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Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer

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Abstract

The low success rate of animal cloning by somatic cell nuclear transfer (SCNT) is believed to be associated with epigenetic errors including abnormal DNA hypermethylation. Recently, we elucidated by using round spermatids that, after nuclear transfer, treatment of zygotes with trichostatin A (TSA), an inhibitor of histone deacetylase, can remarkably reduce abnormal DNA hypermethylation depending on the origins of transferred nuclei and their genomic regions [S. Kishigami, N. Van Thuan, T. Hikichi, H. Ohta, S. Wakayama. E. Mizutani, T. Wakayama, Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids, Dev. Biol. (2005) in press]. Here, we found that 5–50 nM TSA-treatment for 10 h following oocyte activation resulted in more efficient in vitro development of somatic cloned embryos to the blastocyst stage from 2- to 5-fold depending on the donor cells including tail tip cells, spleen cells, neural stem cells, and cumulus cells. This TSA-treatment also led to more than 5-fold increase in success rate of mouse cloning from cumulus cells without obvious abnormality but failed to improve ES cloning success. Further, we succeeded in establishment of nuclear transfer-embryonic stem (NT-ES) cells from TSA-treated cloned blastocyst at a rate three times higher than those from untreated cloned blastocysts. Thus, our data indicate that TSA-treatment after SCNT in mice can dramatically improve the practical application of current cloning techniques.

Keywords: Mouse; Nuclear transfer; Trichostatin A; NT-ES; Full term development

The success of somatic cell cloning in the mouse gives promise to applications such as species preservation, livestock propagation, and cell therapy for medical treatment by nuclear transfer embryonic stem cells (NT-ESCs) [2–5]. However, mouse cloning by somatic cell nuclear transfer (SCNT) has been inefficient since the first cloned mouse, "Cumulina," was born in 1997 [6]. Although we have tried several new methods, so far none has exerted a marked influence on the efficiency of cloning. These include the methods of oocyte activation [7], timing of oocyte activation [8], inhibition of cytokinesis [8], and timing of enucleation or injection of nucleus [9]. We have previously shown that treatment of dimethyl sulfoxide (DMSO) is an important determinant of cloning efficiency [8], but improvement was minimal.

Even after birth, cloned mice are found to carry a variety of abnormalities including placentomegaly, obesity, and a shorter life span [10–14]. Recent molecular analyses of cloned embryos reveal abnormal epigenetic modifications such as DNA methylation and histone modifications [15–18]. Further, abnormal profiles of gene expression have been found in placentas and livers of clones [19–21]. These abnormal epigenetic modifications and gene expression patterns are likely associated with the overall low success rate of cloning. Therefore, the prevention of epigenetic errors such as DNA hypermethylation has been expected to lead to the improvement of the success rate in animal cloning. Actually, pretreatment of donor cells by 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, before nuclear transfer was found to enhance development of

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cloned embryos [22]. Recently, we found a similar phenomenon of DNA hypermethylation in zygotes following round spermatid injection (ROSI) with the abnormal DNA methylation occurring before the end of the first mitosis [1]. That study also showed that treatment of ROSI zygotes with trichostatin A (TSA) rather than 5-azacytidine results in a significant reduction of DNA methylation level associated with histone modifications. TSA is a histone-deacetylase inhibitor (HDACi), which enhances the pool of acetylated histones [23] and DNA demethylation [24].

Based on our recent findings, in this study we challenged a practical improvement of development of somatic cloned embryos using TSA and succeeded in cloning mice with remarkable efficiency by treating with TSA following nuclear transfer in a concentration- and time-dependent manner.

Materials and methods

Animals. B6D2F1 mice (C57BL/6 × DBA/2) were used to prepare oocyte and somatic-cell (cumulus cell, tail tip cells, and spleen cell) donors. In addition, C57BL/6 and 129B6F1 strains were also used for preparation of donor cells. Surrogate females were ICR females mated with vasectomized males of the same strain. All animals (obtained from SLC, Shizuoka, Japan) were maintained in accordance with the Animal Experiment Hand Book at the Center for Developmental Biology.

