

BIX-01294 increases pig cloning efficiency by improving epigenetic reprogramming of somatic cell nuclei

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Abstract

Accumulating evidence suggests that faulty epigenetic reprogramming leads to the abnormal development of cloned embryos and results in the low success rates observed in all mammals produced through somatic cell nuclear transfer (SCNT). The aberrant methylation status of H3K9me and H3K9me2 has been reported in cloned mouse embryos. To explore the role of H3K9me2 and H3K9me in the porcine somatic cell nuclear reprogramming, BIX-01294, known as a specific inhibitor of G9A (histone-lysine methyltransferase of H3K9), was used to treat the nuclear-transferred (NT) oocytes for 14–16 h after activation. The results showed that the developmental competence of porcine SCNT embryos was significantly enhanced both *in vitro* (blastocyst rate 16.4% vs 23.2%, $P < 0.05$) and *in vivo* (cloning rate 1.59% vs 2.96%) after 50 nm BIX-01294 treatment. BIX-01294 treatment significantly decreased the levels of H3K9me2 and H3K9me at the 2- and 4-cell stages, which are associated with embryo genetic activation, and increased the transcriptional expression of the pluripotency genes *SOX2*, *NANOG* and *OCT4* in cloned blastocysts. Furthermore, the histone acetylation levels of H3K9, H4K8 and H4K12 in cloned embryos were decreased after BIX-01294 treatment. However, co-treatment of activated NT oocytes with BIX-01294 and Scriptaid rescued donor nuclear chromatin from decreased histone acetylation of H4K8 that resulted from exposure to BIX-01294 only and consequently improved the preimplantation development of SCNT embryos (blastocyst formation rates of 23.7% vs 21.5%). These results indicated that treatment with BIX-01294 enhanced the developmental competence of porcine SCNT embryos through improvements in epigenetic reprogramming and gene expression.

Reproduction (2016) 151 39–49

Introduction

Pigs have been widely used in biomedical applications for decades as animal models for human diseases and as genetically defined models for surgery and xenotransplantation because these animals have an anatomy and physiology that are similar to humans (Prather *et al.* 2003, Samiec & Skrzyszowska 2011*a,b*). Advances in pig genome annotation, genetic modification (GM) of pig cells and somatic cell nuclear transfer (SCNT), have facilitated the generation of GM pigs for various biomedical applications. However, the establishment of porcine embryonic stem cells (ESCs) has proven elusive, despite decades of intense trials, and a similar lack of success has been observed with other ungulate species (Esteban *et al.* 2010). The generation of GM pigs primarily relies on homologous recombination in somatic cells, followed by SCNT.

SCNT represents a promising technique for the reprogramming of terminally differentiated somatic

cells into totipotent states by transplanting a donor nucleus into an enucleated oocyte (Wilmot *et al.* 1997, Wakayama *et al.* 1998, Hochedlinger & Jaenisch 2002, Geijsen *et al.* 2004, Samiec & Skrzyszowska 2012, Samiec & Skrzyszowska 2014). However, the cloning efficiency of SCNT is extremely low, which hinders the application of GM pigs in biomedical research. Accumulating evidence suggests that defective epigenetic reprogramming of DNA and histone (Dean *et al.* 2001, Kang *et al.* 2001, Santos *et al.* 2003, Ohgane *et al.* 2004, Inoue *et al.* 2010) and abnormal gene expression profiles (Humpherys *et al.* 2002, Inoue *et al.* 2002) are likely associated with the overall low success rate of cloning. In addition to SCNT, the generation of induced pluripotent stem cells (iPSCs) through the ectopic expression of four factors, OCT4/SOX2/KLF4/C-MYC or OCT4/SOX2/NANOG/LIN28, provides another strategy for reprogramming terminally differentiated somatic cells into pluripotency and shows great potential for

their use in producing GM animals (Takahashi & Yamanaka 2006, Takahashi *et al.* 2007, Yu *et al.* 2007, Hanna *et al.* 2008). However, several studies have suggested that somatic reprogramming through SCNT is more reliable than the ectopic expression of reprogramming factors (Deuse *et al.* 2014, Li *et al.* 2014, Ma *et al.* 2014).

Therefore, it is necessary to investigate the underlying somatic reprogramming mechanism in SCNT to increase the cloning efficiency and facilitate the study of iPS. A number of strategies to improve porcine cloning efficiency have been investigated. One of the most successful strategies involves the application of small molecules to regulate epigenetic modifications. Histone deacetylase inhibitors (HDACi), such as Scriptaid, significantly increase the overall cloning efficiency through improvements in the histone acetylation on Histone 4 at lysine 8 (H4K8ac) in a pattern similar to that of the IVF embryos (Zhao *et al.* 2010). Other HDACi, such as *m*-carboxycinnamic acid *b*ishydroxamide (Song *et al.* 2014), trichostatin A (Li *et al.* 2008) and valproic acid (Kim *et al.* 2011), have also been demonstrated to be beneficial to the *in vitro* development of porcine SCNT embryos (Miyoshi *et al.* 2010, Zhao *et al.* 2010, Samiec *et al.* 2015).

Histone lysine methylation plays an important role in the organization of chromatin domains and the regulation of gene expression. The aberrant reprogramming of histone methylation on histone 3 at lysines 4, 9 and 27 (Zhang *et al.* 2009, Matoba *et al.* 2014) has been observed in clone embryos. BIX-01294 (diazepinquinazolin-amine derivative) has been identified as a pharmacological inhibitor of G9A histone-lysine methyltransferase (HMT), and this inhibitor selectively impairs the generation of H3K9me2 *in vitro* (Kubicek *et al.* 2007). The inhibition of G9A HMT through BIX-01294 improves the transducing efficiency of pluripotency genes for reprogramming somatic cells into iPSCs (Shi *et al.* 2008a,b, Feng *et al.* 2009).

