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Epigenetic Modification of Cloned Embryos Improves *Nanog* Reprogramming in Pigs

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Abstract

Incomplete reprogramming of pluripotent genes in cloned embryos is associated with low cloning efficiency. Epigenetic modification agents have been shown to enhance the developmental competence of cloned embryos; however, the effect of the epigenetic modification agents on pluripotent gene reprogramming remains unclear. Here, we investigated *Nanog* reprogramming and the expression patterns of pluripotent transcription factors during early embryo development in pigs. We found that compared with fertilized embryos, cloned embryos displayed higher methylation in the promoter and 5′-untranslated region and lower methylation in the first exon of *Nanog*. When 5-aza-2′-deoxycytidine (5-aza-dC) or trichostatin A (TSA) enhanced the development of porcine cloned embryos, *Nanog* methylation reprogramming was also improved, similar to that detected in fertilized counterparts. Furthermore, our results showed that the epigenetic modification agents improved the expression levels of *Oct4* and *Sox2* and effectively promoted *Nanog* transcription in cloned embryos. In conclusion, our results demonstrated that the epigenetic modification agent 5-aza-dC or TSA improved *Nanog* methylation reprogramming and the expression patterns of pluripotent transcription factors, thereby resulting in the enhanced expression of *Nanog* and high development of porcine cloned embryos. This work has important implications in the improvement of cloning efficiency.

Introduction

Although somatic cell nuclear transfer (SCNT) has been achieved in many species, overall cloning efficiency is still low, thus limiting the application of cloning technology in basic research, agriculture, and medicine (Galli et al., 2012; Lee and Prather 2013; Rodriguez-Osorio et al., 2012). It is generally believed that low cloning efficiency is due mainly to abnormal epigenetic reprogramming (Zhao et al., 2010). The reprogramming of pluripotent genes could influence the developmental competence of cloned embryos, because pluripotent genes play critical roles in the establishment and maintenance of pluripotency during early embryo development (Dejosez and Zwaka, 2012; Lee et al., 2013).

Nanog is one of the critical pluripotency regulators and responsible for the pluripotency of embryonic stem cells and early embryos (Pan and Thomson, 2007; Lee et al., 2013). During somatic cell reprogramming, Nanog serves as an activator of multiple target genes and can overcome reprogramming barriers (Costa et al., 2013; Theunissen et al., 2011; Zhang et al., 2011). However, in cloned embryos, *Nanog* expression is abnormal, probably leading to the poor development of cloned embryos (Huan et al., 2013).

Our previous studies have shown that a DNA methylation inhibitor [5-aza-2′-deoxycytidine (5-aza-dC)] or histone deacetylase inhibitor [trichostatin A (TSA)] could improve the development of cloned embryos (Huan et al., 2013; Kong et al., 2011). However, the mechanism underlying the developmental improvement of cloned embryos is still poorly understood. Because *Nanog* expression is regulated by epigenetic mechanisms involving DNA methylation and histone modifications and *Nanog* activation is essential for early embryo development (Lee et al., 2013; Miyamoto et al., 2009; Xu et al., 2013), it is thought that *Nanog* reprogramming must be improved efficiently in these treated embryos. However, the effect of the epigenetic modification agents on *Nanog* reprogramming during early embryo development remains unknown.

To understand *Nanog* reprogramming during SCNT, the epigenetic modification agents 5-aza-dC and TSA were

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employed to enhance the development of cloned embryos. We found that compared with fertilized embryos, cloned embryos displayed incomplete methylation reprogramming of *Nanog*, whereas *Nanog* methylation reprogramming was improved in 5-aza-dC– or TSA-treated embryos, similar to that detected in fertilized counterparts. Moreover, 5-aza-dC or TSA improved the expression levels of *Oct4* and *Sox2* and effectively promoted *Nanog* transcription in cloned embryos. These results demonstrated that *Nanog* reprogramming was associated with the development of cloned embryos and would have important implications in the improvement of cloning efficiency.

Materials and Methods

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

All experiments 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 culture

Porcine fetal fibroblast (PFF) cell culture has been described previously (Huan et al., 2013). Briefly, a 35-day-old fetus was recovered and rinsed five times with Dulbecco's phosphate buffered saline (D-PBS). After removal of the head, internal organs, and limbs, the remaining tissues were finely minced. The minced tissues were digested with a 0.25% trypsin and 0.04% EDTA solution at 37°C for 45 min, followed by dispersal in high-glucose-enriched Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. The dispersed cells were centrifuged, resuspended, and cultured in high-glucose-enriched DMEM containing 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin at 38.5°C in a 5% CO_2 atmosphere and saturated humidity. Until confluence, PFFs were digested, centrifuged, and resuspended in FBS (GIBCO) containing 10% dimethyl sulfoxide and stored in liquid nitrogen until use. Prior to SCNT, PFFs were thawed, cultured, and used in three to five passages.

