Zygote **24** (December), pp. 909–917. © Cambridge University Press 2016
doi:10.1017/S0967199416000241 First Published Online 3 October 2016

Aggregation of cloned embryos in empty zona pellucida improves derivation efficiency of pig ES-like cells

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Date submitted: 10.08.2015. Date revised: 27.07.2016. Date accepted: 06.08.2016

Summary

The development of embryonic stem cells (ESCs) from large animal species has become an important model for therapeutic cloning using ESCs derived by somatic cell nuclear transfer (SCNT). However, poor embryo quality and blastocyst formation have been major limitations for derivation of cloned ESCs (ntESCs). In this study, we have tried to overcome these problems by treating these cells with histone deacetylase inhibitors (HDACi) and aggregating porcine embryos. First, cloned embryos were treated with Scriptaid to confirm the effect of HDACi on cloned embryo quality. The Scriptaid-treated blastocysts showed significantly higher total cell numbers (29.50 ± 2.10) than non-treated blastocysts (22.29 ± 1.50, $P < 0.05$). Next, cloned embryo quality and blastocyst formation were analyzed in aggregates. Three zona-free, reconstructed, four-cell-stage SCNT embryos were injected into the empty zona of hatched parthenogenetic (PA) blastocysts. Blastocyst formation and total cell number of cloned blastocysts increased significantly for all aggregates (76.4% and 83.18 ± 8.33) compared with non-aggregates (25.5% and 27.11 ± 1.67, $P < 0.05$). Finally, aggregated blastocysts were cultured on a feeder layer to examine the efficiency of porcine ES-like cell derivation. Aggregated blastocysts showed a higher primary colony formation rate than non-aggregated cloned blastocysts (17.6 ± 12.3% vs. 2.2 ± 1.35%, respectively, $P < 0.05$). In addition, derived ES-like cells showed typical characters of ESCs. In conclusion, the aggregation of porcine SCNT embryos at the four-cell stage could be a useful technique for improving the development rate and quality of porcine-cloned blastocysts and the derivation efficiency of porcine ntESCs.

Keywords: Aggregation, Empty zona, Porcine embryo, Somatic cell nuclear transfer

Introduction

There has been a great deal of interest in the possible application of embryonic stem cells (ESCs) in regenerative medicine, because ESCs could allow for cell replacement therapies. Unfortunately, ESCs

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derived from fertilized blastocysts carry a risk of immunorejection after transplantation. ESCs derived from cloned embryos from the host patient's own cell nuclei could prevent the problem of rejection, as any replacement cells would be genetically identical to the host's own somatic cell nuclei (Gurdon & Colman, 1999; Mombaerts, 2003; Wakayama, 2004). There have been reports of success in generating stem cell lines from cloned embryos (ntESCs) in cows (Cibelli *et al.*, 1998), mice (Munsie *et al.*, 2000), humans (Tachibana *et al.*, 2013), and pig (Siriboon *et al.*, 2015). These cell lines are proved to have strong proliferation and differentiation potential, similar to those of *in vitro* fertilized, embryo-derived stem cells. Unfortunately, derivation of ntESCs is less efficient; therefore, the production of high-quality cloned blastocysts derived from somatic cell nuclear transfer (SCNT) is needed to improve ntESCs.

Despite improvements in *in vitro* culture systems for embryos (Biswas & Hyun, 2011; Naruse *et al.*, 2007), cloned embryos still have lower quality blastocysts than *in vitro* fertilized (IVF) blastocysts due to low cell number and poor development (Koo *et al.*, 2009; Terashita *et al.*, 2011), especially in pigs.

To produce high-quality cloned embryos, embryo aggregation has been used to increase total cell numbers and developmental abilities of embryos produced *in vitro* (Boiani *et al.*, 2003). Aggregation of bovine embryos increased the number of cells in the inner cell mass (ICM) and the whole blastocyst, as well as the proportion of cells composing the ICM (Misica-Turner *et al.*, 2007; Zhou *et al.*, 2008). Additionally, aggregation of porcine embryos produced by IVF increased blastocyst formation rate and total cell numbers of blastocysts (Lee *et al.*, 2007).

