การสร้างเซลล์ที่คล้ายเซลล์ต้นกำเนิดจากตัวอ่อนสุกร: ผลของแหล่งที่มาของตัวอ่อนและสภาวะการเลี้ยงเซลล์ต้นกำเนิด

นางสาว ศศิธร พนโสภณกุล

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

THE ESTABLISHMENT OF PORCINE EMBRYONIC STEM (pES)-LIKE CELLS: EFFECTS OF EMBRYONIC SOURCES AND CULTURE CONDITIONS

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ศศิธร พนโสภณกุล : การสร้างเซลล์ที่คล้ายเซลล์ต้นกำเนิดจากตัวอ่อนสุกร: ผลของแหล่งที่มาของตัวอ่อนและสภาวะการเลี้ยงเซลล์ต้นกำเนิด (THE ESTABLISHMENT OF PORCINE EMBRYONIC STEM (pES)-LIKE CELLS: EFFECTS OF EMBRYONIC SOURCES AND CULTURE CONDITIONS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ. ดร. มงคล เตชะกำพุ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ. ดร. ธีรวัฒน์ ธาราศานิต, 97 หน้า

การทดลองที่ 1 ศึกษาผลของวิธีการกระตุ้นโอโอไซต์สุกรด้วยกระแสไฟฟ้า (PA) ในขนาดที่แตกต่างกัน อัตราส่วนจำนวนตัวอสุจิต่อโอโอไซต์ในช่วงการปฏิสนธิภายนอกร่างกาย (IVF) และผลของสารที่ออกฤทธิ์ในการยับยั้ง (inhibitor; i) การทำงานของ MEK และ GSK-3 ต่อความสามารถในการพัฒนาและคุณภาพตัวอ่อนที่ได้ กระตุ้นกลุ่มโอโอไซต์ระยะเมตาเฟส II ด้วยกระแสไฟฟ้าที่แตกต่างกัน 3 วิธี คือ วิธีที่ 1 กระแสไฟฟ้าขนาด 1.36 kV/cm นาน 30 msec 2 ครั้ง วิธีที่ 2 กระแสไฟฟ้าขนาด 1.50 kV/cm นาน 60 msec 2 ครั้ง และวิธีที่ 3 กระแสไฟฟ้าขนาด 1.0 kV/cm นาน 80 msec 3 ครั้ง ภายหลังการเลี้ยงตัวอ่อนนาน 7 วัน พบกลุ่มที่ถูกกระตุ้นด้วยวิธีที่ 3 มีอัตราการเจริญของตัวอ่อนระยะบลาสโตซิสสูงกว่ากลุ่มที่ถูกกระตุ้นด้วยวิธีที่ 1 และ 2 ($p<0.05$) แต่ไม่พบความแตกต่างของค่าเฉลี่ยจำนวนเซลล์ตัวอ่อนในแต่ละกลุ่มทดลอง ทำการปฏิสนธิกลุ่มโอโอไซต์ที่เจริญเต็มที่และไม่มีส่วนของเซลล์คูมูลัสที่อัตราส่วนจำนวนตัวอสุจิต่อโอโอไซต์ ดังนี้ 1000:1, 2000:1 และ 4000:1 นาน 6 ชั่วโมง พบว่ากลุ่มที่ปฏิสนธิที่ 2000 และ 4000 ตัวอสุจิต่อโอโอไซต์ มีอัตราการผ่านเข้าปฏิสนธิของตัวอสุจิมากกว่ากลุ่มที่ปฏิสนธิที่ 1000 ตัว ($p<0.05$) อย่างไรก็ดี การปฏิสนธิที่ 1000 ตัวอสุจิต่อโอโอไซต์ให้อัตราการเข้าปฏิสนธิของตัวอสุจิหนึ่งตัวต่อโอโอไซต์สูงกว่าที่ 4000 ตัวอสุจิ ($p<0.05$) และมีอัตราการเจริญของบลาสโตซิสมากกว่ากลุ่มที่ปฏิสนธิที่ 4000 ตัวอสุจิต่อโอโอไซต์ ($p<0.05$) ทำการเลี้ยงกลุ่มโอโอไซต์ที่ปฏิสนธิภายนอกร่างกาย หรือ กระตุ้นด้วยกระแสไฟฟ้า ในน้ำยาเลี้ยงตัวอ่อนที่เสริมด้วย 0.5μM MEKi และ/หรือ 3.0μM GSK-3i ภายหลังการเลี้ยงตัวอ่อนนาน 6 วัน พบอัตราการเจริญของตัวอ่อนระยะคลีเวทและบลาสโตซิสเพิ่มขึ้น ($p<0.05$) ในกลุ่มตัวอ่อน IVF ที่เลี้ยงในน้ำยาที่เสริมด้วย MEKi นอกจากนี้ยังพบค่าเฉลี่ยจำนวนเซลล์ของกลุ่มมวลเซลล์ชั้นใน (ICM) และอัตราส่วนของจำนวน ICM:จำนวนเซลล์โทรโฟบลาส (TE) ที่สูงขึ้นในกลุ่มตัวอ่อน IVF และ PA ที่เลี้ยงในน้ำยาที่เสริมด้วยสารยับยั้งตัวเดียวกัน ($p<0.05$) พบมีการแสดงออกของโปรตีน Nanog ในเซลล์ ICM ที่เพิ่มขึ้นในกลุ่มที่เลี้ยงในน้ำยาที่เสริมด้วย MEKi มากกว่ากลุ่มทดลองอื่น สรุปว่าการกระตุ้นโอโอไซต์ด้วยวิธีที่ 3 และ การปฏิสนธิภายนอกร่างกายด้วยอัตราส่วนตัวอสุจิ 1000 ตัวต่อโอโอไซต์ ให้ผลการเจริญของตัวอ่อนบลาสโตซิสดีที่สุด และการยับยั้งกลไกของ ERK signaling โดยฤทธิ์ของ MEKi เพิ่มจำนวนตัวอ่อนบลาสโตซิสและ pluripotency ในเซลล์ ICM

การทดลองที่ 2 ศึกษาเปรียบเทียบคุณภาพของตัวอ่อนที่ผลิตได้จากวิธี IVF, PA (ผลการทดลองที่ 1) และการปฏิสนธิภายในร่างกาย (IVV) ต่อการสร้างเซลล์ ES ในสุกร ทำการเลี้ยงตัวอ่อนบลาสโตซิสที่ไม่มีเปลือกหุ้ม IVF, PA และ IVV บนเซลล์พี่เลี้ยงชนิด STO ในน้ำยาเลี้ยง พบว่าอัตราการยึดเกาะบนเซลล์พี่เลี้ยงของตัวอ่อน IVV สูงกว่าตัวอ่อนที่ผลิตได้จากภายนอกร่างกาย ($p<0.01$) ส่วนลักษณะของกลุ่มเซลล์ที่คล้ายเซลล์ ES แรกเริ่มพบมีการเจริญในกลุ่มตัวอ่อน IVV มากกว่า ตัวอ่อน IVF แต่ไม่พบในกลุ่มตัวอ่อน PA ($p<0.01$) ภายหลังการเพิ่มจำนวน (passage) กลุ่มเซลล์ดังกล่าวที่เจริญจากตัวอ่อน IVV สามารถพัฒนาเป็น ES เซลล์ไลน์ได้มากกว่ากลุ่มที่เจริญจากตัวอ่อน IVF และแสดงคุณลักษณะที่จำเพาะต่อการเป็นเซลล์ ES สรุปว่าตัวอ่อนที่ผลิตจาก IVF สามารสร้างเซลล์ที่คล้ายเซลล์ต้นกำเนิดตัวอ่อนสุกรได้

การทดลองที่ 3 ศึกษาประสิทธิภาพของเซลล์พี่เลี้ยงต่อการแยกและเลี้ยงเซลล์ ES ในสุกร ทำการเลี้ยงตัวอ่อนบลาสโตซิสที่ไม่มีเปลือกหุ้มบนเซลล์พี่เลี้ยงที่ต่างชนิดกัน ดังนี้ PESF, PTSF, HFK, MEF และ STO กลุ่มตัวอ่อนที่เลี้ยงบนเซลล์พี่เลี้ยง HFK และ STO มีจำนวนร้อยละของกลุ่มมวลเซลล์ชั้นในที่เจริญมากกว่ากลุ่มที่เลี้ยงบนเซลล์พี่เลี้ยง PESF ($p<0.05$) และพบมีการเจริญของกลุ่มเซลล์ที่คล้ายเซลล์ ES แรกเริ่มและจำนวนเซลล์ไลน์ในกลุ่มตัวอ่อนที่เลี้ยงบนเซลล์พี่เลี้ยง PESF ลดลงเมื่อเปรียบเทียบกับกลุ่มที่เลี้ยงบน HFK ($p<0.05$) แต่ไม่มีความแตกต่างจากกลุ่มอื่น อย่างไรก็ดีพบว่า 1 และ 3 เซลล์ไลน์ที่เจริญบนเซลล์พี่เลี้ยง PTSF และ STO ตามลำดับ สามารถเพิ่มจำนวนและคงลักษณะการเป็นเซลล์ ES ไว้ได้ สรุปว่าเซลล์พี่เลี้ยง PTSF มีคุณสมบัติในการใช้เลี้ยงเซลล์ที่คล้ายเซลล์ต้นกำเนิดตัวอ่อนสุกรได้ดีกว่าเซลล์อื่น

การทดลองที่ 4 ศึกษาผลของการเสริมซีรั่ม FBS และ KSR ต่อการแยกและเลี้ยงเซลล์ ES-like ในสุกร ทำการเลี้ยงตัวอ่อนบลาสโตซิสที่ไม่มีเปลือกหุ้มในน้ำยาเลี้ยงที่เสริมด้วย FBS:KSR ในอัตราส่วน 20:0, 10:10 และ 0:20 พบว่าอัตราการเกาะของตัวอ่อนในกลุ่มที่เลี้ยงด้วย FBS:KSR ที่ 20:0 และ10:10 สูงกว่ากลุ่มที่เลี้ยงด้วยอัตราส่วน 0:20 อย่างไรก็ตาม บลาสโตซิสที่เลี้ยงในน้ำยาที่ส่วนประกอบของ FBS และ KSR มีแนวโน้มของอัตราการเจริญของกลุ่ม ICM มากกว่ากลุ่มทดลองอื่น พบมีการเจริญของกลุ่มเซลล์ที่คล้ายเซลล์ ES แรกเริ่มในกลุ่มตัวอ่อนที่เจริญใน FBS:KSR ที่ 10:10 สูงกว่ากลุ่มการทดลองอื่น 3 และ 6 เซลล์ไลน์สามารถสังเกตพบได้ในกลุ่มที่เลี้ยงด้วย FBS:KSR ที่ 0:20 และ10:10 ตามลำดับ โดยยังคงลักษณะการเป็นเซลล์ ES ไว้ได้ขณะทำการเลี้ยงเพื่อเพิ่มจำนวน สรุปว่าการเสริมด้วย FBS และ KSR ในน้ำยาเลี้ยง ESสามารถรักษาสภาพการเป็น ES และเพิ่มจำนวนครั้งของการ passage ได้ดีกว่าการเสริมด้วย FSB หรือ KSR เพียงอย่างเดียว

ภาควิชาสูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SASITHORN PANASOPHONKUL: THE ESTABLISHMENT OF PORCINE EMBRYONIC STEM (pES)-LIKE CELLS: EFFECTS OF EMBRYONIC SOURCES AND CULTURE CONDITIONS. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorate de 3^{e} cycle. THESIS CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., 97 pp.

EXP. I aims to examine the effects of parthenogenetic activation (PA) protocols, sperm:oocyte ratio at fertilization *in vitro*(IVF), and MEK and GSK-3 inhibitors (i) on the developmental competence and quality of embryo. Groups of MII oocytes were stimulated with three different PA protocols as: I) 1.36 kV/cm, 30 msec, 2 pulses; II) 1.50 kV/cm, 60 msec, 2 pulses; and III) 1.0 kV/cm, 80 msec, 3 pulses. Blastocyst development was significantly greater in protocol III-activated oocytes than in protocol I- and II-activated oocytes (p<0.05), however, no significant difference in the mean number of blastocyst cells among the groups. Groups of denuded mature oocytes were fertilized with three different sperm:oocyte ratios as 1000:1, 2000:1, and 4000:1 for 6 h. Sperm penetration rate significantly increased when the oocytes were fertilized with 2000 and 4000 sperm:oocyte (p<0.05), by contrast, insemination with 1000 sperm:oocyte gave a significant higher rate of monospermic zygotes than 2,000 and 4,000 sperm:oocyte (p<0.05). Blastocyst development increased significantly in the group fertilized with 1,000 sperm:oocyte compared to those of 4,000 sperm:oocyte (p<0.05). *In vitro* fertilized and activated oocytes were cultured in IVC medium supplemented with 0.5µM MEKi and/or 3.0µM GSK-3i for 6 days. Cleavage and blastocyst rates increased significantly in IVF embryos treated with MEKi alone (p<0.05), however, both IVF- and PA-derived blastocysts cultured in MEKi had a significant higher cells number of ICMs and ICM:TE ratio than other groups (p<0.05). Nanog expression significantly increased in ICM cells of blastocysts cultured in MEKi rather than other groups (p<0.05). In conclusion, embryo development was optimal when three 80-msec consecutive pulses of 1.0 kV/cm and the ratio of sperm per oocyte at 1000:1 were used for PA and IVF, respectively, and suppression of ERK signaling using MEKi improves the development and quality of porcine embryos, and can promote the pluripotency in the ICM cells.

EXP. II aims to compare the quality of blastocysts produced from IVF, PA (by using the optimal protocol resulting from our previous study; Chapter II), and *in vivo* fertilization (IVV) on establishment of pES-like cells. Zona pellucida (ZP)-free blastocysts derived from IVF-, PA-, and IVV were cultured on STO feeder. The rate of blastocysts attachment was significantly greater in IVV embryos than those derived from *in vitro* (p<0.01). The primary colony formation was also significantly greater (p<0.01) in IVV than IVF blastocysts, on the contrary, none of the primary colony was formed in cultured PA blastocysts. After continuous culture, the greater number of putative ESC lines were significantly developed from IVV than IVF blastocysts (p<0.01). These colonies showed all characteristics of typically ES cells including positive AP activity and expressions of pluripotent markers and genes. In conclusion, embryos produced from IVF technique could be served as alternative source for porcine ES-like cell establishment as *in vivo* counterpart.

EXP. III aims to examine the effect of FBS and KSR supplementations on the derivation of pES-like cells. *In vivo* hatched or ZP-free blastocysts were cultured in ES medium supplemented with different (volume/volume) ratios of FBS:KSR (20:0, 10:10, and 0:20). The attachment rate was significant higher (100%) in blastocysts cultured in media supplemented with 20% FBS and 10% FBS+10% KSR than those cultured in KSR alone (64.4%). Blastocysts cultured in a mixture of FBS and KSR showed a higher tendency rate of ICM outgrowths than other groups. The formation of primary ES-like colonies was significantly greater in blastocysts growing in 10% FBS+ 10% KSR than those cultured in 20% FBS and 20% KSR (64.4, 17.8 and 33.3%, respectively). Three (5.88%) and six (40%) putative ES cell lines were observed from groups cultured in 20% KSR and 10% FBS+ 10% KSR, respectively. These colonies showed all the characteristics of typically ES cells. In conclusion, the culture condition supplemented with equal ratio at 10% of FBS and KSR is more suitable for the isolation and culture of pES-like cells than that supplemented with FBS or KSR alone.

EXP. IV aims to evaluate the efficiency of feeder cells on the isolation and culture of undifferentiated pES-like cells. *In vivo* hatched or ZP-free blastocysts were cultured on different feeders as PESF, PTSF, HFK, MEF, and STO. Attached blastocysts cultured on HFK and STO feeder layers showed a higher percentage of ICM outgrowths than those cultured on PESF (76.7, 72.9 and 38.9%, respectively; p<0.05) The rates of primary ES-like colony formation (30.6 vs 76.7%) and the number of putative ES cell lines (1 vs 7) were significantly decreased when ICM outgrowths were cultured on PESF compared to those cultured on HFK, respectively; p<0.05). Only colonies from one (25%) and three (50%) cell lines derived respectively on PTSF and STO feeder layers could be maintained in undifferentiated stage by presenting all the characteristics of typically ES cells. In conclusion, feeder type plays an important role in establishing ES cells, while PTSF and STO cells were the best in maintaining porcine ES-like cells in an undifferentiated stage.

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Student's signature……..……
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LIST OF ABBREVIATIONS

AI	artificial insemination
Akt	protein kinase B
ALP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic proteins
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
COCs	cumulus-oocyte complexes
CL	corpus luteum
CB	cytochalasin B
Cdx2	caudal type homeobox transcription factor 2
cm	centimeter
CO_2	carbon dioxide
DAPI	4‘-6‘-diamidino-2-phenylindole
6-DMAP	6-dimethylaminopurine
DMEM	Dulbecco‘s modified Eagle medium
DMSO	dimethyl sulfoxide
DC	direct current
DNA	deoxyribonucleic acid
EB	early blastocyst
ExB	expanded blastocyst
EGF	epidermal growth factor
eCG	equine chorionic gonadotropin
ESCs	embryonic stem cells
ERK	extracellular signal-related kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
Fz	Frizzled protein receptors
GAPGH	glyceraldehydes phosphate dehydrogenase
Gp-130	glycol-protein 130
GSK	glycogen synthase kinase
HB	hatched blastocyst
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFK	human foreskin fibroblast
HCl	hydrochloric acid
h	hour
hCG	human chorionic gonadotropin
ICM	inner cell mass
i.m.	intramuscular
IVF	*in vitro* fertilization
IVC	*in vitro* embryo culture

IVM	*in vitro* maturation
IVP	*in vitro* embryo production
IVV	*in vivo* production
IU	international unit
JAK	Janus kinase non-receptor tyrosine kinase
KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
KSR	knockout serum replacement
kV	kilovolt
L	litre
LEF	lymphoid enhancer factor
LRP	low density lipoprotein
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
TCM199	TCM199 with Earle‘s salts
MEF	mouse embryonic fibroblast
MEK	mitogen-activated protein kinase
MAPK	mitogen activated protein kinases
MEM	Minimum Essential Media
MPF	maturation promoting factor
MII	metaphase II
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
mM	millimole
mOsm	milliosmole
mRNA	messenger ribonucleic acid
mg	milligram
min	minute
ml	milliliter
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NEAA	non essential amino acid
NCSU	North Carolina State University
Oct4	octamer-binding transcription factor
PBS	phosphate buffered saline
PFA	paraformaldehyde
PA	parthenogenetic activation
PESF	porcine ear-skin fibroblast
pFF	porcine follicular fluid
PI3K	phosphatidyl inositol 3 kinase
PTSF	porcine tail-skin fibroblast
PN	pronuclear
PVA	polyvinyl alcohol

PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RT	reverse transcriptase
PCR	polymerase chain reaction
sec	second
SSEA	stage-specific embryonic antigen
SEM	standard error of the mean
STAT	signal transducers and activators of transcription
STO	SIM mouse embryonic thioquanine and ouabain resistant
SOF	synthetic oviductal fluid
TBE	Tris, Borate, EDTA
TCF	T-cell factor
TE	trophectoderm
TGF-β	transforming growth factor β
v	volume
w	weight
wk	week
µM	micromole
µg	microgram
µm	micrometer
µsec	microsecond
β-ME	β-Mercaptoethanol

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Stem cell biology, nowadays, has a great potential to facilitate modern clinical medicine because it theoretically plays a major role in the treatment of a number of incurable diseases via transplantation therapy. Stem cells are defined by their unique capacity for self-renewal and differentiation into multiple cell lineages and tissue types. In general, stem cells can be broadly classified into two categories, embryonic and adult stem cells, depending on the developmental stage from which they are obtained. Between the two cell types, adult stem (AS) cells show a multipotent property which has a limited potential to differentiate into specific cell types of their tissue of origin. For instance, bone marrow-derived hematopoietic stem cells can differentiate only into progenitors of all the blood-cell lineages such as red and white blood cells (Mayhall *et al*., 2004; Figure 1). Furthermore, restriction of cell division and slow growth rate may affect their utilization for tissue engineering (Vogel, 2001). On the contrary, embryonic stem (ES) cells represent an alternative source of cells due to the ability to propagate unlimitedly and their potential to differentiate into all cell types in our body (Figure 1).

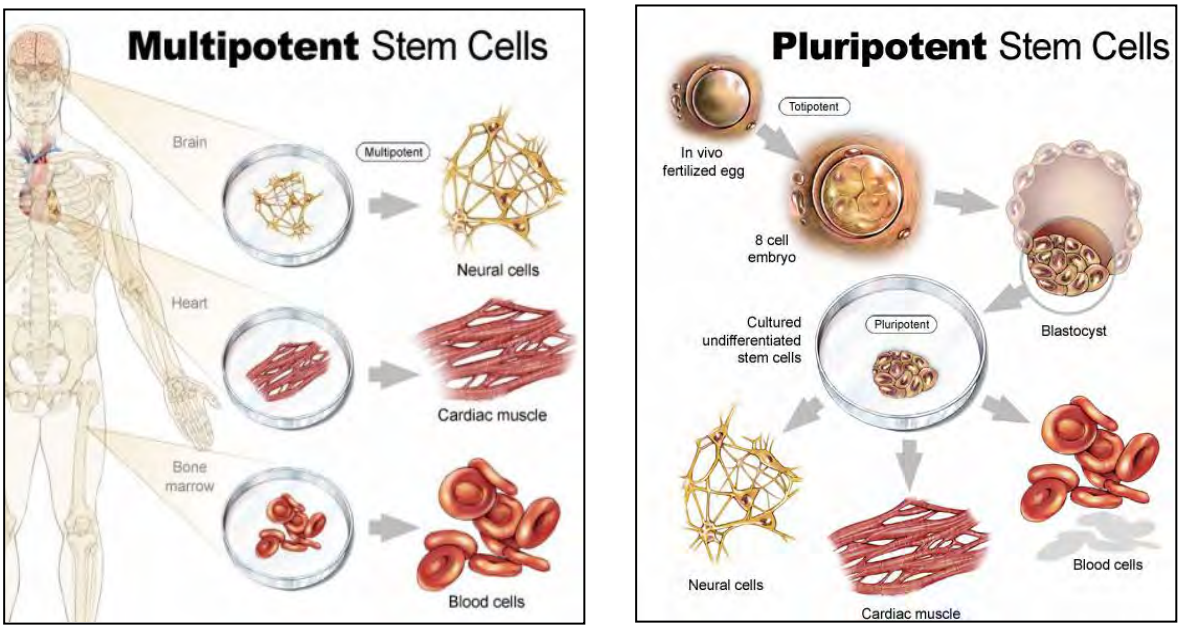

Figure 1 A property of stem cells to differentiate into specific cell types. Adult stem cells (Left) represent a multipotent property which has a limited potential to differentiate into specific cell types of their tissue of origin. Embryonic stem cells (Right) represent a pluripotent property which has an unlimited potential to differentiate into the variety of cell types in the body. (http://www.swineflucaretips.blogspot.com/2010/10/stem-cell-therapy-miraculous-power-of.html.; http://www.hyscience.com/archives/2006/03/stem_cell_innov.php)

Human (h) and mouse (m) ES cell lines have been the two predominant models over the past one (Thomson *et al*., 1998) and two decades (Evans and Kaufman, 1981; Martin, 1981), respectively, leading to more than 1000 ES cell publications per year being launched from different countries between 1981 to 2010. However, differences in physiology and organ‘s size between laboratory animals like mouse and human have unfortunately become a major problem when clinical applications of those ES cells are required. Furthermore, ES cells derived from mice and humans differ considerably such as the cell signaling pathway and gene expression (Darr and Benvenisty, 2006; Henderson *et al.*, 2002). The establishment of hES cells is also considered as immoral or unethical due to a human life would be sacrificed for the production of the ES cell lines. Giving that the development of ES cell lines from large mammals would serve a better model for pre-clinical applications than laboratory animals: pig is presented as a suitable animal model because of the similarity of cell physiology, such as cardiovascular, renal and gastro-intestinal functions, when compared to human (Schuurman and Pierson, 2008). Indeed, some of bio-products derived from pig such as insulin, have been widely used in human suggesting the immunological similarity between humans and pigs. Therefore, porcine (p) ES cells establishment has increasingly become a valuable model for human medicine as well as for veterinary medicine. Despite these benefits, stable pluripotent pES cell lines have yet to be reported. This likely involves several factors that affect the derivation of pluripotent ES cell in pig including the age and the source of embryos, specific mechanisms that maintain the pluripotency, and optimization of culture conditions.

Generally, mammalian-derived ES cell lines including pig are usually obtained from *in vivo* produced blastocysts which are expected to be of very high quality. However, animal intervention and labor-intensive techniques limit the availability of embryos to be used for ES study. Thus, it would be desirable to use embryos produced from *in vitro* matured and fertilized oocytes. In the pigs, some techniques of *in vitro* embryo production (IVP) such as parthenogenetic activation (PA), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) have been used to produce *in vitro* derived blastocysts, an alternative biomaterial, for ES cells establishment. Unfortunately, only a few ES cell lines have until recently been established, and it remains unclear whether these lines show the characteristics of ES cells like in the mouse (Li *et al*., 2004[a]; Kim et al., 2007; Ock *et al*., 2005). Nevertheless, there are great prospects for the development of pESCs which could be used to help abate the organ shortage crisis currently endured.

1.2 Literature Review

1.2.1 *In vitro* embryo production

In vitro embryo production (IVP) places major demands on oocytes and embryos with regard to developmental transitions including oocyte maturation, fertilization with or without sperm, and embryo development. Several essential factors such as signal transduction mechanisms, cytoskeletal constituents and related genes have to be activated in order to successfully negotiate and complete all the necessary developmental process of oocytes and embryos *in vitro* (Kidson, 2005). Nowadays, there is a tremendous interest to produce large quantities of matured pig oocytes and embryos through many reproductive technologies, such

as *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), and parthenogenetic activation (PA) for basic as well as biomedical research purposes, including cell replacement therapy by using ES cell technology. However, the achievement of pig IVP through these techniques depends on the efficient culture systems either *in vitro* oocyte maturation (IVM) or *in vitro* embryo culture (IVC) to generate the good quality of matured oocytes and embryos, respectively (Abeydeera, 2002).

In general, the oocyte maturation process can be broadly divided into two aspects, nuclear and cytoplasmic maturation. Nuclear maturation refers to the resumption of meiosis and progression to the metaphase II (MII) stage (Swain and Pool, 2008; Figure 2). Cytoplasmic maturation is a broader term that refers to other maturational events for both meiotic processes and other events that prepare the oocyte for fertilization and pre-implantation development, including penetration block of more than one and supporting of sperm head decondensation (Ducibella, 1998). Therefore, the synchronization of nuclear and cytoplasmic maturation is essential for establishing optimal oocyte developmental potential. The follicular growth is necessary for full meiotic potential and subsequently developmental competence of oocytes. When cumulus-oocyte complexes (COCs) are harvested from medium follicles of 3 to 8 mm in diameter, most of them can reach to MII stage, form pronuclei, and develop to blastocysts if compared with small (<3 mm) follicles (Yoon et al., 2000; Lucas et al., 2003). Furthermore, success of pig IVM can be accomplished in culture medium with various supplements including follicular fluid (FF), gonadotropins, growth factors, and thiol compounds (Funahashi and Day, 1993; Dode and Graves, 2001; Abeydeera et al., 1998; Jeong and Yang, 2001). These supplements improve nuclear and cytoplasmic maturation, including male pronuclear (MPN) formation.

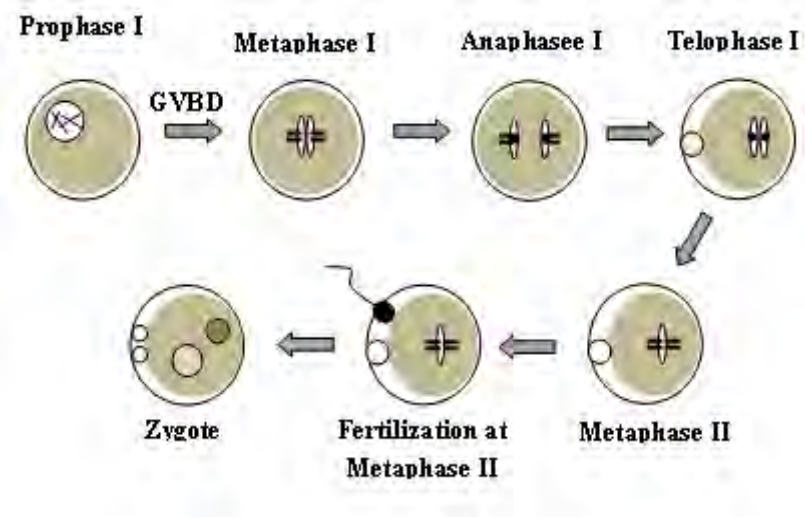

Figure 2 A diagram represents the meiosis resumption event of oocyte during nuclear maturation. An oocyte undergoes consecutively to germinal vesicle breakdown (GVBD), metaphase I, anaphase I, telophase I, and arrested at metaphase II (MII). MII oocytes ovulate and complete their meiosis after fertilization (Modified from Swain and Pool, 2008).

At the same time, the optimal culture system is another aspect that ought to be considered for production of a large number of good quality blastocysts. In the past decade, an *in vitro* developmental block of porcine embryos is frequently observed at the 4-cell stage (Jarrell et al., 1991). Nevertheless, this was overcome by adapting the available culture media that suit the development and specific needs of the pig embryos. Normally, formulation of

various culture media including, North Carolina State University (NCSU)-23 and NCSU-37 media (Petters and Wells, 1993), modified Whitten's medium (mWM; Beckmann and Day, 1993), Beltsville Embryo Culture Medium (BECM)-3; Dobrinsky et al., 1996), and porcine zygote medium (PZM)-3 and PZM-4 (Yoshioka et al., 2002) have provided the high percentage (>70%) of derived *in vivo* developmental embryos to reach the blastocyst stage. However, the varying degrees of success with IVP embryos in these media have been reported (Day et al., 2000). Currently, NCSU-23 has been proved and wildly used when compared with other media in order to enhance the proportion of IVP blastocyst development (Swain et al., 2001; Yi and Park, 2005). Moreover, some supplements such as bovine serum albumin (BSA), fetal bovine serum (FBS), non-essential amino acids (NEAA) have been also applied according to the stage of embryos (Koo et al., 1997; Cui et al., 2004; Hong et al., 2004). The blastocyst yields are the ultimate parameter as well as blastocyst quality for evaluating the success of a given porcine IVP system. For ES cell establishment, some parameters have been commonly used to define embryo quality including blastocyst morphology, blastocyst cell number (total, inner cell masses; ICMs, and trophectoderm; TE cells), and chromosomal abnormalities, all of which substantially affect the success of ES cell technology. In general, *in vitro* produced blastocysts differ distinctly from their *in vivo* counterparts, with the former containing much lower number of ICM, TE and total nuclei (Macháty et al., 1998). This relates to the methods of fertilization and IVM-IVC systems which may induce poor nuclear and cytoplasmic maturation of the oocytes (Wang et al., 1999[b]).

To date, attempts to establish pluripotent ES cell lines from the pig require a large number of blastocyst-developed embryos. This is a crucial obstacle because most porcine ES cells have been produced from *in vivo* producing embryos. However, this technique is time consuming, expensive, and the embryo recovery rate is limited. Therefore, the use of technology for producing embryos *in vitro* such as IVF and PA has become an increasingly alternative choice for generating the ES cell lines in pigs.

1.2.1.1 In vitro fertilization technique

The *in vitro* developmental competence or viability of porcine IVM-IVF oocytes to the blastocyst stage was first reported by Mattioli et al. (1989). It is generally known that pig embryo production via IVF technique has been retarded by two major problems, (i) poor male pronuclear formation (MPN) and (ii) polyspermy (reviewed by Abeydeera, 2002). Various improvements of IVM culture conditions, such as addition of follicular fluid, co-culture with follicular cells, limited exposure to gonadotropins, and supplementation of EGF or cysteine, significantly improved cytoplasmic maturation as evidenced by higher MPN formation after sperm penetration (Abeydeera, 2001). Beside these supporting factors, the ability of the cytoplasm to transform a penetrated sperm nucleus into a MPN depends on the presence of cumulus cells in matured and fertilized oocytes (Ka et al., 1997; Yamauchi and Nagai, 1999), which are known to initiate the acrosome reaction of spermatozoa (reviewed by Van Soom et al., 2002). To date, although the cause of poor MPN formation is partially corrected, problems of polyspermy and embryonic quality remain unsolved.

