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ORIGINAL CONTRIBUTIONS

Genetic variation in C57BL/6 ES cell lines and genetic instability in the Bruce4 C57BL/6 ES cell line

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Abstract Genetically modified mouse strains derived from embryonic stem (ES) cells are powerful tools for gene function analysis. ES cells from the C57BL/6 mouse strain are not widely used to generate mouse models despite the advantage of a defined genetic background. We assessed genetic variation in six such ES cell lines with 275 SSLP markers. Compared to C57BL/6, Bruce4 differed at 34 SSLP markers and had significant heterozygosity on three chromosomes. BL/6#3 and Dale1 ES cell lines differed at only 3 SSLP makers. The C2 and WB6d ES cell lines differed at 6 SSLP markers. It is important to compare the efficiency of producing mouse models with available C57BL/6 ES cells relative to standard 129 mouse strain ES cells. We assessed genetic stability (the tendency of cells to become aneuploid) in 110 gene-targeted ES cell clones from the most widely used C57BL/6 ES cell line, Bruce4, and 710 targeted 129 ES cell clones. Bruce4 clones were more likely to be aneuploid and unsuitable for ES cell-mouse chimera production. Despite their tendency to aneuploidy and consequent inefficiency, use of Bruce4 ES cells can be valuable for models requiring behavioral studies and other mouse models that benefit from a defined C57BL/6 background.

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Introduction

There are several reasons why the C57BL/6 strain is favored for some gene-targeting studies. The availability of the C57BL/6J mouse genome sequence facilitates the generation of isogenic targeting vectors. C57BL/6J mice

are an excellent model for metabolic and diabetes research because they are known to be susceptible to diet-induced hyperglycemia, hyperinsulinemia, insulin resistance, and obesity-associated diabetes (Black et al. 1998; Molnar et al. 2005; Petro et al. 2004). They are also the model of choice for behavior and cognition (Kelly et al. 2003; Nguyen et al. 2000; Ohl et al. 2003) and for studies of the immune system (Blier et al. 1998; Chang et al. 2005; Kato et al. 1994; Kontgen et al. 1993). As the standard inbred mouse strain in these fields, new data from C57BL/6J mouse models can be interpreted in the context of a large body of research. Comparisons can be made directly with research in other mouse strains and also with genetically engineered models produced by transgenesis and gene targeting.

Transgenic mice are often used in dominant gene models, while gene modification by homologous recombination in embryonic stem (ES) cells allows for models of recessive genetic diseases. Transgenic mice can be prepared in a pure C57BL/6J background through DNA microinjection into the pronuclei of fertilized mouse eggs, although the process is approximately eightfold less efficient than when F_1 hybrid eggs are used (Auerbach et al. 2003; Brinster et al. 1985). Similarly, gene targeting can be carried out in ES cells from a C57BL/6 mouse strain, although the efficiency of obtaining modified mice is reduced relative to commonly used ES cells derived from mice heterozygous for 129 substrains (Nagy et al. 1993).

ES cell lines derived from 129 strains of mice account for the vast majority of published studies on gene-targeted mouse models (Seong et al. 2004). However, there are several limitations to the use of 129-derived ES cells. First, there is considerable genetic variation among the different 129 mouse strains (Simpson et al. 1997; Threadgill et al. 1997). Genetic and phenotypic variations of gene-targeted mice made with 129 mouse ES cells are well known (Linder 2006). For example, deletion of *Egfr* in different 129 mouse ES cell lines resulted in different phenotypes that depended on genetic background (Sibelia and Wagner 1995; Threadgill et al. 1995). Corpus callosum dysgenesis is commonly observed in 129 mouse strains, which confounds studies of behavioral and cognitive phenotypes (Crawley 1996; Gerlai 1996). The genetic complexity introduced by the use of 129 ES cell lines for gene knockouts is an impediment to immunologists who rely on the extensively characterized C57BL/6 immune response (Bauler et al. 2007; Rolink et al. 2004; Schwarz et al. 2003). Stem cell biologists use C57BL/6 mice for transplantation studies which require that mouse knockouts be backcrossed onto C57BL/6 (Yilmaz et al. 2006). This common approach to reduce genetic variation (Silva et al. 1997) is time consuming and expensive, potentially exceeding the cost of producing the initial gene-targeted mouse strain. Importantly, even after numerous backcrosses a significant portion (10 cM) of the donor 129 chromosome can persist, potentially confounding research results (Armstrong et al. 2006).

