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Effects of Aggregation of Nuclear-transferred Mouse Embryos Developed from Enucleated Eggs Receiving ES Cells on *In Vitro* and *In Vivo* Development

Akiko YABUUCHI¹⁾, Yoko KATO¹⁾ and Yukio TSUNODA¹⁾

¹⁾Laboratory of Animal Reproduction, College of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan

Abstract. Nuclear-transferred mouse eggs receiving embryonic stem cells develop into blastocysts at a high rate, but their potential to develop into fetuses is very low. Therefore, the present study examined the effects of aggregating of two nuclear-transferred eggs at the 8-cell stage to increase the cell number of nuclear-transferred embryos. The proportion of implantation sites on gestational day 10.5 after transfer of aggregated nuclear-transferred embryos (53%) was significantly higher than after single nuclear transfer (29%) or serial nuclear-transfer (37%). The proportion of implantation sites with fetuses did not differ significantly among the three groups (7 to 17%). These results demonstrate that the low potential of nuclear-transferred embryos with embryonic stem cells to develop into fetuses is not due to the lower cell number of nuclear-transferred embryos.

Key words: ES cells, Nuclear transfer, Serial nuclear transfer, Aggregation

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Cloned mice were recently produced after nuclear transfer of embryonic stem (ES) cells [1-7]. The potential of nuclear-transferred eggs receiving ES cells to develop into blastocysts *in vitro* is relatively high (11 to 86%), but the proportions of live young that are born after transfer to recipients are low, and there are large variations depending on the nuclear transfer procedure used and the genetic background of the ES cells (1 to 21%). Serial nuclear transfer of ES cells by membrane fusion into unfertilized eggs and again into fertilized 2-cell embryos increased the probability of developing into blastocysts [3].

Due to the abnormal expression of imprinted genes [6], the placentae of cloned mice are two to three times heavier than those of controls, a large proportion of young die soon after birth due to respiration problems [1-4]. The rate of postnatal

death is low if ES cells with a hybrid genetic background are used as nuclear donors, although the reason for this is not clear [2, 5]. The developmental potential of nuclear-transferred eggs receiving ES cells with a hybrid genetic background, however, is higher than those with an inbred background [5]. The preimplantation embryos from an inbred mouse strain are generally more sensitive than those from F1 strain mice, as demonstrated by the "*in vitro* 2-cell block" phenomenon [8]. The low potential of cultured embryos with an inbred background to develop into young might be due to the lower cell number of the embryos compared with F1 embryos [9]. Zhou *et al.* (2001) reported that the cell number of blastocysts that developed from nuclear-transferred eggs with ES cells was lower than that of blastocysts fertilized *in vivo*. In the present study, we aggregated two nuclear-transferred embryos to increase the cell number and compared

this procedure with single and serial nuclear transfer with regard to their potential to develop into fetuses.

Materials and Methods

Donor embryonic stem cells

The ES cell line, established from a male blastocyst of ROSA 26 transgenic mice [10] with a nonalbino inbred 129/sv background, was used at passages 18 to 26.

Nuclear transfer

Chromosomes at the second metaphase of unfertilized eggs recovered from superovulated F₁ (C57BL/6 × C3H) females were mechanically removed, and the eggs were used as the recipient eggs [11,12]. A single metaphase ES cell synchronized with nocodazole treatment [3] was fused with an egg from which the maternal chromosomes had been removed using inactivated Sendai virus (HVJ). Fused eggs were cultured for 1 h in M16 medium [13] supplemented with cytochalasin B and then cultured in Ca²⁺-free M16 medium containing 10 mM Sr²⁺ for 5 h. Nuclear-transferred eggs with one pronucleus and a second polar body were cultured for 1 or 4 days to the 2-cell or blastocyst stage in M16 medium.

