Dnmt3l-knockout donor cells improve somatic cell nuclear transfer reprogramming efficiency

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Abstract

Nuclear transfer (NT) is a technique used to investigate the development and reprogramming potential of a single cell. DNA methyltransferase-3-like, which has been characterized as a repressive transcriptional regulator, is expressed in naturally fertilized egg and morula/blastocyst at pre-implantation stages. In this study, we demonstrate that the use of Dnmt3l-knockout (Dnmt3l-KO) donor cells in combination with Trichostatin A treatment improved the developmental efficiency and quality of the cloned embryos. Compared with the WT group, Dnmt3l-KO donor cell-derived cloned embryos exhibited increased cell numbers as well as restricted OCT4 expression in the inner cell mass (ICM) and silencing of transposable elements at the blastocyst stage. In addition, our results indicate that zygotic Dnmt3l is dispensable for cloned embryo development at pre-implantation stages. In Dnmt3l-KO mouse embryonic fibroblasts, we observed reduced nuclear localization of HDAC1, increased levels of the active histone mark H3K27ac and decreased accumulation of the repressive histone marks H3K27me3 and H3K9me3, suggesting that Dnmt3l-KO donor cells may offer a more permissive epigenetic state that is beneficial for NT reprogramming.

Introduction

Nuclear transfer (NT) is one of several approaches that have been developed to reprogram terminally differentiated somatic cells toward pluripotency or totipotency in an effort to better understand epigenetic reprogramming and generate in vitro models for disease research (Nishikawa et al. 2008, Jullien et al. 2011). NT consists of transferring a differentiated somatic cell into an enucleated oocyte to reacquire totipotency. Embryonic stem cells generated from NT-derived blastocysts (NT-ESCs) serve as a potential resource for regenerative medicine. However, the low efficiency and developmental abnormalities of cloned embryos remain important challenges in NT-associated research and applications (Yang et al. 2007, Rodriguez-Osorio et al. 2012, Long et al. 2014).

Insufficient reprogramming of the genome is thought to be a critical cause of the developmental failure of cloned embryos. Compared to fertilized embryos, cloned embryos display higher levels of DNA methylation and incomplete chromatin remodeling (Zhang et al. 2009, Mason et al. 2012, Rodriguez-Osorio et al. 2012). To improve NT efficiency, several approaches, including the use of chemical inhibitors such as HDAC inhibitors (HDACi) and 5-aza-C, have been devised to facilitate the reprogramming of somatic nuclei and establish embryonic epigenetic patterns (Ogura et al. 2013). HDACi treatment for altering the epigenetic status of donor cells or cloned embryos can significantly
increase cloning efficiency. Treatment of donor cell with HDACi, trichostatin A (TSA), increases the developmental efficiency and quality of the resulting cloned embryos (Enright et al. 2003, Enright et al. 2005, Ding et al. 2008), suggesting that the initial chromatin status of the somatic cell contributes to the development of the cloned embryo. Furthermore, reduction of the repressive mark H3K9me3 in donor cells also significantly increases cloning efficiency, indicating that the global reduction of the repressive histone modification is beneficial to the onset of reprogramming in cloned embryos (Antony et al. 2013, Matoba et al. 2014). In addition to treatments of donor cells, TSA-treated cloned embryos also displayed lower repressive epigenetic marks, enhanced histone acetylation, remodeling of pericentromeric heterochromatin and increased nascent RNA transcription (Kishigami et al. 2006, Rybouchkin et al. 2006, Maalouf et al. 2009, Bui et al. 2010). These results suggest that establishing an accessible chromatin state can significantly improve genomic reprogramming.

DNA methyltransferase-3-like (DNMT3L) is an epigenetic factor that contributes to the establishment of DNA methylation and histone modifications (Chedin et al. 2002, Suetake et al. 2004, Kao et al. 2014). DNMT3L lacks catalytic activity but acts as a co-factor for the establishment of CG methylation and non-CG methylation (Ichijiyagi et al. 2013, Shirane et al. 2013, Vlachogiannis et al. 2015). DNMT3L can interact with histone H3 tail when its lysine 4 is unmethylated and induce de novo DNA methylation (Jia et al. 2007, Ooi et al. 2007). In addition, DNMT3L can recruit HDAC1 into the nucleus to perform histone deacetylation (Aapola et al. 2002, Deplus et al. 2002) and can facilitate HDAC1/TRIM28/SETDB1 complex formation in the nucleus, which accompanies the accumulation of repressive chromatin modification and transcriptional repression without triggering de novo DNA methylation (Kao et al. 2014).

