Review article

Derivation of porcine pluripotent stem cells for biomedical research

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**Abstract**

Pluripotent stem cells including embryonic stem cells (ESCs), embryonic germ cells (EGCs), and induced pluripotent stem cells (iPSCs) are capable of self-renewal and limitless proliferation \textit{in vitro} with undifferentiated characteristics. They are able to differentiate \textit{in vitro}, spontaneously or responding to suitable signals, into cells of all three primary germ layers. Consequently, these pluripotent stem cells will be valuable sources for cell replacement therapy in numerous disorders. However, the promise of human ESCs and EGCs is cramped by the ethical argument about destroying embryos and fetuses for cell line creation. Moreover, there are still carcinogenic risks existing toward the goal of clinical application for human ESCs, EGCs, and iPSCs. Therefore, a suitable animal model for stem cell research will benefit the further development of human stem cell technology. The pigs, on the basis of their similarity in anatomy, immunology, physiology, and biochemical properties, have been widely used as model animals in the study of various human diseases. The development of porcine pluripotent stem cell lines will hold the opportunity to provide an excellent material for human counterpart to the transplantation in biomedical research and further development of cell-based therapeutic strategy.

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**1. Introduction**

Pluripotent stem cells are cell lines established from embryos including embryonic stem cells (ESCs) and epiblast stem cells (EpiSCs) or from the primordial germ cells (PGCs) in the genital ridge of fetus as embryonic germ cells (EGCs)\textsuperscript{[1]}. Also, from somatic cells reprogrammed by ectopic coexpression of various defined transcription factors\textsuperscript{[2]} are approached to generate pluripotent cells, which possessed comparable characteristics of ESCs and nominated as induced pluripotent stem cells (iPSCs; Fig. 1). Embryonic stem cells are firstly isolated from preimplantation mouse embryos [3,4]. Thereafter, isolation of putative pluripotent ESC lines other than murine ES cells (mESCs) has been attempted in various mammalian species (refers to review in [5]), including human [6]. Defined ESCs are able to self-renew and proliferate continuously \textit{in vitro} with the undifferentiated characteristics. In responding to suitable conditions, they can be induced to differentiate into cells of all three primary germ layers. There were many remarkable results in directing the human ES cells (hESCs) differentiation into neuronal cells.
pancreatic β cells [8], and cardiomyocytes [9]. These results reveal a potential for clinical application of hESCs in the treatment of diseases such as Parkinson’s disease, spinal cord injury, diabetes, and heart diseases.

Domestic swine are demonstrated to be very similar to the human in anatomic, immunologic, and physiological characteristics, and the sizes of their organs are fairly comparable to those of human. Moreover, swine, especially the miniature pigs, has been demonstrated as excellent animal model in therapeutics development for various human diseases, including congenital heart disease, hypertension, organ transplantation, pharmacology, and toxicology [10,11]. Therefore, the study of porcine pluripotent stem cells might serve as an excellent model in development of biomedical and regeneration medicine in humans [12].

2. Derivation of porcine ESC (pESC) lines from embryos of different origins

Although the establishment of ESC lines from domestic species is much more difficult than that in murine species [5,13,14], putative pESCs have been derived from inner cell mass (ICM) of the ex vivo blastocysts, in vitro-produced blastocysts, parthenogenetic blastocysts, and the blastocysts derived from somatic cell nuclear transplantation with diverse derivation efficiency [5,14,15]. In general, almost all the blastocyst-derived putative pESC lines possessed an epithelial-like morphology, were feeder-dependent, and expressed alkaline phosphatase (AP). However, the capability of these blastocyst-derived cells in self-renew, proliferation, maintenance of undifferentiated status in vitro, and pluripotency is varied among the cell lines reported, and most of them lose their pluripotency subsequently [5,12,15]; if any which retain the capacity of repeated proliferation and pluripotency, they possess very limited capacity in chimera generation [16,17], and so far, no germline transmissions are obtained when injected into a blastocyst (Table 1).

