tions are unclear, it might be beneficial to start the touch-up segment at lower temperatures.

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## Establishment and Chimera Analysis of 129/SvEv- and C57BL/6-Derived Mouse Embryonic Stem Cell Lines

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### ABSTRACT

*Hundreds of new mutant mouse lines are being produced annually using gene targeting and gene trap approaches in embryonic stem (ES) cells, and the number is expected to continue to grow as the human and mouse genome projects progress. The availability of robust ES cell lines and a simple technology for making chimeras is more attractive now than ever before. We established several new ES cell lines from 129/SvEv and C57BL/6 mice and tested their ability to contribute to the germline following blastocyst injections and/or the less expensive and easier method of morula-ES cell aggregation. Using morula aggregation to produce chimeras, five newly derived 129/SvEv and two C57BL/6 ES cell lines tested at early passages were found to contribute extensively to chimeras and produce germline-transmitting male chimeras. Furthermore, the two 129S/vEv ES cell lines that were tested and one of the C57BL/6 ES cell lines were able to maintain these characteristics after many passages in vitro. Our results indicate that the ability of ES cells to contribute strongly to chimeras following aggregation with outbred embryos is a general property of early passage ES cells and can be maintained for many passages. C56BL/6-derived ES cell lines, however, have a greater tendency than 129-derived ES cell lines to lose their ability to colonize the germline.*

### INTRODUCTION

Gene targeting is a well-established technology used to produce mouse mutants and models of human diseases (2,9). Injection of genetically altered embryonic stem (ES) cells into blastocysts from inbred mice (C57BL/6 or BalbC) has been used most widely to produce chimeras (8). This technique, however, requires both expensive equip-

ment and specialized microinjection skills. Aggregation of ES cells with outbred morulae is a less expensive alternative to making ES cell chimeras, and the technique requires little training (6,12). To date, primarily one cell line, R1 (7), has been used to produce germline chimeras by aggregation with outbred (CD1) embryos. It was suggested by Simpson et al. (10) that the ability of R1 cells to produce germline chimeras by aggregation could be due to its mixed genetic background (129/Sv × 129/SvJ). To test whether this is the case or whether such a property is common to all early passage ES cell lines, we produced several new ES cell lines from 129/SvEv and C57BL/6 mice using a novel technique and tested their ability to produce germline chimeras by aggregation with outbred and inbred embryos.

Historically, most ES cell lines have been established from different substrains of 129 mice. Since not all substrains are readily available, chimeras carrying a new mutation are often crossed to a different 129 substrain than the one from which the ES cell line was derived. This mixing of genetic backgrounds can have important implications for subsequent analysis of the mutant phenotype because some of the substrains have different characteristics, including coat color, reproductive performance and behavior (3). Furthermore, extensive DNA polymorphism has been observed between some 129 substrains, and this is thought to be due to genetic contamination during establishment of the substrains (10). We chose to make new ES cell lines from the129/SvEv line of mice, which, unlike many 129 substrains, breeds fairly well and does not show place-learning disabilities (3). In addition, we made ES cell lines from C56BL/6 mice because this strain of mice has been widely used in genetic studies, many mutants are maintained on this background and it is generally preferred over 129 for behavioral and immunological studies.

## MATERIALS AND METHODS

### Establishment of ES Cell Lines

The W series of 129/SvEv cell lines and the WB series of C57BL/6 cell lines (both from Taconic, Germantown, NY, USA) were established using a new rapid and large-scale approach recently described in Matise et al. (4). Embryos were flushed from the uterine horns of 3.5 days postcoitus (dpc) females (48 blastocysts and 31 morulae for the W series, 60 blastocysts and 24 morulae for the WB series) or 6.5 dpc ovariectomyzed females (32 blastocysts for the WB series) in M2 medium (Sigma, St. Louis, MO, USA), washed in ES cell medium and plated on 96- or 4-well tis-

sue culture plates coated with primary embryonic fibroblast (EMFI) feeder layers in ES cell medium (4) containing 25%–30% fetal calf serum (FCS) (Gemini, Calabasas, CA, USA) and 2000 U/mL leukemia inhibitory factor (LIF) (Life Technologies, Rockville, MD, USA). After several days, the inner cell mass-derived clumps of cells were disaggregated by trypsinization and allowed to reattach on the same plates. Plates containing undifferentiated cells were trypsinized every 2–3 days and either left on the same plates or transferred into new EMFI-containing or gelatinized plates. In this process, undifferentiated ES cells were separated from differentiated ones and from old fibroblasts, and also expanded in number. Clones that were not confluent were replated on the same plate. When trypsinizing cells from a 35-mm dish for the first time for a particular clone, half the cells were frozen and half seeded onto a 60-mm dish. This was considered the first passage. For cell line expansion, cells were trypsinized at 80% confluency. Serum and LIF concentrations were reduced to 15% and 1000 U/mL, respectively, in two steps during passages 2–4. To produce delayed blastocysts, superovulated females at 2.5 dpc were ovariectomyzed and administered with 1 mg progesterone. Nine 129/SvEv ES cell lines were established from the 3.5-dpc blastocysts and one from the 3.5-dpc morulae. Two C57BL/6 ES cell lines were established from 3.5-dpc blastocysts and one from the 6.5-dpc delayed blastocyst.

