High-Efficient FLPo Deleter Mice in C57BL/6J Background

Yingjie Wu1, Chuxin Wang2, Hui Sun1, Derek LeRoith1, Shoshana Yakar1*

1 Endocrinology/Diabetes and Bone Disease Division, Mount Sinai School of Medicine, New York, New York, United States of America, 2 Biochemistry Section, SNB/NINDS, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Conditional gene manipulation in mice becomes a routine for genetic studies of mammalian gene functions. Additional site-specific recombinases such as FLP or δ31 provide one more level of gene manipulation flexibility. The recombination activity of the currently available FLP deleter mice remains low. We generated a new FLP deleter mouse line with the mouse codon-optimized FLPo gene in C57BL/6J background, which showed superior recombination efficacy in comparison to FLPe deleter mice. 100% complete removal of FRT-flanked Neo cassette was observed in all F1 progeny mice carrying both FLPo and Neo cassette, which can be transmitted to F2 generation independent of FLPo activity. Our new FLPo transgenic mice (on pure C57Bl/6 background) will largely facilitate the gene targeting process and is valuable for conditional gene manipulation.

Results

pPGKFLPoA expresses de novo synthesized mouse codon-optimized FLP (FLPo) under the PGK promoter (Fig. 1A), and is highly efficient in inducing recombination in mouse embryonic stem cells [12] and human HCT116 cells (unpublished data). To generate a FLPo deleter mouse line, we
injected the linearized PGK-FLPoA fragment into pronuclei of fertilized eggs of the C57BL/6 mice. Seven transgenic founder mice were identified by PCR genotyping (Fig. 1B), six of which transmitted the FLPo transgene through the germ cells. The PGK-1 promoter activates transgene expression as early as in 3.5d and in adults it drives target genes expression ubiquitously in all tissues including the gonads [13]. FLPo expression levels were analysed in various tissues, including gonads by RT-PCR. As shown in Fig. 1C, the expression of the FLPo transgene can be detected in all tissues examined with the strongest level in testis and ovary.

To determine the efficiency of FLPo-mediated recombination, 4 heterozygous PGK-FLPo transgenic founder mice (FLPo10, FLPo17, FLPo15 and FLPo6) were crossed with mice that carry the FRT-flanked PGK-Neo cassette (GHRNeo/+ in intron 5 of the growth hormone receptor GHR gene (Fig. 2A). As a comparison, GHRNeo/+ mice were also crossed with the FLPe deleter mice. In the F1 generation, 4 genotypes of mice were expected: wild type (+/+); PGK-FLPo/+; GHRNeo/+ and GHRlox/PGK-FLPo. FLPo-mediated recombination can only be evaluated in mice carrying both the FLPo transgene and the Neo cassette. In these mice, if FLPo exerts its enzymatic activity in all cells and results in a complete removal of the FRT-flanked Neo cassette through recombination-based FRT site excision, the genotype of these double transgenic mice should become GHRlox/PGK-FLPo. However, if FLPo exhibits partial enzymatic activity, it will result in a mosaic of cells retaining the Neo cassette or lacking Neo cassette. In this case, a chimera of GHRNeo/GHRlox/PGK-FLPo will be detected. The ratio between GHRlox and GHRNeo can be used for estimation of the efficiency of FLPo-mediated recombination in vivo.

As shown in Fig. 2B, the FLPc primer set can amplify both FLPc and FLPo transgenes whereas the FLPo primer set specifically amplifies the FLPo transgene. All chosen samples carry the FLP transgene (either FLPo or FLPe) for the simplicity of analysis. S6/LoxP-A primer set specifically amplifies the intact FRT-Neo cassette (PCR positive for this primer set, hence, can be scored as “Neo” allele). S6/D452 primer set can distinguish Neo and Lox allele from wild type (990 bp and 840 bp, respectively) and S6/R2 primer set can differ between wild type allele and Lox allele (817 bp and 1019 bp, respectively; Neo allele cannot be amplified under our PCR conditions). The upper band (1019-bp) is scored as a “Lox” allele, and was confirmed by subsequent sequencing (Fig. 2C). The Lox allele can only be produced when FLP-mediated recombination occurs. In F1 pups derived from crosses with FLPo15, FLPo17 and FLPo6 transgenic mice, none had the Neo-cassette removed completely, among the mice carrying the FLPo transgene (Table 1 and Fig. 2B). Partial removal was seen with FLPo15 and FLPo17 transgenic mice as indicated by the presence of both Neo and Lox alleles, indicating mosaic recombination. In contrast, when crosses were made with FLPo-10 transgenic mice, all F1 pups carrying the FLPo transgene have Lox alleles, but not the Neo allele (Table 1 and Fig. 2B). This indicates a 100% removal of FRT-flanked Neo cassette by FLPo-mediated recombination in all cells and FLPo-10 mice can be used as a very efficient deleter mouse line. FLPe deleter mice showed less than 10% complete recombination and less than 20% partial recombination as shown by the chimera of Neo and Lox allele (Table 1 and Fig. 2B).

