

**Establishment of Long-Term Culture and Elucidation of Self-
Renewal Mechanisms of Primitive Male Germ Cells in Cattle**

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Abstract

Spermatogonial stem cells (SSCs) are the unique adult stem cells in the testis that can transmit a legacy from one generation to the next and maintains continuity of life by the process of spermatogenesis. In dairy cattle, gene targeting has potential application in agricultural and biomedical sciences. The major hindrance in a practical application of this research is the lack of an optimal long-term culture system supporting for self-renewal of SSCs. Understanding of the species-specific requirements of growth factors and signaling pathways involved in self-renewal will be helpful in optimization of culture conditions and establishment of cell lines in cattle.

Firstly, we examined growth factors, matrix substrates and serum-free supplements to develop a defined system for culturing germ cells from neonatal bovine testis. Poly-L-lysine was a suitable substrate for selective inhibition of the growth of somatic cells and made it possible to maintain a higher germ cell/somatic cell ratio than could be maintained with gelatin, collagenase and DBA substrates. Among the serum-free supplements tested in our culture medium, knockout serum replacement (KSR) supported the proliferation and survival of germ cells better than the supplements B-27 and StemPro-SFM after sequential passages of colonies. Under our optimized culture conditions consisting of 15% KSR-supplement on poly-l-lysine-coated dishes,

stem cell and germ cell potentials of cultured germ cells were maintained with normal karyotype for more than 2 months (over 13 passages).

Next, we performed experiments to understand molecular mechanisms contributing to self-renewal and maintenance of pluripotency of bovine germ cells in culture. We confirmed the glial cell line-derived neurotrophic factor (GDNF)-mediated self-renewal of germ cells. The addition of GDNF to the culture enhanced the phosphorylation of MAPK1/2 and induced the activation of the MAPK signaling pathway. The inhibition of MAPK signaling by pharmacological inhibitor PD 0325901 reduced germ cell proliferation and abolished colony formation in culture. However, inhibition of phosphoinositide 3-kinase-AKT (PI3K-AKT) signaling, a dominant signaling pathway for the self-renewal of mouse germ cells, by LY294002 did not show any effects on cell proliferation or colony formation efficiency of bovine germ cells in culture. The expression of cell cycle-related regulators cyclin D2 and cdk2 (cyclin dependent kinase 2) were downregulated upon inhibition of MAPK signaling. These results indicate that the activation of MAPK plays a critical role in the self-renewal of bovine germ cells in culture via cyclin D1 and cdk2.

In this study, we proposed culture system for long-term expansion of bovine germ cells. In addition, we show that the different signaling pathways required for the

self-renewal of germ cells in mice (PI3K-AKT and MAPK) and bovines (MAPK). Our results will pave the ways towards establishment of germ cell lines and generation of genetically modified animals in domestic species.

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Table of Abbreviations

AFP	α -fetoprotein
AI	Artificial Insemination
AKT	Protein kinase B
ANOVA	Analysis of variance
ART	Assisted Reproductive Technologies
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
DAPI	4',6-diamidino-2-phenylindole
DBA	Dolichos Biflorus Agglutinin
ECM	Extracellular matrix
EGC	Embryonic germ cells
EGF	Epidermal growth factor
ESC	Embryonic stem cells
ETT	Embryo transfer technology
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
GEL	Gelatin
GS	Germ stem cell
IMDM	Iscove's modified Dulbecco's medium
KITL	Kit ligand
KSOM	Embryo Culture media
KSR	Knockout serum replacement
LIM	LIM homeobox1
MACS	Magnetic-activated cell sorting
MAPK	Mitogen activated protein kinase
MEM	Minimum essential medium
MEXT	Ministry of Education, Culture, Sports, Science and Technology of Japan

MMLV	Reverse transcriptase product
NEAA	Non-essential amino acid solution
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cells
RIPA	Radioimmunoprecipitation assay
RNA	Ribo Nucleic Acid
RNaseH	Ribonuclease H
SSC	Spermatogonial stem cells
TBS	Tris buffered saline
UCHL1	Ubiquitin carboxyl-terminal esterase L1

Chapter 1

General Introduction

1.1 Spermatogenesis

Mammalian spermatogenesis is a sequential, organized process of self-renewal and differentiation of spermatogonial stem cells found in the testis, resulting in the continuous production of spermatozoa throughout the life of male (Russell et al. 1990, Huckins et al. 1971, Clermont et al. 1972, Meistrich and van Beek et al. 1993). Spermatogenesis protects the genomic integrity and plays essential role in species preservation and genetic diversity (Wistuba et al. 2007). This process of spermatogenesis is conserved among mammalian species. Although the duration of spermatogenesis from self-renewing stem cells to resulting mature spermatozoa among species is unique and unchangeable, 32 days in mice (Oakabeg et al. 1956), 74 days in human (Russell et al. 1990) and 63 day in bull (Hochereau et al. 1964). In this duration, SSCs undergo mitotic multiplication, meiotic recombination of genetic material and maturation of spermatozoa (Ehmcke et al. 2006). This is a highly productive process start at the age of puberty of animals producing 100 million spermatozoa in adult men (Sharp et al 1994) and 6000 million sperm in mature bull (Amann et al. 1974). Male fertility completely relies on the steady state of spermatogenesis in pubertal animals.

1.2 Spermatogonial stem cells

Spermatogonial stem cells (SSCs) are the adult stem cells found in mammalian testis, which provides the foundation of spermatogenesis. These cells are originated from gonocytes, which are the derivative of primordial germ cells (PGC). PGC is germline lineage cells that arise from extra embryonic mesoderm at the posterior end of the primitive streak and migrate to the urogenital ridge, which forms gonads (Lawson and Hage 1994). PGCs ceased proliferation in only male genital ridge and called primitive male germ cells. After birth, gonocytes resume proliferation, migrate to the basement membrane of the seminiferous tubules, and transform into spermatogonial stem cells (SSCs). The transition of gonocytes to SSCs after birth occurs at 3 day in mice (McLean et al. 2003) and 20 weeks in bull (Curtis and Amann 1981).

SSCs have the unique ability in both self-renewal and differentiation characterization. The existing SSCs self-renewal model is originally proposed by (Huckins 1971 and Oakberg 1971). This model proposes that only A single (As) spermatogonia acts as stem cells and gives rise to committed cells that divide into A paired (Apr) and A lined (Aal) during spermatogenesis. The extended studies of As model using genetic labeling, lineage analysis and live imaging has put forward striking observation that As spermatogonial cells represent heterogeneity (Nakagawa et al. 2010) and also show that the population of Apr and Aal SSCs changes their behavior

during regeneration and acquires stem cell potential. The actual cell number of SSCs having stem potential is very low ~2000 cells per testis as calculated using the pulse-labeling strategy (Nakagawa et al. 2010) and ~3000 cells per testis using serial transplantation assay (Nagano et al. 2003). This number is very low as compared to the As model based on morphological characteristic (Tegelenbosch and de Rooij 1993), estimated about ~35000 cells per testis. These findings support the heterogeneity of As spermatogonial stem cells in state of their morphological similarity.

1.3 Spermatogonial stem cell niche

Adult stem cells can self-renew only in the specialized microenvironment called niche, which provides architectural support, growth factors and extrinsic stimuli (Schofield. 1978, Spradling et al. 2001). SSCs reside in the basement of the seminiferous tubules and constitute niche surrounded by Sertoli, Leyden and myoid cells (de Rooij et al. 2009). The Sertoli cells seem to be a particularly important component of the SSC niche, as numerous factors such as glial cell-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), kit ligand (KITL), activin A and bone morphogenic protein 4 (BMP4) are produced by Sertoli cells and affect self-renewal, proliferation and differentiation of SSCs (Boyer et al. 2012). Recent evidence suggests that As, Apr and Al spermatogonia find along the peritubular blood vessels and preferentially locate in a

specific compartment might serve as niches (Chiarini-Garcia et al. 2001, Yoshida et al. 2007).

1.4 Identification of SSCs

1.4.1 Transplantation assay

The first transplantation assay for identification of SSCs in mice was given by (Brinster and Zimmermann et al. 1994). Recipient mice are depleted for endogenous SSCs, and transplanted donor-derived SSCs that resulted in complete spermatogenesis. This assay gives functional and quantitative analysis of SSCs, in which donor-derived colonies are generated from single transplanted SSCs (Nagano et al. 2003). In addition, cross-species transplantation between mice and rat (Clouthier et al. 1996), mice and hamster (Ogawa et al. 1999) result into complete spermatogenesis and production of healthy offspring. Surprisingly, the transplantation of germ cells from non-rodents species, i.e. rabbits and dogs (Dobrinski et al. 1999), pigs, cattle and horse (Dobrinski et al. 2000), shows colonization of cells in mice testis, but lacking complete spermatogenesis. This finding raises the question of transplantation as bioassay for determination stem cell potential of non-rodent species (Dobrinski et al. 2005).

1.4.2 Biochemical characterization of SSCs

In recent years, several molecular markers have been identified for SSCs in rodents are summarized in (Table 1.1). Most of these markers are not associated with functions, the level of gene expression and express by other undifferentiated SSCs (Apr and Aal) (Oatley et al. 2013). In search of functional markers, Oatley et al. (2013) identified ID4 as a marker, which only restricted to As spermatogonia in the testis and a protein essential for normal spermatogonial stem cell renewal both in vitro and in vivo. Some of these markers are identified as SSCs markers in domestic animals (Table 1.1) and are conserved among mammalian species.

Table 1.1 Overview of spermatogonial markers in domestic species

Molecular Markers	Species					
	Mice	Bovine	Porcine	Sheep	Goat	Buffalo
VASA/DDX4	+ Sakai et al.2004	+ Fujihara et al. 2011	ND	+ Borjigin et al. 2010	ND	+ Goel et al. 2010
UCHL1	+ Kwon et al.2003	+ Herrid et al. 2007	+ Luo et al. 2006	+ Rodriguez-Sosa et al.2006	+ Heidari et al. 2012	+ Goel et al. 2010
DBA	+ Kim et al. (Up)	+ Izaydar et al. 2002	+ Goel et al. 2007	ND	ND	+ Goel et al. 2010
PLZF	+ Buass et al. 2004	+ Reding et al. 2010	+ Goel et al. 2007	+ Borjigin et al. 2010	ND	ND
THY1	+ Kubota et al.2004	+ Reding et al. 2010	ND	ND	+ Abbasi et al. 2013	+ Rafeeqi et al. 2013
Plzf	+ Buass et al. 2004	+ Reding et al. 2010	ND	ND	ND	ND
Pouf1	+ Pesce et al.1998	+ Fujihara et al. 2011	ND	ND	ND	+ Goel et al. 2010
Nanog	ND	+ Fujihara et al. 201	ND	ND	ND	ND
Etv5(Erm)	+ Oatley et al. 2007	ND	ND	ND	ND	ND
Lhx1	+ Oatley et al. 2007	ND	ND	ND	ND	ND
Bcl6b	+ Oatley et al. 2006	ND	ND	ND	ND	ND
Gfra1	+ Naughton et al. 2006	ND	ND	ND	ND	ND
Ret	+ Naughton et al. 2006	ND	ND	ND	ND	ND

+: expression of protein in undifferentiated SSCs, ND: Not determined, UP: unpublished

1.5 Spermatogonial stem cell culture

1.5.1 Isolation and enrichment of SSCs

The isolation and enrichment of SSCs is the first step towards the establishment of GS cell lines. The isolation of SSCs are challenging because of their limited numbers. The two-step enzymatic digestion was first proposed by Davis and Schuetz (1975), which is the most widely used techniques for isolation of SSCs in rodents. For further enrichment of spermatogonial stem cells, different approaches such as differential plating (Dym et al. 1995), percoll gradient (Van Pelt et al.1996), magnetic-activated cell sorting (MACS) or fluorescence activated cell sorting (FACS)) have been used independently or in combination.

In livestock species, SSCs isolation and enrichment methods has been progress tremendously during last few years. The differential plating is a better method for the enrichment of SSCs in comparison with MACS and FACS for bovine SSCs (Herrid et al. 2009).

1.5.2 Establishment of culture system and germ cell (GS) lines

In 2003, the long-term cultures of SSCs have been successful established in mice (Kanatsu-shinohara et al. 2003). These cells proliferated over a 2-year period (>1085-fold) in the presence of glial cell line derived neurotrophic factor (GDNF) and

restored spermatogenesis following transplantation into the seminiferous tubules of infertile recipient mice. The details are discussed in chapter 2.

1.5.3 Multipotent germ cell (mGS) line

SSCs under certain culture condition acquired embryonic stem (ES) cell like characteristics called multipotent germ cell (mGC) lines have been first generated from germ cells in neonatal mouse testis without introduction of exogenous reprogramming factors (Kanatsu-Shinohara et al. 2004). These cell populations fail to form colonies following testicular transplantation, showing that they are devoid of spermatogonial potential and have the ability to differentiate into three germ layers. Later on, successful evidence of generation of mGS cell line has been shown from adult mice (Guan et al. 2006, Seandel et al. 2007), and human testicular cells (Conrad et al. 2008).

1.5.4 Status of SSCs culture in livestock species

Although SSCs from many mammalian species proliferate for more than six months in the seminiferous tubules of immunodeficient mice (Kubota et al. 2006), to date no culture systems have been established in livestock species. Current culture systems (Table 1.2) for bovine SSCs fail to achieve a long-term culture and the establishment of germ cell lines (Izadyar et al. 2002, 2003, Oatley et al. 2004, Aponte et

al. 2006, 2008, Fujihara et al. 2011). In porcine, culture SSCs cannot survive more than a week (Dirami et al.1999, Goel et al. 2009).

Table 1.2 Overview of culture condition for SSCs in domestic species

	References	Culture conditions	Age of donor	Culture term
Bovine	Izadyar et al. 2003	Compare MEM and KSOM medium+0 to 10 % FCS	5 month	MEM+2.5% FCS is effective for germ cell survival[]than KSOM, no expansion, showing differentiation during 150 days culture
	Oatley et al. 2004	DMEMF+10%FB S+GDNF	1 to 2 month	2 week
	Aponte et al. 2006	MEM+2.5% FCS+GDNF	4 to 6 month	25 days, no passage, differentiation
	Aponte et al. 2008	StemPro-SFM+ GDNF, EGF and FF	4 to 6 month	25 days, no appearance of colonies after passage
	Fujihara et al. 2011	DMEMF12+10% FCS	1-10 days	1.5 month
Porcine	Dirami et al. 1999	DMEMF12+10% FCS	2 month	1week
	Goel et al. 2009	DMEMF12+10% FCS	1-10 days	3 week, reduction of germ cells every passage
	Kujik et al 2009	StemPro SFM+ GDNF, EGF and FF	3 to 4 days	9 passage (30 days), reduction of germ cells every passage

1.6 Signaling pathways regulating the fate of spermatogonial stem cells

The establishment of GS and mGS cell lines from SSCs attracts to study their regulatory mechanisms, particularly signaling pathways regulating self-renewal and differentiation of germ cells as well as conversion to pluripotent like cells. The fate decision of SSCs in culture cells is regulated by autocrine and paracrine pathways regulated interaction between germ cells and niche (He et al. 2009). Contrast to this, recent reports using transplantation assay show that germ cells alone are regulated spermatogonial differentiation through autocrine pathways (França et al. 1998).

The signaling molecules and pathways that regulating spermatogonial stem cell fate in culture has been well documented in rodents are summarised in Fig.1.1. The details will be discussed in chapter 3.

