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Effects of Scriptaid on Cell Cycle and Histone Acetylation of Ovine Nuclear Donor Cumulus Cells and their Ability to Support the Development of Somatic Cell Nuclear Transfer Embryos

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ABSTRACT

Compelling evidence suggests that histone deacetylase inhibitor (HDACi) influences the development of somatic cell nuclear transfer (SCNT) embryos. The current study was conducted to determine the effect of pretreatment of donor cumulus cells with Scriptaid (a novel HDACi) on cell cycle, histone acetylation and cloning embryos development in ovine. First, we optimized the efficiency of Scriptaid in a dose (0, 0.1, 0.2, 0.4 and 0.8 $\mu\text{mol/L}$) and time-dependent (0, 12, 24, 36, and 48 h) manner on the developmental capacity of these embryos. Then, we quantitatively assessed the alterations of acetylation levels in histone H3 lysine 9 (acH3K9) and histone H4 lysine 12 (acH4K12) of cumulus cells and SCNT embryos by immunofluorescence staining. Furthermore, we detected the proportion of G0/G1 phase cells in cumulus cells. We found a significantly improved blastocyst development rates of cloning embryos derived from donor cumulus cells pretreated with a mild dose (0.2 $\mu\text{mol/L}$) of Scriptaid for 24 hours (21/86 [24.39%] vs. 11/85 [12.91%]; $P < 0.05$). Meanwhile, the levels of acH3K9 and acH4K12 were also improved significantly in cumulus cells and SCNT embryos ($P < 0.05$). Moreover, more cumulus cells pretreated with Scriptaid were in G0/G1 phase compared with control group (84.22% vs. 75.96%, $P < 0.05$). In conclusion, donor cumulus cells treated with Scriptaid is beneficial to early development of SCNT embryos, ascending acH3K9/acH4K12 and G0/G1 phase cells proportion of cumulus cell. Scriptaid can be used to improve the efficiency of somatic cell nuclear transfer in ovine.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) in mammals is an assisted reproductive technique that can produce a large number of genetically identical valuable offspring. Hence, this technology has been proven to enhance breeding of domesticated animals [1]. At the same time, it is also a valuable tool for research in the fields of reproduction and biomedical sciences [2,3]. However, a low cloning efficiency has restricted the extensive use of SCNT [4]. One of the most important reasons of SCNT inefficiency is the inadequate epigenetic reprogramming of donor nuclei [5]. Previous studies have shown that the incomplete reprogramming of SCNT embryos results in abnormal gene expression patterns [6]. As a consequence, the cloned embryos may stop developing and subsequently be aborted [7].

Histone modification is a major mechanism of epigenetic reprogramming, with lower histone acetylation levels common in cloned embryos [8]. Overcoming abnormal epigenetic modifications, for example, by artificial elevation of histone acetylation level, has been proposed as a means to improve cloning efficiency. Histone deacetylase inhibitor (HDACi) has been proved to increase

the acetylation level in somatic cells or in nuclear transfer embryos because it makes the chromatin more flexible thereby allowing the combination of transcriptional factors [9-11]. Scriptaid, one of the histone deacetylase (HDAC) inhibitors, has a high histone acetylation activity and low cellular toxicity [12]. Several studies have shown that treating early reconstructed embryos with Scriptaid can significantly improve preimplantation developmental competence in mice [13], pigs [14,15], cattle [16], buffaloes [17], rabbit [18] and sheep [19]. Although many studies were carried out on reconstructed embryos treated with Scriptaid, to the best of our knowledge, there has been no research conducted on the effect of Scriptaid pretreatment of ovine donor cells on the development of SCNT embryos. Moreover, the cell cycle and acetylation level of donor cells pretreated with Scriptaid have not been investigated.

The objectives of the present study were to determine (1) the optimal concentration of Scriptaid and optimal duration of treatment for ovine donor cumulus cells; (2) the effects of Scriptaid on the relative fluorescence intensity of acH4K12 and acH3K9 in cumulus cells and acH4K12 in SCNT embryos; and (3) the effect of Scriptaid pretreatment on G0/G1 phase cell proportion in cumulus cells.

MATERIALS AND METHODS

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. All experimental protocols and animal handling procedures were reviewed and approved by the Laboratory Animal Care and Use Committee of Hebei Province.

