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—Original Article—

Suberoylanilide hydroxamic acid during *in vitro* culture improves development of dog-pig interspecies cloned embryos but not dog cloned embryos

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Abstract. This study was conducted to investigate whether the treatment of dog to pig interspecies somatic cell nuclear transfer (iSCNT) embryos with a histone deacetylase inhibitor, to improve nuclear reprogramming, can be applied to dog SCNT embryos. The dog to pig iSCNT embryos were cultured in fresh porcine zygote medium-5 (PZM-5) with 0, 1, or 10 μM suberoylanilide hydroxamic acid (SAHA) for 6 h, then transferred to PZM-5 without SAHA. Although there were no significant differences in cleavage rates, the rates of 5-8-cell stage embryo development were significantly higher in the 10 μM group (19.5 ± 0.8%) compared to the 0 μM groups (13.4 ± 0.8%). Acetylation of H3K9 was also significantly higher in embryos beyond the 4-cell stage in the 10 μM group compared to the 0 or 1 μM groups. Treatment with 10 μM SAHA for 6 h was chosen for application to dog SCNT. Dog cloned embryos with 0 or 10 μM SAHA were transferred to recipients. However, there were no significant differences in pregnancy and delivery rates between the two groups. Therefore, it can be concluded that although porcine oocytes support nuclear reprogramming of dog fibroblasts, treatment with a histone deacetylase inhibitor that supports nuclear reprogramming in dog to pig iSCNT embryos was not sufficient for reprogramming in dog SCNT embryos.

Key words: Cloning, Dog, Histone deacetylase inhibitor, Interspecies somatic cell nuclear transfer, Pig

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Interspecies somatic cell nuclear transfer (iSCNT) is a technique used for donor cell transplantation into a recipient enucleated oocyte derived from a different species, family, order, or class. Live births of clones using iSCNT have been reported when oocytes and donor cells share closely-related genetic backgrounds, such as cloned gaur with cattle oocytes [1], cloned European mouflon with sheep oocytes [2], cloned sand cat with domestic cat oocytes [3], and cloned Asian wolf with dog oocytes [4]. Although no offspring have been produced from iSCNT embryos reconstructed with interfamily, interorder, or interclass cell-oocyte couplets, oocytes derived from some species have been frequently used as recipient cytoplasm regardless of the donor cell species to produce hybrid blastocysts for studying the interaction between an oocyte and a somatic nucleus. For example, interfamily SCNT embryos reconstructed by the nuclear transfer of rabbit fetal fibroblasts or cattle fibroblasts into porcine oocytes developed into blastocysts with 5.3% [5] or 6.3% [6] efficiency, respectively. Moreover, the success of interorder SCNT blastocysts ranging from 0.6 to 24.0% efficiency were observed when Siberian tiger skin fibroblasts [7], dog dewclaw fibroblasts [8], or mouse fibroblasts [6] were injected into pig oocytes. These results indicate that porcine oocytes can support the reprogramming of somatic cells derived from various mammalian species and thus could be a candidate for a universal recipient cytoplasm for iSCNT. A universal recipient cytoplasm is especially valuable for producing iSCNT embryos of species in which oocytes are difficult to obtain, usually wildlife or endangered animals.

Although dogs are widely used as companion and experimental animals, many assisted reproduction technologies (ARTs) that depend on *in vitro* matured oocytes have suffered from unsatisfactory results compared to other domesticated animals such as cows, sheep, and pigs. The first cloned dog, Snuppy, was born using *in vivo* matured oocytes in 2005 [9], and since then, dog SCNT has been one of the basic ARTs used in this species. When Snuppy was produced, the cloning efficiency was only 0.2% [10], but increased to 1.8% in a subsequent study [11]. Many efforts have been made to improve cloning efficiency by controlling *in vitro* processes (donor cell cycle synchronization [12], donor cell fusion conditions [13], primary culture media for donor cells [14], increased histone acetylation of donor cells [15], and activation media [16]), as well as *in vivo* procedures (criteria for oocyte donors and recipients [17], mineral treatment of oocyte donors [18], using mated dogs as recipients [19], and safer delivery methods for cloned dogs [20]). However, the dog cloning

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efficiency in recent studies is still only 1.6% [16] to 4.2% [15].