The pESCs were very similar to hESCs in many characteristics, including colony morphology, feeder-dependent, and refractory to leukemia inhibitory factor (LIF) in culture, and expression of stem cell markers [13,15,16,22]. To our knowledge, isolation and establishment of persistent pESC lines were firstly accomplished and reported by our laboratory in 1999 by adapting the ESC culture system for murine species, with minimum modification [16]. Briefly,

**Table 1**

<table>
<thead>
<tr>
<th>Embryo origin</th>
<th>d.p.i.</th>
<th>Maximum passage number</th>
<th>Method of undifferentiation evaluation</th>
<th>Method of evaluation and type of differentiation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ex vivo</em></td>
<td>7 or 9</td>
<td>&gt;1 y; &gt;50 passages</td>
<td>Morphology, vimentin</td>
<td>Morphology, EB, muscle, nerve, and endoderm</td>
<td>[13]</td>
</tr>
<tr>
<td><em>Ex vivo</em></td>
<td>7–8</td>
<td>&gt;80 passages, two male lines</td>
<td>Morphology</td>
<td>Morphology, neuron, muscle, epithelium, adipocytes, melanocytes, and glandular epithelium</td>
<td>[18]</td>
</tr>
<tr>
<td><em>Ex vivo</em></td>
<td>5.5–7.5</td>
<td>44 passages</td>
<td>Morphology</td>
<td>Morphology: EB, adipocytes, epithelium, neurons, muscles cells, EB, and chimera</td>
<td>[19]</td>
</tr>
<tr>
<td><em>Ex vivo</em></td>
<td>6–8</td>
<td>&gt;35 passages, one male, three female lines</td>
<td>Morphology, AP</td>
<td>Morphology: neuron, smooth muscle, epithelium, EB, and somatic chimera</td>
<td>[16]</td>
</tr>
<tr>
<td><em>Ex vivo</em></td>
<td>6–8</td>
<td>One male line</td>
<td>Oct-4, AP, SSEA-3/4, TRA 1–60, and TRA 1–81</td>
<td>EB, neuron, and used for cell transplantation</td>
<td>[20]</td>
</tr>
<tr>
<td><em>In vitro</em></td>
<td>7–8</td>
<td>One line, 30 passages</td>
<td>Morphology, epithelial-like</td>
<td>Used for nuclear transfer with embryo development to blastocyst stage</td>
<td>[21]</td>
</tr>
</tbody>
</table>

Abbreviations: AP, alkaline phosphatase; d.p.i., day post insemination; EB, embryoid body; ESCs, embryonic stem cells.

early-hatched blastocysts collected from sows on Day 6 to 8 after the first day of estrus and breeding (Day 0) were cultured on mitosis-inactive STO cells (mouse embryonic fibroblasts, ATCC, CRL-1503, Manassas, VA, USA) in ESC culture medium containing 20% fetal bovine serum. A total of 12 putative pESC lines with stable undifferentiated morphology were successfully established, and these epithelium-like cell lines survived after cryopreservation in liquid nitrogen. Three cell lines were maintained in culture for more than 35 passages. Several important characteristics of the pluripotential ESCs were demonstrated in these cells, including morphology, normal karyotype, expression of AP activity, in vitro pluripotency on the basis of behavior of spontaneous and induced differentiation, and formation of embryoid body after suspension culture. These pESCs were able to participate in the formation and development of a chimeric offspring after blastocyst injection although with a very limited efficiency as determined by microsatellite markers. However, no germline contribution was found in this chimera. Recently, establishments of porcine ESC lines other than from in vivo-derived blastocysts also have been achieved from IVF, IVF-aggregated, and parthenogenetic blastocysts [15]. All these with EpiSC-like cell lines are maintained in vitro for more than 41 passages. They express pluripotent markers such as Oct-4, Sox2, Nanog, SSEA4, TRA-1 to 60, and TRA-1 to 81 and are able to form embryoid bodies. However, the capacity of contribution to chimera formation of these cell lines has not been determined. Putative ESC lines have also been successfully established from seeding of intact-cloned blastocysts [23] and ICMs developed from aggregated 4-cell cloned embryos [24]. These ESCs derived from nuclear transfer embryos can be maintained at undifferentiated status in culture for more than 25 passages and still express ESC-specific markers. They can form embryoid bodies and differentiated in vitro into cell lineages of three primary germ layers. Although the in vivo differentiation capability of these cells has not been determined, still they open a window toward the further development of therapeutic cloning. Putative EGCs have been isolated from the genital ridge from porcine fetuses of 24 to 26-days old (refers to review in [12]). These PGC-derived cells express AP activity, having the capability of embryoid body formation and in vitro differentiation spontaneously into cells of three germ layers. When introduced to blastocysts, porcine PGCs and EGCs of early passages can contribute to somatic chimera, but the efficiency is low and no germline chimera resulted [25–27]. Low plating efficiency and difficulty of maintaining undifferentiated status in vitro have also been addressed in the establishment of porcine EGC lines [12,25–27]. Application of porcine PGCs in biomedical study will be limited before these obstacles have been overcome.

