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Isolation and Culture of Embryonic Stem Cells From Porcine Blastocysts

MING LI, DONG ZHANG, YI HOU, LIHONG JIAO, XING ZHENG, AND WEI-HUA WANG*
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing and College of Biology, China Agricultural University, Beijing, China

ABSTRACT This study was conducted to establish embryonic stem (ES) cell lines from porcine blastocysts. Blastocysts were collected from China miniature pigs at day 7–9 of pregnancy. Embryos were either directly (intact embryos) cultured on mitomysin C-inactivated murine embryonic fibroblasts (MEF) as feeder layers, or were used to isolate the inner cell masses (ICM) by enzyme digestive method and then cultured. It was found that enzyme digestive method could isolate ICMs without any damages of cells in all blastocysts (28). All ICMs attached to the feeder layers. Primary cell colonies were formed in 68% of ICM culture and 28% of intact blastocyst culture. Two ES cell lines derived from ICM passed six subcultures (passages). These cells morphologically resembled mouse ES cells and consistently expressed alkaline phosphatase activity. When the ES cells were cultured in a medium without feeder layer and leukemin inhibitory factor, they differentiated into several types of cells including neuron-like, smooth muscle-like, and epithelium-like cells. Some cells formed embryoid bodies in a suspension culture. These results indicate that porcine ES cell line can be established under the present experimental conditions and these ES cells are pluripotent. *Mol. Reprod. Dev. 65: 429–434, 2003.*

Key Words: blastocyst; ES cells; ICM; pig

INTRODUCTION

Embryonic stem (ES) cells derived from inner cell mass (ICM) of blastocysts and embryonic germ (EG) cells from fetal primordial germ cells can maintain their pluripotency in repeated subcultures. Under the appropriate culture conditions, they can differentiate into a wide range of cell types (Pedersen, 1994). Since ES cells were first successfully obtained in the mice (Evans and Kaufman, 1981), attempts to establish embryo-derived cell lines have also been reported in other mammals, including sheep (Handyside et al., 1987; Tsuchiya et al., 1994), Syrian golden hamster (Doetschman et al., 1988; Piedrahita et al., 1990b), mink (Sukoyan et al., 1992), rabbit (Giles et al., 1993; Neimann and Strelchenko, 1994), cattle (Cherny et al., 1994; Strelchenko and Stice, 1994), pig (Gerfen and Wheeler, 1995; Chen et al., 1999), primate (Thomson et al., 1995), and human (Thomson et al., 1998). But only mouse ES cells have been proven totipotency to differentiate into both somatic and germ cell lineages (Pease and Williams, 1990; Kondoh et al., 1999).

The isolation and culture of pluripotential ES or EG cells from domestic animals would provide potential methods for the multiplication of animals with elite genetics, produce transgenic animals and provide valuable models for studying cell development and differentiation (Evans, 1989; Piedrahita et al., 1998). But it is difficult to obtain and maintain undifferentiated ES or EG cells from domestic animals; this limits the associated research and practical use.

Pigs offer some distinct advantages over other species and serve a better research model because they are immunologically and physiologically more similar to the humans (Phillips and Tumbleson, 1986). Therefore, it is believed that isolation and culture of porcine ES cells play important roles in biomedical research, such as biological reactors and xenografting. However, there is limited information on the isolation of ICM and establishment of ES cell line in the pigs. The present study was designed to develop a new method to isolate ICM from porcine blastosysts and then examine its effects on the establishment of ES cell lines.

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 Institute of Zoology, Chinese Academy of Sciences) at day 12–14 of pregnancy were sacrificed by cervical dislocation. Fetuses were separated from uteri and then washed twice in fresh phosphate buffered saline (PBS) to remove any blood. After head and liver were removed, the carcasses were treated in 0.25% trypsin–0.02% ethylenediamine tetraacetic acid (EDTA) solution for 30 min at room temperature until

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*Correspondence to: Wei-Hua Wang, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Science, Beijing 100080, China. E-mail: wangweihua11@yahoo.com

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the carcasses were broken into single cells, murine 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, Grand Island, NY), 5% (v:v) fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin (Sigma, St. Louis, MO), and 0.05 mg/ml streptomycin (Gibco). Feeder layers were prepared from the MEF after 1 to 6 passages. For preparation of feeder layer, the MEFs were inactivated in a medium containing 10 μg/ml mitomycin C (Sigma) for 2.5 hr. 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 1,000 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 (Sigma) coated with 0.1% gelatin (Fluka AG; 48722; Switzerland). Usually, the MEFs were plated one day before porcine embryos or ICMs were seeded.

