DZNep and UNC0642 enhance *in vitro* developmental competence of cloned pig embryos

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Abstract

Somatic cell nuclear transfer in mammalian cloning suffers from a faulty epigenetic reprogramming, which is believed to cause developmental failures in cloned embryos. Regulating the epigenetic-modifying enzymes can rescue the chromatin of cloned embryos from aberrant epigenetic status, thereby potentially promoting cloning efficiency. In this study, we investigated the effect of two histone methyltransferase inhibitors, namely, DZNep and UNC0642, on the *in vitro* developmental competence of cloned pig embryos. We found that (1) treatment with 10 nM DZNep or 5 nM UNC0642 for 24 h after activation had the best promoting effect on the development of cloned embryos (blastocyst rate 10.32% vs 18.08% for DZNep, and 10.44% vs 18.14% for UNC0642); (2) 10 nM DZNep and 5 nM UNC0642 significantly decreased the levels of H3K27me3 and H3K9me2, respectively, at the 2-cell, 4-cell and blastocyst stages; (3) the apoptosis level was lower in the treatment groups than in untreated control; and (4) the transcriptional expression of epigenetic genes (*EZH2*, *GLP*, *G9a*, *Setdb1*, *Setdb2*, *Suv39h1* and *Suv39h2*) was decreased and pluripotency genes (*Nanog*, *Pou5f1*, *Sox2* and *Bmp4*) was increased in treatment groups compared with control. These results indicated that treatment with DZNep and UNC0642 improves the epigenetic reprogramming of cloned embryos, which could render beneficial effect on the embryo quality and aberrant gene expression, and finally improve the developmental competence of cloned pig embryos.

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Introduction

Animal cloning via somatic cell nuclear transfer (SCNT) is an assisted reproductive technology that can reprogram terminally differentiated somatic cells to the embryonic state and further develop them into individual animals (Wilmut *et al.* 1997, Wakayama *et al.* 1998, Onishi *et al.* 2000, Polejaeva *et al.* 2000, Hochedlinger & Jaenisch 2002, Campbell *et al.* 2007). Cloning technology can be used for multiplying elite animal individuals faster and simpler than traditional breeding programs (Galli *et al.* 2012). Additionally, it can be applied to creation of genetically modified animals, conservation of endangered species and therapeutic cloning for regenerative medicine (Prather *et al.* 2003, Galli *et al.* 2012, Tachibana *et al.* 2013, Chung *et al.* 2014). Since the first cloned mammal, ‘Dolly’ the sheep, was born in 1997 (Wilmut *et al.* 1997), more than 20 cloned mammalian species, including nonhuman primate, have been successfully generated (Thuan *et al.* 2010, Liu *et al.* 2018, Matoba & Zhang 2018). Moreover, large-scale commercial cloning has been applied in some livestock, such as pigs and cattle. However, the cloning efficiency remains low in essentially all species. The live birth rate obtained from SCNT embryos is only

1%–5% (Wilmut *et al.* 2002, Campbell *et al.* 2007), which significantly limits practical application of SCNT in a cost-effective way.

Reasons behind the inefficiency of cloning have not been fully understood, but incomplete nuclear reprogramming of donor cell by the oocyte is believed to be the contributor to reduce cloning efficiency (Niemann & Wrenzycki 2000, Dean *et al.* 2001, Kang *et al.* 2001, Rideout *et al.* 2001, Santos *et al.* 2003). The nuclear reprogramming process mainly involves various epigenetic modifications, including DNA methylation and covalent modifications of histones, which are crucial in altering the chromatin structure, DNA accessibility and gene transcription, and consequently into operating ongoing biological processes (Allis & Jenuwein 2016). Studies on epigenetic patterns of embryos have shown abnormal DNA methylation/histone modification dynamics and distribution patterns in SCNT embryos compared with fertilized (*in vitro* or *in vivo* conditions) counterparts across several species (Dean *et al.* 2001, Kang *et al.* 2001, Beaujean *et al.* 2004, Cao *et al.* 2015, Huang *et al.* 2016, Zhang *et al.* 2016). These abnormal epigenetic marks (usually repressive marks) contribute to heterochromatin formation and aberrant gene expression, and therefore, adversely impact the