Collection of oocytes. Mature oocytes were collected from the oviducts of 8–12-week-old females that had been induced to superovulate with 5 IU PMSG, followed by 5 IU hCG 48 h later. Oocytes were collected from oviducts ≈16 h after hCG injection, placed in Hepes-buffered CZB medium, and treated with 0.1% hyaluronidase until the cumulus cells dispersed. The oocytes were then placed in synthetic oviductal medium enriched with potassium (KSOM) containing NEAA and EAA (KSOM AA; Specialty Media, Phillipsburg, NJ) supplemented with 1 mg/ml BSA, covered with paraffin oil (Nacalai Tesque, Kyoto, Japan), and stored at 37 °C (5% CO_2/air).

Production of cloned embryos by using adult somatic cells. Nuclear transfer was performed as described [6]. Enucleated B6D2F1 oocytes were injected individually with an adult tail-tip fibroblast, cumulus cell, ES cell nuclei, or lymphocytes from spleen [1,11,25,26]. ES cells (B6C3F1) used in this study were established from fertilized blastocysts in our laboratory. After nuclear transfer, the reconstructed oocytes were activated by 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium in the presence of 5 g/ml cytochalasin B and cultured for 4 days in KSOM.

NSC collection and culture. On days 1–4, 129B6F1 males (129 × C57BL/6) were sacrificed and brains were extracted. The subventricular zone of the brain was dissected and minced with fine scissors in phosphate-buffered saline (PBS). The cell suspension was washed twice with DMEM and then the cells were resuspended in culture medium, NeuroCult NSC Basal Medium (StemCell Technologies) containing NeuroCult NSC Proliferation Supplements (StemCell Technologies). NSCs were cultured as neurosphere described previously [27]. Freshly isolated neural cells were cultured in a non-coated dish in NeuroCult NSC Basal Medium supplemented with NeuroCult NSC Proliferation Supplements, 20 ng/ml recombinant human epidermal growth factor (rhEGF). The dishes were incubated on a gyratory shaker in CO2 incubators. After four days of culture, colonies were observed as spheres. For nuclear transfer, spheres were collected and single cell suspension was obtained by pipetting techniques.

Trichostatin A treatment. The preparation and treatment of Trichostatin A (TSA) were described previously [1]. Briefly, TSA was dissolved in DMSO and prepared as a 200-fold concentrated stock solution. These TSA stock solutions were added to the activation or culture media at a 1:200 dilution according to each experimental procedure.

Establishment of NT-ES cell lines. Cloned embryos were produced as described above and, when they reached the blastocyst stage, they were used to establish ntES cell lines as previously described [2–5].

Statistical analyses. The data were basically compared using chi-square test analysis with Yates correction for continuity. The data of body and placenta weights were analyzed by Welch's t test. A value of $P < 0.01$ or 0.05 was considered to be statistically significant.

Results

In vitro development following TSA-treatment after NT of the cumulus cells

Our previous study suggests that abnormal DNA hypermethylation of injected genomes occurs within the time point of 10 h after oocyte activation [1] and can be prevented by culturing in the presence of TSA. Therefore, we first treated reconstructed oocytes after nuclear transfer of cumulus cells with various concentrations of TSA for 10 h following oocyte activation as stated in the experimental scheme shown in Fig. 1. To accurately evaluate the improvement of reprogramming after NT, we calculated the in vitro developmental rates from two-cell stage cloned embryos to blastocysts since development from one- to two-cell stage is often impeded by failure of pronuclear for-

