

Conditional Somatic Mutagenesis in the Mouse Using Site-Specific Recombinases

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Abstract In the last decade, site-specific recombinases (SSRs), such as Cre and Flp, have emerged as indispensable tools for the precise in vivo manipulation of the mouse genome. It is now feasible to control, in space and time, the onset of gene knockouts in almost any tissue of the mouse, thus greatly facilitating the creation of sophisticated animal models for human disease and drug development. This review describes the basic principles and current status of the SSR technology, with a focus on strategies for conditional somatic mutagenesis using the Cre/*lox* system and ligand-activated Cre recombinases. Practical hints for generating and analysing conditional mouse mutants will be given and exciting novel applications of the SSR technology will be discussed, such as cell fate mapping and the combined use of Cre, Flp and other biotechnological tools. It will be shown how genetic manipulation of the mouse by site-specific recombination can provide new solutions to old problems in the analysis of human physiology and pathophysiology and how it can be employed for drug discovery and development.

Keywords Somatic mutagenesis · Conditional gene targeting · Mouse models of human disease · CreER recombinase · Tamoxifen

1

Introduction

Although other mammals, such as rats, pigs and primates, might be better models for specific aspects of human physiology and pathophysiology, the laboratory mouse has evolved into the pre-eminent model species, because it is readily amenable to a wide array of methods for genetic modification. In particular, it is the only species to date for which embryonic stem (ES) cells are available that can be genetically manipulated at predetermined sites by homologous recombination *in vitro*, a method known as gene targeting or targeted transgenesis, and then transmitted through the germ line to establish a genetically modified animal. The most popular application of gene targeting is the generation of so-called knockout mice that carry defined loss-of-function gene mutations, but in principle this technique can be used to manipulate any chosen mouse locus in any desired manner (Capecchi 2005). As opposed to gene targeting, foreign DNA (the transgene) can also be integrated into the genome at sites that are not known *a priori*. The *random* integration of transgenes is usually achieved by injection of the transgenic DNA construct into the male pronucleus of a fertilized egg, but other routes are also possible, for example, viral transfer of the transgene into oocytes or transfection of ES cells with the DNA construct. The genetically modified eggs or ES cells are then used to establish a transgenic mouse line that carries one or more copies of the transgene at one or more sites in its genome. Random transgenesis is most commonly used to (over-)express a gene of interest for gain-of-function studies or to produce biotechnological protein tools such as the Cre recombinase (see below).

Without doubt, both random and targeted transgenesis in the mouse have greatly advanced our understanding of mammalian gene function. However, both methods also suffer from a number of limitations because they create genetic modifications that are permanently fixed in the germ line and, therefore, are present in all cells of the animal throughout life. For example, a conventional gene knockout may be embryonically lethal, precluding the analysis of the gene's function(s) at later stages, or the knockout may initiate a cascade of secondary or compensatory responses during pre- and postnatal development, thereby complicating the interpretation of the phenotype. In general, the chronic nature of germ line mutations precludes the analysis of gene function in a specific cell type and at given time. Furthermore, the conventional methods for random and targeted transgenesis are not suitable to engineer complex chromosomal alterations (large deletions, duplications, inversions and translocations) that are often associated with human pathologies. Thus, although conventional germ line mouse mutants have contributed many valuable models of human disease states (Chien 1996; Wynshaw-Boris 1996; Steele et al. 1998; Offermanns and Hein 2004), they are not ideal to reproduce large chromosomal rearrangements and to model acquired diseases that arise during

postnatal life through the interaction of somatic mutations and environmental factors, such as sporadic cancer and probably many other diseases (Jonkers and Berns 2002; Erickson 2003). These limitations were recently overcome by the combination of conventional germ line transgenesis with site-specific recombination technology (Metzger and Feil 1999; Nagy 2000; Tronche et al. 2002; Branda and Dymecki 2004; Glaser et al. 2005; Garcia-Otin and Guillou 2006). Site-specific recombination relies on site-specific recombinases (SSRs) that can cut and paste DNA fragments between short recognition sites, thereby generating defined chromosomal deletions, inversions and translocations. This review begins with an overview on the fundamental properties of SSRs and strategies for advanced genome engineering using SSRs, followed by a discussion of current and potential future applications of the SSR technology in the mouse, with a focus on time- and tissue-specific somatic mutagenesis, to generate more realistic animal models of human diseases.

2

Basic Properties of SSRs

In contrast to homologous recombination that occurs between *any* two homologous sequences through a largely unknown molecular machinery, site-specific recombination is characterized by the reciprocal exchange between two specific DNA recognition sites mediated by a SSR (Sadowski 1986). Site-specific recombination reactions can generate integration, excision and inversion of defined DNA segments. They occur in nearly every organism and cell, and are driven by a primary need to physically join or separate DNA segments. Examples include the integration and excision of bacteriophage λ in the *Escherichia coli* chromosome, the DNA inversion responsible for flagellar phase variation in *Salmonella* and, in a broader sense, also most DNA transposition events as well as VDJ recombination of immunoglobulin genes that contributes to the generation of antibody diversity.

Virtually all identified SSRs fall into two families which have been named after the catalytic amino acid, the tyrosine recombinases (also known as the λ integrase family) and the serine recombinases (also known as the resolvase family). The last years have brought a wealth of new knowledge on the biochemical and structural aspects of site-specific recombination (Van Duyne 2001; Grindley et al. 2006). The minimal components of a site-specific recombination system are (1) a pair of DNA recombination sites (approximately 20–200 bp in length) and (2) a specialized SSR that recognizes these sites, aligns and breaks them and rejoins them in a reciprocal manner (Fig. 1A). The recombination sequences are partially asymmetric, conferring directionality to the recombination process. Consequently, the outcome depends on the location and relative orientation of the recognition sites with respect to one another. If the two sites are on the same DNA molecule, recombination

between sites that are in the opposite orientation causes inversion of the DNA between the two sites (Fig. 1B), whereas recombination between sites that are in the same orientation results in excision of the intervening DNA in the form of a circular product (Fig. 1C). If the sites are on separate DNA molecules, the recombination is intermolecular and can produce DNA integration, for example, in a reaction that is formally the reversal of excision (Fig. 1C). All reactions are reversible, but intramolecular recombination is more efficient than intermolecular recombination. Thus, it is easier to obtain stable DNA

