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ROSA26Flpo deleter mice promote efficient inversion of conditional gene traps *in vivo*

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Abstract

Gene trap mutagenesis in embryonic stem (ES) cells is an important tool to help elucidate gene function in current mouse mutagenesis efforts. Vector systems based on inversion of the gene trap module have recently been devised to allow for conditional mutagenesis. However, additional efforts are needed to improve this technology including improving the efficiency of site-specific recombinases required to manipulate these conditional vectors *in vivo*. Here we describe a mouse line carrying the codon-optimized FLP recombinase Flpo at the ROSA26 locus that functions at higher efficiency than a similar Flpe line in mediating the DNA inversion of a conditional gene trap cassette *in vivo*.

Keywords

Mouse; mutagenesis; gene trap; FLP; site-specific recombinase; ES cells

Introduction

In the post-genomic era, attention has focused on the functional annotation of individual genes in the mouse. Large-scale mutagenesis efforts by KOMP, EUComm, NorComm, and the International Gene Trap Consortium are employing a number of techniques such as conditional and non-conditional gene targeting strategies, as well as gene trapping techniques in mouse embryonic stem (ES) cells to generate mutant alleles (Austin *et al.*, 2004; Friedel *et al.*, 2007; Skarnes *et al.*, 2004).

Gene trapping is an insertional mutagenesis technique that is designed to mutate a gene with a trap cassette that monitors its expression and serves as a molecular tag for its identification. Typically, a retroviral or plasmid gene trap vector contains a splice acceptor (SA) sequence followed by a promoter-less selection/reporter cassette and a transcription terminating polyadenylation (pA) sequence [for a review, see (Stanford *et al.*, 2001)]. One limitation of the gene trap methodology is that most commonly used vectors do not allow for conditional mutagenesis. Since approximately 25% of trapped genes are required for embryonic development (Friedrich and Soriano, 1991; Mitchell *et al.*, 2001), embryonic lethality precludes the study of these genes in the adult mouse, or their function in specific tissues. Recently, conditional gene trapping vector systems, such as the FlEx vectors, have been described and are currently being employed by EUComm to generate mutant alleles in mouse ES cells (Schnütgen *et al.*, 2005). The FlEx vectors feature a gene trap cassette that is flanked by heterotypic recognition sequences in a head to head orientation for two site-

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specific DNA recombinases (SSRs). The FIEEx system relies on sequential use of the Cre and FLP SSRs to conditionally inactivate and reactivate the gene traps. Upon recombination with the first SSR, the gene trap cassette is reversed from a mutagenic direct orientation (DO) to a non-mutagenic reverse orientation (RO). Subsequent use of a second SSR leads to an inversion of the gene trap back to the original mutagenic direct orientation. If this is done in mice, mutagenesis can be conducted in a tissue-specific manner, thus circumventing the potential embryonic lethality.

Both the P1 bacteriophage-derived Cre and the yeast derived FLP have been used in mammals to mediate DNA recombination, including deletions, insertions and inversions (Buchholz *et al.*, 1998; Dymecki, 1996; O’Gorman *et al.*, 1991; Sauer and Henderson, 1988). We have recently shown that a codon-optimized FLP recombinase (Flpo) mediates DNA recombination in ES cells more efficiently than the currently used variant Flpe (Raymond and Soriano, 2007). Here we describe a mouse strain that ubiquitously expresses Flpo and we show that this optimized SSR can be used to efficiently mediate the DNA inversion of a FIEEx conditional gene trap cassette.

Results

We had previously shown that a codon-optimized Flpo gene improved the recombinase efficiency *in vitro* in mouse embryonic stem (ES) cells (Raymond and Soriano, 2007). To test the *in vivo* performance of the optimized recombinase, we targeted the Flpo gene into the broadly-expressed ROSA26 locus (Soriano, 1999) and subsequently generated heterozygous and homozygous ROSA26Flpo mice (Figure 1). These mice were maintained on a 129S4 co-isogenic background, were fertile and did not exhibit any overt phenotype, similar to mice constitutively expressing the Flpe gene from the ROSA26 locus (Farley *et al.*, 2000).

To assess the recombination efficiency of the Flpo gene compared to the Flpe and Cre recombinases *in vivo*, we generated a gene trap mouse line harboring the FIEEx conditional gene trap vector, FlipROSA β geo*, as this vector is responsive to inversions by both Cre and FLP recombinase (Schnütgen *et al.*, 2005) (Figure 2A). We first tested that the functionality of the FIEEx conditional gene trap vector by mating mice heterozygous for the FlipROSA β geo* allele to Meox2Cre (MORE) heterozygous mice. MORE mice express Cre from the Meox2 locus leading to generalized recombinase activity in the mouse epiblast but not in the extra-embryonic tissues (Tallquist and Soriano, 2000). As the gene trap mice only exhibited diffuse lacZ staining, we assessed inversion efficiency by Southern blotting. This analysis on genomic DNA from tail biopsies of the resultant pups from this cross yielded 2 animals harboring both the FlipROSA β geo* and the MORE allele. Both of these mice displayed successful recombinase-mediated inversion of the gene trap allele, with some lack of inversion due to the mosaic expression of Cre recombinase in this strain (Hayashi *et al.*, 2002)(Figure 2Ba).

