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Original Article

Pluripotency Maintenance in Mouse Somatic Cell Nuclear Transfer Embryos and Its Improvement by Treatment with the Histone Deacetylase Inhibitor TSA

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Abstract

Reprogramming of somatic cells to pluripotency can be achieved by nuclear transfer into enucleated oocytes (SCNT). A key event of this process is the demethylation of the *Oct4* gene and its temporally and spatially regulated expression. Different studies have shown that it occurs abnormally in some SCNT embryos. TSA is a histone deacetylase inhibitor known to increase the efficiency of development to term of SCNT embryos, but its impact on the developmental features of SCNT embryos is poorly understood. Here, we have followed the fate of the pluripotent cells within SCNT embryos, from the late blastocyst to the early epiblast prior to gastrulation. Our data show a delay in development correlated with a defect in forming and maintaining a correct number of Oct4 expressing ICM and epiblast cells in SCNT embryos. As a consequence, during the outgrowth phase of embryonic stem cell derivation as well as during diapause in vivo, part of the SCNT blastocysts completely lose their ICM cells. Meanwhile, the others display a correctly reprogrammed ICM compatible with the derivation of ES cells and development of the epiblast. Our data also indicate that TSA favors the establishment of pluripotency in SCNT embryos.

Introduction

Somatic cells can be reprogrammed into pluripotent cells using different processes: mostly by induction of ectopic expression of transcription factors (iPSCs) or nuclear transfer into enucleated oocytes (Hochedlinger and Plath, 2009). Epigenetic modifications are essential in the reprogramming process, as highlighted by the significant enhancement of the efficiency of reprogramming by drugs affecting chromatin modifications (Huangfu et al., 2008; Kishigami et al., 2006b; Mikkelsen et al., 2008; Rybouchkin et al., 2006). After its transfer into the enucleated oocytes, the donor chromatin undergoes profound remodeling so as to bring it to a state closer to the embryonic chromatin (Heffron et al., 2007; Merico et al., 2007). The use of TSA, a histone deacetylase inhibitor, to treat the somatic cell nuclear transfer (SCNT) embryos right after their reconstruction, could enhance this remodeling (Bui et al., 2010; Maalouf et al., 2009; Wang et al., 2007). The expression pattern of a subset of development-related genes and epigenetic modifier genes has been examined in SCNT blastocysts after TSA treatment (Li et al., 2008). Some genes were apparently TSA-responsive, but the results were difficult to correlate clearly with the beneficial effect of TSA on development.

The somatic nucleus has to shut down its differentiation program and to start to reexpress the pluripotent network of genes. Among them, activation of the expression of the endogenous *Oct4*, a key pluripotency factor, is an important event. In somatic cells, *Oct4* is heavily methylated and maintained in a silent state (Marikawa et al., 2005). After nuclear transfer, *Oct4* is reexpressed at the correct time but often incompletely demethylated (Yamazaki et al., 2006). Some SCNT blastocysts fail to express *Oct4* and other pluripotency factors at a correct level (Boiani et al., 2002; Bortvin et al., 2003; Sebastiano et al., 2005). By contrast, Cdx2, a transcription factor essential for the formation and maintenance of the trophoblast lineage, appears to be correctly expressed (Kishigami et al., 2006a). However, these studies

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were done at a stage when the spatial restriction of Oct4 within the inner cell mass (ICM) is not completed in fertilized embryos (Balbach et al., 2010), making it difficult to identify the fate of the pluripotent cells in SCNT embryos. Strikingly, although iPSCs exhibit a gene expression and epigenetic signature distinct from that of embryonic stem cells (ESCs), ESCs generated through SCNT (NT-ESCs) are very close to ESCs derived from fertilized (FT) embryos regardless of the low efficiency of nuclear transfer (Brambrink et al., 2006; Chin et al., 2009; Ding et al., 2009; Kim et al., 2010; Stadtfeld et al., 2010; Wakayama et al., 2006). It is possible that explanting embryos *in vitro* helps reprogramming as it releases them from the necessity to establish a correct crosstalk between the embryonic and extra-embryonic part of the conceptus at the time of implantation. Such crosstalk is essential for the further development of the embryo, its proliferation, and the formation of embryonic axes and germ lineages (Ang and Constam, 2004). Occurrence of many embryonic losses and the development of placentomegaly, which may be due to abnormal interactions between the two parts of the embryo, highlight how critical this period is for NT embryos (Jouneau et al., 2006; Miki et al., 2009; Rielland et al., 2009).

In this study, we have followed the fate of the pluripotent cells within SCNT embryos, from the late blastocyst stage to the early epiblast prior to gastrulation and their maintenance and expansion during NT-ESC derivation. During diapause, or delayed implantation, embryos maintain their epiblast with activation of the Lif/gp130 signaling pathway, as they do during ESC derivation in vitro (Nichols et al., 2001). Therefore, we have also examined the fate of the epiblast cells after induction of experimental diapause. Moreover, we have used TSA to treat the reconstructed embryos and to examine its effect on the reprogramming to pluripotency through NT.

