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Effect of trichostatin A on transfected donor cells and subsequent development of porcine cloned embryos

Fu Bo[2], *Liu Di*[1,2], *Fang Qing-chang*[2], *Ren Liang*[2], *Ma Hong*[3], *Wang Liang*[3], *Guo Zhen-hua*[3] *and Li Zhong-qiu*[3]

Institute of Animal Science, Heilongjiang Academy of Agriculture Science, Harbin; and College of Life Science, Northeast Agriculture University, Harbin, P.R. China

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Summary

Transgenes integrated into mammalian cells are silenced rapidly. This phenomenon correlates with repressed chromatin structure marked by histone hypoacetylation. This study investigated the effect of trichostatin A (TSA; a histone-deacetylase inhibitor) on EGFP expression in transfected cells and embryonic development after somatic cell nuclear transfer (SCNT). Porcine adult fibroblasts were transfected with a pEGFP-C1 vector. Then transfected cells, donor cells for SCNT, were pretreated with TSA, with the untreated cells being used as the control. Expression of EGFP in donor cells and reconstructed embryos was detected when exposed to blue light. Results showed that the percentage of EGFP-positive cells significantly increased when the transfected cells were treated with TSA and the increased expression of EGFP was sustained to at least the morula stage. In addition, the cytotoxic effect of TSA on the transfected cells was dose dependent. In conclusion, TSA can rescue the silenced *EGFP* gene. Even after transferring the TSA-treated cells to enucleated recipient oocytes, TSA retained the ability to rescue a silenced *EGFP* gene. In addition, TSA had an impact on cell proliferation.

Keywords: EGFP, Histone deacetylation, Porcine, Somatic cell nuclear transfer, Trichostatin A

Introduction

Genetic modification of cultured cells combined with somatic cell nuclear transfer (SCNT) provide an effective approach to obtain large transgenic animals, allowing multiple genetic modifications to be undertaken *in vitro* prior to nuclear transfer (Niemann *et al*., 2003). However, regulation of gene expression in mammalian cells is a complex process and epigenetic modifications affect the expression of stable and transient transgenes in mammalian cells. Transgenes stably integrated into mammalian cells are silenced rapidly, probably under the influence of surrounding endogenous condensed chromatin. DNA methylation and histone deacetylation play an important role in epigenetic modification of genes.

Earlier studies have shown that non-methylated transgenes stably integrated into the genome become packaged with acetylated histones, while *in vitro* CpG-methylated transgenes become associated with deacetylated histones (Eden *et al*., 1998; Schübeler *et al*., 2000). Gene silencing may correlate with repressed chromatin structure marked by histone hypoacetylation (Jenuwein *et al*., 2001; Bhaumik *et al*., 2007), because histone hypoacetylation can influence transcription by forming silent chromatin structures and preventing transcription factor binding. Level of acetylation in histone is controlled by equilibrium of histone acetyltransferase and histone deacetylase (HDAC) activities (Kikuchi & Fujimoto, 1973; Csordas, 1990). Trichostatin, a histone-deacetylase inhibitor, can cause a marked increase in histone acetylation (Yoshida *et al*., 1990). It has been demonstrated previously that TSA as a reversible inhibitor of HDAC and can reactivate reporter gene expression when its promoter was methylated in transgenic cells (Grassi, 2003).

[1]All correspondence to: Liu Di. Heilongjiang Academy of Agricultural Science; 368 Xue Fu Road, Harbin City 150086, China. Tel: +86 13845120192. e-mail: liudi1963@163.com
[2]College of Life Science, Northeast Agriculture University, Harbin 150030, P.R. China.
[3]Institute of Animal Science, Heilongjiang Academy of Agriculture Science, Harbin 150086, P.R. China.

