



Different reprogramming ability of fibroblast and cumulus cells after treated with trichostatin A for nuclear transfer

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ABSTRACT

Fibroblasts and cumulus cells are more prone to be reprogrammed than other cell types, and the reprogramming ability of donor cells will be improved after treated with Trichostatin A (TSA). In this study, both sheep fibroblast cells (SFCs) and sheep cumulus cells (SCCs) were treated with different concentration of TSA to investigate its influences on reprogramming ability. The motilities of SCCs were quite low when the concentration of TSA reached 25ng/ml, and 10ng/ml TSA was enough to increase the levels of histone acetylation or decrease DNA methylation significantly ($P < 0.05$), then inhibited the proliferation of SFCs in G_0/G_1 . Moreover, the developmental rate of the NT embryos increased when the SFCs treated with 10 ng/mL TSA ($P < 0.05$), but an opposite result was got in the TSA-treated SCCs group. Consequently, the fibroblast cells treated with 10 ng/ml TSA are more competent as donor cells.

Key words: DNA methylation, Donor cells, Histone acetylation, Reprogramming, TSA

It has been frequently remarked that the efficiency of somatic cell nuclear transfer (SCNT) is quite low, however, the underlying mechanisms of this relatively low efficiency remain unclear (Colman *et al.* 2000). After several hypotheses the consensus reached was that abnormal reprogramming of epigenetic state leads to the inefficiency of SCNT (Hochedlinger *et al.* 2006). The complete reprogramming of donor nucleus depends on the epigenetic state of donor cell (Enright *et al.* 2003), moreover, the reprogramming of methylation patterns is essential for the erasure of unacquired epigenetic modifications (Surani *et al.* 2001), and it was shown in chromatin immuno-precipitation experiments that histone H4 is hyperacetylated in the promoter regions of active genes (Bostick *et al.* 2007, Farthing *et al.* 2008). In somatic cell cloning, donor cells with lower levels of methylated DNA or higher levels of histone acetylation, may be reprogrammed more easily and contribute to improved cloning efficiency (Wakayama *et al.* 2005). Trichostatin A

(TSA), a histone-deacetylase inhibitor, could enlarge the pool of acetylated histones (Kishigami *et al.* 2006). Treatment of donor cells with chromatin modification agents may improve their ability to be reprogrammed.

Emphasis has been laid on the first step of cloning procedure, namely, the selection of nuclear donor cell and differentiation status (Obach *et al.* 2007). As the donor cell, a little flaw in it will be amplified in the subsequent embryonic development (Shi *et al.* 2003). Thus, the type of donor cell plays an important role in SCNT, and each cell type has its own epigenetic marks in the nucleus, followed by the establishment of different sets of marks (Surani *et al.* 2001). Cumulus cells and fibroblasts were found to more prone to reprogramming than other cell types (Wakayama *et al.* 1998).

In the present study, SFCs and SCCs were treated with 10–100 ng/ml TSA, we developed a method to measure the change of DNA methylation, histone acetylation, and cell-cycle stages after the TSA treatment would improved inefficiency of SCNT.

MATERIALS AND METHODS

Donor cell culture and TSA treatment: Fibroblast cells were routinely cultured according to the methods described by Li *et al.* (2009). The cumulus cells were isolated from the cumulus-oocyte complexes (COCs) in synthetic oviductal fluid-Hepes (SOF-Hepes) with hyaluronidase (1 mg/ml). The

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cells were collected into 1.5-ml tubes, centrifuged at 500 g for 5 min and then resuspended in DMEM supplemented with 10% FBS. The cell suspension was transferred to petri dishes and cultured for 2 to 3 days at 38.5°C with 5% humidified CO₂.

TSA dissolved in dimethylsulfoxide (DMSO) was diluted in DMEM medium to prepare different experimental concentrations of 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/mL, respectively. SFCs and SCCs of passage 2 to 4 in logarithmic phase were incubated in the culture media containing different concentrations of TSA for 24 h. After TSA treatment, cell viabilities were measured using trypan blue exclusion test (Weingartl *et al.* 2002).

