Humanized Prion Protein Knock-in by Cre-Induced Site-Specific Recombination in the Mouse

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To establish humanized mice with a knock-in (gene replacement) technique, we constructed a targeting vector which consists of the human prion protein and the loxP sequences. The introduced human prion protein with the loxP system in the embryonic stem cells was transmitted through the mouse germ line. Transient expression of Cre recombinase in the fertilized eggs resulted in the prion protein humanized mice. The Cre-loxP-mediated gene replacement is a simple and efficient method which is generally applicable to make humanized animal models. © 1996 Academic Press, Inc.

Gene targeting in embryonic stem (ES) cells has enabled the construction of mice with null mutations in many genes associated with human disease. However, most of genetic diseases are not attributable to null mutations. In fact, the diversity of missense mutations causes a similar diversity in symptoms and signs. For example, different mutations in the prion protein gene (PRNP) cause familial Creutzfeldt-Jakob disease, various types of Gerstmann-Sträussler syndrome, and fatal familial insomnia(1–3). To design adequate mouse models of human diseases, it is essential to establish a method for gene replacement (knock-in) which disrupt the mouse locus and introduces the human counterpart with mutation. For this purpose, we and other groups presented the double targeting method in the ES cells (4–8). We herein report a single targeting method combined with Cre-loxP mediated recombination in the fertilized eggs.

MATERIALS AND METHODS

Construction of target vector. Human genomic DNA with codon 145 mutation (9) was amplified by PCR using a pair of sense primer (R-1: AGCCGATAACCGGGGCGAG), and antisense primer (R-2: TTTGATACCTGCTTGAGCTTGAGAAAAG). The R-1 primer was designed to have a mismatched Cytosine (underlined) to induce a new Sma I site for the alignment of the prion protein (PrP) frame (10,11), and the R-2 primer has a Nde I site (underlined) used for the Southern blot analysis. The PCR product was cloned into the pCRII vector (Invitrogen), and was sequenced. The cloned PCR product was then inserted into the EcoRI site of pBluescript II SK+ (pHu).

A pair of loxP sequence in the same direction was inserted in the Pst I site of pBSII. Following deletion of the original cloning sites Eco RI through Hind III in the pBSII, synthetic oligonucleotide consisting of multiple cloning sites (Eco RI, Eco RV, Acc III, and Hind III) was inserted between the loxP sequences. Neo gene fragment from pMC1neo polyA (Stratagene) was inserted in the ploxP using Eco RV and Hind III sites (ploxP-neo).

The mouse PrP gene was isolated from a mouse genomic library prepared from 129 SV/J mice DNA (Stratagene). The replacement vector consisted of the 7.5 kbp genomic sequence from Bam HI to Eco RV site around the exon 3 of murine PRNP. The 5′ sequence (3 kbp) from Bam HI to Sma I site was subcloned into pBSII, and ligated with human PrP gene (pHu) using Sma I and Xho I (p5′-Hu). The Bam HI site of the p5′-Hu was deleted by Klenow reaction. The ploxP-neo fragment (Sma I and Xho I) was ligated into the Eco RV and Xho I sites of p5′-Hu (p5′-Hu-loxP-neo). The PGK gpt gene (kindly provided by Dr. Miyoshi) was inserted into the Bam HI site of the p5′-Hu-loxP-neo (p5′-Hu-loxP-neo-gpt). The 3′ sequence (4 kbp) from Apa I to Eco RV were subcloned in Hinc II site of pBSII (p3′). The 3′ fragment was inserted into

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Abbreviations: PrP; Prion protein, PRNP; Prion protein gene, ES cells; embryonic stem cells, gpt; Xanthine/guanine phosphoribosyl transferase.
the p5'-Hu-loxP-neo-gpt with Cla I and Xho I site. Finally, the diphtheria toxin gene (DTA; kindly provided by Prof. Aizawa, Kumamoto University) was added at the 3' end for the negative selection (12). The completed targeting vector is shown in Fig. 1a schematically.

**Homologous recombination in ES cells.** ES cells (4 × 10⁷) were transfected with 50 μg of the Not I linearized targeting vector DNA by electroporation with a setting of 500 μF capacitance, 270 V/1.8 mm (BTX Inc, ECM 6000) (5,13). G418 (250 μg/ml; Sigma) was added to the medium for selection 24–48 hr after the transfection. ES cell lines in which homologous recombination occurred were identified by Southern blot analysis of Eco RV-, Kpn I-, or Nde I-digested genomic DNA using the 5' (Sma I-Bam HI), 3' (Eco RV-Sac I) flanking regions and the neomycin gene (Eco RI fragment).

