Simultaneous Cre catalyzed recombination of two alleles to restore neomycin sensitivity and facilitate homozygous mutations

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ABSTRACT

Cells homozygous for *neo*-expressing mutations can be derived by culturing heterozygotes with elevated G418. We demonstrate that this strategy is significantly less efficient if *hyg* is substituted for *neo*. Therefore, to introduce additional mutations Cre recombinase was used to remove floxed *neo* from both alleles of homozygotes at two different loci. The rate-determining step in Cre excision appeared independent of substrate copy number. Incorporating cytosine deaminase and Herpes simplex virus thymidine kinase allowed negative selection for both targeting and Cre excision. The resulting G418-sensitive homozygous mutants should allow mutagenesis at additional loci and avoid untoward effects of retained selection markers.

Homozygous mutant embryonic stem (ES) cell lines are useful for studying gene function in the cell line itself, during *in vitro* differentiation, or in chimeric animals (1–3). A widely used method for obtaining homozygotes employs a targeting construct containing *neo* to introduce a heterozygous mutation followed by culture in high concentrations of G418 (4) to select homozygotes arising by loss of heterozygosity (LOH). Because mutation of two or more genes may be necessary to investigate overlapping functions or interacting gene products we have investigated methods to target multiple genes in a single cell line.

This may be accomplished by targeting individual genes using multiple positive selectable markers and culture in high concentrations of the corresponding drugs. To test this approach, we produced heterozygous G-protein α_{i2} -null cells with a construct identical to that used previously (4,5) except that the *hyg* marker was substituted for *neo*. However, homozygotes were not observed after culture of heterozygotes for two or six additional passages prior to selection with elevated hygromycin B (8–15 surviving clones, all heterozygotes, per 10⁵ cells plated at 1.0, 1.5 or 2.0 mg hygromycin B/ml) but were only observed after culture for 10 passages prior to selection (16 and 11%, two and three homozygotes of 12 and 28 surviving clones, respectively, in two experiments). Therefore, LOH using *hyg* was significantly less efficient than

previously observed using *neo* in an otherwise identical α_{i2} -null allele and chromosomal context (4).

Even if efficient, mutagenesis using multiple selectable markers has disadvantages. Prolonged culture is associated with 'spontaneous' mutation, aneuploidy and other genetic instabilities that might offset advantages of analyzing combined mutations. Retained selection markers also can affect neighboring genes and alter phenotypes in mice (5,6). This might be more pronounced if more than one marker is retained in a single cell. The number of selectable markers available also limits the number of mutations that can be introduced.

For these reasons we used Cre-lox to restore G418 sensitivity in *neo* homozygotes so this efficient marker could be reused to introduce additional mutations. Cre expressed chronically can remove floxed regions in homozygotes (7,8) and transient expression can modify floxed regions in heterozygotes (9,10). We extended these observations by investigating if transient Cre expression is sufficient to modify both floxed alleles in homozygotes. Additional genes can be targeted in such cells by re-using *neo* suggesting a generally useful system for efficiently modifying a potentially large number of genes in a single cell line. This method does not require two targeting events, which can decrease the efficiency of obtaining homozygotes.

Cre expressed transiently recombines heterozygotes at a few percent to 16% efficiency (10,11). If recombination events are independent and rate limiting, the frequency of homozygous recombination might be only 0.04–2.6% (i.e. the product of the two rates). Therefore, we incorporated two negative selectable markers, HSV-TK and cytosine deaminase (CD) (12), in our targeting construct precursors pCDLNTKL and pTKLNCDL (Fig. 1) to enhance recovery after initial targeting (13) and subsequent Cre-mediated excision.

Constructs for homologous recombination were produced using pTKLNCDL and pCDLNTKL (Fig. 1). These were based on pNTK (14) and used an attenuated form of *neo* (15) that improves the efficiency of G418 selection for LOH (16). CD cDNA was obtained from Mullen (12). Stock 5-fluorocytosine (5-FC) (12.5 mg/ml water) was stored at –20°C for up to 3 months. pCDLNTKL was combined with the identical $G\alpha_{i2}$ genomic sequences used previously (17). PPAR γ genomic sequences were obtained by long polymerase chain reaction following manufacturer's instructions (Perkin Elmer) using 0.1 μ g ES cell genomic DNA and oligonucleotides in

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Figure 1. Homologous recombination constructs and allele structure. PPAR γ exon 2 is not illustrated. Southern analysis restriction enzymes are outlined. (HR, homologous recombinant; Cre, Cre recombinant; B, *Bam*HI; H, *Hind*III; S, *Sac*I; X, *Xba*I).

exons 1 and 3 and in exons 3 and 4 (18). The final PPAR γ construct utilized subclones directing partial deletion of exon 3 and the following intron to ensure gene inactivation.

