A Dissertation
for the Degree of Doctor of Philosophy

Studies on the Development of Culture System for Primary Follicle-derived Embryonic Stem Cells in Mice

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February, 2013

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SUMMARY

This study examined the establishment of culture system for *in-vitro* culture of primary follicles and the difference of some cytosolic organelles in mature oocytes retrieved from different origins in mice. First, appropriate retrieval time of primary follicles was evaluated. The primary follicles retrieved from neonatal females at different ages (7 to 14-day-old) were cultured for obtaining mature oocytes, and assessment on the efficiency of follicle retrieval and mature oocyte generation was subsequently made by employing both cellular and biological parameters. The number of primary follicles was significantly increased from 9 up to 11-day-old mice. When cultured *in vitro*, primary follicles retrieved from 11-day-old mice showed best follicular development and the largest number of mature oocytes per euthanized animals. In conclusion, primary follicles could be cultured in vitro and 11-day-old mice were appropriate as primary follicle donors.

FSH is essential for follicular development such as viable oocyte recovery, oocyte maturation, granulosa cell proliferation, formation of antral-like cavities and cumulus cell expansion. To optimize culture condition for primary follicles, I attempted to decide proper combination of FSH concentration. Morphology of primary follicles, follicular development, oocyte maturation, steroid hormone production and receptor expression of granulosa cells were determined. The best

follicular development and blastocysts formation after IVF and parthenogenetic activation were observed in 200 mIU/ml FSH-treated group during the whole culture period. When FSH was added to culture media, normal follicular development, steroid hormone production, expression of hormone receptors were observed. In conclusion, I established optimal primary follicle-culture system for producing mature oocytes and viable homozygotic, heterozygotic blastocysts.

Embryonic stem cells (ESCs) are derived from inner cell mass (ICM) of blastocyst through *in vitro* culture on MMC-treated feeder layer under appropriate culture conditions. Because of the developmental potential to differentiate into any type of cells which consist of human body, ESCs have been getting spotlight as the clinical purpose such as stem cell therapy. Next, I established several ESC lines from homozygotic and heterozygotic blastocysts derived from primary follicles cultured under optimal condition. To confirm several characters of colony-forming cells as embryonic stem cells, I conducted conventional characterization methods such as ESCs-specific marker staining, expression of stemness-related genes and *in vitro* and *in vivo* differentiation. In conclusion, established cell lines have suitable chracteristics as stem cells and can be utilized to reproductive medicine and stem cell biotechnology.

Finally, I demonstrated that retrieval age and *in vitro* -culture condition may affect the quality of mature oocytes and

afterward developmental competency. In case of mature oocytes derived from early secondary follicles of 8-week-old mice could not develop to the blastocyst stage after parthenogenetic activation. By the analysis of ultrastructure through TEM, the differences in cytosolic organelles among mature oocytes from different origins were observed. These differences may affect the development of embryo and make the distinction among groups. In conclusion, combined stabilized culture protocol of primary follicles and cytoplasmic study will contribute to develop novel artificial reproductive technology and establish experimental model system for elucidating the mechanism of follicular development.

Keywords : Primary follicle, Follicle stimulating hormone (FSH), *In vitro* culture, MII oocyte, Blastocyst, Embryonic stem cells Transmission electron microscopy (TEM).

Student Number : 2006-21500

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LIST OF ABBREVIATIONS

AMH : anti-mullerian hormone

ART : assisted reproductive technology

BMP : bone morphogenic protein

COC : cumulus oocyte complex

CZB : Chatot, Ziomek, and Bavister

EGF : epidermal growth factor

ESC : embryonic stem cell

FBS : fetal bovine serum

FGF : fibroblast growth factor

FSH : follicle stimulating hormone

GDF : growth differentiation factor

GV : germinal vesicle

GVBD : germinal vesicle breakdown

hCG : human chorionic gonadotropin

ICM : inner cell mass

ICSI : intracytoplasmic sperm injection

IGF : insulin-like growth factor

ITS : insulin, transferrin and selenium

IVF : *in vitro* fertilization

KSOM : potassium simplex optimized medium

LH : luteinizing hormone

LHR : luteinizing hormone receptor

LIF : leukemia inhibitory factor

MESA : microsurgical epididymal sperm aspiration

MII : metaphase II

MMC : mitomycin C

PGC : primordial germ cell

PMSG : pregnant mare's serum gonadotrophin

ROSI : round spermatid injection

TEM : transmission electron microscopy

TESE : testicular sperm extraction

TGF : transforming growth factor

TNF : tumor necrosis factor

CHAPTER 1

GENERAL INTRODUCTION

Although numerous preantral follicles, i.e. primordial, primary, and secondary follicles exist in the mammalian ovaries, only less than 1% of them develop into the Graafian follicles that can ovulate mature oocytes (Moore *et al.*,1998). The majority of follicles in ovarian tissue finally enter the degenerating process called as atresia. If follicles doomed to degenerate can be cultured *in vitro*, it will be a good chance for increasing reproductivity and ultimately contribute to develop artificial reproductive technology.

The preantral follicles are classified as three groups; primary follicles (75−99 μm in diameter), early secondary follicles (100−125 μm in diameter) and late secondary follicles (126−150 μm in diameter) (Lee et al, 2007). Some researchers have made efforts to develop *in vitro*−culture systems for preantral follicles to obtain developmentally competent oocytes (Cortvrindt *et al.*, 1996;Lenie *et al.*, 2004). However, there are only limited studies about primary follicle culture. Only mature oocytes were produced from primary follicles by using two−step culture system (Lenie et al., 2004). Beside mouse, several research about primary follicle have been conducted in rat (Nilsson et al., 2001), bovine (Telfer *et al.*, 2000), porcine (Wu *et al.*, 2001) and human (Hovatta *et al.*, 1997).

Embryonic stem cells have ability that can differentiate into every cell types making up human body and can be established from the inner cell mass (ICM) of blastocysts *in*

vitro. Many researchers constantly have developed and yielded developmentally competent oocytes that can be a source for viable blastocysts and embryonic stem cells. Previous studies demonstrated that viable blastocysts and stem cells could be obtained through the *in vitro* culture of early secondary follicles in prepubertal mice (Lee *et al.*, 2007; Gong *et al.*, 2008; Lee *et al.*, 2008). Most researchers have used prepubertal mice for follicle donors because great number of preantral follicles are ceased or become inactivated in adult ovaries. However, the culture of preantral follicles from the adult ovaries can produce viable oocytes and generate embryonic stem cells through *in vitro* fertilization (IVF) (Kim *et al.*, 2009).

A previous study showed that the preantral follicles retrieved from the adult ovaries of mice can be cultured *in vitro* and yielded viable blastocysts only through IVF (Kim *et al.*, 2009). Parthenogenetic activation of mature oocytes from *in vitro*–culture of preantral follicles in adult ovaries could not develop to the blastocyst stage (Kim *et al.*, 2009). Thus, it is necessary to examine the differences between mature oocytes derived from *in vivo* and *in vitro* cultured follicles from a ultrastructure point of view.

Accordingly, in Chapter 3, for *in vitro*–culture of primary follicles, I conducted experiments to decide appropriate retrieval time first. In succession, in Chapter 4, I carried out experiments to determine optimal combination of FSH

concentration for primary follicle culture. Number of mature oocytes, blastocyst formation after IVF and parthenogenetic activation, and E_2 , P_4 production were observed. Moreover, in study undertaken in Chapter 5, from the homozygotic and heterozygotic embryos activated by parthenogenesis and IVF, I established 11 ESCs lines and confirm the specific features as stem cells. Finally, in Chapter 6, through the observation of ultrastructure and numerical analysis, the difference of some organelles in mature oocytes from different origins.

CHAPTER 2

LITERATURE REVIEW

1. Folliculogenesis

1) Development of follicles *in vivo*

The ovary has two major important roles as female reproductive organ: the generation of developmentally competent oocytes and secretion of several steroid hormones required for normal follicular development, fertilization and maintenance of the following pregnancy. Folliculogenesis is a essential process for producing fertilizable mature oocytes.

The start of female gametogenesis is formation of primordial germ cells. Primordial germ cells (PGCs), the embryonic precursors of gametes, arise during gastrulation in the posterior primitive streak and move to the genital ridge by amoeboid movements at E11.5 (Kathleen *et al.*, 2001). Primordial follicles are the earliest form of ovarian follicle and are made up of a primordial, or inactive, oocyte surrounded by a single layer of flattened somatic cells that are probably the progenitors of granulosa cells. There are thousands of primordial follicles present in the neonatal ovaries (Eppig and O'Brien., 1996). During the folliculogenesis only a few primordial follicles (less than 1%) present at the time of birth of an animal will ever proceed to ovulation (Erickson *et al.*, 1966). It means the majority of follicles (more than 99%) degenerated by atresia.

After formation of primordial follicles, some of the primordial oocytes begin to grow, although most spend month or years in the inactive state (Mohammad *et al.*, 2010). Activation of primordial oocytes induces the transformation of their surrounding flattened granulosa cells to a cuboidal shape (Lintern-Moore S, 1979). Also, theca cells and zona pellucida of oocytes are formed in this stage. The follicles at this stage are called primary follicles that containing growing oocytes (in prophase I stage). The granulosa cells gradually multiply and form multilayered secondary follicles. As follicles develop through the primary, secondary, and tertiary (antral) stages, they gain successive layers of granulosa cells and a fluid-filled cavity, called antrum within the granulosa cells.

Finally, follicles at the preovulatory state undergo exiting changes. As the nuclear membrane of oocyte break down, the nuclear elements release into the cytoplasm. At this stage, the oocyte chromosomes condense and complete the first meiotic division and are arrested at the metaphase II stage with the first polar body in the previtelline space. (Hyttel *et al.*, 1986; Hyttel *et al.*, 1989). After LH surge from pituitary, the cumulus-oocyte complex (COC) is ovulated and the remaining follicles undergo luteinization or atresia after being fertilized by the sperm.

2) Cellular interaction between oocyte and somatic cells

Follicle development is a very intricate and cooperative process requiring interaction between intrafollicular oocyte and surrounding somatic cells such as granulosa cells and theca cells. First, the oocyte induces breakdown of follicular cysts and assembly of primordial follicles through the expression of *Nalp* gene family downstream of *Figla* (Mario *et al.*, 2010). Endocrine effectors generated from the ovary may affect this process. IHNBN produced by granulosa cells increases the number of primordial follicles in the mouse ovaries (Bristol-Gould *et al.*, 2006).

As with follicle formation, oocyte derived transforming growth factor(TGF)-β family protein, including growth differentiation factor (GDF)-9 and bone morphogenetic protein (BMP)-15, are expressed in the primordial follicle and seem to play a part in follicle activation (Choi and Rajkovic, 2006). Besides the oocytes, the flattened granulosa cells of the follicles contribute to the activation of follicles. Complex interaction between KIT and KIT ligand (KITL-1 and KITL-2) exhibited by granulosa cells are fundamental for follicular activation and initial growth (Thomas and Vanderhyden, 2006). Also, another TGF-β family protein AMH secreted by granulosa cells has an important role as inhibitor of transition from primordial to primary follicles to hamper premature exhaustion of primordial follicles (Durlinger *et al.*, 2002). Like this, interactions between oocyte and somatic cells via several growth factors are important for initial follicular development.

Preantral folliculogenesis is gonadotrophin independent and should be driven by autocrine and paracrine factors or by other endocrine signals (Mario *et al.*, 2010). GDF-9 is possible candidate paracrine effector. If *GDF-9* is deleted, the proliferation of the somatic cells in primary follicles is hindered. On the other hand, AMH generated by granulosa cells inhibit granulosa cell proliferation (di Clemente *et al.*, 1994) by lessening the responsiveness of preantral follicles to FSH (Durlinger *et al.*, 2001). The expression level of AMH by proliferating follicles decide the acquisition of dominance. AMH may play a role in the selection mechanism. Also, several growth hormones affect the survival of preantral follicles because the number of follicles is diminished as the growth hormone gene is deleted by null mutation (Slot *et al.*, 2006). Likewise, the growth and differentiation of preantral follicles are under control of the cooperation between signals from oocyte and surrounding somatic cells.

With antrum formation, the oocyte gets the capacity to restart meiosis (Eppig and O'Brien, 1996). Then, oocyte have a role to play in antrum formation process. For instance, FGF8 mRNA is highly expressed in oocytes of small and large antral follicle of adult ovary (Valve *et al.*, 1997). FGF8 may have a regulatory effect on this process. Also, somatic cell factors are necessary for antrum formation. Mouse ovaries *IGF-1* knocked out have no follicles with antrum (Baker *et al.*, 1996). The addition of IFG-1 drive antrum formation in culture of bovine follicles under serum free conditions (Itoh *et al.*, 2002).

Moreover, mice with hypomorphic *Kitl* allele show infertility as a result of impediment in follicular development (Donovan *et al.*, 2001). As mentioned above, formation of antrum requires several factors from oocyte and surrounding somatic cells.

Branching off toward two functionally different type of granulosa cells, mural granulosa cells and cumulus cells, is striking phenomenon of transition toward preovulatory follicles. Oocyte stimulates granulosa cell proliferation by promoting the synthesis of DNA in mural granulosa cells via GDF9, TGFβ1 and activin-A expression. (Gilchrist *et al.*, 2006). Furthermore, oocyte and GDF9 cause differentiation of granulosa cells into the preovulatory phenotype (Latham *et al.*, 2004). Also, oocyte-derived factors, notably BMP15 and BMP6, prevent cumulus cell death through constructing a gradient of bone morphogenic proteins (Hussein *et al.*, 2005). Growth factors secreted from the granulosa cells and theca cells are also important in transition into later stage of folliculogenesis (Mario *et al.*, 2010). For instance, IGF-1 stimulates granulosa cell growth and steroid hormone production (progesterone and estradiol) by granulosa cells. Moreover, IGF-1 increase responsiveness of follicles to gonadotrophin and LHR in granulosa cells and theca cells (Silva *et al.*, 2009). In addition, knockout experiments show the important role of FGF2 and FGF7 at this point (Berisha *et al.*, 2004). Besides, vascular endothelial growth factor and TGF-β1 can be mentioned as influencing factors.

3) *In vitro* culture systems for preantral follicles

For several decades, many research groups have made an effort to improve *in vitro* culture systems for preantral follicles. It is worthy to develop culture systems for preantral follicles for various reasons such as conservation of endangered animals, improvement of livestock reproduction, a vast number of oocytes yield and assistant reproductive technology.

Preantral follicle culture has been started in rodents. The advantage of rodents as ovarian donor is that their ovaries are manipulable and contain a large number of follicles within a stromal matrix (Hartshorne *et al.*, 1997). Since Eppig group tried to culture several ovarian sources, namely isolated oocyte, oocyte-ovarian cell, and follicles (Eppig, 1977), various methods have been tried to optimize *in vitro* culture system. For example, through *in vitro* culture of ovaries retrieved from newborn mice, Eppig and O'Brien obtained preantral follicles with two layers of granulosa cells and metaphase II oocytes of which will subsequently develop blastocysts after insemination (Eppig and O'Brien, 1996). It was also identified that preantral follicles with 150 μm diameter could develop toward large antral follicles (400-500 μm diameter) with normal steroidogenesis through *in vitro* culture on a Millicell membrane (Nayudu and Orsborn, 1992). And preantral follicles have been cultured by placing granulosa-oocyte complex onto collagen

treated porous membranes or poly-L-lysine coated dishes (Eppig and Schroeder, 1989; Cain *et al.*, 1995). Boland and colleague obtained mature oocytes from *in vitro* culture of preantral follicles with 180 μm in diameter (Boland *et al.*, 1993). And Cortvrindt and collegues illustrated the pivotal role of FSH in stimulating the in-vitro growth of preantral follicles and inhibiting premature of oocytes by using drop culture method (Cortvrindt *et al.*, 1997). Because FSH is thought as essential factor for normal folliculogenesis, a large proportion of follicle culture media contain it. Recently, preantral follicles isolated from adult mice can be cultured successfully in vitro and mature oocytes derived from them have developmental capacity (Kim *et al.*, 2009). In addition, many research groups have assessed the effect of diverse growth factors such as IGF-1 (Demeestere *et al.*, 2004; Mao *et al.*, 2004; Walters *et al.*, 2006), EGF (Eppig and O'Brien, 1996; Zhou and Zhang, 2005), PVP (Hirao *et al.*, 2004; Cho *et al.*, 2008), Kit-ligand (Joyce *et al.*, 1999; Thomas *et al.*, 2008), GDF-9 (Vitt *et al.*, 2000), BMP-15(Shimasaki *et al.*, 2004), Activin , TNF-α (Chun and Hsueh, 1998), AMH (Durlinger *et al.*, 2001) to improve *in vitro* culture condition of ovarian follicles.

The preantral follicles are categorized as three groups; primary follicles (75-99 μm in diameter), early secondary follicles (100-125 μm in diameter) and late secondary follicles (126-150 μm in diameter) (Lee *et al.*, 2007). Until now, some studies for *in vitro* primary follicle culture have been conducted. Qvist and his colleague cultured small pieces of ovarian tissue

containing 1 or 2 primary follicles from 12-16-day-old mice and developed them into preovulatory stage (Qvist *et al.*, 1990). And Carroll group isolated primary follicles from 10-day-old mice and cultured them in a collagen gel matrix for 6 days (Carroll *et al.*, 1991). Recently, mature oocytes were produced from primary follicles by using two-step culture system (Lenie *et al.*, 2004). Besides mouse, several research about primary follicle have been conducted in rat (Nilsson, 2001), bovine (Telfer *et al.*, 2000), porcine (Wu *et al.*, 2001) and human (Hovatta *et al.*, 1997).

2. Parthenogenesis and in vitro fertilization

1) Parthenogenesis

Parthenogenesis is a striking form of reproductive strategy in several lower species of insects such as flies, ants, and honeybee and vertebrates such as fish, lizard, snakes and amphibians (Hipp and Atala, 2004). In this process, only female gamete develop by itself without paternal contribution. In case of mammals, parthenogenesis is not observed as normal reproductive process if extrinsic signal doesn't stimulate oocyte. As developmental model, parthenotes obtained from in vitro activation can be transferred to uterine of recipient female and its developmental process can be observed. Several studies have been conducted in various mammalian species including

mice (Barton *et al.*, 1985), pigs (Kure-bayashi *et al.*, 2000), rabbits (Ozil *et al.*, 1990), sheep (Loi *et al.*, 1998) and monkey (Marshall *et al.*, 1998).

Because the parthenotes have no paternal genome, it can't help having developmental limitation. This means that parthenogenetic activation leads to an abnormal growth and differentiation chiefly in extra-embryonic lineages, inducing a insufficient support of embryonic development. In case of mouse, it is well known parthenotes can develop after implantation until the formation of forelimb bud (Kaufman *et al.*, 1977). Maternal and paternal genomes are identical in numbers but functionally different, due to the degree of distinct epigenetic modification which cause different pattern of gene expression during embryo development (Surani *et al.*, 1984). The phenomenon which the expression of maternally and paternally originated gene loci is selectively inhibited or promoted is named as genomic imprinting (Paffoni *et al.*, 2008). In murine embryogenesis. *H19* and *Igf2* are the most well-known imprinted gene (Feil *et al.*, 1994). In the maternal chromosome, the *H19* gene is preferentially transcribed while *Igf2* is inactive, whereas the *H19* gene is silent and *Igf2* is activated (Banerjee *et al.*, 2001). In case of maternal chromosome, the differentially methylated domain (DMD) of *H19* gene is unmethylated and enable CTCF to bind that region. And this complex hinder enhancers from interacting with *Igf2*, rather induce interaction with *H19* (Paffoni *et al.*, 2008).

