REPRODUCTION
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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.

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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

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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 methyltransfer-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 (Ichiyanagi *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 preimplantation period, *Dnmt3l* 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 *Dnmt3l* 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 *Dnmt3l* 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 *Dnmt3l*/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 *Dnmt3l* expression was dispensable for cloned embryo development during pre-implantation stages. Instead, we observed that *Dnmt3l*-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/6J × DBA/2J). 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 ($Dnmt3l^{+/-}$) 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–cytoplast 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 nM TSA for 6 h, followed by culture with 10 nM 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% CO_2 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 0.25% Triton X-100 in PBS. 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, cttcccacttactgaaaaggtca, and *Rplp0*-reverse, tcctttgcttcagctttgg (UPL probe no. 72); *Dnmt3l*-forward, agtcagaagcaggagcaagc, and *Dnmt3l*-reverse, agcgggagaaggcagttc (UPL probe no. 79); *MuERVL*-forward, ggtatgggtcaatcctccag, and *MuERVL*-reverse, tcctctactacccattctgtatttcc (UPL probe no. 41); and *IAP*-forward, tgaaggttcagtgtcctagttcc, and *IAP*-reverse, acaggcttttacccagagca (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 $2^{-\Delta Cp}$ 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 (#69504, 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 $2^{-\Delta\Delta Cp}$ 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 (Bløk-PO or Bløk-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-H3K18ac (#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 H_2O. 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 refseq 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

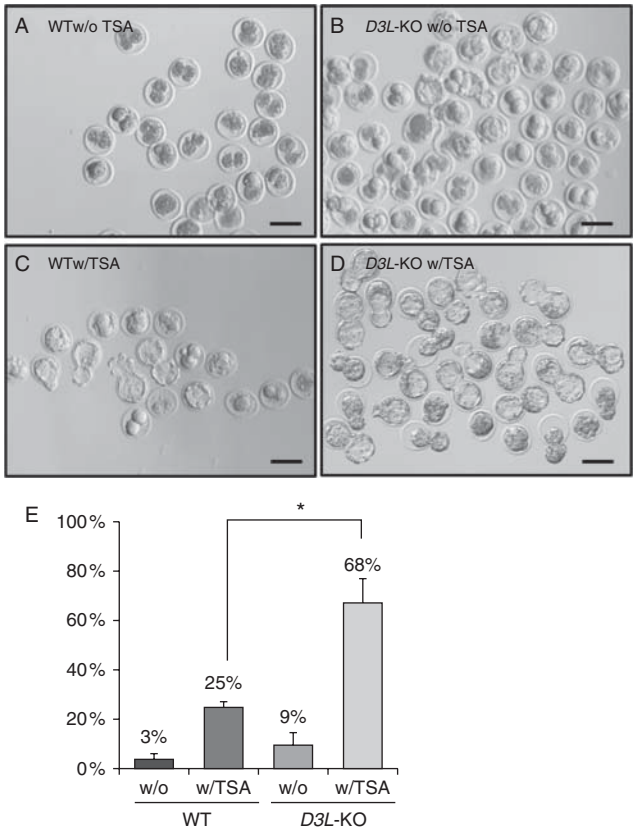

Figure 1 The developmental efficiency of cloned embryos derived from WT and *Dnmt3l*-KO MEF donor cells. Cloned embryos were collected 96 h post-activation. (A and B) Without TSA treatment, most cloned embryos were arrested at the two-cell stage, whether they were derived from WT or *Dnmt3l*-KO MEF donor cells. (C and D) Under TSA treatment, both WT and *Dnmt3l*-KO MEF-derived embryos displayed increased developmental efficiency compared with non-TSA treatment groups. In addition, a significant proportion of *Dnmt3l*-KO NT embryos developed to the blastocyst stage. Scale bar, 100 μm. (E) Quantification results indicated that the percentage of blastocysts derived from *Dnmt3l*-KO MEFs with TSA treatment was the highest compared with the other groups. The results are presented as the means±S.E.M. from at least three independent experiments. *denotes $P<0.05$, Student's *t*-test. w/o, without TSA treatment; w/TSA with TSA treatment. *D3L*-KO, *Dnmt3l*-KO.

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.

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

Table 1 Developmental rate of cloned embryos.

