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A DISSERTATION  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Development of defined culture condition  
for pig pluripotent stem cells  
and their potential application

돼지 만능성줄기세포 배양 조건 개발과  
그 응용 가능성에 대한 연구

August, 2017

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# ABSTRACT

## Development of defined culture condition for pig pluripotent stem cells and their potential application

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Derivation of pluripotent cells can be accomplished by *in vitro*-culture of early embryos. Pluripotent stem cells (PSCs) have been considered as a candidate for regenerative medicine and cell therapy. PSC lines derived from domestic animals such as pigs and cattle are useful tools in the production of transgenic animals. Especially, because of the physiological and immunological similarities between pigs and humans, porcine PSCs have been identified as a useful candidate for a certain human disease. So, in this study, pig PSCs were derived from various origins including embryos and somatic cells to find their application. Firstly, I tried to

analyze stem cells derived from embryos and fetus for unveiling mechanism of pluripotency in pig. So, pig embryonic stem cells (ESCs) were derived from *in vitro*-produced embryos by supplementing FGF2. And reprogramming of PGCs and maintenance of EGCs were achieved by FGF2 signaling. The results showed that FGF2 signaling has a pivotal roles in establishing and maintaining pluripotency in pig both PSCs. Next, pig somatic cells were reprogrammed into pluripotent state using Yamanaka's factors. During reprogramming, FGF2 treatment strongly up-regulated specific pluripotent genes such as *SOX2*, *KLF4*, *REX1*, and epithelial-specific markers when compared to LIF treatment, and blocking FGF2 signaling down-regulated *KLF4* and *NANOG*. Then, optimization of culture media for pig ESCs were conducted by using various metabolic components and signaling molecules. As a result, pig ESCs were successfully established by chemically defined media supplemented with FGF2, ACTIVIN A and WNT activator. These cells expressed pluripotent genes such as *OCT4*, *SOX2* and *NANOG*, and could be maintained for extended periods. Next, transgenic pluripotent cell lines were generated by lentiviral vector harboring enhanced green fluorescence protein (EGFP). Transgenes were successfully introduced into ESCs and transfection was the most efficient under multiplicities of infection (MOI) of 75. It was apparent that the

expression of inserted lentiviral transgenes was controlled by DNA methylation. Neuronal progenitor cells were derived from pig embryonic germ cells. Similar with other species, neuronal progenitor cells were successfully induced by treatment of retinoic acid and these cells expressed neuronal markers such as *PAX6*, *NESTIN* and *SOX1*. Taken together, I found that, as a non-permissive species, pig PSCs are maintained by mainly FGF signaling, and additional signaling molecules such as ACTIVIN and WNT are required for supporting pluripotency. And pig ESCs could be derived using chemically defined media supplementing FGF2, ACTIVIN A and WNT. This study will not only provide basic understanding for mechanism of maintaining pluripotency but also apply stem cell engineering for regenerative medicine. Accordingly, studies on pig PSCs will pave the way for human cell therapy and shed new light on researches of PSCs.

**Key words:** pig, pluripotent stem cells, embryonic stem cells, embryonic germ cells, induced pluripotent stem cells, media optimization, transgenesis, neural differentiation

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# LIST OF ABBREVIATIONS

2i	Two inhibitors; ERK and GSKb inhibitors
ActA	ACTIVIN A
AMD	Age-related macular degeneration
$\alpha$ -MEM	$\alpha$ -Minimum Essential Medium
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BMP	Bone Morphogenetic Proteins
cDNA	Complementary deoxyribonucleic acid
CDs	Cluster of differentiation
CNTF	Ciliary neurotrophic factor
COCs	cumulus oocyte complexes
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
Dox	Doxycycline
DPBS	Dulbecco's phosphate buffered saline
dpc	Days post coitus
E	Embryonic day
EBs	Embryoid bodies
EC cells	Embryonal carcinoma cells

eCG	Equine chorionic gonadotropin
ECM	Extracellular matrix
EGCs	Embryonic germ cells
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EpiSC	Postimplantation epiblast-derived stem cells
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fsk	Forskolin
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
HAR	Hyperacute rejection
hCG	Human chorionic gonadotropin
ICC	Immunocytochemistry
ICM	Inner cell mass
Id	Inhibitor of differentiation
IL	Interleukin
iPSCs	Induced pluripotent stem cells
JAK	Janus kinase
LIF	Leukemia inhibitory factor
LIFr	Leukemia inhibitory factor receptor

LTR	Long terminal repeat
MEFs	Mouse embryonic fibroblasts
MET	Mesenchymal-to-epithelial transition
mRNA	Messenger ribonucleic acid
OSKM	OCT4, SOX2, KLF4 and cMYC
OSM	Oncostatin M
PCR	Polymerase chain reaction
pFF	Pig follicular fluid
PGCs	Primordial germ cells
PI	Propidium iodide
pre-iPSCs	Partially reprogrammed-iPSCs
PSCs	Pluripotent stem cells
PZM	Pig zygote media
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SCNT	Somatic cell nuclear transfer
SSEA	Stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
STO	Shimm's Thioguanine Ouabain resistant

TE	Trophectoderm
TRA	Tumor related antigen
XCI	X chromosome inactivation

CHAPTER 1  
GENERAL INTRODUCTION

Stem cells indicates progenitor cells that are capable of self-renewal and differentiation into several cells. Especially, pluripotent stem cells (PSCs) have *in vivo* and *in vitro* differentiation potentials into three germ layers and can proliferate infinitely. First PSCs, known as embryonic stem cells (ESCs), were derived from preimplantation mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). Subsequently, another pluripotent cell lines, called embryonic germ cells (EGCs), were obtained through *in vitro*-culture of primordial germ cells (PGCs) (Matsui et al., 1992; Resnick et al., 1992). Recently, mouse epiblast stem cells (EpiSCs) and induced pluripotent stem cells (iPSCs) were derived from postimplantation embryos and somatic cells, respectively (Takahashi and Yamanaka, 2006; Tesar et al., 2007). Pluripotent states are divided into “naïve” and “primed” states depending on developmental competency of PSCs (Nichols and Smith, 2009). Naïve PSCs, represented by mouse ESCs and EGCs, are developmental ground state similar with early epiblasts of preimplantation embryos. On the other hand, primed PSCs, including EpiSCs and human ESCs, possess more differentiated pluripotency than naïve cells showing features of late epiblasts in postimplantation embryos. In permissive lines, both state of PSCs can be derived from embryos. However, in non-permissive lines such as human, only primed PSCs are derived in

absence of additional treatment such as genetic manipulation and chemicals (Buecker et al., 2010; Hanna et al., 2009; Park et al., 2013a)).

Because PSCs not only can differentiate into various type of cells and tissues, but also can produce germline–chimera by blastocyst injection (Bradley et al., 1984; Doetschman et al., 1985), PSCs have been considered as cell sources for cell therapy and producing transgenic animals. After establishment of human ESCs (Thomson et al., 1998), various researches for regenerative medicine by tissue engineering have been studied. Degenerative diseases could be treated by replacement of damaged tissues or cells with undamaged normal tissues or cells differentiated from PSCs (Tabar and Studer, 2014). And establishment of induced pluripotent stem cells (iPSCs) and cloned human ESCs allowed patient–specific cell therapies with PSCs (Tachibana et al., 2013; Takahashi and Yamanaka, 2006). In addition, transgenic animals could be generated by blastocyst injection and nuclear transfer using genetically modified PSCs (Capecchi, 2005; Robertson et al., 1986; Thomas and Capecchi, 1986). And, PSCs are more suitable for reprogramming within enucleated oocyte than differentiated cells (Hochedlinger and

Jaenisch, 2007), can provide an indefinite cell source for nuclear transfer.

In domestic animals, the aim of stem cell research is to create an indefinite cell source for transgenic animals used as bioreactors and tissue engineering materials as well as preliminary studies for human research (Keefer et al., 2007). To apply human PSCs as tools for regenerative medicine, preclinical studies with animal models are essential. Several animals such as pig, horse, cow and sheep etc. have been used for PSC researches (Ogorevc et al., 2016). Especially, pigs have been identified as an ideal animal model to study human disease, because of similarities between physiological and immunological features in humans and pigs, together with organ size (Brevini et al., 2007; Hall, 2008). Therefore, many research groups have attempted to derive pig PSCs including ESCs, EGCs and iPSCs, however, authentic pig ESCs have not yet been established (Ezashi et al., 2009; Kues et al., 2013; Park et al., 2013a; Piedrahita et al., 1990b; Son et al., 2009; Yang et al., 2009). Failures of deriving faithful pig PSCs are due to lack of understanding for mechanisms of pluripotency in pigs.

Because it was verified that pig has a different pluripotent networks in early embryos (Liu et al., 2015), it is essential to unveil mechanisms for establishing and maintaining pluripotency for deriving pig PSCs and optimizing pig-specific reprogramming methods. For these reasons, firstly, I attempted to analyze pig PSCs derived from various origins including embryos and somatic cells. In chapter 3 and 4, ESCs and EGCs were derived from pig *in vitro*-produced embryos and fetal gonads, respectively, and iPSCs were generated from pig somatic cells by ectopic expression of Yamanaka's factors. Through an analysis of various PSC lines, it was explored which cell signaling are involved in supporting pluripotency of pig. Then, based on previous chapter, optimizing culture media for pig ESCs was performed by using various metabolic components and signaling molecules. Secondly, for applications of pig PSCs in preclinical study and production of transgenic pig, I tried to develop transgenesis and differentiation methods in pig PSCs. In chapter 6, lentiviral vectors carrying were examined to introduce transgene into pig pluripotent cell lines including ESCs and EGCs. In chapter 7, differentiation ability of pig EGCs into neuronal progenitor cells were investigated.

CHAPTER 2  
LITERATURE REVIEW

## 1. Pluripotent stem cells (PSCs)

Researches on pluripotent stem cells (PSCs) were begun by embryonal carcinoma cells (EC cells) derived from murine gonadal teratomas and teratocarcinoma. Teratomas (benign) and teratocarcinomas (malignant) are tumors which are spontaneously formed in gonad, they are composed of several adult tissues including tooth, bone, muscle, skin and hair etc. When cells from teratocarcinomas were injected intraperitoneally into strain 129 mice, they re-formed teratocarcinomas (Kleinsmith and Pierce, 1964). This findings showed that stem cells which can proliferate indefinitely with differentiation potential into several tissues were existed in teratocarcinomas. In subsequent research, teratocarcinomas were artificially generated by injecting pre- and post-implantation embryos into testis in mouse, which indicated that stem cells in teratocarcinomas are similar with undifferentiated early embryos (Solter et al., 1970; Stevens, 1970) and Stevens developed 129/sv strain which has increased formation rate of gonadal tumor (Stevens, 1981).

In 1970, Martin and Evans succeeded in establishing cell lines

from teratocarcinomas, so called EC cells, by co-culture with feeder cells made of mitotically-inactivated mouse embryonic fibroblasts in vitro (Martin and Evans, 1974). Subsequently, many scientists analyzed EC cells to elucidate their characteristics, which paved the way for researches on PSCs. EC cells possessed differentiation ability into three germ layers, so called pluripotency, and could proliferate indefinitely through symmetric division, so called self-renewal. Similar to teratocarcinomas, when injected subcutaneously into mouse, EC cells could produce teratocarcinomas differentiating into several tissues. When cultured in suspension, the cells aggregated and formed embryo-like structure with cavity, known as cystic embryoid bodies (Martin and Evans, 1974, 1975a; Martin and Evans, 1975b). In 1977, human EC cells were derived from malignant testicular teratomas and, as monoclonal antibodies were developed to detect specific marker protein, it was proven that mouse and human EC cells expressed SSEA1 and SSEA4 respectively (Fenderson et al., 1987; Hogan et al., 1977; Kannagi et al., 1983a; Kannagi et al., 1983b; Stern et al., 1978). Based on their features recapitulating embryonic development, early PSC researches had focused on cellular differentiation and embryology (Martin, 1975).

Although EC cells were potential materials to study pluripotent cells and embryogenesis, they have limitations to study embryology. They have abnormal karyotype and, when micro-injected into recipient blastocysts, couldn't generate germline-chimeras showing restricted differentiation ability (Blelloch et al., 2004; Papaioannou et al., 1978; Papaioannou et al., 1975; Rossant and McBurney, 1982). In 1981, two groups, independently of each other, solved the problems through establishing embryonic stem cells (ESCs) derived by direct seeding of early blastocyst onto feeder cells, not by subcutaneous transplantation into mouse (Evans and Kaufman, 1981; Martin, 1981). ESCs not only possessed similar feature with EC cells in terms of cellular physiology, pluripotency and marker expression, but also could produce germline-chimeras with normal karyotype via blastocyst injection (Bradley et al., 1984; Doetschman et al., 1985). Especially, as gene targeting technologies were developed, transgenic mouse could be generated from genetically modified ESCs, which accelerated studies on gene functions during embryogenesis (Capecchi, 2005; Robertson et al., 1986; Thomas and Capecchi, 1986). On the other hand, because teratocarcinomas are spontaneously derived from germ cells in testis, this concept led to establish pluripotent cell lines from germ cells, as ESCs were derived from embryos (Dolci et al., 1991; Godin et al.,

1991; Matsui et al., 1991). In 1992, these efforts resulted in establishing pluripotent cell lines, so called embryonic germ cells (EGCs) from primordial germ cells (PGCs) (Matsui et al., 1992; Resnick et al., 1992).

In 1998, PSC researches entered on a new phase by establishment of human ESCs (Thomson et al., 1998). The derived human ESCs made people think that pluripotent cells could be used for regenerative medicine by tissue engineering. To take care of degenerative diseases including Parkinson's disease, Alzheimer's disease, diabetes and retinitis pigmentosa etc., damaged tissues or cells should be replaced with undamaged normal tissues or cells, but it is hard to prepare the cells to care patients. However, if PSCs can be differentiated into specific cell lineage, human ESCs could supply normal cells or tissues for transplantation without limitations (Tabar and Studer, 2014). For these reason, PSC researches begun to focus on cell therapy, and were accelerated by establishment of induced pluripotent stem cells (iPSCs) and cloned human ESCs in 2006 and 2013, respectively (Tachibana et al., 2013; Takahashi and Yamanaka, 2006). So, in this chapter, it will be discussed that characteristics of PSCs derived from various origins and recent progress in PSC

research area.

## 1.1 Embryonic stem cells (ESCs)

To retain characteristics of ESCs, self-renewal and pluripotency, various extrinsic and intrinsic factors have to be delicately regulated. Distinct features of ESCs compared with somatic cells are used to identify them. Lack of extrinsic or intrinsic determinants induce differentiation of ESCs losing pluripotency. So, it is important to understand and investigate genes and culture conditions involved in pluripotent circuit.

Microenvironmental surrounding of ESCs, known as stem cell niche, including cytokines, extracellular matrix and metabolic sources is important extrinsic factors for supporting pluripotency. Viability and self-renewal of ESCs in *in vitro*-culture are dependent on stem cell niche. First mouse ESCs were obtained by co-culturing with mitotically-inactivated mouse embryonic fibroblasts (MEFs), so called feeder layer, and EC cell-derived conditioned media (Evans and Kaufman, 1981; Martin, 1981). Main function of feeder layer is

providing physical environment through extracellular matrix (ECM), which affects proliferation and survival rate by activating Cadherin and Integrin signaling in ESCs (Guilak et al., 2009; Nagaoka et al., 2006). And feeder cells activate intrinsic pathways of pluripotency by paracrine effects of several cytokines including LIF, FGF2, BMP4, ACTIVIN A and WNT etc (Eiselleova et al., 2008). Various type of cells including MEFs (Evans and Kaufman, 1981; Martin, 1981), STO cells (Park et al., 2003), and SNL 76/7 cells (Williams et al., 1988) have been used as a feeder cells. Recently, in order to reduce cell-to-cell variation and xeno-contamination occurred by feeder layer, mixture of ECM proteins and synthetic peptides are being used instead of feeder cells (Lee et al., 2010; Mei et al., 2010; Meng et al., 2010).

Together with physical environment, activation of cellular signaling by cytokines is also important to keep pluripotency. Because signaling molecules specifically support pluripotency of ESCs were not known, EC cell-derived conditioned media was used at the beginning of ESC culture (Martin, 1981). Later, it was verified that LIF and BMP4 signaling pathways have a crucial role in supporting pluripotency of ESCs (Smith et al., 1988; Williams et al.,

1988; Ying et al., 2003). Firstly, leukemia inhibitory factor (LIF) is a member of interleukin-6 family. If LIF bound extracellular domain of LIF receptor (LIFr) in plasma membrane, LIFr forms heterodimer with gp130. LIFr/gp130 heterodimer activates receptor-associated Janus kinase (JAK), which results in phosphorylation of STAT3. Phosphorylated STAT3 molecules act as a homodimer in nucleus, which facilitates transcription of pluripotency-related genes. The up-regulated genes prevent differentiation and support self-renewal in mouse ESCs (Boeuf et al., 1997). However, although LIF treatment is sufficient to maintain pluripotency in serum-supplemented media, ESCs differentiated into neural lineage in serum-free media, suggesting unknown factors of fetal bovine serum involved in sustaining pluripotency. Ying and colleagues found that BMP4 support pluripotency with LIF in serum-free media preventing neural differentiation (Ying et al., 2003). BMP4 facilitates expression of inhibitor of differentiation, Id, protein through SMAD signaling and these proteins inhibit neural differentiation. Recently, various small molecules which inhibit cell signaling of differentiation and sustain pluripotent gene networks are used in stem cell culture (reviewed by Ma et al., 2013).

If stem cell niche as mentioned above is prepared in *in vitro*-culture environment, intrinsic determinants of pluripotency are activated in ESCs. As transcription factors, the intrinsic determinants important roles in regulation and maintenance of pluripotent gene networks. Most important genes in regulating pluripotency are *Oct4*, *Sox2* and *Nanog*, which also are crucial for establishing pluripotency and lineage segregation in early embryo. *Oct4* and *Sox2* are starting to express from early blastomere stage and, after cavitation of blastocyst, exclusively express in inner cell mass (ICM) (Avilion et al., 2003; Nichols et al., 1998; Scholer et al., 1989). These two factors form transcriptional complex with various enhancer proteins and work as a complex (Ng and Surani, 2011). Balanced expression of *Oct4* and *Sox2* is important to maintain pluripotency (Radziskeuskaya and Silva, 2014). Overexpression of *Oct4* induce mesodermal differentiation and, in case of *Sox2*, ESCs differentiate into ectodermal lineage. In addition, along with *Oct4* and *Sox2*, *Nanog* as a transcription factor have a crucial role in supporting stemness (Chambers et al., 2003; Loh et al., 2006; Mitsui et al., 2003).

Distinctive epigenetic feature of ESCs is that two X chromosomes are activated in female ESCs. Because, unlikely male

cells, female mammalian cells have two X chromosomes, one X chromosome is randomly inactivated for compensation of genetic materials in female cells (Okamoto and Heard, 2009). As ICM which have two activated X chromosomes undergoes gastrulation, X chromosome inactivation (XCI) is occurred by non-coding RNA, *Xist* (Heard, 2004; Lee and Bartolomei, 2013). In this reason, ESCs as an *in vitro*-counterpart of ICM have two-activated X chromosomes (Nichols and Smith, 2009). Recent researches indicated that pluripotent genes such as *Oct4*, *Sox2* and *Nanog*, have a vital role in sustaining activated status of X chromosomes through regulation of *Xist* and *Tsix*, known as negative regulator of *Xist* during murine preimplantation development (Donohoe et al., 2009; Navarro et al., 2008). Accordingly, several researches are being performed to verify relationship between pluripotency and XCI (reviewed in Minkovsky et al., 2012; Navarro and Avner, 2009).

Feeder cells are also required for culture of human ESCs, similar with mouse (Thomson et al., 1998). In addition, *OCT4*, *SOX2* and *NANOG* play vital roles in establishing and maintaining of pluripotency in human embryos and ESCs (Boyer et al., 2005). However, there are some physiological differences between human

and mouse ESCs. Unlike mouse in which LIF/BMP4 are involved, pluripotency of human ESCs is sustained through ERK and ACTIVIN/NODAL signal pathway activated by FGF2 and TGF- $\beta$  (Pera and Tam, 2010). In female human ESCs, X-linked genes are monoallelically expressed because of inactivation of X chromosome by expression of *XIST* in atmospheric O<sub>2</sub> concentrations (Lengner et al., 2010; Shen et al., 2008). These distinguishable features had been considered as species-specific characteristics for a long time. However, series of experiments discovered that the differences among PSCs are originated by two distinct grades of pluripotency, including “naïve” and “primed” states, based on developmental competence (Hanna et al., 2010b; Nichols and Smith, 2009; discussed in 1.5).

## 1.2 Epiblast stem cells (EpiSCs)

In 2007, two research groups established novel type of pluripotent stem cells, named postimplantation epiblast-derived stem cells (shortly, epiblast stem cells or EpiSCs), from postimplantation embryos (Brons et al., 2007; Tesar et al., 2007). ICM of blastocyst divide into early epiblast and hypoblast through the

lineage segregation. After implantation, epiblast finally develop fetus through a gastrulation, hypoblast differentiate into extraembryonic tissues including visceral and parietal endoderm (reviewed in (Rossant and Tam, 2009)). To derive EpiSCs, late epiblasts were isolated at embryonic day 5.5 before gastrulation and seeded onto feeder cells in media supplemented with FGF2 and ACTIVIN A. After 3–5 days, epiblast explants rapidly grew and from OCT4–expressed compact colonies. Established EpiSCs expressed pluripotent genes such as *Oct4*, *Sox2* and *Nanog*, and teratomas formed when the cells were grafted in immune–deficiency mice. However, EpiSCs showed distinct characteristics compared with ESCs derived from early epiblasts. Expression of ICM–specific genes which are up–regulated in ESCs, such as *Pecam1*, *Tbx3* and *Gbx2*, were decreased in EpiSCs. On the other hand, epiblast and early germ layers–specific genes such as *Otx2*, *Eomes*, *Foxa2*, *T*, *Gata4*, *Sox17* and *Cer1* were highly expressed in EpiSCs. This cell line grew as a monolayer having flattened morphology and relied on ERK and ACTIVIN/NODAL signaling pathway to maintain pluripotency instead of LIF and BMP4 signaling. In epigenetic and developmental aspects, female EpiSCs possessed inactivated X chromosome similar with their *in vivo*–counterpart, late epiblasts. In the same manner, when assessed developmental stage by chimeric assay, although they could not

generate chimeric embryos with morula and early blastocyst, EpiSCs were incorporated within embryos and developed chimeric fetus when engrafted into postimplantation embryos (Huang et al., 2012; Kojima et al., 2014). While murine ESCs could be converted into EpiSCs by treatment of FGF2 and LIF antibody, EpiSC couldn't be converted into ESCs without genetic manipulations, which means steps of mammalian development are irreversible (Bao et al., 2009; Guo et al., 2009; Hanna et al., 2009). Overall, EpiSCs shared defining similarities with human ESCs in terms of gene expression and cellular signaling.

Before derivation of this new type of cell line, it was thought that pluripotent state is fixed. However, this interesting discovery compelled to think that pluripotent states are flexibly altered dependent on culture environments and developmental origins of PSCs. Finally, all these results were sufficient to change paradigm of pluripotency and provoke a new hypothesis/theory for pluripotency status (discussed in 1.5).

### 1.3 Embryonic germ cells (EGCs)

PGCs as an alternative cell source can be used for deriving pluripotent stem cells. When cultured with feeder cells and adequate cytokines, PGCs can be reprogrammed into pluripotent stem cells, named EGCs. As ESCs were established from ICM via *in vitro*-culture, it had been attempted that *in vitro* culture of PGCs, origin of spontaneous teratocarcinomas *in vivo*, for deriving novel pluripotent stem cell line (Dolci et al., 1991; Donovan et al., 1986; Godin et al., 1991; Matsui et al., 1991).

In murine post-implantation embryo at dpc 5.5, PGCs arise from proximal posterior epiblast by stimulation of BMP4, BMP8b and BMP2 from extraembryonic ectoderm and visceral endoderm (Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). In precursor of PGCs, BMP signaling stimulates expression of *Blimp1* known as key transcription factors for specification of germline, which induces repression of somatic program and facilitates expression of pluripotent genes and epigenetic reprogramming (Durcova-Hills et al., 2008; Ohinata et al., 2005). Resulting PGCs migrate from posterior endoderm of the yolk sac at dpc 7.5 via

hindgut and mesentery to genital ridge at dpc 12.5, and primitive gonads and fetal ducts in turn develop into sex organs (Molyneaux et al., 2001). Aposing migration, dozens of cells actively proliferate into thousands and epigenetic remodeling including reconstruction of DNA methylation, imprint erasure and X-chromosome reactivation are occurred (reviewed in (Ewen and Koopman, 2010)). And after arrival at genital ridges, gender-specific imprinting patterns are re-establishment (Lees-Murdock and Walsh, 2008).

When cultured with feeder cells made of STO cell line and cytokines including LIF, FGF2 and SCF, mouse migrating PGCs isolated at dpc 8.5 were reprogrammed and converted into EGCs (Matsui et al., 1992; Resnick et al., 1992). In culture, roles of cytokines are as follows. Stem cell factor (SCF, also known as c-Kit ligand or Steel factor), although PGCs undergo apoptosis in a few days during in vitro culture, prevents apoptosis and promotes viability and proliferation rate of germ cells together with LIF (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). FGF2 up-regulates expression of *Dhx38*, *Myc* and *Klf4* known as targets of BLIMP1 via down-regulation of *Blimp1*, facilitates reprogramming of PGCs into EGCs (Durcova-Hills et al., 2008). Finally, fully-

reprogrammed mouse EGCs cultured with only LIF.

Established mouse EGCs possess similar features with embryonic stem cells in physiological and developmental aspects. They express pluripotent marker genes such as OCT4, SOX2, NANOG and SSEA1, and have developmental competency *in vitro* (embryoid body formation) as well as *in vivo* (teratoma and chimera formation) (Matsui et al., 1992; Resnick et al., 1992). In addition, recent data indicated that there's close similarities between EGCs and ESCs in terms of genetics and epigenetics (Choi et al., 2017). In human, EGCs could be derived from gonad of aborted fetuses culturing with feeder cells and LIF, FGF2 and Fsk (Liu et al., 2004; Shamblott et al., 1998; Turnpenny et al., 2003). Human EGCs expressed several pluripotent markers such as OCT4, SSEA1 and SSEA3/4 and have *in vitro* developmental competency. However, because of ethical problems for using human fetuses, research on human germ cells is not as well performed (Turnpenny et al., 2006).

#### 1.4 Induced pluripotent stem cells (iPSCs)

Developmental biologist Conrad H. Waddington, as known as pioneer of epigenetics, addressed that, because differentiation of cells is like a rolling stones through a downhill, stones reached at bottom of hill are in stable state and cannot move to anywhere (Sieweke, 2015). For a long time, developmental biologists had believed that developmental process of organism are irreversible event, and completely lineage-committed cells cannot be converted into the other type of cells including progenitor cells. In 1962, Sir John B. Gurdon generated tadpoles by nuclear transfer of somatic cells into enucleated eggs (Gurdon, 1962) and, after three decades, Sir Ian Wilmut and colleagues produced first cloned mammal, Dolly the sheep, with same technique (Wilmut et al., 1997). In addition, Japanese research group reported that epigenome of thymocyte is reconstructed into ESCs-like state by fusing with ESCs (Tada et al., 2001). In thymocyte-ESC hybrids, *Oct4-GFP* transgene was expressed and an inactivated X chromosome was reactivated. These serial experiments showed that cellular reprogramming of committed cells into a pluripotent state can be induced by unknown factors in oocytes and ESCs, which means that it could be possible to put stones at the bottom on top of the hill.

In 2006, inspired by previous studies, Yamanaka and colleagues developed new method to easily reprogram somatic cells (Takahashi and Yamanaka, 2006). New PSCs, so called iPSCs, were generated from adult somatic cells by nuclear reprogramming via introducing four genes such as *Oct4*, *Sox2*, *Klf4* and *cMyc*. Comparison of global gene expression between ESCs and iPSCs showed that gene expression pattern of two cell lines was very close. The cells could stably be maintained having normal karyotype and differentiate into three germ layers *in vitro* and *in vivo*. In 2007, human induced pluripotent stem cells were derived by two independent groups (Takahashi et al., 2007; Yu et al., 2007). Yamanaka and colleagues generated human iPSCs by Yamanaka's factors, the other groups used different combinations including *OCT4*, *SOX2*, *NANOG* and *LIN28*. In addition, it was verified that nuclear reprogramming could be achieved by micro RNA and combination of chemicals (Anokye–Danso et al., 2011; Hou et al., 2013a; Miyoshi et al., 2011). The iPS technique was easy to use compared with prior methods (SCNT and cell fusion) and could be apply to adult cells, which accelerated stem cell researches for regenerative medicine and cell therapy using patient–specific PSCs.

However, iPS technique has problems for applying to therapeutic usages. First of all, abnormal features could be occurred by integration of transgenes in genome of cells during maintenance and differentiation of iPSCs. Especially, one of Yamanaka's factors, *cMyc* as a proto-oncogene induced tumor formation, when iPSC/iPSC-derived cells are transplanted *in vivo* (Nakagawa et al., 2008; Nakagawa et al., 2010). And also, because epigenetic memory derived from origin of somatic cells were remained in iPSCs after reprogramming, differentiation of iPSCs were biasedly progressed (Kim et al., 2010a; Polo et al., 2010). And this deficiency of epigenetic reprogramming caused differences between ESCs and iPSCs (Chin et al., 2009; Chin et al., 2010). These problems were solved with integration-free gene delivery systems such as plasmid vector (Okita et al., 2008), episomal vector (Yu et al., 2009), PiggyBack transposon system (Woltjen et al., 2009), adenovirus vector (Stadtfield et al., 2008) and sendai virus vector (Fusaki et al., 2009), and transgene-free methods such as protein- and chemical-mediated systems (Hou et al., 2013a; Zhou et al., 2009). And recent study showed that such variations between ESCs and iPSCs were mainly originated by different genetic background and genetically matched human ESCs and iPSCs were molecularly and functionally equivalent (Choi et al., 2015).

The acquisition of pluripotency in fibroblasts is accomplished by genetic and epigenetic events termed initiation, maturation, and stabilization (Samavarchi–Tehrani et al., 2010). The initiation of reprogramming is defined by mesenchymal–to–epithelial transition (MET), in which epithelial–specific genes are up–regulated and *Tgfb1* (which blocks nuclear reprogramming) is downregulated by expression of reprogramming factors and BMP signaling (Li et al., 2010; Samavarchi–Tehrani et al., 2010). The cells that convert to iPSCs express predictive markers, such as ESRRB, UTF1, LIN28, and DPPA2. Subsequently, NANOG and SALL4 induced by SOX2 activate endogenous pluripotent networks, and pluripotent circuitry is stabilized via epigenetic remodeling such as DNA methylation, histone modification, and X chromosome reactivation (in the case of female cells) (Buganim et al., 2012; Polo et al., 2012). Since *Nanog* as a gateway to pluripotency plays pivotal roles in epigenetic remodeling and X chromosome reactivation, it is important to reactivate *Nanog* in the late stages of reprogramming in mouse (Chambers et al., 2007; Silva et al., 2009). In human as a non–permissive species, NANOG plays an important role in reprogramming and maintaining pluripotency (Hyslop et al., 2005; Yu et al., 2007). Finally, the reprogrammed cells can be maintained

without ectopic expression of transgenes, which indicates that endogenous pluripotent network are fully activated and stabilized (Maherali et al., 2007; Wernig et al., 2007). Because the defective silencing of transgenes after reprogramming affects stability, carcinogenesis, and differentiation ability of iPSCs, silencing or eliminating transgene expression is one of the most important step for fully reprogrammed iPSCs (Okada and Yoneda, 2011; Okita et al., 2007).

As described above, several genetic and epigenetic changes occur during nuclear reprogramming from somatic cells to iPSCs. Achieving faithful pluripotency is required to overcome epigenetic and physiological obstacles such as the epigenetic memory of somatic cells (Kim et al., 2010a; Polo et al., 2010), MET (Li et al., 2010), repressive chromatin (Huangfu et al., 2008), and apoptosis and cell cycle arrest (Kawamura et al., 2009; Li et al., 2009). However, if these barriers are not overcome, silencing of transgenes, epigenetic remodeling, and lack of *Nanog* expression occur, resulting in partial reprogramming of iPSCs (pre-iPSCs) (Okita et al., 2007; Silva et al., 2008). The pre-iPSC lines share several common characteristics, such as incomplete expression of pluripotent genes, inactive X

chromosomes in female cells, and inability to generate germline chimeras, have been observed (Li et al., 2010; Okita et al., 2007; Silva et al., 2008; Silva et al., 2009).

To overcome the barriers of reprogramming, various studies have been performed using small molecules, another reprogramming factor, and nutrient supplements. At this time, drug-inducible vector systems, which can easily turn transgenes on and off, have been used in various studies involving the iPSC generation and the elucidation of reprogramming mechanisms (Buganim et al., 2012; Polo et al., 2012; Wernig et al., 2008). Chromatin remodeling and the erasing of epigenetic memory in somatic cells have been accomplished by inhibiting DNA methylation and using chromatin modifiers (Huangfu et al., 2008). Pre-iPSCs lacking *Nanog* expression were converted into fully reprogrammed iPSCs by inhibiting FGF2 signaling and *Nanog* overexpression (Silva et al., 2008; Theunissen et al., 2011). In addition, serum free media and vitamin C could be used to overcome hurdles and increase reprogramming efficiency (Chen et al., 2010; Esteban et al., 2010). In addition, suppression of TGF signaling, apoptosis, senescence, and cell-cycle arrest are considered useful tools for reducing reprogramming barriers (Banito

et al., 2009; Ichida et al., 2009; Kawamura et al., 2009; Li et al., 2009). Recently, it has been shown that several factors including *Nanog*, *Lin28*, *Nr5a2*, and *Glis1* could be used for nuclear reprogramming instead of Yamanaka's factors (Heng et al., 2010; Maekawa et al., 2011; Yu et al., 2007).

## 1.5 Two pluripotent states based on developmental potency

Human and mouse ESCs have different features as described in Table 1. When human ESCs were firstly derived, it was thought that distinguished features of human ESCs were caused by basic developmental differences between human and mouse (Thomson et al., 1998). However, EpiSCs derived in FGF2/ACTIVIN A-culture media resembled human ESCs, which indicated that PSC could be categorized based on their pluripotent state and developmental potency. Recently, several studies have suggested that the states of PSCs are divided into two categories: naïve and primed (reviewed in (Hanna et al., 2010b; Nichols and Smith, 2009)).

Naïve PSCs derived from early epiblasts in pre-implantation blastocysts as a developmental ground state can generate the chimeric fetus when micro-injected into recipient blastocysts. In addition, primed PSCs derived from late epiblasts in post-implantation blastocyst possess more differentiated pluripotency than naïve cells in terms of developmental capacity, gene expression, and epigenetic signatures. Naïve PSCs, represented by mouse ESCs and EGCs, are characterized by dome-shaped colony morphologies, activation of LIF signaling, and two active X chromosomes in females. By contrast, primed PSCs, including EpiSCs and human ESCs, are defined by flattened colony morphologies and activated FGF signaling pathways. Compared with the primed state, naïve PSCs have developmental and functional ground states showing contributions to blastocyst chimeras and higher transgenic efficiency (Buecker et al., 2010; Hanna et al., 2010a). In permissive lines, both pluripotent states of PSCs can be derived from embryos. Only certain mouse strains, such as 129, C57BL/6 and BALB/C, were categorized as a permissive-line. (Nichols and Smith, 2012; Smith, 2001). However, in non-permissive lines, the stem cells cannot be stabilized in the naïve state, and are instead differentiated and stabilized at the primed state during the establishment process if no additional treatments (including genetic manipulation and chemicals) are performed (Hanna

et al., 2010b; Nichols and Smith, 2009).

Several studies have attempted to establish naïve–state PSCs from non–permissive species such as rats and humans (Buehr et al., 2008; Li et al., 2008). The first case of human naïve PSCs reported that derivation of naïve PSCs were accomplished via ectopic expression of *OCT4* and *KLF4* supplemented with LIF and two inhibitors for GSK and ERK1/2 signaling (Hanna et al., 2010a). However, these cell lines could not be maintained without transgene expression. Recent studies have reported that generation of transgene–free human naïve–like PSCs from primed PSCs could be achieved by several small molecules in addition to 2i (GSK and ERK inhibitors) (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). And Nichols and colleagues successfully derived naïve human ESCs directly from human early embryos by inhibiting PKC and ROCK signaling (Guo et al., 2016b). These cells expressed markers of naïve pluripotency and resembled mouse ESCs in terms of mitochondrial respiration, global gene expression and genome–wide methylation pattern. When transferred into FGF2–supplemented media, naïve cells were converted into conventional human ESCs.

These results demonstrated that *in vivo* counterpart of naïve cells exist in early human embryos and modulating signaling pathways is required for the maintenance of naïve state PSCs in non-permissive species.

Table 1. Characteristics of pluripotent stem cells.

Species	Mouse			Human	
	Permissive species (129, C57BL/6 and BALB/C)			Non-permissive species	
Stem cell types	Embryonic stem cells	Embryonic germ cells	Epiblast stem cells	Embryonic stem cells	Embryonic germ cells
Origins	Preimplantation ICM	Primordial germ cells	Postimplantation epiblast	Preimplantation ICM	Primordial germ cells
Pluripotent states	Naïve		Primed		–
Colony morphology	Domed		Flattened		Domed
Pluripotent signaling	LIF, BMP4 and WNT		FGF2, ACTIVIN and WNT		FGF2, LIF and Fsk
Pluripotent markers	OCT4, SOX2, NANOG, SSEA1		OCT4, SOX2, NANOG and SSEA4		OCT4, SSEA1, SSEA3/4
Chimeric assay	Pre-implantation embryos		Post-implantation embryos		–
X chromosome inactivation in female cells	Two active X chromosomes		One inactive X chromosome and one active X chromosome		–

## 2. Large animal models in biotechnology/stem cell biology: A pig review

Domestic animals such as pigs, cows and chickens have been considered as an ideal model for bioreactor producing medical proteins in by-product form such as milk, urine and egg (Keefer et al., 2007). For these reasons, many research groups have focused on production of transgenic farm animals for bioreactors, disease model and xenotransplantation for a long time (Houdebine, 2009; Pierson et al., 2009). Especially, pigs have a great potential in their application in xenotransplantation and disease model for human and in biopharming, because of its anatomical and physiological similarities to human (Brevini et al., 2007; Hall, 2008).

In order to produce transgenic animals, the somatic cell nuclear transfer (SCNT) and transgenic techniques are essential process. From the first cloned sheep named Dolly in 1997 (Wilmut et al., 1997), a large number of cloned animals including mice in 1998 (Wakayama et al., 1998), goat in 1999 (Baguisi et al., 1999), pig and cattle in 2000 (Kato et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), cat and rabbit in 2002 (Chesne et al., 2002; Shin et al., 2002),

horse, mule and rat in 2003 (Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003), dog in 2005 (Lee et al., 2005), ferret in 2006 (Li et al., 2006), deer, buffalo and wolf in 2007 (Berg et al., 2007; Kim et al., 2007b; Shi et al., 2007), and camel in 2010 (Wani et al., 2010) have been produced. Together with improvement of cloning technique, the way to produce transgenic animals have been also advanced. First generated livestock animal to produce pharmaceutical proteins was transgenic sheep, named Molly and Polly (Schnieke et al., 1997). They were produced by somatic cell nuclear transfer. Transfected fetal fibroblasts carrying human clotting factor IX linked to the ovine  $\beta$ -lactoglobulin gene promoter known as mammary gland-specific promoter were used as donor cells. Lots of transgenic animals have been generated since then, and, in 2009, human antithrombin isolated from transgenic goat milk, named ATryn, was firstly approved by Food and Drug Administration of U.S. (2009; Edmunds et al., 1998; Konkle et al., 2003).

Along with production of bioreactors, development of animal models for human disease have been progressed for a long time. As pig genome project was completed, studies of transgenic pig models for human disease were accelerated. While some of human diseases

are caused by simple genetic mutations, pig genome project verified that pigs and humans share 112 DNA mutations implicated with human diseases (Groenen et al., 2012). So, scientists have made the transgenic pigs carrying human disease including Huntington's disease (Uchida et al., 2001), retinitis pigmentosa (Ghosh et al., 2007), cystic fibrosis (Rogers et al., 2008), diabetes (Renner et al., 2010) and Alzheimer's disease (Kragh et al., 2009; Søndergaard et al., 2012) through gene knockout technique. And porcine organs and tissues anatomically similar with human can be used for transplantation into human body in order to replace a malfunctioning organ. Because of problems caused by increasing imbalance between organ donors and recipients, various research groups are focusing on the xenotransplantation of human organs with those of pigs. Pig heart valves and skins have been transplanted into heart disease patients and burn victims respectively from 30 years ago (reviewed by Schuurman and Pierson, 2008). Moreover, attempt to treat diabetes through a transplantation of porcine pancreatic islet to human has been made by several groups. However, there is an obstacle to overcome such as an immune rejection in order to successful transplantation. Although immunosuppressant drugs are used, inhibition of immune rejection via genetic manipulations is necessary for minimizing side effects. When foreign tissues from other species are grafted into

human body, immune reactions including hyperacute rejection, acute vascular rejection, cellular rejection and chronic rejection are triggered. Hyperacute rejection (HAR) is immediately induced within minutes after the transplantation, and acute vascular rejection and cellular rejection occurred after the HAR (reviewed in (Yang and Sykes, 2007)). To avoid the immune rejection, transgenic pigs for xenotransplantation have been produced by modification of genes such as  $\alpha$  1,3-galactosyltransferase, CAMH, heme oxygenase and human CDs (reviewed in (Niemann and Petersen, 2016)). It was proven that rat organs were successfully generated in mice by interspecies-chimera formation (Isotani et al., 2011; Kobayashi et al., 2010). When rat wild-type pluripotent stem cells (PSCs) were injected into blastocyst of transgenic mouse in which thymus and pancreas could be developed, rat PSCs were developed into various organs including thymus and pancreas while displaying chimerism. Belmonte and colleagues verified that human PSCs could generate chimeric fetuses incorporated with pig embryos (Wu et al., 2017). Accordingly, interspecies chimera technique might be another source for providing therapeutic tissues and organs, if we consider consensus on ethical problems.

PSCs of domestic animals have been identified as promising tools for generating transgenic animals and preclinical researches. In making transgenic animals, because of unique epigenetic milieus, PSCs as a donor cell are more suitable for reprogramming within enucleated oocyte than differentiated cells (Hochedlinger and Jaenisch, 2007). Several studies showed that ESCs were more effective for cloning of mouse than differentiated cells such as immune cells, neuron, and fibroblasts (Blelloch et al., 2006; Blelloch et al., 2004; Eggan et al., 2004; Hochedlinger and Jaenisch, 2002; Inoue et al., 2005). And PSCs have another advantage in application to nuclear transfer. PSCs can proliferate indefinitely maintaining cellular characteristics including karyotype and genomic stability. This property, so called self-renewal, provides an indefinite cell source for nuclear transfer. With genetic manipulations, they can offer stable transgenic cell source for generation of transgenic animals for bioreactors, xenotransplantation and disease models. And also, as PSCs could be incorporated in development of early embryos, blastocyst injection could be apply for generation of transgenic animals. In these reasons, various transgenic piglets and embryos have been produced by using pig PSCs (Ahn et al., 2007; Fan et al., 2013; West et al., 2010; Zhang et al., 2015a).

In 2009, a clinical trial of human ES cell therapy for repairing spinal cord damages was approved by Food and Drug Administration (Alper, 2009). In 2014, first clinical test were operated in Japan (Sugita et al., 2016). Woman who suffered from aged–macular degeneration (AMD) received retinal pigment epithelium (RPE) differentiated from iPSCs, and her vision were partially recovered. However, because spinal cords and eyes are immune–privileged area without immune cells and are not affected to life, clinical tests for organs and tissues which are strongly involved in life, such as heart, brain and pancreas, need strict preclinical test using animal disease models. So, it makes the porcine study involving differentiation and transplantation of stem cells as a preliminary study more important. As a preliminary study for human cell therapy, many researchers are trying to convert the porcine PSCs into several differentiated cells including rod receptors, hepatocytes, endothelium and cardiomyocytes apposing transplantation of the differentiated cells into the pigs (Aravalli et al., 2012b; Gu et al., 2012; Kawamura et al., 2012; Zhou et al., 2011). In these reasons, in this chapter, it will be discussed history, features and research state of pig PSCs.

