Chapter 23

Gene Targeting in Mice: A Review

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Abstract

The ability to introduce DNA sequences (e.g., genes) of interest into the germline genome has rendered the mouse a powerful and indispensable experimental model in fundamental and medical research. The DNA sequences can be integrated into the genome randomly or into a specific locus by homologous recombination, in order to: (1) delete or insert mutations into genes of interest to determine their function, (2) introduce human genes into the genome of mice to generate animal models enabling study of human-specific genes and diseases, e.g., mice susceptible to infections by human-specific pathogens of interest, (3) introduce individual genes or genomes of pathogens (such as viruses) in order to examine the contributions of such genes to the pathogenesis of the parent pathogens, (4) and last but not least introduce reporter genes that allow monitoring *in vivo* or *ex vivo* the expression of genes of interest. Furthermore, the use of recombination systems, such as Cre/loxP or FRT/FLP, enables conditional induction or suppression of gene expression of interest in a restricted period of mouse's lifetime, in a particular cell type, or in a specific tissue. In this review, we will give an updated summary of the gene targeting technology and discuss some important considerations in the design of gene-targeted mice.

Key words Gene targeting, Transgenic mice, Knockout mice, Reporter mice, ES cell lines, Targeting vector, Cre/loxP, FRT/FLP, MultiSite Gateway Cloning

1 The Application of Genetically Modified Mice for the Study of Viral Pathogenesis and Antiviral Immunity

The development of mice with germline genetic modifications has advanced our understanding of the mechanisms of viral pathogenesis and antiviral immune responses during virus–host-interactions enormously. For instance, transgenic mouse models that express antigen-specific, major histocompatibility complex (MHC)-restricted T cell receptor (TCR) transgenes have been used extensively to investigate virus-specific T cell responses. Such transgenic mice include for example mice expressing a TCR transgene specific for the influenza virus hemagglutinin (HA) in the context of the MHC class I or II molecules [1, 2], and transgenic mice expressing

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MHC-restricted TCR with specificity for a lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived T helper cell epitope [3, 4].

In addition, because mice are not susceptible to many human viruses, such as hepatitis viruses, papillomavirus, poliovirus, human immunodeficiency virus-1 (HIV-1), and measles, the generation of transgenic mice that express human receptors specific for such viruses have rendered those transgenic mice susceptible to infection by human viruses of interest and subsequently enabled to investigate their pathogenesis in *in vivo* models (reviewed in ref. 5).

An alternative to mice expressing human virus receptors is provided by transgenic mice with (conditional) expression of individual genes or genomes of viruses of interest. Such (conditional) expression of viral genes in mice imitates viral infection and thus enables *in vivo* investigation of the pathogenesis and immune responses induced by human viruses of interest [5–7].

Moreover, knockout mice with deletions of specific genes or cell populations of interest have been useful to identify and investigate the cellular and molecular components of the adaptive and innate immune system that play a role in controlling viral infections. Such knockout mice include, for example, TCR-β knockout mice that lack T cells [8]; μMT mice that lack B cells [9]; RAG-1 and RAG-2 knockout mice that lack B and T cells [10, 11]; knockout mice with deletions of immune mediators (such as chemokines and cytokines) including knockout mice for type I and type II interferons or interferon receptors [12–15]; knockout mice for immune receptors, such as Toll like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) [16–19]; knockout mice for immunologically relevant transcription factors, such as STAT (Signal Transducer and Activator of Transcription) molecules [20, 21] and interferon regulatory factors (IRFs) [22]; and knockout mice for adaptor molecules (such as MyD88, Rip2, and Trif) that are involved in connecting signals from immune receptors to downstream enzymes and transcription factors [16, 17, 19].

In order to unequivocally identify and investigate the cellular sources of cytokines, major soluble mediators of innate and adaptive immune responses, many cytokine reporter mice have been established that enable to track cytokine production during, e.g., infections [23–25]. A special chapter by Brinkmann and colleagues (*see* Scheibe et al., Chapter 25 of this series) describes in this issue of "Virus-Host-Interactions" the use of a cytokine reporter mouse model, the IFNβ-Luciferase-knockin reporter mice [26], for the analysis of type I IFN induction by mouse cytomegalovirus (MCMV).

2 Techniques to Generate Genetically Modified Mice

While the research of viral pathogenesis and antiviral immunity has taken advantage of genetically modified mice, as described above, it is worth mentioning that, on the other side, it was the use of viruses that opened up the possibility to modify the genome of mice and helped to generate the first transgenic mice in 1976 [27]. To address the question whether exogenous viruses (transmitted horizontally, not hereditarily, from individual to individual) can be converted into endogenous viruses (of which DNA sequences are present in all somatic and germ cells of an individual and passed on to the offspring), Rudolf Jaenisch infected preimplantation mouse embryos (at the 4–8 cell stage) with the Moloney murine leukemia virus (M-MuLV). The mice generated from these infected preimplantation embryos developed M-MuLV-induced leukemia, and the viral DNA was integrated into the germ line of the mice and transmitted to their offspring [27]. Subsequently the direct microinjection of DNA of interest into the pronucleus of fertilized murine eggs was developed as a more commonly used technique to generate transgenic mice [28–32].

However, the generation of genetically modified mice by infection of mouse embryos with retroviruses or microinjection of DNA into fertilized murine eggs results in random integrations of the exogenous DNA into the mouse genome. This in turn can lead to variegated expression of the transgene and inadvertent disruption of genes at the site into which the transgene is inserted. The frequency of phenotypes arising from insertion site mutation by a transgene (almost 10 %) is higher than might be expected from random integration into the genome [33], because introduction of transgenes by pronuclear injection can generate large deletions and complex rearrangements at the site of DNA integration [33]. In contrast, gene targeting by homologous recombination in murine embryonic stem cells (ESCs), a method that was established in the late 1980s, has enabled controlled and specific genetic modification by site-specific integration of exogenous DNA of interest into the genome of mice [34–37].

