

Original

## Effects of Sirtinol on Early Development of the Cloned Murine Embryos

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**Abstract:** Since 1996, when Dolly was born, cloned vertebrates have been produced in many species. However, the success rate of animal cloning has remained very low especially in mice. This low rate is a severe hindrance in the application of animal cloning on various fields of studies. Recently, the treatment of reconstructed embryos with trichostatin A (TSA), one of the inhibitors for classes I and II histone deacetylases (HDACs), has been reported to significantly improve the development of cloned mice. In the present study, we have investigated the effect of sirtinol, an inhibitor for class III HDAC, on the early development of a cloned murine embryos. The embryos, reconstructed with an enucleated oocyte and cumulus cell nucleus, were treated with sirtinol during activation and/or for 12 hr after activation. The rate of blastocyst formation was significantly improved by treatment with 100  $\mu$ M sirtinol for 12 hr after activation. Moreover, six live offspring were born after transfer of the cloned embryos, which were treated with TSA and sirtinol, into surrogate mothers. These results indicate that sirtinol improves the early development of cloned murine embryos without adverse effects on subsequent development.

**Key words:** SCNT (somatic cell nuclear transfer), cloned mouse, early development, HDACi (histone deacetylase inhibitor), sirtinol

### INTRODUCTION

The cloning of vertebrates is carried out by transferring a somatic cell nucleus into an enucleated oocyte. Since 1996, when Dolly, the first cloned vertebrate, was born<sup>1)</sup>, a generation of cloned animals has been reported in several vertebral species including mice, rabbits, horses,

donkeys, pigs, cows, goats, deer, ferrets, cattle and dogs<sup>2)</sup>. Among them, mice have been widely used as laboratory animals for various biological and genetic studies because their generation cycle is relatively short and a large space is not required for breeding. However, there are a few laboratories where the somatic cell nuclear transfer (SCNT) technique and animal cloning are applied to experiments using mice, because the success rate of the SCNT and cloning is still very low in mice although a decade has passed since production of the first cloned mouse, Cumulina, was reported<sup>3,4)</sup>.

Recently, the treatment of reconstructed

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embryos with trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), has been reported to drastically improve the development of the murine cloned embryos<sup>5,6</sup>. HDAC removes acetyl groups from histone tails, causing the DNA to be wrapped more tightly around the histones and interfering with the transcription of genes by blocking access by transcription factors<sup>7,8</sup>. HDAC also deacetylates the non-histone regulatory proteins involved in various cellular functions<sup>7,8</sup>. HDACi inhibits this enzymatic activity resulting in up-regulation of the transcription and/or in modification of function of the regulatory proteins. The improvement of mouse cloning by TSA indicates that the nuclear reprogramming is supported by the acetylated proteins induced by TSA. At least 12 HDACs have been identified and classified into 3 groups: classes I, II and III, depending on the sequence similarity and domain structure<sup>7</sup> (Some researchers define HDAC 11 as class IV because the enzyme has homology to both class I and class II<sup>8</sup>). Moreover, various inhibitors for these HDACs have been reported; inhibitors for classes I and II HDACs include TSA, butyrate and suberic bishydroxamate, and inhibitors for class III HDAC include sirtinol and splitomicin<sup>9-11</sup>. Although little information has been available on differing roles and functions between classes I/II HDACs and class III HDAC in the murine cloned embryo so far, it is possible that the effect of the inhibitor for class III HDAC on the development of murine clone embryos might be different from that of the inhibitor for classes I and II HDACs.

In this context, we studied the effects of the treatment of the reconstructed embryos with sirtinol<sup>11</sup>, an inhibitor for class III HDAC, on the early development of murine cloned embryos generated with the enucleated oocyte and cumulus cell nucleus. The additive effect of

sirtinol on the TSA-treated cloned embryos has been also studied. Furthermore, in order to investigate the effect of the simultaneous treatment of the reconstructed embryos with both compounds on fetal and postnatal development, embryos that were treated by sirtinol and TSA were transferred into pseudopregnant surrogate mothers, and their live offspring were examined.

