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Isolation and culture of pluripotent cells from *in vitro* produced porcine embryos

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Summary

The present study was designed to examine whether *in vitro* produced porcine embryos can be used to establish an embryonic stem (ES) cell line. Porcine embryos were produced by *in vitro* maturation and *in vitro* fertilization. Embryos at the 4-cell to blastocyst stages were cultured in an ES medium containing 16% fetal bovine serum with mouse embryonic fibroblasts as a feeder layer. It was found that ES-like colonies were derived only from blastocysts. When these ES-like colonies were separated in 0.25% trypsin–0.02% EDTA solution and cultured again, ES-like colonies were further observed in the subsequent culture until the fourth passage. The cells from ES-like colonies showed positive alkaline phosphatase activity. Some cells from the colonies differentiated into several types of cells *in vitro* when they were cultured in the medium without feeder layers and leukemin inhibitory factor. Embryoid bodies were also formed when the cells were cultured in a suspension status. These results indicate that porcine ES-like cells can be derived from *in vitro* produced porcine blastocysts and these ES-like cells are pluripotent. The culture system used in the present study is useful to isolate and culture ES cells from *in vitro* produced porcine embryos.

Keywords: Embryo, ES cells, Pig

Introduction

Embryonic stem (ES) cells are useful in the study of cell differentiation, embryonic development and gene regulation, for producing transgenic animals and for other research. Isolation and culture of embryo-derived ES cell lines from preimplantation embryos has been reported in many mammals including mouse (Evans & Kaufman, 1981), sheep (Handyside *et al*., 1987; Tsuchiya *et al*., 1994), mink (Sukoyan *et al*., 1992), cattle (Saito *et al*., 1992; Cherny *et al*., 1994), rabbit (Neimann & Strelchenko, 1994), pig (Gerfen & Wheeler, 1995; Shim *et al*., 1997; Chen *et al*., 1999), primate (Thomson *et al*., 1995) and human (Thomson *et al*., 1998; Shamblott *et al*., 1998). However, ES cell lines have been established only in a few animals, including mouse (Kawase *et al*., 2000), from *in vitro* produced embryos. Little research has been done on other animals because of difficulties and limited practical use.

It is believed that the establishment of ES cell lines and associated studies in the pig offer advantages over other species because pigs are immunologically and physiologically closer to humans (Phillips & Tumbleson, 1986). Thus isolation and culture of porcine ES cells has important applications in medicine, particularly in biological reactors and xenografting. However, limited information can be found in the literature on porcine ES cell line research (Gerfen & Wheeler, 1995; Shim *et al*., 1997; Chen *et al*., 1999). This may be due to the limited supply of porcine embryos, suboptimal embryo culture systems and ES cell culture systems. Usually, embryos used for ES cell research are collected from living animals. However, it is costly and laborious to collect embryos from large animals, such as domestic animals, and it is almost impossible to collect embryos from primates and humans. Thus, *in vitro* produced embryos would provide an approach to conduct such research.

Recently, *in vitro* production of porcine embryos by *in vitro* maturation (IVM) and *in vitro* fertilization

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(IVF) has made it possible to produce high-quality porcine embryos (Wang & Day, 2002). However, it is still difficult to isolate inner cell mass from *in vitro* produced blastocysts as fewer inner cells are present in the blastocysts (Boquest *et al*., 1999). Therefore, in the present study we used intact embryo culture to examine whether such a method can be used to establish ES cell lines in the pig. Our results indicate that it can, and that it may also be applicable to other mammals, such as primate and human.

Materials and methods

Preparation of feeder layers

The feeder cell layers (embryonic fibroblasts) were prepared from fetal mice. Briefly, female mice (Kunming white mouse, provided by the Institute of Zoology, Chinese Academy of Sciences) at day 12–14 of pregnancy were killed by cervical dislocation. Fetuses were separated from uteri and then washed twice in fresh phosphate-buffered saline (PBS) to remove any blood. After the head and liver had been removed, the carcasses were treated in 0.25% trypsin–0.02% ethylenediamine tetraacetic acid (EDTA) solution for 30 min at room temperature until they were broken down into single cells: mouse embryonic fibroblasts (MEF). The cells were resuspended and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v:v) new calf serum (NCS; Gibco, USA.), 5% (v:v) fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin (Sigma, USA) and 0.05 mg/ml streptomycin (Gibco). Feeder layers were prepared after the MEF had one to six passages.

For preparation of the feeder layer, the MEFs were inactivated by incubation in a medium containing 10 μg/ml mitomycin C (Sigma) for 2.5 h. The MEFs were then washed three times in PBS and treated in 0.25% trypsin–0.02% EDTA solution. The trypsinized cells were harvested by centrifugation at 1000 rpm for 3 min and the pellets resuspended in MEF medium. The cell suspension was seeded at a density of 5×10^4 cells per well in a 96-well plate (Sigma) coated with 0.1% gelatin (Fluka, Switzerland). Usually, the cells were plated 1 day before porcine embryos were seeded.

