Use of Coisogenic Host Blastocysts for Efficient Establishment of Germline Chimeras with C57BL/6J ES Cell Lines

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ABSTRACT

Gene targeting in embryonic stem (ES) cells allows the production of mice with specified genetic mutations. Currently, germline-competent ES cell lines are available from only a limited number of mouse strains, and inappropriate ES cell/host blastocyst combinations often restrict the efficient production of gene-targeted mice. Here, we describe the derivation of C57BL/6J (B6) ES lines and compare the effectiveness of two host blastocyst donors, FVB/NJ (FVB) and the coisogenic strain C57BL/6-Tyr$^{c\text{-}2J}$ (c^{2J}), for the production of germline chimeras. We found that when B6 ES cells were injected into c^{2J} host blastocysts, a high rate of coat-color chimerism was detected, and germline transmission could be obtained with few blastocyst injections. In all but one case, highly chimeric mice transmitted to 100% of their offspring. The injection of B6 ES cells into FVB blastocysts produced some chimeric mice. However, the proportion of coat-color chimerism was low, with many more blastocyst injections required to generate chimeras capable of germline transmission. Our data support the use of the coisogenic albino host strain, c^{2J}, for the generation of germline-competent chimeric mice when using B6 ES cells.

INTRODUCTION

The generation of gene-targeted mice by means of homologous recombination in ES cells has become an invaluable research tool. The preference for gene targeting in pure inbred lines has now dictated a demand for ES cell lines from various inbred strains. However, the efficient production of chimeric mice from these strains is not always met. To date, the majority of germline-competent ES cells are from 129-derived lines. More recently, lines from C57BL/6, C3H/He, NOD, BALB/c, and B6D2F1 strains have been reported (3–7,11–13). A persistent problem faced when one is trying to establish germline transmission is finding the best combination of host blastocyst/ES cell line. Here, we describe the establishment of six C57BL/6J (B6) ES cell lines and compare the efficacy of blastocysts from two different host strains, FVB and the coisogenic strain C57BL/6J-Tyr$^{c\text{-}2J}$ (*c^{2J}*), in generating germline chimeras. Germline transmission was greatly enhanced using the coisogenic strain, *c^{2J}*; fewer blastocyst injections were required to produce highly chimeric mice with a high rate of B6 colonization of the germline.

Table 1. Blastocyst Production from FVB and *c^{2J}* Mice

Strain	No. of Females	Age of Females[a]	No. of Blastocysts Obtained
FVB	20	3 weeks	30–80
c^{2J}	20	3 weeks	20–60

[a]Number range of injectable blastocysts obtained at superovulation

MATERIALS AND METHODS

B6 ES Cell Derivation and Culture

B6 ES cell lines were generated using a rapid and large-scale culture approach as previously described (1,9). Mice were kept on a 14-h light/10-h dark cycle. Three-week-old B6 females were superovulated and mated, and blastocysts were collected 3.5 days after hCG injection. Blastocysts were placed in a droplet of KSOM media under oil in an environment of 5% CO_2, 5% O_2, 90% N_2 at 37°C. After 48 h in KSOM, blastocysts were plated individually in 96-well plates containing mitomycin-C-treated (Sigma, St. Louis, MO, USA) mouse embryonic fibroblast (MEF) cells and ES cell culture media (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% FBS (Sigma), 2 mM glutamine, 1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), leukemia-inhibiting factor (LIF) [10 ng/mL; prepared in bacteria (JM109) using the LIF-producing plasmid pGEX-2T-MLIF from John Heath, The University of Birmingham, UK (10)], and an antibiotic mixture containing penicillin/streptomycin/neomycin (Invitrogen). Four to five days after plating, the cells in each well were trypsinized, dissociated to a single-cell suspension, and allowed to grow in the same wells. The wells containing undifferentiated cells were trypsinized every 2–3 days and plated onto growth-arrested, mitomycin-C-treated MEF cells to expand the colony.

Karyotyping and Sexing of ES Cell Lines

Chromosome counts were performed on ES cells that were passaged several times on gelatin plates to eliminate MEF cells. ES cell cultures were treated with 5 µg/mL colchicine (Sigma), incubated at 37°C for 30–60 min, detached from the dish with rubber policemen, and prepared for G-banding analysis as previously described (2).

Sexing of the ES cell lines was performed with genomic DNA prepared from ES cells passaged several times in the absence of MEF cells. The presence of the Y chromosome was determined by Southern blot analysis of genomic DNA using a Y chromosome probe (*Sx-1*) (8).

