Effects of *TET1* knockdown on gene expression and DNA methylation in porcine induced pluripotent stem cells

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Abstract

TET1 is implicated in maintaining the pluripotency of embryonic stem cells. However, its precise effects on induced pluripotent stem cells (iPSCs), and particularly on porcine iPSCs (piPSCs), are not well defined. To investigate the role of *TET1* in the pluripotency and differentiation of piPSCs, piPSCs were induced from porcine embryonic fibroblasts by overexpression of *POU5F1* (*OCT4*), *SOX2*, *KLF4*, and *MYC* (*C-MYC*). siRNAs targeting to *TET1* were used to transiently knockdown the expression of *TET1* in piPSCs. Morphological abnormalities and loss of the undifferentiated state of piPSCs were observed in the piPSCs after the downregulation of *TET1*. The effects of *TET1* knockdown on the expression of key stem cell factors and differentiation markers were analyzed to gain insights into the molecular mechanisms underlying the phenomenon. The results revealed that knockdown of *TET1* resulted in the downregulated expression of pluripotency-related genes, such as *LEFTY2*, *KLF2*, and *SOX2*, and the upregulated expression of differentiation-related genes including *PITX2*, *HAND1*, *GATA6*, and *LEF1*. However, *POU5F1*, *MYC*, *KLF4*, and *NANOG* were actually not downregulated. Further analysis showed that the methylation levels of the promoters for *POU5F1* and *MYC* increased significantly after *TET1* downregulation, whereas there were no obvious changes in the promoters of *SOX2*, *KLF4*, and *NANOG*. The methylation of the whole genome increased, while hydroxymethylation slightly declined. Taken together, these results suggest that *TET1* may play important roles in the self-renewal of piPSCs and the maintenance of their characteristics by regulating the expression of genes and the DNA methylation.

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Introduction

DNA methylation is one of the most important epigenetic markers; it has been well characterized and demonstrated to be involved in many biological processes, including transposable element silencing, genomic imprinting, and X chromosome inactivation (Bird 2002, Surani *et al.* 2007, Sasaki & Matsui 2008, Cedar & Bergman 2009). However, the mechanism underlying DNA demethylation is largely unknown and remains controversial (Ooi & Bestor 2008). Recently, the functions of the Ten-eleven translocation (TET) family of methyl dioxygenases (*TET1*, *TET2*, and *TET3*) have been implicated in DNA demethylation via converting the 5-methylcytosine (5mC) of DNA to 5-hydroxymethylcytosine (5hmC) (Tahiliani *et al.* 2009). *Tet1* is expressed in embryonic stem cells (ESCs) and preimplantation embryos; it is also involved in the self-renewal and the maintenance of pluripotency of the ESCs and the specification of the inner cell mass (Ito *et al.* 2010). *Tet1*-depleted ESCs mediated by RNAi exhibited skewed differentiation toward the

trophectoderm fate, significant reduction in the content of 5hmC, and the loss of their ESC identity (Freudenberger *et al.* 2012).

Induced pluripotent stem cells (iPSCs) exhibit many similarities to ESCs. Since their first generation (Takahashi & Yamanaka 2006), there has been an explosive development of iPSCs because they can be generated from an easily accessible cell source, completely overcoming the ethical drawbacks associated with the use of human ESCs, and they allow patient-customized therapy that avoids immunological rejection (Park *et al.* 2008). Despite their undoubted promise in regenerative medicine, many obstacles must be overcome before using these cells as a source of transplant material; there is an especially strong need for animal models whose anatomy and physiology better resemble those of humans than do those of the mouse to test the efficacy and the safety of the iPSCs transplantation. Pigs are a potentially useful model in this regard because of the low probability of disease transfer between humans and pigs (Cheng & Xiao 2009) and their similarities to

humans in their long life span, organ size, immunology, and physiology (Ezashi *et al.* 2009). Recently, three papers that were published almost simultaneously by different groups described the generation of porcine iPSCs (piPSCs; Esteban *et al.* 2009, Ezashi *et al.* 2009, Wu *et al.* 2009). The piPSCs provide an attractive model to study certain human diseases or assess the therapeutic applications of iPSCs in a large animal model. The process of reprogramming somatic cells to become iPSCs has been shown to be accompanied by changes in DNA methylation and certain epigenetic changes. Thus, piPSCs provide a favorable system to study the epigenetic features that are prerequisite for pluripotency (Mattout *et al.* 2011). In this study, we derived piPSCs and investigated whether TET1 played an important role in the epigenetic regulation of these cells.

Materials and methods

Reagents and animals

Chemicals and media were purchased from Sigma unless otherwise stated. Cell culture-related reagents were purchased from Gibco unless otherwise stated. Specific pathogen-free mice and non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Vital River Laboratories (Beijing, China) and pregnant pigs were purchased from the Jilin University Pig Farm. All the experiments involving animals were approved by and complied with the experimental practices and standards of the Animal Welfare and Research Ethics Committee at Jilin University (approval ID: 20101008-2).

