TET3 overexpression facilitates DNA reprogramming and early development of bovine SCNT embryos

Jian Zhang^{1,2,*}, Linlin Hao^{3,*}, Qian Wei⁴, Sheng Zhang⁵, Hui Cheng¹, Yanhui Zhai¹, Yu Jiang¹, Xinglan An⁵, Ziyi Li⁵, Xueming Zhang¹ and Bo Tang¹

¹College of Veterinary Medicine, Jilin University, Changchun, Jilin, China, ²Department of Biology, Southern University of Science and Technology, Shenzhen, Guangdong, China, ³Department of Radiotherapy, Second Hospital, Jilin University, Changchun, Jilin, China, ⁴Department of Heat Disease, Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, Jilin, China and ⁵Academy of Translational Medicine, First Hospital, Jilin University, Changchun, Jilin, China

Correspondence should be addressed to X Zhang or B Tang; Email: zhangxuem@jlu.edu.cn or tang_bo@jlu.edu.cn

*J Zhang and L Hao contributed equally to this work

Abstract

Somatic cell nuclear transfer (SCNT) has been successfully used for cloning in a variety of mammalian species. However, SCNT reprogramming efficiency is relatively low, in part, due to incomplete DNA methylation reprogramming of donor cell nuclei. We previously showed that ten-eleven translocation 3 (*TET3*) is responsible for active DNA demethylation during preimplantation embryonic development in bovines. In this study, we constructed *TET3*-overexpressing cell lines *in vitro* and observed that the use of these fibroblasts as donor cells increased the blastocyst rate by approximately 18 percentage points compared to SCNT. The overexpression of *TET3* in bovine SCNT embryos caused a decrease in the global DNA methylation level of the pluripotency genes *Nanog* and *Oct-4*, ultimately resulting in an increase in the transcriptional activity of these pluripotency genes. Moreover, the quality of bovine *TET3*-NT embryos at the blastocyst stage was significantly improved, and bovine *TET3*-NT blastocysts possessed more total number of cells and fewer apoptotic cells than the SCNT blastocysts, similar to *in vitro* fertilization (IVF) embryos. Nevertheless, DNA methylation of the imprinting control region (ICR) for the imprinted genes *H19-IGF2* in SCNT embryos remained unaffected by *TET3* overexpression, maintaining parent-specific activity for further development. Thus, the results of our study provide a promising approach to rectify incomplete epigenetic reprogramming and achieve higher cloning efficiency.

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Introduction

Somatic cell nuclear transfer (SCNT) is a powerful tool by which the differentiated somatic cell genome can be reprogrammed back into a totipotent state through a reprogramming mechanism (Gurdon 1962, Wilmot *et al.* 1997). Animal production using SCNT has great potential for the development of therapeutic and regenerative medicine as well as in agriculture and species preservation (Andrabi & Maxwell 2007, Yang *et al.* 2007). However, the efficiency of SCNT for most mammalian species is extremely low. Aberrant reprogramming of the somatic donor nucleus is one of the major causes of the decreased probability of obtaining cloned embryos, which limits the application of SCNT technology in many areas (Dean *et al.* 2001, Armstrong *et al.* 2006).

Various epigenetic abnormalities in SCNT embryos have been identified and often lead to developmental arrest through mechanisms such as DNA methylation,

histone modifications, and genomic imprinting, which are thought to determine activation of the embryonic genome (Dean *et al.* 2001, Wang *et al.* 2007, Okae *et al.* 2014). Therefore, a series of approaches to improve epigenetic reprogramming, such as decreasing DNA methylation levels in the reconstructed embryos by DNA methyltransferase inhibitors, increasing histone acetylation levels by histone deacetylase inhibitors, and reducing histone methylation levels in SCNT embryos by demethylase overexpression, have been reported (Enright *et al.* 2005, Van Thuan *et al.* 2009, Hormanseder *et al.* 2017). These studies offer several effective approaches to improve the efficiency of SCNT embryos to a certain extent.

In mice, the dioxygenase ten-eleven translocation 3 (*TET3*) acts to demethylate the parental genome by converting 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Gu *et al.* 2011,

He *et al.* 2011). By performing inhibition and knockdown experiments on IVF and parthenogenetically activated (PA) embryos, the results of our recent study confirmed that *TET3* is responsible for active DNA demethylation during bovine embryonic development (Zhang *et al.* 2017, Cheng *et al.* 2019). Previous studies have shown that there is an inverse correlation between abnormal DNA methylation and SCNT efficiency (Wei *et al.* 2011). DNA methylation levels have been observed to be abnormally high in SCNT embryos of different species, and aberrant DNA methylation profiles can be identified even after implantation (Peat & Reik 2012, Zhang *et al.* 2016). Therefore, proper DNA methylation reprogramming from the donor cell to the zygote is essential for successful SCNT.

