

# Article

## 5 Generation of Pig-Induced Pluripotent Stem Cells with a Drug-Inducible System

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Domesticated ungulate pluripotent embryonic stem (ES) cell lines would be useful for generating precise gene-modified animals. To date, many efforts have been made to establish domesticated ungulate pluripotent ES cells from early embryos without success. Here, we report the generation of porcine-induced pluripotent stem (iPS) cells using drug-inducible expression of defined factors. We showed that porcine iPS cells expressed alkaline phosphatase, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Oct3/4, Nanog, Sox2, Rex1 and CDH1. Pig iPS cells expressed high levels of telomerase activity and showed normal karyotypes. These cells could differentiate into cell types of all three germ layers *in vitro* and in teratomas. Our study reveals properties of porcine pluripotent stem cells that may facilitate the eventual establishment of porcine ES cells. Moreover, the porcine iPS cells produced may be directly useful for the generation of precise gene-modified pigs.

30 **Keywords:** pluripotency, reprogram, iPS cells, pig, ungulate, embryonic stem cells

## 35 Introduction

Domesticated ungulate pluripotent embryonic stem (ES) cell lines would be useful in various applications, such as creating genetic models for human diseases, precisely engineering transgenic animals for organ transplantation therapies, and improving production traits and disease resistance, as well as biopharming. Ungulate ES cell lines could be used to accomplish these goals either through the chimera technology that is well established in producing genetically modified mice (Thomas and Capecchi, 1987; Capecchi, 1989), or through improving the efficiency of somatic cell nuclear transfer (SCNT) (Campbell *et al.*, 1996; Lai *et al.*, 2002). Among the possible species, the pig has a long-standing reputation as a useful and meaningful model in many branches of medicine. The porcine species displays significant morphological and functional similarities to humans. The organ dimensions are largely similar, which allows for the extrapolation of the acquired information; and the life span of pigs allows for adequate and more targeted experiments. Therefore, isolation and

thorough characterization of porcine ES cell lines have great potential and would represent an advantageous experimental tool for developing therapeutic applications and studying tissue repair. Since mouse ES cell (ESC) lines were first established over two decades ago (Evans and Kaufman, 1981; Martin, 1981), only rodent and primate ESCs, including mouse (Evans and Kaufman, 1981; Martin, 1981), rat (Buehr *et al.*, 2008; Li *et al.*, 2008; Li *et al.*, 2009), monkey (Thomson *et al.*, 1995) and human (Thomson *et al.*, 1998) ESCs have been established to date. Validated ESC lines are yet to be established in domesticated ungulates. As a result, the characteristics of ungulate ESCs, including their morphology, surface markers and pluripotency have not been documented and remain controversial (Brevini *et al.*, 2007; Keefer *et al.*, 2007; Vackova *et al.*, 2007; Talbot and Blomberg, 2008). The lack of such information greatly hampers the establishment and potential application of ungulate ESCs. Another type of pluripotent stem cells, known as iPS cells, can be generated by reprogramming somatic cells using defined transcription factors (Takahashi and Yamanaka, 2006; Qin *et al.*, 2007; Yu *et al.*, 2007; Li *et al.*, 2009; Liao *et al.*, 2009; Liu *et al.*, 2008). Importantly, iPS cells have been demonstrated to be useful in precise genetic engineering *in vivo* (Hanna *et al.*, 2007). Here, we report the generation of pig iPS cell (piPSC) lines by defined factors.

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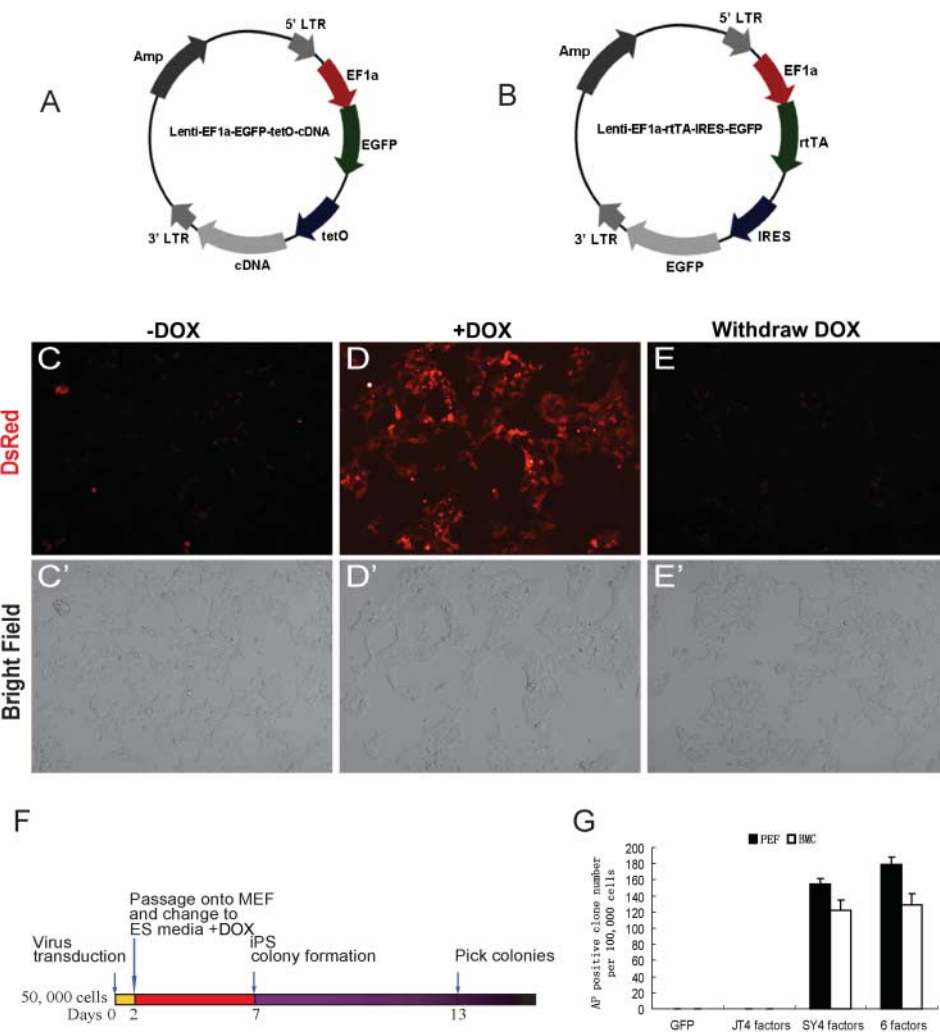
## Results

