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REVIEW

Progress, problems and prospects of porcine pluripotent stem cells

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Abstract Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), can differentiate into cells of the three germ layers, suggesting that PSCs have great potential for basic developmental biology research and wide applications for clinical medicine. Genuine ESCs and iPSCs have been derived from mice and rats, but not from livestock such as the pig—an ideal animal model for studying human disease and regenerative medicine due to similarities with human physiologic processes. Efforts to derive porcine ESCs and iPSCs have not yielded high-quality PSCs that can produce chimeras with germline transmission. Thus, exploration of the unique porcine gene regulation network of preimplantation embryonic development may permit optimization of *in vitro* culture systems for raising porcine PSCs. Here we summarize the recent progress in porcine PSC generation as well as the problems encountered during this progress and we depict prospects for generating porcine naive PSCs.

Keywords induced pluripotent stem cells, embryonic stem cells, pig, reprogramming

1 Introduction

Embryonic stem cells (ESCs) derived from early-stage mammalian embryos are pluripotent stem cells (PSCs) that can self-renew and differentiate into any adult cell type [1,2]. Such capacities have potential applications in animal breeding and clinical medicine, such as cell therapy for Parkinson's disease and spinal cord injury.

Efforts to derive authentic ESCs over the last three decades have been of variable success. Mouse ESCs (mESCs), first isolated from inner cell masses of blastocysts, have been cultured on feeder cells secreting cytokines for self-renewal and pluripotency. These cell lines can be expanded in an undifferentiated state and differentiate into endoderm, mesoderm and ectoderm cells in the form of *in vitro* embryoid bodies (EBs) and *in vivo* teratomas [1,2]. ESCs can even contribute to germline transmittable chimeras [3,4] and fully form ESC mice via tetraploid complementation [5]. Thomson's group [6] derived human ESC lines from embryos with normal karyotypes and the potential to develop into trophoblasts and three embryonic germ layers. Progress has also been made in other species, including pigs [7–9], cattle [7,10–13], sheep [14,15], rabbits [16], horses [17], dogs [18], and cats [19], but these studies did not fulfill all characteristics of mESCs. Also, at this time, researchers have failed to derive genuine ESCs from domesticated ungulates such as pigs, cattle, goats, sheep and derivation of germline transmittable chimeric offspring has proven to be elusive in these species.

Ethical concerns have been raised regarding stem cells generated from human embryos, which must be destroyed for ESC generation, as well as issues regarding immune rejection. Fortunately, iPSCs that somatic cells can be reprogrammed to PSCs by introducing four transcription factors, *Oct4*, *Sox2*, *c-Myc* and *Klf4*, provided a promising method to generate pluripotent cells similar to ESCs [20].

Recent iPSC developments suggest that these cells may help researchers overcome problems with generating pluripotent cell lines in livestock such as the pig. Such cells would represent improvements over mouse ESCs/iPSCs which have limitations (short life spans and frank physiologic differences from humans) for studying human disease.

Furthermore, PSCs can be used to create genetically modified animals for functional genomics studies. Here we depict advancements and problems with porcine PSCs and discuss prospects for deriving naive pluripotent cell lines in the pig, offering a foundation for facilitating the generation of *bona fide* porcine PSCs.

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2 Generation of ESCs and iPSCs in pigs

Putative porcine ESC-like cell lines have been reported since the first paper to describe derivation of mESCs [7–9,14,21–32]. However, it is difficult to maintain porcine PSCs renewal and in the naive state for many passages. Compared to the mESCs, porcine ESC-like cells are not genuine ESCs that fulfill all pluripotency standards. Also, the optimal porcine embryonic stages and the culture systems for deriving ESCs/iPSCs have not been well described.