## 2.1 Pig embryonic stem cells

Because pluripotent cells have great potential as a cell source, research in this area has focused on embryonic carcinoma cells to iPSCs (Martin and Evans, 1974; Takahashi and Yamanaka, 2006). ESC research began in 1981 by the establishment of mouse ESCs and was accelerated by the establishment of human ESCs and iPSCs in 1998 and 2006, respectively (Evans and Kaufman, 1981; Takahashi and Yamanaka, 2006; Thomson et al., 1998). The purpose of PSC research in humans and mice includes the elucidation of basic cellular mechanisms contributing to the maintenance of pluripotency as well as applications in human cell therapies. However, in domestic animals, the research aim is to create an indefinite cell source for transgenic animals used as bioreactors and tissue engineering materials as well as preliminary studies for human research (Keefer et al., 2007). Because of the physiological and immunological similarities that exist between pigs and humans, porcine pluripotent cell lines have been identified as important candidates for preliminary studies on human disease (Brevini et al., 2007; Hall, 2008; Houdebine, 2009). Therefore, many research groups have attempted to derive pig PSCs from early embryos for a long time (Ezashi et al., 2009; Kues et al.,

2013; Park et al., 2013a; Piedrahita et al., 1990b; Son et al., 2009; Yang et al., 2009).

In early studies of pig embryonic stem cells (ESCs), researchers tried to establish stem cells based on teratocarcinoma culture methods as mouse studies did. Various culture materials including serums (fetal bovine serum and calf serum), feeder cells (buffalo liver cells, mouse embryonic fibroblasts, pig embryonic fibroblasts and pig uterine epithelial cells) and conditioned media were tested for maintaining pluripotent inner cell mass (ICM) *in vitro* (Anderson et al., 1994; Piedrahita et al., 1990a, b; Talbot et al., 1993b). Nonetheless, it was hard to derive pig stem cells due to lack of knowledge about cell signaling involved in early embryo development and maintaining pluripotency. When it was verified that LIF and FGF2 are responsible for maintaining mouse and human ESCs respectively (Amit et al., 2000; Smith et al., 1988; Williams et al., 1988), many cytokines like interleukins (IL), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), activin A (ActA) and stem cell factor (SCF) as well as LIF and FGF2 have been used for *in vitro* culture of pig ICM with various combinations and concentrations as summarized in Table 2. Beside,

as culture media were developed for culture of mouse and human ESCs, Knockout–DMEM, F10, F12 and  $\alpha$ MEM have been used for basal media (Table 2). However, although some culture conditions could support *in vitro*–culture of pig ICM for a short term (Alberio et al., 2010; Puy et al., 2010; Vassiliev et al., 2010), cultured ICM lost their own features and in turn differentiated during prolonged culture. Instead of pluripotent cells, during *in vitro*–culture, multipotent stem cells, so called ES–like cells, have been spontaneously obtained by several groups (Brevini et al., 2010; Cheong et al., 2015; Kim et al., 2010b; Uh et al., 2014). To date, it is still endeavoring to establish authentic pig ESCs which have *in vivo* and *in vitro*–developmental competency.

Pig ES–like cells have been derived from various embryo origins as described in Table 2. In early studies, hatched blastocysts were collected from pregnant sow at 6–11 days after artificial insemination. These experiments showed that *in vitro*–survival rate of ICM was increased in older blastocysts (day 10 and 11) but spontaneous differentiation was frequently observed. Although freshly isolated ICM could be formed chimeric piglets after blastocyst injection, cultured ICM lost their developmental competency

(Anderson et al., 1994). Recent studies addressed that FGF2 and LIF supported *in vitro*-culture of ICM (Brevini et al., 2010; Li et al., 2004; Li et al., 2003) and ES-like cells could be derived in FGF2-supplemented media (Alberio et al., 2010; Cheong et al., 2015; Kim et al., 2016; Kim et al., 2010b; Park et al., 2013a). As summarized in Table 2, putative stem cell lines were derived from several *in vitro*-produced embryos including *in vitro*-fertilized, parthenogenetic and cloned embryos. Established pig ES-like cell lines have shown some common features. They express pluripotent markers such as AP, OCT4, SOX2 and NANOG, and have *in vitro*-differentiation ability, but not *in vivo*-developmental competency (chimera and teratoma formation ability). Since restricted differentiation is considered as obstacle for applying degenerative medicine, generating pig ESCs having faithful pluripotency remains ongoing missions in stem cell biology.

Table 2. List of embryonic/epiblast stem cell lines in pigs.

Origin of ICM/epiblasts	Basal media	Serum sources	Cytokines	Markers	References
Day 8–9 <i>in vivo</i> blastocysts	DMEM	10% FBS <sup>a</sup> + 10% CS <sup>b</sup>	–	–	(Piedrahita et al., 1990a, b)
Day 7–8 <i>in vivo</i> blastocysts	DMEM	10% FBS	–	AP <sup>c</sup>	(Talbot et al., 1993a; Talbot et al., 1993b)
Day 6–10 <i>in vivo</i> blastocysts	DMEM	5% FBS + 10% CS	–	–	(Anderson et al., 1994)
Day 7–11 <i>in vivo</i> blastocysts	DMEM	10% FBS	5000 U/ml LIF <sup>d</sup>	SSEA1, Cytokeratin 18 and Laminin	(Wianny et al., 1997)
Day 7 <i>in vivo</i> blastocysts	DMEM/F10	15% FBS	1000 U/ml LIF, 100 ng/ml IL-6 <sup>e</sup> , 2.5 $\mu$ g/ml IL soluble receptor, 100 ng/ml OSM <sup>f</sup> and 100 ng/ml CNTF <sup>g</sup>	–	(Moore and Piedrahita, 1996)
Day 7 <i>in vivo</i> blastocysts	DMEM/F10	15% FBS	1000 U/ml LIF	AP	(Boeuf et al., 1997)
Day 6–8 <i>in vivo</i> blastocysts	DMEM	16% FBS	–	AP	(Chen et al., 1999)

(Table 2–continued 1)

Origin of ICM/epiblasts	Basal media	Serum sources	Cytokines	Markers	References
Day 7–9 <i>in vivo</i> blastocysts	DMEM	16% FBS	20 ng/ml FGF2 <sup>h</sup> and 40 ng/ml LIF	AP	(Li et al., 2003)
Day 3–5 <i>in vitro</i> –fertilized (IVF) blastocysts	DMEM	16% FBS	20 ng/ml FGF2 and 40 ng/ml LIF	AP	(Li et al., 2004)
Day 5.5 <i>in vivo</i> blastocysts	DMEM	16% FBS	–	AP	(Shiue et al., 2006)
Day 7–9 IVF, parthenogenetic, and NT blastocyst	DMEM, DMEM/F10 and DMEM/NCSU23	15% FBS	1000 U/ml LIF	AP	(Kim et al., 2007a)
Day 8 <i>in vivo</i> blastocysts	DMEM/M199	10% FBS	–	OCT4, SOX2, TDGF1, REX1 and NOG	(Blomberg et al., 2008)
Day 6.5–7.5 <i>in vivo</i> blastocysts	Knockout (KO)–DMEM	10% KSR <sup>i</sup> + 10% human plasmanate	10–20 ng/ml FGF2	OCT4, SOX2, NANOG and PGK1	(Puy et al., 2010)
Day 10.5–12 <i>in vivo</i> blastocyst	DMEM/F12 or conditioned DMEM/F12	20% KSR or 20% FBS	5–20 ng/ml FGF2 and ActA <sup>j</sup>	AP, OCT4, NANOG, SSEA1 and NODAL	(Alberio et al., 2010)

(Table 2–continued 2)

Origin of ICM/epiblasts	Basal media	Serum sources	Cytokines	Markers	References
Day 7 IVF & day 6 parthenogenetic blastocysts	DMEM, KO–DMEM or DMEM/F10	15% KSR or 10% KSR +5% FBS	1000 U/ml LIF and 5ng/ml FGF2	OCT4, SOX2, NANOG, REX1 and SSEA4	(Brevini et al., 2010)
Day 7 cloned blastocyst	DMEM/F12	20% KSR	20 ng/ml FGF2	AP, OCT4, SOX2, NANOG, SSEA1, SSEA4, TRA–1–60 and TRA–1–81	(Kim et al., 2010b)
Day 7 IVF blastocysts and day 6 <i>in vivo</i> blastocysts	$\alpha$ MEM	10% KSR	20 ng/ml FGF2, 20 ng/ml EGF <sup>k</sup> , 10 ng/ml ActA and 10 ng/ml LIF	OCT4, NANOG and SSEA1	(Vassiliev et al., 2010)
Day 7 parthenogenetic blastocysts	DMEM/F10	15% FBS	40 ng/ml SCF <sup>l</sup> and 20 ng/ml FGF2	AP, OCT4, SOX2 and NANOG, SSEA1, TRA–1–60 and TRA–1–81	(Uh et al., 2014)
Day 7 IVF & parthenogenetic blastocysts	DMEM/F10	10% FBS	4 ng/ml FGF2	AP and NANOG	(Cheong et al., 2015)
Day 7 aggregated parthenogenetic blastocysts	DMEM, DMEM/F12 or $\alpha$ MEM	15% FBS or 20% KSR	5 ng/ml FGF2, 20 ng/ml LIF and 10 $\mu$ M ROCK inhibitor	AP, OCT4, SOX2, NANOG and REX1	(Siriboon et al., 2015)

(Table 2–continued 3)

Origin of ICM/epiblasts	Basal media	Serum sources	Cytokines	Markers	References
Day 7 aggregated cloned blastocysts	DMEM/F10	15% FBS	20 ng/ml FGF2 and 100 ng/ml heparin	AP, OCT4, SOX2 and NANOG	(Lee et al., 2016)
Day 6 cloned blastocysts	DMEM/F10	15% FBS	4 ng/ml FGF2	AP, OCT4, SOX2, NANOG and REX1	(Kim et al., 2016)

a: fetal bovine serum  
b: calf serum  
c: alkaline phosphatase  
d: leukemia inhibitory factor  
e: interleukin  
f: oncostatin M

g: ciliary neurotrophic factor  
h: basic fibroblast growth factor  
i: knockout serum replacement  
j: activin A  
k: epidermal growth factor  
l: stem cell factor

## 2.2 Pig induced pluripotent stem cells

Because it has proven difficult to obtain authentic pig ESCs, recent studies are focusing on somatic cell reprogramming in pig. Cellular reprogramming of committed cells into a pluripotent state was successfully achieved by ectopic expression of genes. Induced pluripotent stem cell (iPSC) lines in pigs were firstly generated by independent three groups in 2009 (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009). These cell lines were derived from fetal fibroblasts by using viral vectors carrying different reprogramming factors (human *OSKM* (Ezashi et al., 2009), mouse *OSKM* (Esteban et al., 2009), and human *OSKM* + *NANOG* and *LIN28* (Wu et al., 2009)). Interestingly, pig iPSC cell lines have primed pluripotent state in terms of morphologic and molecular features resembling mouse EpiSCs and human ESCs, rather than mouse ESCs. Since then, various FGF2-dependent primed-like porcine iPSCs have been established (Congras et al., 2016; Montserrat et al., 2012; West et al., 2010; Zhang et al., 2015b). In addition, in LIF and small molecules (especially ERK and GSK signaling inhibitors) –supplemented media, naïve-like pig iPSC cell lines have been obtained as described in Table 3. The pig naïve iPSC cell lines showed expression of naïve-

pluripotent markers, two active X chromosomes and differentiation potential into three germ layers (Fujishiro et al., 2013; Zhang et al., 2015b). Compared with the primed state, naïve PSCs have developmental and functional ground states showing contributions to blastocyst chimeras and higher transgenic efficiency (Buecker et al., 2010; Hanna et al., 2010a). For these reasons, many research groups have attempted to generate naïve PSCs for the efficient production of transgenic animals (Fujishiro et al., 2013; Kwon et al., 2013; Telugu et al., 2011; Zhang et al., 2015b). Although genuine pig ESCs couldn't be established, both naïve and primed PSCs have been successfully derived by reprogramming technique. In this reason, it is hard to address whether pig is permissive or non-permissive species. Considering the facts that 1) pig is outbred (Only three inbred mouse strains including 129, C57BL/6 and BALB/C are permissive strain (Hanna et al., 2010b)), 2) inhibitors of ERK and GSK signaling repressed endogenous pluripotent genes during reprogramming of pig cells (Choi et al., 2016; Petkov et al., 2014), pig might be closed to non-permissive species rather than permissive species.

Surprisingly, almost, if not all, reported pig iPSCs have teratoma formation ability when injected into immunodeficient mice.

Tumor formation from iPSCs have been caused by ectopic expression of transgenes (Nair, 2008; Okita et al., 2007), all of pig iPSC lines also depended on expression of the transgenes for the maintenance of pluripotency (Table 3), which means cellular reprogramming was incompletely achieved. Reprogrammed cells can be maintained by activating endogenous pluripotent networks without transgene expression (Maherali et al., 2007; Wernig et al., 2007). The majority of pig iPSC lines were partially reprogrammed, showing dependence on transgenes, lack of *NANOG* expression, failure of epigenetic reprogramming, and inability to form chimeras (Du et al., 2015a; Esteban et al., 2009; Ezashi et al., 2009; Hall et al., 2012; Wei et al., 2015; Zhang et al., 2014b). According to previous studies, the majority of iPS cell lines exhibited dependency of continuous transgene expression, and it was impossible to obtain transgene-independent colonies using a transgene-free system such as drug-inducible, episomal or plasmid vectors (Du et al., 2015a; Ezashi et al., 2009; Telugu et al., 2010; Wu et al., 2009). In recent study using cells carrying OCT4-tdTomato reporter, no dtTomato expression was detected in pig iPSCs induced by human Yamanaka's factors (Lai et al., 2016). Recent reports have suggested the types of cells, promoter and transcription factors for reprogramming can affect the full reprogramming of iPSCs in pigs (Kwon et al., 2013; Petkov et al.,

2013; Zhang et al., 2014b). This may be due to differences in the molecular mechanism during embryo development between mouse and pig (Alberio and Perez, 2012). Nonetheless, in-depth analyses of iPSCs for better understanding of reprogramming mechanism in pig are required for establishing authentic porcine ESCs and obtaining completely reprogrammed iPSCs.

Table 3. List of induced pluripotent stem cell lines in pigs.

Pluripotent state	Treated cytokines/molecule	Type of reprogrammed cells	Transfection methods	Reprogramming factors	References
Primed	4 ng/ml FGF2	Fetal fibroblasts	Lentiviral vector	Human OSKM <sup>a</sup>	(Ezashi et al., 2009)
Primed	4 ng/ml FGF2	Fetal fibroblasts	Retroviral vector	Mouse OSKM	(Esteban et al., 2009)
Primed	–	Fetal fibroblasts	Lentiviral vector	Human OSKM + NANOG and LIN28	(Wu et al., 2009)
Naïve	250 U/ml LIF, 1 $\mu$ M PD <sup>b</sup> and 3 $\mu$ M CH <sup>c</sup>	Fetal fibroblasts	Episomal plasmid vector (nucleofection)	Human OSKM	(Telugu et al., 2010)
–	10 ng/ml FGF2	Mesenchymal stem cells	Lentiviral vector	Human OSKM + NANOG and LIN28	(West et al., 2010)
Primed	5 ng/ml FGF2 and 500 U/ml LIF	Ear fibroblasts	CAG-driven polycistronic plasmid	Human OSKM	(Montserrat et al., 2011)
Naïve	250 U/ml LIF, 1 $\mu$ M Kenpaullone and 3 $\mu$ M CH	Inner cell mass	Lentiviral vector	Human OK	(Telugu et al., 2011)
Primed	5 ng/ml FGF2 and 500 U/ml LIF	Ear fibroblasts	Retroviral vector	Human SKM	(Montserrat et al., 2012)

(Table 3–continued 1)

Pluripotent state	Treated cytokines/molecule	Type of reprogrammed cells	Transfection methods	Reprogramming factors	References
Naïve	10 ng/ml LIF	Fetal fibroblasts	Retroviral vector	Mouse OSKM	(Cheng et al., 2012)
–	20 ng/ml FGF2	Fetal fibroblasts	Lentiviral vector	Inducible human OSKM + NANOG pseudo gene	(Hall et al., 2012)
–	4 ng/ml FGF2	Fetal fibroblasts	PiggyBac vector (electroporation)	Mouse OSKM	(Kues et al., 2013)
Naïve	20 ng/ml LIF	Ear fibroblasts	Lentiviral vector	Human OSKM + NANOG and LIN28	(Kwon et al., 2013)
Naïve	Pig LIF	Fetal fibroblasts	Retroviral vector	Mouse OSKM	(Fujishiro et al., 2013)
–	20 ng/ml FGF2	Ear fibroblasts	Retroviral vector	Human OSKM	(Park et al., 2013b)
Naïve	8 ng/ml FGF2 and 1000 U/ml LIF	Fetal fibroblasts	Retroviral vector	Mouse OSKM + Nr5 $\alpha$ 2 and Tbx3	(Wang et al., 2013)
Naïve	2000 U/ml LIF, 16 ng/ml FGF2, 1 $\mu$ M PD, 3 $\mu$ M CH and 2 $\mu$ M SB <sup>d</sup>	Fetal fibroblasts	Retroviral vector	Mouse OSKM	(Gu et al., 2014)

(Table 3–continued 2)

Pluripotent state	Treated cytokines/molecule	Type of reprogrammed cells	Transfection methods	Reprogramming factors	References
Naïve	LIF, PD and CH	Adipose–derived stem cells	Lentiviral vector	Inducible OSKM	(Zhang et al., 2014b)
–	100 U/ml LIF	Fetal fibroblasts	Retroviral vector	Inducible mouse OSKM + miR 200 and 302	(Ma et al., 2014)
–	10 ng/ml FGF2 and 1000 U/ml LIF	Fetal fibroblasts	Retroviral vector	Human OSKM + TERT	(Gao et al., 2014)
Naïve	1000 U/ml LIF, 1 $\mu$ M PD and 3 $\mu$ M CH	Fetal fibroblasts	Episomal plasmid vector (nucleofection)	Human OSKM + NANOG, LIN28 and miR302	(Du et al., 2015b)
Intermediate	10 ng/ml LIF, 10 ng/ml FGF2, 10 ng/ml BMP4, 3 $\mu$ M CH and 2 $\mu$ M SB	Fetal fibroblasts	Retroviral vector	Human OSKM	(Zhang et al., 2015a)
Naïve or primed	FGF2 or 5 ng/ml LIF, 2 $\mu$ M PD and 3 $\mu$ M CH	Fetal fibroblasts	Retroviral vector	Porcine OSKM	(Zhang et al., 2015b)

(Table 3–continued 3)

Pluripotent state	Treated cytokines/molecule	Type of reprogrammed cells	Transfection methods	Reprogramming factors	References
Naïve	1000 U/ml LIF + HDAC inhibitors	Fetal fibroblasts	Episomal plasmid vector (nucleofection)	Porcine OSKM + pig NANOG and human LIN28	(Petkov et al., 2016)
Primed	8 ng/ml FGF2	Fibroblasts	Sendai virus vector	Human OSKM	(Congras et al., 2016)

a: Oct4, Sox2, Klf4 and cMyc

b: PD0325901

c: CH99021

d: SB431542

## 2.3 Pig embryonic germ cells

Pig germ-line stem cells have been studied for a long time. Porcine primordial germ cells (PGCs) arise in endoderm and mesoderm beside stalk of the yolk sac and allantois during gastrulation (embryonic day (E) 13–14) (Vejlsted et al., 2006). Pig PGCs are starting to migrate from endoderm and mesoderm and arrive at genital ridges in E22 via dorsal mesentery of hindgut (Hyldig et al., 2011b). At around E30, primitive gonads develop into sex organs (Zhang et al., 2016). Global DNA demethylation involving repetitive elements as well as erasing gender-specific imprints are occurred during migration (Hyldig et al., 2011a; Hyldig et al., 2011b). And gender-specific imprinting patterns are re-established between E25–31 (Hyldig et al., 2011a; Petkov et al., 2009). Unlike mouse study, pig embryonic germ cells (EGCs) have been isolated from E25–30 gonadal PGCs (Table 4), while pig migrating PGCs couldn't be converted into EGCs (Petkov et al., 2011). It has been proven hard to derive mouse EGCs with older PGCs (over dpc 12.5) (Labosky et al., 1994), it might be caused by differences in the timing of epigenetic changes between mouse and pig PGCs (Hyldig et al., 2011a). In agreement with mouse PGCs, pig PGCs are

converted into EGCs by FGF2-mediated reprogramming (Zhang et al., 2016). When FGF2 was withdrawn during primary culture of PGCs, number of primary EGC colonies was dramatically reduced (Lee and Piedrahita, 2000). Along with FGF2, membrane-bound/soluble SCF and LIF have pivotal roles in survival and proliferation of pig PGCs during primary culture (Durcova-Hills et al., 1998). Without SCF, *in vitro*-survival rate of PGCs were reduced and, during extended culture, EGC colonies disappeared (Lee and Piedrahita, 2000).

Unfortunately, huge part of knowledge for pig EGCs remains unknown, since pig EGCs have been studied less than those with pig ESCs and iPSCs. In contrast with pig putative ESCs, pig EGCs resembled mouse ES-like morphology showing well-delineated and multilayered dome-shaped colony, and tolerated single-cell dissociation by trypsinization (Lee and Piedrahita, 2000). Pig EGC lines expressed several germ cell/pluripotent marker genes such as BLIMP1, OCT4, SOX2, NANOG, SSEA1 and SSEA4. Interestingly, expression of SSEA1, known as PGC marker, was gradually decreased and disappeared during *in vitro*-culture (Petkov and Anderson, 2008; Petkov et al., 2011). When cultured in suspension,

these cells formed embryoid bodies and differentiated into three germ layers *in vitro*, but couldn't develop into teratomas in immunodeficient mice. In spite of lack of teratom formation ability, pig EGCs could develop into chimeric piglet via incorporation with host embryos by blastocyst injection (Mueller et al., 1999; Piedrahita et al., 1998). In addition to production of chimeras, EGCs can be used as donor cells of nuclear transfer for generating cloned animals. While gonadal PGCs are inadequate for nuclear donors because of unique epigenetic features (Yamazaki et al., 2005; Yamazaki et al., 2003), the cells became suitable for nuclear transfer by epigenetic modifiers during converting from PGCs into EGCs, (Li et al., 2014a). Various transgenic piglets have been born using EGCs as shown in Table 5.

Table 4. List of embryonic germ cells and *in vitro*-cultured PGCs in pigs.

Origin of PGCs	Treated cytokines/molecules	Duration of culture	Markers	References
Day 24–25 GR <sup>a</sup> and DM <sup>b</sup>	1000 U/ml LIF	Over 6 months	AP	(Shim et al., 1997)
Day 25–27 GR	20 ng/ml LIF, 20 ng/ml FGF2 and 40 ng/ml SCF	Over 5 months	AP	(Piedrahita et al., 1998)
Day 25 GR	–	5 days	AP	(Shim and Anderson, 1998)
Day 27 GR	1000 U/ml LIF	8 days	AP	(Durcova–Hills et al., 1998)
Day 25–28 GR	Membrane–bound SCF	Over 23 passages <sup>c</sup>	AP and SSEA1	(Mueller et al., 1999)
Day 25–30 GR	20 ng/ml LIF, 20 ng/ml FGF2 and 40 ng/ml SCF	–	AP and SSEA1	(Lee and Piedrahita, 2000)
Day 25–30 GR	20 ng/ml LIF, 20 ng/ml FGF2 and 40 ng/ml SCF	–	AP and SSEA1	(Lee et al., 2000a)
Day 22–28 GR	–	Over 5 months	AP	(Rui et al., 2004)
Day 24–26 GR	20 ng/ml LIF, 10 ng/ml FGF2 and 20 ng/ml SCF	Over 60 passages	AP, OCT4, SSEA1, SSEA3, SSEA4 and TRA– 1–81	(Petkov and Anderson, 2008)

(Table 4–continued)

Origin of PGCs	Treated cytokines/molecules	Duration of culture	Markers	References
Day 24 DM and day 28 GR	10 ng/ml LIF, 10ng/ml FGF2, 20 ng/ml SCF and 0.5 $\mu$ g/ml BIO	–	OCT4, NANOG and SSEA4	(Wen et al., 2010)
Day 20–24 GR	10 ng/ml LIF, 3 ng/ml FGF2 and 10 ng/ml SCF	Over 30 passages	AP, MYC, SSEA4, TRA– 1–60, TRA–1–81 and BLIMP1, negative for SSEA1	(Petkov et al., 2011)
Day 24–28 GR	10 ng/ml LIF, 3 ng/ml FGF2 and 10 ng/ml SCF	Over 12 passages	AP, OCT4, SOX2, NANOG, SSEA3 and SSEA4	(Cong et al., 2013b)
Day 25–28	10 ng/ml LIF, 25 ng/ml FGF2 and 40 ng/ml SCF	Over 20 passages	AP, OCT4, SSEA1, SSEA3 and SSEA4	(Dong et al., 2014)
Day 24–28	5 ng/ml LIF, 10 ng/ml FGF2 and 10 ng/ml SCF	7 days	AP	(Zhang et al., 2016)

a: genital ridges

b: dorsal mesentary

c: about 5–7 days

### **3. On-going issues in pig stem cell biology**

Overall, advancement of techniques and sources mentioned above will help to improve human welfare in aspect of healthy life and agricultural production via generation of high-production and disease-resistance animals. To accomplish these goals with pig PSCs, there are hurdles to overcome. In this chapter, it will be discussed some issues remained as an ongoing endeavor, and try to suggest strategies to solve the problems in pig stem cell biology.

#### **3.1 Optimization of culture and reprogramming method**

Numerous research groups have attempted to generate authentic pig pluripotent stem cells (PSCs) as discussed above, however, it is still challenging to establish porcine embryonic stem cells (ESCs) and optimize pig-specific reprogramming conditions using typical method because of differences in the molecular mechanisms during embryo development among mouse, human, and pig. During development of the early embryo, which has an inner cell

mass (ICM) considered to be the pluripotent cell population, pig has a prolonged preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in pig embryos, different cell signaling that governs pluripotency reveals differences compared to mouse embryos. Unlike mouse, in which *Oct4* and *Cdx2* are exclusively expressed in ICM and trophoctoderm (TE), respectively, in pig, *SOX2* is specifically expressed in ICM while *OCT4* is expressed in TE until the blastocyst expands on embryonic day 8 (Liu et al., 2015). In ICM and epiblasts of pig blastocysts, the LIF receptor is absent while FGF receptors are specifically expressed, which indicates that FGF signaling may play an important role in the maintenance of pluripotency rather than LIF (Hall et al., 2009). Along with FGF signaling, BMP signaling plays an important role in maintaining pluripotency (Hall and Hyttel, 2014). BMP $\alpha$  and SMADs were strongly expressed in pig ICM and epiblasts as determined by immunostaining and treatment of BMP signaling inhibitor negatively influenced on *in vitro*-survival rate of ICM and epiblasts. It is interesting to note that pig has a unique energy metabolism of preimplantation embryos compared with that of human and mouse. Recent data showed that energy metabolism have pivotal roles in supporting stemness and pluripotency (Folmes and Terzic, 2014; Ito and Suda, 2014). Deep-sequencing indicated that pig ATP-

synthesis and fatty acid metabolisms during embryo development differ from mouse (Cao et al., 2014). Pig, mouse and human preimplantation embryos have different dependency of glucose metabolism for energy production (reviewed in Secher et al., 2016).

Recent studies have increased our understanding of pig somatic cell reprogramming. First, pig induced pluripotent stem cells (iPSCs) could be induced by *KLF4*, *SOX2*, and *MYC* (except for *OCT4*) infection, although transgenes were not silenced (Montserrat et al., 2012). According to this study, the ectopic expression of *OCT4* interfered with reactivation of endogenous pluripotent networks in pig fibroblasts, which indicated that *OCT4* may not be essential for maintaining pluripotency in pig. It was reported that expression of endogenous gene including *OCT4*, *NANOG* and *MYC* was repressed by counterpart exogenous genes (Hall et al., 2012). It has proven that novel reprogramming factors including micro RNAs, *TERT*, *Nr5a2* and *TBX3* work on reprogramming of pig somatic cells (Du et al., 2015b; Gao et al., 2014; Ma et al., 2014; Wang et al., 2013). These studies suggest that further investigation about reprogramming mechanisms and new reprogramming factors or vector systems are required in pig. Generation of pig iPSCs in which

transgenes were silenced was accomplished using combined supplements of three cytokines (LIF, FGF2, and BMP4) and two signaling inhibitors (TGFB1 and WNT inhibitors) (Zhang et al., 2015a). This novel culture condition generated 'intermediate' pluripotent stem cells, showing mixed features of naïve and primed states with inactivation of retroviral transgenes. Taken together, it is possible that pig has unique pluripotent characteristics governed by different cell signaling networks than mouse and human. Accordingly, to derive faithful pig PSCs, it is required to develop pig-specific culture system and reprogramming methods along with identifying universal pluripotent markers across the species based on developmental biology.

### **3.2 Genetic modification of pig PSCs**

To create genetically modified animals by using pig PSCs, genetic manipulation via transgenic technologies has been required in stem cell research. Researchers have successfully delivered transgenes into PSCs using several methods, including electroporation (Eiges et al., 2001), liposomal (Ko et al., 2009) and viral vectors (Ma et al., 2003; Pfeifer et al., 2002), and nucleofection

(Hohenstein et al., 2008). However, stably introducing transgenes in these cells has proven difficult because of low efficiency and cytotoxic side effects. Delivery of transgenes using viral vectors, which are stably expressed, is considered the most useful tool for inducing low cytotoxicity and inserting transgenes into the host genome (Zhang and Godbey, 2006). Moreover, lentiviral vectors belonging to retroviral families are able to infect several types of cells, as well as nondividing cells (Bukrinsky et al., 1993; Naldini et al., 1996). As vectors carrying transgenes, plasmid vector, virus vectors (including lentiviral vector, retroviral vector, sendai virus vector and adenovirus vector (Zhang and Godbey, 2006)), episomal vector (Van Craenenbroeck et al., 2000) and piggyback transposon system (Zhao et al., 2016) have been widely used. If transfected with short hairpin RNA (shRNA)-expressing vector, expression of targeted gene can be reduced instead upregulation (Xiang et al., 2006). In addition to insert transgenes, replacement and disruption of endogenous genes can be accomplished by gene targeting using homologous recombination, known as repair mechanism of DNA double strand break (Bouabe and Okkenhaug, 2013). Transgenic stem cells using the gene targeting were first reported in mouse ESCs (Thomas and Capecchi, 1987). Although it is possible to selectively modify specific gene via gene targeting technique, it has

been proven hard since frequency of homologous recombination is low (Gerlai, 2016). Targeting efficiency was improved through artificially inducing DNA double strand breaks by engineered endonucleases such as zinc finger nuclease (Bibikova et al., 2003), TALE nuclease (Miller et al., 2011), CRISPR/Cas9 (Cong et al., 2013a) and CPF1 (Zetsche et al., 2015). These nucleases were effectively applied for gene targeting in human ESCs (Hockemeyer et al., 2009; Hockemeyer et al., 2011; Hou et al., 2013b).

Transgenesis in porcine PSCs was first reported by Piedrahita and colleagues (Piedrahita et al., 1998). Plasmid vectors carrying humanized GFP were introduced into pig embryonic germ cells (EGCs) via electroporation. When transgenic EGCs were injected into blastocysts, the cells were developed into chimeric fetuses with host embryos. In other study, chimeric piglets were born by blastocyst injection using transgenic EGCs harboring transgene, human growth factor (Mueller et al., 1999). Subsequently, various genes including *EGFP*, *hCD46*, *hCD59* and *hPCSK9* have been transfected into pig EGCs, and several transgenic EGC lines could develop to term when used in nuclear transfer as donor cells as shown in Table 5. Gene delivery in porcine ESCs was first reported

(Yang et al., 2009). In contrast to other reports using somatic cell nuclear transfer (SCNT) with transgenic donor cells (Huang et al., 2011; Tan et al., 2011), the transgene [humanized Renilla green fluorescent protein (hrGFP)] was directly delivered into pESCs via electroporation. Although GFP-expressing pESC lines were established via electroporation, transfection efficiency was very low (only three stably GFP-expression lines from 12 trials), and a GFP-expressing line was not obtained by retroviral and liposome-mediated transfection. In other study, EGFP was successfully introduced into pig ESCs via lentiviral vectors under various multiplicities of infection (MOI), with pluripotency and differentiation potential unaffected after transfection (Choi et al., 2013). Recent studies showed that homologous recombination efficiency is higher in PSCs having naïve state than the cells having primed state (Buecker et al., 2010). In non-permissive species such as human, rat and pigs, conversion of pluripotent state from primed state to naïve state have been accomplished by overexpression of exogenous factors including *OCT4* and *KLF4* and inhibition of cellular signaling pathway through the treatment of signaling inhibition molecules as previously mentioned. In human study, naïve PSCs showed that shorter doubling time and higher plating efficiency colonization rate after single cell dissociation than primed PSCs. Because of these

characteristics, efficiency of homologous recombination in naïve PSCs was more than 200 times higher than in primed PSCs (Buecker et al., 2010). Porcine naïve PSCs also established by several groups as discussed above. This technique could be applied for improvement of generation of transgenic animals.

Interestingly, it was verified that transgene expression in pig ESCs were gradually declined during extended culture by DNA methylation as determined by bisulfite sequencing (Choi et al., 2013). Similarly, silencing of the viral transgenes caused by epigenetic modifications and *trans*-acting factors have been observed in producing transgenic animals (Cherry et al., 2000; Kosaka et al., 2004; Whitelaw et al., 2008). Viral transgenes in transgenic animals were not expressed in some tissues (Hofmann et al., 2006; Park et al., 2010). Silenced transgenes in germ cells did not recover during fertilization and embryo development, and could not be transmitted to the next generation (Hofmann et al., 2006). Because silencing of transgenes are frequently occurred in transgenic animals, to overcome the problems, various approaches have been developed. Using regulatory elements, including woodchuck hepatitis virus response element (WRE) (Zufferey et al., 1999), HIV FLAP (Arhel

et al., 2007), and matrix attachment region (MAR) (Bode et al., 2000), which are responsible for transcript stabilization and the translocation of provirus into nuclear and DNA loop formation, respectively, also improve transgene expression. Accordingly, because the expression level of transgenes is dependent upon the vector construct, transfection methods used and cell types, it is therefore important to consider type of vectors and delivery methods for purpose of transgenic animals.

Table 5. List of transgenic/cloned pigs using pluripotent stem cells.

Type of PSCs	Transfection methods	Transgenes	Production of transgenic animal	References
EGCs	Plasmid vector (electroporation)	Humanized GFP	Blastocyst injection (sacrificed at 25 days of gestation)	(Piedrahita et al., 1998)
EGCs (isolated from transgenic pigs)	–	Human growth hormone	Blastocyst injection (developed to term)	(Mueller et al., 1999)
EGCs	Plasmid vector (liposome-mediated transfection)	EGFP	Aggregation with 4–8 cells (observed at blastocyst stage)	(Rui et al., 2006)
EGCs	Plasmid vector (Effectene transfection reagent)	EGFP	Nuclear transfer (observed at blastocyst stage)	(Ahn et al., 2007)
EGCs	Plasmid vector (Effectene transfection reagent)	Human CD46	Nuclear transfer (observed at blastocyst stage)	(Won et al., 2009)
ESCs	Plasmid vector (electroporation)	hrGFP	–	(Yang et al., 2009)
EGCs	Plasmid vector (Effectene transfection reagent)	Human CD59	Nuclear transfer (developed to term)	(Ahn et al., 2010)
ESCs	Lentiviral vector	EGFP	–	(Choi et al., 2013)

(Table 5–continued)

Type of PSCs	Transfection methods	Transgenes	Production of transgenic animal	References
iPSCs	–	–	Nuclear transfer (developed to term)	(Fan et al., 2013)
EGCs	–	–	Blastocyst injection (developed to term)	(Dong et al., 2014)
EGCs	Sleeping beauty transposon system	Human PCSK9	Nuclear transfer (developed to term)	(Secher et al., 2017)

### 3.3 Development of differentiation method

Human PSCs have identified as pivotal tools for regenerative medicine. This trend was accelerated by establishment of human iPSCs and cloned ESCs in 2007 and 2013, respectively (Tachibana et al., 2013; Yu et al., 2007). Accompanied with generation of human PSCs, methods for differentiation into various type of cells have been developed (reviewed in (Tabar and Studer, 2014)). Recently, in addition to differentiation into specific cell types, putative organs composed of multi-cellular tissues, named organoid, could be generated *in vitro* (reviewed in (Yin et al., 2016)). Various type of tissues including intestinal, brain, eye and kidney have been researched. Because they more closely resemble *in vivo*-organs and tissues than singly type of cells, these organoids might provide *in vitro*-tools for drug screening, disease modeling and organ development models. As a clinical trial of human PSCs for cell therapy as mentioned above, it makes the research of animal PSCs involving differentiation and transplantation study more important as a preliminary.

In this reason, many researchers have been attempted to

convert the porcine PSCs into several differentiated cells with transplantation of the differentiated cells into the pigs (summarized in Table 6). First of all, neuronal differentiation have successfully induced from *in vitro*-cultured ICM and iPSCs. During *in vitro*-culture, pig ICMs were spontaneously differentiated into neural rosette-like structures while couldn't be maintained (Puy et al., 2010). The cells isolated from neural rosette-like structures could be developed into neurons including astrocyte and oligodendrocytes. In other study, researchers successfully induced neural progenitor cells from pig iPSCs (Gallegos-Cardenas et al., 2015). Neural differentiation of pig iPSCs resembles that of hPSCs in terms of gene expression pattern, suggesting pig PSCs could provide valid models for human therapy. In addition, hepatocytes have been derived from pig iPSCs and ESCs (Ao et al., 2014; Park et al., 2015). The differentiated cells from iPSCs and ESCs have molecular and functional similarity with hepatocytes. They have abilities including lipid metabolism, glycogen storage and LDL uptake. Several studies have tried to transplant iPSCs or differentiated cells into pig disease models. Rod photoreceptors and retinal pigment epithelial cells were produced from pig iPSCs, and then these cells were transplanted into subretinal space of pigs (Sohn et al., 2015; Zhou et al., 2011). The engrafted cells were successfully resided in subretinal region.

Another groups used undifferentiated pig iPSCs to treat myocardial infarction and myocardial ischemia (Li et al., 2013; Zhang et al., 2014a; Zhou et al., 2014). The engrafted iPSCs ameliorated symptoms via differentiation into vessel cells (Li et al., 2013). However, undifferentiated PSCs have potential to form tumors when engrafted into body (Lee et al., 2013), it needs to be careful for implant of undifferentiation PSCs. Recent studies showed that PGCs and skeletal myotubes could be induced from pig iPSCs (Genovese et al., 2017; Wang et al., 2016). More diverse type of cells would be derived from pig PSCs and various transplantation models are going to develop in near future. These efforts may lead to success of cell therapy using stem cells.

Table 6. List of differentiation studies using pig pluripotent stem cells.

Origin of PSCs	Target/transplanted cells of differentiation	Injury model	transplantation	References
<i>In vitro</i> -cultured ICM	Neural progenitor cells	–	–	(Puy et al., 2010)
iPSCs (Ezashi et al., 2009)	Rod photoreceptors	Retinal damage	Subretinal space	(Zhou et al., 2011)
iPSCs	Hepatocytes	–	–	(Aravalli et al., 2012a)
iPSCs (Wu et al., 2009)	Undifferentiated iPSCs	Acute myocardial infarction	Myocardium	(Li et al., 2013)
iPSCs	Neural progenitor cells	–	–	(Yang et al., 2013)
iPSCs	Hepatocytes	–	–	(Ao et al., 2014)
iPSCs	Neural progenitor cells	–	–	(Li et al., 2014b)
iPSCs	Undifferentiated iPSCs	Chronic myocardial ischemia	Myocardium	(Zhou et al., 2014)
iPSCs (Wu et al., 2009)	Undifferentiated iPSCs	Myocardial infarction	Myocardium	(Zhang et al., 2014a)
iPSCs	retinal pigment epithelial cells	age-related macular degeneration	Subretinal space	(Sohn et al., 2015)
ESCs	hepatocytes	–	–	(Park et al., 2015)
iPSCs	Neural progenitor cells	–	–	(Gallegos-Cardenas et al., 2015)
iPSCs	Primordial germ cells	–	Mouse testis	(Wang et al., 2016)
iPSCs (Telugu et al., 2011)	Skeletal myotubes	–	–	(Genovese et al., 2017)

This chapter will be published in elsewhere  
as a partial fulfillment of Kwang-Hwan Choi's Ph.D program.

## CHAPTER 3

FGF2 plays an important role in maintenance  
of pluripotency in pig stem cells derived  
from embryonic and fetal origins

## 1. Introduction

Over the last three decades, the establishment of pluripotent cell lines from preimplantation mouse embryos has been considered to be one of the biggest events in developmental biology (Evans and Kaufman, 1981; Martin, 1981). These cells, known as embryonic stem cells (ESCs), have *in vivo* and *in vitro* differentiation potentials into three germ layers and can proliferate infinitely. Subsequently, another pluripotent cell lines, called embryonic germ cells (EGCs), were obtained through *in vitro*-culture of primordial germ cells (PGCs) (Matsui et al., 1992; Resnick et al., 1992). Recently, mouse epiblast stem cells (EpiSCs) and induced pluripotent stem cells (iPSCs) were derived from postimplantation embryos and somatic cells, respectively (Takahashi and Yamanaka, 2006; Tesar et al., 2007). These pluripotent cells are divided into “naïve” and “primed” states by their pluripotent status (Nichols and Smith, 2009). In permissive lines, pluripotent cells can be derived from embryos in both states. However, in non-permissive lines such as human, cells are only derived into the “primed” state, such as epiblast stem cells, if no additional treatments including genetic manipulation and chemicals are performed (Buecker et al., 2010; Hanna et al., 2009; Park et al., 2013a).

Human pluripotent stem cells (PSCs) have considered as pivotal tools for regenerative medicine. This trend was accelerated by establishment of human iPSCs and cloned ESCs in 2007 and 2013, respectively (Tachibana et al., 2013; Yu et al., 2007). Accompanied with generation of human PSCs, methods for differentiation into various cell types have been developed (reviewed in (Tabar and Studer, 2014)). Recently, in addition to differentiation into specific cell types, putative organs composed of multi-cellular tissues, named organoid, could be generated *in vitro* (reviewed in (Yin et al., 2016)). Various type of tissues including intestine, brain, eye and kidney have been researched. Because they more closely resemble *in vivo*-organs and tissues than singly type of cells, these organoids might provide *in vitro*-tools for drug screening, disease modeling and organ development models. As importance of clinical trial for human PSCs are getting larger, an interest for research of animal PSCs including differentiation and transplantation are growing as a preliminary study.

In domestic animals, the aim of stem cell research is to create an indefinite cell source for transgenic animals used as bioreactor and tissue engineering materials as well as preliminary studies for human research (Keefer et al., 2007). Especially, pigs have been identified as an ideal animal model because of similarities between humans and pigs in physiological and

immunological features, as well as organ size (Brevini et al., 2007; Hall, 2008). Therefore, many research groups have attempted to derive pig PSCs including ESCs, EGCs and iPSCs, however, authentic pig ESCs have not yet been established (Ezashi et al., 2009; Kues et al., 2013; Park et al., 2013a; Piedrahita et al., 1990b; Son et al., 2009; Yang et al., 2009). So, in this chapter, I tried to analyze mechanisms for establishing and maintaining pluripotency in pig. Consequently, pig ESCs and EGCs were derived from *in vitro*-produced embryos and fetal gonads, respectively. These cell lines expressed pluripotent markers and have *in vitro*-differentiation ability. It is interesting to note that FGF2 signaling have a pivotal roles in establishing and maintaining pluripotency in both cell lines from preimplantation embryos and PGCs. Overall, pig might be closed to non-permissive species rather than permissive species, and FGF2 signaling plays an important role in generating genuine pig PSCs. This study might be applied for optimization of pig PSC culture condition as well as analysis of pig pluripotent networks.

## 2. Materials and methods

### Animal care

The care and experimental use of pigs and mice was approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-140501-4 for MEFs isolation). A pregnant sow was purchased from animal farm. The sow was taken care exclusively at farm and sacrificed after 30 days from artificial insemination at slaughterhouse (Hanbo, Korea) approved by Korean government. Pregnant ICR mice were purchased from SAMTACO BIO Inc., Korea. The mice were taken care according to standard protocol of Institute of Laboratory Animal Resources and sacrificed by cervical dislocation after anesthesia.

### Isolation and culture of porcine embryonic stem cells (ESCs)

Pig ESCs were derived from *in vitro*-produced blastocysts. *In vitro*-produced and hatched blastocysts were seeded on feeder cells composed of mitotically inactivated mouse embryonic fibroblasts (MEFs) according to our previous studies (Park et al., 2013a; Son et al., 2009). After 5-7 days, primary colonies of ESCs were observed and cultured for approximately 7-10 days longer. Fully expanded colonies were mechanically

dissociated using pulled-glass pipettes and transferred onto new feeder cells for subculture.

