *et al.*, 2004; Shiina *et al.*, 2006; Walters *et al.*, 2007) are essential for female reproduction. Due to an imbalance of steroidogenesis in the ovaries, 5 to 10% of women in reproductive age are infertile resulting from the polycystic ovarian syndrome (Azziz *et al.*, 2004). *In vitro* growth culture of oocytes from growing follicles has potential to be a new method for infertility treatment as well as animal reproduction and improvement. Although some *in vitro* studies also revealed the contribution of steroid hormones to oocyte growth, how they do so has not been understood. *In vitro* growth culture leads us to understand the mechanism of oocyte growth and the roles of steroid hormones in oocyte growth *in vivo*. This study is conducted to examine the effect of steroid hormones on *in vitro* growth of bovine oocytes isolated from early antral follicles and to determine the roles of steroid hormones in the oocyte growth.

CHAPTER II

In vitro growth culture of bovine oocytes with steroid hormones

Introduction

Bovine ovary is estimated to contain 100,000–200,000 oocytes (Erickson, 1966). However, the number of oocytes that grow completely, mature and are ovulated is less than 200 in entire reproductive life in a cow. Other oocytes are eventually degenerated in the ovaries. In order to utilize these oocytes, many approaches have been made for bringing up oocytes *in vitro* (Eppig *et al.*, 1996; Miyano, 2005). While a variety of culture systems have been developed for mouse oocytes (Belli *et al.*, 2012), there are few successful systems for oocytes from large animals.

In the ovary, a follicle is composed of an oocyte and two types of somatic cells, granulosa cells and theca cells. These somatic cells synthesize and secrete different steroid hormones *in vivo*. According to the two-cell two-gonadotropins theory, theca cells are stimulated by LH to synthesis aromatizable androgens such as androstenedione (A_4) and testosterone (Armstrong *et al.*, 1979), whereas the granulosa cells stimulated by follicle stimulating hormone (FSH) convert androgens to estrogens such as 17β-estradiol (E_2) (Dorrington *et al.*, 1975). These steroid

hormones have critical functions in follicle development.

Several studies have revealed the contribution of steroid hormones to *in vitro* growth of oocytes in different species (Murray *et al.*, 1998; Hirao *et al.*, 2004; Hamel *et al.*, 2005; Tasaki *et al.*, 2013). However, there are few culture systems that allow oocytes to acquire meiotic competence in large animals. Culturing bovine oocyte−granulosa cell complexes (OGCs) isolated from early antral follicles for 14 days in the medium with polyvinylpyrrolidone and E_2, oocytes grow and become competent to mature (Hirao *et al.*, 2004); however, the rate of oocytes competent to reach metaphase II is low in this system.

The objective of this chapter is to determine the effects of steroid hormones on *in vitro* growth of bovine oocytes. OGCs from early antral follicles were cultured for 14 days in the presence of both E_2 and A_4, and the effect of combinations of E_2 and A_4 on *in vitro* growth of bovine oocytes was examined. Next, I examined the effect of androgens on *in vitro* growth of bovine oocytes by culturing OGCs with different androgens including non-aromatizable androgen, dihydrotestosterone (DHT) (Springer and Eckstein, 1971; Karakawa *et al*., 1976) (See Figure 2 for chemical structures of steroid hormones used in this study).

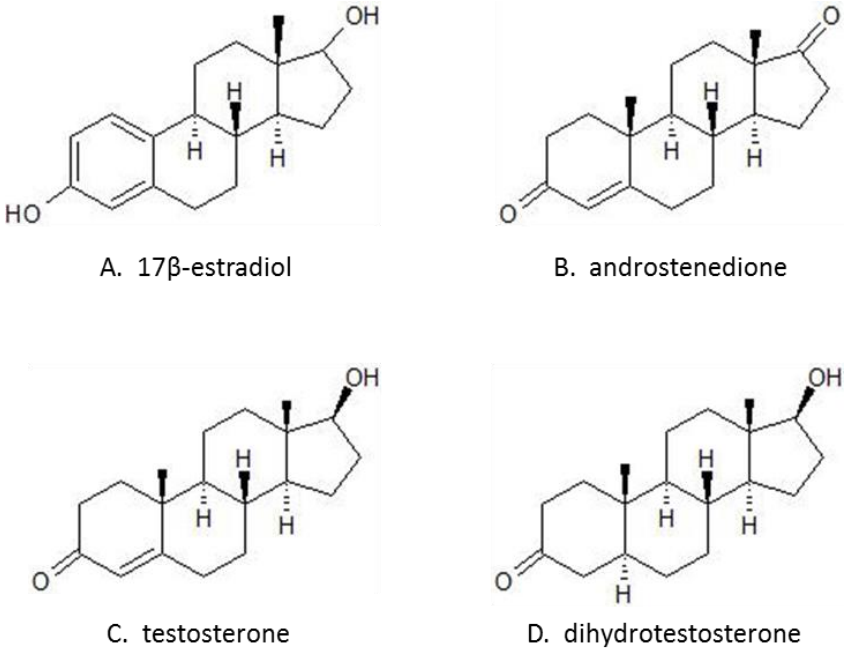

Figure 2 Chemical structures of steroid hormones used in this study. 17β-estradiol (A), androstenedione (B), testosterone (C), and dihydrotestosterone (D) were added to the culture medium for bovine oocyte growth.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of OGCs

Bovine ovaries from Japanese Black Cattle were obtained from a local abattoir and transported to the laboratory. The ovaries were washed once in 0.2% (wt/vol) cetyltrimethylammonium bromide and three times in Dulbecco's phosphate-buffered saline (PBS) containing 0.1% (wt/vol) polyvinyl alcohol (PVA) (PBS–PVA). OGCs consisting of oocytes and cumulus/granulosa cells were collected from early antral follicles and large antral follicles. To collect OGCs from large antral follicles, follicular fluids containing OGCs were drawn up from antral follicles (4–6 mm in diameter) using needles (18 ga; Terumo, Tokyo, Japan) and syringes; these OGCs served as the controls. To collect early antral follicles (0.4–0.7 mm in diameter), ovarian cortical slices (1–1.5 mm) were made using a surgical blade (No. 10; Feather Safety Razor Co. Ltd., Tokyo, Japan) and forceps. Under a dissecting microscope, early antral follicles were dissected from the cortices. The follicles were opened using forceps and a

blade (No. 10) to isolate OGCs in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.1% (wt/vol) PVA, 0.85 mg/mL sodium bicarbonate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 0.08 mg/mL kanamycin sulfate. After measuring diameters of oocytes (excluding the zona pellucida) to the nearest 1 μm using an ocular micrometer attached to an inverted microscope, OGCs that contained oocytes of 90–105 μm diameter were used for *in vitro* growth culture.

In vitro growth culture of oocytes

The OGCs isolated from early antral follicles were subjected to the following two experiments.

Exp. 1 *In vitro* growth culture of oocytes with combinations of E_2 and A_4

The OGCs with growing oocytes from early antral follicles were cultured *in vitro* for 14 days in 0.2 mL culture medium in 96-well culture plates (Biocoat Collagen I Cellware; Becton Dickinson and Co., Franklin Lakes, NJ, USA) under an atmosphere of 5% CO_2 in humidified air at 38.5ºC. The culture medium for oocyte growth was Minimum Essential Medium alpha medium (α-MEM; GIBCO, Invitrogen Co., Scotland, UK) supplemented with 5% (vol/vol) fetal bovine serum (FBS; ICN Biomedicals, Inc., Costa Mesa, CA, USA), 4% (wt/vol) polyvinylpyrrolidone (molecular weight 360,000), 4 mM hypoxanthine, 50 μg/mL ascorbic acid

2-glucoside (Hayashibara Biochemical Laboratories, Okayama, Japan), 55 μg/mL cysteine, 0.05 μM dexamethasone, 1 mM sodium pyruvate, 2.2 mg/mL sodium bicarbonate and 0.08 mg/mL kanamycin sulfate. The medium was also supplemented with E_2 and A_4 (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) either alone (100 ng/mL E_2: $100E_2$ or 100 ng/mL A_4: $100A_4$), or in combinations (10 ng/mL E_2 plus 10 ng/mL A_4: $10E_2$+$10A_4$, 50 ng/mL E_2 plus 50 ng/mL A_4: $50E_2$+$50A_4$, 100 ng/mL E_2 plus 100 ng/mL A_4: $100E_2$+$100A_4$, or 100 ng/mL E_2 plus 10 ng/mL A_4: $100E_2$+$10A_4$). These concentrations were selected based on previous reports (Hirao e*t al.*, 2004; Taketsuru *et al.*, 2012). In addition, because the medium with $100E_2$ or $10A_4$ was reported to be optimal for growth and acquisition of meiotic competence of bovine oocytes *in vitro* by Taketsuru *et al.* (2012), we examined the effect of $100E_2$+$10A_4$.

Exp. 2 *In vitro* growth culture of oocytes with androgens

The OGCs with growing oocytes from early antral follicles were cultured *in vitro* for 14 days. The basic culture medium was same as Exp. 1. The medium was also supplemented with E_2, A_4, testosterone (T; Nacalai Tesque, Kyoto, Japan), and DHT either alone (10 ng/mL E_2: $10E_2$, 10 ng/mL A_4: $10A_4$, 10 ng/mL T: 10T or 10 ng/mL DHT: 10DHT) or in combinations (10 ng/mL E_2 plus 10 ng/mL A_4: $10E_2$+$10A_4$ or 10 ng/mL E_2 plus 10 ng/mL DHT: $10E_2$+10DHT). The OGCs were cultured in 0.2 mL culture medium in 96-well culture plates at 38.5ºC under an

atmosphere of 5% O_2: 5% CO_2: 90% N_2 from Day 0 to Day 6, and an atmosphere of 5% CO_2 in air from Day 7 to Day 14.

In both experiments, the day of OGC isolation was designated Day 0. Half volume of the culture medium was changed with fresh medium every other day after Day 4. On Day 0, 7 and 14, OGCs whose structures had collapsed—those, for example, that exhibited complete detachment of granulosa cells from oocytes and/or contained oocytes that showed cytoplasmic degeneration—were classified as degenerative OGCs. At the end of culture for 14 days, the diameters of oocytes that were enclosed by granulosa cells and showed no sign of degeneration were measured as described above, and subjected to further experiments.

Before and after *in vitro* growth culture, oocytes were denuded and fixed with acetic acid-ethanol (1:3), and stained with 1% (wt/vol) aceto-orcein to assess the stage of meiotic division using the Nomarski interference microscope. The oocytes were classified by the morphology of the chromatin and nuclear envelope according to the classification of Motlík *et al.* (1978) and Hirao *et al.* (1995). The stages for oocytes with an intact germinal vesicle were classified as filamentous chromatin (FC), stringy chromatin (SC), and germinal vesicle I–IV (GV). After germinal vesicle breakdown, stages were classified as metaphase I (MI), anaphase I and telophase I (AI–TI), and metaphase II (MII).

In vitro maturation of oocytes

The OGCs with growing and fully grown oocytes collected from early and large antral follicles, respectively, and with surviving oocytes after 14 days of *in vitro* growth culture were cultured in a 50 μL microdrop of the maturation medium covered with paraffin oil at 38.5ºC under an atmosphere of 5% CO_2 in humidified air for 22 h. The maturation medium was bicarbonate-buffered medium 199 supplemented with 10% FBS, 0.1 mg/mL sodium pyruvate, 0.1 IU/mL human menopausal gonadotropin (hMG; Aska Pharmaceutical Co. Ltd., Tokyo, Japan), 0.08 mg/mL kanamycin sulfate, and 2.2 mg/mL sodium bicarbonate. After culture, the oocytes were denuded mechanically using a small-bore pipette with the help of hyaluronidase (from bovine testes). The oocytes were fixed and stained to assess the stage of meiotic division, as described above. Some of the oocytes activated spontaneously and forming female pronuclei (PN) were classified into the PN stage.

Statistical analysis

The rates of OGC integrity and the frequencies of oocytes at each stage of meiotic division were analyzed using Chi-square test. Differences in the mean (± SEM) diameters of oocytes were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

Exp. 1 *In vitro* growth of oocytes with combinations of E_2 and A_4

In vitro growth of oocytes

Figure 3 shows the typical morphology of OGCs during growth culture with or without steroid hormones. After 1 day (Day 1), granulosa cells of each complex adhered to the bottom of the culture plates and began to grow outward (Fig. 3A a1–c1 and B a1–d1). Over the next few days, OGCs showed gentle development due to horizontal and vertical proliferation of granulosa cells. Some complexes in the medium with steroid hormones had formed cavities in their granulosa cell layers by Day 7 (Fig. 3A b2, c2 and B a2–d2), and the cavities further developed until the end of culture. In the medium without steroid hormones (None), almost all of the oocytes had dissociated from their granulosa cells by Day 14; in contrast, in the medium with steroid hormones, oocytes had been enclosed by surrounding granulosa cells and OGCs retained the dome–like structure (Fig. 3A b3, c3 and B a3–d3).

The integrity of OGCs during growth culture is shown in Figure 4. OGC structures collapsed and oocytes became denuded between Day 7 and Day 14 in the medium without

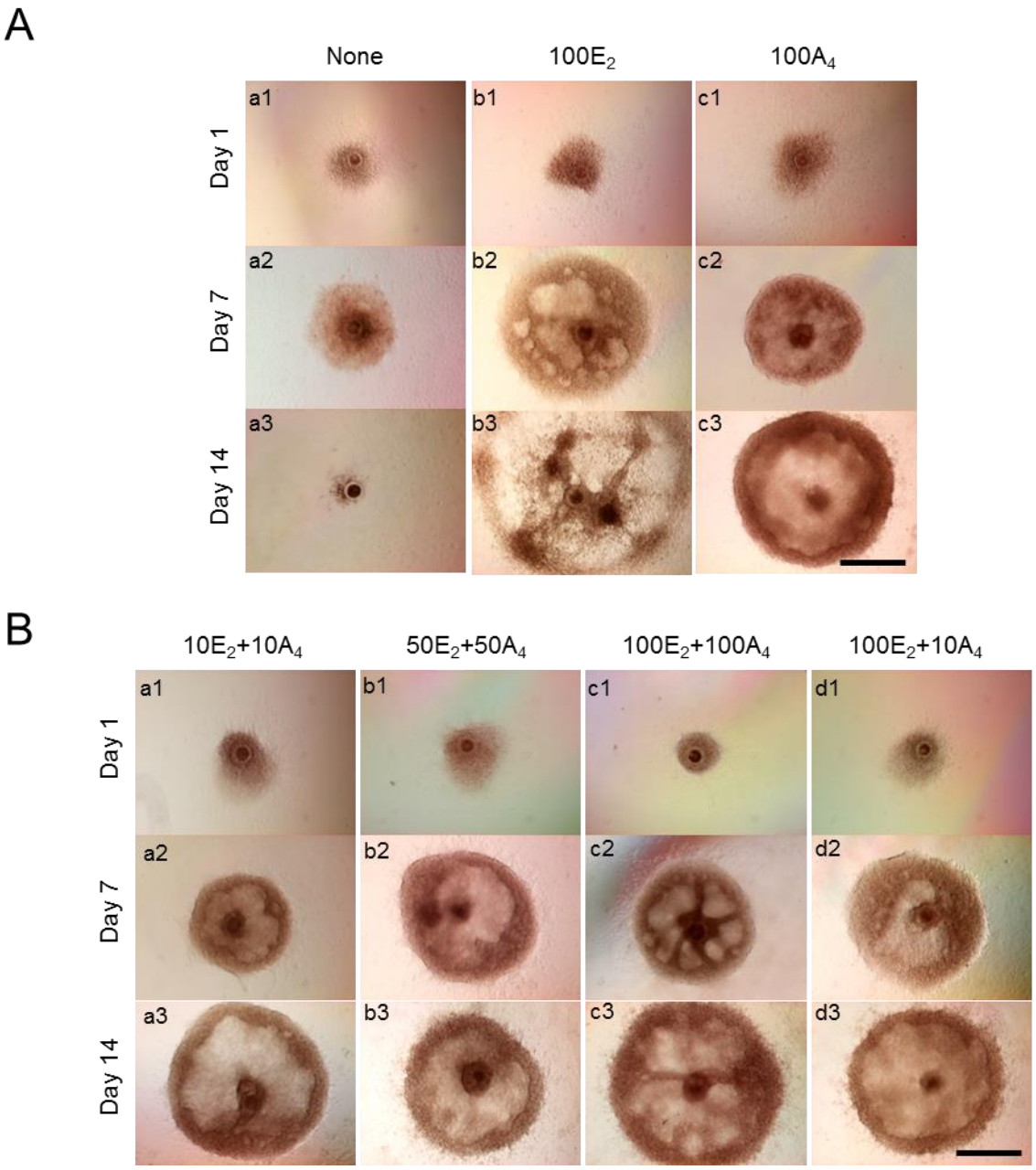

Figure 3 Typical morphology of bovine oocyte–granulosa cell complexes (OGCs) during growth culture. OGCs were cultured for 14 days in the medium with E_2 or A_4 alone (A) or in combinations of E_2 and A_4 (B). Some of the OGCs in the medium with steroid hormones formed cavities in their granulosa cell layers by Day 7 (A b2, c2 and B a2–d2). E_2: 17β-estradiol, and A_4: androstenedione. $100E_2$: 100 ng/mL E_2, $100A_4$: 100 ng/mL A_4, $10E_2+10A_4$: 10 ng/mL E_2 plus 10 ng/mL A_4, $50E_2+50A_4$: 50 ng/mL E_2 plus 50 ng/mL A_4, $100E_2+100A_4$: 100 ng/mL E_2 plus 100 ng/mL A_4, and $100E_2+10A_4$: 100 ng/mL E_2 plus 10 ng/mL A_4. Scale bars represent 500 μm.