Polyspermy is an abnormality of the fertilization process that more than one sperm enter the oocyte due to the failure of initiation or completion of the zona reaction to block the entry of multiple sperm by reduced or delayed release of specific substances from the cortical granules (Cran and Cheng, 1986; Wang et al., 1997[a]). Age of oocyte donor has been

described as a cause of polyspermy phenomenon with subsequent poor development of the porcine oocyte (Marchal et al., 2001; Sherrer et al., 2004). These previous studies, comparing the fertilization and developmental potential of sow- and prepubertal gilt-derived oocytes, indicated that the use of prepubertal gilts as oocyte donors is not the cause of elevated rates of polyspermic fertilization. Nevertheless, sow oocytes are more likely to produce blastocysts *in vitro* and their blastocysts contain more cells. Several studies revealed that sperm concentrations and sperm-oocyte co-incubation periods influence the efficiency of the fertilization *in vitro*. The enhanced frequencies of monospermic penetration and blastocyst quality can be yielded by decreasing of spermatozoa numbers (Abeydeera and Day, 1997; Matas et al., 2003; Gil et al, 2004[a]) but overall fertilization rate was very low, and finding the optimal time for sperm-oocyte co-incubation is difficult (Gil et al., 2004[b]; Alminana et al., 2005; Gil et al., 2007). Most current IVF systems use a 5- to 6-h sperm-oocyte co-incubation time compared to 12- to 18-h used in the original porcine IVF system (Nagai et al., 1983; Toyoda et al., 1984). Furthermore, sources of sperm (freshly ejaculated sperm or frozen-thawed sperm) also influence the sperm penetration and subsequently the polyspermy rate (Sirard et al., 1993; Wang et al, 1994). In the past, freshly ejaculated semen was the main source of sperm for IVF because of the difficulty in cryopreserving boar semen. However, fresh semen characteristics differ between the ejaculates, while the use of frozen-thawed sperm from the same semen collection provides much more reproducible data and the possibility of repeating experiments as well as standardizing the male factor in IVF experiments (Marchal et al., 2002; Gil et al., 2004[a]). In addition, the influences of variation among boars and different fractions within the same ejaculate affect the high frequency of sperm penetration (Xu et al., 1996[a, b]).

In the aspect of developmental competence of embryos, the percentage of blastocyst development as well as the blastocyst cell numbers by means of trophectoderm (TE), these have no difference in both polyspermic- and monospermic- derived embryos. However, blastocysts that developed after polyspermic fertilization have fewer inner cell mass (ICM) numbers as compared with monospermic embryos, caused by retardation at pronuclear stage during early development of oocytes (Giles and Foote, 1995; Han et al., 1999). Consequently, the cell numbers of blastocysts, especially ICM cells, relate to embryos quality which is a crucial source for ES cell establishment.

1.2.1.2 Parthenogenetic activation technique

Parthenogenesis is a process by which a single egg develops without the presence of the male counterpart and is a form of reproduction common to a variety of organisms such as fish, insects, and amphibia (Hipp and Atala, 2004). In mammals, this phenomenon can not occur spontaneously, however, mammalian oocytes can successfully undergo parthenogenesis *in vitro* using artificial activation. Parthenote embryos in various species including mice (Ma et al., 2005; Ibanez, et al., 2005), pigs (Yi and Park, 2005), cattle (Lagutina et al., 2004), and humans (Rogers et al., 2004) have been produced and survived in utero at different developmental stages following embryo transfer to a surrogate uterus. However, full term development has never been established due principally to the poorly developed extraembryonic tissues (Fukui et al., 1992; Marshall et al., 1998; Kure-bayashi et al., 2000; Wu et al., 2006). This advantage, therefore, provides a plentiful source of

preimplantation embryos to create histocompability ESC lines for cell replacement therapy, which minimizes the societal and ethical problems.

In mature pig oocytes, nuclear development is arrested at metaphase of the second meiotic division (metaphase II; MII). This controls by the high activities of maturation promoting factor (MPF) protein kinase (Labbé et al., 1989). The resumption of the meiotic arrest is triggered by the penetrating sperm at fertilization, inducing the increase of intracellular calcium (Ca^{2+}) concentrations and following oocyte activation (Swann and Lai, 1997). This phenomenon also leads to a rapid decline of MPF activity. Parthenogenetic activation (PA) is a technique which mimics the action of sperm by generating transient rises of the intracellular free Ca^{2+} concentrations to activate the developmental program of oocyte (reviewed by Macháty et al., 1999).

Porcine parthenotes can be obtained by electrical stimulation of MII in calcium-containing medium (Zhu et al., 2003; Lee et al., 2004), or by treatment with chemicals, such as ethanol (Didion et al., 1990) and calcium ionophore (Wang et al., 1999[a]). Electrical stimulus is universally effective and has been widely used to produce parthenote embryos. This method evokes the formation of temporary pores in the plasma membrane of cells causing a substantial transmembrane influx of extracellular Ca^{2+} (Rickords and White, 1992). The use of a single electrical pulse usually should be combined with the high field strength and the optimal duration. However, this method frequently induces the degeneration or fragmentation of IVM oocytes after activation (Kure-bayashi et al., 2000; Onishi et al., 2000). Electrical stimulation by multiple pulses has been indicated to be more beneficial to the developmental competence of activated oocytes than a single pulse because the multiple pulses induce the same full suppression of MPF-kinase activity as that recorded after normal fertilization while a single electric pulse results in a transitory drop in MPF-kinase activity (Barnes et. al., 1993; Collas et al., 1993).

The application of multiple pulses have been reported in several species, including mice (Vitullo and Ozil, 1992), rabbits (Ozil, 1990), cows (Collas et al., 1993), and pigs (Liu and Moor, 1997; Cheong et al., 2000; Nánássy et al., 2008) with differences in voltage field strengths, pulse number, and duration. More efficiency of activation can be produced by using multiple pulses combined with lower field strength than 1.5 kV. In addition, the optimal interaction of pulse number (2 to 3 pulses) and duration (30 to 80 μsec) have been suggested affecting the developmental response of activated oocytes and pre-implantation embryos after transplantation into the uterus (King et al., 2002; Zhu et al, 2002; Nánássy et al., 2008). Nevertheless, electrical activation method frequently induces the extrusion of the second polar body (2PB), resulting in haploid parthenotes thereby compromising the developmental competence of activated embryos compared with diploid parthenotes (Henery and Kaufman, 1992; Kim et al., 1997[a]; Liu et al., 2002). To dissolve this problem, both of protein synthesis inhibitors and protein kinase inhibitors have been applied in order to inhibit the extrusion of the 2PB (Nussbaum and Prather, 1995; Dinnyes et al., 1999; Grupen et al., 2002). The inhibition of protein synthesis such as cytochalasin B (CB), an inhibitor of actin polymerization, disrupts microfilaments, and thus effectively inhibits the extrusion of the 2PB without any effect on the formation and movement of pronuclei (Kim et al., 1997[b]; Lee et al., 2003). However, cytochalasin B treatment fails to enhance parthenogenetic development and also produces the tetraploidy or higher ploidy depending on the number of

cytokinases blocked (Petzoldt et al., 1983; Grupen et al., 2002; Somfai et al., 2006). On the other hand, the 6-dimethylaminopurine (6-DMAP), a protein serine/threonine kinase inhibitor, has been shown to preserve the 2PB and accelerate pronuclear formation in mouse (Moses and Masui, 1994), cattle (Shen et al., 2008), and pig (Leal and Liu, 1998) oocytes. Furthermore, incubation of artificially activated oocytes with 6-DMAP induced parthenote development with high efficiency of diploidization (Grupen et al., 2002).

1.2.2 Derivation of embryonic stem cells

ES cells are typically derived from inner cell mass (ICM) or a subset of ICM cells, epiblast, of the blastocysts. These cells can be propagated indefinitely in undifferentiated state, meanwhile they maintain the capability to differentiate into the variety of cell types when cultured in appropriate conditions (Figure 3). Normally, these cells are cultured with standard ES medium on a monolayer cell feeder such as mouse embryonic fibroblasts (MEFs). In order to maintain their pluripotency during the culture, ES medium is supplemented with leukemia inhibitory factor (LIF), a soluble glycoprotein of the interleukin (IL)-6 family, acting through the gp130 signal transduction pathway (reviewed by Prelle et al., 2002; Wobus and Boheler, 2005).

Undifferentiated ES cells are characterized by two unique properties: (i) the unlimited self-renewal capacity and (ii) the capacity to differentiate into terminally differentiated cells. Besides, some properties include a high nuclear:cytoplasmic ratio with prominent nucleoli, normal chromosome numbers, and expression of pluripotency markers as alkaline phosphatase (ALP), transcription factor Oct4 (also known as Oct3, Oct3/4, or Pou5f1) gene,

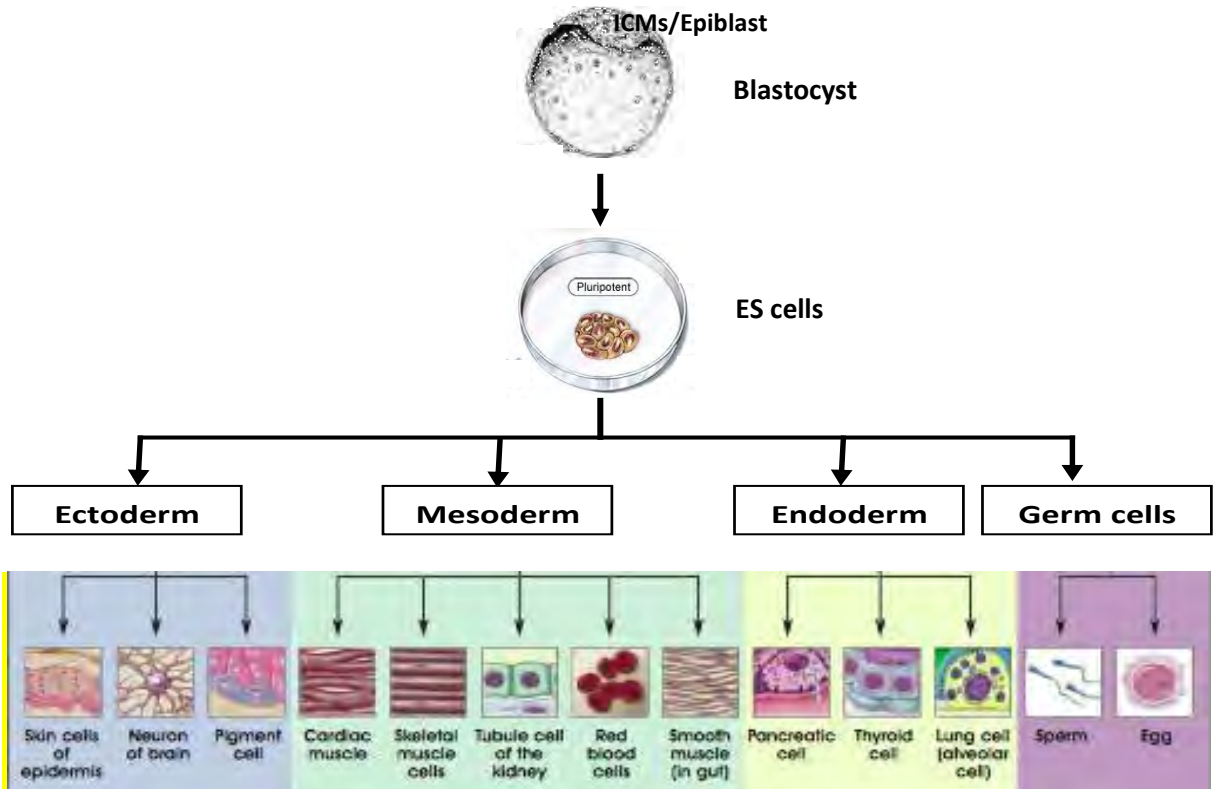

Figure 3 A schematic picture to illustrate cell lineages from ICM-derived cells of blastocyst (Modified from: http// www.stemcells.nih.gov/info/scireport/chapter1.asp)

stage-specific embryonic antigens (SSEAs), and telomerase activity. Recently, another transcription factor, Nanog, has also been recommended as a specific pluripotency marker of ES cells (Mitsui et al., 2003; reviewed by Pan and Thomson, 2007).

The ability of ES cells to spontaneously differentiate *in vitro* can be achieved by the absence of IL-6 family members, removal of feeder cells or elimination of differentiation inhibitory factors, especially, LIF. In addition to spontaneous differentiation in these situations, the culture in form of suspension so-called embryoid bodies (EBs) or inducing with chemicals can lead ES cells to differentiate into specific cell types (reviewed by Vackova et al., 2007). Upon ES cells are introduced by ectopic transplantation (e.g. under renal‘s capsule or into the testis), pluripotent cells give rise to teratocarcinomas, demonstrating their capability to differentiation *in vivo* (Evans and Kaufman, 1981). When incorporation of the ESCs with normal embryos via blastocyst injection or morula aggregation, these cells contribute to all tissues and organs including the germ-line of chimeric individuals, giving rise to functional gametes.

To date, ES cell establishment, apart from the mESC, plays an important role to offer a suitable *in vitro* model of early embryonic developmental events and a powerful tool for innovative gene targeting technologies. The establishment of animal homologues for human diseases has proven to be an invaluable tool for studies of human genetics, infectious disorders and cancer. In particular, pigs offer some distinct advantages compared to the commonly used rodent models because they are immunologically and physiologically more similar to humans and have a more diverse genetic backgrounds (Platt, 1998). However, the derivatives of stable pluripotent ES cell lines from this species have been unsuccessful, resulting from a lack of understanding of species-specific mechanisms that promote or influence cell pluripotency.

1.2.3 Establishment of porcine embryonic stem (pES)-like cells

The establishment of ES cells in pigs has been published over the past 18 years (Evan et al., 1990; Strojeck et al., 1990: Piedrahita et al., 1990[a, b]). However, none of the cell lines described in the literature satisfy all the criteria required for a formally correct definition of embryonic stem cells based on mouse ESCs standards; thus, these lines are often presented as ES-like cells. To establish pES-like cell lines, *in vivo* blastocyst-stage embryos have most often used at various stages. Mostly, these lines obtained from expanded or early-hatched blastocysts between days 6 and 8 of development (Strojeck et al., 1990: Piedrahita et al., 1990[a, b]; Chen et al., 1999; Wianny et al., 1997; Li et al., 2003; Brevini et al., 2006). Nevertheless, the ICMs start to rapidly differentiate later on day 9 of blastocyst development, suggesting the improper stage of ICM isolation in the pigs (Strojeck et al., 1990; Chen et al., 1999). In the pig as well as in other farm animals, even though *in vivo* produced embryos are expected to be of very high quality, this approach is expensive and laborious. Thus, it would be desirable, for economic reasons, to use embryos produced from *in vitro* matured oocytes fertilized with or without sperm. *In vitro* blastocysts-derived ES cell lines can be produced from many IVP techniques such as IVF, ICSI, SCNT or PA (Thomson et al., 1998; Cibelli et al., 2002; Wang et al., 2005; Tielens et al., 2006, Shao et al., 2007), but only two predominant techniques, IVF and PA, have been reported to produce ES cell lines in the pigs

(Miyoshi et al., 2000; Li et al, 2004[a]; Ock et al., 2005; Brevini et al., 2005; Vackova et al., 2007; Kim et al., 2007). These cell lines can be isolated from the stage of either expanded or hatched blastocysts by method of either whole intact blastocysts or immunosurgery. However, most of them differentiated or degenerated after 5 passages.

Maintenance of pES cells in culture is still an important obstacle due to improper culture conditions that potentially induce the differentiation of isolated ICMs. For example, while fetal bovine serum (FBS) has been supplemented as a protein source in ES culture medium, it is also as a potential source of differentiating factors in ES cell culture (Goldsborough et al., 1998). In order to maintain undifferentiated state of ES cells, a defined serum-free supplement, knockout serum replacement (KSR), is recommended instead of using animal serum. **(**Inzunza et al., 2005; Hisamatsu-Sakamoto et al., 2007). The addition of a specific cytokine, leukemia inhibitory factor (LIF), is another factor supporting the derivation and maintenance of ES cells in mouse (reviewed by Ulloa-Montoya, 2005), but its beneficial effect is still unclear in putative pES cell establishment (Hochereau-de-Reviers and Perreau, 1993; Talbot et al., 1993[a, b]; Wianny et al., 1997; Moore and Piedrahita, 1997). In mES cells, LIF functions via a membrane bound of the heterodimerized leukemia inhibitory factor receptor (LIFR) and glycol-protein 130 (gp 130) receptor complex which regulate the undifferentiated state and self-renewal of mES cells through the Janus kinase non-receptor tyrosine kinase (JAK) and signal transducers and activators of transcription 3 (STAT 3) signaling pathway (reviewed by Talbot and Blomberg, 2008). On the other hand, the activity of the LIF receptor and signal transduction pathways have been inconsistently detected in undifferentiated porcine ICMs and in 24 h cultured, undifferentiated epiblast tissue (Blomberg et al., 2008), whereas, LIFR expression has not been found in porcine ES-like cells (Vackova et al., 2007). However, high levels of this supplement prevent a loss of proliferation in epiblast outgrowth cultures, suggesting that its role may aid in continued propagation of pES cells in culture. Other supplements including bovine insulin, platelet-derived growth factor (PDGF), particularly, basic fibroblast growth factor (bFGF) have favorable effects on successful isolation and/or proliferation of pES cells (Hochereau-de-Reviers and Perreau, 1993; Prelle et al., 1995; Miyoshi et al., 2000; Li et al., 2004[b]; Lazzari et al., 2005; Vackova and Madrova, 2006).

Feeder cell type is one of the most important factors affecting ES cell culture. It serves as an attachment matrix for cells and can secrete some kinds of cytokines, such as LIF, that stimulate the proliferation and inhibit the differentiation of ES cells. Murine embryonic fibroblasts, MEFs and STO cells, have been the most predominant feeder cell choice for growth and cultivation of pES cells. There are many attempts to use the porcine embryonic fibroblasts (PEFs) as feeder cells, and it shows negative effect on the establishment and maintenance of pES-like cells (Hochereau-de-Reviers and Perreau, 1993; Wianny et al., 1997; Li et al., 2004[b]). In condition of feeder-free culture, primary pig epiblast cells fail to derive ES cells lines (reviewed by Talbot and Blomberg, 2008). The use of feeder cells from various sources either of fetus or adult, such as foreskin, muscle, skin, uterine endometrium, placenta, and bone marrow have been reported as appropriate feeder cells for hES cells establishment (Richards et al., 2003; Cheng et al., 2003; Inzunza et al., 2005; Genbacev et al., 2005; Lee et al., 2005). In the aspect of ES researches in pigs, these feeder cells have not yet been applied for derivation or maintenance ES cell lines.

All above mentioned, it is indicated that colony outgrowths of ES cells could be established from porcine embryos, but characterization and maintenance of these cells in-vitro, were limited due to the rapid differentiation of the unstable pluripotent state.

1.2.4 Characterization of porcine ES-like cell lines

Because of a limitation of ES cells maintenance in pigs, to date, only some characteristics, including morphology, ALP activity, *in vitro* differentiation, and ES cell markers, have been proven to identify the undifferentiated status of pluripotent pES-like cell lines. Two morphologies have been identified in pES-like cell lines. Most lines are described as "ES-like" when cells are small and rounded and have a large nucleus with one or two prominent nucleoli (Evan et al., 1990; Anderson et al, 1994; Gerfen and Wheeler, 1995; Wianny et al., 1997). Beside this type, others were derived with an "epithelial-like" appearance described as cells with "flattened cuboidal or eventually with polygonal shape, with distinct cell borders and abundant lipid-like vacuoles (Chen et al., 1999; Gerfen and Wheeler, 1995; Moore and Piedrahita, 1997; Miyoshi et al., 2000; Li et al., 2004[a, b]). Interestingly, epithelial-like cell lines were able to survive for a number of passages higher than for the ES-like cell lines (Piedrahita et al., 1990[a,b]). *In vitro* differentiation in both spontaneous differentiation and embryoid bodies formation have been also described. They tend to differentiate into various cell types of three origins including ectoderm, mesoderm, and endoderm. In addition, spontaneous differentiation tends to begin in the center similar to hES cells instead at the periphery of colonies as in mouse (Thomson et al., 1998; reviewed by Talbot and Blomberg, 2008). However, cells derived from these cell lines failed to form teratomas following injection into immunocompromised mice, a common proof of the pluripotency of primate and mouse ESC lines. And also, up to now, the success of germ-line chimeric piglets production by injecting ES-like cells into recipient blastocyst still have not been reported (reviewed by Hall, 2008).

Pluripotent cells of the mouse and human ICM normally express the enzyme alkaline phosphatase and the transcription factors, Oct4, Nanog, Sox2 and Zfp42 or Rex-1 (Loh et al., 2006; Babaie et al., 2007). In the pig, the expression of Oct4 is observed within the ICM of the porcine hatching blastocyst, and localized in the epiblast (Vejlsted et al., 2006; Magnani and Cabot, 2008) until germ layer takes place, where it is then down-regulated. However, its expression has also been detected in the trophectoderm of the porcine blastocyst (Kirchhof et al., 2000). The expression of Oct4 in porcine ES-like cell lines has been also detected in undifferentiated colonies by RT-PCR. This also relates to the positive results of Nanog expression. Interestingly, Oct4 expression starts to downregulate during subcultures, whereas, Nanog is still expressed even in the absence of Oct4 (Brevini et al., 2007). This indicated that self-renewal of pig ESCs can occur upon Nanog expression alone without the strict requirement of the contemporary expression of Oct 4. Besides, another specific-marker, SSEA-1, a pluripotent marker in mES cells, has been exhibited in freshly dissected porcine ICM, but its signal was completely lost as early as after trypsinization. Recently, SSEA-4, a specific pluripotent-marker in human, also has been reported in putative ES cells which a stable expression even though in prolonged culture (Brevini et al., 2010). Apart from these markers, other ES cell-specific genes relating to pluripotency in mouse and primate ESC

lines, such as SSEA-3, TRA-1-60/85, SOX2, and ZFP421 could be considered as good candidates for the study in embryo-derived porcine cell lines.

1.2.5 Signaling pathways controlling pluripotency in porcine ES-like cells

Signaling pathways have been demonstrated to actively control the ES cell self-renewal, pluripotency, and differentiation either directly or through interaction (Figure 4). These pathways are triggered by specific ligands, including growth factors, cytokines, and hormones at singular or multiple entry points to initiate downstream cascades (Renard et al., 2007). Currently, the self-renewal of mES cells primarily depends on two key signaling pathways: 1) JAK/ STAT signaling by activating of LIF, and 2) BMP/Smad signaling by activating of BMP4. However, the cell signaling pathways that govern pluripotency in pig are currently unknown. LIF functions via a membrane bound of the heterodime rized leukemia inhibitory factor receptor (LIFR) and glycol-protein 130 (gp 130) receptor complex which regulate the undifferentiated state and self-renewal of mES cells through the JAK and STAT3 signaling pathway (reviewed by Talbot and Blomberg, 2008). Bone morphologenetic proteins (BMP) are members of the transforming growth factor β (TGF/β) superfamily (Shi et al., 2003), inducing expression of Id (inhibitor of differentiation) genes via Smad pathway and inhibits mES cell differentiation to neuroectoderm. The combination of BMP and LIF can sustain mES cell self-renewal in the absence of serum and feeder cells (Ying et al., 2003). In contrast with mES cell, gp130 and LIF transcripts, two specific subunits of LIF receptors, have been inconsistently detected in undifferentiated porcine ICMs and in 24 h cultured, undifferentiated epiblast tissue (Blomberg et al., 2008; Hall et al., 2009; Brevini et al., 2010), whereas LIFR expression has not been found in porcine ES-like cells (Vackova et al., 2007).

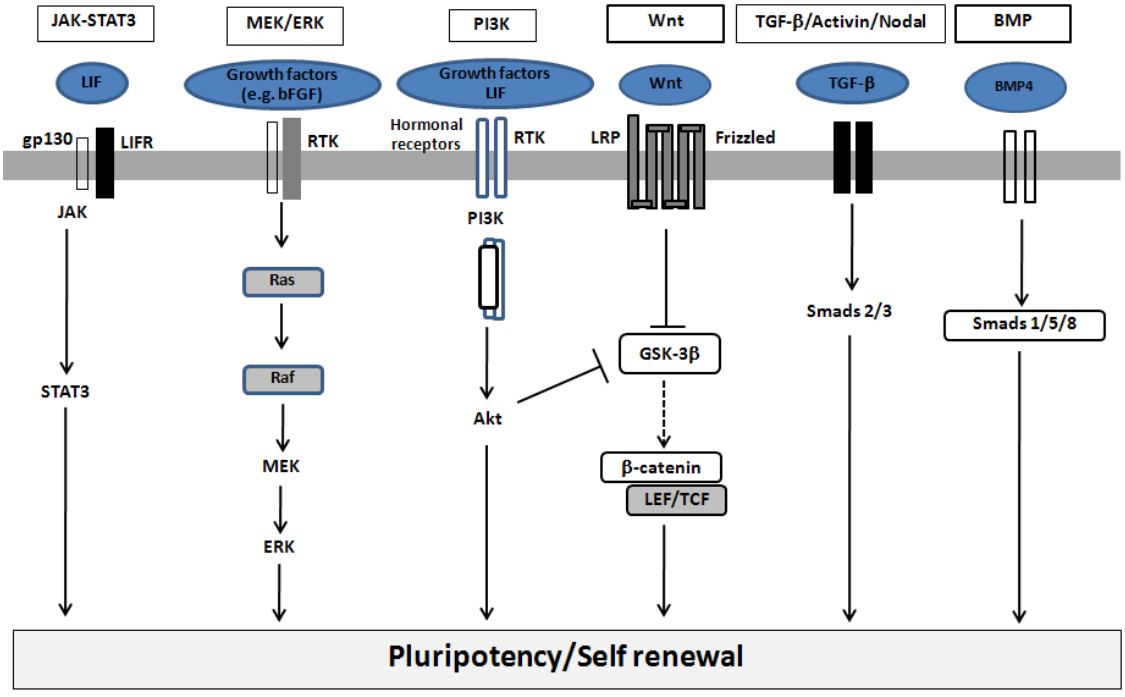

Figure 4 Signaling pathways controlling pluripotency in ES cells (Modified from Sumer et al., 2010)

Furthermore, upregulation of BMP signaling promotes differentiation of porcine ICMs and epiblasts cells during culture (Blomberg et al., 2008; Hall et al., 2009) as been reported in hES cells (Xu et al., 2002).

The other signaling pathways such as TGFβ/Activin/Nodal and MEK/ERK pathways are equally important in maintaining pluripotency in hES cells, but it has been shown to be a negative regulatory pathway for self-renewal in mESCs. TGFβ/Activin/Nodal signaling is activated through the signal transducer SMAD2/3, initiating the intracellular signaling cascade regulating genes such as Nanog involved in self-renewal (Shi and Massague, 2003). TGF-β family members including TGFβ1, growth/differentiation factors, noggin (NOG), activin A, and nodal homolog (NODAL) have all been implicated in the control for stemness of ES cells, promoting pluripotency and self-renewal of hESC (Pucéat, 2007). Much less is known about the important of TGFB factors in pES cells. However, neither activin A nor NOG, BMP4 antagonist, abrogates porcine epiblast differentiation into neuronal cells, indicating inhibition of BMP4 alone is not effective in maintenance of pES cells pluripotency (Talbot and Blomberg, 2008). MEK/ERK pathway regulate the activities of several transcription factors such as c-Myc, as well as regulation of mRNA translation to protein (Binetruy et al., 2007) by activating of growth factors such as basic fibroblast growth factor (bFGF or known as FGF2) These factors, in turn, activate receptor-linked tyrosine kinases (RTK), resulting in the activation of Ras, a small membrane-bound GTPase. Activated Ras activates the serine/threonine kinase Raf which ultimately results in the activation of a ERK. Recently, high relatively expression of the genes encoding FGFR1 and FGFR2, and even FGF2 itself have been found in porcine epiblats from d 11 blastocysts (Hall et al., 2009), although these genes are not the pluripotent markers for pES cells. This may suggest a possible role of FGF signaling in regulating self-renewal and pluripotency of pig ES cells.

The phosphoinositide 3-kinase (PI3K)/AKT pathway in ES cells is activated by growth factors (e.g. FGF, EFG, PDGF) and cytokines (e.g. insulin and LIF) through many different hormonal receptors and transmembrane tyrosine kinase-linked receptors (RTK), resulting in the activation of PI3K through interaction of the p85 subunit with various activating proteins (e.g. protein kinase C, Ras, etc.) and leads to activation of the p110 catalytic subunit. Activated form of PI3K binds AKT protein kinase, leading to the regulation of intracellular pathways involved in proliferation and apoptosis (Takahashi et al., 2005; Okita and Yamanaka, 2006). Inhibition of PI3K/Akt signaling in mouse and human ESCs induces differentiation even in the presence of LIF and feeder cells, respectively (Paling et al., 2004; Kim et al., 2005). While activation of the PI3K/Akt pathway can maintain pluripotency independent of Wnt/β-catenin signaling in mES cells (Watanabe et al., 2006). In respect of pES cells, it has recently been reported that this pathway may be involved in maintenance of self-renewal in porcine cells through the activation of LIF (Brevini et al., 2010).

Nowadays, the pathway as known _Wnt/β-catenin signaling‘ has been reported to involve in the regulation of ES cell pluripotency and self-renewal. Wnt extracellular ligands bind to the extracellular matrix and signal through cell surface frizzled protein receptors (Fz) and low density lipoprotein (LRP5/6) complex (Sato et al., 2004; Nelson and Nusse, 2004). Downstream activation of the Dishevelled family proteins, resulting in suppression of a

glycogen synthase kinase-3 (GSK-3) activity, a negative regulator of Wnt/β-catenin signaling. As a result, β-catenin (known as CTNNB1) is not phosphorylated and remains free from β-TrCP–mediated degradation. The accumulated β-catenin binds the transcription factor of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) family and converts them from repressors to activators, which triggers downstream gene transcription in the nucleus (Peifer and Polakis, 2000; Chan and Struhl, 2002; Bienz and Clevers, 2003). Several studies have indicated that β-catenin is vital for the pluripotency of mESC or hESC and that the Wnt pathway is functional in ESC lines (Sato et al., 2004; Wei et al., 2005; Anton et al., 2007). This pathway can be activated by GSK-3 inhibition, which was revealed by its specific inhibitors such as 6-bromoindirubin-3‘-oxime (BIO) and CHIR99021 (Sato et al., 2004; Ying et al., 2008). Yet even though Wnt signaling is likely important, this pathway per se is not sufficient to maintain hESC renewal (Dravid et al., 2005) and may not be functional in all ES cell lines (Wei et al., 2005).

These findings concluded that pluripotent ES cell lines have recently been isolated from *in vivo*, *in vitro* and parthenogenetic pig embryos. The principal procedures are similar to those developed for mouse and human cell lines. These would be essential to facilitate a better *in-vitro* culture system for the establishment and long-term maintenance of pluripotent pES cells. Most cell lines are characterized mainly on morphological criteria in the absence of species specific molecular probes. However, the current development of molecular markers has been studied to investigate the properties of these cells. Finally, the stable pluripotent pES cell lines have not been yet derived compared with mouse and human ES cells. Thus, the development of porcine embryonic stem cell lines is crucial for continued extension of biomedical research and also for development of stem cell therapy.

