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VPA selectively regulates pluripotency gene expression on donor cell and improve SCNT embryo development

Xinxin Li[1] · Xudong Ao[1] · Li Bai[1] · Dongfang Li[1] · Xuefei Liu[1] · Zhuying Wei[1] · Shorgan Bou[1] · Guangpeng Li[1]

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Abstract

SCNT technology has been successfully used to clone a variety of mammals, but the cloning efficiency is very low. This low efficiency is likely due to the incomplete reprogramming of SCNT embryos. Histone modification and DNA methylation may participate in these events. Thus, it would be interesting to attempt to improve the efficiency of SCNT by using a HDACi VPA. In order to guarantee the effect of VPA and reduce its cytotoxicity, a comprehensive analysis of the cell proliferation and histone modification was performed. The results showed that 0.5 and 1 mM VPA treatment for 24 h were the optimal condition. According to the results, H3K4me3 was increased in 0.5 and 1 mM VPA groups, whereas H3K9me2 was significantly decreased. These are the signals of gene-activation. In addition, VPA treatment led to the overexpression of *Oct4* and *Nanog*. These indicated that VPA-treated cells had similar patterns of histone to zygotic embryos, and may be more favorable for reprograming. A total of 833 cloned embryos were produced from the experimental replicates of VPA-treated donor cells. In 1 mM treatment group, the blastocyst rates were significantly increased compared with control. At the same time, our findings demonstrated the interrelation between DNA methylation and histone modifications.

Keywords VPA · Embryo development · Histone modification · DNA methylation · SCNT

Introduction

Somatic cell nuclear transfer (SCNT) is an extraordinary and important technology for generating transgenic animals (Yum et al. 2018) and preserving species (Gomez et al. 2009). Although several mammalian species have been successfully cloned (Rodriguez-Osorio et al. 2012), its rate remains extremely low. One of the underlying problems is the incorrect or incomplete epigenetic reprogramming of SCNT embryos (Akagi et al. 2014). To overcome this problem, many chemicals associated with epigenetic modifications have been used (Huang et al. 2011; Akagi et al. 2013; Azuma et al. 2018; Liu et al. 2018a).

Valproic acid (VPA), a histone deacetylase inhibitor, is often used as an anti-epileptic drug in patients with brain neoplasms due to its effectiveness and low toxicity profile (Tomson et al. 2016). Researchers have shown that VPA treatment of B6CBAF1 mouse (Costa-Borges et al. 2011), miniature pig (Miyoshi et al. 2010), pig (Kang et al. 2013), and bovine (Song et al. 2014) SCNT embryos can promote their embryonic development. In 2016, Miyoshi K et al. successfully obtained cloned microminipigs derived from SCNT embryos transiently treated with VPA (Miyoshi et al. 2016). Although these studies confirmed the effect of VPA on SCNT, study in 2014 showed that VPA-treated bovine donor cells did not increase the efficiency of SCNT (Sangalli et al. 2014). In 2017, Selokar NL et al. confirmed that VPA could alter the histone acetylation and gene expression in the buffalo donor cells but did not improve the in vitro developmental competence of hand-made cloning embryos (Selokar et al. 2017).

Induced pluripotent stem cells (iPSCs) are self renewable and can differentiate to nearly all kinds of cells (Zeng et al. 2018). Reprogramming may be achieved by employing different cocktails with a number of different transcription factors, application of miRNA and some small molecules such as VPA (Singh et al. 2015). VPA can induce reprogramming

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✉ Guangpeng Li
gpengli@imu.edu.cn

[1] State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Inner Mongolia University, 24 Zhaojun Road, Hohhot 010021, Inner Mongolia Autonomous Region, People's Republic of China

in mouse fibroblasts with only three of the four transcription factors (*Oct-3/4*, *Sox2*, *c-Myc*, and *Klf4*) (Huangfu et al. 2008a). MiR367 and VPA were shown to cooperate in reprogramming mouse and human somatic cells to approach pluripotency (Anokye-Danso et al. 2011). VPA enhances iPSC induction from human bone marrow-derived cells through suppressing reprogramming induced senescence (Chen et al. 2016). VPA also can assist reprogramming of buffalo fibroblasts to pluripotent stem cells (Mahapatra et al. 2017). These findings provide us a hypothesis that somatic cells treated by VPA may be more favorable for reprogramming.

Based on this hypothesis, somatic cells treated by VPA were used to SCNT research. The objective of the present experiment was to assess (1) the effect of VPA treatment on the characteristics, histone acetylation and DNA methylation of bovine fetal fibroblasts and (2) the effect on in vitro development of bovine SCNT embryo. This study will provide a theoretical reference for bovine nuclear transfer technology.