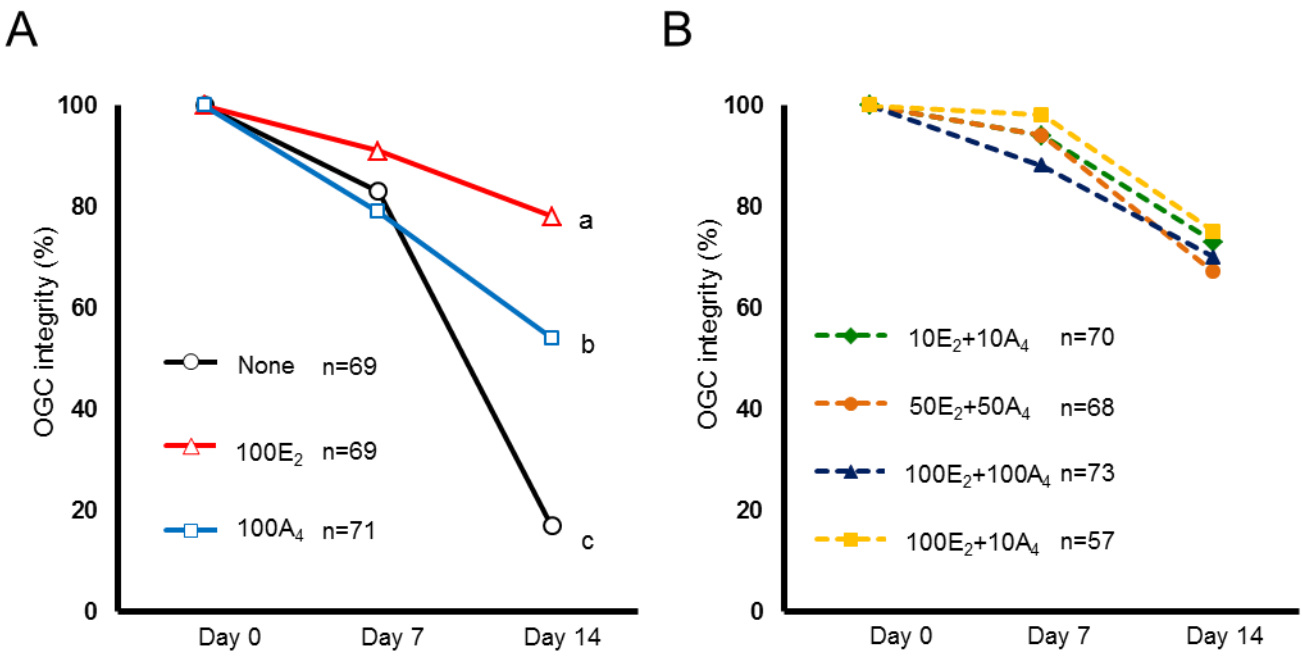

Figure 4 Integrity of bovine oocyte–granulosa cell complexes (OGCs) during growth culture. On Day 0, 7 and 14, OGCs in the medium with either E_2 or A_4 alone (A) or in combinations of E_2 and A_4 (B) that showed degenerative signs, such as cytoplasmic degeneration of oocytes and/or complete detachment of granulosa cells from oocytes, were classified as degenerative complexes. See the footnotes in Figure 3 for the abbreviations. [a–c] Values with different letters differ significantly ($P < 0.05$).

steroid hormones. The OGCs cultured with any steroid hormone maintained the structures that contained surviving oocytes enclosed by granulosa cells throughout the culture period. For the OGCs cultured with E_2 or A_4 alone (Fig. 4A), those in the medium with E_2 showed higher integrity on Day 14 (78%) than those in the medium with A_4 (54%). OGCs also maintained their structures at high rates in the medium with both E_2 and A_4 ($10E_2$+$10A_4$: 73%, $50E_2$+$50A_4$: 68%, $100E_2$+$100A_4$: 70%, and $100E_2$+$10A_4$: 75%) (Fig. 4B).

Before *in vitro* growth culture, the mean diameter of oocytes collected from early antral follicles was 95.8 ± 0.1 μm (white box on the left in Fig. 5). An increase in oocyte size after *in vitro* growth culture was apparent in all culture media. The mean diameters of the oocytes on Day 14 in the medium with E_2 or A_4 alone ($100E_2$ and $100A_4$) were 115.6 ± 0.8 and 113.9 ± 0.9 μm, respectively, and these were larger than oocytes grown without steroid hormones (None: 109.9 ± 2.3 μm) (color filled boxes in Fig. 5). Similarly, in the media with both E_2 and A_4 ($10E_2$+$10A_4$, $50E_2$+$50A_4$, $100E_2$+$100A_4$, and $100E_2$+$10A_4$), the mean diameters of oocytes on Day 14 were increased to 119.6 ± 0.9, 116.8 ± 0.8, 115.6 ± 0.9 and 119.3 ± 0.8 μm, respectively (striped boxes in Fig. 5). Oocytes cultured with $10E_2$+$10A_4$ or $100E_2$+$10A_4$ grew to a size similar to the *in vivo* grown oocytes (119.4 ± 0.4 μm) collected from the 4–6 mm antral follicles (a white box on the right in Fig. 5).

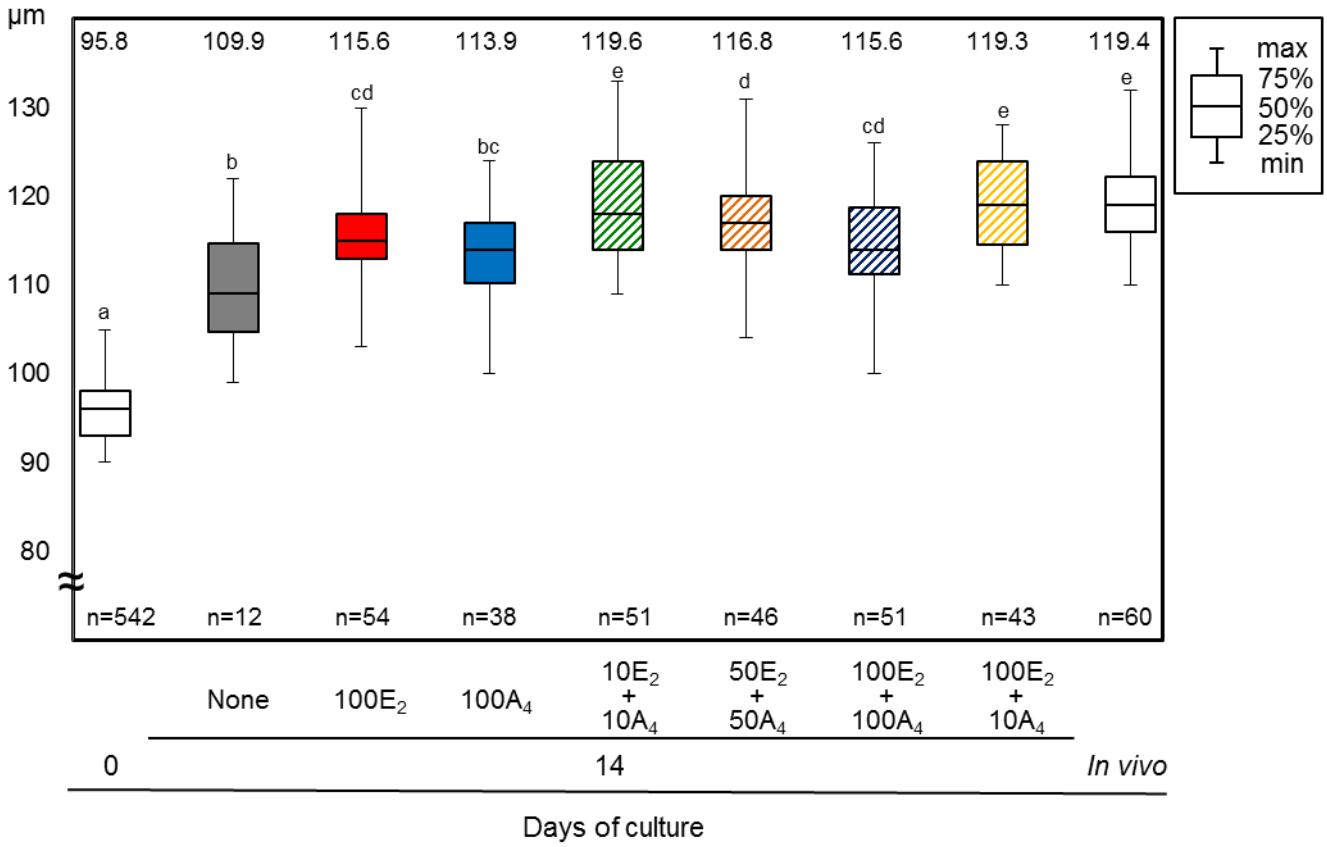

Figure 5 Comparison of bovine oocyte diameters after growth culture for 14 days. The white box on the left represents the diameter of oocytes isolated from early antral follicles (0.4–0.7 mm). The color filled boxes and striped boxes indicate the diameters of oocytes cultured for 14 days in the medium with or without E_2 or A_4 and combinations of E_2 and A_4. The white box on the right represents the diameter of oocytes obtained from antral follicles (4–6 mm). The numbers of oocytes examined are shown at the bottom of each box, and the numbers above the boxes indicate the mean diameters of oocytes (μm). See the footnotes in Figure 3 for the abbreviations. [a–e] Values with different letters differ significantly ($P < 0.05$).

In vitro maturation of oocytes

Growing oocytes isolated from early antral follicles were at 48% FC and 52% SC stages, and all *in vivo* grown oocytes collected from the 4–6 mm antral follicles were at GV (Table 1). After 14 days of growth culture, almost all of the surviving oocytes progressed to GV in all culture media (Table 1).

After 22 h of *in vitro* maturation, 100% of growing oocytes isolated from early antral follicles remained at the germinal vesicle stages (FC–GV); in contrast, 92% of the oocytes grown *in vivo* reached metaphase II (MII) (Table 2). All oocytes cultured *in vitro* for 14 days underwent germinal vesicle breakdown and a portion of them reached MII after *in vitro* maturation culture. Among the surviving oocytes grown in $100E_2$, 39% reached MII, a rate similar to that of oocytes grown without steroid hormones (25%). Oocytes grown in the media with some combinations of E_2 and A_4 showed high percentages of MII oocytes after *in vitro* maturation; in particular, 82% of oocytes grown in $10E_2$+$10A_4$ reached MII, a rate similar to that of *in vivo* grown oocytes (92%). Comparing the rates of MII oocytes to total oocytes initially used for the growth culture, oocytes cultured with steroid hormones matured to MII at higher rates than those cultured without steroid hormones. Fifty-eight percent of oocytes initially used for growth culture reached MII after *in vitro* maturation when cultured for growth in $10E_2$+10A, whereas 5% of oocytes matured to MII when cultured without steroid hormones.

Table 1 Nuclear stage of bovine oocytes after *in vitro* growth culture with E_2 and A_4.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of viable oocytes[3]		No. (%) of oocytes at the stage of [4]					
	E_2	A_4	Day 0	Day 14	FC	SC	GV	MI	AI–TI	MII
0	—	—	—	29	14 (48)	15 (52)	0 (0)	0 (0)	0 (0)	0 (0)
14	0	0	29	4	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)
	100	0	29	23	0 (0)	0 (0)	23 (100)	0 (0)	0 (0)	0 (0)
	0	100	30	15	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)	0 (0)
	10	10	30	23	0 (0)	0 (0)	23 (100)	0 (0)	0 (0)	0 (0)
	50	50	29	19	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)	0 (0)
	100	100	30	20	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)	0 (0)
	100	10	20	14	0 (0)	0 (0)	13 (93)	1 (7)	0 (0)	0 (0)
In vivo	—	—	—	30	0 (0)	0 (0)	30 (100)	0 (0)	0 (0)	0 (0)

[1] Oocyte–granulosa cell complexes collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (14). After *in vitro* growth culture, oocytes were denuded and stained with orcein. Oocytes from early antral follicles (0.4–0.7 mm in diameter) and antral follicles (4–6 mm in diameter) were stained as for *in vivo* control (0 and *In vivo*, respectively).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] After *in vitro* growth culture, viable oocytes were stained with orcein.

[4] FC: filamentous chromatin, SC: stringy chromatin, GV: germinal vesicle I–IV, MI: metaphase I, AI–TI: anaphase I and telophase I, and MII: metaphase II.

Table 2 Meiotic competence of bovine oocytes cultured for growth with combinations of E_2 and A_4 after *in vitro* maturation.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of oocytes used[3]		No. (%) of oocytes at the stage of [4]							
	E_2	A_4	IVG	IVM	FC	SC	GV	MI	AI–TI	MII[5]		PN
0	—	—	—	36	10 (28)	25 (69)	1 (3)	0 (0)	0 (0)	0 (0)		0 (0)
14	0	0	40	8	0 (0)	0 (0)	0 (0)	3 (38)^{ab}	0 (0)	2 (25)^{a}	[5]^{a}	3 (38)^{a}
	100	0	40	31	0 (0)	0 (0)	0 (0)	19 (61)^{a}	0 (0)	12 (39)^{a}	[30]^{b}	0 (0)
	0	100	41	23	0 (0)	0 (0)	0 (0)	8 (35)^{ab}	0 (0)	14 (61)^{ab}	[34]^{bc}	1 (4)^{b}
	10	10	40	28	0 (0)	0 (0)	0 (0)	3 (11)^{bc}	0 (0)	23 (82)^{bc}	[58]^{d}	2 (7)^{b}
	50	50	39	27	0 (0)	0 (0)	0 (0)	7 (26)^{b}	1 (4)	19 (70)^{b}	[49]^{bcd}	0 (0)
	100	100	43	31	0 (0)	0 (0)	0 (0)	12 (39)^{ab}	1 (3)	15 (48)^{ab}	[35]^{bc}	3 (10)^{ab}
	100	10	37	29	0 (0)	0 (0)	0 (0)	9 (31)^{b}	0 (0)	20 (69)^{b}	[54]^{cd}	0 (0)
In vivo	—	—	—	60	0 (0)	0 (0)	0 (0)	4 (7)^{c}	1 (2)	55 (92)^{c}		0 (0)

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* maturation culture before (0) or after 14 days of *in vitro* growth culture (14). OGCs from antral follicles (4–6 mm in diameter) were subjected to *in vitro* maturation culture as for *in vivo* control (*In vivo*).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] After *in vitro* growth culture (IVG), OGCs with surviving oocytes were transferred to *in vitro* maturation culture (IVM).

[4] FC: filamentous chromatin, SC: stringy chromatin, GV: germinal vesicle I–IV, MI: metaphase I, AI–TI: anaphase I and telophase I, MII: metaphase II, and PN: pronuclear stage.

[5] The numbers in [ ] indicate the percentages of the oocytes to the oocyte numbers initially used for IVG.

[a–d] Values with different superscripts in the same column differ significantly ($P < 0.05$).

Exp. 2 *In vitro* growth of oocytes with androgens

In vitro growth of oocytes

Figure 6 shows the typical morphology of OGCs during growth culture with androgens. Similar to Figure 3, granulosa cells of each complex started to grow after Day 1 in all culture media (Fig. 6A a1–d1 and B a1–c1). In some media with steroid hormones, antrum-like cavities were formed in their granulosa cell layers by Day 7 (Fig. 6A b2–d2 and B a2, c2). However, in the medium without steroid hormones (None) or with 10DHT, proliferation of granulosa cells was less than in the medium with other steroid hormones, and oocytes gradually dissociated from granulosa cells by Day 14 (Fig. 6A a3 and B b3).

The integrity of OGCs during growth culture is shown in Figure 7. In the medium without steroid hormones or with 10DHT, OGC structures collapsed and oocytes became denuded between Day 7 and Day 14. The OGCs cultured with other steroid hormones maintained structures that contained surviving oocytes enclosed by granulosa cells throughout the culture period. The OGCs cultured with E_2 ($10E_2$, $10E_2$+$10A_4$ and $10E_2$+10DHT) showed high integrity on Day 14 (72%, 80% and 71%, respectively) compared to the OGCs cultured with androgens alone ($10A_4$: 59%, 10T: 63%, and 10DHT: 28%).

