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Efficient biallelic mutagenesis with *Cre/loxP*-mediated inter-chromosomal recombination

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The isolation of mutant cells with phenotypes caused by random mutagenesis has been hampered in mammalian cells because there are two alleles per gene and the disruption of both alleles is extremely rare. We describe a method for the efficient biallelic mutagenesis in embryonic stem cells. *loxP* sites were introduced near the centromeric regions of a pair of chromosome 1s. A mutant *neo* gene was inserted at the distal part of one of the *loxP* sites so that biallelic mutants would be selected by high-dose G418. Expression of Cre induced the recombination between homologous chromosomes and led to an elevation in the number of biallelic mutants. This system will facilitate phenotype-driven gene function study in the mammalian system.

INTRODUCTION

As we enter the post-genomic era, mutant resources will play a pivotal role in filling the gap between sequence information and understanding gene functions. In a mouse system, the gene-targeting method (Capecchi, 1989) opened the way to introduce desired mutations in any gene of choice and has proved to be a powerful method for analyzing gene function. However, a large-scale phenotype-driven genetic screening could not be achieved because mutational effects are masked until homozygous mutant mice are generated after a time-consuming breeding process.

It is known that homozygous mutant embryonic stem (ES) cells are generated spontaneously during the culture of heterozygous cells (Mortensen *et al.*, 1992). This event can be selected by increasing the concentration of G418, although the proportion of homozygous cells is extremely small (1 in ~10^5 of all cells). It is thought that mitotic recombination of homologous chromosomes during the 4N stage is one of the mechanisms of this event (Lefebvre *et al.*, 2001). The frequency might be increased by using a site-specific recombination system. In fact, the *FLP/FRT* system was used for *Drosophila* to induce inter-chromosomal recombination, and mosaic animals consisting of heterozygous and homozygous cells were successfully generated (Xu and Rubin, 1993). In a mouse system, inter-chromosomal recombination using the *Cre/loxP* system has been reported (Ramirez-Solis *et al.*, 1995; Smith *et al.*, 1995), but it remains to be demonstrated whether homozygous cells can be generated as efficiently as shown in *Drosophila*.

In the present study, we utilized the *Cre/loxP* system to induce recombination of homologous chromosomes and demonstrated that homozygous ES cells could be obtained at high frequency. This approach will facilitate a large-scale genetic screening in mammalian systems.

RESULTS AND DISCUSSION

The basic principle of the strategy is shown in Figure 1A. *loxP* sites are introduced at the same location of a pair of homologous chromosomes, and the cells are subsequently mutagenized. When the recombination occurs at the 4N stage, cells bearing chromosomal homozygosity distal to the *loxP* sites are obtained after chromosomal segregation and cell division. As a result, the mutations are introduced in both alleles at the distal part of the *loxP* sites.

We tested this system in mouse chromosome 1 in ES cells. We inserted *loxP* sites in both alleles of the *Opioid receptor, kappa 1* (*Oprk1*) locus (Nishi *et al.*, 1994), which is located near the centromeric region of chromosome 1 (Figure 1B). The mutant *neo* gene (Yenofsky *et al.*, 1990) was then inserted into the