Materials and Methods

All the experiments using animals are performed strictly in accordance with the Chinese Law regulating the administration of affairs concerning experimental animals and local animal ethical regulations.

Reagents and animals

All the reagents and chemicals used for this study were purchased from Sigma-Aldrich Inc. (St.Louis, MO, USA) unless otherwise mentioned.

B6D2F1 and ICR mice were purchased from Beijing Vital River Company. B6C2F1 female mice were obtained from the Animal Breeding Center of INRA, Jouy en Josas, France.

Nuclear transfer and TSA treatment

B6D2F1 or B6C2F1 mouse were used as the oocyte donors. Metaphase II oocytes were recovered from 8–12-week-old female mouse superovulated by sequential injection with 10 IU of pregnant mare serum gonadotropin (PMSG) and 10 IU of human chorionic gonadotropin (hCG).

Nuclear transfer was performed as previously described (Zhou et al., 2003). Briefly, the manipulation was performed in the cytochalasin B containing HEPES-buffered CZB. Cumulus nuclei were injected into the enucleated oocytes and reconstructed embryos activated in strontium containing CZB medium for 6 h. After activation, the reconstructed embryos were cultured in aMEM medium (GIBCO, Grand Island, NY, USA) up to blastocyst stage.

For TSA treatment, reconstructed embryos were activated for 6 h and cultured for a further 4 h in the same media as nuclear transfer embryos except for containing 5 nM TSA.

Embryo transfer

Embryos were either transferred at the two-cell stage into the oviduct of day 0.5 pseudopregnant ICR females or at the blastocyst stage into the uterus of day 2.5 ICR pseudopregnant females.

Induction of mouse embryonic diapause

Pregnant or pseudopregnant females were ovariectomized at E3.5 after the plug. SCNT blastocysts were transferred just before the ovariectomy. Daily injection of 20 mg of P4 was carried out to maintain pregnancy. Diapause blastocysts were recovered at E7.5 by flushing the uterus.

Blastocyst outgrowth derivation

Nuclear transfer (NT), NT-TSA, or control embryos were cultured in aMEM for 4 days. The zona pellucida of the blastocysts was removed in acid phosphate-buffered saline (PBS) and the embryos were cultured on gelatinized four-well plates in DMEM/F12 (1:1, GIBCO) plus 20% knockout serum (GIBCO), LIF1000U (leukemia inhibitory factor, Chemicon, Temecula, CA, USA), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acid (NEAA; GIBCO).

Analysis of postimplantation embryos

E6.5 or E 7.5 embryos were dissected and the presence of a streak or a neural plate was recorded. Abnormal embryos were classified as previously described (Jouneau et al., 2006) into three categories: (1) embryos with a rounded shape, (2) those with an extra-embryonic region occupying more than half of the volume of the embryo (= large Exe), (3) very small embryos with no morphological demarcation between the extra-embryonic and the embryonic regions.

Immunostaining

Embryos or day 2 outgrowths were rinsed in PBS and fixed in 4% paraformaldehyde at 4°C for overnight. They were permeabilized in PBS containing 0.5% Triton X-100 for 30 min at room temperature. After saturation of nonspecific sites with PBS containing 2% bovine serum albumin (BSA) for 1 h, the samples were incubated overnight at 4°C with Oct4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1/200 or Cdx2 antibody (BioGenex, San Francisco, CA, USA) diluted 1/200 in the PBS containing 2% BSA. After rinsing in PBS three times, the samples were incubated under the same conditions with antigoat IgG coupled to Cy5 or antimouse IgG coupled to FITC (Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were counterstained with propidium iodide (PI) before the mounting the slides. Observations were made with an LSM