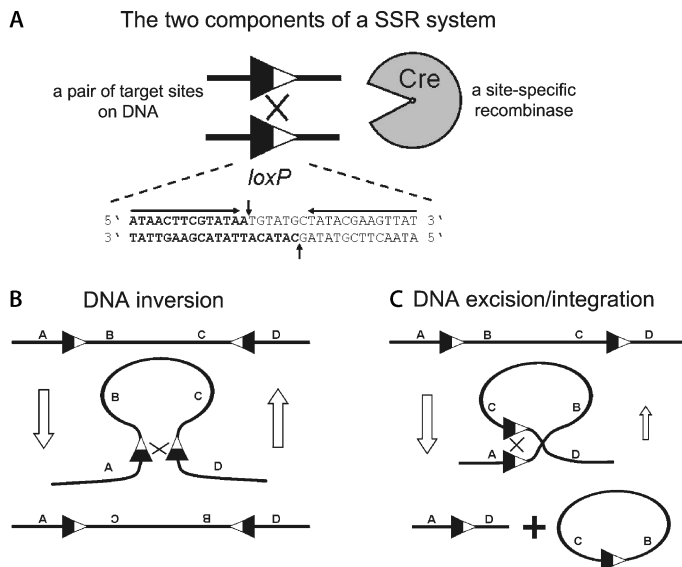


Fig. 1 A–C Basic principles of site-specific recombination as illustrated by the Cre/lox system. **A** The Cre recombinase (*pacman*) promotes reciprocal strand exchange between two 34-bp *loxP* target sites (*triangles*). Each *loxP* sequence consists of two 13-bp inverted repeats (*horizontal arrows*) flanking an 8-bp asymmetric spacer sequence that confers overall directionality. After binding of one Cre monomer to each inverted repeat, the DNA strands are cleaved in the spacer region (*vertical arrows*), exchanged between the two *loxP* sites, and ligated. The two half-sites of the *loxP* sequence that are recombined in a reciprocal manner are indicated by the *black and white segments of the triangles* and by *bold and standard lettering*. Note that the recombination reaction is conservative, i.e. it does not involve any net synthesis or loss of DNA so that two new functional *loxP* sites are generated. **B** Recombination between two *loxP* sites inserted into the same DNA molecule (intramolecular recombination) in opposite orientation leads to inversion of the intervening DNA segment. **C** Recombination between directly repeated *loxP* sites results in excision of the flanked DNA (circular product that is degraded) leaving one *loxP* site behind. When the *loxP* sites are located on separate DNA molecules (*lower part*), intermolecular recombination can lead to DNA integration. For kinetic reasons, DNA excision is strongly favoured over integration and, due to degradation of the circular product, can be considered irreversible. The dimensions of the *white arrows* indicate the relative efficiencies of the respective recombination reactions

excision than stable integration or inversion. The recombination reaction proceeds via covalent recombinase-DNA intermediates with strict conservation of phosphodiester bond energy, and requires no DNA synthesis. The mechanism is analogous to that of DNA topoisomerase, in that DNA strands are broken not by hydrolysis but rather by direct phosphoryl transfer to the nucleophilic hydroxyl group of a catalytic tyrosine or serine residue. The cleaved DNA strands are then rejoined to new partners by reversing the process. Thus, a SSR can be viewed as site-specific endonuclease and ligase in one package. Importantly, SSRs do not require high-energy cofactors such as ATP and many of them work independently of other proteins, although in some cases one or more auxiliary proteins may regulate the timing or outcome of the reaction.

In the first half of the 1990s, several laboratories demonstrated that one site-specific recombination system, the Cre/lox system, works particularly well in the mouse (Lakso et al. 1992; Orban et al. 1992; Gu et al. 1993; Araki et al. 1995), and the seminal work of Klaus Rajewsky's group showed how Cre/lox-mediated recombination can be adapted to generate tissue-specific (Gu et al. 1994) and inducible (Kuhn et al. 1995) knockout mice (see Sect. 3). The Cre (cyclization recombination) recombinase is a 38-kDa protein encoded by bacteriophage P1 that recombines two 34-bp target sites on the P1 genome called *loxP* (locus of crossing-over [X] of P1) without the need for any co-factor (Hoess and Abremski 1990). The *loxP* sequence consists of two 13-bp inverted repeats flanking an 8-bp asymmetric spacer region that confers overall directionality (Fig. 1A). Binding of one Cre monomer to each of the inverted repeats promotes the formation of a synaptic complex of two *loxP* sites and four Cre molecules followed by strand cleavage, exchange and ligation within the spacer regions.

To date the Cre/lox system is the most efficient and advanced tool for site-specific genome engineering in the mouse. Table 1 gives an overview on Cre and various modified Cre recombinases as well as some recent additions to the SSR toolbox with potential utility for in vivo applications. There are also a number of ligand-inducible Cre recombinases available that represent fusion proteins of Cre and mutated ligand-binding domains (LBDs) of steroid receptors. These so-called CreLBD recombinases as well as other strategies that confer inducibility upon the SSR technology will be discussed in Sect. 4. Among the useful non-Cre recombinases is the Flp (flips DNA) recombinase of *Saccharomyces cerevisiae*, which recombines sequences called *FRT* (Flp recombinase target sites). As compared to Cre, the efficiency of Flp-mediated recombination in the mouse is relatively low due to the lower stability of Flp at 37°C (Buchholz et al. 1996). However, the thermostable version Flpe (Buchholz et al. 1998; Rodriguez et al. 2000) and its tamoxifen-activated derivative FlpeER^{T2} (Hunter et al. 2005) might have an in vivo performance comparable to Cre and ligand-activated Cre recombinases, respectively. Based on in vitro studies with cultured mammalian cells, other promising SSR tools include the *Streptomyces* phage-derived Φ C31 recombinase, the bacterial β recombinase, and the Cre-like Dre recombinase (Table 1 and refs. therein). However, further

studies are required to evaluate the usefulness of these latter SSRs for in vivo applications. In general, tyrosine recombinases like Cre and Flp might perform better in eukaryotic cells as compared to serine recombinases like Φ C31 and β recombinase, because the latter require a distinct level of supercoiling of their DNA substrate, which is usually supplied by their prokaryotic host. Certainly, Flp and the other non-Cre SSRs will find their niches for more specialized applications, such as the removal of selectable marker genes and site-specific integration of DNA. In addition, it is expected that combined with Cre they will permit highly flexible engineering strategies, such as multiple independently controlled genetic modifications in the same animal.