Serial nuclear transfer and aggregation of embryos

Serial nuclear transfer at the 2-cell stage was performed in some of the experiments according to previous reports [11, 12]. Briefly, when the nuclear-transferred eggs developed to the 2-cell stage, the nuclei of the embryos were fused with enucleated blastomeres of fertilized 2-cell embryos using inactivated Sendai virus (HVJ). Reconstituted embryos were cultured *in vitro* for 3 days. When nuclear-transferred eggs developed to the 8-cell stage, the zona pellucidae of the embryos were removed by treatment with 0.5% pronase, and two embryos were aggregated and cultured for 2 days.

Cell number of blastocysts and embryo transfer

The number of inner cell mass (ICM) cells and trophectoderm (TE) cells of the blastocysts that developed after single nuclear transfer, serial nuclear transfer, and aggregation were individually counted using double staining methods [14]. Briefly, zona pellucida-free

blastocysts were incubated in M2 medium [15] supplemented with 10% rabbit anti-mouse serum for 30 min, and then incubated in M2 with guinea pig serum at a 1:5 dilution, propidium iodide (10 µg/ml), and Hoechst 33342 (10 µg/ml) for 30 min. Treated embryos were examined under a fluorescent microscope to count the number of ICM cells (stained light blue) and TE cells (stained pink).

Nuclear-transferred eggs that developed into blastocysts were transferred to the oviducts of day 1, or uteri of day 2.5, pseudopregnant ICR strain albino females. Recipients were sacrificed on day 10.5 to examine the potential of the implants to develop into fetuses. Embryos were stained with X-gal staining to confirm their origin according to the procedures reported previously [16].

Statistical analysis

Data were analyzed using χ^2 analysis (Tables 1 and 3) and Student's *t*-test (Table 2). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows the *in vitro* development of nuclear-transferred eggs that received ES cells synchronized at the M phase. The proportion of fused eggs with ES cells was 82%, and most of the eggs formed one pronucleus and a second polar body after parthenogenetic activation (90%). Most of them (97%) cleaved into the 2-cell stage and were then divided into three groups. In the single nuclear transfer group, embryos were cultured for another 3 days and 59% of them developed into blastocysts. In the serial nuclear transfer group, karyoplasts from 350 nuclear-transferred 2-cell embryos were fused with enucleated blastomeres of fertilized 2-cell embryos. In 337 embryos (97%), 2 karyoplasts successfully fused with 2 blastomeres, but in 11 embryos (3%), a single karyoplast fused with one blastomere. Because the potential to develop into blastocysts was not significantly different between these two types of fusion (64% and 56%), the data were combined and summarized in Table 1 (63%). For the last group, 257 aggregates were successfully produced from 516 zona pellucida-free 8-cell embryos and 60% of them developed into blastocysts. The remaining aggregates stopped development at the morula

Table 1. *In vitro* development of nuclear-transferred eggs receiving ES cells

No. of eggs fused /used (%)	No. of eggs with one nucleus and polar body (%)	No. of eggs developed to 2-cell (%)	Type of nuclear-transfer	No. of eggs cultured	No. of eggs develop to (%)	
					morula	blastocyst
1383/1686 (82)	1245 (90)	1208 (97)	single	211	163 (77)	125 (59)
			serial	350	279 (80)	222 (63)
			aggregation	257	–	154 (60)

Table 2. Cell numbers of blastocysts developed from nuclear-transferred eggs

Type of nuclear-transfer	Cell numbers $\pm$ SD (No. of blastocysts)		
	inner cell mass	trophectoderm	total
Single	8.3 $\pm$ 2.0 ^a	29.1 $\pm$ 5.8 ^a	37.4 $\pm$ 6.7 ^a (n=10)
Serial	12.9 $\pm$ 2.7 ^b	32.0 $\pm$ 5.4 ^a	44.9 $\pm$ 7.6 ^b (n=8)
Aggregation	15.7 $\pm$ 5.2 ^b	38.0 $\pm$ 3.6 ^b	53.7 $\pm$ 7.3 ^c (n=6)

Values with different superscripts within the same column are significantly different ($P < 0.05$).

