A Dissertation

For the Degree of Doctor of Philosophy

Pluripotent and reprogramming status of stem cell lines derived from various embryonic origins and somatic cells in pigs

돼지 배아 및 체세포 유래 줄기세포의 만능성 및 리프로그래밍 특성 규명

August, 2013

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이 논문을 박사학위 논문으로 제출함.
2013년 8월

서울대학교 대학원
농생명공학부 동물생식학 전공
박 진 규

박 진 규의 농학박사 학위논문을 인준함.
2013년 8월

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ABSTRACT

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Pluripotent stem cells represent the cells that have a self-renewal capacity and the ability to differentiate into all lineages. Since pluripotent stem cells were first established from murine embryos, a number of attempts have been to derive the pluripotent stem cells from other species including pigs, cattle, rats, sheep, goat, primates and humans. Pluripotent stem cells will help us to understand embryonic biology and improve the studies of regenerative medicine using stem cells. In particular, pigs are considered as the ideal animal model for pre-clinical research, studies of human diseases and the production of bioreactors from transgenic pigs, due to similarities in size, immunology and physiology to human. With these benefits, many researchers have tried to

establish the embryonic stem cells (ESCs) from porcine embryos. However, although several putative ES cell lines has been reported from porcine embryos, authentic ES cell lines don't exist. Meanwhile, as mouse (m) induced pluripotent stem cells (iPSCs) have first been reported by using reprogramming four factors, porcine (p) iPSCs have also been derived in pigs by various methods, such as retrovirus, lentivirus, plasmid vectors and doxycycline inducible lentivirus system. In particular, West et al. showed piPS cells with germ-line transmission, although it is the results by PCR analysis. Most of derived piPSCs showed morphologically and molecularly similar to mouse epiblast stem cells (mEpiSCs) or human (h) ES cells, rather than to mES cells.

Pluripotent stem cells can be divided to naïve and primed state pluripotent stem cells according to their pluripotent state. Only naïve state comprises a full pluripotency or ground state showing the contribution to germ-line transmission. Naïve state is permitted in specific permissive strains or species, such as 129, C57BL/6 and BALB/C strain in mice. However, a number of attempts have been made to derive the naïve state pluripotent stem cell lines from non-permissive species, including human and pig, using various exogenous factors including GSK3β and MEK inhibitors (2i), LIF, hypoxic conditions and up-regulation of Oct4 or klf4. Therefore, this study investigated the process being reprogrammed from somatic cells and various embryonic origins in pig to understand the

difficulties of establishing authentic pluripotent stem cells in porcine species for about 30 years. This study was carried out the following three experiments and could derive the results as follows.

In the first study, I investigated the early process during the derivation of ES-like cells from various blastocysts derived from various embryonic origins. We could observe a surface morphology of blastocyst and the process being attached blastocysts onto feeder cells layer under high magnification. Total 13 ES-like cell lines were derived from blastocyst stage porcine embryos of various origins, including in vitro fertilized (IVF), in vivo derived, IVF aggregated, and parthenogenetic embryos. And this study was analyzed characteristics such as early morphologies, AP activity, chromosome assay and expression of pluripotent markers Oct4, Sox2 and Nanog in each cell lines. Therefore, our results will help to understand the process established putative pESC lines from porcine embryos, although more studies are required to derive authentic ESC lines.

In the second experiment, I performed to understand the pluripotent and reprogramming status of EpiSC-like pESC established in first study from each origins, including IVF, in vivo derived, IVF aggregated, and parthenogenetic embryos and piPSC line newly derived from porcine embryonic fibroblasts. This study was investigated characteristics such as

marker expression, signaling pathways, pluripotency and self-renewal in these EpiSC-like pESC and piPSC lines. In this study, I could confirm the expression of genes associated with the Activin / Nodal and FGF2 pathways along with the expression of pluripotent markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81. Furthermore all of these cell lines showed in vitro differentiation potential, the X chromosome inactivation in female and a normal karyotype. Hence, this study suggests porcine species, which is non-permissive species, undergoes the process reprogramming to primed state during the derivation of pluripotent stem cells from somatic cells and various embryos.

In the final study, I performed to investigate whether a naïve state-like pluripotent stem cell line could be derived from porcine embryonic fibroblasts. Using previous methods performed to derive naïve state pluripotent stem cells in non-permissive species, such as human, NOD mouse strain and rat, we have been able to successfully induce PEFs into a naïve state-like pluripotent stem cell line showing mESC-like morphologies and the expressions of Oct4, Sox2, Nanog, SSEA1 and SSEA4. These cell lines could be maintained a stable morphology for more than 50 passages. In addition, these cell lines could be sequentially re-generated into mESC-like piPSCs from secondary or third fibroblast-like cells differentiated from mESC-like piPSCs by addition of doxycycline (DOX), GSK3β and MEK inhibitors (2i) and LIF. Accordingly, this study

suggests that the porcine species could be induced into mESC-like iPSCs by the introduction of various exogenous factors including continuous transgene expression, 2i and LIF. In these mESC-like piPSC lines, however, the transgenes activated by DOX wasn't able sufficiently to induce the activation of endogenous transcription factors, although it showed the expression of all transgenes, a stable mESC-like morphology and sequential reprogramming by an addition of DOX. Therefore, further studies are required to derive authentic naïve state pluripotent stem cells from porcine species, showing a stable and complete activation of endogenous pluripotency genes by the expression of exogenous transgenes.

In conclusion, I have addressed the studies on the pluripotent and reprogramming status of stem cell lines derived from porcine somatic cells and various embryonic origins in three studies. According to results of three studies, we could confirm that porcine species is reprogrammed to primed state, when establishing the pluripotent stem cells from embryos or somatic cells. And it needs various exogenous factors, such as continuous transgene expression, 2i and LIF, to be induced into mESC-like iPSCs from porcine somatic cells. However, it is hard to derive authentic naïve state pluripotent stem cells, due to insufficient information and understanding of porcine specific mechanism. Therefore, further studies will be required to understand porcine specific mechanism related

to establishing the pluripotent stem cells in pigs. This study will help to approach the goal for not only understanding porcine specific mechanism, but also establishing authentic pluripotent stem cells in pigs.

Key Words: pluripotent stem cells, induced pluripotent stem cells, reprogramming, embryonic stem cells, naïve and primed status, pig

Student Number: 2007-23158

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LIST OF ABBREVIATIONS

2i	GSK3β and MEK inhibitors
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidine salt
BMP 4	bone morphogenic protein 4
DOX	doxycycline
EB	Embryoid body
EC cells	Embryonic carcinoma cells
EG cells	embryonic germ cells
EpiSCs	epiblast stem cells
ES cells	embryonic stem cells
gPS cells	germline-derived pluripotent stem cells
h	human
ICC	immunocytochemistry
ICM	inner cell mass
Id	inhibitor of differentiation
iPSCs	induced pluripotent stem cells
IVF	in vitro fertilized
JAKs	Janus kinases
LIF	leukaemia inhibitory factor

LIFR	LIF receptor
m	murine
MEFs	mouse embryonic fibroblasts
MET	mesenchymal-to-epitherial transition
NBT	nitro blue tetrazolium chloride
NOD	nonobese diabetic
p	porcine
PDGF	platelet-derived growth factor
PEFs	porcine embryonic fibroblasts
PGCs	primordial germ cells
RT-PCR	Reverse transcriptase-polymerase chain reaction
SSCs	spermatogonial stem cells
SSEA-1	stage-specific embryonic antigen-1
Stat 3	signal transducer and activator of transcription 3
TE	trophectoderm
XCI	X chromosome inactivation

CHAPTER 1. GENERAL INTRODUCTION

Embryonic stem (ES) cells represent the cells that have a self-renewal capacity and an ability to differentiate into all lineages (Evans and Kaufman, 1981). As ES cells were established from inner cell mass (ICM) of mouse and human blastocysts (Evans and Kaufman, 1981; Thomson et al., 1998), numerous efforts have been made to derive ES cell lines from various domestic animals, including pigs (Piedrahita et al., 1990a), cattle (Saito et al., 1992b), sheep (Handyside et al., 1987a) and goats (MeineckeTillmann and Meinecke, 1996). Because the research of domestic animal stem cells can be useful tools including the production of transgenic animals and xenografting (Brevini et al., 2007a), stem cell research in domestic animals are progressing steadily by several researchers, although there are not as many as in the area of mouse and human.

Among various domestic animals, because of immunological and physiological similarities with humans, pig has the most potential benefits for clinical research. These advantages have stimulated scientists to utilize pigs as an animal model that can provide therapeutic resource for human diseases. For example, cardiac valves and insulin obtained from pigs (Hall, 2008) and α-1,3-galactosyltransferase knockout pigs that can delay acute immune response were already generated to study the possibility of pig-to-human xenotransplantation (Lai et al., 2002).

Together with these advantages, attempts for the establishment of

porcine embryonic stem cells are in progress since 1990s. Most of the early reports showed relatively limited features of the stemness based on the morphology, AP activity, EB formation, in vitro differentiation potential and limited passage numbers (Nowak-Imialek et al., 2011). In 2000s, several research groups have reported the putative porcine embryonic stem cells from porcine embryos of various blastocyst origins including in vitro produced and in vivo derived embryos, parthenogenetic embryos and somatic cell nuclear transfer embryos (du Puy et al., 2011; Kim et al., 2010; Li et al., 2004; Li et al., 2003; Tan et al., 2011; Vackova et al., 2011; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). However, all of these porcine ES-like cell lines were very different from mouse ES cells with respect to morphology, culture condition and differentiation potential. Because most of ES cell lines established in pigs had lack of pluripotent marker expression and lose their pluripotent ability within a short number of passages, especially with no germline competence, it has been described as “ES-like” or “putative” embryonic stem cells.

Meanwhile, Yamanaka group have reported that pluripotent cells can be induced from somatic cells by using four reprogramming factors *Oct4, Sox2, Klf4,* and c-*Myc*. (Takahashi and Yamanaka, 2006). After establishment of iPSCs from mice and human (Takahashi et al.; Takahashi and Yamanaka), it has been reported the establishment of

iPSC lines from porcine somatic cells using reprogramming factors (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; West et al., 2010; West et al., 2011; Wu et al., 2009). In order to reprogram somatic cell into pluripotent stem cells, Esteban et al. used retrovirus (Esteban et al., 2009), Ezashi et al. used lentivirus (Ezashi et al., 2009) and Wu et al. used doxycycline inducible lentivirus system (Wu et al., 2009). Especially, West and his colleagues reported porcine induced pluripotent stem cells (piPSCs) capable of generating chimeric offspring, although the results were solely grounded on polymerase chain reaction results (West et al., 2010; West et al., 2011). All of these iPSC lines were similar to mouse EpiSCs with respect to morphology and FGF2 culture condition. It has proven difficult to be established into authentic pluripotent stem cells, such as mESCs, in porcine species. It means that the porcine species is hard to be established into authentic pluripotent stem cells, such as mESCs. It means difficult to be established into authentic pluripotent stem cells, showed in mESCs, in porcine species.

This phenomenon is also shown in other domestic animals whose results on pluripotent stem cells represented flattened monolayer colony morphologies and FGF2 culture condition with EpiSC-like characteristics (Wang et al., 2005).

Because there is a very lack of information and knowledge about signaling pathway regulating pluripotency and suitable culture condition

for maintenance of pluripotency, authentic pluripotent stem cells have not been categorized for 30 years in domestic animal. In this study, therefore, I address the question why authentic pluripotent stem cells, such as mESCs showing germ-line competency, have not been categorized for 30 years in domestic animal. To understand the difficulties of establishing authentic pluripotent stem cells in porcine species, this study was performed the following three experiments.

The first experiment in Chapter 3 demonstrated the early establishing ES-like cell lines from various embryonic origins in pigs. We have been able to derive total 13 ES-like cell lines from blastocyst stage porcine embryos of various origins, including in vitro fertilized (IVF), in vivo derived, IVF aggregated, and parthenogenetic embryos. And we analyzed their characteristics for early morphologies, AP activity, chromosome assay and expression of pluripotent markers Oct4, Sox2 and Nanog.

The second experiment performed in Chapter 4 was investigated to understand the pluripotent and reprogramming status of EpiSC-like pESC established from each origin, including IVF, in vivo derived, IVF aggregated, and parthenogenetic embryos derived in chapter 3 and piPSC line newly derived from porcine embryonic fibroblasts. We investigated characteristics for marker expression, signaling pathways,

pluripotency and self-renewal in these EpiSC-like pESC and piPSC lines. In this study, we confirmed the expression of genes associated with the Activin/Nodal and FGF2 pathways along with the expression of pluripotent markers, Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81. Furthermore all of these cell lines showed in vitro differentiation potential, the X chromosome inactivation in female and a normal karyotype.

Final experiment addressed in chapter 5 was performed to investigate whether a naïve pluripotent stem cell line could be derived from porcine embryonic fibroblasts via previous reported various factors. We have been able to successfully induce PEFs into a naïve state-like pluripotent stem cell line displaying mESC-like morphologies and the expressions of Oct4, Sox2, Nanog and SSEA1. All cell lines maintained stemness characteristics and a stable morphology for more than 50 passages. In addition, these cell lines could be re-generated into naïve state-like pluripotent stem cells from secondary and third fibroblast-like cells differentiated from naïve state-like pluripotent stem cells by addition of doxycycline.

Here we suggest that, as a non-permissive species, porcine species undergoes reprogramming into a primed state during the establishment of pluripotent stem cell lines and needs various exogenous factors such as continuous transgene expression, GSK3β and MEK inhibitors (2i) and LIF

to be induced into mESC-like piPS cells.

CHAPTER 2. LITERATURE REWIEW

1. Pluripotent stem cells from various developmental origins

Pluripotent stem cells, having a self-renewal capacity and the ability to differentiate into all somatic lineages (Evans and Kaufman, 1981), could be derived from various developmental origins during mammalian embryonic development, which begins from totipotent zygote. Totipotent embryo initiates cleavage division during the development of embryo, and the first differentiation is generated from the eight cell stage in mouse (Nichols and Smith, 2012). The cell lineages are divided to two lineages from the 16-cell stage. The first is the inner cell mass, which generates the epiblast forming embryo and the hypoblast differentiating into the yolk sac. Evans and Kaufman suggested that embryonic stem cells could be derived from the epiblast of preimplantation blastocyst (Evans and Kaufman, 1981). The second is trophoblast lineage, which generates the placenta.

On the other hand, epiblast stem cells which present the pluripotency were established from the epiblast of early post implantation embryo (Bao et al., 2009; Brons et al., 2007; Tesar et al., 2007). There is another type of pluripotent stem cells termed as embryonic germ (EG) cells. EG cells can be derived by culturing primordial germ cells isolated from embryonic day 8.5 in mouse (Matsui et al., 1992; Resnick et al.,

1992). Meanwhile, pluripotent stem cells could be derived from postnatal or adult stages. Among the pluripotent stem cells, spermatogonial stem cells derived from neonatal precursor cells in testis could proliferate to ES-like cells in vitro, although they could not generate chimeric animals using tetraploid complementation (Kanatsu-Shinohara et al., 2004). In 2010, it was reported that germline-derived pluripotent stem (gPS) cells from adult unipotent germline stem cells displayed in vitro and in vivo differentiation, including the germ-line transmission. However, gPS cells exhibited androgenic pattern of imprinting genes unlike ESCs showing a somatic pattern (Ko et al., 2009).

In 2006, Takahashi and Yamanaka showed induction of pluripotent stem (iPS) cells from mouse embryonic fibroblasts by introducing 4 transcription factors; Oct4, Sox2, Klf4 and c-Myc. The iPS cells showed similar characteristics to mouse ES cells such as the expression of pluripotent marker genes, dome-shaped colony morphologies, LIF and BMP4-mediated signaling pathway, two active X chromosomes in female, teratoma formation, and chimera formation. Various types of pluripotent stem cells are explained more detail in the next section.

1-1. Embryonic stem cells

Embryonic stem cells are the pluripotent stem cells derived from the ICM of pre-implantation blastocyst. Pluripotent stem cells are generally defined to have characteristics such as the capacity for long-term self-renewal without losing any stemness and the ability to differentiate into three germ layers including the germ cell lineage (Evans and Kaufman, 1981; Nagy et al., 1990). The beginning of pluripotent stem cell research was using embryonic carcinoma (EC) cells derived from teratocarcinomas which are consist of the differentiated and undifferentiated cells (Finch and Ephrussi, 1967; Martin and Evans, 1975). EC cells have many features in common with other ES cells. The EC cells could proliferate continuously in vitro culture condition with mitotically inactivated embryonic fibroblasts and differentiate into the three germ layers; endoderm, mesoderm and ectoderm (Kleinsmith and Pierce, 1964; Martin and Evans, 1975). Moreover, when the EC cell was injected into blastocyst for the identification of pluripotency and normal differentiation, it could also contribute to the normal tissue of chimeric mice. However, it showed a malignancy resembling pancreatic adenocarcinoma among all the tissue (Illmensee and Mintz, 1976). In addition, teratocarcinomas-derived EC cells are not suitable to cell-based therapy because most of them have abnormal karyotype (Martin, 1980).

Mouse ES cells were first established from the ICM of pre-implantation blastocyst (Evans and Kaufman, 1981). The ICM have the capacity to differentiate into all the body, however, these pluripotent cells exist transiently during the in vivo development. To maintain the self-renewal and undifferentiated state of these pluripotent cells, a few factors were identified for the differentiation of pluripotent cells. ES cells can be propagated unlimitedly in vitro culture environment without losing pluripotency in the present of leukaemia inhibitory factor (LIF), bone morphogenic protein 4 (BMP4), serum and on a feeder layer of mouse embryonic fibroblasts (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003).

Among these factors, LIF and BMP4 were considered as the key signaling factors required for maintenance of pluripotency in mES cells (Lai et al., 2002; Smith et al., 1988; Ying et al., 2003). LIF belong to a member of the IL-6 whose receptor consists of LIF receptor (LIFR) and the common receptor subunit gp130 (Davis et al., 1993). After the binding of LIF heterodimerizing to the LIFR and gp130, these complexes activate two major signaling pathways; the receptor-associated Janus kinases (JAKs)-signal transducer and activator of transcription 3 (Stat3) pathways and SHP2-Erk pathway. The JAK / Stat3-pathway by the binding of LIF to LIFR/gp130 receptor promote the dimerization of Stat3, dimerized Stat3 translocate to the nucleus for controlling the transcription of genes related

to ES cells self-renewal. Besides, BMP4 signaling promotes the Smad signaling to induce the activation of Id (inhibitor of differentiation) genes. BMP4 seems to suppress the differentiation of ES cells (Ng and Surani, 2011). When ES cells cultured in the ES medium supplemented with LIF and BMP4, it could be maintained to pluripotency in a serum-free medium (Ying et al., 2003).

Many studies showed that there are many key pluripotency-associated factors for maintenance of pluripotency in mES cells, such as Oct4, Sox2, Nanog, Esrrb, Sall4, Dax1, Klf2, Klf4, Klf5, Stat3 and Tcf3 (Ng and Surani, 2011). There are complex transcriptional regulatory networks in mES cells. Among these pluripotency-associated factors, Oct4, Sox2 and Nanog played a crucial role as the transcription factors in mES cells (Chambers et al., 2003; Masui et al., 2007; Niwa et al., 2000b).

In particular, Oct4 appears to be one out of the most important factors that regulate self-renewal of mES cells. The expression of Oct4 is restricted into early embryos, pluripotent stem cells and germ cells in mice (Pesce et al., 1998). A proper expression level is important to maintain the stemness of ES cells (Niwa et al., 2000a). It results in differentiation into mesoderm or endoderm in condition of less than a two-fold increase and differentiation into trophectoderm in condition of less than 50% expression levels (Friel et al., 2005).

Another important factor that regulates self-renewal of mES cells is Nanog. The overexpression of Nanog enables to promote self-renewal in mES cells withdrawal of LIF or with the addition of JAKs inhibitors (Chambers et al., 2003). The enhancer region of Oct4 and Nanog are bound by many transcription factors, such as Sox2, Sall4, Tcf3, Smad1, Esrrb, Klf2, Klf4, Klf5, n-Myc, Oct4 and Nanog. These transcription factors played a crucial role in the regulation of self-renewal as well as diverse cellular functions including transcription regulation, chromatin modifications and post-transcriptional regulation through non-coding RNAs and microRNAs (Ng and Surani, 2011). Therefore, both Oct4 and Nanog are essential to maintain the stemness of mES cells. In addition, as Oct4, Sox2 and Nanog bind to an intrinsic site of the Xist gene which is non-coding RNA, it would regulate X chromosome inactivation (XCI) whose control is important in normal development of mammalian embryos. The condition repressing the transcription of Xist gene is one of the important hallmarks in mES cells (Davis et al., 1993; Navarro et al., 2008).

There are many screening methods for identifying the pluripotency of ES cells such as morphology, marker expression, stable diploid karyotype, in vitro differentiation, teratomas and chimera formation (Smith, 2001). In the case of human ES (hES) cells, they have somewhat different characteristics when compared with mES cells. Morphologically, mES

cells showed small, round and dome-shaped morphologies, while hES cells represent flattened monolayer colony morphologies. Passaging timing is also different to each other between hES cells and mES cells. The hES cells grow slow when compared to mES cells (Friel et al., 2005). There are slight differences in surface marker expression between hES cells and mES cells. The mES cells showed the expression of stage-specific embryonic antigen-1 (SSEA-1), while hES cells express SSEA-4, TRA 1-60 and TRA 1-81 (Thomson et al., 1998). The most striking differences are signaling pathways required for the maintenance of pluripotency. In mES cells, LIF and BMP4 signaling pathways play an important role in the maintenance of pluripotency. Meanwhile, FGF and Nodal / Activin signaling pathways are need for the maintenance of pluripotency in hES cells (Hanna et al., 2010b). Although it showed somewhat different marker expression between mES cells and hES cells, both species have many common characteristics involved in pluripotency, such as the ability to form all somatic lineage and the expression of Oct4, sox2 and Nanog (Evans and Kaufman, 1981; Thomson et al., 1998).

1-2. Epiblast stem cells

Epiblast stem cells (EpiSCs) comprise the pluripotent stem cells derived from epiblasts of post-implantation embryos, embryonic day

(E5.5~7.5), whereas ES cells could be derived from ICMs of pre-implantation embryos in mice (Brons et al., 2007; Tesar et al., 2007). Although mEpiSCs represented pluripotent stem cells, they showed restricted pluripotency when compared to mES cells. For instance, mEpiSCs have different characteristics from mES cells in their morphology, epigenetic status, global gene expression pattern, signaling pathways and poor chimeric contribution (Brons et al., 2007; Han et al., 2010; Hanna et al., 2010b; Tesar et al., 2007).

The mEpiSCs grew as flat colony morphology and showed poor proliferation, when passaged by method of single cell dissociation, unlike mES cells. There are distinct methylation patterns of germ cell marker genes; stella (Dppa3), Vasa and Fragilis (Iftm3) (Han et al., 2010). Oct4 gene has two different regulatory sites; the distal and proximal elements (Yeom et al., 1996). The mEpiSCs and mES cells are regulated by distinct regulatory sites in the promoter region of Oct4 gene. The mEpiSCs primarily utilized epiblast-specific proximal element, whereas mES cells were activated in the distal element of Oct4 promoter during the expression (Tesar et al., 2007). Although EpiSCs expressed similar expression level of Oct4, Sox3 and Nanog genes as compared with ES cells, the comparative results of expression profiles showed that EpiSCs were transcriptionally similar to late epiblasts than ICMs or ES cells in mice. In particular, mEpiSCs and mES cells showed significantly different

expressions from many genes, such as Dax1, Stella, Piwil2, Stra8, Dazl, FGF5 and Nodal (Brons et al., 2007; Tesar et al., 2007). There are differences on differentiation capacity between mEpiSCs and mES cells. The mEpSCs showed the ability of differentiation into extra-embryonic lineages in vitro, unlike mES cells. Interestingly, when EpiSCs were injected into pre-implantation-stage embryos, these cells showed the chimeric contribution of very low efficiency and germ-line transmission was not observed, unlike mES cells (Brons et al., 2007). EpiSCs consist of Oct4-GFP positive and negative populations, distinguished by the expression of specific Oct4-GFP marker (Han et al., 2010). Oct4-GFP positive population which only represents a minor fraction showed the low utilization of a distal element with most of proximal element activation and the contribution of germline, although germline transmission was not observed.

The first and the most importantly, EpiSCs share certain characteristics with human ES cells, such as dependence on Activin / Nodal signaling, morphology and the differentiation capacity of extra-embryonic lineages. Therefore, EpiSCs can be very useful to understand features of developmental biology and therapeutic application in human ES cells (Brons et al., 2007; Tesar et al., 2007).

1-3. Embryonic Germ cells, Spermatogonial stem cells and Germline-derived pluripotent stem cells

Pluripotent stem cells could be derived by culturing fetal primordial germ cells (PGCs). These cells, so-called to embryonic germ (EG) cells, share with ES cells in the aspect of features of pluripotency and self-renewal, including the ability of differentiation into all somatic lineages and the germ-line transmission (Labosky et al., 1994; Matsui et al., 1992; Resnick et al., 1992; Stewart et al., 1994). These EG cells have also derived from humans and pigs (Shamblott et al., 1998; Shim et al., 1997). Precise mechanism of the conversion into pluripotent EG cell from unipotent PGC is still missing. It has just been known that LIF, bFGF and SCF is essential for the conversion into pluripotent status of PGCs, including the survival and proliferation of PGCs. SCF is essential for survival of PGCs. While LIF and bFGF are required to maintain the pluripotency of EG cells and reprogram the PGCs into pluripotent status respectively (Durcova-Hills et al., 2006; Pesce et al., 1993; Sette et al., 2000).

There are two types of stem cells derived from testicular cells. The first is spermatogonial stem cells (SSCs) from newborn and adult male gonads (Kanatsu-Shinohara et al., 2004). The SSC showed ES cell-like characteristics, although they have a limited chimerism because of

memories with a male-specific imprinting pattern. The second is germline-derived pluripotent stem (gPS) cells derived from adult unipotent germ-line stem cells. These cells showed the global gene expression pattern similar to that of ES cells, including differentiation capacity and teratomas and germ-line transmission. However, gPS cells exhibited an androgenic pattern of imprinting gene unlike a somatic methylation pattern in ES cells (Ko et al., 2009).

1-4 Induced pluropitent stem cells

In 2006, pluripotent stem cells could be induced from differentiated murine somatic cells by Takahashi and Yamanaka, so-called induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). Their breakthrough could be inspired and achieved from past great reports, such as reprogramming by nuclear transfer, the concept of master regulator and the generation of pluripotent stem cells (Davis et al., 1987; Evans and Kaufman, 1981; Gurdon, 1962; Schneuwly et al., 1987; Tada et al., 2001; Takahashi and Yamanaka, 2006; Wilmut et al., 1997; Yamanaka, 2012). Previous reports demonstrated oocytes and ES cells contain various factors that can reprogram somatic cells into totipotent and pluripotent cells, taken these results, Takahashi and Yamanaka

hypothesized that somatic cells can be reprogramed into pluripotent cells using 24 candidate transcription factors, including Oct4, Sox2, Nanog, Stat3, E-Ras, c-myc, Klf4 and β-catenin (Resnick et al., 1992; Williams et al., 1988; Wilmut et al., 1997). They could finally derive iPS cells from murine somatic cells by introducing four factors, Oct4, Sox3, c-Myc and Klf4, among the 24 candidate factors (Williams et al., 1988). They showed these iPS cells are similar to ES cells in morphology, marker expression, differentiation capacities, epigenetic status and germ-line transmission.