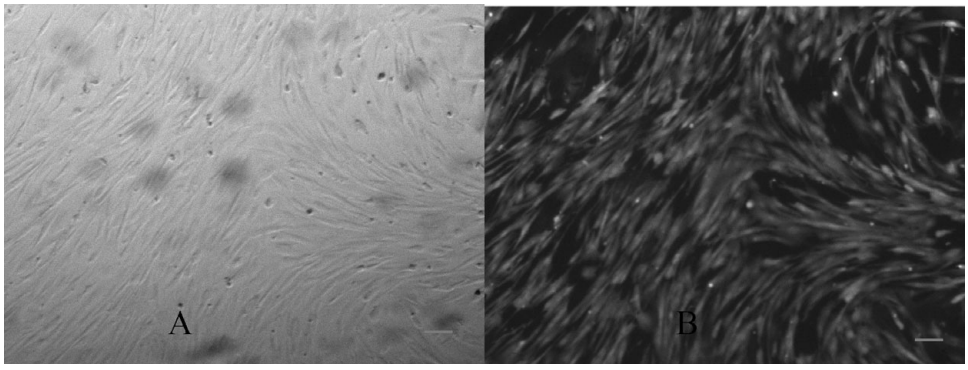

Figure 1 EGFP expression in stable transfected cells. (*A*) Phase contrast images. (*B*) Epifluorescent images. Bar represents 100 μm.

Reporter gene imaging currently represents a powerful approach for studying expression of modified genes in SCNT embryos. Green fluorescent protein (GFP) as a reporter gene traditionally refers to the protein first isolated from the Pacific Northwest jellyfish *Aequorea victoria*. GFP exhibits bright green fluorescence when exposed to blue light (Prendergast & Mann, 1978; Tsien, 1998). Thus, reporter gene imaging, especially EGFP, has been extensively used as a marker for transgene expression in donor cells for SCNT. The issue of reporter gene silencing in SCNT embryos has not been systemically evaluated. We therefore assessed the ability of a histone deacetylation inhibitor (TSA) to rescue the silenced *EGFP* gene and investigated the effects of TSA on EGFP expression and embryonic development after SCNT.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Corp unless otherwise indicated. pEGFP-C1 vector was purchased from Clontech.

Preparation of adult fibroblasts

Porcine adult ear tissue was cut into small pieces with fine scissors, 1–2 mm diameter explants were cultured in DMEM (Gibco) enriched with 20% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin in an atmosphere of 5% CO_2 in air at 38.5°C. Once cells had reached 90% confluency, they were trypsinized and diluted to a concentration of 1×10^6 cells/ml. Cells were then regrown and passaged before being frozen in DMEM containing 20% FBS and 10% dimethyl sulfoxide, and stored in liquid nitrogen.

Transfection of fluorescence protein expression vectors into adult fibroblasts

For transfection, cells were thawed, cultured, and subsequently used, 1×10^6 cells were plated onto 6-well plates 1 day before transfection. Lipofectamine 2000 was used, according to the manufacturer's instructions. 2 μg of DNA (pEGFP-C1 vector) and 4 μl of Lipofectamine^{TM} 2000 were diluted separately with 200 μl DMEM without serum and incubated for 5 min at room temperature. After 5 min incubation the diluted DNA was combined with diluted Lipofectamine^{TM} 2000 (total volume = 400 μl). Cells were then mixed gently and incubated for 20 min at room temperature. The 400 μl of complexes were added to the 6-well plates containing cells and medium. These were then mixed gently by rocking the plate back and forth. After 5–6 h, complexes were replaced by fresh culture medium. The transfected cells were then cultured in DMEM supplemented with 600 μg/ml of G418 to remove the untransfected cells. Stable transfected cells were frozen and were ready for further treatment (Fig. 1).

Trichostatin A treatment, cells proliferation and EGFP expression analysis

Trichostatin A was dissolved in DMSO and stored in 5 μM at −20°C. After being passaged seven times, transfected cells were incubated in DMEM medium containing trichostatin A at the concentrations of 10 nM, 50 nM, 100 nM, 150 nM for 24 h, and then subjected to subsequent cell proliferation and EGFP expression analysis. The quantity of transfected cells in all groups were calculated by cytometry. At the same time, the percentages of EGFP-positive cells were measured.

In vitro maturation of oocytes

Prepubertal porcine ovaries were collected at a local abattoir and transported to the laboratory in 0.9% NaCl

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solution at 35°C. COCs were aspirated from 3 to 6 mm diameter antral follicles by using a 20 ml disposable syringe with an 18-gauge needle. COCs with an evenly distributed cytoplasm and at least three compact layers of cumulus cells were selected and washed three times in TCM199 enriched with 10% (v/v) FBS, and were washed twice with the maturation medium (TCM-199 supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 IU/ml hCG, 10 IU/ml PMSG, 10 ng/ml epidermal growth factor, 10% porcine follicular fluid, 75 μg/ml penicillin G and 50 μg/ml streptomycin). After cultured with maturation medium for 22 h, oocytes were cultured without hormones for 22 h at 38.5°C in 5% CO_2.

