FULL PAPER *Theriogenology*

Extended Exposure to Trichostatin A after Activation Alters the Expression of Genes Important for Early Development in Nuclear Transfer Murine Embryos

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ABSTRACT. The low viability of embryos reconstructed by somatic cell nuclear transfer (SCNT) is believed to be associated with epigenetic modification errors, and reduction of those errors may improve the viability of SCNT embryos. The present study shows the effect of trichostatin A (TSA), a strong inhibitor of histone deacetylase, on the development of murine SCNT embryos. After enucleation and nuclear injection, reconstructed murine oocytes were activated with or without TSA for 6 hr (TSA-6 hr). After activation, TSA treatment was extended to 3 hr (TSA-9 hr), 5 hr (TSA-11 hr) and 18 hr (TSA-24 hr) during culture. As a result, the SCNT embryos in the TSA-11 hr group showed a remarkably higher blastocyst rate (21.1%) when compared with the nontreated embryos (3.4%), while the concentration of TSA did not significantly affect embryonic development. The expressions of histone deacetylase (HDAC1 and HDAC2) and DNA methylation (DNMT3a and DNMT3b) genes decreased in the TSA-11 hr and TSA-24 hr groups, while there was an increase in the expression of histone acetyltransferase (P300 and CBP), pluripotency (OCT4 and NANOG) and embryonic growth/trophectoderm formation (FGF4)-related genes in the same groups. The expression of CDX2, a critical gene for trophectoderm formation was upregulated only in the TSA-24 hr group. Our results show that TSA treatment during the peri- and postactivation period improves the development of reconstructed murine embryos, and this observation may be explained by enhanced epigenetic modification of somatic cells caused by TSA-induced hyperacetylation, demethylation and upregulation of pluripotency and embryonic growth after SCNT.
KEY WORDS: epigenetic modification, pluripotency, somatic cell nuclear transfer, trichostatin A.

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Advances in cloning techniques [4] using somatic cell nuclear transfer (SCNT) started with the birth of the first mammalian clone, Dolly the sheep, in 1997 [55]. SCNT is a powerful tool for the study of cell reprogramming as well as animal cloning. The applications of SCNT technology include the preservation of endangered species, pet or domestic animal cloning and cell therapy using autologous embryonic stem cells [49–51, 54]. However, like other species, mouse cloning by SCNT has demonstrated very low success rates since the first cloned mouse was reported in 1998 [52]. Although a noticeable number of SCNT murine embryos reach the blastocyst stage *in vitro* when produced using published SCNT protocols, the postimplantation development is very limited. The factors affecting the development of SCNT embryos include oocyte activation [19], timing of enucleation and injection of the somatic cell nucleus [48], donor cell pretreatment before nuclear transfer [47] and supplementation of various factors in culture medium [18, 35]. In addition to high abortion and fetal death rates, surviving cloned mice often show a variety of abnormalities including obesity, large placenta and abnormal expression of genes important for development [30, 40, 42, 53]. Operational defects of epigenetic factors may cause these anomalies [33]. In recent studies, molecular analyses of cloned embryos have shown irregular gene expression in the placenta, kidney and liver caused by abnormal epigenetic modifications such as DNA methylation and histone modification [32]. These epigenetic irregularities and changes in gene expression in the cloned mouse result in low success rates for animal cloning. Therefore, the inhibition of abnormal epigenetic changes such as DNA methylation and hypo-acetylation should lead to improvements in animal cloning efficiency. Treatment of donor cells with 5-aza-20-deoxycytidine, an inhibitor of DNA methylation, resulted in an improvement in the development of cloned mouse embryos [9, 35]. Other researchers have suggested that treatment with trichostatin A (TSA), which enhances the pool of acetylated histones and DNA demethylation, leads to 4–5 times higher blastocyst rates after SCNT in mice during oocyte activation, donor cell preparation, and/or culture [11, 18]. The report of Kishigami *et al.* [18] indicates that TSA enhances reprogramming of somatic nuclei in oocytes. This research group also reported that abnormal DNA hypermethylation of spermatid-derived paternal genomes in zygotes following round spermatid injection (ROSI) occurs before the end of the first mitosis and that treatment of ROSI zygotes with TSA results in a reduction of DNA methylation [16]. The hypermethylation of the spermatid-derived genome after ROSI may explain the fact that the development of ROSI embryos is significantly lower than that of the embryos after intracytoplasmic sperm injection (ICSI) [15, 17, 31]. This result demonstrates that the treatment of ROSI zygotes with TSA results in histone modifications associated with the reduction of DNA hypermethylation. As similar abnormal DNA hypermethylation has been observed in SCNT mammalian preimplantation

