ELSEVIER

Mechanisms of Development 82 (1999) 3–21

Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis

Ulrike Müller*

Max-Planck-Institute for Brain Research, Deutschordenstr. 46, D-60528 Frankfurt, Germany

Accepted 21 January 1999

Abstract

Gene targeting, defined as the introduction of site-specific modifications into the genome by homologous recombination, has revolutionarized the field of mouse genetics and allowed the analysis of diverse aspects of gene function in vivo. It is now possible to engineer specific genetic alterations ranging from subtle mutations to chromosomal rearrangements and more recently, even tissue-specific inducible gene targeting with temporo-spatial control has become feasible. This review tries to recapitulate what we have learned in this extremely rapidly expanding field during the past decade. Diverse aspects of the technique will be discussed starting from basic construct design to the analysis of complex phenotypes, including recent advances on inducible expression system. Many examples from different areas of biomedical research are given to illustrate the purpose and limitations of the employed experimental approaches.

Keywords: Homologous recombination; Gene targeting; Knockout; Genetic background; Modifier loci; Inducible gene expression

1. Introduction

Since the development of embryonic stem cell technology in the early 1980s and the generation of the first knockout mice about a decade ago, we have seen an explosive growth in the number of targeted mouse mutants published every year (see Fig. 1; reviewed in Brandon et al., 1995; for database resources see Sikorski and Peters, 1997). It has become feasible to produce mice with designed genetic alterations ranging from simple gene disruptions and point mutations to large genomic deletions encompassing several centimorgans, or even to generate specifically engineered chromosomal translocations. Such mice have since become an invaluable new tool to dissect the functions of individual components of complex biological systems, namely to produce mouse models of human inherited diseases. This review will firstly give a brief introduction into the basic methodology used in generating transgenic mice and highlight potential problems and pitfalls that have become apparent. Secondly, I will discuss, using selected examples, how the phenotype of a mutant may be influenced by genetic background and modifier loci, as well as effects of gene redundancies and compensatory mechanisms. Finally I will focus on new approaches aimed at controlling gene targeting in both, temporal and tissue-specific manner.

1.1. Historical landmarks

Homologous recombination in embryonic stem cells (ES cells) is now a routine technique that is used to modify the mouse genome at any chosen locus. The principles of this technique were developed in the 1970s in yeast, where, contrary to the situation in mammalian cells, the majority of recombinations between introduced vector DNA and genomic DNA occur by homologous recombination as opposed to random integration.

Homologous recombination in mammalian cells (an erythroleukemic and a bladder carcinoma cell line) between an artificial targeting vector and an endogenous gene was first achieved by Smithies et al. (1985) for the β-globin locus, albeit at a very low frequency. In 1981 two groups (Evans and Kaufmann, 1981; Martin, 1981) derived pluripotent embryonic stem cell lines from mouse blastocysts. Bradley

* Tel.: +49-69-96769-459; fax: +49-69-96769-441;
e-mail: umueller@mpih-frankfurt.mpg.de

0925-4773/99/$ - see front matter
PII: S0925-4773(99)00021-0

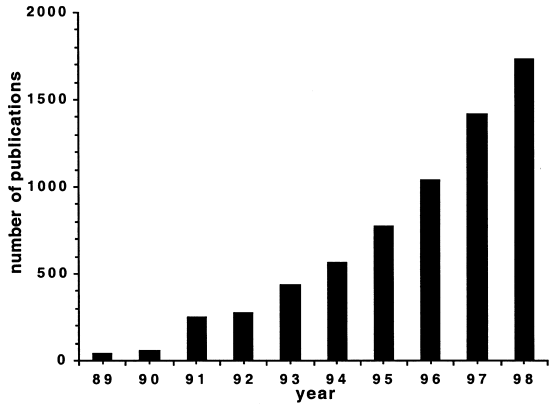

Fig. 1. Number of gene targeting papers. During the past decade the number of publications employing gene targeting has grown exponentially. The above data are extracted from the Science Citation Index Database of the Institute for Scientific Information (ISI, Philadelphia, Pennsylvania) using the following search terms: homologous recombination (higher eucaryotes), gene targeting, knockout mouse, ES cell technology, gene deficiency.

et al. (1984) were able to show that even after prolonged tissue culture such ES cells have the capacity to colonize the germ line of chimeric mice when injected into blastocysts. These experiments paved the way to altering the mouse genome by homologous recombination in ES cells. This was first achieved for the selectable hypoxanthine phosphoribosyl transferase (HPRT) gene locus (Doetschman et al., 1987; Thomas and Capecchi, 1987) and subsequently targeting of non-selectable genes such as int-2 and c-abl (Mansour et al., 1988; Schwartzberg et al., 1989) became possible after enrichment strategies for homologous recombination had been developed.

2. Generation of knockout mice

2.1. ES cells

ES cell lines are derived from pluripotent, uncommitted cells of the inner cell mass of pre-implantation blastocysts and can contribute efficiently to both somatic and germ-line tissues after reintroduction into blastocysts. This capacity crucially depends on culture conditions (for methodical protocols see Joyner, 1993, 1998; Hogan et al., 1994; Torres and Kühn, 1997) that keep the ES cells in an uncommitted, undifferentiated state. These differentiation inhibiting signals can be provided by feeder cells that also serve as a matrix for ES cell adherence and/or by addition of leukaemia inhibitory factor (LIF) to the culture medium (Pease and Williams, 1990).

An important issue that should be considered before starting a lengthy targeting experiment is the genetic background on which the mutation will be studied. The genetic background of the majority of available ES cell lines is 129, a strain from which ES cell lines are most easily established (Kawase et al., 1994). It should be noted however, that the inbred 129-substrains from which these cell lines were isolated, show considerable genetic variation (Simpson et al., 1997) and differ in reproductivity and in behaviour (Festing, 1996; Wehner and Silva, 1996) (see also Section 3.1 for a discussion of strain-specific differences in behavioural tests). ES cell lines from other inbred mouse strains such as C57BL/6 and BALB/c mice (Ledermann and Burki, 1991; Ledermann personal com.) are also available. Recently, two lines of germ line competent ES cells were isolated from hitherto non-permissive CBA/Ca-mice (McWhir et al., 1996; Brook and Gardner, 1997). Although there is considerable interest in ES cell lines from other species, especially rats and commercial livestock, germ line transmission from chimeras (generated by injection of ES-like cells into blastocysts) has not been demonstrated so far for any other species except mice.

2.2. Design of targeting vectors for the generation of null mutants

2.2.1. Vector elements

The first step in a gene targeting experiment is to isolate

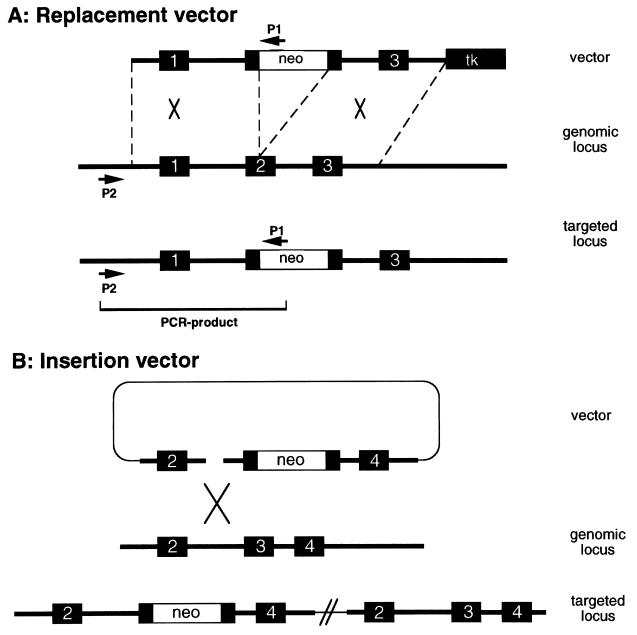

Fig. 2. Classification of targeting vectors. (A) Replacement type vector. The second exon of a target gene is disrupted by a neomycin resistance marker (neo) after homologous recombination with the linearized targeting vector. The HSV thymidine kinase gene (tk) is lost upon homologous recombination (HR). Exons are numbered, filled boxes; stippled lines indicate regions of homology. PCR-primers P1 and P2 are used to screen for homologous recombination. (B) Insertion type targeting vector. The vector in linearized within the region of homology between exon 2 and exon 3 that carries a neo insertion as positive selection marker. Homologous recombination leads to integration of vector sequences and partial duplication of genomic sequences. Genomic target gene sequences are depicted in blue, vector-derived sequences in black. Thin lines represent sequences of the plasmid backbone.

genomic clone(s) containing the gene of interest. The targeting vector should be constructed from a genomic clone of the same genetic background as the ES cells to be used (i.e. isogenic DNA) since this was reported to increase the frequency of homologous recombination (te Riele et al., 1992).

2.2.2. Classification of vectors

Targeting vectors can be classified as either replacement or insertion vectors (Fig. 2). A replacement vector (Fig. 2a) is linearized in such a way that the vector sequences remain colinear with the target sequences. Chromosomal sequences are replaced by vector sequences by a double crossover event involving the flanking homologous regions. An insertion vector (Fig. 2b) is linearized within the region of homology and homologous recombination will lead to a duplication of genomic sequences. The vast majority of null mutants generated to date have employed replacement type vectors.

2.2.3. Factors affecting targeting frequencies

Several parameters have been shown to influence the frequency of homologous recombination: (1) recombination rates increased with the length of total homology between vector and targeted locus to about 10 kb, the minimum length of the short arm of homology being about 0.5 kb (Hasty et al., 1991b; Deng and Capecchi, 1992); (2) recombination rates can increase with isogenic vector DNA (te Riele et al., 1992); (3) absolute frequencies of homologous recombination seem to be locus dependent. This may be due to differences in chromatin structure and thus accessibility for the enzymatic machinery involved.

2.2.4. Selection markers and screening strategies

The most commonly used positive selection marker is a cassette carrying the neomycin resistance gene (neo^r) under the control of a strong promoter. Alternatively, positive selection cassettes conferring resistance to hygromycin, puromycin or histidinol have been utilized (von Melchner et al., 1992; Santerre et al., 1984; Ramirez-Solis et al., 1995). Since ES cell lines are available which are deficient for the hypoxanthin-phosphoribosyltransferase (HPRT), the HPRT gene can also be introduced as a positive selection marker and transfectants can be screened in HAT-medium (Matzuk et al., 1992).

To enrich for clones that have undergone homologous recombination, as opposed to random integration, a strategy termed 'positive/negative' selection was developed by Capecchi et al. (Mansour et al., 1988). A thymidine kinase (TK) gene from HSV is inserted at the end of the linearized-targeting construct (Fig. 2a). Cells that have undergone homologous recombination will have lost the TK gene, whereas cells in which the construct integrated randomly can be eliminated using a toxic nucleoside analogue, such as gancyclovir or FIAU [1-(2-deoxy-2-fluoro-β-D-arabino-furanosyl)-5-iodouracil]. Typical enrichments ($FIAU^r/neo^r$ clones versus neo^r clones) achieved this way range from 3–10 fold. Another negative selection marker successfully used in several studies is the diphtheria toxin A (DT-A) fragment (Yagi et al., 1990) and more recently, immunotoxin-mediated cell killing has been applied as negative selection (Kobayashi et al., 1996).

For genes that are expressed in ES cells, conditional positive selection is another option. In this case the targeting vector is designed in such a way that expression of a selectable marker, e.g. neo^r, depends on homologous recombination to supply a missing regulatory element, such as the promoter/enhancer, or a polyadenylation signal (Schwartzberg et al., 1989; Donehower et al., 1992; Mansour et al., 1993).

Screening for correctly-targeted clones is either done by optimized mini-Southerns (for a protocol see Ramirez-Solis et al., 1992), or by polymerase chain reaction (PCR) with one primer derived from the newly introduced selection cassette and the other primer hybridising to genomic sequences outside the targeting construct (see Fig. 2a). A general drawback of PCR screening is that in order to achieve a robust reaction the neo-flanking 'short-arm' must usually be limited to 1–3 kb in length.

2.3. Considerations for construct design

2.3.1. Insertions

For the generation of null mutants the experimental goal is to design a mutation in such a way that it will prevent any gene expression from the targeted locus. This may be achieved by: (1) inserting the drug resistance marker into an exon critical for gene function; or (2) by making a suitable deletion. In many experiments the gene cassette encoding the resistance marker has been placed behind the ATG-start codon. Introduction of a β-galactosidase (lacZ) gene in-frame with the targeted protein (or a geo-fusion composed of lacZ and neo^r) (Friedrich and Soriano, 1991), will not only allow the disruption of the reading frame but will yield additional information about the spatial transcription pattern of the investigated gene (Mansour et al., 1993). Bicistronic targeting vectors that carry internal ribosomal entry sites (IRES) to drive the translation of lacZ can be used to avoid the construction of in-frame fusions and are more generally applicable (Jones et al., 1997). It should be considered however, that for proteins expressed in polarized cells, for example neurons, or for secreted factors, the expression of lacZ will not necessarily reflect the endogenous pattern of protein expression. In many early reports the selection cassettes were inserted into internal exons, and although this may well result in a functional knockout phenotype, for reasons discussed below, only partial gene ablation has also been observed in several cases.