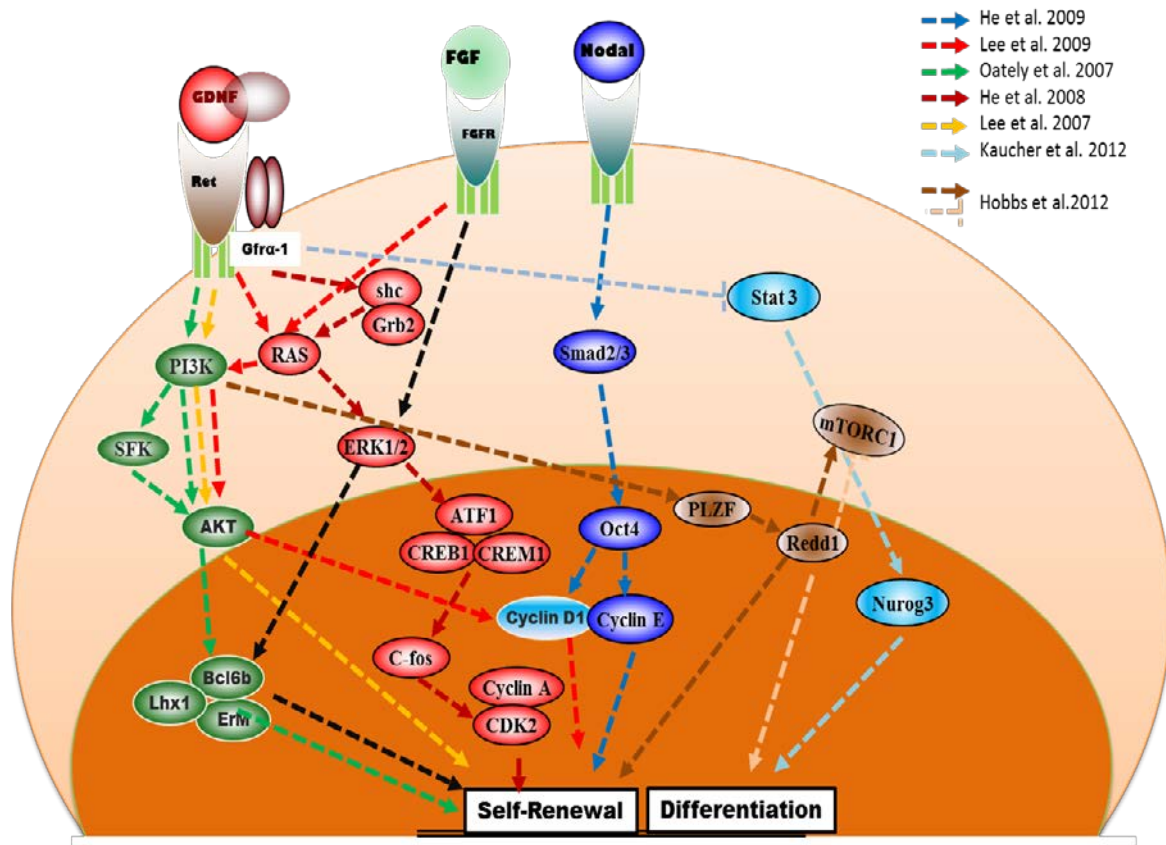


Figure 1.1 Crucial signaling pathways for regulation spermatogonial stem cell self-renewal and differentiation in culture

1.7 Male germ cells transplantation: Emerging tool for generation of transgenesis in livestock

Xeno-transplantation of germ cells into mouse testis and in vitro culture of germ cells allow an alternative method for the development of transgenic and knockout technology in domestic species. These approaches are already available in rodents, direct injection of vectors carrying exogenous genes into the seminiferous tubules of pre-pubertal testis (Sehagal et al. 2011) and viral vector transfection into in vitro cultured germ cells prior to germ cell xeno-transplantation (Nagano et al. 2000, Nagano et al. 2001, Orwig et al. 2002 and Hamra et al. 2002). The germ cell transplantation assay has been developed in pigs (Honaramooz et al. 2002), goats (Honaramooz et al. 2003) and cattle (Izadyar et al. 2003, Hill et al. 2006) (Table 3). In particular, livestock species do not require an immune suppression procedure to allow donor cell survival in recipient testis (Horrid et. al. 2013). The transduction of an adenovirus-associated vector to introduce a transgene into goat germ cells have promising results in generating transgenic goats (Honaramooz et al. 2008). In combination of germ cell transplantation, development of culture system will give birth to emerging technology, having potential application of production of transgenic livestock. The schematic representation potential application of germ cell transplantation is illustrate in Fig. 1.2.

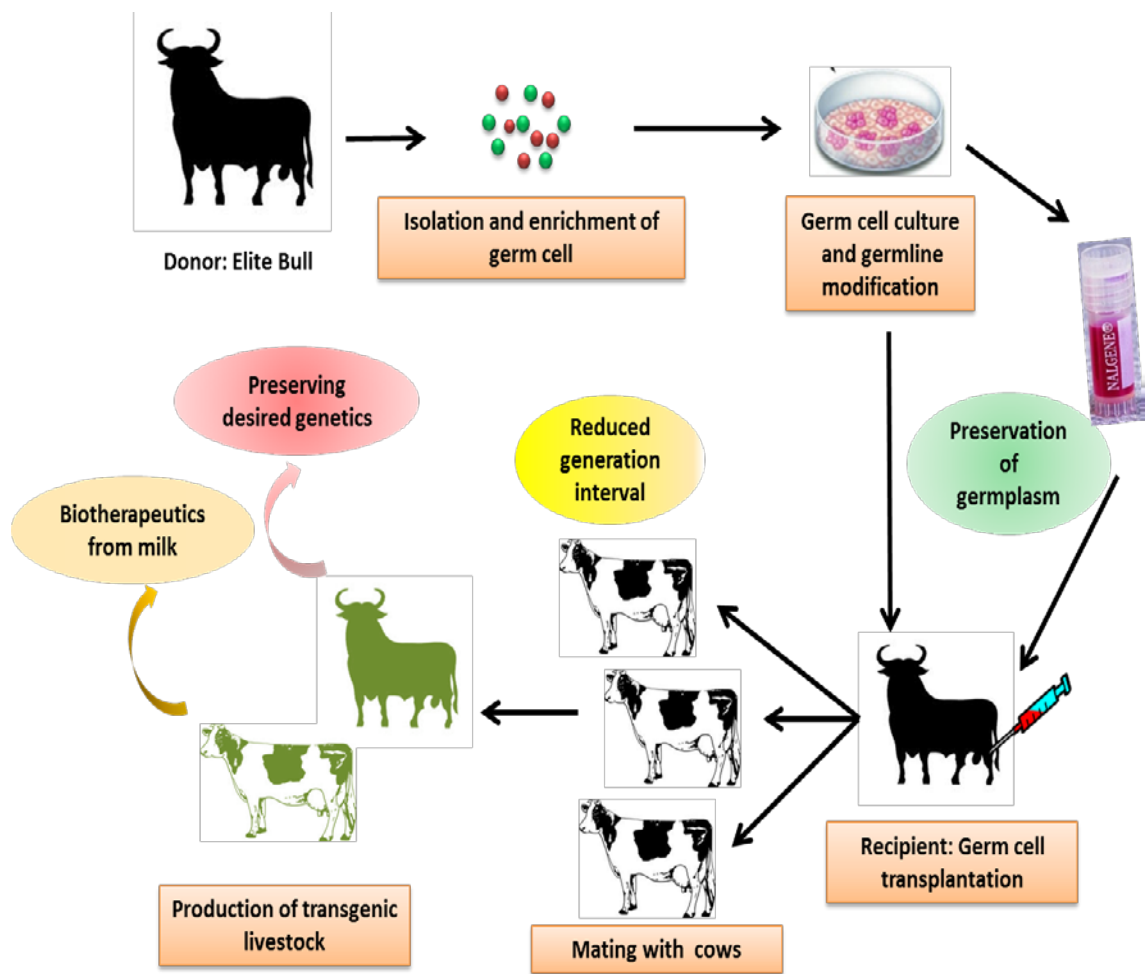


Figure 1.2 Production of male germ cell mediated transgenic animals and their application

Table 1.3 Germ cell transplantation and transgenesis in livestock species

Species	Donor-derived spermatogenesis	Transmission of donor haplotype to offspring	References
Pig (homologous)	Complete	Not determined	Honaramooz et al. 2002
Goat (homologous)	Complete	Yes	Honaramooz et al. 2003
Cattle (autologous)	Complete	Not determined	Izadyar et al. 2003
Cattle (homologous)	Not demonstrated	Not determined	
Cattle (homologous)	Not demonstrated	Not determined	Hill et al. 2006
Goat	Complete with integration of transgene (Adenovirus)	Production transgenic embryo	Honaramooz et al. 2008

1.8 Scope of the thesis

1.8.1 Potential application of male germ cell culture and transplantation in animal breeding and transgenesis

Assisted Reproductive Technologies (ART) has revolutionized impact on animal breeding program along with conventional breeding policies. It can be used to increase the accuracy of selection and therefore enhanced genetic gain to speed up the dissemination of genes from animals with exceptionally high genetic merit to a commercial population. A major benefit of ART is to minimize inbreeding rates and reduce generation intervals. Currently practiced ARTs are Artificial Insemination (AI), Embryo Transfer technology (ETT) and reproductive cloning. The main limitation of the use of current ARTs is its low success rate, high cost to produce an animal and necessity of a large number of females.

The reproductive cloning and gene targeting technologies have opens the door for the generation of transgenic animals for improving traits of agricultural importance and biomedical animals for human therapeutics. Recently scientific community has attracts towards this research to address future challenges of food security as well as bioreactors for producing proteins of high interest in the human pharmaceutical industry. Current approaches i.e. cloning and pronuclear microinjection are expensive and costly

for production of transgenic animals. Unfortunately, despite the advancement of embryonic stem cells in rodents and human, there is unavailability of such proven cell lines in livestock species. Hence, there is immediate need to address this problem and to develop effective technology.

In dairy cattle, germ cell transplantation technology in combination with gene targeting will serve as a time saving and cost effective tool for maximizing genetic gain and for preserving desirable genetics for the production of superior food animals. The major hindrance in a practical application of this research is the lack of an optimal long-term culture system supporting for self-renewal of SSCs in vitro. The understanding of the species-specific requirements of growth factors and signaling pathways involved in self-renewal will be helpful in optimization of culture conditions and establishment of cell lines in cattle.

Chapter 2

Essential Factors for Long-term Culture of Male Germ Cells in Cattle

2.1 Introduction

SSCs are unique adult stem cells in the testis that undergo self-renewal and differentiation to produce spermatozoa throughout a life and transmit genetic information across generations (De Rooij et al. 1999). These cells postnatally ascend from gonocytes, which reside mostly in the center of the seminiferous tubules and remain quiescent (Vergouwen et al. 1993). Gonocytes resume proliferation, migrate to the basement membrane and are transformed to SSCs after arriving at a stem cell niche. The niche is a specialized microenvironment in the seminiferous tubules, and provides extrinsic stimuli that promote the self-renewal of SSCs or their differentiation into meiotic germ cells (Oatley et al. 2008). Pluripotent and multipotent stem cell lines have been developed from unipotent SSCs in mice (Kanatsu-Shinohara et al. 2004, Seandel et al. 2007) and humans (Conrad et al. 2008). The generation of these autologous pluripotent cell lines from SSCs before the initiation of cancer treatment and subsequent autologous transplantation after cancer treatment could be a means of preserving the fertility of male cancer patients (Strijk et al. 2013).

The limited number of SSCs in the testis (Meistrich & Van Beek et al. 1993) hampers studies of the biological characteristics of SSCs. One approach to solving this problem is to develop culture conditions that support the self-renewal of SSCs and that

maintain their pluripotency. Glial-cell-line-derived neurotrophic factor (GDNF) was identified, as a factor that is required for SSCs self-renewal in vivo (Meng et al. 2000). Subsequently, Nagano et al. (2003) developed a short-term culture system supplemented with GDNF that improved the survival of germ cells. These cells complete spermatogenesis after transplantaion into the testis of immunodeficient mice. Longer-term culture of SSCs was achieved by adding other growth factors and hormones in addition to GDNF (Kanatsu-Shinohara et al. 2003). However, the growth factor requirement for proliferation of germ cells is strain specific; in mice, strains C57BL/6 and 129/Sv require FGF and GDNF (Kubota et al. 2004a), while strain DBA requires FGF, GDNF and EGF (Kanatsu-Shinohara et al. 2005). By using species-specific culture components, culture systems and germ cell lines have been established in rat (Hamra et al. 2005, Ryu et al. 2005), hamster (Kanatsu-Shinohara et al. 2008) and rabbit (Kubota et al. 2011).

In livestock species, long-term culture systems for germ cells could reduce the time and money needed to produce transgenic animals and to preserve endangered species, and could be an alternative for pronuclear microinjection and somatic cell cloning (Dobrinski et al. 2006). Although several attempts have been made to develop a culture system for bovine SSCs, most of these studies achieved only short-term cultures

of SSCs from pre-pubertal testis (Izadyar et al. 2002, 2003, Oatley et al. 2004, Aponte et al. 2006, 2008) and neonatal testis (Fujihara et al. 2011). In these studies, serum was used as an important component in the culture medium for survival and self-renewal of culture cells. Some undefined factors in serum induce cell differentiation, while others have detrimental effects on ES cells and germ cell survival in culture (Li et al. 2008, Kubota et al. 2004b). To overcome this problem, serum-free culture systems have been developed for long-term cultures of SSCs in mice (Kubota et al. 2004a, Kanatsu-Shinohara et al. 2011) and rat (Ruy et al. 2005). However, no proven long-term culture system for livestock species has been developed. Here, we describe a culture system that supports continuous proliferation of bovine neonatal germ cells for at least 2 months.

2.2 Materials and methods

2.2.1 Collection of testes and isolation of gonocytes (germ cells)

Testes were collected from 0 to 10-day-old Holstein bull calves in Dulbecco's modified Eagle's medium and Ham's 12 (DMEM/F12; GIBCOBRL Invitrogen, Carlsbad, CA, USA) supplemented with 15 mM HEPES (Wako Pure Chemical, Tokyo, Japan) and were transported to the laboratory on ice within 24 hours.

Germ cells were isolated by a three-step enzymatic digestion method as described previously (Kim et al 2013) with minor modifications. Briefly, the testes were decapsulated, minced and digested with collagenase Type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min with constant agitation. After three washes, tissue fragments of the seminiferous tubules were incubated with collagenase Type IV and hyaluronidase (Sigma-Aldrich), each at a final concentration of 1 mg/ml. The cell suspension was further incubated with a mixture of 0.25% trypsin (Nacalai Tesque, Kyoto, Japan) and DNase I (7 mg/ml; Sigma-Aldrich) for 10 min. After centrifugation the pellete was suspended in DMEM/F12 medium containing 10% FBS to stop enzymatic activity of trypsin. The cell suspension was filtered through 40 µm nylon mesh (Kyoshin Rikou, Tokyo, Japan) and suspended in DMEM/F12 medium containing 5 % FBS.

2.2.2 Enrichment of germ cells for germ cell culture

The cell suspension was subjected to Percoll density gradient centrifugation. Cells from the fraction between 35 to 45 % Percol were separated and plated on 0.2% gelatin-coated dishes (Sigma-Aldrich) for 6 hours in DMEM/F12 medium containing 5% FBS. The supernatant containing germ cells was collected and utilized for further experiments.

The culture medium (the basic medium) for germ cells consisted of DMEM/F12 supplemented with 10 µg/ml apo-transferin (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), 110 µg/ml sodium pyruvate (Sigma-Aldrich), 0.015% sodium DL-lactate (Sigma-Aldrich), NEAA (Non-essential amino acid solution, GIBCOBRL Invitrogen, Carlsbad, CA, USA), 100 µM β-mercaptoethanol (Wako Pure Chemical, Tokyo, Japan), 100 µg/ml Penicillin (Sigma-Aldrich), and 50 µg/ml Streptomycin (Sigma-Aldrich), 40 µg/ml Gentamycin (Sigma-Aldrich) with 1% FBS. The growth factors used in this study were GDNF (40 ng/ml, R&D, Minneapolis, MN, USA) or bFGF (10 ng/µl, Upstate, Temecula, CA, USA) and EGF (20ng/µl, JRH Bioscience, Lenexa, KS, USA).

The serum-free supplement KSR, B-27 (50X) or StemPro-SFM (100X) (all from GIBCOBRL, Invitrogen, Carlsbad, CA, USA) was used as a reduced serum substitution for germ cell culture. The medium is supplemented with the final concentration of KSR(15%), B-27 (1X) and StemPro-SFM (1X). Cells were seeded in various media in 6-well dishes coated with different ECM-substrates (Iwaki, Tokyo, Japan) at a density of 5×10^5 per well at 37°C in 5% CO₂ in air. For long-term culture, cells were plated on poly-L-lysine precoated dishes. Cells were enzymatically passaged every 4 to 6 days at 1:2 to 1:4 dilution of cell concentration. The culture dishes were washed with phosphate

buffered saline (PBS) and incubated in 0.025% trypsin and 0.04% EDTA solution for 7 min followed by vigorous pipetting for 3 min. The trypsin treatment was inhibited by the medium supplemented with 10% FBS. Dissociated cells were counted with a hemocytometer and were plated onto new dishes. Cell smear were prepared for the estimation of germ cell numbers at every passages. Briefly, dissociated cells (1×10^5) were fixed in with 4% paraformaldehyde for 10 min. After washing, 10 μ l of cell suspension were put on poly-L-lysine coated glass slide, air-dried and keep at 4 °C until immunocytochemistry. The colonies were counted manually with an inverted microscope (Nikon, DIAPHOT-300, Japan).

