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—Original—

Improved Isolation and Culture of Embryonic Stem Cells from Chinese Miniature Pig

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Abstract. Pigs serve as a better research model for human beings than other species. The Chinese laboratory miniature pig is a new laboratory animal and is expected to be applicable in many medical research fields. This study was to establish effective technologies to isolate and culture ES cells in Chinese miniature pigs. For isolation of the inner cell mass from blastocysts, an enzyme-digestive method was compared with the traditional immunosurgery. Isolated ICM were cultured in three feeder cell layers: mouse embryonic fibroblasts (MEF), porcine embryonic fibroblasts (PEF) and a continuous cell line of mouse embryonic fibroblasts (STO). Microtubule activity of the three feeder cells was further examined by immunofluorescence. ICM were successfully isolated from 85% of blastocysts by the enzyme-digestive method, compared to only 40% by immunosurgery. When ICM were cultured in three feeder layers for two to three days, 75%, 65% and 20% of ICMs formed primary cell colonies in MEF, PEF and STO, respectively. Colonies were also formed during subcultures after 9, 5 and 1 passage in MEF, PEF and STO, respectively. Microtubules in STO cells were significantly fewer than those in MEF and PEF. When the ES-like cells were cultured in a differentiation medium, they differentiated to neuron-like cells and other types of cells. These results indicate that healthier ICM can be obtained with the enzyme-digestive method. Successful culture of ICM to ES-like cells has been achieved not only in MEF, but also in homologous (pig) feeder layer. The ES cells obtained in the present study were pluripotent.

Key words: Blastocyst, ES cells, Feeder cells, ICM, Miniature pig

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Under appropriate culture conditions, an inner cell mass (ICM) isolated from blastocysts can form embryonic stem (ES) cells. ES cells can be maintained in an undifferentiated status in the culture and have the potential to differentiate to all types of cells. It is believed that ES cells can be used to study cell differentiation, embryonic development, gene regulation and biomedical research [1, 2]. In humans, it is predicted that ES cells can be used to create specialized cells in a differentiated culture condition and these cells can be used to treat a wide range of diseases.

Isolation and culture of embryo-derived cell lines have been reported in many mammals [3, 4–13, 14], including primates [15] and humans [16]. Human ES cell research has provoked considerable debate; many scientists have questioned whether the existing cell lines could be adequate for research or therapy as they are contaminated with other

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biological products, such as antisera for isolation of the inner cell mass (ICM) and the feeder cell layer of mouse fibroblasts for culture. Research on the establishment of new cell lines is necessary but limited. Pigs serve as a better research model for human beings than other species because they are immunologically and physiologically more similar to humans. It is believed that isolation and culture of porcine ES cells play important roles in biomedical research, such as biological reactors and in xenografting. The Chinese laboratory miniature pig is a new laboratory animal and is expected to be useful in many medical research fields.

On the other hand, the feeder cell layer is one of the important factors affecting ES cell culture [17]. It has been reported that feeder layer cells can secret some kinds of cytokines, such as Leukemin inhibitory factor (LIF) [18], which may stimulate ES cell growth and inhibit their differentiation. Currently, continuous cell lines of mouse embryonic fibroblasts (STO), mouse embryonic fibroblasts (MEF) and homologous embryonic fibroblasts (HEF) are the three most commonly used feeder cells. The homologous embryonic fibroblast feeder layer is supposed to be the most suitable feeder layer, especially in humans.

In a previous study, we found that more effective results were obtained when inner cell masses (ICM) isolated from blastocysts by an enzyme-digestive method were used for culture of ES cell lines in pigs than whole blastocyst culture [19]. In the present study, our goal was to 1) compare the enzyme-digestive method for the isolation of ICM from blastocysts with traditional immunosurgery in which a biological product, antiserum, has been used; 2) compare the efficiency of ES cell culture in different feeder layers including a homologous (pig) feeder layer; and 3) examine the differentiation of the ES cells obtained in the present experimental conditions. To analyze the activity of feeder cells, we also examined microtubules in the feeder cells by immunofluorescence and confocal microscopy, which may be related to their functional status and/or secretive activity.