Aggregated embryos have also been used in the derivation of ESCs. In mice, embryo aggregation has been used to improve stem cell derivation efficiency by parthenogenetic embryos (Shan *et al.*, 2012). In addition, one study observed that the outgrowth of blastocysts produced by aggregation of four-cell cloned canine embryos was better formed than that of non-aggregated embryos (Sugimura *et al.*, 2009). Even if cloned embryos have limitations in ESC derivation, this report showed that aggregation could elevate their quality to the proper stage for ntESC derivation. Even though previous studies have revealed that embryo aggregation improves developmental competence and ESC derivation, the conventional aggregation methods, namely the microwell method, have some problems, such as partially aggregated blastocysts (Park *et al.*, 2014). Partially aggregated blastocysts likely form because of lower pressure when embryos are being handled. Our previous data demonstrated that the empty zona (EZ) method reduces the risk of partially aggregated blastocysts by putting embryos into a space, which may allow a greater opportunity for successful aggregation to occur. Furthermore, ICM was significantly higher in the EZ method than in the microwell method (Park *et al.*, 2014).

In our previous study, we confirmed the efficient aggregation and elevation of blastocyst qualities produced by aggregation using the EZ method compared to the microwell method. However, it is unclear whether blastocysts produced by aggregation using EZ methods could efficiently give rise to ESCs. The aim of the current work was to efficiently derive ESCs from cloned porcine embryos aggregated by the EZ method and to improve cloned embryo quality to elevate the derivation efficiency of porcine ntESCs.

Materials and methods

Ethics statement

The experimental use of pigs was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-140328–2).

In vitro maturation

Maturated oocytes were obtained using the method described in our previous study (Hwang *et al.*, 2013). Briefly, the prepubertal gilt ovaries donated by the Sooam Biotech Research Institute (Seoul, Korea) were used in this experiment. The follicular fluid and cumulus–oocyte complexes (COCs) were aspirated using an 18-gauge needle and pooled to obtain sediments. Sediments were washed with TL–HEPES–PVA medium (Funahashi *et al.*, 1997), and the oocytes with compact cumulus cells and granulated cytoplasm were selected for *in vitro* maturation. The washed COCs were cultured in a tissue culture medium (TCM-199; Life Technologies) containing 10 ng/ml epidermal growth factor (EGF), 1 mg/ml insulin, and 10% PFF for 44 h at 39.8°C in 5% CO2 and 100% humidity. The COCs were treated with hormones, 4 IU/ml Q6 equine chorionic gonadotropin (eCG), and human chorionic gonadotropin (hCG) (Intervet, Cambridge, UK) only for the first 22 h. Then the COCs were matured in hormone-free conditions.

Generation of parthenotes and cloned embryos

To generate parthenotes, cumulus-free oocytes were activated by an electric pulse (1.0 kV/cm for 60 ms) in activation medium (280 mM mannitol, 0.01 mM $CaCl_2$, 0.05 mM $MgCl_2$) using a BTX Electro-cell Manipulator (BTX, CA, USA), followed by 4 h of incubation in PZM3 medium containing 2 mmol/l 6-dimethylaminopurine.

https://doi.org/10.1017/S0967199416000241 Published online by Cambridge University Press

Primary cell cultures of porcine embryonic fibroblasts (PEF) for SCNT were derived from fetuses on Day 27 of gestation. Primary cultured cells, at early passages from 2 to 4, were frozen at 2×10^5 cells/vial for use in SCNT. Then, at 3 to 4 days prior to SCNT, cells from one vial were thawed in a four-well dish and cultured until achieving 70–90% confluence. After thawing, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co.) and supplemented with 10% fetal bovine serum (FBS) to approximately 70% confluence (three to five passages).

SCNT was performed as previously described (Park *et al.*, 2012). Briefly, enucleation was carried out in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.4% bovine serum albumin (BSA) and 5 mg/ml cytochalasin B. Matured oocytes were enucleated by aspiration with an enucleation pipette (Humagen, Charlottesville, VA, USA). After enucleation, donor cells were mechanically collected and introduced into the perivitelline space of an enucleated oocyte. Fusion of injected oocytes was induced in a fusion medium (280 mM mannitol, 0.001 mM $CaCl_2$, and 0.05 mM $MgCl_2$) by two DC pulses (1-s interval) of 2.0 kV/cm for 30 ms using a BTX-Cell Manipulator 200 (BTX, San Diego, CA, USA). After 1 h of incubation, reconstructed oocytes were electrically activated as described in the above section. Reconstructed oocytes were treated with 500 nM Scriptaid (Sigma-Aldrich Co.) for 14 h after post-fusion activation, according to a previous study (Zhao *et al.*, 2009). After treatment with Scriptaid, reconstructed oocytes were cultured in PZM3 for 144 h. Embryo culture conditions were maintained at 38.5°C in an air atmosphere containing 5% CO_2, 5% O_2, and 100% humidity.