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embryos, fetal organs and placentas [6, 14, 32], TSA treatment may counteract DNA hypermethylation in the donor cell genome and improve the development of cloned embryos. Histone deacetylase (HDAC) inhibitors are commonly used in medical research as anticonvulsants and anticancer drugs [8]. However, regardless of the improvements observed after TSA treatment in SCNT experiments, TSA should be applied only cautiously because it is a teratogen. HDAC inhibitors such as valproic acid and TSA are known teratogens in zebrafish, domestic animals and mice [12, 38]. It is therefore important to employ optimal exposure in terms of duration and concentration in order to avoid any detrimental effects. Although some other researchers have applied TSA for SCNT in mice after the initial report [52], there has not been a thorough evaluation of the expression pattern of developmentally important genes or the time-dependent effect on development after activation.

In this study, we investigated the effect of TSA on the improvement of SCNT murine embryonic development in a concentration- and time-dependent manner and the effect on the expression of epigenetic modification-related genes, such as HDAC genes (HDAC1, 2), histone acetyltransferase (Hat) genes (CBP, P300) and DNA methylation related genes (DNMT3a, 3b), because histone acetylation and DNA methylation are important epigenetic factors that modify chromatin structure and related-gene expression. We also determined the influence of TSA on the expression levels of several genes associated with pluripotency and early embryonic development such as OCT4, NANOG, FGF4 and CDX2 [21, 37, 39].

MATERIALS AND METHODS

Animals and chemicals: Six-week-old *C57BL6* X *DBA2* F1-hybrid (*B6D2F1*) female mice were used as sources for recipient oocytes and donor cumulus cells. All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated. All culture and handling media used were based on CZB and KSOM [3, 10].

Collection of oocytes and preparation of donor cells: Female *B6D2F1* mice were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (eCG, Intervet, Boxmeer, Holland), and 5 IU human chorionic gonadotropin (hCG, Intervet., Boxmeer, Holland), given 48 hr apart. Oocytes were recovered 15 hr after hCG injection, and the oviducts were removed and transferred to 3 m*l* Hepes-CZB medium (HCZB) supplemented with 300 IU/m*l* hyaluronidase. The ampullae of the oviducts were torn, and the cumulus-oocytes complexes were released. After a 5-min exposure to the hyaluronidase-containing HCZB, the cumulus-free oocytes were washed three times in HCZB before micromanipulation. The cumulus cell suspension in a 5-μl HCZB droplet was mixed with HCZB supplemented with 12% (w/v) polyvinylpyrrolidone (PVP), and the dish was covered with mineral oil. The donor cell and PVP solution mixture (PVP-HCZB) was kept at room temperature for up to 2 hr before nuclear injection [54].

Enucleation: Ten to 15 oocytes were transferred to a 10-μl droplet of HCZB containing 5 μg/m*l* cytochalasin B (CB), which had been placed previously under mineral oil in the operation heat chamber on a microscope stage. The zona pellucida of the oocyte was 'drilled' by applying several piezo pulses to the tip of an enucleation pipette with a diameter of 7 to 10 μm using a piezo-actuated micromanipulator (PMM-150FU, Prime Tech Ltd., Ibaraki, Japan). The metaphase II chromosome–spindle complex was drawn into the pipette with a small amount of accompanying ooplasm and removed from the oocyte. After enucleation, the oocytes were transferred into KSOM, kept for up to 30–60 min before nuclear injection and then moved to a CB-free HCZB droplet for nuclear injection.

Nuclear injection: Nuclear injection was carried out within 30 min to 1 hr after enucleation. In a droplet of PVP-HCZB, a cumulus cell was drawn into an injection pipette with a 5 μm diameter, and the cell was moved in and out with operating piezo pulses until the plasma membrane was broken. Then, the pipette was moved to a CB and BSA-free HCZB droplet where the enucleated oocytes were placed. Before nuclear injection, the zona pellucida was punctured using a laser-assisted piercing device (XYClone^{TM}, Hamilton Thorne Biosciences, Beverly, MA, U.S.A.), as previously described [13]. The pipette containing the donor cell was passed through the puncture made by laser-assisted piercing and then directly injected into the enucleated oocytes through the cytoplast membrane by applying one or two piezo pulses. After the injection, the reconstructed embryos remained in the medium before activation. All micromanipulation processes were performed at room temperature.