Indirect immunofluorescence and scanning confocal microscopy: The cells were immunostained with antibodies against acetylated lysines12 (1: 200) and5-MeC (1: 200). Instrument settings were kept constant for each replicate, and experiments were replicated at least 3-times. The final procedure was counter staining by incubation of 10µg/ml propidium iodide (PI) for 10 min and rinse with PBS. The result of immunofluorescence was observed under a laser scanning confocal microscope and the intensities of fluorescences were determined using an image analyzer system by the fluorescence signal ratios of aCH4K12 and 5-MeC to PI.

Cell cycle analysis: DNA contents of cells were analyzed through fluorescence activated cell sorter (FASC) after staining with PI. Cells were fixed in ice-cold 70% ethanol for 1 h, harvested and resuspended in a solution containing 1 mg/ml RNase and 20 mg/ml propidium iodide to incubate for 30 min. Approximately 1×10⁵ cells were detected and the proportions of cells in G₀/G₁, S, and G₂/M phases were calculated by the manufacturer's software.

Preparation of recipient oocytes and SCNT: Sheep oocytes were matured *in vitro* as previously described (Koo *et al.* 2002). All mature oocytes having an extruded first polar

body with homogeneous cytoplasm were used for nuclear transfer experiments.

Routine procedure of SCNT was described in detail by Wee *et al.* (2006).

Statistical analysis

Three repetitions were performed for each group. The experimental data were analysed using Duncan's multiple range tests following the general linear model procedure in SPSS software. A paired *t*-test was used to compare the values of different immunofluorescence intensities. A value of *P*<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Treatment of donor cells with different concentrations of TSA: Evidence suggested that donor cells which treated with 0.08 µM (25 ng/ml) TSA would improve the development to blastocysts compared to control (Enright *et al.* 2003), and it was certain that high concentration of TSA treatments decrease the blastocyst development of donor cells, since the developmental rates of cloned embryos from donor cells upon treatment with 1.25 µM or 5 µM TSA were lower than those in embryos cloned from control, consequently prior to NT, it was firstly investigated, whether TSA treatment induced the abnormality of SFCs and SCCs. The SFCs were morphologically influenced by different concentration of TSA (Fig.1: B1–B4). Some of the TSA-treated SFCs enlarged in size, flattened and were vague in cell boundary (Fig.1: B5–B8). Morphological degeneration occurred in the SCCs, and the motility rate of TSA-treated SCCs was lower than SFCs significantly when the concentration of TSA exceeded 25ng/ml (Fig.2). We have known that the cumulus cells are relatively difficult to maintain in long-term culture compared with the fibroblasts cells (Dinnyes *et al.* 2001), may be the SCCs is too frail to TSA, and can't afford a little high concentration of TSA.