Accumulating data indicate that DNMT3L is important for the dosage-dependent regulation of imprinted genes and transposable element silencing in germ cell development (Bourc’his & Bestor 2004, Kato et al. 2007, Lucifero et al. 2007, Smallwood et al. 2011, Kobayashi et al. 2012, Hara et al. 2014). During the pre-implantation period, Dmnt3l expression is detected in naturally fertilized eggs but subsequently declines until reaching the morula/blastocyst stage, at which it dramatically increases (Vassena et al. 2005). A recent study demonstrated that deletion of zygotic Dmnt3l resulted in delayed DNA methylation in naturally fertilized embryos (Guenatri et al. 2013). Furthermore, our previous results revealed that DNMT3L facilitates interactions of the transcriptional repressors DNMT3A, SETDB1, TRIM28 and HDAC1 in embryonic stem cells (Kao et al. 2014). Zygotic Dmnt3l expression commences at the morula/blastocyst stage, similar to the H3K9me3 methyltransferase SETDB1 (Dodge et al. 2004, Vassena et al. 2005). SETDB1 is recognized as a transcriptional repressor that can interact with HDAC1 and TRIM28 to silence gene expression (Schultz et al. 2002, Yang et al. 2003). Hence, removal of zygotic Dmnt3l/DNMT3L may disrupt epigenetically mediated gene silencing and result in more accessible chromatin for nuclear reprogramming, and therefore improve the developmental efficiency of NT embryos.

In this study, we used mouse embryonic fibroblasts (MEFs)-derived from Dnmt3l-knockout (Dnmt3l-KO) mice as donor cells for NT to examine the role of Dnmt3l in cloned embryo development. Our results revealed a synergistic effect between Dnmt3l-KO and treatment with TSA to enhance the developmental efficiency and quality of cloned embryos at the blastocyst stage. Our data revealed that zygotic Dmnt3l expression was dispensable for cloned embryo development during pre-implantation stages. Instead, we observed that Dmnt3l-KO donor cells displayed decreased levels of H3K9me3 and H3K27me3, higher levels of the active histone mark H3K27ac and increased cytoplasmic localization of HDAC1, indicative of a permissive epigenetic state beneficial for nuclear reprogramming.

Materials and methods

Animals, oocyte collection and donor cell preparation

Animal care and protocols used throughout the study were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University. C57BL/6 (B6) female mice were bred with DBA/2 males to obtain the hybrid strain B6D2F1 (C57BL/6× DBA/2). For NT experiment, the oocytes at the MII stage used were harvested from female B6D2F1 mice. Superovulation of 8- to 12-week-old mice was induced with 7.5 IU of pregnant mare serum gonadotropin followed by 7.5 IU of human chorionic gonadotropin (hCG) 48 h later. The oocytes were harvested 13–14 h after hCG treatment. The cumulus–oocyte complexes were incubated with 0.1 mg/ml hyaluronidase in M2 medium for 3 min and then washed in CZB-HEPES medium by gentle pipetting with glass pipette to remove the cumulus cells.

For donor cell preparation, C57BL/6 (B6) WT and Dnmt3l-KO MEF cell lines were derived from day 13.5–14.5 embryos via Dnmt3l heterozygote (Dnmt3l<sup>+/−</sup>) intercrosses (Hata et al. 2002). MEFs from passages four to six were used for NT. Near-confluent MEFs derived from WT and Dnmt3l-KO embryos were cultured under serum starvation conditions (0.1% FBS in DMEM) for 5 days. The starved MEFs were trypsinized, incubated with 10% FBS in DMEM and centrifuged to obtain single cell for NT.