Before *in vitro* growth culture, the mean diameter of oocytes collected from early antral

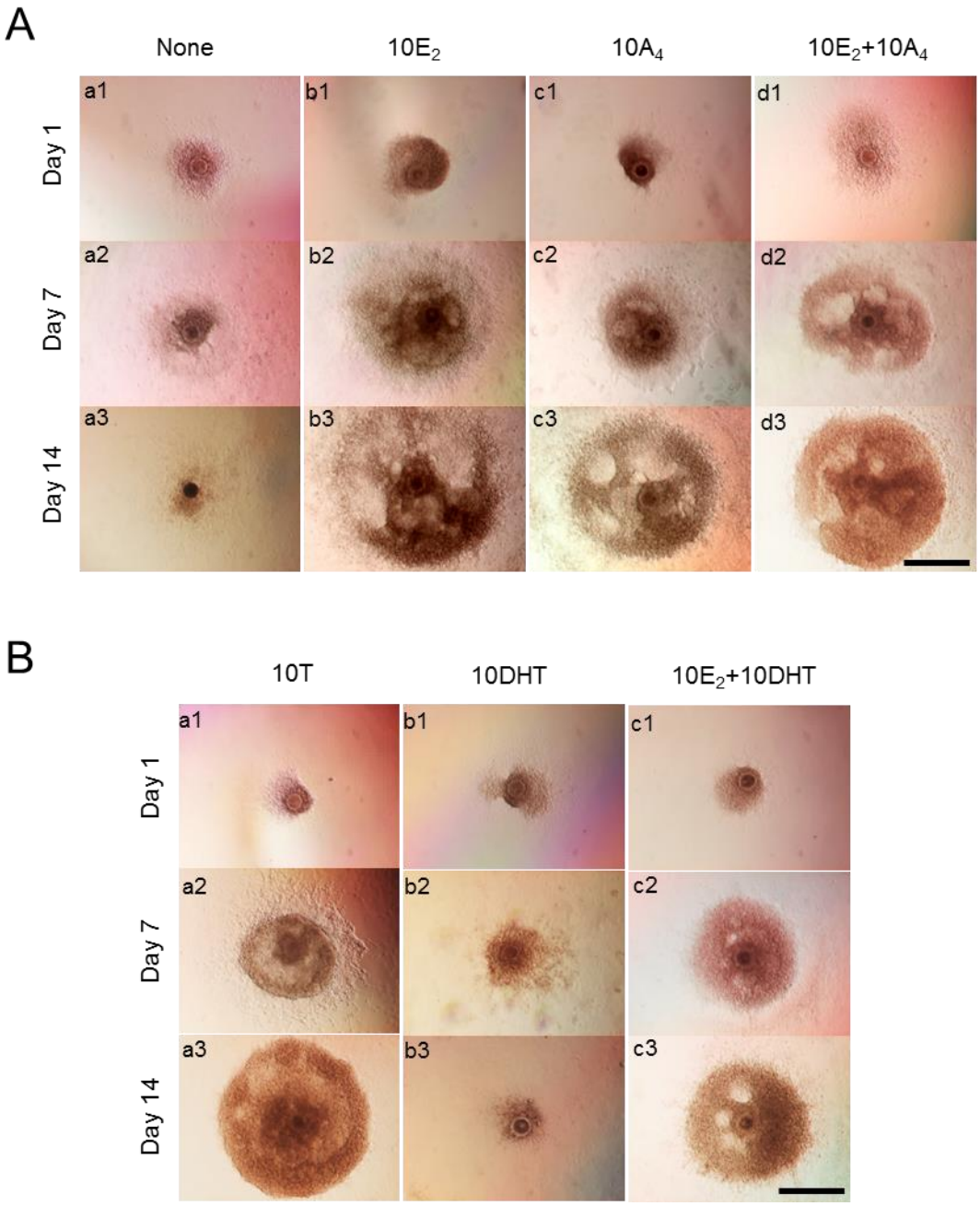

Figure 6 Typical morphology of bovine oocyte–granulosa cell complexes (OGCs) during growth culture with androgens. OGCs were cultured for 14 days in the medium with E_2 and A_4 (A) or T and DHT (B). In the medium with steroid hormones, granulosa cells of each complex proliferated and OGCs developed, except in the medium with DHT alone. E_2: 17β-estradiol, A_4: androstenedione, T: testosterone, and DHT: dihydrotestosterone. $10E_2$: 10 ng/mL E_2, $10A_4$: 10 ng/mL A_4, $10E_2$+$10A_4$: 10 ng/mL E_2 plus 10 ng/mL A_4, 10T: 10 ng/mL T, 10DHT: 10 ng/mL DHT, and $10E_2$+10DHT: 10 ng/mL E_2 plus 10 ng/mL DHT. Scale bars represent 500 μm.

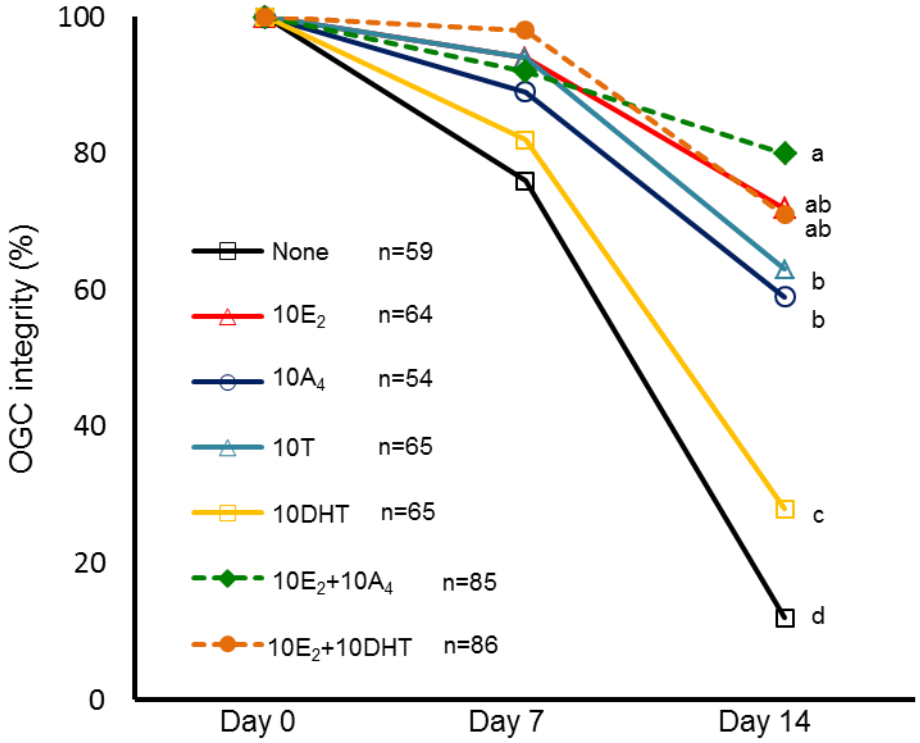

Figure 7 Integrity of bovine oocyte–granulosa cell complexes (OGCs) during growth culture with androgens. On Day 0, 7 and 14, OGCs in the medium with or without steroid hormones that showed degenerative signs, such as cytoplasmic degeneration of oocytes and/or complete detachment of granulosa cells from oocytes, were classified as degenerative complexes. See the footnotes in Figure 6 for the abbreviations. [a–d] Values with different letters differ significantly ($P < 0.05$).

follicles was 94.3 ± 0.1 μm (a white box on the left in Fig. 8). Similar to the results in Exp. 1, oocytes became significantly large in diameter after 14 days of culture in all culture media. The mean diameters of the oocytes on Day 14 in the medium with androgens alone ($10A_4$, 10T and 10DHT) were 119.1 ± 0.9, 118.7 ± 0.9 and 119.0 ± 1.4 μm, respectively, and these were larger than oocytes grown without steroid hormones (None: 114.7 ± 2.3 μm) or with E_2 alone ($10E_2$: 116.0 ± 0.7 μm) (color filled boxes in Fig. 8). In the medium with both E_2 and androgen ($10E_2$+$10A_4$ and $10E_2$+10DHT), the mean diameters of oocytes on Day 14 were increased to 120.0 ± 0.6 and 118.8 ± 0.7 μm, respectively (striped boxes in Fig. 8), and these were similar to the *in vivo* grown oocytes (119.4 ± 0.7 μm) collected from the 4–6 mm antral follicles (white box on the right in Fig. 8).

In vitro maturation of oocytes

Similar to the results in Exp. 1, growing oocytes isolated from early antral follicles were at 57% FC and 43% SC stages (Table 3), and 100% of these oocytes remained at the germinal vesicle stages (35% FC and 65% SC) after 22 h of *in vitro* maturation (Table 4). *In vivo* grown oocytes collected from the 4–6 mm antral follicles were at GV (Table 3), and 75% of the oocytes reached MII after 22 h of *in vitro* maturation (Table 4). Almost all oocytes cultured for growth *in vitro* were at GV; however, some oocytes had resumed meiosis (Table 3). After the

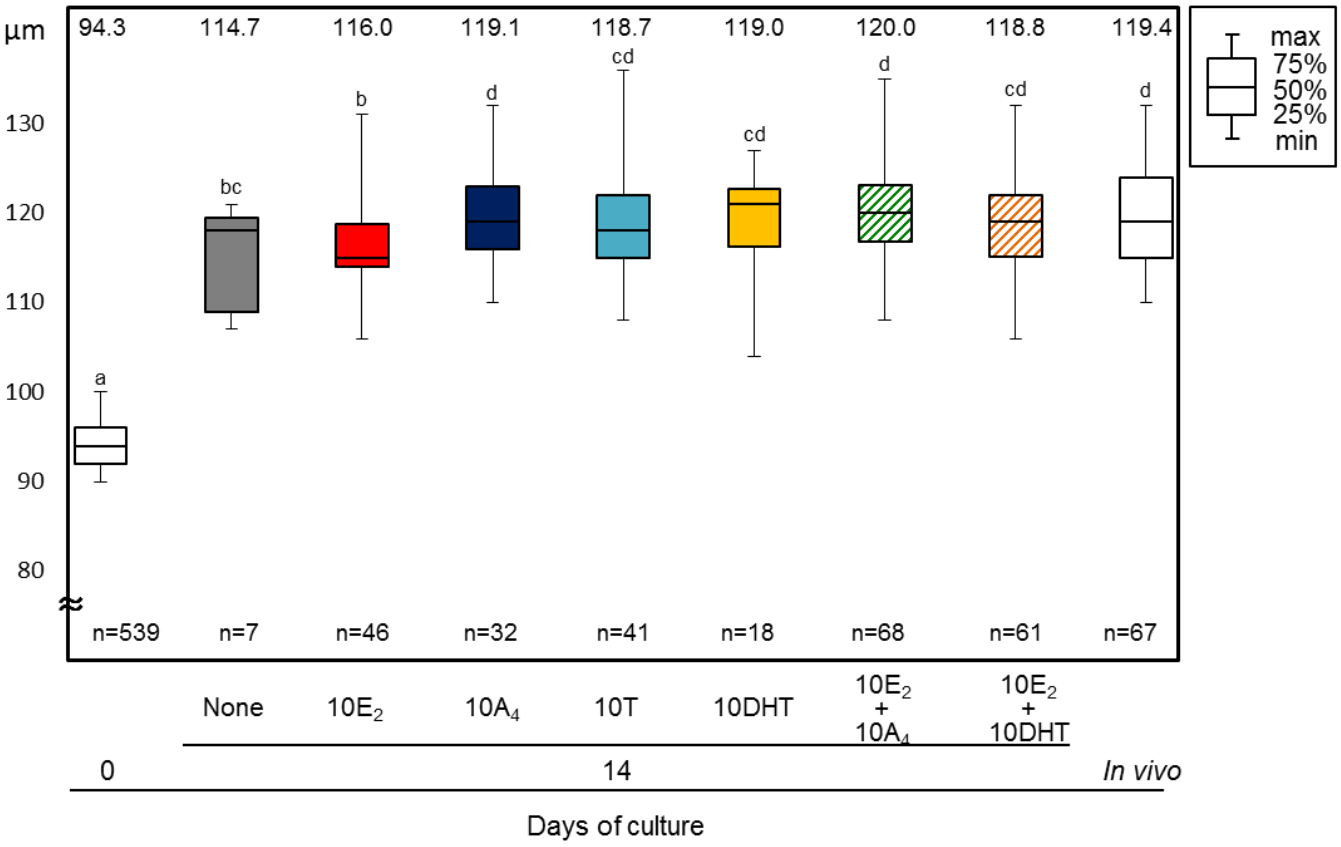

Figure 8 Comparison of bovine oocyte diameters after growth culture for 14 days with androgens. The white box on the left represents the diameter of oocytes isolated from early antral follicles (0.4–0.7 mm). The color filled boxes and striped boxes indicate the diameters of oocytes cultured for 14 days in the medium with or without steroid hormones. The white box on the right represents the diameter of oocytes obtained from antral follicles (4–6 mm). The numbers of oocytes examined are shown at the bottom of each box, and the numbers above the boxes indicate the mean diameters of oocytes (μm). See the footnotes in Figure 6 for the abbreviations. [a–d] Values with different letters differ significantly ($P < 0.05$).

Table 3 Nuclear stage of bovine oocytes after *in vitro* growth culture with androgens.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]				No. of viable oocytes[3]		No. (%) of oocytes at the stage of [4]					
	E_2	A_4	T	DHT	Day 0	Day 14	FC	SC	GV	MI	AI–TI	MII
0	—	—	—	—	—	30	17 (57)	13 (43)	0 (0)	0 (0)	0 (0)	0 (0)
14	0	0	0	0	20	3	0 (0)	0 (0)	2 (67)	1 (33)	0 (0)	0 (0)
	10	0	0	0	20	15	0 (0)	0 (0)	14 (93)	1 (7)	0 (0)	0 (0)
	0	10	0	0	20	12	0 (0)	0 (0)	12 (100)	0 (0)	0 (0)	0 (0)
	0	0	10	0	20	13	0 (0)	0 (0)	12 (92)	1 (8)	0 (0)	0 (0)
	0	0	0	10	20	6	0 (0)	0 (0)	4 (67)	2 (33)	0 (0)	0 (0)
	10	10	0	0	25	17	0 (0)	0 (0)	16 (94)	1 (6)	0 (0)	0 (0)
	10	0	0	10	19	14	0 (0)	0 (0)	14 (100)	0 (0)	0 (0)	0 (0)
In vivo	—	—	—	—	—	29	0 (0)	0 (0)	29 (100)	0 (0)	0 (0)	0 (0)

[1] Oocyte–granulosa cell complexes collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (14). After *in vitro* growth culture, oocytes were denuded and stained with orcein. Oocytes from early antral follicles (0.4–0.7 mm in diameter) and antral follicles (4–6 mm in diameter) were stained as for *in vivo* control (0 and *In vivo*, respectively).

[2] E_2: 17β-estradiol, A_4: androstenedione, T: testosterone, and DHT: dihydrotestosterone.

[3] After *in vitro* growth culture, viable oocytes were stained with orcein.

[4] FC: filamentous chromatin, SC: stringy chromatin, GV: germinal vesicle I–IV, MI: metaphase I, AI–TI: anaphase I and telophase I, and MII: metaphase II.

Table 4 Meiotic competence of bovine oocytes cultured for growth with androgens after *in vitro* maturation.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]				No. of oocytes used[3]		No. (%) of oocytes at the stage of [4]									
	E_2	A_4	T	DHT	IVG	IVM	FC	SC	GV	LD	MI	AI–TI	MII[5]		PN	DG
0	—	—	—	—	—	31	11 (35)	20 (65)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)
14	0	0	0	0	39	4	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	1 (25)^{abc}	[5]^{a}	0 (0)	1 (25)^{ab}
	10	0	0	0	44	31	0 (0)	0 (0)	1 (3)	1 (3)	14 (45)	1 (3)	11 (35)^{ab}	[25]^{b}	1 (3)	2 (6)^{a}
	0	10	0	0	34	20	0 (0)	0 (0)	0 (0)	0 (0)	5 (25)	0 (0)	14 (70)^{c}	[41]^{bc}	1 (5)	0 (0)
	0	0	10	0	45	28	0 (0)	0 (0)	0 (0)	0 (0)	6 (21)	0 (0)	17 (61)^{bc}	[37]^{b}	0 (0)	5 (18)^{a}
	0	0	0	10	45	12	0 (0)	0 (0)	1 (8)	0 (0)	2 (16)	0 (0)	3 (25)^{a}	[7]^{a}	0 (0)	6 (50)^{b}
	10	10	0	0	60	51	0 (0)	0 (0)	0 (0)	0 (0)	15 (29)	0 (0)	31 (61)^{c}	[52]^{c}	1 (2)	4 (8)^{a}
	10	0	0	10	67	47	0 (0)	0 (0)	0 (0)	0 (0)	14 (30)	0 (0)	29 (62)^{c}	[43]^{c}	3 (61)	1 (2)^{a}
In vivo	—	—	—	—	—	36	0 (0)	0 (0)	0 (0)	0 (0)	9 (25)	0 (0)	27 (75)^{c}		0 (0)	0 (0)

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* maturation culture before (0) or after 14 days of i*n vitro* growth culture (14). OGCs from antral follicles (4–6 mm in diameter) were subjected to *in vitro* maturation culture as for *in vivo* control (*In vivo*).

[2] E_2: 17β-estradiol, A_4: androstenedione, T: testosterone, and DHT: dihydrotestosterone.

[3] After *in vitro* growth culture (IVG), OGCs with surviving oocytes were transferred to *in vitro* maturation culture (IVM).

