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Production of viable cloned miniature pigs by aggregation of handmade cloned embryos at the 4-cell stage

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Abstract. The aim of the present study was to improve the quality of handmade cloned porcine embryos by multiple embryo aggregations. Embryos derived from aggregation of three cloned embryos (3×) had a better blastocyst rate than cloned control (1×) embryos (73.6% vs 35.1%, respectively; $P < 0.05$), but did not differ from those produced by aggregation of two cloned embryos (2×; 63.0%). Total cell numbers differed among treatments ($P < 0.05$), with the greatest cell numbers (126) in the 3× group and the lowest (55) in the control group. The ratio of inner cell mass : total cell number was comparable in the 2× and 3× groups (25.1% vs 26.1%, respectively) and was significantly better than that in the control group (15.3%). The proportion of apoptotic cells in 2× and 3× groups was lower than that in the control group (2.7% and 2.2% vs 4.7%, respectively; $P < 0.05$). Expression of *Oct4* and *Cdx2* was higher, whereas that of *Bax* was lower ($P < 0.05$), in the 3× compared with non-aggregate group. Seven piglets were born to two surrogate mothers after embryo transfer of 3× aggregated blastocysts. In conclusion, aggregated embryos had greater total cell numbers and better pluripotency gene expression, with reduced expression of the pro-apoptosis gene *Bax*. Collectively, these improvement may be associated with the development of cloned embryos to term.

Additional keywords: embryonic stem cells; oocyte bisection cloning technique.

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Introduction

Cloning has the potential to enhance the development of animal biotechnology globally, as well as to provide powerful tools for basic and applied research in many different scientific disciplines, including agriculture, wildlife conservation and human medicine. Organ transplantation is now widely viewed as a practical treatment for end-stage organ failure, but the major limitation lies in the availability of transplantable organs. Therefore, the use of animal organs (i.e. xenotransplantation) has been increasingly considered as a potential alternative (Samstein and Platt 2001). That pigs have many physiological functions that are similar to humans has made them a very useful species for biomedical research. Miniature pigs have been used as a model system for human diseases such as the genetically defined model for surgery and xenotransplantation (Prather *et al.* 2003; Vodicka *et al.* 2005). In addition to non-human primates, miniature pigs may be the best model system for xenotransplantation studies (Logan 2000; Perico *et al.* 2002).

The limited success in generating cloned pigs can be attributed mainly to the low cloning efficiency, which includes poor development of cloned embryos and low numbers of cells per embryo. Therefore, the developmental potential of *in vitro*-derived blastocysts is inferior to their *in vivo* counterparts (Thompson 1997; Wang *et al.* 1999). Specifically, a cloned porcine blastocyst generally has lower total cell numbers and a

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lower inner cell mass (ICM) : trophectoderm cell (TE) ratio than *in vivo*-derived blastocysts or an IVF embryo (Koo *et al.* 2004).

In cattle, embryo aggregation does not increase overall blastocyst rate (Zhou *et al.* 2008); however, there were increases in the ICM, total cell numbers (TC) and ICM : TC ratio in aggregated embryos (Misica-Turner *et al.* 2007; Zhou *et al.* 2008). Aggregation of 8- or 16–32-cell cloned bovine embryos improves pregnancy rates, but does not eliminate fetal loss and stillbirth (Akagi *et al.* 2011). In addition, in an attempt to establish embryonic stem (ES) cells, the outgrowth formation of interspecies blastocysts derived by aggregation of 4-cell cloned canine embryos was reported to be better than that of non-aggregated embryos (Sugimura *et al.* 2009).

Embryo quality can be assessed on the basis of TC and the expression of pluripotency-related genes, such as *Oct4*, *Nanog* and *Sox2*, which are crucial for retaining the pluripotency of embryonic cells during early embryogenesis (Boiani *et al.* 2002; Bortvin *et al.* 2003). In addition, apoptotic indices and the expression of apoptosis-related genes, namely *Bcl* (anti-apoptotic) and *Bax* (pro-apoptotic), during preimplantation development are related to cell survival in the pig (Boise *et al.* 1993; Jurisicova *et al.* 1998). Programmed cell death may also play an important role in protecting embryos from embryotoxic conditions by scavenging dead or damaged cells (Xiang *et al.* 1996; Woo *et al.* 1998).

To improve the efficiency of porcine cloning, it is necessary to produce high-quality cloned blastocysts. Some studies suggest that embryo aggregation is a promising way to improve embryo development *in vitro* (Tang and West 2000; Lee *et al.* 2007; Misica-Turner *et al.* 2007). In the mouse, aggregated cloned embryos have increased TC and *Oct4* expression compared with single, non-aggregated cloned embryos, although development is not altered (Boiani *et al.* 2003; Terashita *et al.* 2011). In addition, Lee *et al.* (2007) reported that aggregation of IVF porcine embryos increases blastocyst rates, TC and *Oct4* expression compared with embryos produced *in vivo*. However, there don't appear to be any reports regarding cloned pigs generated by embryo aggregation.

The aims of the present study were to improve both blastocyst rate and the quality of porcine cloned embryos by aggregation of genetically identical cloned embryos at the 4-cell stage and to determine their developmental competence after embryo transfer.

Materials and methods

Oocyte maturation

The IVM of porcine oocytes was performed according to standard protocols (Li *et al.* 2008; Chawalit *et al.* 2012). Briefly, ovaries were collected from peripubertal cross-breed of Landrace, Yorkshire and Duroc (LYD) gilts at a local abattoir and transported to the laboratory in physiological saline (30–35°C) within 2 h. Cumulus–oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) and subjected to IVM for 41–42 h in a 100-μL droplet of maturation medium (TCM-199) supplemented with 10% porcine follicular fluid, 10% fetal bovine serum (FBS), gonadotropins (10 IU mL^{-1} human chorionic gonadotrophin and 10 IU mL^{-1} pregnant mare's serum gonadotropin) at 39°C under 5% CO_2. After IVM, cumulus cells were removed by repeated pipetting in 500 μL Dulbecco's phosphate-buffered saline (DPBS) containing hyaluronidase (1 mg mL^{-1}).