Hence, in the present study, we investigated the role of histone lysine methylation in pig somatic reprogramming by using BIX-01294 to treat nuclear-transferred (NT) oocytes after activation. The results showed that BIX-01294 treatment enhances the developmental competence of porcine SCNT embryos both *in vitro* and *in vivo*. BIX-01294 treatment rescued the somatic cell-inherited nuclear chromatin of cloned embryos from aberrant status of H3K9me and H3K9me2, and improved the transcriptional expression of the pluripotency genes *OCT4*, *NANOG* and *SOX2*.

Materials and methods

Animals

The pigs were raised at the Beijing Farm Animal Research Center and provided *ad libitum* access to water and a commercial pig diet (nutrient levels according to the NRC

during the entire experimental period. All experiments involving animals were performed in accordance with the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

Media and reagents

All chemicals were purchased from Sigma unless otherwise stated. All of the following solutions and media were filtered using a 0.22- μ m filter.

The oocyte *in vitro* maturation (IVM) medium used was TCM 199 (Gibco BRL) supplemented with 0.1% polyvinylalcohol (PVA) (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 1 μ g/ml gentamicin, 0.57 mM cysteine, 0.5 μ g/ml luteinizing hormone, 0.5 μ g/ml follicle-stimulating hormone, and 10 ng/ml epidermal growth factor. The fusion medium contained 0.3 M mannitol, 1.0 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM HEPES, adjusted to a final pH of 7.0–7.4. The embryo culture medium was porcine zygote medium 3 (PZM3), pH 7.4, supplemented with 3 mg/ml BSA (Yoshioka *et al.* 2002). The oocyte manipulation medium contained 9.5 g TCM-199 powder, 0.05 g NaHCO₃, 0.75 g HEPES, 0.05 g penicillin, 0.06 g streptomycin, 1.755 g NaCl, and 3.0 g BSA, in a final volume of 1 l in Milli-Q water (Millipore, Billerica, MA, USA), pH 7.2–7.4 (Lai & Prather 2004).

Primary cell establishment and donor cell preparation

Landrace fetal fibroblast cells (PEFs) were established as previously described (Lai & Prather 2003). Briefly, 35-day-old fetuses were recovered and rinsed three times with PBS. After removal of the head, intestine, liver, heart and limbs, the remaining tissues were finely minced into pieces (1 mm³) using scissors in Dulbecco's phosphate-buffered saline (DPBS, Gibco BRL). The minced tissues were digested with Collagenase (200 U/ml) and DNase I (25 KU/ml) in DMEM containing 15% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) for 4–5 h at 38.5 °C and 5% CO₂ in humidified air. After digestion and rinsing, the cell pellet was seeded in a 75 cm² culture flask and cultured until confluence. After reaching confluence, the cells were frozen in FBS containing 10% DMSO.

In vitro oocyte maturation

The ovaries were collected from prepubertal gilts at a local slaughterhouse, stored in saline and transported to the laboratory at 37 °C. Follicles between 3 and 6 mm in diameter were aspirated using an 18 gauge needle attached to a 10 ml syringe. Cumulus-oocyte complexes (COCs) in follicular fluid were gravity settled at 38.5 °C. The COCs were rinsed three times with HEPES-buffered Tyrode medium containing 0.01% PVA in an incubator at 38.5 °C. Only COCs with multiple layers of intact cumulus cells and a uniform ooplasm were selected for IVM. After washing three times in IVM medium, a group of 70–80 COCs were plated onto four-well cell culture plates (Nunc, Roskilde, Denmark) containing 500 μ l of IVM medium and 350 μ l of mineral oil per well. The COCs were cultured for 42–44 h at 38.5 °C and 5% CO₂ in humidified air (100% humidity). Matured COCs were subsequently vortexed

in 0.1% hyaluronidase in HEPES-buffered Tyrode medium containing 0.01% PVA for 4 min to remove the cumulus cells (Lai & Prather 2003). Only matured oocytes with an extruded first polar body (PB) with uniform cytoplasm were used for further *in vitro* development.

Parthenogenetic activation

The parthenogenetic activation of the matured oocytes was accomplished through two direct current pulses (1-s interval) of 1.2 kV/cm for 30 μ s using a BTX Electro-cell Manipulator 200 (BTX, San Diego, CA, USA) in fusion medium. Subsequently, the activated oocytes were transferred and incubated in four-well plates containing 500 μ l of PZM3 and 350 μ l of mineral oil per well at 38.5 °C and 5% CO₂ in humidified air (Lai & Prather 2003).

IVF

IVF was performed as previously described (Hao *et al.* 2006). Briefly, MII oocytes were washed three times in modified Tris-buffered fertilization medium (mTBM) containing 2 mg/ml of BSA and 2 mM caffeine. Approximately 30–35 oocytes were transferred onto 50 μ l droplets of fertilization medium and covered with mineral oil equilibrated for 48 h at 38.5 °C in 5% CO₂ in humidified air. Porcine semen was washed three times through centrifugation with DPBS, supplemented with 1 mg/ml BSA (pH 7.3) (1900 g, 4 min), and resuspended in mTBM fertilization medium to a concentration of 1×10^6 cells/ml. Approximately 50 μ l of the sperm suspension was added to the fertilization droplets, generating a final sperm concentration of 0.5×10^6 cells/ml. The oocytes were co-incubated with sperm for 4–6 h. After fertilization, the oocytes were washed three times and cultured in 500 μ l PZM3 medium in four-well Nunclon dishes at 38.5 °C in 5% CO₂ in humidified air.

