

# Stem cells: Perspectives for the pig in relation to other species

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The derivation of pluripotent stem cells from mouse and human embryos and the reprogramming of somatic cells into induced pluripotent stem cells (iPSC) has initiated a new era of research in the field of regenerative medicine. The need for these cells to be tested in relevant animal models, as well as their potential to be used in the genetic engineering of livestock, has generated significant interest in the establishment of pluripotent stem cell lines from farm animals, including from the porcine species. Despite that to date no true porcine pluripotent stem cell lines have been established from cultured embryonic stem cells, there have been some significant advances in the area of iPSC and mesenchymal stem cells. This review examines the current state of porcine stem cell culture, with focus on the challenges that still need to be overcome in order to allow the wider use of these cells in biomedical models or in the field of animal biotechnology.

## Introduction

The early embryonic development in all vertebrates begins with the totipotent zygote (which can form all the embryonic and placental tissues), and proceeds in highly organized and controlled fashion whereupon the ability of most cells to form multiple cell types is lost during the process of differentiation. At blastocyst stage, a relatively small group of cells, named inner cell mass (ICM), has retained the capability of specializing into all embryonic lineages but is not able to form the placenta, and is therefore designated as pluripotent. This characteristic is lost in most cells after the onset of gastrulation, where the main germ layers are formed. From this stage on, pluripotency (as induced in certain *in vitro* conditions) is retained in the germ line (primordial germ cells (PGC) and spermatogonial stem cells (SSC)), while throughout the somatic tissues many cells preserve the capability to form a limited number of different cell types (multipotency) and serve mainly as a source of tissue renewal (the so-called adult, or somatic, stem cells).

The preservation and maintenance of the pluripotent or multipotent characteristics of stem cells *in vitro* has been a subject of intense research. In 1981, two groups reported the establishment of the first murine embryonic stem cell (ESC) lines from cultured ICM (Evans & Kaufman 1981, Martin 1981). Seventeen years later, James Thompson and co-workers reported the establishment of the first human ESC (Thompson *et al.* 1998). Usually, the term "ESC" applies for pluripotent cells isolated from ICM *in vitro*; however, embryonic cells with pluripotent potential have been derived also from mouse embryos at later stages, such as epiblast stem cells (EpiSC) (Brons *et al.* 2007, Tesar *et al.* 2007). These cells resembled human rather than mouse ESC in their morphology and culture requirements, and could not form chimeras despite their ability to form teratomas in immunodeficient mice.

Another type of pluripotent embryonic stem cells that has been established are the so-called embryonic germ cells (EGC), which are derived from cultured PGC obtained from fetuses (Matsui *et al.* 1992, Resnick *et al.* 1992). An interesting fact in this case is that murine PGC do not seem to possess pluripotency, since they have failed to produce chimeras (Stewart *et al.* 1994) and to differentiate into different cell types *in vitro*. However, when cultured for certain time in the presence of leukemia inhibitory factor (LIF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF), mouse PGC can become epigenetically reprogrammed to pluripotency (Durcova-Hills *et al.* 2008) and are able to form germ line chimeras (Labosky *et al.* 1994). The establishment of human EGC-like lines has also been reported (Shamblott *et al.* 1998; Turnpenny *et al.* 2005); however, these cells are not considered to be equivalent to their mouse counterparts due to their limited ability to differentiate *in vitro* and inability to form teratomas.

Lastly, stem cells with pluripotent characteristics have been derived from the male germ line in the neonatal (Kanatsu-Shinohara *et al.* 2004) and adult (Ko *et al.* 2009) mouse testis (designated as germ line-derived pluripotent stem cells (gPSC)). Similar cell lines have been also derived from human testicular cells (Kossak *et al.* 2009), but their pluripotency has not been conclusively proven. In addition, the somatic tissues of newborns and adults have been used as sources for isolation of various stem cell types, such as mesenchymal stem cells from bone marrow, umbilical cord and peripheral blood, adipose tissues, uterus, and other tissues. These cells represent heterologous cell populations with multipotent characteristics.

