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# The developmental potential of mouse somatic cell nuclear-transferred oocytes treated with trichostatin A and 5-aza-2′-deoxycytidine

*Yuta Tsuji*[2,3], *Yoko Kato*[2,3] *and Yukio Tsunoda*[1]

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## Summary

To facilitate nuclear reprogramming, somatic cells or somatic cell nuclear-transferred (SCNT) oocytes have been treated with the histone deacetylase inhibitor trichostatin A (TSA), or the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), to relax epigenetic marks of differentiated somatic cells. TSA-treated SCNT oocytes have increased developmental potential, but the optimal treatment period is unknown. Reduced methylation levels in somatic cells have no positive effect on SCNT oocytes, but the treatment of SCNT embryos with 5-aza-dC has not been investigated. We examined the effect of TSA treatment duration on the developmental potential of mouse SCNT oocytes and the effect of 5-aza-dC treatment on their *in vitro* and *in vivo* developmental potential. To determine the effects of TSA treatment duration, nuclear-transferred (NT) oocytes were cultured for 0 to 26 h with 100 nM TSA. SCNT oocytes treated with TSA for 8 to 12 h had the higher rate of development to blastocysts and full-term fetuses were obtained after treatment for 8 to 12 h. When oocytes were treated for 14 h and 26 h, blastocyst rates were significantly decreased and fetuses were not obtained. To examine the effect of 5-aza-dC, 2-cell stage SCNT embryos were cultured with 10 or 100 nM 5-aza-dC for 48 h to the morula stage and transferred. The potential of embryos treated with 5-aza-dC to develop into blastocysts was decreased and no fetuses were obtained after transfer. The findings demonstrated that long-term TSA treatment of SCNT mouse oocytes and treatment with 5-aza-dC inhibit the potential to develop into blastocysts and to fetuses after transfer.

Keywords: 5-Aza-dC, Nuclear transfer, Somatic cells, TSA

## Introduction

Somatic cell nuclei introduced into enucleated oocytes are reprogrammed and then somatic cell nuclear-transferred (SCNT) oocytes begin to develop *in vitro* with the same time schedule as fertilized oocytes. In contrast to fertilized embryos developed *in vitro*, however, a small proportion of SCNT mouse embryos develop to normal live young (Wakayama *et al.*, 1998; Rybouchkin *et al.*, 2006; Kishigami *et al.*, 2006a). Although the morphologic appearance of SCNT embryos is not different from that of fertilized embryos, the expression patterns of developmentally important genes differ between SCNT and fertilized embryos (Li *et al.*, 2005, 2006). Such differences are considered to be due to the inadequate reprogramming of somatic cell nuclei.

To facilitate nuclear reprogramming, somatic cells or SCNT oocytes are treated with a histone deacetylase inhibitor to relax the epigenetic marks of differentiated somatic cells (Enright *et al.*, 2003; Shi *et al.*, 2003). Our previous study and another study demonstrated that the potential of SCNT oocytes to develop *in vitro* and *in vivo* is enhanced by the treatment with a histone deacetylase inhibitor, trichostatin A (TSA) (Rybouchkin *et al.*, 2006; Kishigami *et al.*, 2006a; Zhang *et al.*, 2007). Although the effects of the timing and duration of TSA treatment on the potential of SCNT embryos to develop into blastocysts have been examined (Rybouchkin *et al.*,

[1]All correspondence to: Yukio Tsunoda. Laboratory of Animal Reproduction, College of Agriculture, Kinki University, Nara 631-8505, Japan. e-mail: tsunoda@nara.kindai.ac.jp
[2]Laboratory of Animal Reproduction, College of Agriculture, Kinki University, Nara, Japan.
[3]These authors contributed equally to this work.

2006; Kishigami *et al.*, 2006a; Zhang *et al.*, 2007), the optimal treatment duration to enhance the *in vivo* development potential is not known.