1.3 Objectives of the thesis

1.3.1 To improve the strategy of *in vitro* porcine embryo production using *in vitro* fertilization and parthenogenetic activation techniques

1.3.2 To compare the efficiency of recovery blastocysts derived from IVF, PA and *in vivo* production on the derivation of porcine ES-like cells

1.3.3 To study the efficiency of various feeder cell types on the isolation and culture of pES-like cell lines

1.3.4 To study the effect of different serum-supplemented conditions on the isolation and culture of pES-like cell lines

1.4 Hypothesis

1.4.1 Developmental competence and quality of embryos could be enhanced by renewal IVF and PA strategy

1.4.2 Porcine ES-like cell lines could be established from PA-, IVF-, and *in vivo*-produced blastocysts

1.4.3 Different types of feeder cells affect the establishment efficacy of ES cell lines in pigs

1.4.4 Different serum-supplemented conditions affect the establishment efficacy of ES cell lines in pigs

1.5 Key words: *in vitro* embryo production, embryonic stem cell, embryo source, feeder cell, serum condition, pig

1.6 Research merits

1.6.1 Embryo production from IVP techniques to serve as material sources for ES cell lines establishment in pigs

1.6.2 Establishment the proper culture conditions for derivation and maintenance of porcine ES cells

1.6.3 The knowledge of ES cell establishment as a powerful tool for veterinarian and medical cell therapy

CHAPTER II

IMPROVING STRATEGY FOR *IN VITRO* EMBRYO PRODUCTION USING *IN VITRO* FERTILIZATION AND PARTHENOGENETIC ACTIVATION TECHNIQUES

2.1 Abstract

The present study was conducted to examine: 1) the effect of different parthenogenetic activation (PA) protocols, 2) the influence of sperm:oocyte ratio at fertilization *in vitro*, and 3) the effect of MEK and GSK-3 inhibitors on developmental competence and quality of embryos, the expression of the pluripotency associated transcription factors Oct4 and Nanog. Compacted cumulus-oocyte complexes (COCs) were collected and cultured in maturation medium for 42 to 44 h, subsequently, partially denuded oocytes or MII oocytes were used for IVF and PA experiments, respectively. In the first experiment, MII oocytes were stimulated with three different activation protocols : I) 1.36 kV/cm, 30 msec, 2 pulses; II) 1.50 kV/cm, 60 msec, 2 pulses and III) 1.0 kV/cm, 80 msec, 3 pulses. The blastocysts development on day 7 was significantly greater in protocol III-activated oocytes than in protocol I- and II-activated oocytes ($p<0.05$). However, no significant difference in the mean number of blastocyst cells among the groups was observed. In the second experiment, matured oocytes were fertilized for 6 h with three different sperm:oocyte ratios (1000:1, 2000:1, and 4000:1). Sperm penetration rate significantly increased when the oocytes were fertilized with 2000 and 4000 sperm:oocyte, compared with those fertilized with 1000 sperm:oocyte ($p<0.05$). The oocytes inseminated with 1000 sperm:oocyte had a significantly higher rate of monospermic zygotes than those inseminated with 2,000 and 4,000 sperm:oocyte ($p<0.05$). The development of blastocysts increased significantly ($p<0.05$) in the group fertilized with 1,000 sperm:oocyte compared to those of 4,000 sperm:oocyte. In the third experiment, both fertilized and activated oocytes *in vitro* were randomly cultured in IVC medium supplemented with 0.5 μM PD0325901 MEK and/or 3.0 μM Chir99021 GSK-3 inhibitors (i). Treatment with MEKi and/or GSK-3i did not affect the development of PA-derived embryos, meanwhile cleavage and blastocyst rates increased significantly in IVF embryos treated with MEKi alone ($p<0.05$). Both IVF- and PA-derived blastocysts cultured in MEKi had a significant higher cells number of ICMs and the ICM:TE ratio than other groups ($p<0.05$). The expression of Oct4 was detected in both ICM and TE cells, while Cdx2 was restricted to TE cells. Nanog was localized in some cells of ICMs, which significantly increased in blastocysts cultured in MEKi rather than other groups ($p<0.05$). Our study indicates that embryo development was optimal when three 80-msec consecutive pulses of 1.0 kV/cm and the ratio of sperm per oocyte at 1000:1 were used for PA and IVF, respectively. Furthermore, suppression of ERK signaling induced by MEK inhibitor improves both quantity and quality of porcine blastocysts derived *in vitro* in terms of promoting the pluripotency in ICM cells.

2.2 Introduction

In vitro embryo production (IVP) consisting of oocyte maturation, fertilization, and embryo culture have previously been used to generate a large quantity of embryos for fundamental and applied biomedical researches such as developmental biology of embryos and xenotransplantation. On the one hand, this technology now has also become a powerful tool for ES cell research in many species including pig. Two IVP techniques, *in vitro* fertilization (IVF) and parthenogenetic activation (PA), have been considered as a suitable method producing viable embryos similar to *in vivo*-derived counterpart. These non-invasive and cost-effective techniques have more advantages over *in vivo* embryos by the large-scale embryo production with less time consuming. However, it is important to worthy note that *in vitro* culture of oocytes and embryos has been reported to influence the developmental competence and quality of embryos (e.g. cell metabolism, epigenetic status, and constituent cell numbers [inner cell mass and trophectoderm cells]), involving the derivation of immortal ES cell lines.

Porcine PA embryos can be produced from unfertilized oocytes by artificial activation that mimics the sperm penetration by generating transient rises of intracellular free calcium (Ca^{2+}) concentration either with electrical stimulation or with chemical treatment (Macháty et al., 1999; Lee et al., 2004; Nánássy et al., 2008). In respect of embryo quality, it has been demonstrated that the electrical stimulation protocol is an important factor in determining the quality of embryos after parthenogenetic activation. For example, multiple pulses combined with the optimal field strength and duration are beneficial to the developmental competence of activated oocytes compared with a single pulse because the multiple pulses induce the same full suppression of maturation promoting factor (MPF)-kinase activity as that recorded after normal fertilization while a single electric pulse results in a transitory drop in MPF-kinase activity (Barnes et al., 1993; Collas et al., 1993).

It is generally known that the main problem for pig embryo production via IVF technique is multiple sperm penetration (polyspermy), leading to the poor embryonic development (McCauley et al., 2003; Kikuchi, 2004; Sherrer et al., 2004; Koo et al., 2005), especially at blastocyst stage (Han et al., 1999; Machaty et al., 1998), and decrease of the embryo quality in terms of total cell numbers and inner cell mass (ICM): trophectoderm (TE) ratio when compared with monospermic blastocysts (Giles and Foote, 1995; Funahashi and Day, 1997). Sources of sperm (freshly ejaculated sperm or frozen-thawed sperm) also influence the sperm penetration and subsequently the polyspermy rate (Sirard et al., 1993; Wang et al, 1994). In addition, the effects of variation among boars and different fractions within the same ejaculate affect the high frequency of sperm penetration (Xu et al., 1996[a, b]). These findings suggest that development competence and quality of embryos has been affected by technique or protocol using for embryos production *in vitro* (Wang et al., 1999[b], Nánássy et al., 2008).

It is also clear that, in addition, the existence of pluripotent transcription factors Oct4 (known as Pou5f1) and Nanog is a requisite for early embryo development as well as for ES cell lines establishment (Niwa et al., 2000; Chambers et al., 2003; Chen et.al., 2009). During early embryo development, Oct4 expression is restricted to the ICM of blastocyst and the epiblast of later blastocysts (Palmieri et al., 1994). The inverse of Oct4 expression is the expression of Cdx2 which is restricted to the trophectoderm (TE) (Beck et al., 1995).

However, the expression of Oct4 is not limited only in the ICM but can also be detected in the TE cells of blastocyst (Kirchhof et al., 2000; Kuijk et al., 2008), which contrasts with that in mouse embryo (Palmieri et al., 1994; Niwa et al., 2005). At later blastocyst stages of mouse development, before the evolution of ICM into epiblast and primitive endoderm (PE), the specific precursors for epiblast and PE can early be detected by expression of Nanog and Gata6, respectively, which shows a so-called „pepper and salt" distribution (Chazaud et al., 2006). However, the expression of Nanog in porcine blastocysts can only be detected in low or undetectable level (Kumar et al., 2007; Magnani and Cabot, 2008; Kuijk et. al., 2008). Recently, the application of synthetic inhibitors of the fibroblast growth factor (FGF)/ mitogen-activated protein kinase (MEK)/ extracellular signal-related kinase (ERK) pathway and glycogen synthase kinase 3 (GSK3) has been shown to enhance the derivation of ES cell lines in mouse and rat species (Buehr et al., 2008; Nichols et al., 2009[a]). Furthermore, a combination of these inhibitors also promotes ground state pluripotency in the mouse embryos by increasing the total cell number of ICMs associated with the high uniformly expression levels of Nanog and Oct4 (Nichols et al., 2009[b]).

Thus, the present study was undertaken to determine, (i) the influence of sperm:oocyte ratio at fertilization *in vitro*; (ii) the effect of different activation protocols on fertilization rate, and developmental competence and quality of embryos, and also to investigate (iii) the effect of MEK and GSK-3 inhibitors on the developmental competence and quality of embryos in terms of ICM:TE cells number and the expression of the pluripotency associated transcription factors Oct4 and Nanog.

2.3 Materials and Methods

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

2.3.1 Collection and culture of oocytes

Gilt (Experiment I and II) and sow (Experiment III) ovaries recovered immediately after slaughter from the local abattoirs at Nakornpathom province were transported at room temperature to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from antral follicles with a diameter of 3 to 8 mm and washed twice with pre-warmed (37°C) Hepes-buffered Tyrode-lactate solution supplemented with 0.1% (w/v) polyvinyl alcohol (TL-Hepes-PVA). Groups of 30 to 50 COCs with compact cumulus cells containing at least three uniform layers of cells, and with homogeneous ooplasm (Fig. 5A) were selected and cultured at 38.5°C in a humidified atmosphere of 5% CO_2-in-air for 42 to 44 h in maturation medium (TCM199-IVM; Experiment I and II or NCSU-23; experiment III) as shown in Table 1. For the first 22 to 24 h of oocyte culture, the IVM medium was supplemented with 10 IU/ml equine chorionic gonadotropin (Folligon®, eCG, Intervet-Schering-Plough Animal Health, Boxmeer, The Netherlands) and 10 IU/ml human chorionic gonadotropin (Chorulon®, hCG, Intervet/Schering-Plough Animal Health). The oocytes were then cultured for a further 20 to 22 h in the absence of eCG and hCG. Partial and complete expanded COCs were observed during 22 and 44 h, respectively, after culture (Figure 5B, C).

Table 1 Chemical composition of media used for culturing porcine oocytes (IVM) and embryos (IVC). IVM and IVC were used at the first 42 to 44 h before fertilization and for 6 to 7 days after fertilization or parthenogenetic activation

Chemical	Medium type*			
	IVM		IVC	
	NCSU23 (mM)	TCM199 (mM)	NCSU23 (mM)	SOF (mM)
TCM199 with Earle's salts	-	Commercial (Gibco®, Invitrogen, U.K.)	-	-
NaCl	108.73	-	108.73	107.6
KCl	4.00	-	4.00	7.1
$CaCl_2$. $2H_2O$	1.70	-	1.70	1.7
KH_2PO_4	1.19	-	1.19	1.2
$MgSO_4$. 7 H_2O	1.19	-	1.19	0.9
$NaHCO_3$	25.0	26.2	25.0	25.0
Na-Lactate (60% w/w)	-	-	-	5.3
Glucose	5.55	3.05	5.55	1.5
L-glutamine	1.00	0.7	1.00	2.0
Sodium pyruvate	-	0.91	-	0.3
EGF (ng/ml)	-	10	-	-
Cysteamine	-	0.1	-	-
Cysteine (mg/ml)	0.1	-	-	-
β-mercaptoethanol	10	-	-	-
Taurine	7.00	-	7.00	-
Hypotaurine	5.00	-	5.00	-
MEM NEAA (%)	-	-	1	1
BME NEAA (%)	-	-	-	2
Penicillin (IU/ml)	100	100	100	100
Streptomycin (μg/ml)	100	100	100	100
Phenol read (μg/ml)	-	-	-	0.5
pFF (v/v)	10%	10%	-	-
BSA (mg/ml)	4	-	4	4

*NCSU23, TCM199 and SOF were prepared as previously described by Petters and Wells (1993), Panasophonkul et al. (2010) and Van Wangtndonk-de Leeuw et al. (2000), respectively.

2.3.2 Embryos production

2.3.2.1 Parthenogenetic activation (PA)

After 42 to 44 h of *in vitro* maturation, expanded cumulus cells were gently removed by repeated pipetting; only metaphase II (MII) oocytes with a visibly extruded polar body (Figure 6A) were used for parthenogenetic activation. PA was performed by washing denuded MII oocytes three times in activation medium (Table 2), and then aligned 15 to 20 oocytes between two stainless steel electrodes covered by activation medium (Figure 6B) in a chamber connected to an electrical pulse generator (SD 9 Square Pulse Stimulator; Grass Technologies Ltd., Massachusetts, USA: experiment I or CF-150B Impulse Generator; BLS Ltd., Budapest, Hungary: experiment III). After activation, the oocytes were transferred and

cultured in medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h (Experiment I) or with 5µg/ml cytochalasin B (CB) for 3 h (experiment III).

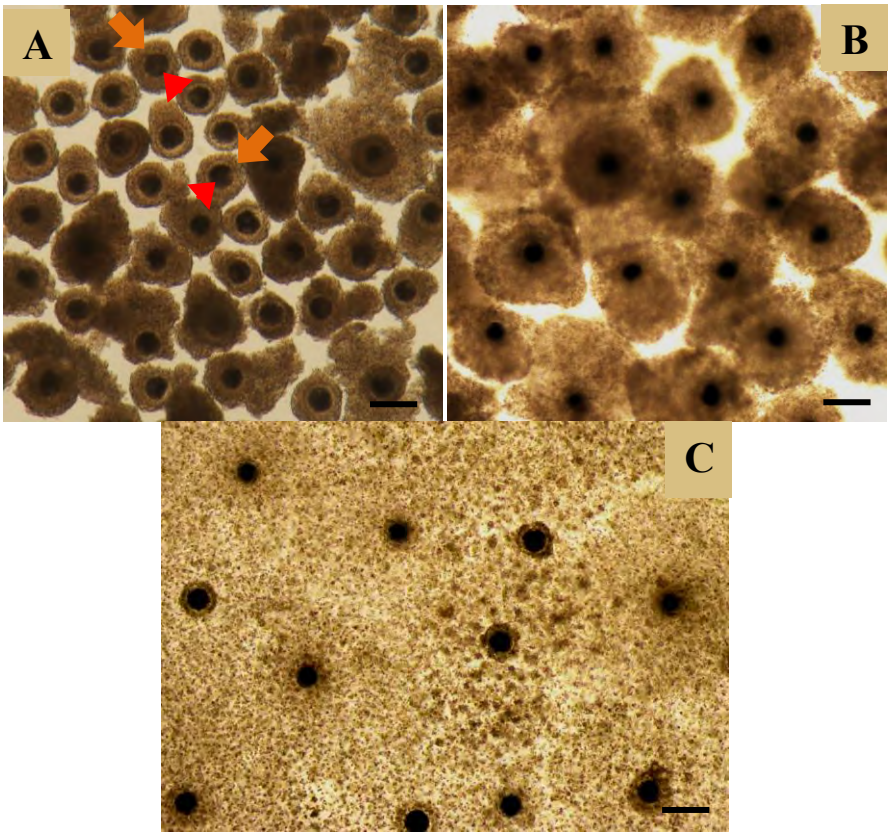

Figure 5 *In vitro* maturation of porcine oocytes. A) Cumulus oocyte complexs (COCs) with a compact cumulus consisting of at least three uniform layers of cells (arrows) and with homogeneous ooplasm (arrowheads) were selected and cultured in IVM medium I. B) COCs with a partially expanded cumulus after 22 to 24 h of culture. C) Mature oocytes with expanded cumulus after 42 to 44 h of culture in IVM II. Scale bar = 500 µm.

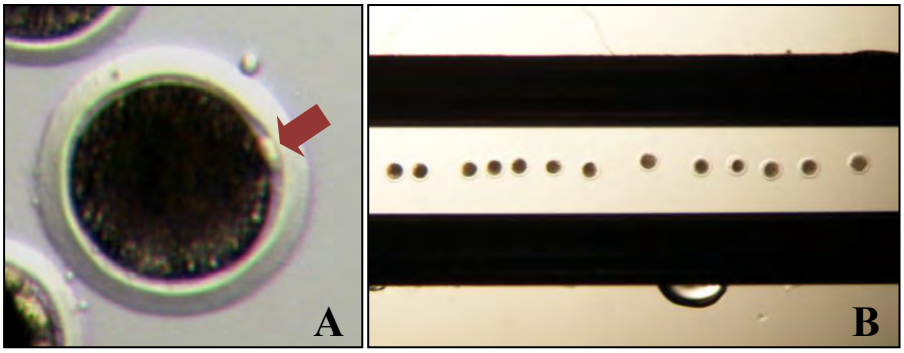

Figure 6 *In vitro* embryo production by parthenogenetic activation (PA). Matured oocyte at metaphase (M) II stage (A) after 42 to 44 h IVM. Note: the arrow indicates an extruded first polar body. Denuded MII oocytes were aligned between two stainless steel electrodes (B) covered with activation medium and stimulated with an electrical pulse generator.

Table 2 Chemical composition of activation media using for PA embryo production by electrical stimulation

Chemical (mM)	Activation medium (AM) type*	
	AM-I (mM)	**AM-II (mM)**
Mannitol	0.3	0.3
$CaCl_2.2H_2O$	0.1	0.1
MgSO4	0.1	-
$MgCl_2.6H_2O$	-	0.1
PVP	-	1 mg.ml
BSA	1 mg/ml	-
HEPES	0.5	0.5

*AM-I and AM-II were prepared as previously described by Panasophonkul et al. (2010) and Hashem et al. (2006), respectively.

2.3.2.2 In vitro fertilization (IVF)

After 42 to 44 h of IVM, expanded cumulus cells were partially removed and then washed three times with pre-equilibrated modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997) supplemented with 5 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin (IVF medium). Groups of 30 to 50 oocytes were placed into a 4-well plate containing 500 μl of IVF medium and incubated for at least 30 min before fertilization. Oocytes were then co-incubated with frozen-thawed spermatozoa for 6 h (experiment II) or with fresh spermatozoa for 20 h (experiment III) at 38.5 ºC in a humidified atmosphere of 5% CO_2-in-air. Next, remaining cumulus cells surrounding oocytes were denuded, and the fertilized oocytes were then cultured for 6 to 7 days depending on the experiment.

2.3.2.3 Sperm preparation

Frozen-thawed sperm: Frozen semen from a single boar (Yorkshire) thawed for 12 sec at 50°C was used in the experiment I. The viable-motile sperm were then selected using a Percoll gradient technique as described by Parrish et al. (1994). Briefly, the contents of semen were layered onto a discontinuous gradient of 45% (v/v) and 90% (v/v) Percoll in a 15 ml-conical tube and then centrifuged at 26 °C, 700 x g for 15 min. The sperm pellet at the bottom of the 90% Percoll fraction was slowly resuspended with 1 ml of IVF medium. After re-centrifugation for 5 min, the sperm pellets were resuspended in IVF medium, and the concentration of spermatozoa was calculated for the experiment. The sperm motility was subjectively examined at a x100 magnification under a light microscope (TS1000 Nikon, Tokyo, Japan). Percoll-treated sperm to be used for the entire experiment had more than 90% progressive motility.

Fresh sperm: Three randomly selected boars were used as sperm source for IVF in the experiment III. An equal volume of fresh extended semen and IVF medium were mixed in a 15 ml-conical and then centrifuged at 26 °C, 700 x g for 5 min. The sperm pellet was resuspended in IVF medium and re-centrifuged under the same condition. The sperm

concentration was calculated and used at 1000 sperm/oocytes. The sperm motility was subjectively assayed by Computer assisted semen analysis (CASA) software.

2.3.3 Embryo culture

Groups of 30 to 50 PA or IVF oocytes were cultured in a 4-wells plate (Nunc, NY, USA) containing with *in vitro* culture medium (NCSU23-IVC; experiment I and II or SOF-IVC; experiment III: Table 1) covered with mineral oil. Supplemented BSA in NCSU23-IVC was substituted with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, USA) between day 5 and 7 of culture. Embryos cultured in NCSU23-IVC were incubated at 38.5ºC in a humidified atmosphere of 5% CO_2-in-air, meanwhile those cultured in SOF-IVC were incubated at 38.7ºC in a humidified atmosphere containing 5% CO_2 and 7%O_2.

2.3.4 Evaluation of fertilization parameters

2.3.4.1 Assessment of sperm penetration

At 18 to 20 h after IVF, 243 of inseminated oocytes were fixed in 4% (w/v) paraformaldehyde at room temperature for 15 min, and then stained with 1 μg/ml fluorescent DNA labeling (4‘6‘ Diamidino-2-phenylindole dihydrochloride: DAPI) for 15 min. To assess the nuclear status of presumptive zygotes, fluorescently labeled oocytes were mounted on a glass microscope slide in a 2-μl droplet of anti-fade medium (Vectashield^{TM}, Vector Lab, Burlingame, CA, USA), and then examined under an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan) at x 200 and x 400 magnifications . Fertilization of porcine oocytes was classified as at least one pronucleus that had formed in the ooplasm. Monospermic fertilization/penetration typified by the presence of only one male pronucleus, while multiple pronucleus (more than 2) indicated the polyspermic fertilization.

2.3.4.2 Differential staining of blastocysts

Differential staining of the inner cell mass (ICM) and the trophectoderm (TE) cells was performed as essentially described by Thouas et al. (2001) with a minor modification. Briefly, blastocysts at 7 days of development were first incubated for 15 sec with 1% (v/v) Triton X-100, 0.01% (w/v) polyvinylpyrrolidone (PVP) and 100 μg/ml propidium iodide (Molecular Probes, Invitrogen, Oregon, USA) in PBS. Blastocysts were transferred into 25 μg/ml *bis*benzimide (Hoechst 33342, Molecular Probes) in an absolute ethanol and then maintained in this fixative at 4°C for overnight. Finally, fluorescently labeled embryos were mounted on to a glass microscopic slide in a droplet of anti-fade medium and sealed with a coverslip. The ICM and TE cell numbers were counted using an epifluorescent microscope. The ICM was classified as a group of embryonic cells that stained with only Hoechst 33342 (blue), while the TE cells were positive to both Hoechst 33342 and propidium iodide.

2.3.4.3 Whole mount immunofluorescence

Blastocysts were fixed in 4% embryo-grade paraformaldehyde (Polysciences, Inc., Warrington, PA, USA) in PBS stored at 4^{o}C until staining. Subsequently, fixed embryos were permeabilized by immersion for 15 min at room temperature in 0.5% Triton X-100 in PBS containing 10% FBS. They were then transferred into blocking solution, which comprised PBS containing 0.1% Triton X-100 and 10% FBS, for 1 h. In the case of Cdx2 staining,

permeabilized embryos were incubated in 2N hydrochloric acid (HCl) for 30 min for antigen retrieval before blocking. Primary antibodies for Oct4, Nanog, Cdx2 and GATA6 were diluted in blocking solution, and the embryos were incubated with an appropriate dilution of the first antibody (see Table 3) at 4°C overnight. They were then rinsed three times in blocking solution for 15 min each, and incubated with the corresponding secondary antibody (Table 3) for 1 h at room temperature. The nuclei were then stained with TOPRO-3 (Molecular Probes, Invitrogen) and the embryos mounted in Vectashield (Vector Lab) before observation. Fluorescent signals were visualized using a Confocal Laser Scanning Microscope (Leica TCS SP5, Wetzlar, Germany), and then reconstructions of three-dimensional images constructed from the confocal images, and cell counts, were performed using Image J software and Adobe Photoshop.

2.4 Statistical Analysis

Data were pooled from at least three independent replicates and expressed as mean ± standard error of the mean (SEM). Differences in fertilization parameters, and embryo development and quality among experimental groups were assessed using one-way ANOVAs and protected least significant different (LSD) analysis. In experiment 3, a chi-square ($\chi 2$) test was used to compare the expression of Nanog between the IVF and PA groups. All statistical analyses were performed using SPSS software (version13.0, SPSS Inc., Chicago, IL). Differences were considered to be statistically significant when $p< 0.05$.

Table 3 Primary and secondary antibodies used for whole mount immunofluorescence

Primary antibody		Secondary antibody	
Type	**Dilution**	**Type**	**Dilution**
Rabbit monoclonal anti-Oct4 (Epitomics, Burlingame, CA, USA)	1:250	Goat anti-rabbit Alexa568-conjugated (Invitrogen)	1:100
Mouse monoclonal Nanog (eBioscience, San Diego, CA, USA)	1:200	Goat anti-mouse Alexa 488-conjugated (Invitrogen)	1:100
Rabbit polyclonal (H-92) GATA6 (Santa Cruz Biotechonology, Inc., Santa Cruz, CA, USA)	1:250	Goat anti-rabbit Alexa 568-conjugated	1:100
Mouse monoclonal anti-CDX2 (Biogenex, CA, USA)	1:50	Goat anti-mouse Alexa 488-conjugated	1:100

2.5 Experimental designs

Experiment I: ***Effect of different parthenogenetic activation (PA) protocols on developmental competence and quality of embryos***

After 44 h of IVM, matured MII oocytes were selected and randomly assigned to stimulate with three different activation protocols as protocol I: two 30-μsec direct current (DC) pulses of 1.36 kV/cm; protocol II: two 60-μsec DC pulses of 1.5 kV/cm and protocol III: three 80-μsec DC pulses of 1.0 kV/cm. The developmental competence in terms of the

formation rates of cleavage and blastocyst was evaluated at 48, 120, 144 and 168 h after culture, respectively. The blastocyst quality was assessed by a presence of the cell numbers of ICM and TE.

Experiment II: Effect of sperm:oocyte ratio during IVF on normal (monospermic) fertilization and embryo development

After 44 h of IVM, TCM199-matured oocytes were fertilized with three different sperm:oocyte ratios (1000:1, 2000:1, and 4000:1) by using frozen-thawed sperm. Some part of presumptive embryos were then fixed at 18 to 20 h after fertilization for investigating pronuclear formation and the rest were further cultured for 7 days in order to determine their developmental competence in terms of cleavage and blastocyst rates at 48 and 168 h after culture, respectively. The blastocyst quality was assessed by a presence of the cell numbers of ICM and TE.

Experiment III: ***Effect of MEK and GSK-3 inhibitors on developmental competence and quality of embryos derived from IVF and PA***

After 42 h of IVM in NCSU23, COCs were either fertilized with fresh sperm at a concentration of 1000 sperm/oocyte for 20 h or parthenogenetically activated by two 80-μsec 0.85 kV/cm DC pulses followed by exposure to cytochalasin B (CB) for 3 h. Subsequently, presumptive zygotes were randomly cultured in SOF-IVC supplemented with 0.5 μM PD0325901 MEK (BioVision, California, U.S.A.) and/or 3.0 μM CHIR99021 GSK-3 (Stemgent, San Dieago, CA, U.S.A.) inhibitors as group I, control; group II, +MEKi; group III, +GSK-3i; and group IV, 0.5 μM MEKi and 3.0 μM GSK-3i (2i). Cleavage and blastocyst rates were evaluated at 48 and 144 h of culture, respectively and the developmental stages of blastocyst were also recorded. Blastocyst quality, in terms of ICM and TE cell numbers, and the localization of Oct4, Nanog, Cdx2 and Gata6 were examined by immunofluorescense microscope.

2.6 Results

2.6.1 *Experiment I: Effect of different parthenogenetic activation (PA) protocols on developmental competence and quality embryos*

After 44 h of IVM, 83.6% (468/560) of oocytes exhibited the 1st polar body extrusion (MII stage). As shown in Table 4, oocytes activated with protocol II and III had a significant higher rate of cleaved embryos than those activated with protocol I (77.2% and 82.4% vs 34.5%, respectively; $p< 0.05$). PA of protocol III gave the highest blastocyst rate of 28.9% and 29.9% on day 6 and 7 of culture, respectively. These values were also significantly higher than those obtained from protocol I (6.9% in both periods) and protocol II (21.4% and 22.5%; $p<0.05$). Although there was no statistical difference in the mean number of blastocyst cells ($p>0.05$) among protocols, the embryos activated by protocol II and III appeared to have a higher ICM cells number than those activated by protocol I (Table 5)

Table 4 Effect of different activation protocols on *in vitro* development of porcine parthenogenetic embryo

Treatment	No. of activated oocytes	No. of cleaved embryos (%mean±SEM)	No. of blastocysts (%mean±SEM)		
			Day 5	Day 6	Day 7
Protocol I	167	56(34.5±2.9)[a]	7(4.3±0.7)[a]	11(6.9±1.0)[a]	11(6.9±1.0)[a]
Protocol II	147	115(77.2±4.8)[b]	20(13.5±2.8)[b]	32(21.4±2.8)[b]	34(22.5±3.1)[b]
Protocol III	154	128(82.4±4.9)[b]	30(18.3±3.8)[b]	45(28.9±2.1)[c]	46(29.9±1.5)[c]

Protocol I: 1.36 kV/cm, 30 µsec, 2 pulses; Protocol II: 1.5 kV/cm, 60 µsec, 2 pulses; Protocol III: 1.0 kV/cm, 80 µsec, 3 pulses
[a, b, c] within a column, different superscripts denote values that differ significantly (p<0.05).

Table 5 Effect of different oocyte activation protocols on blastocyst quality

Treatment	No. of blastocysts	No. of cells (mean±SEM)			ICM : Total (%mean±SEM)
		ICM	TE	Total	
Protocol I	**11**	9.0±1.2	41.3±4.4	50.3±5.2	18.0±1.9
Protocol II	**23**	9.0±0.5	42.0±2.2	51.0±2.5	17.8±0.6
Protocol III	**22**	9.3±0.5	40.0±2.0	49.3±2.2	19.1±1.1

Protocol I: 1.36 kV/cm, 30 µsec, 2 pulses; Protocol II: 1.5 kV/cm, 60 µsec, 2 pulses; Protocol III: 1.0 kV/cm, 80 µsec, 3 pulses; ICM: inner cell mass and TE: trophectoderm.

2.6.2 Experiment II: Effect of sperm:oocyte ratio during IVF on normal (monospermic) fertilization and embryo development

2.6.2.1 Fertilization rate, monospermic penetration and efficiency of fertilization

There was a significant effect of sperm:oocyte ratio on penetration rate, monospermy, and the efficiency of fertilization (Table 6). Our results showed that sperm penetration rate significantly increased when the oocytes were fertilized with 2000 (90.23±2.5%) and 4000 (93.46±3.7%) sperm: oocyte, compared with those fertilized with 1000 (74.08±1.2%) sperm:oocyte (p<0.05). However, the increase in sperm penetration rate was associated with a high rate of polyspermy since the oocytes inseminated with 1000 sperm per oocyte had a significant higher rate of monospermic zygotes (81.79±2.9 %) than those inseminated with 2,000 and 4,000 sperm per oocyte (48.07±6.0 and 31.51±4.9%, respectively; p<0.05, Figure 7). The efficiency of fertilization also was significantly increase when amount of spermatozoa at 1000 per oocyte was used, compared to those at 4000 sperm per oocyte (60.64±2.7 versus 29.36±4.8%; p<0.05). However, the fertilized efficiency was not significantly different between 2000 and 4000 sperm: oocyte groups.

Table 6 Effect of sperm:oocyte ratios on fertilization parameters during IVF of pig oocytes matured *in vitro*

Ratio	No. of inseminated oocytes	Percentage of oocytes (mean±SEM)		% Efficiency** (mean±SEM)
		Penetrated	Monospermic*	
1,000:1	79	74.08±1.2^{a}	81.79±2.9^{a}	60.64±2.7^{a}
2,000:1	79	90.23±2.5^{b}	48.07±6.0^{b}	43.96±6.9^{a,b}
4,000:1	85	93.46±3.7^{b}	31.51±4.9^{c}	29.36±4.8^{b}

* Percentage of the number of monospermic oocytes/total of penetrated oocytes; ** percentage of the number of monospermic oocytes/total of inseminated oocytes.
[a, b, c] within a column, different superscripts denote values that differ significantly (p<0.05 at least).

