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Isolation and initial culture of porcine inner cell masses derived from *in vitro*-produced blastocysts

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Summary

The present study was conducted to isolate and culture inner cell mass (ICM) primarily derived from *in vitro*-produced blastocysts and to develop the culture conditions for the ICM cells. In Experiment 1, immunosurgically isolated ICMs of blastocysts derived from *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT) or parthenogenetic activation (PA) were seeded onto STO cells. Primary colonies from each isolated ICM were formed with a ratio of 28.9, 30.0 and 4.9%, respectively. In Experiment 2, blastocysts collected from IVF were directly seeded onto a feeder layer with or without zona pellucida (ZP), or were subjected to ICM isolation by immunosurgery. Primary colonies were formed in 36.8% of isolated ICMs and 19.4% in intact blastocysts without ZP. In Experiment 3, ICMs from IVF blastocysts were seeded onto STO cells, mouse embryonic fibroblast (MEF) or porcine uterine epithelial cells (PUEC). On STO and MEF cells, 34.5 and 22.2% of primary colonies were formed, respectively. However, no primary colony was formed on the PUEC or in feeder-free condition. In Experiment 4, ICMs from IVF blastocysts were cultured in DMEM + Ham's F10 (D/H medium), DMEM + NCSU-23 (D/N medium) or DMEM alone. When D/H medium or D/N medium was used, 21.7 or 44.4% of primary colony were formed, respectively, while no primary colony was formed in DMEM alone. These cells showed alkaline phosphatase activity and could be maintained for up to five passages. In suspension culture, cells formed embryoid bodies. These results demonstrate that porcine ICM could be isolated and cultured primarily from *in vitro*-produced blastocysts with a suitable culture system.

Keywords: Blastocysts, *In vitro* fertilization, Inner cell mass, Porcine, Somatic cell nuclear transfer

Introduction

In the field of biotechnology, pigs draw more attention than other domestic animals in terms of their great advantages, such as continuous cycling, short generation interval, large litter size and immunological and physiological similarities to human. As reviewed by Wheeler & Walters (2001), genetic modification in swine could have many agricultural and biomedical applications, such as increased growth rate, improved feed utilization, improved carcass composition, increased disease resistance and more environmentally friendly pigs. In the medical field, making specific genetic modifications in pigs provides the possibility of producing recombinant products for biomedical uses, models of human genetic disease for research and drug development and also organ source for xenotransplantation. Some examples are already in existence. Noble *et al*. (2002) produced transgenic gilts expressing bovine alpha-lactalbumin in their milk and Golovan *et al*. (2001) reported the production of pigs expressing salivary phytase making low phosphorus manure. Transgenic pigs expressing the green fluorescence protein (Park *et al*., 2001) and pigs that are deficient in alpha-1,3-galactosyltransferase (Lai *et al*., 2002) were successfully produced by somatic cell nuclear transfer (SCNT).

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The pronuclear DNA microinjection method, routinely used to produce transgenic mice, has been applied for transgenic pigs (Nagashima *et al*., 2003). However, not all injected eggs would develop into transgenic pigs and then not all transgenic pigs would express the transgene in the desired manner. Furthermore, employing the microinjection technology is time consuming and very expensive. Otherwise, SCNT would allow more efficient method for generation of transgenic animals. With the aid of SCNT, existing transgenic animals could be multiplied without going through the germline with its recombination events. This would avoid the time-consuming backcrossing to generate transgenic lines. However, SCNT has low efficiency at birth rate and all somatic cells used as donor cells for SCNT tend to become senescent before sufficient rounds of gene transfer and/or targeting and selection can be performed. This problem may be overcome by isolation of readily transfectable and selectable cells with high proliferative potential and long-term karyotypical normality, like embryonic stem (ES) and embryonic germ (EG) cell lines.