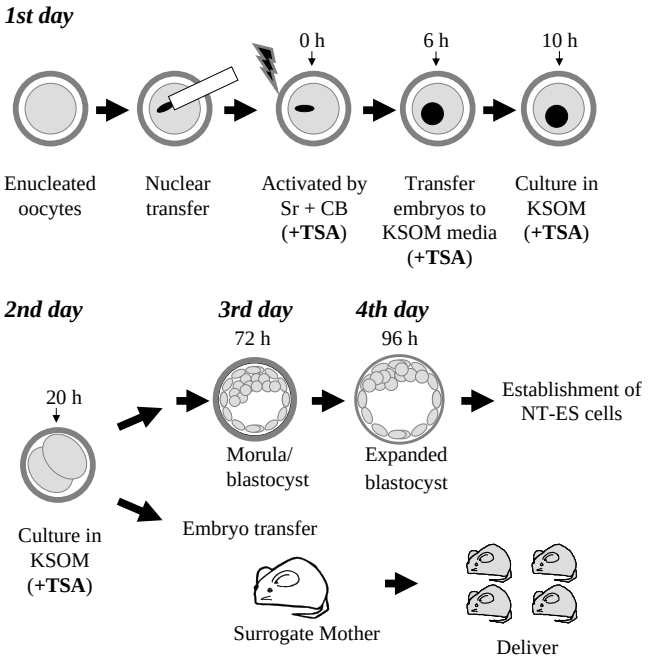

Fig. 1. An experimental scheme of TSA-treatment. According to the standard procedure of mouse cloning [6], donor nuclei from somatic cells were injected into enucleated oocytes. These reconstructed oocytes were activated by culture in the Ca^{2+}-free CZB medium including 5 mM Sr^{2+}, as well as TSA for TSA-treatment, except for the 14 h TSA-treatment used in Fig. 2C. After 6 h activation, the activation medium was changed to KSOM. For 10 h (or 20 h) of TSA-treatment, activated oocytes were cultured in KSOM including TSA for another 4 h (another 14 h) and transferred into KSOM without TSA. These cloned embryos were cultured in KSOM until subjected to embryo transfer into surrogate mother (2nd day), examination of blastocyst formation (3rd or 4th day) or establishment for NT-ES cells (3rd or 4th day).

mation and improper cell-cycle stages of donor cells. At 96 h after oocyte activation, more TSA-treated cloned embryos developed to blastocyst stage depending on TSA concentrations with 5 nM TSA-treated clones showing the highest score, 75% compared with 20% of control embryos (Figs. 2A and B). Next, to optimize the time frame for proper TSA-treatment, we examined different time periods from 6 to 20 h using 5 nM TSA. Interestingly, we found that the 10 h time point for proper TSA treatment is critical for producing the best developmental rates of cloned embryos (Fig. 2C) and that all other time points, both shorter and longer, were less effective in improving developmental rates in cloned embryos. Thus, our results revealed that direct TSA-treatment with proper exposure time and concentration leads to significant improvement of cloned embryonic development.

In vitro development following TSA-treatment after NT of the neural stem cells

To examine the effect of TSA-treatment on other somatic clones, we applied this technique to the cloning of postnatal neural stem cells (NSCs), tail tip fibroblast [25], and lymphocytes from spleen [26]. Cloned oocytes from NSC, tail tip cells and spleen cells, were treated with 5 nM TSA (50 nM TSA for spleen cells) for 10 h following oocyte activation. Normally, less than 10% of NSC cloned embryos develop to morula or blastocyst stage within 72 h after oocyte activation (Fig. 3). However, after TSA-treatment, the success rate increased to nearly 40%. Similarly, in cloned embryos from tail tip and spleen cells, the developmental rates were comparably increased by TSA-treatment from 32% and 22% to 60% and 80%, respectively. Thus, even though the efficiencies of development to the blastocyst stage of those TSA-treated clones varied from one donor type to another, TSA-treatment significantly improved the development of all types of somatic clones examined here.

