

Epigenetic Modification Agents Improve Genomic Methylation Reprogramming in Porcine Cloned Embryos

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Abstract. Incomplete DNA methylation reprogramming in cloned embryos leads to low cloning efficiency. Our previous studies showed that the epigenetic modification agents 5-aza-2'-deoxycytidine (5-aza-dC) or trichostatin A (TSA) could enhance the developmental competence of porcine cloned embryos. Here, we investigated genomic methylation dynamics and specific gene expression levels during early embryonic development in pigs. In this study, our results showed that there was a typical wave of DNA demethylation and remethylation of *centromeric satellite repeat (CenRep)* in fertilized embryos, whereas in cloned embryos, delayed demethylation and a lack of remethylation were observed. When cloned embryos were treated with 5-aza-dC or TSA, *CenRep* methylation reprogramming was improved, and this was similar to that detected in fertilized counterparts. Furthermore, we found that the epigenetic modification agents, especially TSA, effectively promoted silencing of tissue specific genes and transcription of early embryo development-related genes in porcine cloned embryos. In conclusion, our results showed that the epigenetic modification agent 5-aza-dC or TSA could improve genomic methylation reprogramming in porcine cloned embryos and regulate the appropriate expression levels of genes related to early embryonic development, thereby resulting in high developmental competence.

Key words: DNA methylation, Epigenetic modification agents, Pig, Reprogramming, Somatic cell nuclear transfer
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Though somatic cell nuclear transfer (SCNT) has been achieved in many species, overall cloning efficiency is still low, and this limits the applications of cloning technology in agriculture, medicine and basic research [1–3].

It is generally believed that low cloning efficiency is mainly due to incomplete epigenetic reprogramming [4, 5]. To improve epigenetic reprogramming in cloned embryos, various strategies have been used, and epigenetic modification agents, such as 5-aza-dC, TSA, scriptaid and valproic acid, are usually applied and have enhanced the developmental competence of cloned embryos [6–9]. Our previous results also show that 5-aza-dC or TSA could improve cloning efficiency [10, 11]. However, the mechanism underlying the developmental improvement of cloned embryos induced by epigenetic modification agents is still poorly understood.

As the most studied epigenetic modification, DNA methylation could reflect the epigenetic reprogramming degree in cloned embryos; therefore, the mechanism of epigenetic reprogramming

induced by SCNT mainly focuses on DNA methylation [12–14]. Previous studies have shown that compared with that of *in vivo* or *in vitro* fertilized embryos, the genome of cloned embryos is usually highly methylated, leading to poor cloning efficiency [13, 15]. Since epigenetic modification agents could improve the development of cloned embryos, it is thought that DNA methylation reprogramming must be improved in treated embryos. At present, some studies have shown that epigenetic modification agents could rescue the disrupted methylation of imprinting genes [6, 16]. However, the effect of epigenetic modification agents on global methylation reprogramming during early embryonic development has been unclear.

Previous studies have shown that the *centromeric satellite repeat (CenRep)* methylation level could represent the genomic methylation status [17]; thus, *CenRep* was selected to test genomic methylation reprogramming during early embryonic development. In this study, we first treated porcine cloned embryos with 5-aza-dC or TSA to enhance their development, then investigated genomic methylation dynamics during early embryonic development and finally tested the transcripts of DNA methyltransferase, tissue specificity, pluripotency, *zygotie genome activation* and blastocyst quality-related genes in embryos.

Materials and Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and disposable and sterile plasticware was obtained from Nunclon (Roskilde, Denmark), unless otherwise stated. All experi-

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ments were approved by the Animal Care Commission of Shandong Academy of Agricultural Sciences according to animal welfare laws, guidelines and policies.