## MATERIALS AND METHODS

### *Animals*

Mice were purchased from CLEA Japan Inc. (Higashiyama, Tokyo, Japan). Six-week-old female B6D2F1 mice (C57BL/6 × DBA/2, registered as “Jcl:BDF1”), 4-week-old male C57BL/6 mice (registered as “C57BL/6Jcl”), and 6-week-old female and male ICR mice (registered as “Jcl:ICR”) were obtained. The mice were handled according to the Guidelines of the Center for Life Science Research, University of Yamanashi, and the experimental protocol of this study was approved by the Animal Care and Use Committee, University of Yamanashi. The mice were fed food and water *ad libitum* and maintained under 12 hr: 12 hr light-dark cycle.

### *Chemicals and laboratory equipments*

All reagents used in these studies were of the highest grade available. Water was double distilled and autoclaved before use. Sirtinol (Cat. #566320) and TSA (Cat. #647925) were obtained from Calbiochem (San Diego, CA, U.S.A.). Polyvinylpyrrolidone (PVP, 10%) was obtained from IrvineScientific (Cat. #99311, Santa Ana, CA, U.S.A.). Mineral oil was purchased from Purdure Products (Cat. #MIN016, Stamford, CT, U.S.A.). Pregnant mare serum gonadotropin (PMSG, Senotropin<sup>®</sup>) and human chorionic gonadotropin (hCG, Gonat-

ropin<sup>R</sup>) were obtained from Asuka Pharmaceuticals (Shibaura, Tokyo, Japan). BSA (Cat. #A-6003), cytochlasin B (Cat. #C-6762) and the other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

SCNT was carried out using an inverted microscope IX-70 (Olympus, Shinjuku, Tokyo, Japan) and a MO-202ND micromanipulator system (Narishige Minami-Karasuyama, Tokyo, Japan) equipped with a PIEZO drive (Prime Tech Japan, Tsuchiura, Ibaraki, Japan). A glass pipette for enucleation and nuclear transfer was made using a Sutter P97 micropipette puller (Sutter Instruments, Novato, CA, U.S.A.) and a microforge (MF-900, Narishige).

#### *Preparation of oocytes*

8-10-week-old female B6D2F1 mice were superovulated with subcutaneous administration of 5 IU PMSG followed by 5 IU hCG at 48 hr after the PMSG administration. Oocytes collected from oviducts after 14 hr of hCG administration were treated with 0.05% hyaluronidase in modified CZB (Chatot-Tasca-Ziomek) medium (Hepes-CZB, H-CZB)<sup>12)</sup>. Denuded oocytes were transferred into a CZB medium<sup>13)</sup> and stored at 37C under a humidified condition of 5% CO<sub>2</sub>-95% air.

#### *SCNT procedure*

The SCNT was carried out according to the method described in *Ref.14* with some modifications<sup>14)</sup>. The oocyte at metaphase II (MII) was treated with 5 µg/ml cytochlasin B (CB)-1.0% dimethylsulfoxide (DMSO, used as a solvent of CB and HDACis) in H-CZB. The MII chromosome-spindle complex was drawn out using a micropipette (a procedure called "enucleation"). The cumulus cells surrounding the oocytes before hyaluronidase treatment were used as donor cells of the nucleus. The nucleus,

prepared by breaking the plasma membrane of the donor cell in the injection pipette using PIEZO pulses, was injected into the enucleated oocyte to generate the reconstructed embryos. The reconstructed embryos were activated in the activation medium (calcium-free CZB with 10 mM SrCl<sub>2</sub>-5 µg/ml cytochlasin B-1.0% DMSO) for 6 hr. After activation, the embryos were transferred into the CZB medium and incubated.