Induction of piPSCs from porcine embryonic fibroblasts

The 293T cells were plated in T-75 flasks (Corning, New York, USA) containing DMEM/F12 + 10% fetal bovine serum (FBS, HyClone, Utah, USA) until reaching 80% confluency. To package the retroviruses, pMXs retroviral vectors encoding mouse *SOX2*, *KLF4*, *POU5F1* (*OCT4*), and *MYC* (*C-MYC*) (SKOM) or GFP cDNAs were individually used to cotransfect the cells with Vsvg and Gap using Lipofectamine 2000 (Invitrogen). The retroviruses were collected 48 and 72 h later. Porcine embryonic fibroblasts (PEFs) were isolated from the 30-day-old fetuses of purebred Yorkshire pigs and maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin. The cells were seeded at a density of 10^6 per well in six-well plates and maintained in a 5% CO₂ atmosphere at 37.8 °C. After 24 h, the cells were infected with cocktails of the previously packaged retroviruses. A pMXs retroviral vector encoding GFP, which also served as a negative control for iPS cell induction, was used to monitor the transfection efficiency. After 5 days of normal culture, the PEFs were trypsinized (0.25% trypsin, Gibco) for passage onto a feeder layer of mitomycin C mitotically inactivated mouse embryonic fibroblasts (MEFs) in ES medium containing 20% Knockout Serum Replacement, 10 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM GlutaMAX, 1 mM sodium pyruvate, 10 ng/ml

stem cell factor (PeproTech, New Jersey, USA), 10 ng/ml human basic fibroblast growth factor (bFGF, PeproTech), and 1000 U ESGRO (Millipore, Billerica, USA). When ESC-like colonies appeared, they were harvested and maintained by manual passage to expand the cultures every 4–5 days. The colonies that had a normal ES-like morphology were used for alkaline phosphatase (AP) staining. Embryoid bodies (EBs) expressing specific markers of the three germ layers (endoderm, *AFP*; mesoderm, *BMP4*; and ectoderm, *GFAP*) were also detected. The specific primers for the pluripotency genes are listed in Table 1.

siRNA transfection into piPSCs

The piPSCs were cultured on gelatin-coated plates in the ES medium described earlier. siRNAs targeted to *TET1* (siRNA #1,

Table 1 Primers and PCR conditions.

Primer	Primer pair sequences (5'–3')	Annealing temperature (°C) × cycle no.	Product sizes
RT-PCR			
MYC	F-ggattccgcctcgtt R-tctccaagcatcactcg	55 × 40	185
GAPDH	F-acctgccgcctggagaaacc R-gaccatgaggtccaccaccctg	60 × 40	252
GATA6	F-ctggtgtctgccaagcg R-aaggatgagcaggcgtaa	57 × 40	243
HAND1	F-ggcgagaagaggattaaaggg R-caatcaaggatggcagtagt	59 × 40	201
KLF2	F-ggcacgcagcagcgcctcaaca R-cctagcaactccgacaccaagc	60 × 40	186
KLF4	F-cggcaaaactacacgaagagt R-agttcatctgagcgggcaaat	59 × 40	120
LEF1	F-attcaggcaaccctaccc R-agctacgcagcttcgctct	60 × 40	186
LEFTY2	F-gggatgaaatgggctgagaactggg R-tgctgacgatcacgggcaacgag	57 × 40	169
NANOG	F-cttattcaggacagccctgattctc R-aagacggcctccaatacactg	59 × 40	614
POU5F1	F-gtcgccagaaaggcgaac R-cagggtggtgaagtgaagg	57 × 40	125
PITX2	F-cttcagggaagcccgagtc R-cgcccagttgtgtatgagt	57 × 40	171
SOX2	F-cctctgacgtacactccatgac R-ggtgccctgctgcgagta	59 × 40	86
TET1	F-ggcaccctgaagcggatgagc R-gagcaccgagccgtgaatgg	60 × 40	143
Semiquantitative PCR			
AFP	F-tattggagaaatgttcgcagtc R-ccagggtttatgggcatc	56 × 24	349
BMP4	F-gcgtagccccagcatcacc R-atccctctactaccatctcctg	60 × 24	341
GFAP	F-ctcgcgcctcctatgtct R-gcctgtttctgctgctc	59 × 24	291
GAPDH	F-acctgccgcctggagaaacc R-gaccatgaggtccaccaccctg	60 × 24	252
LIN28	F-cagagtaagctgcacatggagg R-gtaggctggcttccctgtg	60 × 24	559
NANOG	F-cttattcaggacagccctgattctc R-aagacggcctccaatacactg	58 × 24	614
POU5F1	F-caaactgaggtgctgccttc R-attgaacttcacctccctcaacc	57 × 24	125
SOX2	F-ggttacctcttctccactcca R-caaaaatagtcctcccaaaaagaag	59 × 24	439

Table 2 Primers and amplicon information of bisulfite sequence PCR.

