The effects of theca cell-derived bone morphogenetic protein-4 on *in vitro* growth, fertilization and subsequent development of bovine oocytes

（内卵胞膜細胞由来骨形成タンパク質 4 が牛卵子の体外発育、受精および胚発生に与える影響）

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Contents

Abbreviations

2PN	two pronuclei
A_4	androstenedione
AFC	antral follicle count
A I/T I	anaphase I/telophase I
ANOVA	analysis of variance
BMPs	bone morphogenetic proteins
BSA	bovine serum albumin
COC	cumulus-oocyte complex
DNase	deoxyribonuclease
D-PBS	Dulbecco’s phosphate buffered saline
E_2	estradiol-17β
EDTA	ethylenediaminetetraacetic acid
ESH	enlarged sperm head
FCS	fetal calf serum
FSH	follicle-stimulating hormone
GC	granulosa cell
GDF	growth differentiation factor
GV	germinal vesicle

HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
HSD	honestly significant difference
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVG	*in vitro* growth
IVM	*in vitro* maturation
M I	metaphase I
M II	metaphase II
mRNA	messenger RNA
OGC	oocyte-granulosa cell complex
OSF	oocyte secreted factor
P_4	progesterone
PCR	polymerase chain reaction
RT-qPCR	quantitative reverse transcription PCR
SEM	standard error of the mean
T	testosterone
TC	theca cell
TCM199	tissue culture medium 199
TGF-β	transforming growth factor-β

Preface

One major role of mammalian ovaries is producing fully-grown and matured oocytes for reproduction purpose. Oocyte starts to grow along with development of ovarian follicles from primordial stage, and reaches its full growth when follicle progresses to antral stage. Fully-grown oocytes are highly competent, as a result, these are widely utilized in human infertility treatment and *in vitro* embryo production in cattle (Neglia *et al.* 2003; Lonergan and Fair 2008). However, *in vivo*, only very few oocytes are selected to grow to their final size, and numerous small-sized oocytes are degenerated at various growing stages. It could be of great significance to develop an efficient *in vitro* growth (IVG) system for these small and immature oocytes as *in vitro*-grown oocytes may contribute to alternative infertility treatments, more embryo production per ovary and effective conservation of genetically-valuable animals. In addition, IVG could also be a valuable *in vitro* study model for clarifying the factors affecting oocyte growth and subsequent maturation/development. However, in large animals, the oocytes derived from IVG system generally show lower developmental competence than *in vivo*-grown oocytes (Hirao and Miyano 2014). There is a need to clarify the causes of low competence in IVG oocytes and develop new IVG culture protocol producing competent oocyte as *in vivo*-grown ones.

Spontaneous luteinization of bovine granulosa cells (GCs), indicated by elevated progesterone (P_4) production and decreased estradiol-17β (E_2) production, is commonly observed in *in vitro* culture (Luck *et al.* 1990; Gutierrez *et al.* 1997). However, in mammals, luteinization is triggered after the preovulatory luteinizing hormone surge when oocyte has completed full growth *in vivo* (Murphy 2000). Along with development of dominant follicles in cattle, follicular E_2 concentration increases concomitantly and the E_2/P_4 ratio also increases; however, subordinate follicles showed

low E_2/P_4 ratios (Kruip and Dieleman 1985; Beg *et al.* 2001). Therefore, luteinization in IVG culture may be one cause for the low competence of IVG oocytes, but few studies have focused on this issue.

A follicle is consisted of an oocyte and two types of follicular cells including GCs surrounding the oocyte and theca cells (TCs) surrounding the follicular wall (outside of basal membrane). TCs play supportive and regulatory functions in follicle growth and development *in vivo*. However, only oocyte-granulosa cell complexes (OGCs) is collected for currently developed bovine IVG culture (Yamamoto *et al.* 1999; Huang *et al.* 2013; Makita and Miyano 2014), and the role of TCs is absent. Some studies have demonstrated that bone morphogenetic proteins (BMPs), largest subgroups of ligands in the transforming growth factor-β (TGF-β) superfamily of extracellular signaling molecules, play important regulatory roles in proliferation, differentiation and steroidogenesis of follicular cells (Glister *et al.* 2004; Shimasaki *et al.* 2004). BMP-4, produced by TCs, is capable of regulating the growth and function of GCs by suppressing apoptosis and enhancing the secretion of E_2, while reducing P_4 secretion *in vitro*, an action consistent with a delay of luteinization (Knight and Glister 2003; Kayamori *et al.* 2009). In addition, it has been suggested that BMP-4 may have a role in preventing ovarian atresia, as a dramatic decrease in the expression of BMP-4 is a feature of atresia (Erickson and Shimasaki 2003). However, these studies mainly focused on the effects of TC-derived BMP-4 on GCs, the effects of BMP-4 on oocyte growth and subsequent maturation/fertilization/development competence is unclear.

These evidences lead to the assumption that TCs or TC-derived BMP-4 may be an important missing factor in IVG culture, which is responsible for maintaining a preferable low-luteinized environment for follicular and oocyte growths. Therefore, the introduction of TCs or TC-derived BMP-4 into current IVG culture may contribute to improve oocyte growth and subsequent development. In this thesis, the author conducted the following studies to examine the effects of

TC-derived BMP-4 on *in vitro* growth, maturation, fertilization and subsequent development of bovine oocytes cultured with GCs.

In Chapter I, the author examined the effects of BMP-4 on *in vitro* growth, maturation, fertilization and developmental competence of bovine oocytes derived from early antral follicles. The steroidogenesis of GCs in IVG culture was also investigated.

In Chapter II, based on the anti-luteinization/atresia function of BMP-4 confirmed in Chapter I, in an attempt to allow oocytes to grow larger and more competent, the author examined the effects of the extension of culture period for the IVG culture in the presence of BMP-4 on growth and subsequent developmental competence of *in vitro*-grown oocytes.

In Chapter III, for TC-derived BMP-4 alone did not improve subsequent developmental competence of IVG oocytes, the author attempted to develop a co-culture system including all components of follicles: oocyte, granulosa and theca cells. The growth and subsequent developmental competence of the co-cultured oocytes and the steroidogenesis in co-culture were investigated.

Chapter I

Effects of bone morphogenetic protein-4 on *in vitro* growth, maturation, fertilization and development of bovine early antral follicle-derived oocytes

Introduction

Fully grown oocytes in antral follicles (more than 2 mm in diameter) are an important source of *in vitro* embryo production in cattle (Neglia *et al.* 2003; Lonergan and Fair 2008). However, the majority of oocytes in an ovary are small oocytes that are either dormant or at various growing stages. It is, therefore, necessary to utilize these small oocytes to make better use of ovaries, especially for species for which ovary samples are extremely rare. Although complete *in vitro* development of oocytes from primordial follicles has been demonstrated in mice (Eppig and O'Brien 1996; O'Brien *et al.* 2003), it has not been achieved in other mammals. Among follicles in developmental stages, early antral follicles show the potential to be a supplemental source of oocytes because they are most similar in size to late antral follicles. Several research groups have successfully produced live calves from oocytes released from early antral follicles (less than 1 mm in diameter) after IVG, *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (Yamamoto *et al.* 1999; Senbon and Miyano 2002; Huang *et al.* 2016). Compared to oocytes grown *in vivo*, however, the meiotic and developmental competence of IVG oocytes are generally lower (Yamamoto *et al.* 1999; Senbon and Miyano 2002; Hirao *et al.* 2004). Therefore, there is a need to improve currently used IVG culture protocol.

TCs are an essential component of growing follicles, supporting follicle growth and

development not only by delivering nutrients and providing the androgens required for conversion into estrogens by GCs but also by producing growth factors that can promote follicular development (Young and McNeilly 2010). BMPs are members of the TGF-β superfamily of extracellular signaling molecules, which play multiple roles in the regulation of the growth, differentiation and apoptosis of numerous cell types. TC-derived BMP-4 has been shown to be capable of regulating the growth and function of GCs by suppressing apoptosis and enhancing the secretion of E_2, while reducing P_4 secretion *in vitro*, an action consistent with a delay of luteinization and/or atresia (Knight and Glister 2003; Kayamori *et al.* 2009). Furthermore, previous *in vivo* studies have shown that BMP-4 mRNA was expressed at high levels in the TCs of developing dominant follicles, while it was very low or undetectable in atretic follicles (Erickson and Shimasaki 2003). This evidence suggests the possibility that TC-derived BMP-4 is related to the emergence and/or maintenance of dominant follicles; thus, it may contribute to IVG, which is aimed at producing healthy oocytes similar to those grown in the dominant follicles. The current IVG protocol for bovine oocytes only uses TC-free OGCs. As a result, the products from TCs, especially growth factors such as BMP-4, are absent. However, the effects of BMP-4 on oocyte growth and subsequent developmental competence are unknown because GCs alone were studies in previous studies (Glister *et al.* 2004; Kayamori *et al.* 2009; Yamashita *et al.* 2011).

It is known that a low antral follicle count (AFC) in ovaries is associated with diminished ovarian function and fertility in both human and cow (Ireland *et al.* 2007; Rosen *et al.* 2011). *In vitro* study also demonstrated that oocytes derived from follicles with low AFC had a lower blastocyst formation rate (Modina *et al.* 2007). Importantly, the P_4 concentration was 68 ng/ml in growing antral follicles with a low AFC, while it was 32 ng/ml in follicles with a high AFC (Modina *et al.* 2007). Preliminary study in author's laboratory found P_4 concentration in the IVG culture medium soared above 60 ng/ml, a high level comparable to that in follicles with a low AFC. Therefore, in

an attempt to reduce P_4 production during IVG culture, in the present study, BMP-4 was added to the IVG culture medium. The effects of BMP-4 addition on OGC growth, P_4 production and subsequent developmental competence acquisition were examined.

