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Epigenetic Modifiers Facilitate Induction and Pluripotency of Porcine iPSCs

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SUMMARY

Inadequate silencing of exogenous genes represents a major obstacle to complete epigenetic reprogramming of porcine-induced pluripotent stem cells (piPSCs) by conventional pluripotency transcription factors (OSKM). We tested the hypothesis that epigenetic modification by active DNA or histone demethylation or by inhibition of histone deacetylase would enhance reprogramming and exogenous gene silencing in piPSCs. piPSCs induced by OSKM in combination with epigenetic factors, specifically Ten-Eleven Translocation (*Tet1* or *Tet3*) or lysine (K)-specific demethylase 3A (*Kdm3a*), expressed higher levels of *Rex1* and other genes representing naive state and exhibited more open chromatin status, compared with those of OSKM controls. *Tet1* also improved differentiation capacity. Conversion with inhibitors of histone deacetylases (HDACi), NaB, TSA, or VPA, further increased *Rex1* expression, while decreasing expression of exogenous genes. piPSCs induced by *Tet1*+OSKM followed by conversion with HDACi show high pluripotency. Together, epigenetic modifiers enhance generation of piPSCs and reduce their reliance on exogenous genes.

INTRODUCTION

Somatic cells can be reprogrammed to pluripotency through the ectopic expression of defined factors such as Oct4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The resultant induced pluripotent stem cells (iPSCs) provide unlimited cell sources in studying disease and in regenerative medicine (Yamanaka, 2012). Pigs show many similarities to humans in organ anatomy, physiology, and metabolism (Vodicka et al., 2005) and could be a suitable source of xenotransplantation and a model for the study of human diseases (Giraud et al., 2011). Derivation of pig iPSCs can complement research on human iPSCs (Montserrat et al., 2010). With fewer ethical concerns, robust transplantation experiments using pigs as a model could test the safety and effectiveness of iPSCs in pre-clinical translational medicine, such as retinal (Zhou et al., 2011) and myocardial therapy (Li et al., 2013).

Currently, the main obstacle in achieving fully reprogrammed porcine iPSCs (piPSCs) is inadequate silencing of exogenous genes (Esteban et al., 2009; Ezashi et al., 2009; Fujishiro et al., 2013; Montserrat et al., 2012; West et al., 2010), even after using non-integrating episomal plasmids (Du et al., 2015) or Sendai virus (Congras et al., 2016).

Epigenetic modification by active DNA demethylation or histone demethylation has been shown to facilitate iPSC induction in human and mouse (Huangfu et al., 2008a, 2008b). Tet1, a DNA methylcytosine dioxygenase, can facilitate iPSC induction by promoting *Oct4* demethylation and re-activation, and even can replace Oct4 to initiate somatic cell reprogramming (Gao et al., 2013). H3K9me3 acts as a block to pluripotency, and Kdm3a/Jmjd1a as a histone H3K9 demethylase, or vitamin C that also can demethylate the histones, enhances reprogramming (Chen et al., 2013; Ma et al., 2008). Tet3 is another dioxygenase of Tet enzymes, and Tet3-mediated DNA hydroxylation is involved in epigenetic reprogramming of the zygotic paternal DNA (Gu et al., 2011); however, it has not been determined whether Tet3 can facilitate iPSC generation.

Recently, a new pluripotent state, called "F-class iPSCs" was found (Hussein et al., 2014; Tonge et al., 2014). The F-class iPSCs is at a Nanog-positive cell state that is stable, occurs frequently, and is dependent on high expression of reprogramming factors, and these cells do not form typical embryonic stem cell (ESC)-like colonies. The F-class cells express significantly reduced levels of many PluriNet genes (Muller et al., 2008), including *Dnmt3b*, *Rex1* (*Zfp42*), and *Tdgf1* (*Cripto*). Nevertheless, they also express many genes at ESC levels such as *Sall4*, endogenous *Oct4*, and *Nanog*. After treatment with histone deacetylase inhibitors (HDACi), sodium butyrate (NaB), or trichostatin A (TSA), these cells could be converted to transgene-independent ESC-like cells capable of contributing to chimeras and the germline (Tonge et al., 2014). We speculated that piPSCs might resemble F-class iPSCs to some extent given that the exogenous genes of piPSCs are not silenced, the edges of early piPSCs appear fuzzy, and piPSCs express relatively high levels of endogenous *Oct4* and *Nanog*, but low level of *Rex1* as one of important naive state marker genes (as seen below).

We tested whether epigenetic factors, including Tet3, Tet1, and Kdm3a, or small molecules that increase histone

acetylation, could enhance epigenetic reprogramming and silencing of the exogenous genes in piPSCs.

RESULTS

Epigenetic Regulatory Factors Activate *Rex1*

Rex1 is a naive pluripotent state marker (Nichols and Smith, 2009), and its expression has been positively linked to increased pluripotency in both mouse (Okita et al., 2007; Toyooka et al., 2008) and human ESCs and iPSCs (Brivanlou et al., 2003; Chan et al., 2009). *Rex1* also is expressed in the inner cell mass of blastocyst and in trophectoderm cells or trophoblast-derived tissues during mouse and porcine embryo development (Liu et al., 2015; Rogers et al., 1991). Under certain conditions, piPSCs acquire features of naive pluripotency, characterized by expression of *Rex1* and *Stella* (Rodriguez et al., 2012). However, pig epiblast stem cell lines (pEpiSC) do not express *Rex1* (Alberio et al., 2010). We also found that piPSCs expressing *Rex1* ($Rex1^+$) showed higher expression levels of many genes associated with pluripotency, including *E-cadherin*, *Utf1*, *Dppa2*, *Cripto*, *Eras*, *Cdc20*, *Nr6a1*, *Tert*, and *Terc*, compared with piPSCs with minimal *Rex1* ($Rex1^-$) (Figure S1A). Moreover, $Rex1^+$ piPSCs also expressed high levels of genes related to pluripotency regulation network in association with *Rex1* (Wang et al., 2006), such as *Nac1*, *Wdr18*, *Prmt1*, *Cdk1*, and *Arid3a* (Figure S1B). Together, high expression levels of *Rex1* can mark high pluripotency of piPSC lines.

To activate *Rex1* and to promote the silence of exogenous genes of piPSCs, we overexpressed epigenetic regulatory factors, including *Tet1*, mouse *Tet3* (*mTet3*) available, or *Kdm3a*, together with OSKM (4F) to generate piPSCs from porcine embryonic fibroblasts (PEF) as precursor cells. To achieve mouse ESC/iPSC-like colonies, we employed the conditions for mouse iPSC induction by adding human leukemia inhibitory factor but without basic fibroblast growth factor, and with NaB (Zhu et al., 2010), S-adenosylhomocysteine (Jeon et al., 2008), and BIX01294 (Shi et al., 2008). These small molecules are known to enhance induction and quality of mouse and human iPSCs.

To confirm the presence of the introduced epigenetic regulatory factors, cells were tested on day 9 of reprogramming for the expression of epigenetic factors. As expected, PEF infected by OSKM together with epigenetic factors expressed high levels of the corresponding factors (Figure S2A). Moreover, *Rex1* was consistently elevated in the piPSC lines induced by 4F + Tet1, and variably activated in piPSC clones generated by addition of other epigenetic regulation factors (Figures S2B–S2E). Expression levels of *Rex1* positively correlated with those of exogenous epigenetic regulatory factors (Figures S2B–S2E).

piPSCs were successfully generated from OSKM (4F, control), 4F + mTet3, 4F + Tet1, 4F + Kdm3a, and 4F + Tet1+Kdm3a. piPSC colonies derived by OSKM with epigenetic factors appeared as round and dome-shaped in contrast to the flattened shape formed by OSKM alone by day 15 (Figure S1C). piPSC clones induced by OSKM were loosened and their boundaries were fuzzy while piPSC clones induced by OSKM with epigenetic factors were compact with visible boundaries (Figure S1C). By randomly picking up a number of colonies, we obtained piPSC clones that resembled typical mouse ESCs in morphology, characterized by dome-shaped compact colonies with large nuclei and clear nucleoli in the cells, distinct from feeder fibroblasts (Figure 1A). Based on relatively high expression levels of *Rex1*, we further characterized the pluripotency of numerous piPSC lines, including two 4F + mTet3, two 4F + Tet1, one 4F + Kdm3a, and two 4F + Tet1+Kdm3a.

piPSCs Induced by Epigenetic Regulatory Factors Express High Levels of Genes Associated with Pluripotency

RT-qPCR analysis revealed that expression levels of endogenous *Oct4* and *Nanog* in piPSCs were much higher than those of PEF (Figure 1B, left). Expression levels of *endo-Oct4* were also higher in piPSC lines induced by OSKM with Tet1 (Figure 1B, left), consistent with the report that *Tet1* can activate *Oct4* (Gao et al., 2013), while *endo-Oct4* expression levels in piPSC lines induced by other epigenetic factors were similar to those of OSKM controls (Figure 1B, left). Expression levels of *Nanog* did not differ among piPSC lines induced by OSKM with epigenetic factors (Figure 1B, left). In addition, *endo-Sox2* and *endo-Klf4* were also activated and expression of *endo-cMyc* reduced to some extent in piPSCs (Figure 1B, middle).