The use of C57BL/6 ES cells for targeted genetic modifications and subsequent production of new mouse strains is an important future goal identified with the knockout mouse project (KOMP; Austin et al. 2004; Auwerx et al. 2004). A survey of 5,344 genetically modified mouse strains curated at the Jackson Laboratory shows that only 70 were generated from C57BL/6 ES cell lines (data available upon request) and that the majority of these were generated with the Bruce4 ES cell line. We sought to examine the genetic stability of gene-targeted Bruce4 ES cell clones and to characterize genetic variation in C57BL/6 ES cell lines. We found minor genetic differences between the C57BL/6J reference genome and four C57BL/6 ES cell lines as well as significant genetic contamination in two ES cell lines. Gene-targeted ES cell clones from the currently available Bruce4 C57BL/6 ES cell line are more likely to be aneuploid than those from 129 mouse ES cell lines such as R1, E14Tg2a.4, and Pat5. Aneuploid ES cell clones are less likely to contribute to the germline of ES cell-mouse chimeras (Longo et al. 1997). Thus, genetic instability inherent in an ES cell line will reduce its germline potency.

Materials and methods

ES cell lines

We established the Dale1 ES cell line from B6(Cg)-Tyr^{c-2J}/J mice (Jackson Laboratory stock number 000058). Briefly, blastocysts were placed in cell culture on primary mouse embryo fibroblast feeder cells and allowed to grow out into ES cell colonies as described (Nagy et al. 2003). Dale1 ES cells were demonstrated to be XY by PCR amplification of the X- and Y-linked *Smcx* and *Smcy* genes (data not shown; Mroz et al. 1999). The genetic background of the Dale1 ES cells is coisogenic with C57BL/6J since the B6(Cg)-Tyr^{c-2J}/J mice differ from C57BL/6J mice by a single nucleotide change described by Le Fur et al. (1996).

BL/6#3 ES cells, derived from C57BL/6J mice (Jackson Laboratory stock number 000664), were a kind gift of Joanne Conover (Schuster-Gossler et al. 2001).

Bruce4 ES cells, derived from a C57BL/6 mouse strain congenic for the Thy1.1 allele from an NZB mouse, were a kind gift of Colin Stewart (Kontgen et al. 1993).

C2 ES cells are derived from C57BL/6NTac mice and were a kind gift of Andras Nagy.

WB6d ES cells are derived from C57BL/NTac mice and were a kind gift of Alex Joyner (Auerbach et al. 2000).

CMTI-2 ES cells are derived from "C57/BL6" mice and were purchased from Specialty Media, Inc. (Phillipsburg, NJ).

Mouse ES cell lines derived from 129 mouse strains used for chromosome counts included CJ7 derived from 129S1/SvImJ mice (Swiatek and Gridley 1993), E14Tg2a.4 derived from 129P2/OlaHsd (Skarnes 2000), GSI-1 derived from 129X1/SvJ (Genome Systems, St. Louis, MO), Pat5 derived from 129X1/SvJ (Domino et al. 2001), R1 derived from (129X1/SvJ × 129S1/SvImJ)F_1 (Nagy et al. 1993), and W4 derived from 129S6/SvEvTac (Auerbach et al. 2000).

C57BL/6 and 129 ES cell lines were maintained in high-glucose Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Harlan, Indianapolis, IN), 4 mM glutamine, 1 μM 2-mercaptoethanol, 1% MEM nonessential amino acids, 50 IU penicillin per ml, 50 μg streptomycin per ml, and 1000 U per ml ESGRO (Chemicon, Temecula, CA) on FVB/N mouse embryonic feeder cells mitotically inactivated by irradiation. Prior to DNA extraction and genotyping, ES cell lines were passaged twice on gelatin-coated dishes to eliminate feeders from the cultures. DNA was extracted from ES cells with standard methods. The presence of FVB/N feeder cell DNA was undetectable by PCR with SSLP primers.

Genotyping ABI PRISM® Mouse Mapping Primers v.1.0 fluorescent SSLP primers were purchased from Applied Biosystems (ABI, Foster City, CA) to amplify selected microsatellite markers. The 286 SSLP markers we tested are listed in Supplementary Table 1. Data are reported for the 275 markers that gave complete and reproducible results. PCR reactions were conducted by the University of Michigan DNA Sequencing Core with ABI reagents according to the manufacturer's conditions. PCR fragment sizes were determined with an ABI Model 3730XL DNA sequencer. SSLP map locations were taken from the Mouse Genome Database, Build 36 (Eppig et al. 2005) or the National Center for Biotechnology Information Map Viewer database (Wheeler et al. 2006).

C57BL/6J mouse reference DNA (stock number 000664) was purchased from the Jackson Laboratory DNA Resource (Bar Harbor, ME). Genomic DNA samples were tested with 286 primer pairs covering SSLPs on chromosomes 1–19 and the X chromosome.

