Porcine pluripotent stem cells: progress, challenges and prospects

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Abstract Pluripotent stem cells (PSCs) are characterized by their capacity for high self-renewal and multiple differentiation potential and include embryonic stem cells, embryonic germ cells and induced PSCs. PSCs provide a very suitable model for the studies of human diseases, drugs screening, regenerative medicine and developmental biology research. Pigs are considered as an ideal model for preclinical development of human xenotransplantation, therapeutic approaches and regenerative medicine because of their size and physiological similarity to humans. However, lack of knowledge about the derivation, characterization and pluripotency mechanisms of porcine PSCs hinders progress in these biotechnologies. In this review, we discuss the latest progress on porcine PSCs generation, evaluation criteria for pluripotency, the scientific and technical questions arising from these studies. We also introduce our perspectives on porcine PSC research, in the hope of providing new ideas for generating naive porcine PSCs and animal breeding.

Keywords embryonic germ cells, embryonic stem cells, induced pluripotent stem cells, pigs, pluripotent stem cells

1 Introduction

Pluripotent stem cells (PSCs) are characterized by their developmental competence to give rise to properties of all three germ layers, including germ cells, but excluding the extra-embryonic tissues. Three types of PSCs have been reported: embryonic stem cells (ESCs), which are derived from the inner cell mass (ICM) of blastocysts and conform to the general standards of pluripotency; embryonic germ cells (EGCs) from primordial germ cells and induced PSCs (iPSCs), which are derived from differentiated cells with forced expression of selected transcription factors. Since PSCs have capabilities of in vitro self-renewal and competence for development of three germ layers both in vivo and in vitro, they are regarded as a powerful model for functional genomics research, and hold great potential in cell transplantation pharmacy, human genetic disease treatment and livestock breeding.

Pigs are important farm animals and also one of the best candidates for human disease models and as xenotransplantation donors because of their well-known similarities to humans in organ size as well as morphology and physiology. The establishment of porcine PSC lines can facilitate the application of pig cells in both biomedical and agricultural fields, especially for the evaluation of efficiency and safety of stem cell related therapies in human. Although great progress on mouse and human PSCs has been achieved in recent decades, research on PSCs in pigs and other large animals has encountered huge difficulties, and there are no porcine PSC lines available that fulfill all the characteristics of mouse ESCs, especially the germline chimeras. This situation indicates that the exploration of PSCs from pig and other large animals has significant theoretical importance for understanding the specific regulation of pluripotency in different species.

In this review, we summarize progress on the derivation of pig ESCs, EGCs and iPSCs, discuss the methods for evaluation of the pluripotency of porcine PSCs, analyze the challenges to the generation of naive porcine PSCs and provide a perspective for future studies.
2 Derivation of porcine embryonic stem cells

Great effort has been expended to derive porcine ESCs (pESCs) since the first report by Evans and Kaufman\cite{1} in 1990 due to their promising potential in both biomedical and agricultural fields. Although pESC-like cell lines that satisfy general pluripotency criteria have been reported, including the capability of extensive self-renewal, expression of alkaline phosphatase (AP), \textit{OCT4} (also named \textit{POU5F1}) and a panel of other markers, as well as the ability to differentiate into derivatives of all three germ layers \textit{in vitro} and in teratomas, there have been few reports of the development of these pESC-like cells into germline chimeras. Furthermore, according to the definition of mouse ESCs, pESCs have not been successfully produced that show the complete characteristics of the naive state.

ESCs have been considered to arise as a result of a selective adaptation process to the culture conditions and to be an artifact rather than a physiological cell type\cite{2} and considerably greater attention needs to be paid to the optimization of \textit{in vitro} culture conditions to acquire pESCs.

2.1 Embryo stage for establishing pESCs

Embryo development stage is an important factor for ESCs derivation. By comparison between human and mouse embryos, the porcine embryo is unique due to its extended preimplantation development. Studies on preimplantation embryo development indicate that the compaction of blastomeres happens around days 4–5 post \textit{in vitro} fertilization or the last \textit{in vivo} insemination, and blastocysts develop with clearly differentiated ICM and trophectoderm (TE) around days 5–6\cite{3}. \textit{OCT4}, one of the key transcription factors governing pluripotency, is expressed in both ICM and TE of porcine embryos, and this expression pattern clearly differs from that of mouse embryos\cite{4}. GATA6\cite{4} and Vimentin\cite{5} begin to be expressed in some of the cells in ICM on days 8 and 9, respectively, which indicates the initiation of the further differentiation of ICM. The species-specific developmental pattern of porcine preimplantation embryos makes it difficult to identify the appropriate developmental stage to use to derive pESCs from the approaches used to produce mouse and human ESCs.

Porcine preimplantation embryos at different developmental stages have been used to establish pESCs, but the results from different researchers are not consistent. Days 7–9 blastocysts were used by Evans et al.\cite{6} in the first reported attempts to derive pESCs, and ESC-like cell lines were derived from these blastocysts. In contrast with this result, ESC-like cell lines or ESC-like colonies that could survive for more than a few passages have generally been derived from expanding or hatched blastocysts, although the day ranges for collection of the blastocysts varied from days 5–9\cite{7–10}. Pre-compaction embryos and morulae have also been used to derive pESCs, but the attachment rates of these embryos were low and none of the embryos began outgrowth\cite{11}. Moreover, it has been reported that hatched blastocysts and elongating blastocysts around days 10–11 have been used to derive pESCs\cite{12} and ESC-like colonies could be derived from 50% of the isolated embryonic discs and could be maintained in culture for at least 8 weeks, while only a few ESC-like colonies were derived from days 5–6 blastocysts in this study. Chen et al.\cite{8} collected days 6–8 porcine embryos for derivation of pESCs. They classified the collected embryos into morulae, early blastocysts, expanding blastocysts, early hatched blastocysts, intermediate hatched blastocysts and late hatched blastocysts according to morphology, and found two types of ESC-like colonies formed from early hatched blastocysts, which were called type A and B. Only type B colonies were isolated from ICM of late hatched blastocysts, and no colonies survived more than five passages in the morulae group. One cell line derived from type A colonies generated a chimeric piglet with overt pigmentation chimerism, although this chimeric piglet did not show germline chimerism, which was suggested by the limited offspring. In contrast with these results, Xue et al.\cite{10} reported ESC-like cell lines derived from expanding to early hatched embryos. The reported ESC-like cells could survive more than 75 passages and retained their pluripotent characteristics and normal karyotype after transfection with fluorescent protein gene DsRed. These ESC-like cells could contribute to the chimeric development of both ICM and TE in expanding blastocysts and chimeric development of both fetus and placenta to day 50 gestation.