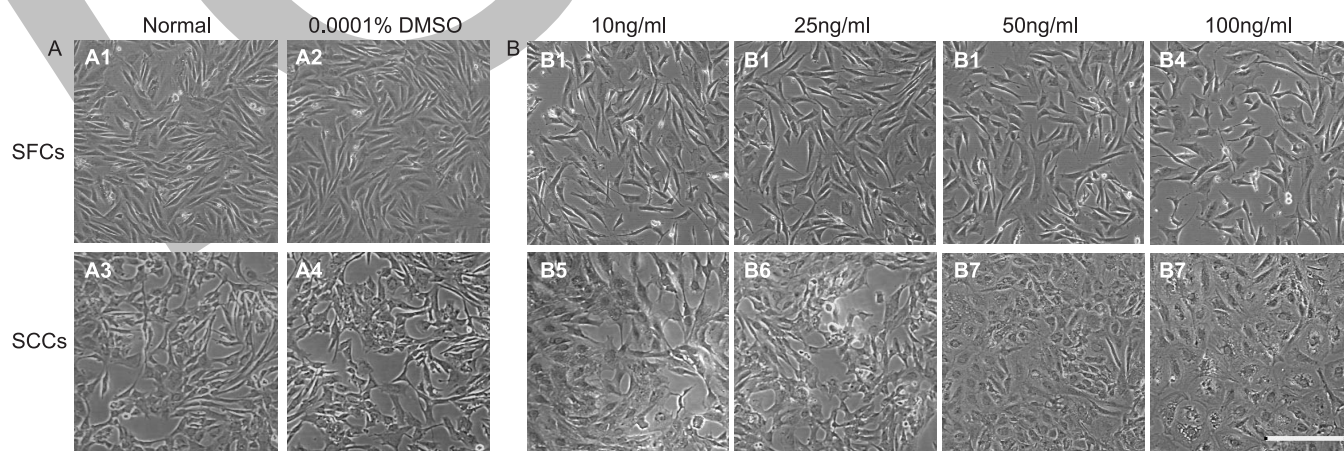


Fig 1. Morphology of TSA-treated cells. (A) Morphology of non-TSA-treated cells. A1 and A3, The normal SFCs and SCCs; A2 and A4, 0.0001%DMSO-treated cells. (B) Morphology of TSA-treated cells. B1, B2, B3, B4, SFCs treated with 10ng/ml, 25ng/ml, 50ng/ml, 100ng/ml TSA; B5, B6, B7, B8, SCCs treated with 10ng/ml, 25ng/ml, 50ng/ml, 100 ng/ml TSA. Scale bar 100 µm.

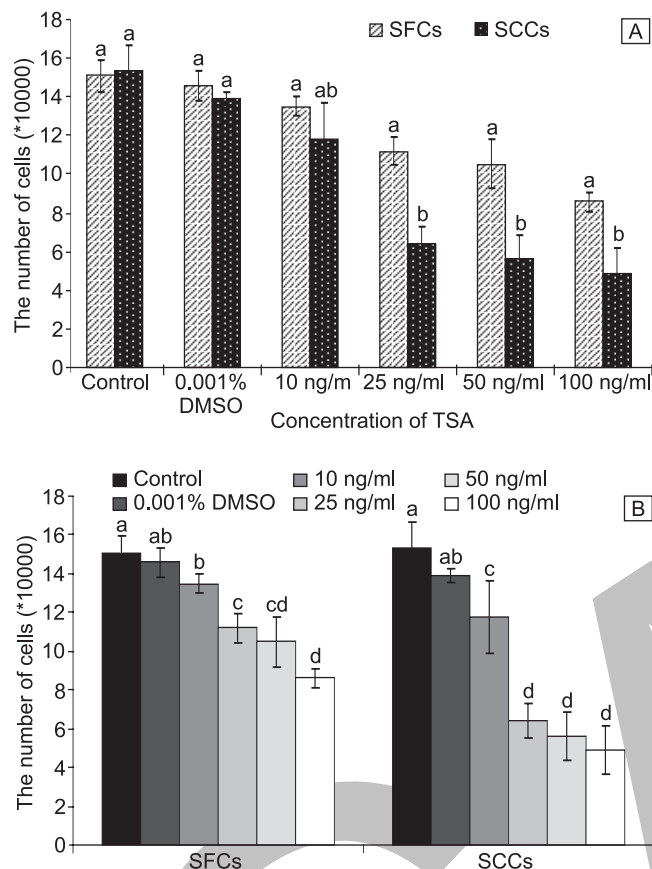


Fig. 2. The motility rate of TSA-treated cells. A: The motility rate of TSA-treated SFCs and SCCs. B: Motility rate of different density of TSA-treated (SFCs and SCCs) cells. Each value is formulated as mean $\pm$ Sem. Different letters mean significant difference among the treatments ($P < 0.05$), the same letters mean nonsignificant difference between treatments ($P > 0.05$).

