Activation-induced cytidine deaminase selectively catalyzed active DNA demethylation in pluripotency gene and improved cell reprogramming in bovine SCNT embryo

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Abstract DNA methylation in mammals is an epigenetic marker and necessary for normal embryogenesis. The global genomic demethylation of 5-methylcytosine occurs during the first cell cycle following fertilization. Activation-induced cytidine deaminase (AID), which is well-known for the function in antibody diversification, has been implicated to play a role in active demethylation, but its role in cell reprogramming and its crosstalk with other DNA demethylation mechanism need to be clarified. In this study, the dynamic epigenetic regulation of cell pluripotency and embryo development by AID in bovine preimplantation embryos was investigated. The analysis of an *AID* overexpressing transgenic cell line showed that *AID* overexpression did not change the global genomic methylation but did change the methylation status of the promoters of the *OCT4*, *NANOG* and *SOX2* genes, thereby causing changes in their expression. The siRNA-mediated *AID* knock-down in early embryonic development indicated that *AID* interference did not affect oocyte maturation or the following embryo development after in vitro

fertilization but influenced the DNA methylation status of *OCT4* and *NANOG*. To clarify the role of AID in preimplantation embryos, SCNT embryos were obtained using *AID*-overexpressing cells as nuclear donors. Compared to the control group, the cleavage and blastocyst rates were both significantly improved in the *AID*-overexpression group. The expression of *OCT4* and *NANOG* was increased in the SCNT embryos, whereas the methylation levels of their promoters were reduced. In conclusion, this study demonstrated that AID selectively catalyzes DNA demethylation of pluripotency genes to play a role in regulation their expression, improves bovine SCNT embryo development by increased expression levels.

Keywords *AID* gene · DNA demethylation · Cell pluripotency · Embryo development

Introduction

Somatic cell nuclear transfer (SCNT) has been employed successfully in a variety of mammals (Baguisi et al. 1999; Kato et al. 1998; Chesné et al. 2002). However, there are still a number of issues need to be resolved, such as the low cloning efficiency, the high abortion and perinatal mortality (Watanabe 2013). Abnormal DNA methylation reprogramming is considered to be one of the causes of the abnormal development of cloned embryos (Dean et al. 2003).

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Active DNA demethylation widely exists in plant and animal (Ooi and Bestor 2008). However, in mammals, no effective DNA demethylase has been identified, and the active DNA demethylation pathways are still disputed. In addition, the demethylation mechanism may exhibit differences among different cells and tissues (Bhutani et al. 2011; Wu and Zhang 2010).

Activation-induced cytidine deaminase (AID, also known as AICDA) was first recognized through its role in the generation of antibody diversity in B lymphocytes (Delker et al. 2009) and was later found to be able to deaminate 5mC into thymine (T) (Maul and Gearhart 2010). In addition, AID was hypothesized to be necessary for the initiation of active DNA demethylation (Bhutani et al. 2010; Morgan et al. 2004; Popp et al. 2010; Rai et al. 2008). Morgan et al. found that *AID* is expressed in pluripotent tissues, primordial germ cells and ovaries (Morgan et al. 2004). Rai et al. found that AID could change 5mC to T through deamination and cause global genomic demethylation in zebrafish (Rai et al. 2008). Thereafter, Bhutani et al. demonstrated the demethylation effect of AID proteins in mouse embryonic stem cells using a heterologous cell fusion model (Bhutani et al. 2010). Christian Popp found that the genomic DNA methylation level of primordial germ cells is 2–3-fold higher in *AID*-deficient mice than in normal mice (Popp et al. 2010).

Moreover, Larijania et al. showed that the deamination efficiency of AID on mC was much lower than that on C, indicating that the demethylation ability of AID was weak (Larijania et al. 2005). Wijesinghe et al. showed that even though AID and the other two of members of the human APOBEC3 family had a strong ability to convert C to U, but only APOBEC3A is an efficient deaminator of 5mC (Wijesinghe and Bhagwat 2012). Nabel et al. (2012) further confirmed that the effect of AID on the deamination of methylated cytosine is not obvious. Thus, the demethylation role of AID remains questionable. In this study, the regulation of the *AID* gene and its role on cell pluripotency and embryo development are discussed.