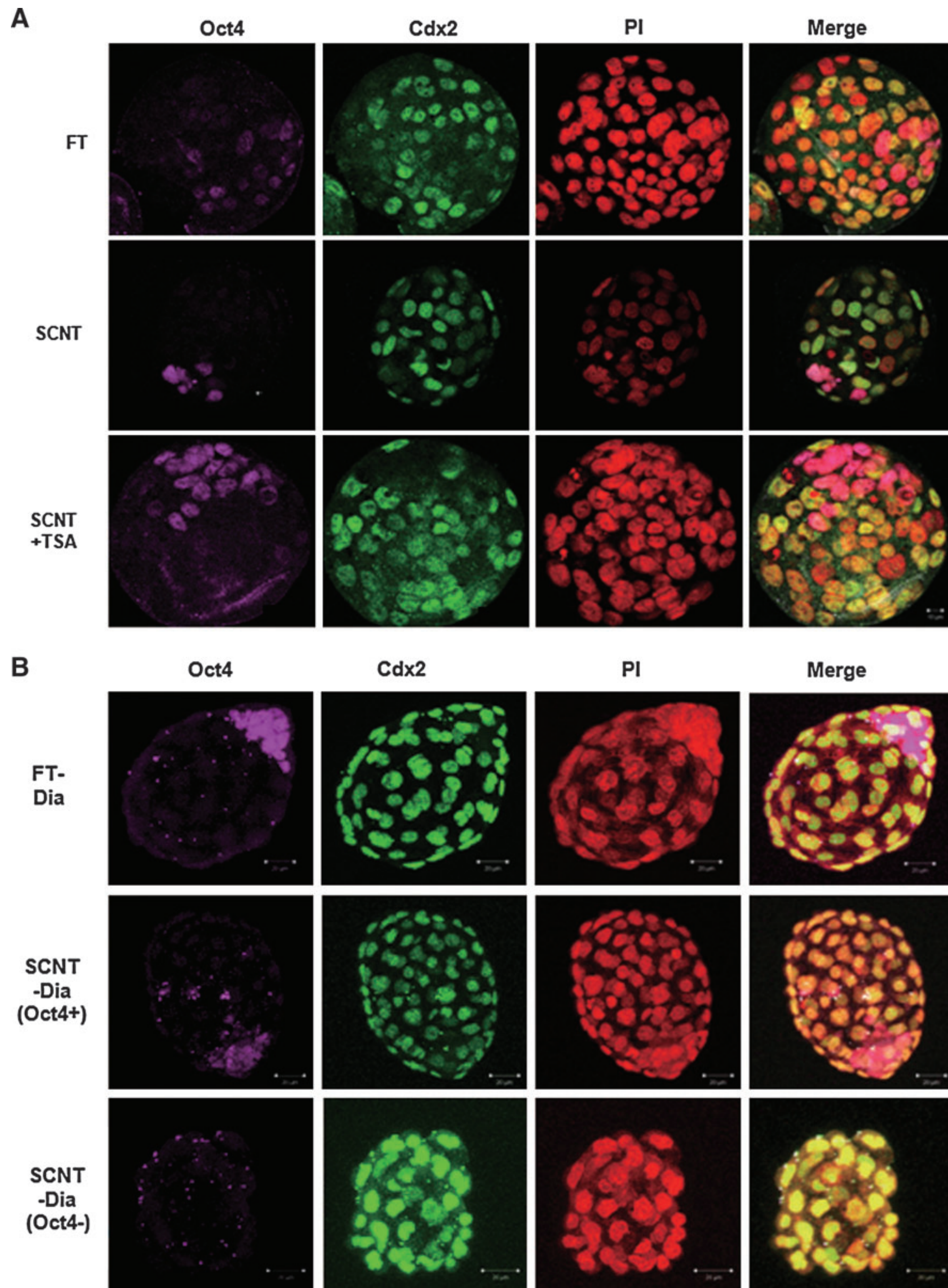

FIG. 1. Morphology of blastocysts assessed by immunostaining for Oct4 and Cdx2. **(A)** Blastocysts recovered after 5 days of *in vitro* culture; **(B)** blastocysts recovered after 5 days of diapause *in vivo*. The images correspond to z-projection of different optical sections obtained from a CLSM microscope.

510 confocal microscope (Carl Zeiss, Thornwood, NY, USA). Cells were counted on z-sections using Image J software (http://rsb.info.nih.gov/ij/index.html).

Detection of proliferation in outgrowths

Outgrowths were incubated with BrdU for 10 min before fixation. Detection of incorporated BrdU was then performed according to the manufacturer's protocol using the BrdU labeling kit (Roche Diagnostics, Indianapolis, IN, USA).

Statistical analysis

Student's *t*-tests and chi-dquare tests were used for statistical analyses. For all, a value of $p < 0.05$ was considered to be statistically significant.

Results

Pluripotency maintenance in SCNT blastocysts in vitro *and* in vivo *during experimental diapause*

To analyze the morphology and quality of SCNT blastocysts, we have used two key markers of the blastocyst lineages: Oct4 for the pluripotent ICM, and Cdx2 for the differentiated trophoblast (Fig. 1A). It has been previously reported that at 96 h of culture, the Oct4 protein is still present in many cells of the trophectoderm in both FT and SCNT embryos (Balbach et al., 2010). Hence, embryos were developed *in vitro* for 120 h, up to the advanced blastocyst stage, when the segregation between Cdx2 in the trophectoderm and Oct4 in the ICM was completed in *in vitro* cultured FT blastocysts. Figure 2 shows the results of cell counting in both compartments—the ICM and the trophectoderm—as well as the ratio of ICM (Oct4 expressing cells) cells. SCNT blastocysts had only half as many cells as FT embryos. It suggests that they are delayed by at least one cell cycle. The reduction of cells was even more pronounced in the ICM, as indicated by the reduced ratio of Oct4 expressing cells among the total cell number (10% for SCNT compared to 19% for FT; see Fig. 2). Nineteen percent of SCNT blastocysts contained only one cell in the ICM (data not shown).

