Production of a Homozygous Mutant Embryonic Stem Cell Line (Double Knockout)

Richard Mortensen[1]

[1]University of Michigan Medical School, Ann Arbor, Michigan

ABSTRACT

Under some circumstances, it may be desirable to produce a mouse cell clone in which both alleles of a desired gene are mutated. This may be because the mutation causes embryonic lethality in homozygous animals, or to test cultured cells before an animal is produced. This protocol details an easy method for obtaining homozygous cells by homologous recombination without the need for two targeting events. *Curr. Protoc. Mol. Biol.* 82:23.6.1-23.6.4. © 2008 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

Homozygous mutant embryonic stem (ES) cell lines have proven very useful in studying gene and protein function, and there are several reasons why this system might be preferred. Because many genes express their phenotype at the level of individual cells, using these cell lines eliminates the time and expense of producing a mutant animal. Homozygous mutant cells can be isolated directly from a heterozygous mutant cell line (*UNIT 23.5*) without rederiving a cell line or using primary tissues from a mutant animal. In some cases, analysis of the phenotype of homozygous mutant cells can be performed more easily in culture. In addition, homozygous mutant ES cell lines may be viable even when a homozygous mutation is lethal to the animal. Finally, microinjection of homozygous mutant ES cells into blastocysts may allow investigation of the developmental potential of cells with such lethal mutations.

Selection of a homozygous cell line depends on being able to increase antibiotic concentrations such that nearly all of the cells containing a single copy of the selectable marker gene will be killed. If the wild-type *neo* gene is used in combination with a strong promoter (e.g., phosphoglycerate kinase; PGK), cells containing one *neo* gene may not be killed by the highest G418 concentrations that can be obtained. For this technique to be effective, therefore, it is recommended that a *neo* gene containing a point mutation that decreases phosphotransferase activity be used in place of wild-type *neo* (Yenofsky et al., 1990). This mutant *neo* gene is readily available because it is contained in some versions of both pMC1-*neo* and p_{PGK}-*neo* (see Fig. 23.5.1). The wild-type hygromycin-B-phosphotransferase gene (*hyg*) used in combination with the PGK promoter, p_{PGK}-*hyg*, has also worked well in the method described in the Basic Protocol.

Alternatively, using a construct with *loxP* sites (such as those derived from pTKLNL or pTKLNCL) or *Frt* sites flanking the *neo* marker allows the *neo* marker to be removed from both alleles simultaneously after homozygous cells are obtained. Using this strategy, one can still use high-G418 selection to isolate homozygous cells, but then remove the *neo* marker to avoid any expression changes from the *neo* sequence (removal of *neo* sequences is shown in Fig. 23.1.7 and 23.1.8).

In this protocol, a homozygous mutant ES cell line is isolated from heterozygous mutant ES cells (*UNIT 23.5*) by culturing the cells in increasing concentrations of G418. The more stringent selection conditions favor cells that contain, as a result of the loss of

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heterozygosity, two copies of the selectable marker gene (e.g., for *neo*), and therefore two mutant alleles of the target gene. Resulting colonies are harvested and screened by northern hybridization or immunoblotting to confirm that the target gene is inactive.

Materials

Heterozygous mutant ES cell line, frozen in liquid nitrogen (*UNIT 23.5*)
ES/LIF medium (*UNIT 23.5*)
G418 (*UNIT 9.5*)
100-mm tissue culture plates, gelatin coated (*UNIT 23.5*)

Additional reagents and equipment for recovery of frozen cell lines (*UNIT 11.9*), ES cell culture (*UNIT 23.2 & 23.3 & APPENDIX 3F*), northern analysis (*UNIT 4.9*), and immunoblotting (*UNIT 10.8*)

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise noted.

1. Thaw heterozygous mutant ES cells. Culture in ES/LIF medium, passaging cells every 2 to 3 days by seeding a 100-mm gelatin-coated tissue culture plate with 1–2 $\times$ 10^6 cells/plate.

   Selection should be performed with more than one heterozygous mutant ES cell line clone because the efficiency of conversion to homozygosity varies among cell lines. In addition, more than one clone should be analyzed for the phenotype.

   Leukemia inhibitory factor (LIF) prevents ES cells from differentiating.

   Some investigators suggest passaging cells at a higher density if blastocyst injection of the cells (UNIT 23.4) is planned (e.g., 1.5 $\times$ 10^6 cells per 25-cm^2 flask). A detailed description of culture techniques for ES cells is found in UNITS 23.2 & 23.3.

2. Begin the selection by plating three 100-mm gelatin-coated tissue culture plates at 10^5 cells/plate using ES/LIF medium and adding 1.0, 1.5, or 2.0 mg/ml G418 (final), respectively.

   It is important to neutralize the G418 to ~pH 7.4.

   Both the neo and hyg genes have been used successfully as selectable markers in mutant ES cells (R.M., unpub. observ.). To select for hyg, use ES/LIF medium containing 1.0, 1.5, or 2.0 mg/ml hygromycin-B (HPH; UNIT 9.5).

3. Incubate cells 7 to 10 days. Change the medium each day, using ES/LIF medium with the appropriate concentration of G418 added, until single colonies are detected.

   If cells overgrow plates and no single colonies are obtained, repeat steps 2 and 3 using a higher G418 concentration (e.g., 2, 4, or 6 mg/ml final).