DNA methylation is also the key gene expression regulatory mechanism and unmethylated DNA is associated with the active chromatin configuration. TSA treatment could also stimulate DNA demethylation (Cervoni & Szyf, 2001; Geiman & Robertson, 2002; Kishigami *et al.*, 2006a), leading to improved reprogramming. This situation is rather unlikely in the case of SCNT oocytes treated with TSA, however, because of the short treatment period during which there was no DNA replication (Rybouchkin *et al.*, 2006). On the other hand, TSA treatment of mouse SCNT oocytes for a short period (Li *et al.*, 2008) or bovine fibroblast cells for 60 h (Wee *et al.*, 2007) decreases the expression of DNA methyltransferase genes in blastocysts.

Compared with differentiated somatic cells, male and female gametes have relatively low levels of DNA methylation (Oswald *et al.*, 2000; Enright *et al.*, 2003). In the mouse, the male pronucleus is rapidly demethylated after fertilization by an active mechanism and female pronucleus is passively demethylated during cleavage. Global DNA methylation is at its lowest level at the morula stage and *de novo* methylation occurs at the blastocyst stage (Yang *et al.*, 2007). In cloned embryos receiving somatic cells with a higher level of DNA methylation, demethylation occurs to some extent by the morula stage, but there is aberrant hypermethylation (Yang *et al.*, 2007). Attempts to decrease DNA methylation levels in somatic cells prior to nuclear transfer have been made by treating them with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC). The developmental potential of oocytes receiving somatic cells treated with 5-aza-dC, however, is not necessarily increased but decreased due to the toxicity (Jones *et al.*, 2001; Viggnon *et al.*, 2002; Enright *et al.*, 2003, 2005; Shi *et al.*, 2003). To our knowledge, however, there are no reports in which SCNT embryos were treated with 5-aza-dC to mimic their DNA methylation patterns of fertilized embryos.

The present study examined: (1) the effect of TSA treatment duration on the developmental potential of mouse SCNT oocytes; and (2) the effect of TSA and 5-aza-dC treatment on developmental potential both *in vitro* and *in vivo*.

## Materials and methods

All experiments and protocols were performed in strict accordance with the Guiding Principles for the Care and Use of Research Animals adopted by the Kinki University Committee on Animal Research and Bioethics. All chemicals were purchased from Sigma-Aldrich Chemical Co., unless otherwise stated.

### Medium

M2 (Fulton & Whittingham, 1978) and KSOM (Erbach *et al.*, 1994) were used for handling and culturing of embryos, respectively. Stock solutions of cytochalasin B and TSA were dissolved in dimethyl sulphoxide at 1mg/ml and 1 mM and used at 5 $\mu$g/ml and 100 nM, respectively. Stock solutions of 5-aza-dC were dissolved in 50% acetic acid solution.

### Nuclear transfer, embryo culture and embryo transfer

Cumulus cells were isolated from ovulated oocytes from the oviducts of mature F1 (C57BL/6 × DBA) females 14 h after hCG injection. Cells were rinsed with M2 after dispersion with hyaluronidase and used as donor cells (Rybouchkin *et al.*, 2006). Chromosomes at the second metaphase of oocytes recovered from superovulated F1 females 14 h after hCG injection were removed mechanically and enucleated oocytes were used as the recipient cytoplasm (Tsunoda & Kato, 1995). A single cumulus cell was directly injected into the enucleated oocyte cytoplasm using a piezo-electric-actuated micromanipulator (Wakayama *et al.*, 1998;Yabuuchi *et al.*, 2004).

NT oocytes were cultured in KSOM with or without 100 nM TSA for 2 h, and then activated in 10 mM $SrCl_2$ and 5 $\mu$g/ml cytochalasin B supplemented calcium-free KSOM with or without TSA for 6 h, according to Rybouchkin *et al.* (2006). NT oocytes in the 10-, 12-, 14- and 26-h TSA treatment groups were further cultured in KSOM supplemented with 100 nM TSA for 2, 4, 6 and 18 h, respectively. After washing with KSOM, they were cultured in KSOM without TSA for 64 h after the start of activation. They were then transferred into KSOM supplemented with 1:200 stock solution of essential and nonessential amino acids (Invitrogen) and 3.5 mg/ml glucose at 64 h, and further cultured for 32 h.

