[Frontiers in Bioscience 11, 1108-1136, January 1, 2006]

Mammalian genome targeting using site-specific recombinases

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1. ABSTRACT

Nowadays, a wide array of procedures in mouse technology has been made available to researchers in order to establish valuable models for the study of gene function. The efficiency of gene transfer and gene targeting as methods for producing genetic changes in mice, in addition to continuous advances in molecular biology tools, has converted the mouse into the major experimental model for the study of mammalian physiology. In recent years, the emergence of site-specific recombinases as tools to engineer mammalian genomes has opened new avenues into the design of genetically modified mouse models. The original Cre and FLP recombinases have demonstrated their utility in developing conditional gene targeting, and now other analogous recombinases are also ready to be used, in the same way or in combined strategies, to achieve more sophisticated experimental schemes for addressing complex biological questions. The properties of site-specific recombinases in combination with other biotechnological tools (tet on/off system, siRNA mediated gene silencing, fluorescent proteins, *et al.*) make them useful instruments to induce precise mutations in specific cells or tissues in a time-controlled manner. This ability can be applied in functional genomics in several ways: from conditional and inducible gene targeting to controlled expression of transgenes and recombination-mediated cassette exchange in mouse models for the study of development or disease phenotypes. This review focuses on the use of site-specific recombinases for mouse genome manipulation. A historical perspective of site-specific recombinases is considered and a number of strategies for achieving inducible or conditional genomic manipulations are contemplated in the context of current techniques for producing genetically modified mice. Finally, several model generation approaches from recent examples in the literature are revised.

2. INTRODUCTION

Recent advances in human genome sequencing and annotation have provided a great wealth of accurate information on gene structure and variability. These data are available to be exploited in the investigation of a wide diversity of biological processes, such as disease physiology and developmental mechanisms, but the function of most human genes has yet to be established.

Various methodologies can be used to determine a particular gene function, and one of the most rewarding is based on the use of model organisms. Among the different existing model organisms, the mouse has become the most successfully used, due to the ease of breeding, the animal's largely well-characterized genome (1), and, above all, the technical ease of introducing modifications into its genome. This is a key point of functional genomics, which emphasizes the analysis of a gene function at the level of the whole organism. Moreover, the current genomic synteny between mouse and human genomes as well as their shared physiological characteristics allow for the cautious extrapolation of findings from mouse models to human.

Phenotype driven approaches for discovering gene function such as N-ethyl N-nitrosourea (ENU) mutagenesis (2,3) and gene trapping (4,5) can take advantage of mouse genome knowledge in order to match straightforwardly a mutant phenotypic trait so as to induce genome changes, and thus establish the function of the affected genes. Consideration of these topics is, however, beyond the aim of the present review and the reader is referred to the reviews cited as bibliographic references (2-5).

Gene driven approaches extract all the available information on genome sequence to produce designed modifications regarding a candidate gene, which may permit the inference of its function. Progress in mouse embryo and murine embryonic stem cell manipulation, together with advances in molecular biology techniques, have allowed for the development of highly effective protocols for the production of genetically modified mice at a reasonable economic cost. A careful design of mouse genomic loci modifications has led to the generation of models for the study of gene function.

The basic approaches to gain insights into the function of a gene of interest consists in achieving a misexpression of the coded protein (either overexpression or expression at the wrong time or location), or in cancelling its activity by disruption of the coding gene. These two kinds of manipulation can be easily brought about by conventional gene transfer and gene targeting strategies, respectively, as means to introduce modifications into the genome that will affect all the cells of the organism, and whose effects may become evident from the earliest developmental stages.

However, due to the effect on the entire organism that this kind of manipulation may have, there are intrinsic limitations that can impair their application in the analysis of a number of biological questions. Such is the case of the appearance of lethal phenotypes during embryonic development that would preclude a postnatal study, or the regulation of genes acting in network that could compensate for the lack of a specific gene and therefore would give rise to a normal phenotype. There is also an unavoidable limitation if the study of the gene function should be restricted to a specific tissue or cell type, because these approaches produce a widespread modification that can affect interrelated physiological systems, thus leading to phenotypes that are difficult to interpret.

Therefore, novel approaches have been developed in order to deal with complex biological questions, including the possibility of inactivating genes in a spatial and temporally controlled way, also referred to as conditional gene targeting. The properties of site-specific recombinases have made them ideal tools to bring about modifications of the genome in specific tissues and at specific moments throughout the development or postnatal life of mice, allowing for the setting up of novel strategies to study gene function.

This review will focus on the use of site-specific recombinases for the development of genetically modified mouse models for the study of gene function in relation to disease and development. A description of recombinase properties will be made, and we will also examine how the mechanism of action of these enzymes allows for precise and controlled modification of a somatic cell genome, providing ways to test hypotheses related to gene function. Finally, several strategies for model generation will be considered. The reader is encouraged to examine some recent reviews that could expand upon the subject treated here (6-15).

3. SITE-SPECIFIC RECOMBINASES

3.1. Cre and FLP recombinases

The P1 bacteriophage Cre recombinase recognizes and mediates site-specific recombination between DNA sequences named loxP (locus of crossover (x) in P1). A loxP sequence expands 34 base pairs (bp) and consists of two 13 bp palindromic sequences flanking an 8 bp non-symmetrical central sequence that defines the orientation for the loxP element. Cre recombinase plays an essential role as resolvase in the P1 bacteriophage replication cycle by cutting and rejoining the duplicated genome of the phage into two similar particles (16).

Cre recombinase is a 343 aminoacid (aa)/38 kDa protein that works as a tetrameric complex with no need for cofactors. The mechanism of recombination passes from the initial exchange of two DNA strands to form an intermediate Holliday junction DNA structure, which is stabilised by the tetrameric Cre complex and a second exchange of DNA strands, which resolves this structure into the excised products. Two recombinase monomers bind at the inverted repeats at each side of the central region of a recombination sequence, forming a tetrameric complex together with another two monomers bound to the

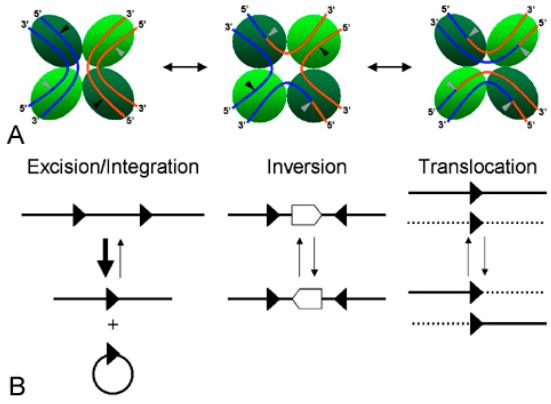

Figure 1. A. Schematic Cre-loxP site-specific recombination. Two Cre subunits bind each loxP site to form a tetrameric structure that stabilizes the synaptic complex. Two opposite subunits cleave and perform the exchange of a first pair of DNA strands to produce a Holliday junction intermediate. Afterwards, an isomerization of the DNA intermediate induces the activation of a second pair of recombinase subunits that catalyse a second cleavage and strand exchange, finishing the recombination. The loxP sites are regenerated in the reaction, becoming new substrates for Cre and making possible the reverse reaction. B. Different outcomes of a Cre-mediated site-specific recombination depending on the position and orientation of loxP sites.

Figure 2. Recombinase target sites. loxP (A) and FRT (B) sites contain 13-bp palindromic sequences (indicated by inverted arrows) flanking a 8-bp non-symmetric core sequence (indicated by an open arrow). One recombinase subunit binds each palindromic element, while the spacer sequence provides the site of strand cleavage, exchange and ligation, as well as an orientation of the whole sequence.

second recombination sequence. The DNA sequences to be recombined are trapped in the complex and adopt an exposed conformation towards the active sites of a pair of opposed recombinase subunits. Recombination is carried out in a stepwise manner through a coordinated succession of events. Nucleophilic tyrosine residues of the first recombinase subunits produce initial DNA cleavages at specific phosphodiester bonds of each trapped DNA helix. Intermediate covalent 3'-phosphotyrosine bonds are formed prior to the first strand exchange, to yield a DNA Holliday junction structure. The isomerization of the Holliday junction results in the activation of the second pair of recombinase subunits, and the subsequent nucleophilic attacks from tyrosine residues produce a second DNA double cleavage in complementary DNA strands, which is followed by the formation of novel intermediate 3'-phosphotyrosine bonds, and by a second DNA strand exchange, completing the recombination (17,18) (Figure 1A).

Cre can recombine two loxP sites if they are situated both in the same DNA strand or in different DNA strands. In the first case, if the loxP elements have the same orientation the reaction gives rise to the excision of the DNA segment flanked by the loxP sequences that remain as a circular particle, and a loxP site remains in each one of the DNA moieties; in contrast, if the loxP elements have opposing orientations the result of the recombination is the inversion of the DNA segment. Recombination between loxP sites situated in different DNA molecules produces strand exchange or translocation. Cre-mediated recombination is a bidirectional process that can lead to the integration of a DNA fragment coming from a plasmid containing a loxP sequence into a loxP site placed in a linear DNA molecule, but the recombination reaction balance is displaced toward excision rather than integration events due to thermodynamic considerations (Figure 1B).

The 2-micron yeast plasmid FLP recombinase is a 424 aa/45 kDa protein that recognizes and mediates site-specific recombination between 34 bp sequences named FRT (FLP Recombination Target). It functions in the maintenance of the 2-micron plasmid copy number in the host yeast *Saccharomyces cerevisiae* (19) and the mechanism of recombination is similar to that described for Cre recombinase (20). The FRT sequence is different from the loxP sequence, but both of them are organized in the same way with two inverted repeats surrounding a non-symmetrical sequence (Figure 2).

3.2. Recombinases in a mammalian environment

Cre and FLP recombinases have been shown to be functional when expressed in heterologous non-mammalian organisms. Both of them have been successfully used to study gene function in fruit fly (*Drosophila melanogaster*) (21,22), zebrafish (*Danio rerio*) (23), frog (*Xenopus laevis*) (24), and several vegetal species (*Arabidopsis thaliana, Oryza sativa, Zea mays, Nicotiana tabacum, Lycopersicon esculentum, Triticum aestiva*), where they have been used essentially to remove selection cassettes from inserted transgenes (25). The recombinase activity of Cre and FLP is also preserved in the mammalian environment, and particularly in mice, allowing their application as tools for experimental manipulation of mammalian cell systems.

Cre recombinase was shown to function *in vitro* in several transformed and primary mammalian cells (26-29). Soon thereafter its functionality was demonstrated *in vivo* in double transgenic mice carrying *Cre* and loxP-flanked beta-galactosidase transgenes (30), opening the way to the use of recombinases as tools for genome manipulation. Mouse embryonic stem cells are not exceptional in acting as 'playgrounds' for Cre and FLP, and this has permitted the introduction of site-specific recombination in gene targeting experiments, at first to eliminate selection cassettes from the targeted locus and

generate clean modifications, and afterwards to develop conditional gene targeting strategies.

Cre recombinase can be used to accomplish recombination in fertilized mouse eggs by transient expression mediated by injection of Cre mRNA or a closed circular Cre expression plasmid (31,32). These approaches were used to remove loxP flanked selection cassettes introduced in gene targeting experiments. The same use for FLP in oocytes has been described (33). Usually the success rate of recombination in microinjected oocytes is nearly 100%, whereas the common rates obtained when this is done in ES cell culture are 5-10%. Nevertheless, the removal of loxP flanked selection cassettes can be accomplished *in vivo* by use of early Cre expression during development in transgenic mouse strains, as discussed in Section 4.2.

It has been observed that Cre-mediated recombination frequency is somehow dependent on the genomic position of the loxP-containing locus (34). A number of factors, such as the distance between loxP sites, may affect recombination rates, but the transcriptional activity and the chromatin structure of the target locus it seems to be particularly important.

It has been shown that Cre recombinase is able to cross the cytoplasmic membrane and reach the nucleus to accomplish recombination (35). This characteristic makes it a potential alternative tool as a transducing protein but its use will be limited to *in vitro* experimentation; moreover a high concentration of Cre is needed to attain satisfactory efficiency. Cre protein uptake is dependent on the cell cycle phase yielding a reduced uptake, and a subsequently lower rate of recombination, when cells are in G1 phase (36).

Although similar or interchangeable potential uses could be envisaged for original Cre and FLP recombinases (37,38), varying recombination efficiency was shown to exist due to the dissimilar thermostability properties of Cre and FLP. While Cre's optimal activity temperature is 37ºC, for FLP it is 30ºC (39). This is the cause of low FLP-mediated recombination rates in mammalian cells, and it was a limitation in the use of this recombinase in mice until a less thermolabile engineered version of the protein was developed (see below).

The DNA-interacting character of recombinases gives rise to the possibility of causing harmful effects at the genomic level if they are not too reliable in recognizing their specific recombination sites. Despite the unlikelihood of a loxP or FRT sequence being present in a mammalian genome, the existence of active recombinase recognition sites for Cre (40) and other recombinases (41) in the mouse and human genomes have been described. These sites are capable of mediating excision with the same efficiency as canonical recombinase recognition sequences in bacterial assays (40). This may be threatening when the use of recombinases is designed to achieve a particular genomic modification while an unattended pseudorecombination sequence is nearby. Conversely, the existence of these sequences may be viewed as an opportunity to perform insertional genomic modifications mediated by recombinases.

Elevated Cre recombinase expression has been shown to be cytotoxic for *Drosophila* proliferating cells (42) and for mammalian cell lines *in vitro* (43). These problems have been circumvented by using self-excising vectors to achieve the pulsed expression of Cre recombinase and avoid high expression toxic effects. *In vivo*, the deleterious effects of Cre recombinase have also been described when expressed in round spermatids under protamine gene promoter, consisting of loxP-independent chromosome rearrangements that lead to unviable embryos (44). Therefore, it is possible that similar alterations could occur in somatic tissues without detection due to the increased difficulty of assessing somatic mutations. Hence, the possible damaging effects of unleashed recombinase activity should be regarded as a risk for some cell types and taken into account when interpreting specific phenotypes.

3.3. Further developments

Wild-type versions of Cre and FLP recombinases have been replaced by modified forms of the proteins in order to improve their performance as mammalian genome manipulation tools. In this way, Cre and FLP have become common and versatile instruments for performing site-specific recombination *in vitro* and *in vivo* in mammalian systems.

Sequence modification to adapt prokaryotic *Cre* gene to codon usage in mammals has been accomplished, resulting in enhanced Cre expression and leading to increased protein levels and a more efficient site-specific recombination. A "mammalian *Cre* recombinase gene" has been synthesized (45), and an improved Cre (iCre) has been developed (46). FLP stability in mammals has also been worked out by cycling mutagenesis in order to render it suitable for carrying out recombination with an efficiency similar to that of Cre. It has been named FLPe (47) and has been shown to work properly in ES cells and transgenic mice (48,49), although its relative activity level is only 25% of Cre activity in transient transfection cell assays, probably due to its ten-fold diminished chromatin activity compared to Cre (50).

Even though Cre recombinase is prokaryotic in origin, it has the ability to reach the nucleus. Although it was accepted that its small size might permit passive entry into the nuclear compartment through nuclear pores or following transient nuclear membrane disorganization during mitosis, it has been shown that Cre protein contains certain determinant sequences that allow for active transport into the nucleus (51). Regardless of this intrinsic capacity to enter the nucleus, successful attempts have been made to improve its efficiency in finding its way to the nucleus by fusing the SV40 large T antigen-derived nuclear localization signal (NLS) at the N-terminal position (52).

Many other modifications have been designed to alter Cre recombinase characteristics by means of exogenous sequence fusion. Among these are modifications designed to enhance its cell transducing properties by

adding to Cre small protein transducing domains such as the hydrophobic FGF (53,54) and the basic TAT peptides (53). However, it has recently been noted that unique N-terminal fusion of a polyhistidine tag and the SV40 large T antigen NLS yields the best degree of solubility and highest activity (55). Other modifications have aimed to detect Cre protein expression and the correlated recombination activity, such as fusion with enhanced green fluorescent (EGFP) protein from *Aequorea victoria* (56), or to adding an epitope tag from the herpes simplex virus allowing for antibody detection (57).

With the purpose of getting control of recombinase activity, chimeric recombinases fused to mutant ligand-binding domains (LBD) of steroid receptors have been created. These LBD are able to interact with synthetic agonists, but incapable of binding to physiologic steroids. In the absence of a synthetic agonist the binding domain interacts with the 90 kDa heat shock protein complex present in the cytoplasm, resulting in impaired recombinase translocation into the nucleus and decreased activity because of steric hindrance. Conversely, the ligand unbound domain does not interact with cytoplasmic proteins and recombination is free to occur as long as cytoplasmic sequestering does not take place. This strategy has been used to activate nuclear factors and oncoproteins (58).

Initial efforts to achieve hormonal control of recombinase activity demonstrated the viability of this approach *in vitro* by using LBD of human estrogen receptor (ER) fused to Cre (59), and human mutant ER (G400V), human androgen receptor or glucocorticoid receptor fused to FLP (60). The use of mutant ER ligand-binding domains that are unable to be activated by their natural ligands but which may be activated by synthetic agonists like 4-hydroxy-tamoxifen (4-OHT) is a key point in attaining tight control, since leaky activity caused by endogenous ligand binding may lead to undesired recombination. Various Cre-ER fusion proteins take advantage of this, such as Cre-ER(T), which carries the mutation G521R (61) and is activated in a similar way by 4-OHT and the antiestrogen ICI 182,780, Cre-ER(T1), which carries the mutations G400V/M539A/L540A and is activated only by 4-OHT, and Cre-ER(T2), which carries the mutations G400V/M543A/L544A and is activated exclusively by ICI 182,780 (62). In order to achieve tighter control of Cre recombinase activity, two ER(T2) domains have been added on to both extremities of iCre (ERiCreER) (63). The murine estrogen receptor (Mer) carrying the mutation G525R has also been used, giving rise to Cre-Mer and Mer-Cre-Mer fusion proteins (64). All of these fusion Cre recombinases are insensitive to 17 beta-estradiol and have been shown to work properly *in vivo* when 4-OHT is exogenously administered (61,65,66). FLPe has also been fused to the ER(T2) domain allowing for hormone control of FLP recombination *in vivo* (67).

The *in vitro* activity of chimeric Cre recombinase fused to ER mutant LBD is similar to wild-type Cre recombinase when the synthetic ligand is present, but in the absence of ligand or with 17-beta estradiol, a background activity as high as 5% is observed (62). A possible explanation for this residual activity may be that proteolysis of the fusion protein separates the regulatory subunit from the enzymatic one, permitting a low level of uncontrolled recombination. Despite the observed *in vitro* background activity for tamoxifen-inducible Cre fusion proteins, no recombination is detected *in vivo* in the absence of 4-OHT (34, 63).

