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Sodium butyrate improves the cloned yak embryo viability and corrects gene expression patterns

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Summary

Interspecies somatic cell nuclear transfer (iSCNT), a powerful tool in basic scientific research, has been used widely to increase and preserve the population of endangered species. Yak (*Bos grunniens*) is one of these species. Development to term of interspecies cloned yak embryos has not been achieved, possibly due to abnormal epigenetic reprogramming. Previous studies have demonstrated that treatment of intraspecies cloned embryos with (NaBu) significantly improves nuclear–cytoplasmic reprogramming and viability *in vitro*. Therefore, in this study, we evaluated the effect of optimal NaBu concentration and exposure time on preimplantation development of yak iSCNT embryos and on the expression patterns of developmentally important genes. The results showed that 8-cell rate, blastocyst formation rate and total cell number increased significantly compared with their untreated counterparts when yak iSCNT embryos were treated with 5 nM NaBu for 12 h after activation, but that the 2-cell stage embryo rate was not significantly different. The treatment of NaBu also increased significantly the expression levels of *Oct-4* and decreased the expression levels of *HDAC-2*, *Dnmt-1* and *IGF-1*; the expression patterns of these genes were more similar to that of their bovine–yak *in vitro* fertilization (BY-IVF) counterparts. The results described above indicated that NaBu treatment improved developmental competence *in vitro* and 'corrected' the gene expression patterns of yak iSCNT embryos.

Keywords: Development, Expression, iSCNT, Sodium butyrate, Yak

Introduction

The yak, known in China as the 'almighty livestock', is well adapted to low temperatures, low oxygen levels, and the low pressure environment found at high altitudes (e.g. at 3500 m above sea level). These animals are able to make full use of alpine grasslands, which other livestock find difficult to utilize. However, this environment provides primitive grazing with vegetation that grows slowly, resulting in low animal production yields, in addition the deteriorating environment makes yak populations vulnerable to environmental pressures. How to accelerate the genetic breeding of yaks, thereby not making them vulnerable to extinction, and to increase the value of their production is an important problem with a worldwide focus.

Much progress has been made towards applying assisted breeding to aid yak conservation, such as hybridization between cattle and yak species. In particular, interspecies somatic cell nuclear transfer (iSCNT) is a novel method that has been shown to protect species that are not subject to genetic variation and are in danger of extinction. The sand cat (Gómez *et al.*, 2008), gaur (Vogel 2001), mouflon (Loi *et al.*, 2001) and grey wolf (Oh *et al.*, 2008) have all been cloned successfully using iSCNT, although the efficiency is still extremely low. The efficiency of iSCNT depends on a variety of factors, such as the status of donor cells, the quality of recipient oocytes and the environment of the *in vitro* operation. However, the low success rate found with this technique has been widely attributed to incomplete reprogramming of epigenetic modifications or to epigenetic errors (Couldrey & Lee, 2010).

It has been reported that histone acetylase levels in cloned SCNT embryos of intraspecies bovine, mouse,

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pig, sheep and rabbit that had been treated with histone deacetylases inhibitors (HDACi), such as TSA and VPA, were improved significantly compared with untreated animals and were similar to their *in vitro* fertilization (IVF) or *in vivo* counterparts (Lager *et al.*, 2008). Furthermore these embryos showed improved development to the blastocyst stage (Beebe *et al.*, 2009; Shao *et al.*, 2009; Costa-Borges *et al.*, 2010; Zhao *et al.*, 2010), plus there was an increase in the number of live offspring after transfer to foster mothers (Maalouf *et al.*, 2009; Costa-Borges *et al.*, 2010), and reduced abnormal phenotypes, with the exception of placental overgrowth in mice (Kishigami *et al.*, 2006). Even though improvements in cloning efficiency have been observed in intraspecies cloned embryos with TSA treatment, conflicting results have been reported when interspecies cloned embryos were treated with TSA (Shi *et al.*, 2008; Srirattana *et al.*, 2008).