2.2.3 Preparation of ECM-coated culture dishes

Culture dishes were coated with four different ECM molecules: 0.2% gelatin (Sigma-Aldrich), 0.001% poly-L-lysine (P2658, Sigma-Aldrich), 20 μ g/ml collagenase (Sigma-Aldrich) or 30 μ g/ml lectin Dolichos Biflorus Agglutinin (DBA Vector Laboratories, Burlingame, CA, USA) for 1 hr at 37°C. Culture dishes were washed once with PBS and utilized for culture experiments.

2.2.4 Immunocytochemistry

The purity of germ cells was estimated by using anti-UCHL1 (1:200; Biomol, Exeter, Exeter, UK) and anti-VIMENTIN (1:100; Sigma–Aldrich). The estimation of

germ cells were calculated using DBA-FITC (1:200, Vector Laboratories, Burlingame, CA, USA) staining on cell smear prepared at every passages. Colonies were double stained with fluorescein isothiocyanate (FITC)-conjugated DBA-Rhodamine (1:100; Vector Laboratories, Burlingame, CA, USA) and stem cell-specific markers anti-OCT3/4 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NANOG (1:200; Chemicon International, Temecula, CA, USA) and anti-E-CADHERIN (1:200; Santa Cruz Biotechnology) antibodies and also germ cell-specific markers anti-DDX4 (1:300; Chemicon International), anti-GFR α (Santa Cruz Biotechnology). The procedure was performed as described previously (Kim et al. 2013). Briefly, cells were fixed with 4% paraformaldehyde for 10 min and incubated with 10% goat serum in TBS (Tris-buffered saline) containing 0.1% Triton X-100, for 1 hr at 37 °C. The samples were washed thrice and incubated with primary antibodies at the optimal concentrations overnight at 4°C. Samples were then washed thrice and incubated with anti-mouse or anti-rabbit IgG antibodies conjugated with FITC (1:200; DAKO A/S, Glostrup, Denmark) as secondary antibodies along with DBA-Rhodamine (1:100). Samples were counterstained with 1 μ g/ml Hoechst 33342 (Sigma-Aldrich) for 5 min and mounted with 50 % glycerol. For DBA staining on cell smear, procedure was performed as above with slight modification. After blocking samples were incubated with DBA-FITC

(1:200) for 2hr in dark. Samples were then washed thrice, counterstained with Hoechst 33342 for 5 min, and mounted with 50 % glycerol. For negative controls, the primary antibodies were omitted and instead the section was incubated with antibody diluents containing 1% BSA in TBS. Photographs were taken with an inverted fluorescent microscope, Eclipse TE2000-U (Olympus BX50, Japan). Cell proliferation was measured by double staining with Ki67 antibody (1:100; DAKO A/S,) along with DBA-Rhodamine (1:100). The samples were processed as described above.

2.2.5 In vitro differentiation assay

Cultured germ cells were harvested by trypsinization and cultivated in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 15% FBS, 2 mM L-glutamine, 1x NEAA and 100 μ M β -mercaptoethanol (Wako) as described for the standard procedure for mouse embryonic stem cells (ESC) differentiation (Guan et al. 1999). Briefly, 1×10^3 cells in 30 μ l differentiation media were placed in one well of an ultra low attachment 96 well dish (Sumitomo Bakelite, Akita, Japan) and incubated for 5 days. Embryoid bodies were collected, trypsinized into single cell and plated on gelatin-coated dishes for 5 days and used for cell differentiation analysis.

Germ cell differentiation was characterized by using primary antibodies to the following proteins: Glial fibrillary acid protein (GFAP, 1:100, DAKO A/S), α -smooth

muscle (ASM, 1:000, Thermo Scientific, USA), and α -fetoprotein (AFP, 1:100, R&D, Minneapolis, MN, USA). Cell smears were immunostained and photographed as described above.

2.2.6 Karyotype analysis

Cultured cells were incubated overnight with 0.1 μ g/ml Karyomax colcemid solution (Gibco). Cells were harvested as single cell suspensions and metaphase spreads were prepared as previously described (Garcia-Gonzalo et al. 2008). The slides were stained with VECTA SHIELD mounting medium with DAPI (Vector Laboratories). Twenty metaphase spreads were examined on two established cell lines. The number of chromosomes was calculated manually.

2.2.7 RNA isolation and Reverse transcriptase-Polymerase chain reaction (RT-PCR)

Total RNAs were isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The DNase activity was inhibited by treating 2U of RNase-free DNase (Roche, Mannheim, Germany). Complementary DNA was synthesized from 1 μ g total RNA using ReverTra Ace (MMLV reverse transcriptase Ribonuclease H (RNaseH); Toyobo, Osaka, Japan). To rule out genomic DNA contamination, the reactions were performed for samples without the addition of

ReverTra Ace (RT-). The PCR amplification was performed using 1 μ L cDNA per 20 μ L PCR reaction mixture containing 2 mM MgCl₂, 0.25 mM dNTPs, 1 $\times$ PCR buffer, 5 pmol of each primer and 1U Taq DNA polymerase (ExTaq; TaKaRa). Nucleotide sequences are obtained from GenBank and primer pairs were design using Primer 3 programme (<http://primer3.sourceforge.net/>) are given in Table 1. PCR amplification conditions were carried out as initial denaturation at 95⁰C for 5 min (1 cycle), followed by 29 cycles of denaturation at 95⁰C for 30 sec, annealing at annealing temperature respective for each primer are given in (Table 1) for 30 sec, extension at 72⁰C for 45 sec, and a final extension at 72⁰C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with 0.5 μ gmL⁻¹ ethidium bromide. All PCR products were sequenced to confirm their identity.

Table 2.1 RT-PCR primer sequences used for amplification of specific genes

Gene Name	Primer Sequence(5'-3')	Annealing Temperature	Product Size(bp)	Accession No.
<i>OCT 3/4</i>	F: CCAGGACATCAAAGCTCTTCA R: AAAACCACACTCGGACCA	60°C	418	NM_1745 80.2
<i>C-MYC</i>	F: AGAGGGCTAAGTTGGACAGTG R: CAAGAGTTCCGTATCTGTTCAAG	58°C	346	NM_0010 46074.2
<i>UCHL1</i>	F: ACCCCGAGATGCTGAACAAAG R: CCCAATGGTCTGCTTCATGAA	60°C	236	NM_0010 46172.1
<i>C-KIT</i>	F: GACCTGGAGGACTTGCTGAG R: AGGGGCTGCTTCCTAAAGAG	60°C	316	AF263827 .1
<i>ACTB</i>	F: CGATCCACACAGAGTACTTGCG R: CGAGCGTGGCTACAGTTCACC	58°C	346	NM_1739 79.3

2.2.8 Statistical analysis

Differences among experimental groups were tested by Analysis of variance (ANOVA) using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean $\pm$ SEM (n=4) in each group from three independent experiments. Differences were considered to be significant at $p < 0.05$. The doubling time was calculated by using online resources (<<http://www.doubling-time.com/compute.php>>).

2.3 Results

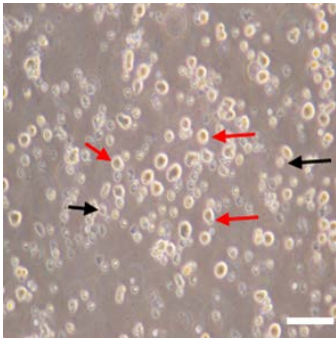
2.3.1 Enrichment of germ cells and selection of coating substrates for germ cell culture

Germ cells were enriched by positive selection through Percoll centrifugation and negative selection on gelation-coated culture dishes (Fig. 2.1A). The overall purity of germ cells, assessed by the co-localization of germ cell marker UCHL1 (Fujihara et al. 2011) and somatic cell marker VIMENTIN (Fig. 2.1B), was approximately 81.00 ± 2.08 % (n=4). This indicates that the ratio of germ cells to somatic cells was 4.46 ± 0.62 .

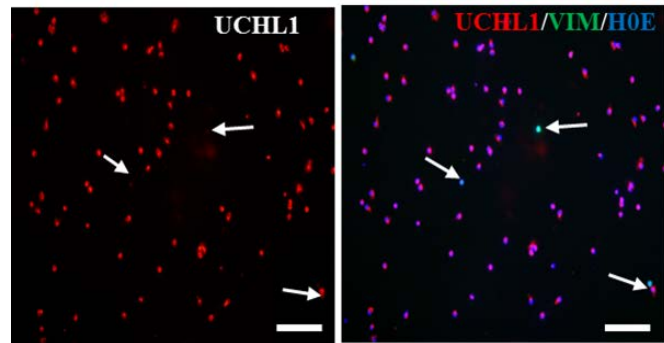
To suppress the overgrowth of somatic cells, we tried coating the culture dishes with different substrates. Colonies appeared on gelatin-, collagenase- and DBA-coated dishes after 3 days of culture and gradually disappeared by 7 days (Fig. 2.1C (a, c and

d)).Colonies emerged on a poly-L-lysine-coated dish at 4 days and gradually increased in size with a distinct dome-like morphology and limited proliferation of somatic cells (Fig. 2.1C (b)). The ratios of germ cells to somatic cells at day 7 of culture were significantly reduced on the gelatin (0.62 ± 0.30), collagen (0.69 ± 0.28) and DBA (1.08 ± 0.28) coated dishes than they were on day 0 (4.46 ± 0.62). The ratio on the poly-L-lysine-coated dish (3.17 ± 0.33) (Fig. 2.1D) was several times higher than the ratios on the other coated dishes. Germ cells to somatic cells ratio is calculated by using DBA staining as germ cell marker shown by (Izadyar et al. 2002) to count actual germ cell numbers (Fig. 2.1E).

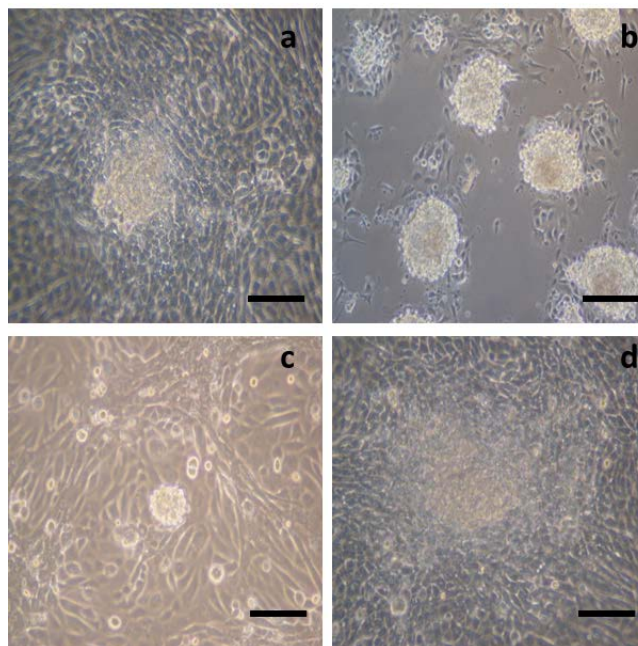
A



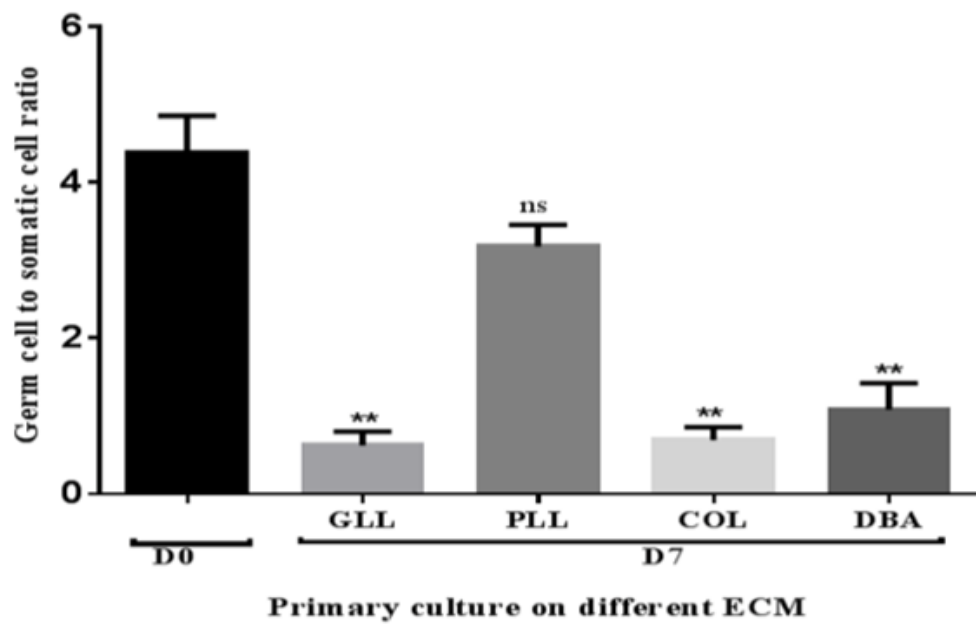
B



C



D



E

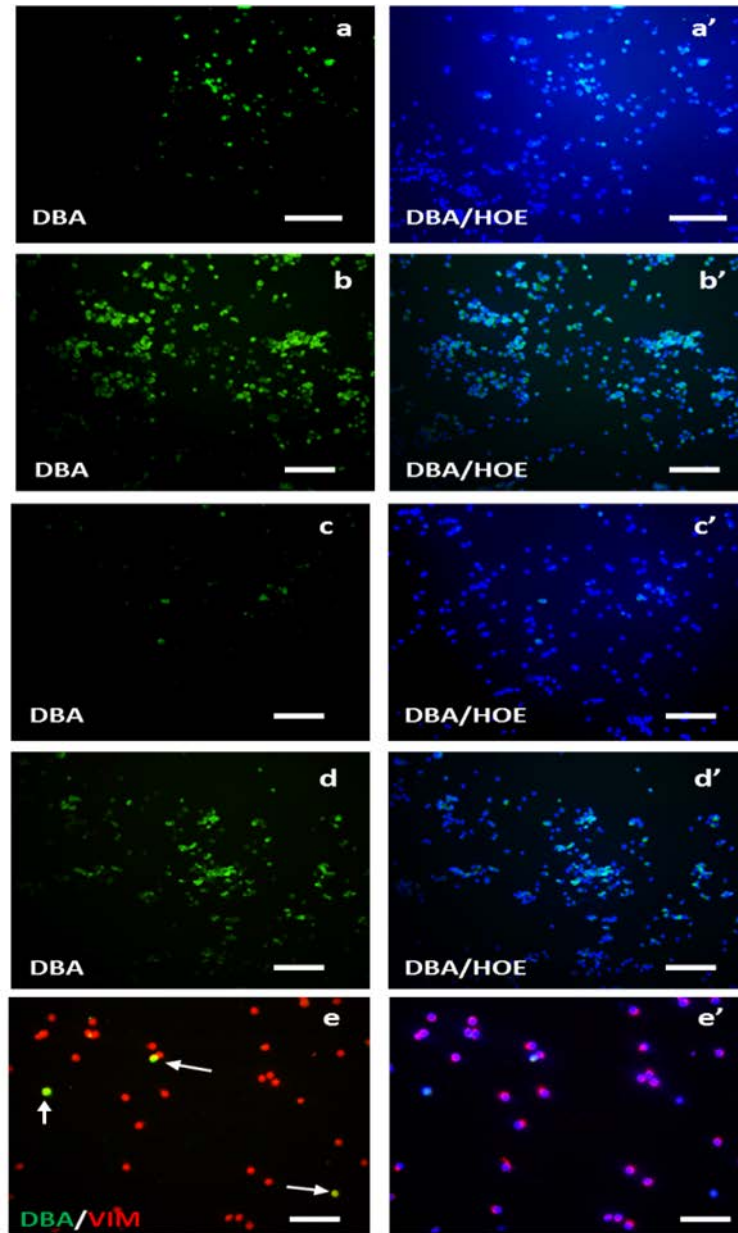


Figure 2.1 Effects of different culture dish coating substrates for culture dish on the proliferation ratios of germ cells in culture. **A)** Phase-contrast micrograph of freshly isolated and enriched germ cells (red arrows) and somatic cells (black arrow). Bar=20 μ m. **B)** Assessment of germ cells by germ cell marker UCHL1

