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Trichostatin A Treatment on Two Types of Donor Cells for Somatic Cell Nuclear Transfer

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Abstract: An increased histone acetylation is associated with more effective formation of DNA replication complexes (Trichostatin A, TSA), a histone deacetylase inhibitor is able to enhance the pool of acetylated histone. Researchers observed the changes of the Sheep Fibroblasts Cells (SFCs) and Sheep Cumulus Cells (SCCs) being treated with TSA, a stronger immunofluorescent signal for acH4K12 was detected in the 1 cell stage ($p<0.05$) also the developmental rates of cloned embryos was increased significantly ($p<0.05$) however, a higher acetylation level were observed in SCCs naturely, no significant effects on acetylation level and developmental rates of cloned embryos were detected after TSA treatment. Moreover, the acetylation distribution in the group of SFC-TSA cloned embryos resembles that in *In Vitro* Fertilisation (IVF) embryos but TSA had no ability to modify the acetylation state of SCCs cloned embryos similar with the IVF embryos, only a hyperacetylate pattern was detected which means a proper pattern of acetylation in cloned embryo is more prone to be reprogrammed than hyperacetylation.

Key words: TSA, histone acetylation, donor cell, reprogramming, IVF, China

INTRODUCTION

An abnormal reprogramming of epigenetic state led to the inefficiency of somatic cell cloning (William *et al*., 2001; Hochedlinger and Jaenisch, 2006). The completely reprogramming of a donor nucleus is up to the donor cells' epigenetic state (Enright *et al*., 2003; Shi *et al*., 2003) and during normal development, early embryos undergo a well-orchestrated series of DNA methylation and histone modification changes that are believed to play an important role in establishing a chromatin state permissive to early embryonic gene expression.

Histone acetylation is heritable epigenetic modifications thus, the histone code may function as an epigenetic marker that is directly associated with transcriptional activation (Kurdistani and Grunstein, 2003; Turner, 2002; Vogelauer *et al*., 2002). It has been supposed that increased histone acetylation levels on most amino acid residues leads to looser binding of the nucleosome to DNA as a result of which the chromatin becomes looser in structure and then enters a transcriptionally permissive state (Zlatanova *et al*., 2000). Moreover, increased histone acetylation is associated with more effective formation of DNA replication complexes (Vogelauer *et al*., 2002). There are more contributing factors in the development of reconstructed embryos which could be improved if to donor cells were treated properly.

As is known, TSA, a histone deacetylase inhibitor is able to enhance the pool of acetylated histone (Kishigami *et al*., 2006). Treatment of donor cells with chromatin modifying agents may improve their ability to be reprogrammed. It has been shown that cumulus cells and fibroblasts are more prone to be reprogrammed than other types (Wakayama *et al*., 1998). Moreover, fibroblasts and cumulus cells of the domestic cat have an inherently long G0/G1 phase and it has been shown that cumulus cells and fetal fibroblasts are more ready to be reprogrammed and were consequently chosen as donor cells.

Although, how the epigenetic state is maintained and stably inherited throughout preimplantation development is unclear, the histone acetylation patterns of cloned embryos is aberrant. So, researchers assessed whether the aberrant histone acetylation patterns of cloned embryos could be corrected by the treatment of donor cells with TSA.

Chromatin immunoprecipitation experiments have shown that histone H4 is hyperacetylated in the promoter regions of active genes selectively recognizes and associates with AcH4K12 (lysine 12; H4K12) to amplify transcription (Kanno *et al*., 2004). So, researchers used an anti-acetyl-histone H4K12 to detect the acetylation level and to reveal the process of epigenetic reprogramming. Staining pattern has been examined in spare sheep embryos. That is from zygotes up to the blastocyst stage.

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Accordingly, in this research we observed the changes of the SFCs and SCCs upon treatment with TSA, through cell cycle, acetylation level and the developmental rates of cloned embryos.