Since iPS cells were first derived from murine fibroblasts with Yamanaka 4 factors, many attempts have been made to understand how the Yamanaka 4 factors work as reprogramming factors for pluripotency. During the reprogramming process, the activation of transgenes induced stochastic epigenetic events that lead to global modification of somatic epigenome into ES-like status including X chromosome reactivation in female miPS cells (Maherali et al., 2007). In addition, it induced the activation of endogenous key factors such as, Oct4, Sox2 and Nanog. And transgenes were gradually down-regulated after the activation of endogenous key factors (Maherali et al., 2007; Wernig et al., 2007). However, the activation of transgenes should be sufficiently activated for the derivation of fully reprogrammed cells with the activation of AP, SSEA1 and endogenous Oct4 and Nanog genes (Brambrink et al., 2008). In order to further understand the reprogramming process, Buganim et

al. analyzed the expression of single cell at different time points during the reprogramming process after transgenes induction (Buganim et al., 2012). They reported that there are two phases in reprogramming process; stochastic phase and deterministic phase. Stochastic phase is the stage including from a mesenchymal-to-epitherial transition (MET), detected at the early stage during the reprogramming process, to the activation of “predictive” markers such as Esrrb, Utf1, Lin28 and Dppa2. Deterministic phase is the activation of endogenous Sox2 that represent final step for acquisition of full pluripotency (Buganim et al., 2012; Pan and Pei, 2012).

On the other hand, the generation of iPS cells is a remarkable discovery in stem cells application, especially in patient-specific therapy using iPS cells which can avoid immune rejection (Takahashi et al., 2007). However, it should be essential to identify the safety of iPS cells for medical application (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Many attempts have been made to avoid the risk of tumorigenity or side-effect of viral integration. These attempts include the generation of integration-free iPS cells such as plasmid, sendai virus, adenovirus, synthesized RNAs and proteins (Fusaki et al., 2009; Kim et al., 2009; Okita et al., 2010; Stadtfeld et al., 2010; Warren et al., 2010).

2. Pluripotent status of two types (Naive and Primed status)

During the embryonic development, epiblast in pre-implantation blastocyst is the developmental ground state that can generates the whole fetus and contribute to all lineages, when micro-injected into recipient blastocyst (Gardner, 1998). Using these pre-implantation epiblast, ES cells were first established at 1981 (Evans and Kaufman, 1981). However, these ES cells, represented ground state pluripotency, can be derived from only specific mouse strains, such as 129, C57BL/6 and BALB/C which are called to “permissive” strains. The isolation of ground state pluripotent stem cells from other strains of mice, such as nonobese diabetic (NOD) mice, has been proven difficult to maintain pluripotency (Hanna et al., 2010b). The derivation of ground state pluripotent stem cells is limited by not only strains but also species. In other words, it is being influenced by the genetic background (Nichols and Smith, 2012; Smith, 2001). Most of ES cell lines being used in many researches came from the 129 background (Nichols and Smith, 2012). It shows that the derivation of ES cells may be strongly dependent to genetic background to meet a full pluripotency including the germ-line transmission.

Nichols and Smith (2009) referred to pluripotent stem cells as two

forms, the “naïve” state of ICM-like ground state stem cells and the “primed” state of post-implantation epiblast-like state stem cells. The first “naïve” state comprises a full pluripotency or ground state. These states of cells consist of mES cells or mEG cells that can contribute to germ-line transmission. The derivation of the naïve state stem cells, as the species or strain-dependent, is restrained to permissive strain. The permissive strains can be maintained a naïve state during the establishment of stem cell lines from naïve epiblast, while non-permissive strain is not easy to maintain a naïve state during the establishment of pluripotent cell lines. These strains are stabilized into a primed state from naïve epiblast. The second “primed” state can be referred to the state of limited pluripotency or prone to differentiate. The mEpiSCs or hESCs can be categorized to “primed’ state of stem cells.

The naïve and primed state are molecularly, epigenetically different including the capacity of differentiation (Hanna et al., 2010a; Hanna et al., 2010b). For instance, naïve state pluripotent stem cells can contribute to germ-line transmission. In addition, these cells can maintain the state activated both X chromosomes (XaXa) in female cells. The *Oct4* distal element is utilized in naïve state cell lines. In contrast to naïve state, primed state pluripotent stem cells undergo X chromosome inactivation (XaXi) during the maintenance of pluripotency and exhibit limited chimerism, when micro-injected into recipient blastocyst. The *Oct4*

proximal element is utilized in primed state cells. These two state stem cells have different morphologies and signaling pathways to maintain stemness respectively. The naive pluripotent cell lines display small, round or dome-shaped colonies morphologies and respond to LIF / Stat3, while primed pluripotent cell lines show flattened monolayer colony morphologies and respond to FGF and Nodal / Activin signaling pathways for maintenance stemness.

In rat, many cell lines have been established by conventional methods used in mice, but these cell lines showed a limited pluripotency (Brenin et al., 1997; Vassilieva et al., 2000). The rat had considered difficult to establish authentic ES cells, because it is non-permissive specie. In 2008, however, authentic rat ES cells can be established by culture medium containing three inhibitors (3i) that target FGF receptor, MEK and GSK3 (Buehr et al., 2008; Li et al., 2008). These rat ES cell lines derived under 2i or 3i showed full pluripotency, such as characteristics of the pluripotent markers expression, in vitro and in vivo differentiation potential including teratomas and germ-line competency shown to only mouse ES cells. In addition, Gue et al. reported EpiSCs derived from post-implantation epiblast can be reverted into naïve ground state by the additional expression of Klf4 (Guo et al., 2009). A naïve pluripotent stem cells from NOD strain mice, as another non-permissive species model, could be induced by using various exogenous factors,

such as GSK3β and MEK inhibitors, LIF and up-regulation of klf4 or c-Myc (Hanna et al., 2009). These reports showed the conversion between the two different types of pluripotent states is possible, although primed state is hard to revert back to naïve pluripotency without various factors, such as 3i, LIF and inducing klf4 or c-Myc.

Using conversion method used in mice and rat, many efforts have been made to derive the naïve state pluripotent stem cells in human and pigs (De Los Angeles et al., 2012; Telugu et al., 2011). In spite of numerous efforts, however, naïve state pluripotent stem cells derived from human and porcine species were not enough to meet the robust nature shown in naïve state of mES cells and miPS cells (De Los Angeles et al., 2012; Nichols and Smith, 2012).

3. Pluripotent stem cells in domestic animals

Domestic ungulates such as pig, cattle, sheep and goat have been considered to be useful for pre-clinical research, studies of human diseases and the production of bioreactors from transgenic animals (Gandolfi et al., 2012; Kues and Niemann, 2011; Niemann and Kues, 2007; Nowak-Imialek et al., 2011). Among these domestic animal, pig can be particularly useful to study human diseases, because of similarities in

size, immunology and physiology to human (Nowak-Imialek et al., 2011). The establishment of ES cells in domestic animals will help us to understand embryonic biology and improve the studies of regenerative medicine using stem cells.

ES cells are defined as the cells that have a pluripotency and self-renewal capacity. ES cells that have the ability to differentiate into all lineages shown in the ICMs of blastocysts(Evans and Kaufman, 1981). Beginning the first differentiation in morula stage during embryogenesis, the cells in embryo can be categorized in two types; ICMs and trophectoderm. The ICMs develop into epiblast and hipoblast. The ICMs or early epibast which is developed into embryo proper can be used as source of ESCs, while EpiSCs can be derived from post-implantation epiblast. Along developmental stages of the embryos, there are categorized into two types of the stem cells. In mouse, ESCs are derived from pre-implantation embryos (E3.5), while EpiSCs are derived from post-implantation embryos (E5.5-E7.5) (Brons et al., 2007).

In domestic animals including pig, however, authentic ES cells couldn’t be derived from pre-implantation embryos. Although several attempts to establish ES cells have been tried from porcine pre-implantation embryos of the various stages (E6-E11) (Chen et al., 1999b; Piedrahita et al., 1990a, b; Strojek et al., 1990; Talbot et al., 1993a; Talbot

et al., 1993b), it couldn't derive the ES cells with characteristics shown in mES cells. Most of ungulate ES cell's papers, including porcine ES cells, derived from pre-implantation embryos were very similar to hES cells which showed monolayer colony morphologies, FGF culture condition and Activin / Nodal signaling pathways. In a recent published our paper, we addressed the comparative study from stem cells being reprogrammed in pigs using EpiSC-like pESC lines derived from porcine embryos of various origins including IVF, *in vivo* derived, IVF aggregated (3X) and parthenogenetic embryos (Park et al., 2013). Our results showed that stem cell lines derived from porcine pre-implantation embryos of various origins exhibited flattened morphologies and FGF and Nodal / Activin signaling and belong to the category of EpiSC-like ES cells rather than authentic ES cells. Meanwhile, Alberio and colleagues reported porcine epiblast stem cell lines displaying the activation of Activin / Nodal signal for maintenance of their pluripotency from E10.5-12 porcine embryos (Alberio et al., 2010b). Thus, putative pES cells were similar to porcine epiblast stem cells and different to mES cells with respect to morphology and activated pathways. These results mean that the condition for the derivation of mES cells isn't suitable for establishing pES cells.

Many reviewers have illustrated in their papers about key points that must be considered for the establishment of authentic pES cells (Alberio

and Perez, 2012; Brevini et al., 2012; Gandolfi et al., 2012; Hall, 2008; Vejlsted et al., 2006). It has been regarded suitable timing of embryonic development as one of the important considerations. Because specific periods of early embryos development are differently present in each species, the optimum stage of the embryos for ESCs derivation could be different for each species (Brevini et al., 2007a; Brevini et al., 2010b). Indeed, the periods of blastocyst implantation are considerably longer in pigs and cows than in mouse and human. In mouse and human, pre-implantation period is around 1 day and 3 days, while pre-implantation period in pig is around 10 days (Brevini et al., 2007a; Hall, 2008). The epiblast that becomes a source for ESCs is formed after hatching and completed around day 12 in pig (Vejlsted et al., 2006). It has been considered that defining the optimum stage of embryos for stem cell derivation is an important key to obtain stable ES cell lines with identifying pluripotent markers and the key signaling pathways that regulate the pluripotency of ES cells in domestic animals. Furthermore, an understanding of species-specific mechanisms and optimal culture conditions needs to maintain stable ES cell lines in domestic animals.

3-1. Porcine pluripotent stem cells

Among domestic animals, pig has been particularly considered as

ideal animal model for biomedical research, as it is immunologically and physiologically similar to human. Pig has a potential for studying regenerative medicine, such as xenografting and cell theraphy (Hall, 2012; Nowak-Imialek et al., 2011; Talbot and Blomberg le, 2008). With these benefits, numerous efforts have been made to establish the pluripotent stem cells from porcine embryos. Although authentic pES cell lines have not been derived, a number of putative pES cells have been isolated from various porcine embryos including in vitro produced and in vivo derived embryos, parthenogenetic embryos, in vitro aggregated and somatic cell nuclear transfer embryos (du Puy et al., 2011; Kim et al., 2010; Li et al., 2004; Li et al., 2003; Park et al., 2013; Tan et al., 2011; Vackova et al., 2011; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). However, most of these reports showed limited pluripotency, when compared to mES cells. In particular, any putative pES cells couldn't show teratoma formation and germ-line chimerism. They have just showed morphology, AP activity, marker expression and in vitro differentiation.

Reasons for these deficient products can be described following explanations, although it is entirely clear. At first, porcine species is termed non-permissive specie, as authentic pluripotent stem cells from porcine embryos can't be derived by conventional method for ES cell establishment used in permissive mouse (Hanna et al., 2010b; Park et al.,

2013). Second, it is not yet identified optimal culture condition to suppress being differentiated into primed state from naïve pre-implantation epiblast in pig. That is because only a few papers have addressed signaling pathway in pES cells with the limited growth factors, such as LIF, platelet-derived growth factor (PDGF) and bFGF. However, combinations of LIF, PDGF and bFGF were not enough to maintain the pluripotency in pES cells (Hall, 2012; Hochereau-de Reviers and Perreau, 1993; Moore and Piedrahita, 1997; Wianny et al., 1997). Third, it is the lack of understanding specific markers for pluripotency in porcine ICMs and ES cells. Oct4, Sox2 and Nanog have been suggested as the pluripotency marker in naïve mES cells (Boyer et al., 2006; Loh et al., 2006; Rodda et al., 2005). The expression of Oct4, as the marker of ICM or ES cells, is detected in the ICMs of murine blastocyst, but not TE. In pig, however, the expression of Oct4 was observed in both the ICMs and TE of early blastocyst (E 5-6). Furthermore, the genes of Nanog and Sox2 were weakly expressed in early porcine epiblasts, while the expression of Oct4, Nanog and Sox2 were exclusively detected in post porcine epiblast (E 10) (Blomberg et al., 2008; Hall et al., 2009; Puy et al., 2010). With these results, it is questionable whether the genes of Oct4, Nanog and Sox2 are the appropriate markers of pluripotency in porcine ICMs and ES cells or not.

Meanwhile, since miPS cell line was first reported by Takahashi and

Yamanaka, a number of research groups have reported porcine iPS cell lines (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; Takahashi and Yamanaka, 2006; West et al., 2010; West et al., 2011; Wu et al., 2009). Esteban et al., Ezashi et al. and Wu et al. have established iPS cell lines from porcine fibroblasts using the retrovirus, lentivirus and doxycycline inducible lentivirus system, respectively. West and colleagues, in peculiar, showed piPS cells with germ-line transmission, although it is the results by PCR analysis. Most of these reports showed piPS cells derived from porcine fibroblast or mesenchymal cells is morphologically and molecularly similar to mEpiS cells or hES cells, rather than to mES cells (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; Park et al., 2013; Takahashi and Yamanaka, 2006; West et al., 2010; West et al., 2011; Wu et al., 2009). Summary of results describing establishment of pluripotent stem cells in pigs are listed in Table 1.

Table 1. Summary of results describing establishment of pluripotent stem cells in pigs

Source		Growth factors	Maximum passage number	Morphology	In vitro differentiation
Murine pre-implantation embryo		LIF	long term	Domed	EB, 3 germ layers
Murine post-implantation embryo		bFGF+Activin	long term	Flattened	EB, 3 germ layers
Human blastocyst		bGFG	long term	Flattened	EB, 3 germ layers
Porcine	In vivo blastocyst	-	> 1 year	ES-like	EB
	In vivo blastocyst	-	> 32	ES- and epithelial-like	EB
	In vivo blastocyst	-	> 80	epithelial-like	neurolike and pigmented cells
	In vivo blastocyst	-	-	Flattened	EB, morphology
	In vivo blastocyst	hLIF, rCNTF	4 days	nonepithelial and epithelial-like	morphology
	In vivo blastocyst	hLIF	2 passages	epithelial-like	CK18, lamine
	In vivo blastocyst	-	> 35	epithelial-like	EB, 3 germ layers
	In vivo, IVF blastocyst	bFGF+hLIF	4 passages	ES-like	EB, morphology
	IVF, PA, NT blastocyst	hLIF	5 passages	ES-like	EB
	In vivo blastocyst	-	-	ES-like	neuro, TE
	In vivo blastocyst	bFGF, mLIF	> 12	Flattened	EB, 3 germ layers
	IVF, PA blastocyst	bFGF, LIF	> 45	ES-like	EB, 3 germ layers
	NT blastocyst	bGFG	> 52	Flattened	EB, 3 germ layers
	In vivo blastocyst	bGFG	24 passages	ES-like	neural differentation
	In vivo, IVF blastocyst	bFGF+hLIF+Actvin A	14 passages	Flattened	EB, 3 germ layers
	In vivo blastocyst	mLIF	7 passages	Domed and Flattened	-
	In vivo, IVF blastocyst	bFGF+hLIF	14 passages	ES-, epithelial- and TE-like	neural, fibroblast and epithelial-like cells
	NT blastocyst	bGFG	-	ES-like	-
	In vivo, IVF, PA, AG blastocyst	bFGF, SCF	> 55	Flattened	EB, 3 germ layers
porcine embryonic fibroblasts (hOKSM)		bFGF	40 passages	Flattened	EB, 3 germ layers
porcine ear cell and bone marrow cells (hOSKMNL)		-	> 48	Flattened	EB, 3 germ layers
porcine embryonic fibroblasts (m/hOKSM)		bFGF, mLIF	> 25	Flattened	-
Porcine mesenchymal cells (hOKSMNL)		bFGF	> 50	defined borders	EB, 3 germ layers

Table 1. Continued

Marker expression	Teratoma	Chimerism	References
Oct4, Sox2, Nanog, SSEA1, AP	O	high/germline	(Evans and Kaufman, 1981), (Thomson et al., 1998), (Brons et al., 2007) (Hanna et al., 2010b), (Gandolfi et al., 2012)
Oct4, Sox2, Nanog, SSEA1, AP	O	low/no germline	
Oct4, Sox2, Nanog, SSEA4, TRA 1-60, TRA 1-81, AP	O	-	
-	-	-	(Evans et al., 1990)
cytokeratin 18	X	X	(Piedrahita et al., 1990a, b)
AP	-	-	(Talbot et al., 1993c; Talbot et al., 1993d)
-	-	X	(Gerfen and Wheeler, 1995)
AP	-	-	(Moore and Piedrahita, 1996, 1997)
SSEA1	-	-	(Wianny et al., 1997)
AP	-	low	(Chen et al., 1999a)
AP	-	-	(Li et al., 2004; Li et al., 2003)
AP	-	-	(Kim et al., 2007)
Oct4, Nanog, Sox2, REXO1, TDGF1	-	-	(Blomberg et al., 2008)
SSEA1, Oct4, Sox2, Nanog, Nodal, AP	-	-	(Alberio et al., 2010a)
Oct4, Sox2, Nanog, Rex1, FGFR2, Stat3, AKT, PI3K, PTEN	-	-	(Brevini et al., 2010a)
AP, Oct4, SSEA1, SSEA4, TRA 1-60, TRA 1-81	-	-	(Kim et al., 2010)
Oct4 and Nanog (early passage), Sox2	-	-	(Puy et al., 2010)
Oct4, Sox2, Nanog, SSEA1	-	low	(Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011)
Oct4, Sox2, Nanog	-	-	(Wolf et al., 2011)
Oct4, Nanog, AP	-	-	(Vackova et al., 2011)
Oct4, Sox2, c-Myc, Klf4	-	-	(Smith et al., 2007)
Oct4, Sox2, Nanog, AP, *	X	-	(Park et al., 2013)
Oct4, Sox2, TDGF1, TERT1, Lin28, Rex1	O	-	(Ezashi et al., 2009)
Oct4, Sox2, Nanog, AP, **	O	-	(Wu et al., 2009)
Oct4, Sox2, Nanog, SSEA4, Rex1, Klf4, AP	O	-	(Esteban et al., 2009)
OCt4, Sox2, AP	-	high	(West et al., 2010)

*: + TDGF1, REX1, bFGF, FGFR1, FGFR2, Activin A, Nodal, SSEA4, TRA 1-60, TRA 1-81, **: + DNMT3b, Lin28, SSEA3/4, TRA 1-60, TRA 1-81, E-cadherin

3-2. Bovine pluripotent stem cells

With the domestic porcine pluripotent stem cells, a number of efforts have also been made to establish the pluripotent stem cells from bovine embryos (Gong et al., 2010; Nowak-Imialek et al., 2011; Pant and Keefer, 2009; Pashaiasl et al., 2010). Various bovine ES cells have been established from nuclear transfer derived, in vivo derived and in vitro derived blastocysts, including parthenogenetic embryos derived blastocysts (Cibelli et al., 1998; Gong et al., 2010; Nowak-Imialek et al., 2011; Pant and Keefer, 2009; Pashaiasl et al., 2010; Saito et al., 1992a; Talbot et al., 1995; Wang et al., 2005). These cell lines were termed to ES-like cells in the aspect of teratoma formation and chimerism ability. In particular, it isn't easy to maintain the undifferentiated state with stable long-term cultivation (Ozawa et al., 2012). Reasons for these deficient results are the lack information of optimal culture condition to maintain the undifferentiated state of bES cells. All of LIF, bFGF and 2i couldn't stably maintain the undifferentiated state of bES cells (Gong et al., 2010; Ozawa et al., 2012). In 2011, Ozawa et al. have first reported the biPS cell lines from bovine fibroblasts using the retroviral infection of bovine 6 factors; bOct4, bSox2, bKlf4, bMyc, bLin28 and bNanog (Han et al., 2011b). These biPS cells showed a mES-like morphology, positive AP activity, the expression of pluripotent markers, in vitro and in vivo differentiation potential. These

results will help to establish bES cell lines in the future.

3-3. Pluripotent stem cells in sheep & goat

Although the sheep was the first cloned mammal by nuclear transfer, authentic ES cells have not been established from ovine embryos yet (Nowak-Imialek et al., 2011). Most of ovine ES-like cells couldn't show a stable and prolonged proliferation, although these cells exhibited positive AP activity and the ability of in vitro differentiation at early passage (Dattena et al., 2006; Handyside et al., 1987b). In the case of ovine iPS cells, pluripotent stem cells derived from sheep fibroblasts showed AP activity, the expression of pluripotent markers and the ability to in vitro and in vivo differentiation into three germ layers, including the capacity of teratoma formation (Bao et al., 2011).

Goats have a potential for the production of human recombinant proteins in mammary gland which can be used in biophaming (Ohkoshi et al., 2003). Unfortunately, only a few papers have been published from goat embryonic stem cells and these ES-like cells showed a limited characterization, such as AP activity, the expression of Oct4 and the capacity of in vitro differentiation (Garg et al., 2012; Pawar et al., 2009). In 2011, however, iPS cell line showing a reasonable pluripotency have been established from goat somatic cells (Ren et al., 2011).

CHAPTER 3. Establishment of porcine embryonic stem-like cell lines from various embryo origins

Introduction

Embryonic stem (ES) cells have a capacity of self-renewal and differentiation into three germ layers; endoderm, mesoderm and ectoderm (Evans and Kaufman, 1981; Friel et al., 2005; Smith, 2001). As ES cells are able to differentiate into all lineages of body under the appropriate environment, it is considered to be the greatest source to treat various diseases (Friel et al., 2005). Pig, showing immunological and physiological similarities to human, has especially been considered an ideal animal model for cell therapy of organ dysfunction syndrome, such as liver, heart and bone marrow (Brevini et al., 2007a; Kano et al., 2003; Klima et al., 2007; Krause et al., 2007; Smith et al., 2007; Zeng et al., 2007). With these benefits, a number of attempts have been made to establish the embryonic stem cells from porcine embryos for three decades after first establishment of murine ES cells. First establishment of porcine (p) ES cells have been made by Piedrahita et al. in 1990 (Piedrahita et al., 1990a, b). They showed the epithelial-like morphology, in vitro differentiation and EB formation potential. However, their ES-like cells couldn't proliferate for more than 10 passages. Most of 1990's papers related to the derivation of pES cells exhibited the similar aspects with that of Piedrahita (Nowak-Imialek et al., 2011).

Until now, many researchers have been making efforts to establish

pES cell lines. So, there are a number of putative pES cell lines using various porcine blastocysts such as in vitro produced, in vivo derived, parthenogenetic, in vitro aggregated and somatic cell nuclear transfer derived blastocysts (du Puy et al., 2011; Kim et al., 2010; Li et al., 2004; Li et al., 2003; Park et al., 2013; Tan et al., 2011; Vackova et al., 2011; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). However, these cell lines have been termed as “putative” or “like” ES cells, because of their limited pluripotency (Gandolfi et al., 2012; Hall, 2008; Hall, 2012; Munoz et al., 2009; Talbot and Blomberg le, 2008). Many reviewers have mentioned reasons for these difficulties of establishing authentic pES cells as the following explanation. One is a lack of information about exact embryonic days to derive ES cells from porcine embryos. The other is that it hasn’t yet been made the optimal culture condition to maintain the undifferentiated state of pES cells. It is considered that is because we don’t know the signaling pathways to maintain the pluripotent state of pES cells. So, many researchers have being tried to understand the porcine specific mechanism related to establishing the ES cells through many researches (Gandolfi et al., 2012; Hall, 2008; Hall, 2012; Munoz et al., 2009).

In this study, therefore, I am trying to address the early process during the derivation of ES-like cells from various blastocysts derived from in vivo, IVF, IVF aggregation and parthenogenetic activation. I could derive ES-like cells from various embryonic origins. And I confirmed positive AP acitivity

and the expressions of the pluripotent markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81 in our cell lines. These results indicate that ES-like cells derived from various embryos possess similar characteristics respectively, such as morphology, AP activity and the expression of pluripotent markers.

Materials and Methods

Collection, production and culture of porcine blastocysts

Methods for the generation of porcine blastocysts, including IVF procedures, *in vivo* collection, embryo aggregation (3X) and parthenogenesis, were performed according to previously described protocols (Lee et al., 2007; Park et al., 2009; Son et al., 2009). Porcine blastocysts were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in pESC medium, a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM low glucose, Gibco Invitrogen, USA, www.invitrogen.com) and Ham's F10 medium (Gibco), supplemented with 15% fetal bovine serum (FBS; collected and processed in Canada; Hyclone, Logan, UT, www.hyclone.com), 2 mM glutamax (Gibco), 0.1 mM ß-mercaptoethanol (Gibco), 1x MEM nonessential amino acids (Gibco), 1x antibiotic/antimycotic (Gibco) containing cytokines, 40 ng/ml human recombinant SCF (hrSCF; R&D Systems, USA, www.rndsystems.com), and 20 ng/ml human recombinant bFGF (hrbFGF; R&D Systems). Two seeding methods were used to establish pluripotent cell lines: intact blastocyst stage embryos were either cultured directly on MEFs or were subjected to mechanical dissection under the microscope using pulled glass pipettes to separate the inner cell mass (ICM) from the trophectoderm (TE) prior to

seeding. Following 5-7 days of culture, I observed ES-like primary colonies derived from day 7 *in vivo*-produced and day 8 *in vitro*-hatched blastocysts. These ES-like cell colonies were mechanically dissociated into several clumps using pulled glass pipettes 10-15 days after seeding. Dissociated clumps were then re-seeded on fresh inactivated MEFs, and subsequent ES-like cell lines were routinely passaged via the pulled glass pipette method every 5-7 days. All cells were cultured in humidified conditions maintained with 5% CO_2 at 37℃.

Alkaline phosphatase (AP) activity and immunocytochemistry (ICC) analysis

For AP staining of ES-like cell lines, cells were fixed with 4% paraformaldehyde for 15 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, Madison, WI, www.roche.com) in a buffer solution for 30 min at room temperature. For ICC analysis of undifferentiated or differentiated cells, fixed cells were washed and permeabilized (for intracellular markers only) with 0.2% Triton X-100 (Sigma, USA, www.sigmaaldrich.com) for 5 min. Washed cells were co-incubated with blocking solution (10% goat serum in PBS) and a primary antibody overnight at 4°C. The primary antibodies used

were Oct4 (SC-9081, Santa Cruz Biotechnology, www.scbt.com 1:100), Nanog (SC-33759, Santa Cruz Biotechnology, 1:100), Sox2 (AB5603, Millipore, Temecula, CA, www. millipore.com, 1:200), SSEA-4 (MAB4304, Millipore, 1:200), Tra 1-60 (MAB4360, Millipore, 1:200), Tra 1-81 (MAB4381, Millipore, 1:200), Nestin (sc-20978, Santa Cruz Biotechnology, 1:200), Cardiac troponin 1 (MAB3152, Millipore, 1:200) and α-fetoprotein (V7049, Biomeda Corp, 1:200). The cells were then washed, incubated with the secondary antibodies. Secondary antibodies were applied and incubated at room temperature for 1 h. The secondary antibody for all reactions was DakoCytomation LSAB 2-system HRP (Dako, Glostrup). Samples were rinsed three times with Tween20/PBS, and 3-3' diminobenzidine (DAB; Vector Laboratories, Burlingame) was used as the indicator.