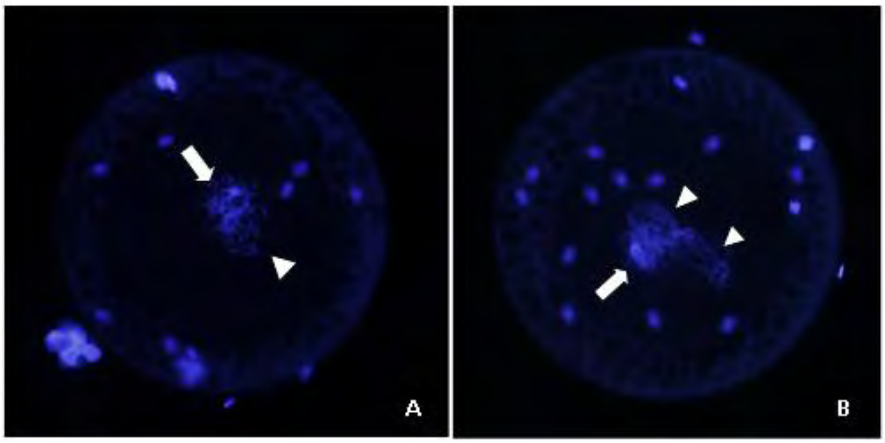

Figure 7 Pronuclear formation of fertilized oocytes at 18 to 20 h post-IVF. **A)** Monospermic fertilization typified by the presence of only one male (arrowhead) and one female pronucleus (arrow) while polyspermic fertilization; B) presents more than one of male pronucleus (arrowheads). (x200).

2.6.2.2 Embryo development and cell number of blastocysts

Cleavage and blastocyst development was observed at 48 and 168 h after IVF, respectively. As shown in Figure 8, the sperm:oocyte ratio during IVF did not affect cleavage rate, while blastocyst rate increased significantly (p<0.05) in the group fertilized with 1,000 sperm:oocyte (29.02±1.8%) compared to those of 4,000 sperm:oocyte (14.00±3.0%). Moreover, we examined the quality of blastocysts in terms of the mean number of cells including ICM and TE. Although there was no statistical difference in the mean number of blastocyst cells (p>0.05) among experimental groups, the blastocysts derived from oocytes inseminated with 1000 sperm:oocyte had a higher tendency of ICM cells number than those obtained from the higher sperm:oocyte ratios (Table 7).

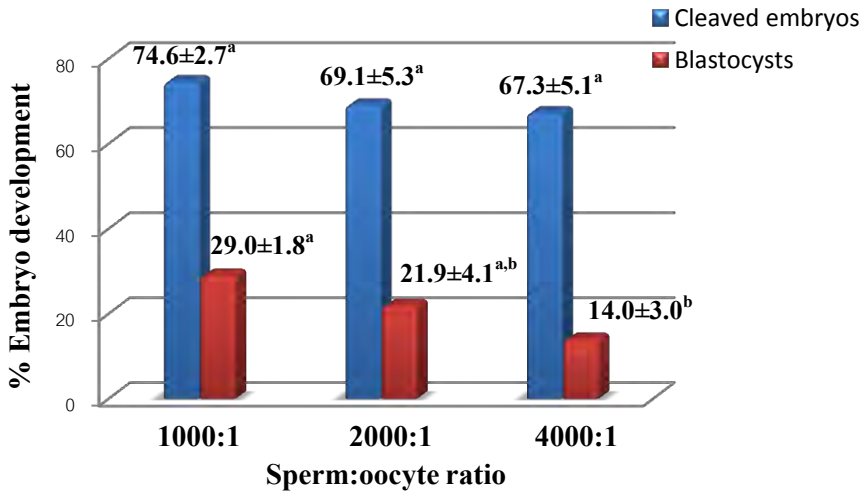

Figure 8 Effect of sperm:oocyte ratios on developmental competence of embryos at cleavage and blastocyst stages after IVF

Table 7 Effect of different sperm:oocyte ratios on the cell numbers of blastocysts

Sperm:oocyte ratio	No. of blastocysts	No. of cells (mean±SEM)			ICM : Total (%mean±SEM)
		ICM	TE	Total	
1000:1	14	10.6±0.6	40.5±2.2	51.1±2.7	20.7±0.8
2000:1	10	9.6±0.7	38.5±2.4	48.1±2.9	19.9±0.7
4000:1	10	9.2±0.6	39.1±2.8	48.3±3.2	19.2±0.8

ICM: inner cell mass, TE: trophectoderm

2.6.3 Experiment III: Effect of MEK and GSK-3 inhibitors on developmental competence and quality of embryos produced by IVF and PA

2.6.3.1 Developmental competence of embryos

Cleavage and blastocyst development were observed at 48 and 144 h, respectively, after IVF and PA. As shown in Table 8, treatment with MEK and/or GSK-3 inhibitors did not affect the percentage of PA oocytes that cleaved or yielded blastocysts. By contrast, the cleavage rate increased significantly (p<0.05) for IVF embryos treated with a MEK inhibitor (MEKi; 85.41±0.6) or the combination of a MEK and a GSK-3 inhibitors (2i; 87.12±2.7) compared to those cultured in GSK-3 inhibitor (GSK-3i; 72.42±4.7) alone or in control condition (65.61±1.9). In addition, oocytes fertilized by IVF and cultured in medium supplemented with MEKi showed a significantly higher rate of blastocyst development than other IVF groups (p<0.05).

We also evaluated the stage of development at 144 h. In this respect, there was no statistical difference in the percentages of embryos produced by PA at the various stages of blastocyst development after 144 h except for the expanded blastocyst (ExB) category. A percentage of developed ExB after MEKi treatment was significantly higher than those

Table 8 Developmental competence of oocytes activated by IVF (upper) or PA (lower) and treated with MEK and/or GSK-3 inhibitors (i) during embryo culture

Experimental group	No. of activated oocytes (n)	No. of cleaved embryos (%)	No. of blastocysts (%)
Control	160	104 (65.61±1.9) ^{a}	50 (31.46±2.4) ^{a}
MEKi	149	127 (85.41±0.6) ^{b}	60 (40.97±3.3) ^{b}
GSK-3i	166	119 (72.42±4.7) ^{a}	55 (32.74±1.8) ^{a}
2i	171	150 (87.12±2.7) ^{b}	56 (32.90±1.6) ^{a}
Control	126	103 (80.10±5.1)	52 (37.73±2.2)
MEKi	154	124 (81.95±5.1)	60 (39.81±3.3)
GSK-3i	154	133 (85.70±5.4)	54 (35.50±3.4)
2i*	155	144 (92.53±1.6)	67 (42.80±1.8)

*Combination of MEK and GSK-3 inhibitors

$^{a, b}$ within a column, different superscripts denote values that differ significantly (p<0.05)

Table 9 The percentage of IVF (upper) and PA (lower) embryos at various stage of blastocyst development after treatment with MEK and/or GSK-3 inhibitors (i)

Experimental group	Percentage of embryos at different developmental stages (Mean±SEM)			
	EB	**B**	**ExB**	**HB**
Control	7.2±3.7 ^{a}	39.08±3.7	43.5±7.9^{a}	10.23±5.4^{a}
MEKi	19.14±4.6^{a,b}	38.8±4.2	31.06±2.5^{a,b}	11.10±2.9^{a}
GSK-3i	21.87±6.8^{a,b}	49.59±4.4	28.53±2.5^{b}	0^{b}
2i	29.59±2.4 ^{b}	49.67±4.5	20.74±2.1^{b}	0^{b}
Control	18.69±1.7	43.54±4.7	26.89±2.0^{a,b}	10.87±5.5
MEKi	8.14±2.8	37.5±12.6	28.97±6.4^{a}	22.34±5.6
GSK-3i	19.97±7.7	59.85±8.8	17.17±4.9^{a,b}	6.35±6.3
2i*	20.40±9.2	52.53±15.6	14.54±1.1^{b}	12.52±6.6

*Combination of MEK and GSK-3 inhibitors

EB= early blastocyst; B= blastocyst; ExB= expanded blastocyst; HB= hatched blastocyst

$^{a, b}$ within a column, different superscripts denote values that differ significantly (p<0.05)

cultured in 2i (28.97±6.4 vs 14.54±1.1; p<0.05). For IVF-derived embryos, the percentage of embryos classified as EBs after 144 h was higher for those incubated in 2i condition (29.59±2.4) than for those cultured in control conditions (7.2±3.7; p<0.05, Table 9). In addition, a higher percentage of embryos cultured in control conditions were classified as

ExB (p<0.05) than those treated with GSK-3i or 2i (43.5±7.9 vs 28.53±2.5 and 20.74±2.1%, respectively). The percentage of hatched blastocysts (HB) was significantly higher in the MEKi-treated and control groups (11.10±2.9 and 10.23±5.4; p<0.05, respectively) than in the GSK-3i and 2i group in which HBs were never observed.

2.6.3.2 Inhibition of MEK pathways and GSK-3 affects ICM cell number and pluripotency state

Blastocyst quality in terms of the mean number of ICM and TE cells was investigated via expression of Oct4 and Cdx2 proteins. We utilized specific antibodies raised against Oct4 to identify ICM cells, and Cdx2, which is specific for TE cells. As shown in Table 10, both IVF- and PA-derived blastocysts cultured in medium supplemented with MEKi had a significantly (p<0.05) higher number of ICM cells (7.69±0.6 and 7.0±0.5) and ICM:TE ratio (17.81±2.1 and 14.96±1.1) than the other experimental groups, although there was no significant difference (p>0.05) in the mean number of TE cells among experimental groups.

Table 10 Effect of MEK (MEKi) and GSK-3 (GSK-3i) inhibitors on the inner cell masses (ICMs) and trophectoderm (TE) cell numbers in blastocysts developed via IVF (upper) and PA (lower)

Experimental group	No. of blastocysts (n)	Cell number (Mean±SEM)		ICM:TE (%)
		ICM	TE	
Control	19	5.26±0.8^a	48.74±4.0	10.46±1.2^a
MEKi	23	7.69±0.6^b	48.22±4.0	17.81±2.1^b
GSK-3i	18	4.55±0.6^a	47.72±2.9	9.66±1.1^a
2i*	22	5.68±0.6^a	48.14±3.6	12.22±1.3^a
Control	25	5.32±0.5^a	47.32±2.9	11.91±1.2^a
MEKi	22	7.0±0.5^b	46.54±2.5	15.21±1.1^b
GSK-3i	20	4.1±0.5^a	43.85±3.3	9.30±0.8^a
2i*	21	4.62±0.4^a	45.52±2.3	10.42±0.9^a

*Combination of MEK and GSK-3 inhibitors

$^{a, b}$ within a column, different superscripts denote values that differ significantly (p<0.05)

Oct4 protein was expressed in all cells of blastocysts produced either by IVF or by PA in all experimental groups, whereas the expression of Cdx2 was restricted to TE cells (Figure 9). We therefore further evaluated the effect of these inhibitors on the localization of Nanog, specific to the primitive ectoderm lineage, and Gata6, which should be specific to primitive endoderm cells. Nanog and Gata6 proteins were detectable in both IVF- and PA-produced porcine blastocysts of all experimental groups. Nanog expression was restricted to a sub-set of ICM cells, whereas Gata6 protein could be detected in subsets of both of ICM and TE cells (Figure 10). Interestingly, the significant existence of Nanog protein was found when blastocysts had been cultured in MEKi compared with the other groups. When IVF- and PA-derived blastocysts were compared, the number of Nanog-positive embryos was significantly higher (p<0.05) for IVF oocytes cultured in medium supplemented with 2i than PA oocytes in the same medium (60 vs 14.28%, respectively; Figure 11).

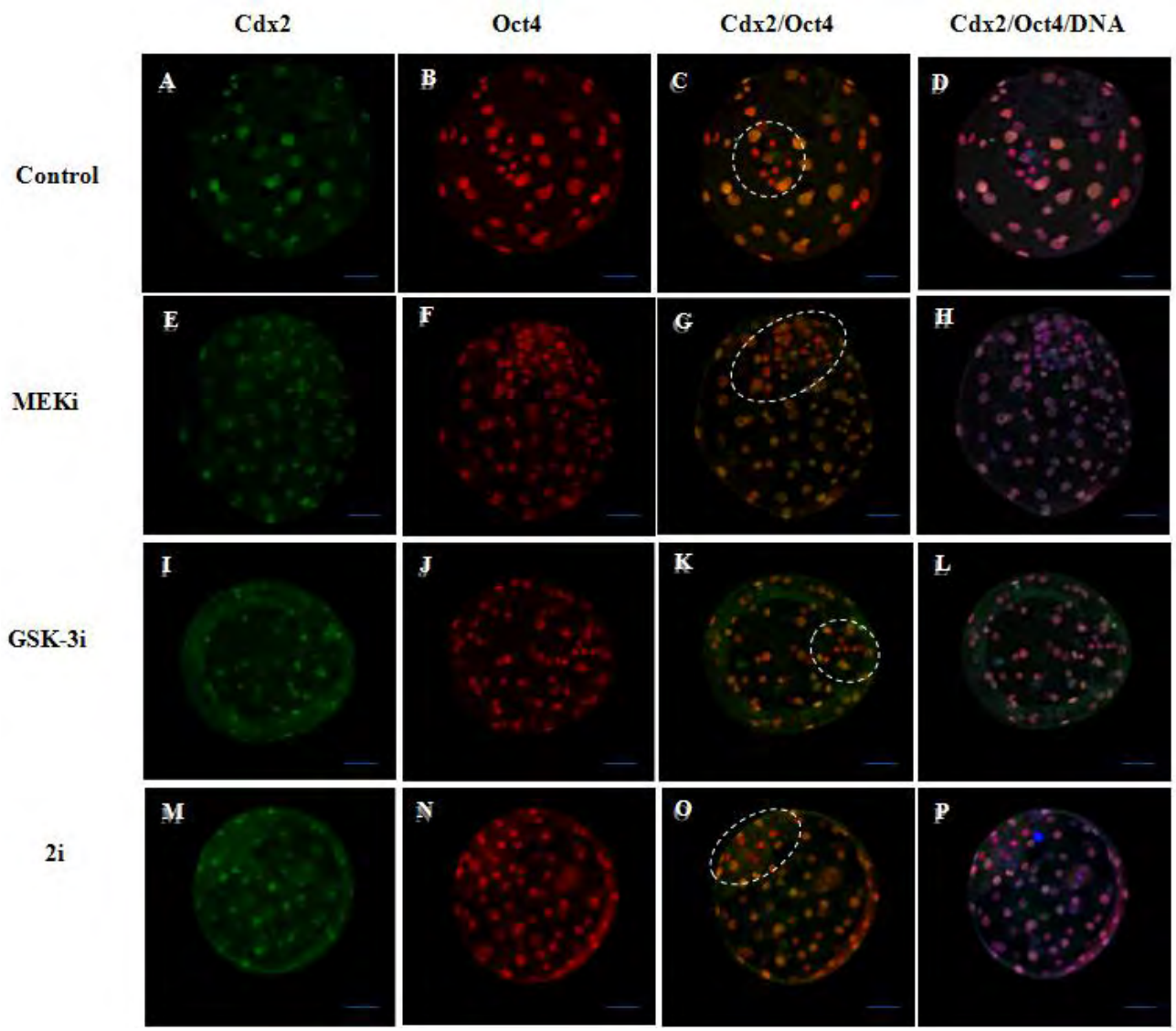

Figure 9 Immunofluorescent staining for Cdx2 and Oct4 of IVF blastocysts exposed to MEK and/or GSK-3 inhibitors. Representative images (from left to right) show Cdx2 protein expression exclusively in TE cells, Oct4 protein expression in both ICM and TE cells, the overlay of Cdx2 and Oct4 expression, and an overlay of Cdx2 and Oct4 together with a DNA stain (TOPRO), (A-D) non-treated embryos (control), (E-H) MEKi-treated embryos, (I-L) GSK-3i-treated embryos, (M-P) 2i-treated embryos. Dashed line denotes the ICM cluster (red). MEKi=MEK inhibitors; GSK-3i=GSK-3 inhibitor; 2i= combination of MEKi and GSK-3i. Scale bar = 50 μM.

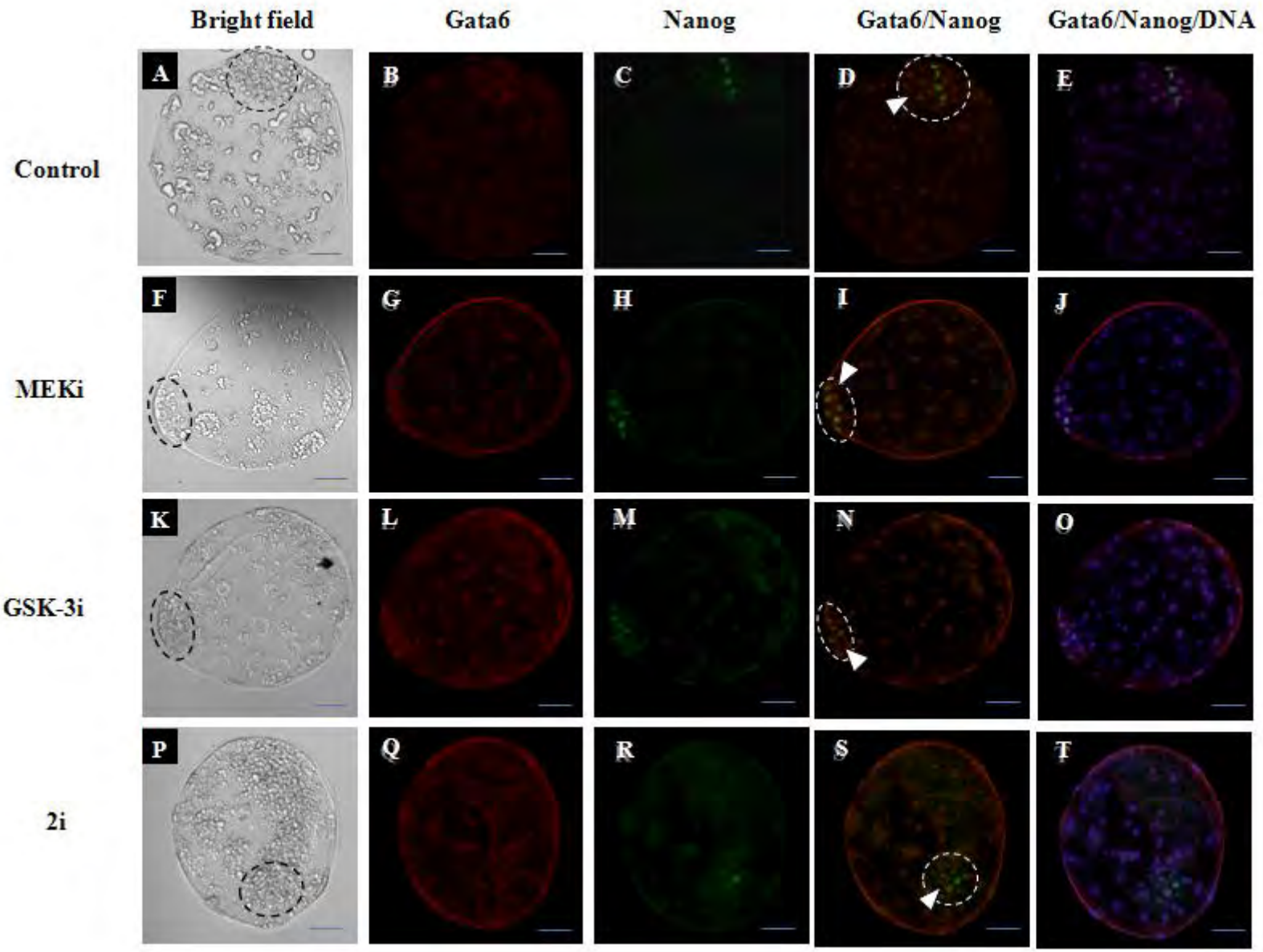

Figure 10 Immunofluorescent staining for Gata6 and Nanog on IVF blastocysts treated with or without MEK and/or GSK-3 inhibitors. Represent images (from left to right columns) show embryos with Gata6 protein expression in both ICM and TE, Nanog protein expression exclusively in the ICM, the overlay of Gata6 and Nanog expressions and an overlay of Gata6, Nanog and DNA staining. A-E) non-treated embryos (control); F-J) MEKi-treated embryos; K-O) GSK3i-treated embryos; P-T) 2i-treated embryos. Dashed line denotes ICM cluster. Arrowhead=Nanog negative ICM cells. MEKi=MEK inhibitors; GSK3i=GSK3 inhibitor; 2i=combination of MEKi and GSK-3i. Scale bar = 50 μM.

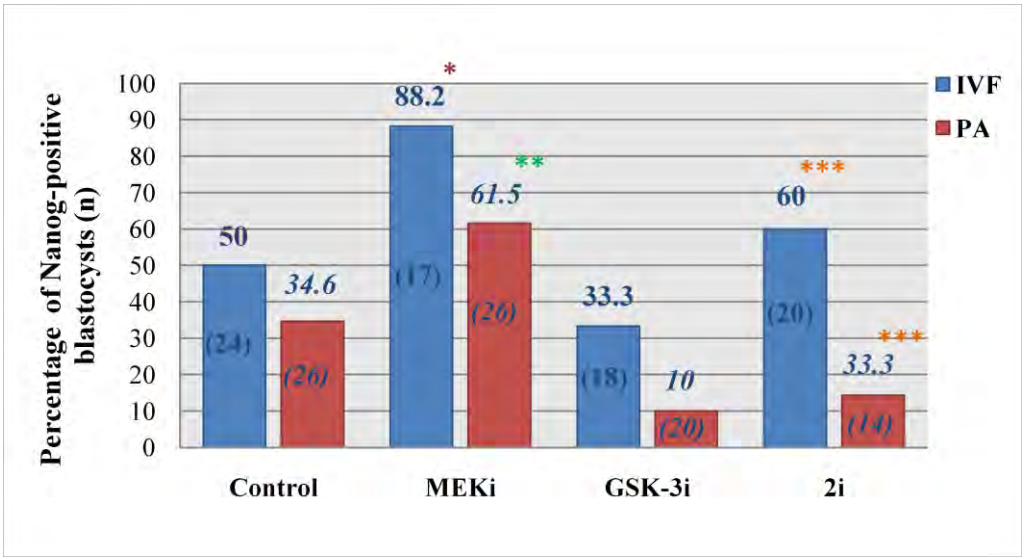

Figure 11 Comparison of percentage of Nanog positive blastocysts in IVF- and PA-derived blastocysts treated with MEK or GSK-3 inhibitors. * significant difference within IVF group; ** significant difference within PA group; *** significant difference between IVF and PA groups ($p<0.05$). MEKi = MEK inhibitor; GSK-3i = GSK-3 inhibitor; 2i = the combination of MEK and GSK-3 inhibitors

2.7 Discussion

Several IVP techniques, particularly IVF and PA, have widely been used to produce a large number of porcine embryos *in vitro*. Their efficiencies for producing a great number of high quality embryos, however depend on a method used. In the present study, different parthenogenetic activation (PA) protocols (experiment I) and different ratios of sperm number per oocyte during IVF (experiment II) were examined in order to find an optimal condition for further porcine ES cell lines establishment. The results demonstrated that PA and IVF porcine embryos can be produced *in vitro* with the high rates of their development and quality by using our culture systems.

Embryo production by activating with SD9 pulse stimulator can induce transient formation of membrane pores that allow the exchange of extra- and intra-cellular calcium (Zimmermann and Vienken, 1982), a process by which mimics the fertilization process. It was found that the oocytes having been activated by the lowest field strength of 1.0 kV/cm with 3 pulses at 80 µsec (protocol III) gave the highest potential of embryo development when compared to those by 1.36 kV/cm, 30 µsec, 2 pulses (protocol I) and 1.5 kV/cm, 60 µsec, 2 pulses (protocol II). This activation protocol gave a high rate of embryo development (30% blastocyst rate) similar to previous report by Zhu et al. (2002) who demonstrated that the developmental rate of PA blastocysts was optimal when three 80 µsec consecutive DC pulses of 1.0 kV/cm were used. However, a large number of oocytes underwent degeneration when a single pulse of higher field strength (1.5 kV/cm) was applied (Kure-bayashi et al., 2000). It is likely that too low field strength (less than 1.2 kV/cm) and low pulse numbers are insufficient to increase calcium influx to the level needed for oocyte activation (Collas et al., 1993). In contrast, multiple pulses of high field strength (more than 1.2 kV/cm) have also been to be detrimental to the oocyte development (Lee et al., 2004). It is therefore obviously demonstrated that there is an interaction between pulse numbers and duration that affects the

developmental competence of electrical activated oocytes (Prather et al., 1989; Ozil and Huneau, 2001; King et al., 2002). In this experiment, we found that activation by three pulses at 80 μsec (protocol III) increased more blastocyst formation rate than those activated by two pulses either at 30 or 60 μsec (protocol I and II, respectively). It could be due to the use of field strengths less than 1.2 kV cause to be less damaging to the oocyte plasma membrane and a combination of longer pulse duration and lower field strength could increase the influx of Ca^{2+} similar to that occurs during *in vivo* fertilization.

When IVF was carried out with different sperm:oocyte ratios for 6 h, our results demonstrated that sperm: oocyte ratio at *in vitro* fertilization affects the efficiency and quality of embryos. A decreasing sperm:oocyte reduced the percentage of polyspermy and also improved the developmental competence. This work is correlated with previous studies reported that excessive numbers of spermatozoa at the time of fertilization increase the percentage of polyspermic penetration (Rath, 1992; Xu et al., 1996[b]; Gil et al., 2007), thereby impairing embryo development. Frozen-thawed semen from a single donor, in the current study, was treated with Percoll gradient density in order to minimize boar-to-boar and sperm quality variations during the experiments. Percoll treatment has been reported to improve sperm viability and quality in bovines (Somfai et al., 2002; Mendes et al., 2003) and porcines (Jeong and Yang, 2001; Matas et al., 2003).

The rates of sperm penetration were significantly enhanced by increasing the ratios of sperm number from 2000 to 4000 per oocyte, compared with the 1000 sperm per oocyte group. However, it found that the monospermic penetration rate (81.79%) increased significantly when partial cumulus-enclosed oocytes were co-incubated with 1000 sperm per oocyte. These results were similar to previous reports indicating that a relative reduction of spermatozoa numbers during *in vitro* fertilization results in greater monospermic penetration rates (Xu et al., 1996[b]; Abeydeera and Day, 1997; Gil et al., 2004). Rath (1992) revealed a high correlation between polyspermy and the absolute number of spermatozoa and oocytes but not between the polyspermy rate and sperm concentration per milliliter. Our results obtained a higher incidence of monospermy (82%) when 1000 sperm per oocyte were used for IVF compared with 50-60% from other studies. The observed differences may have been caused by many factors such as sperm quality (Popwell and Flowers, 2004; Gadea, 2005) and the inter- and intra-boar variability (Suzuki et al., 2003). In addition, suboptimal oocyte maturation and IVF systems caused by culture medium and/or sperm incubation time have also been demonstrated to involve polyspermic penetration (reviewed by Funahashi, 2003). The mechanism preventing the multiple sperm entry, until recently, has not been well understood. It has been demonstrated that normal distribution of intracellular organelles of mitochondria and cortical granules (CG) during IVM plays an important role in preventing polyspermy (Cran and Cheng, 1986; Grupen et al., 1997). More specifically, the CG contents released into the peri-vitelline space (PVS) of the fertilized oocyte induce hardening of the zona pellucida (ZP), thereby preventing polyspermy. In this respect, a delay and incomplete exocytosis of CG due principally to poor cytoplasmic maturation causes an improper pattern of cortical distribution and exocytosis (Wang et al., 1997[b]). In addition, the forming of narrowed PVS in *in vitro* matured oocytes caused by culture medium may also interfere with the distribution of CG contents and could also delay the zona block (Funahashi et al., 1994).

In this experiment, we did not found a difference in the cleavage rates among the

three different sperm:oocyte ratios. However, the number of cleaved embryos that developed to blastocyst stage was significantly higher in oocytes fertilized with the lowest sperm:oocyte (1000:1) ratio than those fertilized with greater sperm:oocyte (4000:1) ratio (29 and 14%, respectively). These observations were similar to a study demonstrating that fewer than 20% of inseminated oocytes developed to blastocyst stage when a high sperm concentration was used (Koo et al., 2005). In respect of embryo quality, our results demonstrate that there was no statistic difference in TE and ICM cell numbers among experimental groups of both PA- and IVF-obtained blastocysts. It is probably that the different quality of pig embryos produced *in vitro* is more influenced by culture medium used for embryo culture rather than IVP technique (Nánássy et al., 2008). Furthermore, it is also likely that embryos have intrinsic ability to control the optimal number of ICM probably via programmed cell death (Hardy et al., 2003) and gene regulating pathways (Marikawa and Alarcón, 2009), affecting ICM segregation and self-renewal.

We further investigated the role of small-molecule inhibitors of the MEK/ERK (PD0325901) and GSK-3 (Chir99021) pathways on the developmental competence of porcine oocytes and quality of pig embryos produced *in vitro*. Our results demonstrated that treatment with MEK and GSK-3 inhibitors during IVP affects the early development and quality of porcine embryos in terms of the cell number and expression of cell lineage specific proteins in ICM and trophectoderm (TE) cells. Oocytes fertilized or activated by IVF and PA were able to cleave and develop to the blastocyst stage during culture in medium containing MEKi and/or GSK-3i at similar rates to control conditions. This supports a previous study in which these inhibitors were reported not to disrupt the formation of trophoblast and ICM cells in mouse embryos (Nichols et al., 2009[b]). We also found that PA tended to yield higher embryo development rates than IVF (Table 8). This may be because differences in method and procedures between IVF and PA affect the developmental competence of the respective oocytes/zygotes. An improvement in the cleavage rate was also observed for IVF-derived embryos treated with MEKi and 2i compared to those treated with GSK-3i or cultured in control conditions, whereas, the blastocyst rate was significantly increased only in the MEKi group. Although, differences in the blastocyst rate were not observed between experimental groups for PA-derived embryos, MEKi and 2i-treatment tended to yield more blastocysts than the other treatments. These findings suggest that suppression of ERK signaling by MEKi may affect pathways regulating embryo development. Activation of the Phosphatidyl Inositol 3 Kinase (PI3K) pathway has been indicated as an important pathway controlling mammalian embryo development during the pre-implantation period in addition to MEK/ERK pathway (Riley et al., 2005; O'Neill, 2008). The PI3K pathway can be activated via Gab1 and PI3 kinase-regulatory subunit p85; alternatively, Ras proteins, a principal mediator in ERK pathway, can also activate PI3K pathway through the GTP-GDP exchange factor SOS (Rodriguez-Viciana et al., 1994; Figure 4). Nevertheless, the activation of the MEK/ERK pathway seems to be lesser important to early embryo development because previous data indicated that mouse embryos lacking the Erk1 and Erk2 genes are viable up to implantation (Pages et al., 1999; Hatano et al., 2003). It is, therefore, more likely that a mechanism downstream of Ras signaling is upregulated by inhibiting the activation of MEK/ERK signaling, leading to the enhancement of PI3K activity promoting embryo development. Contrarily to MEKi, the presence of GSK-3 inhibitor (GSK-3i) during culture appeared to

delay blastocyst development in terms of the percentage reaching the expanded and hatched blastocyst stages (Table 9). It is possible that inhibiting the GSK-3 pathway leads to accumulation of beta-catenin that activates Wnt signaling thereby resulting in a retardation of embryo development. This hypothesis is supported by a previous study by Li et al. (2005[a]) which indicated that inhibition of the GSK-3 pathway with lithium choline (LiCl) decreased the blastocyst hatching rate.