Preparation of donor cells for nuclear transfer

Donor cells were prepared as described by Chawalit *et al.* (2012). Briefly, fibroblasts derived from an ear biopsy of postnatal piglets were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 39°C in a 5% CO_2 incubator. At Passage 3, cells were cryopreserved in DMEM supplemented with 30% FBS and 10% dimethylsulfoxide (DMSO), which was prepared in advance and stored in liquid nitrogen.

Donor cells were thawed and grown for 1 week in DMEM supplemented with 10% FBS. Before nuclear transfer, they were rinsed once with DPBS, trypsinised for 5 min and then resuspended in HEPES-buffered TCM-199 (with 10% FBS; T10). The fibroblast suspension was left at room temperature for 1 h until fusion with recipient cytoplasts.

Production of parthenotes and cloned embryos

For production of parthenogenetic embryos, matured oocytes were activated by a DC pulse (2.2 kV cm^{-1}, 30 μs) in an activation chamber and then incubated in 6-dimethylaminopurine (6-DMAP) for 4 h under the same culture conditions as described by Nguyen *et al.* (2009).

For handmade cloning (HMC) of embryos, matured oocytes with the first polar body were incubated for 20 s in pronase solution (3.3 mg mL^{-1} in HEPES-buffered TCM-199 supplemented with 33% FBS) and washed twice with T10. After washing, oocytes were lined up in 40 μL T10 containing 2.5 μg mL^{-1} cytochalasin B (10 oocytes in each droplet). Oocytes were rotated with a fire-polished glass pipette to identify the membrane protrusion or first polar body for bisection with a microblade (ESE020; Bioniche Animal Health, Pullman, WA, USA) under a stereomicroscope. Following bisection, demi-ooplasts were washed twice in T10.

For cell fusion, a two-step protocol with two consecutive fusions was performed. First, the enucleated cytoplast was transferred to the HEPES–TCM-199 droplet containing 1 mg mL^{-1} phytohemagglutinin A (PHA) for 5 s, before being moved to the T10 droplet holding the fibroblasts, where each cytoplast was allowed to pair with one fibroblast cell. Cytoplast–fibroblast pairs were incubated in the fusion medium containing 0.3M mannitol and 0.01% polyvinyl alcohol (PVA) for 20 s and then transferred to the fusion chamber (with two electrodes, 1 mm apart). Under a 0.6 kV cm^{-1} AC, the pairs were aligned to the wire with the fibroblasts furthest from the wire. Fusion was performed with one DC pulse (2.0 kV cm^{-1} for 9 μs). Thereafter, pairs were transferred from the fusion chamber to the T10 drop and incubated for 1 h before second fusion.

For the second fusion, the remaining cytoplasts and the fused cytoplast-fibroblast pairs were transferred to a droplet containing activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.01% PVA) for equilibration. They were then aligned (0.6 kV cm^{-1} AC) with the fused pairs furthest from the wire, followed by a DC pulse (0.85 kV cm^{-1}, 80 μs) for

fusion and initial activation. Thereafter, cytoplast-fibroblast triplets were incubated in T10 to allow complete fusion before chemical activation with 6-DMAP.

After parthenogenetic activation, HMC reconstructed embryos were washed three times with 200 μL porcine zygote medium-3 (PZM-3) and randomly cultured individually in the well-of-the-well (WOW) system. After 48 h culture, 4-cell embryos were aggregated with various numbers of embryos (1× = single HMC embryo without aggregation; 2× = two embryo aggregation; 3× = three embryo aggregation). For embryo culture, PZM-3 medium supplemented with 3 mg mL^{-1} bovine serum albumin (BSA) was used, and the aggregated embryos was cultured for 5 days in the WOW system, covered with mineral oil at 39°C in an incubator containing 5% CO_2.

Production of IVF embryos

In the present study, IVF was performed as described previously (Nguyen *et al.* 2011). Briefly, 0.25 mL fresh boar semen was washed with 5 mL DPBS supplemented with 10% FBS by centrifuging twice at 300*g* for 2 min at room temperature. After washing, the sperm pellet was resuspended in modified Tris-buffered medium (mTBM) and incubated at 39°C under 5% CO_2 in air until insemination. After 44 h of IVM, oocytes were stripped of cumulus cells by pipetting with 0.1% hyaluronidase and washed three times in mTBM that was pre-equilibrated for at least 12 h at 39°C in 5% CO_2. Oocytes with an even ooplasm and visible first polar bodies were selected for experiments. After washing, 20–30 oocytes were randomly placed in 45-μL droplets of mTBM medium covered with prewarmed mineral oil. After appropriate dilution, 5 μL sperm suspension was added to each 45-μL fertilisation droplet to give a final concentration of 5×10^5 spermatozoa mL^{-1}. Gametes were cocultured for 6 h at 39°C in a humidified atmosphere of 5% CO_2 in air. After 6 h, gametes were removed from the fertilisation droplet and washed three times in PZM-3 to remove excessive spermatozoa. Presumptive zygotes were then cultured for 7 days in 100 μL PZM-3 (20–30 oocytes per droplet) covered with mineral oil.

Embryo culture and aggregation

Embryos were cultured in PZM-3 (supplemented with 3 mg mL^{-1} BSA). Parthenogenetic and IVF embryos were washed three times with 200 μL PZM-3 and then cultured in groups (20 embryos in 100 μL PZM-3) for 7 days.

The HMC embryos were cultured individually for 2 days (up to the 4-cell stage) in the WOW system covered with mineral oil at 39°C in a 5% CO_2 incubator (Chawalit *et al.* 2012). Aggregated embryos were produced by the aggregation of two (2×) or three (3×) 4-celled embryos ('1×' being a single cloned embryo without aggregation) and were cultured continuously for a further 5 days (Fig. 1*a–d*).