Furthermore, piPSC lines expressed higher levels of *Rex1* induced by epigenetic factors, compared with 4F control (Figure 1B, left). Tet1 and Tet1+Kdm3a appeared to be more effective in activating *Rex1* (Figure 1B, left). Notably, immunofluorescence microscopy showed *trans*-localization of Rex1 from the cytoplasm in piPSCs induced by 4F control to the nuclei in piPSCs derived by addition of epigenetic factors (Figure 2A). The nuclear localization of Rex1 may suggest its function in these piPSCs. Western blotting also revealed variable expression levels of Rex1 protein in piPSC lines induced by 4F control and addition of epigenetic factors (Figure S3A).

In addition, piPSCs induced by OSKM with epigenetic factors expressed higher levels of other genes important for naive state, such as *Utf1, Dppa2*, and *Esrrb* (Valamehr et al., 2014) (Figure 1B, right), relative to piPSCs induced by OSKM alone. All three epigenetic factors were able to activate *Utf1* and *Dppa2*, while mTet3, Tet1, and Tet1+Kdm3a significantly activated *Esrrb*, compared with

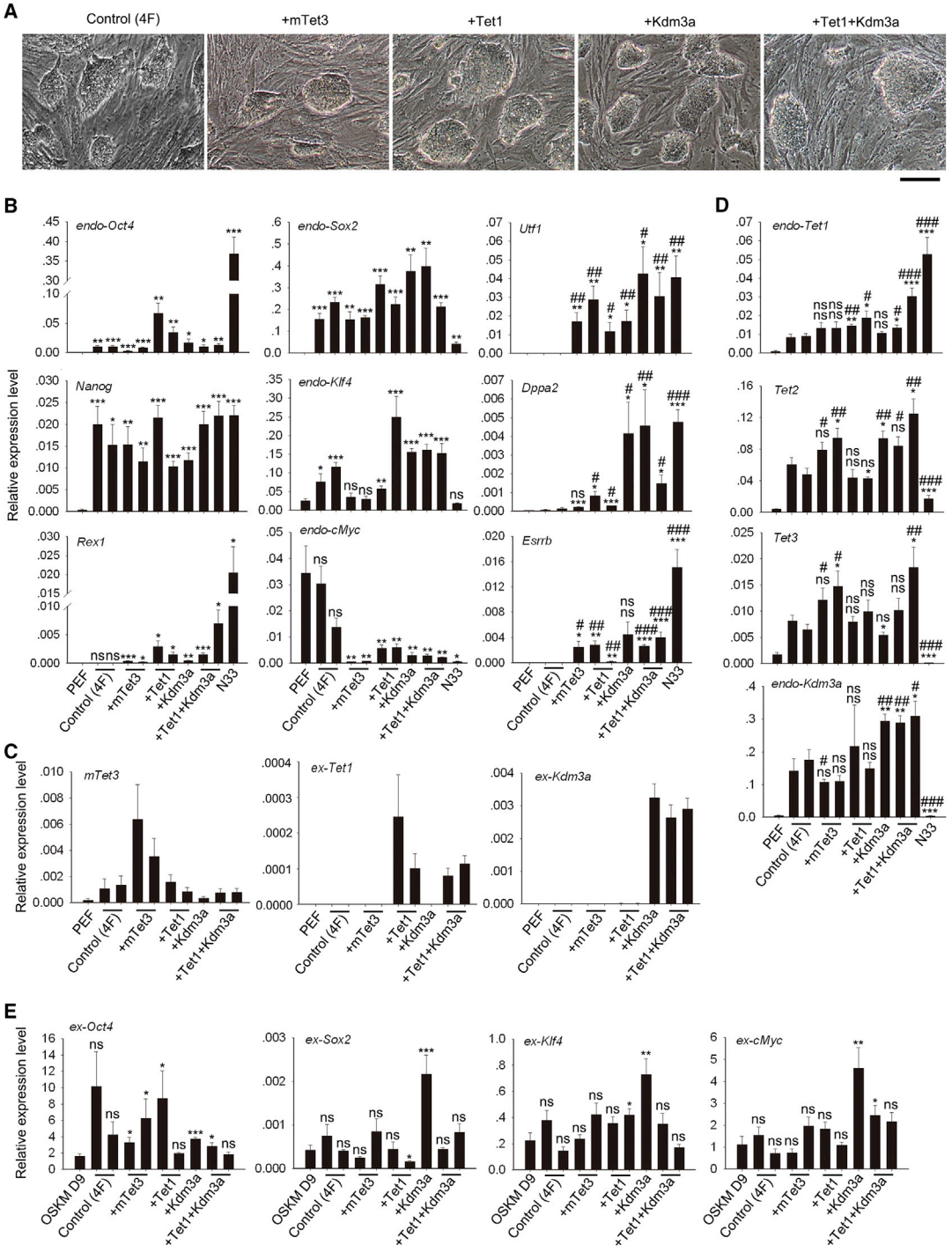


Figure 1. Generation and Characterization of piPSCs Induced by OSKM or OSKM in Combination with Epigenetic Regulatory Factors

(A) Representative images showing piPSCs induced by various conditions. Scale bar, 100 μm.

(B) RT-qPCR analysis of endogenous pluripotent genes. Left and middle, *p < 0.05, **p < 0.01, ***p < 0.001, compared with PEF. Right, */#p < 0.05, **/##p < 0.01, ***/###p < 0.001, compared with control (4F) piPSC lines. Mouse ESCs (N33) served as positive control.

(C and D) RT-qPCR analysis of exogenous (C) and endogenous (D) epigenetic regulatory genes. p values as in (B).

(E) RT-qPCR analysis of exogenous genes. OSKM at day 9 (OSKM D9) served as positive control for exogenous genes. *p < 0.05, **p < 0.01, ***p < 0.001, compared with OSKM D9. Data represent mean ± SEM from three independent experiments. ns, not significant.

See also Figures S1 and S2.