It is difficult to ascertain which developmental stages of porcine embryos are appropriate to use to derive pESCs because there is a lack of consistency in these reports. The post estrus and insemination dates for \textit{in vivo} embryos or post fertilization for \textit{in vitro} embryos are not an accurate criterion to define the developmental stages because the developmental speed of embryos from the same \textit{in vivo} ovulation or \textit{in vitro} fertilization is different and results in embryos with different morphologies at the same time point. Embryo morphology is an alternative criterion for determination of the developmental stages and it has advantages for comparing embryos employed in different studies. According to the morphology criteria, expanding and early hatched blastocysts might be considered first for derivation of pESCs because most of the reported ESC-like cells lines were derived from these two developmental stages regardless of the day of development\cite{7–10}. Also, this suggestion is supported by the results on expression of genes associated with pluripotency in preimplantation embryos\cite{4,13–16}.

Other considerations related to selection of embryos for derivation of pESCs include the origin of the embryos and
the methods for seeding. Progress on these two topics has been comprehensively reviewed\cite{17,18}, so they will not be discussed in this review. The relationship between stages of embryo development and derivation of PSC in mice, humans and pigs are summarized in Fig. 1.

2.2 Development of culture conditions

ESCs derived from embryos are not identical to the pluripotent blastomeres, ICM cells or epiblast cells. To a certain extent, the biological characteristics of ESCs depend on culture conditions. The conversion of mouse EpiSCs into ESCs in 2i/LIF culture system and the conversion of mouse ESCs to expanded PSCs (EPSC), or vice versa, provides clear evidence for the importance of culture conditions\cite{19–22}. Compared to the large number of studies examining appropriate culture conditions for mouse and human ESCs, minimal data are available for ESCs culture conditions of domestic animals\cite{23}. In the early years, ESC cultural conditions were developed mainly for mouse ESCs\cite[6,24,25] and then human ESC culture systems were carefully studied\cite[10,26]. Generally, the culture medium consists of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2-mercaptoethanol, L-glutamine, antibiotics, nucleosides, non-essential amino acids, fetal bovine serum (FBS) and different cytokines, and alternative components. For the basic medium, variations of DMEM from high glucose to low glucose\cite[27,28], α-MEM\cite[29,30] and KO-DMEM\cite[10,31] have been examined. The results from different studies are not consistent, but pESC-like cell lines have predominantly been derived from DMEM medium without consideration of glucose concentrations. FBS has an important but contradictory role in the derivation of pESCs. It is believed that FBS contributes positively to the attachment of embryos to feeder layers and the derivation of outgrowths, but it is also the main source of potential differentiating factors in ESC culture. Defined serum-free replacement (KOSR) was introduced to replace FBS for further improved ESC cultures, and there are increasing reports on the use of KOSR for pESC derivation\cite[10,27,34].

The feeder layer is a fundamental feature for ESC cultures. It is generally believed that the feeder layer provides an attachment matrix for the seeded embryos, the subsequent outgrowth and passaged cells, cytokines such as leukemia inhibitory factor (LIF) to stimulate cell proliferation and inhibit cell differentiation. The wide use of feeder layer conditioned medium indicates the great importance of feeder layers for the release of specific factors and the attachment ability of the matrix and the evidence indicates that little success could be achieved from feeder-layer-free culture systems\cite[32,35]. Although there are reports about the effects of different feeder-layer cells on pESCs derivation\cite[7,9,12,24], STO cell lines or

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**Fig. 1** Derivation of pluripotent stem cells from different embryo development stage.
mouse embryonic fibroblasts are normally used as feeder cell layers because they are easy to prepare and have comparatively few variations in biological characteristics (Table 1).

LIF and basic fibroblast growth factor (bFGF) are two cytokines that have been clearly demonstrated to be crucial for regulating networks of mouse and human ESCs, respectively. There are still debates concerning the exact function of LIF and bFGF in pESC cultures, although results from different reports indicate that both LIF and bFGF can exert a positive influence on the survival, proliferation and self-renewal of pESCs [9, 26, 31, 34]. The function of LIF and bFGF on the pESC-like cell culture has been examined and the results showed that LIF is dispensable for cell survival, and bFGF is necessary for inhibition of cell differentiation [10]. Some other cytokines have also been examined for their potential benefit for the derivation of pESCs, such as transforming growth factor-beta (TGF-β) [43], epidermal growth factor [43] and stem cell factor (SCF) [43], however, these factors did not have any obvious effects on porcine stem cells.

