Nuclear transplantation of embryonic stem cells in mice

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The developmental ability of enucleated mouse eggs that had received embryonic stem cells was examined. In a preliminary study, none of the reconstituted eggs formed a nucleus using inactivated Sendai virus (HVJ) after activation with ethanol. DC pulses were applied at 100 or 140 V mm⁻¹ in addition to this treatment. After electrofusion, 29–40% of reconstituted eggs formed nuclei and 41–60%, 25–44%, 12–24% and 12–18% developed to two-cell and four-cell and morulae and blastocysts, respectively. The ability of reconstituted eggs to form blastocysts did not depend on the age of recipient eggs, except in cases of very young and very old eggs. Although implantation sites were observed, no live fetuses were obtained after the transfer of reconstituted eggs to the recipients.

Introduction

Embryonic stem cells can be established from blastocysts and maintained in culture *in vitro* as a pluripotent cell line. Embryonic stem cells have been injected into blastocysts or aggregated with eight-cell embryos to produce chimaeras (Bradley *et al.*, 1984; Lallemand and Brulet, 1990) and some are germ-line chimaeras. Although embryonic stem cells have thus been effectively used for introducing genes into animals (Robertson *et al.*, 1986), it takes a long time to obtain homologous transgenic animals since at least two matings are necessary. Nagy *et al.* (1990) reported that live fetuses with entirely embryonic stem cell genotype were obtained by aggregation with tetraploid embryos, but died shortly after birth. If embryonic stem cells develop directly into young rather than through chimaeras, if they are totipotent, it should be possible to shorten the time required for obtaining transgenic mice. Moreover, the individuals obtained should be a clone.

The present study was undertaken to examine the developmental ability of nuclear transferred mouse eggs with embryonic stem cells.

Materials and Methods

Preparation of ES cells

The ES cell line (F1/1) used in the present study was established (Tokunaga and Tsunoda, 1992) using the technique described by Doetschman *et al.* (1985) and contained euploid XY chromosomes. The blastocysts obtained on day 3.5 after coitus from C57BL/6 strain female mice mated with CBA males were used to establish the ES cell line. The cells were frozen at –80°C or in liquid nitrogen after 11–20 passages. The frozen-thawed cells were washed and cultured for 1 to 2 days before use. The cells were treated with trypsin and gently disaggregated into single cells before nuclear transplantation. The ES

cells at the same number of passages aggregated with eight-cell embryos to produce chimaeras at high frequency (more than 50%) and most were germ-line chimaeras (Kato and Tsunoda, unpublished).

Nuclear transplantation

Unfertilized eggs were obtained from superovulated F₁ (C57BL/6 × CBA) females 15–17 h after injection of 5 iu hCG. The zonae pellucidae of eggs were cut along 10–20% of their circumference as described by Tsunoda *et al.* (1988). The eggs were stained with Hoechst 33342 (2 µg ml⁻¹) for 5 min and incubated with 5 µg cytochalasin B ml⁻¹ and 3 µg nocodazole ml⁻¹ (Aldrich Chemical Company, WI) for 15 min. The second metaphase chromosomes were mechanically removed under a fluorescence microscope within 15 s according to the procedures reported by Tsunoda *et al.* (1988). By these procedures, the chromosomes could be completely removed and the first polar body was also removed.

In a preliminary study, a single embryonic stem cell (Fig. 1) was fused with enucleated eggs at the second metaphase by inactivated Sendai virus (HVJ) as described by Tsunoda *et al.* (1989). However, none of the eggs formed a nucleus after activation with ethanol. Phytohaemagglutinin (PHA) was used to facilitate fusion of embryonic stem cells with eggs; a single embryonic stem cell was drawn into an injection pipette, moved to PHA solution in M2 (Fulton and Whittingham, 1978) and washed in and out several times before drawing a small volume of HVJ (2600 haemagglutinating activity units ml⁻¹) (Kato and Tsunoda, 1992). Activation was induced within 30 min after embryonic stem cell transfer by exposure to 7% ethanol for 7 min at room temperature (Cuthbertson, 1983). Enucleated eggs with an ES cell were subjected to electrical stimulation to facilitate the fusion and activation of fused eggs. The nuclear transferred eggs were equilibrated in Zimmerman's cell fusion medium (0.28 mol sucrose l⁻¹, 0.5 mmol Mg (C₂H₃O₂)₂ H₂O l⁻¹, 0.1 mmol Ca (C₂H₃O₂)₂ l⁻¹, 0.1 mmol K₂HPO₄ l⁻¹, 0.1 mmol glutathione l⁻¹, 0.01 mg BSA ml⁻¹ and 1 mmol K₂HPO₄ l⁻¹ (Wolfe and Kraemer, 1992) and transferred to a drop of fusion

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Table 1. Effects of intensity of DC pulses on activation and the development of enucleated mouse eggs receiving embryonic stem cells

