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.

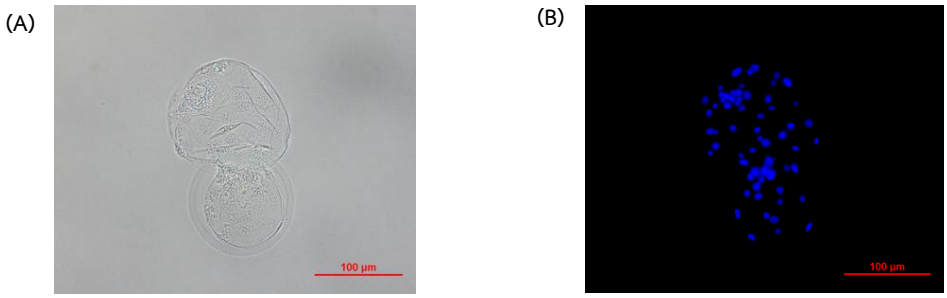

Figure 4.1 **(A)** Representative images of embryos at blastocyst stage **(B)** Representative images of nuclei staining of blastocysts, scale bar = 100 µm.

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 ($P<0.05$).

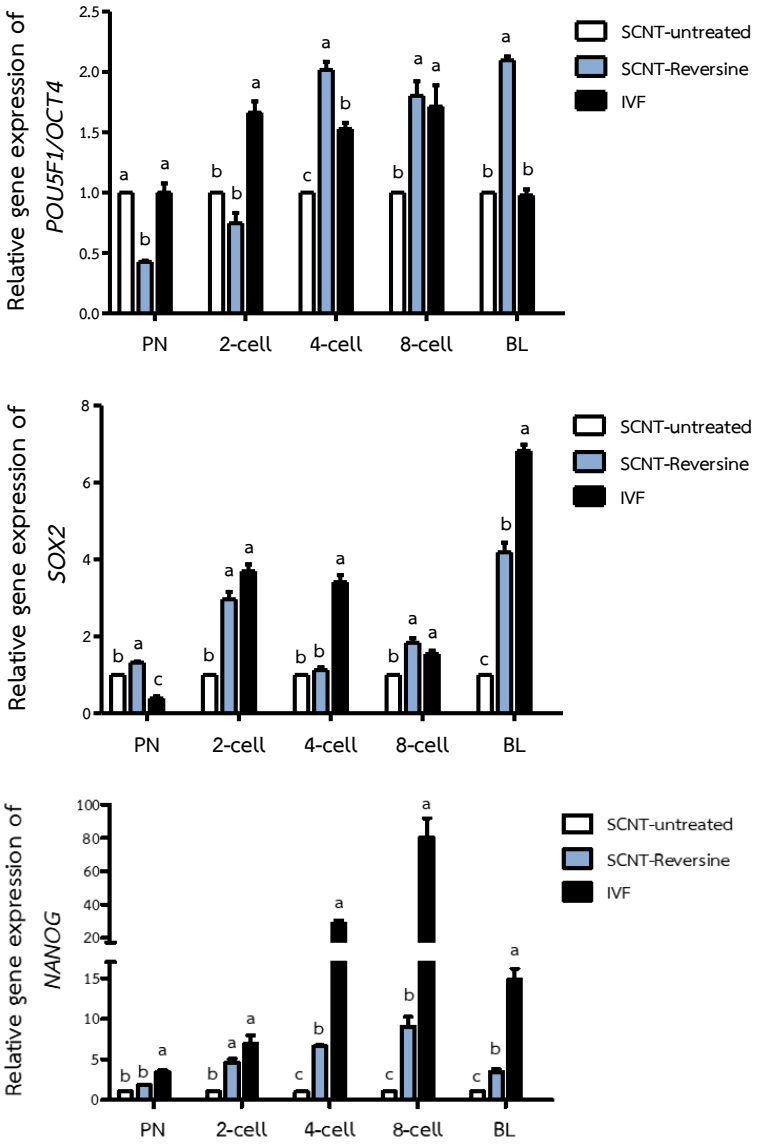

Figure 4.2 Comparison of mRNA expression levels (mean ± SEM) of genes related to developmental competence (*POU5F1/OCT4*, *SOX2* and *NANOG*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment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

