Establishment and Characterization of Novel Porcine Induced Pluripotent Stem Cells Expressing hrGFP

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Abstract

Induced pluripotent stem (iPS) cells have been established in various animal species since 2006. The pig is a potentially useful model in human regenerative medicine, and the characters of porcine embryonic stem (pES) cells were much similar with human embryonic stem (hES) cells. In present study, the traceable humanized recombinant green fluorescent protein expressing porcine induced pluripotent stem (piPS/hrGFP+) cells were generated from porcine ear fibroblasts (pEF) by introducing four human transcription factors (Sox2, Oct4, Klf4, and c-Myc) constructed in lentivirus vectors. The piPS/hrGFP+ cells expressed hrGFP signal continuously and steadily for more than 90 passages. They also retained the typical defined characteristics including continuous proliferation with undifferentiated status, expression of ES pluripotency markers (Oct4, AP, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), and maintenance of a normal karyotype (36 + XY). Three embryonic germ layers were also successfully revealed from in vitro differentiation by embryonic body (EB) formation. Various histological analysis and immunohistochemical staining of the teratomas revealed various tissues derived from three embryonic germ layers, including neural tissues, keratin-containing epidermal tissues, skeletal muscle, smooth muscle, cartilage, adipose tissues, and glandular structures. These results support that piPS/hrGFP+ cells can be generated from pEF by direct reprogramming, and these traceable piPS/hrGFP+ cells would be beneficial for future application on cell transplantation and tissue regeneration.

Keywords: Porcine; Ear fibroblasts (EF); Induced pluripotent stem (iPS) cells; Humanized recombinant green fluorescent protein (hrGFP)

Introduction

Embryonic stem (ES) cells derived from the inner cell mass of the blastocyst were first established from mouse, and they could grow indefinitely with pluripotency and differentiate into all three embryonic germ layers [1]. ES cells, however, face ethical controversies because they derived from blastocysts. Fortunately, induced pluripotent stem (iPS) cells derived from somatic cells, which believed to possess similar ability as ES cells, were established in 2006 by direct reprogramming [2]. By using this technique, we could establish pluripotent cell lines easily and circumvent ethical problems.

The pig, a common livestock species, has the potential to serve as a great research model for human biomedicine, and has been considered an optimal model for preclinical development of therapeutic approaches because the organ size, immunology, and whole animal physiology are similar to human [3-5]. Porcine embryonic stem (pES) cells, like human embryonic stem (hES) cells, were maintained on the feeder layer without supplement of leukemia inhibitory factor (LIF) [6]. Also, the pES cells share similar colony morphology, and expressed the same pluripotency markers including Oct4, AP, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, but not SSEA-1 which is characterized to mouse ES cells [7,8]. Therefore the pig is a potentially useful model in human regenerative medicine.

In our previous studies, we successfully established pES cells expressing humanized recombinant green fluorescent protein (pES/hrGFP+) cells [8]. These cells ameliorated the Parkinson’s disease and spinal cord injury in the rat models by xenotransplantation [9,10], and also the periodontal furcation defects in a porcine model by allotransplantation [11]. In present study, porcine induced pluripotent stem cells expressing hrGFP (piPS/hrGFP+) were generated from porcine ear fibroblasts (pEF) by introducing four human transcription factors (Oct4, Sox2, Klf4, and c-Myc) constructed in lentivirus vectors, and the common criteria for iPS cells were investigated. The main goal for present study was to pave the way for transplantation study, especially allotransplantation. By detecting hrGFP expression, we could easily monitor the growth, differentiation, and migration of grafted cells. In addition, we expect that piPS/hrGFP+ cells not only could be used as cell resources to study the Parkinson’s disease, spinal cord injury, and periodontal furcation defects, but also have the potential for future therapeutic application on regenerative medicine by allotransplantation.