### Reprogramming of pig adult cells by DOX-inducible lentivirus vectors

Initially, we tried to use retrovirus-containing SY4 genes (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) to reprogram adult pig cells. However, we found that we could not transduce the pig cells with a control retrovirus-expressing EGFP (data not shown), suggesting that the pig cells do not express the retrovirus receptor. We then tried to reprogram the adult pig cells with lentivirus. A lentiviral backbone for doxycycline (DOX)-inducible transgene expression was constructed by inserting a tetracycline operator downstream of EGFP (Figure 1A). The cDNAs for DsRed, human *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog* and *Lin28* were subsequently

cloned into this backbone vector. The expression of reverse tetracycline transactivator (rtTA) was under the control of EF1 alpha promoter in a separate lentiviral vector (Figure 1B). We found that the expression of DsRed was inducible by DOX in 293T cells, which were transduced by Lenti-TetO-DsRed and Lenti-EF1 $\alpha$ -rtTA (Figure 1C and D). When DOX was removed from the media, the expression of DsRed became almost undetectable after 5 days (Figure 1E). These observations indicate that the drug-inducible lentiviral system worked well.

The pig primary ear fibroblasts (PEFs) or primary bone marrow cells (BMCs) used in this report were derived from a 10-week-old Danish Landrace breed of pig. A schematic diagram of the reprogramming protocol is shown in Figure 1F, and detailed methods are included in the Materials and Methods section. Viruses



**Figure 1** Generation of porcine-induced pluripotent stem cells from pig primary ear fibroblasts (PEFs) and bone marrow cells (BMCs). (A, B) Vector maps of inducible lentiviral constructs used for reprogramming experiments. (C–E) The expression of DsRed is inducible by DOX in the Lenti-viral vector-based Tet-On system. The 293T cells were transduced by Lenti-TetO-DsRed and Lenti-EF1 $\alpha$ -rtTA. (C) Without DOX, the expression of DsRed was not induced. (D) Robust DsRed expression was observed as early as 1 day after treatment with 1  $\mu$ g/ml DOX. (E) When DOX was removed from the media, the expression of DsRed became almost undetectable after 5 days. C', D' and E' are the bright field view of C, D and E, respectively. DOX, doxycycline. (F) Schematic diagram of the reprogramming protocol used. (G) A comparison of the number of AP<sup>+</sup> embryonic stem cell-like colonies generated from PEFs and BMCs by lentiviral transduction of JT4 (OSNL), SY4 (OSMK) or six genes (OSNLMK) ( $n = 3$ ). Error bars indicate standard deviation.

expressing a cocktail of reprogramming factors were used to transduce the adult cells. These cells were simultaneously infected with lentivirus constitutively expressing the reverse tetracycline transactivator. Two days after transduction, the cells were harvested by trypsinization and plated onto murine embryonic fibroblasts (MEFs) at  $5 \times 10^4$  cells per well in a 6-well plate. The next day, the medium [Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS)] was replaced with medium for ES cell culture (DMEM/F12 + 20%SR + DOX).

Colonies with a human ES cell-like morphology first became visible 7 days after transduction. On Day 13, the ES cell-like colonies were large and round with clear boundaries (Figure 2B). We picked ES cell-like colonies from 50% of the wells and transferred them to 96-well plates for further expansion. The remaining wells were subjected to alkaline phosphatase (AP) staining. The number of AP-positive colonies is shown in Figure 1G. The pig ES-like cells (named as pig iPSCs or piPSCs) could be expanded on MEF feeder cells with ES cell medium plus DOX ( $4 \mu\text{g/ml}$ ).

