Study on the growth of oocytes and steroidogenesis of granulosa cells in cattle: Effects of bone morphogenetic protein-4, follicle stimulating hormone and antral follicle count in ovaries on *in vitro* and *in vivo* follicular development

（牛卵子の発育と顆粒層細胞の性ステロイドホルモン産生の関係:
骨形成タンパク質 4、卵胞刺激ホルモンおよび卵巣内胞状卵胞数が
体外および体内での卵胞発育に及ぼす影響）

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Abbreviations

A_4: androstenedione

AFC: antral follicle count

AMH: anti-Müllerian hormone

ANOVA: analysis of variance

BMP: bone morphogenetic protein

BSA: bovine serum albumin

CMO: carboxymethyloxime

COC: cumulus-oocyte complex

DPBS (–): Dulbecco's phosphate buffered saline without calcium and magnesium

E_2: estradiol-17β

EDTA: ethylenediaminetetraacetic acid

FCS: fetal calf serum

FSH: follicle stimulating hormone

GnRH: gonadotropin releasing hormone

HEPES: 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid

HRP: horseradish peroxidase

HSD: honestly significant difference

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVG: *in vitro* growth

IVM: *in vitro* maturation

LH: luteinizing hormone

mRNA: messenger ribonucleic acid

OCGC: oocyte-cumulus-granulosa complex

OPU: ovum pick-up

P_4: progesterone

P450arom: aromatase

P450scc: cholesterol side-chain cleavage enzyme

PGC: primordial germ cell

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$

SD: standard deviation

SEM: standard error of the mean

SNPs: single nucleotide polymorphisms

StAR: steroidogenic acute regulatory protein

T: testosterone

TCM 199: tissue culture medium 199

TGF-β: transforming growth factor-β

TMB: 3, 3', 5, 5'-tetramethylbenzidine

Contents

Preface

In 1978, Steptoe and Edwards[99] reported the birth of "Louise Brown", the world first test tube baby produced by *in vitro* fertilization (IVF). After that, the technology has been applied to produce offspring in other species such as cattle[10], goat[36], pig[16], sheep[16], cat[33], horse[83], and dog[77]. Nowadays IVF technology has been widely used commercially for producing embryos in cattle[67]. In addition, the bovine genome sequences[20] and the variation of single nucleotide polymorphisms (SNPs)[28] have already clarified, and the "genomic selection" based on SNPs dramatically has shortened the generation intervals[27]. Therefore, the demand for producing embryos from younger heifers before the applicable stage of superovulation and uterine flush for *in vivo* embryo collection is increasing[17, 61, 67]. Ultrasound-guided ovum-pick up (OPU) combined with IVF is widely used to produce embryos in cattle for genetic improvement[67], and the efficiency of embryo production by OPU-IVF is higher than that by *in vivo* embryo production[9, 87]. Therefore, the number of embryos produced *in vitro* has been increased internationally, and become similar to that produced *in vivo*[85]. On the other hand, the developmental competence of oocytes matured *in vitro* by *in vitro* maturation (IVM) is lower than that of oocytes matured *in vivo*[64, 98]. Also, bovine immature oocytes transferred to pre-ovulatory follicles and induced maturation in follicles showed higher developmental competence than oocytes matured *in vitro*[42, 53]. These results indicate that further studies are necessary to investigate the acquisition of oocyte developmental competence *in vivo* and *in vitro* for the improvement of embryo production *in vitro*.

The primary roles of the ovaries are to support the growth and maturation of oocytes for the acquisition of fertilizability and competence for embryonic and fetal developments, as well as the production of sex steroid hormones for inducing the estrous cycle and sustaining pregnancy.

These ovarian functions are regulated by gonadotrophins and steroid hormones. In mono-ovulatory species, the emergence of follicular growth is induced by a surge-like secretion of follicle stimulating hormone (FSH). Then a dominant follicle is selected as the decrease of the level of FSH by the inhibitory effect of estradiol-17β (E_2) and inhibin secreted by follicles themselves. The dominant follicle continues to grow by luteinizing hormone (LH), and results in ovulation[5, 29]. Namely, most of follicles degenerate during follicular growth, and only a small proportion of follicles develops and ovulates[5, 29]. If it is possible to develop a culture system that enables oocytes or follicles in early stages to grow to the ovulatory stage *in vitro*, more oocytes having high developmental competence can be produced for the embryo production, and an experimental model can be established for investigating the mechanisms underlying follicular growth and the acquisition of oocyte developmental competence.

In vitro growth (IVG) culture enables small oocytes derived from follicles in early developmental stages to grow to oocytes having the competence to develop to the blastocyst stage and offspring. In mice, the organ culture of neonatal ovaries, combined with IVG culture of oocyte-cumulus-granulosa complexes (OCGCs), allows pups to be produced from primordial follicles *in vitro*[22, 68, 78]. Recently, differentiation from primordial germ cells (PGCs) to functional oocytes[69] or PGC-like cells derived from embryonic stem cells and induced pluripotent stem cells[39] were realized in mice, resulting in the production of offspring after IVG of these oocytes[39, 69]. On the other hand, in cattle, there are no studies produced calves from follicles earlier than preantral follicles. In conventional *in vitro* embryo production including ultrasound-guided OPU-IVF, oocytes derived from antral follicles lager than 2 mm in diameter are used[80]. After collection, these oocytes can be directly subjected to conventional IVM, IVF, and *in vitro* culture (IVC) for developing to blastocysts[80]. On the other hand, early antral follicles smaller than 1 mm in diameter cannot be detected by general ultrasonography[51], and the oocytes derived from early antral follicles cannot achieve nuclear maturation if they are directly subjected to IVM[40]. Some studies reported the production of bovine calves derived from IVG oocytes that originated from early antral follicles[40, 43, 105]. These results suggest that the follicular

development from early antral follicle stage (<1 mm in diameter) is critical stage for oocytes to acquire maturational and subsequent developmental competences. Therefore, bovine IVG culture system can be utilized as an experimental model for studying factors affecting the acquisition of developmental competence of oocytes from small follicles those cannot be detected by ultrasonography. However, in the conventional bovine IVG system, high concentration of E_2 was added into the media to compensate lack of theca cells, that provide androgens to granulosa cells for E_2 synthesis in the OCGCs[40, 43, 109]. It means that we cannot evaluate the ability of E_2 production in granulosa cells, which is important for the acquisition of oocyte developmental competence.

Bone morphogenetic protein (BMP)-4 is one of a member of growth factors called BMPs included in transforming growth factor-β (TGF-β) family. BMPs are known to regulate FSH-dependent steroidogenesis in ovaries, and to inhibit the luteinization of growing follicles[96]. Especially among them, BMP-4 is produced by theca cells in bovine follicles, that was absent from current bovine IVG, and its receptor is expressed in oocytes and granulosa cells[32]. An *in situ* hybridization analysis of rat ovaries revealed that the expression level of BMP-4 increases during follicular growth[24]. In addition, BMP-4 suppresses the progesterone (P_4) production[106] and promotes FSH-mediated E_2 production[46] of granulosa cells. These findings indicate that BMP-4 has an important role in *in vivo* growth of follicles and oocytes, and that BMP-4 addition to IVG media can be alternative method instead of co-culture with theca cells. In chapter I, therefore, I cultured OCGCs in IVG media without exogenous E_2 but containing androstenedione (A_4), and examined effects of BMP-4 and FSH on maturational competence of oocytes and steroidogenesis of OCGCs. In addition, I also evaluated the possibility of the IVG culture system as a model of follicular growth.

The ovarian reserve, the pool of primordial follicles in a pair of ovaries in individuals, is defined as the potential ability of ovarian function[12, 104] and is known to be an indicator of

female fertility in mono-ovulatory species such as humans[12] and cattle[47]. The peak number of antral follicles in a pair of ovaries during follicular waves counted by ultrasonography (the antral follicle count; AFC) positively correlates with the number of primordial follicles[50] and can be used to estimate the ovarian reserve[13]. Although AFC fluctuates during the estrous cycle and markedly varies between individuals, the peak AFC during the estrous cycle shows high repeatability in individual cattle[13]. In general, cattle with high number of antral follicles in a pair of ovaries showed higher reproductive performance, such as higher fertility[72], a shorter open period[72], and higher responsiveness to superovulation[48] than cattle with low number of antral follicles, even though they were in the same age class. These findings indicate that AFC is related to follicular growth and oocyte developmental competence; therefore, the study on the difference in the functions of oocytes and granulosa cells of cattle having varied AFC can clarify the process of the acquisition of oocyte developmental competence. In chapter II, I examined the relationship between AFC, steroidogenesis of granulosa cells, and oocyte maturational and developmental competences of OCGCs derived from early antral follicles (0.5-1.0 mm in diameter) by using IVG culture system established in chapter I.

In chapter III, I examined bovine follicular growth *in vivo* in cows having varied AFC to investigate the mechanism of the lower E_2 production and lower oocyte competence in the low AFC cows found in chapter II. The follicular growth dynamics observed by ultrasonography, plasma concentrations of FSH and steroid hormones, and steroidogenesis in the dominant follicles in different stages of follicular growth were examined. I also evaluated the concentration of anti-Müllerian hormone (AMH) in plasma, follicular fluid, and IVG media, which has been demonstrated a positive correlation with AFC[50], and affects FSH-mediated E_2 production[14, 15, 18, 34].

Chapter I

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

Introduction

A large number of primordial follicles exist in mammalian ovaries, and granulosa cells multiply and oocytes become developmentally competent as they grow. However, most follicles degenerate during follicular growth, and only a small proportion of follicles develop sufficiently to undergo ovulation[2, 3]. If it is possible to develop a culture system that enables early stage oocytes or follicles to grow to the ovulatory stage, more embryos may be produced, and an experimental model may also be established to investigate the mechanisms underlying follicular recruitment, selection, and ovulation.

The primary roles of follicular cells are to support the growth and maturation of oocytes, as well as the production of sex steroid hormones by granulosa cells. Therefore, in order to mimic *in vivo* follicular growth via an *in vitro* culture system, the growth of oocytes and production of sex steroid hormones by granulosa cells both need to be investigated. A previous study that combined histological observations with the measurement of sex steroid hormones in follicular fluid revealed that E_2 concentrations in growing follicles increased as the follicles grew, with a peak at estrus in cattle, and the degeneration of follicles led to increases in P_4 concentrations[57]. Furthermore, E_2 concentrations in dominant follicles increased concomitantly with follicular development and the E_2/P_4 ratio also increased; however, subordinate follicles showed low E_2/P_4 ratios[7, 57]. These findings indicate that a culture system of OCGCs that produces more E_2 and less P_4 is needed to mimic *in vivo* dominant follicular development. In

conventional IVG, serum has typically been added to culture media to promote cell growth and survival[40, 43, 105]. However, granulosa cells cultured in media containing serum luteinize, compromise E_2 production, and begin to produce P_4[55, 81]. Previous studies attempted to culture OCGCs in serum-free media[94, 95]; however, the oocytes derived from serum-free cultures had low maturational competence and low fertilizability. Thus, a culture system for OCGCs that produces oocytes with high developmental competence and inhibits the luteinization of granulosa cells under culture conditions containing serum needs to be developed. As shown in a previous study, the addition of BMP-4 to the growth medium inhibited the luteinization of granulosa cells[109].

BMP-4 is produced by theca cells in bovine follicles, and its receptor is primarily expressed in oocytes and granulosa cells[32]. An *in situ* hybridization analysis of rat ovaries revealed that the expression level of BMP-4 increases during follicular growth[24]. Based on previous studies of cultured granulosa cells without oocytes, BMP-4 promotes E_2 production by inhibiting apoptosis[54] and promoting aromatase (P450arom) activity in cattle[32]. In addition, P_4 production is inhibited by the suppression of steroidogenic acute regulatory protein (StAR) in cattle[106] and sheep[86] and cholesterol side-chain cleavage enzyme (P450scc) in sheep[86] at the messenger ribonucleic acid (mRNA) and protein levels. Moreover, BMP-4 promotes the FSH-mediated activation of E_2 production, which is increased in the presence of oocytes[46]. On the other hand, P_4 production was inhibited in a manner independent of the presence of oocytes in an *in vitro* study of rat granulosa cells[46]. Therefore, the addition of BMP-4, which compensates for the lack of theca cells, and FSH to the medium of IVG for bovine OCGCs may promote oocyte growth by promoting E_2 production and inhibiting P_4 production. According to the previous report using bovine IVG, the addition of BMP-4 (10 ng/mL) to an IVG culture suppressed P_4 production and did not affect oocyte growth, nuclear maturation, or fertilization, but impaired

subsequent embryonic development, and at a higher concentration (50 ng/mL), even compromised the viability of OCGCs by suppressing the proliferation of granulosa cells[109]. In that study[109], OCGCs were cultured in medium that contained high concentrations of E_2 (1 μg/mL) to increase the E_2/P_4 ratio similar to a dominant follicle[7, 57]; therefore, the effects of BMP-4 and FSH on the E_2 production of OCGCs were unclear. In chapter I, I added BMP-4 and FSH to the growth medium without the addition of E_2 and examined the production of sex steroid hormones from individually cultured OCGCs. I also retrospectively analyzed the correlation between the steroidogenesis of OCGCs during the IVG culture and the nuclear maturation of the corresponding oocytes.

Materials and Methods

Chemicals

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

Collection of OCGCs and the IVG culture

Ovaries of Holstein cows obtained from a local abattoir were stored in plastic bags at 20°C and transported to the laboratory within 6-10 hours of their collection. After the ovaries were washed three times with physiological saline, slices of ovarian cortex tissues (thickness <1 mm) were prepared using a surgical blade (no. 11) and stored in tissue culture medium 199 (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% (w/v) polyvinyl alcohol, 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium, pH 7.4) at 37°C, as described elsewhere[37]. Under a stereomicroscope, early antral follicles (0.5-1.0 mm in diameter) were dissected from sliced ovarian tissues using a surgical blade (no. 20) and fine forceps in a 90-mm petri dish that had a 1-mm scale on its bottom (FLAT Co., Ltd., Chiba, Japan). OCGCs were isolated from early antral follicles using a pair of fine forceps and subjected to IVG

as previously described[45]. The growth medium was HEPES buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (MW 360,000), 50 μg/mL ascorbic acid 2-glucoside (Wako Pure Chemical Industries, Osaka, Japan), 55 μg/mL cysteine, 50 μg/mL gentamicin sulfate, and 10 ng/mL A_4 as a precursor for E_2[103]. OCGCs with oocytes surrounded by a cumulus investment and attached mural granulosa-cell layer (Fig. I-1) were cultured individually in a 96-well culture plate (Primaria 353872; Corning Life Sciences, Tewksbury, MA, USA) with 200 μL of growth medium at 39°C for 12 days in humidified air with 5% CO_2. At the onset of the IVG culture, OCGCs were photographed under an inverted microscope (CK 40; Olympus, Tokyo, Japan) with an attached CCD camera (Moticam 2000; Shimadzu Rika, Tokyo, Japan). The diameters of the oocytes without a zona pellucida were assessed using software (Motic Images Plus 2.2s; Shimadzu Rika). Every 4 days of the IVG culture, half (100 μL) of the growth medium was replaced with the same amount of fresh medium. Spent media collected at 4, 8, and 12 days of the culture were stored at –30°C until steroid hormone assays were conducted.

Evaluation of OCGC morphology

After 4 days of IVG culture, the morphological appearance of OCGCs was examined (Fig. I-1). OCGCs with an evenly granulated ooplasm that were completely enclosed by several layers of healthy cumulus and granulosa cells were defined as normal. When OCGCs had oocytes with an abnormal appearance and/or oocytes were denuded by scattering cumulus and granulosa cells, OCGCs were defined as abnormal.

Evaluation of the characteristics of granulosa cells

The total number, viability, and diameter of granulosa cells after growth culture from morphologically normal OCGCs on days 8 and 12 were assessed using an acridine orange/propidium iodide cell viability kit together with a cell counter (F23001 and L2000, respectively; Logos Biosystems, Gyunggi, Republic of Korea) as previously described[109]. The culture medium in the well of each viable OCGC was removed and replaced with 80 μL of

Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS (–)) supplemented with 0.125% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) to prepare the granulosa cells for counting. After 10 minutes of trypsinization and pipetting several times, 20 μL of FCS were added to stop the digestion. Denuded oocyte was removed from the well and discarded.

Evaluation of nuclear status of oocyte after IVG and IVM

After 12 days of IVG culture, cumulus-oocyte complexes (COCs) were collected from morphologically normal OCGCs by aspiration using a fine glass pipette. Immediately after IVG, some of the oocytes were denuded from cumulus cells by individually pipetting, and fixed in 10 μL of DPBS (–) containing 60% (v/v) methanol in each well of micro-well plates (MiniTrays 163118, NUNC, Roskilde, Denmark) for 30 minutes. After fixation, oocytes were stained with 5 μg/mL Hoechst 33342 in 10 μL of DPBS (–) in each well of the micro-well plate at 37°C for 15 minutes in the dark, as described elsewhere[62]. The nuclear status was then evaluated under an inverted fluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) using an ultraviolet filter (excitation 334-365 nm) to confirm meiotic arrest. Oocytes with a nuclear envelope were defined as being in the germinal vesicle stage. Other oocytes were submitted to individual IVM as previously described[75]. COCs were collected from morphologically normal OCGCs and were washed with IVM medium, which consisted of HEPES buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 20 μg/mL FSH, 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate. IVM of oocytes was performed in each well of micro-well plates filled with 6 mL of IVM medium at 39°C under 5% CO_2 in air for 22 hours. After IVM, oocytes were denuded from cumulus cells by individually pipetting, photographed, and their diameters were measured. Oocytes were mounted individually on a slide glass and fixed with a mixture of acetic acid and ethanol (1:3) for 6 hours. After fixation, oocytes were stained with 1% (w/v) aceto-orcein and the status of their nuclei was examined under a phase contrast microscope, as described

elsewhere[76]. Oocytes that reached metaphase II and had a polar body were defined as mature; oocytes with other nuclear statuses were defined as immature.

E_2 and P_4 assays

Spent media (100 μL) from IVG cultures were assayed to determine the E_2 and P_4 concentrations using a competitive double antibody enzyme immunoassay, as previously described[107]. Samples were subjected to 2- to 2000-fold serial dilutions with assay buffer (145 mM NaCl, 40 mM Na_2HPO_4, and 0.1% (w/v) bovine serum albumin (BSA) (pH 7.2). Diluted samples (20 μL) were incubated with the primary antisera and horseradish peroxidase (HRP)-labeled hormone (100 μL each) in the wells of a 96-well microplate (Costar 3590, Corning, NY, USA) coated with the secondary antiserum for 16-18 hours at 4°C. The primary antisera used for the E_2 and P_4 assays were anti-estradiol-17β-6-carboxymethyloxime (CMO)-BSA (QF-121, Teikoku Hormone Mfg. Co. Ltd., Kanagawa, Japan) and antiprogesterone-3-CMO-BSA (7720-0504, Biogenesis, Poole, England), respectively. Goat anti-rabbit serum (270335, Seikagaku, Tokyo, Japan) was used as the secondary antiserum. After washing all wells 4 times with 300 μL of washing buffer (0.05% Tween 80), 150 μL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (5 mM citric acid, 50 mM Na_2HPO_4, 500 mM urea hydrogen peroxide, 1 mM TMB, and 2% dimethyl sulfoxide) were added to each well and incubated at 37°C for 40 minutes. The absorbance of the solution in the wells was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan) after stopping the chromogenic reaction with 50 μL of 4 N H_2SO_4. All samples were assayed in triplicate. Assay sensitivities were 0.098 pg/well for E_2 and 0.195 pg/well for P_4. The inter- and intra-assay coefficients of variation were 15.8 and 4.0% for E_2 and 17.5 and 3.9% for P_4, respectively.

Experimental design

Experiment 1. Morphology and steroidogenic capacity of OCGCs

OCGCs were cultured in growth medium without BMP-4 or FSH for 4 days to select healthy OCGCs that exhibited steroidogenic activity similar to developing follicles *in vivo* (Fig. I-2). The correlation between the morphological normality of OCGCs and the production of steroid hormones was evaluated by measuring the concentrations of E_2 and P_4 in spent media. OCGCs defined as normal were subjected to subsequent experiments.

