—Original Article—

Valproic Acid Treatment from the 4-cell Stage Improves Oct4 Expression and Nuclear Distribution of Histone H3K27me3 in Mouse Cloned Blastocysts

Yuuki ISAJI[1], Moeko MURATA[1], Naoya TAKAGUCHI[1], Toshita MUKAI[1], Yosuke TAJIMA[1], Hiroshi IMAI[1] and Masayasu YAMADA[1]

[1]Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Abstract. We examined effects of treatment with valproic acid (0, 0.2, 1 or 2 mM, VPA), an inhibitor of class I and IIa histone deacetylases (HDACs), of mouse somatic cell nuclear transfer (SCNT) embryos for 24 h from 48 h (4-cell stage), 24 h (2-cell stage) or immediately after oocyte activation on blastocyst formation rates and qualities of the resultant blastocysts. Blastocyst formation rates (33.4–37.0%) were not improved by VPA treatments compared with the untreated control (35.1–36.4%). However, immunofluorescence staining revealed that Oct4 expression levels, evaluated from percentages of embryos expressing Oct4 strongly and having more than 10 Oct4-positive cells, in blastocysts from SCNT embryos treated with 1 mM VPA for 24 h from the 4-cell stage (VPA-4C) were highest among all the groups and that the proportion of cells with a normal nuclear distribution of histone H3 trimethylated at lysine 27 (H3K27me3), a marker of the state of X-chromosome inactivation, significantly increased in the VPA-4C group (36.6%) compared with the control group (12.4%, P<0.05). Treatments with scriptaid and sodium butyrate, inhibitors of class I and IIa/b HDACs, for 24 h from the 4-cell stage also had beneficial effects on SCNT blastocysts. These findings indicate that treatment with 1 mM VPA from the 4-cell stage improves the Oct4 expression and nuclear distribution of H3K27me3 in mouse SCNT blastocysts and suggest that the inhibition of class I and IIa HDACs from the 4-cell stage plays an important role in these effects.
Key words: H3K27me3, Mouse, Oct4, Somatic cell nuclear transfer, Valproic acid

Somatic cell nuclear transfer (SCNT) to produce cloned animals not only is of great importance to basic research but has practical applications in livestock propagation, species preservation, and cell therapy in regenerative medicine. However, although the cloning of mammals has been conducted successfully for the past decade [1], the success rate remains very low. In order to improve mouse cloning efficiency, several modifications to the original mouse cloning method [2] have been attempted, but they have not resulted in a remarkable improvement [3, 4].

Recently, it was found that treating mouse SCNT embryos with trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), after (2 h) and during (6 h) oocyte activation significantly enhanced *in vitro* development up to the blastocyst stage and led to an increase in cloning efficiency [5–11]. Furthermore, transient treatment with HDACis such as scriptaid (SCR) [12], suberoylanilide hydroxamic acid (SAHA) [9], oxamflatin [9] and m-carboxycinnamic acid bishydroxamide (CBHA) [13] also improved the full-term development of cloned mice, whereas other HDACis, such as aroyl pyrrolyl hydroxamide (APHA) [12], valproic acid (VPA) [9] and sirtinol [14], had little or no positive effect. In general, HDACs are divided into five categories: class I (HDAC 1–3 and 8), class IIa (HDAC 4, 5, 7

and 9), class IIb (HDAC 6 and 10), class III (SIRT 1–7) and class IV (HDAC 11) [15]. TSA, SCR, SAHA, oxamflatin and APHA can inhibit class I and IIa/b [15–18], but APHA is more active against HDAC3 (class I) and 6 (class IIb) than the others [15, 19]. VPA and sirtinol are inhibitors for class I and IIa [16] and class III HDACs, respectively. Therefore, it is suggested that inhibiting class IIb HDACs, particularly HDAC 10, is important for improving mouse cloning efficiency [9].

In contrast, Costa-borges *et al.* [7] reported that VPA treatment before (2–3 h) and during (6 h) oocyte activation in B6CBAF1 mouse SCNT embryos improved *in vitro* and full-term development in comparison with an untreated control. Interestingly, it was recently found that treatment with VPA of miniature pig SCNT embryos for 48 h starting immediately after oocyte activation enhanced the *in vitro* development and expression of Oct4 (also known as Pou5f1) [20] and that when fertilized mouse embryos were treated with 1 mM VPA during progression from the 8-cell to morula stage, the expression of Oct4 was moderately enhanced in the morula stage [21]. Therefore, it seems likely that the effect of VPA on the *in vitro* development as well as Oct4 expression of SCNT embryos varies with the timing of the treatment.

Mouse SCNT embryos have numerous abnormalities that are related to the efficiency of successful cloning, such as aberrant expression of Oct4 in SCNT blastocysts. In fertilized mouse embryos, Oct4 becomes restricted to the inner cell mass (ICM) and downregulated in the trophectoderm (TE) at the blastocyst stage [22]. However, in mouse SCNT blastocysts, Oct4 is often downregulated or abnormally

expressed, suggesting a loss of or reduced pluripotency in the ICM lineage in the cloned embryos [23–26], because Oct4-deficient embryos fail to form a pluripotent ICM [27]. Furthermore, SCNT embryos and offspring have been shown to exhibit aberrations in the state of X chromosome inactivation (XCI) [28–32]. During early embryogenesis, XCI is induced by X-inactive specific transcript (*Xist*) RNA, a noncoding RNA that inactivates one of the two X chromosomes in females [33–35]. Immediately after *Xist* RNA coating begins, the inactivated X-chromosome undergoes various chromatin modifications such as demethylation of histone H3 lysine 4, methylation of histone H3 lysine 9 and trimethylation of histone H3 lysine 27 (H3K27me3), and these changes lead to transcriptional silencing and late replication of one of the X chromosomes [36–39]. So, the state of XCI has often been examined by the distribution of foci of H3K27me3 within cell nuclei in mouse embryonic stem (ES) cells [36–38] and in fertilized and SCNT blastocysts [28, 30]. It was recently found that in mouse cloned embryos, *Xist* is ectopically expressed from the active X chromosome, which causes an aberrant expression of global genes [28]. Thus, attempts were made to prevent inappropriate XCI by using *Xist*-deficient donor nuclei for SCNT. As a result, the abnormal expression of X-linked genes as well as a number of autosomal genes was remarkably improved in the cloned embryos, resulting in an 8- to 9-fold increase in survival rates to term of the cloned embryos (12.7%) when compared with a wild-type control (1.6%) [28].

Meanwhile, it was recently found that pluripotency-associated factors, such as Oct4, Sox2 and Nanog, are involved in the repression of *Xist* expression in ES cells [40–42] and that Oct4 lies at the top of the XCI hierarchy and regulates XCI by triggering X-chromosome pairing and counting [40]. Indeed, depletion of Oct4 blocks homologous X-chromosome pairing and results in the inactivation of both X-chromosomes in female ES cells [40]. Furthermore, Oct4, Sox2 and Nanog bind within *Xist* intron 1 and maintain *Xist* repression in ES cells [41]. It was also suggested that Oct4, Sox2 and Nanog play important roles for control of *Xist* expression in preimplantation embryos [43, 44]. Thus, it is assumed that mouse cloning efficiency could be improved by inducing normal expression of Oct4 in SCNT blastocysts, through which ectopic expression of the *Xist* gene could be suppressed.