To induce parthenogenetic activation in the laboratory, researchers have employed various chemicals and experimental conditions. For example, electroporation (N´an´assy *et al.*, 2008), heat shock (Komar *et al.*, 1973), hyaluronidase (Graham *et al.*, 1970), 6-DMAP. ethanol (Liu *et al.*, 1998), ionomycin (Onger *et al.*, 2001), cyclohexamide, , mineral oil & piroxicam (Martini *et al.*, 2000) and strontium chloride and cytochalasin B. Generally, parthenotes can be obtained from metaphase II stage oocytes treated with an inhibitor of extrusion of the second polar body. The cytochalacins are fungal products and divided to cytochalcin A, B, C and D (Carter *et al.*, 1967). They hinder actin from polymerizing by binding to the extending ends of actin filaments, and induce stoppage of extrusion of the second polar body (Forer *et al.*, 1972).

Practically all methods used for parthenogenetic activation depend on increasing the intracellular free calcium concentration , named as calcium oscillation, for their success as sperm penetration cause that (Jones *et al.*, 1998). Intracellular calcium have an important role as a key regulator in a various cellular events such as cell cycle, differentiation and programmed cell death (Whitfield *et al.*, 1995). However, this phenomenon has been studied in many species, the mechanism has not yet been completely understood. Among parthenogenesis-inducing agents, strontium as the only parthenogenetic agent for mouse oocytes, can induce repetitive intracellular calcium oscillations similar to those that appear during normal fertilization process (Bos-Mikich *et al.*, 1995).

There are several researches about analysis of oocyte activation (Cuthbertson *et al.*, 1981; Kato *et al.*, 2001) and activation of enucleated oocytes for cloning (Wakayama *et al.*, 1998; Ono *et al.*, 2001).

The reason that various researches about parthenogenesis have been conducted is to establish immunologically compatible and patient-specific autologous stem cells for clinical purposes. And stem cells established from parthenogenesis can circumvent ethical issues because there is no requirement of male gametes. Until now, a variety of studies about the parthenogenetic activation of human oocytes (Mai *et al.*, 2007; Revazova *et al.*, 2007). Parthenogenetic embryonic stem cells have several ESC-specific traits, such as self-renewal, expression of pluripotency marker and differentiation potential (Lin *et al.*, 2007; Mai *et al.*, 2007). However, there is risk to use parthenogenetic stem cells as sources for cell therapy because different gene expression pattern and epigenetic instability in comparison with normal human embryonic stem cells. Thus, further studies are required for improving the possibility of parthenogenetic embryonic stem cells as clinical source.

2) In vitro fertilization

In vitro fertilization (IVF) is a kind of assisted reproductive technology (ART) and consists of a succession of

techniques in which oocytes are fertilized by sperm outside the female body, with the purpose of getting viable developing embryos (Paffoni *et al.*, 2008). Since the first birth of baby through IVF and embryo transfer in 1978, millions of babies have been born through the some type of ARTs. Besides experimental purposes, the ultimate goal of ARTs is the provision of solution against the infertility. Infertility affects approximately 12–15% of couples of reproductive age (Palermo *et al.*, 2012).

Until now, several studies for ARTs have been conducted, such as intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992), microsurgical epididymal sperm aspiration (MESA), testicular sperm extraction (TESE) (Silber *et al.*, 1994) and round spermatid injection (ROSI) (Tesarik *et al.*, 1996). In addition, experimental skills such as embryo culture, embryo screening, cryopreservation of gametes, in vitro maturation should be prepared first for successful ARTs.

However, despite of contribution of ARTs to the resolution for infertility, there are some problems to be solved. For example, after ICSI, there is a slightly higher frequency of sex chromosome aberration (Hardy *et al.*, 2002). As the use of immature spermatozoa, spermatid and spermatocyte for ICSI, the papers about congenital abnormality have been reported (Zech *et al.*, 2000). And culture of embryos in serum containing media increase abnormality of imprinted gene (Mann *et al.*, 2004) and cause s small litter (Khosla *et al.*, 2001).

3. Embryonic stem cells

Embryonic stem cells are derived from inner cell mass of blastocyst through *in vitro* culture on feeder layer under appropriate culture conditions. Since Evans and Kaufman succeeded the establishment of ESCs from mouse blastocysts (Evans and Kaufman, 1981), Thomson and his colleague made same achievement in human in 1998 (Thomson *et al.*, 1998). Until now, several studies about ESC establishment in rat (Iannaccone *et al.*, 1994; Buehr *et al.*, 2008), porcine (Strojek *et al.*, 1990; Li *et al.*, 2003), bovine (Cherny *et al.*, 1994), primate (Thomson *et al.*, 1995) and human (Xu *et al.*, 2001; Richards *et al.*, 2002). Because of the developmental potential to differentiate into any type of cells which compose of human body, ESCs have been getting spotlight in the clinical research.

1) Signalings requires for pluripotency

To maintain undifferentiated state of ESCs, appropriate culture conditions including feeder layer and several growth factors are positively necessary. In case of murine ESCs, leukemia inhibitory factor (LIF), a cytokine belonging to interleukin 6 family should be contained culture medium to inhibit differentiation (Smith *et al.*, 1988; Wiliams *et al.*, 1988). Addition of LIF to the culture medium is enough to establish and maintain ES cells even without feeder cells in the presence of

fetal calf serum (Nichols *et al.*, 1990), indicating that LIF has a pivotal role as an extrinsic factor for ES self-renewal.

The LIF receptor is made up of the LIF specific receptor subunit LIFRβ (or gp190) and the signal transducer gp130, which is pooled among the interleukin 6 family members (Taga and Kishimoto, 1997). If LIF binds to this heterodimeric complex, Janus kinase (JAK) phosphorylates $Y_{765/812/904/914}$ of the intracellular domain of gp130 and $Y_{976/996/1023}$ of LIFRβ, and this activates signal transducers and activators of transcription (STAT)1 and STAT3 (Ernst *et al.*, 2001). STAT proteins are transcription factors that upon phosphorylation, dimerize and move into the nucleus where they turn on target genes such as *Klf4* (Niwa *et al.*, 1998)and c-myc (Cartwright *et al.*, 2005). Besides, binding of LIF also activates MAP kinase and PI3K-Akt pathway (Niwa *et al.*, 2009). On the other hands, in primate and human ESCs, LIF-STAT signaling pathway fails to maintain undifferentiate state human ESCs (Dahéron *et al.*, 2004; Reubinoff *et al.*, 2000). One essential role of LIF in murine development is to allow embryonic diapause (Nichols *et al.*, 2001), the transitory capture of blastocyst development in lactating female mice. It enables highly fecund animals to afford best timing of repetitive and multiple pregnancies. Because human blastocysts don't go through this process, ESCs derived from human blastocysts founder to respond to alike human LIF (Dahéron *et al.*, 2004). Moreover, human ESCs express high level of suppressor of cytokine signaling (SOCS) genes which inhibit LIF-mediated signaling and maintain relatively low level

of LIF signaling components (Wei *et al.*, 2005). In case of human ESCs, Fgf2 (bFGF) have profound effect on maintenance of self-renewal via activation of MAP kinase. At this time Activin A, a member of transforming growth factor-β family involves in this mechanism (Wang *et al.*, 2005; Vallier *et al.*, 2005). Activin A activates cellular signaling protein, Smad2/3, and results in promoting transcription of genes encoding transcription factors essential for self-renewal such as Oct3/4 and Nanog (Babaie *et al.*, 2007).

A second pathway involved in mouse ESC self-renewal is bone morphogenic protein (BMP) signaling. BMPs bind to heterodimeric complexes of type I (ALK2,3 and6) and type II (BMPRII, ActRII, ActRIIB) receptor tyrosine kinases (Shi and Massague, 2003). Through activation of Smad1/5/8 and promotion Id gene expression, BMPs involves maintenance of pluripotency and blockade of differentiation pathway (Ohtsuka *et al.*, 2008). Moreover, BMPs contribute to self-renewal of mouse ESCs by suppressing p38 MAP kinase (Qi *et al.*, 2004). On the contrary, BMP promotes differentiation of human ESCs (Xu *et al.*, 2002) and addition of antagonist of BMP signaling, like growth differentiation factor 3, can stimulate pluripotency of human ESCs (Levine *et al.*, 2006).

Besides, phosphatidylinositol 3 kinase (PI3K)/Akt signaling is crucial for self-renewal of mouse ESCs. If PI3K signaling is inhibited by small molecule such as LY294002, mouse ESCs are differentiate even in the presence of LIF

(Watanabe *et al.*, 2006).

The undifferentiated state of *in vitro* cultured ESCs can be confirmed by staining of surface markers and expression of stemness-related genes. Mouse ESCs express surface marker , stage-specific embryonic antigen (SSEA)-1, and don't have SSEA-3 and 4 in their membrane (Lee *et al.*, 2008). While human ESCs are positive to SSEA-3 and 4, they don't express SSEA-1 (Pera *et al.*, 2000). Also, they express not only TRA-1-60, TRA-1-81 as surface markers (Hovatta *et al.*, 2003)but also alkaline phosphatase (Zhang *et al.*, 2006). Through detection of expression level of stemness-related genes such as *Oct-4, Nanog, Sox2, Rex-1, Cripto* and *Bmp4,* established cell lines can be recognized as pluripotent cells.

2) Differentiation potential of embryonic stem cells

Stem cells have the ability to choose between infinite self-renewal and differentiation to other cell types. This selection mechanism is regulated by various intrinsic signals and extracellular microenvironment (Odorico *et al.*, 2001). If factors related to maintain pluripotent state of ESCs are removed from *in vitro* culture conditions, the differentiation of ESCs is induced. For instance, when ESCs are cultured in feeder-free, suspension culture condition, ESCs start to aggregate each other and make embryo bodies (EB's), which resemble early postimplantation embryos. Through adherent

culture of EB's without LIF, mouse ESCs can be differentiated *in vitro* into various cell type of three germ layers (Lee *et al.*, 2010). These include cardiomyocytes, neurons, yolk sac, skeletal myocytes, smooth muscle cells, hematopoietic progenitor cells, adipocytes, chondrocytes, endothelial cells, melanocytes, glial cells, pancreatic islet cells (Rathjen *et al.*, 1998; Doetschman *et al.*, 1985; Baker *et al.*, 1996; Keller *et al.*, 1995; Drap *et al.*, 1997; Rohwedel *et al.*, 1994; Brustle *et al.*, 1999; Yamane *et al.*, 1999; Risau *et al.*, 1988; Poliard *et al.*, 1995; Dain *et al.*, 1999).

Because ESCs have potency to differentiated various cell types which compose our bodies, they have been regarded as ideal source for regenerative medicine and ultimate cell therapy. Through studies in animal disease models, it was demonstrated that the transplantation of pluripotent stem cell derivatives can be used as treatment against a variety disease such as diabetes, Parkinson' disease, traumatic spinal cord injury, Purkinje cell degeneration, liver failure, Duchenne's muscular dystrophy and osteogenesis inperfecta (Soria *et al.*, 2000; McDonalds *et al.*, 1999; Horwitz *et al.*, 1999; Li *et al.*, 2000; Studer *et al.*, 1998; Brustle *et al.*, 1998; Zhang *et al.*, 1996; Lilja *et al.*, 1997; Kobayashi *et al.*, 2000).

CHAPTER 3

GROWTH OF PRIMARY FOLLICLES RETRIEVED FROM NEONATES OF DIFFERENT AGES

1. Introduction

We have made an attempt to developing efficient preantral follicle culture system for generating developmentally competent oocytes, and several culture strategies for secondary follicles have been developed to date (Lee *et al*, 2007; Lee *et al*, 2008; Kim *et al*, 2009). This culture system has been employed neonatal female mice as the follicle donor and has served a recent success on developing of follicles derived from adult ovaries (Kim *et al*., 2009). Viable blastocysts has also been generated from IVF or parthenogenetic activation of the preantral follicle-derived oocytes (Gong *et al*, 2008; Lee *et al*, 2008; Gong *et al*, 2009; Kim *et al*, 2009), which confirms the feasibility of the follicle culture system in reproductive biotechnology and stem cell engineering. However, most follicle culture techniques deal with secondary follicles and a very limited success on the culture of primary follicles has been made to date (Lenie *et al*, 2004). These primary follicles are the most abundant among the growing pool of ovarian follicles (O'Brien *et al*, 2003). Thus, their successful mobilization for generating developmentally competent oocytes can greatly increase feasibility of preantral follicle culture system.

In this study, I attempted to further optimize a primary follicle culture system by evaluating of appropriate retrieval time. The follicles retrieved at different times from neonatal

females were cultured for obtaining mature oocytes, and assessment on the efficiency of follicle retrieval and mature oocyte generation was subsequently made by employing both cellular and biological parameters. Prospective randomized trial was conducted with using of F1 hybrid mice.

2. Materials and Methods

Experimental design

We first evaluated an appropriate time to retrieve primary follicles from neonatal ovaries (experiment 1). The numbers of primary, and early and late secondary follicles retrieved from prepubertal ovaries of 7- to 14-day-old females were monitored and changes in the number of primary follicles were concomitantly evaluated. In experiment 2, primary follicles retrieved from optimal ages of retrieval (9- to 11-day old mice) were cultured for 11 days. Morphological integrity and growth to the pseudo-antral follicles, oocyte mucification, and initiation (germinal vesicle breakdown) and completion (metaphase II plate formation) of meiotic maturation were subsequently monitored.

Experimental animal

As an experimental animal, B6CBAF1 (C57BL6 CBA/ca) mice were employed. All procedures for animal management, breeding, and surgery followed the standard protocols of Seoul National University, Korea. The Institutional Animal Care and Use Committee at Seoul National University approved our research proposal in April 2005 (SNU0050331-

02).

Isolation of preantral follicles

The ovaries retrieved from euthanized females were placed in L-15 Leibovitz-glutamax medium (Gibco Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Preantral follicles were mechanically isolated from neonatal mice by using a 30-gauge needle without enzymatic digestion (Nayudu *et al.*, 1992; Lenie *et al.*, 2004). After retrieval, the follicles were classified according to their size and morphology (Smitz *et al.*, 2002; Lee *et al.*, 2008). Primary follicles selected were placed singly in 10 μl culture droplets overlaid with mineral oil in a 60mm culture dish.

In vitro culture and in vitro maturation

The medium used for the culture of primary follicles is a ribonucleoside and deoxyribonucleoside-free, α-MEM-glutamax medium (primary culture medium), to which 1% (v/v) heat-inactivated FBS, 1% insulin, transferrin and selenium liquid medium (ITS; Sigma-Aldrich Corp.), 200 mIU recombinant FSH (Organon, Oss, The Netherlands), 10 mIU/ml LH (Sigma-Aldrich Corp.) and 1% (v/v) penicillin/streptomycin were added. On day 1 of culture, an additional 10 μl fresh

medium was added to each droplet and half of the culture medium was replaced with fresh medium on day 3. Then, morphological normality and the size of primary follicles were monitored under an inverted microscope at the end of primary culture (day 4).

Only the follicles with normal morphology were further cultured in 10 μl droplets of a ribonucleoside and deoxyribonucleoside-containing α-MEM-glutamax medium (Gibco Invitrogen.) supplemented with 5% (v/v) heat-inactivated FBS, 1% ITS solution, 100 mIU/ml FSH and the antibiotics (secondary culture medium) from day 4 of culture. On day 5, 10 μl of fresh medium was added to culture medium and half of the medium was changed daily with fresh medium from day 7 to the end of secondary culture (day 11). To trigger maturation, 2.5 IU/ml hCG (PregnylTM; Organon) and 5 ng/ml epidermal growth factor (EGF; Sigma-Aldrich Corp.) were added to culture medium 16 h prior to the end of the culture.

Monitoring of follicular growth and oocyte maturation

The number of the follicles formed pseudo-antrum, an intercellular space between granulosa cells in cumulus-oocyte-complex was counted on day 11 of culture. The number of oocytes spontaneously dissociated from granulosa cell mass (defined as mucified oocytes) was evaluated on day 12 of culture. Subsequently, oocytes retrieved at the end of the

second phase culture were freed from cumulus cells by mechanical pipetting and subsequently placed in M2 medium (Lee *et al.*, 2008) supplemented with 4 mg/ml BSA and 200 IU/ml hyaluronidase. A comparison of the development of intrafollicular oocytes into the germinal vesicle breakdown (represent as the initiation of maturation) and metaphase II stage (represent as the completion of meiotic maturation) was made between the ages of retrieval. Meiotic maturation of the oocytes was determined by the expansion of cumulus cells and the presence of a first polar body in the perivitelline space.

Statistical analysis

Numerical data obtained from a randomized, controlled trial were statistically analyzed with a generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS) program. When significant model effect was detected, each treatment effect was compared by the least square method. The level of significance in model effect and pair comparison was determined when p value was less than 0.05.

3. Result

Experiment 1: Number of preantral follicles retrieved from prepubertal females

Overall, mean number of preantral (primary, early secondary and late secondary) follicles per neonatal females of 7- to 14-day-old (n = 24; three in each age) was 10.7, 36.3, 53.7, 71.7, 81, 83.7, 96.3 and 88.7, respectively (Table 1). Significant (p = 0.0005) increase in the number of retrieved follicles was detected from the 9-day-old, which was peaked at the 13-day-old. The number of primary follicles was gradually increased to 35.7 follicles up to 11-day-old, then reduced to 29 follicles at 14-day-old (p = 0.0013). The number of early (2.0 to 54.3 follicles) and late (0 to 14.3 follicles) secondary follicles were also increased continuously up to 13-day-old (p = 0.0052).

Experiment 2: Growth of primary follicles and developmental competence of intrafollicular oocytes matured in primary follicles

As shown in Table 2, more primary follicles retrieved from 10-day-old or 11-day-old females maintained their normal morphological integrity at the end of primary culture than the follicles retrieved from 9-day-old (n = 7 in each age;

p = 0.0086). Of those, number of the follicles developing into the early secondary follicle was significantly higher in the group of 11-day-old than in the others (39% vs. 13 to 29%). Compared with the 10-day-old and 11-day-old, none of the 9-day-old retrieved follicles grew into the late secondary follicles at the end of primary culture. However, there was no significant difference in the number of primary follicles developing into the pseudoantral stage between the retrieval ages at the end of the secondary culture (21 to 30%; p = 0.5222). No significant difference was also detected in the number of oocytes mucified between the retrieval ages (32 to 39%; p = 0.5795).

Next, developmental competence of intrafollicular oocytes from primary follicle culture was monitored (Table 3). Average number of mature oocytes per animal was increased more than 3.3 times in the group of 11-day-old retrieval than in the group of 9-day-old retrieval (0.86 to 2.86; p = 0.0616). However, the initiation of meiosis estimated by germinal vesicle breakdown (17 to 25%; p = 0.2557) and the completion of meiotic maturation (10 to 17%; p = 0.2389) were similar between retrieval ages. Morphology of developing follicles and mature oocytes was depicted in Figure 1, which confirmed morphological normality in each stage of development.

