Transgenic mice for the preparation of hygromycin-resistant primary embryonic fibroblast feeder layers for embryonic stem cell selections

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For the success of directed mutagenesis experiments using homologous recombination in embryonic stem (ES) cells, it is essential that ES cells in culture retain the ability to populate the mouse germline. Culturing the cells in such a way as to maintain this ability presents a technical challenge. Several different methodologies have been employed but one that is commonly used is to culture the ES cells on a feeder layer of mouse primary embryonic fibroblasts (MEFs) (1,2).

The gene targeting technology also requires that ES cells in culture undergo drug selection to identify the cells that integrate the targeting construct. Neomycin-resistance (*neo^R*) is the most commonly used dominant selectable gene for such experiments. To maintain a viable MEF feeder layer during drug selection it is necessary to prepare this layer with drug-resistant cells. Such neomycin-resistant feeder layers are conveniently prepared from transgenic mice expressing the neomycin-resistance gene.

To increase the flexibility of the targeting technology, a second dominant selectable marker, the hygromycin-resistance gene (*hyg^R*), has been employed. Hygromycin B is an aminocyclitol antibiotic that acts by interfering with the ribosomal translocation and aminoacyl tRNA recognition leading to mistranslations (3). The hygromycin B resistance gene is a phosphotransferase that renders the drug inactive by adding a phosphate to position 4 (4,5). With the availability of this second selectable marker, one approach for the inactivation of both alleles of a gene has been to target the first allele with *neo^R* and the second with *hyg^R* (6,10). Additionally, when homozygous targeted mutations of closely linked genes are desired in one animal, crossing the individually targeted animals together to obtain recombinants becomes impractical. Instead, sequential targeting of the two linked genes with two selectable markers becomes the method of choice.

Previously there have been no published examples of ES cells targeted with the *hyg^R* gene successfully repopulating the mouse germline and creating a strain of mice. However, such an experiment has been accomplished in the laboratory of Anton Berns, Netherlands Cancer Institute, thereby demonstrating the feasibility of using the *hyg^R* gene for deriving targeted strains (Anton Berns, personal communication). In addition, we were interested in Dr Bern's strain as a possible source of hygromycin-resistant MEFs. However, in that strain the *hyg^R* gene is under the

control of the endogenous *Pim1* promoter; a promoter with limited endogenous expression and expected not to be active in fibroblasts. Thus, the aim of this study is to derive a hygromycin-resistant transgenic strain of mice, optimal for the preparation of hygromycin-resistant MEF feeder layers, and to demonstrate the usefulness of these feeders for the selection of germline-capable ES cells.

To achieve widespread and high level expression of the *hyg^R* gene in transgenic mice we chose the 3-phosphoglycerate kinase (*Pgk1*) promoter (11). Plasmid pPWL512 has a PGEM-11Zf(+) backbone carrying a modified *hyg^R* gene under the control of the mouse *Pgk1* promoter and polyadenylation signals (Fig. 1A). A 2.0 kb *SfiI* and *XbaI* fragment containing these elements was isolated by electrophoresis, electroeluted, phenol–chloroform extracted, ethanol precipitated, and dissolved in 10 mM Tris, 0.1 mM EDTA pH 8.0. This DNA was microinjected into the male pronucleus of 234 C57BL/6J embryos according to standard procedures (12). Embryo transfer to pseudopregnant females resulted in the birth of 31 mice.

