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ORIGINAL RESEARCH

Generation and Developmental Characteristics of Porcine Tetraploid Embryos and Tetraploid/diploid Chimeric Embryos

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Abstract The aim of this study was to optimize electrofusion conditions for generating porcine tetraploid (4n) embryos and produce tetraploid/diploid (4n/2n) chimeric embryos. Different electric field intensities were tested and 2 direct current (DC) pulses of 0.9 kV/cm for 30 μs was selected as the optimum condition for electrofusion of 2-cell embryos to produce 4n embryos. The fusion rate of 2-cell embryos and the development rate to blastocyst of presumably 4n embryos, reached 85.4% and 28.5%, respectively. 68.18% of the fused embryos were found to be 4n as demonstrated by fluorescent *in situ* hybridization (FISH). Although the number of blastomeres in 4n blastocysts was significantly lower than in 2n blastocysts ($P < 0.05$), there was no significant difference in developmental rates of blastocysts between 2n and 4n embryos ($P > 0.05$), suggesting that the blastocyst forming capacity in 4n embryos is similar to those in 2n embryos. Moreover, 4n/2n chimeric embryos were obtained by aggregation of 4n and 2n embryos. We found that the developmental rate and cell number of blastocysts of 4-cell (4n)/4-cell (2n) chimeric embryos were significantly higher than those of 2-cell (4n)/4-cell (2n), 4-cell (4n)/8-cell (2n), 4-cell (4n)/2-cell (2n) chimeric embryos ($P < 0.05$). Consistent with mouse chimeras, the majority of 4n cells contribute to the trophectoderm (TE), while the 2n cells are mainly present in the inner cell mass (ICM) of porcine 4n/2n chimeric embryos. Our study established a feasible and efficient approach to produce porcine 4n embryos and 4n/2n chimeric embryos.

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Introduction

Recent discoveries in reproductive biotechnology have enhanced the potential uses of genetically modified pigs in the biomedical field with the purpose of improving human lives [1]. Tetraploid (4n) embryos are used to produce chimeras with embryonic stem cells (ESCs) and diploid (2n) embryos, which have been used to substitute microinjection

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of DNA and nuclear transfer for the creation of genetically modified animals [2,3].

4n embryos have been obtained by using many different methods, such as fusion of 2-cell stage embryos, or by using cytochalasin B or colchicine to inhibit cell division [4–7]. The most efficient method to produce 4n embryos in many species seems to be the fusion of 2-cell embryos stimulated by electrofusion [8–15]. However, the conditions for producing porcine 4n embryos needed to be optimized and the development of porcine 4n embryos has not been well addressed.

Directly injecting ESCs into 4n blastocysts or co-culturing ESCs with 4n embryos can generate mice completely derived from ESCs [16,17]. Using inner cell mass (ICM) cells or 2n embryos seem like an approachable way for species in which ESCs are not yet available. Compared to injection of ICM cells into embryos at the blastocyst stage, 4n/2n aggregation is a more efficient approach to produce 4n/2n chimeric embryos [18]. In mice, 4n/2n chimeras typically express a restricted trophectoderm (TE)/ICM distribution [19,20]. Nonetheless, in some uncommon 4n/2n chimeras, 4n cells contributed to the ICM due to the significant differences of the stage and the proportion of aggregated 4n and 2n embryos. The restricted distribution of 4n cells in chimeras has been performed experimentally to directly produce mice from ESCs by 4n complementation [16,17]. However, it has not been demonstrated in other species yet.

In this study, we explored the optimum condition for electrofusion of 2-cell embryos to produce porcine 4n embryos and systematically demonstrated the development of 4n embryos. We also tested the *in vitro* development and distribution pattern of 4n and 2n cells of chimeric embryos, along with the TE/ICM distribution of porcine 4n/2n chimeras.