developmental efficiency of cloned embryos. Among the epigenetic modifications, histone methylation is of particular interest given a diverse set of methylation-relevant biological processes exist, because methylation status at different histone residues can exhibit transcriptional activation or repression effects (Nottke *et al.* 2009, Greer & Shi 2012, Hyun *et al.* 2017). Lysine residue 9 (H3K9) and lysine residue 27 (H3K27) of histone H3 were identified as transcription-repressive marks. Their methylation levels were associated with transcriptional repression and negative regulation of somatic reprogramming (Chen *et al.* 2013, Mozzetta *et al.* 2014, Cao *et al.* 2015, Pan *et al.* 2018). Aberrant H3K9 and H3K27 methylation levels were observed in pig SCNT embryos (Cao *et al.* 2015, Huang *et al.* 2016, Xie *et al.* 2016). The histone lysine demethylases which control the methylation status of lysine residues in the histone H3 (H3K4, H3K9, H3K27) were also observed to be improperly expressed during cell reprogramming in porcine and bovine SCNT embryos compared with that of *in vitro* fertilized (IVF) embryos, proposing an improper establishment of the histone methylation in SCNT embryos (Glanzner *et al.* 2018). Therefore, the histone methylation marks required modulation to sustain normal development in SCNT embryos. As a rescue strategy, overexpression of histone demethylase KDM4A and KDM4D, which specifically target H3K9 methylation (Matoba *et al.* 2014, Chung *et al.* 2015), or inhibition of histone methyltransferase (HMT) G9a, which catalyzes H3K9 methylation (Huang *et al.* 2016), could significantly promote the cloning efficiency in mice and pigs, as well as the human SCNT blastocyst formation and SCNT-derived embryonic stem cell derivation.

Several small-molecule compounds that target epigenetic modifiers have been applied to adjust epigenetic remodeling in order to improve cloning efficiency during SCNT in many species. Three categories of compounds commonly used for this purpose are DNA methyltransferase inhibitors (such as 5-aza-2'-deoxycytidine) (Enright *et al.* 2003), histone deacetylase inhibitors (such as trichostatin A and scriptaid) (Li *et al.* 2008, Zhao *et al.* 2009) and HMT inhibitors (such as BIX-01294 and GSK126) (Huang *et al.* 2016, Xie *et al.* 2016). Three-Deazaneplanocin A (DZNep) (Gannon *et al.* 2013, Ostrup *et al.* 2014) and UNC0642 (Liu *et al.* 2013, Kim *et al.* 2017) are HMT inhibitors displaying excellent separation of functional potency and cell toxicity, thus suitable for cell-based and animal studies. DZNep and UNC0642 can cause selective reductions of H3K27me3 and H3K9me2, respectively. Their effects on the development of SCNT embryos have not been reported. We here investigated the effect of treatment with DZNep and UNC0642 on the developmental rate, epigenetic status, quality and gene expression of cloned pig embryos.

Materials and methods

All chemicals were purchased from Sigma and the media were obtained from Thermo Fisher unless otherwise stated.

Fibroblasts isolation

The nuclear donor cells were adult pig fibroblasts isolated from ear skin. A small piece of skin from pig ear was cut out, and surface sterilized by soaking in 75% ethanol for 5 min. The tissue was subsequently shaved, and diced into millimeter square pieces on a Petri dish. A dissociation buffer including 0.64 g/L collagenase IV, 15% fetal bovine serum, and 2% antibiotic-antimycotic solution in DMEM was used to digest the tissue for 3 h at 38.5°C in a 5% CO₂ incubator. Afterward, the dissociated tissues were collected and pelleted by centrifuge, reseeded on a Petri dish in a DMEM containing 15% FBS and 2% antibiotic-antimycotic solution, and maintained at 38.5°C in a 5% CO₂ incubator with humidity. The fibroblasts began to creep out of the tissues the next day and further spread to reach confluence.

Oocyte collection and *in vitro* maturation

The pig ovaries were collected from a local slaughterhouse, stored in physiological saline with antibiotics, and transported to the laboratory at 37°C. Follicular fluid was aspirated from the follicles between 3 and 8 mm in diameter using a syringe. Cumulus oocyte complexes (COCs) in follicular fluid were allowed to settle at 38.5°C, and then washed three times with HEPES-buffered Tyrode's lactate (HEPES-TL). Only COCs with a uniform ooplasm and at least three layers of intact cumulus cells were subjected to *in vitro* maturation (IVM). COCs were distributed in groups of 50–100 in four-well culture plates containing 500 µL IVM medium covered with 300 µL sterile mineral oil per well, and cultured for 42–44 h under an atmosphere of 5% CO₂ in humidified air at 38.5°C. Matured COCs were vortexed in 0.1% (w/v) hyaluronidase in HEPES-TL for 3 min to strip the cumulus cells. The matured oocytes with an extruded first polar body (Metaphase II, MII) with homogenous cytoplasm were used for SCNT.