In vitro production of porcine embryos

Porcine embryos were produced by IVM and IVF as reported previously (Wang & Day, 2002). Briefly, immature oocytes were aspirated from medium-sized follicles of ovaries collected at a local slaughterhouse and cultured in modified tissue culture medium-199 (Sawai *et al*., 1997) for 44 h at 39 °C, 5% CO_2 in air. Matured oocytes were inseminated with frozen-thawed semen (5×10^6 cells/ml) in a modified Brackett and Oliphant medium (Wang & Niwa, 1997) for 6 h. Fertilized oocytes were transferred into North Carolina State University (NCSU)-23 medium supplemented with 0.4% bovine serum albumin for further culture in the same conditions

Embryo culture

Embryos were cultured in NCSU-23 medium for 2–5 days. Then, embryos with normal morphology at the 4-cell to blastocyst stages were selected. The zona pellucida of embryos was removed in 0.2% pronase (Sigma) solution. Zona-free embryos were washed and individually transferred to 96-well plates containing MEF feeder layer with 200 μl of ES medium. The ES medium consisted of DMEM with 0.1 mM β-mercaptoethanol (Amresco; 3151B55), 100 IU/ml penicillin, 0.05 mg/ml streptomycin, 0.1 mM MEM non-essential amino acids (Gibco), 20 ng/ml recombinant human-fibroblast growth factor-basic (rh-bFGF) (Sigma), 40 ng/ml recombinant human-leukemin inhibitory factor (rh-LIF) (Sigma), nucleosides (Sigma; 0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine) and 16% FBS. Culture medium was changed every day and the growth and development of embryos and colonies were examined daily.

Isolation and passage of ES-like colonies

Approximately 3–5 days after embryos were seeded, ES-like colonies were individually picked off the feeder layer and dissected in a microdrop of 0.25% trypsin–0.02% EDTA for 3–5 min at room temperature. The disaggregated colonies were individually re-seeded onto new mitomycin C-inactivated MEF feeder layers in 96-well plates and continued in culture. Culture medium was also changed every day.

Alkaline phosphatase histochemistry

After different culture periods, the medium was removed from the cultures and the cells (colonies) were fixed with 1% (w/v) paraformaldehyde 7.5%(w/v) sucrose in PBS. After being washed in Tris-HCl (100 mM Tris-HCl, pH 9.5, 50 mM NaCl, 50 mM $mgCl_2$ and 0.1%Tween-20) three times, each for 10 min, a staining solution was added. This solution consists of solutions A and B: solution A is 75 mg/ml nitroblue tetrazolium salt (NBT, Bicm) in 70% dimethylformamide (DMF, Amresco); and solution B is 50 mg/ml 5-bromo-4-chloro-3-indolyphate toluicinium salt (BCIP, Sigma) in 100% DMF. Just before staining, 45 μl solution A and 35 μl solution B were added to 10 ml Tris-HCl buffer. The ES cells were stained purplish-blue and differentiated cells were not stained.

Table 1 Isolation and culture of embryo-derived cells from *in vitro* produced porcine embryos

Embryo stage	No. of embryos		No. of primary ES-like colonies	Passage of ES-like colonies
	Total	Attached (%)		
4-cell	6	1 (16.6)	0	0
8-cell	10	3 (30.0)	0	0
Morulae	36	16 (44.4)	0	0
Early blastocyst	21	13 (61.9)	2	0
Blastocyst	12	9 (75.0)	2	3

Experiments were repeated five times.

Assessment of *in vitro* differentiation of ES-like cells

The ES-like colonies were picked up with a pulled mouth pipette from the feeder layers, and disaggregated by incubation in trypsin/EDTA solution as described above. Dissociated cells or small clumps were transferred into a 96-well or 4-well plate containing ES medium without LIF and without a feeder layer to continue in culture. The medium was changed every other day. Cultures were monitored daily for cell morphology changes and embryoid body (EB) formation.

Results

In vitro production of porcine embryos

Two to five days after insemination, a total of 85 normal embryos were obtained from 5 replicates. Of the embryos, 6 were at the 4-cell stage, 10 at the 8-cell stage, 36 at the morula stage, 21 at the early blastocyst stage and 12 at the blastocyst stage.