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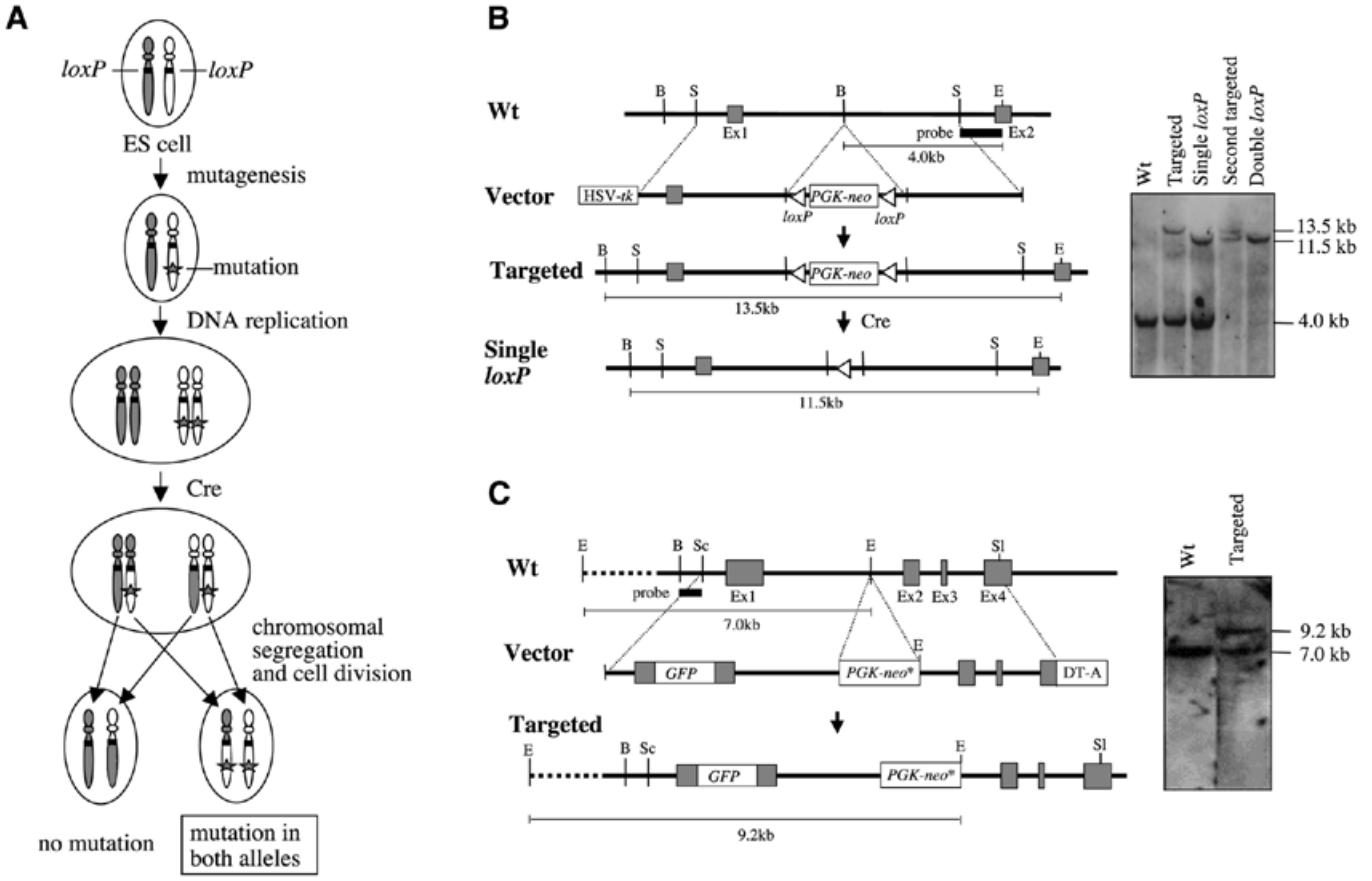

Fig. 1. Strategy to introduce biallelic mutations. (**A**) Principle for the mutagenesis. See text for details. (**B**) Introduction of *loxP* sites into the *Oprk1* locus. The *PGK-neo* gene flanked by two *loxP* sites was inserted into intron 1 of the *Oprk1* gene by homologous recombination. The *PGK-neo* gene was deleted by transient expression of *Cre*, leaving a single copy of the *loxP* site. The same procedure was repeated to introduce a *loxP* site into the other allele of the *Oprk1* gene, and the genomic structure was confirmed by Southern blotting. (**C**) Introduction of a mutant *neo* gene (*neo**) into the *Fasl* locus by homologous recombination. Wt, wild type; Ex, exon; B, *Bam*HI; E, *Eco*RI; S, *Spe*I; Sc, *Sac*I; Sl, *Sal*I; HSV-*tk*, herpes simplex virus thymidine kinase gene; DT-A, diphtheria toxin A fragment.

