Animal Science Papers and Reports vol. 33 (2015) no. 3, 213-222
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Developmental competence of porcine chimeric embryos produced by aggregation*

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(Accepted May 12, 2015)

The purpose of our study was to compare the developmental competence and blastomere allocation of porcine chimeric embryos formed by micro-well aggregation. Chimeras were created by aggregating either two blastomeres originating from 2-cell embryos or two whole embryos, where embryos were produced either by parthenogenetic activation (PA) or handmade cloning (HMC). Results showed that the developmental competence of chimeric embryos, evaluated based on their blastocyst rate and total cell number per blastocyst, was increased when two whole 2-cell stage embryos (PA or HMC) were aggregated. In comparison, when two blastomeres were aggregated, the developmental competence of the chimeric embryos decreased if the blastomeres were either from PA or from HMC embryos, but not if they were from different sources, i.e. one PA and one HMC blastomere. To evaluate the cell contribution in embryo formation, aggregation was made with HMC embryos cloned using EGFP transgenic cells; the cell contribution in the formation of the inner cell mass or trophectoderm was random in chimeric blastocysts. Finally, two blastomeres from 2-cell stage embryos were fused to construct tetraploid embryos, and when diploid and tetraploid embryos were used for aggregation, the cells of the ICM and its close vicinity were mostly formed by blastomeres from the diploid embryo.

KEY WORDS: chimera / co-culture / porcine / somatic cell nuclear transfer / traploidy / WOW

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*This research was supported by grants from Natural Science Foundation of Jiangsu Province (No. BK20130685) and the Fundamental Research Funds for the Central Universities (No. KJQN201521).
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Aggregation by co-culturing embryos or blastomeres is one of the most widely used methods to generate chimeric embryos. When zona-free mammalian embryos are brought into direct contact, blastomeres from each embryo reorganize and produce a single chimeric embryo [McLaren 1975]. Embryo aggregation has also been used to produce transgenic chimeras in mice [Chrenek and Makarevich 2005, Tang and West 2000] and pigs [He *et al*. 2013, Siriboon *et al*. 2014], and aggregation can even cross species boundaries, as sheep-goat chimeric offspring have been born [Fehilly *et al*. 1984, MacLaren *et al*. 1993].

Studies have demonstrated the advantages of aggregation in both enhancing *in vitro* development [Tang and West 2000] and overcoming early developmental obstacles in cloning by somatic cell nuclear transfer [Boiani *et al*. 2003]. Furthermore, in aggregation chimeras the distribution of progenitor cells may be highly influenced by particular cell lineages [Everett and West 1996], probably as a result of cellular competition [West *et al*. 1995]. Exploitation of this influence of cell lineage in aggregation chimeras has facilitated the generation of embryos with trophectoderm (TE) or inner cell mass (ICM) primarily of a single cell origin, while the contrasting tissue may be of mixed or opposite origin [Wells and Powell 2000]. However, the overall efficiency of the procedure in pigs was low compared to that in mice, probably due to physiological differences between the species [Shiue et al. 2006]. An efficient system for porcine embryo aggregation would be useful not only for the above mentioned purposes, but also to study cell-cell interactions, where the genotype or genome status could differ.

In the present study, porcine blastomeres or embryos of different origin (cloned, parthenogenetically activated diploid or tetraploid) were aggregated to test their developmental potential and the possible cell distribution in the chimeric embryos.

Material and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All experiments were performed according to all relevant institutional and national animal welfare laws, guidelines and policies.

Nuclear donor cells

A fibroblast cell line, derived from an ear skin biopsy from a Göttingen minipig, was transfected with a transposon donor vector, pSBT/cHS4.Ubi-GIN.cHS4, which contains a GFP-IRES-neo^r (GIN) cassette derived by the human ubiquitin C promoter (Ubi) [Jakobsen *et al*. 2011]. The DNA constructs were introduced into the cells using the transfection reagent FuGENE 6 (Fugent; LLC, Madison, WI, USA) according to the manufacturer's recommendations. Briefly, FuGENE 6 (3 µl) were pre-incubated in 94 µl serum-free medium and mixed with 0.6 µg of pSBT/cHS4.Ubi-GIN.cHS4 transposon plasmid. The mixture was applied to a 10 cm^2 well containing approximately 40,000 cells. The medium was renewed 24 h after transfection, and then cells were placed in a Petri dish and selected for 8 days with G418-containing