Oocyte collection and in vitro *maturation*

Oocyte maturation has been described previously (Huan et al., 2013). Briefly, porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in physiological saline with antibiotics at 37°C. Follicles with a diameter between 3 and 8 mm were aspirated, and follicular contents were washed three times with HEPES-buffered Tyrode's lactate. Cumulus–oocyte complexes (COCs) with at least three uniform layers of compact cumulus cells and uniform cytoplasm were recovered, washed, and cultured in maturation medium [tissue culture medium-199 (TCM-199) supplemented with 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 0.5 μg/mL follicle-stimulating hormone, 0.5 μg/mL luteinizing hormone, and 100 μL/mL porcine follicular fluid] under mineral oil at 38.5°C in 5% CO_2 atmosphere and saturated humidity. After 42 h, COCs were vortexed in 1 mg/mL hyaluronidase for 3 min to remove cumulus cells. Only oocytes with a visible polar body, regular morphology, and a homogeneous cytoplasm were used.

In vitro *fertilization and SCNT embryo culture, treatment, and collection*

The procedures for *in vitro* fertilization (IVF) and SCNT have been described (Huan et al., 2013; Wei et al., 2011). Briefly, for IVF, the semen was incubated at 39°C, resuspended, and washed three times in D-PBS supplemented with 0.1% bovine serum albumin. The sperm concentration was measured using a hemocytometer, and the proportion of motile sperm was determined. The spermatozoa were diluted with modified Tris-buffered medium (mTBM) to the appropriate concentration. Matured oocytes were washed three times in mTBM, transferred into fertilization medium, and co-incubated with spermatozoa for 6 h at the rate of 1:1000. Then the embryos were washed and cultured in porcine zygote medium-3 (PZM-3) for subsequent development. For SCNT, PFFs were trypsinized, centrifuged, and resuspended in manipulation medium. The matured oocytes and PFFs were placed into manipulation medium supplemented with 7.5 μg/mL cytochalasin B. After enucleation by aspirating the first polar body and adjacent cytoplasm, donor cells were placed into the perivitelline space. Fusion and activation of the cell–cytoplast complexes were induced with two direct pulses of 1.2 kV/cm for 30 μsec in the fusion medium (0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM HEPES), and the fusion rate was confirmed by examination with microscopy. Reconstructed embryos were cultured in PZM-3 for subsequent development.

For 5-aza-dC or TSA treatment (Huan et al., 2013; Kong et al., 2011), reconstructed embryos were treated with 25 nM (optimized) 5-aza-dC (NT-AZA) or 40 nM (optimized) TSA (NT-TSA) for 24 h, washed, and transferred into PZM-3 for further culture. For embryo collection, the one-cell, two-cell, four-cell, eight-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 described in one of our previous reports (Wei et al., 2011). Briefly, pooled samples were digested with proteinase K (PK) and treated with sodium bisulfite to convert all unmethylated cytosines to uracils using an EZ DNA Methylation-Direct™ Kit (compatible with small sample inputs, 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% sodium dodecyl sulfate (SDS), pH 7.2], and incubated in SMB solution supplemented with 40 mM dithiothreitol and 0.3 mg/ml PK at 56°C for 1 h. For samples of 10^3 PFFs, 200 metaphase II (MII) oocytes, and 200, 100, 50, 20, and 10 pooled embryos with the zona pellucidae removed at the one-cell, two-cell, four-cell, eight-cell, and blastocyst stages, respectively, digestion was performed in M-Digestion Buffer supplemented with PK at 50°C for 20 min. After digestion of all the samples, a cytosine-to-thymine (CT) conversion reagent was added 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.

Nested PCR was carried out to amplify the target regions of *Nanog* using the primers described in Table S1 (Supplementary Data are available at www.liebertpub.com/cell/) and Hot Start Taq™ Polymerase (TaKaRa) with a profile of 94°C for 5 min, 45 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, followed by 72°C for 10 min. Products from the first amplification reaction were used in the second PCR reaction, and the optimal annealing temperatures of inner primers (Zhao et al., 2013) were 52°C for promoter and 5′-untranslated region (5′-UTR) and 50°C for the first exon. 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 Vectors (TaKaRa) and subjected to sequence analysis.