In the present study, we examined the effects of treatments with various concentrations of VPA from different developmental stages after oocyte activation in B6D2F1 mouse SCNT embryos on blastocyst formation rates as well as blastocyst quality evaluated by numbers of ICM and TE cells, Oct4 expression levels and the expression and distribution within cell nuclei of H3K27me3, a marker of the state of XCI.

Materials and Methods

Animals

B6D2F1 (C57BL/6J × DBA/2) mice were obtained at 7–10 weeks of age from Japan SLC (Hamamatsu, Japan). All procedures involving animals conformed to the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by and performed according to the guidelines of the Animal Research Committee, Kyoto University.

Collection of oocytes and cumulus cells

Female B6D2F1 mice were superovulated by injection with 7.5 IU of pregnant mare serum gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) followed by 7.5 IU of human chorionic gonadotropin (hCG, Sankyo Zoki, Tokyo, Japan) 48 h later. Cumulus–oocyte complexes (COCs) were collected from the oviducts at 15 h after the hCG injection and treated with 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in Hepes-buffered CZB (Hepes-CZB) medium [45] until the cumulus cells had dispersed. Cumulus-free oocytes were then washed and kept in KSOM medium [46] covered with mineral oil (Sigma-Aldrich) at 37 C in an atmosphere of 5% CO_2 in air prior to use. Cumulus cells were removed from the hyaluronidase drops and placed in an Eppendorf tube with 6% bovine serum albumin (BSA, Sigma-Aldrich)-containing Hepes-CZB medium. Thereafter, they were used as donor cells for SCNT.

Production of cloned embryos by SCNT

After pretreatment of oocytes collected for 10 min in Hepes-CZB medium supplemented with 5 µg/ml cytochalasin B (CB, Sigma-Aldrich), meiotic spindles of oocytes were removed using a Piezo-driven micromanipulator (Prime Tech, Tokyo, Japan). The enucleated oocytes were washed extensively and kept in KSOM medium at 37 C under 5% CO_2 in air for 1–2 h before nuclear transplantation. A cumulus cell from an Eppendorf tube was inserted into the perivitelline space of an enucleated oocyte together with HVJ-E (GenomeONE-CF, Ishihara Sangyo, Osaka, Japan) using a Piezo-driven micromanipulator, and then the oocyte was cultured in KSOM medium for 1 h at 37 C under 5% CO_2 in air, during which time it fused with the donor cell.

The reconstructed oocytes were parthenogenetically activated by incubation in 5 mM $SrCl_2$ (Wako Pure Chemical Industries, Osaka, Japan) and 2 mM EGTA (Sigma-Aldrich)-containing KSOM medium supplemented with 5 µg/ml CB (activation medium) for 6 h. After activation, the oocytes were cultured in KSOM medium, except for the periods during which they were treated with the various reagents indicated below, for 96 h at 37 C under 5% CO_2 in air. The pronuclear formation and development to the 2-cell and blastocyst stages were evaluated at 0 h, 24 h and 96 h after oocyte activation, respectively.

Treatments with HDACis, VPA, SCR and sodium butyrate (NaBu) of SCNT embryos during preimplantation development

SCNT embryos were treated with VPA (0, 0.2, 1 and 2 mM, Sigma-Aldrich) for 24 h from 48 h (4-cell stage, VPA-4C), 24 h (2-cell stage, VPA-2C) or immediately (1-cell stage, VPA-1C) after oocyte activation. SCR (0 and 100 nM, Sigma-Aldrich) and NaBu (0 and 0.5 mM, Wako Pure Chemical Industries) were treated for 24 h from the 4-cell stage (SCR-4C and NaBu-4C, respectively).

Production of parthenogenetic embryos (PE)

Oocytes collected from female B6D2F1 mice at 15 h post-hCG injection were incubated in activation medium for 6 h. After activation, they were cultured in KSOM medium for 96 h until the blastocyst stage.

Production of in vitro fertilized embryos

In vitro fertilization was performed as described previously [47].

Briefly, COCs were collected from female B6D2F1 mice at 15 h post-hCG injection were inseminated with capacitated spermatozoa from adult B6D2F1 male mice. The spermatozoa were capacitated by preincubation for 1 h in TYH medium [48]. Six hours after insemination, the fertilized embryos were washed and then cultured in KSOM medium for 96 h until the blastocyst stage.

Immunofluorescence staining

SCNT embryos were fixed in 3.7% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) overnight at 4 C. After permeabilization with 0.5% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 40 min at room temperature, samples were blocked in a blocking solution (0.02% Tween-20 (Sigma-Aldrich), 1.5% BSA and 0.2% sodium azide (Sigma-Aldrich) in PBS) for 1 h at room temperature. Afterward, the samples were incubated with primary antibodies at 4 C overnight. The primary antibodies used were anti-Oct3/4 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany), anti-Cdx2 (1:100; BioGenex, San Ramon, CA, USA) and anti-H3K27me3 (1:100; Millipore, Ann Arbor, MI, USA). After being washed extensively in the blocking solution, the samples were incubated with the secondary antibody, Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA) or Alexa Fluor 594-conjugated anti-mouse IgG (1:500, Molecular Probes), for 1 h at room temperature. Finally, after further washing with the blocking solution, the samples were stained with 10 mg/ml of Hoechst 33258 (Sigma-Aldrich) for 10 min and mounted on slides in 50% glycerol/PBS. Fluorescence signals were observed using a fluorescence microscope (FSX100, Olympus, Tokyo, Japan). Digital images of Oct4, Cdx2, H3K27me3 and Hoechst signals were acquired on FSX-BSW Software (Olympus) using the same contrast, brightness and exposure settings for all embryos. The immunofluorescence intensities for Oct4 were classified visually by images of nuclear fluorescence intensities corrected by subtracting background fluorescence for the cytoplasm, as described previously [26].

Statistical analysis

Each experiment was repeated at least three times. The values were analyzed using a *t*-test or one-way ANOVA and then Tukey's multiple comparison tests. P values < 0.05 were considered to be statistically significant.