Materials and Methods

Preparation of feeder layers

Three feeder cell layers were used in this study: MEF, pig embryonic fibroblast (PEF) and STO. MEF 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 sacrificed by cervical dislocation. Fetuses were separated from the uteri and then washed twice in fresh phosphate buffered saline (PBS) to remove any remaining blood. After the head and liver were removed, the carcasses were treated in 0.25% trypsin-0.04% ethylenediamine tetraacetic acid (EDTA) solution for 30 min at room temperature until the carcasses were broken into single cells, the MEF. The cells were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new calf serum (NCS; Gibco), 5% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin and 0.05 mg/ml streptomycin. Feeder layers were prepared from the MEF after 1 to 6 passages. For preparation of feeder layers, the MEFs were inactivated 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 were resuspended in MEF medium. The cell suspension was seeded at a density of 5×10^4 cells per well in a 96-well plate coated with 0.1% gelatin (Fluka AG; Switzerland). The MEFs were plated one day before ICMs were seeded. PEF was prepared from fetal pigs. Briefly, female Chinese miniature pigs at 25–26 days of pregnancy were killed at a local abattoir. The uteri and fetuses were transported to the laboratory in warm saline. In the laboratory, the fetuses were separated from uteri and then washed twice in fresh PBS to remove any remaining blood. After that, all treatments and cell cultures were based on the methods for MEF. STO was provided by the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

Blastocyst collection and isolation of ICM

Embryo donors were Chinese laboratory miniature pigs. Blastocysts were collected at 7–9 days after the day of estrus. Embryos were flushed from uteri with PBS containing 5% FBS and then washed three times in fresh DMEM supplemented with 10%NCS, 5%FBS, 100 IU/ml penicillin and 0.05 mg/ml streptomycin. The ICMs were isolated from blastocysts by an enzyme digestive method

[19] or traditional immunosurgery. For enzyme-digestive isolation, the zona pellucida (if present) of the blastocysts was removed with 0.2% pronase (Sigma). Zona-free blastocysts were treated in a drop (0.25–0.5 ml) of 0.25% trypsin-0.04% EDTA solution for several minutes. During the treatment, the embryos were observed under a stereomicroscope. When the trophoblasts began to disperse in the drop, blastocysts were transferred to another drop without trypsin-EDTA. The ICMs were separated from the trophoblasts with the aid of two fine needles and a pulled mouth micropipette. When all trophoblasts were clearly removed with the mouth micropipette, an intact ICM was isolated, as shown in Fig. 1A. The immunosurgery isolation of ICM was based on the method reported previously [20]. The isolated ICMs were individually seeded onto MEF, PEF or STO feeder layers. The cells were cultured in 200 μl of ES medium in a 96-well plate and examined daily with ES medium being changed every day. The ES medium is DMEM supplemented with 0.1 mM β-mercaptoethanol (Amresco), 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 (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine, Sigma) and 16%FBS.

Isolation and passage of putative ES colonies

Approximately 2–4 days after ICMs were seeded, ES-like colonies were identified. ES cell colonies were grown as tightly packed mounds and had abundant lipid-like vacuoles. ES-like colonies were individually packed off feeder cells and dissected in a microdrop of 0.25% trypsin-0.02%EDTA for 3–5 min at room temperature. Under the stereomicroscope, the treated ES-like colonies were partially disaggregated with the aid of two fine needles and a micropipette. The disaggregated colony cells were individually re-seeded onto new MEF, PEF or STO feeder layers in a 96-well plate. Culture medium was changed every day. All colonies were treated and cultured for the next passage until no colony was formed.

Alkaline phosphatase (AP) activity

Expression of AP activity in putative ES colonies was detected by AP staining. For this, the medium was removed from the cultures, and the cells were fixed with 1%(w/v) paraformaldehyde-7.5% (w/v) sucrose. After fixation, the cells were washed 3 times in Tris-HCl buffer (100 mMTris-HCl, pH 9.5, 50 mM NaCl, 50 mM $MgCl_2$, 0.1%Tween-20), each for 10 min. A staining solution was added after the last washing. The staining solution is made up of: Solution A: 75 mg/ml nitroblue tetrazolium salt (Bicm) in 70% dimethylformamide (Amresco); Solution B: 50 mg/ml 5-bromo-4chloro-3-indolyphate toluicinium salt (BCIP, Sigma) in 100% DMF. Just before staining, 45 μl of solution A and 35 μl of solution B were added to 10 ml Tris-HCl buffer. The ES cells were purple-blue and differentiated cells were colorless after staining.