To our knowledge, the effect of pre-treatment of yak iSCNT embryos with NaBu has not been reported previously and the effects of NaBu on the development competence and expression patterns of genes related to development in yak iSCNT embryos after activation have not been thoroughly examined. In the present study, we examined whether treatment of yak iSCNT embryos with NaBu improved developmental competence *in vitro* and 'corrected' expression patterns of genes (*Oct-4, HDAC-2, Dnmt-1,* and *IGF-1*) during development stages *in vitro.*

Materials and methods

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Disposable, sterile plasticware were purchased from Nunclon (Roskilde, Denmark).

All procedures in this experiment were approved by the Animal Care and Use Committee of Southwest University for Nationalities (Chengdu, China) and performed in accordance with animal welfare and ethics.

Nuclear donor cell preparation

Yak fibroblast cells from the ear skin were derived as described previously (Xiong *et al.*, 2012). Briefly, the tissues were minced into pieces (1 mm^3) using sterile scissors in a 35 mm Petri dish, then explants were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 μg/ml EGF, 100 IU/ml penicillin and 100 mg/ml streptomycin under 5.5% CO_2 in air at 38.5°C. The cells were trypsinized and reconstituted at a concentration of 1×10^6 cells/ml after reaching 80–90% confluency. Then 2–5 cells were passaged, as donor cells for iSCNT, and were synchronized in the G_0/G_1 phase by contact inhibition for 2 days before iSCNT.

In vitro maturation (IVM)

Bovine ovaries were obtained from the local slaughter houses and transported to the laboratory within 4 h of slaughter in sterile 0.9% (w/v) NaCl saline at approximately 20°C in a thermos bottle. Cumulus–oocyte complexes (COCs) were aspired from 2–8 mm follicles using a 12-gauge needle and then injected into phosphate-buffered saline (PBS) plus 0.5 IU/ml heparin and 5% (v/v) FBS. COCs showing an even cytoplasm and surrounded by compact cumulus cells were collected from the follicular fluid and PBS mixture, washed twice in PBS and then incubated in TCM-199 (Gibco) supplemented with 10% (v/v) FBS, 1 μg/ml 17β-estradiol and 0.075 IU/ml human menopausal gonadotropin for 20 h at 38.5°C in 5.5% CO_2 in air.

Parthenogenetic activation (PA)

PA was conducted according to the method described previously by Xu & Yang (2001). The matured oocytes were denuded of cumulus cells in PBS supplemented with 0.2% (v/v) hyaluronidase and activated in 5 μM ionomycin for 5 min, followed by a 4 h exposure to 2 mM dimethylaminopurine (6-DMAP) in mSOF. The activated oocytes were washed twice in mSOF and then randomized into five groups, and cultured in mSOF plus either 0 nm (control), 5 nm or 10 nm NaBu for 6 h and 12 h, respectively. After culture with NaBu, the embryos were transferred to NaBu-free mSOF for further culture at 38.5°C in 5.5% CO_2 in air for 7 days.

Interspecies somatic cell nuclear transfer (iSCNT)

iSCNT was conducted according to the method described previously by Xiong *et al.* (2012). Briefly, after IVM, matured bovine oocytes were denuded with 0.2% (v/v) hyaluronidase in PBS to disperse the cumulus cells. Enucleation was performed using a 20-μm (internal diameter) glass pipette and by aspirating the first polar body and a small amount of the surrounding cytoplasm. After enucleation, a single synchronized yak fibroblast cell was introduced into the perivitelline space of the enucleated oocyte and fusion was induced by application of two electrical pulses of 35 V for 10 μs. Reconstructed embryos were stored in mSOF that contained 5 g/l cytochalasin B for 2 h until activation. Then, successfully reconstructed embryos were activated in 5 mM ionomycin for 5 min followed by 4 h of exposure to 2 mM 6-dimethylamino-pyridine (6-DMAP) in mSOF. Activated embryos were cultured in mSOF in randomized groups. After culture

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Table 1 Primer sequences and PCR conditions used for real-time PCR