Pig ESCs were cultured in porcine ESC media (PESM). PESM consisted of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, low glucose) and Ham's F10 media containing 15% fetal bovine serum (FBS; collected and processed in the USA), 2 mM glutamax, 0.1 mM  $\beta$ -mercaptoethanol, 1x MEM nonessential amino acids, and 1x antibiotic-antimycotic (all from Gibco, USA). To support pluripotency and self-renewal, the ESCs were cultured in PESM with the following cytokines: 40 ng/ml human recombinant stem cell factor (SCF; R&D Systems, USA), 20 ng/ml human recombinant fibroblast growth factor 2 (FGF2; R&D Systems), and 100ng/ml heparin sodium salt (Sigma-Aldrich, USA). Media were changed every 24 h and all cells were cultured in humidified conditions with 5% CO<sub>2</sub> at 37 °C. Pig ESCs were sub cultured every 5-7 days using pulled glass pipettes. Expanded colonies were detached from the feeder cells and dissociated into small clumps. These clumps were transferred into new feeder cells containing mitomycin-C-treated (Roche, Germany) MEFs.

## Derivation and culture of pig embryonic germ cells (EGCs)

Pig EGCs were derived from the genital ridges of a porcine dpc 30 fetuses. Pig fetuses collected from pregnant sow were washed in Dulbecco's phosphate-buffered saline (DPBS; Welgene, Korea) supplemented with 0.4 % BSA and antibiotic-antimycotic solution (Gibco, USA). The genital ridges were isolated from mesonephros and mechanically dissociated by mincing with plunger. Minced tissues containing PGCs were seeded on feeder cells composed of mitotically inactivated MEFs and cultured with EGC media. EGC media consisted of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, low glucose) and Ham's F10 media containing 15% fetal bovine serum (FBS; collected and processed in the USA), 2 mM glutamax, 0.1 mM  $\beta$ -mercaptoethanol, 1x MEM nonessential amino acids, and 1x antibiotic-antimycotic (all from Gibco). To promote reprogramming and self-renewal, several cytokines were added in media: 40 ng/ml human recombinant stem cell factor (SCF; R&D Systems, USA), 1000 unit/ml Leukemia inhibitory factor (LIF; Millipore, MA, USA), and 40 ng/ml human recombinant basic fibroblast growth factor (FGF2; R&D Systems). After 7 days, primary colonies of embryonic germ cells were observed. Primary colonies of pig EGCs were dissociated using 0.25% trypsin solution and transferred onto new feeder cells for subculture.

Established pig EGCs were cultured with EGC media supplemented with LIF or FGF2. Subculture was performed every 7 days. EGC colonies were dissociated by treating 0.25% trypsin solution for 5 min and then transferred into new feeder cells. Media were changed every 24 h and all cells were cultured under humidified conditions containing 5% CO<sub>2</sub> at 37°C.

### ***In vitro* differentiation of pig stem cells**

Cultured pig EGCs were dissociated into single cells using 0.25% trypsin/EDTA solution (Welgene) and cultured in Ultra-Low attachment plates (Sigma Aldrich, MO, USA) without cytokines for 5 days. Cultured ESC colonies were detached from feeder cells, and colonies were mechanically dissociated into small clumps. Suspension cultures of these clumps were obtained using the hanging-drop method for 5–6 days with PESM in the absence of cytokines. After suspension culture, dissociated cells were aggregated and formed embryoid bodies. Cultured embryoid bodies were seeded on 0.1% gelatin-coated plates and cultured for 2–3 weeks with DMEM containing 15% FBS. After 2–3 weeks, differentiated cells were fixed with 4% paraformaldehyde and analyzed by immunostaining with the following antibodies: Neurofilament (ectoderm), Vimentin (mesoderm), and Cytokeratin 17 (endoderm), as described below.

## Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA from individual samples was extracted using TRIzol® reagent (Invitrogen, MA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a High–capacity RNA–to–cDNA Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions, producing a final volume of 20 µl. Derived cDNA samples were amplified with 2x PCR master mix solution (iNtRON, Korea) and 2 pmol primers as shown in Table 7. PCR reactions were performed in a thermocycler under the following conditions: 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s (annealing temperatures depended on each primer set), extension at 72°C for 30 s, and a final extension at 72°C for 7 min. Amplified PCR products were visualized using electrophoresis on 1% agarose gel stained with ethidium bromide.

Table 7. RT-PCR primer sets for analyzing pluripotency and differentiation in pig ESCs.

Genes	Sequences	Size
<i>OCT4</i>	5'-AACGATCAAGCAGTGACTATTTCG-3'	153
	5'-GAGTACAGGGTGGTGAAGTGAGG-3'	
<i>NANOG</i>	5'-AATCTTCACCAATGCCTGAG-3'	141
	5'-GGCTGTCCTGAATAAGCAGA-3'	
<i>SOX2</i>	5'-CAACTCTACTGCTGCGGCG-3'	317
	5'-CGGGCAGTGTGTACTTATCCTTC-3'	
<i>CRABP2</i>	5'-CTGACCATGACGGCAGATGA-3'	185
	5'-CCCCAGAAGTGACCGAAGTG-3'	
<i>PAX6</i>	5'-AGAGAAGACAGGCCAGCAAC-3'	169
	5'-GGCAGAGCACTGTAGGTGTT-3'	
<i>NESTIN</i>	5'-TGCCTGGGGGAGGAATCTTTT-3'	252
	5'-CTCTTCAGCCAGGTTGTTCG-3'	
<i>GATA6</i>	5'-GCCACTACTTGTGCAACCG-3'	178
	5'-TTGCACACAGGTTACCCCTC-3'	
<i>AFP</i>	5'-ATGAGACAATTTGGAGCCCGA-3'	142
	5'-CGTTTCCTCTGCAGCATTC-3'	
<i>AMY2</i>	5'-TGCTCTTGAATGTGAGCGGT-3'	206
	5'-TACGGACGCCAACGTTGTTA-3'	
<i>T</i>	5'-GGAAGTACGTGAACGGGGAG-3'	220
	5'-ACCCTCACGATGTGGATTCG-3'	
<i>BMP4</i>	5'-CGTTGGTCTCGAGTATCCCG-3'	106
	5'-AGAGTTTTTCGCTGGTCCCTG-3'	
<i>DES</i>	5'-CCTCAACTTCCGAGAAACAAGC-3'	108
	5'-TCACTGACGACCTCCCCATC-3'	
<i>ACTB</i>	5'-GTGGACATCAGGAAGGACCTCTA-3'	137
	5'-ATGATCTTGATCTTCATGGTGCT-3'	

## Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using the TRIzol® Reagent (Invitrogen, USA) and, cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA). Extracted cDNA samples were amplified with DyNAmo HS SYBR Green qPCR Kit (Thermo scientific, MA, USA) containing 1–2 pmol of each primer set listed in Table 8 in a 10 µl reaction volume. Amplification and detection were conducted using the ABI 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec and annealing/extension for 1 min (annealing/extension temperatures depended on each primer set). The dissociation curve were analyzed and the amplified products were loaded on gels to confirm the specificity of PCR products. The relative expression level was calculated by normalizing the threshold cycle (Ct) values of each gene to that of the ACTB via the  $2^{-\Delta C_t}$  method (Livak and Schmittgen, 2001).

Table 8. Primer sets for detecting pluripotent and germ cell markers.

Genes	Sequences	Size
<i>OCT4a</i>	5'- CTTGGAGAGCCCTGGTTTTACT -3'	159
	5'- GCCAGGTCCGAGGATCAAC -3'	
<i>SOX2</i>	5'- CGGCGGTGGCAACTCTAC -3'	100
	5'- TCGGGACCACACCATGAAAG -3'	
<i>KLF4</i>	5'- GGACCACCTTGCCTTACACA -3'	146
	5'- CTTTCCAGCTGGGTTCCCTCC -3'	
<i>MYC</i>	5'- GAAAAAGACGTGCTGCGGAA -3'	253
	5'- CCAGCCAAGGTTGTGAGGTT -3'	
<i>NANOG</i>	5'- CATCTGCTGAGACCCTCGAC -3'	195
	5'- GGGTCTGCGAGAACACAGTT -3'	
<i>DPPA2</i>	5'- CTTCAAGAGCCGTTACCCCT -3'	144
	5'- GGCGAACCCAACCTTCTGTA -3'	
<i>LIFR</i>	5'- TCTCTCCAGTAGCTGAGCGT -3'	236
	5'- CTGAGGCTTATAGCCTGCCC -3'	
<i>FGFR2</i>	5'- TCATCTGCCTGGTTGTGGTC -3'	281
	5'- CCTAGGCGTGGCACCTTTTA -3'	
<i>DPPA3</i>	5'- CTAGGAGGAAAGGGTGAGGA -3'	115
	5'- CGCTGCTCTCCTGATTTTGTC -3'	
<i>IFITM3</i>	5'- TTCGTGGCTTTCGCCTACTC -3'	161
	5'- CCAGTGGTGCAAACGATGAT -3'	
<i>VASA</i>	5'- GAACCCAGTTGGGGCATTCA -3'	211
	5'- TTTGATGGCATTCTGGGCA -3'	
<i>BLIMP1</i>	5'- GTTCAGGCAGAGGCATCCTT -3'	272
	5'- GAGTGTGCTGGGTTACGTA -3'	
<i>DNMT1</i>	5'- AACTGTCACCAACCCTGAGC -3'	252
	5'- TTGACTTTAACTGAGGCGCT -3'	
<i>DNMT3a</i>	5'- AGGAGACCAACATCGAATCCA -3'	242
	5'- GGTGGACCCCTGGTTTTTCTT -3'	
<i>DNMT3b</i>	5'- CGCTCTAAGGAGGGTGATGA -3'	100
	5'- ACTCCAGGAACCGTGAGATG -3'	
<i>PTEN</i>	5'- CCAGTCAGAGGCGCTATGTG -3'	151
	5'- TGGCAGACCACAACTGAGG -3'	
<i>ACTB</i>	5'- GTGGACATCAGGAAGGACCTCTA -3'	131
	5'- ATGATCTTGATCTTCATGGTGCT -3'	

### **Alkaline phosphatase staining**

Cells were fixed with 4% paraformaldehyde for 30 min. Fixed cells were stained with a solution containing nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) stock solution (Roche) in a buffer solution for 30 min at room temperature. Stained cells were then examined under an inverted microscope.

### **Immunostaining**

Before fixation, cell samples were preincubated for 10 min at 4 °C and fixed with 4% paraformaldehyde for 30 min. After washing twice with DPBS (Welgene), samples were treated for 1 h with 10% goat serum in DPBS to prevent nonspecific binding. Serum-treated cells were incubated overnight at 4 °C with primary antibodies. The primary antibodies used were as follows: OCT4 (Santa Cruz Biotechnology, CA, USA; 1:200), SOX2 (Millipore; 1:200), NANOG (PeproTech, NJ, USA; 1:200), SSEA1 (Millipore; 1:200), SSEA4 (Millipore; 1:200), Neurofilament (Millipore; 1:100), Vimentin (Millipore; 1:100), and Cytokeratin 17 (Millipore; 1:100). When used the antibodies for intracellular proteins such as OCT4, SOX2, and NANOG, fixed cells were treated for 15 min with 0.1% Triton-X100 (Sigma-Aldrich, MO, USA) before serum blocking. After incubation with the primary antibody, the cells were treated for 3 h at room temperature with

Alexa Fluor–conjugated secondary antibodies. Nuclei were stained with Hoechst 33342 (Molecular Probes). Images of stained cells were captured using a LSM 700 Laser Scanning Microscope (Carl Zeiss, Germany) and processed with the ZEN 2012 Light Edition program (Carl Zeiss).

### **Bisulfite sequencing**

Genomic DNA was extracted using the G–spin<sup>TM</sup> Total DNA Extraction Kit (iNtRON) and bisulfite treatment was performed using the EZ DNA Methylation–Gold<sup>TM</sup> Kit (Zymo Research, CA, USA). Bisulfite–treated DNA samples were PCR–amplified with specific primers listed in Table 9. Amplifications were performed using 2x PCR master mix solution containing 1 pmol of each primer in 20 µl reaction volume. The resulting PCR products were separated by electrophoresis and purified from agarose gels using the MEGAquick–spin<sup>TM</sup> Total Fragment DNA Purification Kit (iNtRON). Purified amplicons were cloned into the pGEMT–Easy Vector (Promega, WI, USA) and transformed into *Escherichia coli* (DH5–*a*; Novagen, USA). Plasmids were extracted from the selected positive colonies using the DNA–spin<sup>TM</sup> Plasmid DNA Purification Kit (iNtRON). The extracted plasmids were sequenced using an ABI PRISM 3730 automated sequencer (Applied Biosystems). Finally, the methylation patterns of converted sequences with > 95% cytosine–to–thymine conversion rates were

analyzed by the BIQ Analyzer Program (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/>).

Table 9. Primer sets of bisulfite sequencing for XIST promoter and IGF2/H19 DMR3.

Promoters	sequences	size	CpGs
<i>IGF2/H19</i>	Outer 5'– GGTTTTAGGGGGATATTTTTT –3'	208	12
	5'– TTAAAAAACATTACTTCCATATAC –3'		
	Inner 5'– GATTTTTAGGTTTGTATTATTT –3'		
	5'– CAAATATTCAATAAAAAAACCC –3'		
<i>XIST</i>	Outer 5'– TGGTTAAATGAGGTATTTGGA –3'	429	13
	5'– CCATAAACATAACTAAAACTAAA –3'		
	Inner 5'– TTTGTTATATTGTTTGTGGAAAA –3'		
	5'– CCATAAACATAACTAAAACTAAA –3'		

## **Karyotyping**

Karyotyping of cells using standard G-banding chromosome and cytogenetic analysis was performed at GenDix Laboratories (Korea; [www.gendix.com](http://www.gendix.com)).

## **Statistical analysis**

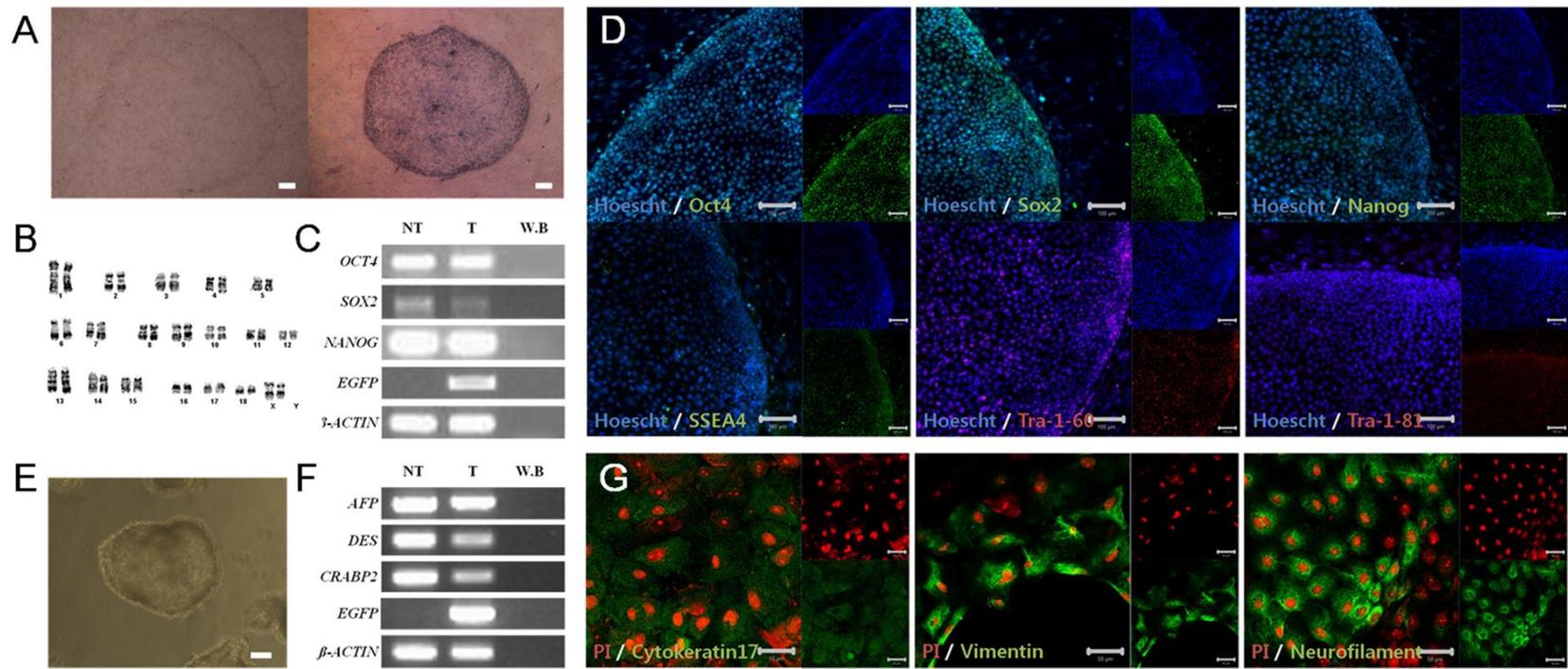
All gene expression data from qPCR analyses were statistically analyzed using GraphPad Prism 6 statistical software (GraphPad Software, CA, USA). Statistical differences between datasets were determined by one-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) tests. Differences were considered significant at  $P < 0.05$ .

### 3. Results

#### Establishment and characterization of porcine embryonic stem cells (ESCs)

It is proven hard to establish authentic pluripotent stem cells (PSCs) in pig species. One of the reasons is due to lack of understanding mechanisms of porcine pluripotency. So, it is important for deriving genuine pig PSCs to analyze various stem cell lines derived from different origins. Firstly, I tried to derive pig ESCs from *in vitro*-produced blastocysts as previously described (Lee et al., 2007; Park et al., 2013a). Two pig ESC lines were established from 42 hatched blastocysts (Derivation efficiency: 4.78% (2/42)). Of these two established cell lines, only one was used for further studies. Established pig ESCs are represented by typical flattened morphologies as previously reported (Park et al., 2013a), similar to mouse EpiSCs and human ESCs. Additionally, they possess AP activity (Fig. 1A) and are stably maintained over long periods (>50 passages in 1 year) with a normal karyotype (Fig. 1B; 36 + XX). These cells were analyzed for pluripotent marker expression and their differentiation ability *in vitro* to verify their pluripotency, according to previously reported standards (Park et al., 2013a). Expression of pluripotency-related transcription factors such as *OCT4*, *SOX2*, and *NANOG* were detected at the mRNA level (Fig.1C).

These factors, as well as pESC surface markers such as SSEA4, TRA-1-60, and TRA-1-81, were also identified at the protein level using immunocytochemistry (Fig. 1D). When these cells were detached from feeder cells and cultured in suspension, they aggregated and subsequently formed embryoid bodies (EBs; Fig. 1E). The generated EBs spontaneously differentiated into three germ layers upon being placed on gelatin-coated plates. In the differentiated cells, the expression of differentiation marker genes on the three germ layers was detected by RT-PCR and immunostaining (ectoderm: *CRABP*, Neurofilament, mesoderm: *DES*, Vimentin, endoderm: *AFP*, Cytokeratin 17; Fig. 1F, G). Thus, it was confirmed that the established cell line was a pluripotent cell line with the differentiation potential to generate the three germ layers.



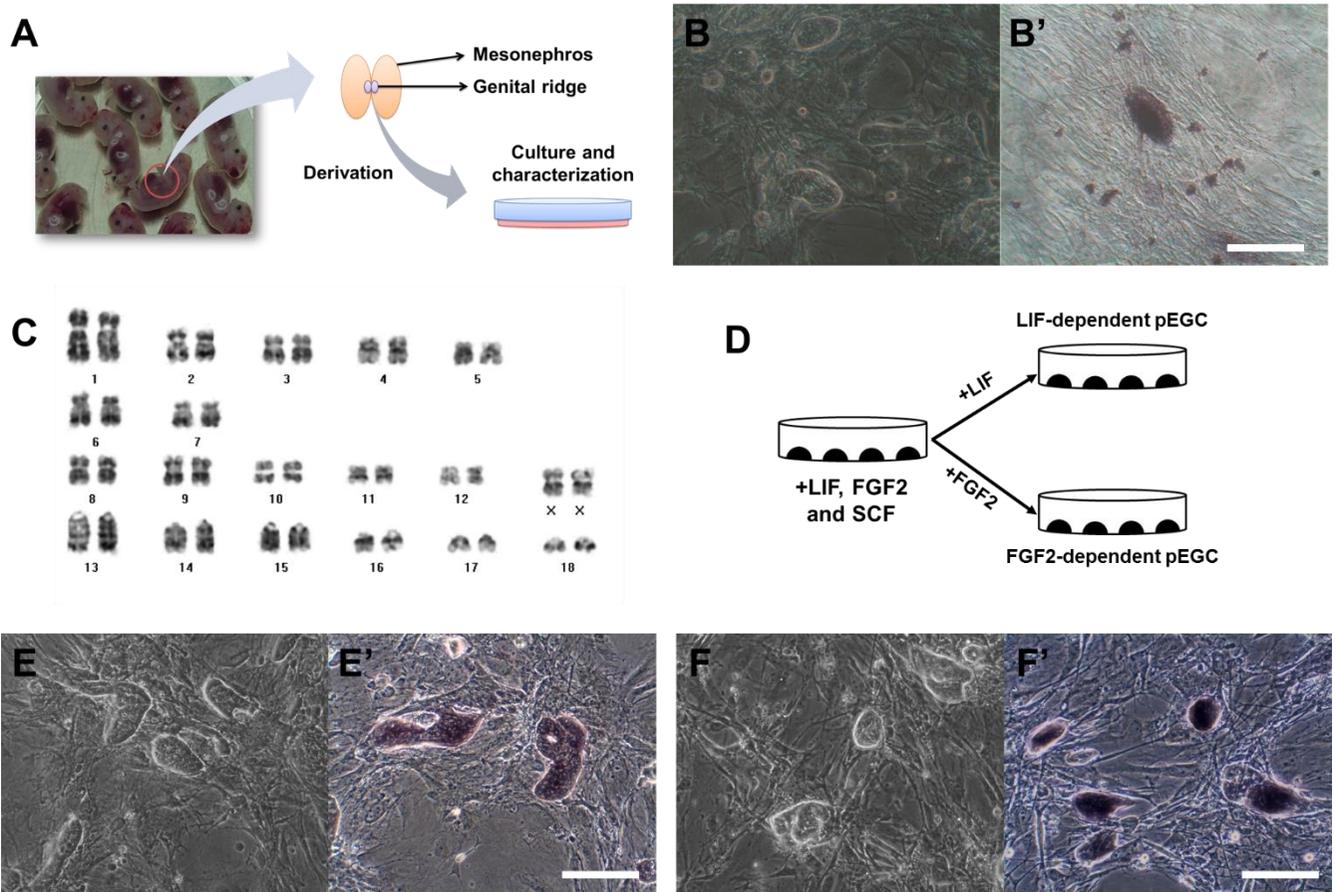
## Figure 1. Derivation and characterization of pig ESCs.

Pig ESCs were derived from *in vitro*-fertilized embryos with FGF2-supplemented media. And pluripotent gene expression and *in vitro*-differentiation ability were examined. (A) Pig ESCs represented typical morphologies of mouse EpiSCs and human ESCs, and have alkaline phosphatase activity (left panel: no-stained colony; right panel: AP stained colony). (B) ESCs have a normal karyotype (36 + XX) and (C) expressed genes related to pluripotency, as determined by RT-PCR (NT: non-transfected pESCs, T: transfected pESCs, W.B.: water blank). (D) Expression of pluripotent markers was detected using immunocytochemistry (passage number: 13). (E) Embryoid bodies were generated in suspension culture and spontaneously differentiated onto culture dishes. (F, G) The differentiated cells expressed differentiation marker genes at the mRNA and protein levels (Ectoderm: *CRABP2*, Neurofilament, Mesoderm: *DES*, Vimentin, Endoderm: *AFP*, Cytokeratin 17; passage number: 17). Scale bars = 100  $\mu\text{m}$ , except for A and G (scale bar = A: 200  $\mu\text{m}$ , G: 50  $\mu\text{m}$ ).

## Derivation of pig embryonic germ cells from fetal gonads

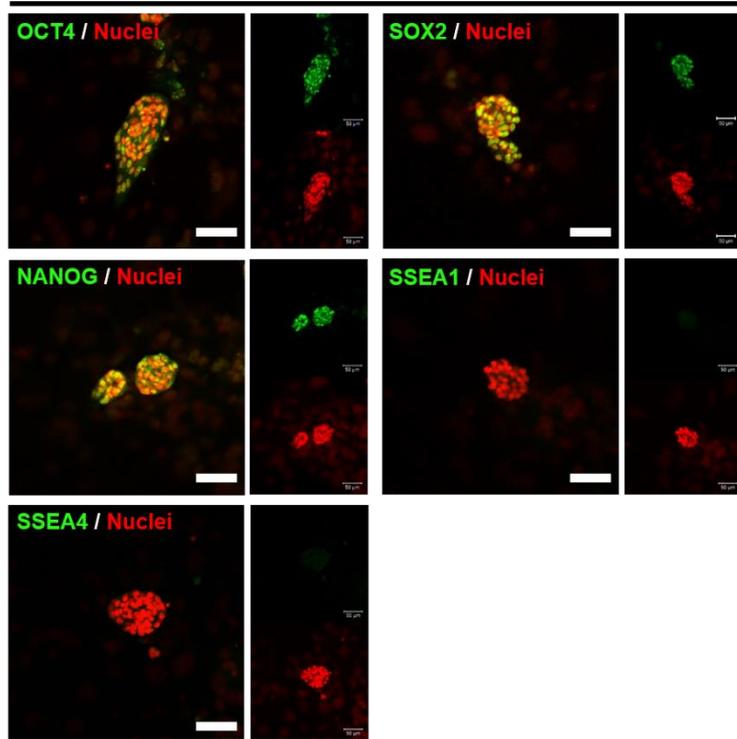
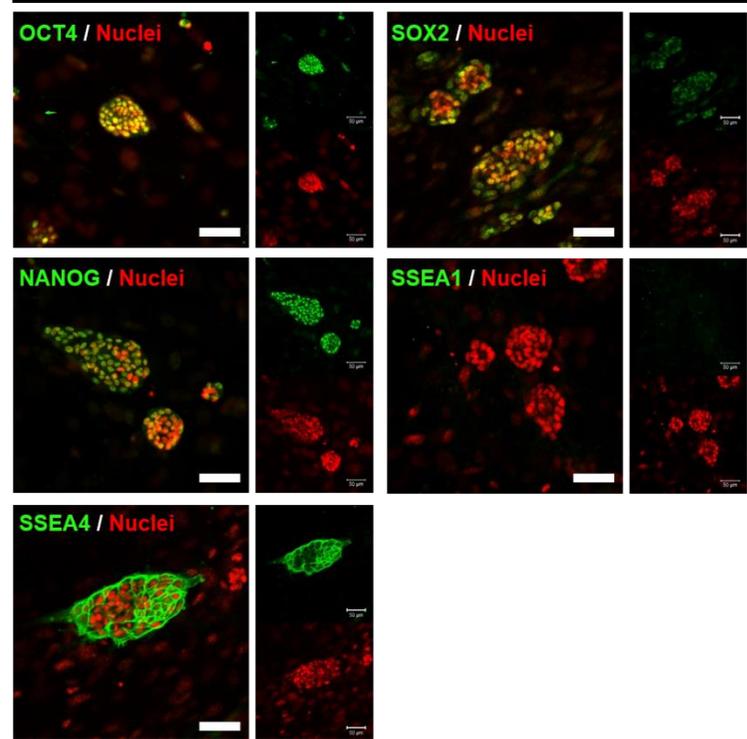
Secondly, I attempted to establish and characterize pig embryonic germ cells (EGCs) from fetal gonads. Primitive gonads were collected from fetuses and mechanically dissociated by mincing with plunger. Mincing tissues containing primordial germ cells (PGCs) were seeded on feeder cells and cultured with EGC media containing LIF, FGF2 and SCF (Fig 2A). After 7 days later, primary colonies of pig EGCs were obtained. Derived cells have alkaline phosphatase activity displaying dome-shaped morphology (Fig 2B). The cells were stably maintained with three cytokines-supplemented media possessing normal karyotype (Fig 2C). Meanwhile, because it has been verified that LIF and FGF2 have an opposite role in maintaining pluripotent state (Hanna et al., 2010b), these cells were separately cultured in LIF- or FGF2-contained media. After several passaging, I could obtain two independent cell lines cultured with LIF- or FGF2-supplemented media (Fig 2D). LIF-dependent EGCs (LIF-EGCs) and FGF2-dependent EGCs (FGF2-EGCs) were maintained over an extended time period and expressed pluripotent marker genes including AP, OCT4, SOX2 and NANAOG (Fig 2E and F; Fig 3A and B). When the both cell lines were cultured in suspension, the cells aggregated and, in turn, formed EBs (Fig 4A). These EBs spontaneously differentiated into three germ layers when placed on gelatin-coated plates. Expression of three germ layer markers was confirmed by RT-PCR and immunostaining (Fig 4B and C).

Epigenetically, *XIST* promoters of both cell lines were similarly hyper-methylated (Fig 5A), however, DMR3 regions of *IGF/H19* in FGF2-EGCs were clearly hemi-methylated while those of LIF-EGCs were incompletely hemi-methylated (Fig 5B). Taken together, embryonic germ cell lines were derived from pig fetal gonad in LIF- or FGF2-supplemented culture media. And the EGCs have different features depend on culture conditions.



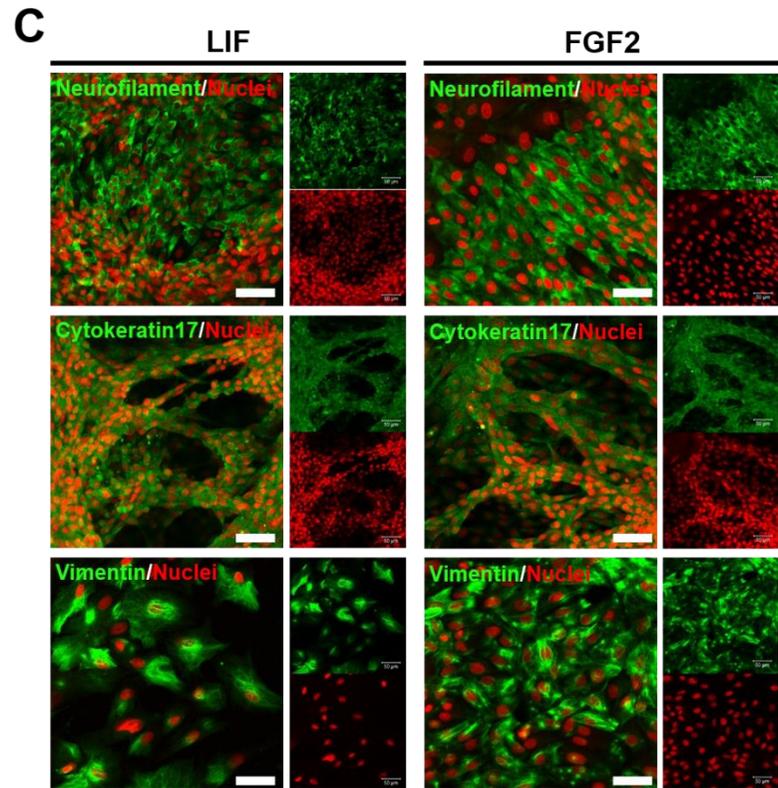
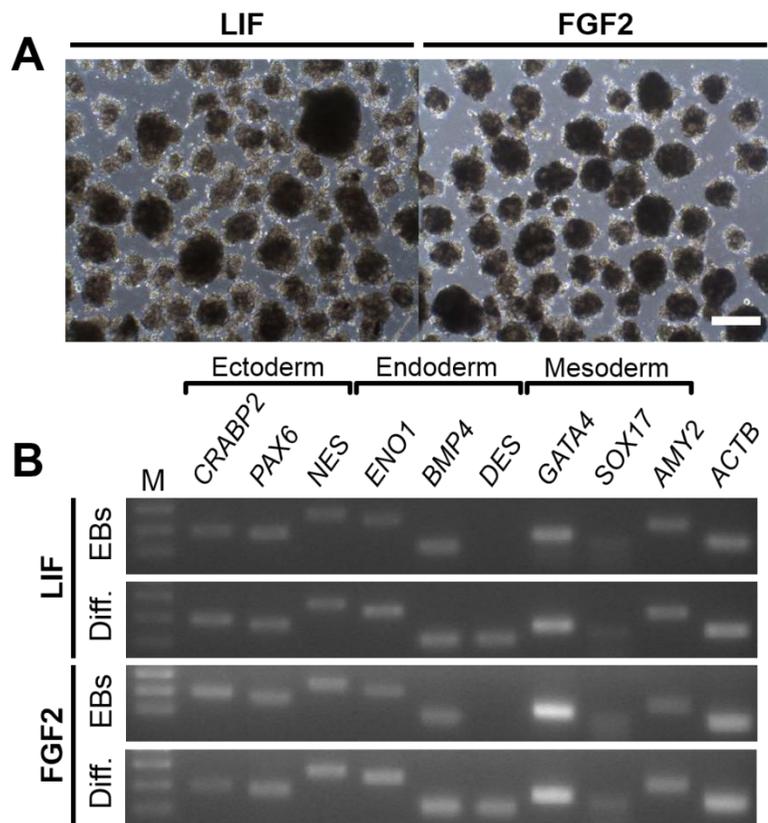
**Figure 2. Derivation of pig EGCs from fetal gonads.**

(A) Primitive gonads were collected from fetuses and mechanically dissociated by mincing with plunger. Mincing tissues containing PGCs were seeded on feeder cells and cultured with EGC media containing LIF, FGF2 and SCF. (B) After 7 days later, primary colonies of pig EGCs were obtained. Derived cells have alkaline phosphatase activity displaying dome-shaped morphology. (C) The cells were stably maintained with three cytokines-supplemented media possessing normal karyotype. (D) These cells were separately cultured in LIF- or FGF2-contained media. After several passaging, I could obtain two independent cell lines cultured with LIF- or FGF2-supplemented media. (E) LIF-dependent EGCs (LIF-EGCs) and (F) FGF2-dependent EGCs (FGF2-EGCs) were maintained over an extended time period having AP activity. Scale bar = 400  $\mu\text{m}$

**A****LIF****B****FGF2**

**Figure 3. Characterization of pig EGCs by immunostaining.**

To analyze differences between LIF- and FGF-EGCs, expression of pluripotent genes in (A) LIF-EGCs and (B) FGF2-EGCs were determined by immunostaining. Scale bar = 50  $\mu$ m



**Figure 4. *In vitro*–differentiation ability of pig EGCs.**

To determine *in vitro*–differentiation ability, pig EGCs were differentiated into three germ layers through EB formation. (A) When the both cell lines were cultured in suspension, the cells aggregated and, in turn, formed EBs. These EBs spontaneously differentiated into three germ layers when placed on gelatin–coated plates. Expression of three germ layer markers was confirmed by (B) RT–PCR and (C) immunostaining (Ectoderm: *CRABP2*, *PAX6*, *NES*, neurofilament, Mesoderm: *ENO1*, *BMP4*, *DES*, vimentin, Endoderm: *GATA4*, *SOX17*, *AMY2*, cytokeratin 17). (scale bar = A: 400  $\mu$ m, C: 50  $\mu$ m)

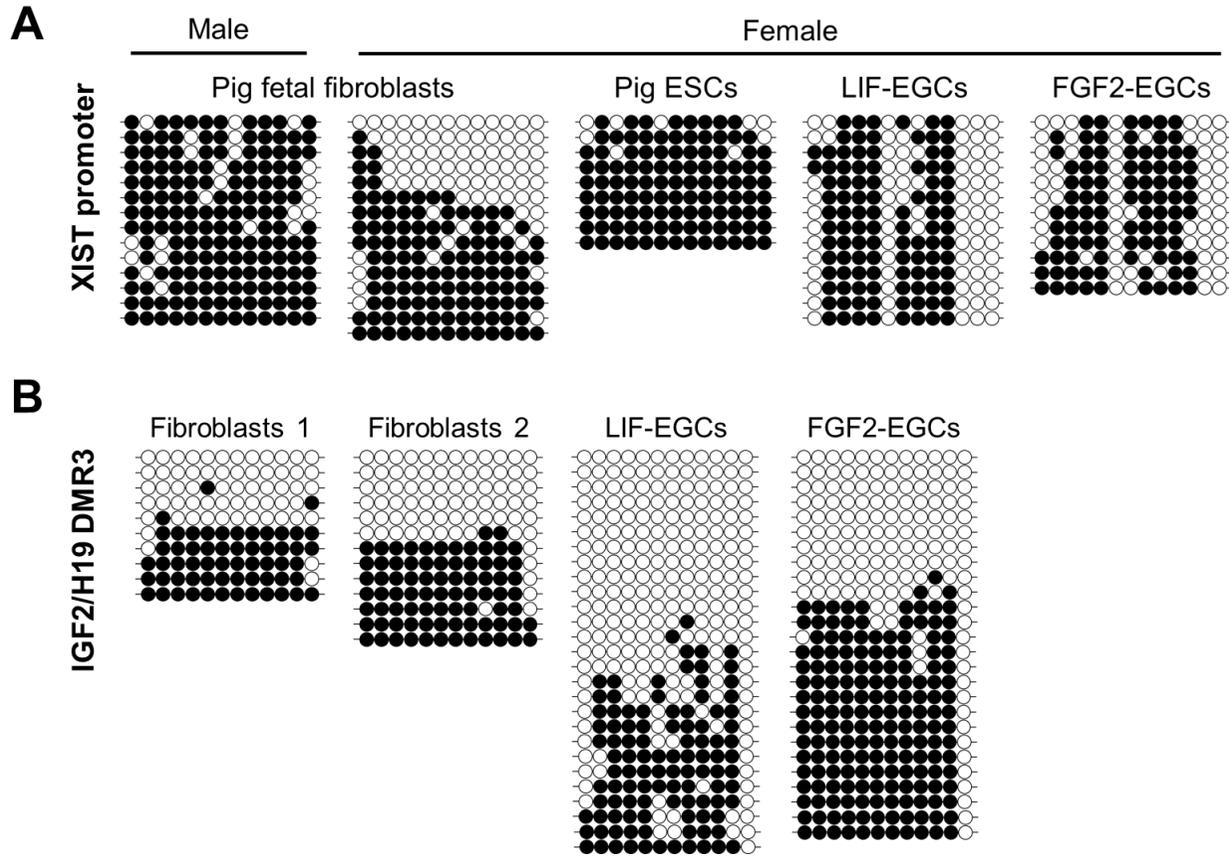


Figure 5. Bisulfite sequencing at DMR region of *IGF2/H19* and promoter region of *XIST*.

DNA methylations of DMR region of *IGF2/H19* and promoter region of *XIST* were analyzed by bisulfite sequencing in order to define epigenetic state of pig EGCs. (A) *XIST* promoters of pig EGC lines were compared with those of pig fetal fibroblasts and ESCs. (B) DMR3 regions of *IGF/H19* in EGCs were analyzed with those of pig fetal fibroblasts. Each circle indicates individual CpG dinucleotides. White and dark circles represent unmethylated and methylated CpGs, respectively. Each row represents one individual clone of amplified PCR products.

## Pluripotency-related genes were differentially expressed in LIF- and FGF2-dependent EGCs

Interestingly, although both cell lines have similar features in terms of morphology and *in vitro* differentiation ability, the cells cultured in LIF- or FGF2-contained media expressed different pluripotency markers. While transcription factors such as OCT4, SOX2, and NANOG were expressed in both cell lines, SSEA4 was expressed only in FGF2-EGCs (Fig 3). Some of cells in LIF-EGCs expressed SSEA4, but majority of cells did not express SSEA4 (Fig 6). And SSEA1, known as PGC and EGC markers in mice, was not expressed in both cell lines (Fig 3). To verify effects of LIF and FGF2 on EGCs, comparative analysis between LIF- and FGF2- treated groups was performed by qPCR. Total RNA of samples were isolated from early stage (after 1 passage from change of LIF- or FGF2-contained media) and late stage (after 5 passages from change of LIF- or FGF2-contained media). First of all, effects of cytokines on germ cell marker gene during extended culture were analyzed. Germ cell markers, *IFITM3* and *STELLA* were expressed at similar level between groups except for *VASA* (Fig 7). In mice, during reprogramming into EGCs, *Blimp1* as a key determinant of germ cells is down-regulated in response to FGF2 (Durcova-Hills et al., 2008), and loss of *Pten* known as reprogramming barriers of PGCs promotes PGC growth and EGC formation efficiency (Kimura et al., 2003). However, pig data showed that *BLIMP1* was up-regulated right after withdrawal of

LIF, and expression of *PTEN* was increased in FGF2–EGCs as culture was progressed, not in LIF–EGCs (Fig 7). Secondly, comparative analysis of pluripotent genes between LIF– and FGF2–EGCs was conducted. Expression of *OCT4* and *NANOG* were higher in FGF2–EGCs, especially, *NANOG* was immediately up–regulated in response of FGF2 (Fig 8A). Expression of *KLF4* and *MYC* as targets of *BLIMP1* were not observed in PGCs (Durcova–Hills et al., 2008), but these genes were highly expressed in both conditions. And these expression were more facilitated by FGF treatment than LIF treatment (Fig 8A). Finally, signaling molecules and DNA methyl transferases (DNMTs) were examined. In LIF–EGCs, *LIFR* was increased during culture with LIF. And in FGF2–EGCs, although expression of *FGFR2* was expressed at similar level with LIF–EGCs, phosphorylated ERK was accumulated by treating FGF2 in time–dependent manner as determined by western blotting (Fig 8B and Fig 9). DNMTs which play important roles in establishing and maintaining DNA methylation were more highly expressed in both cell lines than in somatic control with similar level (Fig 8C). Accordingly, these data showed that several pluripotent genes and germ cell markers were differentially expressed between LIF– and FGF2–EGCs, and most of genes were more highly expressed in FGF2–treated group.

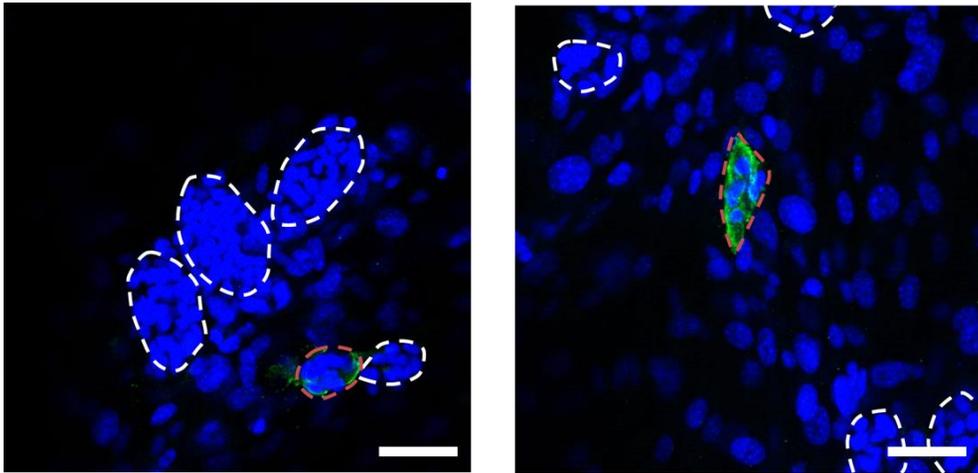
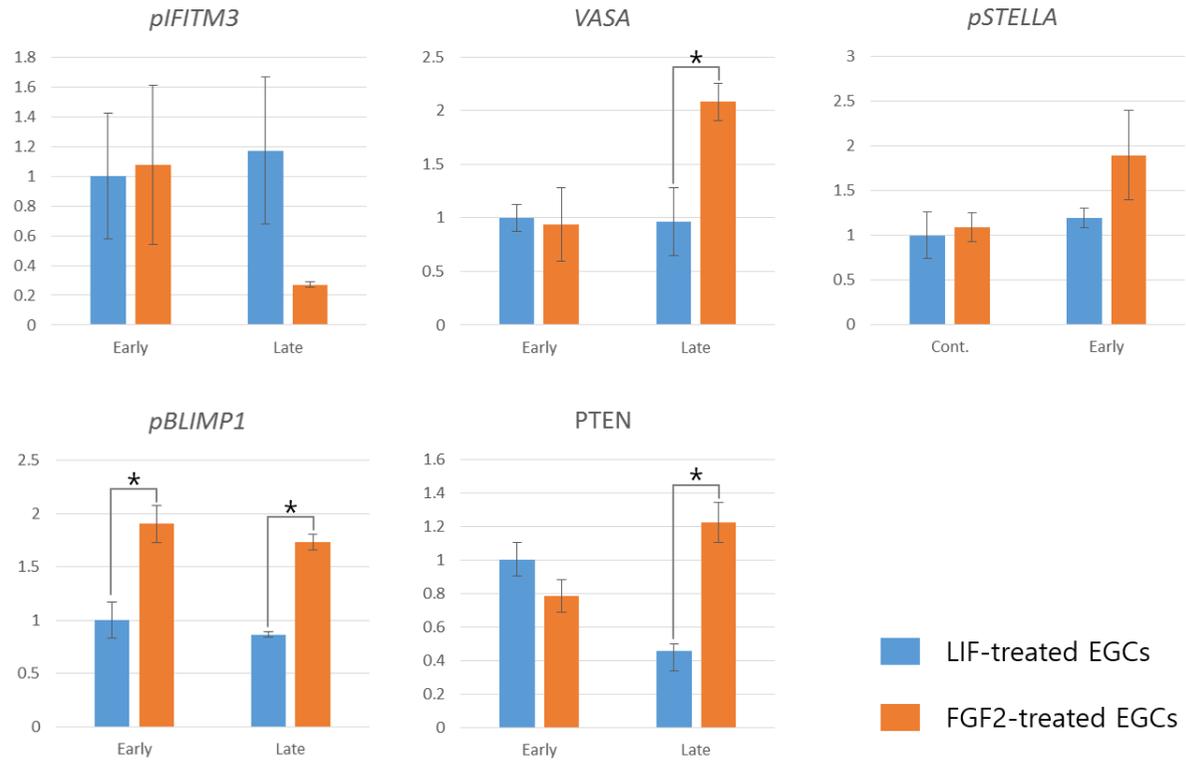


Figure 6. Expression of SSEA4 in LIF-EGCs.