In 2006 - 2007, Shinya Yamanaka and his co-workers established a new milestone in modern science by reprogramming somatic cells back into pluripotency by the induced expression of pluripotency transcription factors (Okita *et al.* 2007, Takashi & Yamanaka 2006). It was shown that the overexpression of only four genes, namely, *Oct4*, *Sox2*, *c-myc*, and *Klf4*, is sufficient to reprogram fibroblasts into pluripotent stem cells (called induced pluripotent stem cells (iPSC)) that are morphologically and functionally indistinguishable from ESC. This method was also efficient in the reprogramming of human somatic cells to iPSC (Takashi *et al.* 2007), while another group used *OCT4*, *SOX2*, *NANOG*, and *LIN28* with similar success (Yu *et al.* 2007). The isolation of iPSC opened up new possibilities in regenerative medicine, since it provides opportunities for derivation of allogeneic pluripotent cells from each individual, thus reducing the risks of immunological rejection. Unlike in ESC, the derivation of iPSC does not require destruction of embryos, which circumvents a major ethical dilemma.

The isolation of ESC, iPSC, and other stem cells are major scientific achievements that have inspired hopes for finding therapies for many currently incurable illnesses and have initiated a new era of research in the regenerative medicine. However, due to the still unpredictable outcomes of pluripotent stem cell transplantation in patients, including considerable risks of tumor formations similar to teratocarcinomas, these cells need to be tested in suitable animal models before they can be used in the clinic. As a widely used farm animal with physiology and organ sizes similar to humans, the domestic pig is a particularly suitable and cost-efficient model for developing of stem cell therapies for the human medicine. In addition, the field of transgenic animal research would benefit significantly from the availability of pluripotent porcine stem cells, which are suitable for use to carry genetic modifications into germ line chimeras, thus simplifying the process of producing genetically modified pigs. Therefore, the derivation of porcine pluripotent stem cells equivalent to mouse or human ESC is an important priority in farm animal research. The purpose of this review is to examine the progress achieved in the derivation of porcine stem cells, and to comment on the problems and perspectives of this field.

### Porcine embryonic stem cells

In general, embryo-derived cell lines need to satisfy certain criteria in order to qualify as ESC, and the germ line-competent mouse ESC have been considered the “golden standard”. First, these cells need to possess pluripotency as shown by the ability to differentiate into all cell types in the body. The most convincing evidence for pluripotency is the formation of chimeras with substantial contribution to all three germ layers and the germ line, either by blastocyst injection or tetraploid complementation (the latter method consists of aggregation of the pluripotent cells with tetraploid embryos, whereupon the resulting organism is derived almost entirely from the donor cells, while the tetraploid recipient cells form primarily the placenta). Another characteristic of ESC is the ability to self-renew indefinitely by symmetric division, and to preserve pluripotency and genetic stability for many numbers of divisions.

To date, no porcine ESC that satisfy all of the above criteria have been established, although the first reports on the culture of porcine blastocysts were published over 20 years ago (Evans *et al.* 1990, Notarianni *et al.* 1990, Piedrahita *et al.* 1990, Strojek *et al.* 1990). The literature on this subject has been already extensively reviewed (Brevini *et al.* 2008, Hall 2008, Keefer *et al.* 2007, Nowak-Imialek *et al.* 2011, Vackova *et al.* 2007). Overall, it has been reported that porcine ICMs can be cultured *in vitro* from 3-5 (Strojek *et al.* 1990) to as many as 80 passages (Talbot *et al.* 1993), although it is unlikely that the pluripotency characteristics have been preserved for the entire period of culture. Most of the reported putative porcine ESC lines have been characterized at early passages for relatively small number of pluripotency markers and have been able to differentiate into different cell types from the three germ layers *in vitro*; however, relatively few groups have characterized their cell lines by *in vivo* differentiation assays. One group had produced teratomas in nude mice (Hochereau-de Reviers & Perreau 1993), while three groups have reported generation of chimeras by blastocyst injection (Chen *et al.* 1999, Gerfen & Wheeler 1995, Vassiliev *et al.* 2010). The contribution of the donor cells to the three germ layers was demonstrated by the presence of coat spotting or by PCR analysis; however, no evidence for germ line chimerism has been shown. It has been estimated that the efficiency of chimera production from putative porcine ESC was low (3% compared with 10-12% from fresh ICM (Chen *et al.* 1999)), while the extent of the contribution of the donor cells to the chimeric piglets has not been determined.