Results

Effects of VPA treatment after oocyte activation on blastocyst formation and Oct4 expression and cell number in the resultant blastocysts

To optimize the time frame for VPA treatment, we examined the effects of treatment with 1 mM VPA for 24 h from various stages of SCNT embryos on blastocyst formation and Oct4 expression in the resultant blastocysts. Pronuclear formation rates were about 80% in all groups, and the rates of 2-cell embryos developed from pronuclei-formed oocytes (cleavage rates) did not differ significantly among all groups (VPA-1C, 85.8%; VPA-2C, 83.1%; VPA-4C, 88.9% and non-treated control (NTC), 82.8%) as well. As shown in Fig. 1A, there were no significant differences in the rates of blastocysts

developed from 2-cell embryos (blastocyst formation rates) among the NTC, VPA-1C, VPA-2C and VPA-4C groups (37.0, 33.4 and 37.0% for VPA-1C, VPA-2C and VPA-4C *vs.* 36.4% for NTC). Then, blastocysts derived from SCNT embryos and PE at 96 h after oocyte activation were subjected to immunofluorescence staining of Oct4, a specific marker for the ICM in mouse blastocysts. All the blastocysts were classified according to the immunofluorescence intensity for Oct4 and number of cells showing positive immunostaining for Oct4. Fig. 2 shows representative staining patterns in SCNT blastocysts: (a) strong expression of Oct4, (b) weak expression of Oct4, (c) less than 10 Oct4-positive cells and (d) more than 10 Oct4-positive cells. As shown in Fig. 1B and C, the percentages of blastocysts expressing Oct4 strongly and having more than 10 Oct4-positive cells were significantly lower in NTC (34.3 and 34.7%, respectively) than in PE (90.6 and 82.9%, respectively, P<0.05). However, VPA treatment increased the proportion of blastocysts expressing Oct4 strongly to the same extent as that in PE (VPA-1C, 85.0%; VPA-2C, 81.5%; VPA-4C, 82.2%; PE, 90.6%). Blastocysts having more than 10 Oct4-positive cells were also observed more frequently in VPA-treated groups compared with the NTC group. Among the VPA-treated groups, the rates were higher for VPA-4C (76.7%) than VPA-1C and VPA-2C (55.0 and 61.1%, respectively). Thus, VPA treatment for 24 h from the 4-cell stage was chosen for the subsequent experiments.

Next, the effects of treating SCNT embryos with different concentrations (0, 0.2, 1 and 2 mM) of VPA from the 4-cell stage on blastocyst formation rates and Oct4 expression in the resultant blastocysts were examined. Cleavage rates did not differ significantly between 0, 0.2, 1 and 2 mM VPA treatments (78.8, 75.7, 88.9 and 78.7%, respectively). As shown in Fig. 3A, there were no significant differences in the blastocyst formation rates between the 0, 0.2 and 1 mM VPA treatments (35.1, 36.3 and 37.0%, respectively), whereas the rate (18.3%) was markedly decreased for the 2 mM VPA treatment. We then classified blastocysts derived from SCNT embryos treated with 0, 0.2, 1 and 2 mM VPA according to immunofluorescence intensity and number of Oct4-positive cells (Fig. 3B and C). Treatment with 1 mM VPA significantly increased the proportion of SCNT blastocysts expressing Oct4 strongly and having more than 10 Oct4-positive cells (82.2 and 76.7%, respectively) to a similar extent to PE compared with the 0 mM group (27.6 and 21.1%, respectively, P<0.05). However, 0.2 and 2 mM VPA treatments had no effect on Oct4 expression. Based on these results, the following experiments were performed using 1 mM VPA.

We then examined the effects of VPA on the total number of cells and number of ICM cells in SCNT blastocysts, as described previously [9, 49, 50]. Each blastocyst was stained with Hoechst and antibodies against Cdx2, a marker for TE cells. The ICM cell number was assessed as the total cell number counted by Hoechst staining minus the TE cell number counted by Cdx2 staining. As shown in Table 1, blastocysts from the VPA-4C group had a significantly higher ICM cell number, which was very similar to that observed in parthenogenetic blastocysts, when compared with those cultured without VPA treatment. However, VPA treatment had no effect on the total cell number in SCNT blastocysts.
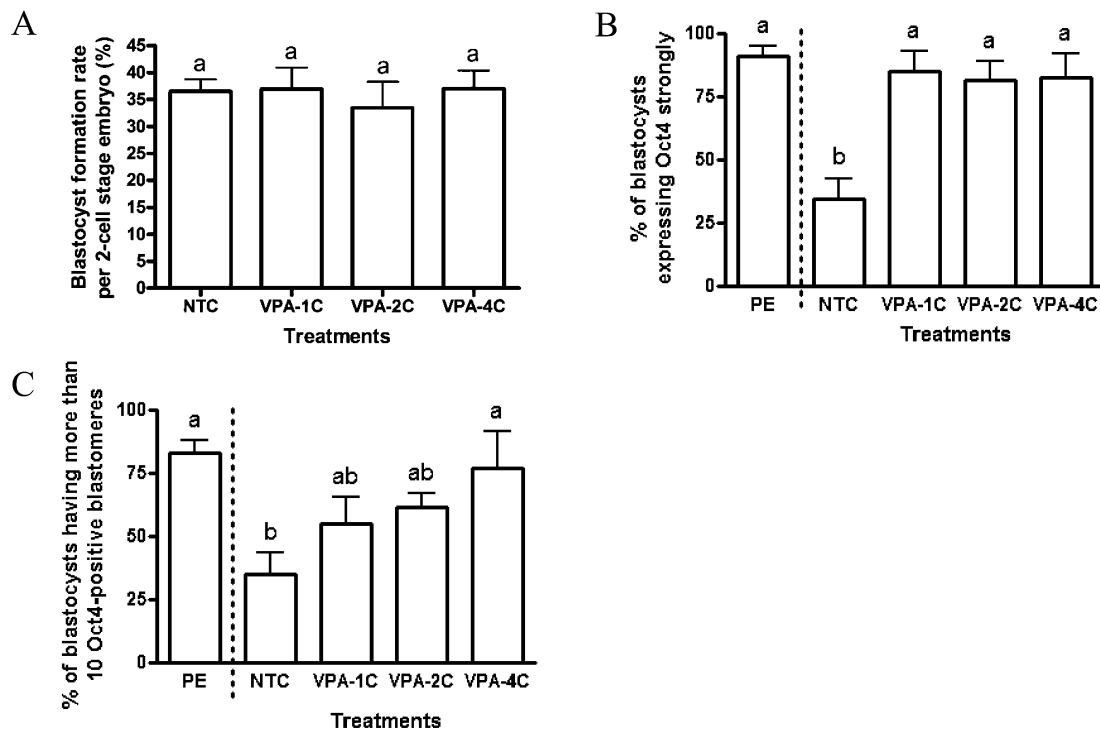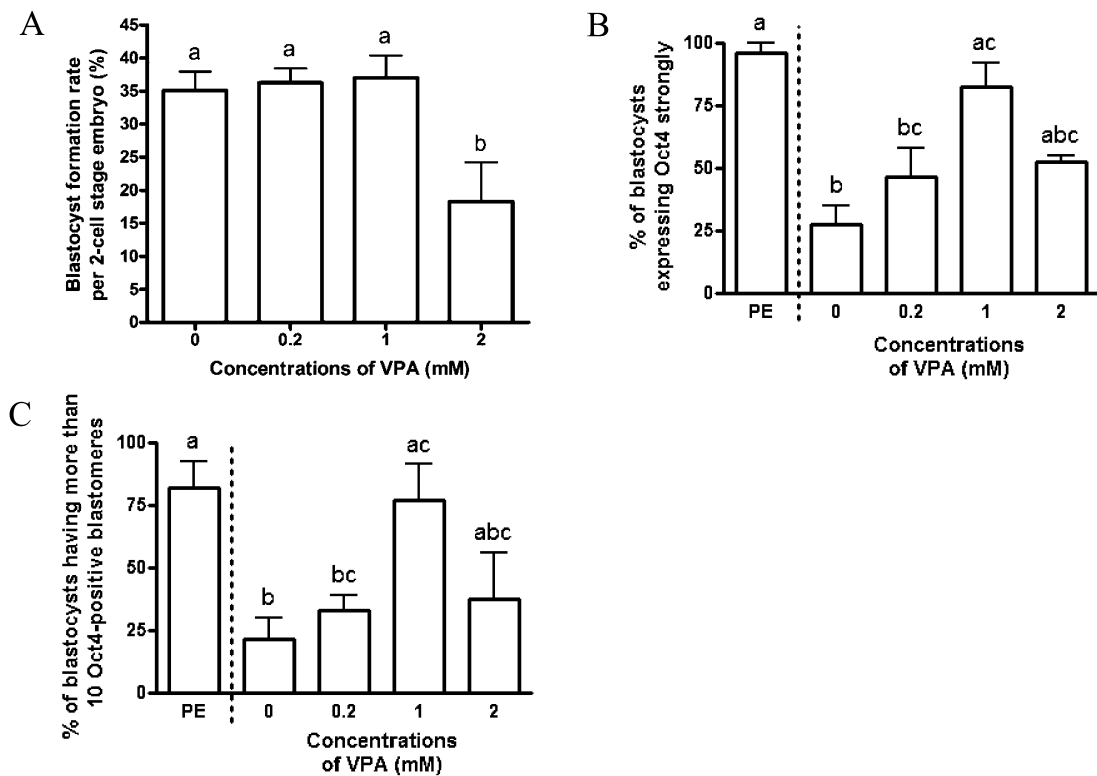
Fig. 1. Blastocyst formation and Oct4 expression at the blastocyst stage in SCNT embryos treated without (non-treated control, NTC) or with 1 mM VPA for 24 h from 48 h (4-cell stage, VPA-4C), 24 h (2-cell stage, VPA-2C) or immediately (1-cell stage, VPA-1C) after oocyte activation. A: Blastocyst formation rate per 2-cell stage embryo at 96 h after oocyte activation. B: Proportion of blastocysts expressing Oct4 strongly. C: Proportion of blastocysts having more than 10 Oct4-positive cells. PE: Parthenogenetic embryos. Error bar represents the standard error of the mean. Values with different superscripts are significantly different at P<0.05.