NT and embryo culture

NT was performed as previously described (Sung et al. 2010). Briefly, cumulus-free oocytes were held with
a holding micropipette in CZB-HEPES medium containing 5 μg/ml cytochalasin B (CB), and the spindle chromosome complexes were removed with a pipette with an inner diameter of 10 μm using a Piezo-drill micromanipulator (PMAS-CT 150, Prime-Tech, Tsuchiura, Japan). The nuclear donor of MEFs with a diameter of 18–20 μm was selected and we inserted the donor cell into the perivitelline space of an enucleated mouse oocyte. After transfer, the cell–cytoplasm complexes were induced to fuse with two DC pulses of 150 V/1 mm for 10 μs using an Electro Cell Manipulator 200 (BTX, San Diego, CA, USA). The reconstituted oocytes were activated in calcium-free CZB medium containing 10 mM SrCl, 5 μg/ml cytochalasin B and 10 mM TSA for 6 h, followed by culture with 10 mM TSA in KSOM+AA medium (MR-121-D, Millipore, Billerica, MA, USA) for 4 h. The reconstructed embryos were cultured in KSOM+AA medium at 37 °C in 5% CO₂ humidified air.

**Immunofluorescence staining**

The embryos were fixed with 4% paraformaldehyde (PFA), washed with 0.1% polyvinyl alcohol in PBS (PVA-PBS) and treated with 0.5% Triton X-100 for 30 min. After incubation with 0.25% Tween-20 in PBS for 30 min, the embryos were blocked with 3% BSA in PVA-PBS for 2 h and then washed with 0.1% PVA-PBS at room temperature. The embryos were incubated at 4 °C overnight with the following primary antibodies: anti-OCT4 (POU5F1) (sc-9081; Santa Cruz Biotechnology, 1:1000 dilution) and anti-CDX2 (MU392A-UC; BioGenex, San Ramon, CA, USA, 1:1000 dilution). After incubation with donkey anti-rabbit-594 (A21207, Invitrogen, 1:500 dilution) and goat anti-mouse 488 (A11029, Invitrogen, 1:500 dilution) secondary antibodies, the embryos were counterstained with DAPI and mounted with a mounting medium.

For immunocytochemistry analysis, the cultured MEFs were fixed in 4% PFA, washed with 0.1% Tween-20 in PBS (PBST) and treated with 0.5% Triton X-100 for 30 min. After washing with PBST, the cells were blocked with 2% BSA in PBST for 1 h at room temperature and incubated at 4 °C overnight with an anti-HDAC1 primary antibody (ab7028; Abcam, Cambridge, MA, USA, 1:5000 dilution). The cells were then washed in PBST, incubated with secondary antibodies, stained with Hoechst33342 (Sigma), mounted with a mounting medium (P36934, Invitrogen) and analyzed using a Leica TCS SP5 II confocal microscope.

**cDNA amplification and quantitative PCR**

The approach utilized for global cDNA amplification and quantitative PCR (qPCR) from a single embryo or oocyte followed that of Kurimoto et al. (2007). In brief, the single embryo at different stages or the oocyte was lysed without purification. First-strand cDNAs were synthesized using a poly(dT)-tailed primer followed by exonuclease treatment to specifically eliminate unreacted primers. The second strands were generated with a second poly(dT)-tailed primer after poly(dA) tailing of the first-strand cDNAs. The cDNAs were amplified by PCR and the products were used for transcript expression analyses by qPCR. The monocolor hydrolysis probe detection system (Roche Diagnostics) was used to quantitate mRNA expression levels. The qPCR primers and corresponding probe numbers from Universal Probe Library (UPL) are as follows: Rplp0-forward, cttctcaatcataagggca, and Rplp0-reverse, tctttgctcagcgg (UPL probe no. 72); Dnmt3l-forward, agctagagcagcaac, and Dnmt3l-reverse, aggaggagaagggagt (UPL probe no. 79); MuERVL-forward, ggtatgggtcaatcctccag, and MuERVL-reverse, tccttacatctctgttattcc (UPL probe no. 41); and IAP-forward, tgaaggttcagtgtcctagttcc, and IAP-reverse, acagggcctccag (UPL probe no. 32). These primer sequences are also listed in Table S1 of the supplementary material, see section on supplementary data given at the end of this article. qPCR was performed in a thermal cycler (LightCycler 480 II Instrument; Roche Applied Science) using the following program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The housekeeping gene Rplp0 was used as a normalization control. The 2⁻ΔΔC樊 method was used to quantify the qPCR results.