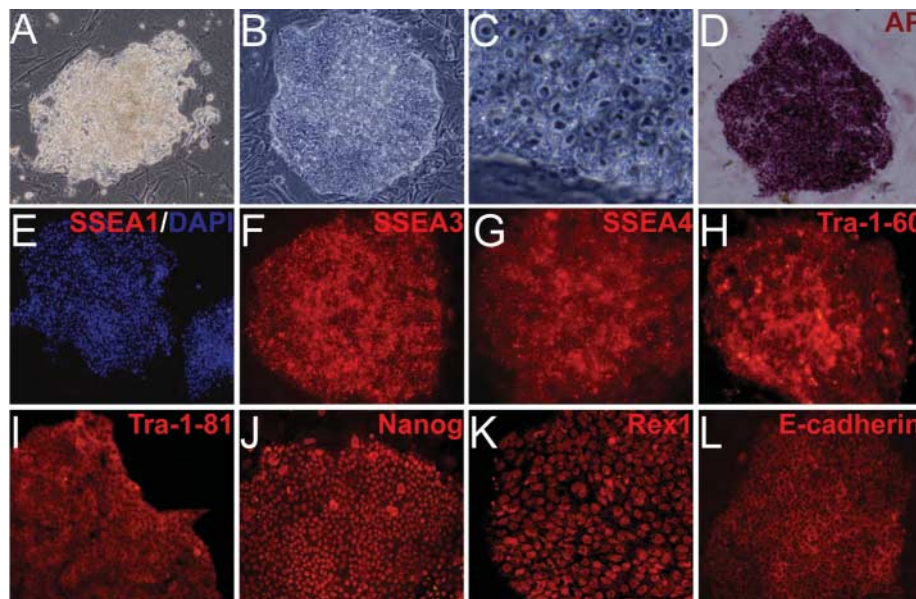
We compared the efficiency of iPSC cell generation side-by-side by transducing with lentiviral vectors expressing SY4 genes (*Oct4*, *Sox2*, *c-Myc* and *Klf4*; OSMK) (Takahashi and Yamanaka, 2006), JT4 genes (*Oct4*, *Sox2*, *Nanog* and *Lin28*; OSNL) (Yu *et al.*, 2007) or a combination of six genes (*Oct4*, *Sox2*, *Nanog*, *Lin28*, *c-Myc* and *Klf4*; OSNLMK) (Liao *et al.*, 2008). We could successfully generate pig iPSCs using SY4 genes or using a combination of six genes, but not using lentiviruses expressing JT4 genes (Figure 1G). We did not observe a significant difference in induction efficiency between SY4 genes and six genes. However, pig iPSCs generated by six genes appeared to grow better and have higher percentage of undifferentiated colonies during the early passages (data not shown).

## Pig iPSC cells exhibit similar morphology and marker expression to human ES cells

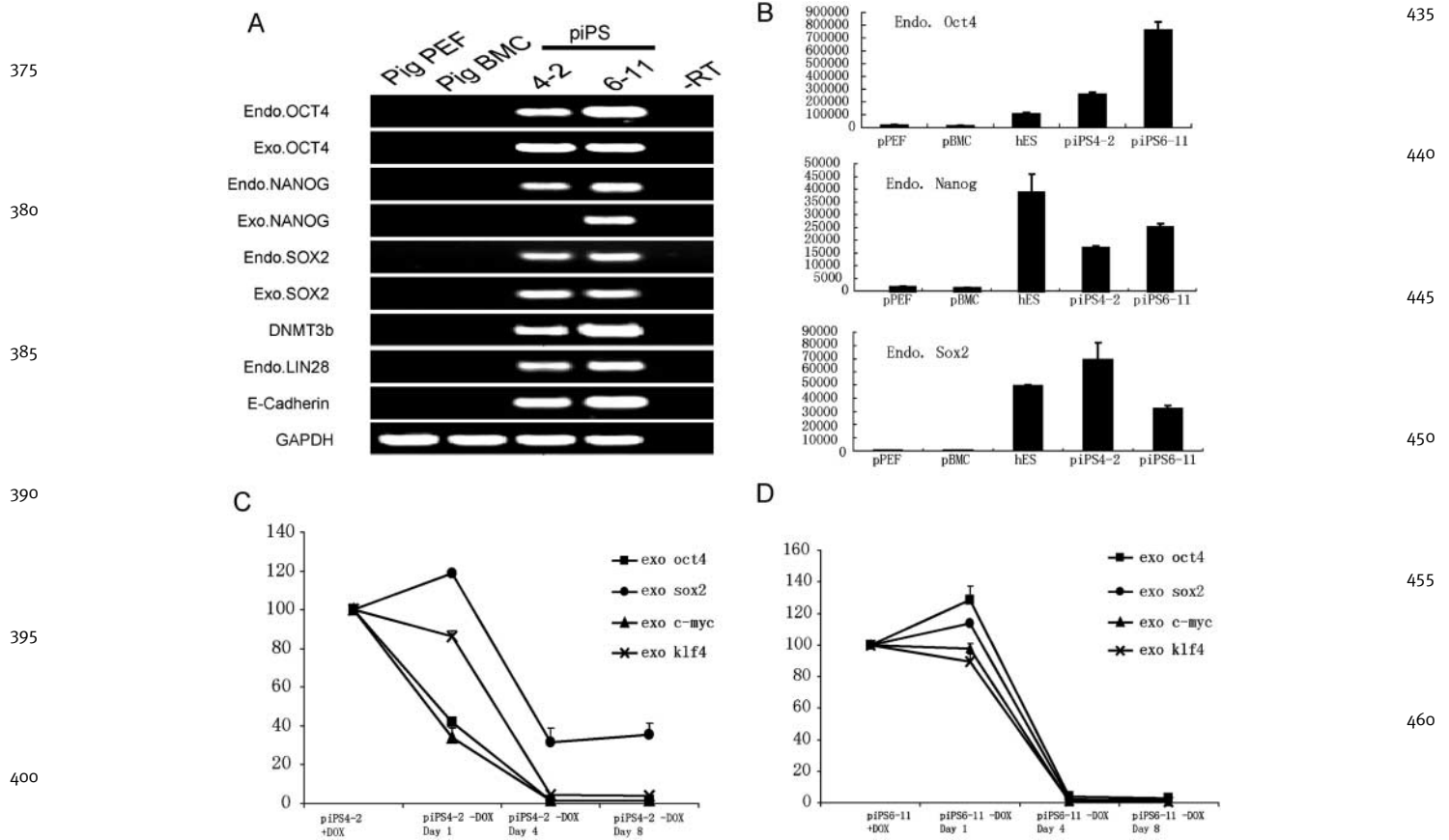
piPSCs exhibited a similar morphology to that of human ES cells (Thomson *et al.*, 1998) and primary pig epiblast cells (Talbot and Blomberg, 2008) characterized by a high nucleus to cytoplasm ratio and prominent nucleoli (Figure 2C). The doubling time of piPSCs was about 24 h, and the plating efficiency was about  $10.1 \pm 0.45\%$  for piPS4-2 and  $8.0 \pm 0.55\%$  for piPS6-11, respectively. PiPS4-2, derived from PEFs by four factors, has now been passaged continuously for more than 48 passages (more than 160 days). PiPS6-11, derived from BMC by six factors, has now been passaged continuously for more than 41 passages (more than 140 days).