The FLP activity in the tail tips may not represent recombination in the germline. Since only removal of Neo cassette occurring in the germline can be transmitted permanently to the

Figure 1. Generation of FLPo transgenic mice. (A) Schematic diagram of FLPo construct used for the generation of FLPo transgenic mice. Two primer sets for genotyping of FLPo transgenic mice are indicated. (B) PCR analysis of FLPo positive founder lines. (C) RT-PCR of FLPo mRNA in germ line [Testis (T) and ovary(O)] and other tissues [brain(B), heart(H), liver(L) and muscle(M)] of transgenic mouse FLPo-10 line. Actin was used as control. doi:10.1371/journal.pone.0008054.g001
next generation, we first sought to examine whether removal of the Neo cassette also took place in other tissues in F1 generation. Of note, a FRT-flanked GFP reporter line for FLP mediated recombination would be optimal to address this question. However, such a reporter line is not available commercially, therefore, we performed PCR in various tissues. As shown in Table 2, removal of Neo cassette was observed in all 10 floxed mice independent of the status of FLPo activity in F2 generation (as only 3 mice segregated the FLPo transgenes in the F2). In contrast, crosses made with the FLPo-15 line, that only showed mosaic removal of Neo cassette in F1 generation, yielded 12 out of 14 flox mice still containing the Neo cassette, indicating limited removal of the Neo cassette in the F2 generation. To further confirm the complete removal of Neo cassette by FLPo-10 line, we also performed similar PCR genotyping in other tissues including gonads and obtained similar data as obtained with tail DNA (Fig. 3B).

The recombination efficiency for each line is summarized in Table 1 and 2. We thus calculated the efficiency of Flp recombination in FLPo-10 line as 100% (22 out of 22 mice).

**Table 1.** Recombination efficiency of different FLP transgenic lines.

| Line    | Number of double-transgenic mice | Number of mice without the Neo-cassette | Number of mosaic mice |
|---------|----------------------------------|----------------------------------------|----------------------|
| FLPe    | 24                               | 2                                      | 4                    |
| FLPo-10 | 22                               | 22                                     | 0                    |
| FLPo-15 | 11                               | 0                                      | 7                    |
| FLPo-17 | 10                               | 0                                      | 9                    |
| FLPo-6  | 9                                | 0                                      | 0                    |

However, such a reporter line is not available commercially, therefore, we performed PCR in various tissues. As shown in Fig. 3A, removal of the Neo cassette was clearly observed in testis, ovary, liver, brain, muscle and kidney. Next, we analyzed whether removal of Neo cassette is independent of co-inheritance of the FLPo transgene in the F2 generation. As shown in Table 2, removal of Neo cassette was observed in all 10 floxed mice independent of the status of FLPo activity in F2 generation (as only 3 mice segregated the FLPo transgenes in the F2). In contrast, crosses made with the FLPo-15 line, that only showed mosaic removal of Neo cassette in F1 generation, yielded 12 out of 14 flox mice still containing the Neo cassette, indicating limited removal of the Neo cassette in the F2 generation. To further confirm the complete removal of Neo cassette by FLPo-10 line, we also performed similar PCR genotyping in other tissues including gonads and obtained similar data as obtained with tail DNA (Fig. 3B).

The recombination efficiency for each line is summarized in Table 1 and 2. We thus calculated the efficiency of Flp recombination in FLPo-10 line as 100% (22 out of 22 mice).
Similarly, the partial recombination was found in FLPo-15, 17 and control FLPe lines. No recombination occurred in progenies derived from FLPo-6 line. It could be due to positional effects that result in differential expression levels of the FLPo transgene. We confirmed the successful recombination by FLPo-10 transgenic line in several other target mice carrying FRT-flanked Neo in various loci. Furthermore, all FRT-cassette removed mice we tested can maintain their neo-deleted status in the next generation and 100% germline-transmitted FLPo recombination was observed in subsequent six generations of backcrosses.

