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.