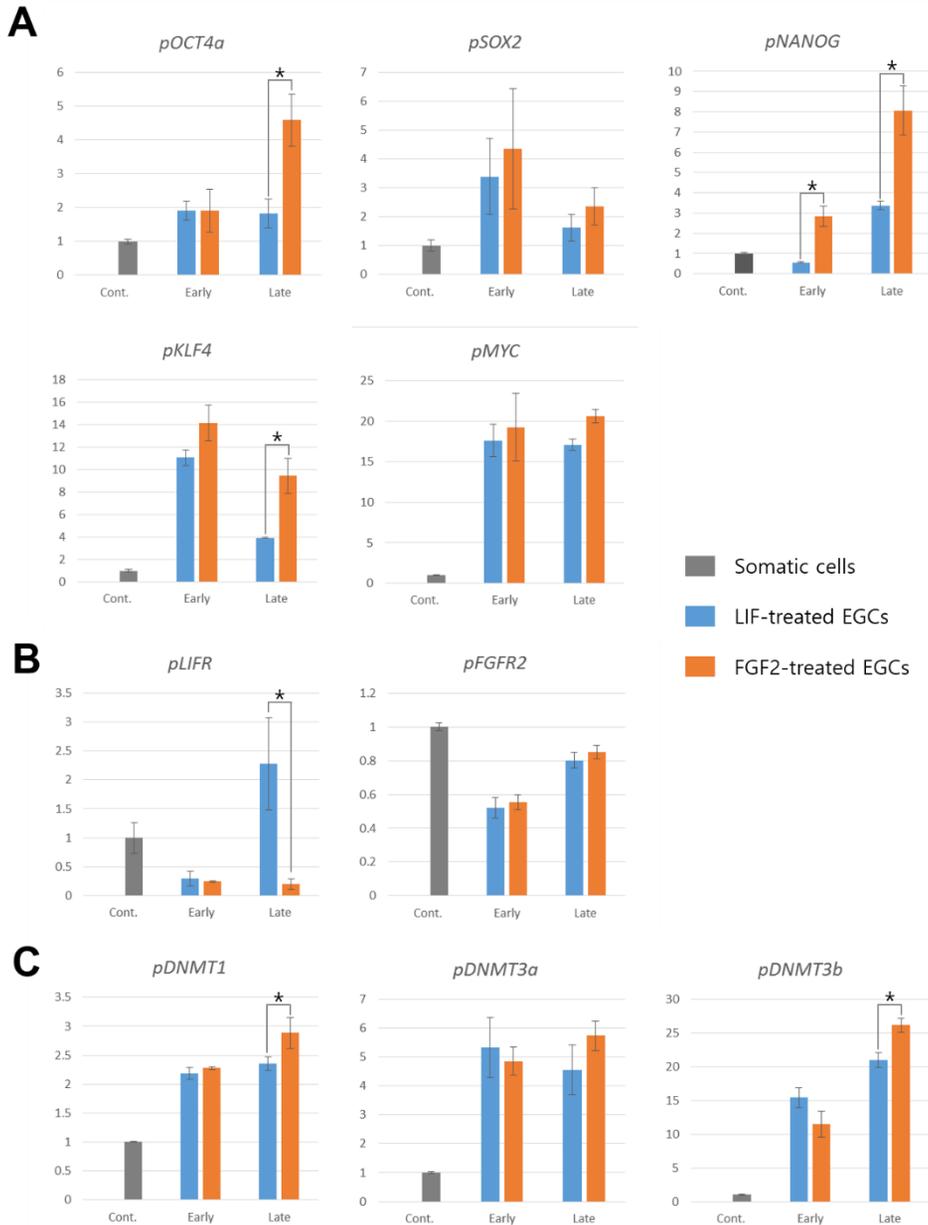
Expression of SSEA4 in LIF-EGCs were determined by immunostaining.

The areas with dashed lines indicate colonies of EGCs (white line: SSEA4 negative; red line: ssea4 positive). Scale bar = 50  $\mu$ m



**Figure 7. Expression of germ cell markers during EGC culture.**

To verify effects of LIF and FGF2 on EGCs during extended culture, comparative analysis between LIF<sup>-</sup> and FGF2<sup>-</sup> treated groups was performed by qPCR. Total RNA of samples were isolated from early stage (after 1 passage from change of LIF<sup>-</sup> or FGF2<sup>-</sup>-contained media) and late stage (after 5 passages from change of LIF<sup>-</sup> or FGF2<sup>-</sup>-contained media). Then, expression of germ cell markers (*IFITM3*, *STELLA*, *VASA*, *BLIMP1* and *P TEN*) was examined. Asterisks indicate a significant difference between groups ( $P < 0.05$ ).



**Figure 8. Expression of pluripotent genes and DNMTs during EGC culture.**

To verify effects of LIF and FGF2 on EGCs during extended culture, comparative analysis between LIF- and FGF2- treated groups was performed by qPCR. Total RNA of samples were isolated from early stage and late stage. Then, expression of pluripotent genes (A; *OCT4*, *SOX2*, *NANOG*, *KLF4* and *MYC*), receptor of signaling molecules (B; *FGFR2* and *LIFR*) and DNMTs (C; *DNMT1*, *DNMT3a* and *DNMT3b*) was examined. Asterisks indicate a significant difference between groups ( $P < 0.05$ ).



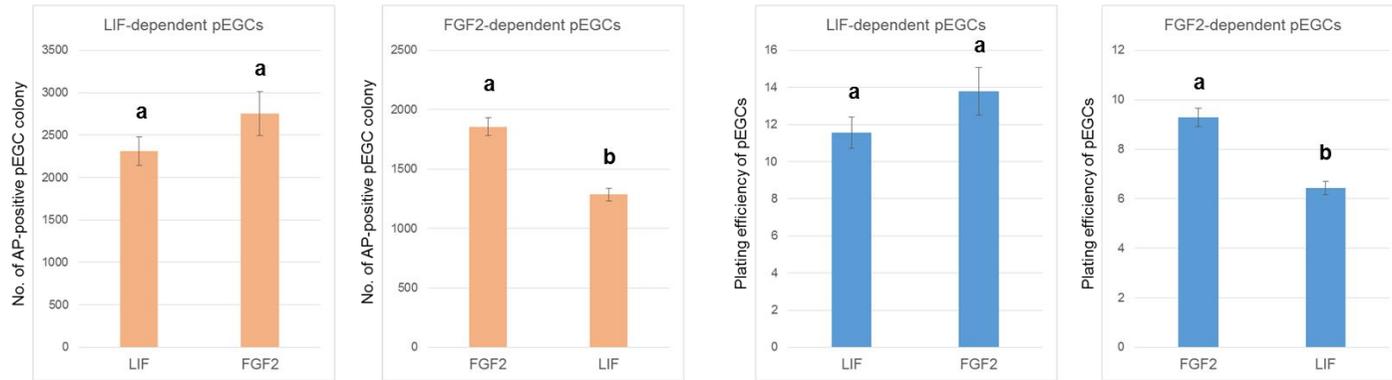
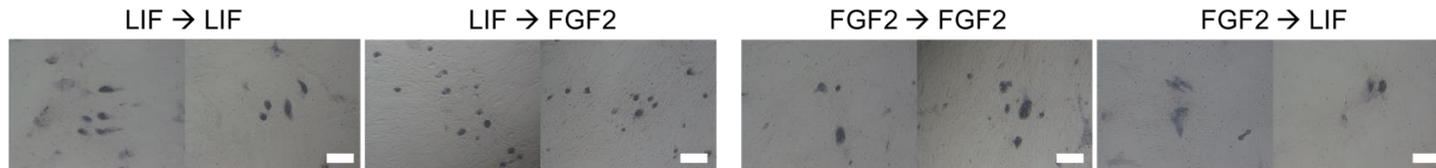
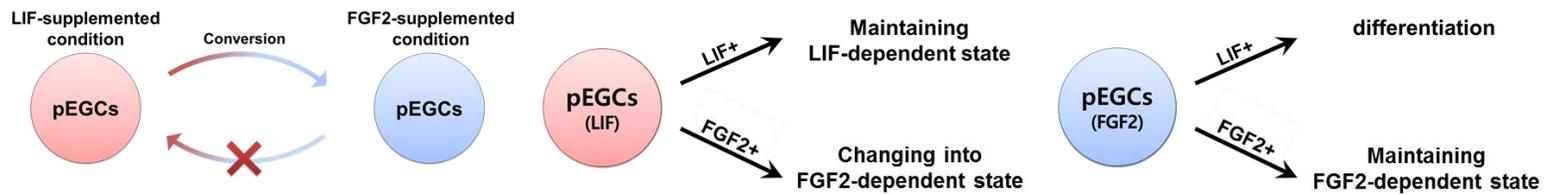
Figure 9. Analysis of ERK signaling in FGF2–EGCs.

To examine activity of FGF signaling in FGF–EGCs, western blotting for ERK1/2 was performed. Before proteins were collected, FGF2 were withdrawal from EGC culture media for 24 hrs. Then, EGCs were treated by FGF2 with various duration (15 min ~ 6 hrs). Finally, ERK1/2 and phosphorylated ERK1/2 were detected by western blotting with specific antibodies.

## LIF-EGCs were irreversibly converted into FGF2-EGCs by treating FGF2

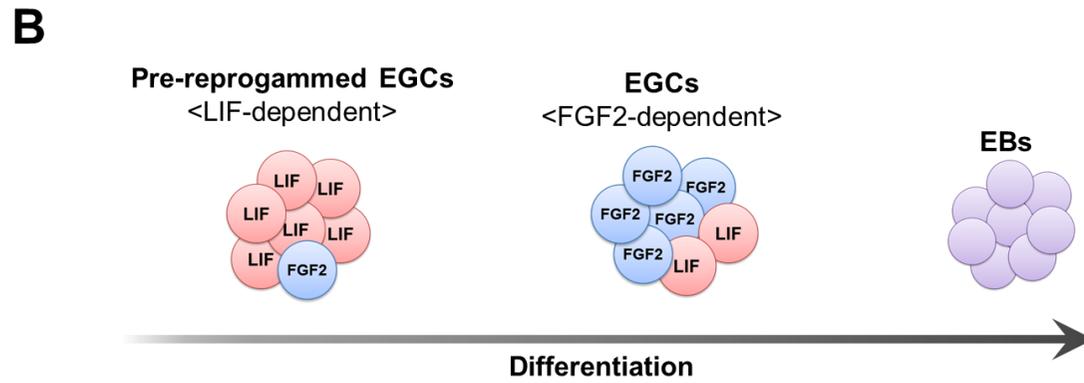
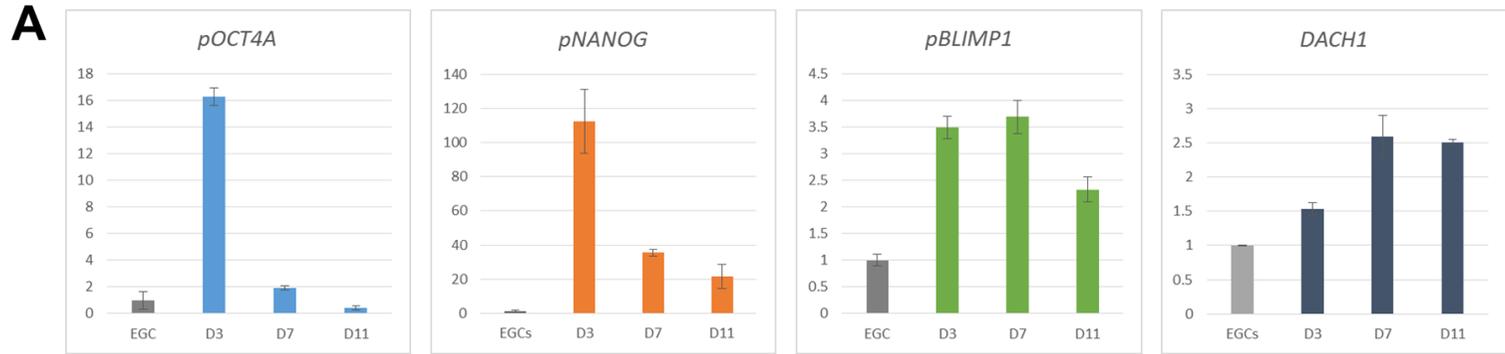
Previous data showed that two lines in different media have differences in aspects of gene expression. So, it was tested whether both cell lines could be converted each other. During subculture,  $2 \times 10^4$  LIF-EGCs were transferred into LIF- or FGF2-contained media and FGF2-EGCs were also subcultured in the same method. Interestingly, although plating efficiency of LIF-EGCs was similar in both conditions, plating efficiency of FGF2-EGCs was decreased when cultured with LIF as measured after AP staining (Fig 10A). AP staining showed FGF2-EGCs cultured with LIF have weak AP activity compared with other groups and displayed abnormal morphologies, which means FGF2-EGCs were differentiated in LIF-contained media (Fig 10B). These data indicated that LIF-dependent cells could be converted into FGF-dependent state but FGF2-dependent cells were differentiated in response of LIF (Fig 10C). So, if differentiation was occurred, it was examined whether LIF-EGCs were differentiated via FGF2-dependent stage or directly differentiated into somatic cells. To prove this question, genes which were differentially expressed between LIF- and FGF2-EGCs were selected, and expression level was analyzed during differentiation process. Surprisingly, expression of *OCT4*, *NANOG*, and *BLIMP1* were immediately increased in early stage of differentiation, and were gradually declined as differentiation was progressed (Fig 11A). Taken together, LIF-EGCs is intermediated state

between PGCs and FGF2-EGCs, and this sequential process from LIF-EGCs to differentiated cells is irreversible (Fig 11B).

**A****B****C**

**Figure 10. Analysis for plating efficiency of pig EGCs.**

It was tested whether both cell lines could be converted each other. During subculture,  $2 \times 10^4$  LIF-EGCs were transferred into LIF- or FGF2-contained media and FGF2-EGCs were also subcultured in the same method. (A) Then, plating efficiency of EGCs was measured by counting AP-positive EGC colonies. (B) AP staining showed FGF2-EGCs cultured with LIF have weak AP activity compared with other groups and displayed abnormal morphologies. (C) These data indicated that LIF-dependent cells could be converted into FGF-dependent state but FGF2-dependent cells were differentiated in response of LIF. Scale bar =  $400 \mu\text{m}$



**Figure 11. Changes of gene expression during differentiation of LIF–EGCs.**

Genes which were differentially expressed between LIF– and FGF2–EGCs were selected, and expression level was analyzed during differentiation process. (A) Expression of *OCT4*, *NANOG*, and *BLIMP1* were immediately increased in early stage of differentiation, and were gradually declined as differentiation was progressed. (B) Taken together, LIF–EGCs is intermediated state between PGCs and FGF2–EGCs, and this sequential process from LIF–EGCs to differentiated cells is irreversible.

## 4. Discussion

### Derivation of pig embryonic stem cells from preimplantation embryos

Because pluripotent cells have great potential as a cell source, research in this area has focused on embryonic carcinoma cells to iPSCs (Martin and Evans, 1974; Takahashi and Yamanaka, 2006). PSC research began in 1981 by the establishment of mouse ESCs and was accelerated by the establishment of human ESCs and iPSCs in 1998 and 2006, respectively (Evans and Kaufman, 1981; Takahashi and Yamanaka, 2006; Thomson et al., 1998). The purpose of PSC research in humans and mice includes the elucidation of basic cellular mechanisms contributing to the maintenance of pluripotency. In humans, applications exist in cell therapies. However, in domestic animals, the research aim is to create an indefinite cell source for transgenic animals used as bioreactors and tissue engineering materials as well as preliminary studies for human research (Keefer et al., 2007). Because of the physiological and immunological similarities that exist between pigs and humans, porcine pluripotent cell lines have been identified as important candidates for preliminary studies on human disease (Brevini et al., 2007; Hall, 2008; Houdebine, 2009). Therefore, many research groups have attempted to derive pig PSCs from early embryos for a long time (Ezashi et al., 2009; Kues et al., 2013; Park et al., 2013a; Piedrahita et

al., 1990b; Son et al., 2009; Yang et al., 2009).

In studies of pig ESCs, researchers tried to establish stem cells based on teratocarcinoma culture methods as mouse studies did. Various culture materials including serums (fetal bovine serum and calf serum), feeder cells (buffalo liver cells, mouse embryonic fibroblasts, pig embryonic fibroblasts and pig uterine epithelial cells) and conditioned media were tested for maintaining pluripotent ICM *in vitro* (Anderson et al., 1994; Piedrahita et al., 1990a, b; Talbot et al., 1993b). In addition, various cytokines and culture components have been tested, however, cultured ICM lost their own features and in turn differentiated during prolonged culture (Alberio et al., 2010; Puy et al., 2010; Vassiliev et al., 2010). Instead of pluripotent cells, during *in vitro*-culture, multipotent stem cells, so called ES-like cells, have been spontaneously obtained by several groups (Brevini et al., 2010; Cheong et al., 2015; Kim et al., 2010b; Uh et al., 2014). To date, it is still endeavoring to establish authentic pig ESCs which have *in vivo* and *in vitro*-developmental competency.

So, it is worth to research various stem cell lines derived from different origins for deriving genuine pig PSCs. Established pig ES-like cell lines have shown some common features. They express pluripotent markers

such as AP, OCT4, SOX2 and NANOG, and have *in vitro*-differentiation ability, but not *in vivo*-developmental competency (chimera and teratoma formation ability) (Brevini et al., 2010; Cheong et al., 2015; Kim et al., 2010b; Uh et al., 2014). In consistent with previous studies, my cell lines have limitations in differentiation ability albeit pluripotent genes were expressed. However, it is interesting to note that expression of pluripotent genes were maintained by activation of FGF2 signaling. In pig reprogramming studies showed that inhibitors of ERK and GSK signaling repressed endogenous pluripotent genes during reprogramming of pig cells (Choi et al., 2016; Petkov et al., 2014). And considering that pig is outbred (Only three mouse strains including 129, C57BL/6 and BALB/C which were highly inbred are permissive strain (Hanna et al., 2010b)), pig might be closed to non-permissive species rather than permissive species. Based on data of pig ES-like cells, in pig as a non-permissive species, FGF2 signaling play an important role in generating genuine pig PSCs.

### **Derivation of pig embryonic germ cells from fetal gonadal primordial germ cells**

PGCs as an alternative cell source can be used for deriving PSCs. When cultured with feeder cells and adequate cytokines, PGCs can be reprogrammed into PSCs, named EGCs (Dolci et al., 1991; Donovan et al.,

1986; Godin et al., 1991; Matsui et al., 1991). Established mouse EGCs possess similar features with ESCs in physiological and developmental aspects. They express pluripotent marker genes such as Oct4, Sox2, Nanog and SSEA1, and have developmental competency *in vitro* (embryoid body formation) as well as *in vivo* (teratoma and chimera formation) (Matsui et al., 1992; Resnick et al., 1992). In addition, recent data indicated that there's close similarities between EGCs and ESCs in terms of genetics and epigenetics (Choi et al., 2017). In human, EGCs could be derived from gonads of aborted fetuses through culturing with feeder cells and LIF, FGF2 and FSK (Liu et al., 2004; Shamblott et al., 1998; Turnpenny et al., 2003). Human EGCs expressed several pluripotent markers such as OCT4, SSEA1 and SSEA3/4 and have *in vitro* developmental competency. However, because of ethical problems for using human fetuses, research on human germ cells is not as well performed (Turnpenny et al., 2006).

During *in vitro*-reprogramming of mouse PGCs, stem cell factor (SCF, also known as c-Kit ligand or Steel factor), although PGCs undergo apoptosis in a few days during *in vitro* culture, prevents apoptosis and promotes viability and proliferation rate of germ cells together with LIF (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). FGF2 up-regulates expression of *Dhx38*, *cMyc* and *Klf4* known as targets of *Blimp1*

via down-regulation of Blimp1, facilitates reprogramming of PGCs into EGCs (Durcova-Hills et al., 2008). Finally, fully-reprogrammed mouse EGCs cultured with only LIF. Similar with mouse PGCs, pig PGCs are converted into EGCs by FGF2-mediated reprogramming (Zhang et al., 2016). When FGF2 was withdrawal during primary culture of PGCs, number of primary EGC colonies was dramatically reduced (Lee and Piedrahita, 2000). Along with FGF2, membrane-bound/soluble SCF and LIF have pivotal roles in survival and proliferation of pig PGCs during primary culture (Durcova-Hills et al., 1998). Without SCF, *in vitro*-survival rate of PGCs were reduced and, during extended culture, EGC colonies were disappeared (Lee and Piedrahita, 2000).

Unlike mouse study, pig migrating PGCs couldn't be converted into EGCs (Petkov et al., 2011). It has been proven hard to derive mouse EGCs with older PGCs (over dpc 12.5) (Labosky et al., 1994), it might be caused by differences in the timing of epigenetic changes between mouse and pig PGCs (Hyldig et al., 2011a). In contrast with pig putative ESCs, pig EGCs resembled mouse ES-like morphology showing well-delineated and multilayered dome-shaped colony, and tolerated single-cell dissociation by trypsinization (Lee and Piedrahita, 2000). Pig EGC lines expressed several germ cell/pluripotent marker genes such as BLIMP1, OCT4, SOX2, NANOG,

SSEA1 and SSEA4. When cultured in suspension, these cells formed embryoid bodies and differentiated into three germ layers *in vitro*, but couldn't develop into teratomas in immunodeficient mice. In spite of lack of teratoma formation ability, pig EGCs could develop into chimeric piglet via incorporation with host embryos by blastocyst injection (Mueller et al., 1999; Piedrahita et al., 1998).

After establishing pig ESCs, I attempted to derive pig ESCs from gonadal PGCs. Pig EGCs derived in this study showed similar features with previous studies in terms of morphology, marker expression and developmental competency. And two independent cell lines were obtained by culturing with LIF- or FGF2-supplemented media. Albeit roles of cytokines during *in vitro*-reprogramming of pig PGCs have been studied, effects during extended culture have not been carried out. So, comparative analysis of pluripotent genes in both cell lines were performed. Notably, like a pig ES cells, pluripotent genes were more highly expressed in FGF2-EGCs than LIF-EGCs, which means FGF2 facilitates expression of pluripotent genes in pig EGCs. And according to qPCR data, it was verified that FGF supplement was more suitable for extended culture of pig EGCs. Taken together, treatment of single cytokine, FGF2, are sufficient for maintaining pig EGCs and FGF signaling plays a pivotal role in facilitating

pluripotency in various pig stem cells.

### **Reprogramming of pig PGCs is achieved in a stepwise manner**

In murine post-implantation embryo at dpc 5.5, PGCs arise from proximal posterior epiblast by stimulation of BMP family from extraembryonic ectoderm and visceral endoderm (Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). In precursor of PGCs, BMP signaling stimulates expression of *Blimp1*, which induces repression of somatic program and facilitates expression of pluripotent genes and epigenetic reprogramming (Durcova-Hills et al., 2008; Ohinata et al., 2005). Resulting PGCs migrate from posterior endoderm of the yolk sac at dpc 7.5 to genital ridge at dpc 12.5, and primitive gonads and fetal ducts in turn develop into sex organs (Molyneaux et al., 2001). Apposing migration, dozens of cells actively proliferate into thousands and epigenetic remodeling including reconstruction of DNA methylation, imprint erasure and X-chromosome reactivation are occurred (reviewed in (Ewen and Koopman, 2010)). And after arrival at genital ridges, gender-specific imprinting patterns are re-establishment (Lees-Murdock and Walsh, 2008). Similar with mouse, porcine PGCs arise in endoderm and mesoderm beside stalk of the yolk sac and allantois during gastrulation (embryonic day (E) 13–14) (Vejlsted et al., 2006). Pig PGCs are starting to migrate from endoderm and mesoderm and

arrive at genital ridges in E22 (Hyldig et al., 2011b). At around E30, primitive gonads develop into sex organs (Zhang et al., 2016). Global DNA demethylation involving repetitive elements as well as erasing gender-specific imprints are occurred during migration (Hyldig et al., 2011a; Hyldig et al., 2011b). And gender-specific imprinting patterns are re-established between E25–31 (Hyldig et al., 2011a; Petkov et al., 2009).

In this study, gender-specific imprinting pattern of pig PGCs was reprogrammed into mono-allelic pattern during *in vitro*-culture. This process make EGCs suitable for nuclear transfer (Li et al., 2014a), while gonadal PGCs are inadequate for nuclear donors because of unique epigenetic features (Yamazaki et al., 2005; Yamazaki et al., 2003). Interestingly, re-establishment process of imprints in LIF-EGCs was less completed than FGF2-EGCs showing incomplete hemi-methylation pattern, which was tempting to speculate that FGF-EGCs are more reprogrammed than LIF-EGCs. In consistent with imprints, expression of DNMTs were more highly expressed in FGF2-EGCs than LIF-EGCs. As shown Fig 8C, plating efficiency test indicated that FGF2-EGCs have upper state than LIF-EGCs and conversion from LIF-EGCs to FGF-EGCs was irreversible. In addition, it has been assessed that SSEA1 was expressed in pig PGCs and EGCs at early passages (Dong et al., 2014; Lee and Piedrahita, 2000; Lee et

al., 2000a; Mueller et al., 1999; Petkov and Anderson, 2008). Expression of SSEA1 was gradually decreased and disappeared during *in vitro*-culture and, finally, only SSEA4 was expressed in pig EGCs (Petkov and Anderson, 2008; Petkov et al., 2011). And efficiency of nuclear transfer using late passage-EGCs was higher than using early passage-EGCs (Li et al., 2014a), it is enticing to think that epigenetic reprogramming was occurred during *in vitro*-culture apposing surface markers were switched. During differentiation of LIF-EGCs, genes which highly expressed in FGF2-EGCs were up-regulated and in turn gradually decreased, which means LIF-EGCs were differentiated via FGF2-EGCs state in stepwise manner. Taken together, LIF-EGCs and FGF2-EGCs represent intermediated state of EGCs and complete reprogrammed EGCs, respectively. And it is verified that reprogramming of pig PGCs into EGCs is conducted in stepwise manner.

### **Candidate model for *in vitro*-reprogramming of pig primordial germ cells**

Based on my data and previous observations, I could establish candidate model for *in vitro*-reprogramming of pig PGCs (Fig 12). Reprogramming of pig gonadal PGCs is progressed in stepwise manner, from PGCs via intermediated EGCs to EGCs. Gonadal/cultured PGCs are positive for AP and SSEA1, and unsuitable for nuclear transfer because of gender-specific imprinting pattern. As reprogramming is progressed, PGCs lose

expression of SSEA1 and gender-specific imprinting pattern. From this stage, PGCs can be used as donor cells for nuclear transfer as previously reported (Ahn et al., 2010). Finally, through the activation of FGF2 signaling, PGCs are converted into EGCs acquiring expression of SSEA4 and mono-allelic imprint pattern. This model would provide insight of reprogramming of pig PGCs and EGCs and aid in the understanding of germ cell biology.

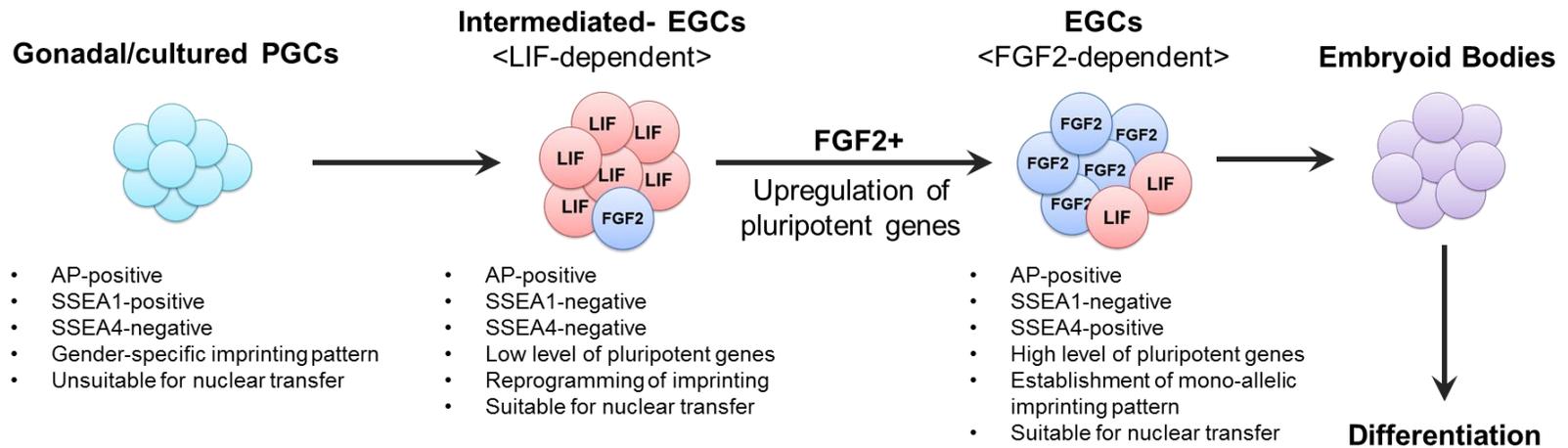


Figure 12. Candidate model for *in vitro*–reprogramming of pig primordial germ cells.

Reprogramming of pig gonadal PGCs is progressed in stepwise manner, from PGCs via intermediated EGCs to EGCs. Gonadal/cultured PGCs are positive for AP and SSEA1, and unsuitable for nuclear transfer because of gender–specific imprinting pattern. As reprogramming is progressed, PGCs lose expression of SSEA1 and gender–specific imprinting pattern. From this stage, PGCs can be used as donor cells for nuclear transfer. Finally, through the activation of FGF2 signaling, PGCs are converted into EGCs acquiring expression of SSEA4 and mono–allelic imprint pattern.

## 5. Conclusion

Here, I was able to successfully derive pig stem cells from preimplantation embryos and fetal gonads. Pig ES cells were stably maintained over an extended period expressing pluripotent markers, and could be differentiated into three germ layers. Pig EGCs derived from fetal gonads, similar with ES cells, were stably maintained and have *in vitro*-differentiation ability. Notably, two independent cell lines cultured with LIF- or FGF2-supplemented media were obtained. As a result of molecular analysis, it was verified that LIF- and FGF2-EGCs represented intermediated state of EGCs and complete reprogrammed EGCs respectively, and reprogramming of pig gonadal PGCs is progressed in stepwise manner, from PGCs via intermediated EGCs to EGCs. Taken together, although *in vivo*-developmental competent PSCs could not be derived, it was assessed that pig might be closed to non-permissive species rather than permissive species, and FGF2 signaling play an important role in generating genuine pig PSCs. Pig pluripotent stem cells could be useful candidates for preliminary studies of human disease as well as a source for generating transgenic animals.

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## CHAPTER 4

Reactivation of endogenous genes and  
epigenetic remodeling are barriers for  
generating transgene–free induced  
pluripotent stem cells in pig

## 1. Introduction

Pluripotent stem cells (PSCs) are a promising tool for human regenerative medicine, and in domestic animals, as useful tools for producing transgenic and disease model animals. PSCs have two distinct grades of pluripotency, “naïve” and “primed” states, based on developmental competence (Nichols and Smith, 2009). Naïve PSCs, represented by mouse embryonic stem cells (ESCs) and embryonic germ cells (EGCs), are characterized by dome-shaped colony morphologies, activation of LIF signaling, and two active X chromosomes in females. By contrast, primed PSCs, including epiblast stem cells (EpiSCs), are defined by flattened colony morphologies and activated FGF signaling pathways. Compared with the primed state, naïve PSCs have developmental and functional ground states showing contributions to blastocyst chimeras and higher transgenic efficiency (Buecker et al., 2010; Hanna et al., 2010a). For these reasons, many research groups have attempted to generate naïve PSCs of domestic animals (especially pig) for the efficient production of transgenic animals (Fujishiro et al., 2013; Kwon et al., 2013; Telugu et al., 2011; Zhang et al., 2015b).

PSCs have been derived from early epiblasts in preimplantation

blastocysts, which are known as ESCs. Induced pluripotent stem cells (iPSCs), as an alternative source of ESCs, can be generated by Yamanaka's factors (Takahashi and Yamanaka, 2006). The acquisition of pluripotency in fibroblasts is accomplished by genetic and epigenetic events termed initiation, maturation, and stabilization (Samavarchi–Tehrani et al., 2010). The initiation of reprogramming is defined by mesenchymal–to–epithelial transition (MET), in which epithelial–specific genes are up–regulated and *Tgfb1* is downregulated by expression of reprogramming factors and BMP signaling (Li et al., 2010; Samavarchi–Tehrani et al., 2010). Subsequently, *Nanog* and *Sall4* induced by *Sox2* activate endogenous pluripotent networks, and pluripotent circuitry is stabilized via epigenetic remodeling such as DNA methylation, histone modification, and X chromosome reactivation (Buganim et al., 2012; Polo et al., 2012). Finally, the reprogrammed cells can be maintained without ectopic expression of transgenes, which indicates that endogenous pluripotent network are fully activated and stabilized (Maherali et al., 2007; Wernig et al., 2007).

As described above, several genetic and epigenetic changes occur during nuclear reprogramming from somatic cells to iPSCs. Achieving faithful pluripotency is required to overcome epigenetic and physiological obstacles such as the epigenetic memory of somatic cells (Kim et al., 2010a; Polo et

al., 2010), MET (Li et al., 2010), repressive chromatin (Huangfu et al., 2008), and apoptosis and cell cycle arrest (Kawamura et al., 2009; Li et al., 2009). However, if these barriers are not overcome, silencing of transgenes, epigenetic remodeling, and lack of *Nanog* expression occur, resulting in partial reprogramming of iPSCs (pre-iPSCs) (Okita et al., 2007; Silva et al., 2008). Although various research groups have attempted to generate pig iPSCs (piPSCs), authentic iPSCs have not been obtained, instead showing features of incomplete reprogramming including dependence on transgene expression, epigenetic remodeling, reactivation of pluripotent genes, and chimera formation (Du et al., 2015a; Esteban et al., 2009; Ezashi et al., 2009; Hall et al., 2012; Wei et al., 2015; Zhang et al., 2014b). This may be due to differences in the molecular mechanisms during embryo development between mouse and pig (Alberio and Perez, 2012). It is important to investigate pre-iPSCs to improve and develop reprogramming methods by understanding reprogramming mechanisms in pig.

Pig has been identified as a valuable candidate model animal for human disease models and xenotransplantation, because of physiological and anatomical similarity between human and pig. In addition, the establishment of authentic iPSCs, which can maintain pluripotency without ectopic expression and have in vivo differentiation ability, is important in pigs for

medical and industrial usages. Here, I derived several pig iPSC lines by introducing Yamanaka's factors using drug-inducible vectors. These cell lines were incompletely reprogrammed, not meeting the criteria of PSCs such as pluripotent gene expression. Accordingly, it was explored the state where pig pre-iPSCs committed to pluripotency through genetic and epigenetic analyses. It was verified that failures of MET and epigenetic remodeling were occurred in pig pre-iPSCs during reprogramming. Expression of exogenous genes could not sufficiently activate the essential endogenous genes for reprogramming into pluripotency in pig. Consequently, further in-depth analyses of pig-specific signaling pathways are required to establish authentic porcine embryonic stem cells and obtain completely reprogrammed iPSCs.

## 2. Materials and methods

### Animal care

The care and experimental use of pigs and mice was approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-140501-4 for MEFs isolation and SNU-140328-2 for PEF isolation). A pregnant sow was purchased from animal farm. The sow was taken care exclusively at farm and sacrificed after 27 days from artificial insemination at slaughterhouse (Hanbo, Korea) approved by Korean government. Pregnant ICR mice were purchased from SAMTACO BIO Inc., Korea. The mice were taken care according to standard protocol of Institute of Laboratory Animal Resources and sacrificed by cervical dislocation after anesthesia.

### Generation and culture of porcine induced pluripotent stem cells (iPSCs)

Pig fetal fibroblasts (PFFs, mixed breed) and mouse embryonic fibroblasts (MEFs) were obtained from approximately 27-day-old and 14-day-old fetuses after artificial insemination, respectively. The head, limbs, and internal organs were removed. The remaining tissue was minced and cultured in DMEM (Welgene, Korea) supplemented with 10% fetal bovine

serum (FBS; collected and processed in the United States; Genedepot, TX, USA),  $1 \times$  glutamax (Gibco), 0.1 mM  $\beta$ -mercaptoethanol (Gibco), and  $1 \times$  antibiotic/antimycotic (Gibco). Pig iPSC derivation was conducted using lentiviral vectors with inducible systems containing human OCT4, SOX2, KLF4, and MYC. Lentiviral vector production and transduction were performed as described previously (Choi et al., 2013). Five plasmids were used for the production of lentiviral vectors: FUW-tetO-hOCT4, FUW-tetO-hSOX2, FUW-tetO-hKlf4, FUW-tetO-hMYC, and FUW-M2rtTA. Cultured female PFFs were infected with lentiviral vectors for 48 hours. Infected PFFs were transferred onto feeder cells composed of mitotically inactivated MEFs and cultured with reprogramming media for 2 weeks. The reprogramming media contained DMEM (Welgene) supplemented with 15% FBS, 2 mM glutamax, 0.1 mM  $\beta$ -mercaptoethanol,  $1 \times$  MEM non-essential amino acids (Gibco),  $1 \times$  antibiotic/antimycotic, 2 ng/ml doxycycline (dox), and 1000 unit/ml Leukemia inhibitory factor (LIF; Millipore, MA, USA). Two weeks post-infection, primary colonies of piPSCs were stained with AP live stain kit as described below, and AP-positive colonies were selected for further analyses and culture. Established pig iPSCs were cultured under culture media supplemented with 1000 unit/ml LIF or 1000 unit/ml LIF, 3  $\mu$ M CHIR99021 (Cayman chemical, MI, USA) and 1  $\mu$ M PD0325901 (Selleckchem, TX, USA; inhibitors for GSK3 and MEK/ERK respectively; 2i) or 10 ng/ml basic fibroblast growth factor (FGF2; R&D Systems, MN, USA).

Media were changed every day and all cells were cultured under humidified conditions with 5% CO<sub>2</sub> at 37 °C. When colonies of pig iPSCs were grown sufficiently for passaging, cells were subcultured into new feeder cells containing mitomycin-C-treated (Roche, Switzerland) MEFs.

### **Embryoid body (EB) formation and in vitro differentiation**

To evaluate the in vitro differentiation ability, embryoid bodies were generated from pig iPSCs. Cultured pig iPSCs were dissociated into single cells using 0.25% trypsin/EDTA solution (Wetgene) and cultured in petri dishes without cytokines for 5 days. After suspension culture, dissociated cells were aggregated and formed embryoid bodies. Cultured embryoid bodies were seeded on 0.1% gelatin-coated plates and cultured for 2–3 weeks with DMEM containing 15% FBS. After 2–3 weeks, differentiated cells were fixed with 4% paraformaldehyde and analyzed by immunostaining with the following antibodies: Neurofilament (ectoderm), Vimentin (mesoderm), and Cytokeratin 17 (endoderm), as described below.

### **Alkaline phosphatase (AP) staining**

Two alkaline phosphatase (AP) staining kits were used for staining both fixed and live cells. For the fixed cells, cells were fixed with 4%

paraformaldehyde for 30 min. After washing, fixed cells were stained with a solution containing nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) stock solution (Roche) in a buffer solution for 30 min at room temperature. Cells were then examined under an inverted microscope. AP staining of live cells was performed using the AP live stain kit (Molecular probes, OR, USA) according to the manufacturer's instructions. The stained cells were examined under a fluorescence microscope and AP-positive colonies were selected for further cell culture and analyses.

### **Immunocytochemistry (ICC) analyses**

ICC analyses were performed according to methods described in Chapter 3. Images of stained cells were captured using a LSM 700 Laser Scanning Microscope (Carl Zeiss, Germany) and processed with the ZEN 2012 Light Edition program (Carl Zeiss).

### **Flow cytometric analyses**

To verify expression of pluripotent genes in pig iPSCs cultured in media supplemented with LIF, LIF + 2i, or FGF2, flow cytometric analyses was performed. Dissociated iPSCs were fixed with 4% paraformaldehyde.

The fixed cells were permeabilized in 0.1% Triton-X 100 (Sigma Aldrich) for 5 min and then incubated with 10% goat serum (blocking solution). The cells were then incubated with primary antibodies at 4°C overnight. The primary antibodies used were as follows: OCT4 (Santa Cruz Biotechnology, 1:200), SOX2 (Millipore, 1:200), and NANOG (Santa Cruz Biotechnology, 1:200). After incubation with primary antibody, the cells were treated for 3 h at room temperature with Alexa Fluor-conjugated secondary antibodies. The stained cells were analyzed using flow cytometry (FACSCalibur) and Cell Quest software (Becton Dickinson, NJ, USA). The resulting data were processed using FlowJo software (Tree Star Inc., OR, USA).

### **Polymerase chain reaction (PCR) amplification**

To determine whether viral transgenes were inserted into the genome, gDNA were PCR-amplified with transgene-specific primers listed in Table 10. Genomic DNA was extracted by the G-spin™ Total DNA Extraction Kit (iNtRON, Korea). Amplifications were performed using 2x PCR Master mix solution (iNtRON) containing 1 pmol of each primer set and 10 ng gDNA in a 10 µl reaction volume. PCR reactions were performed in a thermocycler under the following conditions: 94°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s (annealing temperatures depended on each primer set), and extension at 72°C for 30 s,

with a final extension at 72°C for 7 min. Amplified PCR products were visualized using electrophoresis on 1% agarose gel stained with ethidium bromide.

Table 10. Primers for the detection of transgene insertion in gDNA.

Genes		Sequences	size
Reprogramming factor	<i>hOCT4</i>	5'– CCCCTGTCTCTGTCCACCACT –3'	148
		5'– CCACATAGCGTAAAAGGAGCA –3'	
	<i>hSOX2</i>	5'– ACTTCACATGTCCCAGCACT –3'	180
		5'– CATAGCGTAAAAGGAGCAACAT –3'	
	<i>hKLF4</i>	5'– GACCACCTCGCCTTACACAT –3'	137
		5'– CCACATAGCGTAAAAGGAGCA –3'	
<i>hMYC</i>	5'– CAGCTACGGAACTCTTGTGC –3'	125	
	5'– CCACATAGCGTAAAAGGAGCA –3'		
Reference gene	<i>ACTB</i>	5'– CCGGGACCTGACCGACTACC –3'	126
		5'– TCGAAGTCCAGGGCGACGTA –3'	

## Quantitative real-time polymerase chain reaction (qPCR)

To verify the gene expression level in pig iPSCs, qPCR was performed according to methods described in Chapter 3. Primer sets for qPCR are listed in Table 11.

Table 11. Primers for detecting pluripotent and reprogramming markers.