In the present study, we used sodium bisulfite genomic sequencing (BSP) technology to detect and validate the level of DNA methylation in bovine SCNT embryos. We observed aberrantly increased levels of DNA methylation in SCNT embryos compared to IVF embryos. We subsequently produced bovine SCNT embryos using *TET3*-overexpressed fibroblasts with the SCNT technique. The cloned embryos exhibited significantly enhanced transcriptional reprogramming and improved developmental potential. Thus, our results identified a crucial factor associated with unsuccessful nuclear reprogramming and provide a more accurate method to improve the efficiency of bovine SCNT embryos.

Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise specified.

Ethics statement

Bovine ovaries from slaughtered mature cattle were collected from a local abattoir in Changchun, China. Frozen-thawed bovine semen was purchased from Ketian Co., Ltd. (Changchun, China). All experimental materials and procedures were approved by the Animal Welfare and Research Ethics Committee at Jilin University (approval ID: 20151008-1).

Cell culture and transfection

Bovine embryo fibroblasts (BEFs) were isolated and cultured from Yanbian yellow cattle fetuses as previously described (Wang *et al.* 2011). Commercially synthesized full-length *TET3* cDNA was inserted into the vector pHBLV (Hanbio Biotechnology, Shanghai, China), which was then packaged by three-way transfection of HEK-293T cells using the packaging plasmids pSPAX2 and pMD2G.

At 48 h after transfection, the viral supernatant was harvested, ultracentrifuged to remove floating 293T cells, and frozen at -80°C until used to infect target cells. Then,

BEFs at passage 2–4 were seeded in 6-well plates, cultured to 70% confluency and infected with a mixture of virus at an multiplicity of infection (MOI) of 40 in a solution containing 8 $\mu\text{g/mL}$ polybrene. After incubating for 24 h, the viral solution was replaced with fresh complete medium and incubated for another 24 h. Positive transgenic clones were identified with a fluorescence microscope (Nikon) and selected for by the addition of 2 $\mu\text{g/mL}$ puromycin to the culture medium every 2–3 days. After the nontransfected cells were killed, positive transgenic cells were cultured in fresh medium and used for further experiments.

Oocyte collection and in vitro maturation

Bovine ovaries were obtained from a local abattoir (Changchun, China) and transported into the lab in sterile saline within 2–3 h. Cumulus oocyte complexes (COCs) were aspirated from 3–8 mm bovine ovarian follicles using a 10-mL disposable syringe with a 12-gauge needle. COCs with compact cumulus cells were cultured *in vitro* in maturation medium (TCM-199 medium supplemented with sodium bicarbonate, 10% fetal bovine serum, 1 $\mu\text{g/mL}$ follicle-stimulating hormone and 10 $\mu\text{g/mL}$ luteinizing hormone) in a humidified incubator under an atmosphere with 5% CO_2 at 38.5°C for 20–22 h for IVF or 16–18 h for SCNT.

In vitro fertilization (IVF) and embryo culture

Fresh semen was washed two times with 5 mL of Dulbecco's PBS and then centrifuged at 250 *g* for 5 min. The spermatozoa concentration was adjusted to 1×10^6 sperm/mL using a hemocytometer, and the swim-up sperm were then collected to prepare fertilization droplets (100 μL). Mature oocytes were washed two times in TALP medium. Approximately 20–25 oocytes were transferred into 100- μL droplets and incubated with capacitation of sperm for 20 h at 38.5°C under an atmosphere with 5% CO_2 in air. After fertilization, presumptive zygotes were removed from excessive sperm and cultured in synthetic oviduct fluid (Sofaa) containing 10% fetal bovine serum (FBS) at 38.5°C under an atmosphere with 5% CO_2 and 95% humidity.

Somatic cell nuclear transfer (SCNT) and embryo culture

Donor cells for SCNT were derived from BEFs at passages 3–6 and cultured in serum-free DMEM (Gibco) with 1% FBS (Gibco) for 24 h at 37°C under an atmosphere with 5% CO_2 in air to synchronize cells at G0/G1 phase of the cell cycle. The SCNT process was performed as previously described (Stelzer *et al.* 2016). Briefly, mature and enucleated oocytes with the first polar body were placed in TCM-199 medium-HEPES containing 7.5 $\mu\text{g/mL}$ cytochalasin B. After enucleation using a beveled glass pipette, a single donor cell was individually transferred to the perivitelline space of each oocyte. Then, the couplets were fused in mannitol fusion buffer with one direct pulse of 1.8 kV/cm for 10 μs by an Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). All the reconstructed

embryos were activated with 5 μ M ionomycin for 5 min, followed by 4 h of exposure to 2 mM 6-dimethylpyridine in SOF medium containing 10% FBS at 38.5°C under an atmosphere with 5% CO₂ and 95% humidified air. Then, the activated SCNT embryos were cultured as described for IVF (Zhang *et al.* 2017).