Materials and Methods

The vimentin-positive bovine fetal fibroblast cells (BEF) used in this study all came from one cell line. All experiments were performed with a third generation of BEF. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise mentioned. Each experiment was repeated at least three times.

Ethics statement All procedures were approved by the Inner Mongolia University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiment of Inner Mongolia University.

VPA treatment of bovine fetal fibroblasts and cell proliferation assay VPA was dissolved to 1 M stock solution by DMEM/F12, and stored at − 20°C.

Once the BEF reached the logarithmic phase, VPA at a final concentration of 0, 0.25, 0.5, 1, 2, or 4 mM were added into the culture medium (DMEM/F12 + 10%FBS). After dosing for 24 or 48 h, cell proliferation were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H- tetrazolium bromide (MTT) assay. The protocol for the cell proliferation assay is described as follows. The BEF, which is prepared for detection, were plated into 96-well plates. Then, the measuring medium (100 μL culture medium + 20 μL CellTiter 96® AQueous One Solution Reagent (Promega; Madison, WI)) were added to each well and incubated 4 h at 37°C, 5% CO_2. The luminescence was scanned by *VARIOSKAN FLASH* (Thermo Scientific; Waltham, MA). Control wells were only added measuring medium in blank wells to obtain a value for background luminescence. The percentage of cell inhibition was calculated as follows: the inhibition rate (%) = [1 − (OD treatment − OD blank)/(OD control − OD blank)] × 100%.

Karyotyping assay When the BEFs cultured in 100 mm dishes reached to 80–90% confluence, these cells were treated with 0.1 μg/mL colchicine for 4 h at 37°C before being harvested using trypsin-EDTA solution. The BEFs were resuspended in 37°C pre-warmed hypotonic solution (0.075 M KCl) and incubated at 37°C for 30 min. Then, 1 mL freshly prepared fixative (3:1 methanol/acetic acid) was added to pre-fix for 3 min. The BEFs were centrifuged and fixed at room temperature for 20 min by fixative. This process was repeated three times. The harvested cells were dropped onto frozen slides. After these slides were air-dried, and stained with Giemsa solution for 30 min. One hundred cells were observed and photographed by NIS-Elements F 4.30.01 (Nikon).

Flow cytometry cell cycle assay The analysis of the cell cycle was performed using propidium iodide (PI). The cells were analyzed by flow cytometry (BD FACSAria™ III). Ten thousand events were analyzed for each sample. The cell cycle results were analyzed by ModFit LT (Verity Software House; Topsham, ME).

Flow cytometry apotosis assay The analysis of the externalization of phosphatidylserine was performed using TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems; Minneapolis, MN). The cells were analyzed by flow cytometry (BD FACSAria™ III). Ten thousand events were analyzed for each sample: viable cells (FITC−/PI−), early apoptotic cells (FITC+/PI−), late apoptotic cells (FITC+/PI+), and necrotic cells (FITC−/PI+).

Immunofluorescence staining of H3K9ac, H4K5ac, H3K4me2, and H3K9me2 The BEFs were washed three times by phosphate-buffered saline (PBS) with 0.3% bovine serum albumin (BSA) and then fixed in 4% paraformaldehyde for 15 min at room temperature (RT). The cells were permeabilized in 0.1% Triton-X100 for 20 min at RT and then washed five times with PBS (−) + 0.3% BSA before a final soak in PBS (−) + 3% BSA for 30 min at RT. The cells were incubated overnight with 1:500 dilution primary antibodies (Upstate) at 4°C and then washed three times in PBS (−) + 0.3% BSA. The cells were then incubated with a secondary antibody (1:500 dilution) for 2 h at RT and stained with DAPI for 5 min, followed by microscopic observation. The fluorescence values were analyzed using ImageJ software (NIH; Bethesda, MD) as follows: nuclei fluorescence intensity = (nuclear zone fluorescence − background fluorescence) × nuclear area.

Somatic cell nuclear transfer Fresh bovine ovaries were collected from local abattoir. A 10-mL syringe with an 18 gauge

needle was used to aspirate follicles (2–8 mm) from the ovary surface. Cumulus oocyte complexes (COCs) were cultured in pre-equilibrated TCM199 for 18–24 h. Oocyte maturation was conducted under the conditions of 38.5°C, 5% CO_2 and saturated humidity.