As observed in human ESCs, the piPSCs expressed ES cell surface markers, including stage-specific embryonic antigen SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and E-Cadherin (also called cadherin-1, CDH1) (Figure 2F–I, L). The piPSCs also expressed alkaline phosphatase, *Nanog* and *Rex-1* (Figure 2D, J, K). They did not express mouse ES cell-specific surface antigen SSEA-1 (Figure 2E). Reverse transcription–polymerase chain reaction (RT–PCR) analysis showed that the piPSCs expressed many undifferentiated ES-cell marker genes, including *Oct3/4*, *Sox2*, *Nanog*, *DNMT3b*, *Lin28* and *E-cadherin* (Figure 3A). The expression of E-cadherin suggests that the piPSCs are fully reprogrammed beyond the stable ground-state of ‘near pluripotency’ (the state of so-called FAB-SCs) to the state of ‘full pluripotency’ (the state of ES cells) (Chou *et al.*, 2008).

We analyzed the expression of endogenous *Oct4*, *Nanog* and *Sox2* and found that all three were robustly induced (Figure 3A). We used absolute quantitative PCR to calculate the mRNA copy number of *Oct4*, *Nanog* and *Sox2* in pig and human cells and found that the expression of endogenous *Oct4*, *Nanog* and *Sox2* in piPSCs was comparable to that in hESCs



**Figure 2** Porcine-induced pluripotent stem cells (piPSCs) generated by viral transduction. (A) Typical non-embryonic stem (ES) cell-like colony. (B) Typical piPSC colony. (C) High magnification of the piPSCs. These iPSCs express pluripotency markers: (D) alkaline phosphatase; (F) SSEA-3; (G) SSEA-4; (H) TRA-1-60; (I) TRA-1-81; (J) Nanog; (K) Rex-1; (L) E-Cadherin. PIPS cells do not express mouse ES cell-specific surface antigen SSEA-1 (E).



**Figure 3** (A) Reverse transcription–polymerase chain reaction (RT–PCR) analysis of the piPSC lines compared with the gene expression observed in parental pig primary cells. Abbreviation: –RT, minus reverse transcriptase. (B) Quantitative reverse transcription–PCR analyses of endogenous *Oct4*, *Nanog* and *Sox2* expression in hESCs and piPSCs relative to parental somatic cell populations. The y-axis indicates mRNA copy number of the gene of interest per  $10^6$  copies of GAPDH. (C, D) Quantitative RT–PCR analyses of exogenous gene expression when DOX was withdrawn.

(Figure 3B). These observations suggest that endogenous pluripotency genes (e.g., *Oct4*, *Nanog* and *Sox2*) can be fully induced by reprogramming of the pig somatic cells.