Microtubule assessment of feeder cells

The feeder cells (MEF, PEF and STO) were divided into two groups. Cells in the first group were seeded onto cover slips, and the cells in the second group were treated with 10 μg/ml mitomycin C solution for 2.5 h and then cultured on cover slips. After 1 day of culture, the slips were washed three times in PBS, and were fixed in 4% formaldehyde in a PHEM buffer (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 4 mM $MgSO_4$, pH 6.9) for 20 min. The cells were then washed in PBS 3 times and then treated for 10 min with 1% Triton X-100 in PHEM buffer. After washing with PBS, the cells were blocked in PHEM buffer containing 5% goat serum for 1 hr. The cells were then washed three times again in PBS containing 0.5% (v/v) Tween 20 and incubated with 1:50 diluted fluorescein isothiocyanate conjugated anti-α-tubulin for 1 hr in a dark box, followed by staining with 10 μg/ml propidium iodide for nuclear examination. Finally, the cells were examined under a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany).

Evaluation of in vitro differentiation

Differentiation of putative ES cells was examined by prolonged culturing of ES-like colonies on MEF feeder layer for 2 weeks without passage or by culturing of dissociated single cells in a 96-well plate containing ES medium without LIF or feeder layer. The dissociation of ES colonies was conducted in trypsin/EDTA. The cultures were monitored daily and the culture medium was changed every other day.

