Conditional Mutagenesis: An Approach to Disease Models

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Robert Feil and
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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.

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Part I
Tools for Conditional Mutagenesis

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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

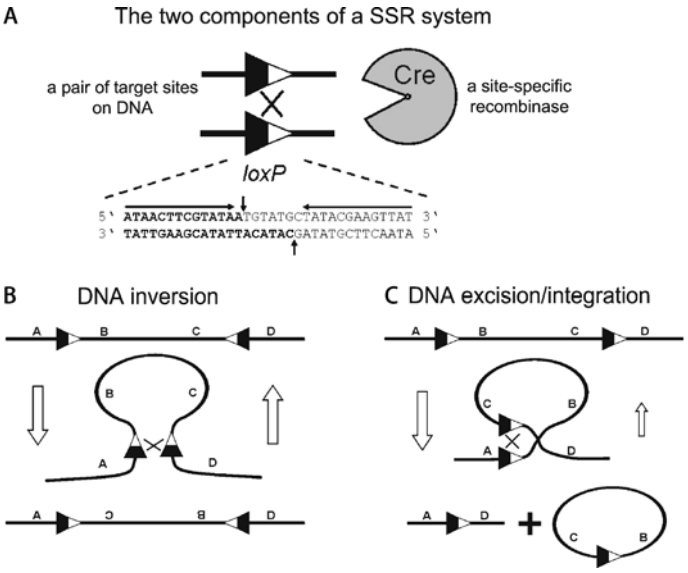

Fig. 1 A–C Basic principles of site-specific recombination as illustrated by the Cre/*lox* system. **A** The Cre recombinase (*pacman*) promotes reciprocal strand exchange between two 34-bp *loxP* target sites (*triangles*). Each *loxP* sequence consists of two 13-bp inverted repeats (*horizontal arrows*) 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 ΦC31 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

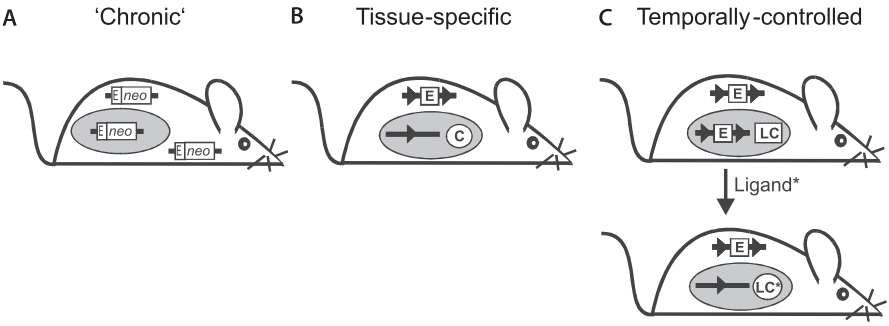

Fig. 2 A–C Conventional vs conditional knockout mice. **A** Conventional gene targeting through germ-line mutation, for example, by the insertion of a neomycin resistance cassette (*neo*) into an essential exon (*E*) of the target gene, produces a chronic gene knockout in all cells. **B** Tissue-specific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

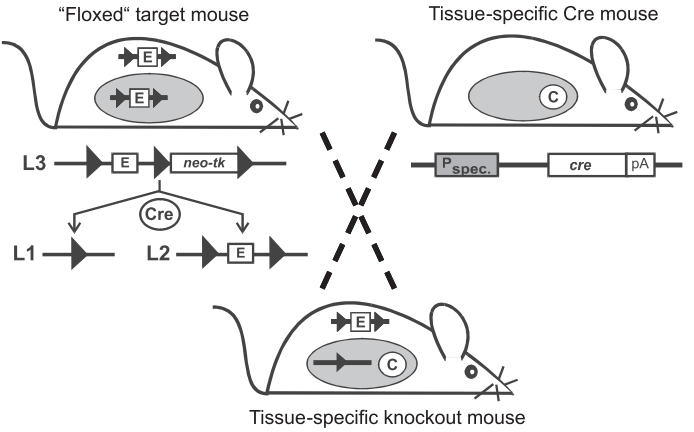

Fig. 3 Generation of a tissue-specific knockout mouse. Two mouse lines are required, a floxed target mouse and a tissue-specific Cre mouse. The floxed target mouse (*left*) is generated by homologous recombination in ES cells. A popular strategy is to integrate a DNA construct that harbours three directly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

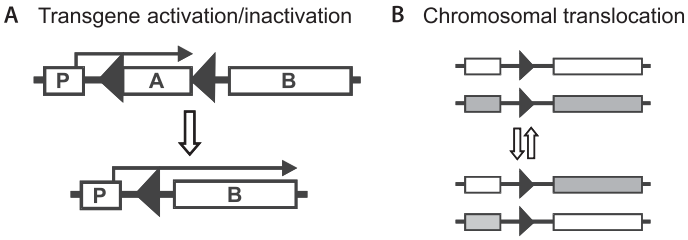

Fig. 4 A,B Advanced Cre/*lox*-assisted genome engineering strategies. **A** Transgene activation/inactivation. A promoter (*P*) drives transcription (*horizontal arrow*) of a *loxP* (*triangle*)-flanked gene A and gene B before and after Cre-mediated excision, respectively. In a popular configuration, 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 CreER^{TM} (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

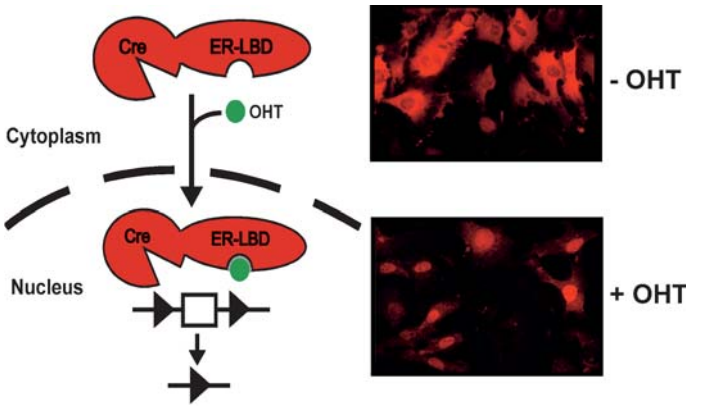

Fig. 5 How do ligand-dependent Cre recombinases work? These recombinases are fusion proteins between Cre and the ligand-binding domains (*LBDs*) of steroid receptors. The LBD has been mutated so that it does not respond to its natural ligand yet binds a synthetic ligand. The scheme (*left*) illustrates 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 ER^{TM} LBDs, has been constructed to eliminate potential background activity of the CreER^{TM} 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)	Col1a1-CreER^{T2}	tg	Collagen 1 α1 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
	GLAST-CreER^{T2}	ki	Astrocyte-specific glutamate transporter	Mori et al. 2006
Neural stem cells	Nes-CreER^{T2}	tg	Nestin	Imayoshi et al. 2006
Schwann cells and oligodenrocytes	PLP-CreER^{T2}	tg	Proteolipid protein	Leone et al. 2003
Schwann cells	P0Cx-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	SM22α	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 FlpeER^{T2} 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-naphtoflavone-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 RXRα 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 CreER^{TM} recombinase under the control of the β-naphtoflavone-inducible *Ah* promoter, whereas recombination could be induced by combined treatment with β-naphtoflavone 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 (Macchiarini 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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Cre/*loxP*-Mediated Chromosome Engineering of the Mouse Genome

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Abstract Together with numerous other genome modifications, chromosome engineering offers a very powerful tool to accelerate the functional analysis of the mammalian genome. The technology, based on the Cre/*loxP* system, is used more and more in the scientific community in order to generate new chromosomes carrying deletions, duplications, inversions and translocations in targeted regions of interest. In this review, we will present the basic principle of the technique either in vivo or in vitro and we will briefly describe some applications to provide highly valuable genetic tools, to decipher the mammalian genome organisation and to analyze human diseases in the mouse.

Keywords Deletion · Duplication · Inversion · Translocation · Mutagenesis

1 Introduction

Large genomic rearrangements have been used for decades and generated routinely in lower organisms such as *Drosophila* or yeast. Obviously, the analysis of lower organisms made it possible to reveal well-conserved molecular, cellular and developmental processes among species. Nevertheless, the understanding of more defined molecular mechanisms and pathologies inherent to humans requires the use of an animal model genetically, morphologically

and physiologically closer. The mouse constitutes a model organism of choice with an anatomy, physiology and genetics highly similar to humans. In addition, 80% of mouse genes have an orthologous counterpart in the human genome (Waterston et al. 2002) and 99% have a sequence match. Less than 1% of the genes of both genomes do not share any homology, and homologous genes are found in conserved syntenic regions in which their order and relative orientation are kept and that encompass large blocks of genomic DNA. Therefore, strategies to produce large rearrangements in the mouse genome offer new perspectives to study gene function, to increase the understanding of the molecular mechanisms and pathways underlying normal and pathological development and to elucidate chromosomal organisation. Chromosome engineering is also crucial for the study of contiguous gene syndromes associated with chromosomal rearrangements that result in gene dosage anomalies.

Different approaches had been used for inducing *chromosome rearrangements* in the mouse such as X-ray irradiation (Green and Roderick 1966) or chemical compounds (Russell et al. 1989). Recently, the use of specific selectable markers and embryonic stem (ES) cells has improved the X-ray irradiation strategy to yield a series of interstitial deletions at a locus of interest (Kushi et al. 1998; Schimenti et al. 2000; Goodwin et al. 2001). However, the chromosome derivatives required further extensive characterisation. Even though deletion of genomic fragments up to 30 kb could be achieved by homologous recombination in ES cells (Gu et al. 1993; Zhang et al. 1994), inducing well-defined larger rearrangements requires the use of the Cre/*loxP* technology either in vitro in ES cells (Ramirez-Solis et al. 1995; Smith et al. 1995; van Deursen et al. 1995) or in vivo (Herault et al. 1998; Puech et al. 2000; Spitz et al. 2005). In this review, we will describe the principle of the in vitro and in vivo approaches for chromosomal engineering and their applications for the study of the function of genes in mammals and the modelling of human diseases.

2
Principles of Cre-Mediated Chromosomal Engineering

The basic principle of chromosomal engineering is to take advantages of *loxP* sites inserted in mouse chromosomes to engineer deletions, duplications, inversions or translocations. Indeed Cre recombinase can act in the mammalian genome without any cofactor, allowing one to generate large chromosomal rearrangements either in culture or in the mouse. The resulting chromosome is modified depending on the position of *loxP* sites (i.e. in *cis*, or in a *trans* configuration), their orientation (direct or inverted) and the localisation in homologous or heterologous chromosomes (Fig. 1).

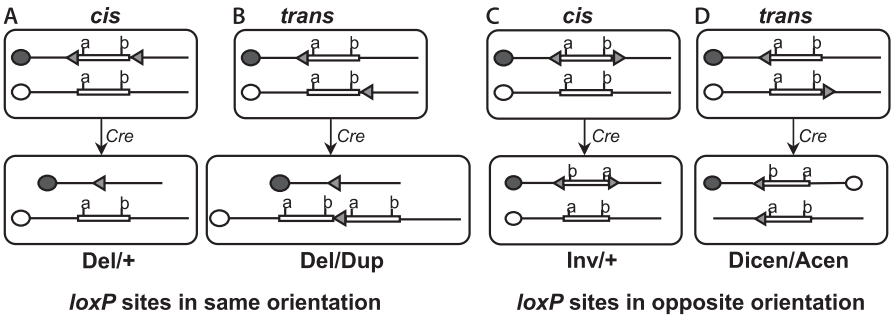

Fig. 1 A–D Different types of rearrangements produced by the Cre/*loxP* recombination system depending on the position and orientation of the *loxP* sites. Whether the *loxP* sites are oriented in the same direction (**A, B**) or in the opposite direction (**C, D**) and if they are on the same chromosome (*cis* configuration; **A, C**) or on different homologues (*trans* configuration; **B, D**), *loxP* recombination will result in different types of chromosome rearrangements (+, wild-type allele; *Del*, deletion; *Dup*, duplication; *Acen*, acentric; *Dicen*, dicentric). This description is valid for both in vitro and in vivo Cre/*loxP*-mediated recombination systems. *a* and *b*, genetic markers. Triangles for *loxP* sites

2.1 In Vitro Technique

In the mid-1990's, several groups independently worked out an in vitro strategy based on the Cre/*loxP* recombination system combined with gene targeting in ES cells in order to generate defined chromosomal *deletions*, *inversions*, *duplications* or *translocations*. For small regions (less than 100 kb), deletions can be easily selected by cloning, starting from ES cells carrying two *loxP* sites in *cis* that are treated with Cre (Zakany and Duboule 1996; Spitz et al. 2001). However, for *loxP* sites that are far apart or on different chromosomes, the frequency of the recombination event is too low, and it should be selected in vitro through the restoration of a positive selectable marker such as *Hprt* (Ramirez-Solis et al. 1995) or the resistance gene for neomycin (Olson et al. 2004), puromycin (Puech et al. 2000; Suemori and Noguchi 2000), or hygromycin (Kudoh et al. 2005). To this end, two targeting vectors that contain the 5′ part or the 3′ part of the selection cassette with *loxP* located downstream or upstream, respectively, are inserted consecutively by homologous recombination at the borders of the genomic interval of interest. Further transient expression of the Cre recombinase in double-targeted clones leads to the restoration of the selection marker. As an alternative, a *tk*-negative selection marker can be deleted in the rearranged locus (Li et al. 1996; Schlake et al. 1999; Zhu et al. 2000; Nobrega et al. 2004). For example, one *loxP- neo-tk* and one *hygro-tk-loxP* cassette were inserted successively at the borders of a genomic interval and were then removed by the recombinase (Nobrega et al. 2004). The availability of a series of vectors containing different kinds of selectable cassettes offers many

possibilities for inducing chromosomal rearrangements in various types of ES cells. Indeed, in the case of the restoration of the *Hprt* gene's function designed in A. Bradley's laboratory, the use of *Hprt*-deficient ES cells such as AB2.2 (Ramirez-Solis et al. 1995) is required. Nevertheless, this is a method of choice given the number of ready-to-use targeting vectors for the *Hprt* selection system available from the Mutagenic Insertion and Chromosome Engineering Resource (*MICER*) and the panel of rearranged chromosomes that could be engineered (Zheng et al. 1999b; Adams et al. 2004). Furthermore, the presence of coat colour markers in those targeting vectors allows an easy discrimination of mice carrying the recombined chromosome (Zheng et al. 1999a).

As the integration of the second *loxP* site, in *cis* or in *trans*, is a random event, one major issue for the Cre/*loxP*-based method is to isolate several (more than six) double-targeted clones in order to get at least one double-targeted clone with *loxP* sites integrated in the correct manner, depending on the rearrangement that is planned. Indeed, deletions, duplications or inversions can be produced depending on the *loxP* relative orientation, its position on the homologous chromosome (i.e. in *cis* or in *trans*), and on the cell cycle stage during which the Cre-mediated recombination occurs (Zheng et al. 2001; Yu and Bradley 2001; Figs. 1 and 2). Whereas recombination between *loxP* sites integrated in the same orientation in a *cis* configuration during the G_1

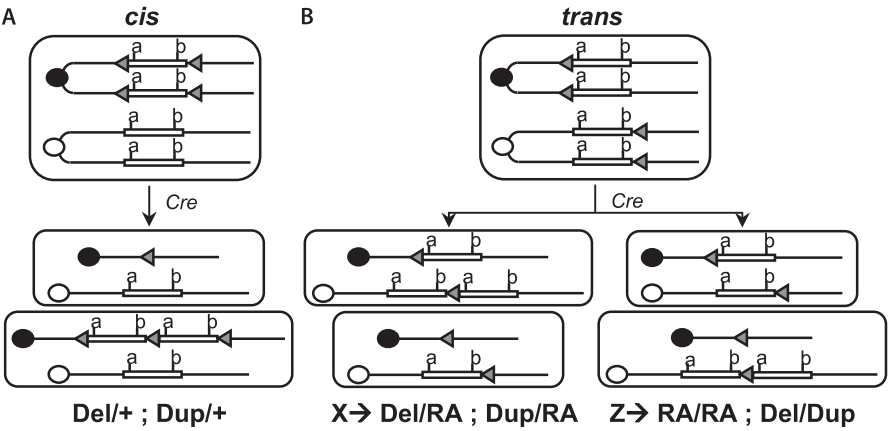

Fig. 2 A,B Cre/*loxP*-mediated recombination after DNA synthesis (G_2 phase). The final recombinant products obtained when the *loxP* sites are in the same orientation are shown here and not the intermediates. We choose not to show recombination events when *loxP* sites are in opposite orientation that only give rise to acentric and dicentric, often nonviable chromosomes. Not all of the rearrangements generated for each case will be found as they are not all resistant to drug selection. This depends on whether the *5′part* and *3′part* halves are outside or inside the *loxP*-flanked region and hence depends on the design of the targeting vectors (see Yu and Bradley 2001). *Del*, deletion; *Dup*, duplication; *RA*, one of the original recombinant alleles; *a* and *b*, genetic markers

phase can only generate a deletion of the region of interest (Fig. 1A), the same configuration in the G_2 phase can result in the creation of a deletion and a duplication (Fig. 2A). The deletion and the duplication can also be obtained from a *trans* configuration in both G_1 and G_2 phases (Figs. 1B and 2B). When the *loxP* sites are oriented in opposite directions, the results from a *cis* - configuration will be an inversion if the Cre is expressed in G_1 (Fig. 1C), while it will more likely give unstable recombined acentric and dicentric chromosomes if the Cre reacts on *loxP* sites after the S phase (not shown) or from a *trans* configuration (Fig. 1D, not shown). Hopefully, only those alleles containing the reconstituted minigene will be retained during the selection among these many different configurations. It is therefore very important to define key parameters such as orientation of the cassette and position of *loxP* sites in order to obtain a defined chromosomal rearrangement for a region of interest. In all cases, recombined ES clones carrying the new genetic configuration should be extensively characterised to verify the engineered chromosome (by Southern blot analysis, normal and quantitative PCR or FISH) before the derivation of the mouse line carrying the new genetic configuration.

ES cells containing both the duplication and the deletion, derived from the recombination of *loxP* sites in a *trans* configuration during G_1 (Fig. 2B), are the best candidates to establish the deleted and duplicated chromosomes in the mouse. Indeed both chromosomes compensate for each other with regard to genetic dosage, reducing the potential consequence of *haploinsufficiency*, and then the two chromosomes will segregate in the progeny of the chimeric mice.

The efficiency of the in vitro technique depends first on the two targeting experiments, the chromosomal context of the bordering loci and more dramatically on the design of the experiment. The Cre recombination efficiency and hence the feasibility of this technique was first evaluated by Zheng et al. (2000), who generated a panel of deletions along chromosomes 4 and 11. They demonstrated that Cre-mediated recombination is able to react on *loxP* sites that are separated by a genomic interval up to 60 cM in size, provided that the rearrangement is tolerated in ES cells. Indeed, some chromosomal deletions may cause ES-cell lethality or growth impairment. This was shown by the presence of compensatory genetic changes in a few ES cells deleted for a 22-cM region on chromosome 11 and in which the Cre-mediated deletion efficiency was low, indicating that this deletion was haploinsufficient and could only be rescued in cells having the remaining wild-type chromosome duplicated (Zheng et al. 2000). Another set of results also indicated that the efficiency is clearly dependent on whether the two *loxP* sites are in *trans* or in *cis* configuration. Moreover, Cre/*loxP* recombination efficiency decreases from 10% to 0.1% over increasing genetic distances with the *loxP* sites in *cis* configuration. For *loxP* sites in *trans* configuration, the efficiency of recombination appears to be no longer dependent upon distance between *loxP* sites but is much lower than recombination in *cis*, reaching 0.1%–0.01% (Liu et al. 1998; Zheng et al. 2000). Recombination in *cis* thus occurs 100–1000 times more frequently than recom-

bination in *trans*. Interestingly, Cre-mediated chromosomal rearrangement appears more efficient (by a factor 10) when the treated ES cells are in the G_2 phase (Yu and Bradley 2001). However, Cre-mediated recombination efficiencies also depend on the level of the Cre expression or activity in ES cells. So the choice of the Cre expression vectors, methods of transfection, or the selection of Cre-expressing cells during the experiment could greatly improve the recovery of the chromosomal targeted event (O'Gorman et al. 1997; Gagneten et al. 1997; V. Besson and Y. Herault, personal communication).

An extension of the chromosome engineering strategy is to generate nested chromosomal deletions starting from a targeted locus. Su et al. (2000) developed a strategy using retroviral integration of a second *loxP* site to induce deletions in ES cells (Fig. 3). They targeted a *5′-Hprt* cassette to the *Hprt* locus that inactivated the locus, providing an *Hprt*-deficient background. They subsequently integrated randomly a *3′-Hprt* cassette by viral infection with an average insertion rate of 1 per 250 kb. After Cre expression, 20% of the resistant clones contained a deletion with an average size of 1 cM, giving a deletion efficiency of 3×10^{-5}. Another experiment done on mouse chromosome 11 led to an average frequency of 1.9×10^{-4} for inducing deletions. LePage and collaborators (2000) used a similar strategy by cotransfecting the cell line containing the first integrated *loxP* site at the *Notch1* locus on chromosome 2, with a sec-

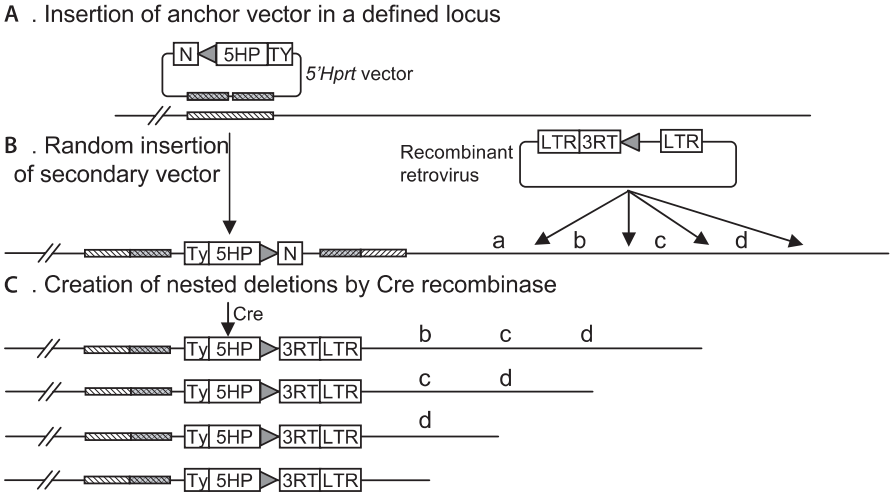

Fig. 3 A–C Strategy for nested chromosomal deletions induced with a retroviral vector. The first deletion endpoint is fixed by targeting the *5′Hprt* cassette (5HP) containing a *loxP* at a determined locus. The second *loxP* site is introduced by random integration using a recombinant retroviral vector containing the *3′Hprt* cassette (3RT), the puromycin resistance gene (*P*) and a *loxP* site. After Cre expression, nested deletions can be identified by the resistance to the HAT drug conferred by the reconstitution of the *Hprt* minigene. *LTR*, long terminal repeat; *Ty*, tyrosinase gene. *a*, *b* , *c* and *d*, genetic markers

ond *loxP* site and a plasmid for transient expression of the Cre. Independent random integrations of the second *loxP* on the same chromosome resulted in multiple nested deletions that could be rapidly mapped due to the original targeting in an F_1 (129S1xCast/Ei) hybrid embryonic stem cell line. The making of nested deletions in ES cells offers an interesting alternative to the direct induction in mice. The possibility of selecting the cell lines before germ-line transmission avoids the problem of finding visible markers and having to generate a large number of mice in order to recover multiple deletions at the same locus.

The Cre/*loxP* technology was also used to engineer reciprocal exchanges between heterologous chromosomes such as translocations observed in models of cancer (Smith et al. 1995; van Deursen et al. 1995). Van Deursen et al. (1995) were able to induce with a frequency of 1/1200–1/2400 a translocation between chromosomes 13 and 2 that corresponds to the recombination of *loxP* sites inserted at two heterologous loci. The resulting product corresponded to the fusion of the *Dek* and *Can* genes commonly found in human acute myeloid leukaemia.

2.2 In Vivo Approach

Cre-mediated recombination can also be induced within the mouse by crossing mice carrying two *loxP* sites with a transgenic line expressing Cre. Several groups succeeded in engineering chromosomes in vivo in order to generate large deletions, duplications, inversions and translocations. This in vivo approach can be carried out between two *loxP* sites in the same relative orientation inserted in *cis* at two different loci in ES cells (Zakany and Duboule 1996; Stemmler al. 2005) or by taking advantage of classical crossing-over to select in mice a chromosome carrying two *loxP* sites in *cis* that can be further combined with a Cre-expressing transgene (Puech et al. 2000; Kmita et al. 2005). Finally, TArgeted MEiotic REcombination (*TAMERE*) offers another alternative to recombine *loxP* sites in a *trans*-configuration (Herault et al. 1998). Similarly, smaller inversions can be generated in vivo (Kmita et al. 2000a; Zakany et al. 2004) with two *loxP* sites in a reverse orientation, located on the same chromosome. The approach was further expanded to large genomic intervals and was renamed Sequential Targeted Recombination INduced Genomic rearrangement (*STRING*; Spitz et al. 2005).

Using the Cre/*loxP*-based technology in vivo, large deletions can be obtained by crossing a mouse carrying *loxP* sites in a *cis* configuration with a transgenic mouse expressing Cre under the control of a ubiquitous or specific promoter. The *loxP* recombination and hence the deletion of the floxed region will occur in the organism and can be transmitted to the progeny or will be restricted to a specific organ or tissue (see following chapters in this volume). More and more Cre transgenic lines are becoming currently avail-

able (http://www.mshri.on.ca/nagy/Cre-pub.html), enabling one to generate conditional gene inactivation and to engineer chromosomes in dedicated tissues (Zheng et al. 2000) or during development (Zakany et al. 2004; Stemmler et al. 2005).

The induction of the deletion in the whole organism in vivo can be achieved using Cre transgenic lines, such as Hprt-Cre (Tang et al. 2002), R26Cre (Soriano 1999) and CMV-Cre (Dupe et al. 1997) in which the Cre recombinase is expressed more or less ubiquitously or at least early during development in most of the tissues. An alternative to the ubiquitous Cre transgenic lines is the ZP3-Cre line (Lewandoski et al. 1997; de Vries et al. 2000), showing a high level of Cre expression during oogenesis before the first meiotic division. However, the recombination efficiency is not always high and this can lead to the generation of mosaic animals (Leneuve et al. 2003). Indeed, in an attempt to get a 550-kb deletion within mouse chromosome 16 using the CMV-Cre line, Puech et al. (2000) ended up with only 3.1% recombination efficiency. We have also noticed in our laboratory a high mosaic effect of the Cre recombinase under the control of the CMV promoter to generate a deletion of 0.7 Mb within mouse chromosome 10. Among the 101 offspring analysed, 26% of the mice showed a mosaic profile (V. Besson and Y. Herault, personal communication). Mersher et al. (2001) noted 17% mosaic mice when they used the ZP3-Cre mouse line to produce a 1.5-Mb deletion within mouse chromosome 16. Indeed, mosaicism is frequent and well-documented, not only for ubiquitous Cre recombinase but also for the tissue-specific ones (Zhong et al. 2005).

Creating duplications/deletions in vivo with *loxP* in *trans* configuration can be achieved by using the TAMERE strategy (Herault et al. 1998). This strategy takes advantage of the homologous chromosome pairing occurring during the first meiotic division of gametogenesis in order to generate trans-allelic recombination. A specific transgenic line expressing the *cre* gene under the control of the *Sycp1* (*Synaptonemal Complex protein 1*) promoter is used, driving the expression of Cre at prophase of meiosis in male spermatocytes when chromatid pairs are closely aligned, in order to facilitate chromatid exchange. The general principle of the TAMERE method is based on successive breedings in order to have in one male, named the trans-loxer, the *Sycp1Cre* transgene and the two *loxP* sites in *trans* configuration, inserted previously in the same orientation at each targeted locus that defines the genetic interval. The last step consists in mating trans-loxer males with wild-type females to generate, in the progeny, individuals with either the deletion or the duplication of the interval delimited by the two *loxP* sites. This strategy has been used extensively for the *HoxD* complex (Herault et al. 1998; Kmita et al. 2000b, 2002a, 2002b) and for a few other loci, showing that the frequency of the recombination events can vary from 1% to 10% for large regions (Genoud et al. 2004). Inversions can also be induced in vivo by using mouse lines with a chromosome engineered in vitro that carries *loxP* sites in a reverse orientation (Kmita et al. 2000a; Zakany et al. 2004) or obtained after classical breeding strategies

using the STRING approach (Spitz et al. 2005). Thus, the in vivo strategy avoids a few complicated steps of cell culture but requires a well-defined breeding strategy.

3 Applications

Chromosome engineering is a powerful tool and its applications are numerous. It can be used for the production of experimental models for contiguous gene syndromes associated with chromosomal rearrangements or for cancer. It also permits one to generate novel genetic configurations for the functional analysis of the genome. Some of these different applications are listed in the following section.

3.1 For a Regional Mutagenesis Screen

Large deletions and inversions are useful tools to improve the genetic analysis and the search for new mutations with the aim to unravel the gene's function. To this end, Cre/*loxP*-mediated chromosomal engineering has a high potential of identifying mutations for regional mutagenesis.

Indeed, in a typical recessive *mutagenesis* program, mutagen-treated males, called F_0, are crossed with wild-type females to generate F_1 males; each of these will found a distinct pedigree after crossing with wild-type females. In the progeny, G_2 females are then backcrossed with the original F_1 males to generate G_3 individuals carrying homozygous mutations that are characterised for specific phenotypes. This screen will lead to the isolation of individuals showing an abnormal phenotype leading to the establishment of a new mouse mutant line after confirmation of Mendelian inheritance. Knowledge of the murine genome allows the identification of the mutations by genetic mapping, the candidate gene approach and sequencing analysis. However, there is a limitation to this technique due to the necessity of analyzing 100–1,000 meioses to localise one mutation with a good resolution (Justice 1999). Thus, the mapping and identification of many mutations is an enormous task. By introducing a deletion, pseudo-recessive screens can be carried out, helping to unravel mutations with phenotypic consequences located inside the deleted interval (Fig. 4A). In addition, coat colour markers associated with the engineered deletion using MICER vectors make it possible to avoid a meticulous genotyping program (Adams et al. 2004). Furthermore, the mapping of recessive mutations can be improved with nested deletions (Yu and Bradley 2001). Deletions can also be used to generate several Cre-expressing lines with different patterns of expression starting from one Cre transgene inserted at a defined locus. Using the TAMERE strategy, Herault et al. (2002) have reallocated

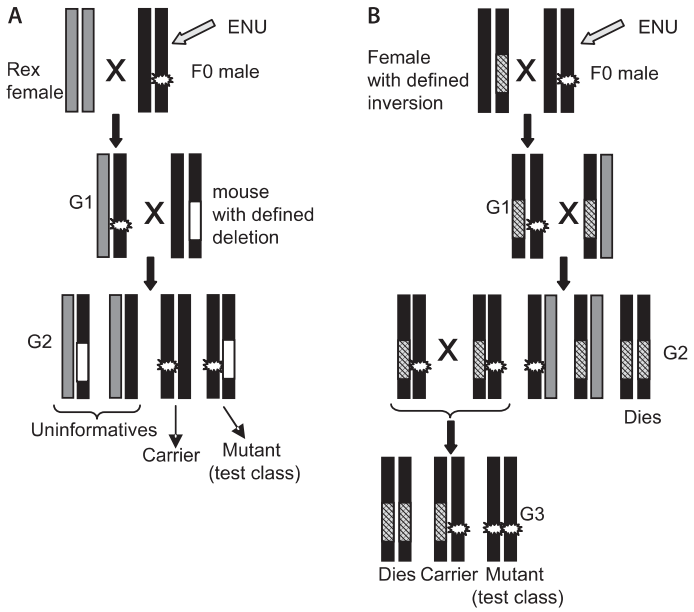

Fig. 4 A,B ENU-induced mutagenesis screens using chromosomal engineering techniques. In the F_0 generation, a wild-type male, here from the C57BL/6J strain (black chromosomes), is injected with ENU to induce random point mutations (*star*) inside the genome. The two screenings are carried out by crossing with genetically engineered mice containing either a deletion (**A**, *white box*) or an inversion (**B**, *striped box*), both tagged with a dominant yellow coat colour marker, K14-Agouti. Both procedures also use a dominant fur marker such as the Rex mutation giving a curly fur (grey chromosome; Kile et al. 2003).**A** deletion scheme. F_0 males are crossed with wild-type females. G_1 animals, heterozygous for the ENU-mutagenized chromosome, are crossed to mice hemizygous for a yellow-tagged deletion. The resulting G_2 offspring can be readily identified: the two classes of animals with grey fur are uninformative; the carrier class has a wild-type aspect (black) and can be used to recover any lethal mutation; the mutant or test class is yellow and straight-haired. If the test class is missing, it indicates that the mutation is probably lethal. **B** Inversion scheme. The F_0 males are mated with females carrying the inversion (balancer chromosome) that suppresses recombination over the inverted interval and is homozygous lethal because of the disruption of some lethal genes at its endpoints. In G_1 offspring, yellow individuals that carry the balancer chromosome plus the mutated one are crossed with animals that bear the balancer plus a normal chromosome carrying the curly-coat mutation (grey). Identifiable yellow, straight-haired mice that contain the balancer and mutated chromosomes in the G_2 generation are intercrossed to give the G_3 generation with the carrier class (yellow fur) that can be bred to maintain the mutant line, and the mutant test class (black) can be analysed

a *Hoxd11Cre* transgene at different positions within the *HoxD* complex, leading to a series of transgenic Cre lines with a progressively more extended Hox-like pattern.

Recessive lethal mutations are difficult to isolate in classical mutagenesis screens, except if a chromosome carrying an inversion is used as a balancer

chromosome. Indeed crossing-over between two homologous chromosomes, one carrying an inversion, will lead to acentric or dicentric nonviable chromosomes, so that the mutation located in the inverted region can be fixed. For example, 88 new mutations, of which 55 induced a lethal phenotype, were isolated from a screen of 735 pedigrees using a balancer chromosome corresponding to a 24-cM region on mouse chromosome 11 (Fig. 4B; Kile et al. 2003). The production of inversions by chromosome engineering offers the advantage of controlling the size of the inverted region. Indeed, the efficiency of the mutagenesis screen increases with the size of the deletion. This size should, however, stay below 30 cM in order to avoid double crossing-over. One interesting issue is to generate an inversion that will induce lethality in homozygous, such as those obtained on mouse chromosome 11 that disrupt the *Wnt3* gene (Kile et al. 2003; Klysik et al. 2004). Alternatively, the combination of several coat colour markers enables the generation of homozygous viable balancer chromosomes that are easily distinguishable (Yu and Bradley 2001). This is a great advantage in the search for embryonic lethal mutations (Nishijima et al. 2003).

The Cre/*loxP* technology can also be used to develop recessive genetic screens for specific chromosomes using ES cells mutated by chemicals or by insertional vectors. Indeed, by using this technology, Liu et al. (2002) were able to induce Cre-mediated *mitotic recombination* (Fig. 5) in culture using ES cells carrying constructs with *loxP* sites inserted near the centromere for mouse chromosomes 7 and 11. For both chromosomes, the authors recovered, after Cre-mediated *Hprt* restoration, ES cells homozygous at all loci located in a telomeric position with respect to the *loxP* site. The frequency of such mitotic events varies from 5.0×10^{-2} to 7.0×10^{-3} depending on the chromosomal context and the number of inserted *loxP* sites (Liu et al. 2002). The recovery of these mitotic recombinations suggests that Cre is preferentially active in G_2 and that the resulting chromatids segregate away from each other (X segregation) in daughter cells. This phenomenon is quiet similar to that observed during FLP-mediated sister chromatid exchange in *Drosophila* (Beumer et al. 1998). A similar approach based on another type of selection (a neo-deficient cassette) was used by Koike et al. (2002) to select ES cells homozygous for the locus downstream of the *Oprk1* gene on mouse chromosome 1.

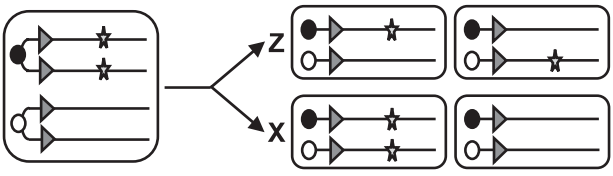

Fig. 5 Cre/*loxP*-mediated mitotic recombination. Cre expression is used to trigger mitotic recombination. X-segregation of the chromosomes leads to the generation of mosaics, whereas Z-segregation produces daughter cells that are phenotypically indistinguishable from heterozygous mothers

The strategies described above make it possible to produce selectively ES cell clones carrying homozygous mutations in vitro. They offer a new and promising alternative for functional recessive screens of mutations induced in ES cells. Moreover, they are opening a new dimension for genetic mosaics in mice.

Genetic *mosaicism* is a powerful approach to decipher cell lineage, cell fate determination and cell autonomy that was extensively used in *Drosophila*. In this organism, this method was based on the FLP/Frt recombinase system to induce mitotic recombinations and took advantage of X segregation. Recently, Zong et al. (2005) developed a similar technique called the Mosaic Analysis with Double Markers (*MADM*) in mice. By utilizing the Cre/*loxP* strategy, they were able to induce recombination events between homologous chromosomes in somatic cells and to mark the resulting daughter cells with different genotypes. They showed that interchromosomal recombination can be induced efficiently in vivo as a consequence of Cre expression in various cell types.

3.2 Exploring the Function and Regulation of Genes and the Genomic Organisation

Well-defined chromosomal rearrangements are powerful tools for the functional analysis of the mouse genome to study the function of large genes, including *App* (Li et al. 1996), *Xist* (Clerc and Avner 1998; Morey et al. 2001), *Nf1* (Schlake et al. 1999), *Dkc1* (He et al. 2002), *TNF/LT* (Kuprash et al. 2002) and *Dystrophin* (Kudoh et al. 2005). Moreover, they have been used successfully to unravel the relation between function, transcriptional regulation and genomic organisation. A good example of such an analysis is the study of the *Hox* genes, mainly done by D. Duboule and collaborators in the last 10 years.

Hox genes encode homeodomain transcription factors that regulate cell identity and positional information along the anteroposterior axis of the embryo and during the formation of the limb bud in embryonic development. They are located in four clusters in vertebrates, namely A, B, C and D, and are expressed with a precise spatial and temporal pattern during development in a colinear way that reflects their genomic organisation in the complex (for a review, see Deschamps and van Nes 2005). Classical mutational analyses of the *Hox* genes revealed a complex network of interactions among genes from different clusters, with individual *Hox* proteins from the same paralogues having redundant and nonredundant functions, as well as synergistic interactions. In order to analyse more precisely the interactions between these genes, new alleles containing deletions of part or all of the genes for the four complexes were generated (Zakany and Duboule 1996; Ramirez-Solis et al. 1995; Medina-Martinez al. 2000; Kmita et al. 2000b, 2005; Suemori and Noguchi 2000). In particular, the specific deletion of the *HoxA* in association with a deletion of the *HoxD* cluster (Kmita et al. 2005) revealed that the recruitment of *Hox* gene

function during limb development was crucial in implementing *shh* signalling. Beyond the study of the function of the *Hox* genes, chromosomal engineering has revealed that the genomic organisation of the *Hox* genes in the complex is reminiscent of the transcriptional regulation of the genes during development. On one hand, the targeted deletion of 90 kb within the *HoxB* complex suggested that *HoxB13* gene expression does not require any element included within the interval of the nine other *HoxB* genes (Medina-Martinez et al. 2000). On the other hand, a more detailed analysis of the *HoxD* complex revealed that several locus control regions are present on both sides of the cluster that control the expression of several genes in the *HoxD* complex in the various embryonic domains (Zakany and Duboule 1996; Spitz et al. 2001, 2005). The action of this global regulatory element is further refined by small regulatory elements located inside the complex (Kmita et al. 2002a, 2002b).

Other gene clusters were analysed by chromosome engineering. The vomeronasal receptor gene cluster was deleted to assess the function of the genes (Del Punta et al. 2002). A fourgenes cluster on chromosome 14 inducing developmental defects and growth arrest when deleted was further analysed by nested deletions to locate the origin of the defects (Semenova et al. 2003). The inversion of the TCRδ was performed to determine its role during the commitment of the αβ T lymphocyte lineage (Khor et al. 2005). Chromosome engineering was also used for functional analysis on a larger scale. Numerous rearrangements (deletions, duplications and inversions) from 1 Mb to 60 cM were generated in ES cells on chromosome 11 to collect functional information about the genes on this chromosome and especially to look for tumour suppressor genes (Liu et al. 1998; Zheng et al. 2000; Biggs et al. 2003).

Thus, chromosomal engineering is a key strategy used to unravel the link between genomic architecture and gene regulation and function. It will be a major step in elucidating the complex mechanisms of transcriptional regulation not only for genes located in clusters but also for genes encompassed in large transcription units such as the *E-cadherin* gene (Stemmler et al. 2005), or in testing the function of genes or large genomic intervals (Zhu et al. 2000; Nobrega et al. 2004).

3.3 Creating Mouse Models for Human Pathologies

The possibility of manipulating large chromosome regions instead of single genes provides the opportunity to develop mouse models of contiguous gene syndromes (CGS). Indeed, many human birth defects are caused by chromosomal abnormalities such as inversions, duplications, deletions and translocations. These chromosomal changes lead to gene dosage errors, resulting in major developmental abnormalities such as Down syndrome (Epstein 1986), Charcot-Marie-Tooth disease (Lupski et al. 1991), Beckwith-Wiedemann syndrome (Waziri et al. 1983) or DiGeorge syndrome (Driscoll 1994).

Df1 was the first mouse model for DiGeorge syndrome (DGS) obtained by chromosomal engineering (Lindsay et al. 1999). DGS is the most prevalent human microdeletion syndrome, affecting the chromosomal region 22q11.2 and inducing cardiovascular, thymus and parathyroid defects as well as craniofacial anomalies and learning difficulties. The 24–30 genes deleted in DGS map in a single region in mouse chromosome 16. The *Df1* mice, heterozygous for a 1.2-Mb deletion between *Es2* and *Ufd1* in mouse chromosome 16, displayed a panel of abnormalities reminiscent of those found in human patients. Other mouse models were subsequently produced in order to decipher the genes involved in DGS. Puech et al. (2000) generated a new deletion partially overlapping with the *Df1* deletion. This new mouse model did not exhibit cardiovascular abnormality, restricting the number of genes that could be implicated in the cardiovascular phenotype. Further studies using nested deletions, BAC transgenesis and gene knockout pointed out the role of the *TBX1* gene in the conotruncal defects observed in the *Dfl1* model and characteristic of DGS (Merscher et al. 2001; Lindsay et al. 2001).

Down syndrome or trisomy 21, probably the most well-known CGS, is caused by an additional entire chromosome 21. Human chromosome 21 orthologues are found in regions of synteny in mouse chromosomes 10, 17 and 16. However, existing mouse models involve only a portion of chromosome 16 and do not recapitulate the major clinical features of the disorder. Whereas the two principal models, Ts65Dn and Ts1Cje (Davisson et al. 1990; Reeves et al. 1995; Sago et al. 1998), were obtained in a random manner, Olson et al. (2004) used chromosome engineering to generate the deletion and the duplication of the Down Syndrome Critical region (DSC) located on mouse chromosome 16 and assessed the morphology of the skeleton. They concluded that the DSCR genes were not necessary to produce the Down syndrome craniofacial phenotype, refuting the concept that suggested that most alterations of the Down syndrome are the product of a few dosage-sensitive genes located on the DSCR.

Following the success of chromosomal engineering to model human CGS in mice, many groups started the creation of mouse models for human pathologies using chromosome engineering (Tsai et al. 1999; Walz et al. 2003; Yan et al. 2004). This technique is likely to be increasingly developed in the future to characterise polygenic diseases such as diabetes, cancer, asthma or obesity, or to study the effect of chromosome loss in mice (Lewandoski and Martin, 1997).

Chromosomal deletions and translocations are crucial events in the formation of many types of tumours, resulting in inactivation of tumour suppressor genes, oncogene overexpression or creation of novel fusion genes. Tumour suppressor genes can be identified using chromosome engineering to produce deletions that encompass a candidate gene, leading to a mouse model that should exhibit an increased risk of developing tumours. In addition, inactivation of the remaining wild-type allele can be used to find the causative gene. This approach provides a method for the functional identification of tu-

mour suppressor genes, without any prior knowledge of gene function (Smith et al. 2002; Biggs et al. 2003). Additionally, interchromosomal translocations generated by introducing *loxP* sites into two non-homologous chromosomes in ES cells and recombining them by transient Cre expression enable the modelling of specific chromosome alterations found in different cancers. Foster et al. (2003) created a mouse model of leukaemia by interchromosomal recombination between the*Mll* gene, whose human counterpart is involved in a spectrum of leukaemias, and the *Enl* gene. *LoxP* sites were introduced into the mouse *Mll* (intron 10) and *Enl* (intron 2) genes to induce a recombination between the two *loxP* sites that results in a *Mll-Enl* fusion gene. The chromosomal translocation was targeted to the bone marrow cells by using a knock-in of the *cre* gene into the hematopoietic regulator *Lmo2*. All the mice carrying the two *loxP* sites and the *Lmo2-Cre* transgene developed leukaemia. Previously, similar translocations between the *Mll* and *Af9* genes (Collins et al. 2000) and also between the *Aml1* and *Eto* genes were induced in various tissues such as the brain with no consequences on mouse phenotype (Collins et al. 2000; Buchholz et al. 2000). These results showed that the Cre transgenic line used should be appropriate, i.e. for the development of a leukaemia, recombination should occur in haematopoietic cells and hence the promoter driving the Cre expression should be active in those cells.

4 Conclusion

Chromosomal engineering is a very powerful strategy to analyse the mammalian genome. It is facilitated now by the availability of more than 15,000 targeting vectors from the MICER system, developed by A. Bradley (Adams et al. 2004) and the development of numerous mice carrying *loxP* sites at various loci in the genome. The crucial step is to define the border of the genetic interval and attempt to avoid the alteration of the neighbouring gene's function. The in vitro or in vivo approaches offer both benefits and drawbacks that should clearly be evaluated before the start of such a genomic manipulation (efficiencies of recombination, time, costs, size of the rearrangements, etc.). Nevertheless, the in vivo approaches, TAMERE and STRING (Herault et al. 1998; Spitz et al. 2005), offer the advantage of combining available mouse lines carrying *loxP* sites with a Cre expressing transgene in a simple breeding design. The crucial issue is the haploid consequences on viability of a particular deletion that could not be resolved a priori. Now any laboratory can easily manipulate large genomic regions without any additional work on ES cells in order to further analyse the genomic organisation or to derive new tools for genetic analysis. This will be further supported by the commitment of the scientific community to highlight the mutagenesis of the mouse genome by gene-trap and conditional vectors with *loxP* sites (Austin et al. 2004; Auwerx et al. 2004).

What emerged from the use of chromosomal engineering is a fascinating aspect of chromosomal organisation, unravelling long-range interactions between genes and regulatory elements. We can speculate that this strategy will be more commonly used to better understand chromosomal structure and architecture in the near future. Of particular interest, too, is the modelling of contiguous gene and aneuploid syndromes in the mouse that will definitely facilitate the understanding of the molecular basis of such diseases, giving insights into the development of therapeutic approaches.

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Tetracycline-Controlled Genetic Switches

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Abstract Unlike recombinase-mediated gene manipulations, tetracycline (Tet)-controlled genetic switches permit reversible control of gene expression in the mouse. Trancriptional activation can be induced by activators termed tTA (Tet-Off) or rtTA (Tet-On) in the absence and presence of Tet, respectively. The Tet-Off and Tet-On systems are complementary, and the decision to choose one over the other depends on the particular experimental strategy. Both systems were optimized over the years and can now be used to develop mouse models.

Keywords Doxycycline · Luciferase · β-Galactosidase · GFP · Rosa26

1 Introduction

The Tet-controlled inducible gene expression systems allow alteration in individual gene activities in intact animals, including insects, fly, mice and rats. In mice, the Tet-controlled gene expression continues to provide fundamental insight on various biological processes such as development, diseases and behaviour.

In recent years, the advent of mouse embryonic stem (ES) cells, bacterial artificial chromosome (BAC) and recombinase techniques have accomplished with great precision functional changes by genetic alterations in selective cell populations. Inducible control of gene expression at specifically chosen time points would further facilitate cross-correlation analyses to reliably link changes in gene activity with changes in phenotypes both in cell physiology and animal behaviour.

Over the past several years, the Tet-controlled gene expression has explored various biological processes in the mouse with impressive detail. Here, we will provide an outline and an overview of different experimental strategies for generating mice with functional Tet-controlled genes.

2 Principles of the Tet-Controlled Gene Expression

2.1 Genetic Elements

Tet-controlled gene expression systems in eukaryotes are derived from the transposon Tn10 Tet-resistance operon. Essential features of this prokaryotic Tet-controlled gene expression systems were modified to be operative in eukaryotic cells. In Gram-negative bacteria, Tet is a potent antibiotic that kills bacteria by blocking protein synthesis (Epe and Woolley 1984). Bacteria can achieve resistance to Tet by expressing the TetA resistance protein, a proton-$[Tet.Mg]^+$ antiporter, embedded in the cytoplasmic membrane (Yamaguchi et al. 1990). Under regular conditions, TetA is not expressed since the tetracycline repressor (TetR) blocks TetA expression. In the absence of Tet, TetR dimers bind in the TetR and TetA promoter regions to the operators tetO1 and tetO2, respectively, which physically hinders transcriptional initiation at the TetR and TetA promoters, thereby down-regulating expression of these two genes. When intracellular Tet concentrations rise, Tet binds to TetRs. This leads to a conformational change of the Tet–TetR complex, rendering it incapable of binding tetOs and thus opening access to transcription initiation. As Tet is effluxed out of cells, TetR regains its ability to bind tetOs and transcription of TetA and TetR genes is down-regulated (Hillen and

Berens 1994). Functional TetR protein binds to tetO sequences as a homodimer. Each polypeptide is composed of 208 amino acids with 10 α-helices (see Sect. 3.2.1) making up interaction surfaces for TetR dimerization and binding sites for tetO and Tet (Orth et al. 2000). The inducers [Tet, doxycycline (Dox) and anhydrotetracycline (ATc)] bind to TetR with very high affinity: $[Tet.Mg]^+$ ($K_a \sim 10^9\ M^{-1}$) $[Dox.Mg]^+$ ($K_a \sim 10^{10}\ M^{-1}$) $[ATc.Mg]^+$ ($K_a \sim 10^{11}\ M^{-1}$), which is about three to five orders of magnitude higher than the affinity of these drugs to prokaryotic ribosomes (Takahashi et al. 1986; Lederer et al. 1996). The binding of two molecules of $[Tet.Mg]^+$ to a TetR dimer reduces its affinity for tetO by about nine orders of magnitude (Lederer et al. 1996). Sensitive and reversible control of gene expression is made feasible by TetR binding to tetO even in the context of competing nonspecific DNA sequences in the genome. Changes in TetR affinity for tetO when Tet binds to TetR is the prerequisite condition for the control of gene expression in higher organisms.

2.2 Tet-Controlled Gene Expression in Eukaryotes

The well-defined elements of the Tn10 Tet operon have been successfully transferred to eukaryotic cells for controlling gene expression by Tet. As mentioned above, the Tet operon operates with a simple genetic circuit that requires three essential components: TetR, tetO and Tets. These three components have been modified in various ways to optimize stringent control of gene expression in eukaryotes.

The major breakthrough occurred by the pioneering work of Gossen and Bujard (Gossen and Bujard 1992), who succeeded in introducing a genetic switch for controlling gene expression in potentially all eukaryotes. First, they converted TetR from a repressor into a Tet-controlled transcriptional activator by fusing the herpes simplex virus transcription activator (VP16) to the C-terminus of TetR. The TetR-VP16 hybrid protein, named tTA, binds to tetO sequences via the TetR domain, while the C-terminal VP16 domain participates in the recruitment of the RNA polymease II (Pol II) transcriptional initiation complex to initiate transcription.

Second, they generated a synthetic tTA-dependent promoter (P_{tet}) where seven tetO sequences were linked to a short stretch of sequences containing the Pol II transcriptional start site derived from the human cytomegalovirus (CMV) immediate early gene IE1 promoter (see Sect. 3.2.2). When tTA binds to P_{tet}, tTA initiates transcription at a defined site in the short CMV-promoter fragment (Fig. 1A). Without tTA bound to tetOs, the short CMV-promoter fragment is transcriptionally inactive and therefore it is also commonly referred to as a CMV minimal promoter (CMV_{min}).

In the presence of Tet, tTA is unable to bind to P_{tet} (Fig. 1B), and transcription initiation at P_{tet} is turned-off (Tet-Off). In this way, tTA allows gene

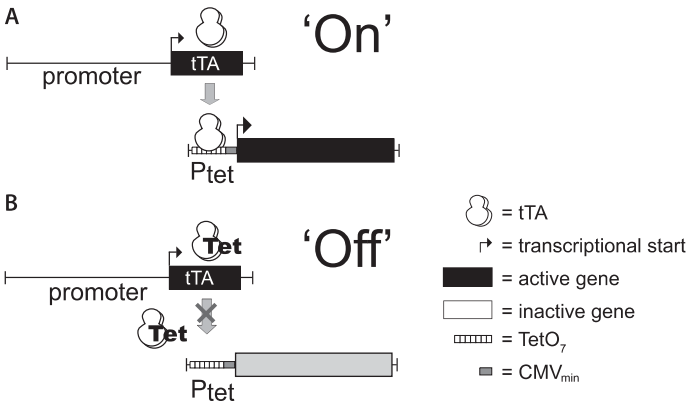

Fig. 1 A,B Principle of the Tet-Off-system. **A** When the constitutively expressed tTA binds to P_{tet}, tTA initiates transcription. **B** In the presence of Tet, tTA is unable to bind to P_{tet} and P_{tet}-controlled gene transcription is turned-off (Tet-Off)

expression to be switched on and off in response to Tet (Gossen and Bujard 1992).

2.2.1 Tet-Controlled Transactivators tTA and rtTA

The original tTA has been functionally improved by eliminating sequences of VP16 to minimal length for transcriptional activation, targeting it to the nucleus of cells and optimizing codon usage (Baron et al. 1997; Kim 2001; Urlinger et al. 2000). Replacement of VP16 activation domain by three copies of 12 amino acid minimal activation F-domains, improved its tolerance in mammalian cells at a higher concentration and have graded activation potentials in range of 1,000-fold (Baron et al. 1997; Kim 2001). Similarly, addition of a nuclear localization signal to tTA improved the efficiency of Tet-controlled gene expression (Kim 2001).

A new quality of the Tet-controlled system was achieved by exchanging the TetR of tTA by a TetR mutant with four point mutations to generate the reverse tTA (rtTA) (Fig. 2). The four point mutations E71K, D95N, L101S and G102D reversed the pharmacology of TetR (Hecht et al. 1993) and now Dox is necessary for rtTA binding to P_{tet} (Gossen et al. 1995).

While both the rtTA- and the tTA-inducible systems can be used in the mouse (Kistner et al. 1996), there are far more functional studies reported with tTA than rtTA (e.g. see Sect. 4.6), which might indicate that Tet-controlled gene expression is more difficult to achieve with rtTA in certain tissues, such as the brain. The comparison of rtTA and tTA in Hela cell cultures showed that with both transactivators gene expression was regulated fast and tightly. However, the regulation factors of 10^5 for tTA and 10^3 for rtTA shows the

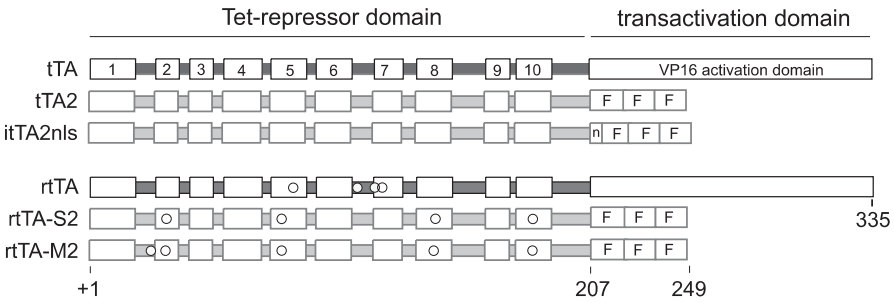

Fig. 2 Schematic drawings for some tTA and rtTA variants, which are currently available. The TetR-core region with 10 α-helices (1–10) contains sites for tetO-binding, Tet-binding and homodimerization. VP16 or minimal F-domains make up the transactivation domains. The position of the nuclear localization domain (*n*) is indicated. Amino acid exchanges in different rtTAs are indicated by *black dots*. Codon improved tTA and rtTA variants are in *light grey*, original tTA and rtTA are in *black*. Amino acid positions are numbered starting with the first amino acid (position *+1*)

better efficiency of tTA. Similarly, 10 ng/ml of Dox was sufficient to fully inactivate tTA-dependent reporter gene transcription in less than 5 min, whereas 1 µg/ml of Dox was needed for full gene activation with the rtTA system. Based on studies in Hela cells, it was estimated that rtTA is approximately 100 times less sensitive than tTA (Gossen et al. 1995). Novel rtTAs with higher sensitivity towards Dox might overcome these limitations. So far, a genetic screen in yeast has identified two rtTA mutants (rtTA-S2 and rtTA-M2) with reduced binding to tetO in absence of Dox and increased Dox sensitivity for rtTA-M2 (Urlinger et al. 2000).

Currently, several tTA and rtTA variants are available, some of which are indicated in Fig. 2. In our hands, the tTA variant itTA2nls and the rtTA-M2nls are suitable choices for mice (Urlinger et al. 2000; Hasan et al. 2001; Kim 2001; Krestel et al. 2004). Both itTA and rtTA-M2nls are codon-improved and lack cryptic splice sites.

2.2.2 tTA- and rtTA-Dependent Promoters (P_{tet}s)

As mentioned above, tTA and rtTA binding to $tetO_7$ initiates transcription at nearby transcriptional start sites (minimal promoters) (Fig. 3). Currently, the CMV_{min} promoter is mostly used in the mouse. Addition of two CMV_{min} promoters on opposite sites of $tetO_7$ produced a bi-directional Tet promoter (P_{tet}-bi), which enabled co-regulated expression of two inversely orientated tTA-controlled transcription units (Baron et al. 1995). Unlike P_{tet}, Tet-controlled P_{tet}-bi constructs are apparently less prone to position-effect-variegation (PEV) (see Sect. 4.4.3).

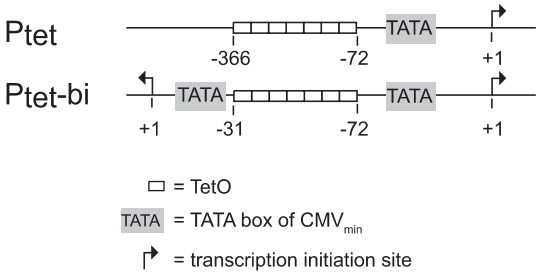

Fig. 3 Uni- (P_{tet}) and bi-directional (P_{tet}-bi) Tet promoters. The different promoter elements of the two tTA- and rtTA-controlled promoters are depicted. Nucleotide positions relative to the transcriptional start sites (position *+1*) are indicated

2.2.3 Tet and Its Derivatives

Tets have been used in both animals and humans and are considered safe. Detailed pharmacological and pharmacokinetic studies are available on these chemicals. Tet, or one of its many derivatives, such as Dox (Fig. 4), with higher membrane permeability and higher affinity to tTA and rtTA, are suitable for gene regulation in animals, including mice. In one study, Tet was found to be cleared from mice relatively faster than Dox. This might be more useful for rapid tTA-dependent re-activation of gene expression upon Tet withdrawal after an extended period of Tet treatment (Robertson et al. 2002). The concentration of Dox required to regulate gene expression in mammalian cells is orders of magnitude lower to create any adverse toxic side effects (Bocker et al. 1981). Dox can be delivered to animals by different means including intraperitoneal injection, drinking water and food pellets. It was estimated that Dox doses of 200 µg/ml yields Dox-blood levels of less than 500 ng/ml, which is far below blood levels used clinically (Chen et al. 1998). Importantly, embryos of Dox-treated pregnant females show normal development, home cage behaviour and life expectancy (Kistner et al. 1996; Krestel et al. 2001). However, offspring from females kept on Dox throughout pregnancy have been reported to show impaired spatial and emotional memories (Mayford et al. 1996).

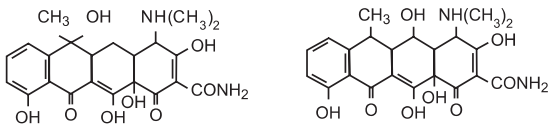

Fig. 4 Chemical structure of Tet (*left*) and Dox (*right*)

3
Dox-Controlled Gene Regulation in Transgenic Mice

For establishing Dox-controlled gene regulation in the mouse, the genetic components of the Tet system have to be introduced into the mouse genome. The components are first engineered into plasmids, cosmids or BACs and then transferred into mice by DNA injection into the pronucleus of fertilized mouse oocytes. Alternatively, genetic components can be inserted at defined chromosomal positions in mouse ES cells, and can be transferred to mice by reconstituting ES cells into early staged embryos (e.g. see the chapter by J.S. Draper and A. Nagy, this volume).

Traditionally, two separate independent mouse lines are generated: a tTA- or rtTA-expressing line (activator) and a line with either the P_{tet}- or P_{tet}-bi controlled gene (responder). Intercrosses between activators and responders give rise to double-positive offspring (containing both the activator and the responder genes) (Fig. 5). These double-positive mice can be tested for tTA- or rtTA-dependent Dox-controlled gene expression.

3.1
tTA and rtTA Minigenes

Expression constructs for the tTA minigene are usually composed of a tissue specific promoter followed by a synthetic 5′-untranslated intron, the tTA or rtTA coding sequences and a polyadenlyation signal (polyA) (Fig. 6). The choice of the promoter for a tTA or a rtTA minigene determines when and where the transgene is expected to be expressed. To readily achieve cell-type specificity, larger promoter fragments can be used with the help of cosmids or BACs. In addition, coding sequences for tTA or rtTA can be inserted directly after promoter regions by replacing the first coding exon of endogenous genes by gene targeting in ES cells (e.g. see the chapter by J.S. Draper and A. Nagy, this volume). However, the latter two experimental approaches have been described in a few reports only (see Sect. 4.7.2).

3.2
tTA- and rtTA-Dependent Responder Genes

A simple responder gene contains P_{tet} followed by a synthetic 5′-untranslated intron, the coding sequences of the gene of interest and polyA (Fig. 6). Popular elements of choice are introns derived from SV40, adenovirus or the β-globin gene and the polyA signal from SV40, growth hormone or β-globin gene. Not all responder constructs must follow this rule (Fig. 6A).

Responders with two co-regulated genes (Fig. 6B) are generated flanking P_{tet}-bi with two transcription units allowing regulated expression of two genes in a tTA- and rtTA-dependent manner (Baron et al. 1995). In transgenic mice, the P_{tet}-bi shows faithful and reproducible co-expression and Dox-controlled regulation of both genes in a P_{tet}-bi module (see Sect. 4.4.3).

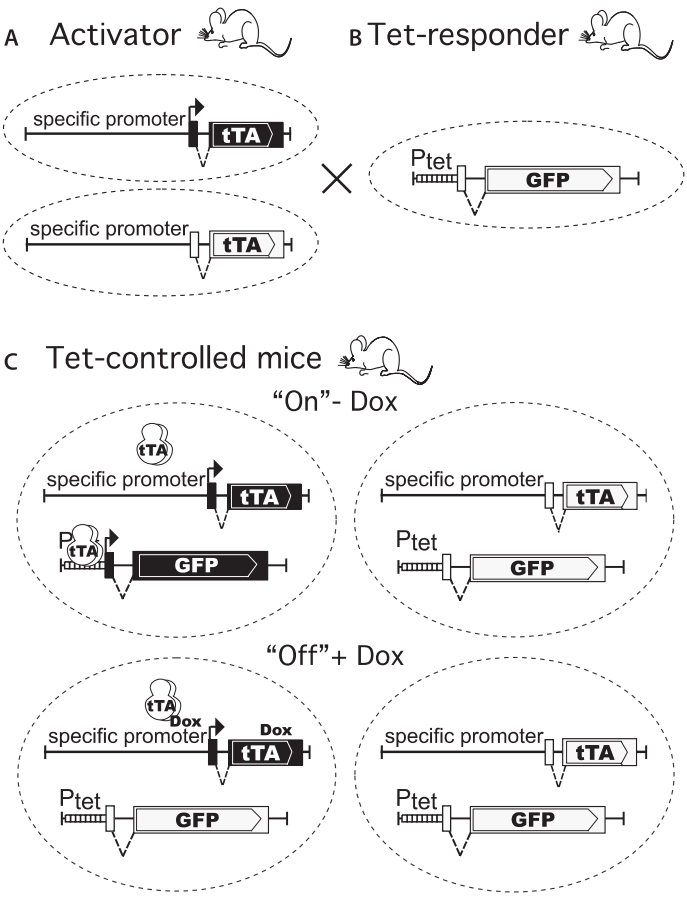

Fig. 5 A–C Scheme for setting up a Tet-regulated gene expression system in the mouse. To operate a Tet-regulated gene in the mouse, an activator line (**A**) has to be crossed with a responder line (**B**) to generate double-positive offspring (**C**) containing both the activator and the responder genes. Activator mice (**A**) express tTA selectively in those tissues and cells, where the promoter of the tTA transgene is active but not in other tissues. Single-positive responder mice (here for P_{tet}-GFP) do not express GFP in any cell of these mice. Double-positive transgenic mice (c) express responder genes specifically in those cells with functional tTA or rtTA expression reminiscent of tissue promoter specific activity. P_{tet}-GFP is turned-off by Dox, since tTA(Dox) is unable to bind P_{tet}. Active genes are given in *black*, inactive genes in *grey*. Gene symbols are the same as in Fig. 6

3.3 Setting up a Tet-Controlled Expression System in the Mouse

For successful generation of functional mouse lines capable of Dox-controlled gene expression, it is very useful to have in hand well-characterized responder and activator mice.

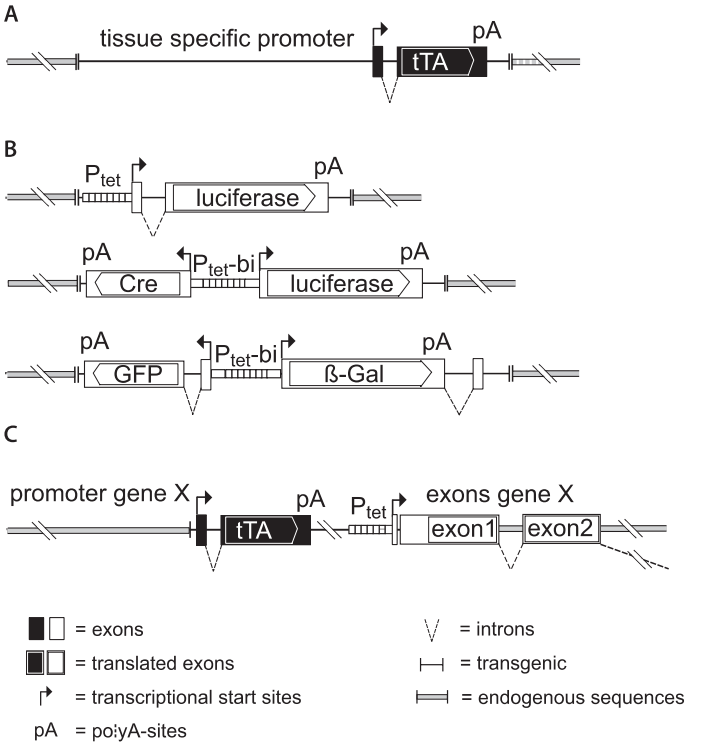

Fig. 6 A–C Schematic representations of gene constructs used in mice for Tet-controlled gene expression. **A** The transgenic expression of tTA is under the control of a tissue specific promoter fragment. **B** Transgenic Tet-reporter genes regulated by P_{tet} and P_{tet}-bi. **C** Gene-targeted insertion of tTA and P_{tet} into the ATG containing exon of an endogenous gene X. The endogenous ATG is now used as translational-initiation site for the targeted tTA expression. In *black* are tTAs expressed constitutively by the tissue specific promoter. In *grey* are the Tet-regulated genes

Transgenic mice harbouring reporter genes under P_{tet} or P_{tet}-bi control (see Sect. 4.4.1) are useful indicators for easy detection of tTA- or rtTA-dependent gene expression by enzymatic activity, bioluminescence or by fluorescence (Kistner et al. 1996; Hasan et al. 2001; Krestel et al. 2001). Usually, intercrosses between activator and responder mice give offspring of which 25% contain both the tTA or the rtTA and the P_{tet}- or the P_{tet}-bi reporter gene. In these double-transgenic mice, the reporter gene activity can visualize and quantify the spatiotemporal profile of functional tTA or rtTA expression. Second, well characterized tTA-activator lines can be used to identify newly generated P_{tet}/P_{tet}-bi responder mouse lines.

3.4 Tet-Controlled Reporter Mouse Lines

3.4.1 Reporter Genes

Reporter mice are needed for identifying functional tTA or rtTA mouse lines. Indicated below are four different reporter genes as possible choices.

3.4.1.1 Luciferase

Amongst the different reporters, the firefly luciferase gives highest sensitivity and allows exact quantitative analysis of gene activity in vivo. Few molecules of luciferase can be detected with very low background activity in mouse tissues. With luciferase half-life of 3–4 h in vivo (Leclerc et al. 2000), fast kinetic studies are possible. In mammalian cells, regulation factors of approximately 10^5 and approximately10^3 can be achieved for tTA and rtTA, respectively (Kistner et al. 1996). With 20 ng/ml of Dox, tTA-dependent gene expression in Hela cells is suppressed in less than 5 min and finally leaving merely a few molecules per cell. More importantly, upon Dox removal from the culture medium, luciferase gene activity can be monitored within 4 h of Dox removal reaching 20% of the steady-state level after 12 h. Techniques are also available for noninvasive imaging of luciferase expression in the mouse (Hasan et al. 2001). Luciferase activity can be easily determined in tissue extracts and tissue explants (Hasan et al. 2004) but so far in these systems has not been demonstrated at a cellular resolution neither by immunohistology nor by optical recording of luciferase activity.

3.4.1.2 Beta-Galactosidase

Although β-galactosidase (β-gal) is less sensitive than luciferase, it can also be quantified in tissue extracts using β-gal enzymatic assays. Now, with β-gal a cellular resolution of enzyme activity can easily be displayed in tissue slices. However, endogenous β-gal hinders detection of very low levels of transgenically expressed β-gal. Variants of β-gal targeted to the nucleus are highly sensitive for detecting β-gal signal in tissue slices. The detection of β-gal can be further enhanced in immunohistochemical reactions using anti-β-gal antibodies.

3.4.1.3 Fluorescent Proteins

Among the different fluorescent proteins (XFPs) which are currently available, the enhanced green fluorescence protein (eGFP) (Chalfie et al. 1994; Cormack

et al. 1996; Zolotukhin et al. 1996) has been used successfully as reporter (Krestel et al. 2001). When expression is analysed by live fluorescence, eGFP has by far the lowest sensitivity since auto-fluorescence of tissue at the wavelength for GFP excitation and emission is quite high. Nevertheless, strong eGFP expression can be easily monitored for macroscopic and microscopic analysis in tissue slices and in the living mouse (Krestel et al. 2001; Hasan et al. 2004). Thus, eGFP reporters can be used in vivo for identifying tTA- and rtTA-expressing mouse lines and for visualizing regulated gene expression by noninvasive imaging in living mice. The sensitivity of eGFP detection can be drastically enhanced by the use of anti-eGFPs antibodies. Immunohistochemical reactions permit a more detailed high-resolution microscopic analysis in tissue slices and immunoblots can be used for quantification of protein levels. Apart from eGFP, other GFP variants under the control of Tet inducible systems have been used in the mouse either by transgenesis (Hasan et al. 2004) or by viral vectors (see Sect. 4.8).

3.4.1.4 Cre Recombinase

Similar to the reporters discussed above, Cre immunostains of tissues can be used to detect tTA and rtTA activity at a cellular level. Quantification in immunoblots from tissue extracts is also achievable. By employing Cre reporters (RosaR26R; see Sect. 4.4.4), tTA- and rtTA-activated Cre expression at earlier stages in development can be historically engraved by Cre-induced β-gal expression (See Sect. 4.4.4).

3.4.2 Responder Mouse Lines with Uni-directional Tet Promoters (P_{tet}) for Dox-Controlled Expression of Either the Firefly Luciferase or the β-Galactosidase

3.4.2.1 Tg(tetL)1Bjd/J

A mouse line, previously called L7, where luciferase gene is under the control of P_{tet}, has been characterized in detail. Mice of line L7 (Tg(tetL)1Bjd/J; see Sect. 4.6) do not show any background luciferase activity and when activated by tTA or rtTA, luciferase activity is expressed at high levels and is tightly regulatable by Dox. In original studies, mice were generated which expressed transgenic tTA or rtTA under the control of the early human CMV_{EA1} promoter (Tg(tTAhCMV)3Bjd/J and Tg(rtTAhCMV)4Bjd/J; see Sect. 4.6). Both activator lines regulated the luciferase reporter of L7 mice to a high degree in various tissues. When these mice were treated with Dox in the drinking water

for 1 week, the regulation factor for luciferase activity was up to 10^5. Similarly, L7 mice crossed with liver-specific tTA-expressing mice (Tg(tTALap)5Bjd/J; see Sect. 4.6) in double-positive mice showed high levels of luciferase activity in the liver, which was tightly regulatable by Dox. By correlating enzymatic activity to the total number of cells, the authors estimated that there are approximately 10^4–10^5 luciferase molecules per cell in the fully induced state and only one molecule per ten cells in the noninduced state (Kistner et al. 1996).

3.4.2.2 Tg(tetNZL)2Bjd/J

Alternatively, a responder encoding luciferase in P_{tet}-bi modules can be used. In Tg(tetNZL)2Bjd/J mice (see Sect. 4.6), both luciferase and β-gal with a nuclear localization signal are controlled by P_{tet}-bi. Tg(tetNZL)2Bjd/J mice were tested with tTA activators of line Tg(tTALap)5Bjd/J (see Sect. 4.6) to demonstrate liver-specific expression of the Tg(tTALap) transgene (Kistner et al. 1996).

3.4.3 Responder Mice with Bidirectional Tet Promoter (P_{tet}-bi) Regulated Co-expression of Green Fluorescence Protein and β-Galactosidase

3.4.3.1 Tg(GFPtetO7lacZ)

Transgenic mice harbouring P_{tet}-bi for dual expression of β-gal and GFP have been used to visualize functional tTA. GFP permit the live analysis of tTA expression down to the cellular level (Krestel et al. 2001). Strong GFP fluorescence in mouse tissues can instantly identify activator lines with functional tTA or rtTA. In cases when no fluorescence signal is detectable, fixed tissues can be stained for β-gal activity by color-based enzyme substrates. Alternatively, immunostaining with anti-GFP and anti-β-gal antibodies display cells and tissues with active tTA. Detailed analyses of GFP/lacZ reporter mice [Tg(GFPtetO7lacZ)] with forebrain-specific tTA-expressing mouse line [Tg(Camk2a-tTA)1Mmay/J, see Sect. 4.6] have shown that two different genes under P_{tet}-bi can be co-expressed and regulatable by Dox in vivo. Interestingly, P_{tet}-bi is less prone to PEV, probably because the TATA-box elements are shielded by flanking DNA sequences: in this case, β-gal and GFP. [Tg(GFPtetO7lacZ] mice are available from R.S.

3.4.4
Responder Mice with Regulated Expression of Cre Recombinase

3.4.4.1
LC1

Another reporter mouse employs P_{tet}-bi controlled Cre and luciferase [line LC1, (Hasan et al. 2001; Schonig et al. 2002)]. In these mice, Cre and luciferase expression occurs only in presence of tTA or rtTA. As mentioned above, Cre expression is historically engraved in the Cre-target gene as soon as Cre is activated and, therefore, uncovers transient tTA or rtTA activities throughout mouse development. For this type of analysis, activator and LC1 mice must be crossed with RosaR26R mice, which contain a gene for Cre-activated β-gal in the Rosa locus (Soriano 1999). In triple-positive mice, ontogenic tTA- or rtTA-induced Cre action is preserved in tissues and detectable by β-gal activity. In addition, immonstains with anti-Cre show tTA or rtTA action at the time when the tissue was analysed (Hasan et al. 2001; Schonig et al. 2002; Krestel et al. 2004).

Most importantly, LC1 mice are suitable for Tet-controlled Cre-mediated gene activations or inactivation, which relies on loxP-modified targeted gene loci (e.g. see the chapter by R. Feil, this volume). Thus, tTA- or rtTA-activated Cre-activity can be restricted to specific cell types in adult mice, thus bypassing phenotypes that might arise when disruption of gene function either by gene deletion or dominant-negative gene expression occurs during early development. In one study, Cre-activated GluR-B(Q) gene was introduced in mice along with LC1 and Tg(Camk2a-tTA)1Mmay/J (see Sect. 4.6). Usually, GluR-B(Q) expression leads to early seizure-mediated death in mice (Brusa et al. 1995), but when GluR-B(Q) expression was suppressed during development, the function of GluR-B(Q) could be studied in adult mice by inducing GluR-B(Q) expression with tTA-activated Cre expression (Krestel et al. 2004).

3.5
tTA- and rtTA-Activator Mouse Lines

With a well characterized tTA-activator mice, both level and pattern of responder gene expression can be analysed. For this analysis, tTA-expressing mice are favored over rtTA activators since tTA function can be analysed without Dox treatment. Comparison of single positive responder mice with mice expressing both the responder and the activator can clearly show whether responder gene expression is tTA-dependent and regulatable by Dox. Over several years, two activator lines have consistently shown robust and reliable expression of tTA. The first mouse line, Tg(Camk2a-tTA)1Mmay/J (see Sect. 4.6), expresses functional tTA in principal neurons in the forebrain.

These mice have been used in numerous studies for controlled gene expression in the brain. The second mouse line, Tg(tTALap)5Bjd/J (see Sect. 4.6), expresses functional tTA in the liver. Both liver and brain tissues can be easily isolated from the mouse and can be analysed for reporter gene expression.

3.6 Tet Mice from the Jackson Laboratory

Some of the Tet-lines published and used in research (for complete lists see Schonig and Bujard 2003 and http://www.tetsystems.com) are available from the Jackson laboratories. Indicated below is a brief description of lines from the Jackson labs. Details and references can be found on the respective homepage. Currently, the Jackson Laboratory offers over 15 strains. The references and details can be found at http://www.jax.org.

tTA expressing mice:

Tg(Ins2-ttTA)2Doi/DoiJ: tTA is expressed in the pancreatic beta cells by the rat insulin promoter (Ins2, commonly designated RIP).

Tg(Camk2a-tTA)1Mmay/J: tTA is under the control of a forebrain-specific promoter derived from a gene encoding for the α-subunit of the calcium/calmodulin-dependent kinase II (α-CaMKII).

Tg(Eno2tTA)5021Nes/J: the rat neuron-specific enolase (*Eno2*) promoter was used for functional tTA expression in the striatum and cerebellum.

Tg(Eno2tTA)5030Nes/J: like line Tg(Eno2tTA)5021Nes/J but the tTA expression pattern is slightly different.

Tg(tTALap)5Bjd/J: the liver-enriched activator protein promoter (P_{LAP}) controls tTA expression in the liver.

Tg(MHCAtTA)6Smbf/J: tTA is under regulatory control of the rat α-myosin heavy chain promoter which directs tTA expression specifically in cardiac myocytes.

Tg(tTAhCMV)3Bjd/J: the human early cytomegalovirus promoter (P_{hCMV}) was used to express tTA in tissues where P_{hCMV} was known to be active (e.g. muscle, kidney, thymus, heart, pancreas).

Tg(MMTVtTA)1Mam/J: the MMTV-LTR was used to target tTA expression to the epithelial cells of secretory organs and skin in transgenic mice.

rtTA expressing mice:

Tg(rtTAhCMV)4Bjd/J: the P_{hCMV} promoter was used to drive rtTA expression to the same organs as described for Tg(tTAhCMV)3Bjd/J.

Tg(Nes-rtTA)306Rvs/J: functional rtTA is expressed by the rat nestin promoter in the neuroepithelium of the developing nervous system. Expression is also observed in some neuron subsets and testes of adult mice.

Tg(Ins2-rtTA)2Doi/DoiJ: the rat insulin promoter (Ins2, commonly designated RIP) was used to express rtTA in the pancreatic beta-cells.

Gt(ROSA)26Sor $^{tm1(rtTA,EGFP)Nagy}$ /J: a genetic module harbouring rtTA and GFP was targeted to the Rosa26 gene locus. Expression of both rtTA and GFP is achieved by the endogenous Rosa26 promoter after a Cre recombinase mediated deletion of the *loxP* -flanked interrupter sequence.

Uni-directional rtTA and tTA responsive mice:

Tg(tetL)1Bjd/J: The luciferase gene is regulated by P_{tet}.

Tg(tetFosb)4468Nes/J: a truncated variant of the *FosB* transcription factor is expressed by P_{tet}.

Tg(tetop-lacZ)2Mam/J: the LacZ gene encoding for β-gal is regulated by P_{tet}.

Tg(tetORo1-lacZ)3Conk/J: P_{tet}-Ro1 and P_{tet}-*lacZ* are co-integrated and under Dox control. Ro1, receptor activated solely by a synthetic ligand.

Tg(tetO-EGFP/FADD)1Doi/DoiJ: a fusion gene between eGFP and the Fas-associated death domain (FADD) is controlled by P_{tet}. The death effector domain of FADD is replaced by EGFP.

Tg(TettTALuc)1Dgs/J: tTA and luciferase genes under the control of P_{tet} were co-integrated in the mouse genome. Expression of tTA is both inducible and autoregulatory and luciferase expression was found in all organs examined (spleen, thymus, lung, liver, kidney, heart, cerebrum, cerebellum, lymph nodes and testes).