Production of transgenic embryos

In order to be used as donor cells for SCNT, transfected cells were thawed and cultured until they reached full confluency. Before SCNT, these transfected cells were treated with TSA for 24 h, with untreated transfected cells as control. After 44 h of maturation, oocytes were denuded of cumulus cells by vortexing in the presence of 0.1% hyaluronidase in DPBS. Only oocytes with first polar body were selected. All manipulations were performed in NCSU-23 buffered with HEPES. Oocytes were exposed to cytochalasin B (5 μg/ml) and Hoechst 33342 (5 μg/ml) for 10 min prior to enucleation. Metaphase chromosomes were removed under very short exposure to UV light with an enucleation pipette. After enucleation, single EGFP-expressed cell was introduced into the perivitelline space of the enucleated recipient oocytes through the hole made during enucleation. For fusion and activation, 1 DC pulses of 2.0 kV/cm for 30 μs were applied by using a BTX-Cell Manipulator 200. The medium used for fusion and activation was 0.3 M mannitol supplemented with 0.1% PVA, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES. Then reconstructed embryos were activated with 2 mM 6-DMAP for 3–5 h. Reconstructed embryos were cultured in NCSU-23 medium for 7 days.

Experimental design

Experiment 1: Effect of trichostatin A on EGFP expression in transfected fibroblast cells

Transfected cells were cultured in medium with TSA at 10 nM, 50 nM, 100 nM, 150 nM for 24 h, the percentages of EGFP-positive transfected cells were determined. At the same time, transfected cells without TSA treatment were used as control.

Experiment 2: Effect of trichostatin A on transfected cells proliferation

DMEM medium with 10, 50, 100, and 150 nM TSA was seeded with 1×10^5/ml adult fibroblasts. At the same time, transfected cells without TSA treatment were used as control. The cells were counted on the third day. Horizontal coordinate data defined concentration of TSA and vertical ordinate data defined cell count.

Experiment 3: Effect of trichostatin A on the development of the cloned embryos derived from transfected cells treated with TSA

Transfected cells were used as donor cells only after reaching full confluency. In addition, the TSA group needed to be treated with 50 nM TSA for 24 h before nuclear transfer. After nuclear transfer and activation, reconstructed embryos derived from transfected cells were transferred into NCSU-23 medium. NT embryos were evaluated for the rate of EGFP-positive embryos on day 2 (activation was done on day 0). The percentages of morula and EGFP-positive morula were determined on day 6.

Statistical analysis

There were at least three replicates for each treatment. EGFP-positive rate in transfected cells was subjected to one-way ANOVA using SPSS (version 17.0 for Windows).

All data, developmental rate to the morula stage, percentage of EGFP-positive embryos on day 2 or percentage of EGFP-positive morula, were subjected to independent-samples *t*-test. A probability value of $p < 0.05$ was considered to be statistically significant.

Results

Experiment 1: Effect of trichostatin A on EGFP expression in transfected fibroblast cells

As shown in Table 1, the proportion of EGFP-positive cells increased with TSA concentration. EGFP-expressing cells significantly increased when TSA concentration was greater than 50 nM ($p < 0.05$). However, with regard to EGFP expression, there was no significant difference among the 50 nM, 100 nM and 150 nM groups. Expression was nearly two-fold higher in the 50 nM, 100 nM and 150 nM TSA groups when compared with the control and 10 nM groups.

