Improved Generation of C57BL/6J Mouse Embryonic Stem Cells in a Defined Serum-Free Media

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Summary: C57BL/6 is a well-characterized mouse strain that is used extensively for immunological and neurological research. The establishment of C57BL/6 ES cell lines has facilitated the study of gene-altered mice in a pure genetic background—however, relatively few such lines exist. Using a defined media supplement, knockout serum replacement (KSR) with knockout DMEM (KSR-KD-MEM), we find that we can readily establish ES cell lines from blastocysts of C57BL/6J mice. Six lines were established, all of which were karyotypically normal and could be maintained in the undifferentiated state on mouse embryonic fibroblast (MEF) feeders. One line was further tested and found to be karyotypically stable and germline competent, both prior to manipulation and after gene targeting. For this cell line, efficiencies of cell cloning and chimera generation were greater when maintained in KSR-KD-MEM.

Twenty-three blastocysts flushed from uterine horns of naturally mated 3.5-day pregnant C57BL/6J females were transferred onto MEF feeder layers and were left undisturbed for 6 days without passage. Of these, 18 blastocysts attached and 15 inner cell masses (ICM) grew. From these cell masses, six cell lines, each derived from an independent blastocyst, were established. The morphology of one such ES cell line is shown in Figure 1a. In contrast, using fetal bovine serum-DMEM (FBS-DMEM), our usual growth media for ES cells, we were unable to obtain any cell lines from 19 blastocysts cultured in the same experiment. A repeat experiment gave similar results with three ES cell lines derived from 24 C57BL/6J blastocysts cultured in KSR-KD-MEM. In a total of five experiments, we cultured 105 C57BL/6J blastocysts in FBS-DMEM from which we were able to see 32 ICMs. However, in our hands we were unable to establish any ES cell lines from these cultures (Table 1).

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Embryonic stem (ES) cells have provided a powerful tool for the study of gene function and regulation, the generation of animal models for human diseases, and the investigation of cell differentiation (Kokron et al., 1997; Shastry, 1998). Most available ES cell lines are derived from various 129 mouse strains. However, given the wide range of 129 substrains and the genetic variability of these strains, many researchers backcross gene-targeted mice to other inbred strains. For a number of reasons, the C57BL/6J mouse strain has been extensively used as a reference strain (Kontgen et al., 1993). The phenotypes of many mouse mutants have been studied in the C57BL/6J strain, including a variety of immunological and behavioral mutants (Crawley et al., 1997). Second, because the genome of C57BL/6J is being sequenced as part of the Human Genome Project, extensive mapping, sequence, and genetic data are available for this strain (Nadeau et al., 2001). Finally, generation of conventional transgenic mice in C57BL/6J. For all of these reasons, investigators have backcrossed mutant mice to C57BL/6J, a laborious process that requires extensive backcrossing even with the use of speed congenics.

Establishment of ES cell lines derived from C57BL/6J strain has been a major step to help facilitate the direct genetic alteration of mice in a pure C57BL/6J genetic background (Auerbach et al., 2000; Kawase et al., 1994; Kontgen et al., 1993; Ledermann and Burki, 1991; Lemckert et al., 1997). However, there are relatively few ES cell lines derived from C57BL/6J mice. In an effort to facilitate the establishment of ES cells lines, we utilized a defined media supplement, KSR, with an optimized KD-MEM. We have found that cells cultured in this media are more readily disrupted upon trypsinization, a critical step in the early culture of ES cells.

 Twenty-three blastocysts flushed from uterine horns of naturally mated 3.5-day pregnant C57BL/6J females were transferred onto MEF feeder layers and were left undisturbed for 6 days without passage. Of these, 18 blastocysts attached and 15 inner cell masses (ICM) grew. From these cell masses, six cell lines, each derived from an independent blastocyst, were established. The morphology of one such ES cell line is shown in Figure 1a. In contrast, using fetal bovine serum-DMEM (FBS-DMEM), our usual growth media for ES cells, we were unable to obtain any cell lines from 19 blastocysts cultured in the same experiment. A repeat experiment gave similar results with three ES cell lines derived from 24 C57BL/6J blastocysts cultured in KSR-KD-MEM. In a total of five experiments, we cultured 105 C57BL/6J blastocysts in FBS-DMEM from which we were able to see 32 ICMs. However, in our hands we were unable to establish any ES cell lines from these cultures (Table 1).
Some.