Materials and Methods

Chemicals

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

Collection of OGCs and IVG culture

Bovine ovaries obtained from a slaughterhouse were kept in plastic bags at 20°C and were transported to the laboratory within 6 to 10 h after collection. After three washes in saline, sliced ovarian cortex strips (<1 mm thick) were prepared using a surgical blade (No. 11). Early antral follicles (0.5-1 mm in diameter) were dissected from cortex strips using a surgical blade (No. 20) under a stereomicroscope in TCM199 (Invitrogen; Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium bicarbonate, and 50 μg/ml gentamicin sulfate (isolation medium, pH 7.4), as described elsewhere (Huang *et al.* 2013). Follicles were punctured to release OGCs using a pair of fine forceps, and the OGCs with a normal appearance were individually cultured for 12 days in 96-well culture plates (Primaria 353872, Corning, NY, USA) with 200 μl of growth medium at 39°C in humidified air with 5% CO_2. The growth medium was HEPES-buffered TCM199 supplemented with 0.91 mM sodium pyruvate, 1 μg/ml E_2, 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000) and 50 μg/ml gentamicin sulfate. Half of the medium was replaced every 4 days, throughout the culture, 10 and 50 ng/ml BMP-4 (HumanZyme, Inc., Chicago, IL, USA) was added to IVG medium while 0 ng/ml BMP-4 group worked as non-treated control. The doses of BMP-4 were selected based on previous *in vitro* studies, in which BMP-4 at these doses was demonstrated to be effective in suppressing GC apoptosis and P_4 production (Glister *et al.* 2004; Kayamori *et al.* 2009).

P_4 assay

The culture medium collected at 4, 8 and 12 days of IVG culture was frozen at -30°C until P_4 assay by using enzyme immunoassay, as previously described (Yanagawa *et al.* 2015). IVG media were loaded directly as a sample to the well of a microplate. Anti-progesterone-3CMO-BSA (KZ-HS-P13, Cosmo Bio Co., Ltd., Tokyo, Japan) was used as the primary antibody, and goat anti-rabbit serum (111-005-003, Jackson Immuno Research Laboratories, Inc., PA, USA) was used as the secondary antibody. The intra- and inter-assay coefficients of variations were 5.8% and 9.8%, respectively.

Evaluation of OGC growth

In the present study, the viability of OGC, as well as the diameter, viability and number of GCs, were considered as OGC growth parameters. Before and after IVG culture, OGC growth parameters were measured. The survivability of OGCs was evaluated by their morphological appearance; oocytes completely enclosed by several healthy GC layers were considered to be viable (Huang *et al.* 2013). The diameter, viability and number of GCs from viable OGCs 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). To prepare dispersive GCs for the counting, culture medium in the well of each viable OGC was removed and replaced with 100 µl of Dulbecco's phosphate buffered saline without calcium and magnesium (D-PBS (-)) supplemented with 0.125% trypsin and 0.05% EDTA. After 10 min of trypsinization and pipetting several times, 25 µl of FCS was added to stop the digestion, and then, the denuded oocyte was removed from the well and discarded.

IVM of in vivo-grown and IVG oocytes

In vivo-derived oocytes, serving as control, were collected from antral follicles (2-8 mm in diameter) were submitted to IVM as described previously (Takahashi *et al.* 1996). Briefly, cumulus-oocyte complexes (COCs) were incubated in droplets of IVM medium (approximately 10 COCs/50 μl) and were then covered with paraffin oil for 22 h at 39°C in a humidified atmosphere with 5% CO_2. The maturation medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml follicle-stimulating hormone (FSH, from porcine pituitary), 1 μg/ml E_2, 10% FCS, and 50 mg/ml gentamicin sulfate.

For IVG oocytes, COCs were isolated from the viable OGCs for pre-IVM and IVM as previously described (Huang *et al.* 2016). Briefly, oocytes were individually cultured in individual wells of micro-well plates (MiniTrays 163118; NUNC, Roskilde, Denmark) that contained 6 ml of pre-IVM medium, and then, 6 ml of IVM medium for 10 and 22 h at 39°C in a humidified atmosphere with 5% CO_2 (Nagano *et al.* 2013). Pre-IVM medium, which is modified from IVM medium, contained additional 0.5 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and a lower FSH concentration (2×10^{-6} units/ml) (Huang *et al.* 2016).

Before IVG and after IVM culture, all 92 oocytes were measured for their diameters as previously described (Huang *et al.* 2013). Briefly, the diameter of each denuded oocyte was measured using an inverted microscope (CK40, Olympus, Tokyo, Japan) connected to a CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan) and image processing software (Motic Images Plus 2.2s, Shimadzu).

IVF and IVC

IVF was performed according to a previously described procedure (Takahashi and Kanagawa 1998). Briefly, COCs were co-incubated with frozen-thawed motile sperm (5×10^6 sperm/ml)

separated by a Percoll gradient (45 and 90%). Approximately 10 COCs were cultured in a 100-μl droplet of modified Brackett and Oliphant isotonic medium containing 3 mg/ml fatty acid–free bovine serum albumin (BSA) and 2.5 mM theophylline for 18 h at 39°C in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2.

Only 10 ng/ml BMP-4 treatment were investigated for IVC because a low survival rate in 50 ng/ml BMP-4 treated OGCs was observed during IVG. IVC was performed as previously described (Takahashi *et al.* 1996). Briefly, presumptive zygotes were denuded by vortexing and washed three times in culture medium, and then approximately 30 zygotes were cultured in a 30-μl droplet of culture medium for 6 days at 39°C in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, twelve essential amino acids for basal medium Eagle, seven nonessential amino acids for minimum essential medium, 10 μg/ml insulin, and 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid-free BSA.

Evaluation of oocyte nuclear maturation and fertilization

Following IVM or IVF, oocytes were denuded from cumulus cells by vortexing and were then stained with 1% aceto-orcein. To evaluate nuclear maturation, the nuclear status was classified as germinal vesicle (GV), metaphase I (M I), anaphase I/telophase I (A I/T I), and metaphase II (M II) by observation under a phase-contrast microscope. The diameter of each denuded oocyte after IVM (before fixation) was measured and was considered the oocyte diameter after IVG culture. To evaluate fertilization, the oocytes were considered to be penetrated by sperm when they had one or more enlarged sperm head(s) or male pronucleus(ei) with corresponding sperm tail(s). Monospermic penetration was defined as normal fertilization in which oocytes have a single male pronucleus and a female pronucleus, or a single enlarged sperm head with the

chromosomes at A II/ TII. Cleavage and the blastocyst rates were determined after 2 days and 6 days of IVC, respectively, and the cell numbers in blastocysts were counted using an air-drying method (Takahashi and First 1992).

Statistical analysis

All statistical analyses were performed using JMP software version 12.0.1 (SAS Institute, Cary, NC, USA). The effects of the BMP-4 treatments on the GC diameter, viability and number, oocyte diameter and blastocyst cell number were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer's honestly significant difference (HSD) test as a post hoc test. The effects of the BMP-4 treatments and culture duration on the P_4 concentration were analyzed by two-way ANOVA followed by Tukey-Kramer's HSD test as a post hoc test. The effect of the BMP-4 treatments on the OGC viability, oocyte nuclear maturation and fertilization, and blastocyst rate were analyzed by a chi-square test.

Results

Growth of OGC

Table 1-1 shows the OGC growth parameters before and after IVG culture with or without BMP-4 treatments. After 12 days of IVG culture, 50 ng/ml BMP-4 treated OGCs showed lower viability (48.2%, $P < 0.05$) compared to others (~ 70%). The viability, diameter and number of GCs significantly increased ($P < 0.05$) compared to those of the pre-IVG culture. The GC viabilities in all of the experimental groups were similar; however, the GC number in the 50 ng/ml BMP-4 treated group was lower than that of the control group ($P < 0.05$). In both BMP-4 treatments, a smaller mean diameter of GCs (11.2 and 11.0 μm) was observed compared to that of the controls (12.0 μm) ($P < 0.05$).

Table 1-1. Effects of BMP-4 in IVG medium on the growth of OGCs from early antral follicles

IVG Culture (day)	BMP-4 (ng/ml)	% of OGC viability (n)	Granulosa cell		
			Number (× 10^3 cells/OGC)	% of viability (n)	Diameter (μm)
0	-	100.0^{a} (30)	2.0 ± 0.5^{c}	88.7 ± 2.8^{b} (30)	9.3 ± 1.1^{c}
12	0	67.8^{b} (242)	85.0 ± 33.7^{a}	92.8 ± 3.4^{a} (26)	12.0 ± 0.8^{a}
	10	70.4^{b} (250)	69.0 ± 23.5^{ab}	93.2 ± 2.9^{a} (25)	11.2 ± 0.9^{b}
	50	48.2^{c} (247)	49.6 ± 12.4^{b}	93.7 ± 3.2^{a} (14)	11.0 ± 0.9^{b}

^{abc} Values with different superscripts in the same column are significantly different ($P < 0.05$).

Data are presented as mean ± SD.

P_4 production of OGC

As shown in Fig. 1-1, the P_4 concentration in the culture medium of the control group increased throughout the duration of IVG culture ($P < 0.05$). However, in the BMP-4 treated groups, the P_4 concentration was decreased on days 8 and 12 of IVG culture in comparison to control ($P < 0.05$). There was no difference in P_4 production between BMP-4 treated groups.

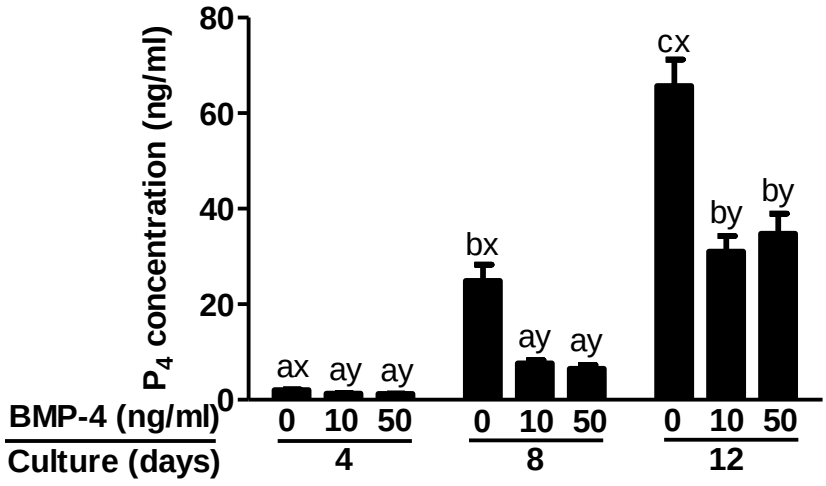

Fig. 1-1. Effects of BMP-4 on progesterone (P_4) production by GCs during IVG culture.