Real-time quantitative PCR

Measurement of gene expression with real-time quantitative PCR (qPCR) was performed (Huan et al., 2013, Wei et al., 2011). Briefly, total RNA was extracted from 30 pooled embryos at each stage using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality was confirmed by the ratios of A260/A280 (all between 1.8 and 2.0), and only RNA samples that did not show signs of degradation were used in this study. Reverse transcription was performed using a PrimeScript® RT Reagent Kit (TaKaRa). The 20-μL reaction volume contained 1 μL of 100 μM random hexamer primer, 4 μL of 5× reverse transcriptase (RT) buffer, 1 μL of RT enzyme mix, 1 μL of total RNA (< 500 ng), and 13 μL of RNase-free distilled H_2O. The reaction parameters were: 37°C for 15 min and 85°C for 5 sec, and the cDNA was stored at −20°C until use.

For real-time qPCR, 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). Each reaction mixture (50 μL) contained 1 μL (< 25 ng) of cDNA solution, 1 μL of 10 μM of each specific primer, 1 μL of 50× SYBR Green Dye, and 25 μL of 2× ExTaq. Thermal cycling conditions were 95°C for 30 sec, followed by 40 two-step cycles of 95°C for 5 sec and 60°C for 34 sec, and a dissociation stage consisting of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec.

The specificity of the PCR reaction was confirmed by a single peak in the dissociation curve and also by a single band in agarose gel electrophoreses. As negative controls, cDNA was omitted during the real-time reaction. For each sample, the cycle threshold (Ct) values were obtained from three replicates. The primers used for amplification of target and internal reference genes are shown in Table S1. The comparative Ct method was used for relative quantification of target gene expression levels. Each pair of primers was confirmed for equal amplification efficiency to primers of the endogenous control (18S ribosomal RNA). Ct value was calculated by the Sequence Detection System software (Applied Biosystems). The ΔCt value was defined as Ct (target gene) − Ct (18S rRNA). The ΔΔCt value was defined as ΔCt (sample) − ΔCt (calibrator). The relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

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

Results

Incomplete Nanog *methylation reprogramming in porcine cloned embryos*

It is known that DNA methylation in a promoter or 5′-UTR silences gene expression, whereas gene body methylation positively regulates gene transcription (Szyf, 2010). Here, the distribution of CpG sites in the *Nanog* promoter, 5′-UTR, and the first exon was analyzed with the MethPrimer program. The result showed that no CpG island existed in the promoter; there were seven CpG sites in the promoter, one CpG site in the 5′-UTR, and four CpG sites in the first exon (Fig. 1A).

The methylation status of *Nanog* in sperm and MII oocytes was examined. The methylation levels of the promoter, 5′-UTR, and the first exon were 92.86%, 81.82%, and 36.11% in sperm and 5.16%, 27.27%, and 17.36% in MII oocytes, respectively (Fig. 1B and C). After fertilization, *Nanog* demethylation did not occur at the one-cell stage in comparison with the mean methylation of sperm and oocytes (Figs. 1 and 2). During the development of IVF embryos (Figs. 2 and S1), *Nanog* methylation levels in the promoter and 5′-UTR decreased, especially from the one-cell to two-cell stage. In the first exon, *Nanog* methylation levels showed an upward trend from the two-cell to blastocyst stage. Thus, IVF embryos displayed active demethylation in the promoter and 5′-UTR and a remethylation pattern in the first exon of *Nanog*.

In donor cells, *Nanog* methylation levels in the promoter, 5′-UTR, and first exon were 54.42%, 58.33%, and 21.53%, respectively. After SCNT, no significant differences in *Nanog* methylation levels were observed between donor cells and the one-cell-stage embryos (Figs. 1 and 2). In cloned embryos (Figs. 2 and S1), gradual demethylation in the promoter and 5′-UTR was observed from the one-cell to blastocyst stage and active demethylation did not occur. In the first exon, the change of *Nanog* methylation levels was not obvious. When compared with the individual developmental stage of IVF embryos (Fig. 2), the methylation levels of the *Nanog* promoter and 5′-UTR in cloned embryos were generally higher, especially at the four-cell stage. For the first exon, the methylation levels in cloned embryos from the four-cell to blastocyst stage were lower than those in fertilized counterparts. These results suggested that *Nanog* methylation reprogramming in porcine cloned embryos was incomplete.