#### *HDACi treatment (Experiments 1-3)*

In experiment 1, to know the optimal concentration of sirtinol, reconstructed embryos were incubated for 12 hr after activation in CZB containing 100 µM sirtinol-0.4% DMSO (termed the "S100" group), 10 µM sirtinol-0.4% DMSO (the "S10" group), or 0.4 % DMSO (the "S0" group) (Fig. 1A).

In experiment 2, to know the effective treatment period of sirtinol, the embryos were treated with 100 µM sirtinol during activation (6 hr) and/or after activation (12 hr). In the "S-S" group, the embryos were activated in the activation medium containing 100 µM sirtinol-1.4% DMSO and incubated for 12 hr after activation with CZB containing 100 µM sirtinol-0.4% DMSO. In the "S-N" and "N-S" groups, the embryos were treated with sirtinol only during and only after activation, respectively. In the "N-N" group, only DMSO was added in activation buffer and CZB using in incubation of 12 hr after activation (Fig. 2A).

In experiment 3, the embryos were activated with the activation medium containing 5 nM TSA-1.5% DMSO, followed by 12 hr of incubation in the CZB containing 5 nM TSA-0.9% DMSO (the "T-T" group) or 5 nM TSA-100 µM sirtinol-0.9% DMSO (the "T-TS" group) (Fig. 3A).

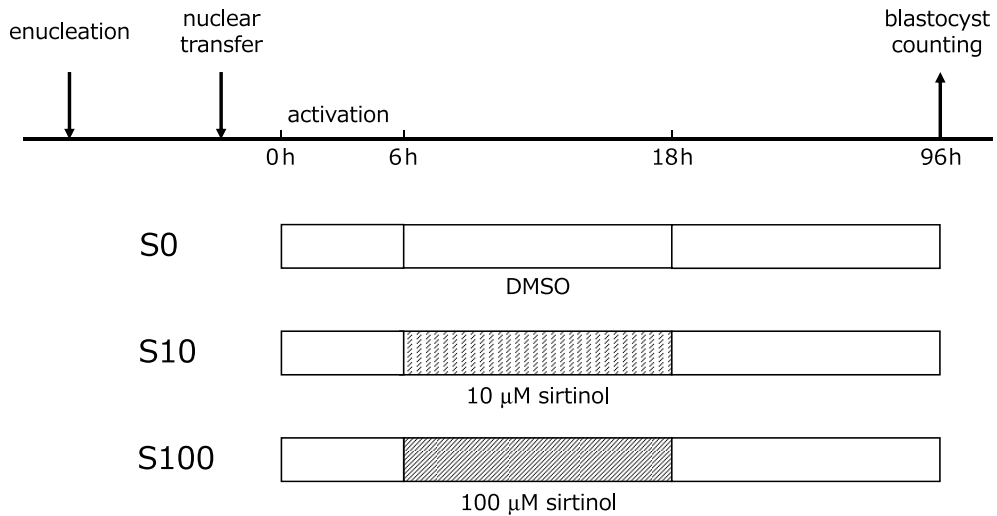


Fig. 1. A: The reconstructed embryos were incubated for 12 hr after activation in CZB containing 100 μM sirtinol-0.4% DMSO (“S100” group), 10 μM sirtinol-0.4% DMSO (“S10” group), or 0.4% DMSO (“S0” group). B: The rate of formation of the total blastocyst (grades I-III) of the “S100” group was significantly higher than that of the “S10” group and “S0” group”.