Voltage of pulses (V mm ⁻¹)	Duration of pulses (μs)	Number of eggs used	Number of eggs with a nucleus (%)	<i>In vitro</i> development of eggs with nucleus (%)	
				2-cell	Blastocyst
100	30	62	26 (42)	10 (38)	4 (15)
	50	100	22 (22)	11 (50)	6 (27)
140	30	56	15 (27)	10 (67)	1 (7)
	50	94	19 (20)	10 (53)	3 (16)
	100	55	10 (18)	6 (60)	0

Table 2. Effects of age of recipient egg on the activation and development of enucleated mouse eggs receiving embryonic stem cells

Age of oocytes (h after hCG)	Number of eggs with nucleus/Total (%)	Number of eggs (%) with					<i>In vitro</i> development of eggs with nucleus(ei) (%)			
		1 nucleus	1 nucleus with polar body	2 nuclei	Immediate cleavage	Fragment	2-cell	4-cell	Morula	Blastocyst
13–14	6/111 (5)	4 (67)				0	1 (25)	0	0	0
			2 (33)			0	0			
18–19	45/154 (29)	25 (56)				0	11 (44)	9 (36)	4 (16)	3 (12)
			19 (42)			0	15 (79)	11 (58)	7 (37)	5 (26)
				1 (2)		0	1 (100)	0		
25–26	60/158 (38)	44 (73)				0	12 (27)	10 (23)	4 (9)	4 (9)
			12 (20)			0	9 (75)	3 (25)	2 (17)	2 (17)
				3 (5)		0	3 (100)	3 (100)	2 (67)	1 (33)
					1 (2)	0	—	1 (100)	0	
25–26*	213/556 (38)	71 (33)				0	19 (27)	8 (11)	6 (8)	5 (7)
			133 (62)			0	70 (53)	42 (32)	28 (21)	23 (17)
				4 (2)		0	4 (100)	4 (100)	3 (75)	1 (25)
					5 (2)	0	—	0		
28–29*	25/63 (40)	12 (48)				0	3 (25)	3 (25)	1 (8)	1 (8)
			13 (52)			0	10 (77)	5 (38)	2 (15)	2 (15)
31–32*	7/95					59 (62)	0			
41–42*	7/111					41 (37)	0			

The table shows the number of eggs at each specific stage of development as the proportion of eggs in which nuclei were observed 6–7 h after fusion.

*These oocytes were recovered and enucleated at 21 to 22 h, but used at the time indicated.

medium in a fusion chamber composed of two wire electrodes mounted on a glass slide 1000 μm apart. The enucleated eggs and embryonic stem cell were aligned by pipetting and fusion was induced by DC pulses of 100 or 140 V mm⁻¹ for 30, 50 or 100 μs. The enucleated eggs with ES cells were then washed with M2 and cultured in M16 medium (Whittingham, 1971) in 5% CO₂ and 95% air at 37°C. They were again subjected to the same DC pulse 1 h after the first treatment (Collas and Robl, 1991) and then cultured for 5 to 6 h and examined for the formation of a nucleus. Eggs with a nucleus(ei) were further cultured for 4 to 5 days. The development of nuclear transferred eggs was examined daily to determine the number of eggs at specific stages of development.

To examine the effects of ageing of recipient oocytes, the following oocytes were used: those recovered at (1) 13–14 h, (2) 18–19 h, (3) 25–26 h after injection of hCG and immediately enucleated; those recovered and enucleated at 21 to 22 h after hCG but used as recipient oocytes at (4) 25–26 h, (5) 28–29 h, (6) 31–32 h and (7) 41–42 h after hCG injection.

Some eggs that developed to the blastocyst stage were transferred to the oviducts of day 1 pseudopregnant CD-1 strain females and killed on days 12.5 and 18.5 to determine the presence of implantation sites and fetuses, or were transferred to pregnant CD-1 strain females mated with F₁ (C57BL/6 × CBA) males to monitor the success of pregnancy (McLaren and Michie, 1956), which was subsequently allowed to proceed to term.

Fig. 1. (a) ES cells before nuclear transfer are shown. (b) and (c) Enucleated eggs at the second metaphase are fused with a single ES cell, and one nucleus was formed without (b) or with (c) a polar body. (d–h) Development *in vitro* of enucleated eggs receiving an ES cell is shown: (d) two-cell stage; (e) four-cell stage; (f) eight-cell stage; (g) morula and (h) blastocyst.

Statistical analysis

The data were analysed by χ^2 analysis.

Results

Eggs exposed to DC pulses at 100 V mm^{-1} for $30 \mu\text{s}$ were activated more frequently ($P < 0.01$ – 0.001) than those in the other groups (42% versus 18 to 27%, respectively) (Table 1). The proportion of eggs the embryonic stem cells of which were degenerated was high at 140 V mm^{-1} DC pulses applied for 50

and $100 \mu\text{s}$. The proportion of eggs that had ES cells and developed into blastocysts was not significantly different except in cases of treatment with 140 V mm^{-1} for $100 \mu\text{s}$.