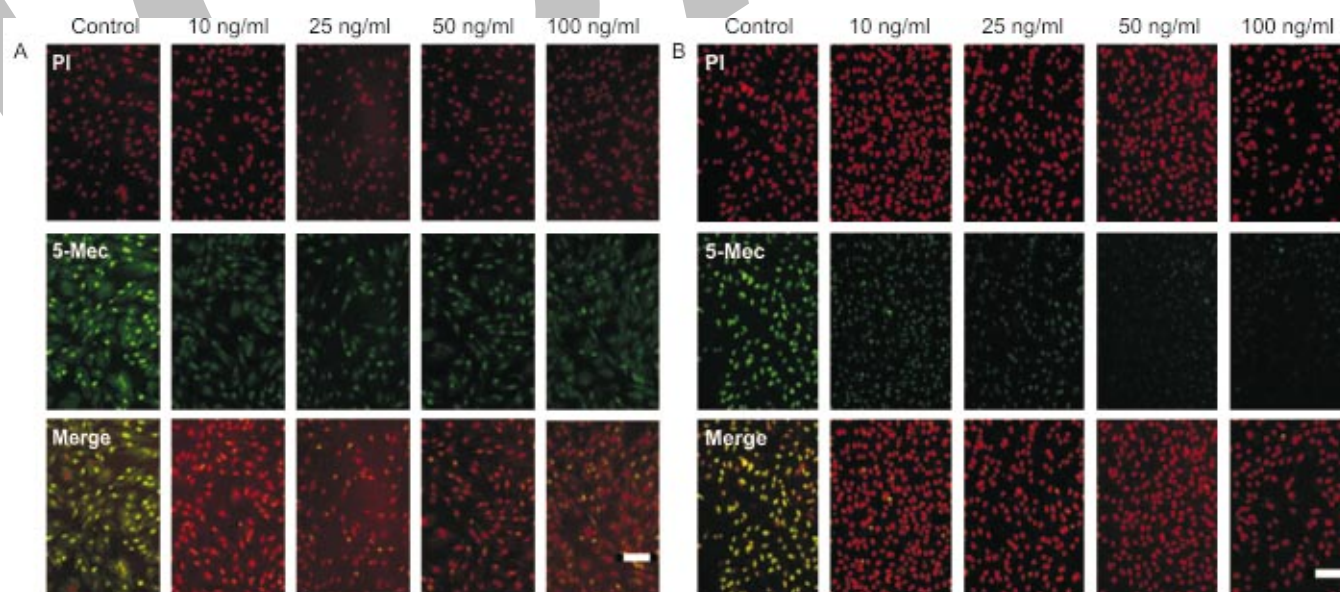


Fig 3. Intensities of 5-MeC signals in TSA-treated cells and non-treated ones. A, Intensities of 5-MeC SFCs; B, Intensities of 5-MeC signals in SCCs. Scale bar 100µm.

DNA methylation and histone acetylation in TSA-treated cells: In somatic cell cloning, fewer epigenetic modifications in donor cells, such as lower level of methylated DNA or higher level of histone acetylation, might be helpful to reprogramming and contribute to the improvement of cloning efficiency (Wakayama *et al.* 2005).

The immunofluorescence experiment was performed to determine the changes in levels of DNA methylation and histone acetylation in TSA-treated SFCs and SCCs. Both TSA-treated SFCs and SCCs had stronger fluorescent signals of acH4K12 compared with non-treated groups ($P < 0.05$). By comparison, the fluorescent signals of SCCs are stronger significantly (Fig. 4, Fig. 5, $P < 0.05$), indicating that histone-acetylation in SCCs might be more sensitive than that in SFCs. Weak fluorescent signals of 5-MeC were detected in the TSA-treated cells, while weaker in the SCCs (Fig. 6, Fig. 7, $P < 0.05$).

In fact, a similar reprogramming of histone acetylation in donor cells has been reported (Yang *et al.* 2007), to demonstrate that alteration of the histone acetylation do affect the development of cloned embryos, rabbit fetus fibroblasts (RFFs) as donor cells are treated with sodium butyrate (NaBu), another kind of histone deacetylase inhibitor, which significantly increases the level of acH3K9/14 concentrations. Each immunocytochemical experiment was repeated thrice.

Cell-cycle stage distribution: For NT, it is generally accepted that the efficiency is higher when the donor nuclei are in G1 or G0 phase (Alessi *et al.* 1998). Because the cell viability of SCCs reduced greatly when the concentration of TSA exceeded 25 ng/ml, 10 ng/ml TSA was used to treat the donor cells, which inhibited the proliferation of SFCs, and in the TSA-treated SFCs, the proportion of cells in the S

