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—Full Paper—

Acetylation Level of Histone H3 in Early Embryonic Stages Affects Subsequent Development of Miniature Pig Somatic Cell Nuclear Transfer Embryos

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Abstract. Successful cloning by somatic cell nuclear transfer (SCNT) requires a reprogramming process in which the epigenetic state of a differentiated donor nucleus must be converted into an embryonic totipotent state. However, this epigenetic reprogramming is incomplete in SCNT embryos, causing low production efficiency. Recently, it has been reported that trichostatin A (TSA), an inhibitor of histone deacetylase, potentially enhances cloning efficiency. The aim of the present study was to optimize the TSA treatment for miniature pig SCNT embryos and investigate the effect of the acetylation level of histone on developmental competence of SCNT embryos. In order to optimize the TSA treatment, we examined the developmental competence of SCNT embryos under various exposure times (0–50 h) and concentrations (0–500 nM). Treatment with 5 nM TSA for 15 and 20 h beginning at the start of activation significantly increased the blastocyst formation rate (34.6 and 32.4 *vs*. 18.2%, respectively) and mean cell number (57.0 ± 2.7 and 56.6 ± 2.7 *vs*. 43.5 ± 2.1, respectively) as compared with the non-treated group (0 h). We then investigated the acetylation levels of histone H3 in SCNT embryos treated with or without TSA (TSA (+) or TSA (–)) as compared with *in vitro*-fertilized (IVF) embryos. The acetylation levels of the TSA (–) SCNT embryos at the pseudo-pronuclear and 2-cell stages were significantly lower than those of the IVF embryos at the same developmental stages. In contrast, the acetylation levels of the TSA (+) SCNT embryos were similar to those of the IVF embryos. There was no difference in the acetylation levels of all groups at the blastocyst stage. Our data therefore suggests that the acetylation level of histone H3 at the pseudo-pronuclear and 2-cell stages is positively correlated with subsequent development of SCNT embryos, which may be an important event for the vital development of SCNT embryos in miniature pigs.

Key words: Embryo development, Histone acetylation, Miniature pig, Nuclear transfer, Trichostatin A

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Although many studies have been performed to improve the developmental competence of miniature pig SCNT embryos [1–4], the overall efficiency remains low. To obtain sufficient developmental competence for SCNT embryos to develop to term, the differentiated cell nucleus should be subjected to epigenetic reprogramming processes including chromatin remodeling and DNA methylation during preimplantation development. However, reprogramming of a differentiated nucleus to an embryonic state is reportedly delayed and incomplete in SCNT embryos [5]. In fact, previous studies have demonstrated that epigenetic modifications such as DNA methylation and histone acetylation are abnormally reprogrammed in SCNT embryos during early development [6, 7]. Incomplete reprogramming of epigenetic modifications causes abnormal gene expression including imprinted genes [8], resulting in deleterious effects on the development of SCNT embryos [9].

Histone acetylation is known as one of the epigenetic modifications [10, 11]. Hyperacetylation of histones increases the access of some transcription factors to nucleosomes [12, 13]. In both yeast and mammalian cells, it has been demonstrated that lysine 18 is a major acetylation site in histone H3 *N*-terminal tails [14, 15]. Previous studies have reported that DNA methylation and histone H3 lysine 18 acetylation are reasonable markers of nuclear reprogramming, correlating with the developmental potential of SCNT embryos [9, 16]. Moreover, increasing the histone acetylation level in donor cells and/or SCNT embryos improves their developmental competence [16–18]. The level of histone acetylation can be increased easily by histone deacetylase inhibitors such as trichostatin A (TSA) [18], scriptaid [19] and sodium butyrate [20]. Furthermore, TSA treatment significantly improves not only the blastocyst rate but also the production efficiency of viable cloned offspring in mice [21, 22]. These findings indicate that alteration of the epigenetic state of a donor nucleus might improve the developmental ability of SCNT embryos. However, there is limited knowledge about the manner in which the developmental competence of SCNT embryos is enhanced by alteration of the epigenetic state in SCNT embryos, such as by histone acetylation, let alone in miniature pigs.

