Putative Embryonic Stem Cell Lines from Pig Embryos

Irena VACKOVA1,2), Andrea UNGROVA1) and Federica LOPES3)

1)Institute of Animal Science, Pratelstvi 815, 104 00 Praha Uhrineves, Prague, 2)Center for Cell Therapy and Tissue Repair, Charles University, Prague, Czech Republic and 3)Dipartimento di Scienze Biomediche Comparate, Teramo University, Piazza Aldo Moro 45, 64100 Teramo, Italy

Abstract. Embryonic stem cells (ES cells) were first established in the mouse, and they represent a population of pluripotent, undifferentiated cells derived from early embryos that is capable of proliferating without any limitation in an undifferentiated state. These cells retain the ability to differentiate in vitro or in vivo into derivates of all three germ layers, and when injected into blastocysts, they can participate in the formation of all tissues, including gonads (germ-line chimeras). It is possible to transfect them with a gene of interest, and the resulting transgenic cell lines can also be used for production of chimeras. Unfortunately, mammalian germ-line chimeras that can carry an inserted gene into their progeny have only been produced in the mouse. Logically, before application of stem cell therapies into a human medicine, it is necessary to verify the efficiency and safety of these methods with an acceptable animal model. The pig is currently used as a very convenient animal for pre-clinical applications, and therefore establishment of porcine ES cell lines is highly needed; unfortunately, no convincing ES cell lines have been produced in this species (and other domestic animals) to date. In this article, we discuss the recent advances in this field, especially oriented on possible reasons and obstacles why derivation of porcine ES cell lines is still unsuccessful.

Key words: Chimera, Embryonic stem cells, Porcine
other hand, in most ungulates, including pigs, implantation occurs only after a considerable delay during which the trophoderm proliferates rapidly and the inner cell mass (ICM) forms a quiescent embryonic disc [14].

In our review, we are thus oriented especially on the progress in derivation of embryonic stem cell lines in the pig, the factors affecting their successful establishment, propagation and culture and on their characteristics and the possibility to differentiate them in vitro and in vivo.

The Age and the Source of Embryos

ES cell lines are conventionally isolated from the inner cell mass of blastocysts and eventually (but rather exceptionally) from earlier embryonic stages, i.e., single blastomeres [15], precompaction embryos [16] or morulae [17], and recently, pregastrula stage embryos [18]. To our knowledge, pig ES-like lines have only been established from blastocysts. There have been several exceptional, yet unsuccessful, attempts at derivation from 4- and 8-cell stage embryos and morulae produced in vitro [19]; however, the attachment rates of these preblastocyst stage embryos were low, and none of the embryos began outgrowth. Chen et al. [20] were also unable to derive undifferentiated cell lines from porcine zona-free morulae, in spite of the fact that morulae formed flattened primary colonies after seeding under an STO feeder layer. Typically, as mentioned above, blastocysts at different stages of development are used for derivation of porcine ES-like cell lines. Day-5–6 postestrus in vivo blastocysts were used by Anderson et al. [21], Hocherau de Reviers and Perreau [20], Chen et al. [20] and Shiue et al. [23].

Anderson et al. [21] attempted to assess whether porcine blastocysts contain undifferentiated cells that are capable of further multiplication. ICMs from day-6 blastocysts did not survive the first passage, and only 2% of them began outgrowth. Nevertheless, his group was able to establish ES-like cultures using ICMs from older blastocysts (d7–d10). The authors assumed that the factor(s) that may affect survival in culture was the size of the isolated ICMs; ICMs with fewer cells had a reduced chance of survival. Chen et al. [20] were unable to isolate a stable cell line from pre-hatching blastocysts; colonies from early blastocysts grew slowly after passaging and all consequently degenerated, and those from expanding blastocysts produced only differentiated cells that were all lost by passage 8. Hatched blastocysts older than 9 or 10 days that have already begun to elongate or are already elongated have been found to be unsuitable for establishment of ES cells due to their fast differentiation in culture [20–22, 24, 25]. This finding was confirmed by Prelle et al. [13], who reported that the porcine embryonic disc begins to differentiate into mesodermal cells around day 9 (d9). On d9, about 50% of evaluated embryos expressed intermediate filament protein vimentin. On d7 and d8, vimentin was found neither in the trophoderm nor the ICM, indicating that the porcine ICM is still pluripotent at this stage. As development proceeds, the epiblasts of ovoid and tubular d10 and d11 embryos showed an intense vimentin staining in all cases. These results suggest that, in the pig, d9 is the latest stage at which undifferentiated cells are still available in the ICM, and thus it is recommended that older blastocysts not be used. However, these observations are not in agreement with those published by Strojek et al. [26], who compared d9 and d10 blastocysts and did not find any outgrowth in the culture for d9 blastocysts, whereas d10 blastocysts formed colonies of ES-like cells. Generally, most authors have used expanded or early-hatched blastocysts [6, 20, 23, 24, 27–32]. This essentially corresponds with the results of Chen et al. [20], indicating that in the pig, an obvious increase in the size of the ICM begins during the early-hatched blastocyst stage. Thereafter, proliferation of this cell population slows down and reaches a more quiescent state, whilst the trophoderm remains mitotically active.