Experiment 2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa cells, growth and maturation of oocytes, and steroidogenesis

Normal OCGCs were randomly divided into 6 groups after 4 days of IVG culture to evaluate the effects of BMP-4 and FSH on the functions of OCGCs. I cultured OCGCs for an additional 8 days (a total of 12 days of IVG culture) in growth medium supplemented with different concentrations of BMP-4 (0, 10, or 50 ng/mL; HZ-1045, Humanzyme, Chicago, IL, USA) and FSH (0 or 0.5 ng/mL) (Fig. I-2). The concentrations of BMP-4 and FSH were determined according to previous studies in which granulosa cells were cultured *in vitro*[54, 86, 93]. In these studies, 10 and 50 ng/mL BMP-4 promoted cell viability[54] and suppressed P_4 production[86], whereas 0.5 ng/mL FSH enhanced the expression of the P450arom mRNA and E_2 production[93]. After 8 and 12 days of IVG culture, the viability of OCGCs was defined by the normality of their morphological appearance (Fig. I-1); *i.e.*, morphologically normal OCGCs were defined as having survived, whereas morphologically abnormal OCGCs were defined as dead. In addition, antrum formation in the granulosa cell layer[40] was noted on day 12 of the IVG culture. On day 12 of the IVG culture, 233 oocytes that were judged as having survived were subjected to IVM and nuclear maturation was examined (5-20 oocytes/replicate). However, 12 oocytes were accidently collapsed during the pipetting procedures for denudation from cumulus cells. Other oocytes that were not subjected to IVM were used to evaluate meiotic arrest immediately after the IVG culture. *In vivo*-derived oocytes collected from antral follicles

of 2-8 mm in diameter were also subjected to IVM and served as *in vivo* controls. Oocyte growth was evaluated as the difference in oocyte volume calculated based on diameters[41] before the IVG culture and after IVM.

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

Steroid hormone production (ng) = 0.2 (mL) × Concentration at the end of the period (ng/mL) - 0.1 (mL) × Concentration at the start of the period (ng/mL)

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

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

Statistical analysis

All statistical analyses were performed using software (JMP version 10, SAS Institute, Cary, NC, USA and StatView 4.51, Abacus Concepts, Inc., Calabasas, CA, USA). Differences in steroid hormone production from OCGCs which had normal or abnormal appearance were analyzed using Student's *t*-test. The effects of BMP-4 and FSH on the viability of OCGCs and the nuclear maturation of IVG oocytes were analyzed using a chi-square test. The differences in oocyte volumes between experimental groups cultured with the same FSH concentrations were analyzed using Tukey-Kramer's honestly significant difference (HSD) test. Volumes of oocytes

cultured with the same BMP-4 concentrations were analyzed using Student's *t*-test. Oocyte volumes measured before and after IVG were compared using Dunnett's test. The volume before IVG served as a control. The differences in the production of steroid hormones and the characteristics of granulosa cells between experimental groups within the same culture period were compared using a one-way analysis of variance (ANOVA) followed by Tukey-Kramer's HSD test. The differences in steroid production and the characteristics of granulosa cells between days 4-8 and days 8-12 of culture were analyzed using Student's *t*-test. In the retrospective analysis, the relationship between antrum formation and steroid production was analyzed using Student's *t*-test. The characteristics of OCGCs (oocyte volume and steroid production) between OCGCs that produced mature and immature oocytes were compared using Student's *t*-test. The difference in antrum formation between OCGCs that produced mature and immature oocytes was analyzed using a chi-square test.

Results

*Experiment 1. Morphology and steroidogenic capacity of OCGC***s**

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

Experiment 2. Effects of BMP-4 and FSH on the viability of OCGCs, the characteristics of granulosa cells, growth and maturation of oocytes, and steroidogenesis

Eight hundred fifty-one OCGCs were cultured in media containing BMP-4 (0, 10, or 50 ng/mL) and FSH (0 or 0.5 ng/mL), and 718 OCGCs were cultured until day 12; the remaining 133 OCGCs were cultured until day 8 to evaluate the characteristic of granulosa cells. The viabilities of OCGCs on days 8 and 12 of IVG culture are shown in Table I-1. After 8 days of culture, the group cultured with 10 ng/mL BMP-4 in the absence of FSH showed higher viability than the group cultured with 50 ng/mL BMP-4 in the absence of FSH ($P < 0.05$). In the presence of 50 ng/mL BMP-4, the group with 0.5 ng/mL FSH showed higher viability than the group cultured without FSH on days 8 ($P < 0.05$). The viability of the group cultured with 10 ng/mL BMP-4 and 0.5 ng/mL FSH was similar between days 8 and 12; however, the viability of other groups decreased from days 8-12 ($P < 0.05$). As shown in Fig. I-4, on day 8, the group cultured with 10 ng/mL BMP-4 and 0.5 ng/mL FSH showed the highest total number of granulosa cells (approximately 68,000 cells) , which was larger than that in the group cultured with 50 ng/mL BMP-4 and 0.5 ng/mL FSH (approximately 42,500 cells, $P < 0.05$). Also, the number was slightly larger than that in the group cultured with 10 ng/mL BMP-4 in the absence of FSH (approximately 47,000 cells, $P = 0.06$). On day 12, no significant differences were observed in the total number of granulosa cells between the experimental groups. The total numbers of granulosa cells observed on days 8 and 12 were similar in the same experimental groups. The viability of granulosa cells on day 8 was approximately 100% in all experimental groups. On day 12, viability was greater than 90% in all groups and small differences were observed between the experimental groups. The highest viability was observed for cells cultured with 50 ng/mL BMP-4 in the absence of FSH (98.9%), and the lowest viability was observed for cells cultured with 50 ng/mL BMP-4 and FSH (92.5%). The addition of FSH to the groups cultured with 10 ng/mL BMP-4 (0 ng/mL FSH, 11.5 μm; 0.5 ng/mL FSH, 12.4 μm; $P < 0.01$) and 50 ng/mL BMP-4 (0 ng/mL FSH, 11.1 μm; 0.5 ng/mL FSH, 12.0 μm; $P = 0.05$) increased the diameters of

granulosa cells measured on day 8; however, no significant differences were observed between experimental groups on day 12.

The mean volumes of oocytes in all groups before the IVG culture were similar; therefore, the mean values of all groups were used as controls prior to the IVG culture. As shown in Fig. I-5, the mean volumes of oocytes in all groups were larger after IVG culture than before IVG ($P < 0.05$). In addition, the mean volumes of oocytes were similar in the groups cultured with 0 and 10 ng/mL of BMP-4 regardless of whether FSH was added following IVM. However, in the groups cultured with 50 ng/mL BMP-4, oocyte volumes were larger in the group cultured without FSH than in the group cultured in the presence of FSH ($P < 0.05$).

Two hundred thirty-three oocytes were studied to evaluate effects of BMP-4 and FSH on nuclear maturation. Immediately after IVG, before IVM culture, all oocytes were arrested at the stage of the germinal vesicle. The results are summarized in Table I-2. After the IVM culture, only oocytes derived from OCGCs cultured with 50 ng/mL BMP-4 in the absence of FSH showed a maturation rate similar to oocytes grown *in vivo*, whereas oocytes derived from other cultures had lower rates of nuclear maturation compared to the *in vivo* control ($P < 0.05$).

E_2 and P_4 productions were evaluated in 364 cultures with BMP-4 (0, 10, or 50 ng/mL) and FSH (0 or 0.5 ng/mL), and results are shown in Fig. I-6. E_2 was produced at a lower level from days 8-12 than from days 4-8, whereas P_4 production increased in all groups during culture ($P < 0.05$). From days 4-8, E_2 production did not significantly differ between the groups cultured without FSH, regardless of the presence or absence of BMP-4. In the presence of FSH, production decreased in the group cultured with 50 ng/mL BMP-4 ($P < 0.01$). Additionally, the addition of FSH to the groups cultured with 0 and 10 ng/mL of BMP-4 increased P_4 production from days 4-8 ($P < 0.05$). Between days 8 and 12, the addition of BMP-4 decreased E_2 production, regardless of whether FSH was added ($P < 0.05$). FSH decreased E_2 production in

the group cultured with 50 ng/mL BMP-4 ($P < 0.01$). The group cultured with 10 ng/mL BMP-4 in the absence of FSH exhibited the lowest production of P_4, regardless of the culture period.

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

Discussion

In the present study, OCGCs that had a normal appearance 4 days after the initiation of the IVG culture produced a large amount of E_2 and less P_4 during the initial period of the IVG culture. During the *in vivo* development of a dominant follicle, E_2 concentration increases as follicles grow[7, 57]. Thus, I only used healthy OCGCs in subsequent experiments to evaluate the effects of BMP-4 and FSH on steroidogenesis and oocyte maturation.

On day 8 of the IVG culture, the viability of the group cultured with 10 ng/mL BMP-4 was higher than the group cultured with 50 ng/mL BMP-4 ($P < 0.05$), when OCGCs were cultured

without FSH. The low viability of OCGCs cultured with 50 ng/mL BMP-4 may stem from a decrease in granulosa cell numbers, as reported previously[109]. Actually, the lowest number of granulosa cells was observed on day 8 in the group cultured with 50 ng/mL BMP-4 (approximately 41,000 cells/well) among all groups tested (approximately 47,000 cells/well) in the absence of FSH, although the values were not significantly different. On the other hand, when 0.5 ng/mL of FSH was added to growth medium, the viability of OCGCs in the group cultured with 50 ng/mL BMP-4 improved on days 8 and 12. In the present study, I only examined OCGCs that had survived; therefore, I could not find the increase of granulosa cell number. I speculate that the result may be attributed to the increased proliferation of granulosa cells by the addition of 0.5 ng/mL FSH from days 4-8[35], and was particularly apparent in the group cultured with 10 ng/mL BMP-4 and FSH, which showed the highest viability on day 12 (approximately 68,000 cells/well).

Between days 4 and 8 of the IVG culture, the addition of 10 ng/mL BMP-4 and 0.5 ng/mL of FSH did not affect E_2 production by OCGCs; however, this supplementation regime decreased P_4 production compared to cultures with 0.5 ng/mL FSH in the absence of BMP-4. On the other hand, no significant differences were noted between the number of granulosa cells in both groups; therefore, under the present culture conditions containing serum, 10 ng/mL BMP-4 may inhibit P_4 production by granulosa cells by suppressing StAR[86, 106] and P450scc[86] at the mRNA and protein levels. However, P450arom may not be enhanced, because previous studies showed that P450arom activity is increased in granulosa cells cultured without serum[32, 35]. A study that cultured granulosa cells for 6 days in the presence of serum showed that expression of the P450arom gene was suppressed, but not StAR and P450scc[55]. These findings indicate that the luteinization of granulosa cells induced by serum counteracts the stimulatory effects of BMP-4 on E_2 production. These results demonstrated that this supplementation regime permitted

OCGCs to partially mimic developing follicles, which secrete E_2 as follicles develop and the E_2/P_4 ratio increases[7]. However, 0.5 ng/mL FSH increased P_4 production from days 4-8 and particularly from days 8-12. Previous studies reported that *in vivo*-grown large luteal cells (38.4 μm in diameter) originated from granulosa cells and *in vitro*-luteinized granulosa cells (38.4 μm) are larger than granulosa cells in pre-ovulatory follicles (10.6 μm)[65, 79]. Under culture conditions in the present study, the mean diameter of granulosa cells on day 8 increased with the addition of FSH to medium containing 10 ng/mL BMP-4 (0 ng/mL FSH, 11.5 μm vs. 0.5 ng/mL FSH, 12.4 μm; $P < 0.01$) or 50 ng/mL BMP-4 (0 ng/mL FSH, 11.1 μm vs. 0.5 ng/mL FSH, 12.0 μm; $P = 0.05$). FSH appears to have enhanced the luteinization of granulosa cells under the present IVG conditions, as has been reported previously[97]. On day 12 of the IVG culture, no significant differences were observed in the diameters of granulosa cells between experimental groups; however, the group cultured with 50 ng/mL BMP-4 in the absence of FSH showed a larger diameter (12.2 μm) than the same group on day 8 of the IVG culture (11.1 μm, $P < 0.05$). These results indicate that the anti-luteinizing effects of BMP-4 were lost when the culture period was extended.

A previous study that cultured granulosa cells for 6 days in serum-free media demonstrated that E_2 and P_4 productions increased as the culture period was extended[92]. According to results in the present study, the production of E_2 from cultured OCGCs was maintained at least until day 8, even when the medium contained serum. However, granulosa cells are apparently unable to stably produce E_2 for 12 days of IVG culture. High densities of granulosa cells have been shown to inhibit E_2 secretion and P450arom expression and also to increase P_4 secretion and the levels of mRNA encoding progestogenic enzymes, such as StAR and P450scc[88]. It is previously reported that the number of granulosa cells peaked on day 12 of the IVG culture and then decreased[44]. These findings indicate that luteinization progresses as

the density of granulosa cells increases.

OCGCs cultured with 50 ng/mL BMP-4 in the absence of FSH showed the lowest viability; however, the surviving oocytes derived from these OCGCs had the largest volumes and highest levels of meiotic competence. The meiotic competence of bovine oocytes grown *in vivo* increases as oocytes[25, 82] and follicles[4] grow. The administration of exogenous BMP-4 during IVG (50 ng/mL)[109] and IVM (100 ng/mL)[26, 60] did not affect oocyte nuclear maturation in previous studies. Thus, BMP-4 does not directly promote oocyte nuclear maturation. However, OCGCs, which can produce oocytes with higher maturational competences, may survive in the presence of higher concentration of BMP-4 in the absence of FSH, and the highest viability of granulosa cells was also observed on day 12 (98.9%). Based on these results, I speculate that OCGCs producing oocytes with high intrinsic developmental competence may survive in the presence of high concentrations of BMP-4. In further studies, the relationship between oocyte competence and the function of granulosa cells needs to be examined.

In addition, FSH did not promote the nuclear maturation of oocytes in the present study. A previous study that cultured bovine OCGCs for 14 days with a higher concentration of FSH (3.5 mg/mL) showed that no oocyte progressed to the metaphase II stage and 73.3% of oocytes degenerated after IVM[58]. According to another study, the addition of 10 μg/mL FSH improved cumulus expansion, whereas the proportion of oocytes at metaphase II after IVM did not significantly differ from cultures that did not include FSH[38]. These findings and the present results suggest that FSH does not affect the maturational competence of bovine oocytes, although FSH improves granulosa cell proliferation[35] and cumulus expansion[38].

In the present study, OCGCs that formed antra produced more E_2 and less P_4 than those without an antrum. As reported in the study by Endo *et al*.[21], OCGCs that formed antra exhibited similar levels of gene expression to healthy follicles that grew *in vivo*. Thus, antrum

formation in the granulosa cell layer is related to the steroidogenesis of OCGCs. On the other hand, I was unable to detect a relationship between oocyte maturation and antrum formation in the granulosa cell layer, as has been described in a previous study[21]; however, OCGCs that produced mature oocytes produced slightly more E_2 and less P_4 during the IVG culture than OCGCs that produced immature oocytes. Furthermore, some of the OCGCs that produced mature oocytes secreted extremely large quantities of E_2. In the present study, I added A_4 to medium instead of E_2 used in the previous study[103], and did not observe any effects of BMP-4 on nuclear maturation. These results suggest that the ability to produce E_2 by granulosa cells has an important effect on the acquisition of oocyte ability. In future studies, we need to investigate the relationship between the maturational competence of oocytes, the steroidogenesis of granulosa cells and antrum formation, and the expression dynamics of growth factors and their receptors, such as BMP-4, in more detail.

Based on the results of the present study, BMP-4 inhibits the luteinization of granulosa cells and FSH enhances the proliferation of granulosa cells, viability of OCGCs, and the luteinization of granulosa cells. Moreover, cultured OCGCs with antra that produce a large amount of E_2 and less P_4 are similar to follicles that are grown *in vivo*. However, E_2 production increased until day 8 of the culture and then decreased. In conclusion, an IVG culture with 10 ng/mL of BMP-4 in the absence of FSH partially mimics *in vivo* steroidogenesis and the development of growing follicles until day 8 of culture. The same conditions also mimic the steroidogenesis of degenerating follicles from days 8-12 of the culture, particularly in the presence of FSH.

The addition of serum to the growth medium may have enhanced the luteinization of granulosa cells in the present study; however, oocytes derived from serum-free cultures had low maturational competence and low fertilizability in previous studies[94, 95]. In further studies, we

should develop an IVG system that does not use serum but enhances oocyte competence, or a system that inhibits the luteinization of granulosa cells even when the growth medium contains serum.

Tables and figures

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

Experimental groups		Day 8		Day 12	
BMP-4 (ng/mL)	FSH (ng/mL)	No. of oocytes (replicates)	% of viable OCGCs	No. of oocytes (replicates)	% of viable OCGCs
0	0.0	158 (14)	72.1^{ab}	132 (11)	62.1^{ab}
	0.5	172 (16)	76.8^{abx}	154 (13)	65.6^{ay}
10	0.0	120 (10)	80.8^{ax}	100 (7)	67.0^{ay}
	0.5	103 (8)	80.6^{ab}	83 (5)	71.1^{a}
50	0.0	153 (10)	69.9^{bx}	127 (7)	53.5^{by}
	0.5	145 (10)	80.0^{ax}	122 (7)	65.6^{aby}
Total		851* (68)	76.3	718 (50)	63.6

^{a,b} Different superscripts indicate differences between groups in the same culture period ($P < 0.05$).

^{x,y} Different superscripts indicate significant differences between days 8 and 12 in the same group ($P < 0.05$).

* One hundred and thirty-three OCGCs were used to evaluate the granulosa cell characteristics on day 8.

Table I-2. Effects of bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) on the nuclear maturation of oocytes after the maturational culture.

Experimental groups		No. of oocytes (replicate)	% of nuclear maturation
BMP-4 (ng/mL)	FSH (ng/mL)		
0	0.0	32 (4)	78.1[b]
	0.5	47 (5)	72.3[b]
10	0.0	46 (4)	60.9[b]
	0.5	44 (3)	66.7[b]
50	0.0	31 (4)	80.6[ab]
	0.5	33 (3)	75.8[b]
In vivo-grown oocytes		79 (7)	92.4[a]

[a,b] Different superscripts indicate significant differences within a column ($P < 0.05$).

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

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

Values are presented as means ± standard deviation (SD).

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

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

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

Values are presented as means ± SD.

* Oocytes at metaphase II were defined as mature.

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

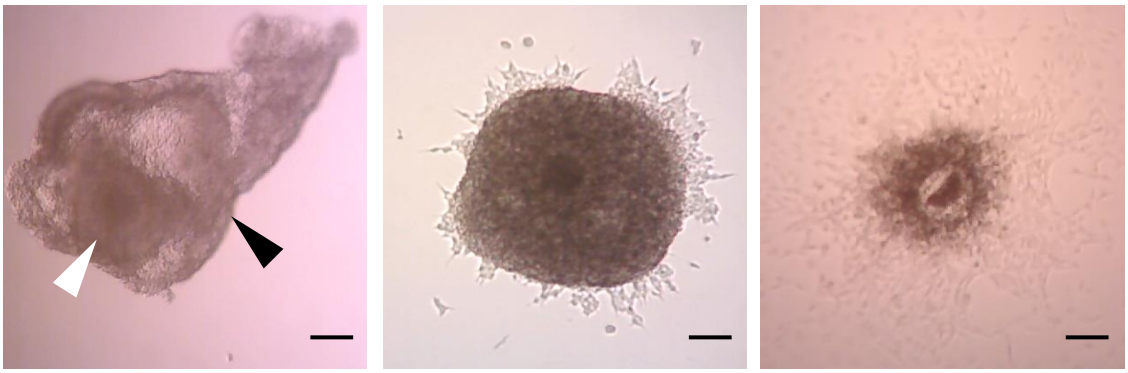

Fig. I-1. Oocyte-cumulus-granulosa complexes (OCGCs) before and after day 4 of the growth culture.