To gain insight into the relationship between histone acetylation and the developmental competence of miniature pig SCNT embryos, we conducted three experiments. First, to determine the

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most suitable TSA concentration and exposure time for miniature SCNT embryos, the preimplantation development of SCNT embryos treated with TSA (0–500 nM) for 0–20 h beginning at from the start of activation was examined. Next, under the optimized TSA treatment, we investigated the pseudo-pronuclear formation rate of SCNT embryos during TSA treatment (0–20 h) to understand the relationship between nuclear state and the positive effect of TSA treatment on developmental competence. Finally, we measured the acetylation levels of histone H3 lysine 18 in *in vitro*-fertilized (IVF), non-treated (TSA (–)) and TSA-treated SCNT (TSA (+)) embryos at the pronuclear or pseudo-pronuclear, 2-cell and blastocyst stages. In this study, we improved the developmental competence of miniature pig SCNT embryos by optimizing the protocol for TSA treatment. Furthermore, we revealed the fluctuation in the acetylation level of histone H3 lysine 18 in miniature pig SCNT embryos treated with TSA. These results provide useful information for improvement of cloning efficiency and elucidation of the reprogramming mechanism in pigs.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation of oocytes

Pig cumulus-oocyte complexes (COCs) were isolated as described by Yamanaka *et al*. [3]. In brief, COCs were aspirated from non-atretic ovarian follicles (3–5 mm in diameter) obtained from prepubertal gilts at a local slaughterhouse. After washing in PB1, about 50 COCs were cultured in a four-well multidish (Nunc, Roskilde, Denmark) containing 500 μl of bovine serum albumin (BSA)-free NCSU-23 medium for 44 h at 38.5 C in a humidified atmosphere of 5% CO_2 in air. The culture medium used for the first 22 h of maturation contained 10 IU/ml of pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Zoki, Tokyo, Japan), 10 IU/ml of human chorionic gonadotropin (hCG; Puberogen, Sankyo, Tokyo, Japan), 0.6 mM cysteine, 5 μg/ml of insulin (Gibco BRL Life Technologies, Grand Island, NY, USA), 20 μM β-mercaptoethanol, 1 mM of 2–0-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP) and 10% (v/v) porcine follicular fluid. For the subsequent 22 h, the COCs were cultured in the same medium without dbcAMP and hormonal supplementation. After cultivating the cells up to maturation, expanded cumulus cells were removed by vortexing in PB1 buffer containing 1 mg/ml of hyaluronidase. The oocytes were observed under a stereomicroscope, and mature oocytes, that is, those containing the first polar body, were selected and used for production of SCNT embryos. The mature oocytes were placed in PB1 until use.

In vitro fertilization of porcine oocytes

After *in vitro* maturation, cumulus-free mature oocytes were washed 3 times with TU medium (113.1 mM NaCl, 3.0 mM KCl, 10.0 mM $CaCl_2 \cdot 2H_2O$, 25.07 mM $NaHCO_3$, 11.0 mM glucose, 5.0 mM Na-pyruvate, 2.0 mM caffeine-benzoate and 1 mg/ml BSA [23]), and 30–40 oocytes were transferred into 100-μl drops of TU medium. Cryopreserved semen was thawed, and the spermatozoa were washed 2 times by centrifugation (at 1000 × *g* for 4 min) in Dulbecco's PBS (Nissui, Tokyo, Japan) supplemented with 1 mg/ml BSA. The spermatozoa were resuspended in the TU medium, and 20–30 μl of this suspension was added to a fertilization drop containing *in vitro*-matured oocytes to yield a final concentration of 7.5×10^6 cells/ml. Coincubation of oocytes with sperm was carried out for 6 h post-insemination. The oocytes were then washed 3 times, and 20–30 oocytes were cultured in 100-μl drops of modified porcine zygote medium (PZM)-5 [24, 25] for 6 days at 38.5 C in 5% CO_2 in air. The rates of cleavage and blastocyst development were assessed on days 2 and 6 of *in vitro* culture, respectively.