Results

4n embryo production and chromosomal analysis

To optimize the conditions for production of porcine 4n embryos, different electric field intensities, including 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 kV/cm were tested to fuse 2n 2-cell embryos. Compared to 0.3, 0.6, 2.1 kV/cm voltages, the fusion rates of 0.9, 1.2, 1.5 and 1.8 kV/cm voltages were significantly higher ($P < 0.05$), and the rates of development to blastocyst of presumably 4n embryos were significantly higher when using 0.9 and 1.2 electric field intensities ($P < 0.05$). However, delayed 4n embryos were found when 1.2 kV/cm was used in comparison to when 0.9 kV/cm was used ($P < 0.05$; **Table 1**). These results indicate that the optimum condition for electrofusion of 2-cell embryos to produce porcine 4n embryos is 2DC pulses at 0.9 kV/cm for 30 μs. Furthermore, we evaluated the ploidy of control embryos, presumably 2n and fused embryos at the blastocyst stage by FISH (**Table 2**; **Figure 1**). Among the 20 control blastocysts subjected to chromosomal analysis, 19 embryos were diploid and one embryo was a mosaic of diploid and tetraploid cells. In fused presumably 4n embryos, 15 out of 22 embryos were tetraploid, six embryos were diploid, and one embryo was a mosaic of diploid and tetraploid cells. These results indicate that a majority of embryos fused under the optimum condition were tetraploid embryos.

Development of 4n embryos

2n embryos were recovered at the 1-cell stage while 4n embryos were obtained by fusion of the newly recovered 2-cell embryos. The developmental characteristics of the 2n and 4n embryos were compared *in vitro* (**Table 3**). Under the optimum conditions, the development of 2n embryos was significantly faster than 4n embryos at the cleavage stage ($P < 0.05$). However, the timing of blastocyst formation of 2n and 4n was very similar and the rates of blastocyst formation between 2n and 4n were not significantly different (23.61 *vs.* 22.84, respectively; $P > 0.05$). As expected, the number of blastomeres in 4n blastocysts was significantly lower than that in the 2n embryos ($P < 0.05$), indicating that the small number of blastomeres did not affect *in vitro* development capability of 4n embryos. Taken together, these data suggest that the competence of 4n embryos for developing into blastocyst is the same as 2n embryos.

Production of 4n/2n chimeras and distribution pattern analysis

Pairs of 4n and 2n zona-free embryos at different development stages were aggregated in individual micro-wells to produce 4n/2n chimeric embryos (**Figure 2**) and the development characteristics of chimeric embryos were checked. The blastocyst formation rate and the cell number of 4-cell (4n)/4-cell (2n) chimeric embryos were significantly higher than other groups (**Table 4**; $P < 0.05$), suggesting that the optimum time frame to obtain chimera by aggregation is when embryos are at the 4-cell stage. To clearly demonstrate the distribution pattern of 4n and 2n cells in porcine chimeric embryos, two distinct series of 4n/2n (GFP4n/2n and 4n/GFP2n) were cultured *in vitro* and analyzed at the blastocyst stage. In 4n/GFP 2n chimeras, the GFP positive cells mainly contributed to the ICM, whereas in the five GFP4n/2n chimeric blastocysts examined, GFP positive cells were present in the TE (**Figure 3**). These results indicated that the development pattern of porcine 4n/2n chimeric embryos is similar to that of mouse 4n/2n chimera, in which the majority of 4n cells contribute to the TE, whereas the 2n cells mainly locate in the ICM.

Discussion

The electrofusion of 2-cell embryos prove to be a simple and efficient way of generating mammalian 4n embryos. Many factors affect the fusion efficiency, such as fusion medium, electric field intensity and fusion pulse length [10,21–23]. In mice, production of 4n embryos by electrofusion has already been investigated [4], however, further investigation of optimum conditions to produce porcine 4n embryos is still needed. In this study, the optimum condition for generating porcine 4n embryos by electrofusion of 2-cell embryos is 2DC pulses of 0.9 kV/cm for 30 μs, which leads to a comparably high fusion rate. Thus, it appears to be more stable to produce 4n embryos by fusing freshly recovered 2-cell embryos in pigs. Furthermore, the cell number in 4n blastocysts was significantly lower than control 2n embryos. This may be attributed to the one less cell cycle of 4n embryos developing to blastocysts compared to 2n embryos, though the blastocyst formation rate

Table 1 Optimum electrofusion protocol for porcine 2-cell embryo fusion

Electric field intensities (kV/cm)	No. of 2-cell embryos	No. of embryos fused (%)	No. of blastocysts (%)	No. of retarded embryos (%)
Control	200	–	73 (36.5)[a]	n.a.
0.3	160	40 (25.0)[a]	13 (32.5)[b]	2 (5.0)[a]
0.6	151	108 (71.5)[b]	34 (31.5)[b]	11 (10.2)[b]
0.9	164	140 (85.4)[c]	40 (28.5)[c]	18 (12.9)[b]
1.2	135	110 (81.5)[c]	31 (28.2)[c]	30 (30.9)[c]
1.5	144	116 (80.6)[c]	28 (24.1)[d]	51 (43.9)[d]
1.8	150	120 (80.0)[c]	9 (7.5)[e]	102 (85.0)[e]
2.1	147	95 (64.6)[b]	4 (4.2)[f]	82 (86.3)[e]

Note: Values with different superscripts within columns denote significant difference ($P < 0.05$). n.a. means not available.