Bi-directional rtTA/ tTA responsive mice:

Tg(tetNZL)2Bjd/J: LacZ with a nuclear localization signal and luciferase genes are under the control of P_{tet}-bi.

3.7
tTA- and rtTA-Dependent Gene Expression in the Mouse

3.7.1
Transgenic Approaches

Detailed analysis of Tet-controlled reporter gene expression in the mouse have revealed several issues.

First, expression of tTAs and rtTAs as well as responders is influenced by where and how the transgene is inserted into the mouse chromosome. An early report employing Dox-controlled gene expression system in the mouse, clearly demonstrated that two mouse lines [Tg(tTALap)] expressing tTA under control of a liver-specific promoter induced responder gene expression either exclusively in the liver or also in brain tissues. Again, tTA-dependent β-gal expression was mosaic in hepatocytes (Kistner et al. 1996). Similar results were also obtained in mouse lines with the neuronal specific enolase promotor driving tTA-dependent gene expression in the brain [see Sect. 4.6: Tg(Eno2tTA)5021Nes/J and Tg(Eno2tTA)5030Nes/J; Chen et al. 1998]. Integration-dependent alteration in gene expression pattern was also described for P_{tet}-responder mouse lines (Mayford et al. 1996). Three different independent responder mouse lines were crossed with a forebrain-specific mouse line (line B) to generate three different combinations of double-positive mice. In line B20 line, the P_{tet}-responder [α-CamKII (T-D)] was expressed in forebrain, hippocampus, striatum and amygdala. In line B22, there were moderate levels of α-CamKII (T-D) expression in the hippocampus, subiculum, striatum and amygdala and little expression in the neocortex. The expression pattern of line B21 was very much restricted to the amygdala. The results show that insertion sites and copy number of P_{tet}-controlled transgenes can influence gene expression patterns. The integration-dependent alteration in gene expression pattern was less pronounced in P_{tet}-bi responder mice Tg(GFPtetO7lacZ). In four different Tg(GFPtetO7lacZ) mouse lines, brain-specific P_{tet}-bi-controlled gene expression was very similar but levels of GFP and β-gal expression were variable and mosaic (Krestel et al. 2001). Similar results were obtained in transgenic mice harbouring P_{tet}-bi constructs expressing fluorescent calcium indicator proteins (Hasan et al. 2004).

Thus, random integration of transgenes in the genome, often as multiple copies, makes them susceptible to surrounding transcriptional control elements such as enhancer and silencer sequences. The observed variegation of gene expression levels from cell to cell, known as position-effect-variegation (PEV), can be explained by different degrees of crosstalk between the inserted transgene and heterochromatin and euchromatin during development.

Responder and activator transgenes engineered into larger DNA fragments, such as BACs, which are less prone to PEV, might allow better recapitulation of the endogenous gene activity pattern (Robertson et al. 2002; Heintz

2004). However, more studies are needed before it becomes clear how well the BAC-delivered components would improve Tet-controlled gene expression in mice.

Hence, whenever transgenic responder or activator lines are generated, it is always necessary to screen the expression of tTA, rtTA and activated responder genes in offspring from all founders. Therefore, founders need to be crossed to activator or reporter mice, and in double-positive offspring the Tet-regulated gene expression has to be evaluated. Only those founders with good expression are selected to establish a mouse line. Using P_{tet}-bi constructs, we achieved a high success rate and in all our experiments obtained reliable Tet-regulated genes (Jerecic et al. 2001; Krestel et al. 2001; Mack et al. 2001; Hasan et al. 2004). Nevertheless, the expression pattern has to be monitored over several generations since sometimes the transgenes are unstable or silenced. Also, by changing the genetic background of the mouse, alterations in transgene expression can occur. In one study, Tet-controlled gene expression was described to be highly variable between mice in the CBA/Ca genetic background but it became more uniform, with a higher proportion of expressing cells, in the C57Bl/6 J background (Robertson et al. 2002).

3.7.2 Gene Targeting Approaches

To overcome disadvantages of the transgenic systems, the genetic elements constituting Tet regulation can be targeted to defined genetic loci by homologous recombination in ES cells for the generation of genetically modified mice. The tTA or rtTA genes can be inserted as single copy at a precise site in the genome. In this way, instability of transgenes and variability between founders can be minimized and the analysis of multiple founders is not necessary. In an elegant study, two alleles of the endothelin-B receptor gene were targeted either by tTA/rtTA or the P_{tet}-Ednrb minigene, respectively, and the P_{tet}-Ednrb gene showed expression patterns similar to the endogenous gene and could be regulated by Dox (Shin et al. 1999).

Dox regulation of an endogenous gene can also be achieved by co-inserting tTA and P_{tet} in a single construct and targeting it into a defined allele by homologous recombination in ES cells. A good example for this case comes from a study where the tTA and P_{tet} was inserted by gene targeting into the 5′UTR of the gene for the potassium channel subunit, SK3 (Fig. 6). In the mouse, the targeted SK3 gene was overexpressed and resulted in a phenotype with an abnormal respiratory responses to hypoxia and compromised parturition. Both conditions could be corrected by down-regulating SK3 gene expression with Dox (Bond et al. 2000). Furthermore, tTA and rtTA have also been targeted into the ubiquitously expressed gene locus, Rosa26 (Zambrowicz et al. 1997), permitting Tet-controlled gene expression in most tissues (Belteki et al. 2005; Masui et al. 2005; Yu et al. 2005).

Thus, targeting of tTA/rtTA to endogenous promoter regions can consistently provide mice which should express tTA/rtTA in tissues where the endogenous promoter is normally active. Although ectopic rTA/tTA-dependent gene activities have not been described, it remains to be seen whether mosaic expression is an issue in the gene targeting approach as well.

Another issue with the gene targeting approach is that it cannot predict the expression level of Tet-controlled genes but fine adjustment of gene expression is possible by keeping animals on specified Dox concentrations (Bond et al. 2000; Bejar et al. 2002).

3.7.3 Kinetics of Tet-Controlled, Regulated Gene Expression in the Mouse

3.7.3.1 Peripheral Tissue

Tet-controlled mice for luciferase expression [Tg(tetL)1Bjd/J] crossed with mice with rtTA under control of the hCMV-promoter [Tg(rtTAhCMV)4Bjd/J] has allowed detailed kinetic studies of Dox-induced luciferase expression (Kistner et al. 1996; Hasan et al. 2001). Mice treated with Dox in the drinking water for various periods of time show activation of luciferase activities after 4 h in most organs and full induction was achieved in 24 h. Mice kept on Dox (20 µg/ml) for 1 week showed partial induction after 24 h in most tissues (tongue, heart, thymus and pancreas) and full gene activities were apparent in 1 week. At a lower concentration (0.2 µg/ml), luciferase expression was induced in the pancreas, but not in kidney. Injecting mice with a single dose of Dox (2 mg) leads to maximum luciferase expression after eight h, which eventually subsides to undetectable levels after 48 h (Hasan et al. 2001).

3.7.3.2 Brain

In neurons of the brain, which are protected from easy drug access by the blood brain–barrier, Dox control of rtTA-dependent gene expression was also achieved, but with slower kinetics. Induction of rtTA-dependent gene expression in cerebellar granular cells was obtained within 3 days when mice were treated with 2 mg /ml of Dox in drinking water and 6 mg /g of Dox in food pellets. Dox-controlled regulated gene expression was strongly reduced after 7 days of Dox withdrawal and after two more weeks it was decreased down to undetectable levels (Yamamoto et al. 2003). A different study also reported that 6 mg /g of Dox in food pellets was sufficient to induce gene expression in the hippocampus, septum striation and cortical layers within 6 days (Mansuy et al. 1998).

For tTA-dependent gene expression in the brain, lower doses of Dox are sufficient for regulating expression levels.

In neurons of tTA activator mice [Tg(Eno2tTA)5021Nes/J], it has been reported that tTA-dependent gene expression was turned-off by 200 μg Dox/ml in drinking water. Similar levels of down-regulation were also observed with 25 μg /ml of Dox, and even lower Dox doses substantially, albeit partially, down-regulated tTA-controlled gene expression. This showed that the level of Tet-regulated transgene expression is adjustable in vivo (Chen et al. 1998).

Full reactivation of Dox-suppressed transgenes was reported in earlier studies for adult mice which were kept under 1 mg/ ml of Dox in the drinking water for 2–3 weeks (Mayford et al. 1996). Similarly, cycles of reporter gene inactivation (+Dox, 2 mg/ml, 5days) and re-activation (–Dox, 10 days) can be achieved multiple times in single individual mice as monitored by noninvasive imaging of luciferase activity (Hasan et al. 2001) .

However, it is necessary to treat developing animals early, in utero, with lower Dox doses if rapid and efficient tTA-dependent re-activation is warranted at a later time point after Dox withdrawal. Mice kept on high-Dox (2 mg/ml) throughout development showed only partial re-activation of tTA-dependent responder genes after more than 8 weeks of Dox withdrawal (Chen et al. 1998; Mack et al. 2001; Krestel et al. 2004). In contrast, mice treated with low-Dox (50 μg/ml) showed gene re-activation to more than 50% of maximal levels within 2 weeks of Dox withdrawal. Even more rapid induction was observed in mice kept on 25 μg/ml of Dox. Similarly, mice treated with a low-Dox diet (0.040 mg Dox/g chow) for more than 3 weeks show no expression of the Dox-controlled gene. After removing Dox from the food, tTA-induced expression was readily apparent within 2 days and is present at high levels at day 14 after switching to a Dox-free diet (Bejar et al. 2002).

3.8
Transfer of Tet-Controlled Gene Expression Via Viral Systems

The virus mediated gene transfer (e.g. see the chapter by P. Osten et al., this volume) is an attractive alternative to deliver Tet-regulated genes into the mouse and other laboratory animals. The tissue selectivity is obtained by local virus injection and by promoter elements used in virus constructs. The two components (activator and responder) of the Tet-inducible system can be incorporated into a single or two separate viral vectors.

In a Parkinson mouse disease model, an adenovirus-based tTA system was used to examine the effects of tyrosine hydroxylase (hTH1) expression on the dopaminergic nigrostriatal system. The tTA gene was expressed by the ubiquitous mouse phosphoglycerate kinase gene promoter (PGK) and hTH1 was placed under the control of P_{tet} (Corti et al. 1999a). A similar vector also mediates Dox-controlled expression of hTH-1 in brain grafts of human neural progenitors (Corti et al. 1999b). In these studies, both components of the Tet system (PGK-tTA and P_{tet}-hTH-1) were placed in the head-to-tail orientation. The complete adenovirus construct (AdPGK.tet.hTH-1) was used to produce

virus stocks and infected in human neural progenitor cells. High levels of hTH-1 expression were observed in infected cells and hTH-1 activities were completely repressed with Dox. These cells were then transplanted into rodent brain. Four weeks after transplantation, untreated and Dox-treated animals (1 mg/ml in the drinking water) showed no difference in the size and quality of transplanted cells. In untreated animals, grafted cells showed intense immunoreactivity for hTH-1, whereas no detectable reactivity was apparent in the Dox-treated group. After 1 week of Dox treatment (1 mg/ ml drinking water) hTH-1 immunoreactivity diminished significantly and was undetectable after 2 weeks of treatment. Removal of Dox for a period of 11 weeks resulted in the appearance of hTH-1 expression. Further gene reactivation was observed 4 weeks after Dox removal in mice treated with low-Dox (50 µg/ml) in the drinking water (Corti et al. 1999b). In another study, two independent adenoviruses were used to express the tTA or rtTA placed under the control of the hCMV promoter (abbreviated as AdTet-On or AdTet-Off). The second adenovirus vector contained the EGFP under the control of the minimal P_{tet}-promoter element (abbreviated as AdTRE-EGFP). Both viruses (adTet-On or AdTet-Off plus AdTRE-EGFP) were co-infected into the dentate gyrus of hippocampus with a ratio of 1:20 (Harding et al. 1998). This ratio was chosen in order to avoid any potential nonspecific activation of the transgene in the AdTRE-EGFP construct. With the AdTet-Off system, EGFP was visible 3 days after the stereotaxic injection of virus in rats. After 5 days of Dox treatment, EGFP was significantly reduced in the hippocampus and was undetectable after 10 days of treatment. When Dox was removed for a period of 3 months, the EGFP signal was fully visible. With the AdTet-On system, EGFP was visible by Dox treatment and was reduced significantly after 7 days of treatment. After 10 days, it was undetectable. Upon re-administration of Dox, re-induced EGFP expression was visible within 3 days and after 10 weeks of Dox treatment, the EGFP signal was present with similar intensity. These results show that the adenovirus-based Tet-inducible system is able to mediate long-term regulated gene expression.

Retrovirus and lentivirus systems were used with success to deliver genes in mammalian systems. Retroviral vectors capable of carrying transactivators and tetO driven genes have been engineered (Hwang et al. 1996; Hwang et al. 1997). Cultured dividing cells infected with these viruses show Dox-dependent gene expression with regulation factor of more than 400-fold. By employing tetO-driven autoregulatory system to drive tTAs with different activation potential, it has been possible to reduce transactivator-related cellular toxicity thereby increasing infectivity in hepatocytes both in vitro and in vivo (Kuhnel et al. 2004). The suitability of retroviruses to infect dividing cells make them ideally suited for cancer therapy. The possibility of infecting both dividing and nondividing cells became possible by the employment of HIV-based lentiviruses. Lentiviral systems harbouring tTA and luciferase as reporter have already proven highly successful (Vigna et al. 2002). For example, ex vivo transduction of human $CD34^+$ hematopoietic cells transplanted into NOD/SCID mice showed mul-

tiple Dox-dependent on/off cycles for up to 20 weeks. In a different study, tTA-dependent expression of ciliary neurotrophic factor (CNTF) under two separate lentiviral vectors was tested in a rat model for Huntington disease (Regulier et al. 2002). Rat with high levels of CNTF expression were neuroprotected from quinolinic acid-induced neuronal damage and showed improved behavioral performance compared to animals when CNTF was either switched-off by Dox or in control animals not expressing CNTF. More recently, it was also demonstrated that single lentiviral vector with the Tet-regulated system elements can achieve Dox-controlled, long-term tissue-specific expression (Vogel et al. 2004).

The recombinant adeno-associated viruses (rAAV) can also provide Tet-controlled gene expression in vitro and in vitro. Two Tet-inducible viral systems were constructed for Dox-controlled regulated GFP expression. In the first case, two AAV2 viruses were generated, each carrying activator genes and P_{tet}-response elements, respectively (McGee Sanftner et al. 2001). In the second case, a single AAV2 virus was equipped with both tTA and P_{tet}-GFP-response genes (Folliot et al. 2003). In both systems, retinal GFP expression was monitored in vivo with a noninvasive fluorescence imaging method. GFP expression was initially observed 1 week after infection. GFP levels were Dox-controlled and multiple on/off cycles of regulated gene expression could be performed. The Dox-doses were also varied in vivo and showed a correlation to GFP expression levels. Thus, transduction of retinal cells with Tet-inducible rAAV delivered genes allows for tight regulation of gene expression.

3.9 Closing Remarks

The temporal and spatial regulated gene expression in various mouse tissues is opening up unique possibilities to systematically dissect complex biological processes. Essential components of the Tet-inducible system, transactivators and Tets, do not appear to have toxic side effects in mice and Dox can regulate tTA and rtTA-dependent gene expression to a high degree in various tissues. With the employment of improved Tet-regulated modules by BACs and gene knock-in methodologies, it would be feasible to target specific cell-types for Tet-controlled gene expression, precisely and reliably to study temporally-controlled changes in physiological conditions in the mouse.

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Novel Gene Switches

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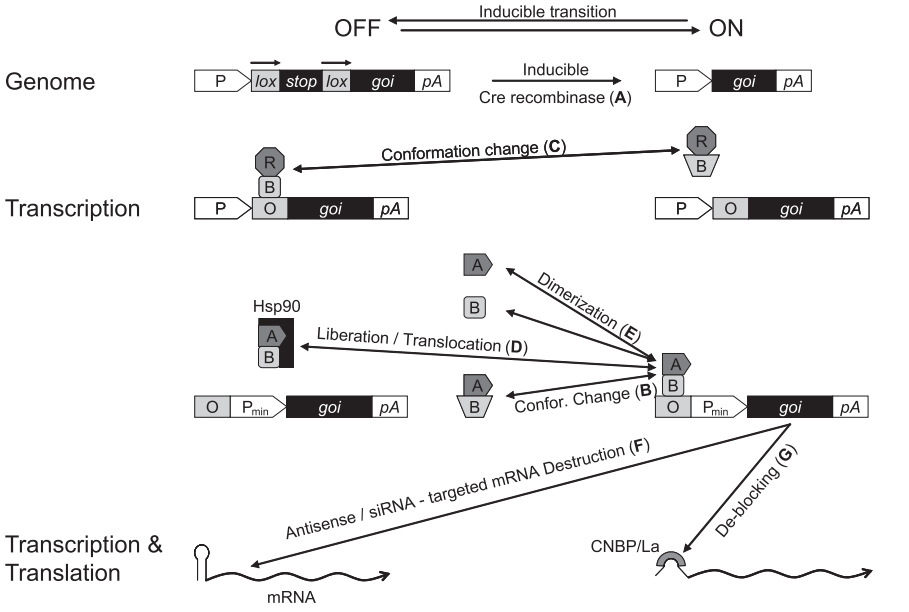
Abstract Controlling gene activity in space and time represents a cornerstone technology in gene and cell therapeutic applications, bioengineering, drug discovery as well as fundamental and applied research. This chapter provides a comprehensive overview of the different approaches for regulating gene activity and product protein formation at different biosynthetic levels, from genomic rearrangements over transcription and translation control to strategies for engineering inducible secretion and protein activity with a focus on the development during the past 2 years. Recent advances in designing second-generation gene switches, based on novel inducer administration routes (gas phase) as well as on the combination of heterologous switches with endogenous signals, will be complemented by an overview of the emerging field of mammalian synthetic biology, which enables the design of complex synthetic and semisynthetic gene networks. This article will conclude with an overview of how the different gene switches have been applied in gene therapy studies, bioengineering and drug discovery.

Keywords Gene Switch · Inducible expression · Gene therapy · Biopharmaceuticals · Bioengineering

1 Introduction

The control of (trans)gene expression is an important tool for gene-function analysis (Malleret et al. 2001), drug discovery (Aubel et al. 2001), biopharmaceutical manufacturing (Fussenegger et al. 1998b; Boorsma et al. 2000), the design of synthetic gene networks (Elowitz and Leibler 2000; Gardner et al. 2000; Atkinson et al. 2003; Kramer et al. 2004b), gene therapy (Auricchio et al. 2002) and engineering of desired cell phenotypes (Niwa et al. 2000). The ideal gene regulation system:

Fig. 1 Strategies for regulated gene expression and target protein production at different biosynthetic levels. *A:* Excision of a stop cassette by an inducible site-specific recombinase (Cre) results in a functional expression unit. *B:* Conditional activation of minimal promoters by inducible binding of a DNA-binding protein fused to a transcriptional activator. *C:* Conditional repression of promoters by inducible binding of a DNA-binding protein fused to a transcriptional repressor. *D:* In the absence of an inducer the transactivator (a+b) is sequestered by heat shock protein 90 (HSP90), but can be released by administration of the inducer, which results in activation of a minimal promoter. *E:* Dimerization of a DNA-binding domain and a transcriptional activator result in a functional transcription factor for activation of minimal promoters. *F:* Expression of antisense mRNA or short interfering RNAs results in destruction of homologous target mRNA. *G:* In the absence of CNBP or La, translation is blocked by a stem-loop structure (TOP) in the 5′ untranslated region of the mRNA. Expression of CNBP or La results in resolution of the TOP secondary structure, thus de-repressing translation. *H:* Inducible RNA-dependent RNA-polymerase-driven amplification of (+)strand mRNA from a subgenomic promoter results in templates for translation. *I:* Chemically induced dimerization of eIF4G subunits results in reconstitution of functional eIFG4, thus restoring translational initiation. *J:* In the absence of inducer, the translation initiation factor 4G (eIF4G) is farnesylated and attached to cellular membranes. Inhibition of farnesylation by farnesyltransferase inhibitors results in non-membrane-bound eIF4G, thus restoring translation initiation. *K:* Small molecule-binding to a 5′UTR positioned aptamer structure results in inhibition of translation. *L:* Ribozyme-mediated self-cleavage of mRNA is prevented by ligand-binding, thus preserving functional mRNA for translation. *M:* Chemically-induced dimerization of inactive enzyme subunits results in functional enzymes. Accordingly, membrane receptors can be activated by chemically induced dimerization or multimerization. *N:* The protein of interest is fused to an aggregation domain leading to protein multimers in the rER, thereby preventing further processing and secretion. Chemically induced deaggregation of rER sequestered protein results in further processing and in cleavage of the aggregation domain prior to secretion into the culture medium. *5′UTR* 5′ untranslated region, *A* transcriptional activation domain, *B* DNA-binding domain, *CNBP* cellular nucleic acid binding protein, *eIF4G* eukaryotic translational initiation factor 4G, *goi* gene of interest, *La* La autoantigen, *lox* recognition site for Cre recombinase, *P* promoter, *pA* polyadenylation signal, P_{min} minimal promoter devoid of any enhancer binding sites, *R* transcriptional repressor domain, *rER* rough endoplasmatic reticulum, *siRNA* short interfering RNA, *stop* transcriptional stop signal


Genome
OFF
Inducible transition
ON
P
lox
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lox
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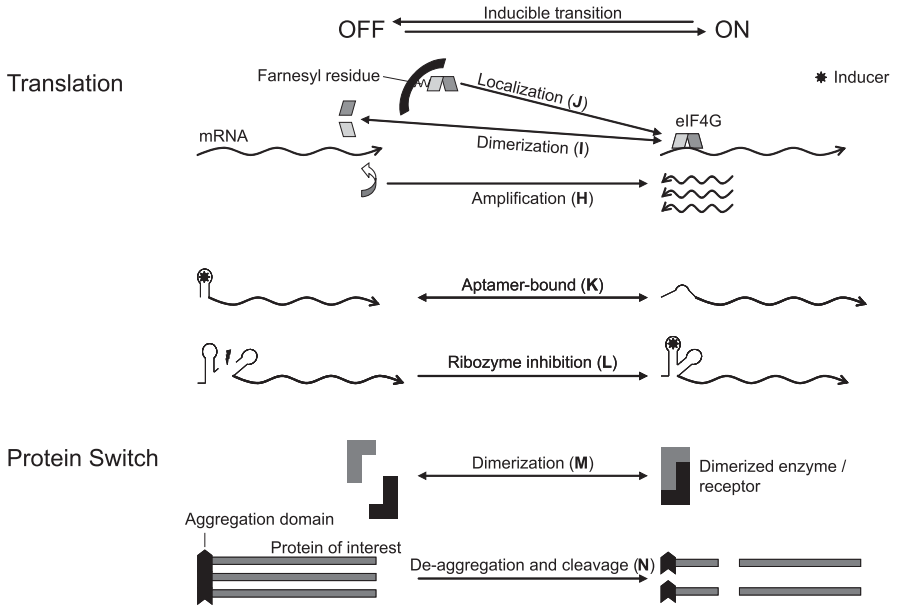
pA
Inducible
Cre recombinase (A)
Transcription
Conformation change (C)
R
B
O
Hsp90
Liberation / Translocation (D)
Dimerization (E)
Confor. Change (B)
Pmin
Transcription &
Translation
Antisense / siRNA - targeted mRNA Destruction (F)
De-blocking (G)
CNBP/La
mRNA


Translation
OFF
Inducible transition
ON
Farnesyl residue
Localization (J)
Inducer
eIF4G
mRNA
Dimerization (I)
Amplification (H)
Aptamer-bound (K)
Ribozyme inhibition (L)
Protein Switch
Dimerization (M)
Dimerized enzyme /
receptor
Aggregation domain
Protein of interest
De-aggregation and cleavage (N)

- Is of heterologous origin to ensure interference-free operation and its heterologous components fail to evoke an immune response
- Enables seamless integration into the regulatory and metabolic network of the target cell
- Provides all expression levels from very high to virtually no expression in response to a bioavailable inducer, including clinically licensed or other inert molecules or physical conditions without side-effects
- Is compatible with current viral and non-viral gene-transfer technologies
- Supports configurations for tissue- or target-specific interventions.

Since the development of the first transcriptional expression control systems more than a decade ago (Baim et al. 1991; Gossen and Bujard 1992), a variety of concepts are now available for modulating gene expression and protein production at almost any stage in the protein biosynthetic pathway (Fussenegger 2001; Weber and Fussenegger 2002; Weber and Fussenegger 2004a, Fig. 1). Highest in the hierarchy and at the level of the genome, the presence and absence of single genes or functional expression units can be modulated (irreversibly) by conditionally active recombinases such as the hormone-analog-inducible CRE or FLP variants (Branda and Dymecki 2004). At the transcriptional level, a variety of concepts have been developed to establish a functional link between a transcriptional modulator (activator/repressor) and minimal or constitutive promoters, either by allosteric DNA-binding proteins, chemically induced dimerizers or inducible translocation of transcription factors between the subcellular compartments (Fussenegger 2001; Weber and Fussenegger 2002). Further downstream, at the mRNA level, switching can be performed by inducible destruction, by amplification of the messenger or by controlled translation initiation (Boorsma et al. 2000; Malphettes and Fussenegger 2004). At the protein level, the activity and availability of the target proteins can be modulated either by pharmacologically controlled retardation in subcellular compartments or by inducible enzyme activation (Rossi et al. 1997; Jin et al. 1998; Rivera et al. 2000).

While these first-generation concepts are modular building blocks that translate small molecule or temperature stimuli into altered gene and protein activity, a second generation of gene switches is currently emerging, represented by higher-order regulatory networks, which are finally connected to cellular signals and thus enable the cell to self-trigger a genetic response for therapeutic or bioprocessing applications.

This chapter outlines the first generation of gene switches and how these modular building blocks can be used to construct second-generation switches through integration into the host network together with a comprehensive overview of applications for gene switches.

2
First-Generation Gene Switches

2.1
Regulation at the Genome Level

The most stringent but irreversible control of gene expression can be achieved by the inducible integration or removal of the transgene into the host genome by site-specific recombinases (SSRs, Branda and Dymecki 2004). The most widely used SSR Cre is derived from the bacteriophage P1 genome and catalyzes the recombination between two *lox* P sequences in an orientation-dependent reaction (Branda and Dymecki 2004): an inverted orientation of two *lox* P sites on the same DNA strand provokes inversion of the intermittent sequence, whereas a consecutive arrangement results in elimination of the *lox* P-flanked (floxed) region (Fig. 1, A; Branda and Dymecki 2004). The inverse reaction can also be used to insert a *lox* P-encoding plasmid into a *lox* P site within the genome, which, however, is less effective since the excision reaction is thermodynamically favored. For such insertion, the recombinase-mediated cassette exchange (RMCE, Lauth et al. 2002; Branda and Dymecki 2004) has been developed based on *lox* P mutants (*lox*P*) that enable recombination with another *lox* P* but not with wild-type (wt) *lox* P. Therefore, a gene of interest, inserted between a *lox* P and *lox* P*, is thus integrated between a *lox* P and *lox* P* sequence previously placed on the genome of the target cell line (Branda and Dymecki 2004).

A major advance in the use of SSRs for conditional mutagenesis was the development of inducible SSR variants based on Cre fusions to the estrogen receptor, resulting in the heat shock protein 90 (Hsp90) -sequestered Cre, which could subsequently be released and activated by the addition of estrogen (Brocard et al. 1997). In order to avoid interference of the endogenous estrogen in animal models, estrogen receptor variants have been engineered so as to be exclusively responsive to the steroid analog 4-hydroxytamoxifen, thus enabling interference-free Cre activity control in transgenic animals (CreER^{T}, Brocard et al. 1997; CreER ^{T2}, Indra et al. 1999). In order to allow dual pharmacologic SSR control, Cre has been fused to a mutated variant of the progesterone receptor that is responsive exclusively to the synthetic steroid RU486 (mifepristone) but not to endogenous progesterone (Brocard et al. 1998). Placing the inducible Cre variants under the control of tissue-specific promoters allows expression control in space and time in transgenic mice. This has become an indispensable tool in the functional analysis of genes that prevent the correct development of the mouse embryo or show pleiotropic effects when expressed in the whole animal (Branda and Dymecki 2004).

In addition to the most widely used Cre recombinase, the *Saccharomyces cerevisiae* Flp has been established recently for use in mammalian cells by increasing the enzymatic performance by directed evolution (Flpe, Buchholz et al. 1998) and by fusion to the tamoxifen-responsive receptor domain

(FlpeER^{T2}, Hunter et al. 2005). The number of mutually compatible recombination events has been increased to three by adapting the *Streptomyces* phage-derived *Φ*C31 SSR, catalyzing recombination of the heterotypic *attB* and *attP* sites (Ginsburg and Calos 2005), for use in embryonic stem (ES) cells (Belteki et al. 2003) or in tissue engineering for the regeneration of human skin to correct junctional epidermolysis bullosa (Ortiz–Urda et al. 2003).

2.2 Regulation at the Transcriptional Level

The majority of recently developed gene switches focus on blocking or activating mRNA synthesis by inducible coupling of transcriptional repressors or activators to constitutive or minimal promoters. Repression can be achieved either by binding the repressor proteins, which sterically block transcriptional initiation, or by actively repressing transcription through transcriptional silencers such as the Kruppel-associated box protein (KRAB, Bellefroid et al. 1991), which exerts its repressing function when bound downstream and upstream of a constitutive promoter (Fig. 1, C; Malphettes and Fussenegger 2004). Activation of mammalian or viral enhancerless minimal promoters can be achieved by the inducible coupling to an activation domain such as human p65 (from NF-κB, Schmitz and Baeuerle 1991; E2F4, Akagi et al. 2001) or the *Herpes simplex* virus VP16 (Triezenberg et al. 1988) at full length or at minimal versions thereof (Baron et al. 1997).

Conditional coupling of transcriptional repressors or activators can be achieved through (a) allosteric proteins that bind the promoters in response to external stimuli, (b) intracellular receptors that are released from sequestrating proteins and, thus, can bind target promoters, or (c) chemically induced dimerizers, where the addition of small molecules induces heterodimerization of a DNA-binding domain and a transcriptional repressor or activator, which can thus bind the target promoter (reviewed in Fussenegger 2001).

2.2.1 Transcriptional Regulation with Allosteric Proteins

The majority of transcription control systems is based on prokaryotic proteins that change their operator-binding activity in response to external stimuli and can, thus, be fused to transcriptional activators or silencers for the conditionally regulating transcription of promoters harboring specific operator sites (Fig. 1, B, C). Table 1 provides an overview of the different allosteric proteins that have been used successfully to modulate transcriptional activity in mammalian cells in response to antibiotics (Gossen and Bujard 1992; Fussenegger et al. 2000; Weber et al. 2002a), bacterial quorum-sensing messengers (Neddermann et al. 2003; Weber et al. 2003b; Shiner et al. 2004; Weber et al. 2005b), catabolites (Baim et al. 1991; Malphettes et al. 2005), as well as to the cultivation temperature (Weber et al. 2003a, Table 1).

Table 1 Gene switches depending on allosteric proteins to change their DNA-binding characteristics in the presence of the inducing molecule

Regulatory protein	Transcription factor	Inducer	Response to inducer	Acronym, reference
CymR	CymR	Cumate	De-repression	Q-mate, http://www.krackeler.com/products/fid/2755
E	ET1 (E-VP16)	Macrolides	De-activation	E.REX; Weber et al. 2002a
E	ET4 (E-KRAB)	Macrolides	De-repression	E.REX; Weber et al. 2002a
HdnoR	NT (HdnoR-VP16)	6-Hydroxynicotine	De-activation	NICE; Malphettes et al. 2005
LacI	LAP267 ($LacI^{N\text{-}term}$-VP16-$LacI^{C\text{-}term}$)	Dual-responsive to IPTG and temperature	De-activation	Baim et al. 1991
Mutated TetR (rTetR)	rtTA (rTetR-VP16)	Doxycycline	Activation	TET; Gossen et al. 1995
Mutated TetR ($TetR^{mut}$)	$rtTA^{S2}$-M2 ($TetR^{mut}$-VP16)	Doxycycline	Activation	TET; Urlinger et al. 2000
PIP	PIT (PIP-VP16)	Streptogramins	De-activation	PIP; Fussenegger et al. 2000
PIP	PIT4 (PIP-KRAB)	Streptogramins	De-repression	PIP; Fussenegger et al. 2000
Rex	Rex-VP16	NADH	De-activation	REDOX; Weber et al., personal communication
RheA	CTA (RheA-VP16)	Low temperature	Activation	TIGR; Weber et al. 2003a
RhlR, LasR	RhlR-NLS-VP16, LasR-NLS-VP16	Homoserinelactones	Activation	Shiner et al. 2004
ScbR	SCA (ScbR-VP16)	SCB1	De-activation	QuoRex; Weber et al. 2003b
ScbR	SCS (ScbR-KRAB)	SCB1	De-repression	QuoRex; Weber et al. 2005b
TetR	TetR	Tetracycline	De-repression	T-REX; Yao et al. 1998
TetR	tTA (TetR-VP16)	Tetracycline	De-activation	TET; Gossen and Bujard 1992
TraR	TraR-p65	N-(3-oxo-octanoyl)-homoserine lactone	Activation	Neddermann et al. 2003

IPTG, isopropyl-β-D-galactopyranoside; KRAB, kruppel-associated box-derived transcriptional silencer; NADH, reduced nicotinamide adenine dinucleotide; NLS, nuclear localization sequence; p65, activation domain of human NF-κB; SCB1, racemic 2-(1'-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide; VP16, *Herpes simplex* viral protein 16-derived transactivation domain

The main novelties of the past 2 years include the emergence of catabolite- and quorum-sensing-based expression systems. Catabolite-based systems, relying on bacterial repressors, which control catabolic genes for alternative carbons sources, have been transferred to mammalian cells for (a) the *Pseudomonas putida* F1-based Q-mate technology (http://www.krackeler.com/products/fid/2755), where repression of target promoters is achieved by cumate-responsive binding of the repressor CymR (Eaton 1997) and (b) the *Arthrobacter nicotinovorans* pAO1-derived expression system relying on the activation of chimeric promoters (P_{NIC}) by 6-hydroxynicotine-responsive binding of the prokaryotic repressor HdnoR, fused to the *Herpes simplex* VP16 transactivation domain (Malphettes et al. 2005). One advantage of these catabolite-based systems is the high tolerance of mammalian cells to the inducing molecules, even at high doses (>100 μg/ml), as well as the absence of interference with host physiology. However, these systems were not shown to function in mice, which might be due to the high effective cumate concentrations (100 μg/ml range) or to the extremely rapid renal clearance of 6-hydroxynicotine from the organism ($t_{1/2} \leq$36 min; Malphettes et al. 2005).

The quorum-sensing-based expression systems are derived from prokaryotes that manage intra- and interpopulation communication by quorum-sensing molecules, which bind to receptors in target cells and initiate specific regulon switches by modulating the receptors' affinity to cognate promoters (Viretta and Fussenegger 2004). Over 50 species of Gram-negative bacteria produce quorum-sensing molecules that slightly differ in structure but are selectively recognized by specific proteins (Bassler 2002). For the following reasons, quorum-sensing systems are attractive options for adaptation for use in mammalian cells: (a) prokaryotes and eukaryotes have a long history of co-evolution; the signaling molecules, therefore, probably do not have an immunogenic impact on higher organisms. (b) Due to the specificity of signaling molecules and their recognition by cognate sensor proteins, a variety of quorum-sensing-based gene regulation systems could be constructed. Three such systems have been developed: (a) The *Agrobacterium tumefaciens* TraR protein was fused to the eukaryotic activation domain NF-κB p65, thus generating a novel chimeric transactivator (Neddermann et al. 2003). In the presence of the quorum-sensing molecule *N*-(3-oxo-octanoyl)-homoserine lactone, TraR-p65 activates expression from a minimal promoter fused to the TraR-specific operator sequence (Neddermann et al. 2003). (b) The QuoRex systems are based on the *Streptomyces coelicolor* A3(2) ScbR repressor that binds its cognate operator O_{ScbR} in the absence of the butyrolactone SCB1 (Takano et al. 2001). ScbR has been fused both to repressing KRAB and to activating VP16, thus enabling SCB1-inducible as well as SCB1-repressible gene expression in mammalian cell culture and in mice implanted with QuoRex cells (Weber et al. 2003b, 2005b). (c) The *Pseudomonas aeruginosa* quorum-sensing repressors RhlR and LasR were fused to the SV40 T-antigen nuclear localization sequence and the *Herpes simplex* VP16 domain and were shown to activate promoters containing

specific operator sequences (*las* boxes) in the presence of homoserine-derived inducers in COS-1 cells (Shiner et al. 2004).

2.2.2 Transcriptional Regulation with Cytoplasmic and Nuclear Receptors

The second strategy for inducible approaching activators and operators linked to minimal promoters relies on the release of transcription factors from sequestering and inhibiting proteins by the addition of small molecules (Fig. 1, D). This concept is commonly found in steroid-regulated design concepts (Fussenegger 2001), where hormone receptors fused to natural or artificial transcription factors are released from HSP90 in the cytosol, thus migrating into the nucleus to activate promoters harboring cognate operator sites (Reviewed in Fussenegger 2001).

In order to avoid crosstalk by endogenous steroid hormones, mutant receptors have been engineered that are exclusively regulated by synthetic steroid analogs, such as the estrogen receptor variant responsive to 4-hydroxytamoxifen or a progesterone-receptor mutant inducible by RU486 (mifepristone). Interference with endogenous promoters is avoided by fusing the receptors to heterologous DNA-binding domains such as yeast Gal4, thereby activating artificial promoters assembled from a Gal4 operator fused to a minimal mammalian or viral promoter (reviewed in Fussenegger 2001).

A nuclear receptor-derived rosiglitazone-responsive transcription switch based on the human nuclear peroxisome proliferator-activated receptor γ (PPARγ) has been recently described. PPARγ heterodimerizes with the retinoic acid receptor RXR and activates PPAR-responsive element (PPRE)-containing promoters in the presence of natural or synthetic ligands such as the type II diabetes drug rosiglitazone (Darteil et al. 2002). In order to avoid interference with endogenous PPARγ-responsive promoters, a mutant PPAR* with altered DNA-binding specificity was constructed. It was shown that the mutant did not induce myogenic differentiation, as was observed by ectopic PPARγ expression (Tascou et al. 2004).

A further variant of steroid-responsive receptors is the recently developed RheoSwitch, based on a modified *Choristoneura fumiferana* ecdysone receptor and the mouse retinoid X receptor (RXR) fused to the Gal4 DNA binding domain and the VP16 transactivator, which in the presence of synthetic ecdysone analogs bind and activate a minimal promoter fused to several repeats of the Gal4-response element (Palli et al. 2003). However, despite efficient regulation in mammalian cells, the use of synthetic, uncharacterized small molecules may bear considerable risks in future gene therapy scenarios.

2.2.3 Transcriptional Regulation Using Chemically Induced Dimerizers

The third approach for reconstituting functional transcription factors relies on chemically induced dimerization of a transcriptional activator and a constitu-

tive DNA-binding protein, which thus activates artificial promoters assembled from a cognate operator fused to a minimal core promoter (Fig. 1, E). The most prominent system of this class is based on the rapamycin-inducible dimerization of FKBP with FRB and is converted into a transcriptional control system by fusing FRB to the p65 transactivator and FKBP to a zinc finger domain specific for cognate operator sites placed upstream of an engineered minimal interleukin-12 promoter (Rivera et al. 1996). In order to overcome immunosuppressive side-effects by regulating rapamycin or FK506, immunoneutral small molecules have been synthesized and are currently being tested in first clinical studies (Iuliucci et al. 2001). A ligand-reversible dimerization strategy has also been developed by mutating FKBP (F_M) so that it will dissociate in the presence of FK506 (Rollins et al. 2000). In this configuration, F_M fused to a DNA-binding domain (ZFHD1 $_{BD}$) is bound to F_M fused to the p65 transactivator and thus activates promoters containing 12 tandem repeats of the ZFHD1 motif in the absence of F_M-dissociating FK506 (Pollock and Clackson 2002; Weber and Fussenegger 2002). The FKBP-derived dimerizer and de-dimerizer systems have been applied mainly in prototype gene therapy studies, as summarized at the end of this chapter (Table 2).

Another inducible dimerizer system is based on the bacterial gyrase B subunit (GyrB), which dimerizes in the presence of the antibiotic coumermycin and dissociates with novobiocin (Zhao et al. 2003). Fusion proteins of the bacterial lamda repressor-binding domain, GyrB, and the p65 activation domain (λRΔ-GyrB-p65) dimerize in the presence of coumermycin and can, thus, bind tandem lamda operator repeats upstream of the cytomegalovirus minimal promoter, thereby activating transcription of downstream genes. However, addition of coumermycin results in the dissociation of the λRΔ-GyrB-p65 dimer, thereby eliminating DNA-binding activity and leading to transcriptional silence (Zhao et al. 2003).

2.3 Translation Regulation Via Transcriptional Control

The classic method for inducible posttranscriptional expression shutdown relies on conventional transcription control systems (see above) for the inducible expression of antisense RNAs leading to translation-inaccessible mRNA duplexes and mRNA degradation (Fux et al. 2001; Singer et al. 2004). However, since RNA dublexes longer than 30 bp induce an interferon response resulting in nonspecific mRNA degradation and inhibition of protein synthesis, full-length antisense mRNA expression is of limited use for many applications (Luo et al. 2004). This obstacle can be overcome in mammalian cells by using short interfering RNAs (siRNAs, < 30 bp), which can be delivered either exogenously as synthetic nucleic acid or endogenously as transcripts driven by polymerase III (Wadhwa et al. 2004) or modified polymerase II (Xia et al. 2002) promoters. These siRNAs stimulate the RNA-induced silencing

Table 2 Gene switches used in gene therapy-relevant in vivo studies

System	Application	Reference
AIR	Tobacco smoke-induced regulated gene expression in mice	Weber et al. 2005e
Dimerizer	Adenoviral intratumoral delivery of inducible caspase-1 resulted in significantly reduced tumor growth and volume compared to mice, which did not receive the dimerizing drug	Shariat et al. 2001
Dimerizer	Elimination of fat cells through apoptosis by dimerization-induced caspase 8 expressed under control of an adipocyte-specific promoter. Omission of the dimerizer drug resulted in adipocyte restoration	Pajvani et al. 2005
Dimerizer	In vivo controlled proliferation of erythroid cells by dimerizer-inducible jak2-based growth induction	Zhao et al. 2002
Dimerizer	Inducible antiangiogenesis by inducible dimerization and activation of caspase-9	Nor et al. 2002
Dimerizer	Inducible erythropoietin control for over 2.5 years in the eye of non-human primates after AAV-mediated gene transfer	Lebherz et al. 2005
Dimerizer	Long-term pharmacologic erythropoietin regulation following AAV-based gene transfer in primates for over 6 years and 26 induction cycles.	Rivera et al. 2005
Dimerizer	Pharmacologically controlled expansion of human pancreatic islets and subsequent implantation into mice resulted in corrected hyperglycemia.	Kobinger et al. 2005
Dimerizer	Pharmacologically regulated production of suicide retroviruses specific for tumor cells	Crittenden et al. 2003
Dimerizer	Regulated in vivo expansion of genetically modified CD34+ cells in dogs by controlled dimerization of the thrombopoietin receptor	Neff et al. 2002
Dimerizer	Regulation of exocrine protein secretion following adenoviral transduction of rat saliva glands	Wang et al. 2004
E.REX	Macrolide-responsive control of erythropoietin and SEAP from bi-directional promoters in mice using encapsulated cells.	Fux et al. 2003
RU486	Inducible expression of vascular endothelial growth factor (VEGF) and erythropoietin from an autoregulated RU486-inducible expression vector transfected as plasmid DNA into the hind-limb muscle of adult mice	Abruzzese et al. 2000
RU486	RU486-inducible expression of caspase-3 in bcl-x_L-expressing tumor cells resulted in tumor eradication in mice	Pollett et al. 2003
Tamoxifen	Proliferation control of cynomolgus bone marrow CD34(+) cells in monkey by tamoxifen-inducible activation of a fusion between the granulocyte colony-stimulating factor (G-CSF) receptor and the estrogen receptor	Hanazono et al. 2002
TET-ON	Doxycycline-controlled leptin expression in the rat brain regulates food uptake and body weight	Wilsey et al. 2002

Table 2 (continued)

System	Application	Reference
TET-ON	Liver-specific interleukin 12 expression following naked plasmid DNA transfer resulted in eradication of liver metastasis in mice and prolonged survival in the presence of regulating doxycycline	Zabala et al. 2004
TET-ON	Regulated expression of human factor IX for treatment of hemophilia B using adenoviral gene delivery	Srour et al. 2003
TET-ON	Reversal of beta-thalassemia by regulated expression of erythropoietin over several weeks	Samakoglu et al. 2002
TET-ON	TET-controlled interleukin-10 expression reduced incidence and severity of collagen-induced arthritis in mice and was shown to be functional for more than 8 weeks	Apparailly et al. 2002

complex (RISC) and result in the degradation of homologous mRNA (Luo et al. 2004).

Inducible siRNA expression can be achieved by adapting the classic transcription control systems for use with polymerase III (Kuninger et al. 2004) or modified polymerase II (Malphettes and Fussenegger 2004) promoters (Fig. 1, F). Inducible siRNA expression has been described for the tetracycline (Kuninger et al. 2004; Malphettes and Fussenegger 2004) and macrolide (E.REX) systems (Malphettes and Fussenegger 2004) as well as for the OFF- and ON-type QuoRex designs (Weber et al. 2003b, 2005b). Regulated siRNA expression from lentiviral vectors using the tetracycline system enable inducible posttranscriptional gene knockdown in difficult-to-transfect cells such as nondividing or primary cells (Miyake et al. 2005).

A novel approach for regulating translation via a transcriptional control system relies on the *Xenopus* terminal oligopyrimidine element (TOP), which blocks translational initiation by forming hairpin structures in the 5′ untranslated region (Schlatter and Fussenegger 2003). Translation was resumed by resolving the TOP structures either by tetracycline-inducible expression of CNBP (cellular nucleic acid binding protein) or La or by exogenous administration of an anti-TOP oligonucleotide (Fig. 1, G; Schlatter and Fussenegger 2003).

2.4 Translation Regulation

The first translation control system that does not rely on inducible DNA-dependent promoters is based on a noncytotoxic alphavirus replicase mutant, which, at temperatures below 35°C, functions as an RNA-dependent RNA polymerase for the amplification of target mRNA from subgenomic promoters, thus providing a template for subsequent target protein translation (Fig. 1, H; Boorsma et al. 2000). While this system has been shown to

be suitable for bioprocessing applications in the expression of toxic proteins (Boorsma et al. 2002), the temperature switch is of limited use in therapeutic and animal studies. In these cases, pharmacologic control systems rather than altered cultivation temperatures are preferable.

The first pharmacologic mammalian translation control system is based on inducible dimerization of an N-terminal (eIF4G$^{\Delta}$) and C-terminal ($^{\Delta}$eIF4G) fragment of the translation initiation factor eIF4G by means of the rapamycin-dependent FKPB-FRP interaction (eIF4G$^{\Delta}$-FKBP and FRP-$^{\Delta}$eIF4G), thus reconstituting 5′cap-dependent as well as IRES (internal ribosome entry site) -dependent translational initiation (Fig. 1, I; Schlatter et al. 2003). The reconstituted eIF4G was shown to be superior to heterologous wild-type eIF4G, thereby leading to a novel metabolic engineering strategy for increased productivity by more efficient translation initiation (Schlatter et al. 2003).

Boutonnet and co-workers (Boutonnet et al. 2004) describe an alternative pharmacologic translation approach by which eIF4G is fused to the bacteriophage R17 RNA-binding protein and to the H-Ras farnesylation site. This induces the attachment of the 15-carbon farnesyl isoprenoid domain and subsequent anchoring and sequestration of the fusion protein in the plasma membrane. Administration of farnesyltransferase inhibitors, which are commonly used in anti-cancer studies (Sebti 2005), prevent H-Ras farnesylation and result in free cytoplasmic eIF4G fusion proteins, which bind mRNA at R17-specific sites placed in the proximity of IRES elements, thus allowing eIF4G-mediated translation of downstream target genes (Fig. 1, J; Boutonnet et al. 2004). Despite good regulation characteristics, the farnesyltransferase inhibitor displays a narrow application window; while 1 µM showed the highest induction, 5 µM already decreased expression levels, probably due to cytotoxic side effects.

While all of the aforementioned control systems require the presence of heterologous proteins, which may be immunogenic in animal studies or therapeutic applications (Latta-Mahieu et al. 2002), two approaches have been developed where the pharmacologic inducer interacts directly with its target mRNA. Werstuck and Green (Werstuck and Green 1998) selected a Hoechst dye (H33342)-binding aptamer and engineered it into the 5′ untranslated region upstream of the SEAP reporter gene. They showed that SEAP expression was unaffected by the aptamer; however, upon addition of 10 µM of H33342, the expression levels dropped by more than 90%, indicating that the dye-bound aptamer efficiently prevented translation initiation but did not have adverse side effects on constitutively expressed genes (Fig. 1, K, Werstuck and Green 1998). The second approach relies on inducible mRNA self-cleavage by a *Schistosoma mansoni* -derived engineered ribozyme placed in the 5′ untranslated region, thereby cleaving the 5′cap and preventing translation of the downstream target gene (Yen et al. 2004). Cell-based high-throughput screening revealed toyocamycin to be a potent inhibitor of the ribozyme, thereby resulting in noncleaved mRNA and, thus, restored target gene translation (Fig. 1, L). This system was validated in mice, which were injected with an adeno-associated viral vector

encoding a luciferase gene downstream of the inducible ribozyme, resulting in toyocamycin-inducible luciferase expression in the transduced mouse tissue (Yen et al. 2004).

2.5 Regulation at the Protein Level

Despite excellent regulation performance of the previously described genetic switches, which rely on the control of transcription or translation, induction kinetics are rather slow due to rate-limiting mRNA or target protein biosynthesis. Recently, kinetics of active protein formation were sped up significantly by the pharmacologic reconstitution of functional enzymes or by boost-like liberation of organelle-sequestered target proteins.

Enzymatically inactive β-galactosidase and luciferase subunits were dimerized via the rapamyin-induced FKBP–FRB complex and, thus, rapidly resulted in maximum enzymatic activity (Fig. 1, M, Luker et al. 2004). This split-enzyme concept enabled the design of rapid-readout bioassays, which had previously relied on a transcription control-based, time-consuming two-hybrid method, as demonstrated in the analysis of interacting proteins such as the positive cell-cycle regulating protein phosphatase Cdc25C and its binding partner 14-3-3ε or the homodimerization of the STAT transcription factors in response to Janus kinase-1-mediated phosphorylation (Luker et al. 2004).

Reconstitution of functional protein complexes via pharmacologic multimerization has also been exemplified in the activation of transmembrane receptors, which trigger downstream cascades for complex cellular responses such as growth, differentiation, and cell death, as shown by pharmacologically regulated Fas-mediated death of adoptively transferred T cells in a non-human primate model (Berger et al. 2004) or the self-renewal of primary multipotential hemopoietic cells, induced by the tyrosine kinase JAK2 complemented by a second signal from c-kit or flt-3 (Zhao et al. 2004).

A similar approach for pharmacologically induced cell proliferation by dimerization of the granulocyte colony-stimulating factor receptor (Gcr) was achieved through Gcr fusions with bacterial gyrase B, which is dimerized by the antibiotic coumermycin and dissociated with an excess of novobiocin, thereby allowing for fast reversal of receptor activation (Kume et al. 1999).

An alternative strategy for delivering maximum doses of pharmacologically active protein within minutes after induction was designed by the multimerization of insulin, fused to a mutated reverse FKBP variant (F_M, see above), leading to sequestration within the endoplasmic reticulum (Rivera et al. 2000). Boost-like release was achieved by adding a small molecule ligand, which resulted in F_M dissociation and migration into the Golgi field, where the F_M-tag was cleaved off by the trans-Golgi-specific furin protease prior to insulin secretion into the extracellular space (Fig. 1, N). Cells engineered for controlled insulin secretion were implanted into hyperglycemic mice and serum glucose levels

were normalized transiently by administering the synthetic small-molecule dissociating ligand AP21998 (Rivera et al. 2000).

3
Second-Generation Gene Switches

Based on the powerful gene regulation concepts, relying on isolated/heterologous regulatory elements for the expression of distinct genes as detailed above, a second generation of conditional molecular interventions is currently emerging, based either on more compliant novel inducer administration routes (e.g., via the gas phase) or direct hook-up to host cell signals, thereby allowing adequate integration of the gene switch into cellular physiology. Sophisticated interconnection of heterologous regulation concepts with cell physiologic signals enable synthetic processing of information by logic gates for complex genetic interventions in the correction of disease genotypes, in reprogramming cell differentiation or in biomanufacturing.

3.1
New Inducer Administration Routes

The dependence on small solute-based inducers for gene induction has been overcome recently by the development of the first gas-phase controlled expression system, AIR (acetaldehyde-induced regulation; Weber et al. 2004). AIR relies on the *Aspergillus nidulans* AlcR transcription factor (Felenbok et al. 2001), which, in the presence of gaseous or liquid acetaldehyde at nontoxic concentrations, specifically activates the P_{AIR} promoter assembled from AlcR-specific operators fused to the minimal human cytomegalovirus promoter. AIR enables a multitude of novel applications such as automatic formation of expression gradients in dose-activity studies through self-controlled gas diffusion (Weber et al. 2004) or the noninvasive and reversible control of gene expression in large-scale bioreactors by gassing in or stripping out the gaseous inducer, as exemplified for inducible interferon-beta expression on a 10 L scale (Weber et al. 2005d). A recent report (Weber et al. 2005e) describes the AIR system induced in mice by tobacco smoke, thereby offering new possibilities for studying smoke-related diseases or engineering inducible genetic protection systems against smoke-related insults (Weber et al. 2005e).

3.2
Higher-Order Regulatory Networks

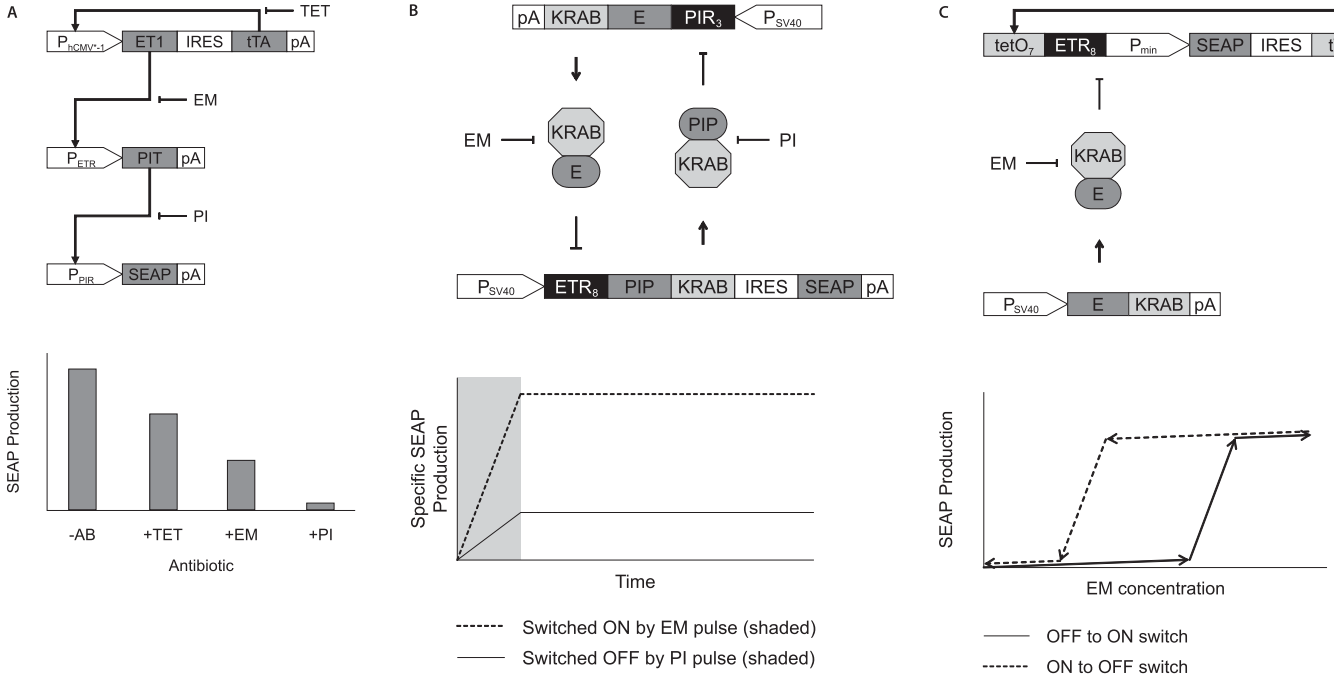
The increasing number of mutually compatible inducible expression systems enables the control of more than one transgene, as exemplified by the independent regulation of up to three different transgenes within a single cell

using macrolide, streptogramin, and tetracycline-responsive promoters (Weber et al. 2002a). Especially these antibiotic-based systems, either in the OFF or ON configuration, have been applied recently for the construction of higher-order artificial networks by interconnecting the regulatory elements in logic topologies similar to those in the information-processing domain.

Pioneering logic regulation scenarios in mammalian cells (Aubrecht et al. 1996; Moser et al. 2001; for a review, see Weber and Fussenegger 2002) show switching characteristics similar to an OR gate or to a combination of an OR and a NOT gate. The first comprehensive analysis of such mammalian cell-compatible logic gene circuits (BioLogic Gates) has been performed by the sophisticated interconnection of promoters responsive to three different activators or repressors now providing integrated circuit-like building blocks comprising NOT, AND, IF, NAND, OR, NOR as well as INVERTER-type gates (Kramer et al. 2004a).

While these logic gates show digital all-or-nothing responses, an analog-to-digital converter has been designed by the sequential interconnection of the macrolide-, streptogramin-, and tetracycline-responsive expression systems in a cascade-like configuration, where one promoter indirectly activates the next one via expression of corresponding transactivators (Fig. 2A; Kramer et al. 2003). The addition of different antibiotic combinations (digital input)

Fig. 2 A–C Synthetic regulatory networks. **A** Cascade-like configuration of the TET-OFF, E-OFF, and PIP-OFF systems results in an analog-to-digital-like converter. Tetracycline (*TET*)-responsive expression of ET1 activates erythromycin (*EM*)-responsive expression of PIT, which in the absence of pristinamycin I (*PI*) activates SEAP expression. Administration of different antibiotics results in a graded expression output. **B** Epigenetic memory by mutual inhibition of the E-ON and PIP-ON systems. Expression of one system remains stable since expression of the other is actively repressed. Switching can be performed by transient EM- or PI-mediated inactivation of the transcriptional repressors E-KRAB or PIP-KRAB, respectively. After a short EM or PI pulse (shaded area), specific expression levels remain stable even in the absence of regulating antibiotics. **C** Hysteresis in a mammalian gene network. The antibiotic concentration at which the system switches between ON and OFF or OFF and ON depends on whether the system was ON or OFF prior to the switch. *Arrow* activation, *arrowhead* inhibition, *AB* antibiotic, *E* macrolide-responsive repressor (also mphR(A)), *EM* macrolide antibiotic erythromycin, *ET1* macrolide-responsive transactivator (E-VP16), *ETR* E-specific operator, *IRES* internal ribosome entry site, *KRAB* kruppel associated box protein (transcriptional silencer), *pA* polyadenylation signal,P_{ETR} macrolide-responsive promoter (ETR-$P_{hCMVmin}$), $P_{hCMV*-1}$ tetracycline-responsive promoter ($tetO_7$-$P_{hCMVmin}$), $P_{hCMVmin}$ minimal human cytomegalovirus immediate early promoter, *PI* streptogramin antibiotic pristinamycin I, *PIP* streptogramin-responsive repressor, *PIR* PIP-specific operator, *PIT* streptogramin-responsive transactivator (PIP-VP16), P_{min} minimal promoter, P_{PIR} streptogramin-responsive promoter (PIR-$P_{hCMVmin}$), P_{SV40} simian virus 40 promoter, *SEAP* human placental secreted alkaline phosphatase, *TET* tetracycline, *tetO* TetR-specific operator, *TetR* tetracycline-responsive repressor, *tTA* tetracycline-responsive transactivator (TetR-VP16), *VP16 Herpes simplex* viral protein 16


A
TET
P hCMV*-1
ET1
IRES
tTA
pA
EM
P ETR
PIT
pA
PI
P PIR
SEAP
pA
SEAP Production
-AB
+TET
+EM
+PI
Antibiotic
B
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PIR3
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KRAB
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P SV40
ETR8
PIP
KRAB
IRES
SEAP
pA
Specific SEAP Production
Time
Switched ON by EM pulse (shaded)
Switched OFF by PI pulse (shaded)
C
TET
tetO7
ETR8
P min
SEAP
IRES
tTA
pA
EM
KRAB
E
P SV40
E
KRAB
pA
SEAP Production
EM concentration
OFF to ON switch
ON to OFF switch

resulted in a graded (analog) response of the reporter gene (Fig. 2A) under the control of the terminal streptogramin-responsive promoter, in agreement with the *in silico* -predicted expression ratio (Kramer et al. 2003). A similar cascade-like approach was designed by configuring the rapamycin-inducible dimerization-based expression system to drive the tetracycline-dependent transactivator tTA, which in turn activates transcription of the reporter gene, which is under the control of the tTA-responsive promoter $P_{hCMV*-1}$ (Sudomoina et al. 2004).

An artificial mammalian long-term memory has been realized through an epigenetic transgene switch, where one given expression state can be locked even if the initial stimulus is no longer present (Kramer et al. 2004b). This bistability was achieved by configuring the inducible E-ON and PIP-ON systems to mutually repress each other (Fig. 2B). Therefore, if the first system is induced, then it actively represses the second system and the first system thus maintains stable expression levels. Switching between the two states can be achieved by adding an antibiotic to relieve repression of the second system, which will then stably repress the first one. Bi-stable expression behavior was validated in stable cell lines in culture and in mice and may provide new opportunities in gene therapy applications requiring transgene expression for a defined period of time with subsequent sustained expression shut-down (Kramer et al. 2004b).

A memory-like switch, where the expression response depends on the cell's transgene expression history has been implemented by constructing a hysteretic gene switch (Fig. 2C; Kramer and Fussenegger 2005). Hysteresis was obtained by a positive feedback loop, consisting of the tetracycline-dependent transactivator (tTA), which stimulates its own expression via a tTA-responsive promoter which can also be repressed by a macrolide-dependent transrepressor (Fig. 2C). The macrolide concentration, which triggers a quasi-discontinuous switch between the ON and OFF states, was shown to depend on the transgene expression history of the cell, i.e., whether it was cultivated in the presence or absence of erythromycin. This hysteretic switch, which mimics the switching characteristics of different biological processes such as eukaryotic cell-cycle progression or nutrient utilization, might prove to be a useful tool in establishing or repairing such systems, both in research and in gene therapy (Kramer and Fussenegger 2005).

3.3 Integration of Transgene Switches into the Host Regulatory Network

A current trend in gene regulation is the integration of natural and artificial regulation modalities. This is achieved either by specifically targeting endogenous promoters with inducible expression elements or by hooking up heterologous control units to physiological signals and endogenous promoters.

3.3.1 Gene Targeting

The recent development in designing zinc-finger proteins (ZFPs) with predictable specificity through assembly of modular zinc-finger building blocks enables the construction of DNA-binding proteins that bind only once within the genome at the desired position. This approach makes all sequences a potential operator and can be used for a multitude of genetic interventions (see Jamieson et al. 2003 for a review), as exemplified by inducible activation of the endogenous vascular endothelial growth factor (VEGF) promoter (Pollock et al. 2002): a VEGF-promoter-specific ZFP was fused to an FKBP-derived dimerizer domain, which, in the presence of a rapamycin analog, recruits FRAP fused to an activator domain and thus results in transcription of the endogenous *vegf* gene.

3.3.2 Linking Transgene Switches to Physiologic Signals

Gene therapeutic applications relying on the above-mentioned (heterologous) expression systems, require external stimuli for orchestration of the target genes. While this approach might be patient-compliant in acute treatments, long-term chronic genetic diseases, on the other hand, would require self-modulation, where pathologic physiologic signals in the patient are translated into activation or repression of corrective therapeutic genes.

Such a hook-up to endogenous signals has been recently achieved by connecting heterologous transcription control to the endogenous oxygen tension signaling unit. The first such network was constructed by placing the tetracycline-responsive transactivator tTA under a dual oxygen control by (a) tTA expression from a hypoxia-inducible promoter and (b) degradation of tTA under normoxia by fusion to the Hif1α-derived oxygen degradation domain (ODD). This two-step cascade was validated in mouse tumors, where the endogenous hypoxic signal resulted in elevated luciferase expression, which could then be fine-tuned by administering doxycycline (Payen et al. 2001), a potential new strategy for killing tumor cells when the luciferase is replaced by a cytotoxic or apoptosis-inducing gene (Payen et al. 2001). In a similar study (Kramer et al. 2005), a three-step cascade was constructed: First, an oxygen-responsive promoter drives expression of the pristinamycin-responsive transactivator PIT, which activates its cognate promoter P_{PIR} for expression of the tetracycline-responsive transactivator tTA, finally driving a reporter gene under control of the tTA-responsive promoter $P_{hCMV*-1}$. Depending on the oxygen state as well as on the presence of regulating antibiotics, expression could be locked to six discrete levels, thereby allowing a graded (analog) response to the binary input (hypoxia/normoxia; antibiotic addition or omission).

Similarly, hypoxia-responsive transcription factors have been engineered for the treatment of ischemia by fusing a Gal4 DNA-binding domain and the

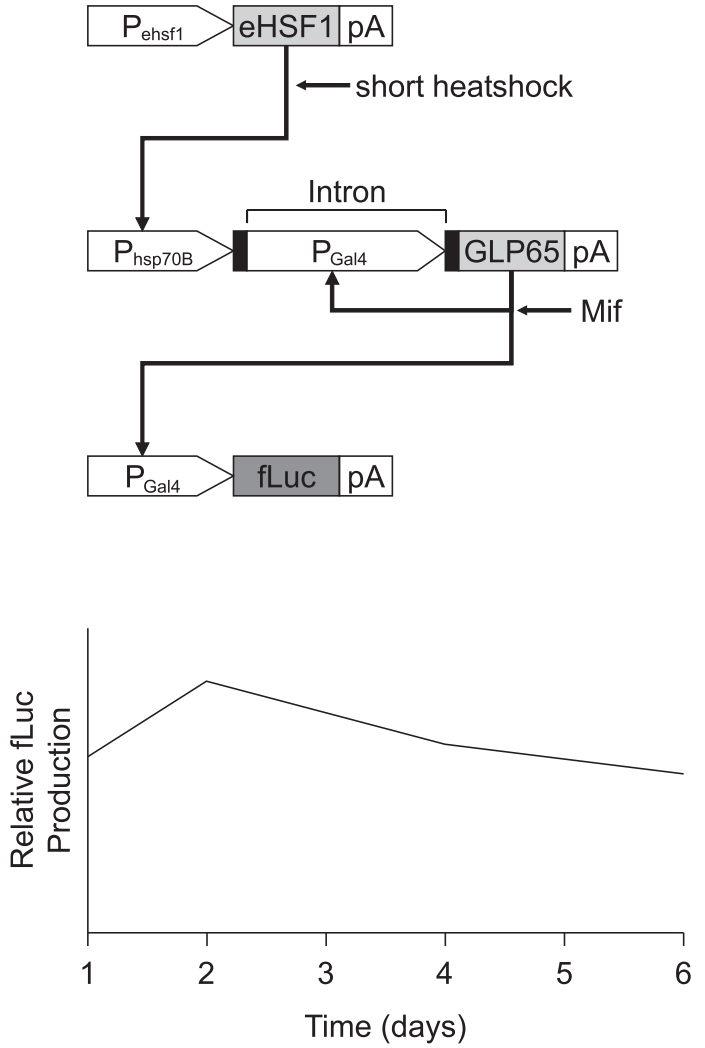

Fig. 3 Semisynthetic gene regulatory network resulting in a short-term cellular expression memory. A transient heat-shock activates transcription from P_{hsp70B} via endogenously expressed eHSF1. P_{hsp70B} drives expression of GLP65, which in the presence of mifepristone (*Mif*) activates its own expression via the intron-encoded P_{Gal4} as well as the expression of the reporter gene firefly luciferase (*fLuc*). A short heatshock (2 h, 43°C) induces fLuc expression, which is sustained for up to 6 days. *eHSF1* endogenous heat shock factor 1, *GLP65* transactivator containing the p65 transactivation domain and a Gal4-binding motif fused to the mifepristone-responsive estrogen-receptor version, P_{ehsf1} endogenous promoter driving expression of eHSF1, P_{Gal4} minimal Gal4 promoter, P_{hsp70B} heat shock protein 70B promoter; see also legend to Fig. 2 for explanation

Table 3 Gene switches used in drug discovery

System	Application	Reference
Dimerizer	Screening for drugs disrupting protein dimerization	Zhao et al. 2004a
E-ON	Detection and discovery of macrolide antibiotics	Weber et al. 2005a
PIP-ON	Mammalian cell-based screening for noncytotoxic cell permeable streptogramin antibiotics	Aubel et al. 2001
Translation control	Screening for farnesylation inhibitors for cancer therapy	Boijoux et al. 2005

p65 activator to the HIF1α-derived oxygen degradation domain (ODD), leading to proteasome-mediated degradation of the chimeric protein under normoxia. However, during hypoxia the transactivator accumulates and activates promoters harboring Gal4-operator sequences, thus resulting in the expression of protective heme oxygenase-1. Transfection of this hypoxia-inducible vigilant vector system into the anterior wall of the left ventricle in mice with subsequently induced ischemia resulted in significantly reduced apoptosis in the infarct area and improved cardiac functions (Tang et al. 2005).

Two recent publications report a similar approach using the radiation-inducible promoters WAF1 or *egr-1* for tumor eradication by expression of toxic inducible nitric oxide synthase (iNOS) or the tumor necrosis factor α (TNFα) (Senzer et al. 2004; Worthington et al. 2004). The latter approach was validated in a clinical study where patients received different doses of an adenoviral vector for $P_{egr\text{-}1}$ responsive TNFα expression followed by radiation leading to objective tumor responses in 70% of the patients.

An elegant design of an integrated physiologic and artificial network has been described recently, in which the endogenous thermosensitive heat shock protein 70 (HSP70B) promoter (P_{hsp70B}) was used to kick-start a positive feedback loop by expression of P_{hsp70B}-activating HSF1(+). In this configuration, a short heat shock (42°C, 1 h) resulted in sustained expression for up to 4 days, whereas a control configuration without the positive feedback loop returned to basal levels within 3 days (Vilaboa et al. 2005). The system was further refined by P_{hsp70B}-driven expression of the mifepristone-responsive transactivator GLP65, which, in the presence of the inducing steroid, activated its own expression in a positive feedback loop as well as that of reporter genes under control of a separate GLP65-responsive promoter. Using this composite system (Fig. 3), a short heat shock (43°C, 2 h) provoked persistent gene expression for up to 6 days in the presence of mifepristone. However, mifepristone withdrawal as a safety switch stopped expression almost immediately (Vilaboa et al. 2005).

Table 4 Gene switches used in biopharmaceuticals manufacturing

System	Application	Reference
AIR	Reversible gas-inducible IFN-β expression in CHO cells in a 10-L bioreactor by gassing in and out the inducer acetaldehyde; determination of thermodynamic and kinetic parameters	Weber et al. 2005d
AIR	Inducible expression of $p27^{KIP1}$ for achieving an inducible growth arrest in HEK EBNA cells	Werner et al. 2005
AIR	Validation of gas-inducible transgene expression in a 1.3-L bioreactor using CHO cells	Weber et al. 2004
Ecdysone	Inducible expression of $p27^{KIP1}$ in CHO cells for increasing the productivity of s-ICAM	Meents et al. 2002
LacI	IPTG-inducible expression of $p21^{CIP1}$ in CHO cells for increasing the productivity of an IgG	Bi et al. 2004
$pCYT^{TS}$	Cold-inducible protein production of different therapeutic and toxic proteins in BHK-21 cells in different bioreactor types	Boorsma et al. 2002
$pCYT^{TS}$	Development and validation of the cold-inducible expression technology in BHK-21 cells	Boorsma et al. 2000
TET-OFF	Controlled expression of $p27^{KIP1}$ for boosting specific protein productivity in CHO cells	Fussenegger et al. 1998b

Coupling the metabolic state of a mammalian cell to a transcriptional readout was recently achieved by fusing the *Streptomyces coelicolor* Rex repressor (Brekasis and Paget 2003), which dissociates from DNA at elevated NADH concentrations (Sickmier et al. 2005), to the *Herpes simplex* VP16 transactivation domain. Thus, the minimal promoter ($P_{hCMVmin}$) cloned downstream of the Rex operator *rop* is activated under nutrient deprivation, resulting in lowered intracellular NADH concentrations, whereas cultivation under hypoxia induces higher NADH concentrations, dissociation of REX-VP16 from *rop* and transcriptional shutdown. This simple binary system thus allows one-step monitoring of the two most important parameters – oxygen and nutrient supply – in large-scale cell culture and tissue engineering by simple reporter gene analysis (Weber et al. 2006). Furthermore, it was shown that the Rex-based transcription control is suitable for investigating the effect of compounds, which interfere with the respiratory chain or induce oxidative damage, as validated by altered transcription readouts after the addition of cyanide (higher NADH, lower transcription), 2,4-dinitrophenol (lower NADH, higher transcription), as well as hydrogen peroxide (oxidative stress, lower NADH, higher transcription).

Table 5 Gene switches in tissue engineering

System	Application	Reference
ΦC31	Recombinase ΦC31-mediated genomic integration of laminin 5β3 into primary human keratinocytes from junctional epidermolysis bullosa patients for regeneration of functional human skin on immunodeficient mice	Ortiz–Urda et al. 2003
Tamoxifen	Adenovirus-mediated inducible endostatin expression in the mouse retina for inhibition of VEGF-mediated retinal neovascularization and detachment	Takahashi et al. 2003
TET/PIP	Differential differentiation of C2C12 cells into adipocytes or osteoblasts by inducible dual-regulated expression of C/EBPα or BMP-2	Fux et al. 2004b
TET/PIP	Myogenic/osteogenic and adipogenic lineage control of C2C12 cells by dual-regulated expression of myoD and msx1	Fux et al. 2004a
TET-ON	Regulated BMP-2 expression following AAV-mediated gene transfer into mice with critically sized calvarial defects for bone regeneration	Gafni et al. 2004

4 Utility of Gene Switches

Gene switches are applied in virtually all fields of mammalian cell biology, from gene therapy and tissue engineering to bioengineering and drug discovery to the construction of transgenic mice for establishing gene function correlations in basic and applied research.

In the area of gene therapy, inducible expression systems are intensively validated for the treatment of inherited or acquired diseases such as cancer. The majority of such studies, especially in large animals, such as non-human primates, are conducted using the dimerizer approach (Table 2). In drug discovery, inducible systems responsive to pharmacologically active core structures, such as streptogramin antibiotics or farnesylation inhibitors, can be used to screen libraries for new inducers of the gene switch, which have also been shown to exert desirable pharmacologic functions (Table 3). In biopharmaceuticals manufacturing, gene switches are mainly applied for the expression of toxic proteins or to impose a productivity-boosting growth arrest by controlled overexpression of cell-cycle regulators (Fussenegger et al. 1998b). However, the use of pharmacologically induced switches entails purification and validation hurdles, which can be elegantly circumvented by the use of the temperature-

Table 6 Molecular toolboxes for straightforward engineering of gene switches

System	Application	Reference
AIR	Design and validation of bidirectional, multicistronic and lentiviral expression vectors for gas-inducible transgene expression	Hartenbach and Fussenegger 2005
Dimerizer	Construction and validation of a compact retroviral one-vector design for rapamycin-inducible gene expression	Pollock et al. 2000
E.REX	A step-by-step protocol for the implementation of macrolide-responsive gene expression in mammalian cells and mice	Weber and Fussenegger 2004b
E.REX	Design of a lentiviral-based toolbox for rapid implementation of streptogramin-controlled transgene expression in vitro and in vivo	Mitta et al. 2005
E.REX/ TET/PIP	Design of a flexible vector toolbox for expression of up to three genes under control of a macrolide-responsive promoter or for independent expression of three different genes under control of the macrolide, streptogramin, and tetracycline-responsive promoters	Weber et al. 2002b
PIP	Design of a lentiviral-based toolbox for rapid implementation of streptogramin-controlled transgene expression in vitro and in vivo	Mitta et al. 2004
PIP	Development and validation of tricistronic vectors for streptogramin-controlled synchronized expression of up to three genes	Moser et al. 2000
QuoRex	Development and validation of bidirectional and multicistronic expression vectors inducible by the quorum-sensing messenger SCB1	Weber et al. 2005c
Tamoxifen	Development and in vivo validation of the tamoxifen-responsive expression system in gutless adenoviral vectors	Zerby et al. 2003
TET	Bidirectional expression of two transgenes by two divergently orientated minimal promoters placed around tetracycline-responsive operator sites	Baron et al. 1995
TET	Design of tetracycline-responsive lentiviral expression vectors using the new rtTA^{2S}-M2 transactiva-	Pluta et al. 2005
TET	Development of tetracycline-dependent transactivators tor with graded transactivation potential derived from the minimal VP16 transactivation domain	Baron et al. 1997

Table 6 (continued)

System	Application	Reference
TET/ Ecdysone	Development and validation of tricistronic vectors for tetracycline or ecdysone-controlled synchronized expression of up to three genes	Fussenegger et al. 1998a
TET/PIP	Development and validation of a vector toolbox for dual-regulated inducible expression of two genes using streptogramin- and tetracycline-responsive promoters	Moser et al. 2001
TET/PIP	Development and validation of dual-regulated adenoviral vectors for independent expression of two transgenes under control of the tetracycline and streptogramin-responsive promoters	Gonzalez–Nicolini and Fussenegger 2005

sensitive pCyt^{TS} technology or the recently validated gas-induced AIR system (Table 4). In tissue engineering, gene switches can be used to control cellular differentiation, preferably in combination with viral vectors for the transduction of primary cells or micro- and macrotissues (Kelm et al. 2004; Weber and Fussenegger 2004a; Table 5). For efficient installation of gene switches in a variety of different cell types and tissues (from regulated expression of single genes up to multiregulated multigene metabolic engineering strategies), a large toolbox of suitable expression vectors has been developed (Table 6).

5 Outlook

Inducible gene expression by external control at different checkpoints is today a mature technology since the multitude of available systems in different expression configurations (viral vectors, multicistronic, multiregulated) ensures an ideal system for a given application. A current focus is on inducible expression in gene therapy applications. It is a matter of time before one of the mature regulation concepts is validated in a clinical study.

The currently emerging second-generation gene switches, based on novel inducer routes or the integration of complex heterologous gene networks into the cell's physiological background, will probably dominate the gene regulation community for the next few years and will offer unprecedented opportunities for cell- and patient-compliant regulation scenarios.

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Improved Embryonic Stem Cell Technologies

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Abstract Murine embryonic stem (ES) cells have become an indispensable tool for investigating genetic function both in vitro and, importantly, in vivo. Recent advances, including tetraploid aggregation, new site-specific recombinases and RNAi, have enabled more sophisticated manipulation of the ES cell genome. For instance, it is now possible to control gene expression in both a temporally and spatially restricted manner. Such new technologies are answering complex questions surrounding the function and interaction of an increasing number of genes. This chapter will review both the history and recent technological progress that has been made in mouse ES cell derivation, genetic manipulation and the generation of ES cell-derived chimaeric animals.

Keywords ES cell · Homologous recombination · Transgenesis · Tetraploid complementation · Chimaera

1 Introduction

From the moment of fertilisation, subsequent mammalian development can be crudely summarised as proliferation concomitant with ever-increasing specialisation of daughter cells until terminal, functional cell types are generated. These cells are characterised by a choice available to them each time they undergo cellular division: to proliferate, generating identical daughter cells, or to differentiate into a more specialised cell type (Weissman et al. 2001). During development, a vast number of different cell types emerge, sometimes

only transiently. One such population of cells, found for only a fleeting time in the early embryo, or blastocyst, are termed the inner cell mass (ICM). In mice, the ICM becomes evident at around 3.5 days postcoitum (dpc). It is evident as a small cluster of cells on the inside surface of the hollow ball of trophectoderm cells. One day later, the ICM is comprised of two cell types, the epiblast, which will become the embryo, and the hypoblast which will differentiate into the yolk sac (Beddington and Robertson 1999). In parallel, the trophectoderm layer will differentiate into the placenta. When explanted into specific *in vitro* conditions, the cells comprising the ICM can be outgrown and maintained as a permanent cell line, called embryonic stem (ES) cells (Evans and Kaufman 1981; Martin 1981). The early stage from which ES cells are derived makes them arguably unique in their ability to differentiate into all cell types found later in the adult organism. Indeed, unlike all other stem cells so far isolated, ES cells reproducibly demonstrate their pluripotency when reintroduced into the developing blastocyst, contributing to all of the embryonic lineages, including the germ cells, allowing for germline transmission of the ES cell genome. This ability combined with sophisticated genetic modification techniques has turned these cells into a powerful tool for investigating gene function. This chapter will discuss some of the related technologies.

2
Blastocyst-Derived Stem Cells

Some 25 years after the first reports of murine embryonic stem cell derivation (Evans and Kaufman 1981; Martin 1981), it has become a routine exercise to generate new ES cell lines from some inbred strains of mice, particularly 129Sv and C57BL/6. The principle of ES cell derivation is simple: explant the ICM onto a substrate permissive for attachment and proliferation (frequently mid-stage mouse embryo-derived feeders) whilst maintaining the cells in a media comprising key nutrients and growth factors.