Genes	GenBank ID	Location	Primer sequence	CpG sites	Length
KLF4	NW_003609455	439364–439701	F-ttgattataaattaaggggaggg R-caaccacaaaatctcaaaacattt	17	336
NANOG ^a	GU433187.1	16–515	F-ggagatttaaaggagtttaggttaagaaa R-tctcctccaaataaataatcaaaaa	10	500
POU5F1	NW_003610613	53168–53453	F-tggttgagttttaaggggtttt R-ccaactctccttatccaactt	24	286
SOX2	NW_003611770	123967–124348	F-ggggggataaaaggttttta R-aaactcaaacctctctcccttc	23	382
MYC	NW_003609985	895195–895492	F-ttgtttgatagggtgaaaga R-aaaacccatttataaaaacacc	16	298

^aZhao MT, Rivera RM & Prather RS 2013 Locus-specific DNA methylation reprogramming during early porcine embryogenesis. *Biology of Reproduction* **88** 48.

siRNA #2, siRNA #3, and siRNA #4), non-sense siRNA (negative control), and FAM-fluorescein-labeled non-sense siRNA (FAM-siRNA) oligonucleotides were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). The oligonucleotide sequences are listed in Table 2. The transfection efficiencies of different ratios of siRNA to Lipofectamine 2000 were determined using FAM-siRNA. The transfection of specific siRNAs was performed under the optimized conditions (Table 3). The dissociated piPSCs were seeded on gelatin-coated 24-well plates in ES medium (400 µl/well). The cells in each well were transfected with 30 pmol siRNAs in ES medium for each well. Briefly, Lipofectamine (2 µl) and siRNAs (30 pmol) were premixed with Opti-MEM (50 µl, Invitrogen) respectively. Then they were mixed and incubated at room temperature for 15–30 min before applying to the cells. The transfected cells of each well were split into three wells of 24-well plate the next day and cultured in ES medium for 24 h. The total RNAs were extracted from the cells 48 h after transfection with siRNA. To analyze *TET1* downregulation, the dissociated piPSCs were plated in one well of a 24-well plate; 24 h after transfection, the cells were replated in another 24-well plate for 24 h, after which they were used for RT-PCR.

Semiquantitative PCR and real-time PCR analysis

The total RNAs from the piPSCs were extracted using RNAiso Plus (TaKaRa, Shiga, Japan). A RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) was used to synthesize the first-strand cDNAs, according to the manufacturer's instructions, immediately after RNA isolation. Semi-quantitative PCRs were performed in a 50 µl volume containing 2 µl cDNA, 25 µl rTaq premix (TaKaRa), 22 µl RNase-free water, and 0.5 µl each of the forward and reverse primers (10 pmol) for each gene. To determine optimal cycles of amplification, PCR products were obtained from 18, 20, 22, 24, and 26 cycles respectively. All the products were conducted on ethidium bromide-stained agarose gels. When the cycles exceeded 24 cycles, the intensity of PCR bands did not change any more. Real-time PCRs were performed in a 25 µl volume containing 2 µl cDNA, 12.5 µl SYBR Green Master Mix (TaKaRa), 9.5 µl RNase-free water, and 0.5 µl each of the forward and reverse primers (10 pmol) for each gene. A reaction mix was formulated for the sample and for the non-reverse transcriptase control reactions. The program used for

the amplification of each gene consisted of a denaturing cycle of 3 min at 95 °C and 40 cycles of PCR (95 °C for 20 s, 55 °C for 45 s, and 95 °C for 1 min) and cooling at 4 °C. Relative gene expression was determined using real-time PCR and the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Triplicate PCR amplifications were performed for each sample and the values were normalized to the internal control of porcine *GAPDH*.

Immunocytochemical staining of piPSCs

The piPSCs colonies were plated in 24-well plates that were previously seeded with a feeders layer for 1 day. After washing with cold PBS and fixation in 4% paraformaldehyde for 15 min, the cells were rinsed with cold PBS 3×5 min, permeabilized with 0.1% Triton X-100 in cold PBS for 15 min, and blocked with blocking solution (PBS containing 5% BSA+0.1% Triton X-100) for 1 h before incubation with primary antibodies overnight in a humidified chamber at 4 °C. The primary antibodies used in this study included antibodies against OCT4 (1:100, Abcam, Cambridge, UK ab18976), SOX2 (1:100, Sigma, AV38232), NANOG (1:100, Abcam, ab80892), REX1 (1:100, Abcam, ab50828), SSEA4 (1:100, Abcam, ab16287), and TET1 (1:100, Santa Cruz, sc-163443). After three washes in PBS, the cells were incubated with secondary antibodies (Alexa Fluor 568 goat anti-mouse, 1:400, Invitrogen; Alexa Fluor 488 goat anti-rabbit, 1:400, Invitrogen) for 2 h. Finally, the cells were incubated with Hoechst 33342 for 15 min and again washed three times with PBS. Images were acquired using an Axiovert 200 (Nikon) and the AxioVision Software.