MATERIALS AND METHODS

Preparation of donor cells: 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^{-1}). The 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 2-3 days at 38.5°C with 5% humidified CO_2.

TSA treatment: TSA dissovled in dimethylsulfoxide was diluted in media to yield solutions of different concentrations and the suitable handling time and concentration was discussed in the previous study (Koo *et al*., 2002). SFCs and SCCs of passage 2-4 in logarithmic phase were incubated in the culture media containing 10 ng mL^{-1} TSA for 24 h.

***In Vitro* Fertilisation (IVF):** Oocytes were washed three times in modified Tris-buffered medium (IVF medium). Ten to fifteen oocytes were transferred into a 45 mL droplet of IVF medium overlaid with paraffin oil. Fresh semen collected from boars at an artificial insemination centre was centrifuged twice at 1500 g for 5 min. The spermatozoa were resuspended in IVF medium at the concentration of 1.0×10^6 cells mL^{-1}. Finally, 5 mL of the sperm suspension was added to each droplet and cultured in a humidified atmosphere with 5% CO_2 in air at 39.8°C for 6 h.

Preparation of recipient oocytes and SCNT: The oocytes were matured *in vitro* as previously described (Li *et al*., 2011). 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). The NT embryos were cultured in 20 μL medium drops and individually in 3 mL drops of SOF with 4 mg mL^{-1} BSA in a humidified atmosphere with 5% CO_2 at 38.5°C.

Detection of acH4K12 immunofluorescence IVF and cloned embryos: All staining procedures were performed at room temperature in 10 mM isotonic PBS supplemented with 1% BSA. The IVF and cloned embryos in groups of 5 for 3 independent repeats each were fixed for 1 h in freshly-prepared 4% paraformaldehyde in PBS without BSA. They were then washed and permeabilized for 20 min with 0.5% Triton X-100, washed again for 20 min and incubated with the primary anti-acetyl antibodies: anti-acetyl-histone H4K12 at 2 μg mL^{-1} (1:200 in stock; Santa Cruz Biochemistry) for 2 h. After incubation and being washed for 20 min, the embryos were incubated with FITC conjugated mouse anti rabbit secondary antibodies at 4 μg mL^{-1} (1:100 in stock; Santa Cruz Biochemistry) for 1 h. They were then washed and mounted for observation with PI containing mounting medium. Finally, the samples were mounted between a coverslip and a glass slide supported by 4 columns of a mixture of petroleum jelly and paraffin (9:1, vol/vol).

Slides were scanned by using a confocal laserscanning microscope (Nikon TE-2000-E, Japan) with an argon-krypton laser at 488 and 563 nm and 2-channel scanning for detection of FITC and PI, respectively. Exposure time of exciting lights was uniform for respective channels.

The nuclear intensities of integrated fluorescence were measured by manually outlining all nuclei, 20 nuclei per morula and 30 nuclei per blastocyst (Suteevun *et al*., 2006). The total fluorescence intensity emitted by each nucleus was measured on antibody stained images after background subtraction by ImageJ Software (image processing and analysis in Java, http://rsb.info.nih.gov/ij/) and was averaged per embryo (Carmona *et al*., 2007; Mitalipov *et al*., 2002).

The result of immunofluorescence was observed under a laser scanning confocal microscope (Nikon Inc., Japan) and the intensities of fluorescences were determined using an image analyzer system, EZ-C1 3.7 Free Viewer (Nikon Inc., Japan) by the fluorescence signal ratios of acH4K12 to PI.

Statistical analysis: Three repetitions were performed for each group. The experimental datas were analysed using Duncan's multiple range tests using the General Linear Model procedure in SPSS Software (SPSS Inc., Chicago, IL). 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

Histone acetylation status of donor cells transferred into non-activated oocytes: After enucleation, donor cells were individually inserted into the perivitelline space of single enucleated oocytes then fused but not activated.