After maturation, oocytes with the first polar body were selected as recipient cytoplasts. All receptor oocytes were randomly grouped. Single cells were transferred to the perivitelline space of the recipient cytoplasts. The couplets were fused in mannitol fusion buffer by two electric DC pulses of 1.8 kV/cm for 20 μs, delivered from a Voltain cell fusion system (Cryoslogic; Blackburn, Australia). The fused embryos were activated by 7% ethanol for 7 min at RT and treated with 2 mM 6-dimethylaminopurine for 4 h. Following activation, the embryos were cultured in 40 μL of synthetic oviduct fluid (SOF) droplets overlaid with mineral oil at 38.5°C with 5% CO_2 in a humidified atmosphere. The cleavage, morula and blastocyst rates were recorded on days 2, 5, and 7, respectively.

DNA methylation status and gene expression analysis DNA methylation status was determined by bisulfite sequencing PCR (BSP). The protocol is described as follows: Genomic DNA of VPA-treated bovine fetal fibroblast was extracted by Wizare Genomic DNA purification (Promega), digested with *Xho*I (Takara; Ichikawashi, Japan), and subjected to bisulfite conversion according to EZ DNA Methylation-Gold Kit (Zymo Research; Irvine, CA). Bisulfite-converted DNA was amplified using the primers listed in Table 1, and the products were sequenced. The primer sets for these genes were described previously (Lan et al. 2011), which is closely correlated with the expression (Khan et al. 2012).

Gene expression was detected by quantitative real-time PCR (qPCR). Total RNA extraction and reverse transcription were performed as previously reported (Ao et al. 2016). qPCR was performed with the primers listed in Table 1. The qPCR was performed in triplicate using an ABI Prism 7500 Instrument (Applied Biosystems; Foster City, CA) with QuantiTect SYBR Green (Qiagen; Hilden, Germany) following the manufacturer's protocol. The thermal cycling conditions included initial sample incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The expression of each target gene was presented as the ratio of the target gene to *Gapdh*, which was expressed as $2^{-\Delta CT}$, where Ct is the threshold cycle and $\Delta CT = CT^{target} - CT^{Gapdh}$.

Statistical analysis All statistical analyses were performed with SPSS 16.0 software for Windows (SPSS Inc.). The results of the histone modifications and qPCR assays were analyzed by one-way ANOVA, and χ^2-analysis was used to analyze the DNA methylation status between groups. A p value of less than 0.05 was considered to be statistically significant.

Table 1. Primer sequences and product sizes

	Primers	Sequence	Products (bp)
BSP-PCR	*Oct4*	forward 5′-GATTTGGATGAGTTTTTAAGGGTT-3′ reverse 5′-ACTCCAACTTCTCCTTATCCAACTT-3′	293
	Nanog	forward 5′-TTTTTTAATTATAATTTGATGGGGT-3′ reverse 5′-CTAACACACCTTAAATAAACAAACC-3′	288
	Sox2	forward 5′-TATTAAAAGAGTAAATTTAAGATTAAGTT-3′ reverse 5′-CAAATTAATAAACAACCATCCATATAAC-3′	361
	Fgf4	forward 5′-TTTATTTGAAGAAAGTGTATTAAGGGG-3′ reverse 5′-AATACAAATTCAAAAAAATCCTCCTC-3′	223
	Rex01	forward 5′-AGTAGTTTGAGGATAGAGGTTAGGG-3′ reverse 5′-ACATACACCTAATAATCTAAAAAATCC-3′	299
Real-time PCR	*Oct4*	forward 5′-CTGCTGCAGAAGTGGGTGGA-3′ reverse 5′-CTCACTCGGTTCTCGATACTCGTC-3′	119
	Nanog	forward 5′-ATCTGCTGACACCCTCGACAC-3′ reverse 5′-GGGTCTGCGAGAACACAGTTCTAA-3′	194
	Sox2	forward 5′-TCAGATGCAGCCCATGCAC-3′ reverse 5′-GGTGCCCTGCTGAGAATAGGAC-3′	121
	Fgf4	forward 5′-TCTTCGTGGCCATGAGCAG-3′ reverse 5′-CACTCGTAGGCGTTGTAGTTGTTG-3	109
	Rex01	forward 5′-TGCGCATCTTCAACGAGTCA-3′ reverse 5′-TGCCCAGGAAACAGTGTGG-3′	127
	Gapdh	forward 5′-GCCATCATGACGTTCAAAGA-3′ reverse 5′-CGCAAGTCATCAACCTCGTA-3′	161

Results

Optimization of VPA treatment for bovine donor cells To optimize the VPA concentration, BEFs were cultured in the culture medium (DMEM/F12 + 10% FBS) supplemented with 0, 0.25, 0.5, 1, 2, and 4 mM VPA for 24 and 48 h, respectively. MTT was used to analyze cell proliferation. As shown in Fig. 1, the cell inhibition rate were significantly increased in 48 h treatment. In 24 h treatment, the curve of cell inhibition rates showed that 2 and 4 mM were significantly higher than the others (Fig. 1). In order to decrease the cytotoxicity, 0.25, 0.5, and 1 mM VPA treated 24 h may be appropriate.