In the pig as well as in other farm animals, the majority of authors have used in vivo produced blastocysts from superovulated animals. Even though in vivo produced embryos are expected to be of very high quality, this approach is expensive and laborious. Thus, it would be desirable, for economic reasons, to use embryos produced from in vitro matured and fertilized oocytes. However, up to now, only a few ES-like lines have been established in farm animals from in vitro produced material [33–35]. The first attempt to use in vitro blastocysts in the pig was described by Miyoshi et al. [36]. Their results indicate that a porcine cell line can be isolated from an in vitro produced hatched blastocyst and maintained in vitro for a relatively
ICM Isolation

Whole intact blastocysts without the zona pellucida or isolated ICMs can be used for establishment of ES cell lines. Intact porcine blastocysts were used by Evans et al. [14], Piedrahita et al. [6], Hocherau de Reviers and Perreau [22], Gerfen and Wheeler [27], Miyoshi et al. [36], Li et al. [19] and Kim et al. [39]. On the other hand, Talbot et al. [47] does not recommend culture of intact pig blastocysts because this approach results in attachment and growth of the trophoblast and primitive endoderm, whilst the embryoblast typically does not grow and instead degenerates or differentiates. They and others [14, 22] suggest that successful establishment of porcine stem cell lines requires isolation of the epiblast from the trophoderm and primitive endoderm. However, it is still difficult to isolate ICMs from *in vitro* produced blastocysts due to not so prominent ICMs and ICMs containing only a few cells [19, 48]. The commonly used method for isolation of ICMs free of the trophodermal cells is immunosurgery [49]. This approach has also been successfully used in the pig [6, 20, 21, 23, 28, 29, 37, 38]. Wianny et al. [25] developed another immunosurgical method when using a monoclonal antibody SN1/38 specifically raised against porcine trophodermal cells [50]. Other methods used include treatment with calcium ionophore [51], microsurgery [22, 24, 32, 48] and a digestive method using 0.25% Trypsin/EDTA [30, 31].

Talbot et al. [47] suggested that pig ICM is composed of an inner core (epiblast) surrounded by multiple layers of outer cells. The outer layers seem to be the primitive endoderm because in culture they can only give rise to vesicles and dome-forming cells. They reported that immunosurgery removed most or eventually all of the trophodermal cells but little or none of the endodermal layer forming the outer layer of the ICMs. The innermost layer of endoderm is closely adhered to the epiblast core, and it can only be removed after two days in culture. Hence, Talbot et al. [47, 52, 53, 54] recommended a 3-step dissection protocol consisting of immunosurgery followed by short culture and subsequent physical dissection of the ICMs from the rapidly growing endodermal layer. This leads to isolation of the epiblast not only from the trophoblast but also from the endoderm.

The use of frozen-thawed (FT) and ICSI produced blastocysts for production of porcine stem cell lines has yet to be reported in spite of the fact that the above techniques have been used (with and without success) for production ES cell lines in other species (horse FT [10]; sheep FT [35]; primate ICSI [43]; human ICSI [44]; human FT [45]). Another possibility is to establish ES cell lines from blastocysts produced by nuclear transfer. In theory, this approach means production of patient-tailored (compatible) ES cells. However, this approach has only been successful in mice [46] and cattle [34] and has failed in porcine [20, 39].
Culture Conditions

Maintenance of porcine ES cells in culture is still difficult and suboptimal because of the total absence of suitable culture conditions capable of inhibiting differentiation of isolated ICMs. The culture systems that are generally used are based on mouse ES cell culture methods [55] and are not optimal for porcine ES cell line derivation. The most probable reason is that the mouse has a short invasive preimplantation period, while the pig exhibits a longer non-invasive preimplantation period that is characterized by rapidly dividing trophoblastic cells and a rather quiescent state in the ICM [29]. Nevertheless, most laboratories use a culture system similar to the mouse method, consisting of DMEM supplemented with L-glutamine, 2-mercaptoethanol, antibiotics, nucleosides, non-essential amino acids and different sources and doses of serum [5, 6, 14, 19–31, 37, 47, 51].