Table 1. Number of preantral (primary, early secondary and late secondary) follicles retrieved from prepubertal mice of different ages.

Ages (days)	Mean no. (±SD) of follicles retrieved (total)	Mean no. (±SD) of retrieved follicles at the stage of		
		Primary (75−99 μm)	Early secondary (100−125 μm)	Late secondary (<126 μm)
7	10.7± 8.1[a]	8.7±7.0[a]	2.0±2.0[a]	0.0±0.0[a]
8	36.3±23.2[ab]	18.0±9.6[ab]	17.0±11.5[ab]	1.3±2.3[ab]
9	53.7±5.1[bc]	31.0±3.6[c]	18.3±2.1[ab]	4.3±4.5[ab]
10	71.7±17.2[cd]	32.7±4.6[c]	30.7±10.4[bc]	8.3±3.2[bc]
11	81.0±20.7[cd]	35.7±5.5[c]	38.0±15.1[bc]	7.3±1.5[abc]
12	83.7±12.1[cd]	26.7±3.2[bc]	48.0±10.6[c]	9.0±1.7[bc]
13	96.3±31.4[d]	27.7±1.2[bc]	54.3±21.7[c]	14.3±9.6[c]
14	88.7±30.0[cd]	29.0±9.2[bc]	46.3±14.2[c]	13.3±2.1[c]

Total 24 female B6CBAF1 mice were euthanized for retrieving preantral follicles.
Model effects of the treatment on the mean number of follicles retrieved, and the mean number of retrieved follicles at the stage of primary, early secondary and late secondary were 0.0005, 0.0013, 0.0012 and 0.0052, respectively.
[a−d]Different superscripts within the same parameter indicate significant difference among the treatments, $p<0.05$.

Table 2. Follicular growth during in-vitro culture of primary follicles retrieved from the mice of different ages

Ages at retrieval[a] (days)	No. (%)[b] of primary follicles		Diameter (μm) of normal follicles[c]			No. (%)[d] of	
	Cultured	Normal morphology	75-99 (%)[d]	100-125 (%)[d]	>125 (%)[d]	Pseudo-antral follicles	Oocytes mucified
9	109	63 (58)[f]	49 (45)	14 (13)[f]	0 (0)[f]	14 (21)	20 (32)
10	157	113 (72)[g]	60 (38)	45 (29)[g]	8 (5)[g]	34 (30)	44 (39)
11	158	118 (75)[g]	53 (34)	61 (39)[h]	4 (3)[fg]	31 (26)	46 (39)

Model effects of the treatment on the number of primary follicles with normal morphology, the diameter of normal follicles with 75-99 m, 100-124 m and more than 125 m, and the number of pseudoantral follicles and follicles mucified, which were indicated as p values, were 0.0086, 0.1698, less than 0.0001 and 0.046, 0.5222 and 0.5795, respectively.

[a]Total 21 female F1 mice were euthanized and each treatment replicated 7 times.

[b]Percentage of the number of primary follicles cultured.

[c]Only primary follicles with normal morphology on day 4 of culture were transferred to the secondary culture medium and their sizes were measured at the end of culture.

[d]Percentage of the number of follicles with normal morphology.

[e]Percentage of the number of follicles mucified.

[fgh]Different superscripts within the same parameter indicate significant differences among the treatments, $p<0.05$.

Table 3. Maturation of intrafollicular oocytes by *in-vitro* culture of primary follicles retrieved from the mice of different ages

Ages at retrieval (days)	No. of females euthanized	No. of primary follicles with normal morphology	No. of intrafollicular oocytes		Mean no. of mature oocytes per female
			Developed to the germinal vesicle breakdown stage (%)[a]	Matured (%)[a]	
9	7	63	12 (19)	6 (10)	0.86[b]
10	7	113	19 (17)	13 (12)	1.86[bc]
11	7	118	30 (25)	20 (17)	2.86[c]

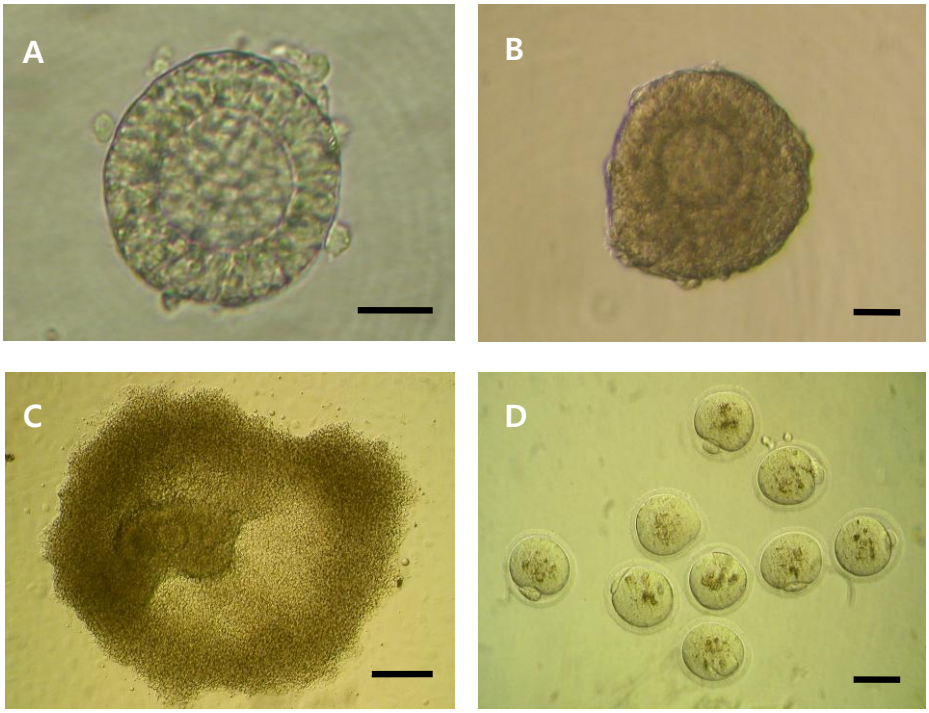
Primary follicles were cultured by a two-step method.

Model effects of the treatment on the number of intrafollicular oocytes developed to the germinal vesicle breakdown stage and matured, and mean number of mature oocytes per female, which were indicated as p values, were 0.2557, 0.2389 and 0.0616, respectively.

[a] Percentage of the number of primary follicles with normal morphology.

[bc] Different superscripts within the same parameter indicate significant differences among the treatments, $p<0.05$.

Figure 1. Morphology of primary follicles during in vitro culture and the follicle-derived oocytes. Primary follicle immediately after isolation (A), the cultured follicle (day 4) at the proliferative stage (B), the cultured follicles developing to the pseudoantral stage (C), mature (metaphase II stage) oocytes derived from in vitro-cultured primary follicles (D). (A and B: bar = 25 μm; C to D: bar = 50 μm).



4. Discussion

The results of this study demonstrated that there is a remarkable difference in in vitro-growth of primary follicles retrieved from neonatal mice of different ages, which suggests a positive correlation between number of retrieved follicles and derivation of mature oocytes per animal. Continuous increase in retrieval number of preantral follicles was observed as the age was increased up to 14-day-old and, among those follicles, primary follicles yielded developmentally-competent oocytes following our in vitro-culture. The best efficiencies in follicle retrieval and oocyte maturation was obtained at 11 day old females and percentile values of intrafollicular oocytes to initiate and complete meiotic maturation, however, are similar among the ages (9- to 11-day-olds).

Although many efforts for the establishment of in vitro culture system for primary follicles have been made (Eppig and Schroeder, 1989; Cortvrindt *et al.*, 1996; O'Brien *et al.*, 2003; Lenie *et al.*, 2004; Sadeu *et al.*, 2008), there was no report for the derivation of mature oocytes from primary follicles except for the experiment of Lenie *et al.* (2004). The report showed that primary follicles were retrieved 8- to 14 day-old females and yielded mature oocytes with a two-step follicle culture system, which consists of a 6 days of primary and a 12 days of secondary culture, respectively. Our study further suggests the optimal time for the retrieval of primary follicles from neonatal

female was 11-day-old and the total culture time for the two-step system was 11 days including a 4 days of primary and a 7 days of secondary culture. This discrepancy may result from the differences of medium composition and miscellaneous culture regimes. Accordingly, primary follicles of 9- to 11-day-old that used in this study may require different microenvironment for optimal growth in vitro, compared with the previous report with the use of 8- to 14-day-old primary follicles.

It is hypothesized that primary follicle may be more sensitive to culture environment than secondary follicles of advanced stage. The fact that primary follicle is encompassed with only single or several layers of primitive granulosa cell layer may induce high susceptibility to exogenous environment. In other words, the culture outcome of primary follicle may be more influenced by cell-to-cell interaction or exogenous environment during in vitro-manipulation than that of secondary follicles. Accordingly, the optimization of manipulation protocols such as retrieval time, miscellaneous change of medium composition and culture duration definitely contributes to enhancing the capacity of primary follicle to derive developmentally competent oocytes in vitro.

There are various paracrine factors involved in regulating follicle growth (McGrath *et al.*, 1995; Dube *et al.*, 1998; Gilchrist *et al.*, 2001; Markstrom *et al.*, 2002). Moreover, it has been suggested that the growth and maturation of

intrafollicular oocytes in preantral follicles are significantly influenced by cell to cell interaction between oocytes and surrounding follicular cells (granulosa and theca cells) through paracrine manner (Eppig *et al.*, 2002). For instance, paracrine factors secreted by oocytes (i.e. growth differentiation factor 9 and bone morphogenic protein 15) stimulate growth of granulosa cells and expression of folliculogenesis-related genes (Dube *et al.*, 1998; Sadeu *et al.*, 2008) and c-kit ligand stimulates the growth of intrafollicular oocytes retrieved from neonatal mice (Packer *et al.*, 1994; Driancourt *et al.*, 2000). Indeed, maturational status of follicular cells is one of the critical factors for successful culture of primary follicles. In this study, I used culture media supplemented 200 mIU FSH during culture period and observed a great expansion of follicle cells with changing their morphology, which might imply functional acquisition of follicular cells by in vitro-culture of primary follicles. Further study is needed for refining some factors which work more critically in growth of primary follicles than that of secondary follicles.

In conclusion, I provide further information on optimizing of primary follicle culture protocol in terms of follicle retrieval. Since a significant difference in primary follicle retrieval and the number of mature oocyte per the follicle donor is observed during neonatal period, selection of retrieval time is definitely necessary to increase the efficiency. Therefore, the follicle retrieval during mid neonatal (approximately less than 3 weeks after birth) period can be recommended for the

establishment of in vitro primary follicle culture system. This suggested guideline will contribute to establishing a new ART strategy, as well as to developing novel regenerative medicine employing embryo-derived, pluripotent cells. I am now confirming the feasibility of oocytes derived from in vitro-cultured, primary follicles for generating viable embryos and for establishing stem cell-like cells.

CHAPTER 4

DERIVATION OF DEVELOPMENTALLY COMPETENT OOCYTES AND BLASTOCYSTS FROM PRIMARY FOLLICLES IN OPTIMAL CULTURE CONDITION

1. Introduction

Approximately 300,000 immature follicles at different developmental stages are present in each human ovary, and the number decreases gradually with age (Forabosco *et al.*, 1991;Faddy *et al.*, 1992). Among these, only about 400 follicles yield mature oocytes during a lifetime, whereas the others undergo atresia (Nicholas *et al.*, 2001; Schwartzman *et al.*, 1993) Recently, attempts have been made to utilize immature follicles to produce developmentally competent oocytes. Live offspring and homozygous or heterozygous embryonic stem cell (ESC) lines have been generated by manipulating secondary follicles (Eppig *et al.*, 1989;Lee *et al.*, 2008;Gong *et al.*, 2008;Kim *et al.*, 2009) but only limited success producing mature oocytes has been made using primary follicle culture (Lenie *et al.*, 2004).

In previous chapter, primary follicles derived from 11-day-old prepubertal mice generate the largest number of mature oocytes. So I used primary follicles retrieved from 11-day-old mice to generate viable homozygotic and heterozygotic embryos. Primary follicles (70-100 μm in diameter) consist of cuboidal granulosa cells, a basement membrane attached to theca cells, and a premeiotic oocyte (Smitz *et al.*, 2002;Pedersen *et al.*, 1968). To increase the efficiency of the culture system, we employed a sequential culture protocol and stimulated follicles using exogenous follicle-stimulating

hormone (FSH). The effect of adding FSH to the culture medium on follicle growth, oocyte maturation, follicular morphology, steroid hormone production, and receptor numbers was determined.

2. Material and Method

Experimental design

In the first series of experiment, primary follicles were sequentially cultured in the medium, to which 0, 100 or 200 mIU recombinant FSH was added in various combinations (no addition as the control treatment, 0 and 100, 200 and 100, 100 and 200, and 200 and 200 mIU for the primary and secondary culture, respectively). Follicle growth was monitored by enumerating the morphologically normal primary, early secondary and late secondary follicles. The follicles forming a pseudoantrum, and the number of diffused and matured oocytes were subsequently monitored. Steroid hormone production of the follicles exposed to 200 mIU FSH, or not throughout the culture was measured. Additionally, the number of FSH and LH receptors in the follicles cultured with or without 200 mIU FSH was quantified by Western blotting and immunocytochemistry. In the second series of experiments, development of inseminated or parthenogenetically activated oocytes derived from the follicles cultured in the medium supplemented with 0, 100 or 200 mIU FSH was monitored. The first cleavage, blastocyst formation and the mean number of blastocysts obtained per animal were evaluated. The blastocysts obtained were subsequently used for the isolation of embryo-derived stem cells.

Animals

Eleven-day-old B6CBAF1 (C57BL/6 x CBA/Ca) female mice served as donors of preantral follicles. All procedures for animal management, breeding, and surgery followed the standard protocols of Seoul National University, Korea. The Institutional Animal Care and Use Committee at Seoul National University approved our research proposal in April 2005 (approval number: SNU0050331-02).

Isolation of follicles

The ovaries retrieved from euthanized females were placed in L-15 Leibovitz-glutamax medium (Gibco Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). A non-enzymatic, mechanical method was employed for retrieving the preantral follicles. Mouse preantral follicles were isolated by using a 30-gauge needle without enzymatic digestion (Lee, 2008). Primary follicles were selected from the preantral follicles by the conventional criteria (Smitz, 2002;Pedersen, 1968) and were subsequently used in the experiments designed.

In vitro-culture of follicles

The total duration of primary follicle culture was for 11 days, which was consisted of the first 4 day and next 7 day cultures. Different supplementation of culture medium was made for each culture step. At the end of primary culture on day 4, the follicles cultured were transferred into new culture dish.

The primary follicles were placed singly in 10 μl culture droplets overlaid with washed-mineral oil (Sigma-Aldrich Corp.) in 60x15 mm Falcon plastic Petri-dishes (Becton Dickinson, Franklin Lakes, NJ). The medium used for primary culture of primary follicles was a ribonucleoside and deoxyribonucleoside-free α-MEM-glutamax medium (primary culture medium), to which 1% (v/v) heat-inactivated FBS, 5 μg/ml insulin, 5 μg/ml transferrin and 5ng/ml selenium liquid medium (ITS; Sigma-Aldrich Corp.), 100 or 200 mIU recombinant FSH (Organon, Oss, The Netherlands), 10 mIU/ml LH (Sigma-Aldrich Corp.) and 1% (v/v) penicillin/streptomycin were added. On day 1 of culture, an additional 10 μl fresh medium was added to each droplet and a half of the medium was changed on day 3. Morphological normality and integrity of the primary follicles cultured were monitored during culture, and the size of the follicles was measured under an inverted microscope on day 4 of culture. The number of primary follicles developing into early and late secondary follicles was counted at the end of primary culture on day 4.

The primary follicles that maintained their morphological normality on day 4 were then cultured in 10 μl droplets of a ribonucleoside and deoxyribonucleoside-containing α-MEM-glutamax medium (cat. no. 12571-063, Gibco Invitrogen.) supplemented with 5% (v/v) heat-inactivated FBS, 5 μg/ml insulin, 5 μg/ml transferring and 5ng/ml selenium liquid medium (ITS; Sigma-Aldrich Corp.), 100 and 200 mIU/ml FSH and the antibiotics (secondary culture medium). On day 5, 10 μl of fresh medium was added to the culture medium and a half of the medium was changed daily from day 7 to the end of culture. In other words, total culture period is 11 days. To trigger maturation, 2.5 IU/ml hCG (Pregnyl™; Organon) and 5 ng/ml epidermal growth factor (EGF; Sigma-Aldrich Corp.) were added to the culture medium 16 hours prior to the end of the secondary culture on day 11.

Enzyme-linked immunosorbent assay (ELISA) of estradiol (E_2) and progesterone (P_4)

To improve the accuracy of data, all experiments were replicated three times and standard operation protocol for sample collection, storage and measure was employed. The media used for culture of primary follicles were collected on days 3, 4 (the end of primary culture), 7, 9 and 11 (the end of secondary culture). After being centrifuged twice with 300g, the supernatant was collected and stored at −20° C for analyzing estradiol and progesterone. Estradiol (E_2) and

progesterone (P_4) were measured using an estradiol EIA Kit (Oxford biomedical research, Rochester Hills, MI) and a progesterone enzyme immunoassay Kit (Oxford). Colorimetric analysis was conducted with a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 650nm. The ELISA-calorimetric analysis detected as less as 0.02 ng/ml of each hormone, which was calculated by our preliminary results (data not shown).

Gonadotrophin receptor analysis by Enzyme-linked immunosorbent assay (ELISA) kit

Concentration of LHCGR and FSHR were measured using an mouse LHCGR ELISA Kit (Cusabio, Hubei, China) and mouse FSHR ELISA Kit (Cusabio). Colorimetric analysis was conducted with a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 450nm. Follicle homogenates (7 μg/ml) were employed for analysis. After assay procedure, Curve Expert 1.4 program was used to create standard curve by mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. After creating the standard curve, the concentration of LHCGR and FSHR about each experimental groups was calcurated.

Derivation of mature oocytes, and IVF and parthenogenetic activation

The number of the follicles forming pseudo-antrum, an intercellular space between granulosa cells, was determined on day 11 of culture. The number of the oocytes spontaneously dissociated from the follicles (defined as mucified oocytes) was evaluated. Some of mucified oocytes remaining as intact cumulus-oocyte-complexes (COCs) were used for in vitro-insemination with epididymal semen. Potassium simplex optimized medium (KSOM) was employed as the basal medium for in vitro-insemination and oocytes were incubated with the sperm for 4 to 6 hours. In the case of parthenogenetic activation, mucified oocytes were freed from cumulus cells by 200 IU/ml hyaluronidase solution and the number of oocytes matured was subsequently evaluated. Mature oocytes were parthenogenetically activated in KSOM supplemented with 10 mM $SrCl_2$ and 5 μg/ml cytochalacin B for 4 hours (Gong, 2008).

In vitro-culture of oocytes inseminated in vitro or activated parthenogenetically

Inseminated or parthenogenetically activated oocytes were subsequently cultured in a 5 μl droplet of modified Chatot, Ziomek and Bavister (CZB) medium consisting of 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$,

1.7 mM $CaCl_2$•$2H_2O$, 25.1 mM $NaHCO_3$, 31.3 mM sodium lactate, 0.3 mM sodium pyruvate, 1 mM glutamine, 0.1 mM EDTA, antibiotics and 5 mg/ml BSA. Putative embryos were cultured at 37 ° C in 5% CO_2 in air, and cleavage and blastocyst formation were monitored under an inverted microscope (Eclipse TE-3000; Nikon, Tokyo, Japan) 48 and 124 hours after in vitro-insemination.

