Review Article

Porcine Embryonic Stem Cells and Their Application: A Review

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Abstract

Embryonic stem cells (ESCs) represent a promising tool for regenerative medicine, tissue repair and cell therapy. They are having high plasticity and possess very unique characteristics due to their unlimited self-renewal ability which allow them to differentiate into all embryonic tissues. The establishment of ESC lines in domestic livestock species could have great impact in the veterinary and biomedical fields. Derivation of pig ESC would find important applications in improving health and production traits through genetic engineering because of its immunological, morphological, physiological and functional similarities to the human which makes pig a very effective and suitable animal model for biomedical studies and pre-clinical trials. Pluripotent stem cells such as ESCs and induced pluripotent stem cells (iPSCs) will provide potential cell sources for gene editing and in the field of regenerative medicine. Here we provide a brief introduction and history of embryonic stem cell, timing of isolation, the use of different culture conditions, the pluripotency-related molecular markers in the pigs and future prospective.

Key words: Embryonic Stem Cells, Pluripotency, Pig, Regenerative Medicine

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Introduction

The embryonic stem (ES) cell line was first obtained from mouse (Evans and Kaufmann 1981; Martin 1981) and human (Thomson and Marshall, 1998; Shamblott *et al*., 1998) embryos which revolutionized cell and developmental biology. The pig is considered to be the most potential source of cells and organs for xenotransplantation (West and Stice, 2011) because of its immunological, morphological, physiological and functional similarities to the human. The isolation and thorough characterization of porcine ESC lines have a great potential which represents no doubt as experimental tool for the development of therapeutic applications and tissue repair. It would also be useful in the precise genetic engineering of the pig for

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improved production traits and products, for disease resistance and for bio-pharming (Keefer *et al.*, 2007). Successful establishment and improving the efficacy of establishing putative porcine ES cell lines was done (pES1 and pES3 cells) by using inner cell mass (ICM) of quality blastocysts produced by oocyte bisection cloning technology and embryo aggregation and these cells could maintain undifferentiation and display typical ES cell pluripotency markers, embryoid bodies forming capacity and differentiation into cell lineages of three germ layers (Siriboon *et al*., 2015).

Embryonic Stem (ES) Cell Lines

ES cell lines have the property of immortality and they are termed as continuous cell lines (Suda *et al*., 1987; Amit *et al*., 2000; Shah *et al*., 2014). These cells can differentiate into derivative of all three germ layers (Bradley, 1987; Hubner *et al*., 2003; Geijsen *et al*., 2004) and they are competent to contribute to all cells of the developing fetus which self-renew as stem cells. Some of the stem cell traits such as the ability to create live-born young are seemingly lost during early passage (Nagy *et al*., 1993). Karyotypic abnormalities become more common within the population over further passages and cell line competence for germ line chimera contribution get reduced (Bradley, 1987; Robertson, 1987). A method for derivation and evaluation of porcine ESC-like cultures was established (Rasmussen, 2010) and when these cell cultures subjected to passage, the ESC-like cells quickly lost expression of *OCT4* along with other markers. They opined that these findings have brought the pig one step closer as a model of human stem cell therapy.

Characteristics of Embryonic Stem Cell

There are three essential characteristics of embryonic stem cell. Firstly, they are derived from the ICM or the epiblast of pre-implantated or peri-implantated embryos. Secondly, they are capable of prolonged undifferentiated proliferation (self-renewal) and lastly, they are able to form derivatives of the three embryonic germ layers namely, ectoderm, mesoderm and endoderm as well as the germ line (Thomson and Marshall, 1998).

The History of Porcine Embryonic Stem Cells

Porcine embryonic stem cells were obtained from *in vivo* hatched blastocysts (removed from the uterus between 7-10 days). Murine embryonic fibroblast cell line is commonly used as a feeder layer for mouse embryonic stem cells. Most colonies were considered as ‘‘ES like’’ when cells ‘‘were small and rounded and had a large nucleus with one or two prominent nucleoli’’ (Piedrahita *et al.*, 1990a). An ‘‘epithelial-like’’ cell similar in appearance with ‘‘flattened cuboidal shape which are grown to confluence and they tend to form structures reminiscent of epithelial sheets’’ was also found (Piedrahita *et al.*, 1990b). Epithelial-like cell lines are able to replicate for a number of passages higher than ES-like lines and have the ability to form embryoid bodies if cultured in suspension (Talbot *et al.*, 1993a). Alkaline phosphatase