Immunofluorescence staining

The zona pellucida (ZP) of bovine embryos was dissolved by treatment with 0.5% Pronase and fixed with 4% paraformaldehyde for 30 min at room temperature. After three washes with PBS-PVA, embryos and cells were permeabilized with 0.1% Triton X-100 prepared in PBS-PVA for 20 min and then blocked for 1 h at room temperature in PBS-PVA containing 2% BSA. Following incubation overnight at 4°C with primary antibodies targeting FLAG (diluted 1:100; Beyotime; Shanghai, China), *TET3* (diluted 1:50; Santa, CA, USA), 5mC (diluted 1:200; Eurogentec, Liege, Belgium) and 5hmC (diluted 1:200; Active Motif, Carlsbad, CA, USA), embryos and cells were washed with PBS-PVA and stained at 37°C for 1.5 h with Alexa Fluor 488 (1:200; Invitrogen) and Alexa Fluor 594 (1:200; Invitrogen). For 5mC/5hmC staining, two additional treatments were performed before blocking; embryos and cells were incubated with 4 N HCl for 30 min and then Tris-HCl buffer (100 mM, pH 8.5) for 20 min. DNA was stained with 10 μ g/mL DAPI for 10 min. All samples were immediately mounted and observed under a fluorescence microscope (Nikon), and images of embryos and cells were taken using the same microscope setting and exposure times. The average fluorescence intensity of embryos was evaluated using ImageJ (National Institutes of Health, Bethesda, MD) based on procedures described elsewhere (Aflalo *et al.* 2005). The data shown are representative of at least three independent experiments.

Western blot analysis

Cells were collected and lysed with RIPA buffer, and protein concentration was measured using a BCA protein assay kit (Thermo Scientific). The proteins were separated via SDS-PAGE and then transferred to PVDF membranes, which were subsequently blocked in 5% BSA and incubated with primary antibodies targeting FLAG (diluted 1:200; Beyotime; Shanghai, China) and Gapdh (diluted 1:500; BOSTER; CA, USA) overnight. The membranes were washed three times in TBST and then incubated for 1 h with HRP anti-rabbit (diluted 1:2000; BOSTER) and HRP anti-mouse (1:2000; BOSTER) antibodies. Then, the membranes were washed three times in TBST and visualized using ECL Detection Reagents (Pierce;

Thermo Scientific). Images were obtained using an LAS-4000 Imager (Fuji).

RNA isolation and RT-qPCR

Total RNA was extracted from BEFs using TRIzol reagent and from embryos at different stages using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using a cDNA synthesis kit (Promega). Real-time PCR was performed in 96-well optical reaction plates using SYBR® Premix Ex Taq™ reagents (TaKaRa) and the primers listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article) in a LightCycler 96 Real-Time PCR System (Roche). Each 20- μ L PCR mixture contained 2 μ L of cDNA, 10 μ L of SYBR Green premix, 6 μ L of RNase-free water, and 1 μ L each of the forward and reverse primers for each gene. The following program was used to amplify all genes: denaturation at 95°C for 60 s, 42 cycles of PCR for quantitative analysis (95°C for 5 s and 60°C for 30 s), melt curve analysis (95°C for 5 s, 60°C for 60 s, and 95°C for 1 s), and cooling at 4°C. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Quantitative PCR analysis was performed three times per sample. Values were normalized to bovine *GAPDH* mRNA expression.

Sodium bisulfite genomic sequencing

Bisulfite sequencing was used to measure the locus-specific DNA methylation status of BEFs, oocytes, and IVF and SCNT blastocysts as previously described (Zhang *et al.* 2016). In short, genomic DNA was subjected to bisulfite transformation, and PCR was performed using the primers shown in Table 1. PCR products were gel recovered using an Axy Prep DNA Gel Extraction kit (Axygen, Beijing, China) and then cloned into the vector PmdTM18-T (TaKaRa). The recombinant plasmid was transformed into DH5 α competent cells (TaKaRa), and at least 30 positive clones were selected for sequencing. BiQ analysis software was used to evaluate the DNA methylation level as the ratio of the methylated CpG number to the total CpG number (Bock *et al.* 2005).

TUNEL assay

To determine the number of apoptotic cells in blastocysts, the DeadEnd Fluorometric TUNEL System (Roche) was used as previously described (Lu *et al.* 2014). Samples were observed with a fluorescence microscope (Nikon) with the same exposure time and image capture settings to enable relative

Table 1 Effect of TET3 overexpression on bovine SCNT embryonic development *in vivo*.

Group	No. of embryos examined	No. of 2-cell cleavage (%)	No. of 8-cell cleavage (%)	No. of Blastocyst cleavage (%)
IVF	233224	204 (87.8 $\pm$ 1.8) ^a	145 (62.3 $\pm$ 4.1) ^a	78 (33.8 $\pm$ 1.7) ^a
SCNT		130 (61.2 $\pm$ 3.6) ^b	66 (31.1 $\pm$ 2.9) ^b	25 (12.1 $\pm$ 0.9) ^b
TET3-NT		188 (84.3 $\pm$ 4.1) ^a	124 (55.7 $\pm$ 2.4) ^a	67 (29.9 $\pm$ 2.1) ^a

Data are the mean $\pm$ S.E.M. of at least three trials.

^{a,b}Different superscript letters within the same column express significant differences ($P < 0.05$).

comparisons. Each experiment was carried in at least three biological replicates.

