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Original Article

Nuclear remodelling and early development in cryopreserved, porcine primordial germ cells following nuclear transfer into *in vitro*-matured oocytes

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ABSTRACT Nuclear transfer was conducted in the pig using, as karyoplasts, primordial germ cells which had been cryopreserved. The cytoplasts were presumptive S- or MII-phase, *in vitro*-matured oocytes, which had been enucleated mechanically. Enucleation was effective in 94.3% of cases. Karyoplasts were introduced into the perivitelline space, in close contact with the cytoplasts, and the complexes fused by electrical stimulation and activation. Activation was successful in 82-88% of nonmanipulated, pulsed oocytes, and in 55% of germ cell-oocyte complexes. The reconstituted embryos were examined for nuclear remodelling and cleavage *in vitro*. Nuclear swelling was more prominent when MII-phase cytoplasts, rather than S-phase, cytoplasts, were used. After 24 h in culture, the cleavage rate was not significantly different whether blastomeres or primordial germ cells were used as karyoplasts, and whether MII-phase or S-phase cytoplasts were used. However, after 72 h in culture, the developmental rate was higher when MII-phase cytoplasts (75%) were used for the recipients of blastomeres compared with S-phase cytoplasts (38.5%, $p<0.05$). Similar tendencies were observed with germ-cell nuclear transfer when inositol was used as medium for electrofusion (60% vs 27.8%, $p<0.05$). Furthermore, when MII-phase cytoplasts were used, the nuclear transferred embryos derived from blastomeres developed at a significantly higher rate than from primordial germ cells (37.5%, $p<0.05$). We conclude that cryopreserved primordial germ cells are competent to undergo nuclear remodelling and cleavage during 72 h of incubation *in vitro* to the 4-cell stage, following nuclear transfer to enucleated, activated (S-) or MII-phase oocytes. This experimental system may help to elucidate events in the early development of pig embryos following nuclear transfer using germ cells as karyoplasts.

KEY WORDS: *nuclear transfer, nuclear remodelling, pig, primordial germ cells*

Introduction

Nuclear transfer in embryos is a technique using the ability of nuclei from early cleavage stages to be reprogrammed to function as zygotic nuclei following transfer to enucleated, activated metaphase II (MII) oocytes. Much effort is currently being made in the application of nuclear transfer technology with a view to the production, ultimately, of cloned livestock. It has been established that pig embryos can undergo the experimental manipulations necessary for nuclear transfer (Prather *et al.*, 1989a). Important parameters for nuclear transfer in the pig have been defined (Niemann and Reichelt, 1993), for successful fusion, activation and cleavage of reconstituted embryos in that species. Nonetheless, reconstituted pig embryos have undergone poor development in experiments to date, and only one normal, live pig has been produced, from transfer of blastomeres to MII-phase oocytes which had been matured *in vivo* (Prather *et al.*, 1989a). Reasons which have been suggested for the inefficiencies of the nuclear transfer procedure include abnormal nuclear development and RNA synthesis (Hyttel *et al.*, 1990, 1993).

Primordial germ cells (PGCs) of amphibians have been used successfully as sources of karyoplasts in nuclear transfer, and live animals have been obtained (Smith, 1965; Lesimple *et al.*, 1987). However, progress in the mammals has been limited. In this class, the PGCs migrate from the base of the allantois, through the hindgut epithelium and dorsal mesentery, to colonise the gonadal anlage (Eddy *et al.*, 1981). PGCs of the livestock species, including the pig at 20 to 57 days of gestation, are viable for a limited period in culture, and degenerated after 24 h

Abbreviations used in this paper: EFM, electrofusion medium; MII, metaphase II; PGC, primordial germ cell; S, DNA synthesis.

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TABLE 1

ELECTRICAL ACTIVATION OF PORCINE OOCYTES MATURED *IN VITRO*

Medium	Time of maturation (h)	No. of oocytes	No. activated (%)
Mannitol	48-49	40	30 (75%)
	53-54	38	34 (89.5%)
	Total	78	64 (82.1%)
Inositol	48-49	53	45 (84.9%)
	53-54	28	26 (92.9%)
	Total	81	71 (87.7%)

(Leichthammer *et al.*, 1990). These authors showed that PGCs from the pig could be successfully cryopreserved, so that 60% of thawed PGCs from the pig were viable 24 h following thawing and culture. PGCs from the pig can be successfully cultured for up to 96 h (Leichthammer and Brem, 1990). In the mouse, Tsunoda *et al.* (1989) examined the developmental potential of enucleated eggs receiving male PGCs. Development *in vitro* was achieved up to blastocyst stages in 6 to 20% of cases, based on the reconstituted eggs with pronuclear formation. The overall success rate was 0-6% blastocysts when taking into account the total number of attempts at introducing germ cells. Most blastocysts obtained frequently did not show an inner cell mass. Live fetuses were not obtained. However, the rate of production of blastocysts using PGCs, and enucleated eggs, was higher than that attained using later 2-cell to 8-cell or innercell mass cells (0-3%).