If a whole blastocyst is plated onto a feeder layer, it is inevitable that several cell types will grow out in addition to ES cells. Included in these are trophoblast and extraembryonic endoderm derivatives. Permanent stem cell lines from both of these murine extra-embryonic tissues have now been established: trophoblast stem (TS) cells from the extra-embryonic ectoderm and extra-embryonic endoderm (XEN) cells from the primitive endoderm (Tanaka et al. 1998; Kunath et al. 2005). Both TS cells and XEN cells display many of the traits exhibited by ES cells: apparent indefinite self-renewal, the ability to differentiate into multiple cell types and the capacity to re-enter development when introduced back into the murine blastocyst. TS cells can contribute to all of the extra-embryonic ectoderm lineages when used to make chimaeras. Early experiments indicate that when XEN cells are injected into the blasto-

cyst they display a bias toward parietal endoderm differentiation, although whether this is an artefact associated with the injection process remains to be elucidated. Notwithstanding, the derivation of TS and XEN cells presents significant progress in our understanding of embryonic development. The extra-embryonic tissues play a major role in the specification and patterning of the cell lineages present in the embryo (Beddington and Robertson 1999). Obtaining the molecular profile of the extra-embryonic tissues during discrete developmental stages should facilitate a better understanding of the genetic pathways that govern differentiation of embryonic tissues. Taking advantage of these patterning interactions could lead to higher efficacy in the differentiation of therapeutically useful tissues from ES cells by co-culturing with TS or XEN cells.

Until TS and XEN cells were isolated, ES cell derivation was typically the primary goal for the majority of researchers; extra-embryonic differentiation was regarded as unwanted contamination, and several techniques were employed to reduce the occurrence of these lineages. Principle amongst the techniques used to achieve this selection is immunosurgery, or complement mediated cell lysis, which kills the surrounding trophectoderm cells of the blastocyst (Solter and Knowles 1975). The remaining ICM can then be plated and the outgrowing cells passaged until a stable ES cell line is established. However, routine ES cell derivation from permissive strains like 129 does not require the use of immunosurgery. Plating down whole blastocysts, picking ICM outgrowths by morphology and then mechanically disaggregating them before transferral to a fresh culture well is a far simpler way of establishing lines (Evans and Kaufman 1981; Nagy et al. 1993). Nonetheless, it is claimed that ES derivation from less permissive strains can be greatly enhanced when immunosurgery is used (Brook and Gardner 1997).

A substantial proportion of the mouse ES cell work performed in labs across the world, and hence transgenic animals generated, utilise only a couple of inbred strain-derived ES cell lines, especially 129 and less frequently C57BL/6. It remains unknown why 129 is particularly permissive in generating ES cell lines, although one explanation may lie with the observation that this strain has a tendency to produce spontaneous testicular teratocarcinomas at a small but appreciable frequency of about 1% (Stevens and Little 1954). Teratocarcinomas contain a stem cell component, the embryonal carcinoma cell, which are remarkably similar in morphology and gene expression to murine ES cells (Evans and Kaufman 1981); it is likely that the genetic components responsible for the appearance of teratocarcinomas in 129Sv is also responsible for the high efficacy with which ES cells can be derived from this particular inbred strain. It is worth noting that for derivation as a whole, different labs can achieve different levels of efficiency; however, it has been reported that approximately 30% of 129Sv blastocysts will give rise to ES cell lines when plated onto inactivated feeder layers (Robertson 1987). As mentioned, C57BL/6 can be used to derive ES cells, but they do so with less efficiency than 129 and are less

likely to generate chimaeric mice than 129, thus reducing the chance germline transmission of a genetic modification (Ware et al. 2003).

Accumulative experience and some technology improvements in the way ES cells are cultured and derived, including the discovery of the ES cell self-renewal-associated cytokine LIF (Gearing et al. 1987; Smith and Hooper 1987; Smith et al. 1988), have made it possible to derive ES cells from mouse strains and crosses previously found to be refractory. Mouse ES cell responsiveness to LIF, a member of the interleukin-6-related family of cytokines, appears to be a physiological adaptation to a process called diapause, whereby the development of fertilised mouse embryos can be arrested at the blastocyst stage for 1 week, providing better separation between consecutive litters (Nichols et al. 2001). Several pathways that promote self-renewal and retard differentiation, including those modulated by STAT3 and MAPK, respectively, have been identified as targets of LIF-based signalling (Burdon et al. 1999a). LIF activates these pathways by binding a complex composed of the LIF receptor and gp130, recruiting JAK kinases enabling activation of STAT3 and MAPK pathways (Taga and Kishimoto 1997; Niwa et al. 1998; Burdon et al. 1999b). The capacity of LIF to induce self-renewal in mouse ES cells, even without the support layer of embryonic fibroblasts, is now routinely exploited in tissue culture laboratories (Smith et al. 1988; Williams et al. 1988). Recently, it has been shown that by preconditioning ES cell derivation media by co-culture with a rabbit fibroblast line expressing rabbit LIF, ES cell lines to be derived and subsequent germ line transmission can be achieved from virtually all strains so far tried, including those which have previously resisted derivation attempts (Schoonjans et al. 2003). Moreover, the same conditioned media appears to be able to rescue established ES cell lines which currently show poor chimaera contribution, including those from less permissive mouse strains. Undifferentiated mouse ES cells cultured in the conditioned media display a rounded morphology compared to the flatter appearance of ES cells which have apparently begun to differentiate. These rounded undifferentiated ES cell colonies are less adherent to the culture surface, permitting their selective enrichment by tapping the culture vessel and collecting these floating clumps (Rabbit LIF conditioned media is commercially available under the RESGRO trademark from Chemicon, Temecula, CA, USA).

As understanding of the genetic interactions underlying the self-renewal and pluripotency of mouse ES cells (reviewed in Boiani and Scholer 2005) has expanded, so have the opportunities to utilise these mechanisms in the derivation of new ES cell lines. For instance, analysis of LIF downstream effectors has demonstrated that certain chemicals, including PD98059, a MAP kinase kinase (MEK) inhibitor, can up-regulate the expression of the pluripotency associated transcription factor, Oct-4, in ES cells (Burdon et al. 1999b); low levels of Oct-4 have been demonstrated to inhibit the derivation of pluripotent ES cells lines (Buehr et al. 2003). These observations have led to the successful derivation

of ES cell lines from a previously refractive mouse strain in the presence of PD98059 (Buehr and Smith 2003).

The decoding of some of the regulatory pathways governing the maintenance of mouse ES cells has had a positive impact upon the derivation of germline competent cell lines from different genetic backgrounds in the mouse. There is an increasing demand for the generation of mutants in C57BL/6 background, partly driven by the vast amount of biological characterisation performed on this stain and the availability of the C57BL/6 genome sequence. By capitalising upon the ground work performed upon the 129 strain, the generation of such mutants in the C57BL/6 strain now approaches that of 129.

3
ES Cell Functionality: Genetic Modification

Although many aspects of ES cell genetic manipulation are discussed elsewhere in this book, it is worth briefly summarising the reasons why ES cells have become such a ubiquitous tool for transgenesis.

Random transgene integration has helped elucidate the role of a number of genes. During development, the expression of many genes is controlled in a tissue-specific manner, both temporally and spatially by the endogenous promoter associated with the gene. By introducing into a cell a stretch of DNA which artificially associates the coding region of the gene under investigation with a constitutive promoter, for instance those derived from CMV, Sv40 or the ubiquitin gene, it is possible to drive expression of the gene at levels and in tissues beyond that typically found in development. Such over-expression studies have been successful in revealing genes which can act as master fate regulators, controlling the lineage into which cells will develop (Pevny et al. 1998; Fujikura et al. 2002; Chambers et al. 2003; Niwa et al. 2005).

Targeted mutation or knockout of genes has become the tool of choice for investigating the phenotypic consequences associated with gene ablation (Hasty et al. 2001). Gene targeting requires the construction of a targeting cassette containing at least three main elements: DNA sequence for insertion or replacement of the target loci, a selectable marker for reclamation of the targeting event from the background of nontargeted cells and flanking arms that are homologous to the endogenous regions surrounding the target locus. Introduction of the targeting cassette to the cell will occasionally lead to the substitution of the endogenous loci with the incoming targeting cassette which can be distinguished from wild-type cells by Southern blot or PCR analysis. Whilst gene targeting was perfected in cell lines other than murine ES cells (Smithies et al. 1985), it soon became clear that ES cells were ideally suited for this task (Thomas and Capecchi 1987; Doetschman et al. 1988). ES cells exhibit several properties which are particularly important in facilitating their functionality in transgenic research: firstly, the rapidity with which these cells

proliferate (cell cycle time of ~12 h) (Stead et al. 2002) and consequently the number of cells, for instance $>1\times10^7$ cells per 10-cm-diameter plate, that can routinely be obtained. These extremely large numbers allow the relatively frequent occurrence of very rare recombination or integration events in a single experiment. Secondly, clonal sublines can be created from a single cell with high efficiency (~10%). This permits these rare recombination or integration events to be retrieved, typically via drug resistance selection, from the background of wild-type ES cells that have not undergone the desired modification. Finally, after transgenic manipulation, ES cells can retain pluripotency and, most importantly, germ line competence, enabling the heritable transmission of the genetic alterations. Initial experiments indicated that these traits could be exploited in order to generate transgenic ES cells; random integration of cassettes including drug resistance genes was demonstrated, along with subsequent germline transmission from chimaeric animals (Lovell-Badge et al. 1985; Gossler et al. 1986; Robertson et al. 1986).

Not long after the initial gene targeting experiments in mouse ES cells, the first mice were generated from ES cells that had undergone targeted mutation of a specific gene (Koller et al. 1989). The number of genes which have been targeted in a similar way now exceeds 7,000 (Capecchi 2005); a number which highlights the importance of mouse transgenesis in our understanding of gene function. Notwithstanding, the generation of targeted gene knockouts is a laborious process, taking many months to construct the targeting vector, target the allele in ES cells and generate transgenic animals from the ES cells. The discovery that an endogenous cellular mechanism could be subverted to silence gene expression has lead to a faster way of reducing target gene expression. RNA interference (RNAi) capitalises upon a cellular response to the presence of double-stranded RNA in which endogenous mRNAs are inhibited or degraded if they share sequence homology to the double-stranded RNA (dsRNA) (reviewed in Mittal 2004). By introduction of small synthetic dsRNAs or through production of dsRNA in the form of hairpin RNAi from a transgene, it is possible to significantly reduce the levels of the complementary mRNA in ES cells. This technology has been utilised to analyse gene function *in vitro* and *in vivo*, enabling recapitulation of traditional gene knockout phenotypes (Henkemeyer et al. 1995; Niwa et al. 2000; Kunath et al. 2003; Velkey and O'Shea 2003). RNAi can be far quicker to implement in ES cells, although gene knockdown levels can be variable compared to the complete knockout attainable by traditional gene targeting (Lickert et al. 2004). Nevertheless, the generation of libraries containing dsRNA sequences for targeting a substantial proportion of the mouse genome is currently being undertaken (see http://www.broad.mit.edu/genome_bio/trc/).

Once protocols had been established for altering the DNA of ES cells and the subsequent generation of live animals carrying these changes, more elaborate methods for analysing gene function became possible. Most notable amongst these technologies was the application of site-specific recombinases

(Nagy 2000; Sorrell and Kolb 2005; Garcia-Otin and Guillou 2006). Recombinases are a broad family of proteins which are sub-classified into two distinct groupings: integrases, which utilise a tyrosine residue for DNA binding, and resolvases/invertases, which use a serine residue (Stark et al. 1992). Site-specific recombinases utilise short DNA sequences as both recognition sites for the recombinase and substrates for the recombination event. The length of recombinase recognition sites is typically on the order of several dozen bp, making the sequence sufficiently unique to occur very infrequently in most mammalian genomes. The lack of such pseudo-recombination sites in the target genome is an important aspect of high-fidelity, efficient recombination (Nagy 2000).

Most notable in the family of recombinases are Cre and Flp. Several observations have established that enzymes, which originate in yeast, bacteria or bacteriophages, could work in mammalian cells (Sauer and Henderson 1988, 1989, 1990; O'Gorman et al. 1991). Subsequently, Cre has become the dominant recombinase used in mammalian transgenics; Flp is less prevalent, likely due to the lower efficacy displayed by Flp in mammalian cells (Andreas et al. 2002). Mutagenesis, mammalian codon optimisation or the addition of nuclear localisation signals has increased the efficiency of both Flp and Cre in mammalian cells (Buchholz et al. 1998; Koresawa et al. 2000; Andreas et al. 2002). Nevertheless, the Cre remains around ten times more efficient than Flp.

Cre and Flp function by binding two identical recombination sites. The sequence of the recognition sites differs between Cre and Flp, designated LoxP sites in the case of the former and FRT sites for the latter, permitting the two systems to be used in parallel. By changing the orientation of these recognition sites with respect to each other, it is possible to invert, insert or excise intervening DNA. The Cre/loxP system has been successfully applied to modify transgenes in mice (Lakso et al. 1992; Orban et al. 1992), but this system has been most notably used in the generation of Cre recombinase excision conditional null alleles of genes (Gu et al. 1993). Homologous recombination permits the loxP sites to be inserted into the genome, flanking the target sequence. Expression of the recombinase deletes all or part of the gene, disabling the gene functionality. To retain the functionality of the pre-Cre conditional allele, the selectable marker must be removed (Fiering et al. 1995), which in often performed using the Flp/FRT system. The generation of a null allele from the conditional one can be achieved by crossing the conditional Cre mouse to a mouse expressing Cre from a powerful constitutive promoter, for instance the CAG promoter (Niwa et al. 1991). Alternatively, site-specific recombination can be achieved by transient transfection of a Cre vector into ES cells before transgenic animals are generated. This has become a common approach to delete drug-resistance cassettes used to select for homologous recombination events in the classical gene targeting approach as well (Meyers et al. 1998; Nagy et al. 1998), following observations that their retention could be deleterious to neighbouring gene function (Braun et al. 1994).

Many genes are re-used several times, frequently in disparate tissues, during development. In some cases, deletion of a particular gene in all tissues, e.g. VEGF, may prove lethal to the early embryo before the functionality of the gene can be assessed in later-stage tissues (Eremina et al. 2003). The solution to this problem was the generation of mouse strains expressing a recombinase under the control of an endogenous promoter, the expression of which is limited to discrete spatial locations in development (Tsien et al. 1996). Such Cre lines have allowed detailed analysis of gene function in a range of tissues and organs. The number of Cre mouse lines enabling tissue-specific Cre-mediated recombination is now in the hundreds, covering the majority of organs and tissues; the CreXMice database contains references to most Cre lines available (http://nagy.mshri.on.ca/PubLinks/indexmain.php). These Cre lines permit spatial control of Cre expression.

Other developments have added temporal control of Cre expression. The most common system used for inducible gene expression in mammalian cells is the Tet system. Briefly, the Tet inducible system relies upon the insertion of two transgenic elements into a cell: the tetracycline transactivator and the tetracycline-responsive element. The system can be arranged to either turn off (Tet-Off) or turn on (Tet-On) gene expression driven by the tetracycline-responsive element in the presence of the inducer doxycycline (Dox) (Gossen and Bujard 1992; Gossen et al. 1995). Dox can readily transverse the membrane of a cell, enabling the activation of the Tet system either by addition of Dox to the cell growth media or through oral ingestion by a mouse transgenic for the Tet system. By utilising the Tet system to control the expression of Cre, it is possible to choose the developmental time point at which genetic modification is induced. Moreover, by further combining this system with tissue-specific control of the tetracycline transactivator, full spatial and temporal control of genetic changes can be achieved (Belteki et al. 2005).

An alternative approach to the temporal control of Cre activity was accomplished by utilising a fusion protein between Cre recombinase and part of the estrogen receptor (Cre-ER), forcing localisation of the Cre-ER protein to the cytosol (Feil et al. 1996; Brocard et al. 1997; Feil et al. 1997). When the drug tamoxifen is applied to the cells or fed to animals transgenic for this system, the Cre-ER protein can translocate from the cytosol to the nucleus and catalyse Cre-mediated recombination. Spatial control of the Cre-ER system has also been reported by expression of the Cre-ER under the control of a tissue-specific promoter (Indra et al. 2005; Schuler et al. 2005).

Together, the versatility of Cre/loxP and Flp/FRT systems has enabled significant advances in the understanding of gene function; however, these two recombinases do have their limitations. One significant restriction is that, following a successful recombination event, both loxP and FRT sites remain a substrate for further recombination events (Garcia-Otin and Guillou 2006). This makes insertion of DNA sequences via Cre or Flp recombination problematic due to the very low efficiency (Araki et al. 1997); the recombinases

will subsequently catalyse the excision of the inserted DNA. Some progress has been made to negate the reversibility of the Cre reaction by generating mutated lox sites (Albert et al. 1995; Araki et al. 1997). Furthermore, some novel integrases may show more promise for inserting sequences into genomic DNA. For example, *Φ*C31 integrase is derived from the *Streptomyces* bacteriophage, catalyses the insertion of the phage genome into the host bacterial genome via two recognition sites: AttP, found in phage, and AttB, present in the bacterial genome. Importantly, after the integration event has occurred the remaining sites, AttL and AttR, are no longer substrates for further recombination events (Thorpe and Smith 1998). When the AttP and AttB sites lie on the same DNA strand, *Φ*C31 integrase can act to excise the intervening DNA, albeit at a lower efficiency than Cre (Andreas et al. 2002). However, demonstrations of the ability of *Φ*C31 integrase to insert DNA sequences, containing AttB sites, into pseudo-AttP sites found in the genomes of flies, amphibian and mammals illustrated new transgenic possibilities (Thyagarajan et al. 2001; Groth et al. 2004; Allen and Weeks 2005). Capitalising upon these and other experiments, *Φ*C31 integrase was recently shown not only to be a viable tool for inserting sequences into transgenic targeted locations, or dock sites, but that the *Φ*C31 integrase system is compatible with normal mouse development (Belteki et al. 2003). By establishing and characterising the expression of transgenes from these dock sites, it should be possible to negate some of the problems, for instance position variegation effect (Pravtcheva et al. 1994; Robertson et al. 1995), frequently associated with random transgene insertions.

4
ES Cells and Chimaeric Animals

In the prelude to the establishment of ES genetic modification protocols, much of the work performed on ES cells centred on the in vitro differentiation capabilities of these cells. In comparison to the restricted differentiation displayed by most murine EC cells (Martin and Evans 1974; Martin et al. 1977), it quickly became apparent that murine ES cells were routinely capable of differentiating into cell types representative of all three germ lineages and that they represented an excellent model for studying murine differentiation in vitro (Axelrod 1984; Wobus et al. 1984; Doetschman et al. 1985). However, the full potential, both development and technological, of ES cells was only realised when ES cells were successfully re-introduced back into mouse development (Bradley et al. 1984). These experiments showed that it was possible for ES cells to fully participate in development and, importantly, that it was feasible for the genome of the ES cells to be passed onto subsequent offspring via ES cell germline colonisation.

The ability to generate chimaeric mice has been available for some time, for instance by mixing cells from early-stage embryos (Tarkowski 1961; Gardner

1968); however, generating chimaeric animals by injection of ES cells into the blastocoele cavity of blastocyst-stage embryos quickly became dominant due to the enormous potential of ES cells. The technique – although relatively straight forward (Robertson 1987) – does require expensive equipment, and cannot easily be scaled up to high throughput. A less labour- and cost-intensive alternative involves the aggregation of ES cells with morula-stage embryos (Wood et al. 1993). Lately, this technique has become an ever more popular alternative (Nagy et al. 2003).

The level of chimaerism and number of chimaeric animals is highly dependent upon the cell line and host embryo used, since those ES cells that do contribute also have to compete with the developmental potential of the ICM cells within the host embryo.

4.1 Tetraploid Embryos as Chimaera Components

Capitalising on observations that extra-embryonic, but not generally embryonic, development could tolerate ploidy greater than 2n (reviewed in Eakin and Behringer 2003), combined with the finding that ES cells injected into blastocysts contributed most often to the embryonic portion of the embryo (Beddington and Robertson 1989), it became evident that a neat trick was available to force ES cells to contribute almost solely to the embryonic portion of the fetus. Experiments in the 1970s demonstrated that disruption of cytokinesis in the early embryo could be achieved by pulsed incubation with cytochalasin B, and if timed correctly, a two-cell-stage embryo could be induced to become uniformly 4n, or tetraploid (Snow 1973; Tarkowski et al. 1977). Similarly, others showed that it was possible to generate tetraploid embryos by electrofusion; a brief pulse of electricity will fuse the adjacent cell membranes of two-cell-stage embryos, creating a tetraploidy (Kurischko and Berg 1986). Of major importance was the observation that, whilst some murine extra-embryonic tissues are comprised of polyploid cell types (Varmuza et al. 1988), the majority of embryonic tissues demonstrate an overall diploid chromosomal complement (Keighren and West 1993). The developmental potential of tetraploid mouse embryos is limited: most die soon after implantation (Tarkowski et al. 1977), some spontaneously abort at around midterm, although occasionally some embryos have been observed at 15 dpc (Kaufman and Webb 1990). A single report of live tetraploid pups exists (Snow 1975), although this work remains to be repeated. Recent work indicates that tolerance of 4n cells in the murine embryo appears to be mouse strain-specific (Eakin et al. 2005), explaining why some experiments gave better survival for 4n embryos.

It was not, however, until ES cells were combined with tetraploid embryos that the full implications could be demonstrated. ES cells placed adjacent to tetraploid embryos in the correct tissue culture environment combine together to become a single viable embryo. The embryos generated from these mixing

experiments displayed clear segregation of the two components: ES cells contributed almost solely to embryonic development and the tetraploid component showed nearly exclusive contribution to extra-embryonic tissues (Nagy et al. 1990). Further, the congruous combination of ES cells with tetraploid embryos could ameliorate the lethality associated with 4n embryos. The protocol for performing aggregations with tetraploid embryos (see Fig. 1), whilst requiring practice, does not necessitate the same level of investment in equipment as ES-injection experiments (detailed protocols are available in Nagy et al. 2003). Variations upon the tetraploid aggregation theme have been explored by other groups, typically employing the injection of ES cells into a tetraploid blastocyst-stage embryo (Wang et al. 1997; Amano et al. 2001; Eggan et al. 2001). Techniques which rely upon mixing ES cells, whether by injection or aggregation, with tetraploid embryos are collectively referred to as tetraploid complementation assays; little data exist analysing their comparative efficacy.

However tetraploid complementation is achieved, the early development of tetraploid embryo ↔ ES cell chimaeras, and, indeed, the early development of all diploid embryo-ES chimaeras, remains, at heart, a struggle for supremacy. This struggle pits the endogenous cells of the embryo epiblast against the intruding ES cells, with the latter attempting to integrate with or, ideally, depose the former. Recently, it has become apparent that, irrespective of the number of ES cells actually re-introduced into the embryo during diploid injection/aggregation or tetraploid complementation, only a few of these ES cells, typically a maximum of three, actually contribute to the somatic lineages of the chimaeric offspring (Wang and Jaenisch 2004). Intriguingly, by use of fluorescent labelling and imaging of live cells, it has now emerged that numerous 4n cells in tetraploid complementation embryos persist in the early embryonic epiblast, although these cells are typically displaced and lost from the embryo around the time of gastrulation (Eakin et al. 2005).

Whilst initial experiments demonstrated that tetraploid embryo ↔ ES cell chimaeras favour the ES cell component in the embryo proper, all of the offspring failed to survive after birth; the ES cell-derived live pups born quickly died, typically of respiratory failure immediately after birth (Nagy et al. 1990). The lack of surviving births from tetraploid complementation experiments was a setback. Nonetheless, the technique could still be used to address certain biological questions in which live pups were not required, for instance, testing the functionality of ES cell-derived tissues (Forrester et al. 1991).

The neonatal mortality observed in tetraploid complementation assays was eventually resolved by the use of ES cells derived from embryos that were the F1 progeny of two separate inbred sub-strains of mice. Thus far, all of the early tetraploid complementation experiments had used ES cells derived from embryos of 129 inbred strains of mice. However, by crossing 129/S1 with 129/X1, Nagy and colleagues generated a 129 substrain hybrid ES cell line, termed R1 (see Fig. 1). R1 ES cells could, when used in tetraploid complementation assays,

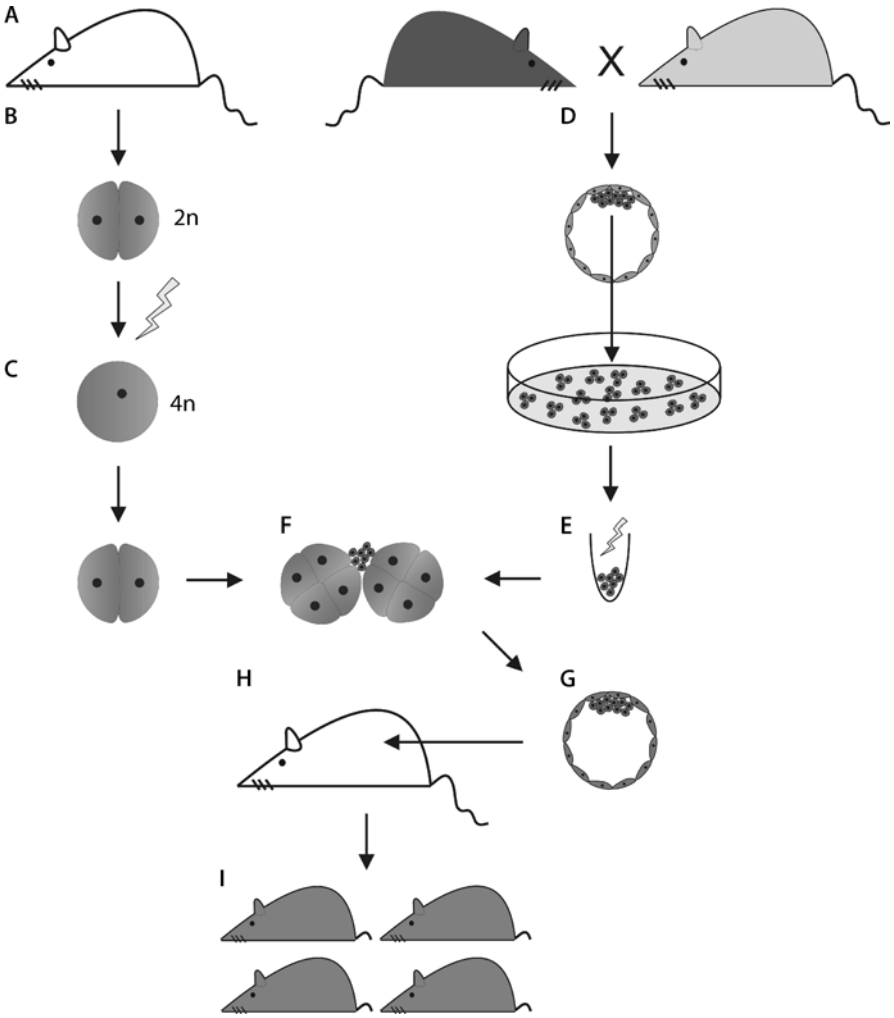

Fig. 1 Generation of transgenic mice by tetraploid aggregation. Plugged females (*A*) are sacrificed and cleavage-stage embryos recovered. Diploid (2n) two-cell-stage embryos are electrofused (*B*) and the resultant tetraploid (4n) embryos (*C*) cultured. Hybrid ES cells, derived from nonisogenic strain crossings (*D*), are genetically modified (*E*), in this case to contain a mutation causing a short tail phenotype. During tetraploid aggregation, a small clump of mutant ES cells are sandwiched between two four-cell-stage tetraploid embryos (*F*) and cultured until a single blastocyst stage embryo develops (*G*). Tetraploid-ES chimaeric embryos are then transferred into a pseudo-pregnant recipient (*H*); the offspring are completely ES-cell-derived and hence all carry the short-tail phenotype (*I*)

produce healthy and fertile offspring completely derived from the R1 ES cell line (Nagy et al. 1993). This was the first demonstration that ES cells alone can support the normal development and life of a mouse.

4.2 Tetraploid Complementation Implications

The ability to derive completely ES cell-derived embryos/animals with high efficiency involves a number of issues.

Primarily, tetraploid complementation technology substantially reduces the breeding cycles that are required when dealing with the variable chimaerism levels displayed by embryos from traditional ES ↔ diploid embryo experiments. Tetraploid complementation facilitates the generation of viable, fertile mice in which all tissues are derived from the parent ES cells in the initial litter. For many applications, there is no requirement for prolonged rounds of breeding, enabling phenotypic analysis within the life span of the first offspring. Contrast this with ES cell chimaeric embryos derived from diploid host embryos: the resultant mixed chimaeras require further rounds of breeding before offspring can be generated that are wholly derived from the transgenic ES cells, typically adding months to the study duration. Indeed, in the case of generating homozygous knockout mice from heterozygous targeted ES cells, tetraploid complementation can substantially reduce the number of crossings that are required to establish male and female heterozygous carriers, which are then bred to produce homozygous offspring, saving time and money. The timeline for deriving homozygous offspring can be further enhanced by using pseudo-female ES cells derived from male ES cells that have lost a Y chromosome, an event that happens at a small but appreciable frequency in culture (Eggan et al. 2002; Eggan and Jaenisch 2003). By using both the diploid male ES cells and the pseudo-female ES cells to generate male and female offspring in tetraploid complementation experiments, it is possible to breed the progeny to homozygosity in a single subsequent mating round.

Secondly, the data from the R1 experiments indicated that ES cells from inbred strains of mice appeared to benefit from a nonisogenic DNA component when participating in tetraploid complementation, or so-called hybrid vigor (Nagy et al. 1993; Eggan et al. 2001). This is probably not surprising: the ubiquity of ES cells from inbred strains of mice such as 129 originates mainly from the ease of derivation and not necessarily upon the efficacy of ES cells to re-enter embryonic development. Indeed, ES cell derivation requires cellular properties which are more akin to cancer fitness: the ability to survive transferral from the comfort of the blastocyst to the rather alien environment of the tissue culture dish, whilst concomitantly forcing sustained proliferation into a precursor cell type, the epiblast, which is present for a short while during normal development (Beddington and Robertson 1999). The ease of ES cell derivation from the 129 strain has had other implications: reliance upon a few inbred strains for much of the mouse research conducted worldwide, raising questions about the transferability of results gained from some of the experiments conducted upon these animals (Wolfer and Lipp 2000). Such concerns are especially evident when, for instance, certain gene knockout phenotypes, including p53

and EGF, are substantially affected by the strain background upon which they are bred (Donehower et al. 1995; Sibilia and Wagner 1995). Notwithstanding, such strain-to-strain variation can open the way to identifying modifiers of gene action which otherwise might not have been found.

The advantage of hybrid genetic background over that of purely inbred background during tetraploid complementation was highlighted by experiments that sought to understand the neonatal lethality observed in many mice cloned by nuclear transfer (Eggan et al. 2001). Similar to the early attempts at tetraploid complementation from inbred ES cells, many cloned animals generated from nuclear transfer can have lethal respiratory problems at birth, often as part of a larger range of abnormalities collectively referred to as large offspring syndrome (Wakayama et al. 1999; Wakayama and Yanagimachi 1999; Rideout et al. 2000). Comparative experiments using ES cells derived from inbred mice or the F1 progeny of two inbred strains demonstrated that the latter produced consistently higher numbers of pups surviving to adulthood when used in tetraploid complementation assays or as the nuclear donor for somatic cell nuclear transfer (Eggan et al. 2001). Indeed, the hybrid vigour displayed by these ES cells does not deplete with extended culture periods in vitro nor by successive rounds of gene targeting and selection.

Finally, ES cell-derived embryos/animals generated using tetraploid embryo complementation can answer biological questions that are simply not possible in traditional ES cell-derived chimaeras generated from diploid host embryos.

As mentioned earlier, many genes are used several times during development and later adulthood, frequently in functionally distinct tissues. For instance, the achaete-scute transcription factor family member Mash2 is expressed in the extra-embryonic ectoderm as well as many tissues of the embryo proper including the germ lineage (Tanaka et al. 1997; Rossant et al. 1998; Kury et al. 2002). However, the role of Mash2 in later embryogenesis and adulthood could not be studied due to the embryonic lethality observed in Mash2$^{-/-}$ knockout offsping; Mash2$^{-/-}$ mice die from placental failure at around 10 dpc (Guillemot et al. 1994). By use of tetraploid complementation, a wild-type tetraploid embryo was used as the host for Mash2$^{-/-}$ ES cells; the wild-type tetraploid embryo contributed to the extraembryonic lineages, rescuing the mid-gestation lethality previously seen in Mash2$^{-/-}$ embryos, permitting the observation that loss of Mash2 appeared to have no overt phenotype in later embryogenesis or adulthood. Similarly, embryos from tetraploid complementation assays permitted verification of Hnf-4 functionality in the visceral endoderm (VE) role as a patterning organiser during embryonic gastrulation (Duncan et al. 1997). Hnf-4 expression is restricted to the VE at the time of gastrulation; Hnf-4$^{-/-}$ embryos arrest at this point, suggesting that Hnf-4 is a functional requirement in the VE for gastrulation to occur. Rescue of this early embryonic phenotype was observed in embryos derived from aggregations of wild-type tetraploid embryos with Hnf-4$^{-/-}$; the VE was derived from the wild-type tetraploid component, conclusively demonstrating that gastru-

lation arrest was not cause by a Hnf-4 deficiency with the embryo proper but by the Hnf-$4^{-/-}$ VE.

Tetraploid complementation, therefore, offers a number of significant benefits over established techniques for transgenic mouse generation. New hybrid lines and further investigation into the factors regulating hybrid vigour should further improve this already robust technique.

5 Concluding Remarks

In the intervening 25 years since the first derivation of mouse ES cells, significant technological advances have been made in the utilisation of these cells. These advances have enabled considerable progress in the understanding of both gene function and the generation of genetic models of human diseases. Two principle areas have permitted this: the genetic modification of ES cells and the generation of ES cell chimaeras. Both of these areas are still subject to continued innovation. New ES cell lines are available with excellent germ line compatibility and increased efficacy in generating mice directly from ES cells. Novel ways of target vector building based on long range PCR amplification of the homology arms (Randolph et al. 1996), BAC transgenesis (Testa et al. 2003; Yang and Seed 2003) or bacteriophage recombineering (Copeland et al. 2001; Muyrers et al. 2001) are making the time-consuming component of gene targeting now high-throughput. Similarly, faster techniques are reducing the time required to genotype and sex mutant mice from transgenic litters (Henneberger et al. 2000; McClive and Sinclair 2001; Linask and Lo 2005).

This chapter discussed a range of tools that are facilitating gene function discovery at an ever-increasing rate. Combinations of several genetic modification technologies are answering questions which, until recently, have proved unfeasible. For instance, the temporal and spatial control of gene knockout means that the function of a particular gene in a selected organ can now be addressed at all points of embryonic and adult life. Indeed, the adoption of new recombinases, such as ΦC31 integrase, which can be used concurrently with Cre and Flp, combined with increasing the number of transgenic alterations made to a single mouse will further increase the complexity of the questions that we can ask. Moreover, fast RNAi-based gene expression knock down combined with conditional or inducible transgenesis in ES cells and subsequent tetraploid embryo complementation assay offers additional acceleration.

All of the advances described here have brought the mouse transgenic field to a position where it is feasible to systematically mutate all of the genes in the murine genome. Indeed, an ambitious international project supported by the European Community, Genome Canada and the NIH will attempt to reach this goal in mouse ES cells. The distribution of these mutated ES cell lines to the research community for characterisation will eventually lead to a substantial

genetic knowledge base, which will pay substantial dividends in our attempts to understand and cure human disease.

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Gene Trap Mutagenesis

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Abstract Our ability to genetically manipulate the mouse has had a great impact on medical research over the last few decades. Mouse genetics has developed into a powerful tool for dissecting the genetic causes of human disease and identifying potential targets for pharmaceutical intervention. With the recent sequencing of the human and mouse genomes, a large number of novel genes have been identified whose function in normal and disease physiology remains largely unknown. Government-sponsored multinational efforts are underway to analyze the function of all mouse genes through mutagenesis and phenotyping, making the mouse the interpreter of the human genome. A number of technologies are available for the generation of mutant mice, including gene targeting, gene trapping and transposon, chemical or radiation-induced mutagenesis. In this chapter, we review the current status of gene trapping technology, including its applicability to conditional mutagenesis.

Keywords Gene trapping · Mouse · Knockout · Genome · Function

1 Introduction

The recent sequencing of the human and mouse genomes (Lander et al. 2001; Venter et al. 2001; Waterston et al. 2002) underscores the need for the functional annotation of thousands of novel genes. For the most part, humans and mice share an almost identical set of genes and physiology. These similarities, along with the development of robust and precise mouse mutagenesis

techniques over the last 25 years, make the mouse the centerpiece model system for the interpretation of human gene function. Recognizing the central role of mouse genetics in the functional annotation of the human genome, government-sponsored initiatives, such as the Knockout Mouse Project, are being launched to knockout every gene in the mouse and analyze their function through the study of the resulting phenotype (Austin et al. 2004; Auwerx et al. 2004). These efforts call initially for the generation of null alleles in all genes through embryonic stem (ES) cell technologies, namely gene targeting and gene trapping. Gene targeting is a widely used technique in which a DNA construct, or targeting vector, is introduced into ES cells to generate the desired mutation through homologous recombination between sequences in the vector and the target locus (Bradley et al. 1992; Joyner 2000; Mansour et al. 1988). Gene targeting allows for the generation of virtually any desired mutation, such as deletions, insertions, point mutations (Hasty et al. 1991), and large chromosomal deletions and rearrangements (Ramirez-Solis et al. 1995), but requires significant upfront effort to generate a different targeting vector for each locus, as well as downstream efforts in the screening of multiple ES cell clones (typically hundreds) in order to identify the relatively rare homologous recombinants.

Gene trapping is a method of random mutagenesis in which the insertion of a DNA element into endogenous genes leads to their transcriptional disruption. Gene trapping elements can be endogenous in nature, such as transposable elements (Wilson et al. 1989), or exogenous recombinant DNA constructs (Gossler et al. 1989). Unlike gene targeting, the insertional nature of gene trapping does not allow for the generation of all types of mutations, such as deletions or point mutations. Its main advantage, however, is that a single gene trapping vector can be used to mutate and identify thousands of genes in ES cells (Zambrowicz et al. 1998; Wiles 2000). This scalability makes gene trapping very suitable for high-throughput, large-scale, and cost-effective mutagenesis programs like the ones being proposed by governmental agencies around the world. In fact, large-scale gene trapping efforts in mouse ES cells have already taken place, demonstrating the ability to mutate at least 60% of all mouse genes in ES cells, including potential pharmaceutical targets such as the *Wnk1* protein kinase (Zambrowicz et al. 2003).

2
Generating Null Alleles Through Gene Trap Mutagenesis

2.1
Types of Gene Trapping Vectors

Gene trapping vectors are typically designed to lack an essential transcriptional component, such as an enhancer (O'Kane and Gehring 1987), promoter (Hicks et al. 1997), or polyadenylation signal (polyA) (Niwa et al. 1993), ren-

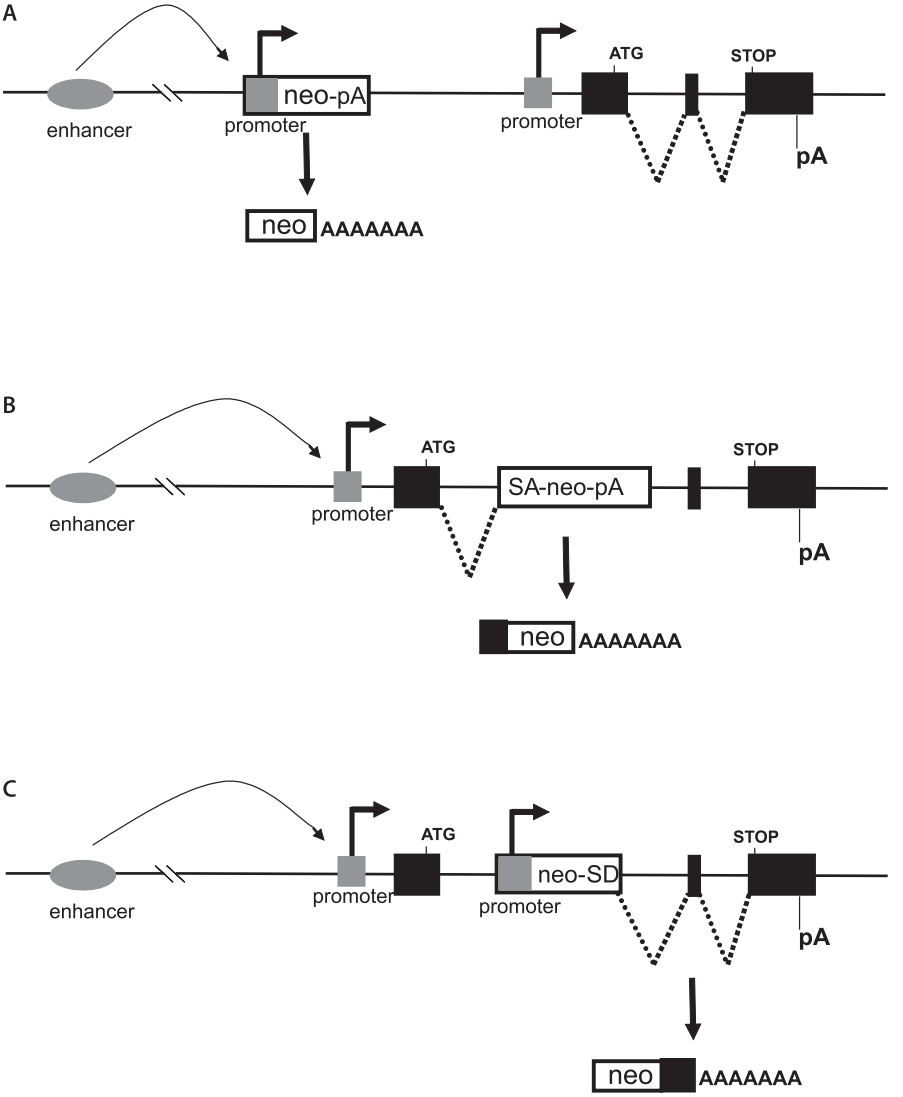


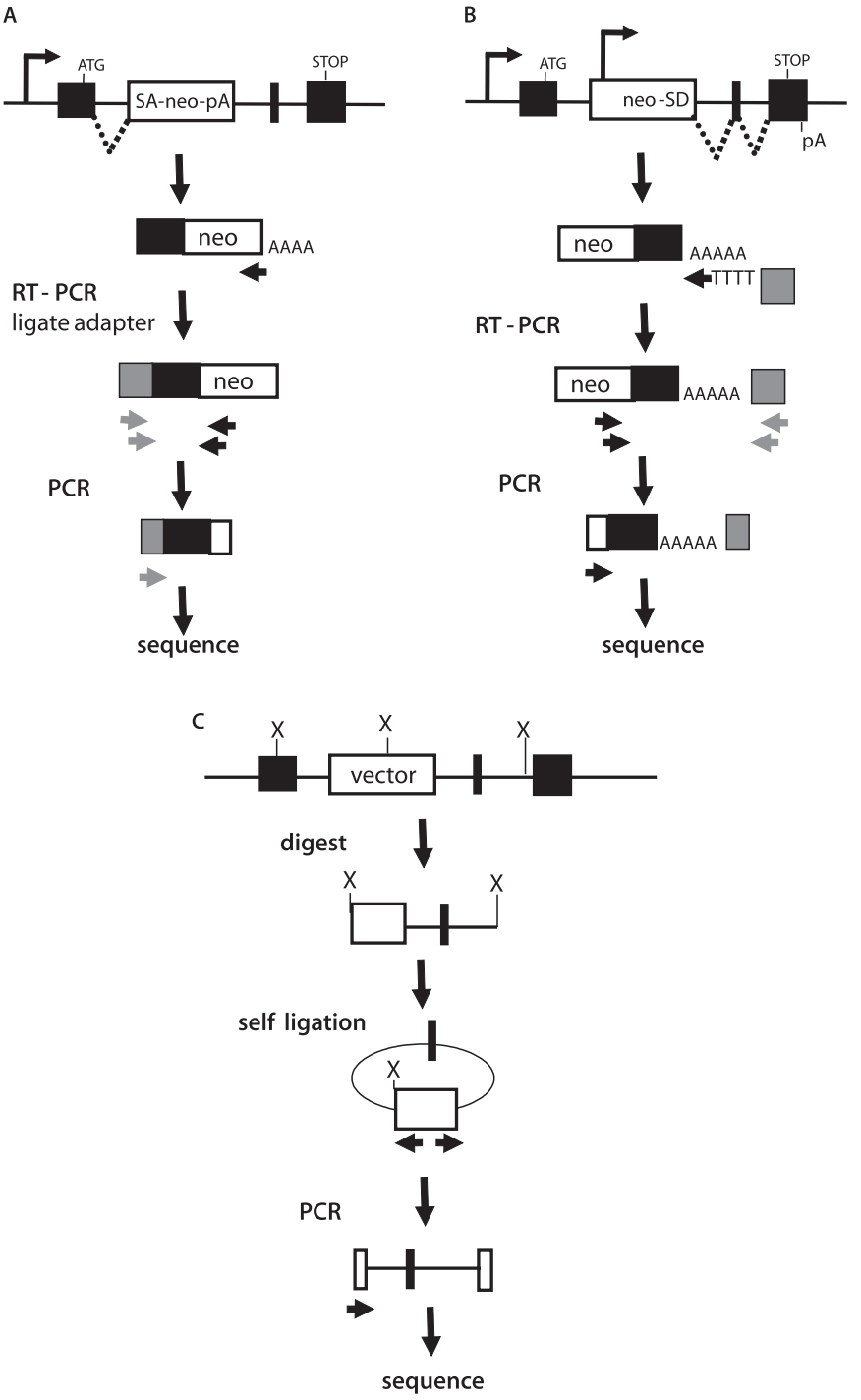
Fig. 1 A–C Different types of gene trapping strategies. **A** An enhancer trap, showing a gene trapping vector containing its own promoter, neomycin resistance (*neo*) gene and polyadenylation signal (*polyA*). The promoter in the vector requires the action of an enhancer to drive transcription of the *neo* mRNA. **B** A promoter trap, showing a vector containing a splice acceptor sequence (*SA*), followed by the *neo* gene and a polyA signal. Insertion in a gene, downstream of the endogenous promoter, leads to expression of the *neo* mRNA fused to the upstream exons (*black boxes*) of the trapped gene. **C** A polyA trap, showing a vector containing its own promoter, the *neo* gene and a splice donor sequence (*SD*). Insertion in a gene, upstream of the endogenous polyA, leads to expression of the *neo* mRNA fused to the downstream exons of the trapped gene

dering them transcriptionally active only when inserted into an endogenous gene (Fig. 1). Vectors containing a splice acceptor and a selectable marker but lacking a promoter element, also known as promoter traps, are the most commonly used type of gene trapping vector. Promoter trap vectors can be designed to contain a reporter gene for in vivo analysis of the expression of the trapped genes (Friedrich and Soriano 1991; Gossler et al. 1989). Constructs are introduced into the cells of interest, such as mouse ES cells, through retroviral infection, or by electroporation. Random insertion of the vector into a transcribed gene leads to expression of the selectable marker and resistance to antibiotics. Insertion of the vector into the gene also leads to its transcriptional disruption. Vectors lacking polyadenylation sequences, also known as polyA traps, can be used to trap untranscribed genes, since they contain their own promoter driving expression of the selectable marker.

2.2 Identification of Trapped Genes

Unlike other random mutagenesis methods, such as chemical (Brown and Peters 1996) or radiation-induced mutagenesis (You et al. 1997), gene trapping vectors can be used to readily identify the mutated gene (Fig. 2). This is typically accomplished through 5′ or 3′ RACE (rapid amplification of cDNA ends) of fusion transcripts generated by the splicing of endogenous exons into promoter traps and out of polyA traps, respectively (Fig. 2A, B). It is important to note that 5′ or 3′ RACE allows for the identification of the mutated gene by virtue of its cDNA sequence, but does not pinpoint the exact location of the insertion within the genome. RACE was virtually the only tool available for identifying a trapped gene prior to the availability of the mouse genome sequence. With the sequencing of the mouse genome, however, a short sequence tag of the genomic DNA immediately flanking the gene trapping vector is sufficient, in most cases, to determine its precise chromosomal location. Flanking genomic sequence can be obtained through a variety of methods, the most common of which is

Fig. 2 A–C Identification of the trapped gene. **A** 5′ rapid amplification of cDNA ends (5′ RACE). After RT-PCR, an adapter oligonucleotide is ligated to the 5′ end of the cDNA for the transcript produced by a promoter trap construct. Primers complementary to the vector and the adapter and then used to amplify the fusion transcript and identify the trapped gene by sequencing. A nested PCR strategy is typically used. **B** 3′ RACE. A polyT primer with a unique sequence tail is used for RT-PCR. Nested PCR with primers complementary to the vector and the unique tail is then used to amplify the fusion transcript generated by a polyA trap construct. **C** Inverse Genomic PCR (IPCR). Genomic DNA from individual gene trapped clones is cleaved with one or more restriction enzymes (X) to produce a vector–genomic junction fragment that can be ligated to produce a circular template. Two vector-specific primers can be used to amplify the flanking genomic DNA. Sequence of the flanking genomic DNA allows for the precise mapping of the insertion within the genome


A
ATG
SA-neo-pA
STOP
neo
AAAA
RT - PCR
ligate adapter
neo
PCR
sequence
B
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RT - PCR
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AAAAA
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vector
digest
X
X
self ligation
X
PCR
sequence

inverse genomic PCR (IPCR) (Silver and Keerikatte 1989). In this technique, genomic DNA from gene-trapped ES cells is cleaved with a restriction enzyme that cuts within the vector, producing a vector–genomic junction fragment that is then circularized by ligation at low DNA concentrations that favor intramolecular ligation. The mouse genomic DNA adjacent to the vector can then be amplified by PCR using outward-facing primers complementary to the vector. The use of multiple enzymes or combinations of enzymes increases the probability of generating a circular template of appropriate size for efficient PCR amplification (Fig. 2C). IPCR is ideally suited for large-scale retroviral gene trap insertion site identification because it requires no prior knowledge of the disrupted gene sequence and can be used repeatedly for the same gene trapping vector. Furthermore, since retroviral insertion mutations are discrete in that they are not associated with deletions or alterations of the inserted vector or the genomic sequence at the site of insertion (Varmus 1988), IPCR can be successfully applied to all gene trap mutations. There are several advantages to this approach for gene identification. First, the technique is more robust since it uses DNA rather than RNA as a template. Second, it provides more accurate data with which to predict gene disruption. This is particularly important when the gene of interest contains multiple promoters or transcriptional start sites, in which case particular attention must be paid to the exact location of the gene trapping vector with respect to each transcript. In these instances, RACE data often fails to provide adequate confirmation of the transcript classes affected by the gene trap. Third, the precise genomic insertion site of the vector is essential for PCR-based genotyping of mice generated through gene trapping (Fig. 3). For large-scale, publicly available gene trap libraries, it is imperative that such data be available.

2.3 Mutagenicity of Gene Trapping Vectors

Although the number of publications describing mutant mice generated through gene trapping lags significantly behind the number of gene-targeting publications, an increasing volume of evidence shows that gene trap insertions in genes are mutagenic and tend to result in null alleles (Skarnes et al. 1992; Voss et al. 1998) (also, see Table 1). A number of groups around the world (Hicks et al. 1997; Zambrowicz et al. 1998; Wiles et al. 2000; Hansen et al. 2003) have carried out efforts to mutate large numbers of genes in mouse ES cells through gene trapping, and initiatives like the Knockout Mouse Project (Austin et al. 2004) will only increase the number of gene-trapped mouse lines characterized in the future. Thus, a detailed assessment of the mutagenicity of gene trap mutations is an important step at this juncture. We have used gene trapping to generate OmniBank, a library of more than 270,000 frozen mouse ES cell clones uniquely identified by their corresponding OmniBank Sequence Tag (OST). The insertion mutations contained in this gene trap library represent

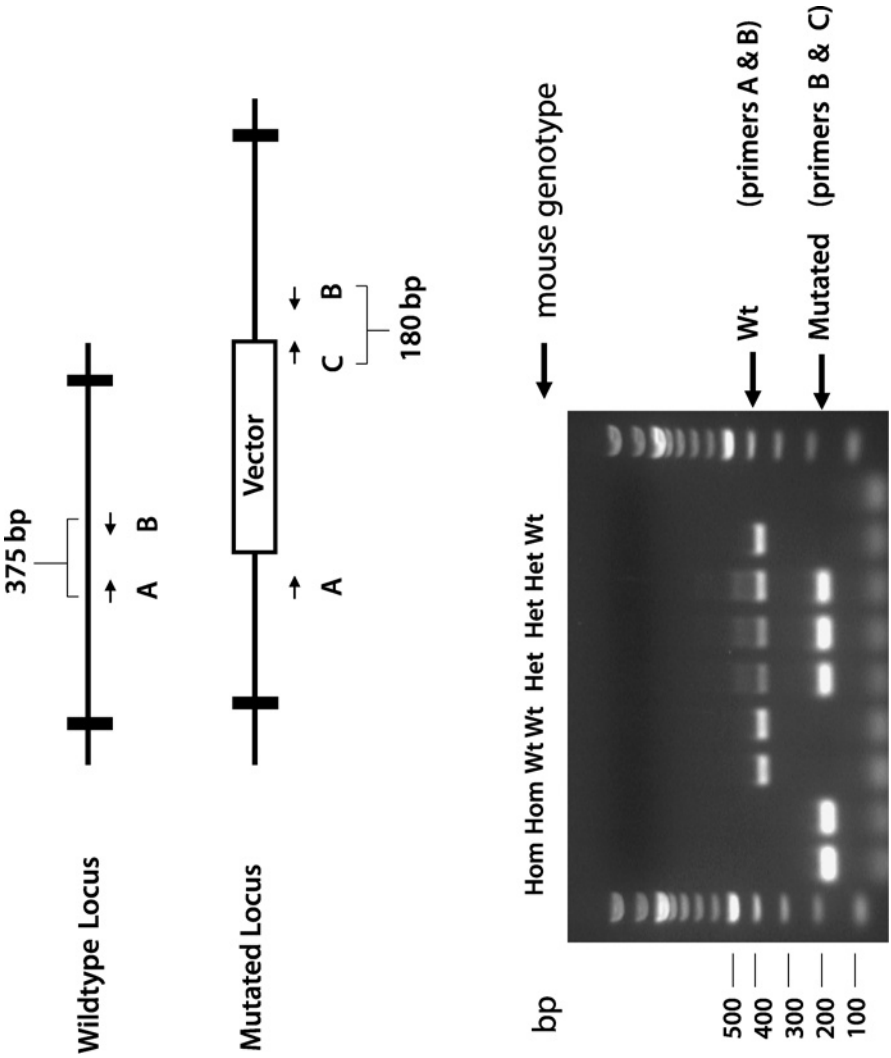

Fig. 3 Genotyping strategy for gene trap mutations. Primers A and B flank the genomic insertion site and amplify a genomic DNA product for the wild-type allele. Primer C, complementary to the gene trapping vector, and primer B amplify the mutated allele. *Black boxes* represent exons of the trapped gene. *bp* base pairs

mutations in approximately 60% of all mouse genes (Zambrowicz et al. 2003). OSTs were generated by 3′ RACE (Fig. 2B). As mentioned above, the cDNA sequence data provided by the OST is useful for predicting the relative location of the gene trap event within a gene of interest; however, it does not pinpoint the exact insertion site in the genome nor facilitate the design of specific PCR genotyping assays. Therefore, OmniBank gene trap mutations are further

Table 1 OmniBank gene trapped mouse lines: efficient generation of null alleles

OmniBank trapped gene	Published confirmation of null allele	Reference
Thioredoxin 2 (*Trx-2*)	- No protein detected by Western blot	Nonn et al. 2003
Mdm4 (*Mdmx*)	- No protein detected by Western blot	Migliorini et al. 2002
Insulin-degrading enzyme (*IDE*)	- No protein detected by Western blot	Farris et al. 2003
Insulin-degrading enzyme (*IDE*)	- No message detected by RT-PCR - No protein detected by Western blot - No IDE enzymatic activity detected	Miller et al. 2003
tRNA synthetase p38 subunit	- No message detected by Northern blot - No protein detected by Western blot	Kim et al. 2002
Wave1	- No protein detected by Western blot	Dahl et al. 2003
Testis–brain RNA-binding protein (*TB-RBP*)	- No protein detected by Western blot	Chennathukuzhi et al. 2003
Insulin-like growth	- No message detected by RT-PCR	Hansen et al. 2004
factor II mRNA-binding protein 1 (*IMP1*)	- No message detected after extended exposures of whole-mount in situ hybridizations	
Calpain 3 (p94)	- No message detected by RT-PCR - No protein detected by Western blot	Kramerova et al. 2004
GYS1 glycogen synthase	- No protein detected by Western blot - No GYS1 enzymatic activity detected - No glycogen detected	Pederson et al. 2004
Tektin-t	- No message detected by Northern blot - No protein detected by Western blot	Tanaka et al. 2004
Phosphatidylinositol 5-phosphate 4-kinase	- No message detected by RT-PCR - No protein detected by Western blot	Lamia et al. 2004

characterized prior to mouse knockout production using IPCR (Fig. 2C). By identifying the precise genomic insertion site of the vector, it is possible to eliminate ES clones in which the gene trapping vector inserts within the promoter region or upstream of the gene, but for which an OST was still generated through splicing into the second exon of the gene. These gene trap insertions are unlikely to be mutagenic.

To evaluate the mutagenicity of OmniBank gene trap mutations in vivo, all nonembryonic lethal OmniBank mouse lines are subjected to a sensitive RT-PCR quality control step. We check for the loss of the endogenous transcript in homozygous mutant animals relative to wild-type controls. RNA is isolated from selected tissues known to normally express the transcript and subjected to RT-PCR using primers complementary to exons flanking the insertion site of the vector (Fig. 4).

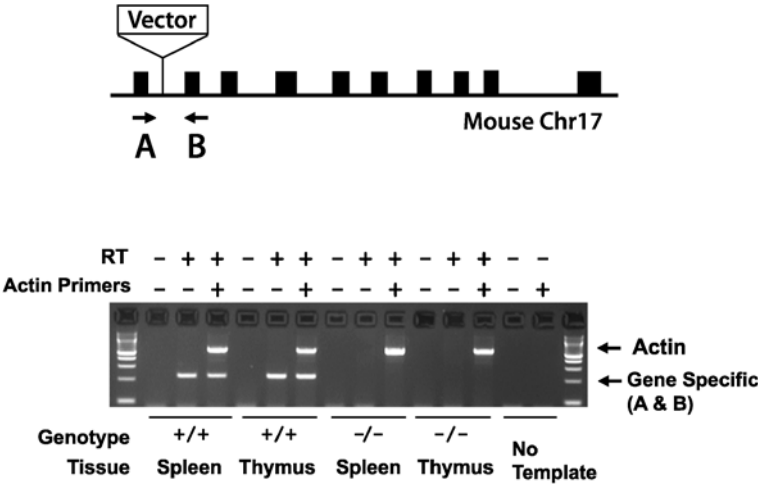

Fig. 4 Assessing the mutagenicity of gene trap insertions by RT-PCR. Primers D and F are complementary to exons flanking the insertion site in the *PolH* gene in mouse chromosome 17 (accession number NM_030715). RT-PCR using primers D and F shows absence of endogenous message in the spleen and thymus of homozygous animals. Control primers to the murine *beta actin* gene were used (accession number M12481)

To date, we have generated and analyzed 1,155 OmniBank mouse lines in which the vector insertion was confirmed by IPCR to be within the gene of interest. Of these, 370 (32%) were not analyzed by RT-PCR due to embryonic lethality (315 lines) or perinatal lethality or reduced viability (55 lines). Of the remaining 785 lines, 706 (90%) showed no detectable wild-type transcript by RT-PCR and 79 (10%) showed drastically reduced levels of wild-type transcript. For selected lines showing reduced but detectable expression, quantitative PCR (QPCR) analysis has generally shown message levels of less than 5% of wild-type controls. Further evidence that gene trap mutations produce null alleles is shown in Table 1. In this table, we have gathered independently published, recent analyses of OmniBank mouse lines where the authors used methods other than RT-PCR to confirm that the allele resulted in a null mutation. Taken together, this data demonstrates that intragenic insertions efficiently disrupt gene transcription in vivo.

Even for the severe hypomorphs in which RT-PCR is able to detect greatly reduced levels of wild-type transcript, the mutation is likely to be useful for the elucidation of gene function, as exemplified in the case of the *Slit3* mutation. In an independently published analysis of a *Slit3* OmniBank gene trap mutation (Liu et al. 2003), the authors were unable to detect any message by in situ hybridization. When the more sensitive RT-PCR technique was used, greatly reduced expression was detected. QPCR revealed 1.48% and 1.27% of wild-type message levels in the homozygotes, depending on the QPCR primers used. Importantly, the phenotype of this OmniBank line (congenital diaphragmatic

hernia, kidney agenesis, and cardiac defects) is the same as the one described for a published *Slit3* knockout generated through gene targeting and predicted to lead to a null mutation (Yuan et al. 2003). Taken together, sequential QC processes such as IPCR of the ES cells in vitro and RT-PCR of the mice in vivo provide a robust method for production of null alleles through gene trapping.

Despite the ability of gene trap events to disrupt the transcript in which they are inserted, it is important to note that, just as with gene targeting, careful attention must be paid to any potential alternative splicing of the gene of interest. Depending on the location of the insertion, mRNAs transcribed from alternate promoters or containing multiple polyadenylation signals may not be affected by the gene trap (Li et al. 2003). The sequencing of the human and mouse genomes, along with the mapping of mRNA and expressed sequence tags (EST) data to public genome-viewing software, can greatly aid in the choice of a gene trap that will disrupt all transcripts of the gene of interest. As the number of publicly available gene trap events increases, the chance of finding an insertion at the desired location will also increase. Alternatively, there may be cases when it may be of interest to disrupt only a subset of transcripts. An example that illustrates the potential benefit of transcript-specific gene traps is that of the *Nogo* gene. *Nogo* codes for a putative inhibitor of axonal regeneration. Alternative splicing and an alternate promoter lead to the production of three *Nogo* transcripts encoding three distinct proteins. A mutation generated through gene targeting that disrupts all transcripts resulted in embryonic lethality (Zheng et al. 2003). Interestingly, mice carrying a gene trap mutation that selectively disrupts two of the three transcripts were viable, allowing for the study of axonal regeneration in adult mice (Kim et al. 2003).

3 Conditional Gene Trapping

The ability of gene trapping to mutate genes on a genome-wide scale has also prompted investigations into the use of this approach to generate more custom mutations such as conditional alleles. Recombinase sites, the essential elements of conditional allele construction, can be incorporated in various configurations within a gene trapping vector to allow for removal or inversion of its functional components. This provides some level of control over the transcriptional disruption of the target gene; however, it hinges upon the assumption that intronic gene trap insertions can be transcriptionally silent when an inverted or partially excised gene trapping vector is present within an intron. At present there is scant data available to properly address this issue; therefore it remains to be seen whether the gene trap approach will be generally useful for conditional allele creation. Regardless, the current demand for mutant alleles and the ease of producing these alleles through gene trapping will continue to push such efforts forward.

Several approaches for generation of conditional gene trap alleles have been reported to date (Li et al. 2005; Schnutgen et al. 2005; Xin et al. 2005). The com-

plexity and flexibility of each approach varies widely, from the most simplistic, in which the splice acceptor element is flanked by *lox* P sites, to elaborate approaches using nested recombinase sites to allow multi-step modifications. The most versatile of these vectors was recently reported by Schnutgen et al. (2005). Their vectors contain multiple nested site-specific recombinase sites allowing gene trapping, allele repair, and allele reinduction through successive recombination steps. Their vectors contain a standard splice acceptor and selection cassette flanked by four nested sets of heterotypic target sequences for the Cre and Flp recombinases. This design allows conditional induction of the mutant allele or conditional reversion of the wild-type allele. In this report, the authors also show that, at least in vitro, their gene trap elements are silent when inverted. Using RT-PCR, they evaluated the transcription of two gene trap mutations in endogenous loci and found no detectable disruption of the wild-type allele when the gene trap elements were inverted. This data provides a first step toward the production of a useful conditional resource, but more data is needed, particularly in vivo studies. As a general resource for either null or conditional alleles, this library also lacks data for germline transmission rates for these ES cell clones and does not currently include genomic insertion site sequence data to confirm the location of these mutations and enable genotyping of the mouse lines.

A single report is currently available describing the function of conditional gene trapping vectors within an endogenous gene in vivo. This report describes an approach that allows conditional allele repair through the excision of the splice acceptor element within an otherwise standard gene trapping vector (Li et al. 2005). An insertion of a gene trapping vector containing *lox* P sites flanking the splice acceptor element within the Myocardin-related transcription factor b (*Mrtf-b*) gene was used to study the requirements for this gene within a specific cell lineage. *Mtrf-b* is essential for embryonic development. Homozygous null *Mtrf-b* embryos die late in gestation or shortly after birth due to defects in cardiac outflow tracts. It was hypothesized that these defects were cell autonomous and therefore might be rescued by the presence of wild-type transcript within this cell lineage. This was confirmed by crossing this line with a mouse line that expresses the Cre recombinase protein specifically in the neural crest lineage (Jiang et al. 2000). Homozygous mutants carrying the *Wnt-1 Cre* transgene survived to birth and showed no obvious phenotypic defects several weeks after birth. This suggests that Cre excision of the splice acceptor element of the gene trap vector was successful in restoring wild-type *Mtrf-b* splicing, and that the presence of the partially excised vector in this instance did not significantly impede the production of wild-type transcript. This is a promising first step in the application of this approach; however, additional studies and much more detailed analysis of the transcriptional effects of these insertions need to be done in order to conclude that conditional gene trap resources will provide all of the function desired from these types of alleles.

4 Other Applications of Gene Trapping

In addition to its ability to generate null or conditional mutations in mice for the study of gene function, gene trapping has been successfully utilized for a number of other purposes. The high-throughput nature of gene trapping allows for the rapid generation of large numbers of mutants. With the proper assay in place, the effects of these mutations on the cellular process of interest can be probed. In general, however, these screens are limited by the fact that most gene trapping mutations will be heterozygous. This hurdle can be overcome in certain hypodiploid cells containing large regions of functional haploidy, such as certain Chinese hamster ovary (CHO) cells. Hubbard et al. used a promoter trap screen and wheat germ agglutinin selection to identify CHO cells deficient in glycosylation (Hubbard et al. 1994), including cells nullizygous for GlcNAc transferase I (Chang et al. 1993). In another attempt to overcome the heterozygosity of gene trap mutations, a gene trapping system was designed to express antisense RNA to the trapped gene from a transactivated promoter. In principle, successful knockdown of the remaining wild-type allele would result in functional homozygosity and the ability to directly screen for recessive phenotypes in vitro. This system was used to screen for gene trap mutants with a transformed phenotype and the identification of TSG101, a putative tumor suppressor gene (Li and Cohen 1996). Finally, a promising development toward the use of gene trapping for in vitro screens has been the development of ES cells deficient for the Bloom syndrome (*Blm*) gene. These cells display a greatly elevated rate of mitotic recombination, which increases the chance that any given gene trap insertion will become homozygous (Guo et al. 2004; Yusa et al. 2004).

Another use of gene trapping is the identification of genes that are differentially regulated under certain conditions, such as differentiation of specific cell types, or expressed in a specific tissue or organ of interest. Promoter traps with selectable markers and/or reporter genes can be used to select for and follow the expression pattern of the trapped gene in the desired context, without the need for homozygosity. Positive or negative selection for a resistance marker placed under the control of endogenous promoters by the gene trap events can be used to identify genes that are up- or down-regulated in a process of interest. This strategy has been used to screen for genes that are differentially regulated during myoblast differentiation in vitro, such as lysosomal cysteine protease cathepsin B (Gogos et al. 1996), and genes that are either up- or down-regulated by germ-cell signaling in Sertoli cells (Vidal et al. 2001). Gene trapping screens have also been designed to identify genes encoding membrane and secreted proteins (Skarnes et al. 1995), genes displaying specific patterns of expression during development (Gossler et al. 1989; Friedrich and Soriano 1991), genes that control neuronal connections (Leighton et al. 2001), and genes expressed in cardiovascular lineages (Kuhnert and Stuhlmann 2004) or specific tissues, such as the hippocampus (Steel et al. 1998) and inner ear (Yang et al. 1997).

Thus, although the current emphasis is in the use of gene trapping as a tool for the large-scale generation and phenotypic analysis of mice carrying null mutations, it is important to recognize the versatility of gene trapping as a powerful in vitro and in vivo screening tool.

5 Targeted Trapping

A recent variation of the gene trapping approach has combined the directed approach of gene targeting through homologous recombination with the efficiency of mutating genes in ES cells through gene trapping (Friedel et al. 2005). This technique, dubbed targeted trapping, uses gene trap constructs for homologous recombination by flanking these cassettes with genomic sequences, thereby targeting them precisely to a chosen intron (Fig. 5). Standard homologous recombination approaches, as mentioned previously, are relatively inefficient at producing correctly targeted ES cells. The advantage of this approach is that the authors report surprisingly high frequencies of correctly targeted events provided the gene of interest is expressed at sufficient levels in ES cells (>50%). In addition, the authors define a threshold of expression in ES cells that appears to be necessary for successful gene trapping as well as targeted trapping. This suggests that although targeted trapping could dramatically reduce at least one of the hurdles associated with homologous recombination (targeting efficiency), it will likely only be useful within the subset of genes that have already been disrupted through large-scale gene trap efforts. As such, this approach will likely not be significant with respect to the Knockout Mouse Project. A limitation to this approach relative to traditional gene targeting is that the high targeting efficiency is apparently lost when a genomic deletion is engineered along with the insertion of the gene trapping components. Furthermore, this approach requires that the arms of homology not include elements of the promoter, which limits the ability for placement of the gene trap cassette near the first exon of many genes. It could prove useful, however, in creating additional allele variety for specific genes, particularly in cases where few or no desirable gene trap alleles are available. It may also be of use in combination with, and extension of, the most recent advances in conditional gene trap approaches allowing the development of a more tractable method for creating conditional alleles.

6 Concluding Remarks

Functional annotation of the mammalian genome is the next essential step in our effort to better understand human physiology and identify new treatments for human disease. The most widely accepted experimental approach

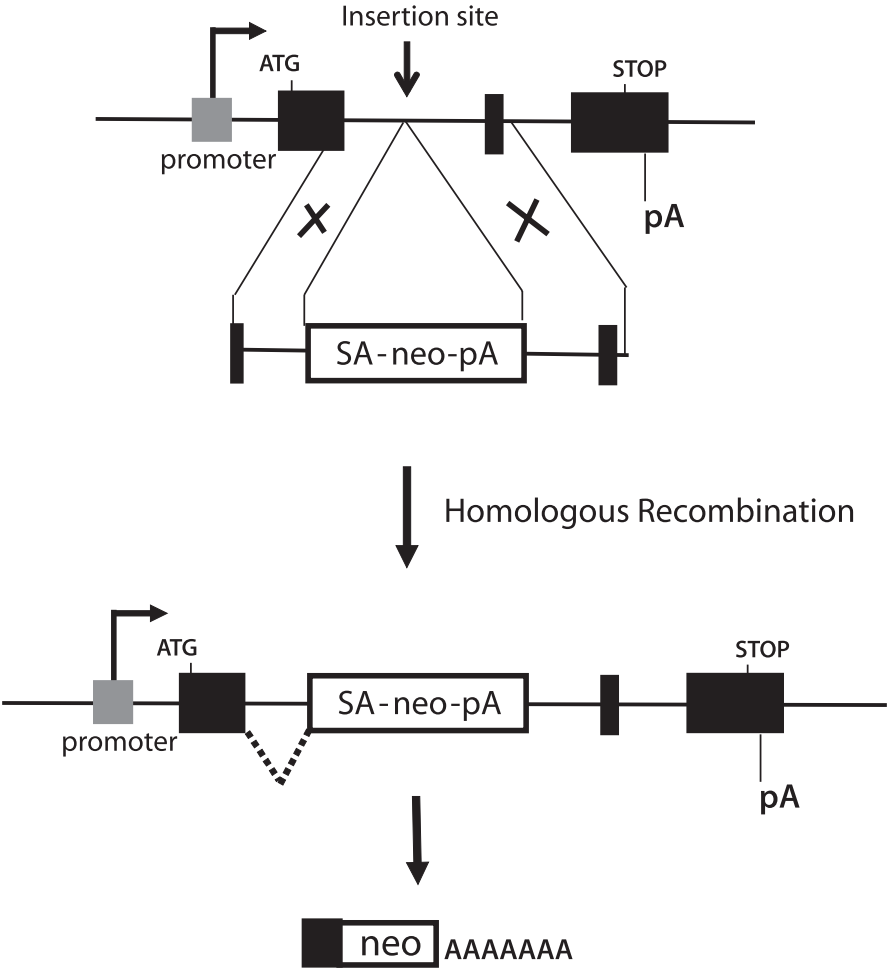

Fig. 5 Targeted gene trapping. A promoterless gene trapping construct containing a splice acceptor sequence (*SA*) followed by the *neo* gene and a polyadenylation signal (*pA*) is flanked by uninterrupted arms of homology to facilitate insertion into the first intron of a hypothetical target gene through homologous recombination. Correctly targeted insertion of the gene trapping cassette leads to expression of the *neo* mRNA fused to the upstream exons (*black boxes*) of the trapped gene

for functional annotation is the gene knockout in a whole mammalian model, the mouse. Gene trapping has gained prominence in recent years as the most efficient high-throughput approach for generating gene knockouts in ES cells, and sufficient data is now available to show that gene trapping vectors are effective at generating null alleles. Phenotypic annotation of the entire complement of mammalian genes will require a combination of multiple gene knockout techniques, and the most efficient combination of these will speed

our progress toward discovery of novel targets for pharmaceutical development and ultimately, disease treatment.

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RNA Interference in Mice

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Abstract Silencing of gene expression by RNA interference (RNAi) has become a powerful tool for functional genomics in mammalian cells. Furthermore, RNAi holds promise as a simple, fast and cost-effective approach to studying mammalian gene function in vivo and as a novel therapeutic approach. This review provides an overview of the progress of RNAi in vivo, with emphasis on systemic/local siRNA delivery, viral shRNA vectors, shRNA vector transgenic mice and conditional systems to control shRNA vectors. Taken together, the data from 80 in vivo studies show that RNAi is a useful tool that offers new opportunities for functional genomics in mice.

Keywords RNAi · Mouse · Functional genomics · Transgenic mice · siRNA

1 Introduction

Silencing of gene expression by RNA interference (RNAi) has become a powerful tool for functional genomics in mammalian cells. RNAi is a sequence-specific gene-silencing process that occurs at the messenger RNA (mRNA) level. In invertebrate cells, long double-stranded RNAs (dsRNA), which are processed into short interfering RNAs (siRNA) by the ribonuclease Dicer, induce efficient and specific gene silencing. In this sequence-guided process, the siRNA antisense strand serves as a template for the RNA-induced silencing complex (RISC). RISC recognises and cleaves the complementary mRNA,

which is then rapidly degraded. In mammalian cells, long dsRNAs (> 30 bp) elicit an interferon response resulting in the global inhibition of protein synthesis and nonspecific mRNA degradation. However, it has been shown that short synthetic dsRNAs (< 30 bp) trigger the specific knockdown of mRNAs in mammalian cells without interferon activation, if their length is below 30 bp (Elbashir et al. 2001). Such synthetic siRNAs can be easily introduced into cultured cells and induce a transient knockdown that makes it possible to study mammalian gene function within a short time frame. Due to advances in the delivery and design of siRNAs, gene silencing has now developed into a routine method for in vitro use. Large siRNA libraries can now perform genome-wide phenotype-driven screens (Sachse et al. 2005). Shortly after the establishment of siRNA-mediated transient gene silencing, DNA-based expression vectors were developed that allow the endogenous production of small dsRNAs in mammalian cells (Brummelkamp et al. 2002; Lee et al. 2002; Paddison et al. 2002). The vector-derived transcripts are designed to contain a sense and antisense region that is complementary to a selected mRNA segment. These transcripts can fold back into a stem-loop structure and form short hairpin RNAs (shRNAs) that are processed by Dicer. Since shRNA expression vectors can be stably integrated into the genome, they allow permanent, long-lasting gene silencing in cell lines and organisms.

Soon after these technologies were introduced for use in cultured cells, it became an obvious challenge to explore RNAi-mediated gene silencing in mice. The RNAi mechanism and the design, efficiency and delivery of siRNAs and shRNA vectors into mammalian cells has been covered by a number of review articles (Dykxhoorn et al. 2003; Mittal 2004; Hannon and Rossi 2004; Huppi et al. 2005). This review provides an overview of the technologies that have been tested for performance in vivo and the progress made towards long- or short-term gene silencing in mice. Oriented along the methodological aspects, this chapter is divided into four sections that cover the delivery of synthetic siRNAs, the use of viral shRNA vectors, shRNA vector transgenic mice and the development of conditional shRNA vectors. All of these technologies involve conditional gene silencing in mice since siRNAs and viral vectors are applied locally at a given time point and conditional shRNA vectors allow the construction of conditional knockdown mice. In the last section, the specific advantages and limitations of in vivo RNAi are discussed and compared to knockout mouse technologies.

2
Somatic RNAi

2.1
Systemic and Local siRNA Delivery

The direct administration of siRNA or shRNA vectors into adult mice or mouse embryos is a straightforward experimental approach that can be completed

within days to weeks. Although gene silencing in a specific organ occurs transiently and is usually not complete, direct siRNA delivery could become a routine method for experimental settings that require a short-term test of gene function in vivo. The experimental design represents a form of conditional mutagenesis that enables inducible, but not cell-type-specific gene silencing. In this section, the literature on systemic and local siRNA administration is discussed separately and summarised in Tables 1 and 2.

The systemic administration of siRNA into mice (Table 1) is performed by intravenous or intraperitoneal injection to elicit a widespread effect in multiple organs. In the frequently used hydrodynamic transfection procedure, 5–50 µg siRNA or shRNA plasmid are rapidly injected in a large volume into the tail vein. This protocol provokes the uptake of siRNA in up to 70% of liver cells and leads to a transient silencing effect that persists for several days. The results from several studies demonstrated that hydrodynamic transfection of siRNA can serve as a short-term assay to test hepatocyte physiology during viral infection and also confirmed that siRNA, in principle, has a potential as a therapeutic against viral replication. In the second strategy of systemic gene silencing, siRNAs are introduced, with or without transfection reagents, by standard intravenous or intraperitoneal injection (Table 1). Most of these studies investigated whether a selected gene is a suitable target to inhibit or slow down the growth and spread of transplanted tumour cells in vivo. As can be expected from the incomplete and transient gene silencing through siRNAs, these reagents alone are unable to reverse tumour growth but could be useful in combination with established chemotherapeutics. The siRNAs directed against tumour cells have also been delivered by local, intratumoural injection (Table 2). Although it is worth testing the efficiency of siRNA and transfection reagents that are optimised for in vitro conditions in mice, it is likely that these reagents do not perform optimally to induce RNAi in vivo. The work of Soutschek et al. (2004) describes optimised siRNAs that have been stabilised against nuclease degradation by a phosphothioate backbone and methyl sugar modifications. In addition, these molecules were conjugated with cholesterol to improve their pharmacokinetic properties. With these modified siRNAs, the authors efficiently silenced the expression of apolipoprotein B in liver and jejunum leading to reduced plasma cholesterol. These results demonstrated that siRNAs have a therapeutic potential to silence disease-causing genes and may be of particular value for targets that are not amenable to conventional drugs.