Blastocyst Collection and Culture

Embryo donors were China miniature pigs. Blastocysts were collected at 7–9 days of estrus. Embryos were flushed from uteri with PBS containing 5% FBS and then washed three times in fresh DMEM medium supplemented with 10% NBS, 5% FBS, 100 IU/ml penicillin, and 0.05 mg/ml streptomycin. The embryos were divided into two groups. Embryos in the first group were used for intact embryo culture. For this, zona pellucida (if present) was removed in 0.2% pronase solution. Zona-free blastocysts were washed and transferred individually into a 96-well plate containing feeder layer with 200 μl of ES medium. The ES medium is consisted of DMEM with 0.1 mM β-mercaptoethanol (Amresco; 3151B55), 100 IU/ml penicillin, 0.05 mg/ml streptomycin, 0.1 mM MEM nonessential amino acids (Gibco; 08540), 20 ng/ml recombinant human-fibroblast growth factor-basic (rh-bFGF, Sigma; F0291), 40 ng/ml recombinant human-leukemin inhibitory factor (rh-LIF; Sigma; L-5283), 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. The growth and development of colonies from each attached embryo were examined daily. Culture medium was changed every day. The embryos in the second group were used to isolate ICM.

Isolation of Inner Cell Masses (ICMs)

The zona pellucida (if present) of blastocysts was removed by 0.2% pronase. Zona-free blastocysts were treated in a microdrop of 0.25% trypsin–0.04% EDTA solution for several minutes. During treatment, embryos were observed under the stereomicroscope. When the trophoblasts began to disperse in the microdrop, the blastocysts were transferred to another drop with culture medium and ICMs were isolated from the blastocysts with the aid of two fine needles and a pulled mouth micropipette. The isolated ICMs were then individually seeded onto MEF feeder layers. The culture was conducted in 200 μl of ES medium in a 96-well plate and was examined daily and ES medium was changed every day.

Isolation and Passage of Putative ES Colonies

Approximately 2–5 days after blastocysts or ICMs were seeded, 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 feeder layers in a 96-well plate. Culture medium was changed every day. All colonies were treated and cultured for next passage until no colony was formed.

Alkaline Phosphatase (AP) Staining

Expression of AP activity in putative ES colonies was detected by AP staining. For this, medium was removed from the cultures, and the cells were fixed with 1% (w/v) paraformaldehyde–7.5% (w/v) sucrose. After fixation, cells were washed three times in Tris-HCl buffer (100 mM Tris-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 as follows: 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, add 45 μl of solution A and 35 μl of solution B to 10 ml Tris-HCl buffer. The ES cells were purplish blue and differentiated cells were colorless after staining.

Evaluation of In Vitro Differentiation

Evaluation of the differentiation of putative ES cells was conducted by prolonged culture and suspension culture. In prolonged culture, some ES-like colonies were cultured on MEF feeder layer for 2 weeks without passage. In suspension culture, putative ES colonies were picked up from the feeder layers, disaggregated in trypsin/EDTA solution and the dissociated single cells or small clumps were transferred into a 96-well or 4-well plate containing ES medium without LIF and feeder layer. Some small clumps were cultured in a drop of ES medium without LIF (suspension culture). The suspension cultures were monitored daily for embryoid body (EB) formation, with medium being changed every other day.