The pig is an excellent preclinical model for studying human diseases because of its similarities with human in physiology and anatomy. In the past 30 years, many laboratories have generated porcine ESC-like cell lines from embryos [7–9,14,21–24,28–38]. However, porcine ESC-like cells were maintained in an epithelial-like state or in an EpiSC-like (Epiblast stem cell-like) state, instead of in a mESC-like state [32,39]. Blastocysts used to generate porcine ESC-like cell lines have been at various stages of development, including post-estrous days 5–6 [25,28] post-estrous days 7–9 [7,28], and embryonic days 7–10 [30]. Porcine uterine cells [9], embryonic fibroblasts [32] or STO cells [8,9,32] have been used as feeder cells. ESC-like cells derived from different stage-embryos have been established via either immune-surgery or mechanical dissection. Some putative porcine ESC lines were positive for alkaline phosphatase (AP) [33,40] and some formed teratomas *in vivo* [30]. Other cells produced chimeric coat color animals without germline transmission [33]. However, passaging these for extended times like mESCs is difficult. Miyoshi and colleagues reported the isolation of putative ESCs from porcine blastocysts which were maintained *in vitro* for more than 30 passages. They also indicated that reconstructed embryos could develop into blastocysts *in vitro* [29], but the cells had flattened and epithelial-like morphology and did not form chimeras. Recently, porcine ESC-like cells have been reported to express pluripotent markers such as *Oct4, Nanog*, *SSEA-4*, *TRA1-60*, and *TRA1-81*, but teratomas have not been produced to date [21].

Recent iPS technology provides a new method to derive PSCs and various methods have been used for reprogramming somatic cells, including approaches with pluripotency-associated factors, recombinant proteins, synthetic microRNAs, and cocktails of diverse chemical compounds [20,41–45]. Yamanaka overexpressed exogenous genes for reprogramming by retroviruses [20,46], and others have generated mouse or human iPSC using lentiviruses [47,48] and inducible lentiviruses [19,49]. In 2013, Deng's team used chemically based reprogramming and this was independent of exogenous genes [42].

Mouse iPSCs have the same *in vivo* developmental ability as ESCs, so iPSCs may replace ESCs for animal breeding and clinical applications. Livestock iPSCs such as porcine iPSCs (piPSCs) are the first induced pluripotent stem cell line established from livestock [21,50–57] and these cells have been described by different research groups who used diverse strategies for reprogramming somatic cells to livestock pluripotent cells, such as piPSCs (Table 1), bovine iPSCs (Table 2), and iPSCs in sheep and goats (Table 3). To generate such cells, either human or mouse pluripotent factors *Oct4*, *Sox2*, *c-Myc* and *Klf4* were combined. Other pluripotent factors such as *Nanog* and *Lin28* have been overexpressed in the reprogramming process to derive piPSCs with high quality and efficiency [57]. Combination pluripotent factors and microRNAs including miR-302a, miR-302b and miR-200c have been reported to improve the efficiency of porcine somatic cell reprogramming [65]. Lentiviral and retrovirally mediated reprogramming methods have been used more frequently to generate piPSCs [50,51,52,55]. Livestock iPSCs have only been derived from differentiated cells via overexpression of transcriptional mouse, human, or livestock factors. The piPSCs resembled ESC-like cells with respect to morphology, pluripotent gene expression, and *in vivo*-formed teratomas, but they cannot form chimeras with germline transmission [21,50–57].

3 Characteristics of porcine PSCs

Mouse ESCs are defined as PSCs in the naive state, characterized by compact, round and dome-like colony morphologies that can produce chimeras with germline transmission and could fully form mESCs via tetraploid complementation. In addition, other PSCs are flattened monolayer colonies (referred to as primed state or EpiSC-like state). Human ESCs (hESCs) and mouse epiblast stem cells (mEpiSCs) represent this type of ESCs. The naive or the primed state of PSCs affects their *in vitro* differentiation and *in vivo* developmental abilities, even their clinical application potential [77,78].