Great attention is paid to small molecules in the study of pESC derivation. Some small molecules were used to derive iPSCs from precompaction differentiated somatic cells without introducing transcription factors into cells [21, 44] or EPSCs from precompaction rat embryos [22]. Reports on these small molecules have included glycogen synthase kinase 3 beta inhibitor (GSK3β) [45] and KLF4 substitute Kenpaullone (KP) [46], and CHIR99021 (CH), Erk signaling inhibitor PD184352 [47], BMP4 inhibitor dorsomorphin (DMS) [48], inhibition of cell differentiation [10]. Some other cytokines are dispensable for cell survival, and bFGF is necessary for inhibition of cell differentiation [10]. Some other cytokines have also been examined for their potential benefit for the derivation of pESCs, such as transforming growth factor-beta (TGF-β) [43], epidermal growth factor [43] and stem cell factor (SCF) [43], however, these factors did not have any obvious effects on porcine stem cells.

However, there are also contradictory reports on the effects of these small molecules when employed in pESC cultures [45, 47]. It is clear that small molecules have shown great potential for derivation of ESCs, and intensive screening is needed to achieve optimal concentrations and combinations of the small molecules to support authentic pESCs derivation.

3 Pluripotent stem cells derived from germlines

3.1 Derivation of pluripotent stem cells from primordial germ cells

Primordial germ cells (PGCs) are embryonic cells that migrate from the root of the allantois to the genital ridge, where they ultimately give rise to gametes [48]. PGCs do not belong to the stem cell population at any stage during embryonic development, but they can indefinitely proliferate under certain in vitro culture conditions and generate a PSC population. Using component defined culture systems, porcine PGCs derived from 24 to 28 dpc genital ridge can proliferate steadily and are known as embryonic germ cells (EGCs) [39, 49]. In 1992, it was first reported that mouse unipotent PGCs can be converted into EGCs [36, 50]. Subsequent research also demonstrated that EGCs could be established from human PGCs [39]. EGCs share several important characteristics with ESCs, including their morphology, pluripotency and capability of contributing to germline chimeras when injected into blastocysts [51, 52].

Unlike ESCs, EGCs are inseparable from the feeder cell type. Growth factors including LIF, bFGF and SCF are essential for the derivation of porcine EGCs [39]. PGCs could be reprogrammed into iEGCs using small molecules and transcription factors of OCT4 and C-MYC [53]. In addition, hypoxia induces reprogramming of PGCs by deregulating expression of OCT4 [54]. In human PGCs, the expression of endogenous KLF4 and C-MYC is similar to EGCs, but the expression levels of SOX2 and OCT4 are lower than ESCs. Thus, the reprogramming of PGCs into iPSCs can occur only by employing two transcription factors, SOX2 and OCT4 [55]. Only one study has found that

Table 1  Pluripotent stem cells derived from primordial germ cells

| Species | Cell source | Culture system | Differentiation potential | Reference |
|---------|-------------|----------------|---------------------------|-----------|
| Mouse   | 8.5 dpc PGCs | STO feeder layer + SCF, LIF, bFGF | Chimera | [36] |
|         | 7.0 dpc PGCs | STO feeder layer + SCF, LIF, bFGF | ES-like cells, teratomas | [37] |
|         | 11.5–13.5 dpc PGCs | MEF feeder layer + LIF, SB431542, Kenpaullone | Chimera | [38] |
| Human   | 5–9 weeks PGCs | STO feeder layer + LIF, bFGF, Forskolin | EG-like cells, all three germ layer cells | [39] |
|         | 106 dpc PGCs | DMEM + 10% NBS | | [40] |
|         | 4–13 week PGCs | Knockout DMEM + 20 KSR + LIF + bFGF + Forskolin | ES-like cells, all three germ layer cells | | |
| Porcine | E25–27 PGCs  | STO feeder + SCF + bFGF + LIF | ND | [41] |
|         | E25–27 PGCs  | Using a growth-factor-defined culture system supplemented bFGF | Chimera | [42] |

Note: ND, not determined.
a porcine iPSC line could be established by transfecting six human reprogramming factors (OCT4, SOX2, NANOG, KLF4, LIN28 and C-MYC) which possessed the ability to produce chimeric offspring (Table 1). However, there have been no subsequent reports on the production of porcine chimeras. Most iPSC lines fulfilled the criteria of pluripotency, but the cells could not contribute to chimeras or generate cloned piglets.

### 3.2 Derivation of pluripotent stem cells from testis

Spermatogonia stem cells (SSCs) are the only type of stem cells in the body that transmits genetic information to offspring. They can continuously generate differentiating spermatogonia. Generally, it is considered that SSCs are equal to A-single (As) cells. However, the number of SSCs qualified by transplantation is only 0.002%, which indicates that not all As spermatogonia function as SSCs. To date, SSCs are the only adult stem cells showing significant OCT4 expression, which is a specific marker for pluripotent and germ cells. The role of OCT4 in germ cell development was elucidated by the demonstration that knockout of this gene led to apoptosis. During the SSCs culture process, germ line stem cell (GSC) clones could be observed, which were typically grape-like clusters. (Table 2). Using neonatal Sertoli cells as the feeder and DMEM/F-12 culture medium supplemented with 10% KSR and four cytokines, the undifferentiated spermatogonia could proliferate in vitro for at least 2 months without loss of stemness. Our preliminary study demonstrated that Peptide-coating 2D is beneficial to the long-term culture of porcine male germ cell-derived clones (pGDCs), and lipid seems effective in prolonging the culture time of pGDCs in vitro (unpublished data of Jinlian Hua research group). In addition, germine-derived PSCs (gPSCs) can be observed with a low frequency under certain culture conditions for both mouse and human cells, being morphologically similar to mouse ESCs. These ESC-like cells were phenotypically similar to ESCs/GSCs except for their genomic imprinting pattern. Kossack et al. found that conversion of GSCs into gPSCs did not alter their imprinting status. gPSCs did not result in the birth of pups after tetraploid complementation (0/82), which was likely due to the imprinting status of gPSCs, whose DMRs of H19 and ICRs of Igf2r are maintained as androgenetic patterns. This result was also supported by a report showing that DNA methylation of imprinted genes is critical for fetal development. It is important to note that paternal imprinting patterns of H19 and Igf2r in gPSCs are not altered, even after 20 passages. Unlike PSCs from the testis of newborns, gPSCs from adult GSCs still maintained an androgenetic pattern in DMRs of H19. Furthermore, these ESC-like cells formed chimeras when injected into blastocysts. All these studies support the notion that, compared to somatic cells, germ cells have the distinct potential to be converted into ESC-like stages without the introduction of exogenous reprogramming factors. The molecular mechanisms underlying the natural shift from a unipotent to a completely pluripotent cell during the establishment of mouse ESC-like cells from SSCs are not yet completely understood. However, in cultures with growth factors, the cell density of SSCs during culture, the time period after initiation of the culture, and the length of the culture might all be key factors in the transition process. Furthermore, this phenomenon seems to be age-dependent. Several studies of long-term cultivation for SSCs failed to prevent this spontaneous shift of SSCs to pluripotent ESC-like cells.