Southern analysis of tail DNA, restricted with *NcoI* and probed with the injected 2.0 kb fragment (Fig. 1B), resulted in the identification of one male mouse with a single copy transgene insertion. The transgene is cut by *NcoI* and results in 5 and 3 kb bands on Southern analysis. The founder was bred to C57BL/6J females, and transgenic offspring were interbred to generate homozygous animals. Candidate homozygotes were initially detected by quantitative analysis of Southern band intensity using a BAS1000 Bioimaging analyzer (Fuji Photo Film Co., Tokyo) (Fig. 1B). The endogenous *Pgk1* gene, which shares homology to the probe, results in 7 and 6 kb X chromosome bands, which after compensating for copy number based on the sex of the mouse, were used to standardize the DNA loading. In Figure 1B, lanes 1, 3 and 4 demonstrate the presence of the transgene; 1 and 4 being from candidate homozygous mice. Homozygosity was confirmed by test mating candidates to C57BL/6J animals to generate at least 14 offspring all of which carried the transgene. Homozygous animals were then interbred to establish the strain C57BL/6J-TgN(pPWL512hyg)1Ems, which is maintained by brother–sister mating. None of the homozygous transgenic animals has any apparent phenotype.

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Figure 1. (A) A map of the plasmid pPWL512, from which the 2.0 kb *SfiI*-*XbaI* fragment was excised for pronuclear injection. pPWL512 was constructed by cloning a *SmaI*-*HindIII* fragment derived from pHA57 (Courtesy of Dr H. te Riele, Netherlands Cancer Institute) and treated with Klenow, into the PGK-CAS plasmid (Courtesy of Dr N. van der Lugt, Netherlands Cancer Institute), which had been digested with *SmaI* and treated with Calf Intestine Alkaline Phosphatase. pHA57 contains a modified *hyg^R* gene generated by PCR and cloned into the *Bam*HI and *Sal*I sites of pUC18. The modification creates an optimal Kozak mammalian translation initiation signal. The sequence from the *Bam*HI site to the *Sal*I site in pHA57, with the *Hyg^R* translation start and stop signals underlined, is as follows: GGATCCAGCCGCCACCATG... 1020 bp of *hyg^R* coding sequence ...TAGTCGAC. PGK-CAS is a pGEM11-based plasmid with modified polylinker sequence containing the mouse *Pglk1* promoter (507 bp) and polyadenylation signals (452 bp) separated by a *SmaI* linker. PGK-CAS is described as the PGK expression vector in van der Lust *et al.*, 1991 (13). The *SmaI* site was lost during the insertion of the *hyg^R* gene, but the adjacent *Bam*HI site in the *hyg^R* insert remains intact. (B) Southern analysis of transgenic mice. *NcoI* restricted tail DNA was probed with the 2.0 kb *SfiI* to *XbaI* fragment from pPWL512. This fragment contains the *Pglk1* promoter, *hyg^R*, and the *Pglk1* polyadenylation site. Lanes 1-3, DNA from female mice; lanes 4 and 5, DNA from male mice. The transgene is detected in lanes 1, 3 and 4 with band intensity revealing twice the signal in lanes 1 and 4, compared with 3. The relationship between signal and phosphoimage is linear (BAS1000 Bio-imaging analyzer, Fuji Photo Film Co., Tokyo).

To characterize the suitability of the C57BL/6J-TgN(pPWL512hyg)1Ems strain for the preparation of hygromycin-resistant MEF feeder layers, heterozygous transgenic animals were intercrossed to produce an anticipated 1:2:1 ratio of wild-type, heterozygous and homozygous embryos, respectively. Thirteen to fifteen day fetuses were individually homogenized in 3 ml of feeder medium (10% Fetal Calf Serum in D-MEM) using a sterilized Brinkmann polytron homogenizer. Cell suspensions were plated in 15 ml of feeder medium/embryo in a T75 flask and incubated overnight at 37°C with 5% CO₂. The following day, cultures were trypsinized and replated in their original flasks to achieve maximal cell dispersal and to rid the cultures of debris. Confluency was typically achieved 2-3 days after the initial plating. At this point, cells from individual embryos were trypsinized, and aliquots replated in 24-well plates in non-selective medium.

