Establishment of Porcine Embryonic Stem Cells by Aggregation of Parthenogenetic Embryos

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ABSTRACT

The pig has been considered to serve as an appropriate model of human disease. Therefore, establishment of porcine embryonic stem cell lines is important. The purpose of the present study was to further work in this direction. We produced porcine parthenogenetic embryos, and separately aggregated two of each of two-cell (2×2), four-cell (2×4), and eight-cell (2×8) embryos derived by parthenogenesis. After culture for 4 days, the developmental ability of the aggregates and total blastocyst cell numbers were evaluated. The percentage of blastocysts was significantly higher in both 2×4- and 2×8-aggregated embryos (58.3±1.9% and 37.2±2.8%, respectively) than in the control or 2×2-aggregated embryos (23.6±1.1% and 12.5±2.4%, respectively). Total blastocyst cell numbers were increased in the 2×4- and 2×8-aggregated embryos (by 44±3.0% and 45±3.3%, respectively) compared with those of control or 2×2-aggregated embryos (30.5±2.1% and 30.7±2.6%, respectively; *p*<0.05). The levels of mRNA encoding Oct-4 were higher in both the 2×4- and 2×8-aggregated embryos than in the control. When blastocysts derived from 2×4- aggregated embryos or intact normal embryos were cultured on mouse embryonic fibroblast feeder cells to obtain porcine stem cells, blastocysts from aggregated embryos formed colonies that were better in shape compared with those derived from intact blastocysts. Together, the data show that aggregation of porcine embryos not only improves blastocyst quality but also serves as an efficient procedure by which porcine embryonic stem cells can become established.

(Key words : Porcine, Embryonic stem cell, Aggregation)

INTRODUCTION

Embryonic stem cells are pluripotent in nature, are capable of virtually unlimited self-renewal and long-term propagation, and have a broad differentiation potential. Such cells are of great value in biomedical research and various clinical applications (Murry and Keller, 2008). Human and mouse embryonic stem cell lines have been established over the past two decades. Currently, many attempts are underway to produce embryonic stem cell lines from pigs, because these animals offer distinct advantages compared with other species as a research model; pigs are immunologically and physiologically similar to humans (Li *et al.*, 2003). Moreover, porcine embryonic stem cells share many characteristics with human embryonic stem cells; these include colony morphology, a requirement for a feeder layer, and a dependence on similar culture medium factors (Kikuchi *et al.*, 1999). The isolation and thorough characterization of porcine embryonic stem cell lines are goals worthy of pursuit. Experimental tools derived from such work could be used in the development of therapeutic applications and techniques for human tissue repair. The possible approaches include construction of biological reactors and organ xenotransplantation (Pedersen, 1994). Unfortunately, no porcine embryonic stem cell line described to date has satisfied all required criteria. *In vitro* production of high-quality porcine blastocysts is essential for production of porcine embryonic stem cell lines. Blastocysts derived from *in vitro* fertilization (IVF) are of poor quality and are low in total cell numbers (Han *et al.*, 1999); cells of both the inner cell mass (ICM) and the trophectoderm (TE) are few (Machaty *et al.*, 1998). In efforts to improve the quality of porcine blastocysts, aggregation of embryos has been shown to enhance development *in vitro* (Tang and West, 2000). Although culture conditions have been im-

* This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MEST) (No. 2010-0001356), and by the BioGreen 21 Program (No. 20070401034031) and Cooperative Research Program for Agriculture Science & Technology Development (No. PJ00779306) of the Rural Development Administration, Republic of Korea.

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proved (Kikuchi *et al.*, 1999, 2002; Betthauser *et al.*, 2000; Yin *et al.*, 2002), the *in vitro* developmental abilities of blastocysts remain inferior to those derived *in vivo* (Thompson, 1997; Wang *et al.*, 1997). Several types of *in vitro*-constructed blastocysts have been reported to form primary colonies; the required embryos have been obtained by parthenogenesis, *in vitro* fertilization, and somatic nuclear transfer (Kim *et al.*, 2007).