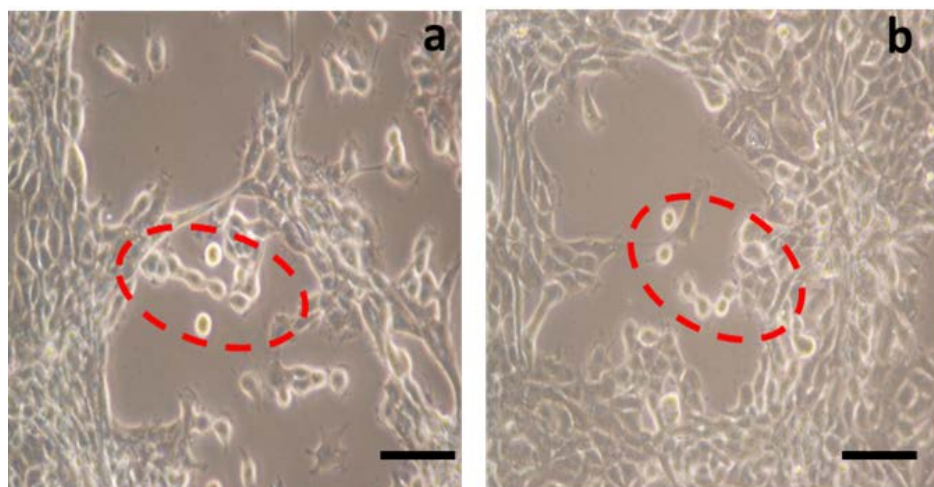
(arrow, left panel) and somatic cell marker VIMENTIN (VIM, arrow, right panel). Bar=20 μ m. C) Appearance of colonies on different coating substrates at 7 days after the initiation of culture; a) gelatin, b) poly-L-lysine c) collagenase and d) DBA. D) Germ cell- to- somatic cell ratios at the initiation of culture (day 0; D0) and at day 7 (D7) on dishes coated with different substrates; GLL (gelatin), PLL (poly-l-lysine), COL (collagenase) and DBA. The germ cell- to- somatic cell ratio was estimated by the germ cell-specific marker DBA (n=4). Data are presented as mean $\pm$ SEM. **P<0.05 and ns (not significant) compared with the germ cell/somatic cell ratio at day 0. Bar=50 μ m. E) Cells were stained with DBA-FITC conjugate to identify germ cells at day 7 of culture from dishes coated with different substrates; a and a') GLL, b and b') PLL, c and c') COL and d and d') DBA. The cells from PLL-coated dish were co-stained with germ cell marker DBA (arrows) and somatic cell marker VIMENTIN (VIM). Bar = 50 μ m

2.3.2 Effects of different serum-free supplements on germ cell culture

To optimize culture conditions for survival and self-renewal of bovine germ cells, we tested different media formulations using poly-l-lysine-coated dishes in short-term culture. Based on the preliminary standardization (data not shown) and published reports (Kanatsu-Shinohara et al 2003, Oatley et al. 2004; Aponte et al. 2006), the medium was supplemented with 1% (FBS) and GDNF (40 ng/ul). The addition of growth factors bFGF and EGF to the medium enhanced the proliferation of somatic cells and formation of chain-like morphology of differentiated germ cells (Fig. 2.2Aa and b). Therefore, to suppress the overgrowth of somatic cells in culture, bFGF and EGF were excluded from the culture medium in further experiments.

To find suitable serum-free supplements for a long-term culture of bovine germ cells, we evaluated KSR, B27 and StemPro-SFM supplements in the culture medium. Germ cells in the medium containing KSR showed 51.34 ± 10.62 -fold expansion as compared to control (no serum-free supplement, 20.18 ± 1.95 -fold), B-27 (29.50 ± 1.19 -fold), StemPro-SFM (24 ± 5.3 -fold) during 17 days of cultures (passage 3) (Fig. 2.2B).

A



B

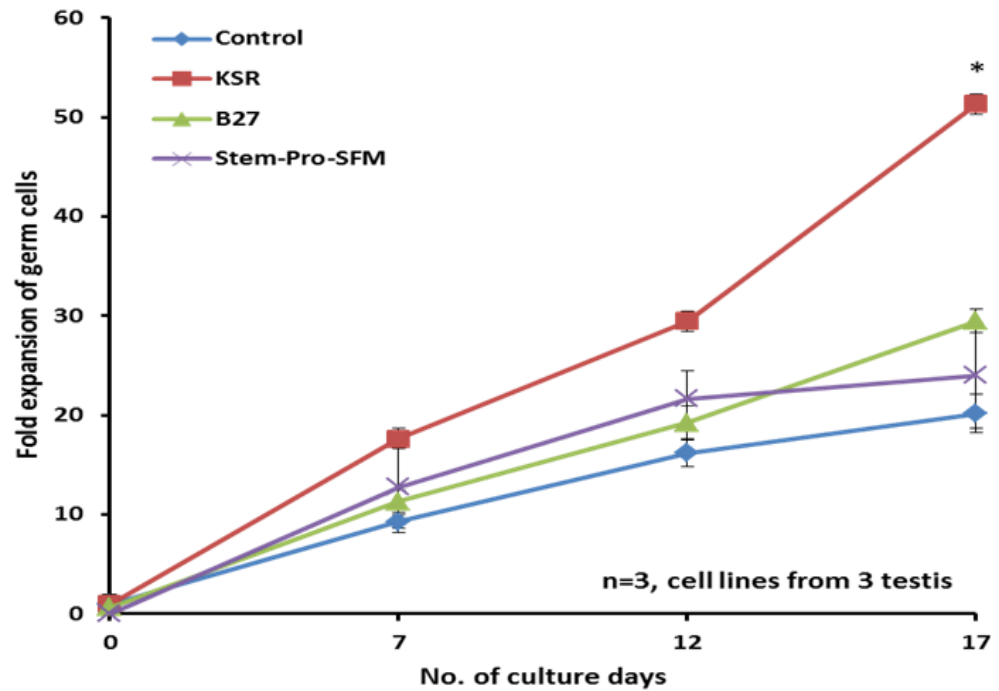


Figure 2.2 Comparison of the effects of different serum-free supplements on the growth of germ cells in poly-L-lysine-coated dishes. A) Appearance of chain-like SSCs during a short-term culture supplemented with growth factors (a) bFGF and (b) EGF. B) Proliferation of germ cells in different culture conditions at different culture days. The fold expansion of germ cells at each passage was determined by counting the DBA-positive cells. Data are from three independent experiments using three different testes. Control (no supplement added to the medium). KSR, B27 and StemPro-SFM (the medium supplemented with the respective serum-free supplement). Data are presented as mean $\pm$ SEM. *P<0.05.

2.3.3 Long-term culture of germ cells in KSR-supplemented medium

KSR-supplemented medium, which had given promising results in our preliminary culture, was utilized for long-term culture of germ cells. The cultured cells were passaged enzymatically using trypsin-EDTA into small clumps every 4 to 5 days. Distinct ES-like colonies were appeared at every passage with expansion of germ cells. The morphologies of colonies of representative passages are shown in Fig 2.3, a-d. The number of germ cells increased 140 fold in 67 days (Fig. 2.4), corresponding to a doubling time of 7.1 days.

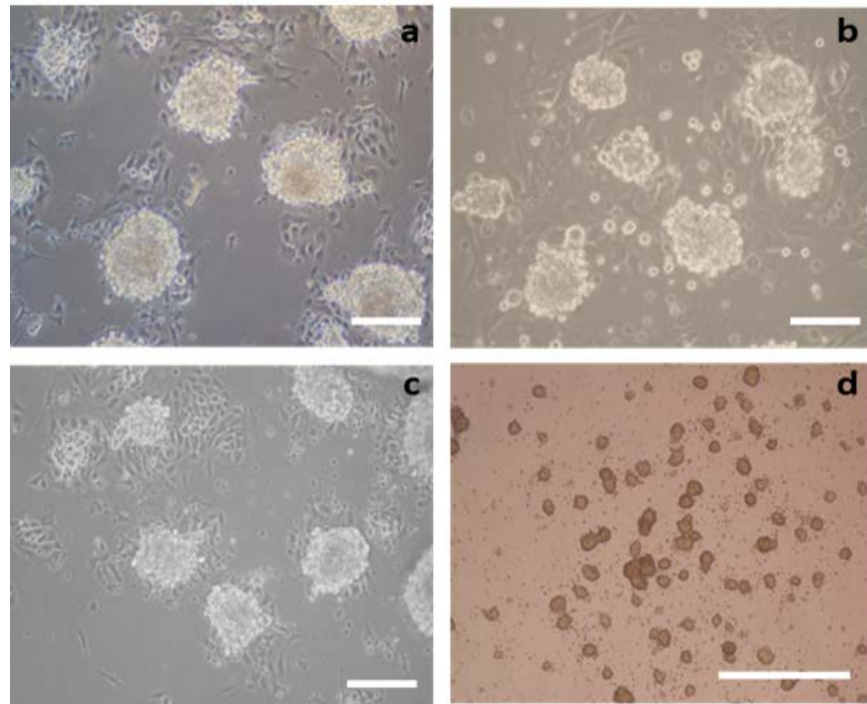


Figure 2.3 Phenotypes of colonies in medium supplemented with KSR on poly-L-lysine-coated dishes at different passages. a) 7 days after initiation of culture, b) passage 5, c) passage 13 and d) passage 5. Bar= 50 μ m.

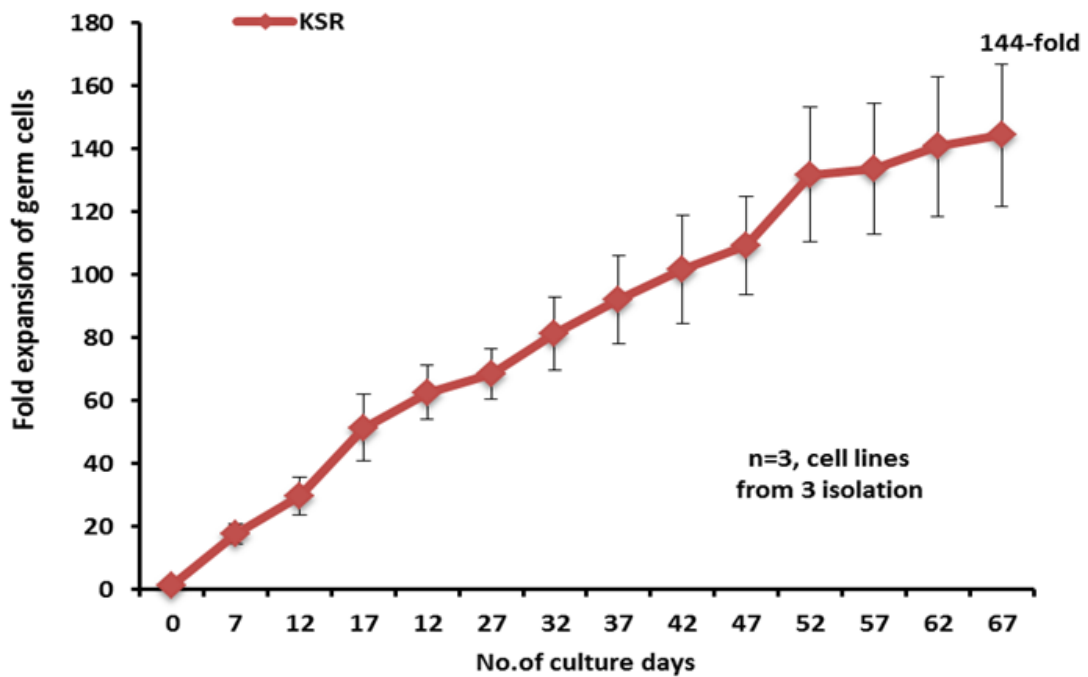


Figure 2.4 Long-term culture of germ cells in medium supplemented with KSR on poly-L-lysine-coated dishes. The total numbers of germ cell at every passage were counted by germ cell marker DBA. The fold expansion of germ cells were plotted. Data are from three independent experiments using three different testes. The number of cells increased by 144 fold over a 2 months period.

2.3.4 Phenotypic characterization of germ cells in a long-term culture

Distinct ES-like colonies stably appeared at successive passages during long-term culture. As shown by reverse transcriptase-polymerase chain reaction (RT-PCR) gene expression profiles, the cells expressed pluripotent markers *OCT3/4* and *c-MYC* and germ cell marker *UCHL1* at passages P0, P5, P10 and P13. The expression of germ cell differentiation marker *c-KIT* was not detected in culture cells (Fig. 2.5). At passage 10 (corresponding to more than 2 months of culture), 75 to 80% of the cells from twenty metaphase spreads count from each of two cell lines had a normal karyotype (60 chromosomes) (Fig. 2.6).

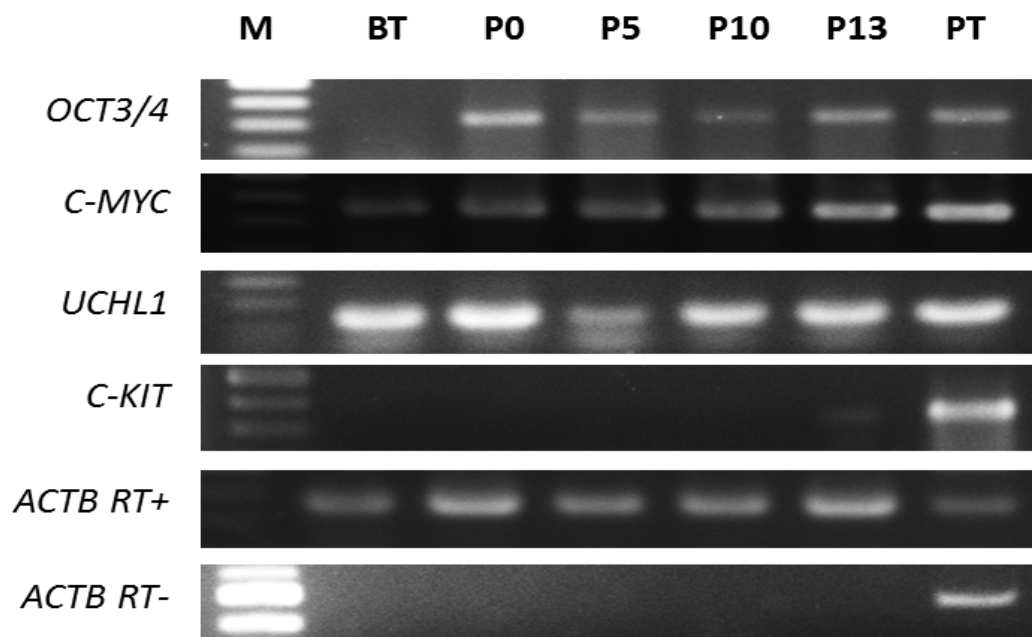


Figure 2.5 RT-PCR analysis of gene expressions of cultured cells at different culture periods. Colonies were collected at the time of passages P0, P5, P10 and P13, respectively. M: 100bp DNA ladder, BT: Neonatal testis (10 days old), PT: Positive control (Genomic DNA).

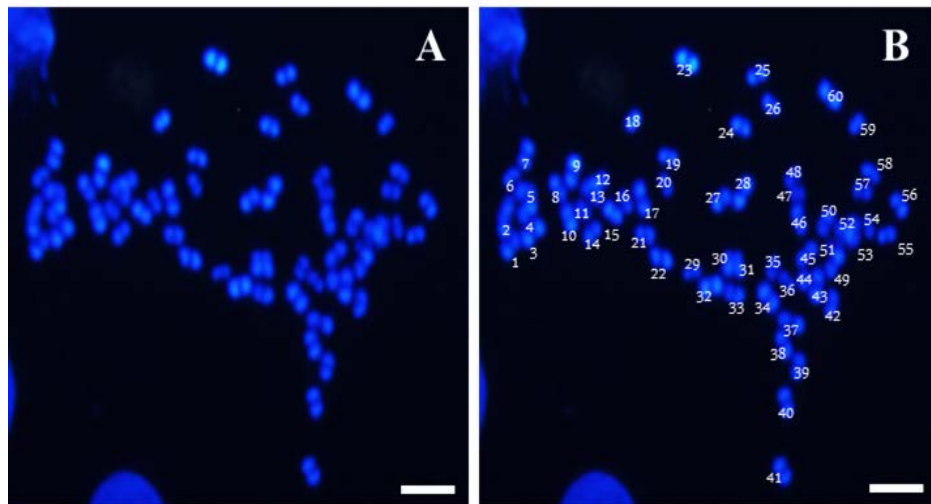


Figure 2.6 Chromosomal analyses of cultured cells. A) Metaphase spread at passage 10 from representative cell lines, B) Representation of image (A) showing marked chromosome numbers from 1 to 60. The images showed normal karyotype with 60 chromosomes. Bar= 20 μ m.