Uterine embryo transfer

Blastocysts developed from in vitro fertilized oocytes derived from cultured primary follicles were co-injected with ICR-derived blastocysts to the recipients' uterine by using mouth pipette. After 20 days, the number of offspring and pregnant spots examined.

Statistical analysis

All experiments were replicated at least more than three times, and the same replication of each treatment was conducted within one experiment. Numerical data obtained from three-time or more replicates were statistically analyzed with a generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS) program. When significant model effects were detected, each treatment effect was compared by the least square method. The level of significance in model

effect and pair comparison was less than 0.05 of P value.

3. Results

A total of 450 primary follicles were collected, and 84% (379/450) of those maintaining morphological integrity were subsequently used in culture (Table 4). A significant model effect ($p < 0.0367$) was detected for primary follicle growth *in vitro*. Significantly more ($p = 0.0367$) follicles developed to the early secondary (40 to 53% vs. 32 to 39%) and to the late secondary (26 to 53% vs. 0%) stages after exposure to 200 mIU FSH during even a partial culture period than after exposure to 0-to-0 mIU and 0-to-100 mIU FSH. We have cultured early secondary follicles in the media added 100 mIU FSH (Lee *et al.*, 2008). So we decided FSH dosage as 100 or 200 mIU. Significantly more ($p < 0.0001$) follicles in the 100-to-200 mIU, 200-to-100 mIU, and 200-to-200 mIU FSH exposure groups developed to the pseudoantral stage (34 to 40% vs. 0 to 10%) resulting in mucified oocytes than in groups exposed to no FSH (3 to 37% vs. 61 to 76%). Exposure of primary follicles to 200 mIU FSH yielded the best follicle growth and oocyte maturation results.

In a preliminary experiment, we evaluated the viability differences between two groups (no treatment vs. 200-to-200 mIU FSH) by ELISA using the Cell Counting Kit 8 on different days of culture and overall, but no significant difference in follicular viability between the optimal 200-mIU FSH exposure

and no exposure was observed (Fig. 2). However, the granulosa cell layer of the primary follicles did not expand (Fig. 3B), and granulosa cells appeared flattened (Fig. 3A). Premature detachment of oocytes from the follicular cell matrix (Fig. 3D) was observed in the group not exposed to FSH. In contrast, primary follicles treated with 200 mIU FSH contained proliferating granulosa cells (Fig. 3C) and formed a pseudoantrum (Fig. 3E). Increased 17β-estradiol (E2) production (Fig. 4A) was detected from day 7 of culture in the FSH-exposed group compared with that in follicles not exposed to FSH (0.007 vs. 0.385 ng/ml). Regardless of FSH exposure, E2 production remained at undetectable levels on days 3 and 4 of culture. Progesterone (P4) production was also higher in follicles exposed to FSH than in those that were not exposed (Fig. 4B). Unlike E2, P4 production was detected on days 2 and 3 of culture (0.042–0.117 ng/ml). A decrease in P4 production on day 7 of culture was observed, followed by an increase ($P < 0.05$). P4 production on days 9 and 11 was 0.117 ng/ml, 0.021 ng/ml, and 0.266 ng/ml in the FSH-exposed group and 0.042 ng/ml, 0.003 ng/ml, and 0.068 ng/ml in the unexposed group on days 3, 7, and 11 of culture, respectively. The immunocytochemistry and ELISA assay to detect differences in follicle-stimulating-hormone receptors (FSHR) and luteinizing-hormone receptors (LHR) after FSH treatment revealed strong signals, reflecting the abundance of FSHR and LHR in follicles from the FSH treatment group compared with those from the control group (Fig. 5 and 6). We conducted an ELISA analysis to clarify the differences in LHR and FSHR

between the two groups (Fig. 7). The results showed significant differences in LHR (p = 0.0213) and FSHR (p = 0.0058) concentration between the treatment groups. Higher LHR expression was detected in follicle homogenate of primary follicles cultured without FSH. On the contrary, more FSHR were detected in group cultured with FSH.

As shown in Table 5, only mature oocytes (Fig. 8A) derived from primary follicles exposed to FSH developed into blastocysts (Fig. 8D), and normal developmental stages (Fig. 8B and C) occurred after IVF (31-38%), resulting in a significant increase in the number of blastocysts obtained per animal after culture of primary follicles in FSH-containing medium (9.3 vs. 12.3 blastocysts/animal). However, no significant differences in mucified oocytes (91 vs. 93%) before IVF, at the first cleavage (47 vs. 53%), or during blastocyst formation were observed among follicles in the various FSH doses (Table 5). Embryo development patterns after parthenogenetic activation were similar to those seen after IVF (Table 6). Although no significant model effects were detected among FSH doses ($p < 0.0582$), more oocytes developed into blastocysts after parthenogenetic activation in the group exposed to 200 mIU FSH than in the other groups (29 vs. 18%).

Table 4. Effects of recombinant FSH added at different culture stages on follicle growth and maturation of intrafollicular oocytes in primary follicles.

Conc. of FSH (mIU) in		No. (%)[a] of primary follicles		Follicles grown to[b]			No. (%)[c] of		No. of matured oocytes (%)[d]
Primary culture	Secondary culture	Cultured	With normal morphology	Primary (%)[c]	Early secondary (%)[c]	Late secondary (%)[c]	Pseudoantral follicles	Mucified oocytes	
0	0	86	61 (71)[e]	37 (61)[e]	24 (39)[ef]	0 (0)[e]	0 (0)[e]	2 (3)[e]	0 (0)
0	100	67	62 (93)[f]	42 (68)[e]	20 (32)[e]	0 (0)[e]	6 (10)[e]	23 (37)[f]	5 (22)
100	200	66	58 (88)[f]	3 (5)[f]	30 (52)[fg]	25 (43)[g]	20 (34)[f]	44 (76)[g]	16 (36)
200	100	163	141 (87)[f]	29 (21)[g]	75 (53)[fg]	37 (26)[f]	52 (37)[f]	86 (61)[h]	26 (30)
200	200	68	57 (84)[f]	4 (7)[f]	23 (40)[eg]	30 (53)[g]	23 (40)[f]	41 (72)[gh]	17 (41)

Primary follicles were cultured by a two-step method.

P values for model effect of the treatment on the number of primary follicles with normal morphology, the number of follicles grown to primary follicle, early secondary follicles and late secondary follicles, the number of pseudoantral follicles and mucified oocytes, and the number of matured oocytes were 0.0025, less than 0.0001, 0.0367, less than 0.0001, less than 0.0001, less than 0.0001 and 0.3892, respectively

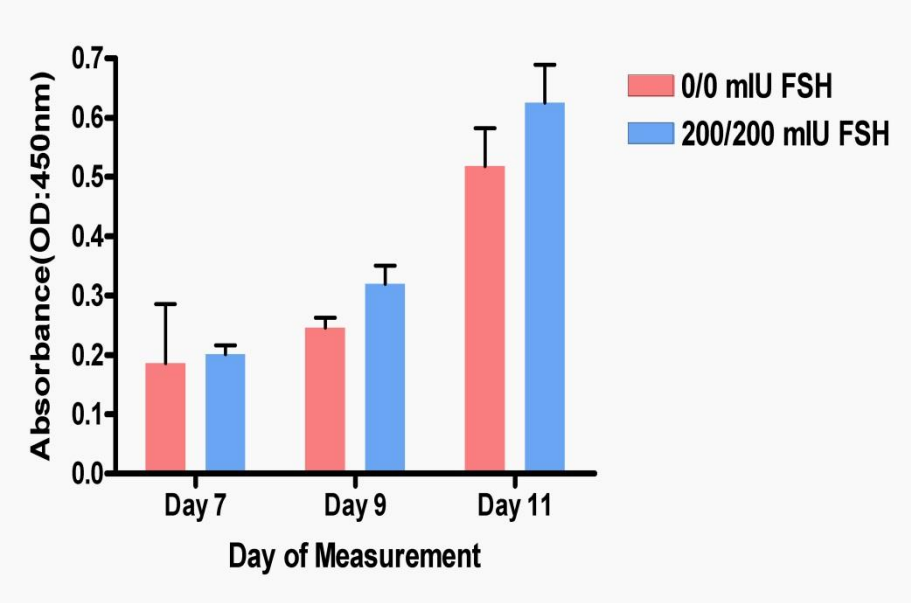
[a]Percentage of the number of primary follicles cultured.

[b]Only primary follicles with normal morphology on day 4 of culture were transferred to the secondary culture medium and the size of the follicles cultured were measured at the end of culture.

[c]Percentage of the number of primary follicles with normal morphology.

[d]Percentage of the number of mucified oocytes.

[efgh]Different superscripts within the same parameter indicate significant differences among the treatments, $p<0.05$.



Figuer 2. The difference of viability between two groups was measured using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt].

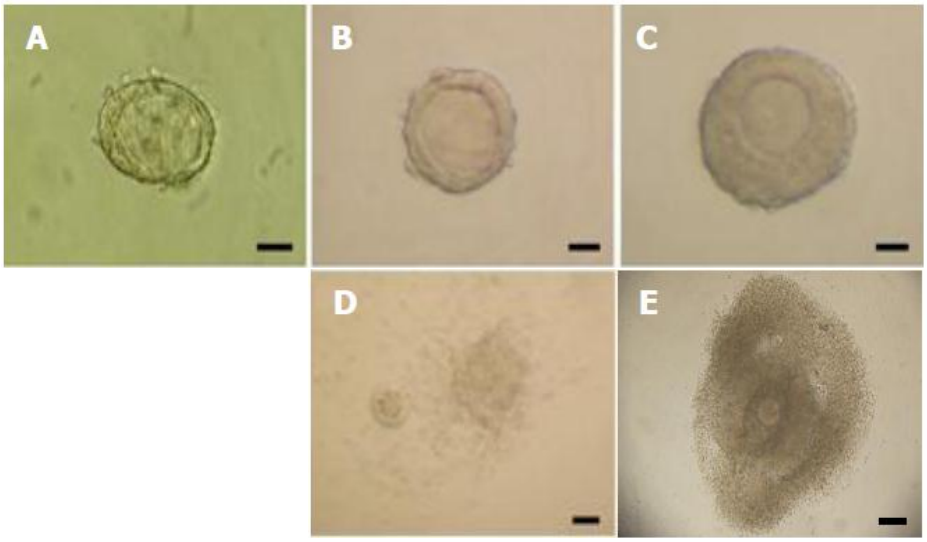


Figure 3. Primary follicles were cultured in the media supplemented with or without 200 mIU FSH. Morphology of a primary follicle cultured in the medium supplemented with 200 mIU FSH (C, E) or without (B, D) FSH on day 4 (B, C) and day 11 (D, E), respectively. A is immediately isolated single-layered follicle. Decreased proliferation compared with FSH addition and breakdown of follicle structure were noticed in the group not exposed to FSH [scale bar=25 μm (A, B and C), 50 μm (D and E)]

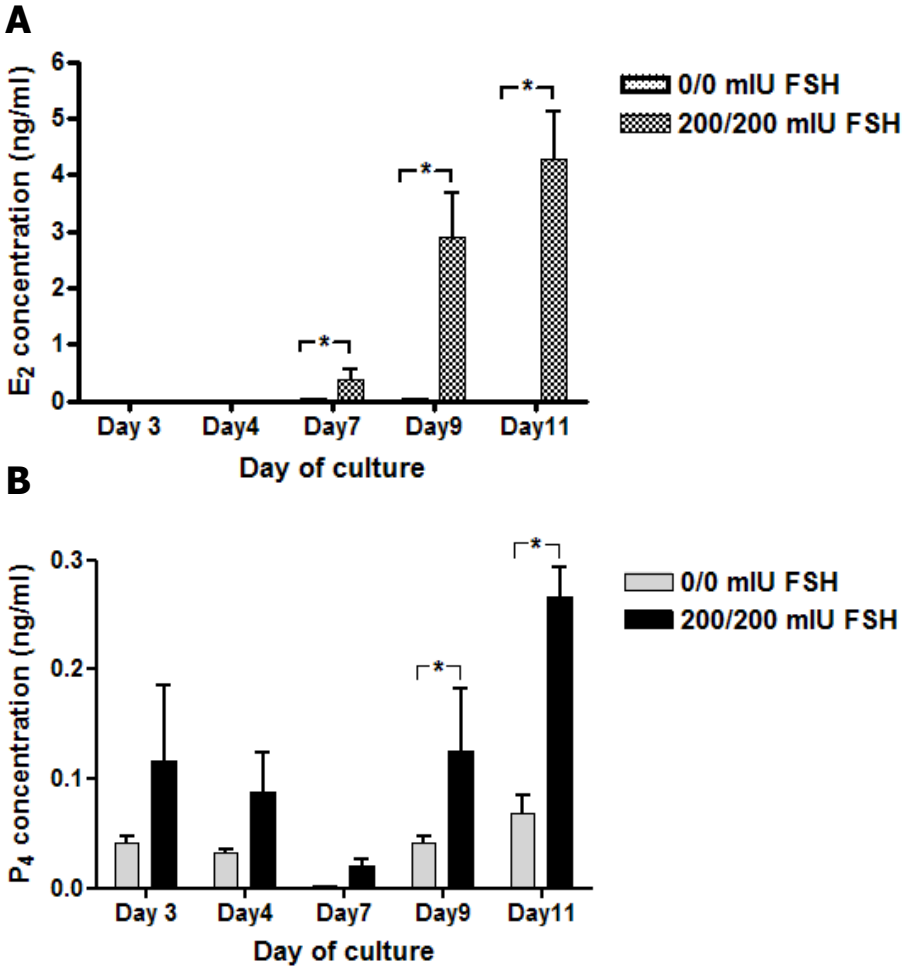


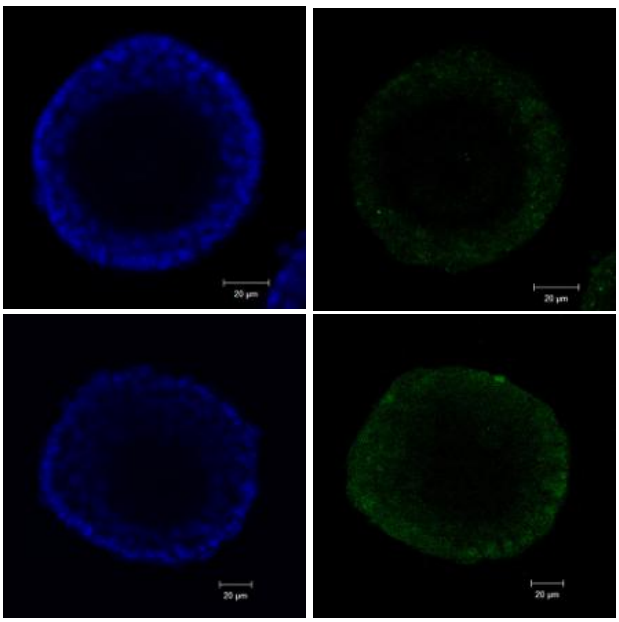
Figure 4. Production of estradiol (E_2) and progesterone (P_4) during culture of primary follicles. Primary follicles were cultured in the media supplemented with or without 200 mIU FSH. The medium used for primary follicles was collected on day 3, 4 (the end of primary culture), 7, 9 and 11 (the end of secondary culture). Changes in E_2 (A) and P_4 (B) production (ng/ml) caused after addition of FSH. Differences marked by asterisk were significant, $P<0.05$, and data were indicated as Mean±SD.

Figure 5. Expression of FSH receptors (FSHR) in the granulosa cells at the end of primary culture (on day 4) by immunocytochemistry. Primary follicles were cultured in the medium supplemented with 200mIU FSH (200/200) or not (0/0). Localization of FSH receptors was detected using the Alexa Fluor 488-conjugated anti-mouse antibody (green). N.C=negative control. Scale bar=20 μm

FSHR

0/0

200/200



N.C

0/0

200/200

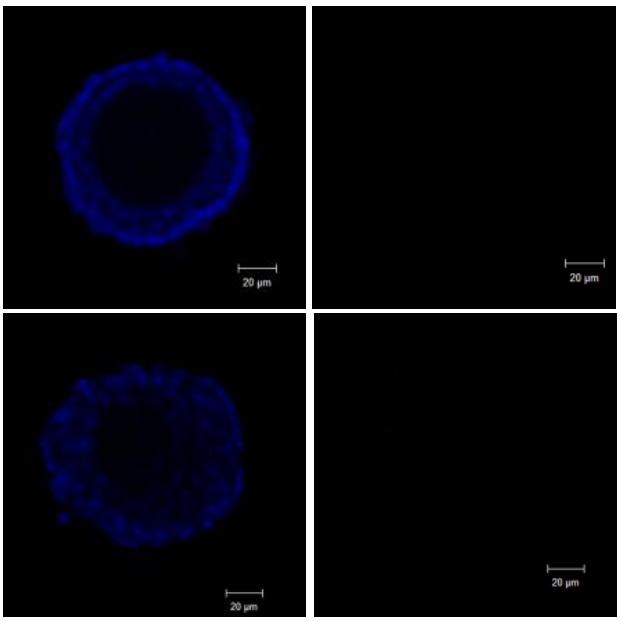


Figure 6. Expression of LH receptors (LHR) in the granulosa cells at the end of primary culture (on day 4) by immunocytochemistry. Primary follicles were cultured in the medium supplemented with 200mIU FSH (200/200) or not (0/0). Localization of LH receptors was detected using the Alexa Fluor 568-conjugated anti-mouse antibody (red). N.C=negative control. Scale bar=20 μm

