Factors Affecting Primary Culture of Nuclear Transfer Blastocysts for Isolation of Embryonic Stem Cells in Miniature Pigs*

Minjeong Kim, Kwang Sung Ahn, Young June Kim** and Hosup Shim†,**

Department of Physiology, Dankook University School of Medicine, Cheonan 330-714, Korea

ABSTRACT

Pluripotent embryonic stem (ES) cells isolated from inner cell mass (ICM) of blastocyst-stage embryos are capable of differentiating into various cell lineages and demonstrate germ-line transmission in experimentally produced chimeras. These cells have a great potential as tools for transgenic animal production, screening of newly-developed drugs, and cell therapy. Miniature pigs, selectively bred pigs for small size, offer several advantages over large breed pigs in biomedical research including human disease model and xenotransplantation. In the present study, factors affecting primary culture of somatic cell nuclear transfer blastocysts from miniature pigs for isolation of ES cells were investigated. Formation of primary colonies occurred only on STO cells in human ES medium. In contrast, no ICM outgrowth was observed on mouse embryonic fibroblasts (MEF) in porcine ES medium. Plating intact blastocysts and isolated ICM resulted in comparable attachment on feeder layer and primary colony formation. After subculture of ES-like colonies, two putative ES cell lines were isolated. Colonies of putative ES cells morphologically resembled murine ES cells. These cells were maintained in culture up to three passages, but lost by spontaneous differentiation. The present study demonstrates factors involved in the early stage of nuclear transfer ES cell isolation in miniature pigs. However, long-term maintenance and characterization of nuclear transfer ES cells in miniature pigs are remained to be done in further studies.

(Key words : Nuclear transfer, Blastocyst, Embryonic stem cells, Miniature pig)

INTRODUCTION

Embryonic stem (ES) cells are undifferentiated pluripotent cells isolated from the inner cell mass (ICM) of blastocysts and are capable of differentiating *in vivo* into the full range of tissues, including germ cells. Since their first successful isolation in mice (Evans and Kaufman, 1981; Martin, 1981), ES cells have been used extensively as experimental models to study differentiation and gene function and for genetic manipulation via transgenic/knockout mouse production. Although putative ES cells demonstrating pluripotency to a certain extent have been isolated in various mammalian species, true pluripotency to yield germ-line chimerism has been limited in mice (reviewed by Melo *et al.*, 2007).

Due to their short gestation period, large litter size, and anatomical and physiological similarities to humans, pigs have been of interest for biomedical applications (Wheeler and Walters, 2001). Especially miniature pigs, selectively bred pigs for characteristics such as small size, gentle disposition, intelligence, and reproductive performance, offer several research advantages over large breed pigs, especially for experiments where controlled conditions are preferred (Johnson *et al.*, 2002).

In this study, factors affecting the initial stage of ES cell isolation from somatic cell nuclear transfer (NT) embryos in miniature pigs, including attachment of plated blastocysts/ICM on feeder layer and formation of primary colonies were investigated. Since ES cells can indefinitely proliferate in culture, these cells may genetically be modified and used for the production of transgenic/knockout animals. In particular, the isolation of ES cells from NT embryos may allow the establishment of disease-specific ES cells and production of animals carrying multiple transgenes. Hence, NT-ES cells in miniature pigs would offer valuable tools for human genetic disease model and xenotransplantation.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

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** Department of Nanobiomedical Science and WCU Research Center for Nanobiomedical Science, Dankook University.

† Corresponding author : Phone: +82-41-550-3865, E-mail: shim@dku.edu

In Vitro Maturation of Oocytes

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to laboratory in a warm box (25 to 30℃) within 2 h. Follicular fluid and cumulus-oocyte complexes (COC) from follicles of 5 to 6 mm in diameter were aspirated using an 18-gauge needle attached to 5-ml disposable syringe. Compact COC were selected and washed six times in HEPES-buffered tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA). The *in vitro* maturation (IVM) medium was TCM-199 supplemented with 10 ng/ml epidermal growth factor, 10 IU/ml equine chorionic gonadotropin (eCG; Intervet, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (hCG; Intervet), and 10% (v/v) porcine follicular fluid. A group of 50 COC was cultured in 500 μl of IVM medium at 39 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, COC were transferred to eCG- and hCG-free IVM medium and cultured for another 20 h. At the end of the maturation, oocytes were freed from cumulus cells by repeated pipetting in the IVM medium containing 0.5 mg/ml hyaluronidase for 1 min.