Effect of treatment with HDACis other than VPA from the 4-cell stage of SCNT embryos on blastocyst formation and Oct4 expression at the blastocyst stage

To examine whether other HDACis exert similar effects to VPA, we employed SCR and NaBu, an inhibitor of class I and IIa HDACs [51]. Cleavage rates in the SCR-4C, NaBu-4C and NTC groups were not significantly different (78.4, 83.0 and 78.8%, respectively). As shown in Fig. 4A, SCR-4C and NaBu-4C did not improve blastocyst formation rates (31.9 and 29.4%, respectively) compared with the NTC group (28.1%). However, as shown in Fig. 4B and C, percentages of SCNT blastocysts expressing Oct4 strongly and having more than 10 Oct4-positive cells were higher in the SCR-4C and NaBu-4C groups than in the NTC group (84.8 and 55.0% for SCR-4C and 90.0 and 67.5% for NaBu-4C *vs.* 39.0 and 20.8% for NTC, respectively, P<0.05). Furthermore, the ICM cell numbers in the blastocysts from the SCR-4C and NaBu-4C groups were significantly higher than those in NTC group (Table 2) and very similar to those in the VPA-4C and PE groups. Although total cell numbers in the SCR-4C group were significantly higher than those in the NTC group, there were no significant differences in TE cell numbers between the SCR-4C, NaBu-4C and NTC groups (Table 2).
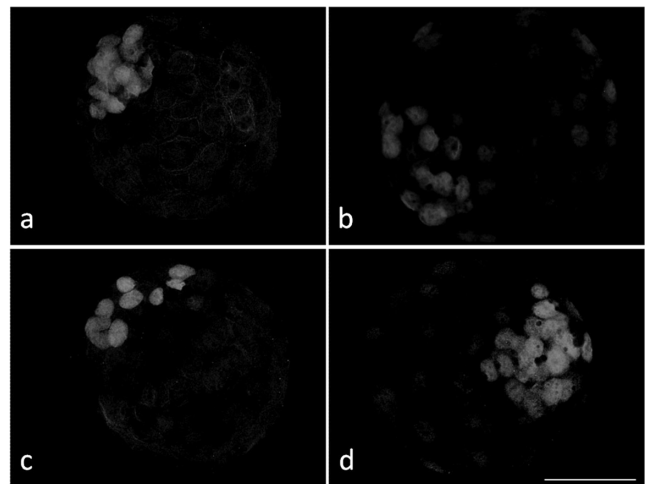
Fig. 2. Representative patterns of Oct4 expression in SCNT blastocysts. Blastocysts were stained using antibodies against Oct4. The classification was based on the immunofluorescence intensity of Oct4 (a, b) and number of cells with positive immunostaining for the Oct4 protein (c, d). (a) Blastocysts expressing Oct4 strongly. (b) Blastocysts expressing Oct4 weakly. (c) Blastocysts having less than 10 Oct4-positive cells. (d) Blastocysts having more than 10 Oct4-positive cells. Scale bar: 50 μm.

A

B

C

Fig. 3. Blastocyst formation and Oct4 expression at the blastocyst stage in SCNT embryos treated with various concentrations of VPA from the 4-cell stage. A: Blastocyst formation rate per 2-cell stage embryo at 96 h after oocyte activation. B: Proportion of blastocysts expressing Oct4 strongly. C: Proportion of blastocysts having more than 10 Oct4-positive cells. PE: Parthenogenetic embryos. Error bar represents the standard error of the mean. Values with different superscripts are significantly different at $P<0.05$.

Table 1. Characterization of blastocysts developing from SCNT embryos treated with 1 mM VPA for 24 h from the 4-cell stage (VPA-4C)

Treatment	No. of SCNT blastocysts examined	No. of cells counted by Hoechst staining	No. of TE cells counted by Cdx2 staining	No. of ICM cells counted by Hoechst & Cdx2 staining [a]
NTC *	11	44.9 ± 3.8 [b]	37.4 ± 3.8 [ab]	7.5 ± 1.2 [b]
VPA-4C	12	44.0 ± 4.4 [b]	30.4 ± 3.8 [b]	13.6 ± 1.8 [a]
PE **	14	60.9 ± 3.0 [a]	48.1 ± 2.9 [a]	12.7 ± 0.9 [a]

[a] Number of ICM cells was estimated by total cell number minus TE cells. * NTC: Non-treated control. ** PE: Parthenogenetic embryos. Within a column, values with different superscripts are significantly different at $P<0.05$.