### Epigenetic status of pig iPS cells

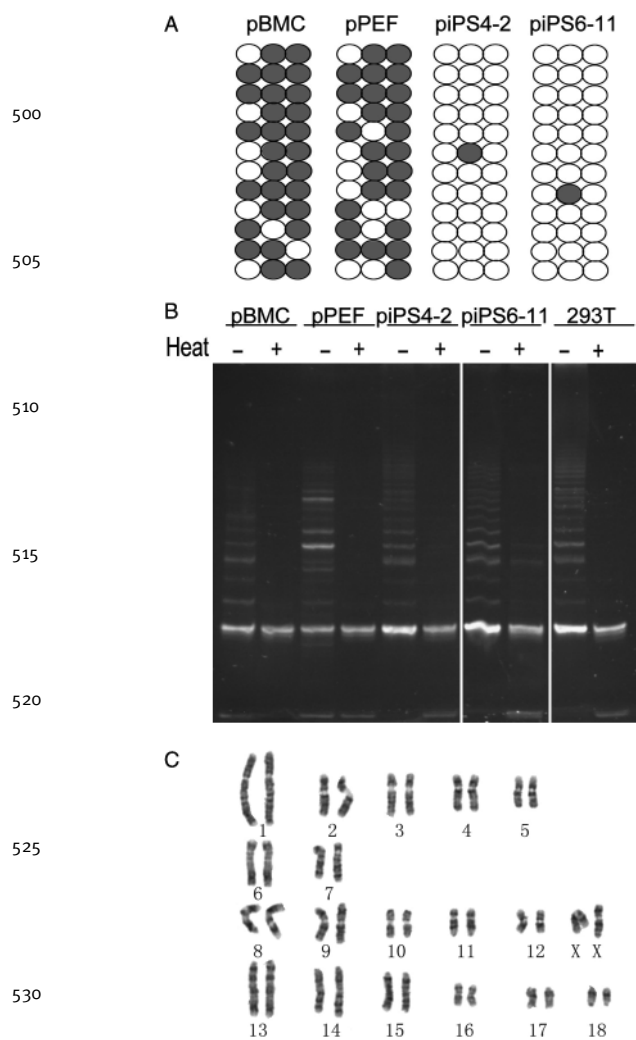
Bisulfite genomic sequencing analyses of the *Oct4* promoter showed that it was highly unmethylated in pig iPS clones, whereas CpG dinucleotides in these regions were highly methylated in parental PEFs and BMCs (Figure 4A). These results are consistent with the fact that the *Oct4* promoter is active in pig iPS cells.

Pig iPS cells also showed high levels of telomerase activity (Figure 4B). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span. Telomerase is present at high levels in germ-line and embryonic tissues (Burger *et al.*, 1997). The high level of telomerase activity expressed by pig iPS cells suggests that their replicative life-span will exceed that of somatic cells. We also performed karyotyping after passage 15, and our analyses suggested that the pig iPS clones showed a normal karyotype of 38XX (Gustavsson, 1988) (Figure 4C).

The expression of exogenous genes was well maintained by DOX in the piPSCs (Figure 3A). When DOX was withdrawn, the exogenous genes were quickly downregulated, and the piPSCs differentiated and no longer exhibited ES cell morphology (Figure 3C and D and data not shown), suggesting that certain growth factors or chemical inhibitors would be required in the culture medium in order to maintain the piPSCs in a pluripotent state, as reported by Li *et al.* (2008).

### Pig iPS cells are able to differentiate into three germ layers *in vitro* and *in vivo*

In order to determine the differentiation capacity of pig iPS cells *in vitro*, we allowed the pig iPS cells to differentiate for 7 days and analyzed the presence of differentiation markers. RT–PCR analysis confirmed that the pig iPS cells could differentiate into all three germ layers in embryoid bodies, as evidenced by the expression of alpha-anti-trypsin (endoderm), AFP (endoderm), Amylase (endoderm), Enolase (mesoderm), Osteonectin (mesoderm), NeuroD (ectoderm) and GFAP (ectoderm) (Figure 5A). In contrast, endogenous *Oct3/4*, *Sox2* and *Nanog* expression was decreased (Figure 5A). At the same time, the transgenes were



**Figure 4** (A) Bisulfite genomic sequencing of the promoter regions of *Oct3/4*. The open and closed circles indicate unmethylated and methylated CpGs, respectively. (B) Detection of telomerase activity by the TRAP method. 293T cell line was used as positive control. Heat-inactivated (-) samples were used as negative controls. (C) The piPSCs at passage 16 showed a normal 38XX karyotype.

silenced because of the absence of DOX (Figure 5A). These data demonstrate that pig iPSCs can differentiate into three germ layers *in vitro*.

To test pluripotency *in vivo*, pig iPSCs were injected intramuscularly into non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice. Four weeks after injection, we observed tumor formation. Histological examination showed that the tumor contained various tissues of three germ layers, including gut-like epithelial tissues (endoderm), bone (mesoderm), cartilage (mesoderm) and neural tissues (ectoderm) (Figure 5B–E).

### Comparison of pig iPSCs with other pluripotent stem cells by gene-expression profiling

To gain a global view of the reprogramming, microarray analysis was performed to compare the patterns of gene expression

exhibited by pig iPSCs, other pluripotent cells (mouse ES cells, rat iPSCs and human ES cells) and non-pluripotent cells. We found that the gene-expression profiles of pig iPSC cell lines piPS4-2 and piPS6-11 were significantly different from the adult pig cells. However, they were very similar to each other. These observations suggest that pig iPSC cell lines piPS4-2 and piPS6-11 were reprogrammed to a similar state. A hierarchical cluster analysis of 604 orthologous genes in pig, rat, mouse and human was performed based on the gene-expression profiles (Figure 6). The results showed that the pluripotent cells, although derived from different species, cluster together, whereas the non-pluripotent cells cluster separately. This analysis also revealed that the pig iPSCs cluster more closely with mouse ES cells, rat iPSCs and human ES cells than to the non-pluripotent populations. These data suggest that the pig iPSCs were reprogrammed faithfully to a pluripotent state similar to that of mouse and human ES cells. The detailed gene-expression profiles are provided in the supplemental material.