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(AP) was a reliable marker for undifferentiated embryonic stem cells in pig and sheep and it represented the first fundamental step to obtain a molecular marker (Talbot *et al.*, 1993b). A very limited stage-specific embryonic antigen-1 (SSEA-1) expression and by the absence of laminin and intermediate filaments like vimentin and cytokeratins 8/18 which are not present in the inner cell mass and epiblast was also demonstrated (Wianny *et al.*, 1997). The birth of chimeric piglets was reported after injecting stem cells derived from primordial germ cells but no germ line transmission was observed (Shim *et al.*, 1997; Piedrahita *et al.*, 1998; Mueller *et al.*, 1999 and Rui *et al.*, 2004). Formation of teratomas using porcine embryonic stem cells was also reported from the cells derived from days 5 to 6 and 10 to 11 blastocysts but only the cells derived from the older embryos formed tumours when injected in nude mice (Hochereau-de *et al.*, 1993). Embryonic stem cell lines derived from days 7 to 8 embryos failed to form teratomas (Piedrahita *et al.*, 1990b) and confirmed the difficulty in obtaining teratomas from the earlier stages of pig embryonic development, however formation of teratoma was observed only after injecting ESCs isolated from days 11 to 12 blastocysts (Anderson *et al.*, 1994).

Isolation of Embryonic Stem Cells from Pig

In comparison to laboratory mice or rats, pigs are more commonly used in biomedical research because of their larger morphology, organ size and physiological similarities with humans (Brevini *et al.*, 2007a; Brevini *et al*., 2008). The two main techniques used for isolation of embryonic cells are from the inner cell mass in pigs namely immunosurgery (Chen *et al*., 1999) and enzymatic digestion (Lie, 2003). Putative pluripotent stem cell lines do not fulfill the requirements of classical embryonic stem cells (Brevini *et al.*, 2010a). The first attempts were conducted in the early 1990s with the establishment of primary cultures of epiblastic cells from 7 to 11 days post conception blastocysts (Evans and Kaufman, 1981; Strojek *et al.*, 1990). These attempts evaluated the embryonic stem cells only on subjective morphologic features based on high nuclear: cytoplasmic ratio and had a tendency to form clumps or the absence of vimentin expression (Piedrahita *et al., 1998*; Strojek *et al*., 1990). Immunosurgical procedures aimed for isolation of ICM was rarely used that time and therefore, plating the whole embryo was the most common approach (Strojek *et al*., 1990). Generation of a chimeric pigs at a relatively high efficiency (72%) using cell lines was maintained up to 44 passages (Wheeler, 1994). Only early-hatched blastocysts could generate stable cell line and those cell lines contributed to host morulae and early blastocyst after evaluation at 24 and 40 hours (Chen *et al.*, 1999). Isolation of porcine embryonic stem cells has started to utilize specific tools for a more exhaustive molecular characterization of the cell lines generated (Miyoshi *et al*, 2000). The expressions of molecular markers like OCT4 (Octamer-binding transcription factor 4), NANOG, SOX2 (Sex determining region Y-box 2), SSEA-1(Stage –specific embryonic antigen-1), SSEA-4(Stage –specific embryonic antigen-4), etc. have been demonstrated in putative porcine embryonic stem cells which were isolated *in*

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vivo (Kim *et al.*, 2010). Pig blastocysts were taken from day 5–6 to day 10–11 of gestation and found that day 10–11 blastocysts yielded ES-like cell cultures (Piedrahita *et al.*, 1998). Confirmation on the possibility of establishing stable pluripotent cell lines using day 6–7 blastocysts was done both *in vivo* and *in vitro* (parthenogenetically) derived embryos (Brevini *et al.*, 2010b). The use of serum replacement (SR) as a substitute for fetal bovine serum (FBS) demonstrates the ability for these conditions to sustain cell growth and keep putative porcine ESCs up to 14 passages (Vassiliev *et al.*, 2010a; Vassiliev *et al.*, 2010b). They also demonstrated that the use of whole *in vivo* derived embryos produced a relatively high percentage of cell lines (10%) with the capacity to contribute to chimeras (Vassiliev *et al.*, 2010b). Leukemia Inhibitory Factor (LIF) was neither essential for culture of putative porcine embryonic stem cells nor it contributed to improve the culture condition (Chen *et al.*, 1999; Kim *et al.*, 2010). A study also used an interesting approach which combines the technique utilized for cellular reprogramming and isolation of embryonic stem cells for the isolation of LIF-dependent putative porcine embryonic stem cells (Telugu *et al.*, 2011). Porcine embryonic stem-like cells (pESLCs) derived by seeding the isolated ICM with basic fibroblast growth factor (bFGF) promoted the establishment of porcine naive ES cells (Hou *et al.*, 2016).