According to the immunofluorescence results, H3K9ac and H4K5ac were significantly increased in 4 mM VPA. There were no significant differences among other groups (Fig. 2*A*, *B*, supplement Figs. 1 and 2). H3K4me3 increased with the increase of VPA concentration, and the highest fluorescent signal was observed at 2 mM VPA treatment (Fig. 2*C*, supplement Fig. 3). However, H3K9me2 was significantly decreased with the increase of VPA concentration (Fig. 2*D*, supplement Fig. 4). A comprehensive analysis of the cell proliferation and histone modification was conducted not only to guarantee the effect of VPA but also reduce its cytotoxicity, and subsequent experiments were handled 24 h using 0.5 and 1 mM VPA.

Characteristics of bovine donor cell after VPA treatment In order to ensure the VPA-treated cells can be used to SCNT, the characteristics of these cells were detected. In cell cycle, there were no significant differences among 0, 0.5, and 1 mM groups (Fig. 3*A*). After apotosis assay, we found that the early and late apoptosis rate was significantly decreased in 1 mM group. Unlike early apoptosis, the late apoptosis rates also significantly declined in 0.5 mM groups (Fig. 3*B*). However, the necrotic cell rates were significantly increased in VPA treatment groups (Fig. 3*B*). This suggests that the treatment of VPA can accelerate the death of late apoptotic cells.

The chromosome status of BEFs treated by 0.5 and 1 mM VPA were detected by a conventional karyotyping method. BEFs contain 2n = 60 chromosomes, with 29 pairs of telocentric chromosomes, an acrocentric chromosome X, and a small telocentric chromosome Y (Fig. 3*C*). The chromosomal euploidity ratios of 0.5 and 1 mM treatment group were 85 and 82%, respectively (Fig. 3*D*, *E*), whereas the ratio of the 0 mM group was 88%. The above results showed 0.5 and 1 mM treatment groups satisfied SCNT.

Effects of VPA on SCNT embryonic development Bovine SCNT was performed using 0.5 and 1 mM treatment groups as donor cells, untreated cells as controls. There were no difference in the cleavage rate among the VPA treatment and control groups. However, in the 1 mM treatment group, the blastocyst rates were significantly increased compared with 0 mM treatment group (30.4% ± 0.7 vs 29.1 ± 1.0%, $p < 0.05$) (Table 2). The produced blastocysts in both groups were stained with DAPI to evaluate whether the treatment affected their development (supplement Fig. 5). The result showed VPA did not alter the cell number of cloned blastocysts.

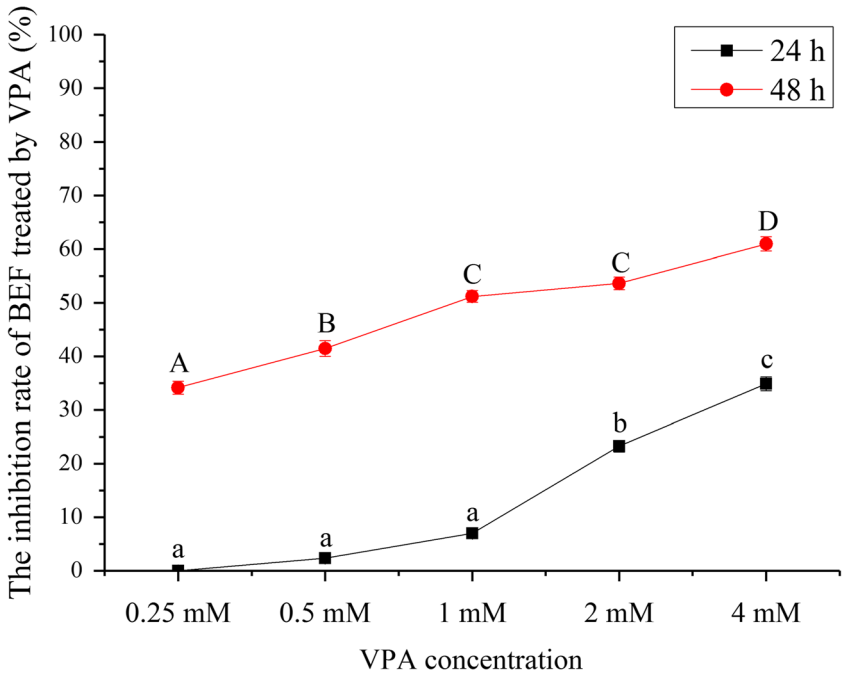

Figure 1. The inhibition rate of BEFs treated by VPA. *Different letters* represent significant differences.

