EFFECTS OF REVERSINE AND SAHA ON DEVELOPMENT AND EPIGENETIC REPROGRAMMING OF CLONED PORCINE EMBRYOS

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ผลของ Reversine และ SAHA ต่ออัตราการเจริญ และ epigenetic reprogramming ของตัวอ่อนสุกรโคลนนิ่ง

นางสาวภัทรวดี น้อยตา

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

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ภัทรวดี น้อยตา : ผลของ Reversine และ SAHA ต่ออัตราการเจริญ และ epigenetic reprogramming ของตัวอ่อนสุกรโคลนนิ่ง (EFFECTS OF REVERSINE AND SAHA ON DEVELOPMENT AND EPIGENETIC REPROGRAMMING OF CLONED PORCINE EMBRYOS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 80 หน้า.

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การเจริญจนครบกำหนดคลอดที่ต่ำ หลังการย้ายฝากตัวอ่อนสุกรที่ผลิตจากเทคนิคการย้ายฝากนิวเคลียสของเซลล์ร่างกาย หรือการโคลนนิ่ง เกิดจากความผิดปกติของการปรับโปรแกรมเอพิเจเนติกส์ มีโมเลกุลขนาดเล็กที่เกี่ยวกับข้องการปรับโปรแกรมเอพิเจเนติกส์หลายชนิดได้ถูกนำมาใช้เพื่อปรับปรุงการปรับโปรแกรมและความสามารถในการเจริญของตัวอ่อนสุกรโคลนนิ่ง การทดลองนี้มีวัตถุประสงค์เพื่อตรวจสอบผลของการเติม Reversine และ SAHA ต่อการเจริญและสถานะการปรับโปรแกรมเอพิเจเนติกส์ของตัวอ่อนสุกรโคลนนิ่ง ทำการตรวจสอบผลของความเข้มข้นและระยะเวลาที่เหมาสมในการเติม Reversine และ SAHA ต่อการเจริญของตัวอ่อน ระดับของการเปลี่ยนแปลงของอีพิเจเนติกส์ โดยการย้อมสีอิมมูโนไซโตเคมี (ICC) และ การแสดงออกของยีน โดยการทำพีซีอาร์เชิงปริมาณ จากการทดลองพบว่าการเติมด้วย Reversine ที่ความเข้มข้น 1 ไมโครโมลาร์ เป็นเวลา 6 ชั่วโมงในระหว่างการกระตุ้นและการเพาะเลี้ยงตัวอ่อนโคลนนิ่ง ส่งผลให้ตัวอ่อนมีการพัฒนาไปสู่ระยะบลาสโตซิสต์สูงที่สุด ถึง 39.3 เปอร์เซ็นต์ การเติมด้วย Reversine ช่วยลดระดับของอะซิทิลเลชันของฮิสโทนที่ 3 ไลซีนที่ 9 (H3K9ac) ในระยะ 4 และ 8 เซลล์ และลดระดับของไตรเมทิลเลชัน ของฮิสโทนที่ 3 ไลซีนที่ 9 (H3K9me3) ในระยะโพรนิวเคลียส (PN) และระยะ บลาสโตซิสต์ของตัวอ่อนโคลนนิ่ง นอกจากนี้การเติมด้วย Reversine ยังช่วยควบคุมการแสดงออกของยีนต่างๆ ในทางบวก โดยเพิ่ม *OCT4*, *SOX2* และ *OCT4* และยับยั้ง *HDAC1*, *DNMT1* และ *DNMT3A* ในระยะบลาสโตซิสต์ของตัวอ่อนโคลนนิ่ง นอกจากนี้ การเติม SAHA ที่ความเข้มข้น 1 ไมโครโมลาร์ เป็นเวลา 12 ชั่วโมงระหว่างการกระตุ้นและการเพาะเลี้ยงตัวอ่อนสุกรโคลนนิ่ง ช่วยเพิ่มอัตราการเจริญของตัวอ่อนไปสู่ระยะบลาสโตซิสต์เมื่อเทียบกับกลุ่มควบคุม ($P<0.05$) และเพิ่มระดับ H3K9ac ในระยะ PN, 8-เซลล์ และ บลาสโตซิสต์ และยังเพิ่มระดับฮิสโทนที่ 3 ไลซีนที่ 14 (H3K14ac) ในระยะบลาสโตซิสต์ ลดระดับดีเฮ็นเอเมทิลเลชัน และยับยั้งการเกิด 5-เมทิลไซโทซีน (5-mC) ในระยะ 2 เซลล์และบลาสโตซิสต์ และลดระดับการเกิด H3K9me3 ในระยะ PN ของตัวอ่อนโคลนนิ่ง นอกจากนี้การเติม SAHA ทำให้เพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับการเจริญ (*NANOG*) อย่างมีนัยสำคัญทางสถิติ และลดการแสดงออกของยีนที่เกี่ยวข้องกับการเมทิลเลชันของดีเอ็นเอ (*DNMT1* และ *DNMT3A*) และการเกิด ดีอะซิทิลเลชันของฮิสโทน (*HDAC1* และ *HDAC2*) ในระยะ บลาสโตซิสต์ของตัวอ่อนโคลนนิ่ง จากผลการทดลองนี้ ชี้ให้เห็นว่า Reversine และ SAHA มีผลร่วมในการปรับปรุงความสามารถในการเจริญของตัวอ่อนสุกรโคลนนิ่ง โดยการควบคุมการปรับโปรแกรม · อีพิเจเนติกส์ ดังนั้นข้อมูลจากการทดลองนี้อาจมีส่วนช่วยในการผลิตตัวอ่อนสุกรโคลนนิ่ง เพื่อการผลิตลูกสุกรสำหรับการประยุกต์ใช้ทางการแพทย์ และการอนุรักษ์สัตว์ที่ใกล้สูญพันธุ์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ภัทรวดี น้อยตา

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PHATTARAWADEE NOITA : EFFECTS OF REVERSINE AND SAHA ON DEVELOPMENT AND EPIGENETIC REPROGRAMMING OF CLONED PORCINE EMBRYOS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D. 80 PP.

Keyword: Porcine/Reversine/SAHA/Development/Epigenetic reprogramming/Cloning

The low full-term live offspring of porcine somatic cell nuclear transfer (SCNT) or cloned embryos after transfer to recipient is caused by aberrant epigenetic reprogramming. Several epigenetic remodeling small molecules have been used to improve reprogramming and the developmental competence of porcine SCNT embryos. In this study, we investigated the effects of Reversine and SAHA on the development and epigenetic reprogramming status of porcine SCNT embryos. The optimal concentration and duration of Reversine and SAHA treatments on embryonic development was investigated by measuring various levels of epigenetic reprogramming by immunocytochemistry staining (ICC) and quantitative polymerase chain reaction (qPCR). The rate of blastocyst formation was 39.3% when SCNT embryos were treated with 1 μM Reversine for 6 hours during activation and culture. In SCNT embryos, Reversine treatment decreased acetylation of histone 3 lysine 9 (H3K9ac) levels at the 4- and 8-cell stages, as well as trimethylation of histone 3 lysine 9 (H3K9me3) levels at the PN and blastocyst stages. Furthermore, Reversine treatment positively regulated the mRNA expression of genes in SCNT blastocysts by promoting *OCT4*, *SOX2* and *NANOG*, but repressing *HDAC1*, *DNMT1* and *DNMT3A*. In addition, 1 μM SAHA treatment for 12 h during activation and culture of SCNT embryos could improve blastocyst formation rates compared to the un-treated group ($P<0.05$). SAHA enhanced H3K9ac levels at pronuclear (PN), 8-cell and blastocyst stages and histone 3 lysine 14 (H3K14ac) levels at blastocyst stage, reduced global DNA methylation levels as well as anti-5-methylcytosine (5-mC) at 2-cell and blastocyst stages, and H3K9me3 at PN stage of SCNT embryos. Moreover, SAHA treatment significantly upregulated the expression of the development-related gene (*NANOG*), but downregulated the expression of genes related to DNA methylation (*DNMT1* and *DNMT3A*) and histone deacetylation (*HDAC1* and *HDAC2*) in SCNT blastocysts. These results suggest that Reversine and SAHA have synergistic effects on improving developmental competences by regulating epigenetic reprogramming. Therefore, these strategies may contribute to the generation of porcine SCNT embryos to produce live offspring for biomedical applications and endangered species conservation.

School of Biotechnology
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Student's Signature Phattarawadee
Advisor's Signature

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Phattarawadee Noita

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LIST OF ABBREVIATIONS

-	=	Minus
%	=	Percentage
°C	=	Degree Celsius
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
µs	=	microsecond
5-mC	=	5-methylCytocine
6-DMAP	=	6-Dimethylamino purine
ANOVA	=	Analysis of variance
bp	=	Base pair
BSA	=	Bovine serum albumin
BTS	=	Beltsville Thawing Solution
$CaCl_2.2H_2O$	=	Calcium chloride dihydrate
cDNA	=	Complementary deoxyribonucleic acid
Class I	=	Class 1
Class IIa	=	Class 2a
Class IIb	=	Class 2b
Class III	=	Class 3
Class IV	=	Class
CO_2	=	Carbon dioxide
COCs	=	Cumulus-oocyte complexes
dbcAMP	=	Dibutyryl cAMP
DMSO	=	Dimethyl sulfoxide
DNMT1	=	DNA methyltransferase 1
DNMT3A	=	DNA methyltransferase 3 alpha
DNMT3B	=	DNA Methyltransferase 3 Beta
DNMTs	=	DNA methyltransferases
DNMTs	=	DNA methyltransferases
EDTA	=	Ethylenediaminetetraacetic acid
EGF	=	Epidermal growth factor
FBS	=	Fetal bovine serum

LIST OF ABBREVIATIONS (Continued)

GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GV	=	Germinal vesicle
h	=	Hour
H1	=	Histone 1
H2A	=	Histone 2A
H2B	=	Histone 2B
H3	=	Histone 3
H3K14ac	=	Lysine14 acetylation of Histone3
H3K27me3	=	Lysine 27 trimethylation of Histone
H3K4me3	=	Lysine 4 trimethylation of Histone 3
H3K9	=	Histone 3 Lysine 9
H3K9ac	=	Lysine9 acetylation of Histone3
H3K9me3	=	Lysine9 trimethylation of Histone3
H4	=	Histone 4
H4K8	=	Histone 4 Lysine 8
HAT	=	Histone acetyltransferase
HCG	=	Human chorionic gonadotropin
HDAC	=	Histone deacetylase
HDAC1	=	Histone deacetylase 1
HDAC2	=	Histone deacetylase 2
HDAC3	=	Histone deacetylase 3
HDACi	=	Histone deactylase inhibitors
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hoechst 33342	=	Bisbenzimide
HSD	=	Honest Significant Difference
ICC	=	Immunocytochemistry
IU	=	The international unit
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation
Kdm	=	Lysine demethylase
LOS	=	Large offspring syndrome
mDPBS	=	Modified Dulbecco's phosphate buffered saline
me	=	Monomethylation

LIST OF ABBREVIATIONS (Continued)

me2	=	Demethylation
me3	=	Trimethylation
MEK	=	Mitogen-activated protein kinase
$MgSO_4.7H_2O$	=	Magnesium Sulfate Heptahydrate
MI	=	Metaphase I
MII	=	Metaphase II
mM	=	Milimolar
mm	=	Millimeter
mRNA	=	Messenger ribonucleic acid
N_2	=	Nitrogen
NaCl	=	Sodium chloride
NANOG	=	Nanog Homeobox
ng	=	Nanogram
NMMII	=	Nonmuscle myosin II
OCT4	=	Homeodomain transcription factor of the POU family
PBS	=	Phosphate-buffered saline
PcG	=	Polycomb group
Pig-FM	=	Pig fertilization medium
PMSG	=	Pregnant mare serum gonadotropin
PN	=	Pronuclear stage
POM	=	Porcine oocyte medium
POU5F1	=	POU Class 5 Homeobox 1
PVP	=	Polyvinylpyrolidone
PZM-3	=	porcine zygote medium-3
qPCR	=	Quantitative polymerase chain reaction
Reversine	=	2-(4-morpholinoanilino)-6-cyclohexylamino-purine analogue
RNA	=	Ribonucleic acid
SAHA	=	Suberoylanilide hydroxamic acid
SCNT	=	Somatic cell nuclear transfer
SEM	=	Standard error of the mean
SOX2	=	SRY-Box Transcription Factor 2
Suv39H1/2	=	Histone Lysine Methyltransferase of variegation 39H1/2
TCM199	=	Medium 19
Tet	=	Ten-eleven translocation

LIST OF ABBREVIATIONS (Continued)

TrxG	=	Trithorax group
TSA	=	Trichostatin A
V	=	Voltage
XCI	=	Chromosome inactivation
αMEM	=	Alpha modification

CHAPTER I
INTRODUCTION

1.1 Background and significance

Cloning is a useful technology for basic and applied research in agriculture and biomedicine. Especially, porcine cloning has been studied for xenotransplantation or "pig-to-human organ transplantation" due to the similar physiology, immunology and anatomy of pigs and humans (Prather et al., 2003; Whyte and Prather, 2011). The low numbers of live offspring of cloned animals were observed with many defects, including large offspring syndrome (LOS) as well as placental abnormalities, an enlarged placenta and failure acute respiratory (Loi et al., 2016). Cloning by somatic cell nuclear transfer (SCNT) is a powerful technology that allows the reprograming of terminally differentiated cells into totipotent cells (Gurdon and Wilmut, 2011). To date, a global effort of research has improved the efficiency of cloning, but the efficiency remains extremely low due to incomplete epigenetic reprogramming of donor cells or donor nuclei. (Dean et al., 2001; Matoba and Zhang, 2018; Simmet et al., 2020; Wang et al., 2020).

Small molecules such as Trichostatin A (TSA), Reversine and SAHA have been used to improve epigenetic reprogramming and the developmental capacity of porcine cloned embryos (Jeong et al., 2021; Miyoshi et al., 2010; Whitworth et al., 2015; Sun et al., 2020). The 2-(4-morpholinoanilino)-6-cyclohexylamino-purine analogue known as Reversine was reported to have a concentration of 1–10 μM, which can induce myogenic progenitor cells to become multipotent mesenchymal progenitor cells that can proliferate and re-differentiate into bone cells (osteoblast) and fat cells (adipocytes) (Chen et al., 2004). A previous report found that Reversine can inhibit nonmuscle myosin II (NMMII) and mitogen-activated protein kinase (MEK)1 barriers, enhancing the hyperacetylation that is associated with transcriptional activity and euchromatin maintenance in mammalian cells (Chen et al., 2007). Reversine can modulate the development of cloned embryos in many species, such as miniature pigs (Miyoshi et al., 2010) and cattle (Yoisungnern et al., 2011). Suberoylanilide hydroxamic acid (SAHA) commonly known as histone deactylase inhibitors (HDACi), SAHA could enhance the developmental efficiency of clone embryos in many species, including mice (Ono et al., 2010), pig (Whitworth et al., 2015; Sun et al., 2022) and cattle (Yoisungnern et al., 2012). Several studies have reported positive effects of

Reversine and SAHA promotes histone acetylation on developmental competence in cloned embryos. However, it is necessary to investigate the mechanisms of action of these novel compounds in activation and *in vitro* culture on the improvement of porcine cloned embryos development. In the present study, we investigated the optimal concentration and duration of Reversine and SAHA treatments on development of porcine cloned embryos (cleavage rate, blastocyst formation rate, total cell number) and confirmed the effects of Reversine and SAHA on changing of development and epigenetic reprogramming during porcine SCNT embryos development using quantitative polymerase chain reaction (qPCR) and Immunocytochemistry staining (ICC).

1.2 Research objectives

1.2.1 To investigate the effects of Reversine and SAHA treatments on the development of porcine cloned embryos.

1.2.2 To investigate the effects of Reversine and SAHA treatments on the levels of specific genes related to epigenetic reprogramming of porcine cloned embryos.

1.2.3 To investigate the effects of Reversine and SAHA treatments on the histone acetylation, histone methylation and global DNA methylation of porcine cloned embryos.

1.2 Research hypothesis

1.3.1 Optimal concentration and duration of Reversine and SAHA supplemented in activation and culture medium can enhance cleavage rate, blastocyst formation rate and total cell number in porcine cloned embryos compared to a group without treatments.

1.3.2 Optimal concentration and duration of Reversine and SAHA supplemented in activation and culture medium can enhance the levels of specific genes related to epigenetic reprogramming in porcine cloned embryos compared to a group without treatments.

1.3.3 Optimal concentration and duration of Reversine and SAHA supplemented in activation and culture medium can increase histone acetylation but reduce histone methylation and global DNA methylation of porcine cloned embryos compared to a group without treatments.

1.4 Scope and limitations of the study

To find the optimal concentration and duration of Reversine and SAHA supplemented in activated and IVC medium on *in vitro* development porcine SCNT embryos. Subsequently, the optimal conditions of Reversine and SAHA were chosen and used for further investigations. Then, porcine embryos derived from IVF and SCNT with and without treatment at PN, 2-, 4-, 8-cell and blastocyst stages were evaluated the effects of Reversine and SAHA on development and epigenetic reprogramming using quantitative polymerase chain reaction (qPCR) and Immunocytochemistry staining (ICC).

1.5 Research methodology

1.5.1 Instrumentation

The provider of all the supplies and instruments was Embryo Technology and Stem Cell Research Center, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

1.5.2 Location of research

The experiments were conducted at Embryo Technology and Stem Cell Research Center, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

CHAPTER II
LITERATURE REVIEW

2.1 Somatic cell nuclear transfer (SCNT)

The technique known as somatic cell nuclear transfer (SCNT) has great potential for cloning desirable livestock species phenotypes and genotypes. (Men et al., 2012; Polejaeva et al. 2000). The several crucial steps involved in SCNT including (1) genetic selection of donor cells (2) aspiration or bisection of a MII-arrested oocyte to remove metaphase chromosomes (Enucleation) (3) transfer of donor cell to an enucleated oocyte (Injection) and fused by an electrical pulse (Fusion) (4) activation of the reconstructed embryos (Activation) (5) embryo culture and transfer into recipients (Lai & Prather, 2003). According to Cooper et al. (2002), Lai et al. (2002), Perota et al. (2019), and Kemter et al. (2020), the procedure is technically challenging and requires high-cost production, making it less likely to be adopted by professionals in the field. Animal production by SCNT has been applied in agriculture and medicine, and the association with genome editing is both practical and commercial. Currently, porcine SCNT to generate cloned embryos is of great importance and has been used for basic research and biomedical science. Because its immunology, metabolism, physiology, pathology and organ size are highly similar to those of humans. Porcine is considered an excellent source for xenotransplantation. However, the extremely low cloning efficiency and low developmental rate, these associated with incomplete reprogramming in the resulting embryos have been still enormous problem in porcine SCNT.

2.2 Low efficiency of cloning underlies incomplete epigenetic reprogramming

"Epigenetics" was first named in 1942 by Waddington (Waddington, 2012), a Cold Spring Harbor summit in 2008 that the scientific community agreed on its definition: a stable heritable phenotype arising from chromosome modifications without changes to the DNA sequence is called an epigenetic trait (Berger et al., 2009). The SCNT is a potential technique that allows the reprogramming of terminally differentiated cells (adult cells) to the totipotent state (embryonic cells) (Gurdon and Wilmut, 2011). During embryonic development, totipotent embryos differentiate into

pluripotent stem cell and after that develop into differentiated involving DNA methylation, histone modification (histone methylation, histone acethylation), genomic imprinting and X chromosome inactivation (XCI) (Reik et al., 2003). The crucial significance in the development of SCNT embryos is these are inherited variations in gene expression without modification in genomic DNA sequences occur during the process from cells of the newly formed zygote start dividing to embryo (Niemann, 2016).

2.2.1 DNA methylation

DNA methylation (5-methylCytocine, 5-mC) is commonly linked to transcriptional silence and occurs at cytosine residues in the CpG sites of DNA sequence regions (Bird, 2002). In order to activate or inactivate particular genes in accordance with the necessities of organism growth and development, the genome goes through DNA methylation maintenance, which includes DNA demethylation and DNA remethylation (Li & Zhang, 2014). DNA methyltransferases (DNMTs) such as DNMT1, are required for de novo DNA methylation, while DNMT3 (DNMT3A and DNMT3B) is crucial for maintaining DNA methylation during embryogenesis (Chen and Zhang, 2019). Following DNA demethylation repair mechanism, ten-eleven translocation (Tet) activation-induced cytidine deaminase and DNA glycosylases are examples of oxidative DNA demethylation enzymes (Ito et al., 2010; Iqbal et al., 2011; Shen et al., 2013). There are several ways in which active DNA demethylation takes place throughout the early stages of embryonic development. (Wang et al., 2014), and when zygote enter to the blastocyst or later implantation stage, genomic DNA is remethylated (Reik et al., 2001; Yang et al., 2007). The genome of SCNT embryos also undergoes de- and re-methylation; the genomic DNA of somatic cells used as donor cells in the SCNT process is highly methylated, and DNA methylation reprogramming, particularly DNA demethylation, is crucial for proper embryonic development. However, these cause incomplete reprogramming when compared with normal embryos or embryos derived from fertilization (Bourc'his et al., 2001; Dean et al., 2001; Yang et al., 2007).