Moore and Piedrahita [29] compared a commonly used murine ES cell medium (DMEM with high glucose = D medium) with a commonly used media used in culture of livestock embryos (DMEM with low glucose and Ham’s-F10 1:1=D/H medium). Their results demonstrate that D/H medium might be superior compared with the commonly used D medium, especially when establishing long-term cultures of porcine embryonic cell lines. Low glucose DMEM/F10 medium has also been successfully used for establishment of ES cell lines from PA blastocysts [38] and is superior to DMEM alone [39].

Maintenance of mouse ES cells depends either on the presence of a feeder layer that is capable of synthesizing and secreting leukemia inhibitory factor (LIF) or on exogenous supplementation with purified LIF protein and eventually on the use of conditioned media [29]. Previous reports have shown only little or no beneficial effect of addition of hLIF on the maintenance of porcine stem cells in an undifferentiated state [22, 25, 28, 29, 47, 52]. Thus, it would be desirable to compare the efficiency of hLIF with the effect of homologous porcine LIF [52]. Some authors have attempted to use other cytokines and growth factors to improve the culture conditions. It has been found that addition of bovine insulin [22, 26], platelet-derived growth factor, PDGF [22], basic fibroblast growth factor, bFGF [19, 30–32, 36, 48, 56], Ewing sarcoma growth factor, ESG [9], and Buffalo rat liver (BRL) cell-conditioned medium [36] have favorable effects on successful isolation and/or proliferation of porcine stem cells.

Some factors that were found to suppress the differentiation of mouse embryonic cells have no differentiation-inhibiting effect on porcine stem cells, including transforming growth factor beta (TGFβ), epidermal growth factor (EGF) [22], erythroid differentiation factor (hrEDF) [47], insulin-like growth factor 2 (IGF-2) [51], conditioned medium [24], interleukin 6 (hIL-6), oncostatin M (hOSM) and ciliary neurotrophic factor (rCNTF) [28].

According to Goldsborough et al. [57], fetal bovine serum is the main source of potential differentiating factors in ES cell cultures. To maintain ES cells in an undifferentiated state, they recommend KNOCKOUT™ Serum Replacement (KO-SR; Gibco, Grand Island, NY, USA), a defined serum-free supplement, be used instead of animal serum. In addition, an optimised DMEM formulation, KNOCKOUT DMEM, can further improve ES cell cultures. KO-SR was used only rarely for isolation and maintenance of porcine embryonic stem cell lines [32, 38, 48]. Other authors have used fetal bovine serum (FBS), fetal calf serum (FCS), newborn calf serum (NCS) or their combinations. Only Strojek et al. [26] compared the influence of serum supplementation on the formation of morphologically undifferentiated cells from porcine ICM outgrowths. The viability of embryos and their potency to form ICM-derived colonies was highest when porcine serum was used in combination with fetal calf serum compared with calf serum only or human cord serum supplementation, respectively.

Because the typical expansion and elongation of porcine blastocysts does not occur under in vitro culture conditions, Ropeter-Scharfenstein et al. [24] attempted to create a culture system that would allow the embryo to survive without differentiation up to the onset of ICM proliferation. To simulate in vivo conditions, the embryos were cultured on a gelatinous collagen matrix. Their two-stage culture system consisted of preculture in NCSU23-medium containing insulin, transferrin and Na-seleinite on a three-dimensional collagen matrix for 4 days with subsequent culture in DMEM on an MEF feeder layer not inactivated by mitomycin. The embryos increased to three times in size and appeared to be perfectly viable, and ICM cells taken from precultured embryos survived longer than control cells (up to 13 passages).
The method of cell passaging is another important factor that is crucial for establishment and maintenance of ES cells in culture. For this purpose, trypsin has been successfully used for murine ES cells [58]. In other species, similar attempts have led to loss of proliferation and/or to induction of cell differentiation, including in cattle and rabbits [59], pigs [47], monkeys [43], and humans [60]. However, the majority of researchers have reported successful trypsinization of porcine pluripotent ES cells [14, 19, 21–23, 25–27, 30, 31, 36, 37, 51, 61] by treatment with trypsin or trypsin/EDTA.