[4] FC: filamentous chromatin, SC: stringy chromatin, GV: germinal vesicle I–IV, MI: metaphase I, AI–TI: anaphase I and telophase I, MII: metaphase II, PN: pronuclear stage, and DG: degeneration.

[5] The numbers in [ ] indicate the percentages of the oocytes to the oocyte numbers initially used for IVG.

[a–c] Values with different superscripts in the same column differ significantly ($P < 0.05$).

subsequent *in vitro* maturation culture, some oocytes cultured for growth *in vitro* reached MII. Among surviving oocytes on Day 14, oocytes grown in $10A_4$ and 10T showed high percentages of MII oocytes (70% and 61%, respectively) compared to those in $10E_2$ (35%) (Table 4). In terms of the percentage of MII oocytes to total oocytes initially used for growth culture, however, there was no significant difference between the rates grown in $10E_2$, $10A_4$ and 10T. Comparing the rates of MII oocytes to total oocytes initially used for the growth culture, oocytes grown with steroid hormones matured to MII at higher rates than those cultured without steroid hormones (5%), except those grown in 10DHT (7%). Fifty-two percent and 43% of oocytes grown in $10E_2$+$10A_4$ and $10E_2$+10DHT reached MII, whereas 25% of oocytes matured to MII when cultured with E_2 alone ($10E_2$).

Discussion

I have shown here that culturing bovine OGCs in the medium with combinations of E_2 and A_4, especially $10E_2$+$10A_4$, resulted in higher oocyte survivability and maturation rates than with E_2 or A_4 alone. The integrity of OGCs was not maintained in the medium with DHT alone, while OGCs maintained their structure in the medium with E_2, A_4 or T alone. Additionally, higher integrity of OGCs was shown in the medium with E_2 than with A_4. These findings indicate E_2 has a beneficial effect on prevention of OGC collapse, or oocyte survival in this culture condition. A recent study shows that E_2 promotes antrum formation of pig preantral follicles (Tasaki *et al.*, 2013). The cadherins are a family of integral membrane glycoproteins and are involved in regulating granulosa cell adhesion. Studies in rats show E_2 enhances the expression of cadherins in granulosa cells *in vitro* (Blaschuk and Farookhi, 1989; Farookhi *et al.*, 1997). Thus, it is inferred that E_2 promotes proliferation and adhesion of granulosa cells so that OGCs maintain their structures.

During *in vitro* growth culture, the essential factors secreted from oocytes or granulosa cells can diffuse into medium before they come into contact with target cells (Eppig *et al.*, 1996). In the medium without steroid hormones, the oocytes became denuded as culture days went by, whereas the OGCs in the medium with E_2, A_4 or T maintained the antrum-like

structure throughout the culture period. The antrum-like structure formed in the present study might provide the microenvironment that prevents the factors from diffusing into medium. Granulosa cells in preantral follicles undergo differentiation into two types of cells, cumulus cells that surround oocytes and mural granulosa cells that line the follicle wall when antral cavities are formed and the follicles become antral follicles (Diaz *et al.*, 2007). In mice, cumulus cells and mural granulosa cells seem to be well-differentiated and there are some cumulus cell or mural granulosa cell specific factors; for example, factors involved in cumulus expansion in cumulus cells, C-type natriuretic peptide in mural granulosa cells, and its receptor, natriuretic peptide receptor 2 in cumulus cells (Tsuji *et al*., 2012). In large animals, there are few reports about the differences between cumulus cells and mural granulosa cells. Dome-like structures formed during *in vitro* growth culture may mimic the follicle structure *in vivo*; however, further studies are needed to investigate the differentiation of granulosa cells during growth culture in large animals.

Although the supplementation of E_2 into the medium resulted in high survivability of oocytes, E_2 alone was not sufficient to promote the growth and the acquisition of meiotic competence of bovine oocytes. The culture conditions used in this study did not include theca cells. Theca cells produce aromatizable androgens (Armstrong *et al.*, 1979). Androgens added to the medium might have compensated for the lack of theca cell in this study. The oocytes

cultured in the medium with androgens alone (10 A_4, 10T or 10DHT) or combinations of E_2 and androgens ($10E_2$+$10A_4$, $100E_2$+$10A_4$ or $10E_2$+10DHT) grew to a size similar to that of *in vivo* grown oocytes collected from 4–6 mm antral follicles. This finding indicates that androgens make a contribution to oocyte growth. In addition, the rate of MII oocytes was higher in oocytes grown with combinations of E_2 and androgens than in those grown with E_2 alone. It is noteworthy that oocytes grown not only with A_4 but also with DHT in the presence of E_2 showed a significant increase in oocyte diameter and high maturation rates. Since DHT is not converted to estrogens, androgens added into the medium appear to affect oocyte growth and maturation directly. The expression of insulin-like growth factor (IGF)-1 and IGF receptor in granulosa cells and oocytes has shown to be regulated by androgens in primates (Vendola *et al.*, 1999a, 1999b). It is reported that IGFs play essential roles in folliculogenesis (Adashi, 1998), and stimulate granulosa cell proliferation (Savion *et al.*, 1981) and differentiation (Baranao and Hammond, 1984).

In conclusion, a combination of E_2 and androgens supported growth of bovine oocytes isolated from early antral follicles to the final size and promoted the acquisition of meiotic competence of the oocytes after 14 days of culture. It was inferred that there are different roles of steroid hormones in oocyte growth; E_2 has a major role in oocyte survivability, while androgens promote the acquisition of meiotic competence of oocytes directly.

CHAPTER III

Roles of steroid hormones in bovine oocyte growth

Introduction

Steroid hormones have crucial roles in folliculogenesis. The results in Chapter II showed that E_2 and androgens supported bovine oocyte growth to the final size and promoted the acquisition of meiotic competence. However, exactly how these steroid hormones contribute to oocyte growth is unclear. The objective of this chapter is to determine the roles of steroid hormones in oocyte growth.

Bovine oocytes grow from 30 μm to their final size 120−125 μm in diameter during growth phase in the ovary (Hyttel *et al.*, 1997). Throughout oocyte growth *in vivo*, oocytes and surrounding granulosa cells interact with each other (Eppig, 2001). Oocytes promote granulosa cell proliferation (Hayashi *et al.*, 1999; Otsuka *et al.*, 2000; Vitt *et al.*, 2000) and differentiation (Eppig, 2001), and granulosa cells support oocyte growth (Brower and Schultz, 1982) and regulate meiotic arrest and resumption of oocytes (Chesnel *et al.*, 1994). This bidirectional communication between oocytes and surrounding granulosa cells is integral to follicle development. Some of the intercellular communications between growing oocytes and

surrounding granulosa cells are facilitated by transzonal projections (TZPs) that extend from the granulosa cells through the zona pellucida to the oolemma. TZPs support oocyte growth by allowing for the exchange of small molecules, amino acids, metabolites and nucleotides between granulosa cells and oocytes (Brower and Schultz, 1982; Anderson and Albertini, 1976). In Chapter III-1, effects of steroid hormones on the connections between oocytes and granulosa cells were examined by assessing the number of TZPs after 14 days of growth culture.

For many years, androgens have been considered damaging for ovarian function (Parker and Mahesh, 1976; Bagnell *et al.*, 1982; Mahajan, 1988; Conway *et al.*, 1990). However, recent *in vivo* and *in vitro* studies have revealed that androgens are important for normal follicle development (Murray *et al.*, 1998; Wang *et al.*, 2001; Hu *et al.*, 2004; Shiina *et al.*, 2006; Walters *et al.*, 2007; Sen *et al.*, 2014). *In vitro* culture of mouse ovarian tissues showed that androgens increase the follicle diameter and enhance the development of preantral follicles (Murray *et al.*, 1998; Wang *et al.*, 2001; Sen *et al.*, 2014). Although there have been a few reports concerning the maturation of *in vitro* grown oocytes from large animals, it has recently reported that A_4 promotes the acquisition of meiotic competence in growing bovine oocytes *in vitro* (Taketsuru *et al.*, 2012). In that study, however, whether androgens affect the acquisition of meiotic competence of oocytes directly is unclear because A_4 can be aromatized to estrogens by granulosa cells. Although the experiment using DHT in Chapter II indicates the direct effect

of androgens on the oocytes growth, further study using androgen receptor (AR) inhibitor is needed to elucidate the androgen actions on the oocyte growth. In Chapter III-2, I examined the ability of AR inhibitor to antagonize the effects of androgens on the oocytes.

III-1 Effects of steroid hormones on oocyte–granulosa cell connections

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Collection of oocyte–granulosa cells complexes

The collection methods of OGCs from bovine ovaries were described in Chapter II.

In vitro growth culture of oocytes

OGCs isolated from bovine early antral follicles (0.4–0.7 mm in diameter) were cultured for 14 days *in vitro*. The culture methods and basic medium were described in Chapter II. OGCs were cultured in growth culture medium with E_2 and A_4 alone (100 ng/ml E_2: $100E_2$ and 100 ng/ml A_4: $100A_4$) or in combinations (10 ng/mL E_2 plus 10 ng/mL A_4: $10E_2$+$10A_4$) under an atmosphere of 5% CO_2 in humidified air at 38.5ºC. These concentrations of steroid hormones were selected based on the results in Chapter II.

Fluorescence microscopy

To assess the association between oocytes and surrounding granulosa cells, the fibrous actin that composes TZPs was stained. The OGCs with growing, fully grown and *in vitro* grown oocytes after 14 days of culture were collected and stained as described below. After being washed twice in PBS–PVA for 15 min each, the OGCs were fixed in 4% paraformaldehyde (PFA) in PBS–PVA for 60 min. Fixed OGCs were washed twice in PBS–PVA and oocytes were denuded mechanically with a fine pipette. Denuded oocytes were then stored in PBS–PVA containing 1 mg/mL bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan; PBS–PVA–BSA) at 4ºC overnight. The oocytes were treated with fluorescein isothiocyanate-labeled Phalloidin (2 μg/mL in PBS–PVA–BSA; Phalloidin-FITC) in an atmosphere of 5% CO_2 in air at 38.5ºC for 90 min. The oocytes were then washed three times for 15 min each in PBS–PVA–BSA before being mounted on glass slides with ProLong Gold Antifade Regent with 4',6-diamidino-2-phenylidone (DAPI) (P36931; Molecular Probes, Invitrogen, Carlsbad, CA, USA) and observed under a confocal laser scanning microscope (FV1000-KDM; Olympus Co., Tokyo, Japan). The number of actin-based TZPs in the widest cross-section of the oocytes was counted using software (Image J).

Statistical analysis

Differences in the mean (± SEM) diameters of oocytes and numbers of TZPs were analyzed by Student's *t*-test. The rate of OGC integrity was analyzed using Chi-square test. Values of $P < 0.05$ were considered significant.

Results

In vitro growth of oocytes

Table 5 shows the integrity of OGCs and mean diameters of oocytes before and after growth culture. In the medium with steroid hormones, more OGCs maintained their structures that contained oocytes enclosed by granulosa cells on Day 14 than those without steroid hormones. In particular, OGCs cultured in the medium with a combination of E_2 and A_4 ($10E_2$+$10A_4$) showed high integrity on Day 14 (75%), while 28% of OGCs maintained their structures in the medium without steroid hormones (Table 5).

Before growth culture, the mean diameters of oocytes collected from early antral follicles were around 95 μm. After 14 days of culture, the mean diameters of oocytes were increased. Compared to the mean diameter of oocytes cultured without steroid hormones (106.4 ± 1.6 μm), oocytes cultured with steroid hormones grew to larger size ($100E_2$: 117.6 ± 1.2, $100A_4$: 115.0 ± 1.5, and $10E_2$+$10A_4$: 119.5 ± 1.5 μm). Similar to the results in Chapter II, oocytes cultured in the medium with a combination of E_2 and A_4 ($10E_2$+$10A_4$) grew to a size similar to the *in vivo* grown oocytes (119.4 ± 0.4 μm) collected from large antral follicles (Table 5).

Table 5 The integrity and mean diameters of bovine oocytes cultured for growth with E_2 and A_4.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of viable oocytes (%)[3]		Mean diameters of oocytes (μm ± SEM)[4]	
	E_2	A_4	Day 0	Day 14	Day 0	Day 14
0	—	—	65	—	95.3 ± 0.4	—
14	0	0	46 (100)	13 (28)[a]	94.3 ± 2.3	106.4 ± 1.6[a]
	100	0	47 (100)	32 (68)[bc]	95.5 ± 0.4	117.6 ± 1.2[bc]
	0	100	48 (100)	25 (52)[b]	96.1 ± 0.5	115.0 ± 1.5[b]
	10	10	48 (100)	36 (75)[c]	96.7 ± 0.5	119.5 ± 1.5[c]
In vivo	—	—	—	30	—	119.4 ± 0.4[c]

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (14). Oocytes from early antral follicles (0.4–0.7 mm in diameter) and antral follicles (4–6 mm in diameter) were examined as for *in vivo* control (0 and *In vivo*, respectively).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] OGCs that exhibited complete detachment of granulosa cells from oocytes and/or contained oocytes that showed cytoplasmic degeneration were classified as degenerative OGCs.

[4] At the end of the growth culture, the diameters of oocytes that were enclosed by granulosa cells and showed no sign of degeneration were measured.

[a–c] Values with different superscripts in the same column differ significantly ($P < 0.05$).

Transzonal projections between oocytes and granulosa cells

Fibrous actin in TZPs between oocytes and granulosa cells became visible by phalloidin staining. There were many TZPs distributed uniformly throughout zona pellucida of growing oocytes isolated from 0.4–0.7 mm follicles before culture and *in vivo* grown oocytes isolated from 4–6 mm follicles (Fig. 9a and b, respectively). Although some OGCs maintained their structures after 14 days of growth culture without steroid hormones, there were few TZPs between oocytes and granulosa cells in these OGCs (Fig. 9c). When oocytes were cultured with E_2 or A_4 alone or in combination, they maintained many TZPs throughout the zona pellucida, indicating that they remained attached to the granulosa cells (Fig. 9d–f).

To compare the association between oocytes and granulosa cells, the number of TZPs in the widest cross-section of oocytes was counted. Before *in vitro* growth culture, growing oocytes isolated from 0.4–0.7 mm follicles and *in vivo* grown oocytes isolated from 4–6 mm follicles had 94.8 ± 5.4 and 125.0 ± 2.8 TZPs/section in their zona pellucida, respectively (Table 6). There was a significant decrease in the number of TZPs in oocytes after 14 days of culture without E_2 or A_4. Such TZP loss was prevented when oocytes were cultured with E_2 or A_4 ($100E_2$: 98.2 ± 5.3, $100A_4$: 63.0 ± 4.9, and $10E_2$+$10A_4$: 85.0 ± 6.1 TZPs/section). Oocytes cultured in $100E_2$ or $10E_2$+$10A_4$ maintained a similar number of TZPs as oocytes before *in vitro* growth culture.

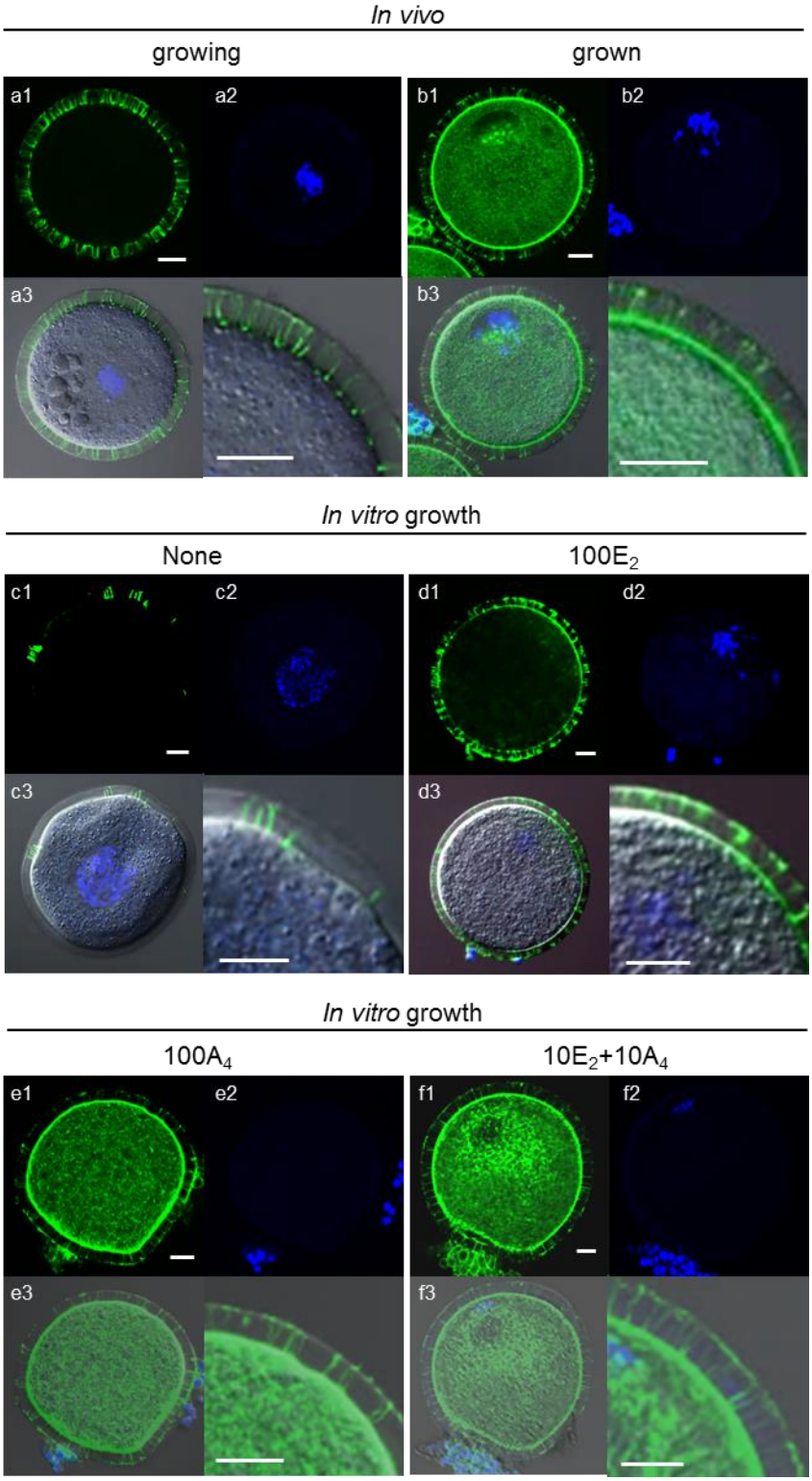

Figure 9 Fluorescence staining of transzonal projections (TZPs) of bovine oocytes. There were many TZPs in growing oocytes collected from early antral follicles (0.4–0.7 mm in diameter) before culture (a1–a3) and *in vivo* grown oocytes isolated from antral follicles (4–6 mm in diameter) (b1–b3). After 14 days of *in vitro* growth culture, oocytes grown without steroid hormones had few TZPs (c1–c3). In contrast, oocytes grown in the presence of steroid hormones maintained many TZPs (d–f). Magnifications show TZPs in the zona pellucida. Phalloidin-FITC staining marks fibrous actin in green. DAPI staining marks the chromatin in blue. Scale bars represent 20 μm.