Activation, in vitro culture and TSA treatment: Reconstructed murine oocytes were exposed to an activation medium consisting of 10 mM $SrCl_2$ with 5 μg/m*l* CB in calcium-free CZB for 6 hr. In the first series of experiments, 5 nM TSA was supplemented in the activation medium. After activation, the activated oocytes were moved to KSOM for *in vitro* culture (TSA-6 hr). In some embryos, exposure to TSA was extended for 3, 5 or 18 hr during the early embryonic culture period. These groups were referred to as TSA-9 hr, TSA-11 hr and TSA-24 hr, respectively. No TSA treatment during the activation was labeled as TSA-0 hr. Then, the embryos were transferred to KSOM and cultured for 102 hr (TSA-24 hr) to 120 hr (TSA-0 and 6 hr) at 37°C in a humidified 5% CO_2 in air. As a comparison group, parthenogenetic embryos that were not manipulated were used and treated in the same way (activation, TSA treatment and culture, but no enucleation or nuclear injection) as the nuclear transfer embryos. The detailed experimental design is shown in Fig. 1. In the second series of experiments, we evaluated different concentrations of TSA treatment (1, 5, 25 and 50 nM). The reconstructed oocytes were exposed to TSA of various concentrations during the activation and additional 5 hr postactivation, and then the embryos were transferred to KSOM for *in vitro* culture for 115 hr.

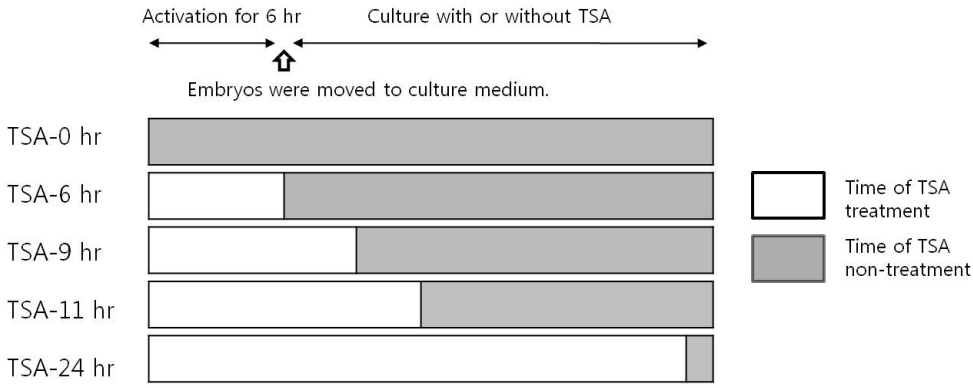

Fig. 1. The experimental scheme for the duration of TSA treatment. TSA treatment was administered during and after activation. After TSA treatment, embryos were moved to conventional *in vitro* culture conditions and developed *in vitro* for up to 120 hr.

Real-time polymerase chain reaction: The cDNAs for quantitative PCR were obtained from pooled blastocysts in each experimental group. The genes of histone modification (HDAC1, HDAC2, P300 and CBP), DNA methylation (DNMT3a, DNMT3b), pluripotency and early embryonic development (OCT4, NANOG, FGF4 and CDX2) were analyzed by real-time RT-PCR. Amplification of target genes was performed with the specific primers listed in Table 1. All reactions were performed using the Fluorescent Real-time Cycler System (Abcam Bio., Cambridge, MA, U.S.A.) with SYBR Green I (Invitrogen, Carlsbad, CA, U.S.A.) as a double-stranded DNA-specific fluorescent dye. Quantification was performed using the $^{\Delta\Delta}$CT method.