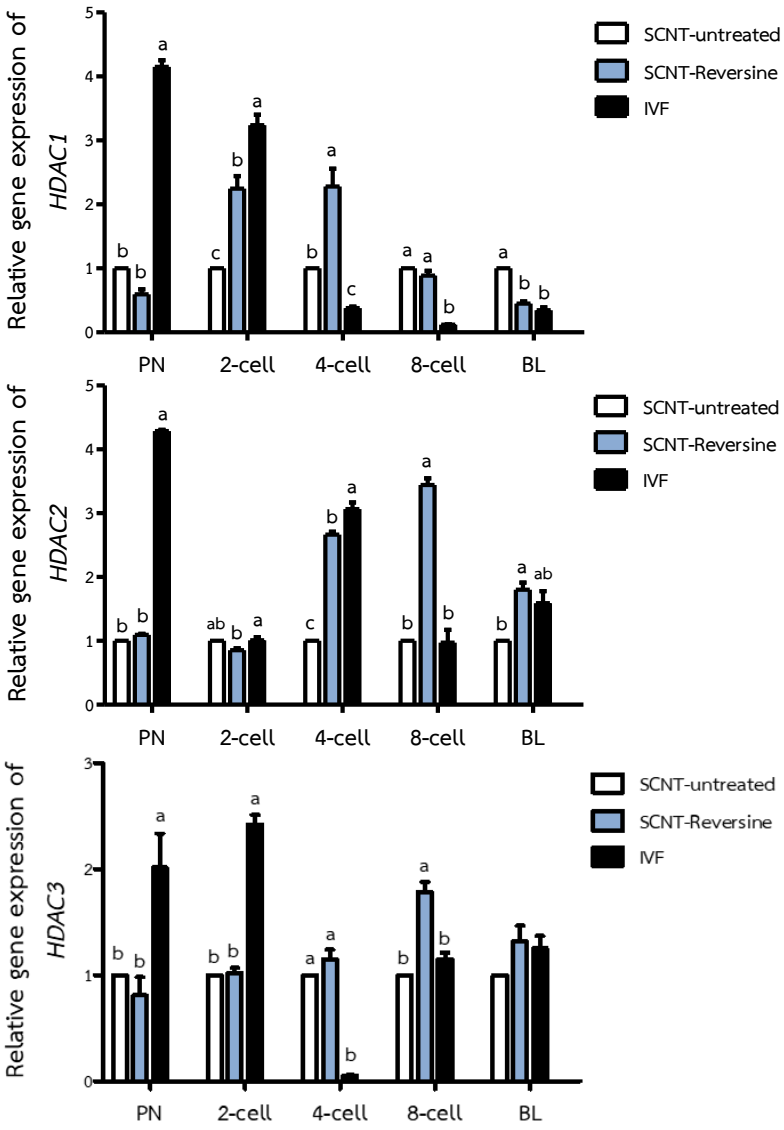

Figure 4.3 Comparison of mRNA expression levels (mean ± SEM) of genes related to Histone acetylation (*HDAC1*, *HDAC2* and *HDAC3*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages.The independent experiment was carried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

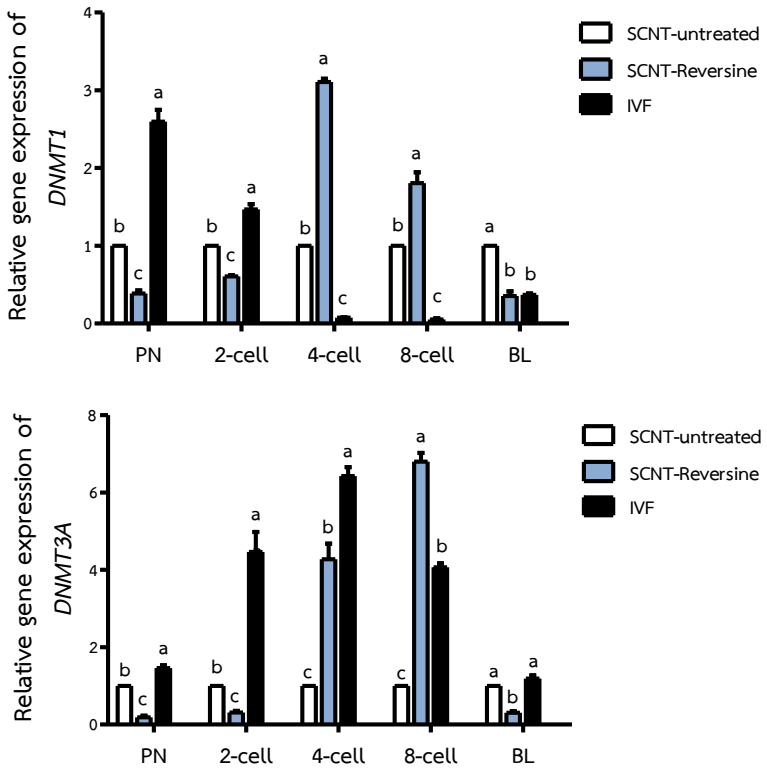

Figure 4.4 Comparison of mRNA expression levels (mean ± SEM) of genes related to DNA methylation (*DNMT1* and *DNMT3A*) between the SCNT-Reversine (1 μM for 6 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for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

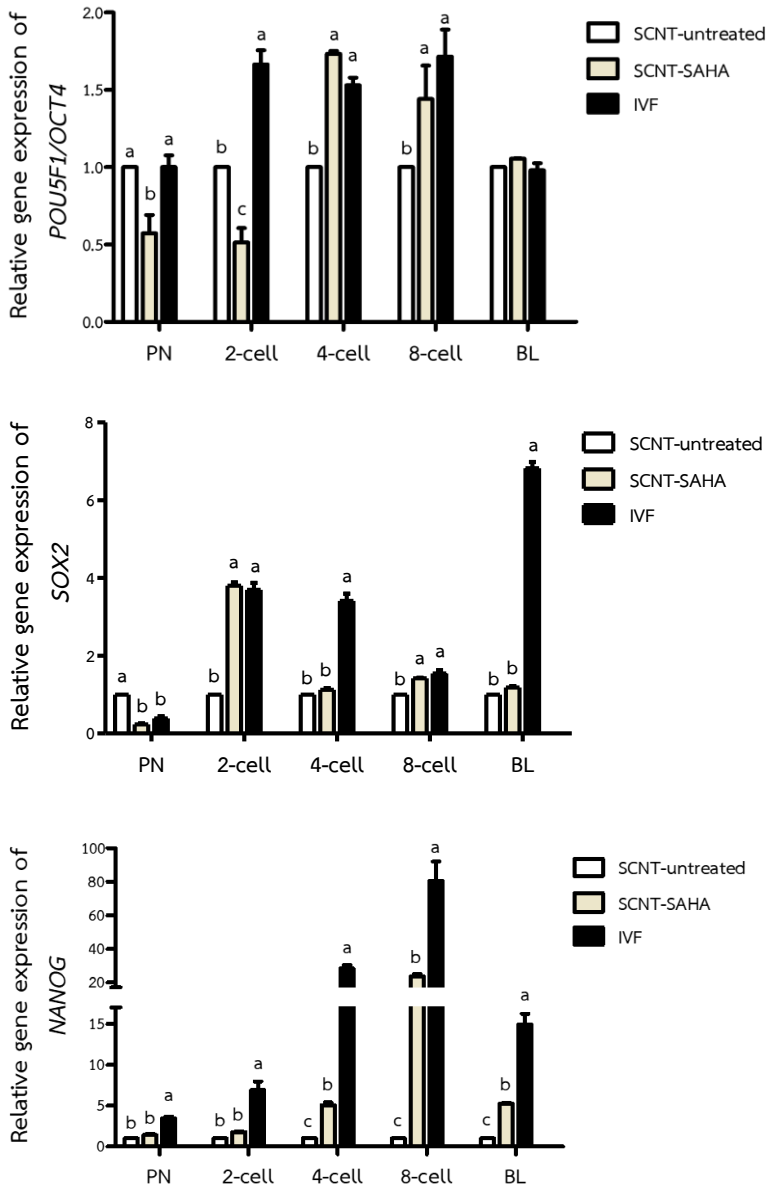

Figure 4.5 Comparison of mRNA expression levels (mean ± SEM) of genes related to developmental competence (*POU5F1/OCT4*, *SOX2* and *NANOG*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment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