2.3.2. Deletions

Replacement-type targeting vectors may also be routinely applied to generate genomic deletions from several kb up to about 20 kb, without drastically lowering the targeting fre-

quency (Matzuk et al., 1992). In this way, clustered genes or several widely spaced exons of large genes, may be removed in a single targeting step. Deletions encompassing the promoter of a transcription unit will eliminate gene expression at the level of RNA transcription and should therefore preclude the expression of any protein from the remaining exons. A number of null-mutants (e.g. for the β-amyloid precursor protein APP) (Zheng et al., 1995) were generated this way, however this requires at least some preliminary information about the crucial promoter elements.

2.3.3. Potential problems and pitfalls

Although the generation of a simple knockout is often relatively straightforward, several pitfalls in experimental design have only become apparent as the number of publications describing targeted mouse mutants has increased. The following section is intended to highlight some of these difficulties, and to show how they may be avoided, if the targeting constructs are designed appropriately.

2.3.4. Incomplete knockouts

Before the presumed null phenotype of a mutant can be interpreted in a meaningful way, one must assess whether any residual protein is expressed from the targeted locus. As long as coding sequences remain present in the genome, truncated or mutant forms of the protein may still be expressed. In cases where the selection marker has been inserted into internal exons, protein fragments truncated at the C-terminus may still be formed. Although the mutation may be designed in a way that should destroy the presumed function of a protein, the truncated polypeptide may acquire new properties, such as transdominant interactions with other proteins. Transdominant properties of truncated proteins may, however, also be exploited in a positive way, as shown by Jones et al. (1997) for the knockout of the $GABA_A$-receptor α6 subunit gene. $GABA_A$-receptors are anion channels formed by pentameric assemblies of different subunits (α1–6, β1–3, γ1–3, δ, and ϵ). The composition of native receptors is still under debate and the rules governing subunit assembly are still unclear. When Jones et al. (1997) generated an α6-knockout mouse truncated after the second (TM2) of four transmembrane domains they found that this was accompanied by a severe reduction of the expression level of the δ-subunit, providing genetic evidence for a specific interaction between both subunits.

Other possibilities by which such a 'leaky' mutation with residual protein expression may arise are: (1) the use of alternative or of cryptic promoters; (2) read-through transcription through the inserted selection marker followed by initiation of translation from downstream AUGs; or (3) skipping of the mutated exons due to aberrant splicing which may lead to the expression of modified proteins.

Read-through transcription beyond the polyA-site of the selection cassette is a frequently occurring problem and in many cases it was reported that low amounts of mutant mRNAs were generated, from which the exon harbouring the selection marker had been spliced out (e.g. Tanaka et al., 1997). As long as no functional protein is produced from these mRNA variants this may not cause problems. However, if aberrant splicing occurs under retention of the reading frame, modified polypeptides with internal deletions will thus be expressed. Examples of knockout attempts for which residual protein expression due to exon skipping was observed include the DNA methyltransferase gene (Li et al., 1992), the CD18- gene (Wilson et al., 1993), the β-amyloid precursor protein (APP) gene (Müller et al., 1994a), the CFTR-locus (Dorin et al., 1994) and the L1 gene (Dahme et al., 1997). Although it was presumably not intended by the investigators, some of these mutants are allelic variants with interesting phenotypes of their own, showing, for example, partial rescue of a more severe null phenotype (Wilson et al., 1993; Dorin et al., 1994). A further striking example was described by Moens et al. (1992) who generated a targeted N-myc allele (designated as N-myc^{9a}) from which low amounts of wild-type protein were still expressed in several tissues. Whereas N-myc null mutants are lethal at embryonic stages (Stanton et al., 1992), mice with N-myc^{9a} alleles survived up to birth, demonstrating a function of N-myc in lung and spleen morphogenesis that was not revealed in the respective null mutant.

2.3.5. Removal of coding regions or of regulatory elements of other genes

Removal of all coding exons may circumvent the problems of residual protein expression. On the other hand the generation of large genomic deletions may also lead to the unintended loss of: (1) as yet unidentified genes, such as removal of genes residing in introns or encoded by the opposite strand; or (2) of regulatory elements governing the expression of unrelated genes. The latter possibility may be especially relevant for clustered genes, as seen in the striking case of the myogenic regulatory factor 4 (MRF4) knockouts, carried out by three independent groups. Three different alleles of similar design (deletions encompassing different parts of the MRF4 coding region) resulted in phenotypes ranging from complete viability to lethality. These differences in phenotype were subsequently attributed to the deletion of positive regulatory elements that led to a reduced expression of the neighbouring MRF5 gene, with the homozygous lethal MRF4 allele unexpectedly representing a functional MRF4$^{-/-}$/MRF5$^{-/-}$ double knockout (Olson et al., 1996).

Similarly, targeted disruption of the CD3-eta locus, a component of the T-cell receptor, also unintendedly affected the expression of the partially overlapping Oct-1 gene and led to an unexpected lethal phenotype (Ohno et al., 1994).

2.3.6. Selection cassette interference

There has been considerable concern that transcription from the strong promoters driving the introduced selection

markers may interfere with the expression of neighbouring genes. That this can indeed happen was dramatically shown by experiments carried out by Fiering et al. (1995) who designed a targeted deletion of the 5′-DNAse hypersensitive site 2 (5′HS2) of the locus control region (LCR) of the β-globin locus. When 5′HS2 was deleted and replaced by a PGK-neo cassette globin gene expression was considerably reduced and homozygous mutant mice died in utero at the time point of foetal liver haematopoiesis. Removal of the selection marker by FLP-mediated site-specific recombination (see Section 2.4), however, restored viability and globin expression was essentially normal. This indicates that the phenotype was due to effects exerted by the selection marker and not caused by the deletion of 5′HS2 itself. Further examples in which selection cassette interference became apparent are the Hoxd-10 knockout (Rijli et al., 1994) and the granzyme B gene cluster mutants. For the latter long range effects on genes over a distance of 100 kb away from the mutation were observed (Pham et al., 1996). An elegant study in which selection cassette interference was intentionally used to create an hypomorphic allele was described by Meyers et al. (1998), who used Cre- and FLP-mediated recombination of the same construct to create a series of phenotypically different FGF8-mutant mice.

It is now widely accepted that, whenever possible, selection marker cassettes should be removed after homologous recombination. This can be done either by applying recombinase systems, or using a strategy called ‘hit and run’, both methods described in the following section.

2.4. Introduction of subtle mutations

2.4.1. Hit and run

Subtle mutations like the insertion of stop codons or amino acid substitutions may be achieved by several different strategies which are outlined in Fig. 3. In the ‘hit and run’ approach (also called ‘in and out’, see Fig. 3a) a mutation is introduced in a two-step procedure (Hasty et al., 1991a; Valancius and Smithies, 1991). The first step (‘hit’ or ‘in’) involves homologous recombination using an insertion type targeting vector that leads to a duplication of genomic sequences carrying the mutation and the concomitant integration of two selection markers (e.g. neo as the positive marker and TK as the negative marker). In the second step (‘run’ or ‘out’) selection against TK will enrich for a rare intrachromosomal recombination event that excises the integrated vector sequences including the negative selection marker. Depending on the actual position of this crossover, the mutation introduced in the first step will thus remain incorporated in the genome, or the wild-type allele may be restored.

2.4.2. Tag and exchange

The ‘tag and exchange’ strategy involves two sequential gene targeting steps using two different replacement type vectors (see Fig. 3b). The first step of homologous recombination results in the introduction of a positive and a negative selection marker, however, only positive selection is applied at this stage. In the second round of gene targeting, a vector is used that carries a subtle mutation in the flanking region of homology, and negative selection is employed to enrich for replacement of the sequences introduced in the first round. The ‘tag and exchange’ strategy may save time if a series of mutants of the same locus is envisaged. As an example, a series of mouse strains with different alterations of the prion protein have been generated by Moore et al. (1995) using a double replacement strategy. Both strategies, however, critically depend on the efficiency of negative selection for which either TK or HPRT (in *hprt*$^-$ ES cells)

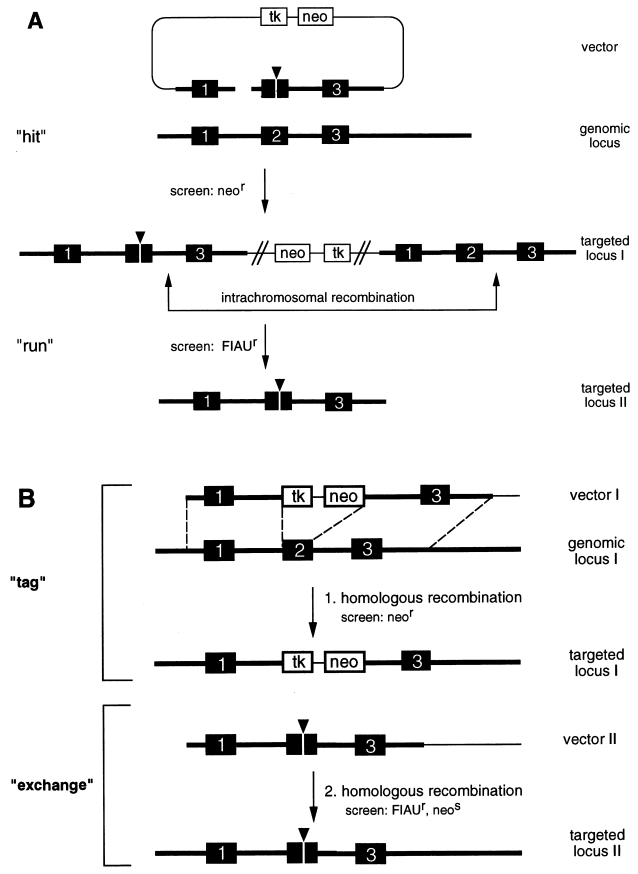

Fig. 3. Introduction of subtle mutations by gene targeting. (A) ‘hit and run’ approach. Homologous recombination with an insertion type targeting vector leads in the first step (‘hit’) to complete integration of vector sequences (black) and concomitant introduction of a point mutation (depicted as white line and filled triangle). In the second step (‘run’) intrachromosomal recombination leads to excision of the integrated vector sequences including the TK gene. Clones that underwent this second recombination step can be enriched by FIAU. Genomic target gene sequences are depicted in blue, vector-derived sequences in black. (B) ‘tag and exchange’ approach. In the first step (‘tag’) homologous recombination with a replacement type targeting vector leads to the replacement of exon 2 with a positive (neo) and a negative (tk) selection marker. Neomycin-resistant clones that have undergone homologous recombination are subjected to a second round of gene targeting (‘exchange’) in which the inserted markers (neo and tk) are replaced by exon 2 harbouring a point mutation (white line and filled triangle). neo^r: neomycin-resistant, neo^s: neomycin-sensitive.

have been used as markers (Askew et al., 1993; Stacey et al., 1994). A modification of the 'tag and exchange' protocol, termed 'plug and socket', was developed to circumvent negative selection, which may be hampered by a background of clones that carry a non-functional negative marker gene without having undergone homologous recombination. In the first step a neo^r gene is introduced together with a second positive, but non-functional selection marker, such as a deletion mutant (Δhprt) of the HPRT-gene. Via recombination with HPRT-sequences, provided by the second targeting vector, functionality of the HPRT gene is restored allowing positive selection for the second targeting step (Detloff et al., 1994). This strategy, however, has the disadvantage that the hprt selection cassette is cointroduced with the mutation.

2.4.3. Recombinase-based approaches

The most versatile and widely applied strategy to introduce non-selectable mutations is based on the Cre/lox recombination system (for reviews see Kilby et al., 1993; Torres and Kühn, 1997) as depicted in Fig. 4. This approach involves the use of the site-specific recombinase Cre from phage P1 that recognizes and binds to a 34-bp long, partly palindromic target sequence called loxP (*l*ocus *o*f crossover *x* in *P*1). Cre recombinase has the ability to efficiently excise any sequence placed between two loxP-sites of the same relative orientation by intramolecular recombination. As a result, one loxP site remains within the genome and one loxP site is found on the excised circularized fragment. If the loxP sites are arranged in opposite orientation (tail-to-tail configuration), recombination will result in an inversion of the intervening sequence.