The aim of this study was to test the suitability of cryopreserved PGCs to function as karyoplasts for nuclear transfer to enucleated, S- or MII-phase oocytes, and their subsequent capacity to undergo early development. The advantage gained by cryopreservation is that a single batch of germ cells was used for the duration of these experiments. It is generally considered that the phenomenon of nuclear remodelling involves nuclear structural changes, such as nuclear envelope breakdown, premature chromosome condensation (PCC) and pronuclear development and swelling (Tsunoda *et al.*, 1989; Prather *et al.*, 1990; Stumpf *et al.*, 1993). Nuclear remodelling is thought to be a result of the exchange of proteins between the nuclear and cytoplasmic compartments (Prather and First, 1990) and is pre-requisite to or is part of reprogramming of gene expression (Collas and Robl, 1991; Hyttel *et al.*, 1993; Niemann and Reichelt, 1993). Biochemical changes include (lamin) protein segregation between nucleus and cytoplasm (Prather *et al.*, 1989b), and regulation of RNA synthesis (Hyttel *et al.*, 1993). Here we show that successful nuclear remodelling and early cleavages, to a degree which resembles that achieved using blastomeres, can be achieved using cryopreserved porcine PGCs, which therefore are a suitable source of karyoplasts for further studies in development following nuclear transfer.

Results

Activation rate in nonmanipulated oocytes matured in vitro

Table 1 shows the rate of activation in non-manipulated, MII-phase oocytes following different times of maturation. Activation rates, as indicated by pronuclear formation, were similar whether oocytes were matured for 48-49 h or 53-54 h, and whether mannitol (82.1%) or inositol (87.7%) were used as the medium during electrofusion ($P>0.05$).

Efficiency of fusion and remodelling of cryopreserved PGCs

Following maturation, it was observed that most oocytes examined had MII-stage chromosomes very near the polar body (Fig. 1A). The average rate of enucleation was 94.3% (264/280). Presumptive S-phase cytoplasts were obtained by activation at 49 h, followed by fusion at 54 h with PGCs; MII-phase cytoplasts, by simultaneous fusion and activation at 54 h.

The percentages of fusion of PGCs to S- and MII-phase cytoplasts following electrical stimulation, using EFM in both cases, were 34.9% and 35.1%, respectively (Table 2, and see Fig. 1B and C). Furthermore, after 15 h of incubation of fused complexes the percentage of pronuclear swelling was similar, at around 55%, whether S-phase or MII-phase cytoplasts were used. However, the degree of nuclear swelling was less pronounced in S-phase (1.5x) than in MII-phase cytoplasts (2.2x; Fig. 1D,E and F).

Compared to the percentage of fusion achieved with blastomeres (81%; see Table 3), those obtained with PGCs (26-40%) were significantly lower ($P<0.001$).

Early development following nuclear transfer of PGCs

Fusion with blastomeres is similar using S or MII cytoplasts (81% and 80%, respectively), and the cleavage rate (to 2 cells or more) is approximately 54% and 50%, respectively (Table 3). This shows that whichever cytoplast is used, there is no significant difference in the cleavage of nuclear transfer embryos using blastomeres.

Using PGCs as karyoplasts and EFM as the fusion medium, the rate of fusion was lower than achieved with blastomeres, whether cytoplasts were S-phase (26.1%) or MII-phase (39.5%). When inositol was used as the electrofusion medium, percentages of fusion were 36.7% for S-phase, and 31.3 for MII phase, which do not represent significantly different values. The cleavage rate for PGCs after 24 h is 41.7% and 37.5% in EFM, and 44.4% and 60% in inositol, when using S and MII-phase cytoplasts, respectively. This compares to 54% and 50% in blastomeres. Therefore there is no significant difference in cleavage rates between blastomeres and PGCs. After 72 h of culture, both types of embryos can develop to the 2 to 4 cell stage. Using blastomeres as karyoplasts, a higher rate of development was achieved when MII-phase cytoplasts were used (75%), compared with S-phase cytoplasts (38.5%, $p<0.05$), and similar tendencies were observed with PGCs as karyoplasts. This demonstrates that the developmental capacity is similar using cryopreserved PGCs and blastomeres, although the rate of development is higher with blastomeres. Also, simultaneous fusion and activation is beneficial for the early development of nuclear transfer embryos in the pig.