Chromosome counting Chromosome counts were made on ES cell clones obtained from gene targeting experiments as described (Domino et al. 2001). All gene-targeted ES cell clones and chromosome counts were performed in the University of Michigan Transgenic Animal Model Core. Classification of clones as euploid or aneuploid was based on the results of chromosome counts. For chromosome counts, 5×10^6 ES cells were plated onto 10-cm gelatin-treated culture plates in ES cell medium. After overnight culture, cells were treated with 0.02 μg/ml colcemid (Invitrogen) for 120 min. Cell cultures were treated with trypsin to obtain a single-cell suspension. Cells were resuspended in 10 ml of room-temperature 75 mM KCl and incubated for 15 min at 37°C. Cells were collected by centrifugation and resuspended in room-temperature fixative (3:1 methanol, glacial acetic acid). Cells were incubated on ice for 30–120 min and washed twice in cold fixative. Chromosome spreads were prepared by dropping fixed cells onto slides and staining for 15 min in Gurr's Giemsa in Gurr's Buffer (Invitrogen). Chromosome spreads were visualized at 1000× magnification with a 100× oil objective on an E800 microscope (Nikon, New York). Spreads were photographed and chromosomes were counted in at least 20 spreads for each ES cell clone.

ES cell subcloning

The earliest available passage of an ES cell clone was plated at 3×10^6 cells on 100-mm dishes of feeder cells to allow for the growth of colonies from individual ES cells. Five to six hours after initial plating the dishes were carefully but vigorously rinsed several times with ES cell medium to dislodge loosely attached cells. ES cell clones were selected on the basis of undifferentiated appearance, small tightly packed cells in a smooth, dome-shaped structure, with high birefringence, absence of single cells, and no halo of pavement-like cells around the colony. Colonies were picked and expanded as described (Nagy et al. 2003) until they could be cryopreserved and their chromosomes counted.

Results

Genetic variation

We used 286 primer pairs to identify SSLPs on chromosomes 1–19 and X in C57BL/6J reference DNA and six C57BL/6-derived ES cell lines. We obtained complete data for 275 SSLPs. The results show that no ES cell line was completely identical to C57BL/6J at all 275 SSLP markers. Amplification products for 39 primer pairs differed between the reference genomic DNA (C57BL/6J) and the ES cell lines (Table 1). The Bruce4 and CMTI-2 ES cell DNA samples differed from C57BL/6J DNA at 34 different SSLP markers. Bruce4 and CMTI-2 are identical to each other for every SSLP marker tested (Table 2), but they are heterozygous instead of homozygous for C57BL/6J markers on 12 chromosomes. Most notably, marker analysis identified heterozygous regions 7.2–10.0 cM in length on chromosomes 3, 7, and 9 (Fig. 1). These chromosome segments accounted for 18 of the 34 primer pairs that

Table 1 SSLP marker sizes (shown as bp) that differ between C57BL/6J genomic DNA and C57BL/6-derived ES cells for 275 SSLPs

				ES cell lines					
Marker	Chromosome	Position (cM)	C57BL/6J	Bruce4	CMTI-2	WB6d	C2	BL/6#3	Dale1
D1Mit159	1	81.6	206	206, ***208***	206, ***208***	206	206	206	206
D1Mit206	1	95.8	135	***128, 133***	***128, 133***	133	133	***131***, 135	135
D2Mit1	2	1	219	***216***, 219	***216***, 219	***216***	***216***	219	***216***, 219
D2Mit411	2	77.6	131	***126***, 131	*126*, 131	131	131	131	131
D3Mit51	2	35.2	241	241, ***258***	241, ***258***	241	241	241	241
D3Mit98	3	39.7	92	92, ***96***	92, ***96***	***96***	***96***	92	92
D3Mit311	3	45.2	117	***110***, 117	***110***, 117	117	117	117	117
D3Mit320	3	71.8	112	***105***, 112	***105***, 112	112	112	112	112
D5Mit123	5	0	198	198, ***204***	198, ***204***	198	198	198	198
D5Mit314	5	59	122	122, ***126***	122, ***126***	122	122	122	122
D7Mit69	7	24.5	241	***237***, 241	***237***, 241	241	241	241	241
D7Mit323	7	50	224	224, ***228***	224, ***228***	224	224	224	224
D7Mit71	7	65.2	140	***122***, 140	***122***, 140	140	140	1***36***, 140	140
D7Mit109	7	66	112	***91***, 112	***91***, 112	112	112	112	112
D7Mit259	7	72	243	***219***, 243	***219***, 243	243	243	243	243
D7Mit223	7	72.4	83	***77***, 83	***77***, 83	83	83	83	83
D8Mit124	8	6	155	***153***, 155	***153***, 155	***153***	***153***	155	155
D9Mit90	9	9	112	***108***, 112	***108***, 112	***108***	***108***	112	112
D9Mit1001	9	15	127	127, ***129***	127, ***129***	127	127	127	127
D9Mit285	9	21	234	***213***, 234	***213***, 234	234	234	234	234
D9Mit129	9	26	130	130, ***143***	130, ***143***	130	130	130	130
D9Mit71	9	29	116	***105***, 116	***105***, 116	116	116	116	116
D9Mit97	9	29	163	163, ***172***	163, ***172***	163	163	163	163
D9Mit336	9	35	175	***158***, 175	***158***, 175	175	175	175	175
D9Mit151	9	73	107	107	107	107	107, ***109***	107	107
D10Mit213	10	11	257	257, ***259***	257, ***259***	257	257	257	257
D10Mit31	10	36	135	135, ***141***	135, ***141***	135	135	135	135
D10Mit233	10	62	133	133	133	133, ***135***	133	133	133
D10Mit103	10	70	173	173, ***179***	173, ***179***	173	173	173	173
D11Mit71	11	1.1	215	***197***, 215	***197***, 215	215	215	215	215
D11Mit86	11	28	86	***84***, 86	***84***, 86	86	86	86	86
D13Mit16	13	13	200	200, ***203***	200, ***203***	200	200	200	200
D13Mit275	13	16	110	110, ***122***	110, ***122***	110	110	110	110
D13Mit88	13	21	133	133, ***141***	133, ***141***	133	133	133	133
D14Mit126	14	5	131	131, ***135***	131, ***135***	131	131	131	131
D14Mit228	14	46	192	192	192	192	192	192	***188***, 192
D15Mit143	15	21.4	133	133	133	133	133	***133***, 152	133
D19Mit92	19	54	229	229, ***233***	229, ***233***	229	229	229	229
DXMit121	X	67	123	123	123	123	123	123	***105***