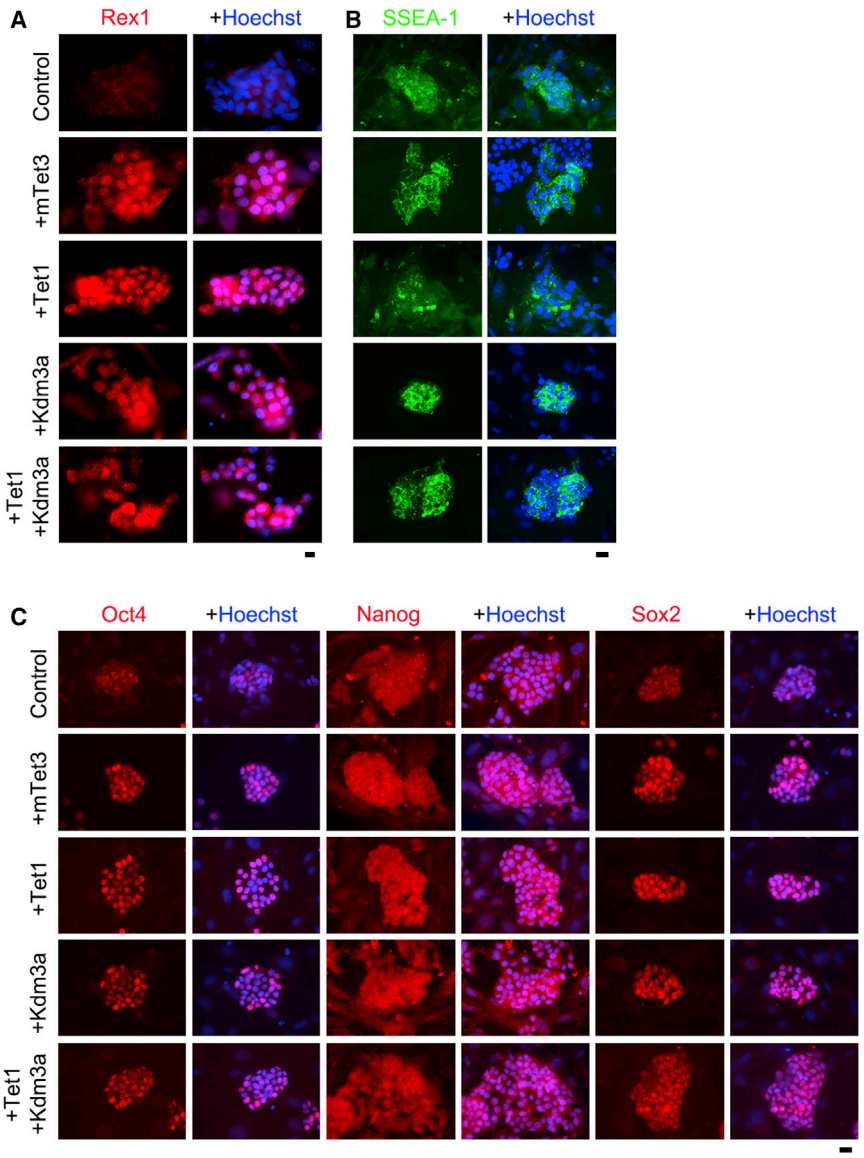


Figure 2. Characterization of piPSCs by Immunofluorescence Microscopy

(A) Translocation of Rex1 from cytoplasm to nuclei in piPSCs induced by epigenetic factors.

(B) piPSCs induced by leukemia inhibitory factor without basic fibroblast growth factor express SSEA-1.

(C) Expression of pluripotent marker genes, Oct4, Nanog, and Sox2 in nuclei of piPSCs. Scale bars, 10 μm (A) or 20 μm (B) and (C). See also Figure S3.

4F-piPSCs. Higher expression levels of *Rex1*, *Utf1*, *Dppa2*, and *Esrrb* in piPSCs generated by additional epigenetic modifiers suggest their pluripotency toward naive state.

The exogenous epigenetic factors were able to activate their corresponding endogenous genes. Expression levels of *endo-Tet1* were much higher in piPSCs induced by OSKM in combination with Tet1 or Tet1+Kdm3a (Figure 1D). Expression levels of *Tet2* were also higher in piPSCs induced by epigenetic factors except for two 4F + Tet1 piPSC lines (Figure 1D). Expression levels of *Tet3* in 4F + mTet3 and 4F + Tet1+Kdm3a piPSC lines and of *endo-Kdm3a* in 4F + Kdm3a and 4F + Tet1+Kdm3a piPSC lines also were higher than those of controls (Figure 1D). Furthermore, these piPSCs expressed multiple pluripotent stem cell markers revealed by immunofluorescence, including Oct4, Nanog, and Sox2 in the nuclei and SSEA-1 on cell surface (Figures 2B and 2C).

By western blot, H3K9me3 protein levels declined in mTet3, Tet1, and Kdm3a-derived piPSCs, while H3K27me3 declined only in Tet1-derived piPSCs (Figure S3A). Unexpectedly, combination of Tet1 and Kdm3a did not reduce the protein levels of H3K9me3 and H3K27me3 (Figure S3A). These results suggest that different epigenetic factors further open chromatin to induce activation of pluripotency genes, although they may affect histone modifications differently.

Importantly, most piPSCs maintained normal karyotypes (Figure S3B) and can be propagated by single cells using TrypLE for more than 30 passages. Furthermore, the piPSCs derived by epigenetic factors showed transgene

copy numbers at slightly higher but comparable levels with 4F control (Figure S3C). Thus, high expression levels of endogenous pluripotency genes in piPSCs induced by epigenetic factors are not likely due to differences in infection efficiency or transgene integration, but to the roles of these epigenetic factors in induction of piPSCs.

Various Expression Levels of Exogenous Genes in piPSCs

Exogenous *mTet3* was exclusively expressed in piPSCs induced by 4F + mTet3, *ex-Tet1* in piPSCs by 4F + Tet1 or 4F + Tet1+Kdm3a, and *ex-Kdm3a* in piPSCs by 4F + Kdm3a or 4F + Tet1+Kdm3a (Figure 1C). Exogenous transcription factor genes were either unchanged or upregulated in most of the piPSC lines regardless of addition of epigenetic factors, compared with those of OSKM at day 9 of induction (Figure 1E). Higher expression of *ex-cMyc* may compensate for the low expression levels of *endo-cMyc*. Together, exogenous genes were not effectively silenced in most of piPSCs induced by epigenetic factors, especially in Kdm3a-derived piPSCs (Figure 1E). Thus, while epigenetic factors can enhance reprogramming and activation of naive pluripotency genes in piPSCs, their effects on silencing exogenous genes are variable.

Pluripotency of piPSCs by Teratoma Test

To examine the developmental potential of piPSCs generated by epigenetic regulation factors, we performed an in vivo differentiation test by teratoma formation (Lensch et al., 2007; Maherali and Hochedlinger, 2008). piPSCs induced by 4F, 4F + Tet1, and 4F + mTet3 formed teratomas following subcutaneous transplantation into non-obese diabetic/severe combined immunodeficiency mice, whereas 4F + Kdm3a and 4F + Tet1 + Kdm3a did not (Figure 3A). The 4F + Tet1-piPSCs formed teratomas faster and larger than did 4F- or 4F + mTet3-piPSCs (Figures 3A and 3B). In addition to the delayed formation, teratomas from 4F + mTet3-piPSCs were even smaller than those of OSKM (Figures 3A and 3B). The teratomas formed from these piPSCs showed the characteristic three embryonic germ layers, including neural epithelium (ectoderm), muscle (mesoderm), and gland epithelium (endoderm) (Figure 3A). Immunofluorescence microscopy validated that these teratomas expressed markers representative of three germ layers, including nestin (ectoderm), smooth muscle actin (mesoderm), and alpha 1-fetoprotein (endoderm) (Figure 3C), consistent with the results by immunohistochemistry (Figure S3D). Notably, piPSCs induced by Tet1 combined with OSKM exhibited enhanced differentiation capacity, while mTet3 seemed to reduce the differentiation potential of piPSCs (Figures 3A–3C).

Conversion of piPSCs by HDACi

piPSCs failed from silencing of exogenous genes presumably are similar to the F-class iPSCs described recently, which, however, can be converted to naive iPSCs by HDACi (Tonge et al., 2014). To further reduce expression of exogenous genes and achieve the naive state, we attempted to convert two relatively high-quality piPSC lines, induced by epigenetic factors shown above (4F + Tet1 and 4F + Kdm3a), by adding HDACi, NaB, TSA, or valproic acid (VPA). Following culture in HDACi for three passages, piPSCs induced from 4F + Tet1 or 4F + Kdm3a maintained their original morphology (Figures 4A and 4C). With regard to the expression of exogenous genes, expression of *ex-Oct4* was maintained in piPSCs induced by 4F + Tet1 after six passages by treatment with NaB or VPA but significantly declined after three and six passages by exposure to TSA (Figure 4B). Expression levels of *ex-Sox2* remained unchanged after exposure to TSA or VPA (Figure 4B). piPSCs induced from 4F + Kdm3a showed lower expression of exogenous genes with increasing passages (Figure 4D). Expression levels of both *ex-Oct4* and *ex-Sox2* in 4F + Kdm3a were noticeably reduced after six passages in the presence of TSA or VPA, compared with controls (Figure 4D). Thus, exogenous genes were downregulated by HDACi.

HDACi Increases Expression of Pluripotency Genes in piPSCs

In piPSCs induced from 4F + Tet1, *endo-Oct4* was upregulated but *Tert* expression minimally affected in the presence of HDACi. NaB significantly upregulated *endo-Oct4*, *Nanog*, *Rex1*, and *E-cadherin* (Figure 4B). Similarly, piPSCs induced from 4F + Kdm3a exhibited further increased expression levels of genes related to naive pluripotency following HDACi treatment. Both *endo-Oct4* and *endo-Nanog* were upregulated (Figure 4D) and expression levels of *Tert* and *Terc* were maintained by HDACi (Figure 4D). *Rex1* and *E-cadherin* also were upregulated by NaB (Figure 4D).

We also tested effects of 5-azacytidine and vitamin C on conversion of piPSCs. Consistent with a recent finding (Tonge et al., 2014), these two small molecules did not silence exogenous genes in piPSCs (Figure S4).

