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IV.　研究成果の刊行物・別冊

Chapter 2

Genetic Manipulations Using Cre and Mutant *LoxP* Sites

Kimi Araki and Ken-ichi Yamamura

Abstract

The bacteriophage P1-derived Cre/*lox* recombination system has been extensively used to engineer the genome of cultured cells and experimental animals. Cre recombinase recognizes the *lox*P site, which is composed of two 13-bp inverted repeats and an 8-bp spacer region, and mediates both intramolecular (excisive) and intermolecular (integrative) recombination between two *lox*P sites. The excision reaction is efficient and can be used in conditional knockout strategies. On the other hand, integrative recombination is inefficient because the integrated DNA retains *lox*P sites at both ends and is easily excised again if the Cre recombinase is still present. However, integrative recombination is expected to be a powerful tool for genome engineering in mouse embryonic stem (ES) cells because it allows precise and repeated knock-in of any DNA into *lox* sites placed in the genome. To promote integrative recombination, two kinds of mutant *lox* systems have been developed and successfully used in ES cells to produce exchangeable (multipurpose) alleles. In this chapter, we describe a Cre/mutant *lox* system for integrative recombination, and we present an application of this system to gene targeting. By incorporating mutant *lox* sites into gene targeting vectors, we can first produce a null allele. Subsequently, any gene of interest, including the Cre recombinase gene itself, fluorescent genes, luciferase genes, mutated cDNAs, and human cDNAs, can be inserted and expressed under the endogenous promoter of the targeted gene. By combining other recombination systems, such as Flp/*FRT*, we can also convert null alleles into conditional alleles.

Key words: Site-specific recombination, Cre, Mutant *lox*, Exchangeable gene targeting, Flp/*FRT*, Embryonic stem cell

1. Introduction: Cre/*Lox* Systems

1.1. Brief Summary of the Cre-LoxP System

The Cre/*lox* recombination system is derived from bacteriophage P1 and arose to prominence as the most powerful tool for genome engineering (1, 2). Cre recombinase catalyzes reciprocal site-specific recombination between two specific 34-bp sites, called *lox*P sites. Each *lox*P site is composed of two 13-bp inverted repeats that serve as Cre binding sites, and an 8-bp central spacer region that participates in strand exchange during recombination (3, 4).

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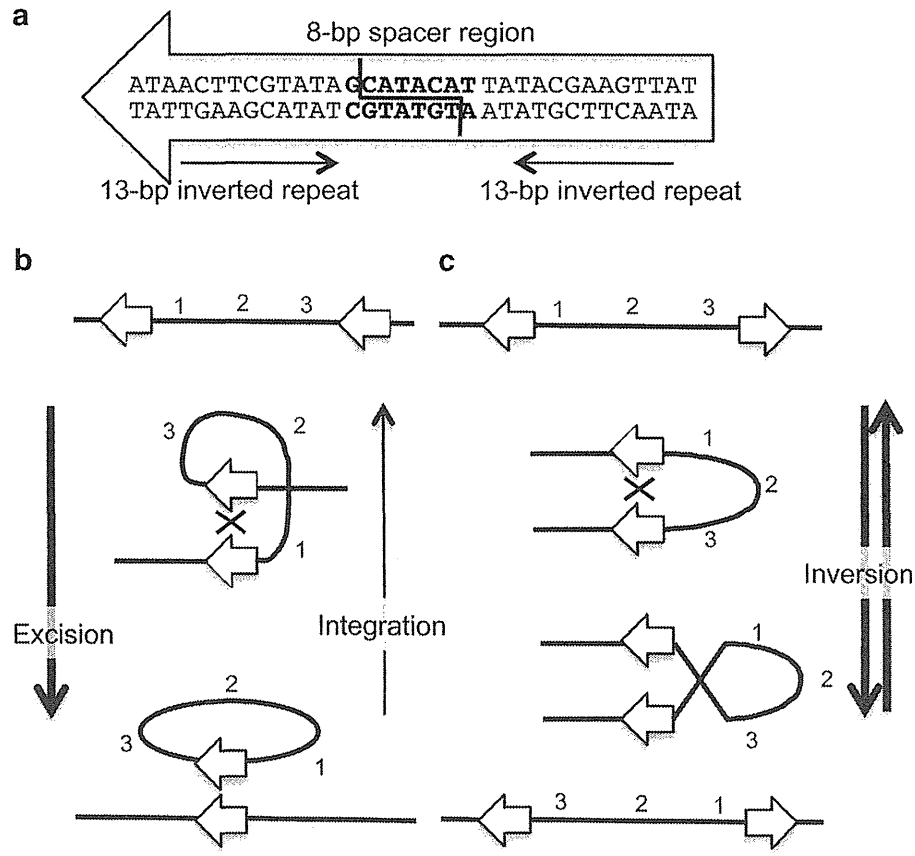

Fig. 1. Cre/*lox* recombination system. (**a**) Wild-type *lox*P sequence. The 34-bp *lox*P site is composed of two 13-bp inverted repeat regions and an 8-bp asymmetric spacer region. The asymmetric spacer region dictates the orientation of the *lox*P sequence, as indicated by the *large arrow*. (**b**) Recombination between two *lox*P sites with the same orientation. Excisive intermolecular recombination occurs efficiently; however, integrative intermolecular recombination rarely occurs due to reinsertion. (**c**) Recombination between two *lox*P sites in the inverse orientation. In this case, recombination results in inversion of floxed DNA.

The sequence of the spacer region is asymmetric and confers directionality to the *lox*P site (Fig. 1a) (5). Depending on the orientation of the *lox*P sites with respect to one another, the recombination can result in excision, inversion, or integration (Fig. 1b, c). Integrative recombination is very powerful for genome engineering in mouse embryonic stem (ES) cells, because it allows precise and repeated knock-in of any exogenous DNA into chromosomally located *lox* sites that have been introduced by gene targeting. However, integrative recombination between wild-type *lox*P sites is inefficient due to re-excision through intramolecular recombination in the presence of Cre recombinase (6). Studies of mutated *lox*P sites have revealed that two classes of mutations can promote Cre-mediated integration or replacement.

1.2. Left Element/Right Element (LE/RE) Mutant Strategy

One class of mutant *lox*, originally reported by Albert et al. (7), is the left element/right element (LE/RE) mutant strategy that uses an LE mutant *lox* site carrying mutations in the left-inverted repeat region and RE mutant *lox* site carrying mutations in the right-inverted repeat region. Recombination between an LE mutant *lox* site and an RE mutant *lox* site results in the production of a double mutant *lox* site having mutations in both ends and a wild-type *lox*P site (Fig. 2a). Since the binding affinity of the double mutant *lox* site for Cre recombinase is severely decreased, the integrated DNA is stably retained.