The earliest reports on the isolation of pig embryonic stem cells used blastocysts hatched *in vivo* (Evans *et al.*, 1990; Notarianni *et al.*, 1990; Piedrahita *et al.*, 1990; Strojek *et al.*, 1990). However, this method is labor-intensive. In contrast to *in vivo*-derived embryos, embryos obtained using *in vitro* fertilization and parthenogenesis are easy to collect, and are thus associated with low cost. Some problems cannot be readily resolved using these embryos; these include optimization of blastocyst total cell numbers (particularly those of the ICM and TE), and exploration of the extent of pluripotent gene expression. For example, it is known that *Oct-4* expression level plays a major role in maintenance or loss of pluripotency in both pre-implantation embryos and stem cells (Niwa *et al.*, 2002).

In the present study, we sought to improve both the developmental competence and the quality of porcine blastocysts, and to enhance the expression levels of genes required for continued pluripotency *in vitro* by aggregating embryos derived by parthenogenesis. In addition, we compared the efficiency of porcine embryonic stem cell establishment after aggregation of blastocysts with that obtained using isolated (thus non-aggregated) blastocysts.

MATERIALS AND METHODS

Chemicals

Unless noted otherwise, all chemicals were purchased from Sigma Chemicals (St Louis, MO).

In Vitro Maturation

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% (w/v) saline supplemented with 75 $\mu g\ mL^{-1}$ potassium penicillin G and 50 mg mL^{-1} streptomycin sulfate, and were maintained at 25~30℃. Cumulus-oocyte complexes (COCs) were separated from follicles (3~6 mm in diameter) by aspiration through 18-gauge needles into disposable 10 mL syringes. After three washes in TLH-EPES medium (Funahashi *et al.* 1994), approximately 50 oocytes were allowed to mature in 500 μL IVM medium in a four-well multi-dish (Nunc, Roskilde, Denmark) at 38.5℃ under 5% (v/v) CO_2 in air. NCSU-23 medium was used for oocyte maturation (Petters and Wells, 1993); the medium was supplemented with 10% (v/v) follicular fluid, 0.57 mM cysteine, 10 ng mL^{-1} β-mercaptoethanol, 10 ng mL^{-1} epidermal growth factor (EGF), 10 IU mL^{-1} pregnant mare serum gonadotropin (PMSG), and 10 IU mL^{-1} human chorionic gonadotropin (hCG). After culture for 22 h, oocytes were washed three times with maturation medium and further cultured for another 22 h in maturation medium without hormones.

Activation of Porcine Oocytes and *In Vitro* Culture

After *in vitro* maturation, cumulus cells were removed by repeated pipetting in TL-HEPES supplemented with 0.1% (w/v) PVA and 0.3% (w/v) hyaluronidase. Oocytes were transferred to activation solution, consisting of 0.3 M D-mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.01% (w/v) PVA in water, and washed three times in the same medium. Oocytes were next stimulated using a direct current pulse of 15 kV/cm for 30 μsec employing a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA). After activation, oocytes were washed and transferred into 50 μL of culture medium, and covered with mineral oil, in a dish 60 cm in diameter (Nunc). Cells were cultured at 38.5℃ under 5% (v/v) CO_2 in air, for 7 days. The IVC medium was Porcine Zygote medium-3 (PZM-3) (Yoshioka *et al.*, 2002).

Aggregation of Embryos and *In Vitro* Culture

After 2 days, the zonae pellucidae of four-cell-stage embryos were removed by treatment with acidic Tyrode's solution. Depression of clusters in the bottom of the culture dish, generated by gentle pressure using a darning needle (BLS, Budapest, Hungary; Nagy and Rossant, 1993) were covered with microdrops (each consisting of 40 μL of NCSU-23 medium), overlaid with oil, and incubated for at least 1 h prior to use. Aggregation was accomplished by placing zona-free embryos into microwells. Two of each of two-cell (2×2)-, 4-cell (2×4)-, and 8-cell (2×8)-stage aggregated embryos were cultured in parallel in separate drops within the same dish (Fig. 1). Aggregates were cultured at 38.5℃ under a 5% (v/v) CO_2, 5% (v/v) O_2, and 90% (v/v) N_2 atmosphere for 2 days, and next added to microwells containing 10% (v/v) fetal bovine serum (Life Technologies, CA, USA) (Kim *et al.*, 2004). Embryo development was observed daily under an inverted microscope (Nikon, Tokyo, Japan). After embryo aggregation, later examination employed epifluorescence microscopy.