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

To evaluate differentiation potential, ES-like cell lines were removed from MEFs, mechanically dissociated with glass pipettes and cultured in pESC medium without cytokines using the hanging drop method. After five days, ES-like cells formed typical EBs, which were transferred to confocal dishes coated with 0.1% gelatin and allowed to further differentiate during 2-3 weeks of culture.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

To analyze the gene expression patterns of undifferentiated or differentiated cells, total RNA from individual samples was extracted using TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Forster City, CA, www.appliedbiosystems.com) according to the manufacturer's instructions, producing a final volume of 20 μl. PCR amplifications were performed using a 2x PCR Master Mix Solution (i-MAX II, iNtRON Biotechnology) with a total reaction volume of 20 μl, containing 1 μl cDNA, 1 μl of each primer, and 7 μl distilled water. The conditions and primers used are listed in Table 2.

Karyotyping

Standard G-banding chromosome and cytogenetic analysis was carried out at Samkwang Medical Laboratories (Korea, www.smlab.co.kr).

Results

Derivation of ES-like cells from various embryo origins

To identify the early process being derived primary ES-like cell's colony from blastocysts, I observed a surface morphology of blastocyst and the process being attached blastocysts onto feeder cells layer under high magnification. As shown in Figure 1A and 1B, there are giant cells in surface section of blastocyst, so-called TE. And the mass of cells, so-called ICMs, exist in a part inside blastocyst. The size of cells in ICMs is smaller than that of TE. In two to three days after the seeding of blastocysts, giant cells shown in TE initially proliferated and then gradually died out or disappeared after a few days, except the mass of cells which is presumed to be ICMs (Figure 1C and 1D). After around 7 days, I was able to observe a small primary colony with ESC-like cells morphology and a large primary colony, which can be passaged, at around 14 days post seeding (Figure 1E and 1F).

I have been able to establish ES-like cell lines from various embryo origins, such as in vivo derived, in vitro fertilized (IVF), IVF aggregated (3X) and parthenogenetic blastocysts. Information and efficiency of established all cell lines is summarized in Table 3 and Table 4. I confirmed similar morphologies of primary colonies and typical morphologies among these cell lines. All our cell lines showed positive AP activity (Figure 2).

Table 2. Primers used and conditions for RT-PCR

Genes	Primer sequences	Annealing Temperature (℃)	Products size (bp)	Accession Number
OCT4	5′-AACGATCAAGCAGTGACTATTCG-3′ 5′-GAGTACAGGGTGGTGAAGTGAGG-3′	60	153	AF074419
NANOG	5′-AATCTTCACCAATGCCTGAG-3′ 5′-GGCTGTCCTGAATAAGCAGA-3′	60	141	DQ447201
SOX2	5′-CGGCGGCAGGATCGGC-3′ 5′-GAGCTCCGCGAGGAAAA-3′	60	113	EU519824
ß-ACTIN	5′-GTGGACATCAGGAAGGACCTCTA-3′ 5′-ATGATCTTGATCTTCATGGTGCT-3′	60	137	U07786

Table 3. Information of established porcine ES-like cell lines.

Cell lines	Origins (porcine)	Initial culture methods	Karyotype	Passaging
pESI1	in vitro fertilization(IVF) blastocysts	Whole Explant	-	more than 51
pESI2	in vitro fertilization(IVF) blastocysts	Whole Explant	-	more than 62
pESI3	in vitro fertilization(IVF) blastocysts	Whole Explant	55 XY, abnormal	more than 49
pESI4	in vitro fertilization(IVF) blastocysts	Whole Explant	38 XX, normal	more than 36
pESI5	in vitro fertilization(IVF) blastocysts	Whole Explant	38 XX, normal	more than 43
pESI6	in vitro fertilization(IVF) blastocysts	Whole Explant	38 XX, normal	more than 46
pESP1	parthenogenetic blastocysts	Whole Explant	38 XX, normal	more than 44
pESP2	parthenogenetic blastocysts	Whole Explant	38 XX, normal	more than 42
pES3X1	IVF aggregated (3X) blastocysts	Whole Explant	38 XX, normal	more than 53
pES3X3	IVF aggregated (3X) blastocysts	Whole Explant	38 XY, normal	more than 45
pES3X4	IVF aggregated (3X) blastocysts	Whole Explant	-	more than 39
pES11	in vivo produced blastocysts	Whole Explant	38 XX, normal	more than 41
pES12	in vivo produced blastocysts	Mechanical dissection	38 XX, normal	more than 45

Table 4. Efficiency of porcine ES-like cell lines derived from various embryo origins

Embryo origins	No. of blastocysts	ESC lines (%)
In vitro fertilization(IVF) blastocysts	120	6 (5)
Parthenogenetic blastocysts	99	3 (3)
IVF aggregated (3X) blastocysts	32	3 (9.4)
In vivo produced blastocysts	3	2 (66.7)

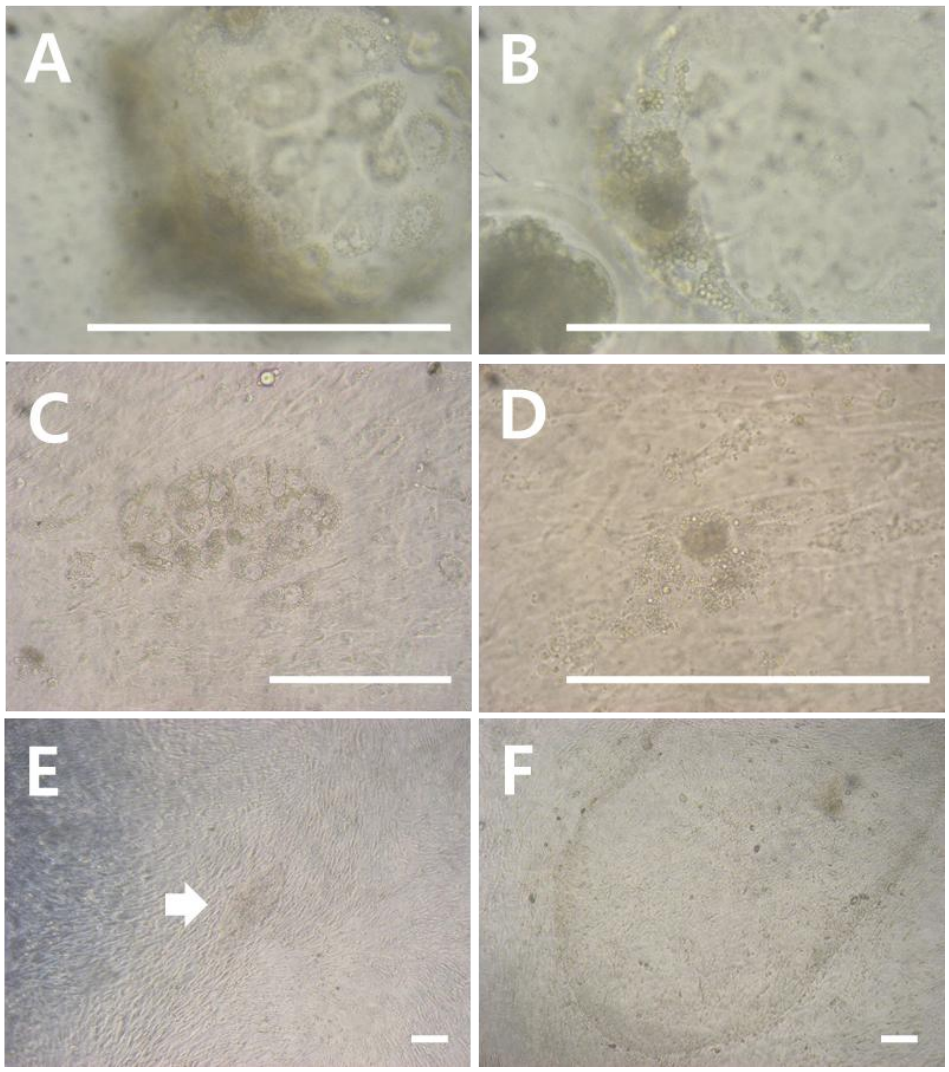

A
B
C
D
E
F

Figure 1. Primary derivation of ES-like cells from embryo origins

(A) Morphology of TE-like giant cells in surface section of blastocyst. **(B)** Morphology of ICM-like the mass of cells in a part inside blastocyst **(C)** In two to three days after the seeding of blastocysts, giant cells shown in Figure 1B are attached on the feeder layer cells **(D)** The mass of cells which is presumed to be ICMs shown in Figure 1C are attached on the feeder layer cells . **(E)** Morphology of ESC-like primary colony derived from porcine embryo. **(F)** Late morphology of ESC-like primary colony derived from porcine embryo. Scale bars = 100μm.

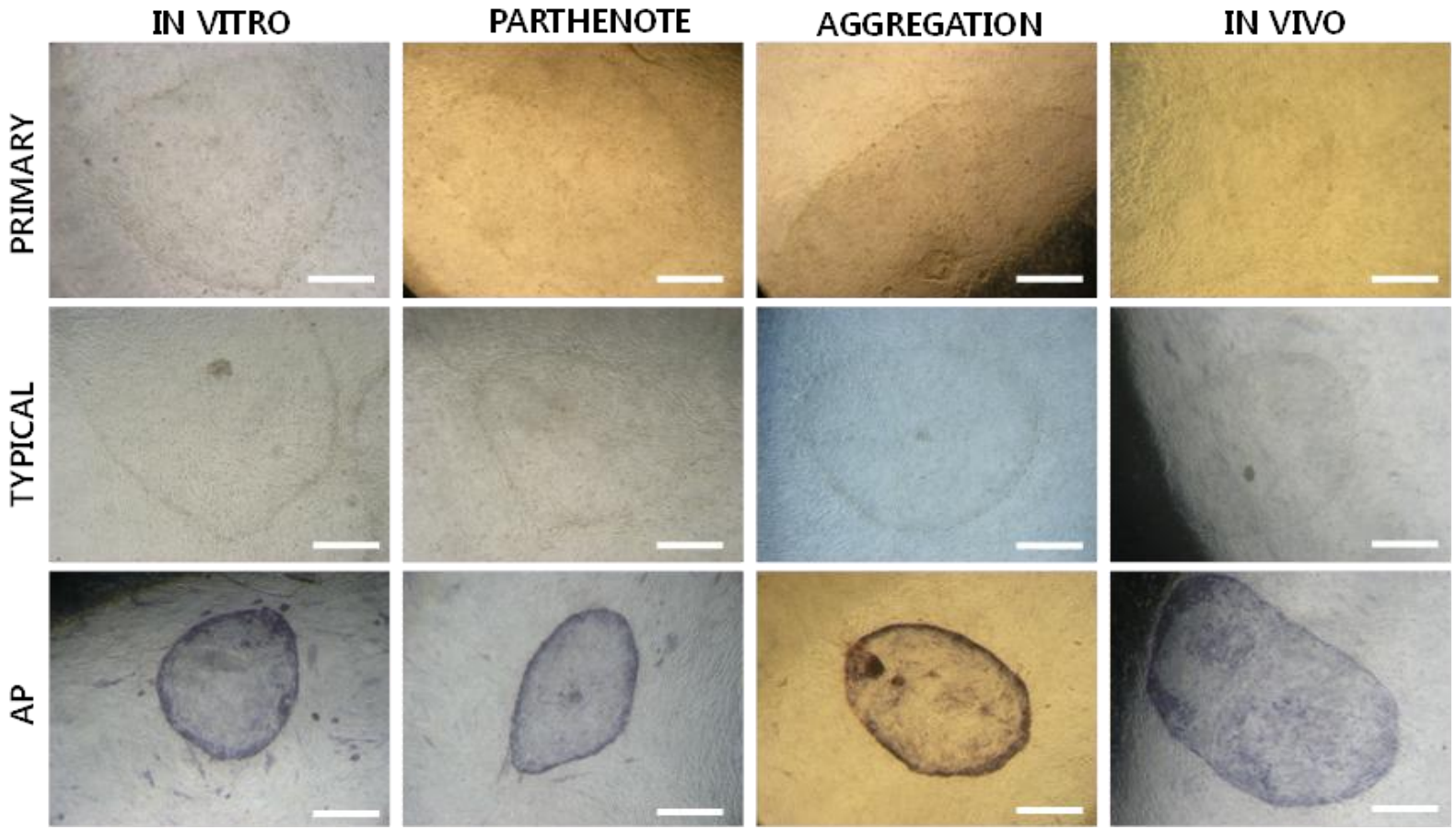

IN VITRO
PARTHENOTE
AGGREGATION
IN VIVO
PRIMARY
TYPICAL
AP

Figure 2. Derivation of ES-like cells from various embryo origins.

Primary morphologies, typical morphologies and AP activity of ESC-like pES cells derived from various porcine embryo origins; in vitro fertilized (IVF), parthenogenetic, IVF aggregated (3X) and in vivo derived blastocysts. Scale bars = 200μm.

Verification of undifferentiated state in various ES-like cells

I invested the expression of pluripotent markers in all cell lines. As shown Figure 3, all cell lines expressed the gene of pluripotent marker; Oct4, Sox2 and Nanog, which have reported to play a crucial role as the transcription factors in mES cells (Chambers et al., 2003; Masui et al., 2007; Niwa et al., 2000b). As shown in Figure 4, I are able to confirm the expression of the pluripotency and surface markers in our cell lines, such as Oct4, Nanog, SSEA1, SSEA4, TRA 1-60 and TRA 1-81, Whose expressions have reported as a feature of hESCs (Thomson et al., 1998). These results indicate that our ES-like cell lines derived from various embryos possess similar characteristics with hES cells. And our cell lines showed similar morphologies of primary colonies and typical morphologies, AP activity and the expression of pluripotent markers, among each other's cell lines.

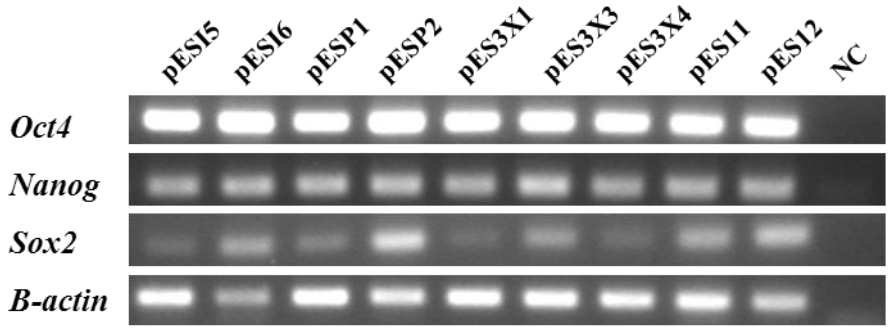
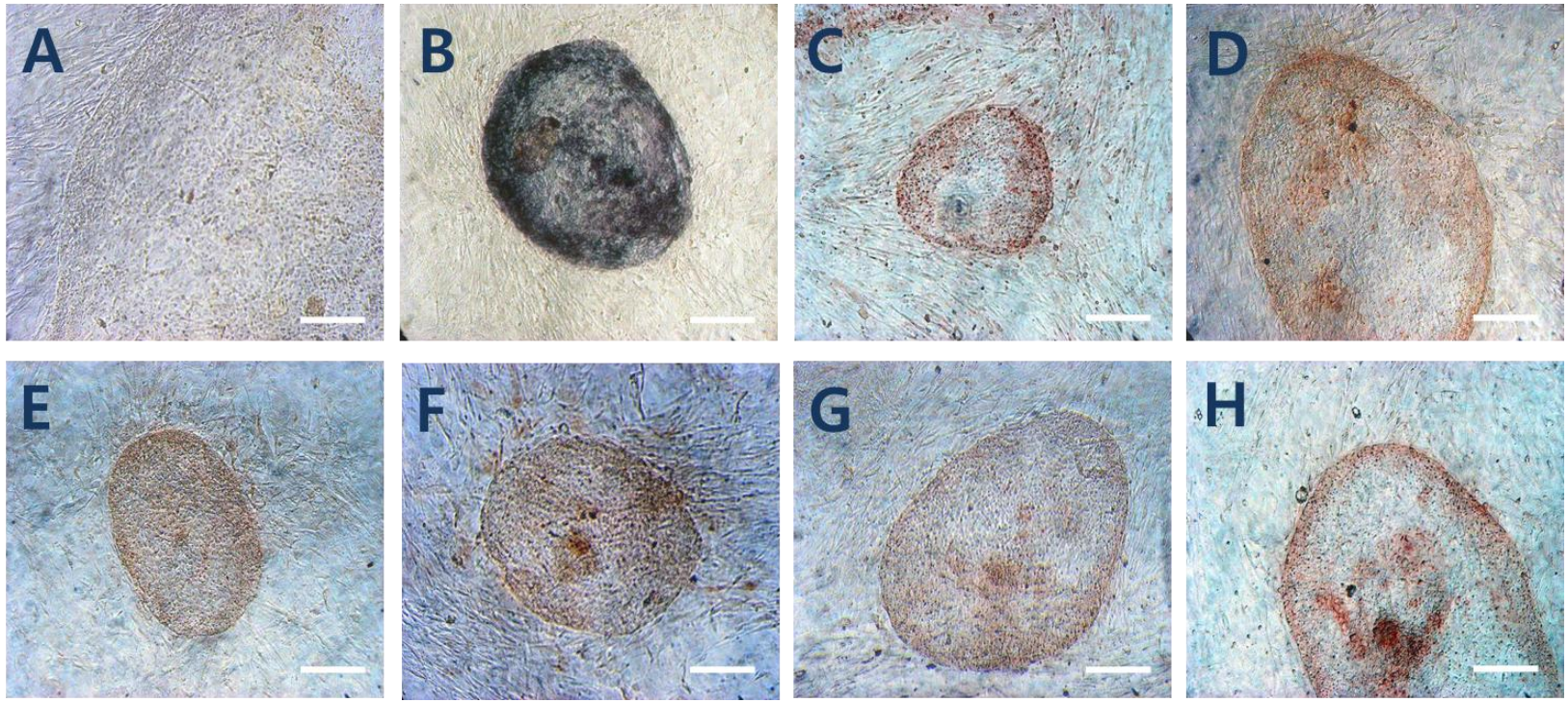


Figure 3. Gene expression analysis of ESC-like pES cell lines.

All of cell lines expressed *Oct4*, *Nanog and Sox2*, which have reported to play a crucial role as the transcription factors in mES cells.


A
B
C
D
E
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G
H

Figure 4. The expression of pluripotent markers in ESC-like pES cells by immunocytochemistry analysis. AP activity and the expression of pluripotent markers and surface markers in ESC-like cells; **(A)** Negative, **(B)** AP activity, **(C)** Oct4, **(D)** Nanog, **(E)** SSEA-1, **(F)** SSEA-4 **(G)** TRA 1-60 and **(H)** TRA 1-81. Scale bars = 200μm.

Differentiation potential and chromosome assay in various ES-like cells

To identify the differentiation potential, I investigated the EB formation and differentiation capacity into three germ layers, such as endodermal, mesodermal and ectodermal germ layers, in our cell lines. As shown in Figure 5A, it showed our cell lines have the potential of EB formation when it was induced to differentiate following five days of culture using the hanging drop method. When EBs were cultured onto plates coated with 0.1% gelatin, I could observe a variety of differentiated cells after 1-2 weeks. As shown in Figure 5B-D, I could confirm the expressions of three germ layer markers, including Nestin (ectoderm), Cardiac troponin 1 (mesoderm) and α-fetoprotein (endoderm), from differentiated cells using immunocytochemical analysis. I investigated normality or abnormality of chromosome in our cell lines using karyotyping assay. As shown in Figure 6, I could observe normal karyotyping in our cell lines except the cell line pESI3 which showed a chromosome aberration (Figure 6J).

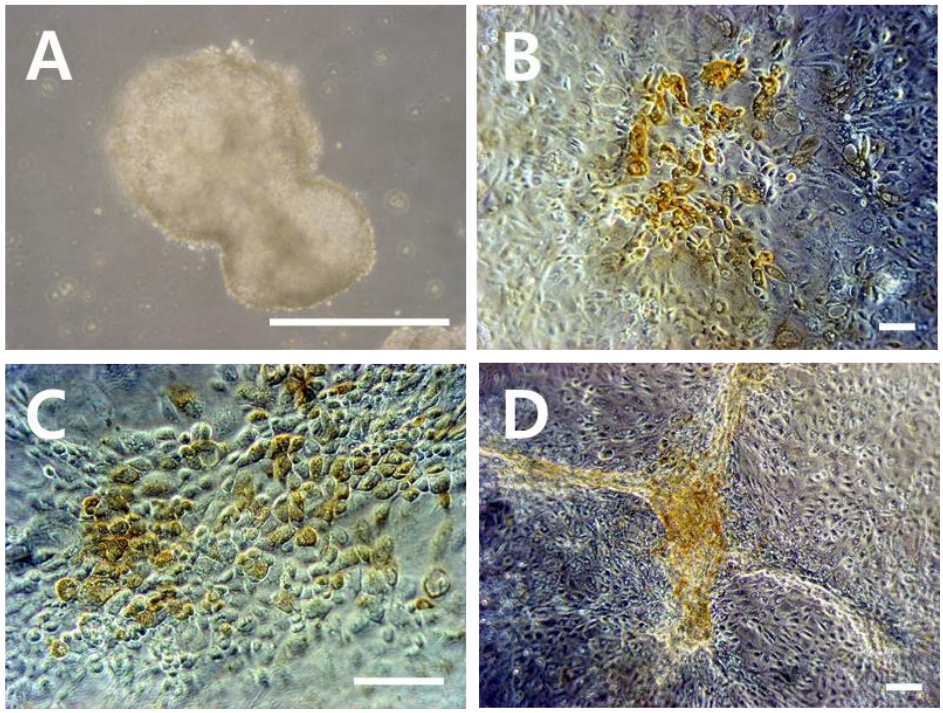

Figure 5. Defferentiation potential of ESC-like pES cell lines.

(A) Representative embryoid bodies derived from EpiSC-like pES cell lines through the culture for 5 days by hanging drop method. When EBs cultured continue onto culture plates, I could observed a variety of differentiated cells. I could confirm the expression of marker genes **(B)** AFP (endoderm), **(C)** CARDIAC (mesoderm) and **(D)** NESTIN (ectoderm) involving differentiation. Scale bars = 100μm.

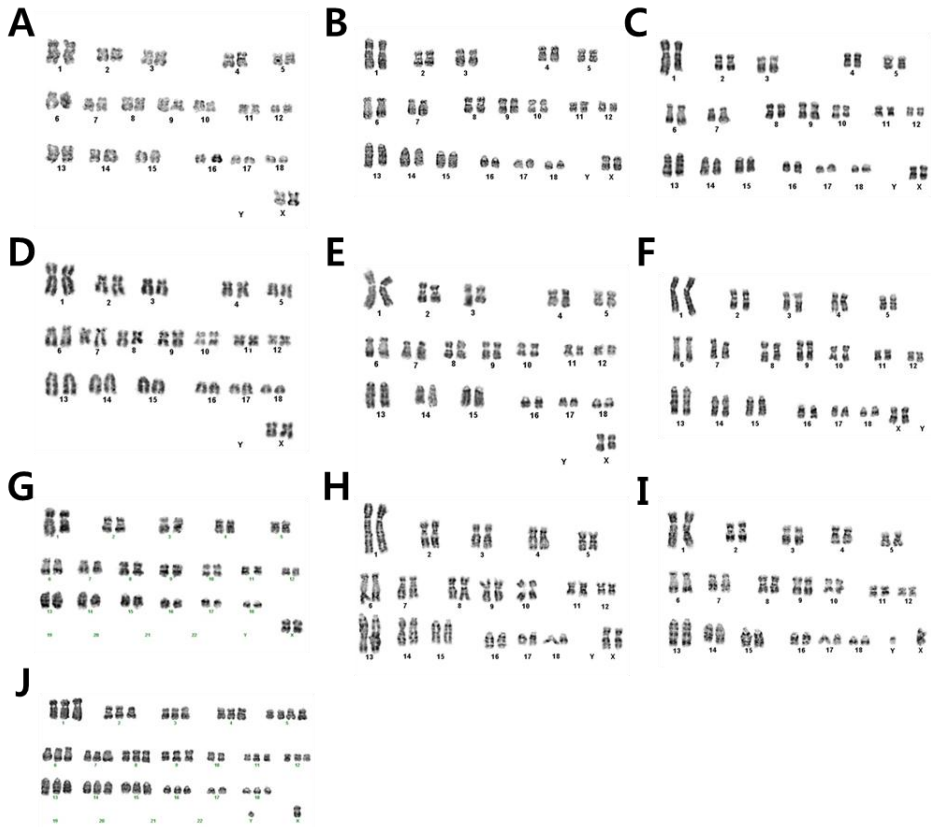

Figure 6. Karyotyping results of ESC-like pES cell lines.

Normal chromosomes were observed in all of cell lines except the cell line pESI3 which showed a chromosome aberration. **(A)** pESI4 (36+XX), **(B)** pESI5 (36+XX), **(C)** pESI6 (36+XX), **(D)** pESP1 (36+XX), **(E)** pESP2 (36+XX), **(F)** pES11 (36+XX), **(G)** pES12 (36+XX), **(H)** pES3X1 (36+XX), **(I)** pES3X3 (36+XY), and **(J)** pESI3 (53+XY).

Discussion

During the embryogenesis, blastocysts comprise the population of two lineages (Friel et al., 2005; Rippon and Bishop, 2004). One is the inner cell mass, which subsequently develop into epiblast and hypoblast. The epiblast in ICMs forms embryo or fetus. Therefore, the epiblast is referred as the population of pluripotent state, due to capacity to generate all lineages in body, and it has been utilized for the source of ES cells. The other is the trophoblast, which exists in an outer layer of cells in blastocyst and forms the placenta. With these backgrounds, in order to understand the process being established ES cell line from porcine embryos, I observed the early process being derived ES cells from various embryos. As shown in Figure 1A and 1B, I could observe two types of cells in porcine blastocysts under high magnification. There are giant cells in an outer layer of TE, and ICMs-like small cell mass exist in the interior of blastocyst. These two types of cells could be observed onto the feeder cell layer in two to three days after the seeding of blastocysts (Figure 1C and 1D). However, TE-presumptive cells were gradually disappeared and ES-like primary cells came out onto feeder cells in around 7 days after the seeding of embryos. These pattern generated to ES cells from embryo was observed in all our cell lines, including ES-like cell lines derived from in vivo derived, IVF, IVF aggregated (3X) and parthenogenetic embryos. As it was hard to identify ICM from most of In vitro-produced blastocysts, I used the whole seeding

method to derive initial primary colony and could establish ES-like cell lines from various porcine blastocysts using this method. Ming Li et al. reported a whole seeding method to derive pluripotent cell lines from in vitro produced porcine embryos which is difficult to be discriminate ICM from trophectoderm (Li et al., 2004). Moreover, Nicco Heins et al. (Heins et al., 2004) also reported the establishment of human ES cell lines from hatched human blastocysts by whole seeding method. Therefore, a whole seeding method could be used to establish the pluripotent stem cell lines from porcine blastocysts like that by former reported whole seeding method.