To induce local gene silencing, siRNAs or shRNA plasmids were delivered directly into the brain, tumours, embryos, lung, eye, testis, muscle, kidney or joints of mice and rats (Table 2). For local delivery, siRNAs or shRNA vectors are directly injected, with or without transfection reagents, into the tissue or introduced by local electroporation. With these methods, rates of gene silencing in the range of 20%–50% in brain and up to 90% in lung and testis were achieved. Most of these studies aim to validate local siRNA delivery

Table 1 RNAi in vivo: systemic siRNA administration

Target	Animal model	Doses/Administration	Results	Reference
Luciferase, SEAP, EGFP	Mouse (ICR; Tg-EGFP)	5–50 µg siRNA, hydrodynamic i.v. injection	90% Reduction of luciferase in liver, spleen, lung; 83% inhibition of SEAP in serum, reduced EGFP expression in hepatocytes	Lewis et al. 2002
Luciferase	Mouse	40 µg siRNA, hydrodynamic i.v. injection	Co-injection of luciferase reporter: 80% inhibition of luciferase expression	McCaffrey et al. 2002
β-catenin	Mouse tumour model	250 pmol siRNA with oligofectamine, 4× intraperitoneal	Prolonged survival in xenograft tumour model	Verma et al. 2003
FAK	Mouse tumour model	4 µg siRNA, 12× intravenous	Reduction of tumour growth in xenograft model	Duxbury et al. 2003
VEGF	Mouse tumour model	3 µg siRNA, 16× intraperitoneal	66% Reduction of tumour growth by day 16	Filleur et al. 2003
TNFα	Mouse sepsis model	siRNA with transfection reagent, 1× intraperitoneal	Inhibition of sepsis upon lipopolysaccharide injection	Sorensen et al. 2003
HBV RNA	HBV mouse model	5 µg shRNA plasmid, hydrodynamic i.v. injection	85% Reduction of viral surface antigen in serum, 99% reduction of viral core antigen in hepatocytes	McCaffrey et al. 2003
Fas receptor	Mouse Hepatitis model	50 µg siRNA, hydrodynamic i.v. injection	Reduction of Fas in hepatocytes for 10 days, survival of Fas-siRNA-treated mice for 10 days in hepatitis model	Song et al. 2003
HBV	HBV mouse model	5–25 µg siRNA, hydrodynamic i.v. injection	Co-injection of HBV plasmid: significant inhibition of viral transcripts, antigens and DNA in liver and serum	Giladi et al. 2003

Table 1 (continued)

Target	Animal model	Doses/Administration	Results	Reference
Caspase 8, LacZ	Mouse (Balb/c; Tg-lacZ)	0.6 nmol siRNA/g, hydrodynamic i.v. injection	Tg lacZ-mice: three- to fourfold reduction of β-galactosidase, 70% of hepatocytes show siRNA intake; siRNA-caspase 8 prevents Fas-mediated apoptosis of hepatocytes	Zender et al. 2003
Luciferase	Mouse (Balb/c)	10 µg siRNA, hydrodynamic i.v. injection	Co-injection of luciferase reported: 85% reduction of luciferase, no influence of 2'-F siRNA-modification on magnitude or duration of response	Layzer et al. 2004
Fas	Mouse	50 µg siRNA, hydrodynamic i.v. injection	Fourfold reduction of Fas in kidney, reduced apoptosis, increased survival in a renal ischemia-reperfusion model	Hamar et al. 2004
RRM2	Mouse tumour model	3 µg siRNA, 12× intravenous	Suppression of tumoural RRM2 expression in model of pancreatic adenocarcinoma	Duxbury et al. 2004
NF-κB p65	Mouse tumour model	250 pmol siRNA twice a week, multiple doses intravenous with CPT11	Significant reduction of tumour growth in combination with CPT11 (irinotecan)	Guo et al. 2004
Influenza virus	Mouse (C57BL/6)	60 µg siRNA, 1× intravenous together with polyethylenimine	siRNAs specific for influenza virus reduce virus production in lungs of infected mice	Ge et al. 2004
ApoB	Mouse (C57BL/6)	50 mg/kg cholesterol conjugated, modified siRNA, 3×intravenous	Decrease in ApoB in liver and plasma, cholesterol reduction	Soutschek et al. 2004
Caspase 8 Fas	Mouse sepsis model	50 µg siRNA, hydrodynamic i.v. injection	Reduced caspase 8 and Fas mRNA and protein; decreased apoptosis and liver damage; improved survival in sepsis model	Welsche-Soldato et al. 2005

Table 1 (continued)

Target	Animal model	Doses/Administration	Results	Reference
PI3kinase p110	Mouse	25 µg siRNA with atelocollagen, intravenous injection	Inhibition of metastatic tumour growth in bone tissues	Takeshita et al. 2005
HBV RNA	HBV mouse model	3 mg/kg/day modified siRNA in liposomes, intravenous injection	Tenfold reduction of viral DNA in serum	Morrissey et al. 2005
HIV-1 gag c-myc	Mouse	Intravenous injection of protamine-HIV antibody fusion protein	Specific targeting of siRNAs to HIV envelope expressing tumour cells	Song et al. 2005
Coxsackievirus 2A protease	Mouse (IFNR-/-)	0.6 nMol/g siRNA, 2×hydrodynamic i.v. injection	Reduction of viral titers, attenuated tissue damage and prolonged survival	Merl et al. 2005
Vasopressin V2 receptor	Mice (C57BL/6)	50 µg siRNA with transfection reagent, 1× intravenous injection	40% Reduction of V2 receptor in kidney	Hassan et al. 2005
ErbB2	Mouse	siRNA complexed with polyenthylenimine, intraperitoneal injection	Reduction of tumour growth	Urban-Klein et al. 2005

EGFP, enhanced green fluorescent protein; FAK, focal adhesion kinase; HBV, hepatitis B virus; i.v., intravenous; NF-κB, nuclear factor-kB; RRM2, M2 subunit of ribonucleotide reductase; SEAP, secreted human placental alkaline phosphatase; Tg, transgenic; VEGF, vascular endothelial growth factor

Table 2 RNAi in vivo: local siRNA administration

Target	Animal model	Doses/Administration	Results	Reference
AGRP	Mice (C57BL/6)	7 µg siRNA or shRNA plasmid, 1×injection into hypothalamus	50% Reduction of mRNA, increased metabolic rate, reduced body weight	Makimura et al. 2002
GFP LacZ	Mice (Tg-EGFP)	50–250 µg esiRNA, electroporation into neural tube of day-10 embryos	90% Reduction of reporter gene expression in neuroepithelial cells	Calegari et al. 2002
VEGF EGFP	Mouse (C57BL/6)	20 pMol siRNA with transfection reagent, 1× subretinal	Reduction of EGFP in eyes, inhibition of hypoxia-induced VEGF levels in eyes	Reich et al. 2003
DCX	Rat	1.5–4.5 µg shRNA plasmid, embryo electroporation in utero	Disruption of neocortical development	Bai et al. 2003
P2X3	Rat	400 µg siRNA/day, intrathecal infusion for 7 days	Reduction of mRNA in dorsal root ganglia and protein in spinal cord, inhibition of neuropathic pain response	Dorn et al. 2004
HO-1	Mouse (C57BL/6)	50 µg siRNA, 1× intranasal	Inhibition of protein expression, enhanced I-R-induced lung apoptosis	Zhang et al. 2004
VEGF	Primate	70–350 µg siRNA, 1× Intravitreal	Inhibition in neovascular area	Tolentino et al. 2004
CSF-1, CSF-1 receptor	Mouse (Balb/c-nu/nu)	10 µg siRNA, 5× intratumoural	Reduction of mRNA and protein, reduction of tumour growth	Aharinejad et al. 2004
Luciferase FGF-4	Mouse (Balb/c nu/nu)	2.5 µg siRNA with atelocollagen, 1× intratumoural, intratesticular	70% Reduction of tumour growth, 50% reduction of FGF4 with FGF-4 siRNAs	Minakuchi et al. 2004
VEGF	Mouse (nu/nu)	1–10 µM siRNA with atelocollagen, 4× intratumoural	Reduction of VEGF in tumour, tumour volume and vessel density	Takei et al. 2004
TGFβ receptor II	Mouse ocular inflammation model	200 nMol siRNA with transfection reagent, 1× subconjunctival	Reduction of inflammatory response	Nakamura et al. 2004
S1P1	Mouse xenograft tumour model	50 µg esiRNA with liposomes, intratumoural injection	Suppression of angiogenesis and tumour growth	Chae et al. 2004

Table 2 (continued)

Target	Animal model	Doses/Administration	Results	Reference
EGFP Dopamine transporter	Mouse (Balb/c)	400 µg siRNA/day, intraventricular infusion for 1–2 weeks	10%–50% EGFP reduction in various brain regions, 50% reduction of DAT protein in dopaminergic projection areas, hyperlocomotor response	Thakker et al. 2004
Serotonin transporter	Mouse	400 µg siRNA/day, intraventricular infusion for 2 weeks	40% Reduced SERT mRNA in raphe nuclei, antidepressant-related response in the forced swim test	Thakker et al. 2005
Huntingtin	Mouse Huntington's disease model	0.2 µg siRNA with transfection reagent, intraventricular into newborns	Decrease of intranuclear inclusions in striatum, improved longevity and motor function. slowed down weight loss	Wang et al. 2005
TNFα	Mouse arthritis model	Electroporation into joint tissue	Inhibition of joint inflammation	Schiffelers et al. 2005
RSV RNA PIV RNA	Mouse (Balb/c)	70 µg siRNA with transfection reagent, intranasal application	Reduction of pulmonary virus titers by 99%	Bitko et al. 2005
EGFP	Mouse (Tg-EGFP)	2 µg siRNA, 1× intramuscular electroporation	Reduced EGFP expression in muscle	Golzio et al. 2005
EGFP TGF-β1	Rat	50 µg siRNA, 200 µg shRNA plasmid, electroporation into kidney	Suppression of EGFP and TGF-β1 in glomerular mesangial cells	Takabatake et al. 2005
EGFP Dmc1	Mouse (ICR)	1–4 µg shRNA plasmid, 1× intratesticular electroporation	Disrupted development of spermatocytes as seen in Dmc1 knockout mice	Shoji et al. 2005

AGRP, agouti-related peptide; CSF, colony-stimulating factor; DCX, Doublecortin; EGFP, enhanced green fluorescent protein; esiRNA, enzymatically prepared siRNA; FGF, fibroblast growth factor; HO-1, heme oxygenase-1; I-R, ischemia reperfusion; PIV, parainfluenza virus; RSV, respiratory syncytial virus; S1P1, sphingosine1-phosphate receptor-1; Tg, transgenic; TGF, transforming growth factor; VEGF, vascular endothelial growth factor

as a therapeutic approach against tumour growth, inflammation and viral replication or use local RNAi as a rapid tool for target validation. Although the local delivery methods lead only to transient and incomplete gene silencing, all studies report significant biological effects and provide a first confirmation of a putative gene function. Therefore, the simplicity and speed of this approach may well compensate its limitations for specific applications. As compared to systemic RNAi, local siRNA delivery targets many organs and tissues in adult mice or rats and greatly extends the utility of transient RNAi in vivo.

Since the reagents and the biological readout of the studies on systemic or local siRNA administration are diverse and barely comparable, it is presently difficult to judge which of these protocols is most efficient and has a potential to become a routine method. Future and more systematic studies are desirable to clarify the value of diverse siRNA formulations and to explore the potential for improvements in siRNA delivery to somatic tissues.

2.2 Viral shRNA Vectors

Three types of viral delivery systems based on adeno-associated virus, lentiviral and adenoviral vectors have been used to introduce shRNA expression units into somatic tissues of mice or rats (Table 3). As compared to the methods of somatic siRNA delivery discussed in the previous section, viral vectors allow the persistent silencing of target genes in cells that are transduced and express the viral shRNA vector. While lentiviral vectors integrate into the genome of infected cells, adeno-associated virus and adenoviral vectors persist episomally. Each of these vector systems can be applied to organs that are largely composed of nondividing cells such as the adult brain. Lentiviral vectors are preferentially used for the transduction of cycling haematopoietic or cancer cells. In addition, lentiviral shRNA vectors generate germline transgenic animals by zygote infection (Table 4). Local virus delivery into the brain has been accomplished by intracerebral injection, virus delivery into the liver by intravenous injection and to haematopoietic and tumour cells by ex vivo infection, followed by cell transfer. As compared to the direct injection of siRNA (Sect. 2.1), the experimental work with viral vectors in vivo requires more time (weeks or months) and more preparation. An efficiently working shRNA vector must be identified, viral supernatants have to be produced and the viral particles must be concentrated to obtain a high titer preparation. For reasons of biological safety, lentiviral and adenoviral vectors must be handled in special facilities. These requirements will probably restrict the use of viral shRNA vectors to specialised laboratories and to research areas for which they offer unique advantages. These are, as obvious from Table 3, hematopoietic stem cell, cancer and brain research. In particular, viral shRNA vectors make it possible to study the meliorating effects of silencing genes in-

volved in neurodegenerative diseases (Xia et al. 2004; Harper et al. 2005; Singer et al. 2005) and provide a new model of RNAi-based gene silencing therapy.

3 shRNA Vector Transgenic Mice

Shortly after the routine use of shRNA vectors in cell lines, the first vector transgenic mice were described by Hasuwa et al. (2002). The number of reports on RNAi in transgenic mice has increased by now to 16 (Table 4), representing all standard methods to generate transgenics. The shRNA transgenic mice were produced by pronuclear DNA injection, the infection of zygotes or ES cells with lentiviral vectors, by random integration into ES cells and targeted transgenesis (knock-in) of single vector copies into ES cells through recombinase-mediated cassette exchange or homologous recombination. The majority of these studies employ constitutively active shRNA expression vectors based on the U6 or H1 promoter; three groups express long double-stranded RNA from RNA polymerase II-driven promoters (CMV, Zp3). Conditional vectors controlled by the Cre/loxP recombination system have been applied in four reports (Table 4). As determined by the scientific focus germ cells, embryonic stages, young or adult mice were analysed for gene silencing efficiency and phenotypes, either in a single or in multiple organs. The targets for RNAi in transgenic mice include reporter genes but in most cases endogenous genes were silenced. The phenotypes of knockdown and the corresponding knockout mice were compared in several instances and found to be identical or very similar (Table 3). The efficiency of gene silencing in transgenic mice reaches knockdown levels in the range of 90% or higher in most, but not all cases.

In general, it can be concluded that vector-based transgenic RNAi provides a tool to achieve efficient gene silencing during embryonic development as well as in organs of adult mice. As compared to the delivery of siRNA or viral vectors to somatic tissues, for which the uptake or infection rate is a critical issue, transgenic animals harbour the shRNA expression vector in all cells and provide a more defined experimental setup. However, if transgenic mice are generated by pronuclear injection, random integration into ES cells or viral infection a variable number of vector copies integrates into unknown chromosomal locations that could interfere with vector expression. From studies with mice transgenic for RNA polymerase II-driven promoters, it is well known that transgene expression can be highly variable among individual lines such that it is necessary to raise several independent lines for each construct and to characterise transgene expression in each line. In case of promoters that are strongly influenced by the chromosomal surrounding, random integration becomes a laborious and time-consuming approach. From the published reports on U6 and H1 promoter transgenic mice (Table 4), it is not clear to which extent these regulatory regions are influenced by the integration site. Most studies include only one transgenic strain and do not

Table 3 In vivo application of viral shRNA expression vectors

Viral vector	Target	Promotor	Animal model	Administration	Results	Reference
AAV	Tyrosine hydroxylase	U6	Mouse	Injection into substantia nigra	Persistent TH knockdown, behavioural defects, reduced response to psychostimulant	Hommel et al. 2003
AAV	Ataxin-1	H1	SCA1 mouse	Intracerebellar injection	Reduction of ataxin-1 expression, improved motor coordination, restored cerebellar morphology	Xia et al. 2004
AAV	CaMKIIα	U6	Rat	Infusion into hippocampus	Reduced expression of CamKIIα, impaired place learning	Babcock et al. 2005
AAV	EGFP	H1	Rat	Intravitreal injection	Down-regulation of EGFP in retinal ganglion cells	Michel et al. 2005
AAV	Htt mutant	U6	HD mouse model	Injection into striatum	Reduced Htt expression in brain, improved behavioural and neuropathological abnormalities associated with HD	Harper et al. 2005
LV	GM-CSF receptor	H1	Mouse (NOD/SCID)	Transfer of transduced $CD34^+$ cells	Inhibition of GM-CSF receptor function	Scherr et al. 2003
LV	EGFP	U6	Mouse (C57BL/6)	Injection into striatum	Suppression of EGFP expression	van den Haute et al. 2003
LV	CD8	U6	Mouse	Transfer of transduced haematopoietic stem cells	90% Reduction of CD8 expression in T lymphocytes	Rubinson et al. 2003
LV	p53	H1	Mouse (RAG2-/ -IL-2Rγc-/-)	Intraperitoneal injection of transduced $CD34^+$ cells	Inactivation of p53 in mature T cells developed from transduced cells, resistance to p53-dependent apoptotic stimuli	Gimeno et al. 2004
LV	Braf, Braf (V599E)	U6	NOD/SCID mouse	Subcutaneous implantation of transduced melanoma cells	Growth inhibition of melanoma cell lines, decrease of Braf protein	Sumimoto et al. 2004
LV	SOD1 (human)	H1	SOD1-G93A mouse	Intramuscular injection	Reduced SOD1 expression, improved survival of motor neurons, improved motor performance, delayed onset of ALS symptoms	Ralph et al. 2005

Table 3 (continued)

Viral vector	Target	Promotor	Animal model	Administration	Results	Reference
LV	SOD1 (human)	H1	SOD1-G93A mouse	Intraspinal injection	Retardation of onset and progression rate of ALS	Raoul et al. 2005
LV	STAT3	H1	Mouse (Balb/c)	Transfer of transduced breast cancer cells into fat pad	Blockage of tumour formation	Ling et al. 2005
LV	BACE1	H1	AD mouse model	Intracerebral injection into hippocampus	Reduced amyloid production and neurodegenerative and behavioural deficits in AD mice	Singer et al. 2005
LV	CD81	U6	Rat	Injection into tegmentum and nucleus accumbens	Down-regulation of CD81, suppression of cocaine-induced behavioural changes	Bahi et al. 2005
Ad	Met	U6	Mouse (Balb/c)	Intratumoural injection	Reduction of tumour growth	Shinomiya et al. 2004
Ad	EGFP laminA/C annexin AII	human SP-C	Rat	Endotracheal administration	Reduced expression of EGFP, lamin A/C and annexin in alveolar type II cells	Gou et al. 2004
Ad	Skp-2	U6	Mouse (NOD/SCID)	Intratumoural injection	Inhibition of growth of subcutaneous tumour	Sumimoto et al. 2005
Ad	IRS-1, IRS-2	U6	Mouse (C57BL/6)	Intravenous injection	Reduced IRS expression in liver, influence on hepatic metabolism	Taniguchi et al. 2005
Ad	HBV	U6	Mouse (HBV transgenic)	Intravenous injection	Suppression of HBV gene expression and replication	Uprichard et al. 2005
MCSV	P53	U6	Mouse (Tg-Eμ-myc)	Transfer of transduced haematopoietic stem cells	Development of lymphoid hyperplasia and lymphomas	Hemann et al. 2003

AAV, adeno-associated virus; Ad, adenovirus; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BACE, beta secretase; EGFP, enhanced green fluorescent protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBV, hepatitis B virus; HD, Huntington's disease; Htt, huntingtin; IRS, insulin receptor substrate; LV, lentiviral vector; MCSV, murine stem cell virus; SCA1, spinocerebellar ataxia type 1; SOD1, superoxide dismutase; SP-C, surfactant protein C promotor; STAT3, signal transducer and activator of transcription 3

Table 4 shRNA vector transgenic mice

Transgenesis	Target	Promotor	Animal model	Results	Reference
Pronucleus injection	EGFP	H1	Mouse, rat (Tg-EGFP)	80%–90% Reduction of EGFP in embryos, newborns and adult tissues	Hasuwa et al. 2002
ES cells: random integration	Neil1	hU6	Mouse, adult	80% Reduction of Neil1 in ES cells and liver	Carmell et al. 2003
ES cells: random integration	RasGAP	H1	Mouse, ES cell tetraploid embryos (E9–10)	Strong reduction of RasGAP protein in embryos, phenocopy of the RasGAP knockout phenotype	Kunath et al. 2003
Pronucleus injection	Ski	CMV, long dsRNA	Mouse, embryos (E9–12)	Partial phenocopy of the Ski knockout phenotype	Shinagawa et al. 2003
Zygote: lentiviral infection	CD8 p53	mU6	Mouse, adult	~90% Reduction of CD8 in thymus/spleen and p53 in brain and liver of adult mice	Rubinson et al. 2003
Pronucleus injection	Mos	Zp3, long dsRNA	Mouse, oocytes	90% Reduction of mos mRNA, parthenogenetive activation of metaphase eggs as in mos knockout mice	Stein et al. 2003
Zygote: lentiviral infection	GFP	H1	Mouse, embryos (E13), newborns	Reduction of EGFP protein in E13 embryos and newborns	Tiscornia et al. 2003
Pronucleus injection	ABCA1	mU6 loxP conditional	Mouse adult	Inducible down-regulation of ABCA1 in liver, cholesterol accumulation in liver	Chang et al. 2004
Pronucleus injection	CTCF	Zp3, long dsRNA	Mouse, oocytes	60%–99% Reduction of CTCF, depending on Tg line, increased H19 methylation	Fedoriw et al. 2004
ES cells, lentiviral infection	CD8	mU6 loxP conditional	Mouse, adult	50% Reduction of splenic $CD8^+$ cells upon Cre-mediated recombination	Ventura et al. 2004

Table 4 (continued)

Transgenesis	Target	Promotor	Animal model	Results	Reference
ES cells: random integration	Fgfr2	mU6 loxP conditional	Mouse, embryo (E11–13)	95% Reduction of Fgfr2 mRNA, embryonic lethality	Coumoul et al. 2005
Pronucleus injection	PLCζ	mU6	Mouse, gametes (E11–13)	40% Reduction of PLCζ in sperm, defect in egg activation	Knott et al. 2005
ES cells: random integration	Grsf1 Fragilis2	H1	Mouse, ES cell tetraploid embryos (E9–10)	Strong reduction of Grsf1 and Fragilis2 mRNA, developmental defects	Lickert et al. 2005
ES cells: integration into HPRT locus	Bfl-1/A1	mU6 loxP conditional	Mouse, adult	90% A1 reduction in thymocytes, ~ 50% reduction in mature B and T lymphocytes	Oberdoerffer et al. 2005
ES cells: integration into Rosa26 locus	Luciferase β-Galactosidase; leptin receptor	H1/hU6	Mouse, adult	40% B 95% silencing efficiency, depending on organ	Seibler et al. 2005
ES cells: random integration into Rosa26 locus	Baf60c	H1	Mouse, ES cell tetraploid embryos (E9–10)	>90% Reduction of Baf60c protein, defects in heart morphogenesis	Lickert et al. 2004

ABCA1, ATP binding cassette subfamily A member 1; GFP, green fluorescent protein; hU6, human U6; mU6, murine U6

comment on the variation among individual lines. In the case of random vector integration into ES cells and ES cell expression of the target gene, it is possible to preselect ES cell clones that exhibit efficient gene silencing. The successful use of this strategy in combination with tetraploid embryo complementation (Kunath et al. 2003; Lickert et al. 2004, 2005) shows that shRNA expression can be maintained in such integration sites at least up to day 10 of embryonic development. The potential problems associated with random vector integrations can be avoided by the targeted insertion of a single vector copy into a predetermined genomic locus. However, the caveat of this approach is whether a single vector copy within a given locus leads to shRNA levels that are sufficient to elicit gene silencing in diverse tissues of mice. Oberdoerffer et al. (2005) inserted a conditional shRNA vector (see Sect. 4) into the widely expressed, X-linked HPRT locus by homologous recombination. Upon Cre-mediated activation of this vector, the authors observed an efficient knockdown of the target genes in thymocytes but not in mature lymphocytes, despite the presence of comparable shRNA levels in both cell types. It is not clear, however, whether a higher copy number of this vector could improve gene silencing in lymphocytes or whether these cells are intrinsically insensitive to RNAi. In a similar approach by Seibler et al. (2005), shRNA vectors with specificity to luciferase, β-galactosidase and leptin receptor were inserted into the ubiquitously expressed Rosa26 locus by site-specific recombination. While in most of the analysed organs of adult mice, the knockdown reached 80%–90%, gene silencing was less efficient (∼50%) in spleen and testis. A comparison of the silencing efficiencies achieved by the H1 or human U6 promoter within Rosa26 showed that both promoters exhibit similar activity.

In contrast to the frequent use of the U6 and H1 promoters for shRNA expression, Shinagawa et al. (2003) developed a CMV promoter-based expression system for long double-stranded (ds) RNA which lack the 5′ Cap structure and the poly(A) tail that are typical for RNA polymerase II transcripts. The authors suggest that this dsRNA is not transported to the cytosol and is unable to induce an interferon response. In a similar approach the oocyte specific Zp3 promoter has been used to transcribe long dsRNA in female germ cells that are apparently unable to undergo an interferon response (Stein et al. 2003; Fedoriw et al. 2004).

Taken together, the reports on RNAi vector transgenic mice represent a collection of diverse, mostly single gene validation experiments. It is presently hard to foresee which of these techniques has potential as a routine procedure to investigate the biological function of new genes in mice. It must be further considered that a consistent phenotype description of knockdown mice requires the analysis of two strains expressing different shRNAs directed to the same gene (e.g. see Lickert et al. 2004). Data derived from a single strain could be regarded as preliminary since potential off target effects of an individual shRNA cannot be ruled out. Due to this requirement it is likely that the only methods that allow the rapid and reproducible production of RNAi-expressing

mice, along the lines of Kunath et al. (2003), Oberdoerffer et al. (2005) or Seibler et al. (2005), will be repeatedly used in future.

4
Conditional shRNA Vectors

4.1
Cre/lox Controlled Vectors

The commonly used U6/H1 promoter-based shRNA vectors are ubiquitously active and do not allow further control of the level or timing of gene silencing. To enable the controlled production of shRNAs, conditional expression vectors were developed that offer a similar analytical refinement as conditional knockout mice or mice with doxycycline-regulated transgenes. Thus, conditional shRNA vectors should restrict gene silencing in mice to a specific cell type or regulate shRNA expression in response to a small molecule inducer. To gain control on their transcriptional activity, the U6 or H1 promoter regions were modified in two ways, utilising either the Cre/loxP recombination system or the reversible binding property of Tet repressor to the Tet operator DNA sequence. To control shRNA production via the Cre/loxP system, the expression vector must be initially disrupted through insertion of a loxP flanked DNA segment that interferes with promoter activity or leads to a truncated, nonfunctional transcript (Fig. 1B). Upon removal of the stop segment through Cre-mediated deletion (Fig. 1C), a single loxP sequence remains in the expression unit, the presence of which should not interfere with shRNA production. To solve this task, stop segments were inserted into shRNA expression vectors at five different positions, either within the promoter region or as part of the transcribed region (Fig. 1A) (Table 5). As stop elements, reporter or resistance genes 1–2 kb long were used that function by their size and by the presence of thymidine repeats, the termination signal for RNA polymerase III. The insertion of a loxP flanked stop segment into the loop of the shRNA separates the antisense from the sense region of the vector (Kasim et al. 2004; Fritsch et al. 2004). While the latter sequence is still transcribed in the nonfunctional configuration the antisense strand, which elicits RNAi, is excluded from the transcript such that functional hairpins are not produced. Upon Cre-mediated deletion of the stop segment, a single loxP sequence of 34 nucleotides remains within the vector and becomes part of the shRNA loop region. Although loop regions of 3–9 residues are commonly used, the presence of a considerably enlarged loop did not affect the potency of conditional shRNAs (Kasim et al. 2004; Fritsch et al. 2004). In the second approach, a loxP flanked stop segment was inserted between the start of transcription and the shRNA sense/antisense region (Chang et al. 2004; the exact configuration is not described). The removal of the stop leads to the production of an elongated shRNA that includes 34

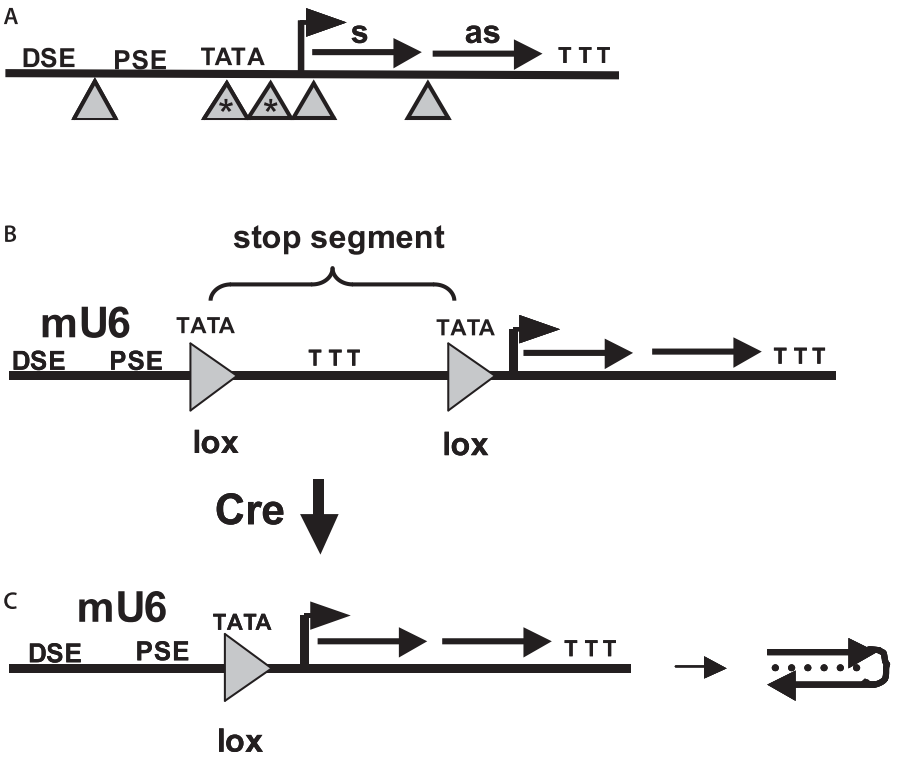

Fig. 1 A–C Cre/loxP-mediated control of shRNA expression vectors. **A** Scheme of U6 or H1 promoter-driven shRNA constructs showing essential regulatory elements (*DSE, PSE, TATA*), the transcriptional starting point (*arrowhead*), shRNA sense (*s*) and antisense (*as*) strands and the termination signal (*TTT*). The positions at which loxP (*triangle*) or modified lox sites (*triangles with asterisks*) were inserted are indicated. **B** An example of a conditional shRNA vector in the inactive configuration. The mouse U6 promoter (*mU6*) is separated from the shRNA region by the insertion of a stop segment flanked by modified lox sites that include a TATA sequence. The TATA box as such is insufficient for transcription of shRNA. **C** The vector depicted in **B** upon Cre-mediated deletion of the lox flanked DNA segment. The shRNA region can be transcribed from the reconstituted U6 promoter

additional nucleotides upstream of the sense sequence. In the tested case, this addition did not interfere with shRNA processing or gene silencing in cell lines and transgenic mice.

Besides placement into the shRNA region, lox sites have been inserted at three different positions into the U6 promoter such that the transcribed region remains undisturbed (Fig. 1A). In the strategy described by Tiscornia et al. (2004) and Ventura et al. (2004), a modified lox site replaces 34 bp of the mouse U6 promoter including the essential TATA box. Due to the sequence modification, the lox site provides a surrogate TATA element that leads, together with the upstream DSE and PSE elements, to a level of transcription that is com-

Table 5 Conditional shRNA vectors

Control element	Promoter	Insertion site	Target	Integration	Cell line/species	Reference
Cre/loxP	U6	Loop	Luciferase	Transient	HeLa	Kasim et al. 2004
Cre/loxP	H1	Loop	GFP p53	Transient	HeLa, U20S, mouse muscle	Fritsch et al. 2004
Cre/loxP	mU6	U6/shRNA	ABCA1	Random integration	HepG2, Tg mice	Chang et al. 2004
Cre/loxP	mU6	DSE/PSE	EGFP, Fgfr2 survivin	Transient/ random integration	WO69, ES cells, Tg mice	Coulmoul et al. 2004, 2005
Cre/lox* (35%)	mU6	TATA	GFP p53, NFκB	Lentiviral transduction	293 MEF	Tiscornia et al. 2004
Cre/lox* (35%)	mU6	TATA	p53, Npm, Dnmt1, CD8	Lentiviral transduction	MEF, ES cells, Tg mice	Ventura et al. 2004
Cre/lox*	mU6	TATA/shRNA	Bfl-1/A1	Integration into HPRT locus	ES cells, Tg mice	Oberdoerffer et al. 2005
TetR/tetO	hU6/7SK	TATA/shRNA	PI3Kinasep110	Random integration	PC3, mouse	Czauderna et al. 2003
TetR/tetO	H1	TATA/shRNA	βcatenin myc	Random integration	CRC cells, HEK	van de Wetering et al. 2003
TetR/tetO	hU6	DSE/TATA	Dnmt1	Random integration	HCT116	Matsukura et al. 2003
TetR/tetO	H1	TATA/shRNA	P300	Adenoviral infection	HeLa	Kuninger et al. 2004
tTR-KRAB/tetO	H1	5'H1	GFP P53 Lamin	Lentiviral transduction	HeLa, MCF7	Wiznerowicz et al. 2003
tTS/tetO	hU6	1–9 tetO copies at various positions	CXCR4	Random integration	Breast cancer cells	Chen et al. 2003
rtTA/TRE	CMVmin	–	Glutaredoxin	Random integration	NIH3T3	Wang et al. 2003
Gal4-Oct2/Gal4	U6	DSE	P53 MyoD	Retroviral transduction	U87MG mHEVc	Gupta et al. 2004

ABCA1, ATP binding cassette subfamilyA member 1; CXCR4, CXC chemokine receptor-4; DSE, distal sequence element; ES, embryonic stem cells; GFP, green fluorescent protein; hU6, human U6; lox*,modified loxP site; MEF, mouse embryonic fibroblasts; mU6, murine U6; PSE, proximal sequence element; TATA, TATA box; tetO, tet operator; TetR, Tet repressor; Tg, transgenic

parable to the activity of the wild-type U6 promoter (Fig. 1C). The insertion of a lox-flanked stop segment at this position separates the TATA/shRNA region from the essential DSE/PSE elements and abrogates shRNA production since the TATA box alone is insufficient to initiate transcription (Fig. 1B). The inactive vector configuration is reversed upon Cre-mediated deletion of the stop segment (Fig. 1C). Although the modified lox sites are known to be less efficiently recombined (∼35%) as compared to wild-type loxP sites, Ventura et al. (2004) observed complete recombination of such a vector in Cre transgenic mice. In a variation of this strategy, a truncated loxP sequence was placed immediately downstream of the TATA box such that their sequences overlap within the ATA triplett (Fig. 1A) (Oberdoerffer et al. 2005). Since the transcriptional start of the mouse U6 promoter is located 26 nucleotides downstream of TATA, the last five residues of loxP were replaced by shRNA sequence. In combination with a second, full-length loxP site placed at the other side of the stop segment, the authors observed efficient recombination of this vector in vitro and in vivo. In the configuration described by Coulmoul et al. (2004, 2005), a loxP-flanked stop segment was placed between the upstream regulatory elements of the U6 promoter such that the DSE element is separated from the PSE/TATA/shRNA region (Fig. 1A). Due to the integrity of the latter region, residual transcription may be possible even in the intended off state of such a vector. This was ruled out by the authors, who found no background transcription and efficient gene silencing in vitro and in vivo upon Cre-mediated activation of the conditional vector.

Four of the Cre/lox-controlled vector types described above have been tested in vivo (Table 5) by crossing the conditional shRNA mice to cell-type-specific or germline deleter Cre strains. These results show that recombinase-mediated, conditional RNAi is feasible in the tested embryonic and adult mouse tissues (liver, limb, thymocytes). In mature lymphocytes, however, gene silencing is inefficient despite the continuous expression of shRNA (Oberdoerffer et al. 2005).

4.2 Tet-Operator-Controlled Vectors

Besides Cre/lox, the second regulatory system to control shRNA vectors is based on transcriptional repressor or activator proteins that are regulated by small molecule inducers. These vectors have been tested in cultured cell lines but not yet in vivo; since this could be a future application, these vectors are briefly described below and included in Table 5. In the negative regulatory approach, Tet repressor (TetR) protein binds to the Tet operator (TetO) DNA that is inserted into the promoter region used for shRNA expression. In most cases, TetO has been placed between the TATA box and the start of transcription (Czauderna et al. 2003; van de Wetering et al. 2003; Matsukura et al. 2003; Kuninger et al. 2004). The binding of TetR at this position suppresses, presumably by physical hindrance, transcriptional initiation at the modified U6 or H1 promoter (Fig. 2). In the presence of doxycycline, TetR is released from TetO

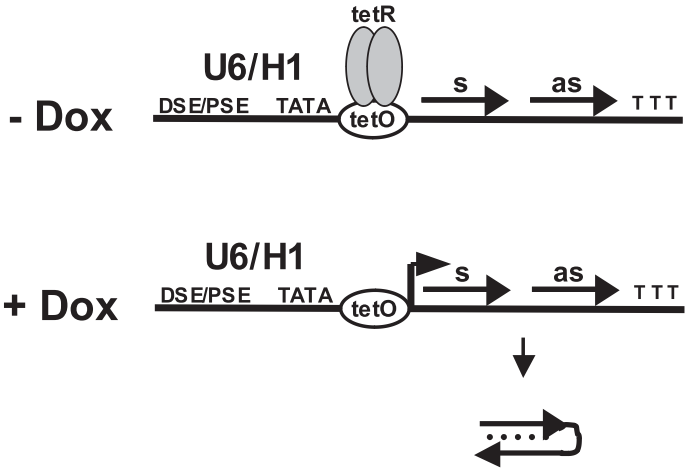

Fig. 2 Tet Repressor-mediated control of shRNA expression vectors. Scheme of Tet Repressor controlled U6 or H1 promoter driven shRNA constructs showing essential regulatory elements (*DSE*, *PSE* , *TATA*), shRNA sense (*s*) and antisense (*as*) strands and the termination signal (*TTT*). In the absence of doxycycline (*Dox*), the Tet operator sequence (*tetO*), placed between *TATA* box and the start of transcription, is occupied by a Tet repressor dimer (*tetR*) that interferes with transcription. In the presence of doxycycline, Tet repressor is released from its binding site and restores the production of shRNA

such that the downstream shRNA region can be transcribed in the activated status of the vector (Fig. 2). In a variation of this principle, TetR is fused with a silencer protein domain that acts as negative enhancer on RNA polymerase III-driven promoter regions (Wiznerowicz et al. 2003; Chen et al. 2003). This strategy of active promoter silencing increases the flexibility towards the positioning of TetO within the promoter region. To maintain the off status of TetR controlled vectors, it is necessary to introduce a second transgene that maintains a continuous high level expression of repressor protein.

In positively controlled, inducible shRNA vectors, truncated CMV or U6 promoter regions are activated through binding of a transcriptional activator protein that is fused to a specific DNA binding domain. Wang et al. (2003) used the reverse Tet-regulated gene expression system that has been developed for the RNA polymerase II-driven CMV minimal promoter, while Gupta et al. (2004) replaced the DSE element of the U6 promoter by a Gal4-binding sequence. Transcription from this modified U6 promoter is activated through binding of a Gal4-Oct2 fusion protein under control of an ecdyson inducible expression system.

Although Tet repressor controlled shRNA vectors enable the reversible on/off switch of gene silencing, their use in mice is less straightforward than the Cre/lox system. One reason for this situation may be the easy access to established Cre transgenic mouse strains while TetR transgenic mice are not

available. In addition, the ubiquitous expression of TetR in mice would allow only global on/off control in all tissues, while cell-type-specific TetR expression will lead to gene silencing by default in all tissues lacking the TetR protein.

5 Conclusion and Outlook

A chapter on RNAi within this book that covers mainly knockout mouse technology, may raise the question whether RNAi is able to induce sufficient gene and protein silencing to cause a mutant phenotype in mice. In other words, is RNAi able to replace the time-consuming construction of knockout mice? One argument that seems to speak against the use of RNAi is the incompleteness of gene silencing that leaves approximately 10% residual gene expression. By comparison of heterozygous and homozygous knockout mice, it is well known that 50% reduction in gene expression in heterozygote mutants rarely results in a detectable phenotype. In case of the gradual suppression caused by RNAi, it would be important to know which level of inhibition is sufficient to cause a functional deficiency that results in an obvious phenotype. Presently this question cannot be answered on the basis of many data but a reasonable assumption can be made. First, a conclusion can be drawn from the previous sections that review the use of RNAi in vivo. It is evident from the work on shRNA vector transgenic mice that 10 of the 11 studies that search for a specific deficiency in knockdown animals were able to detect a phenotype. In three cases, the phenotypes of knockdown and the corresponding knockout mice were compared and found to be a complete or partial phenocopy. The knockdown levels in shRNA transgenic mice fall into the range of 80%–95% in most cases, but values of 40%–50% were found in some organs such as spleen. Secondly, a vast number of publications report on the use of siRNAs in cultured mammalian cells, some of which also employed RNAi for large-scale functional screens (Downward 2005). Since numerous phenotypes have been reported from the use of RNAi in vitro, it is beyond the question of whether gene silencing in principle is a useful approach. Although it is presently unknown which fraction of phenotypes remains undetected as a result of incomplete gene silencing this issue may be clarified in the future. Using modern algorithms to identify target sequences, it is now possible to design large siRNA libraries that exhibit a mean silencing efficacy of approximately 90% (Sachse et al. 2005). For screens that study well-known cellular processes such as the cell cycle, many genes can be predicted as positive hits. The proportion of positive controls that will be identified should reveal the depth of RNAi-based gene silencing.

Taken together, the results from shRNA transgenic mice and the widespread and successful record of in vitro gene silencing suggest that RNAi also provides a powerful tool to identify relevant phenotypes in mice. At the level of an individual gene, RNAi may be regarded as less precise approach in comparison to gene targeting. Under the consideration of limited resources, this can be well

compensated by significant savings of time and labour offered by the RNAi technology that can describe larger numbers of phenotypes. The published data on RNAi in mice suggest several methodologies that have potential for routine use:

- Systemic or local delivery of siRNAs is an option for focused questions with short-term readouts. The siRNA experiments can be conducted within days to weeks, require little investment and give a first hint about the in vivo function of a gene. It could be of particular value for the rapid validation of drug target candidates.
- Viral shRNA vectors require greater effort than the use of siRNA and require a period of weeks to months to complete an experiment. The long-lasting knockdown is of particular advantage for stem cell research, cancer studies and gene therapy.
- ShRNA transgenic mice can be used to knock down target genes either body-wide or in a conditional manner. Depending on the method used, a period of several months to 1 year will be required to obtain the experimental animals. To study embryonic development, ES cell clones with stably integrated shRNA vectors can be used together with tetraploid complementation for the production of ES-cell-derived embryos. To study adult mice, it could be beneficial to focus on a defined genomic locus and pretested shRNA vectors to obtain reproducible levels of gene silencing.

By following one of these approaches, RNAi technology offers more flexibility towards the investments required for in vivo experiments as compared to gene targeting. Therefore, it can be expected that RNAi will be increasingly used in mice for specific applications for which gene silencing offers substantial advantages. Thus, RNAi will not replace the use of knockouts but offers new additional opportunities for functional genomics in mice.

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Viral Vectors: A Wide Range of Choices and High Levels of Service

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Abstract Viruses are intracellular parasites with simple DNA or RNA genomes. Virus life revolves around three steps: infection of a host cell, replication of its genome within the host cell environment, and formation of new virions; this process is often but not always associated with pathogenic effects against the host organism. Since the mid-1980s, the main goal of viral vectorology has been to develop recombinant viral vectors for long-term gene delivery to mammalian cells, with minimal associated toxicity. Today, several viral vector systems are close to achieving this aim, providing stable transgenic expression in many different cell types and tissues. Here we review application characteristics of four vector systems, derived from adeno-associated viruses, adenoviruses, retroviruses and herpes simplex virus-1, for in vivo gene delivery. We discuss the transfer capacity of the expression vectors, the stability

of their transgenic expression, the tropism of the recombinant viruses, the likelihood of induction of immunotoxicity, and the ease (or difficulty) of the virus production. In the end, we discuss applications of these vectors for delivery of three molecular systems for conditional mutagenesis, two for inducible transcriptional control of transgenic expression (the tet and the dimerizer systems), and the third one for inducible control of endogenous gene expression based on RNA interference.

Keywords Recombinant viral vectors · Gene delivery · Conditional gene expression · Conditional gene knock-down

1 Introduction

The immense promise of viral gene delivery for treatment of human diseases has been the driving force behind the development of the various recombinant viral vectors that are available today. Whereas the full potential of viral gene therapy has yet to be realized, viral vectors have become widely used in both basic and clinically oriented research. The latest generations of several vector systems offer highly advanced methodology that can be flexibly fine-tuned for specific experimental applications. Thus with an appropriately chosen system, it is possible to achieve gene delivery to many different cell types and tissues in different animal species.

An ideal viral vector, which admittedly has yet to be realized, should include several basic features:

- It should not induce any toxicity or immune response in the host organism.
- The resulting transgenic expression should be long-lasting and with a rapid onset.
- The vector should have a large packaging capacity, in order to include one or more promoter and transgene (P+G) cassettes, together with posttranscriptional and other regulatory elements for efficient and tissue- or cell-specific expression.
- The vector should be able to infect both dividing and nondividing cells, with a choice of broad or selective tropism.
- The viral particles (virions) should be small in size, so they can easily penetrate extracellular space in tissues and organs.
- The recombinant viruses should be easy to produce to high titers.

Clearly, each of these criteria may have different importance for different applications; for example, experiments in animal models may tolerate some toxicity and/or induction of an immune response, whereas the same may not be acceptable for human applications; or, as a different example, large size virions may be easily applied in some tissues, such as liver or lungs, whereas

Table 1 Comparison of different viral vector types

Vector	Toxicity	Persistence of express.	Capacity (kb)	Postmitotic cells	Virion size	Difficulty of production[a]
rAAV	No	>1 year	≤5[b]	Yes	20 nm	Medium
Ad	Some	>1 year[c]	≤37	Yes	80 nm	Difficult
MoMLV	No	Persistent	≤8	No	100 nm	Easy
Lentiviral	No	Persistent	≤9	Yes	100 nm	Easy
HSV-1	Some	Months	≤150[d]	Yes	150 nm	Difficult

[a]Production protocols can be found in the following references: rAAV (Zolotukhin 2005); Ad (Palmer and Ng 2003); retroviral vectors (Yee et al. 1994); HSV-1 amplicon (Saeki et al. 2001) [b]For split genome vector, capacity ≤10 kb [c]Expression gradually declines from a peak level at around 4–6 weeks after infection [d]For replication-defective HSV-1 vector, capacity ≤40 kb

their penetration would be strongly hindered in the brain where extracellular space is less than 40 nm. In this chapter, we discuss five viral families with respect to their applicability for conditional expression of gene products and short interfering RNAs (siRNA) in animal models. Table 1 lists characteristics of these vectors with regard to the six ideal-vector criteria outlined above.

2 Adeno-associated Viruses

Adeno-associated viruses (AAVs) are single-stranded DNA viruses that belong to the Parvoviridae family, genus *Dependovirus*; the virion is a nonenveloped capsid of about 20 nm in diameter, the DNA genome size is 4.7 kilobases (kb) (Büchen-Osmond 2001). AAVs rely on helper viruses, such as adenovirus (Ad) or herpes simplex virus (HSV), for replication of their genome (hence the name *Dependovirus*). AAVs are highly endemic among humans, primates, and several other species.

AAV genome consists of two genes, rep and cap, flanked by 145 nucleotide (nt) palindromic sequences, called inverted terminal repeats (ITRs); the ITRs contain all *cis*-acting sequences critical for virus packaging, replication, and integration. The rep gene contains two promoters and each transcript is regulated by internal splicing, resulting in production of four nonstructural Rep proteins, Rep78, Rep68, Rep52, and Rep40. The Reps regulate replication, viral transcription, packaging of the AAV genomes and site-specific integration. The cap gene encodes the three structural proteins, virion protein 1, 2, and 3 (VP1, VP2, and VP3), from two alternative transcripts and an alternative translation initiation codon. The VPs form the capsid of the virus. Upon infec-

tion of human cells the AAV genome stays either episomal or integrates into a specific region on the human chromosome 19 (*19q13.3-qter*), a site termed AAVS1 (for review see McCarty et al. 2004). AAV then stays in a repressed state until reactivated by superinfection with a helper virus.

2.1 Recombinant AAV Vectors

Recombinant AAV (rAAV) vectors hold great promise in gene therapy, mainly because of their nonpathogenic viral origin; however, their main limitation for use in both gene therapy and basic research lies in the small size of AAV genome, and hence small packaging capacity of the vectors (but see below for AAV split-genome vectors). The rAAV vector design is quite simple. First, the AAV ITRs contain all *cis*-acting elements necessary for replication and packaging in the presence of a helper virus; the so-called gutless expression vector thus contains only the two flanking ITRs, and have a packaging capacity close to 5 kb (Fig. 1A). Second, both AAV rep and cap genes and all necessary adenoviral genes are expressed in trans, either From one or two helper plasmids (Grimm et al. 1998; Matsushita et al. 1998; Xiao et al. 1998). The expression of the Ad genes from a plasmid thus bypasses the requirement for co-infection with wild-type (wt) adenovirus, and the production of rAAV virions now involves only a simple co-transfection of human embryonic kidney (HEK) cell line with the gutless expression plasmid and one or two helper plasmids.

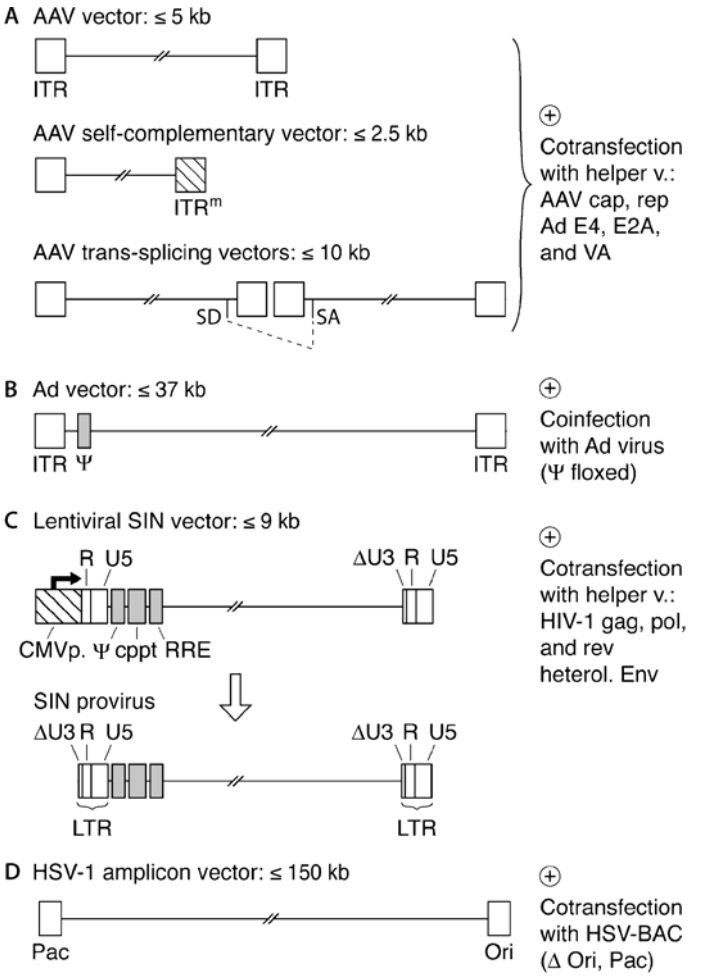
In the host cell environment, rAAV vectors stay mostly episomal (both in human and nonhuman cells). Despite the lack of integration, heterologous

Fig. 1 A–D Simplified schematics of recombinant viral vectors and virus production for gene delivery in an animal model. **A** Basic rAAV vector comprises two inverted terminal repeats (*ITR*); in the self-complementary vector, one ITR is mutated (ITR^{m}), resulting in an initiation of replication only at the wt ITR and reverse read-through the ITR^{m}. In the trans-splicing vectors, splice donor and acceptor (*SD*, *SA*) sites are used to assemble the full gene product from a head-to-tail rAAV DNA dimer. rAAV virus is produced by co-transfection with helper plasmid(s) coding for essential AAV and Ad genes. **B** Ad vector comprises two ITRs and a packaging signal sequence (Ψ); the recombinant virus is produced by co-infection with wt Ad virus (its Ψ is floxed, flanked by *lox* P sites) in Cre recombinase-producing cells. **C** Lentiviral SIN vector. The 5′ CMV promoter drives genomic vector RNA in cells co-transfected with one helper plasmid expressing gag, pol, and rev genes and a second helper plasmid expressing the heterologous env gene. In a host cell, the vector RNA is reverse transcribed to double-stranded DNA (*arrow pointing down*), resulting in a duplication of the 3′ ΔU3 region (lacking promoter activity) to the 5′ region of the provirus. Polypurine tract (cppt) facilitates nuclear import of the vector DNA. *RRE* rev-responsive element, *LTR* long-terminal repeats. **D** HSV-1 amplicon vector comprises two necessary elements: origin of replication (*Ori*), and packaging and cleavage signal sequences (*Pac*). Virus is produced by transfection with bacterial artificial chromosome (*BAC*) expressing complete HSV-1 genome minus the Ori and Pac sequences. **A–D** Packaging capacity of the vectors is indicated next to the vector name

expression from these vectors appears to be stable for extended time periods (in some cases tested for over 1 year) in different tissues, including the brain (Paterna and Büeler 2002), and different cell types in the eye (Martin et al. 2002) or the muscle (Wang et al. 2005).

2.1.1 rAAV Expression Vector

In order to accommodate the small (< 5 kb) capacity of the rAAV vectors, a number of studies compared different combinations of compact promoters, small synthetic introns and other enhancer elements for efficient transgene expression in different tissues. The selection of a promoter is particularly important, as it directly affects both the level and cell type specificity of transgenic



expression. The most commonly used promoter is the human cytomegalovirus (CMV) promoter, a traditional choice in heterologous expression systems because of its strong transcriptional activity in many cell types (Baskar et al. 1996). However, for some applications, such as gene delivery to the central nervous system (CNS), cell type-specific promoters were shown to drive stronger and longer-lasting expression. Efficient promoters for rAAV-based expression in the CNS (and elsewhere) include a chimeric promoter combining the CMV enhancer element in front of a chicken β-Actin promoter (CBA promoter), neuron-specific enolase (NSE) promoter, platelet-derived growth factor β-chain (PDGF) promoter, and a compact (480 nt) human Synapsin I promoter; these are often combined with enhancer elements, such as the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) or short intronic sequence, for improved transport of the transgene mRNA from nucleus to the cytoplasm (Paterna and Büeler 2002; Tenenbaum et al. 2004; Shevtsova et al. 2005 and references therein).

The efficiency of rAAV vectors also depends on factors related to AAV life cycle, such as endocytosis of the virions, intracellular trafficking, and host cell-mediated synthesis of double-stranded DNA from the viral single-stranded genome. The requirement for the second strand synthesis is bypassed in the so-called self-complementary vectors; a mutation introduced into one ITR causes a selective initiation of replication from the second wt ITR; the transcription then proceeds through the mutant ITR and reverses, using the opposite strand as a template; this creates an inverted repeat molecule with two wt ITRs at each end and the mutant ITR in the middle, which folds as a double-stranded DNA in the infected host cell (McCarty et al. 2003; Z. Wang et al. 2003). The self-complementary vector has an increased transduction efficiency and a faster onset of transgenic expression. However, because of the inverted genome, this vector has only half of the wt packaging capacity (< 2.5 kb).

A strategy to increase the packaging size of rAAV vectors was developed based on naturally occurring heterodimerization of AAV double-stranded DNA. After infection of a host cell, episomal rAAV vectors undergo recombination to grow circular forms and concatemers (McCarty et al. 2004). The so-called split (or trans-splicing) rAAV vectors use two expression vectors for delivery of one gene product, relying on assembly of the split gene by formation of head-to-tail DNA dimers in double-infected cells (Nakai et al. 2000; Sun et al. 2000; Yan et al. 2000). The packaging limit of these vectors is thus approximately 10 kb, double that of wt rAAV.

2.1.2 rAAV Helper Vectors: Tropism of AAV Serotypes

As outlined above, rAAV helper vectors express AAV cap and rep genes together with three Ad genes (Grimm et al. 1998; Matsushita et al. 1998; Xiao et al. 1998). Depending on the cap gene, eight different serotypes of AAV

have been identified (serotype refers to a lack of antigenic cross-reactivity: one serotype is neutralized by serum generated against its own capsid but not by sera raised against capsids of the other AAV serotypes) (for review see Grimm and Kay 2003). The capsids of different serotypes bind to different cell surface-expressed proteins, which means that each serotype has more or less its own distinct tropism. Thus cross-packaging (also called transcapsidation) of an expression vector with different cap genes changes the tropism of the resulting rAAV virions. To this end, all eight AAV cap serotypes have been demonstrated to successfully produce rAAV virions with AAV2-based expression vector and rep gene (Gao et al. 2002; Rabinowitz et al. 2002; Grimm et al. 2003).

The AAV2 capsid binds primarily to heparan sulfate proteoglycan (HSPG) (Summerford and Samulski 1998), which accounts for its broad cell tropism. The AAV5 capsid binds to the platelet-derived growth factor receptor (PDGFR) (Di Pasquale et al. 2003), and high PDGFR expression in the brain, lung, and retina accounts for high AAV5 tropism in these tissues (Davidson et al. 2000; Rabinowitz et al. 2002). Whereas cell surface receptors for AAV1, 6, 7, and 8 have yet to be identified, infection studies with cross-packaged rAAV virions showed that AAV1 has a high tropism for muscle (Chao et al. 2000), AAV6 for lung (Halbert et al. 2001), AAV7 for muscle and liver, and AAV8 for liver (Gao et al. 2002). The AAV8 serotype was also shown to be very efficient in crossing the blood vessel barrier, resulting in high rate infection of muscles after systemic delivery (Wang et al. 2005). In addition, co-transfection of two helper plasmids with different cap genes creates mosaic serotypes, which can be used to combine distinct aspects of the individual serotypes (Hauck et al. 2003). For example, combination of serotypes 2 and 5 creates capsids that can be easily purified on heparin chromatography due to the inclusion of the AAV2 cap gene (Clark et al. 1999; Zolotukhin et al. 1999), but which also retain their high brain and eye infectivity due to the AAV5 cap gene (Davidson et al. 2000; Rabinowitz et al. 2002).

In summary, rAAV vectors offer an easy method for long-lasting gene expression in a number of tissues, including brain, liver, lung, muscle, and retina, and are void of any apparent cellular toxicity or immune response. These vectors have been adapted for use with several conditionally regulatable expression systems, overview of which is given in section 6.

3 Adenoviruses

Adenoviruses are double-stranded DNA viruses from Adenoviridae family, with a linear genome of approximately 36 kb (Büchen-Osmond 2001). The adenoviral (Ad) virion is a nonenveloped capsid approximately 80 nm in diameter. Human adenoviruses are respiratory, ocular, and gastrointestinal pathogens.

The Ad genome is flanked by inverted terminal repeats (ITRs) of 140 bp, which function as replication origins. Ad genes are encoded on both DNA strands, and are regulated by complex RNA processing resulting in a high number of viral proteins (for example, over 70 proteins for the well-studied Ad5 serotype). The early transcription units (E1A, E1B, E2, E3, and E4) function in transcriptional activation and replication of the Ad genome, as well as in subversion of host cell immune response. The late transcription units (L1–L5) encode structural proteins for the capsid and internal core. In addition, the E3-11.6K protein (adenovirus death protein) is produced in the late phase of infection, and aids in the host cell lysis (for review see McConnell and Imperiale 2004).

There are 51 known human Ad serotypes, classified into six groups, called A through F. The most studied adenoviruses are serotypes Ad2 and Ad5 from group C. The Ad capsid, similarly to AAV capsids (see Sect. 2), determines the tropism of the virus. Groups A and C–F bind primarily to the widely expressed coxsackievirus B and adenovirus receptor (CAR); this accounts for their high infectivity in many tissues (for review see Mizuguchi and Hayakawa 2004). Group B binds to CD46, a ubiquitous complement-regulatory protein (Gaggar et al. 2003); this makes group B viruses infectious to some cell types that are refractory to other adenoviruses, such as hematopoietic stem cells, dendritic cells, and malignant tumor cells (Mizuguchi and Hayakawa 2004).

3.1 Helper-Dependent Ad Vector

In contrast to the earlier generations of Ad vectors, which relied primarily on deletion of the E1 transcripts that are essential for replication of the virus, the helper-dependent (HdAd) Ad vector was designed in a similar way as the gutless rAAV vector (see Sect. 2.1) and contains only two flanking ITRs and a packaging sequence (ψ) (Fig. 1B) (Schiedner et al. 1998). This gives the HdAd vector its high capacity, up to about 37 kb for Ad2, which allows for packaging large size promoter fragments and genes, or even whole genomic loci (for review see Palmer and Ng 2005). The replication of HdAd DNA and assembly of virions during virus production is done by coinfection with helper adenovirus. However, the main limitation of this system lies in the difficulty of separating the vector and the helper virus DNA. Presently, the most efficient strategy is to use helper viruses with the packaging sequence flanked by *loxP* or *frt* sites, and to propagate the HdAd vectors in cells expressing Cre or Flp recombinase, respectively; this results in deletion of the packaging sequence selectively from the helper virus (Palmer and Ng 2005 and references therein). The reported percentage of helper virus contamination in these systems is quite low ($< 0.01\%$) (Palmer and Ng 2003). Whereas this may still be a concern for human gene therapy, for applications in basic research this level of contamination may be acceptable. This said, the probability of viral immunogenic toxicity, either due

to the helper virus contamination or as an acute immune response triggered by the viral capsid (for review see Muruve 2004), should be considered and tested for when using the HdAd vectors for animal experiments. The acute immune response is dose-dependent and is typically associated with administration of high vector doses.

Because of their broad tropism and large packaging capacity, Ad vectors have been used to delivery various types of P+G cassettes to many different tissues. The vector DNA stays mainly episomal in the host cell genome, but the expression with HdAd vectors has been demonstrated to be persistent for months, and even more than one year. A typical profile of HdAd-driven expression has a peak about 4–6 weeks after infection, and then gradually declines. This has been demonstrated, for example, in rodent liver (Kim et al. 2001), muscle (Gilbert et al. 2003), and brain (Zou et al. 2001). In addition, the study by Kim et al. (2001) demonstrated that expression of transgenes in their native genomic structure, taking advantage of the large packaging capacity of the HdAd vectors, may result in a higher and longer expression compared to expression of cDNA minigenes (Kim et al. 2001).

Finally, a promising strategy to enhance the specificity of Ad-based gene delivery is to modify the surface of the recombinant capsid in order to achieve cell type-specific targeting (for review see Mizuguchi and Hayakawa 2004; a similar strategy is also being developed for AAV and other types of vectors, however, the Ad targeting is presently the most advanced). Such modifications are based either on genetic alterations of the Ad capsid or on antibody-mediated conjugations of specific ligands to the capsid surface; often the strategy is aimed not only at changing the tropism of the Ad vectors, but also at reducing the acute immune response elicited by the Ad capsid.

In summary, the main advantage of the HdAd vector is its large packaging capacity; the remaining downsides of this system include triggering an acute immune response (especially if using large virus doses) and somewhat difficult production relying on co-infection with wt adenovirus.

4 Retroviruses

Retroviruses are enveloped RNA viruses with diploid single-stranded genome of 7–11 kb, which is reverse transcribed to double-stranded DNA in host cell cytoplasm; the virion size is about 80–100 nm. The Retroviridae family is divided into two subfamilies, Orthoretrovirinae and Spumaretrovirinae, and seven genera (Büchen-Osmond 2001). Retroviruses infect reptiles, birds and mammals, and cause chronic infections which may lead to immunodeficiency, anemia, encephalitis or malignancy.

The retroviral genome contains three essential genes, termed gag, pol, and env, flanked by long terminal repeats (LTR); additional accessory genes are

included depending on a type of retrovirus (see, for example, lentiviruses below). The gag gene codes for structural proteins, termed capsid, matrix, and nucleocapsid, which form the viral core; the pol gene codes for viral protease, reverse transcriptase and integrase; and the env gene codes for envelope glycoproteins, which mediate interaction of the virus with host cell surface proteins (all proteins are proteolytically processed from precursor polypeptides). After infection, the RNA genome is reverse transcribed into double-stranded DNA, which is transported to the host cell nucleus and chromosomally integrated.

The development of retroviral vectors is based mainly on work with the Moloney murine leukemia virus (MoMLV), which has a simple gag-, pol-, and env-containing genome (Pages and Bru 2004 and references therein). The most recent generations of retroviral vectors include a number of modifications designed to enhance the safety as well as the efficiency of the system (see Fig. 1C). The main limitation of most retroviruses, with the remarkable exception of lentiviruses, is their inability to infect nondividing cells; this is because the preintegration complex containing the viral double-stranded DNA does not cross intact nuclear membrane (in dividing cells the nuclear membrane is disassembled during mitosis). Due to the ability of lentiviruses to infect both dividing and nondividing cells, we focus on the description of lentiviral vectors as a representative for the retroviral system.

4.1 Lentiviral Vectors

Lentiviruses have a more complex genome containing, besides gag, pol, and env, additional regulatory and accessory genes. The most studied lentivirus is the human immunodeficiency virus type 1 (HIV-1), which infects CD4-positive T lymphocytes and macrophages (CD4 antigen acts as the primary surface receptor for HIV-1) and causes chronic immune depletion known as the acquired immune deficiency syndrome (AIDS) (for review see Frankel and Young 1998). HIV-1 genome contains two additional regulatory genes, termed tat and rev, and four accessory genes, termed vpr, vpu, vif, and nef; the lentiviral genome is 9.2 kb. The Tat protein enhances transcriptional activity from the promoter region in the 5′LTR of an integrated provirus; the Rev protein enhances transport of HIV unspliced messenger RNA (mRNA) from the nucleus; the accessory proteins mainly enhance virulence against host organism. The development of HIV-1-based vectors was primarily motivated by the ability of lentiviruses to infect postmitotic cells. Conveniently, the vector design based on other retroviruses, such as the self-inactivating MoMLV vector (Yu et al. 1986), was directly transferable to the lentiviral vectors (see below).

In the latest generation of HIV-1-based vectors, all accessory genes and the regulatory tat gene are deleted (Naldini et al. 1996; Dull et al. 1998); the regulatory genes necessary for virion production are expressed from two helper vectors, one containing the gag, pol, and rev genes, and the other express-

ing the env gene (Zufferey et al. 1997). The self-inactivating (SIN) expression vector contains a deletion of the U3 promoter in the 3′LTR, a region which is copied to the 5′ end of the viral double-stranded DNA during reverse transcription; the SIN modification thus results in transcriptional inactivation of the integrated provirus, which serves to limit the possibility of mobilization of and recombination with latent retroviral sequences in the host cell genome (Miyoshi et al. 1998; Zufferey et al. 1998) (Fig. 1C). Transgenic expression from the SIN vector is driven by an internal promoter. The most commonly used promoters include the ones already mentioned with respect to rAAV vectors (see Sect. 2). Recently, a 1.3-kb promoter fragment of calcium/calmodulin-dependent protein kinase II was shown to be particularly efficient for transgenic expression in the brain in pyramidal neurons (Dittgen et al. 2004).

Tropism of lentiviral vectors is determined by the type of envelope protein that is used for the viral production; the use of heterologous Env proteins is called pseudotyping. The most commonly used Env protein is the vesicular stomatitis virus glycoprotein (VSV-G), which allows easy concentration of the virus to high titers by ultracentrifugation and gives the virus a broad tropism (VSV-G binds to ubiquitous phospholipid components of plasma membrane) (Burns et al. 1993). VSV-G-coated lentiviral particles were shown to infect numerous tissues and cell types, including brain, lung, liver hepatocytes, the hematopoietic system, and pancreatic cells (Wiznerowicz and Trono 2005 and references therein), and their tropism often overlaps with that of rAAV vectors. The main advantage of lentiviral vectors is their larger packaging capacity (but see split-genome rAAV vectors, Sect. 2.1.1). On the other hand, rAAV vectors have a distinct advantage in infecting larger areas or more cells during direct injections into different tissues (due to the small size of the rAAV virion, 20 nm vs 100 nm for lentivirus). In addition, specific rAAV serotypes can efficiently infect some tissues, such as muscle or liver, by systemic application (see Sect. 2.1.2).

One clear advantage of lentiviral vectors over any other recombinant vector system is their applicability for efficient generation of transgenic animals. In this procedure, usually single-cell embryos are injected with lentivirus into the perivitelline space and subsequently implanted into the uterus of pseudopregnant females (Lois et al. 2002). In contrast to other retroviral vectors, such as the MoMLV vectors, expression from integrated lentiviral vectors is not silenced during development. In addition, expression from cell-specific promoters was shown to remain restricted to relevant tissues in the transgenic animals, such as muscle-specific expression from the myogenin promoter or thymus-specific expression from the T lymphocyte lck promoter in a mouse (Lois et al. 2002). Other transgenic species that were generated with lentiviral vectors include pig, cattle, and chicken (Pfeifer 2004 and references therein).

Finally, next to the HIV-1-based systems, other primate as well as nonprimate lentivirus-based vectors have been developed (for review see Poeschla 2003). Of these, nonprimate vectors derived from feline immunodeficiency

virus (FIV) (Poeschla et al. 1998) and equine infectious anemia virus (EIAV) (Mitrophanous et al. 1999) were both shown to efficiently infect neurons and other cells types in vitro and in vivo. Although it is not clear if these vectors offer any advantage in terms of biosafety in comparison to HIV-1-based vectors, the EIAV vectors are much more efficient for gene delivery to CNS or peripheral nervous system (PNS) via axonal retrograde transport when pseudotyped with rabies glycoproteins (Mazarakis et al. 2001; P. Osten and A. Cetin, unpublished data).

In summary, lentiviral vectors offer an easy to use system for stable delivery of inserts up to about 9 kb. They are ideally suited for local delivery via injections into defined regions, such as distinct brain regions and nuclei. Their use in production of transgenic animals should allow generation of new animal models, which may be more suitable for a specific purpose than the traditionally used mouse models.

5 Herpes Simplex Virus-1

Herpes simplex virus (HSV-1) is an enveloped DNA virus, belonging to the Herpesviridae family, subfamily Alphaherpesvirinae; the virion is 120–200 nm in diameter, and contains one copy of double-stranded DNA genome (Büchen–Osmond 2001). The genome is 152 kb in length, arranged into unique long and unique short segments (U_L and U_S), each flanked by inverted repeats; it encodes over 80 gene products. HSV-1 is a common human pathogen; primary infection occurs in childhood via oral mucosa, and leads to lytic mucosal cell death; trigeminal ganglia sensory neurons become infected via their axonal endings by the viral progeny, and the virus establishes a latent infection which can lead to orolabial cold sores upon reactivation.

Both the lytic and latent forms of infection served as a basis for development of HSV-1 recombinant vectors. The so-called conditionally replicating vectors contain viral genome that does not replicate in neurons but retains its lytic replication in rapidly dividing cancer cells; these vectors are being tested as a treatment for brain tumors (for review see Shah et al. 2003). In contrast, replication-defective vectors and amplicon vectors are being developed for gene delivery to neurons; the main promise of these vectors lies in the high neurotropism of HSV-1 infection, in the possibility to deliver genes to CNS or PNS via retrograde axonal transport (however, this is also possible with pseudotyped EIAV vectors, see Sect. 4.1) and the potential for delivery of large genomic inserts, up to 150 kb in the case of the HSV-1 amplicons.

The development of the replication-defective vectors was based on progressive deletion of genes essential for replication of the virus; a vector which has all immediate early genes deleted was found to be nontoxic and able to establish

persistent latent infection (during virus production, essential genes are provided in *trans* in packaging cell lines) (Samaniego et al. 1998). The amplicon vectors, on the other hand, have all viral genes deleted and contain only HSV-1 origin of replication and DNA packaging/cleavage signal sequences (Spaete and Frenkel 1982; Geller and Breakefield 1988). The most advanced protocol for production of the amplicon vectors, the so-called helper virus-free system, uses co-transfection with bacterial artificial chromosome containing the entire HSV-1 genome but lacking the replication and packaging signals (Fig. 1D) (Saeki et al. 1998; Stavropoulos and Strathdee 1998). Both the latest replication-defective vectors and the HSV-1 amplicons infect neurons with high efficiency and establish latent episomal infection, with no viral toxicity and only a limited immune response (Bowers et al. 2003 and references therein). However, a remaining challenge in the development of HSV-1 vectors concerns the persistence of the transgenic expression. This is because of a strong transcriptional repression which occurs during latent HSV-1 infection, and which also applies to the replication-defective and even the amplicon vectors; thus the use of strong heterologous promoters, such as the CMV promoter (Samaniego et al. 1998), results only in transient expression. A promising strategy to achieve long-term expression is based on the use of the latency-associated promoter (LAP), which normally drives noncoding latency-associated transcripts (LATs) and is active throughout infection (Goins et al. 1999). Another possibility may be to use whole genomic loci containing cell-specific promoters, which may be less silenced compared to relatively short promoter fragments (Wade-Martins et al. 2001).

In summary, HSV-1 vectors hold great promise for gene delivery of large genetic constructs; other vectors, including rAAV and lentiviral vectors, may offer more convenient means for delivery of constructs with a size limit of about 10 kb.

6
Applications of Viral Vectors for Conditional Mutagenesis

The development of conditional gene-regulation systems and that of recombinant viral vectors has been closely linked over the last 10 years or so, and accordingly most conditional systems established to date have been adopted into one or more of the viral vectors described in this chapter. During the phase of initial testing and refining of different molecular prototypes for conditional gene regulation, viral vectors – typically rAAV or lentiviral vectors – offer a convenient delivery method for experiments in vitro in cultured cells and, even more importantly, in vivo in different animal models. Furthermore, due to the high spatiotemporal control over the introduced genetic manipulations, which can be achieved with local delivery of recombinant viruses to specific tissues, advanced viral vector systems offer an attractive alternative to

traditional mouse genetics for conducting studies aimed at modeling different conditions of human pathology (Fig. 2). The main goal of our chapter was to outline advantages and disadvantages of the different viral vectors available today for long-term gene expression, in order to help choose the most favorable vector for a specific conditional mutagenesis application. In the rest of the text, we briefly outline the use of viral vectors for conditional gene regulation based on (a) two inducible transcription systems—the Tet and the Dimerizer systems—applicable for regulatable transgenic expression and (b) inducible expression of short-interfering RNAs for regulatable downregulation of endogenous genes.

6.1 Expression of Inducible Transcription Systems

Molecular systems for regulatable transcription, which allow one to turn on and off expression of a gene of interest, have great potential for study of essentially any cellular function in living organisms, either physiological or with respect to modeled human pathology. Since the pioneering work of the Bujard laboratory (University of Heidelberg) in the early 1990s (Gossen and Bujard 1992), several mammalian systems for transcriptional control of transgenic expression have been developed (fore review see Weber and Fussenegger 2004). The two systems described here, with respect to their use from viral vectors, are based on allosteric regulations of transcription by small molecules, namely tetracycline and rapamycin; both these molecules can be delivered systemically in drinking water, reaching all tissues and organs including the brain (after crossing the brain–blood barrier). The tetracycline-regulated Tet system is based on one transactivator molecule and thus requires delivery of only two components: the transactivator and the responder cassette with a gene of interest under the control of the transactivator. In the rapamycin-regulated dimerizer system, the transactivator is split into two proteins that are reconstituted upon addition of rapamycin acting as a dimerizer; this system requires delivery of three components (two proteins for the transactivator and the responder cassette), which makes it somewhat more cumbersome for viral vector-based expression. At the same time, the dimerizer may offer a tighter control over background (leak) expression of the transgene (see below).

6.1.1 Viral Vectors for the Tet System

The tet system is based on the prokaryotic tetracycline repressor (tetR), a protein which dissociates from its native DNA binding sequence (tet-operator; tetO) in the presence of the antibiotic tetracycline. The tetracycline-controlled transactivator (tTA) was constructed by fusing tetR to VP16 transcriptional activation domain: binding of tTA to the tetO sequence placed in front of

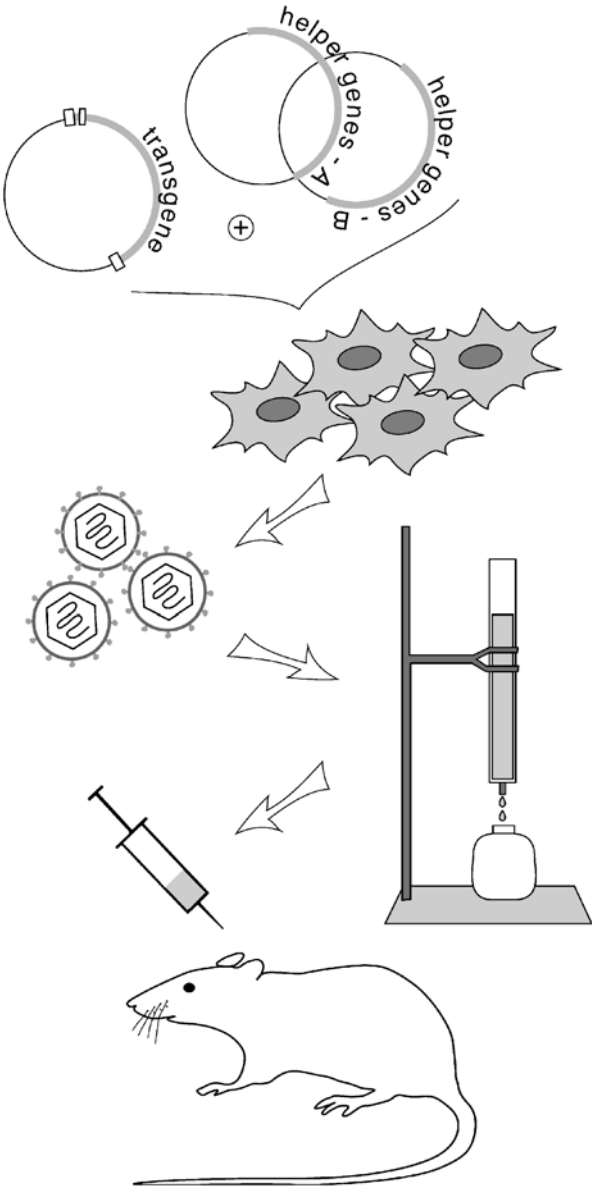


Fig. 2 Typical scheme of recombinant virus production: co-transfection of an expression vector (transgene) with one or two helper vectors (helper genes) in a cell line (usually HEK cells) leads to production of the virus (either secreted to cell medium or remaining inside the transfected cells). The collected medium of cell lysate is purified (filtrated, in some cases run over chromatography columns) and concentrated. The virus can be locally or systemically injected into an animal (typically rat, mouse, or primate)

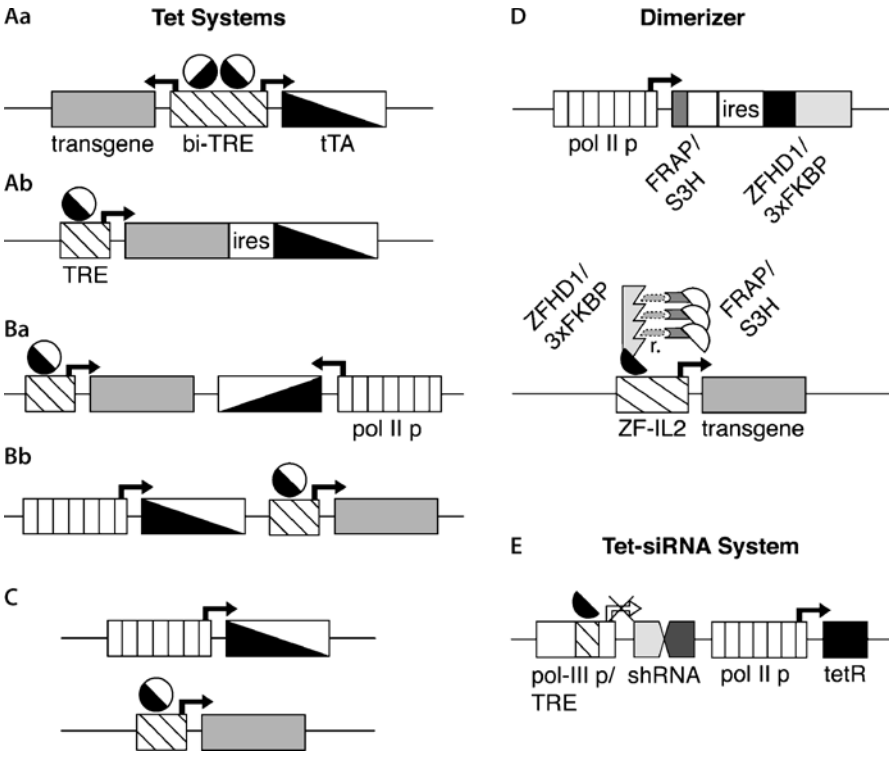
a minimal CMV promoter induces transgenic expression, whereas inhibition of binding in the presence of tetracycline (or its more commonly used derivative doxycycline) leads to an inactivation of the tetO-CMV promoter (Gossen and Bujard 1992) (see the chapter by R. Sprengel and M.T. Hasan, this volume). As a refinement of the system, a reverse mutant of tetR (rtTA) was developed a few years later; rtTA binds tetO in the presence of doxycycline, which allows for timed activation of transgenic expression by adding the drug (Gossen et al. 1995). Both the tTA and rtTA systems, as well as an analogous system based on the Pip (pristinamycin-induced protein) repressor regulated by the antibiotic streptogramin (Fussenegger et al. 2000) (see the chapter by W. Weber and M. Fussenegger, this volume), have been adapted for expression in AAV, Ad, retroviral, and HSV vectors. The two basic components of the system, the transactivator and the responder cassette expressing the transgene, are either

Fig. 3 A–E Schemes of three conditional gene regulation systems adapted to viral vectors. **A–C** Tet systems adapted to rAAV and retroviral vectors. The DNA components of the system are drawn as boxes: *gray box*, transgene; *hatched box*, tet responsive element (*TRE*), which consists of the operon (*tetO7*) and minimal CMV promoter; *bi-TRE* bidirectional TRE promoter; *split black/white box* tTA (or rtTA), with *black* indicating the tet repressor and *white* the VP16 domain; *ire*s internal ribosome entry site; *striped box* polymerase II promoter (pol II p). tTA protein is indicated as *black/white circle* for tet repressor/VP16 fusion protein. **A** Self-regulatory one-vector systems rely on tTA (or rtTA) leak expression from the TRE promoter, which perpetuates itself by self-induction in the absence (tTA) or presence (rtTA) of doxycycline. **Aa** MoMLV vector (Unsinger et al. 2001); rAAV vector (Fitzsimons et al. 2001). The latter study showed that flanking insulator sequences suppress background expression in rAAV vector. **Ab** lentiviral vector for rtTA-based expression (Markusic et al. 2005). **B** Single vector systems with constitutive tTA (or rtTA) expression driven form a pol II (CMV or cell-specific) promoter. **Ba** rAAV vector (Bohl et al. 1998; Jiang et al. 2004); **Bb** lentiviral vector (Kafri et al. 2000; Vigna et al. 2002); rAAV vector (Haberman et al. 1998) (Chenuaud et al. 2004); **C** Two-vector system, with tTA (or rtTA) expression driven from a pol II promoter in lentiviral vector (Vigna et al. 2002; Regulier et al. 2003); AAV vector (Apparailly et al. 2002). **D** The dimerizer system. *Top* Pol II promoter drives expression of a bicistronic construct; the first cistron codes for a fusion protein between FRAP domain and S3H activation domain and the second cistron codes for a fusion protein between ZFHD1 DNA-binding domain and three FKBP domains. *Bottom* The transgene of interest in under the control of the ZF-IL2 promoter, consisting of 12 repeats of ZFHD1-binding sites and a minimal interleukin-2 promoter. The transcription factor is reconstituted from the two products of the bi-cistronic construct in the *top* of the figure by the addition of dimerizer rapamycin (*r.*) heterodimerizing the FKBP and FRAP domains. This system has been adapted to retroviral vector Pollock et al. 2000), rAAV (Rivera et al. 1999; Ye et al. 1999; Auricchio et al. 2002b), Ad (Auricchio et al. 2002a), and HSV-1 (S. Wang et al. 2003) vectors. **E** Tet-regulated siRNA expression. The 5′ region of pol III promoter contains one or two tet-binding operons; the presence of tetR interferes with pol III transcription of shRNAs; release of tetR by addition of tetracycline reverses the block and induces siRNA-based gene silencing. The tet-siRNA system has been described in lentiviral (Wiznerowicz and Trono 2003), and Ad (Hosono et al. 2004) vectors

included in one vector or split into two vectors, and a number of studies examined different designs aimed at achieving the highest induction in the ON state with the lowest background expression in the OFF state (Fig. 3). The two-vector system typically offers better control over the background expression; however, its clear disadvantage is the requirement for double infection of the host cell.