Materials and reagents: All of the following solutions and media were filtered using a 0.22 mm filter (Millipore Ireland Ltd., Tullagreen, Carrigtwohill, Co., Cork). The osmolarity in the media was 280 milliosmoles and pH was maintained between 7.2 and 7.4. The cell culture medium was Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% (V/V) fetal bovine serum (HyClone, Logan, UT). The oocyte IVM medium was TCM199 (Gibco) supplemented with 10% fetal bovine serum (HyClone), 10^5 $\mu\text{g}/\text{mL}$ FSH, 10^5 $\mu\text{g}/\text{mL}$ LH, 10^4 $\mu\text{g}/\text{mL}$ E_2 , 1 mmol/L L-glutamine, and 20 ng/mL EGF. The embryo culture medium was synthetic oviduct medium with amino acids (SOFaa) supplemented with 1% nonessential amino acids, 1% essential amino acids, 10 ng/mL EGF, 8 mg/mL BSA, and 1 mmol/L L-glutamine.

Collection of oocytes and in vitro maturation: Ovine ovaries were collected from a local abattoir and within 3 hours after collection, were transported to the laboratory at 30 °C in physiologic saline containing 100 IU/mL penicillin and 0.05 mg/mL streptomycin. Follicles from 3 to 6 mm in diameter were sliced using a razor blade. The cumulus-oocyte complexes (COCs) in the follicle fluid were collected from the drained off follicular fluid at 37 °C; only COCs with more than three layers of evenly distributed cumulus cells and uniform ooplasm were selected for IVM. After the COCs had been washed three times in IVM medium, 80 COCs were placed into each well of four-well cell culture plates (Nunc, Roskilde, Denmark) containing 500 μL of IVM media equilibrated in a 5% CO_2 incubator for at least 4 hours. The COCs were matured for 19 hours at 38.5 °C and 5% CO_2 in humidified atmosphere. In general, the mean interval between collection of ovaries and incubation of COCs was 4.5 hours.

Preparation of donor cells: Cumulus cells were collected from matured oocytes using 0.1% hyaluronidase in Dulbecco's PBS (Gibco) with 0.5% FCS (HyClone). After centrifugation at 300 $\times\text{g}$ for 5 minutes, cells were seeded and cultured in the wells of a 24-well plate containing DMEM (Gibco) with 10% FCS (HyClone) at 38.5 °C in a humidified atmosphere of 5% CO_2 . When confluence was reached, attached cells were dissociated with 0.5% trypsin in DMEM for 3 minutes, and the recovered cells were centrifuged at 300 $\times\text{g}$ for 5 minutes. The resulting pellets were resuspended in the above medium and subcultured (1×10^4 cells per well on a 24-well plate). Individual cumulus cells (passage 2) were obtained by 0.5% trypsin digestion and were used as donor cells.

Treatment of SCR: The SCR (catalog no. S7817) was dissolved in dimethyl sulfoxide (DMSO; catalog no. D2650) to achieve a solution at 500 $\mu\text{mol}/\text{L}$, were stored at -20 °C and added to synthetic oviductal fluid (SOF) at different concentrations using serial gradient dilutions. Any remaining stock solutions were stored at 4 °C for no more than 2 weeks. To determine the optimal concentration for treatment, the ovine cumulus cells were treated with 0, 0.1, 0.2, 0.4, 0.8 $\mu\text{mol}/\text{L}$ Scriptaid for 24 h. To determine the optimal treatment duration, the ovine cumulus cells were cultured with 0.2 $\mu\text{mol}/\text{L}$ Scriptaid for 0, 6, 12, 24 and 48 h.