For the generation of the MPI series (Millenium Pharmaceuticals) of ES cell lines, female C57BL6/J-HPRT (b-m3) or 129/SvEv mice (Taconic) were ovariectomyzed at 2.5 dpc. Blastocysts were flushed from the uterine horns six days after ovariectomy and plated on EMFI (for C57BL/6) or SNL76/7 (for 129/SvEv) feeder cells (5) in 6-well plates containing ES cell culture medium supplemented with 1000 U/mL LIF. Three days after plating, the inner cell mass outgrowths were trypsinized and replated on fresh 6-well plates, and the resulting ES cell clones were picked and expanded.

The number of chromosomes and the sex of each cell line were determined by karyotype analysis as described by Benn and Perle (1). At least 20 spreads were analyzed for each ES cell clone.

### Chimera Production

Morula-ES cell aggregation chimeras were produced essentially as described by Nagy and Rossant (6). Briefly, morulae were obtained by flushing oviducts of superovulated 2.5 dpc outbred (CD1 or SW) or BalbC (C57BL/6 lines only) inbred mice. The zona pellucida was removed by dissolving it in acid Tyrode's solution (Sigma). Each embryo was placed into a microdepression made in a tissue culture plate in a drop of M16 medium (Sigma) and co-incubated overnight with a small clump (8–12 cells) of ES cells. Blastocysts or compacted morulae, which had incorporated ES cells, were transferred into the uteri of pseudopregnant females on the next day. C57BL/6-derived ES cells were injected into BalbC blastocysts, and 129-derived ES cells were injected into C57BL/6 blastocysts to produce chimeras using standard methods (8).

## RESULTS

### Establishment and Analysis of 129/SvEv-Derived ES Cell Lines

In one series of experiments (W series), 10 ES cell lines were produced from 85 129/SvEv blastocysts or delayed morulae. Seven of the ES cell lines had a normal euploid (40 N) karyotype at passage 5–6 (Table 1). Nine of the lines were male, although in one clone (C11), the Y chromosome was lost at a high frequency. One line had one chromosome duplicated (41 N), and the one female line (H5) lost one chromosome at a high frequency. Five of the euploid male lines were tested after 5–8 passages in culture for their ability to contribute to chimeras after aggregation with CD1 embryos. The degree of chimerism was assessed by coat color (Table 1). The strongest chimeras were mated with outbred CD1 or Swiss Webster (SW) females to test for germline transmission. All five lines were able to contribute extensively to chimeras and produce germline-transmitting male chimeras. Two lines were tested further