Materials and methods

Unless otherwise mentioned, all of the reagents used were purchased from Sigma Chemical Co. (St. Louis,

MO, USA). Each experiment was repeated at least three times.

Construction of the overexpression vector of *AID*

Specific reverse transcription PCR primers were designed to clone the *AID* gene according to the bovine *AID* mRNA sequence (GenBank accession no. NM_001038682). The PGK promoter was obtained from plasmid pL45I by *Bgl*II and *Sac*I digestion and inserted into the pCDsRed-CMV vector to construct the intermediate vector pCDsRed-P. Then, the pL45I termination signal *polA* was inserted into the pCDsRed-P vector to construct the intermediate vector pCDsRed-Pb. Finally, the cloned *AID* gene was connected to the pCDsRed-Pb vector to obtain the expression vector pCDsRed-PbAID.

AID gene transfection into fibroblast cells

Bovine fetuses were collected from a local abattoir. The tissue was washed thoroughly and minced, then the 2–3 mm³ tissues were seeded in 60-mm dishes followed by cultivation for 4–5 h at 37 °C in humidified air containing 5 % CO₂. Then the DMEM/F12 (Gibco Inc., Grand Island, NY, USA) supplemented with 15 % fetal bovine serum (FBS; Gibco Inc.) and antibiotics was added to the dishes. The bovine fetal fibroblast cells (BEF) were passaged and frozen when they reached ≥80 % confluence.

The pCDsRed-PbAID plasmid was linearized by *Sac*II and transfected into bovine fetal fibroblast cells by using Lipofectamine[®] 2000 (Invitrogen[™], Carlsbad, CA, USA). Forty-eight hours after *AID* transfection, the cells were collected by trypsin digestion, seeded in 100-mm dishes at a density of 2×10^4 cells/dish, and cultured in DMEM/F12 with 10 % FBS for 24 h. Then, the neomycin-resistant clones were selected in medium containing an optimal concentration of G418, and individual resistant clones expressing red fluorescence were isolated using cloning cylinders after 15 days culture. The isolated clones were plated onto 24-well plates and passaged when the confluence level reached 80 %.

The growth curve and karyotype of these cells were performed as previously reported (Gao et al. 2013).

In vitro fertilization and nucleus transfer

Bovine oocytes matured in vitro (IVM) and fertilized in vitro (IVF) as previously described (Gao et al.

2013). In brief, bovine cumulus oocyte complexes (COCs) were aspirated from 3 to 8 mm diameter follicles from ovaries, which were obtained from a local abattoir. Only COCs with a compact and homogenous ooplasm were selected. The COCs were matured in TCM-199 (Gibco Inc.) supplemented with 10 % FBS, 25 µg/mL gentamicin, 10 mM Hepes, 0.01 g/mL E2, 0.01 U/mL follicle-stimulating hormone (FSH; NIH-FSH-S17), and 0.01 U/mL luteinizing hormone (LH; USDA-bLH-6) in four-well dishes (Nunc, Roskilde, Denmark) containing 0.5 mL of medium and 50 oocytes per well at 38.5 °C, in air containing 5 % CO₂ under a saturated humidity. Oocytes with the first polar body were considered to be maturation.

When the oocytes matured 20–22 h, frozen bull semen (purchased from Inner Mongolia Livestock Improvement Station, Hohhot, China) was thawed in a 37 °C water bath and washed twice in BO medium containing 10 mM caffeine with centrifugation at 3000 r/min for 5 min. Then the swim-up sperms were collected to do fertilization droplets (100 µL), each droplet contained 30–40 matured oocytes. Seven h after being fertilized, the fertilized oocytes were removed from their cumulus and put into synthetic oviduct fluid (SOF) medium supplemented with 8 mg/mL bovine serum albumin (BSA, fraction V), 1 % (v/v) nonessential amino acids (100×, Gibco Inc.), 1 mM L-glutamine, 0.4 mM sodium pyruvate and 50 µg/mL gentamycin at 38.5 °C with 5 % CO₂ in a humidified atmosphere. The cleavage, morula and blastocyst rates were recorded on days 2, 5 and 7, respectively.