[abc] Different letters represent differences among the same doses of BMP-4 ($P < 0.05$).

[xy] Different letters represent differences within same day of culture ($P < 0.05$).

Data are expressed as the mean values and error bars mean SEM.

Subsequent developmental competence

As shown in Tables 1-2 and 1-3, the oocyte diameter, nuclear maturation and fertilization were not significantly affected by the BMP-4 treatments, although there was a decreasing tendency of total penetration in the 50 ng/ml BMP-4 treated group. IVG oocytes, regardless of BMP-4 addition, were smaller and had lower rates of normal fertilization and total penetration than *in vitro*-grown oocytes ($P < 0.05$). As shown in Table 1-4, the cleavage rates after IVF were similar between BMP-4 treated and non-treated IVG oocytes; however, BMP-4 treated oocytes had lower blastocyst rates, based on both inseminated oocytes and cleaved oocytes ($P < 0.05$). Compared to the *in vivo*-grown oocytes, IVG oocytes showed lower cleavage and blastocyst rates ($P < 0.05$).

Table 1-2. Effects of BMP-4 in IVG medium on the nuclear maturation and diameter of IVG oocytes

Oocyte	BMP-4 (ng/ml)	No. of oocytes cultured (replicates)	Oocyte diameter (μm)		% of nuclear status		
			Before IVG	After IVM	M I	A I/ T I	M II
In vitro grown	0	33‡ (4)	98.9 ± 3.4	116.9 ± 4.1[b]	6.5	0	93.5
	10	29 (4)	99.4 ± 3.0	117.7 ± 3.5[b]	10.3	0	89.7
	50	30 (4)	99.1 ± 3.1	116.8 ± 2.9[b]	3.3	3.3	93.4
In vivo grown†	-	32 (3)	-	120.2 ± 3.1[a]	6.3	0	93.8

M I, metaphase I; A I/T I, anaphase I/telophase I; M II, metaphase II.

† Oocytes collected from antral follicles (2-8 mm in diameter) served as *in vivo*-derived controls.

‡ For the evaluation of nuclear maturation, 31 oocytes in the 0 ng/ml BMP-4 treated group were used because 2 oocytes were lost during fixation.

[ab] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Data are presented as mean ± SD.

Table 1-3. Effect BMP-4 in IVG medium on the fertilization of IVG oocytes

Oocyte	BMP-4 (ng/ml)	No. of oocytes (replicates)	% of normal fertilization			% of polyspermy	% of total penetration
			ESH	2PN	subtotal		
In vitro-grown	0	73 (9)	16.4	43.8[b]	60.2[b]	6.8	67.0[b]
	10	91 (9)	13.2	48.4[b]	61.6[b]	9.9	71.5[b]
	50	53 (9)	5.7	41.5[b]	47.2[b]	7.5	54.7[b]
In vivo-grown[†]	-	83 (3)	6.0	80.7[a]	86.7[a]	7.2	93.9[a]

ESH, enlarged sperm head; 2PN, two pronuclei.

[†] Oocytes collected from antral follicles (2-8 mm in diameter) served as *in vivo*-derived controls.

[ab] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 1-4. Effect of BMP-4 in IVG medium on the development of IVG oocytes after IVF

Oocyte	BMP-4 (ng/ml)	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
				IVF oocytes	Cleaved oocytes	
In vitro-grown	0	257 (8)	54.9[b]	9.3[b]	17.0[b]	91.4 ± 35.8[b] (24)
	10	239 (7)	47.3[b]	1.3[c]	2.7[c]	142.0 ± 82.0[‡] (2)
In vivo-grown[†]	-	150 (5)	76.7[a]	25.3[a]	33.0[a]	164.6 ± 76.6[a] (38)

[†] Oocytes collected from antral follicles (2-8 mm in diameter) served as *in vivo*-derived controls.

[‡] One blastocyst was lost during cell counting; as a result, no statistical comparison was conducted.

[abc] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Data are presented as mean ± SD.

Discussion

In the current study, BMP-4 suppressed P_4 production from GCs, in agreement with the results of other *in vitro* studies (Glister *et al.* 2004; Yamashita *et al.* 2011). It has been reported that the inhibition of the acetylation of histone H3 associated with the steroidogenic acute regulatory protein promoter region by BMP-4 may be one of the underlying molecular mechanisms of the inhibition of P_4 synthesis in GCs (Yamashita *et al.* 2011). Because increased P_4 synthesis is a characteristic of the GC luteinization process (Murphy 2000), BMP-4 showed an effect of anti-luteinization. This effect was further verified by the significantly smaller diameter (~ 11 μm) of GCs in the BMP-4 treated groups after IVG culture compared to non-BMP-4 treated control (12.0 μm). It has been reported that both *in vivo*-grown large luteal cells originated from GCs and *in vitro*-luteinized GCs have similar sizes, which are larger than that of GCs in preovulatory follicles (mean diameter, 38.4 vs.10.6 μm, respectively) (O'Shea *et al.* 1989; Meidan *et al.* 1990). Therefore, the delay in GC enlargement observed in the present study may be attributed to delayed luteinization, which is associated with reduced P_4 synthesis in GCs treated with BMP-4. In atretic follicles, GCs were under luteinization and P_4 production increases (Hsueh *et al.* 1994; Jolly *et al.* 1994). In the present study, however, BMP-4 treatment reduced P_4 to a level (~ 33 ng/ml) close to that of follicular fluid (~ 32 ng/ml) in growing antral follicles with high AFC (Modina *et al.* 2007). These data indicate the possible development of an IVG model with P_4 production similar to that in follicles of high quality.

Despite the similarity in the ability to delay luteinization, the 50 ng/ml BMP-4 treatment caused a decrease in the GC number relative to the controls, while 10 ng/ml BMP-4 did not. It appears that BMP-4 impaired proliferation, but did not induce apoptosis of GCs, as the viability of GCs was high (more than 90%) in all of the experimental groups. This finding is inconsistent with

previous studies in which 50 or 100 ng/ml BMP-4 did not affect GC proliferation over 6 or 4 days of GC culture, respectively (Glister *et al.* 2004; Yamashita *et al.* 2011). I did not expect the GC number decrease with BMP-4 treatment because it has been demonstrated that BMP-4 could reduce apoptosis of GCs by suppressing the action of caspase-activated DNase induced by Survivin, a member of the inhibitor of apoptosis family (Kayamori *et al.* 2009). One major difference between the present and the previous studies is that I cultured OGCs, rather than GCs alone. The involvement of oocytes complicates the culture system and may be responsible for the discrepancies. It is now widely recognized that oocyte secreted factors (OSFs), such as growth differentiation factor-9 (GDF-9) and BMP-15, direct the functions of their surrounding GCs, including the promotion of cell growth and prevention of cell death and luteinization (Vanderhyden and Macdonald 1998; Eppig 2001; Gilchrist *et al.* 2004; Hussein *et al.* 2005). Because the types of receptors for the transforming growth factor-β superfamily are limited, ligands of the BMP/GDF subfamily bind to receptors in a shared manner (Mueller and Nickel 2012). Among the limited receptors, BMP type-II receptor is the sole type-II receptor presenting in GCs for GDF-9 and BMP-15 and is also an important receptor for BMP-4 (Shimasaki *et al.* 2004). In follicles, GDF-9 and BMP-4 could induce GCs to produce gremlin, which is known to be effective at antagonizing BMP-4 actions without affecting oocyte-derived GDF-9 and BMP-15 (Pangas *et al.* 2004; Sudo *et al.* 2004; Hussein *et al.* 2005). As a result, it is proposed that oocyte could maintain its surrounding microenvironment, which is important for oocyte development, from the actions of theca-derived BMPs (Hussein *et al.* 2005). Taking into consideration of the fact that a 20-fold excess of gremlin to BMP-4 was needed to completely block BMP-4 action (Pangas *et al.* 2004), exogenous BMP-4 at the doses studied (10 and 50 ng/ml) may be too high and probably impaired the function of OSFs by competitively binding receptors against OSFs, resulting in decreased GC proliferation in a dose-dependent manner. A smaller number of GCs in the 50 ng/ml BMP-4 treated group, in

turn, may lead to the decreased OGC viability when consider the fact that oocytes also relay on the support of GCs for long-term growth.

In the present study, BMP-4 treatment did not promote oocyte growth. However, BMP-7, another theca-derived growth factor, increased the oocyte diameter and volume during IVG, in which OGCs were cultured in groups on membrane inserts (Hirao *et al.* 2013). The culture system may partially cause the different results, but unknown differences in the biological function between BMP-4 and -7 may also be responsible (Shimasaki *et al.* 2004). Our study showed that BMP-4 impaired embryo development of IVG oocytes without affecting oocyte nuclear maturation and fertilization, indicating that BMP-4 may inhibit the cytoplasmic maturation of oocytes. Another study reported that BMP-4 addition during IVM of COC had no effect on bovine oocyte nuclear maturation and subsequent embryo development (Fatehi *et al.* 2005). The differences in the growth stage of oocytes and exposure time to BMP-4 may have led to the discrepancy. Through OSFs, oocytes appear to control their neighboring somatic cells, directing them to perform functions required for the appropriate development of oocytes. The presence of this regulatory loop was demonstrated by the fact that the neutralization of OSFs by antagonists of BMP-15 and GDF-9 during IVM impaired the developmental competence of COCs (Hussein *et al.* 2006). Although there was no BMP-4 addition during pre-IVM and IVM in the present study, the presumed interruption of the regulatory loop by BMP-4 during IVG appeared to cause a lasting adverse effect, as shown in the subsequent developmental competence of oocytes. Further investigations are necessary to verify this type of receptor competition between BMP-4 and OSFs and its consequential effects.