Next we tested the ability of Flpe driven from the ROSA26 locus (FlpeR) to mediate the inversion of the gene trap allele. Both of the mice that were positive for the Flpe and the gene trap allele only displayed a low level of recombinase mediated inversion to the non-mutagenic reverse orientation, while the majority remained in the forward mutagenic orientation (Figure 2Bb). Last, we tested the recombination efficiency of the optimized Flpo recombinase *in vivo* by mating heterozygous ROSA26Flpo mice to mice heterozygous for the FlipROSA β geo* gene trap allele. Animals positive for both the Flpo recombinase and gene trap alleles displayed a complete inversion of the FlipROSA β geo* cassette to the non-mutagenic reverse orientation (Figure 2Bc). As expected, these mice have also been shown to be efficient for mediating deletion of sequences flanked by FRT sites in the same

orientation (not shown). These results indicate that the optimized Flpo gene is functional *in vivo* and displays enhanced recombination activity in mediating DNA inversions compared to Flpe when expressed from the ROSA26 locus.

Discussion

In this report, we show that a mouse codon-optimized Flpo gene displayed enhanced efficiency in mediating DNA recombination in mice compared to Flpe, as it does in ES cells. Our results are consistent with two recent reports that have shown increased efficiency of Flpo relative to Flpe in mice for mediating deletions of sequences flanked by FRT sites in the same orientation (Kranz *et al.*, 2010; Wu *et al.*, 2009). However comparison of Flpo and Flpe efficiencies was complicated by the use of transgenic lines with random insertions rather than knock-ins as well as differing promoters. We now demonstrate by direct comparison that Flpo also exhibits enhanced activity compared to Flpe *in vivo*. The ROSA26Flpo mice were efficient in mediating the inversion of a FIEEx conditional gene trap vector. As large ES cell libraries of these conditional gene traps are currently being constructed by the European Conditional Mouse Mutagenesis (EUCOMM) initiative, this new mouse strain will prove to be an invaluable resource in fully-enabling this technology. Furthermore, tissue-specific Flpo or Flpo-ER lines may provide in future a set of tools that complements the existing resource of tissue-specific Cre lines. The ROSA26Flpo mouse strain can be obtained from the Jackson Laboratory (JR7844) and will be made freely available to the academic scientific community.

Materials and Methods

Recombinase and conditional gene trap constructs and mice

The optimized Flpo coding sequence was blunt cloned into the pROSA26-1 vector to generate mouse strains that broadly express this SSR from the ROSA26 locus as previously described (Raymond and Soriano, 2007). Consistent to the recommendations of the International Committee on Standardized Genetic Nomenclature for Mice, the strain name is: 129S4-Gt(ROSA)26Sor^{tm2(FLPo)Sor/J}. The Meox2Cre and Flper mouse strains were previously described (Farley *et al.*, 2000; Tallquist and Soriano, 2000).

The conditional gene trap vector pFlipROSAβgeo* was a kind gift from F. Schnütgen (Schnütgen *et al.*, 2005). Gene trap retrovirus was produced from the pFlipROSAβgeo* plasmid in GP+E86 helper cells, and AK7 ES cells were infected with the viral-containing supernatant at a multiplicity of infection of 0.1 as described previously (Soriano *et al.*, 1991). G418-resistant ES cell lines were subsequently used to generate conditional gene trap mouse lines to be used for analysis.

Southern Blotting

Genomic DNA was isolated from mouse tail biopsies and digested with NcoI. Southern blot analysis was performed with a neomycin probe to determine the direct- or reverse-orientation of the FlipROSAβgeo* conditional gene trap cassette.

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Figure 1. Strategy for conditional gene trap inactivation

Diagram of the FLPo knock-in vector and Southern blot of EcoRV digested ES cell DNA hybridized with the ROSA26-5' probe (Soriano, 1999), displaying successful gene targeting to the ROSA26 locus.

Figure 2. Comparison of the efficiency of 3 recombinases in mediating DNA inversions in vivo
A) Diagram of the FlipROSAβgeo* gene trap vector featuring a splice acceptor sequence (SA), β-galactosidase/neomycin phosphotransferase fusion gene (βgeo), and bovine growth hormone polyadenylation sequence (pA). The SAβgeopA gene trap cassette is flanked by heterotypic binding sites for Cre (loxP, lox5171) and FLPe/FLPo (FRT, F3) site-specific DNA recombinases in the FIEEx configuration as previously described (Schnütgen *et al.*, 2005). The Cre and FLPe/FLPo recombinases mediate a DNA inversion/excision reaction that converts the gene trap cassette from the mutagenic direct orientation (DO) and locks it into the non-mutagenic reverse orientation (RO). **B)** Southern blot analysis displaying incomplete inversion of the FlipROSAβgeo* gene trap cassette from the mutagenic direct orientation (DO) into the non-mutagenic reverse orientation (RO) when mediated by **a)** Cre and **b)** FLPe DNA recombinases. In contrast, there is complete DNA inversion of FlipROSAβgeo* from DO to RO when mediated by **c)** FLPo recombinase. DNAs were digested with NcoI, which cuts within the neomycin phosphotransferase gene. As a result, two fragments hybridize with the neo probe. Approximate fragment sizes are indicated in kb. The fragments originating from the recombinase allele are indicated.