A general strategy for the Cre/loxP-mediated introduction of a non-selectable mutation is outlined in Fig. 4b. In a replacement-type targeting vector, a positive selection marker followed by a negative selection marker is flanked by two loxP sites and the non-selectable mutation (e.g. a point mutation or any other modification) is contained within one arm of the targeting vector. After homologous recombination and screening for the co-transfer of the mutation (e.g. by a newly incorporated restriction site), Cre recombinase is introduced by transient transfection. ES cell clones that have excised the loxP-flanked markers can subsequently be enriched by negative selection (e.g. against TK or HPRT).

Since the frequency of co-transfer between the positive selection marker and the introduced mutation declines over distance, the non-selectable mutation should not, regardless of the approach applied, be placed further than about 10 kb away from the positive selection marker (Torres and Kühn, 1997). Obviously the loxP sites have to be introduced into a silent genomic region (an intron or a region downstream of the polyA-site), or ideally, a control vector carrying only the loxP site without the co-transferred mutation can be used to generate a 'control' mouse.

As an alternative to the Cre/loxP system, the FLP/frt

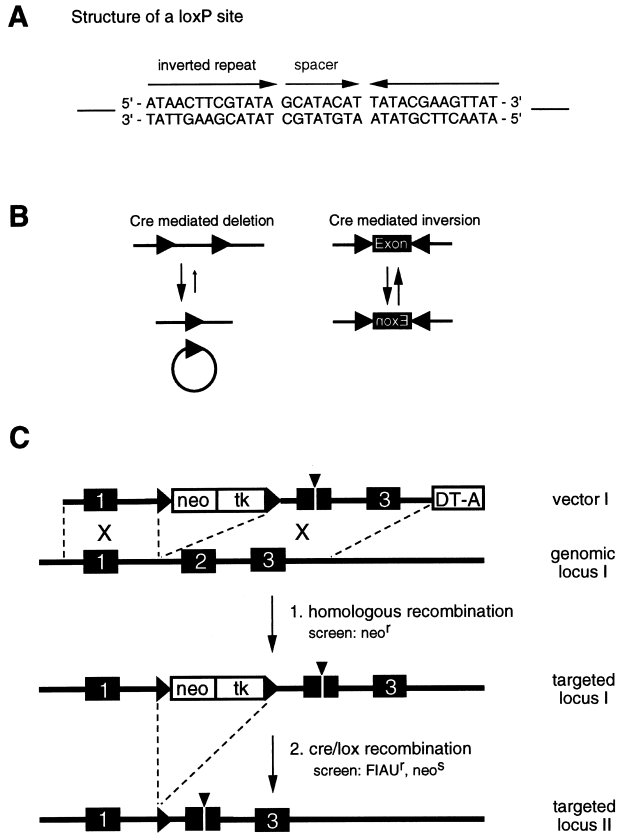

Fig. 4. The Cre/laxP recombination system and making subtle mutations. (A) Structure of a loxP site. The binding site of Cre recombinase, termed loxP consists of two 13-bp inverted repeats flanking an 8-bp spacer region (red arrow) that determines the orientation. (B) Cre-mediated recombination reactions. Recombination between directly repeated loxP sites (large filled triangles) leads to excision and recircularization of the intervening gene segment, leaving one loxP site on each reaction product. Note that excision (large arrow) is much more efficient than reinsertion (small arrow). Recombination between loxP sites of opposite orientation leads to inversion of the intervening gene segment. (C) Introduction of a point mutation and removal of selection markers by Cre mediated recombination. Firstly, a replacement type targeting vector is used to introduce a point mutation into exon 2 (white line and filled triangle). The Diphtheria toxin A gene fragment (DT-A) is lost upon homologous recombination, but is retained in cells that have integrated the vector randomly and will kill those cells. Secondly, selection markers flanked by loxP sites are removed by Cre-mediated recombination in ES cells, leaving one loxP site in the genome. Clones that lost the TK gene can be enriched by their resistance to FIAU.

recombinase system from yeast has been utilized in ES cells and transgenic mice, but with lower efficiencies (Fiering et al., 1995; Dymecki, 1996; Meyers et al., 1998), which may be due to different optimal reaction temperatures (37°C for Cre versus 30°C for FLP), for both enzymes (Buchholz et al., 1996).

2.5. Genome engineering: large deletions and rearrangements

2.5.1. Cre/lox-mediated strategies

Chromosomal rearrangements such as translocations and

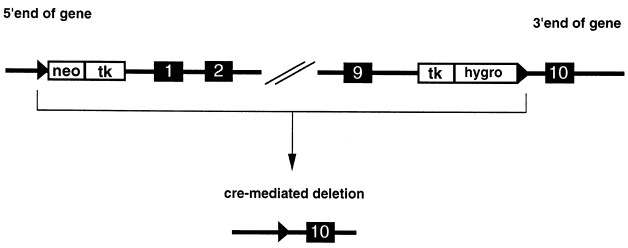

Fig. 5. Generation of large genomic deletions by Cre-mediated recombination. Two loxP sites are inserted by homologous recombination at the 5′- and 3′- proximal regions of a target gene. Two separate targeting vectors with two different positive selection markers (neo and hygro) are used (not shown). Correctly targeted clones are then transfected with a Cre expression plasmid and clones that have excised the loxP-flanked gene segment, including the TK gene, are enriched by their resistance to FIAU.

loss of heterozygosity, are a major cause of inherited diseases and foetal loss. Defined genomic deletions, on the other hand, can be used as a valuable genetic tool to screen for recessive mutations, and by introducing large engineered deletions, even whole gene clusters may be removed. This way, a given locus may be tested for the presence of a tumour suppressor gene, or a chromosomal translocation, known to be involved in tumourigenesis, may be engineered to obtain an animal model of the disease. Site-specific recombinases have been used for this purpose in *Drosophila* (Golic, 1991), plants (Medberry et al., 1995), and more recently also in ES cells (Ramirez-Solis et al., 1995; Li et al., 1996).

Whereas deletions encompassing a few kb can be routinely obtained with conventional replacement-type targeting vectors, a drastic drop in the targeting frequency (below 0.12% of neo^r/FIAU^r-clones) was reported in an attempt to delete 200 kb of the β-APP gene (Li et al., 1996). Strategies based on Cre-mediated recombination were therefore developed (see Fig. 5). In two separate rounds of gene targeting, two loxP-sites are introduced at distant genomic regions, using targeting vectors with two different positive selection markers (e.g. neo^r and hygro^r) in each round of homologous recombination. At least one TK-gene is also introduced with one of the targeting constructs and can be used as a marker to enrich for clones from which the intervening genomic region was subsequently excised by Cre-mediated intrachromosomal recombination (Li et al., 1996). As an alternative to negative selection against TK, reconstitution of a functional positive selection marker was employed by Ramirez-Solis et al. (1995) who used targeting vectors carrying two complementary (hprtΔ5′ and hprtΔ3′), but nonfunctional fragments of a HPRT minigene cassette. Cre-mediated recombination thus restored resistance to HAT-medium in HPRT-deficient ES cells. Using either strategy, deletions of 200 kb or up to several centimorgans were achieved. Deletions by intrachromosomal recombination will, however, only occur if both loxP sites are present on the same chromosome, whereas interchromosomal recombination between loxP sites located on different chromosomes results in translocations and gene duplications. The situation becomes more complex for large genomic intervals, since frequently the gene order and relative orientation of the two loci are not known, thus requiring separate targeting constructs to mimic both possible orientations (Ramirez-Solis et al., 1995). Programmed translocations between non-homologous mouse chromosomes are also feasible (Smith et al., 1995; Van Deursen et al., 1995).

2.5.2. Chromosomal deletions by irradiation of ES cells

An approach that allows the creation of a whole series of overlapping large genomic deletions (between 0.5–10 cM) was developed by You et al. (1997) to engineer the mouse t-complex on chromosome 17. A TK gene was first introduced by homologous recombination at a defined genomic locus followed by γ-irradiation, which has previously been shown to cause large genomic deletions. ES cells in which the irradiation-induced deletion affected the targeted locus were then identified by their resistance to gancyclovir, and the size of the deletions were subsequently mapped using polymorphic flanking markers. The advantage of this approach is that it requires only minimal characterization of the locus around which the deletions are to be made and that a single, straightforward targeting experiment will lead to several mutant alleles. It remains to be shown however, whether additional mutations, unintentionally co-introduced at unrelated sites, are an inherent problem. However, if these are not linked, they may be eliminated by outcrossing after producing mice.

2.6. Generation of transgenic mice using ES cells

Transgenic mice are usually generated by pronuclear injection of oocytes with naked DNA leading to random integration of usually multiple concatemerized vector copies. Although this well established and highly efficient technique allows the generation of a large number of founder animals within a relatively short period of time it is hampered by several major drawbacks: (1) vector integration occurs randomly and sequences neighbouring the integration sites may strongly influence transgene expression; (2) the copy number of integrated transgenes is highly variable; (3) the injected DNA must contain all regulatory elements in order to reliably reproduce endogenous gene expression. These regulatory elements may, however, be located at a great distance from the coding sequence or within introns of complex genes; (4) transgenes with a transdominant lethal phenotype cannot be studied. Although ES cell-based techniques are more elaborate, they may overcome several of these limitations and are sometimes the only way to address certain questions (for a review see Jasin et al., 1996). Lethal phenotypes caused by a transdominant transgene, or by loss of both alleles of an essential gene, can usually be rescued using ES cell-derived chimeras. In addition, ES cell-based techniques offer two other major advantages, namely precise control of the integration site, via homologous recombination, and pre-screening of ES colonies with randomly-integrated vector copies

for copy number and to some extent, for the expression of the transgene.

One kind of engineered mouse mutants that are derived from ES cells are mice harbouring targeted lacZ-fusions which have been widely used as a reporter system for expression analysis in transgenic mice or in gene trap approaches (reviewed in Evans et al., 1997).

Homologous recombination may not only be used to inactivate (knockout) a gene but also to generate 'knock-in' mice in which, for example, the coding region of a different gene or a regulatory element is introduced into the locus (for construct design see Torres and Kühn, 1997). Several applications of these ES cell-derived transgenic mice are given below. In order to achieve tissue-specific knockouts (for which gene inactivation will be restricted to certain tissues, see Section 4.1) Cre recombinase may be targeted to different endogenous promoters, as for example the CD19 locus for B-cell specific expression (Rickert et al., 1997). Furthermore, genes may be replaced by their homologues from different species, to produce, for example, humanized antibodies or to exchange murine genes for disease-associated alleles from humans (Zou et al., 1994; Moore et al., 1995). Similarly, complementation studies within gene families can be conducted by replacing family members by each other, as shown for engrailed1/2 and myogenin/myf5 (Hanks et al., 1995; Wang et al., 1996).

In light of the frequently occurring silencing of expression after random integration, one would ideally like to reuse a well-characterized integration site for many different transgenes. Bronson et al. (1996) generated a 'knock-in' at the HPRT-locus using an *hprt*$^-$ ES cell line carrying a 5′-deletion of the HPRT gene and selected for correction of the mutant HPRT gene upon homologous recombination with HPRT-sequences provided by the targeting vector. In addition, the targeting vector contained a bcl-2 transgene adjacent to the HPRT selection cassette. Using this approach, two lines of mice with identical transgene integration sites were generated that expressed bcl-2 under the control of two different promoters.

Alternatively, one might employ site-specific recombinases for targeted transgenesis. Cre/lox-mediated recombination can be used to integrate a vector carrying a loxP site into the genome via recombination with a genomic loxP site (see Fig. 4b, back reaction). To shift the equilibrium of this reversible reaction against the Cre-mediated re-excision reaction, which is normally much more efficient than integration, a pair of mutant loxP sites has been used (Sauer and Henderson, 1990; Albert et al., 1995; Bethke and Sauer, 1997). In this way, targeted integration has been achieved in various cell lines and has recently also been applied to ES cells (Araki et al., 1997). It should be considered, however, that selection cassettes cointroduced with the transgene may interfere with endogenous gene expression and should therefore be removed, e.g. by applying the frt/FLP-recombinase system in ES cells or transgenic mice (Meyers et al., 1998).