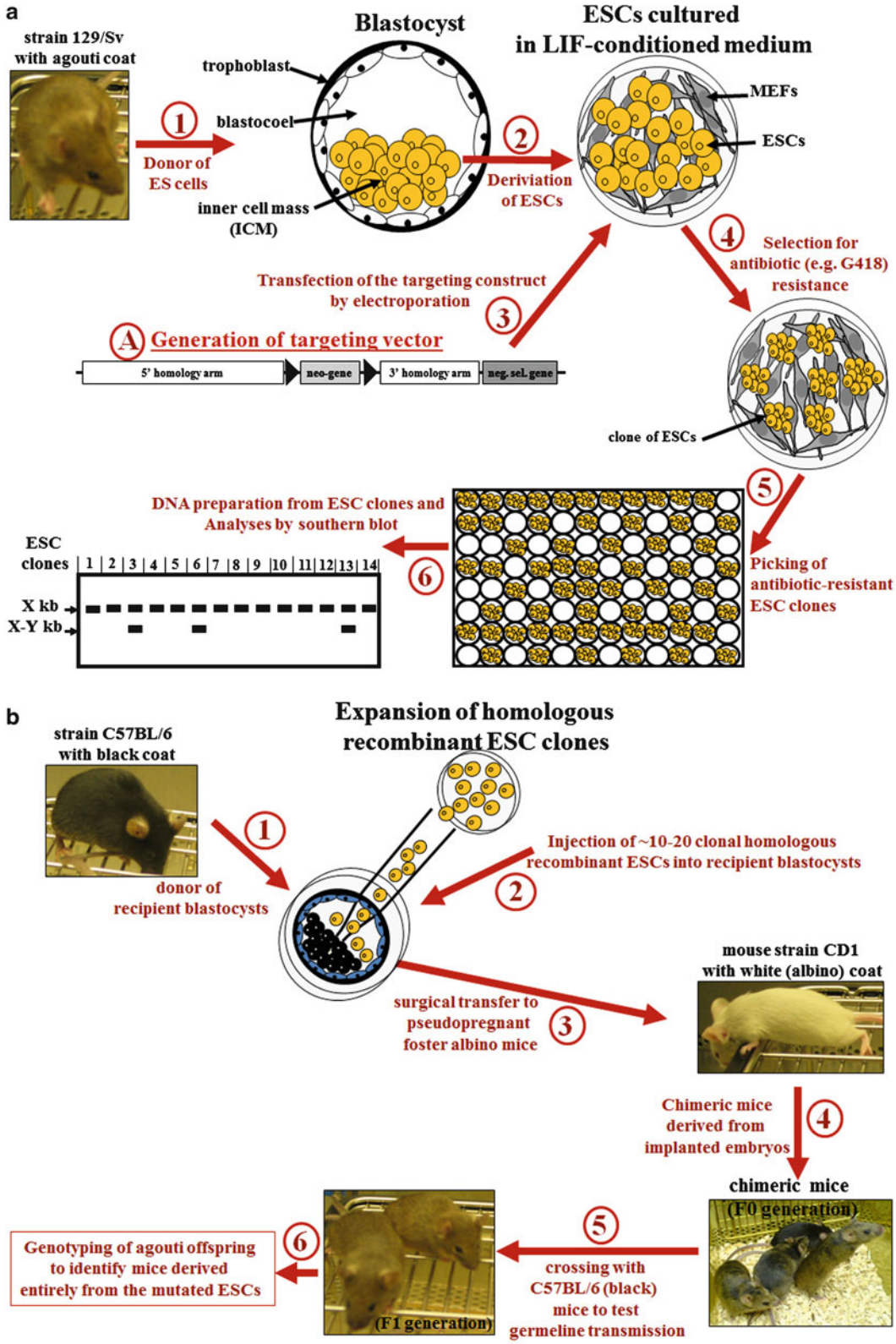
3 Generation of Mutant Mice from Genetically Modified Embryonic Stem Cells

The realization that genes of interest can be specifically modified in a whole mouse was developed from extensive work to determine whether cultured mammalian cells can mediate homologous recombination between their endogenous DNA and exogenously added DNA molecules [38–40]. Simultaneously, murine embryonic stem cells (ESCs) were successfully cultured *in vitro* without losing their pluripotent potential. Thus, when introduced into a

preimplantation embryo they could contribute to the germ line [41–43]. ESCs have subsequently been widely used as vehicles to transfer site-specific genetic modifications of interest to the mouse germline [34–37, 44–46].

ESCs are derived from the pluripotent inner cell mass (ICM) of blastocysts, a structure formed in the early stage of embryogenesis (3.5 days old pre-implantation mouse embryo), and thus ESCs can contribute to all embryonic tissues, including the germ cells, in developing mice (Fig. 1). Isolated ESCs have to be cultured in special culture conditions to maintain their multiplication (self-renewal) capacity without loss of pluripotency. ESCs are typically cultured on a feeder layer of mitotically inactivated (mytomicin C treated or gamma-irradiated) mouse embryonic fibroblasts (MEFs) in order to obtain the necessary factors for self-renewal and pluripotency. Leukemia inhibitory factor (LIF), Wingless/Integrated (Wnt), and ligands of the TGF-β/BMP signaling pathway are among factors supplied by the fibroblasts and were found to influence the state and pluripotency of murine ESCs [47]. Supplementation of ESC culture medium with recombinant LIF helps to increase the maintenance of pluripotency. Recently, it has been shown that the maintenance of pluripotency can also be