Preparation of donor cells

Donor cells were obtained from miniature pig fetuses (Gottingen; Chugai, Suwa, Japan) collected from a pregnant gilt on the 56th day of pregnancy. The cells from a single fetus were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and were passaged 4–9 times. The cells were cultured for 1 week after confluence and then used as donor nuclei.

Nuclear transfer

The oocytes were stained with 5 mg/ml of Hoechst 33342 at 38.5 C for 5–10 min and manually enucleated in PB1 containing 7.5 mg/ml of cytochalasin D. Enucleation was performed by aspirating both the first polar body and adjacent cytoplasm using a beveled pipette driven by a piezo-actuated unit (Prime Tech, Ibaraki, Japan) and confirmed by visualizing the cytoplasm under ultraviolet (UV) light. A single donor cell was injected into the perivitelline space of each oocyte and electrically fused using two direct current pulses of 150 V/mm for 50 μsec in 280 mM of mannitol supplemented with 0.1 mM magnesium sulfate ($MgSO_4$) and 0.01% (v/v) polyvinyl alcohol (PVA). The fused oocytes were cultured in modified PZM-5, which was obtained by adding 4 mg/ml of BSA instead of 3 mg/ml of PVA to PZM-5, for 3 h before activation. For activation, oocytes were incubated in 15 μM ionomycin for 20 min and then in 5 μg/ml of cycloheximide and 2.5 μg/ml of cytochalasin D for 5 h. We then cultured 20–30 embryos in 100 μl of modified PZM-5 separately under mineral oil for 6 days at 38.5 C in an atmosphere of 5% CO_2 in air. The rates of cleavage and blastocyst formation were assessed on Days 2 and 6, respectively. At the end of the culture period, the number of nuclei in all blastocysts was counted under UV light after staining with 5 μg/ml of Hoechst 33342.

Trichostatin A treatment

Preparation and dilution of TSA stock solution was performed according to Kishigami *et al*. [21]. Briefly, TSA was dissolved in dimethyl sulfoxide (DMSO), and the concentrated stock solution was stored at –20 C until use. The TSA stock solutions were added to the activation or culture media at each concentration according to the experimental procedure. Three hours after fusion, reconstructed embryos were randomly distributed and transferred into 100-μl drops of modified PZM-5 supplemented with 15 μM ionomycin and different concentrations of TSA for 20 min; they were

subsequently transferred into drops supplemented with 5 μg/ml of cycloheximide, 2.5 μg/ml of cytochalasin D and different concentrations of TSA for 5 h at 38.5 C in 5% CO_2 with maximum humidity. After repeated washing in modified PZM-5, the reconstructed embryos were further cultured in modified PZM-5 medium containing the same concentration of TSA as before, but without cycloheximide and cytochalasin D, for an additional 5–45 h; following this, the culture medium was changed to modified PZM-5 without TSA.

Assessment of the nuclear state of embryos

To assess the nuclear state, embryos were fixed for 48 h in 25% (v/v) acetic acid in ethanol at differing time points, 0, 5, 10, 15 and 20 h beginning at the start of the activation treatment, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and then examined for pseudo-pronuclear formation under a phase-contrast microscope.

Confocal microscopic analysis of histone acetylation

An immunofluorescence analysis was performed to examine histone acetylation. Briefly, embryos were fixed in 4% (v/v) paraformaldehyde (PFA) for 2 h at room temperature. After fixation, the embryos were washed for 1 h in PBS with 0.1% (v/v) Tween-20 and then permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min at 4 C. The embryos were then washed three times (10 min per wash) and blocked in PBS containing 2% (w/v) BSA. They were then incubated with acetyl-histone H3 lysine 18 primary antibody (rabbit polyclonal antibody against histone H3 acetyl lysine 18, 1:100 dilution; Cell Signaling Technology, Beverly, MA, USA) for 1 h at 37 C. After washing in PBS with 0.1% (v/v) Tween-20 for 3 h, the embryos were labeled with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (goat anti-rabbit IgG FITC-conjugated antibody, 1:200 dilution; Southern Biotech, Birmingham, AL, USA) for 30 min at 37 C. After washing three times in PBS, the DNA was counterstained with 10 μg/ml of propidium iodide (PI) for 30 min. The embryos were mounted on glass slides with a drop of mounting medium and examined with a Bio-Rad MRC-1024 confocal microscope (Hercules, CA, USA) equipped with a 40× objective. Images were obtained with the same exposure times and adjustments of the microscope. Fluorescence was measured by analysis of the embryo pictures with the LaserSharp Processing software (Bio-Rad, Hercules, CA, USA). The mean pixel intensity values were measured in five different random regions of each nucleoplasm excluding nucleolar regions. In IVF embryos at the pronuclear stage, the mean pixel intensity values for both male and female pronuclei were recorded. Autofluorescence of primary antibodies and non-specific binding of secondary antibodies were verified as negative controls. To quantify fluorescence intensity, the values of SCNT embryos were presented relative to the mean value of IVF embryos.