Time to Derive Pig Embryonic Stem Cell

The blastocyst contains three cell types at the time of implantation namely, epiblast, trophectoderm and primitive ectoderm which are present in all eutherian species. Epiblast formation begins at hatching and it is completed around day 12 in the pig (Vejlsted *et al.*, 2006). No defined epiblast is likely to be present in pig blastocysts before hatching (Hunter, 1974). Implantation takes place around 17 days in pig but a large variation in size is observed when post-hatching embryos are used. Recently hatched blastocysts showed fewer trophectoderm cells and a less flattened ICM than late hatched blstocysts (Chen *et al.*, 1999). Pluripotent epiblast cell cultures were obtained from early pig blastocyst stage (7–8 days post-coitus) or from later stage embryonic discs (12–14 days post-coitus). Putative pig embryonic stem cell lines were also isolated from *in-vitro* pig embryos (Li, 2003).

Cell Culture Properties of Embryonic Stem Cell Lines in Pig

Embryonic stem cell lines have the similar cell culture properties in all the species but distinct morphological characteristics of colonies. The ultra-structural study of the inner cell mass of *In vivo* pig blastocysts and of primary cultures of pig epiblast cells showed that the pig epiblast cells develop relatively robust complex junctions/tight junctions shortly after blastocyst formation and have well-developed apical adhesion belt structure associated with actin filament bundles (Talbot and Garrett, 2001).

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Cell Culture Condition for Embryonic Stem Cell and Dissociation

Culture conditions for pig embryonic stem cells were mainly developed on the basis of mouse. The feeder-layer fibroblasts was essential for survival of embryo as without feeder-cell support, cultures of primary pig epiblast cells failed to grow and died over a 10–14 days period (Keefer *et al.,* 2007). The ICM which was cultured on gelatin alone grew very slowly and tended to differentiate and enlarge in dimensions but the presence or the absence of LIF in the culture medium did not prevent cell decay which indicated that the growth factor cannot substitute feeder cells for pig embryonic stem cells line establishment at least in their early culture period (Moore and Piedrahita, 1997). The presence of feeder cells appeared to be necessary in order to ensure good culture conditions. Pig embryonic stem cells are grown on a feeder-layer in medium supplemented with various other nutrients like basic fibroblast growth factor (Strelchenko, 1996 and Yadav *et al.*, 2005), LIF (Strelchenko,1996; Iwasaki, 2000; Saito *et al.*, 2003; Yadav *et al.*, 2005), epidermal growth factor (EGF) and stem cell factor (SCF) (Saito *et al*., 2003). Pig cell lines did not express LIF receptor which indicated that the addition of the cytokine was not essential for the maintenance of pluripotency. The presence of LIF in the culture medium seems to inhibit the differentiation process since it prevented embryoid bodies' formation. Rupture and lysis of primary cultures of pig epiblast cells occur only after 5 minutes of exposure to Ca^{2+}/Mg^{2+}free phosphate buffer solution (PBS) and cells completely disintegrate within 30–60 minutes (Brevini *et al.,* 2007b).

Molecular Markers of Embryonic Stem Cells of Pig

***OCT4* (Octamer-binding transcription factor 4)**

OCT4 gene is located close to the major histocompatibility complex on chromosome 7 in pig (Chardon *et al*., 2000). It does not seem to be specific for totipotent cells as immunocytochemical analysis revealed the presence of *OCT4* protein in all the cells of the blastocyst, including the ICM and trophectoderm (Kirchhof *et al*., 2000; Spencer, 2006; Keefer *et al*., 2007; Kuijk *et al*., 2008; Hall *et al*., 2009). OCT4 is confined to the inner cell mass in embryos at the expanding hatched blastocyst stage (Vejlsted *et al.*, 2006). Presence of OCT-4 plays an indispensable role in plating and early culture of pig epiblasts, but they may be replaced by other pluripotency factors like *NANOG*.