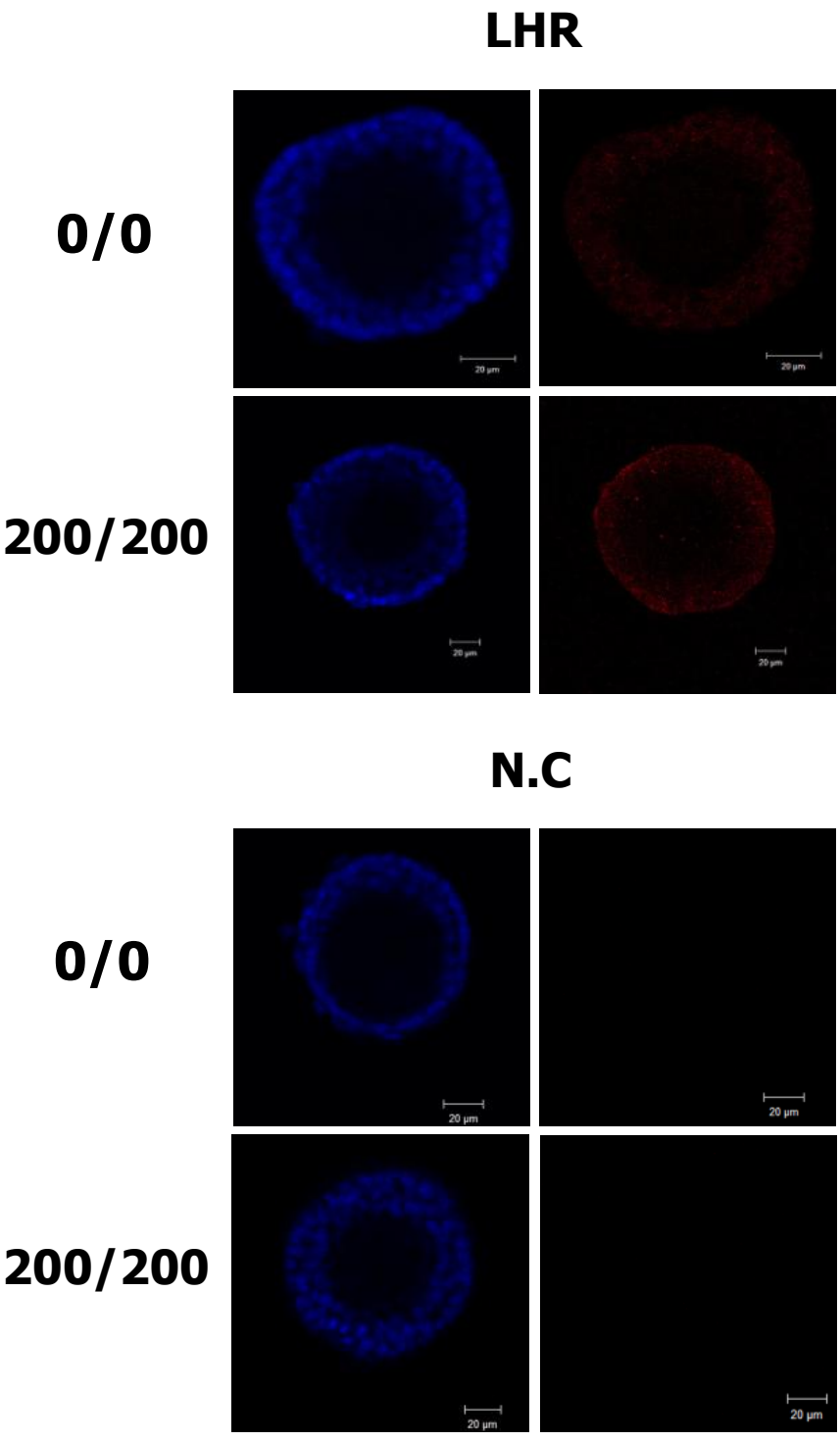
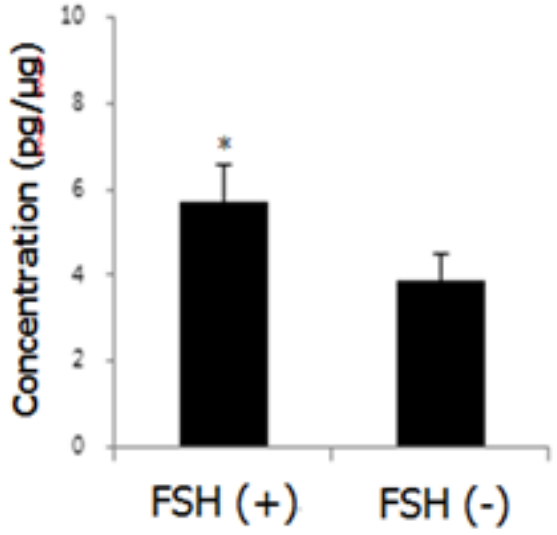

LHR
0/0
200/200
N.C
0/0
200/200
20 μm

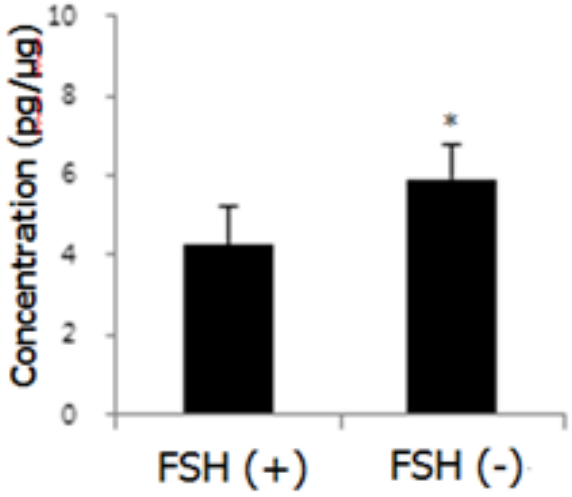
Figure 7. Protein concentration of LHR and FSHR in cultured primary follicles. Total lysates were subjected to ELISA analysis. Primary follicles were collected from 11-day old female B6CBAF1 and cultured for 4 days in the medium supplemented 200mIU FSH [FSH (+)] or not [(FSH(-)].Significant differences between the treatments ($p<0.05$) are indicated by asterisk.

FSH receptor



P=0.0058

LH receptor



P=0.0213

Table 5. Growth of primary follicles exposed to 100 or 200 mIU recombinant FSH at different periods and further development of oocytes matured in in vitro-cultured follicles after IVF.

Conc. of FSH (mIU) in		No. of primary follicle				No. of			Mean no. of BLSTs per animal
Primary culture	Secondary culture	Cultured	With normal morphology (%)[a]	Grown into secondary follicle (%)[b]	Formed pseudoantrum (%)[b]	Mucified oocytes (%)[b]	Cleaved oocytes (%)[c]	Developing BLSTs (%)[c]	
0	0	102	83 (81)[d]	34 (41)[d]	1 (1)[d]	6 (7)[d]	1 (17)	0 (0)	0[d]
100	200	115	99 (86)[de]	87 (88)[e]	65 (66)[e]	90 (91)[e]	48 (53)	28 (31)	9.3[e]
200	100	115	104 (90)[e]	98 (94)[e]	68 (65)[e]	97 (93)[e]	46 (47)	31 (32)	10.3[e]
200	200	115	104 (90)[e]	98 (94)[e]	87 (84)[f]	97 (93)[e]	51 (53)	37 (38)	12.3[e]

BLSTs; blastocysts

Twelve female F1 mice were sacrificed.

Primary follicles developing to more than 100 μm in diameter were considered as secondary follicles.

P values for model effect of the treatment on the number of primary follicles with normal morphology, follicles grown into secondary follicle, follicles formed pseudoantrum, mucified oocytes, cleaved oocytes and developing BLSTs, and the mean number of blastocysts per animal were 0.1451, less than 0.0001, less than 0.0001, less than 0.0001, 0.314, 0.2327 and 0.0162, respectively.

[a]Percentage of the number of primary follicles cultured.

[b]Percentage of the number of primary follicles with normal morphology.

[c]Percentage of the number of muclfied oocytes.

[def]Different superscripts within the same parameter indicate significant differences among the treatments, $p<0.05$.

BLSTs=blastocysts

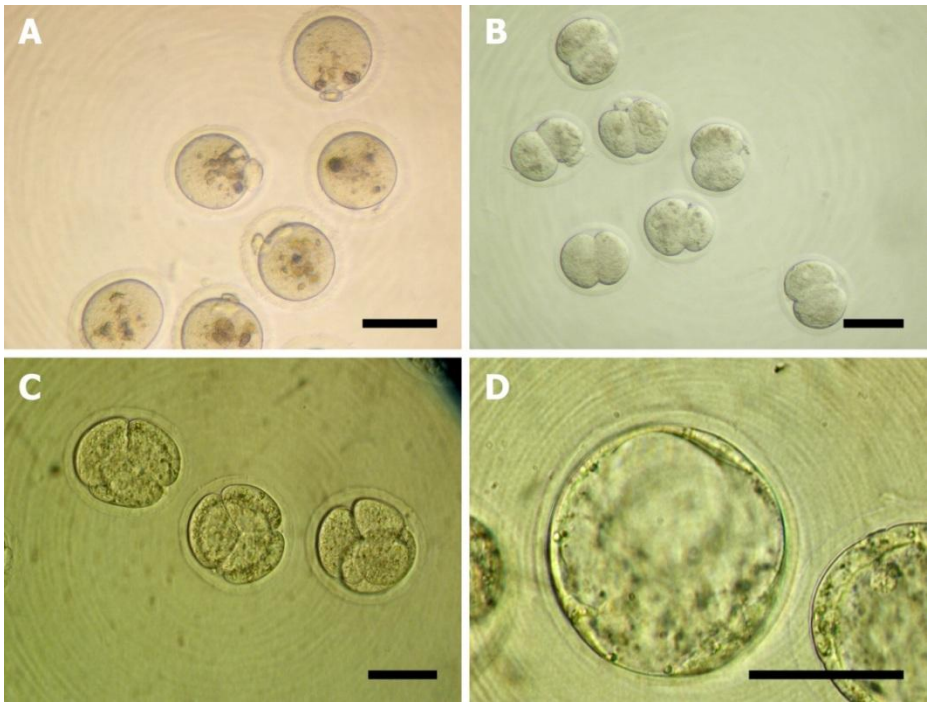


Figure 8. Morphology of the developing embryos derived from IVF and parthenogenetic activation of oocytes matured in in vitro-cultured primary follicles. (A) Mature oocytes freed from cumulus cells before parthenogenetic activation. Embryos developing into the 2-cell (B), 4-cell (C) and blastocyst (D) stages after IVF. Scale bar=50μm

Table 6. Growth of primary follicles exposed to 100 or 200 mIU recombinant FSH at different periods and further development of oocytes matured in in vitro-cultured follicles after parthenogenetic activation.

Conc. of FSH (mIU) in		No. of normal follicles	No. (%)[c] of		No. (%)[d] of matured oocytes	No. (%)[e] of oocytes forming pronucleus	No. (%)[d] of	
Primary culture	Secondary culture		Pseudoantral follicles	Mucified oocytes			Cleaved oocytes	Developing blastocysts
100	200	143	98 (69)	135 (94)	74 (55)	54 (73)	57 (42)	24 (18)
200	100	117	72 (62)	108 (92)	69 (64)	45 (65)	45 (42)	22 (20)
200	200	149	112 (75)	136 (91)	89 (65)	67 (75)	62 (46)	40 (29)

Primary follicles were cultured by a two-step method and intrafollicular oocytes matured in in vitro-cultured follicles were parthenogenetically activated with $SrCl_2$ and cytochalasin B.

P values for model effect of the treatment on the number of pseudoantral follicles, mucified oocytes, matured oocytes, oocytes forming PN, cleaved oocytes and developing blastocysts, which were indicated as p value, were 0.0578, 0.5838, 0.1599, 0.3635, 0.7914 and 0.0582 respectively.

[c]Percentage of the number of primary follicles with normal morphology.

[d]Percentage of the number of mucified oocytes.

[e]Percentage of the number of matured oocytes.

[ab]Different superscripts within the same parameter indicate significant difference among the treatments, $p<0.05$

Table 7. Transfer of embryos derived from in vitro cultured primary follicles under optimal culture conditions.

Trial	Primary-derived BL	ICR-derived BL	Uterine injection	Offspring
1	7		7	N.E
2	12	3	15	N.E
3	5	10	15	1, spot (4)
4	12	3	15	N.E
5	10	5	15	1 (immediately dead)
6	8	7	15	N.E
7	8	7	15	N.E
8	10	-	10	N.E
9	10	-	10	N.E
10	11	-	11	N.E

4. Discussion

We demonstrated that primary follicles can generate developmentally competent oocytes in a two-step, in vitro culture system, which contributed to producing viable homozygous and heterozygous embryos. The primary follicle culture system used in this study was reproducible. However, the developmental competence of the primary follicle-derived oocytes remained low, demonstrating that further improvements in the follicle-culture system are necessary. Studies of ovarian follicle culture conducted in various species such as hamsters (Roy *et al.*, 1987), sheep (Muruvi *et al.*, 2009) cattle (Yang *et al.*, 2006;Yang *et al.*, 2007), humans (Hovatta *et al.*, 1997;Hovatta *et al.*, 1999), and mice (Eppig *et al.*, 1989;Cortvrindt *et al.*, 1996a;Cortvrindt *et al.*, 1996b) have demonstrated limitations in the follicle-culture system. Eppig and his colleagues produced live offspring in mice after IVF of mature oocytes derived from in vitro secondary follicle culture (Eppig and Schroeder, 1989). Cortvrindt *et al.* established a simplified in vitro culture system for early preantral follicles using a drop-culture system (Cortvrindt *et al.*,1996b). Using this system, homologous ESC lines were successfully established following parthenogenetic activation of oocytes from in vitro preantral follicle culture (Lee *et al.*, 2008;Gong *et al.*, 2008;Kim *et al.*, 2008). The development of primary into secondary follicles using neonatal ovarian cultures has been reported by Sadeu *et al.* (Sadeu *et al.*, 2008). The derivation of

developmentally competent oocytes by culturing primary follicles was reported by Lenie *et al.* (Lenie *et al.*, 2004). However, viable embryo production has not been previously reported. In this study, we established a primary follicle-culture system for producing viable homozygotic and heterozygotic blastocysts.

FSH is necessary for viable oocyte recovery (Rosen, 2009), oocyte maturation (Cortvrindt *et al.*, 1998), granulosa cell proliferation (Cortvrindt *et al.*, 1997), formation of antrum-like cavities (Adriaens *et al.*, 2004) and cumulus cell expansion (Eppig *et al.*, 1997;Eppig *et al.*, 1998). Exposure of primary follicles to exogenous FSH is a prerequisite for producing developmentally competent oocytes and stimulates E2 and P4 production in follicles. In this study, the addition of FSH to culture medium containing primary follicles increase the number of FSHR or LHR, and a significant ($P < 0.05$) increase in E2 and P4 production was detected. These results suggest that FSH- and LH-related signals may be stimulated by exogenous FSH and subsequently increase the abundance of relevant receptors. According to previous studies, E2 works in concert with FSH to increase the abundance of FSHR and LHR per granulosa cell (Simoni *et al.*, 1997;Ascoli *et al.*, 2002). Primary follicles may express a sufficient number of FSHR and LHR to stimulate follicle growth and oocyte maturation. These observations suggest that normal primary follicles can be used for in vitro culture. As long as a suitable environment is created, primary follicles can produce mature oocytes in vitro.

A slight decrease in P4 production was detected during days 3 to 7 of culture. Although this decrease was not significant, such a decline in P4 production might reflect a shift in granulosa cell function. In fact, granulosa cells differentiate with increased proliferation during folliculogenesis *in vivo* (van den Hurk *et al.*,2005;Eppig *et al.*, 2001;Eppig *et al.*, 2002) and we also found significant changes in the structure of the follicular matrix during in vitro primary follicle culture. However, it was unclear why steroid production decreased during the early stage of follicle differentiation in vitro. Such aberrant secretion may result from differences between the *in vivo* and in vitro environments, or an error may have been made in hormone measurement, as P4 levels were lower than those reported in other studies. Our standard laboratory-operation protocol was employed for sample collection, storage, and measurement of the hormones, and the experimental design and management was strictly executed to avoid data errors between treatments. Accordingly, the presence of fewer granulosa cells in primary than in secondary follicles may have resulted in the reduced hormone production observed. The lower P4 concentrations could also be due to the use of supernatants for the analysis or to different operating protocols. In this study, we used conditioned media to non-invasively analyze hormone secretion. The level of P4 in conditioned medium was less than the minimal level of detection. In our preliminary experiment, the minimal concentration of P4 was similar to that of E2. Nevertheless, the data showing no detectable P4 may not indicate decreased P4 secretion by follicular cells due to the

medium used for the analysis.

CHAPTER 5

Establishment of Embryonic Stem Cells by In Vitro Fertilization and Parthenogenetic Activation of Oocytes from In Vitro Cultured Primary Follicles

1. Introduction

Embryonic stem cells (ESCs) are derived from inner cell mass (ICM) of blastocyst through *in vitro* culture on MMC-treated feeder layer under appropriate culture conditions. Since Evans and Kaufman succeeded the establishment of ESCs from mouse blastocysts (Evans and Kaufman, 1981), Thomson and his colleague made same achievement in human in 1998 (Thomson *et al.*, 1998). Until now, several studies about ESC establishment in rat (Iannaccone *et al.*, 1994; Buehr *et al.*, 2008), porcine (Strojek *et al.*, 1990; Li *et al.*, 2003), bovine (Cherny *et al.*, 1994), primate (Thomson *et al.*, 1995) and human (Xu *et al.*, 2001; Richards *et al.*, 2002) have been conducted. Because of the developmental potential to differentiate into any type of cells which compose of human body, ESCs have been getting spotlight as the clinical purpose such as stem cell therapy.

Up to now, our research group established embryonic stem cells from oocytes yielded from in vitro culture of follicles retrieved from adult mice (Kim *et al.*, 2009) and autologous embryonic stem cells through parthenogenetic activation of oocytes matured within in vitro-cultured preantral follicles in prepubertal mice (Lee *et al.*, 2008). Through parthenogenetic activation, it is possible to establish autologous and patient-specific stem cells from mature oocytes without immune rejection after transplantation. Besides human (Revazova *et al.*,

2007), autologous stem cells were established in various species such as mice (Shan, 2012), buffalo (Sritanaudomchai *et al.*, 2007), primates (Cibelli *et al.*, 2002) and human (Turovets *et al.*, 2011).

In case of primary follicles, only limited success producing mature oocytes has been made using primary follicle culture (Lenie *et al.*, 2004). I retrieved primary follicles from the 11-day-old prepubertal female mice and cultured in vitro to generate developmentally competent mature oocytes under 200 mIU FSH. And the developmental potential of oocytes derived from *in vitro* primary follicle culture was evaluated. From the homozygotic and heterozygotic embryos activated by parthenogenesis and IVF, I established 11 ESCs lines for the first time. To investigate several characters of colony-forming cells as embryonic stem cells, I conducted conventional characterization methods such as ESCs-specific marker staining, gene expression of stemness-related genes and differentiation potential *in vitro* and *in vivo*.

In conclusion, established cell lines have various chracteristics as stem cells and can be utilized to reproductive medicine and stem cell biotechnology.

2. Material & Method

Animals

Eleven day old B6CBAF1 (C57BL/6 x CBA/Ca) female mice served as donors of preantral follicles. All procedures for animal management, breeding, and surgery followed the standard protocols of Seoul National University, Korea. The Institutional Animal Care and Use Committee at Seoul National University approved our research proposal in April 2005 (approval number: SNU0050331-02).

Establishment of colony-forming cells by subculturing of inner cell mass (ICM) cells

The zona pellucida of the blastocysts derived from IVF and parthenogenetic activation were removed using acidic Tyrode's solution and the zona-free blastocysts were subsequently cultured on a feeder layer of mouse embryonic fibroblasts (MEFs). The MEFs were treated with mitomycin C (Chemicon, Temecula, CA) and cultured in gelatin-coated, 4-well multidishes. Modified knock-out Dulbecco's minimal essential medium (KDMEM; Gibco Invitrogen) was used for initial culture of the blastocysts and on day 4 of culture, ICM cell-derived, colony-forming cells were removed mechanically

from the primary feeder cells and replated on new feeder cells in 35x60 mm tissue culture dishes.