3. Interpretation of the phenotype

Phenotypes of targeted mouse mutants are not always those predicted from the presumed function of a given gene product and/or the pattern of expression of the gene. In some instances, unexpected phenotypes such as lethality due to inflammatory bowel disease in interleukin-2 knockout mice (Sadlack et al., 1993) have been observed, whereas other null mutants revealed either very minor, or no apparent defects such as in the case of HPRT-deficient mice (Wu and Melton, 1993). Lack of phenotype may be due to functional redundancies at the single gene level or at the genetic pathway level (see Section 3.2). In several cases the same gene has been knocked out by independent groups but disparate phenotypes have been reported. On one hand, these discrepancies can be related to the use of different targeting constructs, which may have unintentionally also affected adjacent or overlapping genes (Section 2.3). On the other hand, phenotypic variation may be caused by different genetic backgrounds and the presence or absence of allelic variants of genes, called modifier loci.

3.1. Genetic background and modifier loci

Both naturally occurring and targeted mutations can have very diverse phenotypes when studied on different genetic backgrounds (Erickson, 1996 and Table 1). The majority of targeted mouse mutants to date have been generated using ES cells derived from 129 substrains and in many cases an initial analysis was performed with animals of mixed 129 × C57BL/6 (or 129 × BALB/c) genetic background. The 129-derived chimeras are bred with C57BL/6 females to monitor germ line transmission by coat-colour markers and also to speed up breeding, since 129 strains are poor breeders (Festing, 1996). Heterozygous F1 animals are then intercrossed to generate homozygous mutant mice and wild-type littermates as controls (see Fig. 6). Although the genetic background of these F2 mice is on average 50% of 129 (from the ES cells) and 50% of C57BL/6 origin, the individual mice, however, are all different with regard to the composition of background alleles. F1 descendants of the chimera still have a homogenous background because they obtain one set of chromosomes from each parental strain. If selected homozygous mutants (or wild-type controls) are interbred for several generations, substrains will arise and background alleles may be lost or may become fixed. An example in which the interpretation of a phenotype may have been confounded by background effects is the reported protection of p53$^{-/-}$-mice of mixed (129/Sv × C57BL/6) genetic background from kainate-induced neurodegeneration (Morrison et al., 1996). The conclusion that p53 deficiency causes reduced sensitivity towards kainate was recently challenged by experiments conducted by Schauwecker and Steward (1997), who analysed the kainate response in the respective wild-type strains and showed that C57BL/6 mice are already resistant to kainate and some

Table 1

Examples of knockout mice with background dependent phenotypes

Targeted gene	Phenotypic variation	Dependence on genetic background (strains)	References
Keratin 8	Penetrance of embryonic lethality	1.6% viable (C57BL/6)	(Baribault et al., 1994)
		55% viable (FVB/N)	
EGF-Receptor	Time of lethality	lethal at E 7.5 (CF-1) lethal at midgestation (129/Sv) lethal perinatally (CD-1) lethal postnatally (129/Sv x C57BL/6)	(Threadgill et al., 1995) (Sibilia and Wagner, 1995)
CFTR	Time of lethality	death within 10 days after birth (DBA/2J) major loss at weaning (C57BL/6J) prolonged survival and gradual loss (BALB/cJ)	(Rozmahel et al., 1996)
TGFβ1	Time of lethality	loss before organogenesis (C57BL/6J/Ola) loss at midgestation (F1 NIH/Ola x C57BL/6J/Ola)	(Bonyadi et al., 1997)
p53	Tumour spectrum, Rate of tumour formation	accelerated rate of tumour formation (129/SvEv) half of males develop teratomas (129/SvEv) very few males develop teratomas (129/SvEvx C57BL/6)	(Donehower et al., 1995)
APP	Incidence of corpus callosum abnormalities	100% abnormal, 95% with complete agenesis (129/SvEv)	(Müller et al., 1994a)
		20–80% complete agenesis (129/SvEvx C57BL/6) no agenesis (C57BL/6)	(Magara et al., 1999)
hoxb-4	penetrance of lethality, sternum defects	100% lethal at around birth, with split sternum (129/SvEv); 50% loss around birth, various degrees of sternum defects (129/SvEv x C57BL/6)	(Ramirez-Solis et al., 1993)

subset of genes in 129/SvEMS × C57BL/6 hybrids is suffice to confer protection from excitotoxicity. Moreover, $p53^{-/-}$ mice of a pure 129/SvEMS genetic background behaved like congenic wild-type controls (Schauwecker and Steward, 1997), suggesting that not p53 deficiency itself, but a certain combination of background alleles was most likely responsible for the phenotype observed in $p53^{-/-}$ mice of mixed genetic background.

If the analysis of animals with mixed genetic background cannot be avoided (e.g. in certain behavioural studies) the F2 animals should at least be obtained from a large collection of F1 breeding pairs in order to randomize the influence of background alleles. However, even using this method, it is possible that polymorphic genes linked to the targeted locus may still be unequally distributed between F2 mutants and wild-type littermates, because the region surrounding the mutation is virtually always derived from the ES cells in the mutants, but derived from the other strain in the wild-type littermates. In order to generate better wild-type control animals more sophisticated breeding schemes employing the use of polymorphic markers linked to the targeted locus, but specific for either parental strain, have been suggested (Gerlai, 1996). The issue of background variability and its impact on the analysis of mouse mutants has gained widespread attention (Crawley, 1996; Gerlai, 1996; Lathe, 1996) and recently recommendations concerning appropriate strain derivation have been proposed (Silva, 1997).

To eliminate confounding effects caused by mixed genetic backgrounds, mutants should be maintained and studied on a pure genetic background. This can readily be achieved by breeding chimeras with mice of the same genetic background as the ES cells (see Fig. 6) or by backcrossing the mutation to standard inbred strains. Backcrosses using marker-directed breeding, termed ‘speed congenics’ may limit the number of necessary generations (Lander and Schork, 1994; Matouk et al., 1996).

Although it is generally accepted that mutations should be maintained on a congenic background, some phenotypes may be impossible to analyse on pure genetic backgrounds, since the inbred strain is already affected. For example, DBA and 129 strains perform poorly in spatial learning tasks, which may obscure the analysis of additional effects caused by a targeted mutation, as shown for a study involving β-amyloid precursor protein mutant mice (Wolfer et al., 1997). In contrast, C57BL/6 mice are good spatial learners, but poor avoidance learners (Schwegler and Lipp, 1983) and in addition, BALB/c and C3H mice have visual problems (Upchurch and Wehner, 1988).

Placing a mutation in the context of several different genetic backgrounds allows the study of aspects of gene function that are not apparent on a single background and

would therefore be missed (see Table 1). For example, in APP knockout mice, agenesis of the corpus callosum is only seen with high penetrance on a 129-specific background, suggesting that so far unidentified 129-specific alleles interact with the APP-mutation (Müller et al., 1994b; Magara et al., 1999). Mapping and cloning of these strain-specific modifier genes may also be important for the generation of better models for complex human genetic diseases (Erickson, 1996; Rozmahel et al., 1996; Bonyadi et al., 1997). In an animal model of cystic fibrosis, disease severity and survival rate were shown to be modulated by a secondary genetic factor that can partially compensate for the lack of CFTR-encoded chloride channel function in CFTR knockout mice. The major modifier locus has recently been mapped near the centromere of mouse chromosome 7 and electrophysiological studies showed a linked upregulation of chloride conductance that may mediate prolonged survival (Rozmahel et al., 1996).

3.2. Gene redundancy and compensatory mechanisms

Apart from effects exerted by the genetic background, several other factors may complicate the interpretation of a specific phenotype. For example, defects in early development may indirectly also compromise functions in the adult. Conversely, developmental plasticity may compensate for an early loss of a gene function. This may occur at the single gene level, the genetic pathway level or involve systemic adaptive mechanisms. Within gene families, related proteins may functionally compensate for deficits associated with the loss of one family member. The comparison of phenotypes caused by single, as compared to combined, gene deficiencies allows the dissection of specific and redundant functions of individual family members, for example in the case of src-related tyrosine kinases (Stein et al., 1994), myogenic factors (Rudnicki et al., 1992), transcription factors (Hummler et al., 1994), utrophin/dystrophin (Grady et al., 1997), or neurotrophic factors (Sendtner et al., 1996). A striking example of functional compensation is, for example, seen in the β-APP gene family consisting of the ubiquitously expressed APP-protein and the two closely related APP-like proteins APLP1 and APLP2. Whereas gene ablation of individual family members resulted only in a very minor phenotype (Müller et al., 1994a; Zheng et al., 1995; Li et al., 1996, von Koch et al., 1997) combined gene deficiencies for APP/APLP2 (von Koch et al., 1997; Müller, 1998, unpublished result) and APLP1/APLP2 (Müller, 1998, unpublished result) proved postnatally lethal. In contrast, APP/APLP1-deficient mice were viable and showed no obvious anomalies (Müller, 1998, unpublished result).

Functional redundancy may also be revealed at the genetic-pathway level as, for example, for the interferon (IFN) system (Müller et al., 1994b; van den Broek et al., 1995). Two independent receptor systems, termed type I and type II IFN-systems, control the activation of a partly

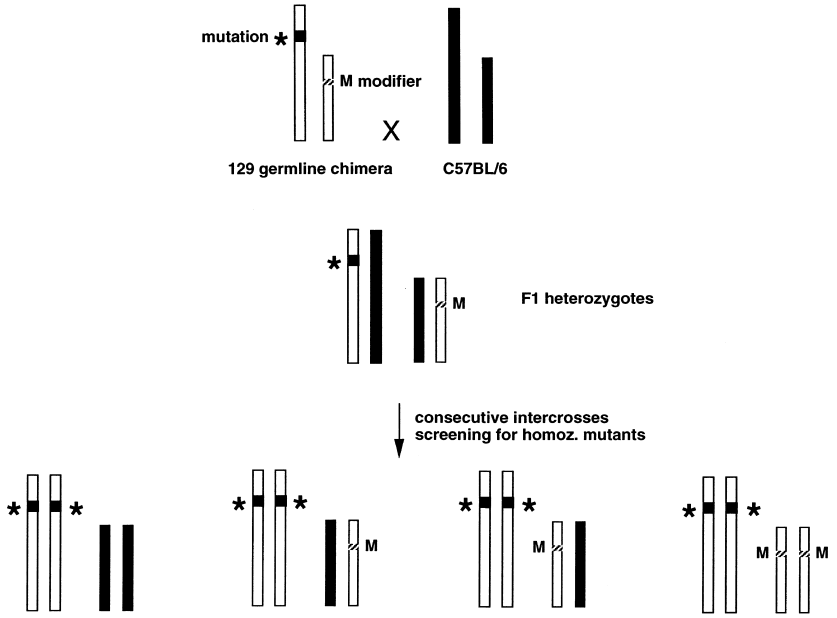

Fig. 6. Occurrence of substrains in knockout mice with mixed genetic background. A germ line chimera derived from ES cells of 129 genetic background is crossed to C57BL/6 wildtype females. Two chromosomes derived from the chimera are depicted by open rectangles and C57BL/6 chromosomes by filled rectangles, respectively. One of the ES cell-derived chromosomes harbours the targeted mutation (indicated by an asterisk) and the other chromosome contains a modifier allele (indicated by M), that is specific for the 129 background. In the F1 generation each animal obtains one set of chromosomes from either parental strain. After consecutive intercrosses the allele carrying the mutation is identified by screening, whereas all other chromosomes are randomly distributed. This results in the generation of substrains and background-specific modifier alleles may become fixed or lost. In addition, crossover events between sister chromatide occur during meiosis (not shown). Heterogenous backgrounds can be avoided if the chimera is crossed with the same strain as the ES cells used.

overlapping set of response genes mediating immunomodulatory responses and innate protection against viruses and other pathogens. Knockouts for either the type I or the type II IFN-receptor, respectively, revealed an essential role of mainly the type I IFN-system for the efficient protection against most viruses tested. For the protection against some viruses, however, both systems are indispensable and seem to interact in a functionally non-redundant way. Complete absence of both IFN-systems, generated by crossing the respective single mutants, showed an additive phenotype with respect to antiviral response (Müller et al., 1994b; van den Broek et al., 1995).