medium (1 μg/ml). The resistant cell colonies were isolated individually, and each colony was grown to approximately 200,000 cells under continuous G418 selection. EGFP positive cells (with green fluorescence confirmed under an inverted microscope by fluorescent light of excitation and emission wavelengths of 480 nm and 510 nm, respectively) were cryopreserved in freezing medium that was supplemented with 10% DMSO; the cells were immediately placed at -70°C for 24 h, and subsequently stored at -135°C. One week before nuclear transfer the EGFP positive cells or non-transgenic cells (a fibroblast cell line established in the same manner, but without the transgene) were prepared as described previously [Kragh *et al.* 2005; Li *et al*. 2009].

Oocyte collection and in-vitro maturation

Oocytes were derived as previously described [Du *et al*. 2007, Kragh *et al*. 2005, Li *et al*. 2009]. Briefly, ovaries were collected from a slaughterhouse and transported at 30-35°C in physiological saline with antibiotics to reach the laboratory within 4 h after slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles using 18-gauge injection needles. Selected COCs were matured for 42–44 h in groups of 50 in 400 μl bicarbonate-buffered TCM-199 (Invitrogen, Carlsbad, USA) supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) pig follicular fluid, 10 IU/ml PMSG and 5 IU/ml hCG at 38.5°C and in 5% CO_2 in air at maximum humidity.

Somatic cell nuclear transfer by HandMade Cloning (HMC)

In the morning of nuclear transfer (designated as Day 0), a non-transgenic fibroblast cell line and a transgenic EGFP positive fibroblast cell line were prepared as previously described [Li *et al*. 2009, Kragh *et al.* 2005, Jakobsen *et al.* 2011]. After removing the cumulus cells using 1 mg/ml hyaluronidase, the matured oocytes with partially digested (in 3.3 mg/ml pronase), but still visible zonae pellucidae (ZP) were lined up in a T2 drop (T refers to Hepes-buffered Tissue Culture Medium 199 [TCM-199; BioWhittake, Walkersville, MD, USA], while the number refers to serum concentration), supplemented with 2.5 μg/ml cytochalasin B (CB). With a glass pipette oocytes were rotated to find the polar body (PB) on the surface. Subsequently, oriented bisection in relation to the PB was performed manually with a micro-blade (AB Technology, Pullman, WA, USA) under a stereomicroscope. Thus half of the cytoplasm close to the PB was removed from the remaining putative cytoplast. Cytoplasts were washed twice in T2 drops and were then collected in a T10 drop.

Subsequently, a two-step fusion procedure was used, including the initiation of activation in the second step. Step 1: After treatment with 1 mg⁄ml of phytohaemaglutinin (PHA; ICN Pharmaceuticals, Australia) for 5 s, one cytoplast was attached to one fibroblast cell. In the fusion medium [0.3 M mannitol and 0.01% (w⁄v) PVA] the cytoplast-cell pairs were aligned to one wire in a fusion chamber (BTX micro slide 0.5 fusion chamber, model 450; BTX, San Diego, CA, USA) with the somatic cell farthest from the wire, using an alternating current (AC) of 6 V⁄cm. Then a single direct current (DC) pulse of 200 V⁄cm for 9 μs was supplied for the first fusion. Step

2: One hour later, cytoplast–fused pairs were aligned sequentially to the wire applying a 6 V/cm AC in the activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01% [w/v] PVA). A single DC pulse of 86 V/cm for 80 µs was used for the second fusion and initiation of activation. After 15 min. reconstructed HMCg (cloned embryos with EGFP green cells) and HMCn (cloned embryos with non-transgenic cells) embryos were chemically activated for 4 h in porcine zygote medium 3 (PZM-3; Yoshioka et al. 2002), supplemented with 5 µg/ml CB and 10 µg/ml cycloheximide (CX). After being washed thoroughly with PZM-3 medium, embryos were *in vitro* cultured for 5-6 days in the PZM-3 medium at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 at maximum humidity.