The following sections will discuss the current state and future potential of SSR technology, focussing on Cre/*lox*- mediated somatic mutagenesis in the mouse as a means to faithfully model acquired human diseases. Other issues of SSR technology, such as the use of modified SSR target sites to achieve stable DNA integration or inversion, and its application for conditional gene trapping and large-scale mutagenesis screens have been excellently reviewed in other chapters of this book (e.g., see the chapters by V. Brault et al. and by A. Abuin et al., this volume) as well as in the recent literature (Branda and Dymecki 2004; Glaser et al. 2005).

3

Genome Engineering Strategies Using SSRs

The basic strategy for SSR-directed genetic engineering is to insert the SSR recognition sites into the chromosomes, and then to deliver the SSR to recombine them as required. As opposed to conventional gene targeting that produces permanent mutations in the germ line and, thus, in every cell of the animal (Fig. 2A), SSR technology allows for the conditional generation of predetermined genetic alterations in selected somatic cells (Fig. 2B, C). Currently, the major tool to create conditional somatic genome modifications in vivo is the Cre/*lox* system, and its most popular application is the generation of so-called conditional knockout mice by time- and tissue-specific deletion of *loxP*-flanked gene segments. The tissue specificity of the gene knockout is achieved by directing Cre expression to the cell type of interest (Fig. 2B), and additional temporal control over the knockout can be obtained by using ligand-inducible Cre recombinases (Fig. 2C, for details, see Sect. 4).

In general, a Cre-mediated tissue-specific gene knockout is produced by crossing two transgenic mouse lines; one line carries a conditional or *loxP*-flanked version of the target gene (floxed target mouse; Fig. 3, left), and the other one expresses Cre selectively in the tissue of interest (tissue-specific Cre mouse; Fig. 3, right). To generate the floxed target mouse, normally an essential exon of the target gene is tagged for excision by inserting two directly repeated *loxP* sequences into the flanking introns by homologous recombination in ES

Table 1 SSRs and some of their derivatives useful for mouse SSR technology

SSR / target site	Properties and application(s)	Reference(s)
A) SSR systems with proven efficiency in cultured mammalian cells as well as in mice		
Cre/loxP	Biological function: DNA excision for dimer reduction of bacteriophage P1 plasmids Most efficient and widely used SSR tool in vitro and in vivo	Sternberg et al. 1981 See text
EGFP-Cre	Fusion with an N-terminal EGFP; facilitates recombinase detection	Le et al. 1999
iCre	Codon-improved version for expression in mammalian cells	Shimshek et al. 2002
Cell-permeable Cre	Fusion with membrane translocation sequences such as the basic HIV-TAT peptide; the efficiency of cell-permeable Cre proteins in vivo is not clear (see the chapter by C. Patsch and F. Edenhofer, this volume)	Jo et al. 2001; Joshi et al. 2002; Peitz et al. 2002
CreLBDs	Various fusions with mutated steroid receptor LBDs; inducible by synthetic ligands of the LBD	See Sect. 4
Flp/FRT	Biological function: DNA inversion for amplification of yeast 2- μ m plasmid Removal of selection cassettes and other more specialized transactions	Vetter et al. 1983 Rodriguez et al. 2000; Schnutgen et al. 2005
Flpe	Mutated version selected in a protein evolution strategy with increased activity	Buchholz et al. 1998; Rodriguez et al. 2000
FlpeER ^{T2}	Tamoxifen-inducible version of Flpe; might perform similar to CreER fusions in mice (see Sect. 4)	Hunter et al. 2005
B) SSR systems with proven efficiency in cultured mammalian cells and potential utility in mice		
ΦC31/att	Biological function: DNA integration and excision of <i>Streptomyces</i> phage Φ C31 Potentially useful for stable integration of transgenes	Thorpe and Smith 1998 Olivares et al. 2002; Belteki et al. 2003
Φ C31-NLS	A version with a C-terminal nuclear localization signal; displays enhanced efficiency	Andreas et al. 2002
β recombinase/six	Biological function: Resolution of plasmid oligomers in Gram-positive bacteria Catalyzes exclusively intramolecular recombination like excision and inversion	Rojo and Alonso 1994 Diaz et al. 1999
β -EGFP	Fusion with a C-terminal EGFP; facilitates recombinase detection	Servet et al. 2006
β -AR, β -EGFP-AR	Fusion with the androgen receptor LBD; inducible with mibolerone; also functional as a triple fusion with a central EGFP	Servet et al. 2006
Dre/rox	Cre-like recombinase encoded by the P1-related bacteriophage D6	Sauer and McDermott 2004

AR, androgen receptor; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; LBD, ligand-binding domain; NLS, nuclear localization signal

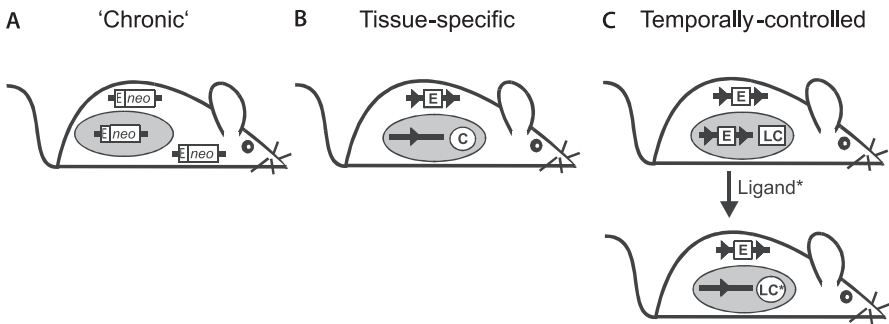


Fig. 2 A–C Conventional vs conditional knockout mice. **A** Conventional gene targeting through germ-line mutation, for example, by the insertion of a neomycin resistance cassette (*neo*) into an essential exon (*E*) of the target gene, produces a chronic gene knockout in all cells. **B** Tissue-specific gene inactivation is based on excision of a *loxP* (triangle)-flanked exon (*E*) in Cre (*C*)-expressing cells (shaded oval). **C** Temporal control over recombination can be obtained by using a ligand-dependent Cre recombinase (*LC*) that is inactive in the absence (boxed *LC*) and active in the presence (circled *LC**) of a synthetic ligand (*). Spatio-temporally controlled somatic mutagenesis can be achieved by tissue-specific expression of a ligand-dependent Cre recombinase