**Table 1. Characteristics of 129/SvEv- and C57B1/6-Derived ES Cell Lines**

| ES Cell Line | Strain | Karyotype | Passage No. | Chimera Type[a] | Host Embryo Strain | No. of Embryos Transferred | Chimerism 0% | Chimerism <40% | Chimerism >40% | No. Males/Total No. Chimeras | No. Germline /No. Tested |
|---|---|---|---|---|---|---|---|---|---|---|---|
| W2 | 129/SvEv | 40 N XY | 5 | Agg | CD 1 | 42 | 9 | 0 | 7 | 7/7 | 3/3 |
| W3 | 129/SvEv | 40 N XY | 5 | Agg | CD 1 | 22 | 2 | 0 | 8 | 6/8 | 3/3 |
| | | | 7 | Agg | CD 1 | 28 | 6 | 1 | 2 | 3/3 | 1/1 |
| W4 | 129/SvEv | 40 N XY | 6 | Agg | CD 1 | 51 | 5 | 4 | 9 | 12/13 | 5/6 |
| | | | 14 | Agg | CD 1 | 16 | 4 | 2 | 0 | 2/2 | nt |
| | | | 14 | Agg | SW | 16 | 1 | 4 | 4 | 8/8 | 2/2 |
| | | | 21 | Agg | CD 1 | 17 | 0 | 4 | 3 | 7/7 | 1/3 |
| W5 | 129/SvEv | 40 N XY | 6 | Agg | CD 1 | 54 | 9 | 2 | 7 | 8/9 | 3/3 |
| | | | 8 | Agg | CD 1 | 15 | 8 | 1 | 3 | 4/4 | 1/1 |
| W12 | 129/SvEv | 40 N XY | 5 | Agg | CD 1 | 30 | 6 | 1 | 8 | 9/9 | 4/5 |
| | | | 8 | Agg | CD 1 | 18 | 1 | 0 | 1 | 0/1 | nt |
| | | | 15 | Agg | SW | 16 | 4 | 0 | 2 | nt | nt |
| | | | 22 | Agg | CD 1 | 17 | 9 | 1 | 0 | nt | nt |
| | | | 25 | Agg | SW | 28 | 7 | 0 | 2 | 2/2 | 1/2 |
| WB6a | C57BL/6 | 40 N XY | 2 | Agg | SW | 18 | 2 | 1 | 3 | 4/4 | 0/3 |
| | | | 5 | Agg | SW | 30 | 8 | 8 | 1 | 6/9 | 0/5 |
| | | | 7 | Agg | SW | 32 | 8 | 4 | 4 | 6/8 | 0/5 |
| WB6b | C57BL/6 | 40 N XY | 4 | Agg | BalbC | 4 | 4 | 2 | 4 | 5/6 | 1/2 |
| | | | 11 | Agg | SW | 11 | 1 | 0 | 0 | | |
| WB6d | C57BL/6 | 40 N XY | 4 | Agg | SW | 18 | 2 | 1 | 3 | 3/4 | 1/2 |
| | | | 4 | Agg | BalbC | 36 | [b] | | | | |
| | | | 10 | Agg | BalbC | 20 | 2 | 0 | 1 | 1 | nt |
| | | | 14 | Agg | SW | 28 | 8 | 1 | 4 | 5/5 | 1/4 |
| | | | 10-12 | Inj | BalbC | 181 | 50 | 5 | 25 | 23/30 | 3/10 |
| MPI 65-3 | C57BL/6 | 40 N XY | 7-10 | Inj | BalbC | 81 | 10 | 3 | 21 | 18/24 | 3/5 |
| | | | 8-10 | Agg | SW | 86 | 2 | +some dead | | | |

[a]Agg, chimeras made by aggregation of ES cells with morulae of host embryo strain.
Inj, chimeras made by injection of ES cells into blastocysts of host embryo strain.

[b]All newborns were eaten.

for their ability to retain the potential for germline transmission after 21 or 25 passages in culture and aggregation with CD1 or SW morulae. Both lines made strong chimeras following aggregation with SW morulae and maintained their ability to contribute to the germline. One of the cell lines (W4) has been successfully targeted at passage 10–12 with 38 different targeting constructs from 129/Sv, 129/SvJ, 129/Ola or 129/SvEv genomic DNA. ES cell clones from 28 of the targeting vectors were used to produce chimeras by aggregation with SW morulae, and all targeted alleles were transmitted through the germline. On average, W4-targeted ES cell clones have given rise to germline chimeras at a rate of approximately three out of four clones.

### Establishment and Analysis of C57BL/6-Derived ES Cell Lines

In a second series of experiments (WB series), three new ES cell lines were produced from 92 C57BL/6 blastocysts (Table 1, WB series). All three cell lines at early passage (P2-4) produced chimeras at a rate similar to the 129/SvEv W series of ES cell lines following aggregation with morulae from either SW or BalbC mice. For two of the lines, the chimeras transmitted the ES cell genotype to 100% of their offspring at early passages. Only one of these two lines (WB6d) maintained the ability to produce germline-transmitting chimeras after aggregation at later passages (P14). This line also efficiently made germline-transmitting chimeras by injection into BalbC blastocysts (Table 1).

In an independent series of experiments (MPI series), we compared the germline competence of C57BL/6 to 129/SvEv-derived ES cells. Five MPI C57BL/6 and four MPI 129/SvEv ES cell lines were established from delayed

**Table 2a. Chimeras Produced by Blastocyst Injections**

| Strain | Clone | Fertile Male Chimeras % | No. of Germline/No. of Fertile Chimeras[a] | No. of Germline/Total No. Pups from Transmitting Chimeras (%) |
|---|---|---|---|---|
| 129/SvEv | MPI-17A | 4/4 (100%) | 3/4 | 28/29 (96%) |
| | MPI-12D | 3/4 (75%) | 3/3 | 39/44 (89%) |
| | MPI-17E | 4/5 (80%) | 3/4 | 46/46 (100%) |
| | MPI-12G | 3/3 (100%) | 3/3 | 36/36 (100%) |
| C57BL/6 | MPI-71.6 | 5/5 (100%) | 1/5 | 5/32 (16%) |
| | MPI76.11 | 7/7 (100%) | 1/7 | 16/25 (64%) |
| | MPI53.1 | 6/6 (100%) | 2/6 | 53/53 (100%) |
| | MPI-48.1 | 3/3 (100%) | 1/3 | 1/17 (6%) |
| | MPI65.3 | 5/5 (100%) | 3/5 | 46/46 (100%) |
| | WB6d | 7/10 (70%) | 3/7 | 14/46 (30%) |

[a]129/SvEv ES cells were injected into C57BL/6 blastocysts, and male chimeras were bred with C57BL/6 females. Agouti offspring were scored positive for germline transmission; black were scored non-germline. C57BL/6 ES cells were injected into BalbC blastocysts, and male chimeras were bred with C57BL/6 females. Black offspring were scored positive for germline transmission; agouti were scored non-germline.