Albert et al. introduced five nucleotide changes into the left 13-bp element (*lox*71) or into the right 13-bp element (*lox*66) (Table 1) and succeeded in producing *lox*-site-specific integration

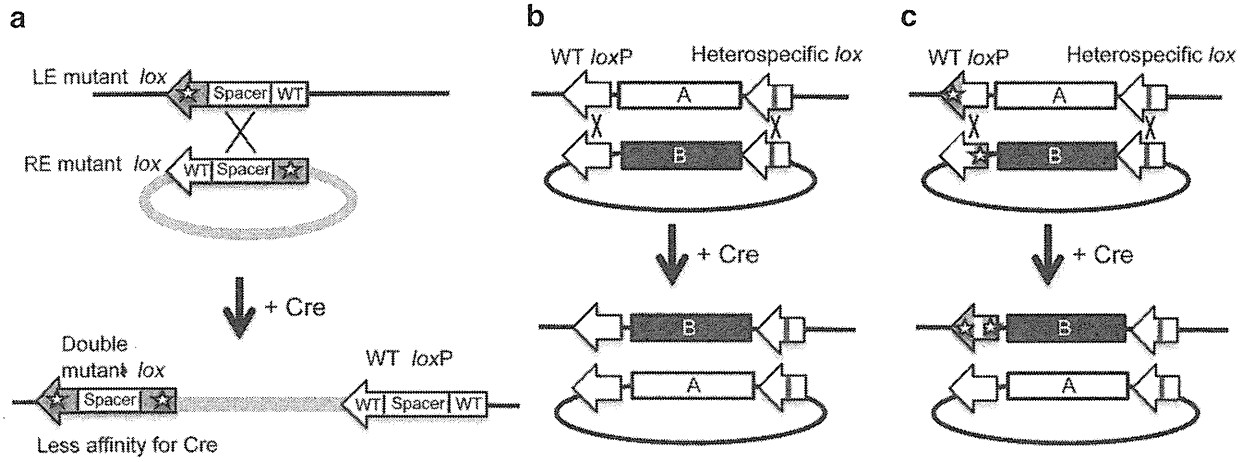

Fig. 2. Cre/mutant *lox* recombination system. (**a**) Integrative recombination using LE/RE mutant *lox* sites. Recombination between LE mutant *lox* and RE mutant *lox* sites results in the production of a double mutant *lox* site having mutations in both ends and a wild-type *lox*P site. *Asterisk* represents mutation. (**b**) RMCE using heterospecific *lox* sites. The heterospecific *lox* site is indicated by an *arrow* with a *center line* representing a mutation in the spacer region. A chromosomal cassette (*open box*) flanked by two heterospecific *lox* sites is replaced through Cre-mediated recombination with another cassette (*solid box*) located on a plasmid. (**c**) Combinational RMCE with LE/RE mutant *lox* and heterospecific *lox* sites. Although the recombination efficiency is almost identical to that of *lox*P and heterospecific *lox*, the replaced cassette is quite stable since it is never recombined by Cre.

Table 1
Mutant *lox* sites

LE mutant *lox* site	Sequence of the left-inverted repeat region	RE mutant *lox* site	Sequence of the right-inverted repeat region
*lox*71	TACCGTTCGTATA	*lox*66	TATACGAACGGTA
*lox*JT15	AATTATTCGTATA	*lox*JTZ2	TATACGAATACCT
*lox*JT12	AGTTGTTCGTATA	*lox*JTZ17	TATAGCAATTTAT
*lox*JT510	TAACGTTCGTATA	*lox*KR3	TATACCTTGTTAT

using tobacco cells (7). We used ES cells and assessed the frequency of *lox*-site-specific integration over random integration. The efficiency of *lox*-site-specific integration with LE/RE mutant *lox* sites was 2–16%, which was much higher than the 0.2% efficiency of *lox*P-*lox*P recombination (8). Recently, Thomson et al. performed mutational analysis of LE/RE mutant *lox* sites using *Escherichia coli* and identified a novel LE/RE mutant *lox* pair, *lox*JT15 and *lox*JTZ17 (Table 1), that showed approximately 1,500-fold higher integration rates than *lox*71and *lox*66 (9). The combination of *lox*JTZ17 and *lox*71 produced a frequency that was tenfold higher than *lox*71 and *lox*66. To confirm whether such large differences in frequency are also observed in ES cells, we compared six RE mutant *lox* sites, including *lox*JTZ17, focusing on their recombination efficiency with *lox*71. Unlike in *E. coli*, all of the RE mutant *lox* sites showed similar recombination efficiency in ES cells. However, two RE mutant *lox* sites, *lox*JTZ17 and *lox*KR3, produced more stable (inactive) double mutant *lox* sites with *lox*71 than did *lox*66/71 (10). These two mutant RE *lox* sites would, therefore, be more suitable than *lox*66 for Cre-mediated integration or inversion in ES cells.

1.3. Recombinase-Mediated Cassette Exchange (RMCE) Using Heterospecific Lox Sites

A second class of mutant *lox* site is a heterospecific *lox* site carrying mutation(s) in the central spacer region (5, 11, 12). Cre cleaves the DNA of the spacer region to generate a 6-bp staggered cut, and sequence homology in the 6-bp single-stranded region is essential for recombination between *lox* sites. Therefore, recombination does not occur between two *lox* sites that differ in the spacer region, whereas *lox* sites with identical spacer regions can be recombined efficiently. Recombination using heterospecific *lox* sites is termed recombinase-mediated cassette exchange (RMCE), in which a chromosomal cassette flanked by two heterospecific *lox* sites is exchanged for another cassette located on a plasmid by Cre-mediated recombination (Fig. 2b) (13). To date, *lox*511 (14), *lox*2272 (15), and *lox*5171 (16) (Table 2) have been successfully used for RMCE in ES cells.

We compared the recombination efficiencies of insertion by the LE/RE mutant *lox* strategy and RMCE in ES cells and found that RMCE was two- to threefold more efficient (17). In a comparison

Table 2
Heterospecific *lox* sites

Heterospecific *lox* site	Sequence of spacer region
*lox*511	GTATACAT
*lox*2272	GGATACTT
*lox*5171	GTACACAT

between *lox*2272 and *lox*511 sites, the former showed approximately 1.5-fold higher efficiency, probably due to unfavorable recombination between *lox*P and *lox*511 (17).

1.4. Combination of LE/RE Mutant Lox Sites and Heterospecific Lox Sites

LE/RE mutant *lox* and heterospecific *lox* sites can be used simultaneously in RMCE, as shown in Fig. 2c. The recombination efficiency of this combinational RMCE is almost the same as that of usual RMCE with wild-type *lox*P and heterospecific *lox* sites. The merit of combinational RMCE is high stability of the recombined product even in the presence of Cre protein. Taking advantage of this stability, we successfully integrated the *cre* gene under the control of a strong promoter through combinational RMCE using *lox*71-*lox*2272 and *lox*66-*lox*2272 cassettes in ES cells (17). The inserted *cre* gene was strongly expressed and stably maintained. Cre-expressing mouse lines were established from the ES clones, and the inserted *cre* gene was stably maintained in sequential generations. Thus, the *cre* knock-in system using combinational RMCE should be useful for the production of various Cre-driver mice.