Effects of VPA treatments on distribution of H3K27me3 foci within cell nuclei of SCNT blastocysts

Next, we examined whether the VPA treatments influenced the state of XCI in the SCNT blastocysts using immunofluorescence staining of H3K27me3, a marker of the state of XCI. First, it was found that in fertilized mouse blastocysts, a single signal-positive focus, which shows H3K27me3 accumulation on the inactivated X chromosome, was observed in each cell nucleus in half of the blastocysts examined, the remaining half not showing any foci, presumably representing female and male, respectively (Fig. 5A, a and b). On the other hand, in SCNT blastocysts, we observed different numbers of signal foci within each cell nucleus. Thus, the cells composing a single blastocyst were classified by the number of H3K27me3 foci within each cell nucleus. In NTC SCNT blastocysts, only 12.4% of the cells (the total number of blastocysts examined was 6) exhibited single nuclear foci (Fig. 5B), whereas nuclei without the signal or with two or multiple signal foci were observed in the remaining cells (Fig. 5A, c), suggesting aberrant inactivation of the X chromosomes in most of the cells of the control SCNT blastocysts. However, in SCNT blastocysts from the VPA-4C group, the proportion (36.6%, the total number of blastocysts examined was 9) of cells with nuclei exhibiting single H3K27me3 foci was significantly higher than that
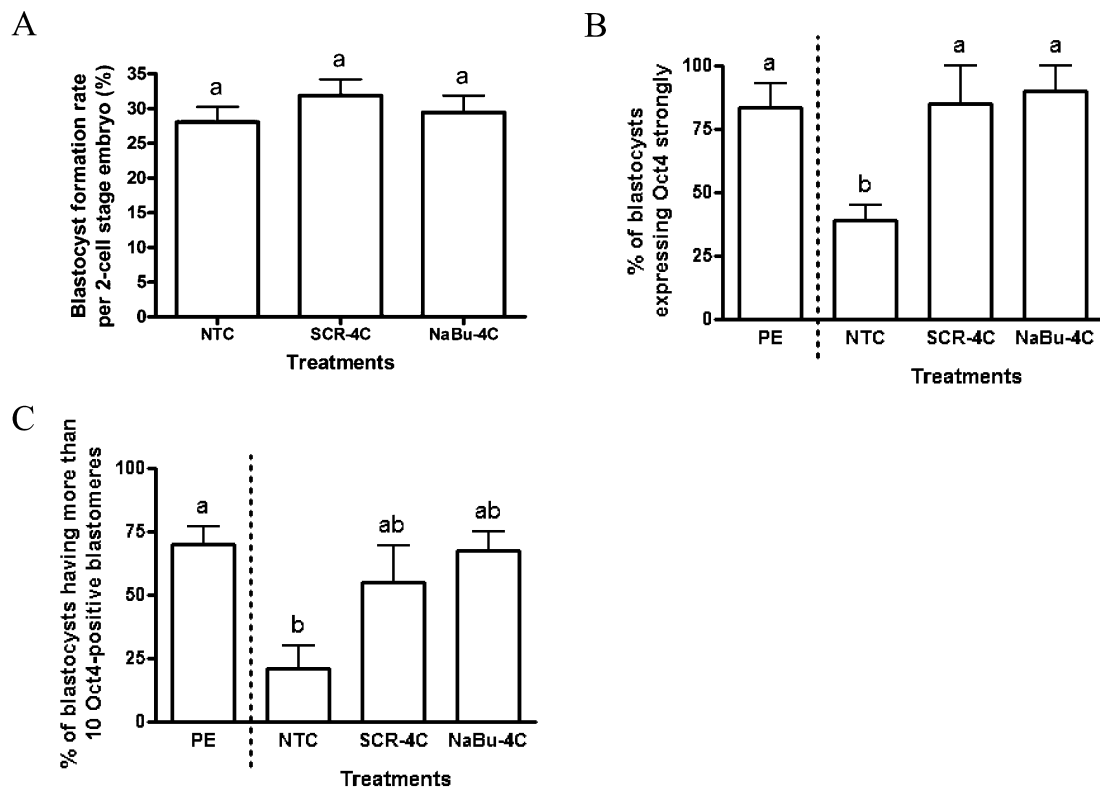
Fig. 4. Blastocyst formation and Oct4 expression at the blastocyst stage in SCNT embryos treated without (NTC) or with another HDACis, scriptaid (SCR) and sodium butyrate (NaBu) from the 4-cell stage (SCR-4C and NaBu-4C, respectively). A: Blastocyst formation rate per 2-cell stage embryo at 96 h after oocyte activation. B: Proportion of blastocysts expressing Oct4 strongly. C: Proportion of blastocysts having more than 10 Oct4-positive cells. PE: Parthenogenetic embryos. Error bar represents the standard error of the mean. Values with different superscripts are significantly different at P<0.05.

Table 2. Characterization of blastocysts developing from SCNT embryos treated with or without SCR and NaBu for 24 h from the 4-cell stage (SCR-4C and NaBu-4C, respectively)

| Treatment	No. of SCNT blastocysts examined	Total No. of cells counted by Hoechst staining	No. of TE cells counted by Cdx2 staining	No. of ICM cells counted by Hoechst & Cdx2 staining [a]
NTC *	33	38.8 ± 3.0 [b]	33.1 ± 2.7 [a]	5.6 ± 0.6 [b]
SCR-4C	17	53.6 ± 4.7 [a]	42.9 ± 3.7 [a]	10.7 ± 1.3 [a]
NaBu-4C	9	47.0 ± 6.2 [ab]	36.9 ± 5.6 [a]	10.1 ± 1.0 [a]

[a] Number of ICM cells was estimated as total cell number minus TE cell number. * NTC: Non-treated control. Within a column, values with different superscripts are significantly different at P<0.05.

in control SCNT blastocysts (Fig. 5A, d and 5B).

Discussion

Mouse SCNT embryos have numerous abnormalities, most caused by epigenetic errors, including abnormal DNA methylation or histone modification, acetylation and methylation [52–55], resulting in their impaired development. In the present study, we demonstrated that treatment with 1 mM VPA of B6D2F1 mouse SCNT embryos for 24 h from the 4-cell stage improved the qualities of the resultant blastocysts, especially 1) the Oct4 expression levels, as evaluated by increases in immunofluorescence intensity for the Oct4 protein and proportions of cells with positive immunostaining for Oct4, 2) the ICM cell numbers and 3) the proportions of cells with a normal nuclear distribution of H3K27me3 foci. However, despite the increased blastocyst formation rates in SCNT embryos treated with VPA before and during oocyte activation [7], the rates were not affected by VPA treatment after oocyte activation. The reason why developmental potential to the blastocyst stage in SCNT embryos varies with the timing of VPA treatment remains to be elucidated. Furthermore, the present study clarified that the effects on the Oct4 expression and ICM cell numbers in SCNT blastocysts were strongest for treatment