DISCUSSION

We show that epigenetic regulatory factors can enhance reprogramming of piPSCs and addition of HDACi can further downregulate exogenous genes. Consistent with the notion that *Rex1* is an indicator of the naive pluripotent state (Nichols and Smith, 2009), piPSCs induced by epigenetic factors, especially Tet1, show significantly activated

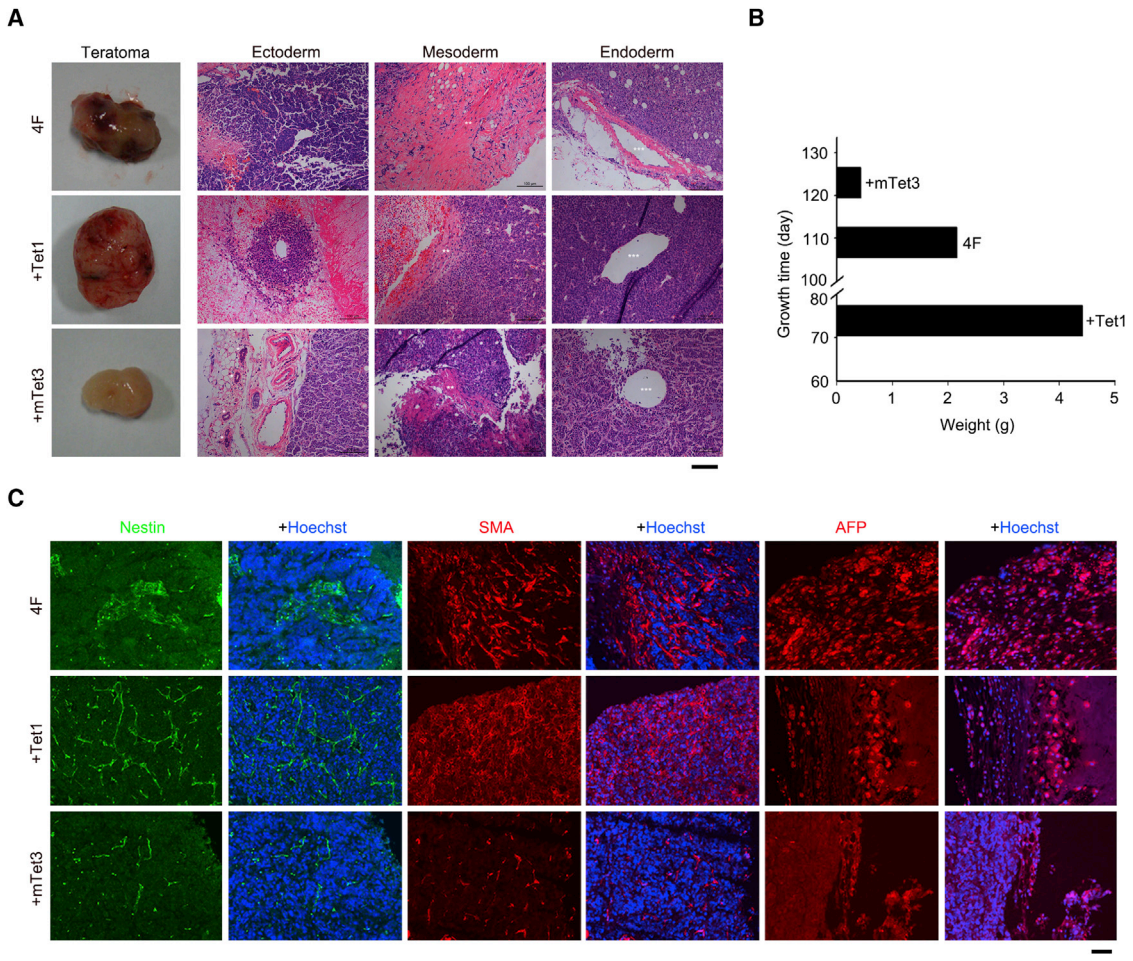


Figure 3. Differentiation Potential of piPSCs Induced by Addition of Epigenetic Factors

(A) Left, differentiation in vivo of piPSCs by teratoma formation test following injection into non-obese diabetic/severe combined immunodeficiency mice. Right, H&E staining of teratoma tissues derived from piPSCs. *, neural epithelium (ectoderm), **, muscle (mesoderm), ***, gland epithelium (endoderm).

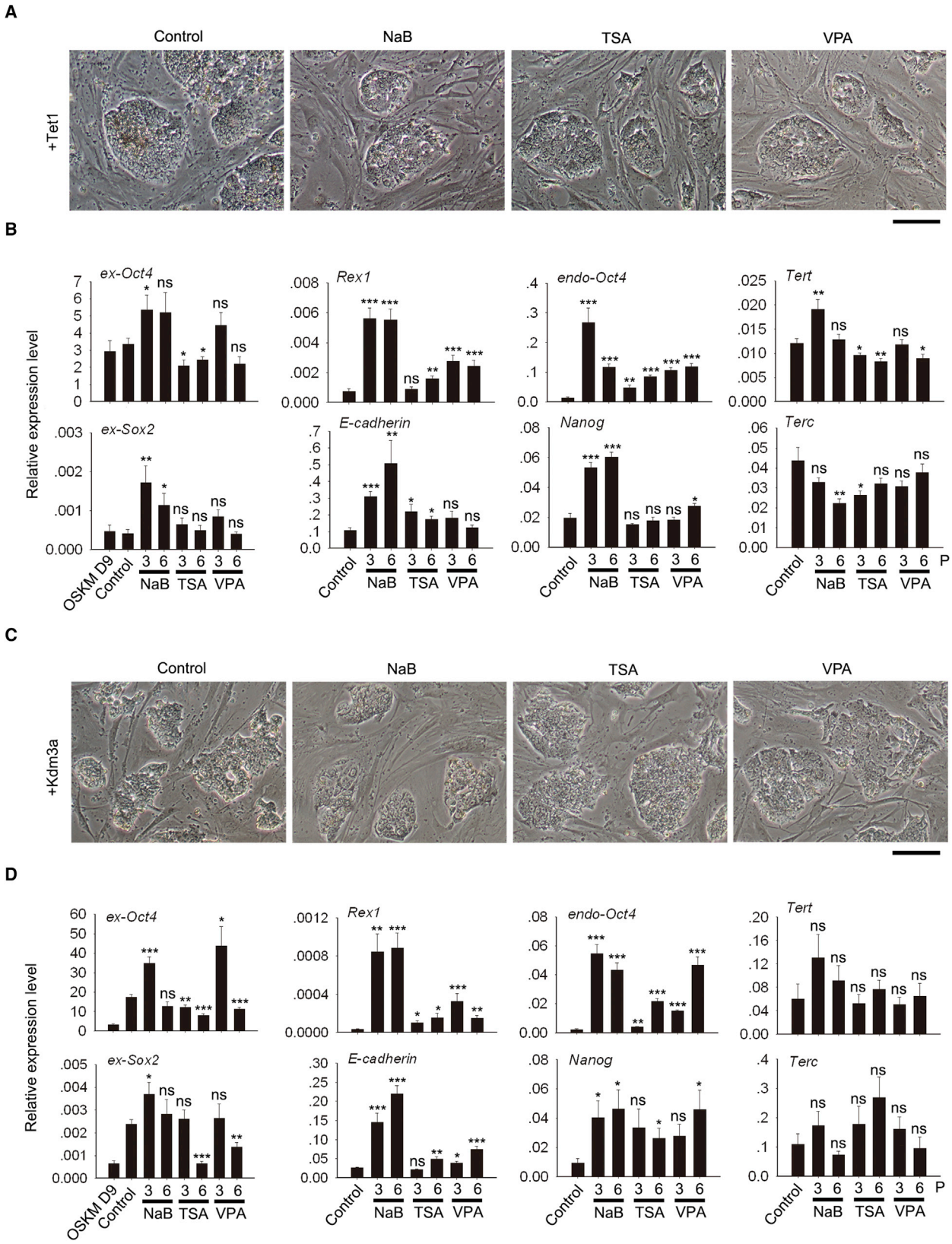
(B) Weight and time of teratomas formed from piPSCs.

(C) Immunofluorescence of the teratomas showing markers representative of three germ layers, nestin (ectoderm), smooth muscle actin (SMA, mesoderm), and alpha 1-fetoprotein (AFP, endoderm). Scale bars, 100 μm (A) or 50 μm (C). See also Figure S3.