Epigenetic modification agents improved Nanog *methylation reprogramming in porcine cloned embryos*

After cloned embryos were treated with 5-aza-dC or TSA, *Nanog* methylation reprogramming was investigated (Figs. 2 and S1). In the NT-AZA group, the *Nanog* methylation level in the one-cell-stage embryos did not differ obviously from that in donor cells. From the one-cell to blastocyst stage, particularly the one-cell to two-cell stage, the *Nanog* promoter and 5′-UTR underwent demethylation, and the

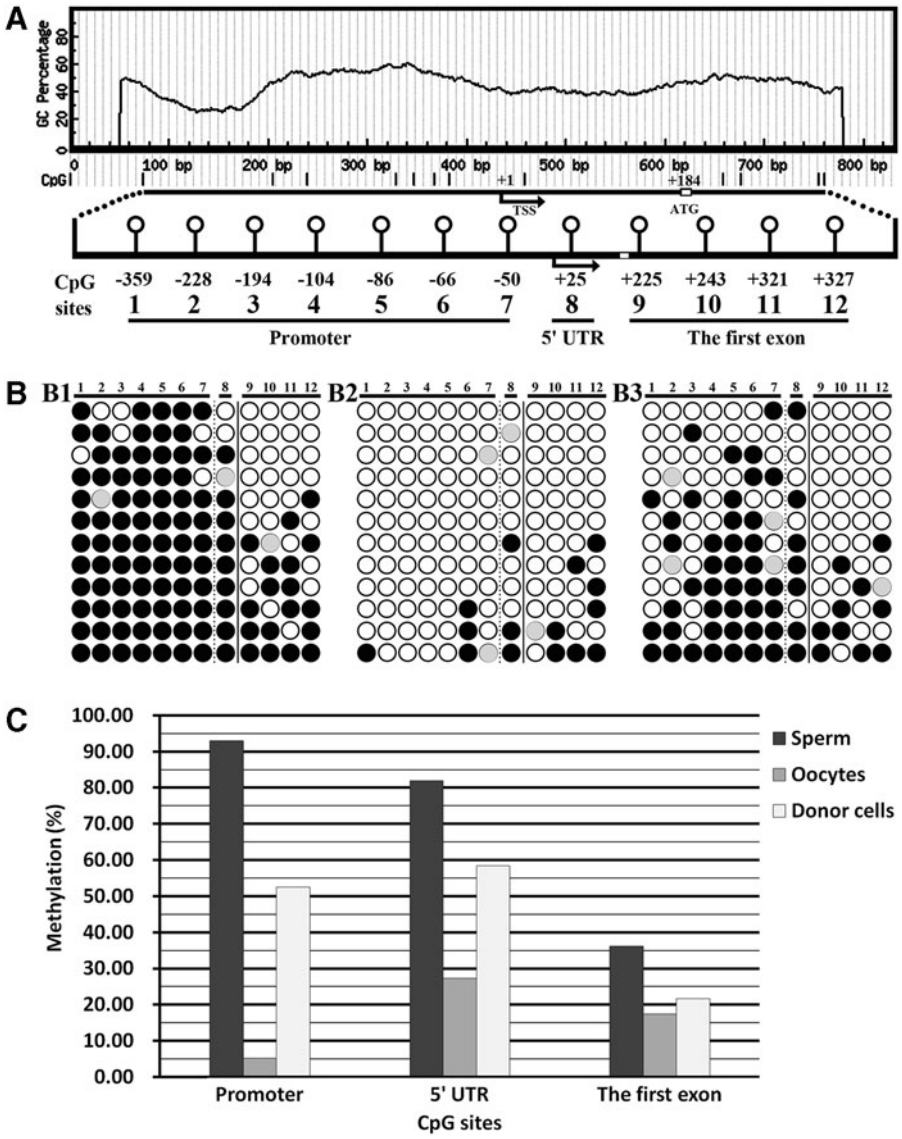

FIG. 1. Prediction and analysis of *Nanog* methylation status. (**A**) Twelve CpG sites (seven in the promoter, one in the 5′-UTR, and four in the first exon, respectively) were analyzed in the *Nanog* sequence around the transcription start site (TSS) by the MethPrimer program. TSS was designated as +1. (**B**) *Nanog* methylation status in sperm (**B1**), oocytes (**B2**), and donor cells (**B3**). (Black or white circles) Methylated or unmethylated CpG sites; (gray circles) mutated and/or single-nucleotide polymorphism (SNP) variation at certain CpG sites. (**C**) Average methylation levels of the *Nanog* promoter, 5′-UTR, and the first exon in sperm, oocytes, and donor cells.

first exon of *Nanog* displayed demethylation from the one-cell to four-cell stage and remethylation from the four-cell to blastocyst stage. As for the differences between the NT-AZA and NT-CON groups, *Nanog* demethylation was shifted earlier in the NT-AZA group and the methylation levels of *Nanog* were lower than those in the NT-CON group, with the exception of 5′-UTR and the first exon in blastocysts. *Nanog* methylation status in the NT-AZA group was closer to those in the IVF group. These results indicated that 5-aza-dC could improve *Nanog* methylation reprogramming in cloned embryos.