Statistical Analysis

Experiments were replicated five times and the data were pooled for statistical analysis by χ-square test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Formation of ES Cell Colonies From Intact Blastocysts and ICM

When 20 intact embryos (five early blastocysts, Fig. 1A; and 15 hatched blastocysts, Fig. 1B) were

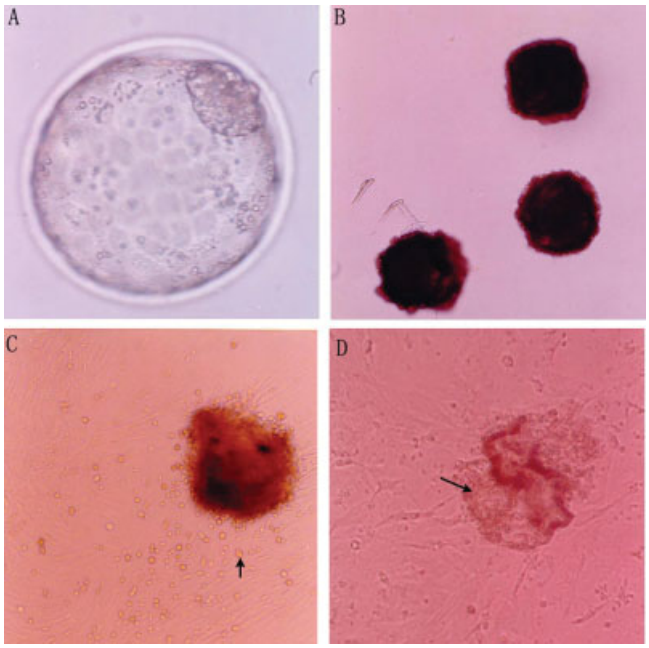

Fig. 1. Morphology of intact porcine blastocyst after collection and during the primary culture. **A**: An early blastocyst collected at 7 days of pregnancy (×400); (**B**) a hatched blastocyst collected at 9 days of pregnancy (×100); (**C**) a blastocyst adhered to the feeder layer after 2 days of culture (arrow indicates trophoblastic cell colony; ×100); (**D**) a primary ES colony, which was obtained after 3 days of culture (arrow indicates primary ES colony ×100).

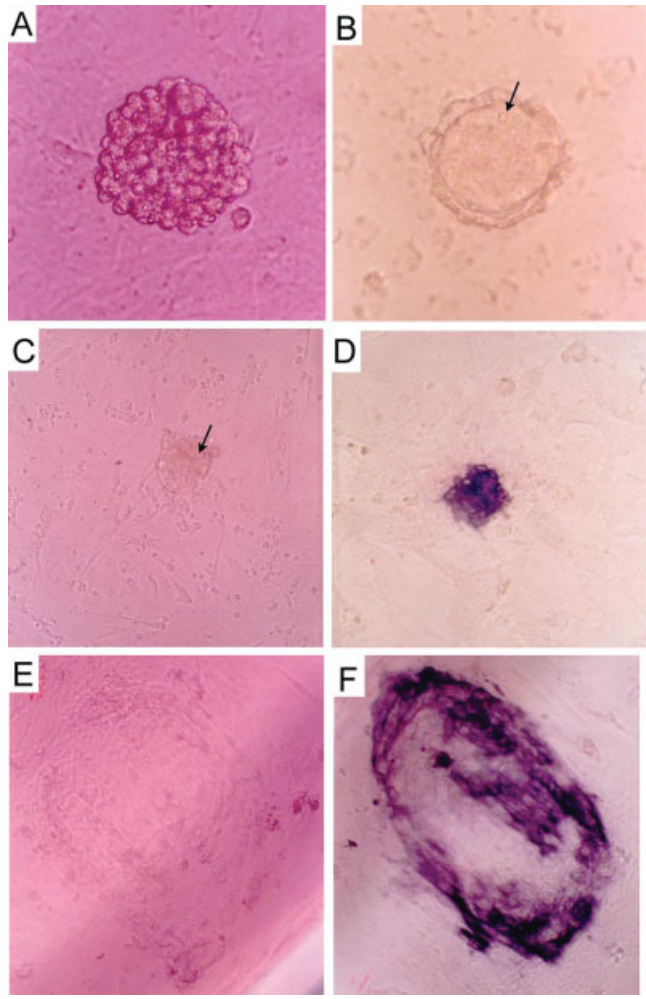

Fig. 2. Morphology of ICM after isolation and during the primary culture. **A**: A ICM, just after seeding to the feeder layer (×400); (**B**) ES colony after 2 days of culture (arrow indicates lipid-like vacuole; ×400); (**C**) ES colony after 2 days of culture (arrow indicates lipid-like vacuole, ×200). **D**: Positive AP staining of a ES cell colony (×200). **E**: ES-like cells grew as a "col"; and (**F**) the same cells in (E) show positive AP staining (×400).