	Genes	Sequences	size
Reprogramming factor	<i>hOCT4</i>	5'– CCCCTGTCTCTGTCACT –3'	148
		5'– CCACATAGCGTAAAAGGAGCA –3'	
	<i>hSOX2</i>	5'– CATTAACGGCACACTGCCC –3'	132
		5'– CATAGCGTAAAAGGAGCAACAT –3'	
	<i>hKLF4</i>	5'– GACCACCTCGCCTTACACAT –3'	137
		5'– CCACATAGCGTAAAAGGAGCA –3'	
	<i>hMYC</i>	5'– CAGCTACGGAACCTTTGTGC –3'	125
		5'– CCACATAGCGTAAAAGGAGCA –3'	
	<i>OCT4a</i>	5'– CTTGGAGAGCCCTGGTTTTACT –3'	159
		5'– GCCAGGTCCGAGGATCAAC –3'	
<i>SOX2</i>	5'– CGGCGGTGGCAACTCTAC –3'	100	
	5'– TCGGACCACACCATGAAAG –3'		
<i>KLF4</i>	5'– GGACCACCTTGCCTTACACA –3'	146	
	5'– CTTTCCAGCTGGGTTCCCTCC –3'		
<i>MYC</i>	5'– GAAAAAGACGTGCTGCGGAA –3'	253	
	5'– CCAGCCAAGGTTGTGAGGTT –3'		
<i>NANOG</i>	5'– CATCTGCTGAGACCCTCGAC –3'	195	
	5'– GGGTCTGCGAGAACACAGTT –3'		
<i>REX1</i>	5'– TCTGAACCCCTCGTGAAGA –3'	100	
	5'– AGCTTGCTGTAAGCACCTGT –3'		
<i>TGFB1</i>	5'– CGTGCTAATGGTGGAAAGCG –3'	122	
	5'– AGAGCAATACAGGTTCCGGC –3'		
<i>CDH1</i>	5'– ATTCTGGGAGGCATCCTTGC –3'	117	
	5'– GTTGTCCCGGTGTCATCTT –3'		
<i>EPCAM</i>	5'– TGCTCTTTGAATGCGCTTGG –3'	172	
	5'– AGAGCCCATCGTTGTTCTGG –3'		
<i>OCN</i>	5'– CAGTGTAACCTGGAGGCGT –3'	104	
	5'– CCGTCGTGTAGTCTGTCTCG –3'		
<i>DPPA2</i>	5'– CTTCAAGAGCCGTTACCCT –3'	144	
	5'– GGCGAACCAACCTTCTGTA –3'		
<i>ACTB</i>	5'– GTGGACATCAGGAAGGACCTCTA –3'	131	
	5'– ATGATCTTGATCTTCATGGTGCT –3'		

## Genome methylation assay

To analyze methylation patterns in porcine OCT4, NANOG, and XIST promoter regions, genomic DNA of pig iPSCs was analyzed by bisulfite sequencing. Primers for bisulfite sequencing are listed in Table 12.

Table 12. Primers of bisulfite sequencing for promoters of *OCT4*, *NANOG* and *XIST*.

Promoters	sequences	size	CpGs
OCT4	5'- GAAGAGGGGTTTAATATTTGGTTTT -3'	281	15
	5'- CCACCCACTAACCTTAACCTCTAA -3'		
NANOG	5'- GGAGATTTAAAGGAGTTTTAGGTTAAGAAA -3'	500	10
	5'- TCTCCTCCAAATATTAATAATATCAAAAA -3'		
XIST	5'- TGGTTAAATGAGGTATTTGGA -3'	525	13
	5'- CCATAAAACATAACTAAAACTAAA -3'		
	5'- TTTGTTATATTGTTTGTGGAAAA -3'	429	
	5'- CCATAAAACATAACTAAAACTAAA -3'		

## **Karyotyping**

Karyotyping of cells using standard G-banding chromosome and cytogenetic analysis was performed at GenDix Laboratories (Korea; [www.gendix.com](http://www.gendix.com)).

## **Statistical analyses**

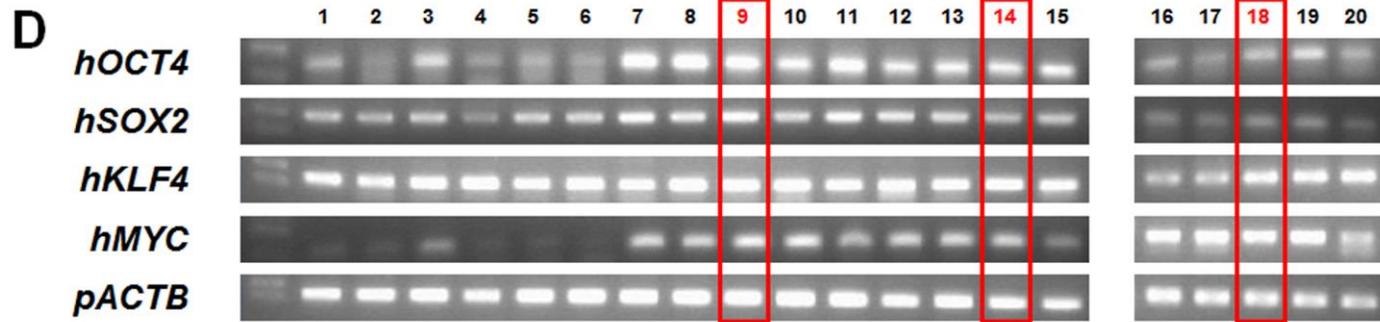
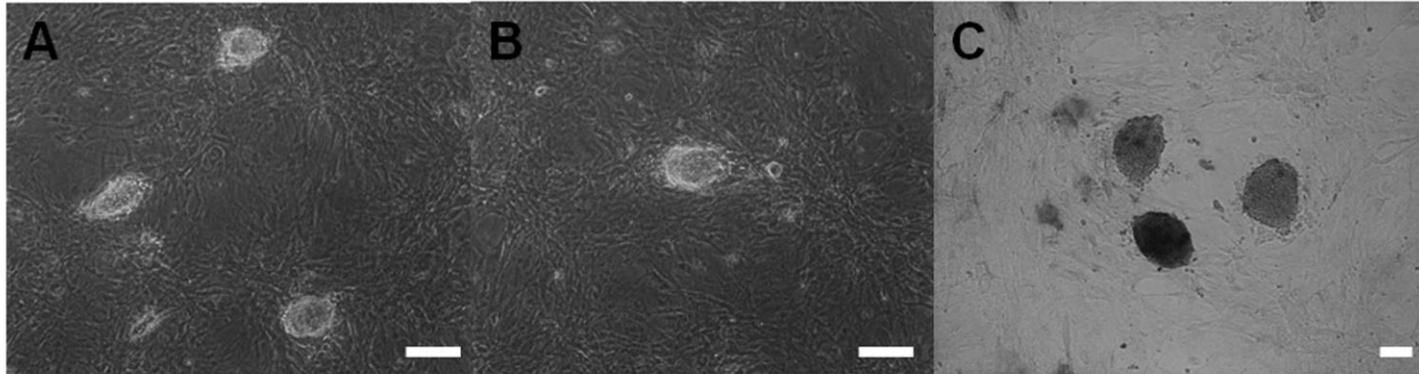
All gene expression data from qPCR analyses were statistically analyzed using GraphPad Prism 6 statistical software (GraphPad Software, CA, USA). Statistical differences between datasets were determined by one-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) tests. Differences were considered significant at  $P < 0.05$ .

### 3. Results

#### Derivation of pig iPSCs from PEFs with a drug-mediated inducible gene expression system

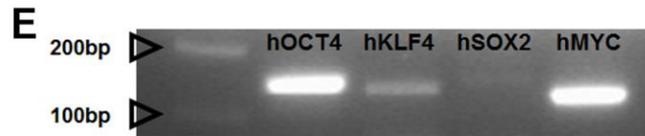
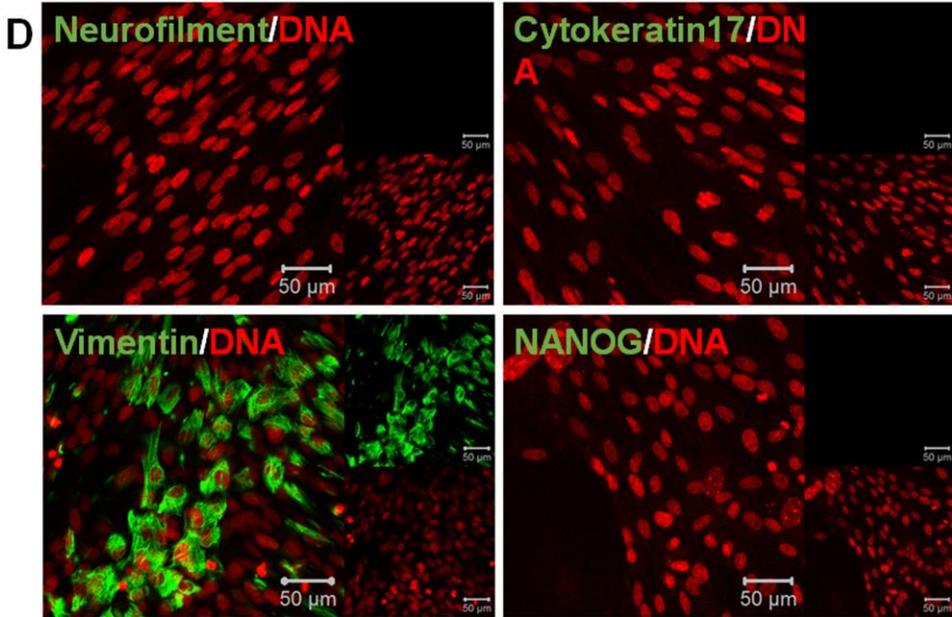
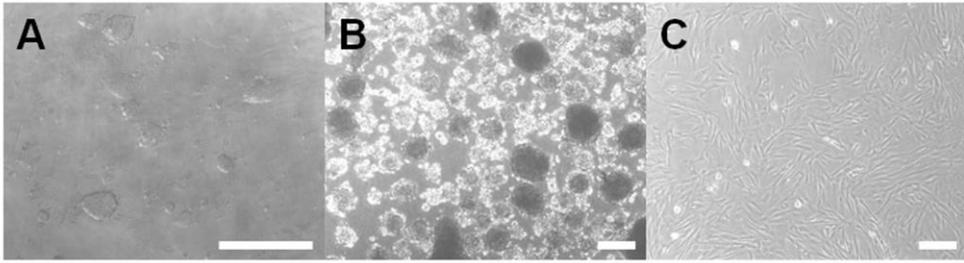
Because gene expression is easily regulated by drugs, drug-inducible vector systems have been used for iPSC studies on the generation of iPSCs, secondary iPS systems (Wernig et al., 2008), reprogramming mechanisms (Buganim et al., 2012; Polo et al., 2012), and inducing naïve PSCs in humans (Buecker et al., 2010; Hanna et al., 2010a). Thus, I used a doxycycline-mediated inducible gene expression system for generating pig iPSCs. Two weeks post-infection, several primary colonies were observed (Fig 13A and B). Because AP-positive and negative colonies were observed simultaneously, AP-positive colonies were selected using AP live stain kit under a fluorescence microscope (Fig 13C). Twenty colonies were picked to confirm the integration of four transgenes into the genome (Fig 13D). The 20 confirmed colonies showed various morphologies, including fussy and naïve types, and differing numbers of transgenes. Three cell lines were selected (one fussy type, piPS-9, and two naïve types, piPS-14 and 18) for further analyses (Fig 15A). When the cells were cultured in suspension, the cells aggregated and subsequently formed embryoid bodies (Fig 15A). These EBs differentiated into three germ layers when placed on gelatin-

coated plates. Expression of three germ layer markers including Cytokeratin17 (endoderm), Vimentin (mesoderm), and Neurofilament (ectoderm) was confirmed by immunostaining (Fig 15B). Interestingly, selected AP-negative colonies possessed no *in vitro* differentiation potential and reverted to fibroblasts with no hSOX transgenes (Fig 14). Of the three cell lines, piPS-14 showed better morphology and differential potential with larger EBs, and was selected for further analyses and culture. Additionally, it was confirmed that the integration of four transgenes into the genome (Fig 15C). The piPS-14 cell line could be stably maintained with normal karyotypes for > 50 passages (Fig 15D). Thus, I generated iPSCs with similar *in vitro* differentiation potential and self-renewal to typical pluripotent stem cells.



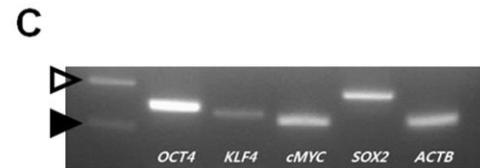
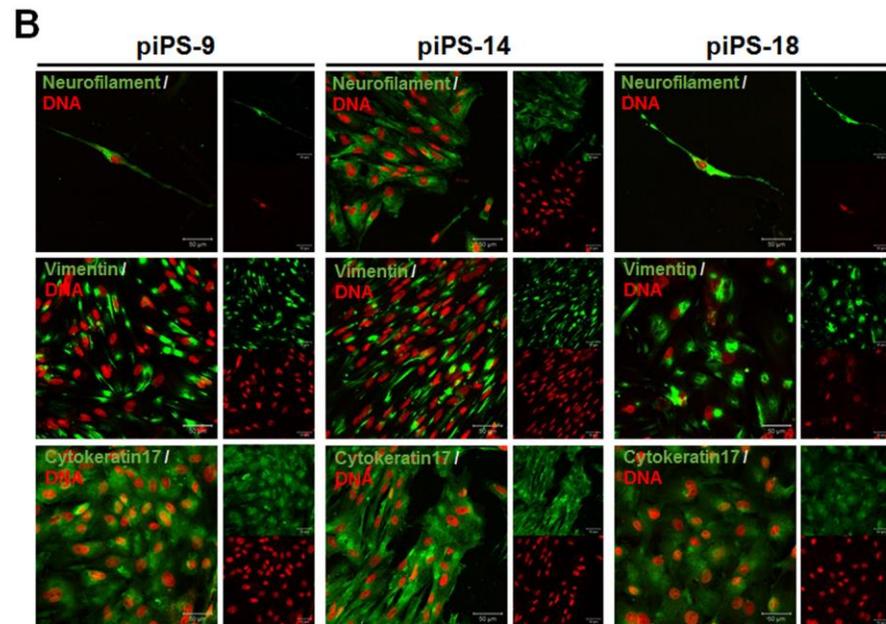
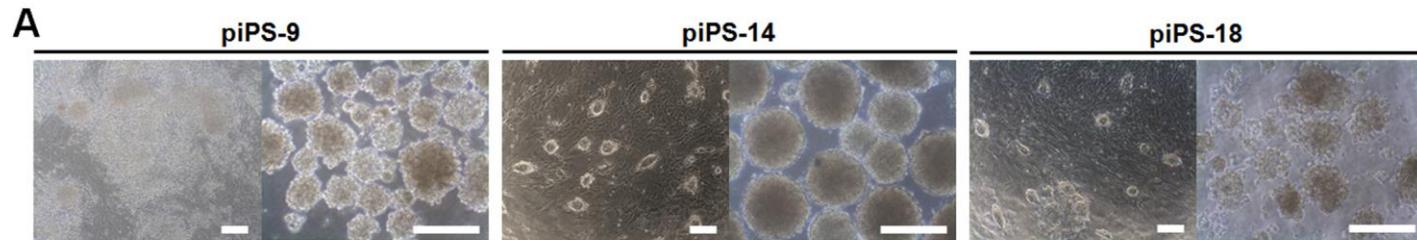
**Figure 13. Generation of pig induced pluripotent stem cells using drug-inducible vectors.**

A doxycycline-mediated inducible gene expression system was used for generating pig iPSCs. (A, B) Two weeks after infection, several primary colonies were observed. (C) Because AP-positive and negative colonies were simultaneously observed, AP-positive colonies were selected using AP live stain kit under a fluorescence microscope. (D) Twenty colonies were picked and confirmed the integration of four transgenes into genome. For further analyses, three cell lines were selected. The selected cell lines are indicated by a red box. Scale bar = 200  $\mu\text{m}$



**Figure 14. Characterization of the AP–negative cell line.**

AP–negative colonies were collected and cultured for characterization. (A) The cells have similar morphologies to AP–positive cell lines. (B) When cultured in suspension, the cells could form embryoid bodies. (C, D) However, the cells could not differentiate into three germ layers; only to mesodermal fibroblast–like cells, as determined by immunostaining. (E) The *hSOX2* was not integrated into the genome of cells. Scale bar = 200  $\mu\text{m}$  in A, B and C; 50  $\mu\text{m}$  in D.



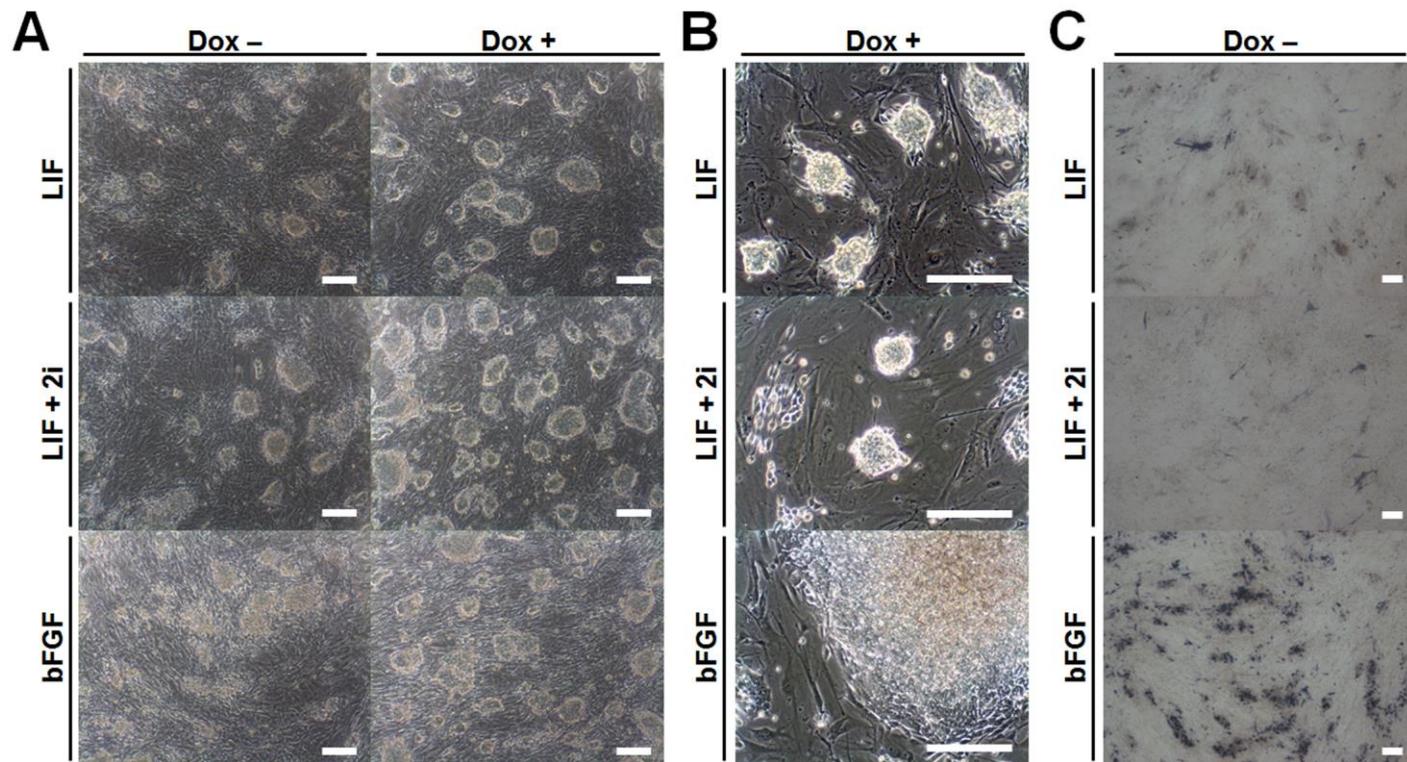
**Figure 15. Characterization of three selected iPSC lines.**

(A) Three cell lines, including one fussy type, piPS-9 and two naïve types, piPS-14 and 18, were selected. When the cells were cultured in suspension, the cells aggregated and subsequently formed embryoid bodies. (B) The formed EBs differentiated into three germ layers after being placed on gelatin-coated plates. Expression of three germ layer markers including Cytokeratin17 (endoderm), Vimentin (mesoderm), and Neurofilament (ectoderm) was confirmed by immunostaining. Of the three cell lines, piPS-14 (showing better morphology and differential potentials with larger EBs than other cell lines) was selected for further analyses and culture. (C) The integration of four transgenes into the genome was confirmed. Black and hollow arrows indicate 100 bp and 200 bp size markers, respectively. (D) The piPS-14 cell line could be stably maintained with a normal karyotype (36 + XX). Scale bar = 200  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in B.

## The pluripotent state of pig iPSCs could be modulated by culture conditions

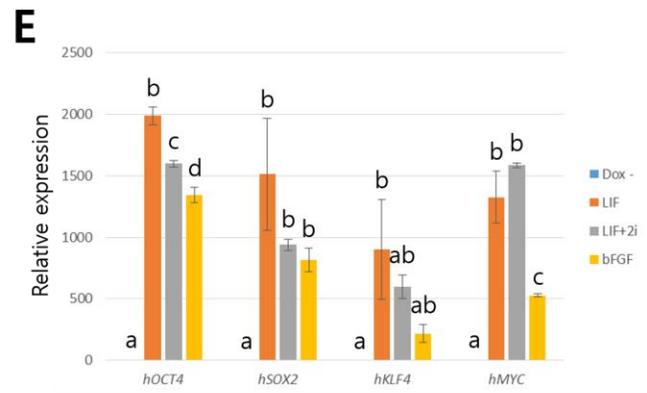
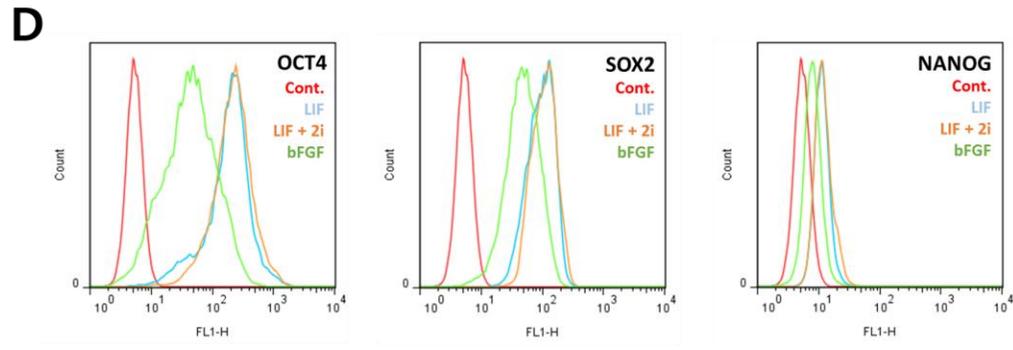
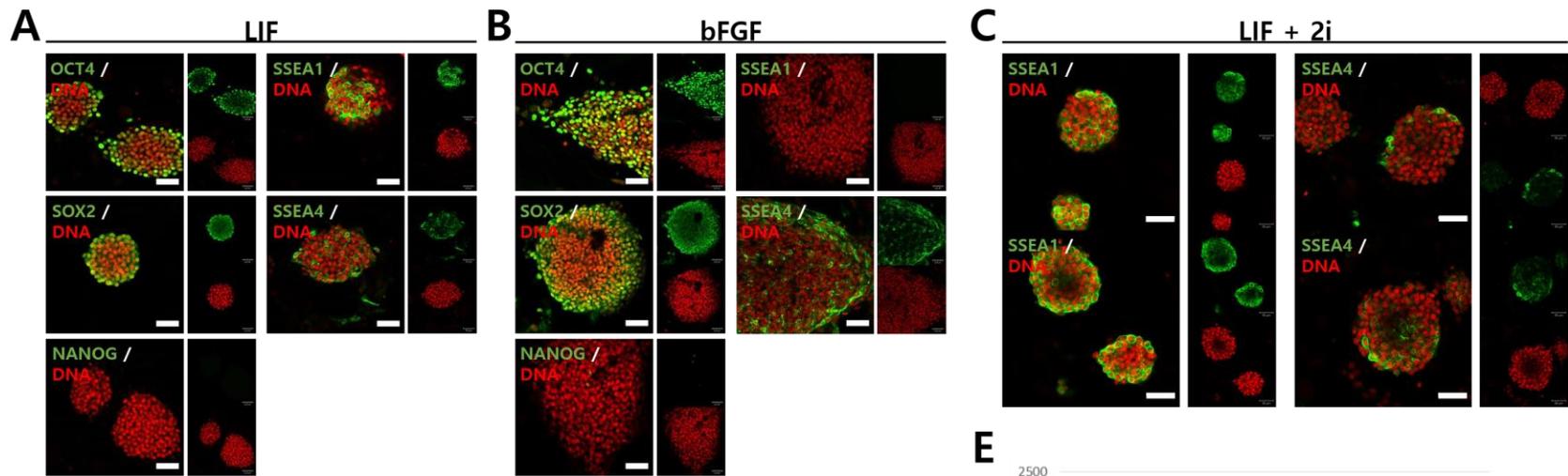
The generated piPS-14 cell line under LIF-supplemented conditions showed a naïve-like pluripotent state with short term subculture (3 days), single cell colonization, and dome-shaped morphology. It was explored whether the pluripotent state of pig iPSCs could be modulated based on culture conditions. The piPS-14 cells cultured in LIF conditions were transferred to two different media supplemented with LIF + 2i or FGF2. Four days after changing the culture media, the cells cultured in FGF2 started to change morphologically, becoming flattened (Fig 16A). After extended culturing, the cells cultured with FGF2 showed a primed-like flattened morphology, while cells cultured with LIF or LIF + 2i still showed compact dome-shaped morphology (Fig 16B). In addition, when cultured in the absence of dox, the number of colonies gradually decreased within 4 days for cells under all three conditions, and AP-positive colonies were absent after subcultures (Fig 16C). Immunostaining analyses revealed two representative results based on gene expression patterns. First, NANOG expression was not detected under any conditions. NANOG, a key pluripotent gene, is reactivated during the late stages of reprogramming (Polo et al., 2012; Silva et al., 2009). However, no cells under any culture conditions

expressed NANOG, though OCT4 and SOX2 were highly expressed, as determined by ICC and flow cytometric analyses (Fig 17A, B and D). Second, pluripotent markers such as SSEA1 and SSEA4 were differentially expressed depending on culture conditions (Fig 17A and B). According to previous studies, SSEA1 and SSEA4 are specific markers for the naïve and primed pluripotent state, respectively (Nichols and Smith, 2009). Consistent with previous studies, SSEA1 and SSEA4 were exclusively detected in cells cultured with LIF and FGF2, respectively. Notably, in naïve-like cells, SSEA1 expression patterns were heterogeneous within colonies, and parts of the cells still expressed SSEA4 as a primed-pluripotent marker (Fig 17A). It is possible that FGF2 from feeder cells could affect SSEA4 expression under LIF-supplemented conditions. When treated with 2i to enhance the naïve state by inhibiting ERK/MEK and GSK signaling, although SSEA1 was expressed homogeneously, SSEA4 was still expressed in specific cell regions (Fig 17C). When injected into immunodeficient mice to verify potential for in vivo differentiation, no cultured cell lines could form teratomas (data not shown). These results indicate that the pluripotent state of derived pig iPSCs can be modulated by manipulating culture conditions, but that these cells may be partially reprogrammed and depend on the transgenes.



**Figure 16. Morphological changes of pig iPSCs in response to culture conditions.**

The piPS-14 cells cultured under LIF conditions were transferred to two different media supplemented with LIF + 2i or FGF2. (A) Four days after changing culture media, the cells cultured in FGF2 started to change morphologically, becoming flattened. (B) Later, the cells cultured with FGF2 showed a primed-like flattened morphology, while those cultured with LIF or LIF + 2i still showed compact dome-shaped morphology. (C) When cultured in the absence of dox, in all cells under the three conditions, the number of colonies gradually decreased within 4 days, and AP-positive colonies were absent after subcultures. Scale bar = 200  $\mu$ m.



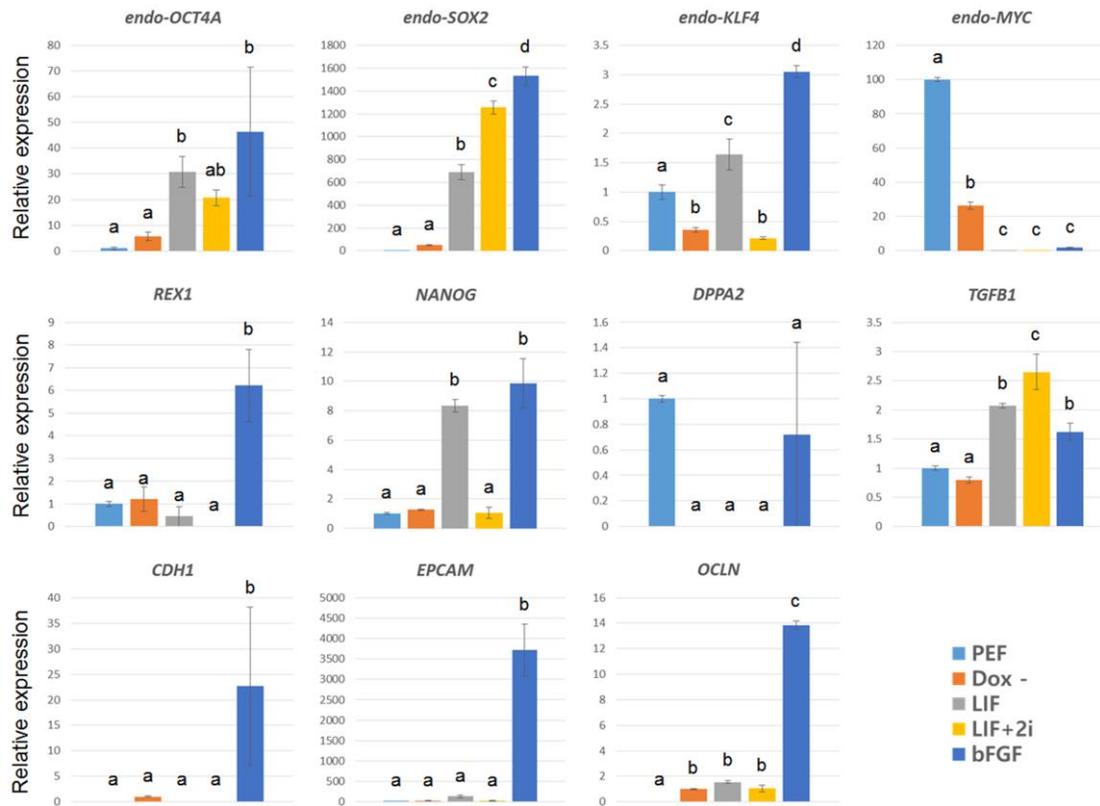
**Figure 17. Expression of pluripotent markers in piPSCs.**

Expression of endogenous and exogenous pluripotent genes was determined by immunostaining and qPCR. (A) OCT4, SOX2, SSEA1, and SSEA4 were expressed in naïve-like pig iPSCs cultured with LIF. (B) OCT4, SOX2, and SSEA4 were expressed in primed-like pig iPSCs cultured with FGF2 (C) When treated with 2i, SSEA4 was still expressed in naïve-like iPSCs. (D) Expression of NANOG was not detected under any culture conditions as determined by flow cytometric analysis. (E) Exogenous transgenes were highly expressed when treated with doxycycline in iPSCs, while transgenes were not expressed in the absence of doxycycline. Scale bar = 50  $\mu\text{m}$

## Endogenous genes related to pluripotency were not sufficiently reactivated by exogenous factors

Based on the above results, generated pig iPSCs were incompletely reprogrammed and depended on transgenes. For complete reprogramming, endogenous pluripotent genes must be reactivated without transgene expression (Maherali et al., 2007; Wernig et al., 2007). During the reprogramming process from fibroblasts to iPSCs, several events occur, including mesenchymal-to-epithelial transition (MET) (Li et al., 2010) and reactivation of endogenous pluripotent genes (Buganim et al., 2012). To verify the effects of transgenes on endogenous genes, expression levels of endogenous genes associated with pluripotency and MET (pluripotent genes: pig (p) *OCT4a*, *pSOX2*, *pKLF4*, *pMYC*, *NANOG*, *DPPA2*, and *REX1*; epithelial-to-mesenchymal transition (EMT) inducer: *TGFB1*; epithelial-specific markers: *CDH1*, *EPCAM*, and *OCN*) were determined by qPCR (Fig 18). In infected cells, exogenous transgenes were highly expressed when treated with doxycycline, while transgenes were not expressed in the absence of doxycycline (Fig 17E). As reprogramming was processed by exogenous genes, endogenous pluripotent genes (*pOCT4a*, *pSOX2*, *pKLF4*, *NANOG*, and *REX1*) and epithelial-specific markers (*CDH1*, *EPCAM*, and *OCN*) were up-regulated, as reported previously (Buganim et al., 2012; Li et al., 2010). However, *pMYC* and *DPPA2*, known predictors of reprogramming, were not reactivated, while TGFB1 blocked reprogramming

and was not efficiently shut down by transgene expression. Thus, although exogenous *MYC* was expressed as determined by RT-qPCR (Fig 17E), lack of endogenous *MYC* (a repressor of *TGFBI* during MET) (Li et al., 2010) led to insufficient downregulation of *TGFBI*. Interestingly, some genes, such as *pSOX2*, *pKLF4*, *REX1*, and epithelial-specific markers, which were preferentially expressed under culture conditions containing FGF2, and *pKLF4* and *NANOG*, were downregulated when treated with 2i. Taken together, these data demonstrate that pig iPSCs were incompletely reprogrammed because endogenous genes were insufficiently reactivated by transgene expression.



**Figure 18. Expression of pluripotent and MET-related genes as measured by qPCR.**

To verify the effects of transgenes on endogenous genes, expression levels of endogenous genes related to pluripotency and MET (pluripotent genes: *pOCT4a*, *pSOX2*, *pKLF4*, *pMYC*, *NANOG*, *DPPA2* and *REX1*; epithelial-to-mesenchymal transition (EMT) inducer: *TGFBI*; epithelial-specific markers: *CDH1*, *EPCAM* and *OCN*) were determined by qPCR. As reprogramming is processed by exogenous genes, endogenous pluripotent genes (*pOCT4a*, *pSOX2*, *pKLF4*, *NANOG*, and *REX1*) and epithelial-specific markers (*CDH1*, *EPCAM*, and *OCN*) were up-regulated. However, *pMYC* and *DPPA2* (known predictors of reprogramming) were not reactivated, while *TGFBI* (blocks reprogramming) was not efficiently shut-down by transgene expression. Some genes, such as *pSOX2*, *pKLF4*, *REX1*, and epithelial-specific markers, were preferentially expressed in culture conditions containing FGF2, and *pKLF4* and *NANOG* were downregulated when treated with 2i.

## Bisulfite sequencing showed that pig iPSCs were not epigenetically reprogrammed

The pig iPSCs were partially reprogrammed as determined by qPCR and immunostaining. To confirm whether epigenetic reprogramming occurred due to the expression of exogenous genes, DNA methylation patterns at promoter regions of pig *OCT4*, *NANOG*, and *XIST* were evaluated using bisulfite sequencing. Although endogenous *OCT4* (a key factor in pluripotency) (Shi and Jin, 2010) was expressed in pig iPSCs as detected by qPCR, *OCT4* promoter regions were highly methylated (Fig 19A). In the late stages of reprogramming, X chromosome reactivation occurred as *NANOG* expression was elevated in naïve PSCs (Hanna et al., 2010a; Navarro et al., 2008; Silva et al., 2009). However, in pig iPSCs, *NANOG* promoter regions were methylated at levels similar to somatic cell control (Fig 19B), and X chromosome reactivation did not occur in naïve-like pig iPSCs (Fig 19C). As shown in Figs 17 and 18, although *NANOG* expression was detected at the mRNA level, it was not detected at the protein level as determined by immunostaining. Consistent with these results, *DPPA2* (a pluripotent gene interacting with *NANOG* (Buganim et al., 2012; Du et al., 2010)) was not expressed in pig iPSCs (Fig 18). These data indicate that transcribed *NANOG* mRNA can be not translated or that the amount of translated *NANOG* protein may be too low to affect reprogramming. Bisulfite sequencing combined with gene expression data

showed that epigenetic reprogramming of pig iPSCs did not occur with incomplete reactivation of endogenous genes.

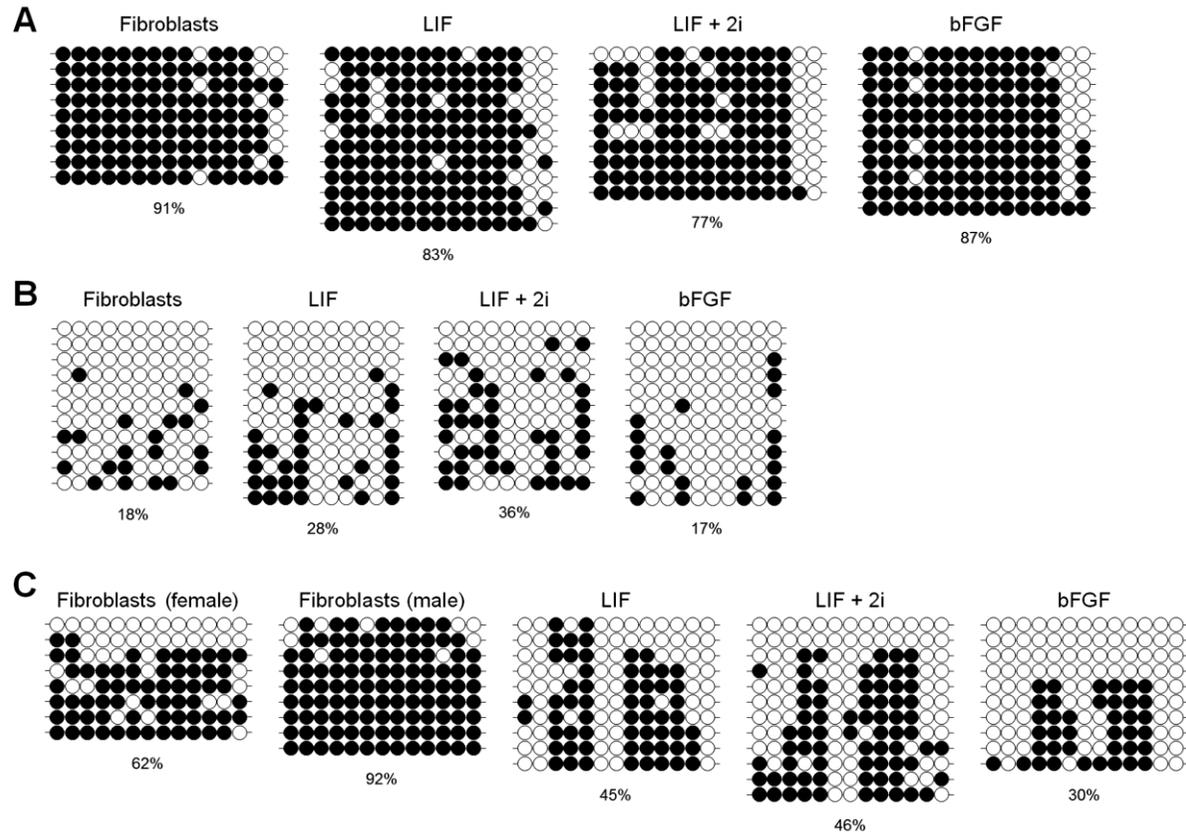


Figure 19. Bisulfite sequencing at promoter regions of *OCT4*, *NANOG*, and *XIST*.

To verify whether epigenetic reprogramming occurred by expression of exogenous genes, DNA methylation patterns at promoter regions of pig *OCT4*, *NANOG*, and *XIST* were evaluated by bisulfite sequencing. (A) *OCT4* core promoter: *OCT4* core promoter regions were highly methylated. (B) *NANOG* promoter: Promoter regions of *NANOG* were methylated to levels similar to somatic cell control. (C) *XIST* promoter: X chromosome reactivation did not occur in naïve-like pig iPSCs. Each circle indicates individual CpG dinucleotides. White and dark circles represent unmethylated and methylated CpGs, respectively. Each row represents one individual clone of amplified PCR products.

## 4. Discussion

### **Pluripotent stem cells of pigs as a non-permissive species.**

Recently, several studies have suggested that the states of PSCs are divided into two categories: naïve and primed. Naïve PSCs derived from early epiblasts in pre-implantation blastocysts as a developmental ground state can generate the chimeric fetus when micro-injected into recipient blastocysts. In addition, primed PSCs derived from late epiblasts in post-implantation blastocyst possess more differentiated pluripotency than naïve cells in terms of developmental capacity, gene expression, and epigenetic signatures. In permissive lines, both pluripotent states of PSCs can be derived from embryos. However, in non-permissive lines, the stem cells cannot be stabilized in the naïve state, and are instead differentiated and stabilized at the primed state during the establishment process if no additional treatments (including genetic manipulation and chemicals) are performed (Hanna et al., 2010b; Nichols and Smith, 2009). It has been suggested that, as a non-permissive species, pig epiblasts are reprogrammed into primed state cells during the establishment of PSCs from embryos (Park et al., 2013a). In addition, pig iPSCs derived from porcine somatic cells are morphologically and molecularly similar to mouse EpiSCs or human ESCs, rather than to mouse ESCs (Esteban et al., 2009; Ezashi et

al., 2009; Montserrat et al., 2012; Park et al., 2013a; West et al., 2010; West et al., 2011; Wu et al., 2009).

Several studies have attempted to establish naïve–state PSCs from non–permissive species such as rats and humans (Buehr et al., 2008; Li et al., 2008). The first case of human naïve PSCs reported that derivation of naïve PSCs were accomplished via ectopic expression of *OCT4* and *KLF4* supplemented with LIF and two inhibitors for GSK and ERK1/2 signaling (Hanna et al., 2010a). However, these cell lines could not be maintained without transgene expression. Recent studies have reported that generation of transgene–free human naïve–like PSCs could be achieved by several small molecules in addition to 2i (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). These results demonstrated that modulating signaling pathways is required for the maintenance of naïve state pluripotent stem cells in non–permissive species.

Consistent with previous studies involving pig ESCs and iPSCs, my data show that pig is a non–permissive species. During reprogramming, FGF2 treatment more strongly up–regulated specific pluripotent genes such as *pSOX2*, *pKLF4*, *REX1*, and epithelial–specific markers compared to LIF

treatment, and blocking FGF2 signaling downregulated *pKLF4* and *NANOG* rather than completing reprogramming, as reported in mice (Fig 18) (Silva et al., 2008). Furthermore, although SSEA1 and SSEA4 were exclusively expressed in LIF– and FGF2– supplemented conditions, respectively, specific cell regions cultured with LIF expressed SSEA4 (Fig 17A and B). Inhibiting ERK/MEK and GSK signaling did not block differentiation of pig iPSCs from the naïve state into the primed state (Fig 17C). In previous studies, several LIF–dependent naïve–like PSC lines have been derived by ectopic expression of transgenes supplemented with LIF and 2i (Fujishiro et al., 2013; Kwon et al., 2013; Telugu et al., 2010; Telugu et al., 2011; Zhang et al., 2015b). These naïve–like pig PSC lines were not epigenetically reprogrammed and still relied on transgene expression for the maintenance of pluripotency. Overall, pig is less permissive of generating naïve pluripotency than human, because pig iPSCs are ready to enter a primed state when cultured with 2i, while human ESCs could be converted into the naïve state by LIF, 2i, and transgene expression (Hanna et al., 2010a). Therefore, as a non–permissive species, pig somatic cells are preferentially reprogrammed into the primed state depending on FGF2 signaling.

**Incomplete reprogramming was induced by the failure of endogenous gene reactivation**

To improve efficiency nuclear reprogramming from somatic cells to pluripotent cells, reprogramming mechanisms and gene expression patterns have been elucidated by several studies. At the early stage of reprogramming, mesenchymal-to-epithelial transition (MET) is occurred upregulating epithelial-specific markers and downregulating *Tgfb1* known as a blockade of nuclear reprogramming by expression of Yamanaka's factors (Li et al., 2010; Maherali and Hochedlinger, 2009). The cells to undergo into iPSCs start to express predictive markers such as *Esrrb*, *Utf1*, *Lin28* and *Dppa2*. At the late stage, reprogramming are completed with epigenetic remodeling through upregulating endogenous genes by *Sox2* and reactivation of X chromosomes (in case of female cells) by *Nanog* (Buganim et al., 2012; Polo et al., 2012). Simultaneously, full reprogramming of the pluripotent state requires the activation of endogenous pluripotency genes along with silencing of transgenes for stable maintenance of the pluripotent state. (Maherali et al., 2007; Wernig et al., 2007). The defective silencing of transgenes after reprogramming affects stability, carcinogenesis, and differentiation ability of iPSCs (Okada and Yoneda, 2011; Okita et al., 2007).

It has been reported that incompletely reprogrammed iPSCs resulted in defective reprogramming processes including insufficient downregulation of *Tgfb1*, lack of *Nanog* expression, and failure of transgene

silencing. Comparing the pre-iPSC lines, several common characteristics such as incomplete expression of pluripotent genes, inactive X chromosome in female cells, and incompetence to generate germline chimaeras have been exhibited (Li et al., 2010; Okita et al., 2007; Silva et al., 2008; Silva et al., 2009). In porcine studies, it has been proven that almost of pig iPSC lines were partially reprogrammed showing incomplete silencing of transgenes, lack of *NANOG* expression, failure of epigenetic reprogramming, and inability of chimera formation (Du et al., 2015a; Esteban et al., 2009; Ezashi et al., 2009; Hall et al., 2012; Wei et al., 2015; Zhang et al., 2014b). According to previous studies, majority of pig iPSC lines have exhibited dependency of transgene continuous expression, even it was impossible to get the transgene-independent colonies using transgene-free system such as episomal vectors and plasmid vectors (Du et al., 2015a; Ezashi et al., 2009; Telugu et al., 2010).