4. Screen colonies for homologous recombinants as described in *UNIT 23.5* (Basic Protocol).

   The hybridizing fragment indicative of the unaltered target gene will be completely absent in homozygous mutant cells. Typically, 50% of the clones are homozygous, but because the process is random, individual heterozygous clones may yield homozygous clones at a frequency of 4% to 100%.

5. Perform a northern hybridization analysis (*UNIT 4.9*) of mRNA or an immunoblot analysis of protein (*UNIT 10.8*) to confirm that the target gene is inactive.

   No normal mRNA or protein should be found.

6. If the constructs contain *loxP* sites flanking the *neo* gene, remove the *neo* by transient expression of Cre (see *UNIT 23.5*, Support Protocol).

   Both copies of neo are removed with the same efficiency as one copy.

COMMENTARY

Background Information

Comparison of the phenotype of wild-type and homozygous mutant cells should provide information concerning the function of the targeted gene, provided that the gene functions in the cell assayed. ES cells offer a unique opportunity because they are capable of differentiating into any cell type. Many cell types can be produced through manipulation of in vitro cultures, including beating cardiocytes, skeletal muscle cells, neurons, glial cells, and vascular endothelial cells. Provided that the phenotype can be analyzed with a single cell or with a few cells, these differentiated cells can then be used to analyze the phenotype of the disrupted gene.

Because the normal number of chromosomes is maintained in ES cells (Mortensen et al., 1992), this method should be applicable to the study of gene function in development. ES cells can also differentiate within an organism when injected into normal blastocysts. Homozygous mutant cells offer no technical advantage over heterozygous cells if the goal is to obtain a mutant mouse line through germline transmission; however, if homozygous mutant cells are tagged by introducing a gene that has a histochemically detectable product (e.g., β-galactosidase), then the fate of the homozygous mutant cells in the animal can be determined easily. Studies of embryos derived from blastocysts injected with tagged mutant ES cells may reveal the role of the target gene during development even if the mutant gene is lethal for the animal when homozygous.

In some cases, the phenotype of the disrupted gene may be tested by introducing heterologous proteins into the cultured cells. For example, an intracellular signaling pathway may be investigated by expression of the particular receptor that creates an intracellular response to an applied agonist.

Cells produced using this protocol are only resistant to *neo* and its analogs. Thus, further genetic manipulations of the cells, such as the targeting of other genes using homologous recombination, can be performed using other drug selections (e.g., HPH; *UNIT 9.5*).

The suitability of this technique for the production of homozygous mutant cell lines other than ES cells is less certain. Homologous recombination and spontaneous loss of heterozygosity occur in some other cell lines and these same methods have been used to produce homozygous mutant cell lines; however, the methods may not be widely applicable, as other types of immortalized cells (e.g., lymphocytes or fibroblasts) may not undergo homologous recombination of a targeting construct at the same frequency as ES cells. Furthermore, many immortalized cells are markedly polyploid or aneuploid. If more than two copies of the target gene are present, this method for producing homozygous mutant cells may not be suitable; however, because these cells often lose or gain chromosomes, some clones might be isolated with no endogenous gene remaining.

The loss of heterozygosity seems to occur by several different mechanisms that vary with cell type. The mechanism by which this conversion occurs is unknown. Some possibilities include nondisjunction, chromosomal loss and duplication, or gene conversion.

Critical Parameters and Troubleshooting

Homozygous mutant ES cells will not be obtained if the mutation is lethal for the ES cell. The critical technical factor in this method is determining the amount of G418 needed to select homozygous over heterozygous cells. Differences in G418 levels required for different constructs can arise from two sources: the strength of the promoter and the influence of the genomic site on *neo* expression. In addition, the resistance to G418 may vary, depending on the enzymatic activity of a wild-type or mutant *neo* gene (Yenofsky et al., 1990). If the heterozygous mutant ES cells are resistant to the highest concentration of antibiotic (i.e., 2 mg/ml G418), the G418 concentration should be increased (e.g., up to 6 mg/ml). The endpoint should be survival of individual colonies with death of nearly all cells.

It should be noted that this selection method allows only a minority of the homozygous clones to survive. In one case, homozygous mutant cells constituted 25% of the cells (presumably due to the chance conversion of a heterozygous to homozygous cell early in the life of the clone). Nevertheless, <1% of the cells survived 1 mg/ml G418 (R.M., unpub. observ.).

It is also possible that loss of heterozygosity may not occur for all genomic sites, although no such genomic sites have yet been identified. Because the inability to isolate homozygous cells may lead to the erroneous conclusion that a homozygous mutation in a gene is lethal, the

lethality of the mutation should be verified. A rescue experiment in which a third copy of the gene (or expressed cDNA) is introduced into the heterozygous mutant cells, followed by selection of the homozygous mutant, would distinguish between lethality and no loss of heterozygosity.

An alternative method for producing homozygous mutant cells is to disrupt the second allele of the target gene using a second targeting construct that uses a different positive selectable marker. For example, if *neo* was used in the first-round construct, the *hyg* gene can be used and cells selected in HPH as described in *UNIT 23.5*.

Anticipated Results

The hybridizing fragment indicative of the altered gene will be absent in homozygous mutant cells. Typically, 50% of the clones are homozygous, but because the process is random, individual heterozygous clones may yield homozygous clones at a frequency of 4% to 100%.

Time Considerations

Expanding and selecting clones requires ~2 to 3 weeks. Screening clones takes several days.

Literature Cited

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