3.3. Rescue by complementation

The ultimate proof that a specific phenotype is caused by a targeted mutation is to revert the phenotype to wild type by reintroducing a functional gene copy. Reverse genetics (Weissmann et al., 1979) may further extend the analysis of knockout phenotypes by complementation with homologues from different species, expression in a tissue-specific or ectopic way, or by reintroducing various mutants which will help to elucidate structure-function relationships. In the mouse, transgenic complementation is not always a straightforward experiment, since expression of the reintroduced transgene is influenced by factors discussed in Section 2.6. Furthermore, genetic background is important, and if possible, oocytes from the knockout strain should be used for transgenic injections. Despite this, the approach may still be very powerful as exemplified by the reverse genetic dissection of different aspects of transmissible spongiform encephalopathies (TSEs), also known as prion diseases. The major genetic factor determining susceptibility to prions, the infectious agent causing TSE, is the PrP gene of the host, and PrP knockout mice have been shown to be resistant to infection by prions (Bueler et al., 1992). This phenotype could be reversed by transgenic overexpression of wild-type PrP in $Prnp^{-/-}$ mutant mice, which restored susceptibility to infection and, in addition, considerably reduced incubation times (Fischer et al., 1996). Transgenic mice overexpressing PrP carrying a mutation associated with the human prion disorder GerstmannSträussler–Scheinker (GSS) disease developed neurodegeneration, however with a wide variation in the age of onset (Hsiao et al., 1990). Upon crossing the mutant transgene onto a PrP knockout background, however, mice displayed a highly synchronous onset of spontaneous disease at a much younger age (Telling et al., 1996). Moreover, these mice had a more severe form of neurodegeneration, and brain extracts prepared from these spontaneously ill transgenic mice, transmitted the disease to transgenic indicator mice, expressing the mutant transgene (Telling et al., 1996).

The issue of species barriers, prolonged incubation times observed after inoculation of infectious agents isolated from different species, was addressed by transgenic expression of human PrP, or chimeric forms of PrP from humans and mice, in either wild-type or PrP knockout mice that were then challenged by prion inoculation (Telling et al., 1994; Telling et al., 1995). Tissue-specific expression of PrP has led to further insight into the mechanisms of pathogenesis (Raeber et al., 1997) and finally, an in vivo structure-function analysis was initiated by introducing a series of PrP-deletion mutants (Fischer et al., 1996; Schmerling et al., 1998) into $Prnp^{-/-}$ mutant mice.

4. Conditional gene targeting

Conventional gene targeting leads to inactivation or modification of a gene in all tissues of the body from the onset of development throughout the whole lifespan. More recently, methods have been developed aimed at controlling gene targeting in a time- and tissue-dependent manner. These so called conditional gene targeting approaches are particularly useful in cases where complete gene inactivation leads to a lethal or otherwise adverse phenotype that prevents a more detailed analysis. Moreover, if a given gene has a widespread pattern of expression, tissue-specific gene inactivation may define physiological roles of the gene product in a certain tissue, without compromising other functions in the organism. Control of gene targeting in a time dependent manner allows the differentiation between effects of chronic versus acute depletion of a protein and also the analysis of functions at different time points in development. Many knockout mice revealed an unexpectedly minor phenotype that was attributed either to gene redundancy, or to adaptive mechanisms mediating developmental plasticity, as was suggested for brain regions involved in learning and memory processes. Gene inactivation at a specific time point in the adult, leaving gene function intact throughout ontogeny, should prevent these adaptive responses and therefore phenotypes are expected to be more severe in conditional, as opposed to conventional, knockout mice.

4.1. Tissue-specific knockouts

Tissue-specific gene inactivation may be achieved by first generating transgenic mice that express Cre (or FLP) recombinase under the control of a tissue-specific promoter. These mice may then be crossed to target mice that contain loxP sites (or frt sites) flanking the genomic region to be deleted or modified. Double transgenic mice will carry a modified gene copy, due to Cre-mediated recombination, in all cells in which Cre was sufficiently expressed. Target mice harbouring appropriately engineered loxP sites can be obtained by homologous recombination in ES cells (see Fig. 7), whereas Cre-transgenic mice are either generated by conventional oocyte injection, or alternatively, by ‘knock-in’ gene targeting behind a suitable promoter exhibiting the desired expression pattern (Table 2). Recently, a database (http://www.mshri.on.ca/develop/Nagy/Cre.htm) has been established as a resource of information covering Cre trans-

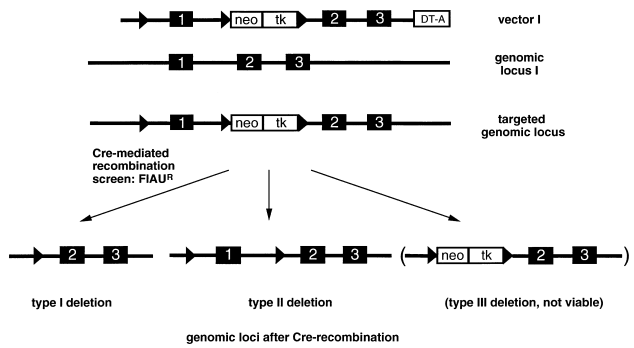

Fig. 7. Generation of target mice for conditional knockout strategies. In the first step, three loxP sites are introduced by homologous recombination into silent regions (e.g. introns) of a target gene. Two of the loxP sites flank the neo/tk selection cassettes, another one is located upstream of exon 1. Upon Cre-mediated recombination in ES cells and FIAU selection, two different genomic deletions (type I and II, type III is not viable in FIAU) are obtained, depending on the loxP sites used. Type I deletions may represent non-functional alleles, whereas mice solely harbouring two loxP sites in silent regions (type II deletion) should display no phenotype. These mice may then be crossed to Cre transgenic mice and double transgenic mice will undergo Cre-mediated recombination.

genic mouse lines that are already available, or are currently being generated. Oocyte injection will lead to random integration of multiple transgene copies and several founder lines of mice will have to be tested for their temporal and spatial pattern of Cre expression. This can be done by Western blotting or immunofluorescence using a monoclonal antibody directed against Cre recombinase (Schwenk et al., 1997). Functional Cre activity may also be directly assessed by determining the ratio of deleted, versus intact, gene segments after subjecting tissue from double transgenic mice to Southern blot analysis. Alternatively, one may cross Cre expressing mice to β-galactosidase indicator mice, that carry a loxP-flanked stop codon between an ubiquitous promoter and the lacZ gene. In all tissues expressing Cre the stop codon will be removed and therefore Cre activity can be monitored by X-Gal staining (Araki et al., 1995; Tsien et al., 1996; Zinyk et al., 1998). The expression level of Cre recombinase will determine the efficiency of gene modification, while temporo-spatial control is mainly dependent on the type of promoter driving Cre expression (see Fig. 8a).

The first example of a tissue-specific knockout was reported by Gu et al. (1994) who used a Cre transgenic line expressing the recombinase under the control of the lck promoter to delete, specifically in the T-cell compartment, a portion of the DNA polymerase β gene. Since then, conditional targeting approaches have been pursued in many laboratories and Table 2 gives examples of mouse lines that express Cre (or FLP) in a tissue-specific way. It is noteworthy that efficient Cre-mediated recombination may even be obtained in non-dividing cells like, for example, postmitotic hippocampal neurons (Tsien et al., 1996).

Several strains of mice have been created that express Cre (sometimes unexpectedly) in the early embryo prior to germ cell development. These so called deleter strains will, when crossed with target mice, give rise to offspring with a modified gene copy in most tissues, including the germ line. This strategy can be used as an alternative to Cre-mediated recombination in ES cells, thereby circumventing prolonged propagation of ES cells in tissue culture that might lead to differentiation. Ideally, as discussed in Section 3.1, the deleter strain should be of the same genetic background as the target mice. Alternatively, injection of fertilized eggs with a circular expression plasmid for Cre recombinase (Araki et al., 1995) or with in vitro transcribed Cre-RNA (de Wit et al., 1998) may lead to the transient expression of Cre in the preimplantation embryo.

The generation of mosaic mice, by Cre-mediated recombination, may be used as an elegant way to bypass lethality of a mutation, as shown by Betz et al. (1996) for the DNA polymerase β gene. In this study, a transgenic mouse line

Table 2

Examples of recombinase expressing mouse strains

Promoter	Site of expression (recombinase)	Reference
lck	T-cells (Cre)	(Gu et al., 1994; Hennet et al., 1995)
α-crystallin	Eye lens (Cre)	(Lakso et al., 1992)
CD19	B-cells (Cre)	(Rickert et al., 1997)
CamKII	Hippocampal neurons (Cre)	(Tsien et al., 1996)
En2	Mid/hindbrain (Cre)	(Zinyk et al., 1998)
Mx1	Ubiquitous, inducible (Cre)	(Kühn et al., 1995)
WAP	Mammary gland (Cre)	(Wagner et al., 1997)
aP2	Adipose tissue (Cre)	(Barlow et al., 1997)
protamin-1	Sperm (Cre)	(O'Gorman et al., 1997)
PO, POMC, IRBP	Schwann cells, pituitary, retina (Cre)	(Akagi et al., 1997)
nestin	Early embryo, mosaic expression (Cre)	(Betz et al., 1996)
PECAM-1	Early embryo, deleter strain (Cre)	(Terry et al., 1997)
CMV minimal	Early embryo, deleter strain (Cre)	(Schwenk et al., 1995)
Adenovirus EIIa	Early embryo, deleter strain (Cre)	(Lakso et al., 1996)
huβ-actin	Early embryo, deleter strain (Cre)	(Meyers et al., 1998)
huβ-actin	early embryo, deleter strain (FLP)	(Dymecki, 1996)

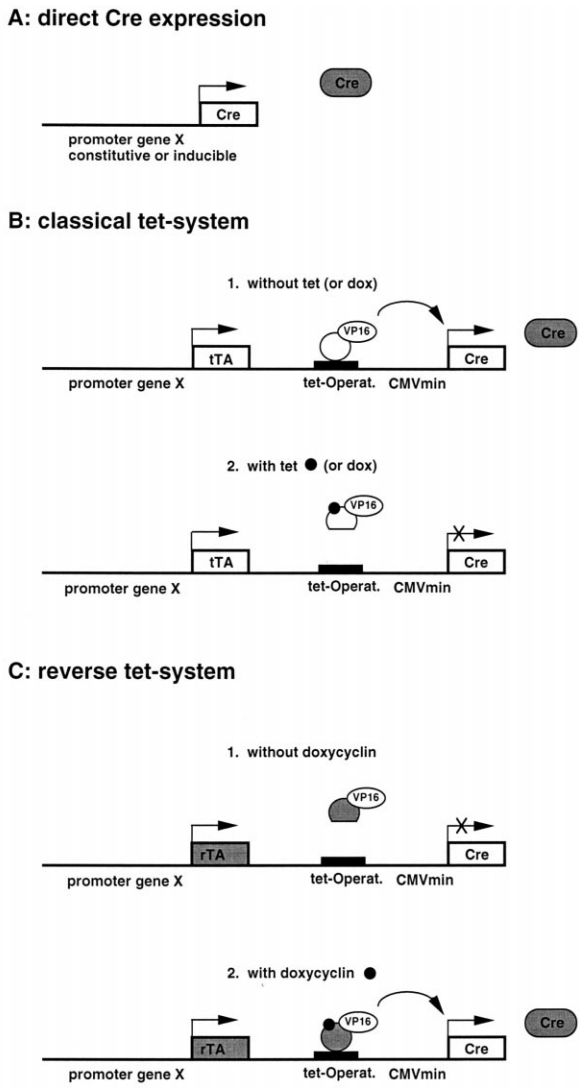

Fig. 8. Transcriptional regulation of Cre activity. (A) Cre expression in transgenic mice. Transgenic mice are generated that express Cre under the control of tissue-specific or inducible promoters. Note that the expression also depends on the integration site and copy number of the transgene. (B) Cre expression under the control of the classical tet system. The system consists of a transactivator, named tTA, that binds in the absence of tetracycline (tet) to an engineered tet-operator (depicted as filled rectangle), thereby activating the transcription of the response gene Cre. The transactivator tTA is a fusion protein composed of the bacterial tet repressor and the transactivation domain of the viral VP16 protein. Expression of tTA is controlled by the promoter X, which may be tissue-specific. The Cre responder gene is expressed in the absence of the ligand and silenced upon induction by ligand. CMVmin: cytomegalovirus minimal promoter. (C) Cre expression under the control of the reverse tet system. In this system a modified transactivator (rTA represented by hatched symbols) is used that binds to DNA only in the presence of ligand. Therefore Cre responder gene expression is normally silent, but can be switched on by ligand administration.

expressing Cre under the control of the nestin promoter was established, originally with the intention to target genes specifically in the central nervous system (CNS). Unexpectedly however, expression was not CNS-restricted and various degrees of Cre-mediated deletion of the target gene segment were detected in all tissues examined. These mice can be used instead of chimeras to bypass lethality and to identify cell lineages for whose development a given target gene is crucial.

Infection of somatic tissues of target mice with a viral vector expressing Cre recombinase is another possibility which would also permit some degree of temporo-spatial control (Rohlmann et al., 1996). Shibata et al. (1997) recently applied this strategy to generate a conditional, colorectal-specific knockout of the APC gene, which is a tumour suppressor gene involved in familial and sporadic forms of colorectal cancer and for which a complete deficiency is embryonic lethal. Upon infection with a Cre-expressing recombinant adenovirus, the target mice had excised the loxP-flanked APC-gene fragment and developed numerous colorectal adenomas.