Generation of Chimeric Mice

Blastocysts were collected from the uterine horns of three-week-old FVB and *c^{2J}* females as described above and placed in KSOM media in 5% CO_2 at 37°C. B6 ES cells (passages 9–17) were thawed from liquid N_2 stocks 3–5 days before injection and cultured in 10% CO_2 at 37°C in ES culture media on growth-arrested MEFs. ES cells were passaged one day before microinjection and prepared for microinjection by trypsinizing the monolayer with 0.05% trypsin (2 mL) for approximate-

Table 2. Comparison of Germline Transmission of C57BL/6-Derived ES Cell Lines Following Injection into Either c2J or FVB Host Blastocysts

ES Cell Line (Passage No.)	Blastocysts Injected	Medium-To-High Chimeras/Total Pups [% Medium-To-High Chimeras]	Germline Transmission Mice Tested	% Transmission
c^{2J} host blastocysts				
693 (p13–19)	64	3/16 [19]	1[a]	100
694 (p12–14)	54	3/21 [14]	3	14, 100, and 100
736 (p12–17)	68	2/8 [25]	1[a]	100
738 (p12–18)	95	3/17 [18]	2[a]	33 and 100
739 (p9–15)	78	3/33 [10]	3	0, 13, and 71
742 (p10–16)	78	2/13 [15]	2	86 and 100
FVB host blastocysts				
693 (p10)	83	3/38 [8]	3	0, 0, and 12
694 (p12)	132	2/52 [4]	2	0 and 0
736 (p12)	101	0/29 [0]	0	_
738 (p9)	82	9/46 [20]	7[b]	0, 0, 0, 0, 0, 0, and 0
739 (p9)	131	2/77 [3]	2	0 and 100
742 (p9–20)	49	0/65 [0]	0	_

[a]Denotes tested chimeras.
[b]Two males were sterile.

ly 2 min at 37°C. ES culture media (2 mL) was added, and the suspension was pipetted to produce a single-cell suspension, to which an additional 3 mL culture media were then added. The ES cell suspension was transferred to a new 60-mm tissue culture plate and incubated for 20–25 min at 37°C to eliminate unwanted MEFs. The supernatant containing the ES cells was collected, and the ES cells were pelleted by centrifugation at 650× *g*. The pellet was washed once with PBS and then resuspended in 2 mL ES culture media supplemented with 10 mM HEPES (Invitrogen). Eight to 12 ES cells were microinjected into either FVB or *c*^{2J} blastocysts at 12°C and, subsequently, a total of 12–16 blastocysts were transferred into either the oviducts or the uterine horns of day 0.5 post-coitum or day 2.5 post-coitum pseudopregnant BALB/cByJ × B6 F1 females, respectively. Chimeric mice were identified by coat color. The male chimeras were mated to female *c*^{2J} mice to determine germline transmission. A total of 50 pups were analyzed from each chimeric male.

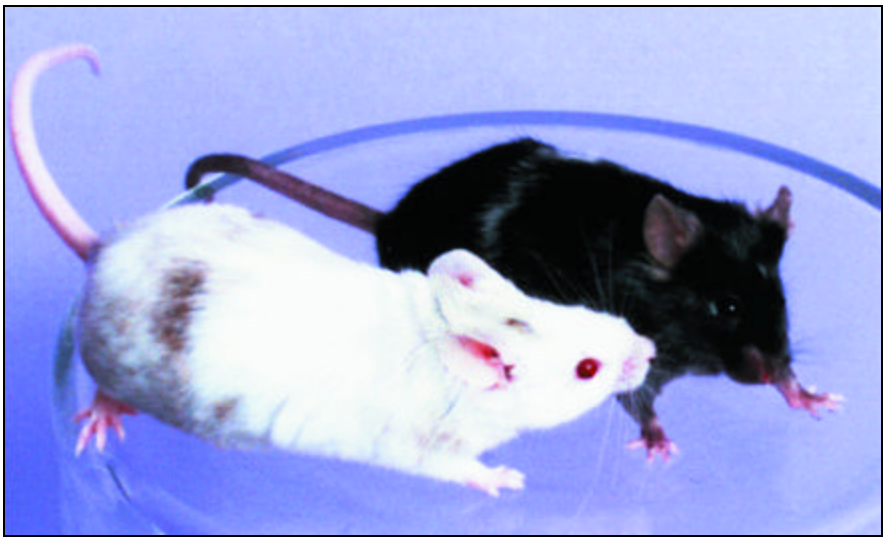

Figure 1. Typical examples of chimeric mice produced from the injection of B6 ES cells into FVB (left, low coat-color chimerism) or *c*^{2J} (right, high coat-color chimerism) host blastocysts.

RESULTS AND DISCUSSION

Fourteen ES cell lines were established from B6 blastocysts grown in culture. The male lines were selected by screening for the presence of the Y chromosome by Southern blot analysis (8). Karyotype analysis revealed that all lines, with one exception, were normal based on chromosome number and low frequency of abnormalities, as determined by G-banding. Line 738 was the exception, with 40% of the spreads exhibiting abnormalities (including translocations, Robertsonian fusions, telomere fusions, and acentric and cen-

tric fragments). Six lines were tested for germline transmission using host blastocysts from mice of either the coisogenic strain c^{2J} or strain FVB.

