Histone H3 lysine 27 trimethylation acts as an epigenetic barrier in porcine nuclear reprogramming

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Abstract

Aberrant epigenetic reprogramming is the main obstacle to the development of somatic cell nuclear transfer (SCNT) embryos and the generation of induced pluripotent stem (iPS) cells, which results in the low reprogramming efficiencies of SCNT and iPS. Histone H3 lysine 27 trimethylation (H3K27me3), as a repressive epigenetic mark, plays important roles in mammalian development and iPS induction. However, the reprogramming of H3K27me3 in pig remains elusive. In this study, we showed that H3K27me3 levels in porcine early cloned embryos were higher than that in IVF embryos. Then GSK126 and GSK-J4, two small molecule inhibitors of H3K27me3 methylase (EZH2) and demethylases (UTX/JMJD3), were used to regulate the H3K27me3 level. The results showed that H3K27me3 level was reduced in cloned embryos after treatment of PEF with 0.75 μM GSK126 for 48 h, incubation of one-cell reconstructed oocytes with 0.1 μM GSK126 and injection of antibody for EZH2 into oocyte. Meanwhile, the development of the cloned embryos was significantly improved after these treatments. On the contrary, GSK-J4 treatment increased the H3K27me3 level in cloned embryos and decreased the cloned embryonic development. Furthermore, iPS efficiency was both increased after reducing the H3K27me3 level in donor cells and in early reprogramming phase. In summary, our results suggest that H3K27me3 acts as an epigenetic barrier in SCNT and iPS reprogramming, and reduction of H3K27me3 level in donor cells and in early reprogramming phase can enhance both porcine SCNT and iPS efficiency.

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Introduction

Somatic cell nuclear transfer (SCNT) and induced pluripotent stem (iPS) cells are two of the primary routes to reprogram differentiated cells back to pluripotent state. Both of the two reprogramming methods have great significance: SCNT holds great potential for agriculture, biomedical industry and endangered species conservation as this technique allows the generation of a whole organism from a single differentiated somatic cell (Yang et al. 2007b). iPS cells can serve as a valuable patient specific cell source for in vitro disease modeling and cell/tissue replacement therapies because they do not have ethical issues (Park et al. 2008). However, the efficiencies of SCNT and iPS induction are extremely low, which limit their practical use. For example, the overall reproductive cloning efficiency in all species is remarkably low (1–5%) except bovine species (5–20%) (Rodriguez-Osorio et al. 2012). And in general, the efficiency of iPS colony formation with the four Yamanaka’s factors (Oct4, Sox2, Klf4 and c-Myc; OSKM) is less than 1% of the starting population (Silva et al. 2008).

Aberrant epigenetic reprogramming is believed to be the main reason for the low reprogramming efficiencies of SCNT and iPS (Mason et al. 2012, Buganim et al. 2013). It could occur on many aspects, including DNA methylation and histone modifications (Dean et al. 2001, Kang et al. 2001, Santos et al. 2003, Ohgane et al. 2004). For example, cloned embryos always show higher DNA methylation level compared to their fertilized counterparts (Dean et al. 2001, Beaujean et al. 2004, Yang et al. 2007a). And more recently, H3K9me3 was identified as a critical epigenetic barrier in SCNT reprogramming. The reprogramming resistant regions (RRRs) that could not be activated in cloned embryos as that in fertilized embryos were enriched for H3K9me3 in donor cells, and its removal by deletion of H3K9me3 methylase in donor cells and by ectopically expressed H3K9me3 demethylase in cloned emryos not only reactivated the majority of RRRs, but greatly improved SCNT efficiency as well (Matoba et al. 2014).
Similarly, abnormal epigenetics reprogramming also often occur in these areas during iPS induction, and the reprogramming efficiency could be improved by regulating these epigenetic status both in SCNT and iPS (Kishigami et al. 2006, Huangfu et al. 2008, Onder et al. 2012, Huan et al. 2013, Matoba et al. 2014).

