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Selection of primary cell cultures with Cre recombinase induced somatic mutations from transgenic mice

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ABSTRACT

Deletion of genes in defined cell types has been achieved using a combination of gene targeting techniques and the Cre–*lox*P recombination system. Here we present a method to selectively isolate genetically altered primary cell cultures based on the permanent activation of a drug-resistance gene by the Cre recombinase. Transgenic mice were generated harboring a dormant form of the *hygromycin resistance* gene. This mouse line was crossed with mice carrying a constitutive *Cre* gene and an endogenous floxed allele. Primary fibroblasts established from triple transgenic embryos displayed not only hygromycin resistance but also recombination of the endogenous floxed allele. These results prove the potential of this approach.

Gene targeting has recently been combined with the Cre–*lox*P recombination system for cell type specific deletion of genes. The Cre recombinase from the bacteriophage P1 recognizes two *lox*P repeats, which are introduced into the gene of interest by homologous recombination, and excises the intervening DNA sequence (1–8). Mice carrying this floxed allele are mated with transgenic mice harboring the Cre recombinase transgene under a tissue-specific promoter for loss of function studies in defined tissues. An important complement to these *in vivo* functional studies has been the use of cell lines or primary cell cultures derived from mutant mice in research areas including signal transduction (9), transcription factors (10) and structural proteins (11). However, the isolation of a homogenous population of genetically altered cells has been constrained by the frequently observed mosaic expression of the Cre recombinase in transgenic mice. Here we describe a new genetic approach allowing the isolation of somatic cells which have undergone Cre mediated recombination, based on the permanent activation of a drug-resistance gene.

A selection cassette, named *dormant-hyg*^{R}, was constructed (Fig. 1A). The *dormant-hyg*^{R} cassette contains the *neo*^{R} and *hyg*^{R} genes which confer resistance to G418 and hygromycin, respectively. The *hyg*^{R} gene is separated from the constitutive murine 3-phosphoglycerate kinase (PGK) promoter by PGK*neo*^{R}, flanked by two *lox*P repeats. In cells expressing the Cre recombinase, PGK*neo*^{R} is excised thereby placing the PGK-promoter and the *hyg*^{R} gene together, resulting in an *active-hyg*^{R} allele (Fig. 1A). To test whether the *hyg*^{R} gene is dormant prior to Cre recombinase activity, we transfected the *dormant-hyg*^{R} cassette either alone or together with the Cre recombinase expression vector pMCcre (4) into ES cells. After hygromycin selection, drug-resistant clones were obtained only in double transfected cells but not in cells carrying the *dormant-hyg*^{R} cassette, demonstrating that the *hyg*^{R} gene is silent without Cre activity (data not shown).

Mice harboring the *dormant-hyg*^{R} cassette were generated by zygote injection. Four transgenic founders were identified and one line, named dh4, was randomly chosen and kept for further analysis. To test if the *dormant-hyg*^{R} cassette is recombined *in vivo* we mated dh4 mice with a Cre recombinase transgenic mouse line, named cre, expressing the enzyme ubiquitously (K. Zeh and H. Baribault, unpublished results). *In vivo* recombination of the *dormant-hyg*^{R} cassette in the progenies was confirmed by PCR analysis. Genomic DNAs isolated from heart, liver, small intestine, skeletal muscle, kidney, spleen and brain were amplified using primers for the PGK-promoter and the *hyg*^{R} gene. The predicted PCR product for the *dormant-hyg*^{R} allele is 2.2 kb, while the amplified fragment for the *active-hyg*^{R} allele is 0.36 kb (Fig. 1A and B). Analysis of the PCR showed that Cre mediated recombination was found in all tissues of double transgenic mice confirming that the *dormant-hyg*^{R} cassette can be recombined *in vivo* (Fig. 1B).