Table 2 Ploidy of control and fused porcine embryos

Embryos	No. of total blastocysts examined	No. of blastocysts with respective ploidy (%)		
		2n	4n	2n/4n
Fused	22	6 (27.27%)	15 (68.18%)	1 (4.5%)
Control	20	19 (95.0%)	0 (0)	1 (5.0%)

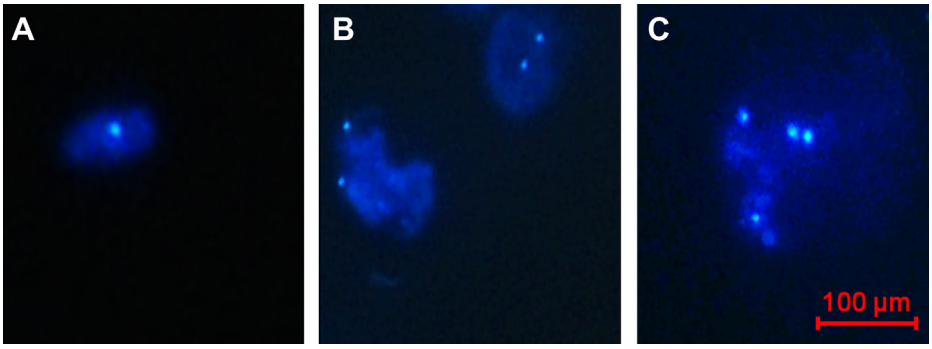

Figure 1 FISH analysis of ploidy of porcine IVF embryos
A. sperm; **B**. 2n cells of blastocyst; **C**. 4n cells of blastocyst. Scale bar, 100 μm.

of 4n embryos was similar to the control. Similar to other studies, we confirmed the high preimplantation development capacity of porcine 4n embryos as other mammalian 4n embryos [24].

Karyotyping analysis showed that the electrofused 2-cell embryos exhibited significant species variances in their ploidy. Uniform 4n cells were found at the blastocyst stage of fused embryos in mice and rats [15]. However, in cattle, only some embryos displayed uniform 4n cells at the morula and blastocyst stage, whereas most of the embryos were 2n or 2n/4n mosaics [25]. In pigs, about 50% of blastocysts produced by electrofusion of 2-cell embryos were 4n status [13]. These results indicate that 68% of the fused embryos display a deviation of the predicted ploidy and 27% of the fused embryos remained 2n. The reasons for discrepancy among these species are not quite clear. It has been proposed that the functional centrosomes exist during the early stages of cleavage in fused porcine and bovine embryos, which may render the embryos more prone to forming a disorganized bipolar or tripolar spindles. However, lack of centrioles in mouse embryos, up until the blastocyst stage, may cause the 4n embryos to develop after fusing normally. So the high rate of dipoid embryos may occur in fused bovine and porcine embryos by unsuccessfully combining the two sets of 2n chromosomes after fusion.

4n embryos cannot complete normal development independently [8,26]. However, when complemented with the ICM, ESCs or 2n embryos, 4n embryos can develop into conceptuses, in which embryonic lineages are derived entirely from the ICM, ESCs or 2n embryos, while the 4n component largely form extraembryonic lineages [20,27]. In this study, the porcine 4n embryos were complemented by aggregation with normal 2n embryos. It has been reported that embryo development stage, cell number and cell size could affect the aggregation of embryos [28–30]. In mice, aggregation of 2n embryos is often performed at the 8-cell stage, just before compaction, and 4n/2n chimera are often produced with the 8-cell stage of 2n embryos and 4-cell stage of 4n embryos. Also, it has been reported that high yields of chimeric blastocysts could be achieved by aggregating the ICM with 4–8 cell-stage or morulae parthenogenetic embryos in pigs [31]. In this report, by evaluating the developmental rate and the cell number of the blastocyst, we found that the combination of 4-cell stage 4n