Establishment of embryonic stem cell (ESC) lines from SCNT embryos: To generate an ESC line, zona-free SCNT blastocysts were transferred onto an STO feeder layer in gelatinized 4-well tissue culture plates (Nunc, Rochester, NY, U.S.A.) in ESC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; high-glucose formation; Invitrogen) supplemented with 15% fetal bovine serum (FBS; Hyclone, Logan, U.S.A.), 1% nonessential amino acid stock (NEAA; Invitrogen), 0.1 mM β-mercaptoethanol, and 1,000 units/m*l* ESGRO leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA, U.S.A.). After 3 or 4 days of culture, the outgrown clumps derived from the inner cell mass were counted under a stereomicroscope. Once the inner cell mass outgrowth was evaluated, the cell clumps were further trypsinized with 0.05% trypsin-EDTA and seeded onto gelatinized new 4-well dishes with fresh ESC medium in order to generate the ESC line. When the colonies appeared, they were considered passage 0 ESC. The putative ESC lines from SCNT blastocysts (SCNT-ESC) were obtained after culturing over 20 passages and were also characterized by alkaline phosphatase (ALP), a pluripotency marker, staining at or after passage 8. The staining was performed according to the manufacturer's instructions (Chemicon; SCR 001). The ESCs were fixed with 4% paraformaldehyde for 2 min and rinsed for 5 min with rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.05% Tween-20). During rinsing, reagents for ALP staining were prepared as follows: Fast Red Violet with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio. After the stain solution was added, the cells were incubated in the dark for 15 min and washed with PBS and then observed.

Table 1. Primer sequences (5'–3') used in real-time RT-PCR analysis

Gene name		Primer sequence
HDAC1	Forward	GAGGAGGACCCTGACAAACG
	Reverse	AGAGTTCTTGCGACCACCTTCT
HDAC2	Forward	AAACTTTTAACTTGCCATTGCTGAT
	Reverse	TGGCAACTCATTGGGAATTTC
DNMT3a	Forward	GATGTTCTTTGCCAATAACCATGA
	Reverse	CAGGAGCCCTGTAGCAATCC
DNMT3b	Forward	CCTGCCCGCAAAGGTTTATA
	Reverse	GGCCACAACATTCTCGAACAT
P300	Forward	GGCATGAATCCTGGAATGTTG
	Reverse	CTGCATTTGGGTACTGCATGTT
CBP	Forward	ACCCCAAACGAGCCAAACT
	Reverse	TCATCAGGAAGGTCATTTTCCA
CDX2	Forward	TGGACGCTGCGAGAATCC
	Reverse	TGTCCTCAATCTCCATCAGTAGATG
OCT4	Forward	CGGTGTGAGGTGGAGTCTGG
	Reverse	GCGATGTGAGTGATCTGCTG
NANOG	Forward	GAAATCCCTTCCCTCGCCAT
	Reverse	CTCAGTAGCAGACCCTTGTA
FGF4	Forward	ATGGCGTCCGCGAGAAG
	Reverse	AGGTACCGGTTGGCACACA

Statistical analysis: Each experiment was replicated at least four times. Cleavage and further embryonic development between treatments were analyzed by the Chi-square test. Data for important gene expression of blastocyst embryos were analyzed by one-way ANOVA using the SPSS software (Version 12.0; SPSS Inc., Chicago, IL, U.S.A.). $P<0.05$ was considered significant.

Animal ethics: All animal experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-061023–1).

RESULTS

Time-dependent manner of TSA treatment for in vitro development of SCNT and parthenogenetic murine embryos: Among the experimental groups, the TSA-11 hr group showed the highest rates of blastocyst formation (21.3%) compared with the other groups (TSA-0 hr, 3.4%; TSA-6 hr, 4.8%; TSA-9 hr, 10.6%; TSA-24 hr, 17.1%; Table 2). The two-cell cleavage rates were not significantly different among groups. The parthenogenetic comparison groups did not show any effects of TSA treatment, although the TSA-24 hr group had a lower blastocyst formation rate (60.0%) when compared with the TSA-0 hr or TSA-11 hr groups (84.0% and 85.0%, respectively; Table 2). Two putative SCNT-ESC lines were only generated from TSA-11 hr group (Table 2). The putative SCNT-ESC colonies showed ALP positive staining as well as general ESC morphology (Fig. 2, at passage 8), and both cell lines were cultured over passage 20 and kept frozen for other experiments.

TSA treatment causes changes in specific gene expression in embryo development: Time-dependent changes in gene expression in blastocysts after TSA treatment are described in Fig. 3 and 4. The expression of histone deacetylase (HDAC1 and HDAC2) and DNA methylation (DNMT3a and DNMT3b) genes is decreased in TSA-11 hr and TSA-24 hr groups, while histone acetyltransferase (P300 and CBP), pluripotency (OCT4 and NANOG) and embryonic growth/trophectoderm formation (FGF4)-related genes were increased in the same groups. The expression of CDX2, a critical gene for trophectoderm formation, was upregulated only in the TSA-24 hr group.