Until now, a number of studies have been reported to establish ES cell lines from porcine embryos (Brevini et al., 2007b; Vackova et al., 2007). However, most of papers showed it is difficult to establish an authentic ES cell line. Instead, it tends to grow differentiated epithelial or ES-like cells during the process being derived ES cells from porcine embryos (Brevini et al., 2007b; Talbot and Blomberg le, 2008; Telugu et al., 2010; Vackova et al., 2007). I have also been able to derive ES-like cells from porcine embryos of various origins.

In conclusion, I were able to establish the ES-like cell lines from porcine embryos of various origins including IVF, in vivo derived, IVF aggregated (3X) and parthenogenetic embryos. All our ES-like cell lines derived from various embryos showed similar morphologies of primary

colonies and typical morphologies. And these cell lines, coincident with results of previous reports, showed the AP activity, the expression of the pluripotent markers, such as Oct4, Sox2, Nanog, SSEA1, SSEA4, TRA 1-60 and TRA 1-81, EB formation, normal karyotyping and differentiation capacity into three germ layers. These results suggest that ES-like cell lines showing similar characteristics respectively are derived, when ES cell lines were established from various embryo origins. And these ES-like pES cells showed similar characteristics with hES cells.

CHAPTER 4. A comparative analysis of primed pluripotent cell lines derived from various embryonic origins and somatic cells in pigs

Introduction

Pluripotent stem cells are capable of differentiating into all three embryonic germ layers and maintain their pluripotency and self-renewal capacity over a long period of time (Thomson et al., 1998). Since pluripotent cells were first derived from the inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman, 1981), attempts to establish embryonic stem (ES) cell lines have been tried from various mammal species, including pigs (Piedrahita et al., 1990a), cattle (Saito et al., 1992), rats (Buehr et al., 2008), primates (Thomson et al., 1995) and humans (Thomson et al., 1998).

Among these various ES cell lines, different characteristics concerning morphology, signaling pathways and cell surface marker antigens are observed across species (Brevini et al., 2007b; Hall, 2008; Talbot and Blomberg le, 2008). A recent study has reported that these pluripotent stem cells exist in one of two forms and can be categorized according to their pluripotent state. The first is a “naïve” state, which is characterized by small, round or dome-shaped colony morphologies, LIF and BMP4 signaling pathways, the expression of stage-specific embryonic antigen-1 (SSEA-1) as a cell surface marker and two active X chromosomes in female; mouse ES cells (mESCs) represent this type of ES cell. A second “primed” state has also been described and is possible in mouse epiblast stem cells

(mEpiSCs) or human ES cells (hESCs) (Hanna et al., 2009; Hanna et al., 2010b; Nichols and Smith, 2009). These primed state pluripotent stem cells display flattened monolayer colony morphologies, FGF and Activin/Nodal signaling pathways, expressions of SSEA4, TRA 1-60 and TRA 1-81 surface marker antigens and X chromosome inactivation in female (Hanna et al., 2010b; Nichols and Smith, 2009; Vallier et al., 2005).

It has been suggested that the porcine model provides the most ideal non-primate system for clinical research regarding potential human therapeutic use, an observation based on immunological and physiological similarities with humans. In addition, porcine embryonic stem cells (pESCs) would provide powerful experimental tools, such as the production of transgenic pigs and xenografting (Brevini et al., 2007a). However, for many domestic species including the pig, authentic ESCs have not yet been categorized. Given the prospective advantages and current limitations, many reviewers have recently emphasized important details that must be considered for the establishment of validated pESCs. It has been noted that defining the optimum stage of embryonic development for stem cell derivation is key to obtaining stable ES cell lines, together with elucidation of pluripotent markers and the key signaling pathways that regulate the pluripotency of pESCs. Furthermore, an understanding of species-specific mechanisms and optimal culture conditions must be achieved in order to maintain stable ES cell lines (Hall, 2008; Munoz et al., 2009; Oestrup et al.,

2009; Vackova et al., 2007). Considering these challenges, in recent years, several research groups have reported putative pESCs from embryos produced in vivo, in vitro fertilization, parthenogenetic activation and somatic cell nuclear transfer (du Puy et al., 2011; Kim et al., 2010; Tan et al., 2011; Vackova et al., 2011; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). It has been identified that these pESC lines showed EpiSC-like characteristics such as flattened monolayer colony morphologies and activin/nodal signaling pathway (Alberio et al., 2010). I have also attempted to establish pluripotent cell lines from porcine embryos; however, like many others, I am so far unable to derive what could be described as authentic pESC lines. However, during our research, I have been able to derive EpiSC-like pESC lines from various porcine blastocysts derived from in vivo, IVF, IVF aggregation, and parthenogenetic activation.

Recently published papers have reported that pluripotent cells can be induced from somatic cells using four commonly cited reprogramming genes (Oct4, Sox2, Klf4, and c-Myc) (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; Takahashi and Yamanaka, 2006; West et al., 2010; West et al., 2011; Wu et al., 2009). Especially, West and colleagues reported porcine induced pluripotent stem cells (piPSCs) capable of generating chimeric offspring (West et al., 2010; West et al., 2011). However, the piPSCs has not been characterized in detail for the pluripotent state, although their ability to produce chimeric offspring had

been reported (West et al., 2010). I have also been successful in deriving piPSCs using these factors and previously described methods (Okita et al., 2010).

The main purpose of the present study was to investigate characteristics such as marker expression, signaling pathways, pluripotency and self-renewal in these EpiSC-like pESC and piPSC lines. In this study, I confirmed activation of the Activin/Nodal/FGF pathway and the expressions of the pluripotency markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81 in all of our cell lines. Furthermore all of these cell lines showed in vitro differentiation potential; the X chromosome inactivation in female and a normal karyotype. Our results suggest that pluripotent stem cells derived from embryos and iPSCs derived from embryonic fibroblasts in the porcine model possess a primed pluripotent state similar to that of mEpiSCs or hESCs, rather than to that of mESCs.

Materials and Methods

Collection, production and culture of porcine blastocysts

Methods for the generation of porcine blastocysts, including IVF procedures, *in vivo* collection, embryo aggregation (3X) and parthenogenesis, were performed according to methods described in Chapter 3.

Generation and culture of piPSCs

The derivation of piPSCs was conducted using previously described methods (Okita et al., 2010). Briefly, a pCX-OKS-2A plasmid containing *Oct4*, *Klf4*, and *Sox2* and a pCX-cMyc plasmid containing *c-Myc* were obtained from Addgene (plasmids 19771 and 19772, respectively; www.addgene.org). Plasmid DNAs were purified from transformed E-coli using a plasmid DNA purification kit (iNtRON Biotechnology, Korea, www.intronbio.com) and were introduced into porcine embryonic fibroblasts (PEFs) in a 35 mm dish with Opti-MEM (Invitrogen) in a total volume of 500 µl, consisting of 2 µg pCX-OKS-2A, 1 µg pCX-cMyc, 6 µl Lipofectamine™ LTX (Invitrogen), and 2 µl Plus™ Reagent (Invitrogen).

Plasmid transfection was performed a total of four times at two-day intervals. PEFs (2 x 10^5 cells) were cultured in pESC medium on mitotically

inactivated MEFs in 35 mm dishes for 2-3 weeks. Transfected PEFs were transferred daily to fresh pESC medium until colonies sufficiently large to passage were observed. EpiSC-like colonies were mechanically dissociated into several clumps using pulled glass pipettes. The resulting piPSCs were routinely passaged every 5-7 days.

Alkaline phosphatase (AP) activity and immunocytochemistry (ICC) analysis

Methods for AP staining and ICC analysis of EpiSC-like pESCs and piPSCs were performed according to methods described in Chapter 3. For ICC analysis of undifferentiated or differentiated cells, the primary antibodies used were Oct4 (SC-9081, Santa Cruz Biotechnology, www.scbt.com 1:100), Nanog (SC-33759, Santa Cruz Biotechnology, 1:100), Sox2 (AB5603, Millipore, Temecula, CA, www. millipore.com, 1:200), SSEA-4 (MAB4304, Millipore, 1:200), Tra 1-60 (MAB4360, Millipore, 1:200), Tra 1-81 (MAB4381, Millipore, 1:200), Neurofilament (MAB1615, Milllipore, 1:200), Desmin (MAB3430, Millipore, 1:200), Cytokeratin 17 (MAB1625, Millipore, 1:200) and Anti-trimethy-Histone H3 Lys27 (07-449, Millopore, 1:500).

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

Methods for the EB formation and in vitro differentiation were performed according to methods described in Chapter 3.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and Real-time PCR

Methods for RT-PCR analysis were performed according to methods described in Chapter 3. The conditions and primers used are listed in Table 3. Real-time PCR amplification was conducted using the ABI 7300 Real-time PCR System (Applied Biosystems). A QuantiTect SYBER Green PCR kit (Finnzymes, Espoo, Finland, www.finnzymes.fi) was used to provide real-time quantification of the desired PCR product. The real-time PCR reaction mixture was comprised 1 µl cDNA, and 2 µl of each primer (Table 5) in a total volume of 20 µl. Four replications were conducted and the mRNA level of each sample was normalized to that of b-actin mRNA level. The relative levels of mRNA were analyzed by the delta-Ct method (Livak and Schmittgen, 2001).

Flow cytometric analysis

Colonies of EpiSC-like pES and piPS cells were mechanically detached from MEFs. After washing, detached colonies were dissociated

into single cells using TrypLE™ Express (Gibco). Dissociated cells were fixed, permeabilized and incubated with primary antibodies overnight at 4°C. The primary antibodies used were Oct4 (Santa Cruz Biotechnology, 1:10), Nanog (Santa Cruz Biotechnology, 1:10), and Sox2 (Millipore, 1:20). After washing, the cells were incubated with a suitable FITC-labeled secondary antibody. All samples were single-color stained using 10,000 cells for each marker, and the resulting data were analyzed using FACScalibur and Cell Quest software (BD Biosciences, CA, www.bd.com).

DNA isolation and Methylation Analysis

Isolation of genomic DNA from samples was carried out using G-spin Genomic DNA extraction kit (iNtRON) according to the manufacturer's instructions. The bisulfite treatment of genomic DNA samples was performed with the EZ DNA Methylation-Gold^{TM} kit (Zymo Reserch, USA, www.zymoresearch.com) according to the manufacturer's instructions. The XIST promoter region of converted DNA samples was amplified using PCR with 2x PCR Master Mix Solution (iNtRON Biotechnology) and XIST BS primers listed in Table 3. The genomic data of XIST were derived from "UCSC Genome Bioinformatics" site (www.genome.ucsc.edu) and a converted primer set was designed by "Methprimer" program (www.urogene.org). PCR products were cloned into the pGEMT-Easy vector (Promega, WI, USA, www.promega.com) and transformed into E.

coli cells (Novagen, USA) and at least 10 insert positive plasmid clones were sequenced by an ABI PRISM 3730 automated sequencer (Applied Biosystems). The methylation patterns were analyzed in sequences derived from clones.

Microarray Analysis

Total RNAs from individual samples were extracted using RNeasy Mini Kit (QIAGEN, www.qiagen.com) based on the manufacturer's instructions. The microarray data had been submitted to GEO database (GSE32506). To compare global gene expression of our pESCs with hESCs and mESCs, I downloaded the human and mouse cell information, hESCs (GSM628197-9, GSM525424-6), mESCs (GSM64922, GSM64924, GSM64926, GSM72622, GSM72624 and GSM72626) from NCBI GEO. We processed RMA of Affy (2.7) software package of the R statistical package to perform background subtracting and quantile normalization on each three species (Gautier et al., 2004). I made a gene expression orthologous set, by using microarray probe and orthologous information of pig (Sscrofa9), human (GRCh37.p3) and mouse (NCBIM37) from Ensembl Genes. I performed quantile normalization on 2606 orthologous probes using preprocess Core package (Bolstad et al., 2003). The gplots package of the R statistical package was employed to generate heatmap plot (Warnes et al.).

Karyotyping

Methods for chromosome analysis were described in Chapter 3.

Statistical analysis

Data are presented as the mean ± s.e.m. and were analyzed using Student's t-test. All analyses were performed using R software. $P < 0.05$ was considered significant.

Results

Generation of EpiSC-like pESCs and piPSCs

I have been able to derive EpiSC-like pESC lines from various porcine blastocysts and a piPSC line from porcine embryonic fibroblasts. Information of our cell lines is summarized in Table 6. As it is difficult to identify and exclusively isolate ICM cells from intact *in vitro* produced blastocysts, I employed a method of whole blastocyst seeding directly onto MEFs to generate our pluripotent cell lines. Two to three days following the seeding of day 8 *in vitro* hatched blastocysts, giant cells originating from trophectoderm initially proliferated and then gradually died out or disappeared. After 5-7 days, I was able to observe primary colonies with an EpiSC-like morphology (Figure 7A). Primary colonies large enough to passage (Figure 7C) were mechanically dissociated into several clumps using pulled glass pipettes at 10-15 days post seeding. I could derive various cell lines from *in vitro* produced blastocysts using this method; however, I could not be entirely sure that these primary colonies originated from the ICM and not the trophectoderm.

To determine whether these primary colonies represented ICM-derived cell lines, I used a mechanical dissection technique to eliminate trophectoderm cells prior to seeding. The cell line pES11 was derived using

the whole blastocysts seeding method from day 7 *in vivo* produced blastocysts, whereas cell line pES12 was derived using the mechanical dissection method. Following dissection, the dissected ICM and trophectoderm were plated onto separate culture plates and cultured under the same culture conditions. I found that it was only possible to derive primary colonies from ICM tissues; trophectoderm seeding onto feeder cells routinely failed to generate any primary colonies, with cells initially proliferating before dying out or disappearing (data not shown). To avoid controversies regarding the origins of pluripotent cell lines, I compared the characteristics of pluripotent cell lines derived from whole blastocysts with those of cell line pES12 derived from ICM tissue alone. The results showed that our pluripotent cell lines derived through the whole seeding method are similar to both cell line pES12 and cell line piPS-1. This whole seeding method has been previously used to generate pluripotent cell lines from *in vitro* produced porcine embryos as it is often difficult to distinguish between ICM and trophectoderm tissues in these embryos (Li et al., 2004). Moreover, Heins et al. also reported the establishment of hESCs from hatched human blastocysts via whole seeding, showing that this is a viable pluripotent cell derivation technique (Heins et al., 2004). Our results further demonstrate the usefulness of the whole seeding method in establishing pluripotent stem cell lines from *in vitro* produced porcine blastocysts. All of the cell lines derived from various embryos, including pES11 and pES12, showed similar primary colonies with typical morphology and AP activity, as shown

in Figures 7A, B, and C.

I also attempted to derive piPSC line from PEFs via plasmid transfection using four reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-MYC*). After transferring onto feeder cells, the PEFs with introduced reprogramming factors gradually demonstrated outgrowth, as shown in Figure 7D. I observed cells with an EpiSC-like morphology 2-3 weeks after seeding (Figure 7E). Cell line piPS-1 was derived via plasmid transfection and was routinely passaged every 5-7 days using pulled glass pipettes. The cell line piPS-1 displayed typical EpiSC-like morphology and AP activity (Figure 7F). In preliminary experiment, it was identified that transgenes (OKSM) were integrated into the genome of piPS-1.

Following the initial subculture of primary colonies, I used a mechanical dissociation method with pulled glass pipettes for routine subculture. When attempting to subculture our cells using enzymatic dissociation, I found that the proliferation efficiency and AP activity of these lines were significantly reduced (data not shown). These features are consistent with those of human or primate ES cells (Thomson and Marshall, 1998). Therefore, a mechanical dissociation method is essential for the stable and long-term maintenance of EpiSC-like pESCs and piPSCs.

All cell lines maintained stemness characteristics (Figure 7 and 8) and a stable morphology for more than 45 passages, as shown in Table 6, and were cryopreserved in liquid nitrogen using EM grids. After thawing, all six

lines proliferated without loss of stemness or normal morphology.

Table 5. Primers used and conditions for RT-PCR.

Gene	Primer sequences	Annealing Temperature (℃)	Product size (bp)	Accession Number
OCT4	5'-AACGATCAAGCAGTGACTATTCG-3' 5'-GAGTACAGGGTGGTGAAGTGAGG-3'	60	153	AF074419
NANOG	5'-AATCTTCACCAATGCCTGAG-3' 5'-GGCTGTCCTGAATAAGCAGA-3'	60	141	DQ447201
SOX2	5'-CGGCGGCAGGATCGGC-3' 5'-GAGCTCCGCGAGGAAAA-3'	60	113	EU519824
TDGF1	5'-CAGGAGGAGCCTGCAATTCG-3' 5'-CCCCCATTCAGACAGCAGGT-3'	60	101	TC207301
REX1	5'-TTTCTGAGTACGTGCCAGGC-3' 5'-GAACGGAGAGATGCTTTCTCAGAG-3'	60	201	TC206552
bFGF	5'-GCGACCCTCACATCAAACT-3' 5'-CAGTGCCACATACCAACT-3'	55	214	AJ577089.1
FGFR1	5'-ACTGCTGGAGTTAATACCACCG-3' 5'-GCAGAGTGATGGGAGAGTCC-3'	55	125	AJ577088
FGFR2	5'-GGTGTTAACACCACGGACAA-3' 5'-CTGGCAGAACTGTCAACCAT-3'	55	139	AJ439886.1
Activin-A	5'-TGCGCATTGACATGTACGCC-3' 5'-AGCTCCTCCAAGGACGGGTG-3'	60	136	NM001005350
NODAL	5'-ATCAGGTCCCACCCGACTGC-3' 5'-AGCTCCCCAGGGTGCTTCAG-3'	60	142	XM001928024
C-kit Ligand	5'-GTTGGATAAGCGAAATGGTGG-3' 5'-GTGACACTGACTCTGGAATCTTTT-3'	60	320	L07786
C-kit Receptor	5'-CCTGGGATTTTCTCTTCGTC-3' 5'-GACGAGGAAAAGCTTCTCAGG-3'	60	341	FJ938289
AFP	5'-CGCGTTTCTGGTTGCTTACAC-3' 5'-ACTTCTTGCTCTTGGCCTTGG-3'	60	483	NM214317
CRABP2	5'-CTGACCATGACGGCAGATGA-3' 5'-CCCCAGAAGTGACCGAAGTG-3'	60	185	NM001164509
DES	5'-CCTCAACTTCCGAGAAACAAGC-3' 5'-TCACTGACGACCTCCCCATC-3'	60	108	NM1001535
XIST	5'-GAAGCATCAGCCAGCAACAC-3' 5'-TCATAACCATCACTAGTACCCAAACC-3'	58	82	AJ429140
XIST BS	5'-TAAGAAGTAGGATGGTTTAAGGAAGG-3' 5'-CAACAAACAAAAACACCAACAATAC-3'	58	210	UCSC Genome Bioinformatics[a]
ß-ACTIN	5'-GTGGACATCAGGAAGGACCTCTA-3' 5'-ATGATCTTGATCTTCATGGTGCT-3'	60	137	U07786

a : The exact information was referred by materials and methods

Table 6. Information of established porcine pluripotent cell lines.

Cell lines	Origins (porcine)	Initial culture methods	Karyotype	Passaging
pESI6	in vitro fertilization(IVF) blastocysts	Whole Explant	38 XX, normal	more than 46
pESP2	parthenogenetic blastocysts	Whole Explant	38 XX, normal	more than 42
pES11	in vivo produced blastocysts	Whole Explant	38 XX, normal	more than 41
pES12	in vivo produced blastocysts	Mechanical dissection	38 XX, normal	more than 45
pES3X3	IVF aggregated (3 X) blastocysts	Whole Explant	38 XY, normal	more than 45
piPS-1	embryonic fibroblasts	Plasmid-transfection (4 factors)	38 XY, normal	more than 55

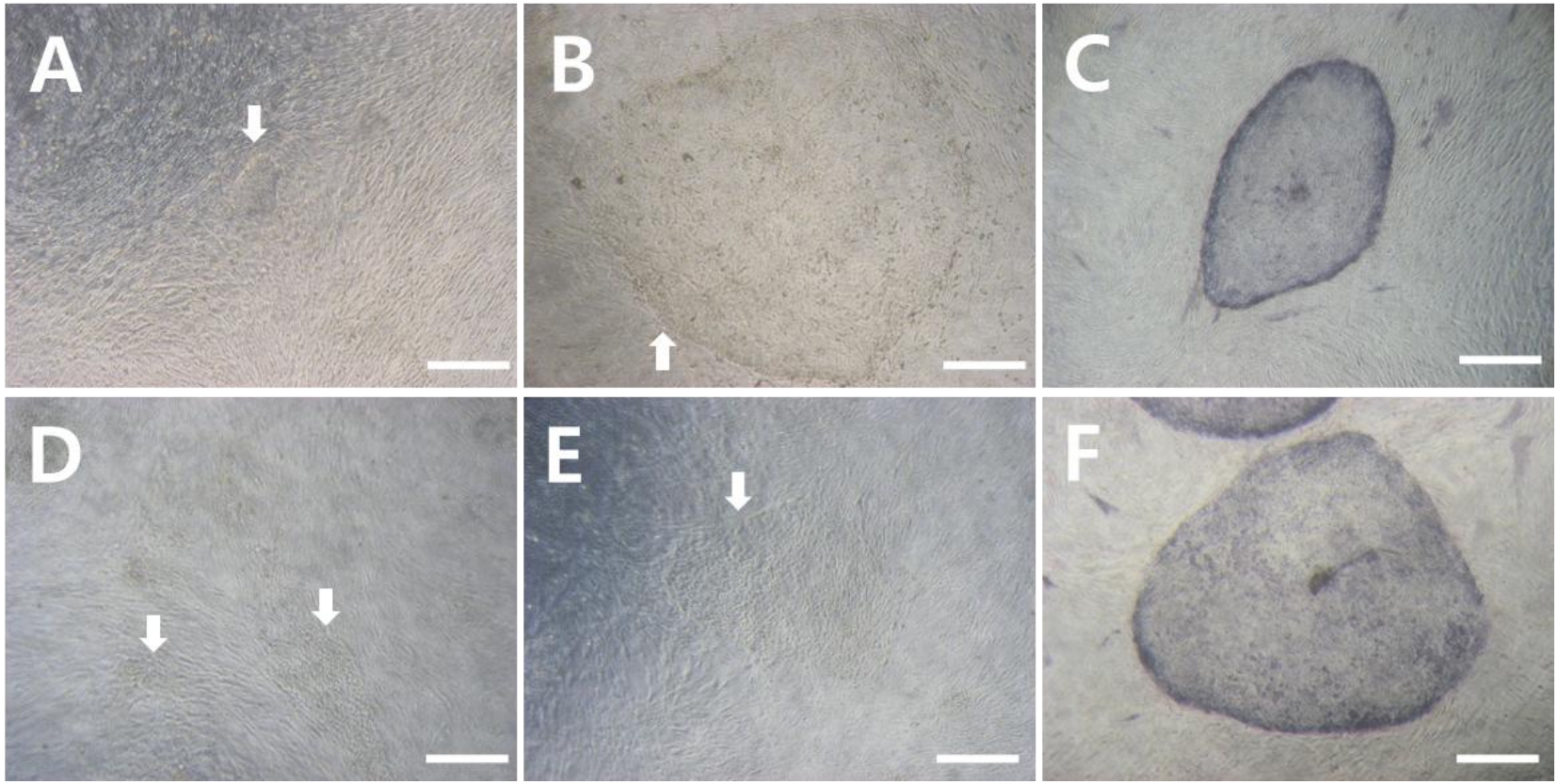
A
B
C
D
E
F

Figure 7. **Derivation of porcine pluripotent cells**

(A) Early morphology of EpiSC-like primary pESCs derived from porcine blastocysts. **(B)** Late morphology of EpiSC-like primary pESCs derived from porcine blastocysts. **(C)** AP activity of typical EpiSC-like pESCs. **(D)** Early morphology of EpiSC-like primary piPSCs derived from porcine embryonic fibroblasts. **(E)** Late morphology of EpiSC-like primary piPSCs derived from porcine embryonic fibroblasts. **(F)** AP activity of typical EpiSC-like piPSCs. **Scale bars = 200μm.**

Verification of pluripotency markers in EpiSC-like pESCs and piPSCs

I investigated the protein and mRNA expression levels of pluripotency markers in six cell lines that showed similar morphologies and AP activities. All of these cell lines expressed *Oct4*, *Sox2*, *Nanog, TDGF1* and *Rex1,* which are known early embryonic and undifferentiated pluripotency cell markers (Figure 8). I also identified the expressions of *bFGF*, *FGFR1*, *FGFR2*, *Activin-A* and *Nodal*, which are commonly activated in epiblast or epiblast stem cells (Alberio et al., 2010b; Greber et al., 2010; Hall et al., 2009; Hanna et al., 2010b) in all lines. All of the pluripotent cell lines, including line piPS-1, were cultured in the presence of hrbFGF and hrSCF as described in Materials and Methods. The addition of hrSCF to culture medium containing hrbFGF showed a greater positive effect on the stable maintenance of porcine pluripotent cells than did the addition of hrbFGF alone (data not shown), although further study is required to identify whether the addition of hrSCF is essential for the maintenance of stemness in porcine pluripotent cells. Interestingly, I found that mRNA expression of the *c-kit* ligand was high in all six cell lines (Figure 8). This may be because of autocrine or paracrine effects among porcine pluripotent cells or to a synergistic effect of hrSCF itself, although factors secreted from the feeder cells should also be considered.

I investigated the expressions of the pluripotency and surface markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81 in all six cell lines,

since expressions of these markers are a feature of hESCs (Thomson et al., 1998). As shown in Figure 9A-F, expressions of these markers were confirmed in all cell lines.

I also measured the relative expression levels of the pluripotency markers Oct4, Sox2 and Nanog among cell populations within the six cell lines using flow cytometric analysis. As a negative control, the cell lines were prepared without primary antibodies. All cell lines showed high expression levels of the pluripotent markers Oct4, Sox2 and Nanog. The results shown in Figure 10 were single-color stained, analyzed using FACScalibur with 10,000 cells per marker and overlapped using Cell Quest software (BD Biosciences, CA). As shown in Figure 10, the pluripotency markers Oct4, Sox2 and Nanog were expressed in 84.91%, 97.93% and 93.01% of cells in the cell line pESI6; 84.4%, 98.3% and 97.54% of the cells in pESP2; 87.08%, 97.57% and 97.74% of pES3X3; 99.56%, 94.14% and 99.8% of pES11; 98.11%, 99.78% and 99.14% of pES12; and 98.04%, 99.2% and 98.82% of piPS-1.