Table 3. *In vivo* development of nuclear-transferred eggs receiving ES cells

Type of nuclear-transfer	No. of embryos transferred	No. of embryos implanted (%)	No. of fetuses (%)	No. of fetuses with beating heart (%)
Single	62	18 (29) ^a	7 (11)	4 (6)
Serial	71	26 (37) ^a	5 (7)	3 (4)
Aggregation	77	41 (53) ^b	13 (17)	7 (9)

a and b are significantly different ($P < 0.05$).

stage. The potential of nuclear-transferred eggs to develop into blastocysts was not significantly different among the three groups.

As shown in Table 2, the ICM and TE number and the total cell numbers of blastocysts in the single nuclear transfer group were significantly lower than those of blastocysts in the aggregation group. The ICM cell number and total cell numbers of blastocysts in the serial nuclear transfer group were significantly higher than those in the single nuclear transfer group, but the TE cell number and total cell number in the serial nuclear transfer group were lower than those in the aggregation group.

The implantation rate of blastocysts on day 10.5 was significantly higher in the aggregation group than in the other two groups (Table 3). The proportion of implantation sites with fetuses, however, was not significantly different between

the groups. The proportion of fetuses with a beating heart and the average number of somites (23.6, 23.6, and 23.5 respectively) were not significantly different among groups. All fetuses were positive for X-gal staining.

Discussion

The hypothesis of the present study was that aggregation of two nuclear-transferred embryos would increase the potential to develop into fetuses/offspring, because blastocysts that develop from nuclear-transferred eggs with ES cells have lower cell numbers [7]. The present study demonstrated that the implantation rate of aggregated embryos on day 10.5 was significantly higher than that of embryos after single or serial nuclear transfer. The proportion of implantation

sites with fetuses, however, was not different among groups, and more than half of the fetuses did not have a beating heart. This is the first report to demonstrate that the low potential of nuclear-transferred eggs with ES cells to develop into fetuses/offspring is not due to the low cell numbers of the nuclear-transferred embryos. The proportion of fetuses after transfer of nuclear-transferred eggs with ES cells gradually decreased soon after implantation: 48% on day 6.5, 20% on day 8.5 (Amano, Kato, and Tsunoda, unpublished findings), and 11% on day 10.5 (present study). Although the nuclear-transferred eggs with ES cells developed normally into blastocysts at a high rate, the blastocysts did not develop into fetuses at a high rate. The inferior potential of both ICM cells and TE cells of nuclear-transferred blastocysts underlies the low developmental rate of nuclear-transferred eggs, because both tetraploid blastocysts injected with ICM cells of nuclear-transferred blastocysts and nuclear-transferred tetraploid blastocysts injected with ICM of diploid blastocysts have low developmental potential [17].

Humpherys *et al.* (2001) reported that imprinted gene expression, such as H19, Igf2, Peg1, Meg1, and Gapdh, in ES cells is extremely unstable during maintenance in culture and there are also variations of imprinted gene expression in healthy cloned mice, as in the case of the methylation pattern in somatic-cell cloned mice [18]. The low potential of nuclear-transferred eggs to develop into fetuses and into young might be due not only to a single gene modification but also to a cumulative effect of the modification of multiple genes. The extent and type of abnormal gene expression might determine the timing of the retardation of embryos during pregnancy. This might be why aggregated nuclear-

transferred embryos with a high ability to implant in the present study did not develop to fetuses at a high rate; gene modification was doubled due to the aggregation. Thus, if a primary culture of somatic cells were used as nuclear donors, the results might be different. The present study did not confirm the previous findings of Amano *et al.* (2001a), in which serial nuclear transfer of ES cells increased the potential to develop into blastocysts. This discrepancy might be due to differences in the ES cell lines used for nuclear transfer, the F2 genetic background in the previous report, and the inbred genetic background in the present study.

In contrast to ES cells with an inbred genetic background, the potential of nuclear-transferred eggs receiving ES cells with a hybrid genetic background to develop into fetuses/offspring was relatively high and the postnatal death rate was low, when a direct injection method in which a small volume of cytoplasm was incorporated into the recipient eggs [5] was used. To increase the viability of nuclear-transferred eggs, further methodological research into the effects of different ES-cell-lines will be required.

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