Another mutant hormone binding domain successfully used to produce chimeric inducible Cre is the progesterone receptor (PR) LBD. A carboxy-terminal truncated form of PR (hPR891) responsive to the synthetic steroid RU486 but not to progesterone (68) was fused to Cre carrying an NLS resulting in a tightly controlled induction ability (69). Cre-PR1 chimeric protein behaves as well as the well-established Cre-ER fusion proteins with a reduced basal activity (70) and has been used *in vivo* (70,71). The main concerns with this system are the antiprogesterone properties of RU486, which cause infertility (72), and other side effects resulting from antiglucocorticoid activity (73), since mice must be treated with daily injections of 2.5 mg RU486 to attain the highest recombinase activity *in vivo*.

Recently, other LBDs have also been demonstrated to be helpful in induction of recombinase activity *in vitro*. This is the case with a modified androgen receptor LBD from the LNCap prostate cell cancer that has been fused to Cre recombinase, which is induced to the same extent both by androgen agonists (mibolerone, DHT, 17-beta estradiol) and the antiandrogen OH-flutamide (74).

Cre- and FLP-mediated recombination relies on the specificity of site recognition, and this feature has been exploited to expand the uses of site-specific recombination in ways that will be treated later. Mutant lox sequences have been shown to be functional with no cross-reaction with wild-type loxP sites. Mutations in the spacer region produce non-compatible sites that are unable of recombine with wild-type loxP. This is the case with the lox511 (75), lox2272, and lox5171 sequences (76,77), although they are capable of loose recombination with wild-type loxP to a certain degree. On the other hand, mutations in the inverted repeats usually can drive recombination, but the resulting lox sequence after excision is completely inactive; this is the case with the lox71 and lox66 sequences (78). Genetic screenings in search of mutant spacer lox sites have yielded several totally non-compatible sequences (loxm2, loxm3, loxm7, loxm11) that can be useful in applications as recombinase mediated cassette integration (79).

Following this reasoning pathway, an alternative approach is to produce mutated recombinase versions with a shifted affinity toward non-canonical site-specific recombination sequences. The technical methodology of directed molecular evolution has been used to obtain Cre mutants with a high affinity for a sequence chosen from human chromosome 22 termed loxH yet insensitive to wild-type loxP sequences (80). Similarly evolved FLP variants have been obtained either with a completely altered sequence specificity (81) or capable of targeted recombination as heterodimeric partners (82).

Table 1. Summary of recombinase features

	Host	Site denomination	Site length	Type	Activity in mammals			References
					Episomal/Cell lines	Chromosomal/ Cell lines	Mouse	
Cre recombinase	E. coli (phage P1)	loxP	34 bp	Tyrosine	yes	yes	yes	16, 26, 27, 31
FLP recombinase	*S. cerevisiae*	FRT	34 bp	Tyrosine	yes	yes	yes	19, 33, 39
Phage phi 31 integrase	*Streptomyces* spp.	attB/attP	313 bp/ 224bp	Serine	yes	yes	yes	41, 50, 87-91
Phage R4 integrase	*Streptomyces* spp.	attB/attP	50 bp/64 bp[1]	Serine	yes	yes	nd	92-93
Phage TP901-1 integrase	*L. lactis cremoris*	attB/attP	31 bp/50 bp[1]	Serine	yes	nd	nd	94
Phage lambda integrase mutants	*E. coli*	attB/attP	45 bp/245 bp	Serine	yes	yes	nd	95-97
Phage HK022 integrase	*E. coli*	attB/attP	21 bp/234 bp[1]	Serine	yes	nd	nd	98
beta-recombinase	*S. pyogenes*	six	~90 bp	Tyrosine	yes	yes	nd	99-103
R recombinase	*Z. rouxii*	RS	31 bp	Tyrosine	nd	nd	nd	104-107
gamma-delta resolvase mutants	*E. coli (Tn1000)*	res	~115 bp	Tyrosine	yes	nd	nd	108-109
Dre recombinase	*E.coli*	rox	32 bp	Tyrosine	yes	nd	nd	110
phi Rv1 integrase	*M. tuberculosis*	attB/attP	-	Serine	nd	nd	nd	111

nd: not described; [1]: minimal requirement

An alpha complementation for Cre recombinase activity, similar to that of beta-galactosidase enzyme, has been assayed. In this approach the Cre enzyme is split into two independent polypeptides that are inactive on their own, but when coexpressed they can associate and show an *in vitro* recombinase activity of approximately 30% of that observed for wildtype Cre recombinase (83). Further sophistication in this direction is a rapamycin-induced complementation of two engineered inactive fragments of Cre recombinase fused to FKBP12 and FRB domains, respectively, showing very low background activity in the absence of ligand (84). This idea can be applied *in vivo* to achieve a highly specific recombination pattern by expressing the two pieces under the control of different promoters.

3.4. Novel recombinases

Cre and FLP belong to the lambda integrase family of recombinases, which includes over 100 members by virtue of sequence similarity and their sharing of a similar mechanism of action, which uses a tyrosine as the catalytic residue (85). However, this is not the only family of site-specific recombinases; the resolvases/invertases use a different mechanism to cut and rejoin duplex DNA molecules, and the active site is occupied by a serine residue. While tyrosine recombinases do not usually need cofactors to catalyse DNA cleavage and strand exchange, most serine recombinases do (86).

Integrases are present in archaea, eubacteria and yeast, where they carry out diverse functions, such as integrative and excisive recombination of viral and plasmid DNA into and out of the host chromosome, conjugative transposition, resolution of catenated DNA circles and regulation of plasmid copy number. Irrespective of the function they fulfil *in vivo*, the common action mechanisms based on recognizing specific DNA sequences and catalysing DNA strand cut and paste make them potential tools to be used in like manner to Cre and FLP recombinases.

Several integrases have shown their suitability to join the currently limited array comprised of Cre and FLP recombinases. The availability of novel exploitable site-specific recombinases would allow the formulation of more sophisticated experimental designs to deal with biological complexity. In the following paragraphs a number of integrases/recombinases that have shown promising features for use as precise genome engineering tools are considered. The basic features of these recombinases are summarized in Table 1.

PhiC31 integrase is a phage serine integrase that mediates unidirectional site-specific recombination between attP sequences present in the phage genome and attB sequences present in the host bacterial genome (*Streptomyces* spp.). This integrase does not need cofactors to catalyse the integration event, generating two different sites, attL and attR, which are not substrates for the integrase (87). This integrase has been shown to mediate site-specific integration into a genomic endogenous site in human cells (88). It has also been fused to an NLS in C-terminal position to enhance its shuffling into the nucleus, achieving a level of recombinase activity comparable to Cre and utility as a chromosomal deletion tool, by placing an attB and attP site at both sides of the sequence to be removed (50). Recently, PhiC31 integrase has also proven to be useful in site-specific cassette exchange in transgenic mice by placing plasmids bearing attB sequences into attP sequences (41,89). However, when designing insertional strategies using attB sequences, one should be aware of the existence of endogenous pseudo-attP sites in mammalian genomes that could mediate undesired results (90). In order to improve frequency integration into particular pseudo-attP sites, a directed evolution scheme has been used to obtain mutant PhiC31 integrases that will be useful to modify mammalian genomes (91).

Phage R4 integrase is a 469-aminoacid protein also termed *sre* (for site-specific recombinase). It is actually a DNA resolvase-DNA invertase which mediates excision of the prophage genome from the host *Streptomycces* spp., and it can additionally mediate integration of the R4 phage genome into the *Streptomycces parvulus* chromosome (92). *In vitro* evidence has been produced that this integrase is functional in human cells, where it performs the precise recombination between extrachromosomal R4 attB and attP sites, as well as between integration sites randomly placed in the human genome (93).

The phage TP901-1 infects *Lactococcus lactis* subsp. *Cremoris*. It carries out integration by the action of a serine integrase which has been proven to promote efficient intramolecular recombination on episomal DNA in mammalian cells through minimal attB and attP sequences previously defined in an *Escherichia coli* system (94). To date no evidence has been presented to the effect that this enzyme can achieve recombination in a chromosomal framework, but its stability and the kinetics of catalysed reaction at 37ºC make it a potential new tool for genome engineering.

Lambda phage integrase (Int) is a serine integrase that carries out both the integration and the excision of the phage genome in and out of the *Escherichia coli* genome. Although it requires the presence of additional protein cofactors to perform recombination between attB/attP specific sequences, there are mutants capable of mediating recombination in the absence of any auxiliary factor, such as Int-h (E174) and Int-h/218 (E174K/E218K) (95). These mutant integrases are able to catalyse site-specific intramolecular integrative recombination in human cells, either in transient cotransfection experiments or in reporter human cell lines (96), and also to eliminate selection cassettes in murine ES cells (97), demonstrating the utility to join Cre and FLP recombinases as genome engineering tools. The closely related integrase of bacteriophage HK022 has been shown to promote site-specific recombination in reporter plasmids inside COS-1 cells without the need for the natural cofactors in *Escherichia coli*, implying that in the mammalian environment endogenous proteins can contribute to its activity (98).

The beta recombinase is encoded by the *Streptococcus pyogenes* plasmid pSM19035, and it is involved in the resolution of plasmid multimers and in DNA inversion between specific recombination sequences (six sites) depending on their relative orientation (99,100), with an efficiency comparable to Cre and FLP recombinases. Although protein host cofactors are necessary for the recombinase activity of beta-recombinase (101), it is proficient in recombination in mammalian cells, both in extrachromosomal sequences and in six sites inserted in various chromosomes (102). It is thought that eukaryotic proteins may act as adjuvant factors to fully develop their activity; one of these is the HMG1 chromatin-associated protein (101,103).

The site-specific recombinase R, encoded by the yeast *Zygossacharomyzes rouxii* plasmid pSR1 is able to catalyse recombination between RS sequences (104,105). Its resemblance to FLP recombinase is great, and the mechanism follows the canonical steps established for FLP and Cre (106). However, no reports on the use of this enzyme in mammalian systems have been published even though it has been used in plants (107). Probably due to its yeast origin, the stability of the protein in a mammalian context is not reliable enough to provide good recombination frequencies.

The gamma-delta resolvase encoded by the transposon Tn1000 is a resolvase/invertase of the tyrosine family. It resolves the DNA structure generated during transposition by recombination between res sequences in a conservative manner, in the same way as Cre and FLP, but with the requirement of negative supercoiling to accomplish full recombination (108). Two mutant forms of gamma-delta resolvase (E124Q and E102Y/E124Q) are able to produce recombination between res sequences in relaxed DNA and have worked in CHO cells assays on reporter plasmids with an efficiency equivalent to that observed for Cre (109).

Recently a highly homologous Cre recombinase has been found by comparison of the pac-c1 region of P1-related phages. It was named Dre, and it recognizes specific sequences different from loxP that are designated as rox sequences (110). This new recombinase catalyses both integrative and excisive recombination in mammalian cells without the participation of other protein factors. Further characterization of Dre recombinase will surely reveal a valuable genome engineering tool.

Finally, it may be worth bearing in mind that original knowledge is actually being generated on recombinase and integrase mechanisms, such as the case of phiRv1 serine integrase from *Mycobacterium tuberculosis* (111), which could eventually be used to develop novel tools for genome manipulation.

4. TECHNOLOGY TO MANIPULATE THE MOUSE GENOME

4.1. Gene transfer

The gene transfer methodology, also known as insertional transgenesis, relies on the microinjection of linearized DNA engineered fragments (the transgene) into the pronuclei of fertilized eggs obtained from donor females which are then reimplanted in acceptor pseudopregnant females that will give birth to pups whose genome will have eventually incorporated the injected DNA fragment (Figure 3A). The genomic modification will be present in all the derived cells from the fertilized egg and, therefore, it will be transmitted to the offspring of the resulting animals (112).

Virtually any mice strain can be used to obtain a fertilized egg to be microinjected, allowing the introduction of transgenes into well-determined genetic backgrounds. However, the poor reproductive performance of most inbred mice strains has led to the routine use of F2 hybrid zygotes derived from F1 hybrids from common inbred strains, unless the genetic background is critical for the model (113).

The DNA fragment containing the transgene is usually incorporated only in one place into the cell genome at randomly produced double-strand breaks and usually not as a single piece but rather as a concatenation of multiple head-to-tail arranged repeats (114). Occasionally, transgene integration can take place at more than one site if insertion happens at a two-cell or later stage embryo, giving raise to mosaic animals and potentially to non-mendelian transmission. The randomness of the insertion point and the

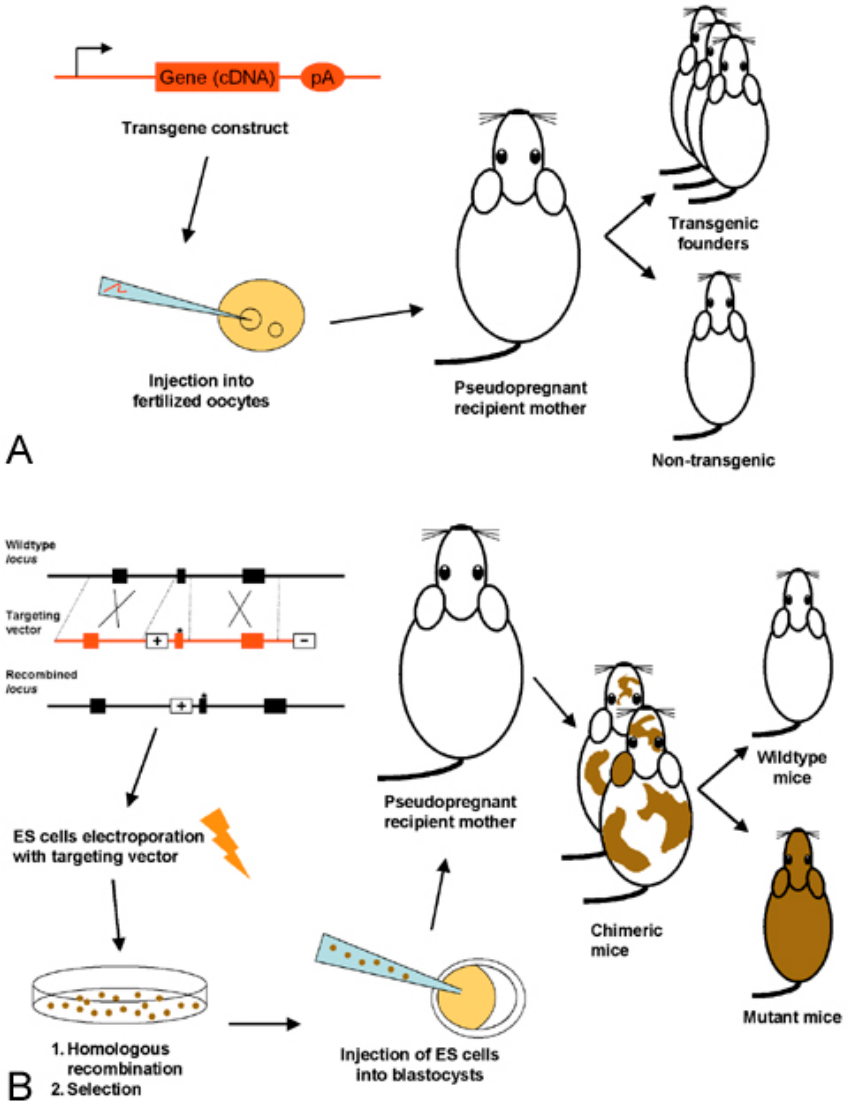

Figure 3. A. Outline of standard gene transfer procedure: a transgene construct is injected into fertilized oocytes that subsequently are implanted into pseudopregnant recipient mothers. Offspring must be screened for the presence and expression pattern of the transgene. B. Outline of standard gene targeting procedure: a targeting vector is electroporated into ES cells and *in vitro* selection is carried out to isolate homologously recombined ES clones. These ES cells are microinjected into blastocysts and implanted in pseudopregnant recipient mothers. Chimeric mice are generated and, if germline transmission has been successful, their offspring will carry the targeted mutation.

transgene copy number are crucial for the expression level of the foreign DNA in the host genome. The intensity of transgene expression should be proportional to the integrated copy number, but genomic position effects modulate the output and can even produce silencing or altered patterns of expression in different tissues. In this way, each injected egg may develop into a founder animal with a different expression level of the transgene, or even a different tissue pattern expression, as a consequence of the genomic context in which the transgene has been inserted. Another significant risk with this methodology is the possibility that the insertion point may disrupt an endogenous gene, resulting in a mutant phenotype not produced by the transgene expression. However, fortuitous inactivation of endogenous genes by transgene insertion has sometimes led to the determination of important gene functions (115) and to the generation of useful models (116).

The previous considerations make it indispensable that there be characterization of several founder lines of mice in order to discard spurious effects of site insertion and to analyse the expression level of the transgene. The overall success rate of the whole pocedure, from egg microinjection to transgene carrier newborn, ranges from 5-10%, yielding from 2 to 4 founder lines in a

typical experiment. Recently, a proprietary technology called Safe Transgenesis™ was developed that relies on *in vitro* preselection of founder characteristics; and claims for founder generation rates over 10 founder lines per standard experiment (http://www.genoway.com/). An alternative to classical microinjection procedures for transgenic generation is the use of lentiviral vectors, which have shown a high efficiency and versatility in transducing early embryos and embryonic ES cells (117).

The simplest composition of a transgene is usually a more or less tissue-specific promoter driving the expression from cDNA of the gene of interest and a polyadenilation signal sequence to promote proper transcriptional termination and polyadenilation. Nevertheless, this minimal configuration for a transgene does not guarantee a good level of expression, and a number of elements can be incorporated into the construct to improve its performance *in vivo*. The use of a consensus Kozak sequence, a short stretch of 5' UTR and intronic sequences, can ensure appropriate translation, and the inclusion of chromatin organising elements, such as locus control regions (LCR) and matrix/scaffold attachment regions (MAR/SAR), may circumvent positional effects by providing open chromatin structures around the transgene. But the efficacy of this is not complete (118). A different element that as been used in transgene construction is internal ribosomal entry sequences (IRES), which allow for the expression of two proteins from single mRNA species (119).

The use of BAC vectors in transgenesis was initiated as a complementary approach to positional cloning by designing complementation experiments in mutant mouse strains (120). Afterwards it was shown that transgene expression from large vectors was position-independent and copy number-dependent, making this a desirable way to achieve correct patterns of expression. The large genome region contained in a BAC usually contains all the required elements that are implicated in tissue specificity gene expression (121,122). The main disadvantage associated with BAC usage for transgenesis has been the inherent need for careful manipulation as well as the difficult modification by conventional restriction-based methods in order to introduce, for example, reporter genes or targeted mutations.