The left panel shows an OCGC before the growth culture. OCGCs with oocytes surrounded by a cumulus investment and an attached mural granulosa-cell layer were subjected to the growth culture. The white arrow head indicates the cumulus investment. The black arrow head indicates the mural granulosa-cell layer. The central panel shows an OCGC with an evenly granulated ooplasm and enclosed by several layers of healthy granulosa cells. The right panel shows an OCGC that has an abnormal appearance with a degenerated oocyte. Scale bars indicate 100 μm.

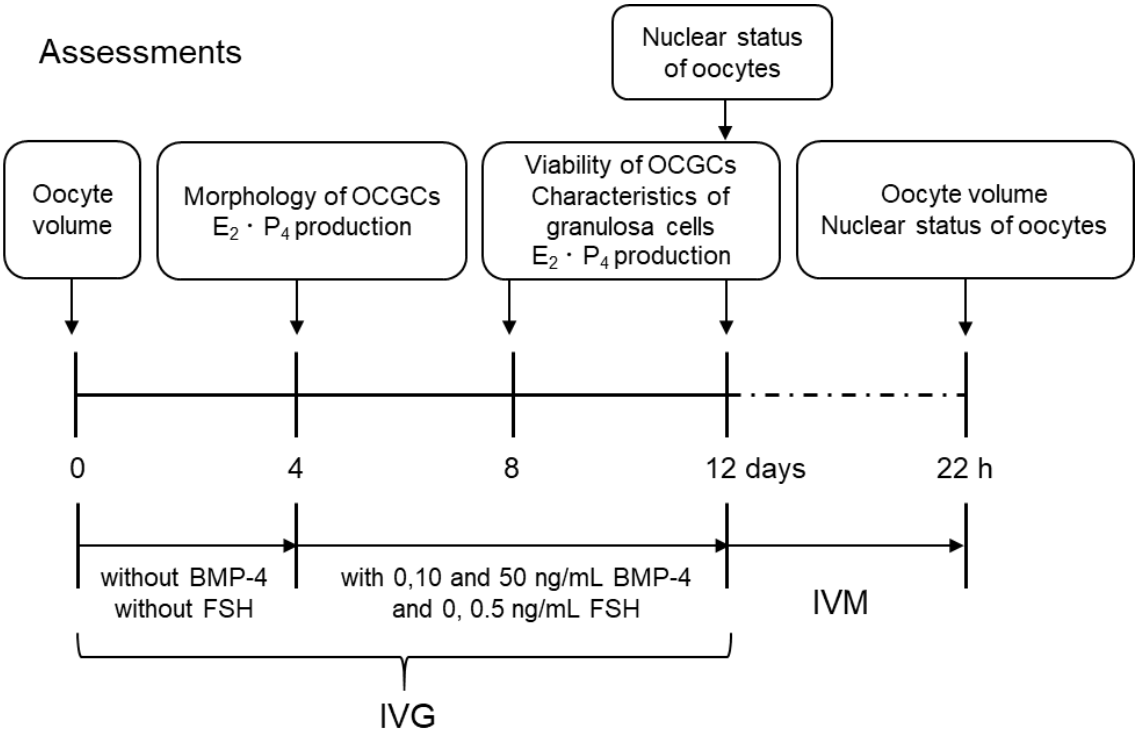

Fig. I-2. Schematic of experimental design.

Firstly, oocyte-granulosa cell complexes (OCGCs) were cultured in growth medium without bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) for 4 days. Morphologically normal OCGCs were cultured for an additional 8 days in growth medium supplemented with different concentrations of BMP-4 (0, 10, or 50 ng/mL) and FSH (0 or 0.5 ng/mL). On day 12 of the IVG culture, oocytes judged as having survived were subjected to IVM. The assessments described in the schematic were conducted at days 0, 4, 8, and 12 and after IVM.

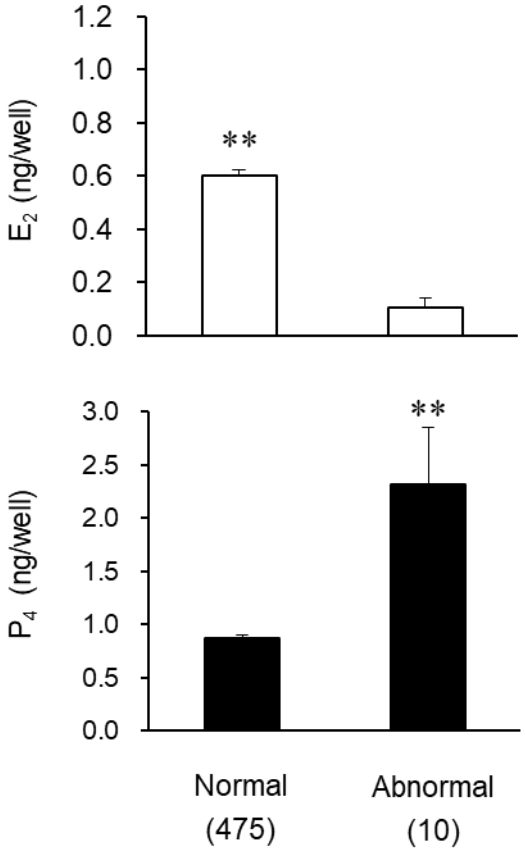

Fig. I-3. Steroidogenesis of oocyte-cumulus-granulosa-complexes (OCGCs) on day 4 of the growth culture.

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

Numbers in parentheses indicate the number of OCGCs evaluated.

Error bars indicate the standard error of the mean (SEM).

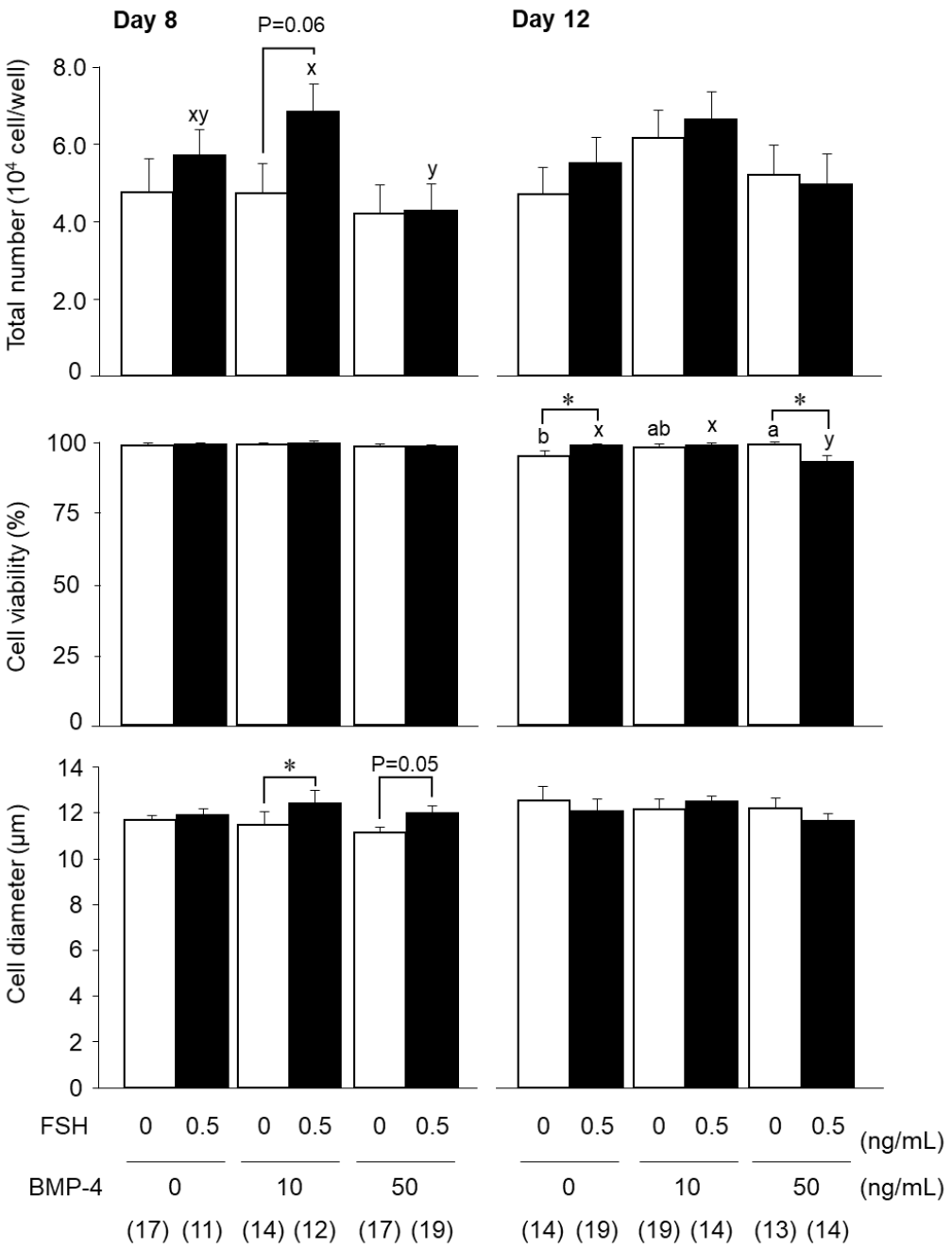

Fig. I-4. Effects of bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) on the number, viability, and diameter of granulosa cells at different culture period.

[a,b] Different letters indicate significant differences between OCGCs cultured without FSH in the same culture period ($P < 0.05$).

[x,y] Different letters indicate significant differences between OCGCs cultured with FSH in the same culture period ($P < 0.05$).

* Asterisks indicate significant differences between OCGCs cultured with the same BMP-4 concentration in the same culture period ($P < 0.05$).

Error bars indicate SEM.

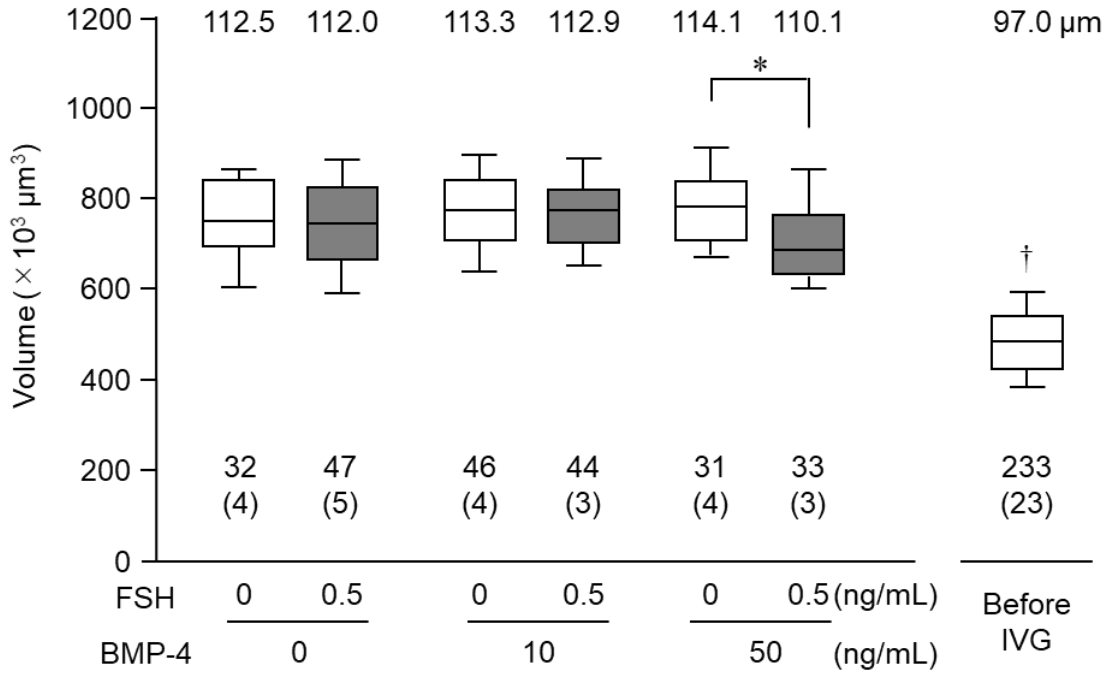

Fig. I-5. Effects of bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) on the oocyte volume after the growth culture.

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

Values under boxes indicate the numbers of oocytes examined and replicates in parentheses.

Lines on the boxes delineate the 25th, 50th, and 75th percentiles, while the whiskers depict the 10th and 90th percentiles.

* An asterisk indicates a significant difference between groups with and without FSH in the presence of the same BMP-4 concentration ($P < 0.05$).

† A dagger indicates a significant difference between the values measured before and after IVG in all groups ($P < 0.05$).

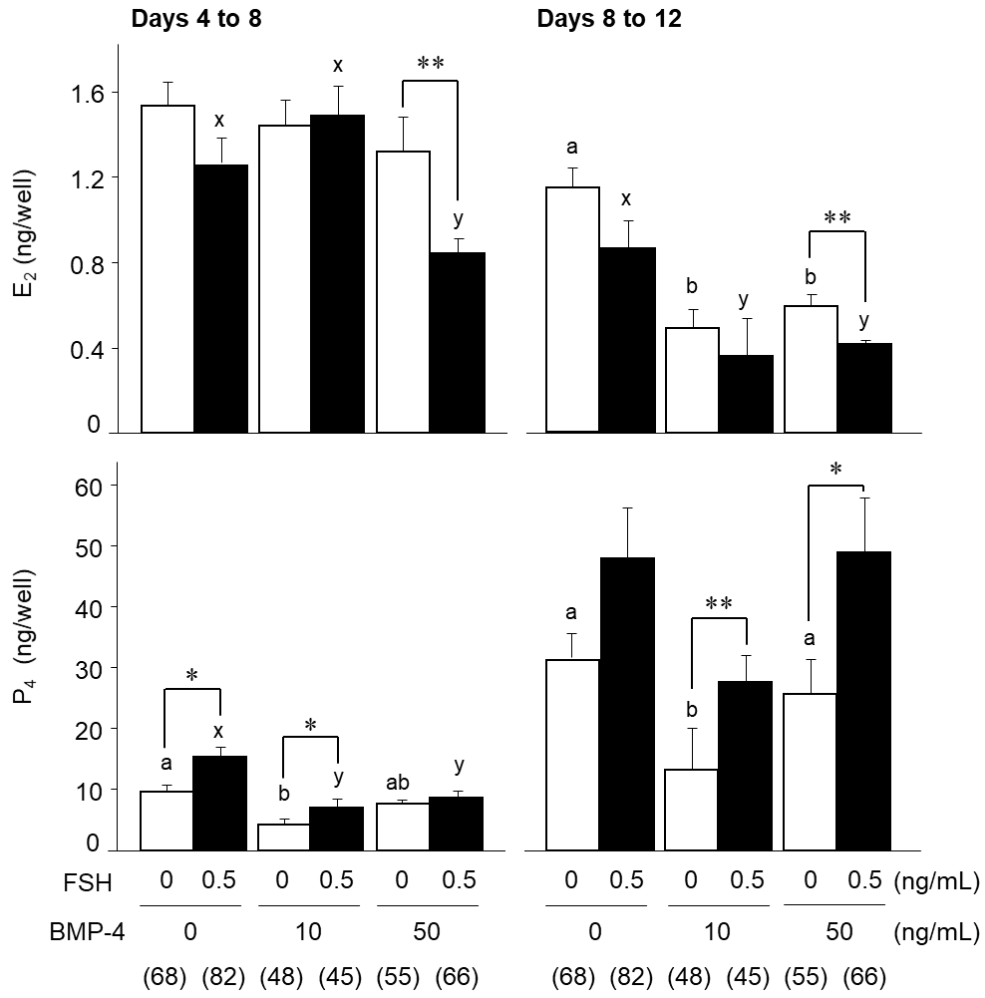

Fig. I-6. Effects of bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) on steroidogenesis from oocyte-cumulus-granulosa complexes (OCGCs) at different culture periods.

In all experimental groups, E_2 was produced at a lower level between days 8 and 12 than between days 4 and 8 ($P < 0.05$); P_4 was produced at a higher level between days 8 and 12 than between days 4 and 8 ($P < 0.05$).

[a,b] Different letters indicate significant differences between OCGCs cultured without FSH in the same culture period ($P < 0.05$).

[x,y] Different letters indicate significant differences between OCGCs cultured with FSH in the same culture period ($P < 0.05$).

*,** Asterisks indicate significant differences between OCGCs cultured with the same BMP-4 concentration in the same culture period (*$P < 0.05$; **$P < 0.01$).

Error bars indicate SEM.

Summary

Bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) play important regulatory roles in follicular growth and steroidogenesis *in vivo*. The purpose of this study was to investigate the effects of BMP-4 and FSH on *in vitro* growth (IVG) and steroidogenesis of bovine oocyte-cumulus-granulosa complexes (OCGCs). I cultured OCGCs collected from early antral follicles (0.5-1.0 mm) in medium without BMP-4 and FSH for 4 days and investigated the appearance of OCGCs and their steroidogenesis. During the first 4 days of IVG, morphologically normal OCGCs produced more estradiol-17β (E_2), but less progesterone (P_4). Morphologically normal OCGCs were subjected to an additional culture in medium supplemented with BMP-4 (0, 10, and 50 ng/mL) and FSH (0 and 0.5 ng/mL) until day 12. I examined the viability and steroidogenesis of OCGCs after 8 and 12 days of culture. Oocyte growth, characteristics of granulosa cells, and the maturational competence of oocytes were also investigated. On day 8, the viability of OCGCs cultured without FSH was higher in the 10 ng/mL BMP-4 group than in the 50 ng/mL BMP-4 group ($P < 0.05$). No significant difference was observed in the viability of groups cultured with FSH, regardless of the addition of BMP-4, and FSH improved the viability of 50 ng/mL BMP-4 group similar to 10 ng/mL BMP-4 group. The total number of granulosa cells was larger in 10 ng/mL BMP-4 group cultured with FSH than in 50 ng/mL BMP-4 group cultured with FSH on day 8 ($P < 0.05$). E_2 production decreased from days 8-12, and P_4 production increased throughout IVG culture, regardless of the addition of BMP-4 and FSH ($P < 0.05$). No significant differences in E_2 production were observed between groups from days 4-8, regardless of whether BMP-4 was added without FSH; however, E_2 production in the group cultured with 50 ng/mL BMP-4 was suppressed by FSH. BMP-4 suppressed E_2 production from days 8-12, regardless of whether FSH was added. The group cultured with 10 ng/mL BMP-4 without FSH showed the lowest P_4 production among all groups

for all culture periods. OCGCs that produced mature oocytes tended to secrete more E_2 and less P_4 than OCGCs that produced immature oocytes. In conclusion, until day 8 of the IVG culture, P_4 production by OCGCs was suppressed by the addition of 10 ng/mL BMP-4 in the absence of FSH, without inhibiting E_2 production. These conditions appear to mimic growing follicles until day 8 and mimic degenerating follicles from days 8-12 of culture.

Chapter II: Relationship between the antral follicle count in bovine ovaries from a local abattoir and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes

Introduction

The ovarian reserve, the number of primordial follicles in a pair of ovaries in individuals, is defined as the potential ability of these functions of ovaries such as to support the growth and maturation of oocytes, as well as the production of sex steroid hormones[104] and is known to be an indicator of female fertility in humans[12] and cattle[47]. The peak AFC positively correlates with the number of primordial follicles[50] and may be used to estimate the ovarian reserve[13]. Although AFC fluctuates during the estrus cycle and markedly varies between individuals, the peak AFC during the estrous cycle shows high repeatability in individual cattle[13]. Between 15 and 20% of individual cattle in a herd were generally classified according to the peak AFC during follicular waves into the low AFC group (15 or fewer follicles), while 15-20% were in the high AFC group (25 or greater follicles), and the remainder were in the intermediate group (16-24 follicles)[13, 47]. In general, cattle with high number of antral follicles in a pair of ovaries showed higher reproductive performance, such as higher fertility[72], a shorter open period[72], and higher responsiveness to superovulation[48] than cattle with low number of antral follicles, even though they were in the same age class.