Histone H3 lysine 27 trimethylation (H3K27me3) is another repressive epigenetic mark. It is catalyzed by enhancer of zeste homolog 2 (EZH2), and can be removed by H3K27me3-specific demethylase of Jumonji domain containing protein 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat X (UTX) (Bogliotti & Ross 2012). And EZH2 and JMJD3/UTX can be specifically suppressed by two small molecule inhibitors of GSK126 and GSK-J4 respectively (Kruidenier et al. 2012, McCabe et al. 2012). H3K27me3 has a great effect on embryonic development. For example, the embryonic development was reduced after inhibition of H3K27me3 removal in bovine (Canovas et al. 2012). Similarly, iPS reprogramming process was also accompanied by genome wide H3K27me3 changes, and the aberrant dynamics of H3K27me3 would impair iPS cells induction (Mikkelsen et al. 2008, Hawkins et al. 2010, Mansour et al. 2012). Recently, defective reprogramming of H3K27me3 has been observed in cloned mouse embryos (Zhang et al. 2009). However, the reprogramming of H3K27me3 has not been reported in other species of cloned embryos.

In this study, we investigated the reprogramming of H3K27me3 in porcine cloned embryos, and assessed the impact of H3K27me3 level on SCNT and iPS efficiency through regulating H3K27me3 level by treatment of small molecules GSK126 and GSK-J4 respectively. We believe the results would advance the understanding of reprogramming mechanism of SCNT and iPS.

### Materials and methods

The reagents and media used in our study were purchased from Life Technologies, R&D, Millipore and Bioind (Kibbutz Beit Haemek, Israel) unless otherwise stated.

### Animals

The PEF lines were derived from Landrace which were from Northeast Agricultural University. Embryo Engineering Laboratory Experimental Pig Base. ICR mice for mouse embryonic fibroblast cells were purchased from Vital River Company, Beijing, China. The porcine ovaries were obtained from DaZhongRouLian Slaughterhouse Company (Harbin, Heilongjiang, China). All studies adhered to procedures consistent with the Northeast Agriculture University of Biological Sciences Guide for the care and use of laboratory animals.

### SCNT, parthenogenetic activation and IVF

The procedure for porcine SCNT was the same as we described previously (Liu et al. 2008). After 42 h of maturation culture, the oocytes were treated with 1 mg/ml hyaluronidase to remove the surrounding cumulus cells. Oocytes with a clearly extruded first polar body were selected as recipient cytoplasm. Cumulus free oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a glass pipette 25 μm in diameter in TC199-Hepes plus 0.3% BSA and 7.5 μg/ml cytochalasin B. A single donor cell was injected into the perivitelline space and electrically fused using two direct pulses of 120 V/mm for 30 μs in fusion medium. Fused eggs were cultured in porcine zygote medium-3 (PZM-3) medium for 6.5 days in an atmosphere of 5% CO₂ and 95% air at 39 °C. The cleavage and blastocyst rates were assessed at 48 and 156 h after activation, and the number of blastocyst cells was examined by nuclear staining with 5 μg/ml Hoechst 33342. For anti-EZH2 group, ten picoliters of EZH2 antibody (3147, Cell signaling) was injected into the blastocoele and 144 h respectively.

For IVF, freshly ejaculated sperm rich fractions were collected from fertile boars, and following a short incubation at 39 °C, the semen was resuspended and washed three times in DPBS supplemented with 0.1% (w/v) BSA by centrifugation at 1500g for 4 min. The spermatozoa concentration was measured using a hemocytometer, and the proportion of motile sperm was determined. The spermatozoa were diluted with modified Tris-buffered medium (mTBM) to an optimal concentration. Cumulus free oocytes were washed three times in mTBM. Approximately 30 oocytes were inseminated in 50-μl drops of mTBM at a final sperm concentration of 3 x 10⁷/ml for 6 h. Then embryos were washed and cultured in PZM-3 medium for 6.5 days in an atmosphere of 5% CO₂ and 95% air at 39 °C. SCNT and IVF embryos, one-cell, two-cell, four-cell and blastocyst (B) stages, were collected at 6/12, 24, 48 and 144 h respectively.

