Developmental competence of interspecies cloned embryos produced using cells from large Japanese field mice (*Apodemus speciosus*) and oocytes from laboratory mice (*Mus musculus domesticus*)

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Abstract. The large Japanese field mouse (*Apodemus speciosus*) is endemic to Japan and may be used as an animal model for studies related to environmental pollution, medical science, and basic biology. However, the large Japanese field mouse has low reproductive ability due to the small number of oocytes ovulated per female. To produce experimental models, we investigated the in vitro developmental potential of interspecies somatic cell nuclear transfer (iSCNT) embryos produced by fusing tail tip cells from the large Japanese field mouse with enucleated oocytes from laboratory mice (*Mus musculus domesticus*). Only a small number of iSCNT embryos developed to the 4-cell (0–4%) and blastocysts (0–1%) stages under sequential treatment using trichostatin A (TSA) and vitamin C (VC) supplemented with deionized bovine serum albumin (d-BSA). This sequential treatment led to the reduction in H3K9 trimethylation and did not affect H3K4 trimethylation in at least the 2-cell stage of the iSCNT embryos. Moreover, iSCNT embryos that received tail tip cells with exposure treatment to ooplasm from cell fusion to oocyte activation or VC treatment prior to cell fusion did not exhibit significant in vitro development improvement compared to that of each control group. This suggests that large Japanese field mice/laboratory mice iSCNT embryos that received sequential treatment using TSA and VC with d-BSA may have slightly better developmental potential beyond the 4-cell stage. Our results provide insights into the reprogramming barriers impeding the wider implementation of iSCNT technology.

Key words: Epigenetics, Genetic resource, Interspecies somatic cell nuclear transfer, Large Japanese field mouse, Reprogramming

Wild mice, including the large Japanese field mouse (*Apodemus speciosus*, 2 n = 46 or 48), possess the potential to be used for research in the fields of medical science and basic biology [1]. In Japan, there are 23 species of mice across four genera that are native to the area [2]. The large Japanese field mouse, a nocturnal species endemic to Japan, inhabits forests, plantations, dry riverbeds, paddy fields, and cultivated fields throughout Japan, excluding Okinawa and parts of the Nansei Islands [2]. Large Japanese field mice are environmentally responsive to humid and warm islands due to their lifestyle which is well-adapted to the ecological systems on the southern islands of Japan. Furthermore, the large Japanese field mice act as an indicator species for evaluating the environmental effects of radiation damage [3] or for assigning regional characteristics to an area [4]. For these reasons, the large Japanese field mice are a novel resource. However, this species exhibits poor reproductive efficiency due to the small number of ovulated oocytes produced from female mice [5]. Recent techniques involving in vitro fertilization using fresh and cryopreserved sperms are being investigated to address this problem [6]. Thus, development of a more advanced reproductive technology such as somatic cell nuclear transfer (SCNT) could be useful for producing mice with a homogeneous genetic background.

An interspecies SCNT (iSCNT) technology that involves transferring somatic cell nuclei from one species into enucleated oocytes of a different species can directly produce reconstructed embryos and interspecies cloned offspring [7]. This approach is valuable for wild or threatened species, as it allows to maintain the species population when the number of available oocytes is limited. Additionally, the iSCNT method can be used to assist the reproduction of rare species [8] or to revive extinct species [9]. It may also be used to establish
nuclear transfer embryonic stem (ntES) cells derived from the somatic cells of animals from which induced pluripotent stem (iPS) cells are difficult to establish. However, the reconstructed oocytes created using iSCNT often fail to progress through embryonic development after oocyte activation. However, this has met limited success so far in producing live offspring [10, 11]. Therefore, it is important to develop technologies that allow for improving the development of viable iSCNT embryos, particularly in mammalian species in which females produce a small number of ovulated oocytes.