reveals 40 XY chromosomes. The arrow indicates the Y chromo-

somal fibroblasts (magnification ×50). b: DAPI stained karyotype

reveals 40 XY chromosomes. The arrow indicates the Y chromo-
some. c: Spectral karyotyping analysis demonstrates a normal
karyotype.

To further characterize the properties of the cell lines
established, we examined them by conventional (Fig. 1b) as well as spectral karyotyping (SKY) (Fig. 1c). All of
the ES cell lines were euploid, with 40 chromosomes.

Three of the ES cell lines examined were male (XY) and the remaining three female (XX). Since male embryonic
stem cells are usually more karyotypically stable and provide an advantage by sex-converting the embryo, we
selected one of the XY cell lines (line HGTC-8) for
further characterization.

Wildtype male HGTC-8 ES cells maintained in KSR-
KDMEM were injected into BALB/c blastocyst recipients;
15 out of 16 pups born were chimeras with high degrees of contribution from the ES cells (Fig. 2a) and 14 out of
15 chimeras were male, suggesting that efficient sex-
conversion of the embryos had occurred. Since BALB/c are wildtype (A/A) at the agouti locus and BALB/c ×
C57Bl/6 F1 progeny are agouti, only the F1 progeny
derived from the C57Bl/6 ES cells will have a black
nonagouti coat color. To determine whether the
C57Bl/6/F6 ES cells could contribute to the germline, nine
male chimeras were bred to C57Bl/6/F6 wildtype females.
Seven of the nine chimeras tested were germline trans-
mitters by coat color. Of these, five transmitted to 100%
of their progeny as determined by coat color (Fig. 2b),
and the other two were partial germline transmitters.
Thus, HGTC-8 ES cells demonstrated a high efficiency of
germline transmission in the unmanipulated state.

To evaluate gene targeting in the HGTC-8 ES cell line, we
generated a C57Bl/6/F6 gene-targeting construct that dis-
rupted Rlk, a gene that we have previously targeted in a
Δ to 12956 cell line (Schaeffer et al., 1999). The linear-
ized Rlk construct was electroporated into the HGTC-8
ES cell line and selected for resistance to G418 Sulfate and
1-2'-deoxy-2'-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU).
In all, 240 colonies were isolated, from which two positive recombinants at the Rlk locus were ob-
tained, similar to the frequency we previously observed
using an isogenic construct in a Δ to 12956-derived cell
line. A second independent C57Bl/6/F6 isogenic DNA con-
struct targeting the NEMO gene gave four positive gene-
disrupted clones out of 120 clones screened (J.C., L.G-B,
P.L.S, unpubl. results).

Two recombinant clones from each construct were
microinjected into BALB/c blastocysts. Both constructs
resulted in chimeras and, to date, one Rlk clone has
given rise to a germline chimera as evidenced both by
coop color and by genotyping (Fig. 3). These results

demonstrated that gene targeting in the HGTC-8
C57Bl/6/F6 ES cells was successful.

In these initial gene-targeting experiments, we trans-
fected HGTC-8 cells that we had propagated and ex-
panded in FBS-DMEM for several generations and the
selection of clones was performed in media with FBS.
However, we noted that the chimeras obtained in these
experiments exhibited lower percentage chimerism
than in our original studies. To determine whether use of
KSR would improve cell cloning and chimera produc-
tion, we transfected in parallel cells that either had been
maintained only in KSR-KDMEM or had been switched to
FBS-DMEM containing media. Cells were transfected
with the pPNT plasmid (Tybulewicz et al., 1991) and
colonies were selected and expanded in each transfec-
tion in the respective media. Cells transfected and grown
in KSR-KDMEM gave rise to more than 2-fold greater
numbers of colonies and 3-fold more chimeras per in-
jected blastocysts compared to the same cells grown in
FBS-DMEM media (Table 2). Although we obtained germ-
line transmission with cells grown in either media, the
chimeras produced from cells in KSR media exhibited
higher levels of chimerism and germline transmission
than those derived from cells grown in FBS containing
media (Fig. 4a,b).