Fas antigen ligand (*Fasl*) locus (Takahashi *et al.*, 1994) located in the middle of chromosome 1 (Figure 1C). The mutant *neo* gene serves as a marker for the efficiency of *Cre*-mediated recombination because the cells containing multiple copies of the mutant *neo* genes can be selected under high-dose G418 (Mortensen *et al.*, 1992). To test the effect of the *Cre/loxP*-mediated inter-chromosomal recombination, we transfected the expression vector containing the *Cre* gene under the control of the *PGK* promoter into ES cells. Stable cell lines expressing Cre protein were established from cells bearing both the mutant *neo* gene and the *loxP* sites (*loxP*+/*Cre*+) and from cells bearing the mutant *neo* gene without the *loxP* sites (*loxP*–/*Cre*+). There were ~1000 colonies from 2×10^6 *loxP*+/*Cre*+ ES cells after the selection with 750 μg/ml G418 (Figure 2A and B), and Southern blot analysis verified that the majority of G418-resistant clones had the mutant *neo* gene in both alleles of the *Fasl* locus (Figure 2C). Absence of either the *Cre* or *loxP* site resulted in a small number of colonies (5–100 colonies, Figure 2A), and the introduction of the biallelic mutations was not predominant (Figure 2C). The results showed that biallelic mutagenesis was induced by *Cre/loxP*-mediated inter-chromosomal recombination. Considering that the plating efficiency of ES cells bearing the mutant *neo* gene at both alleles of the *Fasl* locus was 20% under the condition of 750 μg/ml G418 (data not shown), we speculate that the absolute frequency of biallelic mutagenesis event was ~0.25% [$(1000/2 \times 10^6)/0.2 = 0.0025$].

The rate at which resistant colonies appeared was examined by fluctuation analysis (Luria and Delbruck, 1943; Table I). The rate in the *loxP*–/*Cre*+ ES cells was 7.2×10^{-6}/cell per generation, whereas that of the *loxP*+/*Cre*+ ES cells was 1.1×10^{-4}/cell per generation. Therefore, the efficiency of biallelic mutagenesis was increased 15-fold in a *Cre/loxP*-dependent manner (Table I).

To facilitate our phenotype-driven gene function study in mammalian cells, we developed a system to obtain a large number of homozygous mutant ES cells by utilizing *Cre/loxP*-mediated inter-chromosomal recombination. Two methods have been mainly used to obtain homozygous mutants in mammalian cells. One is sequential gene targeting, in which the standard gene targeting procedure is repeated to mutate each allele separately. This method can be applied only to pre-selected genes and cannot be used for a large number of genes. The other consists of the selection of high-dose G418 for isolating homozygous mutant cells without utilizing the *Cre/loxP* system (Mortensen *et al.*, 1992). Although this is a straightforward approach, the