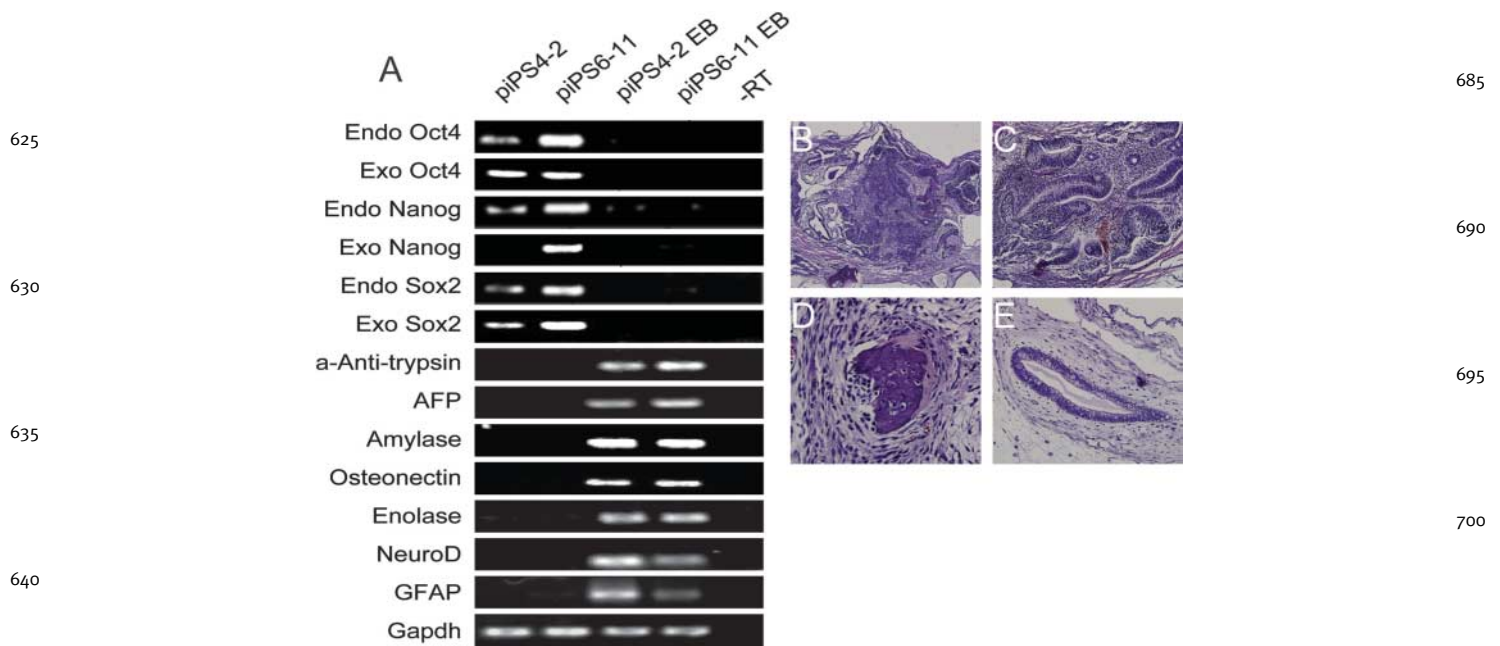
### Discussion

The results presented here have a number of important implications. First, our results may provide insight into the properties of pig ES cells, including their morphology, surface markers and pluripotency, which have not yet been established to date (Brevini *et al.*, 2007; Keefer *et al.*, 2007; Talbot and Blomberg, 2008). This information should facilitate the establishment of pig ES cells in the future. Our study should be informative for the identification of appropriate stem cell markers of pig ES cells, and thus may help resolve the longstanding question about the phenotype of pig ES cells. Previous reports on the establishment of pig ES-like cells has led to a lot of controversy. The putative markers for these cells have never been well documented. Our study suggests that the morphology of pig ES cells may resemble human ES cells instead of mouse ES cells. Pig pluripotent stem cells express SSEA3, SSEA4, Tra-1-60 and Tra-1-81, which mimics the property of human ES/iPSCs.

Second, we have demonstrated a proof-of-principle that the iPSC cell technology provides a feasible approach for establishing pluripotent stem cells for ungulate species in which ES cells have proven difficult to establish from early embryos. Pluripotent stem cell lines that can proliferate for long periods of time without differentiation are vital for targeted gene modification *in vivo* (Capecchi, 1989). ES cell lines from species such as rat, cow, goat and sheep are highly desirable, but many efforts have been invested in producing them without much success over the past two decades (Keefer *et al.*, 2007). We propose that the iPSC cell technology could be used to generate pluripotent stem cell lines for species in which ES cell lines are desirable but cannot be established from early embryos.