The efficacy of generating gene-targeted mice relies on many factors, including the superovulatory responsiveness of the host blastocyst source strain, the quality of the blastocysts for microinjection, and the ability of the ES cells to colonize the host blastocyst efficiently. We found that, while superovulated FVB females produced higher yields of blastocysts, superovulated c^{2J} females did produce limited but adequate numbers of blastocysts (Table 1). For both c^{2J} and FVB strains, we found that three-week-old females produced the highest yields of blastocysts. No striking difference was noted in the ease of injection or the quality of blastocysts from either FVB or c^{2J} mice.

A comparison of the receptiveness of host blastocysts from c^{2J} and FVB to colonization by ES cells from six different B6 ES cell lines was made. The injection of six B6 ES cell lines into the coisogenic c^{2J} blastocysts resulted in the production of more medium-to-high coat-color chimeras (>50% black coat color) per total number of pups born than did the injection of the same B6 ES cell lines into FVB host blastocysts (Figure 1) (Table 2). Germline transmission was tested by mating only medium-to-high coat-color male chimeras to c^{2J} females. Colonization of the germ cells by the B6 ES cell line was indicated by the black coat color of the offspring. All of the cell lines tested were germline-competent. While all but one B6/c^{2J} chimera (11/12) produced ES cell-derived offspring, only two B6/FVB chimeras (2/14) produced ES cell-derived pups (Table 2). Germline efficiency, the number of chimeric mice transmitting to 100% of their progeny, was markedly higher when c^{2J} host blastocysts were used (6/12) (50% medium-to-high chimeras transmitted to 100% of their progeny) (Figure 1) (Table 2). Only one of the 14 medium-to-high coat-color chimeras obtained using FVB host blastocysts resulted in 100% germline transmission (Figure 1) (Table 2; 7%). In all cases, germline-transmission analysis was terminated when more than 50 pups were born to a chimeric male.

To date, BALB/c embryos are the most commonly used source of host blastocysts for B6 ES cell injections. However, using standard procedures, few blastocysts can be collected from BALB/c females because of a delay in embryonic development (7). To overcome this limitation, embryos can be collected at the morula stage and matured in culture (7). We found that the blastocyst yield from c^{2J} females was comparable to that from BALB/c females, following the 24-h in culture

procedure of Lemckert et al. (7) (3.5/BALB/c female and 1–3/c^{2J} female). When analyzed as a percentage of the total pups born, fewer chimeric offspring were generated when c^{2J} host blastocysts were used versus BALB/c host blastocysts. Ledermann et al. (6) report a rate of 42% chimeras compared to our range of 18%–37% (for six different B6 ES cell lines analyzed) for all levels of chimerism (data not shown). When analyzed in terms of percent of medium-to-high chimeras generated per total number of embryos injected, Auerbach et al. (1) report 17% and 30% medium-high chimeras generated for two different B6 ES cell lines and Lemckert et al. (7) report a range of 5%–7% chimeras per embryo injected with three different B6 ES cell lines. We obtained a range of 2.5%–5.5% medium-to-high chimeras per embryo injected with six different B6 ES cell lines (Table 2). When we analyzed germline efficiency, the number of chimeric mice that transmit to 100% of their progeny, we found 50% of the chimeras tested transmitted to 100% of their progeny (Table 2). This appears to be slightly higher than what Auerbach et al. (1) and Ledermann et al. (6) found using BABL/c host blastocysts, which was 40% and 38%, respectively.

In summary, we found that the use of the coisogenic strain c^{2J} for host blastocysts supports the development of B6 ES cells and permits a high level of germline transmission resulting from B6 colonization of the germ cells. Although blastocyst production is low in the c^{2J} strain, adequate numbers can be obtained. We conclude that c^{2J} blastocysts are a more efficient host than FVB blastocysts and a comparable alternative to BALB/c blastocysts for B6 ES cell injections for generating germline chimeras.

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Transfection-Mediated Cell Synchronization: Acceleration of G1-S Phase Transition by Gamma Irradiation

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ABSTRACT

We have previously provided evidence that the uptake of DNA into cells is cell cycle specific following transfection. We show here that, immediately after transfection, successfully transfected cells are greatly enriched for cells in early G1 or G0 phase and that, upon removal of the DNA precipitates, cells progress through G1 and enter S phase in a synchronous fashion. We also demonstrate that this approach can be utilized in meaningful cell-cycle experiments, and we show that gamma irradiation accelerates the G1-S phase transition in a cell line with a functionally inactive p53 protein.