Rex1 and other genes important for naive state, including *Utf1*, *Esrrb*, and *Dppa2*.

piPSCs are maintained dependent on exogenous genes, more like the "F-class" iPSCs (Hussein et al., 2014; Tonge et al., 2014). By exposure to HDACi, the two selected piPSC lines (4F + Tet1 and 4F + Kdm3a) show reduced expression of exogenous genes, especially in 4F + Kdm3a, and increased expression of endogenous genes associated with pluripotency. It is possible to convert F-class to naive-like state in some piPSCs. piPSCs in the presence of HDACi, including NaB, VPA, and suberoylanilide hydroxamic acid, express higher levels of *Oct4*, *Nanog*, *Utf1*, *Rex1*, *Epcam*, and *Esrrb*, compared with control (Petkov et al., 2016), confirmed in our study. VPA has been used for conversion of piPSCs from primed to naive-like state (Telugu et al., 2010). Moreover, we find that HDACi can moderately reduce expression of exogenous genes in piPSCs and increase expression of genes associated with pluripotency. Although exogenous genes are not effectively silenced, these approaches provide the basis for optimizing derivation and culture of piPSCs.

Despite the fact that epigenetic modifiers facilitate reprogramming and reduce reliance on exogenous genes, exogenous genes are still required to maintain pluripotency of piPSCs, likely suggesting that activation of endogenous genes is inadequate to maintain pluripotency of piPSCs, such that continued expression of exogenous genes complements the deficiency. It is also possible that



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some key pluripotency regulators in pigs may differ from the conventional reprogramming mixture. It is anticipated that methods that can fully activate endogenous pluripotent genes would allow piPSCs to self-renew without reliance on exogenous genes. Development of naive porcine ESCs and appropriate culture conditions become more critical for comparison of expression levels of endogenous pluripotent genes.

EXPERIMENTAL PROCEDURES

Mice Used for Teratoma Tests

The care and use of mice for this research followed the guidelines and protocols for animal research approved by the Institutional Animal Care and Use Committee of Nankai University.

Generation of piPSCs

Retroviruses were produced and harvested following the protocol described previously (Ji et al., 2013).

For the induction of piPSCs, 1×10^5 PEF at passage 1–2 were plated in a 6-well dish prior to infection. Cells were infected with corresponding pMXs-based retroviral vectors (Oct4, Sox2, Klf4, c-Myc, mTet3, Tet1, and Kdm3a, 100 μL of concentrated virus suspension for each factor) for 12 hr twice with a 24-hr interval. Cells were induced to iPSCs in induction medium, and the medium changed daily. About 9 days after infection, 5×10^4 cells were passaged on to mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder cells in a 60-mm dish. ESC-like colonies were picked at days 18–20 following a standard protocol. See also Supplemental Experimental Procedures.

Cell Culture

The piPSCs were maintained on MEF treated with mitomycin C and cultured in piPSCs culture medium. piPSCs were passaged using TrypLE at a ratio of 1:10 every 3–4 days. See also Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE87361.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.11.013.

AUTHOR CONTRIBUTIONS

L.L. designed the research; J.M. performed the main experiments; W.D. performed the H&E staining; Q.Z., H.W., K.L., H.F., and Q.Z. helped with the experiments; X.W. performed the RNA-seq experiments; L.L. and J.M. wrote the paper.

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Figure 4. Conversion of piPSCs by HDACi

(A and C) Representative microscopy images showing morphology of piPSCs induced by 4F + Tet1 (A) or 4F + Kdm3a (C) after HDACi treatment for three passages, compared with control without HDACi. Scale bar, 100 μm.

(B and D) RT-qPCR analysis of selected exogenous genes and pluripotency genes of 4F + Tet1 (B) or 4F + Kdm3a (D). P, passaging number after exposure to HDACi. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant, compared with control. Data represent mean ± SEM from three independent experiments.

See also Figure S4.

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Stem Cell Reports, Volume *8*

Supplemental Information

Epigenetic Modifiers Facilitate Induction and Pluripotency of Porcine iPSCs

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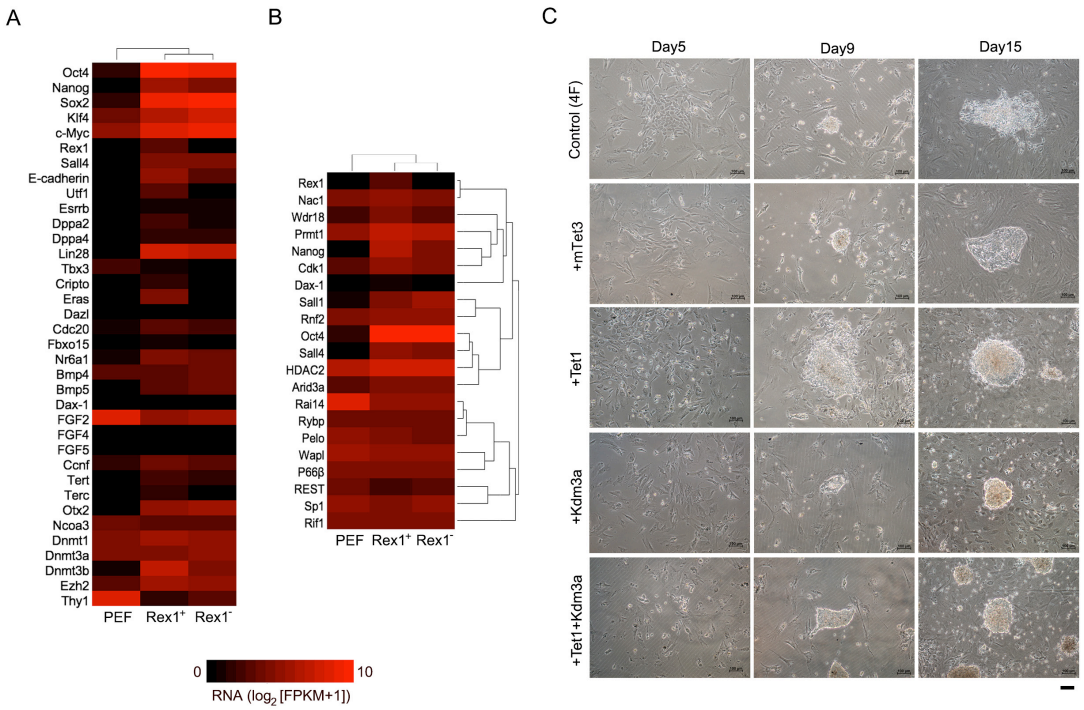


Figure S1. Related to Figure 1. *Rex1* expression and pluripotency network in piPSCs and epigenetic factors for improving piPSCs. (A) Heat-map showing gene expression of PEF and piPSCs with ($Rex1^+$) or without expression ($Rex1^-$) of *Rex1*. (B) Heat-map showing expression of genes related to pluripotency network in association with *Rex1* of PEF and $Rex1^+$ or $Rex1^-$ piPSCs. (C) Morphological change of PEF during induction of iPSCs and their primary iPSC-like colonies following transfection of OSKM (control) or in combination with epigenetic regulatory factors. +mTet3, +Tet1, +Kdm3a, +Tet1+Kdm3a represent OSKM added with mTet3, Tet1, Kdm3a, Tet1 and Kdm3a, respectively. Scale bar = 100 μm.