cells (Fig. 3, left). To select the ES cells, a positive selection marker such as *neo*^r is co-integrated along with the *loxP* sites into the target locus. However, the cassette should later be removed, because it might downregulate the expression of the target gene producing a hypomorphic allele, or otherwise disturb the expression of the target gene and/or nearby genes and, thereby, confound the analysis of the animal's phenotype (Olson et al. 1996). In the tri-*lox* strategy, three *loxP* sites are introduced such that they flank both the exon and the selection cassette (Fig. 3, left). This potentially hypomorphic tri-*lox* allele (L3) can then be manipulated by Cre-mediated recombination in ES cells and/or in mice. Selective excision of the selection cassette converts the L3 allele into a conditional allele with two *loxP* sites (L2), and further excision creates a null allele with one *loxP* site left behind (L1). Thus, an allelic series of the target gene, from hypomorphic (L3) to conditional (L2) to null (L1) can be generated from a single construct. An alternative strategy for removal of the selection marker cassette is to use *FRT*-flanked (flrted) cassettes that can be excised by Flpe (not shown). The tissue-specific Cre mouse is mostly established by random integration of a Cre transgene driven by a tissue-specific promoter (Fig. 3, right). By intercrossing the floxed target mouse and the Cre transgenic mouse, both components of the SSR system are brought together in the offspring, so that the target exon will be deleted in all Cre-expressing cells and a tissue-specific gene knockout is established (Fig. 3, bottom).

In addition to the inactivation of endogenous target genes, the Cre/*lox* system is a powerful tool for a number of other applications. For instance, Cre-mediated DNA excision can be used to switch irreversibly between the

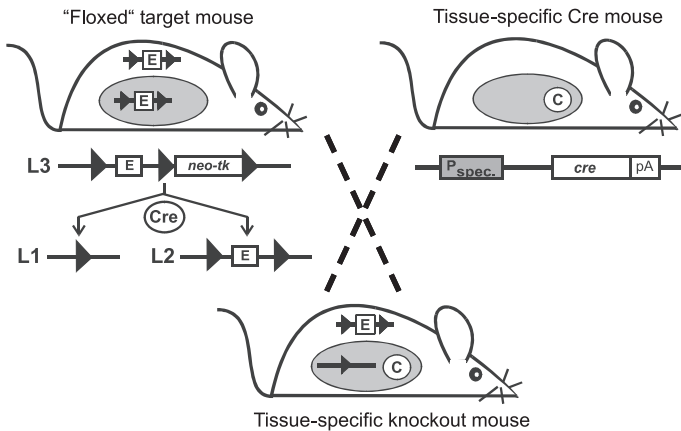


Fig. 3 Generation of a tissue-specific knockout mouse. Two mouse lines are required, a floxed target mouse and a tissue-specific Cre mouse. The floxed target mouse (*left*) is generated by homologous recombination in ES cells. A popular strategy is to integrate a DNA construct that harbours three directly repeated *loxP* sites (*triangles*) flanking an essential exon (*E*) together with a selectable marker cassette (*neo-tk*) into the target locus, thereby generating a potentially hypomorphic tri-*lox* (*L3*) allele. The next step is to express Cre in the correctly targeted ES cell clones (or later in the respective mice) in order to convert the *L3* allele by selective excision of the selection cassette to the conditional floxed (*L2*) allele. Note that complete excision generates a null (*L1*) allele that can be used as an alternative to a conventional gene knockout (see Fig. 2a). Whereas the *neo* gene (neomycin phosphotransferase) is used to select for ES cells that have integrated the DNA construct (positive selection with G418), the *tk* gene (herpes simplex virus thymidine kinase) is useful in the second step to select for cells that have undergone Cre-mediated excision of the *neo-tk* cassette (negative selection with ganciclovir). The tissue-specific Cre mouse (*right*) is in most cases generated by random integration of a *cre* transgene (containing a polyA signal sequence, *pA*) that is driven by a tissue-specific promoter (*P_{spec.}*) to express Cre in the cell type of interest (*shaded oval*). Intercrossing of the floxed target mouse and the tissue-specific Cre mouse results in offspring (*bottom*) in which the floxed target exon is being excised in all Cre-expressing cells (*shaded oval*), thereby generating a tissue-specific knockout mouse

expression of two transgenes (Fig. 4A). Also, large-scale chromosomal rearrangements can be generated such as translocations between homologous chromosomes or chromatids and, though very inefficiently, even between non-homologous chromosomes (Fig. 4B) (Herault et al. 1998; Forster et al. 2003; Spitz et al. 2005; Zong et al. 2005). A detailed discussion of Cre/*lox*-mediated chromosome engineering is presented in the chapter by V. Brault et al., this volume.

Critical to the success of conditional somatic mutagenesis is the availability of Cre transgenic mouse strains in which Cre expression/activity is tightly controlled in space and time. However, two general problems inherent to the transgenic technology, namely leaky and mosaic expression of the transgene,

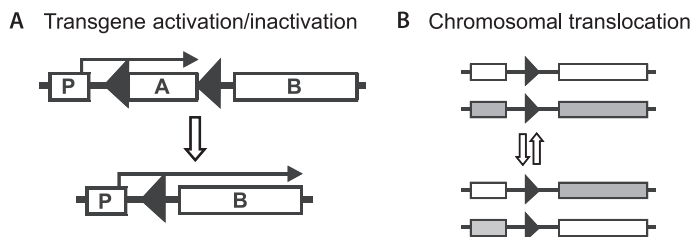


Fig. 4 A,B Advanced Cre/lox-assisted genome engineering strategies. **A** Transgene activation/inactivation. A promoter (*P*) drives transcription (*horizontal arrow*) of a loxP (*triangle*)-flanked gene A and gene B before and after Cre-mediated excision, respectively. In a popular configuration, gene A contains a transcriptional STOP sequence whose Cre-mediated removal activates the expression of gene B. Gene B can encode, for instance, a reporter protein (e.g. β -galactosidase or EGFP) for monitoring of Cre activity or for cell fate mapping, a tetracycline-dependent transactivator for tetracycline-regulated gene expression, a diphtheria toxin receptor for cell ablation, a small hairpin RNA for gene knockdown via RNA interference, as well as an oncogene or tumour suppressor for studying cancer. By placing the inducible cassette into a widely expressed locus (e.g. *ROSA26*), tissue-specific expression of gene B can be achieved simply by crossing to a tissue-specific Cre mouse as required. **B** Chromosomal translocation. By placing the loxP sites (*triangles*) on different chromosomes, chromosomal translocations with specific breakpoints can be created, for example, to model certain human cancers. Cre-mediated translocations are feasible between homologous or heterologous chromosomes. Note, however, that the efficiency of Cre-mediated interchromosomal rearrangements, in particular in the case of nonhomologous chromosomes, is fairly low, presumably reflecting chromosomal position within the cell during interphase and mitosis