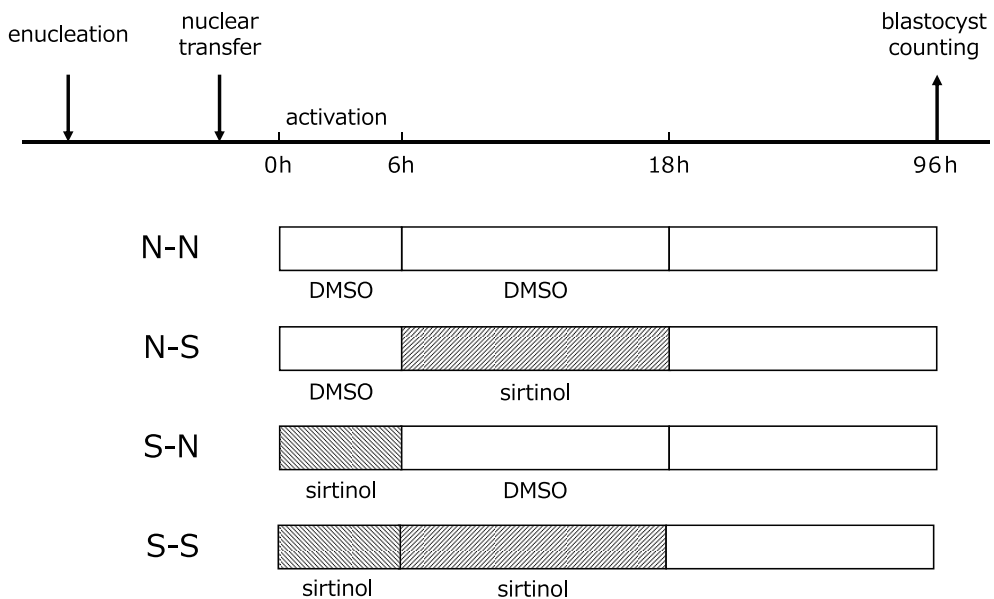


Fig. 2. A: In “S-S” group, the reconstructed embryos were activated in the activation medium containing 100 μM sirtinol-1.4% DMSO and incubated for 12 hr after activation with CZB containing 100 μM sirtinol-0.4% DMSO. In “S-N” and “N-S” groups, the embryos were treated with sirtinol only during and only after activation, respectively. In “N-N” group, only DMSO was activated in the activation medium containing 1.4% DMSO and incubated for 12 hr after activation with CZB containing 0.4% DMSO. B: The rate of formation of the total blastocysts (grades I-III) of “N-S” group was significantly higher than that of “N-N” group and “S-N” group. The rate of “S-S” group was significantly higher than that of “N-N” group and “S-N” group.

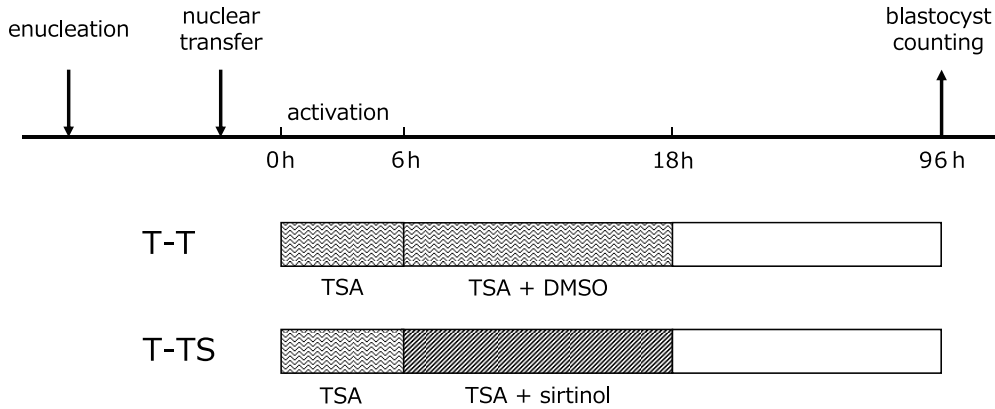


Fig. 3. A: The reconstructed embryos were activated with the activation medium containing 5 nM TSA-1.5% DMSO, followed by 12 hr of incubation in the CZB containing 5 nM TSA-0.9% DMSO (“T-T” group) or 5 nM TSA-100  $\mu$ M sirtinol-0.9% DMSO (“T-TS” group). B: The rate of formation of grades I-III blastocysts of “T-TS” group was not statistically different from that of the “T-T” group. However, the rate of formation of the grades II and III blastocysts of the “T-TS” group was significantly higher than that of the “T-T” group.