Experiment 2: Effect of trichostatin A on transfected cell proliferation

Based on Fig. 2, it appears that the cytotoxic effect of TSA on the transfected cells was dose dependent. Most of the cells died when TSA concentration was greater than 50 nM ($p < 0.05$). In addition, there was no significant difference between 100 nM and 150 nM groups. The cells changed morphologically after TSA treatment for 24 h. TSA-treated cells elongated in size

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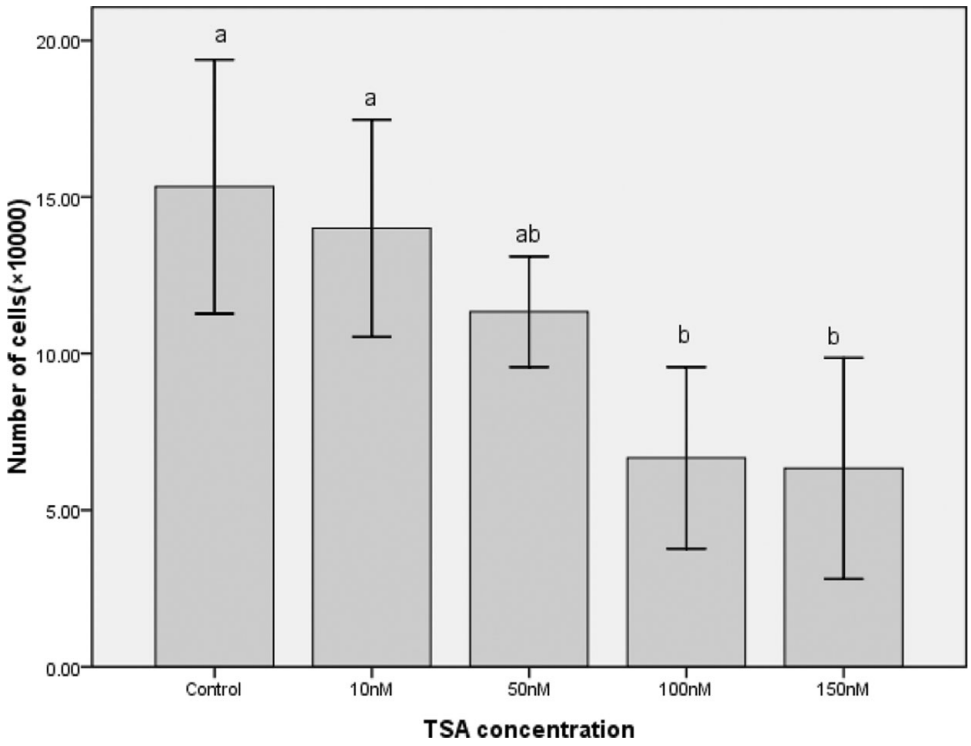

Figure 2 Cytotoxic effect of TSA on the transfected cells. Quantity of transfected cells treated with TSA at 10 nM, 50 nM, 100 nM, 150 nM was calculated respectively. Transfected cells without TSA treatment were used as control. Values with different letters indicate significant difference between the treatments ($p < 0.05$), the same letter indicates no significant difference between treatments ($p > 0.05$) mean ± SE.

Table 1 Effect of TSA on the expression of EGFP gene in transfected cells.

Treatment	EGFP cells (% ± SE)
0 (control)	14.8 ± 1.8^{b}
10 nM	15.3 ± 1.1^{b}
50 nM	26.2 ± 2.9^{a}
100 nM	25.3 ± 1.2^{a}
150 nM	26.3 ± 2.9^{a}

Note: Transfected cells were treated with TSA at 0, 10 nM, 50 nM, 100 nM, 150 nM respectively. The percentage of cells that expressed the *EGFP* gene was determined. ^{a,b}Different letters in the same row mean significant difference between the treatments ($p < 0.05$), same letter in the same row means no significant difference between treatments ($p > 0.05$).

and were vague in outline (Fig. 3). In addition, some cells flattened.

Experiment 3: Effect of trichostatin A on the development of the cloned embryos derived from transfected cells treated with TSA

The proportion of EGFP-positive embryos on day 2 of the 50 nM TSA treatment group was significantly higher (75.8 ± 3.0%) than in control groups (57.5 ± 2.8) ($p < 0.05$) (Table 2) (Fig. 4). With regard to morula rate, there was no significant difference between treatments ($p > 0.05$). However, 75.3% of the morulas derived from the 50 nM TSA-treated transfected cells were EGFP-positive, which was significantly higher than those from TSA-untreated transfected cells (57.0 ± 2.0%) (Table 2).