A

B
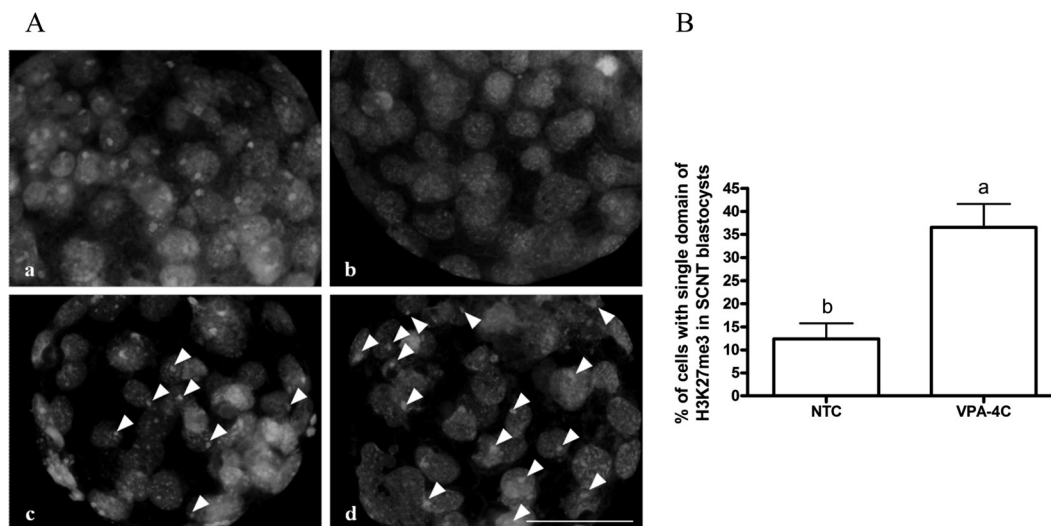
Fig. 5. Distribution of trimethylated histone H3 at lysine 27 (H3K27me3) within cell nuclei of SCNT blastocysts. A: Immunostaining for H3K27me3 and staining with Hoechst 33258 in fertilized blastocysts (a and b), non-treated control (NTC) SCNT blastocyst (c) and SCNT blastocyst treated with VPA from the 4-cell stage (VPA-4C SCNT blastocyst, d). Arrowheads in (c) and (d) indicate single nuclear foci of H3K27me3 within cell nuclei in SCNT blastocysts. Scale bar: 50 μm. B: Percentages of cells having single nuclear foci of H3K27me3 in NTC and VPA-4C SCNT blastocysts (total numbers of NTC and VPA-4C SCNT blastocysts examined were 6 and 9, respectively). Error bar represents the standard error of the mean. Values with different superscripts are significantly different at P<0.05.

with 1 mM VPA for 24 h from the 4-cell stage, as compared with the 2-cell stage or immediately after oocyte activation. When the concentration of VPA was lowered by 0.2 mM, no increase in the level of Oct4 in the SCNT blastocysts was observed. However, when the concentration was increased by 2 mM, developmental potential to the blastocyst stage was extremely reduced, suggesting that 2 mM of VPA is deleterious to B6D2F1 mouse SCNT embryos.

Ono *et al.* [9] demonstrated that treatment with TSA, SCR, SAHA or oxamflatin of mouse SCNT embryos after (2 h) and during (6 h) oocyte activation, probably through inhibition of class IIb HDACs or most likely HDAC 10, is important for improving preimplantation and full-term development. In the present study, treatment with NaBu or SCR for 24 h from the 4-cell stage increased Oct4 expression and ICM cell numbers in the resultant blastocysts but not blastocyst formation rates, as was the case with VPA treatment. VPA and NaBu are inhibitors for class I and IIa HDACs [16, 51], whereas SCR is an inhibitor for class I and IIa/b [9, 15], suggesting that inhibiting class I and IIa HDACs from the 4-cell stage is somehow involved in improving the Oct4 expression and ICM cell numbers of mouse SCNT blastocysts. However, the reason for the beneficial effects on SCNT blastocysts remains to be defined.

VPA is also known to be an inhibitor of glycogen synthase kinase-3 (GSK3) [56]. A substrate of GSK3, β-catenin, is the effector molecule of the Wnt/β-catenin signaling pathway. GSK3-mediated phosphorylation of β-catenin results in its ubiquitination and pro-teasomal degradation. It was recently found that stabilized β-catenin forms a complex with and enhances the activity of Oct4 in ES cells [57, 58]. Therefore, it is possible that inhibition of GSK3 with VPA prevents the degradation of β-catenin, leading to an increase in

Oct4 expression. In addition, it was reported that GSK3 is present in bovine embryos at the 2-cell to blastocyst stages, and an accurate regulation of GSK3 activity during the developmental stage is important to achieve normal mouse embryo development [59]. Thus, it is assumed that the effects of VPA treatment on Oct4 expression of SCNT embryos might be exerted via a molecular pathway mediated by GSK3 inhibition. Further studies are needed to clarify whether VPA treatment improves the Oct4 expression and ICM cell numbers in SCNT blastocysts via the β-catenin signaling pathway.

Finally, the present study demonstrated that Oct4 expression and the distribution of H3K27me3 foci within cell nuclei in SCNT blastocysts are improved by VPA treatment. As mentioned above, Oct4 is involved in the repression of *Xist* expression in ES cells [40–42], and moreover, Oct4 lies at the top of the XCI hierarchy and regulates XCI by triggering X-chromosome pairing and counting [40]. From these findings, the significant reduction in the proportion of cells showing an abnormal nuclear distribution of H3K27me3 foci in SCNT blastocysts following VPA treatment can be attributed to increased levels of Oct4 expression, which might be associated with the correction of SCNT-specific aberrant *Xist* expression, because H3K27me3 is responsible for the repressive chromatin state in the inactive X chromosome [30, 36–38]. It has been found that *Xist* is ectopically expressed from the active X chromosome in cloned mouse embryos of both sexes and that the ectopic expression of *Xist* first appears at the 4-cell stage [28] and increases up to the blastocyst stage [60, 61]. Accordingly, since the effects of VPA on the Oct4 expression, ICM cell numbers and nuclear distribution of foci of H3K27me3 in mouse SCNT blastocysts are greatest with treatment for 24 h from the 4-cell stage, as compared with the 1-cell or 2-cell

stage, the optimum timing and duration of VPA treatment may be associated with the timing of the ectopic expression of *Xist*. Taken together, it is expected that VPA treatment may also have a beneficial influence on full-term development as well as preimplantation development of mouse SCNT embryos.

In summary, the present study indicates that treatment with 1 mM VPA for 24 h from the 4-cell stage in mouse SCNT embryos greatly improves the quality of the resultant blastocysts, with increases in Oct4 expression, ICM cell numbers and the proportion of cells with a normal nuclear distribution of H3K27me3 foci. Because treatment with SCR and NaBu from the 4-cell stage also increased Oct4 levels and ICM cell numbers in the SCNT blastocysts, inhibiting class I and IIa HDACs from the 4-cell stage is important for improving blastocyst quality. These findings provide new insights into improving the success rate for mouse cloning using HDACis.