Embryos that had developed to the blastocyst stage (for experiment 1) or morula stage (for experiment 2) were transferred into oviducts of day 1.0 pseudopregnant (in the afternoon on the day when a vaginal plug was observed) CD-1 strain females. Recipients were killed on day 18.5 (for experiment 1) or day 10.5 (for experiment 2) to examine implantation sites and fetuses.

### Analysis of histone acetylation and DNA methylation

Histone acetylation and DNA methylation levels of SCNT oocytes or embryos at the morula stage were examined, according to previous reports (Rybouchkin

*et al.*, 2006; Kishigami *et al.*, 2006b). Briefly, SCNT oocytes or embryos in five for two independent repeats were fixed overnight at 4 °C in 4% paraformaldehyde. The fixed oocytes or embryos were permeabilized with 0.5% Triton X-100 for 30 min. For histone acetylation analysis, oocytes were incubated with primary rabbit antibodies against acetyl H3 lysine 14 (1:500 of stock, Upstate Biotechnology), acetyl H4 lysine 12 (1:1000 of stock, Upstate Biotechnology). For DNA methylation detection, SCNT morulae were treated with 2N HCl for 30 min, neutralized for 10 min in 100 mM Tris–HCl buffer (pH8.5) and incubated with monoclonal antibody against 5-methylcytosine (Epigentek Inc.) for 1 h.

SCNT oocytes and morulae were stained with secondary goat anti-rabbit fluorescein isothiocyanate-conjugated antibody or goat anti-mouse fluorescein isothiocyanate antibody (Molecular Probes) at 1 : 500 for 1 h. They were then stained with 10 $\mu$g/ml Hoechst 33342 (Wako Chem. Co.) and observed under a Nikon UV Eclipse E800m microscope equipped with UV-1A and F-R filter sets. The level of histone acetylation and DNA methylation were not measured quantitatively, but the nuclear intensities were categorized as high (++), middle (+), low (±) or no (–) expression.

### Experimental design and statistical analysis

*Experiment 1*

To determine the effects of TSA treatment duration on the development of NT embryos into blastocysts and fetuses, two series of experiments were performed. NT oocytes were randomly cultured in two groups (for 0 and 8 h treatment) in the first series or in five groups (for 8, 10, 12, 14 and 26 h treatment) in the second series with 100 nM TSA-supplemented medium.

*Experiment 2*

To examine the effect of 5-aza-dC on the *in vitro* and *in vivo* development of NT embryos, 2-cell embryos developed from SCNT oocytes treated with 100 nM TSA for 8 h were cultured in KSOM supplemented with 10 or 100 nM 5-aza-dC for 48 h to the morula stage, washed and further cultured in KSOM supplemented with amino acid solution and 3.5 mg/ml glucose for 6 h before embryo transfer.

Data were analyzed by the chi-squared test. A $p$ value of less than 0.05 was considered to represent a statistically significant difference.

## Results

### Timing of histone deacetylation in non-activated and activated NT oocytes

The dynamics of deacetylation of lysine 14 of histone H3 (H3K14) and lysine 12 of histone H4 (H4K12) in NT oocytes before and after activation are shown in Table 1. Among all the NT oocytes, there was moderately reduced staining for both acetylated H3K14 and H4K12 1 h after NT, compared with the intensity of staining 15 min after NT. The staining was barely visible 2 h after NT. After parthenogenetic activation, the staining level for acetylated H4K12 gradually increased, but the staining level for H3K14 remained low to moderate 1 to 6 h after activation.

When NT oocytes were cultured for 2 h before activation and for 6 h after activation in 100 nM TSA-supplemented medium, the staining intensity for both acetylated residues was high and somewhat increased from the initial level immediately after NT.