Compared to *in vivo*-grown oocytes, IVG oocytes showed inferior competences for fertilization and development to blastocyst, although their nuclear maturation was similar to *in vivo*-grown ones, indicating that the cytoplasmic maturation of IVG oocytes might be inadequate.

In conclusion, BMP-4 addition during IVG culture suppressed P_4 production from GCs and decreased the diameter of GC, suggesting its effect on steroidogenesis; importantly, it did not affect oocyte growth, nuclear maturation and fertilization. However, BMP-4 impaired embryonic development maybe due to insufficient cytoplasmic maturation and in higher concentration (50 ng/ml) even compromised OGC viability by suppressing GC proliferation.

Summary

Bone morphogenetic proteins (BMPs) play important roles during folliculogenesis. Theca cell (TC)-derived BMP-4 may be beneficial to *in vitro* growth culture of early antral follicle-derived oocyte-granulosa cell complexes (OGCs), which is lacking in TC-derived products. In the present study, BMP-4 (0 [control], 10 and 50 ng/ml) was added to growth culture medium. Growth, steroidogenesis and the subsequent developmental competence of OGCs derived from bovine early antral follicles (0.5-1 mm) were examined. At 4, 8 and 12 days of growth culture, P_4 production by granulosa cells (GCs) was suppressed by the addition of BMP-4 compared to the control ($P < 0.05$). At 12 days, both the OGC survivability and GC number in the 50 ng/ml BMP-4 treated group were lower than those of control (48.2 vs. 67.8%; 4.96×10^4 vs. 8.5×10^4 cells; $P < 0.05$, respectively), while no difference was found between 10 ng/ml and the control. The mean diameters of GCs in the BMP-4 treated groups were smaller than that of the control ($P < 0.05$). However, the GC viability, oocyte diameter, oocyte nuclear maturation rate and normal fertilization rate were similar in all of the experimental groups, regardless of the amount of BMP-4 addition ($P > 0.05$). BMP-4 treated *in vitro*-grown oocytes showed lower blastocyst rates than untreated ones ($P < 0.05$). In conclusion, BMP-4 addition during *in vitro* growth culture suppressed P_4 production and decreased the diameter of GCs, suggesting its effect on steroidogenesis; importantly, it did not affect oocyte growth, nuclear maturation and fertilization. However, BMP-4 impaired subsequent embryonic development, and in higher concentration (50 ng/ml) even compromised OGC viability by suppressing proliferation of GCs.

Chapter II

Effects of extension of the culture period for the *in vitro* growth of bovine oocytes in the presence of bone morphogenetic protein-4 on oocyte growth and subsequent developmental competence

Introduction

In Chapter I, I confirmed the anti-luteinization effect of BMP-4 on GCs co-cultured with bovine oocytes, and the oocytes showed a healthy appearance after a 12-day IVG culture with BMP-4. However, oocytes did not grow to a similar size to *in vivo*-grown oocytes, and their developmental competence was low. An adequate IVG culture duration of oocytes is a prerequisite for better subsequent developmental competence acquisition. A previous study reported that oocytes grew to a larger size after a 16-day IVG culture in the presence of fibroblast growth factor 7, which is another TC-derived growth factor (Cho *et al.* 2008). Development to blastocyst from oocytes cultured for 14 days followed IVF or parthenogenetic activation have also been reported in other groups (Hirao *et al.* 2012; Oi *et al.* 2015). Therefore, I speculate that maintaining the “health” of GCs using BMP-4 may allow oocytes to grow for a longer period (for 16 days) and reach 120 μm, a diameter indicating full developmental competence (Otoi *et al.* 1997). The present study was undertaken to investigate the effects of an extension in the IVG culture period in the presence of TC-derived BMP-4 on oocyte growth and their subsequent developmental competence.

Materials and Methods

Collection of OGCs and IVG culture

The OGCs were collected from bovine early antral follicles (0.5-1 mm in diameter) and submitted for IVG, as described in Chapter I. In order to evaluate the effects of the IVG culture duration and BMP-4 treatment on OGC viability, OGCs were cultured for 12 or 16 days with or without 10 ng/ml BMP-4 (HumanZyme). Half of the medium was replaced at 4, 8, and 12 days of culture. OGCs cultured for 12 days with BMP-4 served as an *in vitro*-grown control. OGC viability was assessed at the end of the IVG culture. Morphologically normal OGCs enclosed by several compact layers of GCs were considered viable (Fig. 2-1). In addition, the diameters of IVG oocytes were measured before and after the IVG culture as described in Chapter I. The diameter of artificially denuded oocytes following an IVM culture was considered to be the diameter after the IVG culture.

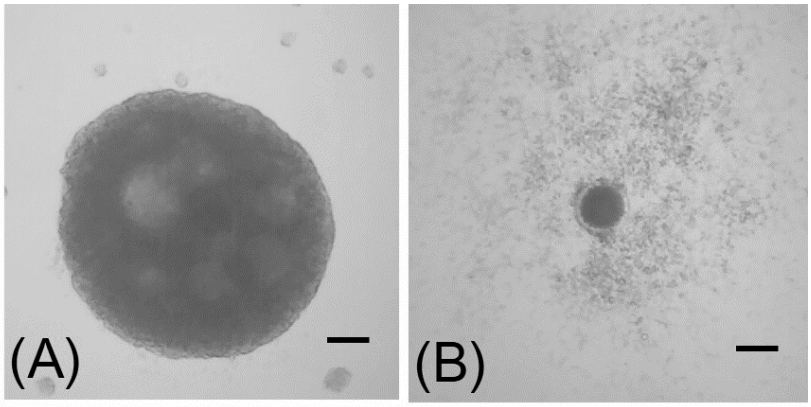

Fig. 2-1. Representative morphology of OGCs after 16 days of IVG culture.

OGCs assessed as viable and degenerated were shown in A and B, respectively. Scale bar = 100 μm.

IVM of IVG oocytes

After IVG, the IVM was conducted to evaluate nuclear maturation of IVG oocytes, as described in Chapter I. For evaluating the competence of nuclear maturation of oocytes immediately after IVG culture, I did not carry out a 10 h pre-IVM culture in this chapter, because the pre-IVM culture significantly improved nuclear maturation rate (approximately 90%) and the difference between BMP-4 treated groups became unclear, as reported in Chapter I.

IVF and IVC

Groups cultured for 12, 14 or 16 days with BMP-4 were used to evaluate *in vitro* developmental competence because 16-day IVG oocytes without the BMP-4 treatment showed low OGC viability. After the IVG culture, a 10-h pre-IVM culture 22-h IVM culture, followed by IVF and IVC, were performed as described in Chapter I. Cleavage and blastocyst rates were assessed after 2 and 6 days of culture, respectively. Cell numbers in blastocysts, as a parameter of embryo quality, were counted using an air-drying method (Takahashi and First 1992).

In vivo-grown oocytes collected from antral follicles (2-8 mm in diameter) were submitted to IVM, IVF, and subsequent culture as an *in vivo* control. In IVM, they were cultured in droplets (approximately 10 COCs/50 μl) covered with paraffin oil (Nagano *et al.* 2013).

Statistical analysis

Multiple sets of data were compared by one-way analysis of variance with Tukey-Kramer's HSD test. Two sets of data were compared with the Student's *t*-test. Statistical analyses were performed using JMP software version 12.0.1 (SAS Institute).

Results

OGC viability

As shown in Fig. 2-2, OGC viabilities were around 85% in 12 days of IVG culture in the presence or absence of BMP-4. When the duration was extended from 12 to 16 days, OGCs showed similar viability in the presence of BMP-4 (83.2%, $P > 0.05$) to that of oocytes cultured for 12 days with BMP-4 (83.0%). However, in the absence of BMP-4 (58.9%, $P < 0.05$) in the 16-day culture, viability was lower than those in the absence of BMP-4 for 12 days and in the presence of BMP-4 for 12 and 16 days.

Oocyte diameter and nuclear maturation

After the 16-day IVG culture, oocytes derived from the BMP-4 treatment had a larger diameter than that cultured for 12 days with BMP-4 ($P < 0.05$), although the diameters of oocytes cultured for 12 and 16 days without the BMP-4 treatment were similar (Table 2-1). No significant differences were observed in oocyte diameter or nuclear maturation between 16-day IVG oocytes with and without the BMP-4 treatment ($P > 0.05$).

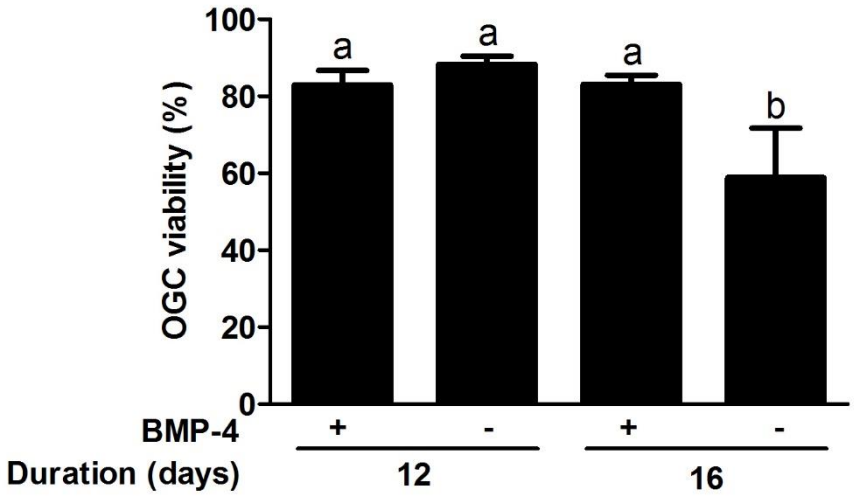

Fig. 2-2. Effects of the IVG culture duration and BMP-4 treatment on OGC viability.