The relative proportions of ICM and TE cells were investigated by the expression of Oct4 and Cdx2, respectively. Both IVF and PA embryos cultured in MEKi alone showed a higher number of ICM cells and a higher ICM:TE ratio than in other groups (Table 10). This findings resembles a previous study in mouse embryos in which it was demonstrated that the naïve epiblast, a subset of ICM cells, can develop and expand in conditions in which FGF/ERK signaling is inhibited (Nichols et al., 2009[b]). However, no reduction in TE cell number was observed in the current study. The number of ICM cells tended to decline when embryos were cultured with GSK-3i alone. This may relate to the retardation of embryo development by this inhibitor. In addition, embryos cultured in 2i (a combination of MEK and GSK-3 inhibitors) did not differ from untreated controls, suggesting that activation of the Wnt signaling pathway may not be implicated in porcine embryo development during the preimplantation period. In the present study, presumptive pluripotent cells located in the ICMs of *in vitro*-produced D6 porcine embryos expressed Oct4 protein, however Oct4 was not specific to the ICM but was also detected in some TE cells (Figure 3). This resembles previous studies that demonstrated the expression of Oct4 in TE and suggest that Oct4 is not involved in the segregation between TE and ICM in the pig (Kirchhof et al., 2000; Kuijk et al., 2008; Hall et al., 2009). On the other hand, Cdx2 was restricted to TE cells suggesting a role for this factor in the formation of porcine TE. The localization of Nanog and Gata6, which are specific for epiblast and PE lineages in the mouse, were also investigated in this study. We found some Nanog-positive cells in blastocysts derived from IVF and PA, and all experimental groups (Figure 4). This is contrast with previous studies that failed to detect Nanog protein expression in *in vitro* or *in vivo* porcine blastocysts (Kuijk et al., 2008; Hall et al., 2009). On the other hand, high Nanog mRNA expression levels have been reported in D6 and D7 *in vivo* and *in vitro* porcine blastocysts (Kumar et al., 2007; Magnani and Cabot, 2008). It is therefore possible that the gene transcription and translation processes may be influenced by the quality of embryos from different conditions. In this study, we found that the number of Nanog-positive embryos was significant increased by treatment with MEKi. However, the number of Nanog-positive cells were similar among MEKi, GSK-3i and 2i conditions, although they tended to be higher in all treatment groups than in control embryos derived by either IVF (3.9, 4, 3.8 vs 2.4) or PA (4.3, 3, 3 vs 2) (data not shown). These findings suggest that both suppression of ERK signaling by MEKi and accumulation of β-catenin as promoted by GSK-3i may increase the number of pluripotent (ICM) cells in porcine embryos.

Gata6 expression could be detected in sub-sets of both of some ICM and TE cells in all conditions including controls. Previously it was reported that expression of Gata6 could be detected in some ICMs cells of *in vivo*, but not *in vitro*, derived blastocysts (Kuijk et al., 2008). In mice, Gata6 presence is thought to indicate the formation of the primitive endoderm (hypoblast) lineage, and can be eliminated by cultivating mouse embryos in medium

containing 2i (Nichole et al., 2009). However, elimination of presumptive hypoblast cells (Gata6 positive) was not promoted in our porcine embryos by culture in 2i. It is likely that the timing (developmental stage) of the translation and transcription processes for genes involved in cell fate decisions (e.g. ICM segregation) differs between species. In mice, the ICM differentiates into both the hypoblast and the epiblast between days 3.5 and 4.5 of development, whereas, in the pig, the ICM forms at around days 5 to 6 of development, while epiblast formation is not initiated until the blastocyst begins hatching between days 6 and 7 of development (Vejlsted et al., 2006). Furthermore, differences in embryo origin (*in vivo* vs. *in vitro*) should also be considered as a factor influencing the timing of gene and protein expression.

Our study demonstrates to three main conclusions. Firstly, we show that an electrical stimulation can be alternatively used to produce parthenogenetic embryos and the high blastocysts formation rate was obtained when three 80-msec consecutive pulses of 1.0 kV/cm were used. Secondly, we show that optimization of sperm:oocyte ratio during *in vitro* fertilization improves fertilization rates and, in particular, monospermic penetration and the quality of blastocysts. Nevertheless, other factors involved in the IVM-IVF system should be considered to increase the success rates of porcine IVF. Lastly, we show that the use of selective inhibitor of MEK/ERK signaling and activation of Wnt pathways such as PD0325901 MEK, and CHIR99021 GSK-3 inhibitors can promote ground state pluripotency in the porcine embryos produced by IVP techniques. This approach may increase a chance for establishment of stable ES cell lines in pigs. However, the true signaling pathways regulating pluripotent self-renewal in porcine ES cells remain largely unknown.

CHAPTER III

ESTABLISHMENT OF PORCINE EMBRYONIC STEM (pES)-LIKE CELL LINES FROM PA-, IVF-, AND *IN VIVO*-PRODUCED EMBRYOS

3.1 Abstract

To create porcine (p) ES-like cell lines, optimization of embryos source remains one of the key factors essential for isolation and culture of these cells. The present study was, therefore, undertaken to determine the efficiency of pES-like production from IVF and PA blastocysts produced by using the optimal protocol resulting from our previous study (Chapter II) comparing with *in vivo* (IVV) derived-blastocysts. Zona pellucida (ZP)-free blastocysts derived from IVF-, PA-, and IVV were cultured on mitotically inactivated STO feeder cell layers. The primary ES-like colonies were observed and passaged further on new feeder layers. The rate of ICMs attachment and outgrowth, primary colony formation, and the characteristics of pES cell including morphology and pluripotent markers [alkaline phosphatase (AP), Oct4, Nanog, and SSEA4] were examined. The specific gene expressions of OCT4, NANOG and SOX2 were determined by RT-PCR. The attachment rate was significantly different ($p<0.05$) between *in vitro* derived embryos, either in PA (75.0%) or in IVF (81.73%), and *in vivo* derived embryos (100%), but did not found between embryo groups produced *in vitro*. The primary colonies formation was significantly greater ($p<0.05$) in IVV (66.67%) than IVF (12.55%) blastocysts, on the contrary, none of the primary colony was formed in cultured PA blastocysts. Nevertheless, the greater number of putative ES cell lines were significantly developed from IVV than IVF blastocysts (41.6 vs 7.7%, respectively; $p<0.05$). These colonies grew and expanded, forming dome-shape with well-defined boundary as described in mES cells, and also showed positive AP activity associated with high expression of Oct4, Nanog and SSEA-4 proteins. The expression of OCT4, NANOG, and SOX2 genes was also detected in the PCR results. However, at least five passages can be maintained from these cell lines, all obviously differentiated into various cell types with loss of AP activity after passage 6^{th}. Our study indicates that embryos production using IVF technique could be served as an alternative source for porcine ES-like cell establishment as *in vivo* counterpart, however, their undifferentiated stage were maintained for limited passages.

3.2 Introduction

Embryonic stem (ES) cells represent a promising tool for cell therapy, regenerative medicine and tissue repair. At the same time they constitute an invaluable model for basic investigations in developmental biology, nuclear reprogramming and differentiation process. Assessment of ES cell capabilities in species different from the mouse is an ongoing topic of interest, and is crucial in view of their potential use as experimental models in pre-clinical applications. The development of ES cells in pig, therefore, is considered as the first priority for supporting this purpose due to its immunological, morphological, physiological and functional similar to the human (Yang et al., 2000; Aleem Khan et al., 2006). Currently, many researchers all over the world have increasingly attempted to generate the porcine

embryonic stem (pES) cells using *in vivo* and *in vitro* derived embryos (Hall, 2008), although overall success has markedly restricted to only porcine embryonic stem-like cells (Li et al., 2004[a]; Brevini et al., 2010).

Pig as well as other farm animals, isolation and culture of pES cell lines has frequently used *in vivo* produced blastocysts from superovulated animals (Evan et al., 1990; Strojeck et al., 1990; Piedrahita et al., 1990[a]). This embryonic source is expected to be of very high quality, however, the production of in vivo embryos is labor-intensive, limits embryo recovery rate, and the animals need to be euthanized at the time of embryo collection. Thus, it would be desirable, for economic reason, to used embryos produced from *in vitro* matured and fertilized oocytes. Even though other sources such as intra cytoplasmic sperm injection (ICSI), and nuclear transfer (NT) produced blastocysts have been reported for production ES cell lines in other species (primates ICSI [Suemori et al., 2001]; humans ICSI [Suss-Toby et al., 2004]; humans FT [Park et al., 2004]; mice NT [Wakayama et al., 2006]; cattles NT [Wang et al., 2005]), *in vitro* fertilization (IVF)- and parthenogenetic activation (PA)-produced blastocysts have remain principally been recommended for ES cells establishment in porcine species. Li et al. (2004[a]) used IVF derived porcine embryos for the production of ES-like cell lines. They were able to maintain these cells in culture only from one blastocyst and maximally only for three passages. This finding is similar to the previous report of Kim et al. (2007). The effectiveness of IVF, PA and *in vivo*-produced porcine embryos has been compared by Ock et al. (2005). Nevertheless, formation of primary colonies was low in all cases, and just one ES-like cell line was established from *in vivo* derived blastocyst. Contrarily with the previous study of Brevini et al. (2005) that putative ES cell lines can be generated from PA blastocysts. Until now, only a few pES-like cell lines have been established from PA blastocysts. These findings indicate that different techniques and culture conditions of *in vitro* embryo production influence the success of ES cell establishment.

The present study was, therefore, undertaken to determine the efficiency of pES-like production from IVF and PA blastocysts obtained from our previous study (Experiment 1 and 2 in Chapter II) by comparing with blastocysts derived from *in vivo*.

3.3 Materials and Methods

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

3.3.1 Collection and culture of oocytes

The ovaries were obtained from a local abattoir in Nakorn Pathom province and were transported at room temperature to the laboratory within 3 to 4 h in 0.9% (w/v) saline solution supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Cumulus oocyte complexes (COCs) were collected from antral follicles with a diameter of 3 to 8 mm and washed twice with pre-warmed (37°C) Hepes-buffered Tyrode-lactate solution supplemented with 0.1% (w/v) polyvinyl alcohol (TL-Hepes-PVA).

Groups of 30 to 50 COCs with homogeneous cytoplasm surrounded with at least three uniform layers of compact cumulus cells were selected and cultured at 38.5°C in a humidified atmosphere with 5% CO_2 in air for 44 h in maturation (IVM) medium consisting of TCM199

with Earle‘s salts, 3.05 mM glucose, 26.2 mM sodium bicarbonate, 0.69 mM L-glutamine, 0.91 mM sodium pyruvate, 0.1 mM cysteamine, 10 ng/ml epidermal growth factor (EGF), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) porcine follicular fluid (pFF). The first 22 h of oocyte culture, the IVM medium was supplemented with 10 IU/ml equine chorionic gonadotropin (Folligon®, eCG, Intervet-Schering-Plough Animal Health, Boxmeer, The Netherlands) and 10 IU/ml human chorionic gonadotropin (Chorulon®, hCG, Intervet/Schering-Plough Animal Health). The oocytes were additionally cultured for further 22 h in an absence of eCG and hCG.

3.3.2 Embryo production

3.3.2.1 Parthenogenetic activation (PA)

After 44 h of *in vitro* maturation, expanded cumulus cells were gently removed by repeated pipetting and only metaphase II (MII) oocytes with extruded a polar body were selected for parthenogenetic activation. Denuded MII oocytes were washed three times in activation medium (300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes and 0.01 mg/ml BSA), aligned between two stainless steel electrodes covered with activation medium and connected to an electrical pulsing machine (SD 9 Square Pulse Stimulator; Grass Technologies Ltd., Massachusetts, USA), and then stimulated with three 80-μsec DC pulses of 1.0 kV/cm. After activation, the oocytes were cultured for 4 h in *in vitro* culture (IVC) medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) before further culture in normal medium for 7 days.

3.3.2.2 In vitro fertilization (IVF) and sperm preparation

To produce IVF embryos, expanded cumulus cells were partially removed from matured oocytes after 44 h of culture, and then washed three times with pre-equilibrated modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997) supplemented with 5 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin (IVF medium). Groups of 30 to 50 oocytes were placed into a 4-well plate containing 500 μl of IVF medium and incubated at least 30 min before fertilization. Oocytes were fertilized with frozen-thawed semen at concentration 1000 sperm per oocyte and coincubated for 6 h at 38.5 ºC in a humidified atmosphere with 5% CO_2 in air. Subsequuently, oocytes surrounded with cumulus cells were denuded and further cultured in IVC medium for 7 days.

For sperm preparation**,** the frozen semen from a single boar (Yorkshire) was thawed for 12 sec at 50°C. The viable-motile sperm were then selected using a Percoll gradient technique as described by Parrish et al. (1994). The sperm motility was subjectively assayed at x100 magnifications under a light microscope (TS1000 Nikon, Tokyo, Japan). Percoll-treated Sperm to be used for the entire experiment had more than 90% progressive motility.

3.3.2.3 In vitro embryo culture

Groups of 30 to 50 PA or IVF oocytes were cultured by transferring into a 4-well plate (Nunc, NY, USA) containing with North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993) supplemented with 1% non-essential amino acid (NEAA) and 4 mg/ml bovine serum albumin (BSA), and then covered with mineral oil. During the 5^{th} to 7^{th}

day of embryo culture, the BSA in NCSU-23 was substituted with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen, USA). In all cases, embryos were cultured at 38.5 ºC in a humidified atmosphere with 5% CO_2 in air.

3.3.2.4 In vivo embryo (IVV) production

In vivo produced-embryos were obtained from the crossbred gilts (at least 100 kg body weight). Gilts were treated with a combination of 400 IU equine chorionic gonadotropin (eCG) and 200 IU human chorionic gonadotropin (hCG) (PG 600®, Intervet International BV, Boxmeer, The Netherlands) by intramuscular injection and were artificially inseminated twice at the first day of estrus (day 0 of pregnancy) and 12 h later with freshly diluted semen. Blastocysts were collected between day 6 and 7 of pregnancy by retrograde flushing each uterine horn with PBS containing 4%heat-inactivated FBS, 100 IU/ml penicillin, and 100µg/ml streptomycin (Figure 12). Morula and early blastocyst stage were further cultured in NCSU-23 medium supplemented with 1%NEAA and 10% heat-inactivated FBS for 1 to 2 days at 38.5 ºC in a humidified condition of 5%CO_2 in air until they develop to expanded or hatched blastocysts.

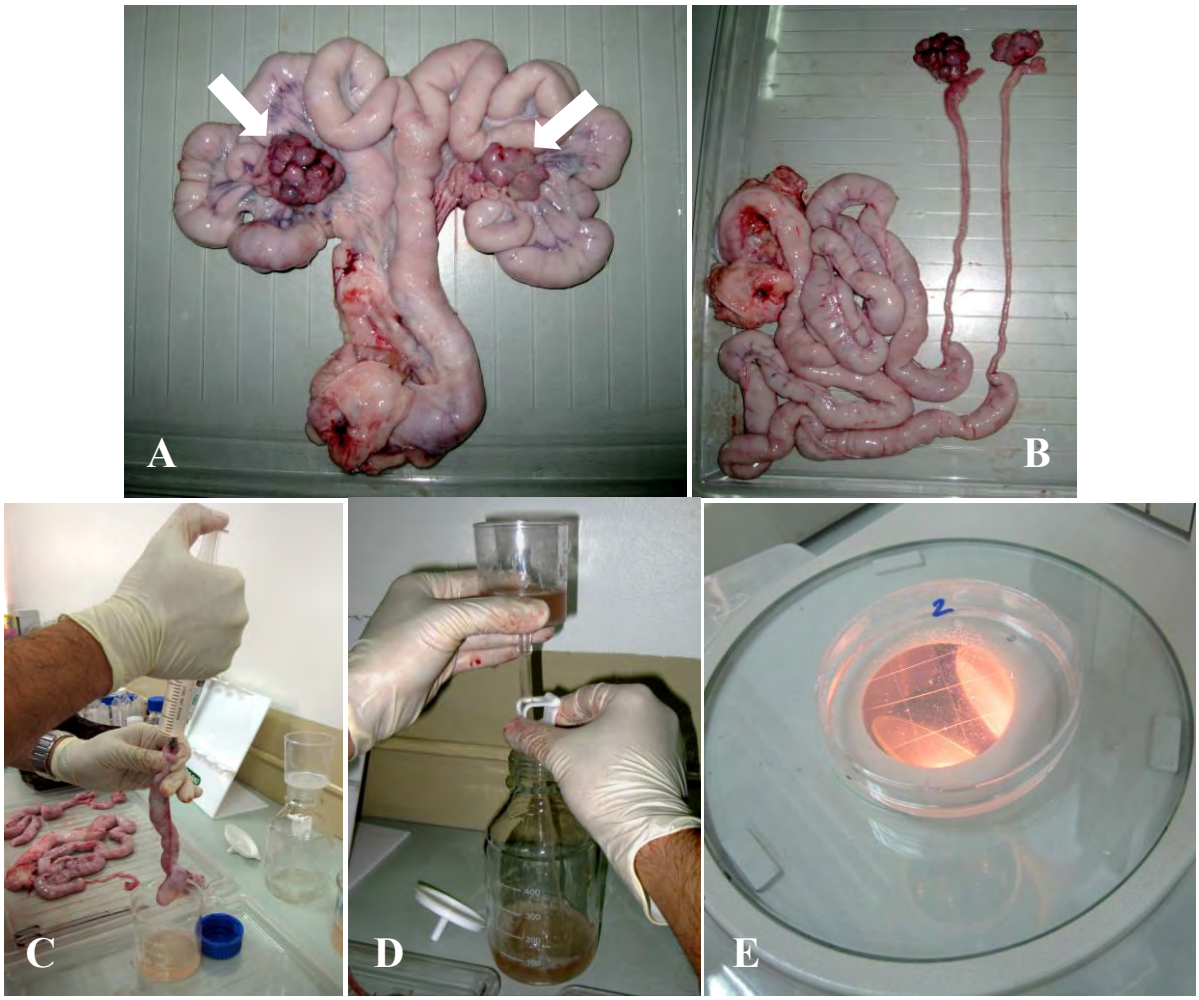

Figure 12 Procedure of *in vivo* embryo collection. (A) Reproductive tract of gilt on day 7 of pregnant; note: the appearance of corpora lutea (CLs; arrows) developed on both ovaries. B) Uterus and ovaries were dissected before collecting embryos; C) Blastocysts were collected by retrograde flushing each uterine horn with PBS containing 4%heat-inactivated FBS; D) Flushed blastocysts were filtrated via embryo filter and recovery embryos were examined using stereo microscope (E).

3.3.3 Preparation of feeder layer

A continuous cell line of SIM mouse embryo-derived thioquanine and ouabain resistant (STO; CRL-1503, ATCC, Manassas, USA) was digested as feeder layer for culturing pES cells. STO cells were mitotically inactivated with 10 µg/ml mitomycin-C for 2 to 3 h, and trypsinized with 0.05% (w/v) trypsin-EDTA solution (Gibco). The resuspended cells were cultured at a density of 55,000 cells/cm^2 on 0.1% (w/v) gelatin-coated plates in FM overnight before plating embryos or ES cells.

3.3.4 Cultivation of ICM and porcine ES-like cells establishment

Intact hatched or intact zona pellucida (ZP)-free blastocysts were used to isolate the ICM. The ZP of expanded blastocysts was digested with 0.25% (w/v) pronase in Hepes buffered NCSU-23. The ICM was isolated by culturing whole intact ZP-free blastocysts on a monolayer of feeder cells in ESC establishment medium at 37°C under a humidified atmosphere with 5% CO_2 in air. This medium composed of knockout-DMEM (Invitrogen) supplemented with a 1:1 mixture of 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 10% (v/v) knockout serum replacement (KSR; Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (Invitrogen), 1% (v/v) non-essential amino acids, 1% (v/v) penicillin-streptomycin, 1000 IU/mL recombinant human leukemia inhibitory factor (rhLIF; Chemicon International, Temecula, CA, USA) and 20 ng/mL recombinant human basic fibroblast growth factor (rbFGF; Chemicon International, Temecula, CA, USA). The medium was not changed for the first two days of embryo culture but 0.5 ml of medium was thereafter added every day. The blastocyst attachment and ICM outgrowths were daily examined. At approximately 4 to 6 days of culture, the ICM outgrowths were mechanically removed from trophectoderm (TE) with a _hand-made‘ capillary glass pipette and re-plated on a new feeder layer for further expansion. The primary ES-like colonies obtained were mechanically dissociated using the mechanical transfer method as described by Oh et al. (2005). The resulting colonies were continuously passaged until they were differentiated or no colony was obviously formed.

3.3.5 Characterization of putative pES cells

3.3.5.1 Alkaline phosphatase (ALP) staining

ALP activity was used as a generalized ES cell marker. The putative ES cells were rinsed with PBS, fixed in 4% (w/v) paraformaldehyde (PFA) for 5 min at room temperature (RT). ALP staining technique was performed using an ALP histochemistry kit (Sigma) according to the manufacturer‘s instruction. The putative pES cells were finally visualized using an inverted phase contrast microscope (Olympus CKX41, NY, USA). An ES-like colony demonstrating bright pink-to-red color indicated ALP positive cells.

3.3.5.2 Immunostaining

The putative ES cells were immunocytochemically characterized using antibodies against markers of undifferentiated porcine/human ES cells. In brief, putative ES cells were fixed in 4% PFA for 15 min at RT and then washed three times with PBS. Samples were permeabilized with 0.1% Triton X-100 in PBS for 1 h at RT, and then transferred into blocking solution which comprised PBS containing 0.1% Triton X-100 and 10% FBS for 1 h

at RT. For Oct4 staining, mixture of 5% donkey serum and 5%FBS was substituted for 10%FBS in the blocking solution. Primary antibodies [goat polyclonal Oct4 (Santa Cruz Biotechonology, Inc., Santa Cruz, CA, USA; 1:250), rabbit polyclonal Nanog (Abcam, Cambridge, MA,USA; 1:500), mouse monoclonal [MC813] SSEA-4 (Abcam; 1: 250)] were diluted in the blocking solution and incubated overnight at 4°C. Putative ES cells were incubated in fluorescent-labeled secondary antibodies [anti-goat IgG-FITC (Jackson ImmunoResearch Europe Ltd. Newmarket, Suffolk, UK); anti-rabbit IgG-FITC (Abcam, Cambridge, MA,USA); anti-mouse IgG-TRITC (Abcam, Cambridge, MA,USA), respectively, at a dilution 1:200 for 1 h at RT. The nuclei were visualized with 4‘,6-diamidino-2-phenylindole (DAPI) staining. After fluorescent labeling, the putative ESC colonies were examined under a fluorescent microscope (BX51 Olympus, Shinjuku, Japan).

3.3.5.3 RNA extraction and reverse transcriptase-polymerase chain reaction

ESC-like colonies were isolated from each passage and then submitted to reverse transcriptase-polymerase chain reaction (RT-PCR) for screening the expression of pluripotent genes. Total RNA was extracted using an Absolutely RNA^{TM} Nanoprep kit (Stratagene, CA, USA) according to the manufacturer‘s instruction. Briefly, colonies were lysed using a lysis buffer containing 0.7% (v/v) β-Mercaptoethanol (β-ME). Cell lysate was added with an equal volume of 80% sulfolane, and then transferred to RNA-binding nano-spin cup and centrifuged at ≥ 12,000 x g for 60 sec. DNA removal was eliminated by adding DNase solution (mixture of RNase-free DNase I and DNase digestion buffer) onto the fiber matrix inside the spin cup and incubated at 37 ˚C for 15 min. RNA was eluted with 15 µl RNase-free water at room temperature for 3 min, subsequently, centrifuged at ≥ 12,000 for 5 min. The extracted RNA was assessed for quality and quantity using spectrophotometer (Nanodrop ND-2000, Wilmington, Delaware, USA) and finally stored at -80 ˚C.

RNA yields were typically 3 to 100 ng/µL, with a total elution volume of approximately 15 µL. Total RNA was synthesized into cDNA and amplified using Sensiscript® RT kit (Qiagen, Valencia, CA, USA) and Go Tag® Green Master Mix (Promega, WI, USA), respectively. Amplifications were carried out in an automated thermal cycler (Nyx Technik A6, Ramsey, MN, USA) using 35 amplification cycles of 60 sec 95°C denaturation, 60 sec 55°C annealing, 60 sec 70°C elongation. For the final extension, one cycle of 15 min at 70°C was used. The PCR products were run on a 1.5% (w/v) agarose gel (Bio-Rad, CA, USA) in 1xTBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8) containing 0.4 mg/mL ethidium bromide (Promega, WI, USA). The separated products in agarose gel were visualized under UV light (Syngene, CB, UK). The oligonucleotide primers used for RT-PCR reaction are listed in Table 1. The housekeeping (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) gene transcript was used as an internal control.

3.4 Statistical Analysis

Data was pooled from at least 4 independent replicates and expressed by mean ± standard error of the mean (SEM). The efficacy of putative ES cells derivation among experimental groups were compared by ANOVA and protected least significant different (LSD) statistical test with SPSS version13.0 software (SPSS Inc., Chicago, IL). A value of $p< 0.05$ was considered to be statistically significant.

Table 11 List of primers and source of primer sequences*

Gene product	Accession no.	Primer sequence	Product size (bp)
OCT4	NM_001113060.1	Forward: AGGTGTTCAGCCAAACGACC	335
	(GenBank, NCBI)	Reverse: TGATCGTTTGCCCTTCTGCG	
NANOG	DQ447201.1	Forward: CGAATGAAATGTAAGAGGT	162
	(GenBank, NCBI)	Reverse: CCAGCTCTGATTACCCCAC	
SOX2	TC2698881	Forward: CCAGAAGAACAGCCCAGA	203
	(DFCI Pig Gene Index)	Reverse: GCAGCGTTCGCAGCCG	
GAPDH	AF017079.1	Forward: TCGGAGTGAACGGATTTG	219
	(GenBank, NCBI)	Reverse: CCTGGAAGATGGTGATGG	

*Primers were specifically designed from sequences available from GenBank, NCBI, or DFCI Pig Gene

3.5 Experimental design

The study was designed to evaluate the efficiency of embryonic sources on the isolation and derivation of pES-like cell lines. *In vitro* embryo production by IVF and PA was conducted by the optimal protocol resulting from our previous study (Expriment I and II in Chapter II). Intact blastocysts containing a cluster of ICM cells were used for establishing pES cells. Whole intact ZP-free blastocysts derived from IVF, PA and IVV were cultured on mitotically inactivated STO feeders. The rates of attached blastocysts and primary colony of ES-like cells were recorded. Established ESC lines were characterized by morphological appearance, generalized pluripotent markers (alkaline phosphatase activity), immunostaining and RT-PCR. *In vivo*-derived blastocysts (n=12) were used as a control group in this study.

3.6 Results

3.6.1 Morphology of embryos derived from different sources

Blastocyst-stage embryos produced by parthenogenetic activation (PA), *in vitro* fertilization (IVF) and *in vivo* fertilization (IVV) differed in embryonic appearance. A cluster of ICM cells within the blastocoels was clearly observed in IVF and IVV embryos compared to those PA embryos (Figure 13A, C, E). Although appearance of ICM cells was found in both IVF and IVV, the embryo quality, by means of size and ICM cells number, was better in IVV than those IVF-produced blastocysts. In our study, less than 5 and 10% of blastocysts developed from PA and IVF, respectively, hatched from zona pellucida (ZP) during *in vitro* culture. On the other hand, more than 90% of hatched blastocysts (Figure 13F) were obtained from collected IVV blastocysts after 24 to 48 h of culture (data not shown). Zona pellucida (ZP), therefore, had been removed from both PA and IVF blastocysts before using them for ES cell establishment. Most PA-derived blastocysts presented a shrinking of TE cells after ZP removal, but not found in blastocysts derived from IVF (data not shown; Figure 13B, D).

3.6.2 Derivation of ES-like cell lines from different embryo sources

The blastocysts derived from IVV adhered onto feeder layers (within 36 h) faster than those derived from IVF and PA (approximately 48 to 60 and 48 to 72 hrs, respectively) after initiation of culture. The attachment rate was significantly different ($p<0.05$) between *in vitro* produced embryos either by PA (75.0±4.3%) or IVF (81.73$\pm$3.7%), and *in vivo* produced embryos (100%; $p<0.05$). However, no significant difference was found between PA and IVF groups (Table 12). The outgrowth of ICMs was observed in both IVV and IVF groups within 1 to 2 and 2 to 4 days, respectively, after blastocysts attachment (data not shown). However, the growth rate of some ICMs developed from IVF, but not from IVV, gradually declined and then stopped to proliferate or differentiated during culture (Figure 14 A, B). The primary colonies formation was significantly greater ($p<0.05$) in IVV (66.67±8.3%) than IVF (12.55±2.5%) blastocysts, on the contrary, none of the primary colony was formed in cultured PA blastocysts. All primary ES-like colonies expanded and formed a dome-shape with well-defined boundary. The cell morphology in these colonies exhibited obvious a large nuclei with one or more prominent nucleoli, and were tightly packed within the multiple layers as described in mouse (m) and human (h) ES cells.

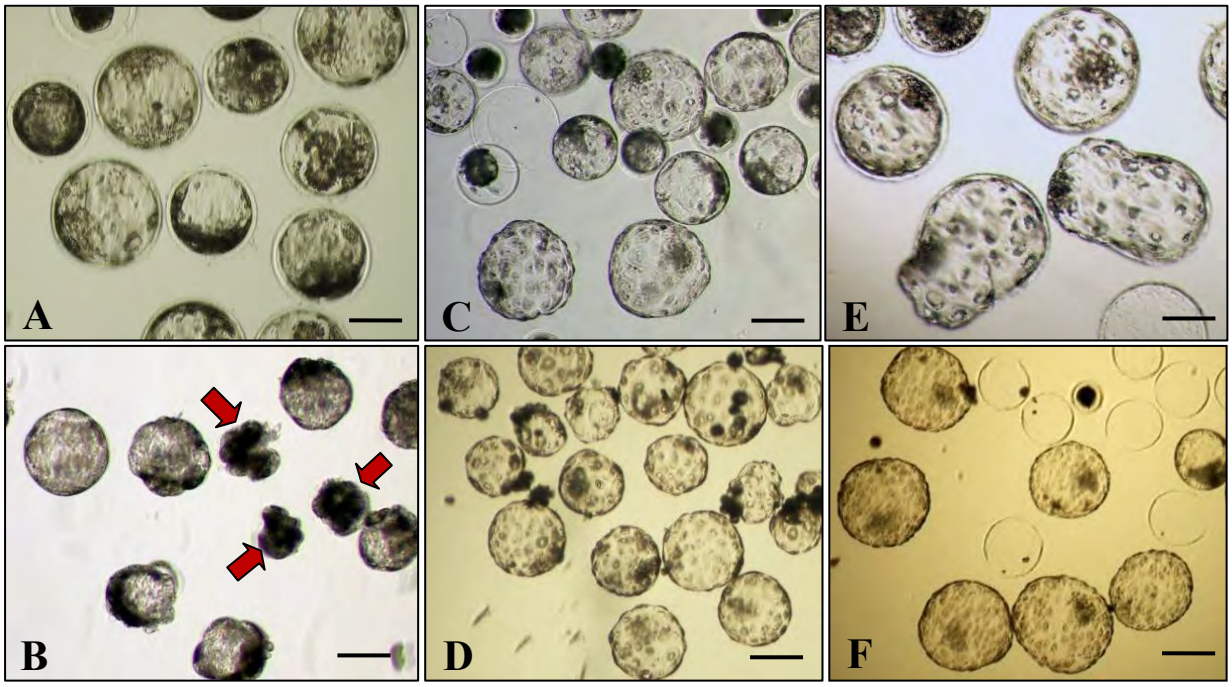

Figure 13 Morphology of blastocysts derived from different embryo sources. The first row represents development of blastocysts during *in vitro* culture. The second row represents morphology of ZP-free blastocysts after enzymatic lysis/spontaneous hatching. (A, B) PA blastocyst developed on day 7 of culture. Note: a shrinking of blastocysts after ZP removal (arrows); (C, D) IVF blastocysts developed on day 7 of culture; (E, F) IVV blastocysts developed after 24 and 48 h of culture. PA: parthenogenetic activation; IVF: *in vitro* fertilization; IVV: *in vivo* fertilization. Scale bars = 100 μM.

Table 12 Effects of embryo sources on derivation of porcine embryonic stem-like cells

Experimental groups	No. of cultured blastocyst (n)	No. of attached blastocysts (%mean±SEM)[a]	No. of primary ES-like colonies (%mean±SEM)[b]	No. of pES-like cell lines (%mean±SEM)[c]
PA	44	33 (75.0±4.3)[d]	0	0
IVF	49	40 (81.73±3.7)[d]	5 (12.55±2.5)[d]	3 (7.78±2.6)[d]
IVV	12	12 (100)[e]	8 (66.67±8.3)[e]	5 (41.67±8.3)[e]

[a] The number of attached blastocysts/total number of cultured blastocysts; [b] the number of primary ES-like colonies/ total number of attached blastocysts; [c] the number of pES-like cell lines/total number of attached blastocysts

[d,e] Different superscripts within the same column indicate significant differences ($p<0.05$).