It is reported that the fertilizability of oocytes collected by ultrasound-guided OPU was greater in high AFC cattle with 30 or more follicles in a pair of ovaries at the time of OPU than low AFC cattle with less than 30 follicles at a 3- or 4-day interval of OPU[73]. In contrast, when the interval of OPU was extended to 7 days, the fertilizability of oocytes in high AFC cattle was impaired and became less than that in low AFC cattle[73]. These findings indicate that the growth

dynamics of antral follicles differ between high and low AFC cattle, and the degeneration of antral follicles at the selection phase in the follicular wave may occur earlier in high AFC cattle than in low AFC cattle. To investigate the differences underlying follicular growth dynamics and the acquisition of oocyte competence between high and low AFC cattle, a study was conducted using an IVG culture of bovine oocytes[74], a culture system that enables bovine oocytes without maturational competence from early antral follicles to grow to the stage acquiring competence for maturation and development to the blastocyst stage[40, 43, 109]. Consequently, OCGCs derived from early antral follicles (0.5-1.0 mm in diameter) in the high AFC group with more than 25 follicles (≥2.0 mm in diameter) in an ovary collected at a slaughterhouse showed higher oocyte maturational competence and fertilizability as well as the higher proliferation of granulosa cells than those in the low AFC group (less than 25 follicles)[74]. However, in the previous study[74], OCGCs were cultured in medium containing a high concentration of E_2 (1 μg/mL) to increase the E_2/P_4 ratio, similar to a dominant follicle[57]; therefore, the relationships between AFC, the steroidogenesis of granulosa cells, and the developmental competence of oocytes have not yet been examined. In addition, I suggested that granulosa cells surrounding *in vitro*-grown oocytes having higher maturational competence secreted more E_2 and less P_4 than the granulosa cells surrounding less competent *in vitro*-grown oocytes using medium containing A_4 instead of E_2 in chapter I.

In chapter II, I investigated the relationship between AFC and the steroidogenesis of granulosa cells using a bovine IVG culture without the exogenous application of E_2. In addition, I confirmed the oocyte competence of growth, maturation, and subsequent development to blastocysts.

Materials and Methods

Collection of OCGCs and the IVG culture

Collection and IVG bovine OCGCs was performed as described in chapter I. Briefly, slaughter-house derived ovaries were sliced at approximately 1 mm thickness. Under a stereomicroscope, early antral follicles (0.5-1.0 mm in diameter) were dissected from sliced ovarian tissues. OCGCs isolated from early antral follicles were cultured individually in a 96-well culture plate with 200 μL of growth medium at 39°C for 12 days in humidified air with 5% CO_2. Every 4 days of the IVG culture, half of the growth medium (100 μL) was replaced with the same amount of fresh medium.

Evaluation of OCGC morphology

Morphology of OCGCs was evaluated as described in chapter I (Fig. II-1). Briefly, every 4 days of IVG culture, the viability of OCGCs was assessed by the morphological appearance; *i.e.*, OCGCs having an evenly granulated ooplasm that were completely enclosed by several layers of healthy cumulus and granulosa cells were defined as survived. OCGCs having oocytes with abnormal appearance and/or oocytes were denuded by scattering cumulus and granulosa cells were defined as dead. Simultaneously antrum formation in the granulosa cell layers, which is an indicator of higher ability for E_2 production, were also recorded.

Evaluation of the characteristics of granulosa cells

The characteristics of granulosa cells (total number, viability, and diameter) after growth culture from survived OCGCs on days 0, 4, 8, and 12 were examined as described in chapter I. Briefly, granulosa cells were stained using an acridine orange/propidium iodide dual fluorescence staining after trypsinization and pipetting several times. Characteristics of granulosa cells were evaluated by a cell counter.

E_2 and P_4 assays

Spent media at days 4, 8, and 12 were assayed for E_2 and P_4 concentrations using a competitive double antibody enzyme immunoassay as described in chapter I with slight modifications. The primary antisera used for the E_2 and P_4 assays were anti-estradiol-17β-6-CMO-BSA (FKA204, Cosmo bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13, Cosmo bio), respectively. Goat anti-rabbit serum (111-005-003, Jackson Immuno Research, West Grave, PA, USA) was used as the secondary antiserum. Assay sensitivities were 0.049 pg/well for E_2 and 0.391 pg/well for P_4. The inter- and intra-assay coefficients of variations were 16.9 and 4.0% for E_2 and 7.0 and 3.9% for P_4, respectively. Production of steroid hormones during each period (days 0-4, days 4-8, and days 8-12) was calculated as described in chapter I.

Evaluation of the growth, nuclear maturation, and developmental competence of oocytes

The growth and nuclear maturation of oocytes were evaluated as described in chapter I. Briefly, diameters of oocytes of OCGCs were measured by an inverted microscope with an attached CCD camera and an image analysis software at the onset of the IVG culture. Volumes of oocytes based on their diameter were calculated. After 12 days of IVG, COCs were collected from survive OCGCs and submitted to IVM in each well of the micro-well plates filled with 6 mL of IVM medium at 39°C under 5% CO_2 in air for 22 hours. After IVM, oocytes were denuded from cumulus cells by pipetting and their diameters were measured. Oocytes were stained with 1% aceto-orcein and their nuclear status was examined under a phase contrast microscope. Oocytes that reached metaphase II and had a polar body were defined as mature; oocytes at other nuclear statuses were defined as immature. After IVM, some COCs were subjected to IVF as previously described[102]. Briefly, frozen semen collected from a Holstein bull was used for IVF. After thawing the semen in a 37°C water bath for 40 second, motile

sperm (5×10^6 sperm/mL) separated by a Percoll gradient (45% and 90%) were co-incubated with COCs in a 100-μL droplet (8 to 12 COCs per droplet) of modified Brackett and Oliphant isotonic medium[11] containing 3 mg/mL fatty acid-free BSA and 2.5 mM theophylline[100] for 18 hours at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. IVC of inseminated oocytes (presumptive zygotes) was performed as previously described[101, 102]. Briefly, after a co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing for 5 minutes and washing three times in culture medium. Cumulus-free zygotes were cultured for 6 days in 30-μL droplets (8 to 12 zygotes per droplet) of culture medium at 39°C under 5% CO_2, 5% O_2, and 90% N_2. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 μg/mL insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/mL fatty acid-free BSA. During IVF and IVC, oocytes derived from different ovaries were pooled and cultured in a same droplet. Cleavage and blastocyst production rates were measured after 2 days (approximately 30 hours) and 6 days (150 hours) of IVC, respectively. The total live cell numbers of blastocysts obtained after 6 days of IVC were counted using an air-drying method[100].

Statistical analysis

All statistical analyses were performed using software (StatView 4.51, Abacus Concepts, Calabasas, CA, USA). Data on the viability of and antrum formation by OCGCs and the nuclear maturation rate were analyzed by the chi-squared test. Other data were analyzed using a two-way ANOVA followed by the Student's *t*-test or Tukey-Kramer's HSD test.

Experimental design

A schematic of the experimental design was shown in Fig. II-2. AFC in ovaries from the local abattoir was assessed by the number of antral follicles (≥2 mm in diameter), and I

allocated ovaries having 25 or more follicles to the high AFC group and others to the low AFC group as described elsewhere[74]. As shown in Fig. II-3, I collected more OCGCs from a higher number of antral follicles (≥2 mm in diameter) in ovaries ($P < 0.05$, Tukey-Kramer's HSD test). I used 186 ovaries as the low AFC group and 70 ovaries as the high AFC group. I collected 388 OCGCs in the low AFC group and 410 OCGCs in the high AFC group and subjected them to evaluations that have been shown in Tables II-1 and II-2. However, as shown in Table II-1, 4 oocytes in the low AFC group and 4 oocytes in the high AFC group were accidentally lost after IVM during the pipetting of oocytes for the denudation of cumulus cells. Three oocytes in the low AFC group and 2 oocytes in the high AFC group were accidentally lost after IVF during the vortexing procedure (Table II-1). As shown in Table II-1, 193 OCGCs in the low AFC group and 225 in the high AFC group were subjected to IVG followed by IVM for the evaluation of oocyte volumes on day 0 of IVG and after IVM and the percentage of metaphase II oocytes after IVM. E_2 and P_4 concentrations were measured in spent media used for the IVG culture of 95 OCGCs in the low AFC group and 110 OCGCs in the high AFC group, which were subjected to IVM. Steroid hormone production during each period (days 0 to 4, 4 to 8, and 8 to 12) was calculated based on concentrations at the beginning and end of the period as described in chapter I. After IVM, 44 OCGCs in low AFC group and 44 OCGCs in high AFC group were subjected to IVF and IVC for the evaluation of developmental competence. As shown in Table II-2, the remaining 151 OCGCs in the low AFC group and 141 OCGCs in the high AFC group were subjected to an evaluation of the characteristics of granulosa cells on days 0, 4, 8, and 12. The viability of OCGCs was calculated based on all cultured OCGCs. The percentage of antrum formation in the granulosa cell layer was calculated based on OCGCs judged as surviving on day 12. I compared differences in the viability of OCGCs, antrum formation in the granulosa cell layer, the total number, viability, and diameter of granulosa cells, production of E_2 and P_4, the

E_2/P_4 ratio, and volume, nuclear status, and developmental competence of oocytes between the AFC groups.

Results

As shown in Fig. II-4, the viabilities of OCGCs in the high and low AFC groups decreased throughout the culture period ($P < 0.05$) and did not significantly differ between the two groups. The percentages of OCGCs having antra increased throughout the culture period, and became higher in the high AFC group (78.8%) than in the low AFC group (67.7%, $P < 0.05$) on day 12.

As shown in Fig. II-5, the two-way ANOVA showed that the interactions between AFC and the culture duration on E_2 production and the E_2/ P_4 ratio ($P < 0.01$), and E_2 production and the E_2/P_4 ratio were affected by AFC ($P < 0.05$) and the culture duration ($P < 0.01$). P_4 production was affected by the culture duration, but not by AFC. E_2 production from days 4 to 8 showed the highest values in all culture periods, and E_2 production from days 4 to 8 was higher in the high AFC group than in the low AFC group ($P < 0.05$). P_4 production increased with the extension of the culture period ($P < 0.05$), and did not significantly differ between the two groups. The E_2/P_4 ratio in the high AFC group did not decrease until day 8, and was higher than that in the low AFC group ($P < 0.05$) on day 8; however, the E_2/P_4 ratio decreased with the extension of the culture period ($P < 0.05$) in the low AFC group.

As shown in Fig. II-6, the two-way ANOVA showed that the culture duration affected the total number, viability, and diameter of granulosa cells ($P < 0.01$) and AFC affected the diameter of granulosa cells ($P < 0.05$); however, there were no interactions between AFC and the culture duration on the total number, viability, and diameter of granulosa cells. The total number of granulosa cells increased throughout the culture period ($P < 0.05$) and did not significantly

differ between the two groups. The viability of granulosa cells was higher than 97% throughout the culture and similar between the two groups. The mean diameters of granulosa cells were larger in the low AFC group than in the high AFC group during all culture periods ($P < 0.05$), and became larger on day 4 of the culture than that on day 0 (before IVG) ($P < 0.05$).

As shown in Fig. II-7, the two-way ANOVA showed that IVG culture and AFC affected the volume of oocytes ($P < 0.01$); however, there was no interaction between AFC and IVG culture on the volume of oocytes. The volume of oocytes became larger after than before IVG in each group ($P < 0.05$), and the high AFC group showed a larger volume than the low AFC group after IVG ($P < 0.05$). The nuclear maturation rate was higher in the high AFC group (78.9%, 105/133) than in the low AFC group (66.4%, 71/107) ($P < 0.05$). As shown in Table II-1, cleavage rates were similar between the low AFC (63.2%, 12/19) and the high AFC group (68.2%, 15/22), but no oocytes (0/19) in the low AFC group developed to the blastocyst stage after IVF and IVC, while 9.1% (2/22) oocytes in the high AFC group developed to blastocysts.

Discussion

In the present study, E_2 production was greater in the high AFC group than in the low AFC group. Furthermore, I confirmed a higher nuclear maturation rate and blastocyst development in the high AFC group. These results on oocyte competence support a previous findings showing that oocytes derived from early antral follicles in ovaries with high AFC had a greater maturational ability, and were assumed to have greater fertilizability than ovaries with low AFC[74]. Moreover, the present study suggests that greater E_2 production and a higher E_2/P_4 ratio contributed to superior oocyte competence in the high AFC group.

Granulosa cell numbers and viabilities were similar between the two groups in the present study; however, the proliferation of granulosa cells was greater in the high AFC group

than in the low AFC group in the previous study[74]. I speculate that this difference in granulosa cell proliferation may be related to the addition of different steroid hormones to culture media in the two studies. In the previous study, a high concentration of E_2 was used[74], whereas I used A_4 in the present study. Taketsuru *et al.*[103] reported that the viability of OCGCs was approximately 80% when OCGCs were cultured in E_2-supplemented medium for 14 days, but approximately 65% when OCGCs were cultured in A_4-supplemented medium. These findings suggest that the effects of A_4 on the enhancement of granulosa cell proliferation were weaker than those of E_2. The mean number of granulosa cells was 8.5×10^4 cells after 12 days of IVG in a previous study using E_2-supplemented medium[109], but were 5.6×10^4 and 6.6×10^4 cells in the low and high AFC groups, respectively, in the present study. Although the previous study evaluating the effects of AFC on the granulosa cell number using E_2-supplemented medium[74] showed lower values (approximately $4.0\text{-}5.0 \times 10^4$ cells) than the present results, granulosa cells were collected and counted after retrieving COCs[74], thereby reducing the number of remaining granulosa cells in a well.

The mean diameter of granulosa cells was larger in the low AFC group (11.3 ± 0.4 μm) than in the high AFC group (10.2 ± 0.3 μm) even before IVG. Previous studies reported that *in vivo*-grown large luteal cells (38.4 μm in diameter) originated from granulosa cells and *in vitro*-luteinized granulosa cells (38.4 μm) were larger than granulosa cells in preovulatory follicles (10.6 μm)[65, 79]. In addition, Scheetz *et al.*[93] suggested that P_4 production and the expression level of the oxytocin-neurophysin I precursor, both of which are markers of granulosa cell luteinization, were higher in granulosa cells from low AFC ovaries having 15 or fewer follicles in a pair of ovaries than from high AFC ovaries having 25 or greater follicles; however, they did not measure the diameter of granulosa cells. The difference in the diameter of granulosa cells may support luteinization or luteinization-like changes in the low AFC group, although P_4

production was not significantly different between both groups in the present study. Endo *et al*.[21] reported that E_2 promoted the growth and maturational competence of oocytes derived from early antral follicles using bovine IVG. Moreover, as described in chapter I, I suggested that E_2 production by OCGCs producing matured oocytes after IVM was slightly higher than that of OCGCs producing immature oocytes after IVM. In the present study, the development rate to blastocyst was 9.1% in the high AFC group, and the developmental competence was similar to that of bovine oocytes with <115 μm in diameter (11.9%) in a previous study[1]. On the other hand, I could not produce blastocysts from the oocytes in the low AFC group, although the cleavage rate after IVF was similar between both groups. The result may indicate the impaired developmental competence of oocytes in the low AFC group. In the previous study[74], some oocytes in the low AFC group developed to blastocysts when we used E_2 (1 μg/mL) for the growth medium instead of A_4 to increase the E_2/P_4 ratio like a dominant follicle[57]. E_2 addition may enhance the granulosa cell proliferation because the numbers of granulosa cells were relatively greater in the previous study (8.5×10^4 cells/well at day 12)[109] compared to this results, 5.6 and 6.6×10^4 cells/well in the low and high AFC groups, respectively. These results suggest that the impaired E_2 production of granulosa cells in the low AFC group had a negative impact on oocyte growth, maturation, and developmental competence. In addition, 10-hours culture of *in vivo*-grown oocytes with low FSH containing medium before IVM culture (pre-IVM) were performed in the previous study[74]. Also, it is reported that pre-IVM improved the developmental competence of bovine oocytes derived from IVG[43] and oocytes with <115 μm in diameter[1]. In further study, the effects of E_2/P_4 ratio during IVG culture and FSH treatment before IVM on the acquisition of developmental competence of *in vitro*-grown oocytes should be examined.

Scheetz *et al*.[93] also reported that FSH-mediated E_2 production was lower in granulosa cells from low AFC ovaries than from high AFC ovaries by culturing granulosa cells under serum-

free conditions. However, I demonstrated that the addition of FSH did not enhance E_2 production and accelerated P_4 production in chapter I, which may have been due to the addition of serum to the medium for the IVG culture. Granulosa cells cultured in media containing serum were found to have luteinized, compromised E_2 production, and begun to produce P_4[55, 81]. Previous studies attempted to culture OCGCs in serum-free media[94, 95]; however, the oocytes derived from serum-free cultures had low maturational competence and low fertilizability. In the future, we need to develop an IVG system that does not use serum, but enhances oocyte competence, or that inhibits the luteinization of granulosa cells even when growth medium contains serum.

In conclusion, E_2 production by bovine granulosa cells cultured as OCGCs was greater in the high AFC group than in the low AFC group, whereas P_4 production by granulosa cells was similar in each group. The diameter of granulosa cells was larger in the low AFC group than in the high AFC group. These results indicate that granulosa cells in the low AFC group are starting luteinization, and the reduced production of E_2 by granulosa cells in the low AFC group may impair the growth and meiotic competence of oocytes. In future studies, we need to identify possible factors inducing luteinization-like changes in the low AFC group.

Tables and figures

Table II-1. Number of oocyte-cumulus-granulosa complexes (OCGCs) cultured for the evaluation of nuclear maturation, production of estradiol-17β (E_2) and progesterone (P_4), and developmental competence.

Groups	No. of OCGCs used for oocyte volume and nuclear maturation				No. of OCGCs used for oocyte developmental competence				
	Cultured	Survived	Evaluated	E_2 and P_4 measurement	Cultured	Survived	Evaluated	Cleaved (%)	Blastocysts (%)
Low	193	111	107 [a]	95	44	22	19 [b]	12 (63.2)	0 (0.0)
High	225	137	133 [a]	110	44	24	22 [c]	15 (68.2)	2 (9.1)

[a] Four oocytes were lost during pipetting after *in vitro* maturation.

[b] Three oocytes were lost during vortexing after *in vitro* fertilization.

[c] Two oocytes were lost during vortexing after *in vitro* fertilization.

The numbers in parentheses indicate rates of cleaved oocytes or oocytes developed to blastocysts after *in vitro* fertilization and *in vitro* culture.

The cell numbers in blastocysts were 65 and 88 in the high AFC group.

Table II-2. Number of oocyte-cumulus-granulosa complexes (OCGCs) cultured for the evaluation of granulosa cell characteristics.