3. Porcine ESCs are much more similar to hESCs than mESCs

The development of nonhuman ESC lines is crucial for research and development of stem cell therapies and regeneration medicine. Among the species in which establishment of ESC lines have been declared, the capacity of germline transmission has been demonstrated only in naive ESCs isolated in mice and rats [28]. The mESC and hESC lines have been the two major animal models in the investigation of regeneration medicine. However, considerable differences in both morphology and development between hESCs and mESCs have been revealed, especially in the gene expression profiles of pluripotency maintenance and differentiation [29]. The mESCs are of a naive status and LIF-dependent, having a compact dorm-like appearance, and they can be enzymatically passaged even to a single cell clone and form germine chimera after induced into blastocysts with a high efficiency. On the other hand, hESCs having a flat epithelial-like morphology are trypsin-sensitive and possess reduced developmental competence when compared to naive mESCs [14]. The expressions of pluripotent cell markers were quite different between the ESCs of these two species. The expression of SSEA1 can be detected in the mESCs but not in hESCs, whereas SSEA3, SSEA4, TRA-1 to 60, and TRA-1 to 81, and GCTM-2 are found only in the hESCs [30]. It is believed that hESCs, also derived from the blastocyst-derived naive mESCs and much similar to the murine EpiSCs derived from epiblast at later stage [31]. In addition, mice with targeted genetic modification do not always exhibit predictive human phenotypes [32,33]. These differences would impair the translation of information gained from mESCs directly to hESCs.

Swine has been demonstrated as an excellent animal model in therapeutics development for various human diseases [11,12]. Furthermore, porcine products including insulin, heart valves, and skin have been widely applied to human [33]. Therefore, it will be of great advantage in the production of ESC lines from swine, which are more physiologically similar and more relevant for clinical translation to the human compared to these of mice [29,32].

In the previous study, we found that the pESCs had an epithelial-like colony morphology very similar to hESCs, and they were both feeder-dependent and refractory to LIF in culture (Table 2) [16]. Both of them expressed Oct-4, AP, SSEA-3/4, TRA-1 to 60, and TRA-1 to 81 (Table 3); however, they did not express SSEA1 that was expressed in naive mESCs [15,29]. Therefore, the pESCs could provide as a suitable preclinical animal model in the study of regenerative medicine in therapeutic approaches.

4. Generation and application of traceable pESC lines in regeneration studies

4.1. Generation of traceable pESC lines

For the application of pESCs as a hESC model in the study and development of regeneration medicine in human, a traceable pESC line is far beyond necessary, especially in therapeutic approaches involving cell transplantation. In our laboratory, three stable green fluorescent protein (GFP) expressing pESC (pESC/GFP–) lines were obtained by electroporation with pAAV-hrGFP Control Plasmid (Stratagene, CA, USA.) [20]. Despite survived repetitive freezing and thawing and subcultures for more than 90 passages over
4.2. Application of traceable pESC lines in rat Parkinson’s disease model

The pESC/GFP<sup>+</sup> cells were subjected to directed differentiation into neural lineages for investigating therapeutic potential in a rat model of Parkinson’s disease [34]. Directed differentiation was induced by single-cell suspension culture in medium containing retinoic acid, sonic hedgehog, and fibroblast growth factor (FGF) combinations, without going through the protocol of embryoid body formation. The expression of TH, ChAT, and GABA specific markers was observed in these pESC/GFP<sup>+</sup>-derived neurofilament 70 kDa-positive neural cells. The undifferentiated pESC/GFP<sup>+</sup> cells and their neuronal differentiation derivatives were transplanted into the striatum of Sprague–Dawley rat’s brain. The GFP-fluorescent signals from the injection site of Sprague–Dawley rats’ striatum could be detected through the experimental period of 3 months. The Parkinson’s disease model rats exhibited stably and significantly decreased asymmetric rotations (P < 0.05) after transplantation with pESC/GFP<sup>+</sup>-derived D18 neuronal progenitors. The dopaminergic differentiation of grafted cells in the brain was further confirmed by immunohistochemical staining with anti-TH, anti-DA, and anti-DAT antibodies. These results suggested that the differentiation approach we developed would direct pESCs to differentiate into DA-neuron lineages and benefit the development of novel therapeutics for Parkinson’s disease involving stem cell transplantation.