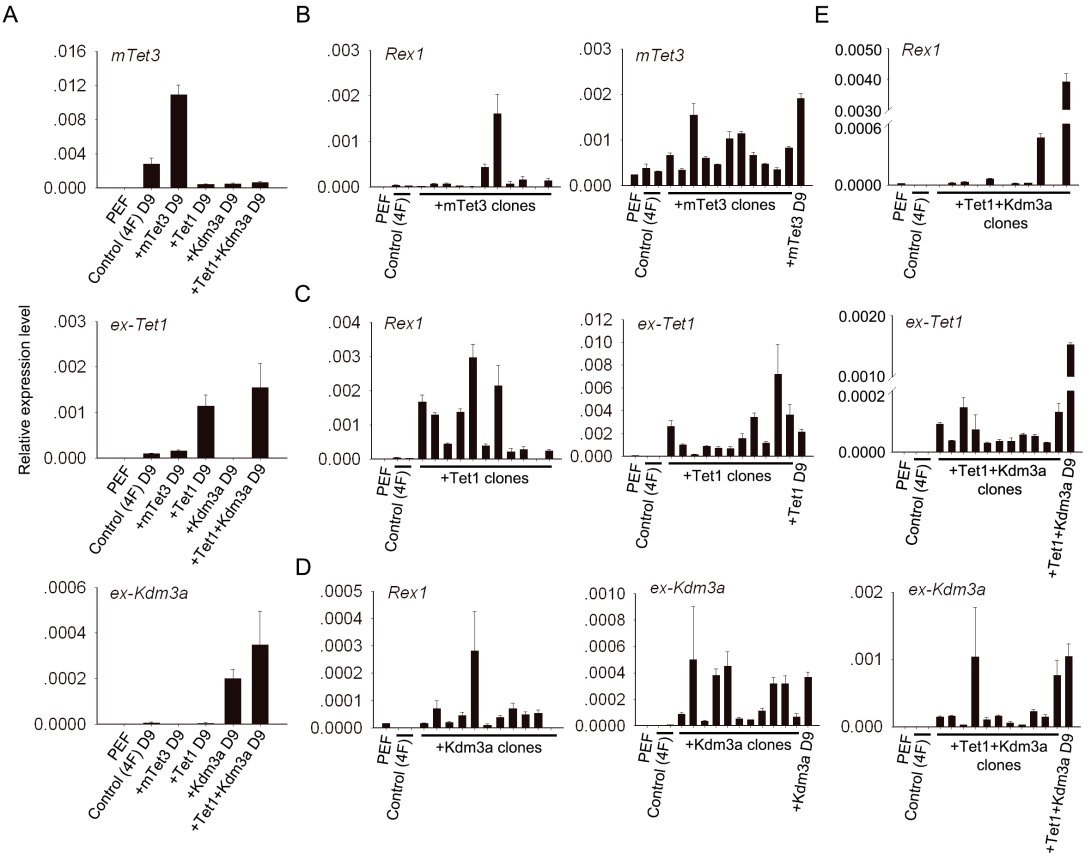
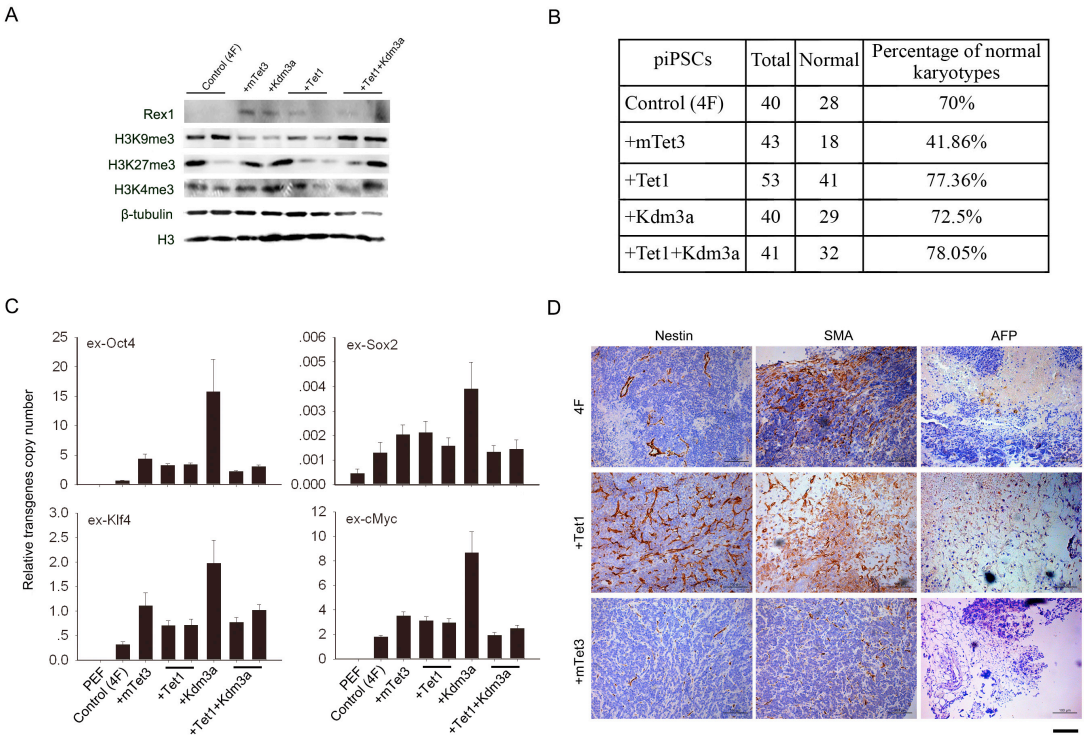


Figure S2. Related to Figure 1. Relative expression of exogenous epigenetic regulatory factors and *Rex1* by RT-qPCR in various piPSC clones. (A) RT-qPCR analysis of exogenous epigenetic regulatory factors in PEF transfected by OSKM or OSKM added with epigenetic regulatory factors on day 9 (D9) during reprogramming. Data represent mean ± SEM from three independent experiments. (B-E) RT-qPCR analysis of *Rex1* and corresponding exogenous epigenetic regulatory factors in various clones of piPSC induction by 4F (OSKM)+mTet3 (B), 4F+Tet1 (C), 4F+Kdm3a (D) and 4F+Tet1+Kdm3a (E). PEF and 4F served as controls. The clones were picked randomly. Based on these data, we selected two 4F+mTet3 piPSC lines, two 4F+Tet1 piPSC lines, one 4F+Kdm3a piPSC line and two 4F+Tet1+Kdm3a piPSC lines for further studies. Data are representative of n=11 biological replicates.



piPSCs	Total	Normal	Percentage of normal karyotypes
Control (4F)	40	28	70%
+mTet3	43	18	41.86%
+Tet1	53	41	77.36%
+Kdm3a	40	29	72.5%
+Tet1+Kdm3a	41	32	78.05%

Figure S3. Related to Figures 2 and 3. (A) Western blotting analysis of protein levels in piPSCs induced by OSKM or OSKM in combination with epigenetic regulatory factors. H3 and β-tubulin served as loading control. (B) The karyotype analysis of piPSCs induced by OSKM and OSKM added with epigenetic regulatory factors. (C) Relative copy number of the four reprogramming factor transgenes (Oct4, Sox2, Klf4 and c-Myc) was determined in piPSCs induced by OSKM or OSKM in combination with epigenetic regulatory factors by quantitative real-time PCR. PEF served as negative control. Data represent mean ± SEM from three independent experiments. (D) Immunohistochemistry analysis of teratomas formed from piPSCs induced by 4F (control), 4F+Tet1 and 4F+mTet3. All of the teratomas expressed representative markers of three embryonic germ layers: Nestin (ectoderm), smooth muscle actin (SMA, mesoderm), and alpha 1-fetoprotein (AFP, endoderm). Scale bar = 100 μm.