OGC collected from early antral follicles (0.5-1 mm in diameter) were cultured for a normal culture duration (12 days) with BMP-4 or for a prolonged duration (16 days) with or without BMP-4 (10 ng/ml). OGC viability was assessed at the end of the IVG culture (see Fig. 2-1).

Data are expressed as mean values (*n* = 4) and error bars mean SEM.

[ab] Different letters indicate significant differences (P < 0.05).

Table 2-1. Effect of the IVG culture duration and BMP-4 treatment on oocyte diameter and nuclear maturation

IVG culture duration (days)	BMP-4 treatment	No. of oocytes (replicates)	Oocyte diameter (μm)		% nuclear maturation†
			Before IVG	After IVM	
12	+	76 (4)	97.5 ± 5.5	111.7 ± 5.7[b]	67.1 ± 11.7
	-	73 (3)	97.7 ± 3.1	113.2 ± 4.1[ab]	47.7 ± 13.5
16	+	69 (4)	98.6 ± 3.9	114.6 ± 3.5[a]	67.7 ± 15.3
	-	48 (4)	97.8 ± 3..9	113.9 ± 3.4[a]	50.7 ± 11.2

[ab] Values with different superscripts in the same column are significantly different (P < 0.05).

† Oocytes that develop to metaphase II after IVM culture.

Data are presented as mean ± SD.

Subsequent developmental competence

As shown in Table 2-2, 16-day IVG oocytes with the BMP-4 treatment had significantly lower cleavage rate (13.8%) than those of 12- and 14-day IVG oocytes (50.3 and 36.0%, respectively; $P < 0.05$). Blastocyst rates of 14- and 16-day IVG oocytes (1.8 and 0%, respectively) were also lower than that of 12-day IVG oocytes with the BMP-4 treatment (9.0%; $P < 0.05$). All IVG oocytes had lower cleavage rates, blastocyst rates, and cell numbers in blastocysts than those of the *in vivo* control ($P < 0.05$).

Table 2-2. Effect of the IVG culture duration on the developmental competence of BMP-4 treated-oocytes after IVF

Oocyte	IVG culture duration(days)	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts/ oocytes	Cell no. in blastocysts (n)
In vitro-grown	12	82 (3)	50.3 ± 13.2^{b}	9.0 ± 3.7^{b}	78.3 ± 38.7^{b} (7)
	14	97 (4)	36.0 ± 12.5^{b}	1.8 ± 3.5^{c}	70.0 ± 29.7^{b} (2)
	16	80 (4)	13.8 ± 7.5^{c}	0^{c}	-
In vivo-grown†	-	82 (4)	87.5 ± 5.4^{a}	36.4 ± 2.2^{a}	145.3 ± 61.5^{a} (30)

† Oocytes collected from antral follicles (2-8 mm in diameter) served as the *in vivo*-grown control.

^{abc} Values with different superscripts in the same column are significantly different ($P < 0.05$).

Data are presented as mean ± SD.

Discussion

In the present study, an extension of the culture period from 12 to 16 days for the IVG of OGCs in medium including 10 ng/ml BMP-4 maintained their viability and enhanced oocyte growth. One possible reason for the positive effects of BMP-4 on oocyte growth *in vitro* may be the anti-luteinization function of BMP-4, which has been confirmed in dispersed GCs (Glister *et al.* 2004; Yamashita *et al.* 2011) and GCs enclosed in OGC in the previous chapter. Since GCs from atretic bovine follicles were reportedly more luteinized than cells from “healthy” follicles in culture (Henderson *et al.* 1987), the BMP-4 treatment may contribute to maintain OGC with “healthy” GCs, resulting in high OGC survival. Another possible reason for improved OGC survival may be the anti-apoptotic effects of BMP-4. BMP-4 has been shown to reduce GC apoptosis by suppressing the release of caspase-activated DNase via Survivin, a member of the inhibitor of apoptosis family (Kayamori *et al.* 2009).

Bovine early antral follicles (0.5-1 mm) take 8-10 days to grow to antral follicles (8 mm) (Adams *et al.* 2008); however, bovine oocytes appear to grow slower *in vitro*. Previous studies comparing 10, 12, and 14-day IVG cultures found that 10 days was inadequate for oocyte growth and nuclear maturation, while 14 days impaired oocyte viability and subsequent developmental competence (Huang *et al.* 2013; Huang *et al.* 2014). Therefore, under culture conditions without BMP-4, I concluded that the optimal IVG duration for bovine oocytes derived from early antral follicles was 12 days based on previous findings (Huang *et al.* 2013; Huang *et al.* 2014). In mice, oocyte diameter was found to increase linearly with extensions in the length of the culture duration for oocytes (Hirao and Miyano 2008). Furthermore, in cattle, a 16-day IVG culture achieved a diameter of approximately 120 μm (Cho *et al.* 2008), which indicated that oocytes had full developmental competence (Otoi *et al.* 1997). Thus, by maintaining high OGC viability, a longer

culture duration may result in better oocyte growth and the subsequent acquisition of competence. As expected, the 16-day IVG culture with BMP-4 maintained OGC survival and resulted in further oocyte growth; however, the size of oocytes did not achieve that of *in vivo*-grown oocytes, which averaged 120 μm in diameter as described in Chapter I. In addition, oocyte nuclear maturation was not improved and developmental competence was impaired as culture duration has been extended to 14 or 16 days. Oocytes have been suggested to suffer aging problems if they are cultured for longer than necessary (Hirao and Miyano 2008). Although 16-day IVG oocytes under the present IVG system showed similar nuclear maturation competence, the developmental competence of embryos derived from 16-day IVG were inferior to those from 12-day IVG, suggesting that oocyte aging is closely associated with impaired cytoplasmic maturation. During 12 to 16 days of IVG culture, oocytes grew at a much slower rate (0.2-0.7 μm/day) than that (approximately 1.2 μm/day) in the initial 12 days. The retarded oocyte growth may be a sign of aging problems. Cho *et al.* (2008) showed an increase in oocyte diameters after a 16-day IVG culture; however, they did not report the viability of OGCs or their developmental competence. These results suggest that extending the culture period to more than 12 days is not an appropriate strategy to promote oocyte growth in cattle.

In the present study, the developmental rate to blastocysts (9.0%) of oocytes derived from 12-day IVG culture without the BMP-4 treatment was higher than that (1.3%) in Chapter I. In the present study, I utilized different bull semen from that used in the previous chapter. I speculate that the difference stems from the difference of intrinsic developmental competence of sperm as described previously (Palma and Sinowatz 2004).

In conclusion, the present study showed that a 16-day IVG culture in the presence of BMP-4 maintained oocyte survival and promoted oocyte growth. However, it did not improve the competence of the nuclear maturation of oocytes, and impaired their developmental competence;

also 14-day IVG with BMP-4 impaired the developmental competence. An extension in the culture duration is not a good strategy to promote oocyte growth, presumably due to cytoplasm-associated aging problems in oocytes.

Summary

Bone morphogenetic protein-4 (BMP-4) inhibits luteinization of granulosa cells (GCs) during *in vitro* growth (IVG) culture of bovine oocytes; however, oocytes derived from a 12-day IVG were less competent for development than *in vivo*-grown oocytes. I herein investigated whether an extended IVG culture with BMP-4 improves oocyte growth and development to blastocysts after *in vitro* fertilization. OGCs were cultured for 14 or 16 days with BMP-4 (10 ng/ml), while a 12-day culture with BMP-4 served as the *in vitro* control. OGC viability was maintained for the 16-day culture with BMP-4 (83.2%), but was significantly lower without BMP-4 (58.9%) than the control (83.0%). Prolong-cultured oocytes at 16 days had statistically greater diameter (114.6 μm) than the control (111.7 μm). IVG oocytes with BMP-4 for the 16-day culture had a similar nuclear maturation rate to the control (approximately 67%); however, blastocyst rates in BMP-4 treated oocytes of 14- (1.8%) and 16-day (0%) IVG were statistically lower than that of 12-day IVG (9.0%). In conclusion, BMP-4 maintained OGC viability and promoted oocyte growth in a prolonged culture, but impaired the developmental competence of oocytes. Prolonged culture may not be an appropriate strategy for enhancing the developmental competence of IVG oocytes.

Chapter III

The steroidogenesis and oocyte growth in the *in vitro* growth culture of bovine oocytes co-cultured with theca cells, and the subsequent development of resultant oocytes

Introduction

In the previous two chapters, the supplementation of TC-derived BMP-4 into culture medium suppressed luteinization of GCs and maintained oocyte survivability in prolonged IVG culture; however, the developmental competence of resultant oocytes was not improved. These results lead to the assumption that introduction of TC into IVG culture system may provide more comprehensive and balanced TC-derived factors for oocyte growth and subsequent development. A previous attempt to culture bovine whole follicles including all follicular cells in serum-free medium resulted in oocyte grow and nuclear maturation after subsequent maturation culture, suggesting the supportive roles of TCs in oocyte growth (Senbon and Miyano 2002). However, whole follicle culture made direct observation of oocyte growth difficult and the reported nuclear maturation rate (23%) after culture for IVM was low (Senbon and Miyano 2002). In contrast to the previous “closed” culture system, an observable co-culture system including OGC and isolated TCs may be an alternative. However, to the best of our knowledge, such kind of co-culture system has not been reported previously in cattle. Steroid hormones such as estrogen and androgen were essential to oocyte growth *in vitro* (Makita and Miyano 2014); however, whether the co-cultured cells could produce steroid hormones by themselves as *in vivo* was still unclear. Therefore, this study was conducted to investigate whether a co-culture system including OGC

and TCs in steroid hormones free medium could produce steroid hormones properly, support oocyte and TC growth, and result in subsequent oocyte development.