PCR product sizes that differ from C57BL/6J are shown in bold

returned heterozygous results for Bruce4 and CMTI-2 DNA. The remaining SSLP markers were scattered over nine other chromosomes. Thus, only 8 of 20 chromosomes in Bruce4 and CMTI-2 gave SSLP results identical to the C57BL/6J reference DNA. The presence of a heterozygous region on chromosome 9 was expected because Bruce4 ES cells were derived from a Thy1.1 congenic mouse strain (Kontgen et al. 1993). Thy1.1 maps to cM 26.0 of chromosome 9. Accordingly, we found that *D9Mit129* (cM 26.0) and flanking markers are heterozygous (Table 1). We were not expecting to identify other regions of genetic heterozygosity in Bruce4 cells. The detection of

heterozygous regions on chromosomes 3 and 7 is consistent with persistence of residual heterozygous regions in congenic mouse lines unless they are excluded by testing with markers spaced 5–10 cM apart (Armstrong et al. 2006).

The WB6d and C2 C57BL/6 ES cell lines each differed from the C57BL/6J reference DNA at six SSLP positions, respectively (Table 1). The differing SSLPs were scattered over seven different chromosomes and no chromosome showed more than one SSLP difference when compared to C57BL/6J. These two ES cell lines differed from each other at two SSLPs. Both WB6d and C2 ES cell lines were derived from C57BL/6NTac, a mouse strain that was derived from the C57BL/6J stocks sent to the NIH from the Jackson Laboratory in 1951 (Taconic, Hudson, NY). Consequently, some differences with C57BL/6J were not surprising.

The BL/6#3 and Dale1 ES cell lines each differed from the C57BL/6J reference DNA at three SSLP positions (Table 2). These two ES cell lines differed from each other by six SSLPs that are scattered over six chromosomes. The BL/6#3 ES cell line was derived directly from C57BL/6J mice and the Dale1 cells were derived from coisogenic B6(Cg)-Tyr^{c-2J}/J mice obtained from the Jackson Laboratory. The B6(Cg)-Tyr^{c-2J}/J mouse strain used to derive Dale1 ES cells carries a mutation in tyrosinase, which maps to cM 44.0 on chromosome 7 (Le Fur et al. 1996). We might expect heterozygosity in SSLPs around the *Tyr*$^{c-2J}$ locus in Dale1 DNA if the strain were not coisogenic. We found that all 15 chromosome 7 SSLP primer pairs tested on Dale1 produced homozygous products identical to those from the C57BL/6J reference DNA (data not shown). This includes the markers flanking tyrosinase, *D7Mit350* at 41.0 cM and *D7Mit323* at 50.0 cM.