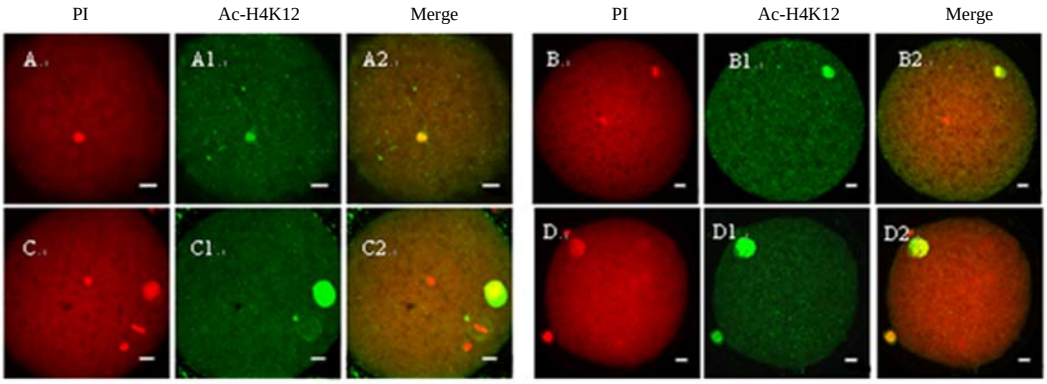

Fig. 1: The immunofluorescent staining of donor cell acetylation transferred into non-activated oocytes, A: The immunofluorescent staining of SFC acetylation, PI, red; A_1: anti-H4K12 acetyl immunofluorescent, Green; A_2: Merge, the same as below; B: TSA treated SFC; C: the immunofluorescent staining of SCC acetylation, D: TSA treated SCC. Scale; Bar = 20 μm

Researchers got the non-activated oocytes after the fused oocytes cultured in the SOF for 1 h. Then, we obtained a similar conclusion with the histone acetylation status of SFCs (Fig. 1 and 2). Surprisingly, in the SCCs group, to the contrary of a strong immunofluorescence signal detected in SCCs TSA-treated oocytes, there were nearly no change in histone acethylation of control embryos in another words, a significant higher level of histone acetylation was only found in the TSA-treated SCC derived oocytes rather than control (Fig. 2).

Histone acetylation patterns of IVF embryos and cloned embryos: For successful development of cloned embryos, epigenetic status of donor cells should be reprogrammed to that of normal embryos. It was therefore, investitgated whether the histone acetylation patterns of cloned embryos could be corrected by the treatment of donor cells with TSA and the results suggested the patterns were just like those in IVF embryos. Consequently, the acetylation state in SFC-TSA cloned embryos were resemble that in IVF embryos, suggesting that TSA had the ability to modify the acetylation pattern of SCNT embryos and reprogramming of this epigenetic mark was aberrant in cloned embryos and could be corrected by the treatment of donor cells with TSA (Fig. 3 and 4).

The cloned embryos from SFC-TSA cells showed a similar acetylation pattern as IVF embryos. While SFC-cloned embryos, moderate acetylation levels were maintained at all stages, there is no significant fluctuate which is bad for the embryos development. This explained why the development rate of TSA-treated SFC derived embryos was close to that of the IVF embryos.