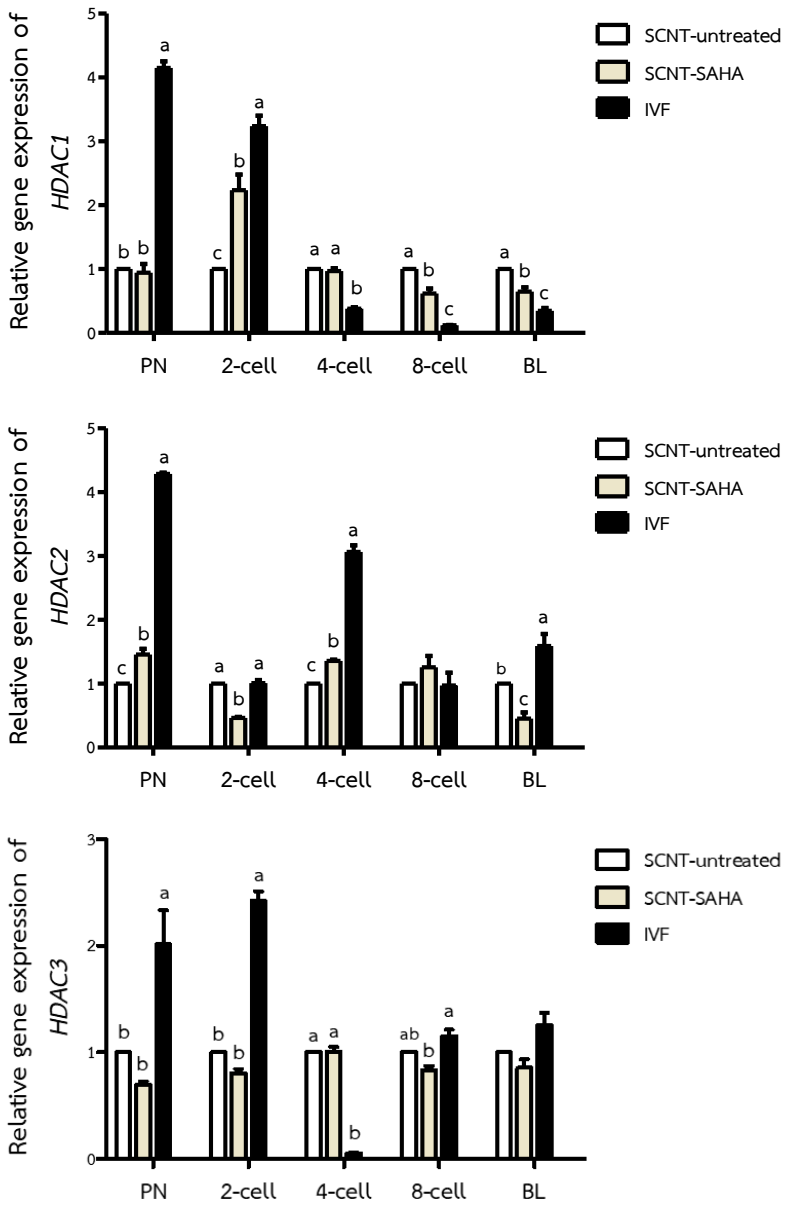

Figure 4.6 Comparison of mRNA expression levels (mean ± SEM) of genes related to histone acetylation (*HDAC1*, *HDAC2* and *HDAC3*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried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

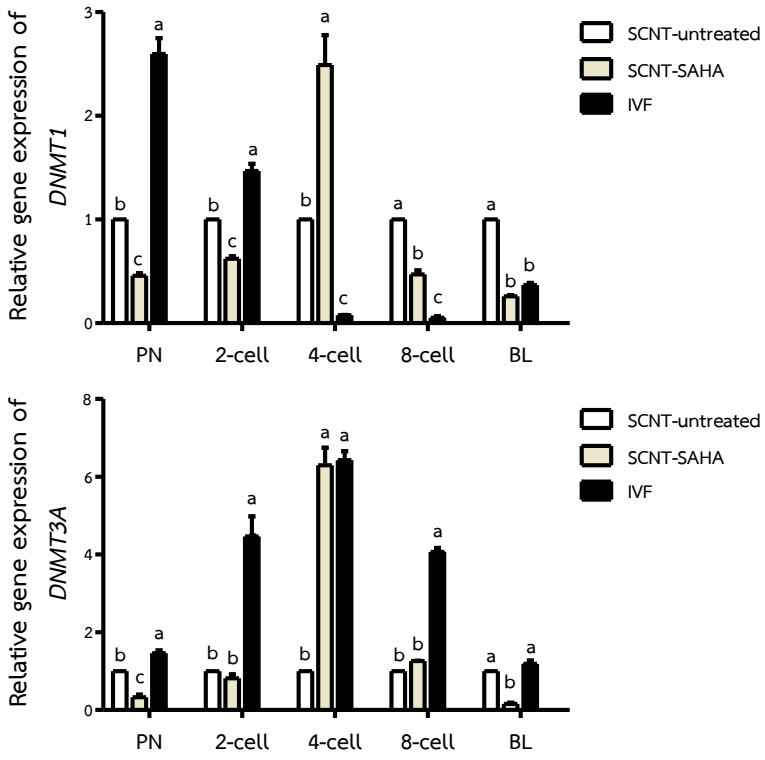

Figure 4.7 Comparison of mRNA expression levels (mean ± SEM) of genes related to DNA methylation (*DNMT1* and *DNMT3A*) between the SCNT-SAHA (1 μM for 12 h), SCNT-untreated and IVF groups at pronuclear (PN), 2-, 4-, 8-cell and blastocyst (BL) stages. The independent experiment was carried out for three 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 ($P<0.05$).