often complicate the analysis of the mutant phenotype (Dobie et al. 1997). The leakiness of Cre expression from a cell type-specific or inducible promoter can lead to recombination in unwanted cell types and/or at the wrong time. Indeed, the Cre/lox system can be considered an extremely sensitive method to monitor and integrate the activity of a given promoter over a defined time frame. If the promoter driving the Cre transgene is ectopically active during early embryogenesis, recombined DNA might be present in most adult tissues. On the other hand, mosaic expression of Cre prevents recombination from taking place in *all* cells of the cell type of interest. Depending on the experimental strategy, mosaic recombination can be a problem or an advantage. Consider, for example, the tissue-specific knockout of a secreted protein. In this case, the presence of even very few wild-type cells, that remain in the target tissue and still secrete the factor, can prevent the development of a phenotype. In contrast, for the modelling of sporadic genetic diseases such as cancer, the presence of both wild-type and mutant cells in the same tissue reproduces the pathological features more accurately. The use of more sophisticated technologies for Cre expression, such as bacterial artificial chromosomes (BACs) and knock-in strategies, should help to obtain reliable and tightly controlled Cre activity in transgenic mice (Giraldo and Montoliu 2001; Ristevski 2005).

Today, hundreds of Cre transgenic mouse lines are available, covering almost every tissue and cell type, and efforts are underway to establish a comprehensive and dynamic Cre mouse line database (<http://www.mshri.on.ca/nagy/>). For the proper analysis of mutant phenotypes produced by a given Cre mouse, it is crucial to know its spatio-temporal recombination pattern at the cellular level. Note that the absence of Cre itself in a given cell at a given time does not necessarily reflect a lack of recombination, which could have occurred by transient Cre expression during earlier stages. Thus, functional analysis of Cre activity is needed to properly characterize a Cre mouse. An elegant way to monitor Cre-mediated recombination with single cell resolution is the use of Cre reporter mice that carry a floxed DNA segment which, when deleted by Cre, induces the expression of a cellular marker protein such as β -galactosidase or EGFP (Fig. 4A). An accurate readout of recombination is only obtained, however, if the promoter driving reporter gene expression is active in all recombined cells. Today, a number of useful Cre-responsive, and more recently also FLP-responsive, indicator strains are available (Branda and Dymecki 2004), the most popular one being the so-called R26R line that produces β -galactosidase after Cre-mediated excision of a STOP cassette from the broadly expressed *ROSA26* locus (Soriano 1999). Although it is highly recommended to use only Cre mouse lines whose recombination properties have been validated by reporter gene studies, it is important to note that the efficiency of Cre-mediated recombination can be locus-dependent and, therefore, the recombination pattern obtained with a particular reporter gene does not necessarily predict that of other floxed genes (Vooijs et al. 2001). Thus, when performing a conditional gene knockout experiment, it is mandatory to monitor the expression of the target gene, preferably at the cellular and protein level. Depending on the rate of mRNA and protein turnover, it may take several weeks until the gene product has disappeared in the recombined cells. Last but not least, it is important to control for potential phenotypes caused by the presence of the Cre transgene alone (Schmidt et al. 2000; Loonstra et al. 2001; Lee et al. 2006).

4

Ligand-Activated SSRs

In many cases, tissue-specific genome modifications would be more informative if they could be induced at will at a chosen time during the life of the animal. Furthermore, a temporally-controlled Cre/*lox* system would allow one to limit unwanted Cre activity and associated side effects, for instance, ectopic recombination due to transient Cre expression during development or potential toxic effects due to prolonged high levels of Cre activity (Schmidt et al. 2000; Loonstra et al. 2001), although the collective experience with hundreds of Cre transgenic mouse strains suggests that Cre toxicity is more likely an exception than the rule.

Currently, the standard approach for the external control of the temporal onset of site-specific recombination is the use of ligand-dependent SSRs that are selectively activated by synthetic drugs (Fig. 2C). Based on the observation that the activity of a number of proteins can be controlled by a ligand when fused to the ligand-binding domain (LBD) of a steroid hormone receptor (Picard 1994), chimeric FlpLBD (Logie and Stewart 1995) and CreLBD (Metzger et al. 1995) recombinases were developed that are indeed activated by ligands of the respective steroid receptor LBD. Further refinement by introducing specific mutations into the LBDs led to CreLBD recombinases that are responsive to synthetic but not natural LBD ligands. Fusion of Cre with mutated LBDs of the estrogen receptor (ER), progesterone receptor (PR) or glucocorticoid receptor (GR) resulted in tamoxifen-activated CreER (Feil et al. 1996, 1997; Zhang et al. 1996), RU486-activated CrePR (Kellendonk et al. 1996) or dexamethasone-activated CreGR (Brocard et al. 1998) recombinases, respectively. How do these ligand-dependent Cre recombinases work? The current model proposes that in the absence of ligand the chimeric CreLBD recombinase is retained in the cytoplasm, and that binding of the cognate ligand to the LBD results in the translocation of the recombinase into the nucleus where it can recombine its *loxP*-flanked DNA substrate (Fig. 5); in other words: ligand binding appears to regulate primarily the *localization* of the recombinase rather than its enzymatic activity per se.