Epigenetic modification is defined as the alteration of chromatin structure to its open or closed state to facilitate changes in genomic activation that can affect gene expression patterns. Modifications include DNA methylation, DNA hydroxymethylation, and post-translational histone modifications (methylation, acetylation, and ubiquitination). Histone acetylation is often associated with an open chromatin structure and active transcription, while histone deacetylation is associated with a closed chromatin structure and repressed transcription. Histone deacetylase inhibitors (HDACi) disturb the role of histone deacetylase, which removes acetyl groups from histone tails, and induce an open chromatin structure. There have been several reports that HDACi could increase cloning efficiency by changing the epigenetic status of cloned embryos [21, 22]. Therefore, in this study, we attempted to develop a protocol to increase the acetylation of dog cloned embryos to improve cloning efficiency.

An optimal *in vitro* oocyte maturation culture system has not yet been established [23]. It is currently difficult to obtain large numbers of *in vivo* matured oocytes at the same time, due to the lack of an estrus synchronization protocol, an average of only 11–12 oocytes per dog in estrus [17], and the long period of anestrus in dogs, which lasts up to 6 months. To overcome the limited number of oocytes available for nuclear transfer experiments and the lack of an *in vitro* culture system for dog embryos, we investigated whether a chromatin-modifying agent used in dog to pig iSCNT that improves nuclear reprogramming could be applied to dog SCNT. Based on a previous report of successful blastocyst formation using dog to pig iSCNT embryos [8], we hypothesized that pig oocytes could be used to assess the *in vitro* reprogramming and developmental competence of dog fibroblast nuclei. A protocol was developed to determine the appropriate concentration and duration of suberoylanilide hydroxamic acid (SAHA), an HDACi, treatment to improve reprogramming of dog to pig iSCNT embryos, and applied to dog SCNT embryos for comparison.

Materials and Methods

Animal use

Animal experiments were performed following a standard procedure established by the Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Seoul National University (approval number is SNU-121123-13). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Collection and in vitro maturation of pig oocytes

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 3 h in sterile saline at 32–35°C. Cumulus oocyte complexes (COC) were collected from follicles of 3–6 mm diameter by aspiration with an 18-gauge needle. Oocytes with homogeneous ooplasm surrounded by several compact layers of cumulus cells were selected and washed three times with tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA) containing 5 mM sodium hydroxide, 2 mM sodium bicarbonate, 10 mM N-[2-Hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), 0.3% polyvinyl alcohol (PVA), and 1% Pen-Strep (Invitrogen). The COCs were placed into *in vitro* maturation medium (IVM) containing TCM-199 supplemented with 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μl/ml insulin-transferrin-selenium solution (ITS-A) 100X (Invitrogen), 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluid (v/v), 10 IU/l equine chorionic gonadotropin (eCG), and 10 IU/ml human chorionic gonadotropin (hCG), and incubated at 38.5°C under 5% CO_2 in 95% humidified air. Following 21–22 h of maturation with hormones, the COCs were washed twice in fresh, hormone-free IVM medium and then cultured for an additional 21–22 h.

Donor cell preparation for somatic cell nuclear transfer

Skin tissues were aseptically collected from a 6-year-old German Shepherd dog and brought to the laboratory within 2 h in sterile saline at 4°C. Tissues were washed three times in phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA, USA), and minced in a culture medium composed of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and antibiotics. Cells were cultured at 39°C in a humidified atmosphere of 5% CO_2 and 95% air, until confluence. Then, the cells were retrieved by trypsinization and cryopreserved with dimethyl sulfoxide supplemented with 10% (v/v) FBS in liquid nitrogen. The cryopreserved cells were thawed and cultured with DMEM supplemented with 10% FBS, and donor cells were retrieved as single cells by trypsinization just after denuding the *in vitro* matured pig oocytes or recovering the *in vivo* matured dog oocytes. Cells between passages 2 and 6 were used as donors for the following studies.

Dog to pig somatic cell nuclear transfer

Porcine oocytes were denuded by gentle pipetting with 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP) medium with HEPES buffer after 44 h of IVM. Denuded oocytes were stained with 5 μg/ml of bisbenzimide in TALP-HEPES for 10 min, then nuclear material was aspirated from oocytes with a first polar body in TALP medium droplets containing 7.5 μg/ml of cytochalasin B under a fluorescence microscope (Nikon, Tokyo, Japan). A single dog fibroblast with a round shape and smooth margin was selected and injected into the perivitelline space of each enucleated oocyte. The oocyte-cell couplets were equilibrated in fusion solution (0.28 M mannitol containing 0.5 mM HEPES and 0.1 mM $MgSO_4$), and then fused in the fusion solution with a single DC pulse of 200 V/mm for 30 μsec using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After incubation in porcine zygote medium-5 (PZM-5, Funakoshi, Tokyo, Japan) for 30 min, fused couplets were equilibrated in activation solution (0.28 M mannitol containing 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$). Then, the couplets were activated with a single DC pulse of 1.5 kV/cm for 60 μsec using a BTX Electrocell Manipulator 2001 (BTX, San Diego, USA) in a chamber containing two electrodes overlaid with activation solution. Reconstructed embryos were cultured with fresh PZM-5 droplets containing 0, 1, or 10 μM SAHA covered with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 6 h, then transferred to PZM-5 droplets (day 0). Cleavage and early development rates were recorded on day 2 and day 3, respectively.