### 3.3 Derivation of pluripotent stem cells from ovary

Female GSCs (FGSCs) isolation from neonatal and adult mice and long-term culture have attracted considerable interest in stem cell biology. FGSCs have been isolated independently by at least two research groups and from a number of species (human, mouse and rat). Wang et al. found that stably proliferating FGSCs from neonatal or prepubertal mouse ovaries can be converted to female ESC-like cells within one month under ESCs culture conditions. These cells exhibited ESC-like characteristics such as ESCs morphology, expression of pluripotency markers and had a normal karyotype. Also, they could differentiate into the three germ layers in vitro, form teratomas in vivo and contribute to chimeras and the germline (Table 3). Dissected cells from porcine thecal layers maintained similar characteristics to mouse FGSCs.

| Table 2 | Pluripotent stem cells derived from testis |
|---------|------------------------------------------|
| Species | Cell source | Culture system | Differentiation potential | Reference |
| Mouse | Neonatal testis | MEF feeder layer + standard ESCs culture conditions | Chimera | [68] |
| Human | Testicular cells | hESCs culture conditions | All three germ layers, no teratoma | [69] |
| Porcine | Neonatal testicular cells | DMEM/F-12 + 10% KSR and four cytokines | Colonize in vivo and differentiate | [67] |

| Table 3 | Pluripotent stem cells derived from mouse ovary |
|---------|-----------------------------------------------|
| Cell source | Culture system | Differentiation potential | Reference |
| Neonatal mouse ovary cells | ESCs culture conditions | All three germ layers, Teratomas, Chimera (dead) | [85] |
and ESCs over 4 months of in vitro culture. At present, however, controversy remains over the biological significance of these cells.

## 4 Induced pluripotent stem cells

### 4.1 Overview and progress on induced pluripotent stem cells

iPSCs with a gene expression profile and developmental potential similar to embryonic stem cells can be generated from mouse somatic cells using a cocktail of four transcription factors [86], and the four factors OCT4, SOX2, KLF4 and C-MYC, called Yamanaka factors [87]. The generation of iPSCs with OSKM has been described as direct reprogramming in contrast to reprogramming via nuclear transfer. Chimeric mice produced by microinjection of iPSCs into a blastocysts [88] and iPSC-mouse generated by tetraploid complementation, suggest that the pluripotency of these cells is equivalent to that of ESCs cells [89].

### 4.2 Progress on inducing pluripotent stem cells of pig and other large animals

Lines of iPSCs have been generated from some domesticated ungulates, such as sheep [90,91], pigs [87,92,93] and cattle [94,95]. Porcine iPSCs have been produced in many laboratories using various induction methods and show pluripotency to some degree [59,93,96,97]. Porcine iPSCs could pass the test of germline chimera production at the molecular genotyping levels using PCR [42,98], but stable molecular genotyping levels using PCR could pass the test of germline chimera production [99]. Until recently, most of iPSCs were generated through tetraploid embryo complementation. The generation of iPSCs with OSKM has been described over 4 months of in vitro culture. At present, however, controversy remains over the biological significance of these cells.

### 5 Criteria for evaluating pluripotency in porcine stem cells

It is important to establish good criteria to evaluate the pluripotency of porcine stem cells. Most evaluation criteria for porcine PSCs are based on those used for mouse PSCs. We summarize below the methods used to evaluate the pluripotency of porcine stem cells in recent decades.

#### 5.1 Morphological parameters

#### 5.1.1 Morphology of stem cells

Most porcine PSCs have an epithelial-like colony morphology (Tables 4–6), which is very similar to human ESCs or mouse epiblast stem cells (EpiSCs) [29,115,121,138,147,148]. They show a large, flat and round (polygonal in rare cases) shape with compact colonies and distinct borders, with relatively small diameters and a high nucleus-to-cytoplasm ratio, a single nucleus with multiple nucleoli, and are sensitive to trypsin. These cells grow more slowly and have limited capability to be integrated into host blastocysts [92,120,122,134,141,143]. Another type of porcine PSCs was shown to have a mouse ESCs morphology, i.e., small, not flat and with a compact, glistening, doom shaped appearance. These cells have a high nuclear-to-cytoplasmic ratio with short cell cycle interval, grow more vigorously and are capable of undergoing successful differentiation both in vivo and vitro, and are considered to have high developmental potential and show naive status of porcine PSCs [29,49]. In general, morphology of stem cell represents a basic indicator for pluripotency of
### Table 4  Characteristics of porcine embryonic stem cells