Porcine fetal fibroblast cell (PFF) culture

PFF culture has been described previously [11]. Briefly, PFFs were isolated from a 35-day-old fetus. After removal of the head, internal organs and limbs, the remaining tissues were finely minced into pieces, digested with 0.25% trypsin-0.04% ethylenediaminetetraacetic acid solution (GIBCO) and then dispersed in high glucose enriched Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin-streptomycin (GIBCO). The dispersed cells were centrifuged, resuspended and cultured in DMEM. Until confluence, PFFs were digested, centrifuged, resuspended in FBS containing 10% dimethyl sulfoxide, and they were then stored in liquid nitrogen until use. Prior to SCNT, PFFs were thawed and cultured, and they were subsequently used in 3 to 5 passages.

Oocyte collection and in vitro maturation (IVM)

Oocyte maturation has been described previously [11]. Briefly, porcine ovaries were collected from a local slaughterhouse and transported to the laboratory. Follicles were aspirated, and follicular contents were washed with HEPES-buffered Tyrode's lactate. Cumulus-oocyte complexes (COCs) with at least three uniform layers of compact cumulus cells and a uniform cytoplasm were recovered, washed and cultured in maturation medium under mineral oil at 38.5 C in a 5% CO₂ atmosphere and saturated humidity. After 42 h, COCs were vortexed in 1 mg/ml hyaluronidase to remove cumulus cells. Only oocytes with a visible polar body, regular morphology and a homogenous cytoplasm were used.

IVF and SCNT embryo culture, treatment and collection

The procedures for porcine IVF and SCNT have been described in one of our previous reports [18]. Briefly, for IVF, the semen was incubated, resuspended and washed in DPBS supplemented with 0.1% (w/v) BSA. The spermatozoa were diluted with modified Tris-buffered medium (mTBM) to the appropriate concentration. Matured oocytes were washed in mTBM, transferred into fertilization medium and co-incubated with spermatozoa. Then the embryos were washed and cultured in porcine zygote medium-3 (PZM-3) for subsequent development. For SCNT, matured oocytes and PFFs were placed in manipulation medium. After enucleation, donor cells were placed into the perivitelline space. Fusion and activation of the cell-cytoplasm complexes were induced by electroporation, and the fusion rate was confirmed by microscopic examination. Then reconstructed embryos were cultured in PZM-3 for subsequent development. The cleavage and blastocyst rates of IVF and SCNT embryos were evaluated at 48 h and 156 h, respectively.

For 5-aza-dC or TSA treatment [10, 11], cloned embryos were cultured in PZM-3 supplemented with 25 nM (optimized) 5-aza-dC (NT-AZA) or 40 nM (optimized) TSA (NT-TSA) for 24 h, washed and then transferred into PZM-3 for further culture.

For embryo collection, the 1-cell, 2-cell, 4-cell, 8-cell and blastocyst stage embryos in the IVF, NT-CON (cloned), NT-AZA and NT-TSA groups were collected at 6 h, 24 h, 48 h, 72 h and 156 h, respectively.

Bisulfite sequencing

Bisulfite sequencing has been reported [18]. Briefly, pooled samples were digested with Proteinase K and then treated with sodium bisulfite to convert all unmethylated cytosine to uracil using an EZ DNA Methylation-Gold™ Kit (Zymo Research) according to the manufacturer's protocol. For semen, the sperm was collected by centrifugation, washed in SMB solution (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl and 2% SDS, pH 7.2) and then incubated in SMB solution supplemented with 40 mM dithiothreitol and 0.3 mg/ml Proteinase K at 56 C for 1 h. For samples of 10³ PFFs, 200 MII oocytes and 150, 80, 30, 20 and 10 pooled zona pellucida-removed embryos at the 1-cell, 2-cell, 4-cell, 8-cell and blastocyst stages, respectively, digestion was performed in M-Digestion Buffer supplemented with Proteinase K at 50 C for 20 min. After digestion, a CT (cytosine to thymine) conversion reagent was added to purified genomic DNA at 98 C for 10 min and 64 C for 2.5 h. Then the samples were desalted, purified and diluted with M-Elution Buffer. Subsequently, PCR was carried out to amplify *CenRep* (Z75640) using the reported primers [17] and Hot Start Taq™ Polymerase (TaKaRa) with a profile of 94 C for 5 min and 45 cycles of 94 C for 30 sec, 55 C for 30 sec and 72 C for 1 min, followed by 72 C for 10 min. Then the amplified products were verified by electrophoresis and purified using an Agarose Gel DNA Purification Kit (TaKaRa). The purified fragments were cloned into pMD18-T Vector (TaKaRa) and subjected to sequence analysis.