Nuclear transfer, activation, and embryo culture: After 19 hours of IVM, COCs were stripped of their cumulus cells with 0.1% hyaluronidase in Dulbecco's PBS supplemented with 0.5% FCS, and chemically assisted enucleation was performed as reported [20], with small modifications. Briefly, oocytes with visible first polar bodies were incubated at 38.4 °C for 30 minutes in modified Dulbecco's PBS containing 0.5% fetal calf serum and 0.5 $\mu\text{g}/\text{mL}$ colchicine before micromanipulation. The first polar body and cytoplasmic protrusions were removed under a fluorescence microscope equipped with a micromanipulator unit in the manipulation medium (Dulbecco's PBS supplemented with 2% BSA) at 37 °C. Successful enucleation was confirmed by Hoechst 33342 staining. The micropipette shaped from glass capillaries were made in our laboratory. A micropipette (15-20 μm inner diameter) containing the donor cell was introduced through the slit of the zona pellucida made during enucleation, and the cell was inserted between the zona and the cytoplasmic membrane to facilitate close membrane contact and subsequent fusion.

Cytoplasts and cumulus cells were electrically fused using Eppendorf Multiporator (Eppendorf, Hamburg, Germany) in fusion medium composed of 0.3 mol/L mannitol, 0.05 mmol/L CaCl_2 , and 0.1 mmol/L MgSO_4 , as described [21]. Reconstructed embryos were manually aligned between a pair of platinum microelectrodes of the fusion chamber so that the contacting membranes of the cytoplast and donor cell were parallel to the electrodes, and a double direct current pulse of 1.25 kV/cm for 40 ms with an interval of 1 second was applied.

Reconstructed embryos were first exposed to 2.5mmol/L ionomycin for 1 minute at room temperature and then incubated at 38.5 °C under 5% CO₂ in humidified air in 2 mmol/L 6-dimethylaminopurine for 2 hours [22]. After activation, reconstructed embryos were cultured in embryo culture medium at 38.5 °C under 5% CO₂ in humidified atmosphere for varying intervals and then transferred to the SOFaa medium after being washed three times. Generally, 30 to 50 embryos were cultured in 500 mL medium per well of the four-well plates. Half of the culture medium was replaced every 48 hours and the total duration of culture was 168 hours.

Detection of histone acetylation levels in donor cells and SCNT embryos: Confluent cumulus cells at passage 2 and SCNT embryos at 2-cell and blastocyst stage were used to evaluate their histone acetylation levels. Cumulus cells/SCNT embryos were immunostained with antibodies against acH3K9 (Cell Signaling Technology, Beverly, MA) and acH4K12 (Upstate Biotechnology, Lake Placid, NY) as previously described [19]. Briefly, cumulus cells/embryos were washed in phosphate buffered saline (PBS), then fixed with 4% paraformaldehyde in PBS for 30 min, and permeabilized with 0.5% Triton X-100 in PBS for 30 min. Cells/embryos were blocked in 1% bovine serum albumin (BSA) in PBS at room temperature for 60 min. Thereafter, cumulus cells were treated with anti-acetylated H3K9 (1:200) or H4K12 antibody (1:300) at 4 °C for 12 hours, SCNT embryos were treated with acH4K12 antibody (1:300) only. Then cells/embryos were washed three times for 5 min in PBS and incubated for 1 h in the presence of 1:100 diluted fluorescein isothiocyanate (FITC)-labeled secondary antibodies (goat antirabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA). DNA was stained with 10 µg/mL Hoechst 33342 (cumulus cells) or 10 µg/mL propidium iodide (SCNT embryos) for 10 minutes. As a negative control, immunostaining was performed without primary antibodies. After another three washes, cumulus cells/embryos were mounted on slides and fluorescence was detected with a Nikon spectral confocal scanning microscope (Nikon Corporation, Tokyo, Japan). Images were acquired using a 20 objective. The system settings were kept constant for all examinations, and the analysis of each group was repeated three times.

The fluorescence intensities were quantified using EZ-C1 Free Viewer software (Nikon) as described [23], with some modifications. Briefly, the pixel value of fluorescence was measured within a constant area from nuclei area and cytoplasm area, and the cytoplasmic value subtracted from the nuclear value was the fluorescent intensity of each cumulus cells. At least 30 cells/10 embryos were measured each time.

Cumulus cells treatments and flow cytometric analysis of the cell cycle: The cell cycle was analyzed by flow cytometry as described [24,25]. Briefly, Cumulus cells (5×10^4) were exposed to Scriptaid in 6-well, flat-bottomed plates for 24 hours. Total cells, both in the suspension and adherent, were collected, washed, and suspended in cold PBS. Cells were fixed in chilled 70% (v/v) ethanol and stained in PBS containing 0.1% (v/v) Triton X-100, 0.1 mg/mL of RNase A and 0.05 mg/mL of propidium iodide (PI), for 15 minutes at 37 °C. The cell cycle distribution was measured by a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the Modify program (Verity Software House, USA). All experiments were performed independently at least three times in triplicate per experimental point.