Researchers generally agree that livestock PSCs chiefly have two different colony morphologies similar to mESCs or hESCs, but that they lack *in vivo* developmental potential. Also, porcine putative ESCs have been generated with features of pluripotent cells, such as an ESC-like morphology, high AP activity, and the ability to differentiate *in vitro* [26,28,79]. These porcine embryo-derived cells can differentiate spontaneously mediated by EBs and can be induced to differentiate chemically. Most EBs in suspension differentiated into ectodermal, endodermal, and mesodermal cell types [80]. When ESC-like cells were injected subcutaneously into nude mice, teratomas were obtained around the injection site on the back of the mouse [81]. EpiSC-like porcine putative ESCs from IVF, IVF aggregated, *in vivo* derived, and parthenogenetic embryos expressed Activin/Nodal and FGF2 signaling pathway genes in addition to pluripotent genes such as *Oct4*, *Sox2*,

Table 1 Generation of piPSCs

Species	Original cell	Factor	Morphology	Karyotype	Pluripotent marker	Differentiation	Chimera	Reference
Pig	Porcine fetal fibroblasts	Human OSKC	Human ESC-like	Normal	Oct4/Sox2/Klf4/c-Myc/Nanog /TERT/AP /SSEA-1	Embryoid bodies/Teratomas	No	[51]
	Porcine fetal fibroblasts	Human/ Mouse OSKC	Human ESC-like	Normal	Oct4/Sox2/TERT/Lin28/AP/Rex1/SSEA-4	Embryoid bodies/Teratomas	No	[50]
	Primary ear fibroblasts/ Bone-marrow cells	Human OSKC / OSKC NL	Human ESC-like	Normal	Oct4/Nanog/Sox2/Lin28 /CDH1/AP/SSEA-3/SSEA-4/TRA1-60/ TRA1-81/Rex1	Embryoid bodies/Teratomas	No	[58]
	Porcine mesenchymal stem cells	Human OSKC NL	Human ESC-like	Normal	Oct4/Sox2/SSEA-4	Embryoid bodies	Yes	[57,59]
	Porcine adult fibroblasts	OSKC	Human ESC-like	Normal	Nanog/SSEA-4/TRA1-60	Embryoid bodies/Teratomas	No	[60]
	Porcine adult fibroblasts	Mouse SKC	Human ESC-like	Normal	Oct4/Sox2/Nanog/AP/ SSEA-4/ TRA1-60/TRA1-81	Embryoid bodies/Teratomas	No	[55]
	Minipig fetal fibroblasts	Human OSKC	Mouse ESC-like	Not mentioned	Oct4/Sox2/Klf4/ c-Myc/Nanog/ SSEA-1/SSEA-4	Embryoid bodies	No	[61]
	Porcine mesenchymal stem cells	Pig Oct4 /Klf4	Mouse ESC-like	70% normal	Oct4/Nanog/Klf4/c-Myc /Bmp4/bFGF/AP	Embryoid bodies/Teratomas	No	[54]
	Porcine embryonic fibroblasts	Mouse OSKC	Mouse ESC-like	Nomal	Oct4/Nanog/Eras/Sox2/ Lin28/Stella/SSEA-1/SSEA-3/SSEA-4	Embryoid bodies/Teratomas	No	[62]
	PFX/ NM /SWF/LFF /PEF/ HH / PEFL	Mouse /Human /Porcine OSKC	Mouse ESC-like	Normal	Oct4/Nanog/ SSEA-3/SSEA-4/AP	Embryoid bodies/Teratomas	No	[63]
	Porcine fetal fibroblasts	Human OSKC NL	Human ESC-like	Normal	Oct4/Sox2/Nanog/AP/ SSEA-1	Embryoid bodies/Teratomas	No	[64]
	Porcine fetal fibroblasts	OSKC /miR-302a /miR-302b /miR-200c	Human ESC-like	Normal	Oct4/Sox2/Klf4/c-Myc/ REX1/NANOG/ SSEA-4	Embryoid bodies/Teratomas	No	[65]
	Porcine adipose-derived stem cells	Human OSKC	Mouse ESC-like	Normal	Oct4/Sox2/ NANOG/AP/ SSEA-3/SSEA-4/ TRA1-60/TRA1-81	Embryoid bodies/Teratomas	No	[66]

Note: PFX, newborn porcine ear fibroblast; NM, mesenchymal cells form new born porcine bone marrow; SWF, embryonic porcine fibroblast; LFF, embryonic porcine fibroblast; PEF, porcine embryonic fibroblast; HH, adult pig era fibroblast; PEFL, porcine embryonic fibroblast.