Materials and Methods

Collections of OGCs and TCs

The OGCs were collected as described in Chapters I and II. In the present chapter, TCs were collected from antral follicles (1.5-3 mm in diameter) of bovine ovaries obtained from a slaughterhouse and isolated according to the methods previously reported with modifications (Bendell *et al.* 1988; Meidan *et al.* 1990; Spicer *et al.* 2008). Briefly, antral follicles were cut into halves, and then GCs and oocyte were removed using fine tip-curved forceps. The theca interna layer was collected by gentle peeling it away from the theca externa with fine forceps and incubated for 10 min in D-PBS (-) supplemented with 0.25% trypsin and 0.02% EDTA at 37℃ followed by vortexing for 1 min to dissociate any remaining GCs. The theca interna layers were torn into small pieces and were digested, aided with several times of pipetting, for 40 min at 37°C in isolation medium containing 2 mg/ml collagenase, 1 mg/ml hyaluronidase and 30 IU/ml DNase in a dish. The dispersed cells were washed 2 times in isolation medium by centrifugation at 700 × *g* for 6 min and then resuspended in 0.5 ml growth medium. The cells were then tested for viability by using trypan blue exclusion method. TC viability isolated by this method was above 90%.

IVG of oocytes

Isolated TCs were counted and diluted to 2.0×10^5 cells/ml in growth medium. OGCs were individually cultured in the each well of 96-well culture plates (Primaria 353872, Corning) containing 200 µl of TC-containing growth medium (4.0×10^4 cells/well) at 39°C in humidified air with 5% CO_2. The growth medium was HEPES-buffered TCM199 supplemented with 0.91 mM sodium pyruvate, 5% FCS, 4 mM hypoxanthine, 4% polyvinylpyrrolidone and 50 µg/ml gentamicin

sulfate. In 12-day IVG culture, half of the medium was replaced every 4 days (4 replicates for every 4 days). For detailed investigation of A_4 production in TC only culture, culture medium was collected at days 1, 2, 3 and 4 (3 replicates for each day). Replaced/collected medium was stored at -80°C until hormone assay. Oocytes grown in growth medium lacking TCs but with A_4 (10 ng/ml) supplementation were served as positive control; oocytes grown without TCs or A_4 were served as negative control. TC only culture was also set for comparison purpose. Morphologically normal OGCs at the end of IVG culture, which were enclosed by several compact layers of GC, were considered viable. Survivability of OGC were compared between co-cultured oocytes and controls. Cell number was counted in co-culture, A_4-supplemented culture and TC only culture.

Hormone assay

The replaced medium collected at 4, 8 and 12 days of IVG culture was frozen at -80°C until P_4, A_4, testosterone (T) and E_2 assays using competitive double-antibody enzyme immunoassay, as previously described (Yanagawa *et al.* 2015). Properly diluted IVG media were loaded directly as a sample to the well of a microplate. The primary antisera used for assay of P_4, A_4, T and E_2 were anti-progesterone-3-CMO-BSA (KZ-HS-P13, Cosmo Bio), anti-androstenedione-3-CMO-BSA (FKA138-E, Cosmo Bio), anti-testosterone-3-CMO-BSA (FKA102, Cosmo Bio) and anti-estradiol-17β-6-CMP-BSA (FKA204, Cosmo Bio), respectively. Goat anti-rabbit serum (111-005-003, Jackson Immuno Research Laboratories) was used as the secondary antibody. The intra- and inter-assay coefficients of variations were 6.5% and 7.7% for P_4, 8.1% and 7.7% for A_4, 7.4% and 3.6% for T, 9.9% and 3.2% for E_2, respectively. The assays were repeated for three or four times. Steroid hormone production during each period (days 0 to 4, days 4 to 8 and days 8 to 12) was calculated using the following formula:

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

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

A_4 production during the first 4 days (days 0 to 1, days 1 to 2, days 2 to 3 and days 3 to 4) was calculated using the following formula:

Steroid hormone production (ng/well) = 0.2 (ml) × Concentration at the end of the period (ng/ml) − 0.2 (ml) × Concentration at the start of the period (ng/ml).

Quantitative PCR

RT-qPCR was used to examine the purity of isolated TCs and their expression level of *CYP17A1*, a gene encoding a key enzyme in the pathway of biosynthesis of A_4. During IVG culture, TCs were collected at 0, 12 and 24 h after cell culture. Cell lysates was made and then cDNA was synthesized directly using SuperPrep Cell Lysis & RT kit for qPCR (TOYOBO, Osaka, Japan) in a thermal cycler (T-Gradient Thermoblock, Biometra, Germany). Quantitative PCR was performed using KAPA SYBR FAST qPCR kit Master Mix Universal (KAPA BIO, MA, USA) in Eco Real-Time PCR System (Illumina, CA, USA) as following the manufacturer's instruction. Primer sequences were *CYP17A1*: ACCATCAGAGAAGTGCTCCGAA (forward) and CCACAACGTCTGTGCCTTTGT (reverse); *GAPDH*: TTCAACGGCACAGTCAAGG and ACATACTCAGCACCAGCATCAC (reverse). The transcript levels were calculated relative to the transcription of the internal control GAPDH.

The purity of TCs purity was determined by comparing the expression levels of TC-specific *CYP17A1* and GC-specific FSH receptor (*FSHR*) in both freshly isolated TCs and GCs, as described previously (Spicer *et al.* 2008). GCs were collected from the same antral follicles used for TC isolation. The purity of TCs used in the present study was 97.9-99.6%. The primer sequences were *FSHR*: AACCTGCTATACATCGACCCTGAT (forward) and GCTTAATACCTGTGTTGGATATTAACAGA (reverse).

IVM

After IVG, oocytes were submitted to IVM culture without pre-IVM in micro-well plates (MiniTrays 163118; NUNC), as described in Chapter II. The *in vivo*-grown oocytes, serving as *in vivo* control, were collected and submitted to the IVM procedure as described in Chapter I and II.

Measurement of oocyte diameter and examination of nuclear maturation

After IVM, parts of oocytes were denuded for measurement of oocyte diameter and examination of nuclear maturation, as described in the previous chapters. The diameters of IVG oocytes were also measured before IVG culture. Oocytes proceed to M II stage were defined as matured oocytes.

IVF and IVC

IVF and IVC was performed as described in Chapter I, and approximately 20 cumulus-free presumptive zygotes were cultured in a 30-μl droplet for IVC. The developmental competence of oocytes grown *in vitro* with TC co-cultured or A_4 supplementation, and *in vivo*-grown oocytes were compared. Cleavage rate, blastocyst rates and cell numbers in blastocysts were determined as described in Chapter I.

Statistical analysis

All statistical analyses were performed using JMP software version 12.0.1 (SAS Institute). The oocyte nuclear maturation, cleavage rate and blastocyst rate were compared by a chi-square test. Other multiple sets of data were compared by one-way ANOVA followed by Tukey-Kramer's HSD test.

Results

TC and OGC growth in co-culture

The dynamics of TC and GC cell numbers in co-culture and A_4-supplemented culture were shown in Fig. 3-1. TC number in co-culture decreased to about 68% (2.7×10^4 cells/well) of the initial seeding number (4.0×10^4 cells/well) during the first 4 days and increased gradually to about 88% (3.5×10^4 cells/well) of the seeding number at the end of culture. In addition, TC only culture had a similar TC proliferation pattern (data not shown). GC number in co-culture was increasing from around 0.3×10^4 cells/well at day 0 to around 1.7×10^4 cells/well at day 8, and decreased slightly to 1.5×10^4 cells/well at day 12 of culture. GC number in A_4-supplemented IVG culture started to increase from day 4 to around 1.9×10^4 cells/well at day 12 of culture. Majority of OGCs in both co-culture (82.7%) and A_4-supplemented cultures (83.1%) survived 12-day IVG culture, while only a few (6.2%) survived in culture without androgen source ($P < 0.05$) (Fig. 3-2).

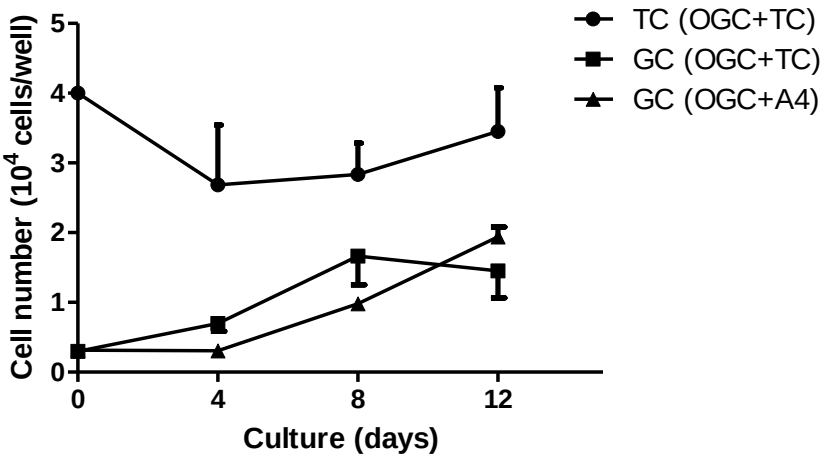

Fig. 3-1. Numbers of TCs and GCs in co-culture (3 replicates) and GCs in A_4-supplementd culture (4 replicates) during IVG.

Data was expressed as the mean value and error bars mean SEM.

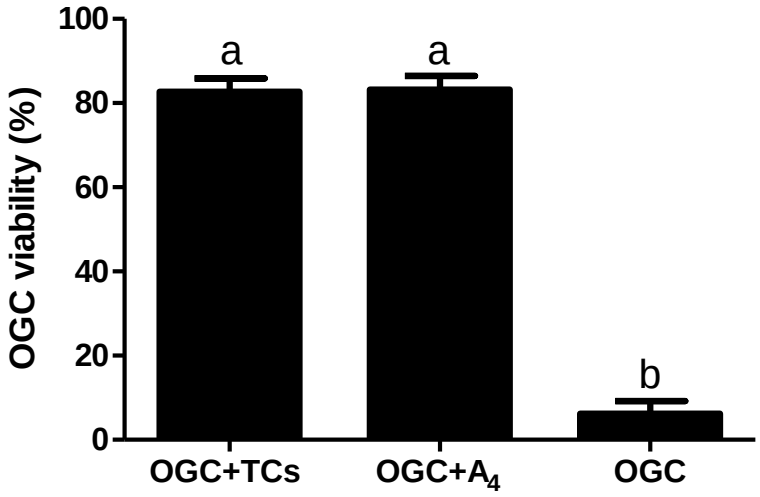

Fig. 3-2. OGC viability in co-culture with TCs (8 replicates), A_4-supplementd culture (4 replicates) and OGC only culture (7 replicates) after IVG.