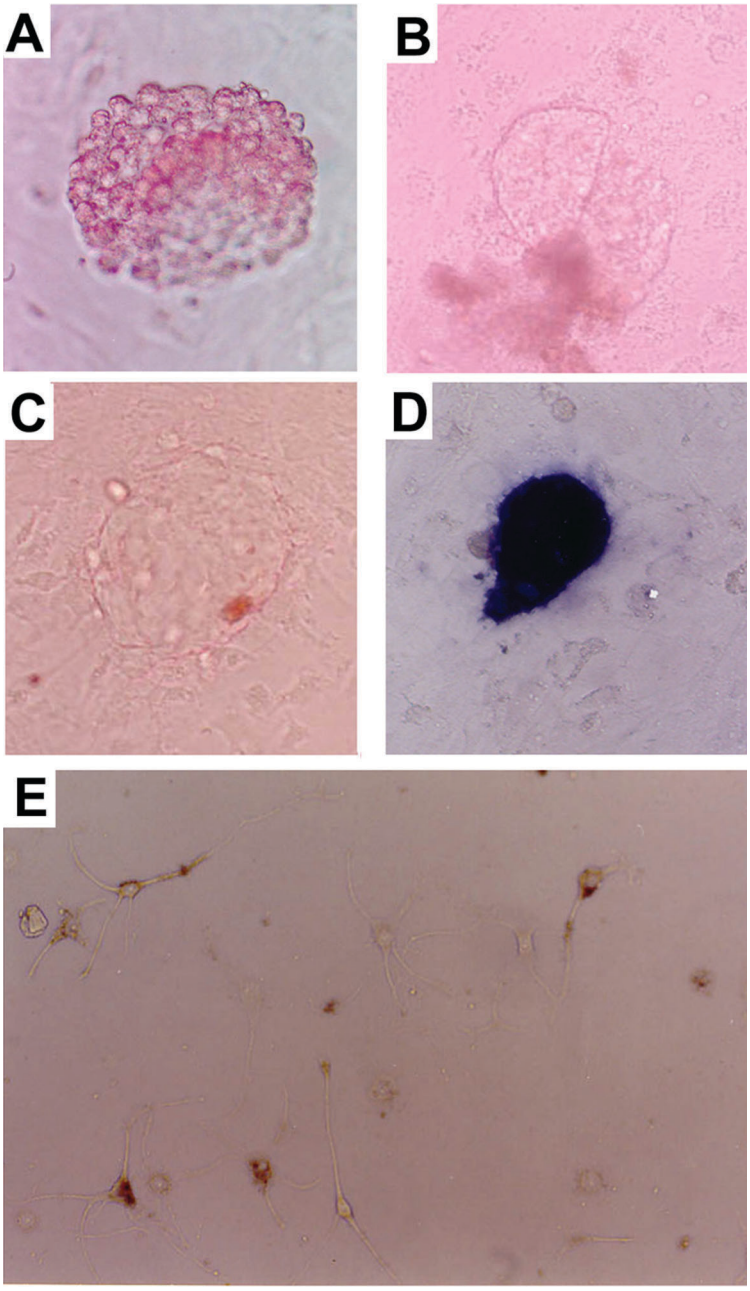

Fig. 1. Isolation and culture of ES cells from Chinese miniature pigs. (A) An ICM isolated by the enzyme-digestive method that had been seeded to the MEF feeder layer. (B) A primary ES-like colony was formed after 2 days of culture. (C) An ES-like colony was formed after 2 days of subculture. (D) Positive AP staining of an ES cell colony. (E) Neuron-like cells were differentiated from a single ES cell culture.

Statistical analysis

The data were analyzed statistically by X-square test. A value of $P<0.05$ was considered to be statistically significant.

Results

ICM isolation

As shown in Table 1, intact ICMs (Fig. 1A) were

Table 1. Isolation of ICM from blastocysts and the effects on ES cell culture*

ICM isolation methods	No. of blastocysts	No. (%) of ICM isolated	No. (%) of primary ES-like colonies
Enzyme-digestive	20	17 (85)[a]	13 (76.5)
Immunosurgery	20	8 (40)[b]	6 (75)

* Experiments were repeated three times.
[ab] Values within the same column with different superscripts are different significantly, P<0.001.

Table 2. Effects of feeder cells on the culture of porcine embryonic stem cells

Feeder cells	No. of ICM cultured		No.(%) of primary ES colonies	No. of ES cell passages
	Total	Attached (%)		
MEF	32	32 (100%)[a]	24(75%)[a]	9
PEF	17	17 (100%)[a]	11(65%)[a]	5
STO	5	2 (40%)[b]	1(20%)	1

[a,b] Values within the same column with different superscripts are significantly different, P<0.025.

isolated from 85% (17/20) blastocysts by the enzyme-digestive method, while only 40% (8/20) of the intact ICMs were successfully isolated by immunosurgery. All ICMs adhered to the feeder cells when they were cultured for 2–4 days, showing no difference between isolation methods.

ES-like colony formation

When the ICMs were cultured in mouse embryonic fibroblast feeder layer (MFF), as shown in Table 1, primary ES-like colonies were formed from 75% of the ICMs isolated by immunosurgery and 76.5% of the ICMs isolated by the enzyme-digestive method (Fig. 1B). Cell proliferation was usually observed in the first few days and cell colonies were formed after 2–3 days of culturing. The colonies were densely packed and had obvious borders (Fig. 1B). When the colonies were separated and reseeded for the subculture, new colonies were produced within 2–4 days and their morphology was the same as the morphology of primary colonies but there were more lipid-like vacuoles (Fig. 1C). When the colonies from the primary culture and subculture were stained for examination of alkaline phosphatase (AP) activity, we found that more than 95% of the colonies obtained in the primary culture and 90% of the colonies obtained from the subculture showed positive AP activity (Fig. 1D).

Cell differentiation

When the ES-like cells were cultured in a fresh feeder layer (after 2–4 passages) in the medium without recombinant human-leukemin inhibitory factor (rh-LIF), AP activity disappeared quickly in these cells. At the same time, the number of lipid-like vacuoles, decreased as the culture progressed. After 8–10 days of culturing, most ES cells differentiated to fibroblast-like, smooth muscle-like and epithelium-like cells. But, when the ES colonies were separated into single cells and cultured in the medium without both rh-LIF and feeder layer, most cells differentiated to neuron-like cells (Fig. 1E) within 5–6 days.