Donor cell type	TSA treatment	Number of activated oocytes[a]	Number of two-cell embryos (%)	Number of morula embryos (%)	Number of blastocyst embryos (%)
WT	−	268	241 (90±3.0)	13 (5±1.6)*	9 (3±2.3)
	+	206	196 (95±1.5)	94 (46±7.9)‡	51 (25±2.4)§
Dnmt3l-KO	−	317	292 (92±4.0)	48 (16±6.1)*	30 (9±4.8)§
	+	146	138 (95±4.6)	115 (79±5.1)‡	99 (68±9.3)$^{\|\|}$

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 ‖.
[a]These results were compiled from three experimental replicates.

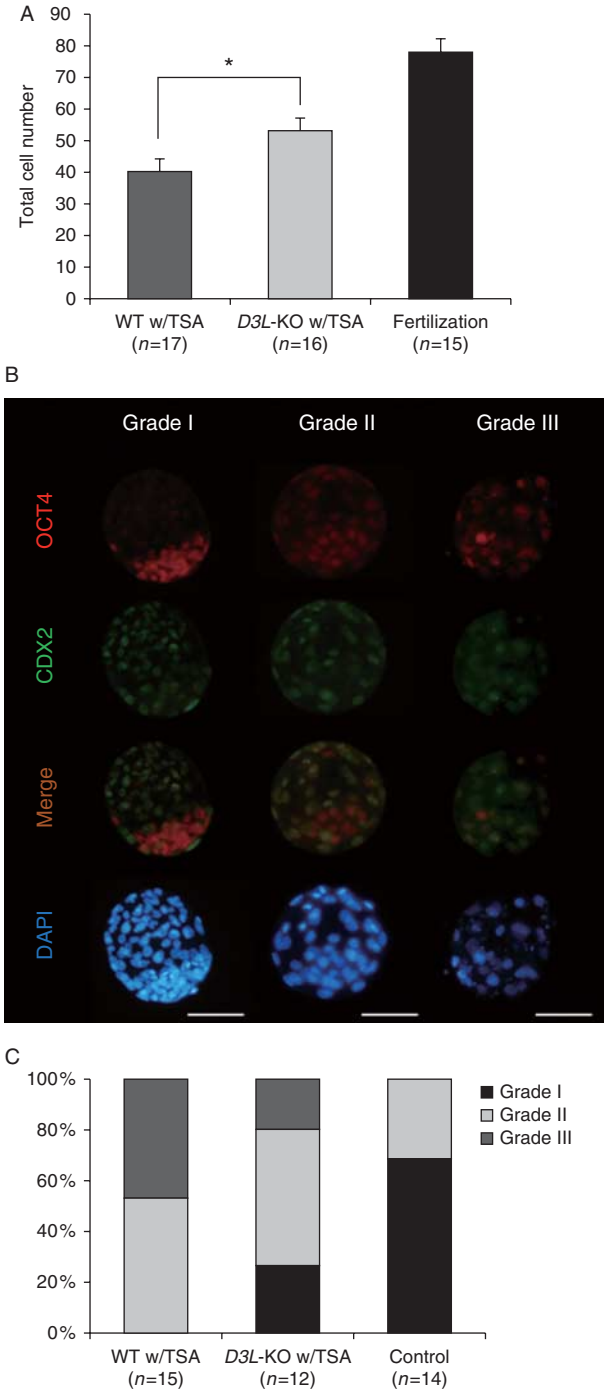

Figure 2 The quality of WT and *Dnmt3l*-KO cloned embryos under TSA treatment. (A) The use of *Dnmt3l*-KO MEFs increased the total cell number of cloned embryos at the blastocyst stage under TSA treatment. The bars indicate the means±S.E.M. from at least three independent experiments. (B) The blastocysts 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.

