

# miRNAs promote generation of porcine-induced pluripotent stem cells

Kuiying Ma · Guangqi Song · Xinglan An · Anran Fan · Wentao Tan · Bo Tang · Xueming Zhang · Ziyi Li

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**Abstract** The pigs have similarities of organ size, immunology and physiology with humans. Porcine-induced pluripotent stem cells (piPSCs) have great potential application in regenerative medicine. Here, we established piPSCs induced from porcine fetal fibroblasts by the retroviral overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. The piPSCs not only express pluripotent markers but also have the capacity for differentiation in vivo and in vitro, including EB and teratoma formation. We supplemented microRNAs during the induction process because miR-302a, miR-302b, and miR-200c have been reported to be highly expressed in human and mouse embryonic stem

cells and in iPSCs. In this study, we found that the overexpression of miR-302a, miR-302b, and miR-200c effectively improved the reprogramming efficiency and reduced the induction time for piPSCs in the OSKM and OSK induction systems. Due to the similar induction efficiency of 4F-induced piPSCs or of three factors combined with miR-302a, miR-302b, and miR-200c (3F-miRNA-induced piPSCs), we recommend the addition of miRNAs instead of *c-Myc* to reduce the tumorigenicity of piPSCs.

**Keywords** Porcine-induced pluripotent stem cells (piPSCs) · microRNA

Kuiying Ma and Guangqi Song have contributed equally to this work.

K. Ma · G. Song · X. An · A. Fan · W. Tan · B. Tang · X. Zhang (✉) · Z. Li (✉)

Jilin Provincial Key Laboratory of Animal Embryo Engineering, The Center for Animal Embryo Engineering of Jilin Province, College of Veterinary Medicine, Jilin University, 5333 Xi An Da Lu, Changchun 130062, Jilin, China  
e-mail: zh xueming@yahoo.com

Z. Li  
e-mail: ziyi\_li86@yahoo.com; ziyi@jlu.edu.cn

K. Ma  
e-mail: mkyamberma@163.com

G. Song  
e-mail: kongki.s@gmail.com

X. An  
e-mail: starlove1999@126.com

A. Fan  
e-mail: fananran2003@163.com

W. Tan  
e-mail: tanwentao1118@163.com

B. Tang  
e-mail: tang\_bo@jlu.edu.cn

## Introduction

Porcine-induced pluripotent stem cells (piPSCs) are a potential resource for application in the studies of heredity and breeding, animal models of human disease and xenotransplantation [1]. The porcine species have similarities to humans in organ morphology and function, and are easier to maintain and breed than monkeys. Despite 20 years of progress, it remains difficult to establish standard porcine ESCs that have the characteristics of true pluripotency [2–4]. Thus, it was difficult to understand the mechanisms of porcine early embryonic development before we were inspired by the first appearance of mouse induced pluripotent stem cells [5]. The retroviral overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (OSKM) is the most classical approach to produce iPSCs, and this approach has been successfully used in mouse, human, monkey, and rat cells [5–8]. In this study, we established porcine-induced pluripotent stem cells through this approach.

MicroRNAs (miRNAs) are small, noncoding RNAs that serve as posttranscriptional regulators of gene expression in

higher eukaryotes. The miRNAs can specifically bind to complementary target mRNAs to effect gene silencing via translational repression or target degradation [9, 10]. Previous studies have shown that several microRNAs are uniquely expressed in human and mouse embryonic stem cells and in pluripotent stem cells. With their crucial role in stem cell development, these miRNAs, which primarily belong to four clusters, miR-290, miR-302, miR-371, and miR-200c [11–13], can strongly promote the reprogramming efficiency of iPSCs. However, the study of whether the miRNAs play a similar role in piPSCs has not been performed.

miR-302 and miR200c are predominant miRNA species in human ESCs and iPSCs [14–17, 19]. According to a previous report, miR-302 may increase the efficiency of cell reprogramming in at least three ways. First, miR-302 inhibits NR2F2 [20] while stimulating the expression of *Oct4* [21]. Second, miR-302 silences *AOF1*, *AOF2*, *MECP1-p66*, and *MECP2* and downregulates *DNMT1*, which results in genome demethylation and iPSC development. Third, miR-302 inhibits TGFBR2 and RHOC expression, leading to the increased efficiency of reprogramming, in part by inducing a more efficient mesenchymal-to-epithelial transition (MET) [22, 23]. MET has been shown as an important early event in somatic cell reprogramming [22, 24, 25]. A recent report found that miR-200c is directly activated by *Oct4* and *Sox2* and significantly represses the expression of zinc finger E-box binding homeobox 2 (*ZEB2*) by directly targeting its 3' UTR to help fibroblasts overcome the MET barrier and promote iPSC generation [26].