Somatic cell nuclear transfer (SCNT)

SCNT was performed as previously described (Lai & Prather 2003). Briefly, MII oocytes were placed in manipulation medium supplemented with 7.5 mg/ml cytochalasin B and enucleated through the aspiration of the PB and MII chromosomes and a small amount of the surrounding cytoplasm using a 17–20 mm beveled glass pipette. A single donor cell was introduced into the perivitelline space and placed adjacent to the recipient cytoplasm. The resulting nuclear donor cell-cytoplasm complexes were placed in electrofusion medium for subsequent induction of simultaneous fusion and electrical activation of reconstituted oocytes. The couplets were washed and incubated for 20 min in PZM3 medium and evaluated for fusion using a stereomicroscope. Reconstructed embryos were cultured in four-well cell culture plates containing 500 μ l of PZM3 medium at 38.5 °C and 5% CO₂ in humidified air.

Postactivation and embryo culture

Stock solutions of BIX-01294 and Scriptaid were separately dissolved in water and DMSO at 10 and 1 mM, respectively, and stored at –20 °C. Following electrical activation or

fertilization, the SCNT or IVF embryos were treated with various concentrations of BIX-01294 and Scriptaid for 14–16 h in PZM3. After treatment, the embryos were washed three times before transferring into a four-well cell culture plate containing 500 μ l PZM3 medium, followed by cultivation at 38.5 °C in 5% CO₂ in humidified air either overnight or for 6 days. Cleavage and blastocyst formation were evaluated on days 2 and 6, respectively, and the day of SCNT or IVF was designated as day 0.

Embryo transfer

Day 1 SCNT zygotes were transferred to the oviducts of surrogates on the day of or 1 day after the onset of estrus. Pregnancy was diagnosed on day 25 (day 0 was the day of SCNT) and regularly assessed at 2-week intervals through ultrasound examination. All of the cloned piglets were delivered through natural labor on day 114 of gestation and were then hand raised. All animals were treated according to a preapproved institutional animal care and use protocol.

Indirect immunofluorescence

Embryos of every stage derived from PA and SCNT were washed in PBS, fixed for 15 min in 4% paraformaldehyde (PFA) in PBS, and permeabilized using 0.1% Triton X-100 in PBS for 30 min. After permeabilization, the embryos were blocked in 5% BSA in PBS for 1 h at room temperature (RT). The samples were stained with primary antibodies against H3K9me (1:500, Abcam, Cambridge, MA, USA) or H3K9me2 (1:500, Abcam) overnight at 4 °C, according to the manufacturer's instructions. After extensive washing with PBS containing 0.1% PVA, the samples were treated with the secondary antibody, Alexa Fluor 488 goat anti rabbit IgG (1:200) for 1 h at RT, according to the manufacturer's instructions (ZSGB-Bio, Beijing, China). After washing three times with PBS containing 0.1% PVA, the embryos were mounted onto slides in mounting medium containing 15 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). Day 6 blastocysts with good morphology were selected for counting the total number of nuclei. After fixation in 4% PFA in PBS for 15 min and permeabilization with 0.1% Triton X-100 in PBS for 30 min at RT, the embryos were mounted onto slides in mounting medium containing DAPI. Embryos stained without primary or secondary or both antibodies were used as negative controls to examine the specificity of the reaction. At least 20 oocytes or embryos were processed for each treatment group, and the experiments were replicated three times. The slides were analyzed using an epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a digital camera. The images were captured and quantified using the Nikon NIS element software. To make relative comparisons, the settings for exposure and image capture remained constant, and all images were assembled without any adjustment in contrast or brightness.

Quantitative real-time PCR

To investigate the abundance of mRNA in porcine embryos, 50–100 embryos were collected for each stage. Total RNA was