Nanog [21], but teratomas could not be formed *in vivo* [21].

Porcine iPSCs were pluripotent due to their ability to differentiate into cell types representative of the three germ layers through both *in vitro* EBs and *in vivo* teratomas [50,51,52,54,55,65]. They had a normal karyotype, but pluripotent marker expression was not stable. For instance, *SSEA-3* [51], *SSEA-4*, *TRA1-60* and *TRA1-81* [55] were positive in some iPS cell lines, whereas others reported negative gene expression [51]. Cell lines positive for *Oct4* and AP staining were have been also described. A main problem with piPSCs was that exogenous genes were not silenced. Furthermore, so far known piPSCs did not have the ability to produce germline transmittable chimeras [57,59].

4 Main problems with porcine PSC

piPSCs have been derived in various ways, but their

Table 2 Generation of bovine iPS cells

Species	Original cell	Factor	Morphology	Karyotype	Pluripotent marker	Differentiation	Chimera	Reference
Cattle	Bovine fetal fibroblasts	Bovine OSKC	Mouse /Human ESC-like	Normal	Oct4/Sox2/Nanog/CDH1/Dppa3 /Stat3/Zfp42/Rex1/AP/SSEA-3 /SSEA4/TRA1-60/TRA1-81	Embryoid bodies /Teratomas	No	[67]
	Bovine fetal fibroblasts	Bovine OSKC	Mouse ESC-like	Normal	Sox2/Nanog/ CDH1 /Dppa-3/Dppa-4 /Sall4/TERT/AP /SSEA-1/SSEA-4	Embryoid bodies /Teratomas	No	[68]
	Bovine adult fibroblasts	Human OSKCN	Mouse ESC-like	Normal	Oct4/Sox2/Nanog /Klf4/c-Myc/Rex1 /AP/SSEA-1 /SSEA-4	Embryoid bodies /Teratomas	No	[69]
	Bovine fetal fibroblasts	Human Oct4 Porcine SK	Human ESC-like	Normal	Oct4/Nanog AP/SSEA-1	Embryoid bodies /Teratomas	No	[70]

Table 3 Generation of ovine and caprine iPS cells

Species	Original cell	Factor	Morphology	Karyotype	Pluripotent marker	Differentiation	Chimera	Reference
Sheep	Ovine primary ear fibroblast	OSKCL/SV40 large T/ hTERT	Mouse ESC-like	Normal	Oct4/Sox2/Nanog/CDH1/ Rex1/ AP /Dppa-4/SSEA-1 /TRA1-60/TRA1-81	Embryoid bodies /Teratomas	No	[71]
	Ovine fetal fibro-blasts	Mouse OSKC	Human ESC-like	Normal	Oct4/Sox2/Nanog AP/SSEA-4	Embryoid bodies /Teratomas	No	[72]
	Ovine fetal fibro-blasts	Human OSKC	Mouse ESC-like	Normal	Oct4/Sox2/Nanog/AP	Embryoid bodies /Teratomas	No	[73]
	Ovine fetal fibro-blasts	Mouse OSKC			Nanog/AP /SSEA-1/SSEA-4	Embryoid bodies /Teratomas	Yes (PCR test)	[74]
Goat	Caprine pri-mary ear fibroblast	Mouse OSKCL/SV40 large T/ hTERT	Mouse ESC-like	Normal	Oct4/Sox2/Nanog/CDH1/Rex1/ AP /Sall4/SSEA1/TRA1-60/TRA1-81	Embryoid bodies /Teratomas	No	[75]
	Fetal primary ear fibroblasts	Human OSKC	Human ESC-like	Normal	Oct4/Sox2/Klf4 /AP/Nanog	Embryoid bodies /Teratomas	No	[76]

morphologies have been inconsistent. As such, these cell lines cannot meet mESC's evaluation standards. During cell programming, somatic mitochondria and bioenergetics — from oxidative to glycolytic metabolism — can be remodeled when PSCs are generated [82]. Differences may exist in energetic metabolism between porcine pluripotent cells and mouse ESCs/iPSCs, as well, but at this time, it is unclear whether current media can meet the metabolic demands of porcine ESCs/iPSCs.