| Cell sources | Morphology | Pluripotency state | Colony formation time/number | Passage | Karyotype | Pluripotency factors | Surface markers | AP | Teratoma | Chimera | EB | Multilineage differentiation potency | Reference |
|--------------|------------|--------------------|-----------------------------|---------|-----------|---------------------|-----------------|----|----------|----------|----|-------------------------------|-----------|
| In vivo and in vitro embryos | ESC-like | 10% | p14 | Normal | OCT4, NANOG | SSEA-1 | – | ND | CP | – | + | + | [114] |
| EpiSC-like | 5–7 d | p > 41 | Normal | OCT4, NANOG | SSEA-1 | – | AP | – | ND | – | + | + | [29] |
| In vivo embryos | ESC-like | 1.6%–9.5% | p > 9 | Normal | NANO | – | AP (weak) | ND | ND | – | + | + | [115] |
| EpiSC-like | 5–7 d | p > 12 | Normal | OCT4, NANOG | — | SSEA-1 | – | ND | ND | – | + | + | [117] |
| In vitro embryos | ESC-like | 5–8 d | p > 48 | Normal | OCT4, NANOG | — | SSEA-1 | – | ND | ND | – | + | + | [118] |
| EpiSC-like | 5.1% | p > 100 | Normal | OCT4, NANOG | SSEA-1, SSEA-4 | AP | – | ND | ND | ND | + | + | [45] |
| | 8–13 d | p > 15 | Normal | OCT4, NANO | SSEA-1, SSEA-4 | AP | – | ND | CB | + | + | [119] |
| | ND | p > 50 | Normal | OCT4, NANO, SOX2, NODAL | SSEA-1, SSEA-4 (strong), SSEA-4 (weak) | AP | – | ND | ND | ND | + | + | [46] |
| ND | 39% | p < 52 | Normal | OCT3, OCT4, NANO | SSEA-4 | AP | + | ND | + | + | + | [120] |
| EpiSC-like | 5–8 d, >26.2% | p > 25 | Normal | OCT4, NANO, SOX2, REX-1 | SSEA-4 | AP | – | ND | + | + | + | [121] |
| | 17.6% | ND | ND | OCT4, SOX2, NANO | SSEA-4 | AP | – | ND | + | + | + | [122] |
| | 13–16 d | p > 75 | Normal | OCT4, SOX2, NANO | SSEA-4 | AP | – | CP | + | + | + | [10] |
| | 10.7% | p > 90 | Normal | OCT4, SOX2, NANO | SSEA-4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | + | [123] |
| | 9 d | p > 36 | Normal | OCT4, SOX2, NANO | SSEA-4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | + | [124] |
| ND | < 29% | ND | ND | OCT4, NANO, SOX2, C-MYC | SSEA-4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | + | [125] |

Note: AP, alkaline phosphatase; EB, embryoid body; ND, not determined; CP, chimeras piglets; CB, chimeric blastocysts.
### Table 5  Characteristics of porcine induced pluripotent stem cells

| Cell sources                  | Morphology | Pluripotency state | Colony formation time/ rate | Passage | Karyotype | Pluripotency factors | Surface markers | AP | Teratoma | Chimera | EB | Multilineage differentiation potency | Reference |
|-------------------------------|------------|--------------------|-----------------------------|---------|-----------|--------------------|-----------------|----|----------|---------|----|-------------------------------|----------|
| Porcine fetal fibroblasts    | ESC-like   | 14 d               | p > 40                      | Normal  | OCT4, SOX2, NANO, LIN28, OCT3, C-MYC | SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | AP | +         | CP      |     | +                             | [97]     |
|                               |            | 6–8 d              | p > 30                      | Normal  | OCT4, SOX2, NANO, TERT | SSEA-4, SSEA-1, TRA-1-60, TRA-1-81 | AP | +         | CB      |     | +                             | [112]    |
|                               |            | 10–12 d            | ND                          | Normal  | REX1, OCT4, SOX2, REX1, NANO | SSEA4 | AP | +         | ND      | +   | +                             | [111]    |
| EpiSC-like                    |            | 5 d                | ND                          | Normal  | OCT4, C-MYC | SSEA4, TRA-1-60 | AP | +         | ND      | +   | +                             | [126]    |
|                               |            | 22 d               | p < 20                      | Abnormal | OCT4, NANO, SOX2, KLF4 | SSEA-1 | AP | +         | ND      |     | +                             | [59]     |
|                               |            | 8–10 d             | p > 25                      | Normal  | NANO, REX1, LIN28, SOX2, OCT3 | SSEA-4 | AP | +         | ND      | ND  | ND                            | [127]    |
|                               |            | 12 d               | p > 90                      | ND      | OCT4, NANO, ZFP42, UTF1, EpCAM, ESRRB | SSEA-1 | AP | -         | -       | +   | -                             | [128]    |
|                               |            | 14–20 d            | p > 20                      | ND      | OCT4, SOX2, NANO, KLF4, C-MYC, LIN28, DPPA2 | SSEA-1, SSEA-4 | AP | +         | CP      | +   | +                             | [66]     |
|                               |            | 10 d               | p > 35                      | ND      | SOX2, OCT4, KLF4, NANO, REX1, TDGF | SSEA-4 | AP | -         | ND      | +   | +                             | [129]    |
|                               |            | ND                 | p > 30                      | Normal  | OCT4, SOX2 | SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 | AP | +         | CB      |     | +                             | [130]    |
|                               |            | 6–7 d              | ND                          | Normal  | OCT4, SOX2, NANO, REX1, KLF4 | SSEA-1 | AP | ND        | ND      | +   | +                             | [34]     |
|                               |            | 7 d                | p > 30                      | Normal  | OCT4, SOX2, NANO, REX1, TBX3, NR5A2 |           | AP | +         | ND      |     | +                             | [131]    |
|                               |            | 8 d                | p > 50                      | Normal  | OCT4, SOX2, NANO | SSEA1, SSEA4, TRA-1-81 (weak), TRA-1-60 (weak) | AP | ND        | ND      | +   | +                             | [132]    |
| Cell sources | Morphology | Pluripotency state | Colony formation time/ rate | Passage | Karyotype | Pluripotency markers | Surface markers | Pluripotency factors | Differentiation in vivo | Differentiation in vitro | Reference |
|--------------|------------|--------------------|-----------------------------|---------|-----------|--------------------|-----------------|----------------------|------------------------|------------------------|-----------|
| Ear fibroblasts | ESC-like | 7 d | p>20 | Normal | OCT4, SOX2, NANOG, REX1 | – | – | + | ND | ND | + | [133] |
| | | 18 d | p76 | Normal | OCT4, NANOG, SOX2, KLF4 | SSEA-1 | AP | + | ND | + | + | [133] |
| | | ND | ND | Normal | – | – | AP | – | ND | + | + | [92] |
| | | 8 d | p>30 | Normal | OCT4, SOX2, NANOG, LIN28 | SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | [134] |
| | | 9 d | ND | Normal | NANOG | SSEA-4, TRA-1-60 | AP | + | ND | + | + | [135] |
| | | 11 d | p>25 | Abnormal | SOX2, OCT4, NANOG, LIN28, REX1, CDH1, DNMT | SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 | AP | ND | ND | + | + | [136] |
| | | 7 d | p>41 | Normal | OCT3, OCT4, NANOG, SOX2, REX1, CDH1 | SSEA3, SSEA4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | [137] |
| Adipose tissue | ESC-like | 8 d | p>30 | Normal | OCT4, SOX2, NANOG, LIN28, ESRRB, DPPA5, UTF1 | SSEA3, SSEA4 | AP | + | ND | + | + | [138] |
| Adipose stromal cells | EpiSC-like | 15 d | p50 | Normal | NANOG, OCT4, SOX2, KLF4 | SSEA-1, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | [139] |
| Testicular fibroblasts | EpiSC-like | 10 d | 12 month | Normal | OCT4, SAHi4, SOX2, NANOG, LIN28, CDH1 | SSEA4 | AP | – | ND | + | + | [140] |
| Dermal fibroblasts | ND | 7 d | p>22 | Normal | SOX2, OCT4, NANOG | SSEA4, TRA-1-60, TRA-1-81 | AP | ND | ND | + | + | [42] |