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

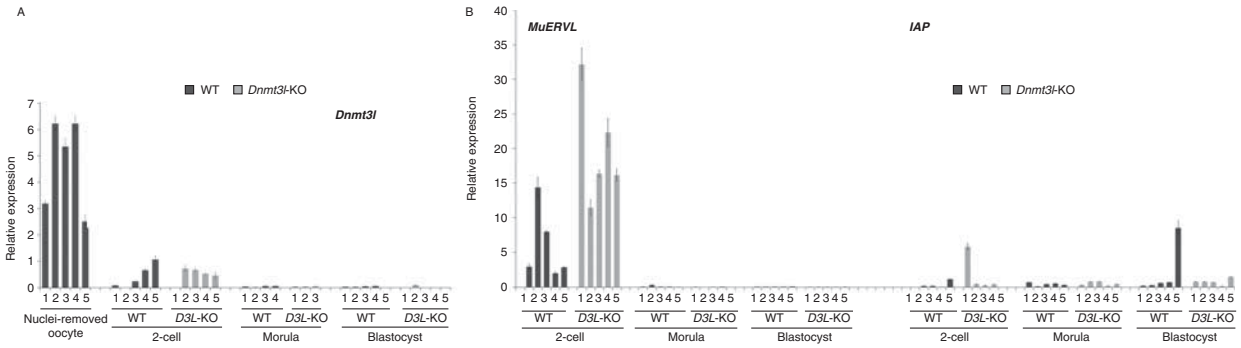

Figure 3 Expression of *Dnmt3l* and transposable elements in cloned embryos at pre-implantation stages. (A) *Dnmt3l* expression in cloned embryos. Single enucleated oocyte or embryo was analyzed by qPCR. *Dnmt3l* expression was readily detectable in both WT and *Dnmt3l*-KO MEF-derived cloned nuclei-removed oocytes but not in morulas and blastocysts. (B) *MuERVL* and *IAP* expression levels in individual cloned embryos by qPCR. No obvious difference in the *MuERVL* or *IAP* expression pattern was observed between the two types of cloned embryos at pre-implantation stages. *Rplp0* was used as an internal control for mRNA expression analysis. Quantification of mRNA expression was performed using the $2^{-\Delta Cp}$ method. Error bars represent the s.e.m. generated from independent embryos with three technical repeats. *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 H3K27ac and decreased H3K27me3 and H3K9me3 levels in Dnmt3l-*KO MEF cells*

Accumulating evidence suggests that the epigenetic state of the donor cell correlates with the developmental efficiency of cloned embryos. Enhanced acetylation modifications on the histone tail improve cloning efficiency, whereas H3K9me3 is recognized as a critical epigenetic barrier in nuclear reprogramming (Enright *et al*. 2003, Antony *et al*. 2013, Matoba *et al*. 2014). We thus examined the levels of several histone marks, including the active marks H3K4me3, H3ac, H3K9ac, H3K18ac and H3K27ac and the repressive marks H3K9me3 and H3K27me3, implicative of the global chromatin state in the two types of donor cells. We did not observe obvious differences in the levels of H3K4me3, H3ac, H3K9ac, H3K18ac between WT and *Dnmt3l*-KO MEFs (Fig. 4E and F). However, compared with WT MEFs, *Dnmt3l*-KO MEFs exhibited significantly increased H3K27ac and decreased H3K9me3 and H3K27m3 levels (Fig. 4E and F). These data suggest that *Dnmt3l*-KO MEFs may present a more accessible chromatin state that facilitates NT reprogramming.

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*, *Pogz* and *Sulf1*, 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-