2.3.5 Molecular characterization of germ cell colonies in a long-term culture

At passage 10, the colonies were positive for DBA with OCT3/4, NANOG and E-CAD (Fig. 2.7). At passage 13, the colonies were positive for proliferation marker Ki-67.

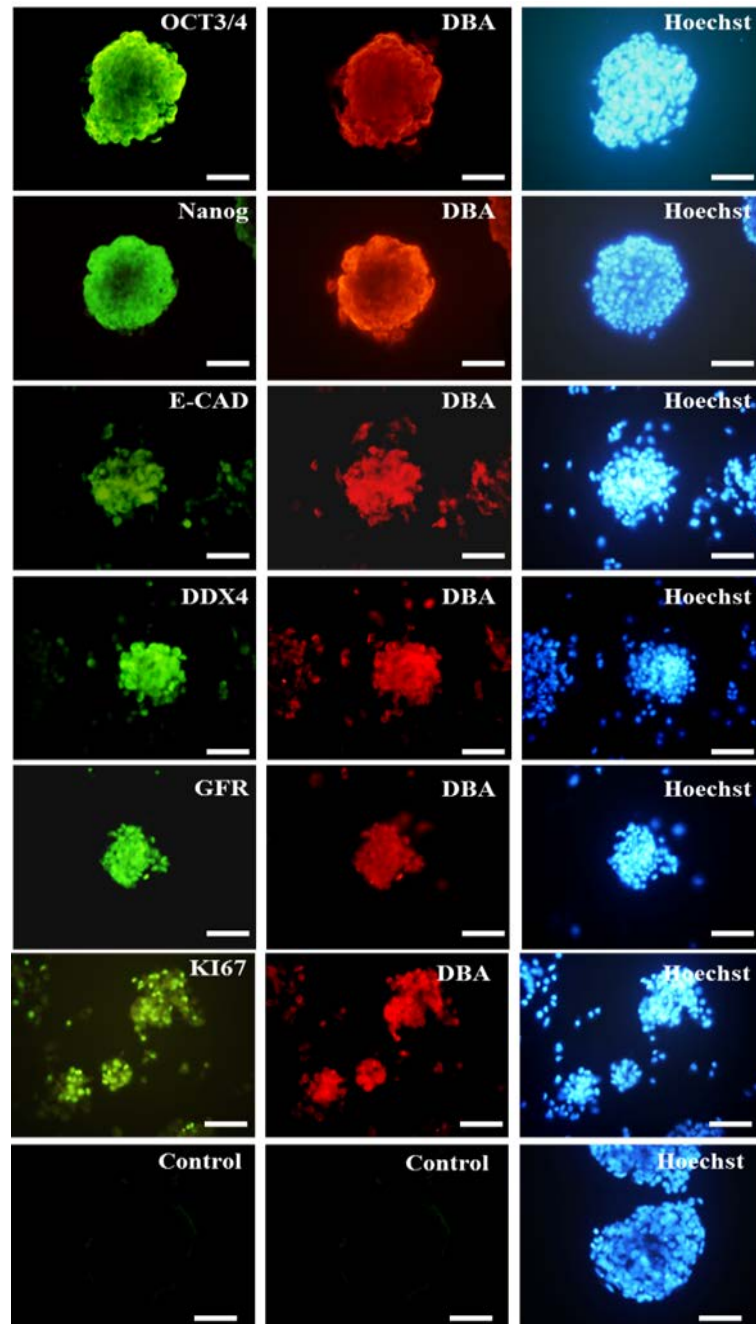


Figure 2.7 Characterization for germ cell colonies in culture. Colonies at passage 10 were double immunostained with DBA and specific marker proteins for stem cells (NANOG, E-CAD and OCT3/4) and germ cells (DDX4 and GFR α -1 and proliferating cell marker (Ki67). Bar=50 μ m.

2.3.6 Differentiation potential of germ cells

Cultured germ cells were used for embryoid body formation. Five days after the start of passage 10, the cells formed embryoid bodies (Fig. 2.8A). The cells were positive for the expressions of α -fetoprotein (AFP), an endoderm lineage protein, α -smooth muscle (ASM), a mesoderm lineage protein, and Glial fibrillary acid protein (GFAP), an ectoderm lineage protein (Fig. 2.8B).

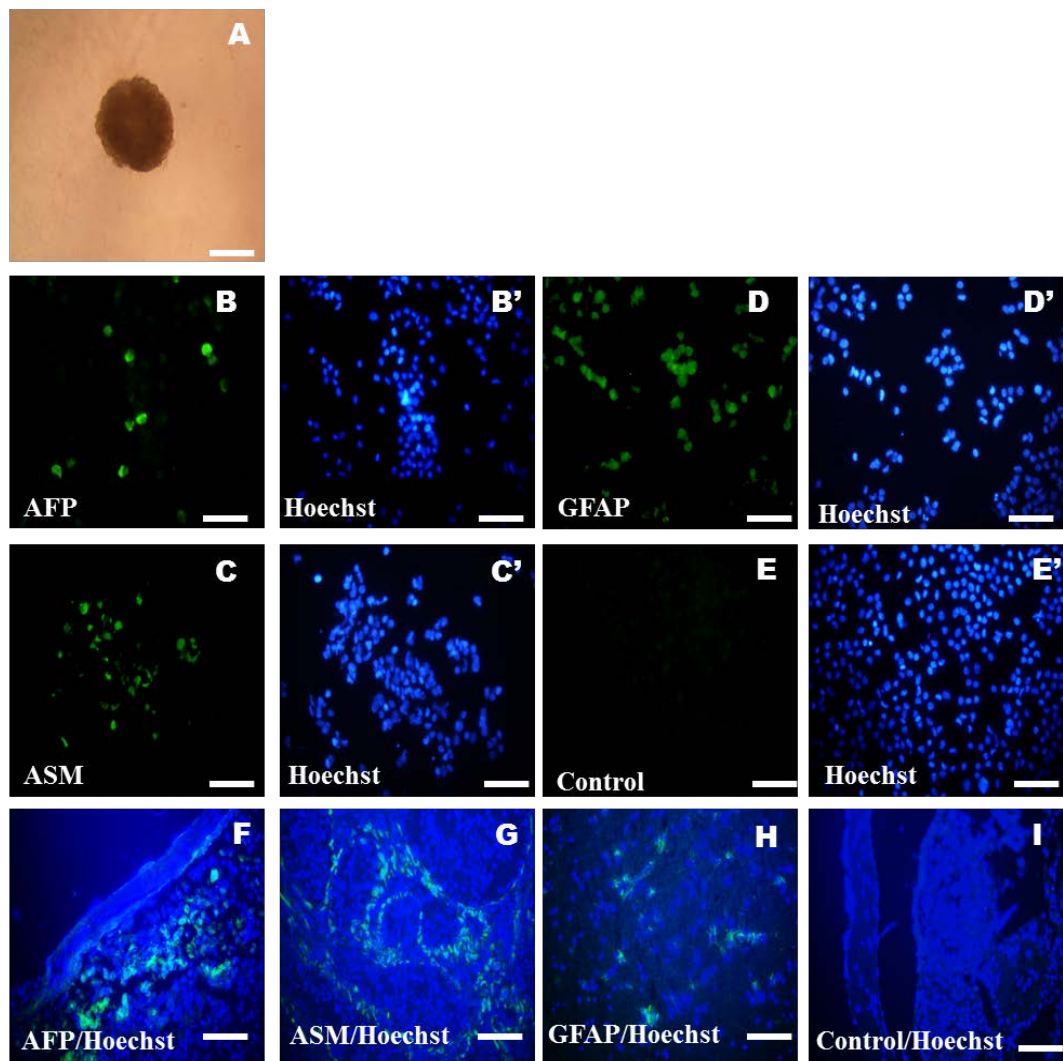


Figure 2.8 Formation of embryoid body and differentiation potential of cultured germ cells A) Phase-contrast image of embryoid body, B-B') AFP and Hoechst, C-C') ASM and Hoechst, D-D') GFAP and Hoechst, E-E') Control and Hoechst. Control tissue section were prepared from teratoma tissue generated from mouse ES cell lines. The tissue section for positive control of AFP (F), ASM (G) and GFP (H) antibodies. The control section was incubated without primary antibody (I). Bar= 50 μm.

2.4 Discussion

The goal of the present study was improve the long-term growth of bovine germ cells by modifying the media and coating the culture dishes. Germ cells exhibit different affinities towards extracellular matrix (ECM) molecules for their self-renewal and differentiation (Oatley et al. 2012).

The growth of male germ cells in culture can be adversely affected by the presence of contaminating somatic cells from the testes. Such cells can be minimized by applying a suitable coating to culture dish (Kubota et al. 2004a). In our study, poly-L-lysine-coated dishes selectively inhibited the proliferation of testicular somatic cells and supported the proliferation of germ cells in culture. Poly-L-lysine is an artificial substrate and the mechanism by which it supported proliferation was unknown. One of the mechanism by which poly-L-lysine promotes attachment and growth of cells, is its highly positive charged nature (McKeehan and Ham et al. 1976). In addition, poly-L-lysine is a key component in a defined culture medium for mouse ES cells (Harb et al. 2008). In short-term culture of bovine germ cells from 3.5-month-old pre-pubertal testis, DBA-coated dishes were found to better support the binding and survival of cells than gelatin-, poly-L-lysine- and laminin-coated dishes (Kim et al. 2013). This indicate that there are age-specific differences in requirements of DBA- and poly-L-lysine-

coated dishes for self-renewal and proliferation between bovine neonatal and pre-pubertal germ cells. Similarly, in mice, pre-pubertal (SSCs) and neonatal (gonocytes) germ cells were found to have different growth requirements (Creemers et al. 2002).

To propagate and maintain the pluripotency of mouse ES cells, extrinsic cell differentiation stimuli need to be suppressed and neutralized (Ying et al. 2008). The differentiation of mouse SSCs is directly influenced by environmental factors, i.e. growth factors and serum (Kanatsu-Shinohara et al. 2011). Growth factors, such as GDNF, bFGF and EGF, are required for the long-term culture of germ cells in mice and rat (Kubota et al. 2004b, Hamra et al. 2009). In our studies, the addition of bFGF and EGF in cattle germ cell culture resulted in the overgrowth of somatic cells and the appearance of differentiated germ cells in short-term culture (Fig 2A). Culture medium supplemented with GDNF, bFGF and EGF did not support the appearance of germ cell colonies during a long-term culture in cattle (Aponte et al. 2008). These results indicate that mice and cattle germ cells need different factors for growth and differentiation. Adding serum to the culture medium reduces germ cell survival, and a reduced concentration of serum is essential for the maintenance of stem-cell activity (Kanatsu-Shinohara et al. 2011). Testicular cells from neonatal bovine testis do not

grow well in medium supplemented with 10 % FBS (Fujihara et al. 2011), indicating that higher concentrations of serum have adverse effects on bovine germ cells.

Serum-free supplement KSR, has been shown to prolong the culture of human ES cells (Amit et al. 2004) mice embryonic germ cells (EGCs) (Horri et al. 2003) and pig EGCs (Petkov et al. 2008). StemPro-SFM is a standard supplement for germ cell culture in mice (Kanatsu-Shinohara et al. 2003), while B27 supplement is required for germ cell culture in rat (Wu et al. 2009). In our experiment, KSR-supplement medium is more effective than that of B-27 or StemPro-SFM supplement for long-term culture of bovine neonatal germ cells. Current studies advocate that Albumax (a lipid-rich BSA) present in KSR-supplement stimulates self-renewal of human ES cells (Garcia-Gonzalo et al. 2008). BSA and lipid-rich BSA are required for the self-renewal of SSCs in culture through a lipid-mediated signaling pathway (Kubota et al 2004a, Kanatsu-Shinohara et al. 2011). The lipid-rich BSA in KSR-supplement, which is not in B27 (Garcia-Gonzalo et al. 2008) or StemPro-SFM (Kanatsu-Shinohara et al. 2003), may contribute to the long-term culture of bovine germ cells. Further studies are needed to elucidate the mechanism by which lipid-rich BSA mediates self-renewal of germ cells.

By using KSR and poly-L-lysine-coated culture dishes, we could propagate germ

cells for over 2 months, with a normal karyotype. We established three cell lines from three different testes isolations. The expansion of germ cells after enzymatic passages suggests that this culture condition is suitable for minimizing the unfavorable effect that are caused by the enzyme treatment. The finding that supports the hypothesis of Ebata et al. (2011) that self-renewal and growth of SSCs rely on frequent cell passages, which disrupt colonies and cell-cell interactions rather than on the presence of growth factors. The estimated doubling time of bovine germ cells was 7.1 days, which is comparable to the doubling times of SSCs reported for mice (5.8 days) (Kubota et al. 2004b) and rats (4 days and 10.6 days) (Hamra et al. 2005, Ryu et al. 2005).

The expression of pluripotent genes *OCT3/4* and *c-MYC* in colonies indicated that cultured germ cells had stem-cell potential, while *UCHL1* expression indicated that they had germ cell potential throughout the term of culture (Fig. 2.5). The absence of *c-KIT* expression indicated that the germ cells sustained an undifferentiated state in culture. Immunocytochemical analysis also revealed stem-cell (*OCT3/4*, *NANAG* and *E-CAD* expressions) and germ cell characteristics (*DDX4* and *GFR* expressions) in the long-term culture. In our studies, cell lines that were derived from cultured germ cells formed embryoid bodies and differentiated into all three germ layers. However, subcutaneously injecting these cells into the backs of immunodeficient mice did not

cause the formation of teratomas.

We established culture conditions for the propagation of undifferentiated bovine germ cells on poly-L-lysine-coated dishes in medium containing KSR. Further investigations are needed to characterize species-specific signaling pathways regulated by cytokines and their association with self-renewal gene cascade for improvement of culture conditions. Optimistically, our culture condition will pave the way towards the establishment of germ stem cell lines in livestock species as well as provide the model for application of this research on conservation of endangered species.

Chapter 3

Mitogen Activated Protein Kinase (MAPK) Signaling is Required for Self-renewal of Cultured Bovine Male Germ Cells

3.1 Introduction

Spermatogonial stem cells (SSCs) are adult stem cells in the testes that maintain the continuity of life by the process of spermatogenesis (Russell et al. 1990). This process solely depends on the continued self-renewal and differentiation of SSCs, by which millions of spermatozoa are produced daily within the testes (Sharp et al. 1994). SSCs are supported by specialized microenvironments referred to as “niches”, which provide architectural support, stimulate growth factors, and provide extrinsic signals to synchronize self-renewal and differentiation (Oatley et al. 2008)

Understanding the niche factor that regulates SSC function in rodents has been greatly aided by transplantation assays to immunodeficient mice and the development of a long-term culture system (Brinster et al. 2007). Culture conditions that support the long-term self-renewal and maintenance of pluripotency of germ cells have been established in various species including mice (Nagano et al. 2003, Kanatsu-Shinohara et al. 2003, Kubota et al. 2004), rats (Hamra et al. 2004), hamsters (Kanatsu-Shinohara et al. 2008) and rabbits (Kubota et al. 2011). Growth factor GDNF was shown to be the critical factor for the self-renewal of cultured germ cells in these culture systems. Global gene expression profiling has identified several intrinsic downstream targets for the GDNF-mediated self-renewal of cultured SSCs. Among these targets, Ets variant 5

(Erm), B cell/lymphoma 6 membrane B (Bcl6b), and LIM homeobox1 (Lhx1) have been identified as core transcription factors associated with the self-renewal of cultured mouse SSCs (Oatley et al. 2006).