Growth of porcine embryos and formation of ES-like colonies in feeder layer

When embryos without a zona pellucida were placed on the feeder layer (Fig. 1A) and cultured for 2–3 days, the proportions of embryos attached to the feeder cell layers were 16.6%, 30.0%, 44.4%, 61.9% and 75.0% for 4-cell embryos, 8-cell embryos, morulae, early blastocysts and blastocysts, respectively (Table 1). At the same time, cell proliferation was clearly observed in these attached embryos and some trophoblastic cell-like colonies were formed (Fig. 1B). Primary ES-like colonies were formed from 2 early blastocysts and 2 blastocysts after 5–6 days of culture, but not from embryos at 4-cell to morula stages. The primary ES-like colonies grew as tightly packed mounds and had abundant lipid-like vacuoles (Fig. 1C).

Evaluation of ES-like colonies

After the colonies were disaggregated and re-seeded onto new mitomycin C-inactivated MEF feeder layers in 96-well plates, cells from one blastocyst continued to divide and new colonies were observed within another 2–3 days of culture (Fig. 1D). The colonies were then disaggregated again, some being used for differentiation experiments or alkaline phosphatase (AP) activity tests while others were re-seeded for subsequent culture. Cell colonies were formed until the fourth passage.

Most colonies obtained during the second and third passages expressed AP activity (Fig. 1E). When cells from the first or second passage were cultured in the medium without LIF and feeder cell layers, it was found that they began to differentiate into other cells after 4–5 days, including fibroblast-like and neuron-like cells (Fig. 1F). Embryoid bodies were also formed when small clumps of cells were cultured for 8–10 days. These embryoid bodies grew further and developed into fluid-filled, cystic embryoid bodies (Fig. 1G).

Discussion

The present study indicates that a porcine ES-like cell line can be established from *in vitro* produced embryos. To our knowledge, this is the first report of pluripotent cells being isolated and cultured from *in vitro* produced porcine embryos. ES cell lines are usually originated from inner cell masses of blastocysts which are collected from living animals and isolated by immunosurgical technique. However, when we used the same technology we could not isolate the inner cell mass from *in vitro* produced porcine embryos because only a few inner cells are present in *in vitro* produced porcine blastocysts. In most cases, there were no obvious inner cell masses. This makes it difficult to use the traditional method for inner cell mass isolation. Similarly, only a few cells from the inner cell mass of *in vitro* produced porcine blastocysts were observed by