**Feeder Cell Layer**

The feeder cell layer is one of the most important factors affecting ES cell culture. It serves as an attachment matrix for cells and can secrete some kinds of cytokines, such as LIF, that may stimulate ES cell growth and inhibit their differentiation [30]. Proliferation of feeder cells must be prevented so that they can be used as nonreplicating viable supporting cells. Mitomycin C and $\gamma$-irradiation treatments are commonly used for inactivation of feeder cells. They inhibit DNA synthesis while RNA and protein synthesis continues; the cells remain vital, although they are unable to divide. Currently, the established cell lines from mouse embryonic fibroblasts STO and MEF and homologous embryonic fibroblasts (HEF) are the most commonly used feeder cells.

Some experiments have been designed to analyze which feeder cells are the most suitable for porcine ES cell cultures, but the results obtained have been rather inconsistent. While Strojek et al. [26] preferred a feeder layer from porcine uterine cells (PUC) and did not recommend STO cells. Piedrahita et al. [5, 6] found an STO feeder layer to be more suitable since it supports the growth of embryo derived colonies for longer than 10 passages. Based on testing of other feeder layers by Piedrahita et al. [5], PUC buffalo rat liver cells (BRL) and epithelial-like porcine embryo-derived cell line (PH3A) do not support attachment of the porcine ICM. Perhaps due to the reduced or missing extracellular matrix in porcine embryonic fibroblasts, PEF does not allow for proliferation of attached cells, and MEF induces quick differentiation into a presumptive endodermal or trophoblastic cell layer. Kim et al. [39] also did not observe any attachment of the ICM in PUCs or under feeder-free conditions. Talbot et al. [47] suggested that culture of STO cells contributes to differentiation of the pig epiblast. Hocherat de Reviers and Perreau [22] compared a feeder-free culture system with culture on a PEF feeder layer. The longest duration of culture of ES-like cells with a morphology resembling mouse ES cells was achieved on the PEF feeder layer. According to Ropeter-Scharfenstein et al. [24], MEFs were the best feeder for ES-like cells, with the highest number of passages obtained. The attachment rate, proliferation or viability of ICMs with the other feeders tested (PEF, neonatal goat testes, porcine trophoblast vesicles, porcine granulosa cells and established cell lines, including LLCPK, 3T3, P19 and BRL) was rather low. When Li et al. [31] compared three different feeders, they found that PEF produced the same results as MEF, and both were superior to STO. The attachment rate and primary colony formation on STO cells were significantly lower, and the majority of the cultured cells differentiated after the 1st passage. They also examined microtubules in the feeder cells and found that the microtubule localization of PEF was the same as that of MEF; however the pattern was significantly different from that in STO. Also, the number of microtubules was higher in MEF and PEF compared with STO. The authors concluded that this could be the reason why STO was unsuitable as a feeder layer because cells with fewer microtubules have reduced secreting activity. Nevertheless, most porcine ES-like cells have been derived using the STO feeder [5, 6, 14, 20, 21, 27, 36–39, 48, 52, 56, 61].

**Characterization of Embryo Derived Porcine Cells**

**Morphology**

The morphology of embryonic stem cells should have two major traits: i) the smallest amount of cytoplasm and large nucleus with one or two prominent nucleoli and ii) the fastest proliferation in the given population [59]. The slower proliferation rate of undifferentiated, stem-like cell explants of porcine embryos compared with those derived from murine embryos [6, 24] may reflect the above-mentioned differences in pre-implantational development between these two species. That is, the period of quiescence in the inner cell mass of...
ungulates lasts up to the time of gastrulation [14]. Due to morphological and developmental differences between murine and porcine embryos, we can also logically expect morphological differences between corresponding colonies [2]. Nevertheless, the majority of porcine embryo-derived cells, as in the mouse, have large, translucent nuclei, one or two prominent nucleoli and relatively little cytoplasm [5, 14, 20–22, 25, 27, 47, 61, 62].

Some authors [19–21, 28–31, 36, 47, 61] have observed, typically soon after seeding onto a feeder layer, the presence of compact colonies growing as tightly packed mounds and containing small rounded cells resembling mouse ES cells. However, maintenance of this morphology is difficult because porcine ICM cells quickly differentiate.

In general, the individual cells in porcine embryo-derived cell lines are morphology similar to mouse ES cells; however, the overall morphology of colonies is more epithelial-like [20]. The epithelial-like colonies are usually characterized as round compact monolayers with clear colony borders growing onto a feeder layer, sometimes dome-forming. The cells of these colonies have a high nucleocytoplasmic ratio. These epithelial-like cells are flat, either cuboidal or eventually with polygonal or irregular in shape and size, with distinct cell borders and abundant lipid-like vacuoles [14, 19, 20, 27–31, 36, 47]. Gerfen and Wheeler [27] noticed the higher resistance of porcine embryonic cells to trypsin disaggregation. This might be an indication of epithelial-like cell characteristics, since many epithelial cell lines have been reported to be poor responders to trypsin in relation to cell dispersion.