ES cell lines have been reported in species other than the mouse (Evans & Kaufman, 1981), such as cattle (Strelchenko, 1996), rabbit (Graves *et al*., 1993), mink (Sukoyan *et al*., 1992), primate (Thomson *et al*., 1995), rat (Iannaccone *et al*., 1994), hamster (Doetshman *et al*., 1988), human (Thomson *et al*., 1998) and pig (Wheeler, 1994). However, at present, the technologies for the derivation and genetic manipulation of murine ES cells are well established and only in the mouse have germline chimeras been produced (Bradly *et al*., 1984). Attempts have been made to develop ES cells in the pig, but this has met with limited success. Although pig embryo-derived cell lines with ES cell-like morphology have been produced (Evans *et al*., 1990), there has been no report of germ-line transmission of the ES cell genotype. Primordial germ cell-derived lines have been isolated from pig fetuses and transfected lines have been shown to contribute to chimera formation when injected into pig blastocysts (Piedrahita, 1998; Mueller, 1999), but in no case has germ line transmission been demonstrated. Nagashima *et al*. (2004) recently reported that chimeric pigs produced by microinjection of ICM clumps into blastocysts showed similar sex differentiation and germ cell formation to mice.

In vitro production (IVP) of embryos by IVF or SCNT has great potential in animal biotechnology, such as providing blastocysts for ES cell isolation. While IVP of bovine embryos is well established, production of porcine embryos *in vitro* is still challenging with low efficiency and quality. Therefore, few studies have been done on isolation of porcine ES cells using *in vitro*-produced blastocysts (Miyoshi *et al*., 2000; Li *et al*., 2004). Therefore, to establish porcine ES cells from IVP embryos successfully, optimizing isolation and initial culture condition of ICMs derived from IVP blastocysts is required.

In the present study, porcine ICM cells were isolated using *in vitro*-produced blastocysts and culture conditions were optimized for the initial culture of porcine ICMs.

Materials and methods

In vitro production of porcine blastocysts

In vitro *fertilization*

Blastocysts were produced using the method described in our previous study (Kim *et al*., 2004).

Somatic cell nuclear transfer

Blastocysts were produced as described in our previous report (Lee *et al*., 2003a). Briefly, *in vitro*-matured gilt oocytes were enucleated, reconstructed with fetal fibroblasts and simultaneously fused/activated using a single pulse of 2.0 kV/cm for 30 μs.

Parthenogenetic activation

Blastocysts were produced by parthenogenetic activation according to the methods of Hyun *et al*. (2003). *In vitro*-matured oocytes were activated using a single pulse of 2.0 kV/cm for 30 μs and then exposed to 6-dimethylaminopurine (6-DMAP) for 4 h.

In vitro *culture and isolation of inner cell masses (ICMs)*

All the fertilized and reconstructed embryos and activated oocytes were cultured in North Carolina State University (NCSU)-23 medium supplemented with 0.5 mM pyruvate and 5 mM lactate for 7–9 days (Kim *et al*., 2004).

Whole blastocysts were cultured with or without zona pellucida (ZP). ZP was removed by acid tyrode solution (Sigma–Aldrich Corp.). ZP-intact or free whole blastocysts were washed and transferred into a 4-well dish containing feeder layers with D/H medium (Moore & Piedrahita, 1997). The D/H medium had the same volume of DMEM (Life Technologies) and Ham's F10 (Life Technologies). The blastocysts in the other group were used to isolate inner cell masses (ICMs). ZP-free blastocysts were transferred to immunosurgery medium consisting of D/H medium, goat anti-pig whole antiserum and guinea pig complement (GPC) (Life Technologies) with the ratio of 3:1:1, respectively, using a mouth pipette, then incubated at 37 °C, 5% CO_2 for 3–4 h. Blastocysts were moved to a D/H drop when embryos became dark and had irregular surface and pipetted gently in and out to remove dead trophoblast cells. Isolated ICMs were transferred through drops of new D/H medium for rinsing and then seeded into a 4-well dish

containing a feeder layer with D/H, D/N and DMEM according to the experimental plan. D/N medium had the same volume of DMEM and NCSU-23. All kinds of culture medium contain 2.0 mM L-glutamine (Life Technologies), 1.0% MEM (Life Technologies), 1.0% penicillin–streptomycin (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), 1000 U/mL recombinant human leukemia inhibitory factor (hLIF) (Sigma) and 15% fetal bovine serum (Life Technologies).