Third, it is intriguing that efforts to establish pig ES cells from early embryos have failed despite many attempts. The maintenance of pluripotency is probably the most critical problem that needs to be solved in establishing pig ESC lines. Past attempts to establish ungulate cell lines that fulfill all the criteria of ES cells may not have been successful due, in part, to the inability to optimize culture conditions. The appropriate culture conditions





**Figure 5** (A) Reverse transcriptase–polymerase chain reaction analyses of various differentiation markers for the three germ layers in the embryoid body. Abbreviation: EB, embryoid body. (B–E) Hematoxylin–eosin staining of teratomas derived from porcine-induced pluripotent stem cells. Teratoma (B) is composed of various types of tissues: (C) neuroepithelium (ectoderm), (D) bone (mesoderm) and (E) gut-like epithelium (endoderm).

have been shown to be essential for the derivation and routine culturing of mouse (Smith *et al.*, 1988; Ying *et al.*, 2003; Chen *et al.*, 2006; Ying *et al.*, 2008), rat (Buehr *et al.*, 2008; Li *et al.*, 2008, 2009) and human ES cells (Amit *et al.*, 2004; Beattie *et al.*, 2005; Xu *et al.*, 2005; Xiao *et al.*, 2006; Wu *et al.*, 2008). The generation of piPSCs, which have properties similar to those of ES cells, provides an opportunity to study the mechanisms essential for the maintenance of pig pluripotent cells and thus may allow for optimization of culture conditions for ES cell maintenance. The drug-inducible system provides a unique platform for identifying growth factors and/or small molecules that stimulate porcine pluripotent stem cell self-renewal, which should facilitate the eventual establishment of true ES cells from porcine blastocysts. One can also attempt to characterize the signaling pathways that regulate the expression of endogenous *Oct4* and *Nanog* in pig iPS cells. The data acquired from these studies might generate insights for achieving the proper culture conditions to derive pig ES cells from the early embryo.

Finally, the pig iPS cells are able to proliferate for a long period of time without differentiation. These properties provide an opportunity for using the homologous recombination technique in pig iPS cells. The successful demonstration of germline transmission (Okita *et al.*, 2007; Wernig *et al.*, 2007) and homologous recombination in mouse iPS cells (Hanna *et al.*, 2007) encourages us to propose that pig iPS cells might also be directly useful for the generation of knock-out and knock-in pigs either through the chimera technology that is well established in producing genetically modified mice, or through improving the efficiency of the SCNT technology (Lai *et al.*, 2002, 2006).

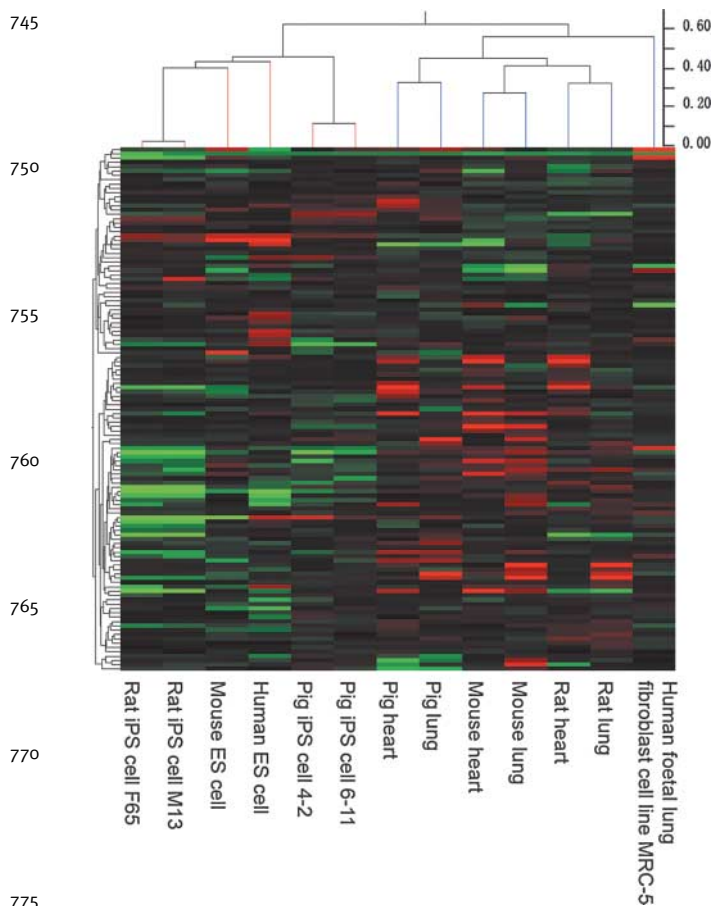
## Materials and methods

### Cell culture

The pig PEFs or primary BMCs used in this report were derived from a 10-week-old Danish Landrace breed of pig. The Danish Landrace is a medium to large breed of pig, white in color with long bodies, fine hair, long snouts and heavy drooping ears. Pig BMCs were cultured in  $\alpha$ -DMEM culture medium (Invitrogen) supplemented with 10% (FBS; Hyclone). Pig PEFs were cultured in DMEM culture medium (Invitrogen) supplemented with 10% FBS (Hyclone). The piPSCs were maintained on irradiated CF-1 mouse embryonic fibroblasts (MEFs) in DMEM/F12 supplemented with 20% KnockOut serum replacer, 0.1 mM non-essential amino acids, 1 mM L-glutamine and 0.1 mM  $\beta$ -mercaptoethanol (ES medium) (all from Invitrogen, Carlsbad, CA, USA), along with 2  $\mu$ g/ml doxycycline. The piPSCs were split with collagenase at a ratio of 1:10 every 4 days. To form embryoid bodies, the piPSCs were dissociated with collagenase and transferred to a Petri dish in a differentiation medium consisting of DMEM (Invitrogen) supplemented with 10% FBS. The doubling time of piPSCs was determined as described by Kim (1995). The plating efficiency was determined as described by Amit *et al.* (2000).