Dosage-dependent effects of TSA treatment on the development of SCNT embryos: Among the experimental groups (1, 5, 25 and 50 nM TSA), treatment with 5 to 50 nM TSA resulted in higher blastocyst formation rates than 1 nM TSA treatment (Table 3). No significant difference was found among the 5 to 50 nM TSA treatment groups.

DISCUSSION

A number of experiments have been performed in an effort to improve the efficiency of animal cloning by SCNT including modification of activation and embryo culture [34, 54, 56], treatment of donor cells or SCNT embryos with specific chemicals such as TSA and 5-aza-20-deoxycytidine [9, 18, 35], application of serial SCNT [50] and removal of the donor cell cytoplasm [45, 46]. The nuclear reprogramming event in a cloned mouse embryo is an unnatural process involving the inactivation of somatic cell genes and reactivation of embryonic genes [2, 25]. In natural reproduction, normal gametes have low levels of DNA methylation, and the methylation levels decline due to active

Table 2. Time-dependent effects of TSA[a)] treatment on the development of SCNT[b)] and parthenogenetic murine embryos[c)]

	Duration of TSA treatment[d)]	Activation	No. (%) 2-cells	No. (%) blastocysts	No. SCNT-ESC[e)]
SCNT embryos	TSA-0 hr	116	44 (37.9)[a)]	4 (3.4)[a)]	0
	TSA-6 hr	164	68 (41.5)[b)]	8 (4.8)[a)]	0
	TSA-9 hr	188	80 (42.5)[b)]	21 (10.6)[b)]	0
	TSA-11 hr	208	92 (44.2)[b)]	44 (21.2)[c)]	2
	TSA-24 hr	140	76 (54.2)[c)]	24 (17.1)[c)]	0
Parthenotes	TSA-0 hr	100	100 (100)[a)]	84 (84)[a)]	N/A
	TSA-6 hr	84	84 (100)[a)]	61 (71.4)[b)]	N/A
	TSA-9 hr	112	108 (96.4)[a)]	87 (78.5)[b)]	N/A
	TSA-11 hr	108	108 (100)[a)]	91 (85)[a)]	N/A
	TSA-24 hr	104	92 (88.4)[b)]	59 (60)[c)]	N/A

a) Trichostatin A. b) SCNT: Somatic cell nuclear transfer. c) Four replicates. d) See Fig. 1 for the detailed experimental design and assignment of group names. e) SCNT-ESC: Putative somatic cell nuclear transfer embryonic stem cell lines.
Values with different superscripts (a, b, c) in the same column are significantly different ($P<0.05$).

Table 3. Dosage-dependent effects of TSA treatment on the development of SCNT embryos[a)]

TSA Concentration	No. activated oocytes	No. (%) 2-cells	No. (%) blastocysts
1 nM	228	108(47.4)[a)]	12 (5.2)[a)]
5 nM	208	92(44.2)[a)]	44 (21.2)[b)]
25 nM	180	84(46.6)[a)]	31 (17.7)[b)]
50 nM	148	64(43.2)[a)]	29 (19.5)[b)]

a) Four replicates.
Values with different superscripts (a, b) in the same column are significantly different ($P<0.05$).

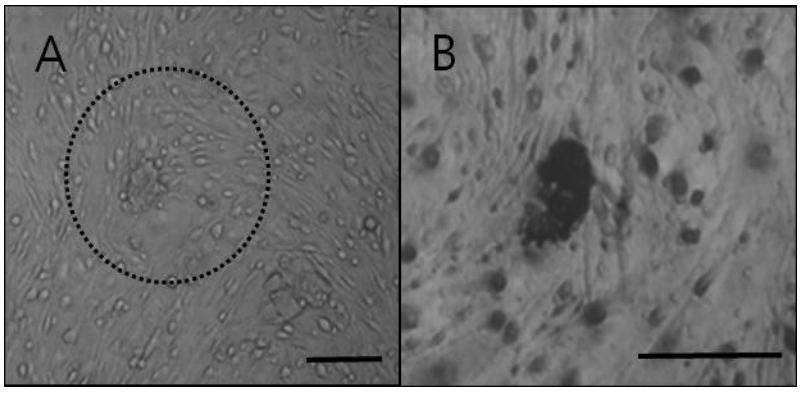

Fig. 2. A Putative SCNT-ESC line from TSA-11 hr group SCNT embryos at passage 8. A: Morphology of a putative SCNT-ESC colony (in the dotted circle). B: Alkaline phosphatase staining of the SCNT-ESC colony. Scale bar: 100 μm.