4.3. Application of traceable pESC lines in rat spinal cord injury model

To investigate therapeutic potential of spinal cord injury in the rat model, the pESC/GFP<sup>+</sup> colonies were subjected to a two-step in vitro induction protocol to differentiate into neural lineage [35]. After suspension culture in neurogenic stimulators consisted of retinoic acid, sonic hedgehog, and EGF, and the pESC/GFP<sup>+</sup> cells were replated and cultured in medium containing EGF and FGF to derive neuron progenitors. The Long Evans rats with contused spinal cord were transplanted with pES/GFP<sup>+</sup> cells and their neuronal differentiation derivatives, and then, the functional recovery of rats was assessed with the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB scale). The spinal cord–contused Long Evans rats grafted with D12 and D18 neuronal progenitors resulted in significant recovery of hind limb function compared to control groups (P < 0.05). Xenotransplantation of pESCs-derived neuronal progenitors into the spinal cord–injured rat model reveals the therapeutic potential of ES cell transplantation in spinal injury treatment.

4.4. Autotransplantation of traceable pESC lines in porcine periodontal furcation defects model

For evaluation of the therapeutic potential of ESCs in the cell-based treatment for periodontal furcation defects, the pESC/GFP<sup>+</sup> cells were transplanted to the experimental periodontitis induced in the buccal furcations of bilateral mandibular second premolars of four 5-month-old female Lan-yu miniature pigs (http://minipigs.angrin.thri.gov.tw/English/). After 3 months of healing, the immunohistochemical staining demonstrated that transplanted pESC/GFP<sup>+</sup> cells differentiated to new periodontal ligament and cementum in the test sites. No obvious teratoma or rejection was seen in any examined animals. The treatment group had significantly better (P < 0.05) clinical parameters regarding clinical probing depth and attachment level, demonstrating the therapeutic potential of using ES cells to improve the regeneration of periodontal furcation defects [36].

5. Derivation and application of porcine iPSC lines from somatic cells

The establishment of hESCs promised a wide application potential in the development of biomedical research. However, the ethical concerns with the creation of hESCs, the safety issues about oncogenic risk, and immunogenicity of hESCs after transplantation preclude the clinical applications of hESCs. In 2006, the successful result of Takahashi and Yamanaka demonstrated that mouse embryonic fibroblasts can be reprogrammed to a pluripotent state comparably to that of ESCs termed iPSCs [2]. Furthermore, the iPSCs generated by the expression of transcription factors Oct-4, Klf4, Sox2, and Myc (OKSM or Yamanaka...
 factors) could also generate germline chimeras in mice showing that the iPSCs and ESCs had almost identical differentiation properties [37,38]. The later successes of creation of iPSCs from human somatic cells [39,40] open a new avenue to the promise of the development in stem cell-based therapy. Thereafter, reprogramming procedures developed for mouse and human cells have also been adapted, and the field of iPSCs has been widely spread in various species, including pigs [15,41–48].

As an alternative of pESCs, porcine iPSCs (piPSCs) might have an excellent opportunity to benefit the development of retailed cell-based therapeutic strategy in humans using the reprogrammed cells from the original donor of somatic cells. Technically, production of piPSCs is much easier than that of pESCs. Since 2009, several groups have created piPSCs from various cellular origins, culture conditions, and reprogramming protocols [41] (Table 4). Only few piPSC lines created chemically by using small molecule inhibitors possessed naive-like iPSCs properties as dorm-like colony morphology and LIF dependency for maintaining pluripotency [17,42]. Most of the resultant piPSCs are adapted, and the developed for mouse and human cells have also been adapted, and the field of iPSCs has been widely spread in various species, including pigs [15,41–48].