Characterization of colony-forming cells

For stem cell characterization with stem cell-specific markers, colony-forming cells maintained by subculturing 20 times were fixed in 4% (v/v) formaldehyde (Sigma-Aldrich). The reactivity of the ESC-like cells to alkaline phosphatase (AP) was assessed with Fast Red TR/naphthol AS-MX phosphate (Sigma-Aldrich). Antibodies against POU5F1 (Oct-4) (BD Biosciences, San Jose, CA, USA), stage-specific embryonic antigens (SSEA)-1 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), SSEA-3 (Developmental Studies Hybridoma Bank), SSEA-4 (Developmental Studies Hybridoma Bank), integrin α6 and integrin β1 (Santa Cruz Biotechnology, Santa Cruz, CA) were provided for the marker staining. Localization of SSEA-1, SSEA-3, SSEA-4, POU5F1 and two integrins was performed using the Alexa Fluor 488- or 568-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR). The reactivity of ESC-like cells to AP was detected by using the Dako Cytomation kit (DakoCytomation, Carpinteria, CA). The expression of stemness-related genes (*Pou5f1*, *Nanog*, *Rexo1*, *Tdgf1* (*Cripto*), *Lif Rc*, *Sox2* and *Gdf3*) and sexing with Y chromosome-specific, *Zfy* were detected by RT-PCR. FACS analysis was also undertaken to confirm the chromosomal

normality of the cell lines established.

Differentiation analysis

To confirm spontaneous differentiation in vitro, the colony-forming cells were treated with 0.04% (v/v) trypsin-EDTA (Gibco Invitrogen), and the dissociated cells were subsequently transferred to 100-mm plastic Petri dishes that contained LIF-free DMEM (Gibco Invitrogen) that was supplemented with 10% (v/v) FBS. When embryoid bodies (Ebs) were formed by the LIF-free culture, they were seeded separately into 4-well culture plates and cultured for 10-14 days. The EBs were subsequently stained with the following specific markers for the three germ layers: nestin (Santa Cruz Biotechnology, Santa Cruz, CA) and S-100 (Biodesign International, Saco, ME) for ectodermal cells; smooth muscle actin (Biodesign International) for mesodermal cells and α-fetoprotein (Biodesign International) for endodermal cells. Antibody localization was conducted with the Dako Cytomation kit (Dako-Cytomation).

For monitoring colony-forming cell capacity to differentiate *in vivo*, $1X10^7$ colony-forming cells were injected subcutaneously into non-obese diabetic/severely compromised immunodeficient mice. Teratomas that formed in the subcutaneous region of the injections were collected 6 weeks post-transplantation and fixed with 4% (v/v) paraformaldehyde

(Sigma-Aldrich). After embedding in paraffin blocks, the tissues were stained with hematoxylin and eosin for examination under a phase-contrast microscope (BX51TF; Olympus, Kogaku, Japan).

Analysis of DNA content in established colony-forming cell lines by PI staining

The DNA content of cells was analyzed after two subcultures. The harvested cells were washed in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS (DPBS; Gibco Invitrogen) and fixed by suspension in 70 % (v/v) ethanol (Sigma Aldrich) for overnight at 4℃. The fixed cells were rinsed within cold DPBS twice and re-suspended in PI solution contained 50 μg/ml propidium iodide (PI; Sigma Aldrich), 0.1% (v/v) triton X-100 (Sigma Aldrich), and 0.2 mg/ml RNase A (Roche, Basel, Switzerland). After 10 minutes at room temperature in dark, the cells were analyzed by BD FACS CaliburTM Flow Cytometer (Becton Dickinson, San Jose, CA). The data was analyzed using the CELL QuestTM software (Becton Dickinson).

3. Result

In a different set of experiments, 12 primary follicle culture programs combined with IVF and 13 combined with parthenogenetic activation were conducted. A total of 570 mucified oocytes (284 for IVF and 286 for parthenogenesis) were used to establish ESC-like colony-forming cells. Overall, 51-58% of these oocytes underwent cleavage, and 41-61% of cleaved oocytes developed into blastocysts. Attachment of the ICM was observed in 94% of seeded blastocysts after IVF or parthenogenesis. A significant difference in the seeding rate (p = 0.011) was detected between IVF and parthenogenesis, whereas no significant differences were found for other parameters. Eleven ESC-like cell lines, four from IVF and seven from parthenogenesis, were established (Table 7). A flow cytometry analysis showed that the cell lines established through IVF (Fig. 9B) and parthenogenetic activation (Fig. 9C) had similar karyotypes compared with those of E14 (Fig. 9A).

Colonies formed in ICM cells by culturing zona-free blastocysts on mitomycin C-treated mouse embryonic fibroblasts (Fig. 10Aa1). ICM cell clumps appeared immediately after subculture (Fig. 10Aa2), and colony-forming cells appeared during subculture of ICM cell clumps with trypsin-EDTA solution (Fig. 10Aa3). All cell lines established were positive for alkaline phosphatase, anti-POU5F1, anti-SSEA-1,

anti-integrin-6, anti-integrin α6, and anti-integrin β1 antibodies (Fig. 10B). As in the control ESCs, no reactivity to anti-SSEA-3 and anti-SSEA-4 antibodies was observed. Various stem-cell-specific genes (*Pou5f1, Nanog, Rexo1, Tdgf1, Lif RC, Sox2*, and *Gdf3*) were expressed by the colony-forming cells (Fig. 11A). We performed RT-PCR using *Zfy1*-specific primers to examine the origin of the established cells (Fig. 11B). The results revealed that the established cell lines possessed the Y sex chromosome and were derived through normal fertilization. The colony-forming cells formed embryoid bodies in leukemia-inhibitory factor-free medium and were positive for markers of the three germ layers (α-fetoprotein, nestin, S-100, and smooth muscle actin; Fig. 12A). When colony-forming cells were transplanted subcutaneously into NOD-SCID mice, they formed teratomas consisting of three germ-layer-derived cells (Fig. 12B).

Table 8. Derivation of colony-forming cells by culture of inner cell mass (ICM) cells of blastocysts derived from IVF or parthenogenesis of oocytes matured in vitro-cultured primary follicles

Rep	Conc. of FSH (mIU)		No. of		No. (%)[d] of blastocysts seeded	No. (%)[e] of	
	Primary culture	Secondary culture	Mucified oocytes	Cleaved oocytes (%)[c]		ICM cells attached	Colony-forming cells
1	100	200	19	11 (58)	3 (27)	3 (100)	0 (0)
1	200	100	20	11 (55)	4 (36)	3 (75)	0 (0)
1	200	200	22	11 (50)	5 (45)	4 (80)	1 (20)
2	100	200	21	10 (48)	6 (60)	5 (83)	0 (0)
2	200	100	23	9 (39)	8 (89)	8 (100)	0 (0)
2	200	200	22	13 (59)	12 (92)	12 (100)	0 (0)
3	100	200	22	11 (50)	7 (64)	7 (100)	0 (0)
3	200	100	21	9 (43)	4 (44)	4 (100)	0 (0)
3	200	200	21	10 (48)	8 (80)	7 (88)	1 (13)
4	100	200	28	16 (57)	12 (75)	11 (92)	1 (8)
4	200	100	33	17 (51)	10 (59)	10 (100)	0 (0)
4	200	200	32	17 (53)	10 (59)	10 (100)	1 (10)
IVF program (n=12)			284	145 (51)	89 (61)[a]	84 (94)	4 (4)

1	100	200	21	7 (33)	1 (14)	1 (100)	0 (0)
1	200	100	20	11 (55)	1 (9)	1 (100)	0 (0)
1	200	200	20	7 (35)	3 (42)	3 (100)	0 (0)
2	200	100	17	10 (59)	1 (10)	1 (100)	0 (0)
2	200	200	20	13 (65)	2 (15)	2 (100)	0 (0)
3	100	200	21	13 (62)	7 (54)	7 (100)	1 (14)
3	200	200	20	11 (55)	9 (81)	9 (100)	0 (0)
4	100	200	38	21 (55)	12 (57)	12 (100)	2 (17)
4	200	100	13	10 (77)	6 (60)	6 (100)	1 (17)
4	200	200	37	23 (62)	14 (61)	14 (100)	3 (21)
5	100	200	18	11 (61)	4 (36)	3 (75)	0 (0)
5	200	100	18	11 (61)	6 (55)	6 (100)	0 (0)
5	200	200	23	18 (78)	12 (67)	8 (67)	0 (0)
Parthenogenesis program (n=13)			286	166 (58)	78 (46)[b]	73 (94)	7 (9)

Rep; replications

P values for model effect of treatments were 0.0943, 0.0110, 0.8308 and 0.2468 in the number of cleaved oocytes, the number of blastocysts seeded, the number of ICM cell attached and the number of colony formation cells, respectively.

[c]Percentage of the number of mucified oocytes.

[d]Percentage of the number of cleaved oocytes.

[e]Percentage of the number of blastocysts seeded.

[ab]Different superscripts within the same parameter indicate significant difference among the treatments, $p<0.05$.

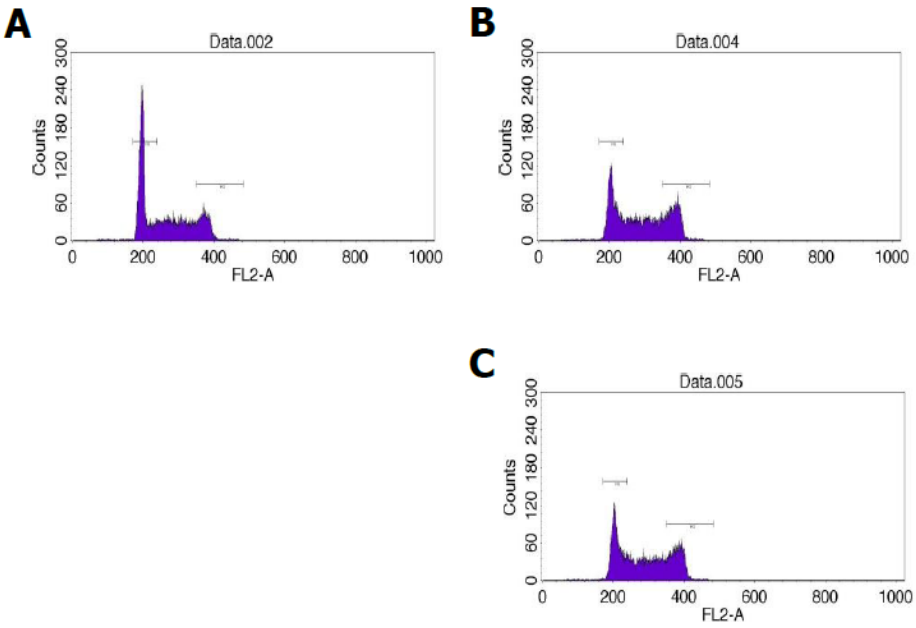
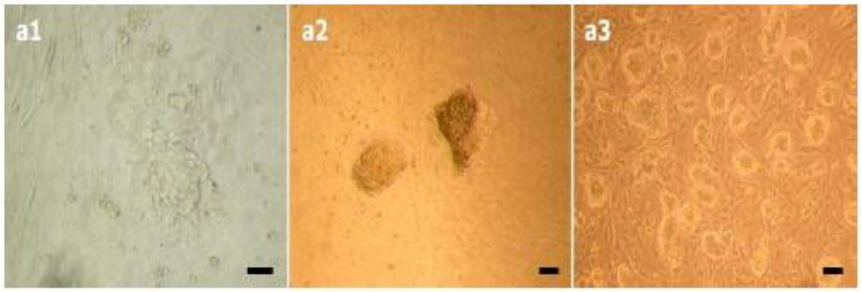


Figure 9. FACS analysis to measure DNA content for E14 ESC cell line (A, positive control), pri-IVF-2, embryonic stem cell (ESC)-like cell line (B) and pri-par-2 ESC-like cell line (C). The cells were analyzed by BD FACS Calibur™ Flow Cytometer. The data was analyzed using the CELL Quest™ software. Similar pattern of DNA content was detected in established ESC-like cell lines.

Figure 10. Establishment of colony-forming cells by the culture of inner cell mass (ICM) cells of blastocysts derived from in vitro-fertilized oocytes matured by in vitro-culture of primary follicles and characterization of colony-forming cells derived from in vitro-culture of primary follicles (pri-ivf-1-4 lines). (A) Colony formation of ICM cells by culture of a zona-free blastocyst on mitomycin C-treated mouse embryonic fibroblasts (a1). ICM cell clumps immediately after subculture (a2) and colony-forming cells formed by subculture of ICM cell clumps with trypsin-EDTA solution (a3). scale bar=50 μm. (B) Marker sensitivity of colony forming cells (pri-IVF-1-4 lines) against pluripotent cell-specific, anti-POU5F1, stage specific embryonic antigen (SSEA)-1 and SSEA-4, integrin α6 and integrin β1 antibodies, and alkaline phosphatase (AP). Scale bar = 50 μm for AP and 20 μm for all other panels.

A



B

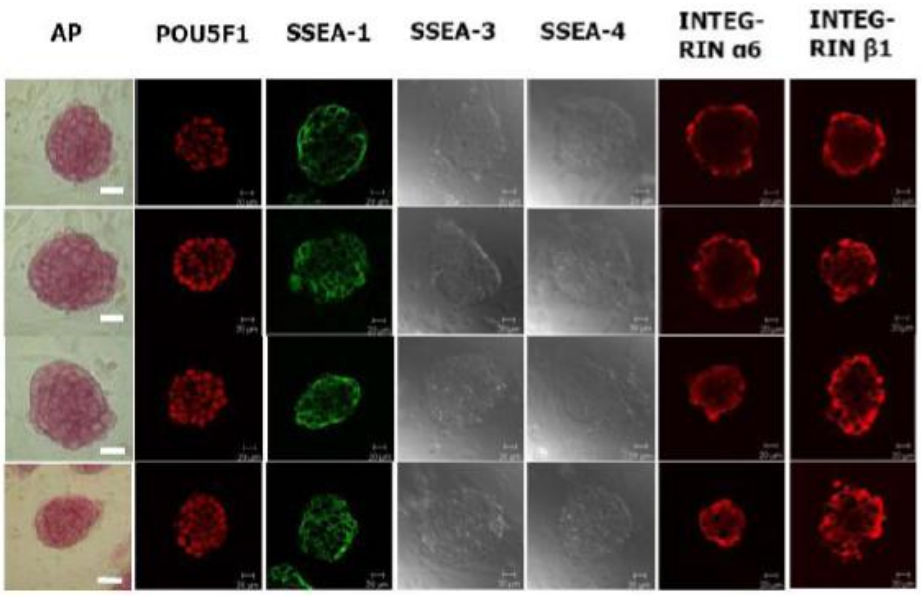


Figure 11. Establishment of colony-forming cells by the culture of inner cell mass (ICM) cells of blastocysts derived from in vitro-fertilized oocytes matured by in vitro-culture of primary follicles and characterization of colony-forming cells derived from in vitro-culture of primary follicles (pri-ivf-1-4 lines). (A) Pluripotent cell-specific, *Pou5f1, Nanog, Rexo1, Tdgf1, Lif RC, Sox2 and Gdf3* expressions in colony-forming cells detected by RT-PCR. (B) Sex-specific *Zfy1* (Y-chromosome specific) expression in pri-IVF-1-4 lines.