Porcine iPSCs are important to help us better understanding porcine embryonic development, to facilitate studies of heredity and breeding, and to establish animal models of human disease. Here, we established piPSCs by the overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. We found that miR-302a, miR-302b, and miR-200c improved the efficiency of cell reprogramming in the OSKM-induced system. These microRNAs were further shown to play a similar role in the OSK-induced system without *c-Myc*.

## Results

### Characterization of piPSCs reprogrammed from fibroblasts

Porcine fetal fibroblasts (PFFs) were infected with retroviral vectors designed to express either four or three reprogramming genes (*Oct4*, *Klf4*, *Sox2*, and *c-Myc*; or *Oct4*, *Klf4*, and *Sox2*) (Fig. 1b). The reprogramming protocol is shown as a schematic diagram (Fig. 1a). The initial time of infection was designated as D0; the cell

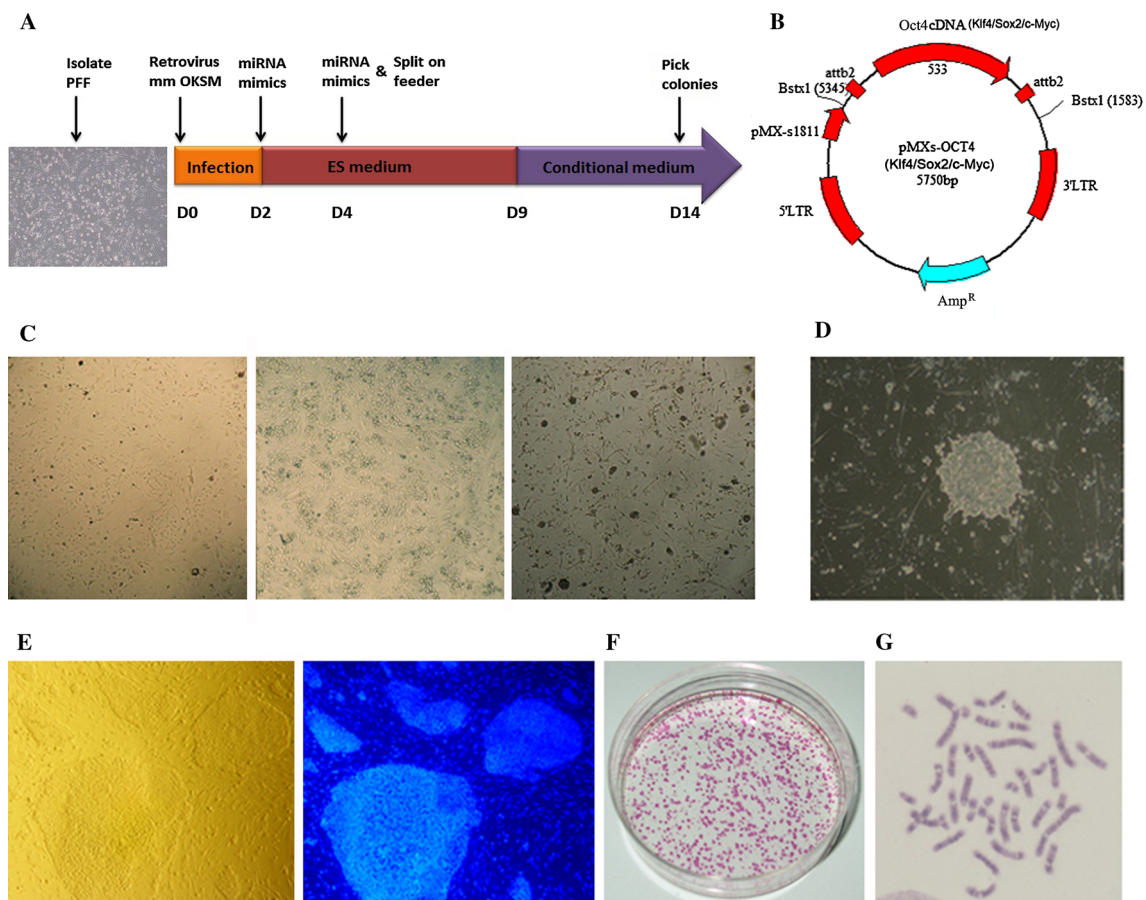
morphology began to change at D5, and progressed until ES-like colonies appeared at D12 (Fig. 1c). Cell staining by DAPI indicated that these colonies had well-defined borders and a high nuclear: cytoplasmic ratio (Fig. 1e) and were positive for alkaline phosphatase (AP) (Fig. 1f). The karyotype analysis of these piPSCs showed that no chromosomal abnormalities were present during cell reprogramming (Fig. 1g). These cells could be routinely passaged on feeders without losing their characteristics, but splitting without feeders induced rapid differentiation.

piPSCs express pluripotency markers and can differentiate both in vivo and in vitro