Somatic cell nuclear transfer

SCNT was performed as previously described with some minor modifications (Lai & Prather 2003). Briefly, we enucleated the MII oocytes via aspiration of the polar body, MII chromosome and a small amount of adjacent cytoplasm using a beveled glass pipette in porcine zygote medium-3 (PZM3) containing 7.5 µg/mL cytochalasin B at 38.5°C. Afterward, we injected a single intact donor cell into the perivitelline space of an enucleated oocyte with the same pipette. The reconstructed nuclear donor cell-oocyte cytoplasm complexes were simultaneously fused and activated by using two direct pulses of 1.2 kV/cm for 30 µs using a BTX Electro-Cell Manipulator. The fusion rate was evaluated by using stereomicroscopic examination. The activated embryos were transferred to four-well culture plates

containing 500 μ L PZM3 and 300 μ L mineral oil per well, and maintained in a humidified 38.5°C, 5% CO₂ incubator for 48 and 156 h to examine the cleavage and blastocyst rates, respectively. For counting cell number of embryos, blastocysts were washed twice in 100 μ L drop of PBS containing 0.1 mg/mL polyvinyl alcohol (PBS-PVA), and then transferred to a 50 μ L microdrop of Hoechst 33342 working solution (1 μ g/mL Hoechst 33342 in PBS-PVA) for nuclear staining for 10 min. The stained blastocysts were washed in PBS-PVA, mounted on slides and analyzed under the fluorescence microscope with a UV filter.

In vivo-derived embryos

A multiparous sow (Duroc) was artificially inseminated after the estrus was examined. The day of insemination was considered as day 1. The sow was sacrificed on day 7 and the uterus was excised and transported to the laboratory in physiological saline with antibiotics at 37°C. The embryos were flushed out of the uterus, and the embryos at blastocyst stage were collected and transferred into PBS-PVA. Afterward, the embryos were rinsed thrice in PBS and subjected to RNA extraction.

DZNep and UNC0642 treatment

DZNep (SML0305, Sigma) and UNC0642 (SML1037, Sigma) were dissolved in DMSO and stored at -20°C. PZM3 medium was used to dilute DZNep and UNC0642 stock solution to obtain the desired working solutions (0, 1, 10, 50 or 100 nM for DZNep, and 0, 1, 5, 10, 50 or 100 nM for UNC0642). For 0 nM treatment groups, DMSO was added into PZM3 as the culture medium. After activation, the reconstructed embryos were cultured in PZM3 containing DZNep or UNC0642 for the indicated times (0, 24, 48 or 72 h). Afterward, the treated embryos were washed thrice and cultured in fresh PZM3 without treatment until reaching the blastocyst stage.

Immunofluorescence

Ten SCNT embryos at each of 2-cell, 4-cell and blastocyst stages were collected from all compound-treated and control groups, washed in PBS-PVA, fixed in 4% paraformaldehyde (PFA) in PBS for 15 min and permeabilized using 0.1% Triton X-100 in PBS for 15 min. The embryos were then blocked in blocking solution consisting of 5% BSA (w/v) in PBS for 1 h at room temperature and stained with mouse monoclonal primary antibodies against H3K27me3 (1:200; ab6002, Abcam), H3K9me2 (1:200; ab1220, Abcam) and H3K9me3 (1:200; NBP1-30141, Novus) overnight at 4°C. After extensive washing with PBS-PVA, the embryos were incubated with the secondary antibody, Alexa Fluor 488 conjugated goat anti-mouse IgG (A11001, Thermo Fisher), for 1 h in the darkness at room temperature. The nuclei of embryos were stained with 1 μ g/mL Hoechst 33342. The images were captured using an inverted fluorescence microscope (Olympus) and the fluorescence intensity was quantified using ImageJ (NIH). For fluorescence intensity quantification, the nucleus region positive for Hoechst 33342 was manually outlined as the

measuring area, in which the green fluorescence intensity was measured after the background subtraction of the cytoplasmic area from the whole image. The values were expressed as the mean fluorescence intensity per nucleus by averaging all nuclei signals of at least three embryos (2-cell, 4-cell embryos and blastocysts).