The attachment and growth of whole blastocysts or ICM were examined daily and culture medium was changed everyday.

Preparation of feeder cells

The mouse STO cells, mouse embryonic fibroblasts (MEF) and porcine uterine epithelial cells (PUEC) were used as feeder cells. To isolate PUEC, uterus epithelium from the 6–8-month-old gilt was scraped with a scalpel, washed with PBS by centrifugation and then cultured for two to three passages until use. All type of cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, 1% non-essential amino acids and 0.1 mM β-mercaptoethanol. For preparation of feeder layer, they were inactivated in DMEM containing 10 μg/ml mitomycin C (Sigma) for 2 h and then the cells 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 5 min and the pellets were resuspended in DMEM. The cell suspension was seeded at a density of 2.5×10^5 cells per well in a 4-well plate (Nunc) coated with 0.1% gelatin (Sigma). Usually, the feeders were plated 1 day before porcine blastocysts or ICMs were seeded.

Passaging of colonies derived from ICMs

Approximately 4–7 days after blastocysts or ICMs were seeded, densely packed ES-like colonies obtained from primary culture were picked from the feeder layer and disaggregated in a microdrop of 0.25% trypsin–EDTA for 5 min at room temperature with the aid of two fine needles and a micropipette under the stereomicroscope. The cells disaggregated from the colonies were seeded onto a fresh feeder layer into a 4-well dish. Putative ES colonies were passaged as described above at 4–7 day intervals until no colony was formed.

Alkaline phosphatase (AP) staining

The AP activity was determined as described previously (Lee & Piedrahita, 2000). After culture, plates were rinsed twice in PBS and fixed in 4% formaldehyde in PBS for 15 min at room temperature, fixed cells were washed twice with PBS and stained in naphthol AS-MX phosphate (200 μg/mL; Sigma) and Fast Red TR salt (1 mg/ml; Sigma) in 100 mM Tris buffer, pH 8.2 for 30 min at room temperature. Washing cultures in PBS terminated staining. Specificity of AP activity was determined by staining in the presence of the AP inhibitor, tetramisole (500 μM; Sigma).

Embryoid body formation of colonies derived from ICMs

ES-like colonies were tested for their ability to differentiate *in vitro* using suspension culture, as well as cultured on plastic in the absence of feeders. ES-like colonies were detached from the plate by gentle trypsinization and cultured on non-adhesive bacterial plates with D/H medium lacking growth factors and with FBS. The suspension culture was observed daily for morphological changes and the medium was changed every other day.

Experimental design

Experiment 1
Blastocysts were derived from IVF, SCNT and PA at day 7–9 of *in vitro* culture and isolated ICMs by immunosurgery. ICMs were then seeded on mitomycin C-inactivated mouse STO cells as a feeder layer.

Experiment 2
Blastocysts collected from IVF were either seeded on mitomycin C-inactivated mouse STO cells directly (whole embryo) with or without ZP (ZP-intact and ZP-free, respectively), or used to isolate ICM by immunosurgery followed by seeding on feeder layer.

Experiment 3
ICMs from IVF blastocysts were seeded on plastic, mitomycin C-inactivated STO cells, MEF or PUEC as feeder layer.

Experiment 4
ICMs from IVF blastocysts were seeded on mitomycin C-inactivated STO cells and cultured in D/H medium, D/N medium and DMEM alone with supplementation of 15% FBS. D/N medium had the same volume of DMEM and NCSU-23.

All blastocysts or ICMs were monitored daily for the attachment to the feeder layer and for colony formation.