The combined approach of RNAi inhibition, microarray analysis, and transplantation assays revealed the cascade of self-renewal and pluripotency in cultured SSCs. Fine tuning of the *ETV5-Bcl6b-Lhx1* expression cascade under the influence of GDNF was shown to be responsible for the self-renewal and maintenance of mouse SSCs (Wu et al. 2011). This mechanism differs from those of mouse ES cells and human ES cells, in which self-renewal and pluripotency maintain the *Oct4-Sox2-Nanog* network (Niwa et al. 2007).. However, the extrinsic signaling pathways for self-renewal and pluripotency respond differently in mice and human ES cells. Instead of different growth factor requirements, common signaling pathways play opposite roles in mice and humans; for example, MAPK inactivation is required for self-renewal in mouse ES cells, while it induces differentiation in human ES cells (Xu et al. 2010). Studies on extrinsic signaling pathways in mice germ cell cultures using a kinase-specific inhibitor demonstrated that PI3K-AKT signaling (Braydich-Stolle et al. 2007, Lee et al. 2007, Oatley et al. 2007) and Ras-mediated MAPK signaling (He et al. 2008, Lee et al. 2009) were involved in the self-renewal and survival of germ cells. Crosstalk between PI3K/AKT

and MAPK signaling was also shown to be essential for the self-renewal of cultured mouse germ cells (Lee et al. 2007).

In domestic species, gene targeting has a potential application in both agriculture and human disease modeling. A combination of gene targeting and SSC research will provide a time saving and cost effective tool for maximizing genetic gain and preserving desirable genetics for the production of superior food animals (Hill et al. 2006). The major hindrance in the practical application of this research is the lack of a long-term culture system supporting the self-renewal of SSCs in domestic species. Although SSCs from many mammalian species have been shown to proliferate for more than six months in the seminiferous tubules of immunodeficient mice (Kubota et al. 2006), no germ cell line has yet been established in livestock species. A possible reason for this is the dearth of understanding on the species-specific requirements of growth factors and mechanisms supporting the self-renewal of cultured SSCs.

In the present study, we focused on exploring the molecular mechanisms responsible for the self-renewal and maintenance of cultured bovine germ cells. Our results indicated that activation of the MAPK pathway was necessary for the self-renewal and maintenance of cultured bovine germ cells via the downstream regulation of cyclin D1 and CDK2.

3.2 Materials and methods

3.2.1 Collection of the testes and isolation of germ cells

The testes were collected from 0 to 10-day-old Holstein bull calves in Dulbecco's modified Eagle's medium and Ham's 12 (DMEM/F12; GIBCOBRL Invitrogen, Carlsbad, CA, USA) supplemented with 15 mM HEPES (Wako Pure Chemical, Tokyo, Japan) from National Livestock Breeding Centre, Fukushima and Gifu Prefectural Livestock Research Institute, Gifu and were transported to the laboratory on ice within 24 hours.

Germ cells were isolated by a three-step enzymatic digestion method as described previously (Kim et al. 2013) with minor modifications. Briefly, the testes were decapsulated and minced, and the minced tissues were digested with collagenase Type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min with constant agitation. After three washes, tissue fragments of the seminiferous tubules were incubated with collagenase Type IV and hyaluronidase (1 mg/ml; Sigma Aldrich). The cell suspension was further incubated with a mixture of 0.25% trypsin (Nacalai Tesque, Kyoto, Japan) and DNase I (7 mg/ml; Sigma Aldrich) for 10 min. After centrifugation, the resulting pellet was suspended in DMEM/F12 medium containing 10% FBS to stop the enzymatic activity of trypsin. The cell suspension was filtered

through a 40 μ m nylon mesh (Kyoshin Rikou, Tokyo, Japan) and suspended in DMEM/F12 medium containing 5 % FBS.

The cell suspension was subjected to Percoll density gradient (20%-60%) centrifugation at 3400 g for 30 min at 21 °C. Cells from the fraction between 35 to 45 % Percoll were separated and plated on 0.2% gelatin-coated dishes (Sigma-Aldrich) for 6 hours in DMEM/F12 medium containing 5% FBS. The supernatant containing germ cells was collected and utilized for further experiments.

3.2.2 Germ cell culture and treatments with cell signaling inhibitors

The culture medium for germ cells consisted of DMEM/F12, which was supplemented with 15% Knockout serum replacement (KSR) (GIBCOBRL, Invitrogen, Carlsbad, CA, USA), 10 μ g/ml apotransferrin (Sigma Aldrich), 10 μ g/ml insulin (Sigma Aldrich), 110 μ g/ml sodium pyruvate (Sigma Aldrich), 0.015% sodium DL-lactate (Sigma Aldrich), NEAA (non-essential amino acid solution, GIBCOBRL Invitrogen, Carlsbad, CA, USA), 100 μ M β -mercaptoethanol (Wako Pure Chemical, Tokyo, Japan), 100 μ g/ml penicillin (Sigma Aldrich), 50 μ g/ml streptomycin (Sigma Aldrich), and 40 μ g/ml Gentamycin (Sigma Aldrich) with 1% FBS. GDNF (40 ng/ml, R&D, Minneapolis, MN, USA) was used as a growth factor in this study.

Culture dishes were coated with 0.001% poly-L-lysine (P2658, Sigma Aldrich) for 1 hr at 37 °C. The dishes were washed once with PBS and utilized for the germ cell culture. Cells were plated at a density of 5×10^5 cells per well of a 6-well dish (Becton Dickinson, Franklin Lakes, NJ, USA) precoated with poly-L-lysine at 37°C in 5% CO₂ in air.

Inhibitors of the MEK (PD098059 (PD), Stemgent, USA) and PI3K (LY294002 (LY), Cell Signaling, Beverly, MA USA) signaling pathways were used at a dose of 10 μ M (Lee et al. 2000). Cells were dissociated by incubation in 0.25% trypsin and 0.04% EDTA solution for 10 min followed by vigorous pipetting. A cell smear was prepared to estimate the purity of germ cells using DBA immunostaining. Colonies were photographed and counted manually using an inverted microscope (Nikon, DIAPHOT-300, Tokyo, Japan).

3.2.3 Immunofluorescence

Cell smears were prepared on poly-l-lysine-coated glass slides. To stain colonies, cells were cultured onto coverslips in 24-well culture dishes (Nunc, DK-4000, Roskilde Denmark). The procedure was performed as described previously (Kim et al. 2013). Briefly, cells were fixed in 4% paraformaldehyde for 10 min and incubated with 10% goat serum in TBS (Tris buffer saline) containing 0.1% Triton X-100 for 1 hr at

37 °C. Samples were washed thrice and incubated with primary antibodies at the optimal concentration overnight at 4 °C. The antibodies used were as follows DBA-Rhodamin (1:100; Vector Laboratories, Burlingame, USA), anti-VASA (1:300; Chemicon, USA), anti-PGP9.5 (1:200; Biomol, Exeter, Exeter, UK), anti-GFR α (Santa Cruz Biotechnology, USA), and anti-RET (1:200; Chemicon International). Samples were again washed thrice and incubated with secondary antibodies such as anti–mouse or anti–rabbit IgG antibodies conjugated with FITC (1:200; DAKO A/S, Denmark) along with DBA-Rhodamine (1:100). The samples were counterstained with DAPI mounting media (Vector Laboratories, Burlingame, CA USA) for 10 min. Primary antibodies were omitted for negative controls and the section was incubated with antibody diluents (1% BSA in TBS). Photographs were taken with the inverted fluorescent microscope, Eclipse TE2000-U (Olympus BX50, Tokyo, Japan).

3.2.4 RNA isolation and RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol. DNase activity was inhibited with 2U of RNase-free DNase (Roche, Mannheim, Germany). Complementary DNA was synthesized from 1 μ g total RNA using ReverTra Ace (MMLV reverse transcriptase RNaseH; Toyobo, Osaka, Japan). To rule out genomic DNA contamination, reactions were performed for samples

without the addition of ReverTra Ace (RT-). PCR amplification was performed using 1 μ L cDNA per 20 μ L PCR reaction mixture containing 2 mM MgCl₂, 0.25 mM dNTPs, 1 $\times$ PCR buffer, 5 pmol of each primer, and 1U Taq DNA polymerase (ExTaq; TaKaRa, Tokyo, Japan). Primer sequences are shown in Table 1. PCR products were separated by 1.5% agarose gel electrophoresis and stained with 0.5 μ g mL⁻¹ ethidium bromide. All PCR products were sequenced to confirm their identity.

Table 3.1 RT-PCR primer sequences of specific genes

Gene Name	Primer sequence (5'-3')	Product Size (bp)	Accession no.
Cyclin D1	F: GCCGAGGAGAACAAGCAGAT R: TCAGATGTTACGTCACGCA	378	NM_00104 6273.2
Cyclin D2	F: GCAGAACTTGCTGACCATCG R: AGGCTTGATGGAGTTGTCGG	319	NM_00103 4709.2
Cyclin D3	F: CACTTGAGGCCCTGCATAA R: GGTAGCATGATGGTCCTCGG	495	NM_00103 4709.2
Cyclin A	F: CCTGCAAAGTGCAGGTTGA R: CTCTGGTGGGTTGAGGAGAG	315	X68321.1
CDK2	F: GGGAACGTACGGAGTTGTGT R: CCAGAAGGATTTCCGGTGCT	491	NM_00101 4934.1
b-Actin	F: CGATCCACACAGAGTACTTGCG R: CGAGCGTGGCTACAGTTCACC	316	NM_17397 9.3

3.2.5 Western blot analysis

Germ cells were cultured for 4 days and were then treated with GDNF, PD and LY for 20 min. These cells were lysed in RIPA (Radioimmunoprecipitation assay) buffer to obtain protein lysates (Abcam, Cambridge, England). Protein concentrations were determined using Coomassie Bradford reagent (Sigma Aldrich). Fifty μ g of total protein was mixed with an equal amount of 2x-SDS loading buffer and resolved by SDS PAGE. Electrophoresis was performed using a Mini electrophoresis system (Biocraft, Tokyo, Japan) at 100V for 60 min. The eluted proteins were transferred to an Immobilon-P transfer membrane (Millipore, Massachusetts, USA) at 60V for 90 min. The transmembrane was blocked for nonspecific antibodies with 5% BSA in TBS-T for 90 min at room temperature with gentle shaking. Blots were probed with the primary antibody anti-rabbit pERK (1:5000; Santa Cruz Biotechnology, USA), anti-rabbit p44/42MAPK (1:5000; Cell Signaling, Beverly, MA USA), or anti-mouse α -tubulin (Sigma Aldrich) overnight at 4 °C with gentle shaking. After a brief wash in TBS-T, membranes were incubated in the secondary antibody ECL-peroxidase labeled anti-rabbit or anti-mouse antibody (1:50000, GE Healthcare, Wisconsin, USA) for 90 min with gentle shaking at room temperature, were washed thrice with TBS-T, and were then developed with an Amersham ECL prime western blotting detection reagent on

x-ray film (GE Healthcare, Wisconsin, USA). Density measurements were taken using Imaj J software on scanned x-ray films.

3.2.6 Statistical analysis

All quantification data were presented as the mean $\pm$ s.e.m. Analysis of variance (ANOVA) and Turkey's multiple comparison tests were performed using Graph Pad Prism 4.0 (Graph Pad Software, Inc. San Diego CA, USA). Differences were considered to be significant at $P < 0.01$. A densitometric evaluation of western blotting results was conducted using Imaj J software with b-actin as an internal control.

3.3 Results

3.3.1 Expression of GFR α -1 and RET in cultured germ cells

Enriched germ cells were cultured overnight in the GDNF-supplemented medium, and revealed the expression of germ cell markers DDX4 (Fig 1A) and PGP9.5 (Fig 1B). We also confirmed the expression of the germ cell marker DBA (Izadyar et al. 2002) and both GFR α -1 (Fig 1C) and RET receptors (Fig 1D), which are targets of the GDNF-induced signaling pathway (Naughton et al. 2006).

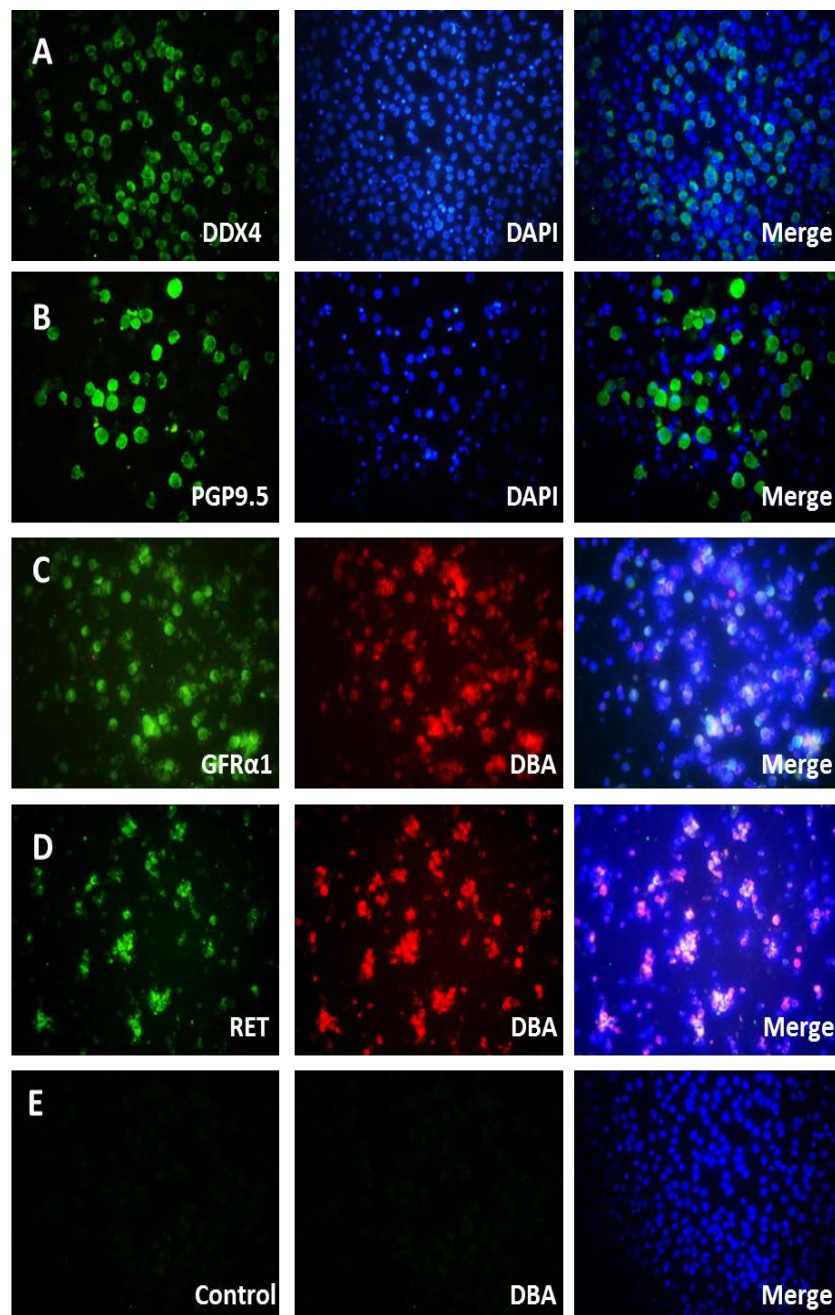


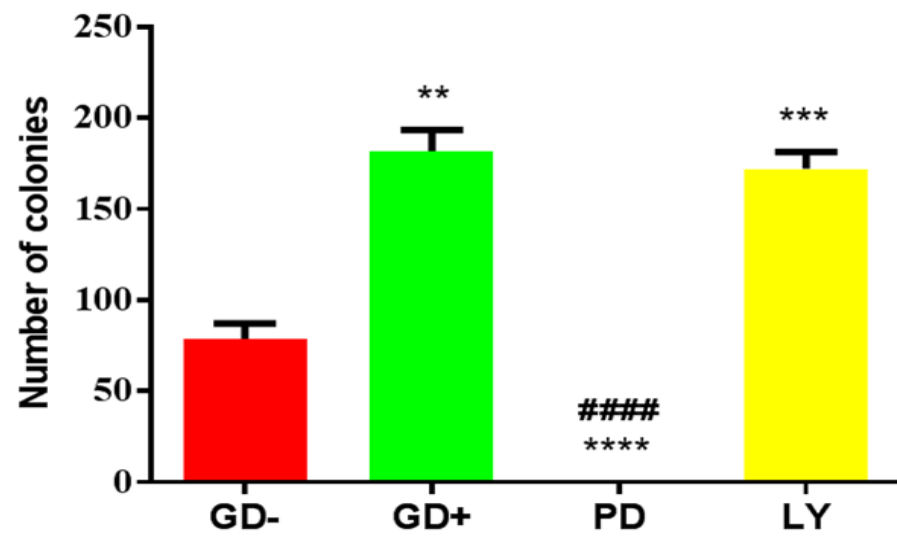
Figure 3.1 Immunocytochemical characterization of cultured germ cells in the presence of GDNF with germ cell-specific markers (DDX4, PGP9.5, GFR α 1, RET, and DBA). A) DDX4 expression with the nuclear marker DAPI, B) PGP9.5 expression with DAPI, C) GFR α -1 expression with the germ cell marker lectin DBA, D) RET expression with DBA E) Control. (Magnification= 40X).