#### Evaluation of early embryonal development

We used a grading system for the murine blastocyst defined by Chen T-C *et al.* in this study; in brief, grade I, II and III blastocysts corresponded to non-expanded, and expanded, and hatched blastocysts, respectively<sup>15</sup>. In experiments 1 and 2, the number of total blastocysts (grades I-III) was counted. In experiment 3 the number of blastocysts of grades II and III as well as total blastocysts (grades I-III) were counted.

#### Generation of offspring derived from the “T-TS” treated embryos

A female ICR mouse, used as a surrogate mother, was mated with a vasectomized male ICR mouse. The “T-TS” treated embryos obtained by the method indicated in experiment 3 were transferred into a surrogate mother. The 2-cell stage and blastocyst stage embryos were transferred into the oviduct and uterus of the mother, respectively. The offspring were delivered by cesarean section after 18.5 days of SCNT, and the body weight and placental

weight of the live offspring and were recorded. The newborn cloned mice were transferred to a foster mother of B6D2F1 strain. Moreover, in order to analyze the fertility of the “T-TS” treated embryo-derived cloned mice, two cloned mice that reached adulthood (the cloned mouse No. 3 named “Chuo”, and No. 6 named “Kai”) were mated with C57BL/6 male mice of the same age, respectively, and the pups of the cloned mice were observed.

#### Statistics

The data were compared by *chi*-square test. A value of  $p < 0.05$  or  $0.01$  was judged as statistically significant.

## RESULTS

#### Experiment 1

In experiment 1, the effect of the treatment of the reconstructed embryos with sirtinol for 12 hr after activation on the early development of the cloned embryos was examined. The rate

of formation of the total blastocysts (grades I-III) of the "S100" group (31.4%, n=204) was significantly higher than that of the "S10" group (20.3%, n=207) ( $p < 0.01$ ) and the control "S0 group" (19.1%, n=199) ( $p < 0.01$ ) (Fig. 1B).

#### Experiment 2

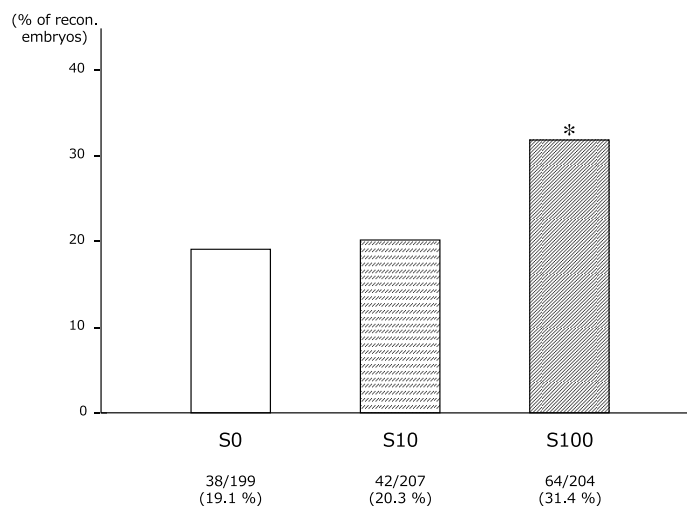
The effect of activation with sirtinol and/or incubation with sirtinol for 12 hr after activation on blastocyst formation of the cloned embryos was examined in experiment 2. The rate of formation of the total blastocysts (grades I-III) of the "N-S" group (30.0%, n=260) was significantly higher than that of the "N-N" group (16.4%, n=261) ( $p < 0.01$ ) and the "S-N" group (18.5%, n=259) ( $p < 0.01$ ). The rate of the "S-S" group (28.0%, n=250) was significantly higher than that of the "N-N" group ( $p < 0.01$ ) and the "S-N" group ( $p < 0.05$ ). No significant differences were observed between the "N-S" group and the "S-S" group, or between the "N-N" group and the "S-N" group (Fig. 2B).