Total and differential cell counts of blastocysts

Reconstructed or parthenogenetic embryos were cultured for 7 days, stained and then examined under a stereomicroscope to determine blastocyst rates and cell numbers (ICM and TE cells) per blastocyst. To determine TC, embryos were stained with Hoechst 33342 (10 μg mL^{-1}) for 10–15 min and observed under an epifluorescence microscope (Nikon, Tokyo, Japan), as described previously (Kragh *et al.* 2005). Differential staining of the ICM and TE cells of blastocysts was performed as described by Machaty *et al.* 1998) and Ju *et al.* (2005), with minor modifications. Briefly, Day 7 HMC blastocysts from the 1×, 2× and 3× groups were rinsed in Tyrode's lactate HEPES (TL-HEPES) containing 0.1% PVA before being exposed to a 1 : 5 dilution of rabbit anti-pig whole serum for 1 h. These embryos were then washed three times (5 min each time) with 0.1% poly-vinylpolypyrrolidone (PVP)–TL-HEPES and placed in guinea-pig complement (1 : 10 dilution) containing propidium iodide (10 μg mL^{-1}) and Hoechst 33342 for 1 h. After a brief rinse in 0.1% PVP–TL-HEPES, stained embryos were mounted on slides under a coverslip and examined under an epifluorescence microscope (Nikon) equipped with an ultraviolet light and wide green (WG) filter set. Cells that were blue and red were identified as the ICM and TE cells, respectively.

Detection of DNA damage by terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling

The terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) assay was used to assess the presence of apoptotic cells (*in situ* cell death detection kit; TMR red; Roche, Mannheim, Germany) according to the manufacturer's instructions (Tseng *et al.* 2006; Nguyen *et al.* 2009). Cloned blastocysts on Day 7 were washed three times with 0.1% PVP in DPBS, fixed in 4% (v/v) paraformaldehyde–DPBS solution for 24 h and then permeabilised with 0.1% Triton X-100 in 0.1% citrate solution before being mounted with DAKO fluorescent mounting medium (S3023; DAKO North America, Carpinteria, CA, USA) on glass slides. An epifluorescence microscope (Nikon) was used to enumerate total and apoptotic nuclei. The apoptotic index was calculated as follows:

$$\text{Apoptotic index} = (\text{no. TUNEL-positive nuclei}/\text{total no. nuclei}) \times 100$$

Expression of pluripotency- and apoptosis-related genes

RNA purification

Quantification of all gene transcripts, including *Oct4*, *Rex01*, *Ccx2*, *Bcl-xL* and *Bax*, was performed as described by Nguyen *et al.* (2011). First, Day 7 cloned embryos of various groups were collected in lysis buffer and stored at −80°C until total RNA was extracted using TRIzol Reagent (Invitrogen, Grand Island, NY, USA) and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Purified RNA was then quantified using an ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and analysed qualitatively (Bioanalyzer 2100; Agilent Technology, Santa Clara, CA, USA). To prepare a cDNA pool from each RNA sample, total RNA (19 ng) was reversely transcribed using the moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA), and the resulting samples were diluted (1 : 2000) with DNase-free water. Each cDNA pool was stored at −20°C pending real-time polymerase chain reaction (PCR) analysis.

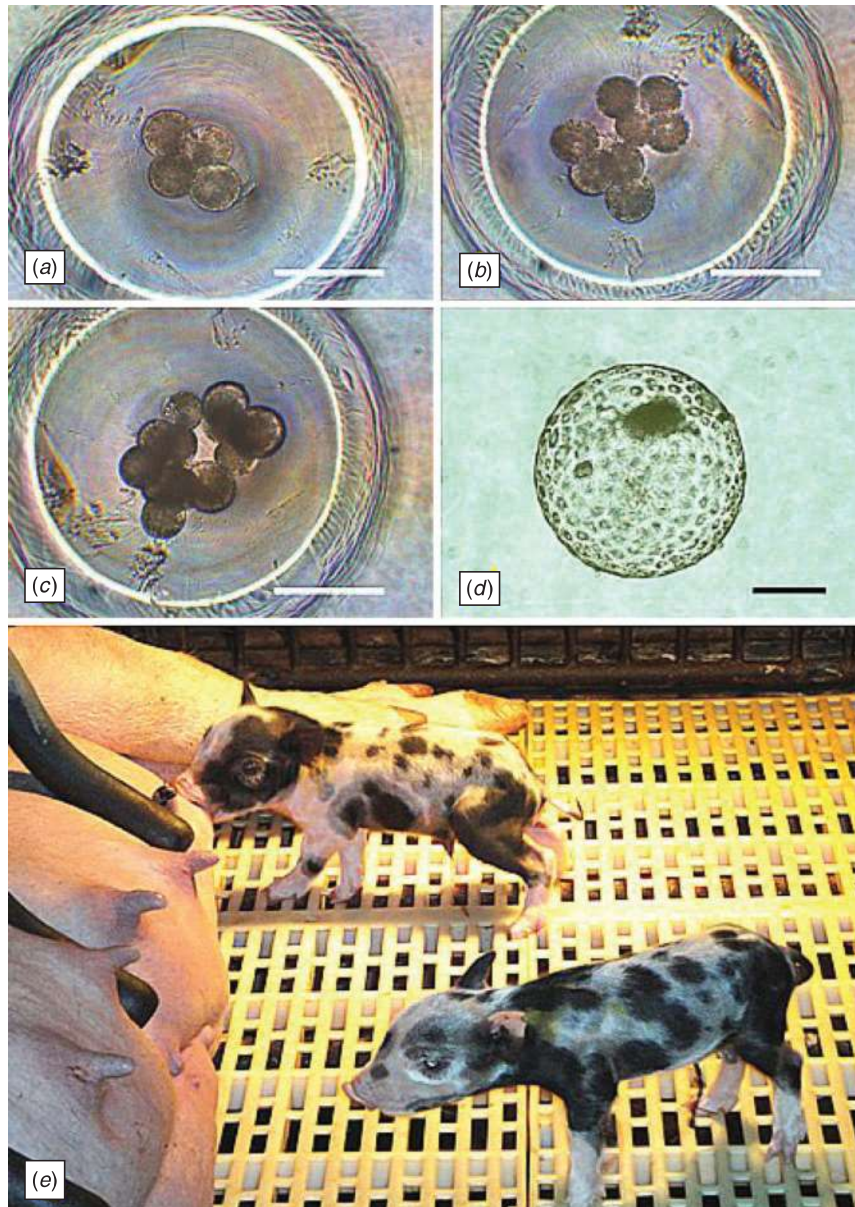

Fig. 1. (*a*) Single handmade cloned (HMC) embryo and aggregates of (*b*) two and (*c*) three porcine HMC embryos (1×, 2× and 3× embryos, respectively). (*d*) Blastocysts derived from 3× embryos. (*e*) Healthy miniature piglets (shown at 2 days of age) were produced by embryo transfer of 3× embryos. Scale bars = 100 μm.