2.2.2 Histone modification

Histone modification is one of major elements that affects associate with chromatin structure and regulate gene expression (Sproul et al., 2005; Yi and Kim, 2018). In eukaryotic cells, the DNA molecule appears in a nucleoprotein complex that resembles chromatin. The basic component of chromatin, which is composed of two molecules of each of the histones H2A, H2B, H3, and H4 as well as H1 acting as

linker, is the nucleosome, which is made up of 147 base pairs of DNA encircled by an octamer

of core histones. Linker DNA, which are short segments, connect each nucleosome to the next (Kurumizaka and Kobayashi, 2019). The regulation of gene expression is influenced by chromatin accessibility, which is governed by chromatin remodeling factors and covalent modification, such as acetylation, methylation, and phosphorylation of amino acids in the histone tail (Kobayashi and Kurumizaka, 2019).

2.2.2.1 Histone acetylation

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are the enzymes that mediate histone acetylation; HAT causes the opening of chromatin to facilitate transcription factor binding and activates gene transcription, whereas HDAC regulates inactivation of gene (Sun et al., 2003). Histone acetylation, including that of histone H3, occurs during fertilization and permits the proper expression of genes involved in early embryonic development (Rybouchkin et al., 2006; Ziegler-Birling et al., 2016). Moreover, Histone deacetylation occurs one step of SCNT during the *in vitro* maturation (IVM) of porcine oocytes, and is catalyzed by Class IIb histone deacetylases (Endo et al., 2008; Endo et al., 2005; Ling et al., 2018). The acetylation level of global histones is highest in germinal vesicle (GV) oocytes and lowest in metaphase I (MI) and metaphase II (MII) oocytes (Endo et al., 2005; Akiyama et al., 2004; Zhou et al., 2017). Histone acetylation is eliminated by HDAC in the cytoplasm (Endo et al., 2008). During the SCNT step of injection, after the nuclear membrane breaks down, the donor cells injected into MII oocytes quickly lose their high levels of acetylation on histones (Endo et al., 2008). Histone acetylation markers, such as Lys9 acetylation of H3 (H3K9ac) at the ZGA stage and H3K14ac at the blastocyst stage, eventually decrease and disappear (Rybouchkin et al., 2006; Wee et al., 2006; Liu et al., 2012; Zhai et al., 2018). In addition, Sun et al. (2020) reported that HDAC in the cytoplasm still continues to eliminate the high levels H3K9ac of donor cell after injection. Broad-spectrum deacetylase inhibitors (HDACi) are used to preserve the acetylation of histones H4K8 and H3K9 in SCNT embryos (Wang et al., 2011; Chawalit 2012). The low cloning efficiency is the result of these disrupted histone modifications, which also impair chromatin accessibility and cause disordered expression of genes necessary for the normal development of cloned embryos (Liu et al., 2012; Xie et al., 2016; Zhai et al., 2018). Hence, one important factor influencing the development of cloned embryos is histone modification.

2.2.2.2 Histone methylation

Histone methylation mainly occurs on lysine and arginine, there are 3 types of methylation patterns: monomethylation (me), dimethylation (me2), and trimethylation (me3) (Izzo and Schneider, 2010). According to Liu et al. (2016)

and Ninova et al. (2019), The most common changes are trimethylation of H3 such as H3K4me3, H3K27me3 and H3K9me3. The H3K4me3 is linked to gene activation and is regulated by the Trithorax group (TrxG) complex, whereas H3K27me3 is mediated by the Polycomb group (PcG) proteins results in gene suppression and H3K9me3 catalyzed by suppressor histone lysine methyltransferase of variegation 39H1/2 (Suv39H1/2) and eliminated by lysine demethylase (Kdm)4. In addition, the depletion of trimethylation of H3, such as H3K4me3 and H3K9me3 improved gene expression patterns and increased blastocyst rates in porcine SCNT embryos (Zhang et al., 2018; Jeong et al., 2021).

2.3 Small molecules for enhancing the development of cloned embryos by ameliorating epigenetic reprogramming

2.3.1 2-(4-morpholinoanilino)-6-cyclohexylamino-purine-analogue (Reversine)

According to Chen et al. (2004), Reversine, the small molecule, induces the plasticity of C2C12 myoblasts at the single-cell level, and Reversine-treated cells gain the ability to differentiate into osteoblasts and adipocytes under lineage-specific inducing conditions. Hence, Reversine functions in cell reprogramming and regenerative medicine have been the focus of numerous investigations (Anastasia et al., 2006; Kim et al., 2007; Saraiya et al., 2010; Jung et al., 2011). The goal of Reversine is to reprogram somatic cells into multipotent, meaning that can differentiate into numerous cell types. Several investigations have shown that Reversine suppresses tumors in human cancer cells by causing apoptosis, cell cycle arrest, polyploidy and autophagy (Lu et al., 2016; Lu et al., 2012; Lee et al., 2012; Kuo et al., 2014). In the first report of Reversine treatment in bovine SCNT embryos, Yoisungnern et al. (2011) investigated the effect of Reversine treatment on the developmental potential of bovine cloned embryos. The result showed that the bovine SCNT embryos after fusion, the reconstructed embryos were cultured in activated and culture medium supplemented with different concentrations of Reversine (0, 1, 5 and 10 μM) and different duration times (0, 6, 12 and 18 h), the blastocyst formation rates of embryos treated with 1 μM Reversine for 6 h and 12 h were significantly higher than those of Reversine treated with 5, 10 μM and without Reversine (P<0.05). These results suggest

that Reversine under optimal condition could enhance development of bovine SCNT embryos. Reversine has been used to treat porcine SCNT embryos with different concentrations (0, 1, 5 and 10 μM) and durations (0, 6, 12, 18 and 24 h) in culture medium. The result showed porcine SCNT embryos treated with 5 μM Reversine for

12 h, the blastocyst formation rate was significantly higher than those without Reversine treatment (P<0.01) (Miyoshi et al., 2010). These results suggest that Reversine under optimal condition could enhance development of porcine cloned embryos. Although, the previous studies indicate that treatment with Reversine can improve the development of SCNT embryos, the underlying histone modification is still unclear.

2.3.2 Suberoylanilide hydroxamic acid (SAHA) or Vorinostat

A histone deacetylase inhibitor (HDACi), vorinostat, also known as SAHA, induces malignant cells to undergo growth arrest, death, or differentiation in both *in vitro* and *in vivo* settings (Roth et al., 2007). Hence, various studies have focused on the roles of SAHA in cell reprogramming and regenerative medicine. According to Wang et al. (2007), a certain kind of HDACi small molecules enhances the genomic reprogramming of somatic cells and positively changes epigenetic abnormalities by raising the levels of H3K9ac and H3K14ac. The HDACs are key enzymes in almost all tissues by the regulation of gene expression, but the expression level of various HDACs are different within different tissue types (Ruijter et al., 2003) and divided into five categories: class I (HDAC1-3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (SIRT1–7) and class IV (HDAC11) (Blackwell et al., 2008). Ono et al. (2010), reported that mouse SCNT embryos treated different concentration of SAHA (0.1, 1, 10 and 100 μM) for 6 h in culture medium. The results showed that when embryos were treated with 1 μM SAHA for 6 h, the blastocyst formation rate was significantly higher than that of embryos from other (P<0.01), suggesting that SAHA is an important HDACi small molecule to reprogram mouse SCNT embryos. Yoisungnern et al. (2012), investigated the effects of SAHA treatment on the development of cloned bovine embryos. The bovine SCNT embryos after fusion, the reconstructed embryos were cultured in activated and culture medium supplemented with different concentrations of SAHA (0, 0.1, 1 and 10 μM) and different duration times (0, 6, 12 and 18 h). The blastocyst rates of embryos treated with 1 μM SAHA for 6 h were significantly higher than those of SAHA-treated embryos (P<0.05), suggesting that SAHA supplemented in activated and culture medium could enhance development of bovine SCNT embryos. In additionally, Whitworth et al., (2015) investigated the effect of SAHA treatment (0, 1 and 10 μM for 14-16 h) in post-

fusion, activation and IVC of porcine embryos. The porcine SCNT embryos treated with 10 μM SAHA for 14-16 h had the highest level of the blastocyst formation rates, upregulated lysosome and successfully produced healthy piglets. However, the level of total cell number 1.0 μM SAHA were significantly higher than non-treatment group

(P<0.021). Sun et al. (2020), investigated the effect of SAHA on the epigenetic modification and preimplantation development of porcine cloned embryos. The mini pig fetal fibroblast was cultured in medium supplemented with different concentration of SAHA (0, 1.5, 3 and 6 μM) and different durations (0 to 96 h). After SCNT, the resulting porcine embryos were treated with 1.5 μM SAHA for 72 h, the blastocyst formation rate and the total cell number were significantly higher than that of embryos without SAHA treatment (P<0.05); Moreover, the H3K14ac levels in the treated SCNT blastocysts were close to that of IVF blastocysts. These results suggest that SAHA treated in fibroblast donor cells could improve histone acetylation and development of porcine SCNT embryos.

2.4 Beneficial application of cloning in pig

Pigs serve as an important agricultural resource and animal model in biomedical research (Prather et al., 2008).

2.4.1 Agricultural applications

Porcine cloned embryos, developed through techniques like SCNT for producing genetically engineered (GE), particularly in enhancing livestock productivity, improving disease resistance (Lee et al., 2020). The distribution of breeding livestock can be expanded genetic resources preserved with the use of cloning (Keefer, 2015).

2.4.1.1 Enhanced Livestock Traits

Cloning in pigs allows the replication of individuals with desirable traits, such as increased growth rates, lean meat production, or resistance to specific diseases. The strategies to produce embryos with selected genetic profiles, breeders can introduce specific advantageous traits into herds faster than through traditional breeding (Niemann and Lucas-Hahn, 2012).

2.4.1.2 Disease Resistance

Cloned embryos enable researchers to introduce genes that can help pigs resist common diseases that threaten livestock such as African swine fever (ASF). ASFV is a large, enveloped double-stranded DNA virus and the single member of the family Asfarviridae (Dixon et al., 2005). Warthogs act as a host to the virus as it causes a non-clinical and persistent infection. For instance, through gene

editing combined with cloning, scientists can engineer pigs to be more resistant to these pathogens, improving herd health and reducing the need for antibiotics.

2.4.1.3 Genetic Preservation

Since the outbreak of African swine fever in European and Southeast Asian worsened the status of local pig breeds (Sánchez-Vizcaíno et al., 2013; Nga et al., 2019). Cloning provides a means to preserve the genetics of valuable animals for biodiversity, sustainable food production, and heritage preservation, ensuring that genetic resources from elite breeding stock or endangered breeds are not lost (Ibtisham et al., 2017). Cloning embryos from high-performing pigs enables the continued replication of these valuable genetics, which can be particularly useful in breed improvement programs.

2.4.2 Biomedical Applications

Pigs have emerged as a critical model, due to physiological similarities between pigs and humans, initially as potential organ donors in xenotransplantation (Lu et al., 2020), which is the transfer of organs across other species such as pig to human (Montgomery et al., 2022; Kavarana et al 2022) and subsequently as models for studying human diseases (Aigner et al., 2010) or as bioreactors for hyperimmune sera production (Reynard et al., 2016). Although the CRISPR/Cas9 technique via zygote microinjection is becoming popular for single-gene editing due to its high efficiency (Lee et al., 2020), SCNT remains the preferred method for complex modifications, such as multiplexed knockouts (KO) and knock-ins (KI) (Niu et al., 2017; Fischer et al., 2016), where edited cells are used.

CHAPTER III
MATERIALS AND METHODS

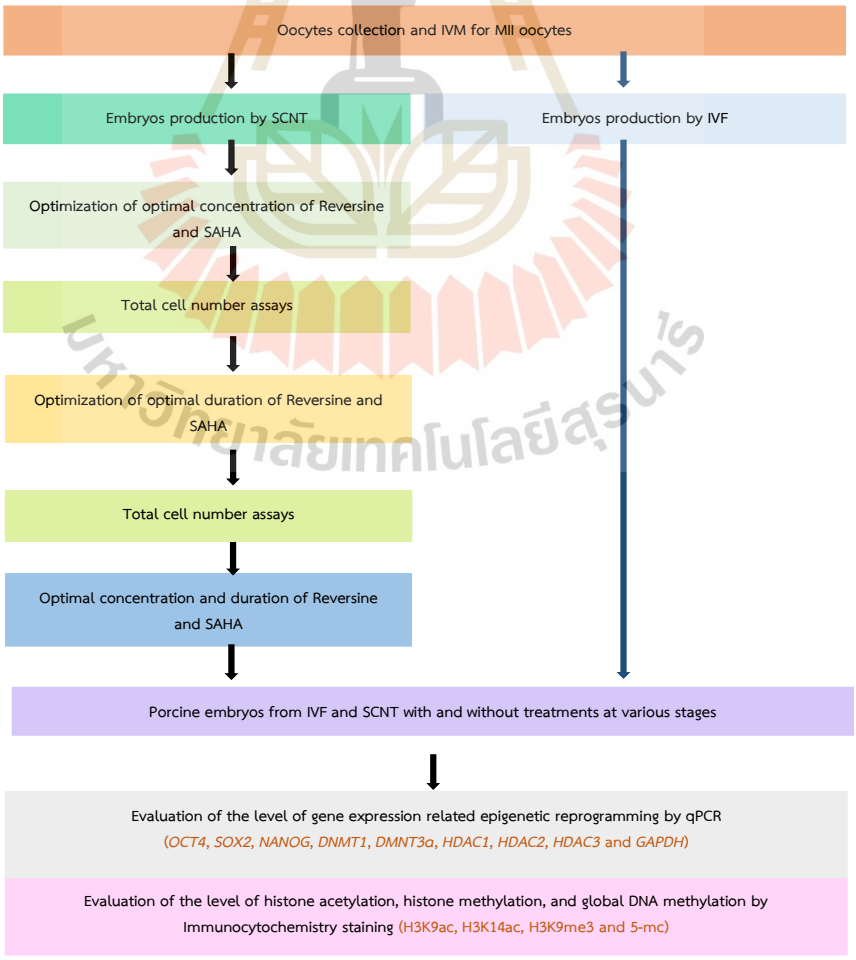
3.1 Chemicals

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

3.2 Ethic statement

Ethical approval for using pig in this study was obtained from the Animal Ethics Committee of Suranaree University of Technology, Thailand.

3.3 Experimental design



3.4 Donor cells preparation

Fibroblast cells were isolated from the ear skin tissues of male piglet. Ear tissue was kept in 0.9% NaCl (Carlo Erba, France, 479687) and was manually cut into small pieces after washed with 70% alcohol and removing root hair and cartilage. The small pieces of ear skin tissue (2×2 mm) were placed on 60 m culture dishes (SPL Life Science, Pocheon-si, Korea) and covered with sterilized glass slide. Then added 4 ml of culture medium in the culture dish and cultured 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 freezing medium containing αMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO; Merck, Germany; 116743). 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 with 3 ml of culture medium under a humidified atmosphere of 5% CO_2 in air at 37°C for 2-3 days.

3.5 Oocytes collection and IVM

Twenty-two to twenty-eight porcine ovaries were collected in 0.9% NaCl from a local slaughterhouse and transported within 2 h to the laboratory. Oocytes were aspirated from antral follicles (2–5 mm in diameter) using an 18-gauge needle attached to a 10 ml disposable syringe. Three hundred cumulus-oocyte complexes (COCs) with uniform oocyte cytoplasm and compact surrounding cumulus cells (CCs) (Fig 3.1) were selected under an inverted microscope (Olympus, Japan, model IX71) and placed in modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinylpyrolidone (PVP). A group of 50 COCs was triple washed and cultured in 4-well dish (SPL Life Science, Pocheon-si, Korea) with 500 µl of *in vitro* maturation medium 1 (IVM-1). IVM-1 consisting of porcine oocyte medium (POM; Yoshika et al., 2008) supplemented with 10 ng/ml epidermal growth factor (EGF), 10 IU/ml dibutyryl cAMP (dbcAMP), 10 IU/ml pregnant mare serum gonadotropin (PMSG, Intervet International GmbH, Unterschleißheim, Germany) and 10 IU/ml human

chorionic gonadotropin (HCG, Intervet International GmbH). COCs in IVM-1 were covered with mineral oil and cultured under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 23 h. After cultured in IVM-1, COCs were triple washed and placed in 4-well dish with 500 µl of IVM-2 consisted of POM supplemented with 1 ng/ml EGF, 10 IU/ml PMSG and 10 IU/ml HCG. COCs were covered with mineral oil and cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 20–22 h (Thouas et al., 2001).

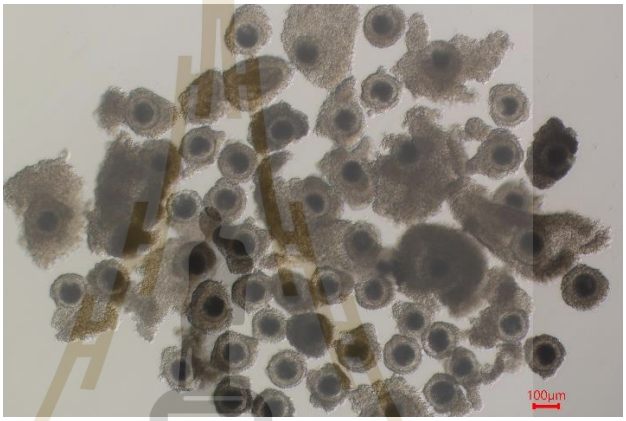


Figure 3.1 Representative image of cumulus-oocyte complexes (COCs) with uniform oocyte cytoplasm and compact surrounding cumulus cells (CCs), scale bar = 100 µm

3.6 Somatic cell nuclear transfer (SCNT) and Activation

At the end of IVM cultured, CCs were removed from the COCs with 0.1% hyaluronidase until the CCs were completely removed. Only metaphase II (MII) oocytes with evenly granular cytoplasm and a visible first polar body (Fig 3.2) (about 50-60% of the total oocytes cultured in IVM medium) were collected under an inverted microscope for SCNT. MII oocytes were incubated in 5 µg/ml cytochalasin B for 5 min. Then the zona pellucida above the first polar body was cut to make a small slit and enucleated with a glass needle squeezed out (about 5–10% of the volume of the cytoplasm with the first polar body). Complete enucleation (about 70-80% of the MII oocytes after enucleation) were confirmed by staining the squeezed-out cytoplasm and first polar body with 5 µg/ml Hoechst 33342 and visualizing under a fluorescence microscope (IX71, Olympus, Tokyo, Japan). A single donor cell was

inserted into the perivitelline space of an enucleated oocyte. The reconstructed oocyte-cell couplets were placed between two wires of fusion electrode covered with fusion medium (0.28 mM mannitol, 0.01 mM bovine serum albumin (BSA), 0.05 mM $CaCl_2.2H_2O$, 0.1 mM $MgSO_4.7H_2O$ (BDH, 101514Y) and 0.1 mg/ml Hepes free acid) (Boquest et al., 2002) and fused with two direct current (DC) pulses of 24V, 16 μs for 5 min using an electro cell fusion machine (SUT F-1, Suranaree University of Technology) for cell-cytoplast fusion. The reconstructed embryos (about 60-70% of complete cell-cytoplast fusion) were triple washed and cultured in TCM199-HEPES supplemented with 10% FBS for 1 h. Then, they were activated by being incubated in 3 μM Ionomycin in TCM199-HEPES for 4 min and then cultured in 2 mM 6-Dimethylamino purine (6-DMAP) under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 3 h (Heytens et al., 2008).

Figure 3.2 Representative image of metaphase II (MII) oocyte with evenly granular cytoplasm and a visible first polar body

3.7 *In vitro* culture (IVC)

At the end of embryos activation, 10 reconstructed embryos were cultured in 100 μl porcine zygote medium-3 (PZM-3, Cao et al., 2012) covered with mineral oil at 38.5°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The cleavage and blastocyst formation rates were examined on days 2 and 6, respectively.

3.8 Reversine and SAHA treatments

3.8.1 Optimization of the optimal concentration of Reversine and SAHA

At the end of cell-cytoplast fusion, reconstructed embryos were cultured in TCM199-HEPES supplemented with 10% FBS for 1 h. Then they were activated and continuously cultured in culture medium supplemented with Reversine

(0, 1, 5 and 10 μM) and SAHA (0, 0.1, 1 and 10 μM) until 12 h before culturing in culture medium without Reversine and SAHA supplementation. The cleavage and blastocyst formation rates were examined on days 2 and 6, respectively.

3.8.2 Optimization of the optimal duration of Reversine and SAHA

At the end of cell-cytoplast fusion, reconstructed embryos were cultured in TCM199-HEPES supplemented with 10% FBS for 1 h. Then they were activated and continuously cultured in culture medium supplemented with optimal concentration of Reversine and SAHA until 6 and 12 h before culturing in culture medium without Reversine and SAHA supplementation. The optimal concentration data came from 3.8.1. The cleavage and blastocyst formation rates were examined on days 2 and 6, respectively.