Preparation of Feeder Cells and Establishment of Embryonic Stem Cells

Mouse embryonic fibroblasts (MEF), used as feeder cells, were prepared from fetal mice. Briefly, mice at day 12~14 of pregnancy were sacrificed by cervical dislocation. Fetuses were separated from uteri and next washed twice in fresh phosphate-buffered saline (PBS)

to remove any blood. After the head and liver were removed, each carcass was treated with 0.25% (w/v) trypsin-0.02% (w/v) ethylenediamine tetra-acetic (EDTA) acid solution for 30 min at room temperature prior to cell culture in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% (v/v) new calf serum (NCS; Gibco, Grand Island, NY), 5% (v/v) FBS (Gibco), 100 IU/mL penicillin (Sigma), and 0.05 mg/mL streptomycin (Gibco). Feeder layers were prepared from mouse MEFs after 1~6 passages. MEFs were inactivated in a medium containing 10 mg/mL mitomycin C (Sigma), for 2.5 h. MEFs were next washed three times in PBS and treated with 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution. Trypsinized cells were harvested by centrifugation at 1,000 rpm for 3 min and pellets were resuspended in MEF medium. Cells were seeded at a density of 5×10^4 cells per well in a 96-well plate (Sigma) coated with 0.1% (w/v) gelatin (Fluka AG; catalog no. 48722; Switzerland). Usually, MEFs were plated 1 day before porcine embryos were seeded in efforts to establish embryonic stem cell lines. To acquire porcine embryonic stem cells, 10 blastocysts were seeded on each feeder layer. During early growth of blastocysts on feeders, DMEM+NCSU-23 medium (volume ratio 1:1) supplemented with 2.0 mM L-glutamine, 0.03 mM nucleoside mix, 20 ng/mL bFGF, and 40 ng/mL h-LIF, was used (Table 1). After colony formation, the basic culture medium was changed to DMEM only, thus without NCSU-23.

Table 1. Components of the medium used for culture of porcine embryonic stem cells

Component	Concentration	Supplier
DMEM+NCSU-23		Gibco
Penicillin-streptomycin	1.0% (w/v)	Sigma
2-Mercaptoethanol	0.1 mM	Sigma
L-Glutamine	2.0 mM	Sigma
NEAA	1.0% (w/v)	Gibco
FBS	15% (v/v)	Gibco
Nucleoside mix	0.03 mM	Sigma
bFGF	20 ng/mL	Sigma
h-LIF	40 ng/mL	Sigma

Real-Time Quantitative Polymerase Chain Reaction

To analyze *Oct-4* mRNA expression, the 2×2-, 2×4-, and 2×8-aggregated embryos, and control blastocysts, were harvested 168 h after fertilization. Total mRNA was extracted using a Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. For reverse transcription, total mRNA from one blastocyst was added to a reaction mix of final volume 20 μL containing 0.5 mg oligo dT, RT buffer (1×), 10 mM dithiothreitol, 10 mM of a dNTP mix, and 10 units of Moloney murine leukemia virus (MMLV) reverse transcriptase. Reverse transcription was performed at 37℃ for 50 min and 70℃ for 15 min, and products were stored at 4℃ until real-time polymerase chain reaction (PCR) analysis was performed. Real-time PCR amplification was conducted using the ABI 7300 Real-time PCR System (Applied Biosystems, Forster City, CA). A QuantiTect SYBR Green PCR kit (Finnzymes, Espoo, Finland) was used to provide real-time quantification of the desired PCR product. Each real-time PCR reaction mixture was composed of 4 μL cDNA, and *Oct-4* forward and reverse primers (Table 2) in a final volume of 20 μL. Replications were performed three times and mRNA levels were normalized to those of the mRNA encoding alpha-actin.

Statistical Analysis

All data were analyzed by one-way ANOVA and the protected least significant difference (LSD) test using the general linear models of the Statistical Analysis Systems (SAS, Cary, NC) software to explore differences among experimental groups. A treatment difference was considered significant when the *P* value was less than 0.05.