Among the various CreLBDs, the CreER^T recombinases, which are insensitive to endogenous β -estradiol but activated by the synthetic ER antagonist 4-hydroxytamoxifen (OHT), proved particularly useful for in vivo applications. From the first demonstration that ligand-activated site-specific recombination is feasible in adult mice (Feil et al. 1996) as well as in the developing mouse embryo (Danielian et al. 1998), the properties of tamoxifen-activated Cre recombinases were continuously improved. Transgenic mice expressing the original CreER^T recombinase (containing the human ER-LBD with a G521R mutation) (Feil et al. 1996) or the CreER^T-like recombinase CreERTM (containing the mouse ER-LBD with a G525R mutation) (Danielian et al. 1998) have the limitation that relatively high doses of tamoxifen (which is converted by the liver to the active inducer OHT) are necessary to induce recombination, which may result in undesired side effects. Consequently, novel tamoxifen-activated Cre recombinases were developed to increase the sensitivity and efficiency of inducible recombination in mice (Feil et al. 1997). One of them, CreER^{T2} (containing the human ER-LBD with a G400V/M543A/L544A triple mutation) is indeed approximately tenfold more sensitive to OHT activation than CreER^T (Feil et al. 1997; Indra et al. 1999). The CreER^{T2} recombinase is currently the sharpest tool in the CreLBD box and its use is highly recommended for temporally controlled somatic mutagenesis in the mouse. Table 2 lists a number of transgenic mouse lines that express CreER^{T2} in specific somatic tissues, and many of them have proven useful in addressing biological questions. It should be noted that the mode of tamoxifen administration (dose, route, frequency) can strongly affect recombination and should, therefore, be optimized for each

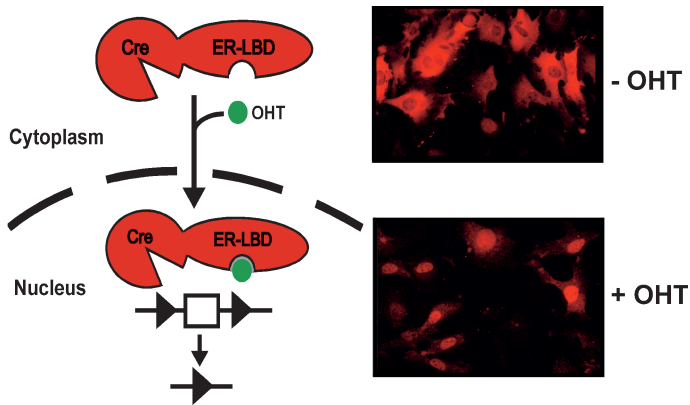


Fig. 5 How do ligand-dependent Cre recombinases work? These recombinases are fusion proteins between Cre and the ligand-binding domains (LBDs) of steroid receptors. The LBD has been mutated so that it does not respond to its natural ligand yet binds a synthetic ligand. The scheme (*left*) illustrates the current model with the tamoxifen-activated CreER^T recombinase (*modified pacman*), a fusion of Cre with a mutated estrogen receptor (ER) LBD that responds specifically to the synthetic drug 4-hydroxytamoxifen (OHT) but not to β -estradiol. In the absence of OHT, the recombinase is located in the cytoplasm. Binding of OHT to the LBD results in the translocation of the recombinase into the nucleus where it can recombine its *loxP* substrates (*triangles*). *Right* Experimental support for this model of tamoxifen-induced nuclear translocation. The CreER^{T2} recombinase was expressed in cultured vascular smooth muscle cells and then detected with a Cre antibody in the absence and presence of OHT (R. Feil, unpublished data, 2006)

application. The spatial control of recombination can be further refined by localized tamoxifen administration, for instance, to a selected region of the skin (Vasioukhin et al. 1999) or, by using a perivascular tamoxifen-eluting cuff, to a defined segment of a blood vessel (Zadelaar et al. 2006). Importantly, it is possible to titrate the rate of recombination by the dose of tamoxifen given to the animal (Kuhbandner et al. 2000). Although most experiments may aim at maximal recombination efficiency, the ability to induce graded levels down to a very low number of recombined cells can be a prerequisite for certain studies, such as the creation of faithful mouse models for sporadic cancer or the analysis of cell lineages by fate mapping (see Sect. 5).

Other ligand-activated SSRs are also useful for inducible somatic mutagenesis in the mouse. The tamoxifen-activated MerCreMer recombinase (Zhang et al. 1996; Sohal et al. 2001), a double fusion of Cre with two ERTM LBDs, has been constructed to eliminate potential background activity of the CreERTM single fusion in the absence of ligand. Although leakiness is not an issue with most CreER transgenic mouse lines, it might be a problem of certain strains expressing the CrePR1 recombinase that responds to the synthetic steroid RU486, but has some degree of activity already in the absence of inducer (Kellendonk et al. 1999). An improved version, termed Cre*PR, displays lower background

Table 2 Examples of mouse lines expressing the CreER^{T2} recombinase

Tissue specificity	Mouse line	Strategy	Promoter	Reference(s)
Bone (osteoblasts and odontoblasts)	Coll1a1-CreER ^{T2}	tg	Collagen 1 α 1 chain	Kim et al. 2004
Endothelium	Tie2-CreER ^{T2}	tg	Tie2 receptor tyrosine kinase	Forde et al. 2002
Epithelium				
Intestinal epithelium	Vil-CreER ^{T2}	tg	Villin	el Marjou et al. 2004
Internal epithelial organs	K18-CreER ^{T2}	tg	Keratin 18	Wen et al. 2003
Renal epithelium	KspCad-CreER ^{T2}	tg	Kidney-specific cadherin	Lantinga-van Leeuwen et al. 2006
Fat (adipocytes)	aP2-CreER ^{T2}	tg	Adipocyte fatty acid binding protein	Imai et al. 2001
Liver (hepatocytes)	SA-CreER ^{T2}	ki	Serum albumin	Schuler et al. 2004
Nervous system				
Astrocytes	GFAP-CreER ^{T2}	tg	Glial fibrillary acidic protein	Hirrlinger et al. 2006
Neural stem cells	GLAST-CreER ^{T2}	ki	Astrocyte-specific glutamate transporter	Mori et al. 2006
Schwann cells and oligodendrocytes	Nes-CreER ^{T2}	tg	Nestin	Imayoshi et al. 2006
	PLP-CreER ^{T2}	tg	Proteolipid protein	Leone et al. 2003
Schwann cells	POCx-CreER ^{T2}	tg	P0 fused to connexin 32	Leone et al. 2003
Skeletal muscle	HAS-CreER ^{T2}	tg (PAC)	Skeletal muscle α -actin	Schuler et al. 2005
Skin				
Keratinocytes	K5-CreER ^{T2}	tg	Keratin 5	Indra et al. 1999
	K14-CreER ^{T2}	tg	Keratin 14	Li et al. 2000
Melanocytes	Tyr-CreER ^{T2}	tg	Tyrosinase	Yajima et al. 2006
	Tyr-CreER ^{T2}	tg	Tyrosinase	Bosenberg et al. 2006
Smooth muscle	SM-CreER ^{T2}	ki	SM22 α	Kuhbandner et al. 2000
Widespread	Rosa26-CreER ^{T2}	ki	Rosa26	Seibler et al. 2003

ki, knock-in; PAC, P1-derived artificial chromosome; tg, transgene

activity and increased sensitivity to RU486 in cultured cells (Wunderlich et al. 2001). However, the *in vivo* performance of Cre*PR has not been reported so far. Recently, a tamoxifen-inducible FlpeERT² recombinase has been developed (Hunter et al. 2005), thus adding temporal control to the Flp/*FRT* system (Table 1).