To demonstrate that a Cre induced *active-hyg*^{R} allele provides hygromycin resistance in primary cell cultures derived from transgenic mice, dh4 and Cre recombinase mice (dh4/cre) were mated and embryos collected between day E14 and E17 of gestation. Embryonic fibroblasts (MEFs) were isolated from dh4 and dh4/cre embryos and subjected to hygromycin selection. MEFs from dh4 embryos grew in non-selective media (Fig. 2A) but were sensitive to hygromycin at a concentration of 0.1 mg/ml (Fig. 2B). MEFs isolated from dh4/cre embryos also grew in non-selective media (Fig. 2C) but in contrast were resistant to 0.1 mg/ml hygromycin (Fig. 2D) and tolerated doses of hygromycin up to 1 mg/ml (data not shown). This result shows that cells expressing the Cre recombinase can be selected from a heterogeneous cell population based on the activation of the *dormant-hyg*^{R} cassette.

To test whether an endogenous floxed allele is processed simultaneously with the *dormant-hyg*^{R} cassette, triple transgenic embryos were generated. These harbor, in addition to the

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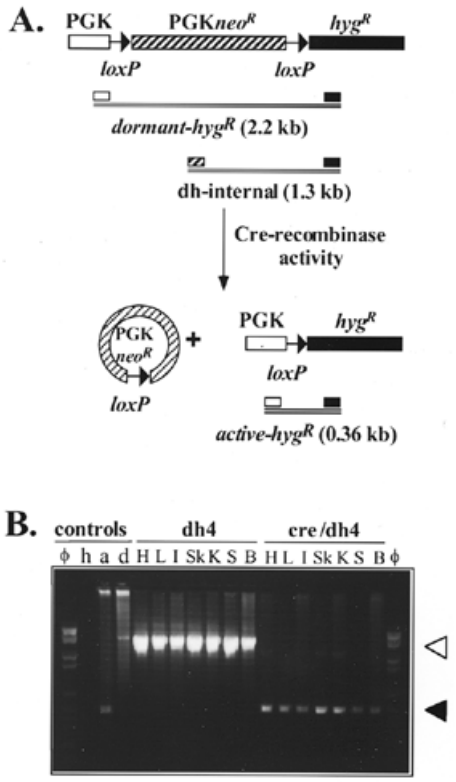

Figure 1. (**A**) Schematic representation of the *dormant-hyg*^{R} and *active-hyg*^{R} alleles. In the *dormant-hyg*^{R} cassette, the PGK-promoter is separated from the *hyg*^{R} gene by PGK*neo*^{R} flanked by two *lox*P repeats. Upon activity of the Cre recombinase the PGK*neo*^{R} cassette is excised and the PGK-promoter and the *hyg*^{R} gene are placed together resulting in an *active-hyg*^{R} allele. PCR primers were chosen in the PGK-promoter (PGK2-5′, CGC GAA GGT CCT CCG GAG CCC G, solid box) and the *hyg*^{R} gene (hygro4-3′, CGG CAC TTT GCA TCG GCC GCG C, hatched box). The expected PCR product prior to Cre activity is 2.2 kb (open arrow) and 0.36 kb (closed arrow) following Cre mediated recombination. To detect the presence of the floxed PGK*neo*^{R} cassette, a PCR assay was performed using a specific primer binding to the *neo*^{R} gene (o-neo-3, CCT GTC ATC TCA CCT TGC TCC TGC C, open box) and the primer hygro4-3′ under the conditions described. The expected size of the fragment dh-internal after amplification of the *dormant-hyg*^{R} cassette is 1.3 kb, while there is no PCR product if the *active-hyg*^{R} allele is present in the samples. PCR fragments are represented by double bars (=). (**B**) PCR based detection of Cre mediated recombination in transgenic mice. To show that the *dormant-hyg*^{R} cassette can be recombined *in vivo*, mice carrying the *dormant-hyg*^{R} cassette alone or dh4/cre littermates harboring the *dormant-hyg*^{R} cassette and a ubiquitously expressed Cre transgene were sacrificed 1 week after birth. Genomic DNAs from heart (H), liver (L), small intestine (I), skeletal muscle (Sk), kidney (K), spleen (S) and brain (B) were isolated and analyzed by PCR (30 cycles, 30 s at 94°C, 30 s at 69°C and 2 min at 72°C). The resulting PCR products had the correct sizes in samples derived from dh4 mice (open arrow) and in samples obtained from dh4/cre mice showing successful recombination of the *dormant-hyg*^{R} cassette (solid arrow). PCR controls are indicated as h (H_2O), a (*active-hyg*^{R} allele), d (*dormant-hyg*^{R} allele) and as ϕ (DNA molecular weight marker ϕX174 *Hin*fI, Gibco BRL).