Table 3 ***In vitro*** **development of 4n and 2n embryos**

Hours after electrofusion	Type of embryos	No. of embryos	No. of embryos developed (%)					No. of blastocyst cells
			2-cell	4-cell	8-cell	Morulae	Blastocyst	
24 h	2n	144	73 (50.69)	69 (47.92)				
	4n	197	30 (15.23)	15 (7.61)				
48 h	2n	144	65 (45.14)	53 (36.81)	14 (9.72)			
	4n	197	31 (15.74)	15 (7.61)	7 (3.55)			
72 h	2n	144	42 (29.17)	30 (20.83)	23 (15.97)	34 (23.61)		
	4n	197	23 (11.68)	19 (9.64)	8 (4.06)	25 (12.69)		
96 h	2n	144	40 (27.78)	25 (17.36)	17 (11.80)	33 (22.92)	12 (8.33)	
	4n	197	21 (10.66)	22 (11.17)	22 (11.17)	18 (9.14)	25 (12.69)	
120 h	2n	144	34 (23.61)	27 (18.75)	17 (11.81)	13 (9.03)	34 (23.61)	
	4n	197	32 (16.24)	20 (10.15)	19 (9.64)	6 (3.04)	38 (19.29)	
132 h	2n	144	28 (19.45)	27 (18.75)	18 (12.5)	16 (11.11)	34 (23.61)	45.34 ± 5.00^{a}
	4n	197	36 (18.27)	19 (9.64)	13 (6.60)	15 (7.61)	45 (22.84)	21.90 ± 4.95^{b}

Note: Values with different superscripts within columns denote significant difference ($P < 0.05$).

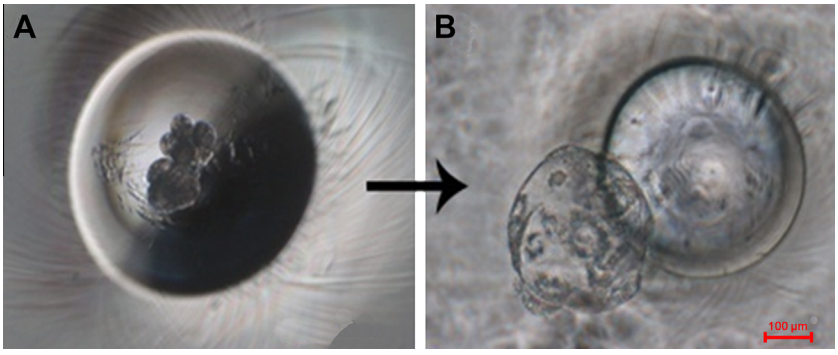

Figure 2 Production of chimeric embryos by 4n/2n aggregation

A. Aggregation of 4-cell (4n)/4-cell (2n) embryos in micro well. **B**. 4n/2n chimeric embryos developed to blastocyst after cultured for 48 h. Scale bar, 100 μm.

embryos with 4-cell stage 2n embryos can provide the best results for generating 4n/2n chimera in pig, which is different from what happens in mice.

By analyzing the 4n/2n chimera development, we found that 4n embryos at the 4-cell stage, when aggregated with 2n embryos at the 4-cell stage, produced the best developmental rate of blastocysts that also have more cells. Although an in-depth study has not yet been performed, we speculate that 4-cell stage embryos may aggregate together with high efficiency, resulting in the same embryo development stage with the same cell number and similar cell size. Comparatively, 2n embryos, at the 8-cell stage, when aggregated with 4n embryos at the 4-cell stage, generated blastocysts at lower development rates and cell numbers *in vitro*. This indicates that embryos at later stages may not be the best for producing chimeric embryos by aggregation compared to early cleavage embryos. However, later stage embryos can be used to form chimeric embryos by using other methods, such as the blastocyst injection method [32].