3.9 Total cell number assay

Blastocysts at day 6 of culture were collected and triple washed in mDPBS containing 0.1% PVA. Then they were permeabilized and stained with 25 μg/ml Hoechst 33342 in 95% ethanol for 3 min at room temperature. Stained embryos were mounted on a glass slide and examined under a fluorescence microscope and total cell numbers in blastocysts were counted.

3.10 *In vitro* fertilization (IVF) and IVC

Embryos from IVF were used as the control group (Hao et al., 2006). After IVM culture (3.3) for 44 h, COCs were triple washed in modified pig-FM medium (Suzuki et al., 2002) containing 10 mM HEPES, 2 mM caffeine, and 5 mg/ml BSA. To prepare spermatozoa, fresh semen from fertile boar at SUT farm (Suranaree University of Technology, Nakhon Ratchasima). The collected semen was diluted in BTS extender (Bwanga et al., 1990) at 15-20 °C and preincubated in sperm washing medium (Kikuchi et al., 1998) under a humidified atmosphere of 5% CO_2 in air at 37.0°C for 30 min. The sperm washing medium consisted of medium 199 (with Earle's salts, Gibco) supplemented with 4.12 mM calcium lactate, 3.05 mM glucose and 12% FBS, pH adjusted to 7.8. After centrifugation, the supernatant was removed and the spermatozoa pellet was resuspended and adjusted with pig fertilization medium (pig-FM) to a final concentration of 1.0×10^6/ml. To fertilize spermatozoa with COCs, a 50 μl droplet of sperm containing 10 COCs in a culture dish that was covered with

mineral oil was co-incubated under a humidified atmosphere of 5% CO_2 in the air at 38.5°C for 5 h. After fertilization, presumptive embryos were removed CCs by gentle pipetting and cultured in *in vitro* culture medium (PZM-3) that was covered with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

3.11 Effects of Reversine and SAHA on development and epigenetic reprogramming of porcine cloned embryos

qPCR and immunocytochemistry staining were performed to evaluate and compare the level of histone acetylation, histone methylation, DNA methylation, and gene expression related development and epigenetic reprogramming of porcine cloned embryos after treatments with the optimal concentration and duration of Reversine and SAHA.

3.11.1 Evaluation of the levels of gene expression related development and epigenetic reprogramming of porcine cloned embryos by qPCR

The mRNA expression of the following genes was determined: *POU5F1/OCT4*, *SOX2*, *NANOG*, *DNMT1*, *DMNT3A*, *HDAC1*, *HDAC2, HDAC3* and *GAPDH*. All samples were washed with PBS and stored at -80 °C until mRNA is extracted. A total of 45-150 embryos from IVF and SCNT with and without treatment at various stages: 150 embryos at pronuclear stage (PN) (8 h post-activation or 12 h post-fertilization), 120 embryos at 2-cell stage (24 h post-activation or post-fertilization), 60 embryos at 4-cell stage (44 h post-activation or post-fertilization), 45 embryos at 8-cell stage and 20 embryos at blastocyst stage (66-144 h post-activation or post-fertilization) were extracted total RNA using the FavorPrep Tissue Total RNA Mini Kit (Favorgen Biotech Crop., PingTung, Taiwan). For cDNA synthesis, RNAs were subsequently reverse transcribed using an iScriptTM reverse transcription (RT) supermix kit (Biorad, Hercules, California, USA). Gene expressions of each stage the sample were evaluated using KAPA SYBR FAST qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Gene expression was examined with a CFX Opus 96 real-time PCR system (Biorad, Hercules, California, USA). Melting curve analysis was also performed to determine the specificity of the specific primers (Table 3.1). *GAPDH* was used as the housekeeping gene to normalize the target genes. Each transcript sample was

quantified using the $2^{-\Delta\Delta CT}$ method. The mean expression level of each gene in control group was set as 1 for comparison.

Table 3.1 List of primer sequences used for qPCR analysis

Genes	Primer sequences (5'- 3')	Product size (bp)	Accession No.
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
POU5F1/OCT4	F: AGCGCTTCAGAAAGATCTCG R: ACTGCAGGAACATGCTCTC	335	NM_001113060
SOX2	F: TGCACAACTCGGAGATCAG R: CATGCTGTAGCTGCAGTTG	323	NM_001123197
NANOG	F: CAGAGAAGAGCACAGACAAG R: TAGAAGCCCGGGTATTCTG	293	NM_001129971
DNMT1	F: TCGAACCAAAACGGCAGTAC R: CGGTCAGTTTGTGTTGGACA	215	NM_001032355
DNMT3A	F: CTGAGAAGCCCAAGGTCAAG R: GTACTGATACGCGCACTCCA	200	NM_001097437
HDAC1	F: TATCGTCTTGGCCATCCTG R: GTCAGAGCCACACTGTAAG	328	XM_013999116
HDAC2	F: TTTACGCATGTTGCCTCA R: TGATCAGCCACATTTCTACG	209	XM_001925318
HDAC3	F: ATGCAAGGCTTCACCAAGAG R: TGAGGTAGAAGGCCTCCTG	310	NM_001243827

3.11.2 Evaluation of the levels of protein expression related to epigenetic reprogramming by immunocytochemistry staining (ICC)

In each group, a total of 15 embryos from IVF and SCNT with and without treatment at various stages, including PN (8 h post-activation or 12 h post-fertilization), 2-cell (24 h post-activation or post-fertilization), 4-cell (44 h post-activation or post-fertilization), 8-cell and blastocyst (66-144 h post-activation or post-fertilization) were collected and triple washed in phosphate-buffered saline (PBS) containing 0.1% BSA, fixed for at least 15 min in 4% paraformaldehyde (PFA) in PBS, permeabilized with 1% triton X-100 in PBS for 20 min at room temperature, triple washed in 0.1% BSA in PBS, transferred into PBS containing 2% BSA (blocking solution) at room temperature for 2 h to block the non-specific sites. After blocking, the 15

embryos from each group were incubated with the primary antibody against histone acetylation H3K9 and H4K9 in PBS containing 2% BSA at 4°C for overnight. Then, embryos were incubated with the conjugated secondary antibody (anti-Rabbit Alexa Fluor 488 and anti-Mouse Alexa Fluor 594) in PBS containing 2% BSA with 1:200 dilution for 2 h at room temperature in dark condition, and DNA was counterstained with 25 µg/ml Hoechst 33342 for 3 min.

To stain histone methylation, fixed 15 embryos from each group were triple washed with PBS containing 0.1% BSA and permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. After this, they were blocked with PBS containing 2% BSA and then incubated with the primary antibody against histone H3K9me3 in PBS containing 2% BSA at 4°C for overnight. Then, embryos were incubated with the conjugated secondary antibody (anti-Rabbit Alexa Fluor 488) in PBS containing 2% BSA with 1:200 dilution for 2 h at room temperature in dark condition, and DNA was counterstained with 25 µg/ml Hoechst 33342 for 3 min.

To stain DNA methylation, 15 fixed embryos from each group were triple washed with PBS containing 0.1% BSA and permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. After this, they were treated with 4 M HCl for 15 min at room temperature, and then neutralized in 100 mM Tris-HCl (pH 8.8) for 10 min before being blocked with PBS containing 2% BSA. The embryos were incubated with primary antibody against 5-mC at 4°C overnight, incubated at room temperature with the secondary antibody (anti-Mouse Alexa Fluor 594) in PBS containing 2% BSA with 1:200 dilution for 2 h in dark condition, and triple washed in 0.1% BSA in PBS, then 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 inverted microscope (Eclipse TE 300, Nikon Imaging Japan Inc.) with NIS-Elements D program (Nikon Imaging Japan Inc., Tokyo, Japan) with the same exposure times and adjustments. Fluorescent intensities were quantified using Basic Intensity Quantification with FIJI software.

Table 3.2 List of primary antibodies and dilutions used for ICC analysis

Type of markers	Primary antibody	Dilution	Company Cat. No
Histone acetylation H3K9ac	Mouse anti-H3K9ac	1:400	GeneTex GTX630554
Histone acetylation H3K14ac	Rabbit anti-H3K14ac	1:200	Abcam ab52946
Histone methylation H3K9me3	Rabbit anti-H3K9me3	1:400	Abcam ab8898
DNA methylation 5-mC	Mouse anti-5-mC	1:1000	Sigma-Aldrid MABE146-(32160702)

3.12 Statistical Analysis

Statistical analysis was performed using GraphPad version 5 (GraphPad Software, San Diego, CA, USA), and data were represented as the mean ± SEM. A value of $P<0.05$ was considered significant with different lower-case letters. 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 two group.

CHAPTER IV
RESULTS

4.1 Effect of Reversine and SAHA treatment on developmental competence of porcine SCNT embryos

4.1.1 Effect of Reversine treatment on developmental competence of porcine SCNT embryos

We evaluated the effect of Reversine treatment on the developmental competence of porcine SCNT embryos. Reconstructed embryos were treated with 0, 1, 5 and 10 μM of Reversine in activation and culture medium for 12 h. The results from table 4.1.1 showed that the treatment with 1 μM Reversine showed the highest level of blastocyst formation rate (38.5% ± 2.4). The total cell number of SCNT blastocysts treated with 1 μM Reversine was significantly higher than the untreated group (50.5 ± 2.0 vs. 43.5 ± 1.1, $P<0.05$). but no significantly different when compared with10 μM Reversine (50.5 ± 2.0 vs. 47.0 ± 1.4). However, the cleavage and blastocyst formation rates were not influenced by Reversine treatment for 12 h

Table 4.1.1 Effects of different concentrations of Reversine treatment on developmental competence of porcine SCNT embryos for 12 h

Reversine concentration (μM)	No. of embryos cultured	No. of cleavage (mean ± SEM, %)	No. of Blastocyst (mean ± SEM, %)	Total cell number in Blastocyst (mean ± SEM)
0	158	143 (90.2 ± 1.3)	53 (33.7 ± 2.4)	43.5 ± 1.1[b]
1	164	154 (94.1 ± 1.5)	63 (38.5 ± 2.4)	50.5 ± 2.0[a]
5	160	149 (93.0 ± 1.0)	53 (33.2 ± 1.9)	44.2 ± 1.9 [b]
10	160	153 (95.7 ± 1.3)	60 (37.5 ± 2.7)	47.0 ± 1.4[ab]

8 replicates were performed

[a, b] Values with different superscripts in the same column are significantly different ($P<0.05$). Clavage percentage; No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage; No. of blastocyst/No. of embryos cultured.

Based on the results of previous experiments, we selected 1 μM of Reversine as the optimal concentration to determine optimal duration treatment developmental competence of porcine SCNT embryos. The reconstructed embryos were treated with

1 μM of Reversine for various durations (0, 6 and 12 h). The percentage of SCNT embryos developed to blastocyst stage in group treated with 1 μM of Reversine for 6 h was significantly higher than in 12 h (39.3 ± 2.3 vs. 30.0 ± 2.6, $P<0.05$). In addition, the percentage of SCNT embryos developed to blastocyst stage did not differ when compared between treated and untreated groups. However, no influence of Reversine was observed on the cleavage rate and total cell numbers (Table 4.1.2).

Table 4.1.2 Effects of different durations with 1 μM of Reversine treatment developmental competence of porcine SCNT embryos

Reversine Duration (h)	No. of embryos cultured	No. of cleavage (mean ± SEM, %)	No. of Blastocyst (mean ± SEM, %)	Total cell number in Blastocyst (mean ± SEM)
0	148	140 (94.4 ± 1.8)	50 (33.9 ± 1.7)[ab]	44.4 ± 2.0
6	150	135 (90.3 ± 2.4)	59 (39.3 ± 2.3)[a]	44.1 ± 1.6
12	150	138 (92.0 ± 1.9)	45 (30.0 ± 2.6)[b]	46.9 ± 2.5

8 replicates were performed

[a, b] Values with different superscripts in the same column are significantly different ($P<0.05$). Clavage percentage; No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage; No. of blastocyst/No. of embryos cultured.

4.1.2 Effect of SAHA treatment on developmental competence of porcine SCNT embryos

We hypothesized that SAHA, a HDACi, will improve the developmental competence of porcine SCNT embryos from aberrant reprogramming of histone deacetylase. From Table 4.1.3, reconstructed embryos were treated with 0, 0.1, 1 and 10 μM of SAHA in activation and culture medium for 12 h, the blastocyst formation rates were 36.9% ± 1.7, 38.8% ± 3.0, 44.1% ± 3.0 and 33.4% ± 2.7 respectively. At various concentrations of SAHA treatment, the blastocyst rates were similar with non-treatment. Additionally, SAHA treatment did not affect the quality of the blastocyst which determined the total cell number compared with untreated group. SAHA treatment had no effect on cleavage rate and total cell number per blastocyst.

Table 4.1.3 Effects of different concentrations of SAHA treatment on developmental competence of porcine SCNT embryos for 12 h

SAHA concentration (μM)	No. of embryos cultured	No. of cleavage (mean ± SEM, %)	No. of Blastocyst (mean ± SEM, %)	Total cell number in Blastocyst (mean ± SEM)
0	168	165 (98.2 ± 1.2)	62 (36.9 ± 1.7)	48.2 ± 1.2
0.1	170	160 (93.9 ± 2.2)	66 (38.8 ± 3.0)	50.5 ± 0.9
1	169	164 (97.0 ± 1.5)	75 (44.1 ± 3.0)	47.4 ±1.1
10	169	158 (93.4 ± 1.6)	56 (33.4 ± 2.7)	48.4 ± 1.6

10 replicates were performed

Data were not significantly different (One-way ANOVA). Clavage percentage; No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage; No. of blastocyst/No. of embryos cultured.

Based on our previous results, SCNT embryos were treated with 1 μM SAHA had the numerically highest level of blastocyst formation rate at 44.1% than embryos treated in the other concentrations. One μM SAHA was used as the optimal concentration of SAHA. To determine the effects of treatment with 1 μM SAHA for different durations (0, 6, 12 h) on the development of porcine SCNT embryos. The results from Table 4.1.4 showed that SCNT embryos treated with 1 μM SAHA for 12 h had significantly higher blastocyst rates than other durations ($P<0.05$). However, no beneficial effect of SAHA was observed on the cleavage rate and total cell numbers. Hence, these data suggest that treatment with 1 μM SAHA for 12 h could improve *in vitro* development of porcine SCNT embryos.

Table 4.1.4 Effects of different durations with 1 μM of SAHA treatment on developmental competence of porcine SCNT embryos

SAHA Duration (h)	No. of embryos cultured	No. of cleavage (mean ± SEM, %)	No. of Blastocyst (mean ± SEM, %)	Total cell number in Blastocyst (mean ± SEM)
0	149	143 (96.1 ± 1.7)	45 (30.4 ± 1.1)[b]	51.6 ± 1.0
6	149	139 (93.6 ± 1.1)	45 (30.4 ± 1.6)[b]	47.7 ± 2.8
12	149	143 (96.0 ± 1.3)	71 (47.4 ± 3.9)[a]	51.9 ± 1.6

8 replicates were performed

[a, b] Values with different superscripts in the same column are significantly different ($P<0.05$). Clavage percentage; No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage; No. of blastocyst/No. of embryos cultured.

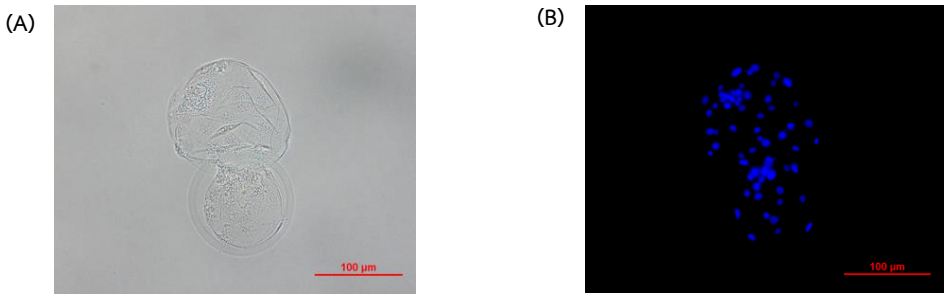


Figure 4.1 **(A)** Representative images of embryos at blastocyst stage **(B)** Representative images of nuclei staining of blastocysts, scale bar = 100 µm.

4.2 Effects of Reversine and SAHA on the relative expression levels of genes related to development and epigenetic reprograming in porcine embryos

4.2.1 Evaluation effects of Reversine on the relative expression levels of genes related to development and epigenetic reprograming by qPCR in PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos.

To elucidate how SAHA affects the reprograming and preimplantation development of SCNT embryos after Reversine treatment (1 µM for 6 h), the relative transcript abundance of three pluripotency genes (*POU5F1/OCT4*, *SOX2* and *NANOG*), three histone acetylation genes (*HDAC1*, *HDAC2* and *HDAC3*) and two DNA methylation genes (*DNMT1* and *DNMT3A*) at PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos were determined by qPCR.

4.2.1.1 Reversine affected the expression of development and pluripotency related genes in porcine embryos.

To explore whether Reversine regulated the expression levels of development and pluripotent genes in porcine embryos including *POU5F1/OCT4*, *SOX2* and *NANOG* genes at PN, 2-, 4-, 8-cell and blastocyst stages between non-treated SCNT embryos served as negative control group (SCNT-untreated), SCNT embryos treated with 1 µM Reversine for 6 h (SCNT-Reversine) and the positive control group was IVF derived embryos (IVF). The expression levels of these genes after Reversine treatment were investigated. From Fig. 4.2, the SCNT-Reversine group showed significantly ($P<0.05$) lower expression of *OCT4* at PN stage in comparison with the SCNT-untreated and IVF groups. *OCT4* transcripts of the SCNT-untreated and SCNT-Reversine at 2-cell

stage showed significantly lower than those in the IVF group (P<0.05). The SCNT-Reversine exhibited significantly higher transcripts of *OCT4* at 4-cell stage in comparison with those in the SCNT-untreated, and also significantly higher than those in the IVF group (P<0.05). *OCT4* transcripts of SCNT-Reversine and IVF groups at 8-cell stage were significantly higher than those in the SCNT-untreated group (P<0.05). In addition, the expression levels of *OCT4* in the blastocyst of SCNT-Reversine group were higher than those in the SCNT-untreated and IVF groups (P<0.05).

At PN stage, *SOX2* transcripts in SCNT-Reversine group was significantly increased than those in SCNT-untreated and IVF groups, but IVF group significantly lower than those in SCNT-untreated group (P<0.05). At 4-cell stage, the expression levels of *SOX2* in the IVF group showed significantly higher levels than those in SCNT-untreated and SCNT-Reversine groups (P<0.05). Although *SOX2* expression at the 2- and 8-cell stage in SCNT-Reversine was still no significant differences with IVF, but significantly higher than those in SCNT-untreated group (P<0.05). However, the SCNT-Reversine group at blastocyst stage showed significantly (P<0.05) lower expression levels of *SOX2* when compared with the IVF group, but significantly higher than those in the SCNT-untreated group (P<0.05).

The expression levels of *NANOG* gene in SCNT-untreated and SCNT-Reversine groups *NANOG* at PN stage showed significantly (P<0.05) lower than those in the IVF groups. At 2-cell stage in the SCNT-Reversine and IVF groups, the expression levels of *NANOG* gene were significantly higher than those in the SCNT-untreated group. Moreover, *NANOG* transcripts at 4-, 8-cell and blastocyst stages in SCNT-Reversine were significantly higher than those in the SCNT-untreated group, but still significantly lower than those in the IVF group (P<0.05). The expression levels of *SOX2* gene in the SCNT-Reversine groups at PN was significantly higher than those in SCNT-untreated and IVF groups (P<0.05).

4.2.1.2 Reversine affected the expression of histone acetylation related genes in porcine embryos.

To explore whether Reversine regulated the expression levels of histone acetylation genes in porcine embryos. The expression levels of *HDAC1*, *HDAC2* and *HDAC3* genes at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-Reversine and IVF groups were investigated. From Fig 4.3, the SCNT-Reversine and SCNT-untreated groups showed significantly (P<0.05) lower expression levels of *HDAC1* at PN stage in comparison with those in the IVF groups. *HDAC1* transcripts of the SCNT-Reversine group at 2-cell stage displayed significantly lower than those in the IVF group, but significantly higher than those in SCNT-untreated group (P<0.05).

The expression levels of *HDAC1* in SCNT-untreated group at 4-cell stage was significantly higher than those in the IVF group, but significantly lower than those in SCNT-Reversine group ($P<0.05$). At 8-cell in SCNT-Reversine and SCNT-untreated groups were significantly higher than those in the IVF group ($P<0.05$). At blastocyst stage, the *HDAC1* expression levels in the SCNT-Reversine and IVF groups were significantly lower than those in the SCNT-untreated group ($P<0.05$).