While chimeric contribution remains an important standard for pluripotency, some caution in interpreting the results of such assays may be required. This was shown by results from experiments where injections of fetal fibroblasts into mouse or sheep blastocysts have resulted in the formation of chimeras with contribution to all germ layers (Karasiewicz *et al.* 2008, Piliszek *et al.* 2007). When careful analysis was performed, it was determined that the mouse donor fibroblasts had formed hybrids by fusion with the recipient cells (Piliszek *et al.* 2007), which suggests that they may have been reprogrammed *in vivo*. The authors note that upon certain conditions tetraploid cells may survive and contribute to various tissues in the embryos, with the exception of the germ line (where normal ploidy is apparently essential for gamete survival). In the light of these findings, it may be necessary to analyze chimeras produced with putative porcine ESC more carefully to exclude the possibility of similar fusion reprogramming events.

Another source for pluripotent cells is the epiblast, which has been used for the isolation of mouse EpiSC. Derivation of porcine EpiSC have been reported by one group (Alberio *et al.* 2010). These authors showed that these cells were similar to human ESC, and depended of bFGF and Activin/Nodal signaling for maintenance of pluripotency characteristics. When induced by Bmp4 *in vitro*, the cells differentiated into cells from all germ layers and trophectoderm, suggesting that they may be equivalent to mouse EpiSC. No teratoma or chimera testing was carried out in order to obtain further proof of pluripotency.

### Porcine embryonic germ cells

The first report regarding the derivation of porcine EGC-like cell lines from cultured PGC using embryos at day 25 of gestation was published by Shim *et al.* (1997). The authors used feeder layers of irradiated mouse fibroblasts, but (unlike culture of mouse ESC) no growth factor supplements. The resulting cell lines formed compact colonies resembling murine EGC, expressed tissue non-specific alkaline phosphatase (AP), and were able to differentiate into various cell types *in vitro*. One chimeric piglet was produced by blastocyst injection, as verified by the presence of a spot on the skin, but germ line chimerism was not confirmed.

Following this report, other groups have reported the derivation of cell lines from cultured porcine PGC (Durcova-Hills *et al.* 1998, Mueller *et al.* 1999, Lee & Piedrahita 2000, Petkov *et al.* 2008, Tsung *et al.* 2003). These cells have been shown to express some pluripotency markers such as AP, SSEA-1, SSEA-3, SSEA-4, and OCT4, and to be able to form embryoid body (EB)-like structures and differentiate *in vitro*. One group has produced chimeric piglets with overt coat chimerism (Mueller *et al.* 1999), and another has shown that transgenic EGC-like cells expressing green fluorescent protein (GFP) contribute to all germ layers in fetuses and stillborn piglets (Piedrahita *et al.* 1998). No germ line chimerism has been confirmed in any of these studies, and the efficiency of generation of chimeras has been low, similarly to putative ESC cells. It was speculated that this low efficiency might be due to the inability of the putative PGC to incorporate into the rapidly developing blastocysts (Rui *et al.* 2004), however, attempts of these authors to improve the results by using slower-developing *in vitro* cultured blastocysts did not produce the desired outcome. The proliferation of the putative EGC lines has been shown to be limited to 13-54 passages as reported by different authors, and the maintenance of pluripotency over long-term has not been proven. In the light of the available results, it can be concluded that true EGC lines that are equivalent to murine EGC have not been yet established in the pig.

### Porcine induced pluripotent stem cells

The establishment of mouse and human iPSC (Okita *et al.* 2007, Yu *et al.* 2007) has awakened significant interest in the reprogramming of porcine somatic cells to pluripotency using Yamanaka's methods. Within a short time from one another, three groups reported the derivation of the first porcine iPSC (Esteban *et al.* 2009, Ezashi *et al.* 2009, Wu *et al.* 2009). Similarly to the generation of mouse and human iPSC, the authors used lentiviral or retroviral expression of four (OCT4, SOX2, C-MYC, KLF4 (Ezashi *et al.* 2009, Esteban *et al.* 2009)), or six (OCT4, SOX2, C-MYC, KLF4, NANOG, LIN28) reprogramming factors (Wu *et al.* 2009) to reprogram porcine fibroblasts. The resulting cell lines formed colonies with human iPSC-like morphology, expressed endogenous pluripotency markers and were able to form EBs and teratomas.