Finally, it is also possible to apply both Cre- and FLP-mediated recombination to introduce specific modifications into the same target gene, which was recently shown for the fibroblast growth factor-8 (FGF8) locus, using transient transfection of either recombinase in ES cells, or transgenic mice expressing Cre- or FLP-recombinase, respectively (Meyers et al., 1998).

4.2. Inducible gene targeting

For many applications it would be desirable to control the function of a gene product in a time-dependent manner, regulated for example by the administration of an inducer. One way to achieve this kind of inducible gene silencing is, in analogy to tissue-specific gene targeting, to use a Cre recombinase based system that may be controlled either at the transcriptional or at the post-transcriptional level. Ideally, any inducible system should fulfil the following requirements: (1) no (or only minor) background activity in the absence of the inducer; (2) fast and efficient response in all tissue; and (3) no toxic, or otherwise adverse, effects should be caused by the inducer.

4.2.1. Transcriptional regulation of Cre activity

Several approaches have been pursued to put Cre expression under the control of inducible promoters (see Fig. 8). In a pioneering study conducted by Kühn et al. (1995) Cre-expression was driven by the Mx1 promoter, which can be induced by recombinant interferon (IFN) or by the IFN-inducer poly(IC). The target mice used in this study carried a loxP-flanked segment of the DNA Polymerase β gene. The efficiency of induced deletion of this gene fragment varied largely between different tissues, ranging from 100% in liver to as little as 8% in brain, the latter presumably due to the inability of IFN to cross the blood-brain barrier. Background activity was undetectable in most tissue, but reached about 10% in spleen, which may be caused by the activation of endogenous IFN in cells of the immune system.

Another system for the inducible control of gene expression in transgenic mice is the tetracycline (tet) regulatory

system developed by Bujard and colleagues (for a review see Shockett and Schatz, 1996). The system consists (see Fig. 8) of a transactivator that binds in a ligand-dependent way to an engineered minimal promoter, thereby activating the transcription of a response gene. The transactivator, named tTA, is a fusion protein composed of the bacterial tet-repressor and the transactivation domain of the viral VP16 protein. In the classical tet system (Fig. 8b) the responder gene is expressed in the absence of the ligand and silenced upon introduction of the ligand. In the reverse tet-system (Fig. 8c), a modified transactivator (rTA) is used that binds to the operator/promoter only in the presence of ligand. Therefore, responder gene expression is normally silent, but can be switched on by ligand administration. Both tTA- and rTA-regulated systems have been shown to control the expression of reporter genes in transgenic mice (Shockett and Schatz, 1996). A modification was introduced by Bluethmann and co-workers, who fused the components of the system (tTA-expression cassette and response cassette) in a single plasmid, thus circumventing the need to generate and cross multiply transgenic lines (Schultze et al., 1996). Tissue specificity can be obtained by placing the transactivator under the control of a suitable promoter as shown in an elegant study conducted by Kandel and co-workers (Mayford et al., 1996). The CamKinase II promoter was used to drive tTA-expression and a transdominant mutant of CamKinase II was utilized as the responder gene. In this way, the expression of the transdominant mutant could be reversibly controlled in a time- and region-specific manner by the administration of the ligand doxycyclin. In the absence of doxycyclin, the mutant CamKinase II was expressed which lead to a loss of long-term potentiation (LTP) and a deficit in spatial memory. Doxycyclin-induced suppression of the mutant transgene reversed both phenotypes.

Applying the classical tet system, with Cre recombinase as the responder gene, St-Onge et al. (1996) demonstrated that, in principle, inducible Cre-mediated recombination can be achieved, although these initial experiments were hampered by a considerable background of non-repressed Cre activity. This may, however, be improved if the reverse tet-system is used instead.

Other ligand-, or drug-inducible expression systems, that may in the future be applied to control Cre-expression, have been developed during the last years (reviewed in Gingrich and Roder, 1998). Such inducing agents include the progesterone antagonist RU486 (Wang et al., 1997), the drosophila hormone ecdysone (No et al., 1996) and ligands of the immunophilins like rapamycin or FK1012 (Rivera et al., 1996). Natural and synthetic ligands of immunophilins have been employed as chemical inducers of dimerization (CIDs) to inducibly regulate the proximity and orientation of proteins (Crabtree and Schreiber, 1996). This strategy has been adapted to control the activity of a transcription factor, that may in turn regulate the expression of a chosen target gene (Rivera et al., 1996). Two transcription factor fusion proteins were generated: one carrying a DNA-binding domain (of GAL4) and the other a transcriptional activation domain (of VP16). Each of these fusion proteins contained, in addition, heterologous ligand binding domains (derived from the immunophilins FKBP12 and FRAP) that permit the interaction with the bivalent ligand rapamycin. In this way, a tripartite complex between rapamycin and the transcription factor fusion proteins is formed. This system has been applied to inducibly express human growth hormone in mice (Rivera et al., 1996). The in vivo use of rapamycin, however, can be complicated by its native biological activity, as an immunosuppressive agent. Recently, the development of rapamycin variants that are not recognized by the endogenous cellular binding proteins has been reported, and ligand binding domains of FRAP with compensatory mutations have been generated (Liberles et al., 1997).

4.2.2. Post-transcriptional regulation of Cre activity

Cre activity may also be inducibly controlled at the post-transcriptional level by employing a strategy that has been successfully used for many intracellular proteins (for a review see Picard, 1994). In this approach (see Fig. 9), Cre recombinase is expressed as a chimeric protein fused to the ligand binding domain (LBD) of steroid receptors. In the absence of the steroidal ligand, heat shock proteins are bound to the Cre fusion protein and thereby mask the enzymatic activity, presumably by steric hindrance. Upon administration of ligand, heat shock proteins dissociate and Cre activity is restored. To circumvent any activation by endogenous steroid hormones, mutant steroid binding

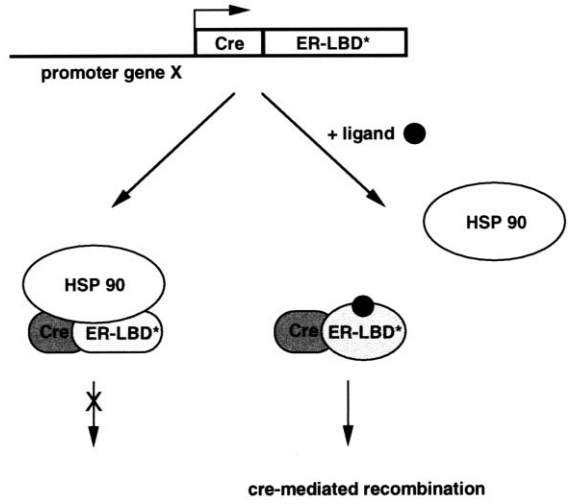

Fig. 9. Post-transcriptional regulation of Cre activity. Cre recombinase is expressed as a chimeric protein fused to the ligand binding domain (LBD) of oestrogen receptor (ER). In the absence of the ligand, heat shock proteins (HSP90) are bound to the Cre fusion protein and thereby mask the enzymatic activity. Upon administration of ligand, heat shock proteins dissociate and Cre activity is restored. To circumvent any activation by endogenous oestrogen, a mutant oestrogen binding domain (ER-G521R, indicated by an asterisk) is used that has a very low affinity for the natural ligand, but binds efficiently to the synthetic steroid 4-hydroxytamoxifen.

domains are used (e.g. oestrogen receptor G521R-mutant), which have a very low affinity for the natural ligand, but bind efficiently to synthetic steroids, like the anti-oestrogen 4-hydroxytamoxifen.

Chimeric Cre fusion proteins with the LBDs of the oestrogen receptor (ER) and the progesterone receptor (PG) have been generated and hormone-inducible Cre-mediated recombination was reported in ES cells (Kellendonk et al., 1996; Zhang et al., 1996) and also in transgenic mice (Feil et al., 1996). Notably, in all systems employed, Cre activity was tightly repressed in the absence of ligand. When transgenic mice were generated that expressed a Cre-ER fusion protein under the control of the largely ubiquitous CMV promoter, the efficiency of ligand-induced Cre-mediated excision reached values ranging from 10% in the brain to about 50% in the skin. Interestingly, the efficiency of recombination seemed to be limited by the level of Cre-expression in the various tissues (Feil et al., 1996).

Both tissue-specific and temporally-regulated Cre activity were recently achieved by Schwenk et al. (1998) who used a B-cell specific promoter to limit the expression of a Cre-ER fusion protein to B-lymphocytes. With this very promising strategy, highly tissue-specific Cre-mediated recombination with an efficiency of about 80% was obtained exclusively in the B-cell compartment.

A current limitation of inducible-gene-targeting approaches is that Cre-mediated recombination does not usually reach 100% efficiency, which results in a mosaic expression of the target gene. This precludes the analysis of knockouts for proteins that act non-cell-autonomously, such as diffusible ligands. On the other hand, knockouts of cell autonomous polypeptides, such as receptors or intracellular signalling molecules, need not necessarily attain 100% recombination rates in order to yield informative (although not null equivalent) phenotypes.

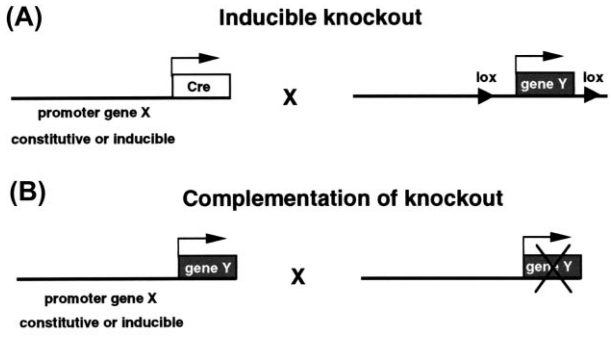

Fig. 10. Comparison of inducible knockout versus complementation strategies. (A,B) Double transgenic mice are generated that express a randomly integrated transgene (Cre or geneY) in addition to a modified allele of the geneY (loxP flanked in A, inactivated in B). (A) Cre expression driven by a constitutive or inducible promoter will lead to the irreversible deletion of loxP flanked target gene segment. (B) In contrast, complementation of a gene knockout by transgenic expression of proteinY may be reversibly controlled.

4.3. Inducible complementation

Inherent disadvantages of Cre-based approaches aimed at gene inactivation are: their irreversibility, and the limitation that no intermediate state of gene silencing can be achieved. Regulated, reversible complementation of a knockout phenotype by inducible expression of a transgene (e.g. via the tet system) might overcome these limitations (see Fig. 10). Exploiting the regulatory expression systems described above, it should become possible to control transgene expression in a way that mimics the endogenous pattern of expression, or that leads to ectopic expression of the transgene on a knockout background. Furthermore, intermediate levels of gene expression may be achieved and should allow the analysis of gene dosage effects.

5. Summary and future directions

Gene targeting techniques have revolutionized the field of mouse genetics and allowed the analysis of diverse aspects of gene function in the context of the whole animal. It has become possible to engineer specific genetic alterations ranging from subtle mutations to chromosomal rearrangements and more recently, even tissue-specific inducible gene targeting with temporo-spatial control has become feasible. With continuous efforts from many laboratories these new approaches are expected to be further optimized in the near future, with the ultimate goal of reversibly switching on and off any gene in a given tissue at a chosen time-point. Currently, tissue-specific conditional gene targeting is restricted by the availability of only a limited number of transgenic mouse lines expressing Cre recombinase in specific organs. Temporally regulated targeting, controlled by the administration of inducers, has become feasible with high efficiency for some organs, but remains to be further improved for other tissues, in particular for the brain. Although gene targeting methods have been refined, and the efficiency of homologous recombination has been optimized in murine ES cells, targeting of most somatic cells (and cell lines) is still much less efficient. There is, however, considerable interest to improve the genetic manipulation of somatic cells, especially with regard to applications in somatic gene therapy. Moreover, work directed at establishing gene targeting in species other than mice such as rats, *Drosophila* and in particular in commercial livestock, may lead to interesting new applications.

Acknowledgements

I would like to thank Charles Weissmann for his advice, continuous support, and many helpful discussions during my time in his laboratory. Furthermore, I am indebted to him for suggesting to write this review and for crucial comments on the manuscript. I am grateful to my colleagues

Antonio Cozzio, Christian Buchholz, Louise Coletta and Rob Harvey for critically reading the manuscript, and to Johann Bauer for support with literature searches.

References

Albert, H., Dale, E.C., Lee, E., Ow, D.W., 1995. Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant Journal 7, 649–659.

Akagi, K., Sandig, V., Voojis, M., Van der Valk, M., Giovannini, M., Strauss, M., Berns, A., 1997. Cre-mediated somatic site-specific recombination in mice. Nucleic Acids Res. 25, 1766–1773.