4.1 There is no optimal *in vitro* culture system for naive porcine PSCs

Maintenance of ESCs and iPSCs from pigs is problematic because little is known regarding optimal culture conditions for these cell lines. Thus, optimal cell culture protocols during iPSC generation may facilitate cell growth, reprogramming, and self-renewal [56]. According to various protocols for culturing mouse and human PSCs, porcine ESC-like cells and iPSCs are grown on feeder cells in medium supplemented with Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, non-essential amino acids (NEAA), β-mercaptoethanol and other compounds such as leukemia inhibitory factor (LIF) [52,54,55,62,83], basic fibroblast growth factor (bFGF) [50,51,57,84], stem cell factor (SCF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [85]. Although mouse and human ESCs can maintain pluripotency without feeder layers, mESCs require LIF and bone morphogenetic protein-4 (BMP4), whereas hESCs needed activin and bFGF [86]. mESC media with LIF and inhibitors was insufficient to elicit a response in human primed ESCs/iPSCs [87].

At present, LIF and bFGF are added to culture medium

based on published papers. In mouse PSCs, LIF was involved in two important signaling pathways: JAK-STAT3 and PI3K-AKT to maintain PSC pluripotency. LIF was insufficient to maintain ungulate pluripotent cells in an undifferentiated state [73,88], but its presence may inhibit cell differentiation [53]. VPA, SAHA and TSA as histone deacetylase inhibitors improved mouse cell reprogramming efficiency [89]. DNA methyltransferase inhibitors including 5-aza and RG108 [89–91] were reported to play a role in enhancing mouse iPSC generation.

LIF-based cell medium with specific protein kinase inhibitors can sustain piPSCs in the mESC state [92]. However, the JAK-STAT3 signaling pathway was not fully activated in porcine PSCs due to the lack of a LIF receptor in the pig [93,94]. The FGF signaling pathway may be present in the porcine epiblast: the presence of LIF, FGF or a combination of these was insufficient for porcine PSC growth and self-renewal, revealing that these growth factors cannot sustain porcine pluripotency [95]. According to published reports, inhibition of MEK1/2 and glycogen synthase-3 signaling pathways may contribute to supporting mouse ESCs/iPSCs pluripotency and self-renewal. Porcine iPSCs, as well as bovine and ovine iPSCs, were also cultured in DMEM-based medium with inhibitors PD0325901 and CHIR9902 [52,67,73].

Compared with mouse and porcine SCNT (somatic cell nuclear transfer) embryos, genes coding for enzymes that regulate fatty acid biosynthesis were highly expressed at the early-embryo stages including the 4-cell and morula stage in normal porcine embryos. Gene ontology analysis suggested that mouse and porcine inner cell mass (ICM) had similar signaling pathways, such as for the cell cycle and cell division, and the transforming growth factor-beta (TGF-beta) receptor pathway for *in vivo* embryonic development and regulation of transcription. However, specific genes in porcine ICM were involved in unique pathways, including heat shock protein binding, and fatty acid beta-oxidation and metabolism. These unique regulation networks may be important in porcine early-embryo development [96].

Glycolysis provides energy for PSC growth and self-renewal. During proliferation, pluripotent cells not only needed NADPH and ATP, but also required carbon, nitrogen and hydrogen products for cytoskeleton integrity [97,98]. Core transcription factors were related to STAT3 (signal reansducer and activator of transcription 3) signaling pathway controlled and regulated by glycolysis [99,100]. Thus, regulation of energetic metabolism must be considered when maintaining PSCs in the naive state. NEAA and L-glutamine were used in the medium as basic compositions. Metabolites of threonine in the medium also had important functions for ESC proliferation and self-renewal [101].