Scanning electron microscopy (SEM) of porcine ES cells has confirmed the presence of rounded or polygonal closely associated cells with numerous microvilli abundant on the apical cell surface [27, 61]. SEM has also shown that undifferentiated ICMs are very compact, with no distinctive cellular junctions [29]. These junctions become more apparent as differentiation proceeds. The advancing differentiation also results in increased cell diameter.

**Immunocytochemistry and PCR**

Markers specific either for undifferentiated or differentiated cell types are commonly used for characterization of ES cell lines. The relative abundance of alkaline phosphatase (AP) is one of the most commonly used markers of the undifferentiated pluripotent state of cells. AP labelling has consistently been found in the earliest developmental stages of embryos and in undifferentiated mouse ES and embryonal carcinoma (EC) cells [52], but this labelling is lost as soon as the cells differentiate. The same also seems to be true for porcine cells [19, 20, 29–31, 52]. The only exception is in neuron-like differentiated cell cultures that still remain positive for AP [20, 52]. In a previous study of porcine blastocysts, in contrast to the trophectoderm, the ICM was uniformly and intensely stained for AP [52]. Positive staining was also detected in porcine embryo-derived cells from the earliest stages of culture up to the time of their differentiation. The intensity of AP staining in the endoderm-like colonies was weak and sporadic.

Another marker that has been shown to function as a key transcription factor in the maintenance of pluripotency in early embryos and ES cells in the mouse and human is the Oct-4 (POU5F1) transcription factor [63]. Surprisingly, Oct-4 protein was present in both the ICM and trophectoderm of not only porcine in vivo and in vitro blastocysts [64], but also in the trophectoderm of cattle and goats [64, 65, 66]. Furthermore, Keefer et al. [65] detected Oct-4 gene expression by RT-PCR in porcine endoderm cell lines and caprine embryonic cell cultures and detected it by immunocytochemistry in the trophectoderm of day 11 porcine ovoid blastocysts. Contrary to this, Vejlsted et al. [67], who examined post-hatching blastocysts 8–9 days postinsemination, reported localization of Oct-4 exclusively in the nuclei of ICMs. The only study that investigated the expression of Oct-4 in porcine ES-like cell colonies was done by Brevini et al. [38]. RT-PCR revealed Oct-4 positive colonies even after the 4th passage. These colonies were also positive for another marker of an undifferentiated phenotype, nanog. Further more, it is well documented that mouse ES cells are positive for the surface antigen SSEA-1. A previous study showed that freshly dissected porcine ICMs also exhibited positive immunostaining for SSEA-1, whilst the surrounding trophectodermal, and endodermal layer were negative [25]. Nevertheless, SSEA-1 labelling was completely lost as early as when these cells were trypsinized.

Intermediate filament protein vimentin, the marker of mesodermal differentiation, has not been found in porcine ES-like cells [6, 14, 24, 51]. Also, another marker of differentiation, cytokeratin 18
(CK 18), which is a component of the cytoskeleton of differentiated polarized cells, has not been detected in the porcine ICM [25, 29]. In contrast, several previous studies have shown that all the cells constituting the trophectodermal and endodermal layers exhibited strong immunoreactivity to cytokeratin and that the expression of CK 18 progressively increased after culturing ICMs in vitro [6, 25]. Expression of laminin, a factor of the extracellular matrix that is expressed in differentiated endoderm-like cells, was not detected by Wianny et al. [25] in the freshly isolated ICM and trophectoderm, but it was detected in the freshly isolated primitive endoderm layer and in ICM cells after plating. Lamins A/C, which are not expressed in undifferentiated mouse and human ES cells [68], have been detected in porcine ES-like cells [69].

