EFFECT OF OCT-4 ACTIVATING COMPOUND 1 (OAC1) SUPPLEMENTATION IN IVC MEDIUM ON DEVELOPMENTAL COMPETENT AND QUALITY OF PORCINE SCNT EMBRYOS

THIDA PRAEKSAMUT

A Thesis Submitted in Partial Fulfillment of the Requirements for the
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ผลของการเสริม Oct-4 activating compound 1 (OAC1) ในน้ำยาเลี้ยงตัวอ่อนต่อการพัฒนา และคุณภาพของตัวอ่อนสุกรโคลนนิ่งด้วยวิธีการย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย

นางสาวธิดา แพรกสมุทร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF OCT-4 ACTIVATING COMPOUND 1 (OAC1) SUPPLEMENTATION IN IVC MEDIUM ON DEVELOPMENTAL COMPETENT AND QUALITY OF PORCINE SCNT EMBRYOS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ธิดา แพรกสมุทร : ผลของการเสริม Oct-4 activating compound 1 (OAC1) ในน้ำยาเลี้ยงตัวอ่อนต่อการพัฒนา และคุณภาพของตัวอ่อนสุกรโคลนนิ่งด้วยวิธีการย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย (EFFECT OF OCT-4 ACTIVATING COMPOUND 1 (OAC1) SUPPLEMENTATION IN IVC MEDIUM ON DEVELOPMENTAL COMPETENT AND QUALITY OF PORCINE SCNT EMBRYOS) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 80 หน้า

คำสำคัญ: ตัวอ่อนสุกรโคลนนิ่ง/โอเอซี1/การพัฒนาของตัวอ่อน/การปรับโปรแกรม/การย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย

การย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย (SCNT) เป็นเทคนิคช่วยในการสืบพันธุ์ที่เกี่ยวข้องกับการฉีดเซลล์ต้นแบบเข้าไปในไข่ที่ปราศจากนิวเคลียส อย่างไรก็ตามประสิทธิภาพของการทำ SCNT ยังคงอยู่ในระดับต่ำ เนื่องจากความผิดปกติในการปรับโปรแกรมของนิวเคลียส งานวิจัยก่อนหน้านี้แสดงให้เห็นว่า ตัวอ่อนระยะบลาสโตซิสต์ที่ได้จากการทำ SCNT มีการแสดงออกของยีน *OCT-4* และยีนที่เกี่ยวข้องผิดปกติ *OCT-4* เป็นปัจจัยควบคุมการถอดรหัสที่สำคัญ มีบทบาทในการพัฒนาตัวอ่อน การคงสภาพพลูริโพเทนซี และควบคุมการสร้างเซลล์สืบพันธุ์ปฐมภูมิ การเพิ่มระดับการแสดงออกของ *OCT-4* ในตัวอ่อน SCNT อาจช่วยปรับปรุงประสิทธิภาพการปรับโปรแกรมของตัวอ่อนได้ การศึกษานี้ได้ตรวจสอบความเข้มข้นที่เหมาะสม และผลของ OCT-4 activating compound 1 (OAC1) ซึ่งเป็นสารโมเลกุลขนาดเล็กชนิดใหม่ที่สามารถเหนี่ยวนำการแสดงออกของ *OCT-4* และ *NANOG* ในตัวอ่อนโค SCNT เปรียบเทียบกับตัวอ่อนที่เกิดจากการปฏิสนธิในหลอดแก้ว จากการศึกษาพบว่า ความเข้มข้นที่เหมาะสมของ OAC1 ที่ 1.5 ไมโครโมลาร์ สามารถเพิ่มอัตราการได้ตัวอ่อนระยะบลาสโตซิสต์และจำนวนเซลล์ทั้งหมด ในตัวอ่อน SCNT ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และยังพบว่า OAC1 ส่งเสริมการแสดงออกของยีน *OCT-4*, *SOX2* และ *NANOG* ในระยะ 8 เซลล์ แต่ไม่มีผลในระยะอื่น ๆ ในส่วนการแสดงออกของยีน *HDAC1-3* พบว่า ตัวอ่อน SCNT ที่เสริม OAC1 ยังไม่ชัดเจนเมื่อเทียบกับตัวอ่อนที่เกิดจากปฏิสนธิในหลอดแก้ว ผลกระทบต่อการปรับเปลี่ยนโครมาตินในตำแหน่ง H3K14ac และ H3K9me3 มีรูปแบบการแสดงออกคล้ายกับตัวอ่อนที่เกิดจากปฏิสนธิในหลอดแก้วในขณะเดียวกันการแสดงออกของยีน *DNMTs* และ การแสดงออกของโปรตีน 5-mC ในระยะแรกของการพัฒนาของตัวอ่อนจากการปฏิสนธิในหลอดแก้วต่ำกว่าตัวอ่อน SCNT และเพิ่มสูงขึ้นในระยะ 8 เซลล์จนถึงระยะบลาสโตซิสต์เกี่ยวข้องกับการทำงานของการสร้าง new genome imprinting จากผลการทดลองนี้สามารถสรุปได้ว่า OAC1 มีผลต่อการพัฒนาของ

ตัวอ่อน และมีส่วนช่วยในการปรับโปรแกรมโดยการควบคุมการทำงานของ DNA Methylation และ Histone modification อย่างไรก็ตามการทำงานของ OAC1 ยังคงต้องได้รับการตรวจสอบอีกมากในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา .....

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา .....

THIDA PRAEKSAMUT : EFFECT OF OCT-4 ACTIVATING COMPOUND 1 (OAC1) SUPPLEMENTATION IN IVC MEDIUM ON DEVELOPMENTAL COMPETENT AND QUALITY OF PORCINE SCNT EMBRYOS. THESIS ADVISOR : PROF. RANGSUN PARNPAI, Ph.D., 80 PP.

Keyword: Porcine SCNT/OAC1/Embryo development/Reprograming

Somatic cell nuclear transfer (SCNT) is an assisted reproductive technique involving transferring a donor cell into an enucleated oocyte. The efficiency remains low, due to abnormal nuclear reprogramming. Previous studies have shown that SCNT blastocysts exhibit aberrant expression of *OCT-4* and *OCT-4* related genes. The OCT-4 transcription factor is crucial for embryonic development, maintaining pluripotency, and regulating primordial germ cell formation. Enhancing *OCT-4* expression in SCNT embryos may improve their reprogramming efficiency. In this study, we investigated the optimal concentration and effects of OCT-4 Activating Compound 1 (OAC1), a novel small molecule reported to induce the expression of *OCT-4* and *NANOG* in bovine SCNT embryos. Comparisons were made with embryos derived from *in vitro* fertilization (IVF). The results of this study indicated that treatment with OAC1 at a concentration of 1.5 μM significantly increased the blastocyst formation rate and total cell number in SCNT embryos ($p < 0.05$). Furthermore, OAC1 enhanced the expression of *OCT-4*, *SOX2*, and *NANOG* specifically at the 8-cell stage, but did not significantly affect gene expression at other developmental stages. The expression of histone deacetylase 1-3 (*HDAC1-3*) genes in porcine SCNT embryos supplemented with OAC1 remain unclear when compared to embryos derived from IVF. In terms of chromatin remodeling, the expression patterns of H3K14ac and H3K9me3 in OAC1-treated SCNT embryos similar to IVF-derived embryos. Regarding DNA methyltransferase (*DNMT*) gene expression and global DNA methylation (5-mC), IVF embryos exhibited lower expression levels than SCNT embryos during early development, with expression levels increasing from the 8-cell to the blastocyst stages involving re-methylated occurs and new genomic imprints are established by activating the methylation. In conclusion, OAC1 appears to exert beneficial effects on embryonic development in SCNT embryos

by promoting reprogramming through modulation of DNA methylation and histone modification. Nevertheless, further studies are necessary to investigate the function of OAC1.

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Student's Signature Thida Praehsarut
Advisor's Signature

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LIST OF ABBREVIATIONS

-	=	Minus
%	=	Percentage
°C	=	Degree Celsius
µg	=	Microgram
µl	=	Microlite
µM	=	Micromolar
µs	=	Microsec
5-mC	=	5-methylcytosine
6-DMAP	=	6-Dimethylamino purine
ANOVA	=	Analysis of variance
BL	=	Blastocyst
bp	=	Base pair
BSA	=	Bovine serum albumin
BTS	=	Beltsville thawing solution
$CaCl_2.2H_2O$	=	Calcium dichloride dihydrate
CB	=	Cytochalasin B
cDNA	=	Complementary DNA
COCs	=	Cumulus oocyte complex
CO_2	=	Carbon dioxide
dbcAMP	=	Dibutyryl cyclic adenosine monophosphate
DC	=	Direct currents
DMNT1	=	DNA methyltransferase 1
DMNT3A	=	DNA methyltransferase 3 alpha
DMNT3B	=	DNA methyltransferase 3 beta
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate

LIST OF ABBREVIATIONS (Continued)

EDTA	=	Ethylenediaminetetraacetic acid
EGF	=	Epidermal growth factor
FBS	=	Fetal Bovine Serum
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
H2A	=	Histone 2A
H2B	=	Histone 2B
H3	=	Histone 3
H3K9	=	Histone 3 lysine 9
H3K9me3	=	Lysine 9 trimethylation of histone 3
H3K14	=	Histone 3 lysine 14
H3K16	=	Histone 3 lysine 16
H4	=	Histone 4
HATs	=	Histone acetyltransferases
H3K14	=	Histone 3 lysine 14
H3K14	=	Histone 3 lysine 14
H3K16	=	Histone 3 lysine 16
H4	=	Histone 4
HATs	=	Histone acetyltransferases
HCG	=	Human chorionic gonadotropin
HCL	=	Hydrochloric acid
HDAC1	=	Histone deacetylase 1
HDAC2	=	Histone deacetylase 2
HDAC3	=	Histone deacetylase 3
HMT	=	Histone methyltransferase
hr.	=	Hour
HoxB4	=	Homeobox B4
HSD	=	Honest significant difference
ICM	=	Inner cell mass

LIST OF ABBREVIATIONS (Continued)

IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation
IU	=	International unit
K	=	Lysine
MII	=	Metaphase II
mDPBS	=	Modified Dulbecco's phosphate-buffered saline
mg	=	Microgram
$MgSO_4.7H_2O$	=	Magnesium sulfate heptahydrate
min	=	Minute
ml	=	Millilitre
mm	=	Millimetre
mM	=	Millimolar
mRNA	=	Messenger ribonucleic acid
N_2	=	Nitrogen
NaCl	=	Sodium chloride
NANOG	=	Nanog homeobox
ng	=	Nanogram
O_2	=	Oxygen
OAC1	=	OCT-4 activating compound 1
OCT-4	=	Octamer-binding transcription factor 4
PB	=	Polar body
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PMSG	=	Pregnant mare serum gonadotropin
PN	=	Pronuclear
PMSG	=	Pregnant mare serum gonadotropin
PN	=	Pronuclear

LIST OF ABBREVIATIONS (Continued)

POM	=	Porcine oocyte medium
POU5F1	=	POU domain, class 5, transcription factor 1
PVA	=	Polyvinyl alcohol
PVP	=	Polyvinylpyrrolidone
PZM-3	=	Porcine zygote medium-3
qPCR	=	Quantitative real-time polymerase chain reaction
SAM	=	S-adenosyl methionine
SCNT	=	Somatic cell nuclear transfer
SEM	=	Standard error of mean
SOX2	=	SRY-Box Transcription Factor 2
SUT	=	Suranaree University of Technology
TCM199-HEPES	=	Medium 199 HEPES modification
TE	=	Trophectoderm
TET	=	Ten-eleven translocation
v/v	=	Volume per volume
w/v	=	Weight per volume
αMEM	=	Minimum Essential Medium, Alpha modification

CHAPTER I
INTRODUCTION

1.1 Background and significance

Somatic cell nuclear transfer (SCNT) involves transferring a donor cell into an enucleated oocyte. Currently, SCNT has been successfully applied to a range of species, such as sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), cats (Shin et al., 2002), rabbits (Chesné et al., 2002), horses (Galli et al., 2003), rats (Zhou et al., 2003) and dogs (Lee et al., 2005). Animal production based on SCNT offers a range of opportunities in basic and applied research, in agriculture, genetic conservation, and human medicine (Campbell et al., 2007). However, SCNT efficiency is still low because of incomplete epigenetic reprogramming of donor cell nuclei (Long et al., 2014). Consequently, the improvement of epigenetic reprogramming is a crucial factor in enhancing the developmental efficiency of SCNT embryos (Qiao et al., 2018).

Oct-4 (Octamer-binding transcription factor 4) or POU5F1 protein (POU domain, class 5, transcription factor 1) is a major transcription factor in embryonic stem cells and primordial germ cells (Looijenga et al., 2003; Rosner et al., 1990) The *OCT-4* gene is essential for complete embryo development (Nichols et al., 1998) and regulating pluripotency in embryonic stem cells (Niwa et al., 2000). Previous SCNT-related studies reveal that the expression level of the *OCT-4* gene in donor cells improves developmental efficiency, promotes nuclear reprogramming of SCNT embryos in bovine and porcine (Goissis et al., 2013; Lee et al., 2014) and enhances the expression level of the *OCT-4* during cleavages resulting in a higher developmental rate of mouse SCNT embryos (Pfeiffer et al., 2010). Oct4-activating compound (OAC1) has been widely applied to increase the expression activity of the *OCT-4* in mouse (W. Li et al., 2012).