AAV vectors, due to their limited packaging size, are perhaps best suited for the two-vector configuration. Such rAAV vectors were used, for example, for regulatable expression of bone morphogenetic protein-2 in a mouse model for bone regeneration (Gafni et al. 2004) or of viral interleukin-10 in a mouse model for rheumatoid arthritis (Apparailly et al. 2002). However, it is possible to use one rAAV vector for tet-regulated expression of a small transgene ($< \sim 3$ kb), and several different vector designs have been tested (Fig. 3A–C). An example of a potential therapeutic application for such rAAV vector was given in two studies, which demonstrated regulatable expression of erythropoietin (600 bp cDNA) in mice and nonhuman primates (Bohl et al. 1998; Chenuaud et al. 2004).

Lentiviral vectors offer larger packaging capacity compared to rAAVs, and accordingly the tet-regulatable lentiviral vector design has been primarily based on one-vector module (Fig. 3A, B). Recently, a promising example for gene therapy application was demonstrated by conditional expression of hu-



man clotting factor IX in a mouse model for severe combined immunodeficiency (SCID) (Vigna et al. 2005). In a different study, a lentiviral two-vector system was used to establish a reversible model for Huntington's disease (Regulier et al. 2003).

Finally, both Ad and HSV-1-based vectors offer even larger packaging capacity compared to retroviruses; however, since the preparation of these vectors is technically more difficult (see legend Table 1 for references on production protocols), they have not been used as routinely as rAAV or lentiviral vectors for the development and testing of the tet systems. In principal, any of the transactivator–transgene arrangements showed in Fig. 3 can also be adapted to Ad and HSV-1 vectors, and several such vectors have been reported, including HdAd vector (Salucci et al. 2002) and HSV-1 amplicon vector (Fotaki et al. 1997).

6.1.2 Viral Vectors for the Dimerizer System

The general principle of the dimerizer system is also quite simple: in contrast to the fusion protein tTA, the dimerizer transactivator is expressed as two separate proteins: a DNA-binding domain and a transcriptional activation domain, each fused to a dimerizing domain (Fig. 3D). The dimerizing domains bind to a specific bivalent molecule, such as rapamycin or FK506; the addition of a dimerizer brings the two proteins together, effectively reconstituting the transactivator (for review see Pollock and Clackson 2002). A transgene of interest is then placed under the control of a promoter containing binding sites for the DNA-binding domain of the transactivator in front of a minimal promoter element, much in the same way as the responder cassette of the tet system.

The most commonly used dimerizer system is based on immunophilin FKBP (FK506-binding protein), its ligand FRAP (FKBP rapamycin-associated protein), and rapamycin, which induces heterodimers between FKBP and FRAP (Pollock and Clackson 2002 and references therein). The inducible transcription system consists of a DNA-binding domain fused with the FKBP domain(s) and a transcription activation domain with the FRAP domain (Rivera et al. 1996). The DNA-binding domain used most commonly in the dimerizer is ZFHD1 (a zinc finger pair and a homeodomain), which binds to a specific ZFHD1-binding DNA sequence. In the original design, ZFHD1 was fused to three FKBP domains, which allowed for simultaneous transcriptional activation by three activation domains derived from the p65 subunit of human NF-κB (Rivera et al. 1996) (Fig. 3D). More recently, the p65 domain was replaced by more potent hybrid activation domain, termed S3H, made as a fusion between human heat shock factor 1 and a longer portion of the p65 domain (Pollock et al. 2000). The responsive transgenic promoter typically consists of 12 ZFHD1 repeats in front of a minimal promoter element from interleukin 2 gene (Rivera et al. 1996).

The dimerizer system has been adapted to rAAV, Ad, retroviral, as well as HSV-1 amplicon vectors, into either one or two vectors, similarly to the tet system (see Fig. 3). So far, there have not been as many publications with the dimerizer compared to the tet system. However, as mentioned above, the dimerizer may offer tighter regulation of background expression. Such strict control over transcription by the dimerizer system has been clearly demonstrated in a study by Pollock et al. (2000), which showed that it is possible to generate a stable cell line with dimerizer-inducible expression of a highly toxic gene (diphtheria toxin A) from a retroviral vector (Pollock et al. 2000).

6.2 Expression of Short-Interfering RNAs

Gene downregulation (knock-down) by RNA interference (RNAi) has become a widely applied method for studying gene functions in many different cell types in vitro and in vivo. In this approach, introduction of double stranded short-interfering RNAs (siRNAs; typically 19-to-21 base pairs) into cell cytoplasm causes an activation of a multiprotein complex termed RISC (RNA-induced silencing complex), and a subsequent degradation of cellular mRNAs containing a region homologous to the siRNA sequence (for review see Meister and Tuschl 2004).

The most common method for induction of RNAi in vivo relies on expression of siRNAs from a polymerase III (pol III) promoter, e.g., small nuclear RNA U6 (snU6) promoter or H1 promoter (Tuschl 2002 and references therein), placed within a viral vector. In this method, siRNA sequence is expressed as a fold-back short hairpin RNA (shRNA), which is posttranscriptionally processed into typical siRNAs by cellular RNase Dicer. Since pol III promoters are quite short (∼200–300 nt), it is possible to include one or many pol III-shRNA cassettes into a viral vector backbone, together with a pol-II promoter expressing green fluorescent protein (GFP) to label the infected cells (Fig. 3E). This strategy has been adapted by a rapidly growing number of laboratories using rAAV, Ad, or lentiviral vectors (Bantounas et al. 2004; Davidson and Harper 2005 and references therein).

Conditional regulation has also been applied to siRNA expression. The regulatory system based on the tet repressor, which is widely used for regulation of pol II-based transcripts (see above, and the chapter by R. Sprengel and M.T. Hasan, this volume), was also adapted for pol III transcription with both U6 and H1 promoters (Ohkawa and Taira 2000; Miyagishi and Taira 2002; Matsukura et al. 2003; van de Wetering et al. 2003). In this system, tet operator is placed within the pol III promoter, near the transcriptional start; in the absence of doxycycline, tet repressor binds to the operator and prevents transcription; in the presence of doxycycline, tet binding is inhibited and the transcriptional block is relieved (Fig. 3E). When combined with rAAV, Ad or lentiviral vectors,

the versatility of application of this system for reversible gene knock-down in vivo is unprecedented. However, an important aspect to keep in mind when designing siRNA-based experiments is that the suppression of the target mRNA is typically, if not always, incomplete, with some remaining residual protein expression. Thus siRNA-based knock-down may be best suited for studying gene functions with expected gene dose-dependent phenotype, such as, for example, up- or downregulation of different gene products involved in cellular transformation in different forms of human cancer (e.g., Chen et al. 2003; Matsukura et al. 2003).

7 Concluding Remarks

The last decade or so has seen great effort devoted to the development of recombinant viral vectors and molecular systems for conditional mutagenesis, and many papers have been published demonstrating the applicability of different vectors and conditional systems for gene expression and inducible gene regulation in different tissues and animal models. More recently, both fields have begun to focus on experimental as well as therapeutic applications of these methods. The viral and molecular techniques described here and elsewhere in the Part 1 of this monograph, when applied to their best advantage, offer excellent tools for establishing disease models in laboratory animals. Next to the high level of spatiotemporal control and relative ease of application, perhaps the main advantage of viral vectors comes from the broad choice of tropism of recombinant viruses, making it easy to carry out experiments in different tissues in mice, rats, or even primates. The capacity to compare the same model conditions in different animal species is likely to greatly enhance the relevance of the observed findings for real human pathology.

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Conditional Mutagenesis by Cell-Permeable Proteins: Potential, Limitations and Prospects

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Abstract The combination of two powerful technologies, the Cre/loxP recombination system and the protein transduction technique, holds great promise for the advancement of biomedical and genome research by enabling precise and rapid control over mutation events. Protein transduction is a recently developed technology to deliver biologically active proteins directly into mammalian cells. It involves the generation of fusion proteins consisting of the cargo molecule to be delivered and a so-called protein transduction domain. Recently, the derivation of cell permeable variants of the DNA recombinase Cre has been reported. Cre is a site-specific recombinase that recognizes 34 base pair loxP sites and has been widely used to genetically engineer mammalian cells in vitro and in vivo. Recombinant cell-permeable Cre recombinase was found to efficiently induce recombination of loxP-modified alleles in various mammalian cell lines. Here we review recent advances

in conditional expression and mutagenesis employing cell-permeable Cre proteins. Moreover, this review summarizes recent findings of studies aimed at deciphering the molecular mechanism of the cellular uptake of cell-permeable fusion proteins.

Keywords Site-specific recombination · Protein transduction · Endocytosis · Protein therapy · Gene therapy

Abbreviations

β-gal	β-galactosidase
ES	Embryonic stem (cells)
FCS	Fetal calf serum
GFP	Green fluorescent protein
NLS	Nuclear localization signal
PTD	Protein transduction domain
SSR	Site-specific recombinase

1 Introduction

The assessment of mammalian gene function is mainly dependent on the use of genetically engineered organisms. So far, mainly DNA transfection and viral transduction have been used to introduce gene functions into cells. Protein transduction developed to a new paradigm based on the observation that short peptides, referred to as protein transduction domains (PTDs), are able to function as carriers to deliver macromolecules. This technology has been used to analyze various gene functions and cellular processes at the organism or cellular level. Cell-permeable proteins and peptides have been used to modulate the cell cycle (Ezhevsky et al. 1997; Gius et al. 1999; Zezula et al. 2001), for manipulating stem cells (Peitz et al. 2002; Krosl et al. 2003; Kwon et al. 2005), and for the rational design of antitumor therapeutics (Takenobu et al. 2002; Kondo et al. 2004; Guelen et al. 2004). These applications are well covered by a series of recent reviews (Kabouridis et al. 2003; Wadia and Dowdy 2003; Dietz and Bähr 2004; Brooks et al. 2005). This review will mainly focus on recent advances in the understanding of the molecular mechanism of protein transduction and the application of cell permeable site-specific recombinases for conditional targeting in mammalian cells. A rational design of an effective protein transduction system can be achieved only with a comprehensive understanding of the mechanism of cellular uptake. The mechanism by which PTD-modified proteins adhere to and cross the plasma membrane of cells as well as the subsequent intracellular trafficking is currently being extensively investigated. What is known about this somewhat mysterious phenomenon of cellular translocation? What are the limitations of the protein transduction technology? Finally, is a rational design of cell-permeable proteins achievable? Besides addressing these questions, we will give an overview of a series of recent applications employing cell-permeable Cre recombinase for conditional

mutagenesis in mammalian cells. We will discuss different Cre protein transduction systems with respect to their abilities to target various cells or cell lines, efficacy in vitro and in vivo, as well as potential toxicity.

2 Principles of Protein Transduction

The plasma membrane is the interface between cells and their environment. The cellular uptake of nutrients and signals is a highly restricted and regulated process. High molecular weight, negatively charged, and hydrophilic bio-molecules can cross the plasma membrane only via selective channels and pumps consisting of specialized membrane proteins. Strategies to deliver biologically active macromolecules into cells by overcoming this barrier have been carried out using detergents, lipids, electroporation, microinjection or recombinant vectors. However, these techniques have limitations such as the inability to deliver to primary nondividing cells, low transfection rates, cellular toxicity, unwanted side effects triggered by chemical or physical stimuli, and the risk of permanent genetic modifications. An alternative noninvasive technique is the direct delivery of biological material into mammalian cells employing protein transduction. This recently developed technology is based on an unsuspected ability of the human immunodeficiency virus type 1 (HIV-1) Tat (trans-activator of transcription) protein. Tat is a key regulator of HIV replication and mediates the pathogenicity of the virus (Madore et al. 1993). Green and Loewenstein (1988) and Frankel and Pabo (1988) independently reported that Tat protein is able to directly enter cells in culture and activate transcription. Fawell et al. (1994) demonstrated that a short part (residues 37–72) of the Tat protein, exhibiting a positively charged domain, promotes cell permeability of several full-length cargo proteins such as β-galactosidase (β-gal) and horseradish peroxidase when chemically cross-linked to the protein. The successful intracellular delivery of heterologous protein was verified by the use of enzymatic analysis. A more refined analysis of the Tat transduction domain revealed that an 11 amino acid peptide, comprising residues 47–57, is sufficient to promote cellular uptake of TAT-p27 ^{Kip1}. As anticipated from the physiological function of the unmodified p27 ^{Kip1}protein, the fusion protein was reported to bind and inactivate cyclinE:Cdk2 complexes. Transduction of TAT-p27 ^{Kip1} in hepatocellular carcinoma (Jakarta) cells induced cell migration. The biological active and cell-permeable fusion protein TAT-p27 ^{Kip1} was expressed in and purified from *Escherichia coli* by Ni-affinity chromatography (Nagahara et al. 1998). Tat-derived transduction peptides turned out to be not the only peptides conferring cell permeability. Since the early 1990s, various peptides, now referred to as protein transduction domains (PTDs), were reported to carry diverse bio-molecules in the cytoplasm (Table 1). A 16 amino acid peptide derived from the third helix of the Antennapedia home-

Table 1 Commonly used protein transduction domains (PTDs) and examples of cargos

Name	Sequence	Cargo	Reference
TAT	RKKRRQRRR	β-galactosidase	Schwarze et al. 1999
Penetratin	RQIKIWFQNRRMKWKK	Biotin	Derossi et al. 1994
HSV-VP22	DAATATRGRSAASRPTER PRAPARSASRPRRPVE[a]	Thymidine kinase, eGFP	Elliott and O'Hare, 1997
Transportan	GWTLNSAGYLLGKINK ALAALAKKIL	Polar compounds	Oelke et al. 1998
K-FGF	AAVLLPVLLAAP	GST	Rojas et al. 1998
Oligoarginine	$R_{(4–12)}$	Human catalase	Jin et al. 2001

[a]Domain of HSV-VP22 essential for protein transduction

odomain protein from *Drosophila melanogaster*, also known as penetratin, has been reported to be internalized by neural rat cells (Derossi et al. 1994). To follow the fate of the peptide, a biotinylated version was monitored by fluoresceinated streptavidin (for review see Derossi et al. 1998). Another PTD was derived from the VP22 of herpes simplex virus type 1 (HSV-1; Elliott and O'Hare 1997). HSV-VP22 is part of the viral tegument and secreted from infected cells and able to enter adjacent cells where it translocates into the nucleus. HSV-VP22-mediated protein delivery mostly involves expression in cells, thereby devaluating the advantage of a direct transduction because donor cells have to be transfected. Protein transduction domains are an expanding family, and besides naturally occurring PTDs artificially derived PTDs have also been discovered by direct approaches. Peptides exhibiting membrane-interacting properties have been tested for their ability to translocate across the plasma membrane such as transportan. This synthetic peptide is a fusion of the N-terminal fragment of the neuropeptide galanin with the toxin mastorpan and was reported to translocate biotin (Pooga et al. 1998). Also hydrophobic PTDs have been reported such as the secretion signal sequence of Kaposi fibroblast growth factor (FGF-4) (Lin et al. 1995). In contrast to this hydrophobic 16 amino acid sequence, most other PTDs are highly basic. In fact, the protein transduction potential of arginine-rich peptides turned out to be particularly potent (Wender et al. 2000). Over the past few years, a wide variety of bio-molecules have been transduced into mammalian cells by direct delivery including oligonucleotides (Astriab-Fisher et al. 2000), fluorescent dyes (Bolton et al. 2000, Ho et al. 2001), peptides (Williams et al. 1997, Dostmann et al. 2000), proteins (Schwarze et al. 1999, Caron et al. 2001, Peitz et al. 2002), antibodies (Cohen-Saidon et al. 2003, Heng et al. 2005), nanoparticles (Lewin et al. 2000), and liposomes (Torchilin et al. 2001). Although protein transduction has been widely applied, the mechanism of cellular uptake remains

poorly understood. However, a comprehensive understanding of the molecular mechanism of cellular uptake is necessary to further improve the efficiency of PTD-mediated delivery of bio-molecules into mammalian cells. Various recent studies elucidated some aspects of the translocation mechanism across the plasma membrane and the intracellular distribution of fusion proteins.

2.1 Mechanism of Protein Transduction

2.1.1 Mechanism of PTD-Mediated Internalization

Initially, labeled PTD-fusion proteins have been used for the analysis of the molecular mechanism of PTD-mediated cellular uptake. These studies revealed a rapid, energy- and temperature-independent uptake into the cytoplasm and nucleus (Derossi et al. 1996; Vivès et al. 1997; Prochiantz 2000). Therefore it was hypothesized that the PTD-mediated uptake could be based on a so far unknown receptor- and endosomal-independent pathway. An endocytosis-independent model of Antennapedia-mediated uptake was reported involving membrane shuttling through inverted micelles (Derossi et al. 1998; Fig. 1A). Inverted micelle formation is limited to small cargoes and requires lipophilic amino acids, which are notably absent in TAT (Table 1). For this reason, this model is unlikely to represent an overall PTD-mediated delivery mechanism. Hence a second model representing a common mechanism was proposed by other groups. According to the penetration/refolding model (Fig. 1B), the PTD-fusion protein is translocated across the plasma membrane by direct penetration. This process is thought to be mainly driven by the energy release during intracellular refolding of the denatured exogenous fusion protein (Nagahara et al. 1998; Becker–Hapak et al. 2001). Indeed, according to initial publications, unfolded denatured protein preparations were necessary for efficient uptake. The reported observation that native preparations displayed poor cell permeability strongly argued for the penetration/refolding model (Schwarze et al. 1999). However, nowadays for most of the reported protein transduction applications, native preparations have been successfully used and therefore it is reasonable to doubt that denaturation is essential (Schwarze et al. 2000). It has been reported that complete denaturation of some proteins in 8 M urea or 6 M guanidine hydrochloride leaves the PTD-fusion protein in a state that has high transduction efficiency but a poor rate of refolding (Schwarze et al. 2000). It remains unclear, however, whether this observation can be confirmed in unfixed, living cells. It seems likely that denaturation and refolding is not essential for the transduction process since several very recent studies provide evidence for a different mode of cellular uptake, namely an endosomal PTD-mediated translocation (Fig. 1C). It has been speculated that earlier observations may have been misinterpreted due to fixation artifacts and the highly positive nature of most PTDs (Leifert et al. 2002). Confocal microscopy of fixed cells

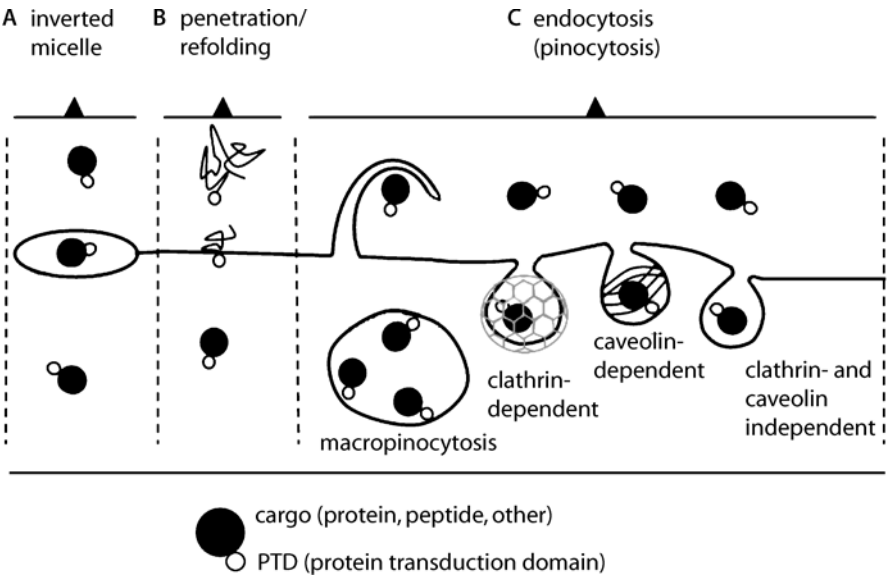


Fig. 1 A–C Different models of cellular uptake of proteins fused to protein transduction domains (PTDs). Translocation across the lipid bilayer was speculated to occur via an inverted micelle mechanism (**A**) or direct penetration of the PTD fusion protein and subsequent intracellular refolding (**B**). However, according to recent studies an endocytotic pathway seems to be most likely (**C**). See text for details

and flow cytometry were mainly used to determine the PTD-mediated cellular uptake and the cellular distribution of labeled protein. Fixation and the highly positive nature of PTDs lead to the assumption of a temperature- and energy-dependent uptake. However, fixatives disrupt the membrane barrier function and may thus increase protein mobility and subsequent redistribution of the PTD-fusion protein to the cytoplasm and nucleus. The high positive charge of the PTD-fusion proteins most likely causes their strong binding to nucleic acids, leading to high accumulation in the nucleus. Energy- and temperature-independent uptake measured by FACS analysis probably results from the nonspecific electrostatic interaction of the highly positively charged PTD and the negatively charged surface of the plasma membrane (Green et al 2003; Richard et al. 2003). Thus, the efficiency of VP22 as a PTD for protein transduction became debatable because of previously misinterpreted observations (Fang et al. 1998; Falnes et al. 2001). Lundberg and Johansson (2001) observed that VP22 as well as VP22-GFP fusion proteins bind to the cell surface and remain attached during washing before fixation. This extracellular VP22 reservoir is released by methanol fixation due to the permeabilization of the cell membrane. Hakkarainen et al. (2005) could not observe intercellular spreading of a fusion protein consisting of VP22, thymidine-kinase, and green fluorescent protein in living cells. In this study, different mammalian

cell lines where analyzed by either fluorescent microscopy and flow cytometry or by negative selection in presence of ganciclovir. In order to avoid artificial observations, Richard et al. (2003) analyzed cellular uptake in living cells by fluorescence microscopy, employing an additional protease digestion step prior to FACS analysis to remove extracellular bound protein. Fluorescein-tagged $(Arg)_9$ peptide and fluorochrome-tagged TAT peptide were transduced into HeLa CHO and Jukart cell lines and analyzed. The cellular uptake of TAT and $(Arg)_9$ at 4°C compared to 37°C was significantly reduced. The internalization of peptides was inhibited with depletion of cellular ATP. The TAT peptide predominantly co-localized with the clathrin-dependent endocytotic marker transferrin. The data obtained strongly suggest a major involvement of endocytosis in the cellular internalization of PTD peptides. These findings are supported by several other reports demonstrating that in presence of metabolic inhibitors such as NaN_3 and low temperature, internalization was significantly decreased (Suzuki et al. 2002; Fittipaldi et al. 2003; Tung et al. 2003; Ferrari et al. 2003; Wadia et al. 2004; Kaplan et al. 2005; Richard et al. 2005).

Despite the probably misinterpreted observations of several early mechanistic studies, the protein transduction technique is a very promising tool for protein delivery, as shown in studies with functional assays. Gius et al. (1999) confirmed the intracellular location of the fusion protein TAT-p16INK4a by the biological function, i.e., inducing cell cycle arrest. Another very prominent example is the derivation of cell-permeable Cre recombinase by Jo et al. (2001) and Peitz et al. (2002). Bioactive TAT-NLS-Cre fusion protein was successfully delivered into fibroblast, primary splenocytes, and murine embryonic stem cells harboring loxP-modified reporter genes. Protein delivery of enzymatic active protein was confirmed by reporter gene activation and, directly at DNA level, by Southern blotting (Peitz et al., 2002; see Sect. 3.3).

2.1.2 Electrostatic Interaction as an Initial Step

According to the endocytosis model, the protein transduction process involves three steps: binding of the PTD-fusion protein, internalization, and endosomal escape (Fig. 2). Electrostatic interaction between the short, highly basic TAT-PTD and the negative charges on the plasma membrane is most likely the initial step for the cellular uptake. Extracellular heparan sulphate proteoglucans (HSPGs) are characterized by a linear polymer structure with repetitive negative charges, which provide efficient binding to basic ligands (Narita et al. 1995; Belting et al. 1999). In fact, the internalization of HIV-1 transactivator of transcription protein has been shown to involve HSPGs and components of low-density lipoprotein receptor family (LRP; Liu et al. 2000). Various studies provide evidence that HSPGs play a key role for protein transduction as cell surface receptors for TAT-peptides (Tyagi et al. 2001) and arginine-rich peptides (Suzuki et al. 2002) such as penetratin (Ghibaudi et al. 2005). Soluble heparin

acts as a competitive inhibitor and heparinase III, which specifically degrades HS chains, resulting in decreased internalization efficacy. Antennapedia and TAT-PTD internalization was also reduced in mutant cell lines deficient in HS or glycosaminoglycan synthesis (Silhol et al. 2002; Mai et al. 2002; Console et al. 2003; Richard et al. 2005). These observations indicate that initial electrostatic interaction is mainly HSPG-dependent. However, due to the lack of complete inhibition by competition, enzymatic digestion, and mutant studies, it may be possible that electrostatic binding is not the only cell surface-peptide interaction.

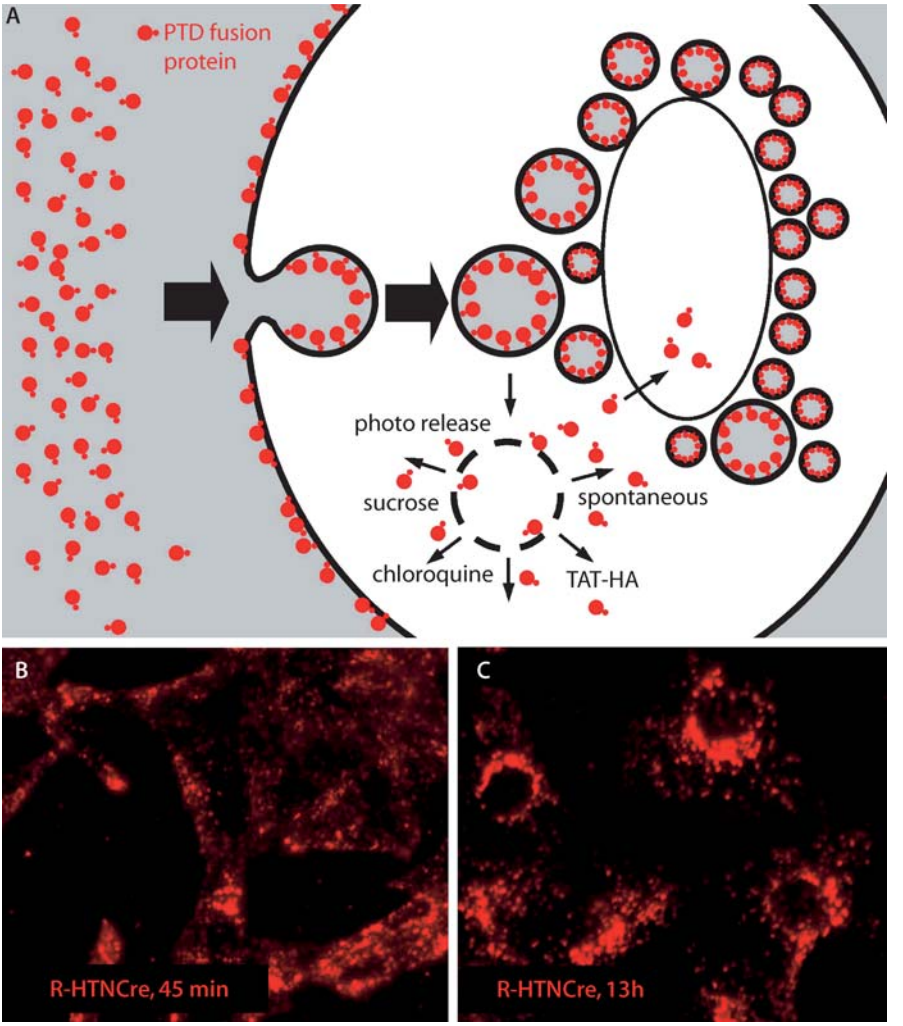
2.1.3 PTD-Mediated Internalization

The initial electrostatic interaction triggers the internalization most probably via endocytosis. Several studies extensively analyzed endocytotic pathways by employing specific metabolic inhibitors and markers as validation tools. Multiple endocytotic pathways exist in mammalian cells. Endocytosis can be divided into phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis is primarily utilized by dendritic cells, macrophages, phagocytes, and neutrophils. In contrast, pinocytosis is performed by essentially all mammalian cells, internalization occurs via clathrin-dependent endocytosis, caveolin-dependent endocytosis, clathrin- and caveolin-independent endocytosis, or macropinocytosis. These pathways differ in their dependence on clathrin, caveolin, and dynamine (Fig. 1C; Conner et al. 2003). In these apparently distinct pathways, some components can be shared (Kirkham and Parton 2005), complicating the interpretation of inhibitor studies. This complex interaction of molecular players represents one of the reasons why the involved pathways are still a matter of debate.

Fig. 2 A–C Cellular entry of TAT fusion proteins. **A** According to an endocytotic three-step model, TAT fusion proteins first bind through electrostatic interaction to the cell surface. Second, subsequent cytoplasmic accumulation results in persistent endosomal vesicles in the perinuclear region. Third, small quantities of PTD fusion protein are released in the cytoplasm by a thus far unknown mechanism. Approaches to bypass the limited intracellular trafficking aim at artificially enhancing the cytosolic release of PTD fusion proteins by disrupting endosome integrity such as application of fusogenic peptide TAT-HA, sucrose, chloroquine, or photo release. **B, C** Fluorescence microscopy analysis of live fibroblasts treated with Rhodamine-labeled cell-permeable His-TAT-NLS-Cre recombinase (R-HTNCre). **B** 45 min after protein transduction most of the R-HTNCre is bound to the cell surface. **C** 13 h after transduction the fusion protein is accumulated around the nucleus visible as punctuate structures. Notably, despite the observation that apparently most of the Cre fusion protein is trapped in endosomal vesicles, spontaneous endosomal release and subsequent nuclear translocation is sufficient to induce Cre-mediated recombination in virtually 100% of the cells

2.1.3.1 Lipid Raft-Mediated Macropinocytosis

Wadia et al. (2004) reported a lipid raft-dependent uptake of a TAT-Cre fusion protein into endosomal vesicles. In this study, the authors used TAT-Cre-mediated recombination of a loxP-STOP-loxP eGFP reporter gene in live mouse reporter T cells. The induction of reporter eGFP served as a functional assay confirming the cytoplasmic release and the subsequent translocation into the nucleus. The labeled TAT-Cre co-localized with FM4-64 a fluorescent marker for general endocytosis. Cholesterol depletion induced by β-cyclodextrin and



nystatin led to a nearly complete inhibition of TAT-Cre uptake, whereas FM4-64 uptake was only decreased up to 20%. Caveolin dependency was excluded because TAT-Cre did not co-localize with labeled caveolin-1 and co-transfection with a dominant negative mutant form of Dyn^{K44A} did not block endosomal vesicle formation. These observations suggest macropinocytosis as an uptake mechanism, which involves the internalization of large (approximately 1 µm diameter) areas of plasma membrane together with significant amounts of fluid. This is in line with a study by Kaplan et al. (2005), who proposed a lipid raft-dependent mechanism at least for the uptake of short TAT peptides (1,000–5,000 kDa). Nakase et al. (2004) reported an inhibition of fluorescent labeled octa-arginine uptake by employing macropinocytosis inhibitors such as EIPA and wortmannin, suggesting that macropinocytosis may be at least a contributing process. However, penetratin and other oligo-arginine uptake was unaffected by these inhibitors, indicating a distinct pathway for these PTDs.

2.1.3.2 Caveolin-Mediated Endocytosis

Fittipaldi et al. (2003) demonstrated an inhibition of TAT-eGFP uptake by cholesterol depletion and cytochalasin D treatment inhibiting actin polymerization. In addition, TAT fusion proteins co-localized with caveolin-1 but not with markers of clathrin-dependent endocytosis such as fluorescein-tagged transferrin, thus endorsing a lipid raft-dependent caveolar endocytosis. According to Ferrari et al. (2003), GST-TAT-eGFP-positive vesicles were initially confined to the periphery of the cell, forming a ring close to the plasma membrane. These punctuate vesicles were found by other groups as well (Fawell et al 1994; Sandgren et al. 2004). The vesicles move subsequently inward and reach the perinuclear region, where they persist as long-term accumulation surrounding the nucleus. As a result of co-localization assays and studies employing specific inhibitors, Ferrari and co-workers proposed that these vesicles were caveolar endosomes (Ferrari et al. 2003).

2.1.3.3 Clathrin-Mediated Endocytosis

In contrast to previous studies, Alexa-labeled TAT co-localized with a marker of clathrin-dependent endocytosis and the cellular uptake was not affected by the inhibition of caveolin-dependent endocytosis (Richard et al. 2005). To explain this apparent discrepancy, the authors suggest that free TAT peptide and TAT conjugated macromolecules utilize different endocytotic pathways. Furthermore, the drug monensin, which is an inhibitor of endosome acidification, increased the fluorescent signal of FITC-labeled TAT, exploiting the fact that the fluorescence of FITC is significantly decreased at pH 5–6. The increased

signal after monensin treatment indicates that a significant fraction of the TAT peptide is delivered into acidic cellular compartments. The latter observation provides further evidence for the hypothesis that TAT peptides utilize different pathways, thereby bypassing the acidic compartments and subsequent acidic hydrolysis (Tünnemann et al. 2006).

2.1.4 Different Cargoes Use Different Entry Pathways

In conclusion, these recent observations imply that the majority of PTD-mediated uptake results from an initial electrostatic interaction between the positively charged PTD and the plasma membrane followed by endocytotic internalization. The subsequent endocytotic pathway may vary depending on the cell type, the biochemical nature of the PTD, and the cargo protein. The lack of complete inhibition by selective drugs and incomplete colocalization with specific markers favors a model of internalization combining several different pathways (Tünnemann et al. 2006). A single general mechanism for all PTD-fusion proteins does not seem reasonable and more than one mechanism for a unique peptide is a possibility. Nevertheless, alternative energy- and receptor-independent mechanism cannot formally be entirely eliminated (Green et al. 2003). Another assumption describes the TAT PTD as an opportunistic peptide adhering to the cell surface and then being internalized through natural cell membrane recycling by any endocytotic mechanism (Brooks et al. 2005). Comparisons between the different reports are difficult due to the wide diversity of the fused cargo molecules. Further studies are required to elucidate the influence of the cargo, in particular its size, biochemical composition, the linker region between PTD peptide and cargo, and its orientation on the involved entry mechanism. A different approach to increase the efficiency of the protein transduction is the use of drugs supporting cytoplasmic release of PTD fusion proteins.

2.2 Approaches to Overcome the Bottleneck of Endosomal Release

As described previously, PTD-mediated cellular uptake results in persistent endosomes sequestering in the periphery of the nucleus. This poor intracellular trafficking is presumably a major limiting factor of the protein transduction technique (Caron et al. 2004; Wadia et al. 2004). Using a Cre/loxP-based functional assay, Caron and colleges reported an increased delivery of TAT-NLS-Cre into the nucleus employing lysosomotropic agents such as chloroquine and sucrose, which are known to disrupt endosome integrity. For the same reason, Wadia et al. (2004) developed a transducible, pH-sensitive, fusogenic dTAT-HA2 peptide that is able to enhance TAT-Cre escape from endosomes referred

to as macropinosomes. Another approach to achieve endosomal disruption is photo release according to Matsushita et al. (2004). In this study, the authors reported that endosomal release of transduced FITC-11 arginine-protein (11R-PTD), TAT-PTD, penetratin-PTD, and functional PTD-fused protein such as FITC-11R-p53 could be artificially increased by exposure to fluorescent light. These reports reveal that the cytosolic release of PTD fusion proteins represents a bottleneck of protein transduction, which can be bypassed by disrupting the endosome integrity (Fig. 2).

3 Conditional Mutagenesis Employing Cell-Permeable Site-Specific Recombinases

3.1 Site-Specific Recombinases

For the most part, two members of the λ integrase family of site-specific recombinases are used for the manipulation of mammalian genomes: Cre (causes recombination) from bacteriophage P1 and Flp (flips DNA sequences) from *Saccharomyces cerevisiae* (Rajewsky et al. 1996; Nagy 2000; Lewandoski 2001; Branda and Dymecki 2004). Since Cre and Flp recombine DNA without accessory factors, these recombinases are particularly useful in heterologous systems. Cre and Flp catalyze recombination of DNA between specific target sequences or recognition sites designated as loxP- (locus of crossover in P1) and FRT-sites (Flp recombinase recognition target), respectively (Hoess et al. 1982; McLeod et al. 1986). Both recombinases share a common mechanism of DNA recombination that involves strand cleavage, exchange, and ligation (Sadowski 1995). The 34-bp recognition sites share an overall structure of two inverted repeats and an 8-base pair core or spacer region. This core confers the directionality of the recombination site. The relative orientation of recombination sites with respect to one another determines the product of the recombination reaction. Cre and Flp catalyze the recombination of two directly repeated recognition sites by excising a circular molecule, resulting in the deletion of the sequence flanked by the recognition sites. This reaction can be used to inactivate genes either by deleting the promoter or simply by deleting essential regions of the gene. However, in a different experimental setting SSRs are also able to activate genes by deleting transcriptional stop sequences that have been introduced previously. If placed in the inverted direction, recombination of two loxP sites results in inverting the flanked sequence. This can be used to switch between two different promoters for one gene. Inversion, however, results in two loxP sites that are indistinguishable from the original loxP pair due to the conservative nature of the recombination event, and recombination continues as long as Cre is present. By using a pair of mutated loxP sites (lox66 and

lox71), the inversion has been shown to work in an almost irreversible manner (Oberdoerffer et al. 2003). There are also other applications beyond conditional gene expression that have been published such as cassette exchange, translocations, or integrations (for review see Bode et al. 2000) demonstrating the efficiency of engineering mammalian genomes by SSR. One main limitation of genetic switching using Cre recombination alone is the irreversibility of the process (resulting from the loss of the circular reaction product). Switching a gene *on* by a SSR mediated reaction leaves a single loxP sequence in the locus with no further possibility of recombination required, for instance, to switch the gene *off* after a given period of time. This limitation can be overcome by combination of two SSR activities such as Cre and Flp. Dual recombinase strategies also enable higher order recombination reactions in more complex genetic settings.

3.2 Conditional Mutagenesis and Inducible Recombination

A gene alteration in the germ line of a mouse may cause an embryonic lethal phenotype resulting in no viable mouse to study the gene function. Moreover, a mutation may lead to multiple effects in different tissues and cell types, resulting in a complex phenotype making it difficult to dissect the gene function in a particular cell type from secondary effects in other cell types. Conditional mutagenesis employing Cre/loxP-recombination has been developed to overcome these limitations (Gu et al. 1994). An increasing number of studies have demonstrated the efficacy of Cre-mediated conditional mutagenesis in mice and cell lines (for review see Lewandoski 2001; Branda and Dymecki 2004). Both Cre and Flp have been used to gain control over the mutation in a spatial (Orban et al. 1992; Lakso et al. 1992; Gu et al. 1994) and/or temporal manner (Kühn et al. 1995; Metzger et al. 1995). Usually a mouse or cell line is generated, in which an essential part of the gene of interest is flanked by two loxP sites (floxed). The loxP sites represent Cre recombination recognition sites and can be used to delete the respective gene segment upon Cre recombination, resulting in a conditioned inactivation or mutation of the gene of interest. In order to gain temporal control over this mutation event, two different approaches have been used (Fig. 3): (a) Cre is delivered into cultured cells either by transfection (Torres and Kühn 1997) or adenoviral infection (Rohlmann et al. 1996; Shibata et al. 1997); (b) Cre recombinase activity is induced by application of an exogenous inducer. Induction can be carried out either at the transcriptional level (e.g., Mx-Cre; Kühn et al. 1997) or tetracycline-controlled Cre expression (Utomo et al. 1999) or at the post-translational level employing fusion proteins of Cre with mutated ligand-binding domains (LBDs) of steroid receptors (Metzger et al. 1995; Logie and Stewart 1995; Feil et al. 1996). Although a number of studies have demonstrated the efficacy of Cre-mediated inducible

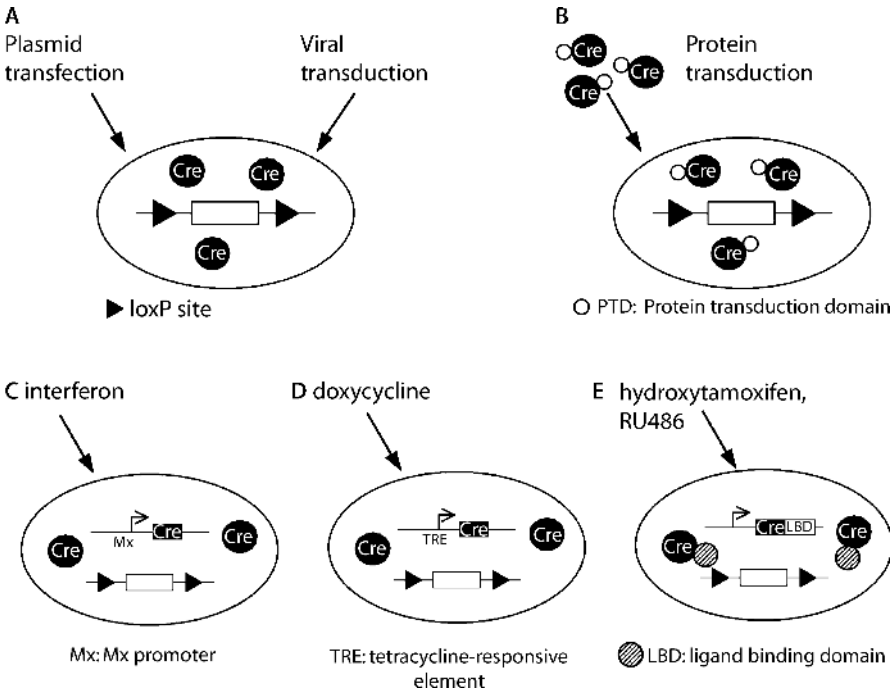


Fig. 3 A–E Overview of experimental strategies to induce Cre recombinase activity in cells. Site-specific recombinases such as Cre can be used to gain control over a mutation in a spatial and temporal manner. Cre recombinase activity in mammalian cells can be induced by (**A**) gene or (**B**) protein transfer. Alternatively, stable Cre transgenic lines can be activated either at the (**C, D**) transcriptional or (**E**) post-translational level. See text for details

mutagenesis, this experimental strategy is still limited by several drawbacks such as leakiness and restricted efficiency. With respect to the use in vivo, extensive mouse breeding causes the experiments to be time-consuming and costly. Moreover, the widely used inducers interferon, hydroxy-tamoxifen, and doxycycline are known to have toxic side effects (Danelian et al. 1998; Vasioukhin et al. 1999) and/or induce unwanted physiological effects that may interfere with the experimental phenotype of the conditional mutation to be analyzed (Lin et al. 1998; Kuzin et al. 2001). In cultured cells, particularly in primary cells, Cre-mediated recombination is limited by poor transfection efficiencies and putative toxicity of the protein (Torres and Kühn 1997; Loonstra et al. 2001; Silver and Livingston 2001). Thus traditional delivery of Cre – by Cre transgenics, viral vectors, or transfection – represents a limiting step of conditional mutagenesis employing Cre/loxP technology. Moreover, any gene transfer method carries the risk of insertional mutagenesis. Ectopic chromosomal integration of foreign DNA is able to disrupt the expression of tumor suppressor genes or activate oncogenes (Glover et al. 2005), resulting in the

malignant transformation of cells, as has been shown in a recent clinical trial (Hacein-Bey-Abina et al. 2003). Protein transduction may help to circumvent these drawbacks by avoiding the requirement of a further genetic manipulation simply by adding modified recombinant protein to the medium (Fig. 3B).

3.3 Engineering a Cell-Permeable Cre Recombinase

PTD-driven induction of Cre-mediated recombination was initially assessed by a chimeric protein consisting of a nuclear localization sequence (NLS), the Cre recombinase, and the Kaposi fibroblast growth factor PTD, as well as an amino-terminal histidine sequence for purification from bacteria (Jo et al. 2001). This fusion protein, designated as NLS-Cre-MTS (MTS for membrane translocation sequence), induced recombination in up to 80% of T lymphocytes in vitro. However, it remained unclear from this study whether the MTS peptide is essential for transduction or even contributes significantly to the cellular uptake of Cre. Another study aimed at evaluating the actual potency of two prominent PTDs, namely FGF and TAT peptides, to promote the translocation of biologically active Cre across the plasma membrane of mammalian cells (Peitz et al. 2002). For control reasons, the potentials of the recombinant proteins to transduce and subsequently recombine loxP-flanked targets in mammalian cells were compared side by side with Cre lacking any particular PTD. It turned out that Cre recombinase carrying a His-tag only exhibits a weak intrinsic potential to transduce into cells. However, the transduction efficiency is very low unless fused to an NLS. Unexpectedly, the fibroblast growth factor-derived MTS peptide turned out to be dispensable for transduction, whereas fusion with the TAT peptide significantly enhanced cellular uptake and subsequent recombination. The improved version of cell-permeable Cre, designated His-TAT-NLS-Cre (HTNCre in brief), resulted in recombination efficiencies of virtually 100% in fibroblast cells as well as murine (Peitz et al. 2002) and human embryonic stem (ES) cells (Nolden et al. 2006). HTNCre induces recombination in a strictly time and concentration-dependent manner. The only recombinant protein analyzed in this comparative study displaying no or weak transduction potential was Cre-His, i.e., the only construct without NLS. This is in line with observations reported in two other studies (Will et al. 2002; Lin et al. 2004). However, the NLS and/or TAT PTD sequence considerably enhanced cell permeability. From this observation, one can conclude that the basic, SV40 type NLS itself can function as a PTD at least in the context of Cre recombinase (Fig. 4). This conclusion is consistent with the observation that basic peptides in general are able to enhance cellular uptake of heterologous proteins or peptides (Wender et al. 2000; Matsushita et al. 2001; Han et al. 2001). Table 2 gives an overview to several TAT-Cre fusion proteins that turned out to be transducible into various mammalian cells, including cell lines and primary cells.

Fig. 4 The pTriEx-HTNCre expression vector (Peitz et al. 2002) encodes a fusion protein consisting of a histidine tag (*H6*), a protein transduction domain derived from HIV Tat (*TAT*), an NLS sequence, and the Cre recombinase. The amino terminal sequence of the fusion protein is given at amino acid level, the TAT PTD is underlined. The expression cassette is driven either by a CAG promoter for expression in mammalian cells (for transfection control experiments) or T7 Lac op promoter (for the bacterial overexpression of the fusion protein)

Table 2 Spectrum of cells amenable to Cre protein transduction in vitro and ex vivo

Target cell	Recombination efficiency	Quantification method	Reference
Thymoma cell linie	Up to 80%	Flow cytometry	Jo et al. 2001
Fibroblast line CV1–5B	> 95%	Southern blot	Peitz et al. 2002
Fibroblast line SC-1	> 50%	Flow cytometry	Will et al. 2002
COS7	ND	ND	Lin et al. 2004
NIH3T3	ND	ND	Lin et al. 2004
Embryonic fibroblasts	> 95%	Southern blot	Joshi et al. 2002
Murine ES cells	> 95%	Southern blot	Peitz et al. 2002
	Very high	Southern blot	Joshi et al. 2002
Primary splenocytes	70%	Southern Blot	Peitz et al. 2002
	50%	LacZ/X-Gal	Jo et al. 2001
Splenic B cells	Up to 67%	Southern blot	Peitz et al. 2002
Splenic T cells	Up to 54%	Southern blot	Peitz et al. 2002
Lymphocytes	Up to 77%	PCR	Demuth et al. 2004
Bone marrow cells	ND	ND	Will et al. 2002
Erythroblastic leukemia line	> 95%	Flow cytometry	Joshi et al. 2002
Postmitotic neurons	> 80%	Southern blot	Haupt et al. 2006
Human ES cells	> 95%	Southern blot	Haupt et al. 2006

ND, not detected

3.4 Conditional Mutagenesis in Cultured Cells by Cre Protein Transduction

Cre protein transduction is highly useful in ES cells not only for the rapid and simple removal of loxP-flanked selection markers, but also for the analysis of gene functions. Cre protein transduction has been used to drive conditional mutagenesis of loxP-modified alleles in various mammalian cell lines (Table 3). TAT-Cre fusion proteins were used to conditionally ablate the B cell antigen receptor ex vivo (Oberdoerffer et al. 2003), the immunoglobulin heavy chain (Iwakoshi et al. 2003), and the nibrin gene (Demuth et al. 2004; Kracker et al. 2005). A model of HTNCre-inducible mTOR (mammalian target of rapamycin) inactivation in ES cells has been reported, revealing a central role of mTOR for cell growth and proliferation (Murakami et al. 2004). In fact, in our laboratory every ES cell line analyzed so far could be modified genetically by HTNCre transduction at low, seemingly nontoxic concentrations, with at least a 90% efficiency (FE et al., unpublished results). HTNCre treatment seems not to interfere with proliferation and viability. Toxicity was observed only at concentrations beyond levels sufficient for maximal recombination. Recently, we demonstrated that HTNCre treated ES cells were still able to contribute to the germ line after blastocyst injection (M. Peitz and F. Edenhofer, unpublished results). This observation indicates that HTNCre transduction does not interfere with the developmental potential of ES cells.

3.5 Cre Protein Transduction In Vivo

3.5.1 Cell-Permeable Cre Protein Displays Limited Activity In Vivo

The potential of Cre protein transduction in vivo was assessed in various studies employing different versions of cell-permeable Cre protein (see Table 4). Jo et al. (2001) used the ROSA26R mouse line carrying a Cre-inducible LacZ reporter gene for intraperitoneal and intravenous injection of cell-permeable Cre fractions. Three days after injection, widespread and strong β-gal activity was observed in all organs examined such as liver, heart, kidney, lung, spleen, and brain, indicating that recombinant Cre is even able to cross the blood–brain barrier. β-galactosidase expression in Cre-injected animals was highest in regions surrounding blood vessels in brain and liver sections but was more evenly distributed in the kidney, consistent with systemic delivery of Cre through the bloodstream. No significant difference was observed between intraperitoneal and intravenous injection. The efficiency of recombination was assessed by analyzing β-galactosidase activity in splenocytes and thymocytes by flow cytometry 5 days after injection. The authors report up to 51% and 37% β-galactosidase-positive thymocytes and splenocytes, respec-

Table 3 Examples of conditional mutagenesis in vitro and ex vivo employing cell-permeable Cre recombinase

Target allele	Target cell	Result	Reference
B cell antigen receptor (BCR)	Splenocytes ex vivo	Irreversible Cre-lox66/lox71-mediated genetic switch	Oberdoerffer et al. 2003
Bl-8	Splenic B cells ex vivo	Inducible inactivation of Ig heavy chain in B cells ex vivo	Iwakoshi et al. 2003
Mammalian target of rapamycin (mTOR)	Murine ES cells	Conditional inactivation of mTOR results in reduced cell size and proliferation arrest	Murakami et al. 2004
NBS1 gene, encoding nibrin	Lymphocytes ex vivo, embryonic fibroblasts	Induction of nibrin-null mutation leads to the loss of the G2/M checkpoint	Demuth et al. 2004
Inducible diphtheria toxin receptor (DTR)	Murine ES cells	Cre-inducible diphtheria toxin receptor-mediated cell lineage ablation	Buch et al. 2005
NBS1 gene, encoding nibrin	B lymphocytes	Nibrin inactivation in activated B cells leads to increased ɣ-irradiation-induced DNA damage	Kracker et al. 2005
Hen egg lysozyme (HEL) transgene	B lymphocytes	Basal immunoglobulin signaling actively maintains developmental stage in immature B cells	Tze et al. 2005

tively (Jo et al. 2001). However, in this study recombination was determined indirectly only, by activation of a LacZ reporter gene. It might well be that there is some recombination activity in vivo and this activity could be strong enough to activate reporter genes in some experimental settings. However, the recombinase activity might be insufficient to result in recombination of the loxP targets in the majority of the cells. To rule out the possibility that such an indirect read-out system could lead to an overestimation of the actual recombination efficiency, another study directly assessed the recombination product at the DNA level by Southern blot analysis. Although using three different mouse lines, each containing different loxP-modified loci, only poor recombination efficiency was detected by Southern blot after either in-

Table 4 Studies aimed at application of Cre protein transduction in vivo

Mode of application	Recombination	Quantification method	Reference
Mice i.p., i.v.	High efficiency, many organs such as liver, heart, spleen, brain	LacZ/X-Gal, LacZ/Flow cytometry	Jo et al. 2001
Mice i.p., i.v.	Poor efficiency, some organs; approx. 10%	Southern blot	Peitz et al. 2002
Mice i.p.	Undetectable, various tissues examined	Southern blot	Joshi et al. 2002
Mice s.c.	Detectable in epidermis, hair, dermis, muscle, adipose tissue	LacZ/X-Gal	Yu et al. 2003
Mice i.p.	Undetectable in liver	PCR	Sun et al. 2005

i.p., intraperitoneal injection; i.v., intravenous injection; s.c., subcutaneous injection

traperitoneal or intravenous injection of cell-permeable Cre. A weak signal was observed representing approximately 10% deletion in a single mouse in the peritoneum close to the site of injection (Peitz et al. 2002). This study unambiguously provided a proof-of-concept of Cre protein transduction in vivo; however, the overall efficiency is limited, most likely because of poor diffusion and/or stability of the Cre fusion protein. In fact, two other studies failed to report successful Cre recombination after in vivo transduction of Cre protein. Joshi et al. (2002) injected adult ROSA26 LacZ reporter mice intraperitoneally with 1 mg of cell-permeable Cre on four consecutive days and then examined various tissues for deletion. No deletion at all was detected by Southern blot analysis in this study. Sun et al. (2005) were unable to detect recombinase activity in liver of transgenic mice following intraperitoneal injection. However, the particular fusion proteins used in these different studies are not completely identical with respect to the amino acid sequence, and they originate from different preparation modes; in particular the protocol used by Joshi et al. (2002) involves denatured protein preparations. These differences might affect stability and, more generally, pharmacokinetics, in vivo.

3.5.2 Influence of Serum on Cre Protein Transduction

In fact, there is evidence that recombinase activity of TAT-modified Cre fusion proteins is inhibited by serum proteins. The data presented by Jo et al. (2001) and Peitz et al. (2002) with regard to in vitro transduction originates from experiments under serum-free culture conditions. Two other reports assessed the influence of serum components on Cre protein transduction. Joshi et al. (2002) report that Cre transduction works best in serum-free medium. In the presence of 1% FCS and bovine serum albumin, a minor inhibitory effect was observed. However, addition of 10% FCS resulted in substantial inhibition, i.e., a three times higher concentration was needed to induce half-maximal efficiency (Joshi et al. 2001). These data are in line with a report by Lin et al. (2004) who demonstrate that Cre transduction is inhibited by up to 60% in media containing 10% FCS. Interestingly, mouse serum has an even stronger effect, inhibiting Cre transduction efficiency by up to 80%. The authors of this study state that serum inhibits protein transduction specifically, since they did not observe an effect on either the stability or activity of the modified Cre protein without showing the data (Lin et al. 2004). However, there is also one report on Cre protein transduction where the authors successfully used the protease inhibitor leupeptin to enhance Cre activity, indicating the limited stability of modified Cre recombinase (Yu et al. 2003). Experiments from our group confirmed this view since we observed that the FCS-inhibiting effect could be partially compensated by leupeptin in cell culture (M. Peitz and F. Edenhofer, unpublished results).

3.5.3 Applications of In Vivo Cre Protein Transduction

Even though serum components inhibit Cre protein transduction, it must be pointed out that even in the presence of 10% FCS virtually 100% recombination efficiency can be achieved if sufficient TAT-modified Cre is added to the culture medium (Joshi et al. 2002; M. Peitz and F. Edenhofer, unpublished results). Whether or not this kind of overcompensation is also possible with regard of an in vivo application remains to be investigated and so far has been limited by the maximal achievable concentration of purified Cre stock solutions available. Very recent results from our group indicate that indeed, by using enhanced highly concentrated HTNCre preparations, one can significantly improve protein transduction in vivo with respect to efficiency and accessibility to various tissues (M. Peitz and F. Edenhofer, unpublished results). In conclusion, there is convincing data that Cre protein transduction works in vivo as well. However, the overall efficiency seems to be far from what can be induced in cultured cells, at least in its present state. This find-

ing precludes Cre protein transduction from the use in mouse models where highly efficient recombination is desired. However, for some experimental settings this mode of Cre in vivo delivery represents an ideal mode of Cre induction, since it provides an invaluable advantage over other inducible Cre systems, i.e., there is definitely no Cre recombinase activity before induction (Fig. 3B). Besides Cre protein transduction, there are two main strategies to induce Cre recombinase activity in living cells, i.e., Cre transfection and viral transduction (Fig. 3A), or ligand-inducible Cre activation (Fig. 3C–E). The latter is carried out by modifying cells genetically with inactivated Cre constructs. Cre activity is induced by the application of an exogenous inducer either at the transcriptional level (Fig. 3C, D) or at post-translational level (Fig. 3E). Unfortunately, the use of these inducible systems is hampered by the leakiness of the systems, resulting in unwanted premature Cre activity. The leakiness of the system is a critical factor because a Cre recombinase that is undesirably active before induction often leads to unwanted side effects such as mosaic recombination and/or selection of recombined or nonrecombined cells both in vivo and in vitro (Kellendonk et al. 1999; Fuhrmann-Benzakein et al. 2000; Minamino et al. 2001). In contrast, the Cre protein transduction system is not limited by undesired premature inactivation since before application there is no form of Cre present in the cell, neither at the genomic nor at the protein level. In other words the conditional genetic switch is unambiguously switched off before induction. Thus, particular applications of conditional mutagenesis that do not tolerate any unwanted recombination before induction, even in very rare cells, such as analyzing tumor inducing factors in vivo, can profit from Cre protein transduction technology. Also, local in vivo application of Cre protein represents a promising technology for conditional mutagenesis in vivo. In fact, subcutaneous injection of Cre protein has been used to study the role retinoblastoma (Rb) protein during oncogenic transformation in adult mice (Yu et al. 2003). Between 1.25 and 5 µg cell-permeable Cre protein was injected into the dorsal skin at the hindlimb of ROSA26R reporter mice, resulting in significant levels of recombination as determined by X-Gal staining (Yu et al. 2003). LacZ expression was detected in the epidermis, hair, dermis, muscle, and adipose tissue, indicating that TAT-modified Cre allows recombination of LoxP-flanked DNA segments in multiple cell types after subcutaneous injection. Then the authors injected Cre protein into mutant RbLoxP/LoxP mice in order to inactivate Rb in the skin. Subcutaneous administration of a single dose of TAT-Cre or control TAT-β-gal proteins into small, demarked areas of RbLoxP/LoxP P10 mice resulted in normal timing of hair growth and hair follicle activation during the postnatal cycle in both control and experimental regions, indicating that normal growth had occurred. However, despite showing normal hair morphology, the hair shafts of TAT-Cre-treated RbLoxP/LoxP mice were severely depigmented, indicating that somatic deletion of Rb results in depigmentation of hair shafts as a consequence of melanocyte loss.

These results demonstrate that local application of cell-permeable Cre protein can serve as an efficient tool for conditional mutagenesis even in adult mice.

3.6 Other Applications of Protein Transduction in Conditional Mutagenesis

Conditional mutagenesis can be further refined by the application of two site-specific recombination systems. One recombinase can be used for selection marker deletion in vitro, the other recombinase could be exploited for conditional mutagenesis in vivo. Very recently, our group developed a cell-permeable version of another commonly used site-specific recombinase, Flp, by protein transduction technology. The recombinant TAT-Flp fusion protein can be readily expressed in and purified from *E. coli* using Ni-(II)-affinity chromatography. TAT-Flp induces recombination of FRT sites in fibroblast cells and ES cells with high efficiency (F. Edenhofer et al., unpublished results). Transducible FLP in combination with cell permeable Cre will greatly expand our ability to manipulate mammalian genomes by dual recombination strategies. Another interesting application is the development of a cell permeable tet repressor (Mortlock et al. 2003).

4 Conclusions and Perspectives

The combination of two powerful technologies, the Cre/loxP recombination system and protein transduction technology, holds great promise for the advancement of biomedical and genome research by enabling precise control over mutation events. Direct delivery of site-specific recombinases circumvents the introduction of nucleic acids into cells, thereby eliminating the risk of insertional mutagenesis and unwanted premature Cre activation. The Cre protein transduction system provides a reliable model to study factors that influence the delivery of proteins since recombination of genomic DNA provides a stable and unambiguous read-out of protein uptake. Despite the fact that Cre protein transduction represents a highly attractive technology to modulate cellular functions in vitro, there are two main limitations for the application in vivo: poor transduction efficiency and lack of organ and/or cellular specificity. Future studies will exploit the potential of additional functional peptides in order to overcome these hurdles. Serum-binding factors may improve the stability as well as enhance pharmacokinetics in vivo. Specific peptides, e.g., identified by phage display, could be used to target cell-permeable fusion proteins to specific cells.

The protein transduction technology holds great promise for the advancement of biomedical research enabling modulation of cellular functions without genetic alteration. So far, mainly DNA transfection and viral transduction are used to introduce genes into cells, carrying the risk of introducing unwanted and/or harmful genetic material and having poor control over levels of transgene expression. By contrast, protein transduction provides a new paradigm for the analysis of mammalian gene functions in living cells. Protein transduction offers precise control over time and dose of intracellular levels of proteins in a reversible manner. However, there are several limitations to the use of protein transduction technology. A rational design of an effective protein transduction system can be achieved only with a comprehensive understanding of the mechanism of cellular uptake. However, as discussed in this review, this mechanism remains elusive and according to recent findings seems most likely to be a complex combination of several cellular processes such as caveolin-, or clathrin-mediated endocytosis and macropinocytosis. Moreover, the mechanism might differ among the various PTD types and the biochemical nature of the cargo molecule as such, which adds an additional level of complexity to the rational design of cell-permeable proteins. Consequently, the process of developing potent transduction systems is still largely empirical. The effect of different PTDs on different proteins is difficult to predict in advance and therefore a series of fusion constructs have to be tested in an elaborate manner. Some recombinant fusion proteins display poor solubility in physiological buffers, others may not exert biological function. Screening of vast numbers of different constructs by using high throughput expression, purification, and, ideally, functional cellular read-outs could enhance protein transduction technology in the future. According to recent studies, the range of proteins and cell types as well as transduction efficiency as such could be enhanced by strategies aimed at increased endosomal release. In this respect, future studies will show whether the application of fusogenic peptides or photo release is of general use.

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Part II
Examples of Conditional Disease Models

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Analysis of Mouse Development with Conditional Mutagenesis

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Abstract Explorations into the molecular embryology of the mouse have played a vital role in our understanding of the basic mechanisms of gene regulation that govern development and disease. In the last 15 years, these mechanisms have been analyzed with vastly greater precision and clarity with the advent of systems that allow the conditional control of gene expression. Typically, this control is achieved by silencing or activating the gene of interest with site-specific DNA recombination or transcriptional transactivation. In this review, I discuss the application of these technologies to mouse development, focusing on recent innovations and experimental designs that specifically aid the study of the mouse embryo.

Keywords Embryonic development · Tissue-specific gene inactivation · Genetic ablation · Fate Map

1 Introduction

In classical genetics, a conditional mutation encodes a product with wild type activity under one set of conditions and with altered (usually reduced) activity under another. The most common examples are microbial temperature-sensitive mutations, which encode products that lose activity with increased temperature. However, as mice are homeothermic, regulating body tempera-

ture, this approach is not available to mouse geneticists. Our solution to this dilemma has been to develop various binary transgenic or "bigenic" approaches, in which the desired phenotype is due to the interactions between two transgenes or alleles (reviewed in Lewandoski 2001). Such interactions generally result in either transcriptional transactivation or DNA recombination of a target gene, converting it to an active or inactive form. These approaches are specific in as far as the machinery involved (e.g., Cre or FLP DNA recombinases or the Tet repressor) is of microbial origin and therefore unlikely to recognize wild-type murine DNA sequences or proteins; usually only planned interactions should occur. [However, the reader should be aware that although such unplanned interactions can sometimes occur with the mammalian genome (Schmidt et al. 2000; Loonstra et al. 2001), such effects are not so likely as to discourage the use of these technologies].

Many excellent reviews (Nagy 2000; Lewandoski 2001; Dymecki et al. 2002; Gossen and Bujard 2002; Sauer 2002; Berger and Bujard 2004; Branda and Dymecki 2004; Toniatti et al. 2004; Garcia-Otin and Guillou 2006 and see the chapters by R. Feil and by R. Sprengel and M.T. Hasan, this volume) are available that focus on the details of these technologies. Here, I shall assume the reader is familiar with the variations of molecular machinery that make conditional gene expression possible and shall refer to such details only when relevant to the primary subject, which is the use of these approaches to address questions in mouse embryonic development that cannot otherwise be answered. Accordingly, I have not organized sections according to the genetic tools used (e.g. Cre, Tet repressor, etc.) but according to the type of questions asked. In doing so I have chosen studies that provide details that illuminate the features of transgene design and experimental approaches that investigators might consider before embarking on similar work.

2
Loss-of-Function Experiments

With the powerful combination of well-established genetics and gene-targeting techniques (see chapter by J.S. Draper and A. Nagy, this volume), an experimental *forte* in mouse development studies is to investigate the role of a given gene through loss-of-function approaches by gene inactivation. However, one of the major insights gained during the last 15 years is that a limited number of signaling and genetic pathways can control a larger number of different stages of embryological development. Thus, it is often the case that a mutant embryo homozygous for a given null allele reveals only the first requirement for that gene; potential phenotypes that may exist at later developmental stages cannot be studied, simply because the mutant embryo does not develop long enough for the investigator to access these stages. Tissue-specific gene inactivation (or knockout), where the gene of interest is inactivated only in specific

tissues or cells in an embryo that is otherwise phenotypically wild type, is an ideal approach to solve this problem. This is achieved by targeting the gene of interest to generate a conditional allele that has wild-type activity but which contains an essential region flanked by the recognition sites for site-specific recombination (Nagy 2000; Lewandoski 2001; Dymecki et al. 2002; Sauer 2002). Thus the essential region is "floxed" or "flrted" if flanked by *loxP* sites (for Cre recognition) or FRT sites (for FLP recognition), respectively. Tissue-specific expression of the corresponding recombinase inactivates the gene of interest only in certain embryonic locations or lineages (Fig. 1). An example of a preferable genetic cross that will generate the number of mutant embryos required by most developmental studies is shown in Fig. 1.

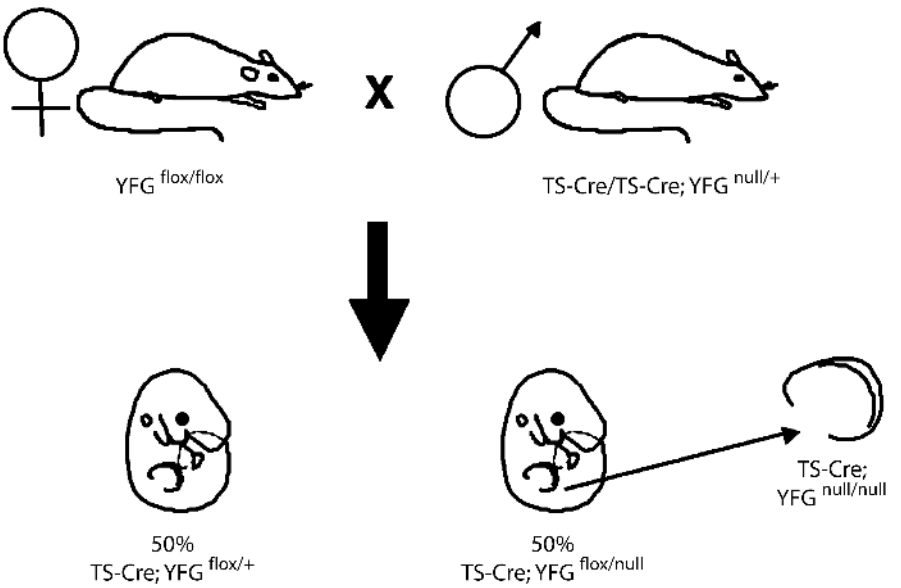


Fig. 1 A preferred genetic cross for generating mouse embryos that carry a tissue-specific gene inactivation. Females that are homozygous for a floxed allele of Your Favorite Gene (*YFG*^{flox}) are crossed to males that are homozygous for a tissue-specific Cre allele or transgene (*TS-Cre*) and heterozygous for an YFG null allele. By choosing these parental genotypes, mutant and control embryos are generated at a 1:1 ratio. Cre inheritance from the male parent has been chosen for two reasons. First, assuming a colony of floxed homozygotes can be maintained to provide a supply of females as needed, this parental configuration requires the least amount of mouse husbandry/genotyping. Second, and more importantly, many tissue-specific Cre transgenes/alleles can be unexpectedly active in the germline (either due to position effects or normal, but previously uncharacterized, germline activity (Voiculescu et al. 2000; Hayashi et al. 2003; Vincent and Robertson 2003). If this occurs in the female germline, Cre protein may be deposited in the egg. In this case, if we reverse the parental genotypes above, this maternal effect can result in recombination of a paternally inherited floxed allele soon after conception (Lewandoski et al. 1997; Hayashi et al. 2003; Vincent and Robertson 2003), thus preempting tissue-specific recombination in the embryonic progeny

Note that Fig. 1 utilizes the Cre/*loxP* and not the FLP/FRT system. Cre was the first system developed for manipulating gene expression in mice and remains the most commonly used system. However, both systems are formally equivalent, largely due to elegant work that produced a modified FLP variant with high activity at the body temperature of mice (Buchholz et al. 1998; Rodriguez et al. 2000). Indeed, there is now a series of FLP variants with a range in recombination efficiencies that can serve different experimental requirements (Dymecki et al. 2002). For example, one study exploited transgenes driving either high- or low-activity FLP variants to reveal the significance of graded *Wnt1* expression levels in the developing hindbrain (Landsberg et al. 2005). Furthermore, the availability of both DNA recombination systems to simultaneously control different targets in the same embryo allows the performance of very sophisticated genetic manipulations.

One such example is seen in the allelogenic strategy of gene targeting, which refers to generating many alleles (Fig. 2). The basic idea is to insert *loxP* and FRT sites in strategic locations in the initially targeted gene so that a series of alleles can be generated from the first generated mouse line by breeding it to Cre and FLP transgenic mice. In the original example, four different *Fgf8* alleles were generated (Meyers et al. 1998). The originally generated allele ($Fgf8^{neo}$) was a hypomorph due to the presence of an intronic neomycin resistance selection cassette (neo^r) that reduced wild-type FGF8 mRNA. Because of the availability of mice carrying this allele, the role of *Fgf8* has been studied in left/right axis formation, heart and nephron development (Meyers and Martin 1999; Abu-Issa et al. 2002; Grieshammer et al. 2005). FLP-mediated deletion of neo^r-generated $Fgf8^{flox}$, which has wild-type activity and is suited for Cre-mediated conditional inactivation. With the appropriate Cre line, this allele has been used to study *Fgf8's* role in nephrogenesis, somitogenesis, limb development, and brain development (Lewandoski et al. 2000; Chi et al. 2003; Perantoni et al. 2005). Finally, by breeding the original line to general Cre deleters (i.e., mouse lines with early Cre activity resulting in widespread or early ubiquitous recombination), two *Fgf8* null alleles were generated (one with and one without a neo^r tag) and used to explore FGF8 signaling during gastrulation (Sun et al. 1999). This *Fgf8* allelic series demonstrates how an allelic series can be very helpful for embryonic studies and provides a model for targeting other loci.

In general, tissue-specific inactivations are now a very basic instrument in the mouse embryologist's toolbox. For example, since the first limb bud-specific Cre-mediated gene inactivation was reported (Sun et al. 2000), there are now at least thirteen published mouse lines that will provide Cre activity in different locations and times during early limb bud development (Kimmel et al. 2000; Lowe et al. 2000; Moon et al. 2000; Sun et al. 2000; Ahn et al. 2001; Logan et al. 2002; Ruest et al. 2003; Ahn and Joyner 2004; Boulet et al. 2004; Harfe et al. 2004; Nelson and Williams 2004; Verheyden et al. 2005). It should be appreciated that a subset of these mouse lines were not originally intended for limb studies.

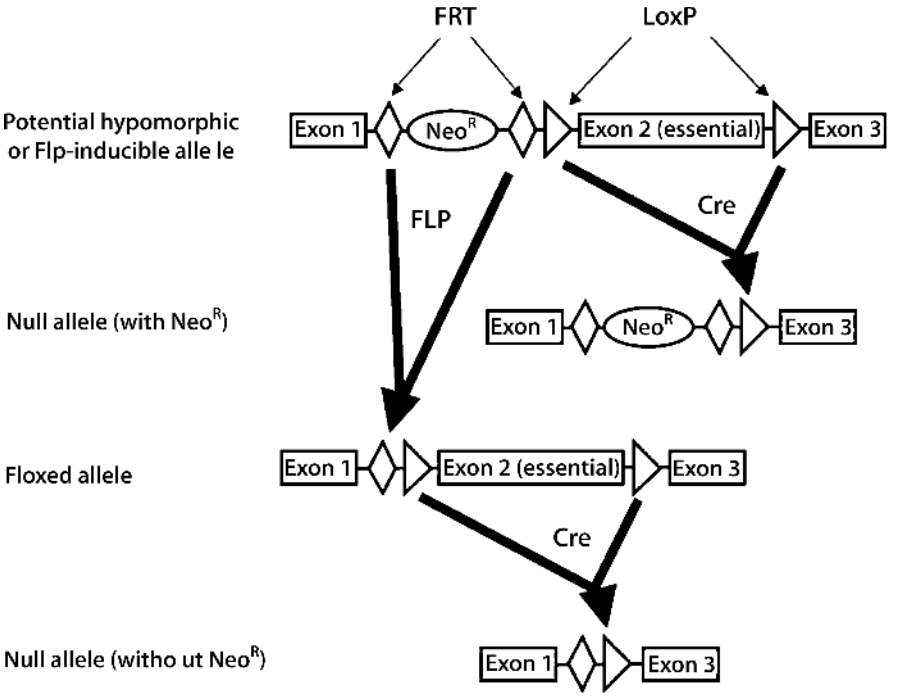


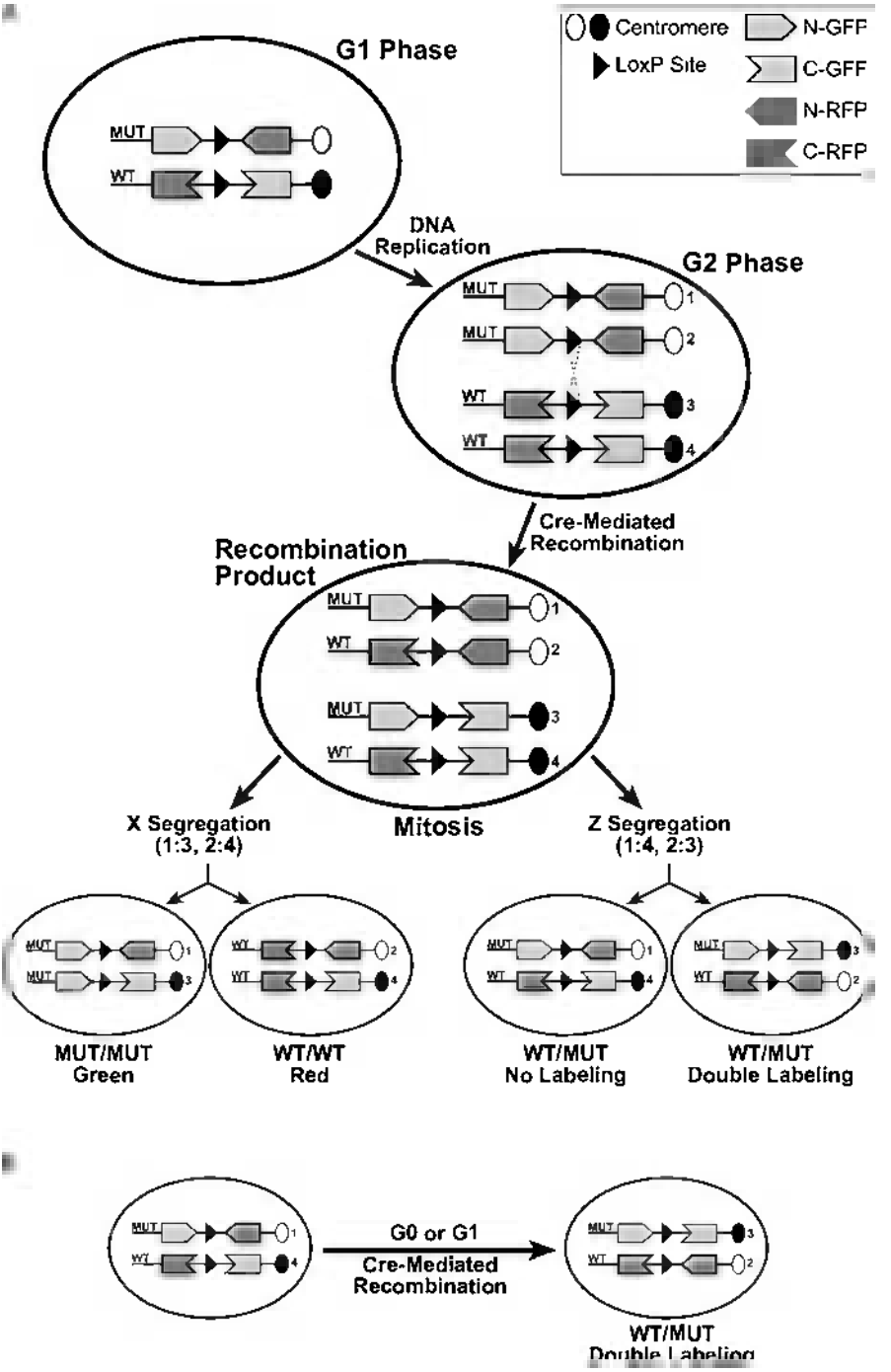
Fig. 2 A strategy for gene targeting that generates multiple alleles from one mouse line. The original mouse line carries an allele in which an essential region is floxed and the selection cassette (*Neo*^{r}) is flrted (*top line*). Because of the presence of the selection cassette, this may be a partial loss of function or a hypomorphic allele. Should the selection cassette cause a complete loss of function, the resulting null allele can be used in experiments in which tissue-specific FLP activity restores gene function (Voronina et al. 2005). With the appropriate crosses with general Cre deleters, two different null alleles (*third and bottom line*) can be generated, one marked with *Neo*^{r}. With the appropriate cross with a general FLP deleter, a conditional allele can be generated that encodes wild-type activity but which can be conditionally inactivated with tissue-specific Cre activity

Thus careful characterization of embryonic Cre expression patterns can result in interesting mouse lines that are useful in unexpected ways. As a case in point, one of these limb-specific Cre lines was later found to have Cre activity in the developing hair follicle (Pan et al. 2004). Also, at first glance Cre activity in some of these lines appears redundant because the Cre is expressed in the same embryonic structure in a second line; but subtle differences often exist in the timing or extent of Cre activity that can be very useful. This is illustrated by the inactivation of *Bmpr1a* in the limb bud ectoderm with either Brn4-Cre or Msx2-Cre mice. In the former case, BMP signaling is shown to be required for the formation of an important signaling tissue called the apical ectodermal ridge (AER) (Ahn et al. 2001), and in the latter case, in which inactivation of the same gene occurs in the same tissue but only hours later (Sun et al. 2000), the

opposite phenotype unfolds: a delay in AER regression (S. Panji Underwood and M. Lewandoski, unpublished observations). This quickly changing role is not necessarily unique to BMP signaling or limb development and illustrates the relative speed with which embryonic events can unfold. Therefore our understanding of how the embryo develops will be aided the more we gather mouse lines to canvass various lineages with Cre and FLP activity.

This goal of ultimately controlling gene regulation in any embryonic lineage is aided by the ability to temporally control site-specific recombination in transgenic embryos by administrating exogenous factors to the mother. One approach is to generate "trigenic" embryos so that Tetracycline (Tet)-controlled transactivators regulate transcription of a Cre transgene, resulting in the recombination of a floxed target (Saam and Gordon 1999; Utomo et al. 1999; Lindeberg et al. 2002). Careful screening of Cre lines can result in tight control in which recombination is entirely dependent upon transactivation (Perl et al. 2002; Schonig et al. 2002). This strategy has been used to study the role of the *Rb* gene in controlling cell fate in the embryonic lung (Wikenheiser-Brokamp 2004). Another method is to utilize Cre or FLP variants that encode fusions with mutated forms of the estrogen receptor (ER) ligand-binding domain (see the chapter by R. Feil, this volume). With such variants, when the synthetic antagonist tamoxifen (TAM) or its active metabolite 4-OH-TAM is delivered, recombination of chromosomal targets is induced through the translocation of Cre-ER (or FLP-ER) protein to the nucleus (Logie and Stewart 1995; Metzger et al. 1995; Feil et al. 1996; Schwenk et al. 1998). Mice have been generated with these variants with nonspecific widespread expression (Guo et al. 2002; Hayashi and McMahon 2002; Seibler et al. 2003) as well as with embryonic tissue-specific expression (Kimmel et al. 2000; Zervas et al. 2004; Sgaier et al. 2005). A concern for the embryologist is controlling the kinetics of recombination during development. One study reported that an intraperitoneal TAM injection to a postcoitus female at E8.5 resulted in dose-dependent recombination in the embryo starting after 6 h and becoming widespread by 24 h (Hayashi and McMahon 2002). Other reports

Fig. 3 A,B Illustration of mosaic analysis with double markers or MADM (Zong et al. 2005). **A** Transheterozygous mice are engineered with chimeric marker genes so that one encodes the N-terminus of the green fluorescent protein (*N-GFP*) and the C-terminus of the red fluorescent protein (*C-RFP*), and the other is reciprocally constructed as indicated. The mice are also heterozygous for a gene of interest, located distally to each reporter (MUT or WT). Interchromosomal Cre-mediated recombination at G2 restores each marker. X segregation (*left*) produces two daughter cells, one green and homozygous for the mutant gene of interest allele and the other red and wild type for the gene of interest. Z-segregation (*right*) produces two daughter cells that are heterozygous for the gene of interest, one unlabeled and one doubly labeled. **B** Cre-mediated recombination at G_0 or G_1 results in doubly labeled heterozygous cells. (Figure adapted from Zong et al. 2005)


Centromere
LoxP Site
N-GFP
C-GFP
N-RFP
C-RFP
G1 Phase
MUT
WT
DNA Replication
G2 Phase
Cre-Mediated Recombination
Recombination Product
Mitosis
X Segregation (1:3, 2:4)
Z Segregation (1:4, 2:3)
MUT/MUT Green
WT/WT Red
WT/MUT No Labeling
WT/MUT Double Labeling
G0 or G1
Cre-Mediated Recombination
WT/MUT

describe TAM delivery by oral gavage and demonstrate that recombination efficiency can be increased with two consecutive doses (Kimmel et al. 2000; Zervas et al. 2004; Sgaier et al. 2005). However, as the TAM dose increases, one must balance a need for high recombination efficiency with nonspecific embryonic lethality caused by TAM (Sadek and Bell 1996; Hayashi and McMahon 2002). For example, less than total recombination may be sufficient for lineage mapping experiments (as described below) but may not generate a mutant phenotype via gene inactivation – especially if the inactivated gene encodes a secreted factor. One solution to this potential quandary is to use recombinase variants with ER domains mutated such that lower TAM doses are required for efficient recombination (Feil et al. 1997; Indra et al. 1999). One such variant, called Cre-ER^{T2}, requires less TAM than its prototype, Cre-ER^{T}, to achieve the same level of recombination in early-stage embryos, thus reducing artifactual embryonic lethality (Zervas et al. 2004; Sgaier et al. 2005). Cre-ER ^{T2} has been used to regulate efficient recombination in (Indra et al. 2000, 2005).