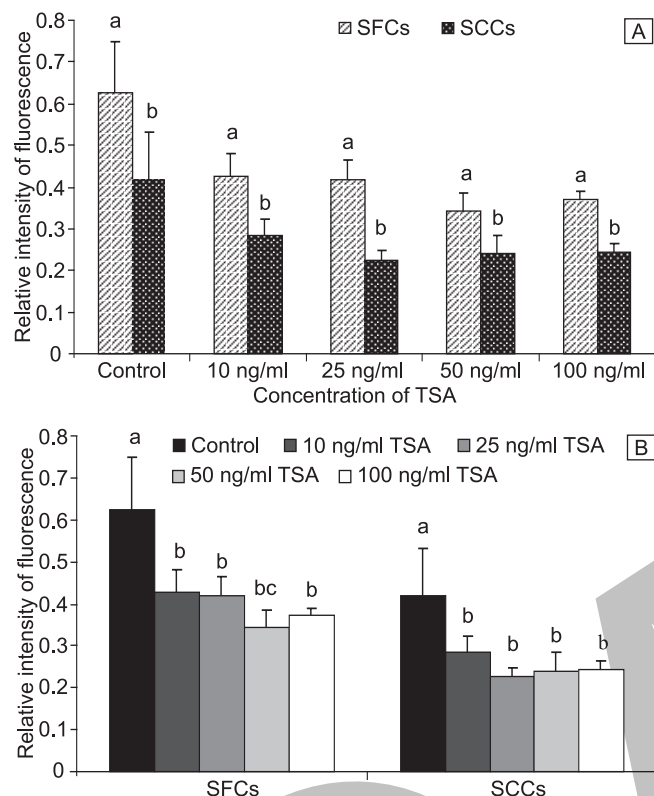


Fig. 4. The quantitative analysis 5-MeC signals of the donor cell after different concentration of TSA. Different letters mean significant differences among the treatments ($P < 0.05$), while same letters mean nonsignificant difference among treatments ($P > 0.05$). Each immunocytochemical treatment was repeated thrice.

phase was reduced, while the proportion of cells in G_0/G_1 phase was enhanced significantly compared with the non-treated group ($P < 0.05$) (Fig. 3). Andrew *et al.* found that

small cells, the majority of which exist in the G_0/G_1 phase, such as the cumulus cells had more advantages in supporting the development of reconstructed embryos than large or medium cells using fetal fibroblasts as donor cells (Andrew *et al.* 1999), only a same increased proportion of cells in G_0/G_1 was deserved in TSA-treated SCCs, and non-significant to the control (Table 1). The results suggested that TSA inhibited the proliferation of SFCs but not the SCCs. 77.48% of SCCs are naturally in G_0/G_1 phase, in accordance with a similar research by flow cytometric cell cycle analysis, it was found that more than 82% of cumulus cells were in G_0/G_1 phase (Mohamed Nour *et al.* 2000). While the percent of TSA-treated cumulus cells in this phase was just reached 81.99%, this is consistent with data regarding bovine cell cycles, in which more than 80% of cumulus cells were in the G_0/G_1 stage of the cell cycle in both short- and long-term cultures (Enright *et al.* 2003b).

Development of SCNT embryos with the TSA-treated donor cells: In the SFCs groups, embryonic development rate increased significantly after TSA treatment ($P < 0.05$). While in the SCCs groups, the development was not improved by using TSA-treated SFCs as donor cells. In contrary, the developmental rate was decreased but not significantly, indicating that TSA was not suitable to treat the cumulus cells. The fibroblast cells treated with 10 ng/ml TSA are more competent as donor cells than cumulus cells.

Embryos cloned from non-treated SCCs show higher developmental rate in previous study. In mouse cloned embryos derived from cumulus cells develop better both *in vitro* and *in vivo* than those originating from fibroblasts (Cervera and Garcia-Ximenez 2003, Wakayama *et al.* 2001). However, the NT embryos from TSA-treated SCCs were not so fortunate, the blastocyst rate reduced compared with the non-treated group (13.5% vs 19.4%). In this study, the levels