Araki, K., Araki, M., Miyazaki, J., Vassalli, P., 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc. Natl. Acad. Sci. USA 92, 160–164.

Araki, K., Araki, M., Yamamura, K., 1997. Targeted integration of DNA using mutant lox sites in embryonic stem cells. Nucleic Acids Res. 25, 868–872.

Askew, G.R., Doetschman, T., Lingrel, J.B., 1993. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol. Cell. Biol. 13, 4115–4124.

Baribault, H., Penner, J., Iozzo, R.V., Wilson-Heiner, M., 1994. Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. Genes Dev. 8, 2964–2973.

Barlow, C., Schroeder, M., Lekstrom-Himes, J., Kylefjord, H., Deng, C.X., Wynshaw-Boris, A., Spiegelman, B.M., Xanthopoulos, K.G., 1997. Targeted expression of Cre recombinase to adipose tissue of transgenic mice directs adipose-specific excision of loxP-flanked gene segments. Nucleic Acids Res. 25, 2543–2545.

Bethke, B., Sauer, B., 1997. Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants. Nucleic Acids Research 25(14), 2828–2834.

Betz, U.A., Vosshenrich, C.A., Rajewsky, K., Muller, W., 1996. Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. Curr. Biol. 6, 1307–1316.

Bonyadi, M., Rusholme, S.A., Cousins, F.M., Su, H.C., Biron, C.A., Farrall, M., Akhurst, R.J., 1997. Mapping of a major genetic modifier of embryonic lethality in TGF beta 1 knockout mice. Nature Genet. 15, 207–211.

Bradley, A., Evans, M., Kaufman, M.H., Robertson, E., 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309, 255–256.

Brandon, E.P., Idzerda, R.L., McKnight, G.S., 1995. Targeting the mouse genome: a compendium of knockouts. Curr. Biol. 5, 625–634 (Part I), 758–765 (Part II), 873–881 (Part III).

Bronson, S.K., Plaehn, E.G., Kluckman, K.D., Hagaman, J.R., Maeda, N., Smithies, O., 1996. Single-copy transgenic mice with chosen-site integration. Proc. Natl. Acad. Sci. USA 93, 9067–9072.

Brook, F.A., Gardner, R.L., 1997. The origin and efficient derivation of embryonic stem cells in the mouse. Proc. Natl. Acad. Sci. USA 94, 5709–5712.

Buchholz, F., Ringrose, L., Angrand, P.O., Rossi, F., Stewart, A.F., 1996. Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. Nucleic Acids Res. 24, 4256–4262.

Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M., Weissmann, C., 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 356, 577–582.

Crabtree, G.R., Schreiber, S.L., 1996. Three-part inventions: intracellular signaling and induced proximity. Trends Biochem. Sci. 21, 418–422.

Crawley, J.N., 1996. Unusual behavioral phenotype of inbred mouse strains. Trends Neurosci. 19, 181–182.

Dahme, M., Bartsch, U., Martini, R., Anliker, B., Schachner, M., Mantei, N., 1997. Disruption of the mouse L1 gene leads to malformations of the nervous system. Nature Genet. 17, 346–349.

Deng, C., Capecchi, M.R., 1992. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol. Cell. Biol. 12, 3365–3371.

Detloff, P.J., Lewis, J., John, S.W., Shehee, W.R., Langenbach, R., Maeda, N., Smithies, O., 1994. Deletion and replacement of the mouse adult beta-globin genes by a 'plug and socket' repeated targeting strategy. Mol. Cell. Biol. 14, 6936–6943.

de Wit, T., Drabek, D., Grosveld, F., 1998. Microinjection of Cre recombinase RNA induces site-specific recombination of a transgene inmouse oocytes. Nucleic Acids Res. 26, 676–678.

Doetschman, T., Gregg, R.G., Maeda, N., Hooper, M.L., Melton, D.W., Thompson, S., Smithies, O., 1987. Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature 330, 57657–57658.

Donehower, L.A., Harvey, M., Vogel, H., McArthur, M.J., Montgomery, C.A. Jr., Park, S.H., Thompson, T., Ford, R.J., Bradley, A., 1995. Effects of genetic background on tumorigenesis in p53-deficient mice. Mol. Carcinogen. 14, 16–22.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A. Jr., Butel, J.S., Bradley, A., 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356, 215–221.

Dorin, J.R., Stevenson, B.J., Fleming, S., Alton, E.W., Dickinson, P., Porteous, D.J., 1994. Long-term survival of the exon 10 insertional cystic fibrosis mutant mouse is a consequence of low level residual wild-type Cftr gene expression. Mamm. Genome 5, 465–472.

Dymecki, S.M., 1996. FLP recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc. Natl. Acad. Sci. USA 93, 6191–6196.

Erickson, R.P., 1996. Mouse models of human genetic disease: which mouse is more like a man?. Bioessays 18, 993–998.

Evans, M.J., Carlton, M.B., Russ, A.P., 1997. Gene trapping and functional genomics. Trends Genet. 13, 370–374.

Evans, M.J., Kaufmann, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., Chambon, P., 1996. Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. USA 93, 10887–10890.

Festing, M., 1996. Inbred strains of mice. Mouse Genome 94, 523–677.

Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D.I., Enver, T., Ley, T.J., Groudine, M., 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 Dev. 9, 2203–2213.

Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A., Weissmann, C., 1996. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. 15, 1255–1264.

Friedrich, G., Soriano, P., 1991. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev. 5, 1513–1523.

Gerlai, R., 1996. Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype?. Trends Neurosci. 19, 177–181.

Gingrich, J.R., Roder, J., 1998. Inducible gene expression in the nervous system of transgenic mice. Annu. Rev. Neurosci. 21, 377–405.

Golic, K.G., 1991. Site-specific recombination between homologous chromosomes in Drosophila. Science 252, 958–961.

Grady, R.M., Teng, H., Nichol, M.C., Cunningham, J.C., Wilkinson, R.S., Sanes, J.R., 1997. Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell 90, 729–738.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., Rajewsky, K., 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265, 103–106.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., Joyner,

A.L., 1995. Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 269, 679–682.

Hasty, P., Ramirez-Solis, R., Krumlauf, R., Bradley, A., 1991. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. Nature 350, 243–246.

Hasty, P., Rivera-Perez, J., Bradley, A., 1991. The length of homology required for gene targeting in embryonic stem cells. Mol. Cell. Biol. 11, 5586–5591.

Hennet, T., Hagen, F.K., Tabak, L.A., Marth, J.D., 1995. T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl-transferase gene by site-directed recombination. Proc. Natl. Acad. Sci. USA 92, 12070–12074.

Hogan, B., Beddington, R., Constantini, F., Lacy, E., 1994. Manipulating the Mouse Embryo, A laboratory manual. Cold Spring Harbour Laboratory Press, USA.

Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F., Schutz, G., 1994. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc. Natl. Acad. Sci. USA 91, 5647–5651.

Hsiao, K.K., Scott, M., Foster, D., Groth, D.F., DeArmond, S.J., Prusiner, S.B., 1990. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science 250, 1587–1590.

Jasin, M., Moynahan, M.E., Richardson, C., 1996. Targeted transgenesis. Proc. Natl. Acad. Sci. USA 93, 8804–8808.

Jones, A., Korpi, E.R., McKernan, R.M., Pelz, R., Nusser, Z., Makela, R., Mellor, J.R., Pollard, S., Bahn, S., Stephenson, F.A., Randall, A.D., Sieghart, W., Somogyi, P., Smith, A.J., Wisden, W., 1997. Ligand-gated ion channel subunit partnerships: GABAA receptor alpha6 subunit gene inactivation inhibits delta subunit expression. J. Neurosci. 17, 1350–1362.

Joyner, A.L. (Ed.), 1993. Gene Targeting: a Practical Approach, 1st edn. Oxford University Press, New York.

Joyner, A.L. (Ed.), 1998. Gene Targeting: a Practical Approach. Oxford University Press, New York. Second edition, in press.

Kawase, E., Suemori, H., Takahashi, N., Okazaki, K., Hashimoto, K., Nakatsuji, N., 1994. Strain difference in establishment of mouse embryonic stem (ES) cell lines. Int. J. Dev. Biol. 38, 385–390.

Kellendonk, C., Tronche, F., Monaghan, A.P., Angrand, P.O., Stewart, F., Schutz, G., 1996. Regulation of Cre recombinase activity by the synthetic steroid RU 486. Nucleic Acids Res. 24, 1404–1411.

Kilby, N.J., Snaith, M.R., Murray, J.A., 1993. Site-specific recombinases: tools for genome engineering. Trends Genet. 9, 413–421.

Kobayashi, K., Ohye, T., Pastan, I., Nagatsu, T., 1996. A novel strategy for the negative selection in mouse embryonic stem cells operated with immunotoxin-mediated cell targeting. Nucleic Acids Res. 24, 3653–3655.

Kühn, R., Schwenk, F., Aguet, M., Rajewsky, K., 1995. Inducible gene targeting in mice. Science 269, 1427–1429.

Lakso, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W., Westphal, H., 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl. Acad. Sci. USA 93, 5860–5865.

Lakso, M., Sauer, B., Mosinger, B., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., Westophal, H., 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. USA 89, 6232–6236.

Lander, E.S., Schork, N.J., 1994. Genetic dissection of complex traits. Science 265, 2037–2048.

Lathe, R., 1996. Mice, gene targeting and behaviour: more than just genetic background. Trends Neurosci. 19, 183–186.

Ledermann, B., Burki, K., 1991. Establishment of a germ-line competent C57BL/6 embryonic stem cell line. Exp. Cell Res. 197, 254–258.

Li, E., Bestor, T.H., Jaenisch, R., 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915–926.

Li, Z.W., Stark, G., Gotz, J., Rulicke, T., Gschwind, M., Huber, G., Muller, U., Weissmann, C., 1996. Generation of mice with a 200-kb amyloid precursor protein gene deletion by Cre recombinase-mediated site-specific recombination in embryonic stem cells. Proc. Natl. Acad. Sci. USA 93, 6158–6162.

Liberles, S.D., Diver, S.T., Austin, D.J., Schreiber, S.L., 1997. Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. Proc. Natl. Acad. Sci. USA 94, 7825–7830.

Magara, F., Müller, U., Lipp, H.P., Weissmann, C., Stagliar, M., Wolfer, D.P., 1999. Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the β-amyloid-precursor protein. Proc. Natl. Acad. Sci. USA, in press.

Mansour, S.L., Goddard, J.M., Capecchi, M.R., 1993. Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. Development 117, 13–28.

Mansour, S.L., Thomas, K.R., Capecchi, M.R., 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336, 348–352.

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634–7638.

Matouk, C., Gosselin, D., Malo, D., Skamene, E., Radzioch, D., 1996. PCR-analyzed microsatellites for the inbred mouse strain 129/Sv, the strain most commonly used in gene knockout technology. Mamm. Genome 7, 603–605.

Matzuk, M.M., Finegold, M.J., Su, J.G., Hsueh, A.J., Bradley, A., 1992. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 360, 313–319.

Mayford, M., Bach, M.E., Huang, Y.Y., Wang, L., Hawkins, R.D., Kandel, E.R., 1996. Control of memory formation through regulated expression of a CaMKII transgene. Science 274, 1678–1683.

McWhir, J., Schnieke, A.E., Ansell, R., Wallace, H., Colman, A., Scott, A.R., Kind, A.J., 1996. Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. Nature Genet. 14, 223–226.

Medberry, S.L., Dale, E., Qin, M., Ow, D.W., 1995. Intra-chromosomal rearrangements generated by Cre-lox site-specific recombination. Nucleic Acids Res. 23, 485–490.

Meyers, E.N., Lewandoski, M., Martin, G.R., 1998. An Fgf8 mutant allelic series generated by Cre- and FLP-mediated recombination. Nature Genet. 18, 136–141.

Moens, C.B., Auerbach, A.B., Conlon, R.A., Joyner, A.L., Rossant, J., 1992. A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. Genes Dev. 6, 691–704.

Moore, R.C., Redhead, N.J., Selfridge, J., Hope, J., Manson, J.C., Melton, D.W., 1995. Double replacement gene targeting for the production of a series of mouse strains with different prion protein gene alterations. Biotechnology 13, 999–1004.

Morrison, R.S., Wenzel, H.J., Kinoshita, Y., Robbins, C.A., Donehower, L.A., Schwartzkroin, P.A., 1996. Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. J. Neurosci. 16, 1337–1345.

Müller, U., Cristina, N., Li, Z.W., Wolfer, D.P., Lipp, H.P., Rulicke, T., Brandner, S., Aguzzi, A., Weissmann, C., 1994. Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell 79, 755–765.