In the NT-TSA group, a trend of demethylation in the *Nanog* promoter and 5′-UTR and remethylation in the first exon of *Nanog* were observed. For *Nanog* methylation differences between the NT-TSA and NT-CON or NT-AZA groups, the NT-TSA group showed a much more similar methylation pattern to the IVF group. Therefore, our results showed that the epigenetic modification agents 5-aza-dC or TSA improved *Nanog* methylation reprogramming in porcine cloned embryos.

Epigenetic modification agents improved Nanog *expression in porcine cloned embryos*

For *Nanog* transcription, a similar expression trend was observed in the IVF, NT-CON, NT-AZA, and NT-TSA groups, showing the initial expression at the four-cell stage, a maximum peak at the eight-cell stage, and a slight decrease at the blastocyst stage (Fig. 3A). When the individual developmental stage was compared among these groups

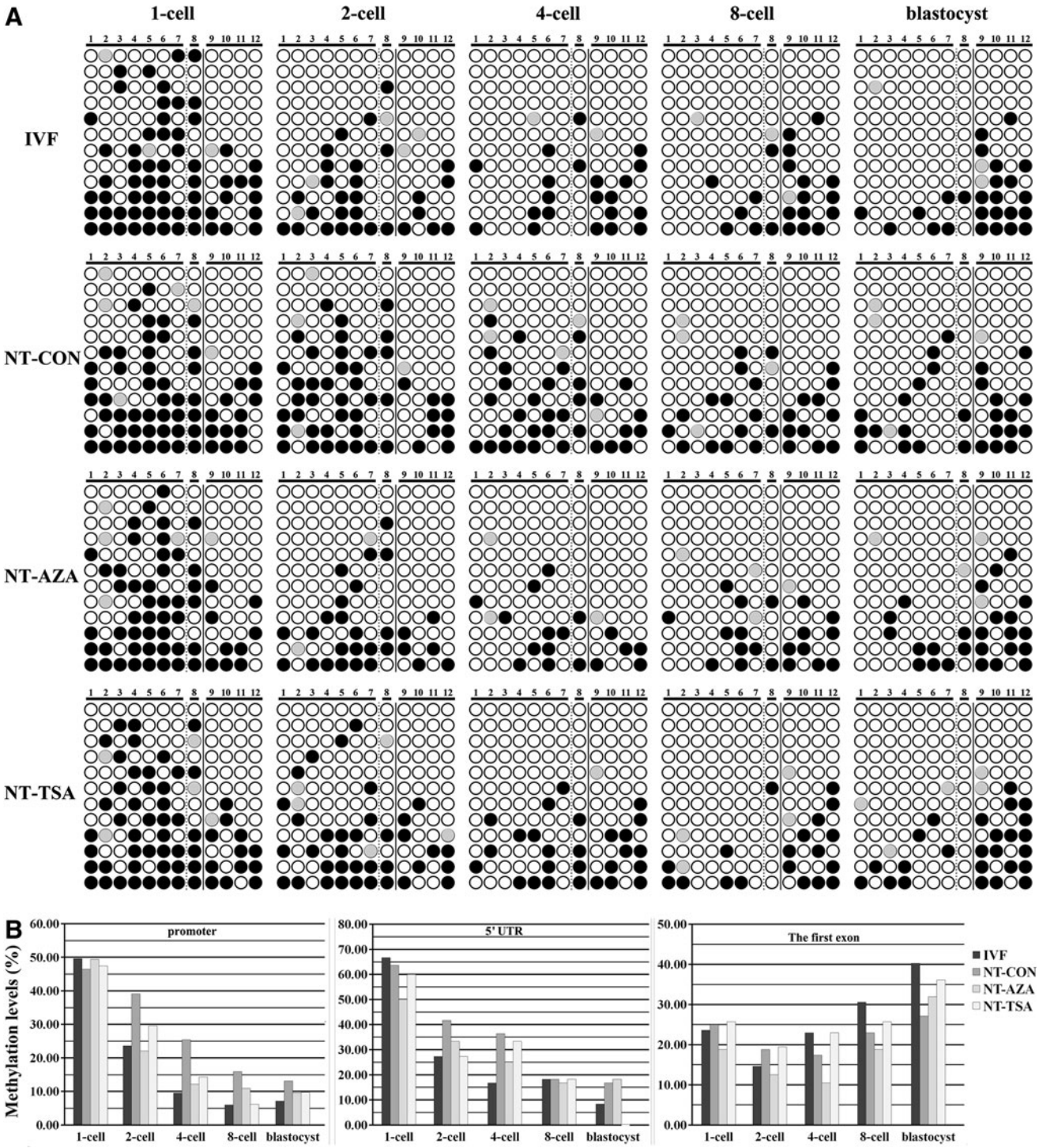

FIG. 2. *Nanog* methylation status in embryos. (**A**) *Nanog* methylation status at the one-cell, two-cell, four-cell, eight-cell, and blastocyst stages of IVF, NT-CON, NT-AZA, and NT-TSA embryos. (Black and white circles) Methylated and unmethylated CpG sites, respectively; (gray circles) mutated and/or single-nucleotide polymorphism (SNP) variation at certain CpG sites. (**B**) Methylation status of *Nanog* promoter, 5′-UTR, and the first exon at the one-cell, two-cell, four-cell, 8-eight cell, and blastocyst stages of IVF, NT-CON, NT-AZA, and NT-TSA embryos.

(Fig. 3A′), *Nanog* transcript levels in the NT-CON group were significantly ($p < 0.05$) lower than those in the IVF group (from the four-cell to blastocyst stage), the NT-AZA group, or the NT-TSA group (from the two-cell to blastocyst stage), and in comparison with that in the IVF group, *Nanog* expression in the NT-AZA or NT-TSA group showed a significant increase at the four-cell stage and a significant decrease at the eight-cell stage ($p < 0.05$). Regarding the differences between the NT-TSA and NT-AZA groups, *Nanog* transcripts in the NT-TSA group showed significantly