Genetic instability of Bruce4 ES cells

A direct correlation exists between the proportion of euploid chromosome spreads in an ES cell clone and germline transmission of targeted genes by ES cell-mouse chimeras (Longo et al. 1997; our unpublished observations). We collected data from 820 gene-targeted clones from different ES cell lines to determine if any of them had a tendency to become aneuploid (Table 3). ES cell clones were classified as euploid or aneuploid by the numbers of chromosomes in ES cell spreads and observations of obvious translocations. We find that ES cell clones with 60% or more euploid spreads (spreads that contain 40 chromosomes and no visible abnormalities) have a better chance of forming germline chimeras than clones with fewer euploid spreads. Thus, in practical terms, we consider an ES cell clone to be aneuploid and unsuitable for the production of ES-cell mouse chimeras when fewer than 60% of chromosome spreads contain 40 chromosomes. We generated and counted 820 gene-targeted ES cell clones generated from six ES cell lines derived from 129 mouse substrains and the C57BL/6-derived Bruce4 ES cell line (Table 3). Chromosome counts performed on 110 Bruce4 clones showed that only 39 were euploid (35%) by our criteria. Chromosome counts of the 710 clones derived from 129 mouse ES cell lines showed that 428 clones were euploid (60%). The proportion of euploid clones derived from Bruce4 was lower than the proportion of euploid clones derived from 129 ES cell lines ($p < 0.001$, two-tailed χ^2 test). To control for such variables such as cell culture conditions, drug resistance cassettes in targeting vectors, and other variables, we performed a series of gene-targeting experiments in parallel with Bruce4 and W4 (129S6/SvEvTac) ES cells (Table 4). Under these conditions we observed more aneuploid gene-targeted in the Bruce4 cells than in the W4 cells ($p < 0.001$ by two-tailed χ^2 analysis). As a result of the higher proportion of aneuploid ES cell clones, more targeted ES cell clones must be generated and characterized to produce gene-targeted mice when Bruce4 cells are used instead of 129-derived ES cells such as W4, R1, E14Tg2a.4, and Pat5. Studies of additional C57BL/6 ES cell lines are required to determine if this is a general characteristic of C57BL/6 ES cell lines.

Table 2 Number of SSLP markers that differed between C57BL/6J mice and C57BL/6 ES cell lines

	ES cell lines						
	C57BL/6J	Bruce4	CMTI-2	WB6d	C2	BL/6#3	Dale1
C57BL/6J	—	34	34	6	6	3	3
Bruce4		—	0	35	35	35	35
CMTI-2			—	35	35	35	35
WB6d				—	2	8	8
C2					—	8	8
BL/6#3						—	6
Dale1							—

Results from 275 SSLPs are shown

ES cell subcloning

ES cell subcloning can be used to generate cell lines that form germline chimeras with greater efficiency than the parental cell line (Nagy et al. 1993). We selected targeted ES cell clones for subcloning from two gene-targeting projects with low targeting frequencies in which only aneuploid clones were identified. We tested the effectiveness of developing euploid subclones from aneuploid clones of R1 (129 mouse strain) and Bruce4 (C57BL/6) ES cell lines.

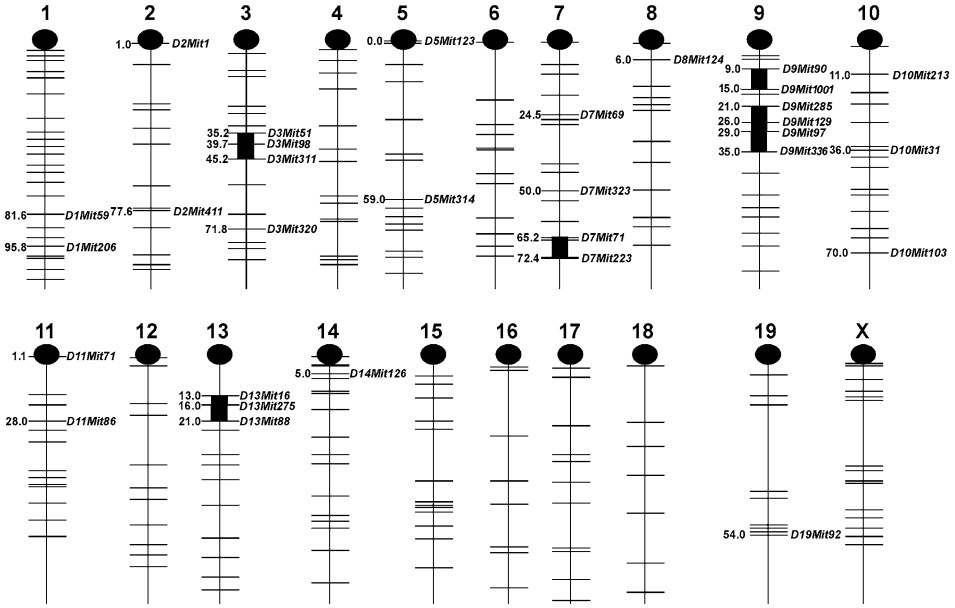

Fig. 1 Location of heterozygous regions in Bruce4 ES cell genomic DNA. Mouse chromosomes 1–19 and X are depicted schematically with centromeres indicated by the black circles; distances are shown in centimorgans. The position of each SSLP that was identical in Bruce4 and C57BL/6J is shown by a thin line. All Bruce4 SSLPs that differed from C57BL/6J in Table 1 are identified by name. The thickened portions of chromosomes 3, 7, and 9 indicate minimum regions of heterozygosity in Bruce4 ES cell genomic DNA