Table 4

<table>
<thead>
<tr>
<th>Cell sources</th>
<th>Reprogram method</th>
<th>In vitro differentiation</th>
<th>In vivo differentiation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary ear fibroblasts</td>
<td>DOX-inducible lentivirus (human OKSM)</td>
<td>EB</td>
<td>Teratoma</td>
<td>[48]</td>
</tr>
<tr>
<td>Primary bone marrow (10 wk)</td>
<td>Retrovirus (human/mouse OKSM)</td>
<td>None</td>
<td>Teratoma</td>
<td>[46]</td>
</tr>
<tr>
<td>Embryonic fibroblasts (Day 37)</td>
<td>Lentivirus (human OKSM)</td>
<td>EB</td>
<td>Teratoma</td>
<td>[41]</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>Lentivirus (human OKSM)</td>
<td>EB</td>
<td>Germline chimera</td>
<td>[43]</td>
</tr>
<tr>
<td>Embryonic fibroblasts</td>
<td>Episomal plasmids (human OKSM)</td>
<td>EB</td>
<td>Teratoma</td>
<td>[42]</td>
</tr>
<tr>
<td>Embryonic fibroblasts</td>
<td>Retrovirus (human OKSM)</td>
<td>EB</td>
<td>Chimeras</td>
<td>[44]</td>
</tr>
<tr>
<td>Embryonic fibroblasts</td>
<td>Transfection (mouse OKSM)</td>
<td>EB</td>
<td>None</td>
<td>[15]</td>
</tr>
<tr>
<td>Ear fibroblasts with hrGFP</td>
<td>Lentivirus (human OKSM)</td>
<td>EB</td>
<td>Teratoma</td>
<td>[47]</td>
</tr>
</tbody>
</table>

Abbreviations: DOX, doxycycline; hrGFP, humanized recombinant GFP; OKSM, Oct-4, Klf4, Sox2, and Myc; piPSCs, porcine-induced pluripotent stem cells.

As an alternative of pESCs, porcine iPSCs (piPSCs) might have an excellent opportunity to benefit the development of retailed cell-based therapeutic strategy in humans using the reprogrammed cells from the original donor of somatic cells. Technically, production of piPSCs is much easier than that of pESCs. Since 2009, several groups have created piPSCs from various cellular origins, culture conditions, and reprogramming protocols [41] (Table 4). Only few piPSC lines created chemically by using small molecule inhibitors possessed naive-like iPSCs properties as dorm-like colony morphology and LIF dependency for maintaining pluripotency [17,42]. Most of the resultant piPSCs are classified as primed iPSCs as hESCs or mouse EpiSCs according to their colony morphology, gene expression profiles, FGF-dependency, limited capacity of chimera formation, and germline transmission. To date, only one study resulted in germ-line transmission after blastocyst injection; however, the efficiency was very low (2/34), and none of these chimeric piglets survived for 3 days [43]. These results might imply that these piPSCs are not true naive pluripotent stem cells as that of the naive mESCs. Besides, the possible epigenetic problems from the incomplete reprogramming and the lack of full silencing of the exogenous reprogramming factors could not be excluded [1,42–44].

Although iPSCs have been successfully generated in many species, there are still issues to be overcome before they can be safely used for clinical application. The risk of oncogenes used for reprogramming such as Klf4 and c-Myc has been avoided by the development of nonintegrating vectors, small molecules, messenger RNA, minicircle DNA, episomal vectors, recombinant proteins, and transposons that integrate into the host genome but can be subsequently excised with low reprogramming efficiency [1,41–48]. Moreover, the purported benefit and high expectation on free of immunogenicity in iPSCs have been under suspicion recently. Zhao et al. [45] demonstrated that iPSCs were immune-rejected or immunogenic in an autologous transplantation model when those cells were reprogrammed by retrovirus or episomal transfection, respectively. It is not clear whether the similar problems existed in human and porcine iPSCs. Further research is needed in multiple lines and species to conclusively demonstrate safety of iPSCs in term of oncogenicity and immunogenicity before they can be used in regenerative medicine.

6. Conclusions

Pluripotent stem cells such as putative ES and IPS cell lines have been successfully established in pigs. These cells survived repeated subcultures and cryopreservation, maintained typical morphology of ES cells, epithelial-like colonies and normal karyotype, and continued expressing pluripotent cell markers. They could form embryoid bodies and differentiate into cell lineages representative to three germ layers spontaneously or after artificial induction. Piglets of somatic but not germline chimera can be obtained by blastocyst injection with pESCs. However, the gold standard for ES cells has not been achieved in the established pESCs and piPSCs because of the incompetency of germline transmission. Nevertheless, therapeutic implications of these cells have been demonstrated in modeling Parkinson’s disease and spinal cord injuries in rats, as well as autotransplantation to porcine model for periodontitis. Owing to the limitations in performing research with pluripotent stem cells directly in humans, there is a great demand for humanized animals, such as pigs, to promote related studies in a safe and efficient manner. The pig shares many similarities with humans in anatomic, immunologic, and physiological characteristics, even the characteristics and behaviors of pluripotent stem cell lines. Therefore, further in-depth study of porcine pluripotent stem cells would immediately benefit the biomedical research and regenerative medicine in humans.

References