### Immunofluorescence analysis

PEF, sperm, oocytes and embryos without zona pellucida were washed twice in PBS, then fixed in freshly prepared 4% paraformaldehyde in PBS, permeabilized in 1% Triton X-100 in PBS, and left in blocking solution (1% BSA in PBS) for 1 h. For immunolabeling, the embryos were incubated overnight at 4 °C with primary antibody for H3K27me3 (07449, Millipore) washed three times and incubated for 1 h with secondary
antibody, FITC-labeled donkey anti-rabbit IgG (A21206, Invitrogen), diluted 1:1000 with blocking solution. Samples were washed and stained with 5 μg/ml of Hoechst 33342. Fluorescence was detected and imaged using a Nikon fluorescence microscope. The average optical intensity (AOI) of the H3K27me3 staining and the number of PEF cells were calculated using the freely available Image J software. The AOI of H3K27me3 nuclear area staining in embryos were normalized by subtracting the AOI of the background areas.

**Western blot**

PEF or embryos removed zona pellucida, stored at −80°C, were lysed with cell lysis buffer for western (Beyotime, Jiangsu, China). Lysates were separated in SDS–PAGE for the routine western blot assay and transferred to PVDF membranes (Invitrogen); nonspecific binding was blocked by incubation in 1% BSA in PBS at room temperature for 1 h. Blots were then probed for overnight at 4°C with primary antibody for H3K27me3 (07449, Millipore). β-Actin (A1978, Sigma) served as loading control. After 1 h incubation at room temperature with secondary antibodies, protein bands were detected by HRP Chemiluminescent Kit (Invitrogen) and Kodak BioMax Light film. The average optical intensity of the H3K27me3 and β-actin bands were calculated by Image J.

**iPSCs generation**

Inducible iPSCs were generated as described previously (Wang et al. 2013). Briefly, GP293T cells were transfected with the pMX plasmids which contained mouse Oct4?Sox2?Klf4, c-Myc, Nr5a2 and Tbx3 together with packaging plasmids VSVG. The medium was replaced 12 h after transfection, and the virus supernatants were harvested after subsequent 24 h. PEF or embryos removed zona pellucida, stored at −80°C, were lysed with cell lysis buffer for western (Beyotime, Jiangsu, China). Lysates were separated in SDS–PAGE for the routine western blot assay and transferred to PVDF membranes (Invitrogen); nonspecific binding was blocked by incubation in 1% BSA in PBS at room temperature for 1 h. Blots were then probed for overnight at 4°C with primary antibody for H3K27me3 (07449, Millipore). β-Actin (A1978, Sigma) served as loading control. After 1 h incubation at room temperature with secondary antibodies, protein bands were detected by HRP Chemiluminescent Kit (Invitrogen) and Kodak BioMax Light film. The average optical intensity of the H3K27me3 and β-actin bands were calculated by Image J.

**Alkaline phosphatase staining**

iPSCs was fixed with 4% paraformaldehyde at room temperature for 1 min and washed three times with PBS. Alkaline phosphatase (AP) staining was performed with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) following the manufacturer’s instructions.

**GSK126 and GSK-J4 treatment**

PEF cells (2×10^5 per well) were seeded into six well tissue culture plates 24 h before treatment. Cells were then exposed to 0.1% DMSO and varying concentrations of GSK126 and GSK-J4 (range 0–15 μM) for 48 h respectively, then the cells were harvested for SCNT or iPS induction. During cloned and PA embryonic development 0.1% DMSO, 0.1 μM GSK126 and GSK-J4 incubated one-cell embryos for 24 h respectively. During iPS induction, 0.75 μM GSK126 incubated PEF for 2–7 days after infection respectively.

**Statistical analysis**

Differences of data (mean±S.E.M.) were analyzed by SPSS statistical software 19.0. The data were analyzed using one way ANOVA. For all analyses, differences were considered to be statistically significant when P<0.05.

**Results**

**The H3K27me3 levels of early cloned embryos were higher than that of IVF embryos**

The global H3K27me3 levels were determined at varying stages of IVF and cloned embryos (Fig. 1A). In pronuclear stage of IVF embryos (6 and 12 h), H3K27me3 signal was found in female pronucleus (red arrowhead). Since the late stage of one-cell, H3K27me3 staining intensity was dramatically reduced and faint in two-cell, four-cell and blastocyst stage. In cloned embryos, similar to IVF embryos, H3K27me3 level gradually decreased with the cleavage, and became faint after four-cell stage. However, in one-cell and two-cell cloned embryos, H3K27me3 levels were higher than that of IVF embryos (Fig. 1A and C).