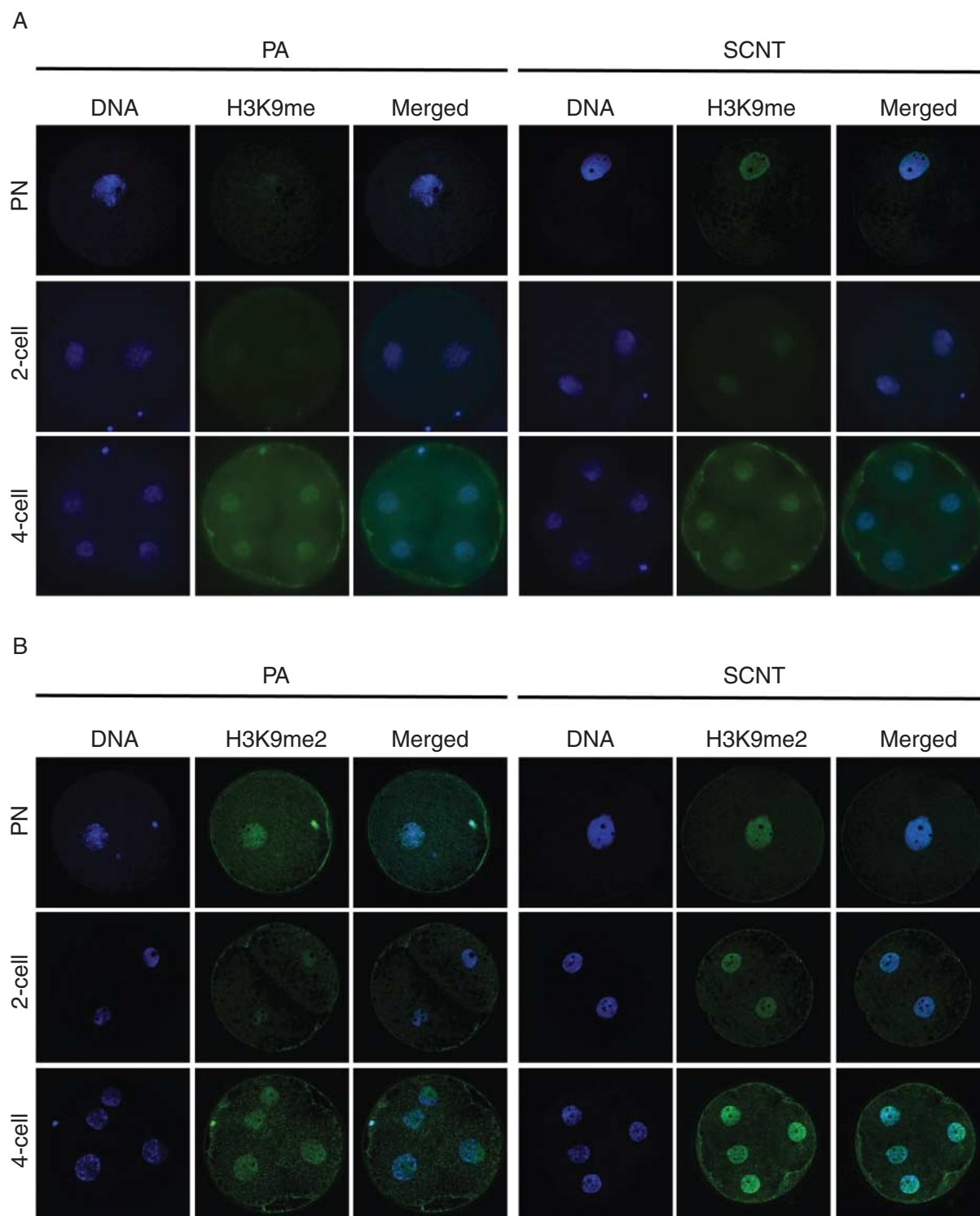


Figure 1 Temporal and spatial abundance of H3K9me and H3K9me2 histone modifications in porcine embryonic development. (A) Immunofluorescence detection of H3K9me in porcine embryo cells at early embryonic stages (PN, 2- and 4-cell) derived from PA and SCNT. H3K9me protein (green) was probed with rabbit anti-H3K4me antibodies (1:500) and detected using Alexa 488-conjugated goat anti-rabbit antibodies (1:200). The nuclei (blue) were labeled with DAPI stain. (B) Immunofluorescence detection of H3K9me2 in porcine embryo cells at early embryonic stages (PN, 2- and 4-cell) derived from PA and SCNT. H3K9me2 protein (green) was probed with rabbit anti-H3K4me antibodies (1:500) and detected using Alexa 488-conjugated goat anti-rabbit antibodies (1:200). The nuclei (blue) were labeled with DAPI stain. The original magnification was $\times 400$ for all embryos. Notably, the aberrant reprogramming of H3K9me and H3K9me2 are observed at the 2- and 4-cell stages in porcine somatic cell-cloned embryos. H3K4me3 was decreased at the 4-cell and blastocyst stages.

extracted from the samples using the Qiagen AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions. After RNA isolation, reverse transcription was conducted using the TIANscript RT Kit (Tiagen, Beijing, China). The synthesized cDNA was used for quantitative real-time PCR. The housekeeping gene, *H2AFZ*, was used as an internal control (primer sequences are shown in Supplementary Table 1, see section on supplementary data given at the end of this article). The PCR was conducted using TaKaRa SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan). Primer validation tests were conducted for each designed primer to verify that the amplification efficiencies were similar for each cycle. The program used for the PCR included an initial temperature of 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Real-time fluorescence data were collected during the extension time. The relative quantification method based on the comparative values for the threshold cycles (Ct) was used to identify the abundance of the message. The transcript abundance of each gene was calculated relative to that of the internal control gene *H2AFZ*. ΔCt was calculated by subtracting the Ct values of each gene from the Ct values for *H2AFZ*. The control group Ct values served as calibrators and were subsequently used to obtain $\Delta\Delta Ct$ values. Fold differences in transcript abundance were obtained using the Default $2^{-\Delta\Delta Ct}$. At least three biological and three experimental replicates were used for each assay. The quantitative real-time PCR results were compared through one-way ANOVA using the Statistical Analysis System software SAS 6.12 (SAS Institute, Cary, NC, USA). Differences with $P < 0.05$ were considered significantly different.

Statistical analysis

All experiments were repeated with at least three replicates. Differences in the relative expression assayed through qPCR, immunofluorescence intensity assayed, average blastocyst cell numbers were tested for significance using one-way ANOVA using SAS 6.12 (SAS Institute). Differences in cleavage, blastocyst rates, percentage of apoptosis cells and clone efficiency were tested for significance using χ^2 . The data were considered significant when the P value was less than 0.05 (*) or 0.01 (**).

Results

Aberrant reprogramming of H3K9me and H3K9me2 are observed at the 2- and 4-cell stages in porcine somatic cell-cloned embryos

To determine whether porcine SCNT-produced embryos exhibit abnormal histone methylation modifications, the levels of H3K9me and H3K9me2 in PA-produced and SCNT-cloned embryos during preimplantation development were investigated. As shown in Fig. 1, the nuclei of both cloned and PA embryos from the PN to the blastocyst stage were all positively marked with the H3K9me and H3K9me2, which is consistent with the results of a previous report (Park *et al.* 2012). SCNT-derived embryos showed no marked difference in

the pattern of H3K9me and H3K9me2 at the PN and blastocyst stages but did show a significantly high abundance of H3K9me and H3K9me2 in 2- and 4-cell stage embryos compared with PA embryos. These results suggest that the aberrant reprogramming of H3K9me and H3K9me2 took place during preimplantation development of somatic cell-cloned embryos.

BIX-01294 treatment improved the developmental competence of SCNT-derived embryos in vitro and in vivo.