Spontaneous and Induced Differentiation of ES Cells

In vitro differentiation

The ability of porcine embryo-derived cells to differentiate in vitro was evaluated using three different strategies: i) spontaneous differentiation, ii) ability to create embryoid bodies and iii) chemically induced differentiation. Usually, when cells are permitted to reach a high density in prolonged culture, their differentiation occurs spontaneously [14, 19–21, 27, 28, 30, 31, 47, 61]. They tend to differentiate into various cell types, including those of mesodermal, neuroectodermal, ectodermal and endodermal origin. Muscle [14, 19, 21, 27, 30, 31, 61], fibroblast [14, 19, 20, 27, 30, 31, 61], adipocyte [27, 61], macrophage [70], neural-like [14, 19, 20, 27, 30, 31, 54, 61], endoderm-like [14, 20, 28], trophectoderm-like [19, 20, 30, 31, 54] and epithelial-like cell types [19–21, 27, 28, 30, 31, 61] have also been described. Overtly differentiated cells normally fail to re-attach and are lost during sub-culture [14, 27]. The amount of lipid droplets decreases in differentiated colonies [19, 20, 30, 31], and the proliferation of colonies gradually decreases [20, 25]. The AP activity of differentiated cell types rapidly disappears [19, 20, 23, 29–31, 52], with the exception of neural-like cells [20, 23, 52].

The possibility of chemically-induced differentiation has been tested after addition of all-transretinoic acid (RA) [20, 25, 61], or dimethyl sulfoxide (DMSO) [61], to culture medium in the presence of a feeder layer. The ability to respond to RA and DMSO treatment by differentiation into a wide range of cell types suggests that porcine ES-like cells are not yet terminally differentiated.

When cells were seeded onto non-adhesive substrates and cultured in suspension, they formed aggregates resembling mouse embryo bodies (EB) [6, 14, 19, 20, 22, 23, 27, 30, 31, 38, 39, 61]. The majority of these cystic embryo bodies differentiated from epithelial (ectodermal) into endodermal and mesodermal cell types, which was confirmed by molecular analysis [38]. The differentiation was much more extensive when EBs were permitted to attach to the new feeder layer [14, 20, 27, 38]. In general, the ability to form embryoid bodies that can give rise to different differentiated cells indicates that the colonies obtained from porcine embryos are at least pluripotent.

In vivo differentiation

To test their pluripotency, ES cells are injected i) into nude mice and the formation of teratomas is examined or ii) into blastocyst-stage embryos to produce chimeras. The first attempt to analyze the eventual formation of teratomas in athymic mice was unsuccessful [6]. Nevertheless, later on when Hocherau de Reviers and Perreau [22] subcutaneously injected nude athymic mice with porcine ES-like cells, teratomas composed of abnormal cartilage mixed with blood cells and hematopoietic tissue surrounded by muscles and epithelia formed. On the other hand, no teratomas were detected in a mouse that had received irradiated porcine fibroblasts.

Up to now, chimeric offspring in the pig have been obtained from blastocysts injected with either fresh ICM cells [21, 71–73], cultured EG cells [74] or ES cells [20, 61] or eventually from injections of ES cells into the perivitelline spaces of 4- to 8-cell stage embryos [75]. Production of chimeric piglets either from blastomere aggregation [76] or morula injection [72] has been unsuccessful. Germ-line chimerism in the pig has only been reported by Anderson et al. [21], Onishi et al. [72] and Nagashima et al. [73], but these authors used freshly isolated ICM cells for injection into the blastocoel.

In spite of the intensive effort to produce chimeras by injecting cultured porcine ES-like cells into recipient blastocysts [6, 20, 21, 23, 27, 61, 75, 77], the birth of chimeric piglets, documented by coat-colour chimerism and DNA markers, has only been
demonstrated in three cases [20, 61, 75]. Notarianni et al. [77] reported chimerism and transgenesis in the pig when using an embryonic cell line transformed by electroporation. Their results represent the first report of transgenesis in the pig at fetal stages, with the exception of the most commonly used method, pronuclear injection. Golueke et al. [75] reported the birth of piglets after injection of cells from transgenic an embryonic cell line into in vivo embryos. Three piglets were confirmed positive for the marker gene in various endoderm and/or mesoderm derived tissues, and the three remaining piglets had positive skin samples, suggesting contribution to ectoderm derived tissues. However, germ-line chimerism has not been confirmed in any cases [20, 61, 75, 77].

Chen et al. [20] analysed not only the in vivo developmental competence of injected embryos but also the integration of ES-like cells into the ICMs in the host embryos. FITC-labelled ES-like cells were incorporated not only within the host ICM but also within the trophoderm of expanding blastocysts that developed from injected early blastocysts. The influence of passage number and duration of in vitro culture on the ability of porcine ICM-derived cells to produce chimeric embryos were determined by Shiue et al. [23]. They found that cultured cells had the potency to integrate into both the ICM and trophoblast of the host blastocyst. They also mentioned that prolonged culture for up to 6 days in each passage has detrimental effects on chimera generation.