In addition, the expression levels of *HDAC2* in SCNT-untreated and SCNT-Reversine groups at PN were significantly lower than those in the IVF group ($P<0.05$). At 2-cell stage, the expression levels of *HDAC2* in IVF group was significantly higher than those in of SCNT-Reversine, but did not differ when compared with SCNT-untreated group. *HDAC2* transcripts of the SCNT-Reversine group at 4-cell stage displayed significantly higher than those in the SCNT-untreated group, but still lower than those in the IVF group ($P<0.05$). the expression levels of *HDAC2* in SCNT-Reversine at 8-cell stage was significantly higher than those in the SCNT-untreated and IVF groups ($P<0.05$). At blastocyst stage, the *HDAC2* expression levels in the SCNT-Reversine group was significantly higher than those in the SCNT-untreated group, but there were no significant differences with IVF groups ($P<0.05$). Moreover, at PN and 2-cell stages, the *HDAC3* expression levels in the SCNT- Reversine group was significantly lower than IVF group ($P<0.05$), but still similar to the SCNT-untreated group. The expression levels of *HDAC3* in SCNT-Reversine and SCNT-untreated at 4-cell stage were significantly higher than those in the IVF group ($P<0.05$). At 8-cell stage, SCNT-untreated and IVF groups were significantly lower than those in the SCNT-Reversine group ($P<0.05$). At blastocyst stage, there were no significant differences in the expression levels of *HDAC3* between the groups.

4.2.1.3 Reversine affected the expression of DNA methylation related genes in porcine embryos.

To explore whether Reversine regulated the expression levels of DNA methylation genes in porcine embryos. The expression levels of *DNMT1* and *DNMT3A* genes at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-Reversine and IVF groups were investigated. From Fig. 4.4, the SCNT-untreated group showed significantly lower expression levels of *DNMT1* at PN and 2-cell stages in comparison with those in the IVF group, but significantly higher than those in the SCNT-Reversine group ($P<0.05$). *DNMT1* transcripts of the SCNT-untreated at 4- and 8-cell stages displayed significantly lower than those in the SCNT-Reversine, but significantly higher than those in the IVF group ($P<0.05$). At blastocyst stage, the *DNMT1* expression levels in the SCNT-Reversine and IVF groups was significantly lower than

those in the IVF group (P<0.05). In addition, the *DNMT3A* transcripts of the SCNT-untreated at PN and 2-cell stages showed significantly lower than that of the IVF group, but higher than those in the SCNT-Reversine group (P<0.05). At 4-cell stage, the *DNMT3A* expression levels in the SCNT-Reversine group was significantly higher than those in SCNT-untreated group, and significantly lower than those in the IVF group (P<0.05). The expression levels of *DNMT3A* in IVF group at 8-cell were significantly higher than those in the SCNT-untreated, but significantly lower than those in the SCNT-Reversine group (P<0.05). At blastocyst stage, the expression levels of *DNMT3A* in SCNT-untreated and IVF groups were significantly lower than those in the SCNT-untreated and IVF groups (P<0.05).

4.2.2. Evaluation effects of SAHA on the relative expression levels of genes related to development and epigenetic reprograming by qPCR in PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos.

To elucidate how SAHA affect the reprograming and preimplantation development of SCNT embryos after SAHA treatment (1 μM for 12 h), the relative transcript abundance of three pluripotency genes (*POU5F1/OCT4*, *SOX2* and *NANOG*), three histone acetylation genes (*HDAC1*, *HDAC2* and *HDAC3*) and two DNA methylation genes (*DNMT1* and *DNMT3A*) at PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos were determined by qPCR.

4.2.2.1 SAHA affected the expression of development and pluripotency related genes in porcine embryos

To explore whether SAHA regulated the expression levels of development and pluripotent genes of porcine embryos including *POU5F1/OCT4*, *SOX2* and *NANOG* genes at PN, 2-, 4-, 8-cell and blastocyst stages between non-treated SCNT embryos served as the negative control group (SCNT-untreated), SCNT embryos treated with 1 μM SAHA for 12 h (SCNT-SAHA) and the positive control group was IVF derived embryos (IVF). The expression levels of these genes after SAHA treatment were investigated. From Fig. 4.5, the SCNT-SAHA group showed significantly (P<0.05) lower expression levels of *OCT4* at PN stage in comparison with those in the SCNT-untreated and IVF groups. The transcripts of *OCT4* at 2 stage in the SCNT-untreated group were significantly higher than those in the SCNT-SAHA, but significantly lower than those in the IVF group (P <0.05). *OCT4* transcripts of the SCNT-SAHA and IVF at 4- and 8-cell stages displayed significantly higher than those in the SCNT-untreated group (P<0.05), but did not differ with those in the IVF group.

Moreover, the expression levels of *OCT4* at blastocyst stage between the SCNT-untreated, SCNT-SAHA and IVF groups showed no significant differences. The transcripts of *SOX2* at PN stage in the SCNT-untreated group were significantly higher than those in the SCNT-SAHA and IVF groups (P <0.05). The transcripts of *SOX2* at 2- and 8-cell stages of SCNT-SAHA and IVF groups were significantly higher than those in SCNT-untreated group (P< 0.05). *SOX2* transcripts at the 4-cell and blastocyst stages in the SCNT-untreated and SCNT-SAHA were significantly lower than those in the IVF group (P< 0.05). The SCNT-untreated and SCNT-SAHA groups showed significantly (P<0.05) lower expression levels of *NANOG* at PN and 2-cell stages than those in the IVF group, and the SCNT-SAHA exhibited significantly higher transcripts of *NANOG* at 4-, 8-cell and blastocyst stages in comparison with those in the SCNT-untreated, but significantly lower than those in the IVF group (P<0.05).

4.2.2.2 SAHA affected the expression of histone acetylation related genes in porcine embryos

To explore whether SAHA regulated the expression levels of histone acetylation genes in porcine embryos. The expression levels of *HDAC1*, *HDAC2* and *HDAC3* genes at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-SAHA and IVF groups were investigated. From Fig. 4.6, the SCNT-SAHA and SCNT-untreated groups showed significantly (P<0.05) lower expression levels of *HDAC1* at PN stage in comparison with the IVF groups. *HDAC1* transcripts of the SCNT-SAHA group at 2-cell stage displayed significantly higher than those in the SCNT-untreated group, but significantly lower than those in the IVF group (P<0.05). At 4-cell stage, the *HDAC1* transcripts in the SCNT-untreated and SCNT-SAHA groups did not differ among groups, but significantly higher than those in the IVF group (P<0.05). The expression levels of *HDAC1* in SCNT-SAHA at 8-cell and blastocyst stages were significantly lower than those in the SCNT-untreated group, but significantly higher than those in the IVF group (P<0.05). In addition, the expression levels of *HDAC2* in SCNT-SAHA at PN and 4-cell stages were significantly lower than those of the IVF group, but significantly higher than those in the SCNT-untreated group (P<0.05). *HDAC2* transcripts of the SCNT-untreated and IVF groups at 2-cell stage displayed significantly higher than those in the SCNT-SAHA group (P<0.05). However, the expression levels of *HDAC2* between the SCNT-untreated, SCNT-SAHA and IVF groups showed no significant differences in transcripts of *HDAC2* at 8-cell. At blastocyst stage, the *HDAC2* expression levels in the SCNT-untreated group was significantly lower than those in the IVF group, but significantly higher with the SCNT-SAHA group (P<0.05). Moreover, at PN, 4- and 8-cell stages, the *HDAC3* expression levels in the SCNT-SAHA group was significantly

lower than those in the IVF group ($P<0.05$), but did not differ with the SCNT-untreated group. The expression levels of *HDAC3* in SCNT-SAHA and SCNT-untreated at 4-cell stage were significantly higher than those in the IVF group ($P<0.05$). At blastocyst stage, there were no significant differences in the expression levels of *HDAC3* between the groups.

4.2.2.3 SAHA affected the expression of DNA methylation related genes in porcine embryos

To explore whether SAHA regulated the expression levels of DNA methylation in porcine embryos. The expression levels of *DNMT1* and *DNMT3A* genes at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-SAHA and IVF groups were investigated. From Fig. 4.7, the SCNT-untreated group showed significantly lower expression levels of *DNMT1* at PN and 2-cell stages in comparison with those in the IVF group, but significantly higher than those in the SCNT-SAHA group ($P<0.05$). *DNMT1* transcripts of the SCNT-untreated at 4-cell stage displayed significantly higher than those in the IVF group, and significantly lower than those in the SCNT-SAHA group ($P<0.05$). At 8-cell stage, the *DNMT1* expression levels in the SCNT-SAHA group was significantly lower than those in the SCNT-untreated group, but significantly higher than the IVF group ($P<0.05$). For the expression levels of *DNMT1* at blastocyst stage in SCNT-SAHA and IVF groups were lower than those in SCNT-untreated group ($P<0.05$). In addition, the SCNT-untreated group showed significantly lower expression levels of *DNMT1* at PN stage in comparison with those in the IVF group, but significantly higher than those in the SCNT-SAHA group ($P<0.05$). *DNMT3A* transcripts of the SCNT-untreated and SCNT-SAHA at 2-and 8-cell stages displayed significantly lower than those in the IVF group ($P<0.05$). At 4-cell stage, the *DNMT3A* expression levels in the SCNT-SAHA and IVF groups were significantly higher than those in SCNT-untreated group ($P<0.05$). However, the expression levels of *DNMT3A* in SCNT-SAHA at blastocyst stages was significantly lower than those in the SCNT-untreated and IVF groups ($P<0.05$).

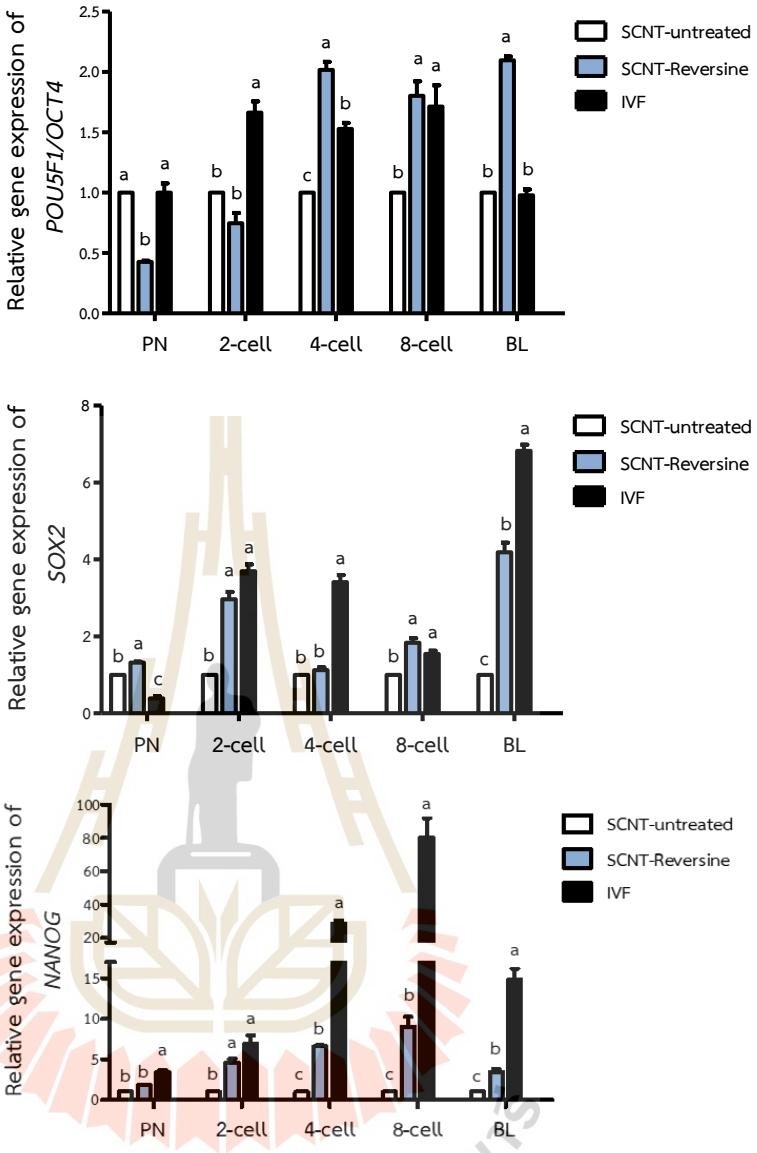


Figure 4.2 Comparison of mRNA expression levels (mean ± SEM) of genes related to developmental competence (*POU5F1/OCT4*, *SOX2* and *NANOG*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three times. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$)

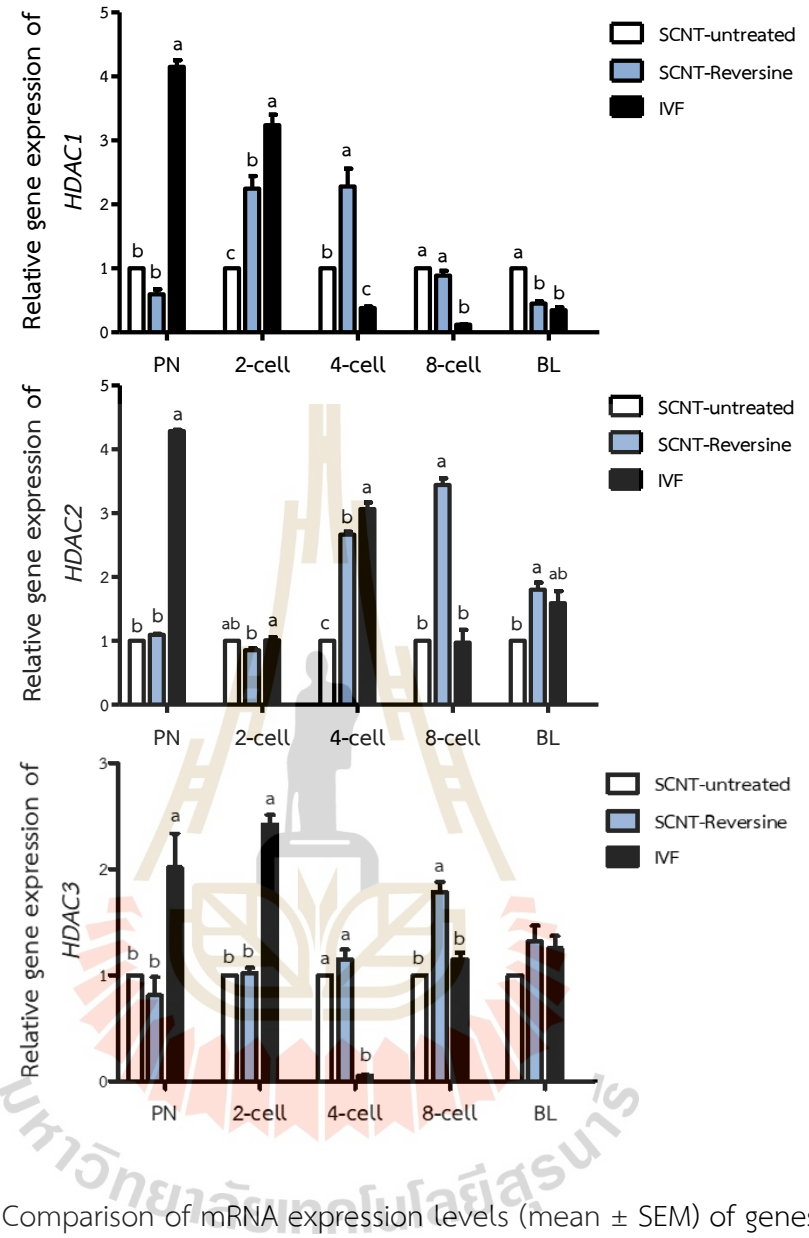


Figure 4.3 Comparison of mRNA expression levels (mean ± SEM) of genes related to Histone acetylation (*HDAC1*, *HDAC2* and *HDAC3*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages.The independent experiment was carried out for three times. Error bar = standard error of the mean. $^{a, b, c}$ Values with different superscripts indicate significant difference (P<0.05).

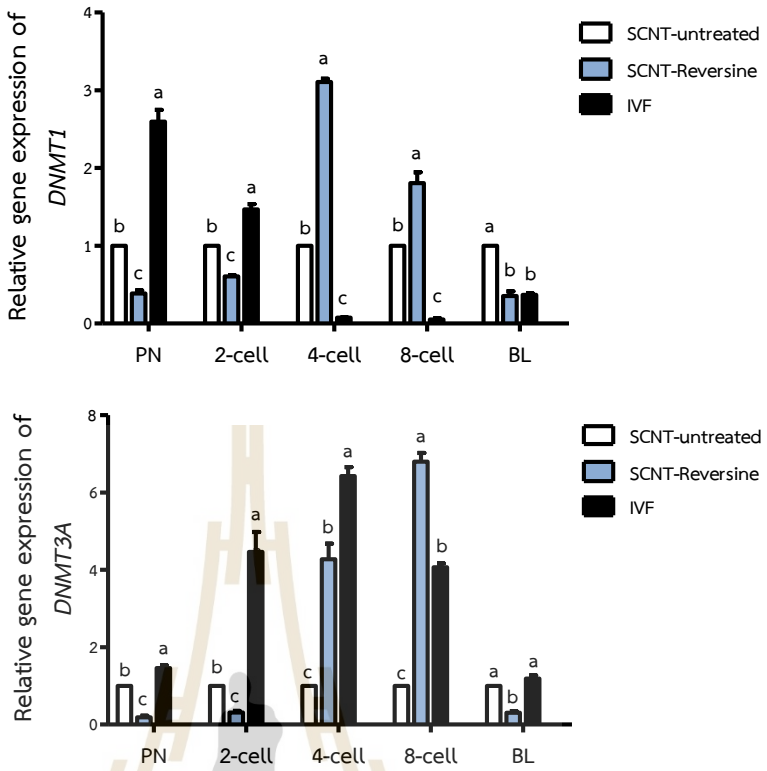


Figure 4.4 Comparison of mRNA expression levels (mean ± SEM) of genes related to DNA methylation (*DNMT1* and *DNMT3A*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three times. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$).

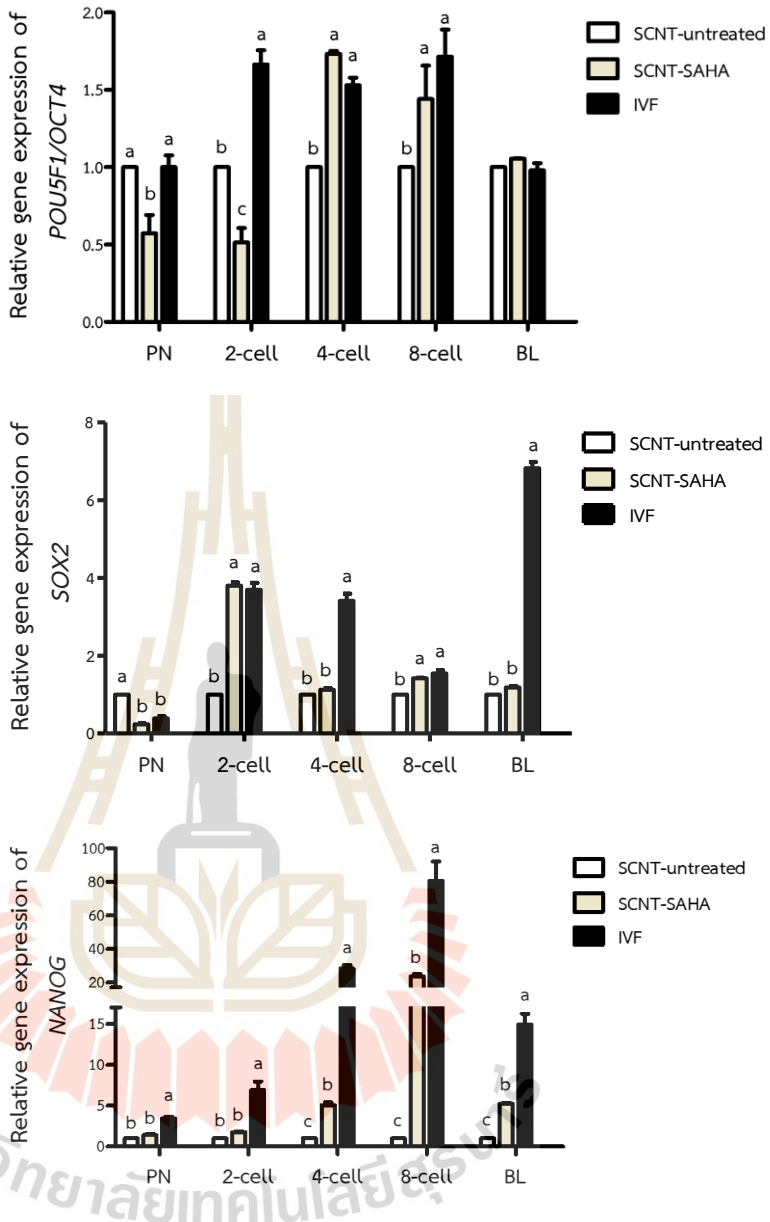


Figure 4.5 Comparison of mRNA expression levels (mean ± SEM) of genes related to developmental competence (*POU5F1/OCT4*, *SOX2* and *NANOG*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three times. Error bar = standard error of the mean. $^{a, b, c}$ Values with different superscripts indicate significant difference (P<0.05).