**DNA extraction and bisulfite sequencing**

Genomic DNA was extracted from MEFs at passage 5 (P5) with a DNeasy Blood and Tissue kit (Qiagen). The genomic DNA was subjected to bisulfite treatment using an EZ DNA methylation kit according to the manufacturer’s instructions (Zymo Research, Orange, CA, USA), followed by PCR amplification and PCR product purification using a QIAquick Gel Extraction Kit (#28704, Qiagen). The PCR products were cloned using the pGEM-T Easy kit for subsequent sequencing (Promega). The sequencing results were analyzed using a BiQ Analyzer (Bock et al. 2005).

**Quantitative RT-PCR**

WT and Dnmt3l-KO MEFs were collected and treated with TRIzol (Invitrogen) to extract RNA. Reverse transcription reactions were performed using the Super-Script First-Strand Synthesis System (Invitrogen). qPCR was performed in a Roche Light Cycler 480II. The expression levels were detected using SYBR Green PCR master mix (KAPA Biosystems, Woburn, MA, USA) by the following program: 50 °C for 2 min, 95 °C for 10 min,
and then 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 45 s. The 2−ΔΔCt method was used to quantify the qPCR results. Gapdh was used as a housekeeping gene for normalization. The primers used are listed in Table S1 of the supplementary material, see section on supplementary data given at the end of this article.

**Western blotting analysis**

Western blotting was performed according to a standard protocol (Sambrook & Russell 2001) using PVDF membranes (Millipore). The cultured MEFs were lysed in RIPA buffer (Millipore) supplemented with 1× protease inhibitor cocktail (591134, Millipore), 1× phosphatase inhibitor cocktail (524629, Millipore), and 1 mM PMSF. The membranes were blocked with a blocker (Blok-PO or Blok-CH, Millipore) and 5% BSA (Sigma). Incubations with the primary antibodies anti-HDAC1 (ab7028, Abcam, 1:10,000 dilution), anti-H3K9me3 (05-1242, Millipore, 1:2000 dilution), anti-H3K9me3 (ab8898, Abcam, 1:3000 dilution), anti-H3K9ac (07-352, Millipore, 1:2000 dilution), anti-H3K27me3 (05-1951, Millipore, 1:2000 dilution), anti-H3K27ac (ab4729, Abcam, 1:2000 dilution), anti-H3ac (06-599, Millipore, 1:2000 dilution), anti-H3K9ac (9675, Cell Signaling Technology, Beverly, MA, USA, 1:2000 dilution), and anti-H3 (06-755, Millipore, 1:2000 dilution) were performed at 4 °C overnight with gentle shaking. After incubating with a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA, 1:10,000 dilution), the proteins were detected using a chemiluminescent detection reagent (Millipore). The images were quantified with ImageJ Software (http://rsbweb.nih.gov/ij/). The antibodies are listed in Table S2 of the supplementary material, see section on supplementary data given at the end of this article.

**RNA sequencing and bioinformatics analysis**

Total RNA for library preparation, sequencing, and RNA-seq analysis was extracted using TRIzol Reagent (Ambion, Austin, TX, USA). Following the manual, after extraction procedures, the total RNA was washed by 80% ethanol, and re-suspended in nuclease-free H2O. The concentration and quality of the RNA were determined using a RNA BR Chip (Agilent Technologies, Palo Alto, CA, USA) with an Agilent 2100 Bioanalyzer (Agilent Technologies). The isolated RNAs were rRNA depleted and purified by a RiboMinus Kit (Invitrogen). Following purification, the rRNA-free RNAs were fragmented into small pieces. The cleaved RNA fragments were copied into first-strand cDNA using random primers, followed by second-strand cDNA synthesis, a single A base addition and adapter ligation using a TruSeq RNA library preparation kit (Illumina, San Diego, CA, USA). The products were then enriched through 12 cycles of PCR amplification to create the final cDNA library. The concentration of final cDNA libraries were determined by a DNA HS Chip (Agilent Technologies) with an Agilent 2100 Bioanalyzer. The high throughput sequencing process was performed by Illumina HiSeq 2500 sequencer (Illumina, Inc., CIC bioGUNE). Notably, all of the cDNA libraries were pre-sequenced with typical TA-cloning methods using an *Escherichia coli* strand (DH5-α) to estimate the rRNA depletion rate before high throughput sequencing.