Table 1. Drug sensitivity of fibroblasts from TgN(pPWL512hyg)1Ems mice

embryo number (cell treatment)	hygromycin B (mg/ml)				
	0	0.2	0.4	0.8	1.6
370-4 ^a	+	-	-		
370-7	+	-	-		
370-9	+	-	-		
375-10	+	-		-	
375-10 (irradiated)	+	-	-	-	-
376-4	+	-			
376-6	+	-			
376-1	+	+			
376-2	+	+			
376-3	+	+			
376-5	+	+			
376-7	+	+			
376-8	+	+			
376-9	+	+			
370-1	+	+	+		
370-1 (irradiated)	+	+	+	-	-
370-2	+	+	+		
370-3	+	+	+		
370-5	+	+	+		
370-6	+	+	+		
370-8	+	+	+		
375-1	+	+		+	
375-2	+	+		+	
375-2 (irradiated)	+	+	+	+	-
375-3	+	+		+	
375-4	+	+		+	
375-5	+	+		+	
375-6	+	+		+	
375-7	+	+		+	
375-8	+	+		+	
375-9	+	+		+	

+, cells unaffected by treatment; -, cells dying or dead; blank, not done.

After at least 12 h, hygromycin B selection was started, with triplicate wells prepared for each embryo at various drug concentrations (hygromycin B, Calbiochem, La Jolla, CA) (Table 1). The medium of each well was changed daily to simulate ES cell selection protocols. Observations were made daily but they did not vary after 1 day of selection nor among the triplicate wells. At the standard hygromycin B concentration used for ES cell selections, 0.2 mg/ml, 22/28 (79%) of the MEF wells from individual embryos showed no effect of the drug (Table 1). Since this approximates the expected value of 75%, we deduce that both heterozygous and homozygous C57BL/6J-TgN(pPWL512;hyg)1Ems embryos produce drug-resistant MEFs.

The concentration of hygromycin B required to achieve selection with some ES cell lines, or to select for multiple *hyg^R* insertion events in one cell, may be higher than 0.2 mg/ml. Thus, a subset of resistant embryos was tested at 0.4 and/or 0.8 mg/ml. MEFs from all the resistant embryos that were retested showed higher levels of drug resistance, 15/22 (68%), supporting the conclusion that both heterozygous and homozygous C57BL/6J-TgN(pPWL512hyg)1Ems embryos are highly drug resistant.

MEFs are usually further expanded and mitotically inactivated, by either mitomycin C or irradiation, prior to use as ES cell feeder layers (1,2). To test whether this protocol affected our

C57BL/6J-TgN(pPWL512hyg)1Ems MEFs, cultures from three embryos were amplified until one embryo yielded ~24 T75 flasks of confluent fibroblasts. At this point the cells were trypsinized, suspended in 40 ml of media in a 50 ml conical tube, and irradiated on ice with 3000 RADs (radiation absorbed dose) in a Shepard Mark 1 irradiator. The drug resistance of an aliquot of these irradiated fibroblasts was then compared to that of their unirradiated counterparts as described above. Table 1 shows that the drug-resistant phenotype was unaffected by this protocol.

Finally, MEFs from the C57BL/6J-TgN(pPWL512hyg)1Ems strain of mice, prepared as described above, were used as feeder layers for the selection of hygromycin-resistant J1 (7) derived ES cells. These ES cells had previously been selected for *neo^R* and were replated for electroporation with a *hyg^R* based targeting vector. ES cell clones were isolated and characterized by Southern analysis to detect *hyg^R* targeting constructs which had inserted into the genome by homologous recombination. Seven such clones were chosen for microinjection into blastocysts and five of these clones gave rise to chimeras. When test mated, chimeras from all five clones resulted in offspring that inherited the ES cell genome. Furthermore, each of the five clones generated two to three chimeras that gave 100% transmission of the ES genome. These results clearly demonstrate that high germline potential was retained during ES cell selection on our hygromycin feeders.

We have successfully derived a strain of homozygous *hyg^R* transgenic mice from which highly drug-resistant MEFs can be prepared. These MEFs permit the maintenance of germline potential during hygromycin-resistant selection of ES cells. We are presently using the same approach to develop an improved strain of neomycin-resistant transgenic animals and a strain of puromycin-resistant animals; both are designed to increase the versatility of the gene targeting technology.

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