TUNEL assay

TUNEL (TdT-mediated dUTP-digoxigenin nick end labeling) assay (KeyGen Biotech) was performed to detect apoptotic DNA fragmentation according to the manufacturer's protocol. Reconstructed embryos were split into three groups and treated with 10 nM DZNep, 5 nM UNC0642 and DMSO after activation, respectively. TUNEL assay was performed with ten blastocysts for each group. Blastocysts were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing in PBS, blastocysts were incubated in TUNEL reaction mixture (equilibration buffer + TRITC-dUTP + TdT enzyme) for 1 h in the darkness at 37°C. Furthermore, the blastocysts were counterstained with 300 nM 4,6-diamidino-2-phenylindole (DAPI) for 5 min to identify the nuclei.

Quantitative PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher) from 20 to 30 blastocysts for each treatment group. Afterward, reverse transcription was performed to synthesize cDNA. The quantitative PCR was conducted with Takara SYBR Premix Ex Taq by using cDNA as template and specific primers for each gene in a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). The amplification of each gene was performed in four replicates and the Ct values were obtained for each reaction. The transcript abundance of each gene was calculated relative to those of the internal control gene β -actin using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

All experiments were repeated at least thrice. Statistical analysis was performed with GraphPad Prism (GraphPad Software) by using one-way analysis of variance (ANOVA). All data are expressed as mean $\pm$ S.E.M. (standard error of mean). The level of significance is fixed at 0.05 (*) or 0.01 (**).

Results

Effects of DZNep and UNC0642 treatments on the in vitro development of reconstructed pig embryos

To investigate the effects of DZNep and UNC0642 treatment on SCNT embryo development, we treated pig SCNT embryos with either DZNep (0, 1, 10, 50 and 100 nM) or UNC0642 (0, 1, 5, 10, 50 and 100 nM) for 24 h. Afterward, the compounds were washed out and the embryos were cultured in fresh PZM3 until day 7. Treatment with DZNep at 1, 10 and 50 nM (Table 1), as well as UNC0642 at 1 and 5 nM (Table 2), showed increasing trends in the blastocyst rate and total cell

Table 1 Effect of 24 h treatment with DZNep at different concentrations on the developmental competence of pig cloned embryos.

Concentration of DZNep (nM) (replicates)	Embryos (n)	Cleaved embryos (n)	Cleavage rate (%)	Blastocysts (n)	Blastocyst rate (%)	Total cell number
0 (4)	148	104	70.05 ± 1.21	15	10.32 ± 1.14 ^b	45.40 ± 2.66 ^b
1 (4)	154	111	72.33 ± 3.01	22	14.35 ± 0.62 ^b	50.38 ± 1.00 ^{a,b}
10 (7)	268	201	74.96 ± 1.36	48	18.08 ± 0.87 ^a	54.70 ± 1.16 ^a
50 (4)	149	105	71.01 ± 3.59	18	12.19 ± 0.87 ^b	48.38 ± 2.33 ^b
100 (4)	167	117	70.20 ± 2.53	14	8.37 ± 0.53 ^c	44.13 ± 1.68 ^b

^{a,b,c}Values with different superscripts within the same column differ significantly ($P < 0.05$).

number of blastocysts compared with 0 nM DZNep and 0 nM UNC0642 treatment, respectively; moreover, 10 nM DZNep and 5 nM UNC0642 treatments yielded the highest blastocyst rate (18.08% vs 10.32% for DZNep, and 18.14% vs 10.44% for UNC0642, $P < 0.05$) and total cell number (54.70 vs 45.40 for DZNep, and 57.46 vs 49.71 for UNC0642, $P < 0.05$) among groups (Fig. 1 and Tables 1, 2). The cleavage rate showed a concomitant increase with the increasing blastocyst rate, but the difference was not significant. We next treated the pig SCNT embryos with 10 nM DZNep or 5 nM UNC0642 at different exposure durations (0, 24, 48 and 72 h). Results showed that 24 h treatment with either compound yielded the highest blastocyst rate (16.67% vs 11.11% for DZNep, and 18.57% vs 9.52% for UNC0642, $P < 0.05$) and total cell number (52.67 vs 42.92 for DZNep, and 50.07 vs 43.27 for UNC0642, $P < 0.05$) among groups, whereas results from the 48 and 72 h treatments were not significantly different from the 0 h treatment group (Tables 3 and 4). Afterward, we treated the cloned embryos with a mixture of 10 nM DZNep and 5 nM UNC0642 for 0, 24 and 48 h. Following the treatment, the three groups showed no significant differences in the cleavage and blastocyst rates and total cell number. However, compared with 0 h treatment, the blastocyst rate and total cell number after 24 h treatment with the mixed compounds showed a tendency to increase from 11.04% to 13.70%, and 47.00 to 52.22, respectively (Table 5).