Acknowledgments

References

1. **Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS.** Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; **385**: 810–813. [Medline] [CrossRef]

2. **Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R.** Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; **394**: 369–374. [Medline] [CrossRef]

3. **Thuan NV, Kishigami S, Wakayama T.** How to improve the success rate of mouse cloning technology. *J Reprod Dev* 2010; **56**: 20–30. [Medline] [CrossRef]

4. **Wakayama T.** Production of cloned mice and ES cells from adult somatic cells by nuclear transfer: how to improve cloning efficiency? *J Reprod Dev* 2007; **53**: 13–26. [Medline] [CrossRef]

5. **Kishigami S, Mizutani E, Ohta H, Hikichi T, Van Thuan N, Wakayama S, Bui HT, Wakayama T.** Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochem Biophys Res Commun* 2006; **340**: 183–189. [Medline] [CrossRef]

6. **Maalouf WE, Liu Z, Brochard V, Renard JP, Debey P, Beaujean N, Zink D.** Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin remodeling as well as developmental potential to term. *BMC Dev Biol* 2009; **9**: 11. [Medline] [CrossRef]

7. **Costa-Borges N, Santaló J, Ibáñez E.** Comparison between the effects of valproic acid and trichostatin A on the *in vitro* development, blastocyst quality, and full-term development of mouse somatic cell nuclear transfer embryos. *Cell Reprogram* 2010; **12**: 437–446. [Medline] [CrossRef]

8. **Rybouchkin A, Kato Y, Tsunoda Y.** Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. *Biol Reprod* 2006; **74**: 1083–1089. [Medline] [CrossRef]

9. **Ono T, Li C, Mizutani E, Terashita Y, Yamagata K, Wakayama T.** Inhibition of class IIb histone deacetylase significantly improves cloning efficiency in mice. *Biol Reprod* 2010; **83**: 929–937. [Medline] [CrossRef]

10. **Tsuji Y, Kato Y, Tsunoda Y.** The developmental potential of mouse somatic cell nuclear-transferred oocytes treated with trichostatin A and 5-aza-2'-deoxycytidine. *Zygote* 2009; **17**: 109–115. [Medline] [CrossRef]

11. **Kishigami S, Bui HT, Wakayama S, Tokunaga K, Van Thuan N, Hikichi T, Mizutani E, Ohta H, Suetsugu R, Sata T, Wakayama T.** Successful mouse cloning of an outbred strain by trichostatin A treatment after somatic nuclear transfer. *J Reprod Dev* 2007; **53**: 165–170. [Medline] [CrossRef]

12. **Van Thuan N, Bui HT, Kim JH, Hikichi T, Wakayama S, Kishigami S, Mizutani E, Wakayama T.** The histone deacetylase inhibitor scriptaid enhances nascent mRNA production and rescues full-term development in cloned inbred mice. *Reproduction* 2009; **138**: 309–317. [Medline] [CrossRef]

13. **Dai X, Hao J, Hou XJ, Hai T, Fan Y, Yu Y, Jouneau A, Wang L, Zhou Q.** Somatic nucleus reprogramming is significantly improved by m-carboxycinnamic acid bishydroxamide, a histone deacetylase inhibitor. *J Biol Chem* 2010; **285**: 31002–31010. [Medline] [CrossRef]

14. **Hirata S, Fukasawa H, Tagaya H, Shoda T, Wakayama T, Hoshi K.** Effects of sirtinol on early development of the cloned murine embryos. *Yamanashi Med J* 2008; **23**: 97–107.

15. **Blackwell L, Norris J, Suto CM, Janzen WP.** The use of diversity profiling to characterize chemical modulators of the histone deacetylases. *Life Sci* 2008; **82**: 1050–1058. [Medline] [CrossRef]

16. **Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT.** Multiple roles of HDAC inhibition in neurodegenerative conditions. *Trends Neurosci* 2009; **32**: 591–601. [Medline] [CrossRef]

17. **Kuhn AN, Van Santen MA, Schwienhorst A, Urlaub H, Lührmann R.** Stalling of spliceosome assembly at distinct stages by small-molecule inhibitors of protein acetylation and deacetylation. *RNA* 2009; **15**: 153–175. [Medline] [CrossRef]

18. **Codd R, Braich N, Liu J, Soe CZ, Pakchung AAH.** Zn (II)-dependent histone deacetylase inhibitors: suberoylanilide hydroxamic acid and trichostatin A. *Int J Biochem Cell Biol* 2009; **41**: 736–739. [Medline] [CrossRef]

19. **Mai A, Massa S, Ragno R, Cerbara I, Jesacher F, Loidl P, Brosch G.** 3-(4-Aroyl-1-methyl-1H-2-pyrrolyl)-N-hydroxy-2-alkylamides as a new class of synthetic histone deacetylase inhibitors. 1. Design, synthesis, biological evaluation, and binding mode studies performed through three different docking procedures. *J Med Chem* 2003; **46**: 512–524. [Medline] [CrossRef]

20. **Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, Sato M.** Valproic acid enhances *in vitro* development and Oct-3/4 expression of miniature pig somatic cell nuclear transfer embryos. *Cell Reprogram* 2010; **12**: 67–74. [Medline] [CrossRef]

21. **VerMilyea MD, O'Neill LP, Turner BM.** Transcription-independent heritability of induced histone modifications in the mouse preimplantation embryo. *PLoS ONE* 2009; **4**: 9. [Medline] [CrossRef]

22. **Palmieri SL, Peter W, Hess H, Schöler HR.** Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol* 1994; **166**: 259–267. [Medline] [CrossRef]

23. **Boiani M, Eckardt S, Schöler HR, McLaughlin KJ.** Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev* 2002; **16**: 1209–1219. [Medline] [CrossRef]

24. **Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC, Jaenisch R.** Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. *Development* 2003; **130**: 1673–1680. [Medline] [CrossRef]

25. **Boiani M, Gentile L, Gambles VV, Cavaleri F, Redi CA, Schöler HR.** Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments. *Stem Cells* 2005; **23**: 1089–1104. [Medline] [CrossRef]

26. **Kishigami S, Hikichi T, Van Thuan N, Ohta H, Wakayama S, Bui HT, Mizutani E, Wakayama T.** Normal specification of the extraembryonic lineage after somatic nuclear transfer. *FEBS Lett* 2006; **580**: 1801–1806. [Medline] [CrossRef]

27. **Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A.** Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 1998; **95**: 379–391. [Medline] [CrossRef]

28. **Inoue K, Kohda T, Sugimoto M, Sado T, Ogonuki N, Matoba S, Shiura H, Ikeda R, Mochida K, Fujii T, Sawai K, Otte AP, Tian XC, Yang X, Ishino F, Abe K, Ogura A.** Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. *Science* 2010; **330**: 496–499. [Medline] [CrossRef]