Embryo aggregation

Empty zonae with various sizes were collected and washed free of any debris in DPBS with 0.2% BSA by gentle pipetting. These were stored in PZM3 with 0.2% BSA under the same embryo culture conditions until use. At Day 2 of culture at the four-cell stage, SCNT embryos had their zona pellucida (ZP) removed by short exposure to acidic Tyrode's solution (Gibco). The denuded embryos were introduced into a slit of EZ and placed into each concave microwell, which were made by a smooth depression using darning needles (BLS, Budapest, Hungary) (Nagy *et al.*, 1993), as described in our previous study (Lee *et al.*, 2007).

The microwell method for embryo aggregation was performed as previously described (Lee *et al.*, 2007). Briefly, after 2 days, the ZPs were removed from the four-cell stage embryos by treatment with acid Tyrode's solution. Clusters of depressions in the bottom of a culture dish, generated by gentle pressure with a darning needle, were covered with microdrops (20 μl) of PZM-3 medium. Aggregation was accomplished by placing the zona-free embryos in groups of one or three into each microwell. Single (control) and three (3X) four-cell stage embryos were cultured in parallel in separate drops within the same dish. Aggregates were cultured at 39.8°C in a 5% CO_2, 5% O_2, and 90% N_2 atmosphere for five days. The development of embryos was observed daily under an inverted microscope (Nikon, Japan).

Total cell number counts

For examining the cell count of blastocysts, the ZPs were removed using Tyrode's acid solution. The numbers of total cells of individual aggregates, single clones, and parthenotes blastocysts collected on Day 7 were counted using Hoechst 33342 (Molecular Probes, USA) staining under a fluorescence microscope.

Isolation and culture of porcine ntES-like cells

ntES-like cells were derived from SCNT blastocysts. Reconstructed (hatched or denuded) blastocysts were seeded on feeder cells composed of mitotically inactivated mouse embryonic fibroblasts (MEFs) and followed by our previous studies (Park *et al.*, 2013; Choi *et al.*, 2013). Briefly, 5 to 7 days after seeding, primary colonies were observed and cultured for approximately 7–10 days longer. Fully expanded colonies were mechanically dissociated using pulled-glass pipettes and transferred onto new feeder cells for subculture. ES-like cells were cultured in porcine ESC media (PESM). PESM consisted of a 1:1 mixture of DMEM and Ham's F10 medium containing 15% fetal bovine serum (FBS; collected and processed in the USA), 2 mM glutamax, 0.1 mM β-mercaptoethanol, 1× MEM nonessential amino acids, and 1× antibiotics or antimycotics (all from Gibco, USA). To support pluripotency and self-renewal, the ESCs were cultured in PESM with the following cytokines: 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF; R&D Systems) and 100 ng/ml heparin sodium salt (Sigma-Aldrich, USA). ntES-like cells were subcultured every 5–7 days using pulled-glass pipettes. Expanded colonies were detached from the feeder cells and dissociated into small clumps. These clumps were transferred into new feeder cells containing mitomycin-C-treated MEFs (Roche, Germany).

Immunocytochemistry (ICC) and alkaline phosphatase (AP) staining

ICC and AP staining were performed to evaluate expression of genes related to pluripotency and AP

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activity. Before staining, all cell samples were pre-incubated for 10 min at 4ºC and fixed with 4% paraformaldehyde for 30 min. After washing twice with DPBS, samples were treated for 1 h with 10% goat serum in DPBS to block nonspecific binding. Serum-treated cells were incubated overnight at 4ºC with the primary antibodies. The primary antibodies included OCT4 (1:200; Santa Cruz Biotechnology, USA), SOX2 (1:200; Millipore, USA) and NANOG (1:200; Peprotech, USA). When we used the antibodies for intracellular proteins, such as Oct4, Sox2 and NANOG, fixed cells were treated for 5 min with 0.2% Triton-X100 (Sigma-Aldrich) before serum blocking. After incubation with the primary antibody, the cells were treated for 3 h at room temperature with Alexa Fluor-conjugated secondary antibodies. Nuclei were stained with Hoechst 33342 (Molecular Probes, USA). Images of stained cells were captured using a LSM 700 Laser Scanning Microscope (Carl Zeiss, Germany) and processed with the ZEN 2013 Light Edition program (Carl Zeiss). For AP staining, fixed ntES-like cells were incubated for 30 min at room temperature in the dark with 2% nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) stock solution (Roche) diluted in buffer solution (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Cells were then examined under an inverted microscope.