Beside that, the H3K27me3 level of PEF cells, sperm and oocyte were also determined. The results showed that H3K27me3 could be detected on metaphase chromosomes of MII oocytes but not in sperm. And PEF possessed strong H3K27me3 signal in nucleus (Fig. 1B). In order to compare the H3K27me3 level of PEF and oocyte more clearly, nuclear transfer was performed without enucleation, and H3K27me3 was detected within 1 h after the fusion. It could be observed clearly that H3K27me3 level of the nucleus derived from PEF (red arrowhead) was obviously higher than that of maternal chromosomes (white arrows) (Fig. 1B).
Improvement of cloned embryonic development by reducing the H3K27me3 level in donor PEF cells

In order to regulate the H3K27me3 level in donor cells, PEF were treated with GSK126 and GSK-J4 for 48 h. It was shown that treatment with GSK126 in the dosage more than 0.5 μM significantly reduced the H3K27me3 level in PEF cells (Supplementary Figure 1, see section on supplementary data given at the end of this article, Fig. 2). And interestingly, when treated with 0.75 μM GSK126, the blastocyst rate of PEF cells was significantly higher than that of control group (DMSO vs GSK126 0.75 μM and 21.99 vs 31.82%, P<0.05; Table 1). Meanwhile, the H3K27me3 level was lower in one-cell and two-cell of treatment group than that of control (Fig. 3A and C). On the other hand, treatment with GSK-J4 in the dosage more than 0.5 μM increased the H3K27me3 level in PEF cells (Supplementary Figure 1, Fig. 2). However, there was no significant difference in the blastocyst rate between 0.75 μM GSK-J4 treatment group and control group (DMSO vs GSK-J4 0.75 μM and 21.99 vs 20.93%, P>0.05) (Table 1). The H3K27me3 level was higher in one-cell but not in two-cell of treatment group than that of control group (Fig. 3A and C).

Improvement of cloned embryonic development by reducing the H3K27me3 level in early embryos

We also incubated one-cell cloned embryos with GSK126 and GSK-J4 to regulate H3K27me3 level.
The results showed that there were no significant effects on parthenogenetic embryonic development after incubating one-cell with 0.1 \mu M GSK126 or GSK-J4 for 24 h (Supplementary Table 1). When one-cell cloned embryos were incubated with 0.1 \mu M GSK126 for 24 h, the H3K27me3 level in two-cell of treatment group were significantly lower than that of control group (Fig. 3B and D). Meanwhile, the blastocyst rate of treatment group was significantly higher than that of control group (DMSO vs GSK126 0.1 \mu M, 21.99 vs 31.82\%, \textit{P}<0.05; Table 2).

In order to verify the influence of H3K27me3 level on the cloned embryonic development, we injected EZH2 antibody in oocyte to interfere EZH2 function. Consistent with GSK126 treatment, the H3K27me3 level in two-cell of anti-EZH2 group was significantly lower than that of control group (Fig. 3B and D). The blastocyst rate of treatment group was significant lower than that of control group (IgG vs anti-EZH2 and 20.60 vs 30.63\%, \textit{P}<0.05; Supplementary Table 2).

**Improvement of iPS efficiency by reducing the H3K27me3 level in donor cells or during early reprogramming phase**

We also examined the iPS efficiency after reducing H3K27me3 level. After incubation with 0.75 \mu M GSK126 for 72 h, PEF cells (PEF1) were infected by Oct4, Sox2, Klf4, c-Myc, Nr5a2, and Tbx3 (m-pMX-OsKMNT). On the 8th day after infection, the numbers of AP-positive colonies were detected. The results showed that the AP-positive colonies of GSK126 treatment group were about 4 times more than that of DMSO group (Table 2).