Nowadays, however, recombinogenic technology allows for easy manipulation of BACs and increases the number of alternatives when designing transgenic experiments. This technique is based on in vivo recombination between a BAC and a linear DNA fragment containing the replacing sequence with flanking homology arms as short as only 50 bp in specific in *E. coli* strains suitable to host BACs. Homologous recombination functions are provided by transient expression of prophage-derived proteins that carry out recombination. Two efficient systems have been described to modify BACs into host bacteria, both of them using proteins derived from prophages. The RecET system uses an arabinose inducible expression plasmid encoding the proteins involved in recombination (123,124), and the Red system uses an engineered *E. coli* strain that carries a defective lambda prophage, from which expression of the proteins required for recombination is attained by a temperature inducible system (125,126). These recombination-mediated genetic engineering methods can be used to replace genes within BAC vectors with mutated versions, or with reporter or recombinase coding genes, offering novel possibilities for generating transgenic mice (124,127).

One of the most recent incorporations to the transgenic technology is the use of RNA interference (RNAi) tools to achieve knockout or knockdown of specific genes. This approach is an easier and faster alternative to homologous recombination-based gene knockout (see below), if complete inactivation of the gene of interest is not necessary. The use of sequence-specific silencing of gene function may constitute a versatile instrument with its possibility of generating graded hypomorphic phenotypes, and even of silencing restricted alternative splicing species.

RNAi is an evolutionarily conserved mechanism to inhibit gene expression at a posttranscriptional level mediated by short pieces of double stranded RNA (small interference RNA: siRNA) (128). The siRNAs have 21-23 nucleotides (nt) with characteristic 2-3 nt 3' overhanging ends that resemble the products of degradation of long double strand RNA by RNAse III (129). siRNAs have been shown to work in mammalian cells (130,131) and this has provided a new and powerful tool for accomplishing gene inactivation in a specific way. Early use of siRNA relied on its administration to cells by classic transfection methods, until the development of plasmidic vectors, with which the expression of short hairpin siRNA precursors is driven by promoters of an RNA polymerase III (U6 or H1 promoter in both human and mouse versions) (132-134). Short hairpin RNAs designed to interfere with specific genes have been used to generate transgenic mice, resulting in highly reduced expression of the targeted endogenous genes (135-140). This gene silencing or knocking-down approach represents a fast procedure for evaluating down-regulation of a gene of interest.

4.2. Gene targeting

The gene targeting methodology enables the precise replacement of genomic segments by engineered DNA sequences carrying altered versions of the gene that causes its inactivation: the gene is knocked out. Alternatively, this procedure can be applied to induce modifications in the rate of expression of endogenous genes or to introduce subtle mutations. Homologous recombination (HR) is the underlying process for this methodology, and it is accomplished *in vitro* in embryonic stem (ES) cells, which are subsequently introduced into blastocysts from donor females and reimplanted in pseudopregnant acceptor females (141). ES cells introduced in the blastocyst will eventually be incorporated into the developing organism tissues, yielding a chimeric animal. If the ES cells are able to colonize the germ line of chimeric mice, their offspring will have the same genetic background of ES cells, except for the engineered mutation (Figure 3B).

Designing vectors for HR requires the inclusion of homology arms surrounding the gene region to be

replaced and the use of positive and negative selection cassettes to increase the likelihood of ES cell clones in which HR is right. Targeting vectors are delivered by electroporation into ES cells as linearized fragments, since linear DNA molecules are a better substrate for HR (142). The length of the homology arms usually encompasses several kb at either side of the genomic segment carrying the targeting modification. The utilization of DNA isogenic with the ES cell line that is going to be used increases the probability of correct HR events. It is also widely accepted that the larger the homology arms, the easier it is to achieve a legitime HR, but correct HR have been obtained with homology arms of less than 1 kb (143). On the other hand, the use of homology arms longer than 8 kb does not improve the efficiency of HR (144). Therefore, although longer homology arms tend to give better results, a good HR depends rather on the locus or targeting gene. The presence of heterologous DNA sequences at the extremities of the homology arms inhibits the process of HR to different degrees (145). Usually, replacement vectors are designed with a long and a short homology arm, in order to use PCR-based approaches for screening, although Southern hybridization must always be used for full confirmation.

The principal technical limitation of gene targeting is that random integration occurs at a very high frequency compared to HR. The inclusion of positive selection cassettes enables the enrichment in cells that have integrated the targeting vector, either at the target locus or at any random position of the genome, and the use of negative selection cassettes eliminates the cells that have undergone random integration of the whole targeting vector. Positive selection cassettes usually consist of an antibiotic resistance gene (G418, hygromycin, puromycin) under the control of an active promoter, such as that of phosphoglycerol kinase (PGK), and they carry a functional poliadenilation sequence at 3' position. Negative selection cassettes are genes that metabolise harmless molecules (ganciclovir) added to the cell culture media into toxic substances that kill the expressing cells, like the herpes simplex virus thymidine kinase gene (HSV-TK), or genes that produce a toxic peptide such as the diphtheria toxin fragment A (DTA). A special case is that of the hypoxantine phosphoribosyl transferase (Hprt), which can support positive selection by HAT (hypoxanthine/aminopterine/thymidine) and negative selection by 6-thioguanine. Since mammalian cells endogenously express the Hprt gene, this system is only useful in ES cells deficient in the *Hprt* gene (146). Other alternatives have been envisaged to select ES cells by high throughput cell sorting techniques using EGFP in the positive selection cassette.

Insertional gene targeting mutagenesis has been shown to be effective in disrupting specific genes due to partial duplication of exons and inclusion of plasmid backbone sequences. Otherwise, the use of simple replacement vectors for gene targeting allowed the disruption of genes by insertion of positive selection cassettes into exonic regions. Later on, this approach was used to introduce subtle modifications into genes, but leaving behind the selection cassette placed in intronic regions (147). The pitfall of this methodology is that the selection cassette can interfere in normal splicing and expression of the targeted gene or adjacent ones. Two-step approaches have been used to avoid the introduction of selection cassettes. The "hit-and-run" method uses an insertion vector carrying both a positive and a negative selection cassette, and it produces a partial duplication of the targeted locus in the first step of HR. Afterwards, intrachromosomal HR resolution at the partially duplicated locus is enriched by negative selection, resulting in a modified locus without insertion of foreign DNA fragments (148). In the "tag-and-exchange" method, two sequential targeting steps are performed with two independent vectors, the first one replacing a segment of the target locus with positive and negative selection cassettes, and the second one replacing these cassettes with a mutated version of the original locus in negative selection conditions (149,150).

The use of site-specific recombinases allows for the elimination of selection cassettes flanked by loxP or FRT sequences, with only a loxP or FRT sequence remaining after deletion. Transient expression of Cre or FLP from plasmid vectors *in vitro* effectively removes selection cassettes in ES cells that have undergone a positive HR event (37) or those in oocytes, as described in Section 3.2. Alternatively, this can also be achieved *in vivo* by using Cre or FLP deleter mice strains; that is, mouse lines where recombinase expression starts very early in development (48,151-153). The advantage of this use of site-specific recombinases is that only replacement vectors are needed for gene targeting, contrasting with more elaborate and time-consuming two-step strategies. Moreover, the introduction of the Cre/loxP and FLP/FRT systems has expanded the possible applications of gene targeting in ES cells, permitting the production of large deletions, the introduction of small mutations or the replacement of genes by means of relatively simple strategies (154) (see below).

Several ES cell lines are available that can be used to perform HR; most of them have been derived from male embryos. This makes them more stable to mutation, and male chimeric mice are easier to breed facilitating the screening of ES cell germline colonization. The genetic background of the ES cells used to perform HR should be a concern regarding the expected phenotype, since significant differences can exist at the morphological and metabolic levels among inbred strains, and comparison is not legitimate in a number of cases. Most of the ES cell lines suitable for gene targeting are derived from 129Sv related strains, but C57BL/6 is the reference strain for most biomedical research fields. This makes it necessary to backcross mice obtained by gene targeting into the desired genetic background. After 10 generations of backcross breeding, the resulting congenic strain is 99.9% similar to the reference strain, except for the chromosomic region harbouring the targeted gene that can encompass up to 300 genes corresponding to the original strain. This fact should be contemplated when setting up the use of right control animals, in that the genes linked to the targeted locus can affect the phenotype, and the generation of valid control

animals should be carefully planned (155). Quite a few ES cell lines derived from C57BL/6 related strains have been reported, although their performance for standard gene targeting seems to be poorer than 129 ES cell derived lines. Recently, a comparison between the performances of a 129-ES cell strain (CJ7) and a C57BL/6-ES cell strain (BRUCE4) has been reported. This report shows a better efficiency of chimera generation for the 129-ES cells, but a decreased germline transmission compared to C57BL/6-ES cells (156).

One of the limiting steps of the standard gene targeting methodology is the uncertain germline transmission potential of the generated chimeras, as well as the accompanying time-consuming breeding phase. One alternative that is becoming common is the aggregation of ES cells with tetraploid embryos (157-159). This method allows the derivation of mice entirely from ES cells, significantly accelerating the timing of the generation of gene-targeted mice (160-163). Embryos at the two-cell stage are forced to fuse, yielding tetraploid cells that will only be able to generate almost exclusively extraembryonic tissues, and the aggregated ES cells coming from homologous recombination *in vitro* will be the only contributors to embryo development, resulting in a completely ES cell-derived animal.

The low efficiency of HR is the principal bottleneck of gene targeting efficiency. Although it can be partially overcome by the use of selection markers, this does not in and of itself improve the occurrence of HR events. It has been shown that HR is principally stimulated by double strand breaks (DSB) and, to a lesser extent, by single nicks at the homology arms (164). This fact has been used to enhance gene targeting in a two-step method that introduces an 18 bp I-SceI meganuclease recognition site in a first targeting of the locus of interest in order to use transient I-SceI expression during the second targeting step (165). I-SceI causes a DSB, which is repaired with the second targeting vector, increasing efficiency 100-fold. This approach can be useful if the same locus must be targeted on repeated occasions, and it allows the use of shorter homology arms in the second targeting plasmid, which facilitates vector construction.

Following this reasoning, the use of chimeric zinc-finger nucleases to stimulate gene targeting in human cells by induction of DSB at specific sequences has recently been described (166). A further development of the idea has used engineered domains to recognize precisely endogenous sequences at the locus to be targeted, obtaining targeting rates of up to 20% of the chromatids and a remarkable 7% of biallelic targeting (167). Although these attempts at obtaining nuclease-assisted HR in somatic cells have a therapeutic aim, the concept may well be applied to gene targeting experiments in mice.

4.3. Nuclear transfer

Mouse nuclear transfer, more commonly termed cloning, was first reported in 1998, when enucleated oocytes were injected with cumulus cells and full development was achieved in pseudopregnant recipient mothers (168). The main drawback of this technique is the very low yield of oocytes that are carried to term (1%) (169), which renders it a nonviable procedure for application in the same manner as transgenesis or gene targeting. Multiple variables contribute to the overall efficiency; it is essential that there be a reprogramming of the nucleus for successful cloning, and there are several technical problems associated with this, such as the cell cycle phase of the donor cells (170) and the procedure to obtain the oocytes (171). Usually, the surviving animals suffer serious phenotypic and gene expression abnormalities caused by defective epigenetic reprogramming of the donor nucleus (172,173). It has been reported that the generation of adult animals by nuclear transfer is more efficient if nuclei from ES cells are used (174,175), which makes possible a genome engineering phase preceding the transference of the nucleus.

At present, nuclear transfer technology is evolving toward greater efficiency and security, but there remain basic questions that must be addressed. When the concerns about efficiency, viability and altered gene expression patterns are resolved, nuclear transfer will be a valid alternative to generating gene targeting models for the analysis of very early developmental processes. Additionally, it may become a cost-effective strategy for generating experimental mouse models with multiple genome modifications that can be performed in ES cells *in vitro* to obtain donor nuclei, instead of carrying out complex breeding schemes.

5. STRATEGIES FOR CONDITIONAL GENE TARGETING

5.1. Generation of floxed alleles

For the sake of simplicity, we will focus in this section on Cre/loxP system applications, but the ideas presented here can be transposed to other recombinase systems, or applied with modifications to integrase systems that use non-conservative attB/attP sites.

Conditional gene targeting experiments are comprised of two elements: the floxed allele (that is, a gene allele modified by insertion of loxP sequences) to be inactivated, and the way by which recombinase activity is achieved in a spatial- and time-controlled manner. A large part of the experimental success depends on careful design of the position where the recombinase recognition sequences should be placed by gene targeting methodology in the gene to be inactivated.

The ideal and certain way to attain inactivation of a gene is to delete it in its totality by flanking the whole gene with loxP sequences, but in fact this is only possible for monoexonic or short genes, because placing loxP sites at distant genomic positions requires time-consuming procedures involving two consecutive steps of homologous recombination or the use of large BAC based vectors. This option has been used to produce large deletions in ES cells to generate germline knockout mice (176), which is not affordable in the use of conventional gene targeting vectors. Moreover, the distance between loxP sites can

affect Cre-mediated recombination efficiency, although deletions of up to 60 cM have been achieved in ES cells *in vitro* at a very low frequency (177). Hence, for conditional gene deletion *in vivo* distant loxP sequences foretell diminished recombination efficiency and poor outcomes.

Alternatively, well-chosen essential exons of the gene are usually floxed through a single homologous recombination step in such a way that, after Cre-mediated deletion, a frameshift mutation appears. If such a mutation does not cause RNA decay, it gives rise to a truncated inactive protein, which will probably be unstable. The position of lox sequences should be chosen carefully in order to not perturb gene expression. Thus, although elimination of the promoter region and the first exon containing the initiation codon would always be a desirable option, a reasonable risk for perturbation of essential transcriptional control elements in the promoter with the presence of a loxP sequence exists, which usually shifts the choice towards other initial exons of the gene to be floxed. Finally, a number of details must be taken into account to make the right selection, such as alternative splicing in the gene, the potential residual functionality of the resulting truncated protein, and the existence of exons composed of nucleotides in multiples of three.

The basic scheme for introducing loxP sites at specific locations in a specific locus was established by Gu *et al.* (178). It consists of the use of a replacement vector for gene targeting carrying the region to be floxed by a loxP site at one side and a loxP flanked positive selection cassette at the other side. In this way, after homologous recombination the targeted locus contains three loxP sites (tri-lox), two of them bordering the positive selection cassette. In a second step performed in ES cells, transient expression of Cre recombinase can lead to three possible excisions: deletion of the selection cassette, deletion of the gene floxed region, or deletion of the intervening region between the more distant loxP sites at each side of the modified locus. ES cell clones that have undergone deletion of the selection cassette, leaving behind the floxed gene, are then used to generate the mouse strain with the floxed allele (Figure 4). Variations on this approach have been envisaged leading to sequence replacement or inversion results (154).

The described procedure has been widely used, but other alternatives for *in vivo* elimination of positive selection cassettes are also possible: the cassette deletion can be achieved with the use of specific Cre transgenic strains (EIIaCre (179), MeuCre40 (180)) that produce mosaicism in the germline, that is, after crossing a tri-lox mouse mouse with one of these mice, their offspring consist of animals harbouring the different excision alternatives, among them the complete knockout and the floxed allele carrier. Another option is to use an FRT-flanked selection cassette, allowing elimination to be driven *in vitro* by ES cell transfection with an FLP-expressing vector, or *in vivo* by an FLP deleter strain (181). These strategies are subjected to the viability of mice carrying a potentially inactivated allele, but usually the presence of the selection cassette does not produce the complete annulation of the gene in which it is inserted, giving rise to useful hypomorphic phenotypes.

The use of mutant lox sequences permits the design of more elaborate floxed alleles. For example, an arrangement of inverted loxP and lox511 sites can be used to perform gene function rescue, subtle mutation introduction or sequence replacement in a conditional way (182).

5.2. Promoter-specific driven recombinase expression

The straightforward way to perform conditional gene targeting is to use transgenic Cre expressing mouse strains under the control of tissue-specific promoters. In this way, Cre expression should be limited to the tissues or cell types where the specific promoter retains full functionality. The drawbacks of transgenesis are applicable to these circumstances: positional effects can perturb the expected expression pattern and ectopic expression can occur due to the absence of regulatory elements in the limited promoter sequence employed. It is necessary to characterize the actual expression pattern by crossing the Cre transgenic strain with reporter strains, in order to indirectly define the tissues and cell types where Cre recombinase activity is present (see Section 6.3). A database of Cre transgenic strains is maintained by A. Nagy at Samuel Lunenfeld Research Institute (http://www.mshri.on.ca/nagy/).

The use of BAC transgenesis offers better control of Cre expression, as previously discussed. Recombinogenic techniques have been used to introduce *Cre* coding sequences in specific BAC vectors, resulting in tightly controlled Cre expression that resembles the substituted gene pattern of expression in tissue and cell targets (63,183-186).

If haploinsufficiency is not a concern, a knockin strategy can be applied to achieve a more accurate Cre expression. There are several reports of successful substitution of endogenous coding regions by *Cre* coding sequences using gene targeting procedures, yielding specific patterns of Cre recombinase activity in particular cell populations and tissues (187-195). An alternative is the use of knockin approaches to introduce constructs carrying an internal ribosomal entry sequence (IRES), followed by a *Cre* coding region into the 3'UTR sequence of a specific gene, in such a way that a bicistronic mRNA is produced and endogenous gene expression is preserved (196-199).