Statistical analysis

Data that were derived from at least three independent experiments and analyzed using two-tailed Student's *t*-test with SPSS Statistics 19.0 (IBM Corporation) are presented as the mean $\pm$ S.E.M. Values of $P < 0.05$ indicated significance; values of $P < 0.01$ indicated extreme significance.

Results

Successful expression of exogenous *TET3* in BEF

In this study, we obtained the *TET3* CDS of bovine endogenous *TET3* and used it to overexpress *TET3* in bovine fibroblasts. To obtain *TET3*-transfected cells, the overexpression vector pHBLV-CMV-*TET3*-3FLAG-EF1-ZsGreen-T2A-Puro was successfully constructed according to the schematic diagram of the vector (Fig. 1A), and the *TET3* gene was expressed with the CMV promoter. After lentiviral transfection and selection with puromycin, the expression of *TET3* was validated through immunohistochemical staining (Fig. 1B). Real-time PCR was used to assess the expression of *TET3* in bovine fetal fibroblasts at 48 h after transfection, and the results demonstrated that *TET3* transcription was significantly increased (by 75-fold) at 72 h after *TET3*

transfection compared to that observed in the Blank group (WT) and the N.C. group (BEFs transfected with an empty vector that did not contain *TET3*) (Fig. 1C). Immunofluorescence results also showed that the *TET3* protein was highly expressed in BEFs stably transfected with *TET3* (E.G.) (Fig. 1D). In addition, the immunofluorescence and Western blot results showed that the FLAG-*TET3* fusion protein was detected in only the nuclei of BEFs transfected with the *TET3* construct (Fig. 1E and F).

Activation of pluripotency genes in BEFs by *TET3* overexpression

To determine whether ectopic *TET3* expression affects other genes associated with epigenetic and pluripotency related genes in fibroblasts prior to nuclear transfer, the expression levels of related genes were assessed by real-time PCR. The results showed that the mRNA expression levels of the pluripotency genes *Nanog*, *Sox2*, and *Oct4* were significantly increased in the *TET3* overexpression group compared to those observed in the Blank and N.C. groups, while *Cdx2* expression was not detected in any of the three groups (Fig. 2A). In addition, the mRNA levels of genes encoding DNA methyltransferases (*DNMT3a* and *DNMT3b*), lysine-specific demethylases (*KDM3A*, *KDM4A*, *KDM4C*, and *KDM5B*) and imprinted genes (*H19* and *Igf2*) were not significantly different

Figure 1 Successful expression of the *TET3* protein in BEFs. (A) Schematic diagram of the overexpression vector pHBLV-CMV-*TET3*-3FLAG-EF1-ZsGreen-T2A-Puro. (B) Representative green fluorescence image of a BEF transfected with *TET3*. (C) Real-time PCR results assessing the expression of Tet family in BEFs 72 h after transfection with *TET3*. (D) Morphology and *TET3* expression of Blank, N.C. and E.G. BEFs. (E and F) FLAG-*TET3* fusion protein was detected in the nuclei of transfected BEFs by immunofluorescence and Western blot analyses. The data are presented as the mean $\pm$ S.E.M. *** $P < 0.001$. Scale bar = 100 μ m.

Figure 2 Relative mRNA abundances of various genes. (A, B, C and D) Expression of DNA methyltransferase (DNMT), lysine-specific demethylase (KDM) and imprinted genes in the Blank, N.C. and E.G. bovine fibroblasts. GAPDH was used as an internal control. The gene expression levels in the Blank were used to calibrate the sample (expression set to 1). The data are presented as the mean $\pm$ S.E.M. ** $P < 0.01$, *** $P < 0.001$.

among the three cell lines; however, the expression of the DNA methyltransferase-encoding gene *DNMT1* was upregulated in the E.G. group compared to that observed in the Blank and N.C. groups (Fig. 2B, C and D).

Overexpression of TET3 improves bovine SCNT embryonic development

Having established that *TET3* was enriched in BEFs, we asked whether *TET3* overexpression would aid in overcoming the DNA methylation reprogramming barrier in bovine SCNT embryos. To this end, we performed SCNT using *TET3*-overexpressing BEFs as nuclear donors, and the GFP protein levels at the reconstructed embryo, 2-cell embryo, 4-cell embryo, 8-cell embryo and blastocyst stages were examined via fluorescence microscopy (Fig. 3A). In addition, WT bovine SCNT embryos and IVF embryos were used as controls. The effects on embryonic development are shown in Table 1. Cleavage rates and 8-cell and blastocyst formation rates in the TET-NT embryos were significantly higher than those observed in the WT SCNT embryos but still much lower than those detected in the IVF embryos (Fig. 3B). As expected, the *TET3* protein was more highly expressed in the TET3-NT embryos than in the WT SCNT embryos and IVF embryos, with little expression detected in the WT SCNT embryos (Fig. 3C and E). Immunostaining results revealed that *TET3* overexpression greatly reduced 5mC levels in the nuclei of bovine SCNT embryos, which showed the same methylation pattern as the IVF embryos (Fig. 3D and F). The results indicated that the reprogramming barrier of DNA methylation in the somatic genome can be eliminated by *TET3* demethylases in bovine SCNT embryos.