seeded on the feeder layer, the trophoblastic cells started to adhere to it and then spreaded out from the attached plot. After that, all cells from the embryos adhered to the feeder layer by the pulling force from trophoblastic cells. Cell proliferation could be found and many cell colonies were formed after 2–3 days of culture. The colonies were densely packed and had obvious border (Fig. 1C). As shown in Table 1, 18 (90%) blastocysts attached to the feeder layers after culture. Five (28%) ES-like colonies were emerged from primary culture of blastocysts (Fig. 1D) after 2–4 days.

All ICMs (28/28) adhered to the feeder layers a few hour after being seeded (Fig. 2A). Putative ES colonies were formed after 2–3 days of culture. The primary colonies grew as tightly-packed mounds and had abundant lipid-like vacuoles (Fig. 2B,C). As shown in Table 1, primary ES colonies were formed in 68% of ICMs, which was significantly ($P < 0.01$) higher than that from intact blastocysts (28%). Most colonies showed positive AP activity (Fig. 2D). When the colonies were separated and reseeded in the culture, new colonies were produced within 2–3 days of subculture. Morphology of most new colonies was similar to the primary colonies, but some, as shown in Figure 2E, spreaded out and grew as "col" shape, but also expressed positive AP activity (Fig. 2F). As shown in Table 1, all colonies originated from intact blastocysts and most colonies originated from ICMs just had 3–5 passages and then differentiated or degenerated, only two colonies originated from ICM continued 6–9 passages. After the 10th passage, no more colonies were formed.

TABLE 1. Isolation and Culture of Embryo-Derived Cells From Porcine Blastocysts

	No. of embryos		No. of primary colonies	No. of more than passage 6
	Total	Attached		
Intact blastocysts	20	18 (90%)	5 (28%)[a]	0
ICMs	28	28 (100%)	19 (68%)[b]	2

[a,b]Values within the same column with the different superscripts are different significantly ($P < 0.01$).

Analysis of Pluripotency

When the cells from colonies were prolonged in the culture on the MEF feeder layer for 10 days, the cells

differentiated into several types of cells. From the cell morphologies, these cells look like trophoblast and epithelial cells (Fig. 3A,B). Single putative ES cells cultured in a 96-well plate without feeder layers began to differentiate to neuron-like cells within 5–6 days (Fig. 3C–E) or smooth muscle-like cells (Fig. 3F). Fluid-filled, cystic embryoid bodies were formed when small clumps of cells were cultured for 8–10 days in a suspension culture (Fig. 3G,H).

DISCUSSION

The present study indicates that culture of ICM from blastocysts isolated by enzyme digestive method is more effective than intact blastocyst culture for establishement of ES cell lines in the pigs. Also the results indicate that isolation of ICM by enzyme-digestive method is a good technology for isolation of ICM from porcine blastocysts. These ICMs can be used to establish ES cell lines under the present experimental conditions. Furthermore, our results indicate that the ES cells obtained in the present study are pluripotental and can differentiate into other cells and form embryoid bodies.

Isolation of ICM is a very important step in the culture of ES cells. Although ES colonies could be produced from intact blastocyst culture, the efficiency was low (Table 1). When in vitro produced blastocysts were used, we found that intact blastocyst culture was better than isolated ICM culture as small or unclear ICMs were present in in vitro produced blastocysts (unpublished data). Therefore, in the present study, we also examined if intact blastocyst culture collected from oviducts can be used for ES cell culture. The results indicate that isolated ICM is superior to intact blastocyst culture when in vivo produced blastocysts were used. In previously studies, ICMs were isolated from blastocysts by immunosurgery (Solter and Knowles, 1975; Wianny et al., 1997; Chen et al., 1999), but it is not always effective when antiserum is used during immunosurgery. In addition, making antiserum is laborious. In this study, an easy method to isolate ICMs from blastocysts was established. We found that it is very easy to isolate ICMs from blastocysts in trypsin/EDTA solution. The most important key in this method is the treatment time. The manipulator must observe the embryos under the stereomicroscope during treatment. When the trophoblasts begin to disperse, the treatment should be stopped and the ICM could be separated easily from trophoblasts with the aid of a pulled mouth pipette and two fine needles under a stereomicroscope. We also compared this method with immunosurgery and found that this method can obtain more healthy ICMs and better subsequent ES cell culture (unpublished data).