Statistical analysis

Experiments were replicated three times and all data were subjected to ANOVA and protected least significant different (LSD) test using general linear models in a statistical analysis system (SAS Institute, version 8.1) program to determine differences among

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Table 1 Effect of the source of blastocysts produced *in vitro* on attachment of blastocysts and colony formation

Group	No. of blastocysts[a]	No. of attached blastocysts (%)[b]	No. of primary colonies (%)[c]
IVF	132	76 (57.6%)	22 (28.9%)[d]
SCNT	65	40 (61.5%)	12 (30.0%)[d]
PA	85	41 (48.2%)	2 (4.9%)[e]

ICMs were isolated from different source of blastocysts by immunosurgery and cultured in the D/H medium on STO feeder cells. Attachment of ICMs and formation of primary colonies were investigated after 1–2 and 4–7 days, respectively.
[a] The rates of blastocyst formation of IVF, SCNT and PA were 20.2, 15.4 and 38.7% from 654, 422 and 220 oocytes, respectively.
[b] Percentage of blastocysts cultured.
[c] Percentage of blastocysts attached.
[d,e] Within the same column, values with different superscripts were significantly different ($p < 0.05$).

Table 2 Effect of seeding methods of blastocysts on attachment of blastocysts and colony formation

Group	No. of blastocysts[a]	No. of attached blastocysts (%)[b]	No. of primary colonies (%)[c]
Immunosurgery	127	87 (68.5%)[d]	32 (36.8%)[d]
Whole mount			
ZP-free	85	36 (42.4%)[e]	7 (19.4%)[e]
ZP-intact	54	15 (27.8%)[e]	0[f]

ICMs or intact blastocysts with/without zona pellucida from IVF were cultured in the D/H medium on STO feeder cells. Attachment of ICMs/blastocysts and formation of primary colonies were investigated after 1–2 and 4–7 days, respectively.
[a] The rate of blastocyst formation was 22.8% from 1167 oocytes.
[b] Percentage of blastocysts cultured.
[c] Percentage of blastocysts attached.
[d,e,f] Within the same column, values with different superscripts were significantly different ($p < 0.05$).

Table 3 Effect of feeder cells on attachment of blastocysts and colony formation

Group	No. of blastocysts[a]	No. of attached blastocysts (%)[b]	No. of primary colonies (%)[c]
None	21	2 (9.5%)[c]	0[c]
STO	97	55 (56.7%)[d]	19 (34.5%)[d]
MEF	44	18 (40.9%)[d]	4 (22.2%)[b]
PUE	21	3 (14.3%)[e]	0[e]

ICMs were isolated from IVF-derived blastocysts by immunosurgery and cultured in the D/H medium on different types of feeder cells. Attachment of ICMs and formation of primary colonies were investigated after 1–2 and 4–7 days, respectively.
[a] The rate of blastocyst formation was 21.5% from 851 oocytes.
[b] Percentage of blastocysts cultured.
[c] Percentage of blastocysts attached.
[d,e] Within the same column, values with different superscripts were significantly different ($p < 0.05$).

Table 4 Effect of culture medium on attachment of blastocysts and colony formation

Group	No. of blastocysts[a]	No. of attached blastocysts (%)[b]	No. of primary colonies (%)[c]
D/H medium	80	46 (57.5%)[d]	10 (21.7%)[d]
DMEM	40	8 (20.0%)[e]	0[e]
D/N medium	48	36 (75.0%)[f]	16 (44.4%)[f]

ICMs were isolated from IVF-derived blastocysts by immunosurgery and cultured in different types of medium on STO feeder cells. Attachment of ICMs and formation of primary colonies were investigated after 1–2 and 4–7 days, respectively.
[a] The rate of blastocyst formation was 19.1% from 878 oocytes.
[b] Percentage of blastocysts cultured.
[c] Percentage of blastocysts attached.
[d,e,f] Within the same column, values with different superscripts were significantly different ($p < 0.05$).

experimental groups. When a significant effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined when p value was less than 0.05.

Results

In determining suitable conditions for the isolation and initial culture of porcine inner cell masses (ICMs) from *in vitro*-produced blastocysts, porcine blastocysts were produced under various conditions. The overall rates of blastocyst formation from IVF, SCNT and PA were 21.1, 15.4 and 38.7%, respectively (Tables 1–4).