Fig. 1 (**a**) Summarized most common steps for isolation of mouse embryonic stem cells (ESCs) and generation of homologous recombinant ESC clones. (1) In the first step 3.5-day-old mouse embryos (blastocysts) are collected from the uterine horn of superovulated (hormone treated) mated female mice with, for example, an agouti coat (strain 129/Sv). (2) ESCs are derived from the inner cell mass of blastocysts and cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs), in ESC medium (supplemented with leukemia inhibitory factor (LIF)). (3) After electroporation with the targeting vector of interest, (4) successfully transfected ESCs are selected by adding appropriate selection agent to the ESC medium; and (5) ESC clones are picked. (6) Homologous recombinant ESC clones are identified by Southern blot. The genomic DNA isolated from ESC clones should be digested with an appropriate restriction enzyme that produce one cut inside the targeting vector and one cut just outside (upstream or downstream) the targeting vector, in the targeted chromosomal region. The use of an "external" probe outside of the targeting construct will produce a band with a size corresponding to unmodified wild-type allele(s), which is here indicated by *X* kb, and, if homologous recombination occurred, a second band of bigger or smaller size corresponding to the targeted allele, which is here indicated by *X–Y* kb. (**b**) Generation of mice with genome modification of interest using homologous recombinant ESC clones. (1) If ESCs are derived from mice with an agouti coat (such as strain 129/Sv), the recipient pre-implantation mouse embryos (blastocysts) should be collected from female mice with black coat (such as strain C57BL/6). (2) The identified and expanded homologous recombinant ESC clones (see Fig. 1a) are injected into recipient pre-implantation mouse embryos (blastocysts) that are collected from female mice with black coat (strain C57BL/6). (3) These injected blastocysts are then surgically transferred to a recipient pseudopregnant foster mother to allow the embryos to develop. Females of CD1 mouse strain make very good mothers, and are thus used by several laboratories as foster mothers. (4) Because ESCs and recipient blastocysts were derived from mouse strains with distinguishable coat-colors, the desired chimeric offspring can be visually recognized by inspection of coat-color chimerism (percentage of black and agouti hair on the mouse black-agouti). (5) Chimeric offspring (usually only the males, because the used ESC lines are usually male) are mated with C57BL/6 mice to produce the F1 generation. (6) The germline transmission is then confirmed by Southern blot analysis or PCR of tail DNA from the agouti (not black) mice of the F1 generation


a
strain 129/Sv
with agouti coat
1
Donor of
ES cells
Blastocyst
trophoblast
blastocoel
inner cell mass
(ICM)
2
Deriviation
of ESCs
ESCs cultured
in LIF-conditioned medium
MEFs
ESCs
4
Selection for
antibiotic (e.g. G418)
resistance
Transfection of the targeting construct
by electroporation
3
A Generation of targeting vector
5' homology arm
neo-gene
3' homology arm
neg. sel. gene
clone of ESCs
5
Picking of
antibiotic-resistant
ESC clones
DNA preparation from ESC clones and
Analyses by southern blot
6
ESC
clones
1 2 3 4 5 6 7 8 9 10 11 12 13 14
X kb
X-Y kb
b
strain C57BL/6
with black coat
1
donor of
recipient blastocysts
Expansion of homologous
recombinant ESC clones
Injection of ~10-20 clonal homologous
recombinant ESCs into recipient blastocysts
2
surgical transfer to
pseudopregnant
foster albino mice
3
mouse strain CD1
with white (albino) coat
Chimeric mice
derived from
implanted embryos
4
chimeric mice
(F0 generation)
5
crossing with
C57BL/6 (black)
mice to test
germeline transmission
(F1 generation)
6
Genotyping of agouti offspring
to identify mice derived
entirely from the mutated ESCs

achieved, in MEF-free culture systems, by use of glycogen synthase kinase (GSK)-3-specific inhibitors, such as 6-bromoindirubin-3′-oxime (BIO) [48], or, optimally, by use of a combination of three inhibitors (3i medium): SU5402 (inhibits FGF receptor tyrosine kinases), PD184352 (inhibits ERK signal cascades) and CHIR99021 (a more selective inhibitor of GSK-3) [49]. Interestingly, Domogatskaya et al. [50] demonstrated recently that pluripotency and self-renewal of mouse ESCs can be achieved in the absence of feeder cells, LIF, or differentiation inhibitors by culturing ESCs on plates coated with a recombinant human extracellular matrix protein, the laminin isoform 511 (LN-511) [50].

Several established ESC lines are in common use. Initially, most used ES cells were derived from the 129 mouse strain [51, 52]. Examples include, among others, E14 cell lines [53], D3 cell lines [54], J1 cell line [55], R1 cell line [56], and AB2.1 cell line [57]. 129 ES cell lines are often used because of their more robust performance in cell culture and higher germline transmission rates compared to ESC lines from C57BL/6J and C57BL/6N mouse strains [58–60].

The C57BL/6 mouse strain is however one of the best characterized inbred strains that is widely accepted as the reference strain for immunological, neurobiological, behavioral, and physiological studies in mice, and is the standard reference library for the mouse genome-sequencing program [58–61]. Therefore, mice derived from 129 ESC lines need to be backcrossed for ten or more generations onto C57BL/6 background, which is a time-consuming process taking 2 or more years.

C57BL/6 ESC lines with efficient germline colonization have been generated and these facilitate the direct generation of genetically altered C57BL/6 mice [58, 59, 61–64]. The requirement for backcrossing mice derived from C57BL/6-derived ESCs is considerably less (though not entirely eliminated as the ESCs may harbor mutations acquired *in vitro*).

ESC lines derived from C57BL/6 mice that can be used include: Bruce4 [64], BL/6-III [63], LK1 [59], and JM8 [61]. The JM8 cell lines have been used for the large-scale mouse knockout program to generate mice with targeted mutations in the C57BL/6 genetic background [61, 65]. JM8 cells can be easily propagated using standard ESC culture conditions, in the presence or absence of feeder cells. In addition, to simplify breeding schemes, the dominant agouti coat color gene was restored in JM8 cells by targeted repair of the C57BL/6 nonagouti mutation enabling visual assessment of coat color contribution and germline transmission [61].