Different letters (a, b) indicate significant differences ($P < 0.05$).

Data are expressed as the mean values and error bars mean SEM.

Table 3-1. Effect of co-culture on diameter and nuclear maturation of IVG oocytes

No. of oocytes (replicates)	Cultured with	Oocyte diameter (μm)		% of nuclear status			
		Before IVG	After IVM	GV	M I	A I/T I	M II
48 (4)	TC	97.2 ± 2.7	112.4 ± 4.1	0	29.2	12.5	58.3
49 (4)	A_4	97.4 ± 4.2	112.2 ± 4.3	4.1	38.8	0	57.1

GV, germinal vesicle; M I, metaphase I; A I/T I, anaphase I/telophase I; M II, metaphase II.

Data are presented as mean ± SD.

The mean diameter of co-cultured oocytes before IVG (97.2 μm) increased to 112.4 μm after IVM, and these diameters were similar to those of A_4-supplemented IVG oocytes (Table 3-1).

Steroidogenesis and expression of CYP17A1

The steroidogenesis in co-culture, A_4-supplemented culture during IVG and in TC only culture was shown in Fig. 3-3. E_2 production was maintained until 8 days of culture and then decreased in co-culture, while it was increasing in A_4-supplemented culture (Fig. 3-3A). E_2 production was higher in co-culture in the first 4 days, but lower at 4-8 and 8-12 days of culture than those in A_4-supplemented culture ($P < 0.05$). Few production of E_2 was observed in TC culture. P_4 production was increasing in both co-culture and A_4-supplemented culture, but was decreasing in TC culture (Fig. 3-3B). Production of P_4 by co-cultured cells, including both GCs and TCs, were more than that by GCs in A_4-supplemented culture throughout 12-day culture or that by TCs in TC only culture from 4 days of culture ($P < 0.05$). E_2/P_4 ratio was maintained until 8 days of culture then declined in co-culture, while it was increasing continuously in A_4-supplemented culture with more productions than those of co-culture ($P < 0.05$; Fig. 3-3C). In TC only culture, E_2/P_4 ratio is minimal. Co-cultured TCs had similar A_4 and T production patterns with TCs in TC only culture (Fig. 3-3D and E). A_4 production was only observed in first 4 days and T production was decreasing in both cultures. Further detailed investigation showed that A_4 production was mainly observed in first day after TC culture (Fig. 3-3F) and the expression of *CYP17A1* decreased markedly in 12 h after culture (Fig. 3-4).

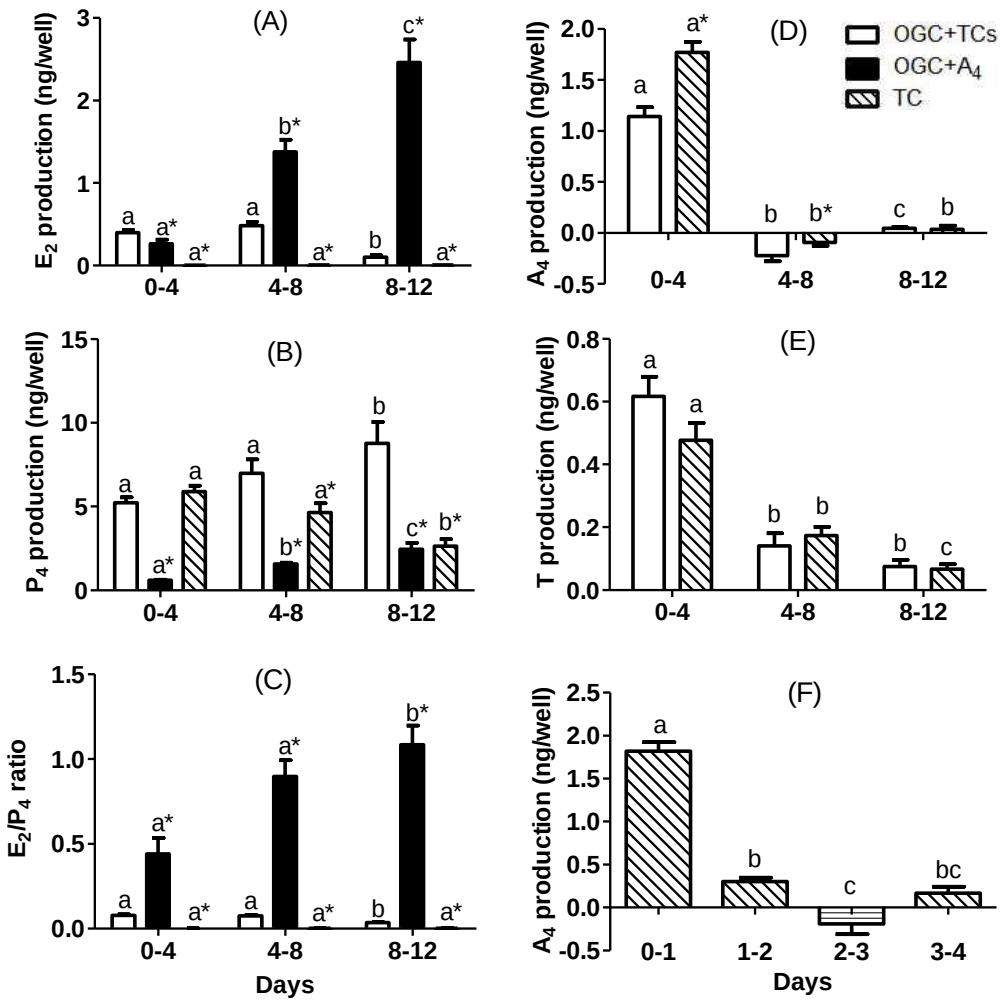

Fig. 3-3. Steroidogenesis in co-culture, A_4-supplemented culture and TC only culture during culture. E_2 production by GCs, P_4 production by both TCs and GCs, and E_2/P_4 ratio in co-culture, A_4-supplementd culture and TC only culture (A-C); A_4 and T productions by TCs in co-culture and TC only culture (D and E, respectively) (n=20). A_4 production in TC only culture during the first 4-day culture (F) (n=9).

[abc] Different letters indicate significant differences within same culture at different times ($P < 0.05$).

Asterisk indicates significant difference from co-culture at the same culture periods ($P < 0.05$).

Data are expressed as the mean values and error bars mean SEM.

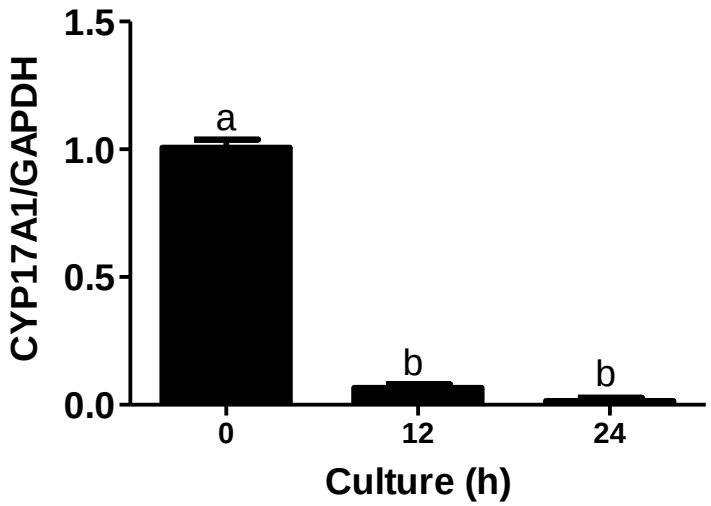

Fig. 3-4. Expression of *CYP17A1* in TCs in the first 24 h after culture.

[ab] Different letters indicate significant differences within same culture at different times ($P < 0.05$).

Data are expressed as the mean values (n=3) and error bars mean SEM.

Nuclear maturation and developmental competence of IVG oocytes

As shown in Table 3-1, the nuclear maturation rate (58.3%) of co-cultured oocytes was similar to that of oocytes derived from A_4-supplemented culture (57.1%). The subsequent development to blastocyst after IVF was shown in Table 3-2. IVG oocytes, regardless of culture methods, had lower cleavage rates (about 57%) and lower blastocyst rates per IVF oocytes (about 15%), in comparison to those (81.3% and 33.8%, respectively) for *in vivo*-grown oocytes ($P < 0.05$). However, blastocyst rates per cleaved oocytes did not significantly differ between *in vitro*- and *in vivo*-grown oocytes. There was no difference in mean cell numbers per blastocyst derived from two different IVG cultures ($P > 0.05$). The cell number in blastocysts derived from co-cultured IVG was similar to that from *in vivo*-grown oocytes ($P > 0.05$), while A_4-supplemented IVG oocytes resulted in a less cell number ($P < 0.05$).

Table 3-2. Effect of co-culture on the developmental competence of IVG oocytes after IVF

Oocyte	Cultured with	No. of oocyte (replicates)	% of cleaved oocytes	% of blastocyst based on		Cell no. in blastocysts (n)
				IVF oocytes	Cleaved oocytes	
In vitro-grown	TC	75 (4)	58.7[a]	17.3[a]	29.5	95.3 ± 31.8[ab] (13)
	A_4	81 (4)	56.8[a]	14.8[a]	26.1	85.1 ± 37.9[a] (12)
In vivo-grown[†]	-	80 (4)	81.3[b]	33.8[b]	41.5	133.3 ± 61.6[b] (27)

[†] Oocytes collected from antral follicles (2-8 mm in diameter) served as the *in vivo*-grown control.

[ab] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Data are presented as mean ± SD.