Recently, RNA sequencing (RNA-seq) analysis has demonstrated that the leading causes of poor developmental competence in SCNT embryos are abnormal gene expression of the 2-cell embryo after SCNT due to the maintenance of histone H3 lysine K9 (H3K9) methylation levels [12]. According to this result, an artificial reduction of H3K9 methylation levels in donor cells has been shown to improve the development of SCNT embryos in mouse, bovine, ovine, and porcine models [12–15]. Thus, these results suggest that the fundamental mechanisms and related factors affecting epigenetic modification may be similar among mouse, bovine, and porcine SCNT embryos. Moreover, as iSCNT embryos have undergone partial reprogramming, the expression of the fibroblast-specific gene in the donor cell is more frequently expressed in the iSCNT embryo than in the SCNT embryo [16]. This can result in incomplete reprogramming that stems from developmental arrest prior to embryonic genome activation (ZGA). Interestingly, lysine (K) demethylase (KDM) families that promote ZGA have been shown to be inactivated in iSCNT embryos compared to those in SCNT embryos [17]. Additionally, supplementing culture medium with the histone deacetylating inhibitor trichostatin A (TSA) to alter epigenetic modifications in a cat–cow iSCNT embryo subsequently has been demonstrated to improve its developmental competence [18]. These observations indicate that it is desirable to improve donor cell status and iSCNT method for reprogramming the donor nucleus to give rise to a totipotent embryo. Indeed, treatment of donor cells with inhibitors of DNA and histone methylation improves the developmental potential of black-footed cat/domestic cat iSCNT embryos [19]. More recently, we demonstrated that sequential treatment using TSA and vitamin C (VC), which individually are well known to act as epigenetic modifiers, in the presence of deionized bovine serum albumin (d-BSA) after oocyte activation in reconstructed SCNT oocytes receiving cumulus cells improves the efficiency of embryonic development with a significant reduction of H3K9 trimethylation (H3K9me3) [20, 21]. However, it has not been evaluated if the sequential treatment using TSA and VC with d-BSA can overcome the reprogramming issues faced in iSCNT.

In this study, we examined the in vitro developmental potential of iSCNT embryos that were reconstructed by fusing the tail tip cells from large Japanese field mice with enucleated oocytes from laboratory mice (Mus musculus domesticus, 2 n = 40) under the sequential conditions using TSA and VC with d-BSA. We then investigated if sequentially supplementing TSA and VC with d-BSA to the culture medium leads to a reduction in H3K9me3 and H3K4me3 at the 2-cell stage of the iSCNT embryos. Finally, we examined if the technical method used to expose the donor cells to the ooplasm of oocytes for 3 h prior to activation and the artificial treatment of the donor cells with VC prior to cell fusion possessed the potential to facilitate nuclear reprogramming of the iSCNT embryos. 

Materials and Methods

Animals

B6D2F1 (C57BL/6NCr × DBA/2Cr) mice were purchased from Japan SLC (Hamamatsu, Japan) and mature large Japanese field mice were reared and provided by Toyama Municipal Family Park Zoo. All mice were maintained in light-cycled and air-controlled rooms when performing the SCNT. This study conformed to the Guide for the Care and Use of Laboratory Animals. The protocols were approved according to the guidelines of the Animal Research Committee from Kindai University, Japan (Permit Number: KAAT-31-002).

Establishment and passage of primary cultured cells from tail tip tissues derived from laboratory mice and large Japanese field mice

The primary cell culture using cells from tail tip tissues were performed as described previously [22, 23]. Briefly, the tail tip tissues from B6D2F1 mice (aged 8–10 weeks) and from large Japanese field mice were obtained from anesthetized animals. The cells were incubated in DMEM medium supplemented with 10% fetal bovine serum and 2.5 µg/ml amphotericin B to allow for cell migration at 37°C under 5% CO2 in air. Cells undergoing migration were used for passaged culture or stocked after freezing using a CELLBANKER 1 (Takara Bio, Shiga, Japan; CB011). As donor cells, the tail tip cells derived from laboratory mouse were used at passages 3 to 5, and the tail tip cells derived from large Japanese field mice were used at passages 8 to 14. The preparation of donor cells for SCNT or iSCNT were performed as previously described [20, 21]. Briefly, the cell pellets were resuspended in Hepes-CZB (HCZB) medium supplemented with 6% d-BSA and placed on ice until cell fusion.