Moradi-Hajidavaloo et al. (2023) reportedthat using 1.5 μM of OAC1 in IVC medium improves the quality of bovine SCNT that using 1.5 μM of OAC1 in IVC medium improves the quality of bovine SCNT embryos. However, the effects of OAC1 on porcine SCNT embryos have not yet been reported.

This study aimed to determine the optimal concentration and the effects of OAC1 as a small-molecule supplemented in IVC medium on cleavage rate, blastocyst formation rate, expression level of *OCT-4, SOX2, NANOG* gene, and epigenetic reprogramming of porcine SCNT embryo.

1.2 Research objectives

1.2.1 To examine the optimal concentration of OAC1 supplemented in IVC medium on developmental competence of SCNT porcine embryos

1.2.2 To investigate the effects of OAC1 on genes related to SCNT porcine embryo development

1.2.3 To evaluate the effects of OAC1 on DNA methylation and histone modification of SCNT porcine embryos

1.3 Research hypothesis

1.3.1 Optimal concentration of OAC1 supplemented in the culture medium can enhance higher embryo development rate, including cleavage rate, blastocyst, and total cell number in porcine SCNT embryos compared to the untreated group.

1.3.2 Optimal concentration of OAC1 supplemented in the culture medium can induce the expression level of genes related to embryo development and improve DNA methylation and histone modification in porcine SCNT embryos, higher than untreated group and similar to *in vitro* fertilization group.

1.4 Scope and limitations of study

Firstly, the study attempted to find an optimal concentration: 0, 1.0, 1.5, and 3.0 μM of OAC1. supplemented in the IVC medium to maximize cleavage and blastocyst rates, and total cell number in SCNT porcine embryos. Next, SCNT untreated and treated with OAC1 and IVF porcine embryos at pronuclear (PN), 2-, 4-, 8-, and blastocyst (BL) stages served as negative and positive controls and the treatment groups were examined the expression of gene-related to the embryo development using quantitative polymerase chain reaction (qPCR) by using IVF embryos as positive control (normal control) group and epigenetic reprogramming using immunocytochemistry staining

CHAPTER II

LITERATURE REVIEWS

2.1 Somatic cell nuclear transfer (SCNT)

SCNT is a unique tool for increasing genetically valuable animals, wildlife conservation, genetically modified animal production, and biomedical applications. SCNT is commonly known as a cloning technique by transferring a somatic cell into an enucleated metaphase-II oocyte for the generation of a new individual, genetically identical to the somatic cell donor (Figure 1) (Tian et al., 2003)

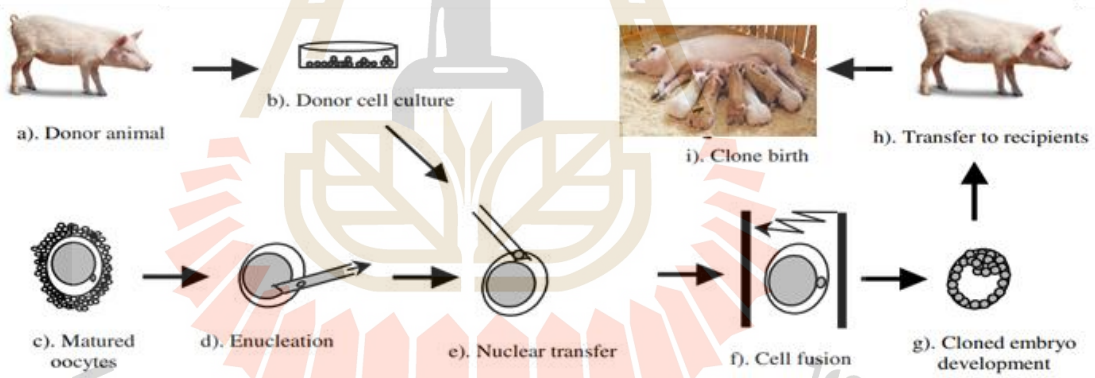


Figure 1. Diagram of somatic cell nuclear transfer (Tian et al., 2003)

Dolly is the first mammalian species to effectively from an adult mammary epithelial cell (Wilmut et al., 1997). This achievement proved that genes suppressed during tissue differentiation can be fully reactivated through a process known as nuclear reprogramming. Somatic cell cloning can be utilized to produce multiple copies of genetically superior farm animals, create transgenic animals for pharmaceutical protein production, or xenotransplantation (Anderson & Seidel, 1998; Polejaeva et al., 2000; Robl, 1999; Stice et al., 1998). For biomedical advancements, including therapeutic cloning and allotransplantation (Lanza et al., 1999) become a

crucial tool for studying gene function (Capecchi, 2000), and genomic reprogramming (Surani, 2001; Winger et al., 2000). However, cloning by SCNT has low efficiency and high developmental abnormalities that come from somatic cell types, the effect of donor age, passage numbers of donor cells, and a major factor is incomplete epigenetic reprogramming of donor cell nuclei (Bourc'his et al., 2001; Kubota et al., 2000). The improvement of epigenetic reprogramming is a crucial factor in enhancing the developmental efficiency of SCNT embryos (Srirattana et al., 2022; Tian et al., 2003).

2.2 Epigenetic reprogramming on the development of SCNT embryos

Epigenetics refers to the activity or function that plays a crucial role in regulating changes in gene expression. Epigenetics not coded in the DNA sequence itself (Bird, 2007; Turner, 2000). Epigenetic mechanisms of gene regulation depend on many factors such as DNA methylation and post-translational modifications of histone proteins (Jaenisch & Bird, 2003). In cloning techniques, epigenetic reprogramming is essential for normal development of the somatic cell nucleus (Solter, 2000). The success of producing cloned animals is currently still low. This involves aberrant reprogramming of the original somatic cell nucleus (Jablonka & Lamb, 2002).

2.2.1 DNA methylation

DNA methylation is associated with transcriptional silencing and changes in chromatin structure (Cho et al., 2001; Eden et al., 1998). DNA methylation is considered a major epigenetic factor inducing gene activities that involve the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine commonly known as a CpG site (Razin & Cedar, 1991).

DNA methylation regulates gene expression through either recruiting proteins involved in gene repression or inhibiting the binding of transcription factors to DNA methyltransferases (Dnmt) catalyzed DNA. DNA methylation is involved in three major enzymes: Dnmt3a, Dnmt3b, and Dnmt1. Dnmt3a and Dnmt3b are responsible for the establishment of a new methylation pattern to unmodified DNA, known as de novo Dnmt (Figure 2a) while Dnmt1 participates in the biosynthesis of the daughter DNA strand from the parental DNA strand (Figure 2b) (Moore et al., 2013)

DNA demethylation, a removal of DNA methylation, can occur through two distinct mechanisms: 1) active demethylation, a fast reaction independent of cell division, and 2) passive demethylation, which occurs when DNA methylation is passively diluted by DNA replication following cell division (Rougier et al., 1998; Zhang et al., 2016). The Ten-eleven translocation (TET) enzymes, i.e. TET1, TET2, and TET3, play a crucial role in the progression of active demethylation by adding a hydroxyl group onto the methyl group of 5mC to form 5hmC in the paternal genome (He et al., 2011; Wossidlo et al., 2011).

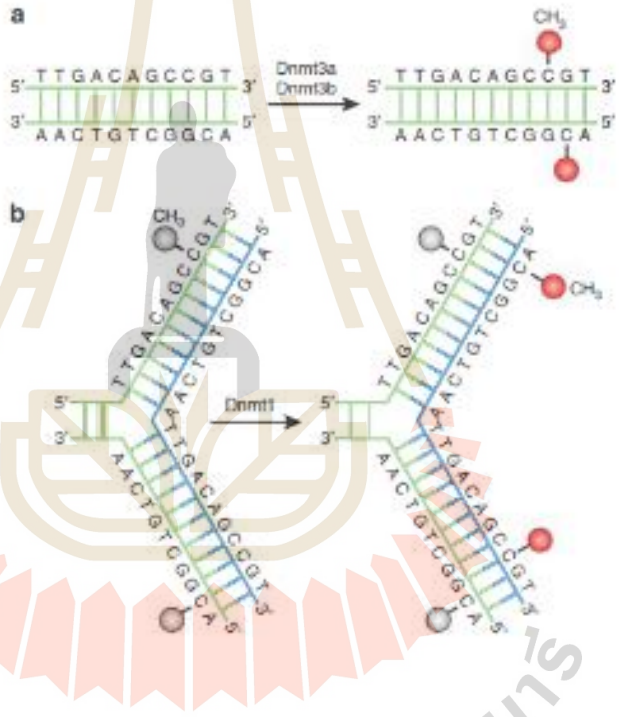


Figure 2. Diagram of (a) Dnmt3a and Dnmt3b are the de novo Dnmts and transfer methyl groups onto naked DNA. (b) Dnmt1 is the maintenance Dnmt and DNA methylation pattern during replication (Moore et al., 2013).

DNA methylation plays an important role in mammalian embryogenesis, such as gene imprinting, X chromosome inactivation, and genome stability (Bestor, 2000; Bird, 2002; Bird & Wolffe, 1999). In the cloning technique, SCNT-derived embryos are highly methylated compared with in vitro fertilized (Dean et al., 2001; Liu et al., 2008) Therefore, abnormal SCNT embryos may be caused by an incomplete

reprogramming of DNA methylation (Armstrong et al., 2006)

2.2.2 Histone modification

In eukaryotic cells, a nucleosome is a basic unit of chromatin comprised of 146–147 base pairs of DNA and a histone octamer H2A, H2B, H3, and H4 dimers (Jenuwein & Allis, 2001). The N- and C-terminal tails of these histone proteins can be post-translationally modified resulting in a change in electronic charge and structures of histone tails binding to DNA responsible for modification of chromatin status and gene expression (Kouzarides, 2007; Strahl & Allis, 2000). Histone modifications have been found to play an important role in the regulation of chromatin structure and gene expression (Zhang et al., 2021).

2.2.2.1 Histone methylation

Histone methylation is one of the most important post transcriptional modifications participating in the addition of methyl group onto histone H3 and H4 at the lysine (K) residues (Greer & Shi, 2012). In general, lysine (K) residues of histone are catalyzed by the histone methyltransferase (HMT) and use S-adenosyl methionine (SAM) as a substrate to transfer methyl group (Murray, 1964). The histones lysine residues existed in 3 forms: mono-, di-, and trimethylated (me1, me2, and me3) and were used as active or repressive markers of gene expression (Black et al., 2012). At early embryo development, modification processes exist using the histone H3K9 trimethylation and demethylation in the parental pronuclei (Santos et al., 2005). The modification plays an important role in X chromosome inactivation in female cells (Bannister et al., 2001; Lachner et al., 2001).

2.2.2.2 Histone acetylation

Histone acetylation is one of the important epigenetic modifications. It is responsible for unfolding chromatin to recruit different transcriptional factors, and is also associated with the activation of gene transcription in cells (Valls et al., 2005). Regulation of histone acetylation is related to maintaining the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) through the reduction of the positive charge of the lysine residues, and inhibition of binding

between histone tails and negatively charged DNA (Bannister & Kouzarides, 2011).

Histone deacetylases Class I (*HDAC1*, *HDAC2*, and *HDAC3*) are a family of enzymes that play an essential role in regulating chromatin structure and gene expression during embryonic development (Murko et al., 2010) *HDAC1* is crucial for embryonic development and in preimplantation mouse embryos (Ma & Schultz, 2008). Loss of *HDAC1* results in a marked decrease in overall deacetylase activity, leading to hyperacetylation of specific histone H3 and H4 residues, along with concurrent alterations in other histone modifications (Lagger et al., 2002). *HDAC2* shares structural similarities with *HDAC1* but performs roles during embryonic development (Ma & Schultz, 2008). In mouse oocytes, *HDAC2* is more critical than *HDAC1*, deletion leads to reduced nuclear accumulation of DNMT3A, resulting in global DNA hypomethylation and impaired establishment of imprinting marks (Ma & Schultz, 2016). HDAC3 plays a crucial role in mesodermal lineage commitment in embryonic stem cells enhances mesodermal differentiation, its function as a repressor of mesoderm-specific gene expression (Lv et al., 2014). The combined deletion of HDAC1 and HDAC2 in mouse preimplantation embryos results in developmental arrest during the transition from morula to blastocyst, this defect is characterized by decreased cell proliferation, elevated apoptosis, and impaired lineage specification. Furthermore, the double knockdown leads to global DNA hypermethylation, associated with increased expression of DNA methyltransferases (Zhao et al., 2020). Histone deacetylase inhibitors (HDACi) enhance the acetylation levels of core histones in cloned bovine embryo suggesting that HDACi promotes hyperacetylation and activates key genes essential for SCNT embryonic development (Oliveira et al., 2010).

SCNT-derived embryos showed high acetylation on specific lysine residues in histone H3, and H4 during oocyte meiosis, and early embryo development (Kim et al., 2003). The acetylated core histones of the somatic cell genome undergo two different processes. The first involves the activation of reconstructed oocytes through histones H3K9, H3K14, and H4K16. The latter exist in the histone H4K8 and H4K12 to generate the condensed chromosomes, and pseudo-pronuclei of SCNT embryos, normal MII oocytes, and zygotic gene activation (Worrad et al., 1995)

2.3 The importance of OCT-4 on the development of the embryo

OCT4 is a transcription factor expressed by early embryo and germ cells. It acts as a central regulator of pluripotency in mammals(Nichols et al., 1998).

OCT-4 is a homeodomain transcription factor of the POU (Pit-Oct Unc) interacting with a specific octameric sequence motif (ATGCAAT) on enhancer and promoter regions of target genes through a POU domain to activate and repress gene expression (Nordhoff et al., 2001; Wu & Schöler, 2014).