In the 15 4n/2n porcine chimeric embryos we analyzed, the 4n cells and 2n cells contributed to the TE and ICM, respectively. This development pattern is consistent with 4n/2n chimera mice. 4n cells in the blastocyst are preferentially selected to locate in the TE, rather than in the ICM. It is still not clear how the ICM is selective against 4n cells. The selective differentiation of these two types of blastomeres in 4n/2n chimera may involve

Table 4 4n ↔ 2n chimera made by aggregation of embryos at different stages

Group	No. of embryo pair	No. of blastocyst (%)	No. of blastocyst cells
2-cell (4n) ↔ 4-cell (2n)	48	$26\ (54.17\%)^{b}$	46.88 ± 10.44^{a}
4-cell (4n) ↔ 8-cell (2n)	52	$18\ (34.62\%)^{c}$	55.67 ± 18.87^{a}
4-cell (4n) ↔ 4-cell (2n)	39	$29\ (74.36\%)^{a}$	67.10 ± 15.39^{b}
4-cell (4n) ↔ 2-cell (2n)	48	$11\ (22.92\ \%)^{d}$	28.25 ± 8.56^{c}
2n	47	$11\ (23.40\%)^{d}$	44.35 ± 10.44^{a}
4n	36	$7\ (19.44\%)^{d}$	24.00 ± 7.69^{c}

Note: Values with different superscripts within columns denote significant difference ($P < 0.05$).

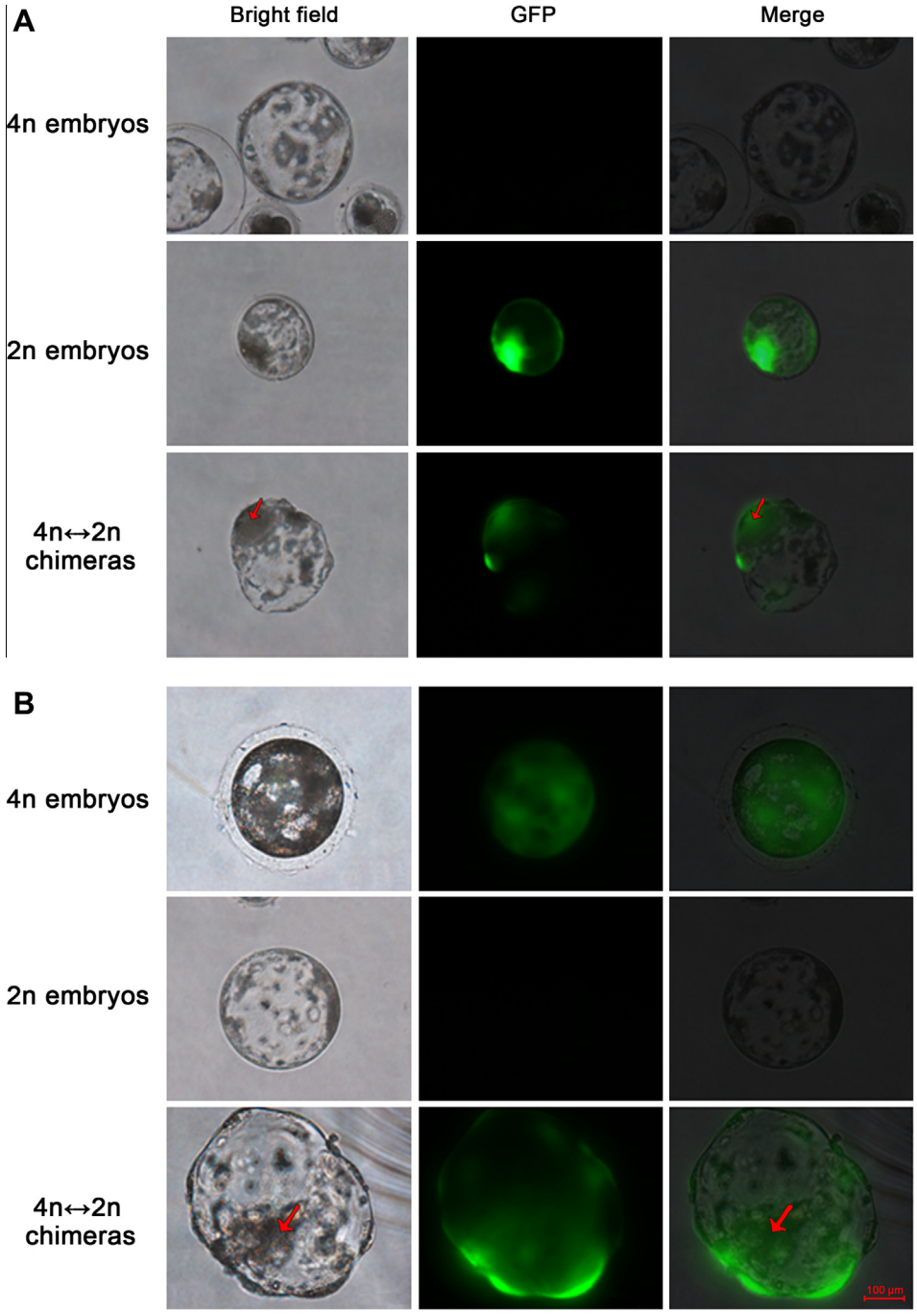

Figure 3 Distribution pattern of porcine 4n/2n chimera
A. 2n embryos were labeled with GFP; **B**. 4n embryos were labeled with GFP. Arrow marked the ICM. Scale bar, 100 μm.