Table 3 Sequences of siRNA oligonucleotides.

Name	Sequences of siRNA (5'–3')
Non-sense siRNA	Sense: UUC UCC GAA CGU GUC ACG UTT Anti-sense: ACG UGA CAC GUU CGG AGA ATT
Non-sense FAM-siRNA	Sense: UUC UCC GAA CGU GUC ACG UTT Anti-sense: ACG UGA CAC GUU CGG AGA ATT
siRNA #1	Sense: GAA GCA GUG UAC ACA UAA UTT Anti-sense: AUU AUG UGU ACA CUG CUU CTT
siRNA #2	Sense: GGC CAG AAC AGA ACA UUC ATT Anti-sense: UGA AUG UUC UGU UCU GGC CTT
siRNA #3	Sense: GCA GCU AGC UAU AGA GUA UTT Anti-sense: AUA CUC UAU AGC UAG CUG CTT
siRNA #4	Sense: GCA GAU GGC CGU GAC ACA AAUTT Anti-sense: AUU UGU GUC ACG GCC AUC UGC TT

AP staining of piPSCs

AP staining was performed as we described previously (Tang *et al.* 2012). Briefly, the piPSCs were cultured at a low density for 5 days. On the fifth day, the medium was discarded and the piPSCs were fixed with 4% paraformaldehyde in PBS for 1–2 min and then washed with rinse buffer. A sufficient volume of staining solution was added to cover each well and the cells were incubated in the dark at room temperature for 15 min. The wells were rinsed with rinse buffer and the cells were covered with PBS to prevent drying. The colonies expressing AP were counted.

Teratoma formation and analysis

To test the pluripotency of piPSCs *in vivo*, pig iPS cells were treated using collagenase IV, harvested, and s.c. injected into dorsal flanks and armpits of NOD/SCID mice ($\sim 5 \times 10^6$ cells per site). After 4 weeks, solid tumors were observed. The tumors were collected, sectioned, and processed for hematoxylin–eosin staining and were histologically analyzed.

Methylation analysis: bisulfite-treated sequencing and touchdown PCR (BSP)

For bisulfite sequencing, both pre-transfected cells and 48-h post-transfected cells were collected by centrifugation at 100 g for 10 min after washing with cold PBS. Genomic DNA was extracted using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China) and subjected to bisulfite conversion using an EZ DNA Methylation Direct Kit (Zymo Research, Los Angeles, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was subjected to PCR. To increase the specificity and sensitivity of the reactions, touchdown PCR was performed using hot-start polymerase. The cycling program involved two separate phases. Phase 1 was the touchdown phase, which started with an annealing temperature at $T_m + 10^\circ\text{C}$ of the primers and then decreased the annealing temperature by 1°C per cycle until $T_m - 5^\circ\text{C}$ of the primers was reached, for a total of 15 cycles. Phase 2 was a generic amplification stage of 15 cycles using the final annealing temperature determined in Phase 1. Finally, the PCR products were cloned into pMD18-T Vector (TaKaRa) and sequenced.

Dot-blot assay

Genomic DNA was isolated both from pre-transfected and 48-h post-transfected cells, and the concentrations were measured. Identical amounts of DNA were denatured at 95°C for 15 min and rapidly transferred into an ice bath before being individually spotted on a nitrocellulose membrane. The blotted membrane was washed three times with $2 \times \text{SSC}$ buffer and air-dried at 80°C for 2 h. The blots were blocked with 5% non-fat milk. After three washes with TBST for 10 min each time, they were incubated with 5mC primary antibody (1:1000, Eurogentec, BI-MECY-1000, Liege, Belgium) or with 5hmC primary antibody (1:5000, Active Motif, Carlsbad, USA) respectively. After five washes with TBST for 10 min each time, the blots were incubated with anti-rabbit or anti-mouse

horseradish peroxidase (HRP) conjugated secondary antibodies (1:4000, Bios, Shanghai, China). The dots were visualized using the SuperSignal Pico Chemiluminescent Substrate detection reagents (Thermo, Waltham, UK).

Statistical analysis

The data were analyzed by a one-way ANOVA, using Statistics Production and Service Solution Software (SPSS, version 16.0). Differences were considered significant at the value of $P < 0.05$ and highly significant at the value of $P < 0.01$.