Taken together, when selecting an ESC line, several considerations are important: (1) the advantages and drawbacks of C57BL/6 versus 129 ESC lines [60]; (2) the genetic variation and stability among ESC lines from different 129 substrains [52] and

C57BL/6 substrains [66], respectively, which might influence, e.g., gene targeting efficiency and phenotype of mice derived from the respective ESC line; (3) the homology arms of the targeting vector should be from DNA that is isogenic to the ESCs used. Furthermore, the most appropriate ESC line to use is dependent on the cell culture conditions and expertise in the respective laboratories.

In vitro cultured ESCs can be genetically modified and then reinjected into the blastocoel (cavity) of blastocysts (3.5 days old pre-implantation mouse embryo) [67, 68] (Fig. 1), or into morula (2.5 days old pre-implantation mouse embryo) [68]. Usually 10–20 ESCs are injected in a blastocyst [67, 68]. Alternatively, chimeric embryos can be generated by aggregating ESCs with morula [69]. The chimeric embryos (usually five to ten) are then surgically transferred into the uterus of recipient pseudopregnant foster females. The genotype of these females is irrelevant, as long as they are good surrogate mothers. That is, they nurse carefully the newborns to weaning age and they accept pups from another mother. For these reasons, CD1 females or C57BL/6 × BALB/c F1 females are recommended for use [68]. Chimeric mice, in which the injected genetically modified ESCs have contributed to the formation of most or all tissues, will be born at frequencies varying from a few percent to the majority of the pups (Fig. 1). If the genetically modified ESCs have contributed to germ cell formation, the introduced genetic modifications can be passed on to offspring from the chimeric mice.

To facilitate the selection of the desired chimeric offspring, the ESCs and recipient blastocysts are derived from different mouse strains with distinguishable coat-colors (each mouse strain homozygous for the corresponding coat-color allele) (Fig. 1b). For example, the recipient blastocysts may be derived from C57BL/6 mice (black coat-color), and ESCs from a 129 mouse strain (agouti/brown coat-color). The extent of the contribution of the ESCs to the formation of the chimeric mouse can be visually recognized by inspection of coat-color chimerism (percentage of black and agouti hair on the mouse black-agouti) (Fig. 1b).

4 Gene Targeting in ESCs

Using current techniques, there are almost no limitations into the types of modifications that can be introduced, ranging from gene insertion, point mutations, short- and long-range deletions, inversions. Conditional knockouts or knockins are generated by placing loxP or FRT sites flanking selected exons (see also text below).

The introduction of site-specific modifications into the genome of ESCs by homologous recombination, a process called gene

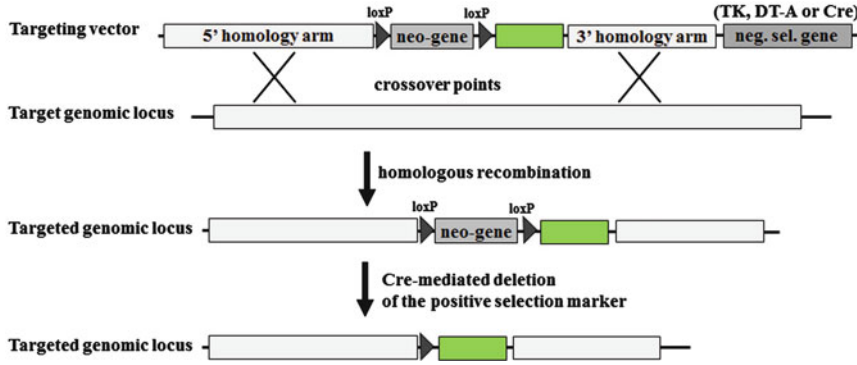


Fig. 2 Typical gene targeting strategy. A targeting vector is typically composed of three basic units: (1) a 5′ homology arm; (2) a gene marker for positive selection (e.g., neomycin resistance gene (neo)); (3) a 3′ homology arm. Furthermore, a negative selection marker (neg. sel. Marker) can be included outside the homology arms, such as thymidine kinase (TK), diphtheria toxin fragment A (DT-A), or, if the positive selection marker is flanked by loxP sites, Cre recombinase gene (Cre). Furthermore, any desired DNA sequence of interest (here *green box*) can be inserted between the homology arms of the targeting vector, in order to introduce it into the target genome by homologous recombination. Homologous recombination between the targeting vector and the target cognate chromosomal region results in the disruption of one genomic copy of the targeted genomic locus and loss of the vector's negative selection marker gene. Crossover points are depicted by "X". The floxed (loxP sites flanked) positive selection marker gene can be removed by expressing Cre recombinase in the recombinant ESCs or by crossing the chimeric mice with Cre-expressing transgenic mice (*see* also Fig. 4a) (Color figure online)

targeting, is achieved through the introduction of a targeting vector into ESCs by electroporation (Figs. 1 and 2).

A targeting vector (DNA construct) is typically composed of three basic units (Fig. 2): (1) a 5′ homology arm, (2) a positive selectable gene marker (such as the neomycin resistance gene (*neo*) or hygromycin (hyg)), (3) a 3′ homology arm. The transfected targeting vector can either insert itself randomly into the genome or be integrated by homologous recombination as determined by the 5′ and 3′ homology arms. Successfully transfected cells are positively selected by culturing ESCs in medium with neomycin (G418) or other appropriate antibiotics, such as hygromycin or puromycin (Fig. 1). If the positive selection marker gene is flanked by loxP or FRT sites, then it can be later removed from targeted loci in ESCs or transgenic mice by expressing Cre recombinase or flippase (FLP) in the recombinant ESCs (through transfection with Cre- or FLP-expressing vector) or by crossing the chimeric mice with Cre- or FLP-expressing transgenic mice (Fig. 2, and *see* also Fig. 4a). This is an important consideration as the introduction of selection marker genes, such as neo, can profoundly affect the expression of endogenous genes neighboring to the targeted gene locus [70–72].