Nuclear Transfer

Fibroblast cells were cultured from ear skin biopsies from adult male specific pathogen-free (SPF) Minnesota miniature pigs maintained at Seoul National University according to the procedure from Lee *et al.* (2007). At 42 h after the onset of IVM, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspirating the first polar body and the second metaphase plate with a small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.4% bovine serum albumin (BSA) and 7.5 mg/ml cytochalasin B. After the enucleation, oocytes were stained with 5 mg/ml bisbenzimide (Hoechst 33342) for 5 min and observed under Nikon TE-300 inverted microscope equipped with epifluorescence (Nikon Instrument, Tokyo, Japan). Oocytes containing DNA materials were excluded from the subsequent experiments. Fibroblast cells were trypsinized into single cells, and transferred into the perivitelline space of enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Then, couplets were fused with double DC pulse of 1.5 kV/cm for 40 μsec using BTX Electro-Cell Manipulator 2001 (Gentronics, San Diego, CA). Following the electrical stimulation, reconstructed oocytes were cultured in NCSU23 supplemented with 4 mg/ml fatty acid-free BSA and 7.5 mg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Then, oocytes were cultured for 4 days in NCSU23 containing 4 mg/ml fatty acid-free BSA and transferred to NCSU23 containing 10% FBS and cultured for another 3 days. All NT embryos were cultured at 39℃ in a humidified atmosphere containing 5% CO_2 in 95% air. Prior to seeding onto feeder layer, blastocysts were treated with 0.2% (w/v) pronase to remove zona pellucida (ZP).

Isolation of Inner Cell Mass

Hatched blastocysts were treated in a microdrop of 0.25% trypsin-EDTA (Invitrogen) for 1 to 2 min at room temperature. When the trophoblasts began to disperse, the blastocysts were transferred into another drop of fresh NCSU23 medium, and ICM was isolated with the aid of a micropipette.

Preparation of Feeder Cells

Feeder cells were prepared and maintained as described by Hogan *et al.* (1994). Briefly, mouse embryonic fibroblast and STO cells were inactivated by incubation in medium containing 10 μg/ml of mitomycin C for 2 h. A day before seeding blastocysts or isolated ICM, inactivated feeder cells were plated at a density of 2×10^5 cells per well in a gelatin-coated 4-well multidish (Nunc, Roskilde, Denmark).

Isolation of Embryonic Stem Cells

Whole ZP-free blastocysts or isolated ICM were seeded onto mitotically inactivated feeder cells. Either porcine ES (pES) or human ES (hES) medium was used for basal culture medium to isolate ES cells. The former consisted of Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 1 mM L-glutamine, 0.1 M MEM nonessential amino acids, 10 μM 2-mercaptoethanol, 100 units/ml penicillin, 0.5 mg/ml streptomycin, 15% ES-screened FBS (HyClone, Logan, UT), and 1,000 units/ml murine leukemia inhibitory factor (LIF; Millipore, Billerica, MA), whereas the latter consisted of DMEM/F12 (Invitrogen) containing 1 mM L-glutamine, 0.1 M MEM nonessential amino acids, 10 μM 2-mercaptoethanol, 100 units/ml penicillin, 0.5 mg/ml streptomycin, 20% (v/v) knockout serum replacement (Invitrogen), 1,000 units/ml murine LIF, and 4 ng/ml basic fibroblast growth factor. Once densely packed putative ES colony appears approximately 4 to 7 days after seeding blastocysts or ICM, they were picked from the feeder layer and disaggregated in a microdrop of 0.25% trypsin-EDTA for 10 to 15 min at 39℃ with the aid of a micropipette. The cells disaggregated from the colonies were seeded onto a fresh feeder layer. Putative ES cells were passaged every 5 to 7 days. All cultures were maintained in 4-well mutidishes at 39℃ in 5% CO_2, 95% air with culture medium changed every other day.

Statistical Analysis

In the comparison of two different types of feeder cells, culture media, and starting materials to be seeded, at least three replicates were conducted for each experiment. Data on the percentages of attachment and primary colony formation in each experiment were subjected to Student's t-test. Differences of $p<0.05$ were considered to be significant.

RESULTS

Effects of Feeder Cells on Attachment of Blastocysts and Colony Formation

As represented in Table 1, more ZP-free miniature pig blastocysts were attached, and their ICM were proliferated to form colonies on STO cells compare to murine primary embryonic fibroblasts. In this study, none of the seeded blastocysts resulted in outgrowth of ICM, so that STO cells were exclusively used for feeder cells for the subsequent experiments.

Effects of Culture Medium on Attachment of Blastocysts and Colony Formation

Culture media commonly used for the isolation of embryonic stem cells in two different species were tested. As summarized in Table 2, hES medium often used for the culture of human ES cells were more effective than pES medium for porcine ES cells. Interestingly, no primary colonies were obtained from miniature pig blatocysts cultured in the medium known to be developed for the culture of porcine ES cells. Hence, the subsequent experiments were carried out using hES medium.

Effects of ICM Isolation on Their Attachment and Colony Formation

Whether isolation of ICM is important for the establishment of porcine ES cells was tested, and the results were shown in Table 3. No statistical differences were found between seeding ZP-free blastocysts and isolated ICM on initial attachment and primary colony formation. Primary colonies obtained from blastocysts seeded on STO cells in hES medium were subcultured on fresh STO feeder layer, and ES-like cells were isolated. The putative ES cells derived from miniature pig NT embryos resembled murine ES cells in their characteristic morphology, including tightly-packed colonies consisting of cells with high nuclear-cytoplasmic ratio and prominent nucleoli (Fig. 1). These ES-like ce-

Table 1. Effects of feeder cells on attachment of blastocysts and colony formation

Feeder cells	No. of blastocysts	No. (%) of attached blastocysts*	No. (%) of primary colony formation**
Mouse embryonic fibroblasts	52	13 (25.0)	0 (0.0)
STO cells	69	33 (47.8)	2 (6.0)

* Percentage from cultured blastocysts.
** Percentage from attached blastocysts.