Discussion

The purpose of these experiments was to determine the competency of cryopreserved primordial germ cells to function as karyoplasts in nuclear transfer, together with factors influencing their development at early stages. A mixture of male and female germ cells was cryopreserved and used as a source of kary-

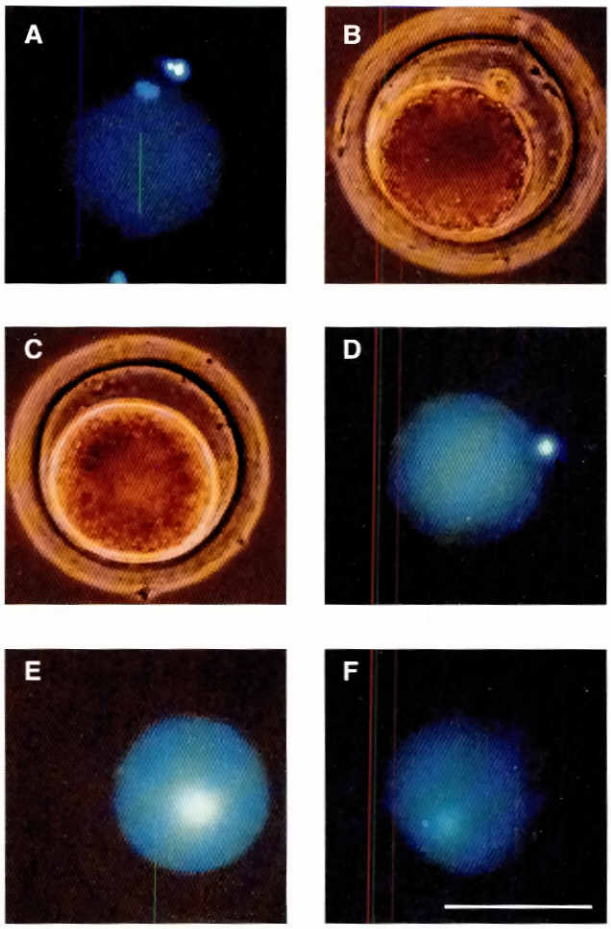

Fig. 1. Nuclear transfer using porcine PGCs. (A) *In vitro-matured oocytes with MII chromosomes located near the polar body.* **(B)** *PGC-cytoplast complexes before electrofusion, as viewed by phase-contrast microscopy.* **(C)** *The fused complexes.* **(D)** *PGC-cytoplast complexes, as viewed by fluorescence microscopy. An arrow indicates the nucleus of the PGC.* **(E)** *Nuclear swelling in MII-phase cytoplasts.* **(F)** *Nuclear swelling in S-phase cytoplasts. Bar, 100 μm.*

oplasts. In order to establish a model system, we employed also blastomeres as karyoplasts, in order to provide controls (Saito *et al.*, 1992). Firstly we compared the suitability of S-phase and MII-phase oocytes as cytoplasts for the germ-cell karyoplasts. It was reported that in porcine zygotes, the S-phase commences 56 h post hCG injection, and lasts 4.5 to 7.5 h: complete pronuclear formation was observable at 55 h after hCG injection, with condensed chromatin visible in the pronuclei (Laurincik *et al.*, 1995). We observed that pronuclear formation began at 5-6 h after electrical activation of *in vitro*-matured oocytes (that is 53-54 h after *in vitro* maturation; data not shown), and that the fusion of PGCs to the cytoplasts typically required 1-2 h for completion. Consequently, presumptive S-phase cytoplasts were obtained by activation followed by incubation for 5 h prior to fusion, and MII-phase oocytes by simultaneous fusion and activation. Percentages of fusion of PGCs to S- and MII-phase cytoplasts, so obtained, following electrical stimulation and using EFM in both cases, were similar. Thus, for the S-phase oocytes, prior electrical stimulation does not adversely affect subsequent fusion. Inositol was tested as a possible variable, as it is suggested to be a better electrofusion medium than mannitol-based EFM (Neil and Zimmermann, 1993). Nonetheless, we found no differences using cryopreserved PGCs. Activation rates for non-manipulated, MII-phase oocytes were uniformly high, whether or not they had been matured for 48-49 h or 53-54 h, using mannitol or inositol. Therefore, in this system, inositol is comparable to mannitol, whether for PGCs or blastomeres.