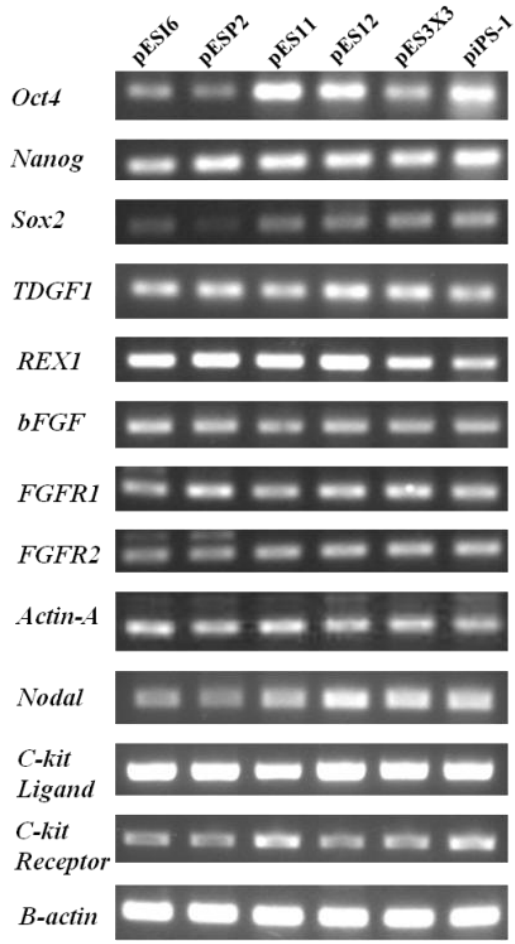

Figure 8. **Gene expression analysis of EpiSC-like pES cell lines and piPS cell line.**

All of porcine pluripotent cells expressed *Oct4*, *Sox2*, *Nanog, TDGF1* and *Rex1* known early embryos or undifferentiated pluripotent cells marker as well as *bFGF*, *FGFR1*, *FGFR2*, *Activin-A*, *Nodal*, which were activated in epiblast stem cells, *C-kit lagand* and *C-kit receptor*, known as the growth factor for self-renewal of stem cells.

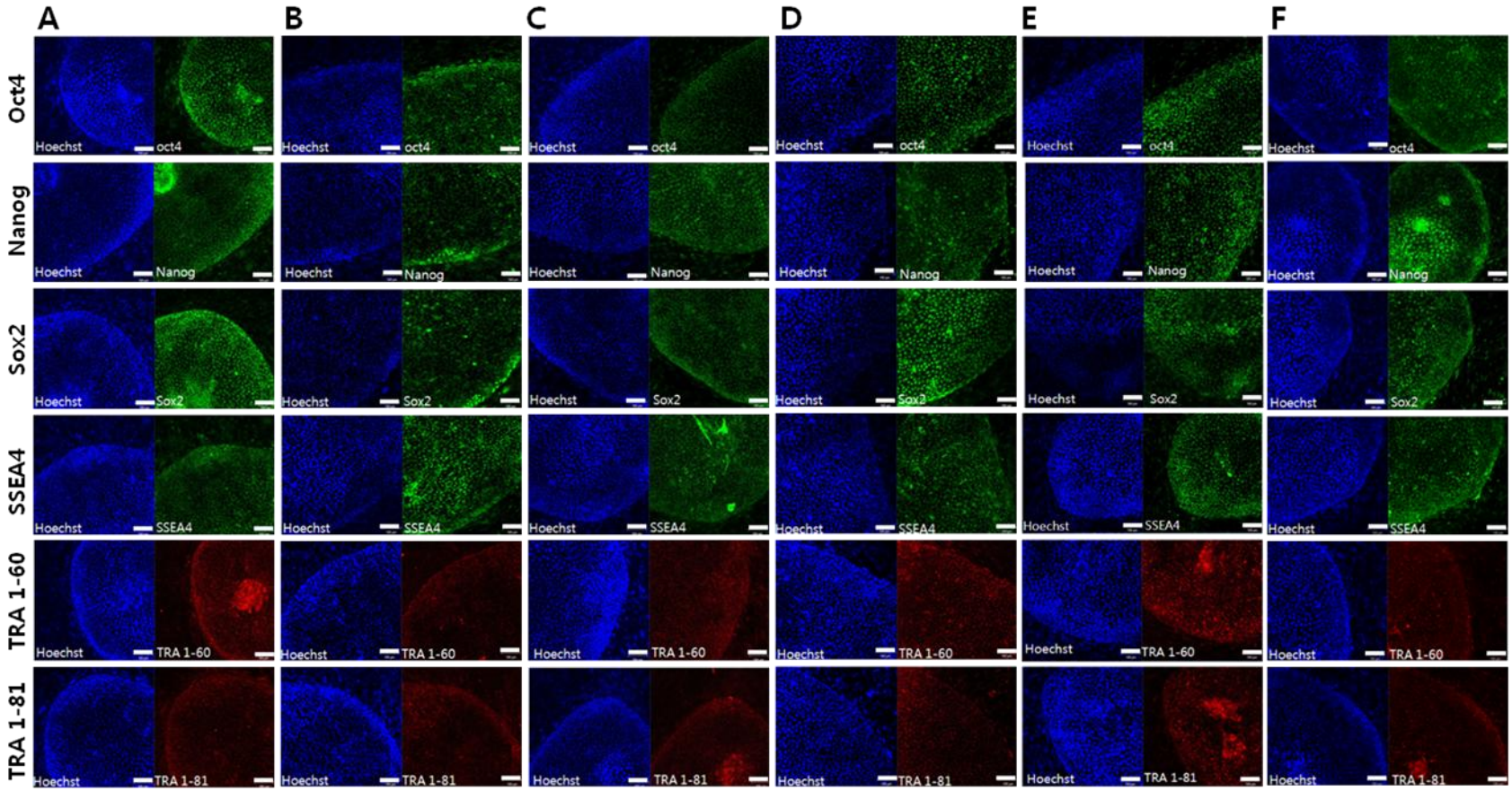
A
B
C
D
E
F
Oct4
Nanog
Sox2
SSEA4
TRA 1-60
TRA 1-81
Hoechst
oct4
Nanog
Sox2
SSEA4
TRA 1-60
TRA 1-81

Figure 9. The expression of pluripotent markers in porcine pluripotent cells by immunocytochemistry analysis.

All six cell lines expressed pluripotent markers and surface markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81; **(A)** pESI6, **(B)** pESP2, **(C)** pES3X3, **(D)** pES11, **(E)** pES12 and **(F)** piPS-1. Scale bars = 100µm.

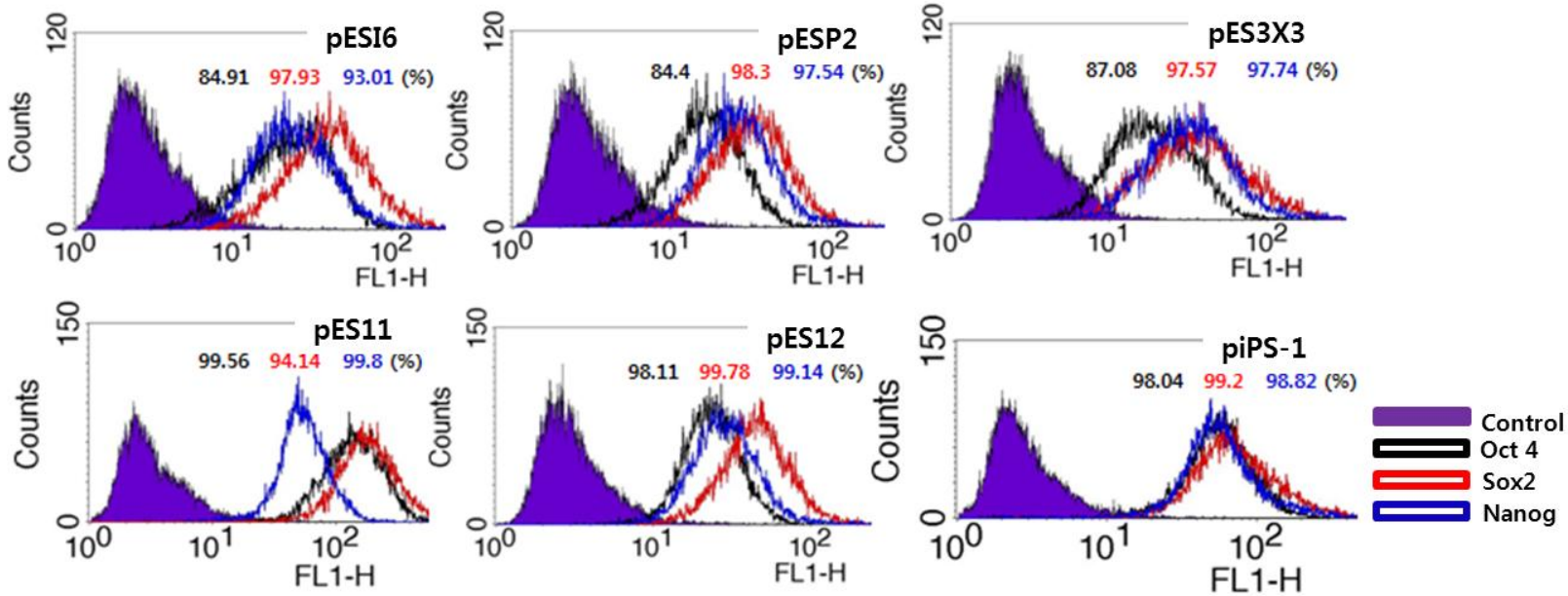
pESI6
84.91 97.93 93.01 (%)
pESP2
84.4 98.3 97.54 (%)
pES3X3
87.08 97.57 97.74 (%)
pES11
99.56 94.14 99.8 (%)
pES12
98.11 99.78 99.14 (%)
piPS-1
98.04 99.2 98.82 (%)
Counts
FL1-H
Control
Oct 4
Sox2
Nanog

Figure 10. Flow cytometric analysis of pluripotent markers on porcine pluripotent cell lines.

The pluripotent markers Oct4, Sox2 and Nanog were respectively expressed into 84.91%, 97.93% and 93.01% in the cell line pESI6, 84.4%, 98.3% and 97.54% in the cell line pESP2, 87.08%, 97.57% and 97.74% in the cell line pES3X3, 99.56%, 94.14% and 99.8% in the cell line pEpiS11, 98.11%, 99.78% and 99.14% in the cell line pES12 and 98.04%, 99.2% and 98.82% in the cell line piPS-1.

Epigenetic characteristics of EpiSC-like pESCs and piPSCs, and global gene expression profile of EpiSC-like pESCs

X chromosome inactivation have been addressed as a hallmark to determine whether pluripotent cell is naïve or primed state.(Nichols and Smith, 2009) Here, I could identify X chromosome inactivation status in our porcine pluripotent stem cell lines. X chromosome inactivation as a feature of primed state is related to the activation of *XIST* gene, the demethylation of *XIST* promoter and histone modification (H3K27-me3) (Lengner et al., 2010).

First, I investigated the expression of *XIST* gene from all our cell lines using RT-PCR. As shown in Figure 11A, I could identify the expression of *XIST* gene in cell line PESI6, PESP2, PES11 and PES12 which are all female. Meanwhile, no expression was identified in male cell lines, PES3X3 and piPS-1. I next assessed relative expression levels of *XIST* gene in individual cell lines by real-time PCR. The results clearly demonstrated that the *XIST* gene in female cell lines was highly expressed when compared to male cell lines (Figure 11B). I also confirmed DNA methylation status of XIST promoter regions in female cell lines pESI6, pES11 and pES12. The result shown that XIST promoter was 33.3~52.8% methylated in female cell lines (Figure 11C). Finally, I observed the methylation of H3K27, which is a hallmark of X chromosome inactivation, using an immunocytochemistry analysis in all cell lines. As shown in Figure 12, nuclear H3K27me3 foci

were detected in all female cell lines PESI6, PESP2, PES11 and PES12 (Figure 12 i, ii, iii and iv). However, nuclear H3K27me3 foci were hardly detected in male cell lines; PES3X3 and piPS-1 (Figure 12 v and vi).

To compare global gene expression of our pESCs with hESCs and mESCs, I conducted microarray analysis on pESCs derived from in vivo produced embryos using the GeneChip® Porcine Genome Array. In Figure 13, I could identify the 3 large groups in the heatmap. Hierarchical clustering result showed the global gene expression pattern of pESCs is more similar to that of hESCs than to mESCs.

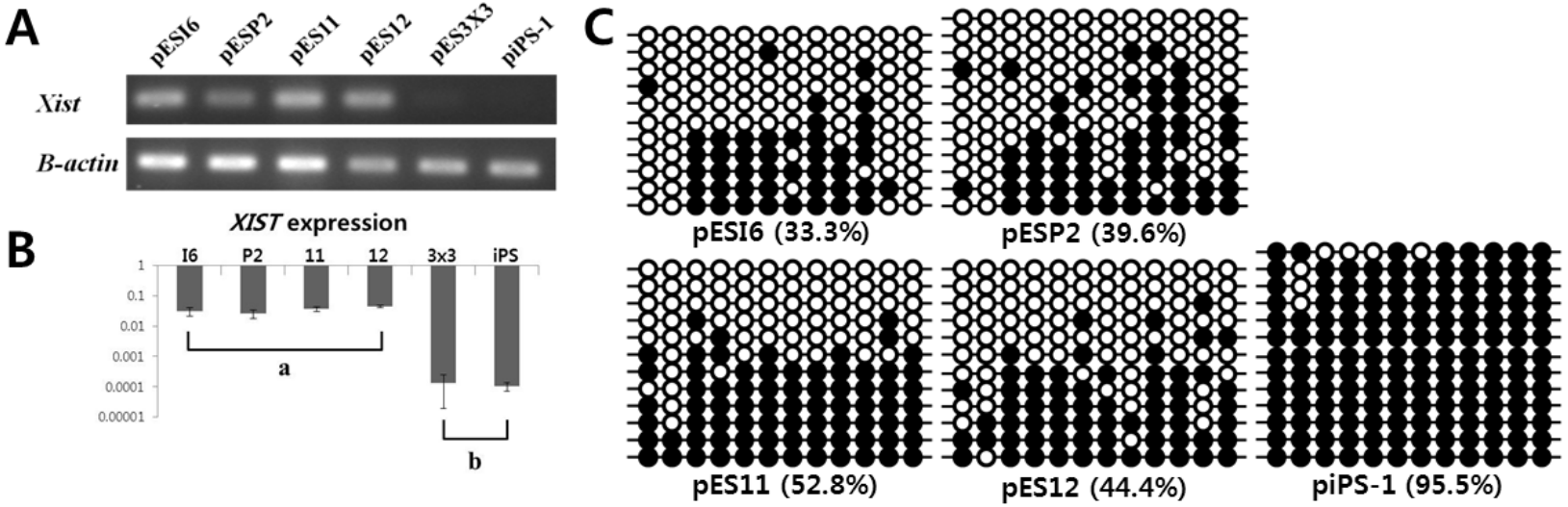
A
pESI6
pESP2
pES11
pES12
pES3X3
piPS-1
Xist
B-actin
B
XIST expression
I6
P2
11
12
3x3
iPS
1
0.1
0.01
0.001
0.0001
0.00001
a
b
C
pESI6 (33.3%)
pESP2 (39.6%)
pES11 (52.8%)
pES12 (44.4%)
piPS-1 (95.5%)

Figure 11. Epigenetic characteristics of porcine EpiSC-like embryonic stem cells and induced pluripotent stem cells

(A) XIST gene was expressed in cell line PESI6, PESP2, PES11 and PES12 which are all female. Meanwhile, no expression was identified in male cell lines, PES3X3 and piPS-1. **(B)** Relative expression levels of XIST gene in individual cell lines clearly demonstrated the XIST gene in female cell lines was highly expressed comparable to male cell lines **(C)** DNA methylation status of XIST promoter regions in female cell lines PESI6, PES11 and PES12. Circles indicated the CpG sites of resion analyzed. Open and closed circles mean unmethylation and methylation status.

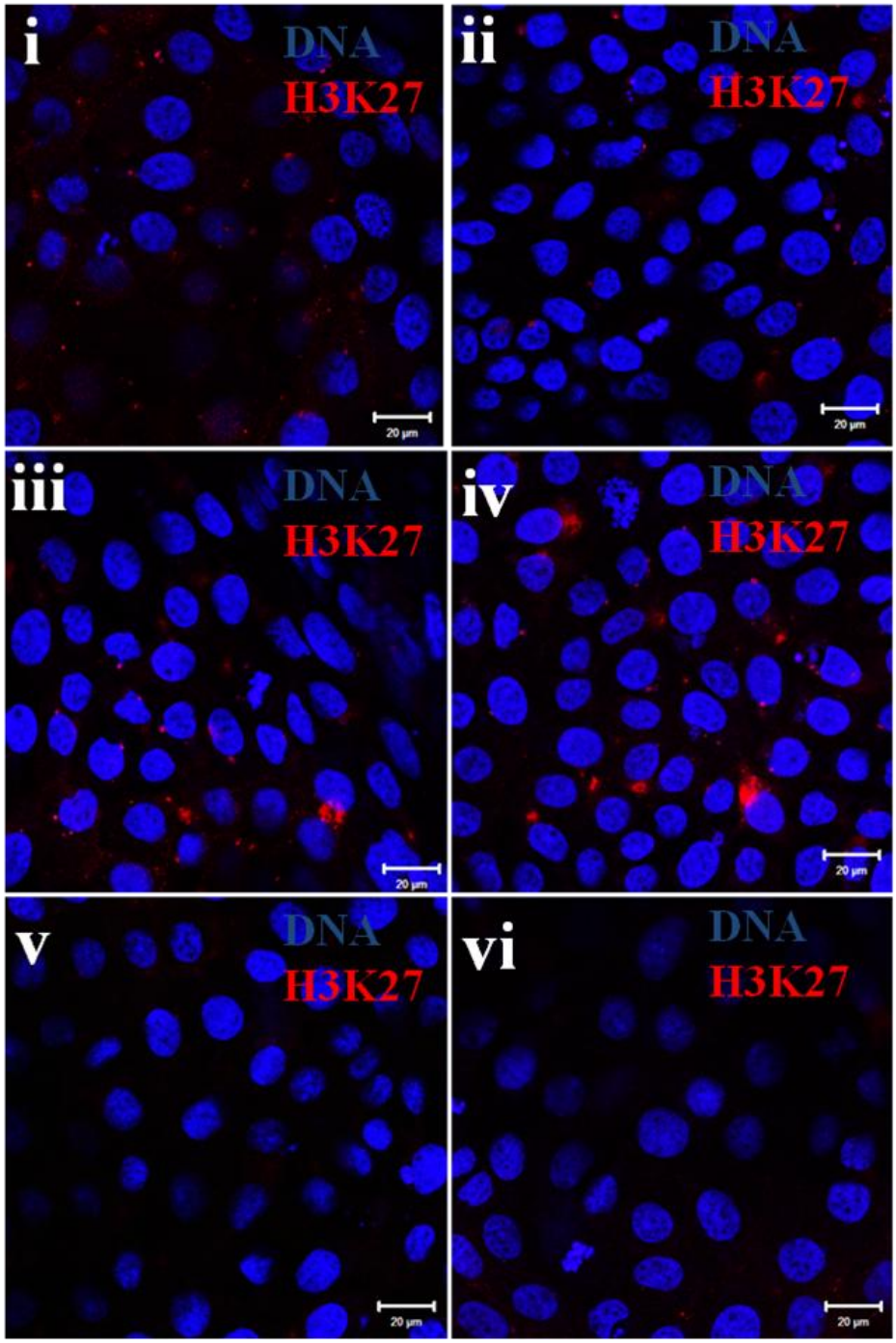
i
DNA
H3K27
20 µm
ii
DNA
H3K27
20 µm
iii
DNA
H3K27
20 µm
iv
DNA
H3K27
20 µm
v
DNA
H3K27
20 µm
vi
DNA
H3K27
20 µm

Figure 12. **Epigenetic characteristics of porcine EpiSC-like embryonic stem cells and induced pluripotent stem cells**.

Nuclear H3K27me3 foci were detected in female cell lines; PESI6, PESP2, PES11 and PES12 (i, ii, iii and iv). However, nuclear H3K27me3 foci were hardly detected in male cell lines; PES3X3 and piPS-1 (v and vi) Scale bars = 20μm.

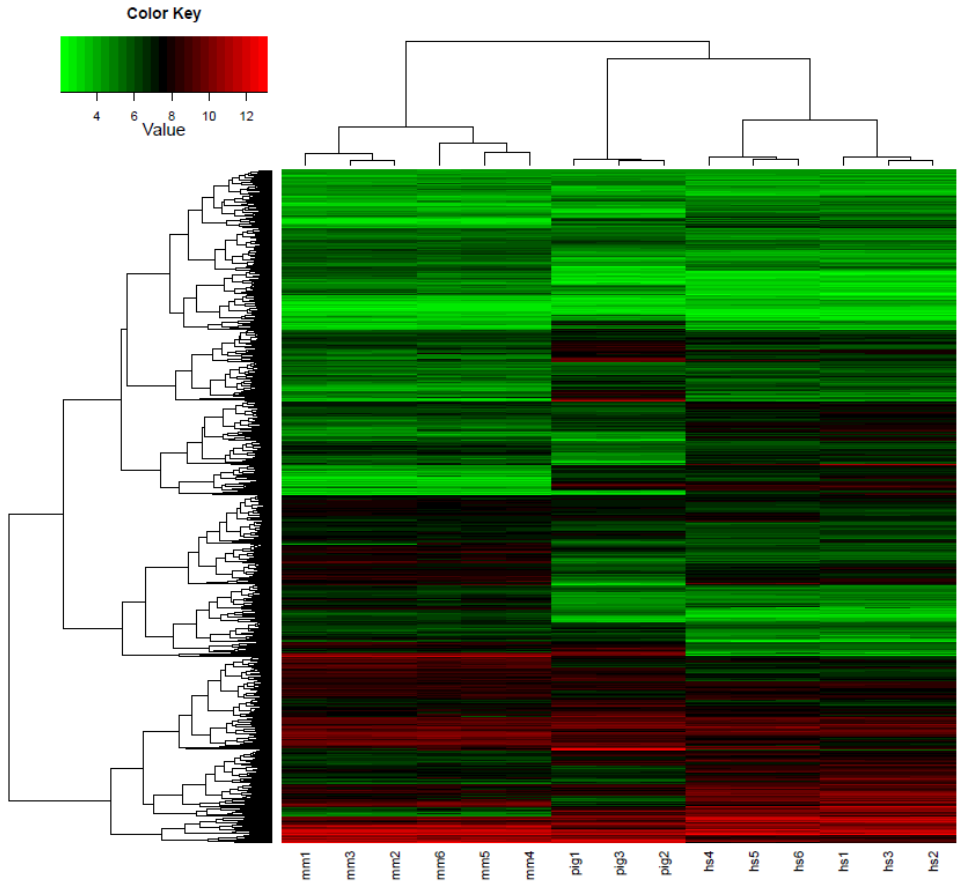

Figure 13. **Global gene expression profile of porcine EpiSC-like embryonic stem cells**.

Each row represents the expression for one gene and columns indicate mESCs, pESCs and hESCs. I could identify the 3 large groups in the heatmap (Figure S1). Hierarchical clustering result showed the expression pattern of pESCs and hESCs is more similar than between hESCs and mESCs. mm 1, 2 and 3 ; R1, mm 4, 5 and 6 ; J1, pig 1, 2 and 3 ; PES5, hs 1, 2 and 3 : HUES6, hs 4, 5 and 6 : H9.

Differentiation potential and karyotyping of EpiSC-like pESCs and piPSCs

I investigated the differentiation potentials of the six cell lines to formation of EB and all three endodermal, mesodermal and ectodermal germ layers. As shown in Figure 14A, all cell lines showed the potential to undergo EB formation following five days of culture using the hanging drop method.

When EBs were cultured onto plates coated with 0.1% gelatin, a variety of differentiated cells were observed after 2-3 weeks (data not shown). I confirmed the expressions of three genes known to be involved in differentiation: *AFP* (endoderm), *DESMIN* (mesoderm), and *CRABP*2 (ectoderm). As shown in Figure 14B, all of the differentiation markers were expressed in all cell lines. I also investigated the expressions of differentiation markers from differentiated cells using immunocytochemical analysis. As shown in Figures 14C and 14D, both EpiSC-like pESCs and piPSCs not only expressed the differentiation markers Cytokeratin 17 (endoderm), Desmin (mesoderm), and Neurofilament (ectoderm), but also demonstrated normal karyotyping.

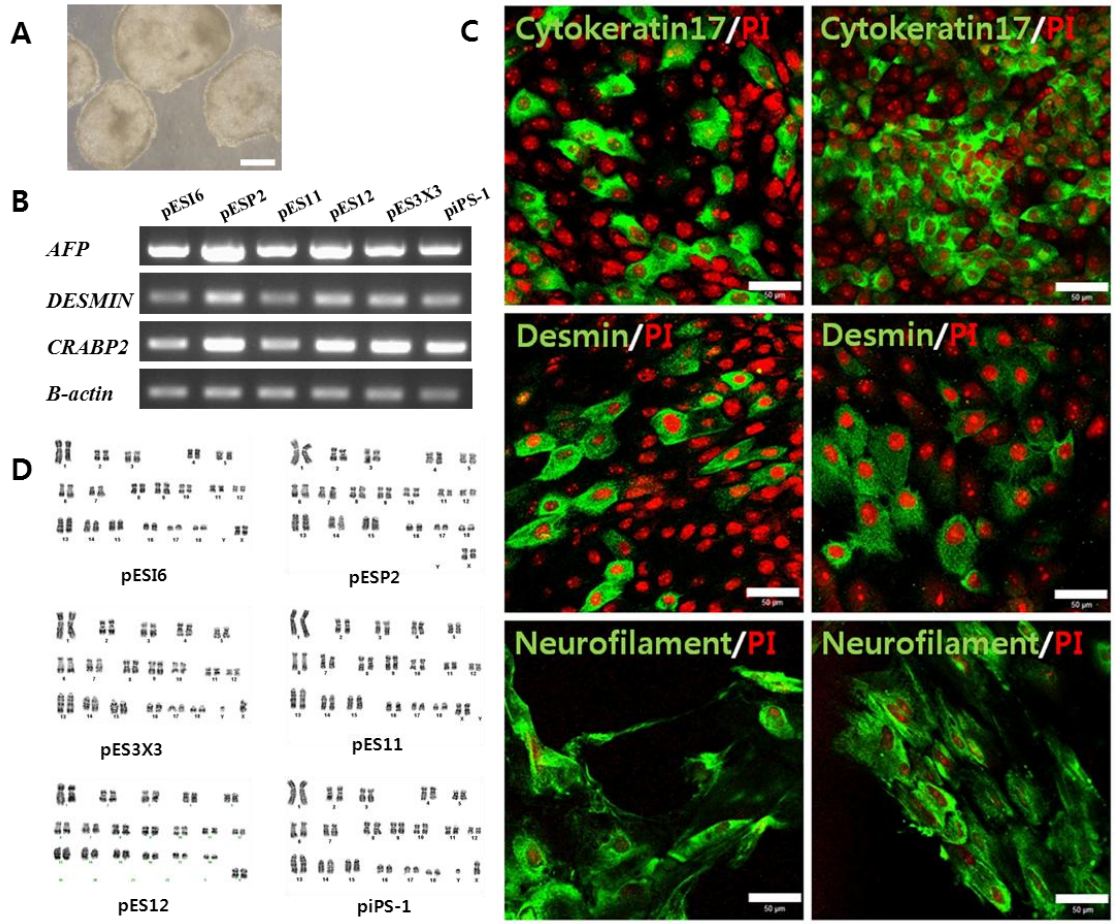
A
B
pESI6
pESP2
pES11
pES12
pES3X3
piPS-1
AFP
DESMIN
CRABP2
B-actin
C
Cytokeratin17/PI
Cytokeratin17/PI
Desmin/PI
Desmin/PI
Neurofilament/PI
Neurofilament/PI
D
pESI6
pESP2
pES3X3
pES11
pES12
piPS-1

Figure 14. Defferentiation potential and karyotyping of EpiSC-like pES cell lines and piPS cell line.