Preparation of deionized bovine serum albumin

Stock solutions of d-BSA were prepared as described previously [21]. Briefly, 1.2 g of BSA (Sigma-Aldrich, St. Louis, MO, USA; A3311) was dissolved into 10 ml of sterile endotoxin-free water (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; 196-15645) at room temperature, and approximately 0.12 g of mixed ion-exchange resin beads (Bio-Rad Laboratories, Hercules, CA, USA; 501-X8(D)) were then added under gently stirring.

Collection of oocytes and inter- and intra- somatic cell nuclear transfer

The SCNT procedure was performed as described previously [21]. Briefly, oocytes were collected from the excised oviducts of female B6D2F1 mice (aged 8–10 weeks) that were superovulated using intraperitoneal injections of 7.5 IU of pregnant mare serum gonadotropin (PMSG; ASKA Animal Health, Tokyo, Japan) followed by 7.5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical, Tokyo, Japan) after 48 h. Cumulus-oocyte complexes were recovered into 0.1% hyaluronidase in HCZB medium at 37°C until the cumulus cells dispersed after approximately 10 min. Cumulus-free oocytes were washed and maintained in mKSO medium until further use. The oocytes were completely enucleated with a small amount of cytoplasm in HCZB medium containing 5 µg/ml cytochalasin B (CB). The enucleated oocytes were fused with the donor cells (cumulus and tail tip cells) using the hemagglutinating virus of orthomyxovirus adhesion assay.
Japan envelope (HVJ-E, GenomONE-CF, Ishihara Sangyo Kaisha, Shiga, Japan; CF001). After incubation, cells that fused with oocytes were observed under a microscope and transferred to an activation medium containing 5 mM SrCl2, 2 mM EGTA, 5 µg/ml CB, and 50 nM TSA in KSOM containing 0.3% d-BSA (mKSOM) for 6 h. The iSCNT and SCNT oocytes were observed for formation of pseudo-pronuclei (PPN) and were subsequently treated with TSA for 2 h in mKSOM medium. Next, the oocytes were transferred to mKSOM medium supplemented with VC and incubated for 7 h. The reconstructed embryos were incubated in mKSOM medium at 37°C under 5% CO₂ in air.

Treatment of cultured cells with VC

VC was dissolved in distilled water as a stock solution. The primary cultured cells were incubated in the culture medium from 0 to 72 h at VC concentrations of 0–100 µM prior to cell fusion.

Immunocytochemistry for H3K9me3 and H3K4me3

H3K9me3 signals were detected in cloned embryos at 28 h after oocyte activation and in VC-treated cells using immunocytochemistry as previously described [20]. Briefly, the embryos were fixed in 3.7% paraformaldehyde (PFA) in PBS for 20 min at room temperature. After permeabilization with 0.5% Triton X-100 in PBS for 40 min at room temperature, the samples were blocked in blocking solution (0.01% Tween-20, 1% BSA in PBS) for 1 h at room temperature. They were then incubated with anti-H3K9me3 antibody (final dilution: 1:100, MBL, Nagoya, Japan; MAB10318) at 4°C in blocking solution overnight. After incubation, the samples were washed and reacted with Alexa Fluor 568-labeled Donkey anti-mouse IgG antibody (final dilution: 1:500, Abcam plc, Tokyo, Japan; ab175472) for 1 h at room temperature. For immunocytochemical staining of H3K4me3, the embryos were fixed in 4% PFA in PBS for 5 min. They were then permeabilized with 0.1% Triton X-100 in PBS for 40 min at room temperature. Those samples were blocked in a blocking solution (0.01% Tween-20, 1% BSA in PBS) for 1 h at room temperature. The anti-H3K4me3 antibodies (final dilution: 1:200, Abcam; ab8580) were diluted in blocking solution and incubated at 4°C overnight. After incubation, the samples were washed and allowed to react with Alexa Fluor 488-labeled Donkey anti-rabbit IgG antibody (final dilution: 1:500, Abcam; ab150073) for 1 h at room temperature. All samples were stained with 0.1 µg/ml 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Thermo Fisher Scientific, Tokyo, Japan; D1306) in PBS. Specimens were mounted on glass slides using VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Finally, the fluorescence images of cells were obtained using a fluorescence microscope and associated analysis software (Keyence BZ-X800, BZ-X800 Analyzer).