Table 3 Genetic stability of gene-targeted ES clones

ES cell line	Clones tested[a]	Euploid clones[b] (%)	No. of experiments[c]	Mouse strain	Reference
Bruce4	110	39 (35%)	17	C57BL/6-Thy1.1 (congenic)	Kontgen et al. 1993
R1	435	281 (65%)	114	(129X1/SvJ × 129S1/SvImJ)F_1	Nagy et al. 1993
CJ7	115	49 (43%)	13	129S1/SvImJ	Swiatek and Gridley 1993
GSI-1	65	36 (55%)	9	129X1/SvJ	Genome Systems, Inc.
Pat5	33	23 (70%)	5	129X1/SvJ	Domino et al. 2001
W4	38	19 (50%)	20	129S6/SvEvTac	Auerbach et al. 2000
E14Tg2a.4	24	20 (83%)	5	129P2/OlaHsd	Skarnes 2000

[a] Number of gene-targeted ES clones for which chromosome counts were made

[b] Number of euploid ES clones and percentage of clones that were euploid. Clones were considered euploid if 60% or more of chromosome spreads contained 40 chromosomes

[c] Number of gene-targeting experiments (electroporations of ES cell lines with targeting vectors) to produce gene-targeted clones for chromosome counting

Table 4 Comparison of aneuploidy in gene-targeted Bruce4 and W4 ES cell clones produced in parallel electroporation experiments

Electroporation number	Gene-targeted ES cell clones			
	Bruce4		W4	
	Euploid	Aneuploid	Euploid	Aneuploid
060807	2	7	2	1
060508	0	4	1	1
060227	4	10	0	1
060630	0	1	7	3
060631	1	1	3	6
Total	7	23	13	12

Four gene-targeted ES cell clones were identified in the course of targeting the *Prop1* locus in R1 ES cells (Nasonkin et al. 2004). In an attempt to obtain a euploid gene-targeted ES cell line for chimera production, we derived six subclones from one of the clones, SC3, which had a ring chromosome. All six SC3 subclones had a greater proportion of euploid spreads than the SC3 parental line, with five meeting our criteria for euploidy, and one of these produced germline transmission of the targeted allele (Table 5).

In a separate gene-targeting project we identified nine targeted Bruce4 ES cell clones that were all aneuploid (data not shown). We derived six subclones from the SE1D9 aneuploid targeted clone. Chromosome counts of the parental SE1D9 clone showed a 1:1:1 ratio of chromosome spreads with a visible translocation, spreads with a modal number of 41 chromosomes, and euploid spreads. Unfortunately, all six subclones were aneuploid. A majority (60%) of the subclones had chromosome spreads that displayed the translocation while 70% of the spreads

from the parental SE1D9 clone had the translocation. None of the SE1D9 subclones met our criteria for ES cell-chimera production (Table 5). The cell passage number of the R1 ES cells used to produce the SC3 parental clone was 18, while the cell passage number of the Bruce4 ES cells used to produce the SE1D9 parental clone was 16. The difficulty in deriving euploid SE1D9 subclones cannot be attributed to a higher passage number, which is known to increase aneuploidy (Longo et al. 1997). The data suggest that cells with euploid chromosome numbers in the SE1D9 subclones were unstable and deteriorated as the subclones were established in culture. The inability to generate euploid subclones from the aneuploid SE1D9 parental clone is consistent with the observation that Bruce4 ES cell clones are more likely to be aneuploid (Table 3).

Discussion

The genetic background of ES cell lines and mouse strains derived from ES cell lines affects phenotypes of mutant mice and should be considered when interpreting data from mouse models (Linder 2006). We performed genome scans on six C57BL/6 ES cell lines to compare their genetic makeup to the C57BL/6J genome. Genomic DNA from C57BL/6J mice and the Bruce4, CMTI-2, WB6d, C2, BL/6#3, and Dale1 ES cell lines are not genetically identical. The six C57BL/6 ES cell lines we analyzed can be divided into three related pairs based on differences with the C57BL/6 reference DNA: BL/6#3 and Dale1, WB6d and C2, and Bruce4 and CMTI-2. BL/6#3 and Dale1 are most closely related to C57BL/6J and to each other (Table 2). The WB6d and C2 cell lines are genetically alike, with minor differences compared to C57BL/6J. Bruce4 and CMTI-2 ES cells are identical to each other and differ the most from C57BL/6J, with significant areas of heterozygosity on chromosomes 3, 7, and 9. The differences between BL/6#3 and Dale1 are not surprising since they were derived from C57BL/6J and coisogenic B6(Cg)-Tyr^{c-2J}/J mice, respectively. Differences between WB6d and C2 are not expected since they were both derived from C57BL/6NTac mice, a distinct substrain of C57BL/6. It may be that the isolated differences between these ES cell lines arose in cell culture after they were derived (see below). Bruce4 and CMTI-2 ES cells were genetically identical at every SSLP tested. This was unexpected since Bruce4 was derived from a Thy1.1 congenic strain (Kontgen et al. 1993) and CMTI-2 was derived from "C57/BL6" mice Specialty Media, Inc. (Phillipburg, NJ). Our data suggest that although these two ES cell lines have different names, were obtained from different sources, and are derived from different mouse strains, they are in fact genetically identical.