Colonies showing similar morphology of embryonic stem (ES) cell were obtained from *in vitro*-produced blastocysts. ICMs were successfully isolated from blastocysts by immunosurgery (Fig. 1*a*). The attachment and colony formation were observed approximately 1–2 and 4–7 days after initiation of culture, respectively (Fig. 1*b*, *c*). Colonies derived form porcine ICMs in initial culture showed typical morphology of murine ES cells, such as compact colonies having well delineated boundaries. All colonies showed positive AP activity (Fig. 1*e*). However, most of the colonies maintained their characteristics as an ES cell until

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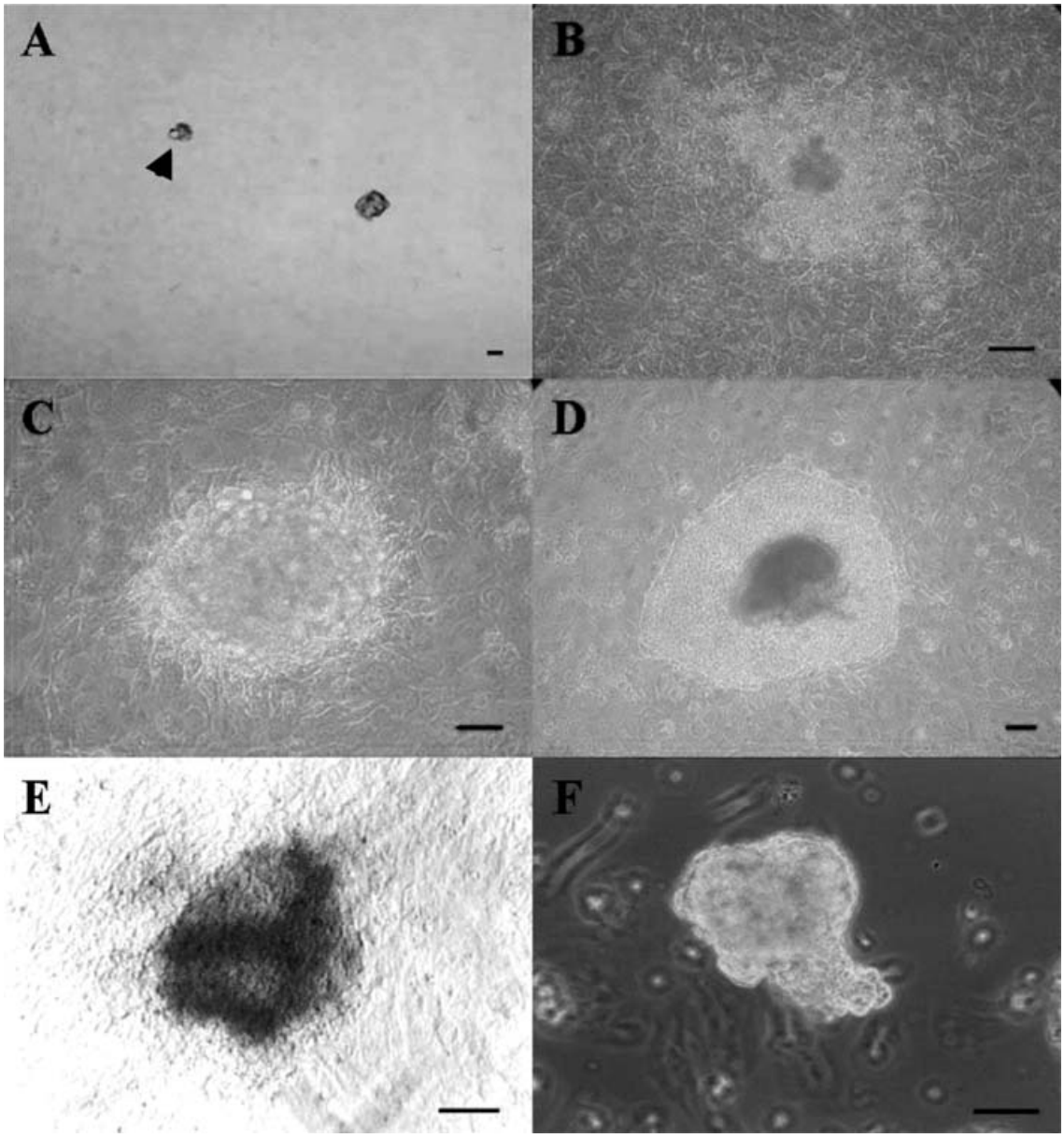