Statistical analysis

For statistical analysis, we used StatView version 5.0 (SAS Institute, Cary, NC, USA) and Microsoft Excel to performed analysis of variance (ANOVA) with an α level of 0.05 to determine possible statistically significant differences between the means of groups.

Results

Effect of sequential combination treatment using TSA and VC with d-BSA on developmental competence and histone methylation status in iSCNT embryos

Previously, we observed that the embryonic development of mouse SCNT embryos was drastically increased by sequential combination treatment using TSA and VC with d-BSA [20, 21], and a reduction of H3K9me3 in mouse 2-cell SCNT embryos was also induced by this treatment [20]. To elucidate the effect of the sequential combination treatment using TSA and VC with d-BSA on in vitro embryonic development in iSCNT embryos, we produced iSCNT embryos using tail tip cells derived from large Japanese field mice as donor cells and enucleated oocytes from laboratory mice as enucleated recipient oocytes. First, we confirmed that the sequential treatment using TSA and VC with d-BSA after activation improved in vitro embryonic development of SCNT embryos receiving cumulus cells (Table 1) and induced a reduction of H3K9me3, which is a heterochromatin-associated histone mark [24], in the SCNT embryos (Supplementary Figs. 1A and 1B; online only) in agreement with previous observations [20]. Furthermore, under the sequential treatment using TSA and VC with d-BSA, SCNT embryos receiving tail tip cells from laboratory mice as donor cells remarkably increased the rate of embryonic development at the blastocyst stage (untreated vs. treated, 28% vs. 65%; Table 1), These results indicate that the sequential combination treatment using TSA and VC with d-BSA results in reprogramming that occurs irrespective of cell types. Next, we examined the effect of the sequential combination treatment using TSA and VC with

Table 1. Embryonic development of somatic cell nuclear transfer oocytes and interspecies somatic cell nuclear oocytes using enucleated laboratory mouse oocytes

| Group       | Donor cell                   | Treatment                  | No. of oocytes formed pseudo-pronuclei | No. (%) of embryos developed to |
|-------------|------------------------------|----------------------------|----------------------------------------|----------------------------------|
|             | Animals                      | Cell-types                 |                                        | 2-cell | 4-cell | Morula | Blastocysts |
| SCNT (Control) | Laboratory mice             | Cumulus cells              | Untreated                               | 63     | 60 (95) | 37 (59) | 34 (54) | 32 (51) |
|             |                              |                            | Treated with TSA and VC                | 64     | 64 (100)| 61 (95) | 61 (95) | 59 (92) |
| SCNT        | Laboratory mice             | Tail tip cells             | Untreated                               | 83     | 70 (84)| 43 (52) | 31 (37) | 23 (28) |
|             |                              |                            | Treated with TSA and VC                | 26     | 24 (92)| 24 (92) | 23 (89) | 17 (65) |
| iSCNT       | Large Japanese field mice   | Tail tip cells             | Untreated                               | 53     | 34 (64)| 0 (0)   | 0 (0)   | 0 (0)   |
|             |                              |                            | Treated with TSA and VC                | 85     | 79 (93)| 2 (2)   | 2 (2)   | 1 (1)   |

SCNT, Somatic cell nuclear transfer; iSCNT, interspecies somatic cell nuclear transfer; TSA, trichostatin A; VC, vitamin C.
d-BSA on iSCNT embryos. Following this sequential combination treatment, the developmental rate of iSCNT embryos to the 4-cell and the blastocyst stages was slightly increased compared to that of the untreated group (untreated vs. treated, 0% vs. 2% at 4-cell stage, 0% vs. 1% at blastocyst stage; Table 1).