Gene	Primer sequences (5′– 3′)	Annealing temperature (°C)	Product size (bp)	Accession no.
Dnmt-1	S: CAGTCGGGAAGCGAATGGATGTCTA; A: CCGTGGGAAATGAGATGTGATGGTG	60	223	BC114063
HDAC-2	S: ACAAGAAGGAAACGGAGGACAA; A: AAGAAAAACAAAAACAAAGCCA	60	271	BC120419
Oct-4	S: GCAGGAGATATGCAAGGCAGAG; A: CGCCAGAGGAGAGGATACGGGT	60	295	NM_174580
IGF-1	S: CTAAATTCAGAGCAGATAGAGCC; A: AGATAGAAGAGATGCGAGGAGGA	60	260	NM_001077828
H2a	S: CATTAGTGTGACATTCAGACGC; A: ACCTAGCAAGACATACCCTTCC	60	247	BC134772

S: forward primer; A: reverse primer.

in mSOF with NaBu for different lengths of time, embryos were transferred to NaBu-free mSOF for further culture at 38.5°C in 5.5% CO_2 in air for 7 days.

In vitro fertilization (IVF)

Frozen–thawed yak semen was thawed, and motile spermatozoa were obtained for fertilization using a Percoll gradient (Pharmacia, diluted into 45% and 90%). Viable sperm were recovered and resuspended. Maturated oocytes were co-incubated with spermatozoa at a concentration of 1×10^6 cells/ml for approximately 24 h in a humidified atmosphere with 5.5% CO_2 in air at 38.5°C. Then bovine–yak IVF (BY-IVF) presumptive zygotes were denuded by treatment with 0.2% (v/v) hyaluronidase in PBS, washed twice in mSOF, and then transferred into mSOF for further culture as yak iSCNT embryos.

Counting of cell numbers

Day 7 blastocysts from BY-IVF and iSCNT were permeabilized in 4% paraformaldehyde in PBS for 20 min, stained with 10 μg/ml propidium iodide (PI) for 15 min, and then mounted on slides in 5 μl glycerol. At least 10 embryos from each group were selected randomly for processing. The total number of cells was counted under an epifluorescence microscope (Nikon, Japan) using a digital camera.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

mRNA was extracted using the RNeasy total extraction kit (Qiagen, Valencia, USA) in accordance with the manufacturer's protocol but with some modifications. Briefly, two embryos from BY-IVF, iSCNT and iSCNT-NaBu groups were chosen randomly, washed twice in Ca^{2+}- and Mg^{2+}-free PBS, and 10 μl lysis buffer was added for 3 min. cDNA synthesis was achieved using the cDNA synthesis kit (Takara, China) in accordance with the manufacturer's instructions.

Real-time RT-PCR was performed using the five primer sets shown in Table 1, primers were designed using the Primer 5.0 software. The specificities of all primers were tested using BLAST analysis against the genomic National Center for Biotechnology Information, USA (NCBI) database, and PCR products were sequenced for verification.

Quantitative real-time PCR (qRT-PCR)

All qRT-PCR reactions were performed in triplicate in a 20-μl reaction volume using the quantitative real-time PCR CFX96 detection system (Bio-Rad, Hercules, USA) and using reaction mixture SYBR Premix Ex *Taq*™ II (TaKaRa, China) that contained 10 μl 2× SYBR Green premix, plus 0.8 μl of forward and reverse primers (20 pmol/ml), 2 μl embryonic cDNA and 6.4 μl RNase- and DNase-free water. Histone H2a was used initially as the housekeeping reference gene. All primers used were optimized to ensure similar reaction efficiencies (96–98%) between target genes and H2a. Thermal cycling conditions were 95°C for 10 min, followed by 40 PCR cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The melting protocol was a step cycle starting at 65°C, and then increased to 95°C with 0.5°C/5 s increments. In addition, a non-template control that contained the above reaction mixture but lacking cDNA was included in each PCR run as a negative control. The relative quantification of gene expression levels was conducted using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The experiment was repeated at least three times for each treatment group. Data are presented as mean ± standard error of the mean (SEM). The 2-cell stage embryo, 8-cell stage embryo, and blastocyst formation,

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Table 2 Effects of treated concentration and expose time of NaBu on the *in vitro* development of parthenotes embryos