Table 6 Number of transzonal projections in bovine oocytes before and after *in vitro* growth culture.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of oocytes used	Number of TZPs (mean ± SEM)[3]
	E_2	A_4		
0	—	—	22	94.8 ± 5.4[a]
14	0	0	12	22.8 ± 7.4[b]
	100	0	22	98.2 ± 5.3[a]
	0	100	21	63.0 ± 4.9[c]
	10	10	18	85.0 ± 6.1[a]
In vivo	—	—	27	125.0 ± 2.8[d]

[1] Oocytes collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to fluorescence staining before (0) or after 14 days of *in vitro* growth culture (14). At the end of the growth culture, only surviving oocytes were stained. Oocytes from antral follicles (4–6 mm in diameter) were stained as for *in vivo* control (*In vivo*).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] Transzonal projections (TZPs) in the widest cross-sections of oocytes were counted using the Image J software.

[a–d] Values with different superscripts differ significantly ($P < 0.05$).

III-2 Effect of the androgen receptor inhibitor on bovine oocyte growth

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Collection of oocyte–granulosa cells complexes

The collection methods of OGCs from bovine ovaries were described in Chapter II.

In vitro growth culture of oocytes

In vitro growth culture was performed according to a procedure described in Chapter II with slight modifications. OGCs isolated from bovine early antral follicles (0.4–0.7 mm in diameter) were cultured for 14 days *in vitro*. Briefly, the OGCs with growing oocytes isolated from early antral follicles were individually cultured for 14 days at 38.5ºC under an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 from Day 0 to Day 6, and an atmosphere of 5% CO_2 in humidified air from Day 7 to Day 14. The culture methods and basic medium were described in Chapter II. Combinations of steroid hormones (0 or 10 ng/ml E_2, A_4, and DHT) and

hydroxyflutamide (OHF; 0, 1 or 5 μg/ml), which is an AR antagonist and is widely used as an AR inhibitor, were added to the growth culture medium.

In vitro maturation of oocytes

The OGCs with growing and fully grown oocytes collected from the early and large antral follicles, respectively, and those with surviving oocytes after 14 days of *in vitro* growth culture were further cultured in 50 μL microdrops of the maturation medium covered with paraffin oil at 38.5ºC under an atmosphere of 5% CO_2 in humidified air for 22 h. The maturation method and medium were described in Chapter II. After culture for 22 h, the oocytes were denuded mechanically using a small-bore pipette with the help of 0.1% (wt/vol) hyaluronidase. The oocytes were then fixed and stained to assess the stage of meiotic division.

Immunofluorescence staining for androgen receptors

The OGCs collected from early antral follicles were stained as follows. After being slightly denuded with a fine pipette, the oocytes with granulosa cells were washed twice in PBS–PVA for 15 min each and fixed in 4% (wt/vol) PFA in PBS–PVA containing 0.2% (vol/vol) Triton X-100 (Nacalai Tesque Inc.) for 60 min. Fixed oocytes were washed three times in PBS–PVA for 15 min each and then blocked in PBS–PVA–BSA at 4ºC overnight. The

oocytes were treated with a rabbit polyclonal anti-androgen receptor antibody diluted with PBS–PVA–BSA (1:5000, sc-816; Santa Cruz Biotechnology Inc., Dallas, TX, USA) for 2 h at room temperature. The binding specificity for ARs was determined by preadsorbing the antibody with a blocking peptide (10 μg/mL, sc-816P; Santa Cruz Biotechnology Inc.). After three 15-min washes in PBS–PVA–BSA, the oocytes were reacted with Alexa Fluor 488-labeled donkey anti-rabbit immunoglobulin G (IgG) (1:400, A21206; Molecular Probes, Invitrogen) for 40 min at room temperature. The oocytes were then washed three times for 15 min each in PBS–PVA–BSA before being mounted on glass slides with ProLong Gold Antifade Reagent with DAPI (P36931) and observed under a confocal laser scanning microscope (FV1000-KDM; Olympus Co.).

The localization of ARs in ovarian follicles was also determined immunohistochemically. Early antral follicles of 0.4–0.7 mm in diameter collected from bovine ovarian cortical slices were fixed in 4% PFA in PBS–PVA containing 0.2% Triton X-100 for 60 min. Fixed follicles were washed three times in PBS–PVA for 15 min each and then treated with PBS–PVA containing 5% (wt/vol) sucrose (PBS–PVA–Suc) for 30 min followed by 10%, 12.5%, 15%, and 20% PBS–PVA–Suc for 30 min each. Next, the follicles were embedded in a mixture of 33% (vol/vol) OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) in 20% PBS–PVA–Suc and frozen in liquid nitrogen. The embedded follicles were cut into 10 μm

sections using a Microtome Cryostat (MICROM International GmbH, Walldorf, Germany). Follicular sections on APS-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan) were refixed with 4% PFA in PBS–PVA containing 0.2% Triton X-100 for 15 min. After washing three times in PBS–PVA for 5 min each, they were blocked with PBS–PVA–BSA for 1 h and subsequently treated with the rabbit polyclonal anti-androgen receptor antibody (1:500, sc-816) diluted with PBS–PVA–BSA at 4ºC overnight. After three 15-min washes in PBS–PVA–BSA, they were reacted with Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:400, A21206) for 40 min at room temperature. Finally, they were washed three times for 15 min each in PBS–PVA–BSA, and treated with ProLong Gold Antifade Reagent with DAPI (P36931) and observed under a confocal laser scanning microscope.

Statistical analysis

For statistical analyses of the OGC integrity and the meiotic division of oocytes, data were subjected to one-way ANOVA followed by the Tukey-Kramer multiple range test (Excel software with the add-in Ekuseru-Toukei 2010; Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences among the mean (± SEM) diameters of *in vitro* and *in vivo* grown oocytes were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple range test. Comparisons of the mean diameters of oocytes between the oocytes before culture and each

in vitro cultured oocyte group were performed with an unpaired *t*-test. Values of $P < 0.05$ were considered significant.

Results

In vitro growth of oocytes

Figure 10 shows the typical morphology of OGCs during growth culture. Similar to the results in Chapter II, each complex contained an oocyte at the center with thin layers of surrounding granulosa cells on Day 1, and the complexes gradually developed after Day 1. In the medium with steroid hormones, the granulosa cells proliferated vigorously, and some complexes formed cavities in their granulosa cell layers in spite of presence or absence of OHF (Fig. 10b3-h3). However, in the medium without steroid hormones (None), proliferation of granulosa cells was less than in the medium with steroid hormones, and oocytes gradually dissociated from granulosa cells by Day 14 (Fig. 10a3).

The integrity of OGCs during growth culture is shown in Figure 11. In the medium without steroid hormones, the OGC structure collapsed, and oocytes gradually became denuded between Day 7 and Day 14. A higher integrity of OGCs was observed in the medium with steroid hormones, and many OGCs in these media exhibited a sustained structure that contained viable oocytes enclosed by granulosa cells throughout the culture period. There were no effects of 1 μg/mL OHF (OHF1) on the morphology of OGCs during culture; however, higher concentration of OHF (OHF5) slightly decreased the OGC integrity (Fig. 11).

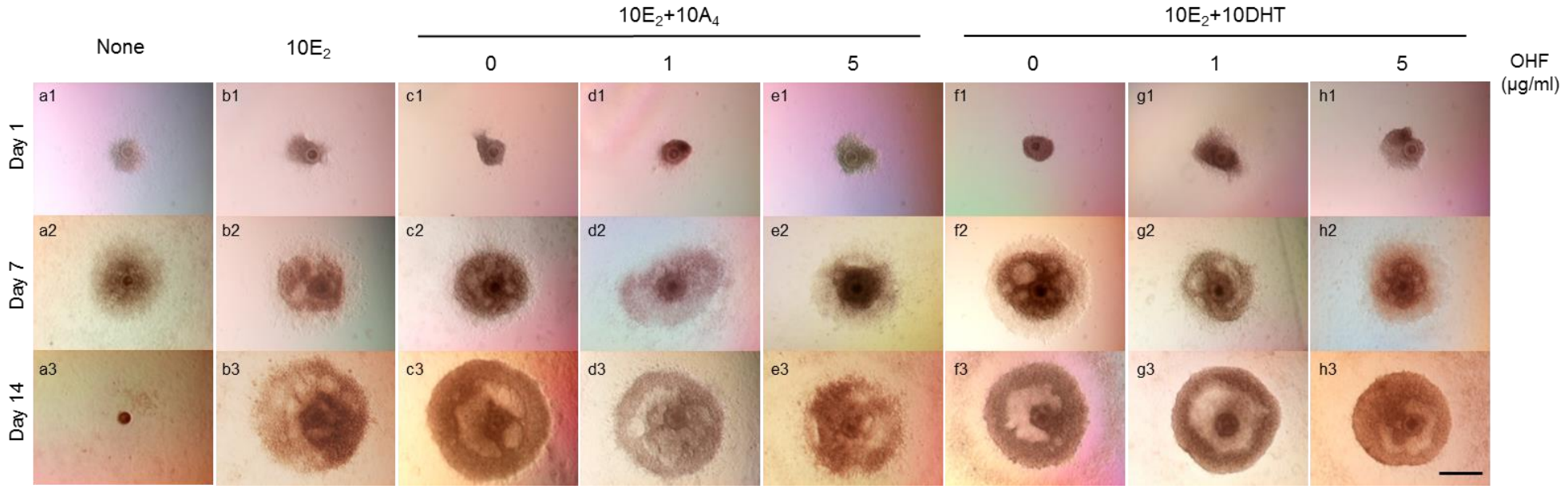

Figure 10 Typical morphology of bovine oocyte–granulosa cell complexes (OGCs) during growth culture. OGCs were cultured for 14 days in the medium with combinations of steroid hormones and OHF. E_2: 17β-estradiol, A_4: androstenedione, DHT: dihydrotestosterone, OHF: hydroxyflutamide, $10E_2$: 10 ng/mL E_2, $10E_2$+$10A_4$: 10 ng/mL E_2 plus 10 ng/mL A_4, and 10 E_2+10DHT: 10 ng/mL E_2 plus 10 ng/mL DHT. Scale bar represents 500 μm.

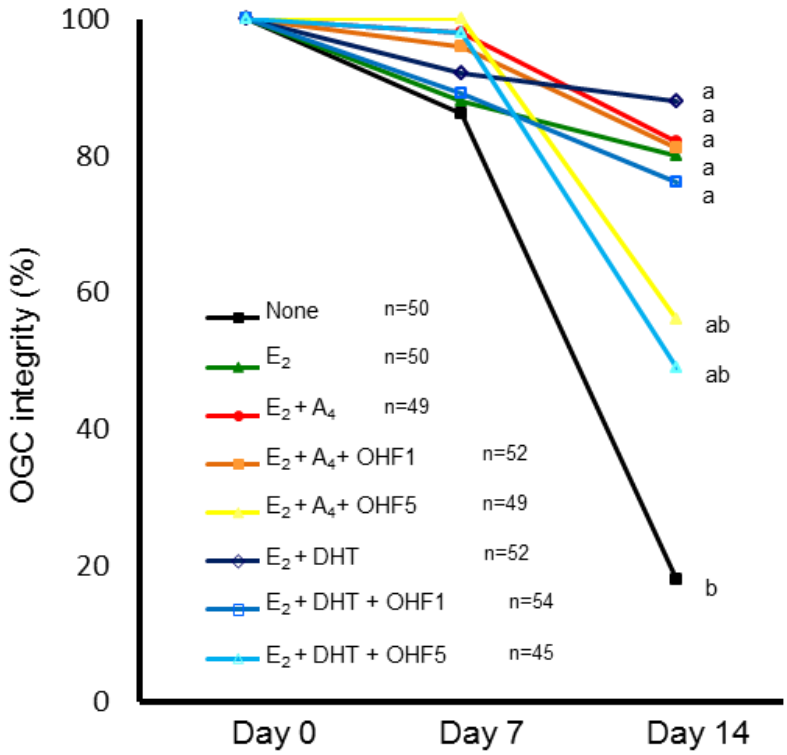

Figure 11 Integrity of bovine oocyte–granulosa cell complexes (OGCs) during growth culture with combinations of steroid hormones and OHF. On Days 0, 7 and 14, OGCs that showed degenerative signs, such as cytoplasmic degeneration of oocytes and/or complete detachment of granulosa cells from oocytes, were classified as degenerative complexes. See the footnotes in Figure 10 for abbreviations. [a,b]Values with different superscripts differ significantly ($P < 0.05$).

The mean diameter of oocytes before and after growth culture is shown in Figure 12. Oocytes became significantly larger in diameter after 14 days of growth culture in all culture media compared with the oocytes collected from 0.4–0.7 mm follicles (a white box on the left in Fig. 12). The increase in oocyte size was especially apparent in the medium containing androgens. Oocytes cultured with both E_2 and androgens (red and blue boxes in Fig. 12) grew to a larger size than those cultured with E_2 alone; the final size was comparable to that of *in vivo* grown oocytes (a white box on the right in Fig. 12). There were no significant differences between the mean diameters of oocytes grown in E_2+A_4 and E_2+A_4+OHF1, or in E_2+DHT and E_2+DHT+OHF1; however, the oocyte size was smaller in the medium containing 5 μg/mL OHF (dotted boxes in Fig. 12).

