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In vitro culture period but not the passage number influences the capacity of chimera production of inner cell mass and its deriving cells from porcine embryos☆

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Abstract

Mammalian embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst. These cells are able to proliferate continuously without differentiation in vitro under suitable conditions. Their capacity of pluripotency in differentiation will be resumed when they are reintroduced into host embryos, when they will contribute to the embryonic development to form chimeric individuals. Manipulation of ES cells has been mainly established from studies in the mouse, and is powerful in the production of transgenic animals. Porcine ICM-derived cell lines possess the same cellular morphology and in vitro behavior as those of murine ES cells, but have lower efficiency in chimera formation when reintroduced into host embryos. This study was to determine the influences of passage number and the duration of in vitro culture on the capacity of porcine ICM-derived cells in the generation of chimeric embryos. The results showed that when passage number of porcine ICM-derived cells was less than 15, there were no detrimental effects on its integration ability. Extending the culture time up to 6 days in each passage of porcine ICM-derived cells impaired its integration capacity into the host blastocyst. Porcine ICM-derived cells cultured for more than 4 days in each

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passage should not be used for blastocyst injection if high efficiency of chimera production is to be achieved.

Keywords: Pig; Embryonic stem cells; Chimera; Passage number; Culture period

1. Introduction

Embryonic stem (ES) cell lines are derived and established from in vitro culturing the inner cell mass (ICM) in blastocysts (Evans and Kaufman, 1981). They have been demonstrated to be pluripotent and are able to renew themselves without differentiation under optimal in vitro conditions (Evans and Kaufman, 1981). An ideal pluripotent ES cell line will extensively contribute to the formation of all somatic tissues as well as the germ line of a chimeric animal, whether produced by blastocyst injection (Stewart, 1993; Peli et al., 1996), morula injection (Tokunaga and Tsunoda, 1992; Stewart, 1993), blastomere aggregation (Tokunaga and Tsunoda, 1992; Peli et al., 1996), or co-culture (Wood et al., 1993).

ES cell technology has evolved from the establishment of murine ES cell lines and has been widely applied in the fields of embryology, developmental biology, as well as the studies of control and regulation of gene expression. Development of ES cell technology in large-size domestic species would greatly benefit both the agricultural and biomedical sciences. Many attempts have been made to isolate ES cell lines from species other than the mouse based on information accumulated from studies on murine embryonal carcinoma (EC) and ES cells. Isolation of putative pluripotent ES lines has been attempted in hamster (Doetschman et al., 1988), mink (Sukoyan et al., 1993), rabbit (Graves and Moreadith, 1993), rat (Ouhibi et al., 1995), non-human primate (Thomson et al., 1995), pig (Talbot et al., 1993; Chen et al., 1999), cattle (First et al., 1994), sheep (Handyside et al., 1987), and human beings (Thomson et al., 1998). Isolation of pluripotent ES cells from domestic animals would provide a method for the manipulation, modification, and multiplication of animals with superior genetic background for growth, production, or disease resistance (Modlinkski et al., 1996). It is a new pathway to produce transgenic animals and modelling the process and mechanisms for differentiation, in addition to modifying the composition of animal products for pharmaceutical and biomedical purposes (Wilmut et al., 1991). In particular, establishment of ES cell lines from an animal with similar body size and physiological conditions to humans, such as pigs, would allow the development of animal models more closely related to the human than the mouse. This would benefit and facilitate the development of new strategies in gene and drug therapies for humans (Moore and Piedrahita, 1997a,b).

One criterion used to evaluate the in vivo differentiation capacity of ES cells is the efficiency of chimera formation after their reintroduction into other host embryos. However, to produce chimeras from putative pluripotent cells in livestock species has been demonstrated to be less efficient than those from the mouse (Mueller et al., 1999). This might result from a prolonged culture period and/or increased passages that the ES cells experienced before being reintroduced into the host embryos. These culture conditions would

impair the affinity between ES cells and the inner cell mass of the host embryo so as to influence the differentiation capacity of the ES cells (Anderson et al., 1994; Notarianni et al., 1997; Chen et al., 1999). The object of this study was to determine the effects of culture period and the passage number of porcine ES cells on their ability to form chimeric embryos.