competition between them. One explanation for the occurrence of this phenomenon is that 4n cells may be less competent in turning into embryonic tissues. This interpretation is supported by observations that independent 4n embryos fail to produce organized embryos after implantation [8,26]. Moreover, preimplantation embryos of pigs, sheep, cattle and humans frequently contain polyploid cells, which are more abundant in the TE than the ICM in the blastocyst [20,33]. With the support of previous data and the results we found in this study, we conclude that in most mammalian embryos, 4n or polyploid blastomeres generally are apt to contribute to the TE. This will be helpful to produce genetically modified animals through the same route widely used in mice.

In conclusion, the results of this study showed the optimum condition for electrofusion of 2-cell embryos to produce porcine 4n embryos and the high capability of 4n embryos to undergo preimplantation development. Moreover, 4n/2n chimeric embryos could be generated by aggregation of 4n and 2n embryos and the distribution of 4n and 2n cells to the TE and ICM, respectively, were demonstrated. Our study encourages research on the production of genetically modified porcine by producing 4n embryos and 4n/2n chimera.

Materials and methods

In vitro maturation of oocytes

The procedure for *in vitro* maturation (IVM) of porcine oocytes was described as followed. Porcine ovaries were received and transported at 30–35 °C, and an 18-gauge needle was used to aspirate follicular fluid from 3 to 6 mm follicles. Cumulus–oocyte complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected and rinsed three times in Tyrode lactate-Hepes plus polyvinyl alchohol (PVA). The COCs were cultured in a 4-well dish containing TCM-199 medium for 42–44 h at 38.5 °C as described previously [34].

In vitro fertilization and embryo culture

Porcine *in vitro* fertilization (IVF) was performed as described previously [35]. Briefly, the semen was washed three times in Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1% (w/v) bovine serum albumin (BSA) by centrifugation at 1500*g* for 3 min. The spermatozoa concentration and the proportion of motile sperm were determined by using a hemocytometer and sperm was diluted with the modified Tris-buffered medium (mTBM). Matured oocytes at 42 h of IVM were washed three times in mTBM. Approximately 30 oocytes were inseminated in 50 μl mTBM containing sperms at a final concentration of 3×10^5/ml for 6 h [35].

The embryos were cultured in porcine zygote medium-3 (PZM-3) at 38.5 °C. The cleavage and blastocyst rates were assessed at 48 h and 156 h after activation, and the number of blastocyst cells was evaluated by nuclear staining with 5 μg/ml Hoechst 33342.

Electrofusion of 2-cell embryos and production of chimeras by embryo aggregation

2-cell embryos of 2n were pre-equilibrated in fusion medium containing 0.3 mM mannitol, 0.1 mM $MgCl_2$ and 1.0 mM $CaCl_2$. Using an AC field of 6 V and 10 s, embryos were aligned in the chamber with their fusion plane parallel to the electrodes. 2DC electro pulses of different electric field intensities (0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 kV/cm) for 30 μs on a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA) were used to induce blastomere fusion. 30 min after electrofusion, the fusion rate was evaluated and the fused embryos were cultured in PZM-3 as described above.

Zona pellucida of embryos was removed by proteinase K (PK). Pairs of zona-free 4n and 2n embryos with different stages were pushed together in individual micro-well in PZM-3 to produce 4n/2n chimeric embryos.