The activity of the *OCT-4* is essential for the identity of the pluripotential founder cell population in the mammalian embryos (Nichols et al., 1998). Expression levels of the *OCT-4* on embryos initiate at the 8–16 cell stage simultaneously with embryonic genome activation (Graf et al., 2014; Kurosaka et al., 2004). However, *OCT-4* expression is low in unfertilized oocytes and localized in the nuclei of preimplantation embryos at cleavage stages. The expression is found only in inner cell mass but not in the trophectoderm of blastocyst after differentiation of the trophectoderm lineage (Palmieri et al., 1994).

2.4 Oct-4 activating compound 1 (OAC1)

Oct-4 activating compound 1 (OAC1) belongs to a structural class of 5-substituted pyrrolo [2,3-b] pyridine- and indole-based benzamides (Figure 2) (Li et al., 2012), that have few reports about its biological activities.

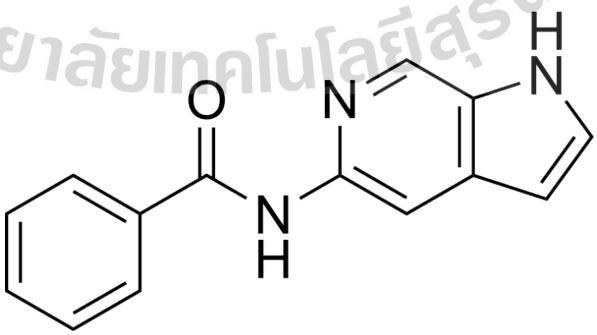


Figure 3. Structure of OAC1 (Huang et al., 2016)

Li et al. (2012) Identified compounds that could activate the Oct-4 and Nanog promoters, named “Oct-4 activating compound (OAC).” They reported that OAC (Oct-4 activating compound analog 1, OAC1) activates OCT-4 and Nanog promoters and enhances epigenetic reprogramming by activating the expression of Tet1, which catalyzes the conversion of 5-methylcytosine (5-mC) to 5 hydroxymethylcytosine (5hmC) of DNA.

OAC1 triggers OCT-4 through upregulation of HOXB4 expression and increases transcription of the Oct4-Nanog-Sox2 triad (Huang et al., 2016; Li et al., 2012). In development of bovine SCNT embryos investigated by Moradi-Hajidavaloo et al. (2023) showed that 1.5 μM of OAC1 supplementation in IVC medium induced the expression level of the *OCT-4* gene, and increased the number of TE/ICM, and total cell number without detrimental consequences on a donor cell, enhancing DNA methylation and histone acetylation.

2.5 Beneficial application of cloning in pig

Pig cloning offers substantial benefits by enhancing agricultural productivity and sustainability, as well as advancing medical research and therapeutic applications.

Agricultural benefits

Enhanced meat production: Cloning enables the replication of pigs with superior traits such as increased growth rates and leaner meat composition, leading to higher-quality pork products (Whyte & Prather, 2011)

Improved disease Resistance: By cloning pigs that exhibit natural resistance to specific diseases, herds can become more resilient, reducing reliance on antibiotics and minimizing economic losses (Prather et al., 2008).

Environmental sustainability: Cloning pigs with efficient feed conversion rates can decrease the environmental footprint of pig farming by reducing waste and resource consumption (Whyte & Prather, 2011).

Medical and biomedical research benefits

Xenotransplantation: Cloned pigs serve as potential organ donors for human transplantation. Genetic modifications can be introduced to make pig organs more

compatible with the human immune system, addressing the shortage of human donor organs (Hryhorowicz et al., 2017; Sykes & Sachs, 2019).

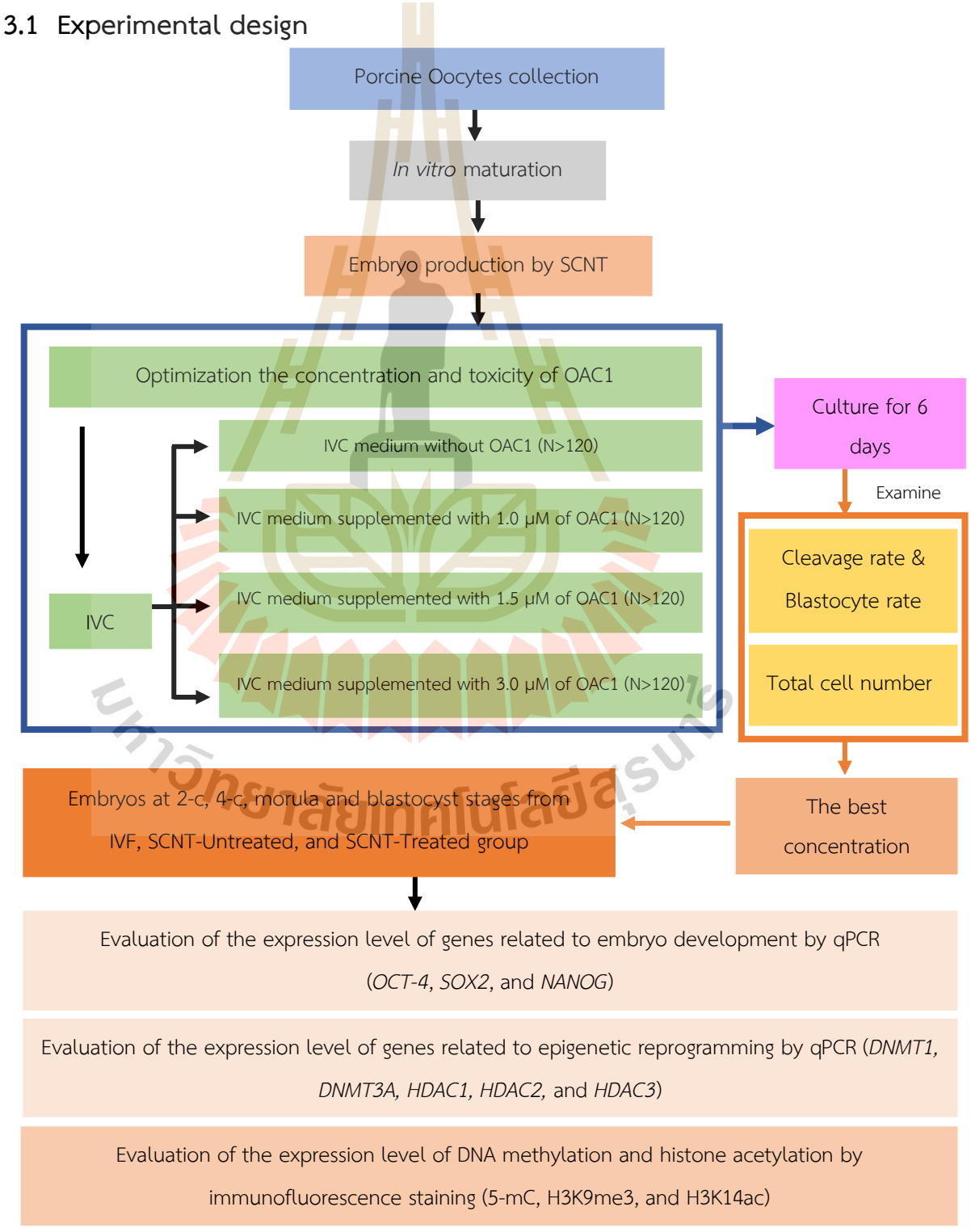
Disease modeling: Cloned pigs can be genetically engineered to develop specific human diseases, providing valuable models for studying disease progression and testing new treatments (Hryhorowicz et al., 2020).

Pharmaceutical production: Transgenic cloned pigs can produce human proteins in their milk or blood, which can be harvested and purified for therapeutic use, such as clotting factors for hemophilia patients (Johnson, 2011; Lanza et al., 1999).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Experimental design



3.2 Chemicals

Chemicals used in this research were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), if not, specify additionally.

3.3 Oocyte collection and *in vitro* maturation (IVM)

Porcine ovaries were obtained from a local slaughterhouse and kept in 0.9% NaCl (Carlo Erba, 479687, France) solution at room temperature during transport to the laboratory within 2 hr. Then COCs were collected from antral follicles (3-6 mm in diameter) using an 18-gauge needle equipped with a 10 ml syringe before placing into a 15 ml conical tube (Figure 4A). COCs with a homogeneous cytoplasm and surrounded by at least 3 layers of intact cumulus cells were placed in modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrrolidone (PVP) (Figure 4B). COCs were cultured in IVM-I medium (Porcine oocyte medium; POM, Yoshioka et al., 2008) supplemented with 10 IU/ml pregnant mare serum gonadotropin (PMSG, Intervet International GmbH, Unterschleißheim, Germany), 10 IU/ml human chorionic gonadotropin (HCG, Invert International GmbH), 10 ng/ml epidermal growth factor (EGF), and 10 IU/ml dibutyryl cAMP (dbcAMP)) on a 4-well dish covered with mineral oil (50 oocytes/500 µl of IVM medium) under humidified atmosphere of 5% CO_2 at 38.5 °C for 23 hr. (Figure 4C), and then, cultured in IVM-II medium (POM) supplemented with 10 ng/ml EGF and 10 IU/ml dbcAMP under humidified atmosphere of 5% CO_2 at 38.5 °C for 22 hr. (Figure 4D)

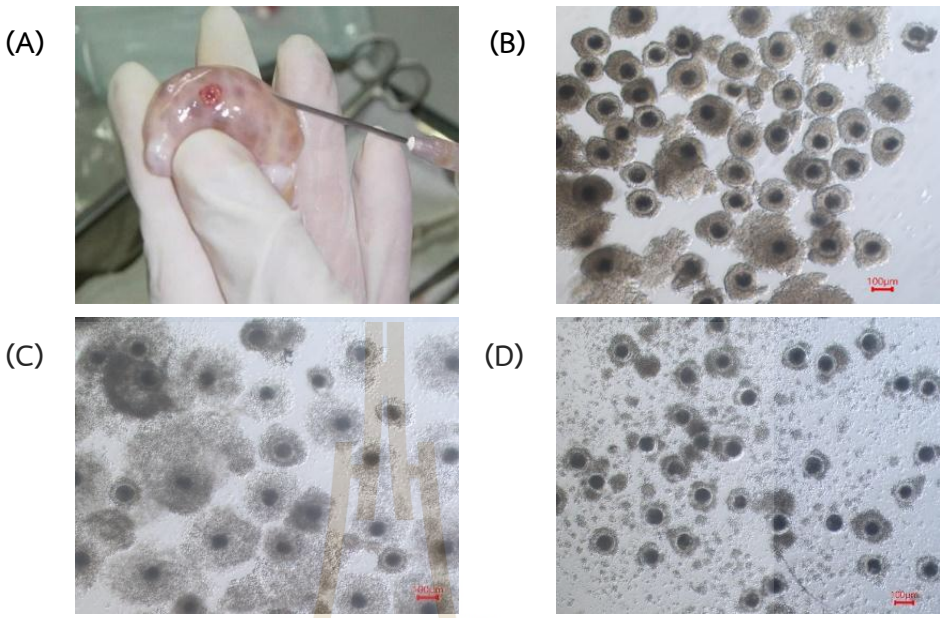


Figure 4. Oocyte collection and in vitro maturation (IVM) A) COCs collected from antral follicles using an 18-gauge needle with a 10 ml syringe, B) COCs with homogeneous cytoplasm and surrounded by at least 3 layers of intact cumulus, C) COCs cultured in IVM-I medium, and D) COCs cultured in IVM-I medium

3.4 Somatic cell nuclear transfer (SCNT)

3.4.1 Donor cell preparation

Fibroblast cells were isolated from the ear skin tissue of a 1-day-old male piglet. The tissue was kept in 0.9% NaCl during transport to the laboratory. The skin was manually cut into small pieces after washed with 70% alcohol and removing root hair and cartilage. The small pieces of ear skin tissue were placed on 60 mm. culture dishes (SPL Life Sciences, Gyeonggi-do, Korea) and covered with a sterilized glass slide, added 4 ml of culture medium in the culture dish and culture under a humidified atmosphere of 5% CO_2 in air at 37°C. The culture medium consisted of Minimum Essential Medium Eagle, Alpha modification (αMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, 10270-098), 1mM L-glutamine, and 100 IU/ml penicillin-G, and 100 µg/ml streptomycin sulfate. The medium was changed every 3 days. After the

fibroblasts reached at least 80% confluency, they were harvested using trypsin/EDTA and then passaged until reaching the third passage. In the third passage, the harvested cells were resuspended in a freezing medium containing αMEM supplemented with 20% FBS, and 10% dimethyl sulfoxide (DMSO; 116743, Merck, Germany). Then they were kept at -80 °C overnight and subsequently placed in liquid nitrogen until they were used. Fibroblasts were prepared for donor cells by thawing and cultured on a 35 mm. culture dish (SPL Life Sciences, Gyeonggi-do, Korea) with 3 ml. of culture medium under a humidified atmosphere of 5% CO_2 in air at 37°C for 2-3 days. Porcine fibroblasts at the 4th passage were used as donor cells.