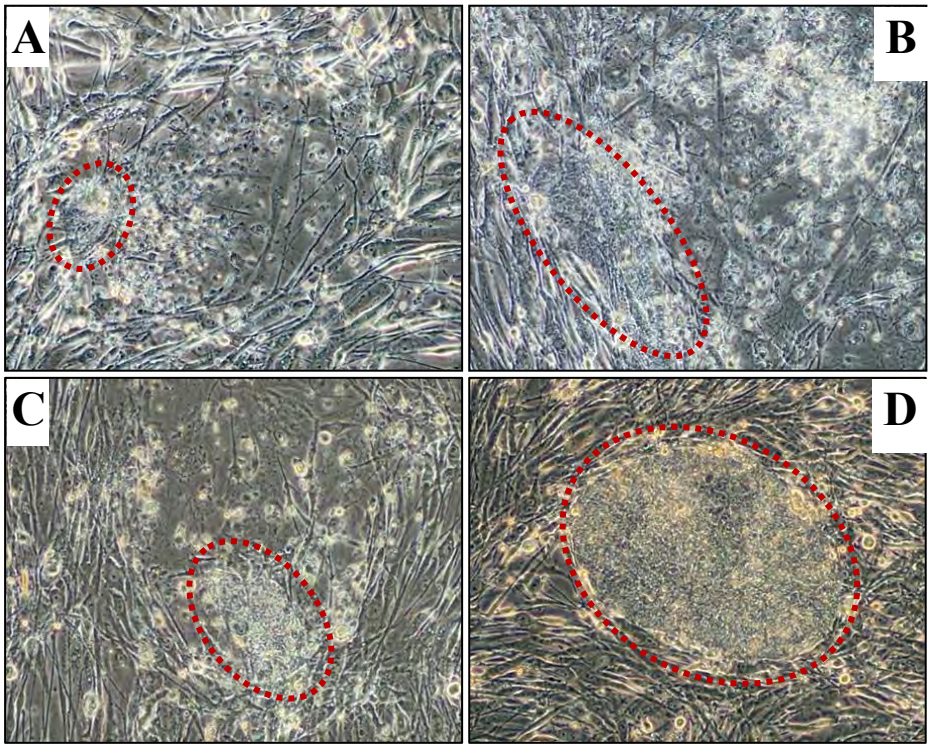

Figure 14 Morphology of ICM grown on STO feeder layer after attachment. Outgrowth of ICM developed from IVF blastocysts at day 2 (A; dash circle) and day 7 (B; dash circle) surrounded with proliferated trophoblast cells (x100). Outgrowth of ICM at day 2 (C; dash circle) and primary ES-like colony at day 5 (D; dash circle) generated from IVV blastocyst. ICM, inner cell mass; IVF, *in vitro* fertilization; IVV, *in vivo* fertilization (x100).

Nonetheless, the greater number of putative ES cell lines were significantly developed from IVV than IVF blastocysts (41.67±8.3 vs 7.78±2.6%, respectively; $p<0.01$). These cell lines showed positive AP activity (Figure 15A) together with a high expression of Oct4, Nanog and SSEA-4 proteins. The expression of OCT4, NANOG, and SOX2 genes was also detected in PCR results (Figure 16). At least five passages could be maintained from these cell lines (Table 13), and then differentiated into various cell types which indicated by AP activity loss (Figure 15B).

Table 13 Efficiency of embryos derived from different sources on propagation of pES-like cell lines

Embryos source	**Passage number (P)**					
	P1	P2	P3	P4	P5	P6
IVF	3/3*	2/3	1/2	0	0	0
IVV	5/5	5/5	4/5	3/4	1/3	0

*The numerator indicates the number of ES-like cell lines survived after passaging, the denominator indicates the total number of ES-like cell lines before passaging. IVF: *in vitro* fertilized embryos, IVV: *in vivo* fertilized embryos.

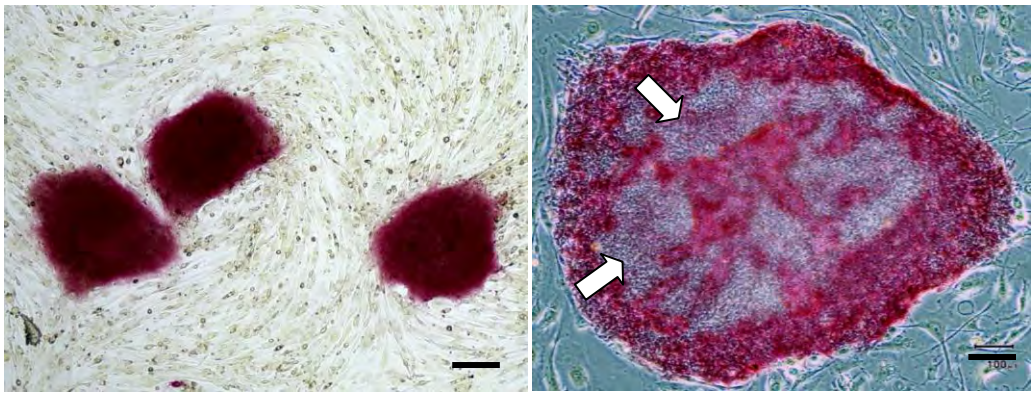

Figure 15 Activity of alkaline phosphatase expressing on porcine ES-like colonies derived from *in vivo* blastocyst. A) AP-positive ES-like colonies at passage 5 (scale bar = 500μM); and B) AP-negative area (arrows) in differentiated colony at passage 6 (scale bar = 200μM)

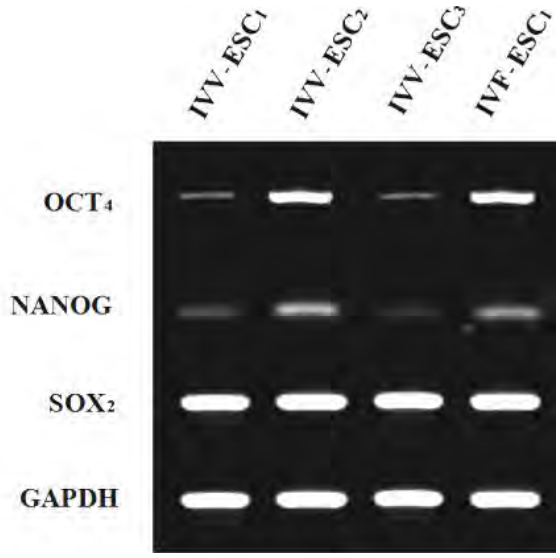

Figure 16 Gene expression of OCT4, NANOG, SOX2 and GAPDH detected in 3 and 1 ES-like cell lines derived from IVV and IVF embryos at passage 4 and 3, respectively. IVV: *in vivo* fertilization; IVF: *in vitro* fertilization.

6.7 Discussion

In the present study, we examined the capability of embryos derived from IVP techniques, parthenogenetic activation (PA) and *in vitro* fertilization (IVF), on the derivation of porcine embryonic stem (pES) cells compared with *in vivo* fertilization (IVV). The development of pES-like cells in our study was achieved when blastocysts produced from IVF and IVV were isolated and cultured on feeder layers. The primary colonies derived from these embryo sources showed typical morphology of ES cells, whileas they revealed the positive effects of AP activity and the expression of pluripotent specific-proteins and genes. Nevertheless, with the same culture condition, the formation of primary ES-like colonies failed to generate from PA blastocysts. This is opposed with previous studies which demonstrated the success of ES cells production by using PA embryos (Brevini et al., 2007; Kim et al., 2007). This failure may cause by the quality of embryos *per se* since the quality of embryo, in terms of trophoblastic and ICM cells, is markedly different between activation protocols (Zhu et al., 2002; Cong et al., 2008) and also between culture conditions, leading to

an alternation of gene expression affecting the activation of ICM outgrowth (Boiani et al., 2005; Cervera et al., 2010). Furthermore, a higher proportion of apoptotic cells between ICM and TE cells has also been reported in blastocysts produced from PA than IVF (Hao et al., 2004; Gómez et al., 2009).

Under our culture system, more than 40% of primary ES-like colonies were successfully isolate from *in vivo* produced porcine embryos, whereas less than 8% of those colonies were produced from IVF embryos (Table 12). Difference in the cell number of ICM between *in vitro* and *in vivo* derived porcine embryos has been indicated to be a primary factor influencing the success of primary ES-like colony appearance. For example, total cell number of *in vivo*-derived blastocysts is reported to be nearly 57 and 93 at day 6 (Yoshioka et al., 2002) and day 7 (Fuente & King, 1997) of development, respectively while *in vitro*-derived blastocysts have less than 55 cells at day 7 (Grupen et al., 2002; Krylov et al., 2005) which related to our previous study (in chapter II). In addition, difference in the expression level of pluripotent-related genes, such as OCT4, NANOG and SOX2, also relates the derivation of pES-like cells (Boyer et al., 2005; Chew et al., 2005; Rodda et al., 2005). This is supported by a previous study of Magnani and Cabot (2008) which demonstrated a higher level of NANOG and SOX2 expression in blastocysts developed from IVV compared to those developed from IVF and PA.

Unfortunately, undifferentiated colonies developed from either IVF or IVV blastocysts were spontaneously differentiated into multiple cell types such as neuronal- and epithelial-like cells, even these colonies were maintained beyond 5th passage. Maintaining of these cells in undifferentiated stage *in vitro* is one of major obstacles of ES cell line establishment in this species, in particular that culture conditions suitable to support mouse and human ESC may be inadequate to maintain ESC in pigs. Relative recently, difference in media components plays a pivotal role in regulating intrinsic and extrinsic factors involved in the control of pluripotency, that means specific pathways may be up-regulated, or down-regulated, in response to the addition of specific molecules which have been shown to be different among species (Hanna et al., 2009; Brevini et al., 2010). These finding are consistent with the requirements of mESC for leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) 4, whereas human ESC needs the presence of fibroblast growth factor (FGF)-2, to preserve their pluripotency in the absence of feeder cells (Qi et al., 2004; Peerani et al., 2007).

In pig, the characterization technique of pES cells has also been hampered by limited information of pluripotent markers that are essentially specific and standardized to pES cells. Characterization of pES cells therefore has been mainly performed by morphology assessment and some molecular markers such as alkaline phosphatase, Oct4, Nanog, Sox2, SSEA-1, and SSEA-4 (Blomberg et al., 2008; Brevini et al., 2010; Du Puy et al., 2010). These cells have a typical characteristic of ES cell morphology as small and rounded cells that have large nucleus with one or two prominent nucleoli (Piedrahita et al., 1990[a,b]). In mouse and human, Oct4 and Nanog transcription factors have been identified as reliable markers for ES cells since these two transcription factors are necessary for maintaining the stemness in ES cells (Nichols et al., 1998; Chambers et al., 2003). Furthermore, recent study demonstrated a close similarity in the existence of pluripotent state through OCT4 and NANOG expression between epiblast presented in mouse blastocyst and embryo-derived cell

lines (Nichole et al., 2009[b]). The OCT4 however expresses throughout the preimplantation stage of porcine embryos and is not specific to only ICM of the embryos (Kirchhof et al., 2000). On the other hand, the expression of Nanog is not certainly detected as previously described (Kumar et al., 2007; Magnani and Cabot, 2008; Kuijk et al., 2008; Hall et al. 2009).

In conclusion, our study indicates that pES-like cell lines could be generated from either IVF or IVV embryos, but a higher efficiency of IVV embryos promoted more chance for ES cell development. However, their undifferentiated stages were maintained for limited passages. Further study, therefore, requires elucidating the networks of gene and factors that potentially regulate the pluripotency pathways of porcine embryonic stem cells and also the specific requirement of pES cells during *in vitro* culture.

CHAPTER IV

INFLUENCE OF CULTURE CONDITION ON ESTABLISHMENT OF pES-LIKE CELL LINES: MAINTENANCE OF pES-LIKE CELL IN DIFFERENT SERUM-SUPPLEMENTED CONDITIONS

4.1 Abstract

To examine the effect of serum supplementation on the derivation and maintenance of pES cells, *in vivo* hatched or zona pellucida (ZP)-free blastocysts were cultured in ES medium supplemented with different (volume/volume) ratios of FBS:KSR (20:0, 10:10, and 0:20). The primary ES-like colonies were observed and passaged further on new feeder layers. The rate of ICMs attachment and outgrowth, primary colony formation, and the characteristic of pES cells including morphology and pluripotent markers such as alkaline phosphatase (AP), Oct4, Nanog, and SSEA-4 were examined. The specific gene expressions of OCT4, NANOG, and SOX2 were determined by RT-PCR. All blastocysts (100%) plated and cultured in both 20% FBS- and 10% FBS plus 10% KSR (10% FBS+10% KSR)-supplemented ES media gave the higher attachment rates within 48 h than those cultured in medium supplemented with 20% KSR (64.4±9.8%; $p<0.05$). Although, there was no significant difference in the ICM outgrowth rates among the experimental groups, blastocysts cultured in medium supplemented with 10% FBS+10% KSR appeared to have a higher rate of ICM outgrowths than other groups. The formation of primary ES-like colonies was significantly greater in blastocysts growing in medium supplemented with 10%FBS+10% KSR (64.4±9.9%) than those growing in media supplemented with 20% FBS and 20% KSR (17.8±1.1 and 33.3±17.6%, respectively; $p<0.05$). No primary colonies, nevertheless, cultured in 20%FBS-supplemented medium could be maintained the undifferentiated state, while 3 (5.88%) and 6 (40%) putative ES cell lines were observed from primary colonies cultured in 20% KSR- and 10% FBS+10% KSR-supplemented media after the first subculture, respectively. The undifferentiated colonies developed on 20% KSR and 10% FBS+10% KSR showed a strong positive AP acitivity, high expression of Oct4, Nanog and SSEA-4 proteins associated with the expression of all specific genes even if all characteristics were disappeared when colonies were continued until the 5^{th} passage (P5). These results indicated that culture condition supplemented with equal ratio at 10% of FBS and KSR is more suitable for isolation and culture pES cells than that supplemented with FBS or KSR alone.

4.2 Introduction

Attempt to establish porcine embryonic stem (pES) cell lines, to date, remains as the challenge for basic research on cell replacement therapy and biomedical applications. These cells would also be beneficial for the agricultural area, allowing efficient genetic engineering of this animal, and also for improving health and production traits. However, the most significant obstacle hindering the establishment of pES cell lines is the inability to control the spontaneous differentiation during culture.

The use of conventional protocols for culture of mouse and human ES cells does not appear to sustain extended growth or pluripotency of cultured pES cells. We have previously shown that the age and the source of embryos as well as feeder cell type influence the isolation and culture of ES cells in the pigs (Panasophonkul et al., 2010), which is in agreement to the previous studies (Wianny et al., 1997; Li et al., 2004[a,b]; Kim et al., 2007). Another factor involving with the mechanism regulating ES cells differentiation is fetal bovine serum (FBS), a protein source in ES culture medium. Undifferentiated growth of mouse (m) ES cells is dependent on FBS. Without serum, mES cells differentiate along the neural pathway. This inhibitory effect of serum is induced by bone morphogenetic proteins (BMPs) through the expression of inhibitor of differentiation (Id) genes via the SMAD pathway (Finley et al., 1999; Ying et al., 2003). On the other hand, human (h) ES cells cultured in either FBS-containing or BMP4-supplemented medium led to spontaneous differentiation towards extra-embryonic lineage (Xu et al., 2002; Qi et al., 2004).

FBS is by nature a complex mixture containing unknown compounds, often resulting in significant batch-to-batch variability. In addition, animal-derived components can introduce known or unidentified animal pathogens into ES cell cultures. A defined serum-free supplement, therefore, referred to as Knockout Serum Replacement (KSR, Invitrogen) has been applied instead of animal serum. (Inzunza et al., 2005; Hisamatsu-Sakamoto et al., 2007). KSR was designed to provide more standardized and better defined supplements to ESC medium. Human ES cells cultured in KSR-containing medium have an increased growth rate compared with those grown in FBS-containing medium (Koivisto et al., 2004). Although, this product contains AlbuMAX, a lipid-rich albumin fraction of bovine serum, which is not free of animal-derived components (Price et al., 1998), it results for supporting pluripotent undifferentiated state in hES cells is better than other commercially available animal-free serum replacement products such as Lipumin, SerEx (PAA Laboratories), Serum Replacement 3 (Sigma) and Serum Substitute Supplement ([Irvine, Scientific], Rajala et al., 2007). In pigs, both FBS and KSR have been used in pES cell establishment. Nevertheless, their properties for isolating and maintaining pluripotency of ES cells in undifferentiated state in this species have yet to be clearly defined.

The objective of this study was to examine the effect of different serum-supplemented conditions in ES culture medium on the derivation and maintenance of pES cells.

4.3 Materials and methods

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

All materials and methods in this experiment were described in **Chapter III** including production of embryos (*in vivo* fertilization; IVV), preparation of feeder layer, isolation and culture of inner cell mass (ICM), characterization of ES cells (alkaline phosphatase activity, imunocytochemistry, and RT-PCR analysis).

4.4 Statistical Analysis

Data represented as mean ± standard error of mean (SEM) was pooled from at least 3 independent replicates, and analyzed by ANOVA to determine statistical differences among

the derivative rate of ES cells. Statistical analysis was carried out with SPSS 13.0 software (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was considered significantly different.

4.5 Experimental design

In order to evaluate the effects of serum supplementation, FBS (Hyclone, Logan, UT) and KSR (Invitrogen), on derivation and maintenance of pES cells, *in vivo* hatched and intact ZP-free blastocysts were cultured in ES medium supplemented with different ratios of FBS:KSR (20:0, 10:10, and 0:20%) at least three replicates. The rates of blastocysts that attached and primary colony of ES-like cells were recorded. The resulting colonies developed from different embryo sources were subcultured and replated continuously on new feeder layers until being differentiated or no colony was obviously formed. The putative ESCs were characterized by morphological appearance, ALP activity, and the expression of generalized pluripotent markers, Oct4, Nanog, and SSEA4, by immunostaining. The expression of specific genes (OCT4, NANOG, SOX2) also were analysed in undifferentiated ES-like colonies by RT-PCR.

4.6 Results

The influence of serum supplementation, FBS and KSR, on the isolation and maintenance of undifferentiated pES cells was examined at different ratios. As shown in Table 14, all blastocysts (100%) plated and cultured in both 20% FBS- and 10%FBS plus 10%KSR (10%FBS+KSR)-supplemented ES media had the higher attachment rates than those cultured in medium supplemented with 20% KSR (64.4±9.8%; $p<0.05$). The outgrowth of ICM cells began around 1 to 2 days after attachment of blastocysts onto the feeder layers. There was no significant difference in the ICM outgrowth rates among these experimental groups. However, blastocysts cultured in medium supplemented with 10% FBS+ 10% KSR tended to have a higher rate of ICM outgrowths than other groups.

Table 14 Effect of different serum-supplemented conditions in ES culture medium on the derivation of porcine ES-like cells

Experimental group	n	No. attached blastocyst (%mean ±SEM)*	No. of ICM outgrowth (%mean ±SEM)**	No. of primary ES-like colony (%mean ±SEM)***
20%FBS	16	16 (100)^{a}	9 (57.8±13.5)	5 (33.3±17.6)^{a,b}
20%KSR	17	11 (64.4±9.8)^{b}	8 (46.7±3.3)	3 (17.8±1.1)^{a}
10%FBS+10%KSR	15	15 (100)^{a}	11 (71.1±10.6)	10 (64.4±9.9)^{b}

* Percentage of attached blastocysts; ** percentage of ICM outgrowth; *** percentage of primary ES-like colonies

^{a,b} Different superscripts within the same column indicate significant differences ($p<0.05$)

During continuous culture, most ICM outgrowths developing in 20% FBS - and 20% KSR - supplemented media spontaneously differentiated into mesoderm and endoderm-like cells, and trophoblastic-like cells, respectively (Figure 17). The formation of primary ES-like colonies was significantly greater ($p<0.05$) in blastocysts growing in medium supplemented with 10% FBS+10% KSR (64.4±9.9%) than those cultured in medium supplemented with 20% FBS and 20% KSR (17.8±1.1 and 33.3±17.6%, respectively). No primary colonies, nevertheless, cultured in 20% FBS-supplemented medium could be maintained in the undifferentiated state, while 3 (5.88%) and 6 (40%) putative ES cell lines were observed from primary colonies cultured in 20% KSR- and 10% FBS+10% KSR-supplemented media after the first subculture, respectively (Table 15). Nonetheless, putative cell lines cultured in 20% KSR-conditioned medium started to differentiate at the 1st passage (P1). By contrast, most cell lines cultured in 10% FBS+10% KSR remain sustained the cell morphology with small and round, and had a large nucleus with one or two prominent nucleoli, similar to mouse and human ES cells. Undifferentiated colonies showed a strong positive AP activity with high Oct4, Nanog, and SSEA-4 (Figure 18) expressions. Furthermore, the expression of OCT4, NANOG and SOX2 genes was also observed by RT-PCR. Unfortunately, all characteristics were disappeared from ES-like colonies maintained in 10% FBS+10% KSR-condition medium when the subculture was continued further than the 5th passage (P5).

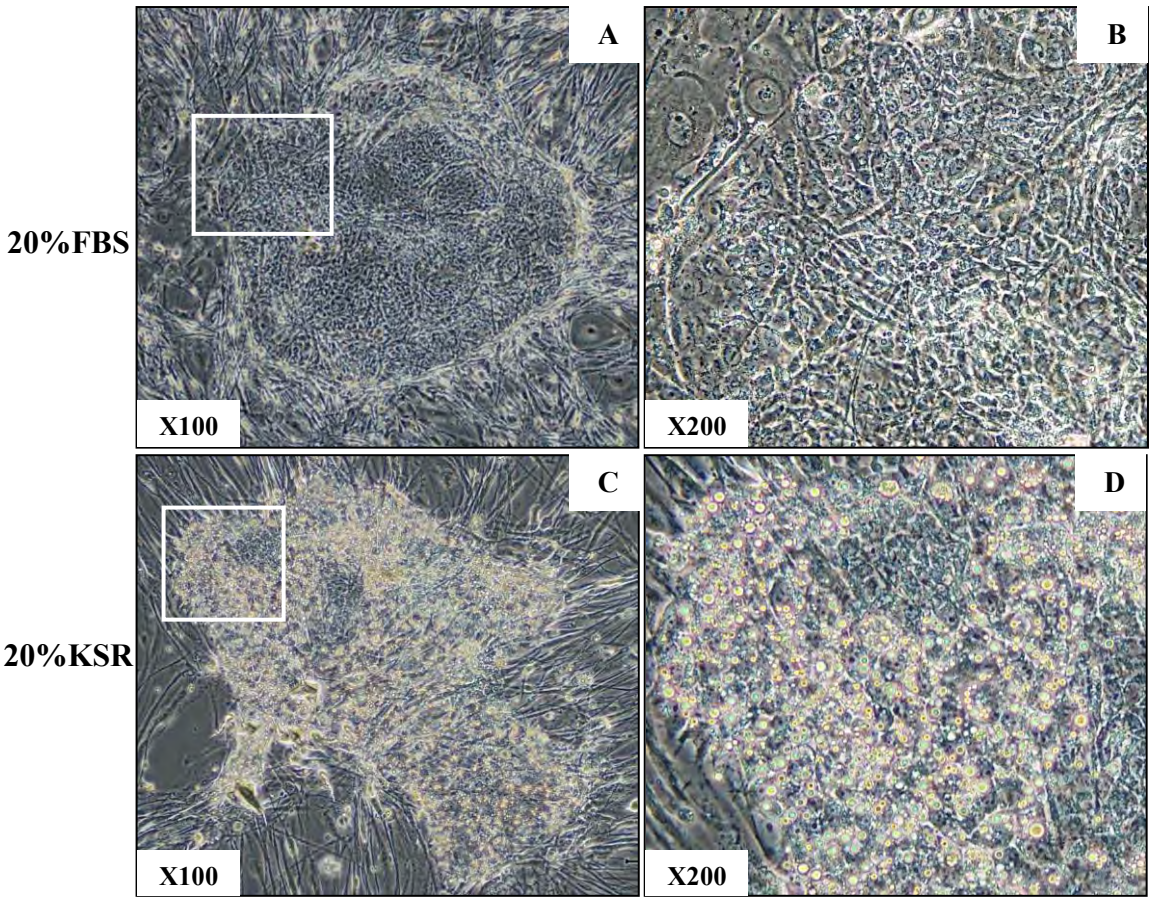

Figure 17 Morphology of spontaneously differentiated ICM outgrowths developed under medium supplemented with FBS (upper row) or KSR (lower row) on day 7 after plating. Colony formation of endoderm-like cells (A, B) and trophoblast-like cells (C, D) cultured in 20% FBS- and 20% KSR-supplemented media, respectively.

Table 15 Effect of different serum-supplemented conditions in ES culture medium on pES cells propagation and derivation of putative cell lines

Serum conditions	Passage number (P)					No. of cell line (%)*
	P1	P2	P3	P4	P5	
20%FBS	0	0	0	0	0	0
20%KSR	1/3**	0	0	0	0	1 (5.88)
10%FBS+10%KSR	6/10	5/10	4/10	2/10	0	11 (40.0)

*Percentage of putative cell line number/total number of plated blastocysts

**Number of putative ES cell lines/ total number of primary ES-like colonies

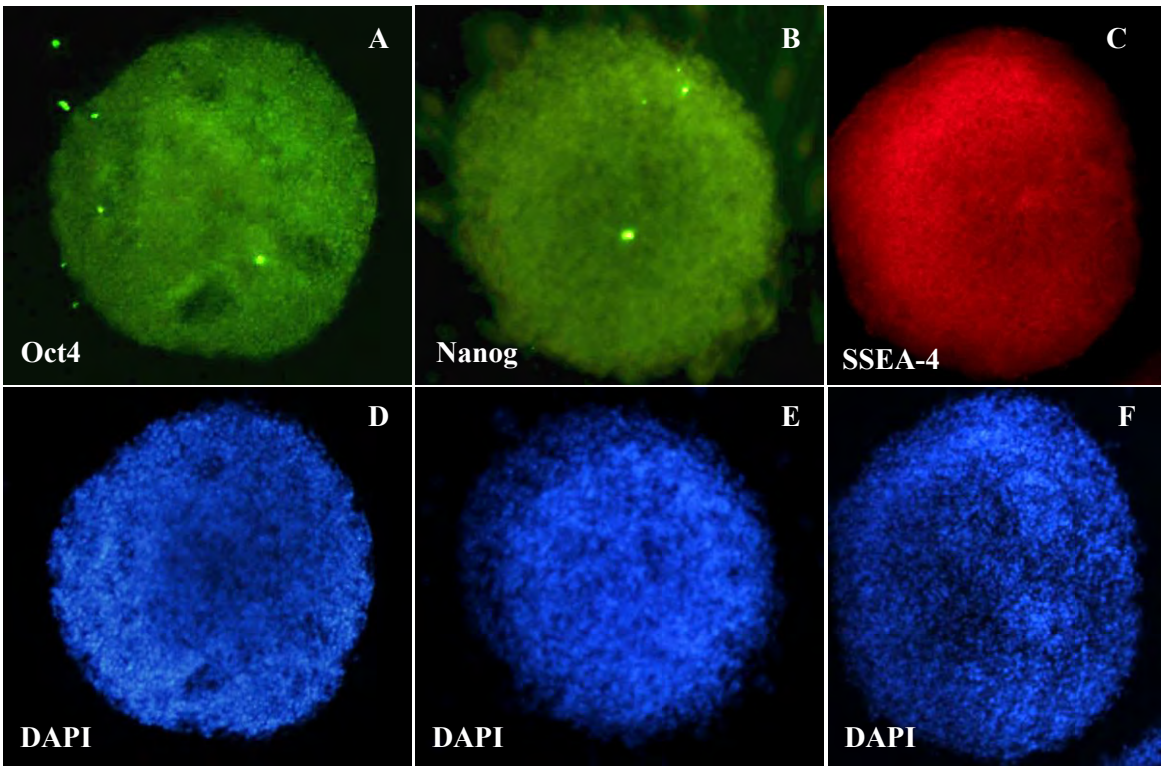

Figure 18 Characteristics of porcine ES-like colonies growing on STO feeder in medium supplemented with 10%FBS and 10% KSR at passage 4. Represent images show (A,B) the expression of Oct4 (A), Nanog (B), and SSEA-4 (C) proteins by immunostaining. Nuclei are counterstained with DAPI (blue: D-F). Oct4 (FITC, green) and Nanog (FITC, green) specifically expressed in the nuclei, and SSEA-4 (TRITC, red) is expressed on the cell surface. Magnification at x200.

4.7 Disscussion

The addition of serum in ES cell culture condition is essential for isolation and proliferation in undifferentiated state of ES cell. However, the adverse effect of serum is also described in ES cell culture. The use of both animal- and synthetic-derived serum such as fetal bovine serum (FBS) and knockout-serum replacement (KSR) have widely been used for ES cell establishment. We, therefore, investigated the potential effect of supplemented FBS and KSR either alone or in a combination in terms of attachment rates, the formation of ICM outgrowths and primary ES-like colonies, colonies expansion and the derivation of cell lines. Our results demonstrated that different serum-supplemented conditions affected the isolation and propagation of porcine ES-like cells.

In this study, we found the viable blastocysts adhering on feeder layers significantly higher when they were cultured in FBS alone or in combination with KSR compared to KSR supplementation. This is in accordance with a recent study in canine ES cells demonstrating that embryos cultured in ES medium containing 15%FBS attached to feeder layer and developed to hatched blastocysts spontaneously. On the other hand, supplementation of medium with 15%KSR limited embryo attachment (Vaags et al., 2009). Moreover, gene expression profiles of hES cells were different between FBS- and KSR-containing media (Skottman et al., 2006). They found a group of 50 adhesion-related genes was upregulated in hES cells cultured under FBS-containing conditions, while their adhesion was decreased when cultured in KSR. FBS is known as a cocktail containing many factors required for cell attachment, growth, and proliferation *in vitro* (Gstraunthaler, 2003), it is therefore likely that these unknown factors increase the expression of adhesion-related genes such as beta integrin-1 and vitronectin in the embryos, leading to stabilize cell to cell adhesion between trophoblast and feeder cells (Giancotti and Ruoslahti, 1999). Despite, FBS supports the blastocysts attachment, no significant difference of ICMs growth rate was observed among experimental groups. During culture, we found that the number of outgrowths cultured in medium containing with FBS and KSR formed primary colonies with ES-like morphology (64.4%) were higher than those cultured in FBS (33.3%) or KSR (17.8%) alone (Table 14). Unfortunately, all colonies maintained in FBS- and KSR- supplemented media appeared rapidly morphology of mixed differentiated cells after early subculture. These results indicated that neither FBS nor KSR alone are sufficient to establish outgrowths and generate self-renewal undifferentiated ES-like cells under *in vitro* culture. Similar results were also observed by others studies where pES cells were cultured in ES medium supplemented only with serum (Ropeter-Scharfenstein et al., 1996; Wianny et al., 1997; Li et al., 2004[b]; Ock et al., 2005) or with serum replacement (Lazzari et al., 2005).