Experimental design

The experiments were designed to study the effects of TSA treatment on *in vitro* development and acetylation level of histone in miniature pig SCNT embryos.

Experiment 1: We evaluated the effect of TSA treatment beginning at the start of activation with different exposure times and concentrations on *in vitro* development and mean cell number of blastocysts in SCNT embryos. To determine the optimal exposure time, the embryos were cultured in modified PZM-5 supplemented with 5 nM TSA for 0, 5, 10, 15, 20 or 50 h from the start of activation, and the developmental rates of each group were compared. To determine the optimal concentration, the embryos were cultured in modified PZM-5 supplemented with 0, 5, 50 or 500 nM TSA for 15 h, and the developmental rates of each group were compared.

Experiment 2: We investigated the nuclear state of SCNT embryos during TSA treatment. Based on the results of Experiment 1, we examined the nuclear state of SCNT embryos for the period (0–20 h) when TSA treatment was effective in improving embryo development.

Experiment 3: The effect of TSA treatment beginning at the start of activation on the acetylation level of histone H3 lysine 18 in SCNT embryos was studied. Based on the results of Experiment 1, SCNT embryos were cultured in modified PZM-5 supplemented with 5-nM TSA for 15 h from the start of activation. Embryos were collected at different embryonic stages, i.e, the pronuclear and pseudo-nuclear stage at 15 h from insemination or activation; 2-cell stage (35–40 h) and blastocyst stage (135–140 h). The acetylation level of histone H3 lysine 18 was analyzed by measuring the immunofluorescence intensity and comparing the values among the IVF, non-treated (TSA (–)) and TSA-treated SCNT (TSA (+)) embryos.

Statistical analysis

All data were obtained from at least four replicates. Developmental rates were analysed by chi-square tests ($P<0.05$). Other data were analyzed by analysis of variance (ANOVA) and then Fisher's protected least significant difference ($P<0.05$).

Results

Optimization of TSA treatment for miniature pig SCNT embryos

We examined the effect of TSA treatment on the *in vitro* development of SCNT embryos. For comparison of the exposure times of TSA treatment, SCNT embryos were cultured in modified PZM-5 supplemented with 5 nM TSA for 0, 5, 10, 15, 20 and 50 h from the start of activation (Table 1). The rates of blastocyst formation and mean cell numbers were significantly higher ($P<0.05$) in the groups treated for 15 and 20 h compared with the group treated for 0 h (34.6 and 32.4 *vs*. 18.2% and 57.0 ± 2.7 and 56.6 ± 2.7 *vs*. 43.5 ± 2.1, respectively; Table 1). Next, in order to determine the optimized TSA concentration in the treatment medium, SCNT embryos were cultured in modified PZM-5 supplemented with 0, 5, 50 or 500 nM TSA for 15 h (Table 2). The rates of blastocyst formation and mean cell numbers were significantly higher ($P<0.05$) in the 5 nM treated group compared with the 0 nM group (35.4 *vs*. 17.0% and 56.7 ± 3.2 *vs*. 41.4 ± 2.6, respectively; Table 2). On the basis of these results, we determined the optimized conditions of TSA treatment; namely, treatment with 5 nM TSA for 15–20 h notably enhanced the developmental competence of SCNT embryos. Thus, the following experiments were performed using this optimized TSA treatment (concentration, 5 nM; exposure time, 15 h).