In vitro maturation of oocytes

After 14 days of growth culture, surviving oocytes were further cultured for maturation for 22 h. Figure 13 shows the percentages of MII oocytes to total oocytes that had been cultured for growth with steroid hormones and/or OHF. Oocytes collected form 0.4–0.7 mm early antral follicles had no competence to mature. The maturation rate of oocytes cultured with E_2 (32%) was comparable to that of oocytes grown without steroid hormones (10%). Oocytes grown with a combination of E_2 and androgens (A_4 or DHT) showed significantly higher percentage of MII

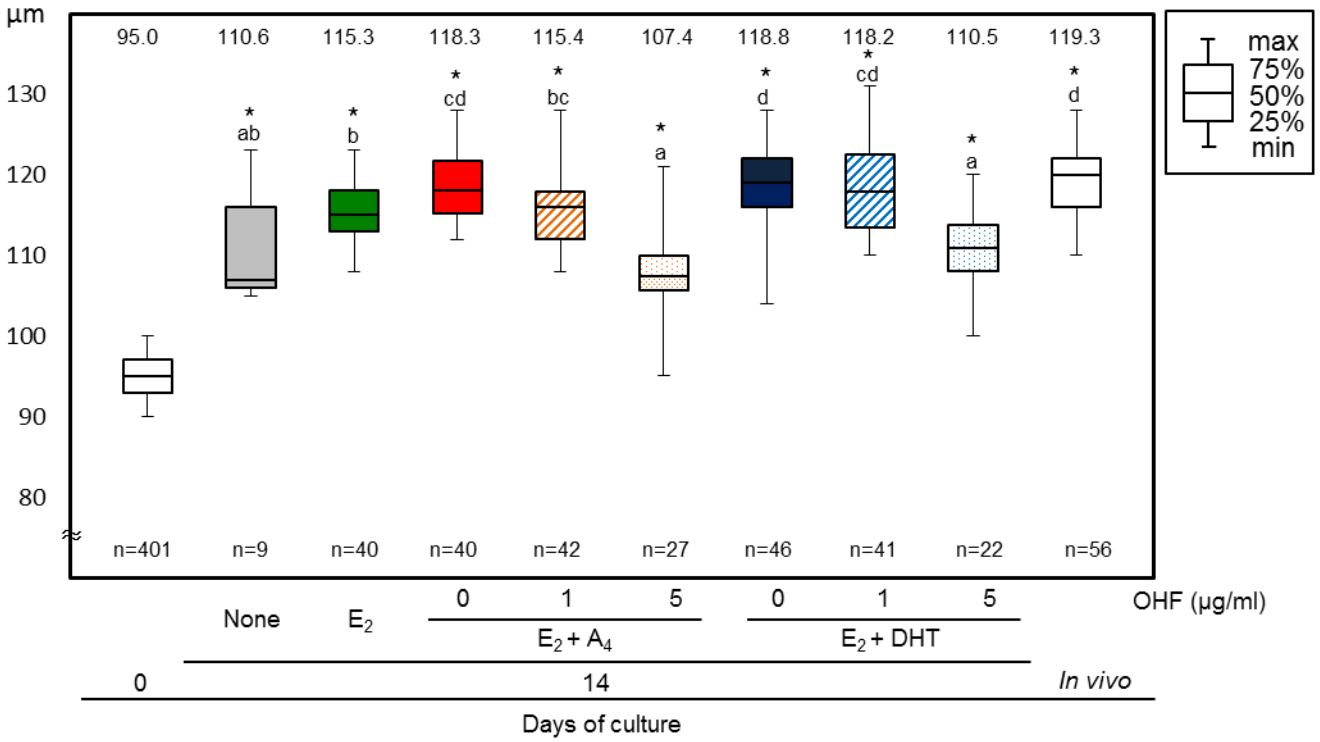

Figure 12 Comparison of the diameters of bovine oocytes after 14 days of growth culture. The white box on the left represents the diameter of oocytes isolated from early antral follicles (0.4–0.7 mm). The color filled, striped and dotted boxes indicate the diameters of oocytes cultured for 14 days in the medium with or without steroid hormones and OHF. The white box on the right represents the diameter of oocytes obtained from antral follicles (4–6 mm). The numbers of oocytes examined are shown below each box (n), and the numbers above the boxes indicate the mean diameters of oocytes (µm). See the footnotes in Figure 10 for the abbreviations.[a–d] Values with different superscripts differ significantly ($P < 0.05$). *Values were significantly different from those of oocytes before culture (a white box on the left, $P < 0.05$).

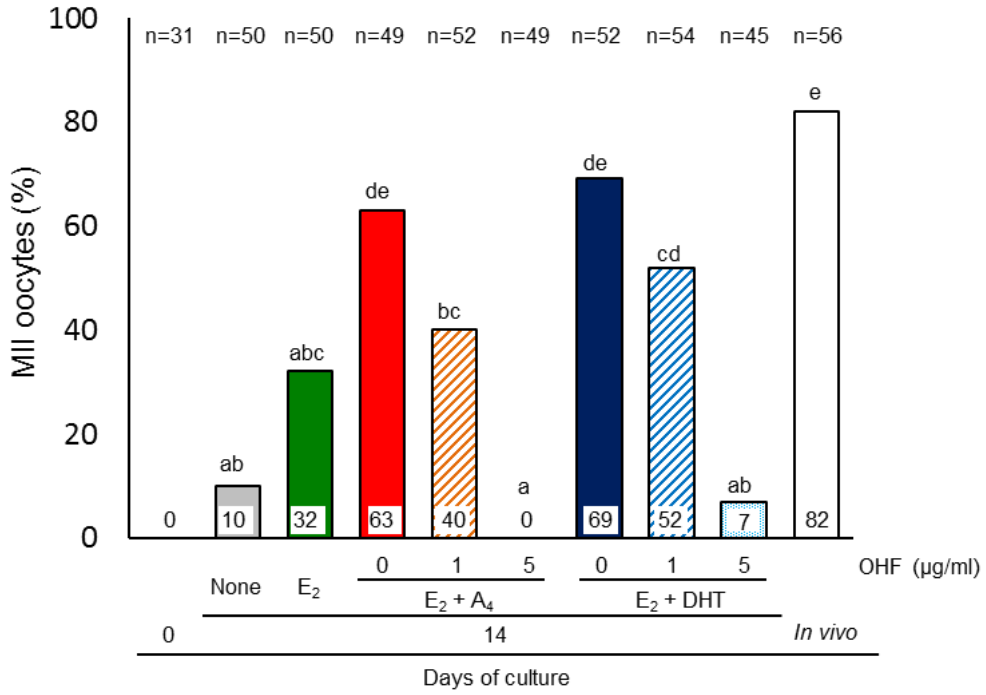

Figure 13 Meiotic competence of bovine oocytes cultured for growth after *in vitro* maturation. The percentages of MII oocytes out of total oocytes initially used for *in vitro* growth culture are shown. The white bars on the left and the right represent maturation rates of oocytes collected from early antral follicles (0.4–0.7 mm) and antral follicles (4–6 mm), respectively. The color filled, striped and dotted bars indicate the maturation rates of oocytes used for growth culture. The numbers above the bars indicate the numbers of oocytes used for growth culture (n). The percentages of MII oocytes are shown at the bottom of each bar. [a–e] Values with different superscripts differ significantly ($P < 0.05$).

oocytes (63% or 69%, respectively) than those grown with E_2 alone (32%); this result is consistent with the result in Chapter II. Moreover, these maturation rates were similar to the rate of *in vivo* grown oocytes. OHF decreased the rates of MII oocytes in a dose-dependent manner; 40% and 52% of oocytes grown with 1 μg/mL OHF matured to MII, while a few oocytes reached MII when cultured for growth with 5 μg/mL OHF. In addition, there were no significant differences between the maturation rates of oocytes grown with E_2 alone and those grown with steroid hormones plus 1 μg/mL OHF.

Expression of androgen receptors in early antral follicles and OGCs

Figure 14 shows the expression of ARs in 0.4–0.7 mm early antral follicles and OGCs collected from these follicles. In early antral follicles, AR expression was found in oocytes, granulosa cells and theca cells (Fig. 14A). Particularly intense AR immunosignals were observed in the nuclei of granulosa cells (Fig. 14Ac). In OGCs isolated from early antral follicles, AR expression in oocytes was clearly visible (Fig. 14B). Oocytes showed overall AR staining in the cytoplasm and nuclei except in the nucleoli, whereas the nuclei of granulosa cells showed intense AR expression. Preadsorbing the AR antibody with the blocking peptide decreased the immunosignals of ARs in OGCs.

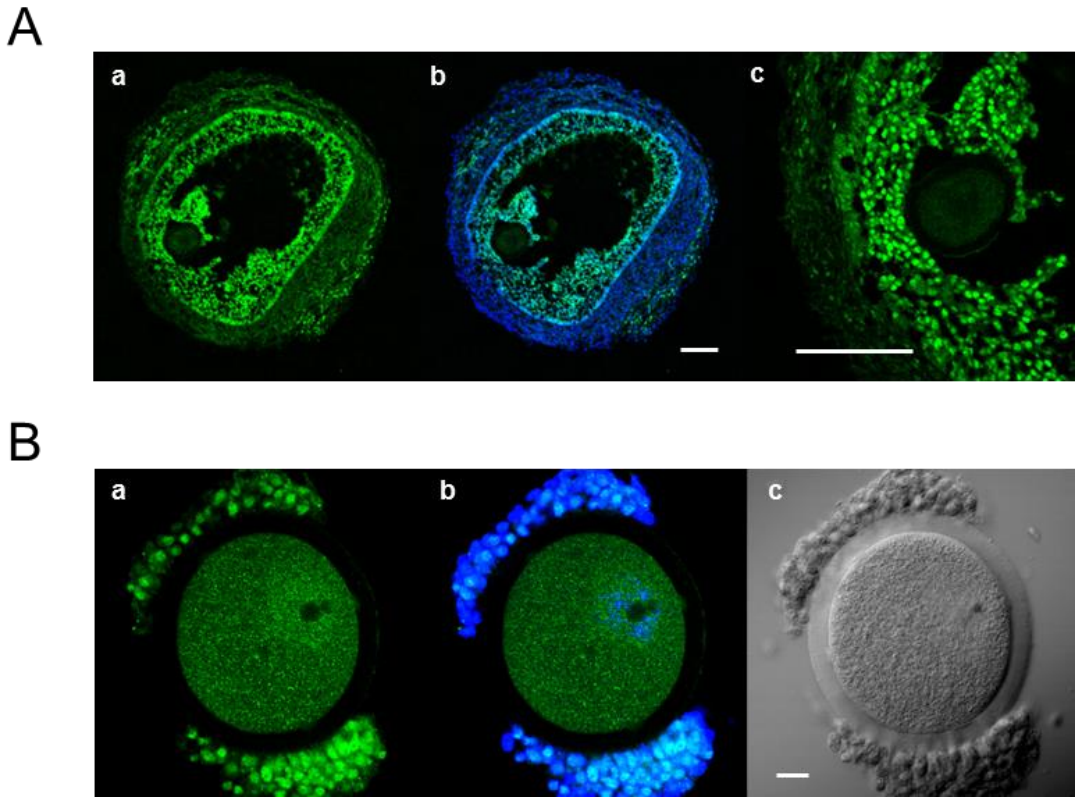

Figure 14 Expression of androgen receptors in bovine early antral follicles and OGCs. (A) Cryosections of 0.4–0.7 mm follicles were treated with anti-androgen receptor antibody and Alexa Fluor 488-labeled anti-rabbit immunoglobulin antibody. Alexa Fluor 488 staining marks androgen receptors in green (a–c). DAPI staining marks the chromatin in blue (b). A magnified image of the cumulus oophorus in (a) is shown in (c). Scale bars represent 100 μm. (B) OGCs isolated from 0.4–0.7 mm follicles were stained. Alexa Fluor 488 staining marks androgen receptors in green (a, b). DAPI staining marks the chromatin in blue (b). A bright-field image is shown in (c). Scale bar represents 20 μm.

Discussion

In this chapter, I examined the roles of steroid hormones on bovine oocyte growth by assessing the connections between oocytes and granulosa cells, and by culturing OGCs with the AR inhibitor.

TZPs are cytoskeleton-enriched structures that originate from granulosa cells and extend through the zona pellucida to the oocytes. Granulosa cells form gap junctions at the tip of TZPs with the oocytes. Gap junctions permit direct transfer of molecules necessary for oocyte growth. TZPs are formed as early as the primary follicle stage, and increase in number as oocyte growth proceeds (Li and Albertini, 2013). In the present study, *in vivo* grown oocytes isolated from 4–6 mm follicles had more TZPs than oocytes isolated from 0.4–0.7 mm follicles. Because bovine oocytes continue to grow in follicles after antrum formation, it appears that they have to keep or increase the number of TZPs to support the nutrient and metabolic demands of the oocytes.

After 14 days of the growth culture, there was a significant decrease in the number of TZPs in oocytes cultured without E_2 or A_4, while the number of TZPs was maintained in oocytes cultured with E_2 alone or in combination with A_4. This result indicates that E_2 plays a role in the maintenance or formation of connections between oocytes and granulosa cells. Several factors have been identified that regulate the formation of TZPs in follicles. Growth

differentiation factor-9 (GDF-9) secreted by oocytes promotes granulosa cell proliferation and differentiation during follicle development and is required for the formation of TZPs in mice (Carabatsos *et al.*, 1998). Expression of GDF-9 receptor in bovine granulosa cells is promoted by E_2 *in vitro* (Jayawardana *et al.*, 2006). Connexin 43 is a major abundant gap junction protein present in the granulosa cells adjacent to the bovine oocyte (Sutovský *et al.*, 1993). Exogenous estrogens lead to the increase in the expression of connexin 43 in the granulosa cells of rats (Merk *et al.*, 1972; Burghardt and Anderson 1981; Risek *et al.*, 1995). Based on these reports, it seems that E_2 supplemented in the growth culture medium promoted the maintenance or formation of TZPs by granulosa cells directly or indirectly, to support oocyte growth. Oocytes cultured with A_4 alone also maintained TZPs at some level. A_4 can be aromatized into estrogens by granulosa cells. While it is unclear whether A_4 affected TZPs directly, our finding that oocytes grown with $100A_4$ had fewer TZPs than those grown with $100E_2$ suggests that not all A_4 was converted into E_2, and that E_2 has a more advantageous effect on TZPs than A_4.

Although the number of TZPs was maintained in the medium with steroid hormones, it was still fewer than that of *in vivo* grown oocytes. In the present study, OGCs were cultured individually in 96-well culture plates. A recent study showed the increase in number of TZPs by *in vitro* culture. When mouse follicles are co-cultured with multiple follicles, which allow follicles to interact with each other like normal follicle development in ovaries, the number of

TZPs is increased in a follicle number-dependent manner (Hornick *et al.*, 2013). Further improvement of culture systems may be needed to increase the number of TZPs in bovine oocytes *in vitro*.

The results from the experiments using androgens for *in vitro* growth culture of bovine oocytes in Chapters II and III-2 suggested the roles of androgens on oocyte growth and maturation through the ARs. To examine the ability of an AR inhibitor to antagonize the effect of androgens on oocyte growth and maturation *in vitro*, I employed a clinically used nonsteroidal AR antagonist, OHF. OHF has been widely used in the treatment of prostate cancer because they competitively block the binding of androgens to ARs with low side effect (Culig *et al.*, 2004; Lenie and Smitz, 2009). In mice, OHF inhibits the acquisition of meiotic competence of oocytes during *in vitro* culture (Lenie and Smitz, 2009). In the present study, addition of OHF to the culture medium containing androgens decreased the mean diameters of oocytes on Day 14. In addition, OHF inhibited the stimulatory effect of androgens on the acquisition of maturation competence in oocytes in a dose-dependent manner. These results indicate that the increase in oocyte diameter and oocyte maturation rate caused by androgens occurs in part through the ARs. That is, androgens themselves, not converted estrogen, are able to promote oocyte growth and the acquisition of meiotic competence. With respect to the integrity of OGCs, the results in Chapter II showed that the OGC structure collapsed and that the oocytes were denuded during

growth culture with DHT alone. In the presence of E_2 and DHT, however, OGCs maintained high integrity throughout culture, suggesting that E_2 plays a role in oocyte survivability by preventing oocytes from being denuded.

Androgens have been shown to upregulate FSH receptors in granulosa cells (Hiller and Tetsuka, 1997), and contribute to granulosa cell differentiation and follicle development (Wang *et al.*, 2001, Sen *et al.*, 2014). Also, the expression of IGFs and their receptor, which play essential roles in folliculogenesis (Adashi, 1998), are regulated by androgens (Vendola 1999a, 1999b; Hickey *et al.*, 2004, 2005). Moreover, GDF-9 requires androgens to promote follicle growth (Hickey *et al.*, 2005; Orisaka *et al.*, 2009). These reports suggest that androgens, acting through ARs in granulosa cells, may regulate the expression and/or action of these important growth factors and contribute to follicle or oocyte growth.

The AR is a member of the steroid hormone receptor superfamily and contains a DNA-binding domain and a hormone-binding domain. ARs are predominantly located within the cytoplasm, activated by binding with androgens and translocated into the nucleus (Georget *et al.*, 1997, Tyagi *et al.*, 2000). Studies across species have reported that ARs reside in granulosa cells and theca cells (Horie *et al.*, 1992; Hampton *et al.*, 2004; Juengel *et al.*, 2006), and that they are also present in mouse (Gill *et al.*, 2004), rat (Szołtys and Słomczyńska, 2000) and pig (Cárdenas *et al.*, 2002) oocytes. In the present study, the nuclei of granulosa cells

showed intense expressions of ARs in early antral follicles, indicating that functional ARs reside in granulosa cells. Since AR expression was also found in the oocytes, androgens have the potential to affect oocytes directly. There are a few reports about androgen action through ARs in oocytes; however, in mice, testosterone induces oocyte maturation through AR-mediated activation of mitogen-activated protein kinase and cyclin-dependent kinase 1 signaling (Gill *et al.*, 2004).

In conclusion, E_2 maintained the physical connections between oocytes and granulosa cells and supported oocyte viability and growth, while androgens in combination with E_2, but not alone, promoted the growth of bovine oocytes and their acquisition of meiotic competence during *in vitro* growth culture. The androgen-induced oocyte growth and maturation were suppressed by the AR inhibitor, indicating that androgens themselves promote the growth of oocytes and their acquisition of meiotic competence, and that these stimulatory effects of androgens are mediated by the ARs in granulosa cells and/or oocytes.