The proportion of eggs with a nucleus(ei) was low in eggs used at 13–14 h after hCG, but not different from those used at 18–29 h after hCG (Table 2). A large proportion of reconstituted eggs used at 31–32 h and 41–42 h after hCG injection degenerated 5–6 h after activation. The proportion of reconstituted eggs that had two nuclei or showed immediate cleavage was low in all groups. The proportion of eggs that formed one nucleus with one polar body (Fig. 1b) was higher in groups IV and V than in groups I–III. The ability of reconstituted eggs with a polar body to develop to blastocysts was high in all groups (15–26%) compared with that of eggs without a polar body (Fig. 1c to 12%). Twenty-three blastocysts that developed from eggs that received ES cells were transferred to fifteen pregnant CD-1 females mated with the same strain males but none produced young from reconstituted eggs. Eighteen reconstituted blastocysts were transferred to four pseudopregnant recipients and one had two implantation sites but no fetus on day 18.5. Fifteen reconstituted blastocysts were also transferred to five pseudopregnant recipients and three of eight had implantation sites, but no fetuses could be detected on day 12.5.

Discussion

In a preliminary study, the nuclear transfer of embryonic stem cells into enucleated eggs was conducted by the same procedures as those for mouse primordial germ cells (Tsunoda *et al.*, 1989; Kato and Tsunoda, 1992). However, none formed a nucleus by HVJ following activation with ethanol. Electrofusion in addition to HVJ and ethanol treatment was thus conducted.

After electrofusion, 29–40% of enucleated eggs that had received embryonic stem cells formed a nucleus and 41–60%, 25–44%, 12–24% and 12–18% developed to the two-cell stage, four-cell stage, morula and blastocyst, respectively. As far as we know, this is a first report on nuclear transfer of mouse embryonic stem cells. The chromosomes at the second metaphase could be completely removed (Tsunoda *et al.*, 1988), thus ruling out the possibility that the blastocysts were of parthenogenetic origin.

Live fetuses were not obtained after transfer of reconstituted blastocysts to pregnant or pseudopregnant recipients. In the present study, only three to five eggs were transferred to each pseudopregnant recipient owing to the limited number of blastocysts that could be obtained in one day. However, this should not have affected their developmental potential, since McLaren (1970) reported that the number of eggs transferred into pseudopregnant mice (in the range 2–12) had no effect on the number that implanted. Although 70% of the embryonic stem cells, F1/1, had 40 chromosomes (Tokunaga, unpublished), chromosome abnormality of embryonic stem cells may explain the low ability of eggs to develop into blastocysts *in vitro* and the failure of enucleated eggs receiving embryonic stem cells to form fetuses. Although it is possible that there are irreversible epigenetic differences among nuclei of various stages, the most plausible explanation for failure would be the inadequency of nuclear transfer techniques. From previous studies (Tsunoda *et al.*, 1987a,b) in which young were obtained after transfer of

isolated blastomeres of eight-cell mouse embryos aggregated with parthenogenone or enucleated two-cell embryos receiving the nucleus of eight-cell embryos, it is clear that nuclei of eight-cell mouse embryos are totipotent. However, the fact that enucleated mouse unfertilized eggs that had received a nucleus from an eight-cell embryo did not develop into blastocysts (Tsunoda *et al.*, 1989; Kono *et al.*, 1991) indicates that the physiological conditions of the nucleus and cytoplasm were not suitable in the mouse nuclear transfer system. Totipotency of inner cell mass nuclei has not been confirmed in mice, although a few enucleated eggs receiving inner cell mass nuclei have been shown to develop to blastocysts (Tsunoda *et al.*, 1989; Kono *et al.*, 1991).

In contrast to mouse eggs, enucleated sheep eggs fused with inner cell mass cells of blastocysts developed into lambs after transfer to recipients (Smith and Wilmut, 1989). The finding that enucleated mouse eggs receiving ES cells developed into blastocysts indicates that enucleated sheep eggs reconstituted with ES cells, if established, could possibly develop into young.

The proportion of reconstituted eggs receiving ES cells that developed into blastocysts did not depend on the age of a recipient egg, except in cases of very young and very old eggs. In contrast to the present results, the developmental ability of enucleated bovine oocytes receiving a blastomere of eight- to 32-cell stage embryos was high when aged oocytes were used (Yang *et al.*, 1991; Leibfried-Rutledge *et al.*, 1992). Although the reason for this discrepancy is not clear, differences in the duration of each stage of the cell cycle between mouse ES cells and bovine embryonic nuclei may be a partial explanation. The importance of synchronization of the cell cycle between the donor nucleus and the recipient cytoplasm has been pointed out in the development of nuclear transferred eggs (Smith *et al.*, 1988; Collas *et al.*, 1992a,b). Collas *et al.* (1992a) reported the enhanced rate of development of enucleated rabbit eggs receiving 32-cell stage nucleus to blastocysts with donor nuclei in the G1 phase (71%), as opposed to the late S phase (15%). An unsuitable stage of the cell cycle of a donor nucleus has also been shown to lead to abnormal chromosome constitution in nuclear transferred eggs (Collas *et al.*, 1992b). Techniques for the development of reconstituted eggs with ES cells *in vitro* and for obtaining young following transfer to recipients should be improved.

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