3.3.2 Effect of the MAPK signaling pathway on self-renewal of cultured germ cells

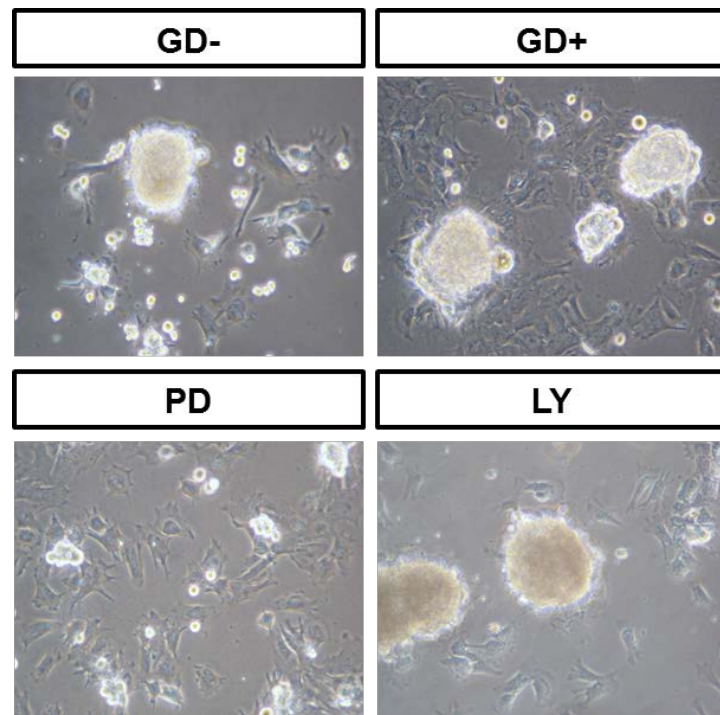
To investigate the signaling pathways responsible for the self-renewal of germ cells, pharmacological inhibitors of the MAPK and PI3K signaling pathways, PD and LY, respectively, were used. Culturing cells in the presence of PD significantly reduced the proliferation of germ cells and failed to form colonies. However, proliferation and colony formation were not influenced by the presence of LY in the culture (Fig. 3.2A and B). The appearance of germ cell colonies was observed in the presence of GDNF, PD, and LY as shown in Fig 3.2C.

Western blot analysis indicated that the level of MAPK phosphorylation induced in the culture was higher in the presence of GDNF than without GDNF (Fig. 3.2D and E). MAPK phosphorylation was blocked by the addition of PD to the culture medium, but was unaffected by LY (Fig. 3.2D and E).

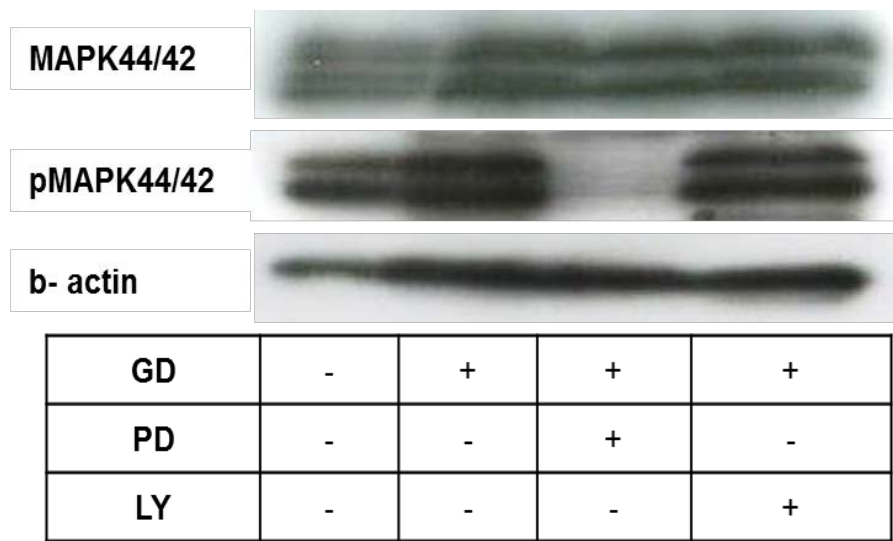
A



B



C



D

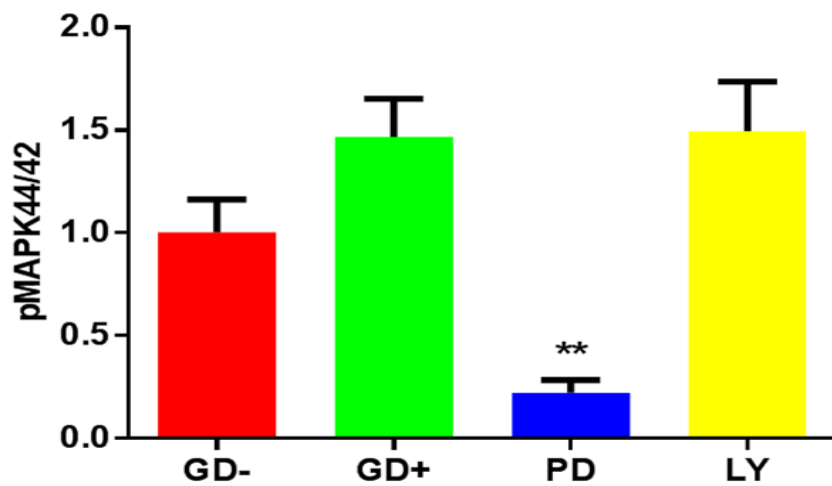
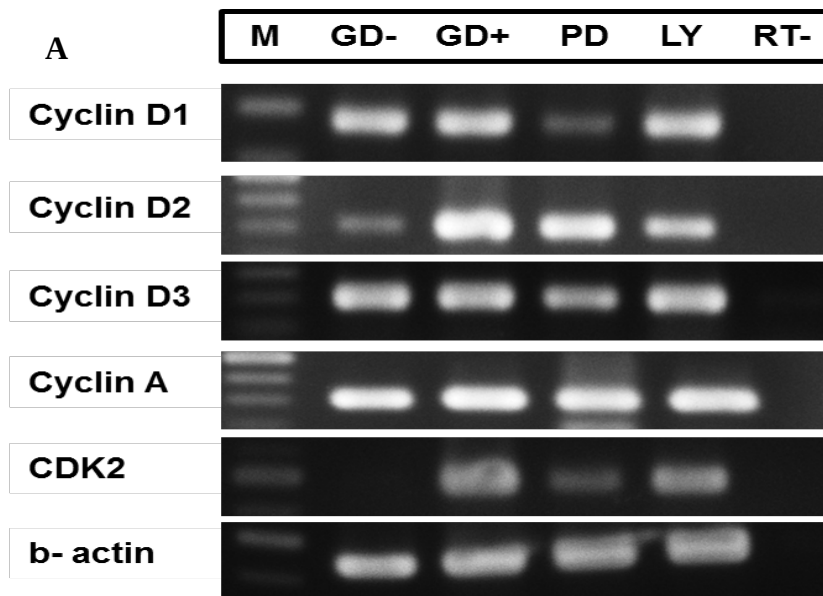


Figure 3.2 Effects of MAPK and PI3K signaling inhibitors on the self-renewal and colony formation of cultured germ cells. A) Cell proliferation of cultured germ cells for 6 days in the presence of MAPK (PD) and PI3K (LY) inhibitors. Cell

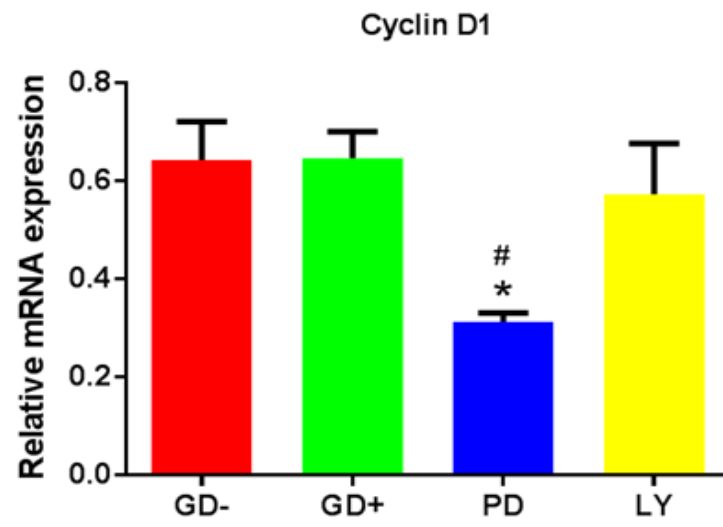
proliferation was significantly inhibited in the presence of PD relative to that in the absence of GDNF as the control (GD-), in the presence of GDNF (GD+) or LY. *P< 0.01 and **P< 0.01 significantly different from GD-, respectively; #####P< 0.01 significantly different from GD+. (Data were collected n=3 in each experiment, from three independent experiments, and indicated as the mean $\pm$ s.e.m). B) Colony formation by cultured germ cells for 6 days in the presence of MAPK/PI3K inhibitors. The number of colonies formed was significantly less in the PD-treated group than in the GD- group. The number of colonies formed was higher in the GD+ culture than in the GD- culture. ****P< 0.01 and **P< 0.01 significantly different from GD-; ####P< 0.01 significantly different from GD+. (Data were collected n=3 in each experiment, from three independent experiments, and indicated as the mean $\pm$ s.e.m). C) Appearance of germ cell colonies in the control, and in the presence of GD+, PD, and LY. D) Western blot analysis of MAPK and phosphorylated MAPK (pMAPK). (Germ cells were cultured for 4 days in the presence of GDNF. Cells were starved for 16 hours without GDNF and treated with no chemicals as the control, GDNF, PD, and LY for 20 min. (Magnification= 100X). E) Estimation of phosphorylated MAPK expression from three independent immunoblot experiments (mean $\pm$ s.e.m). The level of phosphorylated MAPK was significantly lower in PD-treated cells than in GD+-treated cells (**P< 0.01). However, the level of phosphorylated MAPK was not significantly different in the absence of GD (GD-) and in the presence of LY.

3.3.3 Enhanced cell cycle regulation of cultured germ cells

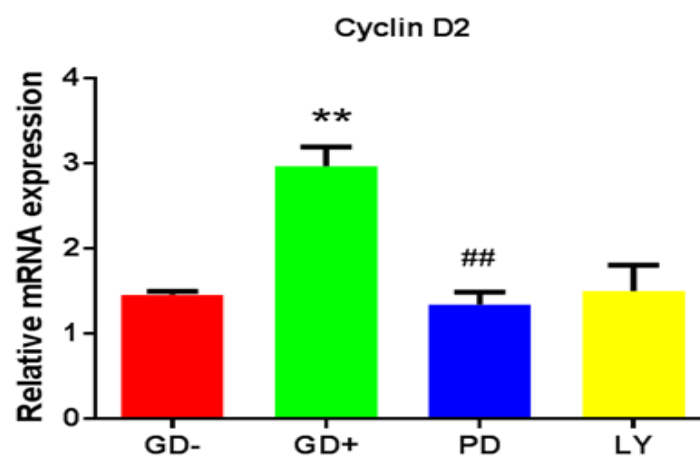
The expression patterns of cell cycle regulators in cultured cells treated with signaling inhibitors were analyzed using RT-PCR (Fig. 3A). The addition of GDNF enhanced the expression of cyclin D2 and CDK2 (Fig. 3B, C, and F, respectively). The expression of cyclin D1 and CDK2 was significantly reduced by the addition of PD to the culture medium (Fig. 3B and F). However, the enhanced expression of cyclin D2 was significantly reduced by the PD treatment (Fig. 3B). The expression of cyclin D3 and cyclin A was unaffected by the addition of GDNF or PD to the culture medium (Fig. 3D and E). Treatment with the LY inhibitor (PI3K signaling) did not influence the expression of these genes.



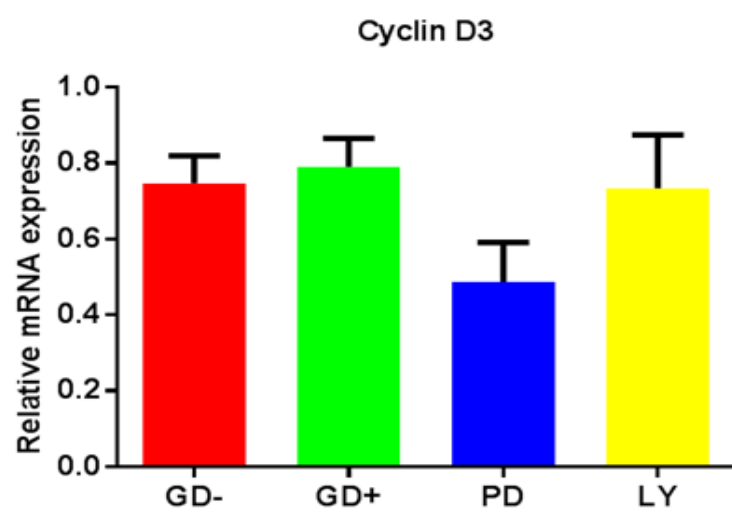
B



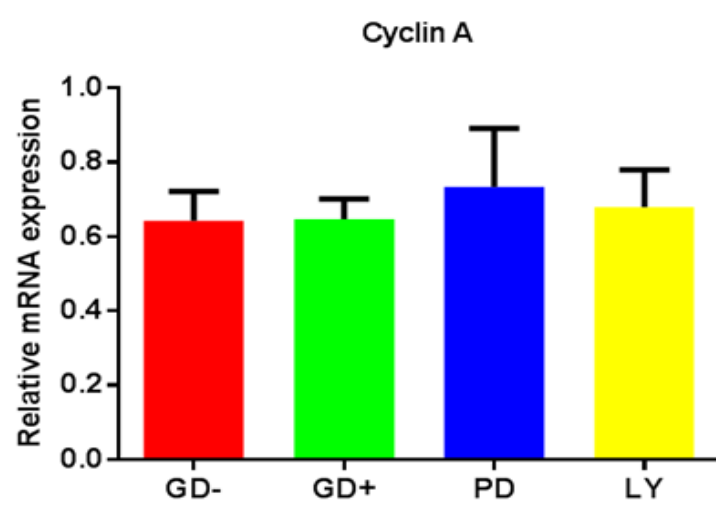
C



D



E



F

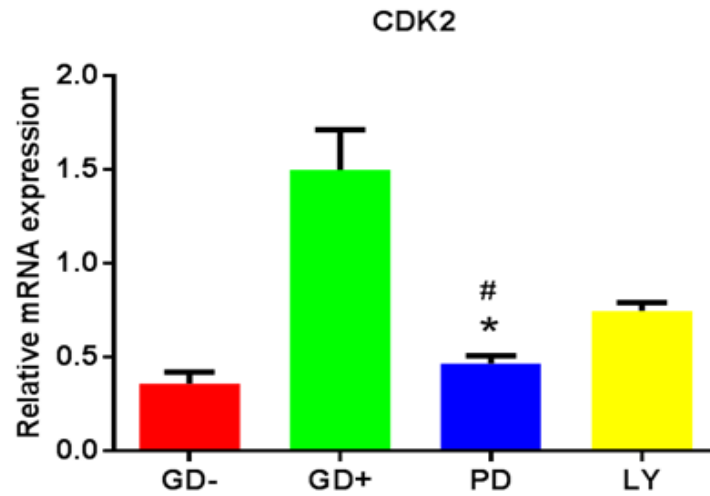


Figure 3.3 Effect of the inhibition of MAPK and PI3K signaling on the expression of cell cycle regulator genes.. A) RT-PCR analysis of cell cycle regulator genes (cyclin D1, cyclin D2, cyclin D3, cyclin, and CDK2) and b-Actin as the housekeeping gene. Relative mRNA expression of chicken D1 (B), cyclin D2 (C), cyclin D3 (D), cyclin A (E), and CDK2 (F) were examined in the presence of GDNF or MAPK/PI3K inhibitors. Data represented from three independent gel images (mean $\pm$ s.e.m). * $P < 0.01$ significantly different from GD-, # $P < 0.01$ significantly different from GD+.

