Generation of mice derived from embryonic stem cells using blastocysts of different developmental ages

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Abstract

We previously showed that increasing the cell number of host tetraploid (4n) embryos by aggregating multiple 4n embryos at two to four-cell stages can improve the birthrate of mice from embryonic stem cells (ES mice). In the present study, we assessed whether *in vitro* aged blastocysts (e.g., E4.5 or E5.5), where their cell number also increased with development, can be used as hosts for generating ES mice. As expected, the cell number of *in vitro* aged 4n blastocysts increased with development, i.e., 26.5 ± 2.4 , 49.6 ± 8.4 , and 84.9 ± 20.9 cells for E3.5, E4.5, and E5.5 respectively. Three independent ES cell lines were injected into 4n aged blastocysts, and their developmental ability was compared with that of E3.5 4n blastocysts commonly used for this procedure. We found that the birthrate of ES mice derived from E4.5 blastocysts were comparable with those of mice generated from E3.5 blastocysts. On the other hand, the birthrates decreased when E5.5 blastocysts were used. These results suggest that not only the cell number but also developmental age is important for producing ES mice. We also discuss a comparison of the present findings with those of our previous study, where ES mice were generated using an aggregation method employing the same ES cell lines.

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Introduction

The production of mouse chimeras is a common step in the establishment of genetically modified mouse strains. Chimeric mice are usually produced by injecting or aggregating embryonic stem (ES) cells into diploid (2n) host preimplantation embryos (Nagy *et al.* 2003) composed of modified donor cells and cells derived from the host embryo. The contribution of donor ES cells to the germ line of chimeric mice allows the generation of mouse strains carrying the haplotype of the ES cells. In general, the production of a mutant strain via this procedure is a time-intensive task, taking more than 12 months before adult mutants can be analyzed. To accelerate the production of mutant mouse lines, a tetraploid (4n) embryo complementation assay has also been established (Nagy *et al.* 1990, 1993). Since 4n embryos are not capable of completing normal development independently (Kaufman & Webb 1990, Eakin & Behringer 2003), ES cells aggregated with 4n embryos develop into conceptuses in which embryonic lineages are derived entirely from ES cells and extra-embryonic lineages arise largely from the 4n component (Nagy *et al.* 1990, 1993). Mice derived from ES cells (ES mice) show normal phenotypes, growth, and fertility (Nagy *et al.* 1993), indicating that ES mice can be used as founders for

establishing mouse lines. Because all germ cells in ES mice are derived from ES cells, germ line transmission of ES cells is easier in ES mice than in 2n chimeric mice. Thus, producing ES↔4n embryos would be effective for analyzing gene function *in vivo*.

Although the methodology for producing ES mice derived from ES cell lines was described more than a decade ago (Nagy *et al.* 1990, 1993), its application remains limited due to the extremely low frequency at which viable ES mice are recovered (Nagy *et al.* 1990, 1993). We previously used a technique where increasing the cell number of host 4n embryos enhanced the production of ES mice (Ohta *et al.* 2008). The important advantage gained from the improvement of this technique, which was achieved by increasing the cell number of host 4n embryos, was that it could be applied to any ES cell lines such as those commonly used for gene targeting. However, additional host 4n embryos are required in order to reconstruct ES↔4n chimeric embryos. If this required number can be reduced, the production of ES mice will become easier and more efficient than our previous procedure.

In the present study, we increased the cell number of host 4n embryos by prolonged *in vitro* culturing, and assessed the functional ability of these *in vitro* aged blastocysts to generate ES mice.

Figure 1 Blastocoel formation of 4n preimplantation embryos of different developmental ages. (A–C) Light micrographs of 4n preimplantation embryos of different ages; (A) E3.5 embryos, (B) E4.5 embryos, (C) E5.5 embryos. Scale bars = 200 μ m.