2. Vector Designs for Exchangeable Gene Targeting

By incorporating mutant *lox* sites into gene targeting vectors, the selection marker gene retained in the targeted locus can be exchanged with any other gene of interest, for example, the *cre* gene, fluorescent protein genes, luciferase genes, mutated cDNAs, or cDNAs of other species. Thus, a targeted null allele can be converted into various knock-in alleles. In this section, several designs of such exchangeable targeting vectors and replacement patterns using Cre-mediated recombination are presented.

2.1. Disruption of ATG-Containing Exons

The purpose of this targeting vector is to insert and express a cDNA (containing an open reading frame) under the control of the promoter of a targeted gene. Therefore, the ATG codon of the targeted gene is required to be disrupted and replaced with an LE mutant *lox* site. The backbone of the targeting vector is shown in Fig. 3a. Using a *lox*P-polyadenylation (pA) signal-heterospecific *lox* cassette, two patterns of replacement are possible, as described in the following sections. The 5′ arm of the targeting vector should extend until just before the ATG codon of the target gene (Fig. 3b). Homologous recombination (Fig. 3c) will result in a null allele due to deletion of the start codon. The selection marker can be removed by transient expression of Cre (18) or by mating with Cre-deleter mice (19) (Fig. 3d). Using the original targeted ES clone, the allele can be further modified in two ways.

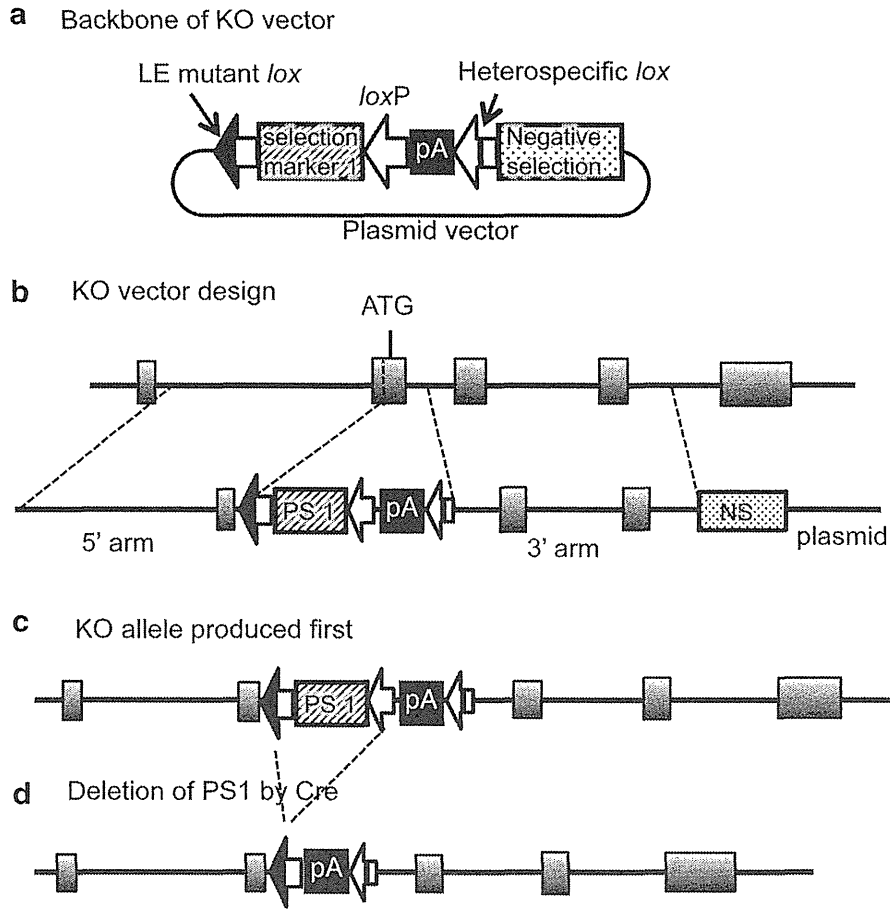

Fig. 3. ATG-containing exon knockout. (**a**) Backbone of KO vector. The transcriptional directions of positive selection marker gene 1 and the negative selection marker gene are not fixed (either direction is acceptable). However, the direction of the polyadenylation signal (pA) should be the same as the direction of the targeted gene. (**b**) Design of KO vector. The positive selection marker cassette (PS1) should be placed just before the ATG codon of the target gene. (**c**) Targeted allele. Since the endogenous ATG codon is deleted, this allele should be null. (**d**) PS1-deleted allele through Cre-mediated recombination.

2.2. Insertion of cDNA

A gene of interest (GoI) can be inserted into the ATG position of a targeted gene (Fig. 4), and the GoI is then driven by the endogenous promoter of the targeted gene. Design of such a replacement vector is shown in Fig. 4a. Importantly, the selection marker in this vector should not contain a pA signal to provide further selection of the recombination event, as described below. The replacement and Cre expression vectors are coelectroporated into the targeted ES clone in their circular forms. The *cre* gene is transiently expressed and mediates recombination. Since the replacement plasmid and the targeting vector in the ES genome both carry two *lox* sites with the same spacer region (RE or LE mutant *lox* and *lox*P), it is expected that intramolecular recombination between the two *lox* sites should occur after coelectroporation, resulting in the production of two intermediate molecules, as shown in Fig. 4b. Integrative recombination then occurs between LE mutant *lox* sites in the genome and RE mutant *lox* sites in the intermediate molecule. ES cells in which