Dynamic DNA methylation patterns of satellite I and α -satellite during the early development of bovine embryos

To further evaluate the global trend in DNA methylation, we also assessed the DNA methylation status of repeat elements (α -satellite and satellite I) using sodium bisulfite genomic sequencing in bovine embryos. The results showed high satellite I and α -satellite DNA methylation levels in bovine oocytes (63.2% vs 55.9%, respectively) and BEFs (85.1% vs 83.4%, respectively), and the DNA methylation of both repeat elements in bovine IVF embryos decreased steadily in 8-cell embryos (23.9% vs 37.5%, respectively) and blastocysts (19.4% vs 25.6%, respectively) (Fig. 4A and C), indicating that the DNA demethylation process took place during IVF. Unlike the IVF group, among WT SCNT embryos, satellite I and α -satellite DNA methylation remained high in 8-cell embryos (78.9% vs 72.0%, respectively) and blastocysts (76.6% vs 70.9%, respectively) (Fig. 4A and C). Subsequently, we tested DNA methylation levels in the TET-NT group and observed that satellite I and α -satellite methylation profiles were similar to those of IVF embryos at the 8-cell and blastocyst stages (Fig. 4B and D), which was consistent with the 5mC staining results (Fig. 3D). These results suggest that the reprogramming barrier, DNA methylation in the somatic cell genome, could be eliminated by overexpression of *TET3* demethylases in bovine SCNT embryos.

TET3 overexpression restores pluripotent gene transcriptome in bovine SCNT embryos

To better elucidate the mechanism associated with the improved development of *TET3*-overexpressing SCNT embryos, we focused on pluripotency-related genes and the methylation status of their promoters. We first detected *TET3* expression in the TET3-NT group, which was significantly upregulated compared to that observed in the WT SCNT and IVF embryos at the 8-cell and blastocyst stages (Fig. 5A). Furthermore, the expression of *Nanog*, *Oct-4* and *Sox2*, three transcription factors that are essential for maintaining pluripotency, were analyzed in these three groups, and the results showed that *TET3* overexpression in transgenic NT embryos could activate the endogenous expression of these transcription factors, as their levels were much higher than those observed in the WT SCNT embryos at the 8-cell and blastocyst stages (Fig. 5B, C and F). However, we observed no significant difference in *Cdx2* mRNA levels between the three groups (Fig. 5D).

DNA methylation, which has been identified as a concomitant abnormal modification in bovine cloned embryos, is known to affect gene expression (Santos *et al.* 2003). Therefore, we used BSP to test DNA methylation levels at the *Nanog* and *Oct-4* gene promoter regions in IVF and SCNT embryos at the 8-cell and blastocyst

Figure 3 Overexpression of TET3 improves the development of bovine SCNT embryos by removing aberrant DNA methylation. (A) Schematic illustration of the bovine SCNT procedures. (B) Preimplantation development rates in the IVF, SCNT and TET3-SCNT groups. Error bars represent the S.E.M. of five biological replicates. (C) TET3 (red) and DAPI staining (blue) in two-cell to blastocyst IVF, SCNT and TET3-NT embryos. Each sample was counter-stained with DAPI to visualize DNA. (D) Methylation patterns were determined based on the relative levels of 5-methylcytosine (5mC) in two-cell to blastocyst IVF, SCNT and TET3-NT embryos. (E and F) Average optical intensity of global TET3 and DNA methylation measured using Image J. The data are presented as the means $\pm$ S.E.M. ($P < 0.05$). Scale bars = 300 μ m (A), 50 μ m (C and D).

stages, BEFs, and bovine oocytes. The results showed that the methylation levels of the *Nanog* and *Oct-4* promoter regions in IVF embryos were lower than those observed in oocytes and BEFs. In addition, *Nanog* and *Oct-4* were more highly methylated in WT SCNT embryos than that observed in IVF embryos and the methylation profiles of *Nanog* and *Oct-4* in SCNT embryos were similar to those observed in donor cells (Fig. 6A and C). These results suggest that incomplete removal of DNA methylation can result in inconsistent activation of pluripotency genes. Furthermore, the methylation profiles in TET3-NT embryos were observed to be similar to those observed in IVF embryos but were much different from those observed in WT SCNT embryos, suggesting that *TET3*

overexpression can restore the global transcriptional status in clone embryos.