Fatty acids store long-term energy supplies and are precursors for other molecules with multiple biologic functions. They may sustain porcine pluripotency as well [96]. Fatty acids may play an important role in the regulation of early embryo development somatic reprogramming. Polyunsaturated fatty acids were contributed to the process of oxidation reduction, and their metabolic products helped maintain “stemness” of pluripotent cells [102]. Arachidonic acid enhanced reprogramming efficiency during iPSC generation [103]. Also, butyrates in the medium improved *Dppa5* expression and increased reprogramming efficiency and clone numbers. Short-chain fatty acids affected ESC pluripotency through epigenetic modification such as histone methylation and acetylation [104–106]. Therefore, culture systems, including a variety of compositions should be studied in-depth to facilitate the generation of genuine porcine PSCs.

4.2 No suitable standards exist for the evaluation of naive porcine PSCs

Pluripotency standards have been based on mouse or human ESCs/iPSCs, which express AP/*Oct4*/*Sox2*/*Nanog*/*SSEA-1*/*SSEA-3*/*SSEA-4*, the potential to differentiate *in vitro* and *in vivo*, and the ability to produce chimeras. MicroRNA profiles and mitochondrial morphology can also be used as markers of the pluripotent stage [107]. However, recent porcine PSCs have not fulfilled the evaluation standards of mouse PSCs. The main reason may be the species diversity among pig, mouse and human.

The process from ICM to epiblast precursors in mouse is controlled by FGF, resulting in the repression of *Nanog* and the expression of *Gata6*, but the mechanism of human epiblast formation is unclear. In cattle, FGF has no effect in regulating *Gata6* expression but it is reported to repress *Nanog* [108]. Studies suggest that *Oct4* was expressed only in ICM during mouse preimplantation development [96,109,110] and similarities in *Oct4* expression patterns were identified between mouse embryos and *in vivo* porcine embryos, indicating that porcine *Oct4* may have an effect on lineage segregation similar to that observed in mouse embryos. In the mouse, increased *Cdx2* expression occurred at the 8-cell stage and it predominated in the trophectoderm (TE) in early embryo-stage development. This also occurred in the pig, but *Cdx2* expression was less than that in mouse. *Nanog* was expressed modestly but was not detected in the morula and blastocyst via immunofluorescence during porcine preimplantation development. Also, *Gata6* and *Sox2* expression in pigs was different from that observed in mice, suggesting that differences in the regulation and control of second lineage segregation events may exist between the mouse and pig [96]. Blastocysts did not express *Nanog* and *Sox2* in porcine embryos on day 5–6 but later (about embryonic day 8.5) embryos expressed them [94]. Different from either mouse or primate, porcine embryos had its own expression pattern

of *Oct4*, *Nanog* and *Sox2*. The hatched porcine embryo was independent on the expression profile compared to hatching mouse and human embryos [95].

Therefore, genes related to pluripotency and three germ layers differentiation are different during early embryo development, and pluripotency-associated genes in ESCs/iPSC *in vitro* may differ in various species. It is of questionable value to assess porcine pluripotent cells according to the standards of mouse ESCs/iPSCs pluripotency.

5 Perspectives and conclusions

Substantial efforts have been made to generate PSCs from livestock such as the pig during the last three decades. Many attempts should be done in different ways so as to generate genuine porcine pluripotent stem cells in the naive state (Fig. 1). Key regulators in porcine early-embryo development must be understood for successfully reprogramming somatic cells into iPSCs via overexpression of Yamanaka's factors and candidate genes. Also, screening and identifying small molecules and fatty acids (as well as other additives) for culture medium may be required. Finally, pluripotency standards must be incorporated such as self-renewal, differentiation potential, metabolic similarities between piPSCs and porcine early embryos, and chimeric offspring. mRNA profiles and mitochondrial metabolism may also require consideration for generating porcine naive PSCs. At this time, scientists can apply iPSC technology to convert somatic cells into PSC, differentiating them for the study of diverse diseases and treatments [111].

The ability to differentiate PSCs into disease-relevant cell types—neurons, hepatocytes and cardiomyocytes—provides an invaluable paradigm in drug development.