Results

Generation of piPSCs

Retroviral vectors overexpressing the classic mouse SKOM cassette were packaged using 293T cells (Fig. 1A) and then employed to reprogram the PEFs. The reprogramming protocol was shown as the schematic diagram (Fig. 1B). The day when PEFs were infected was marked as D0. Colonies with a human ESC-like morphology appeared on approximately the tenth day after transduction. These partially reprogrammed colonies exhibited a dispersed and amorphic appearance (Fig. 1C). On day 14, the ESC-like colonies were large, flattened, and round with clear boundaries (Fig. 1D). The colony-component cells were transferred to 24-well plates coated with feeder layer for expansion and further characterization. These colonies were AP positive (Fig. 1E). Semiquantitative RT-PCR indicated that they expressed endogenous *POU5F1*, *SOX2*, *NANOG*, and *LIN28* (Fig. 1F). The piPSCs differentiated to form EBs on culture in the absence of bFGF and Lif on a nonadhesive substratum (Fig. 1G). When these EBs were subsequently cultured for a longer time on a gelatin-coated surface, they attached to the substratum, began to spread, and displayed overt signs of differentiation (Fig. 1H). The expression of all the markers of the three embryonic layers (endoderm, *AFP*; mesoderm, *BMP4*; and ectoderm, *GFAP*) was detected in the EBs (Fig. 1I). Solid tumors formed after 4 weeks when piPSCs were injected into NOD/SCID mice. Tumors contained tissues derived from three germ layers, including ectoderm-derived neural epithelium (Fig. 1J), mesoderm-derived striated muscle (Fig. 1K) and blood vessel (Fig. 1L), and endoderm-derived crypta-like structures (Fig. 1M). Immunocytochemical staining indicated that colony cells were OCT4, *SOX2*, *NANOG*, and SSEA4 positive (Fig. 2A, B, C and D).

Knockdown of *TET1* in piPSCs by siRNAs

We confirmed that *TET1* was indeed expressed in the piPSCs (Fig. 3A, B, C, D, E, F, G and H). A fluorescein-labeled non-sense control siRNA was used to optimize the transfection conditions for the piPSCs (Fig. 3I and J). The optimal concentration of siRNA was determined to

Figure 1 Generation and characterization of piPSCs. Packaging of the retroviral vectors encoding the cDNAs was evaluated using fluorescence microscopy 72 h after transfection. The expression of GFP indicated that successful packaging of the retroviruses had occurred (A). Schematic diagram of the reprogramming protocol illustrated the process from the infection to colony-picking. PEFs were derived from 30-day-old fetuses and infected twice, on days 0 and 2, with retroviruses. Six days after infection, PEFs were replated on a feeder layer. Starting on day 14, classical (similar to those of human ESC) morphological changes (cells becoming rounded and aggregating) appeared, and the colonies were picked (B). The morphology of PEFs changed during the reprogramming process from the moment of infection to colony-picking (C and D). The day of the first retrovirus infection is D0. Starting on D9, partially reprogrammed colonies with human ESC-like morphology first became visible, but they were dispersed and amorphic (C). On day 14, the colonies were large, flattened, and round with clear boundaries and exhibited the classic ESC-like morphology (D). The piPSC colonies expressed AP (E). Semiquantitative PCR using two selected piPSC lines indicated the expression of the pluripotency genes *NANOG*, *POU5F1*, *SOX2*, and *LIN28* (F). EBs formed when the colonies were cultured *in vitro* (G). When EBs were differentiated for a longer time, they attached to the substratum, began to spread, and displayed overt signs of differentiation (H). The expression of three embryonic layers markers (endoderm, *AFP*; mesoderm, *BMP4*; and ectoderm, *GFAP*) were detected (K). *GAPDH* was used as a loading control, uninfected PEFs were used as a negative control, and ddH₂O was used as a mock control (I). Teratoma formed after piPSCs were injected into NOD/SCID mice, and tumors contained tissues derived from three germ layers, including ectoderm-derived neural epithelium (J), mesoderm-derived striated muscle (K) and blood vessel (L), and endoderm-derived cryptae-like structures (M).

Figure 2 Immunofluorescence staining of pluripotent markers. Immunofluorescence staining of the pluripotent markers OCT4 (A, A', A'' and A'''), SOX2 (B, B', B'' and B'''), NANOG (C, C', C'' and C'''), and SSEA4 (D, D', D'' and D''') in piPSC colonies cultured on MEF feeder layers were shown. Nuclear counterstaining was proceeded with Hoechst 33342.

be 30 pmol. The final condition used to transfect the iPSCs was to suspend them in normal, fully supplemented ES medium with 30 pmol siRNA mixed with 2 μ l transfection agent. To identify whether the each of the four *TET1* siRNAs or the non-sense siRNA was transfected into the piPSCs, the expression of *TET1* was examined 48 h after transfection. The results demonstrated that siRNA #2 downregulated *TET1* more effectively than did the other *TET1* siRNAs (Fig. 3K).

