Conditional Mutagenesis: An Approach to Disease Models

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Robert Feil and
Daniel Metzger

Springer

Handbook of Experimental Pharmacology

Volume 178

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With 64 Figures and 37 Tables

ISSN 0171-2004

ISBN-10 3-540-35108-6 Springer Berlin Heidelberg New York

ISBN-13 978-3-540-35108-5 Springer Berlin Heidelberg New York

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Editor: Simon Rallison, London
Desk Editor: Susanne Dathe, Heidelberg
Cover design: *design & production* GmbH, Heidelberg, Germany
Typesetting and production: LE-TEX Jelonek, Schmidt & Vöckler GbR, Leipzig, Germany
Printed on acid-free paper 27/3100-YL - 5 4 3 2 1 0

Preface

The understanding and treatment of human diseases is one of the biggest challenges of mankind. Driven by new technological developments, biomedical research generates an ever-increasing knowledge about human health and disease. Today, molecular medicine is arguably one of the most exciting research areas bridging the life sciences and medical sciences. It focuses on the molecular dissection of physiological and pathophysiological processes, and uses this information to improve the prevention, diagnosis and treatment of human disease. Molecular medicine is an interdisciplinary research area that brings together people and ideas from various fields, such as biology, biochemistry, physiology and pharmacology, as well as pharmaceutical and clinical sciences. Investigators are increasingly confronted with the generation and/or analysis of genetically engineered mice, which have emerged as the pre-eminent animal models for exploring human biology. Although some aspects of human (patho)physiology might be better reproduced in other mammals, the laboratory mouse is in many cases an excellent experimental system for generating animal models of human diseases. Indeed, the mouse has many anatomical, physiological and metabolic parallels with humans. The similarities range from embryonic development to physiological homeostasis, reproduction and behaviour in adults. Many disease processes in mice accurately mimic those in humans. These similarities are reflected at the genomic level, as virtually every human gene has a counterpart in the mouse. Moreover, mouse housing is relatively inexpensive compared to other mammals. They require relatively little space and have short gestation periods, a brief time to sexual maturity and large litter sizes. Finally, and most important, a number of genetic manipulations are feasible in the mouse. Currently it is the only mammal where it is possible to generate targeted genome modifications, such as conventional and conditional gene knockouts.

Classic techniques for mutagenesis in the mouse were developed some 20 years ago. These methods introduce a permanent genetic modification into the germ line, which is a good mimicry for a hereditary disease. However, germ line mouse mutants are not appropriate to model "acquired" diseases that arise through the interaction of somatic mutations and environmental factors, such as sporadic cancer and presumably many other human diseases. Thanks to

the development of conditional mutagenesis during the last decade, the experimental induction of specific somatic mutations within the living mouse, in a selected cell type and at a given time, has become reality, and can now be done with extraordinary precision. To date, a plethora of conditional mouse mutants has been described covering a great variety of human diseases. The ability to delete, add, replace or modify genes in a spatio-temporally controlled manner allows one to dissect the complex cellular and molecular processes of mammalian pathophysiology. Conditional mouse mutants are useful at several stages in drug discovery and development, such as target identification and validation, as well as preclinical evaluation of drug efficacy and safety. For instance, inducible gene ablation is the method of choice for target validation, because it closely simulates the administration of an antagonist to a given target. Furthermore, it is expected that mouse models that mimic human variation in drug response will play a central role in pharmacogenomic research.

The aim of this book is to provide a timely and comprehensive review of the tools for conditional mutagenesis and their application to generate faithful mouse models for human diseases and drug development. Accordingly, it is organized in two parts. Part I introduces the basic methodologies for generating time- and tissue-specific somatic mouse mutants: site-specific recombination systems, in particular the Cre/lox system; chromosome engineering; tetracycline-controlled and other gene switches; gene trap mutagenesis; RNA interference; viral and protein transduction; and new developments in embryonic stem cell technology. Part II is an up-to-date compilation of conditional disease models ranging from embryonic development to adulthood, including models for cancer and for disorders of the immune, neuronal and cardiovascular system. Moreover, it covers diseases related to the dysfunction of ion channels, G-protein-coupled receptors and nuclear hormone receptors. The chapters have been written by leading experts in the field. They provide an overview on the current state and future developments as well as a detailed discussion of the various mutagenesis methods and disease models. Tables list the most important transgenic mouse lines and existing disease models, and figures illustrate the techniques and major new concepts derived from the mouse models.

There is little doubt that conditional mouse models will play a central role in the field of molecular medicine, in particular in the translation of advances in basic research into drug discovery and, finally, clinical benefit. When compiling the contents of this volume, it was our intention to include chapters that not only cover the application of conditional mutagenesis in biomedical research, but also provide detailed information on the methods behind this powerful technology. As is the case with most tools, a sound understanding of its operating mode, its advantages and potential pitfalls will help in designing the most informative experiments. We do hope that this book will

be a useful guide for both graduate students and advanced scientists working in biomedical research and development. Last but not least, we would like to thank the authors and all those who contributed to the success of this project, especially Susanne Dathe from Springer for her kindness and patience.