### Lentiviral transduction and reprogramming culture

Human cDNAs were inserted downstream of the tet operator in the lenti-vector. Five hundred thousand PEFs or BMCs



**Figure 6** Hierarchical cluster analysis of expression of orthologous genes in pig, rat, mouse and human genomes was performed based on the data of gene-expression profiles. The distances between the samples are shown.

from Danish Landrace pig were transduced with a lentivirus carrying GFP (negative control) or a cocktail of lentiviruses carrying reprogramming factors at Day 0. Two days after transduction, the cells were harvested by trypsinization and plated onto MEFs at  $5 \times 10^4$  cells per well in a 6-well plate. The next day, the medium (DMEM containing 10% FBS) was replaced with the medium used for ESC culture (DMEM/F12 + 20%SR) plus doxycycline (4  $\mu\text{g}/\text{ml}$ ). On Day 13, the iPS colonies were picked and plated onto new culture dishes.

## Immunostaining

Immunostaining was carried out similarly as previously described (Xiao *et al.*, 2006). The primary antibodies used were anti-Nanog (1:150, R&D Systems), anti-SSEA1 (Ascites, 1:500, Developmental Studies Hybridoma Bank), anti-SSEA3 (Ascites, 1:400, Developmental Studies Hybridoma Bank), anti-SSEA4 (Ascites, 1:400, Developmental Studies Hybridoma Bank), anti-Tra-1-60 (1:150, Chemicon), anti-Tra-1-81 (1:150, Chemicon), anti-Rex1 (1:200, Santa Cruz) and anti-E-Cadherin (1:100, BD).

## Reverse transcription–polymerase chain reaction and real-time polymerase chain reaction

Total RNA was prepared using an RNeasy kit (Qiagen) and then used as a template for RT–PCR. Real-time PCR was performed in an Eppendorf Mastercycler® ep realplex real-time PCR system using a SYBR Green-based PCR Master mix (TOBOYO). The PCR primers are listed in Supplemental Table S1. Standard curves were acquired for both the gene of interest and the internal control (GAPDH). The standard curve defines the copy number. The CT data for the gene of interest and the internal control (GAPDH) that were obtained from the real-time PCR were transformed into the copy number using the standard curve. The expression value of each gene was normalized to the amount of GAPDH cDNA in order to calculate the relative amount of RNA present in each sample. The mRNA copy number of the gene of interest was defined as the number of copies per  $10^6$  copies of GAPDH.

## Microarray analysis

Total RNA was extracted with TRIZOL reagent (Invitrogen) and further purified using an RNeasy column (Qiagen, Hilden, Germany; <http://www1.qiagen.com>). The labeling procedure was carried out using an RNA Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA; <http://www.agilent.com>). The sample and control RNAs were labeled with cy-3 and cy-5, respectively. Fragmentation was carried out by incubation at 60°C for 30 min in a fragmentation buffer (Agilent Technologies), and the process was stopped by the addition of an equal volume of hybridization buffer (Agilent Technologies). The fragmented target was applied to an Oligo Microarray for pig (Agilent Technologies). Hybridization was carried out at 60°C for 17 h in a hybridization oven (Robbins Scientific, Sunnyvale, CA, USA). The hybridized array was scanned with an Agilent microarray scanner. The TIFF image generated was loaded into Feature Extraction Software (Agilent Technologies) for feature data extraction, and data analysis was performed with GeneSpring 10.0.

Mouse, rat and human expression data were from GEO omnibus (accession number: GSE13462) (Liao *et al.*, 2009). The signal ratios of orthologous genes were used to perform hierarchical clustering (conditioned tree) with GeneSpring 10.0.

## Bisulfite genomic sequencing

Bisulfite treatment was performed using a CpGenome modification kit (Chemicon) according to the manufacturer's recommendations. The PCR primers are listed in Supplemental Table S2. The amplified products were cloned into the T-vector, and at least 10 randomly selected clones were sequenced.

## Telomerase activity of iPSCs

The telomerase activity of iPSCs and other cells was determined with a TRAPEZE telomerase detection kit (Chemicon) according to the manufacturer's recommendations. The lysates were

heated at 85°C for 10 min and used as negative controls. The reactions were separated by non-denaturing TBE-based 12% polyacrylamide gel electrophoresis and visualized with SYBR gold staining.

## Karyotype analysis

Karyotyping was performed at the Xiangtan Center Hospital using standard protocols for high-resolution G-banding.

## Teratoma formation

The piPSCs were injected intramuscularly into non-obese diabetic/severe combined immune deficient (NOD/SCID) mice (approximately  $5 \times 10^6$  cells per site). In total, four piPSC cell lines at passage >10 were used to produce teratomas. Three to five mice were injected for each cell line. Two tumors were generated for piPSC4-2 and one was generated for piPSC6-2. After 4–6 weeks, tumors were processed for hematoxylin–eosin staining. All animal experiments were conducted in accordance with the Guide for the Care and Use of Animals for Research Purposes and were approved by the SIBS Animal Care Committee.

## Supplementary Data

Supplementary data for this article are available online at <http://jmc.oxfordjournals.org>.

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