Statistical analyses: Experiments were repeated at least three times. Oocytes matured on the same day were used to remove batch effect in each replication. All values are presented as mean standard deviation unless indicated otherwise. The proportional data were normalized by arcsine transformation and transformed values were analyzed by one-way ANOVA combined with Fisher's least significant difference test using the statistical package SPSS 19.0 (SPSS, Inc., Chicago, IL). Unless otherwise noted, P<0.05 was considered significant.

RESULTS

Effect of cumulus cells treated with Scriptaid on in vitro developmental potential of ovine nuclear transferred embryos: Cumulus cells treated with 0.2 µmol/L Scriptaid increased blastocyst and morulae formation rates (168 hours; P<0.05; **Table 1**) compared with no treatment. However, cleavage rate was not significantly different between the Scriptaid-treated and untreated (control) groups. The blastocyst and morulae formation rates of the cumulus cells treated with 0.2 mmol/L Scriptaid for 24 or 36 hours were higher than that of untreated group (**Table 2**). Nevertheless, treatment with Scriptaid for various durations had no significant effect on the cleavage rate.

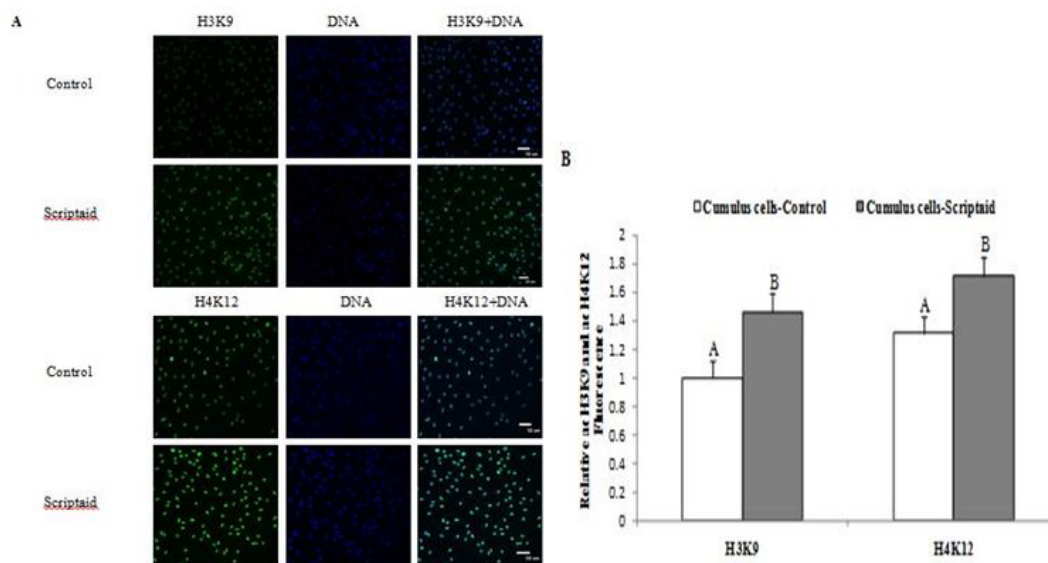
Effect of Scriptaid on the expression of epigenetic markers (acH3K9 and acH4K12) in cumulus cells: After cumulus cells were treated with 0.2 µmol/L Scriptaid for 24 hours, the intensities of two epigenetic histone acetylation markers (acH3K9 and acH4K12) were measured. Treatment with 0.2 mmol/L Scriptaid for 24 hours increased fluorescence signals of acH3K9 and acH4K12 in cumulus cells (**Figure 1**). The acH3K9 and acH4K12 signals in control groups were lower (P<0.05).

Table1. *In vitro* development (no. and mean standard deviation) of cloned ovine embryo from donor cells treated with varying concentrations of Scriptaid for 24 hours.