Epigenetic effects of DZNep and UNC0642 on reconstructed pig embryos

The epigenetic status of the compound-treated SCNT embryos was evaluated by using immunofluorescence

staining and quantitative analysis. The H3K27me3 was significantly reduced following DZNep treatment compared with that in the control (Fig. 2A). Through quantifying the fluorescence intensity, the H3K27me3 marks decreased by approximately 45% ($P < 0.01$), 37% ($P < 0.01$) and 7% ($P < 0.05$) at the 2-cell, 4-cell and blastocyst stages, respectively, compared with control (Fig. 2B). The UNC0642 treatment resulted in decreased H3K9me2 (Fig. 3A and B), but not trimethylated H3K9me3 (Fig. 4A and B) at the 2-cell, 4-cell and blastocyst stages; this was consistent with previous reports, wherein UNC0642 specifically inhibited G9a, which catalyzes dimethylated states of H3K9 (Shinkai & Tachibana 2011, Liu et al. 2013). Fluorescence intensity assay showed approximately 13% ($P < 0.05$), 47% ($P < 0.01$) and 29% ($P < 0.05$) decreases in H3K9me2 marks for the embryos at 2-cell, 4-cell and blastocyst stages, respectively, compared with control (Fig. 3B).

Effects of DZNep and UNC0642 on the apoptosis status of reconstructed pig embryos

We further investigated the apoptosis level of cloned embryos exposed to 10 nM DZNep and 5 nM UNC0642. TUNEL assay of the cloned embryos showed lower TUNEL positivity after DZNep and UNC0642 treatments compared with the DMSO-treated control (Fig. 5A). Moreover, we stained the blastocyst nuclei with DAPI and found markedly higher nuclear fragmentation level in the control cloned embryos than those treated with DZNep or UNC0642 (Fig. 5A). The fragmented nuclei were mostly merged with TUNEL positivity, thereby indicating the occurrence of apoptosis in the stained cells. TUNEL result showed that DZNep and UNC0642 treatment could decrease the apoptotic index of the

Table 2 Effect of 24 h treatment with UNC0642 at different concentrations on the developmental competence of pig cloned embryos.

Concentration of UNC0642 (nM) (replicates)	Embryos (n)	Cleaved embryos (n)	Cleavage rate (%)	Blastocysts (n)	Blastocyst rate (%)	Total cell number
0 (4)	163	113	69.31 ± 0.83 ^a	17	10.44 ± 1.21 ^c	49.71 ± 1.73 ^b
1 (3)	141	101	71.16 ± 1.96 ^a	20	14.13 ± 0.85 ^b	50.60 ± 2.12 ^{a,b}
5 (5)	203	146	71.91 ± 1.79 ^a	37	18.14 ± 1.32 ^a	57.46 ± 2.24 ^a
10 (5)	208	144	69.37 ± 1.66 ^a	22	10.62 ± 0.44 ^c	50.56 ± 2.21 ^{a,b}
50 (5)	237	163	68.67 ± 1.57 ^a	20	8.73 ± 0.62 ^c	47.00 ± 2.83 ^{b,c}
100 (5)	243	148	60.68 ± 2.58 ^b	12	4.69 ± 0.99 ^d	40.75 ± 3.20 ^c

^{a,b,c,d}Values with different superscripts within the same column differ significantly ($P < 0.05$).

Figure 1 DZNep and UNC0642 treatments enhance the developmental efficiency of cloned embryos. The SCNT reconstructed embryos were treated with 10 nM DZNep or 5 nM UNC0642 for 24 h after activation, and DMSO treatment was used as control. The medium was replaced with fresh PZM3 without compound addition until day 7. Upper panel shows the representative images of cloned blastocysts at 156 h after activation from different groups under the bright field. Lower panel shows the representative images of cloned blastocysts stained with Hoechst 33342 for total cell number counting. Significantly increased blastocyst rate and total cell number can be observed in the DZNep and UNC0642 treatment groups compared with control. Scale bars = 100 μ m.

cloned pig embryos. This result was further confirmed by quantifying the expression level of *Bax*, which functions as an apoptosis activator. The blastocysts in the DZNep and UNC0642 treatment groups demonstrated a significant decrease in *Bax* level compared with DMSO-treated control group (Fig. 5B). The *Bax* level of the compound-treated embryos remained higher than that of *in vivo*-derived (IVV) embryos; however, it showed a tendency toward the low apoptotic index, thereby implying the beneficial effects of DZNep and UNC0642 treatments on the quality of cloned pig embryos.