Changes in morphology and expression of stem cell markers and differentiation markers in piPSCs after knockdown of *TET1*

Morphological examination of the piPSCs was conducted after *TET1* siRNA transfection. Changes were found in the siRNA #2 group, in which efficient downregulation of *TET1* was also observed. These piPSCs were differentiated and lost the growth characteristics of colonies; they appeared dispersed and amorphic without clear boundaries between them and the surrounding feeder cells, while no similar outcomes were observed in other three *TET1* siRNA groups and the mock group (Fig. 4A, B, C, D and E), in which the downregulation of *TET1* was not as pronounced (Fig. 3K). To gain an insight into the molecular mechanism underlying this morphological phenomenon and identify whether *TET1* downregulation had an effect on the expression of genes closely related to the piPSCs identity, the expression levels of several pluripotency-related genes and differentiation-related genes were analyzed at 48 h after transfection. The results showed

that the knockdown of *TET1* resulted in upregulated expression of differentiation-related genes including *PITX2*, *HAND1*, *GATA6*, and *LEF1* (Fig. 4F) and down-regulated expression of pluripotency-related genes such as *LEFTY2*, *KLF2*, and *SOX2* (Fig. 4G). Interestingly, the stem cell genes *POU5F1*, *MYC*, *KLF4*, and *NANOG* were actually not downregulated in the *TET1* knockdown piPSCs (Fig. 4G).

Changes in the methylation and hydroxymethylation status in piPSCs after *TET1* downregulation

The methylation status of the promoters for the pluripotency-related genes (SKOM) was examined using BSP after *TET1* siRNA transfection. The results demonstrated that the methylation levels in the promoters of *POU5F1* and *Myc* were obviously increased (Fig. 5A, B, C, D, E and F), while there was no evident changes in the promoters of *SOX2*, *KLF4*, and *NANOG* (Fig. 5G, H, I, J, K, L, M, N and O). Evaluation of the 5hmC and 5mC levels in the whole genomes of the piPSCs after *TET1* siRNA transfection was conducted using the dot-blot assay. The results revealed that after *TET1* knockdown, the total 5hmC content decreased, while the total 5mC content increased (Fig. 5P and Q).

Discussion

Recently, piPSCs were successfully established by three independent groups almost simultaneously (Esteban *et al.* 2009, Ezashi *et al.* 2009, Wu *et al.* 2009). Danish Landrace pigs (Wu *et al.* 2009) and Tibetan miniature

Figure 3 Targeting siRNA selection and downregulation of *TET1*. *TET1* immunofluorescence staining in piPSC colonies cultured on feeder layers demonstrated its expression (A, B, C and D). Nuclear counterstaining was proceeded with Hoechst 33342. The downregulated expression of *TET1* by siRNA #2 transfection caused the piPSCs to differentiate (E, F, G and H). Negative staining of feeder layers, which have larger nuclei also provided another control (*). Detection of FAM-positive cells by FACS (I) indicated that the positive cells comprised ~50% of the population (J). Relative expression of *TET1* detected by RT-PCR indicated that siRNA #2 had the strongest downregulation effect (K). **P* < 0.05 and ***P* < 0.01 (Student's *t*-test).

pigs (Esteban *et al.* 2009) were used as the sources to generate the iPSCs. In this study, Yorkshire pigs were chosen as a source for generating piPSCs. PEFs were used as a target for ectopical overexpression of SKOM to obtain the piPSCs. Whether reprogramming using cells from the different pig strains occurs with the same efficacy when using the same reprogramming system or not is still unknown. Different evolved and restricted environments may have imposed evolutionary changes and thus affect the suitability of different porcine cells for iPSCs generation. Our research of piPSCs from Yorkshire pigs may enrich the knowledge of piPSCs with a different background, but systematic analysis of the susceptibility of cells from the other pig strains to become iPSCs is important as well and remains to be observed. In addition, targeting cell types other than fibroblasts has been demonstrated to have a dramatic impact on iPSCs generation in other species, such as mouse meningeal membrane cells (Qin *et al.* 2008), neural progenitor cells (Eminli *et al.* 2008), and inneural stem cells (Kim *et al.* 2008) and human keratinocytes (Aasen *et al.* 2008), cord blood cells (Giorgetti *et al.* 2009), and adipose stem cells (Sun *et al.* 2009). In our previous study, different porcine cell types exhibited different capabilities of iPSCs generation (Tang *et al.* 2012). This discrepancy may be

due to every particular cell type having a unique transcriptional program, epigenetic profile, and gene expression profile. Thus, efforts should be concentrated on finding a highly susceptible and accessible tissue or cell type that can be used to generate iPSCs for clinical applications.