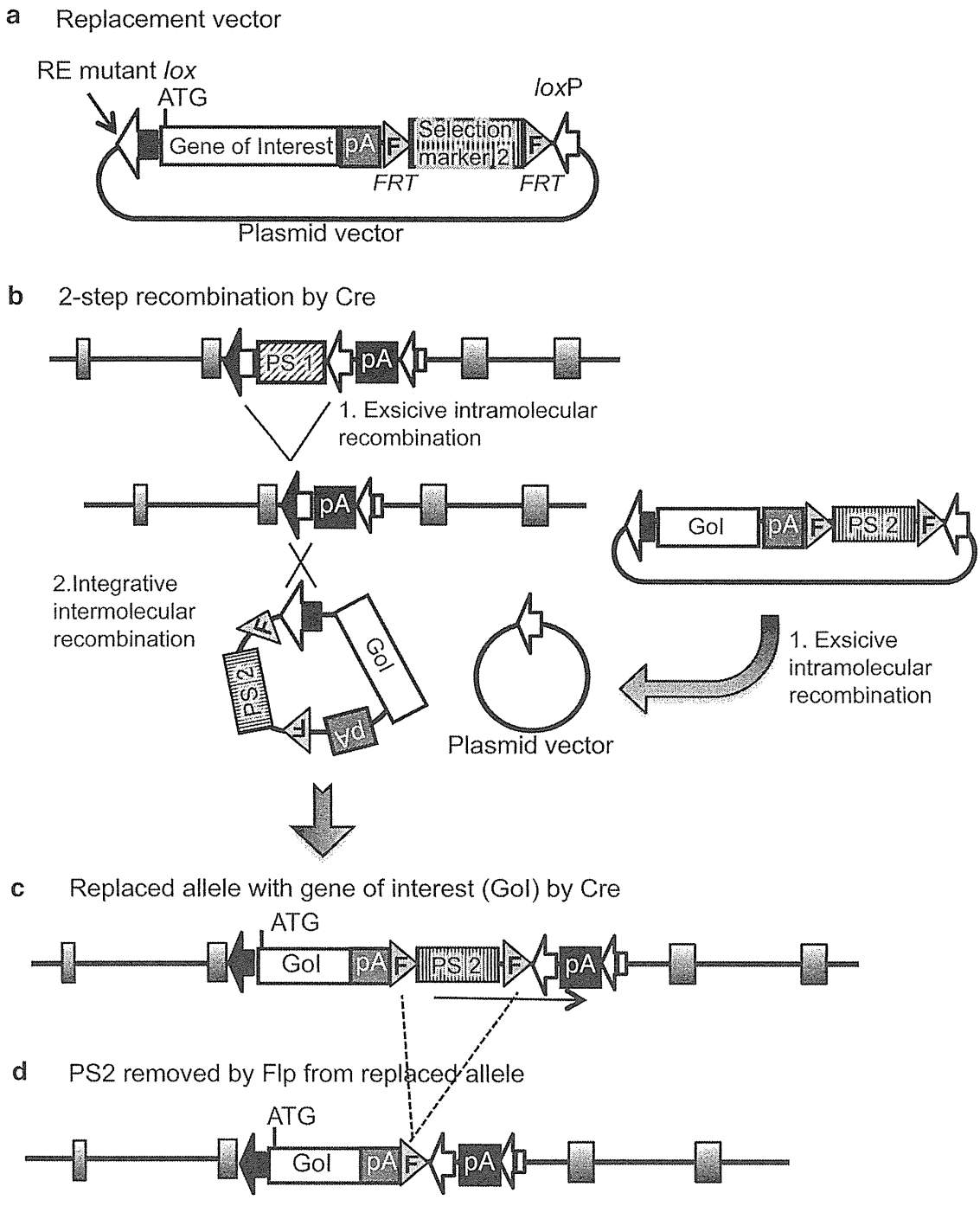

Fig. 4. Knock-in of cDNA through Cre-mediated recombination. (**a**) Replacement vector. The positive selection marker gene 2 (PS2) cassette should not contain a pA signal. (**b**) Intermediate molecules produced through intramolecular recombination. The PS1 cassette is removed from the targeted allele, and the knock-in vector is divided into two circular molecules. Integrative recombination then occurs between LE and RE mutant *lox* sites. Gol, gene of interest. (**c**) Replaced allele. Only upon Cre-mediated site-specific integration does the PS2 gene fuse to the pA signal on the targeting vector, thereby making the cells drug-resistant. (**d**) PS2-deleted allele through Flp-mediated recombination.

the replacement cassette is integrated into the LE mutant *lox* site are selected for with an appropriate drug. Because the selection marker gene in the replacement vector does not contain a pA signal, most of random integrants should be drug sensitive. Only upon

Cre-mediated targeted integration does the selection marker gene fuse to the pA signal on the targeting vector, thereby making the cells drug-resistant (Fig. 4c). This pA trapping strategy enables replaced clones to be obtained at the high frequency of 60–100% (20, 21). If needed, the selection marker gene used for insertion can be removed using another recombination system (Fig. 4d), for example, the Flp/*FRT* system (22). In our experience, inserted genes are expressed efficiently in the presence of the selectable marker gene driven by the mouse *phosphoglycerate kinase-1 (Pgk)* promoter (23). In such cases, it would be better to retain the marker gene including its promoter.

2.3. Insertion of a Tag Peptide

It is often desirable to add a tag peptide to a targeted gene. As shown in Fig. 5a, the ATG codon of a tag peptide should be designed to be in frame with the targeted gene, and the endogenous sequence of splice donor is fused to the tag sequence. To remove a pA signal from the chromosomal targeting cassette, *lox*P and heterospecific *lox* sites are used for recombination, and therefore, a pA signal is required for the selection marker gene which should be flanked with other recombination sites, such as *FRT*. However, the addition of a pA signal dramatically increases the frequency of random integration. We found that the use of the *diphtheria toxin A fragment* (*DT-A*) gene can reduce random integrants by almost half (24). Furthermore, an additional *lox*P site placed between the *DT-A* gene and the plasmid vector sequence is effective in increasing recombination frequency. After electroporation with a Cre expression plasmid, intramolecular recombination between RE mutant *lox* and *lox*P sites occurs, resulting in two circular molecules, as shown in Fig. 5b. Since the size of the targeting DNA molecule becomes smaller, the chance of collision between chromosomal *lox* and plasmid *lox* sites becomes higher. After site-specific recombination (Fig. 5c, d), the selection marker has to be removed, either in ES cells by another recombination system or by mating with recombinase-expressing mice (Fig. 5e).

2.4. Conditional Knockout

Exon replacement, via the engineering of conditional alleles, is a highly effective strategy for the production of mice that model genetic disease. A conditional allele can be produced by flanking a exon of interest with *lox* sites (floxing). The conditional allele can then be exchanged for a mutated exon using a targeting vector, as shown in Fig. 7a. In the exon-exchange targeting vector (Fig. 6a, b), the replacing exon is flanked by *lox*P sites, a heterospecific *lox* site is placed between the coding region of the positive selection marker gene and the pA signal, and the positive selection marker cassette is flanked by other recombination sites such as *FRT*. After homologous recombination (Fig. 6c), the selection marker cassette has to be removed through excising recombination by the other recombination system to produce the conditional allele (Fig. 6d).