**Germ-line transmission and transient expression of Cre recombinase in fertilized eggs.** Chimeric mice were generated by injecting the homologous recombinant ES cells into C57BL/6J blastocysts, and then implanting them into the pseudopregnant recipients. Two agouti coat color chimeric male mice were mated to C57BL/6J female to examine germ-line transmission. The agouti coat color offspring were analyzed by the Southern blot hybridization and PCR with neomycin primers. Eggs from C57 BL/6J females mated with the heterozygous mutant males were collected, and a solution of a circular pCAGGS-Cre (kindly provided by Prof. Miyazaki, Tohoku University) was microinjected into the pronucleus (14). These eggs injected with the Cre plasmid were transferred into pseudopregnant mice.

**PCR analysis.** To detect mice which carry a mutant allele (lox-neo-gpt-lox) in the genome, tail DNA samples were examined by the PCR analysis with a pair of neo primers (neo-1: TCTTGCGAGAAGTATCCA, neo-6: GTCAAGAAG-GCGATAGAAGG). The PCR conditions were 94°C for 7 min. to denature, and then 30 cycles of 94°C for 1 min., 55°C for 1 min., and 72°C for 1 min. To detect a humanized allele (HuKi), tail DNA samples were examined by the PCR amplification with a pair of primers (M-21: TACTGGGCACTGATACCTTG, MT-2: GGCCTGAAGCAAAGAGCAA). The PCR conditions were 94°C for 7 min. to denature, and then 30 cycles of 94°C for 1 min., 55°C for 1 min., and 72°C for 2 min. Theoretically, the wild allele shows 998 bp PCR product, while the humanized allele shows 985 bp. To differentiate the wild and the humanized allele, the PCR products were digested with Kpn I, Pst I or Mae I. Humanized PCR products were cut into 589 and 396 bp by Pst I, and wild PCR product were cut into 671 and 327 bp by Kpn I.

**FIG. 1.** Targeting of the mouse PRNP locus with human prion protein (PrP) knock-in sequence in the embryonic stem (ES) cells. **a.** Genomic structure of murine PRNP with the location of the exon 3 (a large box indicates protein coding sequence; a small box indicates 3' untranslated region). The target vector consists of 5' homology part (Bam HI to Sma I), human PrP,lox-neo-gpt-lox cassette, 3' homology part (Apa I to Eco RV), and diphtheria toxin gene (DTA). Human PrP sequence with a Nde I site is inserted in the Sma I site of the mouse PrP. Thus, N-terminal sequence (39 amino acids) of the target vector derives from the mouse gene. The murine N-terminal amino acid sequence is different from human sequence. However, after N-terminal processing (22 amino acids) with signal peptidase, the mature PrP N-terminal sequence (17 amino acids) is identical between mouse and human (10,11). **b.** Southern blot analysis of the ES cells with the 3' flanking probe. A targeted clone (lox-neo-gpt-lox lane) showed 9 and 10 kbp bands in the Nde I digest, and 6.8 and 8 kbp bands in the Kpn I digest. Untargeted clones (the other 3 lanes) showed a wild type band each in Nde I or Kpn I digestion. The numbers on the left side indicate the molecular weight standard (kbp).
RESULTS AND DISCUSSION

The targeting vector (Fig. 1a) is designed to perform deletion of the coding sequence from the Sma I to Apa I site in the exon 3 of murine PRNP as well as introduction of the human PrP coding region with codon 145 mutation. In the vector, a DNA fragment containing MC1-neo and PGK-gpt (Xanthine/guanine phosphoribosyl transferase) flanked by two loxP sequences was inserted downstream of the human PrP segment. The targeting vector was transfected into ES cells, and 120 G418-resistant clones were screened by the Southern blotting using 3′ (Fig. 1b), 5′ or neo probe (data not shown). One clone out of 120 G418 resistant colonies was found to contain the desired homologous recombination.

For germ line transmission, 2 agouti coat color chimeric male mice were mated to C57BL/6J females. The agouti coat color offspring were analyzed by the Southern blot hybridization (Fig. 2b). The recombinant allele was detected in 12 (52%) of 23 agouti offspring. Cre recombinase was expressed transiently in the fertilized eggs obtained from C57BL/6J females mated with the heterozygous mutant males. The tail DNA of 13 offspring from 2 heterozygous mutant males was examined using PCR analysis with neo primers and Southern blotting. Only one DNA sample showed a positive PCR product for the neo gene. Thus, about half of remaining 12 mice were considered to attain a humanized knock-in allele after site-specific recombination. Southern blot analysis was performed with 3′ flanking probe using Nde I digest (Fig. 2b) or Kpn I digest (data not shown). Six DNA samples showed a wild type band (9 kbp in Nde I, and 6.8 kbp in Kpn I), one sample showed a wild and a mutant (lox-neo-gpt-lox) band (10 kbp in Nde I, and 8 kbp in Kpn I), and 6 samples showed a wild and a humanized knock-in band (6.5 kbp in Nde I, and ~20 kbp in Kpn I). Thus, transient expression of Cre recombinase in the eggs resulted in the site-specific recombination in 6 out of 7 mice.