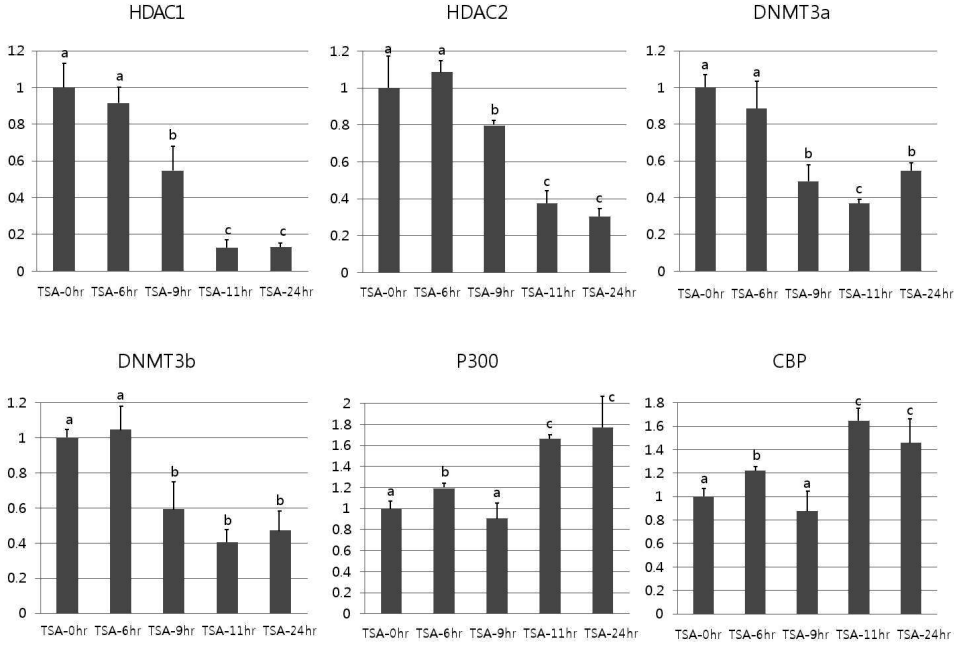

Fig. 3. The expression levels of histone modification (HDAC1, HDAC2, P300 and CBP) and DNA methylation (DNMT3a, DNMT3b)-related genes after TSA treatment for different durations. [a,b,c] Values without a common superscript differ (P<0.05).

methylation of the paternal genome and passive methylation of the maternal genome [47]. The first methylation in an embryo occurs at the early blastocyst stage [23, 59]. However, the levels of DNA methylation in somatic cells are much higher than those in naturally fertilized embryos. There have been many attempts to decrease methylation levels in mammalian somatic cells before nuclear reprogramming [9, 11, 27]. TSA, which enhances the pool of acetylated histones and DNA demethylation, has been used in cloning experiments for many mammalian species. The application of TSA treatment in SCNT has resulted in higher pre-implantation embryonic development in pigs [61], cattle [7] and rabbits [36]. Although the application of TSA has resulted in improvements in the murine SCNT program [18, 44], the effect of TSA on SCNT is still an issue in some animal species [26, 36, 57] and many research groups have suggested that it has various harmful effects on the development of mammalian SCNT embryos [26, 44, 57].

In our experiment, we investigated the optimal duration of TSA exposure during the early reprogramming event in SCNT embryos. We found that 11 hr of TSA treatment from activation and subsequent *in vitro* culture was appropriate for development of SCNT embryos. The first report using TSA for SCNT concluded that TSA-treatment for 10