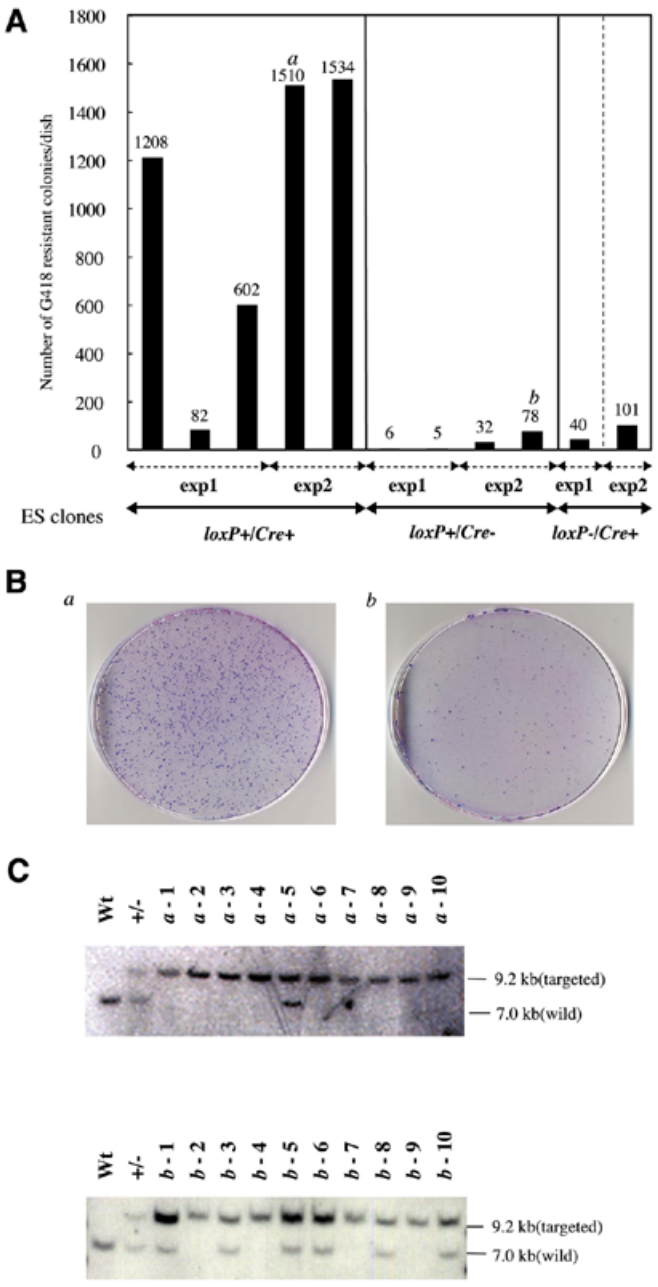

Fig. 2. Introduction of biallelic mutations by *Cre/loxP*. (**A**) Increase of high-dose G418-resistant colonies by *Cre/loxP*. Two million ES cells were plated per 100 mm dish and selected with 750 μg/ml G418 for 10 days. The presence or absence of *loxP* and *Cre* in each clone is indicated at the bottom. Two independent experiments (exp1 and exp2) were performed. (**B**) High-dose G418-resistant colonies stained with Giemsa's solution. Plates *a* and *b* were obtained from the experiments marked in (A) as *a* and *b*, respectively. (**C**) Southern blotting of the high-dose G418-resistant clones at the *Fasl* locus. Clones *a*-1 to *a*-10 and *b*-1 to *b*-10 were derived from the experiments marked in (A) as *a* and *b*, respectively. Wt, wild-type ES cells; +/–, heterozygous ES clone bearing the *neo** gene in a single allele of the *Fasl* locus. The conditions for Southern blotting were the same as in Figure 1C. Some lanes (*b*-1, *b*-5, *b*-6 and *b*-10) showed an intense upper band, suggesting that the *neo* gene was unusually amplified.

Table I. Fluctuation analysis of the resistance to high-dose G418

	loxP+/Cre+	*loxP–/Cre+*
Number of replicate cultures	10	10
Initial number of cells per culture	1	1
Final number of cells per culture (mean)	4.6×10^6	4.3×10^6
Number of cells per sample	1×10^6	1×10^6
Mean of resistant colonies per sample[a]	1026	45
The rate of appearance of resistant colonies	1.1×10^{-4}	7.2×10^{-6}

[a]G418 (750 μg/ml) was used for selection.

number of homozygous cells is too small to accomplish a large-scale biallelic mutagenesis. We compared it with our approach and found that using the *Cre/loxP* system resulted in a 15-fold increase in the efficiency of biallelic mutagenesis. This is probably an underestimate, because only a fraction of the G418-resistant cells (4 out of 10) were homozygous mutants when the *Cre/loxP* system was not used, whereas a majority of high-dose G418-resistant clones (9 out of 10) were homozygous when the system was used (Figure 2C).

For the expression of Cre, we generated stable cell lines that express the Cre protein constitutively. We also tested the effect of the transient expression of Cre but found no increase in the number of high-dose G418-resistant clones (data not shown). *Cre/loxP*-mediated recombination must occur at the 4N stage to generate biallelic mutant cells (Figure 1A), and continuous expression of the Cre protein may be required to increase the chance of recombination at this specific stage.