Figure 1 Isolation and culture of embryonic cell lines derived from *in vitro*-produced blastocysts. (*a*) Isolation of ICM from IVF-derived blastocysts by immunosurgery (arrow indicates ICM). (*b*) Attachment of blastocysts on STO feeder layer after 1 day of culture. (*c*) Primary colony was formed after 5 days after culture of blastocysts on STO feeder layer. (*d*) Colony at passage 5. (*e*) Colony showed positive AP staining (*f*). Embryoid bodies observed when the cells from the colony were cultured in the suspension medium. All colonies and embryoid body were generated from ICMs of IVF, STO feeder and D/H culture. Bar = 100 μm.

three to four passages and then differentiated or degenerated. Only four lines from IVF-derived ICMs, three from the D/H culture and one from the D/N culture, continued up to five passages (Fig. 1*d*). When cells from colonies were cultured for 7 days in a suspension culture, cystic embryoid bodies were formed (Fig. 1*f*).

To determine the optimal conditions for porcine ICM isolation and *in vitro* culture, series of experiments were carried out.

In Experiment 1, blastocysts were employed from various source of *in vitro* production process, such as IVF, SCNT and PA. As shown in Table 1, there are no significant differences in the rate of attachment among the groups. However, more primary colonies were formed in the group of IVF and SCNT than PA (28.9 and 30.0% vs. 4.9%, respectively).

In Experiment 2, IVF-derived blastocysts were divided into three groups according to the seeding method. As in Table 2, when ICMs were isolated

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from blastocysts by immunosurgery, the rate of attachment was significantly higher (68.5%) than whole blastocysts, regardless of the presence of ZP (42.4 and 27.8%). Significant differences were also observed in the rate of primary colony formation among the groups. The rate of primary colony formation in the group of ICM was significantly increased (36.8%) than in the group of ZP-free (19.4%). No primary colonies were formed in the ZP-intact group.

In Experiment 3, several types of cells were tested for feeder layer. As shown in Table 3, the rate of attachment was significantly higher in the group of STO and MEF (56.7 and 40.9%, respectively) than PUEC and none (14.3 and 9.5%, respectively). More primary colonies were formed in the group of STO (34.5%) than in the group of MEF (22.2%). No primary colony was formed in the group of PUEC and none.

In Experiment 4, the effect of different culture media on porcine ICM cells was compared. As in Table 4, more ICMs were attached when they were cultured in D/N medium (75.0%) than in D/H medium and DMEM only (57.5 and 20.0%, respectively). The rate of primary colony formation was also significantly higher in D/N medium (44.4%) than D/H medium (21.7%). No primary colonies were formed in DMEM only.

Discussion

The present study was performed to determine the optimized conditions that could support isolation and primary culture of porcine inner cell masses (ICMs) derived from *in vitro*-produced blastocysts. Our results demonstrate that *in vitro*-produced blastocysts, either by IVF or NT, can be used effectively to culture ICM cells, isolated immunosurgically, with STO cells as a feeder layer and mixture of DMEM and NCSU-23 as a culture medium.

The morphology of the colonies derived from this study showed typical characteristics of murine embryonic stem (ES) cells. Also, cells from the colonies consistently showed positive alkaline phosphatase (AP) activity. AP, a marker of the undifferentiated state, has been used to evaluate the pluripotency of porcine embryonic cells (Chen *et al*., 1999; Li *et al*., 2003). These cells even formed embryoid bodies in a suspension culture.