The piPSCs were positive for the protein expression of pluripotency markers REX1, OCT4, NANOG, and SSEA4 by immunocytochemical staining (Fig. 2a). Porcine iPSCs then underwent 10 days of EB differentiation to assess their ability to differentiate into three germ layers (Fig. 2b). Each of the germ layer markers—the endoderm gene (*AFP*, *alpgaAT*), the mesoderm gene (*BMP4*, *Enolase*), and the ectoderm gene (*GFAP*, *Neurod*)—were expressed in EBs (Fig. 2c). The reprogramming of the PFFs with exogenous factors resulted in the reactivation of endogenous porcine *Oct4*, *Sox2*, *Klf4*, and *Nanog*, which were not expressed in PFFs (Fig. 2d). Semi-quantitative RT-PCR indicated that mRNA products for the exogenous transgenes were not silenced (Fig. 2d). To ascertain their multilineage differentiation, piPSCs were injected subcutaneously into NOD/SCID mice and, after 2.5–4 weeks, resulted in teratomas (Fig. 2f) containing tissues derived from the three germ layers, including ectoderm-derived neural epithelium, mesoderm-derived striated muscle, and endoderm-derived crypt-like structures (Fig. 2g). We also analyzed the methylation of the *Oct4* promoter in piPSCs and PFFs and found obvious demethylation in piPSCs compared to PFFs (Fig. 2e).

miRNAs promote the induction of pluripotent stem cells

To test the role of microRNAs in cell reprogramming, we selected miR-302a, miR-302b, and miR-200c, which have crucial role in maintaining the characteristics of embryonic stem cells and in reprogramming mouse and human iPSCs. We separately transfected 20 pmol of each group of miRNAs into the infected cells on D2 and D4 after the introduction of OKSM and then scored the AP-positive colonies at D15 (Fig. 3a). The induction efficiency with four factors was ~0.02–0.04 %; however, the efficiency of four factors combined with miR-302a and miR-302b, or four factors combined with miR-200c was obviously increased to 0.2–0.3 % (Fig. 3b). The time until the appearance of colonies was shortened from an average of 12 to 10 days



**Fig. 1** Porcine iPSC lines derived from PFFs. **a** Schematic diagram of reprogramming protocol in general, the miRNAs were added in medium at D2 and D4. **b** Vector maps of retroviral constructs used for reprogramming. **c** Cell morphology at D5, D8, and D12 during

infection. **d** Typical ES-like colonies appeared at D14. **e** High nuclear/cytoplasmic ratio of piPSC colonies using DAPI stain. **f** The piPSC colonies were AP positive. **g** Karyotype analysis of piPSCs

(Fig. 3c). The results indicated that miR-302a, miR-302b, and miR-200c could efficiently promote the induction of OSKM-mediated piPSCs. Next, we introduced the combination of miR-302a, miR-302b, and miR200c into the OSK induction system and found that the induction efficiency increased from ~0.003 to 0.02 % (Fig. 3b); the time of appearance of colonies was shortened from an average of 14.5 to 10 days (Fig. 3c). These results suggested that miR-302a, miR-302b, and miR-200c could efficiently improve the induction of both OSKM- and OSK-mediated piPSCs.

3F-miRNA-induced piPSCs are pluripotent and can differentiate both in vivo and in vitro

To further investigate whether the miR-piPSCs induced by three factors reach a pluripotent state, the expression of pluripotent markers was analyzed by semiquantitative RT-PCR (Fig. 4a). These cells were all positive for the protein

expression of pluripotency markers REX1, OCT4, NANOG, and SSEA4 by immunocytochemical staining (Fig. 4b). The analysis of embryonic bodies (Fig. 4c) and teratomas (Fig. 4d, e) indicated the miR-piPSCs have differentiation capacity both in vitro and in vivo. Further studies showed that miR-302a, miR-302b, and miR-200c induced 3F-piPSCs have similar characteristics as OSKM-mediated piPSCs.

## Discussion

The generation of piPSCs has been reported using the traditional four factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* by retroviral vectors [27–29] and by lentiviruses [11], and 4 factors with the addition of *Nanog* and *Lin28* has been reported by DOX-inducible lentiviral vectors [12]. However, low efficiency is still a barrier to obtaining piPSCs.

**Fig. 2** Characteristics and generational abilities of piPSCs in vivo and in vitro.

**a** Immunocytochemical staining confirmed the expression of REX1, OCT4, NANOG, and SSEA4 in piPSCs.

**b** Semiquantitative RT-PCR demonstrated the integration of the four transgenes into the genome of piPSC lines, and the robustly increased expression of endogenous *Oct4*, *Nanog*, *Klf4*, and *Sox2*.