Concentration of treatment (nM)	Time of treatment (h)	No. of embryos cultured (replicates)	No. of embryos		
			2-cell (%)	8-cell (%)	Blastocyst (%)
0	0	160 (4)	118 (73.4 ± 1.1)	50 (42.3 ± 1.6)^{b}	35 (29.7 ± 1.2)^{b}
	6	184 (4)	137 (74.5 ± 0.8)	82 (59.9 ± 1.9)^{a}	44 (32.1 ± 2.1)^{b}
5	12	175 (4)	134 (76.6 ± 0.4)	81 (60.4 ± 0.9)^{a}	60 (44.8 ± 0.8)^{a}
	6	191 (4)	142 (74.3 ± 0.7)	77 (54.2 ± 1.5)^{a}	59 (41.5 ± 1.3)^{a}
10	12	186 (4)	139 (74.7 ± 1.2)	74 (53.2 ± 1.7)^{a}	43 (30.9 ± 0.9)^{b}

^{a,b}Values with different superscripts within a column are significantly different ($P < 0.05$).
2-cell rate: no. of 2-cell stage embryos/no. of embryos cultured. 8-cell rate: no. of 8-cell stage embryos/no. of 2-cell stage embryos. Blastocyst rate: no. of blastocysts/no. of 2-cell stage embryos.

total cell number and the gene expression levels among those groups were tested by one-way analysis of variance (ANOVA) and least significant difference (LSD) test using the SPSS 17.0 software. Differences were considered significant at *P*-values < 0.05.

Results

Effects of NaBu on the *in vitro* development of PA embryos

First, the effect of NaBu on the *in vitro* development of PA embryos was evaluated. In this study, parthenotes were chosen because they were relatively easy to produce in large numbers for the analysis, and the chemical activation protocol of PA embryos was similar to that of iSCNT embryos. NaBu concentrations of 1–10 nM and the treatment times of 4–20 h have been reported in different species (Shi *et al.*, 2008). Parthenotes were exposed to different concentrations of NaBu (0, 5, 10 nM) for 6 h or 12 h, respectively, then cultured *in vitro* for 7 days. As shown in Table 2, NaBu did not have any negative effect on cleavage rates ($P > 0.05$), but there was a significant beneficial effect on 8-cell stage and blastocyst formation rates when PA embryos were treated with 5 nM NaBu for 12 h. However, with the increase in treatment concentration and exposure time, the blastocyst formation rate was in a downward trend that might be due to the toxicity of NaBu. Therefore, a 12 h treatment period plus a 5 nM NaBu concentration was chosen for treating iSCNT embryos.

Effects of NaBu on the *in vitro* development of yak iSCNT embryos

The yak iSCNT embryos were divided randomly into two groups and treated with 5 nM NaBu for 12 h after activation, and then the *in vitro* development and quality of yak iSCNTs were evaluated. As shown in Table 3, although there was no significant difference on the rate of cleavage after NaBu treatment ($P > 0.05$), this treatment significantly improved the rate of 8-cell stage embryo (34.2 ± 1.3% vs. 24.8 ± 1.6%, $P < 0.05$) and blastocyst formation (24.7 ± 1.7% vs. 15.0 ± 1.9%, $P < 0.05$) compared with their controls; the treatment of NaBu had a positive effect on the total cell number per blastocyst (shown in Fig. 1).

Effects of NaBu on gene expression of yak iSCNT embryos

The relative transcript abundance of *Oct-4*, *HDAC-2*, *Dnmt-1* and *IGF-1* was quantified by real-time PCR at various preimplantation developmental stages of BY-IVF and yak iSCNT embryos with or without NaBu treatment to examine if the low cloning efficiency was correlated with gene expression during development *in vitro*. Figure 2 shows that the relative expression level of Oct-4 in yak iSCNT embryos was significant