Porcine embryo-derived cell lines have been isolated using *in vivo*-produced (Strojek *et al*., 1990; Anderson *et al*., 1994; Wianny *et al*., 1997; Chen *et al*., 1999; Li *et al*., 2003) and *in vitro*-produced (Miyoshi *et al*., 2000; Li *et al*., 2004) blastocysts. When isolating ES cells, the quality of blastocysts is a very important factor for the colony formation. Although porcine blastocysts have been obtained successfully using IVF and SCNT (Abeydeera *et al*., 1997; Lee *et al*., 2003b), their quality is still lower than that of blastocysts produced *in vivo*. It is well documented that total cell number close to that of *in vivo*-derived blastocysts can be regarded as a valuable indicator of *in vitro*-produced (IVP) embryo viability (Fuente & King, 1997; Soom *et al*., 1997; Velde *et al*., 1999). The total cell number of *in vivo*-derived blastocysts is reported to be around 57 at day 6 (Yoshioka *et al*., 2002), 93 (Fuente & King, 1997) at day 7, increasing to about 175 just before hatching (Davis, 1985). In our previous report (Kim *et al*., 2004), we employed modified NCSU-23 and new culture systems for culturing blastocysts from IVF, which elevated the total cell number to 73 with simultaneous increase of ICM and TE at day 7. In this study, we additionally obtained 30 blastocysts derived from IVF, SCNT and PA and counted the number of cells which consist of blastocyst of each IVF, SCNT and PA using differential staining method (Kim *et al*., 2004). The average mean cell numbers were 75, 64 and 23, respectively (data not shown). However, in the case of SCNT embryos, we usually transfer embryos to surrogate mother at day 1 or day 2 because of incomplete condition of *in vitro* culture and, in our case, the rates of pregnancy and birth are approximately 30.0 and 15.9%, respectively (data not shown). In our results, the rates of attachment to feeder and colony formation were similar when the blastocysts were derived from IVF and SCNT. The efficiency of colony formation was very low in the group of PA (Table 1). Although blastocysts from SCNT had smaller cell number than IVF, they reached similar to hatching blastocysts easily because of the slit come from manipulation and therefore, they might be influenced less by acidic tyrode solution during removal of ZP.

Two distinct cell lineages, ICM and TE, separate at the blastocyst stage. The ICM, although contributing cells to TE (Fleming *et al*., 1984), is predetermined to give rise to the embryo proper. Therefore, in research with ES cells, usually ICM has been isolated and cultured (Wianny *et al*., 1997; Chen *et al*., 1999; Li *et al*., 2003). Li *et al*. (2003) reported that isolated ICM by enzyme digestion was superior to intact blastocyst culture when *in vivo*-produced blastocysts were used. Also, same research group tried to isolate and culture porcine blastocysts derived from IVF (Li *et al*., 2004). Even though they reported success in culturing porcine pluripotent cells, those cells were not maintained more than three passages possibly due to improper condition.

In the present study, we examined if intact blastocysts can be used for ES cell culture when *in vitro*-produced blastocysts were seeded with or without ZP. Also, efficiency of colony formation was compared between intact blastocysts and isolated ICMs. As a result, more primary colonies were formed from ICMs than

intact blastocysts. This result indicated that immunosurgically isolated ICM was better in terms of colony formation than intact blastocysts both with or without ZP, consistent with the result of other study that employed *in vivo*-produced blastocysts (Li *et al*., 2003). When culturing intact blastocysts, proliferative multinucleated giant cells with broad range were observed, therefore, trophoblast might affect ICM outgrowth in the intact blastocysts culture (Li *et al*., 2003). In the ZP-intact blastocyst culture, no primary colony was formed maybe due to the hardness of the hatching process.

