CHAPTER III
MATERIALS AND METHODS

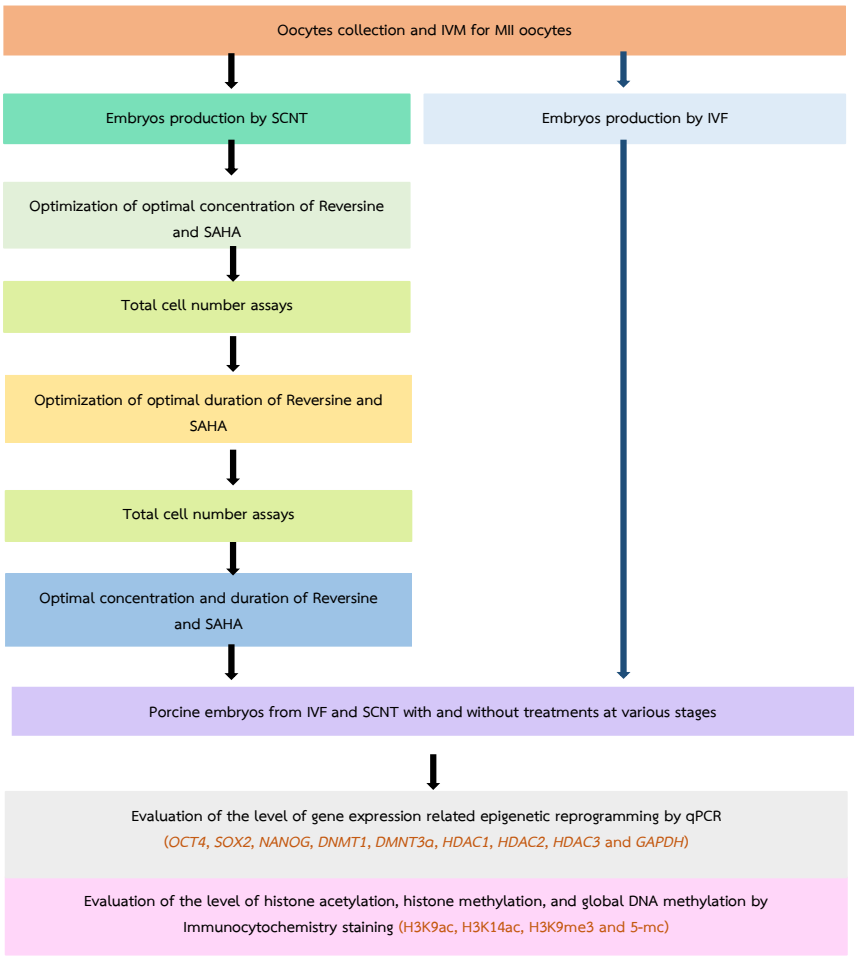
3.1 Chemicals

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).

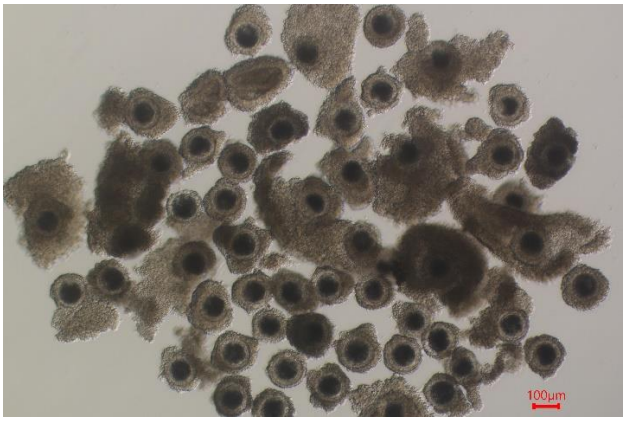


Figure 3.1 Representative image of cumulus-oocyte complexes (COCs) with uniform oocyte cytoplasm and compact surrounding cumulus cells (CCs), scale bar = 100 µm

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).

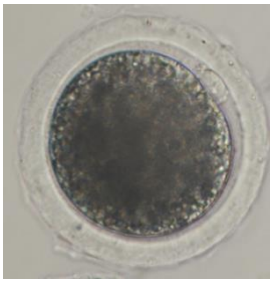


Figure 3.2 Representative image of metaphase II (MII) oocyte with evenly granular cytoplasm and a visible first polar body

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	207	NM_001206359
	R: TTGACGAAGTGGTCGTTGAG		
POU5F1/OCT4	F: AGCGCTTCAGAAAGATCTCG	335	NM_001113060
	R: ACTGCAGGAACATGCTCTC		
SOX2	F: TGCACAACTCGGAGATCAG	323	NM_001123197
	R: CATGCTGTAGCTGCAGTTG		
NANOG	F: CAGAGAAGAGCACAGACAAG	293	NM_001129971
	R: TAGAAGCCCGGGTATTCTG		
DNMT1	F: TCGAACCAAAACGGCAGTAC	215	NM_001032355
\	R: CGGTCAGTTTGTGTTGGACA		
DNMT3A	F: CTGAGAAGCCCAAGGTCAAG	200	NM_001097437
	R: GTACTGATACGCGCACTCCA		
HDAC1	F: TATCGTCTTGGCCATCCTG	328	XM_013999116
	R: GTCAGAGCCACACTGTAAG		
HDAC2	F: TTTACGCATGTTGCCTCA	209	XM_001925318
	R: TGATCAGCCACATTTCTACG		
HDAC3	F: ATGCAAGGCTTCACCAAGAG	310	NM_001243827
	R: TGAGGTAGAAGGCCTCCTG		

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.