(A) Representative embryoid bodies derived from EpiSC-like pES cell lines and piPS cell lines through the culture for 5 days by hanging drop method. **(B)** When EBs cultured continue onto culture plates, I could observed a variety of differentiated cells. I could detect the expression of marker genes *AFP* (endoderm), *DESMIN* (mesoderm) and *CRABP*2 (ectoderm) involving differentiation. **(C)** I also confirmed the expression of differentiation marker Cytokeratin 17 (endoderm), Desmin(mesoderm) and Neurofilament (ectoderm) from differentiated cells by the immunocytochemistry analysis. Left lane is the cells differentiated from EpiSC-like pESCs and right lane is the cells differentiated from EpiSC-like piPSCs. **(D)** Both EpiSC-like pES cell lines and piPS cell line have a normal karyotyping. Scale bars = 50μm.

Discussion

Several studies have been conducted with the aim of establishing ESC lines from porcine embryos of various origins (Brevini et al., 2007b; du Puy et al., 2011; Kim et al., 2010; Tan et al., 2011; Vackova et al., 2011; Vackova et al., 2007; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). However, most researchers to date have found it difficult to maintain an ES-like state in derived cell lines, with the cells showing a strong tendency to differentiate into an epithelial or EpiSC-like state (Piedrahita et al., 1990b; Telugu et al., 2010). Accordingly, the purposes of present study are not only establishing pluripotent stem cells in pig, but also the comprehensive comparison of porcine pluripotent stem cells derived from various embryonic origin (*in vivo* embryos, *in vitro* produced embryos, parthenotes) and iPSCs in the aspect of pluripotent status. In addition, I expected to explain why authentic ESCs have not yet been categorized for many years in pigs. I have been able to derive cell lines of an EpiSC-like state from porcine embryos of various origins and could be stably maintained for long period, saying more than 1 year. All of our cell lines could be described as similar to epiblast stem cells with respect to their morphology and signaling pathways for the maintenance of pluripotency. Previous studies have shown Oct4, Rex1 and TDGF1 to be relatively good markers of porcine epiblast cells (Blomberg et al., 2008), while Sox2, Nanog, bFGF, FGFR1 and FGFR2 are exclusively expressed in porcine

epiblast cells (Hall et al., 2009). Consistent with previous reports, our results showed that all of our cell lines express the pluripotency genes Oct4, Sox2, Nanog, Rex1 and TDGF1 in addition to bFGF, FGFR1, FGFR2, Nodal and Activin-A, which are involved in signaling pathways activated in epiblast stem cells or hES cells. Moreover, our results showed X chromosome inactivation in our female cell lines is consistent with that of mEpiSC (Han et al., 2011a) (Figure 11 and 12). Therefore, I concluded that our cell lines derived from porcine embryos of various origins belong to the category of EpiSC-like ESCs rather than authentic ESCs.

Recently, several studies have reported the establishment of iPSC lines from porcine somatic cells using reprogramming factors (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; West et al., 2010; West et al., 2011; Wu et al., 2009). All of these cell lines displayed characteristics attributed to primed pluripotent state cells, with flattened morphologies and FGF and Nodal/Activin signaling pathways similar to mEpiSCs and hESCs. I have also generated piPSCs from PEFs via plasmid transfection using four reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) and have derived cell lines of a primed state. In addition, our results suggest that cell line piPS-1 is very similar to all of our EpiSC-like pESC lines with respect to morphology, AP activity, the activation of signaling pathways and the expressions of pluripotency markers. Therefore, it can be concluded that piPSC is analogous to EpiSC-like pESCs, and both piPSC and EpiSC-like pESCs possess a primed pluripotent state rather

than a naïve state. Previous reports demonstrated that patterns of gene expression of hESCs share those of mEpiSCs and clustered closely with those of mEpiSCs than to those of mESCs (Hanna et al., 2010a; Tesar et al., 2007). Our cluster analysis of global gene expression showed that the global gene expression pattern of EpiSC-like pESCs is similar to hESCs, although further analysis is required to confirm this (Figure 13).

To investigate *in vivo* differentiation potential, all of our EpiSC-like ESC lines were injected subcutaneously into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Despite numerous attempts using increased cell numbers ($1x10^7$ from $1x10^6$ EpiSC-like pESCs), I was unable to observe teratoma formation with any of our EpiSC-like pESC lines. I do not know why teratoma formation has so far remained impossible with our EpiSC-like pESC lines; however, to date, there are no reports of definite teratoma formation using pluripotent cell lines derived from porcine embryos. Anderson et al. have reported that it is difficult to generate embryonic carcinoma (EC) cells using early stage porcine embryos, unlike in mice and humans (Anderson et al., 1996). I presume that the failure of our EpiSC-like pESC lines to result in teratoma formation reflects the myriad of difficulties faced in EC derivation from porcine species.

Recent studies have reported that pluripotent stem cells exist in one of two forms and can be categorized according to their pluripotent state (Hanna et al., 2010b; Nichols and Smith, 2009). The first is a “naïve” state,

which represents full pluripotency or a ground state. The mESCs or mouse embryonic germ cells (mEGCs) could be categorized as such. The second "primed" state has been described as a state of limited pluripotency, within which mEpiSCs or hESCs can be placed. Nichols and Smith (2009) (Nichols and Smith, 2009) have suggested that the establishment of the naïve state, which is full pluripotency, is species-dependent, and that permissive species are able to maintain a naïve state during stabilization into stem cell lines from naïve epiblast during preimplantation embryo development. In contrast to naïve state species, non-permissive species do not easily maintain a naïve state during the establishment of pluripotent cell lines and instead are stabilized into a primed state from naïve epiblasts.

The differences in the establishments of these pluripotent states result in many differences between the two states. For example, naïve state cell lines form small, round or dome-shaped colonies, with LIF and BMP4 signaling pathways playing an important role in the maintenance of pluripotency. Furthermore, both X chromosomes remain activated (X^aX^a) in female cells and the *Oct4* distal enhancer is still active in naïve state cell lines, as can be seen in early embryonic cells. In contrast, primed pluripotent cell lines display flattened monolayer colony morphologies and show activations of FGF and Nodal/Activin signaling pathways for maintenance of pluripotency, X chromosome-inactivation (X^aX^i) and activation of the proximal element of the *Oct4* enhancer, which represent distinct similarities with primed epiblast cells of post-implantation embryo

stage development (Hanna et al., 2010b; Nichols and Smith, 2009). Interestingly, when pluripotent stem cell lines are established from non-obese diabetic (NOD) mice or rats, which are non-permissive species (Buehr et al., 2003), primed pluripotent cell lines can be derived from blastocyst stage embryos and induced from somatic cells (Hanna et al., 2009).

Recent studies have reported that primed pluripotent stem cell lines could be reverted to a naïve pluripotent state using various exogenous factors including GSK3β and MEK inhibitors, LIF, hypoxic conditions and up-regulation of klf4 (Buecker et al., 2010; Guo et al., 2009; Hanna et al., 2010a; Hanna et al., 2009; Lengner et al., 2010). Recently, Roberts and colleagues reported LIF-dependent pluripotent stem cells derived from porcine embryos by up-regulation of Oct4 and Klf4 (Telugu et al., 2011). I are also trying to create a LIF-dependent naïve pluripotent porcine stem cell line using various exogenous factors and have so far been able to successfully induce PEFs into a naïve pluripotent cell line showing a mESC-like morphology. In the future, I intend to investigate the potential of germ cell specification through the use of chimeras and the comparison of epigenetic states, such as X chromosome and Oct4 enhancer activation, between naïve pluripotent cell lines and primed pluripotent cell lines in pig.

In conclusion, I have been able to derive EpiSC-like pESC lines from porcine embryos of various origins including IVF, *in vivo* derived, IVF

aggregated (3X) and parthenogenetic embryos, in addition to a piPSC line derived from PEFs, via plasmid transfection with reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*). All of our cell lines showed AP activity and expressions of the genes *Oct4*, *Sox2*, *Nanog*, *Rex1*, *TDGF1*, *bFGF*, *FGFR1*, *FGFR2*, *Nodal* and *Activin-A* involved in pluripotency and signaling pathways, X chromosome inactivation in female cell lines, in vitro differentiation potential and a normal karyotype, thus displaying similarities to mEpiSCs or hESCs, which was supported by the comparison of the global gene expression pattern. Therefore, our data suggest that, as a non-permissive species, the porcine embryos and somatic cells undergoes reprogramming into a primed state during the establishment of pluripotent stem cell lines based on genomic background. Our results will help not only to understand the reprogramming into a primed state during the establishment of pluripotent stem cell lines in pigs, but also explain current status of stem cell research in domestic animals.

CHAPTER 5. Dox-inducible induced pluripotent stem cell lines derived from porcine embryonic fibroblasts

Introduction

With the first derivation of ES cells in mouse and human, a number of efforts have been made to establish the ES cell lines from other species, including pig (Evans and Kaufman, 1981; Thomson et al., 1998). However, it was able to show a full pluripotency in only rodent species. In rat, many researchers have attempted to establish authentic ES cell lines by the conventional methods used in mice, but it isn't easy to maintain the pluripotent state which is shown in mice (Brenin et al., 1997; Ueda et al., 2008). Although rat is physiologically very similar to mouse, their embryos showed somewhat distinct differentiation capacity when formed to embryonic carcinoma stem cells (Damjanov and Sell, 1977; Li et al., 2008). And the activation of LIF/Stat3 pathways, which are key regulators for maintenance of pluripotency in mES cells, were not able to maintain the undifferentiated state in establishing the ES cells from rat embryos (Lai et al., 2002; Li et al., 2008; Smith et al., 1988; Ying et al., 2003). In 2008, Buehr et al. and Li et al. confirmed that the addition of three inhibitors (3i), which target FGF receptor, MEK and GSK3, has an excellent effect in inhibition of differentiation and maintenance of undifferentiated state during the establishment of ES cells from rat embryos(Buehr et al., 2008; Li et al., 2008). Besides, Guo et al. showed that EpiSCs, which are pluripotent stem cells differentiated from ground state ES cells, could be reverted to ground state stem cells using the up-regulation of Klf4 (Guo et al., 2009).

Based on these backgrounds, Nichols and Smith (2009) introduced two types of pluripotent state in pluripotent stem cells (Nichols and Smith, 2009). One is “naïve” state which comprises a ground or full pluripotent state. Naïve pluripotent stem cells, represented by mES cells and mEG cells, display small and dome-shaped colony morphologies, activation of LIF and BMP4 signaling pathways and two active X chromosomes in female. These state stem cells can contribute to germ-line transmission. The other is “primed” state. Primed pluripotent stem cells, such as mEpiSCs, show the restricted pluripotency, such as a low chimerism, when compared to naïve pluripotent stem cells. These stem cells are characterized by flattened monolayer colony morphologies and the activation of FGF and Nodal/Activin signaling pathways for maintenance of stemness.

In previous our results, I have reported that porcine species have a non-permissive genomic background and are reprogrammed by their genomic background into a primed state during the establishment of pluripotent stem cell lines (Park et al., 2013). In this study, therefore, I investigated whether a murine (m) ESC-like induced pluripotent stem cell line could be derived from porcine embryonic fibroblasts (PEFs) using various exogenous factors. I have been able to successfully induce PEFs into mESC-like induced pluripotent stem cells. These cell lines showed mESC-like morphologies, the expressions of pluripotency markers, such as Oct4, Sox2, Nanog and SSEA1 and sequential reprogramming by addition

of doxycycline (DOX). Here I suggest that the porcine species could be induced into mESC-like induced pluripotent stem cells by the introduction of various exogenous factors including continuous transgene expression, GSK3β and MEK inhibitors (2i) and LIF.

Materials and Methods

Generation and culture of mESC-like porcine induced pluripotent stem cells

The derivation of mESC-like porcine induced pluripotent stem cells (piPSCs) was conducted using previously described methods. Briefly, a FUW-tetO-hOCT4, FUW-tetO-hSOX2, FUW-tetO-hKlf4, FUW-tetO-hMYC and FUW-M2rtTA were obtained from Addgene. HEK 293 LTV cells (Cell Biolabs, USA) were used as packaging cell line and cultured according to manufacturer's instructions. Four plasmids were used for the production of lentiviral vectors (vector plasmid; FUW-tetO-hOCT4, FUW-tetO-hSOX2, FUW-tetO-hKlf4, FUW-tetO-hMYC and FUW-M2rtTA packaging plasmids: pLP1 and pLP2, envelop plasmid: pLP/VSVG; Invitrogen, USA). These plasmids were transfected into HEK 293 LTV cells using a calcium phosphate coprecipitation method. Before 2 hours from the transfection, the cells were incubated with 25uM chloroquine (Sigma-Aldrich) and after 12 hours of transfection, transfected cells were treated with 15 % glycerol solution for 90 sec and cultured with culture media for 24 h more. And then cultured supernatants were harvested every 12h four times and stored at 4℃. Harvested supernatants were filtrated with 0.45 um pore filters (Nalgene, USA) and concentrated by centrifuge at 18,000g of 4℃ for 5h. The virus pellet was dissolved with PESM and stored at -76℃ until before

using. After 48 h post infection, viral infected PEFs of concentration of $5x10^4$ cells were cultured in pESC medium including DOX, 2i and LIF on mitotically inactivated MEFs in $35mm^2$ plate dish for 2~3 weeks. The pESC medium contains a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM low glucose, Gibco Invitrogen, USA, www.invitrogen.com) and Ham's F10 medium (Gibco), supplemented with 15% fetal bovine serum (FBS; collected and processed in Canada; Hyclone, Logan, UT, www.hyclone.com), 2 mM glutamax (Gibco), 0.1 mM ß-mercaptoethanol (Gibco), 1x MEM nonessential amino acids (Gibco), 1x antibiotic/antimycotic (Gibco). Infected PEFs were transferred to fresh medium on a daily basis until colonies large enough to passage were observable.

Alkaline phosphatase (AP) activity and immunocytochemistry (ICC) analysis

Methods for AP staining and ICC analysis of mESC-like piPSCs were performed according to methods described in Chapter 3. For ICC analysis of undifferentiated cells, the primary antibodies used were Oct4 (SC-9081, Santa Cruz Biotechnology, www.scbt.com 1:100), Nanog (SC-33759, Santa Cruz Biotechnology, 1:100), Sox2 (AB5603, Millipore, Temecula, CA, www. millipore.com, 1:200), SSEA-1 (MAB4301, Millipore, 1:200), SSEA-4 (MAB4304, Millipore, 1:200).

Embryoid body (EB) formation and differentiation into fibroblast-like cells

To evaluate EB formation potential, mESC-like piPSCs were removed from MEFs, dissociated with trypLE (12605, GIBCO) and cultured in petridish under a culture condition without DOX, 2i or LIF. After five days, mESC-like piPSCs formed typical EBs, which were transferred to confocal dishes coated with 0.1% gelatin and allowed to further differentiate during 2-3 weeks of culture.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Methods for RT-PCR analysis were performed according to methods described in Chapter 3. The conditions and primers used are listed in Table 7.

Flow cytometric analysis

Methods for flow cytometry analysis were performed according to methods described in Chapter 4.

Karyotyping

Standard G-banding chromosome and cytogenetic analysis was performed at GenDix Laboratories (Korea).

Results

Derivation of mESC-like porcine induced pluripotent stem cells

I used the previously reported 2i and DOX-inducible lentiviral vectors containing human cDNAs encoding four factors; Oct4, Sox2, Klf4 and c-Myc to derive mESC-like piPS cells from PEFs (Buecker et al., 2010; Buehr et al., 2008; Hanna et al., 2010a; Li et al., 2008). To confirm whether a mESC-like iPS cell line could be derived from PEFs using a DOX-inducible reprogramming method and 2i, lentivirus-mediated transgenes containing FUW-tetO-hOCT4, FUW-tetO-hSOX2, FUW-tetO-hKlf4, FUW-tetO-hMYC and FUW-M2rtTA were introduced into PEFs. Approximately 2 weeks post-infection, mESC-like primary colonies could be observed (Figure 15 E). As shown in Figure 15 C and D, ESC-like primary colony showed the positive AP activity and typical mESC-like dome-shape morphologies. I derived nine mESC-like iPS cell lines with ~0.02% efficiency. The mESC-like iPS cells had high single-cell clonogenic proliferation and were routinely sub-cultured every 3 days by TrypLE. All cell lines were cryopreserved in liquid nitrogen and could be maintained in stable morphologies after thawing. The cell lines could be stably maintained for more than 50 passages.

Table 7. Primers used and conditions for RT-PCR

Genes	Primer sequences	Annealing temperature (°C)	Accession number
OCT4	5'-AACGATCAAGCAGTGACTATTCG-3' 5'-GAGTACAGGGTGGTGAAGTGAGG-3'	60	AF074419
NANOG	5'-AATCTTCACCAATGCCTGAG-3' 5'-GGCTGTCCTGAATAAGCAGA-3'	60	DQ447201
SOX2	5'-CGGCGGCAGGATCGGC-3' 5'-GAGCTCCGCGAGGAAAA-3'	60	EU519824
RT-FUW-hOCT4	5'-CCCCTGTCTCTGTCACCACT-3' 5'-CCACATAGCGTAAAAGGAGCA-3'	58	PMID: 18786421
RT-FUW-hKLF4	5'-GACCACCTCGCCTTACACAT-3' 5'-CCACATAGCGTAAAAGGAGCA-3'	58	PMID: 18786421
RT-FUW-hC-MYC	5'-CAGCTACGGAACTCTTGTGC-3' 5'-CCACATAGCGTAAAAGGAGCA-3'	58	PMID: 18786421
RT-FUW-hSOX2	5'-ACACTGCCCCTCTCACACAT-3' 5'-CATAGCGTAAAAGGAGCAACA-3'	58	PMID: 18786421
ß-ACTIN	5'-GTGGACATCAGGAAGGACCTCTA-3' 5'-ATGATCTTGATCTTCATGGTGCT-3'	60	U07786

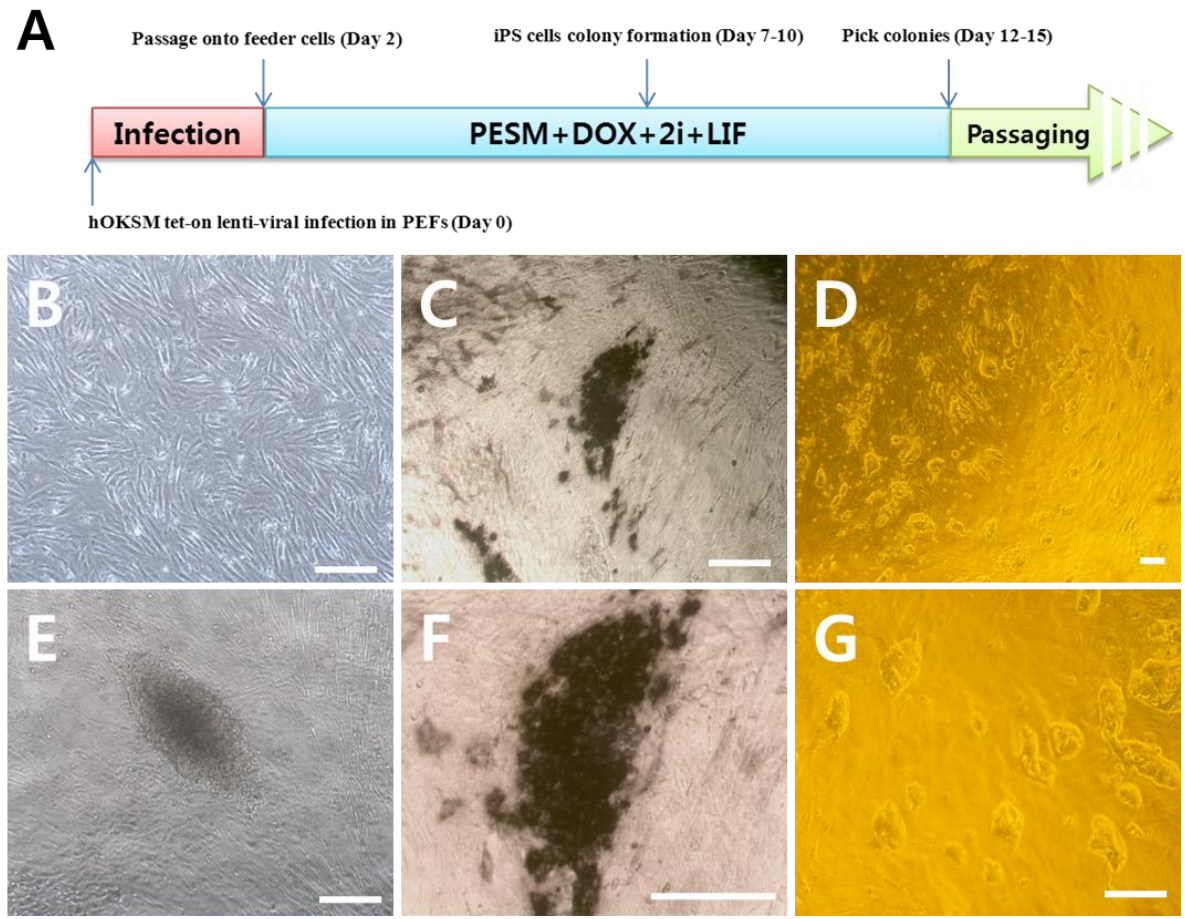
A
Passage onto feeder cells (Day 2)
iPS cells colony formation (Day 7-10)
Pick colonies (Day 12-15)
Infection
PESM+DOX+2i+LIF
Passaging
hOKSM tet-on lenti-viral infection in PEFs (Day 0)
B
C
D
E
F
G

Figure 15. Derivation of mESC-like piPSCs.

(A) Scheme of reprogramming mESC-like piPS cells from PEFs. (B) Morphology of PEFs used for infection of transgenes. (C) AP activity of mESC-like piPSCs. **(D)** Typical morphology of mESC-like piPSCs. **(E)** Morphology of mESC-like primary piPSCs derived from porcine embryonic fibroblsts. **(F)** AP activity of mESC-like piPSCs-high magnification. **(G)** Typical morphology of mESC-like piPSCs-high magnification. **Scale bars = 100μm.**

Verification of undifferentiation markers and chromosome assay in mESC-like porcine induced pluripotent stem cells

To identify the integration of transgenes and stable expression by the addition of DOX, we investigated the integration of genomic (g) DNA and mRNA expression of transgenes in mESC-like iPS cell lines by PCR. As shown in Figure 2A, we confirmed the integration of transgenes, such as Oct4, Sox2 and Myc, but the Klf4 gene was not detected in the iPSn1, iPSn2 or iPSn3 cell lines. However, after addition of DOX, the expression of all transgenes, including the Klf4 gene, could be confirmed (Figure 16B). By identifying endogenous Klf4 gene by PCR analysis of cDNA, the integration of exogenous Klf4 gene by PCR analysis of gDNAs could not be detected due to the low number of copies of the Klf4 gene integrated in the iPS cell lines. As shown in Figure 16C, the expression of endogenous Oct4, Sox2 and Nanog, transcription factors that play important roles in maintaining the pluripotency in mES cells (Chambers et al., 2003; Masui et al., 2007; Niwa et al., 2000b) was also confirmed. I investigated the expression of pluripotent markers Oct4, Sox2, Nanog, SSEA1 and SSEA4 using ICC analysis in piPSn1 and piPSn3 cell lines. As shown in Figure 17, both cell lines expressed Oct4, Sox2, Nanog and SSEA4. The flow cytometric analysis showed the iPS cell lines highly expressed the pluripotent markers (Figure 18). Additionally, the normal karyotyping in the cell lines using a chromosome assay was confirmed (Figure 19). To investigate the effect of

culture condition, mESC-like iPS cells were cultured in three kinds of media containing DOX+2i+LIF, DOX and 2i+LIF. As shown in Figure 20, it could be maintained in only culture condition containing DOX+2i+LIF or DOX. A medium supplemented with DOX+2i+LIF showed the best effect among three culture conditions, while mESC-like iPS cells in condition with 2i and LIF failed to be maintained stable morphology.

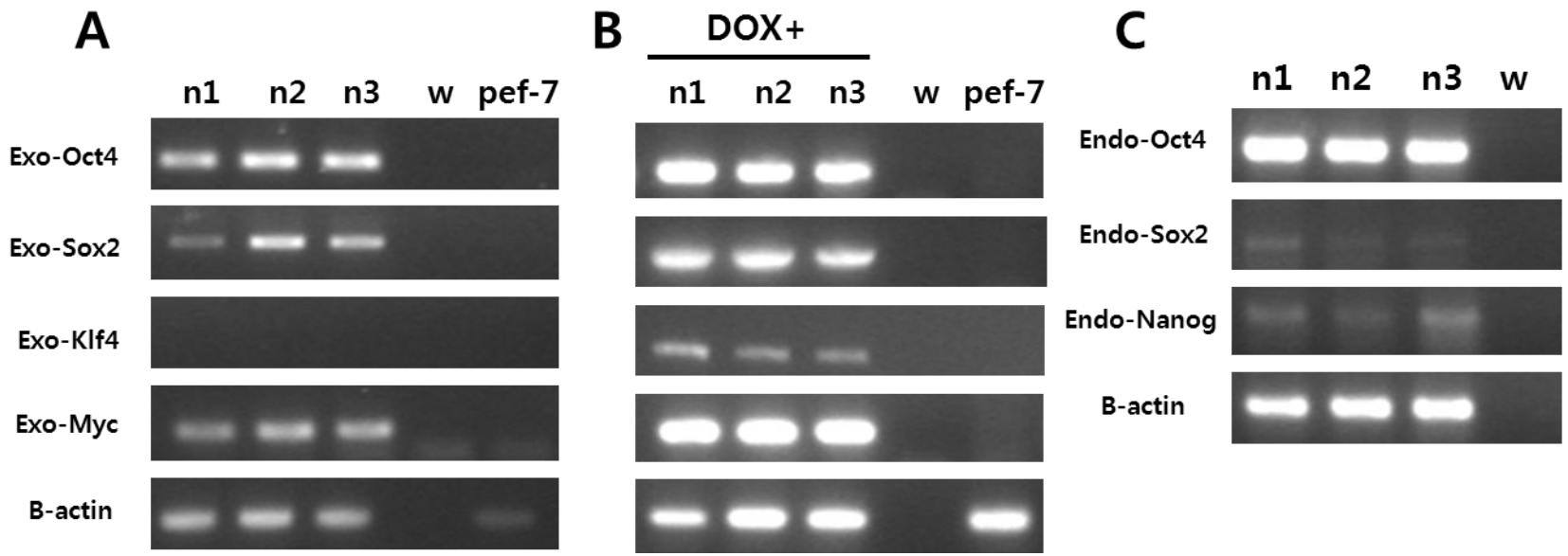

Figure 16. The integration of transgenes and stable expression in mESC-like porcine induced pluripotent stem cells.