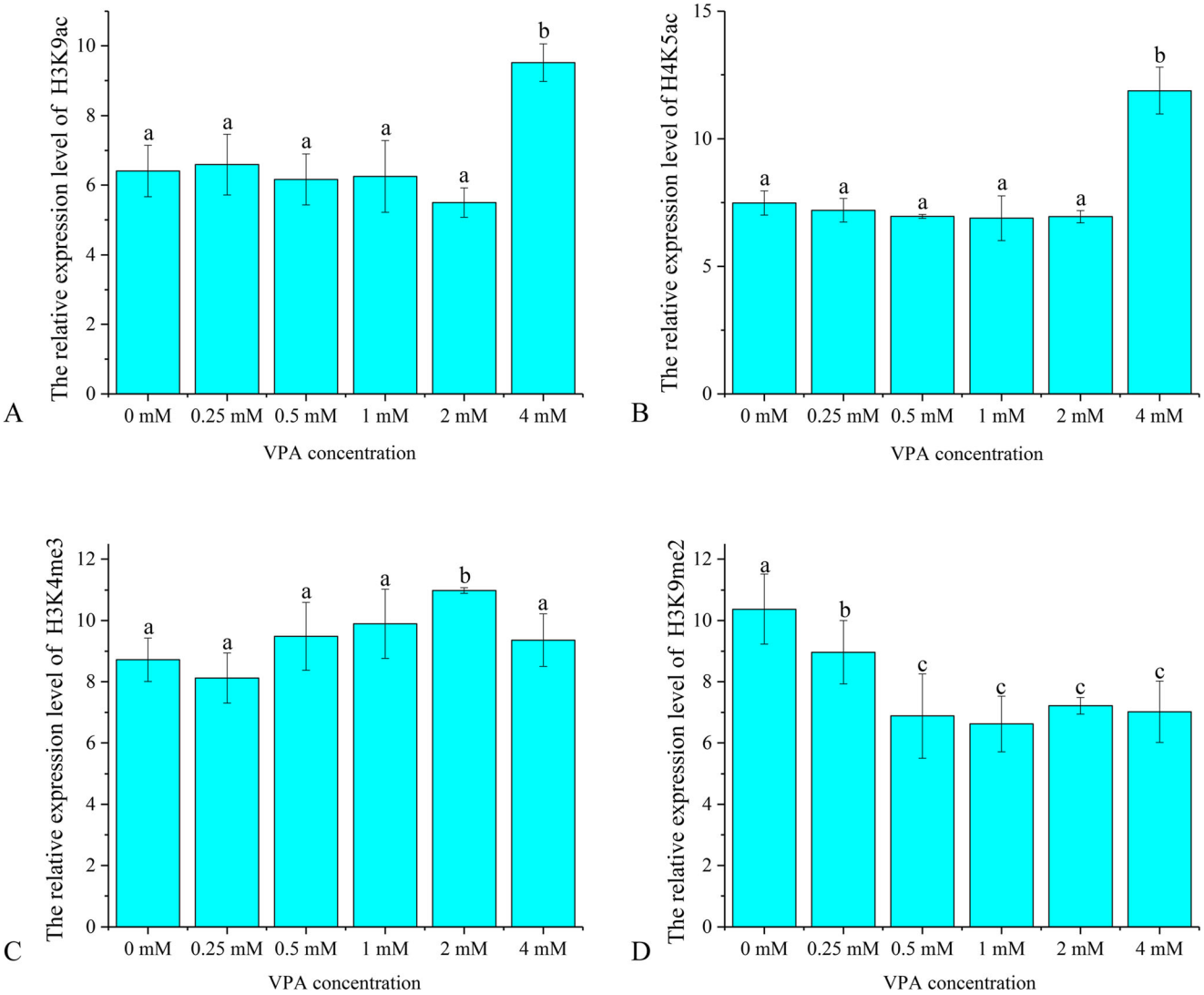

Figure 2. The changes of histone modifications on BEFs after VPA treatment. *(A)* The changes of H3K9ac in BEFs after VPA treatment for 24 h. *Different letters* represent significant differences. *(B)* The changes of H4K5ac in BEFs after VPA treatment for 24 h. *Different letters* represent significant differences. *(C)* The changes of H3K4me3 in BEFs after VPA treatment for 24 h. *Different letters* represent significant differences. *(D)* The changes of H3K9me2 in BEFs after VPA treatment for 24 h. *Different letters* represent significant differences.