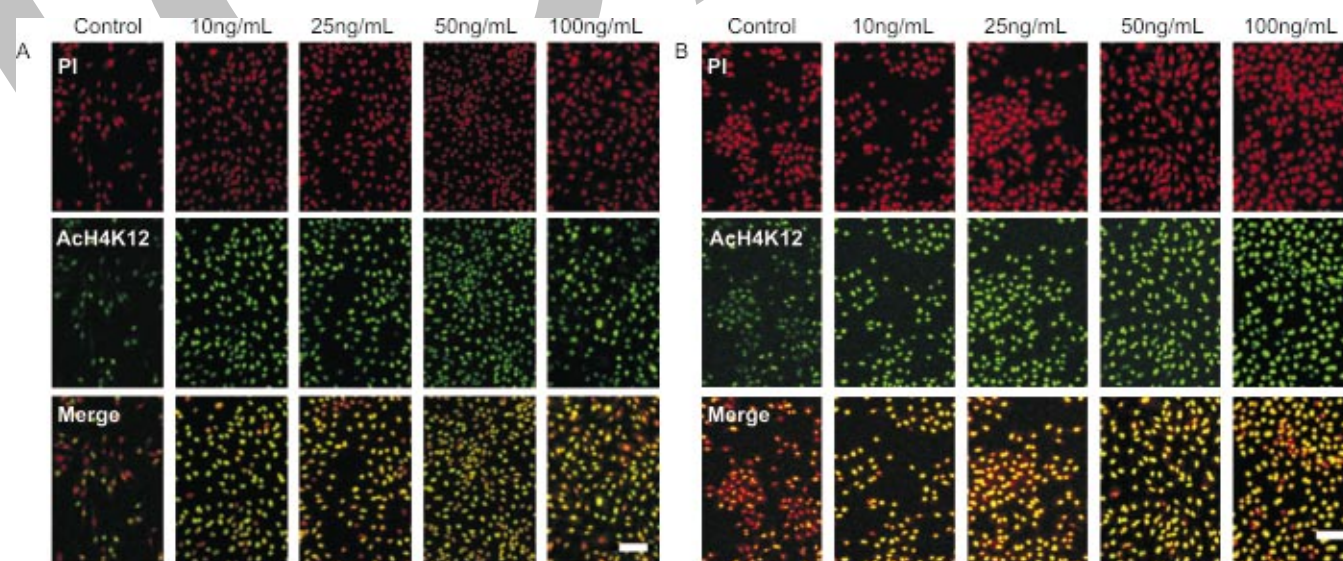


Fig 5. Intensities of acH4K12 signals in TSA-treated cells and non-treated ones. A: Intensities of acH4K12 signals SFCs. B: Intensities of acH4K12 signals in non- and TSA-treated SCCs. Scale bar 100 μ m.

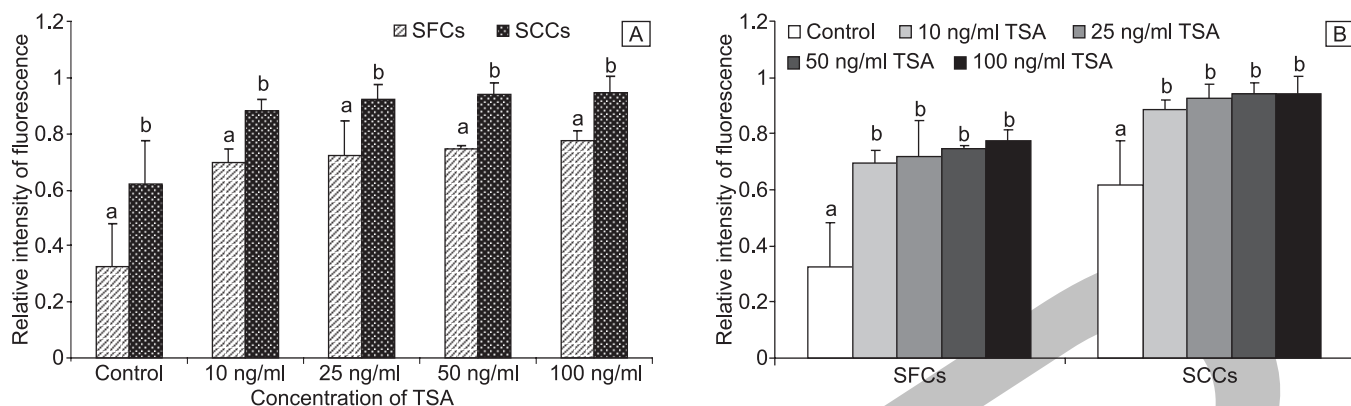


Fig. 6. The quantitative analysis aH4K12 signals of the donor cell after different density of TSA. Different letters mean significant difference among the treatments ($P < 0.05$), while same letters mean not significant difference among treatments ($P > 0.05$). Each immunocytochemical experiment was repeated thrice.