RESULTS

Developmental Ability of Aggregated Parthenogenetic Embryos

After activation of matured oocytes, different stages of parthenogenetic embryos were aggregated in the ab-

Table 2. Primers used in, and cycling conditions of, real-time RT-PCR

Gene	Primer	Sequence	Accession number	Annealing temperature (℃)	Size (bp)
Oct-4	Forward Reverse	AACGATCAAGCAGTGACTATTCG GAGTACAGGGTGGTGAAGTGAGG	AF074419	60℃	153
β-actin	Forward Reverse	GTGGACATCAGGAAGGACCTCTA ATGATCTTGATCTTCATGGTGCT	U07786	58℃	137

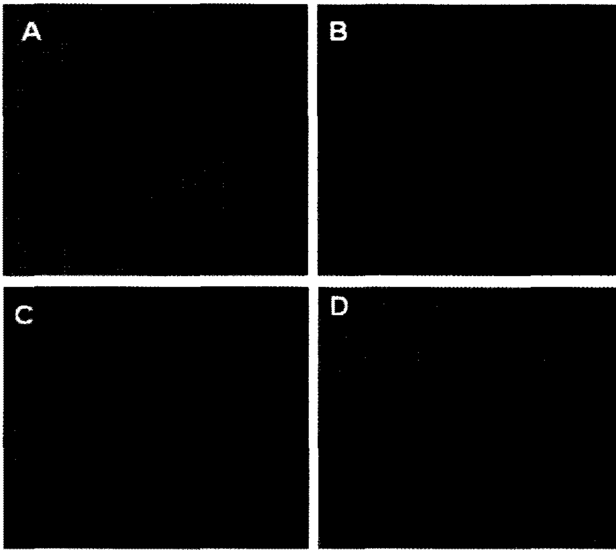

Fig. 1. Aggregation of porcine parthenogenetic embryos using the depression cluster method. A: Aggregation of two 4-cell stage embryos without zonae pellucidae. B: Depression formed by aggregated embryos in the bottom of a culture dish. C and D: Blastocysts derived from aggregated parthenogenetic embryos.

sence of the zonae pellucidae. The 2×2-, 2×4-, and 2×8-aggregated embryos developed into blastocysts, and both developmental rates and total cell numbers were measured (Table 3). Developmental rates were significantly increased in aggregated embryos (by 29.5±2.4% for the 2×2-, 58.3±1.9% for the 2×4-, and 37.2±2.8% for the 2×8-aggregates) compared with non-aggregated control embryos (23.6±1.1% for the control) ($p<0.05$). The blastocyst formation rate of 2×4-aggregated embryos was highest. The developmental morphology of aggregated embryos was normal (Fig. 1).

Total Cell Numbers in Aggregated and Intact Embryos

The nuclear morphology of cells in aggregated embryos was examined by Hoechst 33258 staining (Fig. 2). Significant differences were observed in the total cell numbers of aggregated and non-aggregated embryos. The total cell numbers in blastocysts derived from 2×4- and 2×8-aggregates (44±3.0 and 45±3.3, respectively) were

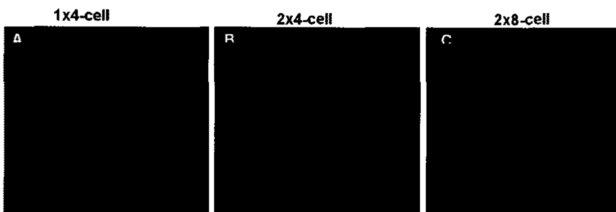

Fig. 2. Hoechst 33258 staining of aggregated porcine parthenogenetic blastocysts. A: Blastocyst derived from a single 4-cell-stage embryo. B: Blastocyst derived from aggregation of two 4-cell-stage embryos. C: Blastocyst derived from a 2×8-aggregate.

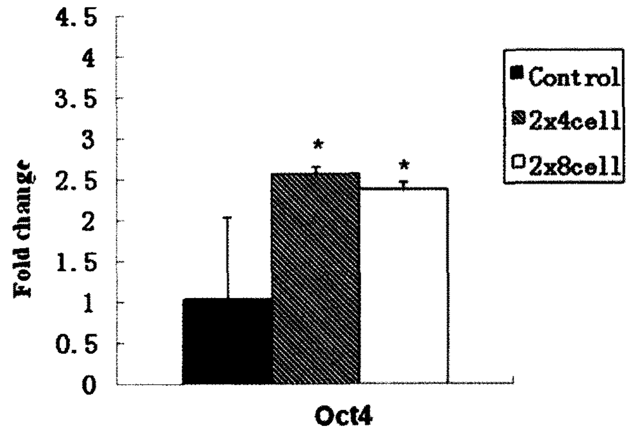

Fig. 3. *Oct-4* gene expression in aggregated parthenogenetic blastocysts. The levels of *Oct-4* expression in 2×4-cell, 2×8-cell, and control blastocysts, 120 h after aggregation. mRNA levels were analyzed by real-time PCR using an ABI 7300 system. Three replications were conducted and the mRNA level of each sample was normalized to that of β-actin. Bars with different superscripts reflect significantly different values ($p<0.05$).