**Table 1** The change of histone acetylation level in SCNT oocytes treated with or without TSA

| | | H3K14 | | H4K12 | |
|---|---|---|---|---|---|
| Time (h) | | Without TSA | With TSA | Without TSA | With TSA |
| Post NT | 0 | + | + | +/++ | +/++ |
| | 1 | ±/+ | +/++ | ±/+ | ++ |
| | 2 | − | +/++ | − | ++ |
| Post activation | 0 | − | ++ | − | ++ |
| | 1 | − | ++ | + | ++ |
| | 2 | −/± | ++ | + | ++ |
| | 3 | −/± | ++ | +/++ | ++ |
| | 4 | −/± | ++ | ++ | ++ |
| | 5 | ± | ++ | ++ | ++ |
| | 6 | ±/+ | ++ | ++ | ++ |

### DNA methylation level in NT embryos

The DNA methylation levels in NT morulae that developed from 2-cell embryos cultured in 5-aza-dC-supplemented medium for 48 h were compared with NT morulae developed from embryos cultured without 5-aza-dC. Irrespective the concentrations of 5-aza-dC, we detected no decrease in the DNA methylation intensity.

### Effect of TSA treatment duration on development of SCNT oocytes

The potential of SCNT oocytes treated with TSA for different periods of time to develop into blastocysts is shown in Table 2. The development rates of NT oocytes from the 2-cell to morula stages were not significantly different among groups. The proportion of oocytes treated with TSA for 8 to 12 h to develop into blastocysts was significantly higher than that of the control group (46 to 56% vs 35%). The blastocyst development rates,

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**Table 2** Effect of TSA treatment duration on the *in vitro* development of NT oocytes

| Treatment duration (h) | No. of oocytes cultured | No. of oocytes developed to (%) | | | |
|---|---|---|---|---|---|
| | | 2-cell | 4–8-cell | Morulae | Blastocyst |
| 0 | 498 | 462 (93) | 346 (69) | 298 (60) | 175 (35)[a,b] |
| 8 | 809 | 730 (90) | 585 (72) | 510 (63) | 376 (46)[c] |
| 8 | 212 | 189 (89) | 160 (75) | 148 (70) | 99 (47)[c] |
| 10 | 120 | 116 (97) | 87 (73) | 75 (63) | 56 (47)[c,e] |
| 12 | 117 | 112 (96) | 89 (76) | 84 (72) | 65 (56)[c] |
| 14 | 99 | 87 (88) | 64 (65) | 55 (56) | 34 (34)[b,e] |
| 26 | 195 | 157 (81) | 113 (58) | 100 (51) | 58 (30)[b,e] |

[a–d]Values with the same letters were not significantly different ($p < 0.05$).

**Table 3** Effect of TSA treatment duration on the *in vivo* development of NT oocytes

| Treatment duration (h) | No. of embryos transferred | No. of recipients | No. of pregnant (%) | No. of implantations (%) | No. of placentas (%) | No. of fetuses on d18.5 (%) |
|---|---|---|---|---|---|---|
| 0 | 82 | 13 | 6 (46) | 13 (16)[a] | 1 (1) | 0 (0) |
| 8 | 236 | 30 | 17 (57) | 76 (32)[b] | 1 (0) | 5 (2) |
| 8 | 92 | 14 | 11 (79) | 59 (64)[c] | 1 (1) | 2 (2) |
| 10 | 52 | 7 | 4 (57) | 16 (31)[b] | 0 (0) | 1 (2) |
| 12 | 62 | 7 | 3 (43) | 20 (32)[b] | 0 (0) | 1 (2) |
| 14 | 31 | 5 | 2 (40) | 8 (26)[a,b] | 0 (0) | 0 (0) |
| 26 | 30 | 4 | 2 (50) | 11 (37)[b] | 0 (0) | 0 (0) |

[a–c] Values with the same letters were not significantly different ($p < 0.05$).

however, decreased when NT oocytes were treated with TSA for 14 h and 26 h (34% and 30%).