To address the issues with epigenetic reprogramming following SCNT under the sequential treatment using TSA, and VC with d-BSA, we compared the trimethylation status of H3K9 and H3K4, which are associated with active transcription [25], between SCNT and iSCNT embryos. Our results showed that the sequential supplementing of TSA and VC with d-BSA to culture media led to a reduction in H3K9me3 but not in H3K4me3 at the 2-cell stage in iSCNT embryos, while similar demethylation profiles were observed for both H3K9me3 and H3K4me3 in SCNT embryos using both donor cells and enucleated oocytes from laboratory mice irrespective of sequential treatment using TSA and VC with d-BSA. Additionally, any improvement in iSCNT embryonic development did not result from differences in time of exposure and concentration of two small molecules (TSA and VC) during the treatment with d-BSA (Supplementary Figs. 2A and 2B: online only).

Overall, our data suggest that the sequential combination treatment using TSA and VC with d-BSA cannot fully reduce species incompatibility between donor cell and recipient cytoplasm in epigenetic reprogramming in iSCNT embryos.

**Effect of exposure duration of donor cells to ooplasm from cell fusion to oocyte activation on the in vitro embryonic development in iSCNT embryos**

In our study, SCNT and iSCNT oocytes were activated within 1 h after fusion of donor cells with enucleated oocytes, and the donor cells were reprogrammed during this period. Therefore, longer exposure of the donor cells to ooplasm from the enucleated oocytes is expected to improve the embryonic developmental potential in SCNT/iSCNT embryos. Indeed, the donor cell nuclei are traditionally exposed for 1–3 h to the ooplasm prior to oocyte activation in mouse cloning using adult somatic cells (Cumulus, Sertoli, and neuronal cells) [26]. Based on this, we investigated the effect of the exposure period of donor cells to the ooplasm on the developmental ability of iSCNT embryos by comparing 1 and 3 h exposure durations (Fig. 2A). First, we reconfirmed that the rate of embryonic development to blastocysts in SCNT embryos receiving cumulus cells as donor cells exposed for 3 h prior to oocyte activation was higher than that in SCNT embryos from cumulus cells exposed for 1 h (Fig. 2B). Next, we investigated this effect in iSCNT embryos receiving the tail tip cells derived from large Japanese field mice. As a result, in iSCNT from the donor cells exposed to the ooplasm of enucleated oocytes for 3 h, 96% (134/139) of iSCNT oocytes formed PPN and developed to the 2-cell stage, while only 1% (1/139) of iSCNT embryos developed beyond the 4-cell stage in iSCNT (Fig. 2C). These results indicate that the exposure duration of donor cells to the ooplasm of enucleated oocytes prior to activation may not significantly affect the reprogramming of iSCNT embryos from the tail tip cells derived from large Japanese field mice.