In vivo development following TSA-treatment after NT of the cumulus cells

To examine whether the TSA-treatment improves the in vivo development of cloned embryos, we transferred TSA-treated two-cell stage embryos cloned from cumulus cells into surrogate mothers. Surprisingly, 5 or 50 nM TSA-treatment for 10 h resulted in more than 6% live offspring (Table 1) and often led to multiple conceptions

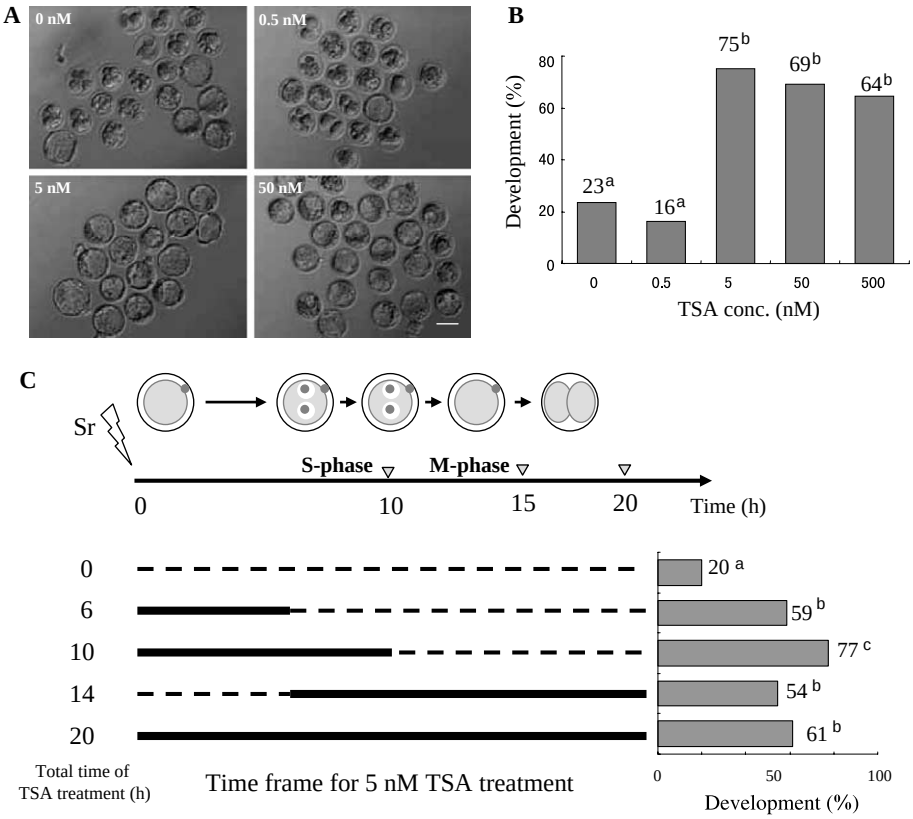

Fig. 2. Effect of TSA-treatment on development of cumulus clones and optimization of exposure time period and concentration of TSA. According to Fig. 1, cloned oocytes from cumulus cells were treated for 10 h with different concentrations of TSA using 0, 0.5, 5, 50, and 500 nM. Ninety-six hours after activation, TSA-treatment increased the number of blastocysts (A). Scale bar = 50 μm (B) The development % was shown as the ratios of (expanded) blastocysts after another 72 h developed from two-cell cloned embryos 24 h after activation ($n > 50$ for each, average = 194). (C) To optimize the exposure time of TSA, 0, 6, 10, 14, and 20 h were used for time period according to Fig. 1 ($n > 50$ for each, average = 98). Fourteen hours TSA-treatment was the same as 20 h treatment, except using the activation medium without TSA. Values with different superscripts are significantly different. $P < 0.05$.