FISH assay

Embryos were washed in TCM199 to remove embryo culture medium and suspended in hypotonic solution (0.075 M KCl). After being fixed with cold acetic acid/methanol solution (v/v 1:3) to dissolve cytoplasm, the embryo was transferred to a slide and frozen in −20 °C for at least 24 h. A 245-bp probe for porcine chromosome 1 was amplified and labeled by PCR using Alexa 546-dUTP (Invitrogen, Carlsbad, CA, USA) with the primer sets 5′GTTGCACTTTCACGGACGCAGC3′ and 5′CTAGCCCATTGCTCGCCATAGC3′. RNase and pepsin were used to treat the embryos on slides, which were then dehydrated in an ethanol series and denatured by incubation in 70% paraformaldehyde in 2× SSC solution at 70 °C for 3 min. After dehydration, the denatured probe was applied onto slides and incubated overnight at 37 °C before washes [36]. Following Hoechst 33342 staining, slides were mounted with Prolong gold antifade reagent (Invitrogen, Carlsbad, CA, USA) and analyzed using Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan).

Embryos labeling and distribution pattern analysis of chimeric blastocysts

PgcFU GFP lentivirus (titer at 2×10^9 IU/ml), purchased from Shanghai Genechem, was injected into the perivitelline space of 1-cell embryos with FemtiJet express Microinjector (Eppendorf, Hamburg, Germany) to label 2n and 4n embryos. The GFP4n/2n and 4n/GFP 2n chimeric embryos were produced by aggregation. Chimeric blastocysts were examined under a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 13.0. Data are shown as the mean ± SD. One-way ANOVA was used to assess any differences between groups. The Duncan method was employed for pairwise comparisons, followed by a Bonferroni correction. $P < 0.05$ (two-tailed) was considered statistically significant [37].

Authors' contributions

WH, QK and ZL conceived and designed the experiments. WH, YS, BX, MJ, TH, SG, and KH performed the experiments. WH, QK and ZL analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors have declared that there are no competing interests.

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References

[1] Luo Y, Lin L, Bolund L, Jensen TG, Sørensen CB. Genetically modified pigs for biomedical research. J Inherit Metab Dis 2012;35:695–713.