Results

Characterization of *in vitro* aged blastocysts

We initially characterized 4n blastocysts at different developmental ages (E3.5, E4.5, and E5.5) before the *in vitro* aged blastocysts were used to produce ES mice. We examined the blastocoel formation of 4n aged blastocysts, and the trophectoderm (TE) and inner cell mass (ICM) cell numbers of 4n aged blastocysts. At E3.5, approximately half of the 4n embryos were formed blastocoel (Fig. 1A; Table 1); therefore, we used and analyzed blastocoel forming E3.5 embryos in the present study. On the other hand, almost all 4n embryos at E4.5 and E5.5 formed a blastocoel (Fig. 1B and C; Table 1).

We then assessed the cell number of *in vitro* aged 4n blastocysts to examine whether this number actually increased. The total cell numbers of E3.5, E4.5, and E5.5 4n blastocysts were 26.5 ± 2.4 , 49.6 ± 8.4 , and 84.9 ± 20.9 respectively (Fig. 2; Table 2), showing an increase with development as expected. The TE cell number of E5.5 4n blastocysts showed an ~ 3.7 - and 1.8 -fold increase from those of E3.5 and E4.5 4n blastocysts respectively. On the other hand, no obvious difference was found in ICM cell number among the blastocysts (Table 2). Consistent with the results of a previous study (Liu *et al.* 2004), the ICM cells in E4.5 (not shown) and E5.5 (Fig. 2D–D'') 4n blastocysts were predominately stained by the POU5F1 antibody, suggesting that the specification and allocation of TE and ICM occurred normally in these 4n aged embryos. Thus, we succeeded in increasing the cell number of host 4n blastocysts with normal TE and ICM allocations by prolonged culturing.

Generation of ES mice using *in vitro* aged 4n blastocysts

To assess whether *in vitro* aged 4n blastocysts can produce ES mice, ES cells were injected into E3.5, E4.5, and E5.5 4n blastocysts. A total of ~ 100 reconstructed embryos were transferred for each donor and host developmental age. We generated ES mice from all three ES cell lines at ~ 2 – 7% using E3.5 host blastocysts (Table 3). The birthrate of ES mice derived from E4.5 blastocysts was similar to that of ES mice generated from E3.5 blastocysts, but was lower when E5.5 blastocysts were used (Table 3). We also confirmed the high contribution of ES cells in the progeny derived from 129B6F1G1 $\leftrightarrow$ E4.5 blastocysts by the presence of green fluorescent protein (GFP)-positive cells (Fig. 3), indicating that ES mice can be generated from E4.5 host

embryos. In addition, the frequency of abnormal pups was similar in both E4.5 and E3.5 blastocysts (Table 4). Thus, although the E3.5 and E4.5 4n blastocysts can generate ES mice, the production of such mice was not improved using E5.5 4n blastocysts even if its cell number was relatively higher than that of the other blastocysts. These results indicate that not only the cell number but also the developmental age of host 4n embryos is important for generating ES mice.

Implantation ability of *in vitro* aged 4n blastocysts and tracing of ES cells injected into host 4n blastocysts

Although our results indicate that the E5.5 4n blastocysts are not an effective means of producing ES mice, the underlying mechanisms are largely unknown. To address this issue, we assessed the implantation abilities of *in vitro* aged 4n blastocysts. Thus, 4n blastocysts were transferred into pseudopregnant females without the injection of ES cells. Since 4n embryos die during early embryonic development (Eakin *et al.* 2005), the analysis was performed at E7.5. The implantation abilities of the 4n embryos were estimated as the numbers of decidual and embryos recovered. As shown in Table 5, the implantation and developmental abilities of the E5.5 4n blastocysts were not lower than those of the other blastocysts examined. This suggests that the low birthrate observed for ES mice from E5.5 4n embryos is not related to the implantation and developmental abilities of the host 4n embryos.

Another possible reason for the low birthrate of ES mice from E5.5 4n blastocysts is that the ES cells injected into the E5.5 4n blastocysts do not contribute to the ICM. To assess the homing efficiency of ES cells into the ICM, ES cells that expressed GFP (129B6F1G1) were injected into E3.5, E4.5, and E5.5 4n blastocysts and these cells were analyzed 24 h later. Host ICM cells were identified based on morphology or immunofluorescence for POU5F1, which was visualized as red fluorescence.