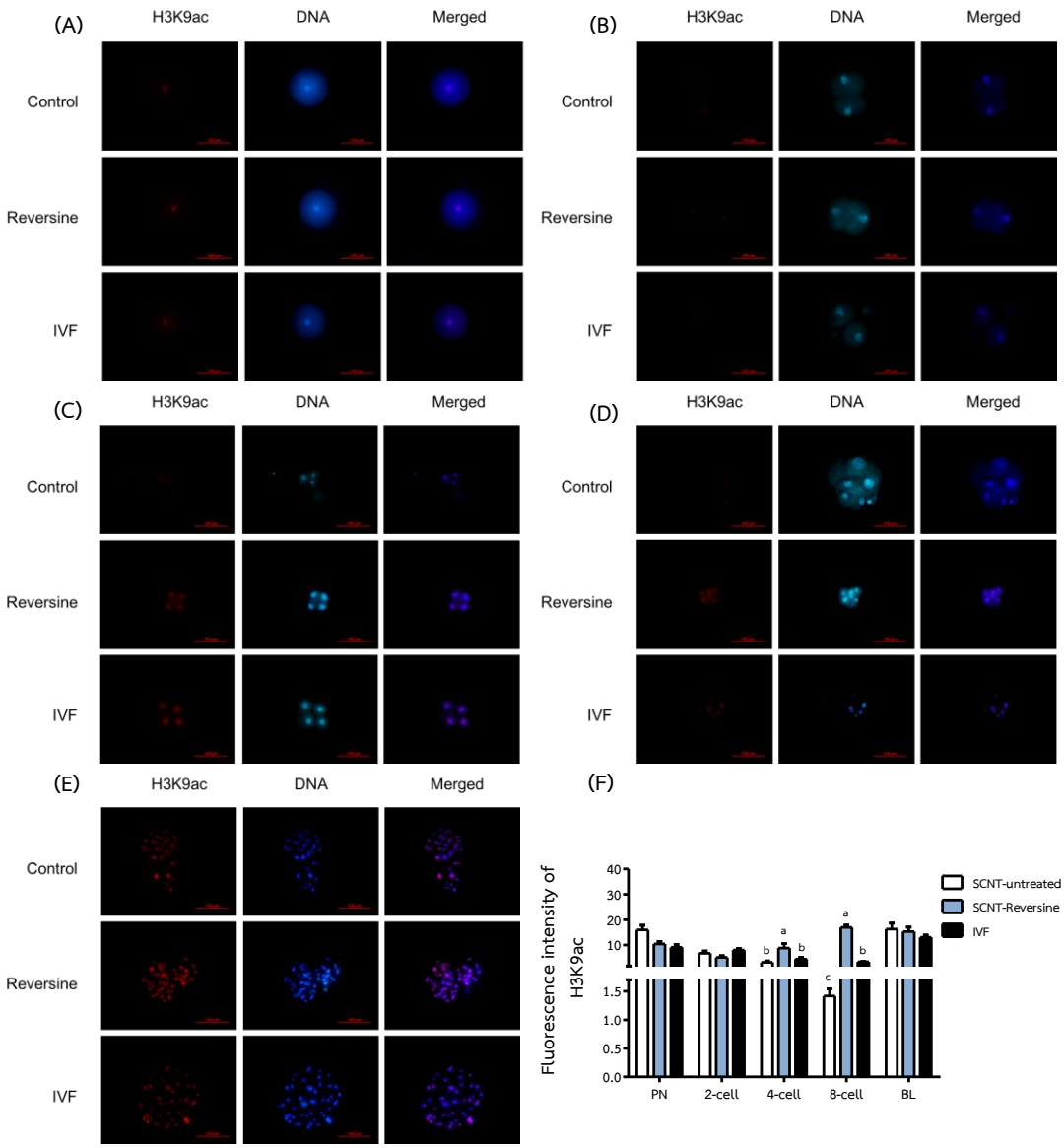

Figure 4.8 Effect of Reversine on the histone acetylation level (H3K9ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K9ac level at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescent intensity 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).