Table 3 Effects of NaBu on the *in vitro* development of yak iSCNT embryos

Embryo groups	No. of embryos cultured (replicates)	No. of embryos			No. of cells in blastocysts
		2-cell (%)	8-cell (%)	Blastocysts (%)	
iSCNT	220 (5)	153 (69.5 ± 1.1)^{a}	38 (24.8 ± 1.6)^{b}	23 (15.0 ± 1.9)^{b}	64.3 ± 2.2^{b}
iSCNT-NaBu	220 (5)	158 (71.8 ± 1.0)^{a}	54 (34.2 ± 1.3)^{a}	39 (24.7 ± 1.7)^{a}	77.5 ± 1.8^{a}

^{a,b}Values with different superscripts within a column are significantly different ($P < 0.05$).
2-cell rate: no. of 2-cell stage embryos/no. of embryos cultured. 8-cell rate: no. of 8-cell stage embryos/no. of 2-cell stage embryos. Blastocyst rate: no. of blastocysts/no. of 2-cell stage embryos.

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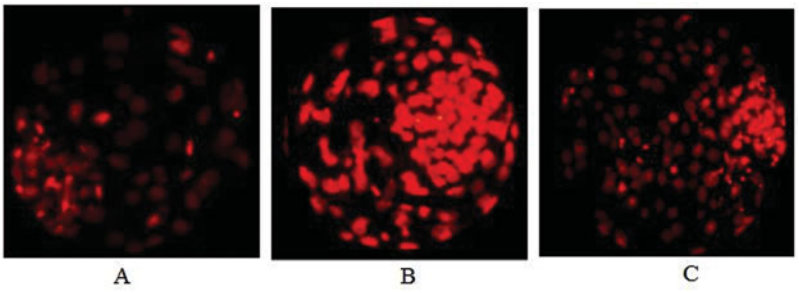

Figure 1 The total cell number of blastocysts derived from interspecies somatic cell nuclear transfer (iSCNT) (*A*), iSCNT-NaBu (*B*) and bovine–yak *in vitro* fertilization (BY-IVF) (C) (×200 magnification).

lower compared with that of their BY-IVF counterparts after the 2-cell stage; after NaBu treatment, the expression levels of Oct4 increased significantly in yak iSCNT embryos, which exhibited similar levels to their BY-IVF counterparts. The expression patterns of *HDAC-2* and *IGF-1* were observed in yak iSCNT and BY-IVF embryos, the NaBu-treated iSCNT embryos showed lower levels of expression for *HDAC-2* and *IGF-1*, and these levels were more similar to their BY-IVF counterparts. The level of DNA methylation related gene, *Dnmt-1*, was higher in iSCNT embryos than in BY-IVF counterparts, and there was no significant difference between iSCNT and iSCNT-NaBu groups up to the blastocyst stage.