The homologous recombination of a targeting vector into a genomic locus of interest occurs at a very low frequency (at a frequency of 10^{-3} to 10^{-4} relative to nonhomologous recombinants)

[73]. In general, the longer the length of the 5′ and 3′ homology arms the higher the targeting frequency is [74, 75]. Another factor that increases homologous recombination frequencies is whether the homology arms of the targeting vector are isogenic with the ESC DNA [74, 76]. Ideally, the homologous arms should be derived from genomic DNA prepared from the ESCs to be used or at least from the same strain of mice that the ESCs were derived from. Linearization of the targeting construct before its transfection into ESCs also enhances the frequency of homologous recombination [77]. Nevertheless, because homologous recombination is a rare event, the screening of at least 200, and often up to 1,000 clones is required to identify a few clones that have undergone homologous recombination.

A method to enrich the selection for homologous recombinant clones uses negative selection markers, such as thymidine kinase (TK) from herpes simplex virus (HSV) [44], or the diphtheria toxin fragment A (DT-A) from *Corynebacterium diphtheria* [78]. The gene encoding for the negative selection marker is included outside the homology arms of the targeting vector (Fig. 2). During homologous recombination, sequences outside the regions of homology to the target genomic locus are usually lost. By contrast, if the gene targeting vector is integrated randomly in the genome, the negative selection marker is often retained. TK renders the cells sensitive to thymidine analogues, such as 5-iodo-2′-fluoro-2′-deoxy-1-β-D-arabino-furonosyluracil (FIAU) or gancyclovir, that are supplemented in the ESC culture medium to eliminate clones with randomly integrated targeting vector. The TK enzyme activates these thymidine analogues, resulting in their incorporation into replicating DNA, causing premature chain termination and cell death [79]. DT-A exerts toxicity by catalyzing the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to a modified histidine residue on the elongation factor 2 (eEF2), thereby inhibiting protein synthesis [78].

Recently, a new simple negative selection procedure using an "auto-selecting targeting vector" has been developed (Fig. 2) [80]. As negative selection marker, a cyclization recombination (*cre*) gene—under the control of herpes simplex virus (HSV) promoter—was placed outside the homology region of the targeting vector. Because the positive selection marker, the neomycin resistance gene (*neo*), was flanked by two loxP sites (floxed), random integration of targeting vector into the genome will often result in the maintenance and expression of Cre recombinase which specifically recognizes the recombining loxP sites and subsequently mediates the deletion of the floxed positive selection marker (*neo*). In this case, the ESCs are not able to grow in medium containing G418 (neomycin) [80]. However, after homologous recombination, the Cre gene will in most cases not integrate and the positive selection marker will be retained (though it can be deleted once positive clones have been identified).

5 The Use of Cre/loxP Recombination System in Gene Targeting

Cre recombinase is a 38 kDa protein from the bacteriophage P1 that mediates intramolecular and intermolecular site-specific recombination between two loxP sites (locus of X-over of P1). The loxP sequence is 34 bp long and consists of two 13 bp inverted repeats separated by an 8 bp nonpalindromic (asymmetric) sequence which dictates the orientation of the overall loxP site (Fig. 3). Two loxP sequences in opposite orientation mediate the inversion of the intervening DNA by Cre recombinase rather than excision while two sites in the same orientation mediate excision of the intervening DNA between the sites after which only one loxP site remains. If the loxP sites are located on different chromosomes, Cre recombinase can mediate a chromosomal translocation (Fig. 4) [81–85].

The Cre/loxP system has emerged as a useful tool in genetic manipulations [86]. Generally, any DNA sequence of interest can be deleted by flanking it with loxP sites. Cre/loxP enables, for

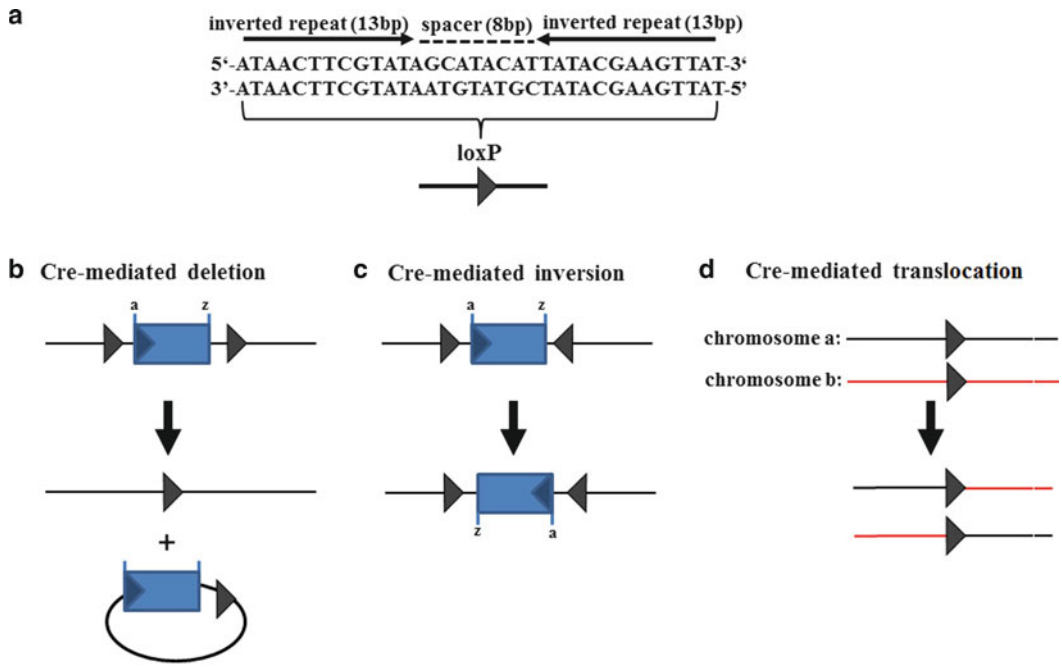


Fig. 3 LoxP structure and Cre recombinase-mediated recombinations. (**a**) Single loxP site that contains two inverted 13 bp repeats, separated by an asymmetric 8 bp long sequence. The type of Cre-mediated recombination is dependent on the orientation and location of the loxP sites: (**b**) Cre excises a circular molecule from between two loxP sites placed in the same orientation; (**c**) Cre inverts the DNA sequence between two loxP sites positioned in opposite orientation; (**d**) Cre-mediated recombination between two different linear DNA molecules (e.g., chromosomes), each containing a loxP site, resulting in the exchange of the DNA regions flanking the loxP sites. Figure was modified from Torres and Kuehn [88]