Table 1. Effect of TSA treatment time on *in vitro* development of SCNT embryos

TSA treatment time (h)	No. of embryos	No. of ≥ 2-cell embryos (%)	No. of blastocysts (%)	No. of cells/blastocyst (mean ± SE)
0	165	112 (67.9)	30 (18.2)[a]	43.5 ± 2.1[a]
5	129	96 (74.4)	24 (18.6)[a]	43.3 ± 3.0[a]
10	76	54 (71.1)	16 (21.1)[a,b]	47.8 ± 4.6[a,b]
15	104	83 (79.8)	36 (34.6)[b]	57.0 ± 2.7[b]
20	139	103 (74.1)	45 (32.4)[b]	56.6 ± 2.7[b]
50	86	65 (75.6)	23 (26.7)[a,b]	44.8 ± 3.4[a]

TSA, trichostatin A; SCNT, somatic cell nuclear transfer. [a,b] Values with different superscripts within each column are significantly different (P<0.05).

Table 2. Effect of TSA concentration on *in vitro* development of SCNT embryos

TSA concentration (nM)	No. of embryos	No. of 2-cell embryos (%)	No. of blastocysts (%)	No. of cells/blastocyst (mean ± SE)
0	112	71 (63.4)	19 (17.0)[a]	41.4 ± 2.6[a]
5	96	72 (75.0)	34 (35.4)[b]	56.7 ± 3.2[b]
50	76	55 (72.4)	17 (22.4)[a,b]	44.2 ± 4.2[a]
500	104	76 (73.1)	24 (23.1)[a,b]	45.7 ± 2.9[a]

TSA, trichostatin A; SCNT, somatic cell nuclear transfer. [a,b] Values with different superscripts within each column are significantly different (P<0.05).

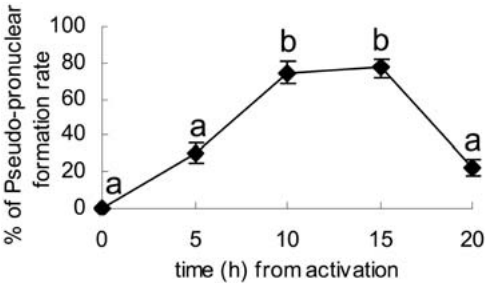

Fig. 1. Transition rate of pseudo-pronuclear formation in SCNT embryos. The nuclear status of SCNT embryos was examined for each period ranging from 0 to 20 h from the start of activation; a and b indicate values that are significantly different (P<0.05).

Duration of TSA treatment required for improving the development of SCNT embryos

As described above, we determined the optimized exposure time for TSA treatment, 15–20 h. Furthermore, to explore whether the state of the donor nucleus during TSA treatment is connected with the positive effect of TSA treatment on development of SCNT embryos, we observed pseudo-pronuclear formation in SCNT embryos at 0, 5, 10, 15 and 20 h after the start of activation (Fig. 1). The rate of embryos at the pseudo-pronuclear stage was 30.0 ± 5.5% at 5 h and reached the peak at between 10 and 15 h (74.7 ± 6.5 and 77.6 ± 4.8%). At 20 h, most SCNT embryos entered mitosis, and the rate of embryos at the pseudo-pronuclear stage was only 22.6 ± 4.3%. Hence, these results, together with the developmental data (Table 1), indicated that treating SCNT embryos with 5 nM TSA until the pseudo-pronuclear stage was beneficial to improvement of the developmental competence to the blastocyst stage.