29. **Nolen LD, Gao S, Han Z, Mann MRW, Gie Chung Y, Otte AP, Bartolomei MS, Latham KE.** X chromosome reactivation and regulation in cloned embryos. *Dev Biol* 2005; **279**: 525–540. [Medline] [CrossRef]

30. **Bao S, Miyoshi N, Okamoto I, Jenuwein T, Heard E, Azim Surani M.** Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to a mouse oocyte. *EMBO Rep* 2005; **6**: 748–754. [Medline] [CrossRef]

31. **Eggan K, Akutsu H, Hochedlinger K, Rideout W, Yanagimachi R, Jaenisch R.** X-Chromosome inactivation in cloned mouse embryos. *Science* 2000; **290**: 1578–1581. [Medline] [CrossRef]

32. **Senda S, Wakayama T, Yamazaki Y, Ohgane J, Hattori N, Tanaka S, Yanagimachi R, Shiota K.** Skewed X-inactivation in cloned mice. *Biochem Biophys Res Commun* 2004; **321**: 38–44. [Medline] [CrossRef]

33. **Brockdorff N, Ashworth A, Kay GF, Cooper P, Smith S, McCabe VM, Norris DP, Penny GD, Patel D, Rastan S.** Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature* 1991; **351**: 329–331. [Medline] [CrossRef]

34. **Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, Tonlorenzi R, Willard HF.** A gene from the region of the human X chromosome inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 1991; **349**: 38–44. [Medline] [CrossRef]

35. **Borsani G, Tonlorenzi R, Simmler MC, Dandolo L, Arnaud D, Capra V, Grompe M,**

Pizzuti A, Muzny D, Lawrence C, Willard HF, Avner P, Ballabio A. Characterization of a murine gene expressed from the inactive X chromosome. *Nature* 1991; **351**: 325–329. [Medline] [CrossRef]

36. **Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, De La Cruz CC, Otte AP, Panning B, Zhang Y.** Role of histone H3 lysine 27 methylation in X inactivation. *Science* 2003; **300**: 131–135. [Medline] [CrossRef]

37. **Kohlmaier A, Savarese F, Lachner M, Martens J, Jenuwein T, Wutz A.** A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. *PLoS Biol* 2004; **2**: e171. [Medline] [CrossRef]

38. **Rougeulle C, Chaumeil J, Sarma K, Allis CD, Reinberg D, Avner P, Heard E.** Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. *Mol Cell Biol* 2004; **24**: 5475–5484. [Medline] [CrossRef]

39. **Keohane AM, Barlow AL, Waters J, Bourn D, Turner BM.** H4 acetylation, XIST RNA and replication timing are coincident and define x;autosome boundaries in two abnormal X chromosomes. *Hum Mol Genet* 1999; **8**: 377–383. [Medline] [CrossRef]

40. **Donohoe ME, Silva SS, Pinter SF, Xu N, Lee JT.** The pluripotency factor Oct4 interacts with Ctcf and also controls X-chromosome pairing and counting. *Nature* 2009; **460**: 128–132. [Medline] [CrossRef]

41. **Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P.** Molecular coupling of Xist regulation and pluripotency. *Science* 2008; **321**: 1693–1695. [Medline] [CrossRef]

42. **Navarro P, Oldfield A, Legoupi J, Festuccia N, Dubois A, Attia M, Schoorlemmer J, Rougeulle C, Chambers I, Avner P.** Molecular coupling of Tsix regulation and pluripotency. *Nature* 2010; **468**: 457–460. [Medline] [CrossRef]

43. **Navarro P, Avner P.** When X-inactivation meets pluripotency: an intimate rendezvous. *FEBS Letters* 2009; **583**: 1721–1727. [Medline] [CrossRef]

44. **Erwin JA, Del Rosario B, Payer B, Lee JT.** An *ex vivo* model for imprinting: mutually exclusive binding of Cdx2 and Oct4 as a switch for imprinted and random X-inactivation. *Genetics* 2012; **192**: 857–868. [CrossRef]. [Medline]

45. **Kimura Y, Yanagimachi R.** Intracytoplasmic sperm injection in the mouse. *Biol Reprod* 1995; **52**: 709–720. [Medline] [CrossRef]

46. **Ho Y, Wigglesworth K, Eppig JJ, Schultz RM.** Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995; **41**: 232–238. [Medline] [CrossRef]

47. **Suzuki T, Minami N, Kono T, Imai H.** Zygotically activated genes are suppressed in mouse nuclear transferred embryos. *Cloning Stem Cells* 2006; **8**: 295–304. [Medline] [CrossRef]

48. **Choi YH, Toyoda Y.** Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. *Biol Reprod.* 1998; **59**: 1328–1333. [Medline]

[CrossRef]

49. **Ohta H, Sakaide Y, Wakayama T.** Generation of mice derived from embryonic stem cells using blastocysts of different developmental ages. *Reproduction* 2008; **136**: 581–587. [Medline] [CrossRef]

50. **Su J, Wang Y, Li Y, Li R, Li Q, Wu Y, Quan F, Liu J, Guo Z, Zhang Y.** Oxamflatin significantly improves nuclear reprogramming, blastocyst quality, and *in vitro* development of bovine SCNT embryos. *PLoS One* 2011; **6**: e23805. [Medline] [CrossRef]

51. **Davie JR.** Inhibition of histone deacetylase activity by butyrate. *J Nutr* 2003; **133**: 2485S–2493S. [Medline]

52. **Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R.** Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc Natl Acad Sci USA* 2002; **99**: 12889–12894. [Medline] [CrossRef]

53. **Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogura A.** Faithful expression of imprinted genes in cloned mice. *Science* 2002; **295**: 297. [Medline] [CrossRef]

54. **Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R, Shiota K.** The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. *Genes Cells* 2004; **9**: 253–260. [Medline] [CrossRef]

55. **Wang F, Kou Z, Zhang Y, Gao S.** Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. *Biol Reprod* 2007; **77**: 1007–1016. [Medline] [CrossRef]

56. **Williams RSB, Cheng L, Mudge AW, Harwood AJ.** A common mechanism of action for three mood-stabilizing drugs. *Nature* 2002; **417**: 292–295. [Medline] [CrossRef]

57. **Kelly KF, Ng DY, Jayakumaran G, Wood GA, Koide H, Doble BW.** β-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. *Cell Stem Cell* 2011; **8**: 214–227. [Medline] [CrossRef]

58. **Li J, Li J, Chen B.** Oct4 was a novel target of Wnt signaling pathway. *Mol Cell Biochem* 2012; **362**: 233–240. [Medline] [CrossRef]

59. **Aparicio IM, Garcia-Herreros M, Fair T, Lonergan P.** Identification and regulation of glycogen synthase kinase-3 during bovine embryo development. *Reproduction* 2010; **140**: 83–92. [Medline] [CrossRef]

60. **Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E.** Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 2004; **303**: 644–649. [Medline] [CrossRef]

61. **Patrat C, Okamoto I, Diabangouaya P, Vialon V, Le Baccon P, Chow J, Heard E.** Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. *Proc Natl Acad Sci USA* 2009; **106**: 5198–5203. [Medline] [CrossRef]