Discussion

We report here the generation of highly efficient FLPo deleter mice in C57BL/6 background. It exhibited 100% complete removal of FRT-flanked Neo cassette in the F1 progeny mice carrying both FLPo transgene and the Neo cassette in different genetic loci. Importantly, the complete removal of Neo cassette was maintained in F2 generation independently of FLPo activity. In the subsequent six generations of crosses with the wild type mice, 100% germline transmission of Lox allele (after Neo cassette removed by FLPo) was observed in each generation in the absence of FLPo transgene, indicating the superior recombination efficacy of our newly developed FLPo deleter mice.

The predominant use of conditional knockout in mouse genetic studies demands additional effective site-specific recombinase other than Cre for more delicate and efficient gene manipulation. Although FLP was the first site-specific recombinase used in genetic engineering of higher eukaryotes, successful site-specific recombination in mammalian cells and mice was first achieved with and mostly involved Cre, largely due to its highly efficient recombination activity in mammals. Despite initial reports that the efficacy of FLP recombination could be as high as 90% in mouse ES cells (AK-7) [14] and 30–78% in CCE ES cells [4], FLP exhibits low recombination activity in germ cells [15]. For example, Meyers et al (1998) reported that complete FLP-

Table 2. Recombination analysis of F2 generation.

| Line                                      | FLPo-10 | FLPo-15 |
|-------------------------------------------|---------|---------|
| Number of mice in the F2 generation       | 13      | 17      |
| Number of mice in the F2 generation Carrying the Flox allele | 10      | 14      |
| Number of mice in the F2 generation Carrying the Flox allele and the Neo cassette | 0       | 12      |
| Number of mice in the F2 generation Carrying the Flox allele without the Neo cassette | 10      | 2       |
| Number of mice in the F2 generation carrying the FLPo transgene. | 3       | 4       |

doi:10.1371/journal.pone.0008054.t002
mediated recombination was not observed in all cells of the F1 pups that inherited both Neo cassette and Flp transgene although some of the mosaic animals transmitted the recombinated allele (which lacks the neo cassette) to their F2 progeny [16].

The improved version of Flp, FlPe exhibits 4–10 fold enhanced thermal stability at 37°C [6]. Nonetheless, FlPe was reported to still show limited recombination rate [7] and 10% efficiency of Cre on chromatin targets [11]. As a consequence, deleter mice generated with this improved FlPEs are much less efficient compare to Cre deleter mice in terms of mediating recombination in vivo. Surprisingly, no data on FlP-mediated recombination rate in F1 generation has been reported with much less stability of FlPe protein levels in different tissues. Surprisingly, a recent report by Kondo et al. showed that FlPe exhibits lower recombination efficiency than Cre [12].

In summary, our FlPo deleter mouse line is valuable for gene manipulation and will facilitate the general process of generation of conditional knockout mice. This line will be donated to The Jackson Laboratory. For information on obtaining the animals, please refer to the URL: http://www.jax.org.

Materials and Methods

Animals

All mouse models used in the study were bred on a C57B6/J background. Animal care and maintenance were provided through the Mount Sinai School of Medicine AAALAC Accredited Animal Facility. All procedures were approved by the Animal Care and Use Committee of the Mount Sinai School of Medicine.

Materials

pPGKFLPobpA plasmid was constructed by Raymond et al. [18] and purchased from Addgene (Addgene plasmid 13793) [12]. The FlPe transgenic mice B6.Cg Tg(AC/FLTFLPe)9205Dym/J (JAX Mice and Services Stock number 005703) were purchased from the Jackson Laboratory [12].

Generation and Characterization of Transgenic Mice

The 2.2-kb Sal I/Nor I fragment from the pPGKFLPobpA plasmid was purified by QIAquick Gel Extraction Kit (QIAGEN Inc. CA, USA). The linearized DNA solution (2 ug/ml) was microinjected into pronuclei of fertilized one-cell embryos of C57BL/6N mice. Injected embryos were transplanted into the pseudopregnant mice. Breeding and maintenance of mice were performed under institutional guidelines. Mice were fed ad libitum with standard laboratory chow and water under a 12-h light/dark cycle. FlPe transgenic mice were genotyped by PCR with a primer set: PGK-F and Flpo-A or Flpo 5 and Flpo3. (Information of primer sequences can be obtained upon request).