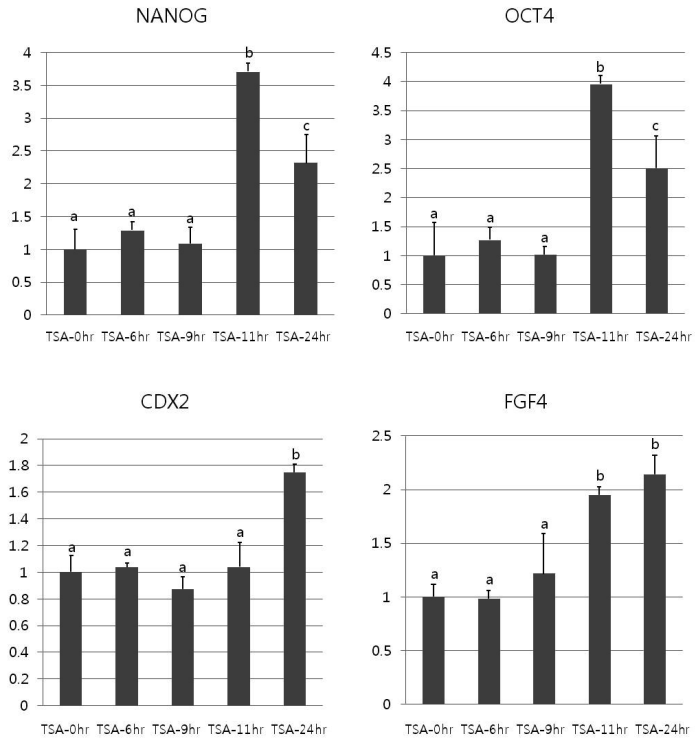

Fig. 4. The expression levels of pluripotency (OCT4, NANOG) and early embryonic development (FGF4, CDX2)-related genes after TSA treatment for different durations. [a,b,c] Values without a common superscript differ ($P<0.05$).

hrs following activation is most efficient [18] whereas our data shows that extended treatment for 24 hr is not detrimental to SCNT embryos in terms of their developmental potential to the blastocyst stage. However, the experiment using parthenotes in the present study (Table 2) also implies that 24 hr of treatment is detrimental to blastocyst development *in vitro*. Although another report using TSA also claimed that there was no significant increase from 6 to 9 hr of TSA treatment [35], the differences among various research groups may be from the different window of TSA treatment, as the latter group additionally treated TSA for 2 hrs prior to activation. We also concluded that treatment with 5 nM of TSA is optimal for SCNT embryo development. Although 5 to 50 nM of TSA treatment did not have a different effect on the reprogramming of SCNT embryos, applications of minimal doses of TSA are recommended, as TSA is teratogenic and higher doses may have negative effects [12, 38]. Although our present study investigating the duration and concentration of TSA treatment is similar to previous work of other research groups [18, 22, 61], we investigated the effect of TSA treatment at the molecular level by studying the expression of several important genes in SCNT embryos after exposure to 5 nM of TSA for different durations, ranging from 0 to 24 hr. We focused on several specific genes related to pluripotency and epigenetic status for the early embryonic development and investigated the correlation between TSA exposure and changes of pluripotency and epigenetic status of SCNT embryos.

Changes in epigenetic status and gene expression profiles affect all aspects of embryo development. Acetylation and deacetylation of the lysines in the histones are controlled by HATs and HDAC enzymes. Histone acetylation is thought to facilitate transcription, and deacetylation has the reverse effect on gene expression. An imbalance between the HAT and HDAC enzymes induces disorder in proliferation and differentiation in normal cells, and leads to initiation of tumor development [1]. Targeted disruption of HDAC1 is lethal to embryos and reduces proliferation, despite an increase in the expression of HDAC2 and HDAC3 [8, 24]. Gene knockouts in mice have demonstrated that homozygous disruption of P300 and CBP, the HAT-related genes, is lethal to embryos, indicating that normal embryogenesis requires the presence of both factors [20, 60]. In the present study, TSA treatment for over 11 hr resulted in inhibition of the expression of HDAC-related genes and activation of the expression of HAT-related genes. Another key epigenetic mechanism that modifies chromatin structure and function is DNA methylation, which is involved in epigenetic imprinting, and abnormal gene expression and silencing [58]. In addition to enhancing acetylation, TSA also induces DNA demethylation even in the absence of DNA methyltransferase (DNMT) inhibitor. The effect of TSA on DNMT