Because the embryo can be such a fast-changing entity, the burden upon the investigator to demonstrate when and where her favorite gene has been inactivated may be great and even essential to understanding how gene loss generates the phenotype. Extrapolating from histological reporter lines (described below) may not always be satisfactory as not all regions of the genome are equally accessible to Cre or FLP activity (Vooijs et al. 2001) and the same Cre line can generate different patterns with different reporters (Hebert and McConnell 2000). A basic approach is to perform RNA in situ hybridization probing with the deleted sequences (e.g., see Perantoni et al. 2005), or to probe for the loss of the protein product if appropriate antibodies are available. A more direct approach is to design the floxed allele so that Cre simultaneously inactivates the gene and activates a histological marker (Moon et al. 2000; Moon and Capecchi 2000). However, the reader should be cautioned that the presence of the histological reporter itself might affect the activity of the conditional allele and cause a phenotype prior to Cre or FLP action thus obscuring analysis of a phenotype caused by tissue-specific inactivation.

A variation on the concept of using site-specific recombination to both generate mutations and label cells is the use of mitotic recombination to create genetic mosaics (Liu et al. 2002; Zong et al. 2005). This idea harkens back to early *Drosophila* genetics (Friesen 1936; Stern 1936) and was updated in that organism to a greater level of control by employing FLP-mediated recombination (Golic 1991). The basic idea, shown in Fig. 3, is that a mouse line contains reciprocally chimeric transgenes, each containing the N terminus of one histological reporter and the C terminus of the other, interrupted by a *loxP* -containing intron. Because these reporters are inserted in the identical loci on homologous chromosomes, Cre-mediated interchromosomal recombination restores reporter activity and subsequent cell division results in a balanced genome. However, heterozygous markers distal to the reporters become ho-

mozygous in those lineages that undergo X-segregation (see Fig. 3). This results in an unambiguous labeling of those lineages homozygous for the allele of interest. The advantage of this approach is that the allele of interest need not be targeted – it can contain a classical or chemically induced mutation. A disadvantage is that the frequency of interchromosomal recombination is likely to be too low to generate a mutant phenotype. However, a lower number of labeled mutant cells in a field of wild-type cells is ideal to address questions of cell autonomy of a given phenotype. As of this writing, only alleles on chromosome 6 distal to the *Rosa26* locus can be studied because this is the only locus appropriately engineered (Zong et al. 2005). However, this impediment should lift as characterization of the mouse genome proceeds and other loci, like *Rosa26*, are altered to provide near-ubiquitous expression.

One measure of the sophistication in the experimental strategies pursued in addressing some developmental questions is the use of Cre to recombine more than one genomic target in an embryo. For example, one may need to follow a specific embryonic cell lineage that has been both mutated and histologically labeled by site-specific recombination (see below). This is the case in which Marquardt et al. determined the role of *Pax6* in restricting retinal cell fate by using the same Cre activity, which was active in a specific subset of the developing retina, to both activate a lineage marker and inactivate the homeobox gene (Marquardt et al. 2001).

Another example is provided by the study of genetic redundancy during embryogenesis. How are redundant functions maintained during evolution? How do they fuel the engine of evolutional change? The answers to these questions are grounded in basic exploration into gene function during development and in many cases can only be addressed with double or triple tissue-specific gene inactivations. This is exemplified by studies in which the limb bud-specific deletion of *Fgf8* or *4* results in a relatively minor or no limb defect, respectively (Lewandoski et al. 2000; Moon et al. 2000; Moon and Capecchi 2000; Sun et al. 2000) but when both genes are deleted, limb development collapses (Sun et al. 2002**;** Boulet et al. 2004). The possibility in such studies that the recombinase may cause rearrangements between target genes on nonhomologous chromosomes, thus scrambling the genome, is not a great one, as this occurs at too low a frequency (10^{-6}–10^{-4}) to be of concern in this context (Buchholz et al. 2000).

3
Gain-of-Function Experiments

Gene function during embryogenesis can be investigated by asking whether the gene product has the capacity to control some aspect of development when it is ectopically or overexpressed. These analyses can help dissect molecular pathways, although the prudent embryologist interprets such experiments cautiously, for simply because a gene can affect a process under aberrant conditions

does not necessarily mean it plays a role during normal development. In the mouse, such experiments are done less frequently than in other experimental model vertebrate embryos, such as *Xenopus* and the chick, because the mouse embryo is relatively inaccessible in utero, and the dominant nature of such genetic manipulations prevents the establishment of stable transgenic lines. Nevertheless, as exemplified below, with conditional methodologies, these approaches are increasingly used in mouse embryology as we seek to combine gain-of-function studies with mouse genetics.

One design for devising target genes that can be activated by site-specific recombination that potentially provides the investigator the flexibility to use any tissue-specific Cre/FLP line is modeled by the R26R (Soriano 1999) and Z/AP (Lobe et al. 1999) histological reporter mouse lines for Cre activity. In these mice, tissue-specific recombination deletes sequences that inhibit the expression of a coding region, allowing it to be driven by a ubiquitous or near-ubiquitous promoter. The novice to mouse transgenesis should note that ubiquitous expression is not a trivial matter of using a housekeeping promoter in transgene construction and then pursuing standard transgenic procedures (i.e., zygotic pronuclear injection); this often does not yield ubiquitous expression. Two approaches for achieving ubiquitous expression are now common, both requiring the production of mice from ES cells. In the R26R model, the transgene is inserted by homologous recombination into a locus known to provide ubiquitous expression: usually *Rosa26* (Soriano 1999) (although sometimes *Hprt* - see Chen and Bradley 2000). In the Z/AP model, a set of ES cells are screened in vitro to identify the few clones where random integration of the transgene, which usually is driven by the strong CAGG promoter (Niwa et al. 1991), has resulted in widespread expression (Lobe et al. 1999).

Two studies analyzing Hedgehog (HH) signaling nicely exemplify each approach (Mill et al. 2003; Jeong et al. 2004). In one study, a mouse line was generated in which a transgene encoding a dominant active form of *Smoothened* , which results in constitutive HH signaling, was rendered Cre-dependent and placed under *Rosa26* regulation by gene targeting. With Wnt1-Cre activation of this transgene, HH signaling in the neural crest was manipulated to study facial development (Jeong et al. 2004). In the other study, transduction of the HH signal through *Gli2* in developing hair follicles was manipulated by Cre-mediated activation of different variants of Z/AP- Gli2 transgenes (Mill et al. 2003). For another gain-of-function example based on the Z/AP model that explores the role of FGF signaling in limb development, see Lu et al. (2006).

A different approach is to use gene targeting to render the locus of interest inducible via site-specific recombination; in this method the gene retains its endogenous tissue specificity. Examples can be found in mouse models of certain human skeletal dysplasias caused by dominant mutations in FGF receptors that cause aberrant signaling in the absence of ligand (see Brodie and Deng 2003; Ornitz 2005 for reviews). To generate these mice, the relevant FGFR codons were converted into the human mutations, resulting in dominant alleles that

were initially repressed due to the presence of an intronic floxed *neo*^{r} cassette. Deletion of the cassette via germline- or chondrocyte-specific Cre activity activated the gene (Chen et al. 1999; Wang et al. 1999; Iwata et al. 2000). This is analogous to cases that occur when generating an allelic series via the allogenic strategy (Fig. 2) in which the flrted selection cassette represses gene expression so severely as to effectively generate a null allele. Such a scenario affords the investigator the opportunity to use FLP-expressing mice to restore wild-type activity in specific tissues, determining where and when the gene is required during embryogenesis (Voronina et al. 2005). However, with this general strategy there is no guarantee that the selection cassette will reduce activity, or the degree to which this may occur. Should the investigator not wish to leave this possibility up to chance, she may use versatile targeting cassettes designed to increase the likelihood of generating such a null allele (Testa et al. 2004).

An elegant target design that exploits a fortuitous gene structure is the *Catnb*$^{lox(ex3)}$ allele, which allows investigators to use Cre-mediated recombination to activate canonical WNT signaling. The wild-type molecular interactions normally triggered by WNT signaling culminate in the stabilization of β-catenin, which together with nuclear T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors regulate WNT target genes (Bienz 2005). In the absence of a WNT signal, phosphorylation of serine and threonine residues in β-catenin results in a series of molecular events that culminates in its ultimate degradation. These negative regulatory residues are encoded by the third exon, in which the number of nucleotides is a multiple of three. Therefore, Cre-mediated deletion of exon 3 produces an allele with exons 2 and 4 in-frame that encodes a protein with enhanced stability and transcriptional activity (Harada et al. 1999). Mice carrying this Cre-inducible *Catnb*$^{lox(ex3)}$ allele were originally generated to model human colorectal cancer caused by similar deletions in β-catenin (Iwao et al. 1998; Sparks et al. 1998) but have been used in several developmental studies (Backman et al. 2005; Kemler et al. 2004). In one report that nicely illustrates the advantage of combining gain- and loss-of-function data, it was concluded that WNT signaling acts downstream of BMP signaling in formation of the limb bud AER, but upstream or in parallel in dorsal-ventral patterning of the limb (Soshnikova et al. 2003).

Conditional systems based on transcriptional transactivation have also been used to activate gene expression in developmental studies. The UAS-Gal4 system (Ornitz et al. 1991), a prominent tool in *Drosophila* genetics, has been utilized in the mouse to activate BMP signaling in the neural tube (Hu et al. 2004) and Indian Hedgehog in chondrocytes (Long et al. 2004). One cautionary report demonstrated that high expression levels in the developing heart of the Gal4 transcription factor per se, driven by the α-myosin heavy chain (α Mhc) promoter, caused perinatal lethality due to cardiomyopathy (Habets et al. 2003). Unlike most promoters used in mouse transgenics, gene expression levels driven by the αMhc promoter is proportional to copy number (Gulick

et al. 1997; James et al. 1999). Taking advantage of this phenomenon, the authors produced a single copy αMhc-Gal4 mouse line with relatively low Gal4 expression levels that displayed no heart defects and provided heart-specific transactivation of target transgenes (Habets et al. 2003). It is not clear if high Gal4 levels in other embryonic lineages are similarly detrimental.

The Tet regulatory system of transcriptional transactivation may arguably be the most advantageous in gain-of-function studies because, unlike UAS-Gal4 and site-specific recombination systems, target gene activation is regulated with an exogenously added tetracycline (usually Doxycline or Dox) and thus is reversible. Additional versatility is provided because the same target gene containing tet operator sequences (*tet*O) can be regulated via different variants of the tetracycline controlled transactivator (tTA or tet-off) or the reverse tetracycline controlled transactivator (rtTA or tet-on) (Baron and Gossen 2000; Gossen and Bujard 2002). These systems have been used to control gene expression successfully in the developing skin, pancreas, and liver (Diamond et al. 2000; Holland et al. 2002; Carpenter et al. 2005). In one case in which transgenic target genes encoding the Kruppel-like factor 4 were regulated by tTA in the developing epidermis, different target lines exhibited variable activation levels that correlated with phenotype, essentially providing a useful allelic series (Jaubert et al. 2003).

One strategy in using this system is to appropriately insert *tetO* sites into the locus of interest, rendering it dependent upon a Tet represser (TetR). An experimental tour-de-force that illustrates several important lessons is available in Shin et al. (Shin et al. 1999). These investigators sought to determine when signaling through endothelin receptor type B (EDNRB) was required for normal neural crest development. Gene targeting was used to replace the endogenous *Ednrb* with one of three constructs: one encoding tTA, one encoding rtTA, and a $Ednrb^{tetO}$ target transgene, thus generating three different mouse lines. This approach served several purposes. First, by placing the two transactivator transgenes in the endogenous locus, these constructs were presumably placed under *Ednrb* regulatory sequences without *a priori* characterization or information of these sequences. Second, by generating both tTA and rtTA alleles the option was created to use Dox to activate or inactivate (depending on the genotype) *Ednrb* expression and thus bypass potential problems with the kinetics of Dox clearance from the animal upon Dox removal. Third, insertion of the $Ednrb^{tetO}$ target gene into this locus may place this target in a favorable open chromatin configuration for transactivator accessibility in the neural crest lineage. Fourth, each targeting event also inactivated the endogenous *Ednrb* gene so that in a compound heterozygote (e.g., $Ednrb^{tTA/tetO}$) the only EDNRB activity was that generated by Dox-regulated transactivation. Lastly, the selection cassette used in these targeting events was floxed, thus allowing its removal via Cre-mediated recombination. In all three transgenic lines, the expression cassette obstructed transgene function, reducing expression. However, in the case of the $Ednrb^{tetO}$ transgenic line, this obstruction was advantageous as

uninduced basal expression of the Cre-mediated transgene was too high to be useful, whereas expression from the *Ednrb*^{tTA} or the *Ednrb*^{rtTA} allele was acceptable only after deletion of the selection cassette. Thus this project succeeded because the investigators provided a removal strategy for the selection cassette but did not remove it until after mouse lines were established. Variations of this approach have been used to generate Tet-regulated alleles of two homeobox genes, *Pdx1* and *Hoxa2* (Holland et al. 2002; Mallo et al. 2003).

Note that this particular strategy straddles the border between loss- and gain-of-function experiments. Repression of a *TetO*-bearing gene within a subset of its endogenous expression domain is useful for finely tuned loss-of-function questions, but the same *TetO*-bearing gene can be used in crosses to ectopically activate the gene with appropriate tTA/rtTA-expressing mice. It is unknown to what degree regulatory cis-acting elements located in the endogenous locus will restrict gene activation in such ectopic expression experiments and is likely to vary between loci.

4
Fate Maps and Pulse-Chase Experiments

A fate map is a drawing of the embryo that identifies regions as the progenitors of specific embryonic lineages or adult tissues and organs. Such maps can be essential to provide a normal context in which to understand a mutant phenotype. These maps are obtained by labeling specific embryonic regions or cells with a marker that can be traced in the lineages derived from the originally labeled cells. Established methods include the quail/chicken chimerism pioneered by LeDourain (Le Douarin 1982), or injecting vital dyes (Keller 1975) or fluorescent (Wetts and Fraser 1991) or retroviral markers (Galileo et al. 1990). However, despite some extraordinary efforts (Tam and Beddington 1987; Lawson and Pedersen 1992), the availability of mouse embryo fate maps have lagged behind that in chicken and *Xenopus* because of the difficulty in accessing and labeling the embryo in utero.

Because site-specific recombination results in a permanent genomic change inherited by all cells derived from the original population that underwent recombination, it is ideal for labeling an embryonic lineage in utero. Furthermore, should the recombinase expression domain recapitulate an endogenous gene expression domain, a special type of fate map can be generated in which the progenitor population is not identified by an anatomical feature or embryonic location but by a gene expression pattern. Thus gene expression can be linked to lineage fate.

To make this powerful approach practical, there are two considerations. First, the recombination event should result in the activation of a histological reporter, such as β-galactosidase or green fluorescent protein (GFP) that is capable of near-ubiquitous expression. Second, the Cre/FLP allele or trans-

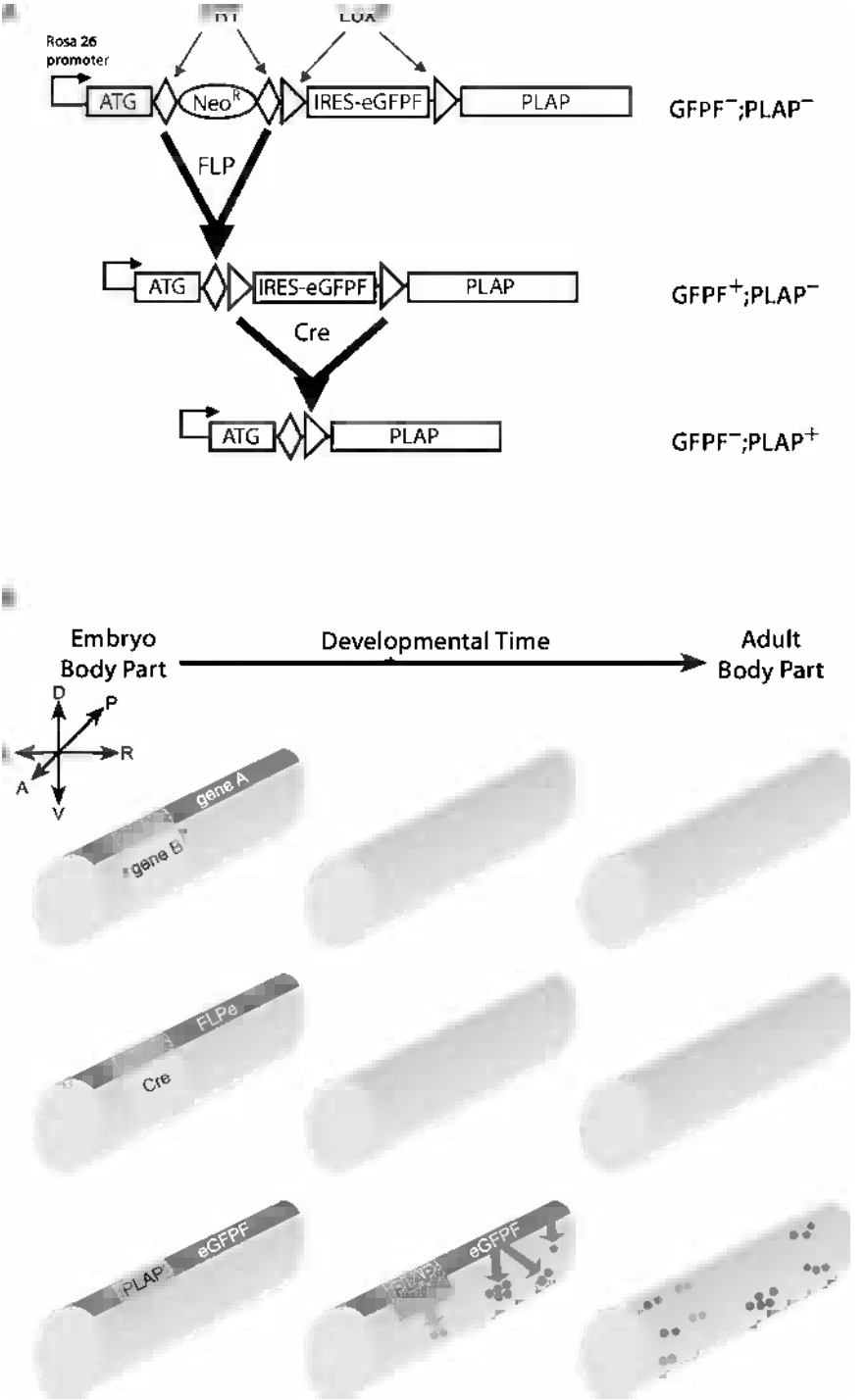
gene expression pattern should faithfully reflect that of the endogenous gene. A series of Cre and FLP reporters is now available that offer near-ubiquitous expression of a variety of histological reporter genes (reviewed in Dymecki et al. 2002; Branda and Dymecki 2004). Most of these reporters were generated by ES-cell based approaches (see discussion of R26R and Z/AP above).

Accurate Cre/FLP expression can sometimes be obtained by using well-characterized control elements in standard transgenesis techniques. For example, a *Wnt1* DNA fragment that drives expression in the dorsal neural tube has been used to map neural crest fate via Wnt1-Cre and Wnt1-FLP activity (Chai et al. 2000; Rodriguez and Dymecki 2000; Awatramani et al. 2003; Matsuoka et al. 2005).

Regulatory elements that successfully recapitulate an endogenous expression pattern are more likely to be established with constructs that use large DNA fragments such as BAC or YAC vectors (Copeland et al. 2001) or by the insertion of sequences that encode the recombinase into an endogenous locus (a knock-in) (Hebert and McConnell 2000; Zervas et al. 2004; Sgaier et al. 2005). However, such approaches are not helpful if the investigator requires a subset of the entire endogenous pattern.

The first examples of this type of fate map characterized the lineages derived from mid/hindbrain progenitors expressing an *En1*-driven Cre transgene (Zinyk et al. 1998) and, in another study, neural crest progenitors expressing a *Wnt1*-driven FLP transgene (Dymecki and Tomasiewicz 1998). The data and biological insights of these initial attempts have been refined and deepened with advances in the technology. Two related landmark studies carefully mapped the morphological movements that convert the simple neural tube into specific regions of the midbrain and cerebellum (Zervas et al. 2004; Sgaier et al. 2005) by analyzing the lineages marked in a variety of Cre mice, including Cre knock-in lines. Because development of the nervous system is dynamic within rather short time windows, a crucial component of these studies was the

Fig. 4 A,B Fate mapping the descendants of a progenitor population that is defined by the expression of two recombinases. **A** The R26::FLAP reporter contains an alkaline phosphatase reporter (*PLAP*) interrupted by the insertion, downstream of its initiation codon (*ATG*), of a flrted *neo*^{r} selection cassette and a floxed IRES-eGFPF cassette. Silence of PLAP is ensured by the presence of multiple polyA sequences within each cassette. FLP-mediated recombination deletes *neo*^{r} and activates eGFPF. FLP- and Cre-mediated recombination restores PLAP activity. **B** A generic embryonic region is represented by a cylinder in which a domain is defined by the intersection of the transient expression domains of genes A and B (*top row*). The *middle row* represents the transient expression domains of FLPe and Cre, driven by regulatory elements from gene A and B, respectively. The resulting FLPe and Cre activities result in the activation of the PLAP reporter such that eGFP labels descendents of FLPe expressing cells and PLAP labels descendants of cells that express both Cre and FLPe. (Figure adapted from Awatramani et al. 2003)


Rosa 26
promoter
ATG
Neo^R
IRES-eGFPF
PLAP
GFPF⁻;PLAP⁻
FLP
ATG
IRES-eGFPF
PLAP
GFPF⁺;PLAP⁻
Cre
ATG
PLAP
GFPF⁻;PLAP⁺
Embryo
Body Part
Developmental Time
Adult
Body Part
D
P
R
A
V
gene A
gene B
FLPe
Cre
PLAP
eGFPF
PLAP
eGFPF

use of TAM-inducible Cre variants, allowing the investigators to label relevant lineages at different embryological stages, thus adding a temporal component to the analysis. In another exciting study, a mouse line was generated in which a histological reporter is activated only in those lineages derived from progenitors that expressed both Cre and FLP (Fig. 4a). By expressing FLP along the anterior–posterior axis of the dorsal neural tube axis and Cre in specific hindbrain segments, the orthogonal intersection of these different expression domains was finely mapped, revealing that unexpected boundaries exist in the hindbrain that contribute to structures such as the choroid plexus (Awatramani et al. 2003). This approach has been used by Farago et al. (2006) to analyze how the cochlear nuclear complex, a brainstem region required for auditory processing, is assembled from different microdomains originating within the developing hindbrain.

Boundary juxtaposition is an important concept in development because the resulting molecular interactions that occur at boundary borders are often essential organizing centers that pattern the embryo (Irvine and Rauskolb 2001). Therefore the uncovering of functional domains via site-specific recombination fate mapping that heretofore have been unknown because they do not correspond to obvious anatomical features are essential to understanding basic embryology. For example, outgrowth of the limb bud requires molecular signals from the AER, which forms at the dorsal/ventral border of the limb bud. By activating inducible Cre at different stages, it was shown that two borders play a role during AER formation, one of which acts transiently (Kimmel et al. 2000). Furthermore, these investigators were able to determine the role of the *Engrailed* (*En1*) gene in AER formation by combining their lineage analysis with *En1* gain of function (via transgenesis) and loss of function (because the Cre knock-in into the *En1* locus also generated a null allele). What is the ultimate fate of the AER? Cre-based fate mapping reveals that the pre-AER domain that generates the AER also contributes to the ventral paw. However, the AER itself is eventually lost, leaving no descendants in the newborn limb (Guo et al. 2003).

A TetR-based system was used in a creative update of another kind of lineage map from classical experimental embryology: the pulse-chase experiment. In the early 1990s skin stem cell candidates were identified in a region of the hair follicle called the bulge. One criterion these cells filled was that they cycled slowly, as demonstrated by the retention of a DNA label generated by a pulse of tritiated thymidine (Cotsarelis et al. 1990). To similarly label these cells in living tissue, Tumbar et al. (2004) first produced mice in which keratinocyte-specific rtTA activity activated a *TetO* -target encoding a histone fused with GFP. Then, Dox administration repressed the histone–GFP target, effectively washing the label from rapidly dividing cells with unlabeled histones. Because the slow cycling bulge cells retained GFP, they then could be isolated by FACS-sorting and subjected to microarray genome profiling. Mice carrying the *TetO*-histone-GFP transgene can be used to label cells within other lineages by using different tissue-specific tTA/tTTA transgenic mice.

5
Genetic Ablation

Studying the consequences of the surgical removal of an embryonic tissue or region is an experimental approach that goes back to the classical work of Spemann on lens induction in frog (*Rana fusca)* embryos (Spemann 1901). With the comparatively inaccessible mouse embryo, this has been accomplished genetically by expressing a transgene that encodes a cell-autonomous lethal product, such as the diphtheria toxin A subunit (DTA) (Breitman et al. 1987; Palmiter et al. 1987). However, given the dominant nature of the transgenic phenotype, these experiments are facilitated with the use of conditional systems. In the first such report, the effect of muscle ablation upon motoneuron survival was studied by using a general Cre deleter line to place DTA under the control of a tissue-specific promoter active in muscle cells (Grieshammer et al. 1998). A similar approach was also used to ablate the roof plate of the neural tube, but in this study tissue specificity was obtained by inserting the DTA coding region into the *Gdf7* locus (Lee et al. 2000).

A perhaps more productive method is to generate a mouse line in which the lethal transgene is ubiquitously transcribed but requires Cre-mediated recombination for activation and thus can be bred with any of the tissue-specific Cre lines in the field. Four groups have reported such an ablater mouse (Chen et al. 2004; Sato and Tanigawa 2005). The CETD mouse line expresses EGFP prior to recombination and activation of DTA. Three of six transgenic founders carrying the CETD transgene died (suggesting some leaky DTA expression) and the survivors expressed EGFP less strongly than expected. Nevertheless, the authors demonstrated some evidence for ablation at embryonic stages (Sato and Tanigawa 2005). In the pu*Δ* tk selector line (Chen et al. 2004), the suicide transgene, pu*Δ* tk, has been inserted into the *Hprt* locus of the X chromosome. After Cre-mediated recombination, ablation can be temporally regulated because cell death requires administration of a prodrug such as ganciclovir or 1-(-2-deoxy-2-fluoro-1-beta-D-arabino-furanosyl)-5-iodouracil (FIAU). Furthermore, X-linkage aids mouse husbandry and provides for the potential for complete ablation in males and partial ablation in females due to X-chromosome inactivation. Lastly, lineages marked via Cre-mediated recombination may be selected for in vitro study, as the pu*Δ* tk fusion gene provides positive selection with puromycin (Chen and Bradley 2000).

Two mouse lines have been generated with a Cre-activatable DTA transgene placed under the regulation of the endogenous *Rosa26* locus for near-ubiquitous expression. Each line employs a different strategy to prevent leaky DTA expression and monitor target transgene expression. In the transgene of the R26:LacZ/DTA line, the ATG initiation codon defines a *lacZ* reporter ORF prior to recombination and a DTA ORF after recombination (Brockschnieder et al. 2004). Analysis of Cre-mediated ablation of postmitotic neurons in the future cortical plate indicated that the time required from Cre expression

to ablation was 36–48 h (Brockschnieder et al. 2004). In the *ROSA26-eGFP-DTA* line, Cre activates DTA by the deletion of an eGFP ORF followed by three polyadenylation sites (Ivanova et al. 2005). The time required for Cre-mediated ablation using this line was estimated to be less than 24 h in the developing heart and 16–20 h in the early neural tube (Ivanova et al. 2005). These studies illustrate the importance of carefully considering the kinetics of Cre-mediated recombination during development.

Transgenic mice have also been generated; they carry a *TetO* -DTA target gene that can be controlled by the Tet-regulatory system (Lee et al. 1998). Like CETD mouse production, the number of transgenic founders was reduced with this construct, presumably due to leakiness of DTA in the absence of transactivation. Nevertheless, in the lines that were established, DTA was transactivated in the adult heart, causing cardiomyopathies (Lee et al. 1998). It is not clear whether these lines are suitable for ablation studies in the embryo.

6
Conditional Gene Knockdown (RNA Interference)

The rapidly emerging technology of RNA interference (RNAi) has begun to see applications in mouse embryology. RNAi exploits a phenomenon that occurs in most metazoans involving the natural processing of double-stranded RNA into microRNAs (21–26 nt) by the RnaseIII enzyme, Dicer (see chapter by R. Kühn et al., this volume). These miRNAs in turn may repress specific genes by a variety of mechanisms (reviewed in Novina and Sharp 2004; Matzke and Birchler 2005; Sandy et al. 2005). miRNAs have been shown to be expressed in compelling embryonic patterns during mouse development (Mansfield et al. 2004) and specific miRNAs have been implicated in hematopoiesis (Chen et al. 2004), brain morphogenesis (in zebrafish) (Giraldez et al. 2005), and heart (Zhao et al. 2005) and limb development (Hornstein et al. 2005). Furthermore, conventional and tissue-specific inactivation experiments reveal that *Dicer* is required for gastrulation and limb and lung development (Bernstein et al. 2003; Harfe et al. 2005, Harris et al. 2006). For a recent review on miRNA and vertebrate development see Harfe (2005).

The current methodology for exploiting RNAi to control gene expression in mice is not yet as standardized as other techniques. One emerging method involves the generation of short hairpin RNAs (shRNA), driven from RNA polymerase III promoters (Brummelkamp et al. 2002; Dykxhoorn et al. 2003). Such constructed transgenes have been introduced into ES cells by either standard DNA electroporation (Kunath et al. 2003) or lentivirus vectors (Rubinson et al. 2003; Tiscornia et al. 2003) or by targeting the *Rosa26* locus (Seibler et al. 2005). Whether such transgenes produce an embryonic phenotype can be determined by analyzing entirely ES-derived embryos generated using tetraploid chimeric techniques (Kunath et al. 2003; Ventura et al. 2004; Seibler et al. 2005). This ex-

perimental paradigm allows for quickly screening a large number of candidate genes. For example, in this manner, potential targets of Wnt/β-catenin signaling, first identified via microarray-based genome profiling, were functionally analyzed to determine whether they were required in early development (Lickert et al. 2005). One consideration in choosing an RNAi approach to control gene expression is that there can be variation in the degree of a knock-down loss-of-function effect (see Sandy et al. 2005 for a good discussion of this); but as discussed above, hypomorphic phenotypes have their own valued place in embryological studies.

Although RNAi/ES cell strategies circumvent the need to establish transgenic mouse lines, should a particular embryonic phenotype generated in this manner require detailed investigation, a transgenic line may have to be generated in order to produce the required number of embryos. However, such an effort is likely thwarted by the dominant nature such a shRNA transgene and therefore, RNAi techniques must be wed to one of the conditional systems for gene expression. To achieve this, RNAi constructs containing the RNA pol III promoter have been modified with *TetO* sites for regulation by the Tet-regulatory system (Chen et al. 2003; Czauderna et al. 2003; Matsukura et al. 2003; van de Wetering et al. 2003). These promoters have also been modified with *loxP* sites so that expression can be activated by Cre-mediated DNA recombination (Kasim et al. 2003; Fritsch et al. 2004; Tiscornia et al. 2004; Ventura et al. 2004; Coumoul et al. 2005).

Besides knocking down the expression of individual genes, an appealing application of RNAi methodology may be to target more than one gene and thus circumvent functional genetic redundancy. This approach has uncovered redundant functions in *C. elegans* by targeting more than one paralog (Fraser 2004). Experiments in plant and Drosophila development indicate that a single shRNA may target the mRNA of two or more genes within a gene family if they share a high enough sequence similarity (Lawrence and Pikaard 2003; Nagel et al. 2004; Kan and Kessler 2005). Time will tell if such strategies will be useful in bypassing genetic redundancy in mouse development.

7 Concluding Remarks

It is well over a decade since the first reports were published describing conditional control of gene expression in the mouse (Gossen and Bujard 1992; Lakso et al. 1992). Mouse embryology has benefited greatly from the development of these systems with questions of gene function, lineage cell fate, and pattern formation being addressed with ever greater precision. This precision increases as the tools of DNA recombination and transcriptional control become sharper through creative innovations within each system as well as compounding the systems with each other. For example, by using tissue-specific Cre-mediated

recombination to activate rtTA/tTA targets, inserted into the *Rosa26* locus, these transactivators become active throughout the lineage derived from the original progenitor population expressing Cre. Gene regulation can then be controlled in these lineages with DOX administration, even though no regulatory elements have been characterized that are specifically active in these lineages (Belteki et al. 2005; Yu et al. 2005).

Mouse embryology has not only benefited from these technologies, but in many cases it has also been the impetus for developing and enhancing them, as investigators strove to overcome the limitations of this experimental organism such as the inaccessibility of the embryo in utero or the time and expense of generating targeted mouse lines. This connection is likely to persist, with the mouse embryo maintaining a central position in vertebrate molecular biology as we continue annotating the genome and fulfilling the promise held out by embryonic and adult stem cell technologies. Lastly, let us not forget that the mouse embryo will always be fascinating in its own right.

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Conditional Mouse Models of Cancer

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Abstract The development of inducible and conditional technologies allowed us to generate transgenic mouse models that faithfully recapitulate human tumorigenesis. It is possible to control, in time and space, the development of tumors in almost every mouse tissue. The result is that now we have available mouse models for all major human cancers. Novel noninvasive approaches to tumor imaging will enable us to follow tumor development and metastasis in vivo, as well as the effects of candidate therapeutic drugs. Such new generation tumor models, which accurately emulate the disease state in situ, should provide a useful platform with which to experimentally test drugs targeted to specific gene products, or combinations of genes that control rate-limiting steps of tumor development. In this review, we focus on the different mouse models for colon cancer.

Keywords Colon cancer · Conditional mouse models

Abbreviations

Rb	Retinoblastoma
min	Multiple intestinal neoplasia
Apc	Adenomatous polyposis coli
HNPCC	Hereditary nonpolyposis colorectal cancer
CRC	Colorectal cancer
FAP	Familial adenomatous polyposis
NSAIDs	Nonsteroidal anti-inflammatory drugs
MRI	Magnetic resonance imaging
CT	Computed tomography

PET	Positron emission tomography
SPECT	Single photon emission tomography
CCD camera	Charge-coupled device camera
MTB Database	Mouse Tumor Biology Database
ACF	Aberrant crypt foci
ER	Estrogen receptor
MMTV	Mouse mammary tumor virus
5-FU	5-Fluoro-uracyl
GFP	Green fluorescent protein
LOH	Loss of heterozygosity
CIN	Chromosomal instability
ENU	Ethyl-nitroso-urea
GI	Gastrointestinal
Fabp	Fatty acid binding protein
IRES	Internal ribosome entry site
MMR	Mismatch repair

1 The Ideal Model for Human Cancer

Mouse models of human cancer have played an important role in formulating modern concepts of multistage carcinogenesis, and are providing us with a new set of tools for testing novel therapeutic approaches to cancer treatment. However, as the scientific and medical community's understanding of human cancer becomes more pronounced, limitations and potential weaknesses of existing models are revealed. There is the expectation that mouse models will mimic human cancer, i.e., tumors developing in transgenic mice should look and behave like the human disease. Mouse tumors should have the same or similar histological features of the corresponding human tumors; they should progress through the same stages and cause the same physiological effects on the host; the same genes and/or pathways should be affected in tumor initiation and progression; the response of a given tumor to a particular therapy in the mouse should accurately reflect the response in human patients; and the results from preclinical testing of experimental therapies in mouse models should predict the efficacy of such therapies in clinical trials in humans.

Certain mouse models recapitulate human disease extremely well. For example, overexpression of *c-Myc* in the mouse leads to similar pathologies (B cell lymphomas) as it does in man (Adams et al. 1985; Macleod and Jacks 1999). However, identical genetic lesions do sometimes produce very different pathologies in the two species. A good example is the retinoblastoma gene product (Rb). Rb transduces antiproliferative signals and is an important tumor suppressor. In humans, loss of Rb leads to the development of retinoblastoma at an early age, followed by osteosarcomas and small cell lung cancer (Jacks et al. 1992). In mice, however, loss of Rb very rarely causes retinoblastoma (Jacks et al. 1992; Macleod and Jacks 1999); Rb-null mice ex-

hibit embryonic lethality, while heterozygotes develop pituitary carcinomas and thyroid tumors at high frequency (Jacks et al. 1992; Harrison et al. 1995; Macleod and Jacks 1999). Thus, there are some considerations that should be kept in mind when designing a mouse cancer model.

1.1 Constitutive Versus Conditional Tumor Models

Genetically engineered mouse models are used primarily to determine whether a particular gene is involved in tumorigenesis, or whether a gene product, involved in specific signaling processes, contributes to cancer in combination with other predisposing conditions. A wide variety of constitutive, transgenic and knockout, models have been developed to study the effects of different oncogenes and tumor suppressors. A common feature of these mouse tumor models is that they represent mainly the early stages of disease development and relatively few of them recapitulate the features of advanced human cancer, including high-frequency metastasis (e.g., *min* mouse and the *Apc* gene; Moser et al. 1990). Despite their limitations, such models can be used to study the effects of early therapeutic intervention or preventive measures. Conversely, some constitutive models that take out whole pathways may be very convenient, because of rapid tumor growth and progression, but inappropriate for tumor prevention focused on early stages. However, beyond simply targeting whole genes, today's methodologies also allow engineering of targeted point mutations, in some cases creating the exact genetic change present in naturally occurring tumors.

The major limitation of constitutive mouse models is that the desired genetic defect is present in every cell of the animal from the start of development, including those cells that constitute the tumor microenvironment. Consequently, the functions of stromal cells in either stimulation or inhibition of tumor growth could be impaired. Many of these models, therefore, do not recapitulate sporadic forms of human cancer. On the contrary, they have more similarities with familial cancers caused by high-penetrance mutations in critical genes. Further, several mutations of tumor suppressor genes cause embryonic lethality in the homozygous state, or a spectrum of tumors and other abnormalities and surprising phenotypes, which are different from their human counterparts. Finally, germ-line mutations can also trigger compensatory pathways during embryogenesis.

In contrast to constitutive models in which the activating mutation occurs in every cell of the organism or tissue, in conditional mouse models, the tumor-initiating genetic event occurs at the level of specific cells (Holland and Varmus 1998; Johnson et al. 2001). Conditional gene expression systems (Cre-Lox and Flp-FRT recombinase systems), allow the spatial and temporal control of somatic mutations, and can be used to target gene disruption as well as activating gene expression, in specific tissues. The development of

such inducible systems has made it possible to generate highly specific tumors in a narrow time window (Meuwissen et al. 2001). These models provide new opportunities to establish the influence of specific genes in the initiation and progression of cancer. They also provide a more accurate model of the clonal origin of naturally occurring spontaneous human tumors. However, it is important to note that even in constitutive models in which all the cells in a given animal or tissue bear the engineered tumor-promoting genetic event, subsequent stochastic events are presumed to occur in one or more premalignant cells, leading to clonal progression of the tumor.

Further, the major advantages of conditional mouse models is that they enable tumor growth to occur in proximity of the genetically wild-type neighboring epithelial and stromal cells, thus in the nonmutated microenvironment. Johnson et al. (2001) describe a model in which an engineered recombination event occurs at a low frequency in mouse somatic cells, resulting in expression of an activated *K-ras* oncogene (Johnson et al. 2001). Another novel approach for creating somatic genetic events involves in vivo virus-mediated gene transfer to tissues in mice expressing a transgene encoding avian-leukosis-virus receptor (Holland and Varmus 1998). Multiple genes can thereby be transferred to a target cell by retrovirus infection, either individually or in combination. In this way, Holland and co-workers have generated multiple mouse models of glial-cell-based tumors. Conditional mutagenesis also overcomes other limitations that are often encountered in conventional mutants, such as embryonic lethality. For example, recombinase-mediated gene disruption is used to circumvent the embryonic lethality associated with germ-line mutations in a number of tumor-suppressor genes, including *Apc*, *Brca1*, *Brca2*, *Nf1*, *Nf2*, *Pten*, *Rb*, and *Vhl*. Further, even when the inactivation of a tumor suppressor gene does not result in embryonic lethality, tissue-specific gene disruption is often necessary to prevent unwanted tumorigenesis outside the tissue of interest.

More sophisticated conditional approaches are based on mice in which the activity of Cre or oncogenes is inducible in a time-controlled fashion with small molecules such as tamoxifen.

Indeed, tamoxifen efficiently induces the Cre recombinase activity of fusion proteins between the Cre recombinase and mutated ligand-binding domains of estrogen receptors (ER) (e.g., Cre-ER ^{T2}; see chapter by R. Feil, this volume), thus allowing Cre-mediated recombination of LoxP-flanked genes in mice. An application of the Cre-ER ^{T2} approach is exemplified by the generation of transgenic mice expressing Cre-ER ^{T2} under the control of the villin promoter, allowing tamoxifen-dependent Cre-mediated recombination in the intestine (el Marjou et al. 2004).

Alternatively, the gene of interest can be fused to the ligand-binding domain of ER, as has been reported for transgenic mice that utilize the involucrin promoter to direct expression of a c-Myc–ER fusion protein in the suprabasal cell layer in the skin. Upon topic application of tamoxifen, these mice develop premalignant skin tumors (Pelengaris et al. 1999).

A similar approach has been used to create an inducible *H-ras–G12V* mouse model of melanoma in an *INK4a* -deficient background (Chin et al. 1999) and a *K-ras G12D* driven model of non-small cell lung cancer (Fisher et al. 2001). However, even inducible mouse models harbor some disadvantages, such as poor inducibility, leading to low recombination penetrance, or leakiness, causing unspecific expression in the absence of the inducer (Jonkers and Berns 2002).

1.2 Importance of Microenvironment and Etiologic Considerations

Cancer develops through a cumulative series of genetic and epigenetic changes that drive the progressive transformation of normal cells into highly malignant derivatives (Hanahan and Weinberg 2000). As discussed earlier, in sporadic cancers, the initiating lesion affects a single cell in an otherwise normal environment. Nevertheless, tumorigenesis is governed not only by the tumor cells per se, but also by the microenvironment. For example, tumor-associated fibroblasts can direct tumor progression (Olumi et al. 1999), and endothelial cells foster tumor angiogenesis (Carmeliet and Jain 2000). Most of the existing mouse models of cancer have mainly focused on the target cells from which the tumor is derived, and less on the microenvironment that fosters tumor growth. New studies are drawing attention to the functions of stromal cells, endothelial cells, and immune-system-derived cells in either stimulation or inhibition of tumor growth. Also, in several mouse models, it has been observed that the genetic background of the mouse strain can alter the tumor spectrum and the observed phenotype. Although difficult to study, the identification of tumor-modifier genes that control these phenotypes may give important insights into the basis of individual human genetic susceptibility to cancer (Balmain and Nagase 1998).

Since it is known that environmental mutagens contribute to human cancer, accurate mouse models should also be subject to external agents, such as exposure to chemical carcinogens. Dietary factors are also known to be contributing factors in the etiology of human cancer. Two studies using *Apc*-mutant mice indicated that Western-style diets resulted in increased tumor incidence and, in one case, tumors were present in the otherwise spared large intestine (Wasan et al. 1997; Yang et al. 1998).

In conclusion, an ideal mouse tumor model should faithfully recapitulate the natural history of tumorigenesis and mimic the pathobiological, genetic, biochemical, etiological, and therapeutic characteristics of its human counterpart. First, the model should allow the induction of well-defined sporadic tumors with high penetrance but low tumor number (so that animals do not die prematurely from overload by multiple benign tumors). Ideally, tumors should develop within a narrow time window, allowing for tumor progression and analysis within the relatively short lifespan of the mouse. Also, the model should enable tumor growth to be measured over time, making it suitable

for testing new therapies. In addition, the model should allow assessment of the contributions of individual mutations to the tumor phenotype. For this purpose, it should be possible to measure the effect of a single variable in an otherwise stable genetic background that contains a fixed number of defined complementing mutations. Besides the establishment of genotype–phenotype correlations, the model should also allow the identification of additional mutations that cooperate with the predefined mutations in tumor formation. No single tumor model can be expected to meet all of these criteria.

Finally, while the genetically engineered mouse models can be used to study the influence of genetic changes on carcinogenesis and the interaction of these mutations with environmental factors, other mouse models such as chemical-induced or xenograft models could be more suitable for other type of experiments. Therefore, it is crucial to use the optimal model to address a specific scientific question.

2 Why Do We Need Mouse Models of Cancer?

Mouse models have allowed us to address fundamental questions in cancer biology, but also preclinical drug screening accompanied by the tumor imaging.

2.1 Use of Animal Models to Study Basic Cancer Biology Questions

While the mice carrying gene ablation/overexpression can be used to study the influence of single genetic changes on tumor progression, they also could be combined to create compound mice containing two or more genetic changes (as is present in humans), and they could be examined for potentially collaborative events in tumorigenesis. Cross-breeding two individual mouse lines that display a mild tumor phenotype in the organ of interest can yield accelerated tumor progression and higher penetrance. For example, separate mouse lines expressing either c-*myc* or v-*ras* under the control of the MMTV promoter were generated and then crossed together (Sinn et al. 1987). Transgenic mice expressing v-*ras* developed hyperplasia of the Harderian gland, adenocarcinomas of the mammary and salivary glands, and the occasional lymphoma. The c-*myc*-expressing transgenic mice developed mammary adenocarcinomas and some lymphomas. When the two strains were interbred, mice co-expressing both oncogenes developed carcinomas of the mammary gland at a much greater rate than either of the transgenic mice expressing only one of the oncogenes. Clearly, c-*myc* co-operated with v-*ras* in mammary gland tumorigenesis in vivo.

In addition, the use of the Tet system to conditionally regulate the expression of several different oncogenes has shown that cancer is a reversible disease

(Chin and DePinho 2000; Pelengaris et al. 2002; Felsher 2003). Even highly genomically complex and/or metastatic tumors are reversible upon oncogene inactivation (Felsher and Bishop 1999; Moody et al. 2002; Karlsson et al. 2003). The stereotypic consequences of oncogene inactivation, regardless of the specific oncogene or type of cancer involved, suggest that there may be a unifying mechanism. Several experimental strategies could be useful in understanding the mechanism by which oncogene inactivation induces tumor regression. Microarray analysis is likely to define the gene expression programs that are generally required for oncogene activation to initiate and sustain tumorigenesis. One way to understand what governs this cell fate switch would be to compare gene expression profiles before and shortly after oncogene inactivation. This comparison may unmask the molecular basis of tumor regression by identifying the early signals that stop cell proliferation, the induction of apoptosis, and differentiation. Genomic strategies should also be useful to investigate the mechanisms by which tumors evolve to escape oncogene dependence. Analysis of tumors that have become independent of an oncogene may identify genes critical to the maintenance of a neoplastic phenotype (Giuriato and Felsher 2003; Karlsson et al. 2003).

Clinically, it has generally been observed that certain cancers that have fully regressed after therapeutic maneuvers relapse and progress, and become unresponsive to treatment. Similarly, using animal models, it has been found that the inactivation of a single oncogene, although sufficient to initially induce tumor regression, does not necessarily prevents a subsequent relapse of the tumor (Giuriato and Felsher 2003). The detailed examination of relapsed tumors may allow the identification of secondary genetic lesions that compensate for the loss of sustained oncogene expression for tumor maintenance. Conditional animal models can be used to refine our understanding of the mechanisms by which tumors can escape the requirement of a particular oncogene to retain their neoplastic features.

To better understand the mechanisms of tumor induction and to mimic what usually is observed in human cancers, several conditional model systems can be used to sequentially turn on the expression of different genes. The controlled sequential acquisition of oncogenic mutations adds an important new dimension to the study of oncogene cooperation. The traditional strategy for assessing the ability of oncogenes to cooperate in tumorigenesis has involved the cross of transgenic mice that express each oncogene in the desired tissue, and then checking for an acceleration in tumor onset in the double transgenic animals. A combination of the different conditional strategies now available might allow the generation of mice in which each oncogene can be regulated independently, providing a way to determine whether the sequential order of gene activation has an impact on the ability of the genes to cooperate, and whether the inactivation of one, the other, or both oncogenes is sufficient to induce sustained tumor regression.

2.2 Use of Animal Models in Preclinical Drug Screening

Animal studies cannot replace human clinical trials, but they can be used as a prescreening tool, so that human trials become more directed, with greater chances of success. A critical need in the development of anticancer agents is in vivo testing for efficacy. Ideally, before a drug or other therapeutic agent is put through the costly and time-consuming process of clinical testing, its activity would be confirmed in an appropriate animal model. Unfortunately, for a variety of reasons, the in vivo models of choice involve the use of established human tumor cell lines growing under the skin of immunocompromised mice. While these xenograft models have a long history in the pharmaceutical industry, and while they are indisputably straightforward to use, their record of accurately predicting the efficacy of anticancer agents in the clinic has been questionable. Among other problems, xenograft tumors do not evolve in situ and, thus, lack the appropriate cellular interactions with the host microenvironment. Therefore, it is of particular importance to establish whether the new breed of cancer-prone strains will provide better preclinical models for therapy and prevention. For example, conditional mouse models will be ideally suited to study drugs that interfere with specifically mutated regulatory pathways promoting tumor growth (Meuwissen et al. 2001). It is important to note that some drugs will have pharmacodynamic or pharmacokinetic properties in mice that will preclude their testing in any mouse models. Others will be highly specific to a human target, which may make mouse models unsuitable for testing. However, for the majority of agents, in vivo testing in an accurate and predictive model is possible and highly desirable.

For experimental therapies and prevention strategies, the ultimate proof of the utility of animal models must await a thorough examination of their performance in the human clinical experience. Until then, in order to provide a benchmark for evaluating the new agents, it would be useful to know how these models respond to established therapies with known clinical response rates. The potential of this approach can be illustrated by an experiment in which tumor cells with amplified c-myc expression and wild-type p53 are susceptible to 5-FU, in contrast to tumor cells with a mutated p53 gene. In accordance with these findings, retrospective analysis of a phase III clinical trial showed that only patients with tumors containing amplified expression of c-myc and p53 responded to therapy with 5-FU (Arango et al. 2001). Also, a comparison was made between the efficacy of agents on the prevention of colorectal carcinoma in mice and polyp recurrence in humans (Bruce 2003; Corpet and Pierre 2003, 2005). Nonsteroidal anti-inflammatory drugs (NSAIDs) strongly decrease the tumor yield in the small intestine of Min mice (Williamson et al. 1999; Corpet and Pierre 2005). This is consistent with epidemiological studies suggesting that NSAIDs might decrease the colorectal cancer incidence in humans.

2.3 Use of Animal Models for Tumor Imaging

Noninvasive imaging techniques allow monitoring of tumor initiation, progression, metastasis, and response to therapy in a single animal by sequential imaging analysis. When compared to classical (two-dimensional) histological analyses, the imaging techniques show improved (three-dimensional) accuracy and reproducibility as well as the enormous advantage of serial measurements. Fewer animals are therefore required to collect statistically reliable results.

Conventional imaging methods that are used in the clinic have been adjusted to visualize mouse tumors (Lewis et al. 2002; Weissleder 2002) including magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET)? and single photon emission tomography (SPECT). Furthermore, fluorescence and bioluminescence imaging techniques that allow for monitoring the growth of tumors expressing either green fluorescent protein (GFP) or luciferase became prevalent since they use relatively inexpensive capture technology.

Ultrasensitive charge-coupled device (CCD) cameras can detect tumor cells that continuously express GFP at high concentrations. The development of colorectal cancer liver metastases has been imaged noninvasively using this method (Chishima et al. 1997; Hoffman 1998; Yang et al. 2004; Yamauchi et al. 2005; Yang et al. 2005). One of the drawbacks of this technique is that the high level of GFP expression that is required for noninvasive imaging may interfere with the growth and survival of tumor cells. Another limitation is that it is difficult to detect the fluorescence emitted from GFP-expressing tumors through the abdominal wall, due to scattering and light absorption. The latter problem can largely be prevented by creating a flap of skin that may be opened while imaging and closed afterwards for multiple measurements of tumor growth in a single mouse. Alternatively, the tumor-bearing tissue can be exposed surgically and in this way it is possible to visualize local tumor–tissue interaction, tumor progression, angiogenesis, and metastasis formation at the single cell level. Despite the disadvantages outlined above, it is expected that with modern laser scanning technologies and improved detection limits and signal-to-noise ratios, it will be possible to follow the growth of fluorescent tumors noninvasively on a routine basis.

An alternative to following tumor growth by fluorescence is to make use of bioluminescence. Tumor cells can be engineered to express luciferase, a photoprotein that generates light following oxidative conversion of its substrate luciferin. A disadvantage of this system is that prior to imaging, the mice must receive an intraperitoneal injection of luciferin. Low-light photon-counting cameras can subsequently detect the light emitted from the tumor cells. This technique is ideal for visualizing tumor growth in a noninvasive and sequential manner, and it has successfully been used to study the growth of colorectal carcinoma cells in the mouse liver.

In conclusion, mouse models enable the development and testing of new approaches to disease prevention and treatment, the identification of early diagnostic markers and novel therapeutic targets, and represent precious tools for the in vivo study of the molecular basis of tumor initiation, promotion, progression, and metastasis.

3 Examples of Conditional Cancer Mouse Models

Many mouse cancer models could be found in The Mouse Tumor Biology (MTB) Database website (http://tumor.informatics.jax.org/mtbwi/index.do), which provides electronic access to:

- Information on endogenous spontaneous and induced tumors in mice, including tumor frequency and latency data
- Information on genetically defined mice (inbred, hybrid, mutant, and genetically engineered strains of mice) in which tumors arise
- Information on genetic factors associated with tumor susceptibility in mice and somatic genetic mutations observed in the tumors
- Tumor pathology reports and images
- References supporting MTB data
- Links to other online resources for cancer.

However, no single model recapitulates every aspect of its corresponding human disease counterpart. This review summarizes some recent mouse models for colon cancer, focusing on examples that mimic human disease.

Available tissue-specific Cre-expressing mice could be found on the Nagy lab website (http://nagy.mshri.on.ca/PubLinks/indexmain.html).

3.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer in the world. The main problem in the treatment of CRC is not so much eradication of the primary tumor, but rather the formation of incurable metastases. Most human adenocarcinomas evolve from aberrant crypt foci (ACF) and adenomas. In polyps smaller than 1 cm in diameter, the morphology of the intestinal cells and their localization in the epithelium appear usually rather normal. The larger the polyp, the more likely it is to contain cells that cannot properly differentiate and that form abnormally organized structures. Sometimes, two or more sectors can be distinguished within a single polyp, the cells in one

sector appearing relatively normal, those in the other appearing cancerous, as though they have arisen as a mutant subclone within the original clone of adenomatous cells. At later stages of the disease, the tumor cells become invasive, first breaking through the epithelial basal lamina, then spreading through the layer of muscle that surrounds the gut, and finally metastasizing to lymph nodes, liver, lung, and other tissues. This model has been progressively enriched, and several interdependent pathways are now known to be involved in colorectal tumorigenesis, based on the analysis of sporadic tumors and two inherited syndromes: the familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancers (HNPCC).

3.1.1 Sporadic Tumors and Familial Adenomatous Polyposis

Germline mutations in the *Apc* gene determines the FAP syndrome. However, most colorectal cancers are sporadic (90%), meaning that those patients do not carry the hereditary FAP mutation. Interestingly, they share with FAP tumors the same early *Apc* mutation, that in 50%–80% of cases has undergone loss of heterozygosity (LOH) in the cancerous cells but not in other tissues.

In most sporadic colon cancers, like in FAP, a direct consequence of *Apc* mutations is the nuclear accumulation of β-catenin. Indeed, APC forms a complex with β-catenin, axin, and glycogen synthase-3β kinase (GSK3β). Axin promotes β-catenin phosphorylation that mediates its degradation in the proteasome. In normal cells, this process is regulated by the Wnt signaling pathway. Mutations in *Apc* prevents the formation of the complex, and the levels of β-catenin rise in the cytoplasm. The stabilized β-catenin protein associates with the transcription factor Tcf4 and translocates into the nucleus, inducing a constitutive activation of *c-myc*, *cyclin D1* , and *c-jun* (Clevers 2004). In tumors where *Apc* is intact, the β-*catenin* gene is mutated, and stabilized β-catenin translocates into the nucleus and triggers the transcriptional activation of the same target genes. The dysregulation of the Wnt/β-catenin/Tcf pathway is thus a major event in most colon cancers. Further, chromosomal instability (CIN), a common feature of 8/10 colorectal cancers (Fodde et al. 2001), is associated with *Apc* mutations. A truncated APC protein may loose its ability to connect chromosomes to microtubules. Defective chromosome segregation, leading to CIN, would thus result once again from *Apc* mutations. In an attempt to discover other mutations responsible for colorectal cancer, tumor cells were screened for abnormalities in genes already known or suspected to be involved in cancer elsewhere. This type of genetic screening has revealed that approximately 40% of colorectal cancers have a specific point mutation in *K-Ras*, activating it as an oncogene, and approximately 60% have inactivating mutations or deletions of the tumor suppressor *p53.* Activated K-Ras is known to induce tyrosine phosphorylation of β-catenin, leading to its release from E-cadherin at the adherens junction. This ultimately results in an increase of the β-catenin pool in the cy-

toplasm, its subsequent translocation to the nucleus, and the transcriptional activation/repression of Wnt downstream target genes.

As mentioned before, a sequence of specific genetic changes underlies the development of intestinal carcinogenesis in the mouse. These include inactivation of the tumor suppressor genes APC, p53, and Smad3 and activation of the proto-oncogene K-Ras. In what sequence do genes undergo their mutations, and how does each of them contribute to the eventual unruly behavior of the cancer cell? There cannot be a simple answer to this question, because colorectal cancer can arise through more than one route. Nevertheless, certain patterns of events are particularly common. Thus, mutations inactivating the *APC* gene appear to be the first, or at least a very early, step in most cases. They can be detected already in small benign polyps at the same high frequency as in large malignant tumors. Loss of *APC* seems to increase the rate of cell proliferation in the colonic epithelium relative to the rate of cell loss, without affecting cell differentiation or their histological localization. Mutations activating the *K-Ras* oncogene appear to take place a little later than those in *APC*; they are rare in small polyps but cells carrying gain of function *Ras* mutations and grown in culture show typical features of transformed cells, such as the ability to proliferate without anchorage to a substratum. Loss of tumor suppressor genes on chromosome 18 and mutations in *p53* are believed to arise even later. They are rare in polyps but common in malignant carcinomas, suggesting that they may occur late in the sequence. Loss of *p53* function is thought to prevent apoptosis of abnormal cells, hence allowing them to rapidly divide and accumulate additional mutations by progressing through the cell cycle when they are not fit to do so, creating many abnormal chromosomes. Other yet unknown genes might have a role in the tumor progression and the development of metastasis.

3.1.1.1 Mouse Models for Familial Adenomatous Polyposis Coli and Intestinal Sporadic Tumors

The Min (multiple intestinal neoplasia) mouse model is one of the best-studied models for intestinal tumors (Moser et al. 1990). It was generated by random ENU mutagenesis and carries a nonsense mutation at codon 850 of the Apc gene, resulting in a truncated Apc polypeptide lacking all the C-terminal domain, involved in the regulation of β-catenin (Su et al. 1992). Somatic mutation analysis of the wild-type Apc gene in these tumors showed that not only Apc itself but the entire chromosome 18 is lost in all investigated lesions (Luongo et al. 1994). *Min* /+ mice develop numerous intestinal and colonic adenomas similar in morphology to the human polyps seen in FAP patients. The incidence of polyps in *Min* mice varies considerably depending on the background of the mouse strain (Moser et al. 1992). On a C57B1/6 background, *Min* mice developed approximately 30 tumors in their intestine, whereas on a mixed AKR × C57B1/6 background, this number decreased to an average of approxi-

mately six tumors and the mice lived considerably longer. These results suggest that the AKR strain of mice carries genes that can reduce polyp formation in *Min*/+ mice. By back-crossing *Min* mice from the AKR × C57B1/6 background to pure C57B1/6 and using simple sequence length polymorphisms (SSLP), the Min modifying locus was mapped to chromosome 4. The locus was named *Mom-1* for modifier of *Min1*. When the AKR allele of *Mom-1* was transferred onto the sensitive C57B1/6 background, the tumor incidence in *Min*/+; *Mom-1*^{AKR}/*Mom-1*$^{B1/6}$ mice was halved. When both alleles of *Mom-1* were derived from the AKR strain, the tumor incidence was four times less. The secretory phospholipase A2 gene (*Pla2*) has been proposed as a candidate for *Mom-1* (MacPhee et al. 1995). *Pla2* is highly expressed in the intestine of resistant mouse strains such as AKR, CAST, and MA mice, but poorly expressed in the intestine of the sensitive strain, C57B1/6. Sequence analysis of *Pla2* showed that the C57B1/6 allele contained a thymidine insertion in exon 3, resulting in aberrant splicing and a frameshift mutation (during Apc expression). This alteration was not present in any of the resistant mouse strains. It has been suggested that *Pla2* may reduce the incidence of intestinal tumor formation by protecting against the harmful effects of dietary fatty acids. These studies clearly highlighted the critical importance that genetic background has on different mouse models, as well as the need to rigorously utilize a homogeneous genetic background when analyzing a specific mouse phenotype.

In Min mice, additional somatic mutations need to be induced by carcinogens in order to achieve a high incidence of intestinal tumors (Shoemaker et al. 1997). In addition, COX-2 and iNOS play an important role in Min mice carcinogenesis, as well as in humans: knockout Min mice with deleted COX-2 or iNOS gene(s) develop fewer adenomas than wild-type Min mice (Ahn and Ohshima 2001). In addition, methylation plays a critical role in Min mice carcinogenesis, since a reduction in DNA methyltransferase activity suppresses polyp formation (Laird et al. 1995). *K-ras* and *p53* mutations are not detected in Min mice tumors, in contrast to human tumors. Nevertheless, the Min mice have been broadly used to study the general principles of carcinogenesis, to test the response to suppressive agents such as aspirin (Williamson et al. 1999; Jacoby et al. 2000) and to test the carcinogenicity of various compounds (Corpet and Pierre 2003).

There are several mouse models carrying specific deletions in the *APC* gene, which develop multiple adenomas throughout the gastrointestinal tract (GI), especially in the small intestine, for example, Apc$^{\Delta 716}$ (Oshima et al. 1995) and Apc$^{+/1638N}$ (Fodde et al. 1994). Somatic mutation analysis of the intestinal Apc$^{+/1638N}$ tumors revealed that approximately 75% of the neoplastic lesions analyzed underwent somatic loss of the wild-type copy of Apc (LOH), while the remaining 25% may have inactivated the wild-type Apc allele by point mutations. The Apc$^{+/1638N}$ tumors are mainly located in the proximal regions of the GI tract, particularly clustering at the transition from the stomach to the small intestine. This is an interesting difference with the Min model, which

shows a higher density of tumors in the second half of the jejunum (Shoemaker et al. 1997). Furthermore, $Apc^{+/1638N}$ mice contain a wide spectrum of extraintestinal abnormalities.

Thus, mice carrying germline mutations in APC represent a powerful model for studying FAP, but are less suitable for studying sporadic CRC. In fact, tumors develop too early for experimental therapeutical approaches, causing a high mortality rate at a young age. Further, they are mainly located in the small intestine and no tumors are detected in the colon. The use of conditional mice models has made it possible to induce temporally controlled and tissue-specific somatic gene mutations. For example, in Apc^{580S} (Shibata et al. 1997) and $Apc^{\Delta 14/+}$ (Colnot et al. 2004) mice, exon 14 of the APC gene is flanked by loxP sites. The gene can thus be deleted by local infection of the colorectal region with an adenovirus expressing the Cre recombinase (Shibata et al. 1997). The main phenotypic difference of the $Apc^{\Delta 14/+}$ model from other Apc models, is the shift of the tumors in the distal colon and rectum, resembling human disease more closely (5–15 polyps develop in the colorectum of these mice, whereas only 0.4–4 in other *Apc* mutants). All lesions, including early lesions, revealed *Apc* LOH and consequently loss of *Apc* gene expression. Further, the severity of the colorectal phenotype is partly due to the particular mutation $\Delta 14$, but also to environmental parameters, since mice raised in conventional conditions developed more colon cancers than those raised in pathogen-free environments. To analyze the immediate effects of Apc loss in the small intestinal epithelium, both in the stem-cell compartment and in postmitotic epithelial cells, $Apc^{\Delta 14/+}$ mice were crossed with transgenic mice expressing tamoxifen-dependent Cre recombinase under the control of the villin promoter (Table 1) (el Marjou et al. 2004). Within 4 days, Apc loss induced a dramatic enlargement of the crypt compartment associated with intense cell proliferation, apoptosis, and impairment of cell migration.

To determine the role of β-catenin in intestinal tumorigenesis, Harada and colleagues constructed a mutant mouse strain $Catnb^{lox(ex3)}$, in which exon 3 of the β-catenin gene was sandwiched by loxP sequences (Harada et al. 1999, 2002). When the germline heterozygote mice were crossed with mice expressing Cre recombinase controlled by the Fabp promoter, they developed adenomatous intestinal polyps resembling those found in $Apc^{\Delta 716}$ mice. Some nascent microadenomas were also found in the colon. Other mice, like $A\,33^{\Delta N\beta cat}$ which carry N-terminal truncation of β-catenin, also develop a few spontaneous ACF in the colon (Orner et al. 2002). These results present experimental evidence that activation of the Wnt signaling pathway can cause intestinal and colonic tumors.

Recently, several new murine models for K-*ras*-induced tumorigenesis have been described (reviewed in Janssen et al. 2005). Using a new gene targeting procedure, called hit-and-run, Johnson and colleagues created mouse strains carrying oncogenic alleles of *K-ras* ($K\text{-}ras^{LA}$) that can be activated only through

Table 1 Conditional mouse lines targeting the intestinal epithelium

Promoter	System	Inducer	Phenotype	Reference
FABP	Cre	No	By embryonic day 16.5, Fabp-Cre induces recombination in all cell layers of the transitional epithelium that lines the renal calyces and pelvis, ureters, and bladder.	Saam and Gordon 1999
	rtTA	Doxycycline	Fabp-rtTA allows temporally and spatially controlled recombination in the gut and at any period during adulthood. This system uses Fabp regulatory elements to direct the expression of a reverse tetracycline-regulated transactivator (rtTA). Another transgene encodes the Cre recombinase under the control of tet operator sequences and a minimal promoter from human cytomegalovirus (tetO-PhCMV-Cre). In the absence of a doxycycline inducer, no basal recombination is detectable in the gut. After 4 days of oral administration of doxycycline, recombination of the reporter is apparent in the small intestinal, cecal, and colonic epithelium. After doxycycline is withdrawn, the recombined locus persists for at least 60 days, indicating that recombination has occurred in epithelial cell progenitors that have long residency times in the proliferative units of the intestine.	

a spontaneous recombination event in the whole animal. In addition to lung cancer, the *K-ras*^{LA} animals developed both thymic lymphoma and skin papillomas. Despite the frequency of *K-ras* mutations in carcinomas of the pancreas and colon in humans, these tumors were not detected in the *K-ras*^{LA} mice. Significantly, however, all of the mutant mice examined had multiple aberrant crypt foci (ACF) in the colon. A mouse model targeting oncogenic K-*ras*^{G12V} under the control of the intestine-specific villin promoter in epithelial cells of

Table 1 (continued)

Promoter	System	Inducer	Phenotype	Reference
Villin 9 kb	Cre, Cre-ERT2	No Tam-oxifen	Genetic recombination is initiated at embryonic day 9 in the visceral endoderm, and by E12.5 in the entire intestinal epithelium, but not in other tissues. Cre expression is maintained throughout adulthood. Transgenic mice bearing a tamoxifen-dependent Cre recombinase (vil-Cre-ERT2) expressed under the control of the villin promoter allow targeted spatiotemporally controlled somatic recombination. After tamoxifen treatment, recombination is detectable throughout the digestive epithelium. The recombined locus can persist for over 60 days after tamoxifen administration, despite rapid intestinal cell renewal, indicating that epithelial progenitor cells are targeted.	el Marjou et al. 2004
Villin 12.4 kb	Cre	No	A 12.4-kilobase region of the mouse villin gene drives high level expression of two different reporter genes (LacZ and Cre recombinase) within the entire intestinal epithelium of transgenic mice.	Madison et al. 2002
Cyto P 450	Cre	β-naptho flavone	Recombination at a lacZ reporter locus showed extensive expression of β-galactosidase in liver, intestine, pancreas, gallbladder, esophagus, and stomach in response to β-napthoflavone treatment. Expression patterns were stable in renewing epithelia for at least 6 months, implying that long-lived stem cells are targeted.	Ireland et al. 2004

the large and small intestine led to the development of tumors mainly localized in the duodenum and the jejunum (Janssen et al. 2002). This animal model recapitulates the histopathologic and genetic features of the human disease and displays a close resemblance to the stages of tumor progression in human colorectal cancer, ranging from preinvasive precursor lesions to invasive adenocarcinoma. The fact that *Apc* was not found mutated in this particular

mouse model suggests that K-*ras* might contribute not only to colorectal cancer progression, but might also be implicated in tumor initiation. The effects of endogenous *K-ras*^{G12D} expression in colonic epithelial cells were examined (Tuveson et al. 2004) by interbreeding *LSL-K-ras*^{G12D} mice (Jackson et al. 2001) with fatty acid-binding protein (Fabp)-cre transgenic mice (Table 2) (Saam and Gordon 1999). All compound mice examined had diffuse hyperplasia and dysplasia of colonic crypts, whereas the parental strains did not. These epithelial hyperplasias and dysplasias are markedly different from the reported lack of effect of an expressed *K-ras*$^{G12V\text{-}IRES\text{-}BGeo}$ allele in colonic epithelial cells (Guerra et al. 2003).

TGFβ-related growth factors have also been implicated in intestinal tumor formation. TGFβ transduces its signal into the cell via the second messengers Smad2, Smad3, and Smad4. It has recently been shown that Smad3 mutant mice develop metastatic colorectal cancer (Zhu et al. 1998). All inbred Smad3$^{-/-}$ mutants present colorectal adenocarcinomas, some of which are highly aggressive; approximately one-third of them invade through all layers of the bowel wall. In several mutant mice, lymph nodes are enlarged and show infiltration by carcinoma.

As discussed earlier, the development of the intestinal tumors requires the sequential acquisition and cooperation between different oncogenic mutations. To mimic this complex process, it is possible to generate mice in which the expression of different genes in the intestine will be sequentially turn on and the acceleration in tumor onset could be monitored. Thus far there have been several examples of this strategy.

Inactivation of the APC gene is observed at early stages of intestinal tumor formation, whereas loss of E-cadherin is usually associated with tumor progression. Since both proteins compete for binding to β-catenin, a possible synergistic mechanism on tumor initiation and progression was studied in the compound *Apc*$^{+/1638N}$ / *E-cadherin* $^{+/-}$ animal model (Smits et al. 2000). Double heterozygous animals showed a ninefold increase in intestinal tumor numbers, compared with Apc^{1638N} animals. However, the intestinal tumors showed no significant differences in grading and staging. Loss of heterozygosity analysis at the Apc and E-cadherin loci revealed loss of the wild-type Apc allele in most cases, whereas the wild-type E-cadherin allele was always retained. This observation led to the conclusion that introduction of the E-cadherin mutation in Apc^{1638N} animals enhances Apc-driven tumor initiation without affecting tumor progression.

In order to study the molecular mechanisms underlying the synergism between mutant *Apc* and *K-Ras*, a compound transgenic mouse model that mimics this common genetic combination has been generated (Janssen et al. 2006). A transgenic line expressing the activated human *K-Ras* oncogene driven by the villin promoter (Janssen et al. 2002) was bred with the*Apc*$^{+/1638N}$ model that carries a targeted mutant allele at the endogenous *Apc* locus. Compound *Apc*$^{+/1638N}$/pVillin- *K-Ras*^{G12V} animals are characterized by a tenfold increase

Table 2 Examples of conditional gene expression/ablation giving rise to cell proliferation dysfunction in the digestive tract

Genes	Promoter	System	Phenotype	Reference
K-ras G12D, overexpression	FABP	Cre	Diffuse hyperplasia and dysplasia of the colonic crypts.	Tuveson et al. 2004
APC, gene ablation	Villin (9 kb)	Cre-ERT2	Dramatic enlargement of the crypt compartment associated with intense cell proliferation, apoptosis, and impairment of cell migration.	Andreu et al. 2005
Notch, overexpression	Villin (9 kb)	Cre	Notch activation amplifies the intestinal progenitor pool while it inhibits cell differentiation. Notch activity is required for the maintenance of proliferating crypt cells in the intestinal epithelium.	Fre et al. 2005
RBPJk, gene ablation	Villin (9 kb) and P450	Cre-ERT2	Rapid, massive conversion of proliferative crypt cells into postmitotic goblet cells. A similar phenotype was observed by blocking the Notch cascade with a γ-secretase inhibitor.	van Es et al. 2005
c-myc, gene ablation	Villin (9 kb)	Cre-ERT2	Failure to form normal numbers of crypts in the small intestine.	Bettess et al. 2005
c jun, gene ablation	Villin (9 kb)	Cre	In the Apc Min mouse model, genetic abrogation of c-Jun N-terminal phosphorylation or gut-specific conditional c-jun inactivation reduced tumor number and size and prolonged lifespan.	Nateri et al. 2005
β-catenin, gene ablation	P 450	Cre	Crypt ablation, increased apoptosis, depleted numbers of goblet cells, and detachment of villus absorptive cells from the villus core as intact sheets.	Ireland et al. 2004
Oct-4, overexpression	M2[a]	rtTA	In the intestine, Oct-4 expression causes dysplasia by inhibiting cellular differentiation in a manner similar to its effect on embryonic cells.	Hochedlinger et al. 2005

[a]M2 and mx1 are not intestine-specific promoters

Table 2 (continued)

Genes	Promoter	System	Phenotype	Reference
Bmpr1a, gene ablation	mx1[a]	Cre	Disturbs homeostasis of intestinal epithelial regeneration with an expansion of stem and progenitor populations, eventually leading to intestinal polyposis resembling human juvenile polyposis syndrome.	He et al. 2004

[a]M2 and mx1 are not intestine-specific promoters

in tumor multiplicity and by accelerated tumor progression when compared with the single transgenic littermates, resulting in strongly enhanced morbidity and mortality. Tumors from compound mutant mice proliferate faster and show lower levels of apoptosis compared with the single transgenic littermates. Several lines of evidence indicate that the observed increase in tumor multiplicity and malignant transformation is caused by the synergistic activation of Wnt/β-catenin signalling in cells with oncogenic *K-Ras* and loss-of-function *Apc* mutations. Accordingly, intestinal tumors from $Apc^{+/1638N}$/pVillin- $K\text{-}Ras^{G12V}$ mice show a significant increase in cells presenting nuclear accumulation of β-catenin, accompanied by a reduction of its membranous staining when compared to $Apc^{+/1638N}$ animals.

To study the role of the ternary complex made of the proto-oncoprotein c-Jun, TCF4 and β-catenin on intestinal tumorigenesis, Nateri and colleagues inactivated c-*jun* specifically in the gut, using villin-cre transgenic mice (el Marjou et al. 2004) (Nateri et al. 2005). A compound animal was generated by crossing $Apc^{Min/+}$ mice with a floxed allele of c-*jun* (Behrens et al. 2002). Inactivation of c-jun seems to protect $Apc^{Min/+}$ mice from intestinal cancer, since a reduced number and size of tumors was observed, accompanied to a prolonged animal lifespan.

The adenomas that develop in the mouse models described above show proper resemblance to the equivalent lesions in FAP patients. Furthermore, the tumors form spontaneously, predictably, and with a high incidence. They are nonimmunogenic and arise in immunocompetent mice (in contrast to the implantation models). A disadvantage of these approaches, though, is that metastases form infrequently (if at all) and in an unpredictable fashion.

3.1.2 Hereditary Nonpolyposis Colorectal Cancer

In addition to the hereditary disease associated with *APC* mutations, there is a second, and actually more widespread, type of hereditary predisposition

to colon carcinoma in which the course of events is quite different from the one we have described for FAP syndrome. In patients with HNPCC, the probability of colon cancer is increased without any correlation with the number of colorectal polyps. The HNPCC syndrome is not attributable to *Apc* mutations, but to mutations in mismatch repair (MMR) genes (*Mlh1*, *Msh2*, *Msh6*, *Pms1*, *Pms2*). The mutation rate is 100- to 1,000-fold greater in MMR-deficient cells than in normal cells. The tumors are characterized by instability at short tandem repeat sequences, also called microsatellites. Most microsatellites are found in noncoding DNA, but some mutations caused by MSI modify genes involved in later stages of carcinogenesis, e.g., transforming growth factor-β receptor II and insulin-like growth factor II receptor. In addition to mutations, human tumors have a general DNA hypomethylation status, and the aberrant hypermethylation of promoter CpG islands leads to transcriptional silencing of key growth-controlling genes and contributes to cancer progression.

3.1.2.1 Mouse Models for HNPCC

As mismatch repair enzymes play a crucial role in the repair of single base mismatches, small insertions or deletions, the increased mutation rate in mismatch repair pathway-deficient mice was examined as a possible tool to increase tumorigenesis in tumor-prone mice.

Indeed, mice harboring homozygous deletions in the mismatch repair enzyme *Mlh1* display accelerated intestinal adenoma formation in a heterozygous *APC* mutant background, and missense and frameshift mutations that are characteristic of *Mlh1* deficiency were demonstrated in the remaining wild-type *APC* allele (Kuraguchi et al. 2000; Shoemaker et al. 2000). Mice homozygously deleted for Mlh-1 or MSH-2 develop lymphomas but are also prone to intestinal neoplasia and therefore are a good model for studying HNPCC development. $Mlh1^{-/-}$ mice develop gastrointestinal tumors in 33% of cases. Moreover, addition of an APC gene mutation into the $Mlh^{-/-}$ background results in a 40-fold increase in the number of GI tumors, leading to 100% GI tumor formation. There were no reports of metastases in these mice (Edelmann et al. 1999). All homozygous *Msh2* -deficient mice succumbed to disease within the 1st year of observation, with lymphomas observed in at least 80% of the cases. The majority (70%) of animals 6 months of age or older developed intestinal neoplasias associated with APC inactivation (Reitmair et al. 1996; de Wind et al. 1998; Prolla et al. 1998). These models have provided insights into the mechanisms underlying the development of CRC. Mice homozygous for mismatch repair (MMR) genes and heterozygous for a defect in the gatekeeper gene APC have shown that the MMR gene enhances APC-mediated intestinal carcinogenesis. Exposure of MMR-deficient cells to endogenous or exogenous mutagens may potentiate tumorigenesis and may be critical in the organ selection in HNPCC (de Wind et al. 1995; Edelmann et al. 1999). These models also revealed that

MMR-deficient cells fail to induce apoptosis in response to alkylating agents. In contrast, the alkylated base damage remains in the DNA, thereby potentiating carcinogenesis. This may have direct implications for chemotherapy of HNPCC patients (de Wind et al. 1995).

At present, transgenic mouse models are the best tools available to researchers to study the pathogenesis of human cancer, identify the genes implicated in the development and progression of the disease, as well as test potential new therapies. Nonetheless, several limitations, some of which are discussed here, still preclude our complete understanding of certain tumors and the results obtained in mice have to be considered with caution when they are applied to human cases. The recent and rapid development of new technologies for transgenesis and for mouse engineering will certainly improve in the near future our ability to understand the molecular mechanisms leading to cancer and will illustrate even further the versatility and advantages of using transgenic mice for cancer research.