Gene expression and DNA methylation of imprinting genes

Abnormal imprinting patterns observed in cloning often lead to maladjustment of parental gene expression. Bisulfite sequencing and real-time PCR were used to determine the methylation status and expression of the *H19/IGF2* ICR (Fig. 7). The results showed that the ICR was highly methylated in BEFs (81.8%). Moderate DNA methylation of the ICR was maintained in bovine IVF embryos at the 8-cell (46.2%) and blastocyst

Figure 4 Dynamic DNA methylation profiles of *satellite I* and α -satellite in oocyte- to blastocyst IVF, SCNT, TET3-NT embryos and BEFs. (A and B) DNA methylation status of *satellite I* measured using bisulfite sequencing in oocytes as well as IVF, SCNT, and TET3-NT embryos and BEFs. (C and D) DNA methylation status of α -satellite measured using bisulfite sequencing in oocytes as well as IVF, SCNT, and TET3-NT embryos and BEFs. The filled (black) circles correspond to methylated cytosines, the unfilled (white) circles indicate unmethylated cytosines, and the small vertical lines without a circle correspond to missing values.

(57.5%) stages, and ICR DNA methylation levels were significantly lower in bovine SCNT 8-cell embryos (59.7%) and blastocysts (38.7%) than was observed in IVF embryos ($62.5 \pm 7.5\%$) (Fig. 7B). As expected for the *H19-IGF2* ICR, the analyzed region was highly

methylated in BEFs (81.8%) but hypomethylated in oocytes (4.5%). In contrast, moderate DNA methylation was maintained in bovine IVF 8-cell embryos (46.2%) and blastocysts (57.5%), and the ICR DNA methylation level was significantly decreased in bovine SCNT

Figure 5 *TET3* overexpression improves the relative expression of pluripotency genes. Relative expression level of *TET3* (A) and the pluripotency genes *Nanog* (B) and *Oct-4* (C), *Cdx2* (D) and *Sox2* (E) in bovine SCNT embryos. The gene expression levels in the IVF 8-cell stage were used to calibrate the sample (expression set to 1). The data are presented as the mean $\pm$ S.E.M. ** $P < 0.01$, *** $P < 0.001$.

Figure 6 Dynamic DNA methylation profiles of pluripotency genes *Nanog* and *Oct-4* in oocyte- to blastocyst IVF, SCNT, TET3-NT embryos and BEF. (A and B) DNA methylation status of *Nanog* measured using bisulfite sequencing in oocytes as well as IVF, SCNT, and TET3-NT embryos and BEFs. (C and D) DNA methylation status of *Oct-4* measured using bisulfite sequencing in oocytes as well as IVF, SCNT, and TET3-NT embryos and BEFs. The filled (black) circles correspond to methylated cytosines, the unfilled (white) circles indicate unmethylated cytosines and the small vertical lines without a circle correspond to missing values.

blastocysts (38.7%) compared to that observed in IVF blastocysts (Fig. 7B). However, no difference in DNA methylation profile was observed in TET3-NT embryos compared to the WT SCNT embryos, which showed 56.4% DNA methylation at the 8-cell stage and 30.6% DNA methylation at the blastocyst stages, respectively (Fig. 7B). Furthermore, *H19* and *IGF2* mRNA levels were detected in the IVF and NT 8-cell embryos and blastocysts. *H19* showed much higher expression in IVF blastocyst embryos, whereas the expression of *IGF2* was lower in bovine IVF embryos than that observed in SCNT embryos. In addition, expression of the *H19* and *IGF2* genes did not differ significantly between WT SCNT and TET3-NT embryos (Fig. 7D and E).

Effects of TET3 expression on cell apoptosis in bovine SCNT embryos

The expression of key genes related to apoptosis was assessed in the embryos using real-time PCR and the TUNEL assays (Fig. 8A). Apoptotic staining results showed that the percentage of apoptotic cells in the TET3-NT blastocysts was significantly lower than that observed in WT SCNT blastocysts but not significantly

different from that detected in IVF blastocysts (Fig. 8B). The transcriptional levels of the apoptotic genes *Bcl-2* and *Bax* were analyzed by RT-qPCR and were observed to be unchanged in embryos at the 8-cell stage. In addition, the expression of *Bax* in TET3-NT embryos was significantly decreased, while *Bcl-2* expression was higher in TET3-NT embryos than that detected in WT NT embryos at the blastocyst stage (Fig. 8C and D).

Discussion

Animal cloning using the SCNT technique has been applied to animal model construction, species conservation, and xenotransplantation. However, SCNT efficiency is still low in essentially all species (Campbell et al. 2007). This finding has been speculated to be related to incomplete epigenetic reprogramming during the process of SCNT (Matoba & Zhang 2018). Therefore, successful SCNT reprogramming should include the reprogramming of histone modification and DNA methylation profiles from the donor cell to zygotes. In previous studies, high levels of H3K9 methylation were reported to inhibit the activation of genes in two-cell embryos, and the removal of H3K9me3 was shown to

Figure 7 Methylation profiles and relative expression of the identified bovine intergenic *IGF2-H19* ICR. (A) Schematic drawing of the bovine *IGF2-H19* region. (B and C) DNA methylation status of *IGF2-H19* ICR measured using bisulfite sequencing in oocytes as well as IVF, SCNT, and TET3-NT embryos and BEFs. (D and E) The mRNA expression levels of imprinted genes *H19* and *IGF2* at the 8-cell and blastocyst stages. The gene expression levels in the IVF 8-cell stage were used to calibrate the sample (expression set to 1). The data are presented as the mean $\pm$ S.E.M. ** $P < 0.01$, *** $P < 0.001$.