3.4.2 Oocyte enucleation

Cumulus cells were removed from the mature COCs at 42-44 h in IVM medium by mechanically repeated pipetting in 0.1% hyaluronidase solution and washed with TCM199-HEPES supplemented with and 10% FBS. Only metaphase II (MII) with homogeneous cytoplasm and extruded the 1st Polar body (1st PB) were collected for the SCNT process. Then, MII oocytes were immersed in TCM199-HEPES supplemented with 10% FBS containing 5 µg/ml of cytochalasin B (CB) for 5 min. The zona pellucida above the 1st PB were cut to make a small slit and enucleated with a glass needle squeeze out (about 5–10% of the volume of the cytoplasm with the 1st PB). Complete enucleation will be confirmed by staining the squeezed-out cytoplasm and 1st PB with 5 µg/ml Hoechst 33342 and visualizing under a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

3.4.3 Doner cells injection, fusion, and activation

A single donor cell was inserted into the perivitelline space of the enucleated oocyte. The reconstructed oocyte-cell couplets were placed between two wires of fusion electrode covered with fusion medium (0.28mM mannitol, 0.01mM BSA, 0.05mM $CaCl_2.2H_2O$, 0.1 mM $MgSO_4.7H_2O$ (BDH, 101514Y, Darmstadt, Germany), and 0.1 mg/ml HEPES free acid) and fused with two direct currents (DC) pulses of 24V, 16 µsec using an electro-cell fusion machine (SUT F-1, Suranaree University of Technology). The reconstructed embryos were triple-washed and cultured in TCM199-HEPES supplemented with 10% FBS for 1 hr. before being examined the success of fusion

under inverted microscope. After that, they were activated by incubating in 3 μM Ionomycin in TCM199-HEPES supplemented with 10% FBS for 4 min at room temperature and then cultured in 2mM 6-Dimethylamino purine (6-DMAP) at 38.5 °C under a humidified atmosphere of 5% CO_2 .for 3 hr. (Heytens et al., 2008)

3.5 *In vitro* embryo culture with OAC1 treatment

After the processes of SCNT, reconstructed embryos were cultured with 500 μl of PZM medium (Porcine zygote medium-3; (Cao et al., 2012)) added with 0, 1.0, 1.5, and 3.0 μM of OAC1. The culture system was carried out in a 4-well culture dish covered with mineral oil (50 embryos/500 μl) and incubated at 38.5 °C under a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 6 days. Development of the embryos was examined on days 2 and 6 to record the number of cleavage and blastocyst stages, respectively.

3.6 Total cell number assay

Total cell number measurements were performed using the Hoechst 33342 staining method. After the IVC process at 144 h post activation, 10 blastocysts collected from each treatment were incubated with 25 μg/ml Hoechst 33342 dilute in PBS without Ca^{2+} and Mg^{2+}(PBS (-)) for 5 min at room temperature. The stained embryos were mounted on a glass slide and observed under a fluorescence microscope.

3.7 *In vitro* fertilization (IVF)

Embryos from the IVF group were used for positive control (Normal control). After IVM culture (3.3) for 44 h, the oocytes were washed three times in a modified pig-FM medium (Suzuki et al., 2002) containing 10 mM HEPES, 2 mM caffeine, and 5mg/ml BSA. To prepare sperm, fresh semen from the SUT farm was diluted in a BTS extender (Bwanga et al., 1990) at 15-20 °C and preincubated in sperm washing medium (TCM 199 (Earle's salts, Gibco), 4.12 mM calcium lactate, 3.05 mM glucose, and 12% FBS pH 7.8 under a humidified atmosphere of 5% CO_2 in air at 37°C for 30 min. Then

sperm were centrifuged and removed a supernatant and the pellet was resuspended and adjusted with pig-FM to a final concentration of 1.0×10^6/ml. To fertilize spermatozoa with oocytes, a 50 µl droplet of sperm containing 10-15 oocytes in a 35 mm culture dish covered with mineral oil was co-incubated under a humidified atmosphere of 5% CO_2 in the air at 38.5°C for 4-6 h. After fertilization, cumulus cells were removed by gentle pipetting and cultured in *in vitro* culture medium (PZM-3) that was covered with mineral oil at 38.5°C under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The embryos at PN, 2-, 4-, 8-cells, and blastocyst stages were evaluated on days 1, 2 and 5-6, respectively.

3.8 Evaluation the expression level of genes related to embryo development and epigenetic reprogramming of porcine SCNT embryo by qPCR

The expression levels of mRNA for the following genes were analyzed including *OCT4*, *NANOG*, *SOX2*, *DMNT1*, *DMNT3A*, *HDAC1*, *HDAC2*, *HDAC3*, and *GAPDH*

IVF and SCNT embryos at PN, 2-, 4-, 8-cells, and blastocyst stages performed in all treatment groups were harvested in PBS (-), and stored at -80 °C until RNA was extracted. Embryos at PN stage (n=150), 2-cell stage (n=120), 4-cell stage (n=60), 8-cell stage (n=40), and blastocyst stage (n=20) were lysed by lysis buffer and mRNA were extracted using FavorPrep™ Tissue Total RNA Mini Kit (Favorgen Biotech Crop., Pingtung, Taiwan) according to the manufacturer's instructions. Next, mRNA was converted to complementary DNA (cDNA) using biotechrabbit™ cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany). Briefly, 1 ng of total RNA was mixed with 4 µl of 5x cDNA synthesis buffer, 2 µl of dNTP, 0.5 µl of Oligo primer, 0.5 µl of RNase inhibitor, 1 µl Revert up II Reverse transcriptase and PCR-grade water up to 20 µl total volume then the mixer was incubated at 25 °C for 30 sec and 52 °C for 30 min respectively After that, the mixer was incubated at 99 °C for inactivating the reverse transcriptase enzyme. Finally, cDNAs were stored at -20 °C until use. To analyze gene expression (specific primers as shown in Table 3.8.1) quantitative real-time PCR (qPCR) was then the mixer was

incubated at 25 ℃ for 30 sec and 52 ℃ for 30 min respectively then the mixer was incubated at 25 ℃ for 30 sec and 52 ℃ for 30 min respectively After that, the mixer was incubated at 99 ℃ for inactivating the reverse transcriptase enzyme. Finally, cDNAs were stored at -20 ℃ until use. To analyze gene expression (specific primers as shown in Table 3.8.1) quantitative real-time PCR (qPCR) was performed using the KAPA SYBR-Green PCR Master mix (Applied Biosystems, Carlsbad, CA, USA). The examination was performed on the CFX Opus 96 real-time PCR system (Biorad, Hercules, California, USA). Finally, the relative gene expression of embryos under different culture conditions was normalized with GAPDH (reference gene) and compared with the IVF embryos (normal control) with $2^{-\Delta\Delta Ct}$ method.

Table 1. Genes used for real-time qPCR

enes	Primer Sequence5' to 3'	Product Length, bp	Accession No.
genes related to embryo development			
OCT4	F: TTTGGGAAGGTGTTCAGCCAAACG R: TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
SOX2	F: ATGCACAACTCGGAGATCAG R: TATAATCCGGGTGCTCCTTC	130	NM_001123197
NANOG	F: GGTTTATGGGCCTGAAGAAA R: GATCCATGGAGGAAGGAAGA	98	NM_001129971
genes related to epigenetic reprogramming			
DNMT1	F: TCGAACCAAAACGGCAGTAC R: CGGTCAGTTTGTGTTGGACA	215	NM_001032355
DNMT3a	F: CTGAGAAGCCCAAGGTCAAG R: GTACTGATACGCGCACTCCA	200	NM_001097437
HDAC1	F: CGCATGACTCACAATTTGCT R: AGCCATCAAATACCGGACAG	211	XM_013999116
HDAC2	F: ACAGGAGACTTGAGGGAT R: CACATTTAGCGTGACCTT	232	XM_001925318
HDAC3	F: GCTGCTGGACGGATGAGA R: CTGGATGGAGCGTGAAGT	108	NM_001243827
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359

3.9 Evaluation the protein expression level related to DNA methylation and histone acetylation of porcine SCNT embryo by immunofluorescence staining

To stain DNA methylation, 15 embryos from SCNT in treated, untreated, and IVF groups at PN, 2-, 4-, 8-cells, and blastocyst stages were collected and washed in PBS containing 0.1% PVA, fixed in 4% paraformaldehyde in PBS for 1 hr, then washed thoroughly with PBS containing 1% PVA, and permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature. After that, they were treated with 3 M HCl for 30 min at 25°C and then neutralized in 100 mmol/l Tris-HCl (pH 8.5) for 20 min before blocked with PBS containing 2% BSA. The embryos were incubated with primary antibody against 5-mC (Table 2) at 4°C overnight, incubated at 25°C with the secondary antibody (anti-Mouse Alexa Fluor 594) in PBS containing 2% BSA (1:200 dilution) for 3 hr, and washed in 2% PVA in PBS, DNA was counterstained with 25 µg/ml Hoechst 33342 for 3 min., stained embryos were mounted on a glass slide and evaluated under a fluorescence microscope (ECLIPSE Ti-s, Nikon Imaging Japan Inc., Tokyo, Japan) with the same exposure times and adjustments. The mean levels of fluorescence intensity were measured by analyzing the FIJI software

To stain histone methylation and acetylation, 15 embryos from SCNT in treated, untreated and IVF groups at PN, 2-, 4-, 8-cells and BL stages were collected and washed in PBS containing 0.1% PVA, fixed in 4% paraformaldehyde in PBS for 1 hr, permeabilized with 1% PVA triton X-100 in PBS for 30 min at room temperature, washed five-time in 1% PVA in PBS, and blocking in PBS containing 2% BSA at 4°C for overnight. After blocking, the embryos were incubated with the primary antibody against histone H3K9me3 and Ac-H3K14 (Table 2) in PBS containing 2% BSA at 4°C overnight. The embryos were incubated with the secondary antibody (anti-Rabbit Alexa Fluor 488) in PBS containing 2% BSA (1:200 dilution) for 3 h at room temperature, and DNA was counterstained with 25 µg/ml Hoechst 33342 for 3 min, stained embryos were mounted on a glass slide and evaluated under a fluorescence microscope with the same exposure times and adjustments. The mean levels adjustments. The mean level

of fluorescence intensity were measured by analyzing the FIJI software.

Table 2. Primary antibodies and dilutions used for immunofluorescence staining

Markers	Primary antibody	Dilution	Company Cat. No.
DNA methylation 5-mC	Mouse anti-5-mC	1:1000	Sigma-Aldrid MABE146-(32160702)
Histone methylation H3K9me3	Rabbit anti-H3K9me3	1:400	Abcam ab8898
Histone acetylation H3K14ac	Rabbit anti-H3K14ac	1:200	Abcam ab52946

3.10 Statistical Analysis

Statistical analysis was performed by GraphPad version 5 (GraphPadSoftware, San Diego, CA, USA), and data were represented as the mean ± SEM. A value of $P<0.05$ was considered significant. The differences between data were indicated using a one-way analysis of variance (ANOVA), followed by the Tukey–Kramer Honest Significant Difference (HSD) Post hoc test to compare differences between the three groups.

CHAPTER IV

RESULTS

4.1 Optimal concentration of OAC1 supplemented in IVC medium on developmental competence and quality of porcine SCNT embryos

We examined the optimal concentration of OAC1 treatment on the developmental competence of porcine SCNT embryos. The reconstructed embryos were cultured in an IVC medium supplemented with 0, 1.0, 1.5, and 3.0 μM of OAC1 for 6 days, and examined for cleavage rate, blastocyst rate, and total cell numbers. From Table 3, the results showed that the cleavage rate of the 1.5 μM OAC1 group was a significantly higher than the untreated group (86.3 ± 1.2% vs 74.2 ± 1.8%, $p < 0.05$) The blastocyst rate of the 1.5 μM OAC1 group was significantly higher than the untreated group (48.1 ± 1.8% vs 36.1 ± 2.9%, $p < 0.05$) but not significantly different when compared to the 1.0 and 3.0 μM group (42.8 ± 1.9%, and 41.53 ± 2.0%, $p > 0.05$). Total cell numbers of the blastocyst derived from 1.5 μM OAC1 group (47.6 ± 0.8%) was significantly higher than the other groups (39.2 ± 1.8%, 37.2 ± 1.1% and 33.2 ± 1.6%, $p < 0.05$, respectively).

Table 3. The effect of different concentrations of OAC1 on the development of porcine SCNT embryos

OAC1 concentration (μM)	No. of embryos cultured	No. of cleaved (mean ± SEM, %)	No. of blastocysts (mean ± SEM, %)	Total cell number in blastocyst (mean ± SEM)
0	156	117 (74.2 ± 1.8) ^{c}	57 (36.1 ± 2.9) ^{c}	53.8 ± 1.6 ^{c}
1.0	158	124 (79.0 ± 1.7) ^{bc}	68 (42.8 ± 1.9) ^{b}	61.1 ± 1.2 ^{b}
1.5	160	140 (86.3 ± 1.2) ^{a}	76 (48.1 ± 1.8) ^{ab}	66.0 ± 1.8 ^{a}
3.0	158	130 (82.5 ± 1.6) ^{bc}	66 (41.53 ± 2.0) ^{bc}	58.2 ± 1.1 ^{b}

9 replicates were performed

$^{a, b,c}$ Values with different superscripts in the same column are significantly different (*P<0.05*).