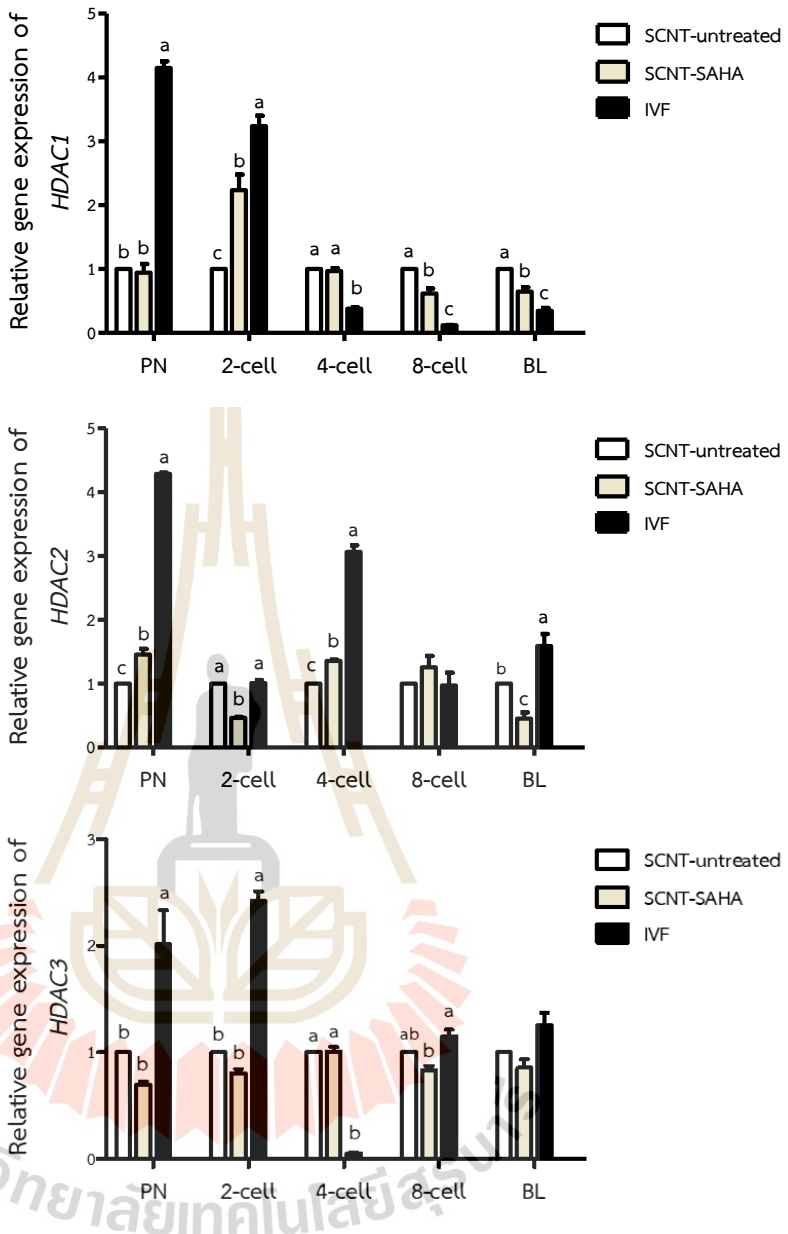


Figure 4.6 Comparison of mRNA expression levels (mean ± SEM) of genes related to histone acetylation (*HDAC1*, *HDAC2* and *HDAC3*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three times. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$).

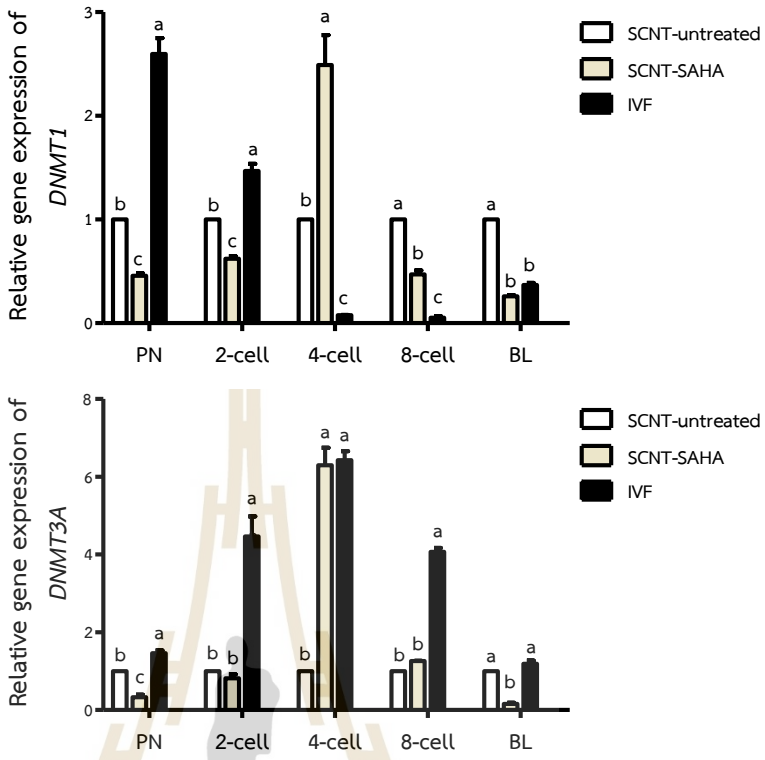


Figure 4.7 Comparison of mRNA expression levels (mean ± SEM) of genes related to DNA methylation (*DNMT1* and *DNMT3A*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three times. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$).

4.3 Effects of Reversine and SAHA on protein expression levels of epigenetic reprogramming in porcine embryos.

4.3.1 Evaluation of effects Reversine on the expression levels of protein related epigenetic reprogramming by immunocytochemistry staining (ICC) in PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos.

To further investigate the effects of Reversine (1μM for 6 h) on epigenetic reprogramming by determining the levels of protein expression of porcine SCNT embryos. The levels of histone acetylation (H3K9ac and H3K14ac), histone methylation (H3K9me3) and global DNA methylation (5-methylcytosine, 5-mc) at PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos were determined by ICC.

4.3.1.1 Reversine affected the protein expression of histone acetylation in porcine embryos.

To indicate whether Reversine regulated histone acetylation in porcine embryos. The fluorescence intensity levels of H3K9ac and H3K14ac at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-Reversine and IVF groups were investigated.

From Fig. 4.8 and 4.9, The H3K9ac level of PN, 2-cell and blastocyst stages in SCNT-Reversine group were no significant differences in intensity between the groups ($P<0.05$). The H3K9ac levels of 4-cell stage in SCNT-Reversine groups were higher than those in the SCNT-untreated and IVF groups ($P<0.05$). The SCNT-Reversine group at 8-cell stage showed a significantly higher level of H3K9ac than the IVF group, and also higher than those in the SCNT-untreated group ($P<0.05$). In addition, The PN stage in SCNT-Reversine and SCNT-untreated groups showed a significantly higher level of H3K14ac than the IVF group ($P<0.05$). The H3K14ac level of 2-cell stage in SCNT- Reversine and IVF groups were no significant differences, but the IVF group showed significantly higher level of H3K14ac than those in the SCNT-untreated group ($P<0.05$). The H3K14ac levels of 4-cell stage in SCNT-Reversine and SCNT-untreated groups were lower than those in the IVF group ($P<0.05$). The SCNT-Reversine at 8-cell stage showed a lower level of H3K14ac than SCNT-untreated and IVF groups ($P<0.05$). At blastocyst stage, SCNT-Reversine group was slightly increased in intensity of H3K14ac between the SCNT-untreated and IVF groups, but there was no significant difference ($P>0.05$).

4.3.1.2 Reversine affected the protein expression of histone methylation in porcine embryos

To indicate whether Reversine regulated histone methylation in porcine embryos. The fluorescence intensity levels of H3K9me3 at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-Reversine and IVF groups were investigated.

From Fig. 4.10, the H3K9me3 levels of PN stage in SCNT-Reversine group was lower than those in the SCNT-untreated and IVF groups ($P<0.05$). The SCNT-Reversine and SCNT-untreated group at 2-cell stage showed lower level of H3K9me3 than those in the IVF group ($P<0.05$). The H3K9me3 level of 4-cell in SCNT-Reversine group were no significant differences between the groups ($P<0.05$). Moreover, the H3K9me3 levels of 8-cell stage in SCNT-Reversine group showed significantly higher H3K9me3 than those in the IVF and SCNT-untreated groups ($P<0.05$). At blastocyst stage, SCNT-Reversine and IVF groups showed a lower level of H3K9me3 than those in the SCNT-untreated group ($P<0.05$).

4.3.1.3 Reversine affected the protein expression of DNA methylation in porcine embryos

To indicate whether Reversine regulated histone methylation in porcine embryos. The fluorescence intensity levels of 5-mC at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-Reversine and IVF groups were investigated. From Fig. 4.11, the 5-mC level of PN stage in SCNT-Reversine group was significantly higher than those in the IVF group, but significantly lower than the SCNT-untreated group ($P<0.05$). The SCNT-Reversine group at 2- and 4-cell stages showed significantly higher levels of 5-mc than those in the SCNT-untreated and IVF groups ($P<0.05$). The SCNT-Reversine group at 8-cell stage showed a similar level of 5-mC with SCNT-untreated and IVF groups. However, the blastocyst stages in SCNT-Reversine and SCNT-untreated groups showed significantly higher levels of 5-mC than those in the IVF group ($P<0.05$).

4.3.2 Evaluation effects of SAHA on the expression levels of protein related epigenetic reprogramming by immunofluorescence staining (ICC) in PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos.

To further investigate the effects of SAHA (1μM for 12 h) on epigenetic reprogramming by determining the levels of protein expression of porcine SCNT embryos. The levels of histone acetylation (H3K9ac and H3K14ac), histone methylation

(H3K9me3) and global DNA methylation (5-methylcytosine, 5-mc) at PN, 2-, 4-, 8-cell and blastocyst stages of porcine embryos were determined by ICC.

4.3.2.1 SAHA affected the protein expression of histone acetylation in porcine embryos.

To indicate whether SAHA regulated histone acetylation in porcine embryos. The fluorescence intensity levels of H3K9ac and H3K14ac at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-SAHA and IVF groups were investigated.

From Fig. 4.12 and 4.13, the H3K9ac levels of PN and blastocyst stages in SCNT-SAHA groups were higher than those in the SCNT-untreated and the IVF groups (P<0.05). The SCNT-SAHA group at 2- and 4-cell stages showed a similar level of H3K9ac with SCNT-untreated and IVF groups (P<0.05). The H3K9ac level of 8-cell in SCNT-SAHA group, showed no significant differences with the IVF group, but the SCNT-SAHA and IVF groups showed significantly higher the level of H3K9ac than those in the SCNT-untreated group (P<0.05). Moreover, the PN stage in SCNT-SAHA and SCNT-untreated groups showed significantly higher levels of H3K14ac than those in the IVF group (P<0.05). The H3K14ac level of 2-cell in SCNT-SAHA group were no significant differences with the IVF group, but the IVF group showed significantly higher level of H3K14ac than those in the SCNT-untreated group (P<0.05). The H3K14ac levels of 4-cell stage in SCNT-SAHA and SCNT-untreated groups were significantly lower than IVF group (P<0.05). The SCNT-SAHA at 8-cell stage showed similar levels of H3K14ac with SCNT-untreated and IVF groups (P<0.05). At blastocyst stage, the level of H3K14ac in SCNT-SAHA showed significantly higher than those in the SCNT-untreated and IVF groups (P<0.05).

4.3.2.2 SAHA affected the protein expression of histone methylation in porcine embryos.

To indicate whether SAHA regulated histone methylation in porcine embryos. The fluorescence intensity levels of H3K9me3 at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-SAHA and IVF groups

From Fig. 4.14, the H3K9me3 levels of PN stage in SCNT-SAHA group was significantly lower than those in the SCNT-untreated and IVF groups (P<0.05). The SCNT-SAHA and SCNT-untreated group at 2-cell stage showed lower level of H3K9me3 than those in the IVF group (P<0.05). The H3K9me3 level of 4-cell in SCNT-SAHA group showed no significant differences between the groups (P<0.05). Moreover, the H3K9me3 level of 8-cell in SCNT-SAHA group were no significant differences with

IVF group, but the IVF group showed significantly higher H3K9me3 levels than those in the SCNT-untreated group ($P<0.05$). At the blastocyst stage in SCNT-SAHA and SCNT-untreated groups showed significantly higher H3K9me3 levels than those in the IVF group ($P<0.05$).

4.3.2.3 SAHA affected the protein expression of DNA methylation in porcine embryos

To indicate whether SAHA regulated histone methylation in porcine embryos. The fluorescence intensity levels of 5-mC at PN, 2-, 4-, 8-cell and blastocyst stages between SCNT-untreated, SCNT-SAHA and IVF groups were investigated.

From Fig. 4.15, the 5-mC level of PN stage in SCNT-SAHA and SCNT-untreated groups were significantly higher than those in the IVF group ($P<0.05$). The SCNT-SAHA group at 2-cell stage showed a lower level of 5-mC than SCNT-untreated and IVF groups. The SCNT-SAHA group at 4- and 8-cell stages showed a similar level of 5-mC with SCNT-untreated and IVF groups. However, the blastocyst stages in SCNT-SAHA and IVF groups showed a significantly lower level of 5-mC than those in the untreated group ($P<0.05$).

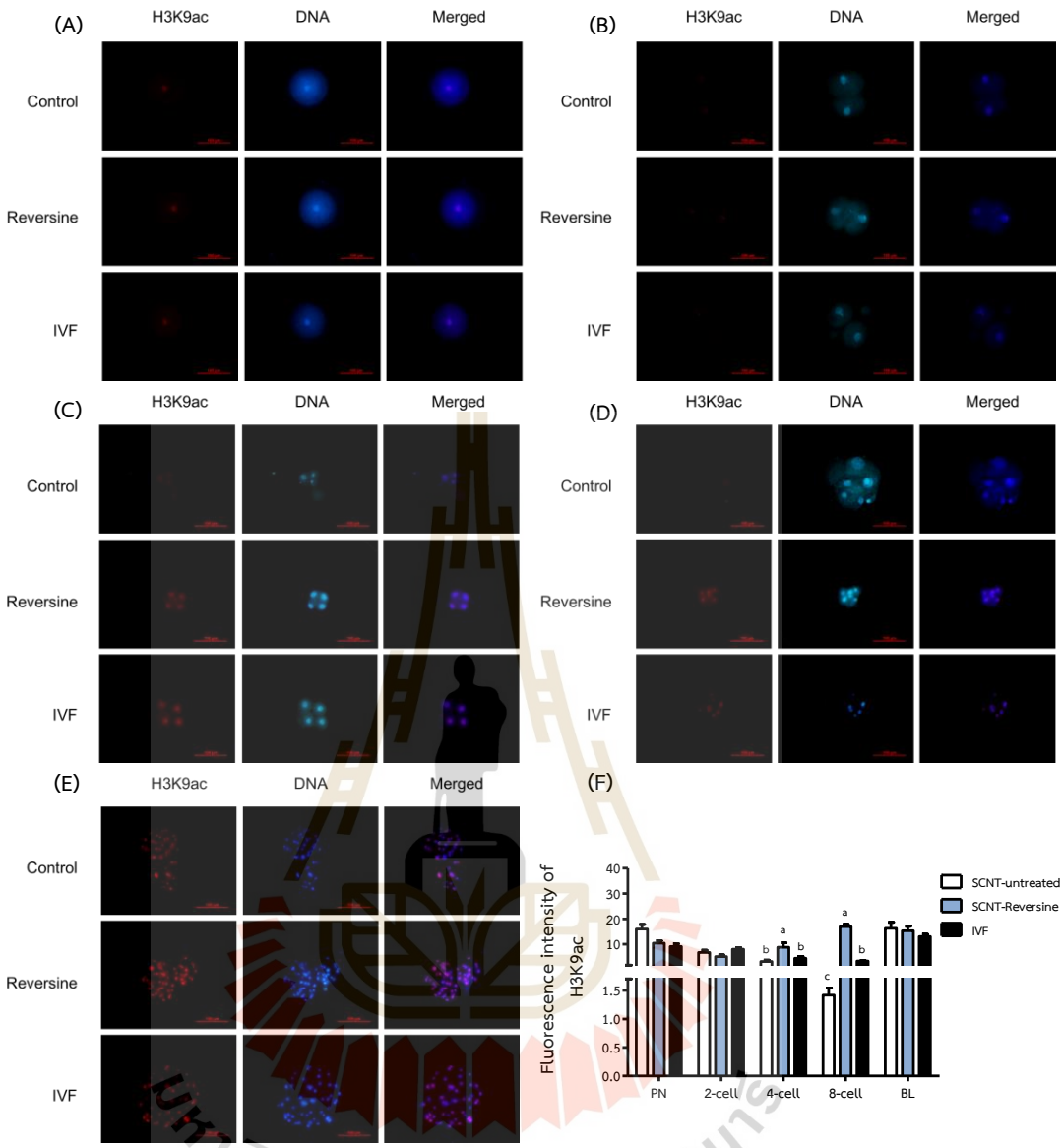


Figure 4.8 Effect of Reversine on the histone acetylation level (H3K9ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K9ac level at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescent intensity of H3K9ac level at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n=15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference (P<0.05).

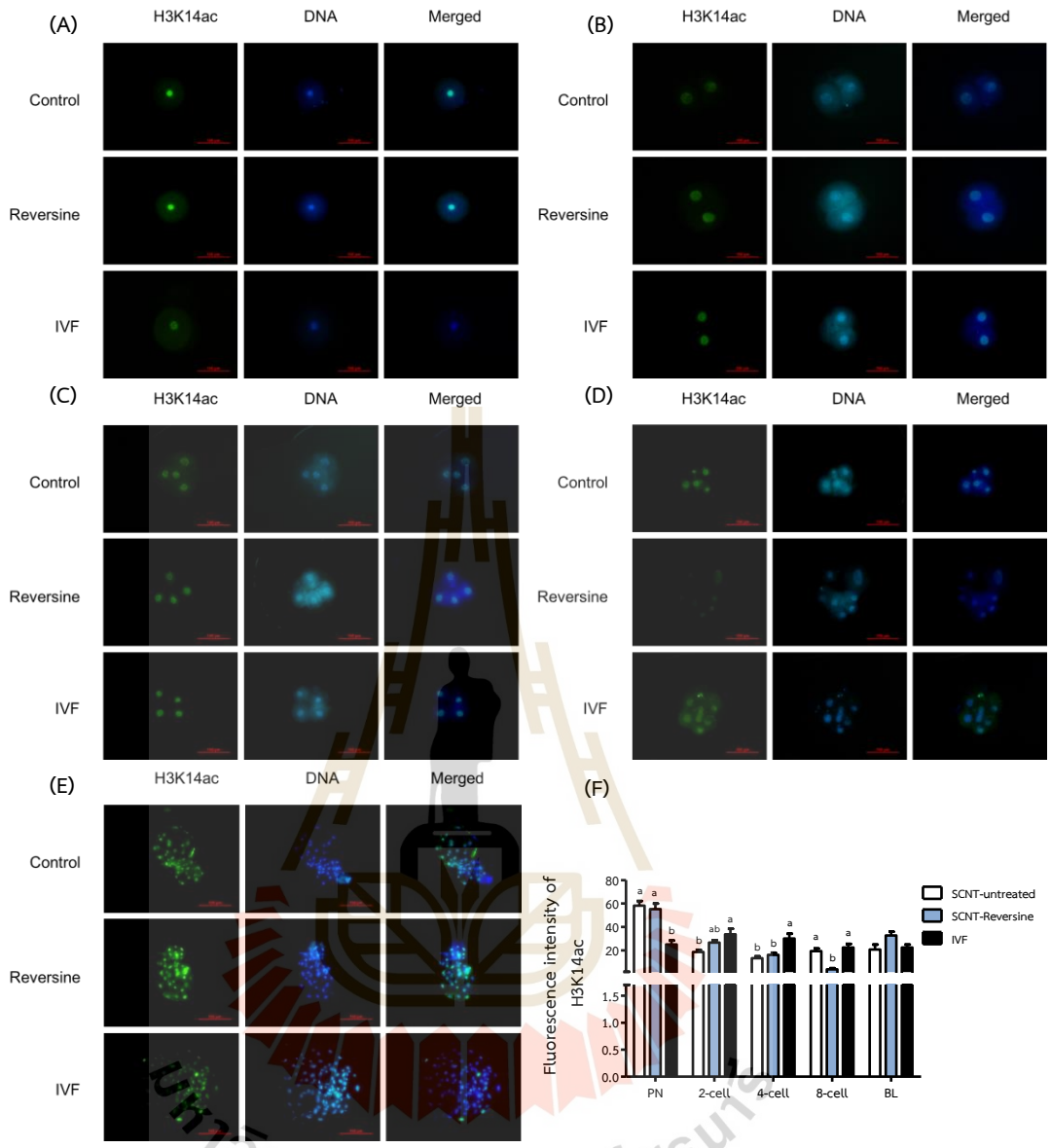


Figure 4.9 Effect of Reversine on the histone acetylation level (H3K14ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of H3K14ac levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$).