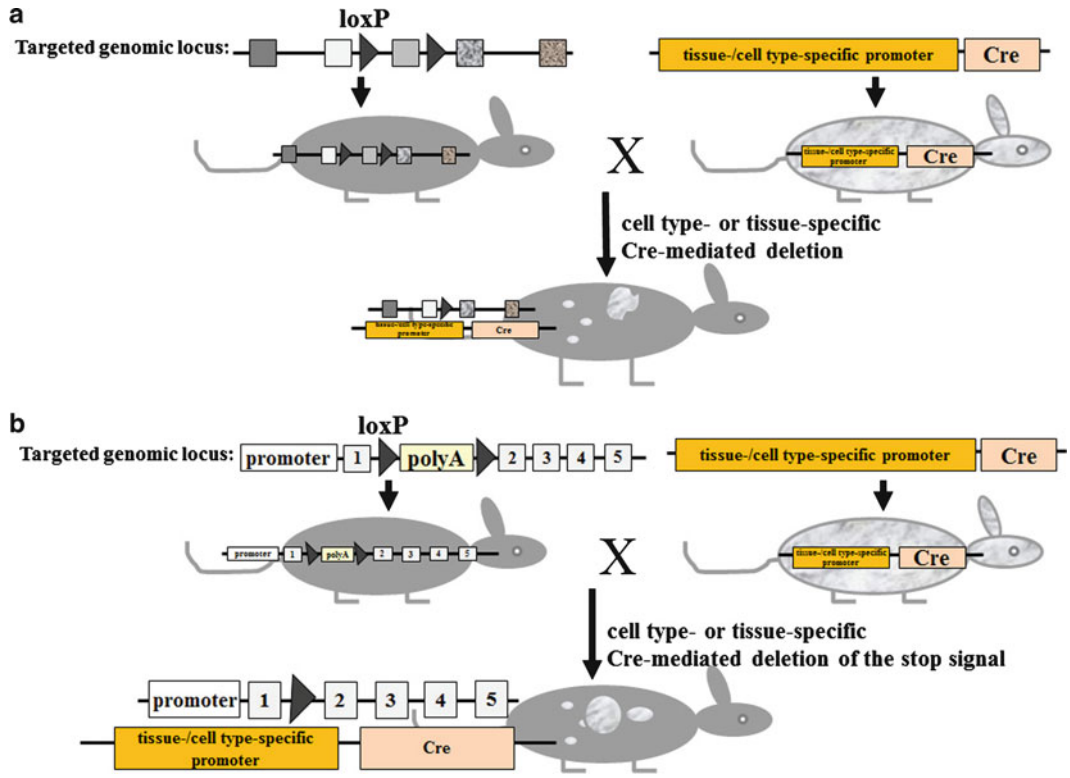


Fig. 4 Conditional gene targeting using the Cre/loxP recombination system. (**a**) Cre-mediated inactivation of a gene of interest. *Left mouse*: introduction of loxP sites into a genomic locus of interest by homologous recombination using ESCs (*see* also Fig. 1). LoxP sites are introduced in a manner that they don't interfere with the function of the targeted gene. *Right mouse*: a transgenic strain that express Cre-recombinase under the control of cell type- or tissue-specific promoter. By crossing the floxed mouse with Cre transgenic mice, Cre mediates the deletion of the floxed genomic sequence, resulting in the inactivation of the targeted gene. Gene deletion is restricted to the "area" (cell types or tissues) where Cre is expressed (*white flecks*). (**b**) Cre-mediated activation of a gene of interest. *Left mouse*: introduction of a floxed intervening sequence (e.g., polyadenylation signal sequences) that prevent the correct transcription of the targeted gene. By crossing the floxed mouse with Cre transgenic mice, Cre mediates the deletion of the floxed intervening sequence, resulting in the reactivation of the targeted gene. Gene activation is restricted to the "area" (cell types or tissues) where Cre is expressed (*white flecks*)

example, the deletion of the selection marker after successful integration of the targeting vector into the genome of ESCs or mice. The loxP sites can be introduced into the genomic locus of interest by homologous recombination as described above. Furthermore, the conditional (timely or spatially controlled) expression of Cre recombinase enables to determine, where (e.g., in which cell type or tissue) and when (at which time of mouse's life or of developmental stage of cells/tissues) the deletion of the floxed DNA sequence should occur (Fig. 4a). Thus, for conditional site-specific genome modification, two mouse lines are usually needed (Fig. 4a):

First, mice that have the DNA sequence of interest flanked by loxP sites (floxed). Second, Cre recombinase transgenic mice, in which Cre is expressed under the control of a promoter that is active in specific cell types or tissues, or Cre is transiently expressed under the control of a promoter that is active at a particular developmental stage of tissues or cells. When crossing the floxed mouse with a Cre transgenic mouse, the floxed DNA sequence is subsequently deleted in the cell types or tissues, where Cre is expressed. Collection databases of several hundreds of Cre transgenic mouse lines expressing the Cre recombinase in specific tissues or cells are available (e.g., http://www.ics-mci.fr/mousecre/; http://nagy.mshri.on.ca/cre_new/index.php; http://www.creportal.org/; http://bioit.fleming.gr/crezoo/; http://creline.org/).