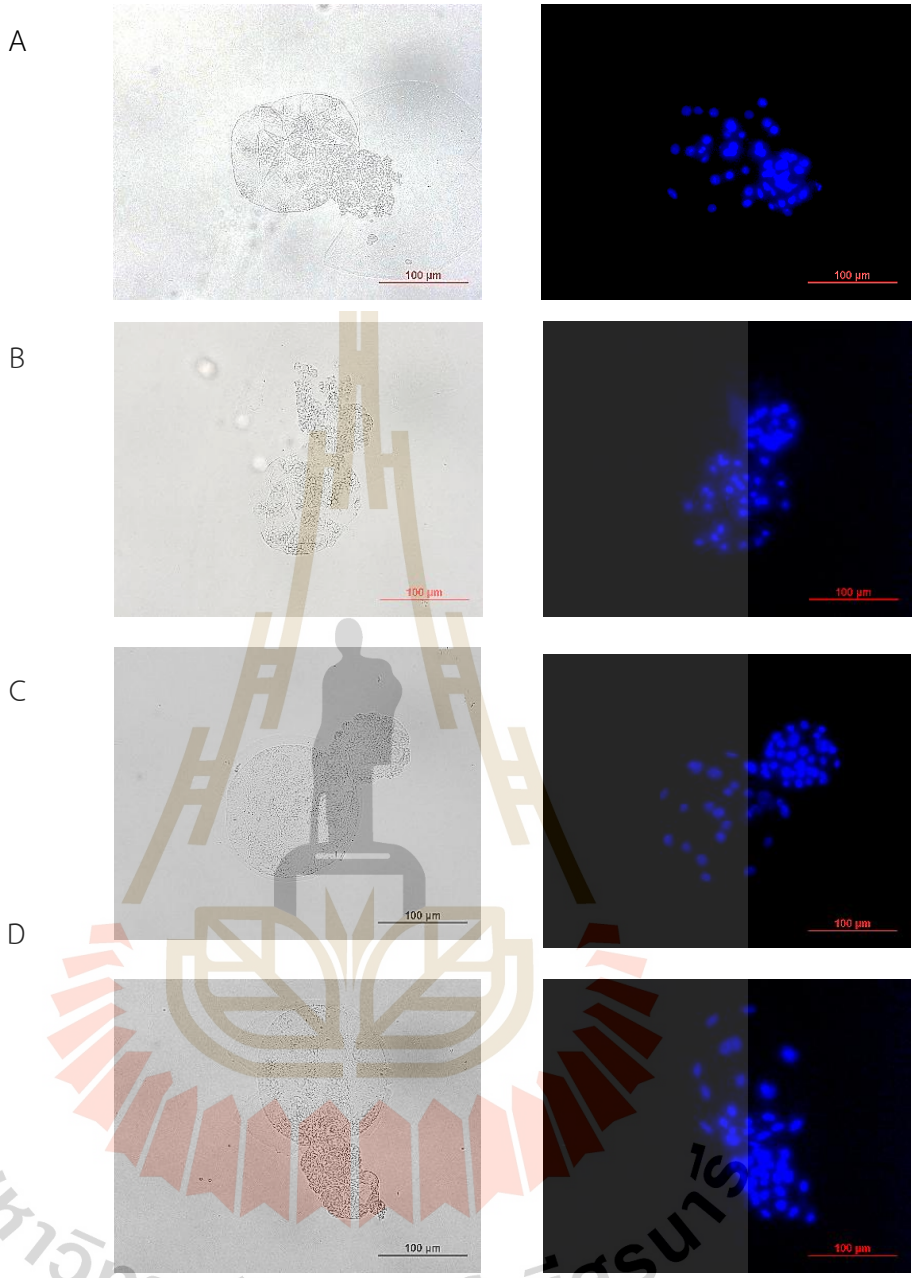


Figure 5. Representative of the porcine blastocyst and Hoechst 33342 stained hatching blastocyst from SCNT treated with different concentrations of OAC1 A) 0 µM of OAC1, B) 1.0 µM of OAC1, C) 1.5 µM of OAC1, and D) 3.0 µM of OAC1, scale bar = 100 µm

Based on the results of the previous experiment, we selected 1.5 μM of OAC1 as the optimal concentration for porcine SCNT embryos. In the next experiment, SCNT embryos treated with 1.5 μM of OAC1 were used to evaluate mRNA expression using the qPCR technique and protein expression through immunofluorescence staining.

4.2 Effect of OAC1 supplemented in IVC medium on developmental competence and quality of porcine SCNT embryos by qPCR

4.2.1 OAC1 affected the expression of genes related to the development and pluripotency of porcine SCNT embryos

We investigated the OAC1 that regulated the mRNA expression level of the development and pluripotency of porcine SCNT embryos, including *OCT-4*, *SOX2*, and *NANOG* genes at PN, 2-, 4-, 8- cells and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control) and SCNT embryos treated with 1.5 μM of OAC1 as a treatment group (SCNT-OAC1) with the results shown in Figure 6.

The expression level of the *OCT-4* gene in the treatment group showed variation across the developmental stages. At PN and 8-cell stages, the *OCT-4* expression level of the SCNT-OAC1 group was significantly higher than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group. At 2- and 4-cell stages, the *OCT-4* expression level of the SCNT-OAC1 group was significantly higher than the SCNT-Untreated and IVF groups ($p < 0.05$). Finally, at the blastocyst stage, the *OCT-4* expression level of the SCNT-OAC1 group had no significant difference when compared with the SCNT-Untreated and IVF groupsThe expression level of the *SOX2* gene in the treatment group showed variation across different stages. At PN stage, the *SOX2* expression level of the SCNT-OAC1 group was significantly higher than SCNT-Untreated and IVF groups ($p < 0.05$). At the 2-cell stage, the *SOX2* expression level of the SCNT-OAC1 group was significantly lower than the SCNT-Untreated, but no significant difference when compared with the IVF groups. At 4-cell and blastocyst

stages, the *SOX2* expression level of the SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but was significantly higher than the SCNT-Untreated group ($p < 0.05$). Finally, at 8-cell stage, the *SOX2* expression level of the SCNT-OAC1 group was significantly higher than SCNT-Untreated and IVF groups ($p < 0.05$), but the IVF group was significantly higher than the SCNT-Untreated group ($p < 0.05$).

The expression level of the *NANOG* gene in the treatment group showed variation across different stages. At PN and 4-cell stages, the *NANOG* expression level of the SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but no significant difference when compared with the SCNT-Untreated group. At the 2-cell stage, the *NANOG* expression level of the SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but was significantly higher than the SCNT-Untreated group. Finally, at 8-cell and blastocyst stages, the *NANOG* expression level of the SCNT-OAC1 group was significantly higher than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group.

4.2.2 OAC1 affected the expression of genes related to the DNA methylation and histone acetylation of porcine SCNT embryos

We investigated the OAC1 that regulated the mRNA expression level of the DNA methylation of porcine SCNT embryos, including *DNMT1*, and *DNMT3A* genes at PN, 2-, 4-, 8- cells, and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control) and SCNT embryos treat with 1.5 μM OAC1 as a treatment group (SCNT-OAC1) with the results shown in Figure 7.

The expression level of the *DNMT1* gene in the treatment group showed variation across different stages. At the PN stage, the *DNMT1* expression level of the SCNT-OAC1 group was significantly higher than the IVF group ($p < 0.05$), but no significant difference when compared with the SCNT-Untreated group. At the 2-cell stage, the *DNMT1* expression level of the SCNT-OAC1 group was significantly higher than SCNT-Untreated and IVF groups ($p < 0.05$), and the SCNT-Untreated group was

significantly higher than the IVF group. At the 4-cell stage, the *DNMT1* expression level of the SCNT-OAC1 group was significantly lower than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with IVF group. Finally, At 8-cell and blastocyst stages, the *DNMT1* expression level of the SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but no significant difference when compared with the SCNT-Untreated group.

The expression level of the *DNMT3A* gene in the treatment group showed variation across different stages. At PN stage, the *DNMT3A* expression level of the SCNT-OAC1 group was significantly lower than the SCNT-Untreated group ($p < 0.05$), but was significantly higher than the IVF group ($p < 0.05$). At 2- and 8-cell stage, the *DNMT3A* expression level of the SCNT-OAC1 group was significantly higher than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group. At the 4-cell stage, the *DNMT3A* expression level of the SCNT-OAC1 group was significantly lower than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group. Finally, at blastocyst stage, the *DNMT3A* expression level of the SCNT-OAC1 group was significantly higher than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group.4.2.3 OAC1 affected the expression of genes related to histone acetylation of porcine SCNT embryos

We investigated the OAC1 that regulated the mRNA expression level of the DNA methylation of porcine SCNT embryos, including *HDAC1*, *HDAC2,* and *HDAC3* genes at PN, 2-, 4-, 8- cells and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control) and SCNT embryos treat with 1.5 μM OAC1 as a treatment group (SCNT-OAC1) with the results shown in Figure 8.

The expression level of the *HDAC1* gene in the treatment group showed variation across different stages. At the PN stag, the *HDAC1* expression level of SCNT-OAC1 group was significantly higher than the IVF group ($p < 0.05$), but no significant difference when compared with SCNT-Untreated. At the 2-cell stage, the *HDAC1*

expression level of the SCNT-OAC1 group was significantly higher than SCNT-Untreated and IVF groups ($p < 0.05$), and the SCNT-Untreated group was significantly higher than the IVF group ($p < 0.05$). At the 4-cell stage, the *HDAC1* expression level of the SCNT-OAC1 group was significantly lower than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with IVF group. Finally, at 8-cell and blastocyst stages, the *HDAC1* expression level of the SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but no significant difference when compared with the SCNT-Untreated group. The expression level of the *HDAC2* gene in the treatment group showed variation across different stages. At the PN stage, the *HDAC2* expression level of SCNT-OAC1 group was significantly higher than the IVF group ($p < 0.05$), but no significant difference when compared with SCNT-Untreated. At the 2-cell stage, the *HDAC2* expression level of SCNT-OAC1 group was significantly higher than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group. At the 4-cll stage, the *HDAC2* expression level of SCNT-OAC1 group was significantly lower than IVF and SCNT-Untreated groups ($p < 0.05$). At the 8-cell stage, the *HDAC2* expression level of SCNT-OAC1 group was significantly lower than the IVF group ($p < 0.05$), but no significant difference when compared with the SCNT-Untreated group. Finally, at blastocyst stage, the *HDAC2* expression level of SCNT-OAC1 group was significantly lower than the SCNT-Untreated group ($p < 0.05$), but no significant difference when compared with the IVF group. The expression level of the *HDAC3* gene in the treatment group showed variation across different stages. At PN, 8-cell, and blastocyst stages, the *HDAC3* expression level of the SCNT-OAC1 was significantly lower than the SCNT-Untreated ($p < 0.05$), but no significant difference when compared with the IVF group. At the 2-cell stage, the *HDAC3* expression level of the SCNT-OAC1 was significantly higher than the SCNT-Untreated group ($p < 0.05$), but was significantly lower than the IVF group. Finally, at the 4-cell stage, the *HDAC3* expression level of the SCNT-OAC1 had no significant difference when compared with all of groups.

4.3 Effect of OAC1 supplement in IVC medium on the protein expression level of porcine SCNT embryos by immunofluorescence staining

Finally, we investigated the effects of 1.5 μM OAC1 supplemented in IVC medium on protein expression including the global DNA methylation (5-mC) and histone modification (H3K9me3 and H3K14ac) at PN, 2-, 4-, 8- cells and blastocyst stages. The protein expression levels were examined by immunofluorescence staining (ICC).

4.3.1 OAC1 affected to the protein expression level of DNA methylation of porcine SCNT embryos

To examine the OAC1 that regulated the global DNA methylation of porcine SCNT embryos. The fluorescence intensity levels of 5-mC at PN, 2-, 4-, 8-cells and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control), and SCNT embryos treated with 1.5 μM OAC1 as a treatment group (SCNT-OAC1) was observed and shown in Figure 9.

The protein expression level of the 5-mC in the treatment group showed variation across different stages. At the PN, 2-, 4-cell and blastocyst stages, the 5-mC expression level had no significant difference when compared with all of groups ($p > 0.05$). In addition, at the 8-cell stage, the 5-mC expression level was significantly lower than the IVF group ($p < 0.05$), but no significant difference when compared with SCNT-Untreated group.

4.3.2 OAC1 affected of the protein expression level of histone methylation of porcine SCNT embryos

To examine the OAC1 that regulated the global histone methylation of porcine SCNT embryos. The fluorescence intensity levels of H3K9me3 at PN, 2-, 4-, 8-cells and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control) and SCNT

Untreated), IVF group as a positive control (normal control) and SCNT embryos treated with 1.5 μM OAC1 as a treatment group (SCNT-OAC1) was observed and shown in Figure 10.

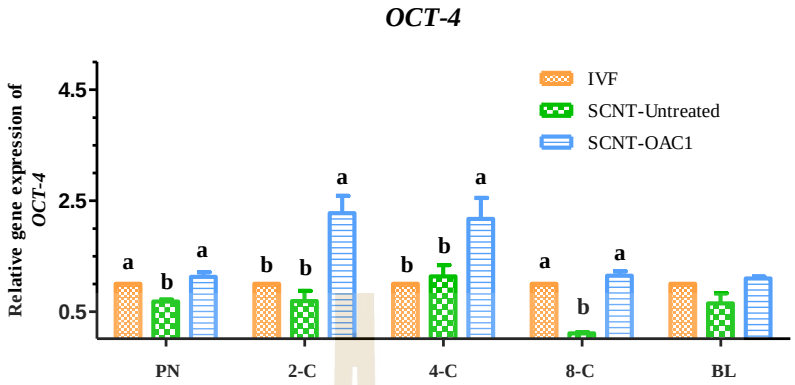
The protein expression level of the H3K9me3 in the treatment group showed variation across different stages. At the PN, 4-, and 8-cell stages, the H3K9me3 expression level was not significant difference when compared with the SCNT-Untreated and IVF groups. At 2-cell stage, the H3K9me3 expression level was significantly lower than the IVF group ($p < 0.05$) but no significant difference when compared with the SCNT-Untreated group. However, at the blastocyst stage, the H3K9me3 expression level was significantly higher than the SCNT-Untreated group ($p < 0.05$) but no significant difference when compared with the IVF group.