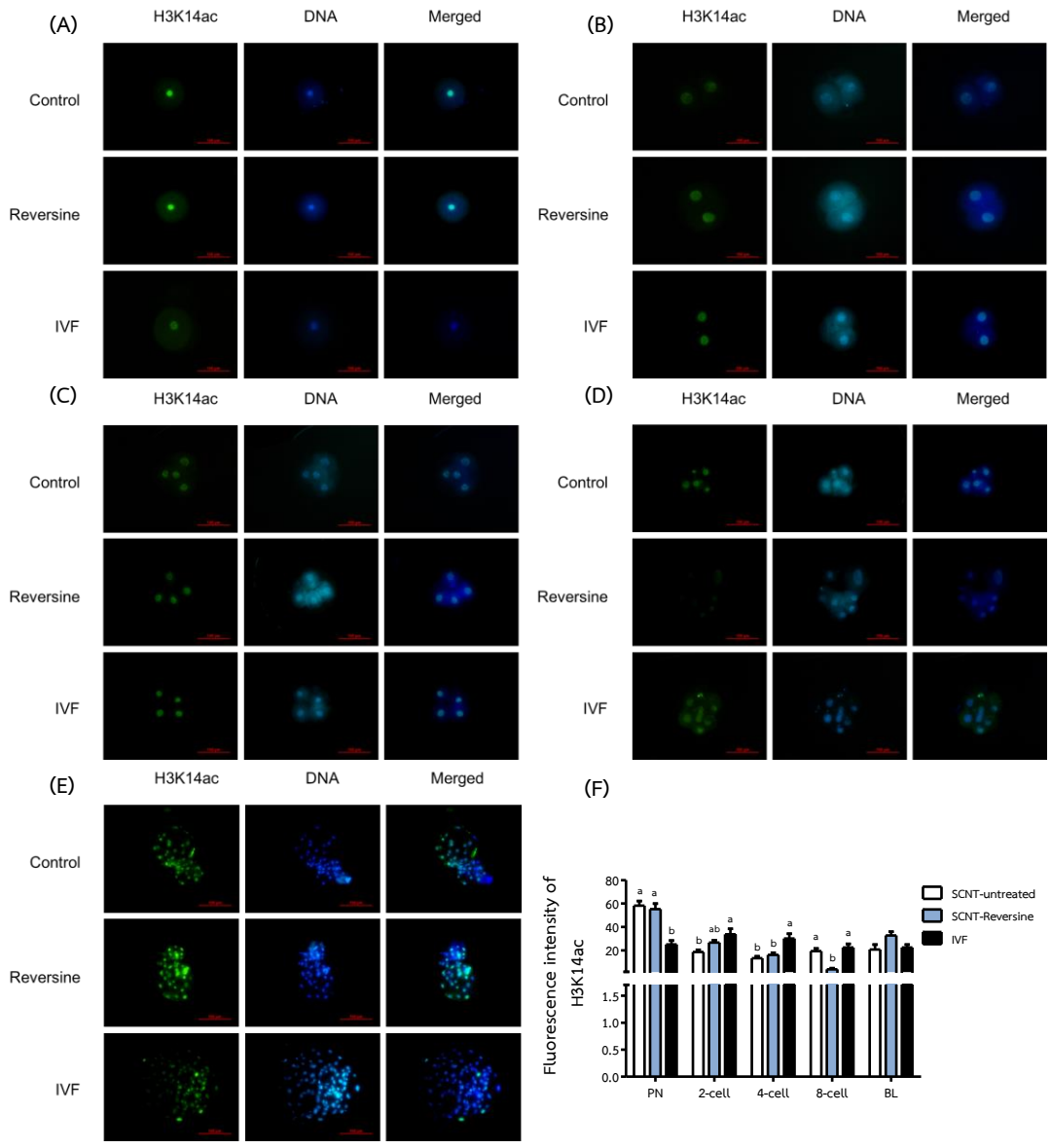

Figure 4.9 Effect of Reversine on the histone acetylation level (H3K14ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).

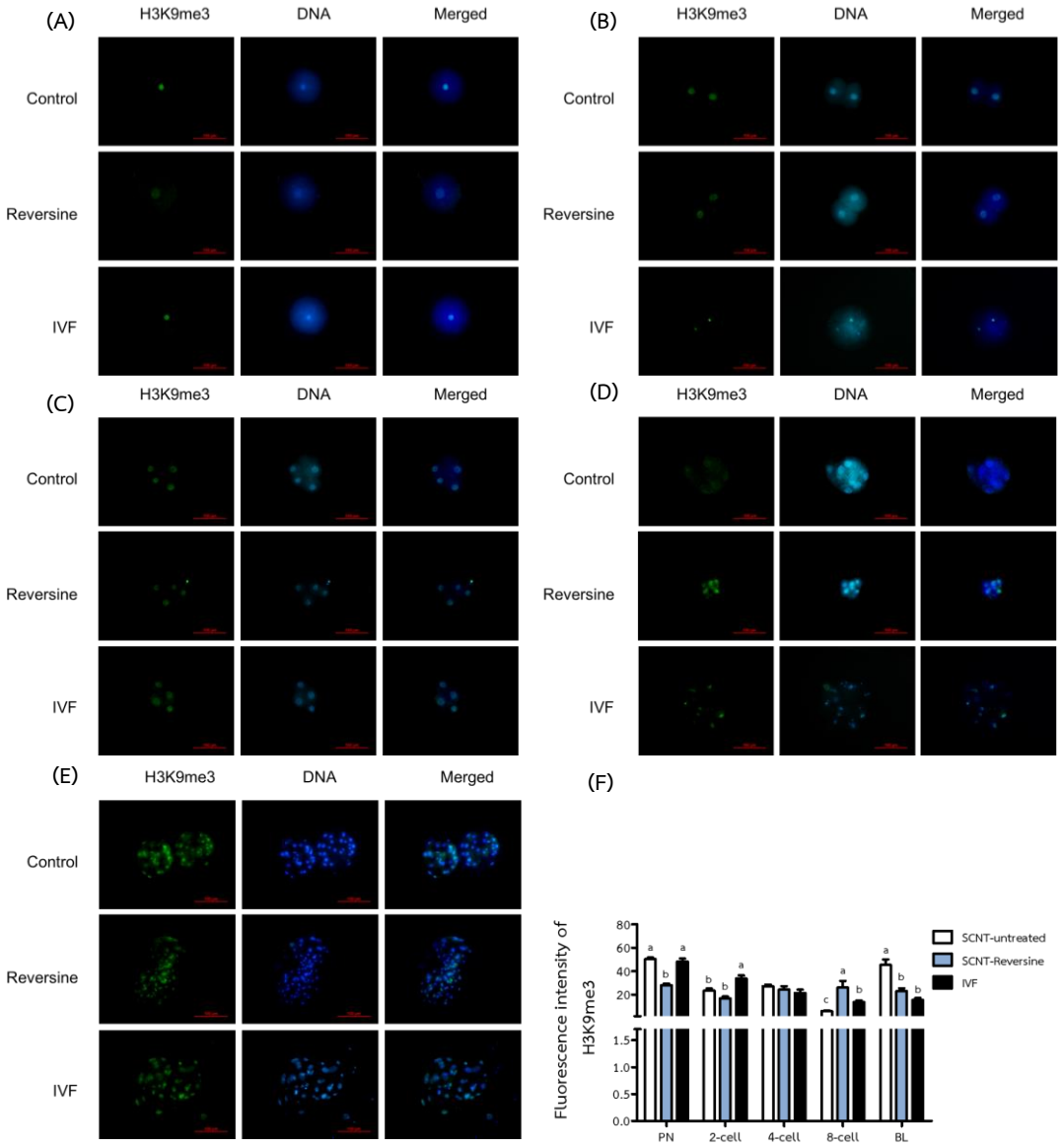

Figure 4.10 Effect of Reversine on the histone acetylation level (H3K9me3) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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 ($P<0.05$).