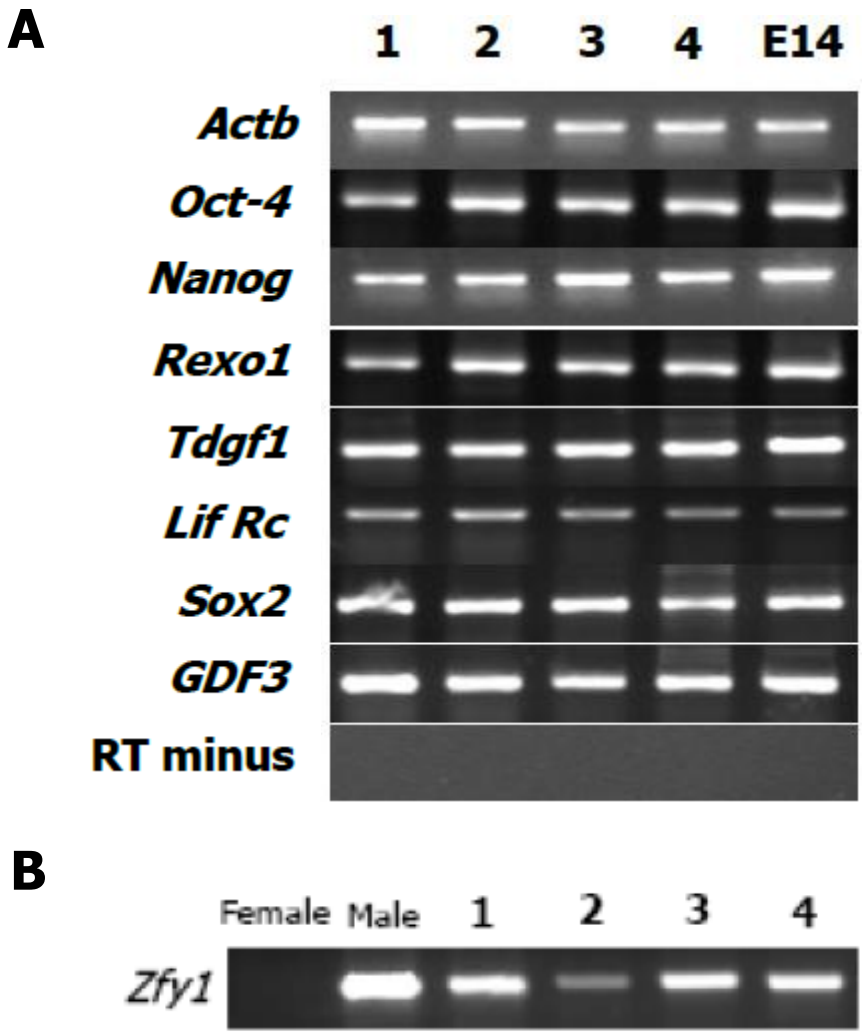
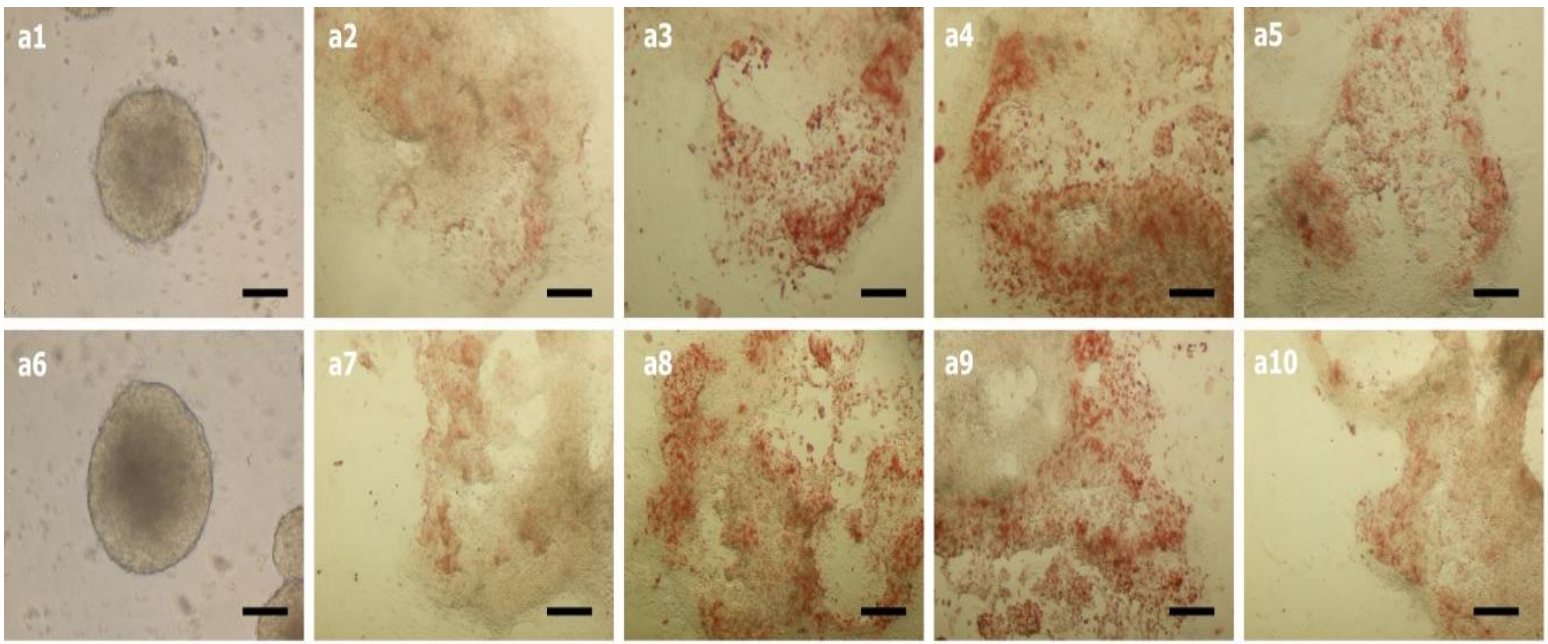
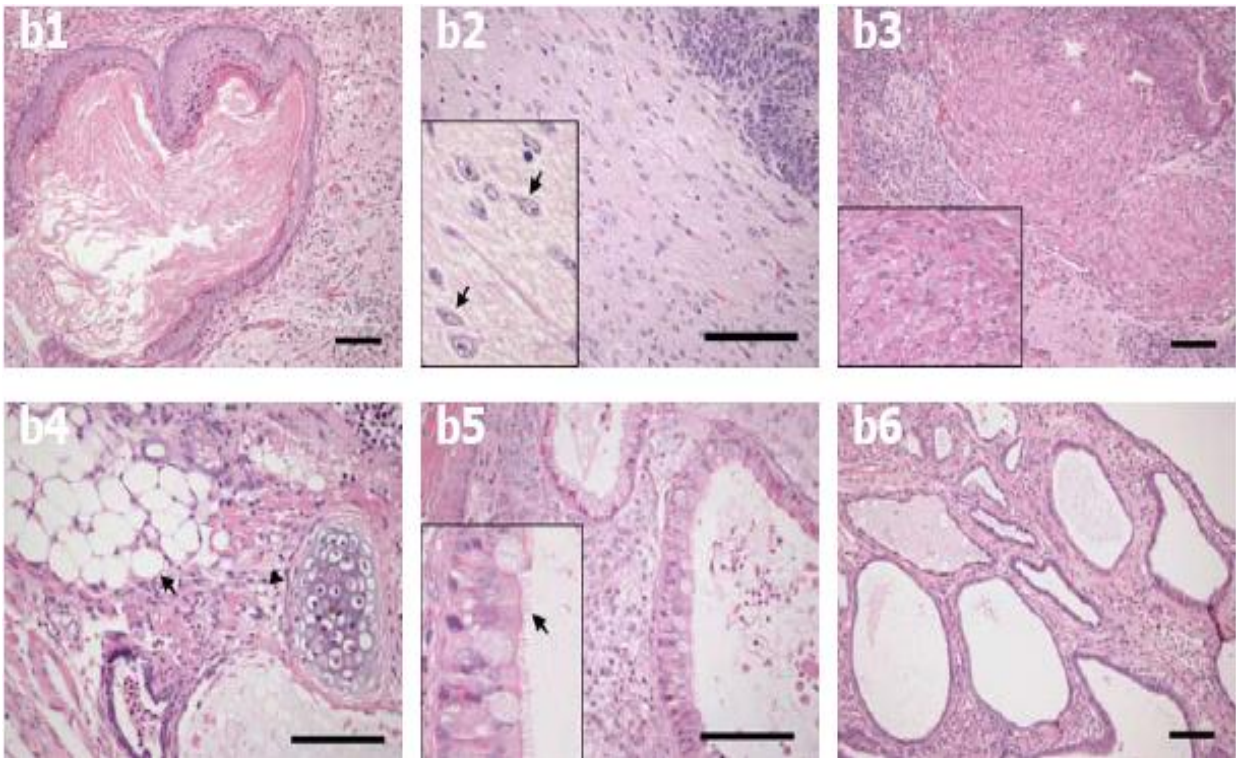

A
1
2
3
4
E14
Actb
Oct-4
Nanog
Rexo1
Tdgf1
Lif Rc
Sox2
GDF3
RT minus
B
Female
Male
1
2
3
4
Zfy1

Figure 12. *In vivo* and in vitro differentiation. (A) Formation of embryoid bodies (EBs) by culture of established embryonic stem cell (ESC)-like cells (pri-IVF-2 and pri-IVF-4 lines) in leukemia inhibitory factor-free culture medium and by transfer to immunodeficient mice. EB formation (a1 and a6) was observed on day 4 of culture and verified by immunohistochemical analysis using three germ layer specific markers: α-fetoprotein (a2 and a7; endodermal), nestin (a3 and a8; ectodermal), S-100 (a4 and a9; ectodermal) and smooth muscle actin (a5 and a10; mesodermal). Scale bar=100㎛. (B) The teratoma(from pri-IVF-2) contains stratified squamous epithelium with keratin (b1) and neural tissue composed of neurons (*arrow*; b2), glial cells embedded with neuropil from ectodermal cells; skeletal muscle bundles (b3) and adipocytes (*arrow*; b4), cartilage (*arrowhead*; b4) from mesodermal cells; respiratory epithelium with cilia (*arrow*; b5) and glandular epithelium (b6) from endodermal cells. Scale bar, 100 ㎛

A



B



4. Discussion

I established ESC-like cells with a normal ESC profile by generating viable blastocysts, which further expands this technique for stem cell engineering. I showed that immature primary follicles can be rescued from atresia and no changes in DNA contents were detected in the ESC-like cells produced. Eleven ESC lines established from IVF or parthenogenesis were successfully maintained *in vitro* for more than 20 passages, and additional characterization demonstrated their stability as ESC cell lines. In addition to the feasibility of the follicle-culture technology for reproductive purposes, the establishment of stem cells followed by parthenogenesis of follicle-derived oocytes can be applied to generate immune-specific therapeutic materials for both animals and humans.

In conclusion, further efforts to improve *in vitro* growth of primary follicles are necessary to generate more developmentally competent oocytes, and the technology used in this study will contribute to the development of novel assisted reproductive technologies in the field of reproductive medicine and domestic animal reproduction, as well as to cell regenerative medicine. Our data confirmed the earlier findings of Lenie et al. (Lenie *et al.*, 2004) on the feasibility of *in vitro* folliculogenesis. Infertile patients suffering from ovarian dysfunction can be treated with this technology. This system

can also be employed to increase reproductive efficiency in endangered species and contribute to understanding various biological procedures for folliculogenesis and granulosa cell differentiation. Stem cell engineering could use this follicle-manipulation technology to generate various ESC-derived biomaterials for therapeutic purposes.

CHAPTER 6

Cytoplasmic Study of Oocytes Derived from Natural Ovulation and *In vitro* Cultured Preantral Follicles

1. Introduction

Although mammal have a lot of follicles in their ovaries, only a minority of them complete their development by ovulation. In case of human, only about 400 follicles yield mature oocytes during a lifetime, whereas the others undergo atresia (Nicholas *et al.*, 2001; Schwartzman *et al.*, 1993) Thus, attempts have been made to utilize immature follicles to produce developmentally competent oocytes through *in vitro* culture. As a results, advance in culture system for in vitro culture of ovarian follicles have been made by several researchers (Lee *et al.*, 2007; Gong *et al.*, 2008).

In the previous chapters, I established culture system for *in vitro* culture for primary follicles and ESCs from homozygotic and heterozygotic embryos. For the common purpose as the improvement of ART, I conducted the experiments about cytosolic organelles of mature oocytes derived from *in vivo* and in-vitro follicle culture. It can be helpful to observe cytosolic organelles to improve the quality of mature oocytes. Because many preantral follicles degenerated or become inactivated in adult ovaries (Hirshfield *et al.*, 1991; O'Brien *et al.*, 200), the most part of research about follicle culture employ prepubertal mice. However, previous study demonstrated that the preantral follicles retrieved from adult mouse ovaries yield developmentally competent oocytes and could be a source of ESCs after IVF. Interestingly, parthenogenesis did not enable activated mature oocytes

derived from *in-vitro* follicle culture to blastocyst stage in adult mice (Kim *et al.*, 2009). Therefore, I hypothesized that different state of organelles in mature oocytes may induce the difference in developmental capacity. To confirm the hypothesis, I compared follicular and embryonic development and cytoplasmic organelles of MII oocytes derived from *in vivo* or *in-vitro* cultured follicles of different ages. Based on these observations, I conducted numerical analysis of the abnormality of cytoplasmic organelles.

2. Material & Method

Experimental animals

Naturally ovulated oocytes were collected by oviduct flushing of B6CBAF1 female in estrus 16h after mating with a vasectomized male. The flushing medium was M2 medium (Sigma-Aldrich, St Louis, MO). Also, female B6CBAF1 (C57BL6 x CBA/ca) mice of F1 hybrid strain were utilized as the experimental animals. Both prepubertal (2-week-old) and aged (8-week-old) mice were provided for the *in vitro* cultivation of preantral follicles. The mice bred in the Laboratory of Stem Cell and Bioevaluation, Seoul National University, were maintained under the condition of controlled lightening (14L:10D), temperature (20-22℃) and humidity (40-60%). All procedures for animal management, breeding, and surgery followed the standard protocols of Seoul National University. The protocols employed in this study were approved by the institution animal care and use committee (approval number; SNU-091028-4). Appropriate management of experimental samples and quality control of the laboratory facility and equipment were also conducted.

Isolation of preantral follicles

The ovaries obtained from 2-week-old prepubertal and 8-week-old adult females were removed aseptically and placed in Leibovitz L-15 medium supplemented with 10% heat-inactivated FBS (Lenie et al., 2004). Then, preantral follicles were retrieved mechanically using a 30-gauge needle. Early secondary follicles (100-125μm) with granulose cells and an intrafollicular oocyte were collected with mechanical isolation. Then, the retrieved follicles were placed singly in 10 μl droplets and overlaid with washed mineral oil in 60 x 15 mm Falcon plastic Petri dishes under the guidance of an ocular micrometer of a stereomicroscope (SMZ-645; Nikon, Tokyo, Japan) at 40x magnification. The follicles were washed three times in 10μl droplets of Leibovitz L-15 medium.

In vitro cultured of early secondary follicles

The early secondary follicles were cultured at 37℃ in an air atmosphere containing 5% CO_2 and placed singly in 10 μl culture droplets overlaid with washed mineral oil in 60 x 15 mm culture dishes. Early secondary follicles were cultured in ribonucleoside and deoxyribonucleoside containing α-MEM-glutamax medium supplemented with 5% (v/v) heat-inactivated FBS, 1% (v/v) ITS liquid medium, 100mIU/ml recombinant human FSH (Organon, Oss, The Netherlands) and 1% (v/v) lyophilized penicillin-streptomycin solution. On day 1 of culture, an additional 10μl of fresh medium was added to each droplet, and half of the medium was replaced with fresh medium every

other day from day 3 to the end of culture.

Collection of mature oocytes derived from in vitro cultured follicles

To retrieve mature oocytes, early secondary follicles of prepubertal and adult mice were cultured for 9 and 13 days, respectively. Oocyte maturation was triggered by exposure to 2.5 IU/ml hCG and 5ng/ml EGF 16 hours before the end of culture. COCs were retrieved from mucified follicles. Then, oocytes were freed from COCs by mechanical pipetting in M2 medium supplemented with 200 IU/ml hyaluronidase. Oocyte maturation to the MII stage was monitored by extrusion of the first polar body and expansion of the cumulus cells.

Transmission electron microscopy

Isolated MII oocytes were placed in modified Karnovsky's solution overnight and were post-fixed with osmium tetroxide for 2 hours. They were dehydrated sequentially in graded ethanol solutions. The samples conducted *en-bloc* staining overnight, and then embedded into spurr resin. Ultrathin section (60 nm thick) was made with an ultramicrotome. The samples were stained with uranyl acetate and Reynolds' lead citrate after being mounted on a grid. The samples were subsequently examined with an energy filtering

transmission electron microscope (LIBRA 120; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All data were replicated at least three times and subjected to statistical analysis. All images were analyzed using the imaging software (NIS-Elements BR 3.0, Nikon, Japan). General observation of oocyte ultrastructure, the number of deformed mitochondria, multivesicular body and the area rate of lipid droplets in ooplasm were counted. A generalized linear model (PROC-GLM) using Statistical Analysis System (SAS Institute, Cary, NC) was used to analyzed the data. When a significant model effect was detected, comparisons among groups were subsequently conducted using the least-squares or Duncan methods. A value of $P < 0.05$ was taken to indicate a significant difference.

3. Results

As shown in Table 8, there was no significant difference in the number of mucified cumulus oocyte complex derived from in vitro cultured early secondary follicles of 2-week and 8-week-old mice (91% vs 96%). The number of mature oocytes was more yielded in 8-week-old mice group than in prepubertal mice group (73% vs 40%). The cleavage rate after parthenogenetic activation was significantly different among three groups. In adult mice group, the lowest rate of 2-cell formation was observed when compared to naturally ovulated and prepubertal mice group (17% vs 84-94%). Also, the rate of blastocyst formation was significantly different among groups (0% vs 62-74%) There was no embryo developed to blastocyst in adult mice group.

The ultrastructure of organelles in mature oocytes derived from different origin was examined. As shown Figure 13, MII oocytes from all experimental group shows similar distribution of normal and vacuolated mitochondria. In adult mice group, the existence of lipid droplet was more remarkable. Numerical analysis based on TEM was also performed to compare state of organelles among groups more detailed. As shown Figure 14, the total number of mitochondria in the ooplasm was significantly greater in MII oocytes from adult mice than other group (38.6 vs 11.3-18.7; P=0.0064). In addition, there was no difference among groups about the

percentage of vacuolated mitochondria per total mitochondria (P=0.5819). The rate of the total lipid droplet area was significantly greater in adult mice group than others (P=0.0403). Moreover, the number of multivesicular bodies was significantly greater in adult mice group than others, too (P=0.0119).

Table 9. Developmental competence of mature oocytes derived from natural ovulation and in vitro cultured preantral follicles in prepubertal and adult mice.

Age of mice	No. (%)[c] of early secondary follicles		No. (%) of		No.(%)[e] of activated oocytes developing to	
	Cultured	Formed psedoantrum	Mucified[c]	No.(%)[d] of MII oocytes	Cleavage	Blastocysts
Natural	N.D	N.D	N.D	28	26(93)[b]	21(75)[b]
2 week	100	89(89)	91(91)	37(40)	31(84)[b]	23(62)[b]
8 week	108	96(89)	104(96)	76(73)	13(17)[a]	0(0)[a]

Model effects of the treatment on the number of cleaved oocytes and developing blastocysts were less than 0.0001 and less than 0.0001 respectively.
[c]Percentage of the number follicles cultured.
[d]Percentage of the number mucified cultured.
[e]Percentage of the number of mature oocytes.
[c,d,e] Different superscripts within the same parameter indicate significant difference among the treatments, $p < 0.05$

Figure 13. Comparison of ultrastructure of oocytes ovulated naturally (A), in vitro cultured preantral follicles of prepubertal (B) and adult (C) mice, seperatly. Specific differences were not observed exccept for presence of more lipid droplet in oocytes from adult mice. M: mitochondria; v-M: vacuolated mitochondria; L: lipid droplet; F: filaments. Scale bar = 2 μm

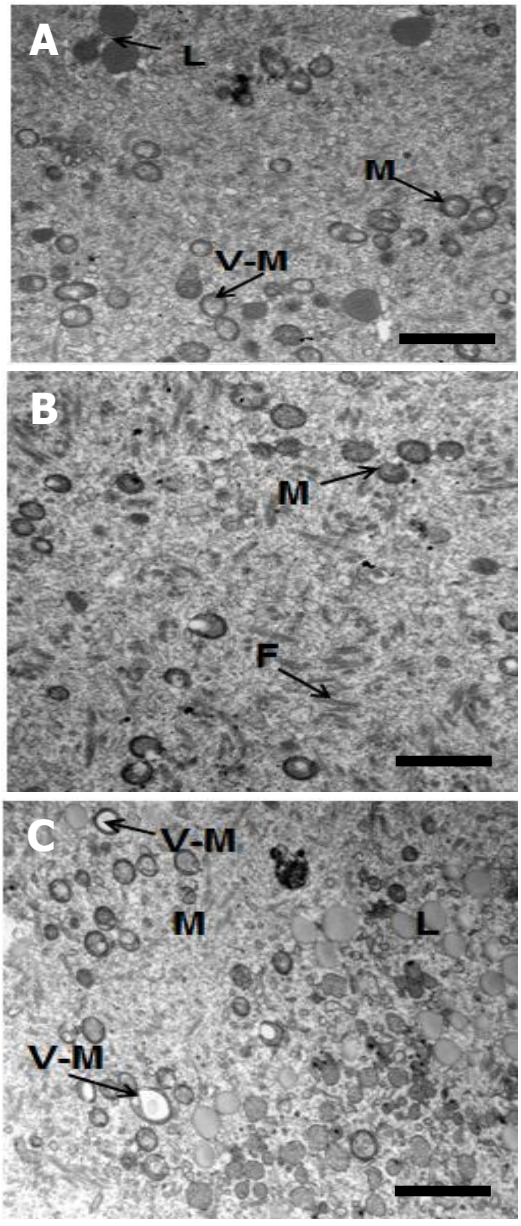
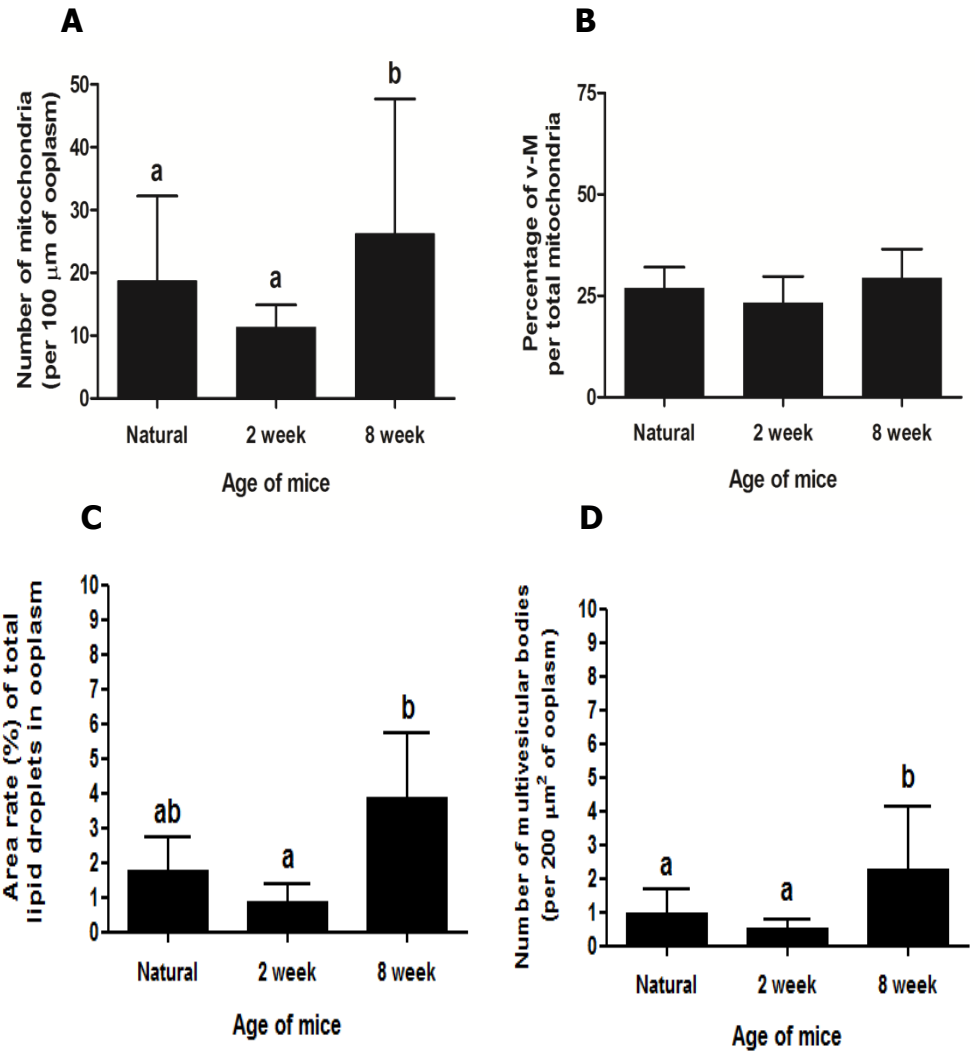


Figure 14. A comparison of oocyte organelles derived from naturally ovulated, in vitro cultured preantral follicles in ovaries of prepubertal (2 week) and adult (8 week) mice. For numerical analysis of the abnormality in ooplasm, the mean number of mitochondria per 100 μm^2 of ooplasm of mature oocytes (A), mean fraction of vacuolated-mitochondria per total mitochondria in 100 μm^2 of ooplasm (B), mean percentage of total lipid droplets area per 100 m^2 of ooplasm (C) and mean number of multivesicular body per 200 μm^2 of ooplasm (D) were counted and compared among groups. Data are presented as mean ± SD. Statistically significant difference were observed among the groups as indicated by different script.