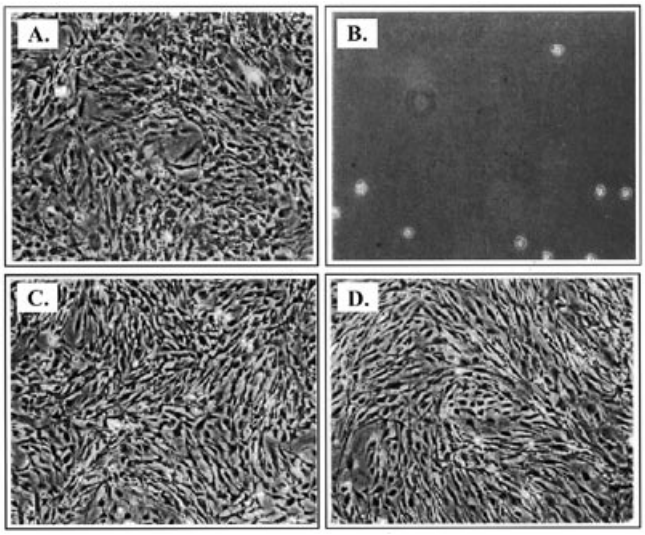

Figure 2. Derivation of primary fibroblast cell cultures from transgenic embryos. dh4 and Cre recombinase mice were mated and embryos collected between gestation day E14 and E17. MEFs were isolated according to standard protocols (13). Double transgenic MEFs and control cells harboring the *dormant-hyg*^{R} cassette only were cultured in the presence of hygromycin at different concentrations (0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml). After 7 days of selection the cultures were analyzed by light microscopy. The phase contrast picture shows MEFs established from dh4 embryos cultivated in the absence (**A**) or in the presence (**B**) of 0.1 mg/ml hygromycin in the media. (**C** and **D**) MEFs cultures from dh4/cre embryos using the same conditions as described for (A) and (B), respectively.

dormant-hyg^R and the Cre recombinase transgenes, a floxed *neo*^{R} gene inserted by homologous recombination into exon 1 of the large subunit of the *RNA Polymerase II* gene. The targeted allele was named P2Bc (12). Upon Cre induced recombination, the *neo*^{R} gene is excised resulting in the recombined P2Br allele. The predicted PCR product for the unrecombined P2Bc allele is 1.7 kb while amplification of the recombined P2Br results in a fragment of 0.25 kb. MEFs isolated from two triple transgenic embryos (#23 and #31) were resistant to 0.1 mg/ml hygromycin in the media (data not shown). To study if both floxed alleles have been recombined successfully, genomic DNAs were isolated from these cell cultures before and after hygromycin selection. PCR analysis showed that only the *active-hyg*^{R} and the P2Br alleles were present (Fig. 3B, upper panels). To further confirm complete Cre mediated recombination, a specific PCR designed to detect the floxed DNA sequences within the *dormant-hyg*^{R} (dh-internal, Fig. 1A) and the P2Bc (P2Bc-internal, Fig. 3A) alleles was performed. Analysis showed that no amplification products were obtained indicating the absence of any floxed fragments in the MEFs (Fig. 3B, lower panels). These results clearly demonstrate that all hygromycin resistant cells underwent Cre mediated recombination of the *dormant-hyg*^{R} allele simultaneously with the endogenous floxed P2Bc allele.