Nanog

"Nanog" was first identified in the mouse in 2003 and which was named after a mythological Celtic land of eternal youth, Tir Nan Og (Wang *et al*., 2003; Chambers *et al*., 2003; Mitsui *et al*., 2003). *Nanog* belongs to a family of proteins containing homeobox domains and it binds to the consensus sequence(C/G)(G/A)(C/G)C(G/C)ATTAN(G/C) where ATTA is a common homeobox DNA binding sequence(Mitsui *et al*.,2003). *NANOG* mRNA expression as well as protein staining was not observed in

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day 6-8 blastocysts in the pig (Blomberg *et al*., 2008; Kuijk *et al*., 2008; Hall *et al*., 2009) but *NANOG* was detected in several adult cells and tissues including porcine umbilical cord cells and porcine fetal fibroblasts (Carlin *et al*., 2006) as well as porcine brain, lung and liver (Blomberg *et al*., 2008), which indicates to a non-pluripotent role of *NANOG* in the pig. NANOG has been observed exclusively in the epiblast of day 9 and 11 blastocysts by using immunocytochemistry (Hall *et al*., 2009) and *NANOG* transcripts were significantly up regulated in *in-vivo* blastocysts compared to in-vitro produced blastocysts (Kumar *et al*., 2007; Magnani and Cabot 2008).NANOG may be able to maintain pig pluripotent cells in an undifferentiated state in the absence of the simultaneous expression of OCT4.

***Sox2* (Sex Determining Region Y-box 2)**

"Sox2" is a member of the sex determination region (SRY)-related HMG box gene family which encodes transcription factors with a single DNA-binding domain (Avilion *et al*., 2003). *Sox2* is expressed in the cells of the ICM and its descendant (epiblast) and have been found to regulate a range of genes associated with pluripotency in collaboration with OCT4 (Catena *et al*., 2004).Though the studies of *SOX2* expression in the pig are limited but it was reported to be exclusively expressed in the epiblast of 9-11 day embryos (Hall *et al*., 2009).

Rex1

"Rex1" is a zinc finger protein transcription factor which is seems to be specific for the epiblast of day-8 blastocysts and could be a useful marker of pluripotency (Blomberg *et al*., 2008).

TRA-1-60 and TRA-1-81

TRA-1-60 and TRA-1-81 are the tumor rejection antigens, normally synthesized in undifferentiated cells and used as markers for hESCs (Xu *et al*., 2001).

***SSEA1, SSEA4* and Alkaline Phosphatase**

The pluripotency markers (SSEA1, SSEA4, alkaline phosphatase) cannot be regarded as definitive markers in the pig but they are considered to be characteristics of embryonic stem cell in other species (Kirchhoff *et al*., 2000 and Keefer *et al*., 2007). SSEA1 has been detected in porcine PGCs (Takagi *et al*., 1997).The cell surface markers are glycoproteins specifically expressed in early embryonic development (Zhao *et al*., 2012). SSEA-1 has shown to express in the day- 7 porcine epiblast but not in the trophectoderm (Wianny *et al*., 1997) and in some cells of the day-12 embryonic disc which could be used as a potential marker of pESCs (Flechon *et al*., 2004). Li *et al*. (2017) discovered a small population of stage-specific embryonic antigen 1 positive cells (SSEA-1+) in Danish Landrace and Göttingen minipig pEFs (porcine embryonic fibroblast populations), which were absent in the Yucatan pEFs. Reprogramming of SSEA-1+ sorted pEFs

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led to higher reprogramming efficiency and reported that SEER (SSEA-1 Expressing Enhanced Reprogramming) cells are more amenable for reprogramming and that the expression of mesenchymal stem cell genes is advantageous in the reprogramming process.

Perspectives of Embryonic Stem Cell of Pig

Although research is going on adult porcine stem cells which is promising as reported for applications in cell therapy of liver (Kano *et al.*, 2003), heart (Smith *et al.*, 2007; Krause, *et al.*, 2007), epidermis (Klima *et al.*, 2007) and bone marrow (Zeng *et al.*, 2007) but many factors are responsible for its establishment as embryonic stem cell lines and the developmental process is very slow. Further investigation is required to identify the optimal time for the initiation of pig ICM cultures and to set up better *In-vitro* culture systems for the establishment and long-term maintenance of porcine embryonic stem cell and also the interactions with the feeder layer need to be fully understood.

Conclusion

Embryonic stem cells are considered as the gold standard for potential use in regenerative medicine because of their pluripotent nature to differentiate into any cell type in the body. These cells can only be derived from embryos during early stage which excludes the possibility to establish of autologous cell lines for patients.

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