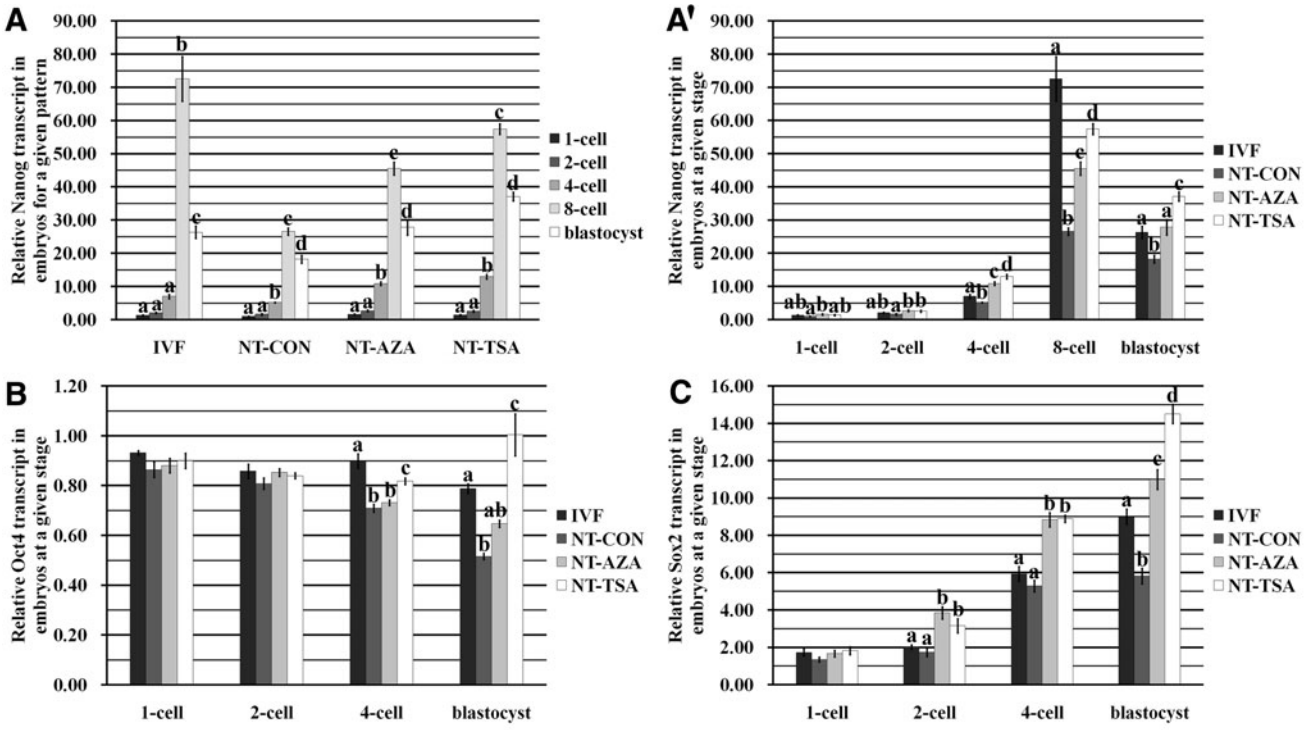

FIG. 3. Transcript levels of *Nanog*, *Oct4*, and *Sox2* in IVF, NT-CON, NT-AZA, and NT-TSA embryos. (**A**) Relative transcript levels of *Nanog* in IVF, NT-CON, NT-AZA, and NT-TSA embryos. (**A'**) Relative transcript levels of *Nanog* at the one-cell, two-cell, four-cell, eight-cell, and blastocyst stages of IVF, NT-CON, NT-AZA, and NT-TSA embryos. (**B**) Relative transcript levels of *Oct4* at the one-cell, two-cell, four-cell, and blastocyst stages of IVF, NT-CON, NT-AZA, and NT-TSA embryos. (**C**) Relative transcript levels of *Sox2* at the one-cell, two-cell, four-cell, and blastocyst stages of IVF, NT-CON, NT-AZA, and NT-TSA embryos. The transcript abundance for each gene in oocytes at the MII stage was considered as the control. The data are expressed as mean ± SEM. [a–d]Values of a given gene in a certain group (**A**) or at a certain stage (**A'**, **B**, and **C**) in columns with different superscripts differ significantly ($p < 0.05$).

($p < 0.05$) higher levels from the four-cell to blastocyst stage, and a significant upregulation of *Nanog* expression at the blastocyst stage was also observed in the NT-TSA group in comparison with that in the IVF group ($p < 0.05$). These results suggested that the enhanced *Nanog* methylation reprogramming induced by 5-aza-dC or TSA improved its expression in cloned embryos.

To further explore the mechanism underlying the improved *Nanog* expression derived from its enhanced methylation reprogramming, transcription factors regulating *Nanog* expression were analyzed with the TRANSFAC and TFSEARCH programs. Sequence analysis revealed that a number of binding sites of potentially important transcription factors were present in the *Nanog* sequence, and the degree of methylation of *Nanog* CpG sites determined the binding capability of these factors, thereby regulating *Nanog* expression (Fig. S2).