Parthenogenetically activated zona-free embryos (PA)

Oocytes were denuded the same way as described above, except that a longer incubation in 3.3 mg/ml pronase was used to remove the ZP completely. Subsequently, the ZP-free oocytes were aligned on the wire of the fusion chamber using an AC of 6 V/cm. A single DC pulse of 86 V/cm for 80 µs was then applied for the parthenogenetic activation. The procedure after the electrical pulse was the same as for HMC reconstructed embryos.

Separation of blastomeres

After washing in PBS medium without Ca^{2+} and Mg^{2+}, embryos at the 2-cell stage (22-24 h) were separated into single blastomeres by gently pipetting in PBS medium. Immediately afterwards, single blastomeres with no visible morphological damage were selected and transferred into a T10 drop for recovery and equilibration.

Construction of tetraploid embryos

Two synchronous blastomeres, separated as described above at the 2-cell stage, were electrically fused to construct tetraploid (2-TET) embryos. Briefly, two single blastomeres were aligned on the wire of the fusion chamber using a continuous AC of 6 V/cm in the activation medium. Electric fusion and initial activation were performed with a single pulse DC of 200 V/cm for 9 µs. The procedure after the fusion was the same as for HMC reconstructed embryos.

Aggregation of embryos or blastomeres

Aggregation was accomplished by placing two single blastomeres or two single embryos into microwells (WOWs; Vajta et al., 2000) made in Nunc four-well dishes as shown in Figure 1 A. Further incubation was performed at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 at maximum humidity until evaluation.

Embryo evaluation

Developmental competence of embryos and aggregates was determined based on blastocyst rates and total cell number on Day 5. To determine the total cell number,

blastocysts were stained with 10 μg/ml Hoechst 33342 for 10-15 min in PBS medium without Ca^{2+} and Mg^{2+}, and then they were mounted on a glass microscope slide in a small droplet of glycerol (<2 μl) before being kept overnight in a refrigerator. Pictures of the stained blastocysts (10-15 in each group) were taken under an inverted microscope (Nikon, Japan), and the total cell numbers were counted on the images. The blastocysts from different groups were transferred into PBS medium, and the allocated fluorescent cells were observed under fluorescent light with excitation and emission wavelengths of 480 and 510 nm, respectively.

Experimental design

Experiment 1. Developmental competence and cell allocation of aggregated whole embryos. Aggregations of two intact, ZP-free early stage (2-cell) embryos were performed in WOWs using the following three combinations: (i) HMCg and HMCn; (ii) HMCg and PA; (iii) PA and PA.

To determine the cell allocations after aggregation of whole embryos, both HMCg and HMCg+HMCn embryos were cultured and observed.

Experiment 2. Developmental competence and cell allocation of aggregated single blastomeres. Aggregations of two single blastomeres derived from 2-cell stage embryos were performed in the following three combinations: (i) HMCg and HMCn; (ii) HMCg and PA; (iii) PA and PA.

Experiment 3. Developmental competence and cell allocation of HMCg and a tetraploid embryo. A single HMCg (diploid) and a single 2-TET embryo (tetraploid), both at the 2-cell stage, were co-cultured.

In all three experiments the blastocyst rates were registered, the cell number per blastocyst was determined, and the allocation of HMCg cells in the ICM and TE was observed.

One-way ANOVA was applied to compare differences among groups.

Results and discussion

In Experiment 1 the developmental competences and blastocyst cell numbers for the three groups are shown in Table 1A. More blastocysts were produced when the aggregation was carried out using two embryos produced in the same way, i.e. HMC+HMC or PA+PA, when compared to either the control group or aggregates of HMC+PA embryos. However, cell numbers of blastocysts from all aggregated groups were increased when compared to the control HMCg embryos.

In the subset of blastocysts derived from HMCg and HMCg+HMCn examined by fluorescence microscopy to determine cell allocation in the ICM and TE, the cells from the HMCg embryo contributed both to ICM and TE of the chimeric blastocysts (Fig. 1 A and B).