**Definition of optimal conditions for reduction of H3K9me3 levels by VC treatment in primary cultured cells from large Japanese field mice**

Artificial manipulation of epigenetic modification in donor cells prior to SCNT to improve its development potential is well-documented [11]. As a preliminary step prior to application for iSCNT, we determined the optimal experimental conditions (time and concentration) for decreasing H3K9me3 levels by VC treatment in primary cultured cells derived from large Japanese field mice (Fig. 3A and D). As a result, the signal intensity of H3K9me3 was gradually decreased in accordance with VC treatment time for 36 h and above, and the lowest signal intensity for H3K9me3 was detected at 72 h after treatment with VC (Fig. 3B and C). As a further experiment, we compared the intensity of H3K9me3 signals in response to various concentrations of VC (0, 10, 25, 50 and 100 µg/ml) at 72 h. The signal intensity of H3K9me3 in the cells treated with 25 µg/ml of VC exhibited the lowest levels at the optimal treatment time selected in our experiment (Fig. 3E and F). These results indicate that optimal conditions for reduction of H3K9me3 levels in primary cultured tail tip cells derived from large Japanese field mice occur at a VC exposure time of 72 h and a VC concentration of 25 µg/ml.

**In vitro development of iSCNT embryos produced using the donor cells treated with VC under the defined experimental conditions**

We next investigated if the optimal experimental conditions as defined in the previous experiments (Figs. 2 and 3) could improve the *in vitro* development of iSCNT embryos produced using tail tip cells derived from large Japanese field mice and enucleated laboratory mouse oocytes. The donor cells were treated with 25 µg/ml of VC for 72 h prior to cell fusion, and those donor cells were exposed to the ooplasm for 3 h until oocyte activation (Fig. 4A). As shown in Fig. 4B, we could not observe any improvement in developmental potential toward the morula and blastocyst stages. These results indicated that more convenient methods focusing on reprogramming of other epigenetic marks, including H3K9me3, must be developed to improve iSCNT efficiency.

**Discussion**

Throughout this study, we observed that none of iSCNT embryos produced using donor cells derived from large Japanese field mice and enucleated oocytes from laboratory mice developed to the 4-cell stage (Table 1), while under the sequential condition using TSA and VC with d-BSA, only a small number of iSCNT embryos exhibited developmental potential to the 4-cell stage (2% [2/85]) in Table 1; 0% [0/37] and 4% [5/139] in Fig. 2C; 2% [2/84] and 2% [5/208] in Fig. 4B) and beyond the 4-cell stage (2% [2/85]) in Table 1; 0% [0/37] and 1% [1/139] in Fig. 2C; 1% [1/84] and 0% [0/208] in Fig. 4B) irrespective of epigenetic status of the donor cells before oocyte activation. This observation indicates that despite limited data in gene expression, large Japanese field mouse/laboratory mouse iSCNT embryos can develop beyond the 2-cell stage that occurs concurrently with major embryonic gene activation (ZGA) in laboratory mice. Previously, it was shown that developmental arrest of SCNT embryos is associated with a failure to activate transcription in the transferred somatic cell...
Fig. 1. Effect of combination treatment using trichostatin A and vitamin C on histone modifications in somatic cell nuclear transfer embryos and interspecies somatic cell nuclear transfer embryos. (A) Schematic diagram of somatic cell nuclear transfer (SCNT) and interspecies somatic cell nuclear transfer (iSCNT) using tail tip cells derived from laboratory mice (*Mus musculus domesticus*) and large Japanese field mice (*Apodemus speciosus*). PCC, premature chromosome condensation; PB1, first polar body; PPN, pseudo-pronuclei; TSA, trichostatin A; VC, vitamin C; Obs, Observation; IC, immunocytochemistry. (B) Immunocytochemistry of H3K9me3 in SCNT and iSCNT embryos. Representative images of embryos stained with anti-H3K9me3 antibody in reconstructed embryos derived from laboratory mouse (upper) and large Japanese field mouse (lower panel). H3K9me3, trimethylation of histone H3 lysine K9; H3K4me3, trimethylation of histone H3 lysine K4. Different letters indicate statistical significances (P < 0.05). (C) Immunocytochemistry of H3K4me3 in SCNT and iSCNT embryos. Representative images of embryos stained with anti-H3K4me3 antibody in reconstructed embryos using tail tip cells derived from laboratory mouse (upper) and large Japanese field mouse (lower panel). All nuclei were stained with DAPI (gray). Merge images show all images combined with DAPI. Ten to twelve 2-cell embryos were examined in each treatment group by three independent repeated experiments. The intensity of fluorescent signals was measured using Image J software. Scale bar = 50 µm.
H3K4me3 acts as a barrier for the reprogramming of pre-implantation embryonic development [25], and the reduction of H3K9me3 creates an epigenetic environment that is permissive for subsequent ZGA [24]. With regard to mouse cloning, the inhibition of H3K9me3 at the 2-cell stage onwards results in these cells becoming blastocysts, thereby demonstrating that the inhibition of H3K9me3 is an important step in epigenetic reprogramming for differentiated cells [12]. However, the questions of how and which epigenetic mechanisms, including gene expression and epigenetic remodeling in iSCNT embryos, remain to be investigated.