#### Experiment 3

In experiment 3, the additive effect of the treatment of the reconstructed embryos with sirtinol for 12 hr after activation on the early development of the TSA-treated cloned embryos was examined. The rate of formation of grades I-III blastocysts of the "T-TS" group (47.4%, n=270) was not statistically different from that of the "T-T" group (42.8%, n=283). However, when the rate of formation of the grades II and III blastocysts was compared, the rate of the "T-TS" group (39.6%) was significantly higher than that of the "T-T" group (31.1%) ( $p < 0.05$ ) (Fig. 3B).

#### Offspring of the cloned mouse derived from the "T-TS" treated embryos

For generation of offspring derived from the "T-TS" treated embryos, SCNT experiments were carried out 26 times between 06/27/2007 and 09/16/2007. We obtained 2405 reconstructed embryos. 1338 embryos at 2 cell stage and 142 embryos at blastocyst stage were transferred into 40 and 8 surrogate mothers, respec-



\* S100 > S10, S0 ( $p < 0.01$ )

Fig. 1B

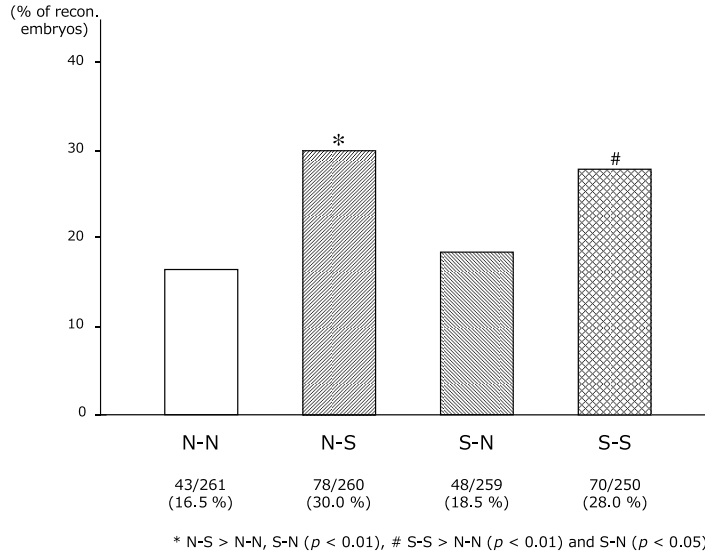


Fig. 2B

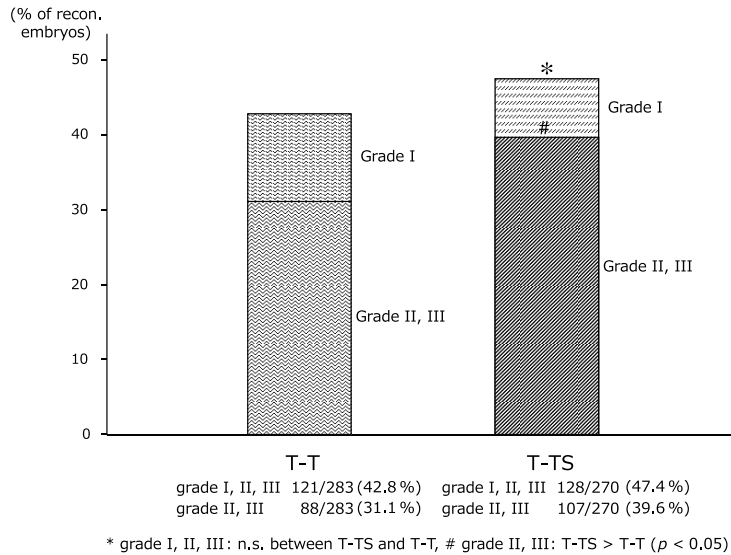


Fig. 3B

tively. Six live born offspring, weighing 1.621 +/- 0.430 g (mean +/- SD), were delivered (Table 1). The weights of the placenta were 0.423 +/- 0.061 g. Of the live born offspring, 2 cloned mice reached adulthood and were named "Chuo" and "Kai". "Chuo" and "Kai"