The Cre/loxP system enables not only the deletion (shut off) of genes of interest, but has been proved to be successful also to specifically turn on (activate) the expression of any gene (or transgene) of interest [87, 88]. For this, the gene of interest is rendered quiescent, e.g., by interposing floxed polyadenylation signal sequences mediating premature transcription arrest within a fundamental site of a gene of interest (Fig. 4b). After intercrossing with a Cre-transgenic mouse or delivery of Cre into floxed transgenic mice using, e.g., Cre-expressing adenoviruses, the floxed polyadenylation signal sequences can be removed by Cre-mediated excision, resulting in the activation of the gene expression in a specific cell type or tissue.

Such Cre/loxP-mediated gene activation has been a useful approach to avoid harmful effects of the transgene during mouse embryogenesis, or the induction of immune tolerance against the transgene product, for example in the case of viral genes. For instance, the application of the Cre/loxP technology has enabled to generate transgenic mice that conditionally express human hepatitis C virus transgenes upon intravenous administration of Cre-expressing adenovirus, and thus enabled the investigation of the immune responses to and pathogenesis of HCV infection [89].

Finally, another useful recombination system is the yeast *Flip*pase (*Flp*)/*F*lp *r*ecognition *t*arget (*FRT*), which is mechanistically identical to that of the Cre/loxP recombination system [90–95], and represents an alternative tool to Cre/loxP. Moreover, the combination of both recombination systems can significantly increase the potentials of conditional gene manipulation in mice.

6 Cloning of the Targeting Vector

The design and construction of the targeting vector is among the most critical steps in generating gene-targeted mice. Many issues should be considered in the design of the targeting vector. These include, among others, the type of the desired genetic

modification and the scientific questions of interest to be addressed (e.g., complete or conditional knockout, insertion of a reporter gene to monitor the expression of a gene of interest, insertion of point mutations, and constitutive or conditional expression of a transgene of interest). Furthermore, the design should consider the option to remove the selection marker cassette at a later stage, and to linearize the targeting vector at a unique restriction site outside the homology arms prior to electroporation. It is also important to design restriction sites and probes that will enable the detection of ESC clones that have undergone homologous recombination. If necessary, new restriction sites have to be inserted that enable easy discrimination of homologous recombinant ESC clones by Southern blotting. Alternatively, homologous recombination can also be detected by long-range PCR or by qPCR reactions designed to detect the loss of an endogenous allele. Thus, accurate design of the targeting vector requires knowledge about the sequence and structure of the gene locus to be targeted (e.g., restriction sites, promoter region, 5′UTR, exons, introns, and intron–exon borders, splice donor and acceptor sequences, and 3′UTR). This has been made considerably simpler by access to the data sequences of the whole mouse genome [96] (http://www.informatics.jax.org/; http://www.sanger.ac.uk/resources/mouse/genomes/).

The construction of a gene targeting vector can proceed by conventional restriction enzyme-based cloning strategies, by using MultiSite Gateway Technology, or by using *Recombination-mediated* genetic engi*neering* (*Recombineering*) based protocols. Generation of targeting vectors using MultiSite Gateway Cloning is described in a separate chapter (*see* Bouabe and Okkenhaug, Chapter 24 of this series).

7 Targeting Transgenes into the ROSA26 Locus

The expression of transgenes can be achieved by microinjection of DNA (e.g., BAC) containing the transgene of interest into fertilized murine eggs. However, this results in variegated expression of the transgene and inadvertent disruption of genes at the site into which the transgene might be inserted.

A controlled insertion and expression of a transgene of interest can be achieved by targeting it into the Rosa26 locus. The Rosa26 locus was identified by analyzing pools of embryonic stem cells infected with the retroviral gene trap vector Gen-ROSAβgeo (bifunctional lacZ/neomycin phosphotransferase gene cassette) at low multiplicity [97]. The gene trap vector has integrated into the Rosa26, a chromosomal region that encodes for three transcripts (only the third transcript, originating from the reverse strand, seems to encode for a protein) [98].

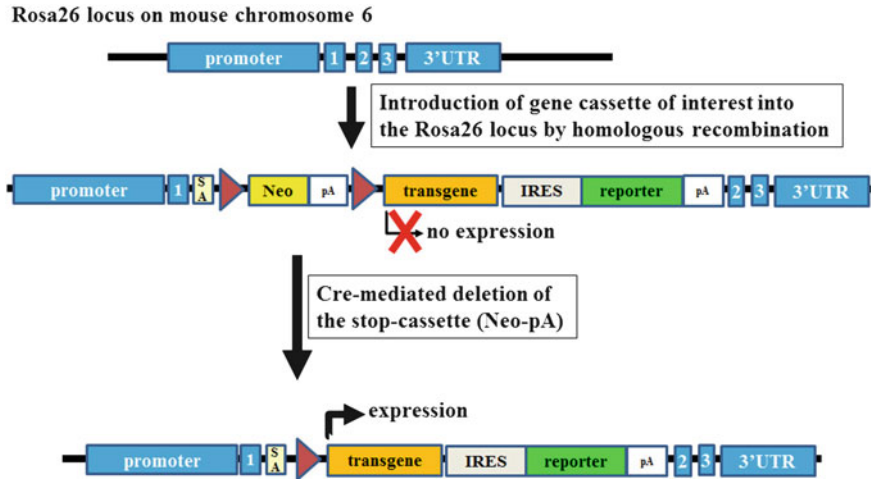


Fig. 5 Targeting transgenes into the ROSA26 locus. Introduction of gene cassettes of interest into Rosa26 locus by homologous recombination in embryonic stem cells. A floxed intervening sequence, neomycin (Neo)-polyadenylation signal sequences (pA), prevents the transcription of the transgene. Cre expression mediates then the deletion of the floxed intervening sequence, resulting in the expression of the transgene and a reporter gene. The reporter gene facilitates to track the expression of the transgene. Numbered (1–3) *blue rectangles*: exons of the Rosa26 locus. *SA* splice acceptor. DNA elements are not drawn to scale (Color figure online)

Rosa26 locus is ubiquitously transcriptionally active, and thus it has become a preferred site for the integration and ubiquitous expression of transgenes and reporter genes. The genes of interest can be introduced into Rosa26 locus by homologous recombination. Conditional expression of Rosa26-targeting genes can be achieved by using Cre/loxP system (Fig. 5 and Subheading 5).