(A) Integration analysis of genomic DNA in mESC-like iPS cell lines by PCR. **(B)** mRNA expression of transgenes in mESC-like iPS cell lines. **(C)** The expression of endogenous Oct4, Sox2, and Nanog in mESC-like iPS cell lines.

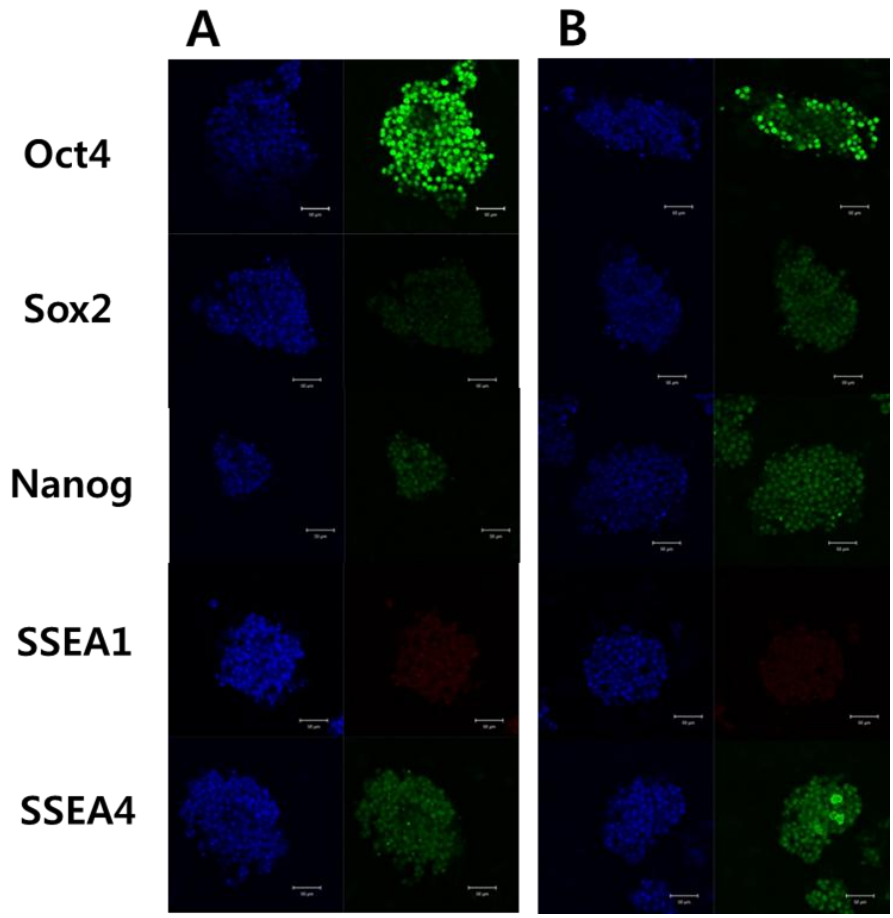

Figure 17. The expression of pluripotent markers in mESC-like piPSC cell lines based on ICC analysis.

mESC-like iPS cell lines expressed of pluripotent markers and surface markers Oct4, Sox2, Nanog and SSEA4; **(A)** piPS-n1 and **(B)** piPS-n3. Scale bars = 50µm.

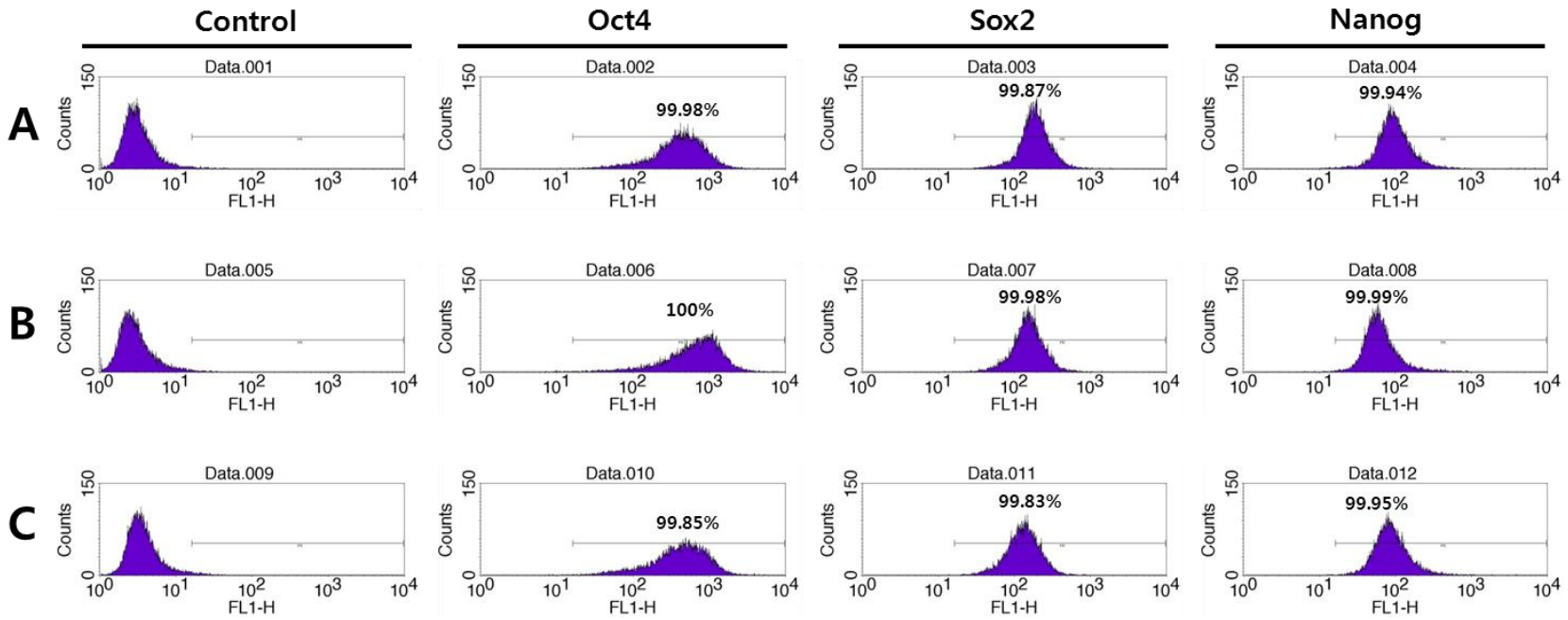

Figure 18. Flow cytometric analysis of pluripotent markers in mESC-like piPSC cell lines.

The pluripotent markers Oct4, Sox2 and Nanog were respectively expressed into 99.98%, 99.87% and 99.94% in the cell line **(A)** piPS-n1, 100%, 99.98% and 99.99% in the cell line **(B)** piPS-n2, and 99.85%, 99.83% and 99.95% in the cell line **(C)** piPS-n3.

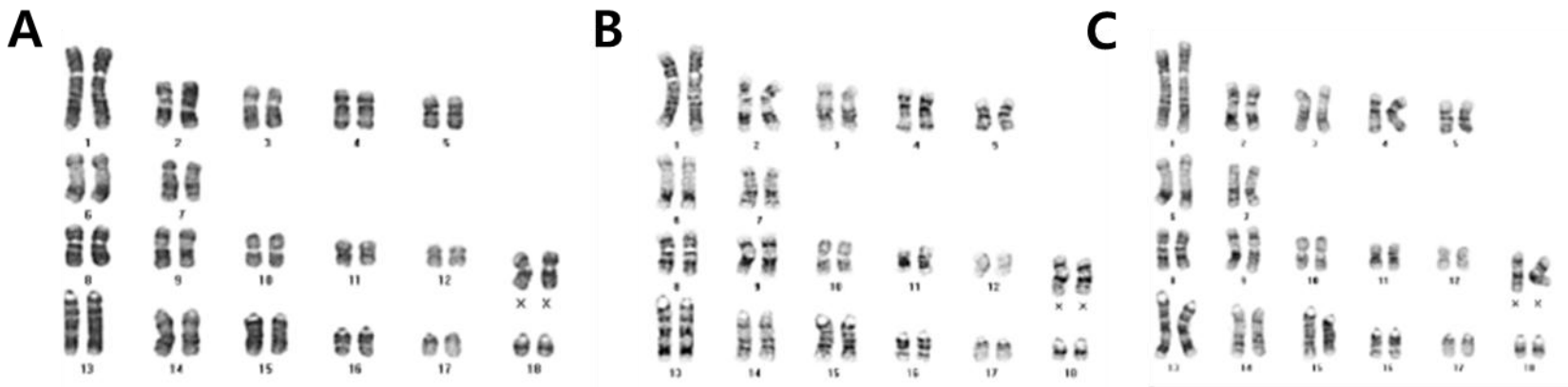

Figure 19. Chromosome assay in mESC-like iPS cell lines.

Cell line **(A)** piPSN1 **(B)** piPSN1 **(C)** piPSN1 have a normal karyotyping

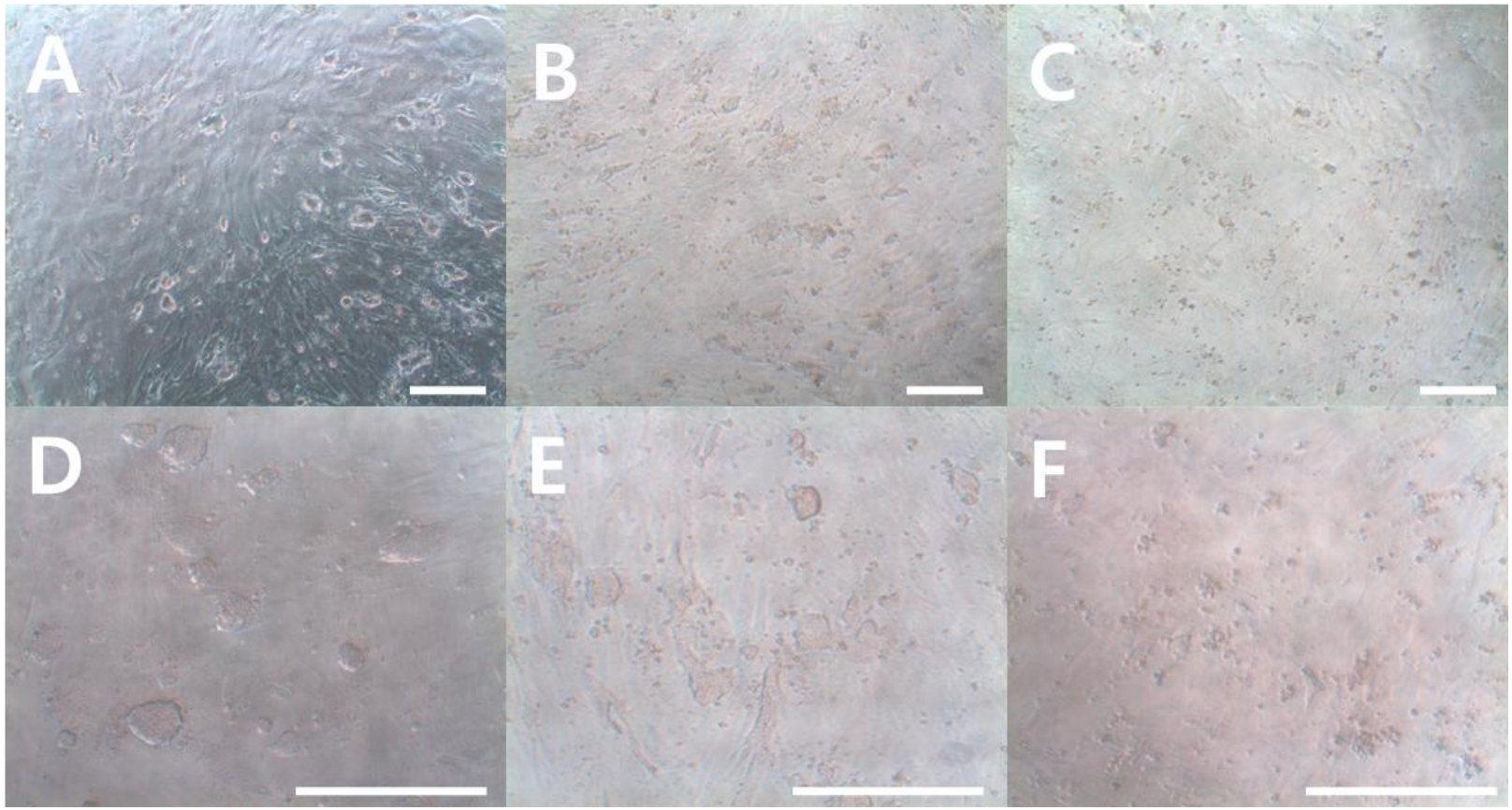
A
B
C
D
E
F

Figure 20. Effect of culture conditions in mESC-like iPS cell lines.

mESC-like iPS cells were cultured in three kinds of media containing **(A)** DOX+2i+LIF, **(B)** DOX and **(C)** 2i+LIF**. (D)**, **(E)** and **(F)** are the high magnification of (A), (B) and (C) in alphabetical order. Scale bars = 200μm.

Sequential reprogramming in mESC-like porcine induced pluripotent stem cells

To investigate DOX-inducible reprogramming from differentiated fibroblast-like cells, we induced the differentiation of mESC-like piPS cells into fibroblast-like cells under a culture condition without DOX, 2i or LIF. First, we induced differentiation of mESC-like piPS cells to generate EBs without DOX, 2i and LIF in petridish for 5 days. The generated EBs were subsequently cultured on plates coated with 0.1% gelatin. Thoroughly differentiated fibroblast-like cells were observed (Figure 21A). Secondary fibroblast-like cells were re-induced to mESC-like piPS cells on the feeder layer cells by culture in the presence of DOX, 2i and LIF. Secondary reprogramming from secondary fibroblast-like cells was confirmed (Figure 21A). Using the same method, we generated tertiary mESC-like piPS cells from tertiary fibroblast-like cells (Figure 21B). The results suggest that our cell lines induced from PEFs are DOX-dependent and could regenerate mESC-like piPS cells from differentiated fibroblast-like cells by addition of DOX, 2i and LIF.

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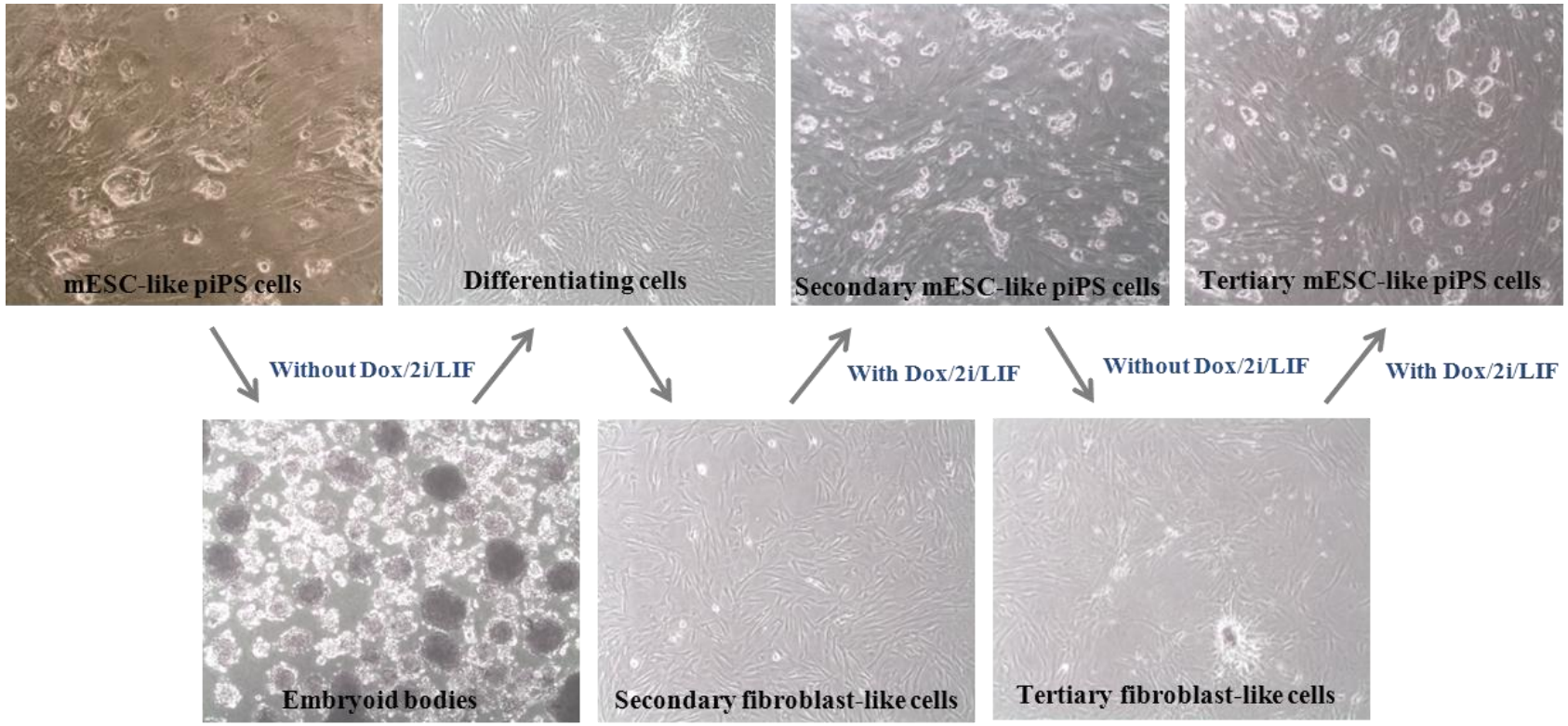
mESC-like piPS cells
Differentiating cells
Secondary mESC-like piPS cells
Tertiary mESC-like piPS cells
Without Dox/2i/LIF
With Dox/2i/LIF
Without Dox/2i/LIF
With Dox/2i/LIF
Embryoid bodies
Secondary fibroblast-like cells
Tertiary fibroblast-like cells

Figure 21. Sequential reprogramming in mESC-like piPSCs.

Differentiation of mESC-like piPS cells induced in Petri dishes to generate EBs for 5 days in the absence of DOX, 2i and LIF. The generated EBs were subsequently cultured on plates coated with 0.1% gelatin. Thoroughly differentiated fibroblast-like cells were observed. Secondary fibroblast-like cells were re-induced to secondary mESC-like piPSCs on the feeder-layer cells by culturing in the presence of DOX, 2i and LIF. Using the same methods, tertiary mESC-like piPSCs were derived from tertiary fibroblast-like cells.

Discussion

Results of Chapter 4 demonstrated that porcine cells were reprogrammed into primed pluripotent stem cells from fibroblasts when using conventional methods for deriving mouse pluripotent stem cells (Park *et al*., 2013). In addition to pig, pluripotent stem cells derived from embryos of non-permissive species including rat, NOD mouse strain and human did not demonstrate full pluripotency shown in naïve mES cells (Hanna *et al*., 2010b; Nichols and Smith, 2009, 2012; Smith, 2001). However, numerous efforts have been made to establish naïve-state pluripotent stem cells from non-permissive species. In rat, naïve pluripotent stem cells have been successfully established from rat embryos using 3i, which targets the FGF receptor and 2i (Buehr *et al*., 2008; Li *et al*., 2008). Culture with 2i, LIF and continuous expression of Klf4 and c-Myc enabled the establishment of naïve-state pluripotent stem cells from NOD strain murine embryos, which is a non-permissive murine species (Hanna *et al*., 2009). In humans, numerous attempts have been made to derive naïve pluripotent stem cells (De Los Angeles *et al*., 2012). The hESCs of two active X chromosomes in females, which is a naïve-state characteristic, were derived from human embryos in 5% oxygen (Lengner *et al.*, 2010). Li *et al*. showed conventional mESC-like hiPSCs by culture in the presence of of LIF, 2i and A-83-01 (Li *et al*., 2009). Hanna and colleagues reported naïve-state hiPSCs from secondary human fibroblasts and hESCs by inducing continuous

expression of transgenes with the LIF and 2i supplemented culture condition (Hanna *et al*., 2010a). In the pig, Telugu *et al*. reported LIF-dependent pluripotent stem cells derived from porcine ICMs by up-regulation of Oct4 and Klf4 (Telugu *et al*., 2011). Additionally, Fujishiro *et al*. reported putative naïve piPSCs with porcine LIF and forskolin. Their putative naïve piPSCs showed two active X chromosomes, differentiation potential into three germ layers and fetal chimerism using derived iPS cells (Fujishiro *et al*., 2013).

I have also attempted to establish naïve-state pluripotent stem cell lines from PEFs. I derived naïve-like pluripotent stem cells that expressed the transcription factors Oct4, Sox2 and Nanog, stable mESC-like morphology and sequential reprogramming using a DOX-inducible system, which we selected for continuous expression of transgenes as in previous reports (Buecker *et al*., 2010; Hanna *et al*., 2010a). However, my cell lines were dependent on transgenes and were induced to differentiate in the absence of DOX (Figure 20). Full reprogramming to reverse the differentiated cells into an ES-like state requires the activation of endogenous pluripotency genes by the expression of exogenous transgenes for stable maintenance of the pluripotent state (Maherali *et al*., 2007; Wernig *et al*., 2007). In particular, activation of endogenous Oct4 and Nanog genes by the expression of transgenes is indispensable for reprogramming of somatic cells into an ES-like state (Wernig *et al*., 2007). However, the regulation of endogenous pluripotency genes alone could not

maintain the pluripotent state without activation of transgenes in my cell lines. In other words, the transgenes activated by DOX did not induce sufficient activation of endogenous transcription factors in mESC-like pluripotent stem cell lines, although the expression of all transgenes, stable mESC-like morphology and sequential reprogramming by the addition of DOX (Figures 16B and 21) were observed. Consistent with my results, Petkov *et al*. reported that the outcome of reprogramming differs according to the type of promoter used to drive the expression of transgenes (Petkov *et al*., 2013). For instance, their results showed that the reprogramming of DOX-inducible tet-on promoters could not sufficiently activate the up-regulation of essential endogenous pluripotency genes in pig compared to other promoters, such as Ef1a and CAG. Therefore, I aim to derive naïve-state pluripotent stem cells from PEFs using a modified method; this will be addressed in a future study.

Recent reviews have suggested that stable naïve pluripotent stem cells, shown in mES cells, don't exist in non-murine species yet. Furthermore, authentic naïve pluripotent stem cells may be a permissive murine-specific phenomenon, although a number of naïve-like pluripotent stem cell lines have been established from non-rodent species, including human and pig (De Los Angeles *et al*., 2012; Nichols and Smith, 2012). In conclusion, I have derived mESC-like piPSCs from PEFs by infection of lentiviral transgenes into PEFs using DOX-inducible tet-on promoters. The cell lines

demonstrated mESC-like morphologies and Oct4, Sox2, Nanog and SSEA4 expression. In addition, these cell lines could be regenerated into mESC-like iPS cells from secondary or tertiary fibroblast-like cells differentiated from mESC-like iPS cells by addition of DOX. Herein I suggest that cells of a non-permissive species, the pig, can be induced into mESC-like iPS cells from PEFs by activation of various exogenous factors, such as continuous transgene expression by a DOX-inducible system, 2i and LIF. However, further work that aims to effectively induce the activation of endogenous transcription factors is necessary to derive authentic naïve-state pluripotent porcine stem cells.

CHAPTER 6. General discussion and Conclusion

Since mES cell lines were first established from mouse embryos in 1981 (Evans and Kaufman, 1981), many attempts have been made to establish ES cell line from other species including pigs (Piedrahita et al., 1990a), cattle (Saito et al., 1992b), rats (Buehr et al., 2008), primates (Thomson et al., 1995) and humans (Thomson et al., 1998). There are somewhat distinct characteristics between mES cells and hES cells, such as morphologies, surface marker expression and signaling pathways activated for maintenance their pluripotency. The mES cells demonstrated dome-shaped morphologies and the expression of SSEA-1. They are activated LIF and BMP4 signaling pathways as playing an important role for the maintenance of pluripotency. Meanwhile, hES cells showed flattened monolayer colony morphologies SSEA-4, TRA 1-60 and TRA 1-81 (Thomson et al., 1998). FGF and Nodal / Activin signaling pathways for maintenance of pluripotency are activated in hES cells (Hanna et al., 2010b). Although they have a slight distinct characteristics, both mES cells and hES cells are pluripotent stem cells, because they show the expression of pluripotent markers Oct4, Sox2 and Nanog and the ability to differentiate into all somatic lineage (Evans and Kaufman, 1981; Thomson et al., 1998).

In the case of domestic animals, although it has been reported that authentic ES cell lines don’t exist, a number of researchers have tried to establish ES cell lines from livestock animals. In pig, so far, several putative pES cell lines have been reported from various porcine blastocysts such as

in vitro produced, in vivo derived, parthenogenetic, in vitro aggregated and somatic cell nuclear transfer derived blastocysts (du Puy et al., 2011; Kim et al., 2010; Li et al., 2004; Li et al., 2003; Park et al., 2013; Tan et al., 2011; Vackova et al., 2011; Vassiliev et al., 2010a; Vassiliev et al., 2010b; Vassiliev et al., 2011). However, the term of “putative” or “like” have been attached in front of porcine ES cells, because of their limited pluripotency and proliferation (Gandolfi et al., 2012; Hall, 2008; Hall, 2012; Munoz et al., 2009; Talbot and Blomberg le, 2008). The reason showing limited pluripotency in putative pES cells have been considered to a lack of optimal culture condition to maintain the undifferentiated state in pig and insufficient information about porcine specific mechanism and signaling pathways to maintain the pluripotent state in pES cells (Gandolfi et al., 2012; Hall, 2008; Hall, 2012; Munoz et al., 2009). Therefore, more studies or information are required to establish authentic pES cells from porcine embryos.

Induced pluripotent stem cells have been first reported using four reprogramming factors (*Oct4, Sox2, Klf4,* and c-*Myc*) from mice and human (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). With miPS cells and hiPS cells, several papers for derivation of piPS cells have been reported from porcine somatic cells using reprogramming factors (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2012; West et al., 2010; West et al., 2011; Wu et al., 2009). Esteban et al. introduced the 4 factors to somatic cells using retrovirus (Esteban et al., 2009), entivirus (Ezashi et

al., 2009), plasmid vector transformation (Park et al., 2013) and Wu et al. used doxycycline inducible lentiviral system (Wu et al., 2009). In particular, West and colleagues showed porcine induced pluripotent stem cells (piPSCs) capable of generating chimeric offspring, although the results were only based on PCR results (West et al., 2010; West et al., 2011).

Pluripotent stem cells can be divided to naïve and primed state pluripotent stem cells according to their pluripotent state. The first “naïve” state comprises full pluripotency or a ground state. Only naïve state comprises full pluripotency or a ground state showing the contribution to germline transmission. Naïve state is permitted in specific permissive strains or species, such as 129, C57BL/6 and BALB/C strain in mice. However, a number of attempts have been made to derive the naïve state pluripotent stem cell lines from non-permissive species, including human and pig, using various exogenous factors including GSK3β and MEK inhibitors (2i), LIF, hypoxic conditions and up-regulation of Oct4 or klf4. There are molecular or epigenetic differences between naïve and primed state pluripotent stem cells (Hanna et al., 2010a; Hanna et al., 2010b). Only naïve state pluripotent stem cells can contribute to germ-line transmission. In addition, these cells show double X chromosomes activation (XaXa) in female cells and the utilization of *Oct4* distal element. Unlike naïve state, primed state pluripotent stem cells demonstrate X chromosome inactivation (XaXi), utilization of *Oct4* proximal element and limited chimerism. The

naive pluripotent cells have exhibited dome-shaped colonies morphologies and respond to LIF/Stat3, whereas primed pluripotent cells have showed flattened monolayer colony morphologies and respond to FGF and Nodal / Activin signaling pathways for maintenance of undifferentiated state. With these backgrounds, in this study, I addressed the process reprogrammed from somatic cells and various embryonic origins in pig to understand the difficulties of establishing authentic pluripotent stem cells in porcine species for about 30 years. I carried out the following three experiment and the results of three studies are as follows.