Pluripotency-related gene expression and methylation status of their promoters in bovine donor cells after VPA treatment

To better understand the mechanism of VPA on the development of bovine SCNT embryos, the pluripotency-related gene expression (Fig. 4) and methylation status of their promoters (Fig. 5) were investigated. The results showed that the expression of *Oct4* were significantly increased after VPA treatment, and no obvious change was observed in *Sox2* and *Rex01* (Fig. 4). *Nanog* was only significantly increased after 0.5 mM VPA treatment (Fig. 4). *Fgf4* was significantly decreased after VPA treatment (Fig. 4).

BSP results showed that there was no obvious change in the methylation status of the *Nanog*, *Sox2* and *Rex01* promoter region after treatment with VPA (Fig. 5). In comparison, more methylation was found in the *Oct4* gene promoter regions after VPA treated. The methylation status of the *Fgf4* gene promoter region was only increased in 0.5 mM group (Fig. 5).

Discussion

In the process of SCNT, the donor cell nucleus in the enucleated oocyte must undergo cellular reprogramming; incomplete reprogramming will lead to a low cloning efficiency and abnormalities in the cloned animals (Dean et al. 2001; Yang et al. 2007; Pagé-Larivièreflorence and Sirardmarc-André 2014). In previous studies, HDAC inhibitors (Guo et al. 2017; Saini et al. 2017) and DNA demethylation (Chen and Riggs 2011) were shown to have a modest effect (two- to five-fold) on the efficiency of reprogramming. VPA, as a type of HDAC