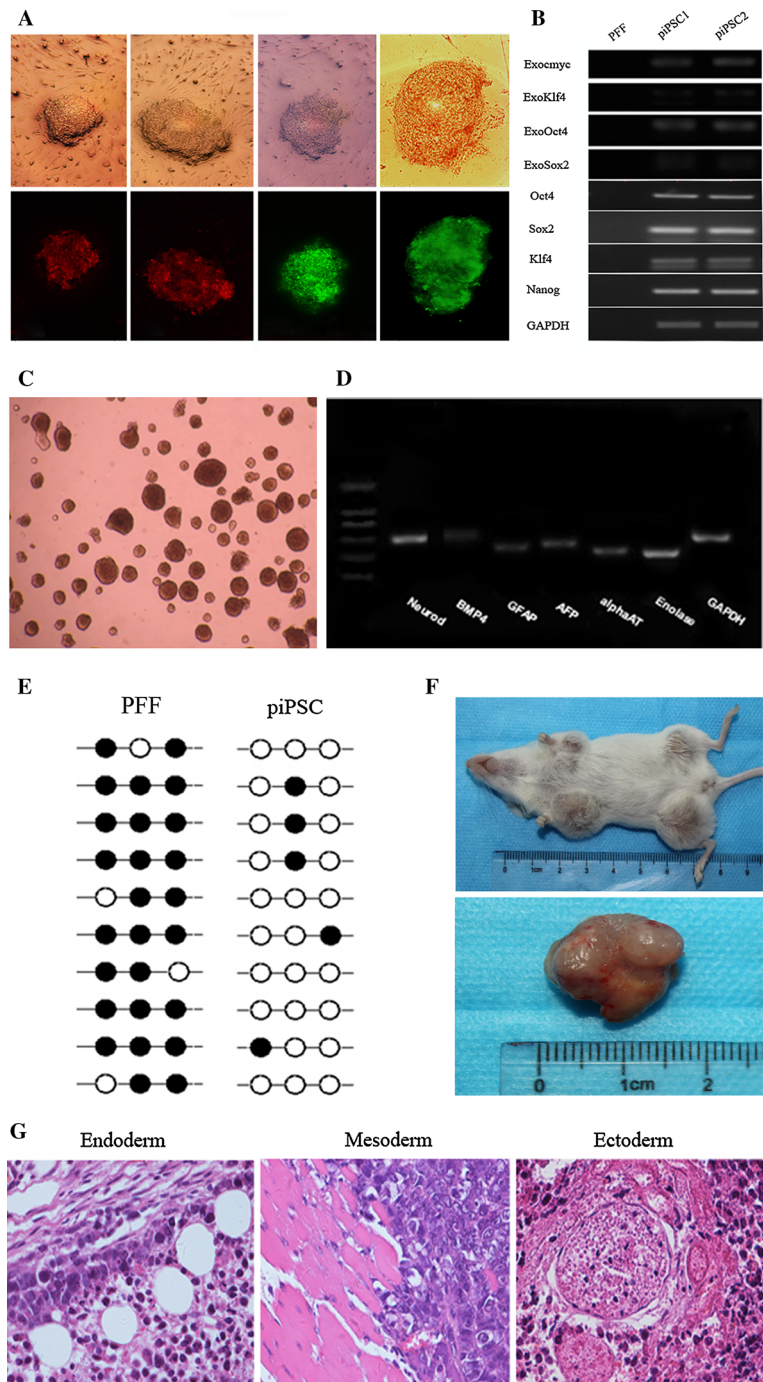
**c** Embryoid bodies derived from porcine iPS cells.

**d** The positive expression of marker genes of the three germ layers was detected from the embryoid bodies: the endoderm gene (*AFP*, *alphaAT*), the mesoderm gene (*BMP4*, *Enolase*), and the ectoderm gene (*GFAP*, *Neurod*).

**e** Bisulfite genomic sequencing of the promoter regions of *Oct4*. The open and closed circles indicate unmethylated and methylated CpGs, respectively.