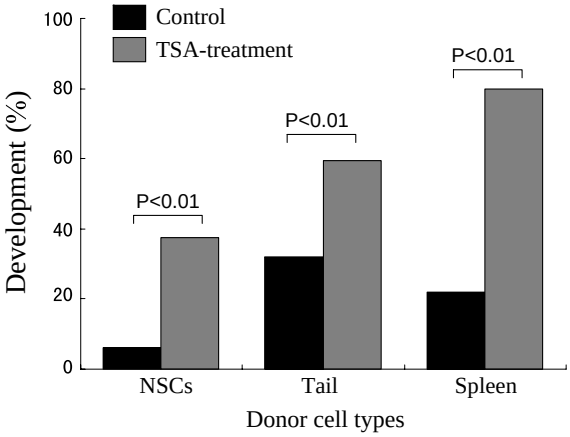

Fig. 3. Effect of TSA-treatment on NSC and tail tip clones. Cloned embryos were treated with 5 nM TSA for 10 h from neural stem cells (NSCs) or tail tip fibroblast cells and with 50 nM TSA for 10 h from spleen cells. The development % was shown as the ratios of blastocysts (or morula) after another 48 h developed from two-cell cloned embryos 24 h after activation. The numbers of examined clones were: 84 (control) and 96 of two-cell embryos for NSC clones, 72 (control) and 79 for tail tip clones, and 87 (control which were cited from our previous data [26]) and 65 for spleen clones, respectively. Regardless of origins of donor cells, TSA-treatment expressed significant improvement for in vitro development of cloned embryos.

(Fig. 4A). These TSA-cloned embryos themselves showed no overt phenotypes except the large placentas also seen in a control clone (Figs. 4B and C), indicating that TSA-treatment cannot rescue the phenotype of placentomegaly in cloned mice. On the other hand, control nuclear transfer without TSA-treatment produced only 1% cloned mice as previously reported (Table 1). However, when higher concentration of or longer exposure time to TSA such as 500 nM and 20 h was used, the success rates of cloning were dramatically reduced (Table 1 and Fig. 2). Further, we examined whether TSA-treatment also raises the success rate of cloning from ES cells, which is already as high as 2–6% [11]. Interestingly, regardless of the high success rate of control ES cloning, we have never obtained pups from TSA-treated ES cloned embryos. Thus, our data suggest that proper TSA-treatment can significantly improve, at least, somatic cloning.

Establishment of NT-ES cells from TSA-treated somatic cloned blastcysts

It has been known that the rates of establishing ES cells from cloned blastocysts (NT-ESCs) are significantly lower than those from fertilized blastocysts although it is relatively better than that of animal cloning [2–6]. Next, we tried to establish the NT-ESCs from TSA-treated cloned blastocysts. Consistent with the improvement of full term development by TSA-treatment, NT-ESCs from TSA-treated cumulus and tail clones were established two to three times more than from control clones (Table 2). Thus, these data suggest that NT-ESCs can be established at higher efficiency after TSA-treatment.

Discussion

In this study, we have examined whether TSA-treatment after SCNT can improve the full-term development of cloned embryos as well as establishment of NT-ES cells, based on our recent findings of the effect of TSA-treatment on DNA methylation. Then, it was found that proper TSA-treatment after SCNT significantly improved the subsequent development of cloned embryos and the establishment of NT-ESCs. Based on this study, the best protocol for TSA-treatment in mice is: (1) reconstructed oocytes should be continuously exposed to TSA from the time point of oocyte activation at least for 10 h but before two-cell stage, (2) TSA concentration of 5–50 nM as is recommended since TSA becomes effective from 5 nM but shows toxicity at 500 nM.

Can TSA-treatment of animal cloning be used for any type of cells? So far significant improvement was observed in cloning from any somatic cells including cumulus, fibroblast, spleen, and neural stem cells. However, we have never succeeded in improving ES-cell cloning but rather, sabotaged development. The genomes of ES cells are