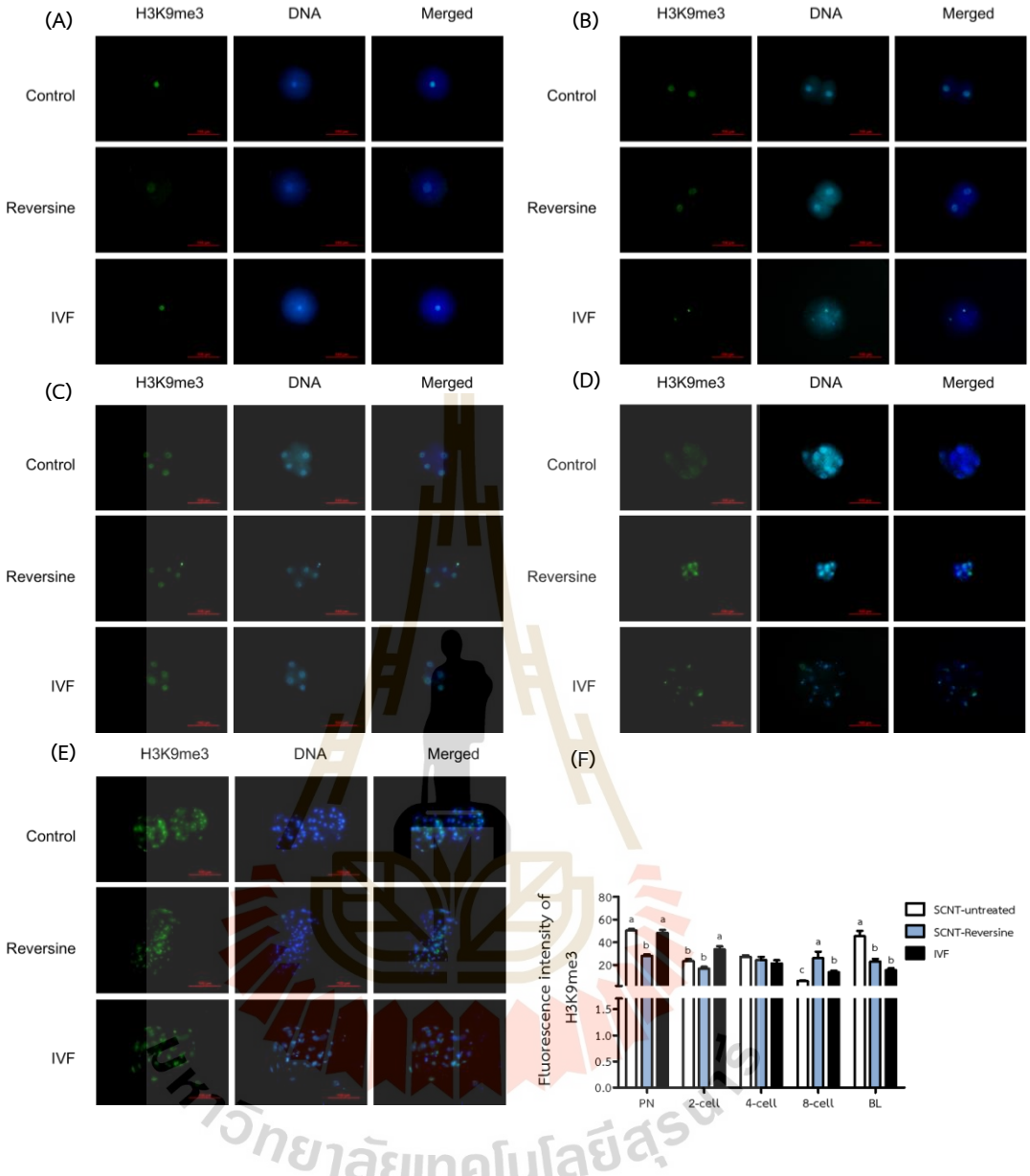


Figure 4.10 Effect of Reversine on the histone acetylation level (H3K9me3) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of H3K9me3 levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference ($P<0.05$).

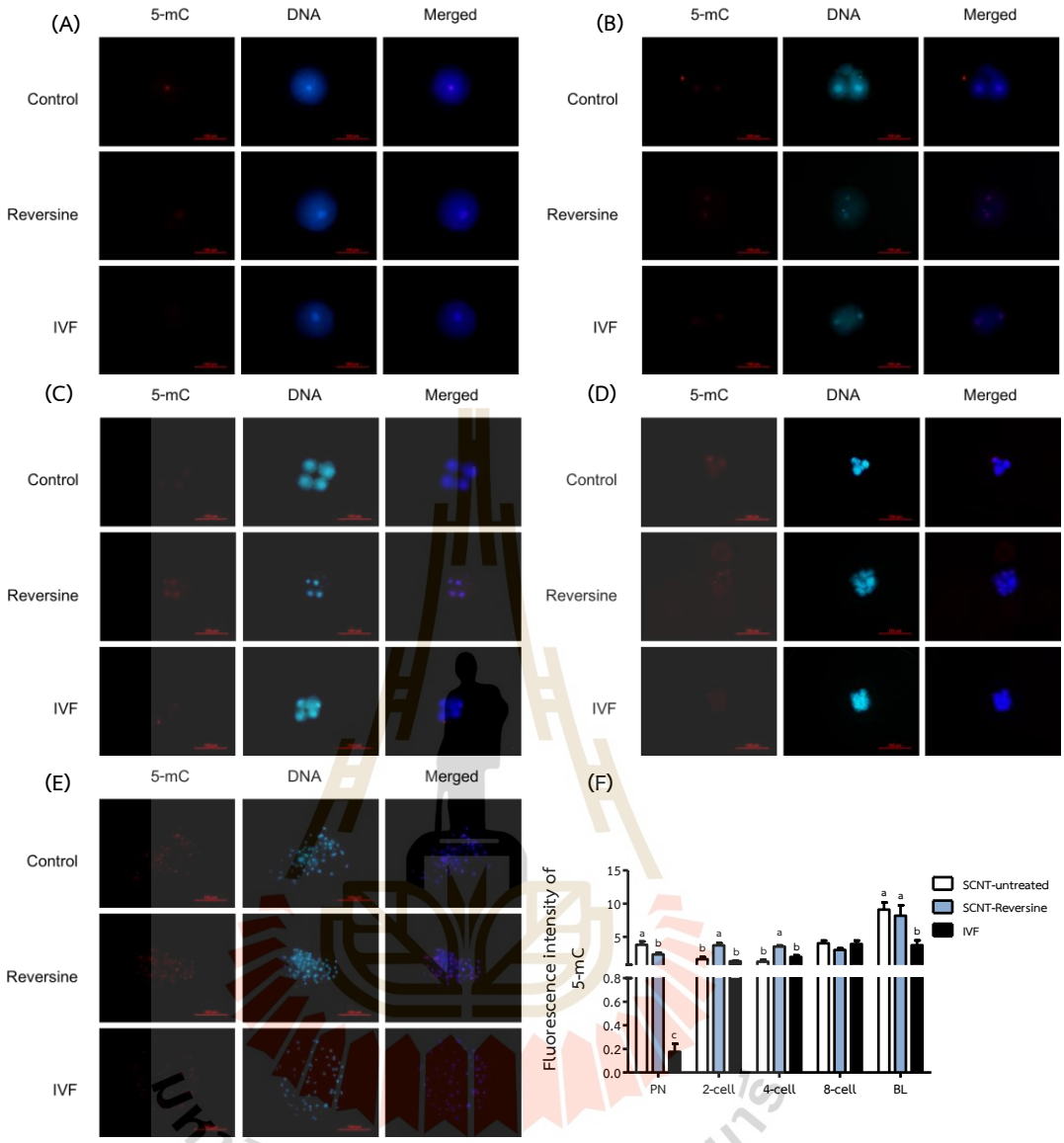


Figure 4.11 Effect of Reversine on the histone acetylation level (5-mC) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of 5-mC level at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference (P<0.05).

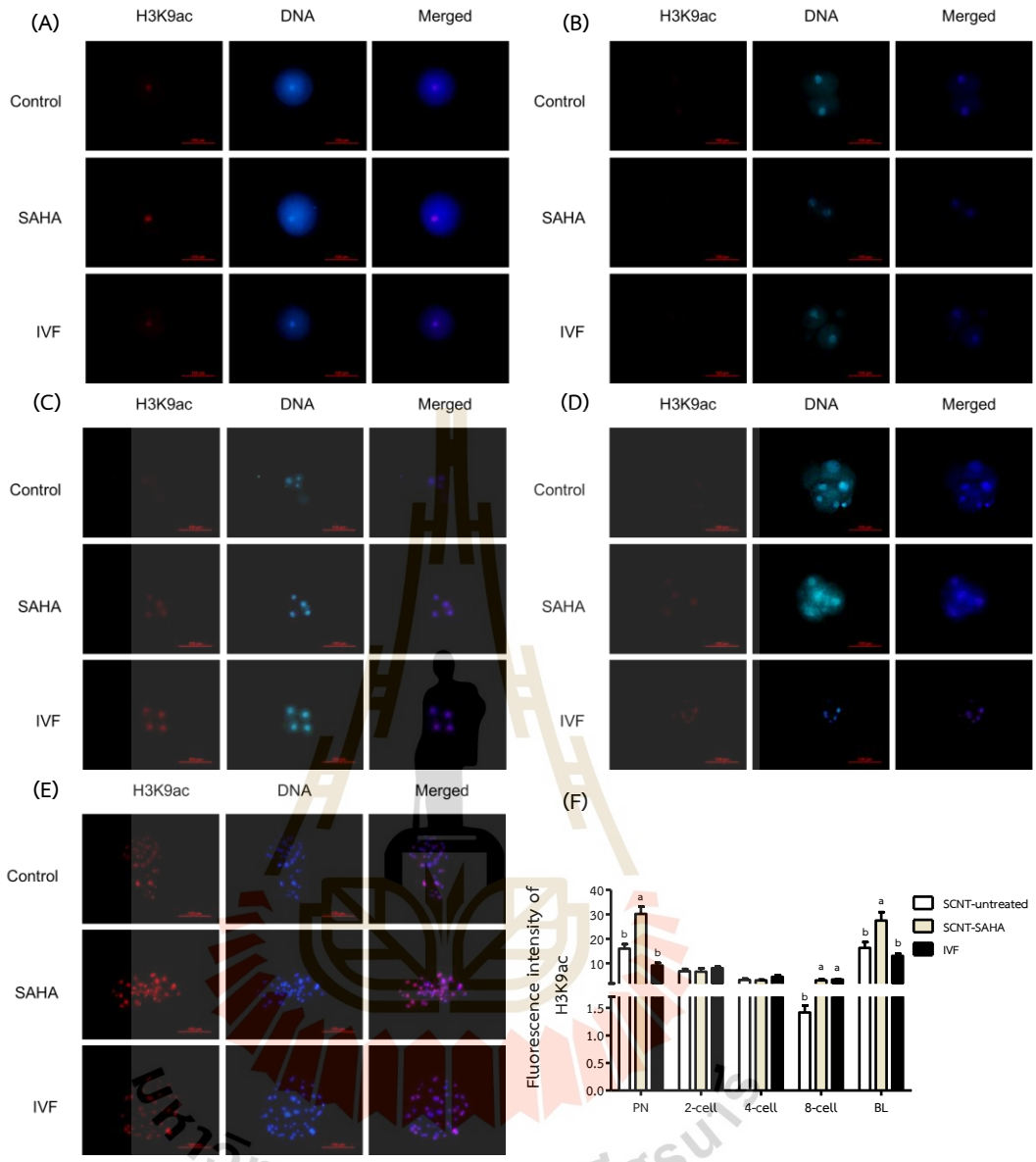


Figure 4.12 Effect of SAHA on the histone acetylation level (H3K9ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K9ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of H3K9ac levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. $^{a, b, c}$ Values with different superscripts indicate significant difference (P<0.05).

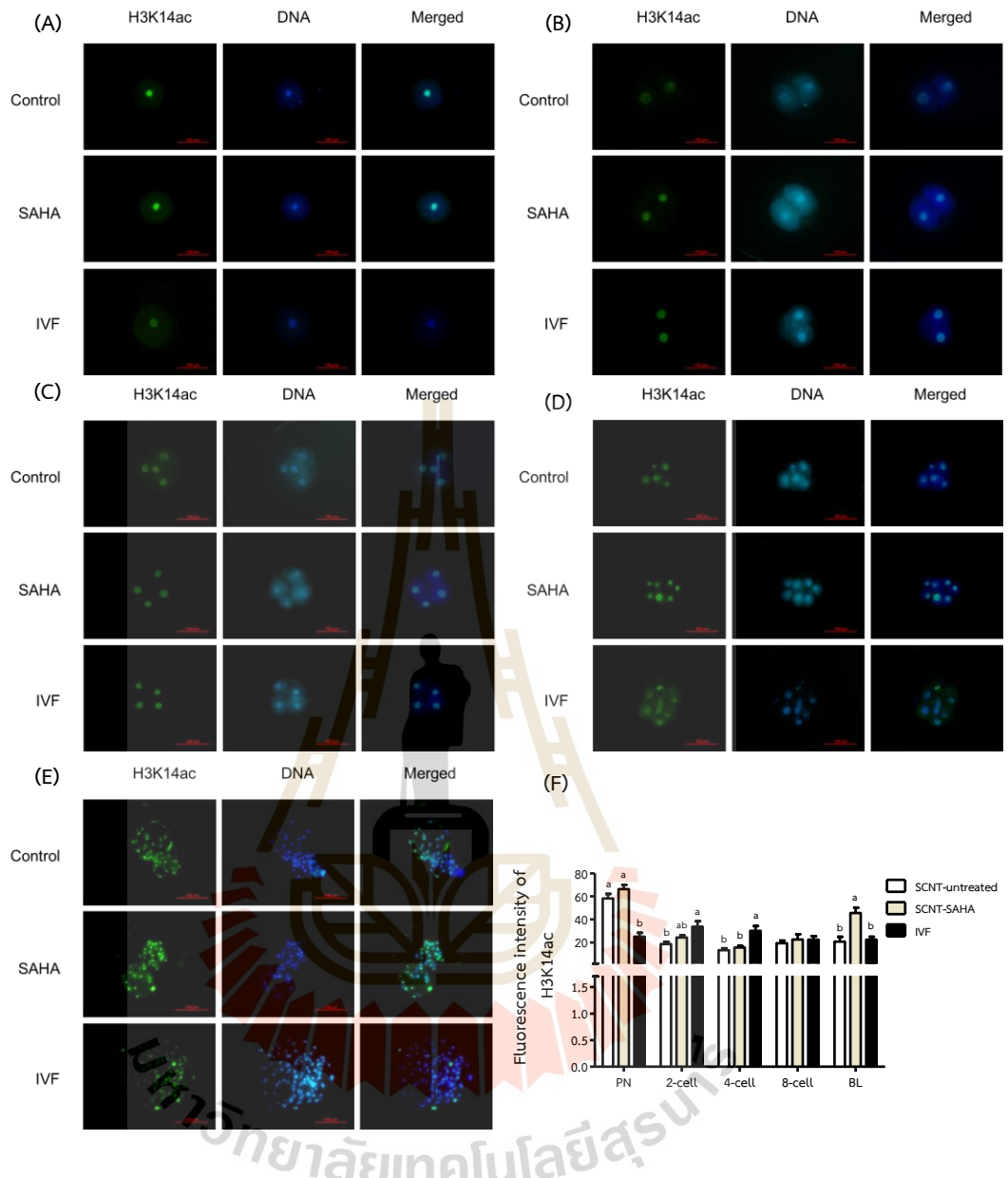


Figure 4.13 Effect of SAHA on the histone acetylation level (H3K14ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of H3K14ac levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. $^{a, b, c}$ Values with different superscripts indicate significant difference (P<0.05).

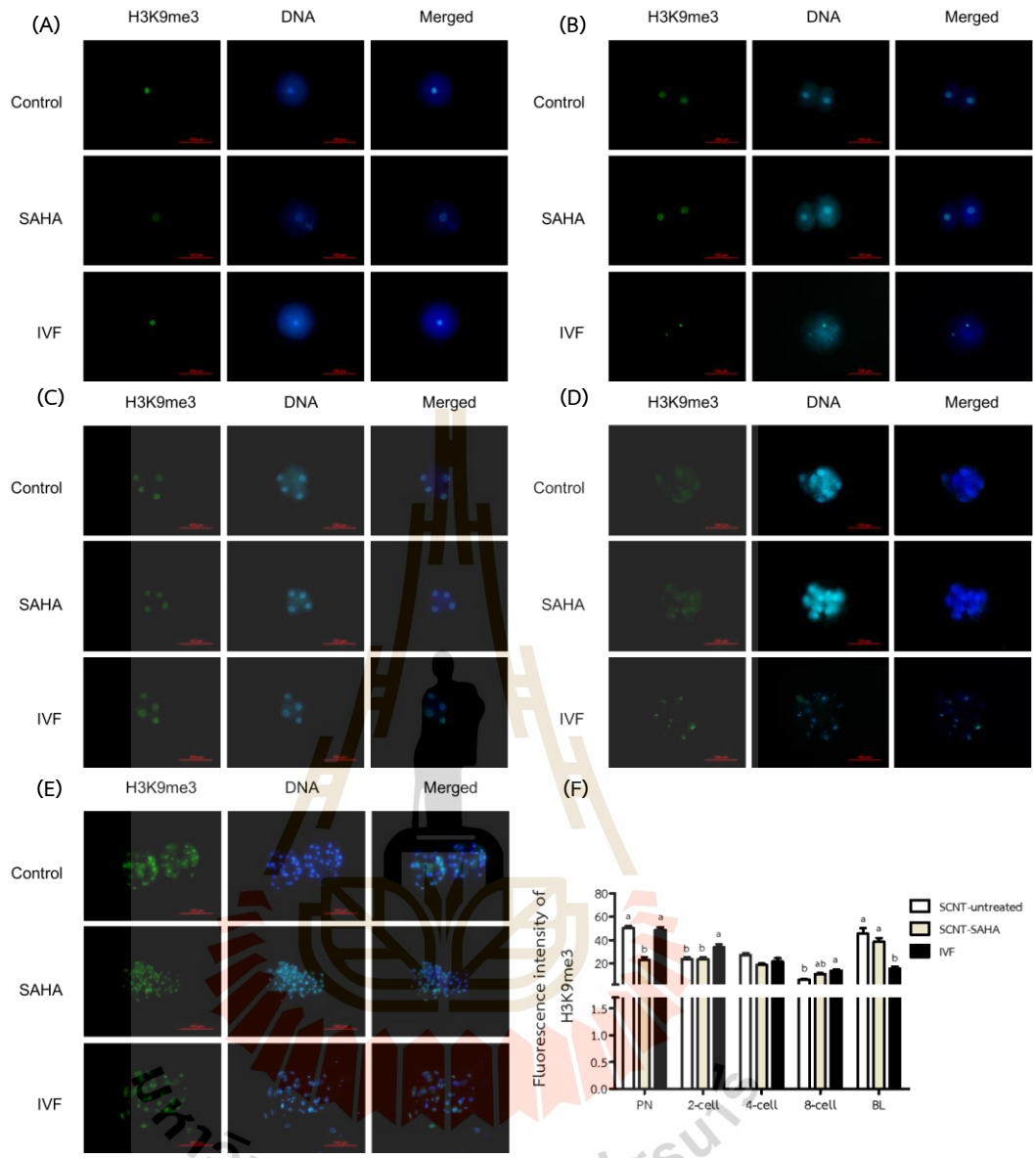


Figure 4.14 Effect of SAHA on the histone methylation level (H3K9me3) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of H3K9me3 levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference (P<0.05).