However, the percentages of fusion are significantly lower using PGCs compared to blastomeres, and similarly high rates were obtained for blastomeres by Prather *et al.* (1989a). Reasons for the lowered percentages of fusion could include: that cells were smaller than blastomeres (approximately 15 μm, compared with 30-40 μm for blastomeres), and would present a lower surface area for contact and need a different field strength; the cells were freeze-thawed and used within 3 h, and appeared viable, but may have had defective membranes which would affect fusion. In fact, cryopreservation of PGCs may have affected their viability following nuclear transfer. However, the advantage gained by cryopreservation is that a single batch of germ cells was used for the duration of these experiments. This is the first report of the use of electrofusion with PGCs in the pig, and use of freeze-thawed PGCs.

Nuclear swelling was achieved using PGCs in 55% of nuclear transfer embryos. It is generally considered that the phenomenon of nuclear swelling (or remodelling) is one essential component of the more complex process of genetic reorganisation (or reprogramming). Indeed, as suggested by Prather *et al.* (1990), this may not necessarily be a sufficient indicator of developmental potential in a reconstructed embryo. The results suggest that the earliest morphological change in the PGC nucleus after nuclear transfer, nuclear swelling, is similar to that of blastomeres.

It is considered that processing of a transplant nucleus from blastomeres in an *in vitro*-matured pig oocyte is influenced by the maturation stage of the oocyte, and the interval between activation and fusion (Stumpf *et al.*, 1993). Artificial activation of oocytes results in the release from meiotic block via the inactivation of MPF, which corresponds to histone H1 kinase activity (Collas *et al.*, 1993). MPF is responsible for germinal vesicle breakdown and PCC and the decline of MPF activity is followed by pronuclear formation. As observed by Kono *et al.* (1994) in the cattle using 16-32-cell embryos as karyoplasts, swelling of the donor nucleus was greater in the aged (MII-phase) than in

TABLE 2

FUSION AND REMODELLING OF NUCLEAR TRANSPLANT EMBRYOS DERIVED FROM PGCS[1)]

Stage of cytoplasts	No. of complexes	No. of fused (%)	PN (pronuclear) swelling		
			No. examined	No. with swollen PN (%)	Relative degree of swelling [2)] (x±SD)
S	63	22 (34.9%)	22	12 (54.5%)	1.5±0.1
MII	154	54 (35.1%)	41	23 (56.1%)	2.2±0.4

[1)]Electrofusion medium (EFM) used was 0.28 M mannitol based medium. [2)]Based on the original diameter of PGC nuclei (as 1.0).

TABLE 3

FUSION AND EARLY DEVELOPMENT OF NUCLEAR TRANSPLANT EMBRYOS DERIVED FROM BLASTOMERES AND PGCs

Donor	Cytoplasts	Medium	No. of complexes	No. of fused (%)	No. of cleaved (%) at 24h	Development at 72h *in vitro* culture	
						No.(%)	Stage
Blastomere	S	EFM	42	34 (81%)[a1)]	14/26* (53.8%)	10 (38.5%)[ac2)]	2-3 cells
	MII	EFM	20	16 (80%)[a]	8 (50%)	12 (75%)[b]	2-4 cells
PGC	S	EFM	46	12 (26.1%)[b]	5 (41.7%)	-	-
		Inositol	49	18 (36.7%)[b]	8(44.4%)	5 (27.8%)[c]	2-4 cells
	MII	EFM	38	15 (39.5%)[b]	3/8*(37.5%)	3 (37.5%)[ac]	2-4 cells
		Inositol	32	10 (31.3%)[b]	6 (60%)	6 (60%)[ab]	2-3 cells

[1)]Values in the same column with different superscripts significantly differ ($p<0.05$). [2)]Based on fused complexes. *The number used for further culture.