2. Materials and methods

2.1. Embryo collection

Maisan gilts aged 8 months were used for donor embryo collection. Following an intramuscular (i.m.) injection of 1000 iu of equine gonadotropin (eCG, Chinese Chemicals, Taiwan), each gilt was given 750 iu of human chorionic gonadotropin (hCG, Chinese Chemicals) 72 h later by i.m. injection. Gilts were artificially inseminated with semen of the same breed 12 (Day 0) and 18 h after hCG treatment. Hatched blastocysts were collected on Day 6.5 using a surgical procedure as described by Chen and Wu (1991). Blastocysts with intact zona pellucida were collected from Lanyu gilts as host embryos by the same procedure as above, except that the day of embryo collection was Day 5.5.

2.2. Derivation and in vitro culture of porcine ES cells

Each inner cell mass was isolated from collected embryos as earlier hatched blastocysts for further culture as previously described by Chen et al. (1999). Briefly, individual intact-hatched blastocysts were cultured onto the mitomycin C-inactivated STO feeder layers in ESM, Dulbecco's Modified Eagle's medium (DMEM, Gibco) containing 16% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β-2-mercaptoethanol (Sigma), 10 mM MEM non-essential amino acids (Gibco), 0.03 mM adenosine (Sigma), 0.03 mM guanosine (Sigma), 0.03 mM cytidine (Sigma), 0.03 mM uridine (Sigma), 0.01 mM thymidine (Sigma), antibiotics (50 units/ml penicillin G and 50 μg/ml streptomycin sulfate, Gibco), and 3.7 g/l sodium bicarbonate.

Each derived ICM-outgrowth was picked up and slightly disassociated in a 0.25% trypsin–0.02% EDTA solution for 30 s and then separated into four aliquot pieces with a glass needle. Two of them were then replanted and cultured onto fresh feeders for cell line establishment. The remaining two pieces were directly subjected to fluorescence labeling for blastocyst injection.

Colonies derived from the in vitro culture of ICM-outgrowths with ES-like morphology were fixed in 80% ethanol and stained for alkaline phosphate (AP) activity using an AP histochemistry kit (Sigma) according to the manufacturer's protocol. After removal of culture medium, the well or plate containing the embryo-derived colonies and their co-cultured feeder layer was washed three times with DPBS and fixed in 80% ethanol (Burehr and McLaren, 1993) for 2–18 h at 4 °C. The fixative was removed, and the well or plate was rehydrated by two changes of deionized water each of 10 min. Following removal of deionized water, the well or plate was stained with freshly prepared stain. The degree of staining was monitored microscopically. When the degree of staining was

satisfactory, the reaction was stopped by rinsing the well or plate twice in deionized water for 10 min.

In vitro differentiation test of the colonies with ES-like morphology was performed by suspension culture to induce the formation embryonal bodies (Chen et al., 1999). Confluent ICM-derived colonies were picked up with a mouth pipette, separated from feeder cells by brief exposure to 0.15% protease E (Sigma) in DMEM, slightly trypsinized and transferred to a suspension culture in a non-adhesive petri dish containing ESM in the absence of a feeder layer. Suspension cultures were monitored daily. When cystic embryoid bodies were found in the suspension cultures, the differentiated structures were transferred and plated onto a dish of fresh feeder layer for further differentiation.

Putative ES cell lines established from these pluripotent colonies were regularly sub-cultured by using 0.25% trypsin–0.02% EDTA and plated onto fresh feeders every 4–7 days. Cells were regularly frozen and thawed as described as elsewhere (Robertson and Bradley, 1986; Chen et al., 1999) for the later usages of the putative ES cells in the study. Briefly, Colonies of exponentially growing cells were harvested and disassociated with trypsin–EDTA into small clumps composed of three to five cells. After neutralization of the trypsin by the addition of DMEM containing 10% fetal calf serum, cells were pelleted and resuspended to a concentration of $1.5–1.8 \times 10^7$ cells/ml in ESM. Two volumes of freezing medium were gradually added to one volume of the cell suspension directly before being dispensed into freezing vials. These vials were immediately placed in a −80 °C freezer and stored for 24 h before being plunged into liquid nitrogen. Thawing of frozen cell stocks was performed at room temperature. Individual vials of frozen cells were placed into 37 °C warm water until ice disappeared. The outer surface of the vial was sterilized by submerging and wiping with 70% ethanol. The contents of the vial were transferred to a 15-ml conical centrifuge tube, and the volume immediately brought up to 10 ml with DMEM containing 10% fetal calf serum. The freezing medium was removed by three repeated steps involving centrifugation and resuspension in DMEM containing 10% fetal calf serum. The resulting pellet then was suspended in ESM and replated onto fresh feeder layers.