Effects of DZNep and UNC0642 on chromatin-modifying enzymes and pluripotency factors

To investigate the underlying mechanism of the improved developmental competence of cloned embryos exposed to DZNep and UNC0642, multiple HMT genes (*EZH2*, *GLP*, *G9a*, *Setdb1*, *Setdb2*, *Suv39h1* and *Suv39h2*) and pluripotency genes (*Nanog*, *Pou5f1*, *Sox2* and *Bmp4*) were determined by using quantitative

PCR at blastocyst stage. Results showed that DZNep treatment significantly decreased the *EZH2* mRNA level, and UNC0642 significantly decreased *G9a* and *GLP* mRNA levels (Fig. 6A). Moreover, we observed decreases in other HMTs in mRNA levels, thereby likely reflecting that DZNep and UNC0642 can unselectively inhibit the expression of multiple chromatin-modifying enzymes. Pluripotency-related genes, namely *Nanog*, *Pou5f1*, *Sox2* and *Bmp4*, exhibited a great increase in compound-treated blastocysts, indicating enhanced pluripotency in SCNT embryos exposed to DZNep and UNC0642 (Fig. 6B).

Discussion

The SCNT process involves a global epigenetic remodeling. However, epigenetic reprogramming of DNA and histone is inefficient in most cloned embryos, thereby affecting cloning efficiency (Long et al. 2014, Matoba & Zhang 2018). Histone methylation plays a crucial role in the maintenance of stem cell pluripotency

Table 3 Effect of 10 nM DZNep treatment for different exposure durations on the developmental competence of pig cloned embryos.

Treatment duration (h) (replicates)	Embryos (n)	Cleaved embryos (n)	Cleavage rate (%)	Blastocysts (n)	Blastocyst rate (%)	Total cell number
0 (3)	99	77	77.78 $\pm$ 2.67	11	11.11 $\pm$ 1.01 ^b	42.92 $\pm$ 1.73 ^b
24 (3)	102	81	79.41 $\pm$ 2.40	17	16.67 $\pm$ 0.98 ^a	52.67 $\pm$ 1.27 ^a
48 (3)	99	66	73.74 $\pm$ 2.67	10	10.10 $\pm$ 1.01 ^b	43.83 $\pm$ 3.28 ^b
72 (3)	103	74	71.86 $\pm$ 1.71	9	9.69 $\pm$ 0.88 ^b	44.57 $\pm$ 2.47 ^b

^{a,b}Values with different superscripts within the same column differ significantly ($P < 0.05$).

Table 4 Effect of 5 nM UNC0642 treatment for different exposure durations on the developmental competence of pig cloned embryos.

Treatment duration (h) (replicates)	Embryos (n)	Cleaved embryos (n)	Cleavage rate (%)	Blastocysts (n)	Blastocyst rate (%)	Total cell number
0 (3)	105	80	76.19±3.43	10	9.52±0.95 ^b	43.27±1.80 ^b
24 (3)	119	94	79.05±2.79	22	18.57±0.99 ^a	50.07±1.54 ^a
48 (3)	126	92	73.02±2.86	15	11.90±1.37 ^b	46.38±2.93 ^b
72 (3)	119	91	72.86±2.25	12	10.08±0.99 ^b	44.43±2.91 ^b

^{a,b}Values with different superscripts within the same column differ significantly ($P<0.05$).

and early embryo development (Greer & Shi 2012, Hyun *et al.* 2017). Studies on mouse embryos have shown that levels of arginine methylation of H3 in four-cell blastomeres is associated with the development of inner cell mass (Torres-Padilla *et al.* 2007). H3K9 methylation has been found to be the primary epigenetic determinant during somatic cell reprogramming into induced pluripotent stem cells (iPSCs), and removal of H3K9 methylation leads to fully reprogrammed iPSCs (Chen *et al.* 2013). Additionally, H3K79me2 acts as a barrier to repress reprogramming, and inhibition of H3K79-specific methyltransferase DOT1L by shRNA-mediated knockdown or small-molecule inhibitor could facilitate iPSC generation (Onder *et al.* 2012). In pig cloning, aberrant histone methylation marks have been observed in SCNT-derived pig embryos (Cao *et al.* 2015, Huang *et al.* 2016, Xie *et al.* 2016). Selective HMT inhibitors could improve cloning efficiency by preventing the nuclear chromatin of cloned embryos from reaching the aberrant methylation status (Huang *et al.* 2016, Xie *et al.* 2016). In contrast, treatment with small-molecule inhibitor of histone demethylases decreased the cloned embryonic development (Xie *et al.* 2016).