(cloned mice No. 3 and 6 in Table 1), mated with C57BL/6 male mice, became pregnant and delivered their pups, and could nurse their pups. The first eight pups of "Chuo" (born on 2007/10/29 weighing 1.333 +/- 0.105 g) at 15 days of age were shown in Fig.4.

Table 1. The live born offsprings derived from the “T-TS” treated embryo

No.	DOB	ET	BW	PIW	age	remarks
1	070720	blast	2.476	0.496	(3 weeks)	died, injured by fight
2	070720	blast	1.433	0.409	(2 days)	died, water trouble
3	070828	2 cell	1.490	0.496	39 weeks	alive and well, named “Chuo”
4	070901	2 cell	1.353	0.344	(1 day)	respiratory failure
5	070908	2 cell	1.360	0.410	(1 day)	respiratory failure
6	071005	blast	1.615	0.384	34 weeks	alive and well, named “Kai”

“T-TS” treatment: the embryos were treated with TSA and sirtinol as indicated in methods of the experiment 3.

DOB: date of birth

ET: type of embryonal transfer, 2 cell: the embryos were transferred at the 2 cell stage into the oviduct of the surrogate mother, blast: the embryos were transferred at the blastocyst stage into the uterus of the surrogate mother.

BW: body weight of new born offspring, 1.621 +/- 0.430 g (mean +/- SD).

PIW: placental weight, 0.423 +/- 0.061 g (mean +/- SD).

age: the age on 6/1/2008. The days or weeks franked by the parenthesis indicated the length of life.



Fig. 4. Two of six live born offspring, which were derived from the “T-TS” treated embryo, named “Chuo” and “Kai” were mated with C57BL/6 male mice. They became pregnant and delivered pups, and could nurse their pups. The photo indicates “Chuo” and her first 8 pups born on 10/29/2007 (The photo was taken on 11/14/2007).

## DISCUSSION

Sirtuins are a family of homologues of Sir-2

(Silent mating type Information Regulation-2) that has been identified in yeast. Sir-2 is known as a “lifespan-extending gene” because overex-



pression of the gene induces deacetylation of histones and results in about a 30% extension of lifespan in yeast<sup>16</sup>). Sirtuins remove acetyl groups from histones and other non-histone proteins in the presence of NAD<sup>+</sup>, and are classified as a class III HDAC. Sirtuins have also been identified in mammals including mice. Sirtinol and splitomicin are general inhibitors for sirtuins, class III HDACs, in mammalian cells<sup>10,11</sup>).

Recent studies revealed that treatment of the reconstructed embryos with TSA, an inhibitor for classes I and II HDACs, significantly improves the development of the cloned mice<sup>5</sup>. Because the substrate proteins of class III HDACs are reported to be different from those of classes I and II HDACs<sup>7,8</sup>), the effects of sirtinol on murine reconstructed embryos are also considered different from that of TSA. Therefore, we hypothesized that the simultaneous treatment of the murine cloned embryos with TSA and sirtinol might induce additional improvement of embryonal and fetal development. In order to verify this hypothesis, the present study was performed.