The adaptor-trimmed reads were aligned against the mm10 mouse reference genome using TopHat2 (v2.0.12) with sequence-specific parameter and were used for transcript fragment assembly with Cufflinks (v2.2.1) (Trapnell et al. 2012, Kim et al. 2013). After the assembly, transcript expression levels were calculated based on eXpress (v1.4.1) (Roberts & Pachter 2013) against the mm10 reseq database to identify differentially expressed genes and transcripts. For cross-sample comparison, expression levels were normalized and analyzed using DESeq2 (v1.8.0) (Love et al. 2014). Gene ontology (GO) analysis was performed using Panther Software (http://pantherdb.org/) (Mi et al. 2013). The sequences have been deposited in the NCBI GEO dataset under accession number GSE69007.

**Results**

The use of Dnmt3l-KO donor cells synergizes with TSA treatment to improve the developmental efficiency of the cloned embryos

To evaluate whether DNMT3L contributes to the developmental efficiency of reconstructed embryos, we analyzed cloned embryos generated with fibroblast donors from WT and Dnmt3l-KO embryos until 96 h post-activation. In the absence of the HDACi TSA treatment, most of the cloned embryos underwent two-cell division but then arrested at this stage (Fig. 1A and B; Table 1). Low percentages of cloned morula and blastocyst embryos were generated from WT MEFs (5 ± 1.6% of morula stage and 3 ± 2.3% of blastocyst stage) or Dnmt3l-KO MEF donor cells (15 ± 6.1% of morula stage and 9 ± 4.8% of blastocyst stage).

To further investigate the potential relationship between Dnmt3l-KO donor cell and HDACi, we performed NT experiments in the presence of TSA. Notably, under TSA treatment, cloned embryos derived from Dnmt3l-KO donor cells exhibited significantly increased blastocyst formation efficiency (68%), which was 2.5-fold higher than the WT group (25%) (Fig. 1C, D and E; Table 1), indicating an improved NT efficiency in the Dnmt3l-KO cloned embryos under TSA treatment. Furthermore, these results suggest that Dnmt3l depletion and TSA treatment synergistically improve the efficiency
of cloned embryos. In the following experiments, we focused on comparisons between the cloned embryos generated from WT and Dnmt3l-KO donor cells under TSA treatment.

**Table 1** Developmental rate of cloned embryos.

<table>
<thead>
<tr>
<th>Donor cell type</th>
<th>TSA treatment</th>
<th>Number of activated oocytes*</th>
<th>Number of two-cell embryos (%)</th>
<th>Number of morula embryos (%)</th>
<th>Number of blastocyst embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>268</td>
<td>241 (90±3.0)</td>
<td>13 (5±1.6)§</td>
<td>9 (3±2.3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>206</td>
<td>196 (95±1.5)</td>
<td>94 (46±7.9)§</td>
<td>51 (25±4.4)§</td>
</tr>
<tr>
<td>Dnmt3l-KO</td>
<td>–</td>
<td>317</td>
<td>292 (92±4.0)</td>
<td>48 (16±6.1)§</td>
<td>30 (9±4.8)§</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>146</td>
<td>138 (95±4.6)</td>
<td>115 (79±5.1)§</td>
<td>99 (68±9.3)§</td>
</tr>
</tbody>
</table>

M, morula embryos were check at day 4 after NT; BL, blastocyst embryos were check at day 5 after NT; the developmental rate was based on activated oocytes. The results are presented as the means±S.E.M. Statistical comparisons were determined by ANOVA with Duncan’s multiple range test. Values with different superscripts are significantly different (P<0.05). * vs †, † vs ‡, § vs ‡.

**Dnmt3l-KO donor cells improve the quality of cloned embryos under TSA treatment**

To compare the quality of the cloned embryos derived from these two types of donor cells under TSA treatment, we calculated the total cell numbers of the cloned embryos at the blastocyst stage. Fertilized embryos were used as a positive control. The results indicated that the blastocysts derived from the Dnmt3l-KO donor cells exhibited significantly higher total cell numbers compared with the embryos generated from WT cells under TSA treatment (Fig. 2A). Next, we classified the cloned blastocysts from grade I to grade III based on their quality, represented by total cell numbers and OCT4/CDX2 expression patterns in the blastocysts (Fig. 2B).