It was previously shown that after treatment with histone deacetylase inhibitors, SCNT embryo development was significantly improved, in terms of both blastocyst and live pup rates (Dai et al, 2010; Kishigami et al., 2006b; Rybouchkin et al., 2006). Therefore, we treated the reconstructed SCNT embryos with TSA to study how it would impact the quality of the blastocysts, and more specifically, the formation of the ICM. The rate of blastocysts improved slightly, but significantly and interestingly, the effect of the treatment was evidenced at the transition from morula to blastocyst (Table 1). Immunostaining of Oct4 and Cdx2 revealed that TSA treatment specifically increased the Oct4 cell number, therefore raising the ratio up to the level of FT blastocysts, whereas the trophectoderm cell number did not significantly change (Figs. 1 and 2A). This suggests that in TSA-treated embryos the cell allocation toward the ICM is favored. Another interesting result showed that, although all the blastocysts were fixed at the same time after activation, 6 out of 22 SCNT blastocysts still displayed double positive (for Oct4 and Cdx2) cells in the trophectoderm compared to only 1 out of 14 after TSA treatment.

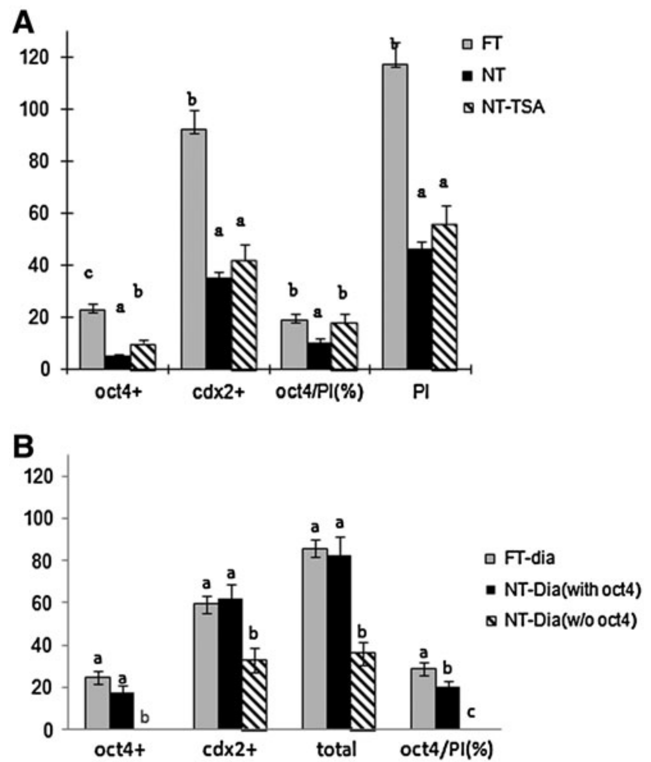

FIG. 2. Cell allocation in blastocysts after in vitro culture **(A)** and diapause **(B)**. Total cell number was evaluated by the number of PI-stained cells and ICM and trophectoderm cell numbers by Oct4 and Cdx2 immunostaining. Data are expressed as mean ± SEM of these embryos. Different superscript letters mean significantly different results between two groups (Student *t*-test, $p < 0.05$). After diapause the NT embryos were separated into blastocysts with Oct4 expressing cells (NT-Dia with Oct4) and blastocysts without Oct4 expressing cells (NT-Dia w/o Oct4). For each group, more than 10 embryos were stained for Oct4 and Cdx2. The data are expressed as mean ± SEM. *Values with a different superscript indicate a difference ($p < 0.05$) between two groups for an individual staining or ratio (*t*-test).

To further check the maintenance of pluripotency in SCNT blastocysts, we induced an experimental diapause in foster mice after transfer of FT or SCNT embryos. If SCNT embryos had a defect in pluripotency maintenance, they should not be able to maintain the expression of Oct4 in the ICM *in vivo* during this period of dormancy. Blastocysts were recovered after 5 days of diapause and immunostained for Oct4 and Cdx2 (Fig. 1B). SCNT embryos segregated into two populations of equal size. In the first one, the blastocysts were only composed of Cdx2-expressing trophoblastic cells with a total cell number being similar to the number before diapause (35–40) (Fig. 2). The other half of the SCNT embryo population displayed a morphology and cell allocation very similar to control diapause blastocysts (Fig. 2). It was noteworthy that, although FT blastocysts did not increase in cell number during diapause as shown before (Batlle-Morera et al., 2008; Given, 1988), these SCNT blastocysts were able to double their cell number (from 40 to 85 cells). This suggests either SCNT embryos do not answer normally to the dormancy signals or the cell cycle arrest following diapause is dependent on the total cell number in the embryo.

Table 1. Preimplantation Development of SCNT Embryos after TSA Treatment

Group	*No. reconstructed*	*No. two-cell*	*No. four-cell*	*No. morulas*	*No. blastocysts (% of two-cell)*
SCNT	620	520	487	367	$207(40 \pm 3)^{a}$
SCNT-TSA	1225	776	662	547	$445(57 \pm 7)^{b}$

$^{a,b}p < 0.01$.
SCNT, somatic cell nuclear transfer.

As the pluripotency cells are substantially few in the SCNT blastocyst and half of the SCNT blastocysts lost the pluripotent cells during subsequent experimental diapause process, we examined the Oct4 expression during the outgrowth formation and ESC derivation to know how the pluripotency of SCNT embryo will be maintained in the *in vitro* artificial culture environment, released from the stress of embryonic development.