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Materials and Methods

Induction and culture of green fluorescent protein expressing porcine ear fibroblasts

The pEF in this study was derived from the ear dissection of Livestock Research Institute Black Pig No. one (a topcrossing breed established from Taoyuan and Duroc pigs, No. 53501) and trypsinized to single cells by 0.25% (w/v) trypsin-0.02 mM EDTA (Invitrogen, Grand Island, NY, USA). The pEF were maintained in Dulbecco’s modified eagle medium (DMEM, high glucose and no pyruvate, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 units/ml penicillin-100 g/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with an atmosphere of 5% CO2 in air. The methods for induction of hrGFP by electroporation-mediated transfection were described in previous study [8]. Briefly, the pEF cells were trypsinized to single cells and adjusted to a concentration of approximately 5×10^4 cells/ml in phosphate buffered solution (PBS). Approximately 20 μg of pAAV-hrGFP Control Plasmid (Stratagene, Santa Clara, CA, USA) were added into the cells suspension in the electroporation cuvette (Cuvettes PlusTM, Model No. 620, BTX, San Diego, CA, USA). Electroporation with condition including 2 DC pulses, 150 V/cm of field strength, and 10 msec duration time was performed by the Electro Cell Manipulator (BTX ECM 2001, San Diego, CA, USA). Approximately 40% of pEF expressed hrGFP after electroporation. When the total cell number was scaled up to 15 million, the pEF were collected for hrGFP positive sorting by flow cytometer (FACS Vantage SE, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). We obtained more than 95% hrGFP expressing pEF and nominated as pEFhrGFP+ cells.

Induction and culture of hrGFP expressing porcine induced pluripotent cells

For the generation of four factors-induced piPS cells, pEF/hrGFP+ cells were cultured in Multidishes Nunclon+ 6-wells (Nunc 140675, Roskilde, Denmark) to a cell number of 80,000/well and infected with human Oct4, Sox2, Klf4, and c-Myc constructed in lentivirus vectors (TLC-TRE-IPS-II, Tseng Hsiang Life Science LTD, Taipei, Taiwan). On day 2 after infection, the infection medium was withdrawn, and the cells were maintained in pES cells culture medium (ESM) at 37°C with an atmosphere of 5% CO2 in air. The ESM consisted of DMEM supplemented with 1 mM L-glutamine, 0.1 mM β-2-mercaptoethanol, 10 mM MEM non-essential amino acids, 0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine, 0.01 mM thymidine (all from Sigma-Aldrich), antibiotics (50 units/mL penicillin G and 50 μg/mL streptomyein sulfate, Invitrogen) and 16% fetal bovine serum (FBS, Invitrogen) as described previously [12].

For isolation and establishment of piPS cells, the colonies exhibited dome-like morphology were picked up about 1 month post-infection. These cells were subsequently expanded and maintained in ESM on mitomycin C (Sigma-Aldrich) inactivated STO feeders (mouse embryonic fibroblasts, CRL-1503, ATCC, Manassas, VA, USA). These putative piPS/hrGFP+ cells were regularly subcultured every 5 to 7 days.

Characterization of the pluripotentcy markers

The putative piPS/hrGFP+ cells were fixed in 10% (v/v) neutral buffered formalin for 30 min, and permeabilized with 0.3% (v/v) Triton X-100 for 10 min after washing with PBS three times. After permeabilization, the cells were incubated with blocking solution [5% (v/v) FBS in PBS containing 0.1% (v/v) Tween-20] for 2 h at room temperature, and then incubated with primary antibody diluted with blocking solution (1:200 dilution) at 4°C overnight. After washing with PBS three times on the next morning, the cells were incubated with secondary antibody diluted with blocking solution (1:200 dilution) for 2 h at room temperature. The cells were then washed twice with PBS again, and stained with 4,6-diamidino-2-phenylindole (DAPI).