4.3.3 OAC1 affected to the protein expression level of histone acetylation of porcine SCNT embryos

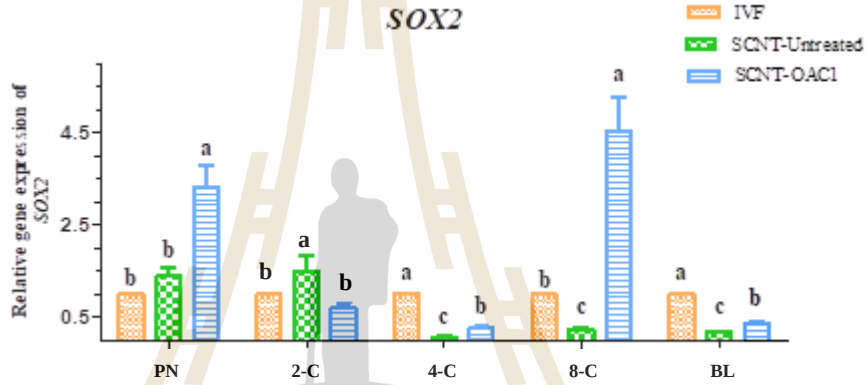
To examine the OAC1 that regulated the global histone methylation of porcine SCNT embryos, the fluorescence intensity levels of H3K14ac at PN, 2-, 4-, 8-cells and blastocyst stages between untreated SCNT embryos group as a negative control (SCNT-Untreated), IVF group as a positive control (normal control) and SCNT embryos treat with 1.5 μM OAC1 as a treatment group (SCNT-OAC1) was observed and shown in Figure 11.

The protein expression level of the H3K14ac in the treatment group showed variation across different stages. At the PN, the H3K14ac expression level was significantly lower than the IVF group ($p < 0.05$) but no significant difference when compared with SCNT-Untreated group. At the 2-cellstage, the H3K14ac expression level was significantly lower than the IVF group and was significantly higher than SCNT-Untreated group.4-, 8-cells and blastocyst stages, the H3K14ac expression level was significantly higher than the SCNT-Untreated group ($p < 0.05$) but no significant difference when compared with the IVF group.

A



(B)



(C)

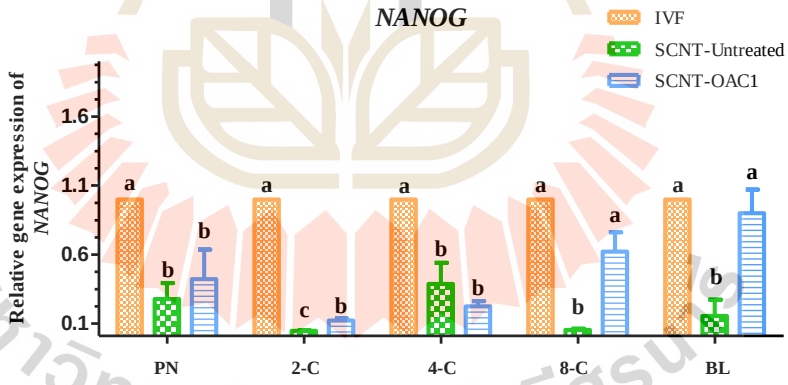
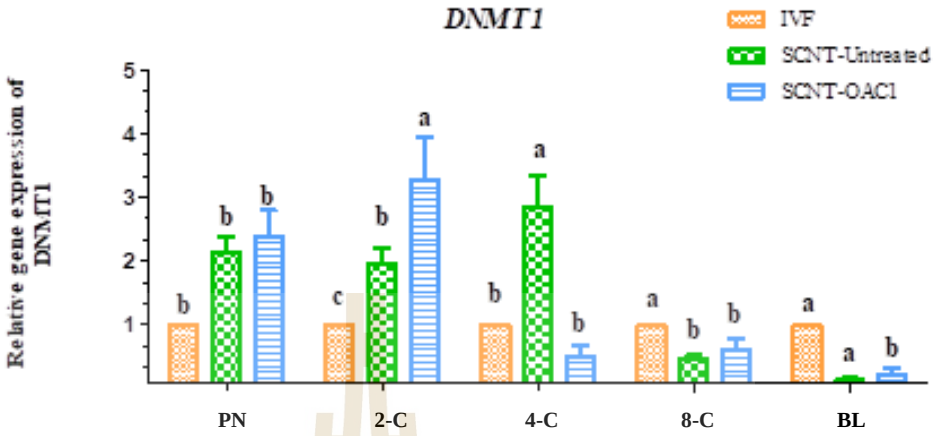


Figure 6. Comparison of mRNA expression levels (mean ± SEM) of genes related to development and pluripotency of porcine embryos between the SCNT-OAC1, IVF, and SCNT-Untreated groups at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages: (A) Expression levels of *OCT-4* gene, (B) Expression levels of *SOX2* gene, and (C) Expression levels of *NANOG* gene. [a, b, and c] Values with different superscripts indicate a significant difference ($p < 0.05$). Error bar = standard error of the mean.

(A)



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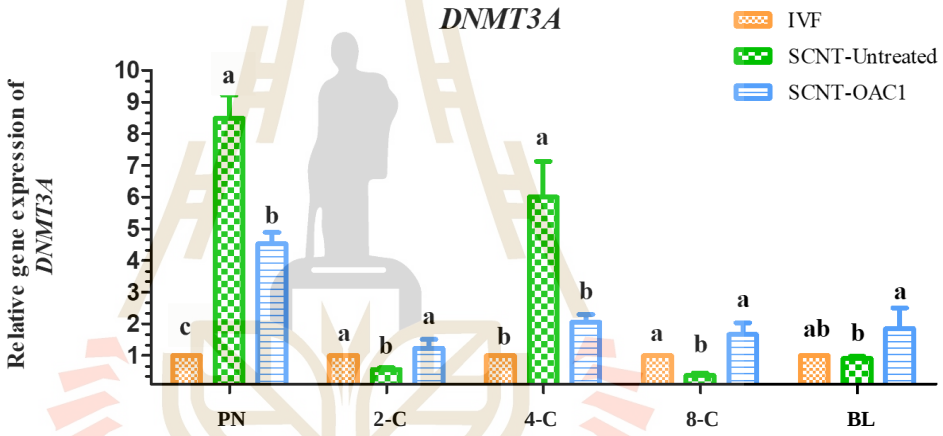
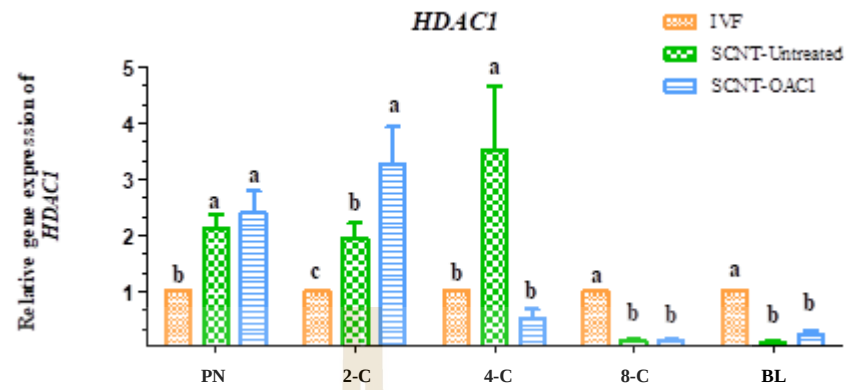
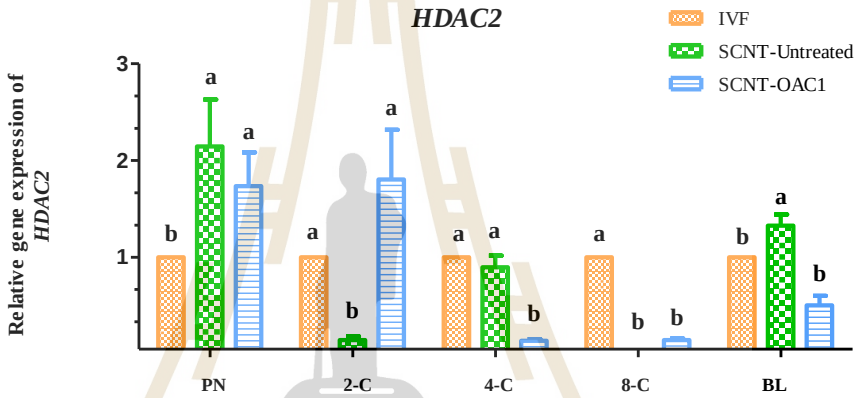


Figure 7. Comparison of mRNA expression levels (mean ± SEM) of genes related to DNA methylation of porcine embryos between the SCNT-OAC1, IVF, and SCNT-Untreated groups at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages: (A) Expression levels of *IDNMT1* gene, (B) Expression levels of *DNMT3A* gene. [a, b, and c] Values with different superscripts indicate significant difference ($p < 0.05$). Error bar = standard error of the mean

(A)



(B)



(C)

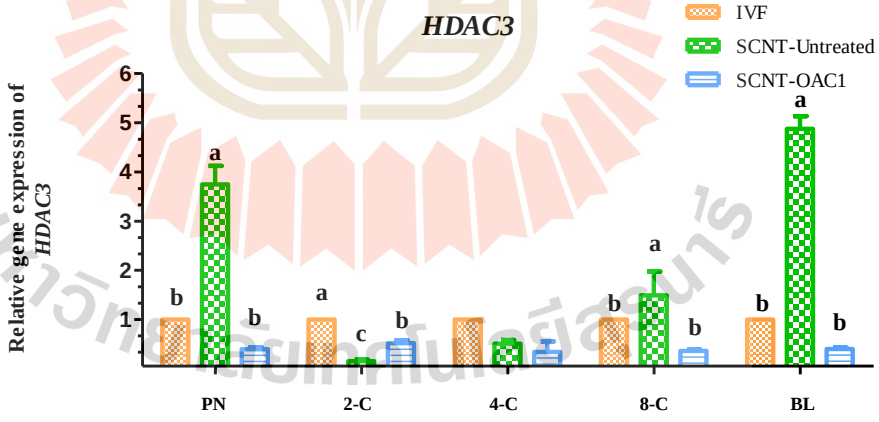


Figure 8. Comparison of mRNA expression levels (mean ± SEM) of genes related to histone acetylation of porcine SCNT embryos between the SCNT-OAC1, IVF, and SCNT-Untreated groups at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages: (A) Expression levels of *HDAC1* gene, (B) Expression levels of *HDAC2* gene, and (C) Expression levels of *HDAC3* gene. [a, b and c] Values with different superscripts indicate significant difference ($p < 0.05$). Error bar = standard error of the mean

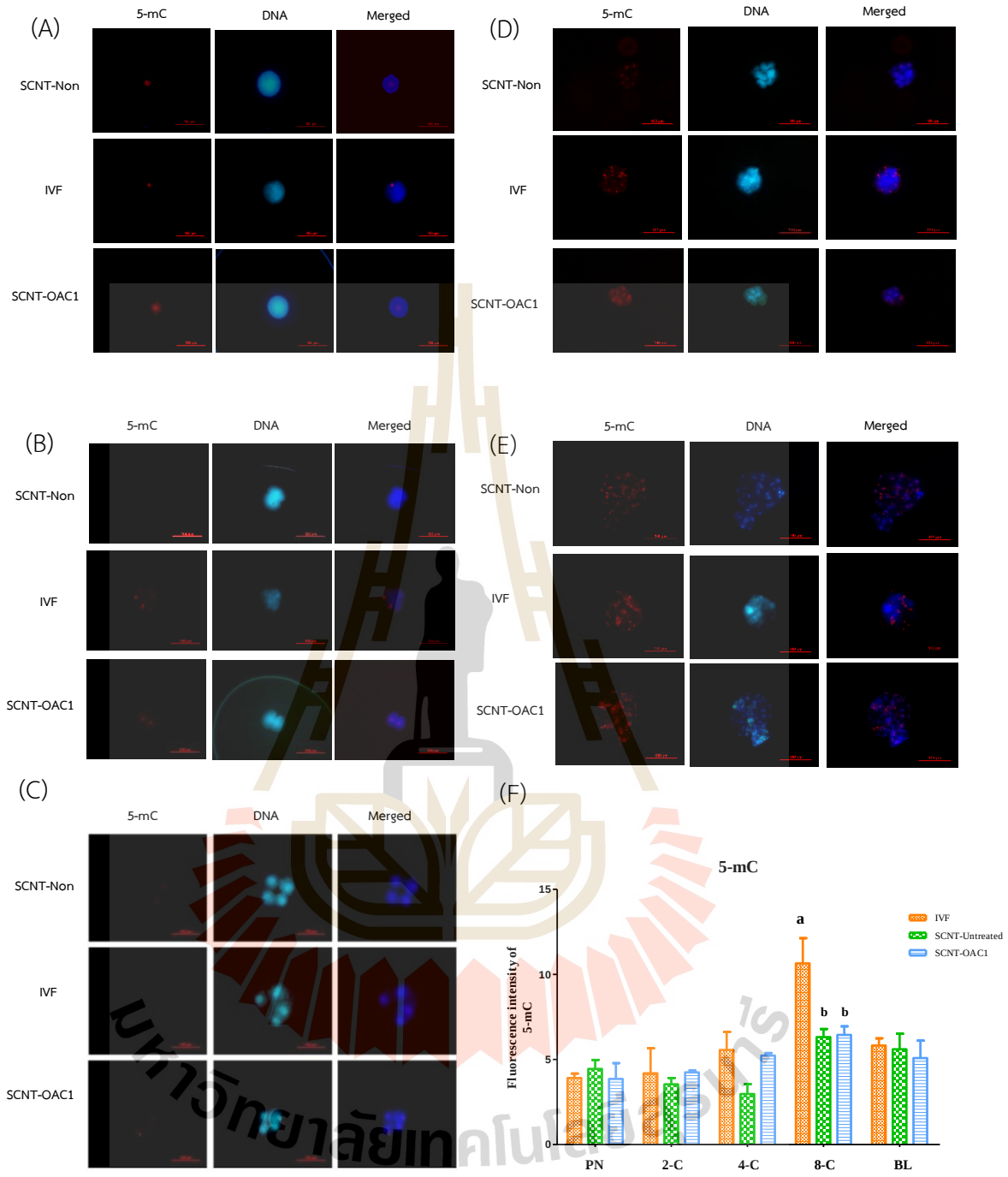


Figure 9. Representative the immunofluorescence images of 5-mC levels of the porcine embryos from the SCNT-OAC1, IVF, and SCNT-Untreated groups at: (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell, (E) blastocyst stage, and (F) Quantification on fluorescence intensity of 5-mC levels at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages, respectively (mean ± SEM). [a and b] Values with different letters