3.4 Discussion

Signaling pathways emanating from niches in the testes, which govern the self-renewal and differentiation of cultured SSCs, have been well documented in mice (He et al. 2009). However, the mechanisms of proliferation of cultured SSCs have yet to be elucidated in species other than mice. The establishment of long-term culture systems for bovine germ cells is a daunting challenge. Although several attempts have been made to develop a culture system for bovine germ cells, they could not achieve colony formation after subsequent passages (Izaydar et al. 2002, 2003, Oatley et al. 2004, Aponte et al. 2008). We previously proposed a long-term culture system for bovine germ cells of more than 2 months (13 passages) (unpublished data). Mouse ES-like colonies

appeared after passages and expressed pluripotency markers (*OCT3/4* and *NANOG*) (Fujihara et al. 2011).

GDNF was shown to be the first molecule that regulates the self-renewal and differentiation of mouse SSCs (Meng et al 2000). GDNF signals act through the multicomponent receptor complex comprised of $\text{GFR}\alpha\text{-1}$ and RET tyrosin kinases in various cell types (Jing et al. 1996). $\text{GFR}\alpha\text{-1}$ and RET have also been recognized as spermatogonial markers expressed in gonocytes, SSCs, and differentiated spermatogonia (Widenfalk et al. 2000). These co-receptors of GDNF-mediated signaling were shown to be necessary for the self-renewal of germ cells in rodents (Naughton et al. 2006). In this study, we identified the nuclear expression of $\text{GFR}\alpha\text{-1}$ and RET proteins in cultured bovine germ cells. We and others (Aponte et al. 2006–2008), demonstrated that GDNF enhanced the germ cell proliferation and colony efficiency of cultured bovine germ cells, which indicated that GDNF-mediated signaling was conserved in germ cell cultures in rodents and cattle.

In the present study, we showed that the inhibition of MAPK pathways by the inhibitor PD98095 impaired cell proliferation and abolished colony formation. The presence of GDNF significantly increased tyrosine phosphorylation of MAPK44/42. This stimulation was blocked by the treatment with PD98059. These results indicate

that the activation of MAPK pathways is essential for the self-renewal of cultured bovine germ cells. In accordance with these results, GDNF signals were previously shown to activate RET phosphorylation and subsequently activate MAPK pathways, which are essential for the cellular growth and proliferation of SSCs in mice (He et al, 2008). Previous studies also demonstrated that FGF2, not GDNF, mediated activation of the MAPK pathway by upregulating the downstream targets ETV5 and Bcl6b in a mouse germ cell culture (Ishii et al. 2012). The addition of FGF to our culture system enhanced somatic cell proliferation and induced the differentiation of bovine germ cells (unpublished work).

PI3K/AKT is known to play an important role in the self-renewal of germ cells in mice through GDNF or FGF stimulation. The activation of PI3K/AKT signaling in mouse germ cells was shown to be completely inhibited by the inhibitor LY294002, which impaired the self-renewal of cultured germ cells (Lee et al. 2007, Oatley et al. 2007). However, the activation of AKT alone was not sufficient for the self-renewal of SSCs (Lee et al. 2007). Src kinase is an alternative activator of PI3K pathways, which results in the upregulation of *N-myc* expression and promotes the proliferation and self-renewal of mouse germ cells (Bryadich –Stolle et al. 2007, Oatley et al. 2007). Our results showed that the inhibition of PI3K/AKT signaling by LY294002 did not affect the cell proliferation

or colony formation efficiency of bovine germ cells. In addition, treatment with the Src inhibitor SU6656 did not have any effect on cultured bovine germ cells (data not shown). This result indicated that AKT-or Src-mediated PI3K signaling did not play a significant role in the self-renewal of germ cells in cattle. This finding is in contrast to that reported in mice, in which PI3K was shown to be the dominant signaling pathway.

The inhibition of MAPK and PI3K signaling was previously shown to result in the downregulation of the pluripotency genes *OCT3/4*, *NANOG*, and *SOX2* in human embryonic cell lines (Li et al. 2007, Eliselleova et al. 2009).which indicated that these signaling pathways play essential roles in maintaining the self-renewal and pluripotency of human ES cells. PI3K/AKT signaling was shown to regulate expression of the self-renewal cascade genes *Bcl6b*, *Etv5*, and *Lhx1* in a mouse germ cell culture (Oatley et al. 2007). Interestingly, the expression of *Oct3/4* was essential for the survival of mouse germ cells, but was not influenced by GDNF and did not play a significant role in self-renewal (Wu et al. 2010). However, the expression of *OCT3/4* and *NANOG* was detected in cultured germ cells and the testes of pigs (Goel et al. 2008) and cattle (Fujihara et al. 2011), suggesting that these pluripotent genes have roles in the maintenance and self-renewal of germ cells in domestic species. In contrast, *Nanog* expression has not been detected in cultured germ cells or the testes of mice (Yamaguchi et al. 2005). Our

previous report (Fujihara et al. 2011) demonstrated that the strong expression of the pluripotency markers *OCT3/4* and *NANOG* in cultured bovine germ cells was associated with the appearance of mouse ES-like colonies. These results indicate that the different expressions of transcription factors in mice and domestic species may lead to different regulatory mechanisms for the self-renewal and colony formation of cultured germ cells. However, the role of these genes has to be elucidated further to understand the MAPK-mediated self-renewal of bovine germ cells.

Activation of the extrinsic MAPK (He et al. 2008, Lee et al. 2009) and PI3K (Lee et al. 2007).signaling pathways in germ cells was previously shown to be involved in the regulation of cell-cycle-related cyclin gene expression. To understand the relationship between signaling pathways and the self-renewal of cultured bovine germ cells, we analyzed the downstream genes potentially involved in cell cycle regulation. Cyclin D1 is essential for entry to the G1/S-phase of the cell cycle in the presence or absence of GDNF and is regulated by the MAPK pathway (Dolci et al. 2001). The expression of cyclin D1 has also been observed in proliferating gonocytes and SSCs in the mouse testes (Beumer et al. 2000). In this study, the expression of cyclin D1 was significantly downregulated after the inhibition of MAPK signaling by PD, but was unaffected by the presence of GDNF. In contrast, cyclin D2 expression was significantly upregulated

upon GDNF stimulation and inhibited upon pre-treatment with the MAPK inhibitor, which indicated that the MAPK pathway was involved in regulating the cell cycle of bovine germ cells. The overexpression of cyclin D2 was previously shown to regulate the self-renewal of germ cells, and was mediated by Ras activation in mice (Lee et al. 2009). CDK2 has been shown to be involved in controlling entry to the S-phase in association with cyclin A. CDK2 was upregulated in the presence of GDNF and controlled entry to the G1/S-phase in mouse C18-4 germ cell lines via MAPK-mediated signaling (He et al. 2008). In this study, CDK2 expression was also significantly upregulated upon GDNF stimulation and the inhibition of MAPK signaling resulted in the downregulation of CDK2 expression. Enhanced CDK kinase activity was previously shown to be essential for the Ras-induced proliferation of cultured mouse germ cells (Lee et al. 2009). Our results suggested that cell cycle-related genes were not influenced by the inhibition of PI3K signaling, which is consistent with a previous report (Lee et al. 2007) in which the inhibition of PI3K signaling did not significantly affect changes in cyclin gene expression in cultured germ cells.

Taken together, these findings reveal the unique and crucial role of MAPK signaling in maintaining the self-renewal and colony formation efficiency of cultured bovine germ cells. In contrast to our findings, cultured mouse germ cells require

crosstalk between MAPK and PI3K signaling pathways for self-renewal. The downstream targets of MAPK signaling that ultimately influence the self-renewal of bovine germ cells need to be determined in future experiments. The present study has revealed marked differences in the control of the self-renewal and maintenance of cultured germ cells from mice and cattle. These results will be useful for identifying optimal culture conditions to establish a long-term culture system and germ cell lines in domestic species.

Chapter 4

Summary and Conclusions

4.1 Summary

Advances in animal reproductive biotechnologies provide a powerful tool for enhanced production of livestock animals and address the future challenges of food security. In dairy cattle, application of currently available ARTs i.e. animal cloning and AI is limited due to a long generation interval and to be too costly to use large number of females. Recent years, male germ cell transplantation is emerging as assisted reproductive tools to address the existing challenges of current technologies. The initial step for fruitful application of male germ cell transplantation is requirement of large number of germ cells for transplantation to recipient testis. Despite advances in rodents germ cell culture, no proven long-term culture system for livestock species has been developed.

In chapter 2, we worked on hypothesis that tailoring culture components

according to species-specific need of bovine germ cells for the establishment of a culture system. We tested four ECM substrate, poly-L-lysine, gelatin, DBA and collagenase for coating culture dish to inhibit somatic cell growth and to support germ cells. Poly-L-lysine-coated dishes selectively inhibited the proliferation of testicular somatic cells and supported the proliferation of germ cells in culture. Previous studies show that the adverse effect of overgrowing somatic cells on germ cells expansion due to presence of high concentration of serum in culture medium favoring somatic cell growth. To address this problem and reduced serum concentration, we tested different commercially available serum-free supplements, KSR, B-27 and StemPro-SFM, which have been proven for mice and rodent germ cell culture. In our experiment, KSR-supplement medium is more effective than that of B-27 or StemPro-SFM supplement for long-term culture of bovine neonatal germ cells. Albumax (a lipid-rich BSA) present in KSR-supplement, but is absent in B-27 or StemPro-SFM, may contribute to the long-term culture of bovine germ cells. Combination of KSR and poly-L-lysine-coated culture dishes, we could propagate germ cells with a normal karyotype for over 2 months. The cultured germ cells show abilities of germ cells and stem cells revealed by molecular markers throughout the culture period.

In final experiments (chapter 3), we focused on exploring the molecular

mechanisms responsible for the self-renewal and maintenance of cultured bovine germ cells. We showed that the inhibition of MAPK pathways by the inhibitor PD98095 impaired cell proliferation and abolished colony formation. We provide the evidence for active state of GDNF is responsible for MAPK-mediated self-renewal. Our downstream gene target analysis indicates that cyclin D1 and CDK2 are involved in MAPK mediated self-renewal.

In summary, we established culture conditions for the propagation of undifferentiated bovine germ cells on poly-L-lysine-coated dishes in medium containing KSR. Also these findings reveal the unique and crucial role of MAPK signaling in maintaining the self-renewal and colony formation efficiency of cultured bovine germ cells.

4.2 Conclusions

In comparison with mice, our study emphasis on requirement of different culture components for bovine germ cell culture (Fig. 4.1). The signaling pathways required for self-renewal mechanism of germ cells in cattle is different from mice (Fig. 4.2). This indicates the striking difference in SSCs biology in rodent and livestock species at molecular level.

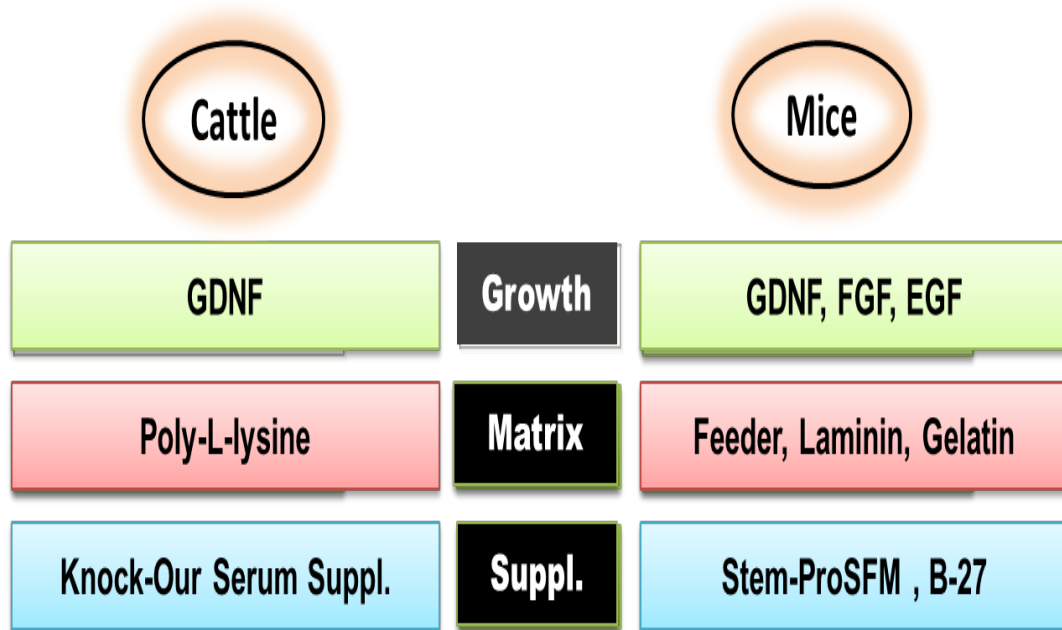


Figure 4.1 Schematic illustration of requirement of different culture components for germ cell culture in cattle and mice

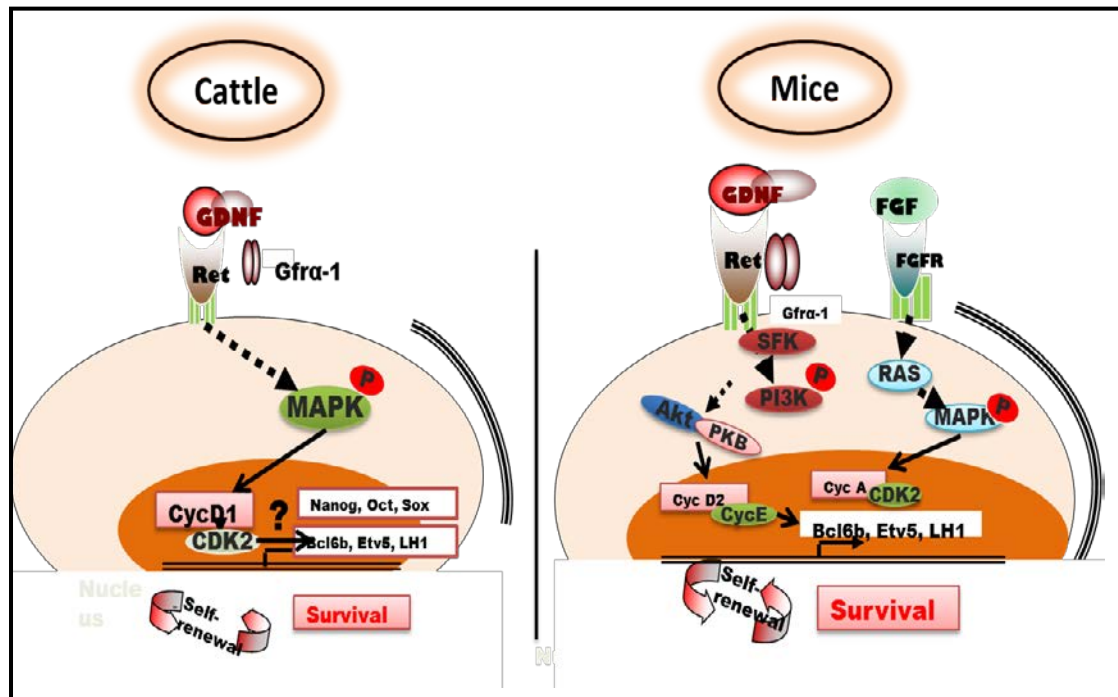


Figure 4.2 Schematic illustration of regulation of self-renewal mechanism in cattle and mice

4.3 Future direction

Our studies provide the foundation for studying different regulatory mechanism of SSCs biology in cattle and rodents. The evaluation of active components and their specific role present in KSR-supplement need to be studies for improvement culture condition. We identified the role of MAPK-mediated self-renewal of bovine germ cell, however, the role of downstream targets (transcription factors) need to be elucidated.

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