In mES cells, culture condition without serum induces differentiation along the neural pathway. This inhibitory effect of serum is promoted by BMP signaling by blocking the activation of phosphorylated p38/MAPK (p-p38/MAPK) (Kunath et al., 2007) and through the expression of inhibitor of differentiation (Id) genes via the SMAD pathway (Finley et al., 1999; Ying et al., 2003). In porcine embryos, the expression of BMP4 and presence of p-p38/MAPK, indicating the beginning of primitive streak formation, have also been demonstrated in the epiblasts (Hall et al., 2009). This is similar with the expression of p-p38/MAPK in hES cells (Lee et al., 2007) which promotes differentiation via BMP pathway in hES cells (Xu et al., 2002; Qi et al., 2004). These findings suggest that BMP

signaling pathway may be involved in differentiation of pES-like cells in culture condition supplemented with FBS. On the one hand, hES cells cultured in medium containing KSR are subjected to high levels of BMP signaling activity (Xu et al., 2005), although bFGF supplement was presented in feeder-free condition. Nevertheless, this pathway could be inhibited by activin secreting from MEF or presenting in MEF-conditioned medium by induction of bFGF, resulting in activation of Activin/Nodal signaling pathway that is essential to support hESC self-renewal (Ogawa et al., 2007). However, our condition used STO cells to serve as feeder layer. It therefore is likely that residing factors in KSR are insufficient to promote pluripotent self-renewal of pES cells via interaction between bFGF and STO feeder cells. On the contrary, we found, in this study, that culture condition supplemented with FBS and KSR could maintain ES-like colonies in undifferentiated state after several passages, with a high activity of AP, expression of Oct4, Nanog and SSEA-4 proteins, and also the expression of specific genes including OCT4, NANOG and SOX2 (Figure 17). This is related to a recent study of Brevini et al. (2010) demonstrated that cultivation of porcine ES cell lines in containing medium with FBS and KSR could promote putative ES cell lines with undifferentiated morphology. By contrast, medium containing with KSR alone decreased the growth rate of ICMs, and also induced differentiated during culture. These data suggest that some factors existing in FBS and KSR may affect the maintenance of pES cells in undifferentiated state by cooperating with specific growth factors or cytokines secreted from STO feeder cells. Although two main growth factors, LIF and bFGF were added in this study, all ES-like colonies derived from six cell lines developed into differentiated cells at the fifth passage. This may be due to the difference in culture conditions between our system and previous study (Brevini et al., 2010) such as culture medium (knockout DMEM vs. DMEM/HAM‘S-F10, nutrient mix (without vs. with nucleoside mix), FBS:KSR volume (5:10 vs. 10:10%) affects pluripotent self-renewal of pES cells as previously described (Chaudhry et al., 2008). Furthermore, batch to batch of FBS, and KSR also have been indicated to influence on the maintenance of ES cells. However, it is noteworthy that self-renewal in ES cell must be a coordinated series of events that involves maintenance of the pluripotent state and the blockade of differentiation pathways which may be dependent upon species–specific difference. Mouse ES cells require BMP and LIF/JAK/STAT3 signaling pathways to sustain pluripotency and maintain self-renewal, whereas, in human and primate ES cells, the FGF pathway is necessary for proliferation and pluripotency (Mummery et al., 1993; Levenstein et al., 2006), and these processes are independent of STAT3 (Daheron et al., 2004; Humphrey et al., 2004; Sumi et al., 2004). BMP pathway also promotes differentiation in hESCs (Xu et al., 2002; Qi et al., 2004). Addition of noggin, a BMP4 antagonist can maintain pluripotency without lossing of long-term self-renewal. (Abeyta et al., 2004; Inzunza et al., 2005; Genbacev et al., 2005; Xu et al., 2005). Nevertheless, the cell signaling pathways that govern pluripotency in the pig are currently unknown. The inconsistent expression of LIFR have been reported in undifferentiated and 24 h cultured porcine ICMs, undifferentiated epiblast tissue (Blomberg et al., 2008; Hall et al., 2009; Brevini et al., 2010), but has not been found in pES-like cells (Vackova et al., 2007). Recent study, however, showed that supplement of LIF may be involved in maintenance of self-renewal in pESCs by activating phosphatidylinositol 3 kinase (PI3K) signaling pathway through protein kinase B (PKB or known as AKT), a major

effector of PI3K pathway, in stead of JAK/STAT3 pathway (Brevini et al., 2010). In addition, the expression of FGFR2 was also recently noted in pES cells, related to the presence of bFGF, FGFR1, and FGFR2 transcripts in porcine epiblasts (Hall et al., 2009). This may involve the FGF pathway in maintenance the pluripotency of ES cell in pigs.

In the present study, we concluded that culture condition containing a combination of FBS and KSR in ES medium improved the efficiency of pES cell derivation. However, further study is required in order to elucidate the networks of gene and factors that potentially regulate the pluripotency pathways of porcine embryonic stem cells.

CHAPTER V

INFLUENCE OF CULTURE CONDITION ON ESTABLISHMENT OF pES-LIKE CELL LINES: EFFICIENCY OF FEEDER CELL TYPES

5.1 Abstract

To evaluate the efficiency of feeder cells on the isolation and cultivation of undifferentiated porcine embryonic stem (pES)-like cells, two [porcine ear- and tail- skin fibroblasts (PESF, PTSF)] allogeneic- and three [cell lines from human foreskin fibroblasts (HFK), mouse embryosnic fibroblasts (MEF) and immortalized mouse embryonic fibroblasts (STO)] xenogeneic-derived feeders were used. *In vivo* derived zona pellucida (ZP)-free blastocysts were cultured on different mitotically inactivated feeder layers. The rate of ICM outgrowth and primary colony formation were observed, and further passaged onto new feeders. The characteristics of pESC including alkaline phosphatase (AP) activity, and pluripotent-related markers (Oct4, Nanog, SSEA-4) and genes (OCT4, NANOG, SOX2) were examined. Attached blastocysts cultured on HFK and STO feeder layers showed a higher percentage of ICM outgrowths than those cultured on PESF (76.7, 72.9 and 38.9%, respectively; $p<0.05$). The rates of primary ES-like colony formation and the number of putative ESC lines were significantly decreased when ICM outgrowths were cultured on PESF, compared to those cultured on HFK (30.6 vs. 76.7%, respectively; $p<0.05$). Only colonies from one (25%) and three (50%) cell lines derived from on PTSF and STO feeders, respectively, were further maintained in an undifferentiated ES morphology, while they presented all the characteristics of typical ES cells. Nonetheless, all characteristics disappeared when colonies cultured on PTSF and STO were continued at the 8^{th} and 6^{th} passage, respectively. It is concluded that feeder type plays an important role in establishing ES cells, while PTSF and STO cell lines were the best in maintaining porcine ES-like cells in an undifferentiated state.

5.2 Introduction

Embryonic stem (ES) cells are pluripotent cells having the capacity of indefinite self-renewal, while maintaining their ability to differentiate into most cell types. These cells are generated from the inner cell mass (ICM) or epiblast of blastocyst stage embryos. The first stable ES cell line was established from a mouse in 1981 (Evans and Kaufman, 1981), after which stable ES cell lines have also been generated in other species including primate monkeys (Thomson et al., 1995) and humans (Thomson et al., 1998). These cells provide an effective approach to studying cell biology during embryogenesis, cell reprogramming and therapeutic applications for regenerative medicine.

Porcine (p) ES cells have been demonstrated to have a remarkable potential for stem cell research from both the agricultural perspective and in biomedical fields due principally to the greater similarities in anatomy, physiology, immunology and lifespan with humans compared to mice and other species (Ibrahim et al., 2006; Matsunari and Nagashima, 2009). Although the generation of pES cell lines has been published since 1990, no conclusive

results have been obtained from the isolation and propagation of putative pES cell lines (Evans et al, 1990; Strojeck et al., 1990; Piedrahita et al., 1990).

Based on the protocols used for derivation of mouse (m) ES cell lines, most isolated ICMs and pES cells have been commonly cultured and maintained on basic feeder layers, mouse embryonic fibroblasts (MEF) and SIM mouse embryo-derived thioguanin- and ouabain-resistant (STO) cell lines (Li et al., 2004; Kim et al., 2007; Blomberg et al., 2008; Panasophonkul et al., 2010). Currently, prolonged culture of undifferentiated human and primate ES cells can be maintained on their allogeneic-derived feeders from various sources of skin fibroblasts with characteristics similar to those ES cells cultured on MEF and STO feeders (Richards et al., 2003; Park et al., 2004; Li et al., 2005). These feeder cells keep ES cells away from the ‗cross-species' pathogens and/or foreign proteins that may be contaminated during ES cell culture. Additionally, the use of feeder cells derived from skin fibroblasts is more practical with a less invasive technique, in particular the ease of obtaining skin biopsies, than using by feeders developed from foetuses or embryos. In pigs, pES-like cell lines have been produced using both allogeneic and xenogeneic feeder cells but the overall success in the establishment of pES cell lines using these feeder cells has been poor and inconsistent among laboratories (Piedrahita et al., 1990; Talbot et al., 1993; Ropeter-Scharfenstein et al., 1996; Li et al., 2004). Furthermore, pES cells cultured in feeder-free conditions failed to continue replication and started senescence or differentiation (Hochereau-de-Reviers and Perreau, 1993; Brevini et al., 2007), suggesting the important role of the feeder in the culture and propagation of ES cells in pigs.

This study aimed to examine the effects of feeder types on the derivation of ICM outgrowths and porcine embryonic stem-like cells.

5.3 Materials and Methods

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

All materials and methods in this experiment were described in **Chapter III** including production of embryos (*in vivo* fertilization; IVV), isolation and culture of inner cell mass (ICM), characterization of ES cells (alkaline phosphatase activity, imunocytochemistry, and RT-PCR analysis). Some different methods from above were described as followed.

5.3.1 Feeder layer preparation

Five types of fibroblast cells including porcine ear-skin fibroblasts (PESF), porcine tail-skin fibroblasts (PTSF), human foreskin fibroblasts cell line (HFK; CRL-2429, ATCC), a continuous cell line of SIM mouse embryo-derived thioquanine and ouabain resistant (STO; CRL-1503, ATCC), and a CF1-mouse embryonic fibroblasts cell line (CF1-MEF; CRL-1040, ATCC) were used as feeder cells in order to compare their efficiency on the derivation of pES cells.

5.3.1.1 Isolation and culture of feeder cells

PESF and PTSF were aseptically established from the ear and tail skins of a 4-week old piglet, respectively. Briefly, both ear and tail pieces were collected from a dead piglet,

washed twice with disinfectant and then preserved in PBS supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (washing solution). The tissue samples were transported at 4 °C on ice to the laboratory. Upon arrival, the skin samples were washed three times in washing solution and individually dissected in Dulbecco‘s modified Eagle‘s medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 1% (v/v) Antibiotic-antimycotic® (Invitrogen). They were cut into small pieces and cultured separately onto 60 mm culture dishes in a culture medium (FM) containing DMEM-high glucose supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 1% (v/v) L-glutamine (Glutamax® 100x, Invitrogen), and 1% (v/v) antibiotic-antimycotic®. The growth of primary cells was observed and the medium was changed every other day. After 10 to 14 days, confluent primary monolayer cells were established. All primary cultures were serially passaged with 0.05% (w/v) trypsin-EDTA (Invitrogen, Carlsbad, USA) and then cryopreserved in a freezing medium containing 90% FM and 10% (v/v) dimethyl sulfoxide (DMSO) using a freezing container (Nalgene®, NY, USA). The feeder cells were then stored in liquid nitrogen until further use. When required, porcine feeder cells were thawed and further expanded for at least 1 passage prior to use.

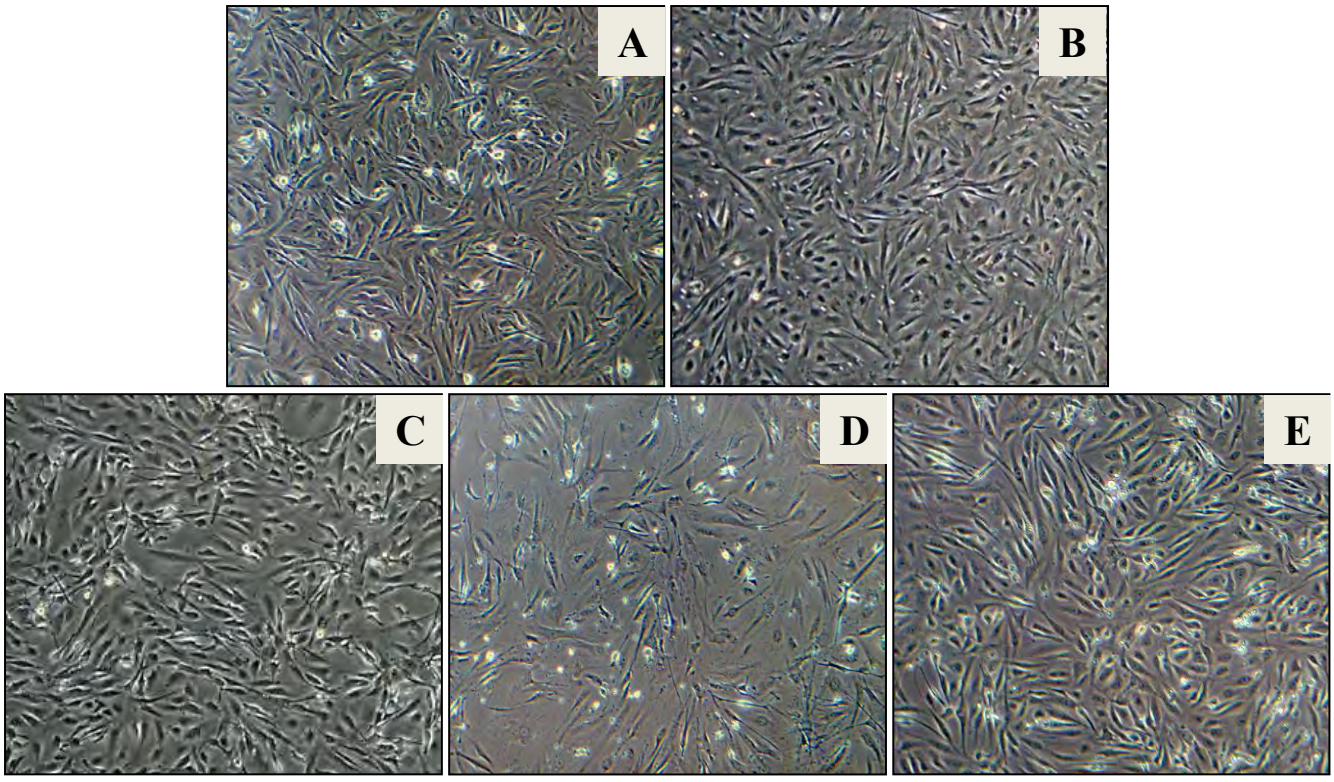

Figure 19 Morphology of mitotically inactivated feeder cells derived from porcine ear skin fibroblasts (PESF, A); porcine tail skin fibroblasts (PTSF, B); Human foreskin fibroblasts (HFK2429, C); CF1-mouse embryonic fibroblast (CF1-MEF, D); a continuous cell line of SIM mouse embryo-derived thioquanine and ouabain resistant (STO, E). Magnification at x100.

The commercial cell lines of HFK, STO, and CF1-MEF were purchased from American Type Culture Collection Institute (ATCC, Manassas, USA). They were thawed and expanded for up to 2 times in the same medium as used for porcine feeder cells in order to multiply the cell numbers.

5.3.1.2 Preparation of feeder layers

All frozen feeder cells were thawed and expanded in FM. They were subcultured and mitotically inactivated with 10 μg/ml mitomycin-C for 2.5 h in FM. Cells were dissociated by trypsinization using 0.05% (w/v) trypsin-EDTA solution. After centrifugation at 1000 rpm for 5 min., the pellet was then resuspended with FM and seeded into a 1-well dish (BD Falcon^{TM}, NJ, USA) pre-coated with 0.1% (w/v) gelatin at a density of $3.5x10^4$ cells/cm^2 for PESF, PTSF, HFK, and CF1-MEF cells and $5.5x10^4$ cells/cm^2 for STO cells (Figure 19). All feeder cell types used as feeder cells were between the 3rd and the 10th passage, except that the CF1-MEF was used between the 2nd passage and the 5th passage as previously described (Villa-Diaz et al., 2009).

5.4 Statistical Analysis

Data was pooled at least 3 independent replicates and expressed by mean ± standard error of the mean (SEM). The results were analyzed by one-way ANOVA and protected least significant different (LSD) statistical tests using SPSS software (version13.0 SPSS Inc., Chicago, IL). A value of $p< 0.05$ was considered to be statistically significant.

5.5 Experimental design

In order to evaluate the efficiency of feeder cells on the derivation of ICM outgrowth and pES-like cells, *in vivo* hatched and intact ZP-free blastocysts were cultured on five different feeder types. The rates of attached blastocysts and the primary colony of ES-like cells were recorded. The putative ES cells were characterized by morphological appearance, the expression of generalized pluripotent markers such as AP activity, and Oct4, Nanog, and SSEA-4 proteins. The expression of specific genes (OCT4, NANOG, SOX2) was also analyzed by RT-PCR.

5.6 Results

5.6.1 Efficiency of feeder cell types on derivation of putative pES cells

The derivation of pES-like colonies was affected by the types of feeder layer. As shown in Table 16, all intact whole ZP-free blastocysts adhered to the feeder layers within 24 to 48 h of plating. Attached blastocysts cultured on HFK and STO feeder layers had a significantly higher percentage of ICM outgrowths than those cultured on PESF (76.7%, 72.9% and 38.9%, respectively; $p<0.05$). These rates however did not differ when compared with PTSF and MEF feeder layers (63.3% and 55.6%, respectively; $p>0.05$). After the removal of ICM outgrowths onto new feeder layers, the rates of primary ES-like colonies formation and the number of putative ES cell lines were significantly decreased when the outgrowths were continuously cultured on PESF, compared to those cultured on HFK (30.6% vs 76.7%, respectively; $p<0.05$).

Table 16 Efficiency of various feeder cell types on the derivation of porcine ES-like cells

Feeder type	No. of cultured blastocysts (n)	No. of attached blastocysts (%)	No. of ICM outgrowths (%mean ±SEM)*	No. of primary ES-like colonies (%mean ±SEM)**	No. of pES-like cell lines (%mean ±SEM)***
PESF	11	11 (100)	4 (38.9±13.9^{a}	3 (30.6±19.4)^{a}	1 (11.0±11.0)^{a}
PTSF	13	13 (100)	8 (63.3±18.6)^{a,b}	7 (56.7±23.3)^{a,b}	4 (31.7±9.3)^{a,b}
HFK	14	14 (100)	11 (76.7±5.1)^{b}	11 (76.7±5.1)^{b}	7 (47.8±7.8)^{b}
MEF	13	13 (100)	7 (55.6±8.0)^{a,b}	7 (55.6±8.0)^{a,b}	2 (17.8±9.7)^{a,b}
STO	15	15 (100)	11 (72.9±2.1)^{b}	10 (66.7±5.9)^{a,b}	6 (39.6±11.9)^{a,b}

* Percentage of ICM outgrowth; ** percentage of primary ES-like colonies; *** the number of pES-like cell lines/total number of attached blastocysts

^{a,b} Different superscripts within the same column indicate significant differences (p<0.05).

In our culture condition, the outgrowths of ICM appeared within 3 to 5 days after plating (Figure 20A, D, G, J, M) and pES-like colonies were initially formed between 6 and 8 days of culture (Figure 1B, E, H, K, N). The onset of ICM outgrowths and formation of ES-like colonies was not influenced by the type of feeder layers but the PTSF and STO feeder

Table 17 Effects of feeder cell types on propagation of porcine ES-like cell lines

Feeder types	Total no. of pES-like cell lines	No. of cell lines with maximum passage; P (%)	
		<P5	≥P5
PESF	1	1 (100)	0
PTSF	4	3 (75)	1 (25)
HFK	7	7 (100)	0
MEF	2	2 (100)	0
STO	6	3 (50)	3 (50)

cells maintained pES-like cells in an undifferentiated state better than other feeder cell types (Table 17). The cell lines grown on PTSF (25%) and STO (50%) remained undifferentiated in culture for more than 5 passages, whereas all colonies cultured on PESF, HFK and MEF spontaneously differentiated or stopped cell replication after a few passages. Putative ES cells growing on various feeder layers developed into colonies with an obvious morphological heterogeneity. Porcine ES-like colonies cultured on STO maintained a tightly packed dome shape and had distinct margins (Figure 20N), while the colonies cultured on PESF, PTSF,

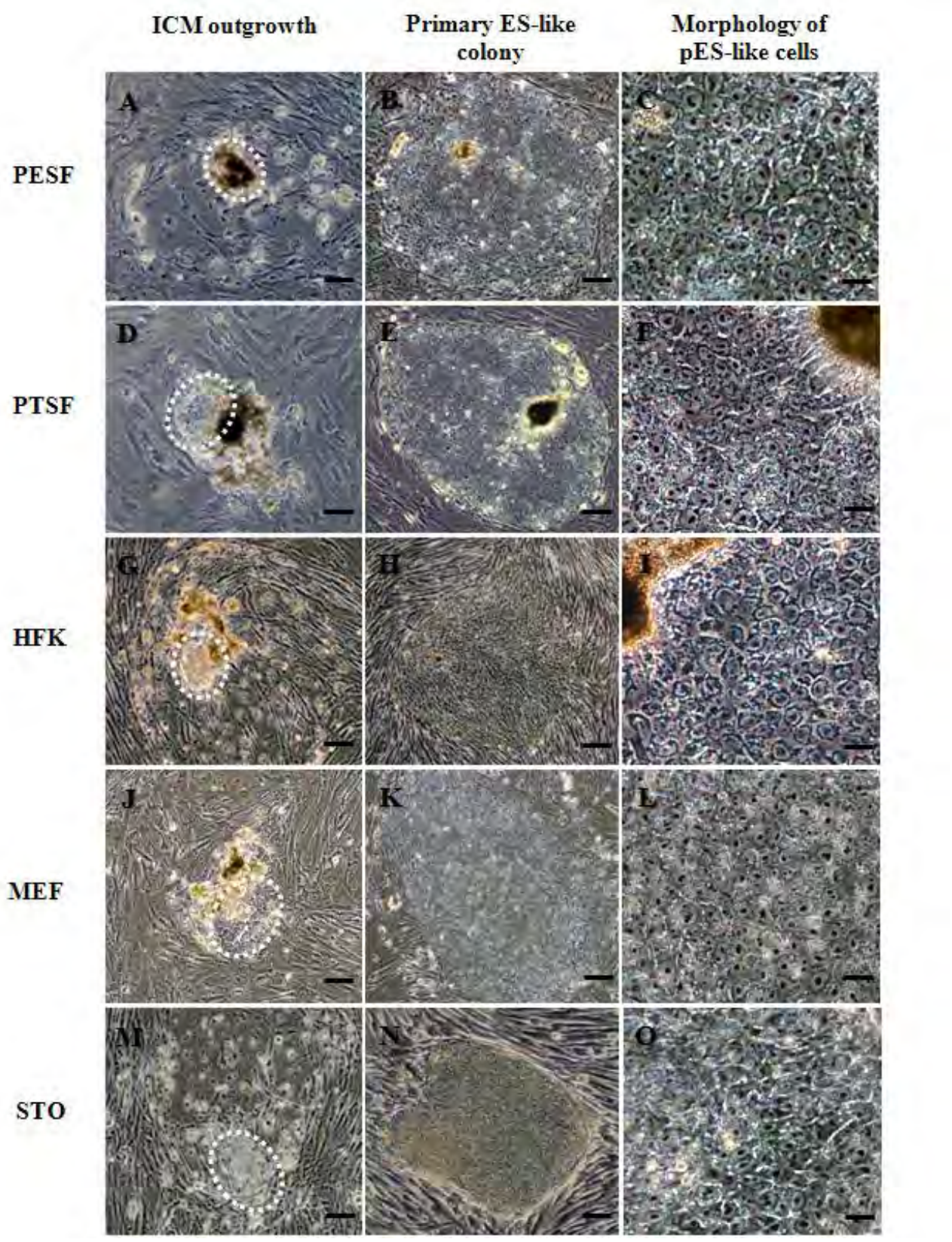

Figure 20 Derivation of porcine ES-like cells cultured on various feeder types. Representative images show the outgrowths of ICM (dash line; 1st vertical line) on day 3, primary ES-like colonies formation (2nd vertical line) on day 7, and the morphology of primary ES-like cells (3rd vertical line) at high magnification, after plating on PESF (A-C), PTSF (D-F), HFK (G-I), MEF (J-L), STO (M-O). All colonies developed on different feeder cell types exhibit morphology typical of ESC with a high nucleus to cytoplasm ratio, prominent nucleoli, and tightly packed cells. Scale bars represented in 1st and 2nd vertical lines = 100 μM, and 3rd vertical line = 50 μM.

HFK and MEF exhibited the same pattern, a flattened shape with obvious borders (Figure 20B, E, H, K). These colonies, however, showed morphological resemblance to human ES or primate ES cells. Although the growth characteristics of pES-like colonies were different among feeder layer types, all colonies were contained with small and round cells, and had a high nucleus to cytoplasm ratio with one or two prominent nucleoli under high magnification (200x; Figure 20C, F, I, L, O). Colonies from one putative ES cells line originated on PTSF maintained an undifferentiated form until 8 passages, and colonies from the three other cell lines developed on STO remained in culture for 5 to 6 passages before cell differentiation was observed. These colonies spontaneously differentiated into multiple cell types such as neural-like cells or neural-rosette formation (Figure 21A), fibroblast-like (Figure 21B), endoderm-like (Figure 21D), trophoblastic-like (Figure 21E), and also large flattened-differentiated cells (Figure 2F). They started the differentiation from the center rather than the periphery of colonies (Figure 21C).

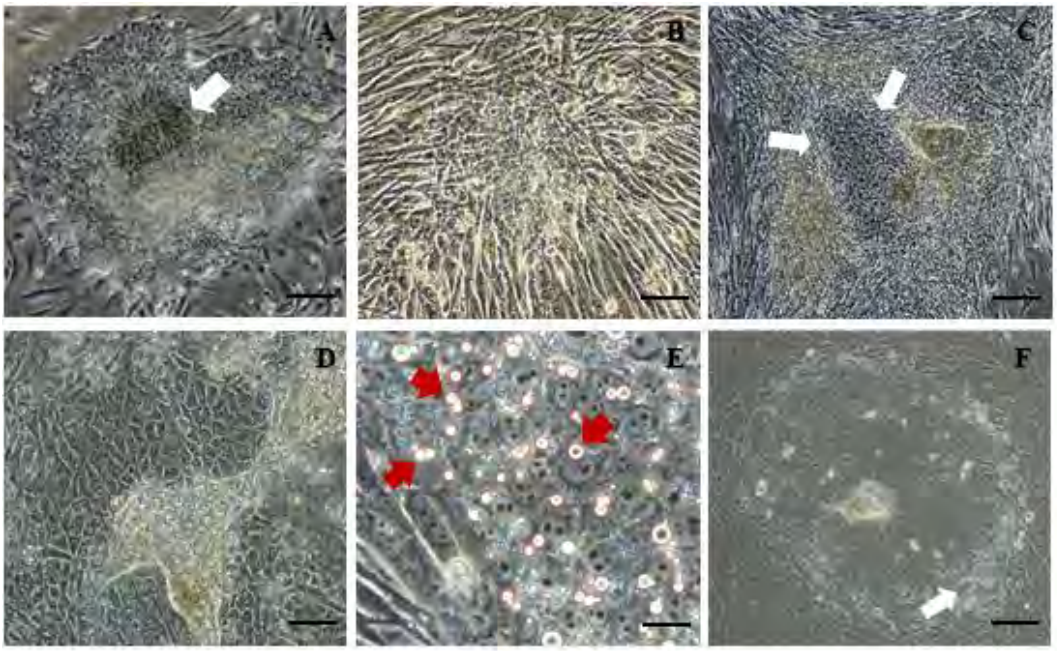

Figure 21 Morphology of spontaneously differentiated colonies developed on different feeder cell types. Porcine ES-like colony cultured on STO formed (A) a neural rossette-like structure (arrow) or differentiated into (B) fibroblast-like cells. (C) pES-like colony developed on HFK shows a group of differentiated cells at the center of colony (arrows) surrounded by undifferentiated area. pES-like colony grown on CF1-MEF differentiated into (D) endoderm-like cells, or (E) trophoblast-like cells; note the characteristic lipid droplets in trophoblast cells (arrows) (scale bar = 50 μM). (F) Colony developed on PTSF, arrow shows a group of undifferentiated cells at the edge of colony with the large differentiated area. Scale bars represented in A-F except E = 100 μM.

5.6.2 Characterization of putative pES cells

Porcine ES-like colonies were characterized by the expression of AP activity, and pluripotent-specific markers. After the first few passages, colonies from most cell lines cultured on various feeder layers presented a low or non-detectable AP activity as well as the

expression of Oct4, Nanog, and SSEA-4 markers associated with appearance of spontaneous differentiation. However, undifferentiated colonies from one and three cell lines derived on PTSF and STO feeder layers, respectively, showed a strong AP activity (Figure 22A, B) with high expression of Oct4 (Figure 22C, D), Nanog (Figure 22E, F), and SSEA-4 (Figure 22G, H) proteins. Furthermore, the expression of specific genes was assessed in these cell lines using RT-PCR. All colonies derived from these cell lines expressed OCT4, NANOG and SOX2 while the expression of these genes was absent in either PTSF or STO feeder cells (Figure 23). Regrettably, all characteristics disappeared when the colonies were cultured on PTSF and STO were continued to the 8th and 6th passages, respectively.

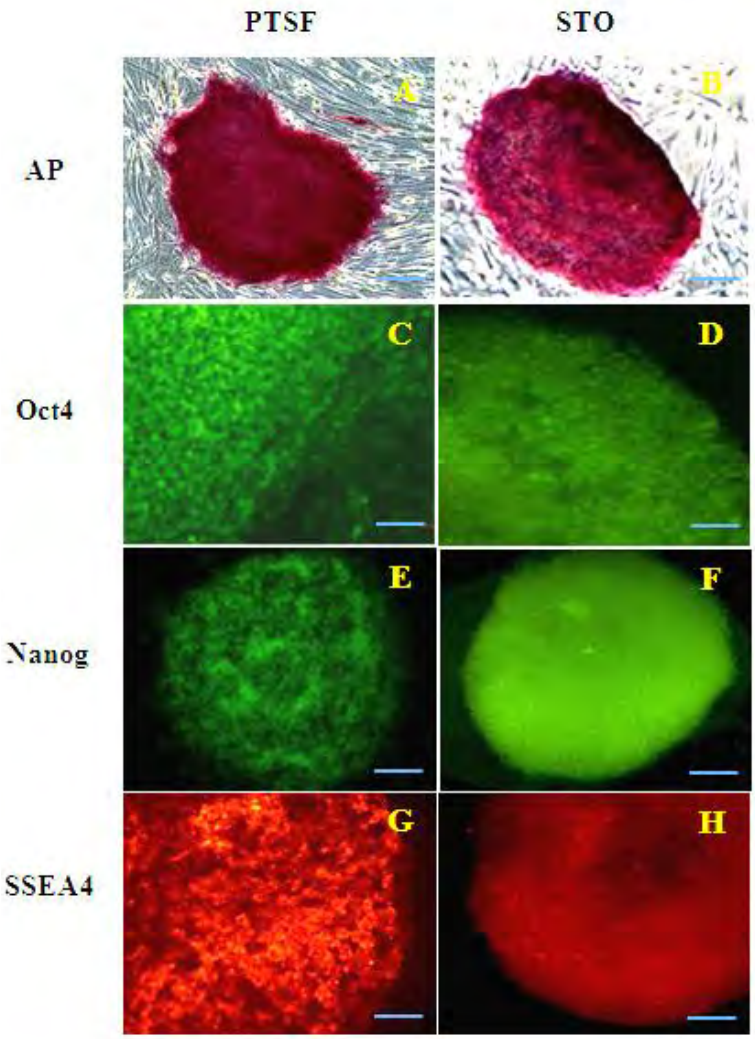

Figure 22 Characteristics of porcine ES-like colonies grown on PTSF (1st line) and STO (2nd line) feeders at passage 5. Representative images show, (A,B) positive alkaline phosphatase activity and immunostaining expression of Oct4 (C,D), Nanog (E,F) and SSEA4 (G,H). Oct4 (FITC, green) and Nanog (FITC, green) specifically expressed in the nuclei, and SSEA-4 (TRITC, red) is expressed on the cell surface. Representing scale bars in staining of AP and Nanog= 100 µM, and Oct4 and SSEA-4=50 µM.