In this article we describe the improved generation of
ES cell clones from C57Bl/6/F6 mice in a defined medium.
Independent of the approach chosen, the establishment
of ES cell lines depends largely on the availability of
optimal culture conditions and on the technical skill of
the investigator. In the establishment of these C57Bl/6/F6
ES cell lines, we have found that KSR-KDMEM provided
several advantages in the initial stage of the work.

First, we noted that we had difficulty growing ES cells
from C57Bl/6/F6 strain in serum-supplemented ES cell
medium after the ICM cells were trypsinized. However, we
have found that cells disassociate more readily upon
tryptsinization after growth in KSR-KDMEM and we ob-
tained better growth post-trypsinization. It has also been
suggested that FBS may contain potential differentiation
and attachment factors. KSR-KDMEM may lack such fac-
tors and provide a differentiation factor-free growing
environment for ES cells. It is possible that the optimized
DMEM formulation knockout DMEM (KDMEM) may further improve ES cell culture. It should be noted that in these experiments the osmolarity of KSR-KDMEM was also lower than the media containing FBS (KSR-DMEM, 306 mOsm versus FBS-DMEM, 334 mOsm). It is also noteworthy that although our KSR-derived cell lines looked normal when grown in FBS-DMEM, they transfected more efficiently and gave rise to greater numbers of clones and chimeras when maintained in KSR-KDMEM.

A second modification in our protocol for generating ES cells was to leave the ICM undisturbed for at least 6 days, even though peripheral cells in the colonies had differentiated into flat endodermal cells. Colonies were allowed to expand as long as the majority of the cells remained undifferentiated. This allowed for the increase of the absolute number of undifferentiated ES cells. By carefully selecting the undifferentiated central mass, we were able to expand cells more readily.

The generation of genetically defined mice by homologous recombination in ES cells has been instrumental in the study of human genetic diseases and basic biology. However, since the evaluation of phenotypes produced from gene targeting may vary, depending on the strain of mice used and the strain to which the transgenic mouse is backcrossed, it has become increasingly important to establish ES cell lines in pure genetic backgrounds (Auerbach et al., 2000; Crawley et al., 1997). The increased availability of ES cell lines from different mouse strains is therefore an important resource for the mouse community. Our results suggest that the use of defined media may more readily permit the establishment of ES cells from different mouse strains.

Our findings may also be useful for the establishment of cell lines from mutant animals. Approximately 12–14% of all knockout mice generated from gene targeting are embryonic lethal (http://research.bmn.com/mkd). To complement the analysis of embryonic lethality, we have developed a method to isolate ES cell lines in different medias, as shown in Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Medium</th>
<th>Feeder cells</th>
<th>LIF</th>
<th>No. of blastocysts cultured</th>
<th>No. of ICM appeared</th>
<th>No. of ES cell lines established</th>
<th>XY</th>
<th>XX</th>
<th>No. of chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FBS-DMEM</td>
<td>MEF</td>
<td>–</td>
<td>20</td>
<td>7</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FBS-DMEM</td>
<td>MEF</td>
<td>+</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>FBS-DMEM</td>
<td>MEF</td>
<td>+</td>
<td>23</td>
<td>7</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>KSR-DMEM</td>
<td>MEF</td>
<td>+</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>FBS-DMEM</td>
<td>MEF</td>
<td>+</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>KSR-DMEM</td>
<td>MEF</td>
<td>+</td>
<td>12</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

*Not tested.

**FIG. 2.** Chimeric mice and F1 offspring. a: Chimeric mice, generated from wildtype C57BL/6J ES cell line, HGTC-8 injected into Balb/C blastocysts. b: 100% black-agouti F1 offspring were produced when a chimeric mouse mated to C57BL/6J wildtype female.