Members of the *Tet* family of enzymes, including *TET1*, *TET2*, and *TET3*, convert 5mC to 5hmC, and plus promote the demethylation of DNA (Tahiliani *et al.* 2009). *TET1* is particularly abundant in mouse ESCs, and increased expression of *TET1* was observed when mouse fibroblasts were reprogrammed into iPSCs (Koh *et al.* 2011). Two recent studies found that the RNAi-mediated knockdown of *TET1* led to loss of the undifferentiated state in mouse ESCs (Ito *et al.* 2010, Freudenberg *et al.* 2012). By contrast, other groups reported that knockdown of *TET1* caused no morphologically distinguishable changes in mouse ESCs (Dawlaty *et al.* 2011, Koh *et al.* 2011). Koh *et al.* (2011) pointed out that *TET1*^{-/-} ESCs supported the development of live-born mice, although these mice had a slightly reduced body size. However, in a more recent report, significant reduction of oocyte numbers and fertility was observed in other *TET1* knockout mice, and univalent chromosomes and unresolved DNA double-strand breaks were also

Figure 4 Morphological and gene expression changes in piPSCs after downregulation of *TET1*. Striking morphological changes were observed in the siRNA #2 group (C), whereas no similar results in other groups (A, B, D and E). It was also in this group that significant downregulation of *TET1* was achieved (Fig. 3E). RT-PCR assays demonstrated that the expression of differentiation-related genes *PITX2*, *HAND1*, *GATA6*, and *LEF1* was upregulated (F), whereas pluripotency-related genes *LEFTY2*, *KLF2*, and *SOX2* were downregulated, and *POU5F1*, *MYC*, *KLF4*, and *NANOG* were not downregulated (G). * $P < 0.05$ and ** $P < 0.01$ (Student's t-test).

observed (Yamaguchi *et al.* 2012). In the same study, it was found that *TET1* deficiency led to defective DNA demethylation and decreased expression of a subset of meiotic genes but did not greatly affect the genome-wide demethylation in primordial germ cells. In addition, new papers have been published that describe the role of *TET1* and *TET2* in methylation reprogramming during fetal germline development and found that *TET1*, *TET2*, and 5hmC are dispensable for the initial global depletion of 5mC from the PGC genome; however, *TET* depletion had a locus-specific role in DNA demethylation in PGCs (Seisenberger *et al.* 2012, Hackett *et al.* 2013, Piccolo *et al.* 2013, Vincent *et al.* 2013). Here, we reported that *TET1* was also expressed in piPSCs. After downregulation of *TET1* using siRNA, colony morphological changes were observed. However, no similar outcome was found in the mock group or in other three *TET1* siRNA groups in which the downregulation of *TET1* was not as pronounced. Therefore, the possibility that the morphological changes were due to that siRNA off-target effects could be ruled out, and the morphological changes with differentiation were indeed induced by significant downregulation of *TET1*. The reasons for the discrepancies among these studies may be due to

differences in the knockdown efficiencies, off-target effects, homeostatic compensation, or different mESC backgrounds. Therefore, efforts should be made in order to find what caused the controversy over whether *TET1* was indispensable for the maintenance of the pluripotent state in mESC.

In addition to causing morphological changes, the downregulation of *TET1* also had an effect on the expression of many genes. Some studies reported that *TET1* knockdown caused more genes to be upregulated than to be downregulated (Williams *et al.* 2011, Wu *et al.* 2011, Xu *et al.* 2011). The paradoxical phenomenon of *TET1* causing both the upregulation of differentiation-related genes and the downregulation of pluripotency-related genes was demonstrated in several recent studies (Ito *et al.* 2010, Ficiz *et al.* 2011, Koh *et al.* 2011, Williams *et al.* 2011, Wu *et al.* 2011, Freudenberger *et al.* 2012), which resembles the effects of some factors that function as both activators and repressors, such as esBAF, a specific chromatin remodeling factor (Ho *et al.* 2009a, 2009b). In this study, the differentiation-related genes *PITX2* and *LEF1* were upregulated, and *HAND1* (trophoblast marker) and *GATA6* (primitive endoderm marker) were also upregulated, whereas the

Figure 5 Methylation changes in piPSCs after downregulation of *TET1*. The levels of methylation of the promoters of *POU5F1* (A, B and C) and *MYC* (D, E and F) increased obviously, whereas it was barely changed for *SOX2* (G, H and I) and *NANOG* (J, K and L), and slightly increased for *KLF4* (M, N and O). The hydroxymethylation levels of the whole genomes decreased after *TET1* siRNA interference (P), whereas the methylation levels increased (Q). The filled (black) circles correspond to methylated cytosines, the unfilled (white) circles correspond to unmethylated cytosines, and small vertical lines without a circle correspond to the missing values. ** $P < 0.01$ (Student's t-test).