Primary antibodies used for determining the undifferentiated status of piPS/hrGFP+ cells were ES cell-specific markers, including Octamer-binding transcription factor-4 (Oct-4, Millipore Cat. #AB3209, Temecula, CA, USA), alkaline phosphatase (AP, Millipore Cat. #AB4349), stage specific embryonic antigen-3 (SSEA-3, Millipore Cat. #AB4303), stage specific embryonic antigen-4 (SSEA-4, Millipore Cat. #AB4304), tumor related antigen-1-60 (TRA-1-60, Millipore Cat. #AB4360), and tumor related antigen-1-81 (TRA-1-81, Millipore Cat. #AB4381). The secondary antibodies were the rhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG (H+L) (for Oct-4 staining, Jackson ImmunoResearch Cat. #111-025-003, West Baltimore Pike, PA, USA), rabbit anti-mouse IgG (H+L) (for AP and SSEA-4 staining, Jackson ImmunoResearch Cat. #315-025-003), rabbit anti-rat IgM (for SSEA-3 staining, Jackson ImmunoResearch Cat. #312-025-020), and rabbit anti-mouse IgM+IgG (for TRA-1-60 and TRA-1-81 staining, Jackson ImmunoResearch Cat #315-025-044). Fluorescent cells were visualized by the inverted fluorescence microscope (DM IRB, Leica, Wetzlar, Germany) equipped with CCD camera (CoolSNAP, Monochrome, Photometrics, Tucson, AZ, USA), and the images were analyzed by RS image software (Photomats).

Karyotype analysis

G-banding was used for karyotyping analysis and carried out as previously described [8,13]. Briefly, The piPS/hrGFP+ cells were then mitotically arrested with colcemid (KaryoMax Colcemid solution, Invitrogen) at a final working concentration of 0.02 μg/mL at 39°C for 30 min. Cells were harvested for hypotonic treatment for 30 min in 0.56% (w/v) KCl aqueous solution following removal from colcemid treatment on dish (in situ method). The cells were pelleted by centrifugation at 800 x g and fixed in cold Carnoy’s fixative (3:1, v/v, of absolute methanol to glacial acetic acid) for 10 min. After a second wash in Carnoy’s fixative, the cells were resuspended in 2 mL fixative. Slides were prepared by dropping the cell suspension onto dry microscope slides prewashed with fixative. Immediately after dropping, the slides were exposed to a flame to burn off the fixative, incubated 30 seconds to 1 minute in a trypsin (1:250) solution (0.1 g trypsin in 100 mL isotonic buffer), rinsed for a few seconds in a jar with FBS (2-3 mL FBS in 50 mL isotonic buffer), rinsed in isotonic buffer, and then stained in 5% Gurr’s Giemsa staining solution (Invitrogen) for 2 min. The stained slides were rinsed, air dried and examined under a microscope at 1,000 × magnification with oil immersion. The images were then analyzed by Applied Images software (AI cytovision 2.8, 2002, Applied Images Group, Gainesville, GA, USA).

Gene expression analysis

For gene expression analysis of porcine endogenous Oct4 (pOct4), Sox2 (pSox2), Klf4 (pKlf4), and c-Myc (pMyc), the total RNA of pEF/hrGFP+, piPS/hrGFP+, and pES/hrGFP+ cells were extracted by PureLink® RNA Mini Kit (Ambion, Grand Island, NY, USA), and reverse-transcribed into cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). RT-PCR was performed on TPersonal RT-PCR system (Biotemta GmbH, Rudolf-Wissell-Str. 30, D-37079 Goettingen, Germany). The conditions of RT-PCR were as follows: 94°C, 5 min/94°C, 30 sec for denaturation; 60°C, 30 sec for annealing; 72°C, 1 min/72°C 3 min for elongation; 4°C pause; followed by 32 amplification cycles. The primers used in the study were listed as Table 1.
Embryonic body formation, differentiation, and embryonic germ layers determination

For the formation of embryoid body (EB), the piPS/hrGFP+ cells were removed from their feeders and subjected to suspending culture by hanging drops in the bacteriological Petri dish [8]. The piPS/hrGFP+ cells were harvested and cultured in 20 μL of ESM on the lid of 100-mm sterile bacteriological Petri. The cells were cultured at 37°C with an atmosphere of 5% CO₂ in air for 7 days. The medium was regularly changed every other day. After 7 days culture, the formed EB was transferred to gelatin-coated 48 well in the same medium for another 14 days to induce in vitro spontaneous differentiation.