Since *Nanog* as a gateway to pluripotency plays pivotal roles in epigenetic remodeling and X chromosome reactivation, it is important to reactivate *Nanog* in the late stages of reprogramming in mouse (Chambers et al., 2007; Silva et al., 2009). In human as a non-permissive species, *NANOG* plays an important role in reprogramming and maintaining pluripotency (Hyslop et al., 2005; Yu et al., 2007). In some pig studies,

*NANOG* upregulation and epigenetic reprogramming were not achieved (Du et al., 2015a; Wei et al., 2015). Consistent with previous studies, it was confirmed that generated iPSCs carrying whole Yamanaka's factors have no *NANOG* expression, and that epigenetic alteration relied on the expression of transgenes for maintenance (Figure 17C and 19). Although *Nanog* could be reactivated by treatment of 2i in mouse pre-iPSCs of permissive species (Silva et al., 2008), *NANOG* mRNA expression level could not be up-regulated by 2i treatment in my pig pre-iPSCs (Fig 18). Furthermore, insufficient downregulation of *TGFBI* due to the lack of endogenous *MYC* resulted in abnormal MET. In addition, during the reprogramming process, I obtained AP-negative cell lines, as reported previously (Hall et al., 2012; Takahashi and Yamanaka, 2006; Wei et al., 2015). These pre-iPSC lines lacking AP activity did not express *SOX2* without integration of exogenous *SOX2* (Fig 14). It is possible that *SOX2* expression is correlated with AP activity. Taken together, Yamanaka's factors may be not sufficient to achieve MET and epigenetic resetting in pig. It is important to identify optimal culture conditions and novel factors for the reprogramming of pig somatic cells.

**New approaches are required to overcome reprogramming hurdles and generate pig iPSCs**

To overcome the barriers of reprogramming described above,

various studies have been performed using small molecules, another reprogramming factor, and nutrient supplements. At this time, drug-inducible vector systems, which can easily turn transgenes on and off, have been used in various studies involving the iPSC generation and the elucidation of reprogramming mechanisms (Buganim et al., 2012; Polo et al., 2012; Wernig et al., 2008). Chromatin remodeling and the erasing of epigenetic memory in somatic cells have been accomplished by inhibiting DNA methylation and using chromatin modifiers (Huangfu et al., 2008). Pre-iPSCs lacking *Nanog* expression were converted into fully reprogrammed iPSCs by inhibiting FGF2 signaling and *Nanog* overexpression (Silva et al., 2008; Theunissen et al., 2011). In addition, serum free media and vitamin C could be used to overcome hurdles and increase reprogramming efficiency (Chen et al., 2010; Esteban et al., 2010). In addition, suppression of TGF $\beta$  signaling, apoptosis, senescence, and cell-cycle arrest are considered useful tools for reducing reprogramming barriers (Banito et al., 2009; Ichida et al., 2009; Kawamura et al., 2009; Li et al., 2009). Recently, it has been shown that several factors including *Nanog*, *Lin28*, *Nr5a2*, and *Glis1* could be used for nuclear reprogramming instead of Yamanaka's factors (Heng et al., 2010; Maekawa et al., 2011; Yu et al., 2007). Signaling inhibitors that elevate pluripotent status to the naïve state have been revealed in human studies (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014). Accordingly, if various tools are properly utilized,

it may be possible to generate fully-reprogrammed porcine iPSCs by overcoming the genetic and epigenetic barriers.

However, it has proven hard to establish porcine ESCs and optimize pig-specific reprogramming condition using typical methods because of several differences caused by genetic backgrounds of mouse, human and pig. During development of early embryo which have inner cell mass (ICM) considered as pluripotent cell population, pig have a prolonged preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in pig embryos, different cell signaling which governs pluripotency reveals some differences with that of mouse embryos. Unlike mouse in which *Oct4* and *Cdx2* are exclusively expressed in ICM and trophoctoderm (TE) respectively, in pig, SOX2 are specifically expressed in ICM while OCT4 are expressed in TE until expanded blastocyst of embryonic day 8 (Liu et al., 2015). In ICM and epiblasts of pig blastocyst, LIF receptor is absence while FGF receptors are specifically expressed, which indicate that FGF signaling might play a pivotal role in maintenance of pluripotency rather than LIF (Hall et al., 2009). Along with FGF signaling, it was also elucidated that BMP signaling have an important role in maintaining pluripotency (Hall and Hyttel, 2014).

Recent studies have increased understanding of pig somatic cell reprogramming. First, pig iPSCs could be induced by *KLF4*, *SOX2*, and *MYC* (except for *OCT4*) infection, although transgenes were not silenced (Montserrat et al., 2012). According to that study, the ectopic expression of *OCT4* interfered with reactivation of endogenous pluripotent networks in pig fibroblasts, which indicated that *OCT4* may not be essential for maintaining pluripotency in pig. However it was reported that endogenous gene expression including *OCT4*, *NANOG* and *MYC* were repressed by expression of counterpart exogenous genes (Hall et al., 2012). Similar phenomenon was also occurred in our study (Fig 18). Endogenous *MYC* was down-regulated as exogenous *MYC* was activated. These studies suggest that further investigation about reprogramming mechanisms and new reprogramming factors or vector systems are required in pig. Another study showed that generation of pig iPSCs in which transgenes were silenced was accomplished using combined supplements of three cytokines (LIF, FGF2, and BMP4) and two signaling inhibitors (TGFB1 and WNT inhibitors) (Zhang et al., 2015a). This novel culture condition generated ‘intermediate’ pluripotent stem cells, showing mixed features of naïve and primed states with inactivation of retroviral transgenes. Taken together, it is possible that pig has unique pluripotent characteristics governed by different cell signaling networks than mouse and human. Accordingly, in-depth analyses of pig-specific signaling pathways are required for establishing authentic porcine

ESCs and obtaining completely reprogrammed iPSCs.

## 5. Conclusion

Based on my results, pig pre-iPSCs were stably maintained over an extended period, and could differentiate into three germ layers. In addition, the pluripotent state of pre-iPSCs was regulated by the culture condition. Pre-iPSCs showed a naive- or primed-like pluripotent state in LIF or FGF2 supplemented culture conditions, respectively. However, pre-iPSCs were not fully reprogrammed and showed transgene dependency. The cultured pre-iPSCs expressed endogenous transcription factors such as *OCT4* and *SOX2*, but not *NANOG* (a gateway to complete reprogramming). In addition, endogenous genes related to mesenchymal-to-epithelial transition (*DPPA2*, *CDH1*, *EPCAM*, and *OCN*) were not sufficiently reactivated and failed to down-regulate *TGFBI*. DNA methylation analysis for promoters of *OCT4*, *NANOG*, and *XIST* showed that epigenetic reprogramming did not occur in female pre-iPSCs. Therefore, Yamanaka's factors per se with typical culture conditions may not be sufficient to achieve MET and epigenetic resetting in pig. Finally, in-depth analyses of pig-specific signaling pathways are required to establish authentic porcine embryonic stem cells and obtain completely reprogrammed iPSCs.

This chapter will be published in elsewhere  
as a partial fulfillment of Kwang–Hwan Choi’s Ph.D program.

## CHAPTER 5

### Culture of pig embryonic stem cells using chemically defined media

## 1. Introduction

Derivation of pluripotent cells can be accomplished by *in vitro*-culture of early embryos (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Pluripotent stem cells (PSCs), represented by embryonic stem cells (ESCs), can be differentiated into various types of cells and tissues in our body, so have been considered as ideal tools for regenerative medicine and cell therapy. Recently, a clinical trial of human ES cell therapy for repairing spinal cord damages was approved by Food and Drug Administration in 2009 (Alper, 2009). In 2014, first clinical test were operated in Japan (Sugita et al., 2016). A woman who suffered from aged-macular degeneration (AMD) received retinal pigment epithelium (RPE) differentiated from iPSCs, and her vision was partially recovered. However, although spinal cords and eyes are immune-privileged areas without immune cells and are not strongly associated with life, clinical tests for organs and tissues which are strongly involved in life, such as heart, brain and pancreas, need tight preclinical tests using animal disease models.

Because of the physiological and immunological similarities that exist between pigs and humans, porcine pluripotent cell lines have been identified as important candidates for preliminary studies on human disease

(Brevini et al., 2007; Hall, 2008; Houdebine, 2009). Therefore, many research groups have attempted to derive pig pluripotent stem cells (PSCs) from early embryos for a long time (Ezashi et al., 2009; Kues et al., 2013; Park et al., 2013a; Piedrahita et al., 1990b; Son et al., 2009; Yang et al., 2009). However, it has been proven difficult to establish genuine pig ESCs which have *in vivo* developmental–competence such as chimera and teratoma formation ability. From 1990s, several scientists have attempted to derive stem cells from embryos, but resulting cell lines did not meet the criteria, especially *in vivo* developmental–competency. One of the reasons is that optimized culture media for pig pluripotent stem cells is not developed. During development of the early embryo, which has an inner cell mass (ICM) considered as pluripotent cell population, pig has a prolonged preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in pig embryos, different cell signaling that governs pluripotency reveals differences compared to mouse embryos (Hall et al., 2009; Hall and Hyttel, 2014; Liu et al., 2015). Recent data indicated that metabolisms such as fatty acid and glucose during pig embryo development differ from mouse and human (Cao et al., 2014; Secher et al., 2016). Accordingly, additional or different combination of signaling molecules would be needed to support pluripotent networks in pig.

Here, I attempted to optimize culture media for deriving pig ESCs by using various metabolic components and signaling molecules. As a result, pig ESCs could be successfully established by chemically defined media supplemented with FGF2, Activin A and Wnt activator. These cells expressed pluripotent genes such as *OCT4*, *SOX2* and *NANOG*, and could be maintained for extended periods. Accordingly, this system will accelerate stem cell researches for regenerative medicine and could be applied to generate PSCs of other domestic animals. And pig PSCs will aid to generate disease models in large animals and help to explore pluripotent network across the species.

## 2. Materials and methods

### Animal care

The care and experimental use of pigs and mice was approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-160120-9-1 and SNU-170223-2 for MEFs isolation). The ovaries used in the present study were donated from local slaughterhouses (Dodram, Korea; Samsung, Korea) only for research. Pregnant ICR mice were purchased from SAMTACO BIO Inc., Korea. The mice were taken care according to standard protocol of Institute of Laboratory Animal Resources and sacrificed by cervical dislocation after anesthesia.

### Collection and *in vitro* maturation of oocytes

The ovaries were collected from prepubertal gilts at a local slaughterhouse within 1 h after extraction. The follicular fluid and cumulus-oocyte complexes (COCs) from 3–6 mm diameter follicles were aspirated using an 18-gauge needle. The follicular contents were pooled and allowed to sediment. After sedimentation, the supernatant was discarded and the sediment was washed once with TL-HEPES-PVA medium (Funahashi et al.,

1997). The COCs with granulate cytoplasm on the oocytes and compact cumulus cells were collected. The collected COCs were matured in tissue culture medium (TCM-199; Life Technologies, Rockville, MD, USA) containing 10 ng/mL epidermal growth factor (EGF), 1 mg/ml insulin, 4 IU/mL equine chorionic gonadotrophin (eCG; Foligon; Intervet, Cambridge, UK), human chorionic gonadotrophin (hCG; Intervet) and 10% (v/v) porcine follicular fluid (pFF). Each group of 50–60 COCs was cultured in TCM-199 medium for 22 h at 39°C in a 5% CO<sub>2</sub> incubator. After 22 h maturation, COCs were transferred to eCG- and hCG-free TCM-199 and cultured for an additional 22 h.

### **Parthenogenetic activation**

To generate parthenotes, cumulus-free oocytes were activated by an electric pulse (1.0 kV/cm for 60  $\mu$ s) in activation medium (280 mM mannitol, 0.01 mM CaCl<sub>2</sub>, 0.05 mM MgCl<sub>2</sub>) using a BTX Electro cell Manipulator ECM2001 (BTX, CA, USA). Parthenogenetic-activated oocytes were washed twice with 0.4% (w/v) BSA-supplemented DPBS (Welgene, Korea), followed by a 4 h incubation in PZM-3 containing 2 mM 6-dimethylaminopurin for post-activation. Post-activated oocytes were cultured in PZM-3 for 168 h under humidified atmosphere containing 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 39° C.

## Derivation of primary colonies

To optimize pig ESC culture media, hatched parthenogenetic blastocysts were seeded on feeder cells composed of mitotically inactivated mouse embryonic fibroblasts (MEFs) with various culture condition. ESC media consists of basal media, replacements of fetal bovine serum (FBS) and signaling molecules. Basal media was composed of KnockOut™ Dulbecco's modified Eagle's medium (KO-DMEM) containing 2 mM glutamax, 0.1 mM  $\beta$ -mercaptoethanol, 1x MEM nonessential amino acids, and 1x antibiotic-antimycotic (all from Gibco, USA). Tested serum replacements were 20 % KnockOut™ Serum Replacement (KSR; Gibco), 1x N2/B27 supplements (Gibco), combination of 20 % KSR and 0.2 % chemically defined lipid concentrate (LC; Gibco), and combination of 5 % KSR and 1x N2/B27 supplements. Treated signaling molecules were 10 ng/ml human recombinant basic fibroblast growth factor (hrFGF2; R&D Systems), 10 ng/ml Actin A (ActA, R&D Systems) and 1  $\mu$ M CHIR99021 (CH; Cayman chemical, MI, USA). After 7 days, primary colonies of embryonic stem cells were observed and then fixed with 4% paraformaldehyde for further analysis. All cultures were performed under humidified conditions containing 5 % CO<sub>2</sub> and 5 % O<sub>2</sub> at 38°C.

## **Culture of pig embryonic stem cells**

Established pig ESCs were cultured with ESC media supplemented with 10 ng/ml human recombinant basic fibroblast growth factor (FGF2; R&D Systems), 10 ng/ml Actvin A (ActA, R&D Systems) and 1  $\mu$ M CHIR99021 (CH; Cayman chemical, MI, USA). ESC media consisted of KnockOut™ Dulbecco's modified Eagle's medium (KO-DMEM) containing 20% KnockOut™ Serum Replacement (KSR), 0.2% chemically defined lipid concentrate (LC; Gibco), 2 mM glutamax, 0.1 mM  $\beta$ -mercaptoethanol, 1x MEM nonessential amino acids, and 1x antibiotic-antimycotic (all from Gibco, USA). Pig ESCs were subcultured every 5–7 days. Expanded colonies were detached from the feeder cells and dissociated into small clumps using pulled glass pipettes. These clumps were transferred onto new feeder cells and cultured with 10  $\mu$ M Y-27632 (Santa Cruz, USA) contained-ESC media for 24 h. After 24h, attached clumps were cultured with ESC media in absence of Y-27632 for 4–6 days.

## **Immunostaining**

Immunocytochemistry was conducted according to methods described in Chapter 3. Images of stained cells were captured using inverted fluorescence microscope.

## Statistical analysis

The data obtained in this study were statistically analyzed using GraphPad Prism 6 statistical software (GraphPad Software, CA, USA). Statistical differences between datasets were determined by one-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) tests. Differences were considered significant at  $P < 0.05$ .

### 3. Results

#### Optimization of culture media for pig embryonic stem cells

To optimize culture media for pig ESCs, basic component of media such as serum-replacement and signaling molecules were examined. Although fetal bovine serum (FBS) have been widely used in cell culture, because of variation between batches and undefined factors, FBS is unsuitable for optimizing culture media. For replacing serum, KSR, N2/B27 supplements, mixture of KSR and LC, and mixture of KSR and N2/B27 supplements were used. Because pig has peculiar features of preimplantation development compared with mouse and human, it was supposed that activation of additional signaling is pivotal in order to maintain pluripotency *in vitro*. Because pig are considered as a non-permissive species (Choi et al., 2016), signaling molecules for maintaining primed pluripotent state including FGF2, Activin A and CHIR99021 were selected (Pera and Tam, 2010). Firstly, it was verified which serum-replacements are suitable for growth of pluripotent inner cell mass (ICM) *in vitro*. As shown in Table 13, attachment efficiency and expansion efficiency of all groups were similar without significant differences. When examined expression of SOX2, known as ICM markers of early blastocyst in pig (Liu et al., 2015), no significant differences in number of SOX2-expressed

primary colonies were observed among four groups. However, as presented in Fig 20, KSR and KSR+LC supplements more efficiently supported growth of SOX2-expressed cells than N2/B27 and KSR+N2/B27 supplements. Especially, in KSR+LC group, all cells were SOX2-positive cells, whereas SOX2-negative cells were observed in other groups. Although KSR and KSR+LC supported growth of ICM-derived cells during culture, long-term cultured cell lines couldn't be obtained with only FGF2-supplemented media. So, in next step, it was examined which signaling molecules are required for extended *in vitro*-culture of ICM. Four combinations including FGF2, FGF2+ActA, FGF2+CH, and FGF2+ActA+CH were treated with KSR or KSR+LC contained media. As presented in Tables 14 and 15, there were no significant differences in all of index among combinations of signaling molecules. And no considerable differences existed in growth pattern of SOX2-positive cells among groups (Figs 21 and 22). Interestingly, while cell lines could not be derived with other media, only in KSR+LC media supplemented with FGF2+ActA+CH, two embryonic stem cell lines were established (Table 15; Fig 23). Additional experiments with approximately one hundred blastocysts have the same result. Accordingly, pig ES cell lines could be derived using chemically defined media supplementing FGF2, ACT A and WNT activator.

Table 13. Effects of serum replacements on outgrowth of primary pig ICM colonies.

Serum replacement*	Replication No.	Seeded Blastocyst No.	Attachment efficiency (%)	Expansion efficiency (%)	Sox2-expressed Colony (%)	Colonies maintained over 2 passages
1	3	30	83.3 ± 8.8**	56.7 ± 8.8	40.0 ± 5.8	ND***
2	3	30	76.7 ± 12.0	46.7 ± 3.3	30.0 ± 0.0	ND
3	3	30	66.7 ± 16.7	46.7 ± 14.5	33.4 ± 8.8	ND
4	3	30	70.0 ± 12.0	56.7 ± 8.8	40.0 ± 5.8	ND

\*:1: 20 % Knockout serum replacement (KSR), 2: N2/B27 supplements, 3: 5 % KSR+ N2/B27 supplements, 4: 20 % KSR + 0.2 % chemically defined lipid concentrate

\*\* : No significant differences existed in all of index among experimental groups.

\*\*\*: non-detected

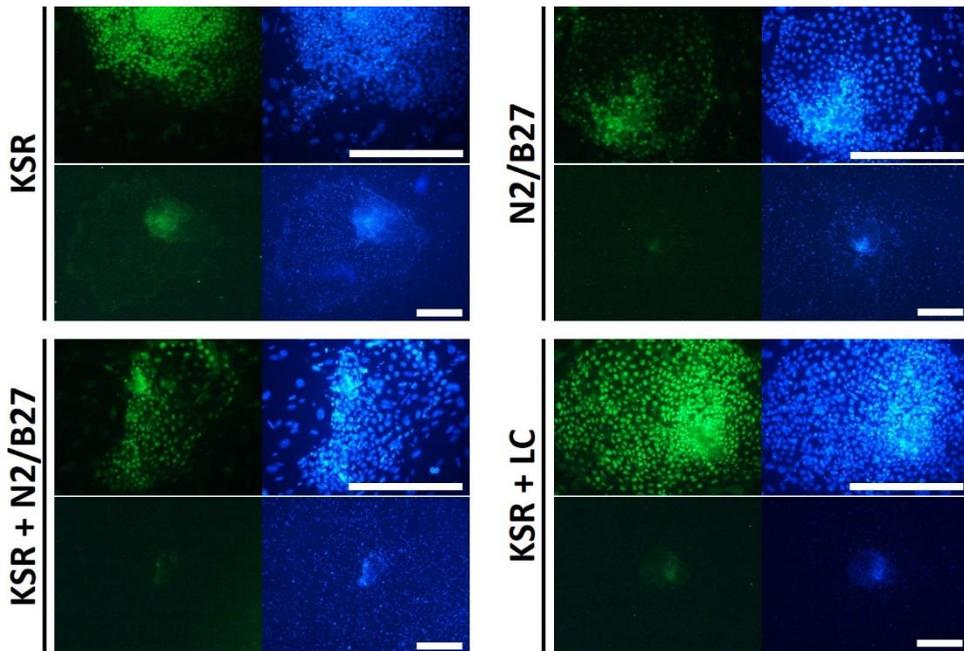


Figure 20. Expression of SOX2 in primary porcine ICM outgrowths cultured with various serum replacements.

To verify which serum-replacements are suitable for *in vitro*-culture of pluripotent ICM, porcine blastocysts were seeded onto feeder cells with several combinations of protein sources including KSR, N2/B27, KSR + N2/B27 and KSR + LC. After 7 days from blastocyst seeding, primary ICM colonies were observed and then fixed with 4% PFA. Expression of SOX2, known as ICM markers of early blastocyst in pig, was examined in primary ICM colonies by immunostaining (Green = SOX2; Blue = DNA). Scale bar = 200  $\mu$ m

Table 14. Effects of cytokines on outgrowth of primary pig ICM colonies in KSR-supplemented media.

Cytokines*	Replication No.	Seeded Blastocyst No.	Attachment efficiency (%)	Expansion efficiency (%)	Sox2-expressed Colony (%)	Colonies maintained over 2 passages
1	3	30	66.7 ± 12.0**	30.0 ± 5.8	30.0 ± 5.8	ND***
2	3	30	60.0 ± 10.0	40.0 ± 5.8	26.7 ± 6.7	ND
3	3	30	70.0 ± 10.0	46.7 ± 13.3	33.3 ± 12.0	ND
4	3	30	70.0 ± 11.5	43.3 ± 24.0	26.7 ± 14.5	ND

\*: 1: 10 ng/ml FGF2, 2: 10 ng/ml FGF2 + 10 ng/ml Activin A, 3: 10 ng/ml FGF2 + 1  $\mu$  M CHIR99021, 4: 10 ng/ml FGF2 + 10 ng/ml Activin A + 1  $\mu$  M CHIR99021.

\*\* : No significant differences existed in all of index among experimental groups.

\*\*\*: non-detected

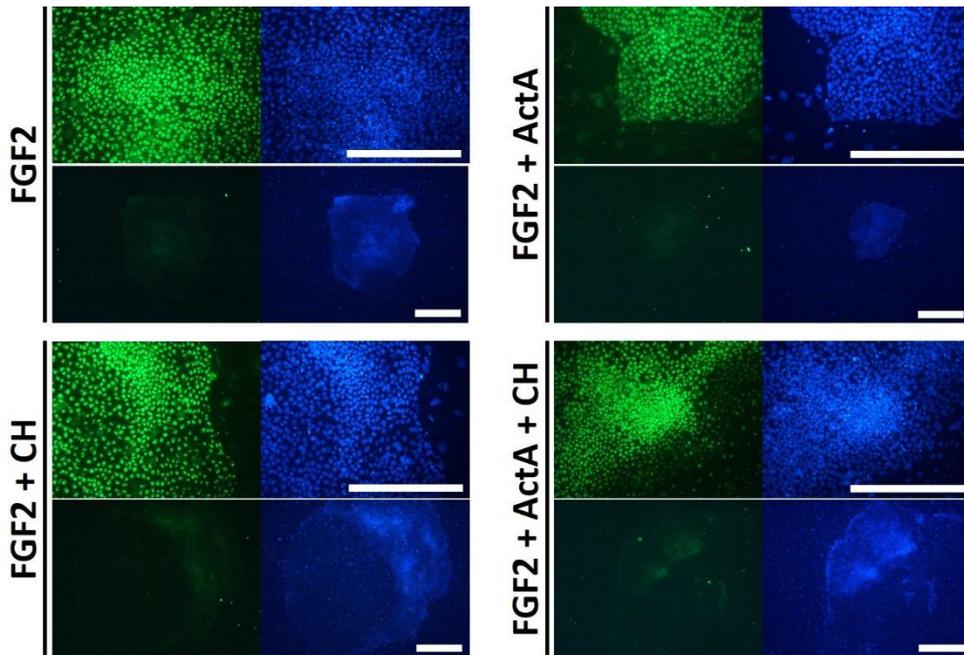


Figure 21. Expression of SOX2 in primary porcine ICM outgrowths cultured with KSR-supplemented media containing various cytokines.

To verify effects of cytokines on *in vitro*-culture of porcine ICM, porcine blastocysts were seeded onto feeder cells and cultured with KSR-supplemented media containing several combinations of cytokines including FGF2, FGF2 + ActA, FGF2 + CH and FGF2 + ActA + CH. After 7 days from blastocysts seeding, expression of SOX2, known as ICM markers of early blastocyst in pig, was examined in primary ICM colonies by immunostaining (Green = SOX2; Blue = DNA). Scale bar = 200  $\mu\text{m}$

Table 15. Effects of cytokines on outgrowth of primary pig ICM colonies in KSR+LC-supplemented media.

Cytokines*	Replication No.	Seeded Blastocyst No.	Attachment efficiency (%)	Expansion efficiency (%)	Sox2-expressed Colony (%)	Colonies maintained over 2 passages
1	3	29	75.2 ± 13.1**	51.5 ± 4.6	44.8 ± 2.9	ND***
2	3	29	82.6 ± 3.8	48.9 ± 10.6	45.6 ± 10.9	ND
3	3	29	85.9 ± 7.1	51.5 ± 4.6	48.1 ± 6.1	ND
4	3	29	79.2 ± 5.8	62.6 ± 11.5	52.6 ± 12.6	2 lines****

\*: 1: 10 ng/ml FGF2, 2: 10 ng/ml FGF2 + 10 ng/ml Activin A, 3: 10 ng/ml FGF2 + 1  $\mu$ M CHIR99021, 4: 10 ng/ml FGF2 + 10 ng/ml Activin A + 1  $\mu$ M CHIR99021.

\*\* : No significant differences existed in all of index among experimental groups.

\*\*\*: non-detected

\*\*\*\*: Derived lines were maintained over 3 months.

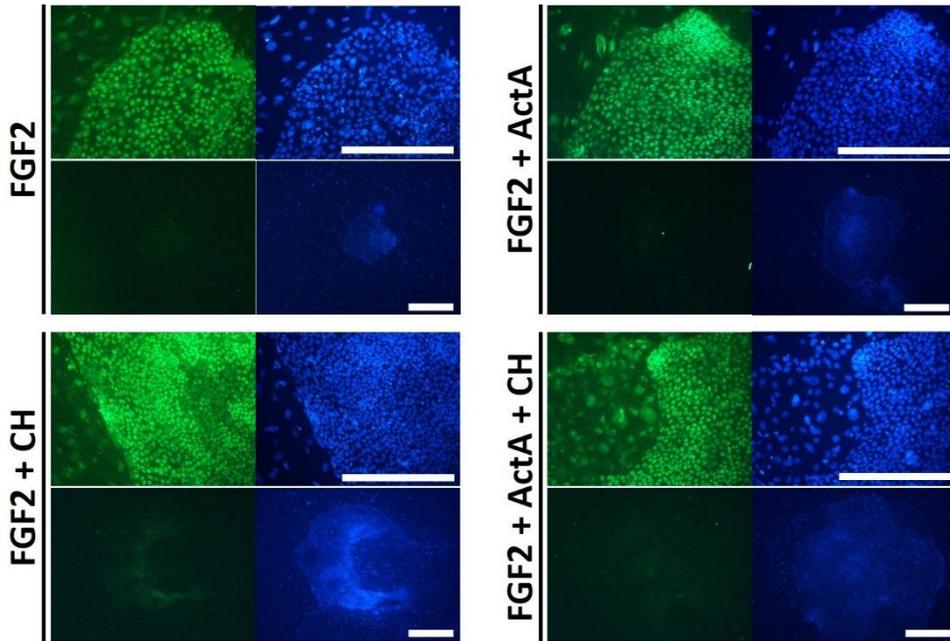
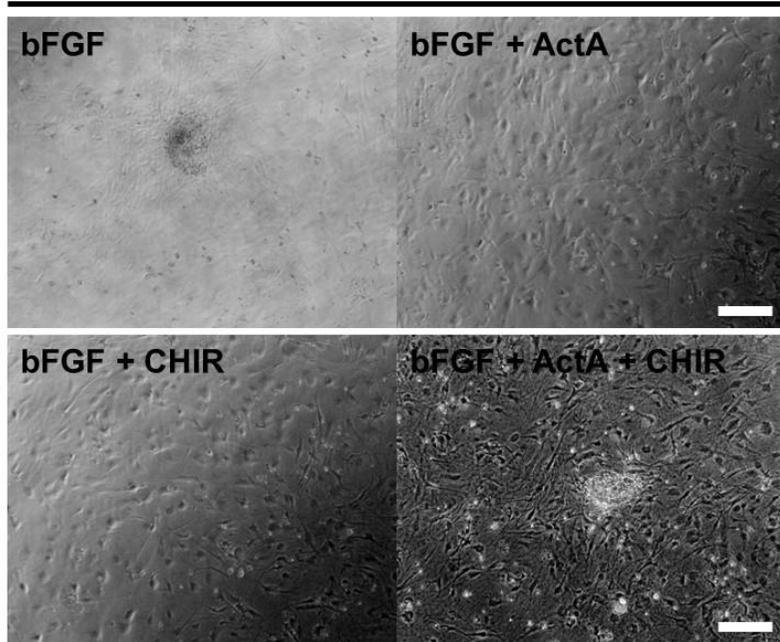


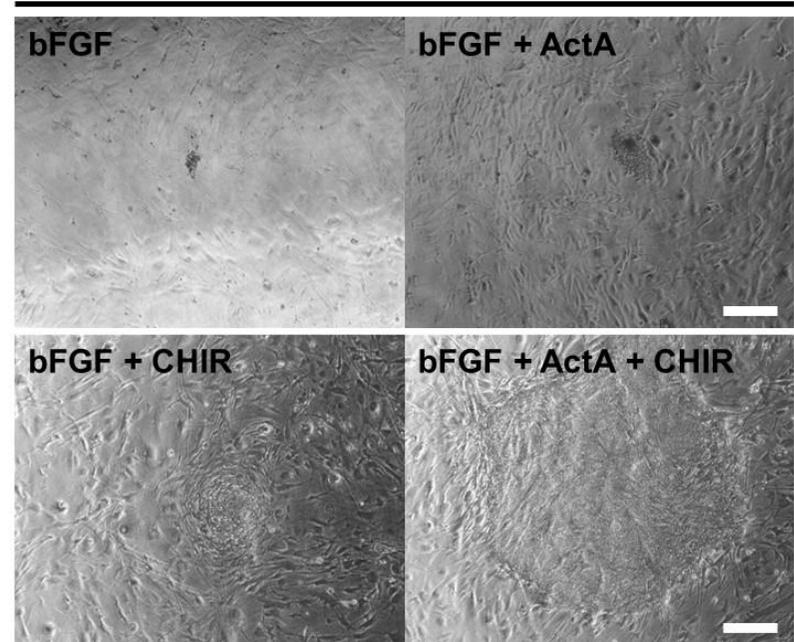
Figure 22. Expression of SOX2 in primary porcine ICM outgrowths cultured with KSR+LC-supplemented media containing various cytokines.

To verify effects of cytokines on *in vitro*-culture of porcine ICM, porcine blastocysts were seeded onto feeder cells and cultured with KSR+LC-supplemented media containing several combinations of cytokines including FGF2, FGF2 + ActA, FGF2 + CH and FGF2 + ActA + CH. After 7 days from blastocysts seeding, expression of SOX2, known as ICM markers of early blastocyst in pig, was examined in primary ICM colonies by immunostaining (Green = SOX2; Blue = DNA). Scale bar = 200  $\mu\text{m}$

**KSR**



**KSR + LC**

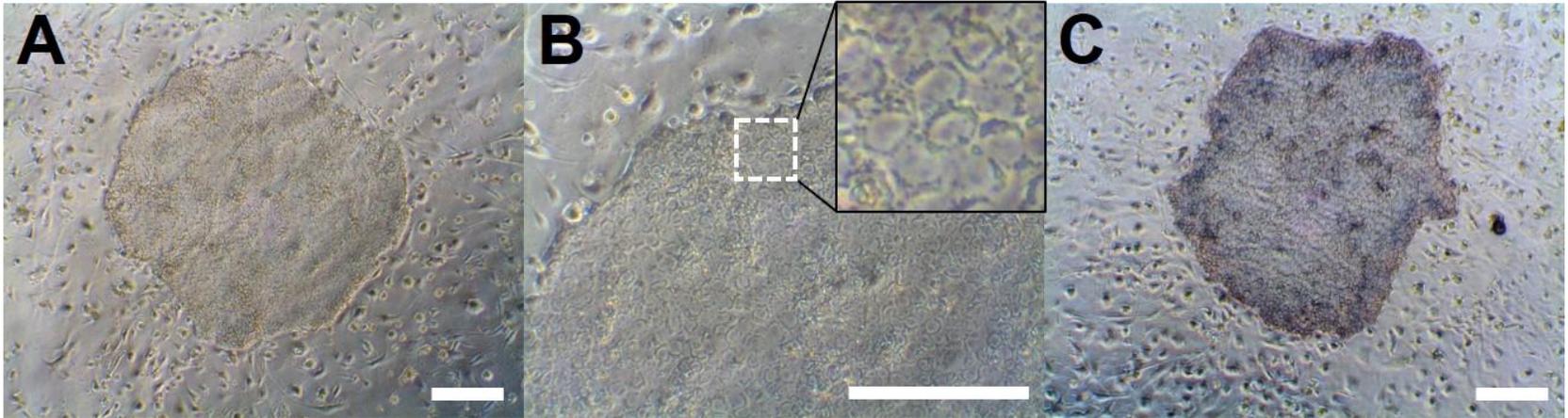


**Figure 23. Long term culture of pig ICM with various combinations of culture media.**

To verify effects of culture media on long-term culture of pig ICM colonies, primary colonies from ICM were subcultured onto fresh feeder cells and maintained for several passages. When pig ICMs were cultured for extended time, two embryonic stem cell lines were established only in KSR+LC media supplemented with FGF2+ActA+CH. Scale bar = 400  $\mu$ m.

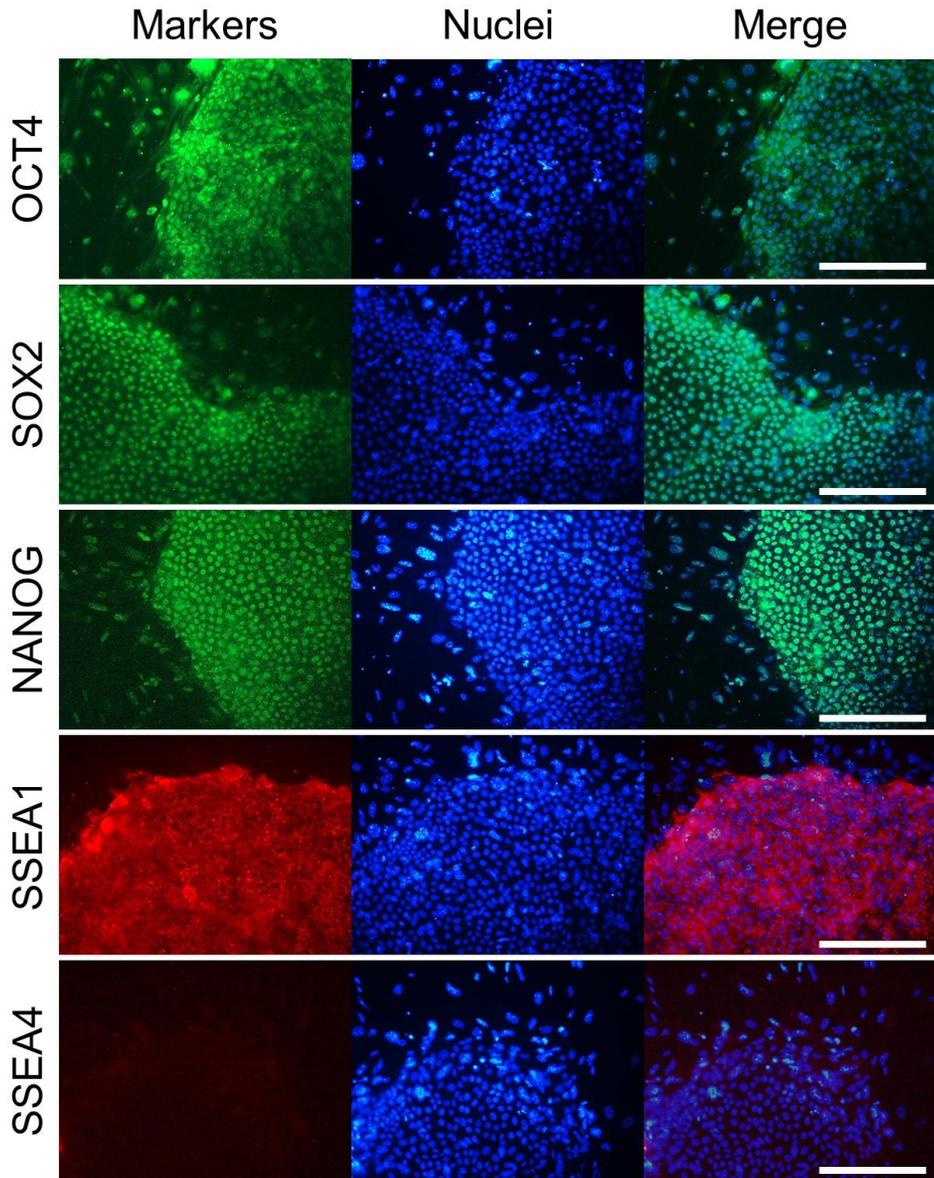
## Characterization of pig embryonic stem cells

Growth pattern of PSCs have two type depends on their pluripotent state (Nichols and Smith, 2009). PSC colonies having naïve state display multi-layered and dome-shaped morphology, and primed PSCs have single-layered and flattened morphology. Colonies of newly derived ESCs showed flattened morphology having AP activity and the single cells exhibited epithelial morphologies with high nucleus-to-cytoplasm ratio (Fig 24). Pluripotent markers including OCT4, SOX2, NANOG, and SSEA1 were expressed in pig ESCs as determined by immunostaining (Fig 25). Especially, SOX2 and SSEA1 known as early ICM markers in pig highly expressed in the cells (Hall et al., 2010; Liu et al., 2015). Taken together, it was confirmed that the established cells were pluripotent possessing features of early ICM in pig.



**Figure 24. Morphology of pig embryonic stem cells derived by chemically defined media.**

New embryonic stem cell lines were established in KSR+LC media supplemented with FGF2+ActA+CH. (A, B) Colonies of newly derived ESCs showed flattened morphology and the single cells exhibited epithelial morphologies with high nucleus-to-cytoplasm ratio. (C) Pig ESCs have alkaline phosphatase activity. Scale bar = 400  $\mu$ m.



**Figure 25. Expression of pluripotent genes in pig ESCs.**

Pluripotent markers including OCT4, SOX2, NANOG, SSEA1 and SSEA4 were expressed in pig ESCs as determined by immunostaining. Scale bar = 200  $\mu$ m.

## 4. Discussion

### **Fig ESCs can be stably maintained in chemically defined media.**

First derivations of mouse and human embryonic stem cells (ESCs) were accomplished by undefined culture conditions composed of feeder cells, fetal bovine serum and embryonal carcinoma-derived conditioned media (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). However, it has been assessed that undetermined culture sources such as serum caused heterogeneity of PSCs and induced differentiation during culture (Galonska et al., 2015; Guo et al., 2016a). In addition, animal-derived components such as feeder cells and serum would hamper clinical use of human PSCs. So, chemically optimization of PSC culture media is pivotal for basic stem cell researches as well as therapeutic applications. In mice, it was verified that LIF and Bmp4 signal pathways have a crucial role in maintaining pluripotency of ESCs (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). And recent study showed that chemically defined media containing inhibitors of ERK and GSK3 $\beta$  signaling could support pluripotency and enhance ground state by reducing heterogeneity in mouse ESCs (Galonska et al., 2015; Guo et al., 2016a). In human cases, unlikely mouse, pluripotency is sustained through ERK and ACTIVIN/NODAL signal pathway activated by FGF2 and TGF- $\beta$  (Pera and Tam, 2010). Chemically optimized culture

conditions supplemented with FGF2 and TGFb could prevent differentiation of human PSCs over 3 months and improve deriving efficiency of iPSCs (Chen et al., 2011; Ludwig et al., 2006).

Numerous research groups have attempted to generate authentic pig PSCs. In early studies of pig ESCs, researchers tried to establish stem cells based on teratocarcinoma culture methods as mouse studies did. Various culture materials including serums (fetal bovine serum and calf serum), feeder cells (buffalo liver cells, mouse embryonic fibroblasts, pig embryonic fibroblasts and pig uterine epithelial cells) and conditioned media were tested for maintaining pluripotent ICM *in vitro* (Anderson et al., 1994; Piedrahita et al., 1990a, b; Talbot et al., 1993b). Nonetheless, it was hard to derive pig stem cells due to lack of knowledge about cell signaling involved in early embryo development and maintaining pluripotency. When it was verified that LIF and FGF2 are responsible for maintaining mouse and human ESCs respectively (Amit et al., 2000; Smith et al., 1988; Williams et al., 1988), many cytokines like interleukins (IL), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), activin A (ActA) and stem cell factor (SCF) as well as LIF and FGF2 have been used for *in vitro* culture of pig ICM with various combinations and concentrations (Alberio et al., 2010; Boeuf et al., 1997; Li et al., 2004; Li et al., 2003; Moore

and Piedrahita, 1996; Siriboon et al., 2015; Vassiliev et al., 2010). Beside, as culture media were developed for culture of mouse and human ESCs, Knockout–DMEM, F10, F12 and  $\alpha$ MEM have been used for basal media (Kim et al., 2007a; Moore and Piedrahita, 1996; Puy et al., 2010; Vassiliev et al., 2010). However, although some culture conditions could support *in vitro*–culture of pig ICM for a short term (Alberio et al., 2010; Puy et al., 2010; Vassiliev et al., 2010), cultured ICM lost their own features and in turn differentiated during prolonged culture. Instead of pluripotent cells, during *in vitro*–culture, multipotent stem cells, so called ES–like cells, have been spontaneously obtained by several groups (Brevini et al., 2010; Cheong et al., 2015; Kim et al., 2010b; Uh et al., 2014).

In this study, it was succeeded in chemically optimization of culture media for maintaining pig ESCs by testing various combinations of cytokines and serum replacements. Newly derived ESCs displayed a typical flattened monolayer colony morphology and was maintained over an extended time period. Pluripotency–related transcription factors including OCT4, SOX2 and NANOG were expressed in pig ESCs as determined by immunostaining (Fig 25). Overall, it was tempting to speculate that pig ESCs have primed pluripotent state resembling human PSCs (Nichols and Smith, 2009). However, unlikely human ESCs, SSEA1, known as surface marker for naïve

state, was highly expressed in pig ESCs as assessed by immunostaining (Fig 25). Interestingly, expression of SSEA1 was observed in pig ICM of early embryos, while SSEA1 was not detected in ICM of human blastocysts (Hall et al., 2010; Liu et al., 2015). In consistent with this data, several studies showed that SSEA1 was expressed in pig *in vitro*-cultured ICM (Alberio et al., 2010; Vassiliev et al., 2010) and iPSCs (Ezashi et al., 2009) instead of SSEA4. Taken together, derived pig ESCs with defined media have primed pluripotent state in terms of physiological features, but highly express SSEA1 unlike human.

**Additional signalings are required to support pluripotency in pig ESCs compared to those of human and mouse.**

It has been proven hard to establish porcine ESCs and optimize pig-specific reprogramming conditions using typical methods because of differences in the molecular mechanisms during embryo development among mouse, human, and pig. During development of the early embryo, which has an pluripotent ICM, pig has a prolonged preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in pig embryos, different cell signaling that governs pluripotency reveals differences compared to mouse embryos. Accordingly, additional or different combination of signaling molecules were needed to support pluripotent

networks in pig. In this chapter, essential signaling molecules for maintaining pluripotency of pig ESCs were defined. Several molecules were pre-screened to assess which molecules improve *in vitro*-survival rate of pig ICMs. Considering that 1) pig is outbred (Only three mouse strains including 129, C57BL/6 and BALB/C which were highly inbred are permissive strain (Hanna et al., 2010b)), 2) inhibitors of ERK and GSK signaling repressed endogenous pluripotent genes during reprogramming of pig cells (Choi et al., 2016; Petkov et al., 2014), pig might be closed to non-permissive species rather than permissive species. So, based on these observations, several signaling molecules, including FGF2, Activin A, Wnt signaling activator (CHIR99021), BMP4 and noggin etc, were chosen. Of those molecules, only FGF2, Activin A and WNT activator could facilitate *in vitro*-survival rate of pig ICM, and finally, pig ESCs could be derived by treating combination of three molecules (Fig 23). Activin/Nodal signaling play an important role in maintaining primed pluripotent state (Hanna et al., 2010b). Wnt signaling is activated in human and mouse early embryos, and pluripotent genes in human and mouse ESCs were elevated by Wnt (Sato et al., 2004). Feeder-free culture of human PSCs could be accomplished by supplementing high concentration of Activin A and Wnt activator (Ludwig et al., 2006; Tomizawa et al., 2011). Although Activin and Wnt signaling participate in maintaining pluripotency, these two factors were not essential to culture human PSCs if feeder cells were underlying (Schatten et al., 2005). However, regardless

of co-culture with feeder cells, simultaneous activations of three cellular signaling were essential to maintain pig ESCs, and if any one factor was absent, ESCs were differentiated (Fig 23). Therefore, it was verified that additional signaling are required to support pluripotency compared to human and mouse.