Embryoid body (EB) formation and *in vitro* differentiation

To evaluate *in vitro* differentiation, EB were generated from ntES-like cells detached from feeder cells, and colonies were mechanically dissociated into small clumps. Suspension cultures of these clumps were obtained using the hanging-drop method for 5–6 days with PESM in the absence of cytokines. After hanging-drop culture, small clumps were aggregated, forming EBs (Fig. 5*B*, *B*′; see later). Cultured EBs were seeded on 0.1% gelatin-coated plates and cultured for 2–3 weeks with DMEM containing 15% FBS. Then, differentiated cells were fixed in 4% paraformaldehyde and analyzed using immunostaining with differentiation specific antibodies including neurofilament (ectoderm; 1:200; Millipore), vimentin (mesoderm; 1:200; Millipore), and cytokeratin 17 (endoderm; 1:200; Millipore). Staining protocols were followed as described in the above section.

Statistical analysis

The data obtained in this study were analyzed using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA). Data on developmental rates were arcsine transformed and then examined using analysis of variance (ANOVA) and a Newman–Keuls multiple comparison test. Relative transcription levels in embryos were analyzed using unpaired Student's *t*-tests. All data are expressed as mean values ± standard error of the mean (SEM). A probability of $P < 0.05$ was considered statistically significant.

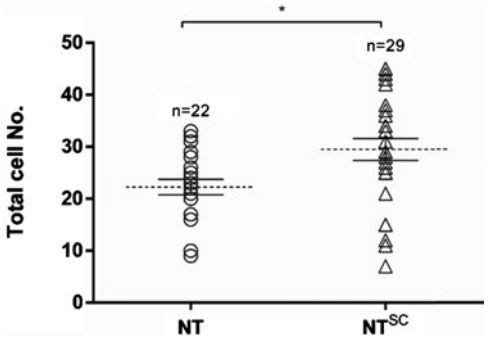

Figure 1. Effect of Scriptaid on cloned blastocysts. Aligned dot plots represent total cell number of individual blastocysts. Asterisks indicate a significant difference between NT and NT^{SC} ($P < 0.05$). NT, cloned embryos produced by SCNT; NT^{SC}, cloned embryos treated with 500 nM Scriptaid for 14 h post-activation.

Results

Scriptaid treatment could rescue developmental competence of cloned embryos

In the present study, we examined the effect of Scriptaid on cloned embryos, specifically whether it can improve the quality and developmental competence of cloned embryos before producing aggregated cloned embryos, as previously reported. Application of Scriptaid to cloned embryos also has positive effects on cell numbers of cloned blastocysts (Fig. 1) and developmental competence of cloned embryos (Table 1). The numbers of cells in blastocysts treated with Scriptaid (NT^{SC}; 29.5) were significantly higher than those of non-treated cloned blastocysts (NT; 22.3, $P < 0.05$) (Fig. 1). Blastocyst formation rate of cloned embryos treated with Scriptaid (31.0%) was significantly higher than that of non-treated cloned embryos (16.1%). Therefore, we treated all groups in the following experiment with Scriptaid.

EZ method efficiently aggregates cloned embryos

To confirm the effect of EZ methods on efficiency of aggregation and developmental potential of aggregates, we compared the aggregates produced by the EZ and microwell methods (Table 2). Aggregates induced by EZ methods formed compact and spherical embryos, while microwell methods either produced incomplete aggregates having separate blastocoel

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Table 1 The effect of Scriptaid on *in vitro* development of cloned embryos

Groups	No. examined	No. (%) cleaved[§]	No. (%) blastocyst[‡]
PA	1335	1095 (81.3 ± 1.9)	661 (49.0 ± 4.8)^{a}
NT	324	228 (72.3 ± 8.1)	52 (16.1 ± 1.1)^{b}
NT^{SC†}	399	273 (71.7 ± 3.1)	134 (31.0 ± 2.8)^{c}

[§]Cleavage rate was counted after two days in culture.
[‡]Blastocyst rate was counted after seven days in culture.
PA, parthenogenetic embryos; NT, cloned embryos produced by SCNT; NT^{SC†}, treated with 500 nM Scriptaid for 14 h after post-activated cloned embryos.
Each group has eight replicates. Values are expressed as mean ± SEM. Within the same column, values with different letters (a, b, and c) are significantly different ($P < 0.05$).