Groups	Total	No. of OCGCs used for granulosa characteristics at each duration						
		Day 0	Day 4		Day 8		Day 12	
			Cultured	Survived	Cultured	Survived	Cultured	Survived
Low	151	26	34	32	38	27	53	25
High	141	27	27	23	37	24	50	28

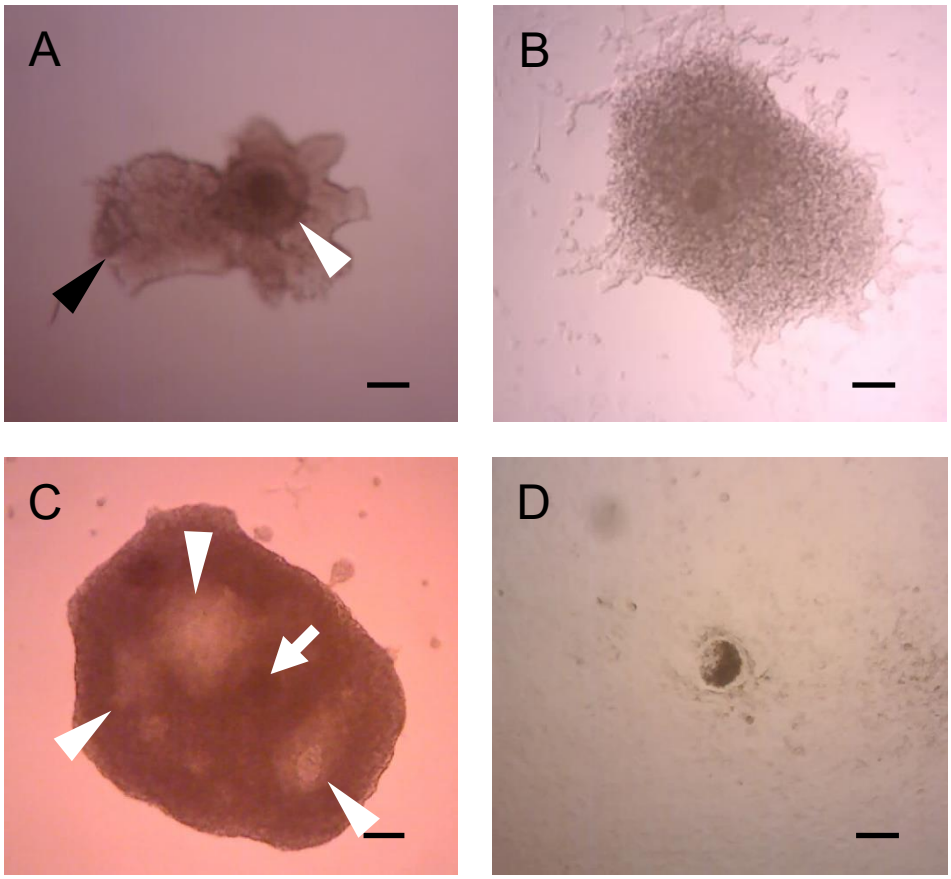

Fig. II-1. Oocyte-cumulus-granulosa complexes (OCGCs) before and after day 12 of the growth culture.

A: An OCGC before the growth culture. The OCGC has an oocyte surrounded by a cumulus investment and attached mural granulosa-cell layer. The white arrowhead indicates the cumulus investment. The black arrowhead indicates the mural granulosa-cell layer.

B: A surviving OCGC without antrum formation in the granulosa cell layer after a 12-day growth culture. OCGCs having oocytes with an evenly granulated ooplasm and enclosed by several layers of healthy granulosa cells were defined as surviving.

C: A surviving OCGC with the formation of antra (white arrowheads) in the granulosa cell layer.

D: A dead OCGC having a degenerated oocyte.

The white arrow indicates an oocyte. Scale bars indicate 100 μm.

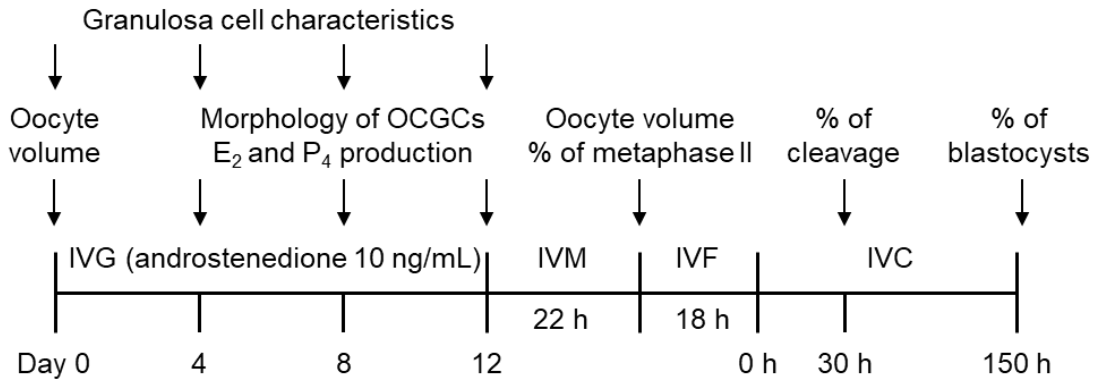

Fig. II-2. Schematic of the experimental design.

Antral follicle count (AFC) in an ovary from the local abattoir was assessed by the number of antral follicles (≥2 mm in diameter), and I allocated ovaries having 25 or more follicles to the high AFC group and others to the low AFC group. Oocyte-cumulus-granulosa complexes (OCGCs) from each ovary were cultured for 0, 4, 8, or 12 days in an *in vitro* growth (IVG) culture. Oocyte volume was evaluated on day 0 of IVG. Granulosa cell characteristics were evaluated on every 4 days of IVG (days 0, 4, 8, and 12). On every 4 days of IVG, the morphology of OCGCs (viability of OCGCs and antrum formation in granulosa cell layers) was evaluated. After 12 days of IVG, oocyte-cumulus complexes (COCs) derived from surviving OCGCs were subjected to *in vitro* maturation (IVM). The concentrations of estradiol-17β (E_2) and progesterone (P_4) in the IVG media of some of these OCGCs at each period of IVG every 4 days (days 0, 4, 8, and 12) were evaluated. After IVM, the volume and nuclear status of some oocytes were evaluated. The remaining oocytes were subjected to *in vitro* fertilization (IVF) and an *in vitro* culture (IVC) for the evaluation of developmental competence to blastocysts.

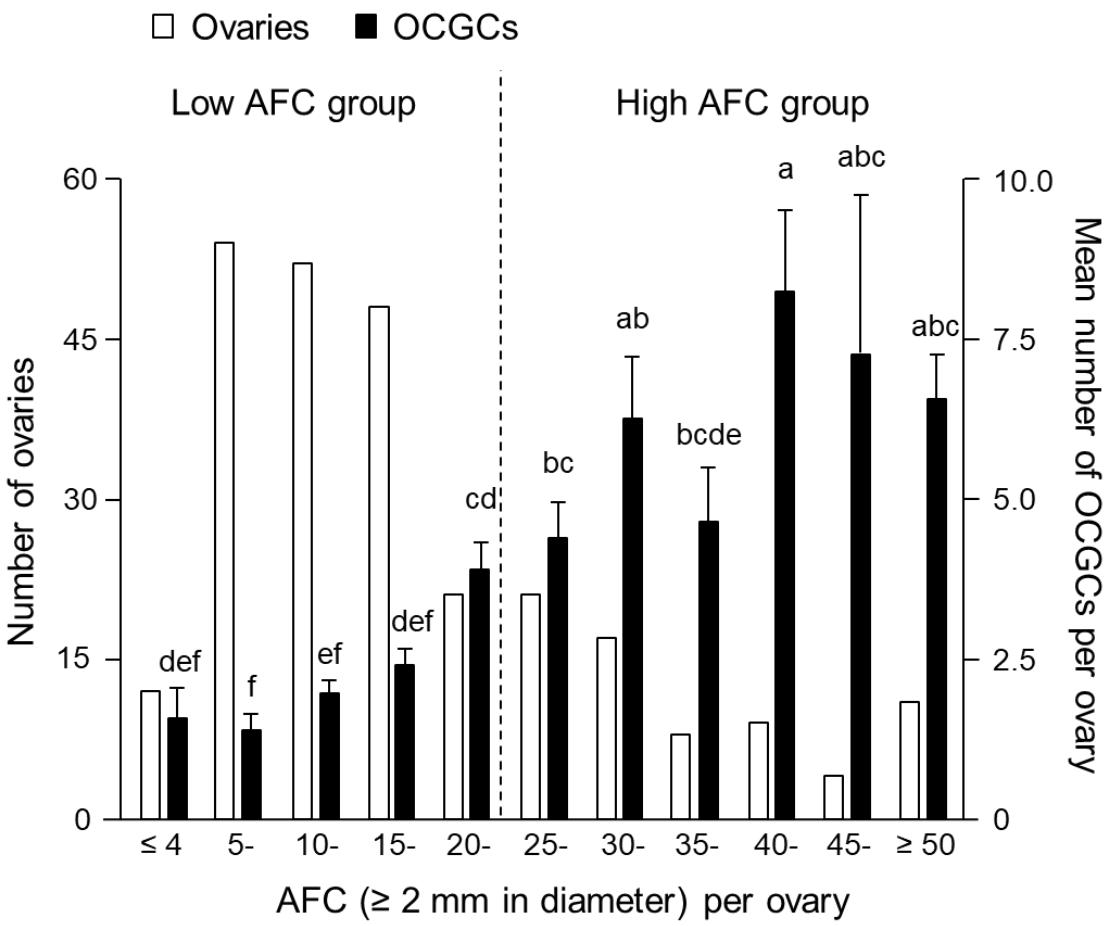

Fig. II-3. Relationship between antral follicle count (AFC) and number of collected oocyte-cumulus-granulosa complexes (OCGCs) in each ovary.

A total of 186 ovaries as the low AFC group and 70 ovaries as the high AFC group were used, and 388 OCGCs in the low AFC group and 410 OCGCs in the high AFC group were collected.

[a-f] Different letters indicate significant differences between different AFC ($P < 0.05$).

Error bars indicate SEM.

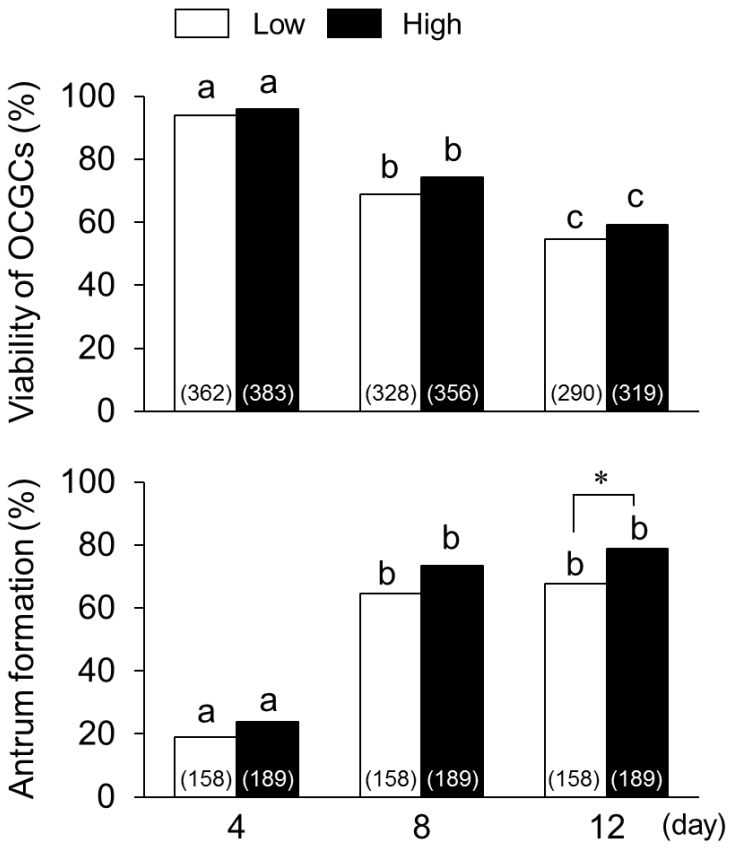

Fig. II-4. Relationships between antral follicle count (AFC), viability, and antrum formation in granulosa cell layers of oocyte-cumulus-granulosa complexes (OCGCs).

Numbers in parentheses indicate the number of OCGCs; namely, the number of OCGCs

[a-c] Different letters indicate significant differences between different culture periods in the same group ($P < 0.05$).

* An asterisk indicates a significant difference between the low and high AFC groups on the same day ($P < 0.05$).

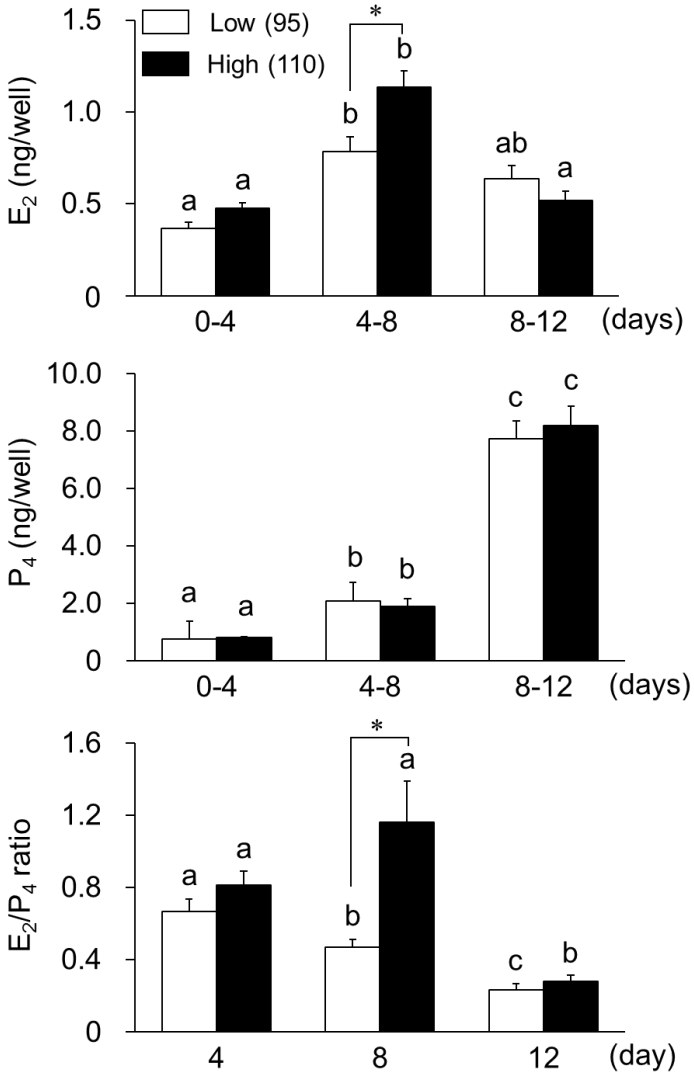

Fig. II-5. Relationships between antral follicle counts (AFC), the production of estradiol-17β (E_2) and progesterone (P_4) by oocyte cumulus-granulosa complexes (OCGCs), and the E_2/P_4 ratio in culture media.

[a-c] Different letters indicate significant differences between different culture periods in the same group ($P < 0.05$).

* An asterisk indicates a significant difference between the low and high AFC groups ($P < 0.05$).

Numbers in parentheses indicate the number of OCGCs on the same day.

Error bars indicate SEM.

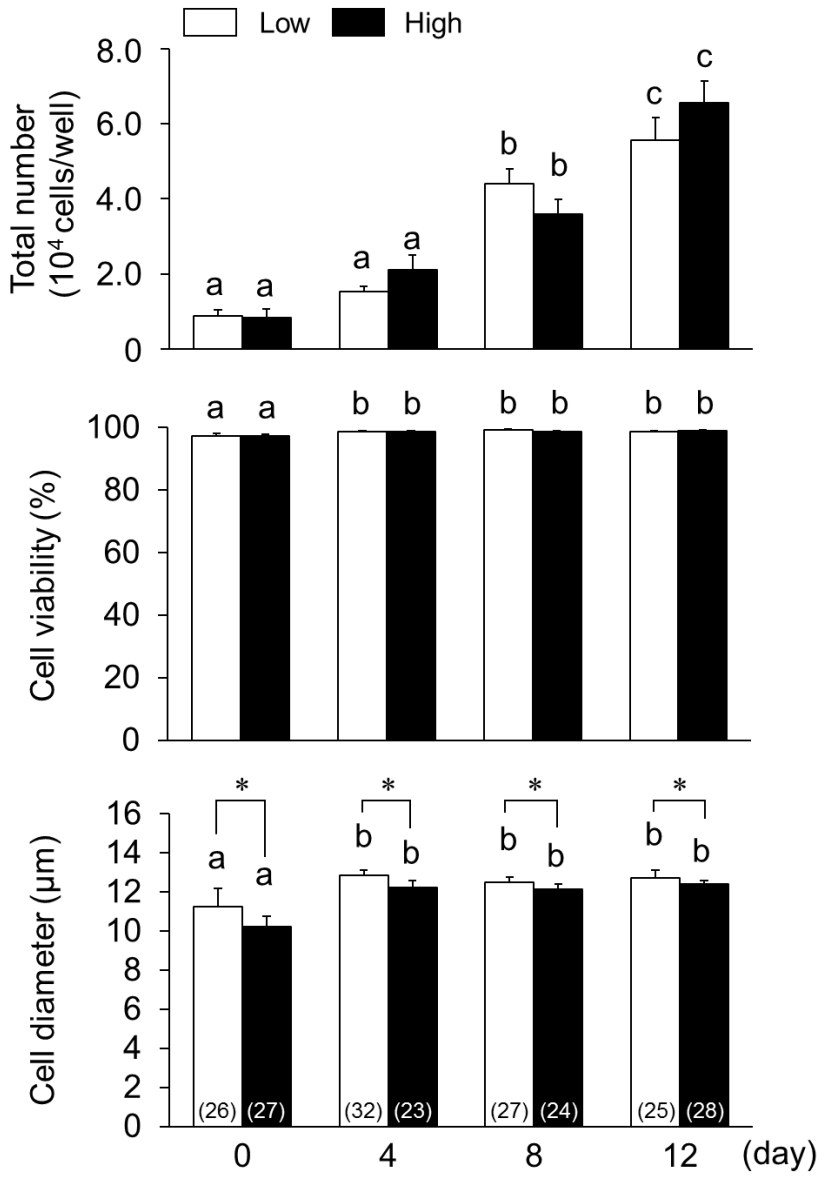

Fig. II-6. Relationships between antral follicle counts (AFC) and the total number, viability, and diameter of granulosa cells of oocyte-cumulus-granulosa complexes (OCGCs).

[a-c] Different letters indicate significant differences between different culture periods in the same group ($P < 0.05$).

* An asterisk indicates a significant difference between the low and high AFC groups on the same day ($P < 0.05$).

Numbers in parentheses indicate the number of OCGCs.

Error bars indicate SEM.

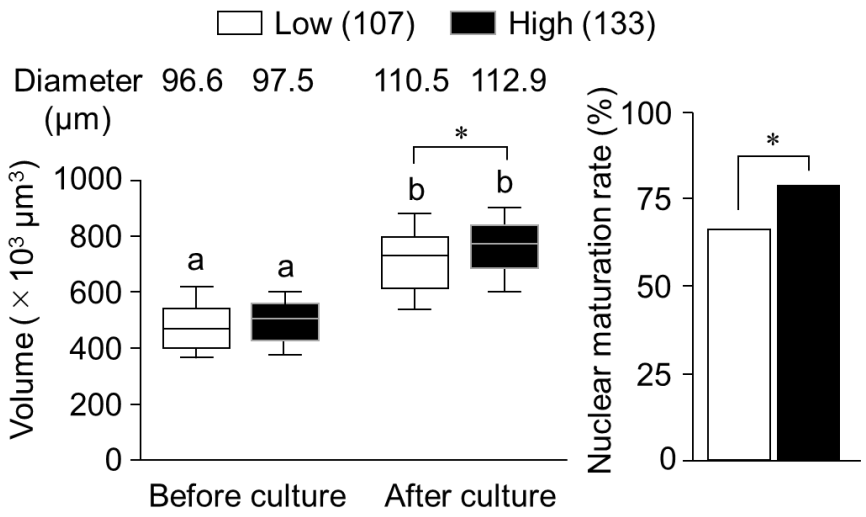

Fig. II-7. Relationships between antral follicle counts (AFC) and oocyte growth and maturation. Values above boxes in the box-and-whisker plot indicate the mean diameters (μm) of oocytes. Numbers in parentheses indicate the number of oocytes submitted to IVM (Table II-1) derived from 193 OCGCs in the low AFC group and 225 in the high AFC group.

Lines on the boxes in the box-and whisker plot delineate the 25th, 50th, and 75th percentiles, while the whiskers depict the 10th and 90th percentiles.

[a, b] Different letters indicate significant differences between before and after IVG ($P < 0.05$).

* An asterisk indicates a significant difference between the low and high AFC groups on the same day ($P < 0.05$).