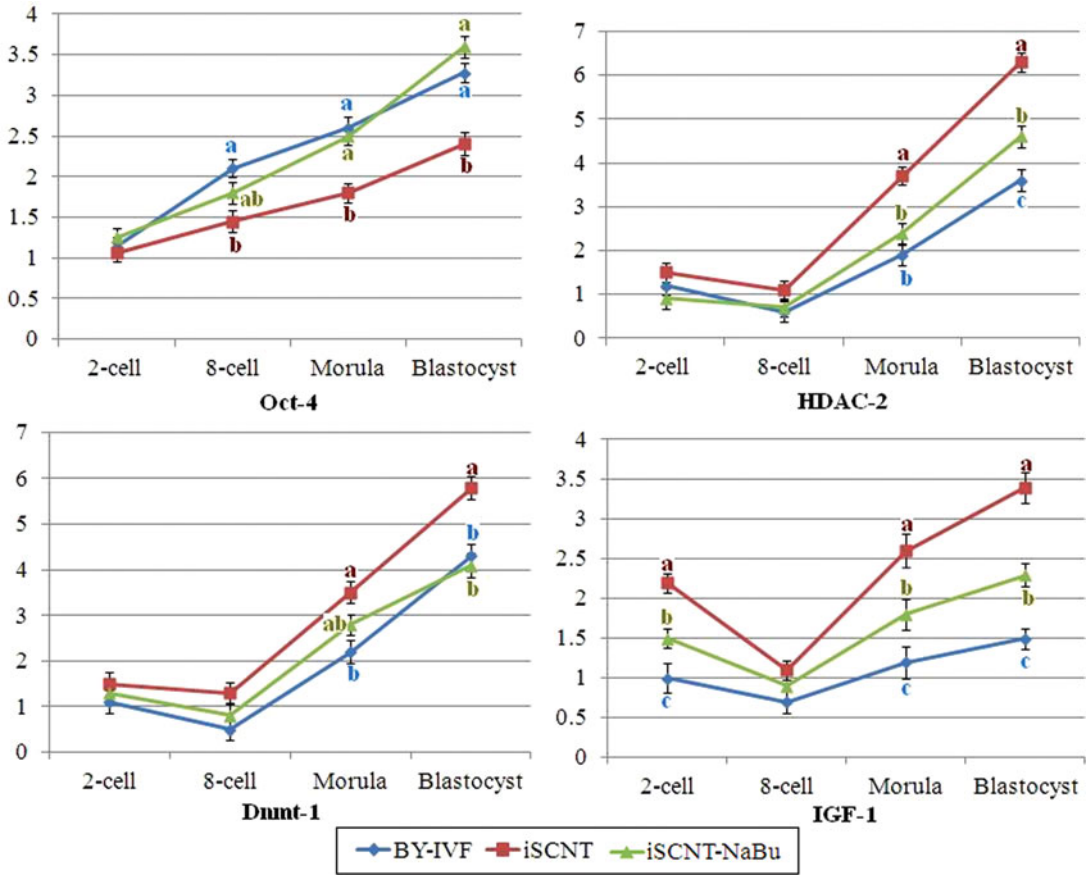

Figure 2 Relative transcript abundance of *Oct-4*, *HDAC-2*, *Dnmt-1* and *IGF-1* genes in embryos produced by bovine–yak *in vitro* fertilization (BY-IVF), interspecies somatic cell nuclear transfer (iSCNT) or iSCNT-NaBu at four stages of embryonic development (2-cell stage embryo, 8-cell stage embryo, morula, and blastocyst). Different letters denote samples that differed significantly within each developmental stage ($P < 0.05$).

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Discussion

Global epigenetic reprogramming has been reported as a major factor that is required to take place following SCNT for normal development and successful cloning. The epigenetic state of the donor nucleus must be erasure and an embryonic epigenetic state pattern should be established in SCNT embryos. Although the mechanism of how epigenetic states modify a donor cell after iSCNT is still a mystery, several studies have shown that nuclear–cytoplasmic incompatibilities between species were the major handicaps for iSCNT (Martha *et al.*, 2011).

There is growing evidence that suggests that proper use of histone deacetylase inhibitor (HDACi) can improve the efficiency of SCNT. Lee *et al.* (2010) reported that treating cat cells with TSA before iSCNT, significantly increased the level of acetylation in histone H3K9, and improved the *in vitro* developmental competence and total cell numbers of iSCNT blastocysts. Our previous study showed that SCNT embryos pre-treated with TSA and Scriptaid significantly improved the efficiency of bovine cloning (Wang *et al.*, 2011). Likewise we observed that in iSCNT yak embryos treated with NaBu, a novel HDACi substantially increased cloned embryo development *in vitro*. Our results are consistent with those of Das *et al.* (2010) and of Mohana *et al.* (2007), who both found that NaBu can increase the blastocyst formation rate in SCNT embryos. However, a discrepancy exists in its effectiveness in donor cells. Das *et al.* (2010) confirmed that there was no effect on cloning efficiency when donor cells were pre-treated with NaBu. This finding was in sharp contrast with the report by Shi *et al.* (2003). This discrepancy may be due to species differences in response to NaBu in these studies. In our study, we also found no positive effect when donor cells were pre-treated with NaBu (data not show). Interestingly, we discovered that most yak iSCNT embryos were arrested at the 2- to 8-cell stage, and that the 8-cell stage embryos increased predominantly when yak iSCNT embryos were treated with 5 nM NaBu for 12 h after activation. Therefore, we concluded that the lower levels of development *in vitro* of iSCNT yak embryos may be related to aberrant gene expression at this stage, and that NaBu could 'correct' the aberrant gene expression of iSCNT yak embryos during development *in vitro*, especially during the maternal zygotic transition (MZT) stage.