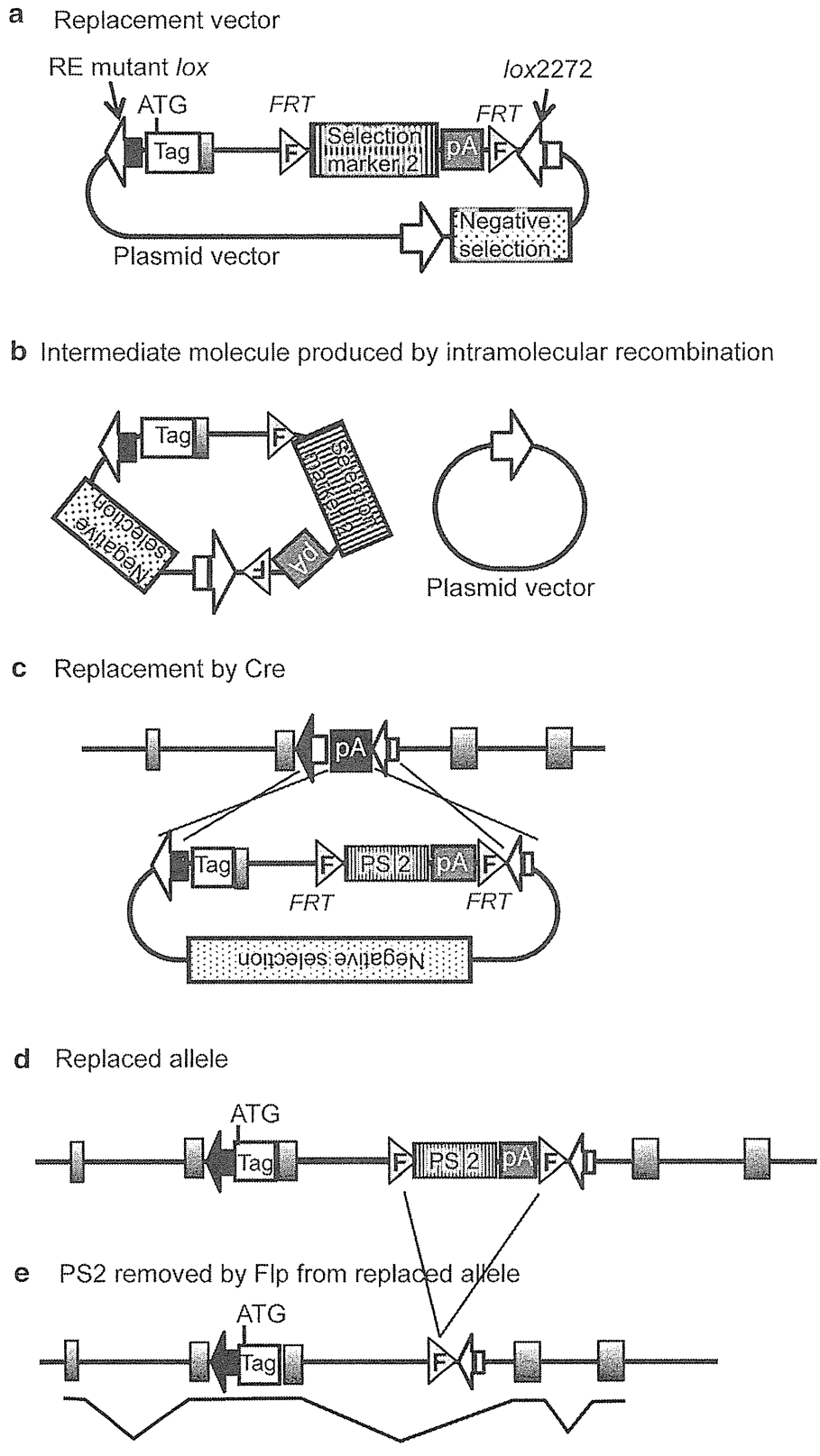

Fig. 5. Production of a tag-fused gene through Cre-mediated recombination. (**a**) Replacement vector. A DNA fragment between RE mutant *lox* and heterospecific *lox* sites is integrated into chromosomal lox sites in the targeting vector. In this case, positive selection marker gene 2 should have a pA signal. To reduce random integrants, addition of a negative selection marker gene and a *lox*P site is effective. (**b**) Intermediate molecules produced through intramolecular recombination. The knock-in vector is divided into two circular molecules. (**c**) Cassette-exchange recombination occurs between LE/RE mutant *lox* sites and heterospecific *lox* sites. PS2, positive selection marker gene 2. (**d**) Replaced allele. Since the PS2 gene exists in the intron, transcription from the endogenous promoter may be disturbed. (**e**) PS2-deleted allele through Flp-mediated recombination. After removal of the PS2 cassette, a fused transcript can be produced.

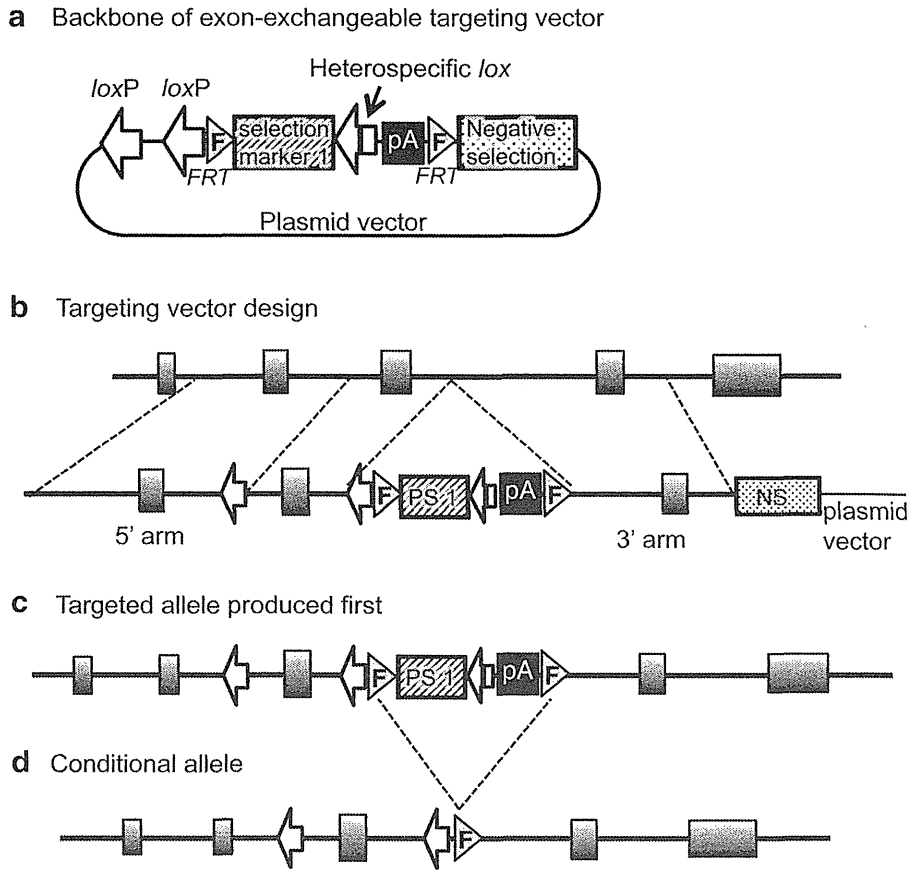

Fig. 6. Exon-exchange targeting. (**a**) Backbone of targeting vector. The transcriptional directions of positive selection marker gene 1 (PS1) and the negative selection marker gene (NS) are not fixed (either direction is acceptable). However, the direction of the polyadenylation signal (pA) should be the same as the direction of targeted gene. (**b**) Design of KO vector. The exon to be replaced in the next step is placed between two *loxP* sites. (**c**) Targeted allele produced first. Since the PS1 gene exists in the intron, transcription of the targeted gene may be disturbed. (**d**) Conditional allele produced by Flp-mediated recombination. After removal of the PS2 cassette, only a *loxP* site and *FRT* site remain in the intron, which should leave a normally transcribed gene.