Table 1 Blastocoel formation of preimplantation 4n embryos of different developmental ages.

E3.5	E4.5	E5.5
39/72 (54.2%)	72/72 (100%)	68/72 (94.4%) ^a

The numbers of blastocysts that form blastocoels are shown.

^aFour embryos degenerated at E5.5.

Figure 2 Immunostaining of 4n blastocysts of different developmental ages with CDX2 or POU5F1 antibodies. The 4n blastocysts of different developmental ages were stained with propidium iodide (PI; A–D) and CDX2 (A'–C'). Merged photographs are shown in (A''–D''); (A–A'') E3.5 blastocysts; (B–B'') E4.5 blastocysts; and (C–C'') E5.5 blastocysts. In D–D'', E5.5 blastocysts were stained with PI (D) or POU5F1 antibody (D'). (A–C'') Stacked serial images of the blastocysts obtained by confocal microscopy are shown. (D–D'') Images were taken under fluorescence microscopy. Scale bars=100 μm.

We found that ES cells injected into E3.5 4n or E4.5 4n blastocysts contributed significantly to the host ICM (Table 6 and Fig. 4A–A''). By contrast, there was a relative weak contribution of ES cells to the ICM in the case of E5.5 4n blastocysts (Table 6, Fig. 4B–B''). These results suggest that the low birthrate of ES mice from E5.5 4n blastocysts is due to inefficient ES cell contribution to the host 4n ICM.

Finally, we examined the differences between E4.5 and E5.5 4n blastocysts. It is known that the ICM differentiates into the primitive endoderm (PrE, the future

yolk sack) and epiblast (future embryo), at around E4.5 in the case of a normal embryo (Kunath *et al.* 2005). Therefore, we examined by immunofluorescence the expression of GATA4, which is one of the markers for PrE, in E4.5 4n and E5.5 4n blastocysts. As shown in Fig. 4, 6% (3/50) of the E4.5 4n blastocysts formed PrE, whereas ~60% (34/56) of the E5.5 4n blastocysts formed PrE. These results indicate that the ICM of E5.5 4n blastocysts has already differentiated into PrE and epiblast, and this may inhibit ES cell contribution to the ICM of E5.5 4n blastocysts.

Table 2 Cell number of 4n blastocysts of different developmental ages.

Type of embryo	Total cell number (PI staining)	TE cell number (CDX2 staining)	ICM cell number (total-TE)
E3.5	26.5 ± 2.4 ^a	20.3 ± 2.1 ^c	6.2 ± 2.1
E4.5	49.6 ± 8.4 ^{a',b}	42.6 ± 7.8 ^{c',d}	7.4 ± 6.7
E5.5	84.9 ± 20.9 ^{a',b'}	75.8 ± 16.3 ^{c',d'}	9.6 ± 7.8

a,a';b,b';c,c';d,d' *P*<0.05 (Student's *t*-test).

Table 3 Birthrate of embryonic stem cell (ES) mice using 4n blastocysts of different developmental ages.

Donor ES	Culture period of 4n blastocysts	Number of transferred embryos (ET)	Number of pups (%)	Imp*	Implantation rate (pup + imp/ET)
E14	E3.5	105	4 (3.8)	25	27.6%
	E4.5	105	4 (3.8)	48	49.5%
	E5.5	106	0	0	0
129B6F1G1	E3.5	105	2 (1.9)	19	20%
	E4.5	124	2 (1.6)	43	36.2%
	E5.5	104	0	0	0
GR14	E3.5	120	9 (7.5)	35	36.7%
	E4.5	104	11 (10.6)	42	51%
	E5.5	104	4 (3.8)	19	22.1%
Total	E3.5	330	15 (4.5) ^a	79	28.5%
	E4.5	333	17 (5.1) ^b	133	45%
	E5.5	314	4 (1.3) ^{a',b'}	19	7.3%

^{a,a',b,b'} $P < 0.05$ (Fisher's exact probability test). *Number of implantations.