Quantitative real-time PCR analysis

Real-time PCR was performed using a Roche LightCycler Instrument 1.5 and a LightCycler FastStart DNA MasterPLUS SYBR Green I kit (catalogue no. 03 515 885 001; Roche). Briefly, the 10-μL reaction mixture contained 2 μL master mix, 2 μL of 0.75 μM forward primer, 2 μL of 0.50 μM reverse primer (each primer sample contained 5% DMSO) and 6 μL cDNA sample. Each sample was run in triplicate. The PCR parameters were 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 10 s. At the end of each running program, a melting curve analysis was performed, data were analysed automatically and an amplification plot was generated for each cDNA sample. From each plot, the LightCycler3 Data Analysis Software (Roche Diagnostics) automatically calculated the crossing point (CP) value, the turning point corresponding to the first maximum of the second derivative curve, regarded as the beginning of an exponential amplification. The comparative threshold cycle (CT) method was used to quantify gene expression, as described previously (Wee *et al.* 2006). Fold changes in gene expression were calculated using the formula $2^{-(\Delta\Delta CT_{Exp} - \Delta\Delta CT_{Control})}$, where:

$\Delta\Delta CT_{Exp} = CT_{Target} - CT_{ref}$ of the treatment group sample
$\Delta\Delta CT_{Control} = CT_{Target} - CT_{ref}$ of the control sample

and CT_{Target} and CT_{ref} are the comparative threshold cycle of target genes and reference genes, respectively.

Primer sequences, GenBank accession numbers and expected amplicon sizes are given in (Table 1).

Embryo transfer

Aggregated compact morulae and blastocysts on Days 5–6 of 3× aggregated blastocysts were transferred surgically to the uterine horn of recipients (Landrace × Yorkshire) 4–5 days after administration of hormones. Pregnancy was diagnosed by trans-abdominal ultrasonography on Day 21 after embryo transfer and confirmed every other week thereafter. Parturition was induced with 175 µg prostaglandin $F_{2\alpha}$ (PLANATE; Intervet Canada, Kirkland, Canada) on Day 113. All experiments involving live animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Technology Institute Taiwan, Chunan, Taiwan.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in SAS Version 9 (SAS Institute, Cary, NC, USA), followed by Tukey's test. Percentile data were arcsine transformed before ANOVA. Differences between treatment groups were considered significant at $P < 0.05$.

Results

In vitro *development of parthenotes and HMC embryos*

Development of parthenogenetic and cloned embryos is summarised in Table 2. The blastocyst rate of cloned embryos was lower than that of parthenogenetic embryos (36.5 vs 48.8%, respectively; $P < 0.05$). However, there were no significant differences between these two groups in terms of cleavage rates (89.9 vs 88.8, respectively) or total cell (TC) numbers (52 vs 55, respectively).

In vitro *development of aggregated HMC embryos*

Blastocyst rates were higher in the 2× and 3× aggregated groups compared with the non-aggregated group (63% and 73.6% vs 35.1%, respectively; $P < 0.05$; Table 3). The TC per blastocyst differed significantly among treatment groups, being greatest in the 3× aggregated group compared with both the 2× and non-aggregated groups (126 vs 86 and 55, respectively; $P < 0.05$).

Based on TC, embryos were arbitrarily split into three groups (i.e. those with <50, 50–100 and >100 TC). In blastocysts with TC >100, most (69.0%) were from the 3× aggregated embryo group, more than twice as much as the sum of the non-aggregated and 2× aggregated groups (2.5% and 30.6%, respectively; Fig. 2). In contrast, only 7.0% and 2.1% of the blastocysts in the 2× and 3× aggregated embryos, respectively, had TC <50, much lower than that in the non-aggregated group (54.8%; $P < 0.05$).

Table 1. Primer sequences, GenBank accession numbers and expected amplicon sizes for quantitative real-time polymerase chain reaction analyses of cloned porcine embryos

Gene	Primer sequence (5′–3′)	GenBank Accession no.	Product size (bp)
Oct4	Forward: CGCAACGAGAGGATTTTGAG	TC_168415	68
	Reverse: CGCCAGAGGAAAGGATACTG		
Rex01	Forward: GGTCTCAGCCTCAGTGTCAGC	TC_317123	105
	Reverse: TGTAGCCAGCGTCCTCTTCC		
Cdx2	Forward: CCTCTCGCCCACAAATGTTCAC	TC_206866	82
	Reverse: TCCAACCGCACCTGTCTTTACC		
Bcl-xL	Forward: GCAGGTATTGAACGAACTCTTCCG	AJ_001203	107
	Reverse: GCATCTCCTTGTCTACGCTCTCC		
Bax	Forward: CTACCAAGAAGTTGAGCGAGTGTC	AJ_606301	85
	Reverse: ACGGCTGCGATCATCCTCTG		
BACT	Forward: CCACGCCATCCTGCGTCT	AK_237086	168
	Reverse: CCATCTCCTGCTCGAAGTCCAG		

Table 2. Comparison of the developmental competence between parthenogenetic and handmade cloned (HMC) embryos

Unless indicated otherwise, data are given as the mean ± s.e.m. ($n = 8$ replicates). Within a column, values with different superscript letters differ significantly ($P < 0.05$)

Embryo	Total	Cleavage rate (*n*)	Blastocyst rate (*n*)	Total cell number
Parthenogenetic	160	88.8 ± 3.0% (142)	48.8 ± 4.3%[a] (78)	52.4 ± 1.3
HMC	178	89.9 ± 1.7% (160)	36.5 ± 2.3%[b] (65)	54.5 ± 2.2

Table 3. Development of porcine handmade cloned embryos before and after embryo aggregation