Culture conditions currently in use for porcine ES cell culture have been mainly developed from mouse ES cell culture and it would appear that they are not very effective for maintaining porcine ES cells. Various components are usually supplemented to ES culture media. One of the components is the cytokine, such as LIF, bFGF, and SCF. Some researchers used only feeder layers and did not add any foreign cytokines in the ES medium (Chen et al., 1991) while others added LIF, bFGF, and SCF to the ES medium and also used feeder layers (Piedrahita et al., 1990a). Since LIF can inhibit differentiation of ES cells, bFGF can stimulate ES cell proliferation (Schmitt et al., 1991), and feeder layer is one of the key factors influencing ES cell culture (Piedrahita et al., 1990a), we added both LIF and bFGF to our ES culture medium and used feeder layers. We found that good results could be obtained when MEFs were used after 2–4 passages. The fresh MEF may have higher secretive activity. It has been reported that MEF secrets some kinds of cytokines (Smith et al., 1988), which may stimulate ES cell growth and inhibit their differentiation. When MEFs were passed four times, their morphology became heterogeneous, thus their secretive activity may decrease. In addition, mitomycin C treat time also affected the MEF activity. If the time was more than 3 hr, the MEFs could not be used as a feeder layer as the cell's ability to adhere to the dishes was decreased significantly.

We found that more primary colonies were formed from ICMs than intact blastocysts. It is probably that trophoblasts affect ICM growth in the intact blastocyst culture and induce ICM cell differentiation in culture at the early stage. Same as previous reports (Evans and Kaufman, 1981; Gerfen and Wheeler, 1995; Chen et al., 1999), we found that most of putative ES cell colonies were mound-shape. However, in very few case, we found that cells from some colonies pushed out feeder layer cells and grew dispersively as a "col." The exact reason is unknown. The cells also showed positive AP staining. These results suggest that pluripotent stem cells may grow in different ways, not always form colony.

AP is one of the markers to evaluate pluripotent cells and has been used to identify stem cells in culture for many years (Wobus et al., 1984; Talbot et al., 1993). In this study, AP activity was consistently expressed in the cells from the colonies in the primary cultures and subcultures. This indicates that the cells derived from the present experiment are ES or ES-like cells. During in vitro differentiation, AP activity disappeared quickly. At the same time, the amount of lipid-like vacuoles, which may be secretive steroids (Gerfen and Wheeler, 1995), decreased as differentiation progressed. Some ES cells differentiated into several types of cells in the culture including neuron-like, fibroblast-like, smooth muscle-like, and epithelium-like cells. Some cells formed embryoid bodies in a suspension culture. These results indicate that porcine ES cells obtained in the present study are pluripotent.

In summary, porcine putative ES cells were successfully isolated and cultured in the present study. These cells exhibit many important features of pluripotent stem cells, including ES-like morphology, positive AP activity, and the ability to differentiate in vitro. These results indicate that these cells are pluripotent. This study also provides an easy method to isolate high quality of ICMs from the blastocysts. Further research is required to maintain and enhance the efficiency of ES cell culture in the pigs.