Quantitative real-time PCR

Measurement of gene expression with quantitative real-time PCR has been applied in our previous studies [11, 18]. Briefly, total RNA was extracted from 30 pooled embryos at each stage using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript® RT Reagent Kit (TaKaRa) with the following parameters: 37 C for 15 min and 85 C for 5 sec, and the cDNA was stored at -20 C until use. For quantitative real-time PCR, reactions were performed in 96-well optical reaction plates (Applied Biosystems) using SYBR® Premix ExTaq™ II (TaKaRa) and a 7500 Real-Time PCR System (Applied Biosystems) with the following conditions: 95 C for 30 sec and 40 two-step cycles of 95 C for 5 sec and 60 C for 34 sec, followed by a dissociation stage consisting of 95 C for 15 sec, 60 C for 1 min and 95 C for 15 sec. For every sample, the cycle threshold (CT) values were obtained from three replicates. The primers used for amplification of target and internal reference genes are presented in Supplementary Table 1 (on-line only). The relative expression levels of target genes were analyzed using the 2^{-ΔΔCT} method.

Statistical analysis

Differences in data (mean ± SEM) were analyzed with the SPSS statistical software. Statistical analysis of data concerning genomic methylation and gene expression levels was performed with one-way analysis of variance (ANOVA). For all analyses, differences were considered to be statistically significant when P < 0.05.