Unless indicated otherwise, data are given as the mean ± s.e.m. ($n = 11$ replicates). Within a column, values with different superscript letters differ significantly ($P < 0.05$). N/A, not applicable; 1×, single cloned embryo, non-aggregated group; 2×, aggregated with two cloned embryos; 3×, aggregated with three cloned embryos

Embryo	No. embryos			Total cell number
	Total no.	Aggregated	Blastocyst	
1×	114	N/A	40 (35.1%)[b]	55.4 ± 3.5[c]
2×	216	108	68/108 (63.0%)[a]	86.3 ± 3.8[b]
3×	318	106	78/106 (73.6%)[a]	125.9 ± 7.2[a]

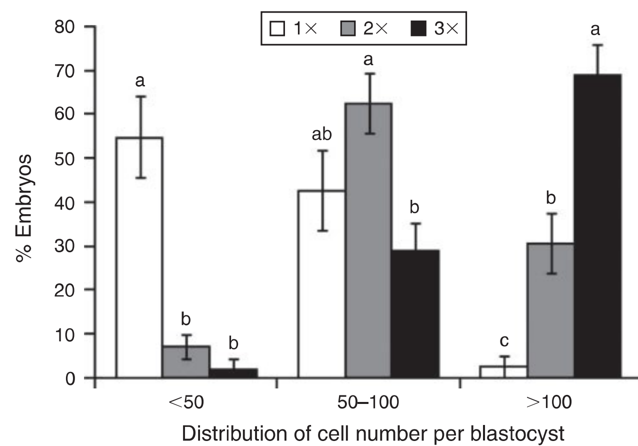

Fig. 2. Distribution of total cell numbers per handmade cloned (HMC) blastocyst from a single HMC embryo (1×) and aggregates of two (2×) and three (3×) HMC embryos. Data are the mean ± s.e.m. Within each grouping, columns with different letters differ significantly ($P < 0.05$).

Table 4. Effect of embryo aggregation on cell allocation to the inner cell mass and trophectoderm in handmade cloned blastocysts

Unless indicated otherwise, data are given as the mean ± s.e.m. ($n = 4$ replicates). Within a column, values with different superscript letters differ significantly ($P < 0.05$). 1×, single cloned embryo, non-aggregated group; 2×, aggregated with two cloned embryos; 3×, aggregated with three cloned embryos; ICM, inner cell mass; TE, trophectoderm

Embryo	Total no. stained embryos	No. cells			
		ICM	TE	Total	ICM/Total (%)
1×	14	8.6 ± 2.1[b]	46.2 ± 7.8[c]	54.8 ± 9.9[c]	15.3 ± 0.8[b]
2×	13	24.5 ± 2.8[a]	72.7 ± 2.4[b]	97.1 ± 2.5[b]	25.1 ± 2.7[a]
3×	15	34.7 ± 3.0[a]	97.5 ± 2.9[a]	132.2 ± 5.3[a]	26.1 ± 1.5[a]

ICM and TE cells in aggregated cloned blastocysts

There were significant differences between aggregated and non-aggregated embryos in terms of the total numbers of ICM and TE cells ($P < 0.05$; Table 4; Fig. 3). The numbers of TC, ICM and TE cells in the 3× and 2× aggregated blastocysts were higher than those in non-aggregated blastocysts (132 and 97 vs 55, respectively, for TC; 35 and 25 vs 9, respectively, for the ICM; 98 and 73 vs 46, respectively, for TE cells; all $P < 0.05$). Comparing 2× and 3× aggregates revealed that the 3× aggregated embryos had significantly more TC and TE cells than the 2× aggregated embryos ($P < 0.05$). However, the number of ICM cells did not differ significantly between the two groups. The ICM : TC ratio in the 2× and 3× aggregates was higher than in the non-aggregated group (26.1% and 25.1% vs 15.3%, respectively; $P < 0.05$).

Apoptotic indices in aggregated cloned embryos

The TC, number of apoptotic nuclei and apoptotic indices of the 1×, 2× and 3× cloned aggregated embryos are given in Table 5. The TC of the 3× group was higher than that of the 2× and non-aggregated groups (126 vs 89 and 57, respectively; $P < 0.05$). The apoptotic index of the non-aggregated group was higher than that of the 2× and 3× groups (4.7% vs 2.7% and 2.2%, respectively; $P < 0.05$). Nevertheless, the occurrence of apoptotic nuclei per blastocyst did not differ among the 1×, 2× and 3× groups (2.7, 2.4 and 2.7, respectively; $P > 0.05$).

Effect of embryo aggregation on gene expression in porcine embryos

The expression of pluripotency- (*Oct4*, *Rex01*, and *Cdx2*) and survival-related (*Bcl-xL* and *Bax*) genes was analysed using quantitative real-time PCR at the blastocyst stage on Day 7. There was no significant difference in *Rex01* expression among the treatment groups (Fig. 4). However, *Oct4* expression was increased in the 3× aggregated blastocysts compared with that in the non-aggregated and parthenogenetically activated (PA) groups (1.51-, 0.66- and 0.70-fold, respectively). There was no significant difference in *Oct4* expression among the 3× and 2× aggregates and the IVF control group. The expression of *Cdx2* in the 3× aggregates (1.32-fold) was similar to that in the IVF control group, but differed significantly different from that in the other treatment groups (0.56-, 0.70- and 0.53-fold in the 1×, 2× and PA groups, respectively).

There was no significant difference in the expression of the survival-related gene *Bcl-xL* among treatment groups. Conversely, *Bax* expression in the non-aggregated (1.41-fold) and the PA (1.24-fold) groups was significantly higher ($P < 0.05$) than in the 2× (0.82-fold) and 3× (0.66-fold) aggregates, with no differences in *Bax* expression among the 2× and 3× aggregates and IVF control group (Fig. 4).

Full-term development of aggregated cloned embryos reconstructed with miniature pig fibroblasts

In all, 239 blastocysts were transferred surgically to four hormonally synchronised recipients (40–70 embryos per recipient), resulting in two pregnant recipients (50% pregnancy rate; Table 6). A total of seven piglets was obtained from the two surrogate mothers (Fig. 1*e*; Table 7). One piglet was stillborn. Another piglet died 3 days after birth; this piglet had abnormalities involving various internal organs (Fig. 5).