Of course, the expression levels of these transcription factors also regulate *Nanog* transcription. Here, the transcription levels of two functional transcription factors (*Oct4* and *Sox2*, identified) were investigated (Fig. 3B, C). Compared with those in the IVF group, significantly lower transcripts of *Oct4* at the four-cell and blastocyst stages and *Sox2* at the blastocyst stage were observed in the NT-CON group ($p < 0.05$). After 5-aza-dC or TSA treatment, the NT-AZA group showed significantly ($p < 0.05$) higher expression levels of *Sox2* from the two-cell to blastocyst stage, and the NT-TSA group displayed significantly higher transcripts of *Sox2* from the two-cell to blastocyst stage and *Oct4* at the four-cell and blastocyst stages in comparison with those in the NT-CON group ($p < 0.05$). *Sox2* transcripts from the two-cell to blastocyst stage in the NT-AZA or NT-TSA group were also significantly higher than those in the IVF group, although *Oct4* expression at the four-cell stage was still significantly lower ($p < 0.05$).

When the expression levels of *Oct4* and *Sox2* were compared between the NT-AZA and NT-TSA groups, the NT-TSA group showed significantly ($p < 0.05$) higher transcripts of *Oct4* at the four-cell and blastocyst stages and *Sox2* at the blastocyst stage, and *Oct4* expression at the blastocyst stage in the NT-TSA group was also significantly higher than that in the IVF group ($p < 0.05$). Thus, these results showed that the epigenetic modification agent 5-aza-dC or TSA improved the expression of transcription factors in cloned embryos. Overall, the improvement of *Nanog* methylation reprogramming and the expression and binding of transcription factors induced by 5-aza-dC or TSA enhanced *Nanog* expression in cloned embryos.