significantly higher ($p<0.05$) than those of control blastocysts (30.5±2.1).

Analysis of *Oct-4* Transcription in Aggregated Blastocysts

The high pluripotency of blastocysts renders embryonic stem cell development feasible, and it was thus necessary to investigate whether embryo aggregation resulted in expression of genes associated with pluripotency. To determine the relative levels of pluripotent gene expression, *Oct-4* mRNA relevant to embryo quality in aggregated blastocysts, real-time PCR was employed. The levels of *Oct-4* mRNA in 2×4- and 2×8-aggregated blastocysts were significantly increased compared with that of the control (Fig. 3). Interestingly, the *Oct-4* mRNA level in 2×8-aggregated blastocysts was not higher than that in 2×4-aggregates.

Establishment of Porcine Embryonic Stem Cells

An appropriate culture medium is critical during stem cell culture. Porcine embryonic stem cells are more similar to human stem cells than are the stem cells of other species. Here, we optimized medium components for culture of porcine embryonic stem cells, with reference to the constituents required for human stem cell development. At early stages of blastocyst growth on feeders, DMEM+NCSU-23 medium (in a 1:1 volume ratio) supplemented with 2.0 mM L-glutamine, 0.03 mM nucleoside mix, 20 ng/mL bFGF, and 40 ng/mL h-LIF, was used (Table 1). After colonies formed, the culture medium was changed to a medium lacking NCSU-23. To obtain porcine embryonic stem cells, 10 intact blastocysts were seeded onto each feeder layer. After cul-

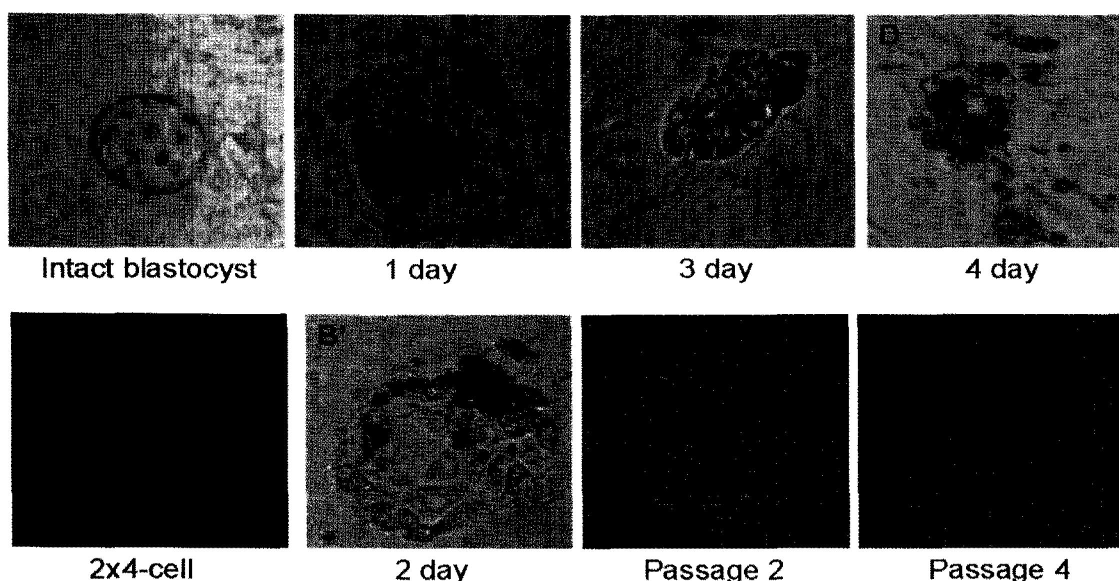

Fig. 4. Establishment of porcine embryonic stem cells by co-culture of aggregated or non-aggregated blastocysts with feeder cells. A: Control blastocysts were cultured on feeder cells. B and C: Cells attached to feeders. D: Differentiated cells. A', B', C', and D': Cultures of aggregated blastocysts on feeder cells and subcultures of embryonic stem cells.