pluripotency-related genes *LEFTY2*, *KLF2*, and *SOX2* were significantly downregulated. The pluripotency-related gene *POU5F1* was slightly downregulated, but without significant difference. The fact that *TET1* promoted the transcription of pluripotency factors may be the result of *TET1*-mediated promoters' hypomethylation excluding the binding of repression complexes that could recognize methylated sites and thus maintaining the expression of a group of transcriptionally active genes. The possibility exists that *TET1* represses the expression of some genes by participating in the repression of Polycomb-targeted genes by the direct association of Sin3a and facilitating the recruitment of PRC2 at the transcription start site (TSS).

It was observed that *TET1* was preferentially bound to the CpG-rich TSSs in gene promoters, especially those of transcriptionally active genes, and that DNA methylation was generally excluded from the TSSs of gene promoters with *TET1* bound to them. By contrast, gene promoters that lacked *TET1* were frequently methylated (Williams *et al.* 2011, Wu *et al.* 2011). Evaluation using the BSP method revealed that the methylation levels of the *POU5F1* and *MYC* promoters were clearly increased, although their expression was not downregulated. The levels of methylation of the *KLF4*, *SOX2*, and *NANOG* promoters were not significantly changed, whereas

SOX2 gene expression was downregulated significantly and that of *KLF4* barely changed. These results confirmed the aforementioned evidence that *TET1* has dual functions but contradict the prevalent notion that hypermethylation is often inversely correlated with gene expression. The methylation levels increased in self-renewal genes *POU5F1* and *MYC* but not changed in *KLF4*, *SOX2*, and *NANOG* following knockdown of *TET1* could possibly be explained by the fact that *TET1* had a locus-specific role in DNA demethylation just as it played in PGCs (Seisenberger *et al.* 2012, Hackett *et al.* 2013, Piccolo *et al.* 2013, Vincent *et al.* 2013). Recently, after fusion mouse B lines that carries a silent *Oct*-GFP with EGC lines, *POU5F1*-GFP was detected from day 3, although at this time the *POU5F1* promoter still had a high methylation level (Piccolo *et al.* 2013). Similarly, in PGCs with a *TET1* knockdown background, the increased methylation at *BLIMP1* did not alter its expression (Vincent *et al.* 2013) and some downregulated genes, such as *Stra8*, showed no obvious change in DNA methylation (Yamaguchi *et al.* 2012). In addition, recent studies found that histone deacetylase inhibitors such as trichostatin A and depsipeptide produced gene reactivation from hypermethylated promoters without any changes in DNA methylation at the promoter level (Pruitt *et al.* 2006, Raynal *et al.* 2012).

Seisenberger *et al.* (2012) found that the global loss of methylation at promoters did not result in a profound shift in transcriptional regulation. So the reason why methylation increases at self-renewal genes following knockdown of *TET1*, while their expression is maintained, may be that DNA methylation and transcription are largely uncoupled. Therefore, DNA methylation does not always lock gene expression. The relationship between DNA methylation and transcription is complex, so their expression may either be regulated indirectly by *TET1* or be regulated in a DNA methylation-independent manner. In one recent study, it was found that after siRNA-mediated knockdown of *TET1*, the total 5hmC levels decreased (Freudenberger *et al.* 2012). The converse has been reported: overexpression of the TET1 catalytic domains greatly reduced the total 5mC (Ito *et al.* 2010). In this study, the total 5mC and 5hmC levels after the knockdown of *TET1* were examined using the dot-blot method, and the results revealed that the level of 5hmC had decreased. The results may be caused by the enzymatic activity of TET1, which can oxidize 5mC to 5hmC, and downregulation of *Tet1* impaired the process of conversion of 5mC to 5hmC. Therefore, TET1 indeed plays important roles in conversion of 5mC to 5hmC. The mild increase in 5mC in our study, combined with what others found in mouse PGC (Seisenberger *et al.* 2012, Hackett *et al.* 2013, Piccolo *et al.* 2013, Vincent *et al.* 2013), could be explained by *TET1* depletion not regulating genome-wide DNA demethylation but having a local effect on promoter and gene body methylation, and this effect was a directional change of hypermethylation.

Taken collectively, *TET1* is expressed in the piPSCs and can serve as a marker of pluripotency. *Tet1* may play important roles in maintaining the molecular identity of the piPSCs. These roles derive not only from its enzymatic activity in converting 5mC to 5hmC, thus changing the methylation metabolism, but also from its dual function in regulating gene transcription. However, the full functional complexity of *TET1* in pluripotent stem cells and development remains to be elucidated.

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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