5.3. Inducible systems

The ability to control the start of recombinase activity is a key point in conditional gene targeting. The approaches to inducing recombinase activity by exogenously delivered molecular inducers have evolved into two different alternatives: control at the transcriptional level or at the posttranslational level. It should be noted that a temporal lag exists between the moment that Cre transcription is started and the actual moment when the recombinase target ceases its activity due to the consecutive processes that must take place (transcription, translation, migration into the nucleus, interaction with recognition

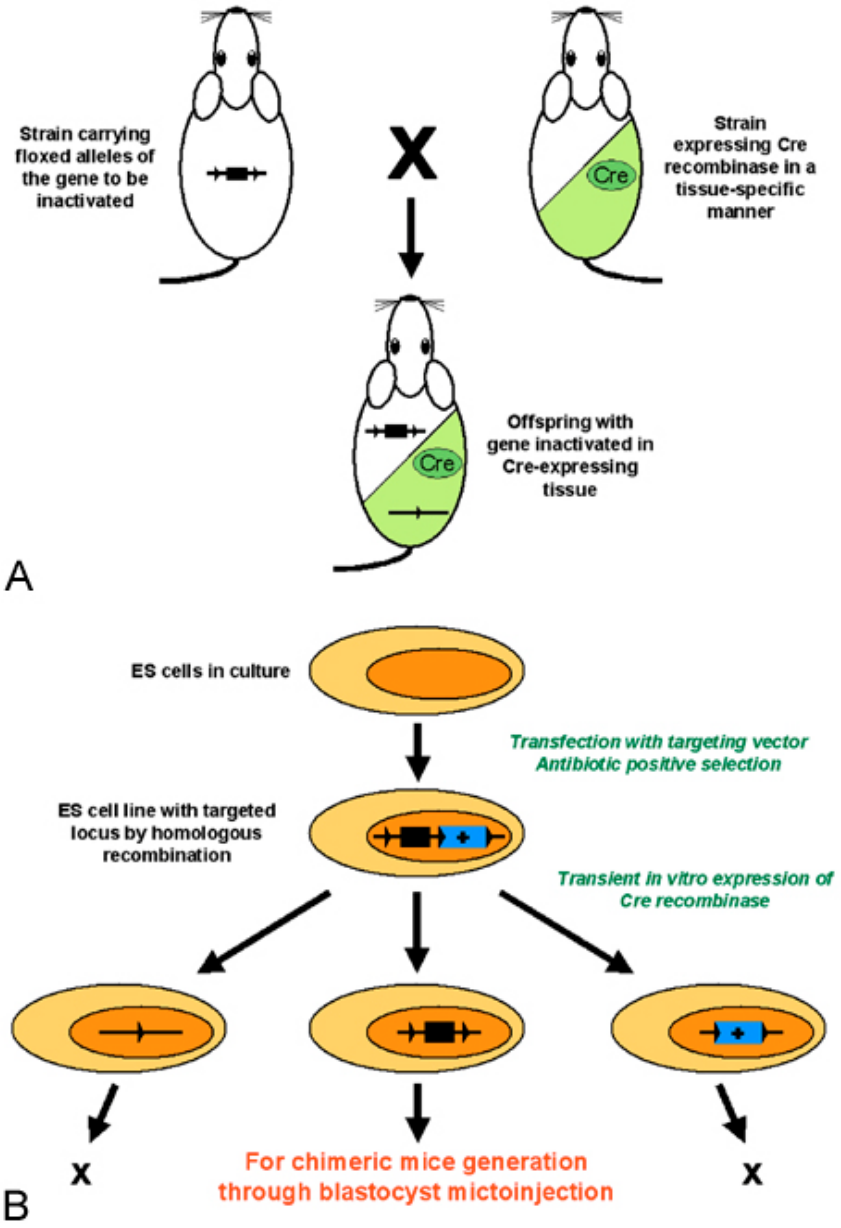

Figure 4. A. The basic scheme for a cell- or tissue-specific gene inactivation is composed of a two-step process consisting of the generation of a mouse strain carrying a floxed allele of the gene of interest, and the use of a suitable Cre transgenic strain, which provides temporal and/or spatial control over gene inactivation in the offspring obtained from the breeding of the two strains. B. One of the most commonly used strategies to produce a floxed allele passes through a tri-lox configuration in cultured ES cells attained by gene targeting procedures, followed by transient Cre expression *in vitro* to give rise to the three possible deletions and subsequent selection of ES cell clones carrying the desired floxed allele without the positive selection cassette. These cells are used to generate chimeric mice by introduction into blastocysts.

sequences, site-specific recombination in the genomic locus, decay of target transcripts/degradation of target protein) (6). The use of inducible recombinase systems adds one step related to the bioavailability of the inducer molecule in the target cells or tissues, in such a way that the lag time can be slightly drawn out if the control is at a transcriptional level, but the effects can appear comparatively earlier after inducer stimulus if posttranslational control is used.

Transcriptional control approaches are based on the use of binary systems, usually provided by two independent transgenic mouse strains, with one expressing a recombinase transgene driven by a responsive promoter

and the other a second transgenic strain for a protein effector (transactivator) under the control of a cell- or tissue-specific promoter whose activating/inactivating ability depends on the presence of inducer molecules. This kind of binary system has been widely used to control the expression of transgenes in a number of experimental models, and is discussed in detail in the accompanying review by M Mallo (200) in this issue.

The most common transcriptional control system used to regulate Cre activity is the tet on/off system, which is based in the use of tetO operator sequences of the tet operon inserted in the promoter controlling the expression of Cre recombinase, and tetracycline binding transactivators consisting of fusions of the VP16 transactivation domain from herpes simplex virus and the tetracycline repressor protein (TetR) from *Escherichia coli*. Two tetracycline-dependent transactivator versions have been developed: tTA and rtTA, which are, respectively, inactive and active when the inducer is present. The inducer molecule is doxycycline, a low-cost tetracycline analogue that effectively activates rtTA and inactivates tTA at low doses (201). Several different transcriptional control systems have been developed (200); one of them based on the use of ecdysone analogues as inducers has been applied to the regulation of FLP activity in mammalian cells *in vitro* (202). The need for double transgenic strains plus the floxed alleles to be inactivated implies complex breeding schemes that can be simplified by introducing in a single transgene a cell/tissue-specific transactivator expressing unit and the Cre expressing cassette connected by a suitably large intronic sequence, thereby preventing transcriptional interference (203).

A growing number of examples of the successful use of the tet on/off system to regulate Cre recombinase activity exists. The tTA transactivator has been used in neurons with interesting effects depending on doxycycline delivery timing (204), but most of applications have used rtTA transactivator versions to achieve regulatable Cre activity in various tissues, such as intestinal epithelium (205), liver (206) and lung (207).

Posttranslational control of Cre activity is enabled by fusion with hormone receptor ligand binding domains, as described in section 3.3. The most commonly used system employs a mutant estrogen receptor LBD, which is activated by 4-OHT delivered to the animals by intraperitoneal injection. A rising number of mouse transgenic lines expressing tamoxifen-inducible Cre is also available. Among the most recent lines reported for inducible Cre activation are the ones in which Cre activity is restricted to skeletal muscle (208), osteoblasts and odontoblasts (209), epidermis (210), liver (211,212), myelinating cells (213,214), podocytes (215), testis (216), fibroblasts (217), endothelium (218) and different epithelia (219-221). All these mouse transgenic lines will be useful to address gene function and physiological process modelling at the corresponding target cells or tissues.

Critical requirements for inducible targeting experiments are the tightly controlled regulation of Cre-ER fusion by 4-OHT at a consistent dosage and the specific expression of the transgene. However, a minimal background activity in the absence of induction is quite possible in the target cell type or tissue, as well as in other tissues (222). This background activity may be the source of confounding phenotypes when sporadic mutation in somatic cells is engineered by inducible Cre activity, or, in the case of targeting cell populations that are in the origin of cell lineages, due to possible clonal expansion of random recombination events. To date, the Cre-ER(T2) system has been shown to offer the most stringent control of uninduced recombinase activity. In addition, the specificity of the promoter region used to drive the *Cre* transgene expression is essential to reduce the background level. Moreover, external factors must be controlled, in that it has recently been reported that cross-contamination with tamoxifen in mouse-breeding facilities results in unattended high background activity (223).

The previously considered inducible systems exert their control at transcriptional and posttranslational stages. Recently, an inducible system has been described in which the control is carried out at the translational level. It is based in the use of a chimeric protein containing a specific RNA binding moiety not present in the human genome, a ribosome recruitment core (elF4G), and a farnesylation box provided by the C-terminal domain of H-ras. The chimeric protein remains sequestered in the cytoplasmic membrane if farnesylated, impairing this way its interaction with ribosomes and remaining in a non-functional status. The use of farnesyl transferase inhibitors at non-toxic doses hinders farnesylation and membrane attachment, in such a way that the chimeric protein is released to the cytoplasm and participates actively in the translation of mRNA species containing the specific sequence recognized by the RNA binding domain (224). This system could be used in combination with transcriptional control systems of recombinase transgenes, in order to decrease the leaky expression that usually produces a basal non-induced level of activity.

5.4. Virus-mediated delivery to specific cell or tissue types

The above-described conditional gene targeting with site-specific recombinases relies on the use of several components, one consisting of the floxed allele (provided by a gene targeted mouse strain) and another of the Cre-expressing transcriptional unit (provided by a transgenic mouse strain). In the case of a transcriptional inducible system, one more component should be added: the transactivator transgene. Obviously, complicated breeding schemes must be organized so as to bring together the different elements in order to achieve the inducible conditional gene targeting, particularly when homozygosity at the floxed locus is required.

An alternative to this approach is the use of viral vectors to deliver recombinases to the target tissues or cells at the chosen time. Even the known tropism of several viruses can allow for highly specific targeting toward particular cell populations. This approach can produce a rapid recombinase-mediated gene disruption without the

need for joining all the elements through mouse breeding. *In vitro* use of adenoviral vectors expressing Cre recombinase (225,226) or FLP recombinase (227) have proven to be useful in achieving site-specific recombination in transgenes. Moreover, the use of adenoviral Cre-expressing vectors in ES cells causes no alteration of germline competence, and thus can be used in early steps of floxed allele generation (228).

In vivo methods of virus delivery to different target organs and tissues include injection into the bloodstream (tail vein injection, jugular vein catheter) (229), estereotaxic injection in the brain (230-232), intratracheal injection to lungs (233), skin injection (234), liver injection (231), aortic root injection (235), and intraductal delivery to prostate (236).

One of the risks of stable Cre expression from a transgene is the potential associated toxic effect. A possible solution to this problem is the use of self-excising vectors, in which the recombinase expression unit is flanked by loxP sites, so that once a determined level of Cre activity is reached, the protein expression is stopped by deletion of the cassette. This kind of vector can achieve a burst of Cre recombinase activity strong enough to inactivate the floxed gene of interest. Several viral vectors have been generated following this approach (237,238).

Different types of viral vectors have been used to deliver Cre recombinase activity *in vitro* and *in vivo*, following their use in gene therapy experiments. For example, herpes simplex virus type 1(HSV-1) is capable of delivering DNA stretches of up to 150 kb. Although they remain as non-replicating extrachromosomal particles and are rapidly diluted in dividing cell populations, they show cytotoxic effects that have been circumvented through generation of mutants (239). Other viral vectors have more desirable characteristics for long term expression in infected cells, such as Epstein Barr derived virus, adenoassociated virus, and retroviruses, since they are capable of episomal replication or chromosomal integration. The generation of hybrid viral vectors encompassing the most appropriate characteristics could lead to widespread use of recombinase for delivery to cells and tissues (240,241).

5.5. Recombinase-activated siRNA expression

Polymerase III promoters have been used to produce transgenic mice expressing short hairpin RNAs, which have successfully yielded null or knockdown phenotypes (135-140), but the ubiquitous transcriptional activity of this kind of promoter does not allow for tissue-specific gene silencing. The Cre/loxP system has been used in the development of two strategies that permit the inducible activation of siRNAs. The first one is based on the introduction of a loxP flanked neomycin resistance gene into the loop of the short hairpin corresponding to the specific siRNA. In this configuration the transcriptional unit does not produce siRNAs, but after Cre-mediated removal of the cassette a short RNA hairpin is successfully produced with a slightly longer loop than normal, which is completely functional for driving RNAi (242,243).

The other strategy relies on the tolerance of the U6 promoter to the presence of inserted loxP sites inside. The promoter contains three essential regions: a distal sequence element (DSE), a proximal sequence element (PSE) and the TATA box. A lentiviral vector has been reported for siRNA expression from a modified U6 promoter by insertion of a lacZ cassette flanked by spacer mutant loxP sequence that resembles the U6 TATA box sequence (244). Alternatively, a U6 promoter with a loxP site substituting for a precise sequence between DSE and PSE retains its functionality and allows the introduction of a loxP flanked stop cassette (245).

The aforementioned experimental schemes have been shown to be useful *in vitro* in a variety of mammalian cell lines including ES cells. They are likely to become equally functional *in vivo,* opening the way for inducible gene knockdown in mice as well as increasing the feasibility of time- and tissue-specific control using double transgenic approaches involving tissue-specific Cre transgenic mice. Until now only one successful attempt has been reported along these lines to knock down CD8 and p53 genes with the use of lentiviral vectors with U6 TATA box regions engineered to contain an EGFP loxP flanked as stop cassette (246).

6. APPROACHES TO MODEL GENERATION

6.1. Switching on/off genes in a space- and time-controlled fashion

Activation or inactivation of transgenes can be performed in a reversible way by use of tetracycline transactivator systems (247). Alternatively, Cre recombinase is a useful tool to achieve irreversible transcriptional activation of transgenes by elimination of loxP flanked stop cassettes placed in their promoter region. These approaches are discussed in detail in the accompanying review by M Mallo (200). One application of this idea consists of activation of oncogenes to produce models of tumorigenesis (248,249).

Recently, a combinatorial system has been described that makes use of tissue-specific Cre recombinase activity to activate the transcription of an rtTA transgene inserted in the ROSA26 locus, which in turn can activate a transgene under the control of a tetracycline-responsive promoter (250). This approach enables space- and time-controlled transgene activation with tight control and provides the opportunity to test transgene activation in various tissues by use of several specific Cre strains or tamoxifen inducible Cre.

Conditional endogenous gene inactivation is the main application of Cre recombinase; the ways to accomplish that end have been treated in Section 5. The recombination event is irreversible in the cells of tissues where Cre recombinase becomes active. The advantages of this system are the possibility of bypassing early lethal phenotypes, and the circumventing of compensatory developmental phenomena in order to obtain a more accurate dissection of gene function by induced loss of its function at specific times.

However, this approach can also be used to study collateral or indirect changes induced by the loss of a particular gene function at the level of the whole organism. For example, the conditional inactivation of hepatic microsomal transfer protein impairs lipoprotein secretion in liver, resulting in a drastic lowering in lipid plasma levels. If this manipulation is performed in a hypercholesterolemic background it could provide a good model for analysing changes associated with the transition from hypercholesterolemia to hypocholesterolemia in the tissues and organs involved (251).

Particularly interesting are the options offered by the use of inducible Cre in specific tissues for the development of models related to disease physiology (252,253), organogenesis (254), and structural relationships (255,256). Functional inactivation of a particular gene provides insights not only into its specific function, but also about the functions of partners in system networks. Tissue-specific inducible Cre can also be used to assess the role of transduction pathways in particular systems by activating transgenes of key effectors in signalling cascades (257,258).

The use of two different recombinases allows the generation of models where a gene can be inactivated and activated in a sequential manner. An example of this application is the experiment performed on the leptin receptor gene, which was targeted by insertion of a neomycin resistance cassette FRT flanked into an exon, and additionally floxed around an exon coding for an essential domain involved in intracellular signal transduction. Mice with the inactive gene present a characteristic obese and diabetic phenotype, but when FLP activity eliminates the *neo* cassette, gene function is restablished and the phenotype reverts to normal. Finally, Cre-mediated deletion of the floxed allele abolishes the gene function and the obesity and diabetes phenotype reappears (259).

6.2. Chromosomal rearrangement

Sporadic chromosomal rearrangements in somatic cells, such as large deletions, inversions, or translocations, underlie almost all malignancies. Particularly, chromosome translocations are involved in the generation of fusion genes whose products acquire enhanced, unregulated, or novel properties that are selected in tumorigenic cells. Cre-mediated intermolecular recombination produces strand translocations, suggesting a way to obtain inducible chromosomal translocations. This was shown *in vitro* as a proof of principle in engineered ES cells containing loxP sequences in different chromosomes, occurring with a frequency of 1 in 1200-2400 cells expressing Cre recombinase (260,261). The frequency of interchromosomal recombination is low compared to deletions and inversions of even several cM in systems with selection towards recombination events (177). Chromosomal engineering strategies in ES cells have been proposed in order to produce models for human diseases (262), and the application of such strategies has led to the generation of mouse models for DiGeorge syndrome, produced by a large deletion in human chromosome 22, which was mimicked by Cre-mediated chromosome translocation between loxP sites located in a similar orientation in different positions in the syntenic region of mouse chromosome 16 (263).

However, the *in vivo* situation was thought to be refractory to this kind of manipulation and only possible in germline cells or early embryo. Cre-mediated Y chromosome loss was achieved by placing two inverted loxP sites in the Y chromosome and using ubiquitous Cre expression in early embryo (264). Cre-mediated targeted recombination is enhanced during the first meiotic division due to the close alignment of chromatids. This has been used to induce interchromosomal recombination between loxP sites placed at different positions in the homeotic *Hoxdl2* locus, leading to deletions and duplications of the involved genes by Cre recombinase expression in spermatozoids, and resulting in striking limb development phenotypes (264). The high frequency of such targeted exchanges *in vivo* makes targeted meiotic recombination (TAMERE) a powerful genetic tool applicable to areas in which complex genomic recombination is required.

To date, several reports have described interchromosomal Cre-mediated translocations in somatic cells of postnatal mice: the first one consists of a translocation between chromosomes 8 and 21, which fuses the *Aml1* and *Eto* genes, leading to acute myeloid leukaemia (266), and the second one uses a similar approach to achieve the fusion of the *Mll* and *Enl* genes by translocation between chromosomes 9 and 17, respectively, producing myeloid tumours (267). In both cases, the pathologic progression of the tumorigenesis is recapitulated. These are valuable models for the human counterpart diseases. Another attempt to generate Cre-mediated translocation events between the *Mll* gene and the *Af9* gene (chromosome 4) only yielded detectable rearrangement in brain tissue, highlighting the importance of Cre accessibility to the target sequences, which seemed to be favoured by the high expression rate of *Af9* gene during brain development (268).

Insertional vectors derived from lambda phage by Cre recombinase excision have been designed to perform gene disruption and to place loxP sequences at specific loci that can be used in chromosome engineering. Actually, two series of vectors have been developed that contain either puromycin or neomycin resistance genes, a partial Hprt gene sequence (*5'hprt* or *3'hprt*) next to the loxP sequence, and either the tyrosinase minigene or the K14Agouti transgene, allowing the selection of mice by coat colour. Libraries of genomic DNA from 129S5 mouse have been constructed and annotated using these two kinds of vectors, thereby allowing the quick retrieval of suitable combinations of targeting vectors to engineer large genomic changes including deletions, duplications, translocations and inversions by double round targeting in Hprt-deficient ES cells. The mean homology region useful for insertional targeting is 7.6 kb for the *5'hprt* library and 9.1 for the *3'hprt* library, providing average efficiencies of 28%. This resource (MICER) is intended to carry out high throughput targeted manipulation of the mouse genome, and the vectors can be easily modified to enable the targeted knockin of transgenes (269,270).

Finally, it is worth mentioning a recent approach to obtaining biallelic gene targeting by taking advantage of Cre-enhanced interchromosomal translocations between single loxP sites placed in the proximity of centromeric regions in ES cells, so that Cre activity and appropriate selective pressure improve the frequency of biallelic mutant ES cell clones (271).