Table 3. Developmental rates of aggregated parthenogenetic porcine embryos at different stages

Embryonic state*	No. of embryos	No. of blastocysts (%)	No. of nuclei (mean±SE)
Control	40	9 (23.6±1.1)[a]	30.5±2.1[a]
2×2	38	5 (29.5±2.4)[a]	30.7±2.6[a]
2×4	42	25 (58.3±1.9)[b]	44±3.0[b]
2×8	48	18 (37.2±2.8)[c]	45±3.3[b]

* Control: intact non-aggregated embryos, 2×2: aggregation of two 2-cell stage embryos, 2×4: aggregation of two 4-cell stage embryos, 2×8: aggregation of two 8-cell stage embryos.
Within the same column, values bearing different letters are significantly different ($p<0.05$).

ture for 1 day, cells of the trophectoderm began to attach to the feeder layer. After culture for 2 days, some cells from embryos adhered to the feeder layer, attracted by the trophoblastic cells. Unfortunately, cells became differentiated after culture for 4 days (Fig. 4A). Blastocysts from the 2×4-aggregates, of high quality and showing enhanced *Oct-4* gene expression, were cultured on feeder cells under the same conditions used for culture of intact embryos. However, cells derived from aggregates proliferated and formed many colonies (Fig. 4). Isolated colonies could still be observed even after four subcultures had been completed.

DISCUSSION

Establishment of porcine embryonic stem cells is more difficult than is the case for stem cells of other species because no optimized *in vitro* culture system for porcine embryonic stem cells has been developed. To enhance the developmental competence of *in vitro*-produced blastocysts, we investigated the effect of embryo aggregation using porcine parthenogenetic embryos. The results showed that aggregation of four-cell stage embryos derived by parthenogenesis improved the *in vitro* development rate, enhanced blastocyst total cell numbers, and stimulated *Oct-4* gene expression; all of these effects may favor the establishment of embryonic stem cells with a high degree of pluripotency. Our results were similar to those of a previous study on aggregation using porcine IVF-derived embryos (Lee *et al.*, 2007). Although the total cell numbers of 2×8-derived blastocysts were higher than those from 2×4-derived blastocysts, neither the developmental competence nor the pluripotency of 2×8-derived blastocysts was significantly enhanced compared with those of 2×4-derived blastocysts.

Aggregation methods have been used to improve the stem cell establishment rates of many mammals *in vitro*. In cattle (Wells and Powell, 2000), mice (Tang and West, 2000), and the rhesus monkey (Schramm and Paprocki, 2004), aggregation of embryos with developmentally asynchronous or synchronous blastomeres has been used to enhance stem cell development *in vitro*. Herein, we have determined optimal conditions for *in vitro* development of aggregated porcine blastocysts. Pluripotent blastocysts of high quality are essential if porcine embryonic stem cell lines are to be efficiently established. Embryos at the four-cell stage were the optimal choice for aggregation. Two-cell-stage embryos had a low aggre-

gation rate, and aggregations of eight-cell-stage embryos developed slowly. Also, we found that total cell numbers and the level of *Oct-4* gene expression in blastocysts derived from eight-cell stage embryos were not superior to those of the other aggregates tested. However, total cell numbers in blastocysts derived from aggregated embryos were greater than those of non-aggregated embryos (Table 1 and Fig. 2).

Embryo aggregation yields good-quality blastocysts, in terms of cell numbers; this may be important for the efficient production of embryonic stem cells. We assessed *Oct-4* mRNA levels using real-time PCR (Fig. 3). High-level expression of *Oct-4* is a valuable marker for identification of mammalian pluripotent cells (Kirchhof *et al.*, 2000). Blastocysts derived from aggregated parthenogenetic embryos have been shown to be more efficient when used to establish embryonic stem cell lines. Our results show that 2×4-aggregated embryos may be effectively used in the establishment of porcine embryonic stem cell lines. A future study will investigate markers of porcine embryonic stem cells derived from aggregated blastocysts; expression of SSEA-1, Nanog, and SOX2 will be evaluated. In addition, the aggregation method may be useful when cloned embryos derived from somatic cell nuclear transfer are used to establish embryonic stem cells.