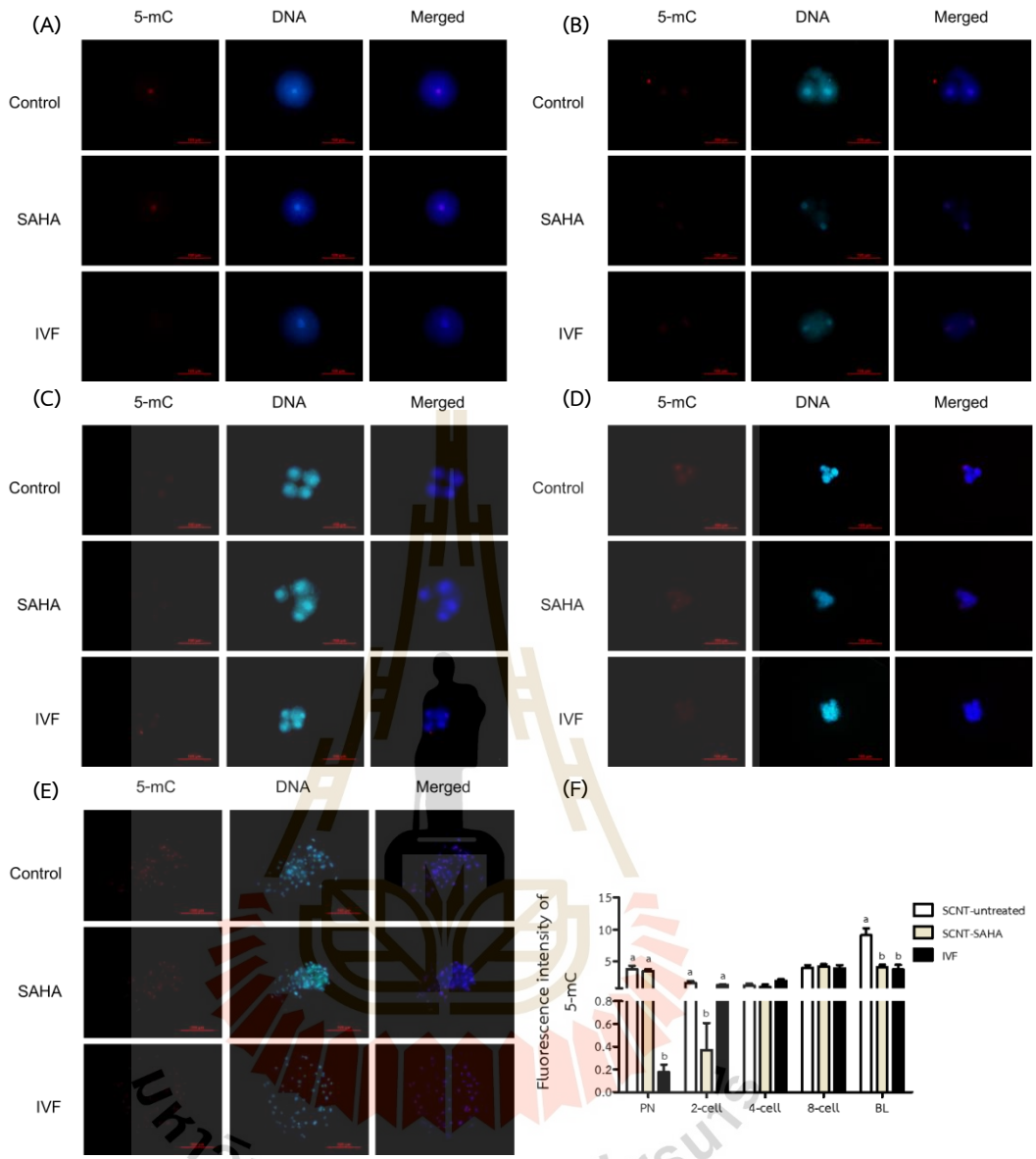


Figure 4.15 Effect of SAHA on the histone acetylation level (5-mC) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity of 5-mC levels at PN, 2, 4-, 8-cell and blastocyst stages, respectively (n= 15 per stage). The data are form three independent experiments and are means ± SEM. Error bar = standard error of the mean. [a, b, c] Values with different superscripts indicate significant difference (P<0.05).

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

To date, previous studies have reported that small molecules, including Reversine altered MEK-dependent signaling to control histone acetylation, which may have an impact on cloned embryo reprogramming events (Chen et al., 2007). SAHA, commonly known as HDACi, significantly enhanced the developmental and reprogramming efficiency of cloned embryos in several species (Ono et al., 2010; Sun et al., 2020)., but the precise mechanism of both small molecules underlying epigenetic reprogramming in mammalian species remains unclear. Developmental defects in SCNT embryos are mostly referable to incomplete epigenetic reprogramming and low efficiency of potential development (Czernik et al., 2019; Jeong et al., 2021). In the present study, with the aim of enhancing SCNT embryonic development, we cultured porcine SCNT embryos in activation and culture medium with various concentrations and durations of Reversine and SAHA to optimize the concentration and duration of Reversine and SAHA. The results of our study demonstrated that optimal conditions of Reversine (1 μM for 6 h) during activation and IVC did not affect the developmental competence of porcine SCNT embryos and the quality of the SCNT blastocysts. Moreover, the other report indicates that treatment with Reversine is not beneficial for increasing the cleavage rate and total cell number in the blastocyst of miniature pig SCNT embryos, but it is still unclear of the underlying histone modification (Miyoshi et al., 2010). In contrast to the first report of bovine cloning using Reversine, the treatment of reconstructed SCNT embryos with 1 μM of Reversine for 6 h resulted in a significant increase in blastocyst formation rates compared to the group without Reversine treatment (Yoisungnern et al., 2010). Hence, from the previous experiment, the optimal conditions of Reversine treatment during activation and IVC was 1 μM of Reversine for 6 h. Suggesting of these results that improving porcine SCNT embryos by small molecules during development may differ according to different species and treatment protocol. Moreover, Reversine treatment has been shown to significantly enhance the blastocyst rate of cloned porcine embryos, but no offspring were born (Miyoshi et al. 2010). For SAHA, it has been shown to significantly increase the blastocyst formation, rate of porcine SCNT embryos that were treated with optimal concentration and duration of SAHA (1.5 μM of SAHA for 72 h) in the minipig fetal fibroblasts (Sun et al.,

2020). Therefore, it would be worth determining the optimal concentration and duration of SAHA treatment in order to improve pig SCNT reprograming, particularly histone acetylation. Our experiment found that after treating reconstructed SCNT embryos with different concentrations of SAHA for 12 h during cultured in activation and IVC medium resulted in 1 μM of SAHA had the highest percentage of blastocyst formation, but no significantly with those in the SCNT-untreated group (Table 4.1.3). In contrast to the effect of different durations with 1 μM of SAHA treatment (Table 4.1.4) showed 1 μM of SAHA for 12 h was significantly higher than those in the SCNT-untreated group. Suggesting of these results that improving developmental competence of porcine SCNT embryos by SAHA treatment may differ according to different quality of oocytes for SCNT method influenced by temperature of each season. Although, there is no beneficial effect of SAHA on the cleavage rate of cloned embryos (Ono et al., 2010; Yoisungnern et al., 2012). Whitworth et al. (2015) reported that 1 μM SAHA in post-fusion, activation and IVC increased total cell number of porcine SCNT embryos. Thus, the optimal conditions of SAHA treatment during activation and IVC was 1 μM of SAHA for 12 h.

The reprogramming of pluripotent genes could influence the developmental competence of cloned embryos (Dejosez and Zwaka, 2012). We investigated the effects of the optimal conditions of Reversine and SAHA-influenced gene transcription-related development of cloned porcine embryos. The pluripotent genes, including *OCT4*, *SOX2* and *NANOG*, play a key pivotal role in the maintenance of pluripotency during early embryonic development (Lee et al., 2013). There are many previous studies showed that the expression of *OCT4*, *SOX2* and *NANOG* in porcine IVF were higher than blastocysts than SCNT blastocysts, IVF embryos as normal embryonic development and reprogramming (Qu et al. 2020; Liu et., 2011; Zhai et al., 2017).

For Reversine treatment, the SCNT embryos after Reversine treatment at blastocyst stages exhibited significantly higher expression of *OCT4*, *SOX2* and *NANOG* than SCNT-untreated groups. This result is consistent with previous studies by Jin et al. (2017), after LQ824 (HDACi) treatment as well as HDACi showed significantly increased mRNA levels of *OCT4, SOX2 and NANOG* in porcine cloned blastocyst. *OCT4/POU5F1* is key to the maintenance of self-renewal of the pluripotent inner cell mass (ICM) for preimplantation embryonic development (Kermari et al., 2010; Park et al., 2012). *SOX2* is also a faithful marker of pluripotency factor (Liu et al. 2015). It cooperates with *OCT4* as well as the *OCT-SOX* enhancer that plays an important role

in regulating the pluripotency of embryonic stem cells (Kermari et al., 2010; Masui et al., 2007). Lower expression of *OCT4* has been observed in porcine SCNT blastocysts than IVF blastocysts (Liu et al., 2011; Sun et al., 2020). Our results showed the higher expression of *OCT4* in porcine SCNT-Reversine blastocysts than IVF and SCNT-untreated blastocysts. This result consistent with previous studies in buffalo cloned blastocysts after treatment with HDACi (m-carboxycinnamic acid bishydroxymide; CBHA) (Agrawal et al., 2018). Hence, the increase in pluripotency-related genes could be a reason for the increase in developmental competence of treated groups. Moreover, the SCNT embryos after Reversine treatment showed no significant difference in the expression of *SOX2* at 4-cell stage were observed between SCNT-Reversine and SCNT-untreated groups. This observation is inconsistent with previous studies in porcine SCNT embryos (Qu et at., 2020). Although, the higher expression of *SOX2* has been reported in SCNT-Reversine at PN, 2-, 8-cell and blastocyst stages than SCNT-untreated. Collectively, these results in the improvement of embryonic development by Reversine treatment enhanced *OCT4, SOX2 and NANOG* markers of pluripotency in porcine SCNT embryos, especially at the blastocyst stage and closed to IVF porcine embryos.

For SAHA treatment, the reported by Sun et al. (2020), they found that the *OCT4* transcription in porcine cloned blastocysts after SAHA treatment was lower compared with IVF blastocysts. In our experiment, the *OCT4* and *SOX2* expression levels in SAHA treatment at blastocyst stage were also similar with IVF and SCNT-untreated blastocysts. Similar to Huo et al. (2014), one of HDACi (Oxamflatin) treatment was not found to result in a direct impact on *SOX2* expression of porcine SCNT embryos and Whitworth et al (2015) also showed the similar *OCT4* expression in porcine SCNT-SAHA and IVF blastocyst and the *NANOG* expression in IVF and SCNT-SAHA blastocysts were higher than SCNT-untreated blastocyst. On the basis of these results, the evaluated expression of *NANOG* gene transcripts after SAHA treatment might contribute to the improvements of porcine cloned embryos at 4-cell, 8-cell and blastocyst stages or after zygote genome activation (ZGA). ZGA occurs in mice at 2-cell stage, pig at 4-, 8-cell stages, and cattle at 8-cell stage (Hyttel et al., 2000; Schultz, 2002). Moreover, the results from previous studies also reported that combination of DNMTi (scriptaid) and HDACi (RG108) can promote *NANOG* transcription of porcine SCNT embryos and enhance their preimplantation (Xu et al., 2013). NANOG is one of the major pluripotent factors, and its expression affects the ability of nuclear reprogramming, indicating that NANOG may be a good marker to evaluate nuclear reprogramming in cloned embryos (Costa et al., 2013; Miyamoto et al., 2009; Stuart et al., 2014). However, SAHA treatment

appeared to have no direct effect on the expression of *OCT4* and *SOX2* after ZGA in porcine SCNT embryos, but affected on the expression of *NANOG* in SCNT blastocysts

and this result is also similar to Whitworth et al. (2015), SAHA treatment in post-fusion and activation of porcine SCNT embryos.

Epigenetic reprogramming, including histone acetylation, histone methylation, and DNA methylation, affects gene transcription and chromatin structure during embryonic development (Matoba and Zhang, 2018). During the ZGA stage, SCNT embryos undergo abnormal gene expression and developmental arrest, which is thought to be caused by aberrant epigenetic reprogramming (Loi et al., 2016). Degradation of maternal proteins and mRNAs and the start of mRNA synthesis in the newly generated zygotic genome are linked to the key developmental transition in ZGA.

Histone acetylation, one of the major types of epigenetic marks, involves an acetyl group being added to lysine residues in the protruding histone tails, and it is catalyzed by the enzymes histone acetyl transferases (HATs) and histone deacetylases (HDACs), which have opposing effects; HATs transfer acetyl groups to lysine, but HDACs remove acetyl groups from lysine (Bannister et al., 2011). Increasing histone acetylation could promote the accessibility of the loose binding of DNA to nucleosomes, chromatin relaxation, and the activation of gene transcription during the early development of embryos (Zhao et al., 2010; Yamanaka et al., 2009). Many efforts have been made to modify SCNT protocols using HDACis to promote cell reprogramming, and the level of histone acetylation under optimal conditions with various HDACis could improve their *in vitro* developmental competence in mammalian species (Bohrer et al., 2014; Wang et al., 2015; Hou et al., 2014; Agrawal et al., 2018). Reversine can regulate acetylation of histone H3 by MEK-dependent signaling (Chean et al., 2007). However, to date, the precise mechanism underlying histone acetylation by Reversine and SAHA has not been fully elucidated. The results of the present study, with the aim of enhancing the epigenetic reprogramming of porcine cloned embryos by Reversine and SAHA. To elucidate the effect of Reversine and SAHA treatments on the level of histone acetylation, among the several types of histones, acetylation of histone H3 is essential for promoting gene expression in SCNT embryos, which may have an impact on the embryo developmental competence (Yamanaka et al. 2009). H3K9ac and H3K14ac at the promoter region of active ES cells significantly improve the transcriptional suppression (Guenther et al., 2007; Liang et al., 2004). Class I HDACs include *HDAC1-3*, which are primarily expressed in oocytes and early stages of embryonic development

and have been identified as potential genes involved in ZGA (Pan et al., 2015). The stable status of histone acetylation is controlled by balancing the expression of HATs and HDACs. The downregulation of these genes-related HDACs influences transcriptional activity and chromatin modification and reprogramming. We investigated the histone acetylation levels of H3K9ac and H3K14ac and the gene expression level of *HDAC1*, *HDAC2* and *HDAC3* these gene-related deacetylases of histone in PN, 2- , 4- , 8-cell and blastocyst of porcine SCNT embryos. The porcine IVF embryos at 2-cell and blastocysts stages showed a higher level of H3K9ac with SCNT-treated, but Zhai et al. (2018) found that the IVF embryos showed significantly lower the level of H3K9ac than those in the SCNT-treated group. Moreover, the H3K14ac levels in porcine IVF embryos was higher at 2-cell stage, lower than at 4-cell stage than those in the SCNT-untreated (Liu et al., 2012) and no significant differences at blastocyst stages. Sun et al. (2020) found the H3K14ac level in porcine IVF embryos was higher at PN and blastocyst stages but lower at 2- and 4-cell stages than those in the SCNT-untreated. The suggestion, the protein expression with specific markers of H3K9ac and H3K14ac in porcine IVF embryos is still unclear of the underlying of different protocol, location and quality of SCNT-untreated comparison. For Reversine treatment, there is the report by Liu et al., 2012 that level of histone activation mark could disappear of H3K9ac at the ZGA stage in porcine SCNT embryos treatment by NaBu (HDACi) and IVF embryos. Surprisingly, from our results, we found that Reversine treatment significantly increased H3K9ac levels in SCNT embryos at ZGA stage when compared with those in the SCNT-untreated group. Although Reversine did not affect the levels of both H3K9ac and H3K14ac till the blastocyst stage of cloned porcine embryos (compared to the untreated group), that is consistent with previous studies in porcine cloned embryos (Jin et al., 2017; Jin et al., 2018). Kim et al. (2014) indicated that Reversine induces a multipotency of C2C12 myoblasts by suppressing miR-133a expression through the exhibition of active H3K14ac. Hence, Reversine influenced histone acetylation by increasing H3K9ac levels at ZGA stage, but not report in H3K14ac levels in porcine SCNT embryos.

For SAHA treatment, this observation is in line with a previous study by Hou et al. (2014) who showed that the level of histone acetylation (H3K9ac) increased at 4-cell and disappeared at blastocyst stage when compared to SCNT-untreated porcine embryos. Additionally, similar to Sun et al. (2020), SAHA treatment in porcine cloned embryos at 2-cell stage had a higher level of H3K14ac in comparison without SAHA treatment. Furthermore, histone acetylation level began to decrease at the 4-cell stage significantly. The results from previous studies also report that histone acetylation marks decreasing and gradually disappearing of H3K9ac at the ZGA stage (Rybouchkin

et al., 2006; Wee et al., 2006). In our study, the same pattern of decreasing of H3K9ac in the 4-cell stage was observed. However, we found significantly enhanced both H3K9ac and H3K14ac levels by SAHA treatment in the blastocyst of porcine SCNT

embryos. This observation is inconsistent with previous studies in porcine SCNT embryos (Jin et al., 2017; Jin et al., 2018; Hou et al., 2014; Liu et al; 2011). Hence, SAHA treatment can enhance histone acetylation by increasing the H3K9ac levels at PN, 8-cell and blastocyst stages and the H3K14ac levels at blastocyst stage in porcine SCNT embryos. *HDAC1-3* can represent the changes in deacetylation, this effect is reversed by deacetylation, which correlates with gene repression in cloned embryos. The porcine IVF embryos at PN and 2-cell stages showed a similar expression level of *HDAC1* with SCNT-treated, but the IVF embryos showed significantly lower the expression level of *HDAC1* than those in the SCNT-treated group (Sun et al., 2018). This result indicated decreasing *HDAC1* expression occur in IVF embryos at ZGA and this observation is consistent with our study. Moreover, the expression level of *HDAC2* in porcine IVF embryos was higher at 2-, 4-cell and blastocyst stages than those in the SCNT-untreated (Liu et al., 2012) and this observation is consistent with our study, increasing *HDAC2* expression occur in IVF embryos at 4-cell and blastocyst stages.

For Reversine and SAHA treatments, when compared with the SCNT-untreated group, it showed significantly downregulated *HDAC1* at blastocyst stage after Reversine treatment and downregulated expression of *HDAC1* at 8-cell and blastocyst stages and *HDAC2* at 2-cell and blastocyst stages after SAHA treatment and low expression of this observation is inconsistent with previous studies in porcine SCNT embryos (Sun et al., 2020; Liu et al., 2011). HDAC1 is more critical than HDAC2 for preimplantation development of mouse embryos (Ma & Schultz, 2016). Interesting, Reversine and SAHA treatment can decrease the expression levels of *HDAC1* at blastocyst stage in preimplantation development of porcine cloned embryos, but *HDAC3* at blastocyst stage of SCNT-Reversine and SAHA treatment groups were similar with those of SCNT-untreated and IVF. Recently, HDAC3 plays a pivotal role within the HDAC family, being essential for embryonic growth and development and its modulates numerous oxidative stress-related processes and molecules, functioning through both its deacetylase and non-enzymatic activities (He et al., 2023). Moreover, HDAC3 and the development of its selective inhibitors still need further explanation in the future. We cannot explain the possibility that *HDAC3* may be contributing to this site-specific hyperacetylation. However, Whitworth et al. (2015) reported that the gene expression of *HDAC1*, *HDAC2* and *HDAC3* were strongly expressed in all blastocyst stage groups (*in*

vivo, *in vitro* fertilization, SCNT-untreated and SCNT-treatment) of porcine embryos. These results indicated blastocyst derived from *in vitro* and *in vivo* of porcine embryo production has same the pattern of *HDAC1-3* expression. In general, these genes were related to the early development of mouse embryos, including PN, 2-, 4- and 8-cell stages (Pan et al., 2005). Hence, the effect of downregulated expression of *HDAC1*, *HDAC2* and *HDAC3* genes on histone acetylation and the full-term development efficiency in porcine SCNT embryos might need further exploration in the future.

The most well-researched of epigenetic mechanism, DNA methylation, occurs on the fifth carbon of cytosine residues in CpG dinucleotides and is necessary for both long-term transcriptional silence and normal mammalian embryo development. Highly methylated somatic cells are used as nuclear donors to produce cloned embryos; the resulting embryos typically have greater levels of DNA methylation, which has been shown to be abnormally hypermethylated (Enright et al., 2003). DNA demethylation occurs during early embryonic development, but in later stages, re-methylation takes place (Reik et al., 2001; Ivanova et al., 2020). DNA methylation reprogramming in early embryos is regulated by DNA methylation and demethylation-related genes (Wu and Zhang, 2014). DNA methyltransferases (DNMTs) are responsible for catalyzing DNA methylation. *DNMT1* is the primary maintenance methyltransferase, while *DNMT3* enzymes are primarily involved in de novo methylation (Wu and Zhang, 2014). Previous investigations have primarily used immunofluorescence quantification, in which methylated DNA is marked with a particular antibody (anti-5mC), to evaluate the dynamics of DNA methylation throughout preimplantation embryonic development (Santos and Dean, 2006). This technique works well for examining the degree of DNA methylation over the whole genome in individual nuclei. Pleat and Reik (2012) have reported that the development of cloned embryos is reduced by incomplete DNA methylation reprogramming mediated by SCNT. The porcine IVF embryos at 4-, 8-cell and blastocysts stages showed a higher level of *DNMT1* with SCNT-untreated than those in the IVF group but inconsistent with the results of Deshmukh et al. (2018) found that showed protein expression of DNMT1 in porcine preimplantation embryos developed in IVF and SCNT. Similarly, the results by Agrawal et al. (2018) found that porcine IVF blastocyst in buffalo cloned embryos had lower expression level of *DNMT1* than those in SCNT-untreated group and consistent with the report of Huan et al. (2014) found that DNA methylation occurs in ZGA stage. From our results found that the expression levels of *DNMT3A* in IVF at PN, 2-, 4- and 8-cell were higher than those in SCNT-untreated and consistent with the results of Huan et al. (2015), found that the

higher expression levels of *DNMT3A* has been reported in IVF at PN, 8-cell and blastocyst stages than those in SCNT-untreated porcine embryos. In our results, the altered DNA methylation (5-mC) occurred in 2-, 4- and 8-cell derived by IVF which were similar within those SCNT-untreated, but in blastocysts was lower than those SCNT-untreated and consistent with the results of Zhang et al. (2018) in porcine cloned

blastocysts. Moreover, the result of related DNA methylation indicates a lack of de novo methylation in porcine IVF blastocysts. For Reversine treatment, the report by Deshmukh et al. (2011) showed that DNA methylation level in IVF one-cell stages was significantly higher than in *in vivo*, PA, and SCNT embryos. Similarly, our results found that Reversine can reduce the *DNMT1* and *DNMT3A* expression in porcine SCNT embryos at PN compared to SCNT-untreated and IVF groups. Surprisingly, Reversine can downregulate the expression of *DNMT1* and *DNMT3A* in SCNT blastocyst stage and close to IVF embryos, consistent with the finding of Deshmukh et al. (2011). The qPCR data suggested that Reversine adversely affected the gene expression related to DNA methylation in blastocyst SCNT embryos. Apart from this, the upregulation of protein expression level of 5-mC induced by Reversine treatment in 2-cell and 4-cell stages, compared to SCNT-untreated embryos, also did not show any effect on decreasing of 5-mC levels in effectively activated after ZGA of SCNT porcine embryos, possibly explaining the cause of incomplete DNA methylation reprogramming and low development of cloned embryos (Huan et al., 2014; Yamanaka et al., 2011). Therefore, enhanced epigenetic reprogramming by regulating global DNA methylation (5-mC levels) could not be preserved till the blastocyst stage but *DNMT1* and *DNMT3A* expression could be preserved till the blastocyst stage by treating 0.1 μM of Reversine for 6 h and close to IVF group, especially *DNMT1* expression. For SAHA treatment, it showed significantly lower levels of *DNMT1* and *DNMT3A* expression were induced by SAHA treatment at PN, 2-cell and blastocyst stages. Similarly, *DNMT1* and *DNMT3A* transcripts were higher in porcine SCNT embryos before the 4-cell stage. The *DNMT1* transcripts were lower, and *DNMT3A* was similar to the SCNT-untreated group at blastocyst stage. The expression level of these genes after ZGA was positively related to the developmental competence of cloned embryos (Huan et al., 2014). In addition, it is well accepted that HDACi could suppress the expression of *DNMT1* and *DNMT3A*, resulting in reduced methylation levels at blastocyst stages of porcine SCNT embryos (Taweechaipaisankul et al., 2019), more similar to those detected in fertilized counterparts and consistent with the results of Whitworth et al. (2015), *DNMT1* was similarly expressed in IVF, SCNT-untreated and SAHA-treatment of porcine blastocyst. In addition, from the 8-cell to the blastocyst stages, normalized DNA methylation

levels continuously increased in porcine SCNT embryos (Fulka et al., 2006). In contrast, the result revealed that treatment with SAHA reduced global 5-mC levels in 2-cell and blastocysts stages when compared to the SCNT-untreated group (P<0.05) and did not affect in 4- and 8-cell stages. Consistent with these findings (Hou et al., 2014; Taweechaipaisankul et al., 2019), the 5-mC levels in SCNT embryos at the 4-cell stage were similar to the control group by treatment with HDACi. It appears that the developmental competence of SCNT embryos can be improved by treatment with 0.1 μM of SAHA during activation and IVC for 12 h, which positively facilitates DNA methylation after ZGA, especially blastocyst stage and close to IVF group.