Fluctuation in acetylation level of histone H3 lysine 18 in SCNT embryos treated with TSA during preimplantation embryogenesis

Next, we compared the acetylation levels of histone H3 lysine 18 in TSA (+) and TSA (–) SCNT embryos with those in IVF embryos at the pseudo-pronuclear or pronuclear, 2-cell and blastocyst stages. As shown in Fig. 2A and 2B, the acetylation levels of histone H3 lysine 18 in the TSA (–) SCNT embryos at the pseudo-pronuclear and 2-cell stages were apparently lower than those in the other embryos, the TSA (+) SCNT and IVF embryos. However, at the blastocyst stage, no difference in the histone H3 acetylation level was observed among the groups (Fig. 2C). This is in the agreement with the results of the immunofluorescence analysis (Fig. 2D–F) and suggests that the histone H3 acetylation levels enhanced by TSA treatment at the pseudo-pronuclear and 2-cell stages are responsible for the improved of developmental competence of the SCNT embryos.

Discussion

Because of many similarities with humans, pig cloning by SCNT may also prove useful in many biomedical applications [26]. However, the production efficiency of normal offspring remains low. Particularly, in pigs, a large number of embryos with high quality is needed to produce cloned offspring, because pigs have a unique reproductive feature necessitating several embryos are needed to establish and maintain pregnancy [27, 28]. On the other