For somatic cell nuclear transfer (SCNT), oocytes with the first polar body were selected as recipient cytoplasts. Single cells (about 10–12 µm) (Li et al. 2004, 2006) were individually transferred to the perivitelline space of the recipient cytoplasts shortly after enucleation. The couplets were fused in mannitol fusion buffer by two electric DC pulses of 1.2 kV/cm for 25 µs delivered from a Voltain cell fusion system (Cryoslogic, Blackburn, Victoria, Australia). The fused clones were activated by 5 µM ionomycin for 5 min, followed by incubation with 10 µg/mL cycloheximide in SOFaa containing 4 % FBS for 5 h at 38.5 °C in air containing 5 % CO₂. Then the activated NT embryos were cultured as IVF described.

2-, 4-, 8- cell, morula and blastocysts of IVF and NT were collected and frozen in liquid nitrogen.

Microinjection

AID interference fragments (Nos. 22, 410 and 558 of the coding sequences (CDS), listed in Table 1) were designed and synthesized by Invitrogen. The three fragments were mixed and diluted to 20 µM with RNase-free H₂O and stored at −80 °C.

The COCs were randomly divided into four groups: (1) normal COCs, (2) denuded oocytes co-cultured with cumulus cells (DOs), (3) denuded oocytes injected with the nonsense fragment and co-cultured with cumulus cells (Nos-siRNA), and (4) denuded oocytes injected with the interference fragment of *AID* and co-cultured with cumulus cells (*AID*-siRNA). The three siRNAs were diluted 3 times by RNase free dH₂O prior to microinjection. 5pL of *AID* siRNA were microinjected (Eppendorf Microinjector, Hamburg, Germany) into GV oocytes kept in TCM-199 containing 0.2 mM IBMX. The same amount of siRNAs was injected in the positive and negative controls. Pipettes were replaced after fifty oocytes were injected to avoid the potential of false negative results. RNAi oocytes were incubated in 500 µL TCM-199 supplemented with 10 % FBS, 25 µg/mL gentamicin, 10 mM Hepes, 0.01 g/mL E2, 0.01 U/mL FSH, and 0.01 U/mL LH for 20–22 h at 38.5 °C in an atmosphere of 5 % CO₂ in air with maximum humidity. Then, they were used to IVF in accordance with the above steps. MII, 2-, 4-, 8-cell, morula and blastocysts were collected and frozen in liquid nitrogen.

Analyses of cellular DNA methylation and gene expression

The genomic DNA of transgenic cells was extracted by Wizare Genomic DNA purification (PROMEGA, Madison, WI, USA) and digested with the methylation-sensitive restriction enzymes *MspI/HpaII* at 37 °C for 8 h. The digestion products were subjected to 1 % agarose gel electrophoresis, and the gray values of the 1- and 15-kb bands were analyzed using the Quantity One software (Bio-Rad) to examine the global DNA methylation status. To assess the loci-specific DNA methylation status, the genomic DNA was digested with *XhoI* (Takara, Tokyo, Japan) and subjected to bisulfite sequencing PCR (BSP) according to a method described previously (Wang et al. 2013). The primer sets for the three genes were described previously (Lan et al. 2010), which is