Discussion

In the present study, we examined the ability of *in vitro* aged blastocysts to generate ES mice. We found that E3.5 and E4.5 4n embryos were similarly efficient in producing ES mice; however, the efficiency was decreased in E5.5 4n embryos (Table 3). Our previous study showed that a larger cell number of host 4n embryos improves the birthrate of ES mice (Ohta *et al.* 2008). In this report, the cell number of host 4n embryos was increased by aggregating multiple host 4n embryos at the two to four-cell stages, i.e., three two to four-cell stage 4n embryos were aggregated to generate one host 4n embryo. Although the E5.5 4n embryos showed an increase in cell numbers (Table 2) similar to the previous study, the use of E5.5 4n embryos was ineffective in producing ES mice. These results suggest that not only

the cell number but also the host embryonic stage is important for generating ES mice.

As stated above, the E5.5 4n blastocysts were inefficient at producing ES mice, even though the cell numbers in the blastocysts were increased (Tables 2 and 3). Interestingly, the implantation and developmental abilities of the E5.5 4n blastocysts were similar to those of the E3.5 4n or E4.5 4n blastocysts (Table 5), which indicate that the low efficiency of E5.5 4n blastocysts is not attributable to dysfunctions in implantation or subsequent development. To further this investigation, we carried out a cell tracing experiment to examine whether the ES cells injected into E5.5 4n blastocysts could contribute to the ICM. We found that the ES cells injected into E5.5 4n blastocysts made a relatively weak contribution to the ICM (Table 6 and Fig. 4A–B'''). These results suggest that the primary cause of inefficient production of ES mice from E5.5 blastocysts is inefficient homing of injected ES cells to the ICM, possibly due to the ICM status of the E5.5 4n blastocysts. As the ICM of E5.5 4n blastocysts has already differentiated into two lineages (epiblast and primitive endoderm; Fig. 4C–D'''), the formation of primitive endoderm may inhibit the integration of injected ES cells into the epiblast. Thus, although the cell numbers of the host 4n embryos are important to produce ES mice (Ohta *et al.* 2008), there is a time window for the integration of the ES cells into the host 4n epiblast. A potential strategy to increase the ES mouse producing ability of host 4n blastocysts is the addition of insulin to the culture medium, as it has been known to increase the cell numbers of 4n blastocysts (Koizumi & Fukuta 1996).

Our results showed that E4.5 4n blastocysts were similarly efficient as those of E3.5 in generating ES mice (Table 3). The advantage in using E4.5 blastocysts is that they can be injected at a more convenient time than E3.5 blastocysts. As shown in Fig. 1 and Table 1, almost all E4.5 4n blastocysts formed a blastocoel, whereas morula-stage embryos were still present in E3.5 4n

Figure 3 Generation of ES mice from E4.5 4n blastocysts. The 129B6F1G1 ES cell line expressing GFP (A and B) was injected into the blastocoel of E4.5 4n blastocysts. (C and D) ES mouse and placenta at 18.5 dpc delivered from 129B6F1G1 ↔ E4.5 4n embryos showing a light photograph (C) and its fluorescence image (D). High-intensity green fluorescence was found in the progeny. Scale bars = 100 μm.

Table 4 Characterization of newborn embryonic stem cell (ES) mice generated from 4n blastocysts of different developmental ages.

Type of host	BW (n) [†]	PW (n) [†]	Number of abnormal pups*				% Abnormal pups
			AH	LO	OELO	OEAH	
E3.5	1.60±0.44 (17)	0.27±0.08 (17)	1	2	–	–	17.6%
E4.5	1.55±0.22 (24)	0.24±0.05 (24)	–	1	–	2	12.5%
E5.5	1.88±0.6 (4)	0.24±0.06 (4)	–	1	1	–	50%

BW, body weight; PW, placenta weight. *OE, open eyelids; AH, abdominal hernia; LO, large offspring; OELO, pup showing both open eyelids and large offspring; OEAH, pup showing both open eyelids and abdominal hernia. [†]Values are expressed as mean±s.d. (g).

embryos. Although some of the ES mice derived from E4.5 4n embryos showed abnormalities such as abdominal hernia or large offspring syndrome (Table 4), the frequency of these defects was similar to that in ES mice generated from E3.5 4n embryos (Table 4), indicating that ES mice can be produced from both E4.5 and E3.5 4n embryos. Thus, our results demonstrate that the use of E4.5 embryos is effective in generating ES mice.