Summary

The antral follicle count (AFC) is used as an indicator of cow fertility. I herein investigated the relationship between AFC and the steroidogenesis of granulosa cells and confirmed the developmental competence of oocytes derived from early antral follicles (0.5-1.0 mm) using *in vitro* growth culture. Slaughterhouse-derived ovaries were divided into high (≥25 follicles) and low AFC groups (<25 follicles) based on AFC (≥2.0 mm). Oocyte-cumulus-granulosa complexes (OCGCs) collected from early antral follicles were cultured for 12 days. The total number, viability, and diameter of granulosa cells and estradiol-17β (E_2) and progesterone (P_4) production during the culture were evaluated. Surviving oocytes on day 12 were subjected to *in vitro* maturation, and their volume and nuclear status were evaluated. Some oocytes were subjected to the evaluation of developmental competence to blastocysts. Although the total number and viability of granulosa cells did not differ between the groups, granulosa cell diameters were smaller in the high AFC group than in the low AFC group ($P < 0.05$). The E_2 and P_4 ratio on day 8 was higher in the high AFC group than in the low AFC group ($P < 0.05$). Oocyte volumes and nuclear maturation rates were greater in the high AFC group than in the low AFC group ($P < 0.05$). The development rate to blastocysts was 9.1% in the high AFC group, while no oocytes developed to blastocysts in the low AFC group. E_2 production by granulosa cells appears to be greater in high AFC cattle than in low AFC cattle, thereby promoting the acquisition of oocyte competence.

Chapter III: Relationship between the antral follicle count, steroidogenesis, and secretions of follicle stimulating hormone and anti-Müllerian hormone during follicular growth in cattle

Introduction

In mono-ovulatory species, the recruitment of follicular growth is induced by a surge-like secretion of FSH. According to the decrease of the level of FSH by the inhibitory effect of E_2 and inhibin secreted by follicles themselves, a dominant follicle is selected. The dominant follicle continues growth by LH, and results in ovulation[5, 29]. On the other hand, most of follicles recruited at the same time as the dominant follicle degenerates[5, 29].

The ovarian reserve, the pool of primordial follicles in a pair of ovaries in individuals, is defined as the potential ability of ovarian function[12, 104] and is known to be an indicator of female fertility in mono-ovulatory species such as humans[12] and cattle[47]. As mentioned earlier, the peak AFC positively correlates with the number of primordial follicles[50] and can be used to estimate the ovarian reserve[13]. The peak AFC between follicular waves seems consistent in individual cattle[13], cattle with high number of antral follicles have been linked to higher reproductive performance, such as higher fertility[72], a shorter open period[72], and higher responsiveness to superovulation[48], than cattle with low number of antral follicles, even though they were in the same age class. A previous report showed that the fertilizability of oocytes collected from cattle by ultrasound-guided OPU was higher in high AFC cows having 30 or more antral follicles in a pair of ovaries at the time of OPU than in low AFC cows having less than 30 antral follicles at a 3- or 4-day interval of OPU[73]. In contrast, at the interval of OPU to 7 days, the fertilizability of oocytes in high AFC cows was impaired and became less than that in low AFC cows, although the fertilizability of oocytes derived from low AFC cows was similar regardless of OPU interval[73]. These findings indicate that the growth dynamics of antral follicles differ between high and low AFC cows, and the degeneration of antral follicles at the selection phase in the follicular wave may occur earlier in the high AFC cows than in the low

AFC cows. However, the reason of this reversal of the relationship between AFC and oocyte fertilizability is uncertain. In addition, I conducted IVG culture of bovine OCGCs in chapter II, which enables bovine oocytes without maturational competence from early antral follicles to grow to the stage acquiring competence for maturation and development to the blastocyst stage[40, 43, 109] and offspring[40, 43]. By using this technology, I investigated the follicular function, the acquisition of oocyte competence, and the steroidogenesis in granulosa cells, and I estimated the follicular growth dynamics from the period which we cannot detect the follicles by ultrasonography *in vivo* to the period which oocytes acquire the developmental competence in high and low AFC cows. Consequently, OCGCs derived from early antral follicles (0.5-1.0 mm in diameter) in the high AFC group having 25 or more antral follicles (≥2.0 mm in diameter) in an ovary collected at a slaughterhouse showed higher oocyte maturational competence and fertilizability than those in the low AFC group having less than 25 antral follicles. Although proliferation of granulosa cells was same in both groups, E_2 production of OCGCs was higher in the high AFC group than in the low AFC group. In addition, I revealed that granulosa cells surrounding IVG oocytes having higher maturational competence secreted more E_2 and less P_4 than the granulosa cells surrounding less competent *in vitro*-grown oocytes using medium containing A_4 instead of E_2 in chapter I.

AMH is one of a member of TGF-β family and produced by granulosa cells of primary to early antral follicles[59]. In AMH deficient mice, premature depletion of primordial follicles occurred[19], and AMH inhibited activation of primordial follicles in cattle[108]. AMH inhibited FSH-stimulated growth of antral follicles and E_2 production by decreasing the sensitivity of preantral and antral follicles to FSH in mice[18], humans[15, 34], and sheep[14]. These results suggest that AMH is an important regulator of follicular activation, follicular growth, and steroidogenesis in growing follicles. Further, the plasma concentration of AMH was positively correlated with the number of primordial follicles and AFC in humans[59] and cattle[50]. In cattle, the concentration of AMH in follicular fluid of antral follicles (≥3 mm in diameter) decreased during follicular growth[66, 90]. Granulosa cells derived from antral follicles (3-5 mm in diameter)

produced more E_2 and AMH in the high AFC cows having 25 or more follicles in a pair of ovaries than in the low AFC cows having 15 or fewer antral follicles regardless of FSH addition in *in vitro* culture of granulosa cells[93]. In follicular fluid of antral follicles (5-7 mm in diameter), immediately before selection of dominant follicles, AMH concentration was similar between in the high AFC heifers and in the low AFC heifers, while E_2 concentration was lower in the high AFC heifers than in the low AFC heifers[49]. On the other hand, E_2 concentration of follicular fluid in ovulatory follicles (approximately 15 mm in diameter) was higher in the high AFC heifers than in the low AFC heifers[70]. These results indicate that AMH regulates FSH-stimulated E_2 production during follicular growth, and the regulation can be different between each stage of follicular growth. However, there is no information about the relationship between AMH concentration in follicles after selection (≥8 mm in diameter) or before recruitment (<4 mm in diameter) and AFC. In chapter III, I investigated the relationship between AFC, follicular growth dynamics, the concentrations of FSH in plasma and steroid hormones, E_2, testosterone (T, one of the precursors of E_2), and P_4 in plasma and follicular fluid as the factors affecting the oocyte developmental competence in high and low AFC cattle. In addition, I also investigated the relationship between AMH and AFC at follicular stages before and after recruitment by IVG of OCGCs derived from early antral follicles (<1 mm in diameter) and ultrasound-guided follicular aspiration, respectively.

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Hokkaido University. The cows were kept at the experimental farm of Hokkaido University (n = 7; 3 lactating and 4 dry cows). Their age and parity were 9.0 ± 4.7 (mean ± SD) and 4.0 ± 2.2, respectively.

Collection of follicular fluid, blood, and ultrasound examination

The schematic drawing of ultrasound-guided follicular aspiration schedule was

described in Fig. III-1. Estrous cycles and follicular waves of the cows were synchronized for collecting follicular fluid from follicles just before the expected time of the LH surge as previously described[91]. Briefly, cows were inserted an intra vaginal P_4 device (1.9 g, CIDR 1900; Zoetis Japan, Tokyo, Japan) (day –18). Five days after insertion of the P_4 devise, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 25 mg, Pronalgon F containing 5 mg/mL of dinoprost; Zoetis Japan) was injected intramusculaly (day –13). Then P_4 devise was removed 8 days after insertion (day –10). Two days later, gonadotropin releasing hormone (GnRH) analogue (200 μg, Conceral injection containing 50 μg/mL fertirelin acetate; Intervet, Osaka, Japan) was injected intramusculaly (day –8). After 8 days, large follicles were ablated under an ultrasound imaging device (HS-2100; Honda Electronics, Aichi, Japan) equipped with a 9.0 MHz long-handled convex transducer (HCV-4710MV; Honda Electronics) for synchronization of the emergence of the follicular wave[8] (day 0). Follicles were aspirated using a single-lumen needle (17-gauge, 490 mm long; Misawa Medical, Ibaraki, Japan) connected to a 50 mL tube (Falcon 2070; Becton Dickinson, Franklin Lakes, NJ, USA) via a silicone tube (100 cm long, 1 mm internal diameter). Four days later, $PGF_{2\alpha}$ was injected intramusculaly (day 4). After 40 hours of $PGF_{2\alpha}$ injection, a follicular fluid of a dominant follicle (ovulatory phase) was collected under the ultrasonography (day 6). For the collection of follicular fluid, the single-lumen needle was connected to a 5- or 10-mL syringe. Two cows had a large subordinate follicle (≥8 mm in diameter) after collection of follicular fluid, respectively, then these follicles were also ablated. After that, GnRH was injected intramusculaly to induce LH surge. Five days after GnRH injection, formation of corpus luteum was confirmed by ultrasonography in all cows (day 11), and 2 days later, follicular fluid was collected from a dominant follicle (luteal phase) (day 13). Then all visible follicles were ablated. Four days later, follicular fluid was collected from the largest follicle (selection phase) (day 17). A cow had 2 large follicles (9.6 and 8.7 mm) and I could not distinguish the dominant follicle that expressed LH receptors[6] under ultrasonography; therefore, I collected follicular fluids from these follicles and pooled as a sample. In three cows (1 low AFC and 2 high AFC), follicular ablation was performed again at day 16 and follicular fluid was collected at day 20. During days 0 to 16,

I daily examined ovaries by the ultrasound imaging device equipped with a 7.5 MHz rectal linear transducer (HLV-575M; Honda Electronics) and moving images of ultrasonography were saved into a video recorder (VR570; Toshiba Teli, Tokyo, Japan). I also daily collected blood by jugular or caudal venipuncture using EDTA-loaded vacuum tubes for hormone measurements. Each tube was centrifuged at 4ºC, 3000 rpm for 10 minutes. Plasma samples were stored at –30ºC until hormone assays were conducted. In addition, I performed ovarian ultrasonography and blood collection at the days of hormone treatments, ablation of follicles, and sampling of follicular fluid. For analyzing follicular growth dynamics, recorded moving images were subjected to frame-by-frame playback using a media player (Windows Media Player; Microsoft, WA, USA). The number of antral follicles were counted, and diameter of each antral follicle was measured by a digital calipers-software (Hakarundesu; Onegland.net, Sizuoka, Japan). Antral follicles were divided into 3 categories according to their diameters (small: <4 mm, intermediate: 4-8 mm, and large: ≥8 mm) since follicles of 4 mm or larger in diameter are usually defined as representing the emergence of follicles[31] and follicles of 8 mm or larger in diameter start to express LH receptors[6].

Collection of OCGCs and the IVG culture

Collection and IVG of bovine OCGCs was performed as described in chapter I. Briefly, slaughter-house derived ovaries were sliced at approximately 1 mm thickness. Under a stereomicroscope, early antral follicles (0.5-1.0 mm in diameter) were dissected from sliced ovarian tissues. OCGCs isolated from early antral follicles were cultured individually in a 96-well culture plate with 200 μL of growth medium at 39ºC for 12 days in humidified air with 5% CO_2. Every 4 days of the IVG culture, half of the growth medium (100 μL) was replaced with the same amount of fresh medium. Spent media of surviving OCGCs collected on days 4, 8, and 12 of the culture were stored at –30ºC until assays of steroid hormone and AMH.

E_2, P_4, FSH, and AMH assays

Steroid hormones in plasma samples were extracted as described previously with slight

modifications for T assay[107]. For the E_2 assay, 2 mL of plasma was extracted with 6 mL of diethyl ether (Kanto chemical, Tokyo, Japan). For the T assay, 1 mL of plasma was extracted with 3 mL of diethyl ether. For the P_4 assay, 200 μL of plasma were extracted with 2 mL of diethyl ether. Then the diethyl ether was decanted into a new tube after freezing the plasma. After evaporating the diethyl ether, 0.5 mL of acetonitrile (Kanto chemical) and 1 mL of hexane (Kanto chemical) were added and mixed well in the extracted samples for E_2 and T assays for delipidation. Thereafter, 1 mL of hexane was added again, and all the hexane was discarded using an aspirator. The acetonitrile was evaporated after repeating delipidation by hexane three times. Samples were reconstituted with 100 μL (E_2) or 200 μL (T) of assay buffer. The extracted samples for P_4 reconstituted with 200 μL of assay buffer without delipidation using acetonitrile and hexane. Follicular fluid samples and spent media were assayed without extraction. Samples were diluted with assay buffer. Extracted samples from plasma were assayed without dilution or subjected to 10-fold dilution. Follicular fluids were subjected to 100- or 1000-fold dilution. Spent media were subjected to 2- to 2000-fold serial dilutions. The concentrations of E_2, T, and P_4 were determined using competitive double-antibody enzyme immunoassays as described in chapter II with slight modification for T assay. The primary antisera used for T assays were anti-teatosterone-3-CMO-BSA (FKA102; Cosmo bio). Amounts of the solution of the primary antisera and HRP-labeled hormone into the wells of a 96-well microplate in T assay were 50 μL. Assay sensitivities were 0.049 pg/well for E_2, 0.195 pg/well for T, and 0.391 pg/well for P_4. The inter- and intra-assay coefficients of variations were 15.1 and 4.0% for E_2, 7.1 and 7.4% for T and 14.9 and 3.9% for P_4, respectively.

The concentrations of FSH in plasma samples were determined using competitive double-antibody time-resolved fluoroimmunoassay using Eu-labeled FSH as probes with slight modifications[52]. A bovine FSH immunoassay kit consisting of bovine FSH antisera (AFP7722291), bovine FSH (iodination grade, AFP-9294C), and reference standard of bovine FSH (AFP-5346D) was provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) National Hormone and Pituitary Program (NHPP) (Dr. A. F. Parlow, NHPP,

Torrance, CA, USA). I mixed 10 μL of bovine FSH solution (500 μg/mL) with Eu-labelling reagent (PerkinElmer, Waltham, MA, USA), and incubated at 37°C overnight according to the manufacturer's instructions. The Eu-labeled FSH was separated from free Eu by gel filtration with a column (1.5 cm inner diameter, 12.0 cm, Econo-Pac column; Bio-Rad Laboratories) of Sephadex G-50 (GE Healthcare, Chicago, IL, USA). The bovine FSH antisera and the reference standard of bovine FSH were diluted by assay buffer (PerkinElmer) containing 0.1% gelatin. The bovine FSH antisera (100 μL) were incubated in the wells of a 96-well microplate (FluoroNunc Modules; Nalge Nunc International, Rochester, NY, USA) coated with the secondary antiserum overnight at 34°C. Goat anti-rabbit IgG (AP132; Merck Millipore, Burlington, MA, USA) was used as the secondary antibody. After the washing of all wells 10 times with 300 μL of washing buffer (0.1% (w/v) Tween 20, 150 mM NaCl, 0.05% (w/v) NaN_3 in 5 mM Tris buffer, pH 7.8), plasma samples without dilution (100 μL) were added into the wells and incubated overnight at 34°C. After incubation, the wells were washed 12 times, Eu-labeled FSH was added to wells, and incubated for 6 hours at 34°C. After the wells were washed 12 times, enhancement solution (100 μL, PerkinElmer) was added to each well and incubated 5 minutes at 34°C. The fluorescence of the solution in the wells was measured using a microplate reader (1420 $ARVO_{SX}$ DELFIA; PerkinElmer). Assay sensitivities was 204.8 pg/mL for FSH. The inter- and intra-assay coefficients of variations were 17.2 and 13.3%, respectively.

The concentrations of AMH in plasma, follicular fluid, and spent media were determined using a commercial-kit (Bovine AMH ELISA; Ansh Labs., Webster, TX, USA) according to the manufacture's instruction. Samples were diluted with a sample diluent in the kit. Follicular fluid was subjected to 100- or 1000-fold dilution. Plasma samples were assayed without dilution or subjected to 4-fold dilution. Spent media were subjected to 100-fold dilution. The absorbance of the solution in the wells was measured at 450 nm with background wavelength correction at 630 nm using a microplate reader (iMark; Bio-Rad Laboratories, Tokyo, Japan). Assay sensitivities was 11.0 pg/mL for AMH. The inter- and intra-assay coefficients of variations were 4.3 and 2.5%, respectively.

Experimental design

The cows were classified into the low AFC group (less than 30 follicles) and the high AFC group (more than 30 follicles) based on the peak number of antral follicles (≥3 mm in diameter) in a pair of ovaries from days 0 to 16 as described in the previous report[73]. The number of small, intermediate, and large follicles from days 0 to 16 were compared between groups and days. In addition, the transition of number of intermediate and large follicles from 1 to 6 days after follicular ablation and sampling at days 0 and 6, respectively, were examined, and I compared the number of antral follicles between groups, days after follicular ablation and sampling. The plasma concentrations of FSH, E_2, T, and P_4 from days 0 to 16 were compared between groups and days. In addition, those concentrations during selection phase (2 to 4 days after follicular ablation at days 0 and 6) were also compared between groups. Plasma concentrations of AMH on the representative date of each stage of follicular growth (selection phase; day 4, luteal phase; day 13, and ovulatory phase; day 6) were compared between groups and each stage of follicular growth. Concentrations of E_2, T, P_4, and AMH in follicular fluid samples were compared between groups and each stage of follicular growth. However, in a session of collection of follicular fluid, the follicular fluid was scattered in the line of the needle and the tube due to the small volume of the follicular fluid. For collecting the follicular fluid, I washed the line by DPBS (–). The total amount of collected solution was 10 mL (cm^3). Therefore, the concentrations of hormones in the follicular fluid were calculated based on the formula as described below.

Concentrations in the follicular fluid (ng/mL) =

Concentrations in the collected solution (ng/mL) × volume of the follicle (cm^3)/10 (cm^3)

Volume of the follicle in the formula was calculated based on a formula for the volume of a sphere and the radius of the follicle measured using ultrasonography.

In IVG study, the OCGCs were divided into the low AFC group (less than 25 follicles) and the high AFC group (25 or more follicles) based on the number of antral follicles (≥2 mm in diameter) in an ovary as described in chapter II. Concentrations of E_2, P_4, and AMH in IVG

media derived from surviving 5 OCGCs after 12-days culture in each group were compared between groups and days of culture (days 4, 8, and 12).

Statistical analysis

All statistical analyses were performed using software (JMP Pro 14, SAS Institute, Cary, NC, USA). All data were analyzed using a two-way ANOVA. The Student's *t*-test or Tukey-Kramer's HSD test were used as post-hoc tests.

Results

The mean diameters of aspirated largest follicles at each stage of follicular growth were similar in the low and the high AFC groups. As shown in Fig. III-2A, the number of small and intermediate follicles were higher in the high AFC group than in the low AFC group ($P < 0.01$). In addition, the numbers of intermediate and large follicles were changed after the days of follicular ablation ($P < 0.01$). The numbers of small (<4 mm) and intermediate follicles (4-8 mm) fluctuated in the high AFC group, while those numbers were stable in the low AFC group. When the transition of the number of small (<4 mm), intermediate (4-8 mm), and large (≥8 mm) follicles in the follicular wave was analyzed as shown in Fig. III-2B, the number of small (<4 mm) and intermediate (4-8 mm) antral follicles did not show the significant change according to the days after follicular ablation in each AFC group, although the number of large antral follicles (≥8 mm) increased in both groups. And the selection of dominant follicles (≥8 mm) occurred on 4 days after follicular ablation in both groups.

As shown in Fig. III-3A, the plasma concentration of FSH was higher in the low AFC group than in the high AFC group ($P < 0.01$), while the plasma concentrations of E_2 and T were higher in the high AFC group than in the low AFC group ($P < 0.01$). There was no difference in the plasma concentration of P_4 between both groups. The plasma concentrations of each hormone during the selection phase (2 to 4 days after follicular ablation) were shown in Fig. III-3B. FSH concentration was higher in the low AFC group than in the high AFC group ($P < 0.05$), while E_2 and T concentrations were higher in the high AFC group than in the low AFC group (P

< 0.01).