**Table 2b. Chimeras Produced by ES Cells Morula Aggregation**

| Strain | Clone | Fertile Male Chimeras % | No. of Germline/No. of Fertile Chimeras[a] | No. of Germline/Total No. Pups from Transmitting Chimeras (%) |
|---|---|---|---|---|
| 129/SvEv | W2 | 3/3 (100%) | 3/3 | 61/75 (81%) |
| | W3 | 4/4 (100%) | 4/4 | 71/106 (67%) |
| | W4 | 8/8 (100%) | 7/8 | 125/125 (100%) |
| | W5 | 4/5 (80%) | 4/4 | 84/102 (82%) |
| | W12 | 5/5 (100%) | 4/5 | 63/85 (74%) |
| C57BL/6 | WB6a | 5/13 (38%) | 0/5 | 0/146 (0%) |
| | WB6b | 1/3 (33%) | 1/1 | 6/6 (100%) |
| | WB6d | 3/6 (50%) | 2/3 | 20/20 (100%) |

[a]129/SvEv ES cells were aggregated with CD1 or SW morulae, and male chimeras were bred with females from same outbred strain. Agouti offspring were scored positive for germline transmission; white were scored non-germline. C57BL/6 ES cells were aggregated with BalbC or SW morulae, and male chimeras were bred with SW females. Black offspring were scored positive for germline transmission; white were scored non-germline.

blastocysts and tested for germline transmission using blastocyst injection at passage 7–10 (Table 2a). In general, the C57BL/6 ES lines were not quite as proficient in producing high-percentage ES cell-derived chimeras as the 129/SvEv lines (data not shown). However, the most striking difference between the two strains was in their efficiency of germline transmission. All of the cell lines tested were germline competent, but whereas virtually all of the 129/SvEv-derived chimeras produced ES cell-derived offspring (34 germline chimeras/38 fertile chimeras or 89%), only a minority of the C57BL/6-derived chimeras from any given line typically produced ES cell-derived pups (11/35 or 31%), and the efficiency of germline transmission was overall lower and much more variable than for 129/SvEv (43% vs. 85%, respectively). Only one of the five C57BL/6 lines (MPI 65-3) produced germline chimeras at an efficiency comparable to that of the 129/SvEv ES cell lines. This cell line has also been successfully used in gene tar-

geting experiments (11). However, this line did not produce chimeras following aggregation with SW morulae (Table 1). For the W and WB series of 129/SvEv and C57BL/6 ES cell lines, similar results were observed when chimeras generated by aggregation were tested for germline transmission (Table 2b). However, in the three WB C57BL/6 ES cell lines tested, the principal impediment to germline transmission was a high percentage of infertility in male chimeras.

## DISCUSSION

Several new ES cell lines were established from 129/SvEv and C57BL/6 embryos that are able to contribute to the germline of chimeras following aggregation with morulae from outbred mouse strains. Our results indicate that the ability of ES cells to contribute strongly to chimeras following aggregation with outbred (CD1 and SW) embryos is a general property of early passage ES cells, and this property can be maintained for many passages. Furthermore, for some ES cell clones that have lost their ability to produce germline-transmitting chimeras following aggregation, such chimeras can still be made using blastocyst injection. Whereas inbred strains of mice (C57BL/6 and BalbC) have been found to be more effective host embryos than outbred strains for producing chimeras by blastocyst injection (data not shown), for aggregation there is a trend towards better chimera production using outbred morulae (Table 2). Finally, the stability and high germline efficiency of the 129/SvEv-derived ES cell lines, the availability of isogenic genomic DNA libraries and the ease of obtaining isogenic inbred 129/SvEv mice make these lines ideal for gene targeting experiments and other genetic manipulations and subsequent transmission of the genetic alterations into mice. In general, C57BL/6-derived ES cells are more difficult to maintain in culture in a state able to produce germline-transmitting chimeras than 129-derived cell lines. Using either aggregation or blastocyst injection, C57BL/6-derived ES cells at early passages can give rise to strong male chimeras at a frequency close to that of 129-derived cell lines (Tables 1 and 2 and data not shown), but their germline transmission efficiency is lower (Table 2). Therefore, for routine gene targeting experiments in which the genetic background is not critical, 129-derived ES cells offer the most efficient route for transmitting mutations into mice.

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