Note: AP, alkaline phosphatase; EB, embryoid body; CP, chimeras piglets; CB, chimeric blastocysts; ND, not determined.
porcine stem cells. However, pPSCs with a hESC-like morphology were also reported to contribute to chimera formation [10].

5.1.2 Colony formation and maintenance of pluripotency

Authentic ESCs are able to self-renew and proliferate continuously in vitro with undifferentiated characteristics. Naive-state stem cells, like mouse ESCs, can be propagated after dissociation to single cells, however, this same treatment can rapidly damage porcine PSCs with colonies that need to be detached from the feeder layer and passaged mechanically. Therefore, colony formation rate and doubling time are important indicators of pluripotency in porcine PSCs. In fact, most studies focus on the primary colony formation time (7–10 d after being plated onto the feeder layer) or rate (percentage of established porcine PSCs from blastocysts) rather than the date of colony formation and doubling time of established pESC and EGC lines [45,46]. This gap in studies of porcine PSCs deserves serious attention because fast and steady proliferation and passage are important characters of PSCs.

5.1.3 Karyotype analysis

Karyotype analysis is important because PSCs, including porcine PSCs, with abnormal karyotype cannot be used for research. Giemsa banding is widely used for porcine PSCs because this method can produce a visible karyotype by staining condensed chromosomes. Most porcine PSCs are normal in karyotype (38 chromosomes), but a few porcine iPSCs have been found with abnormal chromosome numbers and karyotypic instability which occurred with increased numbers of passages [135]. Overall, considering the safety and clinical application of piPSCs, karyotype analysis might be an indispensable assay before clinical studies.

5.2 Pluripotency markers

5.2.1 Pluripotency factors

OCT4 is considered to be key for pluripotency because it is expressed specifically in the ICM of blastocyst and ESCs of mice and humans, and its expression ceases in the subsequently differentiated cells and tissues [15]. However, the expression pattern and regulation mechanism of OCT4 in porcine embryos or PSCs are quite different from those in mice [149,150]. The pluripotency factors, including of OCT4, REX1 and KLF4, are still the main markers widely used in evaluating pluripotency of porcine PSCs (Tables 4–6). In recent years, researchers have begun to realize the limitation of these factors for porcine PSCs and

### Table 6 Characteristics of porcine embryonic germ cells

| Cell sources | Morphology | Pluripotency state | Colony formation time/rate | Passage | Karyotype | Pluripotency factors | Surface markers | Differentiation in vivo | Differentiation in vitro | Reference |
|--------------|------------|--------------------|---------------------------|---------|-----------|---------------------|------------------|------------------------|------------------------|-----------|
| Fetuses (days 17–30) | ESC-like | 7–10 d | p14 | ND | — | — | AP | ND | CP | + | ND | [41] |
| | | 8 d | p54 | Normal | OCT4 | — | — | SSEA-4, TRA-1-81, SSEA-1 | AP | ND | ND | + | + | [141] |
| | | 6–9 d | ND | ND | — | — | SSEA-1 | AP | ND | ND | + | Endoderm | [142] |
| | | 6–9 d | ND | ND | — | — | SSEA-1 | AP | ND | ND | + | ND | [142] |
| | | ND | p=35 | ND | OCT4, SOX2, NANOG, REX1, C-MYC | — | — | SSEA-4, TRA-1-60, TRA-1-81 | AP | + | ND | + | + | [143] |
| | | ND | p12 | Normal | — | — | AP | ND | CP | + | + | [48] |
| | | ND | p21–23 | ND | — | — | AP | — | CP | + | + | [144] |
| | | 5–7 d | p>20 | Normal | OCT4 (weak), SSEA-1, SSEA-3, SSEA-4 | — | — | AP | + | ND | + | + | [145] |
| | | ND | 5–8 d | ND | ND | — | — | AP | ND | ND | ND | ND | [146] |
| | | ND | 7–10 d | p>31 | Normal | — | — | SSEA-1 | AP | ND | CP | ND | ND | [49] |

Note: AP, alkaline phosphatase; EB, embryoid body; ND, not determined; CP, chimeras piglets.
additional pluripotent factors have been identified to evaluate pluripotency of porcine PSCs, such as TGFf1, CK18, STAT3, LIN28 and C-MYC.