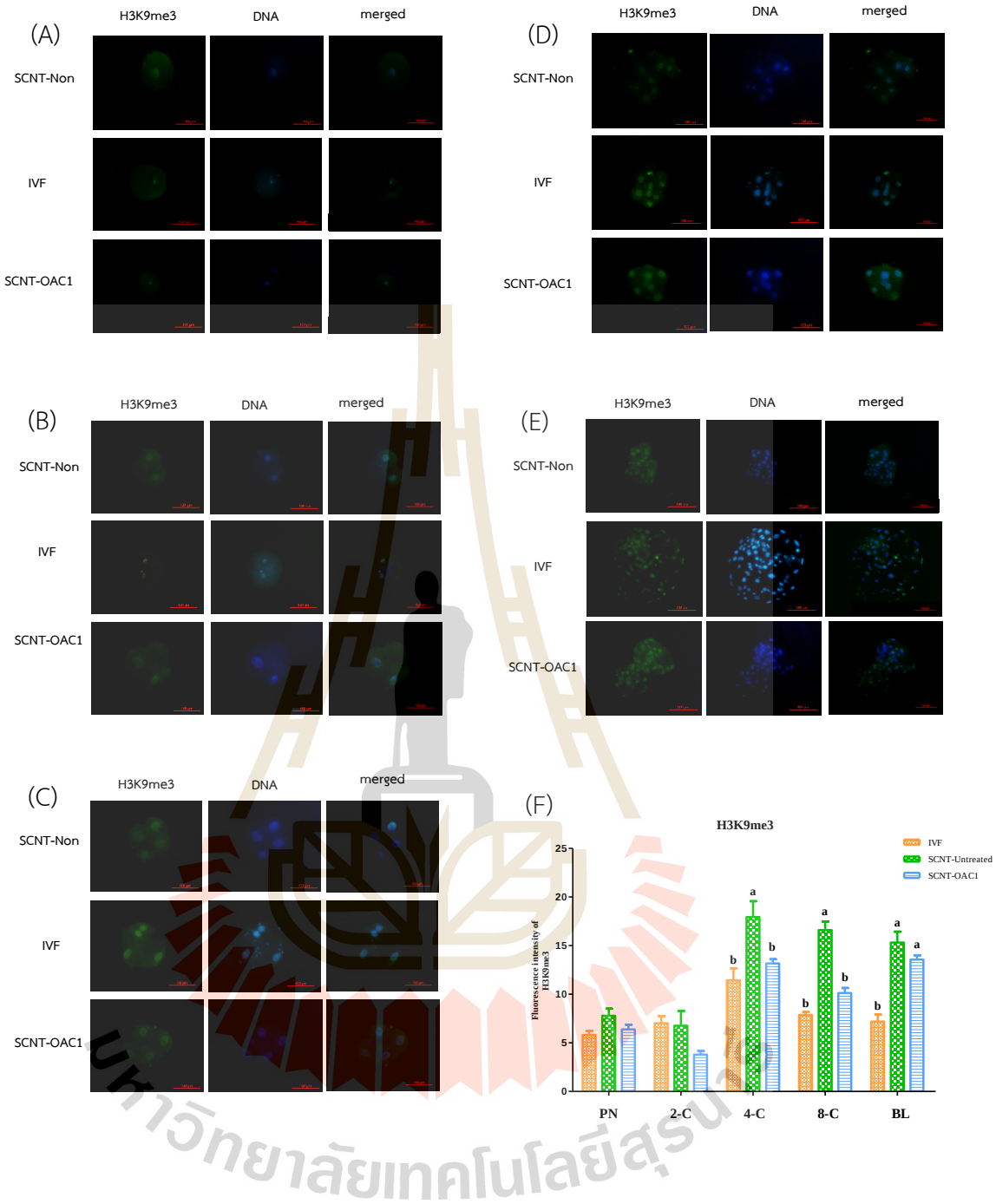


Figure 10. Representative the immunofluorescence images of H3K9me3 levels of the porcine embryos from the SCNT-OAC1, IVF, and SCNT-Untreated groups at: (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell, (E) blastocyst stage, and (F) Quantification on fluorescence intensity of H3K9me3 levels at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages, respectively (mean ± SEM). [a and b] Values with different letters indicate significant differences ($p < 0.05$). Error bar = standard error of the mean

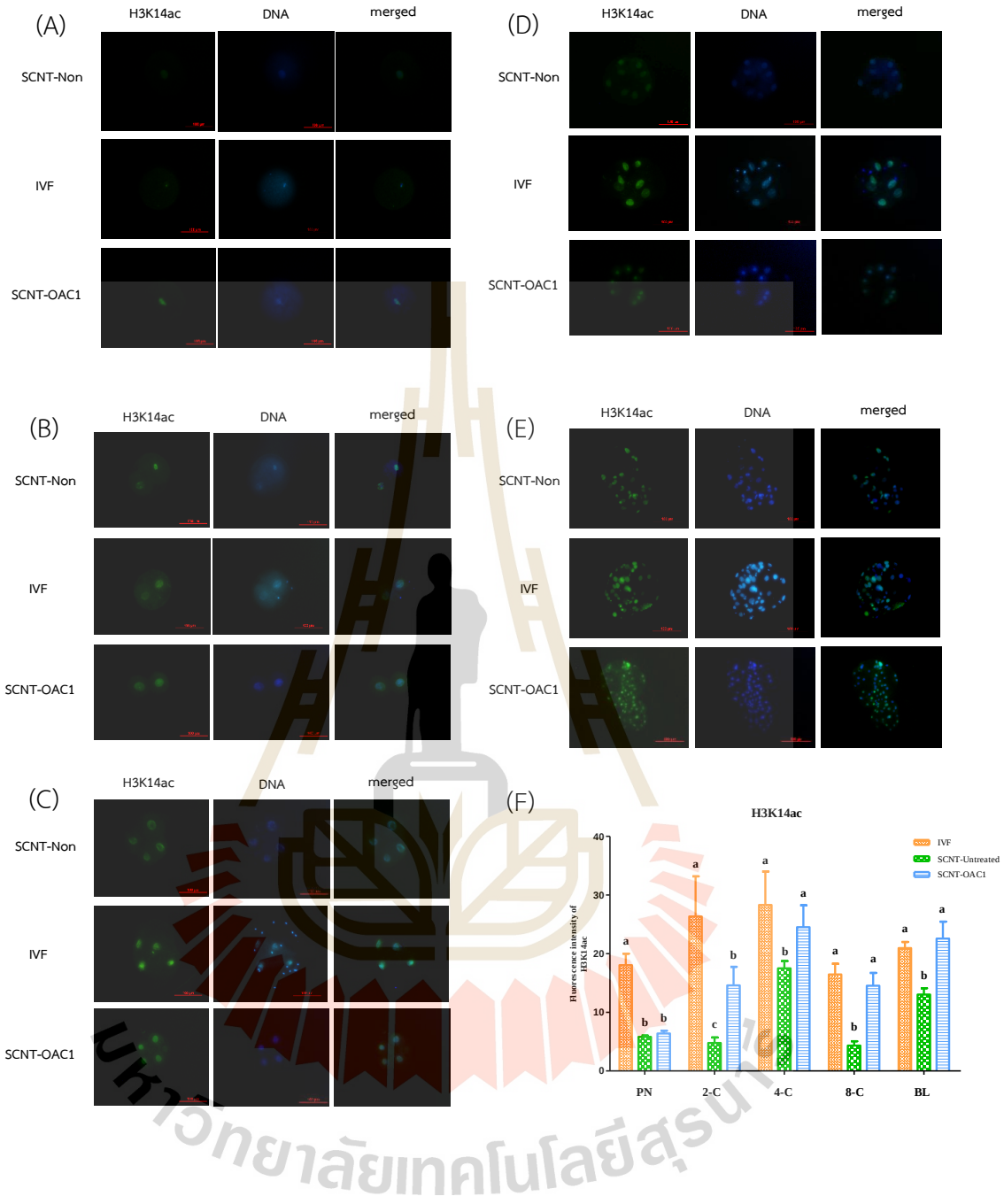


Figure 11. Representative the immunofluorescence images of H3K14ac levels of the porcine embryos from the SCNT-OAC1, IVF, and SCNT-Untreated groups at: (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell, (E) blastocyst stage, and (F) Quantification on fluorescence intensity of H3K14ac levels at PN, 2-cell, 4-cell, 8-cell, and blastocyst stages, respectively (mean ± SEM). [a and b] Values with different letters indicate significant differences ($p < 0.05$). Error bar = standard error of the mean.

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

Since the first report of successful cloning of the mammalian animal (Wilmut et al., 1997). The SCNT technique is used for genetic conservation, animal genetic modification, biomedical applications, and animal model (Loi et al., 2016; Vajta, 2018). However, the major challenge of producing a healthy cloned animal is incomplete nuclear reprogramming (Samiec & Skrzyszowska, 2018; Yang et al., 2007).

During early embryonic development, transcription factors (TFs) play a crucial role in determining the fate of blastomere by regulating the activation or suppression of key development genes *OCT-4* is a core component of the pluripotency regulation network, guiding blastomere to ICM, which later gives rise to embryonic stem cell or toward differentiation into trophoblast cell, with contribution to extra-embryonic structure (Niwa et al., 2000). Several studies have demonstrated a function of *OCT-4* in the embryos development (Boiani et al., 2002; Nichols et al., 1998). Further studies on the transcription regulation of *OCT-4* in porcine improved the developmental efficiency of nuclei transfer embryos, suggesting that high *OCT-4* expression in donor cells are more efficient promote nuclei reprogramming (du Puy et al., 2011; Lee et al., 2014). However, the mRNA expression of *OCT-4* in porcine and bovine SCNT embryos was significantly lower than that derived by IVF (Garcia-Canovas et al., 2024; Long et al., 2007; Zhou et al., 2008). Previous studies have made progress on identifying an OCT-4 promoter-activating compound to promote the *OCT-4* expression to enhance the efficiency of reprogramming (Li et al., 2012) and differentiated cells into pluripotent cells (Huang et al., 2016; Li et al., 2012; Moradi-Hajidavaloo et al., 2023).

In our study, first of all, we treated various concentrations of OAC1 supplemented in IVC medium and cultured for 6 days to examined the optimal concentration by measuring the quality and development of porcine SCNT embryo

(cleavage rate, blastocyst rate, and total cell numbers) between treated and untreated OAC1. The results showed that 1.5 μM of OAC1 gives a significantly increased the embryo development particular at cleavage and blastocyst rates, and the total cell numbers of blastocyst-derived porcine SCNT. The results of this experiment are similar to a SCNT bovine embryo that was treated with 1.5 μM of OAC1, showing an increased developmental rate (Moradi-Hajidavaloo et al., 2023), Next, we used 1.5 μM of OAC1 to investigate the mRNA expression of *OCT-4*, *SOX2*, and *NANOG* genes and the nuclear reprogramming on the mRNA expression of *DNMT1*, *DNMT3A*, *HDAC1*, *HDAC2*, and *HDAC3* and protein expression level on global DNA methylation (5-mC) and histone modification (H3K9me3 and H3K14ac).

The pluripotency markers of embryonic cells, including *OCT-4*, *SOX2*, and *NANOG*, play an important role in self-renewal and maintaining the pluripotency of cloned embryos (Keramari et al., 2010; Park et al., 2012). The effect of pluripotent markers induced by OAC1 has not yet been reported in a porcine SCNT embryo. However, the *OCT-4* expression in our study showed an increase in the SCNT-OAC1 group at the PN until 8-cell stages than the untreated and IVF group but no significant difference was observed at blastocyst stages between the untreated and IVF group. This result is consistent with the report in SCNT porcine embryo, the *OCT-4* expression level is detected until the 8-cell stage from transcription using maternal mRNA, and the zygote *OCT-4* expression being from 8 – 16-cell stage (Kirchhof et al., 2000), In addition, in mouse embryos, the loss of *OCT-4* expression showed at the blastocyst stage due to the occurrence of differentiation of totipotent cells to somatic lineages (Pesce & Schöler, 2001). The *SOX2* expression level of SCNT-OAC1 group showed higher than untreated and IVF groups at PN and 8-cell stages, lower than IVF group at 4-cell and blastocyst stage but no difference at the 2-cell stage. *Sox2* acts cooperatively with *OCT-4* at promoters activating the transcription of several genes, which play important roles in embryo development (Nishimoto et al., 1999; Yuan et al., 1995). The downregulate of *SOX2* expression is possible to decreasing *OCT-4* expression levels influence several transcription factors and induce developmental arrest (Kirchhof et

al., 2000). The *NANOG* expression level in the OAC1 treatment group was lower than in the IVF group at the PN stage until the 4-cell stage, and there was no difference at the 8-cell to blastocyte stages. The results were consistent with the previous study, the *OCT-4* and *SOX2* network stimulates the expression of *NANOG* has shown that the expression of *OCT-4*, *SOX2*, and *NANOG* follows the same trend, with high expression level of *NANOG* at the 8-cell stage to the blastocyst stage (Kalmar et al., 2009; Rodda et al., 2005) and the distinct expression pattern of *OCT-4* compared to *NANOG* at certain stages of embryo development supports previous studies indicating that OAC1 promotes the expression level of *OCT-4* and *NANOG* (Li et al., 2012).