The results of the above-mentioned papers describing ES cells derivation from pig embryos are summarized in Table 1.

**Conclusion**

Establishment and maintenance of ES cell lines from porcine blastocysts is much more complicated than from mouse embryos or primate embryos. To date, there are no suitable culture conditions for preventing of the spontaneous differentiation and occurrence of senescence in putative porcine ES cell lines. Also, it is not yet clear which feeder layer is the most convenient for porcine cells, either for establishment of ES cell lines or maintenance of these cells in the undifferentiated pluripotent state. Two basic types of cell morphologies are observed in porcine embryo derived cultured cells, ES-like and epithelial-like cells. Porcine embryo-derived cells are similar to mouse ES cells; they have large translucent nuclei, one or two prominent nucleoli and a high nucleocytoplasmic ratio. They proliferate more slowly than murine ES cells and have a tendency to differentiate rapidly in in vitro culture. The ability to differentiate in vitro, either spontaneously or after controlled induction, into different cell types indicates that embryo-derived porcine stem cells are at least pluripotent. The in vivo differentiation potential of porcine ES cells has been confirmed by the formation of teratomas after subcutaneous injection of these cells into nude mice [22] and by creation of chimeric offspring after blastocyst injection [61]. Unfortunately, germ-line chimerism in pigs has not been confirmed. However, in spite of great research activity, no ES cells from any vertebrate species other than the mouse [58] and chicken [78] colonize the germ-line.

Embryonic stem cells are considered to be a powerful tool for therapeutic applications, including tissue regenerative medicine and tissue engineering in human medicine. Before these methods are introduced into practice, it is logical that there is the evident requirement to verify their efficiency and suitability and to exclude any detrimental effects. As the mouse physiology, anatomy and life span differ significantly from that of humans, the rodent models seem to be inappropriate [79]. Due to immunological and physiological similarities between pigs and humans and due to a more diverse genetic background, the pig seems to be much better animal model compared with the commonly used rodents. There is also a great interest in using genetically modified pigs as organ donors for xenotransplantation, as well as a model for human diseases [80].