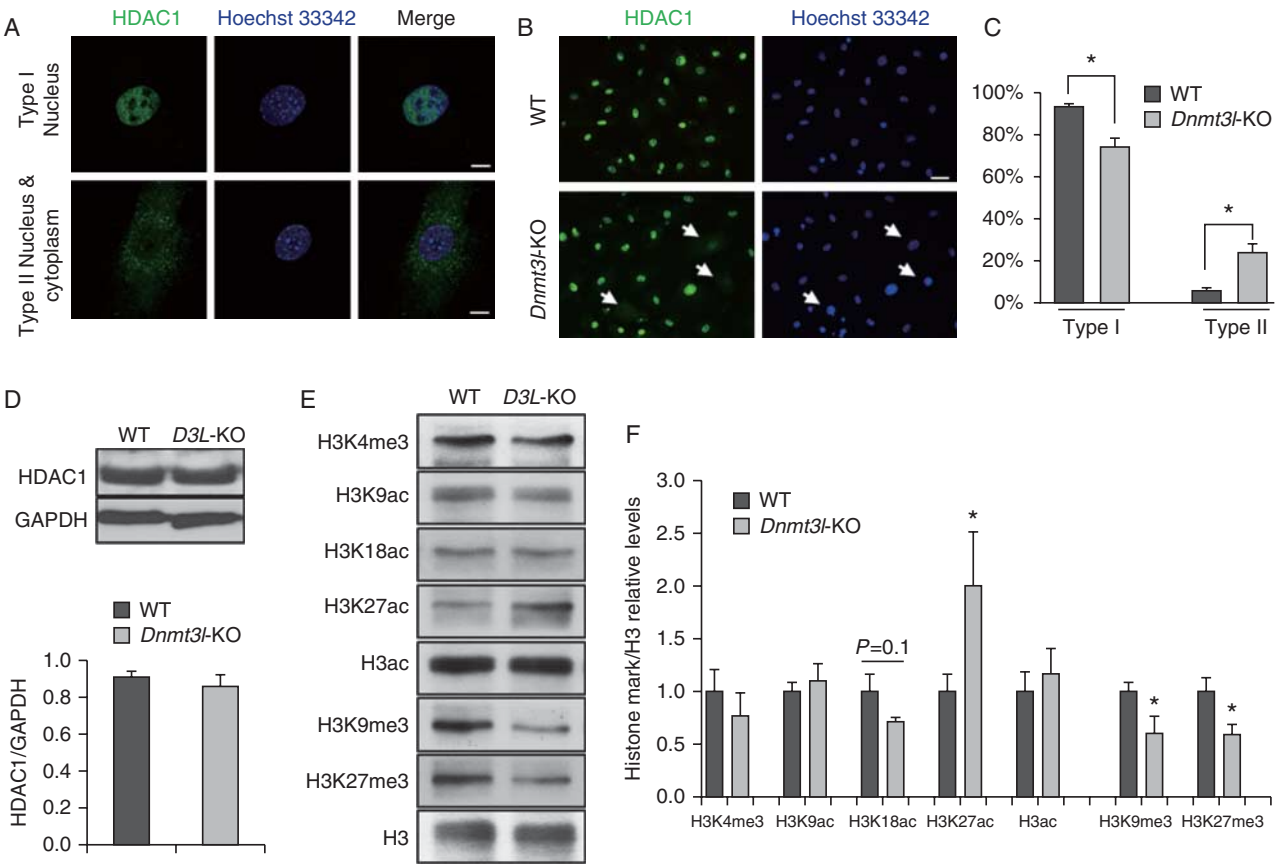

Figure 4 HDAC1 subcellular localization and the relative levels of histone marks in WT and *Dnmt3l*-KO mouse embryonic fibroblast cells. (A) Two patterns of HDAC1 localization were observed in the MEFs: Type I (nuclear localization) and Type II (nuclear and cytoplasmic localization). Scale bar, 10 μm. (B) Immunostaining of HDAC1 in WT and *Dnmt3l*-KO MEFs. Scale bar, 50 μm. (C) A significantly higher percentage of Type II HDAC1 expression was observed in *Dnmt3l*-KO MEFs compared with WT MEFs. The error bars represent the s.e.m. generated from three independent experiments. (D) No obvious change in total HDAC1 protein expression was observed between WT and *Dnmt3l*-KO MEFs. GAPDH was used as an internal control for protein expression. Mean ± s.e.m. were calculated from at least three biological replicates. (E) Western blotting of histone marks in WT and *Dnmt3l*-KO MEFs. Increased H3K27ac and reduced H3K9me3 and H3K27me3 levels were observed in *Dnmt3l*-KO MEFs. Histone H3 was used as an internal control. (F) Bar chart displaying the relative levels of active and repressive histone marks in WT and *Dnmt3l*-KO MEFs. The results are the mean ± s.e.m. from at least three biological replicates. *D3L*-KO, *Dnmt3l*-KO. *denotes $P<0.05$ (Student's *t*-test).

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, which is likely through the creation of a permissive chromatin state (Enright *et al*. 2003, Enright *et al*. 2005, Kishigami *et al*. 2006, Rybouchkin *et al*. 2006, Bui *et al*. 2010). Recent studies have observed that donor cells with decreased H3K9me3 or H3K27me3 levels are associated with improved NT efficiency in cloned embryos (Antony *et al*. 2013, Matoba *et al*. 2014, Saini *et al*. 2015), further suggesting that the establishment of accessible chromatin can facilitate nuclear reprogramming in cloned embryos. In this study, we observed that *Dnmt3l*-KO MEFs display relatively higher levels of H3K27ac and lower levels of H3K9me3 and H3K27me3 compared with WT MEFs. Furthermore, cloned embryos generated from *Dnmt3l*-KO donor cells and treated with TSA significantly increased NT efficiencies at pre-implantation stages. These results suggest that the initial epigenetic state of the donor nucleus contributes to the development of cloned embryos and that the reduction of repressive histone marks in donor cells benefits the reprogramming process.