Table 2 Development of triple-aggregated embryos produced by EZ and conventional microwell methods

Groups	No. examined	No. (%) aggregated[§]	No. (%) blastocyst
Control[†]	37	10[‡] (27.9 ± 4.1)	9 (25.5 ± 4.7)
Microwell	32	23 (55.5 ± 4.2)*	19 (50.9 ± 5.2)*
EZ	37	30 (78.1 ± 6.5)**	29 (76.4 ± 6.5)**

The rates of embryo aggregation and blastocyst formation were calculated on Day 5 at the morula stage and on Day 7 at the blastocyst stage.
Microwell, conventional method for aggregation; EZ, empty zona method for aggregation.
§Aggregates that had only one or two blastomeres were also regarded as successful aggregations.
†Denuded four-cell cloned embryos were individually cultured in microwells.
‡Proportions of embryos that became morulae in the control group. Each group has six replicates. Values are expressed as mean ± SEM. Within the same column, values with asterisks are significantly different with control (∗ $P < 0.05$, ∗∗ $P < 0.0001$).

cavities (partially aggregated) or failed to aggregate after 48 h (Fig. 2*D–F*). The rate of aggregation and blastocyst formation was significantly higher using the EZ method than using the microwell method (Table 2). These results indicate that the EZ method aggregates cloned embryos efficiently and improves the developmental competence of aggregated cloned embryos. Therefore, we selected EZ methods for aggregating cloned embryos in the following experiment.

Aggregation elevates cell number of cloned blastocysts

We compared the cell number of blastocysts produced by singleton and aggregated cloned embryos in order to evaluate whether the number of cells in cloned blastocysts could be increased by aggregation. The number of total cells in blastocysts was significantly higher in aggregated embryos (83.2) than in singletons (PA; 40.6, SCNT; 27.1) (Fig. 3). This result means that embryo aggregation could improve the developmental ability of cloned embryos.

Derivation of ntES-like cells and characterization

Non-aggregated and aggregated embryos were seeded independently onto each feeder layer to derive porcine ntES-like cells. As shown in Table 3, blastocysts from aggregated cloned embryos were attached to the feeder layer at a significantly higher rate than blastocysts from cloned singletons (70.0% vs 47.8%, respectively). After 2–4 days of additional culture of both types of blastocysts, proliferating cell colonies were observed, and they showed positive AP activity. The colonies also showed densely packed morphology with obvious boundaries, typical morphogenic characteristics of porcine ESCs (Fig. 4). Formation rate of the primary colony was significantly higher in aggregates than in singletons (Table 3; 20.2% vs 2.2%, respectively). These results demonstrate that cloned blastocysts generated by aggregating multiple embryos effectively increase the attachment rate to the feeder layer and increase primary colony formation compared with non-aggregated blastocysts under the same culture conditions.

Expression of pluripotent genes, *OCT4, SOX2* and *NANOG*, was examined to characterize the derivate. One primary colony from aggregated cloned blastocysts was selected for further maintenance and analyses. Expression of genes was observed (Fig. 5*A*). Differentiation capacity of the cells was also confirmed (Fig. 5*B*,*C*). Inducing differentiation of the cells formed EB and three germ layers.