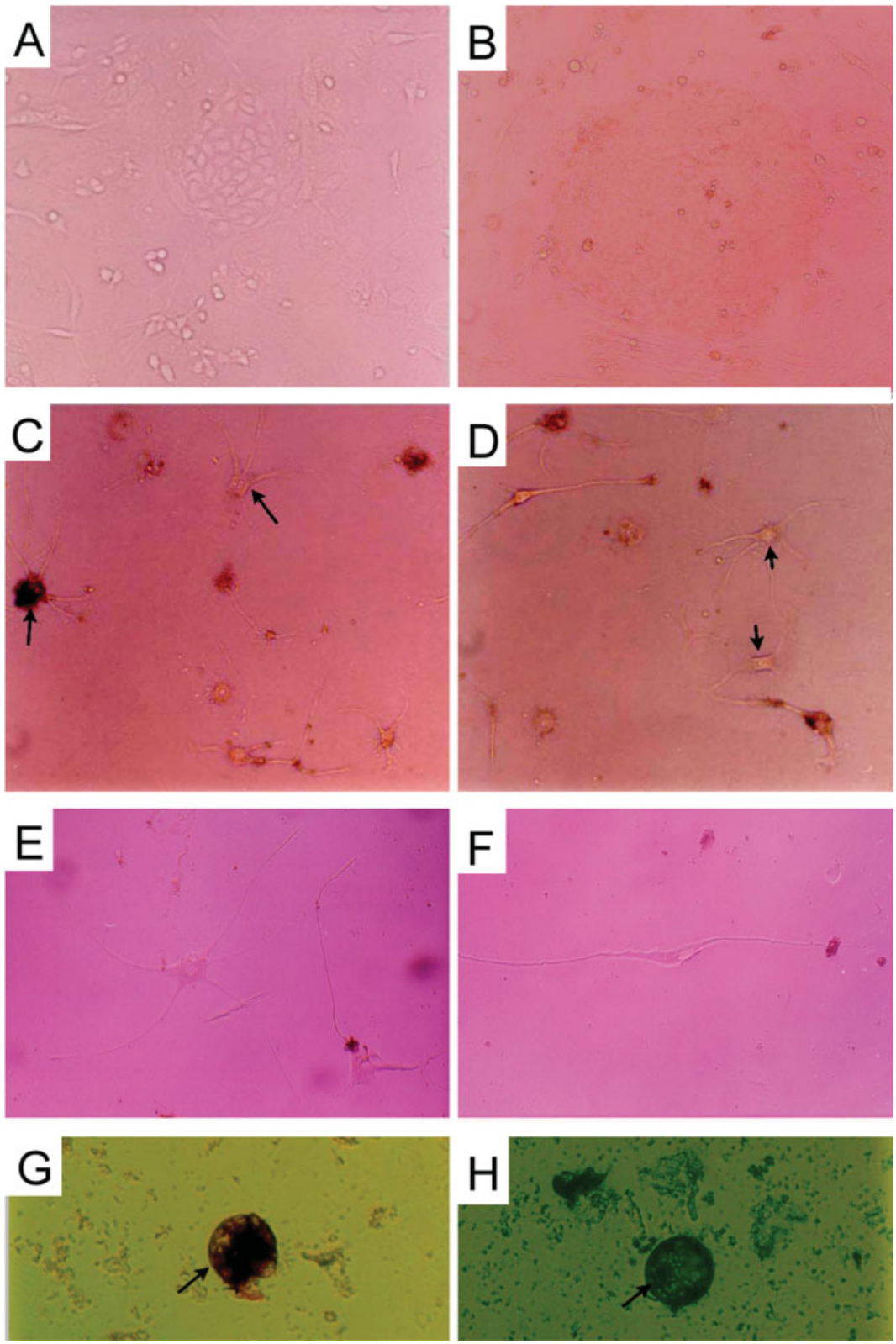

Fig. 3. In vitro differentiation of ES-like cells obtained from ICM colonies. **A** and **B** show epithelium-like cells (×100); **C–E** show neuron-like cells (×100); **F** show smooth muscle-like cells (×200); **G** and **H** show embryoid bodies (arrow) (×100).

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REFERENCES

Chen LR, Wang HP, Wu MC. 1991. In vitro culture of mammalian embryonic stem cells. II. In vitro culture of blastocyst-derived cells in mouse and pigs. J Chin Soc Anim Sci 20:326–339.

Chen LR, Shiue YL, Bertoline L, Medrano JF, BonDurant RH, Anderson GB. 1999. Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology 52:195–212.