8 Considerations for the Type of Genetic Modification

The simplest way to assess the role of a particular gene is to eliminate its expression, either by germline deletion or by inserting loxP sites flanking functionally fundamental parts of the gene to enable its conditional Cre-mediated deletion. This is usually best achieved by deleting the first exon(s). Care must be taken, however, to rule out expression of the gene from internal transcriptional start sites or by alternative splicing of exons. An alternative strategy is to delete exons that encode for the functionally most important part of the protein such as those that control its catalytic activity or essential interactions with other proteins, DNA, or RNA. However, with this strategy there is a risk of generating peptides or truncated proteins which may not be functionally neutral, i.e., they may have dominant negative function preventing activation of other similar proteins in ways that can be difficult to predict and control. A way around this is to introduce point mutations that inactivate a particular enzymatic function or prevent the

protein from interaction with others [99]. Examples of such point mutations in immunologically relevant genes include among others, p110δ^{D910A} (kinase inactive) [100], p110γ^{KD} (kinase inactive) [101], p110γ^{DASA} (loss of interaction with Ras) [102], Vav1^{AA} (lacks guanine exchange activity) [103], Vav1^{R442G} (interrupts lipid binding via the PH domain) [104].

9 Considerations for the Type of Reporter Genes When Generating Reporter Mice

Reporter mice have emerged as important tools that facilitate in/*ex vivo* monitoring of the expression of genes of interest, especially when the gene products are secreted proteins, such as cytokines. In reporter mice the expression of an intracellularly localized reporter protein is linked to that of the endogenous gene of interest by using, for example, an internal ribosome entry site (IRES) or 2A peptides. Alternatively, the reporter gene can be introduced just downstream of the start codon of the endogenous gene of interest, resulting in the expression of the reporter gene instead of the targeted allele [23].

Autofluorescent proteins (AFPs), such as GFP, are the most frequently used reporters because their expression can be detected at single cell level without any invasive treatment of cells and without the need of exogenous substrates. However, a high expression amount of AFP (approximately 10^5 molecules "0.1–1 μM" per cell) is required to detect fluorescence up over background [105]. This is a fundamental limitation when expression of weakly expressed genes, as is the case of many cytokines, should be monitored [24, 106].

A powerful solution to overcome this limitation is to use enzymatic reporters that catalyze strong signal amplification, and consequently a thousand times less catalytic molecules (approximately 10^{-4} μM) than AFP can generate a robust, measurable, reporter signal [107, 108].

An example that emphasizes the importance of considering enzymatic reporters versus AFPs for weakly expressed genes has been revealed recently by studies on IL-10 reporter mice (reviewed in [24]). For instance, in contrast to "regular" or "conventional" IL-10-AFP reporter mice that enabled detection of reporter activity mainly in T cells, because of their small cytosol, low autofluorescence, and high expression level of IL-10 [24], an IL-10 reporter mouse model that is based on the reporter enzyme β-lactamase and the fluorescence resonance energy transfer (FRET) substrate coumarin-cephalosporin-fluorescein 4-AM (CCF4-AM) enabled to easily analyze and quantify IL-10 production at the single-cell level in all myeloid and lymphoid cell types, and thus also in cells exhibiting high autofluorescence and/or low expression level of IL-10 [106].

Table 1
Information was collected from the following references [111–114]

Reporter gene	Substrate	Signal amplification	Cells have to be lysed/permeabilized	Detection by					Endogenous activities similar to the reporter activity
				FACS (at single living cell level)	Fluorescence microscopy	*In vivo* imaging	Fluoro-meter	Lumino-meter	
Autofluorescent proteins (GFP, RFP etc.)	No substrate is needed	No (high detection limit)	No	Yes	Yes	No	Yes	No	No
Firefly luciferase (Luc)	D-luciferin	Yes	Cell lysis	No	No	Yes	No	Yes	No
Secreted alkaline phosphatase (SEAP)	PNPP; FADP; CSPD; PPQ	Yes	Cell lysis	No	No		Yes	Yes	Yes (in some cell types)
Chloramphenicol acetyltransferase (CAT)	^{14}C-labeled chloramphenicol; fluorescent chloramphenicol derivative	Yes	Cell lysis	No	No	No	Yes	Yes	Yes (minimal)
β-galactosidase (LacZ)	ONPG; X-Gal; FDG; DDAOG	Yes	Cell permeabilization	Yes/no	Yes	Yes	Yes	Yes	Yes (high)
β-lactamase (Bla)	CCF2-AM; CCF4-AM; nitrocefin	Yes	No	Yes	Yes	No	Yes	Yes	No

Thus, AFPs and enzymatic reporters along with their corresponding assay systems have specific features, advantages, and limitations that must be carefully considered in choosing a system tailored to the particular questions being studied. Some important properties of commonly used reporter systems are summarized in Table 1.

10 The International Knockout Mouse Consortium

Before embarking on a project to generate a knockout or conditional knockout mouse, it is worth considering the international mouse knockout consortium (IKMC) (http://www.knockoutmouse.org/) whose aim is to generate conditional knockout alleles for all genes in the mouse genome (including also microRNA genes). They can supply targeting vectors, targeted ESCs, or gene-targeted mice [65, 109, 110]. This in turn can result in considerable saving and shortening in time from the design to the execution of experiments involving new gene-targeted strains.

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