Application of this method to mice would be of interest, as a similar method using *FLP/FRT* made it possible to generate biallelic mutation in *Drosophila* (Xu and Rubin, 1993). Combining our method with the recently developed transposon system may facilitate this approach. We (Horie *et al.*, 2001) and others (Dupuy *et al.*, 2001; Fischer *et al.*, 2001) have reported that the *Sleeping Beauty* (*SB*) transposon reconstituted from fish transposes efficiently in mice, i.e. it preferentially transposes to the loci near the original site (Fischer *et al.*, 2001; K. Horie, K. Yusa, J. Odajima and J. Takeda, unpublished data). This feature of the *SB* transposon is very useful for *Cre/loxP*-mediated biallelic mutagenesis, because, when the *SB* transposon is introduced into the chromosome bearing the *loxP* site, the genes on the same chromosome can be biallelically mutagenized. It remains to be determined, however, whether the frequency of biallelic mutagenesis *in vivo* is high enough to make the use of this system worthwhile.

Liu *et al.* (2002) recently demonstrated that biallelic mutants could be obtained by means of *Cre/loxP*-mediated inter-chromosomal recombination, although the selection of mutant cells and chromosomes bearing *loxP* sites was different from ours. Their results, together with ours, validate the biallelic mutagenesis mediated by the *Cre/loxP* system.

Speculation

A large-scale biallelic mutagenesis would be possible in ES cells by combining the *Cre/loxP*-mediated inter-chromosomal

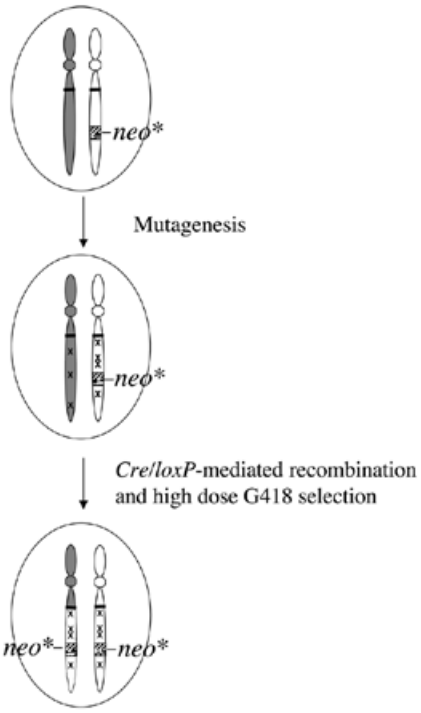

Fig. 3. Scheme of biallelic mutagenesis achieved by combining *Cre/loxP*-mediated inter-chromosomal recombination with chemical mutagenesis. ES cells bearing *Cre*, two *loxP* sites and one copy of the mutant *neo* gene are mutated by chemical mutagens. After selection of cells by high-dose G418, ES cells with biallelic mutations on the chromosomes bearing the *neo* gene can be expected to be obtained. 'x' indicates the mutation introduced by chemical mutagens.

recombination with the gene trap or chemical mutagenesis. In the gene trap (Friedrich and Soriano, 1991), the mutated *neo* gene is in the trap vector, and *Cre*-mediated recombinants are selected by means of high-dose G418. The current limitation is that the G418 resistance varies depending on the integration sites. Utilization of the insulator (Chung *et al.*, 1997) and/or the negative feedback regulation system (Becskei and Serrano, 2000) may solve this problem by achieving constant levels of *neo* gene expression independent of the integration sites. In chemical mutagenesis (Chen *et al.*, 2000; Munroe *et al.*, 2000), ES cells carrying the mutant *neo* gene at a particular locus would then be mutagenized by chemicals. *Cre*-mediated recombinants can be easily selected under high-dose G418, as the conditions for selection can be predetermined (Figure 3).