Table 1 Sequences of primers and the size of the products

Primers	Sequence	Products (bp)
Interference fragments		
410	5'-UAAUCUUUGAACGUCAUGAUGGCGA-3'	
558	5'-AAAUGCAUCCCGCAAGUCAUCAACC-3'	
22	5'-UGAACUGGUAAAGAAACUGUCUCUG-3	
BSP-PCR		
<i>OCT4</i>	Sense primer 5'-GATTTGGATGAGTTTSTAAGGGTT-3' Antisense primer 5'-ACTCCAACCTTCTCCTTATCCAACCTT-3'	293
<i>NANOG</i>	Sense primer 5'-TTTTTTAATTATAATTTGATGGGGT-3' Antisense primer 5'-CTAACACACCTTAAATAAACAAACC-3'	288
<i>SOX2</i>	Sense primer 5'-TATTAAGAGTAAATTTAAGATTAAGTT-3' Antisense primer 5'-CAAATTAATAACAACCATCCATATAAC-3'	361
<i>AID</i>	Sense primer 5'-CGGCTGGAGGAGCAAAAGACCGAAAG-3' Antisense primer 5'-GAGCCACGTCCCTGTCGTCGTCTTC-3'	192
Real-time PCR		
<i>OCT4</i>	Sense primer 5'-GTTTTGAGGCTTTGCAGCTC-3' Antisense primer 5'-CTCCAGGTTGCCTCTCACTC-3'	182
<i>NANOG</i>	Sense primer 5'-GTCCCGGTCAAGAAACAAAA-3' Antisense primer 5'-TCTGGAACCAGGTCTTCACC-3'	163
<i>SOX2</i>	Sense primer 5'-GTTTGCAAAAGGGGGAAAGT-3 Antisense primer 5'-GAGGCAAACTGGAATCAGGA-3	200
<i>CDX2</i>	Sense primer 5'-AAGACAAATACCGGGTCGTG-3 Antisense primer 5'-CTGCGGTTCTGAAACCAAAT-3	155
<i>GAPDH</i>	Sense primer 5'-GCCATCATGACGTTCAAAGA-3' Antisense primer 5'-CGCAAGTCATCAACCTCGTA-3'	161

closely correlated with the expression (Khan et al. 2012). The details on primer sequences were listed in Table 1. The reaction conditions were 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 30 s and a final extension of 7 min at 72 °C. Then PCR products of three independent reactions were mixed together and cloned into a T-vector to sequence.

Total RNA extraction and reverse transcription were performed as previously reported (Wang et al. 2013). Quantitative real-time PCR (qPCR) was performed with the primers listed in Table 1. The qPCRs were performed in triplicate in an ABI Prism 7300 Instrument (Applied Biosystems, Carlsbad, CA, USA) with QuantiTect SYBR Green (Qiagen, Duesseldorf, 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 $2^{-\Delta CT}$. CT is the threshold cycle and $\Delta CT = CT^{\text{target}} - CT^{\text{GAPDH}}$.

Statistical analysis

All statistical analyses were performed with the SPSS 14.0 for Windows software (SPSS Inc, Chicago, IL, USA). The results of the 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 <0.05 was considered to be statistically significant.

Results

The characteristics of *AID*-transfected cells

To produce *AID*-transfected cells, the overexpression vector pCDsRed-PbAID was successfully constructed

according to the blueprint shown in Fig. 1a, and the *AID* gene is triggered by the PGK promoter. Then, it was transfected into bovine fetal fibroblast cell by Lipofectamine® 2000. After *DsRed*⁺ and *Neo*⁺ selective progression, we successfully obtained *AID*-transfected cells. The cellular morphology was not altered (Fig. 1b) and the red fluorescence *DsRed* was well expressed after *AID* transfection (Fig. 1d, e). The growth curve of these cells showed that the growth was slower compared with that of the control cells, but this difference was not significant (Fig. 1f). We then examined 100 cells from the transgenic cells to determine their chromosomal composition (Fig. 1c) and found that 85 cells (85 %) displayed a normal chromosomal composition ($2n = 60$). Real-time PCR was used to assess the expression of *AID*, *OCT4*, *NANOG*, *SOX2* and *CDX2* in bovine fetal fibroblasts 48 h after transfection with *AID* (Fig. 1g) and in *AID*-transfected cells (Fig. 1h). The expression of *AID* was significantly increased by 62-fold 48 h after *AID* transfection. In addition, compared with the control, the pluripotency genes *OCT4* and *NANOG* were significantly increased, and *CDX2* expression was markedly decreased. The *AID*-transfected cells showed a similar change, *OCT4* and *NANOG* were significantly increased, and *CDX2* was obviously decreased. Nevertheless, the expression of *AID* was not modified between the *AID*-transfected cells and the untransfected cells.