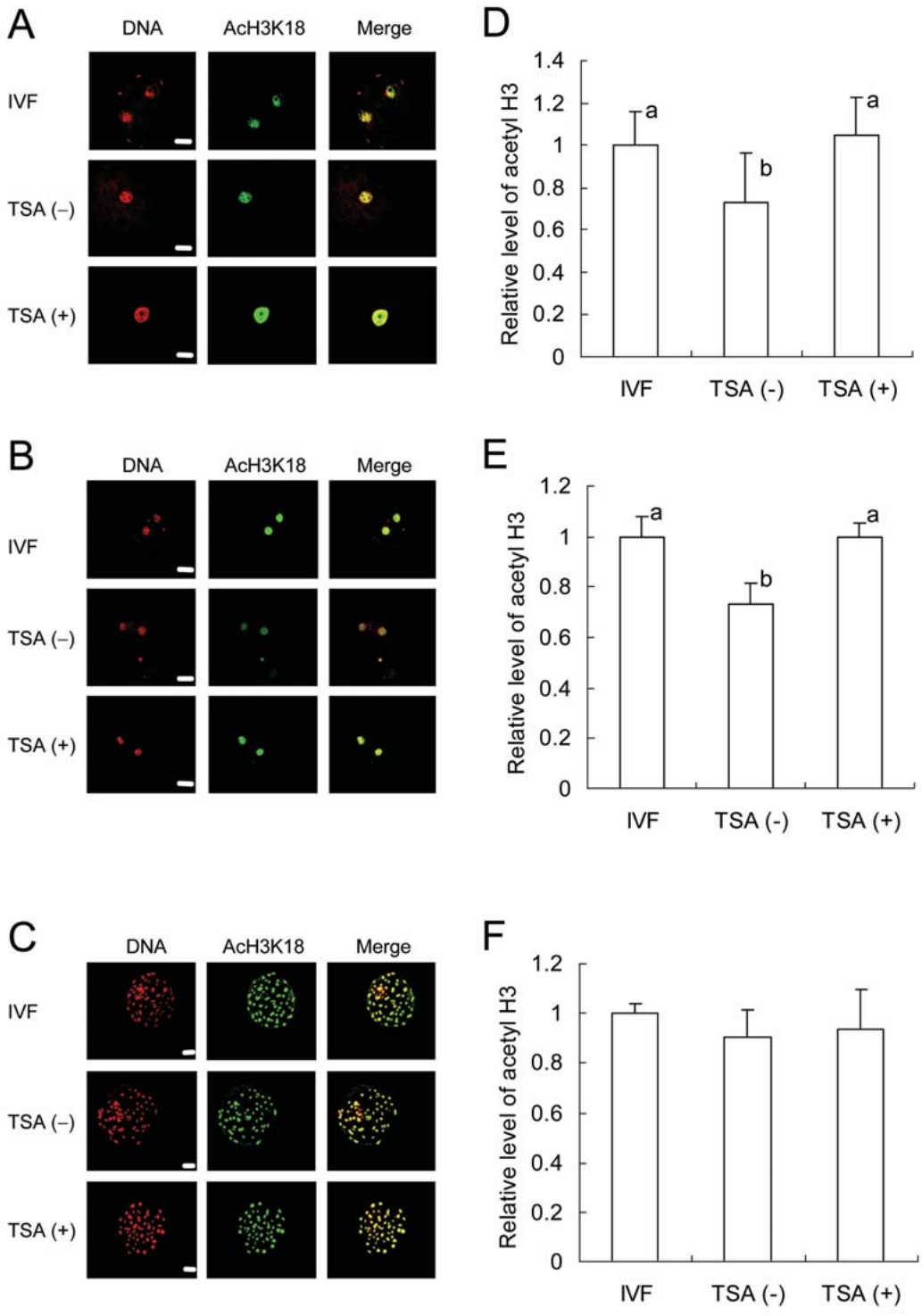

Fig. 2. Confocal micrographs and immunolabeling intensity (mean ± SE) of acetyl histone H3 lysine 18 in IVF and SCNT embryos at the pronuclear or pseudo-pronuclear (A and D), 2-cell (B and E) and blastocyst (C and F) stages. The embryos were labeled for acetyl histone H3 (lysine 18; green) and DNA (red). The merged images of labeled histone H3 and DNA appear yellow. Scale bars=30 μm. Labeling intensity was expressed relative to that of the IVF embryos (arbitrarily set as 100%). TSA (–), non-treated SCNT embryo; TSA (+), trichostatin A-treated SCNT embryo. At least 11 embryos per groups were analyzed in three replicates; a and b indicate values that are significantly different ($P<0.05$).

hand, Suzuki *et al*. [25] recently reported the production of piglets from *in vitro* produced blastocysts by non-surgical embryo transfer into surrogate mothers. Non-surgical embryo transfer is a more effective and economical method compared with surgical embryo transfer, which is mainly used for pigs. Therefore, if SCNT blastocysts with higher quality could be produced *in vitro*, it may also be possible to produce cloned piglets by non-surgical embryo transfer.

In the present study, to improve the developmental competence of miniature pig SCNT embryos, we determined the optimal conditions for TSA treatment and demonstrated that TSA treatment significantly increased acetylation of histone H3 in the early stages and the developmental ability of SCNT embryos. Furthermore, we demonstrated, for the first time, that the important factor for *in vitro* development of miniature pig SCNT embryos is the acetylation levels at the pseudo-pronuclear to 2-cell stages, not at the blastocyst stage. These findings indicate that a higher level of histone acetylation at the pseudo-pronuclear to 2-cell stages may promote reprogramming of the transferred donor nucleus and subsequently enhance the developmental competence of SCNT embryos.

In Experiment 1, we determined the optimal conditions for TSA treatment in miniature pig SCNT embryos. TSA (5, 50 and 500 nM) was used to treat miniature pig SCNT embryos for 5, 10, 15, 20 and 50 h from the start of activation; TSA treatment at 5 nM for 15 and 20 h from the start of activation significantly increased the blastocyst rate and mean cell number in SCNT embryos (Tables 1 and 2). This result is consistent with previous studies reporting improvement of developmental competence in SCNT embryos by TSA treatment [16, 21, 29]. In addition, because hyperacetylation of histones leads to active gene expression [13] and DNA replication [30], it may have contributed to increase in mean cell number in the present study. The optimal conditions determined in this study included a shorter exposure time and lower concentration of TSA compared with the previous studies [16, 31]. This difference might be due to the difference in nuclear transfer protocol and/or culture medium because previous studies have reported that the nuclear transfer protocol [32] and culture medium [33] affect the gene expression of embryos, including DNA methyltransferases, which are related to epigenetic modification. Thus, a difference in expression of epigenetic modification related genes might affect the histone acetylation status in NT embryos, causing a difference in the optimal treatment. Taken together, the optimal TSA treatment was effective for improvement of developmental competence in SCNT embryos.