the activated (S-phase) oocytes, where MPF levels are low. The results presented here using germ cells corroborate the theory that the swelling of the donor nucleus is decreased in the activated (S-phase) ooplast, without compromising early cleavage division. The results also suggest that moderate swelling of the donor nucleus is sufficient to reprogramme and support early development in the nuclear transfer embryo. The S-phase cytoplast is considered to be optimal , and therefore is regarded as the "universal" recipient (Barnes *et al.*, 1993; Campbell *et al.*, 1993, 1994). In these nuclear transfer experiments using early embryos, the cell cycle effect and the importance of normal ploidy was stressed. Nonetheless, the integrity of the nuclear membrane was maintained in the S-phase cytoplasts and no PCC was observed. It can, therefore, be anticipated that in these embryos immediate and extensive nuclear remodelling and reprogramming is limited. It was also reported that PCC of the donor nucleus in nonactivated cytoplasm is an important process for the development of nuclear transplant embryos to term (Cheong *et al.*, 1994). It may be necessary for the nuclei from PGCs to be remodelled extensively in MPF high cytoplasts to obtain better development as PGCs are at more advanced developmental stage than early embryos. According to results presented here, using PGCs and blastomeres, similar developmental capacity was obtained at the early stages after 72 h in culture, before the maternal-zygote transition (at 4-cell stage), whether the cytoplast was S- or MII-phase. However, a high developmental rate was obtained when MII-phase cytoplasts were used. It would be of interest to compare developmental potential at later stages *in vivo*.

Concerning the rates of cleavage at 24 h obtained using blastomeres, the results are comparable to those obtained by Saito *et al.* (1992) in the pig: however, higher rates of cleavage were obtained in this study, after 72 h of culture *in vitro*, without co-culture, compared with 29% *in vivo* by Saito *et al.* (1992). Nevertheless, the activation rate is different in that 68% activation rate was obtained, similar to that reported by Prather *et al.* (1991). Here the activation rate was on average 85%. This may be a factor leading to our higher rates of cleavage. In our experiments we obtained similar cleavage rates irrespective of whether blastomeres or PGCs were used as karyoplasts. After 72 h of culture, both types of embryos can develop to the 2 to 4 cell stage, albeit at different rates. Here we show that successful nuclear remodelling and early cleavages can be achieved using cryopreserved porcine PGCs, to a degree which resembles that achieved using blastomeres, and which therefore are a suitable source of karyoplasts for further studies in development following nuclear transfer.

Materials and Methods

Source of recipient oocytes

Ovaries were obtained from a local abattoir, and transported to the laboratory in Dulbecco's PBS (DPBS) at 25°C. Ovaries were washed once in 0.04% cetyltrimethylammoniumbromide ("Cetab", Sigma Chemical Co.), and then in DPBS three or four times. Follicles were collected from the ovaries by dissection at room temperature in dissection medium (DM), consisting of Medium 199 with 25 mM Hepes (GibcoBRL) supplemented with 1 mg/ml PVA, and 75 µg/ml kanamycin. Only those follicles having diameters of 3-7 mm, containing transparent fluid, and with apparent vessels in the follicular wall, were selected. The follicles were opened using a pair of watch forceps, to release the cumulus-enclosed oocyte complex. Maturation *in vitro* was conducted in 35 mm plastic tissue-culture dishes containing 2 ml of culture medium (CM) and 30 µl of Pergonal Menotrophin Hormone (Serono, UK) preparation, which was reconstituted in DM to give 20 IU/ml FSH and 20 IU/ml LH. Each dish contained one or two everted follicle shells (4-6 mm diameter), and 20-30 cumulus-oocyte complexes. Maturation was performed at 38.5°C in 5% CO_2 in humidified air, with gentle agitation (Staigmiller and Moor, 1984). The CM was Medium 199 (Gibco) supplemented with 2.2 mg/ml $NaHCO_3$, 75 µg/ml kanamycin, 10% heat-inactivated fetal bovine serum (FBS: Imperial, UK), and 2.5% of an additive solution containing 220 mg glutamine, 150 mg ascorbic acid, 10 mg inositol, 220 mg sodium pyruvate, 20 mg transferrin, 20 mg insulin, 4 mg vitamin B_{12}, and 160 µl acetic acid dissolved in 50 ml of spectrum water.

Source of blastomeres

Large White pigs were used as sources of 4-8-cell embryos. Embryos were collected from mated animals at 4 d after onset of oestrus, by perfusion of the oviducts with Ovum Culture Medium (ICN-Flow).