Analysis of Flp Recombination In Vivo

The heterozygous PGK-FlPe transgenic mice Flpo10, Flpo17, Flpo15 and Flpo6 were crossed with mice that carry the FRT-flanked PGK-Neo cassette. The F1 pups from this mating were genotyped for the PGK-FlPe transgene and the FRT-Neo cassette. F1 progeny mice carrying both the FlPe transgene and the Lox allele were backcrossed to wild-type mice to remove the FlPe transgene. Genomic DNA of F1 and F2 progeny was subjected to PCR analysis. S6/D452 primer set was used for distinguish wild type from Neo and Lox alleles (840 bp and 990 bp, respectively). Primer set S6/R2 was used for distinguish wild type and Lox allele (1019 bp and817 bp, respectively). Genomic DNA isolation from tissues and tail is following the standard protocol.

FLPo Expression Analysis by RT-PCR

Total RNA from tissues was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). RNA integrity was verified using Bioanalyzer (Agilent Technologies 2100 Bioanalyzer-Bio Sizing, Version A.02.12 SI292). One ug of RNA was reverse-transcribed to cDNA using oligo(dT) primers with RT-PCR kit according to the
manufacturer’s instructions (Invitrogen Corp., Carlsbad, CA, USA). RT-PCR was performed and b-actin was used as control.

Acknowledgments

We acknowledge Dr. Kevin Kelley at Mouse Genetics Shared Resource Facility Mount Sinai School of Medicine for technical assistance. We also thank Joshua Oppenheimer and Archana Vijayakumar for their help in maintaining the mice and PCR genotyping.

Author Contributions

Conceived and designed the experiments: YW SY. Performed the experiments: YW HS. Analyzed the data: YW CW SY. Contributed reagents/materials/analysis tools: CW DL. Wrote the paper: YW CW SY.

References

1. Nagy A (2000) Cre recombinase: the universal reagent for genome tailoring. Genesis 26: 99–109.
2. Nagy A, Mar L, Watts G (2009) Creation and use of a cre recombinase transgenic database. Methods in molecular biology 530: 365–378.
3. Testa G, Schaft J, van der Hoeven F, Glaser S, Anastassiadis K, et al. (2004) A reliable lacZ expression reporter cassette for multipurpose, knock-out-first alleles. Genesis 38: 151–158.
4. Dynecki SM (1996) Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 93: 6191–6196.
5. Vooits M, van der Valk M, te Riele H, Berns A (1998) Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. Oncogene 17: 1–12.
6. Buchholz F, Angrand PO, Stewart AF (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. Nature biotechnology 16: 637–642.
7. Schaft J, Asherty-Padan R, van der Hoeven F, Gius P, Stewart AF (2001) Efficient FLP recombination in mouse ES cells and oocytes. Genesis 31: 6–10.
8. Farley FW, Soriano P, Stieffen LS, Dynecki SM (2000) Widespread recombinase expression using FLPeR (flipper) mice. Genesis 28: 106–110.
9. Takeuchi T, Nomura T, Tsujita M, Suzuki M, Fuse T, et al. (2002) Flp recombinase transgenic mice of C57BL/6 strain for conditional gene targeting. Biochemical and biophysical research communications 293: 953–957.
10. Kanki H, Suzuki H, Iohara S (2006) High-efficiency CAG-FLPe deleter in C57BL/6J background. Experimental animals/Japanese Association for Laboratory Animal Science 55: 137–141.
11. Andreas S, Schwenk F, Kutet-Laks B, Faust N, Kuhn R (2002) Enhanced efficiency through nuclear localization signal fusion on phage PhiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. Nucleic acids research 30: 2299–2306.
12. Raymond CS, Soriano P (2007) High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. PLoS ONE 2: e162.
13. McBane MW, Staines WA, Bockelheide K, Paity D, Jardine K, et al. (1994) Marine PGK-1 promoter drives widespread but not uniform expression in transgenic mice. Dev Dyn 206: 278–293.
14. Fiering S, Epner E, Robinson K, Zhuang Y, Telling A, et al. (1995) Targeted deletion of 5’HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. Genes & development 9: 2203–2213.
15. Mishina M, Sakimura K (2007) Conditional gene targeting on the pure C57BL/6 genetic background. Neuroscience research 58: 103–112.
16. Meyers EN, Lewandoski M, Martin GR (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nature genetics 18: 136–141.
17. Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, et al. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nature genetics 25: 139–140.
18. Kondo S, Takata Y, Nakano M, Saito I, Kanegae Y (2009) Activities of various FLP recombinases expressed by adenovirus vectors in mammalian cells. Journal of molecular biology 390: 221–230.