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

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Abstract Evolutionarily conserved, widely expressed genes provide the functional backbone of most, if not all, cell types. Although mouse mutants created by germ line gene inactivation are instrumental in establishing the importance of such genes in vivo, distortion of embryonic development or multiple body systems often preclude detailed functional studies. To overcome this limitation, DNA recombination systems such as Cre/loxP of bacteriophage P1, have been adapted for use in mammalian cells. The mutagenic event is restricted to the tissue or cell type in question leaving other body systems undisturbed. Conditional inactivation of Csk or Socs3, for example, established their key role in the prevention of inappropriate inflammation, while unexpected immunoregulatory activities emerged from studies of the NF-κB and AP-1 pathways. Also, cell types responsible for protective or pathogenic TNFα production have been identified. Inactivation of immunoregulatory receptors in leukocyte subsets can provide robust experimental systems revealing the conceptual simplicity

underlying the modulation of complex signaling pathways during homeostatic responses. As illustrated for TGF-β receptor, such system-guided approaches can provide a comprehensive picture of the regulatory events driving in vivo phenotype and specific responses of primary cells. This in turn facilitates the identification of novel regulatory mechanisms, targets for therapeutic intervention and prediction of side effects. With the increasing evidence for a role of somatic mutations in a wider range of human diseases, conditional mouse models are set to play a continuing part in the identification of pathogenic mechanisms for restoration of normal cellular processes in diseases including cancer, inflammation and autoimmunity.

Keywords Inflammation · Autoimmunity · Immune response · Cre/loxP · Regulatory Networks

1 Introduction

Mouse mutants generated by homologous recombination in embryonic stem cells continue to deliver the evidence required to firmly establish gene-function relationships in normal development and disease. The limitation of this approach, however, becomes apparent when widely expressed genes are mutated, many of which control the most fundamental regulatory pathways and functions. In such cases, complex phenotypic consequences or lethality often preclude detailed studies. Hence, it remained practically impossible to determine cell-type specific functions of the most versatile, widely expressed genes until methods to restrict mutagenesis to selected somatic cell types emerged.

Conditional mutagenesis is based on the DNA recombination system that normally supports the life cycle of the bacteriophage P1 adapted to function in mammalian cells (Sauer and Henderson 1988). As summarised previously (Rajewsky et al. 1996; Metzger and Feil 1999), mice can be engineered to express the DNA recombinase Cre in a selected tissue or cell type. The Cre activity is then employed to recombine a target gene sequence flanked by loxP sites (floxed). Development of the animal can proceed normally until Cre expression is switched on in the target tissue leading to the mutagenic recombination of loxP sites and—depending on their relative orientation—to deletion or inversion of the intervening DNA.

Over the past decade, the "zoo" of available Cre-expressing mice has been expanding steadily for applications within and outside the immune system. Mice expressing Cre in leukocyte subsets such as neutrophils and/or macrophages, or at various stages of B and T cell development (Table 1) have been applied to dissect developmental pathways in detail and to uncover, through somatic mutagenesis, the role of widely expressed genes in the protection from infectious and immunopathogenic diseases. Evidence supporting a significant contribution of somatic mutations to a wider range of human diseases is only beginning to emerge (Erickson 2003). Diagnostic advances based on whole genome analysis are likely to boost the growing body of data. As for tumour

Table 1 Some successfully applied mouse lines expressing Cre in haematopoietic cells or target tissues of immunopathogenic responses

Target gene	Target cell	Reference
GEcre	Neutrophils	Thomas et al. 2004
LysMCre	Neutrophils/macrophages	Clausen et al. 1999
Lck-cre	T cells	Orban et al. 1992
CD4-cre	T cells	Lee et al. 2001
hCD2-iCre	T cells	de Boer et al. 2003
Vav-iCre	Haematopoietic cells endothelium	de Boer et al. 2003
CD19-Cre	B cells	Rickert et al. 1995; Rickert et al. 1997
CD21-Cre	Mature B cells	Kraus et al. 2004
Alfp-cre	Hepatocytes	Kellendonk et al. 2000
K14-cre	Keratinocytes	Hafner et al. 2004
Mx1-Cre	Widely inducible	Kühn et al. 1995
Tie2e-Cre	Endothelium	Kano et al. 2003
K5-Cre-ER^{T}	Keratinocytes/skin	Brocard et al. 1997

biology (Jonkers and Berns 2002), conditional mouse mutants will be instrumental in understanding disease processes and in particular those caused by somatic mutations. Some of the examples below also show that unexpected new leads to disease mechanisms can emerge from conditional mutagenesis in cases where the mouse phenotype mirrors the symptoms of a human condition of unknown cause. Given that infectious, inflammatory and autoimmune diseases continue to pose a growing threat to human health worldwide, a full understanding of the molecular and cellular basis of host immunity should be of high priority. Conditional mutagenesis provides the experimental tool to establish gene/function causality in vivo for practically any gene in any functional context within the cellular and molecular networks. Complete integrated data sets are essential to derive models, which truly reflect biological processes facilitating design and optimisation of therapeutic strategies.

2 The Experimental System

Tagging genes with loxP sites is straight forward as it merely requires introduction of short DNA sequence elements into the genome by homologous recombination (Rajewsky et al. 1996; Metzger and Feil 1999). Efficient and specific expression of the Cre recombinase in vivo, however, proves more difficult, because transcriptional control is complex and incompletely under-

stood. Potential driver-loci for Cre can be identified among the genes that are active in specific target cell types. A number of strategies have been employed successfully to impose tissue-specific expression on the Cre recombinase. Classical transgenesis using compact recombinant loci relies on the selection of robust promoter sequences driving Cre expression. However, the random placement of transgenes in the genome often leads to epigenetic deregulation through dominant effects of the chromatin structure at the integration site. This position-dependent expression or variegation can be addressed by including appropriate locus control regions (LCRs), defined as long-range regulatory elements conferring position-independent transgene expression (Festenstein and Kioussis 2000). In many cases, however, the regulatory elements required for bona fide expression are not known. By maximising the chromosomal flanking regions of transgenic loci, the probability of including essential regulatory sites can be increased. Yeast or bacterial artificial chromosomes (YACs, BACs), which provide vector systems to handle large chromosomal loci, are successfully employed to obtain the Cre expression pattern desired. Alternatively, targeted knock-in of the Cre coding sequence into a genomic driver locus can confer specific expression without detailed knowledge of the authentic regulatory mechanisms involved (Rickert et al. 1995, 1997). In cases of known or suspected haploinsufficiency, expression of the modified structural gene can be maintained, for example through a bicistronic configuration of the genomic and Cre coding sequences (Vincent and Robertson 2004).

Unmodified prokaryotic coding sequences, such as Cre, are poorly expressed in mice. Codon usage, GC content and the absence of introns are known to dramatically impair expression in vivo. Attempts to increase the robustness of Cre expression included the adaptation of the Cre codon usage with a 1.5- to 2-fold increase in expression and activity (Shimshek et al. 2002). By removing GC pairs from the coding sequence and creating an alternating exon–intron structure, we found that mRNA levels for recombinant genes including Cre and GFP increased 30-fold compared with the intron-less counterparts (Lacy-Hulbert et al. 2001). Intron insertion not only led to a fivefold increase in protein levels, but also reduced variation of expression among transfected clones. Systematic tests of Cre expression constructs in vivo should help define the optimal approach for robust expression and target gene recombination in vivo. The upper limit of useful Cre expression levels is reached before the documented toxic effects begin to manifest. Nonspecific endonuclease activity, observed at high Cre concentrations, can cause chromosomal aberrations and suppression of cell proliferation, thus calling for careful control of expression levels (de Alboran et al. 2001; Loonstra et al. 2001). Although the levels typically obtained in vivo do not appear to reach the toxicity threshold, the use of an engineered Cre devoid of nonspecific endonuclease activity (Loonstra et al. 2001) may be worth considering.

In addition to tissue specificity, temporal control over the mutagenic event is desirable. Lineage development can then be left undisturbed before inducing

mutagenesis in differentiated somatic cells of the adult mouse. By combining the Cre coding sequence directly with pharmacologically inducible promoters, such as the mouse Mx or Mx1 gene promoter (Kühn et al. 1995), the adapted bacterial Tet system (see the chapter by R. Sprengel and M.T. Hasan, this volume) or nuclear hormone fusion proteins (Brocard et al. 1997), this can be achieved. Along these lines, a system for cell ablation studies developed recently is based on Cre-induced, tissue-specific expression of the diphtheria toxin receptor (Buch et al. 2005). Cre-mediated removal of a transcriptional stop sequence from a ubiquitously active transcription unit causes cell-type-specific receptor expression and toxin sensitivity. Toxin injection leads to efficient lineage ablation, thus offering a versatile system to study the role of specific cell types in vivo, by intercrossing with the appropriate Cre-expressing mouse.

As discussed below, conditional mutagenesis has been instrumental in demonstrating the critical role of widely expressed genes in both cell autonomous and ligand-induced control of the leukocyte activation threshold. The in vivo models not only revealed novel fundamental mechanisms of immunoregulation and the topology of molecular networks involved, but also well defined novel animal models emerged for the systematic testing of therapeutic strategies to prevent or treat infectious and immunopathogenic diseases.

3
Conditional Mutagenesis in the Immune System

3.1
Acute and Chronic Inflammation

Within minutes, neutrophils (polymorphonuclear granulocytes, PMN), phagocytes of the myeloid lineage, are recruited from the blood into infected tissues to ingest and kill microbes. This early acute inflammatory response is followed by influx of macrophages, which provide continuing antimicrobial activity and help resolve the inflammatory focus through uptake of debris and apoptotic cells. Classical knock-out studies not only demonstrated the contribution of neutrophils to immunopathogenic reactions such as endotoxic shock (Tkalcevic et al. 2000), but they also prompted a re-evaluation of the cytotoxic mechanisms employed to kill microbial pathogens (Segal 2005). Mice expressing the Cre recombinase primarily in the neutrophil lineage after insertion into the *ela2* (granulocyte elastase, GEcre) locus (Tkalcevic et al. 2000; Thomas et al. 2004) or in neutrophils and macrophages when driven by the macrophage lysozyme locus (Clausen et al. 1999), have helped identify some key regulatory gene products and cell types, which prevent inflammatory disease by ensuring that the destructive potential of inflammatory cells is contained during the critical recruitment phase before being released to destroy the pathogen (Tables 1, 2).

Table 2 Conditional mutagenesis in myeloid cells and inflammation

Target gene	Cre mouse, target cell	Phenotype	Disease model	Process affected	Reference
Csk	GEcre, Neutrophils	Persistent, acute multifocal inflammation, ↑ Neutrophil responsiveness, endotoxin sensitivity	Sweet's syndrome, granuloma annulare, septic shock	↑ Integrin release, degranulation, adhesion ↓ Migration	Thomas et al. 2004
Rac1	LysMCre, Neutrophils, Macrophages	↓ Neutrophil recruitment	–	↓ Migration, actin assembly	Glogauer et al. 2003
Stat3	LysMCre, Neutrophils, Macrophages	Constitutionally active macrophages, ↑ Proinflammatory cytokines, Th1 activity, endotoxin sensitivity	Chronic enterocolitis, inflammatory bowel disease, septic shock	↓ IL-10 signalling, ↑ Th1 development	Takeda et al. 1999
Hif1α	LysMCre, Neutrophils, Macrophages	↓ Tissue infiltration, microbial killing	Listeriosis	Glycolysis, energy metabolism	Cramer et al. 2003
Socs3	vavCre, haematopietic cells, endothelium	Late-onset neutrophilia, inflammation hind leg paresis ↑ Th1 activity	Multifocal chronic inflammation	↑ Haematopoiesis, granulopoiesis, G-CSF/Stat signalling, Th1 activity	Croker et al. 2003
TNFα	LysMCre	↑ Resistance to endotoxin	Septic shock	TNFα production	Grivennikov et al. 2005
Ikk2	LysMCre	↓ TNFα, IL-10, ↑ Atherosclerosis	Atherosclerosis	↓ NF-κb activation	Kanters et al. 2003

Table 2 (continued)

Target gene	Cre mouse, target cell	Phenotype	Disease model	Process affected	Reference
IκBα	LysMCre	Nonautonomous myeloproliferation	Myeloid premalignancy	↑ Jagged1 expression in hepatocytes, ↑ Notch1 signaling in neutrophils	Rupec et al. 2005
IL4Ra	LysMCre	↑Sensitivity to *S. mansoni* infection, ↑ Liver and gut pathology, ↑ Th1 activity	Schistosomiasis, septic shock	Defective alternative/ anti-inflammatory, macrophage activation	Herbert et al. 2004
Ikk2	K14-Cre	TNFα-dependent inflammatory skin disease	Inflammatory skin disease	NF-κB activation	Pasparakis et al. 2002a
MLL-CBP	Mx-Cre	Myelomonocytic hyperplasia, leukaemia	Mixed lineage leukaemia	Transcription co-factor expression	Wang et al. 2005
Stat3	Tie2-eCre	↑ LPS-induced multifocal inflammation and shock	Septic shock	Stat3-signaling	Kano et al. 2003
Jun	K5-Cre-ER^T	↑ Skin inflammation, cytokine expression arthritis	Psoriasis	AP-1 controlled transcription	Zenz et al. 2005

3.1.1 Csk Controls the Neutrophil Activation Threshold and Prevents Persistent Inflammatory Disease

C-terminal Src-kinase (Csk) inhibits the activity of Src-family kinases, which mediate signal transduction via a wide range of receptors including antigen-, chemokine-, growth factor receptors, and integrins. Csk inactivation in neutrophils of Csk-GEcre mice caused hyper-responsiveness as indicated by a persistent acute microbial inflammatory disease (Thomas et al. 2004). Persistent acute pulmonary inflammation and obstruction, inflammation of eyelids and skin and neutrophil infiltrates in the deep dermis/subcutis of the dorsal extremities were characteristic of the phenotype. Notably, intestinal tissues and joints showed no signs of inflammation. When the animals were treated with antibiotics or maintained under microbiologically controlled conditions they appeared healthy, but continued phagocyte hypersensitivity led to increased susceptibility to LPS-induced shock. The activation threshold of neutrophils had dropped as evident through spontaneous degranulation and release of integrins to the cell surface and hyperphosphorylation of key substrates of the integrin signaling/cell adhesion pathway. At the cellular level, hyperadhesion and impaired migration resulted from this deregulated response, explaining the dense perivascular clustering of neutrophils in vivo. Thus, Csk maintains the normal activation threshold of neutrophils, preventing premature upregulation of adhesion receptors and recruitment.

In T cells, Csk has been shown earlier to prevent the release of useless antigen-receptor-negative cells into the periphery (Schmedt et al. 1998; Schmedt and Tarakhovsky 2001). Similar to its role in neutrophils, Csk seems to maintain an activation threshold by suppressing Src-kinase activity. Expression of a functional antigen receptor is required to overcome the threshold for positive selection of the receptor expressing T cell. The increased Src-kinase activity in the absence of Csk seems to mimic this signal allowing antigen-receptor-negative, Csk-deficient cells to enter the peripheral lymphocyte pool. Csk mediated inhibition thus represents a versatile mechanism to establish distinct critical checkpoints in both innate and acquired immunity.

A number of skin conditions of unknown cause in humans show striking similarities to the inflammatory phenotype seen in Csk-GEcre mice. The localised form of granuloma annulare predominantly affects the extremities, such as the elbows, eyes, and ears, though it can be restricted to the dorsum of the hands with localisation of inflammatory cells to the deep dermis/subcutis (Weedon 1997). Acute febrile neutrophilic dermatosis (Sweet's Syndrome) is thought to represent an immunological hypersensitivity reaction, which may be triggered by respiratory tract infection or treatment with GCSF. Sweet's syndrome also shows the characteristic neutrophilic inflammation of the deep dermis/subcutis. The phenotypic similarities between the Csk-GEcre model

and the human conditions suggest that impaired negative control of granulocyte activation, perhaps due to impaired control of Src family kinases, may play a role in the development of these and similar acute inflammatory diseases. The Csk-GEcre mouse is a useful model to test therapeutic regimen aiming to suppress undesirable acute inflammatory responses by suppressing leukocyte recruitment early on.

3.1.2 Stat3 Suppresses Chronic Inflammatory Disease Via the IL-10 Pathway

The LysMcre mouse (Clausen et al. 1999), a knock-in of Cre into the macrophage lysozyme locus, which is expressed throughout the neutrophil and macrophage lineages, can be used for conditional mutagenesis in these cells. Using LysM-Cre mice to overcome the embryonic lethality of the Stat3 knock-out mouse Takeda et al. (Takeda et al. 1999) showed that this transcriptional regulator is specifically involved in the negative control of macrophage activation. Mutants showed an inflammatory syndrome with characteristic overexpression of proinflammatory cytokines, constitutive activation of macrophages, T helper cell imbalance and chronic inflammatory bowel disease (IBD), with notable similarity to mice deficient in the cytokine IL-10. While responding normally to INF-γ, Stat3-deficient macrophages failed to respond to IL-10, linking Stat3 with this inhibitory signaling pathway. The conditional mutant complements the IL-10 knock-out IBD model (Kühn et al. 1993) for testing specific therapeutic approaches aiming to reconstitute or replace inhibitory Stat activities in vivo.

3.1.3 Socs3 Suppresses G-CSF Signaling and Multifocal Inflammatory Disease

Haematopoietic or neutrophil/macrophage-specific inactivation of Socs3 established the essential role of this pleiotropic suppressor of cytokine signaling in the negative control of granulopoiesis and leukocyte homeostasis. Mice lacking Socs3 in all haematopoietic cells (Croker et al. 2003) were hyperresponsive to G-CSF signaling with multifocal inflammatory disease and extramedullary haematopoiesis. A correspondingly milder defect restricted to increased granulopoiesis and neutrophil numbers resulted from neutrophil/macrophage-specific inactivation (Kimura et al. 2004). The conditional mouse mutants with inflammatory phenotypes described here represent valuable models to test the efficacy of therapeutic strategies aiming to control potentially fatal inflammatory responses. For example, the efficacy of chemokine receptor inhibitors in suppressing certain acute and chronic inflammatory responses in vivo (Kaneider et al. 2005) could be established readily.

3.1.4 Pathogenic Versus Protective Production of TNFα

TNFα is a prime target for the therapeutic intervention in inflammatory diseases (Taylor et al. 2004). While systemic anti-TNF therapy can be beneficial in diseases such as rheumatoid arthritis and Crohn's disease, increased susceptibility to infection is often associated with the treatment. Which leukocyte subsets produce protective or pathogenic TNFα Cell-type-specific inactivation of TNFα in myeloid cells or T cells revealed that TNFα produced by phagocytes seems to drive both anti-microbial resistance and endotoxic shock with a marginal, if any, contribution by T cells (Grivennikov et al. 2005). TNFα inactivation in T cells alone, however, protected from experimental autoimmune hepatitis. The authors therefore suggest that suppression or functional block of T cell-derived TNFα may permit selective suppression of immunopathogenesis while conserving protective immunity.

3.1.5 Unexpected Functional Diversity of the NF-κB Pathway in Inflammation Control

The NF-κB pathway has long been recognised as a central proinflammatory signaling mechanism, which drives the initiation of immune responses by mobilising transcription factors controlling expression of proinflammatory genes. Surprising therefore was the finding that this pathway can, in certain conditions, also suppress inflammation. This was revealed by studying the role of Ikk2 (Pasparakis et al. 2002a), which encodes a kinase essential for NF-κB activation. Its inactivation in macrophages (Kanters et al. 2003) caused a chronic inflammatory condition with increased susceptibility to atherosclerosis. The mechanisms underlying this effect remain unclear, but reduced expression of anti-inflammatory cytokines such as IL-10 most likely contribute to the disease. Interestingly, inactivation of Ikk2 in keratinocytes of the skin provoked a TNF-mediated inflammatory skin disease (Pasparakis et al. 2002a). It seems that Ikk2 expression and thus NF-κB activation can suppress inappropriate inflammation by preventing keratinocytes from initiating recruitment of inflammatory cells prematurely.

Evidence pointing towards possible mechanisms with which a defective NF-κB pathway in one cell type may affect the behaviour and development of another came from a study of IκBα in the context of a myeloproliferative disease (Rupec et al. 2005). IκBα-deficient mice develop a severe myeloproliferative disorder with hyper-granulopoiesis. Surprisingly, conditional inactivation of IκBα in myeloid cells failed to cause a similar disease. However, co-culture of normal bone marrow cells with IκBα-deficient hepatocytes triggered increased colony formation in vitro. This was explained by deregulated expression of Jagged1 in mutant hepatocytes, which in turn caused constitutive activation

of its ligand Notch1 on neutrophils and hyperproliferative lineage expansion. The authors point out phenotypic similarities of their model with some of the currently unclassifiable myelodysplastic/myeloproliferative diseases and suggest that mutations in the *IκBα* locus may represent an underlying cause.

3.1.6 Jun Proteins, AP-1 Transcription Factors and Psoriasis-Like Disease

Psoriasis is a common disease affecting skin and joints with characteristic infiltration by inflammatory cells. *JunB*, a component of the AP-1 transcription factor, is localised near one of six loci linked with the disease in humans. Zenz et al. (Zenz et al. 2005) found that JunB and its proclaimed antagonist c-Jun are downregulated in psoriatic tissue. To test whether loss of Jun expression caused the disease, the authors generated mice in which both JunB and c-Jun could be deleted in the epidermis. They employed the Cre-ER^{T} system, in which the Cre coding sequence is fused to an estrogen receptor domain (Feil et al. 1996). When a keratin promoter mediates specific expression of the transgene in the epidermis, application of an inducer, typically tamoxifen, initiates translocation of the fusion protein to the nucleus and activation of Cre-mediated recombination (Brocard et al. 1997). Induced inactivation of Jun proteins in the epidermis triggered a psoriasis-like disease with arthritis. Combinatorial genetics using mice deficient in lymphocytes or TNF receptor I established that, in contrast to the skin disease, the arthritic phenotype required lymphocytes and TNF receptor signaling. Strikingly, chemotactic S100 proteins, that mapped to a susceptibility region 4 and are known to stimulate neutrophil migration, where strongly induced after Jun deletion. The authors point out that these data support the application of TNFα antagonists in the treatment of psoriatic disease and its suitability for preclinical studies to understand and cure this common inflammatory disease.

3.1.7 Il-4 Receptor Mediates Generation of Anti-inflammatory Macrophages

The cytokine balance during a response to infection is key to a properly tuned, successful anti-microbial response with minimal, potentially fatal destruction of self-tissues. By inactivating the receptor for the Th2 cytokines IL4/13 in macrophages, Herbert et al. have shown that alternative macrophages activated through this pathway play a critical role in the suppression of lethal parasite egg-induced inflammation (Herbert et al. 2004). Anti-inflammatory IL-10 or suppressors of cytokine signaling such as Socs1 and Socs3 did not seem to be involved as their expression was unaffected in the mutants. While the precise mechanisms remain uncertain, it is apparent that IL4/13 signaling in macrophages is vital to suppress exaggerated inflammation that interferes with egg expulsion and vital functions of liver and intestine. The investigators

conclude that macrophages triggered by IL4/13 signals are essential in the implementation of Th2 mediated anti-inflammatory effects required for the down-regulation and resolution of an inflammatory response.

3.2 Lymphocytes

By expressing a diverse antigen receptor repertoire biased for recognition and targeting of non-self determinants, lymphocytes provide a reservoir of cells from which acquired immunity is generated. Binding of foreign antigen triggers a programme of activation, proliferation and differentiation, which activates antigen-specific lymphocytes. Activated T helper cells, for example, can boost the microbicidal activity of macrophages or drive antibody production required for the neutralisation of many pathogens. Stringent control mechanisms are in place to prevent the selection and activation of auto-reactive cells. By conditional mutagenesis in lymphocyte subpopulations, the critical role of some of the more widely expressed regulatory proteins in the prevention of autoimmunity and direction of leukocyte responses has become clear. The inactivation of key regulators of signal transduction, cell survival and homeostasis not only delivered sometimes unexpected models of human diseases, but also genetic clues as to the molecular basis of the reported association of immune deficiency, autoimmunity and tumour formation (Butler and Oskvig 1974; Sleasman 1996).

3.2.1 T Lymphocytes

3.2.1.1 Phosphatase PTEN Links Autoimmunity and T Cell Lymphoma

T cells are endowed with the ability to distinguish self and non-self proteins – a property that is essential for immunological self-tolerance on the one hand and effective pathogen neutralisation by means of adaptive antigen-specific responses on the other. Both B and T cell antigen receptors deliver a positive signal the strength of which determines cell fate and selection into the functional lymphocyte pool. Consequently, modulators of antigen receptor signal strength and cell survival play a pivotal role in this process. Table 3 summarizes some of the studies that contributed to the characterisation of homeostatic mechanisms and generation of novel disease models. The multifunctional lipid phosphatase PTEN has been identified as a key modulator controlling T cell selection and the establishment of the peripheral T cell pool. T cell-specific inactivation of PTEN interfered with the negative selection of auto-reactive cells. Increased phosphorylation of PKB/Akt and Erk indicated enhanced signaling activity associated with enhanced exit of autoreactive cells from the thymus. The distortion of T cell selection caused spontaneous T cell activation

in the periphery, autoantibody production and hyper-γ-globulinaemia (Suzuki et al. 2001). Strikingly, all of the mouse mutants monitored also developed T lymphomas and died before 18 weeks of age. The mouse model is thus not only valuable for the development of therapies controlling lymphomas and autoimmunity, but it also highlights PTEN as a possible link explaining the association of autoimmunity and tumorigenesis in human conditions such as Bannayan-Riley-Ruvalcaba-syndrome.

3.2.1.2
T Cell-Derived IL-10 Prevents Inflammatory Bowel Disease

Proinflammatory cytokines such as Il-2 drive lymphocyte proliferation and activation, while others such as IL-10 play inhibitory roles. Although the phenotype of IL-10-deficient mice unequivocally established the essential role of this cytokine in the prevention of immunopathogenic disease (Kühn et al. 1993), uncertainty remained as to the cellular source of the anti-inflammatory IL-10, which is more widely expressed in leukocytes. To resolve this issue, Roers et al. (Roers et al. 2004) inactivated the *Il10* gene selectively in T cells. Similar to the complete knock-out, the absence of IL-10 in T cells caused inflammatory bowel disease, lethal immunopathology upon infection with *Toxoplasma gondii*, and enhanced contact hypersensitivity to dinitrochlorobenzene. However, impaired cutaneous irritant responses and increased sensitivity to LPS, as seen in IL-10-deficient mice, were not observed. It appears that cells other than T cells, probably of myeloid origin, control these irritant responses and endotoxin sensitivity through the production of IL-10. The data highlight T cells as a preferential target for the therapeutic enhancement of IL-10 production to treat leukocyte hyperactivity in inflammatory bowel disease, without compromising other potentially protective immune functions.

3.2.1.3
Fas/CD95/Apo1 and Pulmonary Fibrosis

The Fas (CD95/Apo1) receptor ligand system, which is widely expressed throughout the body, plays a central role in the downregulation of immune responses through induction of leukocyte apoptosis. Mutations in this receptor–ligand system cause autoimmunity with a lymphoproliferative syndrome in both mice and humans. To investigate the cellular basis of this syndrome, and specifically the role of T cell-expressed FAS, Hao et al. ablated Fas selectively in this cell type (Hao et al. 2004). Animals older than 10 months developed a severe wasting syndrome with pulmonary fibrosis and died before 18 months of age. Expression of the pro-inflammatory cytokine MIP-2 (IL8) and the profibrotic TGF-β1 were dramatically increased in alveolar lavage. Since anti-Fas treatment prevented pulmonary fibrosis and T cell recruitment, the authors conclude that interaction of activated Fas-deficient cells with ligands expressed

Table 3 Conditional mutagenesis in T Lymphocytes

Target Gene	Cre mouse T cells	Phenotype	Disease model	Molecular process affected	Reference
IL-10	CD4-cre	↑ Proinflammatory cytokines, susceptibility to *Toxoplasma gondii*, ear swelling/contact hypersensitivity, inflammatory bowel disease	Inflammatory bowel disease IBD, contact hypersensitivity	IL-10 production by T cells	Roers et al. 2004
Fas	CD4-cre	Progressive loss of T and B lymphocytes, wasting, shortened life span, inflammatory pulmonary fibrosis	Lymphopaenia, idiopathic pulmonary fibrosis	T cell homeostasis	Hao et al. 2004
Pten	Lck-Cre	Premature death CD4 T cell lymphomas, ↓ Thymic negative selection, apoptosis, ↑ Autoreactivity proliferation, cytokines, immunoglobulin production	Autoimmunity, T lymphoma, Bannayan–Riley–Ruvalcaba syndrome	TCR signaling, Akt/Erk phosphorylation	Suzuki et al. 2001
Ikk2	CD4-Cre	↓ Peripheral T cells, regulatory T cells, memory T cells	Lymphopaenia	↓ NF-κB activation	Schmidt–Supprian et al. 2003
TNFα	CD4-Cre	↓ Autoimmune hepatitis	Septic shock	T cell TNFα production	Grivennikov et al. 2005

in the microenvironment drive the fibrogenic process. The mouse model not only implicates deregulated T cell apoptosis as a possible pro-fibrotic mechanism, but it may also prove useful in the development of therapies for common, currently incurable disease such as idiopathic pulmonary fibrosis in humans. It seems tempting to suggest that germ line or somatic mutations affecting T cell apoptosis pathways and perhaps specifically the Fas receptor ligand system may contribute to the disease.

3.2.2
B Lymphocytes

B cells express a clonally distributed diverse repertoire of antigen receptors (BCRs). Antigen binding to the BCR induces vigorous proliferation and differentiation before large amounts of antibodies are secreted by terminally differentiated plasma cells. Both, the vigorous proliferation and the extensive antigen–receptor diversity carry an inherent risk of autoimmunity and tumorigenic transformation. We took advantage of the fact that CD19 – a B cell specific co-stimulatory receptor – is expressed throughout the B cell lineage and generated CD19-Cre mice for conditional mutagenesis in this cell type. Through knock-in of the Cre coding sequence into the CD19 locus (Rickert et al. 1995), a mouse was obtained that recombined floxed target genes efficiently in B cells (Rickert et al. 1997). Widely applied to study developmental aspects of the B cell lineage, CD19-Cre mice have also been instrumental in revealing some of the mechanisms engaged in the maintenance of normal B cell homeostasis and disease prevention in vivo (Table 4).

3.2.2.1
Phosphatase PTEN Guards B Cell Activation and the Spectrum of Antibody Isotypes Produced

Inactivation of the tumour suppressor lipid phosphatase PTEN (see also Sect. 3.2.1.1) in B cells (Suzuki et al. 2003) affects B cell homeostasis and the effectiveness of B cell responses, leading to a phenotype similar to hyper-IgM syndrome in humans. Although B cell numbers and total serum antibodies including autoreactive Ig were increased, immunisation elicited surprisingly poor IgG antibody responses. Impaired germinal centre formation and defective isotype switch explain this observation. Similar to PTEN-deficient T cells, mutant B cells were resistant to apoptosis and hyperproliferated in response to various activating stimuli delivered via the antigen receptor, CD40, or by stimulation with LPS. Furthermore, enhanced in vitro migration was observed. Increased phosphorylation of PKB/Akt confirmed the role of PTEN in the inhibition of this pathway, probably by dephosphorylating PIP3, a PI3 kinase product thought to be the primary substrate of PTEN. Inhibition of the PI3 kinase pathway, which controls multiple processes including activation, proliferation,

Table 4 Application of CD19-Cre mice for conditional mutagenesis in B lymphocytes

Target Gene	Phenotype	Disease	Molecular process	Reference
TGF-β-receptor II	↑ IgM, IgG, B cell responsiveness, anti-DNA antibody, ↓ IgA, chemotaxis	Hyper-ɣ-globulinaemia, IgA-deficiency IgAD	↑ B cell activation, IgG switch, ↓ Homeostasis, chemotaxis, IgA switch	Cazac and Roes 2000 Roes et al. 2003
c-myc	↓ Proliferation, activation	–	↓ Proliferation	de Alboran et al. 2001
Nibrin	↓ Chromosome stability, Ig isotype switch	Nijmegen breakage syndrome	↓ DNA repair, Ig isotype switch	Kracker et al. 2005
Stat3	Lymphomagenesis	Lymphoma ALCL	Cell survival	Chiarle et al. 2005
Pten	↑ B1 cells, marginal zone B cells, IgM, ↓ IgG, IgA	Hyper-IgM	↓Apoptosis Ig isotype switch, ↑ Proliferation, PKB/Akt activation migration	Anzelon et al. 2003; Suzuki et al. 2003
Ikk2	Loss of mature B cells	–	↓ NF-κB activation	Pasparakis et al. 2002b

survival and migration, would explain the profound effects on the cellular response as mentioned above. Surprisingly, in contrast to the T cell-specific PTEN deficiency (see Sect. 3.2.1.1), B cell tumour formation was not observed. The reasons for this difference remain uncertain. More detailed investigations may shed light onto some of the fundamental molecular requirements for tumorigenesis and help identify mechanisms that could be exploited to prevent it. Both the B cell and the T cell model described above should prove useful for testing synthetic inhibitors modulating the PI3 kinase/PKB pathway therapeutically to suppress inappropriate lymphocyte responses and tumorigenesis.

3.2.2.2 TGF-β Receptor Readjusts and Directs B Cell Responses for Mucosal IgA Secretion

The TGF-β/Smad pathway is one of the striking examples of evolutionary conserved multifunctional regulatory mechanisms, which control cell differentiation and homeostasis during embryogenesis and in almost all adult tissues. As a consequence, malfunctions in this pathway are implicated in diverse diseases including fibrotic conditions, tumorigenesis and autoimmunity. To by-

pass embryonic lethality and to determine the role of TGF-β receptor (TβR) in somatic tissues, we conditioned the ligand binding chain of the receptor (TβRII) for Cre-mediated inactivation in mice. TβR inactivation in B cells, by means of CD19-Cre, (TβRII-B) caused B cell hyperresponsiveness, hyper-γ-globulinaemia with anti-DNA reactivity and IgA-deficiency (Cazac and Roes 2000). The effects were most pronounced in the peritoneal cavity and the gut-associated lymphoid tissues. Peritoneal B1 cells were increased fivefold and the profound deficiency in IgA production was over-compensated by a disproportionate increase in IgG1 production. In addition, mutant B cells were hyper-responsive, as they responded readily to immunisation with a normally nonimmunogenic antigen (Cazac and Roes 2000; Borsutzky et al. 2004). The phenotype revealed a prominent role of TGF-β receptors in the control of B cell responses at mucosal sites and body cavities such as the peritoneum where antigen exposure is high. While suppressing inappropriate B cell activation in the mucosal environment, TβR also induces a major shift from IgG1 to IgA production. In contrast to other isotypes, IgA is capable of epithelial transcytosis and mucosal secretion, enabling it to neutralise the pathogen in the exterior space before breaching the protective barrier (see also Sect. 3.3 for more details).

At an incidence of about 1/600, selective IgA-deficiency (IgAD) is the most common primary immune deficiency in the Western world (Cunningham-Rundles 2001). Notably, this type of deficiency is also frequently (up to 40%) associated with autoimmune disorders or abnormal serum antibodies to cells or tissues (Etzioni 2003). A member of the TNF-receptor family, TNFRSF13B/TACI binds the B cell survival factor Baff. Mutations in the receptor were found to co-segregate with the IgA defect in IgAD2 (Castigli et al. 2005; Salzer et al. 2005). Since TβR signals drive IgA production and also support B cell survival, as discussed in more detail below, mutations in the TβR/Smad pathway or its target modules could be involved in other forms of IgAD.

3.3
Tracking Integrated Homeostatic Pathways and Mediators in Primary Cells

As the characterisation of genomes, genes and their function is advancing at an increasing pace, the need becomes apparent for complementary experimental approaches that can determine the physiological connectivity underlying effective biological networks. To begin to understand how large numbers of gene products are functionally integrated to produce complex biological processes, comprehensive observations of ideally all the molecular changes driving a specific cellular response are necessary. To this end, microarray-based techniques provide the means to measure the expression levels of tens of thousands of genes simultaneously, covering virtually complete genomes. Careful design of the experimental system can ensure that the resulting data flood reflects a primary biological process and carries a decipherable message. TβRII-B mice,

which lack the TGF-β receptor (TβR) in B cells, as described above, fulfilled the criteria of a suitable model to determine the gene expression dynamics underlying a typical homeostatic response in vivo. First, TβRs are evolutionarily conserved and mediate their function primarily by modulating genome expression, that is transcription. Second, unlike T cells with a defect in TβR signaling (Gorelik and Flavell 2000), the bulk of splenic TβR-deficient B cells remained in a resting state. Therefore, a meaningful comparative study of normal and mutant B cell responses to various stimuli was possible. For this purpose, mutant B cells provided a critical baseline control to identify true TβR-dependent transcriptome changes in normal cells. Third, the extensive application of genetic methods has led to a detailed picture of the major signaling pathways controlling B cell responses (Kurosaki 1999; Jumaa et al. 2005). Effects on signal transduction and cellular responses inferred from the transcriptome changes could thus be readily confirmed in vitro (Roes et al. 2003). Benefiting from the in vivo system, the molecular response can be mapped onto the TβRII-B phenotype, which has been characterised in considerable detail (Cazac and Roes 2000; Borsutzky et al. 2004). The layout of the experimental approach is illustrated in Fig. 1.

TGF-β modulated about 100/6,500 genes with at least a twofold difference in expression level in primary mouse B cells (Roes et al. 2003). The presence of known TGF-β response genes confirmed the specificity of the response. Moreover, increased IgG and suppressed IgA transcripts reflected the mouse phenotype – hyper-γ-globulinaemia with IgA deficiency – thus confirming the link with in vivo biology. The identity of the modulated genes and their expression changes indicated a surprisingly coherent and comprehensive redirection of B cell responsiveness involving major signaling pathways with profound effects on activation, migration and cell survival. Induction of the lipid phosphatase Ship, or of Socs1/Socs3 pleiotropic suppressors of Jak/Stat and TLR signaling, indicated profound suppression of activation and proliferation, which was reinforced by induction of cell cycle inhibitors and anti-proliferative transcription factors. In contrast, migratory responses were enhanced as evident, for example, from induction of chemokine receptors such as Cxcr4 and Ccr6. Significantly, the apparent shift in responsiveness could be verified at multiple levels including mRNA, signal transduction and cellular responses in vitro (Roes et al. 2003). A qualitative representation of the data is shown in Fig. 2, highlighting some of the key mediators and pathways involved. The striking dichotomy of coordinated suppressing and activating events driving the shift in B cell responsiveness towards chemotaxis is apparent.

The TβR-induced shift described above appears to have a particular impact in the peritoneal cavity and gut-associated lymphoid tissues. It is here where the phenotype is most pronounced (Cazac and Roes 2000; Borsutzky et al. 2004). The number of B1 cells known for their intrinsic association with auto-reactivity (Hayakawa et al. 1999) was fivefold increased in the absence of TβR. Peyer's patches were hyperplastic, with a threefold increase in total cell

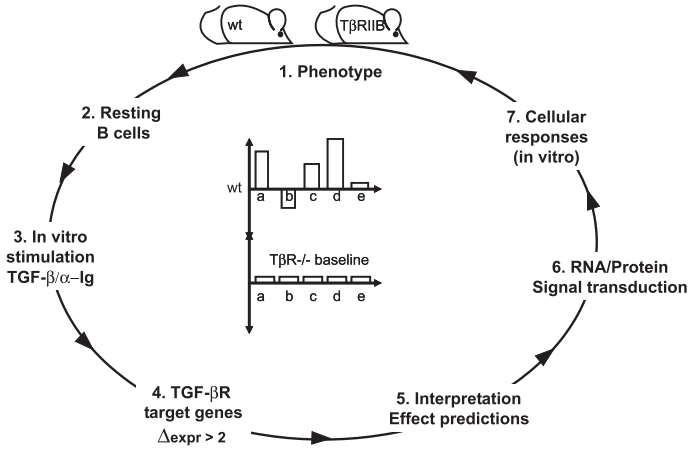

Fig. 1 A system-guided approach for detection of integrated homeostatic networks and mediators in primary B cells. *1* An evolutionarily conserved homeostatic receptor (TGF-βR, TβR) is inactivated by conditional mutagenesis in mouse B cells. The phenotype reveals breakdown of homeostasis, B cell hyperresponsiveness and impaired mucosal IgA production. *2, 3* Mutants (TβRII-B) provide TβR-negative resting B cells as a baseline control for identification of TβR-specific transcriptome changes by comparative analysis within normal cells after in vitro stimulation. *4* Comparative analysis of gene expression profiles reveals TβR-specific transcriptome changes and identifies molecular processes and mediators engaged in a physiological homeostatic response. *5* Predictions of the effects at the level of signal transduction and cellular responses can be made based on the modulation of genes with known function. *6* Validation of selected transcriptome changes at multiple levels in vitro including mRNA, protein expression, signal transduction and cellular responses. *7* Explanation of phenotype by genome expression

numbers and the deficiency in IgA expressing B cells was over-compensated by a 15-fold increase in IgG1-expressing cells. Through integrative analysis of the gene expression profiles with the in vitro and in vivo data, the following scenario emerges (Fig. 3). TβR delivers an inhibitory signal raising the B cell activation threshold. This controls the development of B1 cells, which are known to depend on strong BCR signals for selection (Casola et al. 2004). In the Peyer's patch, the TβR signal also raises the B cell activation threshold through inhibition of BCR, Jak/Stat pathways and proliferation, while sensitising the cells for chemotactic signals and redirecting isotype switch from IgG1 to IgA. How does the enhancement of chemotactic activity facilitate antibody production!mucosal antibody production? TβR induces Ccr6, receptor for Ccl20, a chemokine highly expressed by follicle-associated epithelium of the Peyer's patches (Iwasaki and Kelsall 2000). The underlying sub-epithelial dome defines a site where intestinal antigen is delivered by intraepithelial M cells and where Ccr6+ leukocyte subsets congregate, probably to form cooperative clusters required for effective cellular responses. B cell migration towards the antigenic site is thus encouraged by induction of Ccr6. When the TβR-

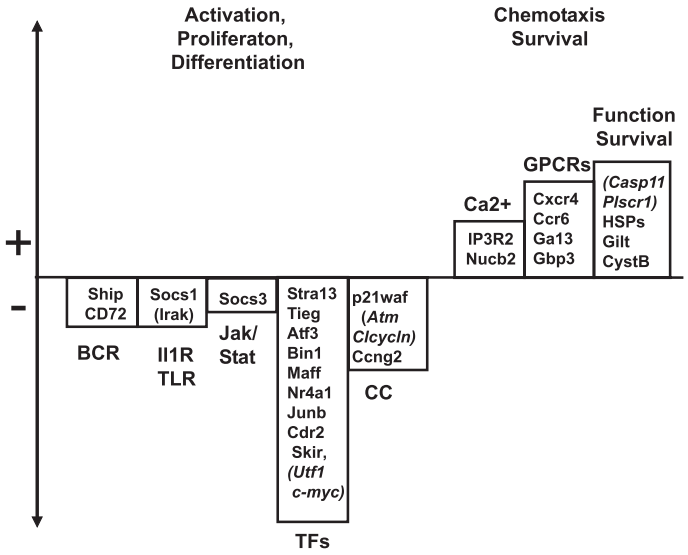

Fig. 2 Qualitative summary of TβR-modulated genes and pathways with known function. The effects on B cells were validated in vitro and explain the phenotypic changes seen in vivo. Induction of an inhibitor and suppression of agonist qualify as inhibitory events, induction of agonist and suppression of inhibitor qualify as activating event. Regular font indicates induced genes, italics in parentheses indicate suppressed genes. *BCR* B cell antigen receptor, *TLR* toll-like receptor, *TFs* transcription factors, *CC* cell cycle regulators, *GPCRs* G-protein coupled receptors

controlled activation threshold is finally overcome by appropriate stimulation and help from accessory cells, the activation programme is induced. The default pathway, leading to IgG1 production as seen in TβRII-B mice, is redirected to IgA by TβR-induced isotype switch. This overall mode of control ensures that the response is of significant affinity, on the one hand, while producing the only isotype, namely IgA, capable of crossing epithelia to neutralise antigens in the intestinal lumen at source. Data from Ccr6-deficient mice, which show defective mucosal lymphocyte homeostasis and impaired IgA production (Cook et al. 2000), retrospectively support this scenario.

As a potential therapeutic target, the multifunctional TGF-β/Smad pathway has attracted considerable attention due to its implications in a wide range of common diseases. In light of the multitude of effects, however, a risk of unforeseen side effects is associated with therapeutic targeting the TGF-β/Smad pathway. While tumour suppression may be achieved by triggering the antiproliferative effects, the concomitant enhancement of chemotaxis may support tumour metastasis (Muller et al. 2001). To begin to exploit the potential of this powerful bioregulatory mechanism for the treatment of disease, detailed knowledge of the molecular genetic network underlying the TGF-β response in distinct cell types is required. With this information, physiological modules

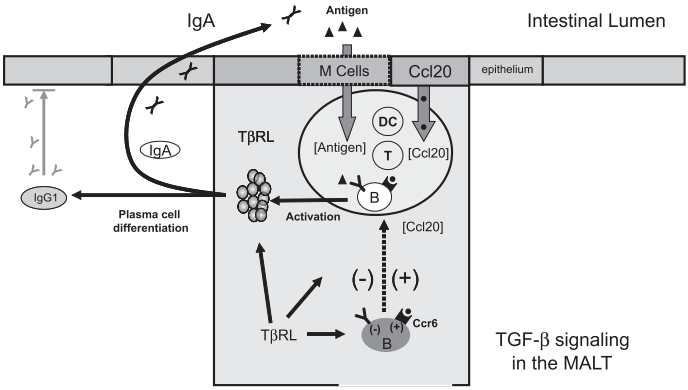

Fig. 3 A model of TGFβ-receptor-mediated control of B cell responses in the Peyer's patch as deduced from the integrated analysis of phenotype, in vitro responses and microarray data. TGFβR signals mediate inhibition of activation while enhancing chemotaxis towards Ccl20 by induction of Ccr6. This prevents premature B cell activation and encourages B cells to seek out the site of antigen concentration sufficiently high to break the activation threshold. Accessory cells such as T helper cells are driven by the same mechanism, congregating with B cells to cooperate in antibody production. When the elevated activation threshold is overcome, TGFβR directs isotype switch to secretable IgA at the expense of the default pathway to nonsecretable IgG1

and mediators that control specific biological processes such as activation, apoptosis, cell cycle arrest or cell migration can be identified for selective targeting by increasing rate-limiting agonists or through suppression using synthetic inhibitors or RNAi (see the chapter by R. Kühn et al., this volume).

4 Concluding Remarks

Conditional mutagenesis in mice is now firmly established as a core technique in the biomedical sciences. As illustrated here for the immune system, the role of widely expressed genes, many of which control the most fundamental of functions, can now be determined by robust genetics in the context of virtually any somatic cell. Conditional mutants can be tailor made to study a basic biological process or to mirror a human disease. Novel disease models may also emerge unexpectedly. Fundamental immunoregulatory mechanisms that drive leukocyte activation, suppress inflammation or modulate complex signaling networks have been revealed. By setting the activation threshold, a tyrosine kinase such as Csk shows a high degree of versatility in guarding distinct processes such as phagocyte recruitment and positive selection of T cells. In contrast to Csk, which seems to operate cell autonomously, TGF-β receptor responds to micro-environmental cues inducing, through modula-

tion of genome expression, the functional adaptation of highly specialised cells to the requirements of specific anatomical sites. This not only ensures effective lymphocyte responses, but also minimises the risk of autoimmunity and tumorigenic transformation by suppressing exaggerated proliferative activity. The role and versatility of transcription factors such as NF-κB and AP-1 in leukocyte recruitment and functional coordination has become apparent, while mouse models for acute and chronic inflammation, systemic autoimmunity, pulmonary fibrosis, lymphomas and psoriasis emerged. Data indicating a role of somatic mutations in a wider range of human diseases are just emerging, but considering the growing application of whole genome diagnosis, further supporting evidence is likely to accumulate at an increasing pace. Conditional mutagenesis is set to provide essential animal models that establish functional links, causality and molecular mechanisms, thereby supporting the rational design of specific protocols to correct disease with minimal side effects.

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Conditional Transgenesis and Recombination to Study the Molecular Mechanisms of Brain Plasticity and Memory

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Abstract In the postgenomic era, a primary focus of mouse genetics is to elucidate the role of individual genes *in vivo*. However, in the nervous system, studying the contribution of specific genes to brain functions is difficult because the brain is a highly complex organ with multiple neuroanatomical structures, orchestrating virtually every function in the body. Further, higher-order brain functions such as learning and memory simultaneously recruit several signaling cascades in different subcellular compartments and have highly fine-tuned spatial and temporal components. Conditional transgenic and gene targeting methodologies, however, now offer valuable tools with improved spatial and temporal resolution for appropriate studies of these functions. This chapter provides an overview of these tools and describes how they have helped gain better understanding of the role of candidate genes such as the NMDA receptor, the protein kinase CaMKIIα, the protein phosphatases calcineurin and PP1, or the transcription factor CREB, in the processes of learning and memory. This review illustrates the broad and innovative applicability of these methodologies to the study of brain plasticity and cognitive functions.

Keywords Conditional transgenesis · Gene targeting · Learning · Memory · Synaptic plasticity

1
Why Employ Conditional Transgenesis or Recombination in Memory Studies?

Cognitive functions are highly complex processes whose molecular mechanisms involve multiple genes with tightly regulated but yet highly dynamic expression profiles. Because most of these genes generally act in a cell- and time-specific fashion, their study requires controllable and flexible genetic tools. Transgenic and gene targeting approaches have been developed to provide such versatility and have been instrumental for studies of gene functions in the nervous system. In their most sophisticated versions, they allow spatial and temporal control over gene manipulations and provide a means to up- or down-regulate specific molecules in selected areas of the brain at will. These features have allowed exquisite analyses of the involvement of candidate genes in specific brain areas, and in distinct types and temporal phases of memory formation and storage.

1.1
Spatial Control over a Genetic Manipulation

The brain is an extremely complex organ with multiple distinct neuroanatomical and functional regions. To investigate the functions of specific genes in the brain using genetic approaches, it is essential that any manipulation of the gene be spatially highly precise. For this, specific promoter sequences have been cloned and used for transgenesis. Most of these promoters were picked for their brain specificity and for their broad, i.e. nestin (Cheng et al. 2004), prion protein (PrP) (Fischer et al. 1996) or neuron-specific enolase (NSE) (Forss-Petter et al. 1990) promoter, or restricted, i.e., Purkinje cell-specific L7 (Oberdick et al. 1990), forebrain neuron-specific Ca^{2+}/calmodulin-dependent protein kinase α (CaMKIIα) (Mayford et al. 1996b), oligodendrocyte-specific proteolipid protein (PLP) (Fuss et al. 2001) or astrocyte-specific GFAP promoter (Brenner et al. 1994) pattern of activity. Although generally stable and reliable, the selectivity of some promoters can vary and be influenced by the site of transgene integration. For instance, a more restricted pattern of gene expression has been observed in transgenic mouse lines carrying the CaMKIIα promoter, a promoter that is normally active in all forebrain neurons but is sometimes restricted to hippocampal CA1 (Tsien et al. 1996a) or striatal (Kellendonk et al. 2006) neurons (see Sect. 2). However, to date, the choice of promoter sequences truly selective for brain sub-regions or nuclei is still slim, and there is no promoter specific for cortical subdivisions such as the frontal cortex, or for hypothalamus or amygdala nuclei. However, promoters can be combined with expression systems to manipulate genes in these regions. For instance, a broadly expressed and drug-dependent transgene can be activated by local stereotactic injection of the drug (see Sect. 4). Such method is however

more invasive and depends on the availability of thus far rare ligand-dependent molecules with appropriate pharmacokinetic properties.

1.2 Temporal Control over a Genetic Manipulation

Higher-order brain functions such as learning and memory have multiple temporal phases that may recruit different signaling pathways and distinct cellular and molecular processes (i.e., spine growth/retraction, structural rearrangements, receptor trafficking, etc.). To distinguish the temporal phase(s) in which a given component of these pathways may act, it is essential to restrict any manipulation of its coding gene to the temporal phase of interest. For this, conditional expression or recombination systems have been developed to allow the induction or inactivation of gene expression at will, often with the possibility for reversal. These systems circumvent many drawbacks of constitutive transgenesis or knockout such as early lethality or developmental defects often induced when genes are manipulated early in life. For conditional transgenesis, systems based on the tetracycline-responsive transactivator (tTA) or its reversed versions (rtTA and rtTA2), whose transactivation activity can be controlled by doxycycline (administered in food or drinking water), have been developed and adapted to the brain. Their inducibility and reversibility were useful to dissect out some of the molecular mechanisms of specific phases of memory such as memory retrieval or consolidation (see Sect. 3). Likewise, conditional gene recombination was developed based on the Cre recombinase, an enzyme that recombines and excises a DNA fragment flanked by two loxP sites (floxed). Cre-dependent recombination was further made inducible by combination with inducible expression systems, i.e., tTA-based, or by fusion of Cre with a tamoxifen-dependent mutated human ligand-binding domain of the estrogen receptor (CreER). Spatial restriction of recombination can be achieved by placing Cre expression under the control of a tissue- or cell-specific promoter or for CreER, by local injection of tamoxifen.

This chapter aims at illustrating how conditional transgenesis and recombination have improved the understanding of the molecular mechanisms of learning and memory. Studies of major proteins suggested to be implicated in learning and memory will be presented (Fig. 1). Mutant models created to investigate the functions of the N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor essential for the initiation of intracellular responses to neuronal activation, will be described. Furthermore, as part of the cascades relaying NMDAR-mediated signaling, the calcium/calmodulin-dependent kinase II (CaMKII), the protein phosphatases calcineurin and PP1, and the cAMP-responsive element binding (CREB) protein involved in synaptic and nuclear events will also be covered. The chapter will end with future perspectives on conditional transgenesis and recombination, and on how further technical improvements may aid forthcoming studies.

2 NMDA Receptor-Dependent Processes

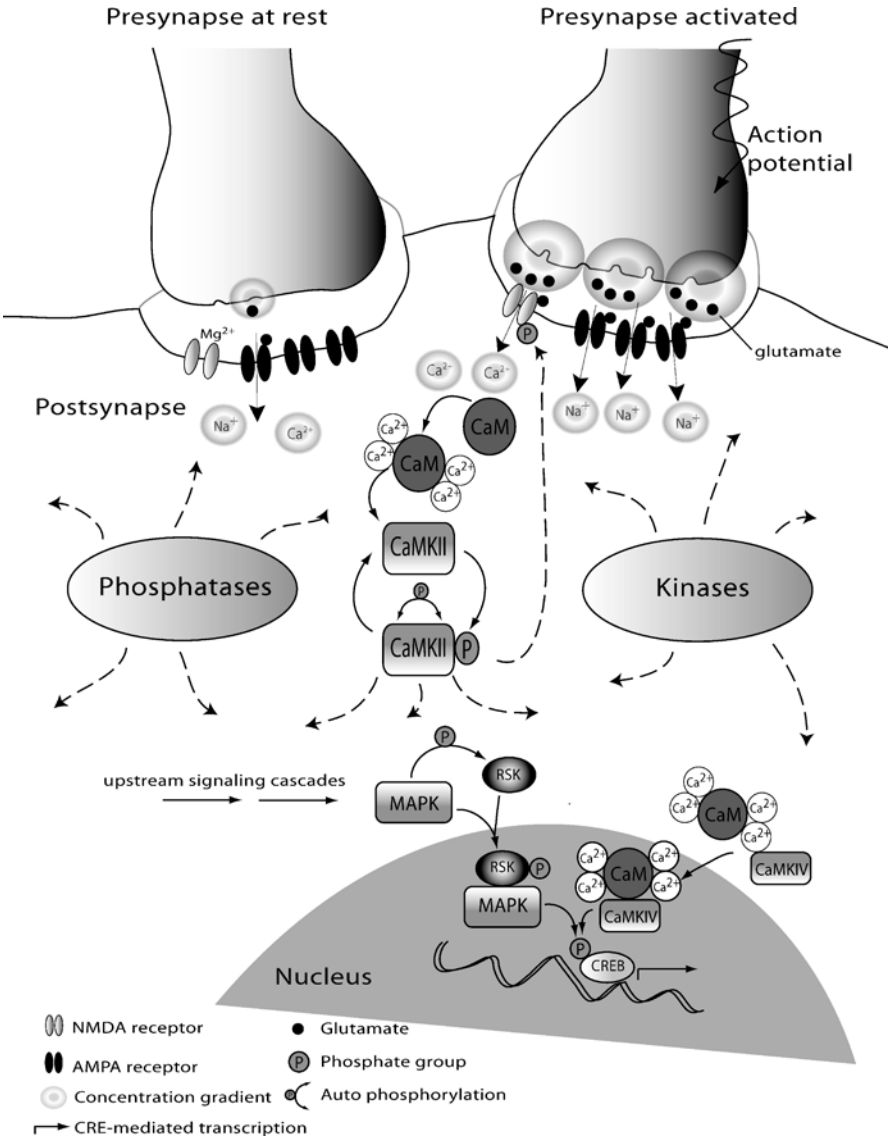
The NMDAR is a glutamate-activated membrane receptor that functions as an ion channel highly permeable to Ca^{2+} and is present essentially on postsynaptic neurons. The NMDAR assembles as a hetero-tetramer of two obligatory NR1 subunits that are ubiquitously expressed and are essential for channel function, and two NR2 subunits, NR2A, B, C or D. NR2 subunits have different profiles of expression and different properties that modulate the characteristics of the NMDAR, for instance its sensitivity to magnesium block, channel conductance or glutamate affinity. The NMDAR is critical for developmental processes in the brain such as neuronal survival (Balazs et al. 1989), differentiation (Blanton et al. 1990), migration (Marret et al. 1996), and for the formation, stabilization, and modulation of synapses and neuronal circuits (Constantine-Paton 1990).

The NMDAR was first implicated in learning and memory when receptor antagonists were found to block the induction of long-term potentiation (LTP) in hippocampal synapses (Collingridge et al. 1983). LTP is a well-characterized form of synaptic plasticity reflecting an increase in synaptic efficacy that is observed in many excitatory synapses in the mammalian brain, in particular CA3-CA1 hippocampal synapses (Bliss and Collingridge 1993). During LTP in CA1 neurons, the NMDAR acts as a coincidence detector that senses simultaneous pre- and post-synaptic activity and ensures efficient and reliable neuronal activity (Fig. 1). LTP in CA3 neurons, however, does not require the NMDAR but rather other types of glutamate receptors such as kainate or metabotropic receptors. The coincidence detector property of the NMDAR was first postulated to be a mechanism for the strengthening of synaptic connections by Donald Hebb (Hebb 1949), and is now widely accepted as one of the prerequisites for the formation and the storage of major forms of memory. However, the precise role of the NMDAR in synaptic plasticity and memory was poorly understood until transgenic and recombination

Fig. 1 Major molecular components of signaling cascades during synaptic transmission. This figure schematically outlines a synapse at rest (*left*) or after activation (*right*) in the brain. When an action potential (AP) reaches a glutamatergic presynaptic terminal, glutamate is released and diffuses across the synaptic cleft to bind to specific receptors on the postsynaptic membrane. This results in an influx of sodium (Na^+) leading to membrane depolarization, which is required for the subsequent influx of calcium (Ca^{2+}) ions. The level of intracellular Ca^{2+} determines which intracellular cascade predominates: a protein kinase cascade activated by high levels of Ca^{2+} that enhances synaptic strength and promotes signal transmission, or a protein phosphatase cascade responsive to low Ca^{2+} that weakens synaptic strength and down-regulates signal transmission. These intracytoplasmic cascades may then transmit the signal to the nucleus where gene expression may be activated for long-term processes

technologies were exploited to manipulate the different NMDAR subunits *in vivo*.

Mutant mice carrying null alleles of NR1 (Forrest et al. 1994), NR2A (Sakimura et al. 1995), NR2B (Kutsuwada et al. 1996) or NR2C (Ebralidze et al. 1996) were first created by classical knockout in the mid-1990s, followed by mice carrying inactivating point mutations or interrupting loxP sites. NR1 null mutants are not viable and die shortly after birth. Likewise, mice with



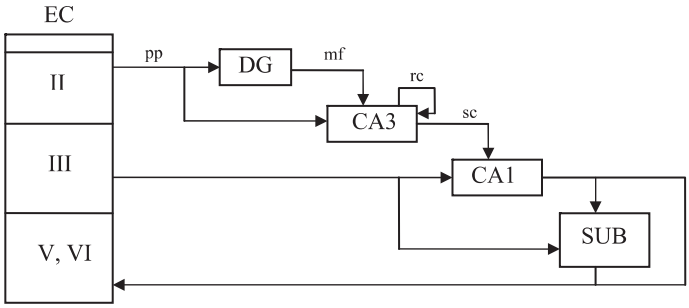


Fig. 2 Main connections of the hippocampal/cortical circuit. *EC,* entorhinal cortex; *DG,* dentate gyrus; *SUB,* subiculum; *pp,* perforant pathway; *mf,* mossy fibers; *rc,* recurrent collateral axons of CA3 pyramidal neurons; *sc,* Schaffer collateral axons

an inactive NMDAR due to a point mutation at asparagine N598, an amino acid required for correct voltage-dependent Mg^{2+} block and Ca^{2+} permeability, die prematurely (Single et al. 2000). Other mutant lines are viable (although NR2B knockout pups need manual feeding to survive) but have impaired NMDAR functions and NMDAR-dependent plasticity. To circumvent the lethality of NR1 inactivation, a conditional manipulation was designed using the late onset forebrain-specific CaMKIIα promoter and the Cre recombinase in transgenic mice. Several mouse lines expressing Cre under the control of the CaMKIIα promoter (CaMKIIα promoter-Cre) were generated and the pattern of Cre-dependent recombination was tested in reporter mice. As expected, gene recombination was induced postnatally but surprisingly in one line of mice, it was restricted to the hippocampus CA1 area (Tsien et al. 1996a). In rodents and human, the CA1 area is part of a tri-synaptic loop that together with the dentate gyrus, CA3 area, and subiculum, constitutes the hippocampal formation. The hippocampal formation receives input from the entorhinal cortex, then the signal is successively processed through dentate gyrus, CA3 and CA1 areas and sent back to the cortex (Amaral and Witter 1989) (Fig. 2). In this circuit, CA1 neurons are particularly important because they express NMDAR-dependent forms of synaptic plasticity including LTP and LTD, and are essential for memory formation (Zola-Morgan et al. 1986). The unusual CA1 restriction of Cre-dependent gene recombination obtained with that CaMKIIα promoter-Cre transgenic line was fortuitous but extremely useful. It allowed the elimination of NR1 not only late in development but also selectively in CA1 neurons, providing convenient temporal and spatial restriction.

Double mutant mice carrying a floxed NR1 gene (exons 3–22 flanked by loxP sites) and expressing the CA1-specific recombining Cre were obtained. Unlike plain knockout animals, these conditional knockout mice were viable, grew and developed normally. However, when adult, they exhibited severe impairments in NMDAR- and hippocampal-dependent functions. They

showed reduced NMDAR-mediated synaptic currents and deficient NMDAR-dependent LTP in area CA1 but normal plasticity in other hippocampal regions such as dentate gyrus. This selective impairment in plasticity was accompanied by a severe deficit in spatial learning, shown by an inability to acquire and remember the location of a hidden platform in a water maze (Tsien et al. 1996b). Nonspatial learning was also affected and performance was severely impaired in both trace- and contextual-fear conditioning, two hippocampal-dependent tasks based on learning of an association between a sound or a context and a foot shock (Huerta et al. 2000; Rampon et al. 2000). In other hippocampal-dependent tests such as object recognition, olfactory discrimination or olfaction-based transverse patterning tasks, performance was also impaired (Rampon et al. 2000; Rondi-Reig et al. 2001). In contrast, nonhippocampal-dependent learning such as cued fear conditioning (the association between a tone and foot shock) was not altered, highlighting overall the essential role of NR1 in hippocampal LTP and multiple forms of learning.

These initial results, however, did not determine whether NR1 is needed for processes following learning needed for the establishment, the consolidation and the storage of memory traces since these processes cannot take place when learning is blocked. To answer this question, it was necessary to inactivate NR1 only after training (allowing normal learning) and examine performance thereafter. This was achieved with a combined conditional approach, with which NR1 deficiency in the CA1-specific knockout animals was rescued by inducible expression of an NR1 transgene in CA1 neurons. The inducible NR1 transgene was assembled with a triple construct composed of a CaMKIIα promoter-Cre transgene, a Cre-dependent tTA gene interrupted by a floxed stop sequence and placed under the control of a β-actin promoter, and a tTA-dependent NR1 transgene fused to a tetO promoter (Fig. 3) (Shimizu et al. 2000). When combined with the endogenous floxed NR1 gene, this system allowed the inducible and reversible rescue of NR1 through tTA-dependent expression of transgenic NR1 in CA1 neurons during learning. In the resulting animals, dox treatment induced NR1 deficiency in CA1 neurons by suppression of transgene expression resulting in a similar defect as in the conditional knockout animals. Transgene expression induced by dox withdrawal fully restored LTP in area CA1, indicating that the NR1 transgene compensated for the absence of endogenous NR1. Suppression of NR1 rescue during learning, however, prevented the animals from acquiring information on the fear conditioning task or the water maze and induced a similar performance deficit as in the knockout animals (Tsien et al. 1996b). Strikingly, when the rescue was suppressed only after learning (for 1 or 2 weeks), performance was similarly impaired, suggesting that NR1 is required not only during but also after learning. This defect may have resulted from a failure in the consolidation of the acquired information and the formation of memory, or in the retrieval of a consolidated memory. To distinguish between these possibilities, NR1 was rescued both during and after learning to allow proper acquisition and

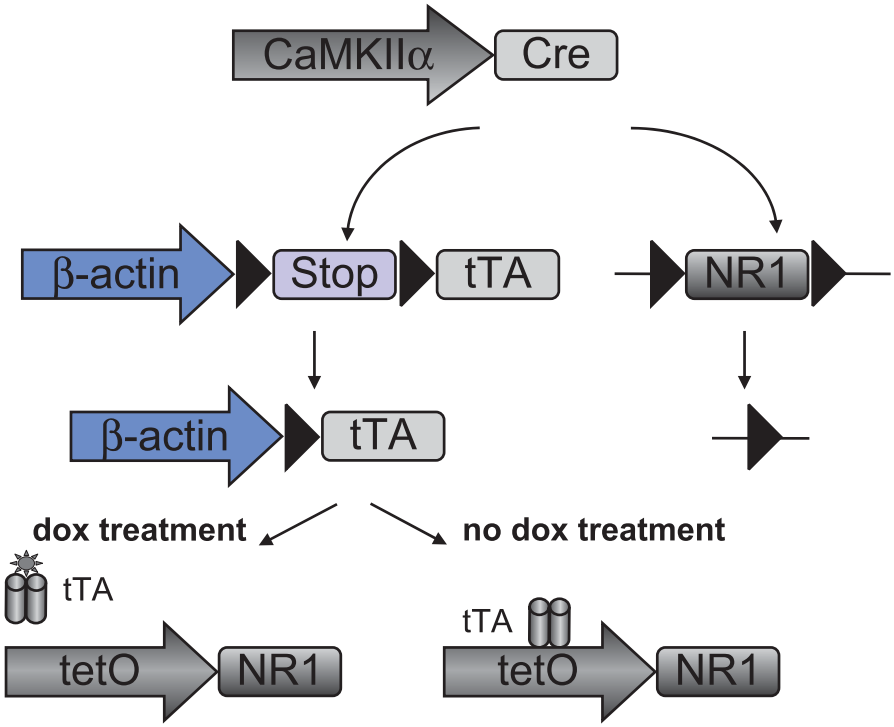


Fig. 3 Strategy to obtain an inducible, reversible, and CA1-specific NR1 knockout in the mouse. Cre expression under the control of the CaMKIIα promoter leads to simultaneous expression of tTA, after excision of a stop cassette 5′ to the tTA transgene by Cre, and inactivation of the endogenous NR1 gene through loxP-directed recombination in CA1 neurons. Regulation of the system is possible through the administration/withdrawal of dox. tTA binds to the tet operon (tetO) in the absence of dox and induces the expression of the NR1 transgene. Dox administration prevents the binding of tTA to tetO and switches NR1 transgene expression off (derived from Shimizu et al. 2000)

memory consolidation, then NR1 deficiency was re-instated by dox-induced NR1 transgene expression selectively during retrieval, i.e. shortly before the memory test that took place 4 weeks after training. This transient deficiency in NR1 at the time of retrieval did not impair retrieval and the animals correctly remembered the learned information, indicating that NR1 is not needed for retrieval *per se* but is required for the consolidation of memory, whether spatial, associative or even gustatory (Shimizu et al. 2000; Cui et al. 2004, 2005).

Since NR1 appeared to be required for the acquisition and the consolidation of memory traces, it was important to determine whether it is also needed after the initial consolidation to maintain memory traces for long periods of time. The conditional NR1 knockout model was ideal to test this possibility (Cui et al. 2004). After NR1 inactivation (for 1 month) by dox treatment 6 months after

learning (when consolidation is generally complete), the content of contextual and cued fear memory, both long-lasting forms of memory, was examined (Shimizu et al. 2000). This prolonged NR1 deficiency impaired performance in both contextual and cued-fear conditioning tests. However, it had no effect when induced for only 7 days, indicating that only prolonged but not transient NR1 absence interferes with the storage of memory. The continuous presence of NR1 is thus indispensable for the stability of stored remote fear memories. Overall, these results provided firm evidence that NR1 is required not only for the acquisition of information but also for the consolidation and the storage of memory traces.

The formation of hippocampus-dependent memory involves the association of complex configurations of stimuli into a memory trace that can be later recalled or recognized. The studies mentioned above examined the role of the NMDAR in different memory phases on the basis of full-cue conditions, meaning that retrieval occurred in the presence of all cues available during learning. In real life, however, memory recall often relies on incomplete or degraded sets of cues and requires that entire memory patterns be reconstructed from these sets. Recall based on associations must then be engaged to optimize retrieval. The CA3 region of the hippocampus has been proposed to be an anatomical basis for building such associations because it has an extensive recurrent connection network. This network has associative features due to its massive recurrent CA3 collaterals (auto-connections of pyramidal neurons) that provide major feedback to CA3, and the associated excitatory input coming from the dentate gyrus through mossy fibers, and from the entorhinal cortex through the perforant pathway (Fig. 2). The involvement of NR1 in pattern completion in CA3 neurons was tested by conditional recombination using the floxed NR1 animals and CA3-specific Cre transgenic mice. In these latter mice, Cre is expressed under the control of a kainate receptor 1 (KA1) promoter active essentially in CA3 neurons (Nakazawa et al. 2002). In mutant mice carrying this Cre transgene and the floxed NR1 gene, NR1 could be eliminated selectively in CA3 neurons about 5 weeks after birth. The loss of NR1 severely impaired LTP at CA3 synapses but not in other hippocampal synapses. In the water maze, it did not perturb spatial learning or the recall of spatial information when memory was tested under conditions of full extramaze cues (all cues used during training were available during memory test). In contrast, when most of the cues were removed, recall was severely impaired and the animals were no longer able to locate the hidden platform. The animals were also impaired in one-trial learning on a delayed matching-to-place version of the water maze, another form of memory thought to implicate the recurrent CA3 network (Nakazawa et al. 2003). The selective deficiency of NR1 in CA3 neurons thus demonstrated the NMDAR in these neurons is required for proper associative memory recall, as well as for rapid hippocampal encoding of novel information and fast learning of one-time experience.

3 Intracellular Signaling Cascades

The major functions of the NMDAR in neuronal transmission, synaptic plasticity, and memory are largely mediated by downstream intracellular cascades activated by Ca^{2+} ions flowing through the receptor. In postsynaptic neurons, Ca^{2+} stimulates numerous Ca^{2+}-sensitive enzymes that relay the signal conveyed by the NMDAR. Among them, several Ca^{2+}-dependent protein kinases and phosphatases are activated depending on their affinity for Ca^{2+} and the level of ambient Ca^{2+}. These enzymes dynamically regulate common or distinct targets in the postsynaptic neuron, and thereby modulate the efficacy of signal transmission (Fig. 1). CaMKII is a moderately Ca^{2+}-sensitive Ser/Thr protein kinase recruited by synaptic stimulation to the postsynaptic density (PSD), an electron-dense structure directly apposed to presynaptic terminals in excitatory glutamatergic synapses. After initial activation by Ca^{2+}, CaMKII has the ability to autophosphorylate at Thr286 to become Ca^{2+}-independent and remain active for long periods of time. Its persisting activity is required for the maintenance of high synaptic efficacy and for synaptic plasticity, in particular for LTP (Lisman and Goldring 1988; Lisman 1994; Pettit et al. 1994; Lisman and McIntyre 2001; Lisman and Zhabotinsky 2001). CaMKII is in part deactivated by dephosphorylation by PP1, whose activity and local distribution are themselves controlled by several regulators such as specific inhibitors and scaffolding proteins (Cohen 2002; Gibbons et al. 2005). One potent PP1 inhibitor is inhibitor-1 (I-1), a peptide activated by phosphorylation by the cAMP-dependent protein kinase A (PKA) and blocked by dephosphorylation by the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (PP2B), a highly Ca^{2+}-sensitive phosphatase. Altogether, CaMKII, PP1, I-1, PKA, and calcineurin form a balance of kinases and phosphatases differentially activated by Ca^{2+}. In this balance, kinases and phosphatases compete and antagonize each other to control intra-cytoplasmic and nuclear signal transduction pathways during neuronal activity. Predominant kinase activity is thought to favor signaling and enhance synaptic activity while predominant phosphatase activity weakens signaling and synaptic efficacy.

Several components of the kinase/phosphatase balance have been investigated *in vitro* and *in vivo* using conditional approaches and were shown to be required for synaptic plasticity, learning, and memory. Initial evidence for a contribution of CaMKII to plasticity and memory was provided when the gene coding for CaMKIIα, a predominant isoform in hippocampus and cortex, was permanently inactivated in the mouse by conventional knockout. The resulting CaMKIIα deficiency diminished NMDAR-dependent LTP in hippocampus area CA1 (Silva et al. 1992b, 1992c). However, this defect was not observed in heterozygous mice expressing half the amount of CaMKIIα, indicating that partial CaMKII activity is sufficient for proper LTP. Moreover, the effect of CaMKIIα deficiency was found to depend on the genetic make-up

of the animal as LTP was only slightly altered in this line of knockout mice with a different genetic background (Hinds et al. 1998). This may be due to compensatory mechanisms such as the recruitment of CaMKIIβ (Elgersma et al. 2002) activated to counteract the lack of CaMKII activity. Such mechanisms, however, may not operate or be less effective in visual or temporal cortex, two brain areas important for long-term memory (LTM), in which LTP was abolished whether CaMKIIα was fully (in homozygous mice) or partially (heterozygous mice) eliminated (Kirkwood et al. 1997; Frankland et al. 2001). Thus overall, CaMKIIα activity is required for cortical plasticity but is dispensable for plasticity in the hippocampus.

Importantly, a lack of Ca^{2+}-dependent kinase activity rather than a CaMKIIα protein deficiency in itself appeared to be the primary cause for the impairment in plasticity. When Ca^{2+}-dependent CaMKII activity was inhibited by blockade of Thr286 autophosphorylation through a Thr to Ala point mutation (CaMKII-T286A introduced by gene recombination), NMDAR-dependent LTP was prevented whether stimulated at high or intermediate frequency (100 or 10 Hz), or by theta bursts (two 100 Hz bursts of four stimuli, 200 ms intervals) (Giese et al. 1998). Likewise in the barrel cortex, *in vitro* LTP was absent whether induced by theta-burst stimulation, spike pairing, or postsynaptic depolarization paired with low-frequency presynaptic stimulation, and sensory-evoked potentials were impaired in vivo (Hardingham et al. 2003). The effect on LTP was dose-dependent and was only observed in the total absence of wild-type CaMKII-Thr286; mice heterozygous for the CaMKII-T286A allele had normal hippocampal LTP (Frankland et al. 2001). Interestingly however, in these mice the LTP defect could be induced in the adult hippocampus when the heterozygous mutation was combined with a drug-dependent approach. When activation of the remaining endogenous CaMKII was prevented by partial blockade of NMDAR-dependent Ca^{2+} influx (see Fig. 1) with a sub-threshold dose of the NMDAR antagonist CPP, LTP was impaired similarly to that in homozygous mice (Ohno et al. 2001, 2002). This effect was observed only when CPP was applied prior to LTP induction but not after. Further, the administered dose of CPP did not alter LTP in control slices whether applied before or after its induction. These results clearly indicated that the LTP impairment is a direct effect of Ca^{2+}-dependent CaMKIIα deficiency and not of a developmental anomaly, confirming the requirement of Ca^{2+}-dependent CaMKII activity for the induction of LTP in the hippocampus.

The importance of Thr286-autophosphorylation for CaMKII function in the brain was further confirmed by expression of a constitutively active and Ca^{2+}-independent CaMKII mutant in the mouse brain. CaMKII-Asp286 (carrying an Asp residue instead of Thr286) was inducibly expressed in the brain of transgenic mice using the dox-dependent tTA system and the CaMKIIα promoter (Mayford et al. 1996a). In these mice, the inducibility and reversibility of the tTA system was exploited to modulate the level of CaMKII-Asp286 expression and obtain a high or low increase in Ca^{2+}-independent activity. When induced

through development via tTA-dependent transactivation, CaMKII-Asp286 expression was strong and yielded high Ca^{2+}-independent CaMKII activity in forebrain neurons. However, when suppressed by dox during development and re-activated only in adulthood by dox removal, transgene expression was lower (only about 60% of wild-type CaMKIIα levels in hippocampus and 20%–30% in striatum) yielding a low level of Ca^{2+}-independent CaMKII activity (Bejar et al. 2002). This partial silencing may have resulted from changes in chromatin structure or DNA methylation following early and prolonged transcriptional suppression that could not be fully reversed after dox removal (Bejar et al. 2002) (our own observation). Strikingly, the resulting high or low level of Ca^{2+}-independent CaMKII activity produced an opposite effect on LTP in hippocampus area CA1. LTP was enhanced when Ca^{2+}-independent CaMKII activity was moderate (Bejar et al. 2002), consistent with the hypothesis that Ca^{2+}-independent CaMKII favors neuronal signaling and enhances plasticity. However, LTP was impaired when Ca^{2+}-independent CaMKII activity was high (Mayford et al. 1996a). High activity actually provoked a general shift of synaptic plasticity toward synaptic depression and increased the threshold for long-term depression (LTD), a form of synaptic plasticity induced by low frequency stimulation reflecting a weakening of synaptic efficacy (Mayford et al. 1996a). This effect was not due to a developmental anomaly resulting from transgene expression because it could be reversed by blockade of CaMKII-Asp286 expression. This finding was unexpected and is inconsistent with the postulated strengthening function of CaMKII on plasticity. It was later explained as resulting from altered expression of dozens of secondary genes including protease inhibitors, Ca^{2+}-binding proteins, growth factors, and transcription factors due to CaMKII-Asp286 expression (Bejar et al. 2002). Such genetic compensation was observed whether transgene expression was low or high but it was much more pronounced (more genes and larger changes) with high expression. The purpose of this transcriptional compensation may be to counterbalance the increased kinase activity but when excessive, it was detrimental to plasticity.

These results were corroborated by another study in which both Ca^{2+}-dependent and Ca^{2+}-independent activity were increased inducibly but over a shorter time window, minimizing genetic compensation. This was achieved by using a mutant form of CaMKIIα designed to be selectively and reversibly inhibited by a specific inhibitor peptide (Wang et al. 2003). This mutant carries a point mutation at Phe89 (F89G) in the ATP-binding pocket that does not affect ATP binding but renders this kinase mutant sensitive to low doses of a designed inhibitor 1-naphtylmethyl-PP1 (NM-PP1, IC_{50} = 32 nM). When expressed in forebrain neurons under the control of the CaMKIIα promoter, CaMKIIα-F89G increased Ca^{2+}-dependent CaMKII activity 2.6-fold and doubled Ca^{2+}-independent activity in the hippocampus. The increase was quickly suppressed by oral administration of NM-PP1 to the animals (5 μM in drinking water) and was fully reversible. The analysis of synaptic plasticity in CaMKIIα-

F89G-expressing mice revealed the expected potentiation in LTP across a broad range of stimulation frequency (from 1 to 100 Hz), that was accompanied by a reduction in LTD (induced by 5-min 3-Hz stimulation) (Wang et al. 2003). Importantly, this effect was specific to postsynaptic CaMKII while presynaptic CaMKII, in contrast, appeared to negatively regulate neuronal efficacy. When presynaptic CA3 terminals were selectively deprived of CaMKII by Cre-dependent recombination with the KA1 promoter, basal neurotransmitter release was increased in response to neuronal activity (Hinds et al. 2003; Nakazawa et al. 2003). This indicated that CaMKIIα serves as a negative modulator of activity in CA3 hippocampal area, contrary to CA1 where it promotes synaptic activity.

Altering CaMKII activity also has a strong impact on cognitive functions. Full elimination of CaMKII by plain knockout, mild or high overexpression by transgenesis interferes with spatial and associative learning and memory. Thus, null, CaMKIIα-T286A or CaMKIIα-Asp286 mutant animals are not able to learn spatial information in the water maze (Silva et al. 1992a; Mayford et al. 1996a; Giese et al. 1998) or on the Barnes maze (Mayford et al. 1996a). Intensive training on the water maze, however, rescues spatial learning in the null mutants (Elgersma et al. 2002), possibly through molecular compensation by recruitment of CaMKIIβ. Partial reduction in CaMKII activity in heterozygous CaMKII-T286A mice impairs short-term associative memory. On the contextual fear conditioning paradigm, heterozygous animals acquire and retain information for 1 day but do not consolidate this information and lose it after 36 days (Frankland et al. 2001). This selective defect in long-term memory correlates with the strong LTP impairment in cortex, a site for remote memory, and with normal LTP in hippocampus, a site for temporary memory storage (Frankland et al. 2001, 2004). One-day fear memory was nonetheless impaired in CaMKII-T286A heterozygous mutants when CaMKII activity was fully eliminated by administration of a subthreshold dose of the NMDAR antagonist CPP (5 mg/kg) (Ohno et al. 2001). When injected before training, CPP prevented the animals from learning the association between a context and a foot shock, but had no effect when injected after learning, highlighting the selective impact of CaMKII deficiency on information acquisition. CPP also did not affect memory in control animals at a sub-threshold concentration, clearly demonstrating that its effect was conditional to partial CaMKII inhibition. Unexpectedly, CaMKII overexpression also alters associative memory. tTA-dependent expression of CaMKII-Asp286 or constitutive expression of CaMKIIα-F89G in forebrain neurons during learning impairs cued and contextual fear conditioning 1 day or even 1 month after training (Mayford et al. 1996a; Wang et al. 2003). This effect was strong and could be produced even when CaMKIIα-F89G expression was induced only after training and for only 1 week (by removal of NM-PP1). However, it had no effect when induced only 2–3 weeks after training (Wang et al. 2003). Transgene expression directly accounted for the memory defects, as restoration of normal CaMKII activity by suppression of transgene expres-

sion with dox in the CaMKII-Asp286 mutants or by NM-PP1 administration in the CaMKIIα-F89G mice fully reversed the memory impairment (Mayford et al. 1996a; Wang et al. 2003).