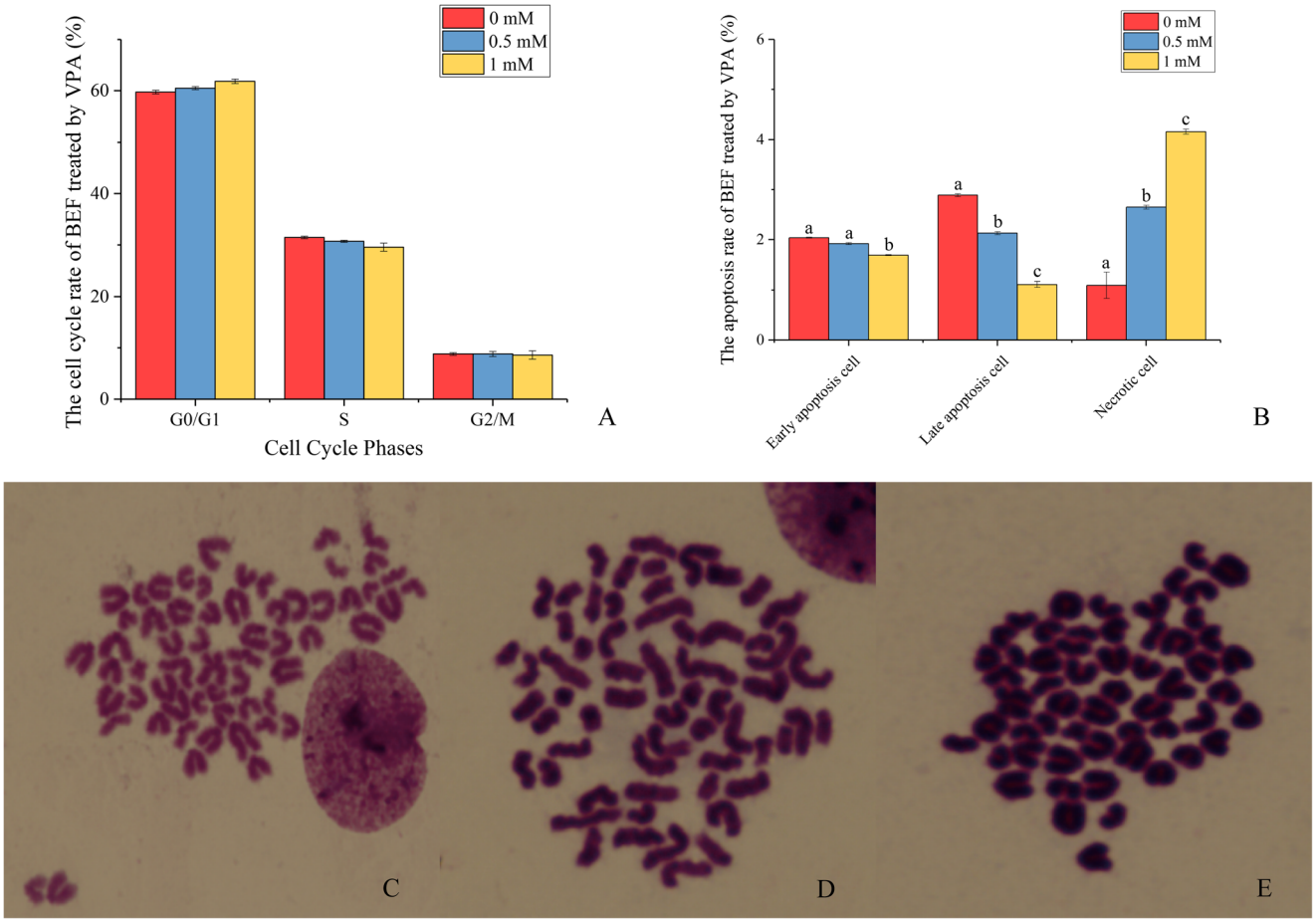

Figure 3. The characteristics of BEFs after VPA treatment. *(A)* Cell cycle rate of BEFs after VPA treatment for 24 h. *(B)* Cell apoptosis of BEFs after VPA treatment for 24 h. *Different letters* represent significant differences. *(C–E)* Representative image of BEFs chromosomes after 0, 0.5, and 1 mM VPA treatment for 24 h (× 1000).

inhibitors, can significantly promote reprogramming (Chen et al. 2016; Mahapatra et al. 2017; Zhai et al. 2015). VPA has been widely used to improve the efficiency of SCNT and the cell number of SCNT embryos in mice (Isaji et al. 2013), cattle (Miyoshi et al. 2016; Xu et al. 2012), and pigs (Miyoshi et al. 2010). Their experiments all directly processed SCNT embryos with VPA, only a few study-treated donor cells with VPA (Sangalli et al. 2014; Selokar et al. 2017). Their result was not improving the efficiency of SCNT. As we know, VPA is

Table 2 Development of cloned embryos derived from VPA-treated cells

Concentration (mM)	Culture no.	Cleavage rate (%)	Blastocyst rate (%)
0	275	231 (84.0 ± 0.2)	80 (29.1 ± 1.0)^{a}
0.5	282	234 (83.0 ± 0.7)	87 (29.8 ± 1.9)^{ab}
1	276	227 (82.2 ± 0.6)	84 (30.4 ± 0.7)^{bc}

Different letter in the same *column* represent significant differences ($p < 0.05$)

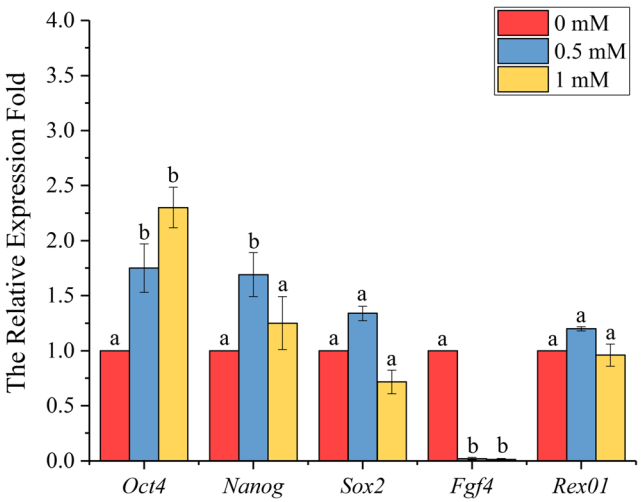

Figure 4. The expression of pluripotency-related gene in BEFs after VPA treatment. Untreated fetal fibroblasts served as controls, and *Oct4*, *Nanog*, *Sox2*, *Fgf4*, and *Rex01* expression was normalized to *Gapdh* expression. Gene expression was then normalized to that of the control fetal fibroblasts, which is represented as 100%. The data shown are from three independent fusion experiments (*Different letters represent* $p < 0.05$).