Concentration (µmol/L)	No. embryos reconstructed	No. cleaved (%)	No. morulae (%)	No. blastocysts (%)
0(control)	93	85 ^a (91.36 ± 3.72)	33 ^a (38.76 ± 3.29)	11 ^a (12.91 ± 1.81)
0.1	86	80 ^a (93.01 ± 2.16)	36 ^a (41.83 ± 2.92)	13 ^a (16.12 ± 2.12)
0.2	91	86 ^a (94.52 ± 1.99)	44 ^b (51.26 ± 2.06)	21 ^b (24.39 ± 2.94)
0.4	87	82 ^a (94.81 ± 1.33)	38 ^{ab} (46.44 ± 3.89)	15 ^a (18.56 ± 2.82)
0.8	84	73 ^a (86.76 ± 2.15)	31 ^a (42.57 ± 3.00)	8 ^a (10.87 ± 1.35)

Table 2. In vitro development (no. and mean standard deviation) of reconstructed ovine embryos from donor cells treated with 0.2 µmol/L Scriptaid at different durations.

Duration of treatment (h)	No. embryos reconstructed	No. cleaved (%)	No. morulae (%)	No. blastocysts (%)
0	96	88 ^a (91.58 ± 3.65)	36 ^a (40.79 ± 2.67)	13 ^a (14.79 ± 2.31)
12	91	84 ^a (92.39 ± 3.32)	40 ^a (47.72 ± 2.96)	14 ^a (16.65 ± 1.87)
24	102	98 ^a (96.16 ± 3.76)	53 ^b (54.03 ± 3.27)	25 ^b (25.49 ± 2.03)
36	96	92 ^a (95.47 ± 3.49)	47 ^{ab} (50.96 ± 3.12)	19 ^{ab} (20.56 ± 2.17)
48	95	86 ^a (90.15 ± 3.22)	36 ^a (41.77 ± 2.95)	13 ^a (15.14 ± 2.26)



Note: The acH3K9 fluorescence intensity of the cumulus cells from the control group was set as 1.

Figure 1. Effects of Scriptaid on acH3K9 and acH4K12 and relative fluorescence intensity of acH3K9 and acH4K12 in ovine cumulus cells. The acH3K9 and acH4K12 in Scriptaid-treated cells were detected by immunofluorescence analysis (Figure 1A) and presented as mean standard deviation (SD) (Figure 1B). Means with different superscript uppercase letter differed ($p < 0.05$). Scale bar represents 50 µm.

Cell-cycle analysis of cumulus cells after exposure to Scriptaid: Cell cycles of scriptaid-treated and untreated cells were analyzed by flow cytometry profiling (Figure 2). Cumulus cells cultured for 24 hours in the presence of Scriptaid showed an accumulation in the G0/G1 phase (0.2 µmol/L of Scriptaid) of the cell cycle. A total of $75.96 \pm 0.19\%$ of the untreated cumulus cells, compared with $84.22 \pm 0.33\%$ of cells cultured with 0.2 µmol/L of Scriptaid, were in the G0/G1 phase (Table 3, $P < 0.05$). These results suggest that the treatment of cumulus cells with Scriptaid resulted in the arrest of cells in G0/G1 phase of the cell cycle.

Effect of Scriptaid on the expression of epigenetic marker in ovine SCNT embryos: To investigate the acetylation level of SCNT embryos, the intensity of acH4K12 at the two-cell and blastocyst stages of SCNT embryos were measured. Compared with the control group, fluorescence signals for acH4K12 in SCNT embryos was increased significantly after donor cells treated with 0.2 µmol/L Scriptaid for 24 hours ($P < 0.05$; Figure 3).

DISCUSSION

Although a large number of species have been cloned successfully using SCNT technology, cloning efficiency is still extremely low [26]. Related studies have provided much evidence that low efficiency of SCNT technology is caused by incomplete reprogramming of the donor cell [27-29]. Histone acetylation is one of the main modification patterns in epigenetic reprogramming. Compared with normal fertilized zygotes, the acetylated levels of SCNT embryos are decreased [8,19]. Thus, pretreatment of donor cells with HDACi was expected to lead to an improvement in the success rate of reprogramming and SCNT efficiency. Kishigami et al. [9] reported that trichostatin A (TSA, a histone deacetylase inhibitor) treatment of donor cells strikingly improved the development ability of cloned embryo without obvious abnormalities in mice. However, there is still a debate as to whether SCNT efficiency is indeed increased by using HDACi or not. Some results indicated that little improvement in the development of SCNT embryos has been achieved by the pretreatment of donor cells with TSA before nuclear transfer [30,31] due to high toxicity of TSA. So an alternative strategy has been to find less toxic drugs to treat somatic cells.