Immunocytostaining

Immunocytostaining was performed based on a previous report [15]. In brief, embryos on day 3 were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS, then blocked with 5% goat serum in PBS for 1 h. Anti-histone H3 acetyl K9 (H3K9) antibody (Abcam, Cambridge, MA, USA) diluted to 1:200 was added to the embryos at room temperature for 1 h. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Abcam) diluted to 1:200 was used as secondary antibody. Embryos were observed with confocal microscopy after 4′,6-diamidino-2-phenylindole (DAPI) staining, and fluorescence was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative FITC intensity was calculated by dividing the mean FITC intensity (the mean intensity of the nucleus subtracted from the mean intensity of the ooplasm) by the DAPI intensity (the mean intensity of the nucleus subtracted from the mean intensity of the ooplasm).

Retrieval of dog in vivo matured oocytes

Serum progesterone concentrations of proestrus female dogs were analyzed with Immulite 1000 (Siemens Healthcare Diagnostics, Flanders, NJ, USA), and ovulation was determined according to our previous study [24]. Approximately 72 h after ovulation, dogs were anesthetized with ketamine and xylazine *via* intravenous injection, and anesthesia was maintained with isoflurane. Dogs were placed in the ventrodorsal position, and the abdominal region was prepared aseptically. Ovaries were exteriorized following a midline incision, and a flushing needle was inserted into the infundibulum orifice. After securing the flushing needle with a ligature, an intravenous catheter was inserted into the caudal portion of the oviduct. The flushing medium, which consisted of HEPES-buffered TCM-199 supplemented with 10% (v/v) FBS, was injected though the catheter, and ovulated COCs in the oviduct were collected through the flushing needle with the flushed medium. The COCs in the flushing medium were loaded into a straw, and transported to the laboratory within 30 min at 37°C.

Dog somatic cell nuclear transfer

Cumulus cells were removed by repeated pipetting of COCs in 0.1% (v/v) hyaluronidase in TCM-199, and denuded oocytes underwent enucleation in TCM-199 containing 5 mg/ml of cytochalasin B and 5 mg/ml of bisbenzimide. The same fibroblasts used for iSCNT were prepared, and a single cell was injected into the perivitelline space of each enucleated oocyte. The oocyte-cell couplets were equilibrated in fusion solution (0.26 M mannitol containing 0.5 mM HEPES and 0.2 mM $MgSO_4$) and then fused in the fusion solution with two pulses of 72 V for 15 μsec using Electro-Cell Fusion apparatus (Nepa Gene). Fused embryos were activated with 10 μM calcium ionophore for 4 min, and then incubated with 1.9 mM 6-dimethylaminopurine containing 0 or 10 μM SAHA for 2 h. Then, the cloned embryos were transferred to fresh PZM-5 droplets containing 0 or 10 μM SAHA for an additional 4 h of culture. After *in vitro* culture, embryos loaded in HEPES-buffered TCM-199 medium were transferred into the end of a 3.5 Fr Tom Cat Catheter (Sherwood, St. Louis, MO, USA), and surgically transferred into an oviduct of a synchronized recipient dog [17]. Pregnancy diagnosis was performed at least 27 days after the embryo transfer by ultrasonography, and serum progesterone concentrations and fetal heartbeat were monitored for safe delivery [20].

Statistical analysis

All experiments were repeated over three times. The data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare the *in vitro* development of the dog to pig iSCNT embryos and the relative intensity levels of H3K9 acetylation among the groups. A chi-square test was used to compare the pregnancy and delivery rates between the two groups. The significance level was $P < 0.05$.