6.3. Cell lineage studies

Cell ablation experiments *in vivo* have usually been carried out with the use of transgenes of HSV-TK driven by a cell-specific promoter, in such a way that, when a nucleoside analogue (gancyclovir, FIAU) is administered in a systemic fashion to the mouse, only TK-expressing cells are affected by the toxic effects of nucleoside metabolites. Employing this kind of transgenes, the elimination of specific T cell subpopulations (272), cerebellar astrocytes (273), mature oligodendrocytes (274), and multipotent neural progenitors (275) has been achieved, providing insights into cell population dynamics and physiology.

Conditional gene targeting strategies make possible the elimination of defined cell populations without the need for external drug administration. The use of transgenes of the diphtheria toxin A fragment with an ubiquitous activity promoter carrying a loxP flanked stop cassette makes it possible to obliterate cells expressing Cre under the control of specific promoters. This approach has been used to show that the interaction of skeletal muscle cells in embryos is essential for motoneuron development (276), and to elucidate lineage relationships among pancreatic cells (277). Recently, the targeting of a conditional expression construct of DTA into the ROSA26 locus has been reported; it has become a useful tool for accomplishing specific cell ablation (278). A related system is composed of a Cre-inducible diphtheria toxin receptor, and the application of intraperitoneal diphtheria toxin has proven to be effective in producing the selective elimination of T cells, B cells and oligodendrocytes (279) by using different Cre-expressing mouse strains. Another tool in this line is an X-linked transgenic line that carries a Cre inducible TK gene, which can be selectively turned on in double transgenic mice expressing a Cre transgene that allows the ablation of Cre-expressing cells when gancyclovir is dispensed (280).

A complementary experimental procedure to specific cell population ablation is the labelling of specific cell subsets with an ontogenic relationship. This approach enhances the use of morphological analysis techniques with the use of markers like beta-galactosidase and green fluorescent proteins. Transient expression in oocytes of Cre recombinase from a vector coinjected together with another dual EGFP/beta-galactosidase reporter vector can be used to track early developmental stages (281).

However, the use of constitutive transgenes encoding marker proteins and containing loxP flanked stop cassettes to obtain their Cre-mediated transcriptional activation has become the most widely-used and versatile model for cell lineage study. These mice strains are more commonly called reporter strains, and they represent a verification tool for the specificity of Cre transgenes, as they can be used to monitor Cre-mediated events. Nevertheless, the observation of recombinase activity in a reporter strain does not imply a similar intensity of recombination in other floxed genes, since chromatin accessibility may be different. The use of reporter strains together with knockin Cre strains has been used to study the fate of cell lineages and a number of morphological questions in several studies (188-193).

Several Cre reporter lines have been established; the first ones were based on the use of lacZ transgene under the control of cytomegalovirus (CMV) promoter (282,283), the CAG promoter, consisting of the chicken beta actin promoter with the CMV-IE enhancer (284), or the PGK promoter (285). They may also be produced by insertion through homologous recombination in the ROSA26 locus, from which constitutive gene expression occurs during embryonic development and adulthood (286,287). Soon after its development, EGFP began to be used following similar strategies and improving sensitivity, both in transgenes driven by the CAG promoter (288) and by targeting into the ROSA26 locus (289). The characteristics of the ROSA26 locus make it a suitable genomic localization to place indicator cassettes, as it seems to be accessible for recombination throughout different stages of development, in a very wide array of tissues. Therefore, it has been used to generate reporter lines that express the GFP emission-shifted variants EYFP and ECFP (290), or luciferase, allowing for non-invasive bioluminescent monitoring of Cre activity (291).

Dual indicator lacZ/EGFP cassettes have been developed, such that in a non-recombined condition one of the two markers is active, while recombination causes a switching to the other marker (182,292). This kind of indicator cassette has been proposed for use in the monitoring of effective Cre-mediated recombination, if integrated in targeting constructs toward genes of interest.

Regarding the FLP reporter lines described to date, the number is quite reduced, comprising a transgenic lacZ-based system under the control of the hydroxy-methyl-glutaryl Coenzyme A (48) and an alkaline phosphatase-based system introduced in the ROSA26 locus (293). No mouse reporter lines have been described for other recombinases/integrases (Section 3.4), but reporter cassettes have been used in mammalian cells, and the principles used to create Cre and FLP reporter strains can be applied.

A smart strategy for the study of cell lineage has recently been devised that makes use of interchromosomal Cre-mediated recombination between partial fluorescent reporters. Recombination induces the reconstitution of functional green or red fluorescent markers that serve to track the cell lineage where somatic recombination has taken place. This strategy relies on the insertion of two loxP sequences in different but homologous chromosomes. These loxP sites are introduced along with two reciprocally chimeric genes, each composed of the N-terminus of one

marker and the C-terminus of the other, and a loxP-containing intron between the two elements. Cre-mediated recombination during the G2 phase prior to mitosis produces cell subpopulations with different patterns of fluorescence labelling, either by generating single colour (green/red) cells, double colour or no labelling, while Cre-mediated recombination during the G0 or G1 phases of cell cycle results in double-labelled cells. This technique, called MADM (mosaic analysis with double markers), has been extensively used in the *Drosophila* model, and this adaptation to mammalian organisms represents a powerful tool for producing conditional mutations in isolated cells and for studying resulting phenotypes with high anatomical resolution. It has permitted the authors to examine the dynamics of cerebellar granule cell progenitors during development and to trace axonal projections and wiring. An additional use for this dual marker design is in the production of conditional mutations if one of the markers is associated with a defined mutation of the gene of interest. A recombination event during mitosis of a hemizygous mutant cell results in two clonal populations of homozygous wildtype and mutated cells that can be distinguished by their differently-associated fluorescent marker (294).

6.4. Humanization of mouse genes

Regardless of the similarity between mouse and human physiological processes, in some cases the mouse does not constitute an accurate model of study. In most cases, despite the high degree of homology between mouse and human genes, small but significant differences are observed, such as ligand binding properties, metabolising rate of substrates, or differentially regulated expression. In these situations, genome engineering techniques permit the replacement of mouse genes with their human homologues. Among the applications of this kind of approach are the generation of better models for human diseases, and the development of therapeutic compounds, to the extent that the effect of a drug targeting a human-specific protein can be assessed *in vivo* and the metabolism and mechanisms of toxicity of drugs can be rapidly evaluated.

Homologous recombination is the way to replace the endogenous mouse gene coding sequence with its homologous human sequence. Approaches without the participation of recombinases have been used (295), but they leave behind the selection cassette and sequences of the endogenous gene, which can potentially lead to an altered expression pattern of the gene of interest, invalidating phenotype analysis. The use of selection cassettes flanked by loxP sites raises the possibility of their removal, as previously described (Section 4.2).

Conversely, a gene replacement strategy has been developed that uses a targeting vector which, together with the replacing sequence, contains a positive selection cassette with only one loxP site, and a second loxP site placed in the limit of the region to be displaced, just before the homology arm of the vector, so that both the selection marker and the endogenous sequences to be replaced are enclosed by loxP sequences after homologous recombination. Therefore, recombinase mediated deletion eliminates the entire genomic segment, yielding a clean gene replacement. This approach was used to replace the constant region of the *Cgamma1* mouse gene, which encodes the constant region of the heavy chain of IgG1 antibodies, with its human counterpart, allowing the production of humanized antibodies (296).

This strategy is valid if the length of the segments to be inserted and deleted is less than 10 kb, due to the decay in the frequency of cointegration of the isolated loxP site with the positive selection cassette. An option to overcome this limitation could be the use of two different selection cassettes flanked at opposite sides by loxP sequences, as a way of increasing the likelihood of exchanging the whole fragment under double positive selection conditions.

A different approach for attaining gene humanization is the use of recombinase-mediated cassette exchange (RMCE) of mouse genes previously targeted with site-specific recombination sequences. In this way, cassettes containing the human genes could be delivered by microinjection in oocytes and would give rise to mice humanized at these loci. This strategy may be more versatile than conventional gene targeted subtitutions, in the sense that the generation of multiple strains with polymorphic alleles could be easily accomplished (297).

6.5. Cassette exchange or integration

Most of the Cre and FLP applications have been based on their excision capability, since the integration potential of these recombinases has an essential limitation due to the reversible nature of the site-specific recombination reaction, which makes it an impractical alternative. However, the development of technical approaches for bringing about the integration of well-characterized expression cassettes in specific chromosomal locations tagged with site-specific recombinase recognition sequences has been achieved. This kind of application is termed recombinase-mediated cassette exchange (RMCE). The use of tagged loci with known transcriptional position effects (either a neutral effect or a strong activity in specific tissues) would make possible the insertion of transgenes directed toward referenced loci with a predictable expression pattern. The tagged loci can be produced by homologous recombination or generated in gene-trap experimental settings. RMCE can be employed to characterize cis-regulatory elements in a controlled genomic environment, to study several protein variants expressed from an equivalent locus, to replace regions of a gene by mutated versions, or to investigate multiple alleles (297).

The earliest use of RMCE in cell cultures relied on the use of two mutant FRT sites to avoid FLP-mediated excision (298, 299). Later on, a method was described using mutant loxP sites (300,301) or inverted loxP sites, enabling cassette exchange in addition to inversion (302). Early methods relied on the presence of a negative selection marker in the tagged locus, but the frequency of RMCE under nonselectable conditions was reasonable, and exchange cassettes expressing GFP enabled the enrichment

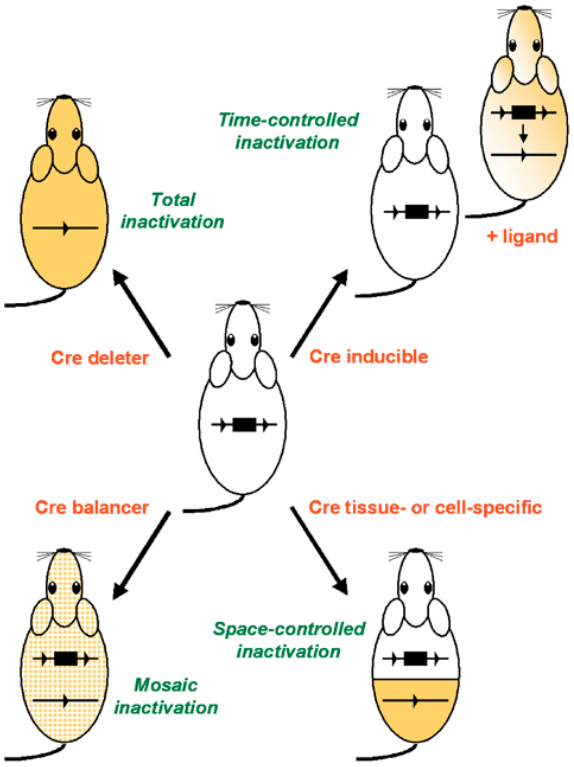

Figure 5. A mouse line carrying a floxed allele can generate several conditionally inactivated models: crossing with a deleter Cre strain results in an ubiquitous gene inactivation, crossing with a balancer Cre strain generates a mosaic pattern of gene inactivation, crossing with a Cre-expressing strain under the control of a tissue- or cell-specific promoter permits a spatial control of gene inactivation, and the use of a Cre-inducible strain allows temporal control of gene inactivation. Simultaneous spatial and temporal control can be achieved by a strain that expresses an inducible Cre under the control of a tissue- or cell-specific promoter.

of positive cells by fluorescence-activated cell sorting (FACS) (296, 298). Further improvements have been proposed to RMCE (303), such as a combined use of loxP/FRT sites and Cre/FLP recombinases, which allows efficient RMCE under non-selectable conditions (304).

These strategies for RMCE can be applied to ES cells (299-301) and oocytes (305), offering a way to generate whole animals with exchanged chromosomal regions from a single source of tagged ES cells or transgenic oocyte donor mice. Gene trapping strategies can incorporate tags to enable subsequent RMCE experiments (306).

The emergence of novel recombinases/integrases (Section 3.4) can enrich the alternatives in RMCE approaches. In particular, the features of integrases make them potential tools for integration of cassettes with an elevated frequency at att sites previously introduced in precise chromosomal regions. As an example of the potential use of phage C31 integrase, a Factor IX expression cassette was inserted from a plasmid bearing an attB site into existing endogenous pseudo-attP sites in mouse genome, producing a ten-fold increase in serum concentration of the protein (307).

7. SUMMARY AND PERSPECTIVES

Conditional gene targeting technology has evolved rapidly, relying fundamentally in the use of site-specific recombinases. Different outcomes can be obtained using a single mouse strain carrying a floxed allele, depending on the Cre transgenic mouse with which is crossed (Figure 5); in this manner the study of gene function can be envisaged from several points of view in successive experiments. The advent of novel recombinases with equivalent properties to Cre and FLP and integrases allowing for integration events more efficiently than classic recombinases opens a wide landscape of generation of mouse models for the study of developmental and physiological processes, as well as disease situations. In addition, the battery of molecular tools is growing with the addition of RNAi, BAC transgenesis, and efficient viral vectors, enabling novel approaches in the generation of mouse models for the study of gene function. The design of mouse models that include conditional alleles may well become a routine practice, due to the versatility they offer, ranging from the production of a total knockout animal to the possibility of RMCE.

Efforts directed toward integrating site-specific potential uses of recombinase to high throughput mutagenic approaches have recently been described by the German Gene Trap Consortium (308). The retroviral-based gene trap strategy employed permits mutation repair and reinduction through site-specific recombination in ES cells. Gene trap vectors rely on the use of two directional site-specific recombination systems designed to invert the gene trap from its mutagenic orientation on the coding strand to a nonmutagenic orientation on the noncoding strand, when successively turned on (182). At the moment a library with one thousand ES cell lines with unique conditionally trapped genes has been established.

The application of these new tools will make possible the study of complex situations with several genes involved in the expression of disease phenotypes, as well as permitting subtle manipulation of the experimental system at the genomic level. Reductionist schemes can be significantly expanded with the possibility of alteration of a number of variables in a controlled way. More elaborated hypotheses for explaining processes during development and disease physiology could be tested through a combination of activation/inactivation of the genes participating in common metabolic or signalling pathways, as well as in the progress of morphogenesis. In addition, experimental humanized models that recapitulate pathogenesis can be created to test therapeutic approaches, either pharmacologically or by using gene therapy. It is also likely that technical advances in the *in vivo* analysis of mouse phenotype, such as magnetic resonance imaging (MRI) or bioluminescence, will add new perspectives in the

design of mouse models that, for instance, could incorporate inducible alleles for the dynamic observation of lesion development.

However, the promising features for most novel recombinases must previously prove to be useful *in vivo*, and the procedures for gene targeting should improve their efficiency to help make of them an even more powerful technology. The combination of mouse high throughput mutagenesis protocols with the kind of manipulations described in this review could greatly enhance development of knowledge concerning gene function. The coming years will undoubtedly offer increased refinement of mouse models in order to gain insights into complex diseases, and a shift in the use of mouse models from addressing basic questions toward application in biomedical issues.

8. ACKNOWLEDGMENTS

Work in our groups is supported by grants CP03/00133 from FIS-ISCIII, RT/C03-01 from RECAVA (FIS-ISCIII) and RT/G03-181 from Red de Hiperlipemias Hereditarias (FIS-ISCIII) (ALGO), and by la Region Centre, INSERM ATC Viellissement (FG).

9. REFERENCES

1. Mouse Genome Sequencing Consortium. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420: 520-562 (2002)
2. Guenet JL. Chemical mutagenesis of the mouse genome: an overview. *Genetica* 122: 9-24 (2004)
3 Justice MJ, JK Noveroske, JS Weber, B Zheng & A Bradley. Mouse ENU mutagenesis. *Hum Mol Genetics* 8: 1955-1963 (1999)
4. Zambrowicz BP & GA Friedrich. Comprehensive mammalian genetics: history and future prospects of gene trapping in the mouse. *Int J Dev Biol* 42: 1025-1036 (1998)
5. Lyons GE, BJ Swanson, MA Haendel & J Daniels. Gene trapping in embryonic stem cells *in vitro* to identify novel developmentally regulated genes in the mouse. *Methods Mol Biol* 136: 297-307 (2000)
6. Nagy A. Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26: 99-109 (2000)
7. Wells T & DA Carter. Genetic engineering of neural function in transgenic rodents: towards a comprehensive strategy? *J Neurosci Methods* 108: 111-130 (2001)
8. Lewandoski M. Conditional control of gene expression in the mouse. *Nat Rev Genet* 2: 743-755 (2001)
9. Ryding ADS, MGF Sharp & JJ Mullins. Conditional transgenic technologies. *J Endocrinol* 171: 1-14 (2001)
10. Bockamp E, M Maringer, C Spangenberg, S Fees, S Fraser, L Eshkind, Foesch & B Zabel. Of mice and models: improved animal models for biomedical research. *Physiol Genomics* 11: 115-132 (2002)
11. Van der Meyden L, DJ Adams & A Bradley. Tools for targeted manipulation of the mouse genome. *Physiol Genomics* 11: 133-164 (2002)
12. Tronche F, E Casanova, M Turiault, I Sahly & C Kellendonk. When reverse genetics meets physiology: the use of site-specific recombinases in mice. *FEBS Lett* 529: 116-121 (2002)
13. Prosser H & S Rastan. Manipulation of the mouse genome: a multiple impact resource for drug discovery and development. *Trends Biotechnol* 21: 224-232 (2003)
14. Gao J, X Wu & J Zuo. Targeting hearing genes in mice. *Mol Brain Res* 132: 192-207 (2004)
15. Giuriato S, K Rabin, AC Fan, CM Shachaf & DW Felsher. Conditional animal models: a strategy to define when oncogenes will be effective targets to treat cancer. *Sem Cancer Biol* 14: 3-11 (2004)
16. Austin S, M Ziese & N Sternberg. A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* 25: 729-736 (1981)
17. Van Duyne GD. A structural view of cre-loxP site-specific recombination. *Annu Rev Biophys Biomol Struct* 30: 87-104 (2001)
18. Ghosh K & GD Van Duyne. Cre-loxP biochemistry. *Methods* 28: 374-383 (2002)
19. Volkert FC, LC Wu, PA Fisher & JR Broach. Survival strategies of the yeast plasmid two-micron circle. *Basic Life Sci* 40: 375-396 (1986)
20. Chen Y & PA Rice. New insight into site-specific recombination from Flp recombinase-DNA structures. *Annu Rev Biophys Biomol Struct* 32: 135-159 (2003)
21. Heidmann D & CF Lehner. Reduction of Cre recombinase toxicity in proliferating Drosophila cells by estrogen-dependent activity regulation. *Dev Genes Evol* 211: 458-465 (2001)
22. McGuire SE, G Roman & RL Davis. Gene expression systems in Drosophila: a synthesis of time and space. *Trends Genet* 20: 384-391 (2004)
23. Dong J & GW Stuart. Transgene manipulation in zebrafish by using recombinases. *Methods Cell Biol* 77: 363-379 (2004)
24. Werdien D, G Peiler & GU Ryffel. FLP and Cre recombinase function in Xenopus embryos. *Nucleic Acids Res* 29: e53 (2001)
25. Srivastava V & DW Ow. Marker-free site-specific gene integration in plants. *Trends Biotechnol* 22: 627-629 (2004)
26. Sauer B & N Henderson. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 85: 5166-5170 (1988)
27. Fukushige S & B Sauer. Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. *Proc Natl Acad Sci USA* 89: 7095-7099 (1992)
28. Beaubonis W & B Sauer. Genomic targeting with purified Cre recombinase. *Nucleic Acids Res* 21: 2025-2029 (1993)
29. Westerman KA & P Leboulch. Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination. *Proc Natl Acad Sci USA* 93: 8971-8976 (1996)
30. Orban PC, D Chui & JD Marth. Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci USA* 89: 6861-6865 (1992)
31. Sunaga S, K Maki, Y Komagata, K Ikuta & JI Miyazaki. Efficient removal of loxP-flanked DNA sequences in a gene-targeted locus by transient expression of Cre recombinase in fertilized eggs. *Mol Reprod Dev* 46: 109-113 (1997)
32. de Wit T, D Drabek & F Grosveld. Microinjection of