**f** NOD/SCID mouse injected with 4F-iPSCs after 20 days.

**g** Teratomas derived from piPSCs revealed a contribution to all three germ layers

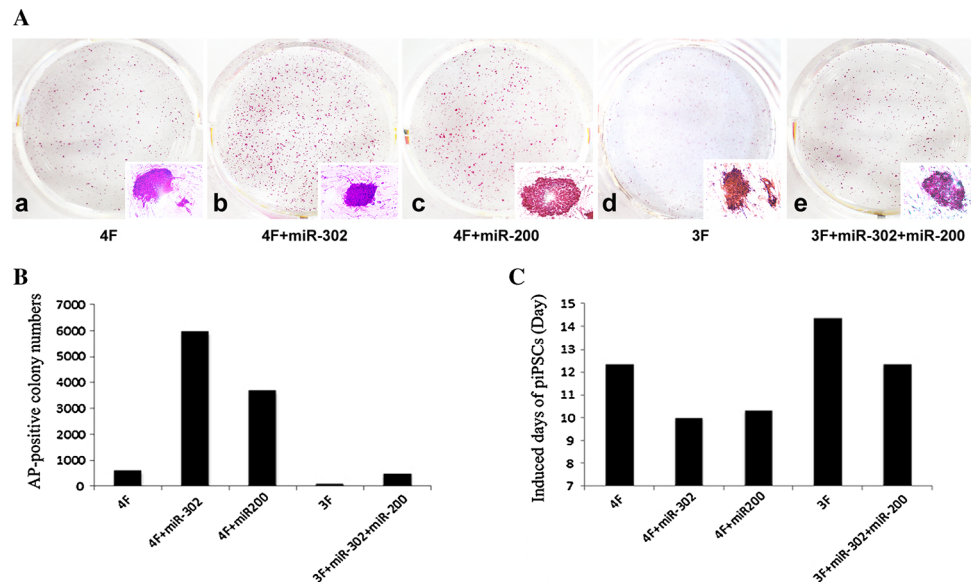


There is a series of roadblocks during the epigenetic transformation from somatic cells to iPSCs, which is well known as the MET [30]. During the reprogramming, microRNA networks regulate MET [31] and have a crucial role in iPSC development.

According to previous reports, the miR-302 cluster enhances the reprogramming of human fibroblasts and hair follicle cells (hHFCs) through multiple targets including

cell cycle regulators, epigenetic modifiers, and MET regulators [23, 32]. Other homologous miRNAs have also been found in hESCs, including miR-17/92, 371, 372, and 294; however, these miRNAs are not as highly expressed as miR-302 in undifferentiated embryonic stem cells [32].

A recent report demonstrated that the miR-200 family promotes the reprogramming of fibroblasts by activating *Oct4* and *Sox2* to overcome the MET barrier [26]. The



**Fig. 3** miRNA302a, 302b and 200c promote the induction of piPSCs. **a** AP staining of 4F-induced piPSCs; 4F+miR-302a+miR-302b induction; 4F+miR-200c induction; 3F induction; 3F+miR-302a+miR-302b+miR-200c induction (4F: *Oct4*, *Klf4*, *Sox2*, *c-Myc*; 3F: *Oct4*, *Klf4*, *Sox2*). **b** Induced efficiency of 4F-induced piPSCs; 4F+miR-302a+miR-302b induction; 4F+miR-200c

induction; 3F induction; 3F+miR-302a+miR-302b+miR-200c induction (4F: *Oct4*, *Klf4*, *Sox2*, *c-Myc*; 3F: *Oct4*, *Klf4*, *Sox2*). **c** Days of piPSC induction, 4F induction; 4F+miR-302a+miR-302b induction; 4F+miR-200c induction; 3F induction; 3F+miR-302a+miR-302b+miR-200c induction (4F: *Oct4*, *Klf4*, *Sox2*, *c-Myc*; 3F: *Oct4*, *Klf4*, *Sox2*)

combination of miR-302, miR-200c, and miR-369 has also been reported to induce pluripotency in mouse and human somatic cells [33]. Our results have shown that miR-302 and miR-200c could also enhance the reprogramming efficiency of porcine OSKM-induced iPSCs.

We further investigated whether these miRNAs could promote piPSCs generation with the OSK system because *c-Myc* is broadly implicated in tumorigenesis [34] and found to be nonessential for iPSC generation, although the speed of reprogramming is twice as slow without *c-Myc* [35]. miR-291-3p, 294, 295, and 302d were reported to enhance mouse iPSCs generation with OSK factors [36], and the expression of these miRNAs is high in ES cells [37] and is controlled by *c-Myc* [38]. These miRNAs directly target multiple cell cycle regulators, including *p21*, to accelerate the G1/S transition [39]; they simultaneously increase the expression of pluripotent transcription factors [40]. Moreover, microRNA clusters 17-92, 106b-25, 106a-363, and 302-367 also enhance mouse somatic cell reprogramming using either three or four factors [41, 42]. In this study, we produced piPSCs without using *c-Myc* and found that the combination of miR-302a, miR-302b, miR-200c, and OSK factors could efficiently enhance the generation of colonies compared to OSK alone to a similar efficiency as OSKM.

In conclusion, we successfully established piPSCs reprogrammed from PFFs with four or three factors (*Oct4*, *Klf4*, *Sox2*, and *c-Myc*; or *Oct4*, *Klf4*, and *Sox2*) and validated them both in vivo and in vitro to have similar

characteristics as embryonic stem cells. Furthermore, we found that miR-302a, miR-302b, and miR-200c could not only enhance the formation of piPSCs colonies but also reduce the induction cycles in both OSKM- and OSK-induced systems.