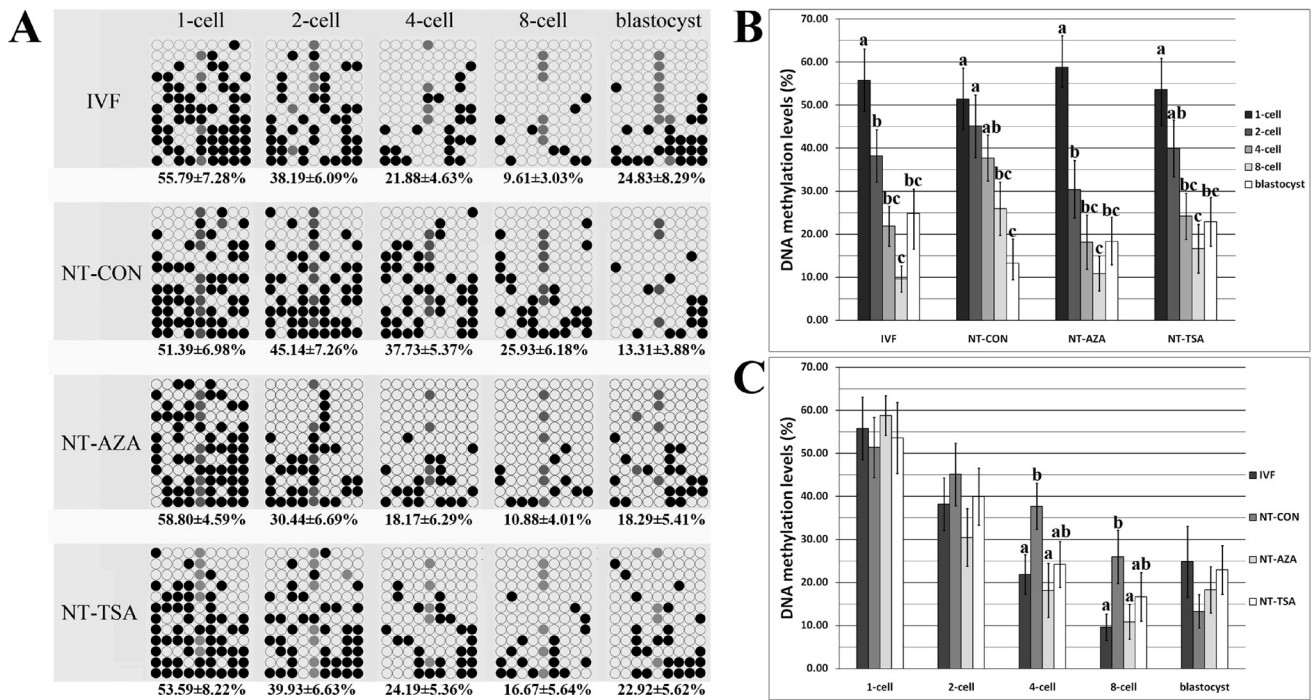


Fig. 1. CenRep methylation status. A: CenRep methylation status at the 1-cell, 2-cell, 4-cell, 8-cell and blastocyst stages of IVF, NT-CON, NT-AZA and NT-TSA embryos; B: dynamic CenRep methylation profiles in the IVF, NT-CON, NT-AZA and NT-TSA groups, respectively; C: CenRep methylation status at the 1-cell, 2-cell, 4-cell, 8-cell and blastocyst stages of IVF, NT-CON, NT-AZA and NT-TSA embryos. Black and white circles indicate methylated and unmethylated CpG sites, respectively, and gray circles represent mutated and/or single nucleotide polymorphism (SNP) variation at certain CpG sites. The data are expressed as means ± SEM. ^{a-c} Values for a given group in columns with different superscripts differ significantly ($P < 0.05$).

Results

Delayed and incomplete genomic methylation reprogramming in porcine cloned embryos

The *CenRep* methylation statuses of sperm and MII oocytes were examined, and a significant difference was observed ($P < 0.05$), with sperm showing 64.58% methylation and MII oocytes showing 28.87% methylation (Supplementary Fig. 1: on-line only). After fertilization, genomic demethylation was not observed in the 1-cell stage embryos in comparison with the mean methylation of sperm and oocytes (Fig. 1 and Supplementary Fig. 1). In the cleavage stage embryos, *CenRep* methylation displayed a continuous decrease from the 1-cell to 8-cell stage, and significant differences were observed between the 1-cell and 2-cell stages or the 4-cell and 8-cell stages (Fig. 1, $P < 0.05$). In blastocysts, the *CenRep* methylation level was higher than that in the 8-cell stage (Fig. 1), indicating that genomic remethylation occurred in blastocysts. Over all, IVF embryos displayed a typical wave of genomic demethylation and remethylation.

The *CenRep* methylation level in PFFs was 52.28%, and after SCNT, no significant differences were found between PFFs and the 1-cell stage embryos (Fig. 1 and Supplementary Fig. 1). In cloned embryos, gradual demethylation from the 1-cell stage to blastocyst stage was observed, suggesting that remethylation did not take place in the blastocyst stage (Fig. 1). When comparing the methylation levels within individual developmental stages between cloned and

IVF embryos, the levels in the 4-cell and 8-cell stage cloned embryos were significantly higher than those in their fertilized counterparts ($P < 0.05$). These results strongly suggested that genomic methylation reprogramming in cloned embryos was delayed and incomplete.

Epigenetic modification agents improved genomic methylation reprogramming in porcine cloned embryos

The epigenetic modification agents 5-aza-dC or TSA could significantly enhance the developmental competence of porcine cloned embryos (Table 1 and Supplementary Fig. 2: on-line only, $P < 0.05$). Here, the alterations of genomic methylation levels in these treated cloned embryos were investigated. In the NT-AZA group, the 1-cell stage embryos did not significantly differ from PFFs, the 1-cell to 8-cell stage embryos underwent DNA demethylation in a gradual fashion, with the level of methylation in the 2-cell stage in particular significantly lower than that in the 1-cell stage ($P < 0.05$), and blastocysts displayed remethylation in comparison with 8-cell stage embryos, which was similar to the pattern in the IVF group (Fig. 1 and Supplementary Fig. 1). When comparing the methylation status within individual developmental stages between the NT-AZA and NT-CON or IVF groups, genomic demethylation was shifted earlier in 2-cell stage, 4-cell and 8-cell stage embryos in the NT-AZA group, with the levels of methylation being significantly lower than those in the NT-CON group ($P < 0.05$), and no significant differences were observed between the NT-AZA and IVF group during early