cre recombinase RNA induces site specific-recombination of a transgene in mouse oocytes. *Nucleic Acids Res* 26: 676-678 (1998)
33. Schaft J, R Ashery-Padan, F van der Hoeven, P Gruss & AF Stewart. Efficient FLP recombination in mouse ES cells and oocytes. *Genesis* 31: 6-10 (2001)
34. Vooijs M, J Jonkers & A Berns. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. *EMBO Rep* 2: 292-297 (2001)
35. Will E, H Klump, N Heffner, M Schwieger, B Schiedlmeier, W Ostertag, C Baum & C Stocking. Unmodified Cre recombinase crosses the membrane. *Nucleic Acids Res* 30: e59 (2002)
36. Jo D, Q Lin, A Nashabi, DJ Mays, D Unutmaz, JA Pietenpol & HE Ruley. Cell cycle-dependent transduction of cell-permeant Cre recombinase proteins. *J Cell Biochem* 89: 674-687 (2003)
37. Fiering S, E Epner, K Robinson, Y Zhuang, A Telling, M Hu, DI Martin, T Enver, TJ Ley & M Groudine. Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that is not essential for proper regulation of the beta-globin locus. *Genes Dev* 9: 2203-2213 (1995)
38. Dymecki SM. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci USA* 93: 6191-6196 (1996)
39. Buchholz F, L Ringrose, PO Angrand, F Rossi & AF Stewart. Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res* 24: 4256-4262 (1996)
40. Thyagarajan B, MJ Guimaraes, AC Groth & MP Calos. Mammalian genomes contain active recombinase recognition sites. *Gene* (2000); 244: 47.
41. Hollis RP, SM Stoll, CR Sclimenti, J Lin, Y Chen-Tsai & MP Calos. Phage integrases for the construction and manipulation of transgenic mammals. *Reprod Biol Endocrinol* 1: 79 (2003)
42. Heidmann D & CF Lehrer. Reduction of Cre recombinase toxicity in proliferating Drosophila cells by estrogen-dependent activity regulation. *Dev Genes Evol* 211: 458-465 (2001)
43. Mahonen AJ, KJ Airenne, MM Lind, HP Lesch & S Yla-Herttuala. Optimized self-excising cassette for mammalian cells. *Biochem Biophys Res Commun* 320: 366-371 (2004)
44. Schmidt EE, DS Taylor, JR Prigge, S Barnett & MR Cappechi. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc Natl Acad Sic USA* 97: 13702-13707 (2000)
45. Koresawa Y, M Miyagawa, M Ikawa, K Katsunami, M Yamada, M Okabe & R Shirakura. A new Cre recombinase based on optimal codon usage in mammals: a powerful material for organ-specific gene targeting. *Transplant Proc* 32: 2516-2517 (2000)
46. Shimshek DR, J Kim, MR Hubner, DJ Spergel, F Buchholz, E Casanova, AF Stewart, PH Seeburg & R Sprengel. Codon-improved Cre Recombinase (iCre) expression in the mouse. *Genesis* 32:19-26 (2002)
47. Buchholz F, PO Angrand & AF Stewart. Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat Biotechnol* 16: 657-662 (1998)
48. Rodriguez CI, F Buchholz, J Galloway, R Seguerra, J Kasper, R Ayala, AF Stewart & SM Dymecki. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet* 25: 139-140 (2000)
49. Farley FW, P Soriano, LS Steffen & SM Dymecki. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* 28: 106-110 (2000)
50. Andreas S, F Schwenk, B Küter-Luks, N Faust & R Kühn. Enhanced efficiency through nuclear localization signal fusion on phage phiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. *Nucleic Acids Res* 30: 2299-2306 (2002)
51. Le Y, S Gagneten, D Tombaccini, B Behtke & B Sauer. Nuclear target determinants of the phage P1 Cre DNA recombinase. *Nucleic Acids Res* 27:4703-4709 (1999)
52. Gu H, YR Zou & K Rajewsky. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 6: 1155-1164 (1993)
53. Peitz M, K Pfannkuche, K Rajewsky & F Edenholfer. Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. *Proc Natl Acad Sci USA* 99: 4489-4494 (2002)
54. Jo D, A Nashabi, C Doxsee, Q Lin, D Unutmaz, J Chen & HE Ruley. Epigenetic regulation of gene stucture and function with a cell-permeable Cre recombinase. *Nat Biotechnol* 19: 929-933 (2001)
55. Lin Q, D jo, KD Grebre-Amlak & HE Ruley. Enhanced cell-permeant Cre protein for site-specific recombination in cultured cells. *BMC Biotechnol* 4:25 (2004)
56. Gagneten S, Y Le, J Miller & B Sauer. Brief expression of a GFP cre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. *Nucleic Acids Res* 25: 3326-3331 (1997)
57. Striklett PK, RD Nelson & DE Kohan. Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase. *Gene* 215: 415-423 (1998)
58. Picard D. Regulation of protein function through expression of chimaeric proteins. *Curr Opin Biotechnol* 5: 511-515 (1994)
59. Metzger D, J Clifford, H Chiba & P Chambon. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc Natl Acad Sci USA* 92: 6991-6995 (1995)
60. Logie C & AF Stewart. Ligand-regulated site-specific recombination. *Proc Natl Acad Sci USA* 92: 5940-5944 (1995)
61. Feil R, J Brocard, B Mascrez, M LeMeur, D Metzger & P Chambon. Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci USA* 93: 10887-10890 (1996)
62. Feil R, J Wagner, D Metzger & P Chambon. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* 237: 752-757 (1997)
63. Casanova E, S Fehsenfeld, T Lemberger, DR Shimshek, R Sprengel & T Mantamadiotis. ER-based double iCre fusion protein allows partial recombination in forebrain. *Genesis* 34: 208-214 (2002)
64. Zhang Y, C Riesterer, AM Ayrall, F Sablitzky, TD Littlewood & M Reth. Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic*

Acids Res 24: 543-548 (1996)
65. Brocard J, X Warot, O Wenling, N Messaddeq, JL Vonesch, P Chambon & D Metzger. Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc Natl Acad Sci USA* 94: 14559-14563 (1997)
66. Tannour-Louet M, A Porteu, S Vaulont, A Kahn & M Vasseur-Cognet. A tamoxifen-inducible chimeric Cre recombinase specifically effective in the fetal and adult mouse liver. *Hepatology* 35: 1072-1081 (2002)
67. Hunter NL, RB Awatramani, FW Farley & SM Dymecki. Ligand-activated Flpe for temporally regulated gene modifications. *Genesis* 41: 99-109 (2005)
68. Vegeto E, GF Allan, WT Schrader, MJ Tsai, DP McDonnell & BW O'Malley. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 69: 703-713 (1992)
69. Kellendonk C, F Tronche, AP Monaghan, PO Angrand, F Stewart & G Schütz. Regulation of Cre recombinase activity by the synthetic steroid RU486. *Nucleic Acids Res* 24: 1404-1411 (1996)
70. Kellendonk C, F Tronche, E Casanova, K Anlag, C Opherk & G Schütz. Inducible site-specific recombination in the brain. *J Mol Biol* 285: 175-182 (1999)
71. Minamino T, V Gaussin, FJ DeMayo & MD Schneider. Inducible gene targeting in postnatal myocardium by cardiac-specific expression of a hormone-activated Cre fusion protein. *Circ Res* 88: 587-592 (2001)
72. Gao Y & RV Short. Fertility control in wild mice after feeding with RU486 or methyl testosterone. *J Reprod Fertil* 101: 483-487 (1994)
73. Youssef JA & MZ Badr. Hepatocarcinogenic potential of the glucocorticoid antagonist RU486 in B6C3F1 mice: effect on apoptosis, expression of oncogenes and the tumor suppressor gene p53. *Mol Cancer* 2: 3 (2003)
74. Kaczmarczyk SJ & Green JE. Induction of cre recombinase activity using modified androgen receptor ligand binding domains: a sensitive assay for ligand-receptor interactions. *Nucleic Acids Res* 31: e86 (2003)
75. Hoess RH, A Wierbicki & K Abremski. The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res* 14: 2287-2300 (1986)
76. Lee G & I Saito. Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* 216: 55-65 (1998)
77. Kolb AF. Selection-marker-free modification of the murine beta-casein gene using a lox2272 site. *Anal Biochem* 290: 260-271 (2001)
78. Albert H, EC Dale, E Lee & DW Ow. Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J* 7: 649-659 (1995)
79. Langer SJ, AP Ghafoori, M Byrd & L Leinwand. A genetic screen identifies novel non-compatible loxP sites. *Nucleic Acids Res* 30: 3067-3077 (2002)
80. Buchholz F & AF Stewart. Alteration of Cre recombinase site specificity by substrate-linked protein evolution. *Nat Biotecnol* 19: 1047-1052 (2001)
81. Voziyanov Y, JH Konieczka, AF Stewart & M Jarayam. Stepwise manipulation of DNA specificity in Flp: progressively adapting Flp to individual and combinatorial mutations in its target site. *J Mol Biol* 326: 65-76 (2003)
82. Konieczka JH, A Paek M Jarayam & Y Voziyanov. Recombination of hybrid target sites by binary combinations of Flp variants: mutations that foster interpromoter collaboration and enlarge substrate tolerance. *J Mol Biol* 339: 365-378 (2004)
83. Casanova E, T Lemberger, S Fehsenfeld, T Mantamadiotis & G Schutz. Alpha complementation in Cre recombinase enzyme. *Genesis* 37: 25-29 (2003)
84. Jullien N, F Sampieri, A Enjalbert & JP Herman. Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Nucleic Acids Res* 31: e131
85. Nunes-Düby SE, RS Thirumalai, HJ Kwon, T Ellenberger & A Landy. Similarities and differences among 105 members of the Int family of site-specific recombinases. *Nucleic Acids Res* 26: 391-406 (1998)
86. Groth AC & MP Calos. Phage integrases: biology and applications. *J Mol Biol* 335: 667-678 (2004)
87. Thorpe HM & MCM Smith. *In vitro* site-specific integration of bacteriophage DNA catalysed by a recombinase of the resolvase/invertase family. *Proc Natl Acad Sci USA* 95: 5505-5510 (1998)
88. Groth AC, EC Olivares, B Thyagarajan & MP Calos. A phage integrase directs efficient site-specific integration in human cells. *Proc Natl Acad Sci USA* 97: 5595-6000 (2000)
89. Belteki G, M Gersenstein DW Ow & A Nagy. Site-specific cassette exchange and germline transmission with mouse ES cells expressing phiC31 integrase. *Nat Biotechnol* 21: 321-324 (2003)
90. Thyagarajan B, EC Olivares, RP Hollis, DS Ginsburg & MC Calos. Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol Cell Biol* 21: 3926-3934 (2001)
91. Sclimenti CR, B Thyagarajan & MP Calos. Directed evolution of a recombinase for improved genomic integration at a native human sequence. *Nucleic Acids Res* 29: 5044-5051 (2001)
92. Matsuura M, T Noguchi, D Yamaguchi, T Aida, M Asayama, H Takahashi & M Shirai. The sre gene (ORF469) encodes a site-specific recombinase responsible for integration of the R4 phage genome. *J Bacteriol* 178: 3374-3376 (1996)
93. Olivares EC, RP Hollis & MP Calos. Phage R4 integrase mediates site-specific integration in human cells. *Gene* 278: 167-76 (2001)
94. Stoll SM, DS Ginsburg & MP Calos. Phage TP901-1 site specific integrase functions in human cells. *J Bacteriol* 184: 3657-63 (2002)
95. Christ N & P Dröge. Alterations in the directionality of lambda site-specific recombinations catalysed by mutant integrases *in vivo*. *J Mol Biol* 288: 825-836 (1999)
96. Lorbach E, N Christ, M Schwikardi & P Droge. Site-specific recombination in human cells catalysed by phage lambda integrase mutants. *J Mol Biol* 296: 1175-1181 (2000)
97. Christ N & P Droge. Genetic manipulation of mouse embryonic stem cells by mutant lambda integrase. *Genesis* 32: 203-208 (2002)
98. Kolot M, N Silberstein & E Yagil. Site-specific recombination in mammalian cells expressing the Int recombinase of bacteriophage HK022. *Mol Biol Rep* 26: 207-213 (1999)

99. Rojo F & JC Alonso. A novel site-specific recombinase encoded by the streptomyces pyogenes plasmid pSM19035. *J Mol Biol* 238: 159-172 (1994)
100. Rojo F & JC Alonso. The beta recombinase of plasmid pSM19035 binds to two adjacent sites, making different contacts at each of them. *Nucleic Acids Res* 23: 3181-3188 (1995)
101. Alonso JC, F Weise & F Rojo. The bacillus subtilis histone-like protein Hbsu is required for DNA resolution and DNA inversion mediated by the beta recombinase of plasmid pSM19035. *J Biol Chem* 270: 2938-2945 (1995)
102. Díaz V, F Rojo, C Martinez-A, JC Alonso, & A Bernad. The prokaryotic beta-recombinase catalyzes site-specific recombination in mammalian cells. *J Biol Chem* 274: 6634-6640 (1999)
103. Alonso JC, C Gutierrez & F Rojo. The role of chromatin-associated protein Hbsu in beta-mediated recombination is to facilitate the joining of distant recombination sites. *Mol Microbiol* 18: 471-478 (1995)
104. Matsuzaki H, H Araki & Y Oshima. Gene conversion associated with site-specific recombination in yeast plasmid pSR1. *Mol Cell Biol* 2: 955-62 (1988)
105. Araki H, N Nakanishi, BR Evans, H Matsuzaki, M Jayaram & Y Oshima. Site-specific recombinase, R, encoded by yeast plasmid pSR1. J *Mol Biol* 225: 25-37 (1992)
106. Yang SH & M Jayaram. Generality of the shared active site among yeast family site-specific recombinases. The R site-specific recombinase follows the Flp paradigm. *J Biol Chem* 269: 12789-12796 (1994)
107. Onouchi H, K Yokoi, C Machida, H Matsuzaki, Y Oshima, K Matsuoka, K Nakamura & Y Machida. Operation of an efficient site-specific recombination system of Zygosaccharomyces rouxii in tobacco cells. *Nucleic Acids Res* 19: 6373-6378 (1991)
108. Dröge P & NR Cozzarelli. Recombination of knotted substrates by Tn3 resolvase. *Proc Natl Acad Sci USA* 86: 6062-6066 (1989)
109. Schwikardi M & P Droge. Site-specific recombination in mammalian cells catalysed by gamma-delta resolvase mutants: implications for the topology of episomal DNA. *FEBS Lett* 471: 147-150 (2000)
110. Sauer B & J McDermott. DNA recombination with a heterospecific Cre homolog identified from comparison of the pac-c1 regions of P1-related phages. *Nucleic Acids Res* 32: 6086-6095 (2004)
111. Bibb LA, MI Hancox & GF Hatfull. Integration and excision by the charge serine recombinase phiRv1 integrase. *Mol Microbiol* 55: 1896-1910 (2005)
112. Brinster RL, HY Chen, M Trumbauer, AW Senear, R Warren & RD Palmiter. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27: 223-231 (1981)
113. B Hogan, R Beddington, F Constantini & E Lacy: Manipulating the mouse embryo: a laboratory manual (2nd ed). CSHL Press, NY (1994)
114. Dellaire G & P Chartrand. Direct evidence that transgene integration is random in murine cells, implying that naturally occurring double-strand breaks may be distributed similarly within the genome. *Radiat Res* 149: 325-329 (1998)
115. Miao GG, RJ Smeyne, G D'Arcangelo, NG Copeland, NA Jenkins, JI Morgan & T Curran. Isolation of an allele of reeler by insertional mutagenesis. *Proc Natl Acad Sci USA* 91: 11050-11054 (1994)
116. Yokoyama T, NG Copeland, NA Jenkins, CA Montgomery, FF Elder & PA Overbeek. Reversal of a left-right asymmetry: a situs inversus mutation. *Science* 260: 679-682 (1993)
117. Pfeifer A. Lentiviral transgenesis. *Transgenic Res* 13: 513-522 (2004)
118. Rülicke T & U Hübscher. Germ line transformation of mammals by pronuclear microinjection. *Exp Physiol* 85: 589-601 (2000)
119. Li X, W Wang & T Lufkin. Dicistronic LacZ and alkaline phosphatase reporter constructs permit simultaneous histological analysis of expression from multiple transgenes. *Biotechniques* 23: 874-882 (1997)
120. Antoch MP, EJ Song, AM Chang, MH Vitaterna, Y Zhao, LD Wilsbacher, AM Sangoram, DP King, LH Pinto & JS Takahashi. Functional identification of the mouse circadian clock gene by transgenic BAC rescue. *Cell* 89: 655-667.
121. Giraldo P & L Montoliu. Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res* 10: 83-103 (2001)
122. Heintz N. BAC transgenesis. *Nat Rev Neurosci* 2:861-870 (2001)
123. Muyrers JP, Y Zhang, G Testa & AF Stewart. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* 27: 1555-1557 (1999)
124. Testa G, Y Zhang, K Vintersten, V Benes, WW Pijnappel, I Chambers, AJ Smith & AF Stewart. Engineering the mouse genome with bacterial artificial chromosomes to create multipurpose alleles. *Nat Biotechnol* 21: 443-447 (2003)
125. Lee EC, D Yu, JM de Velasco, L Tessarollo, DA Swing, DL Court, NA Jenkins & NG Copeland. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73: 56-65 (2001)
126. Warming S, N Costantino, DL Court, NA Jenkins & NG Copeland. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33: e36 (2005)
127. Copeland NG, NA Jenkins & DL Court. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev* 2: 769-779 (2001)
128. Dykxhoorn DM, CD Novina & PA Sharp. Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol* 4: 457-467 (2003)
129. Elbashir SM, W Lendeckel & T Tuschl. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 15: 188-200 (2001)
130. Elbashir SM, J Harborth, W Lendeckel, A Yakin, K Weber, T Tuschl. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-498 (2001)
131. Brummelkamp TR, R Bernards & R Agami. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296: 550-553 (2002)
132. Sui G, C Soohoo, B Affar, F Gay, Y Shi, WC Forrester & Y Shi. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl*