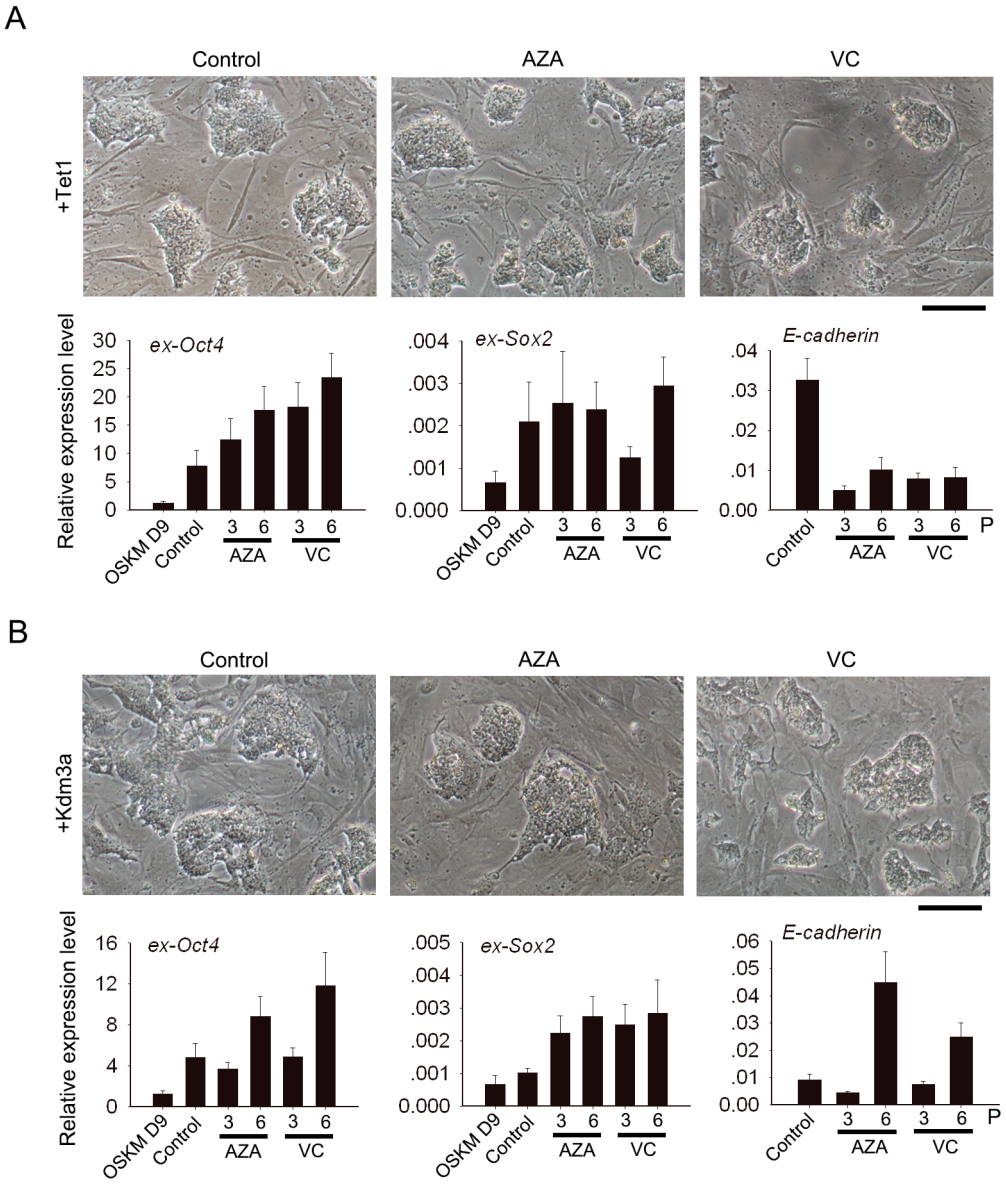


Figure S4. Related to Figure 4. Morphologies and expression of related genes of two piPSC lines (4F+Tet1 and 4F+Kdm3a) following treatment with 5-azacytidine (AZA) or Vitamin C (VC). Scale bar = 100 μm. P represents passage number following AZA or VC treatment. Data represent mean ± SEM from three independent experiments.

Table S1. Primers for RT-qPCR Analysis and Copy Number Analysis of Transgenes

Gene name	Primer	Gene bank accession number
endo-Oct4	F: CTTCACCACCCTGTACTCCTC	ENSSSCT00000001516
	R: GCTTCTCTCCCTAGCTCACC	
endo-Sox2	F: CAGACTTCACATGTCCCAGCACTA	ENSSSCT00000012883
	R: CTTACTCTCCTCCCATTTCCCTCT	
endo-Klf4	F: GAGGGAAGACCAGAATCCCTTGTA	ENSSSCT00000005981
	R: TAGAACCAAGACTCACCAAGCACC	
endo-cMyc	F: CACAGCTTACATCCTGTCTGTCCA	ENSSSCT00000006548
	R: GCCATTCTAGTTCCTCCCTCCAAT	
endo-Nanog	F: CCTACAATCCAGCTCTTTGG	NM_001129971
	R: CTCAGGCATTGGTGAAGATT	
endo-Tet1	F: CTGTGTAGAGTTCCATCTTGTT	ENSSSCT00000027259
	R: TGGTGATATGCTTCCTAATTCC	
Tet2	F: TCTACCACCCATCCATACAC	XM_003129278
	R: GAGCGGAATAAGGAGGAAATG	
Tet3	F: GCCGAGAAGAAGAAGATTCAG	KC137685
	R: GAAGGAGCTAAAGTGGTTCTG	
endo-Kdm3a	F: AACTGTGACTGCTCACCCAG	XM_003124935
	R: GGTACGCAGACAGACACACA	
ex-Oct4	F: GACGGCATCGCAGCAGCTTGGATACAC	a, b
	R: GAGAAGGCGAAGTCGGAAG	
ex-Sox2	F: GACGGCATCGCAGCAGCTTGGATACAC	a, b
	R: GGCTGTTCTTCTGGTTGC	
ex-Klf4	F: GACGGCATCGCAGCAGCTTGGATACAC	a, b
	R: GTCTTTGCTTCATGTGGG	
ex-cMyc	F: GACGGCATCGCAGCAGCTTGGATACAC	a, b
	R: GTTGGTGAAGCTGACGTTG	
mTet3	F: CCGGATTGAGAAGGTCATCTAC	NM_183138
	R: AAGATAACAATCACGGCGTTCT	
ex-Tet1	F: GACGGCATCGCAGCAGCTTGGATACAC	a
	R: GACTAATTTGGAAGGCCTTGCA	
ex-Kdm3a	F: GACGGCATCGCAGCAGCTTGGATACAC	a
	R: TTCCCCACCAATACTGGCCAA	
Rex1	F: CTCTCTCAACAGGTGCTTAC	ENSSSCT00000007665
	R: AAAGTCAGTGCTGGGTATTT	
E-cadherin	F: GAACCCACAGCCTCATGTCA	NM_001163060
	R: CGGTCGTTGAACTCGATGGT	
Utf1	F: CCGCGGGCCCGACCTCACGGAACGC	CN028152.1 (Kues et al., 2013)
	R: CGCCCTCCTGCAGACCTT	

Dppa2	F: GATACAGAAGGTTGGGTTCG	XM_003358822
	R: TAGCACATTCAGGGCATAAC	
Esrrb	F: TGTACATCGAGGATCTGGAG	XM_001928051
	R: TGCAGTTTGATGCTGTAGAA	
Tert	F: GAAAGCCAGAAACGCAGGGAT	NM_001244300
	R: CCAGAAGACAGCTGTAGGTAACG	
Terc	F: GTCTAACCCTAACTGAAAGAGGCG	AF221920
	R: TGTTTCAGACTGGATGGTGGATGG	
β-actin	F: TGCGGCATCCACGAAACTAC	DQ845171
	R: TTCTGCATCCTGTCGGCGAT	
genomic β-actin	F: TCTTCGCCTTAATACTTGTTCT	NW_003609743c
	R: CTCAAGTCAGTGTACAGGTAAG	

[a] Primers for exogenous transcription factors were based on the plasmids.

[b] These primers can be used for the analysis of expression of exogenous genes and relative copy number of the transgenes.

[c] The genomic β-actin was used as internal control for copy number analysis of the transgenes.

Supplemental Experimental Procedures

Package of Retroviruses

The day before plasmid transfection, 7×10^6 293-T cells were seeded per 100-mm dish. Twenty-four hours after the seeding of 293-T cells, pMXs-based retroviral vectors (pMXs-Oct4, Sox2, Klf4, c-Myc, mTet3, Tet1 and Kdm3a), Gag-Pol and VSV-G (10:9:1) were introduced into 293-T cells using lipo-2000 transfection reagent according to the manufacturer's recommendations. A total of 12 μg plasmid was transfected into one 100-mm dish, and the virus collected by 0.45-μm filter at 48 h and 72 h, respectively, after transfection. Virus suspension was centrifuged at 80,000 g, 4 ℃ for 90 min, the supernatant was discarded and virus (packaged from one 100-mm dish) precipitation was dissolved each with 800 μl H-DMEM at 4 ℃ overnight.

Induction and Culture of piPSCs

piPSCs induction medium contained Knock-out Dulbecco's modified Eagle medium (KO-DMEM, Invitrogen), added with 20% knock-out serum replacement (KSR, Invitrogen), 200 Units/ml human leukemia inhibitory factor (hLIF, Millipore), 0.1 mM β-mercaptoethanol (Sigma), 1 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Sigma), and penicillin (100 U/mL)-streptomycin (100 μg/mL) (Invitrogen) with 0.2 mM NaB, 0.4 μM SAH and 0.5 μM BIX01294.

piPSCs culture medium contained KO-DMEM medium supplemented with 10% Knock-out Serum Replacement (KSR, Invitrogen), 10% fetal bovine serum (FBS, ES quality, Hyclone), penicillin (100 U/mL)-streptomycin (100 μg/mL) (Invitrogen), 0.1 mM nonessential amino acids (Sigma), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 200 Units/mL hLIF (Millipore). For treatment with small

molecules, five small molecules were added into the medium respectively at the following final concentrations: 0.5 mM NaB, 10 nM TSA, 0.5 mM VPA, 2 μM AZA, or 50 μg/ml VC. Small molecules used in reprogramming or culture were purchased from Stemgent or Sigma.