Epigenetic characteristics of *AID*-transfected cells

We focused on *AID* as a candidate factor with a role in mammalian DNA demethylation because it has been detected in mammalian pluripotent germ cells (Popp et al. 2010) and implicated in DNA demethylation in zebrafish post fertilization (Rai et al. 2008). To investigate the mechanism underlying the expression of pluripotency genes, the epigenetic characteristics of *AID*-transgenic cells, including loci-specific and global methylation, were examined. The relative methylation status of the genomic DNA (Fig. 2a) was calculated by the gray value ratio of *HpaII* 1–15 kb (Table 2). No obvious changes were detected between the two groups ($p > 0.05$).

Subsequently, the result of BSP showed that the DNA methylation status of *OCT4*, *NANOG* and *SOX2* were significantly decreased (Fig. 2b).

Effects of *AID*-transfected cells on SCNT

In the following experiment, the transgenic cells served as the nuclear donors for SCNT, and untransfected cells cultured under the same conditions were used as the control. Compared with the control group, both the cleavage and blastocyst rates are improved significantly (Table 3). The expression of *OCT4* and *NANOG* were increased compared with the control SCNT blastocysts (Fig. 3a), whereas the expression levels of *AID* and *SOX2* were unchanged.

We then analyzed the DNA methylation status of the promoter of the *OCT4*, *NANOG* and *SOX2* genes in the SCNT blastocysts. Compared with the control SCNT blastocysts, the promoter methylation levels of *OCT4* (Fig. 3b, 53.3 vs. 64.8 %) and *NANOG* (Fig. 3, 9.2 vs. 10.0 %) were insignificantly decreased, whereas that of *SOX2* increased insignificantly.

Effect of *AID* knockdown on bovine early embryonic development

For the *AID* knockdown experiments, the effect of siRNAs were examined before the other experiments (see Supplementary Fig. 1), all three siRNAs were mixed for GV oocytes injection. After injection, both *AID*-siRNA and Nos-siRNA groups showed similar oocyte maturation, cleavage and blastocyst rates (Tables 4, 5). There was a major drop in blastocyst rate after microinjection (from over 28–8.3 %, see Table 5). All groups of experiments were carried out in parallel, the results showed that the cleavage rate and blastocyst rate of *AID*-siRNA injection group, Nos-siRNA injection group and DOs group were significantly lower than that of COCs group, but no significant difference between experimental groups was observed. This showed that the mechanical damage of the microinjection did not cause a decline in the blastocyst rate. *AID*, *OCT4*, *NANOG* and *SOX2* were slightly declined without significant differences in the blastocyst stage compared with Nos-siRNA groups (Fig. 4a). However, the blastocyst subjected to *AID* knockdown using the three siRNAs showed a profound inhibition of CpG demethylation in the *OCT4* and *NANOG* promoters (Fig. 4b).

Discussion

DNA demethylation is essential for overcoming gene silencing and inducing the temporally and spatially

Fig. 1 The characteristics of AID-transfected cells. **a** Schematic diagram of the overexpression vector pCDsRed-PbAID. **b** Representative image of BEF transfected with AID ($\times 100$). **c** Representative image of chromosomes of BEF transfected with AID ($\times 100$). **d** Representative red fluorescent image of BEF transfected with AID ($\times 100$). **e** Representative red fluorescent image of BEF transfected with AID ($\times 100$). **f** Growth curves of AID transgenic cells. BEF which were transfected with an empty vector did not contain AID served as controls. **g** Real-time PCR results using specific primers for assessing the expression of