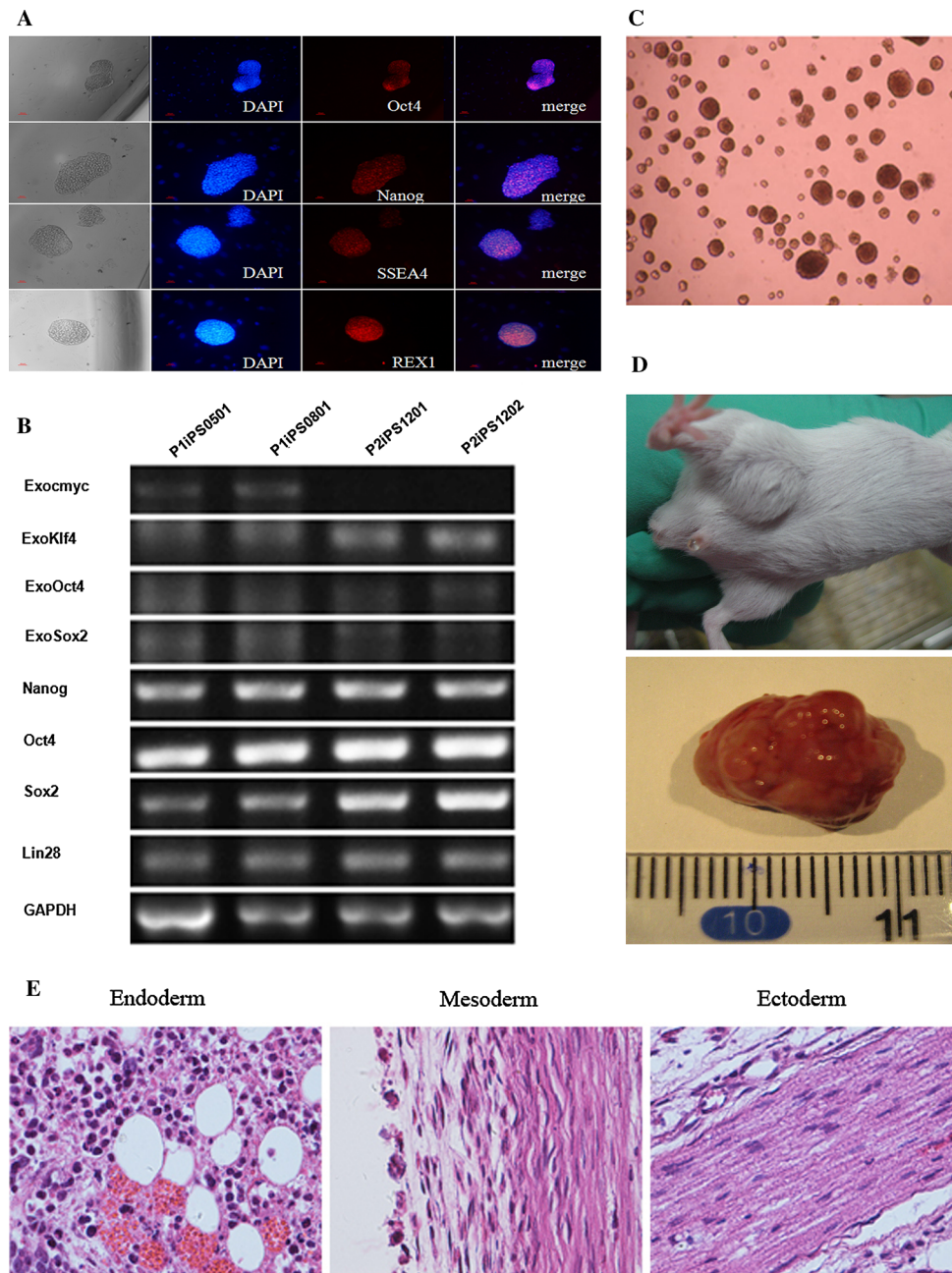
## Materials and methods

### Reagents and animals

Chemicals, media, and cell culture reagents were purchased from Hyclone (US) unless otherwise stated. Pregnant Yorkshire pigs were purchased from the Jilin University Pig Farm. Specific SPF level ICR mice used for feeder cells and nonobese diabetic/severe combined immune deficient (NOD/SCID) mice used for teratoma formation were purchased from Vital River Laboratories (Beijing, China). All 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).

### Cell preparation and culture

Porcine fetal fibroblasts (PFFs) were isolated from Yorkshire pigs at E25-30. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM)



**Fig. 4** Characteristics of 3F-miRNA-induced piPSCs in vivo and in vitro. **a** 3F-miRNA-induced piPSC colonies immunocytochemically stained positive for the human stage-specific embryonic antigen SSEA4 and the transcription factors OCT4, NANOG, and REX1. **b** Semiquantitative RT-PCR demonstrated the integration of the four transgenes into the genome of 3F-miRNA-induced piPSCs lines

(P2iPS1201, P2iPS1202) and 4F-induced piPSCs (P1iPS0501, P1iPS0801), and the robustly increased expression of endogenous *Oct4*, *Nanog*, *Lin28*, and *Sox2*. **c** Embryoid bodies derived from 3F-miRNA-induced piPSCs. **d** NOD/SCID mouse injected with 3F-miRNA-iPSCs after 27 days. **e** Teratomas derived from 3F-miRNA-induced piPSCs revealed a contribution to all three germ layers

supplemented with 10 % fetal bovine serum (FBS), 1 mM GlutaMAX (Invitrogen, USA), and 50 units/ml penicillin–streptomycin (Sigma, USA). Only early four passages of PFFs were used for piPSCs induction. Mouse embryonic fibroblasts (MEFs) were isolated from ICR mice at E12.5.

