Oxamflatin Significantly Improves Nuclear Reprogramming, Blastocyst Quality, and In Vitro Development of Bovine SCNT Embryos

Jianmin Su¹, Yongsheng Wang¹, Yanyan Li¹, Ruizhe Li¹, Qian Li¹, Yongyan Wu¹, Fusheng Quan¹, Jun Liu¹, Zekun Guo², Yong Zhang¹*

¹College of Veterinary Medicine, Northwest A&F University, Key Laboratory of Animal Reproductive Physiology and Embryo Technology, Ministry of Agriculture, Yangling, Shaanxi, People’s Republic of China, ²Department of Biochemistry and Molecular Biology, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, People’s Republic of China

Abstract

Aberrant epigenetic nuclear reprogramming results in low somatic cloning efficiency. Altering epigenetic status by applying histone deacetylase inhibitors (HDACi) enhances developmental potential of somatic cell nuclear transfer (SCNT) embryos. The present study was carried out to examine the effects of Oxamflatin, a novel HDACi, on the nuclear reprogramming and development of bovine SCNT embryos in vitro. We found that Oxamflatin modified the acetylation status on H3K9 and H3K18, increased total and inner cell mass (ICM) cell numbers and the ratio of ICM:trophectoderm (TE) cells, reduced the rate of apoptosis in SCNT blastocysts, and significantly enhanced the development of bovine SCNT embryos in vitro. Furthermore, Oxamflatin treatment suppressed expression of the anti-apoptotic gene Bcl-XL and stimulated expression of the anti-apoptotic gene Bax of satellite I in SCNT blastocysts. In conclusion, Oxamflatin modifies epigenetic status and gene expression, increases blastocyst quality, and subsequently enhances the nuclear reprogramming and developmental potential of SCNT embryos.

Introduction

Somatic cell nuclear transfer (SCNT), has successfully been used to produce cloned animals in several mammalian species [1,2,3,4,5,6,7,8,9,10,11,12]. SCNT is a promising technology with potential applications in both animal science and biomedical application. However, low cloning efficiency and a high incidence of abnormalities in SCNT clones, including respiratory problems, placental deficiency, increased or decreased growth and oversized organs (i.e., large offspring syndrome), obesity, short life span, prolonged gestation, dystocia, fetal edema, hydramnios, and perinatal death [13,14,15], are significant barriers to the use of this technology. It is generally believed that the low cloning efficiency is mostly attributed to aberrant nuclear reprogramming of the donor cell. The nuclear reprogramming process mainly involves various epigenetic modifications, such as DNA methylation and histone modifications, which suggests that epigenetic modifications may be a key factor in improving the cloning efficiency. Hence, the prevention of epigenetic errors may improve the cloning success rate in animals.

Recently, several epigenetic remodeling drugs, such as the histone deacetylase inhibitors (HDACi) trichostatin A (TSA) [16, 17,18,19,20,21,22,23,24,25,26,27,28], valproic acid (VPA) [20, 29], Scriptaid [30,31,32], sodium butyrate [33,34,35], suberoylanilide hydroxamic acid (SAHA) [28], and m-carboxycinnamic acid bishydroxamide (CBHA) [36] have been used to try and improve the developmental competence of SCNT embryos, and results have indicated that the HDACi significantly improves the in vitro and full-term development of SCNT embryos. Oxamflatin, another HDACi, is a novel antitumor compound, which acts by inhibiting mammalian histone deacetylase [37]. A recent study found that Oxamflatin significantly improved the cloning success rate in mice without leading to obvious abnormalities [28]. However, it is not yet known if this novel compound can also improve the development of SCNT embryos in other species, and its mechanisms of action are yet to be investigated.

Thus, we explored the effects of Oxamflatin on the in vitro development of bovine SCNT embryos. To investigate its effects on nuclear reprogramming of somatic cells and the way in which it improves cloning efficiency, global acetylation levels of histone H3 at lysine 9 (AcH3K9) and 18 (AcH3K18) and the quality of bovine SCNT embryos (total, trophectoderm (TE) and inner cell mass (ICM) cell numbers in blastocysts, the ratio of ICM:TE, and the rate of apoptosis in blastocysts) were assessed by immunostaining and TUNEL assay in in vitro-fertilized embryos (IVF group), untreated SCNT embryos (C-NT group), and Oxam-
Results

Experiment 1: Oxamflatin treatment improved the development of bovine SCNT embryos in vitro

To assess whether modification of acetylation could benefit early development of SCNT bovine embryos, we treated SCNT embryos with different concentrations of Oxamflatin and calculated the in vitro developmental rates from the 2-cell embryo to the blastocyst stage (Fig. 1, Table 1). We found that IVF and all SCNT embryos cleaved with a similar rate, around 77–81%, except 5 μM Oxamflatin-treated SCNT embryos. The effect of the Oxamflatin treatment was observed from the morula stage onwards. 0.5 μM and 1 μM Oxamflatin improved the morula and blastocyst rate. A high concentration of Oxamflatin (5 μM) was found to be toxic for development as early as the 2-cell stage.

To optimize the treatment of Oxamflatin, we also tested the developmental rates of blastocyst with various incubation times. The optimum effect was reached when the SCNT embryos were treated with 1 μM Oxamflatin post-ionomycin for 12 h. The developmental rates of blastocyst were 29.05 ± 2.31%, 30.64 ± 0.78%, 40.81 ± 1.10%, 34.34 ± 1.24%, and 31.04 ± 2.61% for incubation times of 0, 6, 12, 18, and 24 h, respectively.