[2] Wang ZQ, Kiefer F, Urbanek P, Wagner EF. Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection. Mech Dev 1997;62:137–45.
[3] Mueller S, Prelle K, Rieger N, Petznek H, Lassnig C, Luksch U, et al. Chimeric pigs following blastocyst injection of transgenic porcine primordial germ cells. Mol Reprod Dev 1999;54:244–54.
[4] Kubiak JZ, Tarkowski AK. Electrofusion of mouse blastomeres. Exp Cell Res 1985;157:561–6.
[5] Snow MH. Tetraploid mouse embryos produced by cytochalasin B during cleavage. Nature 1973;244:513–5.
[6] McLaughlin KJ. Production of tetraploid embryos by electrofusion. Methods Enzymol 1993;225:919–30.
[7] Sembon S, Fuchimoto D, Iwamoto M, Suzuki S, Yoshioka K, Onishi A. A simple method for producing tetraploid porcine parthenogenetic embryos. Theriogenology 2011;76:598–606.
[8] Kaufman MH, Webb S. Postimplantation development of tetraploid mouse embryos produced by electrofusion. Development 1990;110:1121–32.
[9] O'Neill GT, Speirs S, Kaufman MH. Sex-chromosome constitution of postimplantation tetraploid mouse embryos. Cytogenet Cell Genet 1990;53:191–5.
[10] Ozil J-P, Modlinski JA. Effects of electric field on fusion rate and survival of 2-cell rabbit embryos. J Embryol Exp Morphol 1986;96:211–28.
[11] Iwasaki S, Kono T, Fukatsu H, Nakahara T. Production of bovine tetraploid embryos by electrofusion and their developmental capability in vitro. Gamete Res 1989;24:261–7.
[12] Prather RS, Hoffman KE, Schoenbeck RA, Stumpf TT, Li J. Characterization of DNA synthesis during the 2-cell stage and the production of tetraploid chimeric pig embryos. Mol Reprod Dev 1996;45:38–42.
[13] Prochazka R, Vodicka P, Zudova D, Rybar R, Motlik J. Development of in vivo derived diploid and tetraploid pig embryos in a modified medium NCSU 37. Theriogenology 2004;62:155–64.
[14] Hiriart MI, Bevacqua RJ, Canel NG, Fernandez-Martin R, Salamone DF. Production of chimeric embryos by aggregation of bovine egfp eight-cell stage blastomeres with two-cell fused and asynchronic embryos. Theriogenology 2013;80:357–64.
[15] Krivokharchenko A, Galat V, Ganten D, Bader M. *In vitro* formation of tetraploid rat blastocysts after fusion of two-cell embryos. Mol Reprod Dev 2002;61:460–5.
[16] Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M, et al. Embryonic stem cells alone are able to support fetal development in the mouse. Development 1990;110:815–21.
[17] Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 1993;90:8424–8.
[18] Peli J, Schmoll F, Laurincik J, Brem G, Schellander K. Comparison of aggregation and injection techniques in producing chimeras with embryonic stem cells in mice. Theriogenology 1996;45:833–42.
[19] James RM, Klerkx AH, Keighren M, Flockhart JH, West JD. Restricted distribution of tetraploid cells in mouse tetraploid $\Longleftrightarrow$ diploid chimaeras. Dev Biol 1995;167:213–26.
[20] Mackay GE, West JD. Fate of tetraploid cells in 4n $\leftrightarrow$ 2n chimeric mouse blastocysts. Mech Dev 2005;122:1266–81.
[21] Suo L, Wang F, Zhou GB, Shi JM, Wang YB, Zeng SM, et al. Optimal concentration of calcium and electric field levels improve tetraploid embryo production by electrofusion in mice. J Reprod Dev 2009;55:383–5.
[22] Clement-Sengewald A, Brem G. Electrofusion parameters for mouse two-cell embryos. Theriogenology 1989;32:159–69.
[23] Cheong HT, Taniguchi T, Hishinuma M, Takahashi Y, Kanagawa H. Effects of various electric fields on the fusion and in vitro development of mouse two-cell embryos. Theriogenology 1991;36:875–85.
[24] Park MR, Lee AR, Bui HT, Park C, Park KK, Cho SG, et al. Chromosome remodeling and differentiation of tetraploid embryos during preimplantation development. Dev Dyn 2011;240:1660–9.
[25] Curnow EC, Gunn IM, Trounson AO. Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts in vitro. Mol Reprod Dev 2000;56:372–7.
[26] Eakin GS, Behringer RR. Tetraploid development in the mouse. Dev Dyn 2003;228:751–66.
[27] Li X, Yu Y, Wei W, Yong J, Yang J, You J, et al. Simple and efficient production of mice derived from embryonic stem cells aggregated with tetraploid embryos. Mol Reprod Dev 2005;71:154–8.
[28] Lee SG, Park CH, Choi DH, Kim HS, Ka HH, Lee CK. *In vitro* development and cell allocation of porcine blastocysts derived by aggregation of in vitro fertilized embryos. Mol Reprod Dev 2007;74:1436–45.
[29] Tang PC, Ritchie WA, Wilmut I, West JD. The effects of cell size and ploidy on cell allocation in mouse chimaeric blastocysts. Zygote 2000;8:33–43.
[30] Tang PC, West JD. The effects of embryo stage and cell number on the composition of mouse aggregation chimaeras. Zygote 2000;8:235–43.
[31] Nakano K, Watanabe M, Matsunari H, Matsuda T, Honda K, Maehara M, et al. Generating porcine chimeras using inner cell mass cells and parthenogenetic preimplantation embryos. PLoS One 2013;8:e61900.
[32] Gardner R. Mouse chimaeras obtained by the injection of cells into the blastocyst. Nature 1968;220:596–7.
[33] Viuff D, Palsgaard A, Rickords L, Lawson LG, Greve T, Schmidt M, et al. Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. Mol Reprod Dev 2002;62:483–8.
[34] Samuel M, Bonk A, Im G-S, Rieke A, Day BN, Murphy CN, et al. Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells preparation of donor cells. Mol Reprod Dev 2002;306:300–6.
[35] Wei Y, Huan Y, Shi Y, Liu Z, Bou G, Luo Y, et al. Unfaithful maintenance of methylation imprints due to loss of maternal nuclear Dnmt1 during somatic cell nuclear transfer. PLoS One 2011;6:e20154.
[36] Skrzyszowska M, Samiec M, Slomski R, Lipinski D, Maly E. Development of porcine transgenic nuclear-transferred embryos derived from fibroblast cells transfected by the novel technique of nucleofection or standard lipofection. Theriogenology 2008;70:248–59.
[37] Kong Q, Wu M, Wang Z, Zhang X, Li L, Liu X, et al. Effect of trichostatin A and 5-Aza-2′-deoxycytidine on transgene reactivation and epigenetic modification in transgenic pig fibroblast cells. Mol Cell Biochem 2011;355:157–65.