In experiment 1, we examined the effect of the treatment of reconstructed embryos with 100, 10 or 0 (control)  $\mu\text{M}$  sirtinol for 12 hr after activation on early embryonal development (Fig. 1B). The results indicate that sirtinol significantly improves the development of the cloned embryos to the blastocyst stage at the concentration of 100  $\mu\text{M}$  but not at that of 10  $\mu\text{M}$ , indicating that acetylation of the substrate proteins of class III HDACs during 12 hr after activation are involved in the reprogramming of the somatic nucleus. We could not examine the effect of sirtinol at a higher concentration, because it was difficult to dissolve this compound in CZB at 200  $\mu\text{M}$  or higher.

In experiment 2, we studied the effect of the treatment with 100  $\mu\text{M}$  sirtinol during activation

and/or for 12 hr after activation on the early development of a cloned embryos. The results show that the treatment during activation has little or no benefit in contrast to the treatment for 12 hr after activation (Fig. 2B). Therefore, the acetylation of substrate proteins of class III HDACs during activation might not induce nuclear reprogramming. Interestingly, it has been reported that the embryonal development is improved by the treatment with TSA only during activation as well as only during 14 hr after activation<sup>5</sup>), suggesting that the acetylation of the substrate proteins of classes I and II HDACs during activation as well as during 14 hr after activation induce reprogramming. Taken together, it is considered that the acetylated proteins responsible for nuclear reprogramming in the cloned embryos by sirtinol are not the same as those by TSA. Thus, we considered that the treatment with sirtinol after activation could additively improve the development of the TSA-treated cloned embryos. To further investigate this issue, we carried out experiment 3.

In experiment 3, treatment with 100  $\mu\text{M}$  sirtinol for 12 hr after activation in addition to the TSA-treatment ("T-TS" group) did not improve the rate of formation of the total blastocysts (grades I, II and III blastocysts) compared to the TSA-only treated embryos ("T-T" group). However, the rate of formation of the expanded (grade II) blastocysts and the hatched (grade III) blastocysts of the "T-TS" group was significantly higher than that of the "T-T" group (Fig.3B). These results imply that the substrates, which were responsible for nuclear reprogramming, modified after treatment with sirtinol are different from those modified after treatment with TSA, and that the simultaneous treatment of the inhibitor for classes I/II and class III HDAC, TSA and sirtinol, could further improve the success rate of the murine SCNT experi-

ments.

Although the results from experiments 1-3 indicate that sirtinol could improve the early development of murine cloned embryos, it was possible that sirtinol, a general inhibitor for sirtuins, might interfere with the development after the blastocyst stage, because, as mentioned above, sirtuins was thought to have a “lifespan-extending” effect<sup>16)</sup>. In order to investigate this point, we generated offspring derived from the “T-TS” treated embryos. As shown in Table 1, six live offspring were obtained from the embryos, and two of the clones reached adulthood. These two cloned mice, “Chuo” and “Kai”, delivered their pups and could nurse them. These findings indicate that the treatment with sirtinol for 12 hr after activation does not affect embryonal, fetal or postnatal development.

In conclusion, the treatment of the reconstructed embryos with sirtinol for 12 hr after activation improves early embryonal development without adverse effects on subsequent development. This treatment also improves early embryonal development of the TSA-treated cloned embryos, implying that the proteins, which were responsible for nuclear reprogramming, acetylated after the treatment with the inhibitor for class III HDAC would be different from those acetylated after the treatment with the inhibitor for classes I and II in the cloned murine embryo. At the present time, the target substrate proteins of the respective class of the HDACs in the cloned embryo have not been clarified. Since HDACs induce gene transcription<sup>9)</sup>, it is considered that the genes responsible for nuclear reprogramming might be up-regulated by the treatment of HDACs in the reconstructed embryo. However, it is also possible that the HDACs might interact with the non-histone regulatory proteins involved in nuclear reprogramming in the cloned

embryo<sup>9,10)</sup>. Further studies are necessary to clarify the mechanism by which the HDACs improve the development of the cloned murine embryo. Based on such studies, the more improved the murine SCNT technique could be, the more widely the technique would be applied to the various fields of studies in laboratories using mouse as a laboratory animal.

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