Experiment 2: Oxamflatin treatment increased global histone acetylation levels of SCNT embryos

To find the way in which Oxamflatin treatment improved the developmental potential of SCNT embryos, the acetylation levels of two epigenetic markers, H3K9 and H3K18, were studied in 2-cell, 4-cell, 8-cell, and blastocyst stage embryos. No signals were detected in the embryos stained without first or secondary antibodies, indicating the specificity of staining of the first antibody (data not shown). As shown in Fig. 2, 3, and 4, Oxamflatin treatment increased AcH3K9 and AcH3K18 levels in 2-cell, 4-cell, and 8-cell stage embryos. However, at the blastocyst stage, no differences in AcH3K9 and AcH3K18 levels were observed among the groups (Fig. 5).

Experiment 3: Oxamflatin treatment increased total cell numbers, the number of ICM cells, and the ICM:TE ratio in SCNT blastocysts

To determine if the improvement in SCNT embryo development was reflected in blastocyst quality, the number of apoptotic cells was estimated by TUNEL assay. As shown in Fig. 7, the number of apoptotic cells in SCNT blastocysts was significantly lower in the T-NT group than in the C-NT and IVF groups (P < 0.05).

Experiment 5: Oxamflatin treatment affected relative expression of apoptosis and development-related genes

Relative expression levels of 9 different genes were analyzed in IVF, C-NT, and T-NT blastocysts using quantitative real-time PCR (Fig. 8). The expression level of Bax was lower in T-NT blastocysts than in C-NT blastocysts (P < 0.05). The expression levels of Bcl-XL, OCT4 and SOX2 were significantly higher in T-NT blastocysts than in C-NT blastocysts (P < 0.05). The expression level of OCT4 was lower in the C-NT group than in the IVF group (P < 0.05). There were no significant differences in the expression of Caspase-3, Survivin, Gasparne-3, Nanog, and CDX2 among the three groups.

Experiment 6: Oxamflatin treatment reduced DNA methylation levels in the satellite I region

The DNA methylation status of satellite I was analyzed in blastocysts by bisulfite sequencing (Fig. 9). The satellite I sequence of IVF blastocysts (17.92 ± 2.64%) and T-NT blastocysts (31.45 ± 4.61%), had significantly lower methylation levels than that of C-NT blastocysts (53.99 ± 9.11%, P < 0.05).
Discussion

Somatic cells can be reprogrammed into pluripotent cells with various methods, such as induction of ectopic expression of transcription factors (induced pluripotent stem cells; iPSCs) or nuclear transfer into enucleated oocytes using SCNT [38]. It was recently shown that iPSCs display more genetic and epigenetic abnormalities than ESCs or fibroblasts – the cells from which they originate [39,40,41]. Thus, pluripotent cells generated by SCNT technology may have greater therapeutic potential. Besides therapeutic cloning, SCNT is also a promising technology with potential applications in species preservation, livestock propagation, transgenic research, human xenotransplantation, and disease models. The cloning efficiency, however, remains low.

Recently, investigations have focused on the ability of histone deacetylase inhibitors to improve SCNT efficiency. However, studies exploring the mechanism with which HDACi enhances the epigenetic remodeling ability of somatic cell nuclei in SCNT embryos are scarce.

In this study, we examined if Oxamflatin treatment can improve somatic nucleus reprogramming and development of bovine SCNT embryos in vitro. Furthermore, we also explored, for the first time, Oxamflatin’s possible mechanisms of action. We found that Oxamflatin treatment after SCNT modified the epigenetic status and gene expression in SCNT embryos, increased blastocyst quality, and significantly improved the subsequent development of bovine SCNT embryos.

The best protocol for Oxamflatin treatment in cattle was found to be: (1) Oxamflatin concentration of 0.5–1 μM, as Oxamflatin becomes effective from 0.5 μM but shows toxicity at 5 μM, (2) continuous exposure of reconstructed oocytes to Oxamflatin post- ionomycin for 12 h (4 h in DMAP containing Oxamflatin and 8 h in G1.3 medium containing Oxamflatin).

A recent study in mice showed that Oxamflatin treatment (1 μM Oxamflatin for 9 h after nuclear transfer) significantly improved the in vitro and full-term development of cloned mice [28]. In the present study, we found that Oxamflatin treatment (1 μM Oxamflatin post-ionomycin for 12 h) also significantly improved the in vitro development of cloned cattle. Our previous studies on bovine cloning showed that treatment with 5-aza-dC and TSA dramatically improved the development of SCNT bovine embryos in vitro [19] and in vivo [42], thereby significantly increasing bovine cloning efficiency from 2.6% to 13.4% (number of surviving calves at 60 days of birth/number of recipient cows) [42]. Accordingly, we infer that epigenetic modification drugs may also have effects on the in vivo and full-term development of cloned embryos.

Histone acetylation, one of two main types of epigenetic marker, plays a significant role in the process of reprogramming and affects the development of SCNT embryos [19,23,33,36,43,44]. Therefore, we studied the acetylation level of histone H3 in SCNT embryos and found that Oxamflatin treatment increased both AcH3K9 and AcH3K18 levels in 2-cell, 4-cell, and 8-cell SCNT embryos. It is well accepted that increasing global acetylation of histones by HDACi alleviates transcriptional repression by facilitating chromatin remodeling and relieving methylated CpG sites [45,46]. It is believed that hyperacetylation of histones facilitates the access of various factors to nucleosomes [32,47,48]. Therefore, one of the ways in which Oxamflatin treatment improves the developmental potential of SCNT embryos may be that the increased histone acetylation level, caused by inhibition of HDAC activity, may facilitate chromatin remodeling and access of reprogramming-related factors to nucleosomes, alleviating transcriptional repression.