**FIG. 3.** Genotyping F1 of RLK knockout mice by PCR. A 430 bp DNA fragment corresponding to the inserted neo gene was amplified by PCR from genomic DNA of F1 progeny. Lane A1 contains DNA molecular weight markers. Lane A2 a neo-positive control, A3 a neo-negative control, and lanes A4 to A18 are F1 samples. Seven out of 15 F1 samples were positive for neo.
onic lethal phenotypes, investigators often isolate mouse embryonic fibroblasts and other differentiated cell types. However, for early embryonic phenotypes or studies of differentiation in vitro, it is often useful to establish a homozygous null embryonic stem cell line. A null ES cell line may be created by either gene conversion (by increasing the concentration of the selectable marker-G418) or gene targeting of the second allele by transfection. However, homozygous ES cell lines can also be established from mutant blastocysts. Our work suggests that the use of KSR-KDMEM may help facilitate the generation of ES cell lines from many strains and mutants, therefore reducing labor-intensive and time-consuming backcrosses and furthering genetic studies in the mouse.

MATERIALS AND METHODS

ES Cell Medium: KSR-KDMEM and FBS-DMEM
KSR-KDMEM: KDMEM (GIBCO, Gaithersburg, MD; 10829018) was supplemented with 15% KSR (GIBCO; 10828), 1,000 units per ml leukemia inhibitory factor (LIF) (GIBCO; 13275-029), 4 mM L-glutamine (GIBCO; 25030-081), 100 μM NEAA (GIBCO; P332-100), 100 μM 2-mercaptoethanol (GIBCO; 21985-023), and 50 μM penicillin/50 μg/ml streptomycin (GIBCO; 15070-063) (Goldsborough et al., 1998). FBS-DMEM: DMEM (GIBCO; 1178767) was supplemented with LIF, L-glutamine, NEAA, 2-mercaptoethanol, and penicillin/streptomycin as in KDMEM, but 15% fetal bovine serum (FBS; Atlanta Biological, Atlanta, GA; S11550) was used instead of KSR. Osmolarity of media was measured using an Advanced Instruments Micro Osmometer (Norwood, MA; Model 3300).

Preparation of ES Cell Feeder Layers
Mitomycin-treated mouse embryonic fibroblasts (MEF) were derived from 14-day postcoital (dpc) β2 microglobulin -/- embryos. Feeder layers (a total of 4 × 10⁶ feeder cells per 24-well plate (Costar, Cambridge, MA; 3524), 96-well (Costar; 3596), or 6-well plate (Costar; 3527)) were cultured on 0.01% gelatin-coated plates (Sigma, St. Louis, MO; G-2500) in serum-supplemented media overnight. Feeder cells were washed with 1× PBS to reduce serum just prior to plating ES cells in KSR-KDMEM (Goldsborough et al., 1998).

Embryo Recovery, Embryo Culture, and ES Cell Line Isolation
Blastocysts were obtained from natural matings of 6–8 week-old C57BL/6J females (Jackson Laboratory, Bar Harbor, ME) maintained in a pathogen-free facility according to NIH guidelines. Blastocysts were flushed out from uterine horns of 3.5-day pregnant females (Hogan et al., 1994). Embryos were transfected onto MEF feeder layers in the KSR-KDMEM, or FBS-DMEM ES media, in 96-well plates by mouth-controlled glass pipettes and cultured at 37°C in 5% CO₂ in humidified air for 6–7 days without media changes. The ICM, which consists of the