An alternative strategy for the temporal control of recombination is based on the inducible expression of SSRs using the tetracycline-regulated expression system (St-Onge et al. 1996) or other suitable promoters such as the interferon- α/β inducible *Mx1* promoter (Kuhn et al. 1995) or the β -naphthoflavone-inducible *Ah* promoter (Ireland et al. 2004). A general problem of transcriptional regulation is the tight shutdown of recombinase expression before induction, as even a very low level of leakiness of an inducible promoter can result in the expression of SSR molecules sufficient to cause considerable background recombination. A number of tetO-Cre mouse strains have been generated to express Cre under the control of the tetracycline-responsive transactivators, tTA or rtTA (see also the chapter by R. Sprengel and M.T. Hasan, this volume). The tTA binds to the tetO operator sequences and thereby activates transcription from a tetO-linked minimal promoter in the absence but not in the presence of tetracycline (tet-off system), whereas the rtTA (reverse tTA) acts the other way round, being capable of tetO binding and transcriptional activation only in the presence of tetracycline (tet-on system). Interestingly, many tetO-Cre strains express Cre in a tetracycline-independent manner (Leneuve et al. 2003), presumably due to integration of the tetO-Cre transgene nearby endogenous enhancers that activate the tetO-associated minimal promoter. However, it appears that there is at least one tetO-Cre strain, LC-1, in which Cre expression is tightly controlled by tetracycline (Schonig et al. 2002). Combined with tissue-specific expression of tTA or rtTA transgenes and floxed target sequences, the LC-1 line should be useful for time- and tissue-specific mutagenesis.

Spatio-temporally controlled recombination can also be achieved by the administration of Cre-encoding virus particles to mice (see the chapter by P. Osten et al., this volume). The tissue-specificity of recombination can be controlled by the route of virus administration, the spectrum of cells susceptible to infection, and by selection of the promoter driving Cre expression. However, overall control of recombination may not be as precise as with transgenic Cre mice and viral infections may induce side effects.

5

SSR Technology in Biomedicine and Drug Development

As detailed in the foregoing sections, SSR technology offers the ability to control gene activities in the mouse in space and time, thus providing a means to faithfully model the development of human diseases. The first disease models generated by Cre/*lox*-mediated tissue-specific gene knockouts were reported

by the end of the last century; examples include the inactivation of the insulin receptor gene in skeletal muscle (Bruning et al. 1998) and pancreatic β -cells (Kulkarni et al. 1999), which led to new concepts on type 2 diabetes, and the generation of mouse models for human mitochondrial DNA disorders (Wang et al. 1999) and breast cancer (Xu et al. 1999). Shortly after, the utility of CreER recombinases for studying biological questions was demonstrated by the generation of skin abnormalities after temporally controlled ablation of the retinoid receptor RXR α in mouse epidermis (Li et al. 2000). To date, a plethora of time- and tissue-specific mouse mutants have been described, covering a great variety of human diseases. For details on the current state of available conditional mouse models for various signalling pathways and diseases of the cardiovascular, nervous, and immune system, the reader is referred to the second part of this book. Genetically modified mice can also be useful at several points in the drug discovery and development process, including target identification and validation, and preclinical evaluation of drug efficacy and safety (Prosser and Rastan 2003). For instance, inducible gene activation or inactivation is the model of choice for target validation because it most closely mimics the effect of administration of an agonist or antagonist to the target in question and it might also predict potential side effects. Further, it is expected that mouse models that mimic human variation in drug response will play a central role in pharmacogenomic research (Liggett 2004).

Clearly, among the most powerful abilities of the SSR technology is the modelling of human cancer (Jonkers and Berns 2002; Hirst and Balmain 2004). Indeed, one of the first applications of Cre-mediated DNA excision in mice was the tissue-specific activation of an oncogene (Lakso et al. 1992). Many human tumours are associated with specific chromosomal translocations, which cannot be generated with conventional gene targeting technology. Recently, the capacity of the Cre/lox system to engineer chromosomal rearrangements with specific breakpoints (Fig. 4B) has been successfully applied to directly recapitulate naturally occurring human cancer-associated translocations (Forster et al. 2003). Moreover, somatic mutations can now be induced in a tissue-specific and time-controlled fashion, which more faithfully mimics sporadic tumour formation. Today, mouse models of all major human cancers are available and, combined with noninvasive technologies for tumour imaging, these models will enable us to follow tumour progression and metastasis *in vivo*, as well as the effects of candidate therapeutic drugs (see also the chapter by D. Vignjevic et al., this volume).

Beyond modelling of human diseases and drug action, SSR technology can be applied to track specific cell lineages on a wild-type or mutant genetic background (O’Gorman et al. 1991) or to detect cell fusion events *in vivo* (Alvarez-Dolado et al. 2003). Because site-specific recombination results in a permanent genomic change which is stably inherited to all cells derived from the original recombined population, it is ideal for genetic labelling of a cell lineage. Cre-directed cell fate mapping is based on the intercrossing of

a tissue-specific Cre mouse and a Cre indicator mouse (e.g. R26R), resulting in permanent expression of the reporter gene (e.g. β -galactosidase) in all originally recombined cells and their progeny, thereby marking these cells and revealing their contribution to embryonic and adult tissues (e.g. by staining cells blue with X-Gal). Ideally, Cre expression should be under the control of an endogenous gene specifying the cell lineage of interest, whereas the reporter transgene should be linked to a widely active promoter capable of driving its expression in all cell types and at all stages of pre- and postnatal development (see also the chapter by M. Lewandoski, this volume). SSR-mediated fate mapping was first applied by developmental biologists to characterize cell lineages during embryogenesis (Dymecki and Tomasiewicz 1998; Zinyk et al. 1998). An important advance was the introduction of CreLBD recombinases like CreER^{T2} allowing the investigators to label relevant lineages at different developmental stages (Ahn and Joyner 2004; Harfe et al. 2004). Temporally controlled fate mapping using tamoxifen-activated Cre recombinases has also been used to tackle a number of other biological questions that were otherwise difficult to study, for example, the contribution of bone marrow-derived cells to tumour endothelium (Gothert et al. 2004) or the existence of native cardiac progenitor cells in the postnatal heart (Laugwitz et al. 2005). Furthermore, the combination of tamoxifen-controlled gene targeting and cell marking allows one to directly monitor the fate of wild-type vs mutant cells during disease development in adult mice (Wolfsgruber et al. 2003; Feil et al. 2004).