In chapter 3, I addressed the early process during the derivation of ES-like cells from various blastocysts derived from in vivo, IVF, IVF aggregation and parthenogenetic activation. I could confirm positive AP acitivity and the expressions of the pluripotent markers Oct4, Sox2, Nanog, SSEA4, TRA 1-60 and TRA 1-81 in all of our cell lines. As a results, I was able to establish the ES-like cell lines from porcine embryos of various origins including in vitro fertilized (IVF), in vivo derived, IVF aggregated (3X) and parthenogenetic embryos. All ES-like cell lines derived from various embryos showed similar morphologies of primary colonies and typical morphologies respectively. And these cell lines, coincident with results of previous porcine and human reports, showed the AP activity, the expression of the pluripotent markers, such as Oct4, Sox2, Nanog, SSEA1, SSEA4, TRA 1-60 and TRA 1-81, EB formation, normal karyotyping and

differentiation capacity into three germ layers. These results suggest that ES-like cells derived from various embryos possess similar characteristics, such as morphology, AP activity and the expression of pluripotent markers, among each other's cell lines. These results will help understanding the process established putative pES cell lines from porcine embryos. However, I consider that more studies are required to establish authentic pES cell lines from porcine embryos.

The experimental aim of chapter 4 was to investigate the expression of pluripotent markers, signaling pathways and pluripotent status in EpiSC-like pES cells derived from various embryonic origins, including *in vitro* fertilized (IVF), *in vivo* derived, IVF aggregated (3X) and parthenogenetic embryos, and piPS cell derived from PEFs. In this study, all cell lines showed AP activity and expressions of the genes *Oct4*, *Sox2*, *Nanog*, *Rex1*, *TDGF1*, *bFGF*, *FGFR1*, *FGFR2*, *Nodal* and *Activin-A* involved in pluripotency and signaling pathways, X chromosome inactivation in female cell lines, in vitro differentiation potential and a normal karyotype, thus displaying similarities to mEpiSCs or hESCs, which was supported by the comparison of the global gene expression pattern. Therefore, my results suggest that, as a non-permissive species, the porcine embryos and somatic cells undergoes reprogramming into a primed state during the establishment of pluripotent stem cell lines by genomic background. These results will help not only to understand the reprogramming into a primed state during the establishment

of pluripotent stem cell lines in pigs, but also explain current status of stem cell research in domestic animals.

The experimental purpose of final chapter 5 was to investigate whether a mESC-like induced pluripotent stem cell line could be derived from PEFs using various exogenous factors. I have been able to successfully induce PEFs into mESC-like induced pluripotent stem cells. These cell lines showed mESC-like morphologies, the expressions of pluripotency markers, such as Oct4, Sox2, Nanog and SSEA1 and sequential reprogramming by addition of DO). As a result, I have been able to derive mESC-like piPS cell lines from PEFs by infection of lentiviral transgenes using DOX-inducible tet-on promoters. I could confirm mESC-like piPS cell lines demonstrated mESC-like morphologies and the expressions of Oct4, Sox2, Nanog and SSEA4. In addition, these cell lines could be re-generated into mESC-like piPS cells using secondary or third fibroblast-like cells differentiated from mESC-like piPS cells by addition of DOX. These results suggest that the porcine species, as a non-permissive species, could be reprogrammed into mESC-like piPS cells by the activation of various exogenous factors, such as continuous transgene expression by DOX-inducible system, 2i and LIF, although the transgenes activated by DOX wasn't able to sufficiently induce the activation of endogenous transcription factors in derived mESC-like piPS cell lines. Therefore, I think that further studies are required to derive authentic naïve state pluripotent stem cells from porcine species. In present,

I am trying to derive naïve state pluripotent stem cells from PEFs by a newly modified method.

With comprehensive purpose for establishment of porcine pluripotent stem cell lines, I have performed various experiments in chapter 3 to 5. According to results of these experiments, I could confirm that porcine species have been reprogrammed to primed state from embryos and somatic cells, and it is hard to derive authentic pluripotent stem cells, because of insufficient information and understanding of porcine specific mechanism. Therefore, I made arrangements of three further studies in order to understand more information of porcine specific mechanism. First of all, I will use a newly modified method to derive stable naïve state pluripotent stem cells from PEFs. In Chapter 5, I confirmed that the transgenes using tet-on system were not able to sufficiently induce the activation of endogenous transcription factors in derived mESC-like piPS cell lines. For that reason, I have a plan to derive naïve state pluripotent stem cells using nuclear transfer from donor cells, in which transgenes, such as Oct4 and Klf4, will be introduced. In addition, effect of reprogramming according to different kinds of promoters must be verified to understand the porcine specific mechanism during the reprogramming into pluripotent stem cells from somatic cells. Finally, one of the most important things for understanding the porcine specific mechanism is the detection of porcine specific marker in pluripotent status. For the detection of pluripotent

markers in porcine pluripotent stem cells, I have a plan to do comparative study of transcriptomic landscapes between ICMs and pluripotent stem cells in pigs using the next generation RNA sequencing. It will help to understand the porcine specific mechanism, as well as the stemness markers and the control of stem cell differentiation in pigs. Therefore, I am necessary to study more detailed investigation base on results of my experiments.

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SUMMARY IN KOREAN

만능줄기세포는 자가 무한 증식 능과 모든 조직의 세포로 분화할 수 있는 세포를 말한다. 생쥐에서 처음 배아줄기세포가 확립된 이후 돼지, 소, 랫트, 양, 염소, 영장류 및 사람에서 만능줄기세포 확립을 위한 많은 연구가 진행되었다. 만능줄기세포는 배아발생연구와 줄기세포를 이용한 재생의학연구에 도움을 줄 수 있다. 특히, 돼지는 사람과 면역학적 및 생리학적 유사성 때문에 전임상 연구의 활용에 있어서 이상적인 모델동물로 알려져 있다. 이러한 이점을 이유로 돼지 배아줄기세포 확립을 위하여 1990년대 초부터 현재까지 많은 연구가 진행되고 있다. 그러나, 지금까지 유사 돼지만능줄기세포만 존재할 뿐 생쥐만능줄기세포와 같은 완전한 만능줄기세포는 아직 확립이 안 되었다. 한편, 일본의 야마나카 연구진에 의해 연구된 4개의 리프로그래밍 인자 (Oct4, Sox2, Naonog, Klf4)를 이용한 유도만능줄기세포가 처음 확립된 이후 돼지에서도 유도만능줄기세포가 보고되고 있다. 레트로 바이러스, 렌티 바이러스, 플라스미드 벡터 및 독시사이클린 유도 렌티 바이러스 시스템 등 많은 방법을 이용하여 돼지 유도만능줄기세포가 보고가 되고 있으며, 특히 웨스트 연구진은 돼지 유도만능세포를 이용하여 키메라 실험을 통하여 생식선 전이를 보고하였다. 하지만 이 결과는 중합효소 연쇄 반응 분석 결과로서 보다 정확한 키메리즘을 확인하기 위해서는 개체생산의 표현형 확인을 통한 추가 연구가 필요하다. 지금까지 보고된 대부분의 돼지 유도만능줄

기세포는 형태학적 특징 및 분자생물학적 특징이 생쥐배아줄기 상태보다는 착상 후 생쥐배아줄기세포 혹은 인간배아줄기세포와 유사한 특징을 보였다.

만능줄기세포는 만능성 수준에 따라 나이브 상태와 프라임드 상태로 나뉠 수 있다. 두 상태 중 나이프 상태의 줄기세포에서는 생식선 전이능을 충족시킬 수 있는 완전한 만능성을 보여주는 반면 프라임드 상태의 줄기세포는 제한된 분화 능을 지니고 있다. 완전한 만능성을 보여주는 나이브 만능줄기세포는 129, C57BL/6, BALB/C와 같은 특정 생쥐 종에서만 확립이 가능하다. 하지만 많은 연구진에 의해 나이브 상태의 줄기세포 확립이 어려운 종에서 Oct4, Klf4의 유전자 과발현 유도, GSK3β와 MEK 시그널 억제제 (2i)와 LIF, 저산소 배양조건 등 다양한 외부인자를 이용하여 확립을 유도하고 있다. 그러므로 이 연구의 목적은 돼지의 다양한 배아를 이용한 배아줄기세포와 체세포를 이용한 유도만능줄기세포의 리프로그래밍 과정을 분석하여 돼지 줄기세포주 확립과 만능성 수준을 검증하기 위함이다.

첫 번째 연구에서는 다양한 배아 유래의 돼지 배반포를 이용하여 배반포 단계부터 세포주가 확립되는 과정을 분석하여 배반포에 존재하는 두 종류의 세포타입(내부세포괴, 영양외배엽)이 생쥐의 지지세포에 부착하여 세포주가 확립되는 과정을 고 배율의 현미경을 통하여 확인 할 수 있었다. 체외생산 배반포, 체내생산 배반포, 체외생산 배아접합배아와 체

외생산 처녀생식 배반포로부터 전체 13개 유사 배아줄기 세포주를 확립할 수 있었으며 배아줄기세포의 초기배아 확립과정과 알칼라인 포스파타아제 활성, 핵형분석 및 미분화 마커 (Oct4, Sox2, Nanog)의 발현을 확인했다. 그러므로 이 결과는 다양한 배아 유래의 돼지 배반포로부터 유사 줄기세포주 확립과정을 이해하는데 도움을 줄 것이다. 하지만 생쥐배아줄기세포와 같은 완전한 배아줄기세포를 확립하기 위해서는 추가 연구가 필요하다.

두 번째 연구에서 첫 번째 연구에서 확립된 다양한 유래의 돼지 배아줄기세포주들과 돼지 배아 섬유아세포로부터 확립된 유도만능줄기세포주의 특성을 비교 분석하고 만능성 수준을 검증하는 실험을 수행하였다. 돼지 배아줄기세포주들과 유도만능줄기세포에서의 미분화 마커 발현과 시그널 관련 유전자발현 분석을 통하여 Oct4, Sox2, Nanog, SSEA4, TRA 1-60, TRA 1-81등과 같은 미분화 마커의 발현을 확인하였으며 프라임드 특징중의 하나인 Activin / Nodal 과 FGF2 시그널과 관련된 유전자의 발현을 확인했다. 또한, 삼배엽 분화능과 프라임드 특성인 자성 세포주에서 X 염색체 비활성 그리고 모든 세포주에서 정상핵형을 확인 할 수 있었다. 따라서, 이 연구를 통하여 돼지는 배아 혹은 체세포로부터 리프로그래밍되는 과정에서 나이브가 아닌 프라임드 특성을 지닌 만능줄기세포로 확립이 된다는 것을 알 수 있었다.

마지막 연구에서는 나이브상태로의 리프로그래밍이 어려운 돼지로부

터 나이브 상태의 줄기세포 확립을 유도하기 위하여 연구를 진행했다. 최근 여러 연구진들은 돼지와 같이 나이브 상태의 줄기세포 확립이 제한된 종인 사람과 랫트, NOD 생쥐에서 Oct4와 Klf4의 과발현과 2i, LIF의 첨가로 나이브 유사 유도만능줄기세포를 확립하는 결과를 보고했다. 본 실험을 통하여 역시 이전 보고에서 사용되었던 독시사이클린에 의한 외래 유전자의 계속 발현 시스템을 이용하여 생쥐배아줄기세포와 유사한 형태를 보여주는 유도만능줄기세포를 확립했다. 랜티 바이러스를 이용하여 외래 리프로그래밍 인자를 도입 시킨 후 독시사이클린에 의한 외래유전자의 안정된 발현을 확인했다. 이 유도만능줄기세포는 Oct4, Sox2, Nanog, SSEA1, SSEA4의 발현과 50계대배양 이상 안정된 형태를 유지가 가능했다. 또한, 유도만능줄기세포를 배아체 형성을 유도하여 섬유아세포로 완전히 분화 시킨 후 독시사이클린을 다시 첨가하여 2차, 3차 재리프로그래밍이 가능했다. 그러므로, 이 연구는 나이브 상태의 리프로그래밍이 제한된 돼지에서 외래유전자의 계속 발현 유도와 2i, LIF를 이용하여 나이브 유사 상태의 유도만능줄기세포 확립의 가능성을 보여주었다. 하지만 실험에서 사용된 외래 바이러스 프로모터가 돼지세포에서는 내부 만능성 유전자의 활성을 효과적으로 유도해 주지 못했으며 확립된 유도만능줄기세포는 외래 유전자 발현에 의존하는 결과를 보여 주었다. 따라서 외래유전자가 내부유전자의 발현을 효과적으로 유도해줄 수 있는 시스템을 구축하는 추가 연구가 필요하다.

결론적으로 세 가지 실험을 통하여 돼지에서 배아줄기세포 및 유도만능 줄기세포의 확립과 만능성 수준을 검증했으며 연구 결과에 따르면 돼지 종은 만능줄기세포 확립 시 프라임드 유사상태의 만능줄기세포로 리프로그래밍 되고 나이브 상태의 줄기세포 확립을 위해서는 내부 유전자 발현을 효과적으로 유도해 줄 수 있는 외래유전자의 지속 발현 유도와 2i, LIF가 필요하다는 것을 알 수 있었다. 이 연구의 결과는 다른 연구진의 연구결과들과 함께 돼지에서 생쥐 배아줄기세포와 같은 완전한 만능줄기세포 확립으로의 도약을 위해 필요한 자료로 활용 될 수 있을 것이다. 하지만 생쥐줄기세포와 같은 완전한 만능성줄기세포 확립을 위해서는 돼지 특이적 미분화 유지 메커니즘 이해와 리프로그래밍에 관한 추가 연구가 필요하다.

(주요어: 만능줄기세포, 유도만능줄기세포, 리프로그래밍, 배아줄기세포, 나이브 및 프라임드 상태, 돼지)

학번: 2007-23158

감사의 글

실험실 생활을 처음 시작했던 2007년 3월 2일의 기억이 아직 생생한데 벌써 졸업을 앞두고 이렇게 졸업논문 감사의 글을 적고 있는 제 모습에 감개무량해집니다. 6년 반이라는 시간이 주마등처럼 지나가며 많은 생각들이 스쳐가며 무엇보다 제가 졸업하기 까지 도움을 주셨던 많은 분들을 떠올리며 감사의 글을 시작하려 합니다.

사랑과 집념으로 한 갓 돌을 하나의 조각품으로 다듬는 석공의 마음과 같이 많이 부족했던 저에게 학문적 가르침과 지혜를 주셨던 많은 교수님들이 계시기에 지금의 제가 있을 수 있었던 거 같습니다. 특히 학위 과정 동안 가장 큰 영향력을 주신 이창규 지도 교수님께 무한한 감사와 존경을 표합니다. 때로는 엄하시게 때로는 인생의 선배님 혹은 학문적 선배님으로 다가와 제자에 대한 무한한 사랑의 마음을 느낄 수 있었습니다. 종종 늦은 밤 연구실로 오셔서 남은 학생들을 데리고 술 한잔 기울이며 인생 혹은 학문적 고민을 들어주시며 조언해주시던 기억은 평생 잊지 못할 거 같습니다. 또한, 교수님과 함께한 랩 미팅과 저널미팅, 여러 학회 활동을 통해서 연구자로서 갖추어야 할 소양을 함양할 수 있었습니다. 다시 한번 깊은 감사를 표합니다.

학위를 마치는데 많은 가르침을 주신 윤철희 교수님께도 깊은 감사를 전하고 싶습니다. 주말이나 늦은 밤까지 학교에 계시며 연구하시는 교수님을 뵈며 교수님의 열정을 본 받고 싶었습니다. 끊임없이 노력하는 연구자가 되겠습니다. 또한 저희 지도교수님께서 안식년으로 미국에 계실 때에도 지도 학생들처럼 저희를 챙겨주셔서 깊은 감동을 받았습니다. 그리고 학위를 마칠 때까지 학위 심사위원장님으로서 성심 성의껏 학위논문을 지도해주셔서 정말 감사합니다. 처음 논문을 투고하면서 bioinformatics 결과를 얻는데 많은 도움을 주신 김희발 교수님께도 깊은 감사를 전합니다. 항상 학생들을 신뢰하며 격려하고 용기를 북돋아 주시며 감싸주시는 모습에 감동을 받았습니다. 때로는 스승과 제자가 아닌 인생 선배님으로 아낌없는 조언과 가르침으로 교수님의 사랑을 느낄 수 있었습니다. 연대 가학현 교수님께도 감사의 말씀을 전합니다. 학회

에서 언제나 반겨주시며 학문적 조언을 아끼지 않으시며 항상 학문적으로 고민을 하시며 진정한 학자의 길을 몸소 실천하시는 교수님의 모습에 존경을 표하고 싶습니다. 충북대 현상환 교수님께도 감사의 말씀을 전합니다. 학위 심사 중 부족한 부분을 보완할 수 있도록 도움을 주시며 마지막까지 학문적 그리고 학문외적으로 좋은 말씀과 가르침 주셔서 정말 감사의 말씀을 드리며 교수님께서 해주신 좋은 말씀 가슴에 새기어 실천하도록 하겠습니다. 또한, 모든 학생들에게 따뜻하신 하종규 교수님과 수업을 통하여 학문적으로 많은 지혜와 가르침을 주신 최윤재 교수님과 임정묵 교수님 그리고 한재용 교수님께도 깊은 감사의 말씀을 전합니다. 항상 한 식구임을 강조하시며 따뜻하게 맞이해주시며 학자로서의 열정을 몸소 보여 주시며 가르침을 실천하시는 황우석 교수님께도 감사의 말씀을 전합니다. 또한, 불철주야 연구에 몰두하시는 신태영 박사님을 비롯한 여러 수암생명공학연구원 분들께도 감사의 말씀을 전하고 싶습니다. 학부 지도교수님이신 방명걸 교수님께도 감사의 말씀을 전합니다. 학부 인턴 과정에서 익혔던 기초 실험들, 교수님과 방학 때 하던 스터디, 실험 할 때 정확성과 아이디어를 강조하신 가르침이 지금까지 연구자로서 많은 밑거름이 되고 있습니다. 다시 한번 감사 드립니다. 학부 4학년때 류범용 교수님의 강의를 통해서 분자생물학과 줄기세포를 처음 접할 수 있었습니다. 분자생물학 지식이 많이 부족했던 저에게 교수님의 강의는 많은 도움이 되었습니다. 그리고 학회에서 뵐 때 마다 잊지 않으시고 반겨주시며 관심을 가져주셔서 정말 감사 드립니다. 실험을 직접 알려주시며 일과 중에는 엄하시지만 일과 후에는 후배들에게 항상 맛있는 것을 챙겨주시며 좋은 말씀을 많이 해주신 오신애 박사님께도 감사의 말씀을 전합니다. 또한, 물심양면으로 연구하는데 많은 도움을 주신 양병철 박사님과 황성수 박사님께도 감사의 말씀을 전합니다.

실험실 생활 하며 이제는 눈빛만 봐도 무슨 생각을 하는지 알 정도로 하루 24시간 중 가족보다도 더 오랜 시간을 같이 보낸 실험실 선후배님들께도 감사의 말씀을 전합니다. 실험실의 큰 누나로서 학생들에게 실험도 잘 알려주시고 때로는 친 누나처럼 잘 챙겨줬던 혜선 누나에게도 감

사의 말씀을 전합니다. 실험실에 처음 와서 모든 것이 낯설고 어색할 때 아낌없는 조언을 해 주셨던 윤정임 박사님, 많은 격려로 항상 큰 힘이 되어주신 셀 방 1호 박사님이신 손혜영 박사님께도 감사의 말씀 전합니다. 주원석 박사님과 형찬이와 계속해서 행복한 가정 이루시길 빌게요. 항상 열정적으로 실험하시며 좋은 성과 많이 내시어 후배들에게 모범을 보여주시는 박치훈 박사님께도 감사의 말씀을 전합니다. 정박사님과 오래오래 건강하시고 행복하시길 바랍니다. 연구를 위해 파견 때문에 자주 뵐 수는 없었지만 뵐 때마다 귀감이 될 수 있는 좋은 말씀 많이 해 주신 김민구 박사님께도 감사의 말씀을 전합니다. 식약청 근무하시면서 가끔씩 오셔서 맛있는 것도 많이 사주시며 좋은 말씀 많이 해주신 배창준 박사님께도 감사의 말씀을 전합니다. 최근 공동 연구 진행으로 학교에 자주 오셔서 후배들에게 술도 많이 사주시며 좋은 말씀과 다양한 실험을 경험 할 수 있게 도움을 주신 이상구 박사님께도 감사의 말씀 드립니다. 하버드에 가셔서 좋은 성과 많이 내시길 바라고 형수님과 함께 계속해서 건하고 행복하시길 바랍니다. 후배들에게 격려와 동기부여를 함께 일깨워 주신 카리스마 지호 형에게도 감사의 말씀을 전합니다. 학위 과정 중 좋은 성과 많이 내시길 빕니다. 후배들에게 따뜻하게 다가와 좋은 말씀 많이 해주시는 젠틀하신 돈호형에게도 감사의 말씀을 전합니다. 많이 뵙진 못했지만 미주리에서 포닥 중이신 이기호 박사님께도 최근 포닥 준비에 많은 조언을 해 주 신 점 깊은 감사의 말씀 전합니다. 손혜영 박사님과 함께 저에게 셀 실험을 혼자서 잘 해나갈 수 있게 도움을 주신 유진누나에게도 감사의 말씀을 전합니다. 같이 실험실 생활은 못했지만 셀방 선배님이신 정은씨에게도 감사의 말씀을 전합니다. 나이는 한 살 어리지만 선배로서 힘든 시기에 서로 의지하며 많은 추억을 안겨준 상원이에게도 감사의 마음을 전합니다. 늦은 밤까지 함께 실험적으로 서로 고민하고 의지했던 경준이에게도 감사의 마음을 전합니다. 소집해제 후 원하는 꿈 꼭 이루길 바란다. 지금은 낙타 복제의 꿈을 안고 두바이로 떠난 브랜든에게도 감사의 마음을 전합니다. 몸 건강히 지내고 좋은 성과 내어 한국에서든 어디에서든 또 만날 수 있길 기대합니다. 저와 이번에 같이

학위를 받는 한 학기 선배 김형민에게도 감사의 마음을 전합니다. 훌륭한 머리에 열정을 담아 앞으로 좋은 성과 많이 내고 항상 건강하길 바란다. 누구보다 열심히 실험하지만 힘든 테마에 고생 많이 하시는 실험실 맏형이신 동찬이형에게도 감사의 마음을 전합니다. 고생하시는 만큼 빠른 시일 안에 좋은 성과 내시길 빕니다. 이제 곧 박사학위 과정으로 들어오게 될 동갑내기 정민아빠 동경이게도 감사의 마음을 전하며 좋은 성과 많이 내길 바란다. 실험실에서 가장 열정이 넘치는 재연이 계속해서 좋은 성과 많이 내길 바란다. 셀방에서 지금까지 많이 고생하고 있는 광환이, 힘든 시기에 서로 많이 의지하며 많은 도움 줘서 고마웠다. 앞으로 계속해서 좋은 성과 많이 내길 바란다. 수의대에 파견 가있는 지영이도 좋은 성과 많이 내길 바란다. 대학원생 막내로서 고생 많이 하고 있는 종남아 고맙고 이제 곧 박사로 입학하게 되니 좋은 성과 많이 내길 바란다. 이제 곧 입학하게 될 승훈이와 지원이도 실험실 생활 잘 하고 선배를 도와 좋은 성과 많이 내길 바란다. 그리고 지금은 교수님이 되신 공승표 박사님과 준희 박사님, 종일, 정기 등 발생학 연구실 한 분 한 분께 감사의 말씀을 전합니다. 또한 면역학 연구실에서 facs 분석하는데 많은 도움을 주었던 채원, 한울, 영준, 윤철, 정민, 효신 그리고 손영민 박사에게도 고마움을 전합니다. 정보학실 이태헌 박사와 동현에게도 고마움을 전합니다. 학위 중 학회를 통해 많은 이야기를 나눴던 연대 희원이형, 요한이에게도 고마운 마음을 전합니다. 모교에서 학위하고 있는 친구들에게도 고마움을 전합니다. 졸업하고 유펜에서 포닥하고 있는 병각이형과 연희, 좋은 성과 많이 낸 유진이, 미주리에서 학위중인 원희, 곧 졸업하게 될 용안, 용희, 방진이, 기중, 성재, 우성이에게 고마움을 전합니다.

저마다 다양한 분야에서 훌륭하게 일을 해내고 있는 고등학교 친구들 현옥, 호섭, 재훈, 병도, 명환, 기문, 진희, 현석, 영락, 준호, 지환, 용석, 창엽, 보선에게도 고마움을 전합니다. 대학동기 종혁, 장주, 정표, 경일, 임희, 창욱, 준호, 형섭, 근중, 의중, 주희, 윤정이와 석현이형을 비롯한 새품 선후배님들께 고마움을 전합니다. 군생활 동안 서로 의지하며 건강

히 제대하고 제대 후에도 지금까지 만남을 이어오고 있는 군 동기들 현호, 국진, 재근, 영기에게도 고마움을 전합니다. 힘들 때마다 저에게 힘이 되어준 모든 친구들에게 다시 한번 고마움을 전하며 계속해서 우정 이어 가자.

끝으로 저에게 가장 큰 힘이 되어주고 가장 사랑하는 우리 가족, 아버지, 어머니, 누나, 매형에게 감사의 마음을 전합니다. 특히, 지금까지 제가 원하는 공부를 할 수 있게 믿어주시고 지지해주시고 계시는 아버님, 어머님께 진심을 다해 감사 드리며 정말 사랑합니다. 그 동안 받았던 넘치는 사랑 이제는 제가 보답하며 살아가겠습니다. 오래오래 건강하게 사시길 바랍니다. 자랑스러운 아들이 되겠습니다. 누나와 매형 그리고 조카들 듬직한 첫 째 선우, 애교 많은 둘 째 준우, 귀여운 막내 찬우도 저에게 큰 힘이 되었습니다. 고맙습니다. 앞으로 계속해서 건강하고 행복한 가정 이루길 바랍니다. 그리고 살아계셨으면 누구보다 자랑스러워 하고 기뻐하셨을 증조할아버지, 증조할머니, 할아버지, 할머니, 큰 할머니, 큰 고모부님께 감사의 말씀을 전합니다. 그리고 큰 고모, 작은 고모/고모부, 작은 아버지/어머니, 외가친척분들도 이모, 이모부, 삼촌, 외숙모, 할머니, 할아버지, 한 분 한 분께 감사의 말씀을 전해야 합니다만 너무 많으셔서 언급을 못 드려 죄송합니다. 모든 친척 분들께 감사의 마음 전합니다. 다시 한번 학위과정 동안 함께 하며 도움이 되어주신 모든 분들께 머리 숙여 깊은 감사의 말씀을 전하며 이만 마치겠습니다.

2013. 7. 22

박 진 규 올림