Using similar methods, several more groups have reported generation of porcine iPSC lines during the following years (Kues *et al.* 2012, Liu *et al.* 2012, Montserrat *et al.* 2010, West *et al.* 2010). Overall, most of the porcine iPSC lines that have been reported to date have shown morphology similar to human iPSC; however, it has been shown that when the cells are cultured in medium supplemented with LIF and the protein kinase inhibitors CHIR99021 and PD0325901, they resemble mouse ESC, which are considered to be in the so-called naïve pluripotent state (Telugu *et al.* 2011). The pluripotency of most of the reported porcine iPSC lines have been tested by teratoma assay, but relatively few reports contain information regarding *in vitro* differentiation. To date, only one group has produced chimeras from iPSC derived from reprogramming of MSC (West *et al.* 2010), and it has shown evidence for germ line transmission (West *et al.* 2011).

The potential to use porcine iPSC as model for stem cells therapies has been demonstrated by experiments where cell lines have been differentiated *in vitro* into retinal photoreceptors and implanted into damaged porcine retinas (Zhou *et al.* 2011). The successful integration of these cells suggests that they have promising potential as models for retinal regeneration. In another study, iPSC-derived porcine endothelial cells were used for transplantation in mouse myocardial models, where the experimental mice showed improvement of their cardiac function (Gu *et al.* 2012). Additionally, one study has shown that hepatic-like cells can be derived from putative porcine iPSC (Avaralli *et al.* 2012). These achievements, realized in the span of only few years, give hopes that porcine iPSC would prove to be a viable alternative to the still unavailable porcine ESC.

Despite the promising advances in this field, there are some questions that remain to be answered in order to make porcine iPSC reprogramming as efficient, reliable, and reproducible as mouse or human iPSC production. One important question is which pluripotency markers define the truly pluripotent porcine iPSC. This question arise from the finding that some lines have been shown to express SSEA-1, but not SSEA-4 (Ezashi *et al.* 2009), while others express SSEA-4 (Esteban *et al.* 2009). It has been shown that the expression of SSEA-4 is necessary for the porcine iPSC ability to differentiate into the neural lineages (Yang *et al.* 2012); however, it is not clear whether this marker was necessary for pluripotency. The endogenous expression of other common pluripotency markers, such as OCT4, SOX2, NANOG, REX1, LIN28, CDH1, TDH, etc., has been used as marker for pluripotency in most porcine iPSC. Our experience shows that cell lines that express these markers still are not capable of forming teratomas and to differentiate into all three germ layer *in vitro*, although they can readily form trophectoderm (Petkov *et al.* 2013).

Another important question is regarding the optimal culture condition for the reprogramming and maintenance of the pluripotent porcine iPSC. The cell lines reported to date have been isolated in culture conditions normally used for mouse or human ESC/iPSC, and one group has produced iPSC lines without the use of any growth factors (Wu *et al.* 2009). Since research in the field of porcine ESC has shown that these conditions are not able to maintain pluripotency of pluripotent ICM cells *in vitro*, it is then pertinent to ask whether other factors are involved in the process of porcine iPSC maintenance. One such potential factor could be the continuous expression of the transgenes, which are normally silenced in mouse iPSC, but not in any of the reported porcine lines. To clarify this point, it might be necessary to generate transgene-free porcine iPSC and determine whether they preserve their pluripotency in the currently used culture conditions.

### **Germ line-derived pluripotent stem cells**

Similarly to PGC, the unipotent germ line stem cells from the mouse testis can be converted in culture into pluripotent stem cells (gPSC) that are able to differentiate into all three germ layers *in vitro* and *in vivo* and to form germ line chimeras (Ko *et al.* 2009). The derivation of such cells from the porcine testis has not been successful to date, due to the lack of optimal culture conditions. The first report of isolation and culture of A spermatogonia (Dirami *et al.* 1999) showed that the porcine germ line cells could survive for up to 120 hours *in vitro* when cultured with KSOM medium. Another group was able to sustain these cells for 9 days before they lost viability (Marret & Durand 2000), although no information was provided regarding their pluripotent characteristics. The porcine germ line stem cells in the neonatal testis (also called gonocytes), identified by the expression of Dolichos biflorus agglutinin (DBA) antigen, have been maintained in culture for 7 days (Goel *et al.* 2007), have shown expression of

pluripotency markers SSEA-1, NANOG, KLF4, DAZL, and OCT4 in culture, and have been able to form teratomas in nude mice with formation of the three germ layers (Goel *et al.* 2009). Nevertheless, the inability to maintain these cells in long-term culture has limited their application as stem cell models or in the field of animal biotechnology.