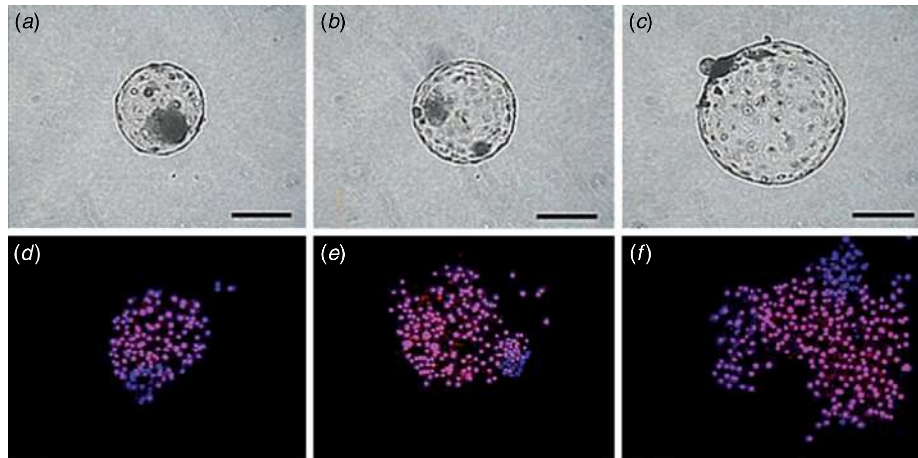

Fig. 3. Aggregated porcine handmade cloned (HMC) blastocysts from a single cloned embryo (1×) and aggregates of two (2×) and three (3×) cloned embryos were examined and photographed using an epifluorescence microscope. The number of inner cell mass (ICM; blue) and trophectoderm (TE; pink–red) cells was determined using differential staining. (*a–c*) Bright field image of 1× and 2× and 3× cloned embryos, respectively, (*d–f*) merged images of the cell allocation pattern of 1×, 2× and 3× cloned embryos following differential staining.

Table 5. Apoptotic indices of cloned blastocysts derived from aggregated and non-aggregated handmade cloned embryos

Unless indicated otherwise, data are given as the mean ± s.e.m. ($n = 4$ replicates). Within a column, values with different superscript letters differ significantly ($P < 0.05$). 1×, single cloned embryo, non-aggregated group; 2×, aggregated with two cloned embryos; 3×, aggregated with three cloned embryos; TC, total cells

Group	No. blastocysts evaluated	No. TC/blastocyst	No. apoptotic nuclei	Apoptotic index
1×	25	57.0 ± 3.0^{c}	2.69 ± 0.35	4.7 ± 0.6^{a}
2×	25	89.1 ± 4.5^{b}	2.37 ± 0.45	2.7 ± 0.5^{b}
3×	28	126.0 ± 8.2^{a}	2.68 ± 0.96	2.2 ± 0.2^{b}

Discussion

The present study appears to be the first report of an efficient *in vitro* production (IVP) system achieving blastocyst rates of PA and HMC porcine embryos up to 48% and 36%, respectively. In previous studies, blastocyst rates of HMC porcine embryos ranged from 20% to 60% (Du *et al.* 2005, 2007; Kragh *et al.* 2005; Lagutina *et al.* 2006; Li *et al.* 2006; Li *et al.* 2008). The lower blastocyst rates of HMC compared with PA embryos may also be associated with some inevitable damage caused by the bisection or prolonged culture under suboptimal conditions (e.g. 2–3 h incubation in HEPES-buffered medium during micromanipulation; Walker *et al.* 2002), which reduced cell numbers. However, the efficiency or viability of cloned embryos created by the somatic cell nuclear transfer (SCNT) technique is generally low, with the birth of normal cloned offspring even lower (1%–10%; Keefer 2008). At present, most of these embryos fail to develop to term. Cloned porcine embryos in particular have a low blastocyst rate with reduced TC. Therefore, to enhance the developmental competence of cloned embryos, the aim of the present study was to increase the TC of cloned embryos by embryo aggregation.

Embryos at the 4-cell stage are considered best for aggregation, whereas those at the 2-cell or beyond the 16-cell stage have a low aggregation or development rate (Lee *et al.* 2007). For an IVP porcine embryo, embryo aggregation improves both blastocyst rate and TC (Lee *et al.* 2007; Terashita *et al.* 2011). Our results confirmed this and demonstrated that the aggregation of embryos, even with clones, increased the blastocyst rate and TC per blastocyst. Apparently, an inadequate culture system prevents embryonic cells from proliferating and this is particularly true of current systems used for porcine embryos (Kikuchi *et al.* 1999; Pomar *et al.* 2005). Therefore, embryo aggregation was used to increase the number of cells in cloned embryos by two- or threefold. We expected that this could enhance the physical contact between the embryonic cells to facilitate cell–cell interactions, because the formation of intercellular gap junctions between blastomeres is critical for normal development during the morula stage (Lee *et al.* 2007). These cell junctions link the cytoplasm of two cells and provide a channel for ion (e.g. K^{+} and Ca^{2+}), intracellular signalling molecule (e.g. cAMP, cGMP, and inositol 1,4,5-trisphosphate) and small metabolite (e.g. glucose) exchanges, allowing electrical and biochemical coupling between adjacent cells (Kanno and Loewenstein 1964; Lawrence *et al.* 1978). It is of note that gap junctions first appear in 8-cell mammalian embryos and increase in number between compaction and blastocoel formation (Lo and Gilula 1979; Goodall and Johnson 1984). In the mouse embryo, communication via gap junctions apparently has an important role in maintaining compaction and differentiation of an organised epithelium within an embryo (Becker and Davies 1995). Collectively, these cell-autonomous and cell–cell interactions may have facilitated development of the aggregated pig embryos (4-cell), with an improved blastocyst rate, in the present study.