2.5. Exchange of an Exon

Design of a replacement vector is shown in Fig. 7a. The selection marker in this vector should not contain a pA signal. The negative selection marker gene and the following *loxP* site are not essential, because the recombination efficiency without these elements is sufficiently high due to pA trapping. After coelectroporation of the replacement and Cre expression vectors, two-step site-specific recombination should occur, intramolecular excising recombination followed by cassette replacement recombination (Fig. 7b). Only upon site-specific recombination does the selection marker gene fuse to the pA signal on the targeting vector, thereby making the cells drug-resistant (Fig. 7c). After removal of the selection marker gene with the other recombination system, the mutated exon is incorporated to the mRNA of the targeted gene.

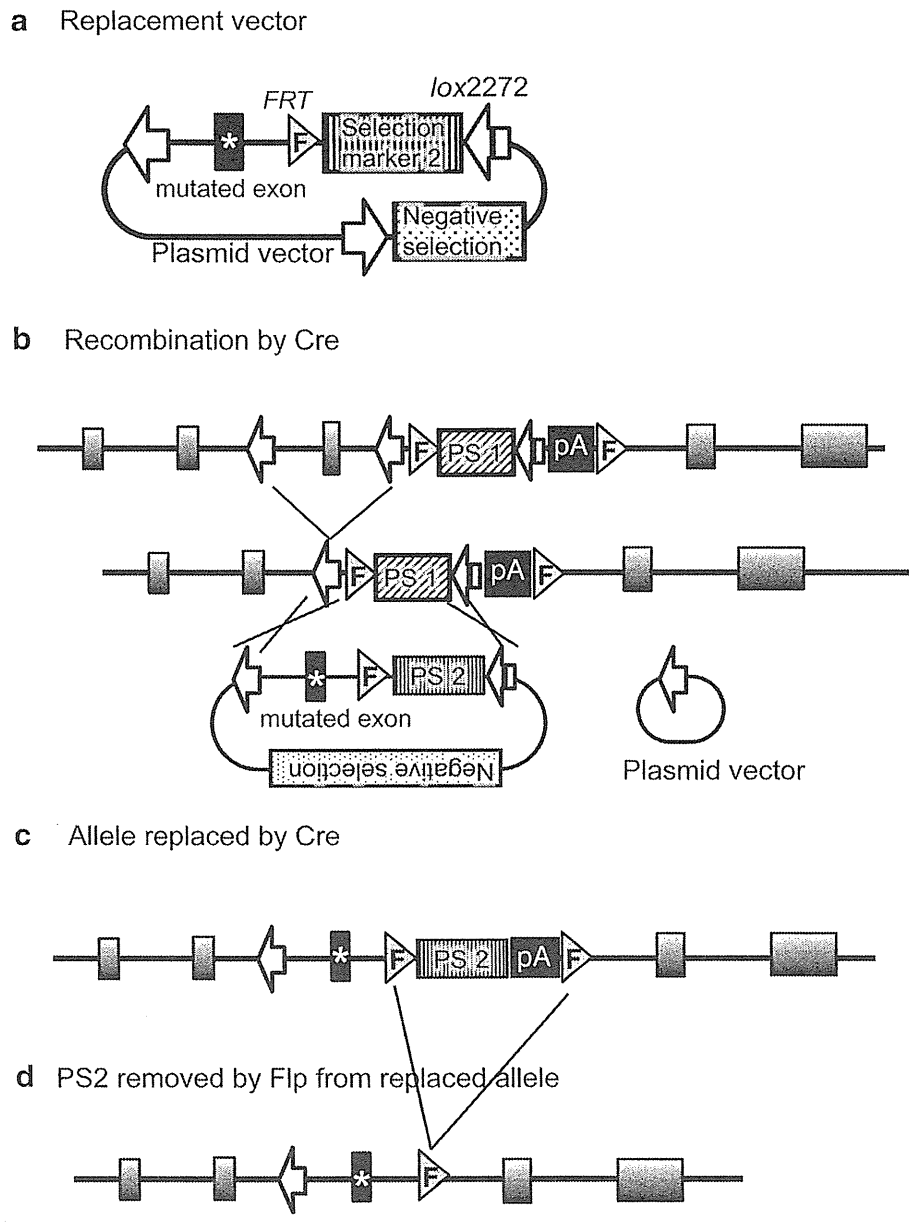

Fig. 7. Exchange of an exon through Cre-mediated recombination. (**a**) Replacement vector. Positive selection marker gene 2 (PS2) should not have a pA signal. A negative selection marker gene and an additional *lox*P site are optional. (**b**) Intermediate molecules produced through intramolecular recombination. First, the floxed exon is removed and the replacement vector is divided into two circular molecules, and then intermolecular cassette-exchange recombination occurs. (**c**) Replaced allele. Since the PS2 gene exists in the intron, transcription from the endogenous promoter may be disturbed. (**e**) PS2-deleted allele through Flp-mediated recombination. After removal of the PS2 cassette, the allele containing the mutated exon is produced.

3. Example of Cre-Mediated Recombination in ES Cells

3.1. Vector Design and Protocol

In this section, we will illustrate how cassette exchange is performed in ES cells by describing a specific example. Figure 8a shows our exchangeable targeting vector containing a *lox*71-*Pgk*-neo^{R}-*lox*P-pA-*lox*2272 cassette (25), and Fig. 8b shows a replacement vector for the insertion of a cDNA fragment. We usually use