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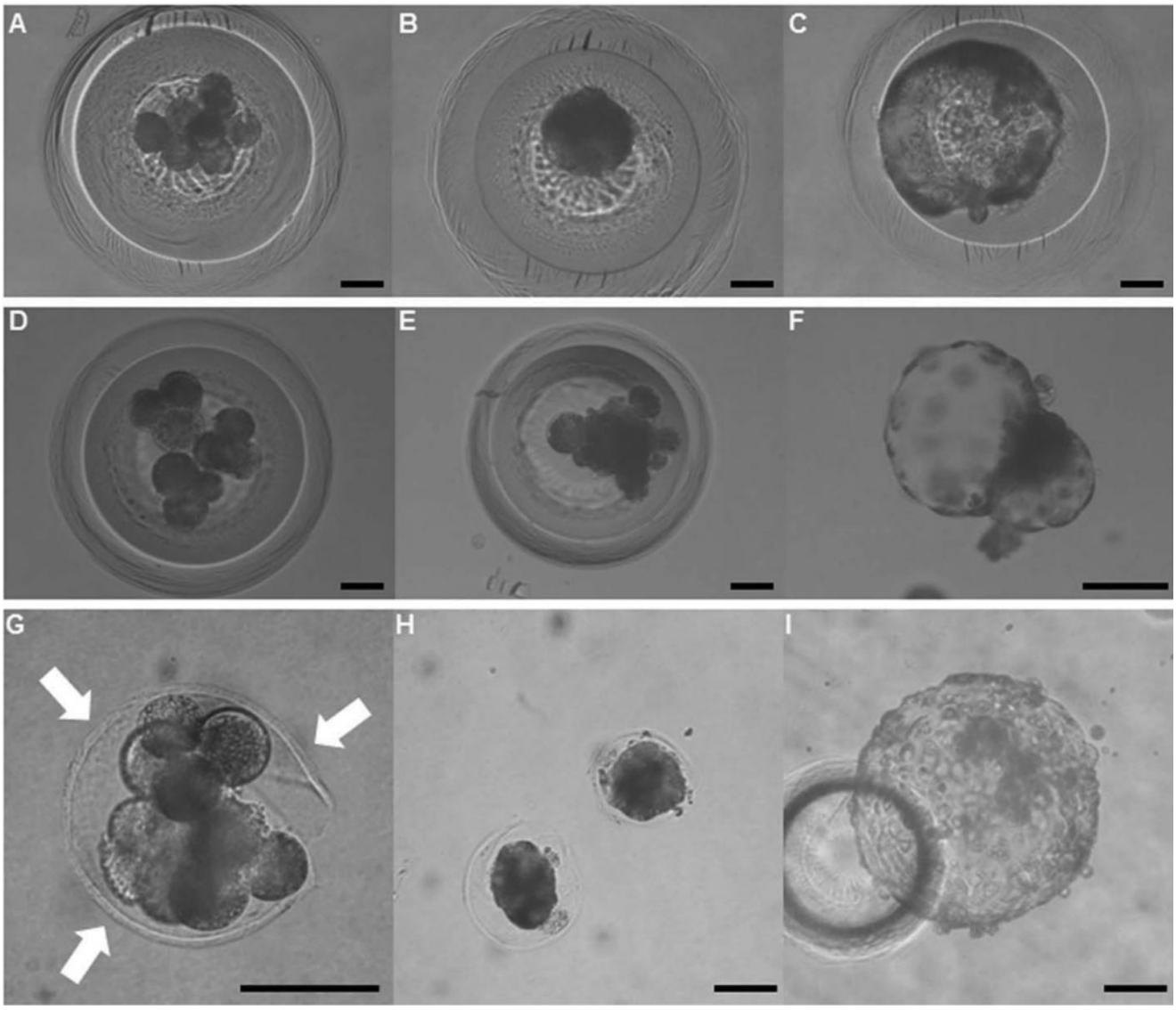

Figure 2. Morphology of aggregated morulae and blastocysts. Chimeric embryos and blastocysts derived from 3X embryos aggregated using the microwell method (*A*–*C*), partially aggregated microwell method (*D*–*F*), and EZ method (*G*–*I*) were photographed using an optical microscope. White arrows indicate boundary of zona. The embryos began to compact on culture at Day 2 after reconstruction. Most of the EZ method group developed normally to the blastocyst stage. After 0 h (*A*, *D*, *G*), 48 h (*B*, *E*, *H*), and 120 h (*C*, *F*, *I*) of aggregation. Bar = 200 μm.

Discussion

ESCs from cloned embryos have been suggested as possible patient-specific therapeutic models; however, despite successful derivation of ESCs from cloned blastocysts in humans (Tachibana *et al.*, 2013), extremely low efficiency has limited their therapeutic application. Low cell numbers in the ICM of cloned blastocysts compared to *in vitro* fertilized blastocysts were confirmed (Terashita *et al.*, 2011), and these low cell numbers likely have an adverse effect on derivation of ESCs (Shan *et al.*, 2012; Sugimura *et al.*, 2009). Therefore, we determined that increasing cell number in cloned blastocysts by aggregating multiple embryos could be a good approach for deriving cloned ESCs.

Recent studies have focused on the ability of HDAC inhibitors to improve cloned embryo quality, especially trichostatin A (TSA). Although TSA treatment can significantly increase the developmental competence of SCNT embryos, negative effects of TSA treatment, such as decreased subsequent developmental potential of cloned embryos, have been reported (Wu *et al.*, 2008; Zhao *et al.*, 2009). Recently, one study confirmed that Scriptaid could more efficiently enhance *in vitro* developmental competence in ovine

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Table 3 Derivation efficiencies of primary colonies from cloned porcine blastocysts

Groups	No. examined	No. (%) adhered blastocysts‡	No. (%) primary colonies
NT^{SC}†	91	43 (47.8 ± 1.4)	2 (2.2 ± 1.35)
NT^{agg}§	17	12 (70.0 ± 2.0)*	3 (17.6 ± 12.3)*

‡Determined by firm attachment to the feeder cells for at least 3 days.
NT^{SC}†, 500 nM Scriptaid were treated for 14 h after post-activated cloned embryos.
NT^{agg}§ , aggregated cloned embryos by the EZ method.
Each group has three replicates. Values are expressed as mean ± SEM. Within the same column, values with different asterisks are significantly different (* $P < 0.05$).