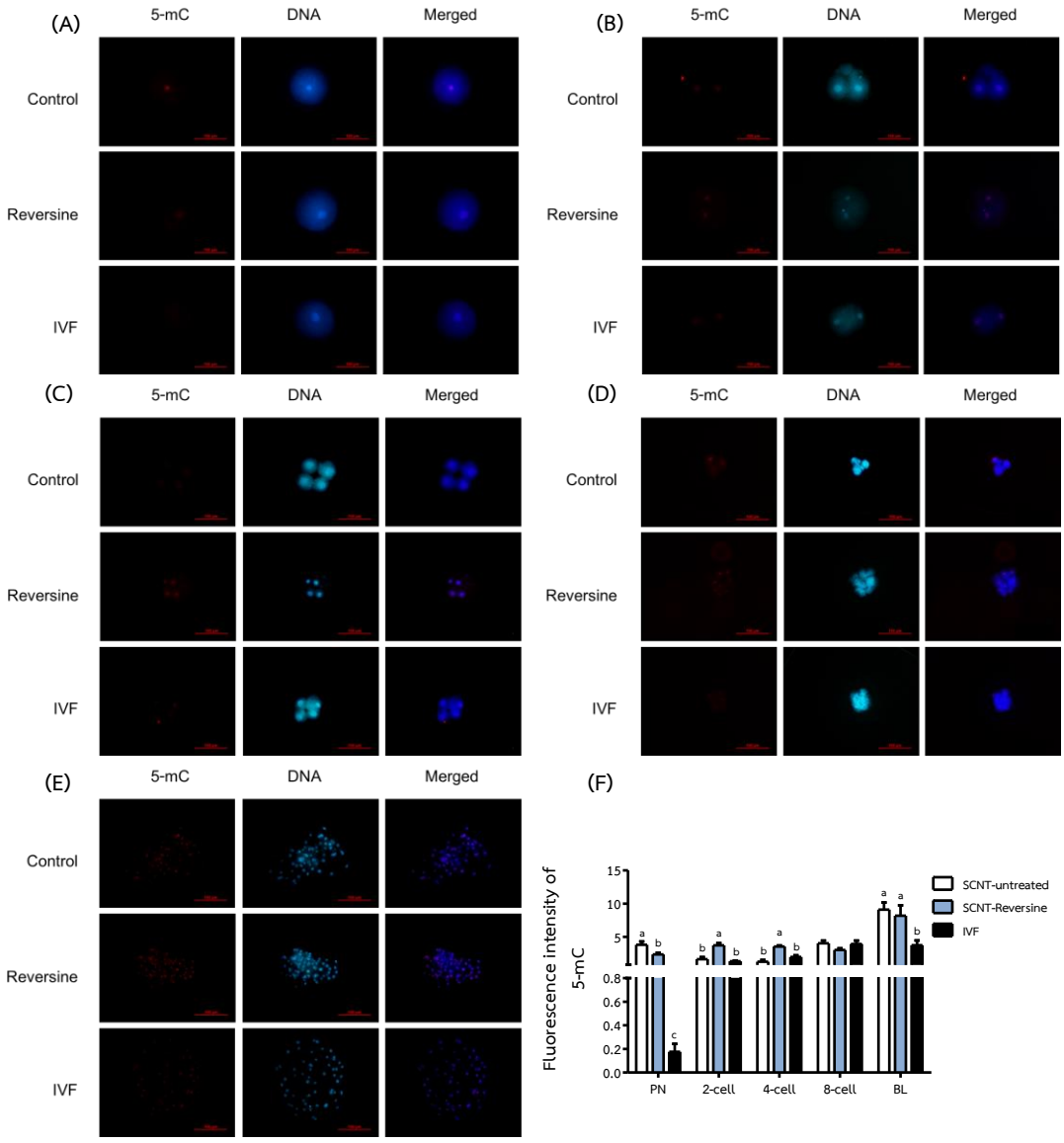

Figure 4.11 Effect of Reversine on the histone acetylation level (5-mC) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).

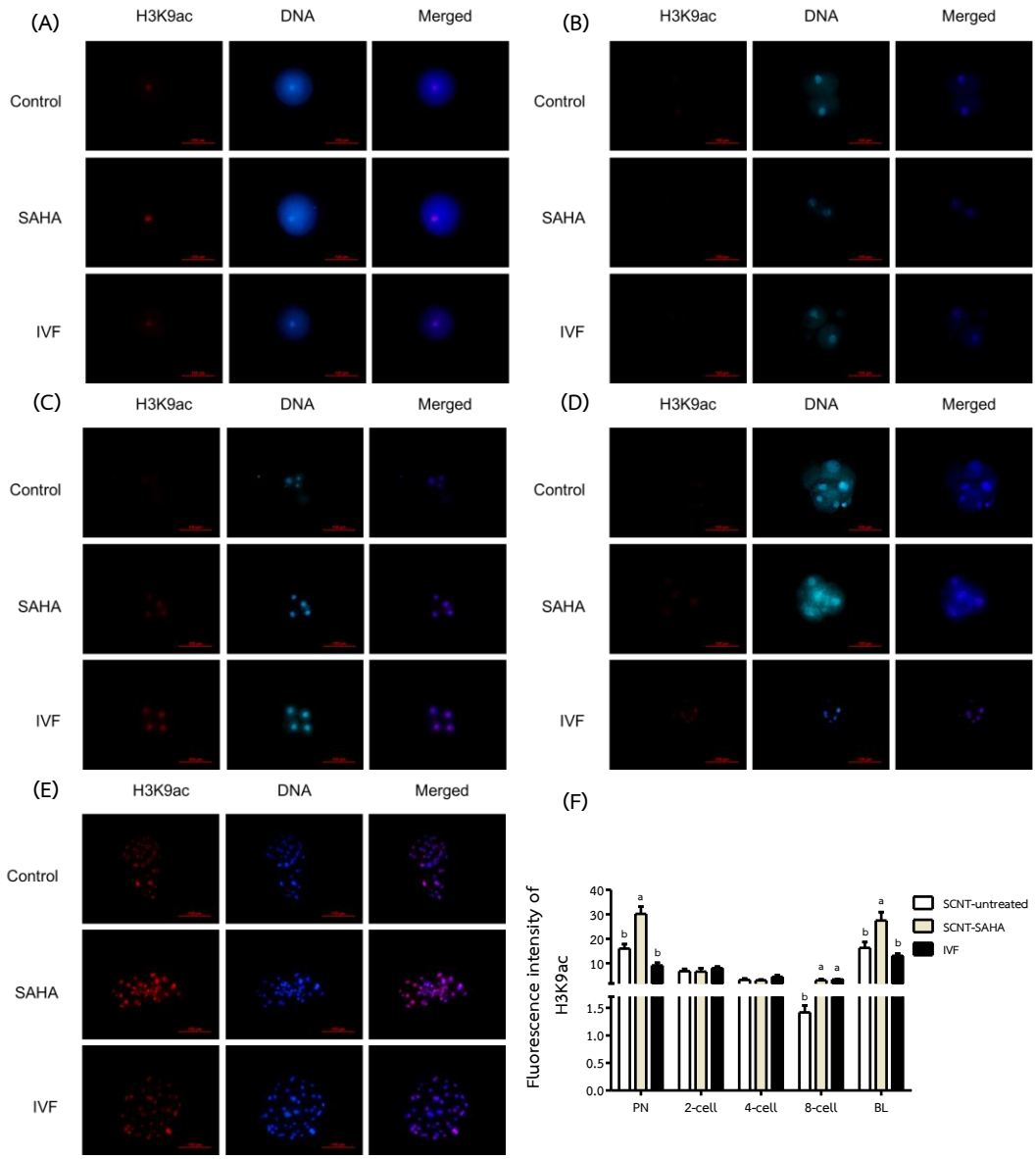

Figure 4.12 Effect of SAHA on the histone acetylation level (H3K9ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K9ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).