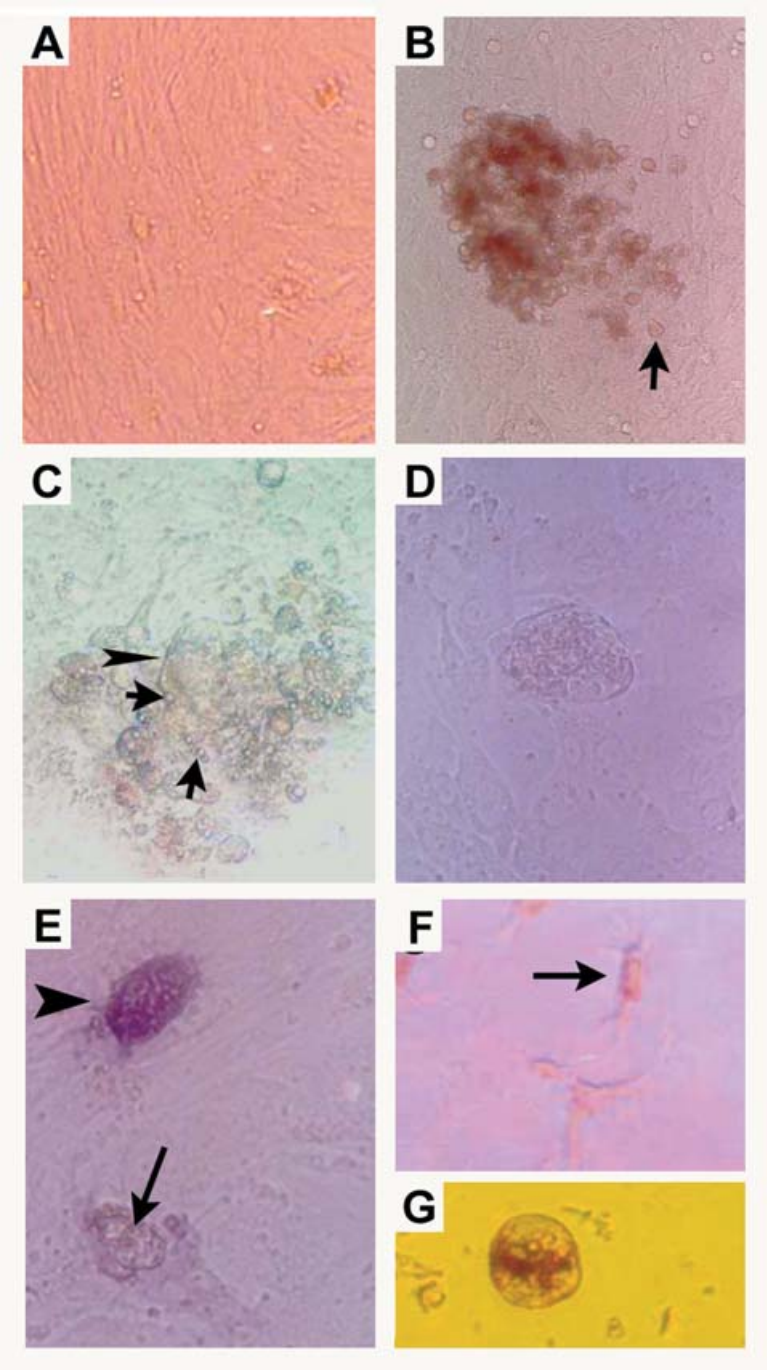

Figure 1 Formation of ES-like colonies from *in vitro* produced porcine embryos. (A) Feeder layer of mouse embryonic fibroblasts (×100). (B) An embryo attached on the feeder layer and trophoblastic cell-like colonies formed from the embryo (arrow; ×200). (C) Primary ES-like colony (arrowhead; ×400) and lipid-like vacuoles (arrows). (D) ES-like colony from a blastocyst after the third passage (arrow; × 400). (E) Alkaline phosphatase (AP)-positive colony (arrowhead; ×200) and differentiated cell colony (AP-negative, arrow; ×200). (F) Neuron-like cell (arrow; ×200). (G) Cystic embryoid body (EB) in suspension culture (arrow; × 100).

Boquest *et al*. (1999). Therefore, in the present study we used an intact embryo culture method to establish an ES-like cell line. The results indicate that intact blastocyst culture is an effective method to establish an ES cell line if *in vitro* produced embryos are used.

Primary cell colonies were observed from early blastocysts and blastocysts. These results suggest that blastomeres may start to differentiate to inner cells at the early blastocyst stage. However, only one cell line was established and maintained in culture for

three passages; the cells from the primary colonies of other embryos differentiated after the first passage. There are two possible reasons for this. First it is probable that only a few or no pluripotent cells were present in *in vitro* produced embryos or blastocysts, thus most blastomeres differentiate during culture.This hypothesis is supported by a previous study indicating that only a few inner cells are present in *in vitro* produced blastocysts (Boquest *et al*., 1999). We found that when *in vivo* produced blastocysts were used to establish the cell lines, most blastocysts (~90%) had a clear inner cell mass and primary colonies could be obtained from most (70%) embryos (Li *et al*., 2003). At or before the morula stage, the low number of uncommitted inner cells might limit the ability to derive colonies suitable for cell-line establishment. It has been reported that the colonies derived from late blastocysts were maintained in culture for a longer period than those derived from early blastocysts (Chen *et al*., 1999), suggesting that late blastocysts may have more inner cells.

Second, the present culture system originates from mouse ES cell culture and may be suboptimal for porcine ES cell culture, thus causing some cells to differentiate quickly during culture and then degenerate. Theoretically, embryonic cells at or before the morula stage are, like inner cell mass cells, totipotent, and should be able to develop to pluripotent ES cells in the appropriate environment. This has been reported in mice (Eistetter, 1989), cattle (Strelchenko & Stice, 1994) and mink (Sukoyan *et al*., 1993). However, we did not obtain an ES cell line from early-stage embryos in the present study. Again, this may be due to the suboptimal culture system. Embryonic cells at different stages may need different growth enviroments. In the present culture system, we used a feeder layer derived from mouse fetuses, which is one of the key factors for ES cell growth (Piedrahita *et al*., 1990) but it may not be ideal for *in vitro* produced porcine embryo development. The effects of other sources of feeder cells are being investigated in our laboratory.

There are many specified characteristics of putative ES cells. The most important of these include morphology, such as colony formation, AP activity and the ability to undergo differentiation. In the present study we used morphology, AP activity and *in vitro* differentiation to evaluate whether the cells in our cell line were ES-like cells. We found that these cells had ES-like colony morphology, showed positive AP activity, differentiated to other cells and formed embryonic bodies in culture. These results indicate that the cells are ES-like cells and pluripotent, although further study is required to enhance the efficiency of ES cell culture in the pig.

In summary, the present study indicates that intact embryo (blastocyst) culture can be used to isolate and establish an ES-like cell line in the pigs. Such a method can be used to culture blastocysts that do not have a clear inner cell mass or only a few cells in the inner cell mass, such as *in vitro* produced blastocysts. It is necessary to improve the culture system for establishment of a porcine ES cell line. The present method may be applicable to other mammals in which high-quality *in vivo* blastocysts cannot be obtained, such as primate and human.

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