RNA-Sequencing and Analysis

RNA from PEF (at passage 5) and piPSCs ($Rex1^+$ and $Rex1^-$) was extracted using a RNeasy Mini Kit (Qiagen). Libraries for sequencing were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) following manufacturer's instruction, and index codes added to attribute sequences to each sample. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instruction. After cluster generation, the library was sequenced using an Illumina Hiseq 2,000 platform and 100 bp paired-end reads were generated. Quality control of raw data (FASTQ format) was firstly processed through in-house Perl scripts. Clean data (clean reads) were obtained by removing reads with adapter and poly-N, as well as low abundant reads from raw data. Subsequent analyses were based on the clean data with high quality. Index of the reference genome was built using Bowtie v2.0.6 which is an ultrafast, memory-efficient short read aligner. Paired-end clean reads were aligned to the reference genome using TopHat v2.0.9 which is a fast splice junction mapper for RNA-Seq reads.

Multi-array log2 transformation, normalization, and t-test were performed to identify the differentially expressed genes between any two selected samples using genomic analysis software suite (http://www.geworkbench.org). Fragments per kilobase of exon per million fragments mapped (FPKM) were calculated for each gene. Hierarchical clustering was built using Pearson's Correlation and total linkage algorithms.

Immunofluorescence Microscopy

Immunofluorescence staining was performed as previously described (Huang et al., 2011). Cells were washed in PBS, fixed in 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with blocking solution, and incubated overnight at 4 °C with primary antibodies Oct4 (1:200, sc9081, Santa Cruz; or sc5279, Santa Cruz), Nanog (1:50, ab77095, Abcam), Sox2 (1:200, ab5603, Millipore), SSEA-1 (1:50, MAB4301, Millipore), SSEA-4 (1:50, MAB4304, Millipore) and Rex1 (1:200, GTX101903, GeneTex). After wash with blocking solution for three times, cells were incubated with a secondary antibody (1:200, goat anti-mouse IgG (H+L) FITC (115-095-003, Jackson), 488 goat anti-mouse IgM (A2103, Invitrogen), goat anti-rabbit IgG (H+L) AlexaFluor® 594 (111-585-003, Jackson) or Alexa Fluor 594 donkey anti-goat IgG (A-11058, Invitrogen)). Nuclei were stained using Vectashield medium (Vector) added with Hoechst 33342 (Sigma). Fluorescence images were captured using a Zeiss fluorescence microscope (AxionVision Z1).

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was purified using a RNA mini kit (Qiagen), treated with DNase I (Qiagen), and the cDNA was generated from 2 μg RNA using Oligo$(dT)_{18}$ primer (Takara) and M-MLV Reverse Transcriptase (Invitrogen). Primers were confirmed their specificity with dissociation curves. All data are normalized using *β-actin* as internal control. RT-qPCR was carried out on a MyiQ detection system (BIO-RAD) using Fast Start Universal SYBR Green Master (Roche). All reactions in duplicate were carried out by amplifying target genes and internal control in the same plate. The amplification was performed for primary denaturation at 95 ºC for 10 min, then 40 cycles of denaturation at 95 ºC for 15 s, annealing and elongation at 60 ºC for 1 min, and the last cycle under 55 ºC-95 ºC for dissociation curve. Relative quantitative evaluation of target gene was determined by comparing the threshold cycles. Most

primers were designed using IDT DNA website (Table S1).

Transgene Copy Number Analysis

The copy number analysis of transgenes was performed as previously described (Leng et al., 2015; Panopoulos et al., 2011). In order to determine the copy numbers of transgenes introduced in reprogramming, a quantitative real-time PCR method was developed to detect transgenic numbers of four reprogramming factors (Oct4, Sox2, Klf4 and c-Myc). Briefly, primer sets specifically detecting each transgene were designed. High quality gDNA samples were prepared using Qiagen DNeasy Blood & Tissue Kit (Qiagen). Real-time quantitative PCR reactions were performed using 35 ng of gDNA with the Fast Start Universal SYBR Green Master (Roche). The relative copy numbers of the four transgenes in piPSCs were normalized to β-actin for each sample. The primers are provided in Supplemental Table S1.

Western Blot

Cells were washed twice in PBS, collected, and lysed in sodium dodecyl sulfate (SDS) sample buffer on ice for 30 min and then sonicated for 1 min at 60 of amplitude with 2 sec interval. After centrifugation at 10,000 g at 4 ºC for 10 min, 100 μl supernatant was transferred into new tubes. The concentration of the protein sample was measured by bicinchoninic acid, and the samples were boiled in SDS Sample Buffer at 99°C for 5 min. Ten micrograms of total proteins for each cell extract were resolved by 10% Bis-Tris Sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred to polyvinylidinedifluoride membranes (Millipore). Nonspecific binding was blocked by incubation in 5% non-fat milk or 5% BSA in Tris-buffered saline and Tween 20 at room temperature for 2 h. Blots were then probed overnight at 4 ºC with anti-β-tubulin (mouse monoclonal, AbM59005-37-PU), H3 (ab1791, Abcam), Rex1 (GTX101903, GeneTex), H3K4me3 (ab1012, Abcam),

H3K9me3 (ab8898, Abcam), H3K27me3 (07-449, Millipore). Immunoreactive bands were then probed for 2 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary anti-Rabbit IgG-HRP (GE Healthcare, NA934V) or goat anti-Mouse IgG (H+L)/HRP (ZSGB-BIO, ZB-2305). Protein bands were detected by Chemiluminescent HRP substrate (Millipore, WBKLS0500).

Teratoma Formation and Histology by Haematoxylin and Eosin (H&E) Staining

Approximately 5×10^6 piPSCs were subcutaneously injected into non-obese diabetic/severe combined immune deficient (NOD/SCID) mice (Avior et al., 2015; Wu et al., 2009). Three mice were injected for each piPSC line. At certain time points, mice were examined for teratoma formation. Teratomas were excised, fixed in 4% paraformaldehyde, washed in 70% ethanol, embedded in paraffin, and sectioned for histological analysis following H&E staining.

Immunocytochemistry and Fluorescence Microscopy of Teratoma Sections

Briefly, after being deparaffinized, rehydration and wash in 0.01 M PBS (pH 7.2-7.4), sections were incubated with 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase, subjected to high pressure antigen recovery sequentially in 0.01 M citrate buffer (pH 6.0) for 3 min, incubated with blocking solution (5% goat serum and 0.1% BSA in PBS) for 2 h at room temperature, and then incubated with the diluted primary antibodies overnight at 4 °C. The following primary antibodies were used for immunocytochemistry: Nestin (1:200, MAB353, Millipore), SMA (1:200, ab5694, Abcam) and AFP (1:50, DAK-N150130, Dako). Blocking solution without the primary antibody served as negative control. After washing with PBS, sections were incubated with appropriate secondary antibodies (1:200, goat anti-mouse IgG (H+L) FITC (115-095-003, Jackson) or goat anti-rabbit IgG (H+L) AlexaFluor® 594 (111-585-003, Jackson)). Nuclei were stained using Vectashield medium (Vector) added with Hoechst 33342 (Sigma) and photographed

with a Zeiss Axio Imager Z1.

Immunohistochemistry (IHC)

Paraffin sections were incubated with 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase, subjected to high pressure antigen recovery sequentially in 0.01M citrate buffer for 3 min, blocked with 5% goat serum with 1% BSA in PBS for 2 h at room temperature, and then incubated with the primary antibodies against Nestin (1:200, MAB353, Millipore), SMA (1:200, ab5694, Abcam) and AFP (1:50, DAK-N150130, Dako) overnight at 4ºC. Blocking solution without the primary antibody served as negative control. After wash in PBS for three times, sections were incubated with secondary antibodies (HRP-Polymer anti-mouse/rabbit IHC Kit; 5010, Maixin Bio) for 15 min at 37ºC, washed in PBS, followed by 3,3'-diaminobenzidine (DAB) reaction. Sections were lightly counterstained with hematoxylin, and then mounted.

Statistics

All the experiments were performed at least three times ($n \geq 3$), and the mean ± SEM or representative images were shown. Statistical analysis of means and variance were compared by Fisher's protected least-significant difference (PLSD) using StatView software from SAS Institute Inc (Cary, NC). Significant differences were defined as *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ or ns, not significant.

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