2.3. Karyotypic analysis

Karyotypic analysis of the putative ES cell lines was performed at each passage following passage 3 as described by Robertson (1984). Exponentially growing cells were exposed to colcemid (KaryoMax Colcemid solution, Gibco) at a final working concentration of 0.02 μg/ml for 4–6 h at 39 °C. Cells were harvested for hypotonic treatment for 30 min in 0.56% (w/v) KCl aqueous solution following removal from colcemid treatment. The cells were pelleted by centrifugation at $800 \times g$ and fixed in cold Carnoy's fixative (3:1, v/v, of absolute methanol to glacial acetic acid) for 5 min. After a second wash in Carnoy's fixative, the cells were resuspened in a small volume of fixative. Slides were prepared by dropping the cell suspension onto dry microscope slides prewashed with fixative. Immediately after dropping, the slides were exposed to a flame to burn off the fixative, and then stained in 5% Gurr's Giemsa staining solution (Gibco) for 2 min. The stained slides were rinsed, air-dried and examined under a microscope at 1000× magnification with oil immersion. At least 50 metaphases from each passage were evaluated for the number of chromosomes and structural abnormalities.

2.4. Fluorescence labeling and blastocyst injection of porcine ICM-derived cells

The porcine ICMs and their derived cells at different culture stages (2, 4, and 6 days after each passage) and of different passages (0, 6th, 9th, 12th, and 15th passages) were harvested directly before blastocyst injection and labeled with fluroescein isothiocyanate (FITC, Sigma) as described by Honig and Hume (1986). Briefly, cells were suspended in 1 ml of ESM, then incubated for 30 min at 37 °C with FITC at a final concentration of 600 μg/ml. Cells were then washed several times until no fluorescence was detected in the supernatant.

Blastocyst injection was performed under an inverted microscope with the aid of a set of micromanipulators. Directly before injection, the host blastocysts were incubated in TL-HEPES medium containing 7.5 μg/ml of cytochalasin B for 15 min. Thereafter, a group of host blastocysts and fluorescence-labeled cells were placed together in a droplet of TL-HEPES medium and covered with mineral oil. After the host blastocyst was held by a holding-pipette (120 μm o.d. and 15 μm i.d.), a clump of approximately 10 cells was aspirated with an injection-pipette (35 μm o.d. and 25 μm i.d.) and then injected into the blastocoel of the host blastocyst.

2.5. Determination of integration between injected cells and the host blastocyst

Immediately after being injected with labeled ICM or ICM-derived cells, the reconstituted host blastocysts that subjected to in vitro culture in modified Whitten's medium supplemented with 10% of fetal calf serum and 1% penicillin/streptomycin at 38 °C, with a humidity atmosphere of 5% CO_2 in air. Those cultured reconstituted host embryos were then examined under a fluorescent microscope after 24 h of incubation. The integrated cells in the host ICM or trophectoderm were readily distinguished by their bright fluorescence under the fluorescence microscope.

2.6. Statistical analysis

The effects of culture period and passage number were analyzed with a Chi-square test and GLM procedures (SAS, 1996).

3. Results

A total of 54 hatched blastocysts were used for the derivation of ICM-outgrowths (Table 1). Forty-six of them attached to the feeder layers and 16 of them developed into primary colonies with an expanding trophoblast monolayer with a visible central ICM-

Table 1
Efficiency of cell line derivation from porcine hatched blastocysts

No. hatched blastocysts plated	54
No. hatched blastocysts attached	46
No. ICM-outgrowths formed	16
No. cell lines derived	2

Table 2
Effects of in vitro culture period on integration of porcine inner cell mass-derived primary cells to the inner cell mass and trophectoderm of the host embryos after blastocysts injection