Effects of feeder layers

As shown in Table 2, it was found that PEF gave the same results as MEF and both were superior to STO. All ICMs cultured in MEF (32/32) and PEF (17/17) attached to the feeder layer after 24–36 h, but only 40% (2/5) of ICMs seeded on the STO feeder layer attached to the feeder layer after 48–60 h. The primary ES colonies were formed in 75% (24/32) of ICMs on the MEF feeder layer and in 65% (11/17) of ICMs on the PEF feeder layer, but only in 20% (1/5) on the STO feeder layer. During the subculture, most cells cultured on STO differentiated after 1 passage, but cells cultured on the MEF and PEF feeder layers continued to form ES-like colonies until 9 and 5 passages. Again, most colonies showed positive AP activity.

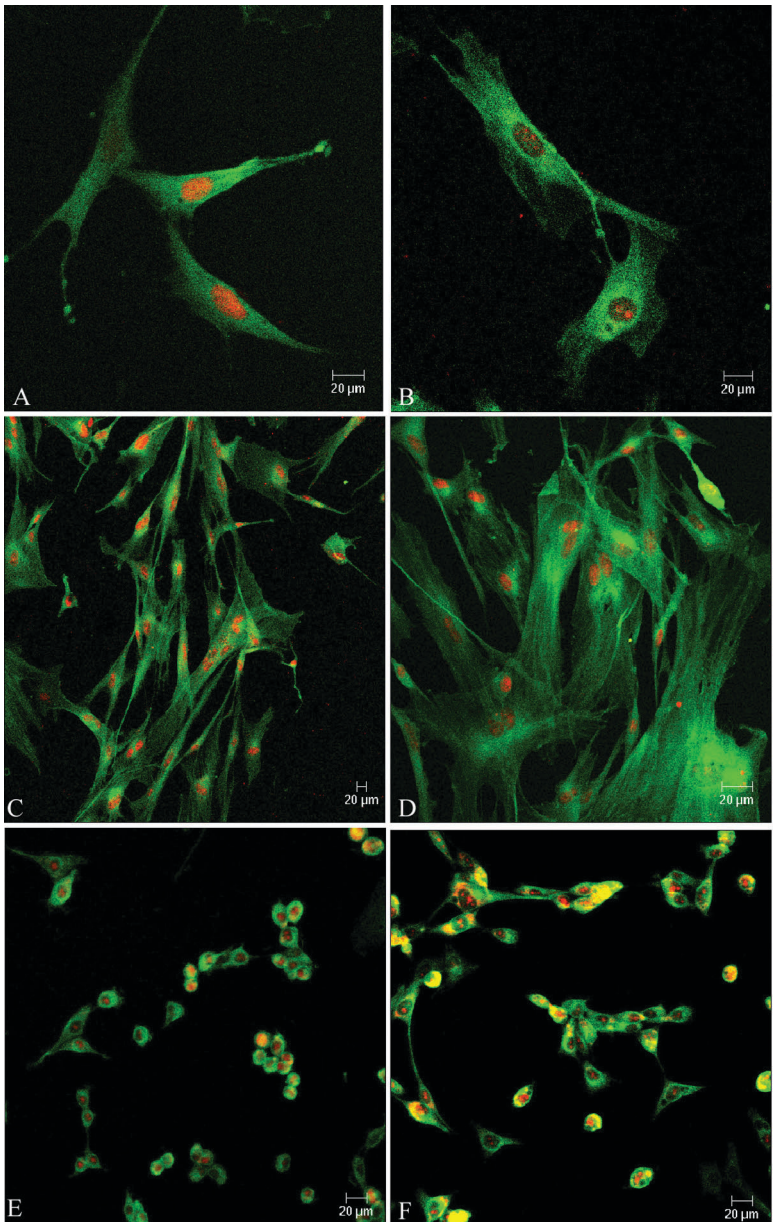

Fig. 2. Microtubule distribution of feeder cells. Microtubules in MEF (A & B), PEF (C & D) and STO (E & F) that had not been treated with mitomycin C (A, C & E) or treated with mitomycin C (B, D & F). Note that the morphology was similar in mitomycin C treated and not treated cells, but different in MEF, PEF and STO. More microtubules were present in MEF and PEF than in STO. Green images indicate microtubules and red indicate nucleus.