Immunocytochemical study was implemented for analysis of embryonic germ lineages of the differentiated EB. The EB was fixed and treated as mentioned above. The primary antibodies for determining ectodermal differentiation were specific against neurofilament light (NFL, Millipore Cat. #AB9568), microtubule associated protein 2 (MAP2, Millipore Cat. #MAB3418), and cytokeratin (Sigma Cat. #C-2562). The primary antibody for determining mesodermal differentiation was specific against atrial natriuretic peptide (ANP, Millipore Cat. #AB9170). The primary antibody for determining endodermal differentiation was specific against α-fetoprotein (AFP, Santa Cruz Cat. #SC-8108, Dallas, TX, USA). The secondary antibodies were the rhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG (H+L) (for NFL and ANP staining, Jackson ImmunoResearch Cat #111-025-003), rabbit anti-mouse IgG (H+L) (for MAP2 and cytokeratin staining, and rabbit anti-goat IgG (H+L) (for AFP staining. Jackson ImmunoResearch Cat #305-025-003).

Teratoma formation and in vivo tracking

All animal experiments in this study were performed in accordance with ethical guidelines and following approval of the Livestock Research Institutional Animal Care and Use Committee (IACUC).

For teratoma formation analysis, five female nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice (Bio-LASCO, Taiwan) at 8 weeks of age were anesthetized with Zoletil/Rompun (severe combined immunodeficiency (NOD-SCID) mice (Bio-LASCO, Taiwan) at 8 weeks of age were anesthetized with Zoletil/Rompun (eight hours post-injection) and then subcutaneously injected with 10⁶ cells of piPS/hrGFP+. The mice were monitored closely for the formation of teratomata. Teratomata were confirmed by histological examination at intervals of 1, 2, 3, and 4 months post-injection. The presence of teratomata was confirmed by the presence of at least one of the three germ layers: endodermal, mesodermal, and ectodermal.

Expression of ES cell pluripotency markers

To investigate the expression of pluripotency markers of piPS/hrGFP+ cells, gene expression was determined by qRT-PCR. The expression levels of the pluripotency markers miRNAs were quantified using the TaqMan MicroRNA Assays (Thermo Fisher Scientific) and analyzed using the ΔΔCt method. The relative expression levels were normalized to the expression levels of the internal reference genes (Actin, β-actin). The statistical significance of the differences in expression levels among the groups was assessed using the Student’s t-test. The results are presented as the mean ± SEM. Significant differences were considered at P < 0.05.

Table 1: Primer sets for RT-PCR.

| Gene   | Sequences       | Length (bp) | Annealing (°C) |
|--------|-----------------|-------------|----------------|
| pOct4  | F: 5’-AGGGTGTCTGCAGCGAACGACC-3’  R: 5’-TGATGTTGGCGCGCTTGGCCG-3’ | 355 330 | 60 |
| pSox2  | F: 5’-GCCAATCTACTGTGCGGGCGG-3’  R: 5’-GCCATGCTGTTGGCCTGCC-3’ | 352 60  | |
| pKlf4  | F: 5’-GGCGGAGAACAGCTTGAAG-3’  R: 5’-GACCTTGTGCCAGCTGGA-3’ | 423 60 | |
| pc-Myc | F: 5’-TCGGAGACCTCTGCGCTCCTGCT-3’  R: 5’-CTGCTAATTGTTGCGTCGTC-3’ | 274 60 | |
| Actin  | F: 5’-CTCTTATTAGTCCAGGACAGGATTTC-3’  R: 5’-GTGGGGGGCCCGAGGCCACCA-3’ | 539 60 | |

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coated 48 well (Figure 4C) and began to differentiate into cells of three embryonic germ layers. The attached cells exhibited various types of morphologies, but the morphology of cells changed frequently. The differentiation timing of each embryonic germ layer was various. Generally, neuron-like cells with obvious Nissl body first appeared on day 3 after successful attachment (Figure 4D), and that gradually differentiated into epithelial cells (Figure 4E). By immunocytochemical staining, the differentiated embryonic germ layers were positive for MAP2 (ectodermal maker), NFL (ectodermal maker), cytokeratin (ectodermal maker), AFP (mesodermal maker), and ANP (endodermal maker) (Figure 4F-J).

hrGFP+ cells, ES cell-specific surface antigens including Oct4, AP, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 were determined. The results of immunocytochemical study showed that the piPS/hrGFP+ cells were positive for those pluripotency markers (Figure 2A). The expression of endogenous pluripotency genes (pOct4, pSox2, pKlf-4, and pc-Myc) were also detected in piPS/hrGFP+ and pES/hrGFP+ cells. In addition, pKlf-4 and pc-Myc were prominently expressed in the pEF/GFP+ cells (Figure 2B).