### Mesenchymal stem cells

Pioneering studies by Friedenstein & Petrakova (1966) first discovered the presence of MSC in rat bone marrow. Subsequent studies have identified MSC as a heterologous population of stromal cells present in a wide variety of tissues, which possess multipotent characteristics as demonstrated by their ability to differentiate into different cell types, such as bone, cartilage, epithelial, muscle, etc. (reviewed by Si *et al.* 2011). Similar to mouse and human, porcine MSC have been isolated by a number of research groups from bone marrow (Bosch *et al.* 2006, Ringe *et al.* 2002), umbilical cord blood (Kumar *et al.* 2007), peripheral blood (Wang & Moutsoglou 2009), skin (Dyce *et al.* 2004, Park *et al.* 2012), adipose tissue (Huang *et al.* 2007), uterine tissues (Miernic *et al.* 2012), and other tissue types. Characteristic of these multipotent cells is the ability to differentiate into the osteogenic, adipogenic, and chondrogenic lineages. In addition, some groups have reported that porcine MSC can be differentiated into the endothelial (Pankajakshan *et al.* 2012), cardiac (Moscoso *et al.* 2012), and neural (Huang *et al.* 2007, Park *et al.* 2012) lineages. Characterization for cell surface antigens has shown that, similar to human, porcine MSC from bone marrow, adipose tissue and peripheral blood express CD29, CD44, CD90, CD105, but lack CD45 (Casado *et al.* 2012); however, there are no markers that are unique to MSC. The cell culture protocols for isolation of porcine MSC are relatively simple and reproducible, which makes them attractive alternative to ESC, where even the most complex culture conditions have not given the desired results. One problem in the culture of MSC is their limited proliferation *in vitro* - some of the longest-proliferating cell lines have reached approximately 100 doublings, while their differentiation potential became limited after 15 passages (Vacanti *et al.* 2005). This problem has been alleviated by immortalization using the pRNS-1 plasmid (Moscoso *et al.* 2012) or by transfection with human TERT (Wei *et al.* 2008).

One important characteristic of MSC is their low immunogenic profile, which accounts for lowered risk of immune rejection. This has been shown in multiple trials, where mostly allogeneic MSC have been transplanted in various tissues, including hearts, spinal cords, muscle, or cartilage in humans or mice (reviewed by Barry & Murphy 2004). An important immunomodulating property of these cells is the ability to prevent immune rejection by suppressing lymphocyte proliferation, which has also been confirmed by studies using pig MSC (Comite *et al.* 2012). These results contrast the finding of another study which shows that, despite their low immunogenicity *in vitro*, porcine MSC can elicit immune reaction *in vivo* (Poncelet *et al.* 2007), although later the same group showed that such cells did not cause immune response when transplanted in the myocardium of fully immunocompetent porcine models (Poncelet *et al.* 2010). Moreover, another group has shown that transplanted bone marrow-derived MSC, together with a transient immunosuppressive treatment, modulated T-cell regulation and suppressed inflammation and graft rejection (Kuo *et al.* 2012). In addition, there have been multiple reports on the successful use of porcine MSC for transplantation, including more recent publications regarding the osteogenic potential of bone marrow MSC transplanted into porcine metaphyseal long-bone defect models (Herten *et al.* 2012), the angiogenic properties of transgenic MSC transplanted into myocardial infarction models (Lu *et al.* 2012), the function-restoring properties of adipose MSC in renal models (Eirin *et al.* 2012), etc.

## Conclusions

Despite some recent achievements in the field of iPSC and MSC culture, some major challenges remain to be overcome in order to make porcine pluripotent stem cells routinely applicable in the fields of regenerative medicine of animal biotechnology. One major challenge is to determine the molecular mechanisms of establishing and maintenance of pluripotency in porcine stem cells, which seem to be different of those in mouse and human stem cells. With improved understanding of these mechanisms, it would be possible to identify the optimal culture conditions for porcine pluripotent cells. Only then it would be possible to maintain them in long-term culture and to differentiate them into the desired cell types as necessary for testing different regenerative therapies, or to use them for the generation of transgenic pigs by chimera production.

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