We hypothesized that the rescue of nuclear donor cell-derived chromatin from aberrant reprogramming of H3K9me and H3K9me2 would improve the developmental competence of cloned embryos. The effects of BIX-01294, a confirmed inhibitor of G9A HMT (HMT of H3K9), on embryo development were examined. After activation, SCNT embryos were treated with various concentrations of BIX-01294 at 0 (control), 5, 50, and 500 nM or 5 μ M for 14–16 h. The results showed that 50 nM BIX-01294 treatment significantly increased the development (defined as the rate of blastocysts) of SCNT embryos compared with untreated and other treatment groups ($P < 0.05$) (Table 1). Therefore, 50 nM BIX-01294 treatment was applied for all subsequent experiments. We observed that 50 nM BIX-01294 treatment had no effect on the 48 h cleavage rate. However, the 144 h blastocyst rate was significantly improved compared with untreated SCNT embryos, similar to the Scriptaid treated group ($P < 0.05$) (Table 2). Additionally, BIX-01294 treatment did not affect the quality of the blastocysts, determined as the total cell number. BIX-01294 and Scriptaid treatment had no effects on porcine parthenogenetic development, determined as the cleavage and blastocyst rates (Table 2).

To examine the effects on full-term development, 50 nM BIX-01294-treated day 1 SCNT zygotes were transferred to surrogates. Two of the three surrogates became pregnant in the BIX-01294 treatment group, and 15 piglets were born with 2.96% cloning efficiency

Table 1 Effect of dose-dependent BIX-01294 treatment on the preimplantation development of SCNT-derived embryos.

Treatment	Replications	No. of embryos cultured	No. of cleavages	No. of blastocysts
0 nM	3	325	288 (88.6) ^a	56 (17.2) ^c
5 nM	3	196	169 (86.2) ^a	32 (18.9) ^b
50 nM	3	196	173 (88.3) ^a	47 (24.0) ^a
500 nM	3	194	170 (87.7) ^a	32 (16.5) ^c
5 μ M	2	97	0	0

Superscript alphabets (a and b) represents values with different BIX-01294 within a column are significantly different ($P < 0.05$). Cleavage percentage: No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage: No. of blastocysts/No. of embryos cultured. In one replication, the 5- μ M treatment was not run.

Table 2 Effect of BIX-01294 treatment at a concentration of 50 nM on the preimplantation development of SCNT- and PA-derived embryos.

Group	Treatment	Replications	No. of embryos cultured	No. of cleavages	No. of blastocysts	Average total cell number
SCNT	BIX-01294	3	336	295 (87.8) ^a	78 (23.2) ^a	32.71 ± 2.008 ^a
	Scriptaid	3	360	306 (88.5)	78 (21.7) ^a	33.70 ± 1.794 ^a
	Control	3	470	416 (88.5) ^a	77 (16.4) ^b	34.00 ± 1.618 ^a
PA	BIX-01294	3	128	119 (93.0) ^a	32 (25) ^a	/
	Scriptaid	3	128	116 (90.6) ^a	25 (19.5) ^a	/
	Control	3	128	118 (92.2) ^a	21 (16.4) ^a	/

Superscript alphabets (a and b) represents values with different treatment within a column are significantly different ($P < 0.05$). Cleavage percentage: No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage: No. of blastocysts/No. of embryos cultured. Cell number in blastocysts: mean ± s.e.m. Average total cell number of blastocyst derived from PA was not tested.

(Table 3). A total of 12 of the 15 piglets were healthy (three died at 2 days after birth). Whereas only one of the three surrogates became pregnant in the untreated group, eight piglets were born with 1.59% cloning efficiency (Table 3). BIX-01294-treated SCNT embryos could successfully develop to term with higher cloning efficiency compared with their counterparts from the non-treated group. Additional embryo transfer studies are needed to further confirm the improved cloning efficiency through BIX-01294 treatment. Both the *in vitro* and *in vivo* results suggested that BIX-01294 improved porcine SCNT embryonic development with no detrimental effects.

BIX-01294 treatment rescued the nuclear donor cell-inherited chromatin of SCNT embryos at 2- and 4-cell stages from aberrant levels of H3K9me and H3K9me2

To test whether the PEF-descended nuclear chromatin of SCNT embryos would be rescued from enhanced levels of H3K9me and H3K9me2 after BIX-01294 treatment, the abundance of H3K9me and H3K9me2 in both BIX-01294-treated cloned embryos and untreated cloned embryos were investigated during their preimplantation development. As shown in Fig. 2, after treatment with 50 nM BIX-01294 for 14–16 h, H3K9me at the 2-cell stage was decreased, and H3K9me2 at the 2- and 4-cell stages was also decreased compared with the untreated group (Fig. 2, $P < 0.01$). These results suggested that the global histone methylation levels of H3K9 in SCNT embryos recovered to the natural reprogramming state after BIX-01294 treatment, likely reflecting the improved development of the SCNT embryos (Table 4).

Combined treatment with BIX-01294 and Scriptaid improved the development of porcine SCNT embryos through a partial rescue of somatic cell-inherited chromatin from decreased histone acetylation triggered by BIX-01294 treatment

Previous studies suggested that epigenetic reprogramming of somatic cell nuclei during preimplantation development of mammalian cloned embryos could be significantly improved after increasing the levels of histone acetylation in donor nuclear chromatin (Zhao *et al.* 2009). Thus, it would be important to determine whether the improved reprogramming of somatic cell-derived nuclear genome that resulted from BIX-01294 treatment of SCNT embryos reflects in the hyperacetylation of lysine residues within histones forming the nucleosomal core of chromatin. The histone acetylation levels of H3K9, H4K8 and H4K12 in PN stage embryos derived from SCNT were examined after BIX-01294 treatment. Interestingly, as shown in Fig. 3, the histone acetylation of H4K8 and H4K12 was decreased at the pronuclear stage after the BIX-01294 treatment. These results suggest that combined treatment with Scriptaid (a histone deacetylase inhibitor) and BIX-01294 might improve the development of SCNT embryos through increased histone acetylation, compared to the treatment with BIX-01294 only. To examine this possibility, Scriptaid (250 nM) was used together with BIX-01294 (25 nM) to treat the reconstructed embryos, and the results confirmed that the combined treatment significantly increased the acetylation profile of H4K8. Nevertheless, we also observed that combined treatment with Scriptaid and BIX-01294 improved SCNT embryo development (blastocyst rate 23.7% vs 21.5%).