Grade I was defined as a blastocyst with more than 60 cells, with OCT4 expression restricted to the ICM and CDX2 expression limited to the trophectoderm. Grade II consisted of blastocysts with cell numbers ranging between 30 and 60; OCT4 was expressed in both the ICM and trophectoderm, and CDX2 was expressed in the trophectoderm. Grade III was characterized as a blastocyst with a low cell number (<30) and weak OCT4 and CDX2 expression patterns, as well as disorganized localization of the OCT4 signal and a lack of a clear ICM structure.

The percentage of good quality (grade I) embryos was higher among the blastocysts derived from Dnmt3l-KO donor cells than in those derived from WT donor cells (Fig. 2C). There was no obvious difference in the percentage of grade II embryos between these two groups. In contrast, compared with WT, the percentage of blastocysts with poor development (grade III) was lower among the Dnmt3l-KO cloned embryos (Fig. 2C). These data suggest that the use of Dnmt3l-KO donor cells improved the quality of the cloned embryos.

**Zygotic Dnmt3l is not required for development and transposable element regulation in cloned embryos during pre-implantation stages**

To investigate whether zygotic Dnmt3l is involved in NT embryo development at pre-implantation stages, we examined the amount of Dnmt3l expression in single enucleated oocyte or cloned embryo by cDNA

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amplification and qRT-PCR analysis. Each nuclei-removed oocyte still contained high levels of Dnmt3l transcripts (Fig. 3A). After injecting the donor cell into the enucleated oocyte, we continued to detect Dnmt3l expression throughout pre-implantation development in the cloned embryos. At the two-cell stage, Dnmt3l transcripts were still readily detectable in most embryos derived from both WT and Dnmt3l-KO MEFs (Fig. 3A). However, Dnmt3l expression became quite low in both WT and Dnmt3l-KO cloned embryos after the two-cell stage. The expression level of Dnmt3l did not increase significantly, even in the WT cloned embryos, until the late blastocyst stage, indicating a lack of significant zygotic Dnmt3l expression in pre-implantation cloned embryos (Fig. 3A).

These results suggest that the upregulation of zygotic Dnmt3l is not required for cloned embryo development at pre-implantation stages and that the improved quality of Dnmt3l-KO MEF-derived cloned embryos may not be primarily attributed to differences in Dnmt3l expression.

Given that proper regulation of transposable elements (TEs) is correlated with pre-implantation embryo development (Kigami et al. 2003, Peaston et al. 2004, Kim et al. 2014) and overexpression of the long terminal repeat retrotransposons, murine endogenous retroviruses-like (MuERVL) and intracisternal A-particle (IAP) retrotransposon are detrimental in stem cell pluripotency, we further investigated the expression levels of MuERVL and IAP in cloned embryo. We detected no obvious differences in the expression of MuERVL or IAP between WT and Dnmt3l-KO cloned embryos from the two-cell stage to the blastocyst stage (Fig. 3B). Therefore, during pre-implantation stages, although no obvious zygotic Dnmt3l expression was detected, MuERVL and IAP expressions were still repressed at the morula and blastocyst stages in most embryos, suggesting that zygotic Dnmt3l may not be required for TE repression in cloned embryos (Fig. 3).

Because zygotic Dnmt3l does not appear to be significantly involved in cloned embryo development at pre-implantation stages, we speculated that different properties of the WT and Dnmt3l-KO donor cells may contribute to the differential reprogramming efficiencies and qualities of the respective cloned embryos. Therefore, we further characterized potential facilitative and obstructive factors in the donor cells.

Reduced HDAC1 nuclear localization in Dnmt3l-KO MEF cells

Accumulating results indicate suppression of chromatin-modifying activities of histone deacetylases, including HDAC1, contribute to NT efficiency improvement (Enright et al. 2003, Rybouchkin et al. 2006, Ma & Schultz 2008). We thus investigated the subcellular localization of HDAC1 in donor cells by immunocytochemistry. Two types of HDAC1 staining were observed in the MEFs. In the cell with Type I pattern, HDAC1