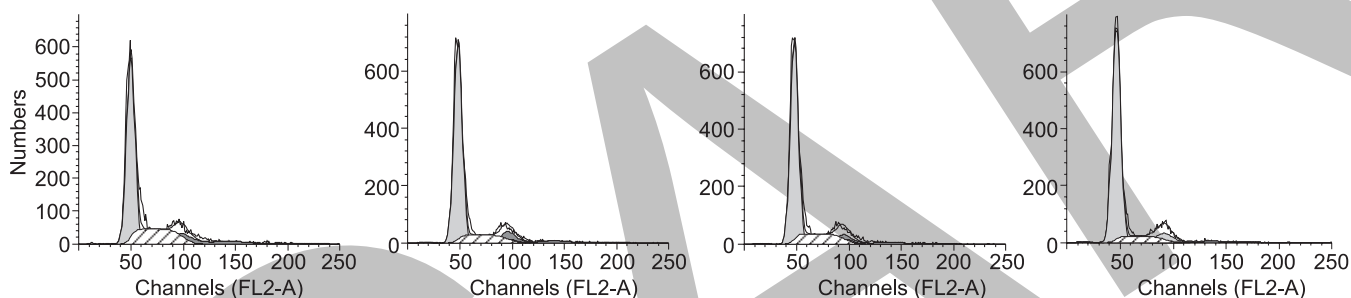


Fig. 7. Proportion of TSA-treated cells at different cell cycle stages. A, Proportion of the normal SFCs cell cycle stages; B, Proportion of the TSA-treated SFCs cell cycle stages; C, Proportion of the normal SCCs cell cycle stages; D, Proportion of the TSA-treated SCCs cell cycle stages

Table 1 Proportion of TSA-treated cells at different cell cycle stages

Group	G ₀ /G ₁ (%±S.D.)	S (%±S.D.)	G ₂ /M (%±S.D.)
SFC	70.51±1.3 ^b	27.46±0.7 ^a	11.06±2.0 ^b
SFC+TSA	82.03±1.6 ^a	15.03±1.2 ^b	2.67±0.7 ^c
SCC	77.48±1.1 ^{ab}	19.07±0.5 ^{ab}	3.45±1.3 ^b
SCC+TSA	81.99±3.3 ^a	2.04±1.7 ^c	6.96±2.1 ^a

SFC, sheep fibroblast cells; SCCs, sheep cumulus cells; S.D., standard deviation; SFC+TSA, SFC treated with 10ng/ml TSA, SCC+TSA, SCC treated with 10ng/ml TSA. a–c values with different superscripts in the same column are significantly different.

of histone acetylation in the SCCs were higher than those in the SFCs naturally, and it might be the epigenetic modifications in SCCs that facilitated the reprogramming. While, TSA, a histone deacetylase inhibitor inducing core histone hyperacetylation, makes corresponding genes overexpressed. There might be some silent genes which are harmful for reprogramming, are expressed in this stage, which prevents the embryos from further development. On the other hand, we have recognized that the cumulus cells are relatively difficult to maintain in long-term culture compared with the fibroblasts (Dinnyes *et al.* 2001), it might

Table 2. Effects of TSA treatment on the development of cloned embryos

Donor cells	No. of embryos cultured	No. of embryos cleaved (%±S.D.)	No. of blastocysts (%±S.D.)
SFCs	293	168 (57.3±8.9) ^c	21 (7.1±7.6) ^c
SFCs+TSA	289	234 (81.2±3.4) ^a	81 (28.2±6.3) ^a
SCCs	288	203 (70.5±6.7) ^b	56 (19.4±8.3) ^{ab}
SCCs+TSA	274	178 (64.9±3.8) ^{bc}	37 (13.5±8.9) ^b

SFC, sheep fibroblast cells; SCCs, sheep cumulus cells; SD, standard deviation. SFC+TSA: SFC treated with 10ng/ml TSA, SCC+TSA: SCC treated with 10ng/ml TSA. a–c values with different superscripts in the same column are significantly different. The cleavage rates and the blastocyst rates were determined at 48 h and 7 days after activation respectively.

account for why the cumulus cells can not afford the TSA treatment.

Our results suggested that treatment of donor cells with Tricostatin A (TSA) may improve their ability to be reprogrammed, however, an adequate concentration of TSA and a donor cell can afford the TSA treatment need more attentions.

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