Observed genetic variation in ES cell lines from the reference C57BL/6J genome may originate in genetic differences that exist between C57BL/6 mouse substrains or in genetic changes secondary to cell culture. The source of genetic variation in Bruce4 (and its genetic twin CMTI-2) is most likely the genetic contribution from the NZB mouse strain used to produce the C57BL/6-Thy1.1^{NZB} congenic mouse used to derive Bruce4 ES cells (Kontgen et al. 1993). Although 34 SSLP markers gave heterozygous results with Bruce4 DNA, only *D1Mit206* gave two PCR products that both failed to match the reference C57BL/6J genome (Table 1). The divergence from C57BL/6J at *D1Mit206* may be the consequence of a genetic change that occurred during Bruce4 cell culture; however, this seems unlikely since both ES cell lines have the identical genotype at *D1Mit206*. The BL/6#3 ES cell line was derived from C57BL/6J mice (Schuster-Gossler et al. 2001); thus, variation from the reference C57BL/J genome is most likely the result of changes during cell culture (3 SSLP markers in BL/6#3 are heterozygous). Like BL/6#3, the Dale1 ES cell line differs at 3 SSLP markers. Because Dale1 is derived from the coisogenic B6(Cg)-Tyr^{c-2J}/J background, we expected to find limited variation. However, without a genome scan of another B6(Cg)-Tyr^{c-2J}/J DNA source, it is difficult to infer whether the differences are inherent in the donor mouse genome or if they are secondary to cell culture. Comparison of WB6d (Auerbach et al. 2000) and C2 ES cell lines (derived from C57BL/

Table 5 Subclones of targeted aneuploid ES cell clones

ES cell line mouse strain	Parental clone	Subclone	Spreads counted[a]	Euploid spreads[b] (%)
R1	SC3		40	11 (28%)
		SC3.A3c	42	46 (91%)
		SC3.A2c	32	35 (91%)
		SC3.H1	39	34 (87%)
		SC3.F1c	43	50 (86%)
		SC3.H4	33	20 (61%)
		SC3.G4	23	13 (56%)
Bruce4	SE1D9		10	3 (30%)
		SE1D9.C3	10	3 (30%)
		SE1D9.D4	10	3 (30%)
		SE1D9.D7	10	1 (10%)
		SE1D9.B2	10	0 (0%)
		SE1D9.C6	10	0 (0%)
		SE1D9.B5	10	0 (0%)

[a] Total number of chromosome spreads counted

[b] Number of chromosome spreads that contained 40 chromosomes

[c] ES cell clones were used to produce ES cell-mouse chimeras that gave germline transmission of the targeted *Prop1* gene (Nasonkin et al. 2004)

6NTac) to each other shows that they are identical except for *D10Mit233* where WB6d is heterozygous and for *D9Mit151* where C2 is heterozygous. Heterozygosity at a single SSLP marker in these two cell lines is most easily explained by genetic changes that occurred during cell culture than by genetic differences in the C57BL/6NTac mice from which they were derived. Future experiments may determine if these genetic variations are due to differences between C57BL/6 mouse substrains or to random genetic changes that are fixed in the cell lines during cell culture.

The genetic diversity of 129 mouse strains and ES cell lines is well known (Simpson et al. 1997; Threadgill et al. 1997). In 129 mouse strains, 129X1/SvJ mice are markedly different in comparison to 14 other 129 mouse strains (55–58 SSLP differences out of 212). Bruce4 ES cells are similarly distinct from four other ES cell lines (34–35 SSLP differences out of 275). The consequences of genetic variation in 129 ES cell lines complicate the interpretation of phenotypes in gene-targeted mice (Silva et al. 1997; Simpson et al. 1997; Threadgill et al. 1997). Compared with the genetic variation among 129 mouse strains (as many as 27.4% of SSLPs were different), the genetic variation between Bruce4, BL/6#3, Dale1, WB6d, and C2 cell lines is not as great (a maximum of 12.7% of the SSLPs were different). Nevertheless, the genetic differences of C57BL/6 ES cell lines should be taken into account during phenotype analysis, particularly in mice derived from the commonly used Bruce4 ES cell line.