4. Discussion

I demonstrated that retrieval age and culture condition may affect the quality of mature oocytes and developmental competency. As shown in Table 8, the rate of blastocysts formation in adult mice group was significantly lower than other groups. I could not obtain viable blastocysts in this group. These results are similar to previous studies that mature oocytes derived from preantral follicles of 2-week-old mice could form blastocysts more than 30% efficiency after parthenogenetic activation (Gong *et al.*, 2008). However, those derived from preantral follicles in 8-week-old mice didn't form blastocysts (Kim et al., 2009).

Through the observation of ultrastructure and numerical analysis, the difference of some organelles in mature oocytes among groups. MII oocytes from all experimental group shows similar distribution of normal and vacuolated mitochondria. But in adult mice group, the existence of lipid droplet was more outstanding. The total number of mitochondria in the ooplasm was significantly greater in MII oocytes from adult mice than other groups. In addition, there was no difference among groups about the percentage of vacuolated mitochondria per total mitochondria. The current discussion about the relation of mitochondria to the acquisition of developmental competence involves the definition of developmental potential itself, which is still controversial (Tarazona *et al.*, 2006). Further experiments

might be needed to demonstrate that point.

Lipids are usually observed in the cytoplasm of matured and developing oocytes and are known to play a role as an energy source for the growth of oocytes and further development (Enders and Schlafke, 1971). As shown in Figure 13 and 14, the total area of lipid droplets in the oocytes derived from *in vitro*–cultured follicles in adult mice had increased compared with other groups. However, further studies about the gene and molecular level might be needed to understand the precise relation between amount of lipid in cytoplasm and developmental competence.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Until now, our laboratory have made efforts to developing efficient preantral follicle culture system for generating developmentally competent oocytes, and several culture strategies for secondary follicles have been developed to date (Lee *et al.*, 2007; Lee *et al.*, 2008; Kim *et al.*, 2009). Viable blastocysts has also been generated from IVF or parthenogenetic activation of the follicle-derived oocytes (Gong *et al.*, 2008; Lee *et al.*, 2008; Gong *et al.*, 2009; Kim *et al.*, 2009), which confirms the feasibility of the follicle culture system in reproductive biotechnology and stem cell engineering. However, most follicle culture techniques deal with secondary follicles and a very limited success on the culture of primary follicles has been made to date (Lenie *et al.*, 2004). Thus, in chapter 3, for *in vitro*-culture of primary follicles, I conducted experiments to decide appropriate retrieval time first. The results indicate primary follicles retrieved from 11-day-old prepubertal mice yielded the most number of mature oocytes per animals. Since a significant difference in primary follicle retrieval and the number of mature oocyte per the follicle donor is observed during neonatal period, selection of appropriate retrieval time is definitely important to increase the efficiency.

Follicle stimulating hormone (FSH) is essential for viable oocyte recovery (Rosen *et al.*, 2009), oocyte maturation (Cortvrindt *et al.*, 1998), proliferation of granulosa cells (Cortvrindt *et al.*, 1997), formation of antral-like cavities (Adriaens *et al.*, 2004) and cumulus cell expansion (Eppig *et al.*, 1997;Eppig *et al.*, 1998). Exposure of primary follicles to

exogenous FSH is a prerequisite for producing developmentally competent oocytes and stimulates E2 and P4 production in follicles. In chapter 4, I conducted experiments to decide optimal combination of FSH concentration for primary follicle culture. Primary follicles cultured under 200/200 mIU FSH yielded most number of blastocyst formation after IVF and parthenogenetic activation. Moreover, FSH increased E_2 and P_4 production and expression of FSHR and LHR in primary follicles. Extrinsic FSH is pivotal factor in development of primary follicles.

Embryonic stem cells (ESCs) are derived from inner cell mass (ICM) of blastocyst through *in vitro* culture on MMC-treated feeder layer under appropriate culture conditions. Until now, several studies about ESC establishment in rat (Iannaccone *et al.*, 1994; Buehr *et al.*, 2008), porcine (Strojek *et al.*, 1990; Li *et al.*, 2003), bovine (Cherny *et al.*, 1994), primate (Thomson *et al.*, 1995) and human (Xu *et al.*, 2001; Richards *et al.*, 2002). Up to now, our research group established embryonic stem cells from oocytes yielded from in vitro culture of follicles retrieved from adult mice (Kim *et al.*, 2009) and autologous embryonic stem cells through parthenogenetic activation of oocytes matured within in vitro-cultured preantral follicles in prepubertal mice (Lee *et al.*, 2008).

In case of primary follicles, only limited success producing mature oocytes has been made using primary follicle

culture (Lenie *et al.*, 2004). I retrieved primary follicles from the 11-day-old prepubertal female mice and cultured in vitro to generate developmentally competent mature oocytes. In chapter 5, from the homozygotic and heterozygotic embryos activated by parthenogenesis and IVF, I established 11 ESCs lines for the first time. To investigate several characters of colony-forming cells as embryonic stem cells, I conducted conventional characterization methods such as ESCs-specific marker staining, gene expression of stemness-related genes and differentiation potential *in vitro* and *in vivo*. Established cell lines have various chracteristics as stem cells and can be utilized to several different purposes.

In the last project, chapter 6, the differences of ultrastructure among mature oocytes derived from various origins. The investigation of ultrastructure can be basic clue to solve the developmental limitation of embryo after parthenogenetic activation. In this experiment, I conducted Transmission Electronic Microscope (TEM) analysis for MII oocytes and then calculate numerical data about some microorganelles. TEM analysis can be a good tool for observing the ultrastructure of diverse type of cells. Number of mitochondria, Percentage of vacuolated mitochondria, rate of lipid droplet and number of multivesicular body were calculated. Percentage of vacuolated mitochondria in MII oocytes derived from early secondary follicles of 8-week-old mice was higher than other group (29.3% vs 25.8-26.8). Moreover, area rate of lipid droplet (3.88% vs 1.49-2.44%) and number of

multivesicular body per 100μm^2 ooplasm (1.13 vs 0.27−0.28) were significantly higher in MII oocytes derived from early secondary follicles of 8−week−old mice than other group. These results indicate that the difference or deformity of ultrastructure can be the cause of developmental retardation. Further studies in gene and molecular level, of course, are needed to demonstrate more definite reasons.

In conclusion, the results obtained from this study would contribute not only to refine in vitro culture system for obtaining mature oocytes with better developmental competency but also to utilize primary follicles as new source for obtaining eggs destined to degenerate and embryonic stem cells. Ultimately, my study will provide support to improve novel artificial reproductive technology and develop patient−specific stem cells and its applications.

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SUMMARY IN KOREAN

본 논문은 마우스 난소 유래 일차난포(primary follicle)의 체외 배양을 통해 성숙 난자를 얻고 체외수정과 단성생식을 통하여 배반포로부터 배아줄기세포주를 확립하는 과정에 대하여 기술되었다. 미성숙 난포의 체외 배양과 배아줄기세포 확립 기술은 동결 보존 기술과 연계하여 항암 치료로 인해 생식 능력을 상실한 여성 환자나 불임 환자의 생식 능력 보존에 기여할 수 있으며 최종적으로 줄기세포를 이용한 세포치료에 주춧돌이 될 수 있는 중요한 기술이다. 일차난포는 다양한 발달단계의 난포 중 휴지기에 있는 원시난포(primordial follicle)를 제외하면 난소 내에 가장 많이 존재하는 난포로써 체외 배양 기술의 확립이 가지는 가치가 크다. 따라서 본 연구를 통해 일차 난포의 효율적인 체외 배양 조건의 정립과 배아줄기세포주의 확립을 진행하였다.

먼저 일차난포를 회수하기 위해서 최적의 마우스 일령을 선택하기 위한 실험을 진행하였다. 7-14일령의 B6CBAF1 mice로부터 일차 난포를 회수한 결과 9, 10, 11일령의 마우스에서 가장 많은 수의 일차난포를 회수할 수 있었고 체외 배양 결과 11일령의 마우스로부터 회수한 일차 난포의 발달률이 가장 좋았으며 체외성숙 후, 마리당 가장 많은 숫자의 성숙 난자를 얻을 수 있었다. 따라서 11일령의 마우스를 실험에 이용하기로 하고 이후 실험을 진행하였다.

FSH(follicle stimulating hormone)은 난구세포(granulosa cell)의 성장을 촉진하고 난자가 정상적으로 발달하는데 중요한 역할을 하며 난포의 체외배양에도 필수적인 호르몬으로 알려져 있다.

따라서 일차난포의 체외 배양에 필요한 최적의 FSH 농도를 결정하기 위한 실험을 진행하였다. 11일령의 B6CBAF1 mice에서 일차난포를 분리하여 각기 다른 농도조합의 FSH 처리 하에서 배양하였을 때, 전체 배양기간 동안 200mIU의 FSH를 처리해준 실험군에서 가장 높은 pseudoantrum 형성률을 보였고, 체외수정(in vitro fertilization)과 단성생식(parthenogenesis) 후에 배반포까지의 발달률이 가장 좋았다.

줄기세포는 끝없는 자기 재생(self-renewal) 능력을 가지고 있으며 인체의 모든 세포로 분화 가능한 전분화능(pluripotency)을 가지고 있는 세포로써 보건의료적, 산업적 가치가 큰 세포이다. 일차난포의 체외배양을 통해 얻어진 성숙 난자로부터 배아줄기세포를 확립했다는 보고는 지금까지 없었기 때문에 관련 실험을 진행하였다. 체외수정과 단성생식을 통해 얻어진 배반포를 mitomycin C 처리된 지지세포(feeder cell) 위에 올리는 방법을 통해 각각 4개, 7개의 배아줄기세포주를 확립하였다. 확립된 세포주들은 세포 표면에 POU5F1, SSEA-1 같은 배아줄기세포 특이적인 마커(surface marker)들을 발현하고 있었으며 *Nanog, Gdf 3* 같은 stemness와 관련된 유전자들을 발현하고 있었다. 또한 확립된 배아줄기세포들은 세포구 (embryo body)와 기형종(teratoma)을 형성했으며 분석 결과 두 실험 모두에서 삼배엽을 이루는 세포들과 조직이 관찰되었다. 일련의 실험들을 통해 확립된 세포들은 배아줄기세포로서의 특징들을 모두 가지고 있음이 검증되었다.

마지막으로 마지막으로 투과전자현미경(transmission electronic microscope) 분석을 통해 자연배란, 2주령과 8주령의 2차 난포로부터 각기 유래한 성숙 난자의 세포 소기관의 차이를

규명하였다. 성숙 난자의 세포 소기관의 차이는 정상적인 수정이나 단성생식 후 배아발달에 영향을 미칠 수 있으며, 그 차이를 규명하는 것은 향후 배아 관련 연구에 있어서 하나의 전기를 마련할 수 있을 것으로 기대된다. 분석 결과 8주령의 2차 난포로부터 유래한 성숙 난자에서 단위 면적당 미토콘드리아 수와 지방(lipid droplet)함유울이 더 많음을 관찰할 수 있었다. 본 연구에서 진행된 실험만으로는 알 수 없지만 후속 실험들을 통해 이러한 차이와 배아의 발달 사이에 어떠한 상관관계가 있는지 밝혀낼 수 있을 것이다.

학위 과정 동안 수행한 일차난포의 효과적인 체외배양을 위한 최적일령 선정 및 난포자극호르몬 (FSH)의 최적농도 선택, 일차난포의 체외배양을 통한 효율적인 성숙난자의 획득, 체외수정 및 단성생식을 통한 배반포 단계까지의 발달, 그리고 마지막으로 배반포로부터 배아줄기세포주의 확립에 관한 일련의 실험들을 통해 도출된 결과들은 줄기세포를 이용한 세포치료기술 개발과 불임치료 및 인공번식을 포함한 인간과 동물의 보조생식술 기술발전에 기여하는 대단히 중요한 연구결과라고 판단된다

감사의 글

청운의 꿈을 안고 대학원에 들어온지도 벌써 7년의 시간이 흘렀습니다. 돌이켜 보면 즐겁고 기뻤던 순간들도 있었고 힘들고 괴로웠던 시간들도 있었습니다. 때론 제 비뚤어진 성품과 부족한 능력 때문에, 때로는 인간에 대한 회의와 절망 때문에 참으로 많은 시간 힘들어 했었습니다. 그런 이유에서 7년 간의 대학원 생활은 저에게 학문을 수양하는 시간 그 이상의 의미로 다가오는 것 같습니다. 아직도 '박사' 라는 수식어를 달기에는 한 없이 부족한 저이지만 제가 무사히 학위 과정을 마칠 수 있도록 도와주신 분들에게 지면을 빌려서나마 감사의 인사를 드리고자 합니다.

먼저 저를 이 땅에 태어나게 해주시고 지금껏 보살펴주신 부모님과 동생에게 너무나 감사하다는 말씀을 드리고 싶습니다. 그 은혜는 제가 백만 번을 다시 태어난다고 해도 다 갚을 수 없을 것입니다. 그리고 기나긴 학위 기간 동안 수 없이 많은 힘든 순간들과 여러 위기에도 불구하고 못난 제 옆을 변함 없이 지켜주고 용기를 불어 넣어주었던 선영이에게 특별히 고맙다는 말을 전하고 싶습니다. 아울러 학위 과정 동안 저를 지도 해주시고 무사히 졸업할 수 있도록 도와주신 임정묵 교수님과 보잘것없는 논문을 다듬어 주시고 조언을 아끼지 않으셨던 한재용 교수님, 이은송 교수님, 한호재 교수님, 그리고 송권화 교수님께도 고개 숙여 감사의 말씀을 드립니다.

아울러 학위기간 동안 함께 동고동락했던 발생공학연구실 선후배님들에게도 감사하다는 말씀을 드립니다. 이은주 박사님, 이승태 박사님, 지금은 다른 곳에 계시지만 항상 학생들을 먼저

챙기셨던 박종흠 박사님, 힘들 때 많이 격려 해주셨던 안지연 박사님, 처음 실험실에 들어왔을 때 실험을 가르쳐 주었던 장미 선배, 항상 열심히 실험했던 승표 선배, 실험실에 있었을 때나 취직을 하고 나가서도 항상 관심을 가져주고 도와주는 길아 선배, 머나 먼 미국 땅에서 꿈을 위해 박사 후 과정을 밟고 있는 정규 선배, 힘들 때에도 언제나 웃음을 잃지 않는 서진 선배, 언제나 스마트한 재희 선배, 변리사가 된 하이톤의 수진이, 실장일 하느라 너무 고생한 애봉이, 후배지만 실험에 대한 열정이 대단한 보햄 (남자랑 결혼함), 언제나 묵묵히 맡겨진 일을 해내는 종특, 이제 본격적으로 데이터를 뽑아낼 준비를 하고 있는 부사수 효숙 (렉시 아님), 요즘 더 댄디해진 정기 (3위), 어마어마한 피지컬을 자랑하는 명욱리, 2NE1도 울고 갈 패션 센스를 자랑하는 김영임이, 말 못할 난치병으로 고생하고 있는 동우, 그리고 힘든 대학원 생활 동안 친구로써 서로 고민을 털어 놓고 서로 위로 해주었던 승민이와 항상 고맙고 미안한 동기 채현이가 없었다면 학위 과정을 무사히 마칠 수 없었을 것입니다. 모두들 정말 감사했습니다. 7년 동안의 소중했던 시간들을 제 마음 속에 깊이 간직 하겠습니다.

이제 저는 실험실을 떠나 새로운 시작을 하려고 합니다. 밖에서 바라보는 대학원과 안에서 제가 경험했던 '대학원 생활' 은 많이 달랐던 것 같습니다. 아직도 많은 대학원생들이 본인의 문제가 아닌 여러 이유로 절망하고 학업의 길을 포기하고 있습니다. 수직적 상하 관계에서 비롯된 소통의 부재, 아니 소통을 가장한 억압과 착취가 빈번하게 일어나고 있으며 여타의 문제점들과 맞물려 학생들이 대학원을 회피하는 결과를 낳고 있습니다. 이는 본인이 대학원생이라는 사실을 자조 섞인 말투로 한탄하는 수준을 넘어 장기적으로는 국가의 과학 기술 발전을 저해하는 걸림돌이 될 것이 자명합니다. 지금 보다 더 나은 상황에서 미래의 학자를

꿈꾸는 사람들이 열심히 연구하고 공부할 수 있는 순간이 오기를 바래봅니다. 저를 생각 해주시고 아껴주시는 모든 분들, 그리고 휴일도 없이 늦은 시간까지 연구실의 불을 밝히고 지치지 않는 열정으로 연구에 혼신의 힘을 다하고 있는 이 땅의 모든 연구자들에게 제 논문을 바칩니다.