MEFs were used for feeder layer cells after treatment with mitomycin C (10  $\mu$ g/ml) for 3 h. HEK293GP cells were maintained in high-glucose DMEM supplemented with 10 % FBS. The piPSCs were grown and passaged on feeder cells in ES medium with knockout DMEM supplemented

with 20 % knockout serum, 1 mM GlutaMAX, 0.1 mM nonessential amino acids, 0.1 mM  $\beta$ -mercaptoethanol (Sigma, USA), 100 U/ml leukemia inhibitory factor (LIF) (Millipore, USA), and 50 units/ml penicillin–streptomycin (Invitrogen, USA). The medium was changed every other day.

#### Retroviral production and generation of piPSCs from PFFs

Retroviral plasmids containing mouse *Oct4*, *Sox2*, *Klf4*, and *c-Myc* were kindly provided by Professor Qi Zhou, Institute of Zoology, Chinese Academy of Sciences. HEK293GP cells ( $1 \times 10^7$ ) were plated in 100 mm culture dishes (Corning, USA). Plasmids were transfected on the following day. A quantity of 10 ml OptiMEM (Gibco, USA) was added to each culture dish when cells reached 80 % confluence. HEK293GP cells were then transduced separately with pCMV-VSV-G plasmid and single-factor retroviral plasmids using Lipofectamine 2000 (Invitrogen, USA). After 6 h, the medium was changed to 10 ml fresh HEK293GP medium. Viruses were collected 48 and 72 h later and filtered (0.45  $\mu$ m pore size) before added onto PFFs.

PFFs were seeded at a density of  $10^6$  cells per well in 6-well plates (Corning, USA) and maintained in 5 % CO<sub>2</sub>, and 95 % air at 37.8 °C. After 24 h, the cells were infected with cocktails of the previously packaged retroviruses. After 5 days, the PFFs were digested by 0.25 % trypsin (Gibco, USA) and moved onto feeder layer cells in ES media. When ESC-like colonies appeared, they were harvested and maintained in ES media (Fig. 1a). The media were changed every other day until the colonies were large enough to be isolated.

#### miRNA mimic composition and induction

The miRNA, FAM positive control, and standard negative control miRNA mimics were purchased from Genepharma Co., Ltd. (Shanghai, China). Mature miRNAs are conserved between different species, so we used human mature microRNAs of miR-302a, miR-302b, and miR200c. The sequences were as follows:

Has-miR-302a-3p: sense(5′–3′): UAAGUGCUUCCAUGUUUUGGUGA

antisense(5′–3′): ACCAAAACAUGGAAGCACUUAU

Has-miR-302b-3p: sense(5′–3′): UAAGUGCUUCCAUGUUUAGUAG

antisense(5′–3′): ACUCAAAACAUGGAAGCACUUAU

Has-miR-200c-3p: sense(5′–3′): UAAUACUGCCGGG UAAUGAUGGA

antisense(5′–3′): CAUCAUACCCGGCAGUAUUAU

A quantity of 20 pmol miRNAs was separately transfected into the infected cells with Lipofectamine 2000 on D2 and D4 after the introduction of OSKM or OSK factors (Fig. 1a); AP-positive colonies were scored at D15 (Fig. 3a).

#### Reverse transcription and semi-quantitative PCR analyses

Total RNA was isolated with Trizol reagent (Invitrogen, USA). The synthesis of the cDNA was performed by Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). Semiquantitative PCR reactions were performed in a 25  $\mu$ l volume containing 1  $\mu$ l of cDNA, 12.5  $\mu$ l of rTaq premix (TaKaRa, Japan), 10.5  $\mu$ l of RNase-free water, and 0.5  $\mu$ l each of the forward and reverse primers (10 pmol) (primers shown in Table 1) for each gene. All the products were resolved on ethidium bromide-stained agarose gels. PCRs were repeated at least twice, and the mean relative expression level was calculated. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers are listed in Table 2.

#### Alkaline phosphatase staining and immunocytochemical staining

The piPSCs were washed twice in PBS (with Mg<sup>2+</sup> and Ca<sup>2+</sup>) and fixed with 4 % paraformaldehyde before staining. The AP staining was performed using AP substrate kit (Chemicon, USA). The piPSCs were immunocytochemically stained with OCT4 (1:100, Abcam, ab18976), SOX2 (1:100, Sigma, AV38232), NANOG (1:100, Abcam, ab80892), REX1 (1:100, Abcam, ab50828), and SSEA4 (1:100, Abcam, ab16287) antibodies. The cells were incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit, 1:400, Invitrogen; Alexa Fluor 568 goat anti-mouse, 1:400, Invitrogen). Photomicrographs were taken with a fluorescent microscope (TE2000-U Nikon/C-SHG1, Japan) and a light microscope (AE20 Motic, German).