We found that targeted ES cell clones obtained from Bruce4 cell lines were more likely to be aneuploid and unsuitable for ES cell-mouse chimera production than 129-derived ES cell lines such as R1, E14Tg2a.4, and Pat5. This is significant because Bruce4 ES cells are the most widely used C57BL/6 ES cell line in gene targeting. A survey of 5,344 gene-targeted mouse strains obtained from the Jackson Laboratory shows that 1.3% of the strains were prepared with C57BL/6 ES cells ($N = 70$) and that 41 of them were derived from Bruce4 ES cells (58.6%, data available upon request). The next most used C57BL/6 ES cell line, BL6-III, accounted for only 14 mouse lines. The tendency to be aneuploid was high for Bruce4 ES cell clones derived from electroporations with gene-targeting vectors and for subclones of gene-targeted ES cell clones. The paucity of gene-targeted models in C57BL/6 ES cell lines in comparison to 129 mouse ES cell lines is likely due to the greater robustness and stability of 129 ES cell lines in culture. Cell culture of C57BL/6 ES cell lines is more demanding than 129 mouse ES cell culture (Auerbach et al. 2000; Hughes and Saunders, unpublished observations). A second difficulty is the need to screen large numbers of targeted clones to identify euploid clones suitable for chimera production (Table 3). A third limitation is that the proportion of C57BL/6 ES cell-mouse chimeras produced by aggregation or blastocyst microinjection is rarely greater than 50% of live births (Auerbach et al. 2000; Lederman and Burki 1991; Saunders, unpublished observations; Schuster-Gossler et al. 2001; Seong et al. 2004) which is significantly less than that typically obtained from 129 ES cells (Auerbach et al. 2000; Fedorov et al. 1997; Pease and Williams 1990; Saunders, unpublished observations; Schwartzberg et al. 1989). A fourth bottleneck is the sensitivity of C57BL/6 ES cells to the genetic background of the blastocyst donor used for microinjection or aggregation to produce germline chimeras. Just as 129 ES cell lines have strain restrictions for germline chimeras production (Schwartzberg et al. 1989). The efficiency of germline transmission is higher when the host blastocyst used to make ES cell chimeras with C57BL/6 ES cells is derived from B6(Cg)-Tyr^{c-2J}/J mice (Schuster-Gossler et al. 2001; Seong et al. 2004) instead of outbred Swiss-Webster mice (Auerbach et al. 2000), BALB/c mice (Auerbach et al. 2000; Ledermann and Burki 1991; Lemckert et al. 1997), or FVB/N mice (Schuster-Gossler et al. 2001). The limited availability and higher cost of B6(Cg)-Tyr^{c-2J}/J mice compared with inexpensive outbred mouse strains such as ICR (Pease and Williams 1990), CD-1, or Swiss Webster (Auerbach et al. 2000) increases the expense of producing ES cell-mouse chimeras.

Despite numerous disadvantages, the production of genetically engineered mouse strains directly in a C57BL/6 gene background remains appealing because mutant phenotypes can be compared with data from other C57BL/6 mouse studies. The use of C57BL/6 ES cell lines instead of 129 mouse ES cell lines reduces the need for backcrossing. Access to the public C57BL/6J mouse sequence and the RPCI-23 C57BL/6J BAC genomic library is a powerful tool that facilitates the production of gene-targeting vectors for the manipulation of genes in C57BL/6 ES cell lines (Liu et al. 2003; Osoegawa et al. 2000). The BL/6#3, Dale1, C2, and WB6d cell lines carry minor differences compared with C57BL/6J mice but have not been widely used to produce gene knockout mice. The Bruce4 ES cell line is one of the most commonly used C57BL/6 ES cell lines. It is also the C57BL/6 ES cell line most genetically divergent from C57BL/6J. Because 12.4% of the SSLP markers tested on Bruce4 DNA are heterozygous, these cells resemble an N3 backcross of a heterozygous line to C57BL/6J. Thus, mice generated from the Bruce4 ES cells should not be considered isogenic with C57BL/6J. Mouse strains derived from the C57BL/6 ES cells described here will not be coisogenic to C57BL/6J mice. Backcrosses to C57BL/6J will be required to transfer gene-targeted mutations to a defined C57BL/6J background, especially for Bruce4-derived strains. If C57BL/6J ES cells were available, then germline transmission from chimeras and

their heterozygous offspring would immediately produce homozygous mutant mice for direct comparison to C57BL/6J baseline data. This will not be possible until C57BL/6J-derived ES cell lines are available.

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