Tübingen and Strasbourg, Robert Feil, Daniel Metzger
September 2006

List of Contents

Part I. Tools for Conditional Mutagenesis

Conditional Somatic Mutagenesis in the Mouse
Using Site-Specific Recombinases 3
R. Feil

Cre/*loxP*-Mediated Chromosome Engineering of the Mouse Genome . . 29
V. Brault, V. Besson, L. Magnol, A. Duchon, Y. Hérault

Tetracycline-Controlled Genetic Switches 49
R. Sprengel, M.T. Hasan

Novel Gene Switches 73
W. Weber, M. Fussenegger

Improved Embryonic Stem Cell Technologies 107
J.S. Draper, A. Nagy

Gene Trap Mutagenesis 129
A. Abuin, G.M. Hansen, B. Zambrowicz

RNA Interference in Mice 149
R. Kühn, S. Streif, W. Wurst

Viral Vectors: A Wide Range of Choices and High Levels of Service . . . 177
P. Osten, V. Grinevich, A. Cetin

Conditional Mutagenesis by Cell-Permeable Proteins:
Potential, Limitations and Prospects 203
C. Patsch, F. Edenhofer

Part II. Examples of Conditional Disease Models

Analysis of Mouse Development with Conditional Mutagenesis 235
M. Lewandoski

Conditional Mouse Models of Cancer 263
D. Vignjevic, S. Fre, D. Louvard, S. Robine

Conditional Mutagenesis Reveals Immunological Functions of Widely Expressed Genes: Activation Thresholds, Homeostatic Mechanisms and Disease Models 289
J. Roes

Conditional Transgenesis and Recombination to Study the Molecular Mechanisms of Brain Plasticity and Memory 315
K. Baumgärtel, C. Fernández, T. Johansson, I.M. Mansuy

A Novel Conditional Knockout Strategy Applied to Serotonin Receptors 347
K.L. Stark, C. Gross, J. Richardson-Jones, X. Zhuang, R. Hen

Conditional Mouse Models for Friedreich Ataxia, a Neurodegenerative Disorder Associating Cardiomyopathy 365
H. Puccio

Animal Models in Cardiovascular Diseases: New Insights from Conditional Models 377
A. Nguyen Din Cat, Y. Sainte-Marie, F. Jaisser

Conditional Animal Models for the Study of Lipid Metabolism and Lipid Disorders 407
H.H. Bock, J. Herz, P. May

Conditional Mouse Models to Study Developmental and Pathophysiological Gene Function in Muscle 441
B.R. Wamhoff, S. Sinha, G.K. Owens

Analysis of Calcium Channels by Conditional Mutagenesis 469
S. Moosmang, T. Kleppisch, J. Wegener, A. Welling, F. Hofmann

Conditional Mutagenesis of G-Protein Coupled Receptors and G-Proteins 491
S. Offermanns

Contribution of Targeted Conditional Somatic Mutagenesis to Deciphering Retinoid X Receptor Functions and to Generating Mouse Models of Human Diseases 511
D. Metzger, P. Chambon

Subject Index 525

List of Contributors

Addresses given at the beginning of respective chapters

Abuin, A. , 129

Baumgärtel, K. , 315
Besson, V. , 29
Bock, H.H. , 407
Brault, V. , 29

Cetin, A. , 177
Chambon, P. , 511

Draper, J.S. , 107
Duchon, A. , 29

Edenhofer, F. , 203

Feil, R. , 3
Fernández, C. , 315
Fre, S. , 263
Fussenegger, M. , 73

Grinevich, V. , 177
Gross, C. , 347

Hansen, G.M. , 129
Hasan, T. , 49
Hen, R. , 347
Hérault, Y. , 29
Herz, J. , 407
Hofmann, F. , 469

Jaisser, F. , 377
Johansson, T. , 315

Kleppisch, T. , 469
Kühn, R. , 149

Lewandoski, M. , 235

Louvard, D. , 263

Magnol, L. , 29
Mansuy, I.M. , 315
May, P. , 407
Metzger, D. , 511
Moosmang, S. , 469

Nagy, A. , 107
Nguyen Din Cat, A. , 377

Offermanns, S. , 491
Osten, P. , 177
Owens, G.K. , 441

Patsch, C. , 203
Puccio, H. , 365

Richardson-Jones, J. , 347
Robine, S. , 263
Roes, J. , 289

S. Sinha, , 441
Sainte-Marie, Y. , 377
Sprengel, R. , 49
Stark, K.L. , 347
Streif, S. , 149

Vignjevic, D. , 263

Wamhoff, B.R. , 441
Weber, W. , 73
Wegener, J. , 469
Welling, A. , 469
Wurst, W. , 149