## 5. Conclusion

Based on results, pig ESCs could be established using chemically defined media. The ESCs expressed pluripotent genes such as OCT4, SOX2 and NANOG, and surprisingly, SSEA1 was highly expressed. It was verified that FGF2, Activin A and Wnt activator could support maintenance of pig ESCs with feeder cells. Although pig ESCs were derived with chemically optimized culture media, to further study, feeder-free culture system of pig PSCs should be developed. These data would accelerate research for PSCs of domestic animals and apply preclinical studies. Finally, deriving pig PSCs will help to improve understanding pluripotency across the species and aid to improve human welfare in aspect of healthy life and agricultural production.

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## CHAPTER 6

### Epigenetic changes of lentiviral transgenes in pig embryonic stem cells

## 1. Introduction

Pluripotent cells are expected to be used as cell therapeutic material in degenerative disorders, and in domestic animals, for generating transgenic animals and xenotransplantation (Keefer et al., 2007). In particular, for transgenic animal and xenotransplantation applications, pigs have been identified as an ideal animal model because of similarities between humans and pigs in physiological and immunological features (Brevini et al., 2007; Hall, 2008). Therefore, many research groups have attempted to create transgenic pigs for producing pharmaceutical proteins and xenotransplantation (Houdebine, 2009; Pierson et al., 2009). In addition, although authentic porcine embryonic stem cells (ESCs) have yet to be established, the characterization of pig ESCs, along with the generation of stable transgenic cell lines, has been studied for a long time (Ezashi et al., 2009; Kues et al., 2013; Park et al., 2013a; Piedrahita et al., 1990b; Son et al., 2009; Yang et al., 2009).

To apply pig ESCs, genetic manipulation via transgenic technologies has been required in stem cell research. Transgenic stem cells using the homologous recombination technique were first reported in mouse embryonic stem cells by Thomas and Capecchi (1987). Subsequently,

researchers have successfully delivered transgenes into pluripotent stem cells using several methods, including electroporation (Eiges et al., 2001), liposomal (Ko et al., 2009) and viral vectors (Ma et al., 2003; Pfeifer et al., 2002), and nucleofection (Hohenstein et al., 2008). However, stably introducing transgenes in these cells has proven difficult because of the low efficiency and cytotoxic side effects. The delivery of transgenes using viral vectors, which are stably expressed, is considered the most useful tool for inducing low cytotoxicity and inserting transgenes into the host genome (Zhang and Godbey, 2006). Moreover, lentiviral vectors belonging to retroviral families are able to infect several types of cells, as well as non-dividing cells (Bukrinsky et al., 1993; Naldini et al., 1996).

Transgenesis in porcine ESCs was first reported by Yang et al. (2009). In contrast to other reports using somatic cell nuclear transfer (SCNT) with transgenic donor cells (Huang et al., 2011; Tan et al., 2011), the transgene [humanized Renilla green fluorescent protein (hrGFP)] was directly delivered into pig ESCs via electroporation. The hrGFP-expressing porcine pluripotent cell lines were successfully established by introducing plasmid vectors via electroporation. Transgenic porcine embryonic germ cell lines were reported by Rui et al. (2006). In this study enhanced green fluorescent protein (EGFP) transgenes were introduced into cells with a

liposomal vector. In other studies involving mouse ESCs, GFP-expressing lines were successfully established using viral vectors (Cherry et al., 2000; Kosaka et al., 2004). However, GFP expression gradually decreased during passaging in mouse ESCs due to DNA methylation. In a similar case of transgenic animal production by lentiviral transduction, transgenes were silenced by DNA methylation in specific cell types (Hofmann et al., 2006; Park et al., 2010).

In previous study, porcine ESC lines were derived from hatched blastocysts, which showed EpiSC-like features. As porcine pluripotent stem cell lines were established, attempt to generate a transgenic pluripotent cell line were conducted. As mentioned above, transgenesis in porcine pluripotent cells is essential for applications such as the production of transgenic pigs and analysis of gene functions. Moreover it is important to characterize and optimize an efficient transfection system. The main purpose of this study was to successfully introduce transgenes into pig ESCs using lentiviral vectors, and to optimize these viral infection conditions. Additionally, it was evaluated the relationship between transgene expression and changes in the DNA methylation status of the inserted lentiviral transgene, particularly in the promoter regions of undifferentiated and differentiated pig ESCs. Consequently, transgenes were successfully

introduced into the cells via lentiviral vectors under various multiplicities of infection. Furthermore, it was confirmed that the expression of inserted lentiviral transgenes was controlled by DNA methylation. This cell line could potentially be used as a donor cell source for transgenic pigs and may be a useful tool for studies of gene functions involving pig ESCs.

## 2. Materials and methods

### Animal care

The care and experimental use of pigs and mice was approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-110509-1 for MEF isolation).

### Culture of porcine embryonic stem cells

Pig ESCs were cultured according to methods described in Chapter 3.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR was conducted according to methods described in Chapter 3. Primers are listed in Table 16.

Table 16. RT-PCR primer sets for pluripotent and differentiation markers in EGFP-transduced pig ESCs

Genes	Sequences	Size
<i>OCT4</i>	5'-AACGATCAAGCAGTGACTATTTCG-3'	153
	5'-GAGTACAGGGTGGTGAAGTGAGG-3'	
<i>NANOG</i>	5'-AATCTTCACCAATGCCTGAG-3'	141
	5'-GGCTGTCCTGAATAAGCAGA-3'	
<i>SOX2</i>	5'-CAACTCTACTGCTGCGGCG-3'	317
	5'-CGGGCAGTGTGTACTTATCCTTC-3'	
<i>CRABP2</i>	5'-CTGACCATGACGGCAGATGA-3'	185
	5'-CCCCAGAAGTGACCGAAGTG-3'	
<i>DES</i>	5'-CCTCAACTTCCGAGAAACAAGC-3'	108
	5'-TCACTGACGACCTCCCCATC-3'	
<i>AFP</i>	5'-CGCGTTTCTGGTTGCTTACAC-3'	483
	5'-ACTTCTTGCTCTTGGCCTTGG-3'	
<i>EGFP</i>	5'-GCGACGTAAACGGCCACAAGTTC-3'	599
	5'-GACCATGTGATCGCGCTTCTCG-3'	
<i>PSIP1</i>	5'-CTCCTCCCTGGGCTTCGGAC-3'	114
	5'-CTCGAGCTGGCCAATGAGGAT-3'	
<i>ACTB</i>	5'-GTGGACATCAGGAAGGACCTCTA-3'	137
	5'-ATGATCTTGATCTTCATGGTGCT-3'	

### **Immunocytochemistry (ICC) and alkaline phosphatase (AP) staining**

ICC and AP staining were performed according to methods described in Chapter 3. Images of stained cells were captured using a LSM 700 Laser Scanning Microscope (Carl Zeiss, Germany) and processed with the ZEN 2009 Light Edition program (Carl Zeiss).

### **Embryoid body (EB) formation and in vitro differentiation**

To evaluate the ability of in vitro differentiation, embryoid bodies were derived from pig ESCs according to methods described in Chapter 3.

### **Karyotype analyses**

Standard G-banding chromosome and cytogenetic analyses were used to karyotype the cell lines. Karyotyping was performed at Samkwang Medical Laboratories (Korea, <http://www.smlab.co.kr/>).

### **Lentiviral vector production**

Lentiviral vectors containing enhanced green fluorescent protein (EGFP) were produced as previously described (Nagano et al., 2002) with

some modifications. HEK 293 LTV cells (Cell Biolabs, USA) were used as the packaging cell line and cultured according to the manufacturer's instructions. Four plasmids were used for the production of lentiviral vectors: self-inactivating lentiviral vector plasmid, pLL3.7; packaging plasmids, pLP1 and pLP2; and envelope plasmid, pLP/VSVG(Invitrogen). These plasmids were transfected into HEK 293 LTV cells using the calcium phosphate precipitation method. Two hours before transfection, the cells were incubated with 25  $\mu$ M chloroquine (Sigma-Aldrich). After 12 h of transfection, the cells were treated with 15% glycerol solution for 90 s and cultured for another 24 h. Culture supernatants were harvested four times for every 12h and stored at 4 °C. Harvested supernatants were filtered using 0.45- $\mu$ m pore filters (Nalgene, USA) and concentrated by centrifugation at  $18,000 \times g$  for 5 h at 4 °C. The virus pellet was dissolved in PESM and stored at -76 °C until use. The viral titer was calculated using the serial dilution method.

### **Lentiviral transgene transduction and flow cytometric analyses**

Three to four days after passaging into new feeder cells, pig ESCs were transduced with lentiviral vectors under various multiplicities of infection (MOIs) of 1-100. Transductions were performed for 24 h in PESM containing 8  $\mu$ g/ml polybrene (Sigma-Aldrich) and concentrated virus.

These transduced pig ESCs were cultured in PESM without virus for another 4–5 days and then passaged. The parts of them were analyzed by flow cytometry. To analyse the EGFP expression level in transduced pig ESCs under each MOI, pig ESC colonies were detached from feeder layers and dissociated into single cells using TrypLE™ Express (Gibco) and fixed with paraformaldehyde. Fixed cells were analyzed using flow cytometry (FACSCalibur) and Cell Quest software (Becton Dickinson, USA). The data were processed using the software FlowJo (Tree Star Inc., USA).

### **Genome methylation assay**

To analyze methylation patterns in CMV promoter regions of lentiviral transgenes, genomic DNA of transduced cells was analyzed by bisulfite sequencing according to methods described in Chapter 3. Primers for the CMV promoter and EGFP regions are listed in Table 17.

Table 17. Primer sets of bisulfite sequencing for CMV promoter and EGFP

	Sequences	Size	CpGs
CMV	5'-ATGATTTTATGGGATTTTTTTATTG-3'	279	14
	5'-ATTCACTAAACCAACTCTACTTATATAAAC-3'		
EGFP	5'- TGGGGTATAAGTTGGAGTATAATTATAATA-3'	259	18
	5'- AACTCCAACAAAACCATATAATC-3'		

## 5-Aza-2'-deoxycytidine (5-AzaC) and trichostatin A (TSA) treatments

Late-passage EGFP-transduced pig ESCs were treated with inhibitors of repressive epigenetic markers to evaluate the relationship between viral transgene expression and epigenetic modifications. 5-Aza-2'-deoxycytidine (5-AzaC; Sigma-Aldrich) and trichostatin A (TSA; Sigma-Aldrich) (DNA methyltransferase and histone deacetylase inhibitors, respectively) were used based on previous reports (Hofmann et al., 2006; Kong et al., 2011), with some modifications. Three to four days after passage into new feeder cells, pig ESCs were treated with 100 nM 5-AzaC (for 48h), 10 nM TSA [for the first 24h and dimethyl sulfoxide (DMSO) only for the second 24h], or DMSO (for 48h; Edwards Life sciences, USA) as a vehicle-only control. After treatments, cells were cultured for 2 more days without inhibitors or DMSO. Finally, treated cells were analyzed using flow cytometry. Bisulfite sequencing was performed with genomic DNA samples extracted as previously described.

## Statistical analyses

All efficiency data from flow cytometric analyses were statistically analyzed using the "R" program (<http://www.r-project.org>). Statistical significance between data was determined by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. Differences were

considered significant when the P-value was less than 0.05.

### 3. Results

#### Lentiviral transgene introduction into porcine embryonic stem cells

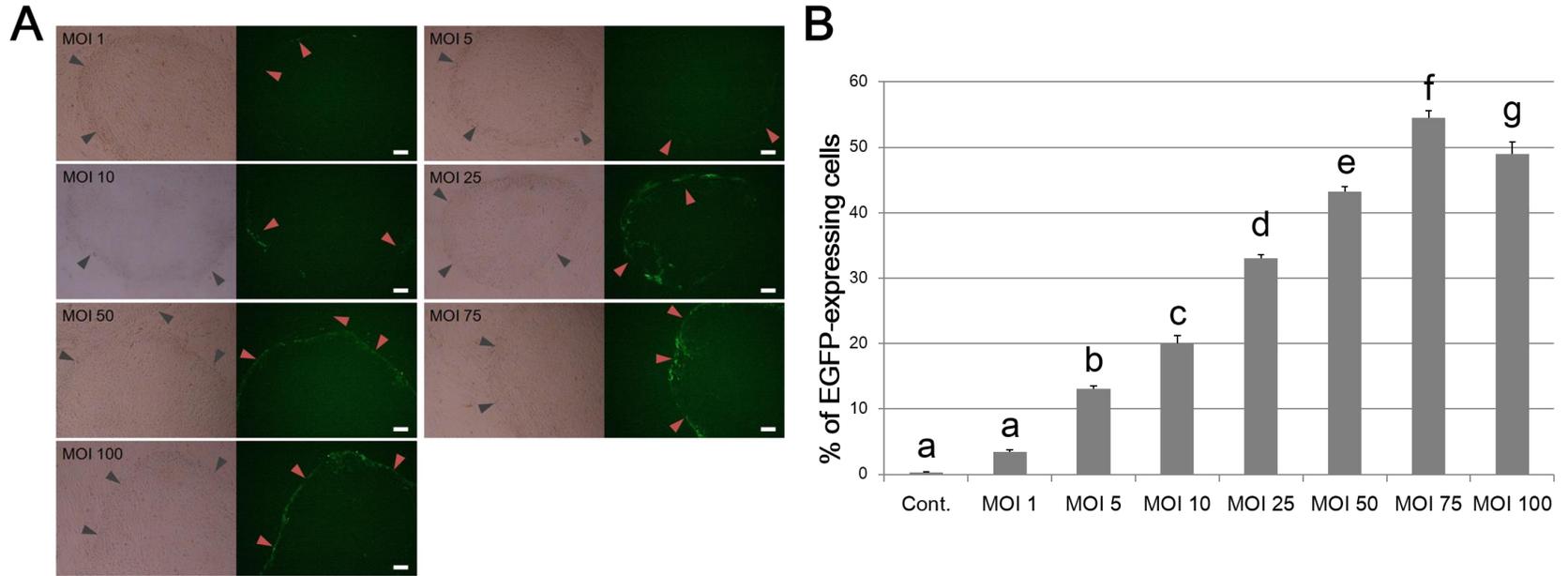
To deliver an exogenous gene into the pig ESCs, lentiviral vectors were employed as a carrier. A lentiviral vector containing EGFP as a reporter was used to investigate the introduction of lentiviral transgenes into pig ESCs. In a previous report, transgenic mouse and mouse ESCs were successfully produced using this lentiviral construct (Rubinson et al., 2003). The estimated titer of produced lentiviral vectors in 293 LTV cells was approximately  $9 \times 10^9$  viral particles/ml. Using these vectors, pig ESCs were transduced for 24 h under several MOIs (1, 5, 10, 25, 50, 75, and 100). Unlike somatic cells, pig ESCs lose their characteristics, including typical morphology and pluripotency, when cultured without feeder cells. Therefore, pig ESCs were transfected while culture on feeder cells and MOIs were calculated based on the number of MEFs and pig ESCs.

EGFP was successfully introduced into the cells via lentiviral vectors under various MOIs. EGFP expression was detected in the cells under an inverted microscope, although expression differences existed depending on the MOI (Fig. 26A). The EGFP expression levels quantified

by flow cytometry significantly increased up to an MOI of 75 in a dose-dependent manner (Fig. 26B). EGFP expression was heterogeneous in the colony of cultured pig ESCs, particularly concentrated on part of the boundary. Therefore, it is possible that expression levels could be increased up to 70–80% in a single colony by selecting the EGFP-expressing part of colonies during subculture (Fig. 27B). Cell characteristics such as viability and proliferation were rarely affected, except at a MOI of 100 in which the cells exhibited cellular toxicity post-transduction.

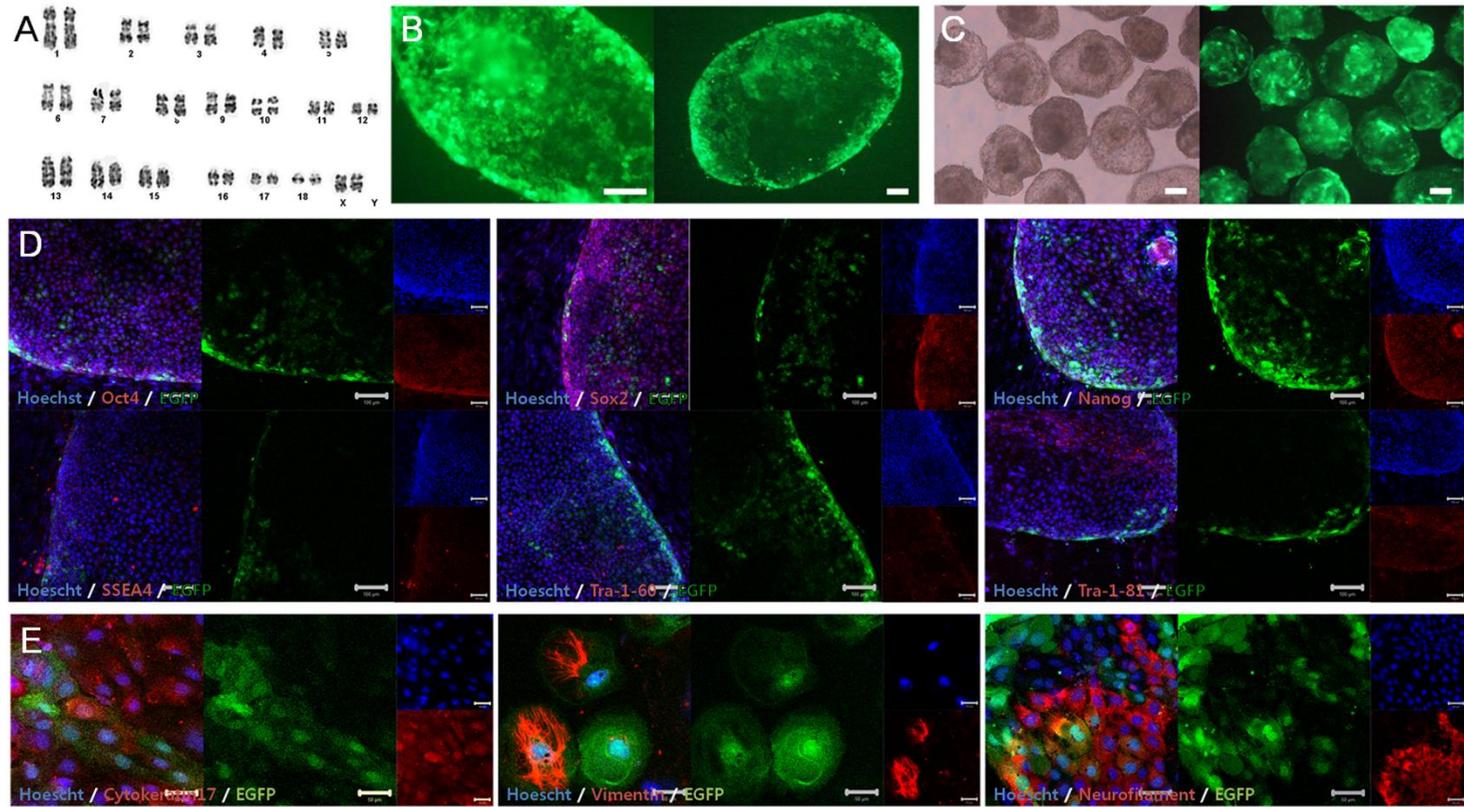
EGFP-transduced pig ESCs were examined whether pluripotency was affected by transduction. Transduced pig ESCs under a MOI of 75 possessing the highest expression levels were used for characterization. Transduced pig ESCs could be stably maintained over an extended time period (>50 passages) using the same general pig ESC culture methods and had an identical karyotype (36 + XX) as before transfection (Fig. 27A). EGFP expression, as well as transcription factors related to pluripotency, was detected in transfected cells at the mRNA and protein levels as measured by RT-PCR and immunostaining, respectively (Fig. 27D). These cells could be differentiated into three germ layers *in vitro* as determined by EB formation (Fig. 27C and E). In brief, transduction-induced abnormalities,

such as physiological features and pluripotency post-transfection, were not detected in EGFP-transduced pig ESCs.



**Figure 26. Lentiviral transduction of pig ESCs.**

Transgene (EGFP) was introduced into pig ESCs using lentiviral vectors (A) The transduced pig ESCs with several MOIs were cultured and passaged stably (left panel: bright field, right panel: EGFP, gray arrows: boundary of colonies, red arrows: EGFP-expressing cells; scale bar = 100  $\mu$ m). (B) Efficiency of transduction was measured using flow cytometry (mean  $\pm$  S.E.M,  $n = 3$ ). Values noted by a-h indicate significant different.



**Figure 27. Characterization of EGFP–transduced pig ESCs.**

Pluripotency and characteristics of EGFP–transduced ESCs were examined. (A) EGFP–transduced pig ESCs have a normal karyotype (36 + XX). (B) The proportion of EGFP–expressing cells increased to 70–80% via selection for the EGFP–expressing part of colonies. (C) Embryoid bodies formed from EGFP–expressing ESCs and expressed EGFP after aggregation (left panel: bright field, right panel: EGFP; scale bar = 100  $\mu\text{m}$ ). Expression of EGFP was measured in undifferentiated (D; scale bar = 100  $\mu\text{m}$ ; passage number: 13) and differentiated state (E; scale bar = 50  $\mu\text{m}$ ; passage number: 23) of ESCs by immunostaining.

## EGFP expression with extended culture and differentiation

The proportion of cells expressing EGFP decreases when cultured for an extended time period without selection. Therefore, additional transfections were attempted to investigate this phenomenon. Thirteen days after transduction, using pig ESCs (passage 12) under a MOI of 75, the proportion of cells expressing EGFP as measured by flow cytometry was  $44.1 \pm 1.28\%$  (Fig. 28A). Decreased EGFP expression was observed to be approximately 10% compared with the results of previous transduction ( $54.5 \pm 1.84\%$  of cells expressed EGFP under MOI of 75; Fig. 26B). Furthermore, when cultured for longer than 13 days without selection, EGFP expression level decreased to  $27.7 \pm 2.42\%$  after 46 days. Unexpectedly, however, the decreased EGFP expression in transduced ESCs was recovered by spontaneous in vitro differentiation. EGFP expression increased from 54.5% and 27.7% to 71.3% and 64.3% in the respective samples as measured by flow cytometry analyses. Note that EGFP expression recovered during differentiation, and the recovered EGFP expression levels of differentiated cells were higher than that of pig ESCs.

Therefore, I hypothesized that these phenomena, including changes in EGFP expression due to extended culture and differentiation, were caused by epigenetic modifications and cellular status. First, to identify the effect of

the cellular state on the expression of transgenes, MEFs and PEFs as somatic cell controls were transduced. Compared to transduced ESCs, the rate of cells expressing EGFP in MEFs or PEFs was  $81.2 \pm 0.70\%$  and  $74.8 \pm 5.71\%$ , respectively, at a MOI of 75, which is similar to the expression level of differentiated cells from pig ESCs (Fig. 29).

To analyze the effects of the epigenetic state among cell types on transgene expression, the DNA methylation patterns of the CMV promoter region in the transgene of each sample were evaluated via bisulfite sequencing. During extended culture, methylation levels in the CMV region increased as the EGFP expression decreased (Fig. 28B). Cells from early passages have much lower methylation levels compared to those from late passage cells. Additionally, early passage cells had irregularly scattered patterns in terms of methylation sites. However, when spontaneously differentiated, the degree of DNA methylation did not change, but GFP expression increased during differentiation. The CMV promoters of differentiated cells were methylated similar to those of non-differentiated cells (Fig. 28B). When transfected somatic cells such as MEFs and PEFs were analyzed, the methylation pattern of the CMV promoter was completely unmethylated (Fig. 30).

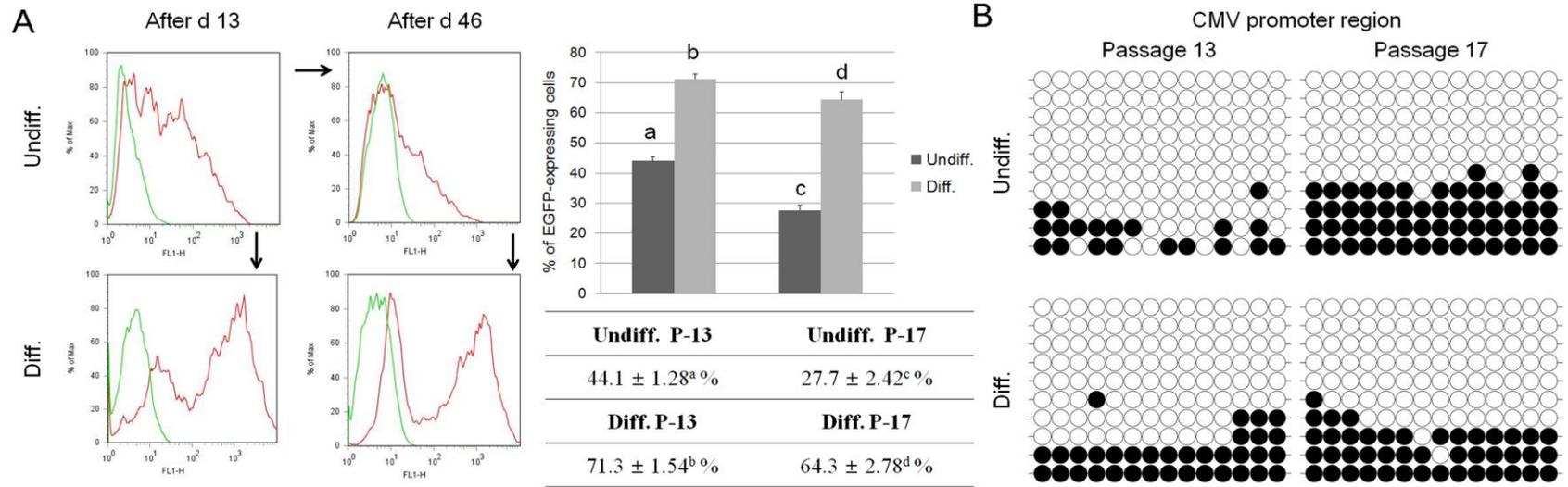


Figure 28. Change in EGFP expression during extended culture and *in vitro* differentiation.

(A) EGFP expression levels of transduced pig ESCs declined during extended culture. The decreased expression recovered during *in vitro* differentiation. (B) To analyze the effects of the epigenetic state on transgene expression, the DNA methylation patterns of the CMV promoter region in the transgene of each sample were evaluated via bisulfite sequencing. Each circle indicates individual CpG dinucleotides. White and dark circles represent unmethylated and methylated CpGs, respectively. Each row represents one individual clone of amplified PCR products. Values noted by a-h indicate they are significantly different. Data are the mean  $\pm$  S.E.M. ( $n=3$ ).

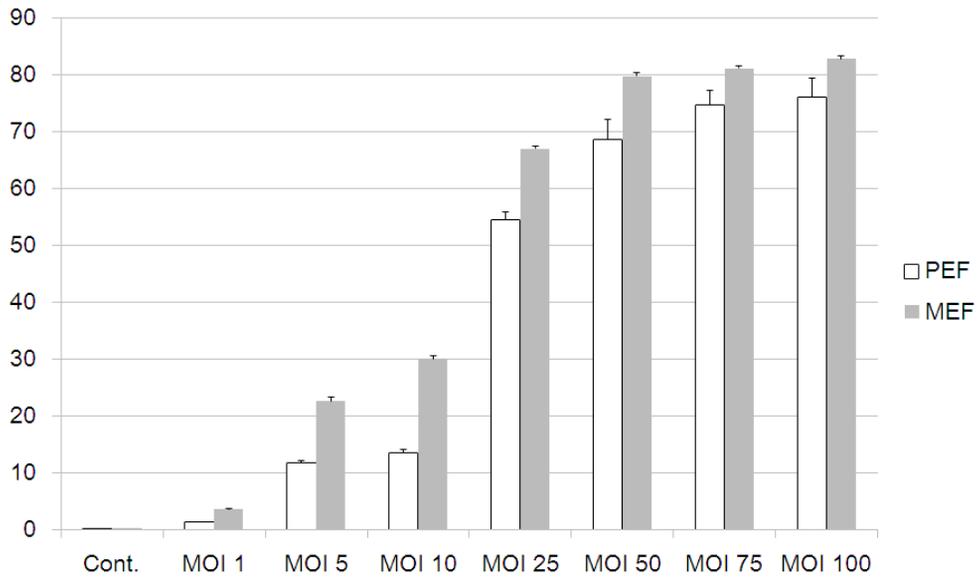
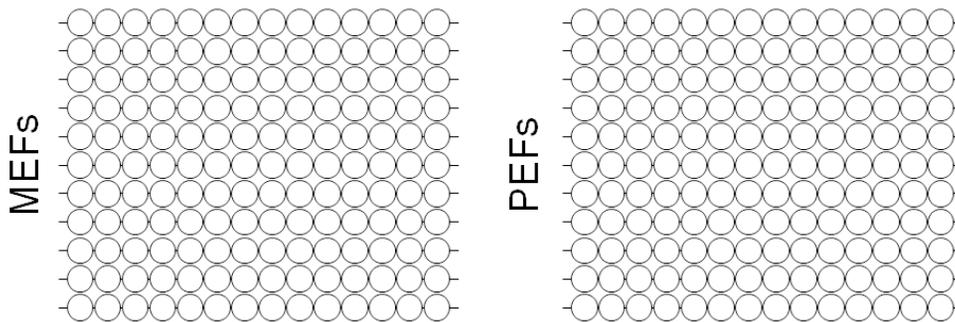


Figure 29. Lentiviral transduction efficiency of MEFs and PEFs at various MOIs.

MEFs and PEFs were transduced by lentiviral vectors carrying EGFPs with various MOIs. Efficiency of transfection was measured by flow cytometry (mean  $\pm$  S.E.M,  $n=3$ ).

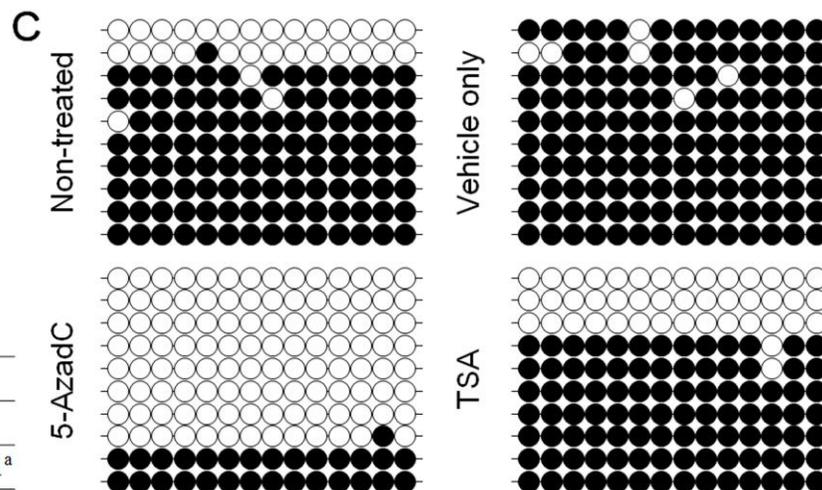
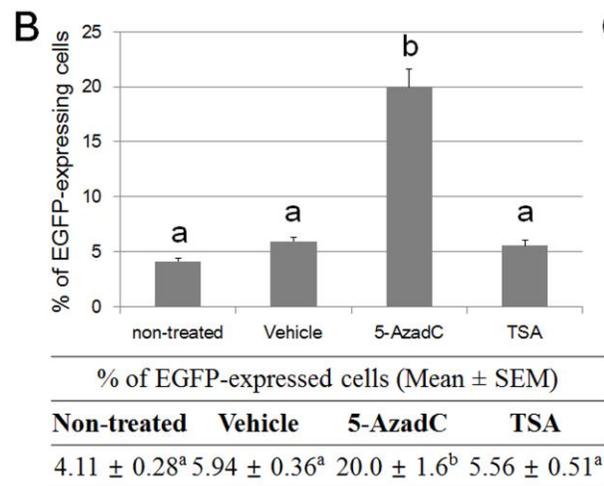
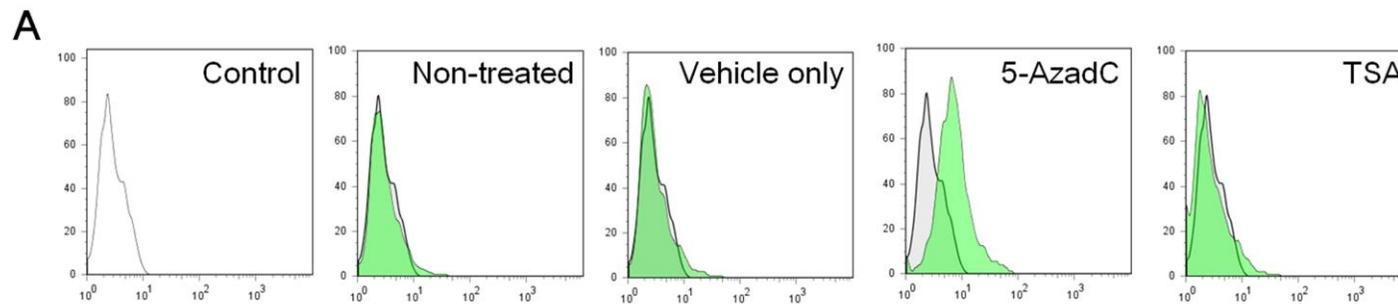


**Figure 30. Methylation level of the CMV promoter region in MEFs and PEFs.**

Methylation level of the CMV promoter region in MEFs and PEFs were examined by bisulfite sequencing. Each circle is individual CpG dinucleotide and white and dark circles indicated unmethylated and methylated CpGs, respectively. Each row is one individual clone of PCR products.

## Decreased EGFP expression during extended culture due to DNA methylation of promoter regions

To evaluate the effect of methylation on EGFP expression during extended culture, pig ESCs were treated with inhibitors of repressive epigenetic marks. Control and experimental groups consisted of four: non-treated, DMSO-, 5-AzadC-, and TSA-treated samples. Late passage (passage 46) pig ESCs expressed low levels of EGFP ( $4.11 \pm 0.28\%$ ). Samples treated with DMSO and TSA did not show a recovery of EGFP expression and showed similar expression levels as nontreated samples. Although these two groups did not affect expression of the transgenes, the silenced EGFP expression recovered to  $20.0 \pm 1.6 \%$  upon treatment with 5-AzadC (Fig. 31A, B). The effect of 5-AzadC on the expression of transgenes was determined via bisulfite sequencing. Among the four groups, CMV promoter region demethylation occurred after 5-AzadC treatment (Fig. 31C).



**Figure 31. Silenced transgenes can be reactivated by treatment with the DNA methylase inhibitor, 5'-aza-2'-deoxycytidine (5-AzadC).**

To evaluate the effect of methylation on EGFP expression during extended culture, pig ESCs were treated with DNA methylase inhibitors. (A) 5-AzadC treatment reactivates the silenced transgene, EGFP. (B) The recovered expression level was measured by flow cytometry. (C) 5-AzadC demethylated the methylated CMV promoter region. Values noted by a-h indicate they are significantly different. Data are the means  $\pm$  S.E.M. ( $n=3$ ).

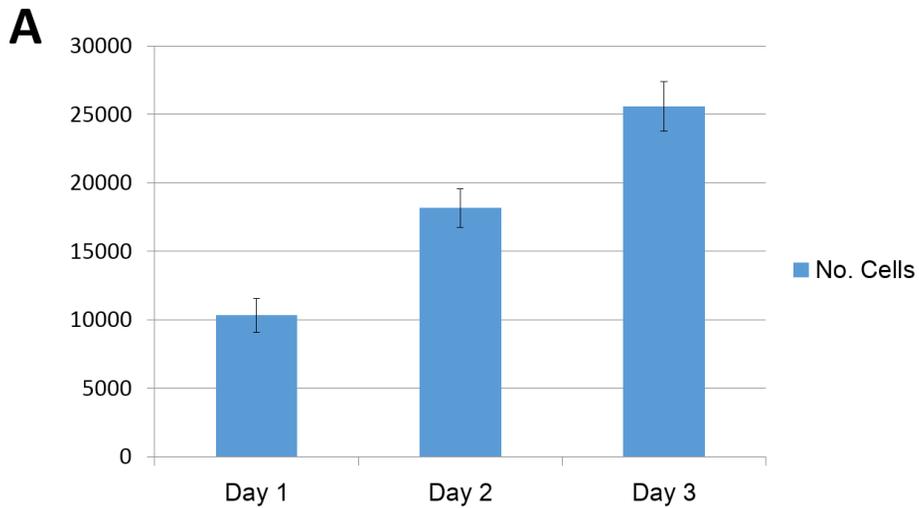
## 4. Discussion

### Transgenesis of porcine pluripotent stem cells

Because of the physiological and immunological similarities that exist between pigs and humans, porcine pluripotent cell lines have been identified as important candidates for preliminary studies on human disease as well as a source for generating transgenic animals (Brevini et al., 2007; Hall, 2008; Houdebine, 2009). Therefore, the establishment and characterization of pig ESCs, along with the generation of stable transgenic cell lines, are vitally important. In previous studies, researchers developed a reproducible method for the establishment of pig ESCs and iPSCs and determined that similar to humans, pig is a non-permissive species and pig pluripotent stem cells show a primed pluripotent state regarding marker gene expression patterns and X chromosome inactivation (Park et al., 2013a; Son et al., 2009).

To achieve such goals, stem cell engineering involving the introduction of transgenes into cells has been developed. In mice and humans, many transfection methods, including viral vectors, liposome-mediated gene delivery, and electroporation, have been studied (Eiges et al., 2001; Ko

et al., 2009; Ma et al., 2003; Pfeifer et al., 2002). In the porcine model, however, much fewer studies have been reported. In the first reported study, EGFP-transduced embryonic germ cells (EGCs) were established by the introduction of plasmid vectors using liposomal vectors (Rui et al., 2006). In a second study, plasmid vectors containing humanized renilla green fluorescent protein (hrGFP) were introduced into pig ESCs via electroporation (Yang et al., 2009). Although GFP-expressing pig ESC lines were established via electroporation, transfection efficiency was very low (only three stably GFP-expression lines from 12 trials), and a GFP-expressing line was not obtained by retroviral and liposome-mediated transfection. Therefore, this study was undertaken to develop a system for efficient introduction of transgenes into embryo-derived pluripotent stem cells. Previous studies showed that pig ESCs have very high cytotoxicity when treated with liposome-mediated and electroporation methods, as well as low colony-forming rates from dissociated single cells, similar to humans (Amit et al., 2000; Park et al., 2013a). Therefore, viral vectors were selected because plasmid vectors are not suitable for generating a stable transgene expression cell line. And because lentiviral vectors can infect dividing cells as well as non-dividing cells, lentiviral vectors were chosen for transfection of pig ESCs having long doubling times of approximately 36h (Fig. 32), similar to human ESCs (Buecker et al., 2010; Naldini et al., 1996).



**B**

	No. cells (Mean ± SEM)	Average No. colonies	Doubling Time
Day 1	10333 ± 1244	7	
Day 2	18167 ± 1402	7	~36.7h
Day 3	25583 ± 1793	6.7	

**Figure 32. Doubling time of porcine embryonic stem cells**

ESC samples were collected for 3 days every 24 hrs for measuring doubling time, and the number of cells was counted with hemocytometer. (A) ESCs in log phase was proliferated with a constant rate. (B) A measured doubling time of pig ESCs is about 36.7 hrs.

## Lentiviral vector is a useful tool for transgenesis of pig ESCs

Historically, although retro- and lentiviral vectors have been widely used for the production of transgenic animals and the establishment of transgenic pluripotent stem cells, silencing of the viral transgenes caused by epigenetic modifications and trans-acting factors remains an obstacle to be resolved (Cherry et al., 2000; Hofmann et al., 2006; Hotta and Ellis, 2008; Kosaka et al., 2004; Laker et al., 1998; Park et al., 2010; Whitelaw et al., 2008). To overcome these silencing problems in cells, particularly embryos and pluripotent cells, various approaches have been developed. The major difficulties post-transfection, the repression of long terminal repeats (LTRs) in the viral genome known as transcriptional regulator by de novo DNA methylation and repressive trans-acting factors (Hoeben et al., 1991; Loh et al., 1990), were resolved using bidirectional or internal promoters such as the CMV promoter, which is strongly expressed in various tissues, (Golding and Mann, 2011; Hamaguchi et al., 2000) and the deletion or modification of LTR sequences to prevent recruitment of repressive trans-acting factors (Laker et al., 1998; Miyoshi et al., 1998). Using regulatory elements, including woodchuck hepatitis virus response element (WRE) (Zufferey et al., 1999), HIV FLAP (Arhel et al., 2007), and matrix attachment region (MAR) (Bode et al., 2000), which are responsible for transcript stabilization and the translocation of provirus into nuclear and DNA loop formation, respectively, also improve transgene expression.

The lentiviral construct used in this study is pLL3.7 consisted of EGFP as a marker gene with the CMV promoter and cis-acting elements including FLAP, WRE, 3'SIN-LTR and multiple cloning site for shRNA. The reason this construct was chosen is as follows: 1) This vector has been proven to work efficiently in mouse embryos, primary cells and embryonic stem cell lines, and transgenic mice have been generated using mouse ESCs and embryos transfected with this vector (Rubinson et al., 2003). 2) In previous transfection studies involving porcine pluripotent cells, the CMV promoter was used for the establishment of transgene-expressed cell lines, these resulting transduced cell lines stably expressed the transgene for more than 90 months (Rui et al., 2006; Yang et al., 2009). When pig ESCs were transfected with this vector under various MOIs, cytotoxicity was not detected up to a MOI of 75. At a MOI of 100, however, cytotoxicity occurred and reduced the number of EGFP-expressing cells (Fig. 26B). Moreover, EGFP expression was stronger at the edge of the colonies (Fig. 26A), likely because of metabolic up-regulation and high cell density due to proliferation of dividing cells at the edge of the colonies.

#### **Lentiviral transgene expression were affected by epigenetical environment**

To examine the decline in EGFP expression during extended culture,

It was assessed whether transgene expression was affected by DNA methylation or histone acetylation, which are known to epigenetically regulate gene expression. Methylated cytosine in DNA represses gene expression via recruitment of proteins associated with heterochromatin such as MeCP2. Acetylation on histone tails activates gene expression by increasing the negative charge of histones (Hotta and Ellis, 2008). Bisulfite sequencing data indicated that the DNA methylation level of the promoter region and expression of the transgene were negatively correlated because DNA methylation in the CMV promoter region increased with a concomitant decrease in EGFP expression (Fig. 28). However, methylation levels of the EGFP region were not related to transgene expression (data not shown), in contrast to a previous report involving transgenic animals (Hofmann et al., 2006). The EGFP region was hypomethylated regardless of EGFP expression.

The expression level of transgenes is dependent upon the vector construct, transfection methods used and cell types. It is therefore important that the characterization of vector activities in various cell types is given attention. In the case of the CMV promoters used in this study, differences in expression patterns have been reported in several papers via the characterization of the transcriptional activities in ESCs. Some studies

showed that the CMV promoter is active in human or mouse ESCs during long term culture (Bagchi et al., 2006; Rubinson et al., 2003; Ward and Stern, 2002; Zeng et al., 2003). Conversely, other studies have reported that CMV-driven transgene expressions are rapidly down-regulated or inactive in human or mouse ESCs. Silenced transgenes were not reactivated during differentiation (Liew et al., 2007; Liu et al., 2009; Norrman et al., 2010; Wang et al., 2008). In porcine studies, CMV-driven expression of GFP was stably expressed during the propagation of pig ESCs (over 20 months) (Yang et al., 2009), and transgenic porcine embryonic germ cell lines were successfully established using a CMV-EGFP construct (Rui et al., 2006). My data has shown that the CMV-driven expression of GFP was progressively down-regulated by DNA methylations and reactivated during differentiation by transacting factors without changes of DNA methylation levels. This result is consistent with previous findings of the latter cases in humans and mice with the exception of the up-regulation of transgenes during differentiations. The differences of my results with previous porcine studies may be as a result of the different vector construct and transfection method used. The reactivation of silenced transgenes during differentiation that observed in this study indicate that trans-acting factors, as well as DNA methylations, affect transgene silencing in undifferentiated pig ESCs.