When we further examined cell microtubules in the feeder cells, as shown in Fig. 2, microtubule localization of PEF was the same as that of MEF but significantly more than that of STO. Mitomycin treatment did not affect the localization or amount of microtubules (Fig. 2).

Discussion

Isolation of the ICM is a very important step in the culture of ES cells. Traditionally, ICMs were isolated from blastocysts by immunosurgery [21] but it is not always effective when antiserum is

used during immunosurgery. In addition, making antiserum is laborious. In humans, contamination of the antiserum may limit its clinical application even an ES cell line has been established. Therefore, a technology without the use of biological products is necessary. In this study, we used an enzyme digestive method to isolate ICM from blastocysts and compared this method with immunosurgery and found that the enzyme digestive method is a much better technology than immunosurgery. We found that it is very easy to isolate the ICMs from blastocysts by the enzyme-digestive method. The most important key in this method is the treatment time of blastocysts in the EDTA solution. The manipulator must observe the embryos under a the stereomicroscope during the treatment. When the trophoblasts begin to disperse, the treatment must be stopped and the ICM is separated easily from trophoblasts with the aid of a pulled mouth pipette and two fine needles under a stereomicroscope.

The feeder layer is one of the most important factors affecting the ES cell culture. It has been reported that feeder cells can secrete some kinds of cytokines, such as LIF [18], which may stimulate ES cell growth and inhibit its differentiation. When we compared three different feeder layers in this study, we found that PEF had the same results as the MEF and both were superior to STO. When we examined cell microtubules in the feeder cells, we found that microtubule localization of PEF was the same as that of MEF but significantly different from that of STO. The different microtubule distribution in the feeder cells may be related to cell function and secretive status. In general, cell secretive activity is related to their microtubules, those cells with more microtubules have higher secretive activity, and it is essential for cell microtubules to transport their secretive cytokines to extracellular fluid [22]. Our results have shown that the amount of microtubules in STO was lower than that in MEF and PEF, which results in lower density of cytokines in STO-culture fluid. This may be the reason why STO was not fit to be used as a feeder layer.

Various components are usually supplemented to ES culture media. One of the components is the cytokine, such as LIF and recombinant human-fibroblast growth factor-basic (rh-bFGF). Some researchers have used only the feeder layer but did not add any foreign cytokines to the ES medium [14], while others added LIF and fibroblast growth factor-basic (bFGF) to the ES medium and also used feeder layers [6]. Since LIF can inhibit the differentiation of ES cells, and bFGF can stimulate ES cell proliferation [18], we added both LIF and bFGF to our ES culture medium and also used feeder layers. We found that good results could be obtained under the present conditions although we did not examine these factors individually.

When the ES-like cells were cultured in a fresh feeder layer (after 2–4 passages) in the medium without rh-LIF, we found that most ES cells differentiated to fibroblast-like, smooth muscle-like and epithelium-like cells but when the ES colonies were separated to single cells and cultured in the medium without either rh-LIF or the feeder layer, most cells differentiated to neuron-like cells. These results indicate that removing LIF from culture medium and having no feeder layer are important for the differentiation of ES cells to neuron-like cells, since the feeder layer cells can secrete some kinds of cytokines [18] and bFGF [23], which may inhibit ES cell differentiation to neuron-like cells. Although the cell differentiation was spontaneously induced in the present study, it would appear that different culture methods could induce the cells to differentiate into different cells. More specific technology is still necessary to the induce majority of the cells to differentiate into the desired cells.

In summary, the present study indicates that enzyme-digestive is a simple but effective method for isolating ICM from blastocysts. Such a method may be useful for isolating ICM from blastocysts the humans and other animals, in which antiserum has been used. Our results also indicate that the STO cell layer is not suitable for the culture of ES cells in Chinese miniature pigs. Furthermore, ES cells obtained in the present study are pluripotent and can differentiate into other types of cells, such as neuron-like cells.

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