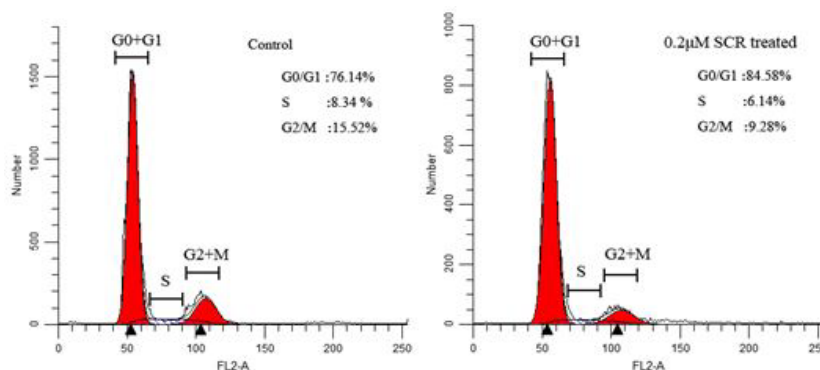


Figure 2. Cell cycle analysis of cumulus cells by flow cytometry. Cumulus cells were cultured with 0.2 µmol/L Scriptaid for 24 h, harvested, and stained with propidium iodide (PI). Control cells were treated with vehicle alone. Cell cycle analysis. Results represent the mean ± SEM. a,b: p<0.05 as determined by the Student's t-test.

Table 3. Effect of Scriptaid treatment on the cell cycle of cumulus cells.

Scriptaid(µmol/L)	Cell cycle stage (mean ± SEM)		
	G ₀ /G ₁	S	G ₂ /M
0(Control)	75.96 ± 0.19 ^a	9.21 ± 0.51 ^a	14.82 ± 0.36 ^a
0.2	84.22 ± 0.33 ^b	6.35 ± 0.59 ^b	9.42 ± 0.85 ^b

Note: Values with different superscripts within the same column are significantly different (P<0.05).

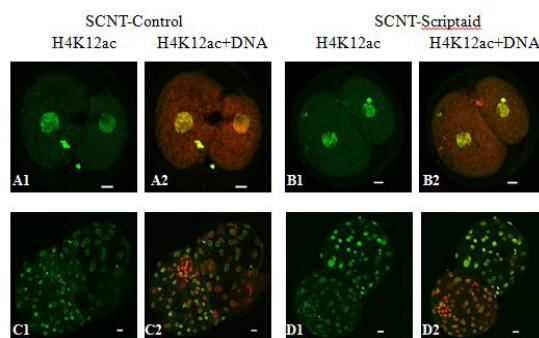


Figure 3. Dynamic changes of acH4K12 level in ovine SCNT-control embryos and SCNT embryos from pretreatment donor cells with 0.2 µmol/L Scriptaid for 24 hours at two-cell and blastocyst stages. (A1, B1, C1, D1) Acetylation of H4K12 (ac H4K12) in two-cell stage and blastocyst stage SCNT embryos (donor cells pretreatment with or without Scriptaid). (A2, B2, C2, D2) Merge of the acH4K12 staining of the same samples. The scale bar represents 20 µm.

Previous studies showed that Scriptaid is a new synthetic compound that shares a common structure but has a relatively lower toxicity than TSA^[12]. It has also been reported that the levels of histone acetylation in donor cells can be modified by Scriptaid treatment, resulting in improved development of cloned embryos to blastocyst in yak^[32]. In the present study, we investigated the effect of ovine donor cumulus cells treated with Scriptaid on the in vitro development potential of ovine SCNT embryos. We found that blastocyst development rate of SCNT embryos was significantly enhanced by the pretreatment of donor cell with 0.2 µmol/L Scriptaid for 24 hours. Therefore, Scriptaid enhanced the developmental potential of cloned ovine embryos as one of the HDAC inhibitor.