Table 1
Full-term development of TSA-treated clones

Donor cells (mouse strain)	Condition of TSA-treatment (nM/h)	No. recon structed	No. (%) PN formation	No. (%) two-cell embryos	No. embryos transferred	No. (%) live offspring	Av. (g) offspring body wt (SE)	Av. (g) placenta wt (SE)
Cumulus (B6D2F1)	0	317	305 (96)	298 (98)	297	1 (0.3)^{A}	1.76	0.28
	DMSO/10	258	247 (96)	235 (95)	235	1 (0.4)^{A}	1.77	0.36
	5/10	324	322 (99)	307 (95)	287	18 (6.3)^{B}	1.54 (0.02)	0.31 (0.02)^{A}
	50/10	178	174 (98)	170 (98)	170	11 (6.5)^{B}	1.59 (0.05)	0.32 (0.02)^{A}
	500/10	196	193 (98)	185 (96)	185	7 (3.8)^{aB}	1.59 (0.08)	0.41 (0.04)^{C}
	5/20	329	317 (96)	308 (97)	274	9 (3.3)^{B}	1.58 (0.10)	0.28 (0.02)^{A}
	50/20	167	138 (83)	128 (93)	81	2 (2.5)^{A}	2.07 (0.38)	0.42 (0.10)^{A}
ES cells (B6C3F1)	0	261	255 (98)	110 (43)	110	3 (2.7)	1.93 (0.18)	0.54 (0.14)
	5/10	167	154 (92)	66 (43)	66	0 (0)	—	—

Values with different superscripts are significantly different. A versus B $P < 0.01$; A versus C $P < 0.05$.
[a] One pup showed umbilical hernia.

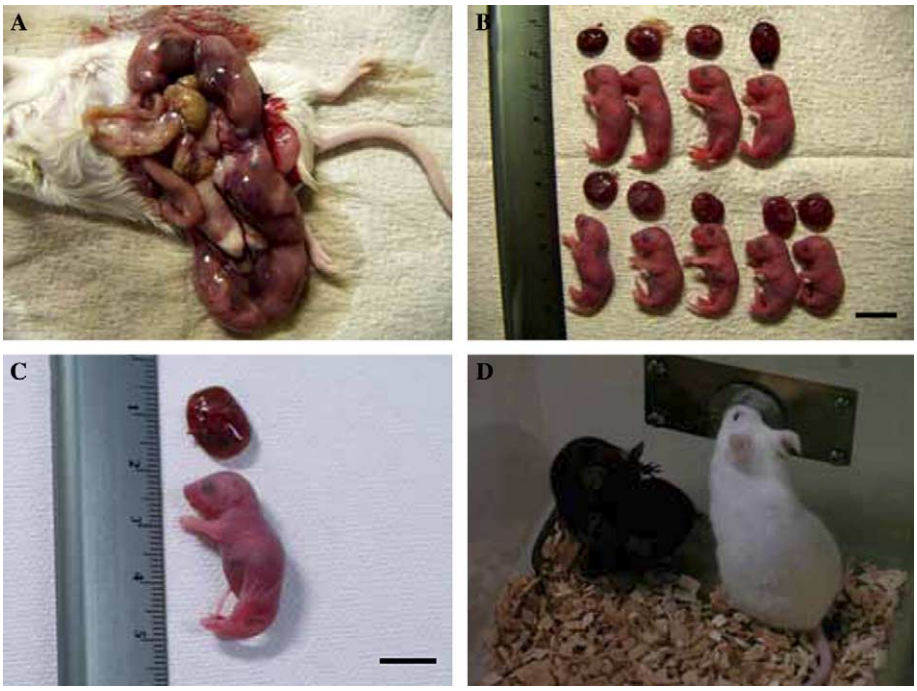

Fig. 4. Production of "normal" cloned mice after TSA-treatment. TSA-treatment often led to multiple conceptions. In one case, five fetuses in one foster were seen at full term (A). Produced cloned mice treated by TSA showed no obvious abnormality with the exception of a large placenta (B) as also seen in "normal" clones (C) without TSA-treatment. Scale bar = 1 cm. After birth, TSA-treated cloned mice were normally fostered and weaned. The first TSA-treated mice from cumulus cells (black) nursed by a foster mother (white) were weaned (D) and fertile. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Table 2
Establishment of NT-ESCs from TSA-treated clones