CHAPTER IV

In vitro fertilization of bovine oocytes grown *in vitro*

Introduction

In 1959, successful *in vitro* fertilization was first reported in rabbits (Chang, 1959). This report has shown that *in vitro* fertilized rabbit eggs developed normally and live offspring was born. After the discovery of sperm capacitation (Austin, 1951; Chang, 1951), many studies about *in vitro* fertilization have been done in variety of species. Yanagimachi and Chang (1963, 1964) first reported *in vitro* fertilization using *in vitro* capacitated spermatozoa in hamster. Based on these studies, *in vitro* fertilization in human succeeded in 1969 (Edwards *et al.*, 1969). *In vitro* fertilization of bovine oocytes was first reported in 1977 (Iritani and Niwa, 1977), and later, the first offspring was produced from *in vitro* fertilization of bovine oocytes using *in vitro* capacitated spermatozoa (Brackett *et al.*, 1982). Nowadays, *in vitro* fertilization is a well-established tool and widely used in agricultural and medical fields (Bavister, 2002).

Many efforts have been made to develop *in vitro* growth culture systems for oocytes in variety of species (Miyano, 2005; Picton *et al.*, 2008). However, *in vitro* grown oocytes usually showed lower development rates after *in vitro* maturation and fertilization compared to that of *in*

vivo grown oocytes. In the cow, live offspring have been produced from oocytes in early antral follicles after *in vitro* growth, maturation and fertilization (Yamamoto *et al.*, 1999; Hirao *et al.*, 2004), but the rates of blastocyst formation in these reports were very low. Furthermore, there is no report that investigated fertilization ability of *in vitro* grown oocytes in large animals.

The *in vivo* or *in vitro* environment in which oocytes grow and mature is a major determinant factor of oocyte quality that represents fertilization ability and developmental competence (Lequarre *et al.*, 2005; Krisher, 2013). In Chapters II and III, the culture system using steroid hormones supported bovine oocyte growth and promoted the acquisition of meiotic competence *in vitro*. To assess whether *in vitro* growth culture systems mimic *in vivo* environment, it is essential to determine the fertilization ability and embryonic development of *in vitro* grown oocytes. In this chapter, the oocytes from early antral follicles were cultured for growth with E_2 and A_4, matured and inseminated *in vitro*. The fertilization ability and subsequent developmental competence were examined.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Collection of oocyte–granulosa cell complexes

The collection methods of OGCs from bovine ovaries were described in Chapter II.

In vitro growth culture of oocytes

In vitro growth culture was performed according to a procedure described in Chapter II with modifications. OGCs were isolated from bovine early antral follicles (0.4–0.7 mm in diameter) and groups of 10–20 OGCs were cultured on Millicell inserts (30 mm diameter, 0.4 μm pore size, Cell Culture Inserts, Merck Millipore, Billerica, MA, USA) placed in Petri dishes (Falcon No. 351008, Becton Dickinson and Co.). In total, 2 mL of medium was put in the dishes; 1 mL on the membrane and another 1 mL under the membrane. The basic medium and culture method were described in Chapter II. According to the results in Chapter II, 10 ng/mL E_2 and 10 ng/mL A_4 were added to the medium. The OGCs were cultured for 14 days at 38.5ºC under an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 from Day 0 to Day 6, and an atmosphere

of 5% CO_2 in humidified air from Day 7 to Day 14.

In vitro maturation of oocytes

The OGCs that contained surviving oocytes after 14 days of *in vitro* growth culture and those contained fully grown oocytes collected from antral follicles (control) were cultured in 50 μL microdrops of the maturation medium covered with paraffin oil at 38.5ºC under an atmosphere of 5% CO_2 in humidified air for 22 h. The maturation method and medium were described in Chapter II. After culture for 22 h, the oocytes were denuded mechanically using a small-bore pipette with the help of 0.1% (wt/vol) hyaluronidase. The oocytes were then fixed and stained to assess the stage of meiotic division. Some OGCs were subjected to subsequent *in vitro* fertilization.

In vitro fertilization and development

For *in vitro* fertilization, frozen semen in straws (Japanese Black, P706, Livestock Improvement Association of Japan, Inc, Tokyo, Japan) were thawed in water at 37ºC. The spermatozoa were washed twice by centrifugation. First, they were washed (760×*g*) in a two-step gradient of 2 mL of 90% (vol/vol) and 2 mL of 45% of Percoll (GE Healthcare UK Ltd., Buckinghamshire, England, UK) in PBS, and then washed (372×*g*) in 3 mL of

insemination medium (IVF100, Research Institute for the Functional Peptides, Yamagata, Japan). The final concentration of spermatozoa was adjusted with insemination medium to 1.0–5.0 $\times 10^6$ sperm/mL. OGCs were then transferred to 100 μL microdrops of sperm suspension and incubated for 6 or 12 h at 38.5ºC under an atmosphere of 5% CO_2 in humidified air.

To assess the fertilization, some of the oocytes were denuded using a small-bore pipette after 6 or 12 h of insemination, and subjected to *in vitro* development for 10 or 4 h, respectively; 16 h after insemination in total. The oocytes were then fixed with acetic acid-ethanol (1:3) and stained with 1 % (wt/vol) aceto-orcein. The oocytes that have two pronuclei and two polar bodies with a sperm tail were classified as normal fertilization. The fertilization other than normal fertilization—for example, the oocytes that have an enlarged sperm head with anaphase/telophase I chromosomes, two pronuclei with a sperm tail and one polar body, or more than two sperm heads or pronuclei—were classified as abnormal fertilization. MI, AI–TI, or MII oocytes and oocytes that have one or two pronuclei without sperm tails were classified as unfertilized oocytes. The oocytes that showed cytoplasmic degeneration were classified as degenerated oocytes.

After *in vitro* fertilization, other oocytes were denuded mechanically and subjected to development culture. The day of *in vitro* fertilization was designated Day 0. Oocytes

(presumptive zygotes) were cultured in 50 μL microdrops of serum free medium (IVD101, Research Institute for the Functional Peptides) for up to Day 8. The rates of blastocyst formation on Day 8 were recorded. After 7 days of development culture, some blastocysts from *in vivo* grown oocytes and *in vitro* grown oocytes cultured with E_2 and A_4 were washed twice in PBS−PVA for 15 min each and fixed in 4% PFA in PBS−PVA for 60 min. Fixed blastocysts were washed twice in PBS−PVA for 15 min each and blocked in PBS−PVA−BSA for 60 min. Blastocysts were then stained with ProLong Gold Antifade Regent with DAPI (P36931) and observed under a fluorescence microscope (BX53F; Olympus Co.) to count the cell numbers.

Statistical analysis

The rate of OGC integrity, the frequencies of oocytes at each stage of meiotic division, the rates of fertilization and blastocyst formation were analyzed using Chi-square test. Differences in the mean (± SEM) diameters of oocytes and cell numbers of blastocysts were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

In vitro growth and maturation of oocytes

Figure 15 shows the morphology of OGCs during growth culture with or without steroid hormones on the Millicell inserts. Similar to those cultured in 96-well plates (Chapters II and III), in the medium without steroid hormones (None), few granulosa cells proliferated during the culture (Fig. 15a). Oocytes in this medium stuck on the membranes and become gradually degenerated. In the medium with steroid hormones (E_2+A_4), proliferation of granulosa cells was notable and antrum-like cavities were formed in the granulosa cell layers (Fig. 15b). The dome-like structures were further developed during the culture.

The OGC integrity and mean diameters of oocytes were shown in Table 7. Similar to the results in Chapter II, OGCs in the medium with E_2+A_4 maintained the structures that contained viable oocytes during growth culture (75%), while in the medium without steroid hormones, most of the oocytes were degenerated on Day 14. The mean diameters of oocytes collected from early antral follicles (0.4–0.7 mm in diameter) were around 95 μm. Although the mean diameters of oocytes were increased after the growth culture, the oocytes cultured without steroid hormones (101.8 ± 1.5 μm) were smaller than the fully grown oocytes collected from antral follicles (4–6 mm in diameter). On the other hand, the mean diameter of oocytes

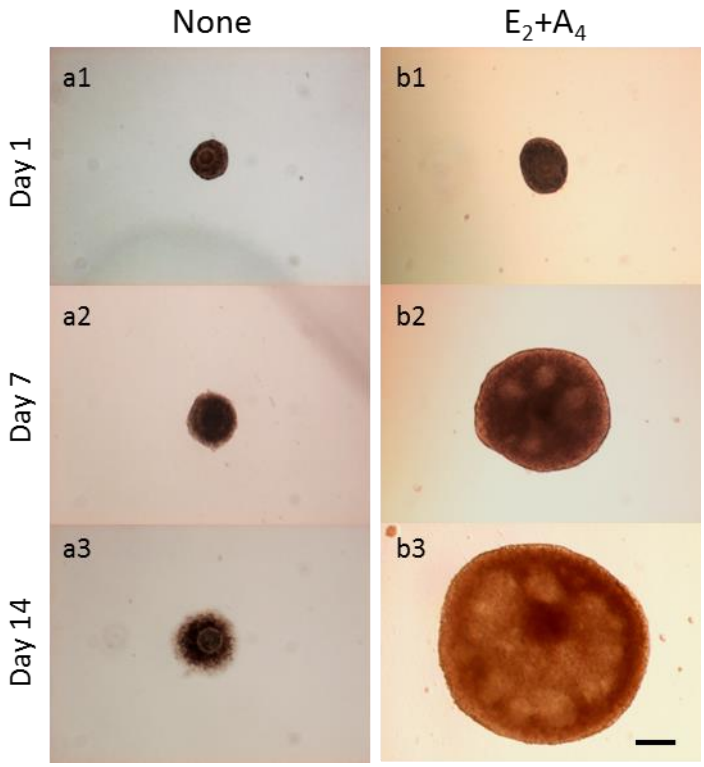

Figure 15 Typical morphology of bovine oocyte–granulosa cell complexes (OGCs) during growth culture. OGCs were cultured for 14 days in the medium with or without E_2 and A_4. E_2+A_4: 10 ng/mL 17β-estradiol plus 10 ng/mL androstenedione. A scale bar represents 200 μm.

Table 7 The integrity and mean diameters of *in vitro* grown bovine oocytes.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of viable oocytes (%)[3]			Mean diameters of oocytes (μm ± SEM)[4]	
	E_2	A_4	Day 0	Day 7	Day 14	Day 0	Day 14
0	—	—	24	—	—	95.5 ± 0.5	—
14	0	0	32 (100)	26 (81)	10 (31)[a]	96.6 ± 0.6	101.8 ± 1.5[a]
	10	10	32 (100)	30 (94)	24 (75)[b]	97.3 ± 0.7	121.0 ± 1.2[b]
In vivo	—	—	—		25	—	123.2 ± 0.9[b]

[1] Oocyte–granulosa cell complexes collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (14). Oocytes from early antral follicles (0.4–0.7 mm in diameter) and antral follicles (4–6 mm in diameter) were examined as for *in vivo* control (0 and *In vivo*, respectively).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] OGCs that exhibited complete detachment of granulosa cells from oocytes and/or contained oocytes that showed cytoplasmic degeneration were classified as degenerative OGCs.

[4] At the end of the growth culture, the diameters of oocytes that were enclosed by granulosa cells and showed no sign of degeneration were measured.

[a, b] Values with different superscripts in the same column differ significantly ($P < 0.05$).

cultured with E_2+A_4 (121.0 ± 1.2 μm) was comparable to that of *in vivo* grown oocytes (123.2 ± 0.9 μm). These results were consistent with the results in Chapters II and III.

Table 8 shows the meiotic competence of *in vitro* grown oocytes. After 22 h of maturation culture, all of the oocytes from early antral follicles remained at FC (54%) or SC (46%) stages, while almost all of the oocytes cultured for growth resumed meiosis. During the maturation culture of *in vivo* and *in vitro* grown oocytes, the OGCs underwent cumulus expansion and some of these oocytes reached MII after the culture. None of them grown without steroid hormones reached MII (0%), however, the oocytes grown with E_2+A_4 showed a high percentage of MII oocytes (79%), and the rate was comparable to that of *in vivo* grown oocytes (84%). With respect to the rates of MII oocytes to total oocytes initially used for growth culture, 59% of oocytes grown with E_2+A_4 matured to MII after maturation culture.

Fertilization ability of oocytes

After maturation culture, oocytes grown *in vivo* and *in vitro* were subjected to *in vitro* fertilization to assess the fertilization ability. After 6 and 12 h of insemination, 38% and 37% of oocytes grown *in vivo* were fertilized normally, respectively (Table 9). All of these oocytes had two pronuclei and two polar bodies (Fig. 16A). There were no significant differences in the rates of unfertilized oocytes after 6 and 12 h of insemination of *in vivo* grown oocytes (52% and

Table 8 Meiotic competence of *in vitro* grown bovine oocytes after *in vitro* maturation.

In vitro growth (day)[1]	Steroid hormones (ng/mL)[2]		No. of oocytes used[3]		No. (%) of oocytes at the stage of [4]						
	E_2	A_4	IVG	IVM	FC	SC	GV	MI	AI–TI	MII[5]	
0	—	—	—	24	13 (54)	11 (46)	0 (0)	0 (0)	0 (0)	0 (0)	
14	0	0	32	10	0 (0)	0 (0)	2 (10)	8 (80)[a]	0 (0)	0 (0)	[0]
	10	10	32	24	0 (0)	0 (0)	0 (0)	2 (8)[b]	3 (13)	19 (79)	[59]
In vivo	—	—	—	25	0 (0)	0 (0)	0 (0)	3 (12)[b]	1 (4)	21 (84)	

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* maturation culture before (0) or after 14 days of *in vitro* growth culture (14). OGCs from antral follicles (4–6 mm in diameter) were subjected to *in vitro* maturation culture as for *in vivo* control (*In vivo*).

[2] E_2: 17β-estradiol and A_4: androstenedione.

[3] After *in vitro* growth culture (IVG), OGCs with surviving oocytes were transferred to *in vitro* maturation culture (IVM).

[4] FC: filamentous chromatin, SC: stringy chromatin, GV: germinal vesicle I–IV, MI: metaphase I, AI–TI: anaphase I and telophase I, and MII: metaphase II.

[5] The numbers in [ ] indicate the percentages of the oocytes to the oocyte numbers initially used for IVG.

[a, b] Values with different superscripts in the same column differ significantly ($P < 0.05$).

Table 9 Fertilization ability of *in vitro* grown bovine oocytes after *in vitro* maturation and fertilization.

Growth[1]	Insemination (h)[2]	No. of oocytes examined	Fertilized oocytes (%)[3]				Unfertilized oocytes (%)[4]				DG[5]
			Normal	Abnormal fertilization							
			2PN2PB	Polyspermy	Others	Total	MI	MII	Others	Total	
In vitro	6	31	1 (3)^{a}	4 (13)	3 (9)	7 (23)^{ab}	3 (10)	11 (35)^{a}	9 (29)^{ab}	23 (74)^{a}	0 (0)
	12	32	11 (34)^{b}	5 (16)	6 (19)	11 (34)^{a}	4 (13)	2 (6)^{b}	4 (13)^{a}	10 (31)^{b}	0 (0)
In vivo	6	50	19 (38)^{b}	1 (2)	2 (4)	3 (6)^{b}	4 (8)	16 (32)^{a}	6 (12)^{a}	26 (52)^{a}	2 (4)
	12	49	18 (37)^{b}	3 (6)	6 (12)	9 (18)^{b}	1 (2)	2 (4)^{b}	17 (35)^{b}	20 (41)^{ab}	2 (4)

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture with 10 ng/mL 17β-estradiol and 10 ng/mL androstenedione for 14 days (*In vitro*). Oocytes from antral follicles (4–6 mm in diameter) were examined as for *in vivo* control (*In vivo*).

[2] After *in vitro* maturation, oocytes were subjected to *in vitro* fertilization with bovine spermatozoa.

[3] Normal fertilization: the oocytes that have two pronuclei and two polar bodies with a sperm tail (2PN2PB). Abnormal fertilization: fertilization other than normal fertilization; for example, the oocytes have an enlarged sperm head with anaphase/telophase I chromosomes, two pronuclei with a sperm tail and one polar body, or more than two sperm heads or pronuclei (Polyspermy).

[4] MI, AI–TI, or MII oocytes and oocytes that have one or two pronuclei without sperm tails were classified as unfertilized oocytes.

[5] Degenerated oocytes.

[a, b] Values with different superscripts in the same column differ significantly ($P < 0.05$).

Figure 16 Morphology of *in vivo* and *in vitro* grown bovine oocytes after *in vitro* fertilization. After 16 h of insemination, both *in vivo* (A) and *in vitro* (B) grown oocytes had two pronuclei and two polar bodies. The arrows indicate sperm tails.

41%, respectively). Among i*n vitro* grown oocytes, only 3% of oocytes showed normal fertilization and most of the oocytes (74%) were unfertilized after 6 h of insemination (Table 9). After 12 h of insemination, however, the rate of unfertilized oocytes, especially MII oocytes, significantly decreased and normal fertilization rate of *in vitro* grown oocytes increased to 34%. These oocytes had two pronuclei and two polar bodies (Fig. 16B), and the rate of normal fertilization was similar to that of *in vivo* grown oocytes. The rates of abnormal fertilization of *in vitro* grown oocytes were higher than those of *in vivo* grown oocytes after both 6 and 12 h of insemination.

Developmental competence of oocytes

After insemination, *in vivo* and *in vitro* grown oocytes were cultured for development up to 8 days (Table 10). After insemination for 6 and 12 h, 23% and 22% of the oocytes grown *in vivo*, respectively, developed to blastocysts until Day 8 of development culture. Although *in vitro* grown oocytes inseminated for 6 h almost never developed to blastocysts after 8 days of culture (1%), 22% of the oocytes developed to blastocysts when they were inseminated for 12 h. This rate of blastocyst formation was comparable to that of *in vivo* grown oocytes.