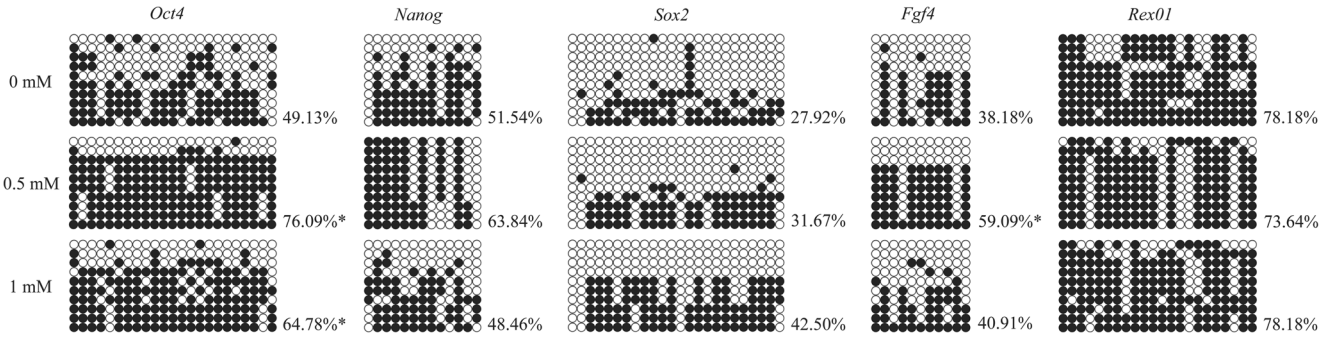

Figure 5. DNA methylation status of pluripotency-related gene in BEFs after VPA treatment. Bisulfite sequencing analysis of the methylation status of the *Oct4*, *Nanog, Sox2*, *Fgf4*, and *Rex01* promoters in bovine fetal fibroblasts treated with VPA. *White and black circles* represent unmethylated and methylated CpG dinucleotides, respectively. * represent significant difference with respect to the control group ($p < 0.05$).

concentration-dependent, the possible reason for this is that the concentration and treatment time of VPA was not reasonable enough to maximize it effect.

In this research, cell proliferation and immunofluorescence of histone were used to optimize the VPA concentration. As shown in the results, the cell proliferation did not change after 24 h treated by 0, 0.25, 0.5, and 1 mM VPA. As an HDACi, the main effect of VPA is to alter histone modifications. H3K9ac, H4K5ac, H3K4me3, and H3K9me2 were detected by immunofluorescence. According to the results, H3K9ac and H4K5ac only changed in 4 mM VPA treatment. H3K4me3 was increased in 0.5 and 1 mM VPA groups, whereas H3K9me2 was significantly decreased. As we know, H3K4me2/3 marks the promoters that genes actively transcribed (Davie et al. 2016), while H3K9me2 is associated with gene silencing (Herrmann et al. 2013). Although there was no change in histone acetylation after 0.5 and 1 mM VPA treatment, histone methylation altered to indicate gene activation. The maternal-to-zygotic transition (MZT) is essential for the embryonic development, including maternal component (mRNAs and proteins) degradation and zygotic genome activation (ZGA). Many types of histone modification have been identified during ZGA (Liu et al. 2018b). H3K4me3 and H3K9me3 are not detected before the MZT. After ZGA, H3K4me3 and H3K9me3 are detectable in two-and eight-cell-stage embryos. In this study, the VPA-treated cells showed high level of H3K4me3 and the low H3K9me2. A decrease in H3K9me2 indicates an increase in H3K9me3, because H3K9me2 is a substrate for H3K9me3 production. These indicated that VPA-treated cells have similar patterns of histone to zygotic embryos, and may be more favorable to reprograming.

A total of 833 cloned embryos were produced from the experimental replicates of VPA-treated donor cells. In 1 mM treatment group, the blastocyst rates were significantly increased compared with control. There are few studies on VPA-treated somatic cells to increase cloning efficiency. But this consisted with the results obtained from Selokar et al. by handmade cloning (Selokar et al. 2013).

After a breakthrough study of Yamanaka, various iPSCs have been produced that derived from a number of different species by the four (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) or six (*Lin28* and *Nanog* in addition to the four previously mentioned factors) transcription factors (Takahashi et al. 2007; Liu et al. 2008; Chang et al. 2010; Honda et al. 2010; West et al. 2010; Li et al. 2011). Thus, these transcription factors especially *Oct4*, *Nanog*, and *Sox2* are very important for cell reprogramming. Previous studies have reported that VPA treatment alone is insufficient to reprogram cells (Huangfu et al. 2008b). However, histone acetylation and global transcriptional changes have been detected in VPA-treated MEFs. These changes promoted reprogramming of somatic cells. In the present study, VPA treatment lead to the overexpression of *Oct4* and *Nanog*, which may facilitate the reprogramming of somatic nuclei. According to the BSP results, except for *Oct4* promoter being hypermethylated after VPA treated, all other genes maintained their methylation status. These results indicated that VPA affect the DNA methylation status of single gene loci by influencing histone modifications.

Conclusions

Our findings provide a proof that VPA can regulate pluripotency gene expression by altering their histone modification and DNA methylation to affect the efficiency of SCNT. At the same time, our findings demonstrate the interrelation between DNA methylation and histone modifications. The histone modifications may alter the chromatin conformation to change the DNA methylation status. These require further study.

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Compliance with ethical standards

All procedures were approved by the Inner Mongolia University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiment of Inner Mongolia University

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