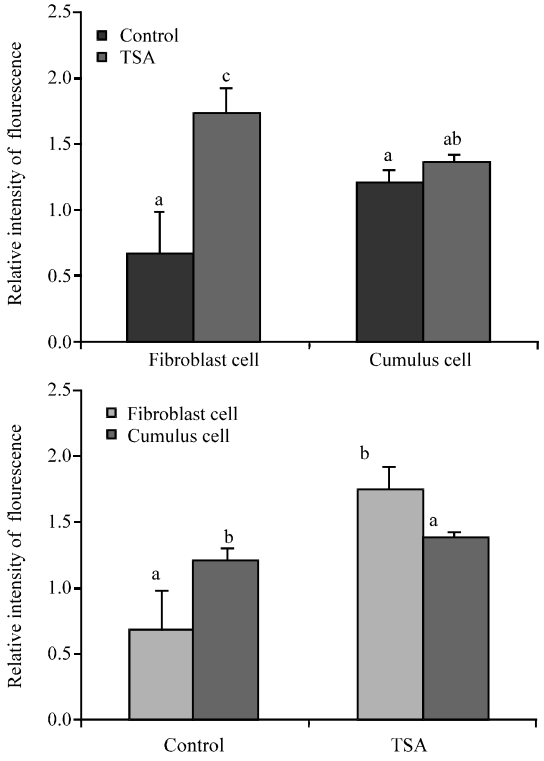

Fig. 2: The quantitative analysis of acH4K12 signals in the donor cell transferred into non-activated oocytes. Each value is formulated as mean±SEM. Different letters means significant difference among the treatments ($p<0.05$) while the same letter means non-significant difference among the treatments ($p>0.05$)

An intensified signal was detected and a much stronger staining was observed in TSA-treated cloned

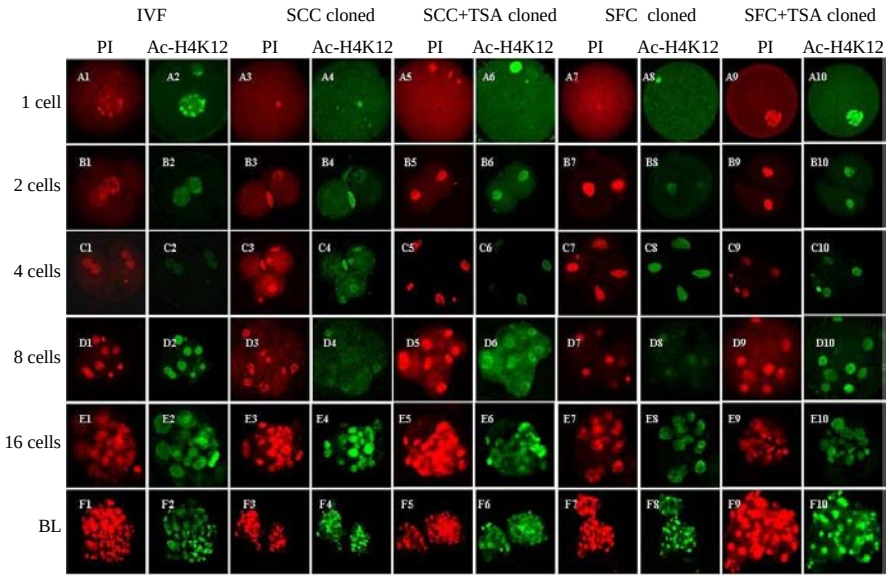

Fig. 3: The immunofluorescent stainingof IVF and cloned embryo acetylation. IVF: 1 cell 13/15; 2 cell 15/18; 4 cell 8/10; 8 cell 13/13; 16 cell 9/9; blastocysts 8/9; SFC: 1 cell 1010; 2 cell 6/8, 4 cell 10/10, 8 cell 7/9; 16 cell 8/10; blastocysts 8/8; SFC+TSA: 1 cell 15/15, 2 cell 10/11, 4 cell 10/10, 8 cell 9/10, 16 cell 9/10, blastocysts 6/7; SCC: 1 cell 11/13; 2 cell 7/9; 4 cell 11/11; 8 cell 8/9, 16 cell 9/11; blastocysts 7/7; SCC+TSA: 1 cell 12/15, 2 cell 12/13, 4 cell 11/12; 8 cell 10/10; 16 cell 9/11 and blastocysts 6/6) IVF: *in vivo* fertilized embryos. SCF and SFC+TSA, SCC, SCC+TSA embryos cloned from, sheep fibroblast cells, TSA-treated sheep fibroblast cells, sheep cumulus cells, TSA-treated sheep cumulus cells respectively; BL: Blastocysts. Cells were immunostained with anti-acH4K12 (green) and counterstained with PI for chromatin (red). The images represent acH4K12 patterns as in the majority of stained embryos. All images are at the magnification x200