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Here is a table illustrating the Table 2: Transfection and Injection of pPNT in B6 ES cells in KSR-ES media or FBS-ES media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Construct</th>
<th>No. of colonies appeared</th>
<th>No. of colonies examined</th>
<th>Karyotypes</th>
<th>Clone injected</th>
<th>No. of blast. transferred</th>
<th>No. of mice born (% of blast. transferred)</th>
<th>No. of chimeras (% of mice born)</th>
<th>No. of males</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSR-ES media</td>
<td>pPNT 25 ug</td>
<td>2880</td>
<td>48</td>
<td>normal</td>
<td>KSR-1</td>
<td>60</td>
<td>18 + 2&quot; (33%)</td>
<td>7 (39%)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KSR-2</td>
<td>36</td>
<td>9 + 1&quot; (27%)</td>
<td>4 (44%)</td>
<td>3</td>
</tr>
<tr>
<td>FBS-ES media</td>
<td>pPNT 25 ug</td>
<td>1038</td>
<td>48</td>
<td>normal</td>
<td>FBS-1</td>
<td>36</td>
<td>4 + 2&quot; (17%)</td>
<td>1 (25%)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FBS-2</td>
<td>96</td>
<td>12 + 3&quot; (16%)</td>
<td>4 (33%)</td>
<td>4</td>
</tr>
</tbody>
</table>

*Mice born but died early before reliable inspection of coat color.
central mass of stem cells and peripheral primitive endoderm-like cells, were identified and transferred into 200 μl of 0.125% trypsin-EDTA solution (GIBCO; 25200-056) into new 96-well plate. The ICM were treated with trypsin for 5 min at 37°C and gently disrupted by pipetting with a 200 μl pipetman. Then 50 μl of the trypsinized ICM were transferred individually and subcultured in 1.5 ml in 24-well feeder plates. Four to five days later, single cell clones of compact stem cell colonies were dissociated as described above and reseeded. Nondifferentiating clonal lines were split and a portion of the cells frozen. The remaining cells were passaged onto 6-well plates and then split after 2–3 generations for further characterization (Hogan et al., 1994).

Karyotype and SKY Analysis of ES Cell Lines

ES cells were cultured until 50% confluency in one 10-cm cell culture dish (Corning, Corning, NY; 430167). Cells were treated with colcemid (Roche, Nutley, NJ; 295892) at a final concentration of 100 ng/ml for 1.5 h, then harvested. After a hypotonic treatment, the cells were fixed in 3:1 ratio of methanol to acetic acid. Slides were stained with DAPI/Antifade (Vector, Burlingame, CA; H-1200) and chromosomes were then analyzed under a fluorescent microscope. Spectral karyotyping (SKY) was performed as described previously (Liyangage et al., 1996)

ES Cell Transfection and Genotyping

Isogenic targeting constructs generated from C57BL/6J BAC DNA and the vector pPNT were linearized by Not I restriction digestion. Two times 10^5 HGT/C-8 ES cells were transfected with 25 μg of linear DNA in 1 ml of PBS in a 0.4 cm cuvette, using a BioRad (Hercules, CA) Gene Pulser (600 volts, 25 μFd, and 0.4 s), and maintained in either FBS-DMEM or KSR-KDMEM. Cells were changed 24 h later to selection medium containing FIAU (0.2 μM) and G418 (280 μg/ml) for the isogenic DNA transfections and G418 only for the pPNT transfection experiment. Seven days later, colonies were selected and expanded for freezing and DNA analysis.

Genotyping of the RLK locus in ES cells was performed as previously described (Schaeffer et al., 1999) F1 progeny of targeted mice were genotyped by PCR with neo-primers: (5’-AGAGGCTATTGGCTATGACT-3’ and 5’-TTGTCCAGATCATCCTGATC-3’). Germline transmitting mice were identified by amplification of a 430 bp PCR product.

Chimera (pPNT Chimeras and Rlk Chimeras) Production

The wildtype and gene-targeted HGT/C-8 ES cells were trypsinized and resuspended into blastocyst injection media (DMEM GIBCO; 12800-017 supplemented with 10% KSR or 10% FBS, 4 mM L-glutamine, pH 7.3). Approximately 15–20 C57BL/6J ES cells were injected into each blastocysts of naturally mated 6–8-week-old BALB/c females. After a recovery period of ~2 h, injected blastocysts were transferred into the uterine horns of 2.5 dpc pseudopregnant Swiss Webster (Taconic Farms, Germantown, NY) recipient mothers (Hogan et al., 1994). Chimeric offspring were identified by eye and coat pigmentation. Chimeric males were bred with C57BL/6J wildtype females for testing germline transmission.

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LITERATURE CITED