Müller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., Aguet, M., 1994. Functional role of type I and type II interferons in antiviral defense. Science 264, 1918–1921.

No, D., Yao, T.P., Evans, R.M., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA 93, 3346–3351.

O'Gorman, S., Dagenais, N.A., Qian, M., Marchuk, Y., 1997. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc. Natl. Acad. Sci. USA 94, 14602–14607.

Ohno, H., Goto, S., Taki, S., Shirasawa, T., Nakano, H., Miyatake, S., Aoe, T., Ishida, Y., Maeda, H., Shirai, T., 1994. Targeted disruption of the

CD3 eta locus causes high lethality in mice: modulation of Oct-1 transcription on the opposite strand. EMBO J. 13, 1157–1165.
Olson, E.N., Arnold, H.H., Rigby, P.W., Wold, B.J., 1996. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell 85, 1–4.
Pease, S., Williams, R.L., 1990. Formation of germ-line chimeras from embryonic stem cells maintained with recombinant leukemia inhibitory factor. Exp. Cell Res. 190, 209–211.
Pham, C.T., MacIvor, D.M., Hug, B.A., Heusel, J.W., Ley, T.J., 1996. Long-range disruption of gene expression by a selectable marker cassette. Proc. Natl. Acad. Sci. USA 93, 13090–13095.
Picard, D., 1994. Regulation of protein function through expression of chimaeric proteins. Curr. Opin. Biotech. 5, 511–515.
Raeber, A.J., Race, R.E., Brandner, S., Priola, S.A., Sailer, A., Bessen, R.A., Mucke, L., Manson, J., Aguzzi, A., Oldstone, M.B., Weissmann, C., Chesebro, B., 1997. Astrocyte-specific expression of hamster prion protein (PrP) renders PrP knockout mice susceptible to hamster scrapie. EMBO J. 16, 6057–6065.
Ramirez-Solis, R., Liu, P., Bradley, A., 1995. Chromosome engineering in mice. Nature 378, 720–724.
Ramirez-Solis, R., Zheng, H., Whiting, J., Krumlauf, R., Bradley, A., 1993. Hoxb-4 (Hox-2.6) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. Cell 73, 279–794.
Ramirez-Solis, R., Rivera-Perez, J., Wallace, J.D., Wims, M., Zheng, H., Bradley, A., 1992. Genomic DNA microextraction: a method to screen numerous samples. Anal. Biochem. 201, 331–335.
Rickert, R.C., Roes, J., Rajewsky, K., 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res. 25, 1317–1378.
Rijli, F.M., Dolle, P., Fraulob, V., LeMeur, M., Chambon, P., 1994. Insertion of a targeting construct in a Hoxd-10 allele can influence the control of Hoxd-9 expression. Dev. Dynam. 201, 366–377.
Rivera, V.M., Clackson, T., Natesan, S., Pollock, R., Amara, J.F., Keenan, T., Magari, S.R., Phillips, T., Courage, N.L., Cerasoli, F. Jr., Holt, D.A., Gilman, M., 1996. A humanized system for pharmacologic control of gene expression. Nature Med. 2, 1028–1032.
Rohlmann, A., Gotthardt, M., Willnow, T.E., Hammer, R.E., Herz, J., 1996. Sustained somatic gene inactivation by viral transfer of Cre recombinase. Nature Biotech. 14, 1562–1565.
Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C., Tsui, L.C., 1996. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. Nature Genet. 12, 280–287.
Rudnicki, M.A., Braun, T., Hinuma, S., Jaenisch, R., 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71, 383–390.
Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A.C., Horak, I., 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75, 253–261.
Santerre, R.F., Allen, N.E., Hobbs, J.N. Jr., Rao, R.N., Schmidt, R.J., 1984. Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells. Gene 30, 147–156.
Sauer, B., Henderson, N., 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol. 2, 441–449.
Schauwecker, P.E., Steward, O., 1997. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc. Natl. Acad. Sci. USA 94, 4103–4108.
Schmerling, D., Hegyi, I., Fischer, M., Blattler, T., Brandner, S., Gotz, J., Rulicke, T., Flechsig, E., Cozzio, A., von Mering, C., Hangartner, C., Aguzzi, A., Weissmann, C., 1998. Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. Cell 93, 203–214.
Schultze, N., Burki, Y., Lang, Y., Certa, U., Bluethmann, H., 1996. Efficient control of gene expression by single step integration of the tetracycline system in transgenic mice. Nature Biotech. 14, 499–503.
Schwartzberg, P.L., Goff, S.P., Robertson, E.J., 1989. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science 246, 799–803.
Schwegler, H., Lipp, H.P., 1983. Hereditary covariations of neuronal circuitry and behavior: correlations between the proportions of hippocampal synaptic fields in the region inferior and two-way avoidance in mice and rats. Behav. Brain Res. 7, 1–38.
Schwenk, F., Kühn, R., Angrand, P.O., Rajewsky, K., Stewart, A.F., 1998. Temporally and spatially regulated somatic mutagenesis in mice. Nucleic Acids Res. 26, 1427–1432.
Schwenk, F., Sauer, B., Kukoc, N., Hoess, R., Muller, W., Kocks, C., Kuhn, R., Rajewsky, K., 1997. Generation of Cre recombinase-specific monoclonal antibodies, able to characterize the pattern of Cre expression in cre-transgenic mouse strains. J. Immunol. Methods 207, 203–212.
Schwenk, F., Baron, U., Rajewsky, K., 1995. A Cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23, 5080–5081.
Sendtner, M., Gotz, R., Holtmann, B., Escary, J.L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P., Thoenen, H., 1996. Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr. Biol. 6, 686–694.
Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., Kanamaru, R., Kanegae, Y., Saito, I., Nakamura, Y., Shiba, K., Noda, T., 1997. Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. Science 278, 120–123.
Shockett, P.E., Schatz, D.G., 1996. Diverse strategies for tetracycline-regulated inducible gene expression. Proc. Natl. Acad. Sci. USA 93, 5173–5176.
Sibilia, M., Wagner, E.F., 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269, 234–238.
Sikorski, R., Peters, R., 1997. Transgenics on the Internet. Nature Biotechnol. 15, 289.
Silva, A.J., 1997. Mutant mice and neuroscience: recommendations concerning genetic background. Banbury conference on genetic background in mice. Neuron 19, 755–759.
Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, M.T., Mobraaten, L.E., Sharp, J.J., 1997. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nature Genet. 16, 19–27.
Smith, A.J., De Sousa, M.A., Kwabi-Addo, B., Heppell-Parton, A., Impey, H., Rabbitts, P., 1995. A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. Nature Genet. 9, 376–385.
Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., Kucherlapati, R.S., 1985. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature 317, 230–234.
St-Onge, L., Furth, P.A., Gruss, P., 1996. Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. Nucleic Acids Res. 24, 3875–3877.
Stacey, A., Schnieke, A., McWhir, J., Cooper, J., Colman, A., Melton, D.W., 1994. Use of double-replacement gene targeting to replace the murine alpha-lactalbumin gene with its human counterpart in embryonic stem cells and mice. Mol. Cell. Biol. 14, 1009–1016.
Stanton, B.R., Perkins, A.S., Tessarollo, L., Sassoon, D.A., Parada, L.F., 1992. Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. Genes Dev. 6, 2235–2247.
Stein, P.L., Vogel, H., Soriano, P., 1994. Combined deficiencies of Src, Fyn, and Yes tyrosine kinases in mutant mice. Genes Dev. 8, 1999–2007.
Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., Okuyama, S., Kawashima, N., Hori, S., Takimoto, M., Wada, K., 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276, 1699–1702.

te Riele, H., Maandag, E.R., Berns, A., 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. USA 89, 5128–5132.

Telling, G.C., Haga, T., Torchia, M., Tremblay, P., DeArmond, S.J., Prusiner, S.B., 1996. Interactions between wild-type and mutant prion proteins modulate neurodegeneration in transgenic mice. Genes Dev. 10, 1736–1750.

Telling, G.C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F.E., DeArmond, S.J., Prusiner, S.B., 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. Cell 83, 79–90.

Telling, G.C., Scott, M., Hsiao, K.K., Foster, D., Yang, S.L., Torchia, M., Sidle, K.C., Collinge, J., DeArmond, S.J., Prusiner, S.B., 1994. Transmission of Creutzfeldt–Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. Proc. Natl. Acad. Sci. USA 91, 9936–9940.

Terry, R.W., Kwee, L., Baldwin, H.S., Labow, M.A., 1997. Cre-mediated generation of a VCAM-1 null allele in transgenic mice. Trans. Res. 6, 349–356.

Thomas, K.R., Capecchi, M.R., 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51, 503–512.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269, 230–234.

Torres, R.M., Kühn, R., 1997. Laboratory protocols for conditional gene targeting. Oxford University Press, Oxford.

Tsien, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R., Tonegawa, S., 1996. Subregion- and cell type-restricted gene knockout in mouse brain. Cell 87, 1317–1326.

Upchurch, M., Wehner, J.M., 1988. Differences between inbred strains of mice in Moriis water maze performance. Behav. Genet. 18, 55–68.

Valancius, V., Smithies, O., 1991. Testing an 'in-out' targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. Mol. Cell. Biol. 11, 1402–1408.

van den Broek, M.F., Muller, U., Huang, S., Aguet, M., Zinkernagel, R.M., 1995. Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. J. Virol. 69, 4792–4796.

Van Deursen, J., Fornerod, M., Van Rees, B., Grosveld, G., 1995. Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. Proc. Natl. Acad. Sci. USA 92, 7376–7380.

von Koch, C.S., Zheng, H., Chen, H., Trumbauer, M., van der Ploeg, L.H., Price, D.L., Sisodia, S.S., 1997. Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. Neurobiol. Aging 18, 661–669.

von Melchner, H., DeGregori, J.V., Rayburn, H., Reddy, S., Friedel, C., Ruley, H.E., 1992. Selective disruption of genes expressed in totipotent embryonal stem cells. Genes Dev. 6, 919–927.

Wagner, K.U., Wall, R.J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P.A., Henninghausen, L., 1997. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Research 25(21), 4323–4330.

Wang, Y., Schnegelsberg, P.N., Dausman, J., Jaenisch, R., 1996. Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. Nature 379, 823–825.

Wang, Y., DeMayo, F.J., Tsai, S.Y., O'Malley, B.W., 1997. Ligand-inducible and liver-specific target gene expression in transgenic mice. Nature Biotechnol. 15, 239–243.

Wehner, J.M., Silva, A., 1996. Importance of strain differences in evaluation of learning and memory processes in null mutants. Ment. Retard. Dev. Disab. Res Rev. 2, 243–248.

Weissmann, C., Weber, H., Taniguchi, T., Muller, W., Meyer, F., 1979. Reversed genetics: a new approach to the elucidation of structure–function relationship. Ciba Foundation Symposium, 47–61.

Wilson, R.W., Ballantyne, C.M., Smith, C.W., Montgomery, C., Bradley, A., O'Brien, W.E., Beaudet, A.L., 1993. Gene targeting yields a CD18-mutant mouse for study of inflammation. J. Immunol. 151, 1571–1578.

Wolfer, D.P., Muller, U., Stagliar, M., Lipp, H.P., 1997. Assessing the effects of the 129/Sv genetic background on swimming navigation learning in transgenic mutants: a study using mice with a modified beta-amyloid precursor protein gene. Brain Res. 771, 1–13.

Wu, C.L., Melton, D.W., 1993. Production of a model for Lesch-Nyhan syndrome in hypoxanthine phosphoribosyltransferase-deficient mice. Nature Genet. 3, 235–240.

Yagi, T., Ikawa, Y., Yoshida, K., Shigetani, Y., Takeda, N., Mabuchi, I., Yamamoto, T., Aizawa, S., 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. Proc. Natl. Acad. Sci. USA 87, 9918–9992.

You, Y., Bergstrom, R., Klemm, M., Lederman, B., Nelson, H., Ticknor, C., Jaenisch, R., Schimenti, J., 1997. Chromosomal deletion complexes in mice by radiation of embryonic stem cells. Nature Genet. 15, 285–288.

Zhang, Y., Riesterer, C., Ayrall, A.M., Sablitzky, F., Littlewood, T.D., Reth, M., 1996. Inducible site-directed recombination in mouse embryonic stem cells. Nucleic Acids Res. 24, 543–548.

Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., 1995. beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81, 525–531.

Zou, Y.R., Muller, W., Gu, H., Rajewsky, K., 1994. Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. Curr. Biol. 4, 1099–1203.

Zinyk, D.L., Mercer, E.H., Harris, E., Anderson, D.J., Joyner, A.L., 1998. Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr. Biol. 8, 665–668.