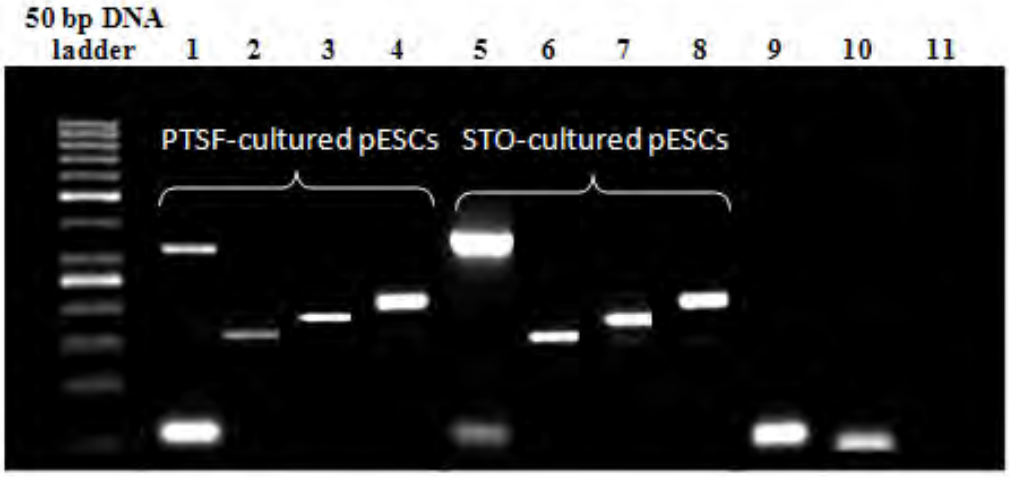

Figure 23 Expression of OCT4, NANOG, SOX2 and GAPDH in putative porcine embryonic stem cell (pESC) lines detected by RT-PCR. These genes were detected in undifferentiated pES-like cells but not detected in differentiated and feeder cells. Lane 1: 50 base pair (bp) DNA ladder; lane 2: PTSF, lane 3-6: undifferentiated PTSF-pESCs; lane 7: differentiated pESCs; lane 8 STO, lane 9-12: undifferentiated STO-pESCs; lane 13: differentiated pESCs; lane 14: negative control.

5.7 Discussion

Maintenance in an undifferentiated state of porcine ES-like cells remains a major obstacle, although several attempts have been made to search for an optimal condition for suppressing the differentiation, and proliferating pES cells during long-term culture. In our study, five different feeder cells derived from both allogeneic (PESF and PTSF) and xenogeneic (HFK, MEF, and STO) origins were used to evaluate their efficiency on promoting ICM outgrowths and maintaining pluripotency in undifferentiated pES cells. The results demonstrated that, under our culture conditions, porcine ES-like cells were generated in an undifferentiated state on these feeder layers but they had varying capabilities in promoting the porcine embryonic development *in vitro*.

The time required for blastocyt attachment onto the feeder cells appears to be the first key in avoiding early death or differentiation of ICM after plating. In this study, we found that hatched blastocysts adhered onto different feeder layers (within 24 h) faster than enzymatic-treated ZP-free blastocysts (between 36 to 48 h), although overall attachment rates (p>0.05) were not different among feeder cell types. This may be due to the enzymatic treatment used may damage the trophectoderm (TE), resulting in delay/reduction of cell-extracellular matrix (ECM) interaction (Richoux et al., 1989; Mecham, 1991) thereby influencing cell attachment and movement between blastocysts and feeder cells.

Several porcine feeder cells prepared from allogeneic fibroblasts such as porcine uterine cells, porcine embryonic fibroblasts, and porcine granulosa cells have been used to isolate ICM cells and maintain their pluripotency (Strojeck et al., 1990; Piedrahita et al., 1990[b]; Hochereau-de-Reviers et al., 1993; Ropeter-Scharfenstein et al., 1996). However, the efficiency of these feeder cells was rather low in supporting blastocyst attachment and only the first few passages could be maintained. This is contradictory to our result that allogeneic feeder cells derived from porcine tail skin fibroblasts (PTSF) were able to produce a

microenvironment in which the derivation of primary colonies and putative pES cell lines was supported. It is likely that each feeder cell type provides a different microenvironment for stem cell survival and proliferation (Nagano et al., 2003). Furthermore, difference in secreted molecules from feeder cells also plays a different role in activating signaling pathways of pluripotency and self-renewal in pES cells. Nevertheless, PESF-derived feeder showed a low efficiency in promoting and maintaining growth and proliferation of pES cells during culture (Table 16). This finding suggests that the origin (i.e. different location) where feeder cells were obtained could also affect the feeder‘s ability on the derivation of pES cells.

In the present study, we found that not only PTSF, but three xenogeneic HFK, MEF, and STO feeders also similarly encouraged the derivation of pES-like cells. However, continuous culture of pES-like cell lines developed on MEF and HFK feeders, represented a quick colony differentiation in the few early passages. In contrast, STO showed a better result for supporting the undifferentiated growth of pES-like colonies, similar to the previous reports (Chen et al., 1999; Ock et al., 2005; Kim et al., 2007). Microenvironments produced from immortal STO cell lines may be more stable than those produced from mortal cell lines as MEF and HFK. Furthermore, the variability between batch and batch of MEF and HFK cell lines may also affect the propagation and maintenance of pES-like cell lines (Park et al., 2004; Villa-Diaz et al., 2009). Only ES-like colonies growing on STO and PTSF demonstrated self-renewal in an undifferentiated state after continuous subculture. They presented all the characteristics of typical ES cells such as morphology, alkaline phosphatase activity, and the expression of pluripotency-related markers and genes. We also detected the expression of surface antigen marker SSEA-4. The high expression of SSEA-4 in undifferentiated colonies is in agreement with a recent study of Brevini et al. (2010) and also previous studies in primates and humans ES cells (Henderson et al., 2002; Li et al., 2005). Although these putative ES cell lines could be continuously passaged in an undifferentiated stage, the mixture of differentiated cells was initially found in these colonies after the sixth to eighth passages even LIF and bFGF were supplemented in this study.

A previous report demonstrated that LIF signaling may not to be an essential pathway for the derivation of pESCs because gp130 and LIF receptor transcripts, two specific subunits of LIF receptors, were absent in pES-like cells (Vackova et al., 2007) These findings has been further confirmed by previous studies of Blomberg et al. (2008) and Hall et al. (2009) indicating that LIFR did not express in the epiblast cells of early embryos. However, Brevini et al. (2010) recently suggested that supplement of LIF may be involved in the maintenance of self-renewal in pES cells by activating phosphatidylinositol 3 kinase (PI3K) signaling pathway through a protein kinase B (PKB or known as Akt), a major effector of PI3K pathway, in stead of JAK/STAT3 pathway. This finding also relates to our observation indicating that pES cells cultured in non LIF-supplemented medium have a slower proliferation than those cultured in medium supplemented with LIF. The expression of FGFR2 was also recently noted in pES cell (Brevini et al., 2010) which related to the presence of bFGF, FGFR1, and FGFR2 transcripts in porcine epiblasts (Hall et al., 2009), nevertheless, the function of bFGF in maintaining self-renewal and pluripotency in pES cells is remain unknown. In the present study, neither supplementation of LIF nor bFGF in culture medium was sufficient to inhibit spontaneous differentiation in putative porcine ES cells. It is possible that suboptimal culture conditions, including medium types, nutrient supplements

and culture techniques affected the maintenance in an undifferentiated growth of pES-like cells. In addition, it is worthy noted that difference in species-specific factors may affect the signaling pathways regulating pluripotency in pES cells as previously described in other mammalian species (Renard et al., 2007). For example, mESC requires BMP4, a member of the transforming growth factor-β (TGF-β) family involved in controlling mESC differentiation. On the contrary, the presence of this factor in hES cells is not only dispensable in maintaining their pluripotency, but is also detrimental as it induces differentiation of hES cell into trophoblast cells (Xu et al., 2002).

In conclusion, feeder cell types affect the establishment efficacy of pES-like cells. Our study also demonstrates that the efficiency of allogeneic PTSF feeder is closely related to that of xenogeneic STO-derived feeder in promoting an undifferentiated growth of pES-like cell lines. Although these cell lines could not retain the capacity for unlimited self-renewal following long-term culture. The variation in the feeders‘ abilities and suitability of culture conditions have been concerned in species-specific difference, leading to the success of pES cell establishment and maintenance of their pluripotency. Therefore, the comparatively transcriptome analysis of ES cell across different species will hopefully identify more fundamental signal transduction pathways for ES cell maintenance in pigs.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Embryonic stem (ES) cells are generally known to provide several benefits for functional cell research and also biomedical applications. To date, cell transplantation and replacement therapy using stem cells have considerably been applied in order to treat a large number of human diseases, such as Parkinson‘s disease, Alzheimer‘s disease, spinal cord injuries and heart failure, all of which can not efficiently be treated with traditional therapy. Because of its significance, more than 1000 ES cell publications per year have been launched from different countries between 1981 and 2010. In fact, technology of the ES cell production has also been developed in Thailand, aimed specifically to produce certain cell lineages for fundamental research and regenerative medicine. Several projects of ES-cell research in Thailand are pursued using only laboratory animals as a model due principally to the availability of biological data of ES-cell culture. However, differences in physiology and organ‘s size between laboratory animals and human have unfortunately become a major problem when clinical applications of those ES cells are required. Several attempts, therefore, try to create ES cell lines from the pig due to its physiology and immunology relatively similar to human, providing a potential source of cells for cell transplantation and for studying diseases and disease mechanisms in humans as well as in domestic animals. Nevertheless, the culture system suitable for mouse (m) ES cells appears not capable of maintaining porcine (p) ES cell self-renewal for long-term culture. In addition to the culture system, the use of *in vivo* blastocysts acquired from the reproductive tracts of inseminated pigs limits the availability of embryos to be used for ES study.

In order to develop and establish ESC research in our laboratory, hence, the improving strategy for *in vitro* embryo production (IVP) using parthenogenetic activation (PA) and *in vitro* fertilization (IVF) techniques was firstly studied in terms of developmental competence and quality of embryos. Subsequently, the efficiency of embryos derived from IVF and PA in comparison to *in vivo* counterparts was investigated in order to generate pES cells. Finally, the derivation of putative ES cell lines was examined through the conditioned effect of serum supplementation and feeder cell types.

6.1 Improving IVP strategy by using PA and IVF techniques

Nowadays, embryo produced from PA and IVF techniques are generally known as an alternative source for supporting several researches including establishment of embryonic stem cells. However, difference in protocols/methods using for PA/IVF among laboratories affect the results of developmental competence and quality of embryos. In the present study, the oocytes activation by SD9 pulse stumulator with the lowest field strength of 1.0 kV/cm with 3 pulses at 80 μsec (protocol III) could promote the high potential of embryonic development compared to those by 1.36 kV/cm, 30 μsec, 2 pulses (protocol I) and 1.5 kV/cm, 60 μsec, 2 pulses (protocol II). Our results related to the previous reports which described that an interaction between pulse numbers and duration affected the development of electrical activated oocytes (Prather et al., 1989; Ozil and Huneau, 2001; King et al., 2002). It is likely

that too low field strength (less than 1.2 kV/cm) and low pulse numbers are insufficient to increase calcium influx to the level needed for oocyte activation (Collas et al., 1993). In contrast, multiple pulses of high field strength (more than 1.2 kV/cm) have also been shown to be detrimental to the oocyte development (Lee et al., 2004). Meanwhile, fertilized oocytes *in vitro* with the lowest ratio of sperm per oocyte at 1000:1 enhanced the monospermic penetration rate and the embryo capability developing to blastocyst stage compared to those fertilized with the higher ratio from 2000 to 4000 per oocyte, indicating that a relative reduction of sperm numbers during *in vitro* fertilization results in greater monospermic penetration rates (Xu et al., 1996; Abeydeera and Day, 1997; Gil et al., 2004). Our results obtained a higher rate of monospermy (82%) when 1000 sperm per oocyte were used for IVF compared with 50-60% from other studies. It is likely to because of sperm quality (Popwell and Flowers, 2004; Gadea, 2005) and the inter- and intra-boar variability (Suzuki et al., 2003). In addition, suboptimal oocyte maturation, leading to an improper pattern of cortical distribution and exocytosis (Wang et al., 1997[b]), including IVF systems caused by culture medium and/or sperm incubation time may be involved in the penetration of polyspermy (reviewed by Funahashi, 2003). The higher incidences of blastocyst formation could be derived from both IVP techniques when PA with three pulses at 80 μsec (protocol III) or IVF with the lowest 1000 sperm per oocyte was used, although the difference in ICM and TE cell number was not observed among experimental groups in both PA- and IVF-derived embryos. This suggests that the different quality of pig embryos produced *in vitro* is more influenced by culture medium used for embryo cultivation rather than IVP technique (Nánássy et al., 2008). Furthermore, it is also likely that embryos have intrinsic ability to control the optimal number of ICM probably via programmed cell death (Hardy et al., 2003) and gene regulating pathways (Marikawa and Alarcón, 2009), affecting ICM segregation and self-renewal.

The role of small-molecule inhibitors of the MEK/ERK pathway (PD0325901) and GSK-3 (CHIR99021), in this study, could increase early development and quality of porcine embryos produced *in vitro*, and simultaneously promote a pluripotent state in the ICM cells. These findings relate to previous studies using these inhibitors for enhancing the derivation of mouse and rat ES cell lines (Buehr et al., 2008; Nichols et al., 2009[a]) and inducing ground state pluripotency in mouse embryos, however, these inhibitors showed different results in the number and stage of blastocyst development. Cultured embryos in MEKi with (2i) or without GSK-3i increased the number of cleaved embryos and blastocysts but was not observed in those cultured only in GSK-3i. This suggests that suppression of MEK/ERK signaling may affect embryonic development rather than Wnt/beta-catenin signaling. In fact, both MEK/ERK and PI3K/Akt pathways have been documented to involve with early mammalian development (Riley et al., 2005; Neill, 2008), and these pathways could be activated via Ras signaling (Rodriguez-Viciana et al., 1994). Nevertheless, the activation of MEK/ERK pathway seems to be lesser important to early embryo development when previous data indicated that mouse embryos eliminated Erk1 and Erk2 genes can viable during preimplantation (Pages et al., 1999; Hatano et al., 2003). It is therefore likely that blockade of MEK activity may upregulate downstream of Ras signaling, resulting in the activation of PI3K signaling, an alterative pathway promoting embryonic growth. The development of ICM and TE cells was exhibited by Oct4 and Cdx2 expressions, respectively. The higher expansion of ICM cells and increasing ratio of ICM:TE cells were also detected in

embryos suppressed activity of MEK/ERK pathway which was similar to a previous study in mouse embryos demonstrated that the naïve epiblast, a sub-set of ICM cells, can develop and expand in conditions in which FGF/Erk signaling is inhibited (Nichols et al., 2009). On the contrary, treated embryos with GSK-3i and 2i did not show the improvement of ICM cells or ICM:TE ratio. This result assured that activation of Wnt signaling pathway may not be implicated in porcine embryo development during preimplantation period. The expression of Oct4 protein in this study was similar to previous studies demonstrating its localization in both ICM and TE cells (Kirchhof et al., 2000; Kuijk et al., 2008; Hall et al., 2009).

The localization of Nanog which is specific for epiblast lineage, a subunit of ICM, was also detected in some embryos treated with MEKi and/or GSK-3 inhibitors. Interestingly, the number of Nanog-positive embryos were significantly expanded in condition treated with MEKi compared to other groups. However the number of Nanog-positive cells observed in these embryos showed similar results among treatment groups, but they showed a higher tendency when compared to untreated control (data not shown). These findings suggest that the pluripotent state of ICM cells may not depend only on signaling of MEK/ERK pathway but may be also involved with the activation of Wnt/β-catenin pathway. The presence of Nanog protein was also detected in untreated embryos which is contrast with previous studies of Kuijk et al. (2008) and Hall et al. (2009). Nevertheless, some studies found Nanog mRNA expression in either D6 or D7 of blastocysts produced *in vivo* and *in vitro* (Kumar et al., 2007; Magnani and Cabot, 2008). It is therefore possible that genes transcription and translation processes may be influenced by the quality of derived embryos from different conditions. The existence of GATA6 in some ICMs cells indicates the formation of primitive endoderm (known as hypoblast) lineage which could be eliminated by cultivating embryos in medium treated with 2i (Nichole et al., 2009). However, hypoblast formation could not be inhibited in our study. It is likely that developmental age of embryos in different species affect the translation and transcription processes of genes involving in fate change of ICM during embryo development. Furthermore, differences in embryos origin (*in vivo* vs. *in vitro*) should also be considered as another factor influence genes and proteins expression.

6.2 Sources of embryo differently affect the pES cell establishment

To produce pES cells, embryonic source is offered as the first criterion necessary for the outgrowth of ICM and primary ES-like colony. In the present study, the effect of different embryo sources on the establishment of pESCs was conducted by using embryos derived from PA, IVF and *in vivo* fertilization (IVV). The results obtained were similar to previous reports that embryo produced *in vitro* could be used to serve as an alternative source for generating pig ES-like cells, although their quality is lesser than those produced *in vivo* (Li et al., 2004[a]; Kim et al., 2007; Ock et al., 2007). However, only blastocysts derived from IVF and IVV, but not from PA, could promote ICM outgrowths and formed primary colonies like ESCs. This failure may be caused by the quality of embryos *per se* that differed between the activation protocols (Zue et al., 2002; Cong et al., 2008) and also between the culture conditions, leading to an alternation of cell number and expression of pluripotency-related genes in ICM (Boiani et al., 2005; Cervera et al., 2010). However, primary ES-like colonies can be produced from IVF blastocysts, the formation rate of these colonies was rather low compared to those developed from *in vivo* counterparts. These findings related to previous

studies indicated a higher amount of Nanog and Sox2 transcripts which increased in abundance at the blastocyst stage of developing embryos derived from *in vivo* than *in vitro* production, although relative abundance of Oct4 is not different among these embryo sources (Lee et al., 2006; Kumar et al., 2007). It is cleared that the high expression levels of these genes affect the derivation of pES-like cells. Unfortunately, undifferentiated colonies developed from IVF and IVV blastocysts spontaneously differentiated into multiple cell types after the 5th passage. Maintenance of these colonies in undifferentiated stage *in vitro* is one of the major obstacles of establishment of ES cell line in this species. In fact, culture conditions suitable to support mouse and human ES cell may be inadequate to maintain ESC in pigs. Difference in medium components plays a pivotal role in regulating intrinsic and extrinsic factors involved in the control of pluripotency, that means specific pathways may be up-regulated, or down-regulated, in response to the addition of specific molecules which have been shown to be different among species (Hanna et al., 2009; Brevini et al., 2010).

6.3 Culture condition affecting the derivation of putative pES cell lines

Apart from embryo quality, technical aspects of ES cell culture are also important since signaling pathways that regulate the pluripotency of porcine ES cells have not yet been clarified. Two principal factors including serum and feeder cells have been focused in this study in order to evaluate their efficacy on derivation of stable pES cell lines. Isolation and maintenance of pES cells were performed in culture conditions containing different ratios of fetal bovine serum (FBS) and knockout-serum replacement (KSR) (20:0, 10:10, 0:20). Our results indicated that the presence of FBS in culture medium enhanced the attachment of viable blastocysts**.** This finding suggests the potential effect of FBS containing several unknown factors required for cell attachment, growth, and proliferation *in vitro* (Gstraunthaler, 2003). These factors increase the expression of adhesion-related genes, leading to cell stabilization and adhesion between trophoblast and feeder cells (Giancotti and Ruoslahti, 1999). Although culture with medium containing KSR alone decreased the rate of attachment and outgrowth of ICMs, some factors existing in KSR also affect the derivation of putative ES cell lines when the higher rate of primary ES-like colony formation was observed in culture medium supplemented with a mixture of FBS and KSR. Furthermore, addition of LIF and bFGF in the present study may cooperate with some cytokines and growth factors existing in FBS and KSR or secreting from feeder cells to support the undifferentiated stage of ES cell through pluripotency-related signaling pathways as described in mouse and human ES cells (Ying et al., 2003 ; Ogawa et al., 2007). Difference in supporting effect of feeder cells for promoting the ICM outgrowths and maintaining pluripotent self-renewal of ES cells has been documented in ES cell culture. We therefore evaluated five types of feeder cells such as porcine ear skin fibroblast (PESF), porcine tail skin fibroblast (PTSF), human foreskin fibroblast (HFK), mouse embryonic fibroblast (MEF), and STO in this study. The results showed that allogeneic feeder cells derived from PTSF was able to produce the microenvironment by which supported the derivation of primary colonies and putative pES cell lines which differed from previous studies. It is likely that each feeder cell type provides different microenvironment for stem cell survival and proliferation (Nagano et al., 2003). Furthermore, difference in secreted molecules from feeder cells also plays different roles on activating the signaling pathways of pluripotency and self-renewal in pES cells. On the

contrary, allogeneic PESF-derived feeder showed a low efficiency in promoting and maintaining the growth and proliferation of pES cells, suggesting that the origin (i.e. different location) where feeder cell were obtained may affect the feeder‘s ability on the derivation of pES cells. Besides PTSF, xenogeneic STO-derived feeder could also support self-renewal and pluripotency of pES cells during continuous culture comparable to the HFK and MEF feeders. Microenvironments produced from immortal STO cell line may be more stabilized than those produced from mortal cell lines as MEF and HFK. Furthermore, the variability between batch to batch of MEF and HFK cell lines may also affect the propagation and maintenance of pES-like cell lines (Park et al., 2003; Villa-Diaz et al., 2009).

However, entire culture conditions used in our study could not maintain undifferentiated pES cell lines over 8 passages, even though supplementation of LIF and bFGF was always presented. This is in contrast with a previous study of Brevini et al. (2010) which may be caused by differences in culture conditions between our and their systems (such as culture medium (knockout DMEM vs. DMEM/HAM‘S-F10, nutrient mix (without vs. with nucleoside mix), FBS:KSR volume (5:10 vs. 10:10%), affecting pluripotent self-renewal of pES cells (Chaudhry et al., 2008). Nevertheless, the truly stable ES cell lines which can generate germ-line transmitted chimeric pigs in this species have not been reported yet. It is most likely that species-specific difference may play a direct role in promoting different mechanisms controlling signaling pathways affecting the maintenance self-renewal, at the same time, the blockade of differentiation. mESCs require BMP and LIF/JAK/STAT3 signaling pathways to sustain pluripotency and maintain self-renewal, whereas, in hES cell and primate ES cell, the FGF pathway is necessary for proliferation and pluripotency (Mummery et al., 1993; Levenstein et al., 2006), and these processes are independent of STAT3 (Daheron et al., 2004; Humphrey et al., 2004; Sumi et al., 2004). BMP pathway also promotes differentiation in hES cells (Xu et al., 2002; Qi et al., 2004). Addition of noggin, a BMP4 antagonist can maintain pluripotency without loss of long-term self-renewal. (Abeyta et al., 2004; Inzunza et al., 2005; Genbacev et al., 2005; Xu et al., 2005). Nevertheless, the cell signaling pathways that govern pluripotency in the pig are currently unknown. The inconsistent expression of LIFR has been reported in undifferentiated porcine ICMs and in 24 h cultured undifferentiated epiblast tissue (Blomberg et al., 2008; Hall et al., 2009; Brevini et al., 2010). Surprisingly, LIFR has not been detected in pES-like cells (Vackova et al., 2007). However, we found that pES cells cultured in non LIF-supplemented medium have a slower proliferation than those cultured in medium supplemented with LIF (unpublished observation). This supposes that other pathways may involve in the propagation and development of porcine ES cell. Recent study showed that supplement of LIF may be involved in maintenance of self-renewal in pES cells by activating phosphatidylinositol 3 kinase (PI3K) signaling pathway through protein kinase B (PKB or known as AKT), a major effector of PI3K pathway, in stead of JAK/STAT3 pathway (Brevini et al., 2010). In addition, the expression of FGFR2 was also recently noted in pESC, related to the presence of bFGF, FGFR1, and FGFR2 transcripts in porcine epiblasts (Hall et al., 2009). This may be also involved with FGF pathway in maintenance pluripotency of ES cell in pigs.

In conclusion, produced embryos *in vitro* using IVF can be served as an alternative source for generating ES cells in pigs. However, the high quality of embryo, by means of the number and the existence of pluripotent stage in the ICM cells, should be considered when

these embryos were produced. Furthermore, techniques and conditions of ES cell culture also influence the success of pES cell establishment and maintenance their pluripotency. These factors have been concerned in species-specific difference. Comparatively transcriptome analysis of ES cell across different species will hopefully identify more fundamental signal transduction pathways for ES cell maintenance in pigs.

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APPENDIX A: Culture Media

All media were prepared using MilliQ water, otherwise indicate. The pH of all media was adjusted to 7.3-7.5 for oocytes and embryos culture, and the osmolarity was also adjusted to 275-290 mOsm/kg. All media were sterilized by filtration immediately after preparation then aliquoted and stored at 4 ˚C or -20 ˚C

Stock solution

EGF	25 µg/mL PBS+0.1%BSA
Cysteine	0.1 mg/ml ddH_2O
DAPI	0.1 mg/mL PBS
Hoechst 33342	10 mg/mL PBS

Oocyte washing (TL-HEPES-PVA) medium

NaCl	6.65 g/L
KCl	0.238 g/L
$NaHCO_3$	0.168 g/L
Na pyruvate	0.028 g/L
$NaH_2PO_4.H_2O$	0.055 g/L
HEPES	2.383 g/L
PVA	1 g/L
Na-lactate (60% syrup)	2.4 g/L
$MgCl_2.6H_2O$	0.102 g/L
$CaCl_2.2H_2O$	0.294 g/L

North Carolina State University (NCSU) -23 medium: for 100 ml solution

NaCl	0.6355 g
KCl	0.0356 g
KH_2PO_4	0.0162 g
$MgSO_4.7H_2O$	0.0293 g
D-glucose	0.1 g
L-glutamine	0.0146 g
Taurine	0.0876 g
Hypotaurine	0.0546 g
$NaHCO_3$	0.2106 g
*$CaCl_2.2H_2O$	0.025 g
Pen/Strep stock	1 µl/ml

Pen/Strep stock: 1,000,000 IU penicillin and 1 g Streptomycin were solubilized in 10 ml ddH_2O.

Oocyte maturation (M-199) medium: for 10 ml soulution	
M-199-HCO_3 buffered	9 ml
D-glucose	0.0055 g
L-glutamine	0.0010 g
0.91 M Na pyruvate	10 µl
Pen/Strep stock	1 µl/ml
pFF	1 ml
100 mM Cysteamine	10 µl
EGF	4 µl

Oocyte maturation (NCSU 23) medium : for 10 ml solution	
NCSU 23	9 ml
Cysteine stock	20 µl
BME stock	10 µl
pFF	1 ml

BME stock: 3.6 µl BME was added to 9.964 ml ddH_2O. Aliquats 20 µl into sterile eppendorf and store at room temperature in the dark.

***Sperm wash and In vitro* fertilization (Modified Tris-buffered) medium**	
NaCl	6.610 g/L
KCl	0.224 g/L
Tris	2.423 g/L
D-Glucose	1.982 g/L
BSA	1 g/L
Caffeine	0.388 g/L
*$CaCl_2.2H_2O$	1.102 g/L
**0.5 M Na pyruvate	200 µl
**Pen/Strep stock	20 µl

*Dissolve $CaCl_2.2H_2O$ separately before adding to the rest of the dissolved components.

** Add to 20 ml of medium on the day of use.

At least 18 h before IVF the medium should be incubated at 38.5°C in 5%CO_2 in air for pH to stabilize.

Synthetic oviductal fluid medium (SOF) – Stock A : for 800 ml solution	
NaCl	6.290 g
KCl	0.534 g
KH_2PO_4	0.162 g
$NaHCO_3$	2.100 g
Na-Lactate (60% w/w)	0.5969 g
$MgSO_4.7H_2O$	0.182 g
$CaCl_2.2H_2O$	0.262 g
MEM NEAA	10 ml
BME EAA	20 ml
phenol red	500 µl

Adjust to osmolarity 300-340 mOsm, store at 4°C up to 9 months.

Synthetic oviductal fluid medium (SOF) – Stock B : for 20 ml solution	
Pen/strep	100 µl
Sodium Pyruvate	0.0036 g
L-Glutamine	0.0300 g
BSA	0.400 g

SOF complete medium : for 5 ml solution	
SOF –stock A	4 ml
SOF –stock B	1 ml

Store at 4°C up to 2 weeks.

Mannitol activation medium-AM I: for 10 ml solution	
0.3 M Mannitol	9.7 ml
10 mM $CaCl_2.2H_2O$	100 µl
10 mM $MgSO_4$	100 µl
BSA (mg/ml)	0.010 g
0.52 M HEPES	100 µl

Mannitol activation medium-AM II : for 10 ml solution	
0.3 M Mannitol	9.7 ml
10 mM $CaCl_2.2H_2O$	100 µl
10 mM $MgCl_2.6H_2O$	100 µl
10% PVP	100 µl
0.52 M HEPES	100 µl
1N NaOH	20 µl

10% PVP: 1 g PVP was dissolved in 10 ml ddH_2O

0.5 M EDTA (pH 8)	
Na_2EDTA	186 g/l

Adjust pH = 8 by 10 N NaOH, then autoclave and store at 4 °C

10 x TBE buffer	
Tris	108 g/l
Boric acid	55 g/l
0.5 M EDTA (pH 8)	40 ml/l

Adjust water up to 1 litr, then autoclave and store at 25 °C (no longer than 1 month)

APPENDIX B: Publications and Conferences

1. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2011. Allo- and Xeno-geneic derived feeder cell types differently promote the establishment of porcine ES-like cells. Anim Reprod Sci. (Submitted).
2. Rungarunlert, S., Rungsiwiwut, R., Suphankong, S., **Panasophonkul, S.**, Pruksananonda, K., Virutamasen, P., Pirity, M.K., Dinnyes, A., Tharasanit, T., Techakumphu, M. 2011. Comparative characterization of four mouse parthenogenetic embryonic stem (pES) cell lines. Thai J Vet Med. (Accepted, in press).
3. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2011. Improvement of normal fertilization rate and embryo development by the reduction of sperm:oocyte ratio during *in vitro* fertilization in pig. Thai J Vet Med. 41: 71-77.
4. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2010. Establishment of porcine embryonic stem-like cells from parthenogenetic and *in vivo* derived Embryos. Thai J Vet Med. 40: 273-280.
5. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2008. Embryo production by parthenogenetic activation of porcine oocytes. Proc. of CU VET Graduate Seminar: Innovations for Animal Health and Production, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 8-9 April: 24-25
6. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2008. Embryo production by parthenogenetic activation of porcine oocytes. Proc. of the 1st Commission on Higher Education (CHE-USDC) Congress, Dusit Thani Pattaya Hotel, Chonburi, Thailand, 5-7 September: 165.
7. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2009. *In vitro* fertilization of porcine oocytes by frozen thawed-semen: effect of sperm:oocytes ratio. Proc. Of the 8th Chulalongkorn University Veterinary Annual Conference, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 3-4 April: 86.
8. Tharasanit, T., **Panasophonkul, S.**, Buarpung, S., Techakumphu, M. 2009. Establishment of feline embryonic stem cells from in vitro produced embryos. Proc. of the 7th Annual Meeting of International Society for Stem Cell Research, Barcelona, Spain, 8-11 July: 105.
9. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2009. Establishment of pig embryonic stem cells from different sources of blastocysts. Proc. of the 2nd Commission on Higher Education (CHE-USDC) Congress, Dusit Thani Pattaya Hotel, Chonburi Province, Thailand, 27-29 August: 202.
10. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2009. Supportive effect of skin fibroblasts derived-feeder layers on isolation and culture of porcine embryonic stem cells. Proc. of the 6th Annual Conference of the Asian Reproductive Biotechnology Society –Innovation in stem cells and endangered animals preservation", Siem Reap, Cambodia, 23-27 November: 69.
11. **Panasophonkul, S.**, Tharasanit, T. and Techakumphu, M. 2009. Effect of *in vitro* fertilized embryos on derivation of porcine embryonic stem cells. Proc. of the 6th

Annual Conference of the Asian Reproductive Biotechnology Society –Innovation in stem cells and endangered animals preservation", Siem Reap, Cambodia, 23-27 November: 68.

VITAE

Miss Sasithorn Panasophonkul was born on October 18th 1975 in Bangkok province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) and Master Degree of Theriogenology from Faculty of Veterinary Science, Chulalongkorn University, in 2000 and 2004, respectively. She is the lecturer in Equine Clinic, Department of Domestic Animals and Wild life, Faculty of Veterary Medicine, Chiang Mai University since 2000. In 2007, she received a scholarship from the Commission on Higher Education PhD Program to perform a PhD Program of Theriogenology at Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her focus research is about *in vitro* embryo production and embryonic stem (ES) cell culture in pigs, which aims to find out the appropriate culture condition for establishing stable ES cell lines in this species as resulted in this thesis.