Expression of Oct4 during outgrowth formation and ESC derivation

The derivation of ESCs can be viewed as a two-step process: outgrowth formation and ESC derivation from these outgrowths. We recorded the rate of SCNT embryos reaching the first and/or the second step and compared it with that of FT embryos. As the TSA increased the pluripotent cells in the blastocyst significantly, we also recorded the *in vitro* behavior of TSA-treated SCNT embryos (Table 2). Our data indicate that SCNT blastocysts have a defect in the ability to form an outgrowth (the first step) rather than impairment in ESC derivation (the second step). Indeed, nearly two-thirds of SCNT blastocysts were unable to form a proliferating outgrowth: as the proliferation is mainly sustained by ICM cells, it suggests that these ICM cells have a proliferation defect that cannot be rescued *in vitro*. However, TSA treatment was able to significantly improve the formation of outgrowth, in good correlation with the increase in Oct4 expressing cells as depicted above (Table 1 and Fig. 2). On the other hand, once an outgrowth was formed its potential to give rise to an ES cell line was similar irrespective of the origin of the blastocyst: FT or NT. Therefore, there is no intrinsic defect in the ICM of SCNT embryos that would preclude the derivation of ESCs. Alternatively, the forming ESCs could come from the few cells correctly reprogrammed within the outgrowth. To explore this possibility, we compared the cell proliferation and the Oct4 expression during the outgrowth phase (2 days after blastocyst attachment) in FT and SCNT blastocysts (Fig. 3). The number of Oct4 expressing cells and their proportion within the outgrowth were recorded (Fig. 3A) and the proliferation was assessed by BrdU incorporation (Fig. 3B). No significant differences were detected between the two types of outgrowth, although a slight decrease of proliferation was observed within the SCNT outgrowths. Taken together, these data do not favor the hypothesis that only a few correctly reprogrammed cells would be selected during *in vitro* culture of the outgrowth.

Postimplantation development of SCNT embryos and improvement after TSA treatment

As TSA could improve the pluripotency in the SCNT embryos, we then examined the impact of the increased pluripotent population in SCNT blastocysts after treatment with TSA on the later development of the epiblast *in vivo*. It has been shown before that development to term was improved, whereas placentomegaly was observed in all cases, as indicated by a similar placental weight whether the SCNT embryos were treated or not with TSA (Kishigami et al., 2006b; Maalouf et al., 2009; Tanaka et al., 2001). Subsequent histology analysis of these placentas revealed a structure very similar to that of untreated NT placentas (data not shown). We had previously shown that different developmental defects arose in the peri-implantation period, with many implanted embryos dying immediately after implantation, the remaining displaying various delays of development and morphological defects (Jouneau et al., 2006; Maruotti et al., 2010). In the present study we investigated the development of SCNT embryos treated with TSA in this critical period by dissecting the recipient mice at 7 days of gestation.

TSA allowed a higher number of embryos to be recovered at E7; however, this improvement was mainly due to the higher rate of blastocyst, as indicated by the comparison of the results after two-cell and blastocyst transfer (Table 3). The high peri-implantation losses were only slightly diminished following treatment with TSA (15 and 21% embryos in deciduas, with and without TSA, respectively). We then classified the recovered embryos according to their stages of gastrulation (Downs and Davies, 1993) (Table 4). NT embryos

Table 2. Outgrowth Formation and ESC Derivation in Fertilized, SCNT, and TSA-Treated SCNT Embryos

				No. ES lines		
Type of embryo	*No. attached blastocysts (%)*	*No. outgrowths (%)*			*% blastocysts*	*% outgrowths*
Fertilized	57	55 (96%)[a]	36 (63%)[a]	21	37%[a]	58%[a]
SCNT	95	80 (84%)[b]	31 (33%)[b]	13	14%[b]	42%[b]
SCNT + TSA	11	11 (100%)[a]	11 (100%)[c]	5	45% [a]	45%[b]

$^{a,b}p < 0.002$; $^{a,c;b,c}p < 0.03$.
SCNT, somatic cell nuclear transfer; ES, embryonic stem cell.