AID, OCT4, NANOG, SOX2 and CDX2 in BEF 48 h after transfection with AID (data from three independent transfection experiments, different letters indicate $p < 0.05$). BEF which were transfected with an empty vector did not contain AID served as controls. **h** AID and pluripotency gene expression in AID transgenic cells as assessed by real-time PCR (data from a single neomycin-resistant clone after G418 selection, different letters indicate $p < 0.05$). BEF which were transfected with an empty vector did not contain AID served as controls

Fig. 2 DNA methylation status of the global genomic DNA and the specific gene locus in transgenic cells. **a** Global genomic DNA digestion by *MspI/HpaII*. BEF which were transfected with an empty vector did not contain *AID* served as controls. **b** Bisulfite sequencing analysis of the methylation status of the *OCT4*, *NANOG* and *SOX2* promoters in *AID* transgenic cells.

controlled expression of mammalian genes; however, no consensus mammalian DNA demethylase has been identified despite years of effort (Ooi and Bestor 2008). It is known that the incomplete or abnormal

BEF which were transfected with an empty vector did not contain *AID* served as controls. The white and black circles indicate unmethylated and methylated CpG dinucleotides, respectively. *Indicates a significant difference with respect to the control group ($p < 0.05$)

reprogramming of somatic nuclei in the recipient cytoplasm is an important reason for the developmental aberration of cloned embryos (Gruppen 2014; Solter 2000). Previous studies have shown that *AID* plays a key role in somatic cell reprogramming (Fritz and Papavasiliou 2010; Kumar et al. 2013). Our data provide new evidence implicating *AID* in active DNA demethylation in mammalian cells and demonstrate that *AID*-dependent DNA demethylation is a loci-specific epigenetic change.

In the past, the researches of *AID* were focused on two central processes in antibody diversification: somatic hypermutation and class-switch recombination. Recent findings have provided that *AID* deamination is not exclusive to immunoglobulin loci; it can instigate DNA lesions in non-immunoglobulin genes (Chandra et al. 2015). *AID* will be able to deaminate 5mC and 5C into thymine (T) in single DNA strand. Then thymine DNA glycosylase (TDG) can excise T

Table 2 Genomic DNA methylation status of *AID* transgenic cells

<i>Hpa</i> II (15 kb)	<i>Hpa</i> II (1 kb)	<i>Hpa</i> II (15/1 kb)	Mean
Ctrl			
0.53	0.78	0.67	0.77
0.62	0.70	0.89	
0.57	0.75	0.76	
<i>AID</i> transgenic cell			
0.62	0.69	0.89	0.82
0.54	0.75	0.72	
0.61	0.73	0.84	

p value 0.09

Table 3 Development of SCNT embryos derived from *AID* transgenic cell

Category	Reconstructed embryos			
	Number of cultures	Cleavage rate (%)	Morula rate (%)	Blastula rate (%)
AID	128	114 (86.9 ± 8.2) ^a	58 (44.0 ± 6.8) ^a	26 (19.1 ± 5.7) ^a
Control	125	68 (54.8 ± 6.8) ^b	33 (26.6 ± 2.2) ^b	16 (12.7 ± 0.6) ^b

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

Fig. 3 Molecular characteristics of nuclear transfer blastocysts derived from *AID* transgenic cells. **a** *AID* and pluripotency genes expression in nuclear transfer blastocysts derived from *AID* transgenic cells, as assessed by real-time PCR. NT blastocysts derived from BEF served as controls. *Indicates a significant difference with respect to the control group ($p < 0.05$).

b Bisulfite sequencing analysis of the methylation status of the *OCT4*, *NANOG*, and *SOX2* promoters in NT blastocysts. NT blastocysts derived from BEF served as controls. The white and black circles indicate unmethylated and methylated CpG dinucleotides, respectively