Heterozygous homologous recombinants were obtained (17) by selection in 0.2 mg/ml G418 and either 2 μ M gancyclovir [cells bearing TKLNCDL (PPAR γ)] or 125 μ g/ml 5-FC [cells bearing CDLNTKL (α_{i2})]. Genotyping by Southern blot analysis (not shown) using enzymes and probes shown in Figure 1 produced predicted restriction fragments in all cases. Homologous

recombination efficiency was similar to that reported previously (17). Enrichment by negative selection was similar using either HSVTK or CD (10–20% of G418-resistant colonies were also FIAU-resistant or 5-FC-resistant, respectively). Complete killing of PPAR γ -CD heterozygotes was observed when 10^3 – 10^4 cells/100 mm plate were cultured in 125 μ g 5-FC/ml and above.

PPAR γ and α_{i2} heterozygotes were then cultured with high concentrations (1–4 mg/ml, active concentration) of G418 and surviving homozygotes were identified by Southern blot analysis (Fig. 1, not shown). Culture in 3–4 mg G418/ml was required to select homozygotes for each mutation. This is several fold higher than required previously at the same α_{i2} locus (4) and may reflect greater *neo* expression due to the additional PGK promoter/enhancer sequences associated with the retained negative selectable markers.

To remove *neo* from homozygotes and determine the rate of single and double excision events, heterozygotes and homozygotes (5×10^6 cells/0.4 cm cuvette, PBS, 20 mM HEPES, pH 7.4) were electroporated (BioRad Genepulser, 450 V, 250 μ F) with 25 μ g pMC1-Cre, plated 10 min later in gelatinized 100 mm plates and selected 48 h later with 2 μ M gancyclovir [cells derived using CDLNTKL (α_{i2})] or 125 μ g/ml 5-FC [cells derived using TKLNCDL (PPAR γ)]. HSVTK-resistant colonies were obtained under all conditions tested while CD-resistant colonies were obtained only in sparsely plated cultures. Below 10^4 cells/100 mm plate 20–30% of colonies survived while none survived at higher densities. This suggests a neighbor effect operating only above 10^4 cells/100 mm plate. This was not reported previously when few cells expressed CD. In our experiments most cells expressed CD and, at higher densities, likely produced sufficient diffusible 5-fluorouracil to kill all cells on the plate.

After transient Cre expression, colonies surviving negative selection were derived with equal frequency from heterozygotes and homozygotes (Table 1). Because all such clones analyzed by Southern blot (Fig. 1, not shown) were homozygous for the Cre-recombined allele (Table 1) recombination of either one or two floxed alleles occurred with equal efficiency. These cells also lacked *neo* sequences and were completely killed by 0.2 mg G418/ml demonstrating restoration of G418 sensitivity (not shown). Therefore, *neo* can be re-used to target additional genes in such cells suggesting a general system for efficiently modifying a potentially large number of genes in a single cell line.

The potential of recently developed human ES cells for cell therapy enhances the importance of genetic manipulation of such cells and their differentiated products. Until human cell lines are widely available murine ES cells will remain the only feasible, verifiable system for investigation in this area.

Table 1. Loss of floxed negative selectable markers after transient expression of Cre recombinase

	Heterozygotes (+/-)		Homozygotes (-/-)	
	% survival in selection (no. with selection/ no. without selection)	% recombined (no. analyzed)	% survival in selection (no. with selection/ no. without selection)	% recombined (no. analyzed)
PPAR γ (TKLNCDL)	25.2% (452/1796)	100% (12)	24.2% (315/1299)	100% (12)
α_{i2} (CDLNTKL)	8.2% (100/1224)	100% (12)	8.3% (116/1390)	100% (11)

These data are the sum of three separate experiments using cells bearing each targeted mutation. Each experiment gave similar results.

Knowledge derived from studies of genetically modified murine ES cells will enhance understanding of the similarities and differences between human disease and transgenic models of disease, as well as the behavior of murine and human ES cells in cell therapy.

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