As shown in Fig. III-4, the concentration of E_2 and the E_2/P_4 ratio in follicular fluid were affected by AFC groups ($P < 0.05$) and the stages of follicular growth ($P < 0.05$). Namely, in the high AFC group, the concentrations of E_2 in follicular fluid were higher in the luteal and the ovulatory phases than the selection phase ($P < 0.05$), while there was no difference in the concentrations of E_2 in follicular fluid in the low AFC group regardless of follicular growth phase. The concentration of E_2 in follicular fluid in the high AFC group was higher than in the low AFC group in the ovulatory phase ($P < 0.05$). E_2/P_4 in follicular fluid was the highest in the ovulatory phase in the high AFC group, and that was higher than in the low AFC group ($P < 0.05$). The concentration of T in follicular fluid was slightly higher in the high AFC group ($P = 0.07$), but not affected by the stage of follicular growth. The concentration of P_4 in follicular fluid was not affected by AFC and the stage of follicular growth.

As shown in Fig. III-5, E_2 production from days 4 to 8 showed the highest values in all culture periods regardless of AFC, and was higher in the high AFC group than in the low AFC group ($P < 0.05$). P_4 production increased with the extension of the culture period ($P < 0.05$), and did not differ between the two groups. The E_2/P_4 ratio in the high AFC group increased from days 4 to 8 ($P < 0.05$), and was higher than that in the low AFC group ($P < 0.05$) on days 8 and 12; however, the E_2/ P_4 ratio decreased with the extension of the culture period ($P < 0.05$) in both groups.

As shown in Fig. III-6A, the plasma concentration of AMH was higher in the high AFC group than in the low AFC group ($P < 0.01$) regardless of follicular growth phase. The concentration of AMH in follicular fluid was slightly higher in the high AFC group than in the low AFC group ($P = 0.08$). As shown in Fig. 6B, the concentration of AMH increased throughout IVG culture in each group ($P < 0.01$) and was higher in the high AFC group than in the low AFC group ($P \leq 0.05$).

Discussion

In the previous study[73], normal fertilizability of oocytes was higher in the high AFC group than in the low AFC group in 3- or 4-day interval of OPU-IVF, while the result became reversed in 7-day interval of OPU-IVF; namely, normal fertilizability of oocytes was higher in the low AFC group than in the high AFC group. In the present study, the number of intermediate follicles increased after follicular ablation then decreased within a few days in the high AFC group; approximately 3 to 4 days after follicular ablation, the number of intermediate follicles became the peak value in the high AFC group (Fig. III-2A). It means that most follicles after 3-4 days after follicular ablation are at growing phase in the high AFC group, resulting in higher fertilizability of oocytes as described in the previous report[73]. However, after 7 days of follicular ablation, follicles already start to regress and the oocyte fertilizability becomes low. On the other hand, in the low AFC group, the number of intermediate follicles were stable regardless of the days after follicular ablation. In the present study and the previous study[13, 71], high FSH concentration in the low AFC cows were shown compared to that in the high AFC group. These results indicate that intermediate follicles in the low AFC group are consistently growing by the high concentration of FSH, resulting in the higher fertilizability in the low AFC group than in the high AFC group at 7-day interval of OPU. The early degradation of intermediate antral follicles can be caused by the higher concentration of E_2 in the dominant follicle in the high AFC group, which should cause degeneration of subordinate follicles[30].

In the present study, the concentrations of E_2 and E_2/P_4 ratio in follicular fluid at ovulatory phase were higher in the high AFC group (1127 ng/mL) than in the low AFC group (332 ng/mL). Mossa *et al.*[70] also showed that the higher E_2 concentration in the dominant follicle of high AFC heifers (588 ng/m) than those of low AFC heifers (435 ng/mL). A study using *in vitro* culture of granulosa cells suggested that the lower expression level of FSH receptors and P450arom resulted in impaired response to FSH and E_2 production by granulosa cells in the low AFC cattle[93]. These results suggest the difference of the response to FSH stimulus between high and low AFC cattle. However, Ireland *et al.*[49] showed that E_2 concentration in follicles (5-

7 mm) at emergence of the follicular wave (24 to 48 hours after the ovulation) was higher in the low AFC heifers (approximately 90 ng/mL) than in the high AFC heifers (approximately 40 ng/mL). In the present study, E_2 concentrations in follicles with >8 mm in diameter at selection phase were 168 ng/mL in the low AFC cows and 203 ng/mL in the high AFC cows. These results may indicate that the function of granulosa cells in the follicles in the low and high AFC cattle alters before and after the expression of LH receptor at around 8 mm[6]. Also, these results suggest that the ability of LH-mediated E_2 production is impaired in the low AFC group, resulting in lower concentration of E_2 in the dominant follicles after selection phase. Endo *et al*.[21] reported that E_2 promoted the growth and maturational competence of bovine IVG oocytes. In chapter I, I indicated that E_2 production by OCGCs producing matured oocytes after IVM was higher than that of OCGCs producing immature oocytes after IVM. In addition, OCGCs derived from high AFC ovaries showed higher E_2 production of granulosa cells and higher oocyte developmental competence than those from low AFC ovaries in chapter II. Consequently, the impairment of E_2 production in the low AFC cattle should have a negative impact on growth, maturation, and developmental competence of oocytes, resulting in lower fertility in the low AFC cattle than in the high AFC cattle.

The concentrations of E_2 and T were higher in the high AFC group than in the low AFC group not only in follicular fluid, but also in plasma, although FSH concentration was higher in the low AFC group than in the high AFC group in the present study. Previous studies reported that the plasma T concertation was higher in the high AFC group than in the low AFC group in heifers and cows[70] and the plasma FSH concentration was higher in the low AFC group than in the high AFC group in heifers[48] and cows[13, 71]; however, the plasma concentrations of E_2 in low and high AFC cattle were similar[13, 48, 71]. One of the possible reasons of the difference in plasma concentrations of E_2 between the present and the previous studies is the difference in the age of cattle used for the experiment. In the present study, we used elder cows (3.7, 11.4, and 14.5 years old in the low AFC cows; 3.9, 4.8, 11.8, and 12.9 years old in the high AFC cows) than cattle used in the previous studies (14-33 months old[48], 3-5 years old[13], and 2.6-10.8 years old[71]).

In cattle, the numbers of primordial and preantral follicles are stable after birth until 4 to 6 years old and then decrease[23]. In human, serum E_2 concentration begin to decrease, and serum FSH concentration increases considerably 2 years before the last menstrual period[89]. In the present study, average E_2 concentration in plasma from days 0 to 16 was similar in the low AFC group (3.2 ± 1.5 pg/mL) and in the high AFC group (3.8 ± 1.9 pg/mL) in cows younger than 10 years old; however, that was higher in the high AFC cows (4.3 ± 1.8 ng/mL) than in the low AFC cows (3.0 ± 1.4 ng/mL) in the cows older than 10 years ($P < 0.01$, Student's *t*-test). These results may indicate the age-related decrease in the plasm concentration of E_2 especially in the low AFC cows, and that the fertility of cows decrease younger ages in the low AFC cows than in the high AFC cows. In addition to E_2, inhibin is one of the major hormones causing negative-feedback to FSH secretion[56]. A previous study using 3-5 years old cows[13] suggested that the serum concentration of inhibin-A was slightly higher in high AFC cows than in low AFC cows at the ovulatory phase ($P = 0.07$), but not at the selection phase of dominant follicles. Another study using 11-13 months old heifers[49] suggested that the inhibin-A concentration in follicles (5-7 mm) at the emergence of the follicular wave (24 to 48 hours after the ovulation) was similar between the high and low AFC heifers. In further study, we should investigate the relationship between AFC, age, and the competence of E_2 and inhibin production in granulosa cells.

In the present study, AMH concentrations of follicular fluids derived from large follicles (≥8 mm) in different stages of follicular growth (selection phase, luteal phase, and ovulatory phase) were slightly higher in the high AFC group than in the low AFC group. In addition, the concentrations of AMH in IVG media of OCGCs derived from 4 to 12 days of culture were higher in the high AFC group than in the low AFC group. Scheetz *et al.*[93] reported that the production of AMH and the expression of mRNA of AMH were higher in cultured granulosa cells derived from high AFC cows than those from low AFC cows. These results indicate that the ability to produce AMH of granulosa cells is higher in the high AFC cows than in the low AFC cows throughout the follicular development. On the other hand, it was reported that AMH decreased the expression of FSH receptor in human granulosa cells[84], and E_2 production was impaired by

decreasing the response of preantral and antral follicles to FSH in mice[18], human[15, 34], and sheep[14]. In the present study and a previous study[70], higher E_2 concentration in follicular fluids was shown in the high AFC group, although AMH concentration which suppressed E_2 secretion was higher in the high AFC group than in the low AFC group. The reason for the contradiction of AMH and E_2 concentrations can be explained by the T concentration in follicular fluid. It was reported that T increased transcription of FSH receptor in bovine cultured granulosa cells[63], and *in vivo* results in the present study showed higher T concentration in the high AFC group. These results may suggest that higher T production from theca cells may counteract the function of AMH in the reduction of FSH-mediated E_2 production in the high AFC cattle. In further study, the roles of theca cells for follicular growth should be investigate in detail.

In conclusion, the plasma concentration of FSH was higher in the low AFC cows than in the high AFC cows, whereas concentrations of E_2 and T were higher in the high AFC cows than in the low AFC cows. These findings suggest that lower function of E_2 production of granulosa cells in the low AFC cows causes low E_2 concentration in systemic level, resulting in high FSH concentration and a consistent development of intermediate follicles in the low AFC cows. Conversely, higher E_2 concentration suppresses FSH secretion in the high AFC cows, resulting in a drastic degradation of intermediate follicles at selection phase. *In vivo* and *in vitro* AMH productions of granulosa cells were higher in the high AFC cows than in the low AFC cows, indicating the existence of stage-dependent regulatory roles of not only AMH but also other factors possibly derived from theca cells on the FSH-mediated follicular growth and steroidogenesis in cattle.

Figures

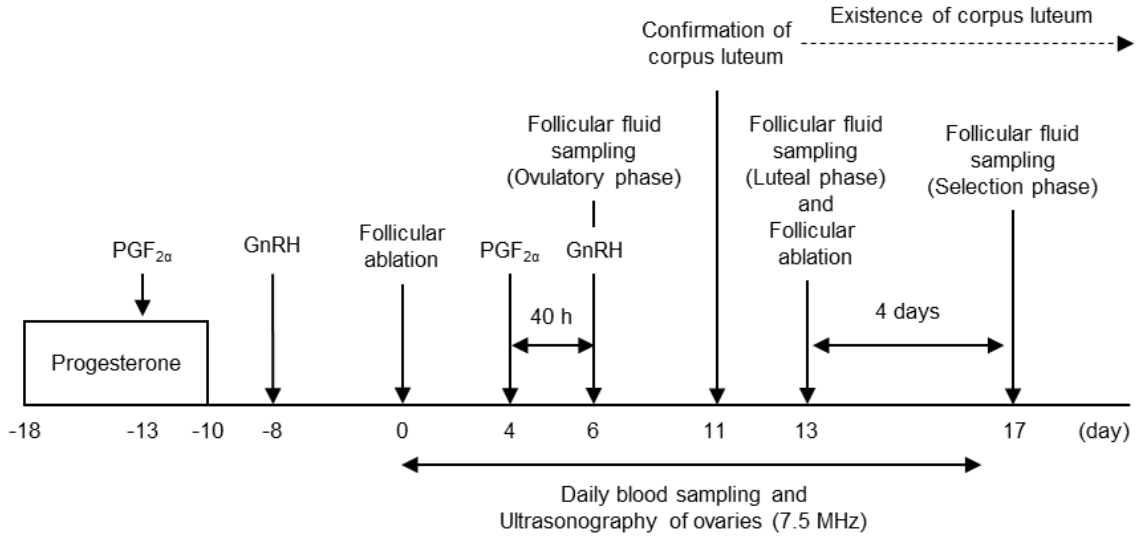

Fig. III-1. Schematic of the experimental design.

Estrous cycles and follicular waves of cows were synchronized using hormonal treatment and follicular ablation between day –18 and day 0[91]. At day 4, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was injected for inducing estrus. After 40 hours, a dominant follicle just before the luteinizing hormone surge was aspirated and a collected follicular fluid was defined as the ovulatory phase (day 6). Soon after follicular aspiration, gonadotropin releasing hormone (GnRH) was injected for inducing luteinization of the dominant follicle. After 7 days, a dominant follicle growing with corpus luteum was aspirated and a collected follicular fluid was defined as the luteal phase (day 13). After that, all visible follicles were ablated. Four days later, the largest follicle was aspirated, and a collected follicular fluid was defined as the selection phase (day 17). In three cows (1 low AFC and 2 high AFC), follicular ablation was performed again at day 16 and follicular fluid was collected at day 20. During days 0 to 16, I daily collected blood sample and monitored ovaries by ultrasonography.

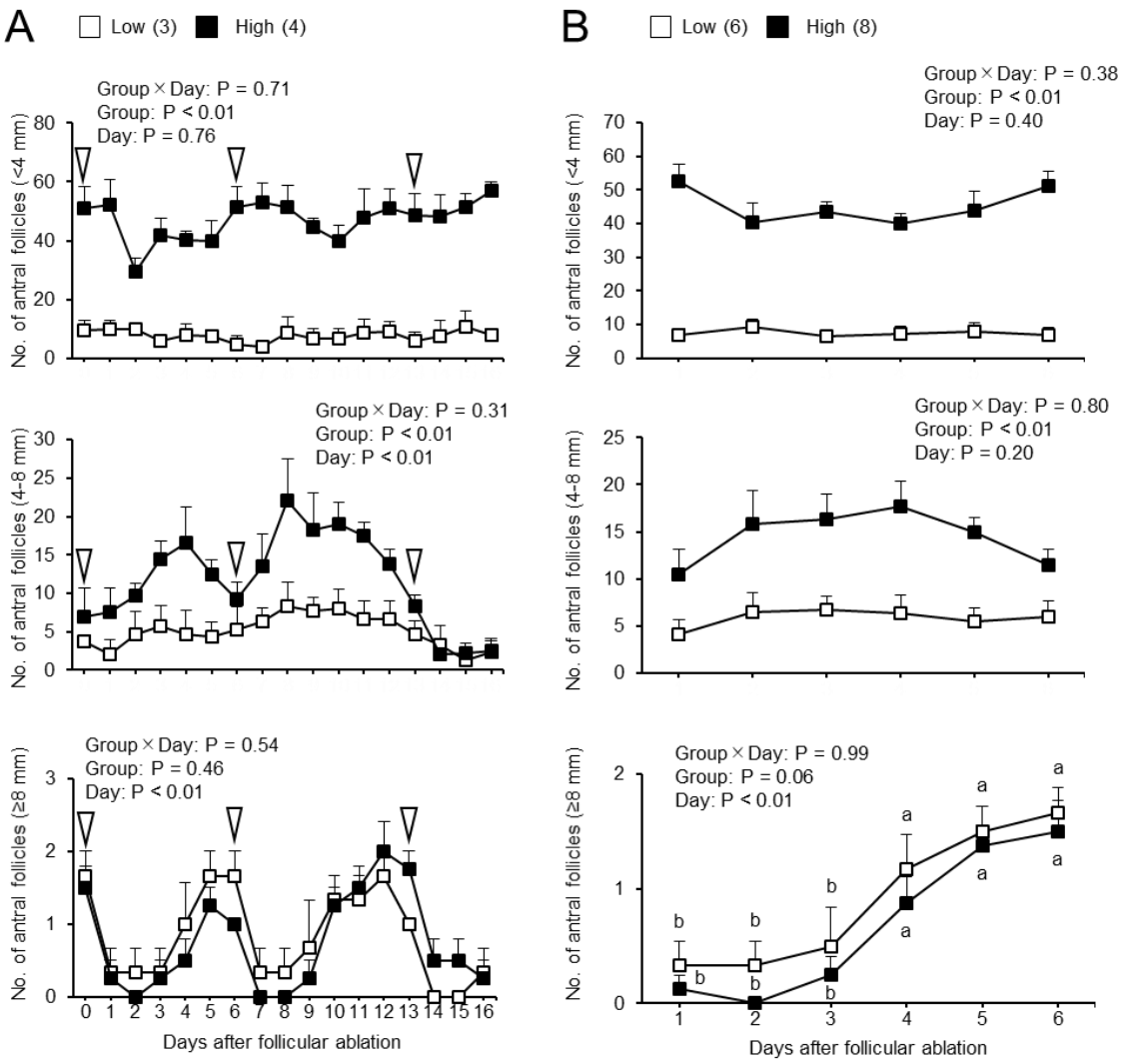

Fig. III-2. Relationship between antral follicle counts (AFC) and follicular growth dynamics monitored by ultrasonography.

A: The number of follicles after the first follicular ablation were monitored by ultrasonography. Diameters of each follicles were measured. Follicles were classified into 3 groups according to their diameters (small: <4 mm, intermediate: 4-8 mm, and large: ≥8 mm). I compared the number of antral follicles in each category between groups and days after follicular ablation. White arrowheads indicate the timings of follicular ablation and the sampling of follicular fluids.

B: The number of small, intermediate, and large antral follicles in two follicular waves from 1 to 6 days after follicular ablation (days 0 and 6) were pooled, and I compared the number of antral follicles between groups and days after follicular ablation.

Results of factorial analysis by two-way ANOVA were shown above each panel.

[a, b] Different letters indicate significant differences between each day ($P < 0.05$).

Numbers in parentheses indicate the number of cows (A) or the number of follicular wave (B).

Error bars indicate SEM.

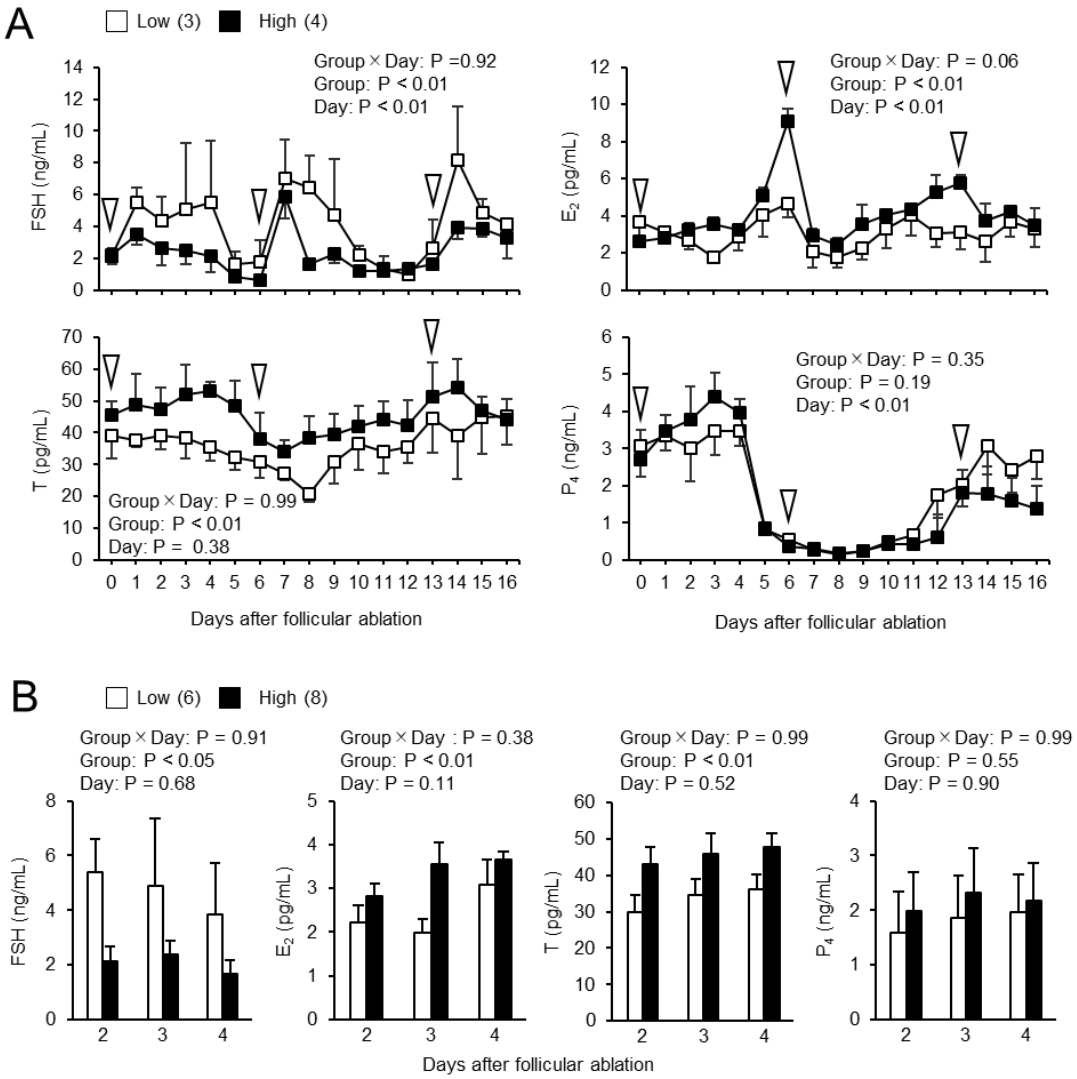

Fig. III-3. Relationship between antral follicle counts (AFC) and plasma concentrations of follicle stimulating hormone (FSH), estradiol-17β (E_2), testosterone (T), and progesterone (P_4).