Table 4 Maturation of bovine oocytes after siRNA-mediated *AID* knockdown

Category	No injection		Injection	
	COCs	DOs	Nos-siRNA	<i>AID</i> -siRNA
Number of cultures	182	193	235	272
Maturation rate (%)	130 (69.3 ± 0.5) ^a	141 (70.5 ± 0.7) ^a	151 (62.6 ± 0.5) ^b	173 (63.2 ± 0.8) ^b

Different letters indicate significant differences ($p < 0.05$)

Table 5 Development of IVF embryos derived from siRNA-mediated *AID* knockdown

Category	No injection		Injection	
	COCs	DOs	Nos-siRNA	<i>AID</i> -siRNA
Number of cultures	256	237	263	302
Cleavage rate (%)	128 (57.3 ± 2.3) ^a	86 (37.8 ± 2.8) ^b	91 (35.3 ± 1.8) ^b	104 (34.2 ± 1.4) ^b
Blastula rate (%)	65 (28 ± 0.3) ^a	23 (8.4 ± 2.1) ^b	21 (8.0 ± 1.9) ^b	26 (8.3 ± 2.6) ^b

Different letters indicate significant differences ($p < 0.05$)

Fig. 4 Molecular characteristics of IVF blastocysts derived from *AID*-siRNA. **a** Real-time PCR using specific primers to assess the *AID*, *OCT4*, *NANOG* and *SOX2* expression in IVF blastocysts derived from the *AID*-siRNA ($*p < 0.05$). IVF blastocysts injected Nos-siRNA served as the controls. **b** Bisulfite sequencing analysis of methylation status of the *OCT4*,

NANOG and *SOX2* promoters in IVF blastocysts derived from the *AID*-siRNA. IVF blastocysts injected Nos-siRNA served as the controls. The white and black circles indicate unmethylated and methylated CpG dinucleotides, respectively. *Indicates a significant difference with respect to the control group ($p < 0.05$)

to initiate base excision repair (BER) pathway. Components of this pathway can act on derivatives of 5mC to orchestrate active demethylation through its replacement with unmodified C (Ficz 2015). Target *AID* activity to specific genomic regions is regulated by the RNA exosome complex (Seisenberger et al.

2013). And substantial evidence supports a role for *AID* in gene-specific demethylation (Ramiro and Barreto 2015).

To date, the demethylation role of *AID* remains questionable, and PGC7/Stella protects the maternal genome from demethylation only after localization to

the nucleus, where it maintains the methylation of several imprinted genes (Nakamura et al. 2006). 5-Methylcytosine (5mC) is converted into 5-hydroxymethylcytosine (5hmC) during the first cell cycle in porcine in vivo fertilization and SCNT embryos but not in parthenogenetically activated embryos. The expression of Ten-Eleven Translocation 1 (TET1) correlates with this conversion (Cao et al. 2014). Sirard et al. detected some AICDA protein in bovine GV oocyte, but did not detect its mRNA. In contrast to TET1 and AID, TET3 dioxygenase is present in the very first embryo stages (Pagé-Larivière and Sirard 2014). Our results confirmed that the active DNA demethylation role of AID acted on loci-specific genes (*OCT4*, *SOX2* and *NANOG*), not on global methylation in *AID* transgenic cell. In addition, a profound inhibition of CpG demethylation in the *OCT4* and *NANOG* promoters was found in the blastocyst from the *AID*-knockdown cells, and these data indicate an important role of AID on DNA demethylation in pluripotency genes.

Vertebrate CpG islands (CGIs) are short interspersed DNA sequences, and most are sites of transcription initiation. Silencing of CGI promoters is achieved through dense CpG methylation or polycomb recruitment, using their distinctive DNA sequence composition (Deaton and Bird 2011). Our BSP amplified regions of three pluripotency genes are located in the neighbourhood of CGIs. According to the findings of expression (Khan et al. 2012) and methylation status (Lan et al. 2010) of these genes, it was confirmed that there is a strong correlation between BSP amplified regions and transcription initiation. In the present study, the expression levels of *OCT4* and *NANOG* were strongly enhanced in *AID* transgenic cells, while the methylation rate of these genes was declined. However, *SOX2* is a special case in this process. The result of SCNT blastocysts showed the same situation as for *AID* transgenic cells. In the *AID* knockdown experiment of bovine GV oocytes, the results showed that *AID* affected the methylation status of *OCT4* and *NANOG*, but not the expression of these genes and the development of these IVF embryos.