Table 3 shows the potential of cloned blastocysts developed from oocytes treated with TSA for different durations to develop into fetuses. The implantation rate in the TSA 8 h treatment group was significantly higher than that of control and also higher than in the TSA 10 to 26 h treatment groups. Fetuses were obtained on day 18.5 when NT oocytes were treated with TSA for 8, 10 and 12 h. There were no differences in the potential to develop into fetuses among groups treated for different durations. Fetuses were not obtained, however, in oocytes treated with TSA for 14 h and 26 h, same as the controls.

### Effect of 5-aza-dC on the development of NT embryos

The potential of NT oocytes treated with 5-aza-dC following TSA treatment to develop *in vitro* and into fetuses is shown in Table 4. The proportions of 2-cell embryos treated with 10 nM 5-aza-dC that developed into the 4-cell and morula stages were not different from controls. When embryos were treated with 100 nM 5-aza-dC, the proportion of embryos developed to morulae significantly decreased. After transfer of cloned morulae to recipients, the implantation rate and fetus development rate on day 10.5 were low in the 5-aza-dC treatment groups.

## Discussion

Our previous study (Rybouchkin *et al.*, 2006) and another study (Kishigami *et al.*, 2006a) demonstrated that the potential of mouse SCNT oocytes to develop *in vitro* and to develop to full term was enhanced by a short-term treatment with the histone deacetylase inhibitor TSA. The beneficial effect of TSA on the development of SCNT oocytes has also been confirmed in porcine (Zhang *et al.*, 2007). We recently reported that TSA treatment of mouse SCNT oocytes decreased the expression of chromatin structure-related and DNA methylation-related genes and increased the expression of *Sox2* and c-*Myc* genes in blastocysts (Li *et al.*, 2008). *Sox2* and c-*Myc* are development-related genes associated with nuclear pluripotency (Takahashi & Yamanaka, 2006) or totipotency (Li *et al.*, 2005, 2006).

In the present study, we examined the effect of TSA treatment duration on development *in vitro* and to full term. We found that the highest rate

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**Table 4** Effect of 5-aza-dC treatment on the development potential of 2-cell embryos derived from NT oocytes treated with TSA for 8 h

| Concentration of 5-aza-dC (nM) | No. of embryos cultured | Developed to (%) | | No. of pregnant/recipients (%) | No. of embryos transferred | No. of implantations (%) | No. of fetuses on d10.5 (%) |
|---|---|---|---|---|---|---|---|
| | | 4–8-cell | Morulae | | | | |
| 0 | 99 | 80 (90) | 68 (69) | 6/6 (100) | 68 | 40 (59)[a] | 9 (13)[a] |
| 10 | 100 | 84 (84) | 69 (69) | 5/6 (83) | 69 | 17 (25)[b] | 1 (1)[b] |
| 0 | 211 | 196 (93) | 173(82)[a] | 4/4 (100) | 43 | 16 (37) | 3 (7) |
| 100 | 235 | 215 (91) | 66 (28)[b] | 6/6 (100) | 43 | 15 (35) | 0 (0) |

[a,b]Significant difference within the same series and same column ($p < 0.05$).

of development of SCNT oocytes to blastocysts was obtained when the oocytes were treated with TSA for 12 h, including 2 h before parthenogenetic activation. When oocytes were treated with TSA for 14 h and 26 h, the blastocyst rates significantly decreased. The development of SCNT oocytes to full term occurred only when SCNT oocytes were treated with TSA for 8, 10 and 12 h. For embryo transfer, the transfer site and the synchronization between embryos and recipients are important. Tsunoda & McLaren (1983) reported that the oviduct is a better site for blastocyst transfer than the uterus for defective embryos, such as half embryos, in the mouse. Because the preliminary study demonstrated that the fetus-development rate in day 10.5 after transfer of SCNT mouse blastocysts to oviducts on day 1.0 pseudo-pregnant females was higher than that after transfer to oviducts on day 0.5 pseudo pregnant females (17.9% vs 2.5%, unpublished observation), we transferred all SCNT blastocysts to oviducts on day 1.0 females.