Histone H3 methylation at lysine 9 (H3K9me) has been related to the formation of heterochromatin in the nucleus and transcriptional repression (Fischle et al., 2003). Porcine SCNT embryos also showed abnormal expression levels of H3K9 and H3K4 methylation (Cao et al., 2015). The down-regulation of H3K9me3 could greatly improve transcriptional reprogramming in mouse, pig and cattle SCNT embryos (Matoba et al., 2014; Liu et al., 2016; Liu et al., 2018). Cao et al. (2015) have reported that during development of IVF and SCNT pig embryos, dynamic patterns of H3K9me3 signal intensity had no remarkable difference between IVF and SCNT PN, 2-cell, 8-cell and blastocyst stages embryos, but at the 4-cell stage around the time of embryonic genome activation was apparently higher than that in IVF counterparts (P < 0.05). These data suggest that there was no significant difference between SCNT and IVF embryos in the intensity of epigenetic modifications during developmental stages, except ZGA in IVF embryos. The high levels of H3K9me3 were detected in PN-stage of porcine SCNT and IVF embryos (Cao 2015 et al., 2015). Interesting, in the present study, H3K9me3 was decreased at PN stages of SCNT embryos by Reversine and SAHA treatment when compared to those SCNT-untreated and IVF embryos. However, H3K9me3 induced by SAHA treatment was similar levels during development stages compared to the SCNT-untreated group. The treatment of reconstructed embryos with SAHA was shown not to promote the completely reprogrammed in SCNT embryos underlying regulation of histone methylation. Additionally, the signal intensity of H3K9me3 had strongly expressed in 8-cell when compared with the 8-cell stage IVF and SCNT-untreated embryos (Liu et al., 2018). The weak signal intensity of H3K9me3 could still be observed in SCNT blastocysts treated with Reversine when compared with the SCNT blastocysts untreated and close to fertilized embryos (P<0.05). These results are consistent with the data from cloned pig embryos reported by Zhang et al. (2018). Suggesting of these results that epigenetic modifications during development

may differ according to small molecules that are used for treatment. Our results indicated that Reversine could greatly improve the quality of blastocysts and porcine SCNT embryos efficiency through reducing histone methylation but SAHA treatment has not been shown.

In addition, we found that Reversine improved development and pluripotency and reduced deacetylation (*HDAC1*) and DNA methylation (*DNMT1* and *DNMT3A*) related genes and regulated H3K9me3 in porcine SCNT blastocysts. SAHA downregulated deacetylation (*HDAC1* and *HDAC2*) and DNA methylation (*DNMT1* and *DNMT3A*) related genes and regulated H3K9ac, H3K14 and global DNA methylation in porcine SCNT blastocysts. These findings suggest that the optimal condition of combination of Reversine and SAHA may which improve development and regulate epigenetic reprogramming of porcine SCNT embryos, enhancing the birth rate of piglets.

5.2 Conclusions

Reversine treatment under optimal conditions (1 μM for 6 h) did not affect the subsequent development of porcine SCNT embryos. However, this improvement appears to be closely related to the enhanced expression of genes related to development including *OCT4* (at 4-, 8-cell and blastocyst stages), *SOX2* (PN, 2-, 8-cell and blastocyst stages) and *NANOG* (at 2-, 4-, 8-cell and blastocyst stages). The repressed expression of genes related to histone acetylation, *HDAC1* (at blastocyst stage) and DNA methylation, *DNMT1* and *DNMT3A* (at PN, 2-cell and blastocyst stages). Treatment with Reversine showed the positive status of global histone activation mark H3K9ac (at 4- and 8-cell stages), but the negative status of H3K9me3 (at PN and blastocyst stages) and 5-mC (at PN stage) was found.

Treatment of SCNT embryos with the optimal conditions of SAHA (1 μM for 12 h) could improve the developmental competence of porcine SCNT embryos and related gene expression patterns of development including *OCT4* (at 4- and 8-cell stages), *SOX2* (2- and 8-cell stages) and *NANOG* (at 4-, 8-cell and blastocyst stages). This improvement seems to be associated with the positive regulation of epigenetic modification status, as shown by reduced dynamics of DNA methylation as 5-mC levels (at 2-cell and blastocyst stages). Treatment with SAHA significantly downregulated the expression levels of *DNMT1* gene (at PN, 2-, 8-cell and blastocyst stages) and *DNMT3A* gene (PN and blastocyst stages). It also enhanced embryonic acetylation, H3K9ac (at PN, 8-cell and blastocyst stages) and H3K14ac (at blastocyst stage) and significantly

decreased the expression levels of *HDAC1* (at 8-cell and blastocyst stages), *HDAC2* (at 2-cell and blastocyst stages) and *HDAC3* (at PN stage).

Moreover, Reversine improved the expression of pluripotency, decreased the expression of DNA methylation and reduced the H3K9me3 levels in SCNT blastocysts. In addition, the expression of DNA methylation, histone deacetylation and the 5-mC levels were decreased and the H3K9ac and H3K14ac levels were enhanced in SCNT blastocyst by SAHA. Interesting, in the further experiments, the combination treatment of Reversine and SAHA under optimal conditions should be determined the affect development and regulate epigenetic reprogramming in porcine cloned embryos. Therefore, the use of Reversine and SAHA in SCNT embryos resulted in improvement in gene and protein expression at various stages of pluripotency and epigenetic reprogramming markers when compared with untreated SCNT embryos and closely with IVF embryos. Reversine and SAHA treatments in cloned embryos should be examined for full-term development. Since several reports have previously shown low numbers of porcine cloned offspring born after embryo transfer.

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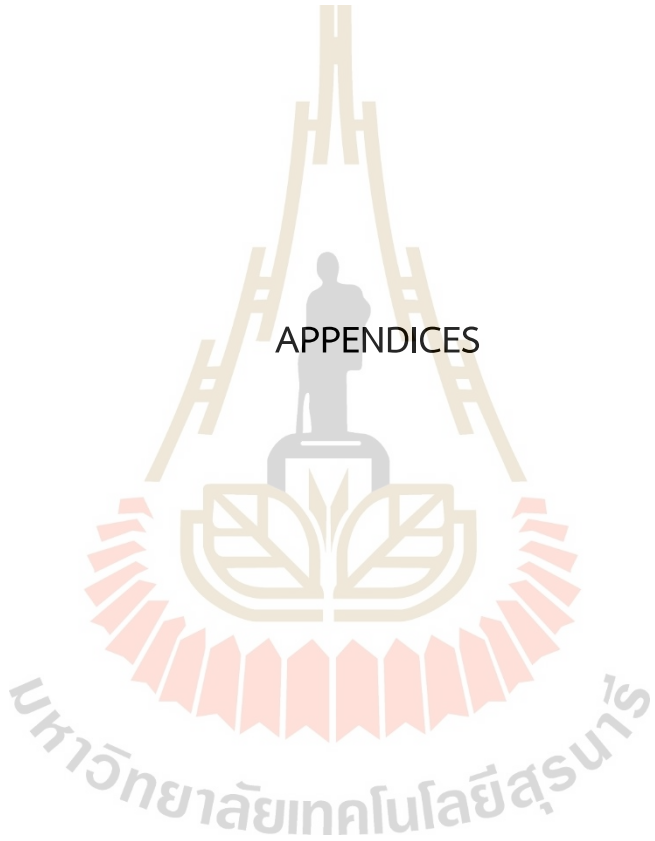
APPENDICES



APPENDIX A

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

Components of reactions

Component	Volume	Final concentration
dNTP Mix (10 mM each)	2 µl	1 mM (each dNTP)
RNase Inhibitor, 40 U/ µl	0.5 µl	1U/ 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	10U/ 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

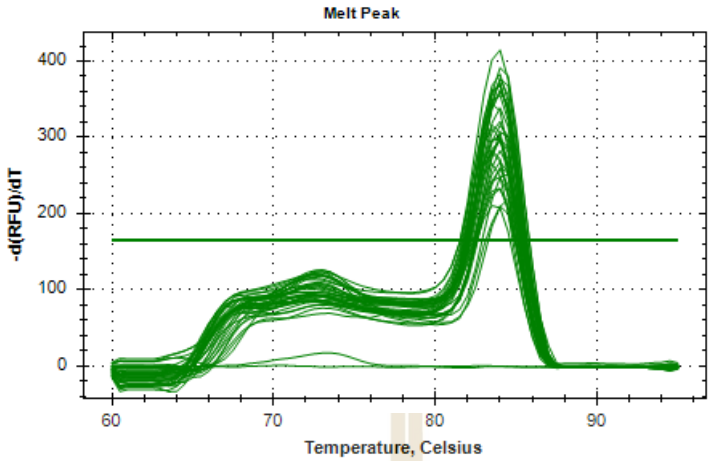


Figure S1 Representative image of *GAPDH* melting curve

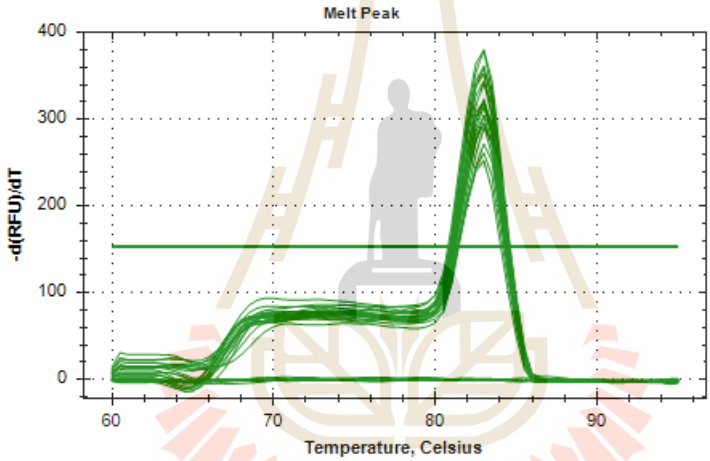


Figure S2 Representative image of *HDAC1* melting curve

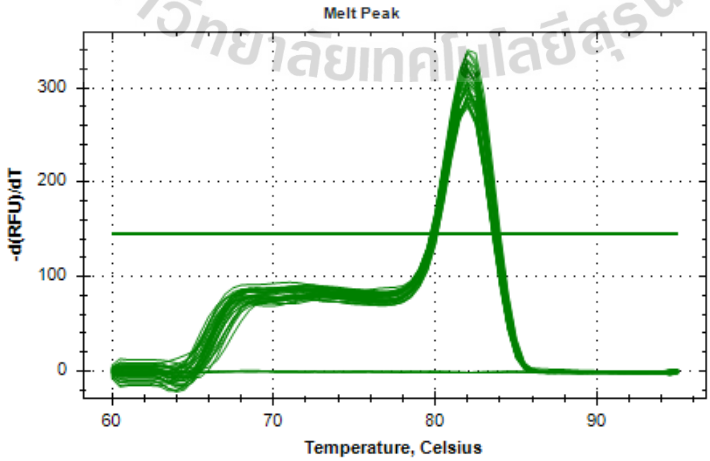


Figure S3 Representative image of *HDAC2* melting curve

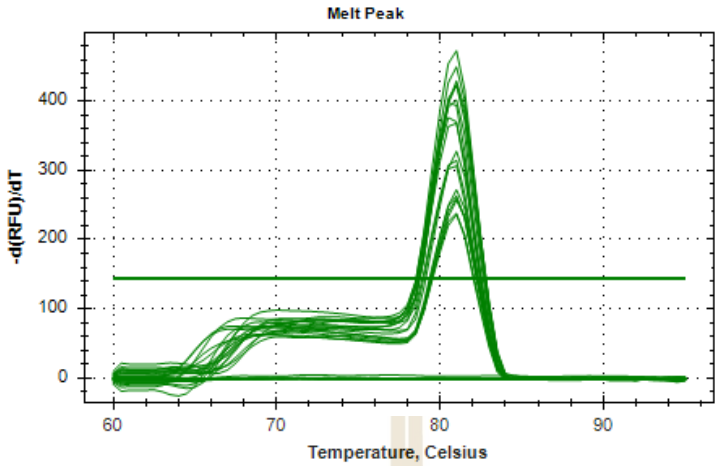


Figure S4 Representative image of *HDAC3* melting curve

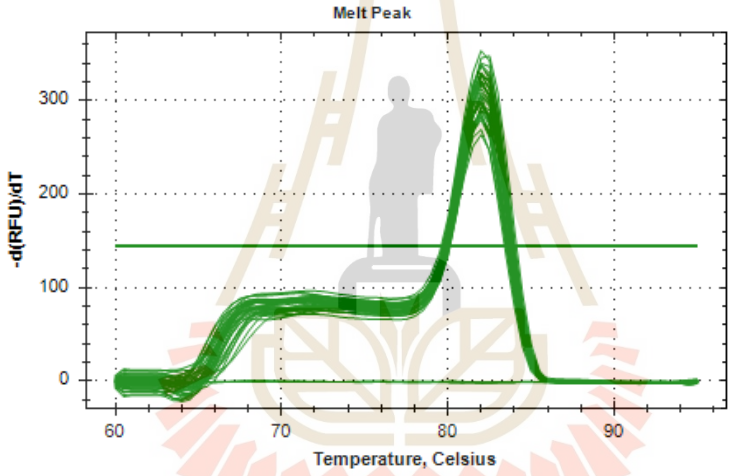


Figure S5 Representative image of *DNMT1* melting curve

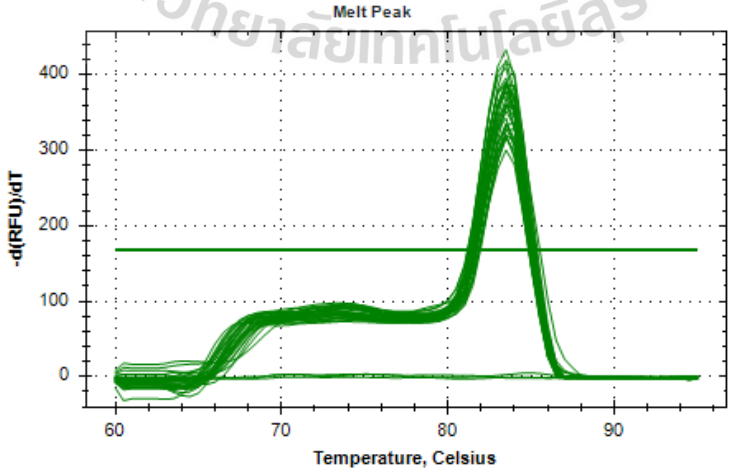


Figure S6 Representative image of *DNMT3A* melting curve

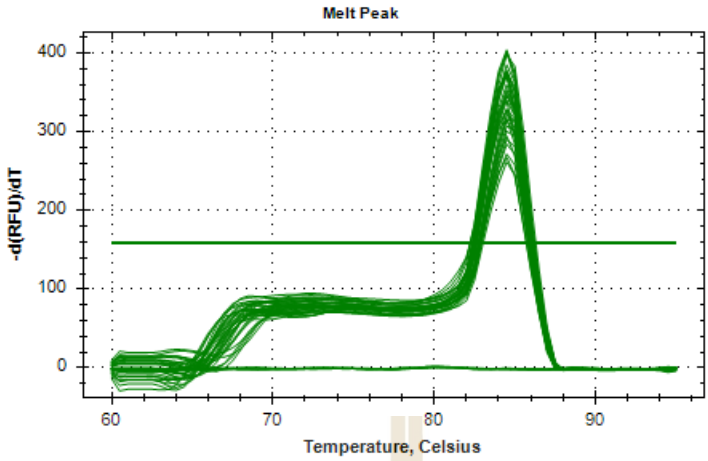


Figure S7 Representative image of *OCT4* melting curve

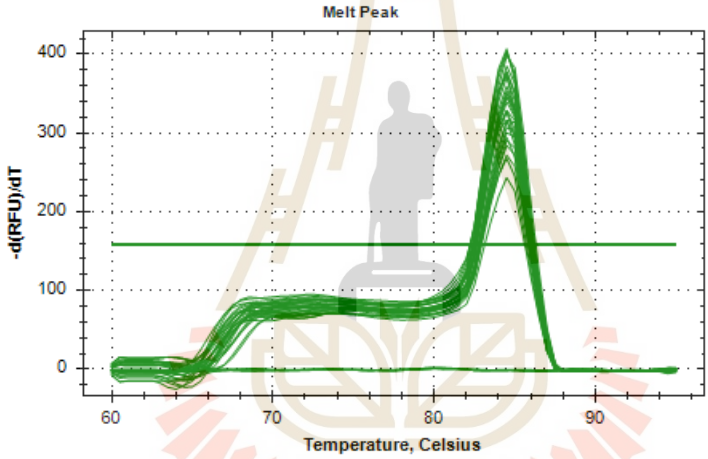


Figure S8 Representative image of *SOX2* melting curve

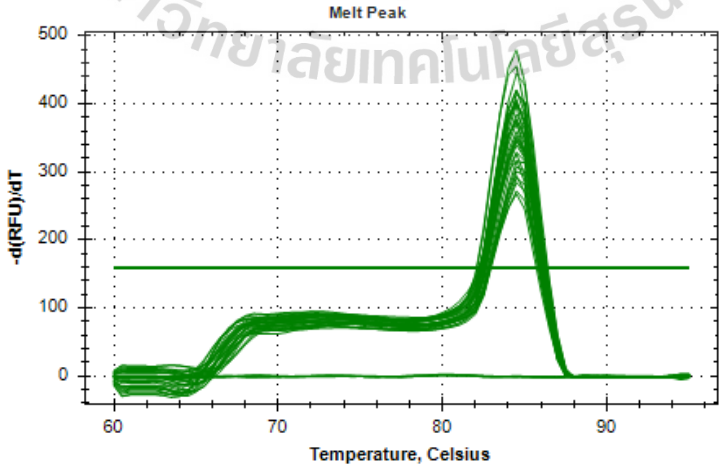


Figure S9 Representative image of *NANOG* melting curve

BIOGRAPHY

Miss Phattarawadee Noita was born in Chiang Rai, Thailand on April 8th, 1999. She finished her high school from Sacred Heart College in Chiang Mai. In 2021, She graduated with a Bachelor of Science in Biotechnology and the research topic is "Antibacterial activity of mucus protein extract from pearl oysters (*Pteria penguin*) and its hydrolysates" from Mae Fah Luang University, Chiang Rai, Thailand. In November 2021, she started studying master degree in Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. She received One research One graduate (OROG) scholarship from Suranaree University of Technology, under supervision of Assoc. Prof. Dr. Rangsun Parnpai The research topic is "Effects of Reversine and SAHA on development and epigenetic reprogramming of cloned porcine Embryos" The results from some parts of this study have been presented as a poster presentation at the 35th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2023) during 26-29 November, 2023.