DNA methylation, another key epigenetic factor, modifies and regulates the chromatin structure and also plays a crucial role in somatic nuclear reprogramming. It was found that TSA not only modifies histone acetylation but also potentially induces DNA demethylation [49]. Bovine SCNT embryos were found to have aberrant hypermethylation in the satellite I region [50]. Wee et al. [43] and we [51] have found that HDACi induces DNA demethylation in the satellite I region. Therefore, we further investigated if Oxamflatin treatment affects DNA methylation in the satellite I region of SCNT embryos. Interestingly, most Oxamflatin treated SCNT embryos had significantly lower DNA methylation levels in the satellite I region than non-treated SCNT embryos. Like TSA, Oxamflatin treatment seems to have a “correcting” effect on the DNA methylation status of the satellite I region.

The cell number, especially the ratio of ICM:TE cells, is one of the criteria for assessment of blastocyst quality [52,53]. Aberrant allocation of ICM and TE cells in SCNT embryos at preimplantation stages may cause placental abnormalities and early fetal loss [54]. Therefore, we counted total blastomere cell numbers, TE and the estimated ICM cell numbers and measured the ratio of ICM:TE in blastocysts. Interestingly, Oxamflatin treatment increased the total number of cells and the number of ICM cells in SCNT blastocysts. The ratio of ICM:TE was also increased in the Oxamflatin treated group. To elucidate the mechanism behind this, we measured the relative expression levels of 4 the development-related genes OCT4, NANOG, SOX2, and

### Table 1. Effect of different concentration of Oxamflatin on the development of cloned bovine embryos in vitro.

| Treatment | No. reconstructed | No. (%) ≥2-cell embryos | No. (%) ≥4-cell embryos | No. (%) ≥8-cell embryos | No. (%) blastocysts |
|-----------|------------------|-------------------------|-------------------------|-------------------------|---------------------|
| IVF       | 206              | 166 (79.68±2.14)a       | 144 (69.91±2.53)a       | 101 (49.03±0.53)b       | 79 (38.39±0.64)b    |
| 0 μM      | 214              | 165 (77.23±2.46)a       | 154 (71.85±1.22)a       | 86 (40.11±0.72)a        | 65 (30.34±0.83)a    |
| 0.05 μM   | 186              | 146 (79.09±1.83)a       | 128 (68.80±2.87)a       | 72 (38.74±1.26)a        | 55 (29.55±1.31)a    |
| 0.5 μM    | 194              | 157 (80.02±1.11)a       | 139 (71.62±1.89)a       | 93 (47.96±2.03)a        | 77 (39.65±2.12)a    |
| 1 μM      | 248              | 201 (81.01±1.87)a       | 181 (72.97±2.10)a       | 121 (68.33±1.38)a       | 101 (40.81±1.18)b   |
| 5 μM      | 188              | 69 (36.73±3.13)b        | 54 (28.78±2.78)b        | 18 (9.56±3.26)c         | 11 (5.84±2.11)c     |

*Within a column, developmental rates with different superscripts are significantly different from each other (P<0.05).*
CDX2, and found up-regulated expression of OCT4 and SOX2 in the Oxamflatin treated group relative to the non-treated group.

OCT4 is a key regulator of pluripotency that is important for maintaining ICM cell fate and pluripotency of ES cells. It has been reported that OCT4 is not only expressed in ICM, but also in TE cells of bovine embryos [55]. In this study, we found similar results. However, Oxamflatin treated SCNT blastocysts had higher OCT4 expression levels than both non-treated SCNT and IVF blastocysts.

SOX2 is another vital regulator of pluripotency. SOX2 acts synergistically with OCT4 in activating OCT–SOX enhancers,

Figure 2. The global AcH3K9 and AcH3K18 levels in 2-cell stage embryos. (A) Staining of AcH3K9 and AcH3K18 (green) in IVF, 0 μM Oxamflatin treated SCNT (C-NT), and 1 μM Oxamflatin treated embryos (T-NT) at the 2-cell stage. Each sample was counterstained with DAPI to visualize DNA (blue). Original magnification was ×200. (B) Quantification of AcH3K9/DNA and AcH3K18/DNA signal intensities in IVF (open bars), C-NT (gray bars), and T-NT (black bars) embryos. Labeling intensity was expressed relative to that of the IVF embryos (set as 100%). Values with different superscripts differ significantly (P < 0.05). The experiments were replicated 3 times. In each replication, n = 10–15 per group.

doi:10.1371/journal.pone.0023805.g002
which regulate the expression of Nanog, OCT4 and SOX2 itself [58]. In this study, we found that the Oxamflatin treatment up-regulated the expression of SOX2 in the bovine SCNT blastocysts.

Both pluripotency-related genes OCT4 and SOX2 are important for maintaining ICM cell fate. Therefore, the up-regulated expression of these two genes in the treated SCNT blastocysts may be associated with the higher ICM:TE ratio. We previously found that 5-aza-dC and TSA treatment also increases the expression levels of OCT4 and SOX2 [51] and the number of ICM cells in the bovine SCNT blastocysts [19].

Apoptosis is another criterion for evaluation of blastocyst quality, as it eliminates cells with nuclear or chromosomal abnormalities [59]. In bovine embryos, apoptosis can be detected in embryos after the 8-cell stage [60]. The high rate of apoptosis in SCNT blastocysts is correlated with a decrease in the total cell number [53]. In this study, the number of apoptotic cells was significantly lower in the Oxamflatin treated blastocysts than in the non-treated and IVF blastocysts. This suggests that Oxamflatin improves the quality of SCNT embryos by reducing cell death in the embryos. This observation is similar to that reported in cloned mice [28]. TSA, another important HDACi, also suppresses apoptosis in bovine SCNT embryos [61] and rat kidney cells [62].