Figure 2 The quality of WT and Dnmt3l-KO cloned embryos under TSA treatment. (A) The use of Dnmt3l-KO MEFs increased the total cell number of cloned embryos at the blastocyst stage under TSA treatment. The bars indicate the means ± s.e.m. from at least three independent experiments. (B) The blastocysts were classified into three classes, grades I–III, based on the expression and localization of OCT4 and CDX2 as well as total cell number. Scale bar = 50 μm. (C) The percentage of different graded blastocysts derived from WT and Dnmt3l-KO MEFs. The results were calculated from at least three independent experiments. Parthenogenetic embryos were used as a control. N, number of embryos; D3L-KO, Dnmt3l-KO.
expression was restricted to the nucleus, whereas the Type II cell exhibited both nuclear and cytoplasmic HDAC1 localization (Fig. 4A). Our assays demonstrated that HDAC1 was largely restricted to the nucleus in WT MEFs (Type I). In contrast, a significantly increased percentage of Dnmt3l-KO MEFs exhibited both nuclear and cytoplasmic localization of HDAC1 (Type II) compared with WT MEFs (Fig. 4B and C). We further examined the protein levels of HDAC1 via western blotting, which revealed no difference in the total amount of HDAC1 protein between WT and Dnmt3l-KO MEFs (Fig. 4D), indicating that the reduced HDAC1 staining signal in Dnmt3l-KO MEFs was primarily due to translocation to the cytoplasm.

**Increased expression of development-related genes in Dnmt3l-KO MEF cells**

From our RNA-seq gene expression profiles of WT and Dnmt3l-KO MEFs, we found that 54 genes were differentially expressed between WT and Dnmt3l-KO MEFs (fold change > 1.5 and $P$ value < 0.05) (Fig. 5A), of which 41 were obviously upregulated in Dnmt3l-KO MEFs. The differentially expressed genes between WT and Dnmt3l-KO MEFs were enriched for the Gene Ontology molecular function terms of nucleic acid binding, catalytic activity and nucleic acid binding transcription factor activity (Fig. 5B). In addition, we found that the expression levels of several genes associated with embryonic development, including Adam19, Chd8, Git1, Lims1/Pinch1, Pogo, and Sul1, were increased in Dnmt3l-KO MEFs (Nishiyama et al. 2004, Zhou et al. 2004, Liang et al. 2005, Holst et al. 2007, Pang et al. 2009). We further investigated the DNA methylation state of CpG islands in the promoter regions of three differentially expressed genes Adam19, Git1 and Lims1. The bisulfite sequencing results revealed that hypomethylation of the CpG islands in the promoter regions of these genes in both WT and Dnmt3l-KO MEFs (Supplementary Figure S1, see section on supplementary data given at the end of this article), suggesting that DNA methylation may not be the primary regulation of these genes in MEFs.

Collectively, these results suggest that the relatively permissive state of chromatin and the increased expression of development-associated genes in Dnmt3l-KO donor cells provide potential benefits for the improvement of NT efficiency.

**Discussion**

*In vitro* studies of epigenetic reprogramming hold great potential to improve our knowledge of natural developmental processes and advance techniques for patient-
specific therapeutic applications (Nishikawa et al. 2008, Cantone & Fisher 2013, Long et al. 2014). However, successful reprogramming occurs only in a proportion of cloned embryos; therefore, improvements in developmental efficiency and our understanding of epigenetic reprogramming are essential. Our results revealed that a synergistic effect between Dnmt3l-KO donor cells and TSA treatment dramatically increased the efficiency and quality of cloned embryos.

Several reports have shown that TSA treatment of the donor cell or the reconstructed embryo can improve NT cloning efficiency, because improvements in developmental efficiency and our understanding of epigenetic reprogramming are essential. Our results revealed that a synergistic effect between Dnmt3l-KO donor cells and TSA treatment dramatically increased the efficiency and quality of cloned embryos.

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In contrast, DNMT3L is not crucial for the modulation of genomic imprinting at pre-implantation stages (Bourc’his et al. 2001, Inoue et al. 2002, Kato et al. 2007, Kamimura et al. 2014). DNMT3L is not strictly required for transposable element silencing in fertilized embryos (Guenatri et al. 2013). Moreover, our results revealed that the repression of TEs, at least MuERV and IAP, occurs normally in Dnmt3l-KO cloned embryos. These results suggest that either the role of DNMT3L in pre-implantation embryos is different from its function in germ cells or that the remaining Dnmt3l transcripts in the recipient ooplasm are sufficient to regulate MuERV and IAP expression.