Acad Sci USA 99: 5515-5520 (2002)
133. Yu JY, SL De Ruiter & DL Turner. RNA interference by expression of shirt-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 99: 6047-6052 (2002)
134. Miyagishi M & K Taira. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 20: 497-500 (2002)
135. Carmell MA, L Zhang, DS Conklin, GJ Hannon & TH Rosenquist. Germline transmission of RNAi in mice. *Nat Struct Biol* 10: 91-92 (2003)
136. Rubinson D, CP Dillon, AV Kwiatkowski, C Sievers, L Yang, J Kopinja, DL Rooney, MM Ihrig, MT McManus, FB Gertler, ML Scott & L Van Parijs. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 33: 401-406 (2003)
137. Stein P, P Svoboda & RM Schultz. Transgenic RNAi in mouse oocytes: a simple and fast approach to study gene function. *Dev Biol* 256: 187-193 (2003)
138. McCaffrey AP, H Nakai, K Pandey, Z Huang, FH Salazar, H Xu, SF Wieland, PL Marion & MA Kay. Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol* 21: 639-644 (2003)
139. Tiscornia G, O Singer, M Ikawa & IM Verma. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* 100: 1844-1848 (2003)
140. Kunath T, G Gish, H Lickert, N Jones, T Pawson & J Rossant. Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotechnol* 21: 559-561 (2003)
141. Thomas KR & MR Capecchi. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51: 503-512 (1987)
142. Wong EA & MR Capecchi. Analysis of homologous recombination in cultured mammalian cells in transient expression and stable transformation assays. *Somat Cell Mol Genet* 12: 63-72 (1986)
143. Hasty P, J Rivera-Perez & A Bradley. The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* 11: 5586-5591 (1991)
144. Lu ZH, JT Books, RM Kaufman & TJ Ley. Long targeting arms do not increase the efficiency of homologous recombination in the beta-globin locus of murine embryonic stem cells. *Blood* 102: 1531-1533 (2003)
145. Kumar S & JP Simons. The effects of terminal heterologies on gene targeting by insertion vectors in embryonic stem cells. *Nucleic Acids Res* 21: 1541-1548 (1993)
146. Hooper M, K Hardy, A Handyside, S Hunter & M Monk. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* 326: 292-295 (1987)
147. Shesely EG, HS Kim, WR Sheshee, T Papayannopoulou, O Smithies & BW Popovich. Correction of a human beta S-globin gene by gene targeting. *Proc Natl Acad Sci USA* 88: 4294-4298 (1991)
148. Hasty P, R Ramirez-Solis, R Krumlauf & A Bradley. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* 350: 243-246 (1991)
149. Askew GR, T Doetschman & J Lingrel. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. *Mol Cell Biol* 13: 4115-4124 (1993)
150. Whyatt LM & PD Rathjen. Introduction of precise alterations into the mouse genome with high efficiency by stable tag-exchange gene targeting: implications for gene targeting in ES cells. *NucleicAcids Res* 25: 2381-2388 (1997)
151. Sakai K & J Miyazaki. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun* 237: 318-324
152. Lallemand Y, V Luria, R Haffner-Krausz & P Lonai. Maternally expressed PGK-Cre transgene as a tool for early uniform activation of the Cre site-specific recombinase. *Transgenic Res* 7:105-112 (1998)
153. Takeuchi T, Nomura T, Tsujita M, Suzuki M, Fuse T, Mori H & M Mishina. Flp recombinase transgenic mice of C57BL/6 strain for conditional gene targeting. *Biochem Biophys Res Commun* 293: 953-957 (2002)
154. RM Torres & R Kühn. Laboratory protocols for conditional gene targeting. Oxford University Press, NY (1997)
155. Wolfer DP, WE Cusio & HP Lipp. Knockout mices: simple solutions to the problems of genetic background and flanking genes. *Trends Neurosci* 25: 336-340 (2002)
156. Seong E, TL Saunders, CL Stewart & M Burmeister. To knockout in 129 or in C57BL/6: that is the question. *Trends Genet* 20: 59-62 (2004)
157. NagyA & J Rossant. Production of completely ES cell-derived fetuses. In: Gene Targeting: A Practical Approach 2nd ed. (Ed. A Joyner). IRL Press. Oxford University Press (1999).
158. Li X, Y Yu, W Wei, J Yong, J Yang, X Xiong, T Qing & H Deng. Simple and efficient production of mice derived from embryonic stem cells aggregated with tetraploid embryos. *Mol Reprod Dev* 71: 154-158 (2005)
159. Eggan K, H Akutsu, J Loring, L Jackson-Grusby, M Klemm, WM Rideout, R Yanagimachi & R Jaenisch. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci USA* 98: 6209-6214 (2001)
160. Misra RP, SK Bronson, Q Xiao, W Garrison, J li, R Zhao & SA Duncan. Generation of single-copy transgenic mouse embryos by tetraploid embryo complementation. *BMC Biotechnol* 1: 12 (2001)
161. Seibler J, B Zevnik, B Kuter-Luks, S Andreas, H Kern, T Hennek, A Rode, C Heimann, N Faust, G Kauselmann, M Schoor, R Jaenisch, K Rajewsky, R Kühn & F Schwenk. Rapid generation of inducible mouse mutants. *Nucleic Acids Res* 31: e12 (2003)
162. Zhou D, JX Ren, TM Ryan, NP Higgins & TM Townes. Rapid tagging of endogenous mouse genes by recombineering and ES cell complementation of tetraploid blastocysts. *Nucleic Acids Res* 32: e128 (2004)
163. Vintersten K, C Monetti, M Gertsenstein, P Zhang, L Laszlo, S Bichele & A Nagy. Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40: 241-246 (2004)
164. Smith GR. How homologous recombination is

initiated: unexpected evidence for single-strand nicks from v(d)j site-specific recombination *Cell* 117: 146-148 (2004)
165. Cohen-Tannoudji M, S Robine, A Choulika, D Pinto, F El Marjou, C Babinet, D Louvard & F Jaisser. I-SceI induced gene replacement at a natural locus in embryonic stem cells. *Mol Cell Biol* 18: 1444-1448 (1998)
166. Porteus MH & D Baltimore. Chimeric nucleases stimulate gene targeting in human cells. *Science* 300: 763 (2003)
167. Urnov FD, JC Miller, YL Lee, CM Beausejour, JM Rock, S Augustus, AC Jamieson, MH Porteus, PD Gregory & MC Holmes. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435: 646-651 (2005)
168. Wakayama T, AC Perry, M Zuccotti, KR Johnson & R Yanagimachi. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394: 369-374 (1998)
169. Wakayama T & R Yanagimachi. Cloning the laboratory mouse. *Semin Cell Dev Biol* 10: 253-258 (1999)
170. Yabuuchi A, Y Yasuda, Y Kato & Y Tsunoda. Effects of nuclear transfer procedures on ES cell cloning efficiency in the mouse. *J Reprod Dev* 50: 263-268 (2004)
171. Hiiragi T & D Solter. Reprogramming is essential in nuclear transfer. *Mol Reprod Dev* 70: 417-421 (2005)
172. Yanagimachi R. Cloning: experience from the mouse and other animals. *Mol Cell Endocrinol* 187: 241-248 (2002)
173. Jaenisch R, K Eggan, D Humphreys, W Rideout & K Hochedlinger. Nuclear cloning, stem cells, and genomic reprogramming. *Cloning Stem Cells* 4: 389-396 (2002)
174. Wakayama T, I Rodriguez, AC Perry, R Yanamigachi & P Mombaerts. Mice cloned from embryonic stem cells. *Proc Natl Acad Sci USA* 96: 14984-14989 (1999)
175. Gao S, M McGarry, H Priddle, T Ferrier, B Gasparini, J Fletcher, L Harkness, P De Sousa, J McWhir & I Wilmut. Effects of donor oocytes and culture conditions on development of cloned mice embryos. *Mol Reprod Dev* 66: 126-133 (2003)
176 . Li ZW, G Stark, J Götz, T Rülicke, U Müller & C Weissmann. 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 (1996)
177. Zheng B, M Sage, EA Sheppeard, V Jurecic & A Bradley. Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic implications. *Mol Cell Biol* 20: 648-655 (2000)
178. Gu H, JD Marth, PC Orban, H Mossmann & K Rajewsky. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* 265: 103-106 (1994)
179. Holzenberger M, C Lenzner, P Leneuve, R Zaoui, G Hamard, S Vaulont & Y Le Bouc. Cre-mediated germline mosaicism: a method allowing rapid generation of several alleles of a target gene. *Nucleic Acids Res* 28: e92 (2000)
180. Leneuve P, S Colnot, G Hamard, F Francis, M Niwa-Kawakita, M Giovannini & M Holzenberger. Cre-mediated germline mosaicism: a new transgenic mouse for the selective removal of residual markers from tri-lox conditional alleles. *Nucleic Acids Res* 31: e21 (2003)
181. Sun X, M Lewandoski, EN Meyers, YH Liu, RE Maxson & GR Martin. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat Genet* 25: 83-86 (2000)
182. Schnütgen F, N Doerflinger, C Calleja, O Wendling, P Chambon & NB Ghyselinck. A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. *Nat Biotechnol* 21: 562-565 (2003)
183. Casanova E, S Fehsenfeld, T Mantamadiotis, T Lemberger, E Greiner, AF Stewart & G Schutz. A CamKIIalpha iCre BAC allows brain-specific gene inactivation. *Genesis* 31: 37-42 (2001)
184. Aller MI, A Jones, D Merlo, M Paterlini, AH Meyer, M Farrant & W Wisden. Cerebellar granule cell Cre recombinase expression. *Genesis* 36: 97-103 (2003)
185. Ohyama T & AK Groves. Generation of Pax2-Cre mice by modification of a Pax2 bacterial artificial chromosome. *Genesis* 38: 195-199 (2004)
186. Zhang XM, AH Ng, JA Tanner, WT Wu, NG Copeland, NA Jenkins & JD Huang. Highly restricted expression of Cre recombinase in cerebellar Purkinje cells. *Genesis* 40: 45-51 (2004)
187. Guo H, S Hong, XL Jin, RS Chen, PP Avasthi, YT Tu, TL Ivanco & Y Li. Specificity and efficiency of Cre-mediated recombination in Emx1-Cre knock-in mice. *Biochem Biophys Res Commun* 273: 661-665 (2000)
188. Voiculescu O, P Charnay & S Schneider-Maunoury. Expression pattern of a Krox-20/Cre knock-in allele in the developing hindbrain, bones, and peripheral nervous system. *Genesis* 26: 123-126 (2000)
189. Moses KA, F DeMayo, RM Braun, JL Reecy & RJ Schwartz. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* 31: 176-180 (2001)
190. Motoike T, DW Markham DW, J Rossant & TN Sato. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. *Genesis* 35: 153-159 (2003)
191. Jeyasuria P, Y Ikeda, SP Jamin, L Zhao, DG De Rooij, AP Themmen, RR Behringer & KL Parker. Cell specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* 18: 1610-1619 (2004)
192. Zhuang X, J Masson, JA Gingrich, S Rayport & R Hen. Targeted gene expression in dopamine and serotonin neurons of the mouse brain. *J Neurosci Methods* 143: 27-32 (2005)
193. Kiwura J, Y Suda, D Kurosawa, ZM Hossain, M Nakawura, M Takahashi, A Mara & S Aizawa. Emx2 and Pax6 function in cooperation with Otx2 and Otx1 to develop caudal forebrain primordium that includes future archipalium. *J Neurosci* 25: 5097-5108 (2005)
194. Lappe-Siefke C, S Goebbels, M Gravel, E Nicksch, J Lee, PE Braun, IR Griffiths & KA Nave. Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. *Nat Genet* 33: 366-374 (2003)
195. Saito H, H Tsumura, S Otake, A Nishida, T Furukawa & N Suzuki. L7/Pcp-2-specific expression of Cre recombinase using knock-in approach. *Biochem Biophys Res Commun* 331: 1216-1221 (2005)
196. Gorski JA & KR Jones. Efficient bicistronic expresión of cre in mammalian cells. *Nucleic Acids Res* 27: 2059-2061 (1999)
197. Stanley EG, C Biben, A Elefanty, L Barnett, F Koentgen, L Robb & RP Harvey. Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-

ires-Cre allele of the homeobox gene Nkx2-5. *Int J Dev Biol* 46: 431-439 (2002)
198. Vincent SD & EJ Robertson Targeted insertion of an IRES Cre into the Hnf4alpha locus: Cre-mediated recombination in the liver, kidney, and gut epithelium. *Genesis* 39: 206-211 (2004)
199. Michael SK, J Brennan & EJ Robertson. Efficient gene-specific expression of Cre recombinase in the mouse embryo by targeted insertion of a novel IRES-Cre cassette into endogenous loci. *Mech Dev* 85: 35-47 (1999)
200. Mallo M. Controlled gene activation and inactivation in the mouse. *Front Biosci* 11: 313-327 (2006)
201. Zhu Z, T Zeng, CG lee, RJ Homer & JA Elias. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modelling. *Semin Cell Dev Biol* 13: 121-128 (2002)
202. Sawicki JA, B Monks & RJ Morris. Cell-specific ecdysone-inducible expression of FLP recombinase in mammalian cells. *Biotechniques* 25: 868-875 (1998)
203. Utomo ARH, AY Nikitin & WH Lee. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* 17: 1091-1096 (1999)
204. Linderberg J, R Mattsson & T Ebendal. Timing of doxycycline yields different patterns of genomic recombination in brain neurons with a new inducible Cre transgene. *J Neurosci Res* 68: 248-253 (2002)
205. Saam JR & JI Gordon. Inducible gene knockouts in the small intestine and colonic epithelium. *J Biol Chem* 274: 38071-38082 (1999)
206. Schönig K, F Schwenk, K Rajewsky & H Bujard. Stringent doxycycline dependent control of CRE recombinase *in vivo*. *Nucleic Acids Res* 30: e134 (2002)
207. Perl AK, SE Wert, A Nagy, CG Lobe, JA Whittsett. Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci USA* 99: 10482-10487 (2002)
208. Schuler M, F Ali, E Metzger, P Chambon & D Metzger. Temporally controlled targeted somatic mutagenesis in skeletal muscles of the mouse. *Genesis* 41: 165-170 (2005)
209. Kim JE, K Nakashima & B de Crombrugghe. Transgenic mice expressing a ligand-inducible cre recombinase in osteoblasts and odontoblasts: a new tool to examine physiology and disease of postnatal bone and tooth. *Am J Pathol* 165: 1875-1882 (2004)
210. Metzger D, M Li & P Chambon. Targeted somatic mutagenesis in the mouse epidermis. *Methods Mol Biol* 289: 329-340 (2005)
211. Schuler M, A Dierich, P Chambon & D Metzger. Efficient temporally controlled targeted somatic mutagenesis in hepatocytes of the mouse. *Genesis* 39: 167-172 (2004)
212. Zhu HZ, JQ Chen, GX Cheng & JL Xue. Generation and characterization of transgenic mice expressing tamoxifen-inducible cre-fusion protein specifically in mouse liver. *World J Gastroenterol* 9: 1844-1847 (2003)
213. Leone DP, S Genoud, S Atanasoski, R Grausenburger, P Berger, D Metzger, WB Macklin, P Chambon & U Suter. Tamoxifen-inducible glia-specific Cre mice for somatic mutagenesis in oligodendrocytes and Schwann cells. *Mol Cell Neurosci* 22: 430-440 (2003)
214. Doerflinger NH, WB Macklin & B Popko. Inducible site-specific recombination in myelinating cells. *Genesis* 35: 63-72 (2003)
215. Bugeon L, A Danou, D Carpentier, P Langridge, N Syed & MJ Dallman. Inducible gene silencing in podocytes: a new tool for studying glomerular function. *J Am Soc Nephrol* 14: 786-791 (2003)
216. Weber P, M Schuler, C Gerard, M Mark, D Metzger & P Chambon. Temporally controlled site-specific mutagenesis in the germ cell lineage of the mouse testis. *Biol Reprod* 68: 553-559 (2003)
217. Forde A, R Constien, HJ Grone, G Hammerling & B Arnold. Temporal Cre-mediated recombination exclusively in endothelial cells using Tie2 regulatory elements. *Genesis* 33: 191-197 (2002)
218. Zheng B, Z Zhang, CM Black, B de Crombrugghe & CP Denton. Ligand-dependent genetic recombination in fibroblasts : a potentially powerful technique for investigating gene function in fibrosis. *Am J Pathol* 160: 1609-1617 (2002)
219. el Marjou F, KP Janssen, BH Chang, M Li, V Hindie, L Chan, D Louvard, P Chambon, D Metzger & S Robine. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 39: 186-193 (2004)
220. Wen F, G Cecena, V Munoz-Ritchie, E Fuchs, P Chambon & RG Oshima. Expression of conditional cre recombinase in epithelial tissues of transgenic mice. *Genesis* 35: 100-106 (2003)
221. Bex A, M Vooijs, S Horenblas & A Berns. Controlling gene expression in the urothelium using transgenic mice with inducible bladder specific Cre-lox recombination. *J Urol* 168: 2641-2644 (2002)
222. Kemp R, H Ireland, E Clayton, C Houghton, L Howard & DJ Winton. Elimination of background recombination: somatic induction of Cre by combined transcriptional regulation and hormone binding affinity. *Nucleic Acids Res* 32: e92 (2004)
223. Brake RL, PJ Simmons & CG Begley. Cross-contamination with tamoxifen induces transgene expression in non-exposed inducible transgenic mice. *Genet Mol Res* 3: 456-462 (2004)
224. Boutonnet C, O Boijoux, S Bernat, A Kharrat, G Fabre, JC Faye & S Vagner. Pharmacological-based translational induction of transgene expression in mammalian cells. *EMBO Rep* 5: 721-727 (2004)
225. Sakai M, K Mitani & J Miyazaki. Efficient regulation of gene expression by adenovirus vector mediated delivery of CRE recombinase. *Biochem Biophys Res Commun* 217: 393-401 (1995)
226. Kanegae Y, K Takamori, Y Sato, G Lee, M Nakai & I Saito. Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase. *Gene* 181: 207-212 (1996)
227. Nakano M, K Odaka, M Ishimura, S Kondo, N Tachikawa, J Chiba, Y Kanegae & I Saito. Efficient gene activation in cultured mammalian cells mediated by FLP recombinase-expressing recombinant adenovirus. *Nucleic Acids Res* 29: e40 (2001)
228. Shui JW & TH Tan. Germline transmission and efficient DNA recombination in mouse embryonic stem cells mediated by adenoviral-Cre transduction. *Genesis* 39: 217-223 (2004)