Table 3 Full-term development of SCNT-derived embryos following BIX-01294 treatment.

Cell type	No. of transferred embryos	BIX-01294 treatment	Pregnancy check	Piglets born	Cloning efficiency
PEFs	152	+	+	4 live	2.96%
PEFs	180	+	+	8 live+3 dead	
PEFs	174	+	–	0	
PEFs	164	–	+	8 live	1.59%
PEFs	181	–	–	0	
PEFs	158	–	–	0	

Clone efficiency: No. of piglets born/No. of transferred embryos.

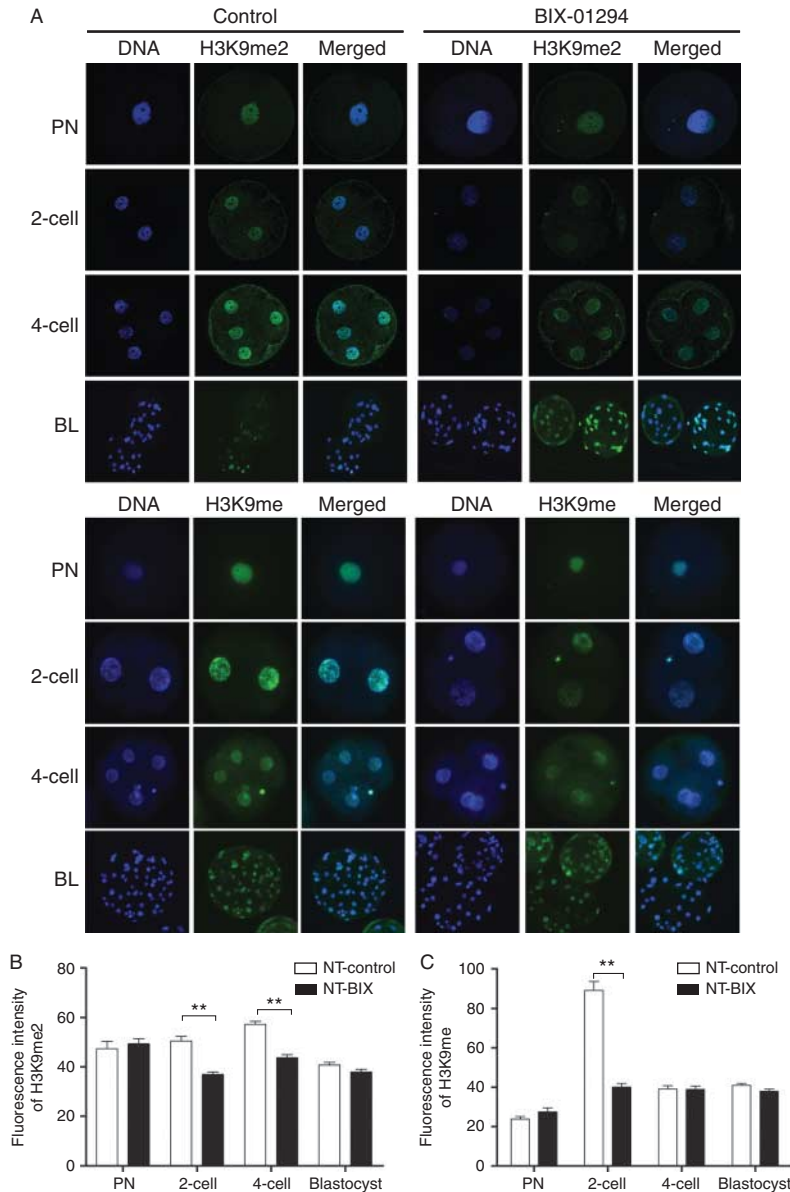


Figure 2 (A) Immunofluorescence detection of H3K9me and H3K9me2 in PN embryos, at the 2-cell, 4-cell and blastocyst stages, derived from non-treated (NT-Control) and 50 nM BIX-01294 treatment groups (NT-BIX). Original magnification was $\times 400$ for PN, 2- and 4-cell embryos and $\times 200$ for blastocyst embryos. (B) Average optical intensity was measured using Nikon NIS element software. The values are presented as the mean \pm S.E.M. H3K9me2 signals after BIX-01294 treatment in 2- and 4-cell embryos are significantly lower than those in non-treated embryos ($P < 0.01$). (C) H3K9me signals after BIX-01294 treatment in 2-cell embryos are significantly lower than those in non-treated embryos ($P < 0.01$). Immunofluorescence intensity assayed were tested for significance using one-way ANOVA for significance. The data were considered significant when the P value was less than 0.01 (**). Note that BIX-01294 treatment rescued the aberrant levels of H3K9me and H3K9me2 in SCNT embryos at the 2- and 4-cell stages.

These results suggest that combined treatment with BIX-01294 and Scriptaid could improve the development of porcine SCNT embryos through the partial rescue of the donor nuclear chromatin from decreased histone acetylation observed after BIX-01294 treatment.

BIX-01294 treatment increased the transcription of pluripotency genes in SCNT embryos

To further investigate the molecular mechanism underlying the increase in the developmental competence of cloned embryos after BIX-01294 treatment, the transcription levels of three pluripotency genes (*OCT4*, *SOX2* and *NANOG*), one imprinted gene (*GTL2*), one DNA methylation gene (*DNMT1*) and eight histone modification genes (*CBP*, *SIRT2*, *HAT1*, *HDAC1*, *HDAC2*, *KDM5B*,

KDM5C and *LSD1*) were determined through quantitative PCR at the blastocyst stage. As shown in Fig. 4, the transcriptional activities of not only pluripotency-related genes *OCT4*, *SOX2* and *NANOG*, but also histone modification-associated genes *HDAC1* and *LSD1*, were all significantly increased after BIX-01294 treatment of SCNT pig embryos, reaching the levels observed in the IVF or PA counterpart groups. These results indicated that BIX-01294 treatment primarily affected the expression of key developmental genes rather than imprinted and histone modification genes in SCNT embryos.