In general, ES mice can be produced using two methods (Nagy *et al.* 2003): injection of ES cells into 4n blastocysts (Wang *et al.* 1997) or aggregation of ES cells with 4n four-cell stage embryos (Nagy *et al.* 1990, 1993). In our previous study, we demonstrated that the use of multiple 4n host aggregates (e.g., three 4n embryos) is more effective in generating ES mice than the use of one or two 4n host embryos (Ohta *et al.* 2008). In this report, the birthrate of ES mice using E14 and 129B6F1G1 strains with three 4n embryos was 14.3 and 9.3% respectively (Ohta *et al.* 2008). As this birthrate was calculated based on the number of transferred ES↔3×4n embryos, that estimated from the number of embryos used was 4.8% for E14 and 3.1% for 129B6F1G1. In the present study, the birthrate of ES mice via blastocyst injection using E3.5 and E4.5 embryos was ~ 3.8% for E14 and 1.8% for 129B6F1G1 (Table 3). Thus, the production of ES mice based on the number of embryos employed using blastocyst injection is similar to that using multiple 4n embryos. These two techniques can be selectively used depending on the research purpose; for example, blastocyst injection is effective for assessing donor cell function since micromanipulation allows the selection of donor cells. On the other hand, the use of 3×4n

embryos is effective in producing ES mice because the procedure is easier to perform. Recently, another procedure for producing ES mice have been reported, in which ES cells were injected into 2n host four- to eight-cell stage embryos (Poueymirou *et al.* 2007). Although we could not compare the efficiencies of this and the above-described procedures, this method may also be suitable for the production of ES mice.

Materials and Methods

Mice

ICR mice were purchased from SLC (Hamamatsu, Japan). All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of the Laboratory Animal Experimentation of the RIKEN Kobe Institute.

ES cell lines

The ES cell line E14 (Hooper *et al.* 1987) was derived from the inbred mouse strain 129/Ola in 1985 by Dr Martin Hooper in Edinburgh, Scotland, and obtained through Dr Peter Mombaerts (Rockefeller University). 129B6F1G1 and GR14 are nuclear transfer-derived ES (ntES) cell lines (Wakayama *et al.* 2001) previously established in our laboratory using Sertoli cells from a 129B6F1 background with GFP (Ohta & Wakayama 2005) and tail tip cells of a male mouse (129BDF2; Wakayama *et al.* 2005) respectively, as donors for nuclear transfer. Karyotype analysis revealed that the ES cell lines used had the normal karyotype at the following percentages (number of metaphases with normal karyotype in parentheses): 46% (12/26) for E14; 35% (9/26) for 129B6F1G1; and 54% (14/26) for GR14.

Table 5 Implantation and developmental abilities of 4n blastocysts.

Type of embryo	Number of embryos transferred	Number of implantations (%) [*]	Number of embryos recovered (%) [*]
E3.5	83	46 (55.4)	5 (6.0)
E4.5	74	26 (35.1)	3 (4.1)
E5.5	116	63 (54.3)	10 (8.6)

The 4n blastocysts of different developmental ages were transferred into pseudopregnant females and assessed at E7.5. No significant difference was found ($P>0.05$; Fisher's exact probability test).

*Percentages relative to the number of embryos transferred were shown in parentheses.

Table 6 Homing efficiencies of embryonic stem (ES) cells for the inner cell mass (ICM) of host 4n blastocysts.

Type of embryo	Number of embryos examined	Number of embryos with GFP positive ES cells (%)	Number of embryos with ES cell contribution to the ICM (%)
E3.5	58	48 (82.8)	41 (85.4) ^a
E4.5	44	37 (84.1)	25 (67.6)
E5.5	52	43 (82.7)	13 (30.2) ^{a'}

^{a,a'} $P<0.05$ (Fisher's exact probability test).