### Table 1 The effect of treatment of donor PEF cells with GSK126 or GSK-J4 on cloned embryonic development

<table>
<thead>
<tr>
<th>Groups</th>
<th>Replicates</th>
<th>Embryos</th>
<th>Cleavage (%)</th>
<th>Blastocyst (%)</th>
<th>No. of blastocyst cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>4</td>
<td>162</td>
<td>133 (82.42±2.87)</td>
<td>35 (21.99±1.54)</td>
<td>36.29±12.65</td>
</tr>
<tr>
<td>GSK126 0.75 \mu M</td>
<td>4</td>
<td>192</td>
<td>167 (86.86±5.73)</td>
<td>61 (31.82±4.04)</td>
<td>35.00±16.92</td>
</tr>
<tr>
<td>GSK-J4 0.75 \mu M</td>
<td>4</td>
<td>211</td>
<td>176 (83.44±1.30)</td>
<td>44 (20.93±2.29)</td>
<td>35.91±8.84</td>
</tr>
</tbody>
</table>

Different superscript indicates \textit{P}<0.05.

### Figure 3

The H3K27me3 level of one-cell and two-cell cloned embryos treated with GSK126 and GSK-J4 respectively (A) The H3K27me3 level of one-cell and two-cell cloned embryos derived from PEF cells incubated with 0.75 \mu M GSK126 and GSK-J4 for 48 h respectively. (B) The H3K27me3 level of two-cell cloned embryos after treating one-cell embryos with 0.1 \mu M GSK126 and GSK-J4 for 24 h respectively. Images shown are representative of about 20 embryos for each group. Bar, 50 \mu m. (C and D) The normalized AOI of H3K27me3 nuclear area staining in one-cell and two-cell cloned embryos. Different superscript in the same figure indicates \textit{P}<0.05.
control group ($P<0.05$; Fig. 4A and B). When PEF1 was incubated with 0.75 μM GSK126 for 2–7 days after infection respectively, the results showed that incubation with GSK126 enhanced iPS induction, which achieved the highest efficiency by incubating for 4 days ($P<0.05$; Fig. 4A and B). We validated the results on the other PEF line (PEF2), and the results showed that GSK126 treatment group had more AP-positive colonies than DMSO treatment group ($P<0.05$; Fig. 4C), which were similar with that in PEF1.

**Discussion**

Epigenetic modifications are important factors that affect the cellular reprogramming efficiencies of SCNT and iPS. But few specific epigenetic locus have been evaluated carefully to determine its role on reprogramming. In this study, our results suggest H3K27me3 acts as an epigenetic barrier in porcine nuclear reprogramming, and reduction of H3K27me3 level in donor cells and in early reprogramming phase can enhance both porcine SCNT and iPS efficiency. This will advance our understanding of the reprogramming mechanisms of both SCNT and iPS.

H3K27me3 is an important repressive epigenetic mark and its level changes dynamically during mammalian embryonic development (Ross et al. 2008, Park et al. 2009, Zhang et al. 2009, 2012, Gao et al. 2010). Here we showed that in porcine IVF embryos, H3K27me3 level decreased drastically after fertilization, which is consistent with previous works (Park et al. 2009, Gao et al. 2010); this was believed to be necessary for normal embryonic genome activation. However, in cloned embryos H3K27me3 levels were significantly higher in one-cell and two-cell cloned embryos compared to that in IVF embryos. And this should be closely related with higher H3K27me3 levels in donor PEF cells indicating that H3K27me3 failed to be reprogrammed normally in porcine cloned embryos, and thus impaired expression of some genes and the development potential of cloned porcine embryos.

Various abnormal epigenetic modifications in cloned embryos have been found. In a recent study, H3K9me3 was identified as a critical epigenetic barrier in murine SCNT reprogramming, which impeded cloned embryonic development. And the SCNT efficiency was greatly improved after reducing H3K9me3 level. Similarly, in this study, we regulated H3K27me3 level by GSK126 and GSK-J4, which have been used in several studies (McCabe et al. 2012, Rao et al. 2015). The results manifested that when the H3K27me3 level of cloned embryos were reduced, the embryonic development was significantly improved, including incubation of

**Table 2** The effect of GSK126 or GSK-J4 treatment on cloned embryonic development

<table>
<thead>
<tr>
<th>Groups</th>
<th>Replicates</th>
<th>Embryos</th>
<th>Cleavage (%)</th>
<th>Blastocyst (%)</th>
<th>No. of blastocyst cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>3</td>
<td>89</td>
<td>81 (91.03±3.78) [a]</td>
<td>18 (20.27±3.74) [b]</td>
<td>37.70±15.67</td>
</tr>
<tr>
<td>GSK126 0.1 μM</td>
<td>3</td>
<td>102</td>
<td>90 (88.26±2.75) [a]</td>
<td>32 (31.33±2.60) [a]</td>
<td>33.20±13.18</td>
</tr>
<tr>
<td>GSK-J4 0.1 μM</td>
<td>3</td>
<td>87</td>
<td>125 (71.26±5.26) [b]</td>
<td>7 (8.05±5.26) [b]</td>
<td>33.60±14.01</td>
</tr>
</tbody>
</table>

Different superscript in same array indicates $P<0.05$.