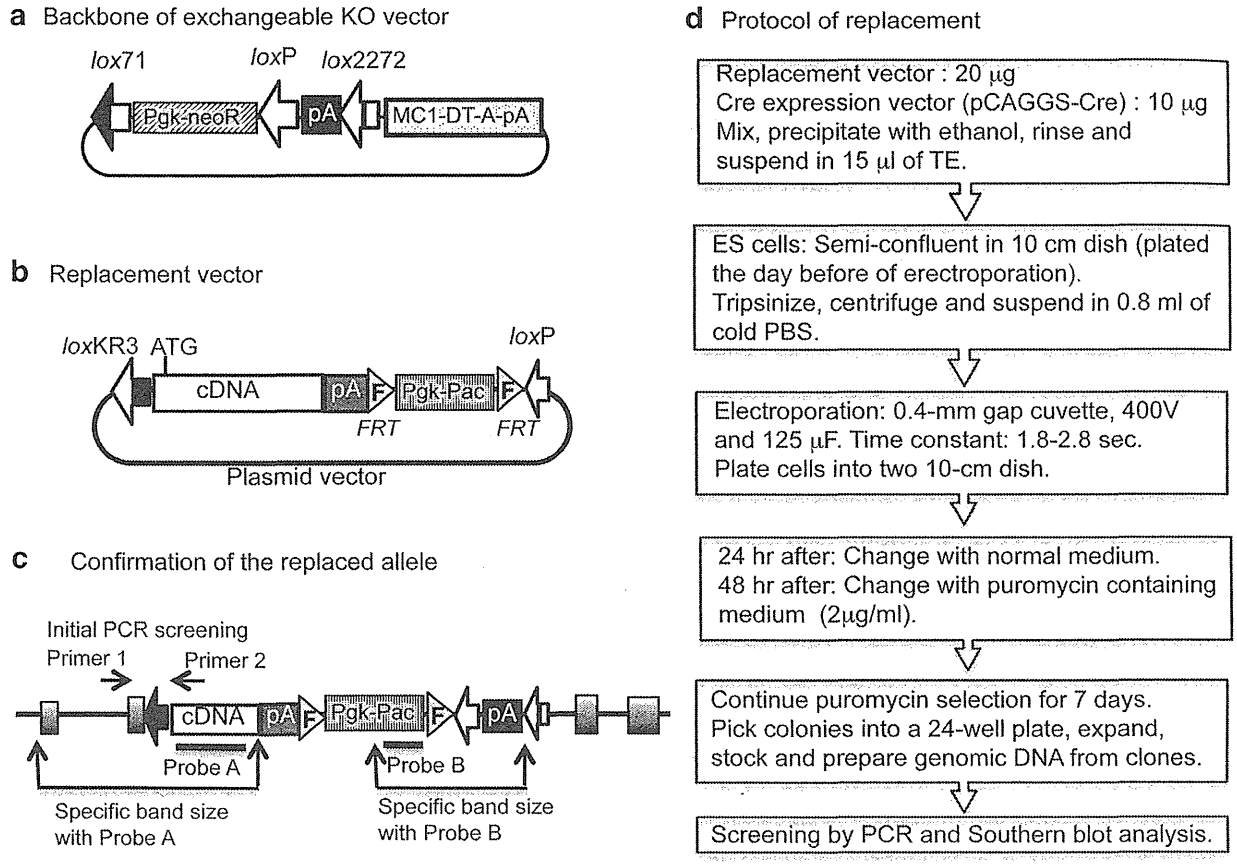

Fig. 8. Example of Cre-mediated recombination. (**a**) Exchangeable KO vector contains a *lox*71-Pgk-neo^R-*lox*P-pA-*lox*2272 cassette. Pgk, the promoter of the mouse *phosphoglycerate kinase-1* gene; neo^R, the open reading frame (ORF) of the *neomycin phosphotransferase* gene; pA, polyadenylation signal. (**b**) Replacement vector to insert a cDNA. The *Pgk* promoter and the *puromycin N-acetyltransferase* (*Pac*) gene are used as a positive selection marker. The *Pac* gene does not have a pA signal. (**c**) Replaced allele. For initial screening, a PCR assay that detects the 5′ junction is used. Then, both junctions should be confirmed by Southern blot analysis. It is also important to confirm that no random integration occurs. (**d**) Electroporation protocol for Cre-mediated replacement.

the *puromycin N-acetyltransferase* (*Pac*) gene as a positive selection marker gene in the replacement vector (26). The *Pac* gene has no pA signal to select recombined clones.

Figure 8d depicts the protocol for coelectroporation and selection. Twenty micrograms of replacement plasmid and 10 μg of pCAGGS-Cre (18), a vector producing strong expression of the *cre* gene, are used in their circular forms. The ES cells are harvested from a 10-cm dish and electroporated with plasmids at 400 V and 125 μF. Selection for 7 days with puromycin at 2 μg/ml is started 48 h after electroporation. Colonies become visible under the microscope from 3 days after starting selection. Since Cre-mediated site-specific recombination fuses the pA signal to the *Pac* gene, we can predict the degree of replacement success from the number of colonies. If the number of colonies is over 50, correctly targeted clones will be successfully obtained, but if the number is under 20, the colonies might all be random integrants.

Genomic DNA from clones has to be examined to determine whether the replacement cassette is correctly inserted. For initial screening, a PCR assay that detects the 5′ junction, as shown in Fig. 8c, is recommended. Next, both junctions should be confirmed through Southern blot analysis using restriction enzyme(s) that digest inside and outside of the inserted cassette to fragments of specific sizes (Fig. 8c). Southern blot analysis is essential to confirm correct targeting with no random integration event.

3.2. Production of Humanized Mice by the Insertion of Human cDNA

Humanized mice can be produced by introducing a homologous human gene or cDNA into the endogenous mouse gene locus. With such an approach, the inserted human gene/cDNA is expressed under the mouse promoter with similar temporal and spatial expression to that of the endogenous mouse gene. Such humanized mice are excellent animal models for human diseases and for genome-based drug discovery.

We conducted humanization of the mouse *transthyretin* (*Ttr*) gene (25). TTR protein is synthesized mainly in the liver, choroid plexus, and retinal pigment cells, and is secreted into plasma, cerebrospinal fluid, and vitreous body, respectively (27–29). It circulates as a tetramer and serves as a transporter for thyroxine and the retinol-binding protein (RBP) (30). The human *transthyretin* (*TTR*) gene spans about 7 kilobases (kb) and is composed of four exons (31). *TTR* has about 110 variants, more than 90 of which are associated with human amyloidosis. To produce humanized mice carrying different TTR variants, we used the exchangeable targeting vector containing a neomycin resistance gene flanked by *lox*71 and *lox*P sites, as shown in Fig. 8a.

The production of the replaced allele was done in two steps. The first step was the production of the targeted KO allele carrying *lox*71 and *lox*P sites by homologous recombination (Fig. 9a). After electroporation with the exchangeable targeting vectors, we isolated and analyzed 98 neo-resistant clones and obtained 5 targeted clones. Two out of the 5 clones produced germ-line chimeras. Therefore, these clones were used for the second step, the Cre-mediated site-specific integration of *TTR* cDNA. We constructed a replacement vector containing *lox*66-exon-intron cassette-hTTR cDNA-pA-*FRT*-Pgk-*Pac*-*FRT*-*lox*P. The targeted ES clones were coelectroporated with the replacement vector and the Cre expression vector, pCAGGS-Cre. Twelve puromycin-resistant colonies were isolated from each clone and were analyzed by PCR and Southern blot analysis for the site-specific recombination event. All 24 puromycin-resistant clones had recombined as expected; the neo cassette was replaced with the *TTR* cDNA and Pgk-Pac cassette (Fig. 9b). Thus, pA trapping upon site-specific integration effectively selected for recombined clones.