Traditionally, CGIs methylation has been thought of as being involved in gene silencing directly or indirectly (Razin and Shemer 1999), which have been challenged by technical advances, especially a variety of deep sequencing-based techniques. A mass spectrometry-based screen and a protein-binding

microarray approach were recently used to identify transcription factors that preferentially bind to methylated CpG sites (Hu et al. 2013; Mann et al. 2013). Wan et al. found two different mechanisms of DNA methylation-dependent gene regulation, which were negative and positive association between tissue-dependent and differentially methylated regions (T-DMRs) and gene expression (Wan et al. 2015). Klug's research showed that active DNA demethylation is a precisely targeted event that parallels or follows the modification of histones, but is not necessarily coupled to alterations in transcriptional activity (Klug et al. 2010). In addition, the transcription of pluripotency genes are regulated by several factors, such as DNA methylation, histone modification and other posttranslational modifications. These results suggest that the mechanism of DNA methylation mediated gene regulation is more complicated. These may be the reason for the expression level of *SOX2* did not correlate with its DNA methylation status in *AID* transgenic cell, and the expression level of *OCT4* and *NANOG* did not correlate with their DNA methylation status in *AID* knockdown embryos. The expression of *OCT4* and *NANOG* was increased significantly along with the decline of their DNA methylation status in *AID* transgenic cell, but their expression was unchanged with the increase of their DNA methylation status in *AID* knockdown embryos. These results suggested that the mechanism of gene expression regulation were different depending on cell types.

As we knew, pluripotency genes such as *OCT4*, *NANOG* and *SOX2* play an important role during early embryogenesis (Goissis et al. 2013; Saadeldin et al. 2012). And overexpression of these molecules can reprogram somatic cells to induce pluripotent cells (Liang and Zhang 2013; Yamanaka et al. 2007). Recent results showed that *NANOG* is the gateway to the pluripotent ground state and plays a role in the establishment of pluripotency (Costa et al. 2013; Silva et al. 2009). In the present study, overexpression of *AID* in bovine fetal fibroblast cell lead to the DNA demethylation and overexpression of *OCT4* and *NANOG*, which may facilitate the reprogramming of somatic nuclei. Our results proved this hypothesis, that the SCNT cleavage and blastocyst rates were significantly increased using *AID*-transfected cells as donor cells. The AID did not affect the development of IVF embryos maybe due to the reduced expression of these pluripotency genes after *AID* knockdown.

In the present study, *AID* was significantly increased by 62-fold 48 h after *AID* transfection but no change was observed between the *AID*-transfected cells and untransfected cells. Previous studies have indicated that exogenous genes are silent during a long period of culture and passage (Krishnan et al. 2006). In addition, DNA demethylation occurred in specific development stages, such as zygote and primordial germ cell, while in somatic cells, there is no obvious DNA demethylation. The internal mechanism of somatic cells may affect DNA methylation of genes and its expression. Another possibly reason was that transcription of the *AID* gene was prevented in some clones because of the modification of chromatin structure depending on the location of insertion on the genome. Despite *AID* was silenced in *AID*-transfected cells, the expression of *OCT4* and *NANOG* was significant increased. At the same time, *OCT4*, *NANOG* and *SOX2* promoters exhibited a low DNA methylation status. All this indicated that although the expression of *AID* was unchanged in *AID*-transfected cells, the pluripotency status of these cells was changed.

In conclusion, this study demonstrated that *AID* selectively catalyzes gene DNA demethylation and indirectly plays a role in the dynamic epigenetic regulation of cell pluripotency, increase in the expression level of *AID* gene improves the SCNT embryo development.

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