Discussion

Transgenic pigs are of great value for research and could provide an alternative source of organs for xenotransplantation. Although GFP represents a good marker to study transgene expression in porcine transgenic models, the issue of *GFP* gene silencing in SCNT embryos has not been systemically evaluated. This study examined the role of TSA on reporter gene silencing during SCNT. Our major findings are as follows: (1) The rate of EGFP-positive cells significantly increased when the transfected cells were treated with TSA. (2) There were cytotoxic impacts on transfected cells treated with TSA. Cell morphological changes resulted from TSA exposure. (3) Percentage of EGFP embryos on day 2 in the 50 nM TSA treatment group was significantly higher than in the control

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Table 2 Developement of cloned embryos derived from transfected cells and TSA-treated transfected cells.

Treatment	No. of oocytes used	No. of EGFP-positive embryos on day 2 (% ± SE)	No. of morula (% ± SE)	No. of EGFP-positive morula (% ± SE)
Transfected cells with 50 nM TSA treatment (TSA)	130	99 (75.8 ± 3.0)[a]	25 (20.0 ± 2.6)[a]	19 (75.3 ± 2.6)[a]
Transfected cells without TSA treatment (no TSA)	120	69 (57.5 ± 2.8)[b]	35 (29.2 ± 2.0)[a]	20 (57.0 ± 2.0)[b]

[a,b]Different letters in the same row mean significant difference between the treatments ($p < 0.05$), same letter in the same row means no significant difference between treatments ($p > 0.05$).

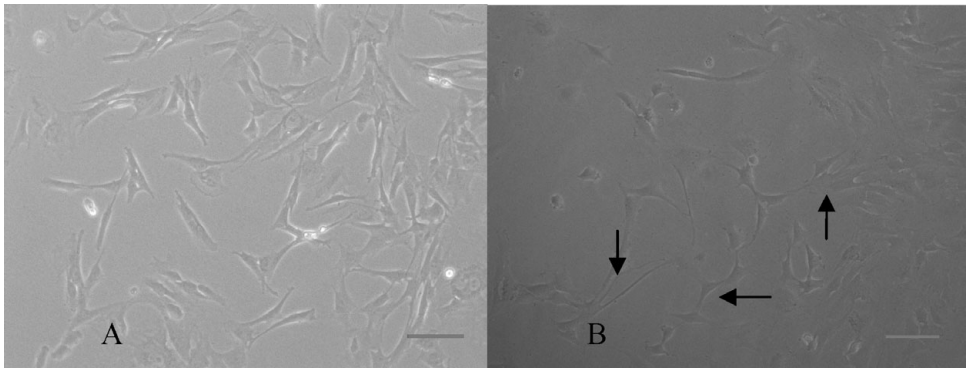

Figure 3 Characterization of TSA-treated transfected cells. (*A*) Morphology of non-TSA-treated cells. (*B*) Morphology of cells treated with 50 nM TSA. (←) Arrow indicates elongated cells, (↑) arrow indicates vague morphology cells and (↓) arrow indicates a flattened cell. Bar represents 100 μm.