Genome-wide epigenetic reprogramming occurs at two developmental stages in the mammalian life cycle: gametogenesis and pre-implantation embryonic development (Sasaki & Matsui 2008, Messerschmidt *et al*. 2014). *Dnmt3l*/DNMT3L is significantly expressed in both pre-implantation embryo and germ cells (Bourc'his *et al*. 2001, Sakai *et al*. 2004, Guenatri *et al*. 2013). In germ

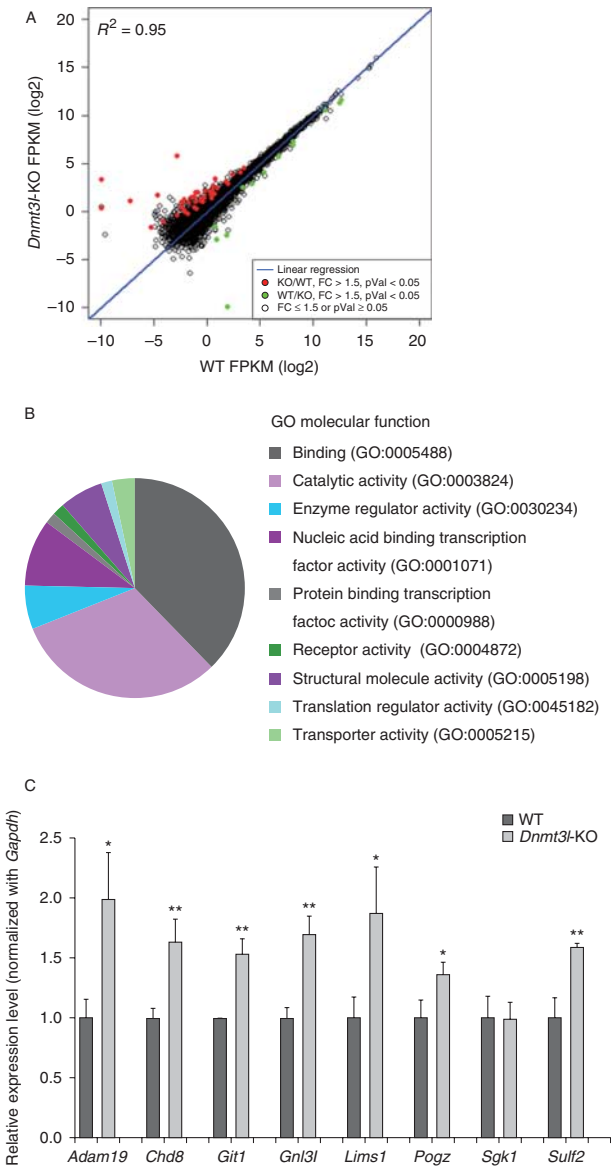

Figure 5 Increased expression of development-associated genes in *Dnmt3l*-KO MEFs. (A) Comparison of the expression profiles of WT and *Dnmt3l*-KO MEFs. Two biological replicates of WT and *Dnmt3l*-KO samples were analyzed. The red and green circles indicate genes that were differentially expressed between WT and *Dnmt3l*-KO MEFs (fold change >1.5 and *P* value <0.05). FC, fold change; pVal, P value; KO, *Dnmt3l*-KO. (B) Gene ontology (GO) term analysis of the differentially expressed genes in WT and *Dnmt3l*-KO MEFs. (C) Bar chart displaying the relative expression levels of differentially expressed genes in WT and *Dnmt3l*-KO MEFs. The qPCR results were quantified by $2^{-\Delta\Delta Cp}$ method and *Gapdh* was used as a housekeeping gene for normalization. The error bars represent the S.E.M. from four to five biological repeats. *denotes $P<0.05$ and **indicates $P<0.01$ (Student's *t* test).

cells, DNMT3L contributes to the monoallelic expression of imprinted genes and TE silencing to safeguard the integrity of the genome for germ cell development (Bourc'his *et al.* 2001, Bourc'his & Bestor 2004, Hata *et al.* 2006). DNMT3L also interacts with DNMT3A to establish oocyte-specific methylation imprints during oocyte growth (Lucifero *et al.* 2007, Hara *et al.* 2014). Furthermore, recent studies using high-throughput analyses of WT and *Dnmt3l*-KO oocytes revealed a role for DNMT3L in establishing DNA methylation patterns on transposable elements (Smallwood *et al.* 2011, Kobayashi *et al.* 2012, Shirane *et al.* 2013). In male germ cells, *Dnmt3l* deficiency leads to a defect in imprinting establishment and silencing of retrotransposons (Bourc'his & Bestor 2004, Hata *et al.* 2006, Kato *et al.* 2007, Liao *et al.* 2014, Vlachogiannis *et al.* 2015).

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 *MuERVL* 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 *MuERVL* 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 generating repressive chromatin for transcriptional inhibition (Kao *et al.* 2014). Compared with WT cloned embryos, the increased developmental efficiency of *Dnmt3l*-KO cloned 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 donor MEFs from *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 gene 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 (NT-hES cells) 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, Saradalekshmi *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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