6

Recent Developments in SSR Technology

Although the SSR technology has rapidly evolved in the last decade to become one of the most advanced tools for genome engineering, there is still room for improvement. So what are the major areas to watch?

An important issue is the further refinement of inducible SSR technology. The leakiness of some temporally controlled SSR systems based on either an inducible promoter or a ligand-activated CreLBD recombinase might be sealed by combining the transcriptional and post-translational level of regulation (Kyrkanides et al. 2003). Indeed, background recombination was undetectable in transgenic mice expressing the CreERTM recombinase under the control of the β -naphthoflavone-inducible *Ah* promoter, whereas recombination could be induced by combined treatment with β -naphthoflavone and tamoxifen (Kemp et al. 2004). Recombination might also be controlled in a light-directed manner by using a CreER recombinase in combination with a photocaged tamoxifen derivative (Link et al. 2005). Another approach to add conditionality to site-specific recombination is based on the model of α complementation in the β -galactosidase enzyme. Interestingly, Cre recombinase can be split into two polypeptides that, when co-expressed, are able to associate into a func-

tional Cre enzyme (Casanova et al. 2003). External control can be provided by a ligand-induced complementation system. To this end, Cre fragments have been modified so that they can be heterodimerized by the drug rapamycin (Jullien et al. 2003). Last but not least, temporal control over the onset of recombination in vivo might also be achieved by relatively simple means, such as administration of a cell-permeable Cre protein (see the chapter by C. Patsch and F. Edenhofer, this volume) or by hydrodynamic injection of a recombinase-expressing plasmid into the tail vein (Olivares et al. 2002; Chen and Woo 2005), although spatial control of recombination is relatively loose with these methods. Further studies will show whether these novel conditional strategies will work efficiently in the mouse in vivo.

As discussed in this chapter, there are more applications for site-specific recombination than there are SSRs. Ideally, each application would have its own recombinase, for instance, Cre for conditional mutagenesis, Flpe for selection cassette removal, a third SSR for chromosome engineering, a fourth for reporter gene activation, and so on. Consequently, new useful SSRs are urgently needed to complement Cre and Flpe. Promising candidates are Φ C31, β recombinase and Dre (Table 1), but their utility for in vivo applications remains to be demonstrated. The combined use of Cre, FLP and other SSRs will permit highly flexible engineering strategies, such as multiple independently controlled genetic modifications. For example, through application of two ligand-dependent SSRs that recombine different target sites and respond to different drugs, such as Cre*PR and FlpeER^{T2}, it should be possible to induce two independent genetic events at selected time points in the same animal.

Another emerging trend is the combination of SSR technology with other biotechnological tools. Advanced methods for conditional gene expression have been developed by combining conditional Cre-mediated DNA excision with the activation of a gene of interest. A popular strategy is to knock-in the gene of interest into the widely expressed ROSA26 locus such that its expression is dependent on Cre-mediated removal of a transcriptional STOP cassette (Fig. 4A), a configuration resembling that of the R26R Cre reporter (see Sect. 3) but with the β -galactosidase gene replaced by the gene of interest. This strategy allows the use of the growing resource of cell type-specific and inducible Cre strains to restrict activation of the gene of interest to specific tissues and time points. Recent examples include the Cre-mediated control of tetracycline-dependent gene expression (Belteki et al. 2005; Mao et al. 2005; Yu et al. 2005), RNAi-mediated gene knockdowns (Yu and McMahon 2006) or diphtheria toxin-mediated cell lineage ablation (Buch et al. 2005; Ivanova et al. 2005), a new approach to studying the role of particular cell types in vivo.

Finally, a limitation of current conditional mutagenesis strategies is the time required to construct targeting vectors and to generate mice that carry the floxed DNA and recombinase transgene, taking in most cases at least 2–3 years. In the future, novel ways of target vector construction based on long-range PCR amplification of homology arms (Randolph et al. 1996), BAC transgen-

ics (Testa et al. 2003; Yang and Seed 2003) or recombineering methodology (Copeland et al. 2001; Muyrers et al. 2001) will speed up gene targeting, and improved ES cell technologies might enable us to circumvent time-consuming breeding steps. One approach requiring less than 50% of the time of traditional breeding strategies and far fewer animals is to generate ES cells with the desired genotype and then establish mice derived completely from these cells by tetraploid blastocyst complementation (see the chapter by J.S. Draper and A. Nagy, this volume).

7

Concluding Remarks

The SSR technology described herein allows one to delete, add, replace, or modify genes in the mouse at will in order to dissect the complex pathways of mammalian physiology and pathophysiology, which is also the key to selecting the right drug targets and developing new drugs for the therapy of human diseases. Although this review focussed on SSR-directed mutagenesis, the reader should be aware of additional strategies for the control of gene expression in the mouse (Lewandoski 2001; Berger and Bujard 2004). Alternative approaches to conditional mutagenesis are based on tetracycline-regulated expression systems, other inducible gene switches, and gene silencing by RNA interference (see the chapters by R. Sprengel and M.T. Hasan, W. Weber and M. Fussenegger, and R. Kühn et al., respectively, this volume). There is little doubt that conditional mouse mutants will be increasingly used to study gene functions *in vivo*, and we can expect them to become central players in the functional genomics arena as well as in biomedicine and pharmaceutical research. However, it is important to note that mouse is not man; in other words, basic principles learned in mice might not always be directly applicable to humans. For example, there is increasing evidence for species-specific drug actions, which has been shown most recently by the devastating effects of the superagonist monoclonal antibody TGN1412 in human volunteers (Wood and Darbyshire 2006). In the future, such problems might be overcome by the development of humanized mouse models that carry partial or complete human physiological systems (Macchiaroni et al. 2005), and the SSR technology is likely to be instrumental in converting mouse genes to their respective human counterparts in order to create humanized mice.

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