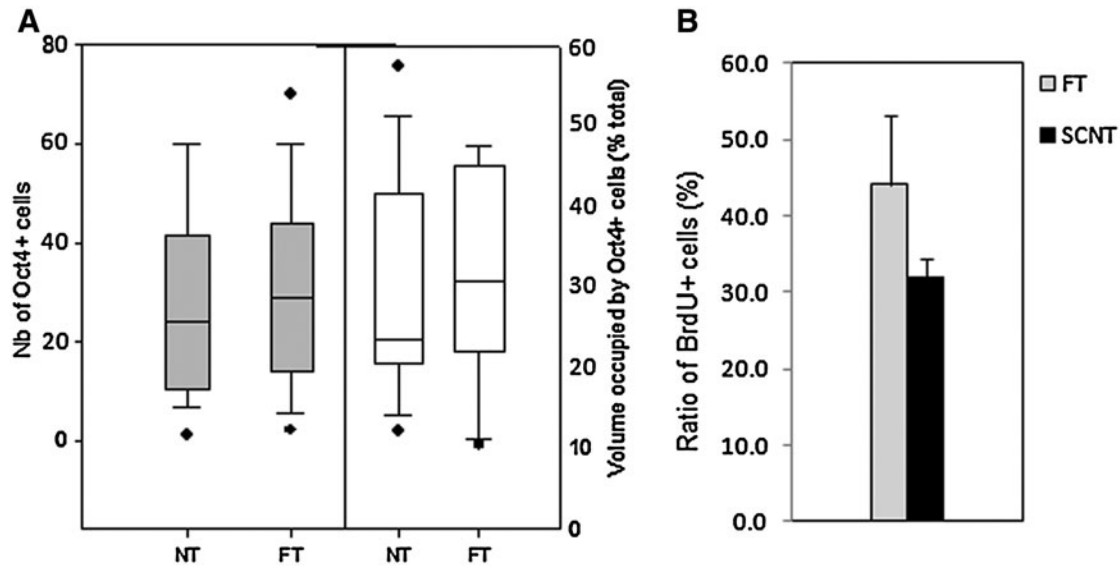

FIG. 3. Assessment of pluripotency and proliferation in day 2 outgrowths obtained from NT or FT− blastocysts. **(A)** Number and ratio of Oct4 positive cells. **(B)** Ratio of BrdU positive cells.

displayed a clear delay in development, as more than half of the fertilized embryos were already at the neural plate stage, whereas none of SCNT embryos have reached this stage. By contrast, TSA-treated embryos have reached the same gastrulation stages as the fertilized embryos. When the morphology of the embryos was examined, a beneficial effect of TSA treatment was observed, as nearly half of the TSA-treated SCNT embryos had a normal morphology (49%) (Table 5), compared to only 12% of SCNT embryos. Among the abnormal ones, the types of abnormalities were found similar to those already described for ESNT and SCNT (see Material and Methods) (Jouneau et al., 2006; Maruotti et al., 2010).

To evaluate more precisely the morphology of the embryos, implanted embryos at E6 were immunostained for Oct4 and the volume occupied by the epiblast was measured. In FT embryos, the volume of the epiblast was always higher than 50% of the whole volume (see example in Fig. 4A). Only half of SCNT embryos displayed the normal ratio (Fig. 4B–D). By contrast, treatment of SCNT embryos with TSA selectively improved the size of the epiblast, restoring a normal proportion within the embryo (Fig. 4D). The beneficial effect of the treatment with TSA on the epiblast, therefore, is observable throughout its development *in vivo*.

Discussion

In this study we have examined how pluripotency is maintained in the developing epiblast of SCNT embryos *in vivo* and *in vitro* during derivation of ESCs. Our main result points out a delay in development in SCNT embryos correlated with a defect in forming and maintaining a correct number of Oct4 expressing ICM and epiblast cells.

Reduced number of pluripotent cells in SCNT embryos and its improvement by TSA

Our data show that most SCNT blastocysts have a reduced number of Oct4-expressing and thus pluripotent cells in the ICM. We performed our analysis in embryos cultured for 120 h in order to get the complete segregation of Oct4 and Cdx2 proteins, at least in FT embryos. In a recent study by Balbach and colleagues (2010), they found that Oct4 level of expression was similar between SCNT and intracytoplasmic sperm injection (ICSI) 16-cell embryos and blastocysts (at a time when Oct4 expression is still not restricted to the ICM even in control blastocysts), whereas Cdx2 expression was highly variable between cells and embryos but frequently higher in control embryos. Interactions between Cdx2 and Oct4 have been shown to regulate the engagement of cells in the trophoblast or epiblast lineages, depending on the relative amount of each protein (Niwa et al., 2005). Altogether these results indicate that there may be an imbalance in favor of Cdx2 expression, resulting in a biased specification of cells to the trophoblast lineage.

When the embryo progresses toward the formation of an egg cylinder around the implantation time, the proliferation

Table 3. Embryo Recovery at E7 after Embryo Transfer

Type of embryo	*No. transferred two-cell*	*No. Implanted (% transferred)*	*No recovered (% implanted)*	*No. transferred blastocysts*	*No. implanted (% transferred)*	*No. recovered (% implanted)*
Fertilized	28	24 (86%)	16 (67%)			16 (15%)[a]
SCNT	1164	312 (27%)[a]	35 (11%)[a]	177	105 (59%)[a]	
SCNT + TSA	489	204 (42%)[b]	34 (17%)[a]	111	66 (59%)[a]	14 (21%)[a]

[a,b] $p < 0.01$.
SCNT, somatic cell nuclear transfer.