Figure 8 Effect of *TET3* overexpression in bovine SCNT embryos on apoptosis. (A) Apoptotic cells (green) in the nuclei (blue) of blastocysts developed from IVF, SCNT and TET3-NT embryos. (B) Percentage of apoptotic cells in blastocysts per treatment group. (C and D) RT-qPCR analysis of the apoptotic related genes *Bax* and *Bcl-2* at the 8-cell and blastocyst stages for the IVF, SCNT and TET3-NT embryos. The expression levels of 8-cell embryos in the IVF group were used for sample normalization (expression set to 1). The data are presented as the mean $\pm$ S.E.M. from three independent experiments; * $P < 0.05$, ** $P < 0.01$. Scale bar = 50 μ m.

substantially improve the transcriptional reprogramming of cloned embryos (Liu *et al.* 2016). In addition, H3K4 methylation was confirmed to be a barrier for SCNT embryo arrest, and reduced H3K4 methylation could markedly improve cloning efficiency (Zhang *et al.* 2018). In mammals, DNA methylation at 5mC is another important epigenetic modification that plays a key role in normal embryonic development (Gao *et al.* 2018). DNA methylation is mediated and maintained by DNA methyltransferases (*DNMTs*) but can be removed by TET protein-mediated oxidation (Gu *et al.* 2011). In mice, DNA is extensively demethylated by the TET3 protein during preimplantation embryo development through a combination of active and passive processes (Shen *et al.* 2014). Research in pigs has shown that *TET1* is involved in DNA demethylation during early embryonic development (Cao *et al.* 2014). Our recent studies revealed that *TET3* expression was higher than that of *TET1* and *TET2* in bovine embryos. Inhibition or knockdown of *TET3* in embryos significantly increased the level of DNA methylation, suggesting that *TET3* is primarily responsible for active DNA demethylation in bovine early embryonic development (Zhang *et al.* 2017, Cheng *et al.* 2019). After determining that *TET3* is a key factor that mediates active DNA demethylation in mammalian embryos, several studies have shown that *TET3* stored in oocytes can be localized to the PPN in mouse SCNT embryos and induce the transformation of 5mC into 5hmC, suggesting that active DNA demethylation does occur in SCNT embryos (Iqbal *et al.* 2011, Wossidlo *et al.* 2011). In this study, we demonstrated that, similar to mice, DNA methylation in the somatic cell genome acts as a barrier for bovine SCNT reprogramming. Elimination of DNA methylation by overexpression of the DNA demethylase *TET3* facilitated epigenetic reprogramming, thereby allowing bovine SCNT embryos to develop more efficiently. Our study thus found that *TET3* is an intrinsic defective regulator that contributes to the unsuccessful removal

of DNA methylation in bovine SCNT embryos and that *TET3* is crucial for this reprogramming process (Fig. 9).

It has been well shown that *TET3* promotes epigenomic or pluripotent genome changes in mammals (Gao *et al.* 2013). Indeed, we observed increased expression of the pluripotency genes *Nanog*, *Sox2*, and *Oct-4* after fluorescent quantitation, the promoter regions of which can be demethylated by the TET protein. Interestingly, expression levels of the methyltransferase *DNMT1* in the *TET3*-overexpression group were higher than those observed in the negative control and untreated groups, revealing an intricate interplay between *TET3*-driven DNA demethylation and *de novo* methylation (Amouroux *et al.* 2016). In addition, our results suggest that overexpression of *TET3* in bovine fibroblasts did not change the transcriptional expression levels of DNA methyltransferases (*DNMT3a/3b*), lysine-specific demethylases (*KDM3a/4a/4c/5b*) or imprinted genes (*H19* and *IGF2*), suggesting that the catalytic capacity of *TET3* is selective and that not all genes are demethylated by *TET3*.

In previous studies, the nuclei of donor cumulus cells were first injected into the enucleated oocytes using a micromanipulator. Then, these SCNT embryos were microinjected with target mRNA after activation (Chung *et al.* 2015, Liu *et al.* 2018). The advantage of this approach is that it does not require screening for positive clones, simplifying the process, but it also raises some questions. First, injecting SCNT embryos after nuclear transplantation will cause further damage to oocytes and affect later embryonic development. More importantly, after direct injection of overexpressed mRNA, the mRNA requires some time for expression. Furthermore, with respect to the proteins responsible for reprogramming somatic cells, only those stored by the oocyte are available, indicating that somatic gene expression from the donor genome may not be immediately regulated by ooplasm. Thus, we conducted somatic nuclear

Figure 9 *TET3* is an epigenetic regulator during embryo development. Schematic representation of the proposed model illustrating the reason for the associated DNA demethylation in IVF embryos and 5mC aberrancies in SCNT embryos. Overexpression of *TET3* can remove the 5mC barriers and lead to successful nuclear reprogramming in bovine SCNT embryos.