activity is still unknown. The present study also showed that the expression of DNMT 3a and 3b in the TSA-treated SCNT blastocysts was significantly suppressed when compared with the control SCNT blastocysts. In particular, the blastocysts from the TSA-11 hr group showed an optimal inhibitory effect on DNMT gene expression. These phenomena indicate that TSA treatment for more than 11 hr during and after activation supports epigenetic reprogramming of the somatic cell nuclei by enhancing activation of the genes that had been silenced before SCNT through enhanced acetylation and demethylation. In the present experiment, HDAC and DNMT gene expression were remarkably inhibited when TSA treatment was extended to 5 hr after activation (TSA-11 hr), and this was similar to a previous report that demonstrated 8 hr of TSA treatment for SCNT [22]. However, our observation of the expression level of HAT-related genes (P300 and CBP) shows complicated results because the expression level was lowest in the TSA-9 hr group, which is similar to the previous report [22], whereas TSA treatment for more than 11 hr elevated the expression level of those genes. Extended exposure to TSA more than 11 hr may enhance HAT activity as well as HDAC and DNMT gene inhibition, and this may activate gene expression for self-renewal and/or maintenance of pluripotency, as 2 putative SCNT ESC lines were only obtained from the TSA-11 hr group. However, the concentration of TSA may not affect HAT activity, as our TSA concentration was only 1/20 of that in the previous report [22].

Blastocyst formation shows the segregation of the two cell lineages in mammalian pre-implantation embryos, the inner cell mass (ICM) and trophectoderm (TE). The ICM lineage is attributed to the function of two important transcription factors, OCT4 and NANOG, the important regulators of pluripotency in mammalian embryos. They are both required for maintaining the ICM cell fate of the embryo and pluripotency of ESCs. Abnormal expression of the pluripotency-related genes induces abnormal extraembryonic endoderm or TE development [28]. In the ESC, OCT4 occupies many promoter elements and cooperates with many pluripotency regulatory genes [28, 39]. In our experiment, the SCNT blastocysts from the TSA-11 hr group had significantly higher expression levels of OCT4 and NANOG than other TSA-treated or non-TSA-treated SCNT blastocysts. Higher expression of the pluripotency-related genes indicates that the use of these embryos may have more of an advantage for ESC generation. As a result, we generated two putative SCNT-ESC lines only from the TSA-11 hr group, and the cell lines showed general characteristics of murine ESCs. Recently, it has become known that P300 is directly involved in modulating NANOG expression [62]. Increased expression of NANOG in the TSA-11 hr group may be caused by elevated expression of P300.

The caudal-type homeodomain protein CDX2 is a key transcription factor required at a distinct stage during early TE lineage development [4, 37]. The CDX2 mutant blastocysts fail to maintain trophoblast differentiation and also fail to implant [5]. CDX2 also promotes trophoblast lineage differentiation in mouse ES cells [43]. In the present study, the SCNT blastocysts from the TSA-24 hr group showed higher expression of CDX2 than those from other experimental groups. This indicates that the expression level of CDX2 is independently affected by TSA treatment after 11 hr, although the expression of this gene is closely related with the expression of OCT4 [29] because our results show that the highest expression level of OCT4 occurs when TSA is treated for 11 hr after nuclear injection. As CDX2 is an important gene for the formation of TE in the preimplantation embryo and subsequent placenta formation in the peri- and postimplantation embryo, our results imply that extended treatment with TSA up to 24 hr may have some positive effect on the formation of TE in SCNT murine embryos. FGF4 is an important factor for the growth of preimplantation embryos and is the marker gene for the TE lineage that contributes to the placenta [41]. Expression of this gene is known to affect the total cell number of preimplantation embryos [21, 41]. In the present study, TSA treatment for over 11 hr after nuclear injection showed a positive effect on the expression of FGF4 in SCNT embryos, which is important for embryonic growth and placental formation in murine embryos.

Exposure of nuclear injected oocytes to TSA for 24 hr in the present study led to a reduction in the blastocyst formation rate, suggesting that prolonged treatment with TSA may cause some defects in early embryogenesis. We found that the expression levels of the genes important for pluripotency, NANOG and OCT4, were highest in the TSA-11 hr group and were significantly downregulated after 24 hr of TSA treatment. This implies that the optimal duration of TSA treatment for ESC generation from SCNT blastocysts is 11 hr, although the expression levels of some other genes important for early embryogenesis and epigenetic reprogramming were not affected by extended treatment with TSA for up to 24 hr.

In conclusion, our results suggest that TSA treatment during the peri- and postactivation periods improves the development of reconstructed murine embryos. This improvement may be due to enhanced epigenetic modification of somatic cells caused by TSA-induced hyperacetylation and demethylation and upregulation of pluripotency and embryonic growth after SCNT.

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