229. Stec DE, RL Davidson, RE Haskell, BL Davidson & CD Sigmund. Efficient liver-specific deletion of a floxed human angiotensinogen transgene by adenoviral delivery of Cre recombinase *in vivo*. *J Biol Chem* 274: 21285-21290 (1999)
230. Thevenot E, F Cote, P Colin, Y He, H Leblois, M Perricaudet, J Mallet & G Vodjdani. Targeting conditional gene modification into the serotonin neurons of the dorsal raphe nucleus by viral delivery of Cre recombinase. *Mol Cell Neurosci* 24: 139-147 (2003)
231. Kaspar BK, B Vissel, T Bengoechea, S Crone, L Randolph-Moore, R Muller, EP Brandon, D Schaffer, IM Verma, KF Lee, SF Heinemann & FH Gage. Adeno-associated virus effectively mediates conditional gene modification in the brain. *Proc Natl Acad Sci USA* 99: 2320-2325 (2002)
232. Sinnayah P, TE Lindley, PD Staber, BL Davidson, MD Cassell & RL Davidson. Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain. *Physiol Genomics* 18: 25-32 (2004)
233. Meuwissen R, SC Linn, M van der Valk, WJ Mooi & A Berns. Mouse model for lung tumorigenesis through Cre/lox controlled sporadic activation of the K-Ras oncogene. *Oncogene* 20: 6551-6558 (2001)
234. Siprashvili Z & PA Khavari. Lentivectors for regulated and reversible cutaneous gene delivery. *Mol Ther* 9: 93-100 (2004)
235. Iwatate M, Y Gu, T Dieterle, Y Iwanaga, KL Peterson, M Hoshijima, KR Chien & J Ross. *In vivo* high-efficiency transcoronary gene delivery and Cre-loxP gene switching in the adult mouse heart. *Gene Ther* 10: 1814-1820 (2003)
236. Leow CC, XD Wang & WQ Gao. Novel method of generating prostate-specific Cre-LoxP gene switching via intraductal delivery of adenovirus. *Prostate* 65: 1-9 (2005)
237. Pfeifer A, EP Brandon, N Koostra, FH Gage & IM Verma. Delivery of Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting *in vivo*. *Proc Natl Acad Sci USA* 98: 11450-11455 (2001)
238. Silver DP & DM Livingston. Self-exciding retroviral vectors encoding Cre recombinase overcome Cre-mediated cellular toxicity. *Mol Cell* 8: 233-243 (2001)
239. Rinaldi A, KR Marshall & CM Preston. A non-cytotoxic herpes simpex virus vector which expresses Cre recombinase directs efficient site specific recombination. *Virus Res* 65: 11-20 (1999)
240. Leblois H, C Roche, N Di Falco, C Orsini, P Yeh & M Perricaudet. Stable transduction of actively dividing cells via a novel adenoviral/episomal vector. *Mol Ther* 1: 314-322 (2000)
241. Oehmig A, C Fraefel, XO Breakefield & M Ackermann. Herpes simplex virus type 1 amplicons and their hybrid virus partners, EBV, AAV, and retrovirus. *Curr Gene Ther* 4: 385-408 (2004)
242. Kasim V, M Miyagashi & K Taira. Control of siRNA expression using the Cre-loxP recombination system. *Nucleic Acids Res* 32: e66 (2004)
243. Fritsch L, LA Martinez, R Sekhri, I Naguibneva, M Gerard, M Vandromme, L Schaeffer & A Harel Bellan. Conditional gene knock-down by CRE-dependent short interfering RNAs. *EMBO Rep* 5: 178-182 (2004)
244. Tiscornia G, V Tergaonkar F Galimi & IM Verma. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. *Proc Natl Acad Sci USA* 101: 7347-7351 (2004)
245. Coumoul X, W Li, RH Wang & C Deng. Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. Nucleic Acids Res 32: e85 (2004)
246. Ventura A, A Meissner, CP Dillon, M McManus, PA Sharp, L Van Parijs, R Jaenisch & T Jacks. Cre-lox regulated RNA interference from transgenes. *Proc Natl Acad Sci USA* 101:10380-10385 (2004)
247. Mallo M, B Kanzler & S Ohnemus. Reversible gene inactivation in the mouse. *Genomics* 81: 356-360 (2003)
248. Lakso M, B Sauer, B Mosinger, EJ Lee, RW Manning, SH Yu, KL Mulder & H Westphal. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci USA* 89: 6232-6236 (1992)
249. Politi K, M Szabolcs, P Fisher, A Kljuijc, T Ludwig & A Efstratiadis. A mouse model of uterine leiomyosarcoma. *Am J Pathol* 164: 325-336 (2004)
250. Belteki G, Haigh J, N Kabacs, K Haigh, K Sison, F Costantini, J Whitsett, SE Quaggin & A Nagy. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res* 33: e51 (2005)
251. Lieu HD, SK Withycombe, Q Walker, JX Rong, RL Walzem, JS Wong, RL Hamilton, EA Fisher & SG Young. Eliminating atherogenesis in mice by switching off hepatic lipoprotein secretion. *Circulation* 107: 1315-1321 (2003)
252. McLean GW, NH Komiyama, B Serrels, H Asano, L Reynolds, F Conti, K Hodivala-Dilke, D Metzger, P Chambon, SG Grant & MC Frame. Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev* 18: 2998-3003 (2005)
253. Gothert JR, SE Gustin, JA van Eekelen, U Schmidt, MA Hall, SM Jane, AR Green, B Gottgens, DJ Izon & CG Begley. Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 104: 1769-1777 (2004)
254. Imai T, R Takakuwa, S Marchand, E Dentz, JM Bornert, N Messaddeq, O Wendling, M Mark, B Desvergne, W Wahli, P Chambon & D Metzger. Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci USA* 101: 4543-4547 (2004)
255. Kostetskii I, J Li, Y Xiong, R Zhou, VA Ferrari, VV Patel, JD Molkentin & GL Radice. Induced deletion of the N-cadherin gene in the heart leads to dissolution of the intercalated disc structure. *Circ Res* 96: 346-354 (2005)
256. Eckardt D, M Theis, J Degen, T Ott, HV van Rijen, S Kirchhoff, JS Kim, JM de Bakker & K Willecke. Functional role of connexin43 gap junction channels in adult mouse heart assessed by inducible gene deletion. *J Mol Cell Cardiol* 36: 101-110 (2004)
257. Kroll J, P Cobo & TN Sato. Versatile inducible activation system of Akt/PKB signalling pathway in mice. *Genesis* 35: 160-163 (2003)
258. Petrich BG, JD Molkentin & Y Wang. Temporal activation of c-Jun N-terminal kinase in adult transgenic heart via cre-loxP-mediated DNA recombination. *FASEB J*

17: 749-751 (2003)
259. McMinn JE, SM Liu, I Dragatsis, P Dietrich, T Ludwig, S Eiden & SC Chua. An allelic series for the leptin receptor gene generated by CRE and FLP recombinase. *Mamm Genome* 15: 677-685 (2004)
260. Smith AJ, MA De Sousa, B Kwabi-Addo, A Heppell-Parton, P Impey & P Rabbits. A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nat Genet* 9: 376-385 (1995)
261. Van Deursen J, M Fornerod, B Van Rees & G Grosveld. Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc Natl Acad Sci USA* 92: 7376-7380 (1995)
262. Yu Y & A Bradley. Engineering chromosomal rearrangements in mice. *Nat Rev Genet* 2: 780-790 (2001)
263. Lindsay EA, A Botta, V Jurecic, S Carattini-Rivera, YC Cheah, HM Rosenblatt, A Bradley & A Baldini. Congenital heart disease in mice deficient for the DiGeorge syndrome region. Nature 401: 379-383 (1999)
264. Lewandoski M & GR Martin. Cre-mediated chromosome loss in mice. *Nat Genet* 17: 223-225 (1997)
265. Herault Y, M Rassoultzadegan, F Cuzin & D Duboule. Engineering chromosomes in mice through targeted meiotic recombination (TAMERE) *Nature* 20: 381-384 (1998)
266. Buchholz F, Y Rafaeli, A Trumpp & JM Bishop. Inducible chromosomal translocation of AML1 and ETO genes through Cre/loxP-mediated recombination in the mouse. *EMBO Rep* 1: 133-139 (2000)
267. Forster A, R Pannell, LF Drynan M McCormack EC Collins, A Daser & TH Rabbits. Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. *Cancer Cell* 3: 449-458 (2003)
268. Collins EC, R Pannel, EM Simpson, A Forster & TH Rabbits. Inter-chromosomal recombination of Mll and Af9 genes mediated by cre-loxP in mouse development. *EMBO Rep* 1: 127-132 (2000)
269. Zheng B, AA Mills & A Bradley. A system for rapid generation of coat color-tagged knockouts and defined chromosomal rearrangements in mice. *Nucleic Acids Res* 27: 2354-2360 (1999)
270. Adams DJ, PJ Biggs, T Cox, R Davies, L van der Weyden, J Jonkers, J Smith, B Plumb, R Taylor, I Nishijima, Y Yu, J Rogers & A Bradley. Mutagenic Insetion and Chromosome Engineering Resource (MICER). *Nat Genet* 36: 867-871 (2004)
271. Koike H, K Horie, H Fukuyama, G Kondoh, S Nagata & J Takeda.Efficient biallelic mutagenesis with Cre/loxP-mediated interchromosomal recombination. *EMBO Rep* 3:433-437 (2002)
272. Minasi LE, Y Kamogawa, S Carding, K Bottomly & RA Flavell. The selective ablation of interleukin 2-producing cells isolated from transgenic mice. *J Exp Med* 177: 1451-1459 (1993)
273. Delaney CL, M Brenner & A Messing. Conditional ablation of cerebellar astrocytes in postnatal transgenic mice. *J Neurosci* 16: 6908-6918 (1996)
274. Mathis C, C Hindelang, M LeMeur & E Borrelli. A transgenic mouse model for inducible and reversible dysmyelination. *J Neurosci* 20: 7698-7705 (2000)
275. Garcia AD, NB Doan, T Imura, TG Bush & MV Sofroniew. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat Neurosci* 7: 1233-1241 (2004)
276. Grieshammer U, M Lewandoski, D Prevette, RW Oppenheim & GR Martin. Muscle-specific cell ablation conditional upon Cre-mediated DNA recombination in transgenic mice leads to massive spinal and cranial motoneuron loss. *Dev Biol* 197: 234-247 (1998)
277. Herrera PL, L Orci & JD Vassalli. Two transgenic approaches to define the cell lineages in endocrine pancreas development. *Mol Cel Endocrinol* 140, 45-50 (1998)
278. Brockschnieder D, C Lappe-Siefke, S Goebbels, MR Boesl, KA Nave & D Riethmacher. Cell depletion due to diphtheria toxin fragment A after Cre-mediated recombination. *Mol Cell Biol* 24: 7636-7642 (2004)
279. Buch T, FL Heppner, C Tertilt, TJ Heinen, M Kremer, FT Wunderlich, S Jung & A Waisman. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat Methods* 2: 419-426 (2005)
280. Chen YT, R Levasseur, S Vaishnav, G Karsenty & A Bradley. Bigenic Cre/loxP, puDeltatk conditional genetic ablation. *Nucleic Acids Res* 32: e61 (2004)
281. Sato M, Y Yasuoka, H Kodama, T Watanabe, JI Miyazaki & M Kimura. New approach to cell lineage analysis in mammals using the Cre-loxP system. *Mol Reprod Dev* 56: 34-44 (2000)
282. Araki K, M Araki, J Miyazaki & P Vassalli. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci USA* 92:160-164 (1995)
283. St-Onge L, PA Furth & P Gruss. Temporal control of Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* 24: 3875-3877 (1996)
284. Akagi K, V Sandig, M Vooijs, M Van der Valk, M Giovannini, M Strauss & A Berns. Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res* 25: 1766-1773 (1997)
285. Thorey IS, Muth K, Russ AP, J Otte, A Reffelmann & H von Melchner. Selective disruption of genes transiently induced in differentiating mouse embryonic stem cells by using gene trap mutagenesis and site-specific recombination. *Mol Cell Biol* 18: 3081-3088 (1998)
286. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21: 70-71 (1999)
287. Mao X, Y Fujiwara & SH Orkin. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci USA* 96: 5037-5042 (1999)
288. Kawamoto S, H Niwa, F Tashiro, S Sano, G Kondoh, J Takeda, K Tabayashi & J Miyazaki. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett* 470: 263-268 (2000)
289. Mao X, Y Fujiwara, A Chapdelaine, H Yang & SH Orkin. Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* 97: 324-326 (2001)
290. Srinivas S, T Watanabe, CS Lin, CM William, Y Tanabe, TM Jessell & F Costantini. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 1: 4 (2001)
291. Safran M, WY Kim, AL Kung, JW Horner, RA De

Pinho & WG Kaelin. Mouse reporter strain for non-invasive bioluminescent imaging of cells that have undergone Cre-mediated recombination. *Mol Imaging* 2: 297-302 (2003)
292. Novak A, C Guo, W Yang, A Nagy & CG Lobe. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28: 147-155 (2000)
293. Awatramani R, P Soriano, JJ Mai & S Dymecki. An FLP indicator mouse expressing alkaline phosphatase from the ROSA26 locus. *Nat Genet* 29: 257-259 (2001)
294. Zong H, JS Espinosa, HH Su, MD Muzumbar & L Luo. Mosaic analysis with double markers in mice. *Cell* 121: 479-492 (2005)
295. Bonaventure P, L Umans, MHM Bakker, P Cras, X Langlois, WHML Luyten, AAHP Megens, L Serneels, F van Leuven & JE Leysen. Humanization of mouse 5-hydroxytryptamine 1B receptor gene by homologous recombination. *Mol Pharm* 56: 54-67 (1999)
296. Zou YR, W Muller H Gu & K Rajewsky. Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. *Curr Biol* 4: 1099-1103 (1994)
297. Nebert DW, TP Dalton, GW Stuart & MJ Carvan. "Gene-swap knock-in" cassette in mice to study allelic differences in human genes. *Ann NY Acad Sci* 919: 148-170 (2000)
298. Schake T & J Bode. Use of mutated FLP-recognition-target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* 33: 12746-12751 (1994)
299. Seibler J, D Schubeler, S Fiering, M Groudine & J Bode. DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker free-constructs. *Biochemistry* 37: 6229-6234 (1998)
300. Soukharev S, JL Miller & B Sauer. Segmental genomic replacement in embryonic stem cells by double lox targeting. *Nucleic Acids Res* 27: e21 (1999)
301. Araki K, M Araki & K Yamamura. Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic Acids Res* 30: e103 (2002)
302. Feng YQ, J Seibler, R Alami, A Eisen, KA Westerman, P Leboulch, S Fiering & EE Bouhassira. Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange. *J Mol Biol* 292: 779-785
303. Baer A & J Bode. Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr Opin Biotechnol* 12: 473-480 (2001)
304. Lauth M, F Spreafico, K Dethleffsen & M Meyer. Stable and efficient cassette exchange under non-selectable conditions by combined use of two site-specific recombinases. *Nucleic Acids Res* 30: e115 (2002)
305. Lauth M, K Moerl, JJ Barski & M Meyer. Characterization of Cre-mediated cassette exchange after plasmid microinjection in fertilized mouse oocytes. *Genesis* 27: 153-158 (2000)
306. Cobellis G, G Nicolaus, M Iovino, A Romito, E Marra, M Barbarisi, M Sardiello, FP Di Giorgio, N Iovino, M Zollo, A Ballabio & R Cortesse. Tagging genes with cassette-exchange sites. *Nucleic Acids Res* 33: e44 (2005)
307. Olivares EC, RP Hollis, TW Chalberg, L Meuse, MA Kay & MP Calos. Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat Biotechnol* 20: 1124-1128 (2002)
308. Schnütgen F, S De-Zolt, P Van Sloun, M Hollatz, T Floss, J Hansen, J Altschmied, C Seisenberger, Norbert B. Ghyselinck, Patricia Ruiz, Pierre Chambon, W Wurst & H von Melchner. Genomewide production of multipurpose alleles for the functional analysis of the mouse genome. *Proc Natl Acad Sci USA* 102: 7221-7226 (2005)

Abbreviations: 4-OHT: 4-hydroxytamoxifen; BAC: bacterial artificial chromosome; bp: base pairs; Cre: causes recombination; DHT: dihydrotestosterone; DNA: deoxyribonucleic acid; DSE: distal sequence element; DTA: diphtheria toxin fragment A; ECFP: enhanced cyane fluorescent protein; EGFP: enhanced green fluorescent protein; ENU: N-ethyl N-nitrosourea; ER: estrogen receptor; ES cells: embryonic stem cells; EYFP: enhanced yellow fluorescent protein; FACS: fluorescence activated cell sorting; FIAU: 2'-fluoro-2'deoxy-5-iodo-1-beta-D-arabinofuranosyluracil; floxed allele: allele with inserted loxP sequences; FLP: flippase; FRT: FLP recombination target; HR: homologous recombination; HSV: herpes simplex virus; IRES: internal ribosome entry sequence; kb: kilobase; kDa: kiloDalton; LBD: ligand binding domain; LCR: locus control region; loxP: locus of crossover in P1; MADM: mosaic analysis with double markers; MAR: matrix attachment region; Mer: murine estrogen receptor; MICER: Mutagenic Insertion and Chromosome Engineering Resource; MRI: magnetic resonance imaging; NLS: nuclear localization signal; nt: nucleotide; PCR: polymerase chain reaction; PGK: phosphoglycerol kinase; PR: progesterone receptor; PSE: proximal sequence element; RNA: ribonucleic acid; RNAi: RNA interference; rtTA: reverse tetracycline transactivator; SAR: scaffold attachment region; siRNA: small interference RNA; TAMERE: targeted meiotic recombination; TetR: tetracycline repressor protein; TK: thymidine kinase; tTA: tetracycline transactivator

Key Words: Mouse, Site-Specific Recombinase, Genetically Modified Mouse Models, Spatial And Temporal Gene Inactivation, Conditional Gene Targeting, Review

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