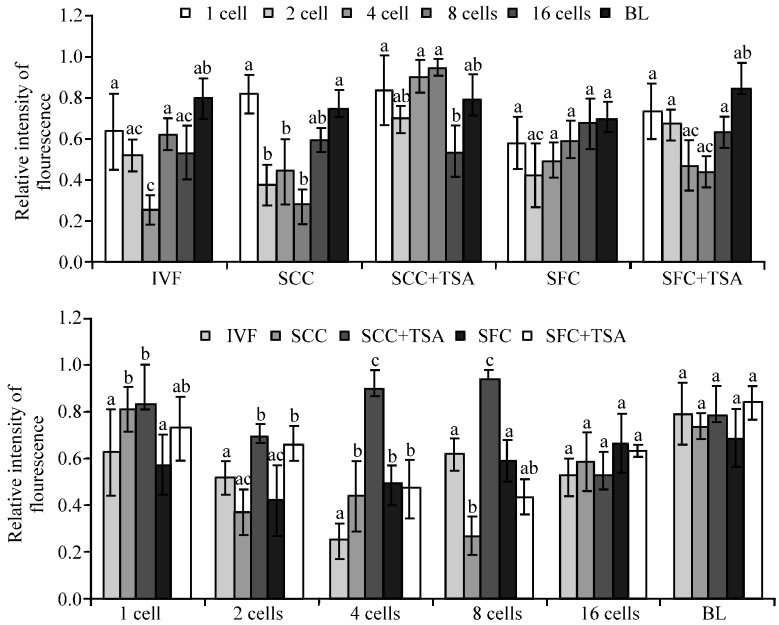

Fig. 4: The quantitative analysis acH4K12 signals of IVF and cloned embryos. Each value is formulated as mean±SEM. Different letters means significant difference among the treatments ($p<0.05$) while same letter means non-significant difference among treatments ($p>0.5$)

embryos than in the normal cloned embryos and especially in the SCC-derived normal cloned embryos, it displayed low levels of acH4k12 at 2-8 cells stages which increases to moderate levels at the 16 cells stages and blastocyst stages however, in embryos cloned from SCC-TSA oocytes, there were hyperacetylated at all stages including 2-8 cells periods (Fig. 4).

The acetylation patterns in SCC-TSA cloned embryos indicated that the hyperacetylation was not suitable for the cloned embryos which would reduce the developmental rates.

Development of cloned embryos and IVF embryos: After SCNT, *in vitro* developmental capacity of cloned sheep embryos with differently treated donors was investigated (Table 1). In the SFCs groups, it was suggested there was a significant increase ($p<0.05$) in developmental rates of TSA-treated SFCs NT embryos. While the TSA-treated SFCs as donor cells was not to improve the development of the NT embryos, on the opposite however, to reduce it but not significantly, indicating that TSA might not be suitable to treat the cumulus cells.

Considering the results showed above, researchers compared the developmental capactity of NT embryos with TSA-treated cells with that of IVF embryos (Table 1). The developmental rate of NT embryos with TSA-treated SFCs was close to that of the IVF embryos (28.2 vs. 32.8%).

The acetylation of nuclear histones is thought to play key roles in the propagation of genomic function information from one cell generation to the next by retaining this information during the mitotic phase (Turner, 2002). Proper patterns of histone acetylation which were characterized by specific chromatin modifications, play a key role in nuclear reprogramming after SCNT and can determine the efficiency of SCNT.