To find the cause of the decreased apoptosis rate, we further analyzed the relative expression levels of five apoptosis-related genes (Bax, Bax inhibitor, Survivin, Bcl-XL, and Caspase-3). During embryogenesis, the pro- and anti-apoptotic members of the Bcl-2 family of proteins regulate the pathways of apoptosis. The pro-apoptotic gene Bax is a positive regulator of apoptosis, whereas the
anti-apoptotic gene *Bcl-XL* acts to protect against apoptosis. In the present study, there was a lower expression level of *Bax* and a higher expression level of *Bcl-XL* in Oxamflatin treated SCNT blastocysts than in non-treated blastocysts, which may have contributed to the reduced apoptosis of cells in Oxamflatin treated blastocysts compared with those in non-treated ones.

In summary, the present study indicates that the histone deacetylase inhibitor Oxamflatin affects the expression of apoptosis and development-related genes, modifies global histone acetylation and DNA methylation in satellite I region, increases the total and ICM cells numbers of SCNT blastocysts, reduces cell death in SCNT embryos, and subsequently enhances the nuclear reprogramming and developmental potential of bovine SCNT embryos.

**Materials and Methods**

**Ethics statement**

The entire experimental procedure was approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. Bovine ovaries of slaughtered mature cattle were collected from Tumen abattoir, a local slaughterhouse of Xi’an, P.R. China. A newborn female Holstein calf was obtained for nuclear donor cell cultures and Beef-breed Angus
Cows were obtained for recipient animals from Yangling Keyuan Cloning Co., Ltd.

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, USA) unless specifically stated otherwise. Disposable, sterile plasticware was purchased from Nunclon (Roskilde, Denmark).

Nuclear donor cell preparation

Nuclear donor cell cultures were established from the ear skin of a newborn female Holstein calf as described previously [51]. Briefly, after the hair was removed, the ear notch was rinsed four times with phosphate-buffered saline (PBS), and minced into 1 mm³ pieces. The tissue pieces were cultivated for 1–2 weeks in 60 mm Petri dishes with DMEM (Gibco, Grand Island, USA) containing 10% FBS (Gibco), 1 mM sodium pyruvate, 100 mg/mL streptomycin and 100 IU/mL penicillin. When fibroblast cells were at 90% confluence, cells were trypsinized, rinsed, and recultivated in 3 new 60 mm Petri dishes for further passaging. Nuclear donor cells for SCNT were derived from passages 2 to 4 and cultured in serum-starved medium (0.5% FBS) for 2 days.

Oocyte collection and in vitro maturation (IVM)

Oocyte collection and in vitro maturation (IVM) were performed as described previously [51,63,64]. Briefly, bovine ovaries were transported from the slaughterhouse to the laboratory within 4 h after the animal was killed in a thermos bottle with sterile saline at

Figure 5. The global AcH3K9 and AcH3K18 levels in blastocysts. Details are described in the legend to Fig. 2. doi:10.1371/journal.pone.0023805.g005
Oxamflatin Enhances Reprogramming

Figure 6. Immunostaining of CDX2. Each blastocyst in the IVF, 0 μM Oxamflatin treated SCNT (C-NT), and 1 μM Oxamflatin treated SCNT groups (T-NT) was stained with DAPI and CDX2, a marker for trophectoderm. Original magnification was ×200. n = 30, 38, and 44 in the IVF, C-NT, and T-NT group, respectively. doi:10.1371/journal.pone.0023805.g006

Table 2. Characterization of day 7 bovine blastocysts.

| Groups | n | DAPI staining | CDX2 staining | DAPI/CDX2 staining |
|--------|---|---------------|---------------|-------------------|
|        |   | Total no. of cells | Range | No. of TE cells | Range | No. of ICM cells | Range | ICM: TE (%) |
| IVF    | 30 | 100.71±8.92a | 67–159 | 71.47±5.63 | 46–111 | 29.27±3.50a | 16–57 | 39.84±2.13a |
| C-NT   | 38 | 85.26±5.32b | 60–149 | 64.79±3.56 | 48–105 | 20.47±2.01b | 13–44 | 31.10±1.79b |
| T-NT   | 44 | 111.45±7.46a | 70–158 | 76.32±5.02 | 52–114 | 35.14±2.61a | 18–56 | 45.87±1.61a |

The cell numbers in blastocysts were estimated by counting the total number of nuclei using DAPI. The number of trophectoderm (TE) nuclei was estimated using immunostaining for CDX2. The ICM cell number was assessed as the total number of nuclei minus the number of TE nuclei. The data are shown as Mean ± SEM. Within columns, values with different superscripts are significantly different from each other (P<0.05).

Table: 0023805.t002

Oxamflatin treatment protocol

Oxamflatin was dissolved in dimethyl sulfoxide (DMSO) to achieve a stock solution of 10 mM (1 mg Oxamflatin in 291 μL DMSO), and stored at −20°C. Working solutions were freshly prepared just before use. First, the 10 mM Oxamflatin stock solution was diluted in SOFaa to obtain 100 μM Oxamflatin. Then 100 μM Oxamflatin was added to the activation or culture media according to the experimental protocol. For the control group (C-NT group), DMSO was added to the culture medium at the same concentration as used in the other treatments.