A: FSH and steroid hormones were measured from days 0 to 16, and I compared concentrations of each hormone in plasma between groups and days after follicular ablation. White arrowheads indicate the timings of follicular ablation and the sampling of follicular fluids.

B: Two to four days after follicular ablation were defined as the selection phase of follicles. The selection phases in two follicular waves after follicular ablation were pooled, and I compared concentrations of each hormone in plasma between groups and days after follicular ablation during the period.

Results of factorial analysis by two-way ANOVA were shown above each panel.

[a, b] Different letters indicate significant differences between each day ($P < 0.05$).

Numbers in parentheses indicate the number of cows (A) or the number of follicular wave (B).

Error bars indicate SEM.

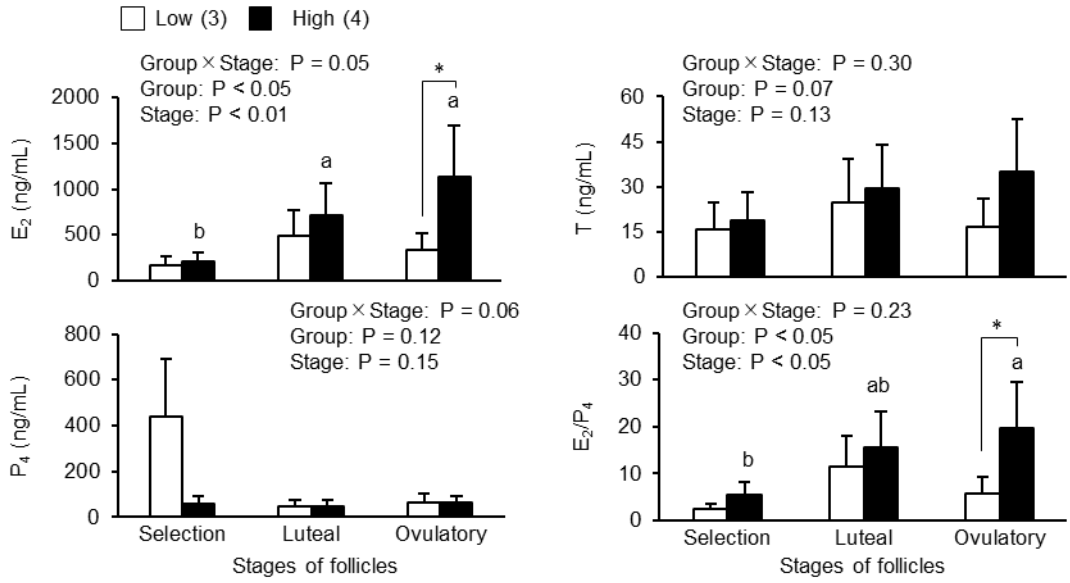

Fig. III-4. Relationship between antral follicle counts (AFC) and concentrations of estradiol-17β (E_2) testosterone (T), progesterone (P_4), and the E_2/P_4 ratio in follicular fluid.

Steroid hormones in follicular fluid collected from largest follicles in each stage of follicular growth (selection, luteal, ovulatory) were measured , and I compared concentrations of each steroid hormone and the E_2/P_4 ratio in follicular fluid between groups and stages of follicular growth.

Results of factorial analysis by two-way ANOVA were shown above each panel.

* An asterisk indicates a significant difference between the low and high AFC groups ($P < 0.05$).

[a, b] Different letters indicate significant differences between each stage ($P < 0.05$).

Numbers in parentheses indicate the number of cows.

Error bars indicate SEM.

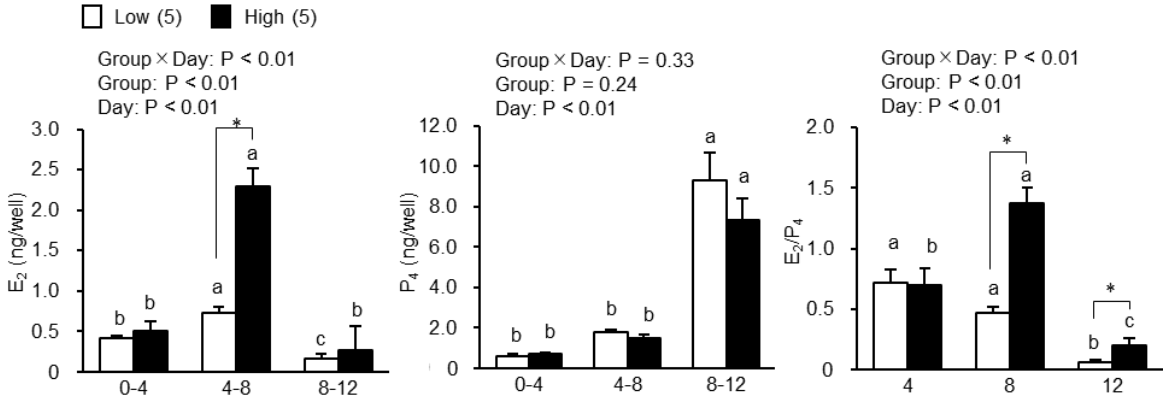

Fig. III-5. Relationships between antral follicle counts (AFC), the production of estradiol-17β (E_2) and progesterone (P_4) by oocyte-cumulus-granulosa complexes (OCGCs), and the E_2/P_4 ratio in culture media

Steroid hormones in IVG media of OCGCs in days 4, 8, and 12 of culture were measured, and the production of E_2, P_4, and the E_2/P_4 ratio were calculated as described in chapter I. I compared concentrations of E_2, P_4, and the E_2/P_4 ratio in IVG media between groups and the day of culture. Results of factorial analysis by two-way ANOVA were shown above each panel.

$^{a\text{-}c}$: Different letters indicate significant differences between different culture periods in the same group ($P < 0.05$).

* An asterisk indicates a significant difference between the low and high AFC groups ($P < 0.05$).

Numbers in parentheses indicate the number of OCGCs on the same day.

Error bars indicate SEM.

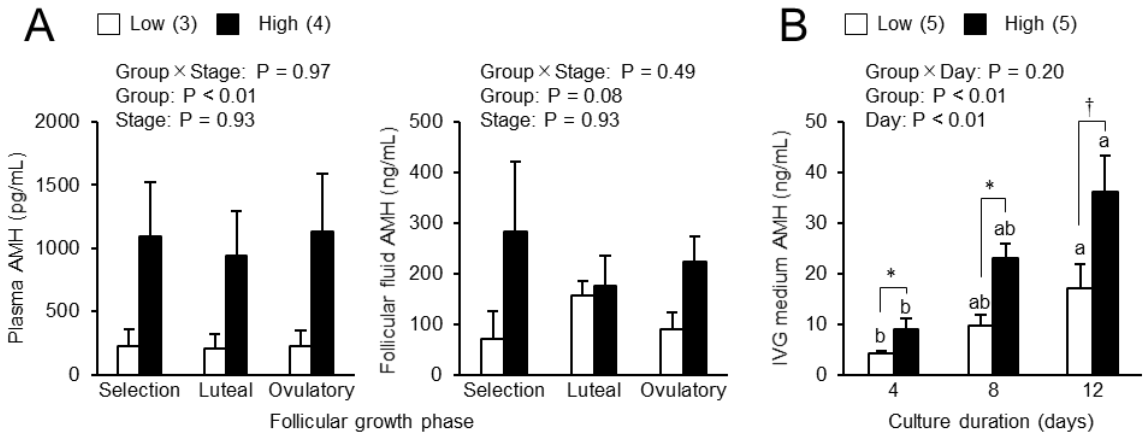

Fig. III-6. Relationships between antral follicle counts (AFC) and concentrations of anti-Müllerian hormone (AMH) in plasma, follicular fluid, and *in vitro* growth (IVG) media.

A: Plasma concentrations of AMH were measured on the representative days of each stage of follicular growth (selection; day 4, luteal; day 13, ovulatory; day 6). Concentrations of AMH in follicular fluid in each stage of follicular growth were measured using same sample as steroid hormones. I compared concentrations of AMH between groups and stages of follicular growth.

B: Concentrations of AMH in IVG media of oocyte-cumulus-granulosa complexes (OCGCs) were measured. I compared concentrations of AMH between groups and day of culture (days 4, 8, and 12).

Results of factorial analysis by two-way ANOVA were shown above each panel.

* An asterisk indicates a significant difference between the low and high AFC groups ($P < 0.05$).

†A dagger indicates a difference between the low and high AFC groups ($P = 0.05$).

[a, b] Different letters indicate significant differences between each day ($P < 0.05$).

Numbers in parentheses indicate the number of cows (A) or the number of OCGCs (B).

Error bars indicate SEM.

Summary

The antral follicle count (AFC) in mammalian ovaries positively correlates with female fertility. To clarify the causes of the difference of fertility between low AFC cows and high AFC cows, I investigated follicular growth dynamics and hormone concentrations in plasma, follicular fluid, and *in vitro* growth (IVG) media in different stages of follicular growth. Seven cows were separated into the high AFC group (n = 4, >30 follicles) and the low AFC group (n = 3, <30 follicles) based on the peak AFC detected by ultrasonography. I ablated their follicles 8 days after estrus and gave prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on day 12. After 40 hours of $PGF_{2\alpha}$ injection, follicular fluid of a dominant follicle (ovulatory phase) was collected, and subordinate follicles were ablated. Then, gonadotropin releasing hormone (GnRH) were injected to induce the luteinizing hormone surge. Seven days after GnRH and the confirmation of corpora lutea, follicular fluids were collected from dominant follicles (luteal phase), and all follicles were ablated. Four days later, follicular fluids were collected from the largest follicles (selection phase). Concentrations of estradiol-17β (E_2), testosterone (T), progesterone (P_4), and anti-Müllerian hormone (AMH) in follicular fluids were measured. I daily examined ovaries by ultrasonography and collected blood for hormone measurements of follicle stimulating hormone (FSH), E_2, T, P_4, and AMH. Concentrations of E_2, P_4, and AMH in IVG media were also measured. The numbers of small (<4 mm) and intermediate (4-8 mm) follicles were larger in the high AFC group than in the low AFC group ($P < 0.05$), whereas numbers of large follicles (>8 mm) were similar in both groups regardless of any phase. Although the number of intermediate follicles was stable in the low AFC group, the number of those follicles fluctuated in the high AFC group. Plasma FSH concentration was higher, but E_2 and T concentrations were lower in the low AFC group than in the high AFC group ($P < 0.05$). Although diameters of aspirated follicles were similar in both groups at each phase, E_2 and E_2/P_4 ratio were higher in the high AFC than in the low AFC group ($P < 0.05$). E_2 and E_2/P_4 ratio in IVG media were also higher in the high AFC group than in the low AFC group on day 8 of culture ($P < 0.05$). Concentrations of AMH in plasma ($P < 0,01$), follicular fluid ($P = 0.08$), and IVG media ($P <$

0.01) were higher in the high AFC group than in the low AFC group. These findings suggest that lower response to FSH of granulosa cells causes low E_2 production in the low AFC group, resulting in high FSH concentration and a consistent development of intermediate follicles. Conversely, higher E_2 concentration suppresses FSH secretion in high AFC group.

Summary and Conclusions

The technology for *in vitro* embryo production in cattle industry has become important in a last decade, but the competence of *in vitro*-derived oocytes is lower than *in vivo*-derived oocytes. Therefore, detailed understanding of factors affecting *in vivo* follicular growth and the acquisition of oocyte developmental competence *in vivo* and *in vitro* are necessary. Although oocytes acquire the developmental competence during follicular development from 1 to 2 mm in diameter, we cannot detect the follicular growth *in vivo* during this stage because of their small diameter. Therefore, in the present study, the author tried to establish an *in vitro* growth (IVG) culture system that can be used as a follicular growth model. In addition, I also examined possible factors affecting follicular growth and oocyte developmental competence by using IVG culture for evaluating small-sized follicular development and by using ultrasonography and the analysis of hormonal dynamics during estrous cycle for evaluating large-sized follicular development.

Bone morphogenetic protein-4 (BMP-4) and follicle stimulating hormone (FSH) play important regulatory roles in follicular growth and steroidogenesis *in vivo*. I conducted a study to investigate the effects of BMP-4 and FSH on IVG and steroidogenesis of bovine oocyte-cumulus-granulosa complexes (OCGCs) in chapter I. I cultured OCGCs collected from early antral follicles (0.5-1.0 mm) in medium without BMP-4 and FSH for 4 days and investigated the appearance of OCGCs and their steroidogenesis. During the first 4 days of IVG, morphologically normal OCGCs produced more estradiol-17β (E_2), but less progesterone (P_4). Morphologically normal OCGCs were subjected to an additional culture in medium supplemented with BMP-4 (0, 10, and 50 ng/mL) and FSH (0 and 0.5 ng/mL) until day 12. I examined the viability and steroidogenesis of OCGCs after 8 and 12 days of culture. Oocyte growth, characteristics of granulosa cells, and the maturational competence of oocytes were also investigated. On day 8, the viability of OCGCs cultured without FSH was higher in the 10 ng/mL BMP-4 group than in the 50 ng/mL BMP-4 group ($P < 0.05$). No significant difference was observed in the viability of groups cultured with FSH, regardless of the addition of BMP-4,

and FSH improved the viability of 50 ng/mL BMP-4 group similar to 10 ng/mL BMP-4 group. The total number of granulosa cells was larger in 10 ng/mL BMP-4 group cultured with FSH than in 50 ng/mL BMP-4 group cultured with FSH on day 8 ($P < 0.05$). E_2 production decreased from days 8-12, and P_4 production increased throughout IVG culture, regardless of the addition of BMP-4 and FSH ($P < 0.05$). No significant differences in E_2 production were observed between groups from days 4-8, regardless of whether BMP-4 was added without FSH; however, E_2 production in the group cultured with 50 ng/mL BMP-4 was suppressed by FSH. BMP-4 suppressed E_2 production from days 8-12, regardless of whether FSH was added. The group cultured with 10 ng/mL BMP-4 without FSH showed the lowest P_4 production among all groups for all culture periods. OCGCs that produced mature oocytes tended to secrete more E_2 and less P_4 than OCGCs that produced immature oocytes. In conclusion, until day 8 of the IVG culture, P_4 production by OCGCs was suppressed by the addition of 10 ng/mL BMP-4 in the absence of FSH, without inhibiting E_2 production. These conditions appear to mimic growing follicles until day 8 and mimic degenerating follicles from days 8-12 of culture.

In chapter II, I investigated the relationship between the antral follicle count (AFC), which is used as an indicator of cow fertility, and the steroidogenesis of granulosa cells. Also, I examined the developmental competence of oocytes derived from early antral follicles (0.5-1.0 mm) collected from ovaries with varied AFC using IVG culture. Slaughterhouse-derived ovaries were divided into high (≥25 follicles) and low AFC groups (<25 follicles) based on AFC (≥2.0 mm). OCGCs collected from early antral follicles were cultured for 12 days. The total number, viability, and diameter of granulosa cells and E_2 and P_4 production during the culture were evaluated. Surviving oocytes on day 12 were subjected to *in vitro* maturation, and their volume and nuclear status were evaluated. Some oocytes were subjected to the evaluation of developmental competence to blastocysts. Although the total number and viability of granulosa cells did not differ between the groups, granulosa cell diameters were smaller in the high AFC group than in the low AFC group ($P < 0.05$). The E_2 and P_4 ratio on day 8 was higher in the high AFC group than in the low AFC group ($P < 0.05$). Oocyte volumes and nuclear maturation rates

were greater in the high AFC group than in the low AFC group ($P < 0.05$). The development rate to blastocysts was 9.1% in the high AFC group, whereas no oocytes developed to blastocysts in the low AFC group. E_2 production by granulosa cells appears to be greater in high AFC cattle than in low AFC cattle, thereby promoting the acquisition of oocyte competence.

In chapter III, I investigated follicular growth dynamics and hormone concentrations in plasma, follicular fluid, and IVG media in different stages of follicular growth to clarify the causes of the difference of fertility between low AFC cows and high AFC cows. Seven cows were separated into the high AFC group (n = 4, >30 follicles) and the low AFC group (n = 3, <30 follicles) based on the peak AFC detected by ultrasonography. I ablated their follicles 8 days after estrus and gave prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on day 12. After 40 hours of $PGF_{2\alpha}$ injection, follicular fluid of a dominant follicle (ovulatory phase) was collected, and subordinate follicles were ablated. Then, gonadotropin releasing hormone (GnRH) were injected to induce the luteinizing hormone surge. Seven days after GnRH and the confirmation of corpora lutea, follicular fluids were collected from dominant follicles (luteal phase), and all follicles were ablated. Four days later, follicular fluids were collected from the largest follicles (selection phase). Concentrations of E_2, testosterone (T), P_4, and anti-Müllerian hormone (AMH) in follicular fluids were measured. I daily examined ovaries by ultrasonography and collected blood for hormone measurements of FSH, E_2, T, P_4, and AMH. Concentrations of E_2, P_4, and AMH in IVG media were also measured. The numbers of small (<4 mm) and intermediate (4-8 mm) follicles were larger in the high AFC group than in the low AFC group ($P < 0.05$), whereas numbers of large follicles (>8 mm) were similar in both groups regardless of any phase. Although the number of intermediate follicles was stable in the low AFC group, the number of those follicles fluctuated in the high AFC group. Plasma FSH concentration was higher, but E_2 and T concentrations were lower in the low AFC group than in the high AFC group ($P < 0.05$). Although diameters of aspirated follicles were similar in both groups at each phase, E_2 and E_2/P_4 ratio were higher in the high AFC than in the low AFC group ($P < 0.05$). E_2 and E_2/P_4 ratio in IVG media were also higher in the high AFC group than in the low AFC group on day 8 of culture

($P < 0.05$). Concentrations of AMH in plasma ($P < 0,01$), follicular fluid ($P = 0.08$), and IVG media ($P < 0.01$) were higher in the high AFC group than in the low AFC group. These findings suggest that lower response to FSH of granulosa cells causes low E_2 production in the low AFC group, resulting in high FSH concentration and a consistent development of intermediate follicles. Conversely, the higher E_2 concentration suppresses FSH secretion in high AFC group.

In the present study, the author has developed the IVG culture system for incompetent bovine oocytes and confirmed the relationship between oocyte developmental competence and the ability of granulosa cells to produce E_2. Also, the author clarified the acquisition of oocyte developmental competence and the control of follicular growth by analyzing the hormonal dynamics in plasma and follicular fluids using *in vivo* derived materials. The detailed information enables us to develop a new procedure of embryo production in cattle and to progress the physiological studies on the follicular development and infertility problems of not only cattle but also many mammals.

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