Table 1. Development of cloned embryos treated with 5-aza-dC or TSA

Groups	No. embryos (Rep.)	No. embryos cleaved (% \pm SEM)	No. blastocysts (% \pm SEM)	Blastocyst cell numbers (mean \pm SEM) ^{&}
NT-CON	242 (5)	209 (85.79 \pm 0.95) ^a	50 (20.50 \pm 0.70) ^a	37 \pm 3 (n=49)
NT-AZA	247 (5)	223 (89.88 \pm 1.14) ^b	67 (27.30 \pm 1.24) ^b	37 \pm 2 (n=55)
NT-TSA	238 (5)	210 (88.82 \pm 1.12) ^{ab}	118 (50.71 \pm 2.21) ^c	38 \pm 2 (n=76)

[&] Blastocyst cell numbers of less than 16 or blastocysts used for molecular analysis (10 or 40 blastocysts in the NT-AZA or NT-TSA group, respectively) were not included. ^{a-c} Values in the same column with different superscripts differ significantly ($P < 0.05$).

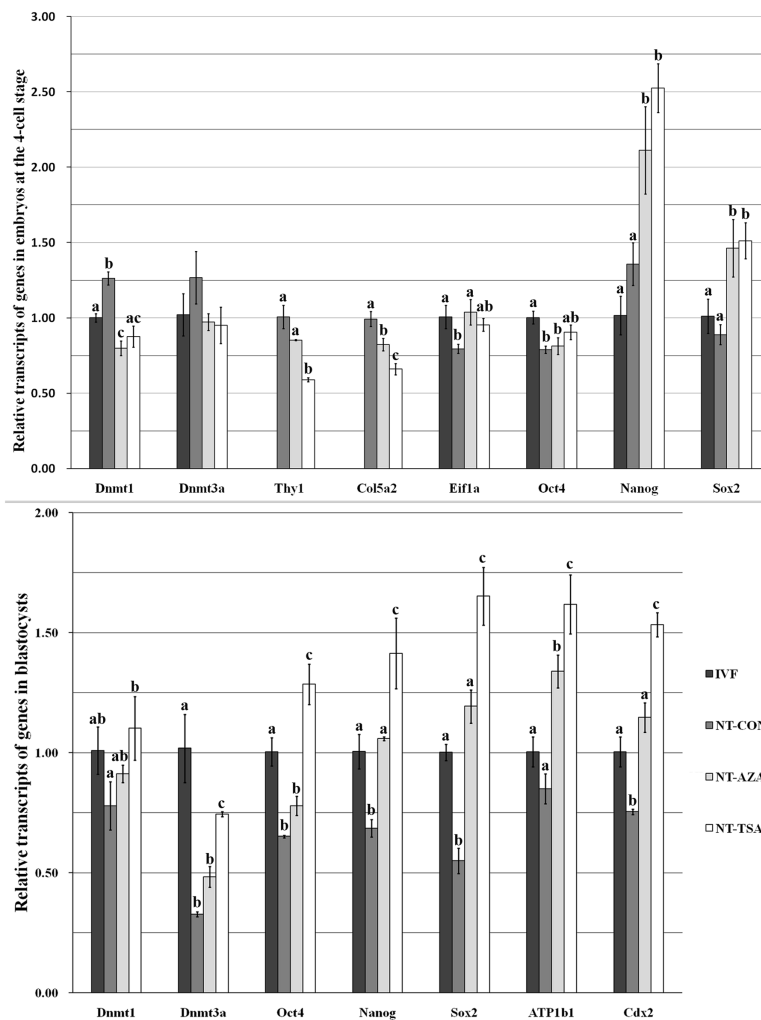


Fig. 2. Transcript levels of early embryo development-related genes at the zygotic genome activation and blastocyst stages of IVF, NT-CON, NT-AZA and NT-TSA embryos. The transcript abundance for each gene (Thy1 and Col5a2 in cloned embryos) in IVF embryos was considered the control. The data are expressed as means \pm SEM. ^{a-c} Values for a given gene at a certain stage in columns with different superscripts differ significantly ($P < 0.05$).

embryonic development. These results indicated that 5-aza-dC could improve genomic methylation reprogramming in cloned embryos.