Karyotype analysis

Karyotyping of the piPS/hrGFP+ cells was performed by G-banding staining for monitoring of their chromosomal normality. The results indicated that the piPS/hrGFP+ cells maintained in culture for more than 90 passages possessed a normal 36 + XY male karyotype (Figure 3).

In vitro differentiation

The piPS/hrGFP+ cells formed ball-shaped EB (Figure 4A) and retained hrGFP signal after 7 days of hanging drops culture (Figure 4B). The EB formation rate was about 93.6 ± 4.7% (190/203, n = 10). Spontaneous differentiation of piPS/hrGFP+ cells was evident when the EBs allowed to grow in gelatin-coated surface. On day 3-5 after adherent culture, the EB in ESM attached to the surface of gelatin-

Figure 1: Induction of piPS/hrGFP+ cells from pEF. (A) Morphology of pEF cells. (B) hrGFP expression of pEF/hrGFP+ Cells. (C) Time schedule of piPS/hrGFP+ cell generation. (D) Aggregation of reprogrammed pEF/hrGFP+ cells. (E) Typical image of ES dome-like colony of reprogrammed pEF/hrGFP+ cells. (F) Typical image of piPS/hrGFP+ colony. (G) hrGFP expression of piPS/hrGFP+ colony.

Figure 2: Expression of ES cell pluripotency markers in piPS/hrGFP+ cells. (A) Immunocytochemistry of piPS/hrGFP+ cells with antibodies to Oct4, AP, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Nuclei were stained with DAPI (blue). (B) RT-PCR analysis of pluripotency genes.
Teratoma formation and in vivo tracking

For determination of the in vivo differentiation capacity, the piPS/hrGFP+ cells were injected into immunocompromised mice. One month after transplantation, all NOD-SCID mice had developed small and solid teratomas of about 0.004 cm³ in size in dorsal flank. The teratomas grew and reached 0.55 ± 0.21 cm³ in size three months after transplantation. The teratomas in the transplantation site were traceable by IVIS 50 through the 3-months experimental period. The relative intensity of fluorescent signal in ROI of treatment groups was 6.95 ± 1.68 folds higher than that of control groups three month after transplantation (Figure 5A). Thereafter, the NOD-SCID mice were sacrificed for histological and immunohistochemical analysis. The dissection of teratomas revealed various tissues derived from the three embryonic germ layers, including neural tissues (ectoderm), keratin pearls (ectoderm), skeletal muscle (mesoderm), smooth muscle (mesoderm), cardiac muscle (mesoderm), cartilage (mesoderm), adipose tissues (mesoderm), and glandular structures (endoderm) (Figures 5B and 5C).

Discussion

The iPS cells were first generated from murine differentiated somatic cells [2], and numerous follow-up researchers also successfully obtained iPS cells from human [14-16], monkey [17], rat [18], pig [19-24], and horse [25]. The pig has been considered an optimal model for human biomedicine and research [3-5,9-11]. Therefore the pig is a potentially useful model in regenerative medicine in human. In present study, the traceable hrGFP-expressing piPS cells were generated and have the potential for future application on regenerative and therapeutic medicine.