Pluripotent cell lines can provide a suitable in vitro model for studying early embryonic development and can be used for gene targeting technologies. Although a germ-line chimera has yet to be obtained in the pig, nuclear transfer technology with transfected ES-cells provides an alternative option for gene targeting. Gene transfer in pigs can thus be focused not only on establishment of suitable animal models for studying human diseases and stem cell therapies but also for production of different tissues and organs for xenotransplantation. These approaches can also lead to further improvement in productivity traits, reproductive performance and disease resistance in
<table>
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<tr>
<th>Authors</th>
<th>Max passage number</th>
<th>Age and source of embryos</th>
<th>Cultivation medium</th>
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<th>Marker expression</th>
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<td>Evans et al., 1990 [14]</td>
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<tr>
<td>Anderson et al., 1994 [21]</td>
<td>10 p</td>
<td>iv 6–10 d</td>
<td>D + FCS, CS</td>
<td>immunosurgery</td>
<td>STO</td>
<td></td>
<td>muscle, epithel, fibrobl</td>
<td></td>
</tr>
<tr>
<td>Prell et al., 1994, 1995 [51, 56]</td>
<td>4 p</td>
<td>iv 8 d</td>
<td>D + different growth factors</td>
<td>calcium ionophore (bFGF)</td>
<td>VIM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheeler, 1994 [63]</td>
<td>44 p</td>
<td>iv blastocysts</td>
<td>SC medium then BRL-CM</td>
<td>STO then feeder free</td>
<td>EB, muscle, fibrobl, adipocyte, neural</td>
<td>coat colour chimerism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerfen &amp; Wheeler, 1995 [27]</td>
<td>cell lines</td>
<td>iv 5–7–5 d</td>
<td>D + FBS</td>
<td>intactor</td>
<td>STO</td>
<td>EB, muscle, neural, adipocyte, epithel, fibrobl</td>
<td>no chimera</td>
<td></td>
</tr>
<tr>
<td>Ropeter-Scharfenstein et al., 1996 [24]</td>
<td>4 p</td>
<td>iv 7–9 d</td>
<td>D + diff. factors or CM</td>
<td>microsurgery</td>
<td>Different kinds of feeder</td>
<td>VIM, keratin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wianny et al., 1997 [25]</td>
<td>2 p</td>
<td>iv 7 or 11 d</td>
<td>D + FCS (LIF)</td>
<td>immunosurgery with SNI/38 or microsurgery</td>
<td>PEF</td>
<td>SSEA1, CK18, laminin</td>
<td>spontaneous differentiation, induced differentiation by RA treatment</td>
<td></td>
</tr>
<tr>
<td>Notariani et al., 1997 [27]</td>
<td>cell lines</td>
<td>iv 8 d</td>
<td>STO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chene et al., 1999 [20]</td>
<td>2 months</td>
<td>iv 6–7 d</td>
<td>D + FBS</td>
<td>intactor immunosurgery</td>
<td>STO</td>
<td>AP</td>
<td>EB, neural, fibrobl, endo, TE, epith, muscle</td>
<td>chimeric piglets, NT - blastocyst</td>
</tr>
<tr>
<td>Miyoshi et al., 2000 [36]</td>
<td>35 p</td>
<td>iv morula or 6D + FBS–8</td>
<td>intactor immunosurgery</td>
<td>STO</td>
<td>AP</td>
<td>EB, neural, fibrobl, endo, TE, epith, muscle</td>
<td>chimeric piglets, NT - blastocyst</td>
<td></td>
</tr>
<tr>
<td>Li et al., 2003, 2004 [30, 31]</td>
<td>9 p</td>
<td>iv 7–9 d</td>
<td>D + FBS (bFGF, LIF)</td>
<td>immunosurgery, digestive method or intact</td>
<td>MEF, PEF, STO</td>
<td>AP</td>
<td>EB, muscle, neural, epithel, fibrobl</td>
<td></td>
</tr>
<tr>
<td>Li et al., 2004 [19]</td>
<td>4 p</td>
<td>iv 7–9 d</td>
<td>D + FBS (bFGF, LIF)</td>
<td>intactor</td>
<td>MEF</td>
<td>AP</td>
<td>EB, fibrobl, neural</td>
<td></td>
</tr>
<tr>
<td>Ock et al., 2005 [37]</td>
<td>4 p</td>
<td>iv 6D + FBS (bFGF, LIF)</td>
<td>D + FBS</td>
<td>immunosurgery</td>
<td>STO</td>
<td>AP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brevini et al., 2005 [38]</td>
<td>9 p</td>
<td>iv 5–7–5 d</td>
<td>D + KOSR + FBS (LIF)</td>
<td>immunosurgery</td>
<td>STO</td>
<td>Oct4, Nanog, TE</td>
<td>EB, mesodermal, endodermal, neuroectodermal</td>
<td></td>
</tr>
<tr>
<td>Lazzari et al., 2005 [48]</td>
<td>no pass</td>
<td>iv 6D + FBS (bFGF, LIF)</td>
<td>immunosurgery or intact</td>
<td>STO</td>
<td>Oct4, Nanog, TE</td>
<td>EB, mesodermal, endodermal, neuroectodermal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shiu et al., 2006 [22]</td>
<td>15 p</td>
<td>iv 5.5–6.5 d</td>
<td>D + FBS</td>
<td>immunosurgery</td>
<td>STO</td>
<td>AP</td>
<td>EB, incorporation into K.M. of the host blastocyst</td>
<td></td>
</tr>
<tr>
<td>Kim et al., 2007 [39]</td>
<td>5 p</td>
<td>iv, ivF, ivNT</td>
<td>D + FBS (LIF)</td>
<td>intactor immunosurgery</td>
<td>STO, MEF, FCS, feeder free</td>
<td>AP</td>
<td>EB, spontaneous differentiation</td>
<td></td>
</tr>
<tr>
<td>Blomberg et al., 2007 [54]</td>
<td>8 d</td>
<td>Iv blastocyst</td>
<td>D/M199 + FBS</td>
<td>3 step dissection</td>
<td>STO</td>
<td>Oct4, Nanog, TE</td>
<td>EB, mesodermal, endodermal, neuroectodermal</td>
<td></td>
</tr>
</tbody>
</table>

animal husbandry.

Due to the broad potential use of ES cells, especially for genetic modifications, there is increasing interest in establishment of true ES cell lines, not only from the pig, but also from other livestock. In this survey, we attempted to review the current results and knowledge concerning ES cell research in relation to only the pig. Unfortunately, it is still difficult to derive and maintain ES cells from embryos other than those of a few strains of mice [65]. Due to the tremendous amount of research in this field, we do not mention the results and progress in other farm animals or the promising results concerning use of porcine primordial germ cells [74, 80, 81] and somatic stem cells [82, 83, 84]. All three of these types of stem cells can serve as a model for application of stem cell therapies to humans, chimera production or nuclear transfer approaches for production of transgenic animals that are able to transfer a modified genotype to their progeny.

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References


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