METHODS

Construction of vectors. The targeting vector for the *Oprk* locus was generated as follows. A 10 kb *Spe*I fragment of the MORG17 genomic clone (Nishi *et al.*, 1994) containing exon 1 and intron 1 of the *Oprk* gene was subcloned into the *Spe*I site of pBluescriptII (Stratagene) in which the *Bam*HI site was disrupted. The 2 kb *loxP*-flanked *PGK-neo* cassette was inserted into the unique *Bam*HI site of the *Oprk* region, resulting in p*PGKneo-OpiR*. An *Eco*RI–*Asp718* fragment of the *PGK-tk* cassette was isolated from the plasmid, which was generated by cloning the *Kpn*I–*Hin*dIII fragment of *pPNT* (Tybulewicz *et al.*, 1991) into pBluescriptII, and was subcloned into the *Eco*RI–*Asp718*-digested p*PGKneo-OpiR*, resulting in the targeting vector for the *Oprk* locus.

The targeting vector for the *Fasl* locus was generated as follows. A 6.5 kb *Sal*I–*Eco*RI (partially digested) fragment of *Fasl* genomic DNA was inserted into pBluescriptII, and the *Sal*I site was used to clone a 1.2 kb *Xho*I–*Sal*I DTA fragment of *pBL-DTA* (Adachi *et al.*, 1995), resulting in *pE/R-DTA*. *pPNT* was digested by *Xho*I–*Bam*HI and filled in, and the 1.8 kb mutant *neo* cassette was inserted into the *Sma*I site of *pE/R-DTA*, resulting in *pE/R-DTAneo*. A *Sac*I–*Eco*RI (blunt-ended) fragment containing the *Fasl* exon 1 region with an in-frame *EGFP* gene was inserted into the *Sac*I–*Not*I (blunt-ended) site of *pE/R-DTAneo*, resulting in the targeting vector for *Fasl* locus.

The *Cre* expression vector p*PGK-Cre-IRES-Puro* was generated as follows. The *Xho*I–*Sal*I fragment of p*PGK-puro* containing the *PGK* promoter was inserted into the *Sal*I site of pBS-*Cre* (Tarutani *et al.*, 1997). As a result, the *PGK* promoter was placed upstream of the *Cre* gene. This plasmid was digested by *Sac*II (blunt-ended), digested by *Not*I, and the *PGK-Cre* fragment was ligated to the *Xho*I(blunt-ended)–*Not*I fragment of pIRES-puro (Clontech), resulting in p*PGK-Cre-IRES-Puro*.

ES cell culture and electroporation. Linearized targeting vectors (10 µg) were electroporated into 1×10^7 R1 ES cells at 600 V/cm, 500 µF using Gene Pulser II (Bio-Rad). ES cells were selected by 150 µg/ml G418 for 7 days from the day after electroporation. GANC selection was performed in the targeting of the *Oprk* locus at a final concentration of 1 µM for 3 days from the second day of G418 selection. Resistant colonies were picked up, and homologous recombinants were analyzed by Southern blot hybridization.

For high-dose G418 selection, 2×10^6 cells were plated per 100 mm dish containing mouse embryonic fibroblasts, and G418 selection was started at the time of plating. G418-resistant colonies were counted after 10 days.

Isolation of stable clones expressing Cre. ES cells were transfected with p*PGK-Cre*-IRES-Puro vector using Transfast (Promega) according to the manufacturer's instructions. ES cells were selected by 1 µg/ml puromycin for 7 days, and resistant colonies were expanded. Cre expression was examined by transient expression of the pCAG-CAT-EGFP reporter plasmid, in which *Cre*-mediated recombination of two *loxP* sites located between the GFP gene and its promoter creates a functional GFP expression unit.

Fluctuation analysis. The rate of biallelic mutagenesis was studied by Luria–Delbruck fluctuation analysis (Luria and Delbruck, 1943). One of the *loxP+/Cre+* or *loxP+/Cre–* clones was plated on a 100 mm dish sparsely, and 10 subclones were picked up, expanded and selected under high-dose G418 (750 µg/ml). The calculations were carried out as described previously (Capizzi and Jameson, 1973).

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