Figure 4 Fate of ES cells injected into E4.5 or E5.5 4n blastocysts. ES cells that express GFP (129B6F1 ES cell line) were injected into E4.5 (A–A''') or E5.5 (B–B''') 4n blastocysts. At 24 h after ES cell injection, the blastocysts were stained with anti-POU5F1 antibody that was visualized with red fluorescence. (A and B) DIC images of E4.5 and E5.5 blastocysts. POU5F1 expression (A' and B') and injected ES cells (A'' and B'') are shown. (A''' and B''') Merged images of A' and A'', B' and B''. Arrows indicate endogenous ICM. In B''', ES cells are indicated as an arrowhead. (C–D''') Primitive endoderm formation by E4.5 and E5.5 4n blastocysts. (C and D) DIC images of E4.5 and E5.5 4n blastocysts. PI (C' and D') and GATA4 expression (C'' and D'') are shown. In C'' and D'', numbers of GATA4 positive 4n blastocysts are shown. (C''' and D''') Merged images of C' and C'', D' and D''.

ES cell culture conditions

ES cells were grown in KNOCKOUT Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich), 1000 units of leukemia inhibitory factor/ml (Invitrogen), and the following reagents: 1% penicillin–streptomycin (Invitrogen); 1% L-glutamine (Specialty Media, Phillipsburg, NJ, USA); 1% non-essential amino acids (Specialty Media); 1% nucleosides (Specialty Media); and 1% β -mercaptoethanol (Specialty Media). Cells were cultured in a gelatin-coated dish in feeder-free conditions and split 1:5 or 1:10 every 48 h.

Generation of ES $\leftrightarrow$ 4n embryos

Blastocysts with an ICR background were used as hosts for 4n embryos. Briefly, superovulated ICR females were mated with ICR males, and two-cell stage embryos were collected from the oviducts at 1.5 dpc. To generate 4n embryos, two-cell stage embryos were electroporated using an electro cell fusion system (Model LF101; Nepagene, Chiba, Japan). Fused embryos were selected and cultured in CZB medium (Chatot *et al.* 1989). Each ES cell line was used within three passages in our experiments. Approximately, 15 ES cells were injected into the blastocysts using a piezo-actuating micromanipulator, using essentially the same procedure as that reported by Wang *et al.* (1997). All blastocysts injected with ES cells were transferred to pseudo-pregnant ICR females at 2.5 dpc (10–16 chimeric embryos/

mouse), which were analyzed at 18.5 dpc. Body and placenta weight were assessed in newborn ES mice. ES mice with open eyelids, abdominal hernia, or large offspring (body weight more than 2.0 g) were categorized as abnormal pups.

Assessment of cell numbers of blastocysts

The total and TE cell numbers of blastocysts were determined. Propidium iodide (PI) staining and immunostaining for CDX2 were used to indicate the total and TE cell numbers respectively. The cell number for ICM was roughly estimated as the TE cell number subtracted from total cell number (total TE). Blastocysts were fixed with 4% paraformaldehyde, washed with PBS containing 1% BSA, and incubated with anti-CDX2 MAB (1:200; BioGenex, San Ramon, CA, USA). The primary antibody was visualized with goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). After immunostaining for CDX2, blastocysts were incubated in PBS containing 1 μ g/ml PI. Serial confocal images were taken using fluorescence confocal microscopy (Yamagata *et al.* 2005), and three-dimensional images of blastocysts were reconstructed using MetaMorph software (Universal Imaging Co., Downingtown, PA, USA). More than ten blastocysts were stained and the total (PI-positive) and TE (CDX2-positive) cell numbers were determined. Immunostaining for POU5F1 or GATA4 was performed employing the same procedure for the CDX2 staining using an anti-POU5F1 MAB (C-10; 1:200; Santa

Cruz Biotechnology Inc., CA, USA; catalog no. sc-5279) or anti-GATA4 polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog no. sc-9053).

Statistical analysis

The Fisher's exact probability test (Tables 3, 5, and 6) and Student's *t*-test (Table 2) were performed. $P < 0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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