In the present study, we established the piPS/hrGFP+ cells from reprogramming of hrGFP-expressing pEF cells. These novel piPS/hrGFP+ cells generated in this study expressed hrGFP signal continuously and steadily for more than 90 passages. Expression of fluorescence can be detected in pEF/hrGFP+ cells and pass to piPS/hrGFP+ cells, EB and teratomas (Figure 1B, 1G, 4B, and 5A). They also possessed the typical defined characteristics of ES cells, including continuous proliferation with undifferentiated status, maintenance of a normal karyotype (Figure 3), and formation of EBs upon suspension culture (Figure 4A and 4B). Expression of the ES cell markers including Oct-4, AP, SSEA-4, TRA-1-60, and TRA-1-81 were also detected in the undifferentiated piPS/hrGFP+ cells, as pES/hrGFP+ cells we described previously [8]. Expression of endogenous pluripotency genes, pOct4, pSox2, pKlf4, and pc-Myc, were detected in piPS/hrGFP+ and pES/hrGFP+ cells. In addition, significant pKlf4 and pc-Myc expression but not pOct4 and pSox2 expression were detected in pEF/hrGFP+ cells (Figure 2B). This phenomenon was also shown in the previous reports of Ezashi et al. (2009) [20] and Fujishiro et al. (2012) [21]. The endogenous Klf4 expression in human fetal endothelial cells was also reported previously, and these cells allowed to be reprogrammed with Oct4 and Sox2 [26]. These results imply that piPS cells might also be able to reprogram from porcine somatic fibroblasts by using transcription factors of Oct4 and Sox2 only.

The EB formation efficiency of piPS/hrGFP+ cells in this study

![Figure 3: The normal karyotype of 36 + XY of piPS/hrGFP+ cells.](image)

![Figure 4: In vitro embryoid body formation and differentiation of piPS/hrGFP+ cells.](image)
was high (93.6 ± 4.7%) and the differentiated cells derived from these embryonic germ layers were detected after adhesive culture of EB. These results demonstrated that the in vitro differentiation capacity of piPS/hrGFP+ cells generated in this study. To our knowledge, there were few reports describing the success in teratomas induction by transplanting pES cells into the nude mice [27-29]. Hochereau-de Reviers and Perreau (1993) [28] reported that only the embryonic disc cells derived from days 10-11 but not days 5-6 blastocysts formed teratomas when transplanted into the nude mice. Similar observation had been depicted by Piedrahita et al. (1990) [29]. They failed to induce teratomas by pES cells derived from day 7-8 embryos. The difficulty in obtaining teratomas from the porcine embryonic cells of earlier stages was also confirmed by Anderson et al. (1994) [27], who demonstrated that teratoma can only be obtained by injecting pES cells isolated from blastocysts of day 11-12. However, in the present study, piPS/hrGFP+ cells formed teratomas after being transplanted into dorsal flank of NOD-SCID mice (n = 5). Other previous studies in the generation of pigs cells also demonstrated the similar results [19-24]. The reason for teratomas formation of pES cells after ectopic transplantation to SCID mice might result from different property of cells in epigenetic background via reprogramming process.

ES cells of ungulate species were rather difficult to establish from early embryos, but iPS cells provide a feasible approach for generating pluripotent stem cells. In our previous studies, transplantation of pES/hrGFP+ cells-derived neuronal progenitors were successfully ameliorated the Parkinson disease [9] and spinal cord injury [10] in the rat models. In addition, regeneration of periodontal furcation defects in a porcine model was improved by transplanted with pES/hrGFP+ cells [11]. In the present study, piPS/hrGFP+ cells were established and possessed very similar property as pES/hrGFP+ cells we established previously [8]. In addition, the intensity of hrGFP signal in piPS/hrGFP+ cells was up to 6.95 ± 1.68 folds compared with control group. This will benefit the transplanted piPS/hrGFP+ cells to easily locate, monitor and traced after transplantation. The therapeutic potential of piPS/hrGFP+ cells in regenerative medicine would be further.

Figure 5: In vivo teratoma formation of piPS/hrGFP+ cells. (A) Teratomas formation tracked by In Vivo Imaging System. (B) Hematoxylin and eosin staining of teratomas derived from piPS/hrGFP+ cells showed multiple tissues, including neural tissues, keratin pearls, skeletal muscle, smooth muscle, cartilage, adipose tissues, and glandular structures. (C) Immunohistochemical staining confirmed that piPS/hrGFP+ cells differentiated into neural tissues, keratin pearl, and cardiac tissues in teratomas.
investigated to compare piPS cells and pES in biomedical applications. In a nutshell, these results implicate that traceable piPS/hrGFP+ cells were successfully established and opened an avenue for biomedical application in pigs.

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