Culture period (day)	No. host blastocysts injected	Position of host blastocyst integrated (%)		
		None	Trophectoderm	Inner cell mass
0	20	0 (0)	7 (35.0)[a]	13 (65.0)[a]
2	14	0 (0)	1 (35.1)[b]	13 (92.9)[b]
4	18	1 (5.6)	1 (35.6)[b]	16 (88.8)[b]
6	15	1 (6.7)	2 (35.3)[b]	12 (80.0)[b]
Total	67	2 (3.0)	11 (35.4)	54 (80.6)[b]

Values within the same row with different superscripts (a and b) differ by $P < 0.01$.

outgrowth. Most of the ICM-derived colonies failed to proliferate and were lost before the fifth passage after being picked up and subcultured. Only two developed into stable cell lines (M698 and M215). Both of them embraced a normal porcine complement of 38 chromosomes after passage numbers 6–15. Their morphology, alkaline phosphatase activity, and the responses to the in vitro differentiation induction were similar to the porcine ICM-derived cell lines as described previously (Chen et al., 1999).

Table 3
Effects of culture period and subculture on integration of porcine inner cell mass-derived cells to the inner cell mass and trophectoderm of the host embryos after blastocysts injection

Passage	Culture period (day)	No. host blastocysts injected	Position of host blastocyst integrated (%)		
			None	Trophectoderm	Inner cell mass
0	2	14	0 (0)	1 (35.1)	13 (35.9)
	4	18	1 (35.6)	1 (35.6)	16 (35.9)
	6	15	1 (35.7)	2 (35.3)	12 (35.0)[a]
	Sum	47	2 (35.3)	4 (35.5)[a]	41 (35.2)
6th	2	16	1 (35.1)	2 (35.3)	13 (35.3)
	4	14	1 (35.3)	2 (35.5)	11 (35.6)
	6	19	4 (35.1)	5 (35.3)	10 (35.6)[b]
	Sum	49	6 (35.2)	9 (35.4)[a]	34 (35.4)
9th	2	15	1 (35.7)	0 (0)	14 (35.3)
	4	16	0 (0)	1 (35.3)	15 (35.8)
	6	20	4 (35.0)	6 (35.0)	10 (35.0)[b]
	Sum	51	5 (35.8)	7 (35.7)[a]	39 (35.5)
12th	2	20	0 (0)	0 (0)	20 (35.0)
	4	21	2 (35.5)	1 (35.8)	18 (35.7)
	6	21	6 (35.6)	1 (35.8)	14 (35.7)[b]
	Sum	62	8 (35.9)	2 (35.2)[b]	52 (35.9)
15th	2	16	0 (0)	0 (0)	16 (35.0)
	4	20	4 (35.0)	0 (0)	16 (35.0)
	6	22	7 (35.8)	0 (0)	15 (35.2)[b]
	Sum	58	11 (35.0)	0 (0)[b]	47 (35.0)
Total		267	32 (35.0)	22 (35.2)	213 (35.8)

Values within the same row with different superscripts (a and b) differ by $P < 0.01$.

ICM-derived cells from different passages of cultures and for different culture periods were fluorescence-labeled prior to blastocyst injection. A total of 287 expanding blastocysts collected from Lanyi pigs were used to determine the capacity of chimeric participation. The integration of ICM-outgrowth-derived primary culture cells to the host blastocyst 24 h after injection is shown in Table 2. The integration rates to the host trophoblast of the primary ICM-outgrowth-derived cells were 35.0, 7.1, 5.6, and 13.3%, after in vitro culture for 0, 2, 4, and 6 days, respectively. In addition, to the host ICMs, their integration rates were 65.0, 92.9, 88.8, and 80.0% after 0, 2, 4, and 6 days of in vitro culture. The cells from the primary culture had greater efficiencies of integration to the trophoblast, but not to the ICM of the host blastocysts ($P < 0.01$).

The integration rates of the porcine ICM-derived cells injected into the host blastocysts are shown in Table 3. The integration rates were 87.2, 69.4, 76.5, 83.9, and 81.0%, to the host ICMs for ICM-derived cells at passages 0, 6, 9, 12, and 15, respectively. No statistically significant differences were identified. The integration rates of ICM-derived cells to the host trophectoderm at passage numbers 0, 6, 9, 12, and 15 were 8.5, 18.4, 13.7, 3.2, and 0%, respectively. The integration efficiencies of the ICM-derived cells to the trophoblast from passages 12 to 15 were less than those from earlier passages ($P < 0.01$). After repeated subcultures were performed to six passages, the ICM-derived cells from the same passage and further subcultured up to 6 days showed a significantly lower ability to be integrated into the host ICM ($P < 0.01$).