Donor cells (mouse strain)	Condition of TSA-treatment (nM/h)	No. recon structed	No. PN	No. (%) developed[a]	No. lines established	From PN (%)	From developed embryos (%)
Cumulus cell (B6D2F1)	0	108	107	67 (63)	12	11.2[A]	17.9
	5/10	28	27	23 (85)	10	37.0[B]	43.5
	50/10	34	32	27 (84)	9	28.1	33.3
	500/10	41	40	25 (63)	6	15.0	24.0
Male tail (C57BL/6)	0	205	191	51 (27)	5	2.6	9.8
	5/10	77	55	19 (35)	4	7.3	21.1

Significant χ^2 comparisons A versus B $P < 0.05$.
[a] Embryos developed to the morula or blastocyst stage after 72 h following oocyte activation.

known to be at a reduced DNA methylation state which may be optimal for cloning. Therefore, TSA-treatment in ES cloning may be toxic as is a high dose TSA-treatment in somatic cloning. On the other hand, TSA-treatment after somatic cloning also led to the success rate of 6–7% which is comparable to that of ES cloning (~5%) [11]. Therefore, it may be possible to interpret that TSA-treatment of somatic cloning brings transferred somatic nuclei close to ES-like nuclei.

TSA itself has been known to be teratogenic [28]. Actually, in our study, when higher concentration or longer exposure time for TSA-treatment led to significant reduction of the success rates of cloning, suggesting that an overdose of TSA may cause developmental defects after implantation. Further, TSA-treatment by 500 nM for 10 h or 50 nM for 20 h resulted in more severe placentomegaly (Table 1). Therefore, for the safe use of this new technique, we still have to carefully optimize the concentration and time period for proper TSA-treatment and also examine how normally TSA-treated clones and NT-ES cells develop. Regardless, so far, more than 30 of the TSA-treated cloned mice, of which the oldest ones are at 11 months of age, have been weaned and are currently healthy with no predicted phenotypes such as obesity and shortened life span [12–14]. Further, we confirmed their normal reproductive ability using at least 3 TSA-treated cloned females.

NT-ESCs have been shown to be equivalent to normal ES cells [5]. Nowadays NT-ESCs are attracting more public attention especially for humans in hopes of advancing regenerative medicine [29,30]. However, similarly to animal cloning, the establishing rates of NT-ESCs have been low.

Our study here indicates that a new technique of TSA-treatment can be applied when establishing NT-ESCs. We have already confirmed their ES marker expression patterns including Nanog and Oct4 using 10 lines of NT-ESCs from TSA-treated cloned blastocysts (SK, data not shown). Further, the contribution of at least three of NT-ES lines to germlines was confirmed. Although more thorough examination of TSA-treated cloned mice and NT-ESCs is required, our data strongly suggest we have established a new technique of TSA-treatment that can be practically used for the improvement of both reproductive and therapeutic cloning.

In this study, we mainly focused on the technical aspect rather than the mechanism of how TSA enhances the reprogramming of somatic nuclei during one cell stage. The proper time period for TSA-treatment of clones determined in this study, 10 h after oocyte activation, is apparently consistent with our recent finding that abnormal DNA methylation after ROSI occurs within the specific time point of 10 h after oocyte activation [1]. Therefore, a similar mechanism may underlie enhancement of reprogramming of somatic nuclei and spermatid nuclei after NT by TSA-treatment during the one-cell stage.

Currently TSA is known to be the most potent inhibitor for HDACs [31]. However, in addition to TSA, we also found that Apicidin [32], another HDAC inhibitor, also improved the in vitro development of cloned embryos (SK, data not shown), suggesting that the regulation of histone deacetylases (HDACs) is a clue for efficient reprogramming. Further study should focus how TSA enhances the reprogramming in terms of DNA methylation and histone modifications in order to elucidate the mechanism of reprogramming.

Our findings here provide for a new approach for the practical improvement of mouse cloning techniques and insight into reprogramming of somatic nuclei. Future study should be focused on the health of TSA-treated cloned mice and the molecular mechanism underlying the improvement and more interesting applications of this method for cloning other species.

Acknowledgments

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