Our previous study indicated that DNMT3L can facilitate interactions among DNMT3A, SETDB1, TRIM28 and HDAC1 in ES cells, suggesting the involvement of DNMT3L in establishing repressive transcriptional silencing (Kao et al. 2014). Compared with WT cloned embryos, the increased developmental efficiency of Dnmt3l-KO embryos indicated improved embryonic gene activation in the absence of zygotic Dnmt3l, as we observed significantly increased cell numbers and more restricted OCT4 expression in the ICM at the blastocyst stage. These results suggested that Dnmt3l-KO embryos possess a more permissive epigenetic environment for nuclear reprogramming. In addition, zygotic Dnmt3l may be dispensable for the function of SETDB1 in ICM establishment, given that disruption of SETDB1 expression results in defective ICM growth in pre-implantation embryos (Dodge et al. 2004); by comparison, Dnmt3l-KO cloned embryos exhibited high-quality blastocysts. These data suggest that zygotic Dnmt3l may not be involved in SETDB1-mediated ICM formation in cloned embryos.

Dnmt3l-KO MEF donor cells displayed increased levels of the active histone mark H3K27ac, reduced nuclear HDAC1 localization and decreased levels of repressive histone marks, suggesting that the chromatin state of these donor cells is more permissive for the reprogramming process. Furthermore, our data revealed that compared with WT MEFs, Dnmt3l-KO MEFs exhibited differential expression of genes associated with embryonic development. The expression of Dnmt3l/Dnmt3L was not detected in MEFS by western blotting or quantitative real-time PCR analysis (data not shown); therefore, the observed effects on HDAC1, histone modifications and gene expression may result from accumulation in stem/progenitor cells when Dnmt3l is expressed.
Relationships between deficiencies of epigenetic regulators and long-term effects at later developmental stages have been previously reported (Bourc'his & Bestor 2004, Gu et al. 2011). For instance, Dnmt3l-KO male germ cells exhibit severe defects at the postnatal spermatocyte stage when Dnmt3l/DNMT3L is not expressed (Bourc'his et al. 2001, Hata et al. 2002). Recently, DNMT3L expression has been observed in several types of stem/progenitor cells (Nimura et al. 2006, Liu et al. 2013, Liao et al. 2014). It will be interesting to investigate how DNMT3L influences the epigenome in stem/progenitor cells and the basis for its potential long-term effects in their descendants.

NT reprogramming in human cell lines has improved in recent years. The recent successful generation of human pluripotent embryonic stem cells (hPESCs) provides hope that NT-derived embryos and NT-ESCs may serve as models for human disease research with significant applications for personalized cell replacement therapies (Tachibana et al. 2013, Chung et al. 2014, Ma et al. 2014, Yamada et al. 2014). Accumulating evidence has revealed that DNMT3L/DNMT3L is expressed in several types of human tissues, including the prefrontal cortex, liver, testis, ovary and thymus (Aapola et al. 2000, Borghese et al. 2012, Lee et al. 2014). DNMT3L mutations have been associated with schizophrenia, subtelomeric hypomethylation, lower intelligence and ovarian endometriosis (El-Maarri et al. 2009, Haggarty et al. 2010, Borghese et al. 2012, Saradaleksimi et al. 2014). Recent studies have shown that the loss of Dnmt3l expression correlates with failed TE silencing and defective genomic imprinting at the Dlk1-Dio3 locus in induced pluripotent stem cells (iPSCs) (Stadtfeld et al. 2012, Tang et al. 2012), which might correlate with reduced neural lineage differentiation potential (Stadtfeld et al. 2012, Mo et al. 2015). Dnmt3l-mutant iPSCs are therefore not suitable for biomedical research. As zygotic Dnmt3l is dispensable for blastocyst development and TE silencing in cloned embryo, NT followed by embryonic stem cell derivation is an alternative method and a potential strategy for DNMT3L-related disease research and pharmaceutical development.

In conclusion, our study suggests that the use of Dnmt3l-KO donor cells benefits SCNT reprogramming efficiency, potentially due to a more accessible chromatin state and reduced HDAC1 activity in Dnmt3l-KO donor cells.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0031.

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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