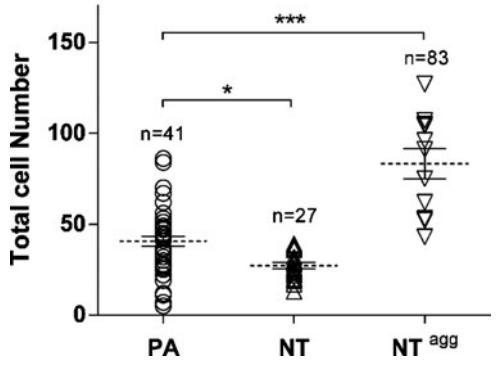

Figure 3. Total cell numbers of *in vitro* cultured blastocysts. Aligned dot plots represent total cell numbers of individual blastocysts. Asterisks indicate significant differences between PA and NT as well as PA and NT^{agg} ($P < 0.05$). PA, parthenogenesis; NT, SCNT embryos which were treated with 500 nM Scriptaid for 14 h post-activation; NT^{agg}, aggregated clone produced by SCNT.

SCNT embryos compared with TSA (Wen *et al.*, 2014). Similar to the previous study, treatment with Scriptaid in the current study improved the formation capacity and cell numbers of cloned blastocysts in pigs (Fig. 1 and Table 1). Even though cell numbers were increased after treatment with Scriptaid, the efficiency of ESC derivation did not differ when compared to non-treated cloned blastocysts (data not shown). This suggests that the elevation of cell number in cloned blastocysts treated with Scriptaid may not be high enough for deriving ESCs efficiently.

Recently, we have developed new methods for embryo aggregation using zona pellucida (ZP) obtained from hatched porcine blastocysts (Park *et al.*, 2012). This method improved the aggregation rate of embryos and reduced blastocysts developed from partial blastomeres, which were frequently observed in conventional aggregation methods using microwells. The benefits of the EZ method on embryo aggregation were also observed in the present study (Table 2 and Fig. 2), and blastocysts were produced by aggregating cloned embryos using the EZ method for successfully deriving ntES-like cells (Table 3). Therefore, this aggregation method can be used for establishing ntESCs derived from cloned blastocysts.

Several studies have reported that aggregation could dramatically elevate cell number and size of blastocysts produced *in vitro* (Lee *et al.*, 2007; Gambini *et al.*, 2012, 2014). Boiani et al. observed that blastocysts from aggregated embryos showed enhanced cell-to-cell communication and expression of the pluripotency gene OCT4 (Boiani *et al.*, 2003). Terashita *et al.* (2011) confirmed that developmental competence of cloned embryos and total cell number of blastocysts were elevated by aggregation in miniature pigs. These reports demonstrated that aggregation could improve the quality of cloned blastocysts. Blastocysts with improved quality due to embryo aggregation may also positively affect full-term development of the fetus (Gambini *et al.*, 2014; Siriboon *et al.*, 2014) and derivation of ESCs (Shan *et al.*, 2012). Outgrowth of canine interspecies-cloned blastocysts was efficiently formed by aggregation (Sugimura *et al.*, 2009), and mouse blastocysts produced by aggregation of parthenotes showed increased efficiency for ESC derivation (Sugimura *et al.*, 2009). Furthermore, a recent study showed that putative ES cells could be derived from cloned embryos by aggregation in pig (Siriboon *et al.*, 2015). These studies support the possibility that derivation efficiency of ESCs could be increased by aggregated embryos. Similar to the previous studies, our results also demonstrated that quality of cloned blastocysts could be rescued by aggregating multiple embryos (Table 2 and Fig. 3) and that the blastocysts could be derived into ESCs more effectively than those cloned from singletons (Table 3). Therefore, our results suggest that aggregation can be used for the efficient derivation of ntESCs.