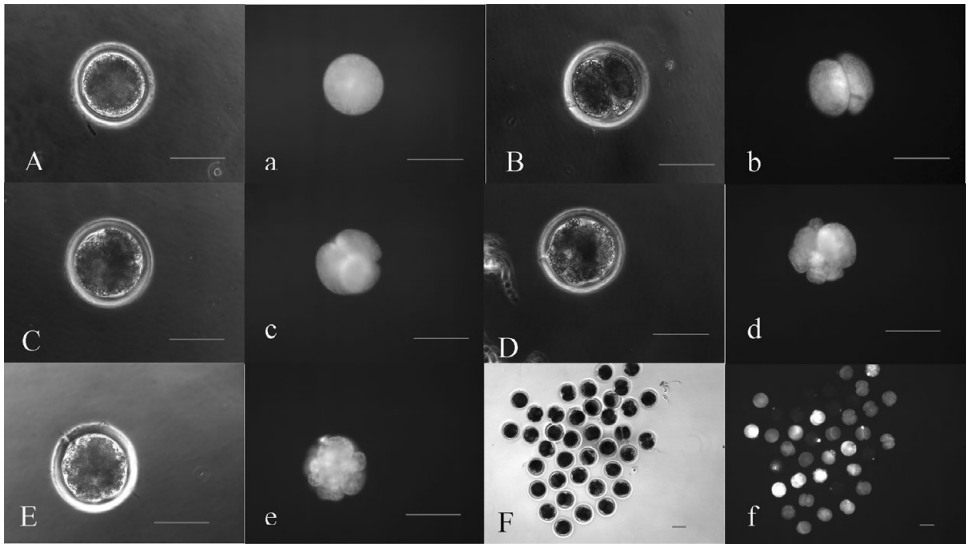

Figure 4 Development of transgenic embryos. (*A–D*), 1-cell stage to 8-cell stage embryos (phase contrast images). (*a–d*), 1-cell stage to 8-cell stage embryos (epifluorescent images). (*E*) Morula (phase contrast images). (*e*) Morula (epifluorescent images). (*F*) SCNT embryos from donor cells transfected with EGFP on day 2 (phase contrast images). (*f*) SCNT embryos from donor cells transfected with EGFP on day 2 (epifluorescent images). Bar represents 100 μm.

group and the percentage of EGFP–positive morula was significantly higher than in the control group. Taken together, the results indicated that transgene silencing in transfected cells could be rescued by TSA treatment during SCNT.

After selection and passaging, stable transfected cells were obtained. With the number of passages increasing, not all transfected cells produced EGFP and the percentage of EGFP-positive cells decreased significantly. In our study, EGFP–positive transfected cells were only 14.8% of the total after seven passages. However, from our results, we can see that treatment of transfected cells with TSA may reactivate *EGFP* gene expression. EGFP expression in the pEGFP-C1

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vector is controlled by cytomegalovirus (CMV), a robust promoter, but many transgenes controlled by viral promoters have been found to be silenced (Verma & Somia, 1997). Although the CMV promoter is a robust expression cassette, it is susceptible to transcriptional inactivation under the influence of several mechanisms, including DNA methylation and histone deacetylation (Löser *et al*., 1998; Meier, 2001). Krishnan confirmed that eight CpG sites within the CMV promoter were regulated by methylation (Krishnan *et al*., 2006). Recent research has shown that TSA can induce genomic DNA demethylation (Ou, 2007). Choi also confirmed that TSA can activate CMV promoter-controlled transgenes (Choi *et al*., 2005). It is probable that TSA, as a histone-deacetylase inhibitor, causes a relaxation of the tight supercoiling of chromatin and enhances accessibility of DNA binding of transcriptional regulatory proteins to promoter regions (Finnin *et al*., 1999). However, TSA is considered too toxic for mammalian cells and the cytotoxicity of these compounds are drawbacks to their use. Our results have shown that the majority of the cells died when the concentration of TSA was over 50 nM and the cells changed morphologically at 24 h after TSA treatment. Due to the cytotoxicity caused by TSA, we treated donor cells with a wide range of TSA concentrations and identified the lowest effective concentration that was capable of rescuing transgene silencing (50 nM).

Results in this study also showed that more cloned embryos derived from TSA-treated transfected cells were EGFP-positive than embryos from the control group. This observation indicates that TSA increases transgene expression, which continues to at least the morula stage. Inhibition of HDAC results in global or regional DNA demethylation in somatic cells (Hu *et al*., 2000). Wee's research demonstrated that the somatic cell nuclei epigenetically altered by treatment with TSA is unyielding to reprogramming during preimplantation development (Wee *et al*., 2007). These findings infer that epigenetic modifications caused by TSA may be maintained and helpful to expression of transgenes in embryos at the morula stage.

In general, histone acetylation is thought to facilitate transcription, which correlates with gene expression including report gene expression. However, as a reversible inhibitor of HDAC, TSA is known to exert a broad and profound impact on cellular traits, including proliferation, apoptosis and differentiation (Li, 2002; Vogelstein & Kinzler, 2004). Thus, their use for rescuing transgene silencing is still limited unless these agents can be designed to specifically target the reversal of a given reporter gene without affecting other genes.

In conclusion, imaging of reporter genes will continue to play an increasingly important role in monitoring the expression of modified gene in SCNT embryos. Although the loss of reporter gene expression poses a big challenge for production of transgenic embryos, we believe that our current pre-SCNT and post-SCNT assay system provides a useful experimental platform to study the mechanisms underlying modified gene silencing in transgenic animals. We have also confirmed the ability of a histone deacetylation inhibitor (TSA) to rescue the silenced *EGFP* gene during SCNT.

Acknowledgements

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