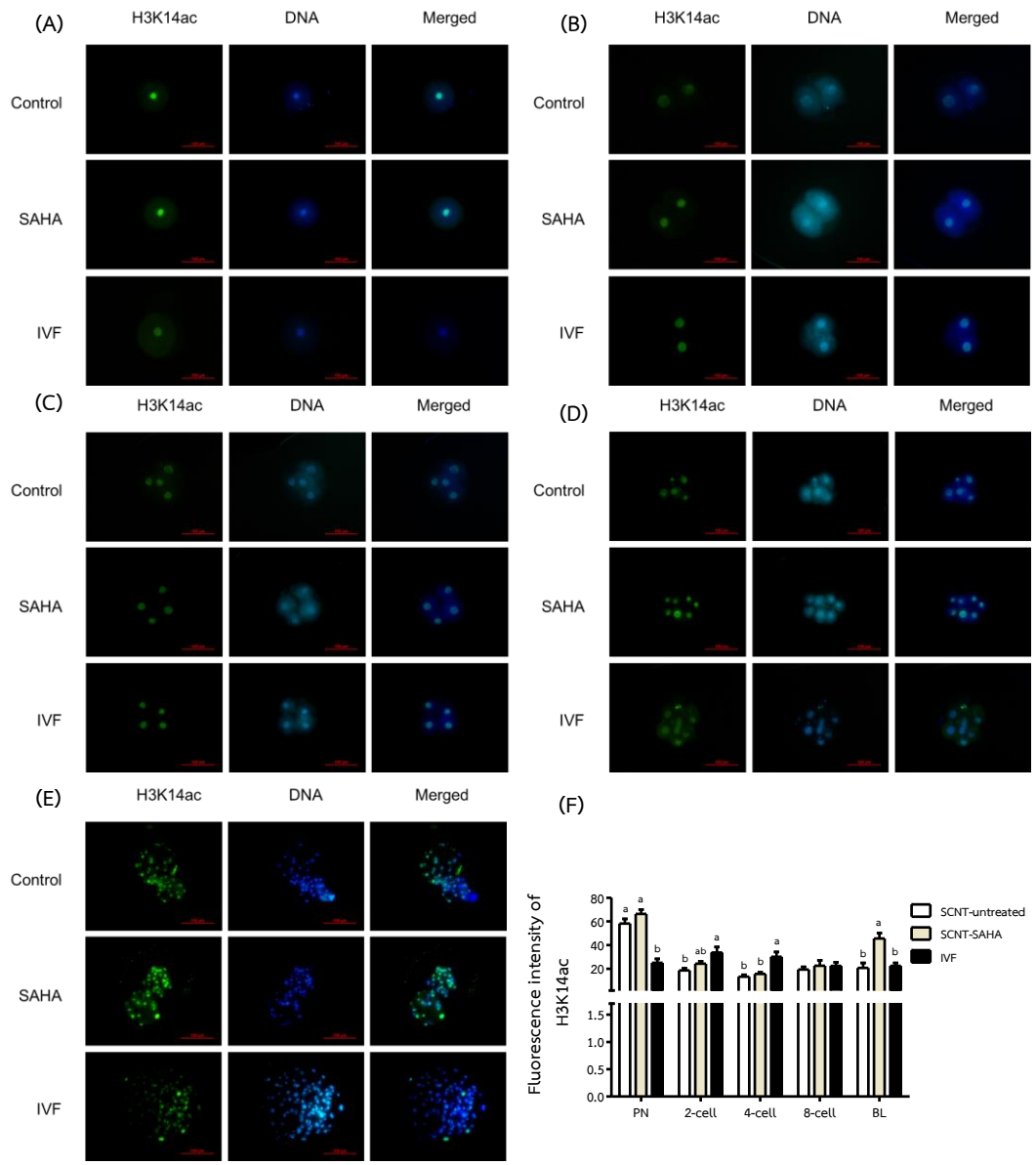

Figure 4.13 Effect of SAHA on the histone acetylation level (H3K14ac) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).