Cherny RA, Tonya M, Merei J, Lom L, Brandon MR, Williams RL. 1994. Strategies for the isolation and characterization of bovine embryonic stem cells. Reprod Fertil Dev 6:569–575.

Doetschman TC, Williams P, Maeda N. 1988. Establishment of hamster blastocyst-derived embryonic stem (ES) cells. Dev Biol 127:224–227.

Evans MJ. 1989. Potential for genetic manipulation of mammals. Mol Biol Med 6:557–565.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156.

Gerfen RW, Wheeler MB. 1995. Isolation of embryonic cell-lines from porcine blastocysts. Anim Biotechnol 6:1–14.

Giles JR, Yang X, Mark W, Foote RH. 1993. Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae. Mol Reprod Dev 36:130–136.

Handyside A, Hooper ML, Kaufman MH, Wilmut I. 1987. Towards the isolation of embryonal stem cell line from sheep. Roux's Arch Dev Biol 196:185–190.

Kondoh G, Yamamoto Y, Yoshida K, Suzuki Y, Osuka S, Nakano Y, Morita T, Takeda J. 1999. An easy assessment of ES cell clone potency for chimeric development and germ-line competency by an optimized aggregation method. J Biochem Biophy Methods 39:137–142.

Neimann H, Strelchenko N. 1994. Isolation and maintenance of rabbit embryonic stem (ES) cell like cells. Theriogenology 41:265.

Pease S, Williams RL. 1990. Formation of germ-line chameras from embryonic stem cells maintained with recombinant leukemia inhibitory factor. Exp Cell Res 190:209–211.

Pedersen RA. 1994. Studies of in vitro differentiatiation with embryonic stem cells. Reprod Fertil Dev 6:543–552.

Phillips RW, Tumbleson ME. 1986. Models in 'swine in biomedical research', Vol. 1. Tumbleson ME, editor. New York: Plenum Press. pp 437–440.

Piedrahita JA, Anderson GB, Bon Durant RH. 1990a. Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34:865–877.

Piedrahita JA, Guillespie L, Maeda N. 1990b. Establishing an embryonic stem (ES) cell system stilizing hamster embryos. Biol Reprod (Suppl) 42:175.

Piedrahita JA, Moore K, Oetama B, Lee CK, Scale N, Ramsoondar J, Bazer FW, Ott T. 1998. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol Reprod 58:1321–1329.

Schmitt RM, Bruyns E, Snodgrass HR. 1991. Hematopoietic development of embryonic stem cells in vitro: Cytokine and receptor gene expression. Genes Dev 5:728–740.

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 366:688–690.

Solter D, Knowles BB. 1975. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci USA 72:5099–5102.

Strelchenko N, Stice S. 1994. Bovine embryonic pluripotent cell lines derived from morula stage embryos. Theriogenology 41:304.

Sukoyan MA, Golubitsa AN, Zhelezova AI, Shilov SY, Vatolin SY, Maximovsky LP, Andreeva LE, McWhir J, Pack SD, Bayborldin SI, Kerkis AY, Mizilova HI, Serov OL. 1992. Isolation and cultivation of blastocyst-derived stem cell lines from American Mink (*Mustela vison*). Mol Reprod Dev 33:418–431.

Talbot NC, Rexroad CE, Purcel VG, Powell AM. 1993. Alkaline phosphatase staining of pig and sheep epiblast cells in culture. Mol Reprod Dev 36:139–147.

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Heabn JP. 1995. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 92:7844–7848.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.

Tsuchiya Y, Raasch GA, Brands TL, Mizoshita K, Youngs CR. 1994. Isolation of ICM-derived cell colonies from sheep blastocysts. Theriogenology 41:321.

Wianny F, Perreau C, Hocherear de Reviers MT. 1997. Proliferation and differentiation of porcine inner cell mass and epiblast in vitro. Biol Reprod 57:756–764.

Wobus AM, Holzhausen H, Jakel P, Schoneich J. 1984. Characterization of a pluripotent stem cell line derived from a mouse embryo. Exp Cell Res 152:212–219.