Table 4. Stages of Embryos Recovered at E7

	Total	Prestreak (%)	Primitive streak (%)	Neural plate/head fold (%)
Fertilized	20	1 (5)	8 (40)	11 (55)[a]
SCNT	21	3 (14)	18 (86)	0 (0)[b]
SCNT-TSA	28	1 (4)	18 (64)	9 (32)[a]

[a,b]$p < 0.01$.
SCNT, somatic cell nuclear transfer.

of cells is greatly enhanced while being tightly regulated, resulting in an epiblast occupying about 60% of the total volume (Jouneau et al., 2006; Power and Tam, 1993). When we measured the volume of the embryos occupied by the epiblast (expressing Oct4), we observed that half of the SCNT embryos displayed a reduced epiblast. These defects affecting the number of Oct4 expressing cells were substantially reduced when the SCNT embryos were treated with the histone deacetylase (HDAC) inhibitor TSA at the time of reconstruction. Increasing the cell number in the ICM and then in the epiblast through TSA treatment correlates positively with the increasing rate of outgrowth formation and a less retarded gastrulation. Indeed, it was shown that reducing the number of blastomeres delayed the onset of gastrulation until the threshold number of cells is reached (Lewis and Rossant, 1982; Power and Tam, 1993). The number of trophoblast cells does not substantially increases after TSA treatment, indicating that the number of epiblast cells is the most critical for the onset of gastrulation.

Formation of outgrowths in vitro *and diapause reveal two types of SCNT embryos*

The induction of an experimental diapause has revealed a major defect in a subpopulation of SCNT blastocysts, as some of them are unable to maintain an ICM as revealed by the absence of any Oct4-expressing cells in half of the diapause SCNT blastocysts. We presume that these degenerating blastocysts come from embryos in which the minimum threshold number of Oct4-expressing cells compatible with their maintenance is not reached. Such blastocysts may also be those that fail to form an outgrowth *in vitro*, in agreement with the report of Boaini and colleagues (2002) showing that outgrowths form mainly from SCNT embryos with strong Oct4–GFP expression. *In vivo*, they may be able to implant as observed for Oct4-null embryos (Nichols et al., 1998). The other blastocysts that maintain an ICM during diapause may not have an intrinsic defect compromising pluripotency. Indeed, during outgrowth expansion, we showed that SCNT-derived Oct4 expressing cells grow at the same rate as FT-derived cells. Similarly, the efficiency of derivation of postimplantation Epiblast Stem Cells (EpiSCs) from SCNT embryos is also not impaired (Maruotti et al., 2010). Overall, our data show that in those SCNT embryos able to generate an outgrowth (from either the ICM or the epiblast), the pluripotent cells are correctly reprogrammed and have at this point the same potential to give rise to an ESC or EpiSC line as an FT embryo. We think our results do not validate the hypothesis of a selection of the few well-reprogrammed cells *in vitro* or an erasure of epigenetic memory during derivation as proposed by Brambrink and colleagues (2006). Our recent study comparing EpiSCs derived from SCNT and FT embryos indicated that abnormal epigenetic modifications occur later during epiblast development and should impair the later survival or differentiation of the fetus without directly affecting pluripotency, consistent with the present study.

After implantation a fine-tuned crosstalk between embryonic and extra-embryonic tissues takes place (Ang and Constam, 2004; Arnold and Robertson, 2009). Any reprogramming errors affecting these relationships may impair development, despite the individual ability of epiblast cells to maintain pluripotency. Functional complementation was shown to occur when SCNT embryos are aggregated together, leading to an improvement of cell allocation to the ICM and postimplantation development (Balbach et al., 2010; Boiani et al., 2003). It suggests that the reprogramming errors affecting SCNT embryos should be noncell autonomous.

How do HDAC inhibitors trigger their benefic effect?

The consequence of the treatment can be observed very early as an improvement of chromatin remodeling and on RNA production at the zygotic genome activation (ZGA) (Bui et al., 2010; Maalouf et al., 2009; Van Thuan et al., 2009). Data recently obtained in zebrafish suggest that bivalent domains, a peculiar epigenetic mark specific to pluripotent cells, are established during ZGA (Vastenhouw et al., 2010). These bivalent domains are known to play an important role in maintaining pluripotency (Bernstein et al., 2006). They seem to be reduced in the ICM of NT blastocysts, whereas differentiation-related gene expression is enhanced (Zhang et al., 2009). These converging data lead us to propose that TSA permits an improvement of reprogramming by modulating the early establishment of epigenetic marks important for establishment and maintenance of pluripotency. Indeed, TSA has been shown to modulate the methylation of lysine 4 and 27 of histone H3 that constitute the bivalent domain (Karantzali et al., 2008). Notably, HDAC inhibitors can enhance nuclear reprogramming of somatic cells toward iPSCs, most likely by promoting a favorable chromatin configuration (Huangfu et al., 2008).

HDAC inhibitors help reprogramming toward pluripotency, therefore allowing SCNT embryos to overcome one

Table 5. Morphology of Embryos Recovered at E7

	Total	Normal (%)	Vesicular (% of abnormal)	Rounded (% of abnormal)	Large Exe (% of abnormal)
SCNT	25	3 (12)[a]	7 (32)[a]	5 (23)[a]	9 (41)[a]
SCNT-TSA	35	17 (49)[b]	6 (33)[a]	2 (11)[a]	6 (33)[a]

[a,b]$p < 0.01$.
SCNT, somatic cell nuclear transfer.