In the NT-TSA group, similar DNA methylation dynamics to the NT-AZA or IVF group were observed (Fig. 1). When comparing the differences between the NT-TSA and NT-AZA groups, the NT-TSA

group showed a methylation pattern that was much more similar to that of the IVF group, as the NT-AZA group underwent faster genomic demethylation and slower genomic remethylation, though no significant differences were observed. Overall, our results showed that the epigenetic modification agents 5-aza-dC and TSA rescued the

disrupted genomic methylation reprogramming in cloned embryos.

Epigenetic modification agents improved the expression levels of genes related to early embryo development

To further explore the mechanism underlying the improved development of cloned embryos treated with 5-aza-dC or TSA, the transcript levels of early embryonic development-related genes in the *zygotic genome activation (ZGA)* and blastocyst stages were investigated (Fig. 2). Compared with the IVF group, the NT-CON group displayed a significantly higher transcript level of *Dnmt1* and lower expression levels of *Oct4* and *Eif1a* at the ZGA stage ($P < 0.05$), and significantly lower transcripts of *Dnmt3a*, *Oct4*, *Nanog*, *Sox2* and *Cdx2* at the blastocyst stage ($P < 0.05$), suggesting that *zygotic genes* were not effectively activated and that the blastocyst quality was poor in cloned embryos.

In the NT-AZA group, the mRNA expression levels of early embryonic development-related genes was improved in comparison to those in the NT-CON group, showing significantly higher transcripts of *Eif1a*, *Nanog* and *Sox2* and lower expression of *Dnmt1* and *Col5a2* at the ZGA stage ($P < 0.05$), and significantly higher mRNA expression of *Nanog*, *Sox2*, *ATP1b1* and *Cdx2* at the blastocyst stage ($P < 0.05$). When compared with the IVF group, the NT-AZA group displayed no significant alterations of *Eif1a* and *Cdx2*, suggesting that 5-aza-dC could improve ZGA and blastocyst quality, though significantly lower expression levels of *Oct4* at the ZGA stage and *Dnmt3a* and *Oct4* at the blastocyst stage were still observed ($P < 0.05$).

In the NT-TSA group, significant downregulation of *Dnmt1* and tissue-specific gene transcripts at the ZGA stage and upregulation of *Nanog* and *Sox2* expression levels at the ZGA stage and DNA methyltransferase, pluripotency and blastocyst quality-related gene transcripts at the blastocyst stage were observed in comparison with the NT-CON group ($P < 0.05$). When compared with 5-aza-dC, TSA was more effective for gene expression regulation, showing significant silencing of the tissue-specific genes at the ZGA stage and activation of *Dnmt3a*, pluripotency and blastocyst quality-related genes at the blastocyst stage ($P < 0.05$). Furthermore, in comparison to those in the IVF group, significant upregulation of pluripotency and blastocyst quality-related gene transcripts was observed in the NT-TSA group ($P < 0.05$), though the *Dnmt3a* transcript level at the blastocyst stage was still significantly lower ($P < 0.05$). Thus, these above results showed that treating cloned embryos with 5-aza-dC or TSA improved the transcription levels of genes related to early embryonic development.

Discussion

Our study showed that treating porcine cloned embryos with 5-aza-dC or TSA could enhance genomic methylation reprogramming and regulate the appropriate transcript levels of early embryonic development-related genes in cloned embryos, thereby resulting in improvement of the development of porcine cloned embryos.