Table 2. Effects of culture medium on attachment of blastocysts and colony formation

Culture medium	No. of blastocysts	No. (%) of attached blastocysts*	No. (%) of primary colony formation**
pES medium	14	4 (28.6)	0 (0.0)
hES medium	55	29 (52.7)	2 (6.9)

* Percentage from cultured blastocysts.
** Percentage from attached blastocysts.

Table 3. Effects of ICM isolation on attachment and colony formation

Group	No. of blastocysts or ICM	No. (%) of attached blastocysts or ICM*	No. (%) of primary colony formation**
ZP-free blastocyst	34	13 (38.2)	2 (15.4)
ICM	74	23 (31.1)	4 (17.4)

* Percentage from cultured blastocysts or ICM.
** Percentage from attached blastocysts or ICM.

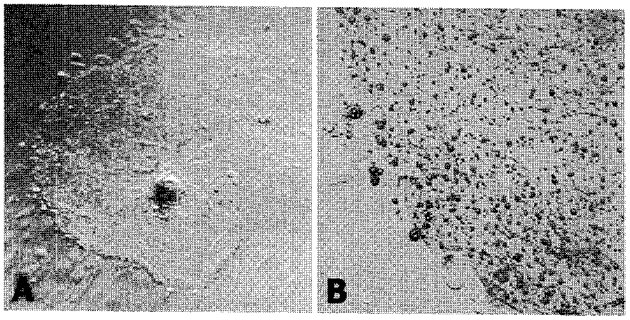

Fig. 1. Putative embryonic stem cells derived from miniature pig blastocyst. A: Colony tightly packed with ES-like cells (100×), B: Putative ES cells with high nuclear-cytoplasmic ratio, prominent nucleoli, and lipid droplets (400×).

lls were maintained in culture until the third passage, but lost due to spontaneous differentiation *in vitro*.

DISCUSSION

Since the swine shares a high similarity in anatomic, immunologic, and physiologic characteristics with humans, it has not only the potential to serve as a great research model for human biomedicine (Phillips and Tumbleson, 1986), but has also been considered as an optimal model for preclinical development of therapeutic approaches (Brevini *et al.*, 2007). In this context, although their germ-line transmission has not yet been demonstrated, porcine ES cells have been isolated from both *in vivo*- (Gerfen and Wheeler, 1995; Chen *et al.*, 1999; Li et al., 2003) and *in vitro*-derived blastocysts (Li *et al.*, 2004a). In addition, ES-like cells maintained in short-term culture have been reported in Chinese laboratory miniature pigs (Li *et al.*, 2004b). In the present study, the isolation of ES cells from Minnesota miniature pig NT embryos was attempted.

The feeder cell is one of the important factors affecting ES cell culture (Piedrahita *et al.*, 1990). It has been reported that feeder cells produce cytokines, including LIF stimulating self-renewal of ES cells while inhibiting their differentiation (Smith *et al.*, 1988). Two most commonly used feeder cells for ES cell culture are primary mouse embryonic fibroblasts and STO cells. As represented in Table 1, STO cells have advantages over mouse embryonic fibroblasts with respect to attachment of blastocysts and colony formation.

Upon testing ES cell culture media, hES medium gave rise to greater attachment of blastocysts on STO feeder cells and subsequent formation of primary colonies (Table 2). Unexpectedly, pES medium, the medium conventionally used for porcine ES cell culture (Piedrahita *et al.*, 1990) did not support any colony formation in the present study. More recently developed hES medium may be more suitable for isolation of ES cells from miniature pig NT embryos due to breed specificity and/or NT embryos.

Isolation of the ICM is often performed prior to seeding embryos onto feeder layer. Traditionally, they have been isolated from blastocysts by immunosurgery (Solter and Knowles, 1975). In the present study, however, ICM were much more conveniently isolated by treatment of enzyme trypsin as described by Li *et al.* (2004b). Plating ICM isolated by this enzymatic procedure resulted in comparable percentage of attachment and primary colony formation to seeding whole ZP-free blastocysts (Table 3).

According to the optimization of procedures, ZP-free blastocyst seeded onto STO feeder cells in hES medium gave rise to two putative miniature pig ES cell lines as represented in Fig. 1. This may reflect appropriate conditions for the isolation of miniature pig ES cells. These cell lines possessed common morphological characteristics of ES cells. Unfortunately, however, these ES cell lines both in miniature pigs have not been maintained beyond passage 3 and lost due to spontaneous differentiation, so that extensive characterization of the cell lines has yet remained for further studies.

In summary, the present study optimized conditions to isolate ES cells in miniature pigs and demonstrates that isolated ICM cultured on STO feeder cells in hES medium could result in the isolation of putative ES cells. However, more refined conditions are remained to be developed in further studies to maintain these ES-like cells in long-term culture by preventing spontaneous differentiation.

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