Various concentration:Immediately following ionomycin treatment, SCNT embryos were incubated for 4 h in SOF medium with 1.9 mM DMAP containing 0, 0.05, 0.5, 1 or 5 μM Oxamflatin. SCNT embryos were then incubated for another 8 h in G1.3 medium supplemented with 0.05, 0.5, 1 or 5 μM Oxamflatin. After treatment, embryos were washed twice with G1.3, and cultured in 150 μL drops of G1.3 medium in a humidified atmosphere with 5% CO₂ in air at 38.5°C.

Various incubation times: 1 μM Oxamflatin was added post-ionomycin for 0, 6, 12, 18 or 24 h.
In vitro fertilization

IVF was carried out in accordance with the methods of our previous study [51].

Immunofluorescence staining of embryos

Embryos were washed 3 times (5 min each) in PBS containing 0.2% PVA, and fixed in Immunol Staining Fix Solution (Beyotime, P0098, Jiangsu, China) for 1 h. All steps were performed at room temperature unless otherwise stated. Embryos were permeabilized with 0.2% Triton X-100 in PBS for 30 min. After 3 washes, they were blocked in the Immunol Staining Blocking Solution (Beyotime, P0102) for 12 h at 4°C and then incubated with the first antibodies for 12 h at 4°C. Antibodies against acetylated histones were diluted 1:500 (AcH3K9, ab10812, P0098, 

Figure 7. Incidence of apoptosis in blastocysts. (A) TUNEL assay of blastocysts (green). Each sample was counterstained with DAPI to visualize DNA (blue). Original magnification was ×40. (B) Number of apoptotic cells in each blastocyst. Values with different superscripts differ significantly (P<0.05). n = 16–20.

doi:10.1371/journal.pone.0023805.g007
Abcam, Cambridge, UK; ACh3K18, ab1191, Abcam), and anti-CDX2 mouse monoclonal antibody (BioGenex, Inc., San Ramon, CA) was diluted 1:200 using Immunol Staining Primary Antibody Dilution Solution (Beyotime, P0103). After 3 washes, the embryos were treated with secondary antibodies of Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (Beyotime, A0423) for ACh3K9 and ACh3K18 or Alexa Fluor 555-labeled Goat Anti-Mouse IgG (Beyotime, A0459) for CDX2. The secondary antibodies were diluted 1:500 with Immunol Staining Secondary Antibody Dilution Solution (Beyotime, P0108). Finally, the DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1005) for 3 min, and samples were mounted on glass slides with a drop of Antifade Mounting Medium (Beyotime, P0126) and analyzed using the Nikon eclipse Ti-S microscope mounted on glass slides with a drop of antifade mounting medium stained with DAPI (Beyotime) for 3 min, and samples were treated with secondary antibodies of Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (Beyotime, A0423) for ACh3K9 and ACh3K18 or Alexa Fluor 555-labeled Goat Anti-Mouse IgG (Beyotime, A0459) for CDX2. The secondary antibodies were diluted 1:500 with Immunol Staining Secondary Antibody Dilution Solution (Beyotime, P0108). Finally, the DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1005) for 3 min, and samples were mounted on glass slides with a drop of Antifade Mounting Medium (Beyotime, P0126) and analyzed using a Nikon eclipse Ti-S microscope equipped with a 198 Nikon DS-Ri1 digital camera (Nikon). The experiments were replicated 3 times, and a total number of 17, 16, and 20 embryos were processed in IVF, C-NT, and T-NT groups, respectively.

Quantitative real-time PCR
A single day 7 blastocyst was used per sample, and 5 to 8 embryos were used for each group. The total RNA of the embryos was isolated using the Cells-to-Signal™ Kit (Ambion Co., USA) according to the manufacturer’s protocol. The RT reaction was achieved using the M-MLV RT included in the Cells-to-Signal Kit. The mRNA levels were quantified using SYBR Premix ExTaq™ II (TaKaRa, Japan) on a CFX96 real-time PCR detection system (Bio-Rad) at the following thermal cycling conditions: 95°C for 1 min, followed by 40 PCR cycles of 95°C for 5 s, 50–60°C (Table 3) for 30 s, and 72°C for 30 s. The melting protocol was a step cycle starting at 65°C and increasing to 95°C with 0.5°C/3 s increments. The primer sequences for all genes were synthesized according to previous reports [22,66,67] (Table 3). Transcripts were quantified in 3 replicates for each sample and calculated relative to the transcription of the housekeeping gene, Histone 2a (H2A) in every sample. The specificity of the PCR reaction was confirmed by gel electrophoresis on a 2.5% agarose gel and by a single peak in the melting curve. For the negative controls, dH2O replaced cDNA in the real-time reaction tubes.

The 2⁻ΔΔCT method [68] was used to quantify the relative mRNA levels. For ease of comparison, the average expression level of each gene from IVF group was set as 1.