Fig. 2. Effect of exposure duration prior to oocyte activation on embryonic development of somatic cell nuclear transfer and interspecies somatic cell nuclear transfer. (A) Brief scheme of two exposure procedures prior to oocyte activation. The donor cells used were cumulus cells from laboratory mice and tail tip cells from large Japanese field mice. PCC, premature chromosome condensation; PB1, first polar body; PPN, pseudo-pronuclei; TSA, trichostatin A; VC, vitamin C. (B) Effects of exposure duration on embryonic development in somatic cell nuclear transfer (SCNT) embryos produced using cumulus cells from laboratory mice. The numbers of examined embryos are shown in each bar. (C) Effects of exposure duration on embryonic development in interspecies somatic cell nuclear transfer (iSCNT) embryos produced using tail tip cells from large Japanese field mice. The numbers of examined embryos are shown in each bar.
Fig. 3. Effect of vitamin C on histone modification in primary cultured cells derived from large Japanese field mice. (A, D) Scheme of the experiment involving the addition of vitamin C to the cell culture medium: treatment concentration of 10 µg/ml (A) and treatment period for 72 h (D). VC, vitamin C. (B, E) Immunofluorescence images of trimethylation of histone H3 lysine K9 (H3K9me3) in untreated and VC-treated cells: treatment concentration of 10 µg/ml (B) and treatment period for 72 h (E). Shown are representative images of tail tip cells derived from large Japanese field mice stained with DAPI (gray) and with anti-H3K9me3 antibody (red) by immunocytochemistry. Merge images show all images combined with DAPI. (C, F) Fluorescent intensity of H3K9me3 in untreated and VC-treated cells: treatment concentration of 10 µg/ml (C) and treatment period for 72 h (F). Fluorescence intensity was detected from more than 100 cells. Different letters indicate statistical significances (P < 0.05).
features are modulated during the reprogramming of donor nuclei remains unknown. In the present study, treated iSCNT embryos that were cultured in the presence of TSA and VC supplemented with d-BSA after oocyte activation exhibited significantly reduced trimethylation levels of H3K9 compared to those in untreated iSCNT embryos; moreover, these levels are similar to those observed in treated SCNT embryos (Fig. 1B). However, the trimethylation levels of H3K4 were not reduced after treatment using TSA and VC with d-BSA in both SCNT and iSCNT embryos (Fig. 1C). Therefore, further studies elucidating the dynamics of the epigenetic landscape during reprogramming in iSCNT embryos will be undertaken in our future experiments.

To our knowledge, this is the first report to demonstrate that iSCNT embryos receiving tail tip cells from large Japanese field mice under sequential treatment using TSA and VC with d-BSA can develop beyond the 4-cell stage. In fact, it has been reported that approximately half of human/mouse iSCNT embryos can develop to the 2-cell stage under the conventional SCNT system but then arrest at the same stage [28]. Therefore, understanding the molecular mechanisms underlying iSCNT reprogramming following sequential treatment using TSA and VC with d-BSA is necessary for further technical improvements of mouse iSCNT, and this more detailed understanding may also provide some insights into technical innovation in mammalian iSCNT.

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