Discussion

In the present study, I have showed that the introduction of TCs into IVG culture without supplementation of steroid hormones resulted in oocyte growth, and subsequent oocyte maturation and development to blastocyst. Furthermore, the OGC viability and subsequent developmental competence of oocytes derived from co-culture with TCs were similar to or even superior than those of oocytes derived from conventional IVG with steroid hormone supplementation.

The TC isolation protocol used in this study has been proved efficient to isolate TCs with low GC contamination (Spicer *et al.* 2008). The purity of TC isolated in the present study were above 97%. In addition, the minimal E_2 production in TC culture has further demonstrate the efficiency of current isolation protocol. The observation of declined TC number after culture is consistent to previous studies (Stewart *et al.* 1995; Yada *et al.* 1999), but different from several other studies (Meidan *et al.* 1990; Roberts and Skinner 1990). This discrepancy may be due to different levels of cell injury caused by isolation protocols and different cell planting efficiency affected by culture medium and culture plate. In the present study, TC number was similar between co-culture and TC culture, and GC number was also similar between co-culture and conventional culture. Based on these results, under current co-culture conditions, co-culture did not affect GC or TC proliferations.

The present study showed the indispensable role of A_4 in maintaining oocyte survival. Without androgen produced by TCs or supplied exogenously, most OGCs were denuded from granulosa cells after 12-day IVG culture. It has been proved that androgens support OGC structure mainly through E_2 aromatized from A_4 by GCs, as a non-aromatizable androgen, dihydrotestosterone, could not support OGC structure (Makita and Miyano 2015). Furthermore, additional androgens

also promote oocyte growth and their acquisition of meiotic competence via androgen receptor during IVG culture with E_2 (Makita and Miyano 2015). The production of A_4 by TCs and the aromatization from A_4 to E_2 by GCs was confirmed in the co-culture, although their concentrations were lower than those in A_4-supplemented culture. Besides, a lower E_2/P_4 ratio in co-culture than A_4-supplemented culture was observed. It has been reported that atretic bovine follicles during follicular stages had a lower E_2/P_4 ratio in follicular fluid than that in non-atretic counterparts (Kruip and Dieleman 1985). In the case of co-culture, therefore, the author speculates that some other TC-derived factors apart from androgens may maintain the high survivability of OGCs. The TC-derived BMPs (for example BMP-4/7) may regulate oocyte and GC functions, as a range of BMP-responsive type-I (BMPR1B, ActRI) and type-II (BMPRII, ActRII, ActRIIB) receptors were found in oocyte and GCs (Glister *et al.* 2005). Further study will focus on the profiles of growth factor excretions from co-cultured TCs.

One major problem in this co-culture system was the immediate loss of androgen-producing ability of TCs. The A_4 was mainly produced in the first day of culture, then decreased markedly to minimal level afterwards. By contrary, P_4 production by TC was observed throughout culture. Although it is difficult to determine the proportions of steroids secreted by TCs *in vivo*, it is estimated that in freshly-isolated non-luteinized TCs, 97% of steroids secreted are androgens and only 1% is P_4 (McNatty *et al.* 1984). The A_4 could be synthesized from P_4 or pregnenolone, the precursor of P_4, with both reactions being catalyzed by *CYP17A1*-encoded P450c17 enzyme (Brock and Waterman 1999). The abundant production of P_4 indicated that there were enough precursors of A_4. Therefore, the sharply decreased *CYP17A1* expression is responsible to the decrease of A_4 production. However, the exact reasons for decreased *CYP17A1* expression is unknown, the regulation of *CYP17A1* expression is complex, involving many factors such as luteinizing hormone, insulin, inhibin, BMP-4//6/7, androgen and E_2 (Young and McNeilly 2010).

The decrease in androgen production and *CYP17A1* expression were signs of luteinization for TCs (Murphy 2000). Apart from steroid productions, the shift of TC phenotype from follicular phenotype to luteinized phenotype also alters its expression pattern of BMPs (Erickson and Shimasaki 2003). I previously expected theca-derived factors apart from androgens could improve oocyte growth *in vitro*. However, the short life span of TCs of follicular phenotype may compromise the contributions of TCs to oocyte growth. Although it is not well clarified, serum that is widely supplemented in culture medium has been believed as a stimulator of spontaneous luteinization of TCs (Engelhardt *et al.* 1991; Gilling-Smith *et al.* 1994). A serum-free culture system for sheep TCs that overcomes the problem of spontaneous luteinization has been reported previously, in which TCs were treated with insulin-like growth factor I and luteinizing hormone (Campbell *et al.* 1998). In future study, it should be examined whether serum-free culture system is applicable in growth culture of oocyte co-cultured with TCs.

In conclusion, the present study demonstrated that bovine oocytes co-cultured with TCs in the absence of steroid hormones could grow, mature and develop as those derived from the IVG culture supplemented with androgen. The androgen produced by co-cultured TCs contributed to oocyte growth and subsequent development; however, TCs lost their function immediately after culture. In further study, an IVG system maintaining TC function should be developed.

Summary

In this chapter, a preliminary steroid hormone-free co-culture system including oocyte-granulosa complex (OGC) and TCs, were examined for TC effects on oocyte growth. OGCs were co-cultured with antral follicle-derived TCs. Approximately 83% OGCs survived 12-day IVG culture with obvious GC proliferation. The co-cultured oocytes increased their sizes to around 112.4 μm after IVG culture, and 58.3% of them could mature *in vitro*. After IVF, around 17.3% of IVG oocytes developed to blastocyst. The growth and developmental competence of co-cultured oocytes was comparable to that of A_4-supplemented IVG culture. A_4 production by TCs and E_2 aromatized from androgen by GCs were confirmed in the co-culture. However, A_4 production could only be observed in the first day of co-culture, accompanied by a dramatic decrease in *CYP17A1* expression in TCs. Without androgen source, most OGCs failed to survive IVG culture. In conclusion, oocytes co-cultured with TCs could grow, mature and develop subsequently. TC-derived androgen may play great roles in maintaining oocyte growth; however TCs could only producing androgen for a short time. The co-culture system with OGC and TCs could be a potential *in vitro* study model for investigating oocyte growth and competence acquisition.

Summary and Conclusions

Most oocytes in ovaries are not able to grow to their full size because most of them are degenerated during follicular development. The IVG system was developed to utilize these valuable oocytes. However, in large animals such as cattle, the oocytes derived from current IVG system are less competent as *in vivo*-grown oocytes. A follicle is consisted of an oocyte, GCs and TCs. In current IVG system, only OGC are collected for culture, while TCs or TC-derived factors are absent. Luteinization of GC in IVG culture is not consistent to the *in vivo* environment of growing oocytes. BMP-4, one major product by TCs, could reportedly suppress luteinization of GC and prevent follicular atresia. In an attempt to improve IVG system and clarify the roles of TC-derived factors in oocyte growth, IVG culture added with BMP-4 was conducted to examine oocyte growth and subsequent development.

In Chapter I, the growth and development of IVG oocytes treated with BMP-4 were examined. The anti-luteinization effect of BMP-4 was confirmed as P_4 production and cell enlargement of GCs was suppressed in the presence of BMP-4. Oocyte nuclear maturation and fertilizability was not affected by BMP-4 treatment, however embryonic development was impaired. The underlying mechanism for the unexpected inconsistence between anti-luteinization function of BMP-4 and impaired developmental competence was not clear. The insufficient cytoplasmic maturation of oocyte may lead to compromised developmental competence.

In Chapter II, whether an extended IVG culture with BMP-4 improves oocyte growth and subsequent development was investigated. Oocytes derived from a 12-day IVG were less competent for development than *in vivo*-grown oocytes. The anti-luteinization/atresia functions exerted by BMP-4 may permit oocyte grow for a longer time without affecting oocyte survival, thus potentially contributing to more sufficient oocyte growth. OGCs were cultured for normal period of

12 days or an extended period of 16 days with BMP-4. Only in the presence of BMP-4, OGC viability was maintained and oocyte diameter gained significantly additional growth. Prolonged culture did not affect oocyte nuclear maturation; however, subsequent developmental competence in BMP-4-treated oocytes for 14 and 16 days was statistically lower than oocytes cultured for only 12 days. These results demonstrated that prolonged culture may not be an appropriate strategy for enhancing the developmental competence of IVG oocytes. The possible aging problem associated with impaired cytoplasmic maturation in oocytes grown in prolonged culture may cause the damages.

In Chapter III, a preliminary co-culture system including OGC and TCs was tested for the effects of TCs on oocyte growth. For the TC-derived BMP-4 alone failed to improve subsequent developmental competence of IVG oocytes, TCs were introduced into the IVG system in the hope to provide better supports for oocyte growth. OGCs were co-cultured with antral follicle-derived TCs without steroid hormone addition, and approximately 83% OGCs survived 12-day IVG culture with obvious GC proliferation. The co-cultured oocytes increased their diameters to around 112.4 μm after IVG culture, and 58.3% of them could mature *in vitro*. After IVF, around 17.3% of IVG oocytes developed to blastocyst. All above mentioned competences of co-cultured oocytes were comparable to those of conventional A_4-supplemented IVG culture. Androgen production by TCs and estrogen aromatized from androgen by GCs were confirmed in the co-culture. However, A_4 production could be observed only during the first day in co-culture, accompanied by a dramatic decrease in *CYP17A1* expression in TCs. Without androgen source, most OGCs degenerated. These results demonstrated that oocytes co-cultured with TCs could grow, mature and develop as oocytes grown in conventional IVG system. TC-derived androgen may play great roles in maintaining oocyte growth. However TCs could only producing androgen for a short time after

culture. The co-culture system with OGC and TCs could be a potential *in vitro* study model for investigating oocyte growth and competence acquisition.

In conclusion, the present studies demonstrated that TC-derived BMP-4 had anti-luteinization and anti-degeneration effects on IVG of bovine oocytes. However, these effects could not improve oocyte development. A co-culture system including oocyte, GCs and TCs supported oocyte growth and development. The co-culture system is a potential direction for improving IVG system.

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