Application of chromatin-modifying small molecules is a simple and effective method to correct aberrant epigenetic reprogramming. Multiple HMT inhibitors have been identified but few have been investigated for their effects on SCNT reprogramming. DZNep can reduce histone methylation level by disrupting the activity of HMT EZH2 (Tan *et al.* 2007). The latter is a functional enzymatic component of the polycomb repressive complex 2 that functions to silence tumor-suppressor genes by catalyzing the addition of three methyl groups to H3K27, which is a modification that leads to chromatin condensation and transcriptional repression (Cao *et al.* 2002, Margueron & Reinberg 2011). UNC0642 can reduce H3K9me2 level by selectively inhibiting HMTs G9a and GLP (Liu *et al.* 2013), which can mono- or dimethylate H3K9 (Shinkai & Tachibana

2011). A previous study has shown that UNC0642 can enhance chromatin accessibility and induce transcriptional activation within the corresponding regions (Kim *et al.* 2017). Both of them harbor potential for activating embryonic chromatin, thereby promoting the development competence of cloned embryos.

In this study, we examined the effects of DZNep and UNC0642 treatments on the *in vitro* development of pig SCNT embryos. The results showed that the two compounds could improve the *in vitro* development of pig SCNT embryos by reducing the levels of H3K27me3 or H3K9me2 at the 2-cell, 4-cell, and blastocyst stages. Incubation of SCNT embryos with varying concentrations of the two compounds revealed that 10 nM DZNep or 5 nM UNC0642 showed the best promoting effect on pig SCNT embryo development, whereas increasing the dosage rendered detrimental effects, as manifested by significantly decreased blastocyst rate. Furthermore, long-term treatment (48 or 72 h) or combined use of the compounds showed less or no improvement to the embryos. This detrimental effect might be attributed to the complete dysfunction of the corresponding chromatin-modifying enzymes, EZH2 and G9a, which are targeted by the compounds, because these enzymes are required for early embryonic development. Either EZH2- or G9a-knockout mice has shown embryonic lethality at early stages of mouse development (O'Carroll *et al.* 2001, Tachibana *et al.* 2002, 2005). These data indicated that an appropriate but nontoxic dose and exposure duration of the chromatin-modifying compounds should be determined before treatment. TUNEL assays showed that treatment using either compound significantly lowered the apoptosis level in cloned embryos compared with the untreated control, which corroborated with the beneficial effect of the two compounds in terms of improved blastocyst rate.

Our findings are in line with previous studies using embryos of mouse, pig and other species, in which loss or inhibition of aberrant histone methylation

Table 5 Effect of combined treatment of 10 nM DZNep and 5 nM UNC0642 for different exposure durations on the developmental competence of pig cloned embryos.

Treatment duration (h) (replicates)	Embryos (n)	Cleaved embryos (n)	Cleavage rate (%)	Blastocysts (n)	Blastocyst rate (%)	Total cell number
0 (3)	91	70	77.18±2.73	10	11.04±1.24	47.00±1.27
24 (3)	124	94	75.80±1.13	17	13.70±0.52	52.22±1.62
48 (3)	106	82	77.33±1.81	12	11.30±1.54	49.43±1.13

Figure 2 H3K27me3 modification is decreased in cloned pig embryos treated with 10 nM DZNep for 24 h. (A) Immunofluorescence analysis of H3K27me3 levels of cloned embryos, at 2-cell, 4-cell and blastocyst stages, treated with 10 nM DZNep and DMSO control. (B) Mean fluorescence intensity quantified for the H3K27me3 level. H3K27me3 signals following DZNep treatment in 2-cell, 4-cell embryos and blastocysts are significantly lower than those in DMSO-treated embryos. *P* values represent the significance of difference in fluorescence signals between DZNep and DMSO treatment groups. **P* < 0.05, ***P* < 0.01. Scale bars = 100 μ m.

Figure 3 H3K9me2 modification is decreased in cloned pig embryos exposed to 5 nM UNC0642 for 24 h. (A) Representative immunofluorescence images showing H3K9me2 levels of cloned embryos, at 2-cell, 4-cell and blastocyst stages, exposed to 5 nM UNC0642 and DMSO control. (B) Quantification of fluorescence intensity showing a significant decrease of H3K9me2 levels in UNC0642-treated embryos compared with DMSO-treated controls. **P* < 0.05 and ***P* < 0.01 between UNC0642 and DMSO treatment groups. Scale bars = 100 μ m.