According to this study, the histone acetylation patterns of cloned embryos are aberrant. Firstly there was a moderate acetylation level at the 1 cell stage, secondly deactylation in the 2, 4 or 8 cells stage and high level of histone acetylation was detected till the 16 cells stage and blastocyst stage. In a recent study, the deactylation-reacetylation transformation occurred in both experiments as well as in other species (Wee *et al*., 2006) and insofar all species shared the erase and rebuild pattern in normal embryo development (Suteevun *et al*., 2006). In the IVF, SCC cloned, SFC+TSA cloned embryos which exhibited more similarities in acH4K12 patterns to the IVF embryos, decreased developmental rates and low acetylation levels were detected at 4 or 8 cells stage indicating that low acetylation level was correlated with the embryos development. Genomic activation in NT or even the TSA-NT embryo is insufficient for subsequent embryonic development because of its low level of histone H4 acetylation at 8 cells stage (Carmona *et al*., 2007). Abnormal DNA methylation and histone acetylation obtained in previous studies which perhaps subsequently prevent the correct expression of crucial genes at 8 cells stage might explain the developmental loss observed in this study at this phase and in a previous report by Mitalipov *et al*. (2002).

The methods of treating donor cells were possibly suitable for one type but not for another. The treatment of SFCs with TSA increased the proportion of NT embryos developing to blastocysts (28.2 vs. 7.1% of non-treated SFCs). However, the TSA-treated SCCs NT embryos were not so fortunate in terms of decreased of developmental rates (13.5 vs. 19.4% with non-treated SCCs). It was found in this study that the levels of histone acetylation in the SCCs are higher than those in the SFCs and it might be the epigenetic modifications in SCCs that facilitated the reprogramming. TSA induces nuclear histone hyperacetylation which would make corresponding genes overexpressed. There might be some genes harmful to reprogramming that should be silent were expressed in this stage which prevented the embryos from further development. On the other hand, researchers have recognized that the cumulus cells were relatively difficult to culture in long term compared with the fibroblasts and those from most individuals could only be subcultured to passage 10 at most but cell lines from a few individuals had no comparable in vitro proliferative potential and their morphology is apt to change (Yang *et al*., 2007) the cumulus cells were might be so fragile that couldn't endure the treatment.

Although, the developmental rates of the cloned embryos are quite different, each of the cloned embryos to the blastocyst stage has the same high level of histone

Table 1: Development of IVF embryos and cloned embryos from different types of somatic donor cells

Embryo type	No. of embryos	No. of cleaved	No. of blastocysts
IVF	265	190 (71.8±5.1)^{b}	87 (32.8±4.5)^{c}
SFC	293	168 (57.3±8.9)^{a}	21 (7.1±7.6)^{a}
SFC+TSA	289	234 (81.2±3.4)^{c}	81 (28.2±6.3)^{bc}
SCC	288	203 (70.5±6.7)^{b}	56 (19.4±8.3)^{b}
SCC+TSA	274	178 (64.9±3.8)^{ab}	37 (13.5±8.9)^{ab}

SFC: Sheep Fibroblast Cells, SCCs: Sheep Cumulus Cells, SD: Standard Deviation, SFC+TSA: SFC treated with 10 ng mL^{-1} TSA, SCC+TSA: SCC treated with 10 ng mL^{-1} 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

acetylation (Fig. 4), either in the TSA-treated group or those non-treated. It is reported that increased histone acetylation is associated with the more effective nucleus reprogramming (Cervera and Garcia-Ximenez, 2003). An interesting discovery from Smith suggested that global gene expression profile of bovine SCNT embryos is similar to that of embryos produced *in vivo*, suggesting that by the blastocyst stage, the SCNT embryos have undergone a relatively complete and correct nuclear reprogramming at molecular level (Smith *et al*., 2005).

CONCLUSION

This research yield important information regarding mechanisms responsible for nuclear reprogramming. However, rather than that all the reprogramming events that should take place in a successful NT are apparent throughout the relatively short embryo culture period and it is possible that they will happen in the fetal survival following embryo transfer during the days of gestation. A key mystery that remains to be unvailed is how reprogramming events happen after embryo transplantation.

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