Cumulus cell nuclei introduced into enucleated oocytes showed rapid histone deacetylation and acetylation of H3K14 and H4K12 was not observed 2 h after nuclear transfer as demonstrated by Kim *et al.* (2003) and Rybouchkin *et al.* (2006). The acetylation level of H4K12 in SCNT oocytes increased 2 h after parthenogenetic activation, even if SCNT oocytes were not treated with TSA and reached the same level as oocytes treated with TSA, however the level of HBK14 in oocytes not treated with TSA was low. These findings suggest that long-term treatment of SCNT mouse oocytes with TSA is not necessary. Considering the low potential of SCNT oocytes to develop into blastocysts (Table 2) and to implant after transfer to recipients (Table 3), long-term treatment with TSA might have adverse effects on the development of SCNT oocytes, as demonstrated by Kishigami *et al.* (2006a). Zhang *et al.* (2007), however, reported that TSA treatment of porcine SCNT oocytes for 24 to 48 h, but not for 10 h, significantly improved the *in vitro* blastocyst production. The optimal duration of TSA treatment might differ among species.

In natural reproduction, male and female gametes have relatively low levels of DNA methylation and the methylation level further declines due to active metylation of the paternal genome and passive methylation of the maternal genome (Oswald *et al.*, 2000; Wade & Kikyo, 2002). Then, *de novo* methylation occurs at the blastocyst stage (Yang *et al.*, 2007). When somatic cells with DNA methylation levels much higher than those of gametes are transferred into enucleated oocytes, reprogramming factor(s) in the oocyte cytoplasm induce DNA demethylation in somatic cell nuclei to some extent but not to the same level as in fertilized embryos. The *de novo* methylation in SCNT mouse embryos might start at the morula stage (Yang *et al.*, 2007). To mimic the fertilized embryo DNA methylation pattern in SCNT embryos, there have been several attempts to decrease methylation levels in somatic cells before nuclear transfer. Jones *et al.* (2001) and Enright *et al.* (2003, 2005) treated bovine somatic cells with the DNA methyltransferase inhibitor 5-aza-dC for several days before use as donor cells for nuclear transfer. Blastocyst rates of SCNT bovine oocytes receiving 5-aza-dC treated somatic cells, however, were not increased compared with those of control SCNT oocytes.

To our knowledge, there are no reports on 5-aza-dC treatment of SCNT embryos. Therefore, we investigated whether treatment of SCNT mouse embryos with 5-aza-dC in addition to TSA has beneficial effects on their *in vitro* and *in vivo* development. Because *de novo* methylation in SCNT mouse embryos seems to occur at the morula stage and not at the blastocyst stage, as in fertilized embryos (Yang *et al.*, 2007), we treated embryos with 5-aza-dC for 48 h from the 2-cell stage, when embryonic genome activation is complete (Flach *et al.*, 1982), to the morula stage. The potential of SCNT embryos treated with 100 nM 5-aza-dC to develop into morulae was significantly lower than that of control embryos and fetuses were not obtained after transfer of 5-aza-dC-treated embryos to recipients. The DNA methylation level of cloned morulae after treatment with 5-aza-dC

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was not changed compared with control embryos. Why we did not observe a decline in the methylation level in the morulae developed from TSA-treated oocytes after 5-aza-dC treatment is not clear, but the most feasible reason is that immunostaining is not a sufficiently sensitive method, as demonstrated in bovine somatic cells (Enright *et al.*, 2005).

Because the preliminary study demonstrated that the potential of fertilized zygotes treated with 100 nM, 500 nM and 5000 nM 5-aza-dC to develop into blastocysts was significantly lower than that of compared with control embryos (15%, 5% and 0% vs 56%), 5-aza-dC is considered to be toxic for preimplantation mouse embryos, which is the same as in somatic cells (Enright *et al.*, 2003; Kumar *et al.*, 2006).

The present study demonstrated that long-term treatment of SCNT mouse oocytes with TSA and treatment of SCNT embryos with TSA and 5-aza-dC inhibits their potential to develop into blastocysts and to fetuses after transfer to recipients.

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