Figure 4 UNC0642 treatment does not affect H3K9me3 levels of cloned pig embryos. (A) Representative immunofluorescence images of H3K9me3 levels of cloned embryos at different developmental stages after UNC0642 treatment. (B) Mean fluorescence intensity quantified for the H3K9me3 levels. No significant difference in fluorescence intensity are observed between UNC0642 and DMSO treatment groups. Scale bars = 100 μ m.

significantly improved cloning efficiency (Matoba *et al.* 2014, 2018, Huang *et al.* 2016, Xie *et al.* 2016, Cao *et al.* 2017). Treatment with 50 nm BIX-01294 (G9a inhibitor) for 14–16 h after activation could enhance the developmental competence of pig SCNT embryos *in vitro* (blastocyst rate 16.4% vs 23.2%) and *in vivo* (cloning rate 1.59% vs 2.96%) (Huang *et al.* 2016). Removal of the H3K9me3 barrier by mRNA injection

of a H3K9me3-specific demethylase, *Kdm4d*, allowed cloned mouse embryos to develop at a rate similar to that of IVF embryos (88.6% blastocyst rate and 7.6% cloning rate using cumulus cells as donor vs 26.0% and 0%, respectively) (Matoba *et al.* 2014). They further improved the mouse cloning efficiency to more than 20% by using a combination of *Xist* knockout donor cells and overexpression of *Kdm4d*. Although such a high

Figure 5 Detection of apoptotic index in cloned blastocysts. (A) TUNEL assay on cloned blastocysts from control, 10 nM DZNep and 5 nM UNC0642 treatment groups. DAPI staining showing significant DNA fragmentations (white arrows) in control group. (B) Significantly decreased *Bax* mRNA expression in DZNep-, and UNC0642-treated blastocysts compared with control. * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m. IVV, *in vivo*-derived embryos.

Figure 6 Relative mRNA abundance of HMT genes (*EZH2*, *GLP*, *G9a*, *Setdb1*, *Setdb2*, *Suv39h1* and *Suv39h2*) (A) and pluripotency genes (*Nanog*, *Pou5f1*, *Sox2* and *Bmp4*) (B) in cloned blastocysts produced following treatment with 10 nM DZNep or 5 nM UNC0642 for 24 h. The expression levels were normalized against *actin*. * $P < 0.05$, ** $P < 0.01$ between the two groups. IVV, *in vivo*-derived embryos.

cloning efficiency can be achieved through combining multiple approaches to remove reprogramming barriers, defective postimplantation development and placental overgrowth were still commonly observed (Matoba *et al.* 2018). This result implies additional barriers exist for high-efficiency animal cloning, and simultaneous modulation of multiple epigenetics marks could facilitate further improvement of cloning efficiency.

Examining the expression levels of the multiple HMTs showed that *EZH2* and *G9a/GLP* expressions in the SCNT blastocysts were significantly inhibited by DZNep and UNC0642 treatments, respectively. However, we also observed inhibitory effects to other HMTs, including *Setdb1*, *Setdb2*, *Suv39h1* and *Suv39h2*. This result suggested that DZNep and UNC0642 may

be nonspecific HMT inhibitors. A previous report has shown that DZNep targets *Setdb1* at the transcription level in human cancer cells, thereby implicating DZNep to inhibit diverse epigenetic targets (Lee & Kim 2013). Another study has also reported that DZNep globally inhibits histone methylation and is nonselective, and the authors observed the nonselective inhibitory effect of some of the other HMT inhibitors on histone methylation (Miranda *et al.* 2009). The mechanism for broad-spectrum inhibition to HMT of the two compounds remains elusive. Inhibition of *EZH2* by DZNep was reportedly achieved through a proteolytic degradation process (Tan *et al.* 2007). However, our data showed these HMTs were regulated at transcriptional level by DZNep, implying a different mechanism of DZNep in regulating the expression of some HMTs. In addition, there is evidence that UNC0642 could inhibit *EZH2*, although less selective than *G9a* and *GLP* (Liu *et al.* 2013). The selectivity of the compounds may vary in different cells or embryos.

Given that epigenetic changes can act as regulatory switches of gene transcription, which is associated with the modulation of pluripotency in the embryos, we determined the expression levels of pluripotency-related genes *Nanog*, *Pou5f1*, *Sox2* and *Bmp4* in the compound-treated SCNT embryos. All these factors showed a marked increase after treatment with either of the two compounds, indicating an enhancement of pluripotency in the SCNT embryos. Improvement of epigenetic reprogramming might result in the increased gene transcripts of pluripotency factors, and thus contribute to the improved developmental competence of SCNT embryos.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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