**Table 1** Induced efficiency of piPSCs

Induced system	AP-positive colony numbers (mean $\pm$ SD)	Induced days of piPSCs (mean $\pm$ SD)
4F	591.67 $\pm$ 154.13	12.3 $\pm$ 0.3
4F+miR-302	5,959.33 $\pm$ 290.49	10 $\pm$ 0.3
4F+miR-200	3,681.33 $\pm$ 559.01	10.3 $\pm$ 0.6
3F	85.50 $\pm$ 24.08	14.4 $\pm$ 0.5
3F+miR-302+miR200	456.50 $\pm$ 101.14	12.4 $\pm$ 0.5

**Table 2** Primer

Gene	Sequence	TM
<i>ExoSox2</i>	Reverse:GCTTCAGCTCCGTCTCCATCATGTTATACAT	64.06
<i>ExoOct4</i>	Reverse:AGTATGCCATCCCTCCGCAGAACTCGTATG	66.77
<i>ExoKlf4</i>	Reverse:AGGATAAAGTCTAGGTCCAGGAGGTCGTTG	65.40
<i>Exoc-Myc</i>	Reverse:AGTCGTAGTCGAGGTCATAGTTCCTGTTGG	65.40
<i>EndoSox2</i>	Forward:GGTTACCTCTTCTTCCCCTCA	60.20
	Reverse:CAAAAATAGTCCCCCAAAAAGAAG	56.98
<i>EndoOct4</i>	Forward:CAAAGTGGAGGTCCTGCCCTTC	61.85
	Reverse:ATTGAACTTCACCTTCCCTCCAACC	60.58
<i>Nanog</i>	Forward:CTTATTCAGGACAGCCCTGATTCTTC	60.75
	Reverse:AAGACGGCCTCCAAATCACTG	57.80
<i>Lin28</i>	Forward:CAGAGTAAGCTGCACATGGAGG	59.99
	Reverse:GTAGGCTGGCTTTCCTGTG	59.50
<i>Neurod</i>	Forward:GACTTGCGTTCAGGCAAAAGC	57.80
	Reverse:GGGCGACTGGTAAGAGTAGG	59.50
<i>BMP4</i>	Forward:GCGTAGCCCCAAGCATCACC	61.55
	Reverse:ATCCCTCTACTACCATCTCCTG	58.13
<i>GFAP</i>	Forward:ATCAGAGAGATGTGACGTCACC	58.13
	Reverse:CTCCATCCGTGGACTGAACTC	59.76
<i>AFP</i>	Forward:TGCCTCCGCCATTCTCTGATG	59.76
	Reverse:CTGGACCCTCTTCTGCAAAGC	59.76
<i>alphaAT</i>	Forward:GACCATTCTGGAACCTATGATC	56.64
	Reverse:CCATGAAGAGGACAGCTTTGG	57.80
<i>Enolase</i>	Forward:TCTGTGATGAATCTATCCAGG	56.26
	Reverse:CTTTTGGGTTACGGAACCTTGCG	57.80
<i>GAPDH</i>	Forward:ACCTGCCGCCTGGAGAAACC	61.55
	Reverse:GACCATGAGGTCCACCCTG	63.72

### In vitro and in vivo differentiation of piPSCs

Porcine iPSCs were harvested by Dispase (Gibco, USA), and were incubated twice on 0.1 % gelatin-coated plates for 30 min to exclude residue feeders. After incubation,  $1 \times 10^6$  cells were injected into nonobese diabetic/severe combined immune deficient (NOD/SCID) mice per site. After 2.5–4 weeks, the sections of tumors embedded in paraffin were stained with hematoxylin/eosin (HE). Porcine iPSCs were digested by 0.25 % trypsin and were incubated twice on 0.1 % gelatin-coated plates for 30 min to exclude residue feeders. Every liquid drop with  $3 \times 10^5$  single cells were cultured in nonadherent bacterial culture plates in KSR-based media without LIF. Embryoid bodies (EBs) were collected after six days. Total RNA derived from plated embryoid bodies on day six was used for RT-PCR analysis.

### Bisulfite genomic sequencing and karyotype analysis

Treatment for piPSCs was performed with a CpGenome modification kit (Chemicon, USA). The amplified PCR products were cloned into the T-Simple vector (T-Simple;

Takara, Japan), and at least ten random clones were sequenced (primers shown in Table 1). Karyotype analysis was performed for G-banding straining [42] and suggested the piPSCs had a normal karyotype.

### Data analyses

Data are shown as the mean and SD. All statistical analyses were performed with Excel 2010 (Microsoft). Differences were considered statistically significant at  $*p < 0.05$ .

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