Results

In vitro development of dog to pig cloned embryos with SAHA treatment

A total of 127, 125, and 123 iSCNT cloned embryos were produced after 0, 1, or 10 μM SAHA treatment, respectively, during *in vitro* culture (Table 1); there were no significant differences in cleavage rates (74.8 ± 1.7, 73.6 ± 3.0, or 72.4 ± 3.4%, respectively). However, the rate of development to a 4-cell stage embryo was significantly higher in the 10 μM SAHA group (48.8 ± 3.1%) than in the control group (37.8 ± 1.4%). The percentage of 5-8-cell stage embryos was also significantly higher in the 10 μM SAHA group (19.5 ± 0.8%) compared to the control group (13.4 ± 0.8%). No blastocysts were formed in any of the experimental groups.

H3K9 acetylation of dog to pig cloned embryos with SAHA treatment

The relative intensity of green H3K9 acetylation fluorescence to blue nucleus fluorescence in 2-cell stage embryos was not different among the 0, 1, and 10 μM SAHA-treated groups (66.9 ± 4.6, 69.0 ± 2.2, and 67.9 ± 2.4%, respectively) (Fig. 1, Fig. 2). However, acetylation fluorescence was reduced during embryo development, and the highest intensity was observed in 4-cell stage embryos and 5-8-cell stage embryos (44.6 ± 1.1 and 27.0 ± 1.2%, respectively) of the 10 μM SAHA group compared to the control (17.4 ± 0.7 and 14.6 ± 0.2%, respectively) or 1 μM SAHA treatment (27.5 ± 1.5 and 16.2 ± 0.1%, respectively) groups.

Production of cloned dogs by SAHA treatment

Based on the embryo development and H3K9 acetylation results, 10 μM SAHA was chosen for dog SCNT. A total of 112 and 77 fibroblast-oocyte couplets were produced in the control and 10 μM SAHA-treated groups. The average fusion rates of those couplets were 79.8 ± 2.2 and 86.7 ± 3.3% in the control and treatment groups, respectively. Within the control and treatment group, 92 and 55 cloned embryos were selected for transfer into the oviducts of five and four recipient dogs, respectively (Table 2). The average number of transferred cloned embryos per dog was 18.4 ± 2.7 and 14.0 ± 1.5 for the control and the 10 μM SAHA-treated group, respectively. About 60 days after embryo transfer, three and one cloned puppies were delivered by cesarean section from two and one recipients in the control and SAHA-treated groups, respectively (Fig. 3). The delivery rate was 3.3% for the control and 1.8% for the SAHA groups. The birth weight of the clone derived from the SAHA-treated group

Table 1. Development of dog to pig interspecies somatic cell nuclear transfer (iSCNT) embryos after 0, 1, or 10 μM SAHA treatment during 6 h of *in vitro* culture

SAHA concentration (μM)	2 cell (%)	4 cell (%)	5-8 cell (%)	Total no. of embryos
0	95 (74.8 ± 1.7)	48 (37.8 ± 1.4) [a]	17 (13.4 ± 0.8) [a]	127
1	92 (73.6 ± 3.0)	52 (41.6 ± 1.7) [ab]	21 (16.8 ± 1.1) [ab]	125
10	89 (72.4 ± 3.4)	60 (48.8 ± 3.1) [b]	24 (19.5 ± 0.8) [b]	123

Within a column, different superscripts (a, b) represent significant differences between treatment groups at $P < 0.05$.

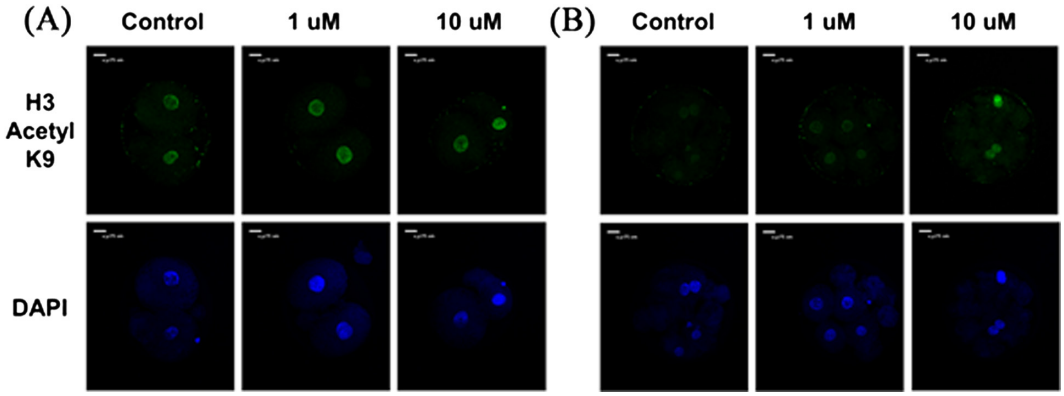

Fig. 1. Immunolocalization of H3K9 acetylation in 2-cell (A) and 4-cell (B) dog to pig interspecies somatic cell nuclear transfer (iSCNT) embryos after 0, 1, or 10 μM SAHA treatment during *in vitro* culture.