Chimeric mice were produced with the humanized ES clones, and mouse lines were established. Humanized mice of all

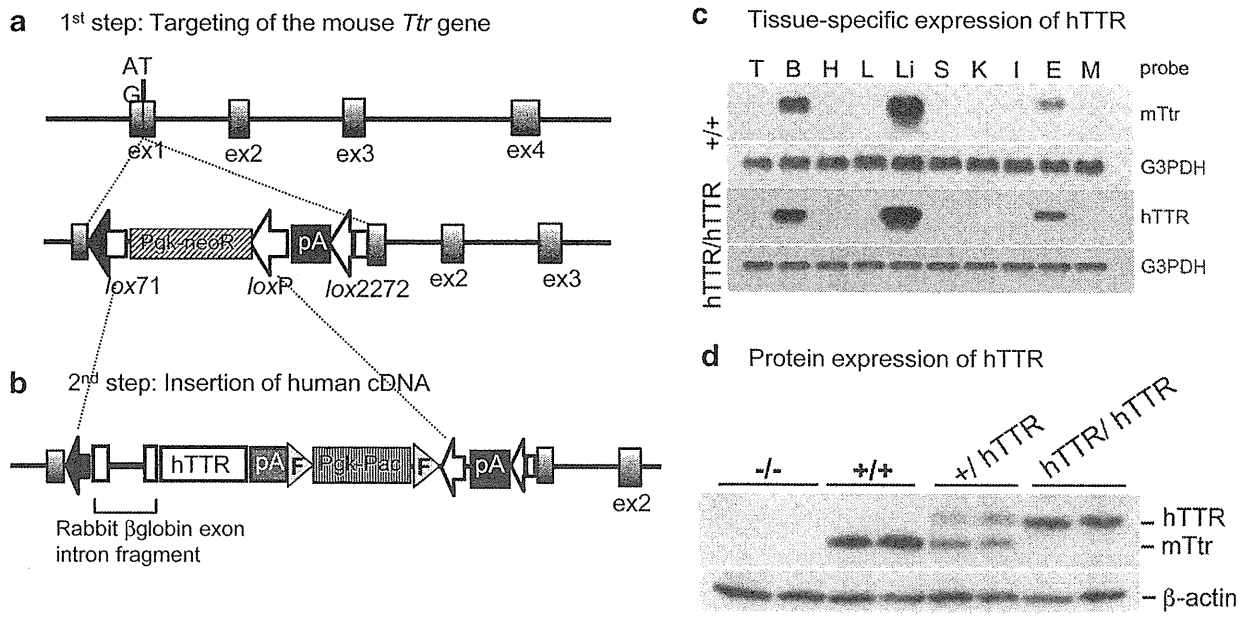

Fig. 9. Production of a humanized allele of the *Ttr* locus. (**a**) Knockout *Ttr* allele produced with a *lox*71-Pgk-neo^R-*lox*P-pA-*lox*2272 cassette. The mouse *Ttr* gene is composed of 4 exons, and the ATG codon resides in exon 1. The ATG codon was removed, and the neo cassette was inserted into the ATG position. (**b**) Replaced allele with a human *TTR* cDNA. To increase expression of the inserted cDNA, an exon-intron cassette was placed before the cDNA. (**c**) Northern blot analysis comparing the expression patterns of the endogenous *Ttr* gene in WT mice and the inserted human *TTR* cDNA in *TTR* allele homozygotes. *T* thymus, *B* brain, *H* heart, *L* lung, *Li* liver, *S* spleen, *K* kidney, *I* intestine, *E* eye, *M* muscle. (**d**) Western blot analysis of liver extracts using an anti-TTR antibody recognizing both human and mouse TTR proteins.

genotypes appeared normal up to at least 6 months of age. To compare the expression patterns of endogenous *Ttr* and the inserted *TTR* cDNA, RNAs were extracted from various tissues of wild-type and humanized mice and analyzed by Northern blotting. In all mice examined, the *TTR* cDNA was consistently expressed in the liver, brain, and eyes (Fig. 9c), which are precisely the same tissues in which endogenous *Ttr* is expressed (32). This suggests that the *TTR* cDNA, located in the *Trt* locus, is correctly regulated under the promoter of the *Ttr* gene.

We analyzed protein levels of human and mouse TTR in KO (−/−), WT (+/+), +/*TTR*, and *TTR*/*TTR* mice using western blotting of liver extracts. As shown in Fig. 9d, both mouse and human TTR proteins were detected in the liver of +/*TTR* mice, but only human protein was detected in the liver of *TTR*/*TTR* mice. The level of TTR in the liver of *TTR*/*TTR* mice was twice that in the livers of +/*TTR* mice. These results showed human TTR protein was expressed in a gene-dose-dependent manner in the humanized mice.

4. Combination of the Cre/*Lox* System with Other Recombination Systems

With the Cre/mutant *lox* system, any DNA of interest can be inserted into the mouse genome; however, re-excision by Cre is impossible, and removal of the inserted DNA requires the use of another recombination system. To date, three recombination systems have been described, although their recombination activities in vivo remain to be examined.

The first is the well-known Flp/*FRT* system (33), which is derived from *Saccharomyces cerevisiae* (34). Flp is a recombinase which recognizes the *FRT* site. Wild-type Flp protein showed limited recombination efficiency in mammalian cells due to thermoinstability (35). Although mutational screening identified Flpe protein, which showed four times higher activity (36), the activity of Flpe is still lower than that of Cre protein. Therefore, the Flp/*FRT* system has been mainly used for deletion of selection marker gene in ES cells. However, in 2007, Raymond and Soriano reported codon-optimized Flp (*Flpo*), which showed high recombination efficiency in ES cells, comparable with that of Cre (37).

The second recombination system is ΦC31 from *Streptomyces lividans*. ΦC31 recombinase recognizes two heterotypic sequences, attB and attP. Raymond and Soriano also improved ΦC31 (ΦC31o) and produced ΦC31o-expressing mice (37). Although the recombination efficiency of ΦC31o in vivo was slightly lower than that of Cre, it would be of use when complete recombination is not required.

The third and most promising recombination system is Dre/*rox*, as reported by Sauer and McDermott in 2004 (38). Dre/rox was identified from the P1-like transducing phage D6, which was isolated from *Salmonella oranienburg*. Dre recombinase recognizes and recombines *rox* sites composed of 32 bp. Recently, Anastassiadis et al. reported that Dre/*rox* is highly efficient in mice as well as in ES cells, similar to Cre/*lox* (39).

By placing two recognition sites of another recombination system on the 5′ and 3′ ends of the inserted DNA, the DNA can be removed conditionally through mating with transgenic mice expressing the recombinase in a tissue-specific manner. Thus, through the combinatorial use of different recombination systems, we can control gene expression in an off-on-off manner.

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