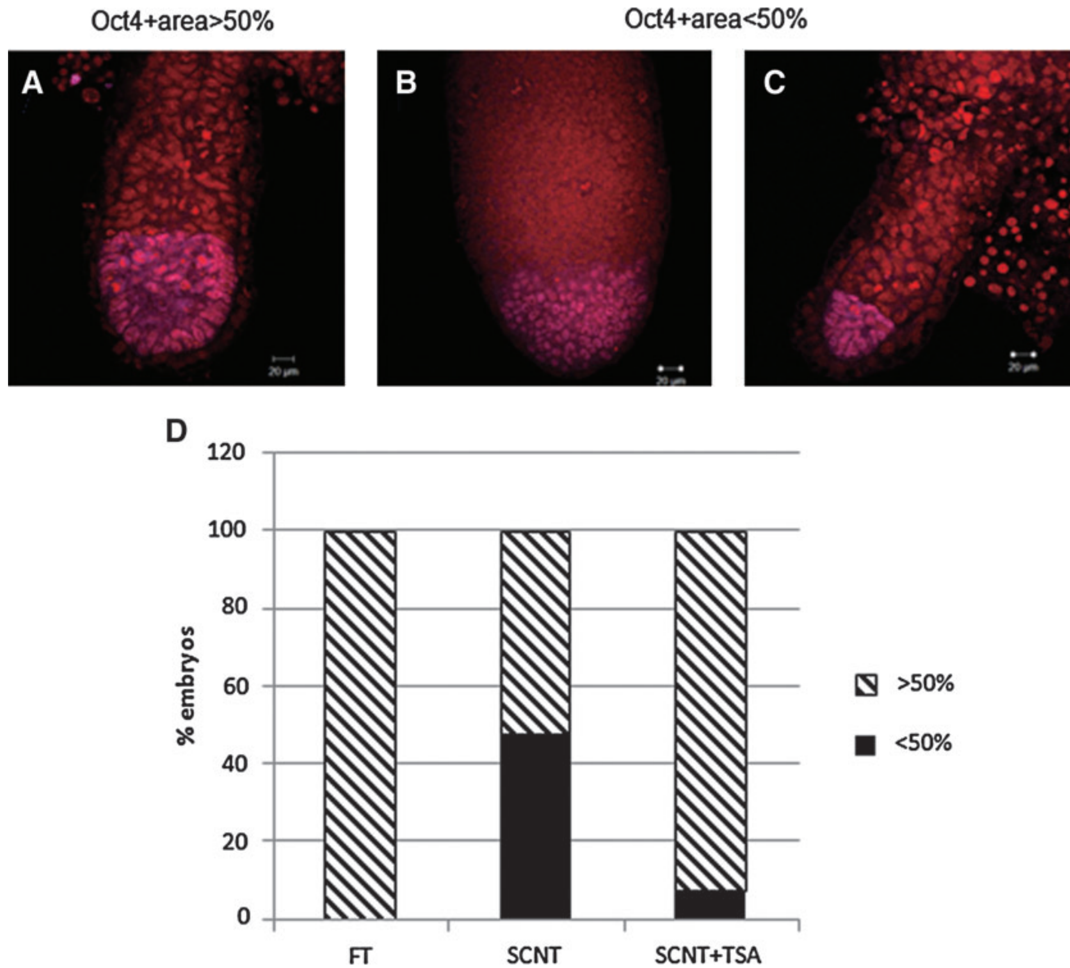

FIG. 4. Oct4 expression in postimplantation embryos and the effect of TSA treatment. **(A–C)** Examples of embryos showing different epiblast sizes assessed by the expression of Oct4: **(A)** normal (ratio >50%); **(B, C)** lower (ratio <50%). Oct4 expressing cells are in pink; red: nuclei stained with PI. **(D)** Histograms showing the distribution of embryos in the normal (ratio >50%) and the abnormal class (ratio <50%).

hurdle. However, the improvement of the rate of development to term, although consistently improved, remains quite low (Kishigami et al., 2006b; Rybouchkin et al., 2006). It reaches the level obtained for ESNT embryos, about 3% (Maalouf et al., 2009; Zhou et al., 2001). This similarity is another hint that TSA helps the somatic nucleus to be turned into a pluripotent one. However, although allowing more NT embryos to develop up to the end of gastrulation, placentomegaly still affects the fetal development of both ESNT and SCNT embryos (Jouneau et al., 2006; Kishigami et al., 2006b). Such abnormal development is, in fact, most likely due to defects not intrinsic to the ICM, but rather to defects affecting the trophoblast and the relationships between epiblast and extra-embryonic tissues (Miki et al., 2009; Rielland et al., 2009). Hence, it seems that histone deacetylase inhibitors only impact the epiblast and not the trophoblast and/or the relationships between the two tissues.

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Author Disclosure Statement

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