It is generally believed that epigenetic modification agents could improve nuclear reprogramming, and previous studies have shown that 5-aza-dC or TSA could enhance the developmental competence of cloned embryos [6, 16, 19]. However, the mechanism underlying the improvement of development is poorly studied. In this study, we

investigated genomic methylation reprogramming in cloned embryos. In comparison with IVF embryos, cloned embryos took on a process of delayed demethylation without remethylation, suggesting that incomplete methylation reprogramming may be one cause of the developmental block or lethality of cloned embryos [13]. As for the reason for incomplete methylation reprogramming in cloned embryos, it is possible that there is a mechanism that causes the donor cell nucleus to preserve its methylation pattern, making oocyte-specific factors incompletely reprogram its nucleus [20]. When 5-aza-dC or TSA were applied, genomic methylation reprogramming was improved and was similar to that in IVF counterparts, showing a typical pattern of demethylation and remethylation. These findings may provide explanations for the observations that 5-aza-dC or TSA enhanced the developmental competence of cloned embryos. As for the improvement of genomic methylation reprogramming, it is possible that 5-aza-dC was incorporated into the genome and that TSA modified the chromatin structure [4]. Of course, other mechanisms also exist [4, 11]. In regard to the differences in genomic methylation reprogramming between the NT-AZA and NT-TSA group, one possible explanation is that the manners of genomic methylation regulation induced by 5-aza-dC or TSA are different, and regulation of histone modification possibly fits better with genomic demethylation and remethylation in cloned embryos [6, 21]. The results concerning embryonic development and the numbers of born and live piglets per surrogate (data not shown) could confirm this explanation. As to how genomic methylation is processed to achieve unimpaired reprogramming in the NT-AZA or NT-TSA group, more information is needed to clarify the mechanism.

In view of the genomic methylation dynamics during early embryonic development, our results also suggest that the partially progressive demethylation possibly results from replication-related passive demethylation and that active demethylation may not occur, even though traditional bisulfite sequencing could not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine [17, 22]. Due to the important role of 5-hydroxymethylcytosine in somatic nuclear reprogramming [23], new technologies such as oxidative bisulfite sequencing will be applied to clarify the genomic demethylation mechanism during embryonic development.

DNA methylation reprogramming is thought to be possibly associated with gene transcription regulation [14, 24]. Our study showed that the transcription level of *Dnmt1* at the ZGA stage in the NT-CON group was significantly higher than that in the IVF group, while the *Dnmt3a* transcript level was significantly downregulated at the blastocyst stage, possibly explaining the cause of failure of DNA demethylation and remethylation in the NT-CON group [13]. Incomplete genomic methylation reprogramming would lead to the disturbed expression levels of genes related to early embryonic development, showing continuous expression of tissue-specific genes, no effective activation of pluripotent genes and downregulation of blastocyst quality-related gene expression in cloned embryos, thereby resulting in low cloning efficiency [25, 26]. When cloned embryos were treated with 5-aza-dC or TSA, the expression levels of *Dnmt1* and *Dnmt3a* in the treatment groups, especially the NT-TSA group, were improved and were much closer to those in the IVF group (Supplementary Fig. 3: on-line only), suggesting that DNA methylation reprogramming in the NT-AZA and NT-TSA groups

would be facilitated [27], and our results showed that genomic methylation reprogramming was improved in the NT-AZA and NT-TSA groups. The results of gene transcription showed that the gene expression patterns in the NT-AZA and NT-TSA groups were also appropriate, which was strongly consistent with the improved genomic methylation reprogramming. Previous studies reported that appropriate transcription of these early embryonic development-related genes is essential for cloned embryo development [28]. Thus, we speculate that the improvement of developmental competence of cloned embryos is probably due to the rescued genomic methylation reprogramming enhancing the restoration of the expression levels of early embryonic development-related genes. Certainly, not all the gene transcription levels were consistent with the overall DNA methylation status during embryonic development, as each gene has its own specific methylation pattern. At present, these gene methylation patterns in cloned embryos have not been well elucidated, and they are very worthy of investigation.

In conclusion, our results showed that treating porcine cloned embryos with 5-aza-dC or TSA improved genomic methylation reprogramming and regulated the appropriate transcripts of genes related to early embryonic development, thereby resulting in improvement of the development of porcine cloned embryos.

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