**Figure 4** Improvement of iPS efficiency by reducing the H3K27me3 level in donor cells or during early reprogramming phase (A) the numbers of AP-positive colonies. −3: PEF1 cells were incubated with 0.75 μM GSK126 for 3 days, and then infected by Oct4, Sox2, Klf4, c-Myc, Nr5a2, and Tbx3 (OSKMNT). 0, +2, +3: PEF1 cells were incubated with 0.75 μM GSK126 for 0 to 7 days after infection. Data represent mean and S.E.M. of 3 replicates experiments; *$P<0.05$. (B) Representative images of AP-staining of −3, 0, +4 groups. (C) The numbers of AP-positive colonies. −3: PEF2 cells were incubated with DMSO and GSK126 (0.75 μM) respectively for 3 days, and then infected by OSKMNT. +4: PEF2 cells were incubated with DMSO and GSK126 (0.75 μM) respectively for 4 days after infection. Data represent mean and S.E.M. of 3 replicates experiments; *$P<0.05$. [Downloaded from Bioscientifica.com at 10/12/2018 05:42:17AM via free access](http://www.reproduction-online.org)
PEF/one-cell with GSK126 and injection of EZH2 antibody into oocyte. On the contrary, when the H3K27me3 level of cloned embryos was increased, the cloned embryonic development was impaired. This demonstrated that high H3K27me3 level is detrimental to porcine embryonic development, and H3K27me3 acts as an epigenetic barrier to porcine SCNT reprogramming. We can speculate that H3K27me3 enriched domain may include genes important for embryonic development, the remained high level of H3K27me3 after SCNT would certainly impede the development. So when H3K27me3 levels were artificially down regulated, the expressions of these genes could be derepressed, as a result, the development of cloned embryos could be improved. However, the improvement of embryonic development could be due to the changes of complicated gene networks, rather than a single gene. For example, in the recent study, it was found that numerous candidate genes expressed differently after SCNT would certainly impede the development. So when H3K27me3 levels were artificially down regulated, the expressions of these genes could be derepressed, as a result, the development of cloned embryos could be improved. However, the improvement of embryonic development could be due to the changes of complicated gene networks, rather than a single gene. For example, in the recent study, it was found that numerous candidate genes expressed differently after regulation of H3K9me3, but most of these genes function were unclear (Matoba et al. 2014). So it was great difficulty, if possible, to determine which genes were responsible for the improvement of embryonic development.

It had been reported that regulating epigenetic status can improve the reprogramming efficiency both in SCNT and iPS (Kishigami et al. 2006, Huangfu et al. 2008, Onder et al. 2012, Huan et al. 2013, Matoba et al. 2014). Therefore, we also examined the iPS efficiency after reducing H3K27me3 level. As expected, we found that iPS induction efficiency could be enhanced by reducing the H3K27me3 level in donor cells and in early reprogramming phase. This suggests that H3K27me3 also acts as an epigenetic barrier to porcine iPS reprogramming. Previous works found that genome wide H3K27me3 changes were accompanied with iPS reprogramming process, and removal of H3K27me3 was required for activation of some genes expression during iPS induction (Hawkins et al. 2010). In this research, during porcine iPS induction, the expressions of these genes might also be facilitated by reduction of H3K27me3 level, and in turn enhance the iPS reprogramming.

In summary, our results suggest that H3K27me3 acts as an epigenetic barrier in porcine SCNT and iPS reprogramming, and reduction of H3K27me3 level in donor cells and in early reprogramming phase can enhance both porcine SCNT and iPS efficiency. In the future, it is exciting to determine whether our results could be generally applied to other animal species, even human.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0338.


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