FIG. 2. Humanization of PrP by the Cre-loxP system in the germ-line transmitted mouse. a. Southern blot scheme of the mouse tail DNA. A lox-neo-gpt-lox line shows the allele from the ES cell with a homologous recombination. A Hu-Ki (human knock-in) line shows the allele obtained following the site-specific recombination by Cre recombinase. A wild line shows the allele from the C57BL/6J mouse. Nde I restriction fragment length polymorphism is useful for the identification of each allele. b. Southern blot analysis of the mouse tail DNA. Tail DNA samples were digested with Nde I, and then analyzed with the 3′ probe shown in a. Wild, wild type allele from 2 agouti coat mice; neo-gpt, wild and mutant alleles from 2 heterozygous mice from the targeted ES clone; Hu-Ki, wild and humanized alleles from 4 heterozygous recombinant mice. Lanes Wild showed a 9 kbp band, lanes neo-gpt showed 9 and 10 kbp bands, and lanes Hu-Ki showed 9 and 6.5 kbp bands. The numbers on the left side indicate the molecular weight standards (kbp).
The humanized knock-in event shown above was further confirmed by the PCR amplification of the PRNP (Fig-3a). The sense primer (M21) is located at the junction site between intron 2 and exon 3, and the antisense primer (MT 2) is in the downstream region from the Apa I site. Therefore, a pair of these primers does not amplify the mutant allele (lox-neo-gpt-lox allele), but amplifies the humanized knock-in allele and the wild allele. The PCR products from the heterozygous humanized mice showed a humanized pattern in restriction fragment length polymorphism (RFLP) with Kpn I or Pst I digestion (Fig. 3b). Mae I RFLP also confirmed the induction of human PrP codon 145

**FIG. 3.** PCR analysis of the PrP gene in the wild and humanized mice. a. A scheme of PCR amplification and the allele-specific restriction enzyme sites. The humanized knock-in allele has a Pst I site, and lacks a Kpn I site, while the wild allele has a Kpn I site, and does not have a Pst I site. b. Restriction fragment length polymorphism of PCR products. Lanes Hu-Ki (4 lanes) correspond to the PCR products amplified for the tail DNA samples from the humanized knock-in heterozygotes. The other lanes are from DNA of the wild-type mice. Pst I cut the PCR products into 583 and 396 bp fragments in lanes Hu-Ki. Kpn I did not cut the PCR products (985 bp) from a humanized allele in lanes Hu-Ki, and did cut the PCR products from a wild allele into 327 and 671 bp fragments.
mutation (data not shown). During preparing manuscript, heterozygous knock-in mice are 3 months old without overt clinical phenotype. Investigations are in progress to determine the pathogenic consequences of these mutant proteins on the function and the transmission study of prion protein.

The Cre-loxP system operates efficiently in mammalian cells. This system is particularly useful in gene-targeting experiments in the mouse, and has already been applied for complete deletions of target gene (15,16), an inducible knock-out (17), and a chromosomal engineering including deletion and translocation (18,19). Recently, the Cre-loxP system was also used in the knock-in strategy directed to induce a partial humanized immunoglobulin (20), or a murine myogenin cDNA (21). In these reports, expression of Cre recombinase was carried out in the ES cells. In the present study, the fertilized eggs from the germ-lined heterozygotes were subjected to expression of Cre recombinase, which led to efficient recombination (6 out of 7 offspring). This method has an advantage to preserve the potentiality of germ-line transmission because it does not need a subsequent electroporation procedure. In the report using the transgenic animal, microinjection of Cre plasmid induced complete recombination before the morula stage, and did not show the mosaicism (incomplete recombination) and the integration of the Cre sequence (14). Therefore, this method is an efficient alternative instead of screening ES cells for successful recombination after transient expression of Cre introduced by electroporation.

For the germ-line transmission, it is important to choose the selection cassette. In the knock-in strategy, the neo-tk (Herpes simplex thymidine kinase) cassette was commonly used (16,20). However, this neo-tk cassette allele did not carry on the germ-line transmission in our previous p53 gene replacement (5) and PRNP gene knock-out (unpublished data). In this study, we used the neo-gpt cassette, and the ES cells carrying this cassette developed into the heterozygotes with the mutant allele. Therefore, the gpt cassette is recommended instead of the tk cassette for the germ-line transmission. In addition, the gpt cassette is useful as the selection marker in the universal knock-in method using the double targeting (5,7). We thus conclude that the lox-neo-gpt-locassette serves as a tool capable of altering the mammalian genome as desired, and has a potentiality to develop the adequate animal model for the human disease.

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