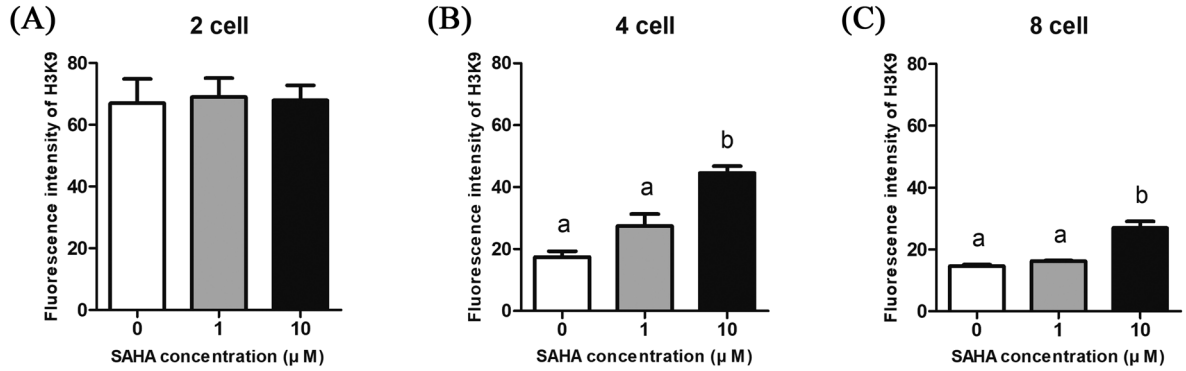

Fig. 2. Relative fluorescence intensity levels of H3K9 acetylation in 2-cell (A), 4-cell (B), and 5-8-cell (C) dog to pig interspecies somatic cell nuclear transfer (iSCNT) embryos after 0, 1, or 10 μM SAHA treatment during 6 h of *in vitro* culture. Different superscripts (a, b) represent significant differences between treatment groups at $P < 0.05$.

was 440 g, which was slightly lower than those from the control group (635, 640, and 560 g), but all pups were healthy, with normal morphology. Their genetic identity with the cell donor was confirmed by microsatellite analysis (Supplementary Table 1: online only).

Discussion

Somatic cell nuclear transfer involves a process called nuclear reprogramming, which is the reversal of the differentiated state of a mature cell to that characteristic of the undifferentiated embryonic state [25]. Although genetic material is not usually lost during the differentiation of a cell, expression is mainly regulated by epigenetic changes such as DNA methylation and histone modification. This suggests that epigenetic modification of a donor nucleus by chromatin-modifying agents could be a crucial factor for improving cloning efficiency [26, 27]. Indeed, treatment of cloned embryos with HDACi has been reported to increase blastocyst formation rate or production of cloned animals. Treatment of porcine cloned embryos with 5 mM

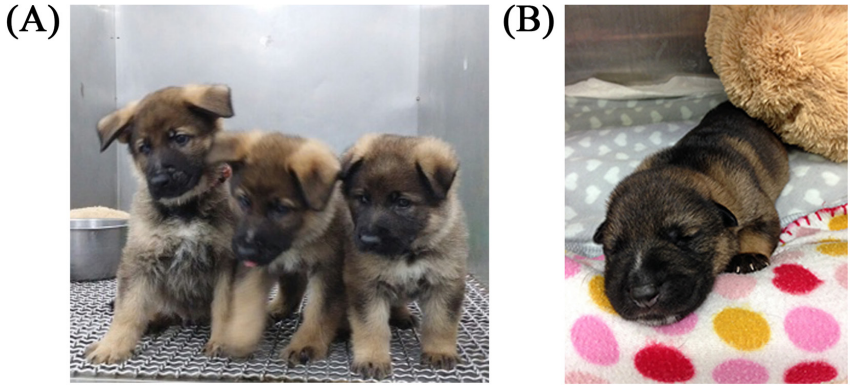

Fig. 3. Photographs of cloned puppies derived from somatic cell nuclear transfer (SCNT) embryos treated with 0 (A) and 10 µM (B) SAHA during 6 h of *in vitro* culture.