Epigenetic mechanisms play an important role in mammalian development by regulating the expression of genes that are essential for pluripotency and differentiation (Dean et al., 2005). DNA methylation occurs at the fifth carbon of cytosine residues within CpG dinucleotides and is essential for long-term transcriptional silencing as well as normal mammalian embryonic development. Somatic cells with high levels of DNA methylation are often used as nuclear donors for cloning, resulting in cloned embryos that typically exhibit increased DNA methylation, which is abnormally hypermethylated (Enright et al., 2003). *DNMT1*, a maintenance methyltransferase, is recruited to the replication fork during the S-phase of the cell cycle, where it methylates the newly synthesized DNA strand (Sharif et al., 2007). DNMT3s enzymes are responsible for establishing *de novo* methylation during gametogenesis and early embryonic development (Okano et al., 1999). DNA demethylation occurs during early embryonic development, followed by re-methylation at later stages (Ivanova et al., 2020; Reik et al., 2003). The reprogramming of DNA methylation in early embryos is regulated by genes involved in both methylation and demethylation processes (Wu & Zhang, 2014). The dynamics of DNA methylation during preimplantation embryonic development have primarily been analyzed using immunofluorescence quantification, in which a specific antibody (anti-5MC) labels methylated DNA. This method is particularly effective for assessing genome-wide DNA methylation levels in individual nuclei (Santos & Dean, 2006). We

investigate the mRNA expression of *DNMT1* and *DNMT3A* and the protein expression of 5-mC involved in the dynamic of DNA methylation. The results showed that the expression of *DNMT1* in the OAC1 treatment group was higher than IVF group at PN, and 2-cell stages, but no significant difference at the 4-cell stage, and significantly lower than the IVF group at 8-cell to blastocyst stages. The expression of *DNMT3A* in the OAC1 treatment group at the PN stage was significantly lower than untreated group but higher than IVF group. At the 2-cell stage had no significant difference from the IVF group but significantly higher than the untreated group. At the 4-cell stage had no significant difference from the IVF group but significantly lower than the untreated group. At 8-cell to blastocyst stages was significantly higher than the untreated group and no significant difference from the IVF group. For the protein expression on 5-mC had no significant difference when observed at the early stage and blastocyst stage, but was significantly lower than the IVF group at the 8-cell stage. Normally, the *DNMTs* expression at the early stage of IVF is lower than SCNT embryos (Kaneda et al., 2004) but it could be higher than SCNT embryos that possible explanation may be the limitation of IVM oocytes to process several spermatozoa entering the ooplasm (Gioia et al., 2005). Inconsistent reports of SCNT embryos can be high DNA methylation expression is partially retained its methylation pattern even after the nucleus has been structurally remodeled into a pronucleus-like state (Deshmukh et al., 2011). Another reason that porcine IVF embryos at early stages still increase more than SCNT embryos is that after fertilization, the parental epigenomes undergo significant changes to restore totipotency in the zygote. The tightly packed paternal genome quickly unravels, sperm protamines are replaced with histones, and DNA is actively demethylated without the need for replication (Abdalla et al., 2009; Fulka et al., 2008). In addition, from the 8-cell to the blastocyst stage, normalized DNA methylation levels continuously increased in IVF embryos, but contrasted to SCNT embryos that decreased DNA methylation is possibly the result of *de novo* methylation related to the activity of *de novo* DNA methyltransferases DNMT3s (Bermejo-Alvarez et al., 2008; Loi et al., 2008). On the another hand, the report of global DNA methylation at an early stage and blastocyst stage does not different between IVF and SCNT embryos

but at the 8-cell stages, the IVF embryo increases more than the SCNT embryo possibly re-methylated occurs and new genomic imprints are established by activating the methylation (Bonk et al., 2008; Deshmukh et al., 2011; Kaneda et al., 2004)

To explain histone modification, chromatin structure plays a crucial role in the post-translational modification of histones at the N-terminal end, which includes acetylation, phosphorylation, methylation, glycosylation, and carbonylation (Ruijter et al., 2003;Tang et al., 2013). The acetylation and deacetylation processes are performed by two enzymes, which are histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs add acetyl groups to the lysine residues to initiate the transcription activity, while HDACs remove these groups (D'Mello, 2020; Wang et al., 2009). Histone deacetylase inhibitors (HDACi) enhance the acetylation levels of core histones in cloned bovine embryos, suggesting that HDACi promote hyperacetylation and activates key genes essential for SCNT embryonic development (Oliveira et al., 2010). In a previous study, the mechanism of OAC1 is unclear on histone modification and its effects of OAC1 on *HDAC1-3* expression levels have not yet been reported in a porcine SCNT embryo. This study investigates whether OAC1 might be an HDACi. Our results showed that the expression level of *HDAC1* in the OAC1 treatment group at the PN stage was not significant difference when compared with the untreated group, but was significantly higher than the IVF group. At 2-cell stage was significantly higher than IVF and untreated groups. At the 4-cell stage, it was significantly lower than the untreated group, but no significant difference when compared with the IVF group. In addition, the *HDAC1* expression level of the treatment group at the 8-cell and blastocyst stages had no significant difference when compared with the untreated group, but was significantly lower than the IVF group. Moreover, the expression levels of *HDAC2* at the PN stage in the OAC1 treatment group had no significant difference when compared with the untreated group, but it was significantly lower than the IVF group. At the 2-cell stage, the OAC1 treatment group had no significant difference when compared with the IVF group, but was significantly lower than the untreated group. At the 4-cell stage, the OAC1 treatment group was significantly lower than the IVF and untreated groups. At the 8-cell stage, the OAC1 treatment group had no significant difference

when compared with the untreated group but was significantly lower than the IVF group. At the blastocyst stage, the OAC1 treatment group had no significant difference when compared with the IVF group, but it was significantly lower than the untreated group. For the expression level of *HDAC3,* at the PN, 8-cell and blastocyst stages in the OAC1 treatment group had no significant difference when compared with the IVF group, but was significantly lower than the untreated group. At the 2-cell stage in the OAC1 treatment group was significantly lower than the IVF group, but was significantly higher than the untreated group. However, at the 4-cell stage in the OAC1 treatment had no significant difference in all of groups.

Our result is consistent with previous studies that report the expression pattern of *HDAC1*, *HDAC2*, and *HDAC3* at early stages is unclear (Salehi et al., 2019). The *HDAC1* expression levels relate to *HDAC2,* which in IVF was lower than SCNT embryos, but the IVF embryos can increase *HDACs* more than SCNT embryos from the 4-cell to the blastocyst stages, because this relates to ZGA and re-methylated occurs interacts with *HDAC* expression (McGraw et al., 2003; Segev et al., 2001). Although *HDAC3* expression at an early stage is unclear, but the 8-cell to blastocyst stage, the expression level of *HDAC3* decreased in IVF embryos because *HDAC3* is responsible for cell proliferation and differentiation (Jiang & Hsieh, 2014). However, the molecular mechanism of OAC1 on *HDAC1-3* on porcine SCNT embryo development needs more investigation.

Moreover, H3K14ac plays a significant role in embryonic development by influencing chromatin structure and gene expression (Tang et al., 2023). In early stage embryos, the re-establishment of constitutive heterochromatin is crucial for genome stability and transposon silencing. H3K14ac facilitates the recruitment of the methyltransferase, which deposits H3K9me3 marks necessary for heterochromatin formation (Tang et al., 2024). In the IVF embryos, H3K14ac is maintained at appropriate levels during early development, supporting normal chromatin remodeling and gene expression necessary for embryogenesis. In contrast, SCNT embryos often exhibit aberrant H3K14ac patterns that are deacetylated in donor somatic cells during the SCNT process, leading to hypoacetylation in the resulting embryos (Li & Sun, 2022). The protein expression level of H3K14ac in OAC1 treatment was significantly lower

than the IVF group at PN and 2-cell stages, and increased similarly to the IVF group and higher than the untreated group supported previous studies. The expression of H3K14ac in IVF embryos was significantly higher than the SCNT embryos (Sun et al., 2020; Zhou et al., 2013). Interestingly, our result on the expression levels of H3K14ac similar to IVF embryo indicated that the OAC1 could improve the expression level of H3K14ac

The last of our results is the effect of OAC1 on H3K9me3, which involved limiting the success of nuclear reprogramming, and indeed, it is associated with the repression of transcription (Hublitz et al., 2009). The porcine SCNT embryo showed abnormal expression of H3K9me3, and the downregulation of H3K9me improved transcriptional reprogramming in mouse, cattle, and porcine SCNT embryos (Cao et al., 2015; Liu et al., 2018; Matoba et al., 2014). The results of this study showed that the protein expression level of H3K9me3 at PN and 2-cell stage had no different in all groups. In addition, at 4-cell and 8-cell stages of OAC1 treatment was significantly lower than the untreated group but no difference from the IVF group. At the blastocyst stage, OAC1 treatment was significantly higher than the IVF group but no difference from the untreated group. In previous studies, the expression of H3K9me3 in IVF embryos was lower than SCNT embryos (Matoba et al., 2014). However, our results showed that protein expression level of H3K9me3 at the early stage had no different in all groups. This may be explained by the presence of asymmetric H3K9me3 signals in the paternal and maternal genomes, suggesting that the imbalance arises not only from differences inherited from gametes but also from distinct reprogramming processes in each parental genome. (Wang et al., 2018). At the 4-cell to 8-cell stages, the protein expression level of H3K9me3 was not differ with IVF embryos, but it was significantly lower than untreated embryos caused by the ZGA process (Weng et al., 2020). Interestingly, our results found that OAC1 treatment similar to IVF indicated that OAC1 could improve the protein expression level of H3K9me3.

5.2 Conclusion

This report examined the effect of the OAC1 supplemented in IVC medium on the development of porcine SCNT embryos. The optimal concentration of OAC1 at 1.5 μM could enhance cleavage and blastocyst rates, as well as the total cell numbers. Additionally, it improved the expression of genes related to the pluripotency of SCNT embryos, including *OCT-4*, *SOX2*, and *NANOG* at the 8-cell stage to the blastocyst stage. Regarding epigenetic reprogramming, the DNA methylation resulting from OAC1 treatment affects *DNMTs* and global DNA methylation expression similarly to IVF embryos from the 8-cell to blastocyst stages. In terms of histone modification, the expression levels of *HDAC1*, *HDAC2*, and *HDAC3* were unclear; however, the expression of H3K14ac was similar to IVF embryos at the blastocyst stage, indicating improved histone acetylation. OAC1 also enhanced the histone methylation expression level of H3K9me3 from the 4- to the 8-cell stages, similar to IVF embryos.

However, more of the OAC1 dynamic on the development and epigenetic modification of porcine SCNT embryos need to be confirmed in the future. The suggestion of the study might be to investigate such as the optimal time to culture OAC1 after the SCNT process, the suitable period of treatment, the IVM or IVC period, the mechanism of OAC1, co-culture with another small molecule, another position of histone modification and the number of healthy offspring after embryo transfer.

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APPENDIX

1. Typical cDNA synthesis reaction by using an iScript™ reverse transcription (RT) supermix kit

Component	Volume	Final concentration
dNTP Mix (10 mM each)	2 µl	1 mM (each dNTP)
RNase Inhibitor, 40 U/µl	0.5 µl	1 U/µl
Oligo (dT), 10 µM	0.5 µl	0.5 µM
5x Reverse Transcriptase Buffer	4 µl	1x
RNA Template	10 µl total RNA	
RevertUP™ II Reverse Transcriptase	1 µl	10 U/µl
MQ water	Variable	
Total volume	20 µl	

2. qPCR reaction by using KAPA SYBR FAST qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA

qPCR reaction	1 reaction
Master mix (2X KAPA SYBR)	5 µl
10 µM F primer	0.1 µl
10 µM R primer	0.1 µl
300 ng/µl cDNA template	1 µl
qPCR grade water	3.8 µl

3. delta delta Ct method ($2^{-\Delta\Delta Ct}$)

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

$\Delta\Delta Ct$ = ΔCt (treated sample) – ΔCt (untreated sample)

$2^{-\Delta\Delta Ct}$ = relative expression

BIOGRAPHY

Miss Thida Praeksamut was born in Samutsakhon, Thailand on September 19th, 1997. She graduated with a Bachelor of Science in Animal Science from Kasetsart University Kamphaeng Saen campus, Nakhon Pathom, Thailand. In August 2022, she started studying for a master's in Biotechnology, at the Institute of Agricultural Technology. She received One Research One Graduate scholarship (OROG) from Suranaree University of Technology, under the supervision of Prof. Dr. Rangsun Parnpai. The research topic is "Effect of oct-4 activating compound 1 (OAC1) supplementation in IVC medium on developmental competent and quality of porcine SCNN embryos", the results from some parts of this study have been presented as a poster presentation at the 2nd International Conference on Biodiversity, Science, and Technology (BioSAT2025) during 16-17 January, 2025.