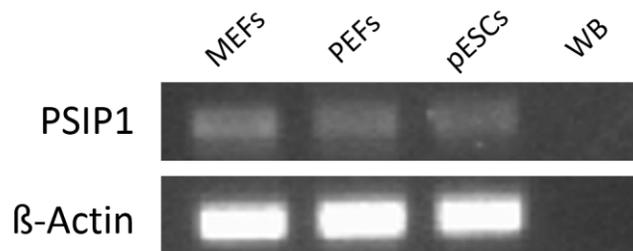
When transduced cells were treated with 5-AzadC and TSA, inhibitors of DNA methylation and histone deacetylases, respectively, 5-AzadC allowed reactivation of silenced expression, but TSA did not (Fig. 31A and B). Bisulfite sequencing of the promoter regions showed that 5-AzadC-treated samples were hypomethylated in CMV promoter regions compared with the other treated groups, which had hypermethylated promoters. These results clearly demonstrate that decreased transgene expression is related to DNA methylation in promoter regions, not histone modifications.

#### **Expression of lentiviral transgenes was regulated by cellular state**

In addition to silencing of gene expression during extended culture, the transfection efficiency of pig ESCs was lower than that of other embryo-originated somatic cells. However, PSIP1, a protein that participates in lentiviral provirus integration into host genomes (Engelman and Cherepanov, 2008), was examined by RT-PCR and no difference was observed among pig ESCs, PEFs, and MEFs (Fig. 33). This result suggests that the low efficiency of transfection is not related to the low integrity of the provirus. In addition, because the copy number of an inserted lentiviral construct could affect EGFP expression, a correlation between EGFP expression level and transgene copy number was verified. However, because of heterogeneity in

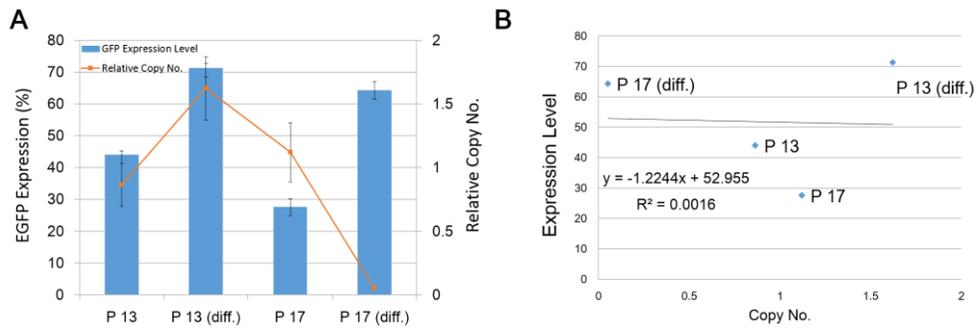
the transduced cells due to the random insertion of multiple copies, it is hard to quantitatively measure the copy number of inserted transgenes. So, transgene copy number was relatively quantified by real-time RCR. Real-time PCR results showed that changes in EGFP expression and transgene copy number are not correlated (Fig. 34). And also reactivation of silenced transgenes during differentiation clearly demonstrated that low expression levels are not due to low integrity (Fig. 28A). Note that DNA methylation levels of the CMV promoter region did not change during differentiation although transgene expression increased. Undifferentiated and differentiated cells at the same passage have similar levels of DNA methylation in the CMV promoter. This suggests that the recruitment of transcription factors in differentiated cells or the down-regulation of repressive trans-acting factors, rather than demethylation of the promoter region, is involved in the unmethylated open chromatin for reactivation of silenced transgenes. In fact, many binding sites for putative transacting factors such as cyclic AMP-response elements (CRE), NF-Kappa B, AP-1, serum response elements exist in the CMV promoter (Meier and Stinski, 1996). These sites could be predicted by the program TFSEARCH ver. 1.3 (<http://www.cbrc.jp/research/db/TFSEARCH.html>). This mechanism is supported by the phenomenon that more methylated cells in late passages showed lower reactivation of transgenes. Therefore, low transgene expression in pluripotent cells is likely because of trans-acting factors as

well as DNA methylation.



**Figure 33. Expression level of *PSIP1* in MEFs, PEFs, and pig ESCs**

To investigate relationship between transgene expression and provirus integration, expression of *PSIP1* in MEFs, PEFs and pig ESCs was measured by semi-quantitative RT-PCR. And, as a reference gene,  $\beta$ -*ACTIN* was used.



**Figure 34. Correlation between the copy number of inserted transgenes and the EGFP expression level**

To assess whether the copy number of an inserted lentiviral construct could affect EGFP expression, a correlation between EGFP expression level and transgene copy number was verified. (A) Expression and relative copy number of EGFP in undifferentiated and differentiated cells (passage 13 and 17) were measured by flow cytometry and qPCR, respectively. (B) A correlation assay between the expression levels and copy numbers was performed.

## 5. Conclusion

In conclusion, I was able to successfully introduce an EGFP transgene into these cells using lentiviral vectors. Transgene expression in pig ESCs was altered during maintenance and differentiation due to epigenetic changes. Although modified lentiviral vectors containing regulatory elements were used, it could not be prevent the transgene silencing that occurred due to DNA methylation and trans-acting factors. Nonetheless the silenced transgene expression was reactivated by differentiation and treatment of 5-AzadC. Therefore, this system could be applied for the short term analysis of gene function in pig ESCs, induction of differentiation, tracking of transplanted cells and the production of chimeras. Finally, this cell line could potentially be used as a donor cell source for transgenic pigs and serve as a useful tool for studies involving pig ESCs and gene therapy in humans, as well as aid in the understanding of epigenetic regulation of transgenes.

## CHAPTER 7

### Generation of neural progenitor cells from pig embryonic germ cells

## 1. Introduction

In 1998, pluripotent stem cell (PSC) researches entered on a new phase by establishment of human embryonic stem cells (ESCs) (Thomson et al., 1998). The derived human ESCs made people think that pluripotent cells could be used for regenerative medicine by tissue engineering. This trend was accelerated by establishment of human induced pluripotent stem cells (iPSCs) and cloned ESCs in 2007 and 2013, respectively (Tachibana et al., 2013; Yu et al., 2007). Accompanied with generation of human PSCs, methods for differentiation into various type of cells have been developed (reviewed in (Tabar and Studer, 2014)). Recently, in addition to differentiation into specific cell types, putative organs composed of multi-cellular tissues, named organoid, could be generated *in vitro* (reviewed in (Yin et al., 2016)). Various type of tissues including intestinal, brain, eye and kidney have been researched. Because they more closely resemble *in vivo*–organs and tissues than singly type of cells, these organoids might provide *in vitro*–tools for drug screening, disease modeling and organ development models. As a clinical trial of human PSCs for cell therapy as mentioned above, it makes the research of animal PSCs involving differentiation and transplantation study more important as a preliminary.

In this reason, many researchers have been attempted to convert the porcine PSCs into several differentiated cells with transplantation of the differentiated cells into the pigs. First of all, hepatocytes have been derived from pig iPSCs and ESCs (Ao et al., 2014; Park et al., 2015). The differentiated cells from iPSCs and ESCs have molecular and functional similarity with hepatocytes. They have abilities including lipid metabolism, glycogen storage and LDL uptake. Several studies have tried to transplant iPSCs or differentiated cells into pig disease models. Rod photoreceptors and retinal pigment epithelial cells were produced from pig iPSCs, and then these cells were transplanted into subretinal space of pigs (Sohn et al., 2015; Zhou et al., 2011). Transplanted cells were successfully resided in subretinal region. Another groups used undifferentiated pig iPSCs to treat myocardial infarction and myocardial ischemia (Li et al., 2013; Zhang et al., 2014a; Zhou et al., 2014). The engrafted iPSCs ameliorated symptoms via differentiation into vessel cells (Li et al., 2013). However, undifferentiated PSCs have potential to form tumors when engrafted into body (Lee et al., 2013), it needs to be careful for implant of undifferentiation PSCs. Recent studies showed that PGCs and skeletal myotubes could be induced from pig iPSCs (Genovese et al., 2017; Wang et al., 2016). More diverse type of cells would be derived from pig PSCs and various transplantation models are going to develop in near future.

Neuron cells derived from PSCs such as dopaminergic neuron and retinal cells have been anticipated as alternative cell sources for cell therapy instead of fetal tissues (Osakada et al., 2008). PSCs have been able to differentiate into various types of neurons including dopaminergic neurons (Lee et al., 2000b), motor neurons (Wichterle et al., 2002), cerebral cortex (Eiraku et al., 2008) and pituitary (Suga et al., 2011) and cortical pyramidal neurons (Espuny–Camacho et al., 2013). In pig, neuronal differentiation have successfully induced from *in vitro*–cultured ICM and iPSCs (Gallegos–Cardenas et al., 2015; Puy et al., 2010). Here, I attempted to derive neuronal progenitor cells from pig embryonic germ cells (EGCs). Similar with other species, neuronal progenitor cells were successfully induced by treatment of retinoic acid and these cells expressed neuronal markers such as PAX6, NESTIN and SOX1. Finally, production of transgenic pig for disease models as well as differentiation methods will provide basic preclinical data for human regenerative medicine and lead to success of stem cell therapy.

## 2. Materials and methods

### **Animal care**

The care and experimental use of pigs and mice was approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-16120-9-1 for MEFs isolation). Pregnant ICR mice were purchased from SAMTACO BIO Inc., Korea. The mice were taken care according to standard protocol of IACUC and sacrificed by cervical dislocation after anesthesia.

### **Culture of pig embryonic germ cells**

Pig embryonic germ cells (EGCs) were derived from the genital ridges of a porcine dpc 30 fetuses. Established EGCs were cultured according to method described in Chapter 3.

### ***In vitro* differentiation into neural progenitor cells**

Differentiation of neural lineage was accomplished according to serum-free floating culture of EB-like aggregates (SFEB) methods with some modifications (Watanabe et al., 2005). Cultured EGCs were

dissociated into single cells using 0.25% trypsin/EDTA solution (Wetgene) and cultured in Ultra-Low attachment plates (Sigma Aldrich, MO, USA) with STEMdiff™ Neural Induction Medium (STEMCELL, Vancouver, Canada) containing 5µM retinoic acid (RA) and SMAD signaling inhibitors (400µM Noggin and 2µM SB431542) for 5 days. After suspension culture, dissociated cells were aggregated and formed embryoid bodies. Cultured embryoid bodies were seeded on BD Matrigel™ (BD Biosciences, NJ, USA)-coated plates and cultured for 8–11 days with STEMdiff™ Neural Induction Medium containing 10ng/ml FGF2, RA, and SMAD inhibitors. After 8–11 days, differentiated cells were used for immunostaining or qPCR analysis.

### **Quantitative real-time polymerase chain reaction (qPCR)**

Total RNA from individual samples was extracted using TRIzol® reagent (Invitrogen, MA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using a High-capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions, producing a final volume of 20 µl. Quantitative PCR was conducted according to methods described in Chapter 3. Primer sets for neuronal markers are listed in Table 18.

Table 18. Primer sets for neuronal markers

Genes	Primer sequence	size
<i>PAX6</i>	5'– AGAGAAGACAGGCCAGCAAC –3'	169
	5'– GGCAGAGCACTGTAGGTGTT –3'	
<i>Nestin</i>	5'– TGCCTGGGGGAGGAATCTTTT –3'	252
	5'– CTCTTCAGCCAGGTTGTCGC –3'	
<i>PLAG1</i>	5'– CAGCCAAGATTGGCCACAATG –3'	116
	5'– AGCCATGTGCCTGATGACAGA –3'	
<i>DACH1</i>	5'– CAGGCTTTCGACCTGTTTCCT –3'	126
	5'– CAGTCCCCTCAAGATGCGAA –3'	
<i>ACTB</i>	5'– GTGGACATCAGGAAGGACCTCTA –3'	131
	5'– ATGATCTTGATCTTCATGGTGCT –3'	

## **Immunostaining**

Differentiated cell samples were preincubated for 10 min at 4°C and fixed with 4% paraformaldehyde for 30 min. After washing twice with Dulbecco's phosphate-buffered saline (DPBS; Welgene), immunostaining was performed according to methods described in Chapter 3. Images of stained cells were captured using an inverted fluorescence microscope.

## **Statistical analysis**

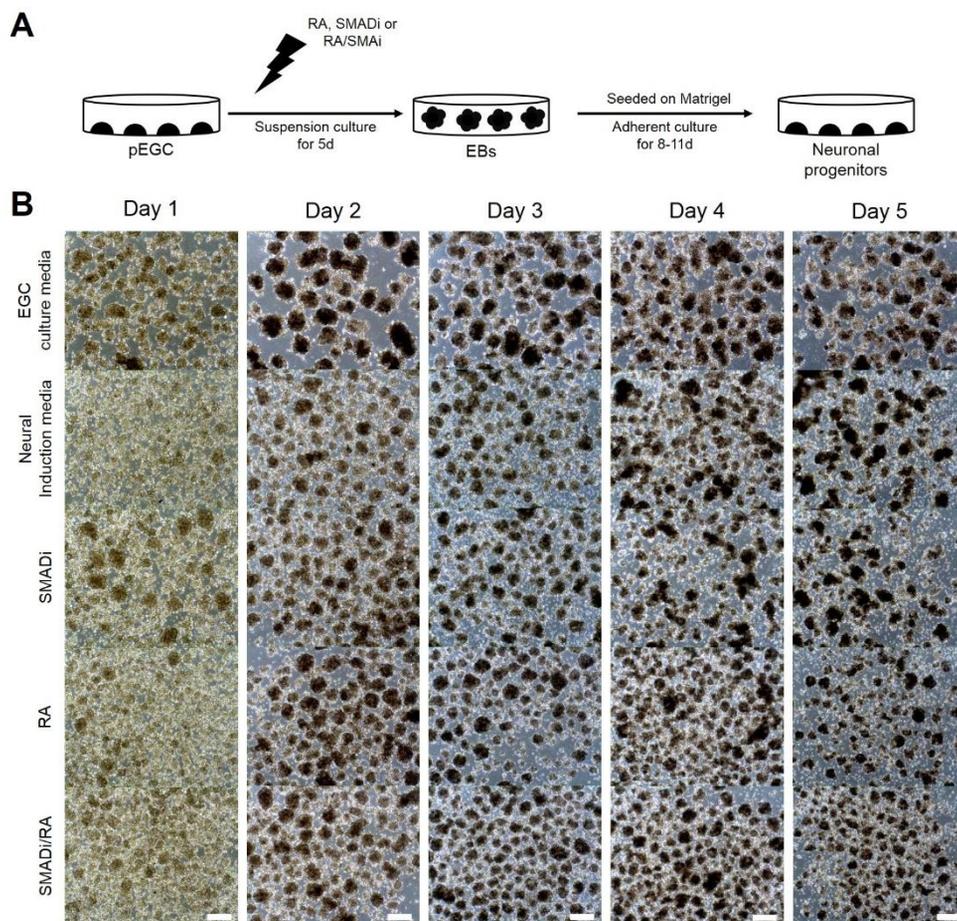
All gene expression data from qPCR analyses were statistically analyzed using GraphPad Prism 6 statistical software (GraphPad Software, CA, USA). Statistical differences between datasets were determined by one-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) tests. Differences were considered significant at  $P < 0.05$ .

### 3. Results

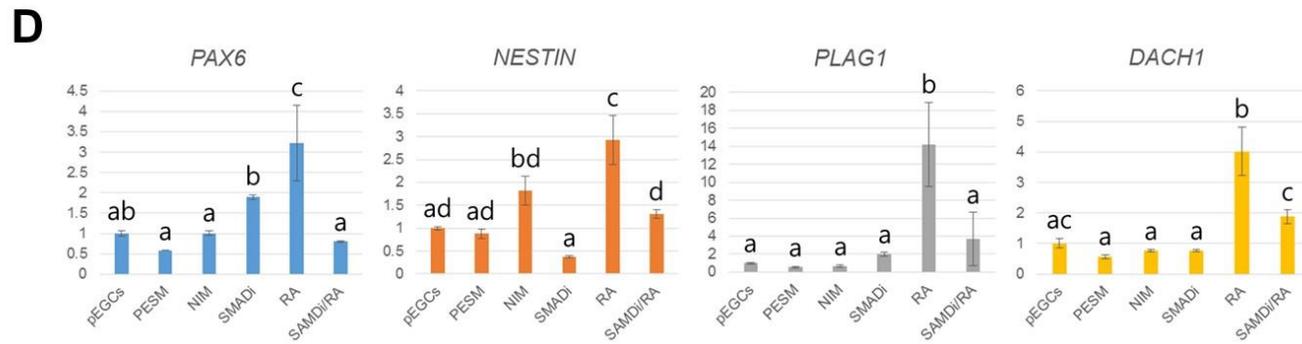
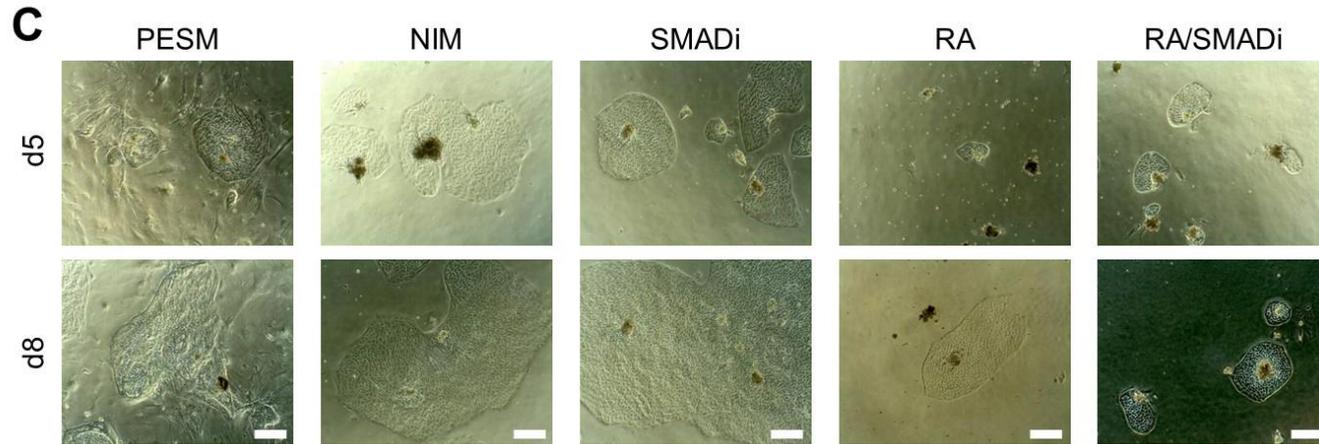
#### **Fig EGCs could be differentiated into neural lineage by treating retinoic acid.**

To apply pig PSCs for therapeutic model, developing methods for induced differentiation into specific lineages are important. In this reason, this study aimed to develop method for inducing neural lineage from pig EGCs. It was verified that several signaling including SMAD inhibition and retinoic acid are involved in differentiation into neural cells from pluripotent cells (Chambers et al., 2009; Parsons et al., 2011). So, firstly, to find which molecules are most efficient in inducing neural cells from pig EGCs, SMAD signaling inhibitors and retinoic acid were selected (Fig 35A). Embryoid bodies were successfully formed by suspension culture as presented in Fig 35B and, after plating onto matrigel, they were cultured for 8 days (Fig 35C). As measured by qPCR, genes expressed in neural progenitors were highly expressed in retinoic acid-treated group compared with other groups (Fig 35D). When examined with various concentration of retinoic acid (Fig 36A and B), expression of neural markers were most efficiently up-regulated in 5  $\mu$ M-treated groups (Fig 36C). Five  $\mu$ M retinoic acid successfully induced neural lineage-related genes during suspension culture (Fig 36D). And also, FGF2 treatment during adherent culture facilitated proliferation of neural progenitor cells (Fig 37). Induced neural progenitor cells expressed neural

markers such as SOX1, PAX6, ZO1 and NESTIN as determined by immunostaining (Fig 38). Taken together, I developed methods for inducing neural lineage from pig EGC by treating retinoic acid.

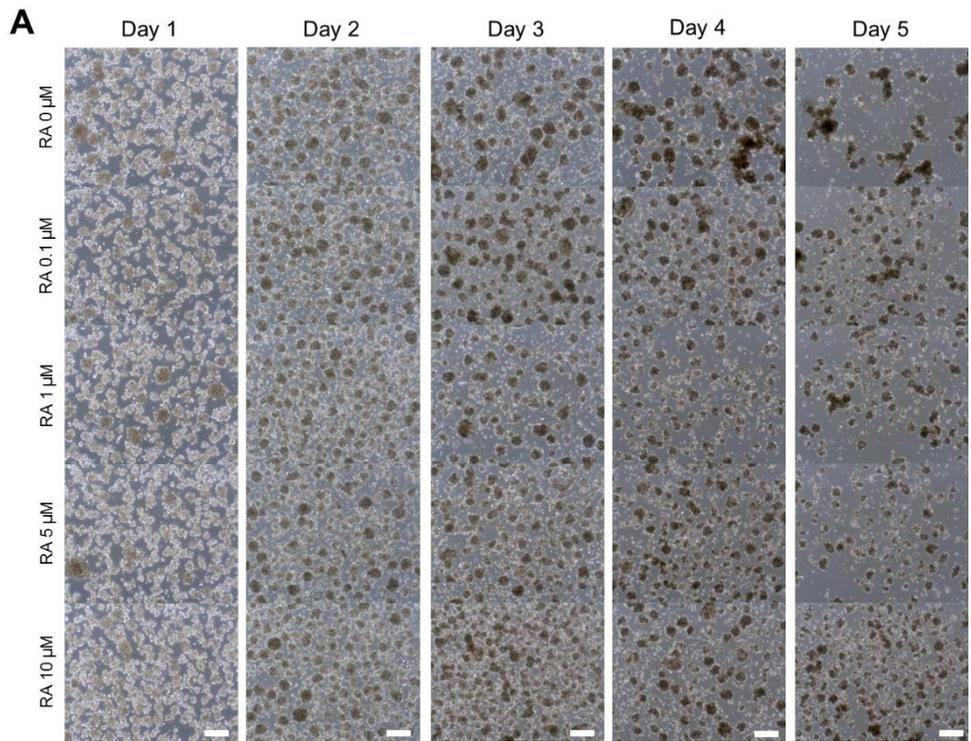


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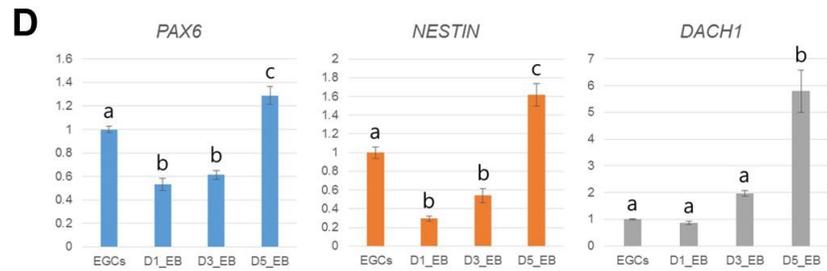
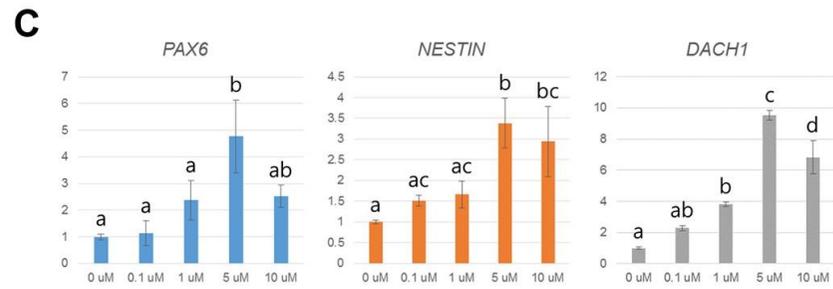
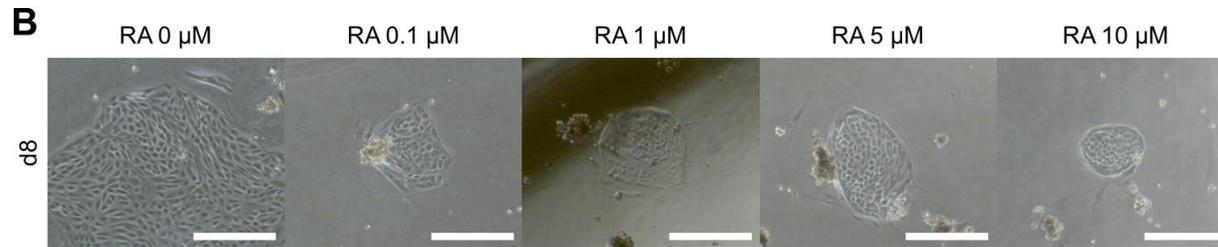


**Figure 35. Effects of culture conditions on neural differentiation in pig EGCs.**

(A) Differentiation of neural lineage was accomplished according to serum-free floating culture of EB-like aggregates (SFEB) methods with some modifications. To find which molecules are most efficient in inducing neural cells from pig EGCs, SMAD signaling inhibitors and retinoic acid were selected. (B) Embryoid bodies were formed by suspension culture. (C) After plating onto matrigel, they were cultured for 8 days. (D) As measured by qPCR, genes expressed in neural progenitors were highly expressed in retinoic acid-treated group compared with other groups. Scale bar = 400  $\mu\text{m}$ .

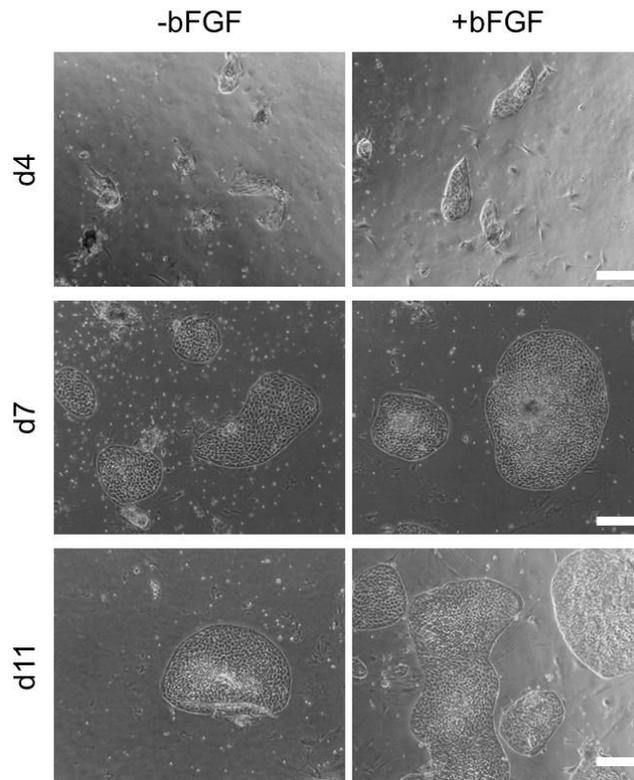


(continued)



**Figure 36. Effects of RA concentration on neural differentiation in pig EGCs.**

(A, B) To optimize culture conditions, embryoid bodies were formed with various RA concentrations and then cultured on Matrigel for 8 days. (C) When examined with various concentration of retinoic acid, expression of neural markers were most efficiently up-regulated in 5  $\mu\text{M}$ -treated groups. (D) Five  $\mu\text{M}$  retinoic acid induced neural lineage-related genes during suspension culture. Scale bar = 400  $\mu\text{m}$ .



**Figure 37. Effects of FGF2 on growth of neural progenitor cells.**

FGF2 treatment during adherent culture facilitated proliferation of neural progenitor cells. Scale bar = 400  $\mu\text{m}$ .

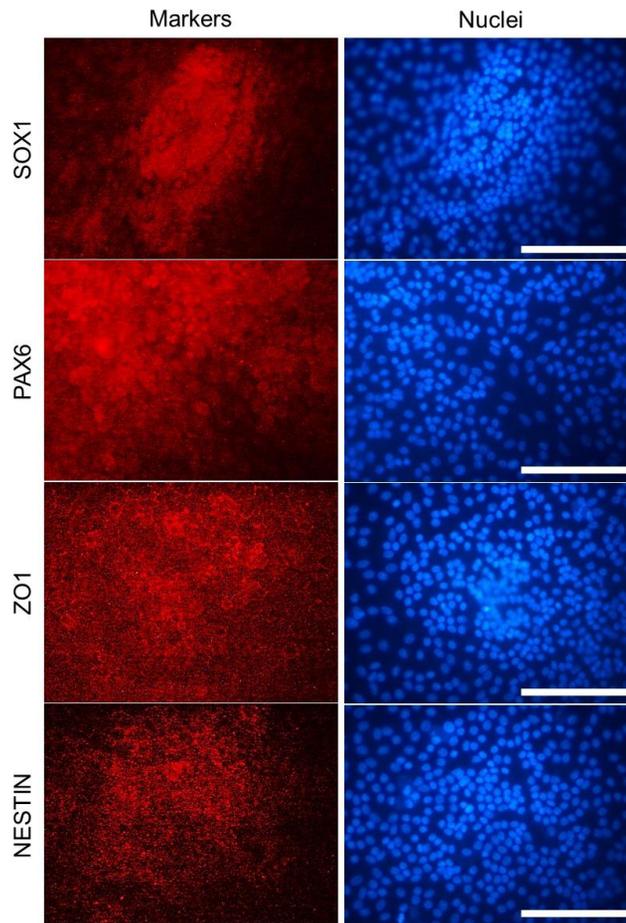


Figure 38. Expression of neural markers as determined by immunostaining.

Expression of neural markers such as SOX1, PAX6, ZO1 and NESTIN was determined by immunostaining. Scale bar = 200  $\mu\text{m}$ .

## 4. Discussion

Human pluripotent stem cell-derived neural cells such as dopaminergic neuron and retinal cells have been anticipated as alternative cell sources for cell therapy instead of fetal tissues (Osakada et al., 2008). Induction of neuronal lineages from pluripotent cells have been accomplished by co-culture with feeder cells made of PA6 stromal cells or culture with serum-free media containing signaling molecules including retinoic acid and inhibitors of SMAD signaling (reviewed in (Schwartz et al., 2008)). Depend on treating combination of signaling molecules, pluripotent stem cells (PSCs) have been able to differentiate into various types of neurons including dopaminergic neurons (Lee et al., 2000b), motor neurons (Wichterle et al., 2002), cerebral cortex (Eiraku et al., 2008) and pituitary (Suga et al., 2011) and cortical pyramidal neurons (Espuny-Camacho et al., 2013). Interestingly, neuroectodermal cells derived from PSCs could recapitulate *in vivo*-development and form multicellular organs, called brain organoid, through three-dimensional culture with extracellular matrix (Matrigel) (Lancaster et al., 2013). Because they more closely resemble *in vivo*-organs and tissues than singly type of cells, brain organoid might provide *in vitro*-tools for drug screening, disease modeling and organ development models (Yin et al., 2016).

Study on stem cells of large animal have been proven important for preclinical researches of human disease. So, many researchers have been attempted to convert the porcine PSCs into neuronal lineages and transplant the differentiated cells into the pigs (Gallegos–Cardenas et al., 2015; Li et al., 2014b; Puy et al., 2010; Yang et al., 2013; Zhou et al., 2011). First of all, neuronal differentiation have successfully induced from *in vitro*–cultured ICM and iPSCs. During *in vitro*–culture, pig ICMs were spontaneously differentiated into neural rosette–like structures while couldn't be maintained (Puy et al., 2010). The cells isolated from neural rosette–like structures could be developed into neurons including astrocyte and oligodendrocytes. In other study, researchers successfully induced neural progenitor cells from pig iPSCs (Gallegos–Cardenas et al., 2015). It was assessed that neural differentiation of pig iPSCs resembles that of human PSCs in terms of gene expression pattern, which mean pig PSCs could provide valid models for human therapy. In addition, Zhou and colleagues tried to transplant differentiated cells into pig disease models. Rod photoreceptors were produced from pig iPSCs, and then these cells were engrafted into subretinal space of pigs (Zhou et al., 2011). The transplanted cells were successfully resided in subretinal region.

In this chapter, I attempted to derive neuronal progenitor cells from pig EGCs according to serum-free floating culture of EB-aggregates (SFEB) method (Watanabe et al., 2005). Similar with other species, neuronal progenitor cells were successfully induced by treatment of retinoic acid and these cells expressed neuronal markers such as PAX6, NESTIN and SOX1. Despite lack of in vivo developmental competency, these data indicated that specific types of somatic cells could be directly induced from pig EGCs by manipulating culture conditions. In consistent with human and mouse, same signaling molecules were involved in neural differentiation of pig EGCs, which means pig EGCs could be applied for human researches. To date, transgenic pigs harboring neurological diseases such as Alzheimer's disease and spinal muscular atrophy by genetic modifications (Kragh et al., 2009; Lorson et al., 2011). If, apposing study on differentiation from PSCs, developing transplantation method into these disease-model animals were achieved, it would lead to success of human cell therapy using stem cells.

## 5. Conclusion

Based on my results, neuronal progenitor cells could be derived directly from pig EGCs through SFEB method. Treating retinoic acid was more efficient for inducing neuronal lineages from EGCs than inhibiting SMAD signaling. Although neuronal markers such as PAX6, NESTIN and SOX1 were expressed, it would be developed to mature neurons such as astrocytes, dendrocytes and dopaminergic neuron, along with functional test. Finally, production of transgenic pig for disease models as well as differentiation methods will provide basic preclinical data for human regenerative medicine and lead to success of stem cell therapy.

CHAPTER 8  
GENERAL DISCUSSION

## **Pluripotent stem cells (PSCs) of pigs as a non-permissive species.**

Because pluripotent cells have considered as potential candidate for a regenerative medicine, research in this area has studied on embryonic carcinoma cells to iPSCs (Martin and Evans, 1974; Takahashi and Yamanaka, 2006). Pluripotent cell research began in 1981 by the establishment of mouse ESCs and was spurred by deriving human ESCs and iPSCs in 1998 and 2006, respectively (Evans and Kaufman, 1981; Takahashi and Yamanaka, 2006; Thomson et al., 1998). Recently, several studies have suggested that the pluripotent states are divided into naïve and primed. Naïve PSCs derived from early epiblasts in pre-implantation blastocysts can generate the chimeric fetus when micro-injected into recipient blastocysts. In addition, primed PSCs derived from late epiblasts in post-implantation blastocyst possess more differentiated pluripotency than naïve cells in terms of developmental capacity, gene expression, and epigenetic signatures. In permissive species, both pluripotent states of PSCs can be derived from embryos. However, in non-permissive species, the stem cells cannot be stabilized in the naïve state, and are instead differentiated and stabilized at the primed state during the establishment process if no additional treatments (including genetic manipulation and chemicals) are performed (Hanna et al., 2010b; Nichols and Smith, 2009).

It has been proven hard to derive porcine PSCs using typical methods because of differences in the molecular mechanisms during embryo development among mouse, human, and pig. So, it is worth to research various stem cell lines derived from different origins for deriving genuine pig PSCs. In this study, I attempted to derive pig stem cells from preimplantation embryos, gonadal primordial germ cells (PGCs) and somatic cells. In chapter 3, pig ESCs and embryonic germ cells (EGCs) were successfully derived from preimplantation embryos and gonadal PGCs respectively. In pig ESCs, expression of pluripotent genes were maintained by activation of FGF2 signaling. Similarly, through the activation of FGF2 signaling, PGCs are converted into EGCs acquiring expression of SSEA4 and mono-allelic imprint pattern. In chapter 4, I tried to reprogram pig somatic cells into pluripotent state by ectopic expression of Yamanaka's factors. During reprogramming, FGF2 treatment more strongly up-regulated specific pluripotent genes such as *pSOX2*, *pKLF4*, *REX1*, and epithelial-specific markers compared to LIF treatment, and blocking FGF2 signaling downregulated *pKLF4* and *NANOG* rather than completing reprogramming, as reported in mice (Fig 18) (Silva et al., 2008). These data revealed that pig might be closed to non-permissive species rather than permissive species. Based on data, it is suggested that, in pig as a non-permissive species, FGF2 signaling play an important role in generating genuine pig PSCs.

### Cell signalings for maintaining pluripotency in pigs.

During development of the early embryo, which has an inner cell mass (ICM) considered to be the pluripotent cell population, pig has a prolonged preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in pig embryos, different cell signaling that governs pluripotency reveals differences compared to mouse embryos. Unlike mouse, in which *Oct4* and *Cdx2* are exclusively expressed in ICM and trophoctoderm (TE), respectively, in pig, *SOX2* is specifically expressed in ICM while *OCT4* is expressed in TE until the blastocyst expands on embryonic day 8 (Liu et al., 2015). In ICM and epiblasts of pig blastocysts, the LIF receptor is absent while FGF receptors are specifically expressed, which indicates that FGF signaling may play an important role in the maintenance of pluripotency rather than LIF (Hall et al., 2009). Along with FGF signaling, BMP signaling plays an important role in maintaining pluripotency (Hall and Hyttel, 2014). BMP $\alpha$  and SMADs were strongly expressed in pig ICM and epiblasts as determined by immunostaining and treatment of BMP signaling inhibitor negatively influenced on *in vitro*-survival rate of ICM and epiblasts. It is interesting to note that pig have a unique energy metabolism of preimplantation embryos compared with human and mouse. Recent data showed that energy metabolism have pivotal

roles in supporting stemness and pluripotency (Folmes and Terzic, 2014; Ito and Suda, 2014). Deep-sequencing indicated that pig ATP-synthesis and fatty acid metabolisms during embryo development differ from mouse (Cao et al., 2014). Pig, mouse and human preimplantation embryos have different dependency of glucose metabolism for energy production (Secher et al., 2016). For these reasons, additional or different combination of signaling molecules were needed to derive PSCs in pig.

In chapter 5, I defined three cellular signaling for maintaining pluripotency of pig ESCs. FGF, Activin and Wnt signaling could facilitate *in vitro*-survival rate of pig ICM, and finally, pig ESCs could be derived by treating combination of three molecules (Fig 23). Activin/Nodal signaling is participated in maintaining primed pluripotent state (Hanna et al., 2010b). Pluripotent genes in human and mouse ESCs were elevated by upregulation of Wnt signaling (Sato et al., 2004). Feeder-free culture of human PSCs could be accomplished by supplementing high concentration of Activin A and Wnt activator (Ludwig et al., 2006; Tomizawa et al., 2011). Although Activin and Wnt signaling involve in maintaining pluripotency, these two factors could be replaced by underlying feeder cells in culture of human PSCs (Schatten et al., 2005). However, regardless of co-culture with feeder cells, simultaneous activations of three cellular signaling were pivotal to maintain

pig ESCs, and if any one factor was absent, ESCs were differentiated (Fig 23). It is enticing to think that dependency of pig PSCs on Activin and Wnt signaling are higher than those of human. Therefore, it is verified that additional signaling are essential to maintain pig PSCs compared to human and mouse.

### **Applications of pig pluripotent stem cells**

Because of the physiological and immunological similarities that exist between pigs and humans, porcine pluripotent cell lines have been proven important as potential candidates for preclinical researches on human disease as well as a source for generating transgenic animals (Brevini et al., 2007; Hall, 2008; Houdebine, 2009). To achieve such goals, stem cell engineering involving the genetic modifications and cellular differentiation has been developed. In chapter 6, I attempted to efficiently introduce transgenes into pig ESCs. Consequently, enhanced green fluorescent protein (EGFP) was successfully introduced into the cells via lentiviral vectors under various multiplicities of infection, with pluripotency and differentiation potential unaffected after transfection. In chapter 7, I could derive neuronal progenitor cells from pig embryonic germ cells according to serum-free floating culture of EB-aggregates (SFEB) method (Watanabe et al., 2005). Similar with other species, neuronal progenitor cells were successfully

induced by treatment of retinoic acid and these cells expressed neuronal markers such as PAX6, NESTIN and SOX1. Accordingly, transgenic stem cell line could potentially be used as a donor cell source for transgenic pigs and may be a useful tool for studies involving pig ESCs as well as aid in the understanding of the epigenetic regulation of transgenes. And differentiation of pig EGCs could provide valid models for human therapy.

## **Conclusion and Perspectives**

Through the research on pig pluripotent stem cells, I found that, as a non-permissive species, pig pluripotent stem cells are maintained by mainly ERK signaling and additional signaling molecules are required for supporting pluripotency. And it is assessed that pig stem cell lines could be used for transgenesis and induced differentiation. Additionally, to apply pig stem cells in preclinical researches, more defined culture techniques including feeder-free culture and transgene-independent iPSCs as well as methods for generating functional cells or tissues from pig stem cells should be developed. And various tools of genetic modifications such as DNA endonucleases have to be adapted in producing transgenic animals with pig stem cells. Thorough the achievement of all, pig models for caring human diseases would be accomplished. As it is growing interests in cell therapy using stem cells, needs for preclinical researches via large animal models

have been increased. Accordingly, studies on pig PSCs will pave the way for human cell therapy and shed new light on researches of PSCs.

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## SUMMARY IN KOREAN

초기배아의 체외 배양을 통해 만능성 줄기세포주의 확립이 가능하다. 만능성 줄기세포는 다양한 세포로 분화가 가능하여 퇴행성 질병치료를 위한 세포 치료에 사용이 가능하다. 또한 경제동물의 만능성 줄기세포는 형질전환 동물 생산을 위해 활용이 가능하다. 특히 돼지 줄기세포의 경우, 생리학 및 면역학적으로 사람과 유사하기 때문에 형질전환동물 생산 이외에도 사람의 질병 치료의 전임상 실험을 위한 모델 동물로써 각광을 받고 있다. 따라서 이 연구에서는 배아, 체세포 등의 다양한 조직으로부터 돼지의 만능성 줄기세포주를 확립하고, 그에 대한 응용에 대해 알아보려고 했다. 먼저, 돼지의 만능성 유지 기작 분석을 위하여, 초기배아와 배아의 생식선에서 돼지의 배아줄기세포주와 배아생식선줄기세포주를 확립하였고 이를 분석하였다. 돼지 FGF2 처리를 통해 배아줄기세포주는 초기배아로부터 확립되었다. 또한 돼지 PGC의 리프로그래밍과 EGC의 만능성 유지는 FGF2 신호전달체계에 의해 이루어 졌다. 이는 FGF2 신호전달체계가 돼지의 만능성 확립과 유지에 중요한 역할을 하고 있는 것을 확인하였다. 다음으로 돼지의 체세포를 ‘아마나카 인자’를 이용하여 만능성 상태로 역분화를 시도하였다. 이를 통해 역분화과정 중 FGF2의 처리가 만능성 유전자의 발현이 높게 증가하는 것을 확인하였고, 이를 억제하였을 때 만능성 유전자의 발현이 감소하였다. 앞선 내용을 바탕으로 다양한 배양인자를 이용하여 돼지 배아줄기세포 배양액의 최적화를 시도하였다. 그 결과, FGF2, ACT A, WNT 활성화인자가 포함된 배양액에서 돼지

배아줄기세포주 확립에 성공하였다. 새로 확립된 세포주에서는 OCT4, SOX2, NANOG와 같은 만능성 인자가 발현을 하였고, 장기간 배양이 가능하였다. 마지막으로 돼지 만능성 줄기세포의 활용을 위하여 형질전환과 분화를 시도하였다. 렌티바이러스 벡터를 이용하여 형질전환 배아줄기세포주의 확립을 성공하였고, 또한 삽입된 외래유전자의 발현이 DNA 메틸화에 영향을 받는다는 것을 확인하였다. 그리고 돼지 배아생식선세포를 신경세포 전구체로 분화를 유도하였다. 다른 종과 마찬가지로 신경전구체는 레티노산에 의해 만능성 줄기세포로부터 성공적으로 유도되었고, 이 세포들은 신경세포 표지인자인 PAX6, NESTIN, SOX1 등을 발현하였다. 모든 결과를 종합하여 볼 때, 이 연구를 통하여 돼지는 비관용종으로서 만능성 줄기세포는 ERK 신호전달체계에 의해 유지가 되며, 이를 위해 추가적인 신호전달 물질들이 필요한 것을 확인하였다. 또한 FGF, ACTIVIN, WNT 가 포함된 최적화 배지를 통해 돼지 배아줄기세포주를 확립 할 수 있었다. 이러한 결과들은 돼지에서 만능성 유지에 대한 기초적 지식을 제공할 것이며, 돼지 만능성 줄기세포의 배양 시스템은 전임상 모델 구축을 위한 줄기세포 공학에 응용 가능하다. 결과적으로 이러한 돼지 만능성 줄기세포의 연구는 사람의 임상적용을 앞당길 것이다.

**주요어:** 돼지, 만능성 줄기세포, 배아줄기세포, 배아생식선줄기세포, 유도만능줄기세포, 배양액 최적화, 형질전환, 신경분화

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