To assess the quality of blastocysts derived from *in vitro* grown oocytes, the blastocysts were stained with DAPI and cell numbers were counted (Fig. 17 and Table 11). On Day 7 of

Table 10 Developmental competence of *in vitro* grown bovine oocytes after *in vitro* fertilization and development.

Growth[1]	Insemination (h)[2]	No. of oocytes used[3]	No. of blastocysts (%)[4]
In vitro	6	95	1 (1)[a]
	12	98	22 (22)[b]
In vivo	6	136	31 (23)[b]
	12	126	28 (22)[b]

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (*In vitro*). Oocytes from antral follicles (4–6 mm in diameter) were examined as for *in vivo* control (*In vivo*).

[2] After *in vitro* maturation, oocytes were subjected to *in vitro* fertilization with bovine spermatozoa.

[3] Number of oocytes used for *in vitro* fertilization.

[4] After *in vitro* fertilization, oocytes were further cultured for development up to 8 days.

[a, b] Values with different superscripts in the same column differ significantly ($P < 0.05$).

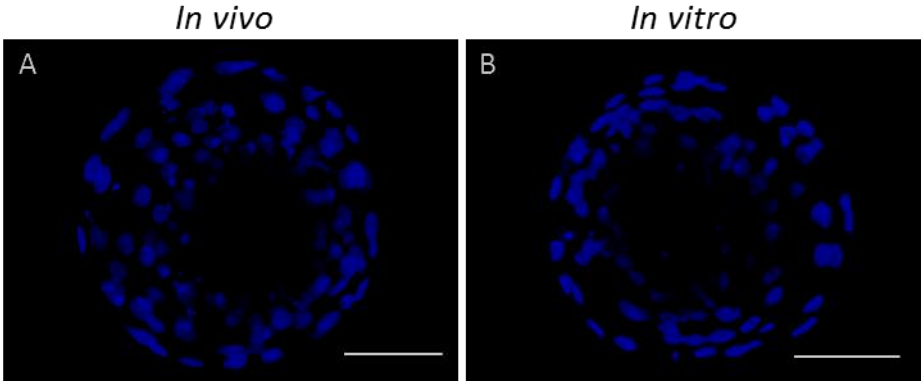

Figure 17 Bovine blastocysts derived from *in vivo* and *in vitro* grown oocytes. Blastocysts (Day 7 of *in vitro* development) derived from *in vivo* grown oocytes isolated from antral follicles (4–6 mm in diameter) (A) and *in vitro* grown oocytes growth-cultured with E_2 and A_4 were stained with DAPI. Scale bars represent 100 μm.

Table 11 Number of cells in bovine blastocysts derived from *in vitro* grown oocytes.

Growth[1]	No. of blastocysts examined[2]	Number of cells (mean ± SEM)
In vitro	9	90.8 ± 5.3
In vivo	8	90.5 ± 4.0

[1] Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were subjected to *in vitro* growth culture for 14 days (*In vitro*). Oocytes from antral follicles (4–6 mm in diameter) were examined as for *in vivo* control (*In vivo*).

[2] *In vivo or in vitro* grown oocytes were subjected to *in vitro* maturation and subsequent fertilization. After 7 days of *in vitro* development, blastocysts were fixed and stained with DAPI.

in vitro development, both *in vivo* and *in vitro* grown oocytes developed to early blastocysts (Fig. 16). The mean numbers of cells that composed the blastocysts derived from *in vivo* and *in vitro* grown oocytes were similar (90.5 ± 4.0 and 90.8 ± 5.3, respectively) (Table 11).

Discussion

In this chapter, OGCs collected from bovine early antral follicles were cultured for growth for 14 days. After maturation culture, the oocytes were subjected to *in vitro* fertilization and development. After 14 days of growth culture, 75% of oocytes were enclosed by granulosa cells and the mean diameter of the oocytes increased to 121.0 ± 1.2 μm in the medium with E_2 and A_4. In addition, 79% of these oocytes matured to MII after maturation culture. Although OGCs were cultured for growth on insert membranes in this chapter, the results of oocyte growth were consistent with those in Chapter II, in which OGCs were cultured individually in 96-well plates.

After *in vitro* maturation and fertilization, 22% of *in vitro* grown oocytes developed to blastocysts during 8 days of development culture. This rate of blastocyst formation is similar to that of *in vivo* grown oocytes. Since the cell numbers that composed blastocysts were also similar between *in vitro* and *in vivo* grown oocytes, the quality of blastocysts derived from *in vitro* grown oocytes appeared to be comparable to that from *in vivo* grown oocytes. Moreover, after 16 h of insemination, more than one third of oocytes grown *in vivo* and *in vitro* were fertilized normally, having two pronuclei and two polar bodies. These results indicate that *in vitro* grown oocytes were able to be fertilized normally and competent to develop to blastocysts.

During the growth phase in the ovary, oocytes increase their volume, acquire maturation competence, and accumulate materials necessary for prospective fertilization and development. The results in Chapters II and III showed that E_2 and A_4 support oocyte growth and promote the acquisition of maturation competence by maintaining the connections between oocytes and surrounding granulosa cells. The results in this chapter indicate that these steroid hormones added to the growth culture medium also supported the acquisition of fertilization and developmental competence during oocyte growth *in vitro*.

There were no significant changes in the rates of fertilization and unfertilized oocytes after 6 and 12 h of insemination of *in vivo* grown oocytes. Among unfertilized oocytes, the rate of MII oocytes was decreased, but the rate of normal fertilization was not increased after 12 h of insemination. These oocytes appeared to undergo parthenogenetic activation. Meanwhile, few *in vitro* grown oocytes were fertilized normally after 6 h of insemination and almost never developed to blastocysts after 8 days of culture; however, the rate of unfertilized oocytes was decreased and 34% of *in vitro* grown oocytes fertilized normally after 12 h of insemination. Since spermatozoa have to penetrate cumulus cell layers and zona pellucida to reach oocyte cytoplasm, cumulus cells and zona pellucida are key factors of fertilization. During oocyte maturation, hyaluronic acid is secreted by surrounding cumulus cells and cumulus expansion occurs (Yokoo and Sato, 2004). Cumulus expansion is essential for normal fertilization (Chen *et*

al., 1993), and the cumulus cells in expanded cumulus induce sperm capacitation (Shimada *et al.*, 2008). The ability of cumulus expansion is acquired during oocyte growth, which is considered to be regulated by oocyte secreted paracrine factors (Elvin *et al.*, 1999). The oocytes grown with E_2 and A_4 maintained connections between oocytes and surrounding cumulus cells; however, the number of TZPs was fewer than that of *in vivo* grown oocytes (Chapter III). It is therefore suggested that although cumulus expansion appeared to occur, *in vitro* cultured cumulus cells might not acquire enough ability to expand completely or to induce sperm capacitation which is needed for fertilization. After penetration to cumulus cell layers, spermatozoa hydrolyze and penetrate zona pellucida to reach oocyte cytoplasm. It is reported that zona pellucida become thick or hard by *in vitro* culture (van Wely and van der Veen, 2011; Bertrand *et al.*, 1995; De Vos and Van Steirtegham, 2000). Since the zona pellucida is secreted and synthesized during oocyte growth, *in vitro* long-term growth culture may influence morphology or function of zona pellucida. It is inferred that because of these factors (cumulus cells and zona pellucida), it took longer time for *in vitro* grown oocytes to be penetrated by spermatozoa than *in vivo* grown oocytes.

Although the normal fertilization rate of *in vitro* grown oocytes after 12 h of insemination was similar to that of *in vivo* grown oocytes, the abnormal fertilization rates of *in vitro* grown oocytes were higher than those of *in vivo* grown oocytes. To be fertilized

successfully, both nuclear maturation and cytoplasmic maturation of oocytes are needed (Krisher, 2004). The oocytes acquire the competence of cytoplasmic maturation as well as nuclear maturation during their growth phase. It is suggested that although MII percentage of *in vitro* grown oocytes cultured with E_2 and A_4 was similar to that of *in vivo* grown oocytes after maturation culture, some of the *in vitro* grown oocytes did not acquire the cytoplasmic maturation competence.

Nonetheless, after fertilization, the oocytes formed two pronuclei with two polar bodies at 16 h of insemination and developed normally to blastocysts after 8 days of culture. The cell number of blastocysts was also normal. Thus, in conclusion, the *in vitro* growth culture system used in this study is able to support not only oocyte growth and the acquisition of maturation competence, but also the acquisition of fertilization ability and developmental competence. It is suggested that *in vitro* grown oocytes are able to be fertilized and develop in the similar way as *in vivo* grown oocytes.

CHAPTER V

General summary

In order to utilize oocytes stored in the ovary, many technologies have been developed. Since 1959 when successful *in vitro* fertilization was first reported in rabbits (Chang, 1959), *in vitro* fertilization has been a well-established tool. After *in vitro* maturation was succeeded in mammals, *in vitro* maturation and subsequent *in vitro* fertilization system have been widely used for studying reproductive biology in mammals. More recently, many efforts have been made to develop *in vitro* growth culture systems of mammalian oocytes. While a variety of systems have been developed for mouse oocytes, there are few successful systems for oocytes from large animals. Recent *in vivo* and *in vitro* studies have revealed the contribution of steroid hormones to female reproduction and folliculogenesis in the ovary. 17β-estradiol (E_2; Hirao *et al.*, 2004) and androstenedione (A_4; Taketsuru *et al.*, 2012) have been reported to support bovine oocyte growth *in vitro*; however, the rate of oocytes competent to reach metaphase II (MII) or develop to blastocysts is low in these systems. Moreover, exactly how these steroid hormones support oocyte growth is not understood. This study was conducted to examine the effects of steroid hormones on bovine oocyte growth *in vitro* and to determine the roles of steroid hormones on the oocyte growth.

In Chapter II, the effect of steroid hormones on *in vitro* growth of bovine oocytes was examined. Oocyte–granulosa cell complexes (OGCs) collected from early antral follicles (0.4–0.7 mm in diameter) were cultured for 14 days with E_2 and A_4 either alone or in combinations. Since A_4 can be converted to estrogens by granulosa cells, I then examined the effect of androgens on bovine oocyte growth by culturing OGCs with testosterone (T) and non-aromatizable androgen, dihydrotestosterone (DHT). In the medium with steroid hormones except for DHT alone, OGCs maintained their structures and dome-like structures were developed during growth culture, while they were never developed in the medium without steroid hormones. The integrity of OGCs on Day 14 in the medium with 100 ng/mL E_2 alone was higher than those with A_4 (100 ng/mL E_2: 78% and 100 ng/mL A_4; 56%). However, the mean diameter of oocytes grown in the medium with 100 ng/mL E_2 alone (115.6 ± 0.8 μm) was smaller than that of *in vivo* grown oocytes (119.4 ± 0.4 μm), and the rate of oocytes matured to MII in subsequent *in vitro* maturation culture (22 h) was 30% in this medium. In the medium containing both E_2 and A_4 (10 ng/mL E_2+10 ng/mL A_4 and 100 ng/mL E_2+10 ng/mL A_4), 70% and 78% of OGCs, respectively, maintained their structures on Day 14, and oocytes matured to MII at high rates after maturation culture (58% and 54%, respectively). Moreover, the mean diameter of oocytes grown in the medium with those combinations of E_2 and A_4 (10 ng/mL E_2+10 ng/mL A_4 and 100 ng/mL E_2+10 ng/mL A_4) was increased to 119.6 ± 0.9 μm and 119.3 ±

0.8 μm, respectively, similar in size to *in vivo* grown oocytes. When OGCs were cultured with androgens alone, 59% and 63% of OGCs cultured with 10 ng/mL A_4 and 10 ng/mL T, respectively, maintained their structures on Day 14, while the integrity of OGCs (28%) was low in the medium with 10 ng/mL DHT alone. In the medium with both DHT and E_2, however, 71% of OGCs were integral on Day 14, and 62% of the oocytes matured to MII in subsequent maturation culture, which was higher than oocytes grown with 10 ng/mL E_2 alone (35%). These results indicate that *in vitro* growth culture of bovine oocytes with combinations of E_2 and androgens resulted in the complete oocyte growth and the acquisition of meiotic competence. It is inferred that E_2 and androgens have different roles in oocyte growth; E_2 maintains OGC structure during oocyte growth, while androgens promote the oocyte growth and the acquisition of meiotic competence. Since dome-like structures were developed only in the medium containing steroid hormones, it is also inferred that they appeared to mimic *in vivo* follicle structures and played important roles in oocyte growth *in vitro*. Further study is needed to determine the roles of dome-like structures in *in vitro* oocyte growth. Whether differentiation of granulosa cells into cumulus cells and mural granulosa cells occurs during *in vitro* culture or not will be a first step to understand the roles of dome-like structure.

In Chapter III, the roles of E_2 and androgens on bovine oocyte growth were determined. In Chapter III-1, the effect of steroid hormones on connections between oocytes and granulosa

cells was determined. To support oocyte growth *in vitro*, the culture systems must meet certain conditions for maintaining connections between oocytes and surrounding granulosa cells. OGCs collected from early antral follicles (0.4–0.7 mm in diameter) were cultured for 14 days in the medium 0, 10 and 100 ng/mL E_2 and A_4. I then assessed the number of transzonal projections (TZPs), which extend from granulosa cells through the zona pellucida to the oocytes. Growing oocytes isolated from early antral follicles had uniformly-distributed TZPs throughout the zona pellucida. After 14 days of culture, there was a significant decrease in the number of TZPs in oocytes grown without steroid hormones, while the number of TZPs was maintained in oocytes grown with steroid hormones. In particular, oocytes grown with 100 ng/mL E_2 alone, or with a combination of 10 ng/mL E_2 and A_4, had numbers of TZPs similar to oocytes before growth culture. These results indicate that E_2 have an advantageous role in formation or maintenance of TZPs during growth culture of OGCs.

Although the experiment using DHT in Chapter II indicates the direct effect of androgens on the oocytes growth, further study using the androgen receptor (AR) inhibitor was conducted to elucidate the androgen actions on oocyte growth in Chapter III-2. OGCs collected from early antral follicles were cultured for 14 days with combinations of 10 ng/mL E_2 and androgens (10 ng/mL A_4 or DHT) and hydroxyflutamide (OHF; 0, 1 or 5 μg/mL), which is an AR inhibitor. In the medium with steroid hormones, OGCs formed dome-like structures during

growth culture, while oocytes were denuded in the medium without steroid hormones. Oocytes cultured with E_2 and androgens (E_2+A_4 and E_2+DHT) grew to similar sizes as *in vivo* grown oocytes, and matured to MII at higher rates (63% and 69%, respectively) after maturation culture than E_2 alone (32%). When OHF was added to the medium, however, the rates of MII oocytes were decreased in a dose-dependent manner. Since ARs expressed in the nuclei of granulosa cells and oocytes in bovine early antral follicles, androgens appeared to affect the oocytes through ARs. These results in Chapter III showed that E_2 maintained the physical connections between oocytes and surrounding granulosa cells to support oocyte viability and growth, while androgens in combination with E_2 promoted the oocyte growth and their acquisition of meiotic competence during *in vitro* growth culture. The androgen-induced oocyte growth and maturation were suppressed by the AR inhibitor, indicating that androgens themselves promote the growth of oocytes and their acquisition of meiotic competence.

In Chapter IV, I examined the fertilization ability and embryonic development of *in vitro* grown oocytes to assess whether *in vitro* growth culture system developed in this study mimics *in vivo* environment. OGCs collected from early antral follicles were cultured for growth for 14 days with E_2 and A_4, matured for 22 h, and inseminated with spermatozoa for 6 or 12 h *in vitro*. Few *in vitro* grown oocytes were fertilized normally after 6 h of insemination and almost never developed to blastocysts after 8 days of culture (1%). Meanwhile, 34 % of *in vitro* grown

oocytes were fertilized normally after 12 h of insemination, having two pronuclei and two polar bodies. In addition, 22% of oocytes inseminated for 12 h developed to blastocysts after 8 days of culture. These rates of fertilization and blastocyst formation were similar to those of *in vivo* grown oocytes. In addition, the numbers of cells that composed blastocysts derived from *in vivo* and *in vitro* grown oocytes were also similar. The results suggest that *in vitro* grown bovine oocytes are able to be fertilized in the similar way as *in vivo* grown oocytes and are competent to develop to blastocysts.

In conclusion, steroid hormones play crucial roles in bovine oocyte growth *in vitro*. The combinations of E_2 and androgens support oocyte growth and promote the acquisition of meiotic competence of oocytes *in vitro*. The results in the present study demonstrate the different roles of steroid hormones in oocyte growth; E_2 has a major role in oocyte survivability by maintaining the connections between oocyte and surrounding granulosa cells, while androgens themselves promote the oocyte growth and their acquisition of meiotic competence through ARs in granulosa cells and/or oocytes. The present *in vitro* growth culture system also supports the acquisition of fertilization ability and developmental competence during oocyte growth. Such i*n vitro* culture systems for large animals would be valuable in agricultural and medical fields.

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