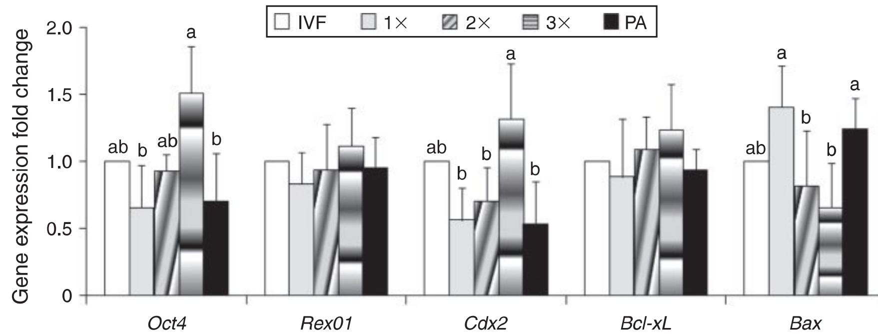

Fig. 4. Relative expression of porcine pluripotency- and apoptosis-related genes (*Oct4*, *Rex01*, *Cdx2*, *Bcl-xL*, and *Bax*) in aggregated handmade cloned (HMC) blastocysts from a single cloned embryo (1×) and aggregates of two (2×) and three (3×) cloned embryos, parthenotes (PA), and IVF blastocysts. The mRNA levels of the genes were analysed using quantitative real-time polymerase chain reaction, with three replicates per sample. The expression of each gene was normalised against that of β-actin mRNA. Data are the mean ± s.e.m. Within each grouping, columns with different letters differ significantly ($P < 0.05$).

Table 6. Full-term development of miniature pigs derived from aggregates of three handmade cloned embryos (3×) using fibroblasts as donor nuclei

+, pregnant; −, not pregnant; N/A, not applicable

Replicates	No. transferred embryos	Status on Day 21[A]	Gestation length, days	No. piglets born	Cloning efficiency[B], %
3× aggregated					
1	40	+	115	1	2.5
2	69	−	N/A	0	0
3	60	+	115	6 (plus one mummified piglet)	10.0
4	70	−	N/A	0	0
Total	239			7	2.9
Non-aggregated[C]					
1	80	−	N/A	0	0
2	83	−	N/A	0	0
3	85	−	N/A	0	0
4	101	−	N/A	0	0
Total	349				0

[A]Pregnancy status on Day 21 was determined by ultrasonography.
[B]Cloning efficiency was calculated as the no. piglets/no. embryos transferred.
[C]Data for the transfer of non-aggregated embryos (control) was obtained in a separate trial.

Table 7. Cloned piglets derived from aggregates of three handmade embryos (3×) using fibroblasts as donor nuclei

N/A, not applicable

Cloned pig ID	Birthweight, kg	Outcome	Pathological findings
100–10	0.7	Died after 3 days	Bleeding in lung tissue
100–11	0.8	Alive	N/A (reached puberty at 8 months with normal oestrous cycles and gave birth to normal offspring)
100–12	0.9	Alive	N/A (reached puberty at 13 months with normal oestrous cycles)
100–13	1.1	Alive	N/A
100–14	1.1	Died after 2 months	No apparent pathology
100–15	1.1	Died after 3 weeks	Lacked one ventricle and had intestinal fibrosis
100–16	0.35	Stillborn	Deformed nose and mouth

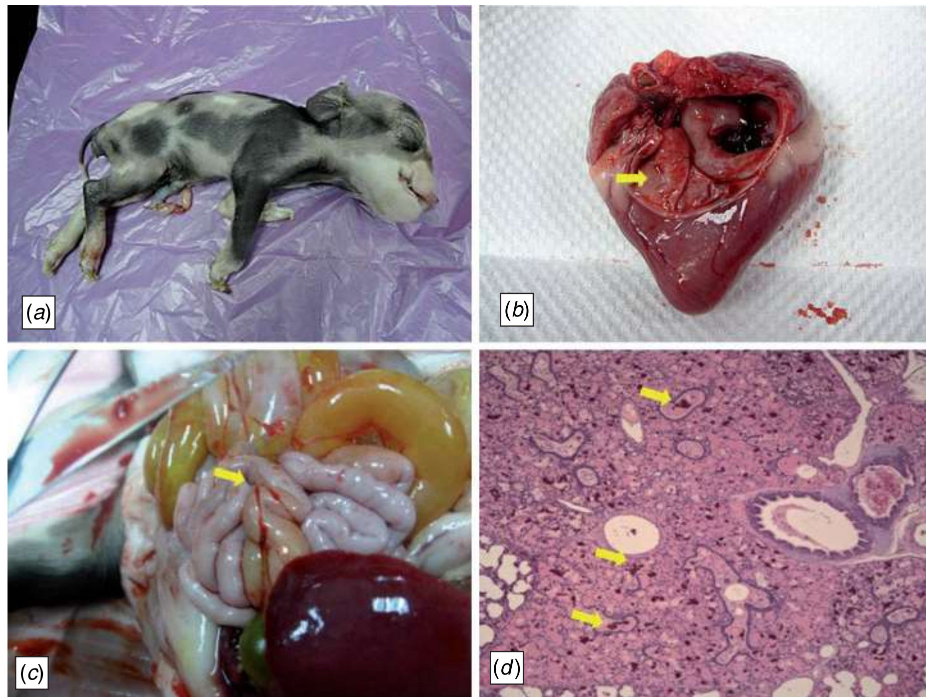

Fig. 5. (*a*) One cloned piglet was stillborn (bodyweight 350 g) and had a deformed nose and mouth. (*b–d*) Abnormalities of the internal organs from this piglet, including the heart lacking one ventricle (*b*), fibrous-like tissues on the surface of the intestines (*c*) and a tissue section of the lungs (*d*) showing blood spots (arrows).

We also demonstrated that embryo aggregation improved the ICM : TC ratio in cloned porcine embryos, consistent with previous reports that aggregation of embryos at the 4-cell stage improved the quality of porcine IVP embryos (Lee *et al.* 2007; Terashita *et al.* 2011). Although the numbers of ICM and TE cells in aggregated blastocysts are greater than in non-aggregated embryos from pigs and cattle, the mechanisms underlying this difference are unclear. It is of note that the diameter of the morula was greater in aggregated embryos during development; this would lead to a greater surface area and volume of embryos (by two- and three-fold, respectively), resulting in a larger embryo with fewer outer cells on its surface than inner cells inside the embryo. Cells on the surface of the morula mainly become TE cells, whereas the inner cells constitute the ICM, resulting in a significant increase in the ICM and TC in aggregated compared with non-aggregated embryos (Misica-Turner *et al.* 2007).