4. Discussion

It seems that chimeric offspring in pigs can only be obtained from blastocyst injection with embryonic cells (Kasiwazaki et al., 1992; Onishi et al., 1994; Anderson et al., 1994; Nagashima et al., 2004), cultured EG cells (Shim et al., 1997), and ES cells (Chen et al., 1999). Producing chimeric pigs either from blastomere aggregation (Hsu et al., 1990) or morula injection (Onishi et al., 1994) have both failed so far. Piglets with germ-line chimera have been reported by way of blastocyst injection with freshly isolated ICMs (Onishi et al., 1994; Anderson et al., 1994; Nagashima et al., 2004). Blastocyst injection of porcine putative ES cells only produced somatically chimeric piglets rather than germ-line chimerism (Chen et al., 1999).

The low efficiency of generation chimeric pigs, when compared to that of mice, might be due to physiological differences among species. The embryonic ectoderm in murine blastocysts is a simple epithelium, but is discoidal and surfaced with more than one layer of polarized epithelium in the porcine blastocyst (Talbot et al., 1993). It was hypothesized that the polarized epithelial sheets in the porcine ICM come with the early differentiation and development of the blastocyst, and this might reduce the incorporation of injected cells into the ICM (Rui et al., 2004).

We have previously documented the establishment of porcine EG (Shim et al., 1997) and ES (Chen et al., 1999) cell lines to generate chimeric piglets by means of blastocyst injection. In this study, we aimed to determine whether the culture conditions, including passage numbers and culture periods would affect the capacity of putative porcine ES cells to be incorporated into the ICM of the injected blastocysts. The integration rates of freshly

isolated cells for the ICM-outgrowth-derived primary cells (passage 0) to the ICMs and trophectoderms in the host blastocysts were 65.0 and 35.0%, respectively (Table 2). This result was comparable to the fresh-isolated ICMs that were injected into the tetraploid blastocysts described by Prather et al. (1996). The incorporation efficiency of ICM-derived cells to the host trophectoderm was significantly higher than those cells from the same passage but cultured for an extended time period ($P < 0.01$; Table 2). This could have resulted from the contamination of trophoblastic cells accompanied with the fresh-isolated ICMs from the donor blastocysts. Even subsequent to subculture to the ninth passage, 13–20% of fluorescence-labeled cells had been integrated into the trophectoderm of the host blastocysts (Table 3). The integration of fluorescence-labeled cells into the host trophectoderm was decreased when the passage number was increased up to the 12th passage ($P < 0.01$). A repetitive screening against trophoblast contamination could only be accomplished after the 12th passage.

At the primary passages, the integration capacities to the ICMs of the host embryos of the ICM-derived cells that had been cultured for 2, 4, and 6 days were similar. However, after being subcultured for six passages, the ICM-derived cells cultured for 2 and 4 days had a significant higher integration rates into the host ICMs than those cultured for 6 days at an individual specific passage. Prolonged in vitro culture up to 6 days at each passage after the primary culture, tended to impair the ICM-incorporating capacity of ICM-derived cells. These results indicated that the capacities of the porcine ICM-derived cells to form chimeric embryos after blastocyst injections would not be affected by the passage number up to the 15th, but prolonged cultures in each passage up to 6 days had detrimental effects on generating chimeras.

Injected cells among the host blastocysts injected with ICM-derived cells from passages 0 to 15, incorporated into the ICM of the host blastocysts for more than 79% of embryos. No efficiency differences in the generation of ICM-chimeric embryos among donor cells from various passages were found. This result indicated that even up to the 15th passage, the porcine ICM-derived cells preserved their capacities to incorporate into the ICM of the host blastocysts. The major cause of low efficiency in the generation of porcine chimeras did not appear to be an integration problem between the injected ES cells and the ICM of the host blastocysts. Alternatively, successfully incorporated porcine ES cells might not be as competitive as the rapidly growing host ICM and they gradually faded away thereafter. Further studies to determine the fates of incorporated ES cells in the resultant chimeric ICM of the host blastocysts during the subsequently embryonic development and body formation are necessary.

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