5.2.2 Stem cell surface markers

Stem cell surface markers, such as stage-specific embryonic antigen SSEA-1 (mouse ESC-specific), SSEA-3 and TRA-1-81 (human ESCs-specific), have been used to characterize both mouse and human PSCs. However, ICM in porcine blastocysts cannot exclusively express the naive-state marker SSEA-1. Neither the blastocysts ICM in porcine blastocysts cannot exclusively express the naive-state marker SSEA-1. Neither the blastocysts (D5/6) nor the epiblasts (D9/10) express any human ESCs-surface markers, suggesting a precluded active state of pluripotency. It is generally considered that porcine PSCs expressing SSEA-1 have a high developmental potential, however, porcine iPSCs have been shown to maintain pluripotency for more than 50 passages and contribute to chimera formation without the expression of SSEA-1. Thus, it is difficult to distinguish which PSCs lines possess a higher pluripotency by detecting stem cell surface markers.

5.2.3 Alkaline phosphatase

Alkaline phosphatase (AP) is the first molecule demonstrated to be a reliable marker for undifferentiated ESCs in pig and some researchers use AP activity as the early detection marker during porcine iPSC establishment. Porcine iPSCs and ESCs with weak AP activity have limited developmental potential and insufficient differentiation ability, suggesting that AP activity is not only an indicator of stem cells but also a potential evaluation marker for pluripotency in porcine stem cells.

5.3 X-chromosome activation

The active X-chromosome (XaXa) state pluripotent cells are considered as naive-state stem cells and are capable of development into chimeras after injection into allogeneic embryos. Therefore, X-chromosome activation is considered to be a key indicator that a pluripotent cell is in a naive or primed state. For porcine iPSCs, three cell lines generated by the expression of transcription factors OCT4, KLF4 and C-MYC showed naive-like iPSC properties with an activated X-chromosome, as well as high embryonic chimera incorporation efficiency. In addition, Haraguchi and colleagues successfully established a unique cell line derived from ICM of porcine embryo that exhibited LIF-dependency but not bFGF-dependency, and could be considered as naive-state cells as mouse ESCs; however, the X-chromosome status in this cell line was XaXf.

XIST is a dominant regulator gene of XCI used to monitor the X-chromosome activation status. However, the regulatory mechanisms and expression patterns in pigs are complicated and not well defined. Also, many studies only determined PSCs to be in the naive state using the expression of XIST or H3K27me3 staining but did not test other capabilities, such as germline chimerism. Thus, application of evaluation X-chromosome activation in porcine PSCs will be limited until these problems have been solved.

5.4 Differentiation ability of stem cell in vivo and in vitro

5.4.1 Teratoma

The efficiency of teratoma formation in established porcine ESCs and EGCs lines was low for all teratoma detection reported for porcine PSC lines (Table 4; Table 6), which suggests that current culture systems cannot maintain the pluripotency of porcine PSCs. However, reprogramming somatic cells to a pluripotent state by the iPSC methods has demonstrated some level of success in teratoma formation (Table 5). The high teratoma formation rate found in porcine iPSCs may be because they can maintain their populations of undifferentiated cells by expressing oncogenes, which raises issues of the safety of application of piPSCs in clinical studies.

5.4.2 Chimera assay

High rates of production of chimeric blastocysts (up to 83.3%) can be obtained through aggregation of blastomeres from early stage embryos with different genetic background and most of the cells will then undergo a further differentiation leading to the formation of trophoblastic cells. However, the efficiency of blastomere aggregation with porcine PSCs is very low (Tables 4–6). Most groups would opt to use early embryo injection and select the early morula without compaction for injection because of the collapse of blastoecels. However, porcine PSCs have been reported to produce chimeric animals based on the coat color and microsatellite examination, but no researchers have been able to obtain chimeras by germine transmission.

5.4.3 Tetraploid complementation

Tetraploid (4N) complementation is considered as a key evaluation criterion for detection of pluripotency in stem cells. Full term development of embryos from stem cells injected into the tetraploid embryos would prove the pluripotency of cells. Unfortunately, no studies have obtained viable piglets by tetraploid complementation using porcine ESCs, EGCs and iPSCs (Tables 4–6), which could be explained by the primed pluripotent state of pPSCs used.
5.4.4 Embryoid body formation

Embryoid body formation (in vitro) is now routinely used to confirm pluripotency of stem cells. In porcine PSCs, embryoid bodies with a similar morphology to that described for mice were formed and they had three germ layer markers (endoderm, mesoderm, and ectoderm). However, the embryonic body generated from porcine EGCs is restricted to a simple embryoid body, not to a cystic form[142].

5.4.5 Multilineage differentiation potency

In porcine ESCs and EGCs, most of the cell lines can differentiate into three embryonic germ layer cell types (Table 4; Table 6). In addition, porcine iPSCs have been coaxed to differentiate into several neuronal lineages[140], cardio myocytes[132,134] and even hepatocytes[160]. Such abilities of porcine PSCs to form different tissues are critical and will of benefit for future development of preclinical studies, but there are still many issues to be resolved before these cells can be used in a safe and reproducible manner.