Previous studies indicate that aberrant ICM : TE or ICM : TC ratios may be a crucial cause of developmental failure (Giles and Foote 1995; Machaty *et al.* 1998). Changes in ICM and TE cell numbers in a blastocyst can alter the expression of pluripotency-related genes, which could affect the developmental competence of the fetus after implantation (Boiani *et al.* 2002). In pigs, *Oct4* and *Cdx2* gene expression was improved in 2× and 3× IVF embryos compared with non-aggregated embryos (Lee *et al.* 2007). Similarly, aggregation of porcine SCNT embryos at the 4-cell stage improved the percentage of *Oct 4*-positive cells in cloned blastocysts (Terashita *et al.* 2011), with similar results in cloned mouse embryos (Boiani *et al.* 2003). In the present study, miniature pig blastocysts derived from aggregation had consistently higher *Oct4* and *Cdx2* expression than non-aggregated blastocysts. Conversely, removal of two blastomeres from the 4-cell embryos had no effects on *Oct4* expression at the blastocyst stage (Boiani *et al.* 2003). It is generally accepted that no cloned embryos or animals are mutually epigenetically identical (Park *et al.* 2002; Boiani *et al.* 2003), and that combining two epigenetically different clones may be able to compensate for non-cell-autonomous defects of epigenetic origin (Rideout *et al.* 2001). Therefore, if some cells in an embryo were deficient in terms of transcriptional activity or developmental competence due to genetic variations, aggregation with a presumably healthy embryo may increase the chances of the aggregated embryo of developing normally due to an increased number of normal cells expressing genes essential for full-term development (Boiani *et al.* 2003).

Apoptosis is a major cause of low cell numbers and a reduction in the quality of developing embryos (Park *et al.* 2004; Carambula *et al.* 2009; Nguyen *et al.* 2009). However, there are few reports regarding the association between embryo aggregation and apoptosis in cloned porcine embryos. In the present study, based on results from the TUNEL assays, apoptotic cell indices were lower in the 2× and 3× aggregated compared with non-aggregated embryos. Furthermore, real-time PCR confirmed that the expressions of *Bax*, a pro-apoptotic gene, was lower in the 2× and 3× blastocysts than in the non-aggregated and PA groups. It has been reported that embryo aggregation reduces TUNEL-positive cells by threefold in 3× aggregated compared with non-aggregated bovine embryos (Yoon *et al.* 2009). In addition, *Oct4*, *Nanog* and *Bcl-2* mRNA expression was

higher, whereas that of *Bax* was lower, in 3× aggregated embryos than in the non-aggregated control (Lee *et al.* 2007; Yoon *et al.* 2009). Collectively, the results suggest that embryo aggregation reduces apoptosis in porcine blastocysts.

In vivo development of the aggregated cloned embryos was further tested in the present study. Two of four recipients sustained pregnancy after the transfer of 3× cloned blastocysts (5–7 days old). One surrogate gave birth to six piglets, resulting in an overall cloning efficiency of 10%. It has long been acknowledged that blastocysts with aberrant gene expression are less likely to develop to term (Boiani *et al.* 2002; Bortvin *et al.* 2003). In that regard, none of the sows receiving non-aggregated blastocysts in the present study was subsequently found to be pregnant. These results are supported by a similar study in the bovine in which pregnancy was not observed at Day 60 after transfer of non-aggregated embryos (Akagi *et al.* 2011). This failure may be due to insufficient ICM cell numbers and disturbed *Oct4* expression under suboptimal culture conditions. In contrast, blastocysts from aggregated embryos frequently exhibit *Oct4* expression and have increased ICM and TE cell numbers, which improve postimplantation development. Therefore, we infer that, in addition to TE cell numbers, an adequate proportion of ICM cells and the expression of *Oct4* and other pluripotency-associated genes facilitated the development of cloned embryos (Boiani *et al.* 2003; Lin *et al.* 2011).

Although many animals can be cloned, efficiency remains low and peri- and postnatal mortality is high in many cases (Hill *et al.* 1999; Kato *et al.* 2000). Reported causes of the early death of cloned piglets include abnormalities of cardiopulmonary function, hepatopneumonic congestion (Park *et al.* 2005) and rigid flexure of the distal joint of the legs (Du *et al.* 2007). In the present study, four piglets died due to abnormal development of the internal organs, including lack of a cardiac ventricle, pulmonary haemorrhage and oedema, and intestinal fibrosis. In cattle, early death of cloned calves due to lymphoid hypoplasia, hepatic congestion, pneumonia, amniotic fluid aspiration and hepatic fibrosis has been reported (Akagi *et al.* 2011). Although there is a lack of similar reports on cloned pigs generated by embryo aggregation, the causes of the abnormalities or deaths may be attributed mainly to insufficient reprogramming or epigenetic modifications of the donor nuclei (Tsunoda and Kato 2000), including treatment of donor cells rather than the aggregation itself (Akagi *et al.* 2011). For example, serum starvation used for synchronisation of donor cells in the present study could induce DNA fragmentation and high perinatal losses, as reported previously (Kato *et al.* 1998; Kues *et al.* 2000; Cho *et al.* 2002; Ideta *et al.* 2007; Akagi *et al.* 2011). Nevertheless, two of the cloned miniature pigs have reached puberty; one was bred and recently gave birth to normal piglets (data not shown).

In conclusion, this is the first report regarding the production of cloned miniature pigs by adopting an embryo aggregation strategy. Furthermore, we demonstrated that cloned miniature porcine embryos produced by aggregation of HMC embryos developed to the blastocyst stage *in vitro* and had improved embryo quality. This aggregation technique enhances the expression of pluripotency-related genes and reduces pro-apoptotic gene expression in cloned porcine embryos during pre- and postimplantation development.

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