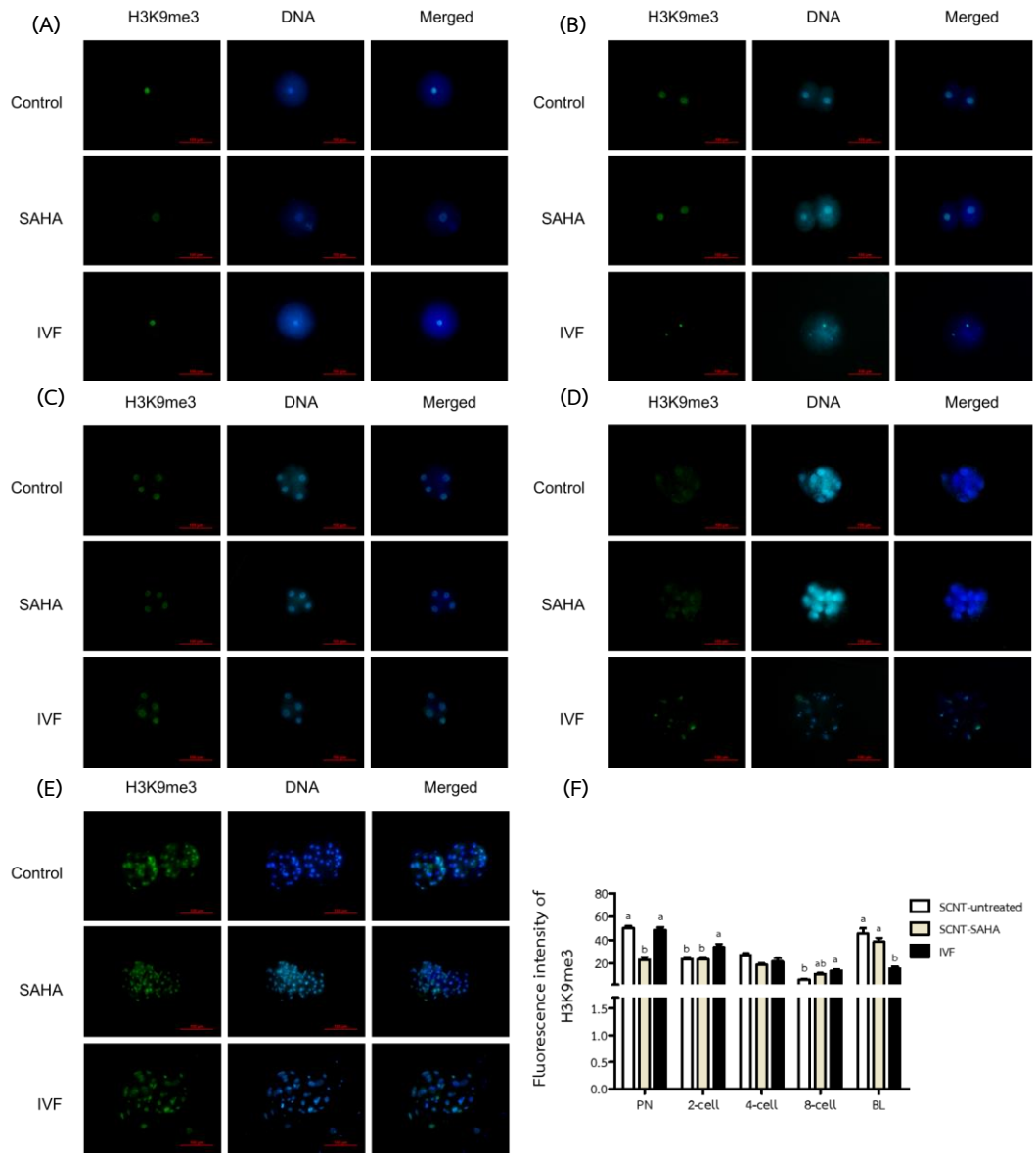

Figure 4.14 Effect of SAHA on the histone methylation level (H3K9me3) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).

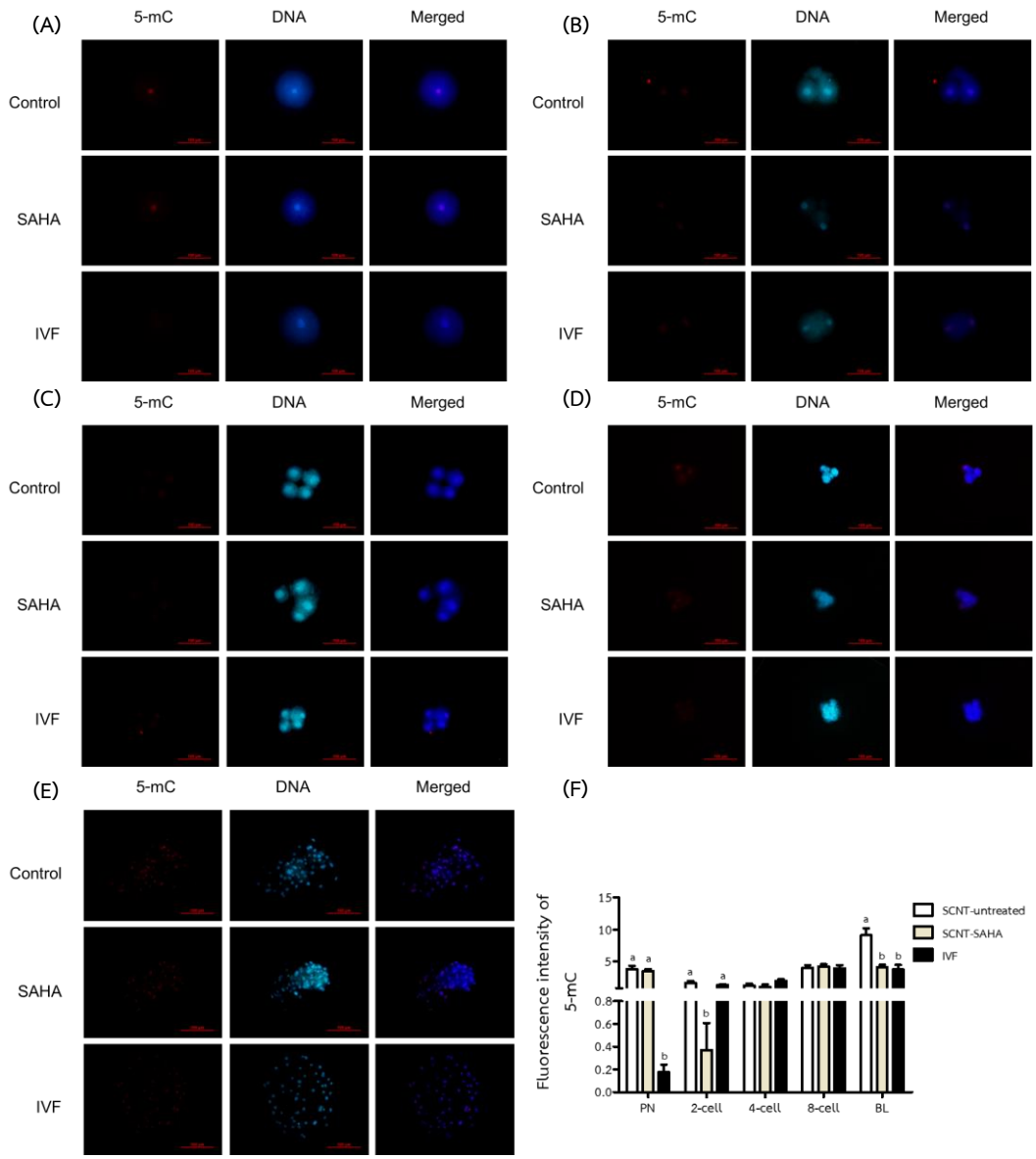

Figure 4.15 Effect of SAHA on the histone acetylation level (5-mC) of porcine SCNT embryos. Representative immunofluorescence images of H3K14ac levels at (A) PN, (B) 2-cell, (C) 4-cell, (D) 8-cell and (E) blastocyst stages in porcine SCNT and IVF embryos. (F) Quantification of fluorescence intensity 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).