In Experiment 2, as shown in Table 1B, fewer blastocysts were generated when the aggregation was carried out using two blastomeres of the same origin, i.e. HMC+HMC

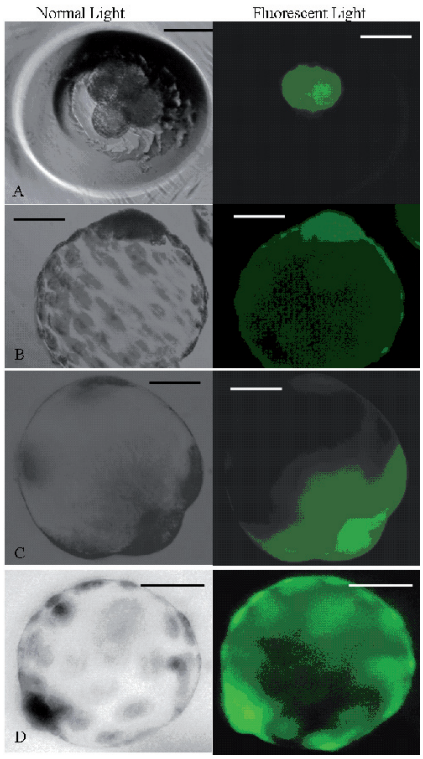

Fig. 1. A – Aggregation performed using WOW for co-culture of two whole embryos (EE-HMCg+n) at the 2-cell stage. B – Blastocyst derived from EE-HMCg+n embryos. C – Aggregated blastocyst of diploid+tetraploid embryos. D – Blastocyst derived from the control HMCg cloned embryos. Scar bar – 100 μm.

or PA+PA, in comparison both to the control group and the aggregates of HMC+PA blastomeres. However, the cell numbers of blastocysts from all aggregated groups were reduced when compared to the control HMCg embryos. The contribution of blastomeres from HMCg embryos to the ICM and TE was random in the resulting chimeric blastocysts, similar to the observation of Experiment 1.

In Experiment 3, the developmental capacity of 2-TET embryos (n=143) was demonstrated by their cleavage rate (90.4±3.3%) and subsequent blastocyst rate (74.6±6.3%), with the cell number per blastocyst of 44±1 (n=13). After 63 aggregates of HMCg+TET embryos were produced the development into chimeric blastocysts was 39.7±8.4% (n=25) with the blastocyst cell number of 93.8±9.1 (n=6). Figure 1 C shows that the cells from the diploid HMC embryo were mostly found in the ICM and its close vicinity, when compared to the control HMCg blastocyst shown (Fig. 1D).

The present study illustrates that the micro-well culture system [WOWs; Vajta *et al*. 2000] together with the removal of zona pellucida was an efficient method to

perform aggregations in order to generate porcine chimeric embryos. Within the WOWs, the zona-free embryos are forced to maintain contact between their blastomeres, and the micro-wells also provide a supporting microenvironment for the developing embryos.

In our study it was confirmed that two diploid embryos may efficiently be aggregated into a chimeric blastocyst, and that aggregation of two diploid embryos enhances total cell numbers in the resulting blastocysts. This beneficial effect of aggregation between two embryos may be attributed to the complementation of differences between clones [Boiani *et al*. 2003], where increasing the cell number in clones by aggregation dramatically improved spatial and temporal gene expression, as well as developmental potential. Several defects regarding reprogramming are known to occur during somatic cell nuclear transfer [Giraldo *et al*. 2008, Hossain *et al*. 2014, Kohan-Ghadr *et al*. 2012], such as aberrant expression of specific genes, aberrant methylation patterns and a lack of telomere restoration of donor chromatin, so aggregated blastocysts could theoretically have a two to three-fold higher chance to contain some fully reprogrammed cells [Misica-Turner *et al*. 2007].