Discussion

The most dominant strategies for reprogramming terminally differentiated somatic cells into pluripotency status

Table 4 Effect of combined BIX-01294 and Scriptaid treatment on the preimplantation development of SCNT-derived embryos.

Treatment	Replications	No. of embryos cultured	No. of cleavages	No. of blastocysts	Average total cell number
Control	5	258	214 (82.9) ^a	36 (14.0) ^c	32.73 ± 2.381 ^b
500 nM Scriptaid	5	270	229 (84.8) ^a	56 (20.7) ^b	41.68 ± 4.321 ^a
50 nM BIX-01294	5	270	227 (84.1) ^a	58 (21.5) ^b	34.91 ± 2.694 ^b
250 nM S + 25 nM B	5	270	229 (84.8) ^a	64 (23.7) ^a	32.61 ± 2.575 ^b

Superscript alphabets (a and b) represents values with different BIX-01294 within a column are significantly different ($P < 0.05$). Cleavage percentage: No. of embryos cleaved/No. of embryos cultured. Blastocyst percentage: No. of blastocysts/No. of embryos cultured.

are SCNT and the generation of iPSCs. After the transfection of pluripotent genes or treatment with small molecules, a fraction of somatic cells can be reprogrammed to a pluripotent state, such as ESCs. However, differences in the epigenetic modification and expression of genes between iPSCs and ESCs have been reported, and the expression of exogenous pluripotent factors likely induces tumors (Bock *et al.* 2011, Menendez *et al.* 2012).

Although SCNT is more powerful and faithful in reprogramming compared with iPSCs, aberrant DNA methylation and histone modification patterns in mammalian SCNT embryos still exist (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001, Beaujean *et al.* 2004, Zhang *et al.* 2009). In the present study, we characterized H3K9me2 and H3K9me patterns in SCNT and PA embryos to determine whether cloned embryos have higher levels of H3K9me and H3K9me2 at the 2- and 4-cell stages compared with those derived from PA. Previous studies have indicated that, during the first cell cycle, SCNT embryos largely maintain the epigenetic characteristics of their somatic cell progenitors, but not the typical epigenetic characteristics of the fertilized zygotes (Bui *et al.* 2008). This high level of H3K9me2 and H3K9me expression was inherited from donor somatic cells and remained globally unchanged throughout zygotic development. The present observations were consistent with previous reports (Bui *et al.* 2008, Wu *et al.* 2011), which showed that fully

matured oocytes could not completely demethylate the transferred somatic cell chromatin, although germinal vesicle stage oocytes exhibited demethylase activity (Bui *et al.* 2008).

BIX-01294 is an inhibitor of G9A HMT that impairs the generation of H3K9me2 *in vitro* (Kubicek *et al.* 2007). In the present study, we examined the effects of BIX-01294 treatment on the *in vitro* and *in vivo* development of porcine SCNT embryos. The results showed that BIX-01294 treatment improved the *in vitro* and *in vivo* development of porcine SCNT embryos through the recovery of the levels of H3K9me at the 2-cell stage and H3K9me2 at the 2- and 4-cell stages. Treatment with lower concentrations of BIX-01294 (5 nM) showed less improvements in the preimplantation development of porcine SCNT embryos (Table 1), whereas higher BIX-01294 concentrations (5 μ M) had detrimental effects on embryo development indicating that it is necessary to determine an appropriate, but non-toxic dose of BIX-01294 before treatment. The embryonic lethality might reflect in the dysfunction of G9A HMT, as G9A is required for mouse embryonic development. G9A-null mice exhibit embryonic lethality, with embryos failing to develop past embryonic day (E) 8.5, and these embryos exhibit reduced H3K9me and H3K9me2 (Tachibana *et al.* 2002). Additionally, the embryonic lethality, triggered by higher BIX-01294 concentrations (5 μ M), was also observed in sheep. In this species, reduced global H3K9me2 levels were confirmed in both cultured

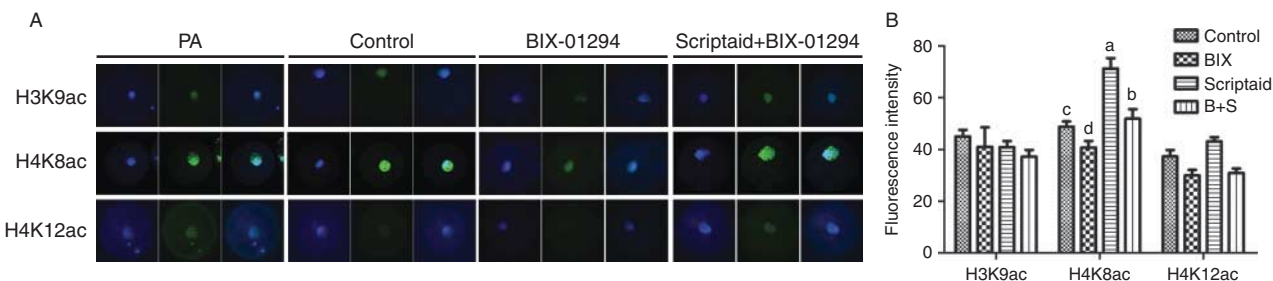


Figure 3 (A) Immunofluorescence detection of H3K9ac, H4K8ac and H4K12ac in PN-stage embryos derived from PA, non-treatment (NT-Control), BIX-01294 treatment (BIX-01294) and combined treatment with BIX-01294 and Scriptaid (Scriptaid+BIX-01294). H3K9ac, H4K8ac and H4K12ac protein (green) were probed with rabbit anti-H3K9ac, anti-H4K8ac or anti-H4K12ac antibodies (1:1000) and detected using Alexa 488-conjugated goat anti-rabbit antibodies (1:200). The nuclei (blue) were labeled with DAPI stain. Original magnification was $\times 400$. (B) The average optical intensity was measured using Nikon NIS element software. The values are presented as the mean \pm s.e.m. Immunofluorescence intensity assayed were tested for significance using one-way ANOVA for significance. ^{a,b,c,d}Values with different BIX-01294 within a column are significantly different ($P < 0.05$). Note that combined treatment with BIX-01294 and Scriptaid partially rescued the decreased histone acetylation observed after BIX-01294 treatment.