Zambrowicz, B. , 129
Zhuang, X. , 347

Part I
Tools for Conditional Mutagenesis

HEP (2007) 178:3–28
© Springer-Verlag Berlin Heidelberg 2007

Conditional Somatic Mutagenesis in the Mouse Using Site-Specific Recombinases

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1 Introduction 4

2 Basic Properties of SSRs 5

3 Genome Engineering Strategies Using SSRs 8

4 Ligand-Activated SSRs 13

5 SSR Technology in Biomedicine and Drug Development 17

6 Recent Developments in SSR Technology 19

7 Concluding Remarks 21

References 22

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

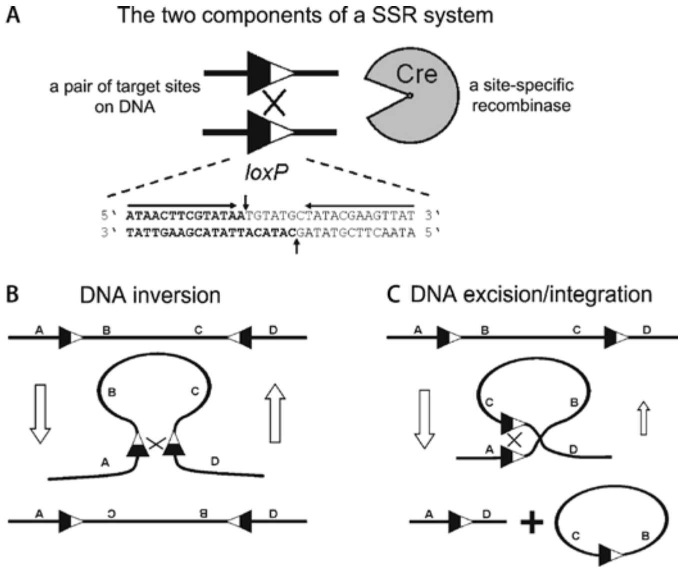

Fig. 1 A–C Basic principles of site-specific recombination as illustrated by the Cre/*lox* system. **A** The Cre recombinase (*pacman*) promotes reciprocal strand exchange between two 34-bp *loxP* target sites (*triangles*). Each *loxP* sequence consists of two 13-bp inverted repeats (*horizontal arrows*) 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	Sternberg et al. 1981
	Most efficient and widely used SSR tool in vitro and in vivo	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-μm plasmid	Vetter et al. 1983
	Removal of selection cassettes and other more specialized transactions	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		
Φ*C31/*att	Biological function: DNA integration and excision of *Streptomyces* phage *Φ*C31	Thorpe and Smith 1998
	Potentially useful for stable integration of transgenes	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	Rojo and Alonso 1994
	Catalyzes exclusively intramolecular recombination like excision and inversion	Diaz et al. 1999
β-EGFP	Fusion with a C-terminal EGFP; facilitates recombinase detection	Servert 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	Servert 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

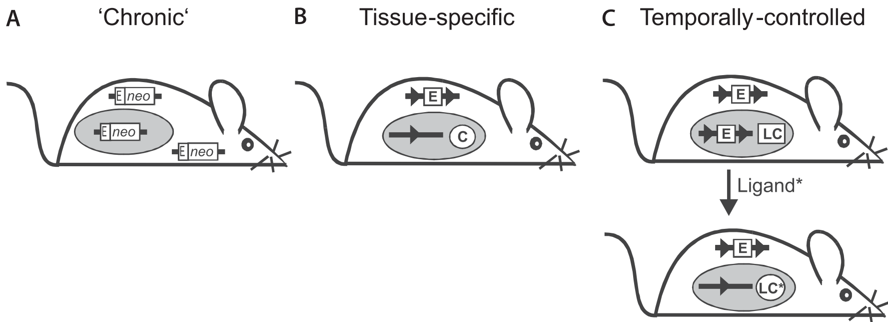

Fig. 2 A–C Conventional vs conditional knockout mice. **A** Conventional gene targeting through germ-line mutation, for example, by the insertion of a neomycin resistance cassette (*neo*) into an essential exon (*E*) of the target gene, produces a chronic gene knockout in all cells. **B** Tissue-specific 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

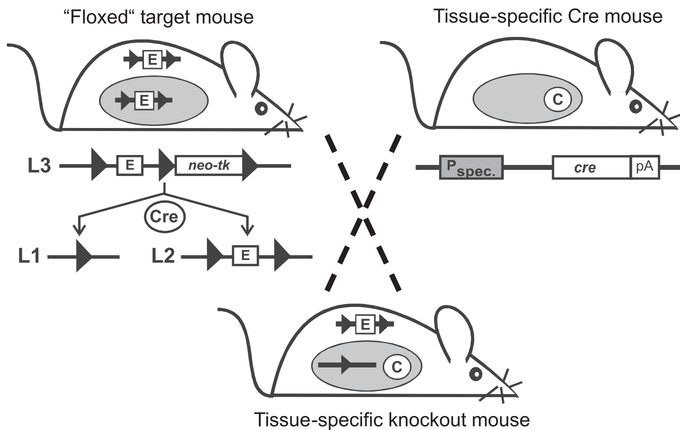

Fig. 3 Generation of a tissue-specific knockout mouse. Two mouse lines are required, a floxed target mouse and a tissue-specific Cre mouse. The floxed target mouse (*left*) is generated by homologous recombination in ES cells. A popular strategy is to integrate a DNA construct that harbours three directly 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 nonhomologous 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,