Source of PGCs

A pregnant gilt at 26 days of gestation was sacrificed and the uterus removed. Fetuses were dissected from the uterus, and genital ridges isolated. At this stage the sex of the fetuses was indistinguishable by morphological criteria of the genital ridges, as the primordia are not yet dif-

ferentiated. The genital ridges were pooled and disaggregated to single-cell suspensions using 1% collagenase and 0.25% (w/v) trypsin in 0.04% (w/v) EDTA (Sigma Chemical Co.), and frozen as suspensions in liquid nitrogen for long-term storage, using freezing medium consisting of 10% fetal calf serum and 10% DMSO in Dulbecco's Modified Eagles Medium. Cryopreservation of genital ridge suspensions was carried out using a refrigerator and a deep-freeze system (-70°C), and finally in liquid nitrogen. Following freeze-thawing of the aliquot suspensions, PGCs were collected using a drawn-out Pasteur pipette, being readily distinguishable from somatic cell types by their characteristic morphology, including refractility and pseudopodia formation (Leichthammer *et al.*, 1990).

Micromanipulation

Following maturation *in vitro*, oocytes were stripped of the surrounding cumulus cells by pipetting, and washed three times in DM. Those oocytes having one polar body, and therefore corresponding to metaphase II (MII), were identified, collected and maintained at 38°C in DM. The procedures of enucleation of oocytes and nuclear transfer were essentially as described for embryo cloning (Yang *et al.*, 1992), except that PGCs were employed as karyoplasts. In brief, the metaphase II oocytes were incubated in Hepes-buffered M199 containing 7.5 µg/ml cytochalasin B (CB; Sigma Chemical Co.), for 15 min prior to enucleation. Enucleation was performed by aspiration using a sharp, bevelled micropipette to remove the polar body together with the adjacent cytoplasm (constituting 20-25% of the oocyte's volume), and the manipulated oocytes were incubated for 1 h, then stained with 10 µg/ml Hoechst 33342 for 10 min and viewed by fluorescence microscopy (for less than 5 sec), to check the efficiency of enucleation. This limited dose of ultraviolet irradiation has been shown to have no harmful effect on the development of embryos following nuclear transfer (Westhusin *et al.*, 1992; Keefer *et al.*, 1993). Those completely-enucleated oocytes were used as recipient cytoplasts for PGCs. The presumptive S-phase cytoplasts were obtained after 5 h of electrical activation (see Results and Discussion). Individual, intact PGCs were placed in the perivitelline space of, and in close contact with, the cytoplasts using the enucleation pipette through the openings made during enucleations. The oocyte-PGC complexes were incubated pending either electrofusion or electrofusion/activation, when MII cytoplasts were used.

A proportion of non-manipulated oocytes were electrically stimulated, cultured and examined for pronuclear formation.

Electrofusion and activation

The electrofusion medium (EFM) was 0.28 M mannitol or inositol (as indicated in the results section), supplemented with 100 µM $CaCl_2$, 100 µM $MgSO_4$ and 10 mM histidine (pH 7.0). Prior to electrical stimulation, the oocyte-PGC complexes were incubated in DM containing PHA (250 µg/ml) for 1-2 min, and in DM: EFM (1:1) for 1-2 min, and then were equilibrated in EFM for 10 min. After three washes in this medium, complexes were placed in the electrofusion chamber and aligned manually with the boundary between cytoplast and PGC parallel to the two platinum electrodes. The electrodes had a diameter of 200 µm, were 500 µm apart, and were covered with the EFM. An initial pulse of alternating current at 5V and 600 KHz was applied for about 5-10 sec, followed by two or three pulses of direct current (DC) at 1.0 KV/cm, for a duration of 50 µsec, at 5 min intervals (using 2 pulses for fusion alone, and three pulses for activation). Electrical current was provided by a Zimmermann Cell Fusion power supply. After pulse treatment, oocyte-complexes were washed in DM and cultured in CM at 38.5°C. Complexes were examined for successful fusion 1.5 h after electrical stimulation.

Culture, and assessment of activation and developmental potential in vitro

In vitro culture was in 2 ml of CM in humidified air at 38.5°C. After 15-16 h of culture, the fused embryos were stained with 10 µg/ml Hoechst 33342 for 10 min, and observed for nuclear swelling by fluorescence microscopy. In order to assess developmental potential, oocytes were examined every 24 h for 3 days in total. At 72 h of culture, the number of nuclei in nuclear transfer embryos were monitored following Hoechst staining, by fluorescence microscopy.

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