Discussion

Aberrant methylation reprogramming has been reported in cloned embryos (Cantone and Fisher 2013; Peat and Reik 2012; Zhao et al., 2010). Nanog is one of the critical pluripotent

factors and its expression influences nuclear reprogramming efficiency, suggesting that Nanog could be a suitable marker to evaluate nuclear reprogramming in cloned embryos (Costa et al., 2013; Miyamoto et al., 2009; Stuart et al., 2014). In this study, *Nanog* methylation in the promoter and 5′-UTR rapidly decreased and was maintained at a low level in IVF embryos, which is consistent with a previous report (Zhao et al., 2013). After SCNT, the methylation levels of the *Nanog* promoter and 5′-UTR were higher than those in IVF embryos, and the methylation of the first exon was also reprogrammed inefficiently, suggesting that incomplete *Nanog* methylation reprogramming could be the cause of the poor development of cloned embryos. As for the reason for incomplete *Nanog* methylation reprogramming, it is possible that there is a mechanism that preserves the methylation pattern of donor cells against reprogramming by oocyte factors (Yamanaka et al., 2011).

Our previous studies have shown that 5-aza-dC or TSA could improve the development of porcine cloned embryos (Huan et al., 2013; Kong et al., 2011). Here, the reprogramming degree of *Nanog* methylation in the NT-AZA or NT-TSA group may explain the results that 5-aza-dC or TSA enhances the development of cloned embryos (Table S2 and Fig. S3). As for the improvement of *Nanog* methylation reprogramming after 5-aza-dC or TSA treatment, it is possible that 5-aza-dC was incorporated into the genome during DNA replication or that TSA loosened the chromatin structure, benefitting the binding of DNA demethylation-related molecules (Huan et al., 2013; Zhao et al., 2010).

Of course, other mechanisms may also exist (Huan et al., 2013; Zhao et al., 2010). In regard to the differences of *Nanog* methylation reprogramming between the NT-AZA and NT-TSA groups, the different manner of regulation induced by 5-aza-dC or TSA may be the cause, and histone modification possibly fits better with *Nanog* methylation reprogramming (Jafarpour et al., 2011; Xu et al., 2013). The results concerning the birth of cloned piglets (data not shown) could also confirm this explanation. As for the detailed mechanism of *Nanog* methylation reprogramming in the NT-AZA or NT-TSA group, further studies are needed.

In view of *Nanog* methylation status during early embryo development, our results suggest that an active demethylation mechanism exists, even though traditional bisulfite sequencing could not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine (Huang et al., 2010). Due to the critical role of 5-hydroxymethylcytosine in somatic cell reprogramming (Wossidlo et al., 2011), new technologies, such as oxidative bisulfite sequencing, would be employed to investigate active demethylation of *Nanog*.

The improvement of *Nanog* methylation reprogramming after 5-aza-dC or TSA treatment should result in its effective activation in cloned embryos. The expression patterns of *Nanog* in cloned embryos support this view and are positively associated with the development of cloned embryos. Thus, we speculate that the improvement of *Nanog* expression derived from its facilitated methylation reprogramming probably enhances the developmental competence of cloned embryos. Certainly, the appropriate expression levels of other early embryo development-related genes are also essential for the development of cloned embryos (Huan et al., 2013); thus, the expression profiles of these genes are also worthy of investigation.

Regarding the improvement of *Nanog* expression in the 5-aza-dC or TSA treatment group, pluripotent transcription factors such as Oct4 and Sox2 should also play a key role (Kuroda et al., 2005; Rodda et al., 2005). Previous studies have shown that the expression levels and binding capability of transcription factors could regulate *Nanog* expression (Kuroda et al., 2005; Palacios et al., 2010; Rodda et al., 2005). Our results showed that the enhanced *Nanog* methylation reprogramming should benefit the binding of transcription factors (Palacios et al., 2010), and the expression levels of transcription factors Oct4 and Sox2 were positively correlated with *Nanog* transcription, suggesting that transcription factors regulate *Nanog* activation in cloned embryos. Interestingly, *Nanog* did not express in MII oocytes, although its methylation was low, and this may be attributed to the lack of the expression of some transcription factors (*e.g.*, Sox2).

Thus, the expression levels and binding capability of transcription factors are crucial for *Nanog* transcription, and 5-aza-dC or TSA could improve this regulation. However, the methylation status of these transcription factors during nuclear reprogramming and how 5-aza-dC or TSA cooperates with these transcription factors to improve *Nanog* expression in cloned embryos remain unclear, needing further investigation.

In conclusion, our results showed that *Nanog* methylation reprogramming was incomplete in cloned embryos, and 5-aza-dC or TSA enhanced *Nanog* methylation reprogramming. Additionally, *Nanog* expression was also improved in the 5-aza-dC or TSA treatment group due to the improvement of *Nanog* methylation reprogramming and the expression and binding of pluripotent transcription factors, thereby resulting in the developmental improvement of cloned embryos.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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