Table 2. Production of cloned dogs using somatic cell nuclear transfer (SCNT) embryos treated with 0 or 10 µM SAHA during 6 h of *in vitro* culture

SAHA concentration (µM)	No. recipients	No. transferred embryos	No. pregnant (% [a])	No. clones (% [b])
0	5	92	2 (40.0)	3 (3.3)
10	4	55	1 (25.0)	1 (1.8)

[a] Pregnancy rate was calculated by dividing the number of pregnant females by the number of recipients. [b] Delivery rate was calculated by dividing the number of clones by the number of transferred embryos.

sodium butyrate for 4 h post-activation enhanced the blastocyst formation rate [28]. The optimal concentration and duration of HDACi treatment is different for different species, including mice [29, 30] and cattle [21, 22], for the improvement of *in vitro* or *in vivo* development. Treatment of iSCNT embryos with HDACi has also been reported to increase development rates, similar to HDACi treatment of SCNT embryos. For example, the blastocyst formation rate was significantly higher in both cat to cow iSCNT embryos and bovine parthenogenetically activated embryos treated with 50 nM trichostatin A (TSA), compared to non-TSA iSCNT embryos [31]. Treatment with 50 nM TSA also improved the development to 8-cell or morula stages in both bovine SCNT and gaur to bovine iSCNT embryos [32]. Therefore, we hypothesized that HDACi treatment may increase the reprogramming of dog nuclei in dog to pig iSCNT embryos and may produce similar results in dog cloned embryos.

Among the various types of HDACi, SAHA was chosen because we previously confirmed that SAHA increases histone acetylation of dog cell nuclei [15]. Because an *in vitro* culture system to support dog embryo development to the blastocyst stage has not yet been established, an optimal concentration of SAHA was determined with dog to pig iSCNT embryos with 6 h of treatment. Although the iSCNT embryos did not reach the blastocyst stage, *in vitro* developmental competence and H3K9 acetylation were higher in the 10 µM SAHA group compared to the control group (Table 1). *Hypo* acetylation has generally been reported in cloned embryos compared with their *in vivo* counterparts, but HDACi has been shown to induce hyperacetylation levels in cloned embryos similar to those seen in fertilized embryos [28, 29, 33–35]. Because hyperacetylation of the paternal genome is linked to the onset of embryonic genome activation [26], the hyperacetylation induced by HDACi could support *in vitro* and *in vivo* embryonic development. The highest expression of H3K9 acetylation in the nucleus of 4-cell stage iSCNT embryos in the 10 µM SAHA group (Fig. 2) may support enhanced embryonic development to the 5-8-cell stage (Table 1).

As a preliminary study, dog to pig iSCNT embryos were cultured past day 7, but no blastocyst formation was observed. Failure of development into blastocysts might due to the different activation protocol and culture media used in our study compared to those reported by Sugimura *et al* [8]. Lagutina *et al.* also reported that dog to pig iSCNT embryos developed up to the 4- to 6-cell stage [36]. Failure of zygote genome activation is one of the main problems found in iSCNT embryos, which leads to embryo development arrest [37]. Because embryonic genome activation of pig and dog embryos occurs at the 4-cell and 8-cell stages, respectively, we focused on the early embryonic developmental competence up to the 8-cell stage in this study. The developmental rate of dog to pig iSCNT embryos up to 8-cells was highest in the 10 µM SAHA group (Table 1), similar to the hyperacetylation results. Therefore, we chose 10 µM SAHA treatment for the dog cloning procedure. Most of the dog cloning procedure was performed by *in vivo* processes due to the lack of established dog ARTs, data from embryo transfer, performed with conditions kept as comparable as possible, were selected and analyzed (Table 2). However, there was no difference between the control and SAHA-treated groups in the *in vivo* development of dog cloned embryos. This implies that, although pig oocytes can support dog nuclei during early embryonic development, the reprogrammed dog nuclei of early stage iSCNT embryos might have a different epigenetic status from that of SCNT embryos. The conflicting results between SCNT and iSCNT embryos is probably due to aberrant nuclear reprogramming and gene expression of iSCNT embryos [36, 38], or perhaps because the HDACi treatment conditions were established based on early embryonic development, not on development to the blastocyst stage.

In conclusion, porcine oocytes partly supported nuclear reprogramming of dog fibroblasts up to the early development of iSCNT embryos, but did not support progress to the blastocyst stage with our activation and culture methods. Additionally, epigenetic modification and embryonic development results with HDACi treatment based on dog to pig iSCNT embryos could not be replicated in dog SCNT embryos.

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