REFERENCES

1. Brevini TAL, Gandolfi F (2008): Parthenotes as a source of embryonic stem cells. Cell Prolif 41:20-30.
2. Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M (2000): Production of cloned pigs from *in vitro* systems. Nat Biotechnol 18: 1055-1059.
3. Chen LR, Shiue YL, Bertolini, L, Medrano JF, Bondurant RH, Anderson GB (1999): Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology 52,195-212.
4. Evans MJ, Notarianni E, Laurie S, Moor RM (1990): Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33:125-8.
5. Han YM, Abeydeera LR, Kim JH, Moon HB, Cabot RA, Day BN, Prather RS (1999): Growth retardation of inner cell mass cells in polyspermic porcine embryos produced *in vitro*. Biol Reprod 60: 1110-1113
6. Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H (1999): Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured *in vitro*. Biol Reprod 60: 336-340.
7. Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T (2002): Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. Biol Reprod 66:1033-1041.
8. Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Scholer H, Niemann H (2007): Expression pattern of Oct-4 in preimplantation embryos of different species. Biol Reprod Sci 98:147-168.
9. Kim HS, Son HY, Kim S, Lee GS, Park CH, Kang SK, Lee BC, Hwang WS, Lee CK (2007): Isolation and initial culture of porcine inner cell masses derived from *in vitro*-produced blastocysts. Zygote 15:55-63.
10. Kim HS, Lee GS, Hyun SH, Lee SH, Nam DH, Jeong YW, Kim S, Kang SK, Lee BC, Hwang WS (2004): Improved *in vitro* development of porcine embryos with different energy substrates and serum. Theriogenology 61:1381-1393.
11. Li M, Zhang D, Hou Y, Jiao L, Zheng X, Wang WH (2003): Isolation and culture of embryonic stem cells from porcine blastocysts. Mol Reprod Dev 65 (4):429-34.
12. Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38(4):431-40.
13. Murry CE, Keller G (2008): Differentiation of embryonic stem cells into clinically relevant populations: Lessons from embryonic development. Cell 132: 661-680.
14. Machaty Z, Day BN, Prather RS (1998): Development of early porcine embryos *in vitro* and *in vivo*. Biol Reprod 59:451-455.
15. Notarianni E, Laurie S, Moor RM, Evans MJ (1990): Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. J Reprod Fertil Suppl 41:51-6.
16. Niwa H, Masui S, Chambers I, Smith AG, Miyazaki (2002): Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. Mol Cell Biol 22:1526-1536.
17. Niwa H, Miyazaki J, Smith AG (2000): Quantitative expression of Oct3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24: 372-376.
18. Pedersen RA (1994): Studies of *in vitro* differentiation with embryonic stem cells. Reprod Fertil Dev 6:543-552.

19. Petters RM, Wells KD (1993): Culture of pig embryos. J Reprod Fertil Suppl 48:61.
20. Piedrahita JA, Anderson GB, Bondurant RH (1990): On the isolation of embryonic stem cells: Comparative behavior of murine, porcine and ovine embryos. Theriogenology 34:879-901.
21. Strojek RM, Reed MA, Hoover JL, Wagner TE (1990): A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. Theriogenology 33:901-13.
22. Tang PC, West JD (2000): The effects of embryo stage and cell number on the composition of mouse aggregation chimaeras. Zygote 8:235-243.
23. Thompson JG (1997): Comparison between *in vivo* derived and *in vitro* produced pre-elongation embryos from domestic ruminants. Reprod Fertil Devel 9:341-354.
24. Wang WH, Abeydeera LR, Han YM, Prather RS, Day BN (1997): Morphologic evaluation and actin filament distribution in porcine embryos produced *in vitro* and *in vivo*. J Reprod Fertil 111:101-108.
25. Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002): Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 66:112-9.
26. Yin XJ, Tani T, Yonemura I, Kawakamia M, Miyamoto K, Hasegawa R, Kato Y, Tsunoda Y (2002): Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. Biol Reprod 67:442-446.

(Received: March 2 2011/ Accepted: March 7 2011)