In conclusion, the method we present here describes the first system that allows to establish primary cell cultures with Cre induced somatic mutations from genetically altered mice by introducing the *dormant-hyg*^{R} cassette into either of the involved mouse lines.

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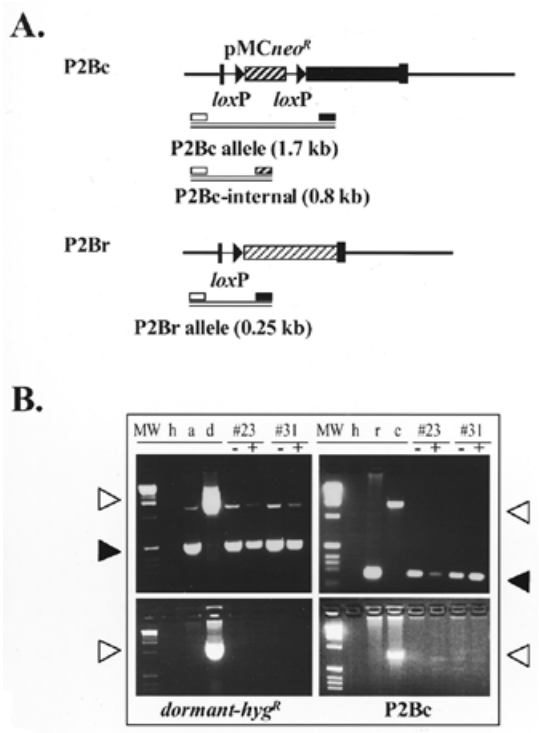

Figure 3. (**A**) Schematic representation of the PCR products for the targeted P2Bc allele before (P2Bc) and after (P2Br) Cre induced recombination as well as for the control reaction, P2Bc-internal. The figure indicates the position of the upstream (P2Bc1-5′ CTT TCC CTC CTC CAG CCT TTC CCT CCC TAT, solid box) and the downstream (P2Bc2-3′ GCG TAA GGT TGA GGA CCC GAG CTT GGC ACT, hatched box) primers as well as primer o-neo-3 (Fig. 1A). The size of the unrecombined P2Bc allele is 1.7 kb (open arrow) and 0.25 kb for the recombined P2Br allele (closed arrow). The PCR was performed under the following conditions: 30 cycles, 30 s at 94°C, 30 s at 63°C and 2 min at 72°C. To amplify the P2Bc-internal fragment primer, P2Bc1-5′ was used together with primer o-neo-3 (28 cycles, 30 s at 94°C, 30 s at 68°C and 1 min at 72°C). Amplification results in a 0.8 kb fragment if the P2Bc allele is the template or in no PCR product if the P2Br allele is present. PCR products are indicated as (=). (**B**) Simultaneous recombination of the *dormant-hyg*^{R} cassette and the endogenous floxed allele by the Cre recombinase. Upper panels: a PCR assay was performed on genomic DNA isolated from MEFs, derived from triple transgenic embryos #23 and #31, before (–) and after (+) hygromycin selection. PCR products had the expected size for the recombined *active-hyg*^{R} allele (left) and the recombined P2Br allele (right), respectively. The additional product in the *dormant-hyg*^{R} PCR represents a background band detectable in all samples regardless of whether they harbor the *dormant-hyg*^{R} cassette or not (data not shown). Lower panels: genomic DNAs described above were amplified for the presence of the dh-internal (left, open arrow) or P2Bc-internal (right, closed arrow) fragments, respectively. No PCR products were detectable indicating the absence of the floxed DNA sequences. PCR controls: h (H_2O); left panel: a (*active-hyg*^{R} allele), d (*dormant-hyg*^{R} allele); right panel: r (P2Br allele), c (P2Bc allele). MW indicates molecular weight marker (1 kb ladder, Gibco BRL).

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