Bisulfite sequencing analysis
A single day 7 blastocyst was used per sample, and 8, 8, and 16 embryo samples were processed for IVF, C-NT, and T-NT groups, respectively. Genomic DNA was extracted from the embryos and subjected to sodium bisulfite treatment using the EZ DNA Methylation-Direct™ Kit (Zymo Research, USA) in accordance with the instruction manual with minor modifications as described previously [51,63]. Briefly, each single blastocyst was washed and transferred to 20 μL digestion mixture. After incubation for 3 h at 50°C, the digested sample was added to
130 µL CT Conversion Reagent for bisulfite conversion and incubated at 96°C for 8 min and 64°C for 3.5 h. Modified DNA was then desalted, purified, and finally eluted with 15 µL of elution buffer. Subsequently, Bisulfite Sequencing PCR (BS-PCR) was immediately carried out using 2 µL of modified DNA per PCR run. The primers of satellite I were synthesized as described.
previously [50] (Table 3). The BS-PCRs were performed using the Hot Start DNA polymerase Zymo Taq™ premix (Zymo Research, USA) with a DNA engine (MJ Research) using the following program: 4 min at 95°C, followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 46°C, extension for 20 s at 72°C, and a final extension at 72°C for 7 min. The PCR products were gel-purified using the TIANgel Midi Purification Kit (Tiangen, China). Purified fragments were subcloned into pMD18-T vectors (TaKaRa, Japan). The clones confirmed by PCR were selected for DNA sequencing (BGI, China). Three independent amplification experiments were performed for each sample. We sequenced 3 to 5 clones from each independent set of amplification and cloning, so there were a total of 9 to 15 clones for each sample. Bisulfite sequencing data and the C–T conversion rate were analyzed by BIQ Analyzer software [69]. To ensure high data quality, only sequences that had a C–T conversion rate >95% were included. Methylation data from bisulfite sequencing were evaluated by computing the percentage of methylated CpGs of the total number of CpGs.

Experimental design

Experiment 1. SCNT embryos were treated with 0, 0.05, 0.5, 1 or 5 μM Oxamflatin post-ionomycin for 12 h. MI I oocytes from the same batch without treatment were in vitro fertilized and used as an additional control group. In vitro development to 2-cell, 4-cell, morula, and blastocyst stages was monitored at 48, 72, 120, and 168 h of culture, respectively (0 h being the time embryos were transferred to G1.3).

SCNT embryos were treated with 1 μM Oxamflatin post-ionomycin for 0, 6, 12, 18, and 24 h to optimize the treatment of Oxamflatin. The developmental rates of blastocyst were tested at 168 h of culture.

Experiment 2. IVF embryos (IVF group) and SCNT embryos treated with 0 μM (C-NT group) or 1 μM Oxamflatin (T-NT group) were collected at the 2-cell, 4-cell, 8-cell, and blastocyst stage for detecting the acetylation level on H3K9 and H3K18.

Experiment 3. The total, TE, and ICM cell numbers in blastocysts of the three groups were estimated to assess the quality of Oxamflatin-treated SCNT blastocysts. The cell numbers in blastocysts were estimated by counting the total number of nuclei using DAPI. The number of trophectoderm (TE) nuclei was estimated using immunostaining for CDX2. The cell number of the ICM was assessed as the total number of nuclei minus the number of TE nuclei [28].

Experiment 4. The rate of cell death in day 7 blastocysts was examined by TUNEL assay to assess the quality of Oxamflatin-treated SCNT blastocysts.

Experiment 5. The relative expression levels of apoptosis-related genes (Bax, Bax inhibitor, Bel-1, Survivin, and Caspase-3) and development-related genes (OCT4, NANOG, SOX2, and CDX2) in blastocysts were compared among the three groups.
Table 3. Primer sequences for BS-PCR and real-time PCR.

| Genes    | Primer sequences (5'-3')                  | T\textsubscript{a} (°C) |
|----------|------------------------------------------|------------------------|
| satellite I | F\textsuperscript{a}: AATACCTCTAATTCTCAACT | 46                     |
|           | R\textsuperscript{a}: TTTGGAAATGTTAGTTAATA |                        |
| OCT4     | F: CACAACCTGCAAGAAATAGGC                  | 60                     |
|          | R: CACACCTGGGACACCAGCTCT                  |                        |
| NANOG    | F: CGTCTTCTGCAAAAGCTCT                    | 60                     |
|          | R: CTGCTTCTCCTTCCTCGCTCT                  |                        |
| SOX2     | F: GGTTGACATCGTTGTTAATATTAATGC           | 60                     |
|          | R: CACAGTAATTTCCATTTGGTTTTTCA             |                        |
| CDX2     | F: GCACAGGAAAAGGAAATTACCAACAA            | 60                     |
|          | R: GGGCTCTTGAGGACCTCTCT                  |                        |
| Bax      | F: GCTCTGAGGAGATCAAG                     | 56                     |
|          | R: AGGCCGCTCTGAAAGAAGTC                   |                        |
| Bax inhibitor | F: GCTCTGAGGAGTTGATT                | 56                     |
|          | R: GCCAAGATCATGATGAC                    |                        |
| Survivin | F: CCTGGCCAGCTCTACCTCAAG                 | 56                     |
|          | R: TAATGGAGCAACAGGAAA                    |                        |
| Bcl-XL   | F: GGATTGCGGAGTCCAGATG                   | 55                     |
|          | R: CAAAGCAGAGCGAGTGAAGAG                 |                        |
| Caspase-3| F: CGATCTGTCAGATGACC                    | 50                     |
|          | R: GCCATCTGCTCTCTCA                      |                        |
| H2A      | F: GCTTGGAGTACCTGACGCC                   | 56                     |
|          | R: ACAACAGGGGCTTCTCTCTGA                 |                        |

\textsuperscript{a}Annealing temperature.  
\textsuperscript{b}Forward primer.  
\textsuperscript{c}Reverse primer.

References

1. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, et al. (1999) Production of goats by somatic cell nuclear transfer. Nature Biotechnology 17: 456–461.

2. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, et al. (2002) Cloned sheep. Reviews of Reproduction 3: 155–163.

3. Ding X, Wang Y, Zhang D, Wang Y, Guo Z, et al. (2008) Increased pre-implantation mortality in embryos derived from fetal and adult mammalian cells (vol 385, pg 810, 1997). Nature 425: 680–680.

4. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, et al. (1998) Eight calves born to its dam twin (vol 424, pg 635, 2003). Nature 425: 680–680.

5. Farin PW, Piedrahita JA, Farin CE (2006) Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 65: 178–191.

6. Kishigami S, Mizutani E, Hikuchi T, Van Thuan N, et al. (2006) Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochemical and Biophysical Research Communications 340: 183–189.

7. Shi LH, Miao YL, Ouyang YC, Huang JC, Lei ZL, et al. (2008) Trichostatin a (TSA) improves the development of rabbit-rabbit intraspecies cloned embryos, but not rabbit-human interspecies cloned embryos. Developmental Dynamics 237: 640–648.

8. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, et al. (2002) A cat cloned by somatic cell nuclear transfer. Cloning and Stem Cells 10: 371–379.

9. Kishigami S, Mizutani E, Ohta H, Hikuchi T, Van Thuan N, et al. (2006) Live Birth of Somatic Cell-Cloned Rabbits following Trichostatin A Treatment and Cotransfer of Parthenogenetic Embryos. Cloning and Stem Cells 11: 203–208.

10. Iqbal AE, Ragina NP, Ross PJ, Beyhan Z, Cunniff K, et al. (2008) Trichostatin A improves histone acetylation in bovine somatic cell nuclear transfer early embryos. Cloning and Stem Cells 10: 371–379.

11. Farin PW, Piedrahita JA, Farin CE (2006) Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 65: 178–191.

12. Ding X, Wang Y, Zhang D, Wang Y, Guo Z, et al. (2008) Increased pre-implantation mortality in embryos derived from fetal and adult mammalian cells (vol 385, pg 810, 1997). Nature 425: 680–680.

13. Shi LH, Miao YL, Ouyang YC, Huang JC, Lei ZL, et al. (2008) Trichostatin a (TSA) improves the development of rabbit-rabbit intraspecies cloned embryos, but not rabbit-human interspecies cloned embryos. Developmental Dynamics 237: 640–648.

14. Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. Reviews of Reproduction 3: 155–163.
Dai XP, Hao J, Hou XJ, Hai T, Fan Y, et al. (2010) Somatic Nucleus
Shi W, Hoeflich A, Flaswinkel H, Stojkovic M, Wolf E, et al. (2003) Induction of
Van Thuan N, Bui HT, Kim JH, Hikichi T, Wakayama S, et al. (2009) The
Zhao JG, Ross JW, Hao YH, Spate LD, Walters EM, et al. (2009) Significant
Zhao J, Hao Y, Ross JW, Spate LD, Walters EM, et al. (2010) Histone
cells to nuclear transfer embryos derived from transgenic Clawed miniature pig cells.
Animal Science Journal: 538–563.
Ono T, Li C, Mizutani E, Terasahita Y, Yamagata K, et al. (2010) Inhibition of
Choi IB Histone Deacetylase Significantly Improves Cloning Efficiency in
Mice. Biology of Reproduction 83: 929–937.
Nakatake Y, Toyooka Y, Shimosato D, Yagi R, et al. (2007) The histone deacetylase inhibitor scriptaid enhances nascent mRNA production and
rescues full-term development in cloned inbred mice. Reproduction 130: 309–317.
Li C, Uhm SJ, Lee HT (2010) Increasing histone acetylation of cloned
embryos, but not donor cells, by sodium butyrate improves their in vivo
development in pigs. Cellular Reprogramming 12: 95–104.
Shi W, Hoeldtch A, Flaswinkel H, Stojkovic M, Wolf E, et al. (2003) Induction of
a senescent-like phenotype does not confer the ability of bovine immortal cells to
support the development of nuclear transfer embryos. Biology of Reproduction 69: 301–309.
Yang FK, Hao R, Kessler B, Brem G, Wolf E, et al. (2007) Rabbit somatic cell
cloning: effects of donor cell type, histone acetylation status and chimeric
embryo complementation. Reproduction 133: 219–230.
Dai XP, Hao J, Hou XJ, Hai T, Fan Y, et al. (2010) Somatic Nucleus
Reprogramming Is Significantly Improved by m-Carboxycinnamic Acid
Bishydroxamide, a Histone Deacetylase Inhibitor. Journal of Biological
Chemistry 285: 31002–31010.
Kim YB, Lee KH, Sugita K, Yoshida M, Horinouchi S (1999) Oxamflatin is a
novel antitumor compound that inhibits mammalian histone methyltransferase.
Oncogene 18: 2461–2470.
Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced
pluripotency. Development 136: 509–523.
Hussein SM, Batada NN, Vuoristo S, Ching RW, Autio R, et al. (2011) Copy
number variation and selection during reprogramming to pluripotency. Nature 471: 58–547.
Gore A, Li Z, Fang HL, Young JE, Agarwal S, et al. (2011) Somatic coding
mutations in human induced pluripotent stem cells. Nature 471: 63–U76.
Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, et al. (2011) Hotspots of
aberrant epigenomic reprogramming in human induced pluripotent stem cells.
Nature 471: 68–U19.
Wang XS, Xiao X, An ZX, Wang LJ, Liu J, et al. (2011) Production of cloned
calves by combination treatment of both donor cells and early cloned embryos with
5-aza-2'-deoxycytidine and triostatin A. Theriogenology 73: 819–825.
Wee G, Sim JH, Koo DB, Chae JI, Lee KK, et al. (2007) Epigenetic alteration of
the donor cells does not recapitulate the reprogramming of DNA methylation in
cloned embryos. Reproduction 134: 781–787.
Yamamaka K, Sugimura S, Waki T, Kawahara M, Sato E (2009) Acetylation
Level of Histone H3 in Early Embryonic Stages Affects Somatic
Development of Miniature Pig Somatic Nuclear Transfer Embryos. Journal of
Reproduction and Development 55: 638–644.
Jones PL, Veenstra GJC, Wade PA, Vermaak D, Kass SU, et al. (1998)
Methylated DNA and Mc2P2 recruit histone deacetylase to repress transcription.
Nature Genetics 18: 187–191.
Nan NX, Ng HH, Johnson CA, Laherty CD, Turner BM, et al. (1998)
Transcriptional repression by the methyl-CpG-binding protein Mc2P2 involves a
histone deacetylase complex. Nature 393: 306–309.
Lee DY, Hayes JR, Pruss D, Wolff AP (1993) A Positive Role for Histone
Acetylation in Transcription Factor Access to Nucleosomal DNA. Cell 72:
73–84.
Li E (2002) Chromatin modification and epigenetic reprogramming in
mammalian development. Nature Reviews Genetics 5: 662–673.
Xiong YN, Dossy SC, Podratz KC, Jin F, Attewell JR, et al. (2005) Histone
deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA
stability and down-regulate de novo DNA methyltransferase activity in human
edometrial cells. Cancer Research 65: 2684–2689.
Kang YK, Koo DB, Park JS, Choi YH, Chung AS, et al. (2001) Aberrant
methylation of donor genome in cloned bovine embryos. Nature Genetics 28:
173–177.
Wang Y, Su J, Wang L, Xu W, Quan F, et al. (2011) The Effects of 5-Aza-2'-
Deoxycytidine and Triostatin A on Gene Expression and DNA Methylation
status in Cloned Bovine Blastocysts. Cellular Reprogramming 13(4): 297–306.
VanSsom A, Boerjan M, Vysbaert MT, DeKeul A (1996) Cell allocation to the
inner cell mass and the trophectoderm in bovine embryos cultured in two
different media. Molecular Reproduction and Development 45: 171–182.
Yu Y, Ding CH, Wang Y, Chen XJ, Li XM, et al. (2007) Pieno-assisted nuclear
transfer affects cloning efficiency and may cause apoptosis. Reproduction 133:
947–954.
Im GS, Seo JS, Hwang IS, Kim DH, Kim SW, et al. (2006) Development and
apoptosis of pre-implantation porcine nuclear transfer embryos activated with
different combination of chemicals. Molecular Reproduction and Development 73:
1094–1101.
Berg DK, Smith CS, Pearson DJ, Wells DN, Broadhurst R, et al. (2011)
Trophectoderm Lineage Determination in Cattle. Developmental Cell 20:
18–27.
Beyhan Z, Forsberg EJ, Edertsen KJ, Kent-First M, First NL (2007) Gene
expression in bovine nuclear transfer embryos in relation to donor cell efficiency
in producing live offspring. Molecular Reproduction and Development 74:
18–27.
Aston KI, Li GP, Hicks BA, Sessions BR, Davis AP, et al. (2010) Abnormal
Levels of Transcript Absundance of Developmentally Important Genes in
Various Stages of Preimplantation Bovine Somatic Cell Nuclear Transfer
Embryos. Cellular Reprogramming 12: 23–52.
Masui S, Nakatate Y, Toyovska Y, Shimosato D, Yagi R, et al. (2007)
Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse
embryonic stem cells. Nature Cell Biology 9: 625–U626.
Hardy K (1997) Cell death in the mammalian blastocyst. Molecular Human
Reproduction 3: 919–925.
Fahrudin M, Oto T, Karja NWK, Mori M, Murakami M, et al. (2002) Analysis of
dNA fragmentation in bovine somatic nuclear transfer embryos using
tUNEL. Reproduction 124: 815–819.
Cui X, Xu YN, Shen XH, Zhang LQ, Zhang JB, et al. (2011) Triostatin A
Modulates Apoptotic-Related Gene Expression and Improves Embryo Viability in
Cloned Bovine Embryos. Cellular Reprogramming 13: 179–189.
Dong GE, Lue J, Kumar V, Dong Z (2010) Inhibitors of histone deacetylases
suppress cispain-induced p53 activation and apoptosis in renal tubular cells.
American Journal of Physiology-Renal Physiology 298: F293–F300.
Su JM, Yang B, Wang YS, Li Y, Xiaog X, et al. (2011) Expression and
methylation status of imprinted genes in placentas of deceased and live cloned
transgenic calves. Theriogenology 75: 1346–1359.
Wang YS, Tang S, An ZX, Li WZ, Liu J, et al. (2011) Effect of mSOF and
G1.1/G2.2 Media on the Developmental Competence of SCNT-Derived
Bovine Embryos. Reproduction in Domestic Animals 46: 404–409.
Takahashi Y, First NL (1992) In vitro development of bovine one-cell embryos:
Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology
37: 963-978.
Jeon K, Kim EY, tài JC, Lee CH, Lee KS, et al. (2008) Survivin protein
expression in bovine follicular oocytes and their in vitro developmental
competence. Animal Reproduction Science 108: 319–333.
Park SY, Kim EY, Jeon K, Cui XS, Lee WD, et al. (2007) Survivin acts as an
apoptotic factor during the development of bovine pre-implantation embryos.
Molecular Reproduction and Development 74: 582–590.
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using
real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:
402–409.
Bock C, Reitcr S, Mikeska T, Pashler M, Walter J, et al. (2005) Bq analyzer:
visualization and quality control for DNA methylation data from bisulfite
sequencing. Bioinformatics 21: 4067–4068.