6 Challenges and prospects

PSCs can differentiate into various tissues and organs in animals, which means they have been widely used in clinical research and breeding, and naive mouse ESCs not only propagate steadily in vitro but also perform tetraploid compensation and germline transmission[161]. However, defects still exist with PSC lines derived from large animals. At present, the embryonic stem cells of existing large animals have encountered similar problems. PSCs cannot be propagated stably in vitro for the long-term and PSCs are unable to produce teratoma and chimera animals although they have certain differentiation abilities[10,35,118,162,163].

Relative to the embryonic stem cell lines, studies on iPSCs in large animals has made more progress, especially for pigs. However, there are still some difficulties, cell propagation is dependent on exogenous genes and cannot produce germline chimeric offspring[59,127,130,164,165]. These problems need to be solved urgently for the study of PSCs in large animals. In addition, the establishment of naive PSCs in porcine and some other kinds of livestock has significant valuable for biomedical research and animal breeding.

6.1 Generation of high quality pluripotent stem cells

Over the last three decades, substantial efforts have been made to generate PSCs from livestock. Reasons for the poor research outcomes are not entirely clear and key questions remain surrounding the basic biology of PSCs, for example: what is the best embryonic stage for isolating pluripotent cells from porcine embryo; what are the pluripotent markers and signaling pathways that regulate pluripotency in pigs, and what are the optimal culture conditions for sustaining long-term in vitro culture of pESC lines? The solution to these problems will facilitate the establishment of pESCs[29,111,166].

Unique regulatory signaling pathways may be associated with porcine ICM development. Mouse, human and pig embryos differ in co-expressed genes related to fatty acid metabolic processes, lipid metabolic processes, the biological aspects of the cytoplasm, nucleus, mitochondria and protein binding. Large numbers of lipids exist in the porcine early embryos and lipids supplement promotes mesenchymal–epithelial transition (MET) through the cAMP/PKA/CREB signal pathway and upregulates the E-cadherin expression during porcine somatic cell reprogramming. These findings may facilitate understanding of the lipid metabolism and lay the foundation for derivation of bona fide porcine embryonic stem cells[167]. Micro-RNAs play a very important role in regulating reprogramming, pluripotency and cell fate decisions and hPiSCs and mpiPSCs under different pluripotent states revealed significant differences in the miRNA signatures. These differentially expressed miRNAs may play important roles in pluripotent regulation in pigs.

Given the prospective advantages and current limitations, many researchers have recently emphasized the importance of establishing validated pESCs. It has been noted that defining the optimum stage of embryonic development for stem cell derivation and a clear understanding of key signaling pathways that regulate the pluripotency of pESCs, would be beneficial for obtaining stable pESC lines[17,168,169].

6.2 Porcine PSCs application in clinical medicine

Given that pigs have immunological and physiological similarities to humans, a porcine model provides the ideal non-primate system for clinical research. Porcine PSCs are important for modeling embryonic development and disease processes in biomedical research, and they are especially important for transplantation medicine, immunology and the study of the circulatory system[3,170].

Inactivation of porcine endogenous retroviruses opens the possibility of porcine-to-human xenotransplantation[171]. Porcine iPSCs can differentiate into photoreceptors which can integrate into the damaged swine neural retina, laying a foundation for retinal stem cell transplantation[172]. Porcine iPSC grafted into the myocardium can differentiate into vessel cells, which result in increased formation of new vessels in an infarcted heart. Direct intramyocardial injection of porcine iPSCs can improve left ventricular function in an immunosuppressed porcine AMI model[173]. Generation of functional hepatocytes
from porcine iPSCs is considered to be a promising therapy for patients with liver diseases\cite{174}, and robust neural differentiation from porcine iPSCs can fill the need for a powerful model to study autologous neural iPSCs therapies\cite{140}. Moreover, insulin produced by pigs is widely used to treat diabetes, pig heart valves have been transplanted for over 50 years and skin transplants have been applied to human burn victims for over 30 years\cite{175}. Transplantation of porcine hearts from $\alpha (1,3)$-galactosyltransferase knockout pigs has increased graft survival over previous methods\cite{176}. Thus, establishment of pESCs provide a useful tool for future cell transplantation and for studying disease mechanisms.

6.3 Porcine PSCs application in animal breeding

For agricultural purposes, PSCs can serve as a valuable genetic engineering tool to improve the generation of livestock through introduction of advantageous genes that are important economically and in disease resistance traits. The potential benefits of transgenic livestock have been discussed and reviewed by many researchers over the past 20 years, but for the most part the promise has remained unfulfilled due to insufficient research effort\cite{177}. As a substitute for pESCs, porcine iPSCs were used to generate cloned animals using somatic cell nuclear transfer\cite{178}, which is a valuable tool for generating transgenic animals. This suggests that application of PSCs in cloning might help reproduce a large number of endangered animals in the near future\cite{179}, and perhaps new animal species can be obtained with heterologous chimeric technology.

In addition, PSCs have great potential to generate primordial germ cells capable of initiating meiosis and generating haploid gametes, oocyte and sperm\cite{180}. The fertility of these in vitro-derived haploid gametes may produce viable and fertile offspring\cite{181}. This would be of substantial value for finally achieving in vitro germ cell induction in domestic species and establishing a new animal breeding system to meet future meat and milk demand\cite{182,183}. In such a system, porcine and other larger animals’ embryonic stem cells can be differentiated into oocyte and sperm. The oocyte and sperm can be fertilized in vitro, then embryos can be used to produce next-generation ESCs, or can be transferred into the uterus to produce live born. This laboratory animal breeding system would greatly shorten breeding time (Fig. 2).

In summary, although we have made a great deal of progress on mammalian pluripotency stem cells, significant problems still persist and many scientific issues need to be explored further.

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