transfer using bovine fibroblasts overexpressing *TET3*. The results indicated that the TET3-GFP fusion protein was stably expressed during zygote division. Combined with *TET3* and FLAG immunofluorescence staining, the results showed that the TET3 protein was stably and highly expressed in embryos at different stages. Previous research has shown that genome-wide DNA methylation reprogramming is a dynamic balance between strong focused remethylation through DNMTs and drastic global demethylation by TET proteins during preimplantation development (Zhu *et al.* 2018). The latest studies have reported that inhibition of DNMTs specifically enhances SCNT efficiency in mice (Gao *et al.* 2018). In this study, *TET3* overexpression in bovine somatic cells before SCNT rescued these demethylation defects and significantly improved the developing rate of cloned embryo, with the blastocyst rate significantly increasing from 12.1 to 29.9%. We also analyzed the DNA methylation levels of *satellite I* and α -*satellite*, representative repeat elements that overlap the majority of the genome, using sodium bisulfite genomic sequencing (BSP). Our results showed that the DNA methylation levels of *satellite I* and α -*satellite* in SCNT embryos were more similar to those of fibroblast cells than of IVF embryos, indicating that global DNA methylation reprogramming in bovine SCNT embryos had not yet been completed during the development of the embryo before implantation. Unlike the dynamic global DNA methylation patterns observed in cows, DNA hypermethylation levels similar to those in fibroblasts were maintained until the 4-cell stage in mouse cloned embryos (Liu *et al.* 2016).

Previous studies have demonstrated that the expression of pluripotency genes plays an essential role in preimplantation embryonic development and that the activation of pluripotency genes can improve reprogramming efficiency (Gomez *et al.* 2012). Overexpression of pluripotency transcription factors, such as *Nanog* and *Oct4*, in donor cells also increased the efficiency of SCNT embryos to some extent (Zhang *et al.* 2011, Goissis *et al.* 2013). In our experiments, *Nanog*, *Oct4* and *Sox2* mRNA transcription was significantly higher in TET3-NT embryos than in SCNT embryos at the 8-cell and blastocyst stages. We subsequently examined the DNA methylation status at the promoters of the pluripotency genes *Nanog* and *Oct4* in IVF embryos, SCNT embryos, and TET3-NT embryos. The results showed that promoter regions of the *Nanog* and *Oct4* loci in TET3-NT embryos were more hypermethylated, similar to IVF embryos, than those in SCNT embryos at the 8-cell and blastocyst stages, indicating that *TET3* overexpression significantly decreased DNA methylation in the *Oct4* and *Nanog* promoter regions and then reactivated pluripotency genes, contributing to an increased SCNT embryo developmental capacity.

Numerous studies have reported deficient epigenetic reprogramming with abnormal imprinted gene expression in SCNT embryos (Young *et al.* 2001, Perecin *et al.* 2009, Suzuki *et al.* 2009). In this study, we analyzed the methylation and transcription levels in the ICR region of the typical imprinted genes *H19/IGF2*. The *H19/IGF2* ICR was hypermethylated in bovine fibroblast cells and hypomethylated in oocytes. However, the *H19/IGF2* ICR in SCNT embryos exhibited lower methylation levels than those observed in IVF embryos at the 8-cell and blastocyst stages. Similar to our previous studies, these results showed that overexpression of *TET3* did not change the level of DNA methylation or expression of the imprinted genes *H19* and *IGF2* in TET3-NT embryos, indicating that the imprinted genes *H19* and *IGF2* ICR resisted demethylation by the TET3 protein in bovine SCNT embryos (Zhang *et al.* 2017). These results showed that DNA methylation of the imprinted genes *H19-IGF2* ICR remains unaffected by this wave of reprogramming in SCNT embryos and maintains parent-specific activity for further development. In addition, we observed that TET3-NT blastocysts possessed more total cells and fewer apoptotic cells than the SCNT blastocysts, similar to IVF embryos, and no difference in apoptotic cell numbers in 8-cell stage embryos was observed between these three groups.

Furthermore, we observed that the transcription levels of the pro-apoptotic gene *Bax* were significantly lower, in TET3-NT embryos at the blastocyst stage than in SCNT embryos, while those of the anti-apoptotic gene *Bcl-2* were higher. These effects may be due to *TET3* overexpression partially preventing the occurrence of apoptosis by promoting anti-apoptotic gene expression and inhibiting that of pro-apoptotic genes.

In summary, the results of our study reveal that aberrant DNA methylation reprogramming is a major epigenetic modification obstruction to the developmental potential of cloned embryos. Overexpression of *TET3* in donor cells could decrease the DNA methylation level and facilitate the activation of pluripotency genes in bovine cloned embryos, thereby improving the efficiency of bovine SCNT embryos. These findings may ultimately be used in promoting application of the large-animal cloning technique.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0021>.

Declaration of interest

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

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Author contribution statement

B T, X Z and Z L conceived and designed the experiments. J Z and L H performed the experiments and analyzed the data. All the authors contributed to interpretation of the results throughout the study and approved the final version of the manuscript.

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