In Experiment 2, to examine whether the nuclear state during TSA treatment is related to the positive effect of TSA treatment on development of SCNT embryos, we investigated the nuclear stage of SCNT embryos at 0, 5, 10, 15 and 20 h after the start of activation. Pseudo- pronuclear formation began to be observed at 5 h and reached the peak at 10–15 h; almost all embryos proceeded to mitosis at 20 h (Fig. 1). These results, together with the data from Experiment 1, indicate that it may be important to treat SCNT embryos with TSA at the pseudo-pronuclear stage in order to increase the developmental competence. After fertilization, the embryo immediately undergoes epigenetic reprogramming for development to term. For instance, the paternal genome undergoes rapid active demethylation within a few hours of fertilization [34]. Histone also undergoes reacetylation at the pronuclear stage following drastic deacetylation at meiotic metaphase [35, 36]. Moreover, a previous study suggested that major events of epigenetic reprogramming during the pronuclear stage are conserved among mammalian species [37]. These findings showed the impotency of epigenetic reprogramming at an early stage for subsequent embryonic development. In fact, round spermatid-injected oocytes showed aberrant epigenetic reprogramming at the pronuclear stage, which causes lower developmental competence to term compared with spermatid-injected oocytes [38, 39]. These findings, combined with our data, suggest that TSA treatment might prevent the aberrant epigenetic reprogramming of the SCNT embryo at the early stages and increase the developmental competence of SCNT embryos.

Next, we investigated the acetylation level of histone H3 in SCNT embryos. Histone acetylation is one of the epigenetic markers that in donor cells [17, 18] and SCNT embryos [16, 29], affect the subsequent development of produced SCNT embryos. Therefore, we also analyzed the acetylation level of histone H3 lysine 18 as an indicator of epigenetic reprogramming; the acetylation levels of histone H3 lysine 18 in the TSA (+) group at the pseudo-pronuclear and 2-cell stages were significantly higher than those of the TSA (–) SCNT embryos but were similar to those of the IVF embryos (Fig. 2). Although the detailed mechanism of improved developmental competence of SCNT embryos with TSA treatment is unclear, hyperacetylation of histones facilitates access of some factors to nucleosomes [12, 13]. Therefore, one of the reasons for the improved developmental competence of SCNT embryos with TSA treatment may be the access of reprogramming-related factors to nucleosomes. In addition, it has been shown that TSA treatment induces not only histone acetylation but also DNA demethylation [39, 40]. In fact, Ding *et al*. [29] suggested that TSA treatment induces a higher level of histone acetylation and lower level of DNA methylation at the 2-cell stage in bovine SCNT embryos, which facilitates epigenetic reprogramming of the transferred somatic cell nucleus. These findings indicate that histone acetylation and DNA methylation interact in a complex manner and are related to epigenetic reprogramming. Hence, modification of these epigenetic marks by TSA treatment may support epigenetic reprogramming of SCNT embryos. On the other hand, no difference in histone acetylation level was observed at the blastocyst stage among the groups. This result is consistent with previous studies reporting that the hyperacetylation effect of TSA treatment lasts for a short period after treatment [16] and that a high level of histone acetylation in blastocysts may not be necessary for embryo development [29]. On the other hand, Li *et al*. [40] demonstrated that treatment of SCNT embryos with TSA influences the expression of chromatin structure- and DNA methylation-related genes at the blastocyst stage and selectively increases the expression level of *Sox2* and *cMyc*, which are responsible for embryonic development, at the blastocyst stage. Hence, these findings, combined with our data, indicate that the higher level of histone acetylation at the early stages with TSA treatment may influence gene expression at later stages, resulting in improvement of developmental competence in SCNT embryos.

In conclusion, we determined the optimal conditions of TSA

treatment (5 nM for 15–20 h) in miniature pig SCNT embryos. Furthermore, enhancement of the histone acetylation level at the pseudo-pronuclear to 2-cell stages could improve developmental competence of miniature pig SCNT embryos. These findings suggest that there is a relationship between developmental competence and the acetylation level of histone H3 in miniature pig SCNT embryos. Thus, this study provides the insight that the developmental competence of SCNT embryos could be enhanced by altering epigenetic modifications. Further research to investigate the relationships among other epigenetic modifications, developmental competence and global gene expression in detail are necessary to elucidate epigenetic reprogramming.

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