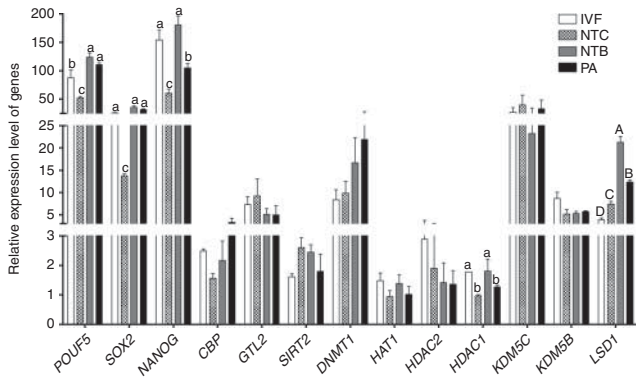


Figure 4 Real-time PCR results for the three pluripotency genes (*OCT4*, *SOX2* and *NANOG*), one imprinted gene (*GTL2*), one DNA methylation gene (*DNMT1*) and eight histone modification genes (*CBP*, *SIRT2*, *HAT1*, *HDAC1*, *HDAC2*, *KDM5B*, *KDM5C* and *LSD1*) in the blastocysts derived from PA, IVF, non-treatment (NTC) and BIX-01294 treatment (NTB). The expression levels were normalized against *H2AFZ*. Note that BIX-01294 treatment increased the transcription of pluripotency genes (*OCT4*, *SOX2* and *NANOG*) and histone modification genes (*HDAC1* and *LSD1*) in SCNT embryos. The quantitative real-time PCR results were compared through one-way ANOVA using the Statistical Product and Service Solutions software. Differences with $P < 0.05$ were considered significantly different. ^{a,b,c,d}Values with different BIX-01294 within a column are significantly different ($P < 0.05$). ^{A,B,C,D}Values with different BIX-01294 within a column are significantly different ($P < 0.01$).

nuclear donor somatic cells and SCNT-derived embryos, but the cloning efficiency was not improved (Fu *et al.* 2012). In the present study, treatment with 50 nM BIX-01294 did not affect the developmental potential of PA-derived embryos, which suggests that dose of 50 nM BIX-01294 is less toxic to *in vitro* cultured pig embryos.

Zhao *et al.* (2009) suggested that increasing the levels of histone acetylation is one of the most successful strategies to improve mammalian SCNT reprogramming. Therefore, it would be worth determining whether BIX-01294 treatment improves SCNT reprogramming via hyperacetylation. The results of the present study showed that the histone acetylation levels of H3K9, H4K8 and H4K12 in SCNT-derived PN stage embryos were decreased after BIX-01294 treatment (Fig. 3). We attempted to enhance histone acetylation level through combined treatment with Scriptaid (a histone deacetylase inhibitor) and BIX-01294, and observed that the combined treatment not only significantly increased H4K8 histone acetylation but also improved SCNT embryo development (blastocyst rate 23.7% vs 21.5%). This effect might result from the partial rescue of somatic cell-inherited nuclear chromatin from decreased histone acetylation that was observed after BIX-01294 treatment.

Embryo development is regulated both genetically and epigenetically. Histone modifications act as a regulatory switch for gene transcription and the histone acetylation and demethylation of H3K9, which are associated with transcriptional activation (Nottke *et al.* 2009). To further explore the effect of BIX-01294 on SCNT embryo

development, we determined the transcriptional expression of 13 critical genes in embryo development at the blastocyst stage after BIX-01294 treatment. The reduced expression of *OCT4*, *NANOG* and *SOX2* in SCNT embryos compared with IVF embryos has been reported in previous studies (Pesce & Scholer 2001). In the present study, BIX-01294 treatment significantly improved the expression of these three genes in SCNT embryos. *OCT4* is important for the survival of primordial germ cells (Scholer *et al.* 1989, Kehler *et al.* 2004) as well as the inner cell mass formation (Pesce & Scholer 2001) and self-renewal of ESCs (Niwa *et al.* 2005, Loh *et al.* 2006). Nottke *et al.* (2009) and Ma *et al.* (2008) showed that H3K9 demethylation might promote pluripotency through a positive-feedback loop involving *NANOG* and *OCT4*. *SOX2* is also a faithful marker of pluripotency in pigs (Liu *et al.* 2015). On the basis of these results, the evaluated expression of *OCT4*, *NANOG* and *SOX2* gene transcripts might contribute to the improvements in epigenetic reprogramming of somatic cell-inherited nuclear genome after treatment of porcine cloned embryos with BIX-01294.

In summary, we observed that BIX-01294 treatment enhanced the developmental competence of porcine SCNT embryos through improvements in epigenetic reprogramming and gene expression. Because various epigenetic defects occurred during somatic reprogramming, further studies are required to elucidate which cluster of genes or epigenetic loci are dysregulated in somatic reprogramming to further improve the cloning efficiency.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0460>.

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.

Funding

This study was financially supported through a grant from the National Natural Science Foundation of China (no. 31172281, 31272440) to J Zhao.

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Received 31 August 2015

First decision 16 September 2015

Revised manuscript received 19 October 2015

Accepted 22 October 2015