Feeder cell layers are essential for both the isolation of ES cell lines and the routine maintenance of established cell lines. Feeder cells encouraged mouse ICM cell outgrowth and prevented them from undergoing spontaneous differentiation, consequently establishing pluripotent ES cells (Nichols *et al*., 1990). As feeder layer is one of the key factors influencing initial ICM cultures, many studies about effects of feeder layer have been done. Strojek *et al*. (1990) compared the mouse STO cells and porcine uterine fibroblasts as feeder cells for the culture of porcine blastocyst and they found that the porcine uterine fibroblast supported the colony formation better than STO cells. It was suggested that a feeder cell type similar to the species of the embryo might be more ideal than of heterologous species (Bongso *et al*., 1994). On the other hand, Anderson *et al*. (1992) showed that hatched bovine blastocysts attached to bovine oviductal epithelial cell monolayers failed to proliferate. In the culture of porcine primordial germ cells, the number of AP positive colonies cultured on porcine embryonic fibroblast was significantly lower than on the other feeder cells (Lee & Piedrahita, 2000). In human, it is known that ES cells are cultured and maintained in feeder-free culture only supplemented growth factor (Amit *et al*., 2004; Rosler *et al*., 2004). In this study, three types of cells as feeder layer were compared, STO cells, MEF, PUEC and feeder-free culture. In terms of attachment and colony formation, STO cells showed the best result. Meanwhile, PUEC could not support the culture of blastocysts outgrowth, while group without feeder, has failed to form colonies. The STO and MEF are well known to secret some kinds of factors which may stimulates ES cell growth and inhibit their differentiation (Smith *et al*., 1988). PUEC was demonstrated not to have ability to support and maintain the attachment and growth of porcine embryonic cell lines in this study.

The culture medium is also an important factor in the colony formation of blastocyst-derived cells. Silcox & Johnson (1988) reported the culture of day 13 porcine embryonic discs in DMEM plus 10% newborn bovine serum formed vesicles and cultured for up to 10 days with a number of developing tissues implying mesoderm differentiation. The effects of hLIF and culture medium on *in vitro* differentiation of cultured porcine ICM were investigated and the results indicated that neither hLIF nor culture medium delayed differentiation of pICMs, but D/H medium promoted the formation of primary colonies (Moore & Piedrahita, 1997). High levels of embryonic development have been obtained with NCSU-23 medium and when 10% FBS was supplemented, more blastocysts were produced and hatched (Kim *et al*., 2004). Miyoshi *et al*. (2000) reported that NCSU-23 was superior to DMEM in the attachment and growth of porcine-hatched blastocysts on feeder layers. Therefore, in this study, the effect of culture medium (D/H medium, D/N medium, DMEM alone) was investigated on attachment of porcine blastocysts on feeder cell and formation of primary colony. As shown in the result, ICMs cultured in D/N medium showed better attachment and colony formation than in other culture media. This might due to the long preimplantation period of porcine blastocysts, so NCSU-23 and DMEM could support synergistically the development the transient status of porcine blastocysts to embryo-derived cells.

Although many primary colonies were obtained during this study, only four lines were survived up to five passages. Although many researchers have tried to isolate porcine ES cells, the largest number of passages that cell lines went through was 10 (Anderson *et al*., 1994), otherwise eight (Li *et al*., 2003), or six (Chen *et al*., 1999). Culture conditions currently in use have been developed from mouse ES cell culture and may not be effective for maintaining the porcine ICM. Moore & Piedrahita (1996) showed that heterologous cytokines are ineffective for maintaining porcine ICMs in an undifferentiated state. Talbot and Garrett (2001) also demonstrated that cultured pig epiblast cells could be more easily lysed by standard cell–cell dissociation methods and exposure to Ca^{2+}–Mg^{2+}-free PBS, therefore physical dissociation method may be suitable for the porcine epiblast cells. However, Gallagher & McWhir (2004) reported that they isolated ES cell lines from the CBA mouse strain with transgenic mice that expressed the selectable neomycin phosphotransferase (*neo*) gene under the transcriptional control of Oct3/4 promoter that is active only in the pluripotent cells and their precursors. Therefore, it may need to develop homologous cytokine or culture condition and to approach with genetic modification to culture porcine ICM cells more efficiently.

In conclusion, porcine ICMs derived from the blastocysts produced *in vitro* were isolated and cultured successfully in term of attachment and primary colony formation. The culture system developed in this study could be used in initial culture of ICM cells to establish porcine ES cells. Further study is required to maintain porcine ICM-derived cells for the long term to the stable cell lines, which is necessary to practical use.

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