In conclusion, we have demonstrated the effect of combining HDACi treatment with embryo aggregation on derivation efficiency of ES cell in porcine embryos. ES cells derived from aggregated cloned blastocysts possess typical characteristics including proliferation, AP activity, pluripotency gene expression, and *in vitro* differentiation capacity. Embryo aggregation significantly improves quality of cloned

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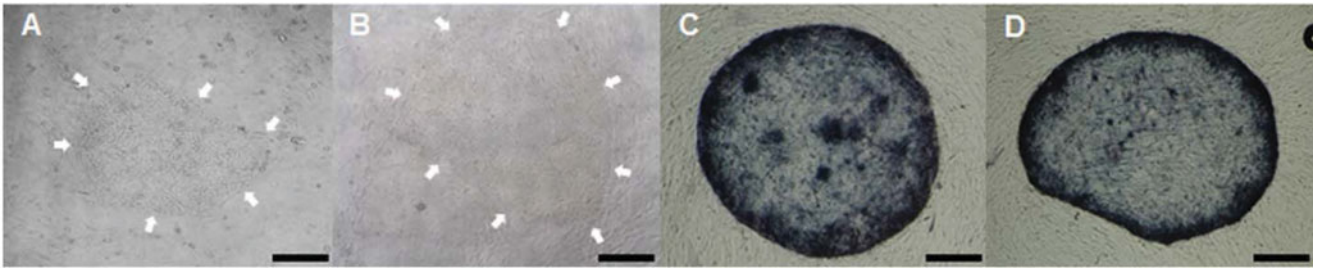

Figure 4. Primary colonies derived from SCNT blastocysts and AP activity. Primary colonies from single (*A*) and aggregated (*B*) blastocysts and positive AP activity of single (*C*) and aggregated (*D*) blastocysts investigated 5–7 days after attachment of blastocysts on the feeder layer. Bar = 200 μm. White arrows indicate boundary of primary colonies.

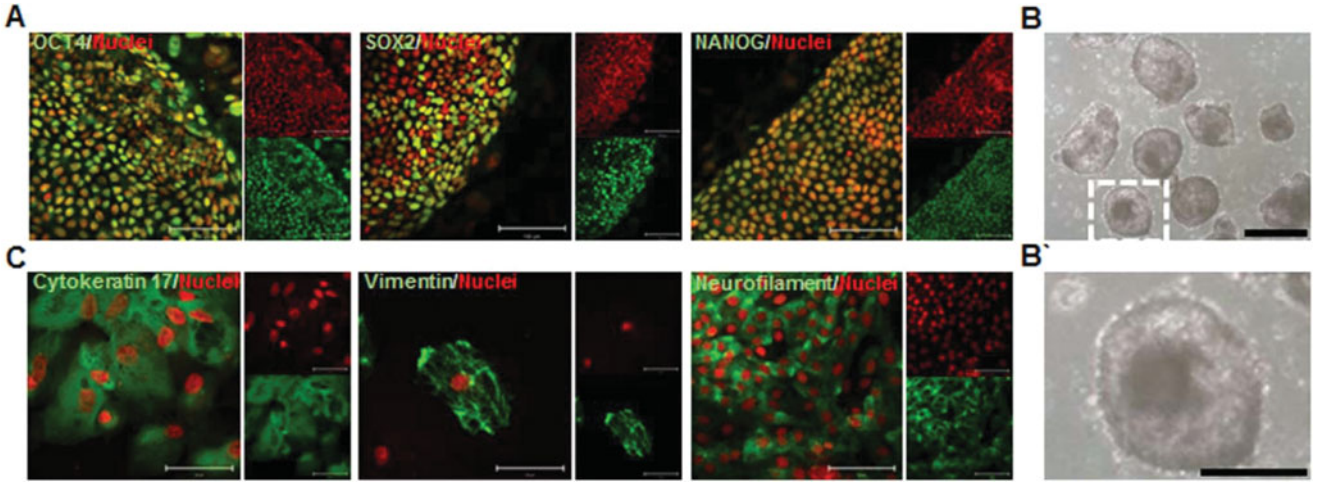

Figure 5. Expression of the undifferentiation and differentiation markers in the ntES-like cells. Expression of pluripotent markers OCT4, SOX2 and NANOG was observed (*A*). Representative embryoid bodies (EB) derived from the ntES-like cells line through the culture for 5 days by the hanging-drop method (*B*, *B′*). When EBs were cultured continuously onto culture plates, a variety of differentiated cells were observed (*C*). We could detect the expression of differentiation marker genes cytokeratin 17 (endoderm) , vimentin (mesoderm) and neurofilament (ectoderm) from differentiated cells by immunocytochemistry analysis. Scale bars represent *A*, *B′* and *C*: 100 μm; *B*: 400 μm.

blastocysts which may help overcome potential problems of ntES cell derivation.

Acknowledgements

The authors are thankful for helpful comments on the manuscript. This work was supported by a grant of Next-Generation BioGreen 21 Program (No. PJ01130012015) Rural Development Administration, Republic of Korea.

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