Table 1. Developmental competence of different types of aggregated porcine embryos

Method	No. of embryos or aggregates (replicates)	Blastocyst rate (mean±SEM, %)	Blastocyst cell numbers (mean±SEM)
HMCg (control)	150 (9)	41.2±3.6[a]	64±3[a]
(A) Embryo-Embryo (EE) aggregates			
EE-HMCg+n	67 (4)	71.6±7.7[b]	103±5[b]
EE-HMCg+PA	117 (4)	39.1±9.4[a]	100±7[b]
EE-PA+PA	190 (9)	61.5±6.4[b]	106±7[b]
(B) Blastomere-Blastomere (BB) aggregates			
BB-HMCg+n	201 (6)	16.3±5.8[b]	51±2[a]
BB-HMCg+PA	236 (6)	49.8±7.1[a]	52±4[a]
BB-PA+PA	170 (6)	10.1±4.5[b]	55±5[a]

[ab]Within columns means bearing the same superscripts differ significantly at P<0.05.
Blastocyst rate – no. of blastocysts/no. of incubated embryos or pairs. Mean value ±SEM (standard error of the mean) is calculated per replicate.
Cell numbers – mean value ±SEM is calculated for all blastocysts in a given group.
HMCg – cloned embryos with GFP transgenic cells; HMCn – cloned embryos with non-transgenic cells; PA – parthenogenetically activated ZP-free embryos.

When the aggregation was performed with two embryos produced in the same way (either HMC or both PA; Table 1A), high developmental rates were obtained; however, when two blastomeres from either HMC or PA embryos were aggregated (Tab. 1B), developmental ability was compromised. This could indicate that developmental defects in in-vitro manipulated embryos were more likely to compensate for each other to improve reprogramming. Cellular interactions are known to play an important

role in beneficial complementarity between individual embryos and blastomeres [Sekirina and Neganova 1995, Misica-Turner *et al*. 2007]. Therefore, when two whole embryos were aggregated, more than one blastomere from embryos of same source could provide some autonomous support, which finally resulted in improved blastocyst rates. In contrast, when two blastomeres were aggregated, any defect blastomere from the different source (i.e. one from HMC and one from PA) could be complemented, but using the same sourced blastomeres (both from HMC or both from PA) this complementarity could not be achieved.

Almost 25-30 years ago, high rates of blastomere fusion and tetraploid development were obtained using electro-fusion techniques [Ozil and Modlinski 1986, Kaufman and Webb 1990]. Since then, the same has been obtained in different species using different aggregation methods, but with varying results with respect to which cells contribute to the ICM and TE cells of the embryo. Our results showed that when two diploid embryos or blastomeres were aggregated resulting in chimeric blastocysts, cells from HMC cloned embryos with green fluorescence were observed randomly in the ICM and TE cells. However, when tetraploid embryos were aggregated with diploid HMC embryos, cells from the cloned embryos were observed to contribute mostly to the ICM and its close vicinity. This is similar to research on chimeric rhesus monkey embryos, where tetraploid blastocysts appeared to be equally capable of contributing to the ICM and TE [Schramm and Paprocki 2004]. In another study it has been reported that the more advanced blastomeres may contribute to the ICM, while the less advanced blastomeres would develop into TE [Rorie *et al*. 1994]. Even though tetraploid blastomeres can produce an ICM and embryo proper [Kaufman and Webb 1990], a strong preference towards the ICM by normal diploid embryos and/or a strong preference against the ICM by tetraploid embryos in aggregated chimeras has been observed [Everett and West 1996]. According to a study on the aggregation of tetraploid and diploid embryos in mice, tetraploid blastomeres contributed only to the primitive endoderm layer of the ICM, persisting only in the extra-embryonic tissues [Everett and West 1996, James *et al.* 1995], while they were clearly excluded from the embryos proper in the developmental process [James *et al.* 1995].

In conclusion, using co-culture in WOWs, porcine zona-free embryos or blastomeres could be aggregated at the early stage and develop into chimeric blastocysts with high developmental efficiency. Aggregation facilitates further investigation into the biological mechanisms underlying the compensating interactions during early embryonic development. It was confirmed that tetraploid embryos could be created efficiently and easily using the electro-fusion technique. Further in-vivo studies are needed to obtain more knowledge concerning viability of these aggregated chimeric embryos.

Acknowledgements. *The authors would like to thank Gábor Vajta and Peter Kragh for crucial reading of and comments to the manuscript.*

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