The histone acetylation of the donor nucleus has an important effect on the developmental potential of cloned embryos produced by SCNT. Both H3K9 and H4K12 are important sites for histone acetylation. In chromatin immunoprecipitation experiments, histone H4 was hyperacetylated in the promoter regions of active genes^[33] and H4K12 acetylation was essential for embryonic gene activation^[34]. Furthermore, an acetylated form of H3K9 was associated with an active chromatin configuration^[35]. Thus, anti-acetyl-histone H4K12 and H3K9 antibodies were used to determine acetylation level of cumulus cells and SCNT embryos in order to assess epigenetic reprogramming competence. In the present study, the acetylation state of donor cumulus cells treated with Scriptaid improved significantly (**Figure 1**). Therefore, we inferred that Scriptaid modified the acetylation pattern of donor cells. Furthermore, we found that the intensity of acH4K12 at 2-cell and blastocyst stage was also increased notably (**Figures 3 and 4**). The findings indicated that a higher level of histone acetylation in donor nucleus can inherit cloned embryos and subsequently enhanced the developmental competence of SCNT embryos. Our result is in agreement with the report that histone hyperacetylation of somatic cells could significantly increase the blastocyst rate in cattle^[36], buffalo^[37] and leopard cat^[38] SCNT embryos. The reprogramming of this epigenetic marker could improve the pre-implantation development capacity of ovine SCNT embryos.

The cell cycle phase of the donor cells is an important factor affecting the development of SCNT embryos^[4], because cell cycle coordination of donor cells and recipient oocytes is essential to maintain ploidy and prevent DNA damage^[39]. Accordingly, cells in G₀- or G₁-phase of the cell cycle have been used in almost all successful reports^[40], although M-phase cells can also be reprogrammed in MII oocytes^[41]. It has also been reported that SCNT embryos made from G₀- or G₁-phase cell showed no

significant difference in the efficiency of blastocyst and full-term development [42-44]. Therefore, we investigated the proportion of G0/G1 phase in the cell cycle of cumulus cells treated with 0.2 μ M Scriptaid for 24 hours (Table 3). Cell cycle analysis showed that the relative percentage of G0/G1 phase cells in Scriptaid-treated group ($84.22 \pm 0.33\%$) was significantly higher than that of the untreated group ($75.96 \pm 0.19\%$, $P < 0.05$). Previous study showed that Scriptaid-induced cell cycle arrest are largely associated with elevation of p21WAF1 and p27KIP1 [45], cyclin-dependent kinase inhibitors, which have important roles in blocking the cell cycle in the G1 phase [46]. This suggests that treatment of cumulus cells with Scriptaid is beneficial to cell cycle arrest in G0/G1 phase (Figure 2) and promotes the cloned embryo development (Tables 1 and 2).

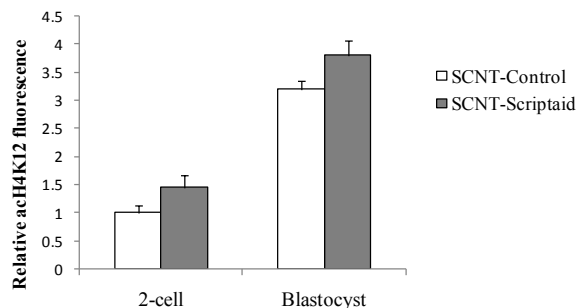


Figure 4. Mean value standard deviation fluorescence intensity of acH4K12 in SCNT ovine embryos (donor cells treated with or without Scriptaid) at two-cell stage and blastocyst stage. a,b Within a cell stage, means without a common superscript differed ($P < 0.05$). Note: The acH4K12 fluorescence intensity of the two-cell stage embryos from the SCNT control group was set as 1.

In summary, the present study indicates that the histone deacetylase inhibitor Scriptaid modifies histone acetylation status of donor cells, arrests donor cumulus cells in G0/G1 phase and subsequently enhances the nuclear reprogramming and development potential of ovine SCNT embryos. The results suggest that Scriptaid-treated cumulus cells can serve as suitable donor cells for ovine somatic cell nuclear transfer.

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