The onset of transcription in embryonic genome activation is one of the most critical events of early embryogenesis. However, previous studies have indicated that the main reason for low efficiency in iSCNT was because correct transcription failed to occur (Bui *et al.*, 2010). This transcriptional activation is a remarkable event, during which a number of genes are activated and there is dramatic reprogramming of embryonic gene expression, including those genes that are important for successful embryo development. Consequently, we determined expression patterns of *Oct-4*, *HDAC-2*, *Dnmt-1* and *IGF-1* that may in part regulate gene expression during early embryogenesis of yak iSCNT embryos. *Oct-4* is a key pluripotency specific gene for which accurate expression is crucial for preimplantation embryo development; reactivation of *Oct-4* expression is a marker of nuclear reprogramming (Miyamoto *et al.*, 2007). *HDAC-2* is a member of the histone deacetylases, and participates in removal of acetyl moieties from histone tails (Murko *et al.*, 2010). *Dnmt-1*, a DNA methyltransferases, is expressed constitutively and is responsible for maintenance of global methylation following DNA replication (Bosak *et al.*, 2003); Deletion of *Dnmt-1* alone results in embryonic lethality (Okano *et al.*, 1999). Insulin-like growth factor 1 (*IGF-1*) is a classic imprinted gene that plays a crucial role in embryo development and viability (Velazquez *et al.*, 2009). Dramatic increase in *Oct-4* expression levels was observed after iSCNT embryos were treated with NaBu, along with significantly decreased levels of *HDAC-2*, *Dnmt-1* and *IGF-1* that were more similar to their BY-IVF counterparts. These results confirmed our hypothesis that NaBu 'corrected' the expression patterns of developmentally important genes in early development of yak iSCNT embryos, and these data were consistent with previous studies (Li *et al.*, 2008; Martha *et al.*, 2011; Ning *et al.*, 2012), all of these reports found that gene expression patterns in cloned embryos were normalized after treatment with HDACi. Abnormal expression patterns in crucial genes at the early developmental stage might explain the block in development observed in the present study between the 2-cell and 8-cell stages. Interestingly, the relative expression level of *IGF-1* was higher in iSCNT yak embryos compared with their BY-IVF counterparts, especially at the 2-cell stage. Therefore, we propose that *IGF-1* can be used as a marker for evaluating the developmental potential of preimplantation iSCNT embryos.

In addition, the 8-cell stage rate, blastocyst formation rate and total cell number in blastocyst of iSCNT embryos were lower compared with their IVF counterparts, and increased significantly after NaBu treatment. This finding indicates that the addition of NaBu improved embryo quantity and quality *in vitro*. NaBu may alter the activity of histone deacetylases, thus changing the epigenetic model and gene expression patterns, to promote the transition of maternal genes to embryo genes and reduce the block in yak iSCNT embryo development. These findings are in contrast with those of Martha *et al.* (2011) who

confirmed that HDACi did not improve the viability of *in vitro* or *in vivo* cloned cat embryos. Different HDACi, treatment time and concentration might be the main reason for this discrepancy. However, future research needs to be carried out to further explore the mechanisms of this phenomenon, and to evaluate the long-term effects of NaBu on the developmental competence of yak iSCNT embryos *in vivo*.

In conclusion, we have demonstrated that NaBu induced improvements in cloning efficiency and gene expression levels at the early developmental stages of yak iSCNT embryos. However, details of the mechanism of nuclear reprogramming of NaBu-treated iSCNT yak embryos are still unclear and need further investigation. On the basis of our results, we propose that the inability of bovine cytoplasm to modify the epigeneticity of yak nuclei after iSCNT resulted in abnormal gene expression during preimplantation development of cloned embryos *in vitro*, and significantly affected epigenetic reprogramming.

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Author disclosure statement

The authors declare that no conflicting financial interests exist.

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