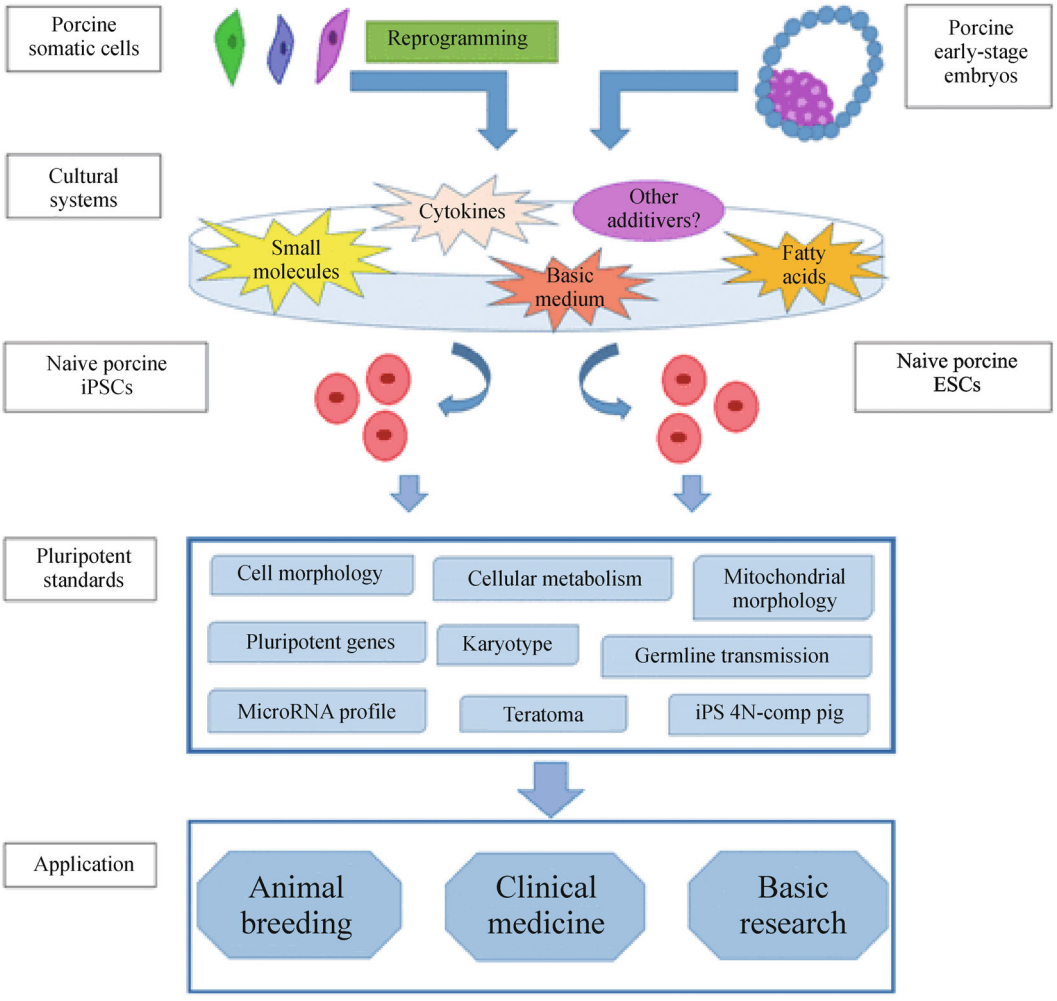

Fig. 1 Attempts to derive authentic piPSCs in the naive state. Chemicals, cytokines, fatty acids and other additives can be supplemented into the basic medium during the course of iPSC generation from somatic cells and ESC derivation from embryos. Various standards can be used to evaluate pluripotency of naive porcine PSCs. Then, these naive cells can be used in different applications such as animal breeding, basic research and clinical medicine

High similarities between humans and pigs in physical and anatomical sides makes porcine PSCs attractive for investigation and as more is known about porcine early-stage embryo development, these cells will be easier to generate and use for clinical and basic research and animal breeding.

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