Altogether, these results indicated that CaMKII activity must be tightly regulated during learning for proper acquisition but also shortly after learning for memory consolidation. They also highlighted the role of autophosphorylated CaMKII as a positive regulator of synaptic plasticity, and the importance of its tight fine-tuning for cognitive functions. In neuronal cells, this tuning is largely provided by protein phosphatases, specifically calcineurin and PP1, that can counteract CaMKII and/or antagonize its activity by dephosphorylation of common targets. The function of calcineurin and PP1 has been examined by conditional transgenesis with dox-dependent expression systems and the CaMKIIα promoter. When the activity of calcineurin or PP1 was reduced (by about 50%–80%) in the mouse forebrain by tTA- or rtTA-dependent expression of selective inhibitor peptides, hippocampal LTP was enhanced both *in vitro* and *in vivo* (Malleret et al. 2001; Genoux et al. 2002). LTP was also less prone to reversal by depotentiation (Jouvenceau et al. 2003; 2006), indicating a general increase in synaptic efficacy by reduced phosphatase activity. Consistently, when calcineurin activity was increased (by 80%–100%) by regulated expression of a partially Ca^{2+}-independent active mutant in forebrain neurons, LTP was impaired in the hippocampal area CA1 (Mansuy et al. 1998b; Winder et al. 1998). These changes in LTP could be reversed in adult animals by suppression of transgene expression, confirming that they were a direct effect of the transgene. Further, reduced calcineurin or PP1 activity in adult animals facilitated spatial learning and memory in the water maze, and improved memory for objects on an object recognition test (Malleret et al. 2001; Genoux et al. 2002). Several temporal components including acquisition, short- and long-term memory were enhanced when phosphatase activity was maintained low during and after training. When normal PP1 activity was restored by transgene suppression right after acquisition, spatial memory remained normal (Genoux et al. 2002). However, when PP1 was inhibited only after acquisition, consolidation was enhanced and memory was more robust and persistent.

In contrast, an increase in calcineurin activity by dox-dependent expression of an active calcineurin mutant during and after training impaired the acquisition of spatial memory in the water maze (Mansuy et al. 1998b). Calcineurin excess was also found to impair memory retrieval. Thus, when calcineurin activity was increased only before retrieval, e.g., after information was properly learned while transgene expression was turned off, the animals were not able to remember the platform position. However, when normal calcineurin activity was subsequently restored (after the failed retrieval attempt), they could find the platform position, indicating that the information had been correctly consolidated and maintained in memory but could not be recollected in the presence of an excess of calcineurin. Altogether, these results highlight the function of calcineurin as a molecular constraint on the acquisition, the con-

solidation, and the retrieval of memory. They are consistent with the model that a tightly regulated kinase-phosphatase balance controls synaptic efficacy, and is essential for learning and memory. When this balance is slightly tilted in favor of kinases or phosphatases, synaptic efficacy and performance are strengthened or weakened, respectively. But if shifted excessively or unduly in either direction, it impairs these processes. This may explain why memory is impaired in mice expressing high levels of CaMKII-Asp286, or in knockout mice deficient for the predominant calcineurin isoform (CNAα) in CA1 hippocampal neurons (Zeng et al. 2001).

4 Nuclear Events and Transcriptional Regulation by CREB

Conditional transgenesis and recombination methods have been instrumental to investigating signaling processes in the nucleus downstream of the NMDAR and intracytoplasmic kinase/phosphatase cascades. Nuclear events such as gene transcription and subsequent cytoplasmic protein translation are essential for the establishment of long-lasting forms of synaptic plasticity and memory. Both memory consolidation and re-consolidation, processes necessary to stabilize (re-stabilize) memory after initial formation or re-activation, respectively, depend on gene expression and protein synthesis. Transcriptional regulation is mediated by transcription factors such as the cAMP-responsive element (CRE) binding protein (CREB). CREB is a ubiquitous factor in mammals activated by two major Ca^{2+}-dependent signaling cascades that control CREB phosphorylation (primarily at serine 133). Upon phosphorylation, CREB binds to CREs in specific genes and recruits a complex of transcriptional activators that promote mRNA synthesis. The initial phosphorylation of CREB is triggered by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), but this phosphorylation is transient and not sufficient to initiate gene transcription. Additional phosphorylation by the mitogen-activated protein kinase (MAPK) is required for persistent CREB activity and transcription. MAPK is activated in the cytoplasm where it subsequently activates pp90 ribosomal protein S6 kinase (Rsk) and as a complex, these proteins translocate to the nucleus where Rsk phosphorylates CREB (Fig. 1) (for review refer to West et al. 2002).

Conditional approaches were first applied to CREB in the fruit fly *Drosophila melanogaster* to investigate the CREB-dependence of long-term forms of plasticity and memory. Olfactory memory in *Drosophila* is a major form of memory that has two distinct phases: anesthesia-resistant (ARM), that is short-term (declines after about 4 days) and independent of protein synthesis, and LTM, a long-lasting and protein synthesis-dependent phase. The importance of CREB in these phases of memory was tested in transgenic fly models taking advantage of the naturally occurring CREB transcriptional repressor dCREB2-b or the activator CREB2-a, and a heat shock promoter for temporal control

(Yin et al. 1994; Yin et al. 1995). Heat shock gene regulation is an endogenous process in *Drosophila* that allows the rapid switching of specific sets of genes upon temperature-induced stress. When combined with the repressor dCREB2-b, the heat shock promoter allowed temperature-controlled repression of CREB-dependent gene expression in adult flies. The effect of repression on memory was examined in a Pavlovian odor avoidance task after dCREB2-b expression was induced by heat shock (42°C). This resulted in a selective blockade of LTM but no alteration of ARM, an effect that was directly due to dCREB2-b expression but not to a developmental anomaly. In contrast, expression of dCREB2-a strongly activated CREB-driven gene expression and enhanced LTM (Yin et al. 1995). Transgenic flies expressing dCREB2-a needed only one training session for optimal performance when wild-type flies needed ten sessions, clearly demonstrating that CREB acts as a positive regulator of memory.

These results were confirmed in the mouse by the generation and analyses of several mutant models in which the CREB gene was inactivated by plain or conditional knockout. Mice deficient for the predominant α- and δ-isoforms (CREB $^{\alpha\delta-/-}$) developed normally and had normal short-term plasticity and memory. However, they exhibited a severe impairment in late-phase LTP in hippocampus area CA1, a form of protein synthesis-dependent plasticity, and in associative and spatial long-term memory (Bourtchuladze et al. 1994). On the fear conditioning test, they were not able to remember the association between a tone or a context and a foot-shock, while on the water maze, they could not recollect the position of an escape platform previously learned. However, the memory deficit appeared to be dependent on gene dosage and genetic background (Gass et al. 1998), and in some cases, it was compensated for by related CREB factors such as CREM or ATF-1 (Hummler et al. 1994). Further, unspecific behavioral abnormalities such as thigmotaxis in the water maze (swimming along the walls of the maze) were also observed and may have confounded the interpretation of the results (Balschun et al. 2003).

Conditional approaches were therefore employed to spatially and temporally restrict manipulations of the CREB gene. One approach based on regulated transgenesis allowed the inducible and reversible inhibition of CREB activity in selected areas of the brain. A CREB dominant-negative mutant, KCREB, carrying an amino acid substitution, was used to block the ability of CREB and related CREM and ATF1 factors to bind to CRE (Walton et al. 1992). When placed under the control of tTA and the CaMKIIα promoter, KCREB expression could be targeted to CA1 neurons in dorsal hippocampus in adult mice (again fortuitously), and interfered with CREB-dependent gene expression selectively in these neurons (Pittenger et al. 2002). The dorsal hippocampus is recognized to be critical for spatial learning and memory and for object recognition, while ventral hippocampus is rather involved in contextual fear conditioning (Moser and Moser 1998; Broadbent et al. 2004). When tested for spatial learning and memory in the water maze or for associative memory on

the contextual fear-conditioning task, mice expressing KCREB in dorsal CA1 showed a severe impairment in spatial LTM but had intact STM and contextual memory (Pittenger et al. 2002). Learning or STM on the object recognition test were also normal but not LTM after 1 day. The memory impairments were fully reversed when CREB activity was restored by suppression of KCREB expression with dox, indicating that they directly resulted from a failure in CREB activity and its related family members CREM and ATF-1. Finally, performance on hippocampal-dependent memory tests was normal in another line of mice not expressing KCREB in hippocampus, but only in striatum and piriform cortex. Consistent with the involvement of CREB in late transcriptional events, CREB inactivation did not impair an early phase of LTP (E-LTP) in hippocampal area CA1 but impaired late phase LTP (L-LTP) induced by forskolin, a drug that stimulates adenylyl cyclase and triggers a PKA-dependent form of LTP, or by pairing a single tetanus with the dopamine agonist chlo-APB. The LTP impairment was directly associated with KCREB expression since it could be reversed when KCREB was turned off by dox administration. Interestingly, KCREB did not interfere with L-LTP induced by high-frequency tetanic stimulation or theta burst potentiation, indicating that CREB-mediated transcription differentially contributes to different forms of L-LTP.

Another conditional study exploited a CREB mutant carrying an amino acid substitution at Ser133 phosphorylation site ($\alpha CREB^{S133A}$) (Gonzalez and Montminy 1989) that competes with endogenous CREB (Kida et al. 2002). $\alpha CREB^{S133A}$ was made inducible by fusion with the ligand-binding domain (LBD) of the human estrogen receptor, itself mutated on G512R (LBD^{G521R}) (Danielian et al. 1993; Logie and Stewart 1995; Feil et al. 1996) to respond only to the synthetic ligand 4-hydroxy-tamoxifen and not to endogenous estrogen. Mice expressing $\alpha CREB^{S133A}$ in excitatory neurons in hippocampus and cortex were generated using the CaMKIIα promoter. In these mice, tamoxifen injection resulted in the rapid translocation of $\alpha CREB^{S133A}$ to the nucleus and disruption of CREB-mediated transcription (within 6 h). This fast inducibility was exploited to study the contribution of CREB to the encoding and the consolidation of information into LTM, and memory retrieval using contextual and cued conditioning tasks. Activation of $\alpha CREB^{S133A}$ by tamoxifen injection before training did not impair short-term contextual or cued fear memory when tested 2 h after training. However, it impaired both types of memory after 24 h, confirming that CREB-mediated transcription is required for LTM but not STM. The defect was not caused by a failure in retrieval since $\alpha CREB^{S133A}$ activation only 6 h before the memory test did not affect performance. Since memory re-consolidation after retrieval also requires protein synthesis and is thought to activate similar mechanisms as initial memory consolidation (Nader et al. 2000), it was examined after $CREB^{S133A}$ activation. For this, transgenic mice expressing $CREB^{S133A}$ were trained for contextual or cued fear conditioning, $\alpha CREB^{S133A}$ was activated 18 h later and then the animals were re-exposed to the context or the tone (alone) 24 h later. Re-exposure

is meant to reactivate memory for context or tone, a process known to make memory traces transiently unstable and susceptible to disruption, and that requires re-consolidation. CREB inactivation at the time of re-consolidation impaired performance, thus providing novel evidence that CREB is needed not only for the consolidation but also the re-consolidation of memory traces after retrieval.

5 Discussion and Future Perspectives

The advent of spatially and temporally restricted genetic manipulations in the mouse brain has been a critical step forward in the understanding of gene functions in synaptic plasticity, learning, and memory. To date, multiple transgenic lines are available for conditional transgenesis in the brain (Tables 1 and 2). The growing popularity of the approach and the need for even further spatial and/or temporal restriction will have to be accommodated in the near future, for instance by the establishment of new promoters.

The limitations and shortcomings of classical transgenesis will also have to be improved. For example, transgene expression is often different and variable in independent mouse lines. This variability generally derives from the randomness of transgene integration, i.e. integration in a region of heterochromatin often correlates with high expression variability (Pravtcheva et al. 1994). Copy number also influences expression parameters and a high number increases the risk for gene silencing (Martin and Whitelaw 1996; Garrick et al. 1998; Henikoff 1998). Although mostly undesired, transgene variability may turn extremely advantageous if the actual pattern of expression is restricted to an area of interest. As discussed in this chapter, mouse lines carrying the same promoter fragment (Table 3) have not always exhibited the same expression pattern, level, or onset depending on whether it was used alone or in combination with tTA(rtTA)- or Cre-dependent systems. That was the case for an 8.5-kb CaMKIIα promoter fragment originally described with a late onset (about 3 days after birth) and neuronal specificity in cortical structures, hippocampus, striatum, and amygdala (Mayford et al. 1996b). However, when combined with tTA or Cre, it was found to be sometimes active during embryogenesis, leading to perinatal lethality as observed with a mutant huntingtin gene (Yamamoto et al. 2000), to drive expression in most forebrain neurons except hippocampal CA1 neurons (with tTA; (Mayford et al. 1996b) or in constrast only in CA1 neurons (with Cre; Tsien et al. 1996a, 1996b).

Different approaches may be used to circumvent the variability of transgene expression. One possibility is to use large fragments of DNA carrying *cis* -regulatory elements including introns, locus control regions, and insulators that generally ensure position-independent and copy number-dependent gene expression (for review, see Giraldo and Montoliu 2001). They require chromosome-type vectors such as bacterial, plasmid, or yeast artificial chro-

Table 1 Different nervous system-specific promoters used to drive the expression of Cre-recombinase

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
D6	Neocortex, hippocampus	van den Bout et al. 2002	-
Emx1	Cerebral cortex, hippocampus	Guo et al. 2000	-
C-kit	CA1, CA2 and CA3 regions of hippocampus, anterior region of the dentate gyrus, ganglion cell layer of retina	Eriksson et al. 2000	-
Nestin	Neuronal and glial cell precursor	Tronche et al. 1999	Fleischmann et al. 2003 Tomita et al. 2003 Golub et al. 2004
CaMKIIα	1: CA1 region of hippocampus/ Hippocampus, cortex and striatum. 2: High level in all forebrain structures; low levels in cerebellum. 3: High levels in hippocampus, cortex and amygdala; low levels in striatum, thalamus and hypothalamus.	1: Tsien et al. 1996a 2: Dragatsis and Zeitlin 2000 3: Casanova et al. 2001	Tsien et al. 1996b Huerta et al. 2000 Rampon et al. 2000 Rondi-Reig et al. 2001b Shimizu et al. 2000 Yu, Saura et al. 2001 Zeng et al. 2001 Schweizer et al. 2003 Bukalo et al. 2004 Kelleher et al. 2004 Saura et al. 2004 Knuesel et al. 2005 Saura et al. 2005
KA1	1: In embryo: most neuronal cells of CNS. In adult: CA3 region and dentate gyrus granule cells of hippocampus. 2: High level in CA3, low in dentate gyrus.	1: Kask et al. 2000 2: Nakazawa et al. 2002	Nakazawa et al. 2002 Nakazawa et al. 2003
mNF-H	Neurons of the brain and spinal cord during late stage of development	Hirasawa et al. 2001	-
NEX	Granule cells of dentate gyrus	Schwab et al. 2000	Kleppisch et al. 2003

Table 1 (continued)

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
PrP (inducible-ERT)	Brain, retina, hippocampus cerebellum	Weber, Metzger et al. 2001	-
Thy-1	CNS and PNS (cortex, cerebellum, spinal cord, retina, dorsal root ganglion)	Campsall, Mazerolle et al. 2002	-
NSE	Embryo: forebrain, midbrain, hindbrain flexure. Adult: cortex, cerebellum, hippocampus, septum	Cinato, Mirotsou et al. 2001	-
Syn-1	Neuron specific (brain and spinal cord)	Zhu, Romero et al. 2001	-

mosomes (BACs, PACs, and YACs) with large cloning capacity (up to 1 Mb). Artificial chromosomes are either microinjected in a linearized form into the pronucleus of fertilized mouse eggs or inserted into embryonic stem cells where they randomly integrate into the host genome. The cloning, handling, and injection of these vectors requires more skill and time than conventional transgenesis because large DNA fragments are more fragile and prone to breakage, and extensive analysis is required after insertion into a host to ensure for the presence of the whole transgene. Furthermore, frequency of integration is lower than with plasmid-based transgenes. However, since the reliability of expression is higher, a smaller number of lines needs to be generated and screened to obtain one with the desired characteristics. A BAC carrying 170 kb of noncoding genomic DNA of the CaMKIIα gene, consisting of approximately 50 kb of genomic sequence upstream of the ATG and 110 kb of downstream sequences was cloned. This BAC allowed faithful expression of genes according to the pattern and onset of endogenous CaMKIIα and expression levels were copy number-dependent (Casanova et al. 2001). Incidentally, its specificity did not differ much from that normally observed with the 8.5-kb promoter, indicating that a shorter fragment in this case was sufficient (Mayford et al. 1996b). Another efficient method to faithfully express a transgene following the pattern, time course, and level of a specific endogenous gene is to knock in the gene of interest into the ORF of a selected locus by homologous recombination (Misawa et al. 2003; Korets-Smith et al. 2004). Knock-ins help prevent expression variability linked to random

Table 2 Different nervous system-specific promoters used to drive the expression of either tTA or rtTA

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
Prnp-tTA	Cerebral cortex, hippocampus, thalamus, hypothalamus, striatum, cerebellum	Tremblay, et al. 1998	Peters et al. 2005
CaMKIIα-tTA	Forebrain, neocortex, hippocampus, amygdala, striatum	Mayford et al. 1996	Mayford et al. 1996 Mansuy et al. 1998a Pittenger et al. 2002 Chen et al. 2003 Huang et al. 2004 Santacruz et al. 2005
CaMKIIα-rtTA	Hippocampus, cortex, septum, striatum	Mansuy et al. 1998b	Mansuy et al. 1998b Malleret et al. 2001 Genoux et al. 2002
NSE-tTA	Striatum, cerebellum, CA1, neocortex	Chen et al. 1998	King et al. 2003
GFAP-rtTA	Astrocytes	Kim et al. 2003	-
GABAα6-rtTA	Cerebellar granule cells	Yamamoto et al. 2003	-

integration and selects for active euchromatin, ensuring efficient expression. However, knock-ins generally yield few positive animals and heterozygosity at the target locus may perturb the full function of the targeted gene. However, this may be alleviated by simultaneous expression of a transgene and the endogenous gene using internal ribosomal entry sequences (IRES) (Michael et al. 1999; Gorski et al. 2002; Funfschilling et al. 2004; Lindeberg et al. 2004).

Several approaches have been used to improve the inducibility and tightness of transgenic manipulations. Improved versions of the rtTA factor with optimized codon use for mammals, enhanced affinity for dox and reduced leakage have been engineered (Urlinger et al. 2000). Transgenic mice expressing one of these new factors, rtTA2S-M2, under the control of the CaMKIIα promoter, have been generated and characterized (Michalon et al. 2005) and will be useful for future studies in the brain. Furthermore, tTA or rtTA expression was made Cre-dependent by insertion of a floxed stop cassette upstream the coding region (Belteki et al. 2005; Yu et al. 2005). Another study combined rtTA with

Table 3 Studies using the CaMKIIα promoter elements in transgenic mice to examine memory and synaptic plasticity

Type of transgene expressed	1995/1997	1998/2001	2002/2005
direct	Bach et al. 1995 Mayford et al. 1995 Kojima et al. 1997 Abel et al. 1997	Tang et al. 1999 Rammes et al. 2000 Kang et al. 2001 Tang et al. 2001 Philpot et al. 2001	Wong et al. 2002 Wang et al. 2003 Wang et al. 2004 Wei et al. 2004 Wood et al. 2005
tTA	Mayford et al. 1996	Mansuy et al. 1998a Jerecic et al. 2001	Bejar et al. 2002 Hernandez et al. 2002 Pittenger et al. 2002 Chen et al. 2003 Fridmacher et al. 2003 Huang et al. 2004 Santacruz et al. 2005
rtTA	-	Mansuy et al. 1998 Malleret et al. 2001	Genoux et al. 2002
Cre	Tsien et al. 1996a	Huerta et al. 2000 Rampon et al. 2000 Rondi-Reig et al. 2001 Shimizu et al. 2000 Yu et al. 2001 Zeng et al. 2001	Vyssotski et al. 2002 Schweizer et al. 2003 Bukalo et al. 2004 Kelleher III et al. 2004 Saura et al. 2004 Knuesel et al. 2005 Saura et al. 2005

a tet repressor tTR that eliminated leakage. tTR binds to tetO promoter and actively represses expression in the absence of dox, but is displaced by rtTA in the presence of dox (Konopka et al. 2005).

Similar to transgenesis, gene targeting has been made conditional by combination with specific promoters and regulated systems. The first example of an inducible knockout made use of the interferon responsive-promoter of the *Mx1* gene fused to Cre and controlled by IFNα or β (Kuhn et al. 1995). More recent examples employed dox-dependent expression systems (Saam and Gordon 1999; Radomska et al. 2002; Schonig et al. 2002; Guo et al. 2005; Yu et al. 2005) that when fused to the CaMKIIα promoter, allowed inducible expression of Cre and recombination in forebrain, similar to the one described above with NR1 (Lindeberg et al. 2002). Interestingly in this latter example, different patterns of gene recombination could be obtained by varying the timing of

dox-mediated shutdown of Cre expression, due to different onset and expression level of tTA in different brain regions. More recently, an inducible version of Cre was designed by fusion of Cre or improved derivatives with the LBD of the estrogen receptor that can be induced with tamoxifen (Metzger et al. 1995; Kellendonk et al. 1996; Brocard et al. 1997; Feil et al. 1997; Danielian et al. 1998; Schwenk et al. 1998; Casanova et al. 2002; Guo et al. 2002; Hayashi and McMahon 2002; Shimshek et al. 2002). While protocols for tamoxifen administration still need optimization, initial reports with high recombination efficiency indicate that the method is promising (Hayashi and McMahon 2002; Zirlinger et al. 2002). The system has been successfully adapted to the brain (Weber et al. 2001; Leone et al. 2003), but its tremendous potential has not yet been exploited for studies of cognitive functions.

Alternative approaches to gene overexpression or recombination may also be based on the design of inducible proteins more amenable to rapid and flexible biochemical modulation. One example in this chapter described tamoxifen-dependent CREB based on CREB fusion with the LBD of the estrogen receptor. Such fusion has been employed with other targets such as the transcription factors c-jun, c-fos, and c-myc (Rossler et al. 2002; Jager et al. 2004) or the cytoplasmic enzyme ornithine decarboxylase (Lan et al. 2005). It was further recently combined with Cre-mediated recombination to control the expression onset of the fusion protein induced by tamoxifen, which resulted in an tightly controlled system (Jager et al. 2004).

In only a little over a decade, conditional transgenesis has evolved to provide an exquisite degree of specificity of genetic manipulations. Further improvements and alternative approaches offering higher spatial resolution, i.e. specific sub-cellular compartments such as recently achieved in the nucleus (Limback-Stokin et al. 2004), enhanced temporal control, and taking into account post-translational modifications are still needed to gain even deeper understanding of protein functions. In light of the rapid technological progress in this field and the rise in popularity of these systems, there are good reasons to believe that these requirements will be met in the near future.

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A Novel Conditional Knockout Strategy Applied to Serotonin Receptors

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Abstract Here we demonstrate the feasibility of a doubly regulatable transgenic mouse design that allows for gene manipulation by both Cre-recombinase and the tetracycline inducible system. Using a knock-in strategy to insert both elements of the tetracycline inducible system and a neomycin (neo) cassette flanked by loxP sequences (floxed) into the wild-type locus, we generated mice that express the 5-HT$_{1B}$ receptor in a conditional manner. In the presence of a floxed neo-cassette, receptor expression was silenced. Removal of this cassette by Cre-mediated recombination led to 5-HT$_{1B}$ receptor expression, which was highly regulatable when doxycycline, a derivative of tetracycline, was administered to the mice. This system allowed for a determination of an in vivo time course of receptor half-life and recovery. Physiological studies also demonstrated that rescued 5-HT$_{1B}$ receptors were functional, and that this functionality was reversible upon treatment with doxycycline. Crossing mice where the 5-HT$_{1B}$, or the 5-HT$_{1A}$, receptors were silenced by the neo-cassette,

with mice expressing either Cre-recombinase or the tetracycline transactivator (tTA) under the control of tissue-specific promoters, led to tissue-specific re-expression of these receptors. Our studies thus demonstrate the potential of this strategy for achieving both a classic knockout, as well as subsequent tissue-specific and/or inducible knockouts.

Keywords Conditional rescue · Conditional knockout · 5-HT receptors · 5-HT1A · 5-HT1B · tTA · Cre · Tissue-specific knockout

1 Introduction

In order to study the contributions of individual genes to various behaviors and functions, our lab and others have created classic knockout mice through homologous recombination in embryonic stem cells. Such knockout mice have provided insight into the roles that these genes may play, but they have some limitations. Specifically, these mice develop throughout life without the protein of interest, and therefore the observed phenotype(s) may be due to developmental effects and not to the acute absence of the protein. We have, for example, recently shown that the anxious-like phenotype of the 5-HT_{1A} knockout mouse results from the absence of the receptor during development rather than from its absence during adulthood (Gross et al. 2002). Similarly, we have shown that 5-HT_{1B} antagonists produce effects that do not mirror the phenotype of a life-long 5-HT_{1B} knockout mouse (Castanon et al. 2000).

A second limitation of the classic knockout has been that it does not allow for dissecting out the contributions of individual neuronal circuits to various behaviors. In order to begin to probe the various functions of specific receptor populations, we were therefore interested in developing a tissue-specific inducible strategy with which to study the function of the serotonin 5-HT_{1B} receptor, among others.

Current techniques for the control of tissue-specific gene expression often rely on the availability of a few well-characterized promoters, such as the α-CAMKII promoter (Mayford et al. 1996). In order to avoid the necessity of characterizing promoters, while still theoretically maintaining the temporal and spatial patterns of expression of a particular protein, a knock-in approach that exploits the endogenous promoter would be advantageous. Furthermore, by combining a knock-in approach with elements of an inducible system, we could hope to achieve endogenous gene expression that would also be regulatable.

In the tetracycline inducible system developed by Gossen and Bujard (Gossen and Bujard 1992), a chimeric transcription factor, composed of the VP16 activation domain fused to the *tet* repressor of the *Escherichia coli* Tn10 tetracycline resistance operon (called tTA, or the tetracycline transactivator), binds to operator sequences (tetO) to activate transcription. In the presence of tetra-

cycline, this binding is inhibited. Early studies with this inducible system demonstrated tight temporal regulation of gene expression, both in cell culture (Gossen and Bujard 1992), and in a two-animal model, as demonstrated with β-galactosidase or luciferase as reporter genes (Furth et al. 1994). A number of benefits had been reported for this system, including a high affinity of tTA for the tetO sequences, low toxicity of tetracycline for mammalian cells, and excellent pharmacokinetic properties of tetracycline such as the rapid uptake into cells and penetration of the blood–brain barrier (Gossen et al. 1993).

A number of more recent studies have now demonstrated the usefulness of this system both in vitro and in vivo. In cell culture studies, including our previously published work (Stark et al. 1998), both one- and two- construct designs have been shown to produce regulatable gene expression. In vivo, the tetracycline system has been applied to create inducible mouse models for a number of studies, including disease models (see review by Yamamoto et al. 2001). In most of these cases, a two-mouse transgenic approach has been used to create inducible gene expression. A different approach, using a single-mouse design, has so far only been previously reported to work in vivo by Bond and colleagues (Bond et al. 2000). This single mouse design has an advantage over the two-mouse design in that breeding time is reduced and that mice are on a more homogeneous background than that which is obtained when two or more mice are bred together. Furthermore, the temporal and spatial pattern of the endogenous protein is theoretically reproduced if a regulatory cassette is placed under the control of the endogenous promoter.

In our present study, we have created a cassette containing the tTA, a neo-resistance gene, and the tetO sequences, and placed them upstream of the serotonin 5-HT_{1B} receptor transcription start site. Through homologous recombination, this cassette was introduced into the 5-HT_{1B} genomic locus in embryonic stem cells. Resulting homozygote mice were found to have a silencing of 5-HT_{1B} expression. Subsequent removal of the neo-selection cassette, and the generation of neo-less mice, led to mice that had regulatable 5-HT_{1B} receptor expression. A similar cassette had also been placed upstream of the 5-HT_{1A} receptor (Gross et al. 2002), although regulatable 5-HT_{1A} expression was not achieved in the one-mouse design after the neo-cassette was removed. In both the 5-HT_{1B} and 5-HT_{1A} loci, however, the presence of the neo-cassette led to a silencing of gene expression. Further manipulations of both of these neo-containing mice demonstrated that it was possible to rescue 5-HT_{1B} and 5-HT_{1A} receptor expression by breeding these mice with other mouse lines expressing either the Cre-recombinase enzyme or the tTA. Our single knock-in strategy therefore produced both an inducible knockout in the case of the 5-HT_{1B} receptor, and a rescuable knockout via breeding with Cre or tTA-expressing mouse lines for both the 5-HT_{1B} and 5-HT_{1A} lines.

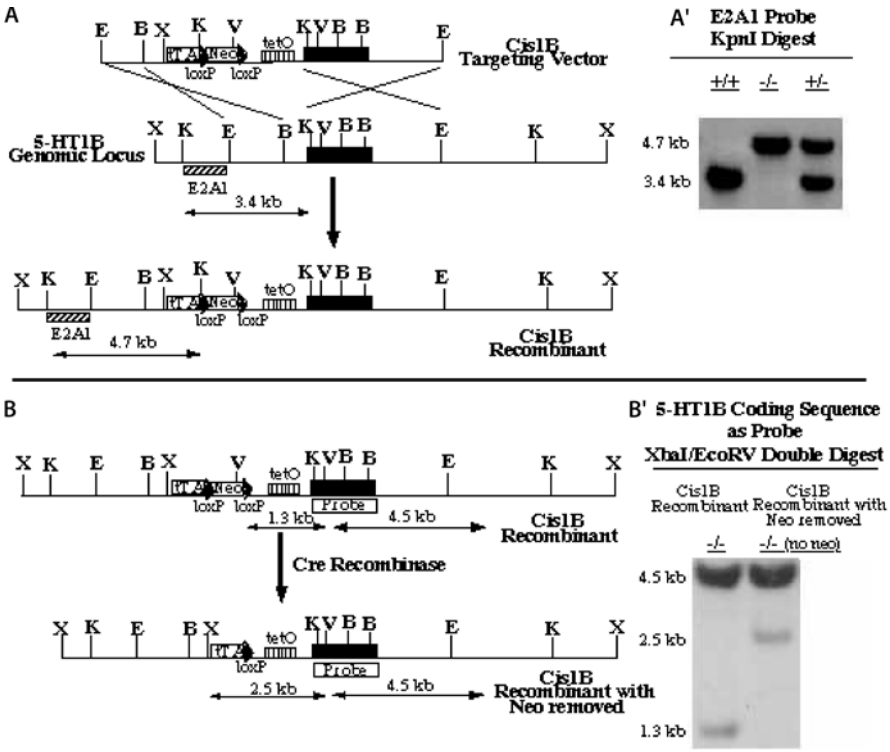


Fig. 1 A,B Homologous recombination with the Cis1B construction and Cre recombinase-mediated excision of the neomycin resistance gene in embryonic stem cells. **A** Cis1B targeting vector and predicted genomic structure after homologous recombination in embryonic stem cells. *Black box* coding sequence of the 5-HT_{1B} receptor. *Triangles* loxP sites. E2A1:1.3-kb external probe used to screen for recombination events after a KpnI digest. **A′** Southern blot analysis of tail DNA from wild-type mice (+/+), mice homozygous for the insertion (–/–), and mice heterozygous for the insertion (+/–). **B** Removal of neo by Cre recombinase treatment in embryonic stem cells. An XbaI/EcoRV double digest distinguishes the recombination event when the 5-HT_{1B} coding sequence is used as the probe. **B′** Southern blot analysis of tail DNA from mice homozygous for the original insertion (–/–) and from mice homozygous for the neo-less construction (–/–; no neo). *tTA,* tetracycline transactivator gene; *tetO,* tetracycline operator sequences; *Neo,* neomycin phosphotransferase gene; *X,* XbaI; *K,* KpnI; *E,* EcoRI; *B,* BalI; *V,* EcoRV

2
The Introduction of a Cis Construct Leads to Regulatable 5-HT_{1B} Receptor Expression

A cassette containing tTA, the floxed neo-selection marker, and the tetO operator sequences and minimal promoter was placed into the 5′ untranslated sequence of the serotonin 5-HT_{1B} receptor gene, 110 bp from the ATG start

site. This construction was introduced into the genomic 5-HT_{1B} receptor locus through homologous recombination in embryonic stem cells (Fig. 1A, 1A′). Analysis of receptor binding with [^{125}I-CYP], a ligand specific for the 5-HT_{1B} receptor under certain conditions (Waeber and Palacios 1992), demonstrated a lack of receptor binding in animals homozygous for the knocked-in construct (Figs. 2, 6D). The subsequent removal of the neo-selection cassette in embryonic stem cells by electroporation of a Cre-recombinase-expressing plasmid, and creation of homozygous mice from these neo-excised cells, led to re-expression of the 5-HT_{1B} receptor (Figs. 1B, 1B′, 2). Expression levels of the

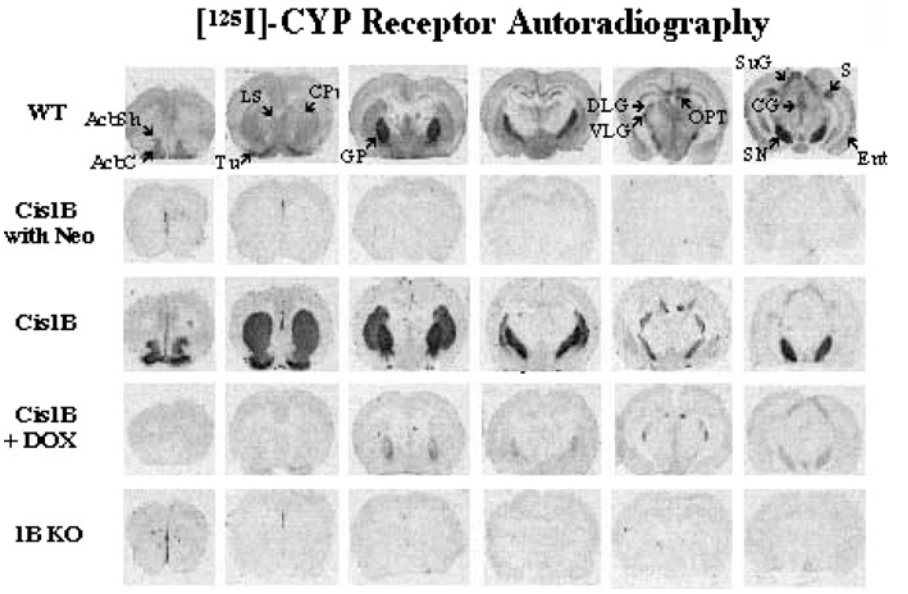


Fig. 2 A 5-HT1B receptor expression pattern in Cis1B mice: before and after doxycycline treatment. [125I]-CYP receptor autoradiography was performed on coronal brain sections of adult wild-type (*WT*) mice, 5-HT_{1B} receptor knockout mice (*1B KO*), Cis1B mice containing the neomycin selection cassette (*Cis1B with Neo*), and Cis1B mice with the neomycin cassette removed (*Cis1B*). The presence of the neomycin selection cassette led to a silencing of gene expression. Removal of this cassette led to a re-expression of gene expression. Treatment of Cis1B mice with 2 mg/ml doxycycline (*DOX*) since conception reveals a differential pattern of regulation of the 5-HT_{1B} receptor between various structures. A long film exposure time (18.5 h) is shown here for the purpose of illustrating structures that show very low expression levels. It should be noted that this exposure minimizes the difference in expression levels between wild-type and Cis1B mice in structures where the 5-HT_{1B} receptor is highly expressed, such as the globus pallidus and substantia nigra, due to saturation of the film. *AcbC,* accumbens nucleus, core; *AcbSh,* accumbens nucleus, shell; *CG,* central gray; *CPu,* caudate putamen; *DLG,* dorsal lateral geniculate nucleus; *Ent,* entorhinal cortex; *GP,* globus pallidus; *LS,* lateral septum; *OPT,* olivary pretectal nucleus; *S,* subiculum; *SN,* substantia nigra; *SuG,* superior colliculus, superficial gray layer; *Tu,* olfactory tubercle; *VLG,* ventrolateral geniculate nucleus

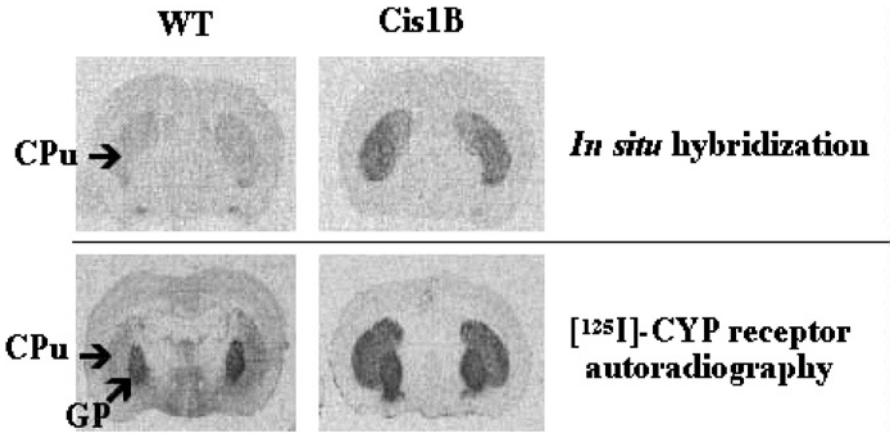
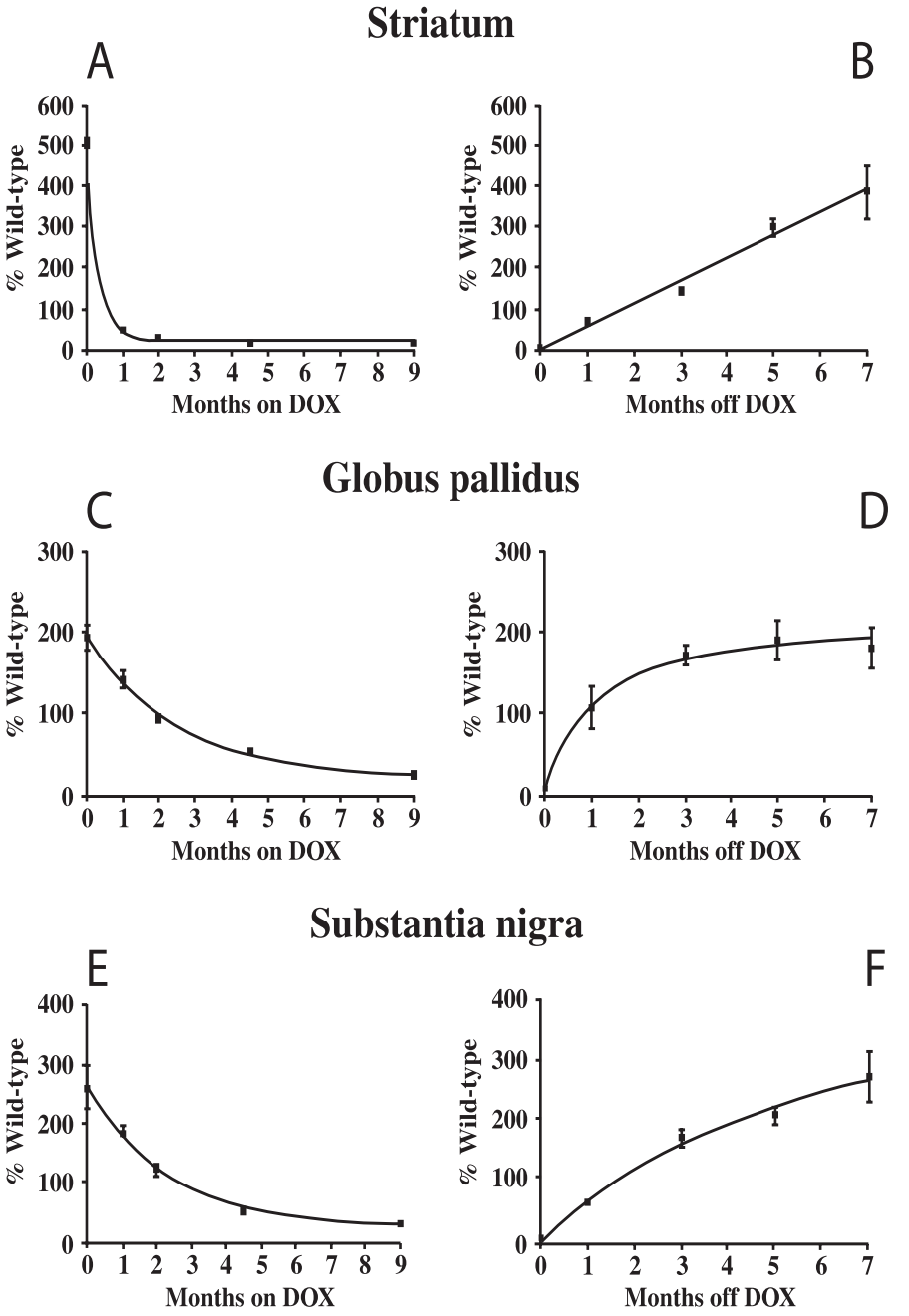


Fig. 3 Comparison of in situ hybridization and receptor binding in wild-type and Cis1B mice. *Top panel* In situ hybridization using a ^{33}P-labeled oligonucleotide probe directed against the 5-HT_{1B} receptor demonstrates an amplification of 5-HT_{1B} receptor mRNA expression in the Cis1B mice as compared to wild-type mice. *Bottom panel* Receptor autoradiography using [^{125}I]-CYP on coronal brain sections of mice demonstrates the targeting of 5-HT_{1B} receptors to the terminals of striatal neurons, here shown at the level of the globus pallidus. *CPu,* caudate putamen; *GP,* globus pallidus; *CYP,* cyanopindolol

5-HT_{1B} receptor were higher than wild-type in some structures, such as the striatum and globus pallidus, and lower in others. This resulting mouse line was called Cis1B.

In order to test the efficacy of gene regulation by the tetracycline system in our mice, we placed Cis1B mice on doxycycline (2 mg/ml) for various periods of time, such that a time course of receptor disappearance could be assessed. Additionally, mice were placed on doxycycline from conception and then, once they were adults, doxycycline treatment was removed so that a time course of receptor reappearance could also be assessed (Figs. 2, 4). 5-HT_{1B} receptor levels were measured in the striatum, globus pallidus, and substantia nigra. In the striatum, base-line 5-HT_{1B} receptor levels were increased fivefold over wild-type. Base-line levels in the globus pallidus and substantia nigra were increased 1.9-fold and 2.5-fold over wild type, respectively (Figs. 2, 4). Doxycycline

Fig. 4 A–F Binding curves for 5-HT1B receptor disappearance and recovery in three brain structures of Cis1B mice. Binding curves obtained from measurements of [^{125}I]-CYP binding of coronal brain sections from Cis1B mice treated with 2 mg/ml doxycycline (*DOX*) for 0, 1, 2, 4.5, or 9 months (graphs **A, C, E**), or from Cis1B mice which were treated with DOX (2 mg/ml) since conception and then as adults had the DOX removed for 1, 3, 5, or 7 months (graphs **B, D, F**). Binding values are represented as % wild-type values


Striatum
A
% Wild-type
Months on DOX
B
% Wild-type
Months off DOX
Globus pallidus
C
% Wild-type
Months on DOX
D
% Wild-type
Months off DOX
Substantia nigra
E
% Wild-type
Months on DOX
F
% Wild-type
Months off DOX

treatment throughout life resulted in an almost complete suppression of 5-HT_{1B} receptor expression (Fig. 2, Cis1B + DOX).

3 Doxycycline Treatment in Cis1B Mice Allows for an Estimation of 5-HT_{1B} Receptor Half-Life and Recovery

5-HT_{1B} receptors have been shown to be expressed predominantly on axon terminals, and previous studies have demonstrated the in vivo transport of the 5-HT_{1B} receptor from cell bodies in the striatum to striatal terminals in the globus pallidus and substantia nigra (Boschert et al. 1994; Ghavami et al. 1999). Figure 3 shows a comparison of in situ hybridization and 5-HT_{1B} receptor binding between wild-type and Cis1B mice, demonstrating that 1B protein is correctly trafficked to axon terminals in our system. This figure also illustrates increased levels of both mRNA and protein in the Cis1B mice as compared to wild types.

The administration of doxycycline (and subsequent suppression of 5-HT_{1B} gene transcription) for 0, 1, 2, 4, 5, and 9 months allowed for construction of 5-HT_{1B} receptor binding curves over time from which the half-life of receptor expression could be estimated (Fig. 4). Additionally, the removal of doxycycline and analysis of 5-HT_{1B} receptor re-expression after 1, 3, 5, and 7 months led to a plot of receptor recovery over time. We found that in both the globus pallidus and substantia nigra, the projection areas of the striatum, the 5-HT_{1B} receptor had an unexpectedly long predicted half-life of 1.8 months. In the striatum itself, a much shorter half-life of 7 days was found, suggesting different proteolytic pathways for the 5-HT_{1B} receptor in the somatodendritic vs axonal compartments.

Analysis of the recovery of 5-HT_{1B} receptor expression after cessation of doxycycline treatment revealed that receptors in the globus pallidus and substantia nigra did not achieve their maximal levels of expression until after a period of several months. A half-life of recovery of 2.8 months in the globus pallidus and 3.7 months in the substantia nigra was predicted. In the striatum, recovery was even more delayed, such that after 7 months of withdrawal from the doxycycline treatment, receptor levels had not yet reached their maximum. This long period of recovery may, in fact, reflect the observation that doxycycline can remain in mouse tissue for quite some time after removal from the diet, and that lower treatment doses of doxycycline may be required to allow a faster recovery of gene expression once doxycycline is removed from the system (Chen et al. 1998). The recovery experiment (Fig. 4) also shows receptor levels in the globus pallidus and substantia nigra reaching a steady plateau before levels in the striatum. This suggests that the high 5-HT_{1B} receptor levels found in the striatum of Cis1B mice are achieved only after saturation of the striatal terminals (globus pallidus and substantia nigra) has occurred.

4
Cis1B Mice Display Physiological Responses Consistent with Functional 5-HT_{1B} Receptors, Which Can Be Reversed upon Treatment with Doxycycline

Previous studies have shown a hypothermic response to 5-HT_{1B} receptor agonists (Hagan et al. 1997; Oerther and Ahlenius 2001). Therefore, to assess the functionality of expressed receptors, we also looked at the effect of the 5-HT_{1B} receptor agonist CP94253 on temperature regulation in our mice. The temperature response to CP94253 (10 mg/kg) was tested in wild-type, Cis1B, and 5-HT_{1B} knockout mice (Fig. 5). In this experiment, rectal temperature was measured before and after administration of the agonist. The same mice were then administered doxycycline (2 mg/ml) and retested 2 months later.

In the 40 min before drug injection, there was no significant difference in body temperature among the three genotypes. This was seen both before doxycycline treatment (ANOVA, $F(2,54) = 0.649$, $p = 0.5266$), and after (ANOVA, $F(2, 54) = 0.271$, $p = 0.7638$). Injections with CP94253 led to differential changes of body temperature across groups. Prior to doxycycline treatment, there was a main effect of genotype (ANOVA, $F(2,51) = 6.391$, $p = 0.0033$) and treatment

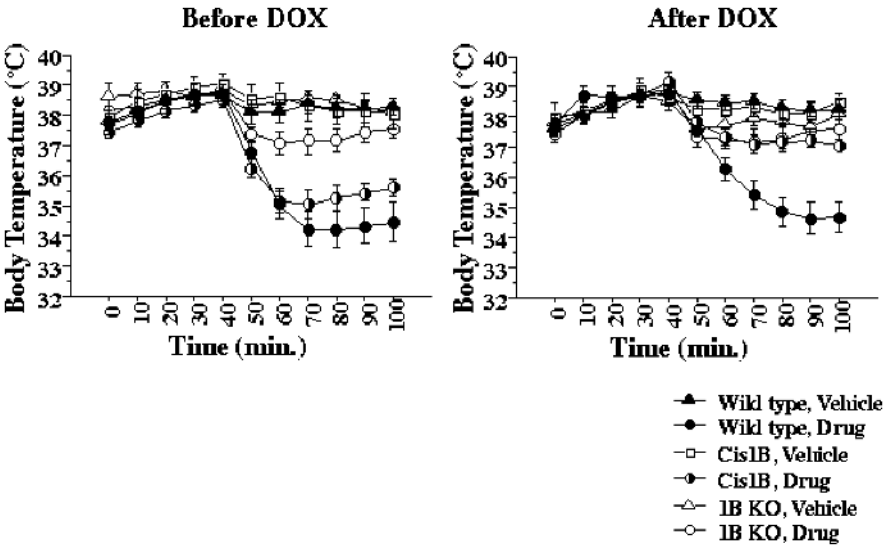


Fig. 5 Hypothermic response to CP94253. Adult male mice were housed overnight in individual cages. The next day, rectal body temperature was recorded every 10 min for 40 min, at which point an injection of CP94253 (10 mg/kg) or vehicle was given. Body temperature was monitored for an additional hour. Mice were then placed on doxycycline (DOX: 2 mg/ml) for 2 months and retested

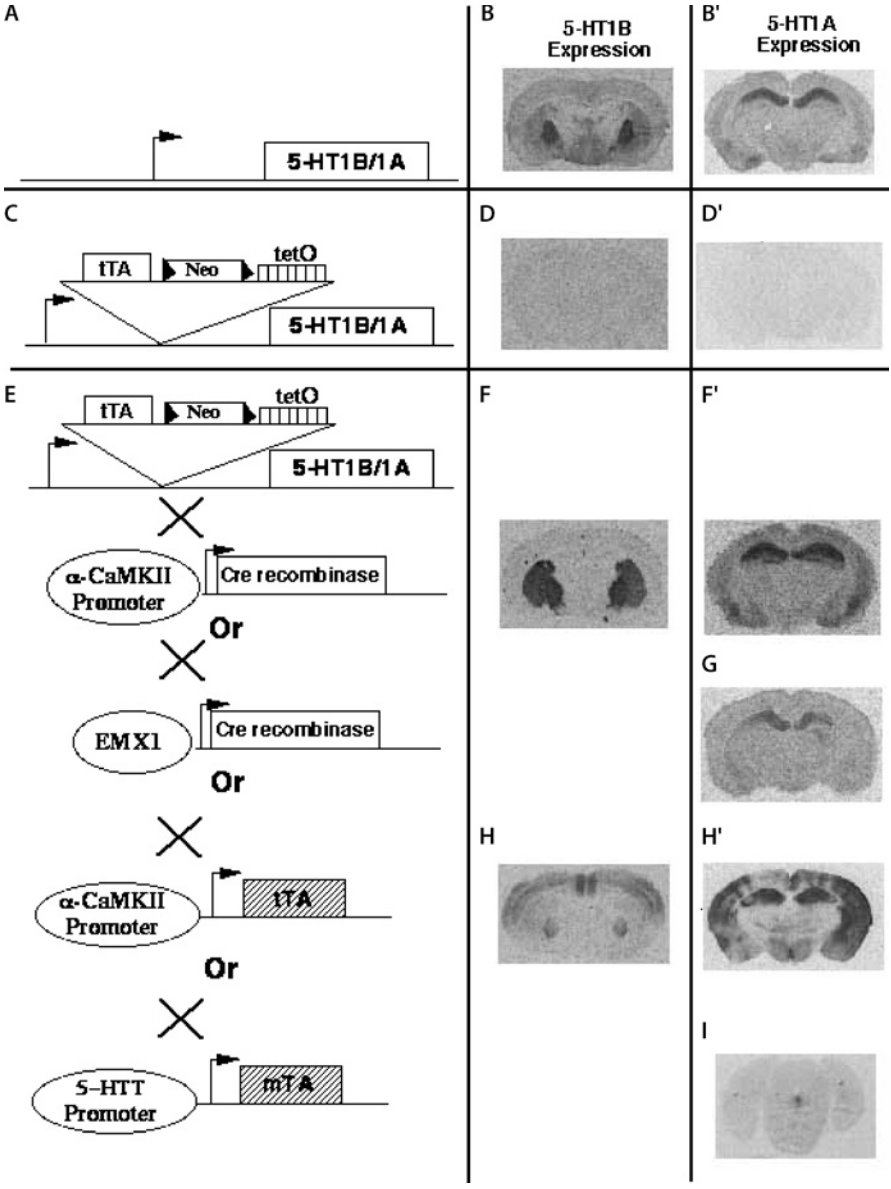
(ANOVA, $F(1,51) = 60.828$, $p < 0.0001$), and a significant interaction between genotype and treatment (ANOVA, $F(2,51) = 5.125$, $p = 0.0094$). Fisher's post-hoc analysis revealed a significant effect of drug ($p < 0.0001$) and a significant difference between 5-HT_{1B} knockout mice and Cis1B mice ($p = 0.0110$), and between wild-type and 5-HT_{1B} knockout mice ($p = 0.0011$). Interestingly, there was no significant difference between wild-type and Cis1B mice ($p = 0.4538$), indicating that the Cis1B mice behaved like the wild-type mice.

In order to further examine the contribution of the 5-HT_{1B} receptor to this phenotype, mice were placed on doxycycline treatment to decrease 5-HT_{1B} receptor expression. After doxycycline treatment, there was still a main effect of genotype (ANOVA, $F(2,51) = 3.59$, $p = 0.0348$), an effect of treatment (ANOVA, $F(1,51) = 29.507$, $p < 0.0001$), and a significant interaction between genotype and treatment (ANOVA, $F(2,51) = 7.791$, $p = 0.0011$). Fisher's post-hoc analysis revealed a significant effect of drug ($p < 0.0001$) and a significant effect of genotype between 5-HT_{1B} knockout mice and wild-type mice ($p = 0.0408$) and between Cis1B mice and wild-type mice ($p = 0.0016$). No significant difference was found between Cis1B and 5-HT_{1B} knockout mice ($p = 0.5633$), indicating that after doxycycline treatment, the Cis1B mice behaved like the knockout mice.

In summary, Cis1B mice responded like wild-type mice to injection of a 5-HT_{1B} agonist. After doxycycline treatment, this effect was suppressed, with

Fig. 6 A–I Rescue strategies in mice containing the Cis construct. **A, C, E** Simplified diagrams of the 5-HT_{1B} and 5-HT_{1A} receptor loci and modifications thereof. **A** Wild-type 5-HT_{1B} and 5-HT_{1A} receptor loci. **C** The insertion of a tTA, tetO, and neomycin-containing construct into these loci. **E** Crosses which were performed to rescue 5-HT_{1B} and 5-HT_{1A} receptor expression. **B, D, F, H** Receptor autoradiography using [^{125}I]-CYP for 5-HT_{1B} receptor binding on coronal brain sections of mice at the level of the globus pallidus. **B′, D′, F′, G, H′** Receptor autoradiography using [^{125}I]-MPPI for 5-HT_{1A} receptor binding on coronal brain sections of mice at the level of the hippocampus or at the level of the raphe nuclei (**I**). **B, B′** Wild-type receptor expression patterns. **D, D′** Insertion of the neomycin-containing construction into the 5-HT_{1B} and 5-HT_{1A} loci led to a silencing of receptor expression. **F** Crossing a mouse with the neomycin-containing construction with a mouse where Cre recombinase is under the control of the α-CaMKII promoter, led to a deletion of the neomycin selection cassette, and a reactivation of receptor expression. **G** Crossing the 5-HT_{1A} mouse with the neomycin-containing construction with a mouse where Cre recombinase is under the control of the EMX1 promoter, led to deletion of the neomycin selection cassette, and a differential reactivation of receptor expression. **H, H′** Crossing with α-CaMKII-tTA mice led to forebrain-specific expression in both lines, and crossing with 5–*HTT*-mtTA mice led to presynaptic raphe-specific expression in the 5-HT_{1A} mouse. *Solid triangles,* loxP sites; *Neo,* neomycin phosphotransferase gene; *tetO,* tetracycline operator sequences; *tTA,* tetracycline transactivator gene; α-*CaMKII,* alpha calcium-calmodulin-dependent kinase II; *CPY,* cyanopindolol; *MPPI,* 4-(2′-methoxy-phenyl)-1-[2′-(*n*-2'pyridinyl)-*p*-iodobenzamido]-ethyl-piperazine; *5-HTT,* serotonin transporter; *mTA,* modified tetracycline transactivator

Cis1B mice now responding like the 5-HT_{1B} knockout mice. These results suggest that, in our system, 5-HT_{1B} receptors are functionally expressed, and that doxycycline treatment can suppress this expression.



5
Specific Manipulations of the Neomycin-Containing Cis1B and 5-HT_{1A} Mice Lead to Re-expression of the 5-HT_{1B} and 5-HT_{1A} Receptors, Respectively

In addition to removing the neo-selection cassette in embryonic stem cells in culture (Fig. 1B, 1B′), we were also able to cross our Cis1B neo-containing mice with a Cre-recombinase-expressing mouse in order to delete the neo-cassette in vivo. By crossing the Cis1B neo-containing mouse with a line of mice where Cre-recombinase is expressed under the control of the α-CAMKII promoter (Dragatsis and Zeitlin 2000), we were able to rescue 5-HT_{1B} receptor expression. The pattern of 5-HT_{1B} expression, however, was indistinguishable from that which had been found for the original Cis1B mouse. This suggests that germline deletion of the neo-cassette had occurred (Fig. 6F). A similar cross was performed with the 5-HT_{1A} neo-containing mouse, resulting in a similar germ-line deletion (Fig. 6F′). Other groups have also obtained unwanted germline deletion when using Cre-recombinase-expressing mice, and alternative breeding strategies have been used to minimize this problem (Minichiello et al. 1999; Iwasato et al. 2000).

Using a conservative breeding strategy, we were able to avoid germline deletion. First, we crossed a mouse expressing Cre-recombinase under the control of the EMX1 promoter (Iwasato et al. 2000) onto a classic 5-HT_{1A} KO background. This line was then crossed with the 5-HT_{1A} neo-containing mice in order to minimize exposure of the floxed locus to Cre-recombinase (Fig. 6G) EMX1 is expressed in the developing forebrain, and the EMX1-Cre lines have been shown to produce hippocampal- and cortical-specific recombination (Iwasato et al. 2000). As shown in Fig. 6, this cross resulted in a 5-HT_{1A} receptor expression pattern that was more restricted than the wild type. Specifically, deletion of the neo-cassette was most efficient in the hippocampus, weak in the cortex, and absent from the septum and raphe nuclei.

Our neo-containing mouse lines also lend themselves to receptor rescue by a trans-tTA, introduced by crossing them with mice expressing tTA under the control of tissue-specific promoters. Crossing the neo-containing Cis1B line and the neo-containing 5-HT_{1A} line with an α-CAMKII-tTA transgenic mouse (Mayford et al. 1996) yielded reactivation of these receptors in a tissue-specific manner (Fig. 6H, 6H′). The α-CAMKII promoter has been shown to produce high levels of expression in the forebrain, including the striatum, hippocampus, and cortex (Mayford et al. 1996). Our mouse crosses thus led to expression of the 5-HT_{1B} and 5-HT_{1A} receptors in forebrain areas, as seen in Fig. 6H, 6h′. 5-HT_{1B} receptor binding was found in the cortex and hippocampus, as well as in the globus pallidus and substantia nigra (projection areas of the striatum). In keeping with the differential targeting of the 5-HT_{1B} and 5-HT_{1A} receptors (Boschert et al. 1994; Ghavami et al. 1999), 5-HT_{1A} receptor binding was found somatodendritically in the cortex, hippocampus, and striatum, but

not in the striatal projections. A second cross with the 5-HT_{1A} neo-containing construct was performed using mTA, a mammalianized version of the tetracycline transactivator (a gift from Dusan Bartsch), which was introduced into the serotonin transporter (5-HTT) locus. This cross gave a rescue of 5-HT_{1A} expression exclusively in the raphe nuclei (see Fig. 6I).

6 Conclusion

Using our unique construct design, we were able to manipulate expression of the 5-HT_{1B} receptor in four distinct ways. First, leaving the neo-cassette in place, we were able to produce a 5-HT_{1B} receptor knockout mouse. Second, by removing the neo-cassette in embryonic stem cells we were able to create our so-called Cis1B mice, which express the 5-HT_{1B} receptor under the control of its own promoter and in a manner regulatable by doxycycline. Third, by crossing a Cre-recombinase-expressing mouse line with our neo-containing mice we were able to demonstrate in vivo excision of this cassette and re-expression of the 5-HT_{1B} receptor. Fourth, by crossing the neo-containing mouse with the tissue-specific α-CAMKII-tTA mice, we were able to demonstrate a second doxycycline-dependent way in which 5-HT_{1B} receptor expression could be rescued in our mouse design.

The Cis1B mouse alone offers the advantage gene of regulatability in a one-mouse design. This is, as far as we know, only the second report of such a tetracycline inducible construction created by homologous recombination and leading to regulatable gene expression in vivo. The previous report by Bond and colleagues (Bond et al. 2000) had reported overexpression for the potassium channel subunit SK3. This group had further found expression to be abolished by doxycycline administration in the diet.

In the Cis1B mice, overexpression was also observed. Doxycycline treatment was able to significantly reduce 5-HT_{1B} receptor expression in most areas, although there was some low residual level of expression in the globus pallidus and substantia nigra. In addition, a few areas, such as the superior colliculus, seemed to be unaffected by doxycycline treatment. The overexpression found in our study, as well as in the Bond study, is likely to be due to an amplification by the tTA/tetO system.

Our Cis1B mouse also allowed for examination of the half-life of the 5-HT_{1B} receptor in vivo by exploiting the regulatable nature of the tetracycline system. We found the half-life in the striatum to be 7 days, an amount of time similar to that which had been found for another G-protein-coupled receptor, the 5-HT_{1A} receptor (Gross et al. 2002). The much longer half-life of 1.8 months in the globus pallidus and substantia nigra, however, was unexpected and may reflect a differential catabolism of the receptor when it is localized in axon terminals (in the globus pallidus and substantia nigra) rather than in the somatodendritic compartment (striatum). After doxycycline was removed, the

differential recovery rate in somatodendritic vs axonal structures may again reflect a different handling of the receptor in these two compartments. 5-HT_{1B} receptors are known to be concentrated in axonal terminals, but the cellular mechanisms underlying this distribution are unknown. Our results suggest that the preferential degradation of the receptor in somatodendritic compartments may contribute to its subcellular distribution. Our regulatable mouse model may provide a means with which to further study this process.

Rescue of 5-HT_{1B} in our system also produced functional receptor activity, as seen by the decrease in body temperature in response to the 5-HT_{1B} receptor agonist CP94253. Doxycycline treatment led to a reversal of this effect, demonstrating functional, as well as well as transcriptional, regulation. Such regulation is essential for future studies of in vivo receptor function and assessment of the contributions of the receptor to behavior.

By using this new inducible strategy to study the 5-HT_{1B} receptor, we have developed a general scheme to create knockout mice that can then be further manipulated to create inducible expression of a gene of interest. Depending on the method of reactivation, endogenous or tissue-specific expression can be achieved (Figs. 2, 6). By placing a floxed neo-cassette upstream of the gene of interest, a classic knockout mouse is created. Subsequent removal of the neo-cassette by Cre-recombinase or reintroduction of tTA through crossing with another tTA-expressing mouse line would lead to reactivation of the gene of interest. The ability to use tissue-specific Cre-recombinase or tissue-specific tTA lines will allow us to begin to dissect the contributions of the 5-HT_{1B} receptor from different receptor populations to various behaviors.

As described earlier, a similar cis-design construct was created and inserted into the 5-HT_{1A} genomic locus (Gross et al. 2002). As in the case of the 5-HT_{1B} receptor, introduction of the tTA-neo-tetO cassette resulted in a silencing the 5-HT_{1A} gene. In addition, removal of neo resulted in re-expression of the 5-HT_{1A} receptor. In this case, however, tetracycline regulation was not obtained, indicating a lack of functional tTA protein, and tTA-independent 5-HT_{1A} expression. We suspect that this may have been due to a cryptic splice site present in the tTA. Use of a modified version of tTA, mTA, which has been mammalianized and has had the cryptic splice site removed (D. Bartsch, personal communication), may alleviate this problem in the future.

In addition to the uses of the cis-mice already demonstrated here, receptor expression in our system can also be regulated by the tTS protein, a tetracycline-sensitive transcriptional silencer that binds to tetO elements (Deuschel et al. 1995). Our cis-1A mouse, in which germline deletion of the neo-cassette yields wild-type expression of the receptor that is not regulatable by doxycycline, is an ideal candidate for pairing with a tTS mouse line. Preliminary studies in our lab have shown that introduction of a ubiquitously expressed tTS (Mallo et al. 2003) onto a neo-deleted 1A background yields a 5-HT_{1A} receptor knockout that can be reversed upon doxycycline treatment. This system has the possibility to achieve an important goal sought in construction of the cis-mice:

preservation of endogenous expression pattern and levels, combined with the ability to temporally regulate expression with doxycycline treatment. tTS has been widely used in cell culture in conjunction with rtTA to control transcriptional leakiness, but is seldom applied to in vivo systems (Zhu et al. 2002). We suspect that this is due to the paucity of available mouse lines containing tetO sequences inserted between the endogenous promoter and coding region of a gene. This proposed use of tTS underscores the versatility of the cis-mouse lines we have created.

Although one other group has also been able to generate a one-mouse inducible model using the tetracycline system (Bond et al. 2000), the function of the one-mouse model seems very locus-dependent and may not work in all cases. In addition to the previously mentioned cryptic splice site present in the earlier version of tTA, a number of other issues may hinder this strategy. The fact that there are several closely located promoters (the endogenous

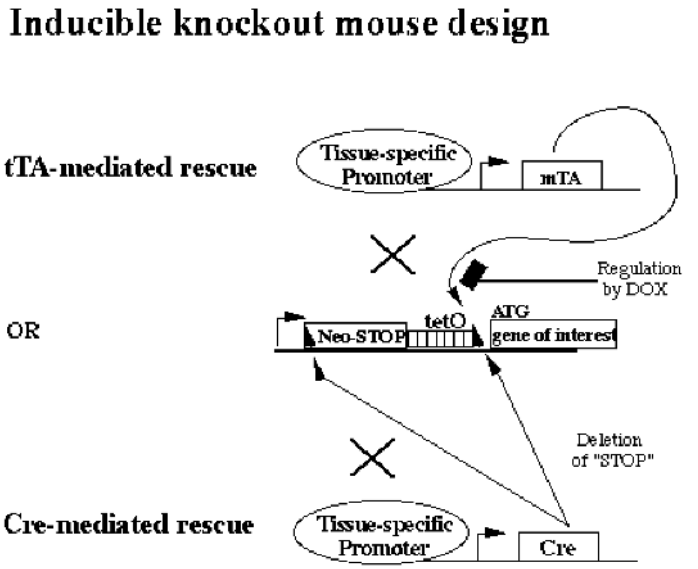


Fig. 7 A,B Inducible knockout mouse design. The introduction of a Neo-STOP-tetO cassette flanked by loxP sequences upstream of a gene of interest should lead to a knockout which is rescuable by two means. **A** Crossing this mouse with a tTA-expressing mouse line should lead to tissue-specific gene expression that would be regulatable by doxycycline administration. **B** Crossing this mouse line with a tissue-specific Cre-expressing line should result in the excision of the regulatory cassette, leading to tissue-specific activation of the gene of interest, under the control of its endogenous promoter. Such a cross would result in gene expression only in the endogenous areas where there was an overlap between the tissue-specific Cre line and the endogenous expression pattern. *mTA,* mammalianized tetracycline transactivator; *Neo,* neomycin phosphotransferase gene; *tetO,* tetracycline operator sequences; *DOX,* doxycycline; *"STOP",* transcriptional/translational stop sequences (Lakso et al. 1992); *Cre,* cre recombinase

promoter, the neomycin promoter, and the CMV minimal promoter located directly after the tetO sequences) is not ideal. Mutual interference may result. Overexpression due to amplification by the tTA/tetO system, as seen in our case with the 5-HT_{1B} receptor, and also seen with in the Bond paper, may also result.

An important difference in the design described in our paper, from the one described in Bond et al., is the fact that we were able to create knockouts with the insertion of our neo-cassette. This property opened the door for a rescue approach. Specifically, both the 5-HT_{1B} and 5-HT_{1A} knockout mice could be reawakened, so to speak, by crossing with various Cre- or tTA-expressing mouse lines.

Since the all-in-one approach does not work in all cases, we suggest that it may be better to create a knockout by placing a neo-selection cassette, followed by a transcriptional/translational stop signal (Lakso et al. 1992) and the tetO recognition sequences, upstream of a gene of interest (see Fig. 7). This stop cassette is an improvement on our current cassette to minimize the possibility of any functional read-through being produced and to ensure that the gene has been silenced. This knockout can then be reawakened by crossing with tTA or Cre-expressing lines. Because of the versatility that is afforded by this technique, it should be considered when designing any knockout strategy.

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Conditional Mouse Models for Friedreich Ataxia, a Neurodegenerative Disorder Associating Cardiomyopathy

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Abstract Friedreich ataxia (FRDA), the most common recessive ataxia, is characterized by degeneration of the large sensory neurons and spinocerebellar tracts and cardiomyopathy. It is caused by severely reduced levels of frataxin, a mitochondrial protein involved in iron–sulfur cluster (ISC) biosynthesis. Mouse models have been important tools in dissecting the steps of pathogenesis in FRDA. Furthermore, animal models that reproduce some of the key events in a pathology are essential for the development of effective therapies, both pharmacological and gene therapy approaches. This chapter presents an overview of the current mouse models that have been developed for FRDA.

Keywords Ataxia · Cardiomyopathy · Mitochondria · Frataxin · Neurodegeneration · Iron–sulfur cluster · Iron

1 Introduction

Many major human neurodegenerative disorders are characterized by delayed onset and slow progression over years and decades. The heterogeneity in the neurodegenerative diseases, both at the clinical and genetic level, prevents a detailed dissection of the primary neuronal insults that lead to neurodegeneration, in addition to the obvious ethical consideration. Animal models, and particularly mouse models, have been important tools in dissecting the differ-

ent molecular steps of pathogenesis in a number of neurodegenerative diseases and have provided important insights into potential mechanisms leading to neuronal cell death. A great advantage of animal models is the potential of determining the early presymptomatic molecular events of a disease. A great advance in recent years in creating animal models has been the availability of new tools allowing for conditional inactivation of genes or conditional expression of mutant proteins implicated in neurodegenerative disease. Furthermore, animal models that reproduce some of the key events in a pathology are essential for the development of effective therapies, both pharmacological and gene therapy approaches.

Although there are several powerful models of neurodegeneration that have been reported over the past 10 years, this review will focus on the recent models that we have generated for the most common form of recessive ataxia, Friedreich ataxia, and the advantages of using conditional approaches to answer very specific questions.

2 Friedreich Ataxia: An Overview

Friedreich ataxia (FRDA) is the most common hereditary ataxia in Caucasians, with an estimated incidence of 1 in 30,000 (Cossee et al. 1997). This neurodegenerative disease is characterized by degeneration of the large sensory neurons and spinocerebellar tracts, cardiomyopathy, and an increased incidence in diabetes (Harding 1981; Pandolfo 1998). FRDA is caused by the partial loss of frataxin, a nuclear-encoded mitochondrial protein thought to be involved in Fe-S protein synthesis. The majority of patients are homozygous for a $(GAA)_n$ triplet expansion within the first intron of the gene, leading to inhibition of transcriptional elongation (Campuzano et al. 1996). A few patients (4%) are compound heterozygotes for the triplet expansion and for a point mutation in the frataxin gene. Frataxin, although highly conserved throughout evolution, has no homology with other proteins, thus preventing a prediction of its functional domain based on its sequence. However, it is its highly conserved nature that has help in defining its role in Fe-S biogenesis (Huynen et al. 2001).

Our understanding of the disease pathogenesis and frataxin function comes from evidence from different models (yeast, cell, and mouse) and from human samples accumulated over the years. Yeast deleted for the frataxin homolog gene, *YFH1* , harbor a petite phenotype suggesting a mitochondrial defect (Wilson and Roof 1997). Moreover, *YFH1* was identified as a high-copy-number suppressor of a yeast mutant deficient in intracellular iron usage, thereby linking the yeast frataxin homolog to iron metabolism (Babcock et al. 1997). The analysis of patient cardiac autopsies and biopsies revealed iron deposits as well as a selective deficit in the activity of a specific set of mitochondrial proteins bearing Fe-S cluster (complexes I, II, III of the respiratory chain and

aconitases) (Rotig et al. 1997). These Fe-S proteins are extremely sensitive to free radicals, and their deficit was initially thought to be a consequence of increased oxidative stress generated through Fenton reaction by the mitochondrial iron accumulation. However, the characterization of mice deficient in frataxin showed that the primary deficit in the disease is the Fe-S protein deficiency followed by the secondary mitochondrial iron accumulation (Puccio et al. 2001; Seznec et al. 2004), and that this mitochondrial iron accumulation does not seem to generate oxidative stress (Seznec et al. 2005). Most recent data coming from yeast models demonstrate a role of frataxin as an iron chaperone closely involved in ISC assembly/protection and heme biosynthesis. Indeed, reconstitutional studies, as well as in vivo studies using a yeast strain deleted for frataxin, demonstrate that frataxin is required, although not essential, for Fe-S cluster biosynthesis (Muhlenhoff et al. 2002). In a direct interaction of frataxin with ISU1 (yeast) /ISCU (mammals), the scaffolding protein involved in Fe-S cluster synthesis was shown, suggesting that frataxin might serve as an iron donor protein in the biosynthesis of Fe-S clusters (Gerber et al. 2003; Yoon and Cowan 2003). Mammalian frataxin has also been shown to interact in vitro with ferrochelatase, a Fe-S cluster-containing enzyme involved in the last step of heme synthesis (Yoon and Cowan 2004). Lastly, both YFH1 and mammalian frataxin have been shown in vivo to interact with the mitochondrial aconitase, a Fe-S cluster-containing enzyme of the Krebs cycle (Bulteau et al. 2004). Taken together, these data strongly suggest that frataxin is involved in delivering iron either for Fe-S cluster biogenesis or for heme synthesis as well as in protecting the Fe-S cluster of aconitase.

3
Mouse Models for Friedreich Ataxia

To study the mechanism of the disease and to test pharmacological therapy, several mouse models have been generated. Because FRDA is a recessive disease, animal modeling depends on the homologous recombination techniques that makes it possible to target a specific gene in embryonic stem (ES) cells to generate either the classical knockout, in which the gene is constitutively inactivated or a knockin model to insert a specific mutation (the GAA mutation in the case of FRDA) or to generate the conditional allele by inserting loxP sites.

3.1
Classical Knockout and Knockin Mice

Our group generated a classical mouse model by constitutive inactivation of frataxin by homologous recombination (Cossee et al. 2000). Homozygous deletion of frataxin causes embryonic lethality a few days after implantation, demonstrating an important role for frataxin during early development. These

results suggest that the milder phenotype in humans is due to residual frataxin expression associated with the expansion mutations. No iron accumulation was observed during embryonic resorption, suggesting that cell death might be due to a mechanism independent of iron accumulation. Dr. Pandolfo's group attempted to generate a mouse model by introducing a $(GAA)_{230}$ repeat within the mouse frataxin gene (knockin model) to mirror the chronically reduced levels of frataxin expression found in the human disease (Miranda et al. 2002). Bred with the Frda knockout, the authors obtained animals expressing 25%–30% of wild-type frataxin levels, an expression level associated with mildly affected FRDA patients. Unfortunately, these mice did not develop abnormalities of motor coordination, cardiomyopathy, iron metabolism, or response to iron loading up to the age of 1 year old. Thus 25%–30% of wild-type frataxin levels seem to be compatible with normal neurological function and iron metabolism in mice. Furthermore, the GAA repeat is meiotically and mitotically stable in the mouse strain they investigated, precluding the generation of mice with larger expansion over different generations.

3.2 Conditional Knockout Mouse Models for Friedreich Ataxia

To circumvent embryonic lethality, our group generated in parallel two different conditional knock-out models, based on the Cre-lox system, in which frataxin was deleted either specifically in skeletal and cardiac muscle (using a transgenic mice expressing the recombinase Cre under the muscle creatine kinase promoter) or a more generalized frataxin-deficient line including neuronal tissues (neuron-specific enolase promoter) (Puccio et al. 2001). Both models are viable and reproduce some morphological and biochemical features observed in FRDA patients, including cardiac hypertrophy without skeletal muscle involvement in the heart and a striated muscle frataxin-deficient line, large sensory neuron dysfunction without alteration of the small sensory and motor neurons in the more generalized frataxin-deficient line, and deficient activities of complexes I–III of the respiratory chain and of the aconitases in both lines. These animals provide an important resource for pathophysiologic studies and testing of new treatments.

The murine FRDA cardiomyopathy is characterized by an early onset of dilatation with development of left ventricular hypertrophy followed by reduced systolic function. A detailed time course experiment in the cardiac model revealed that the Fe-S enzyme deficiencies begin in the initial phase of the pathology, at the onset of the cardiac dysfunction, while the intramitochondrial iron accumulation occurs at the end stage of the disease (Fig. 1). Moreover, the NSE mutant animals do not present any iron deposit, but do have a deficit of Fe-S enzymes. Both models therefore indicate that the Fe-S deficiency and cardiomyopathy are independent of mitochondrial iron accumulation. These results support the necessary role of frataxin for efficient Fe-S cluster synthesis,

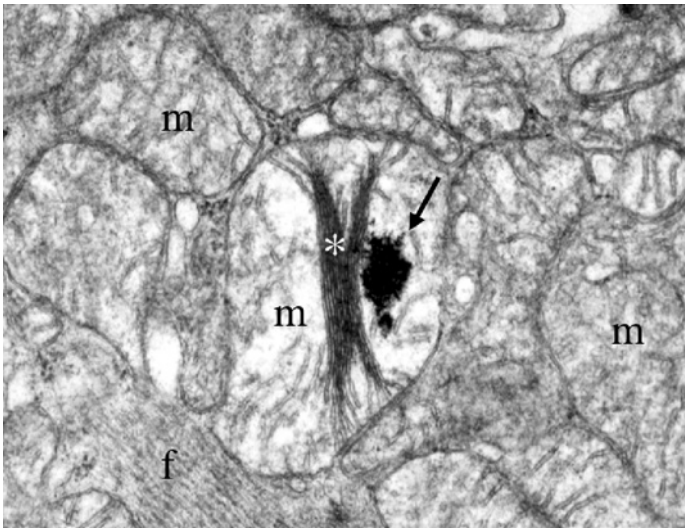

Fig. 1 Mitochondrial iron accumulation in the cardiac tissue of an 8-week-old MCK cardiac mutant animal as seen by electron microscopy. Note the proliferation of mitochondria (*m*), iron accumulation (*arrow*), abnormal central crisae within mitochondria (*asterisk*), and myocardiac fibers (*f*)

although nonessential, since, despite absence of detectable frataxin at birth, there is still 50% Fe-S enzymes activity at 4 weeks of age in the cardiac model (Seznec et al. 2004). Therefore, in agreement with recently published results in the yeast model, in the absence of frataxin, Fe-S cluster biosynthesis would occur at a very reduced rate.

Several reports suggest that continuous oxidative damage resulting from hampered superoxide dismutase signaling participates in the mitochondrial deficiency and ultimately neuronal and cardiac cell death. Indeed, the SOD activity is abnormally low in the diseased mouse (Chantrel-Groussard et al. 2001); however SOD mimetics, contrary to idebenone (see below), have no effect on the survival of the animals and no detectable oxidative stress can be measured (Seznec et al. 2005). These results suggest that free radical production in the murine FRDA model is a minor component in the pathophysiology. Although these results appear at first to be in contradiction with the general agreement, evidence of oxidative damage in FRDA patients is contradictory. These mutant mice therefore are the first mammalian models to evaluate treatment strategies for the human disease.

As previously mentioned, these animal models have been used for therapeutic trials. idebenone is a short-chain synthetic analog of coenzyme Q10 that can function as an electron carrier in the mitochondrial respiratory chain and acts as a potent free-radical scavenger (Gillis et al. 1994; Mordente et al. 1998). During the last 3–5 years, many therapeutic trials have assessed the

clinical value of idebenone, but the results remain controversial, mainly due to the clinical heterogeneity of the disease, and the lack of randomized placebo-controlled studies (Rustin 2003). In view of the methodological difficulties in evaluating the effects of idebenone in patients, the mouse models have become a powerful tool. We found that idebenone effectively delayed the progressive cardiac hypertrophy and dilatation, and preserved ventricular contractility by 1 week, thus increasing life span of the animal by 10% (Seznec et al. 2004). However, in contrast with the recent observation made on one FRDA patient's heart biopsy, idebenone did not restore the Fe-S enzyme activity. The results from the placebo-controlled double-blind trial therefore strengthen the results obtained on the patient trials, as most of them were open trials without placebo controls, and support the use of idebenone for the human disease.

The neuronal-specific mouse model that we have generated developed a movement disorder characterized by gait abnormalities and loss of proprioception (Puccio et al. 2001). Furthermore, electrophysiological studies revealed a specific large sensory nerve conduction defect with normal motor nerve conduction. Although these features mimic the neurological symptoms in FRDA patients, our mouse model is extremely severe, with a life expectancy of 24 days. The severity of this model makes any therapeutical approaches very difficult. Furthermore, this model shows lesions not seen in the human disease (liver and spongiform cortical lesions), preventing cell-specific degeneration mechanisms studies.

A. Pfeiffer's group generated a conditional mouse model with frataxin deficiency selectively in pancreatic β cells (Cre under the rat insulin promoter 2) to better understand the link between frataxin deficiency and type 2 diabetes mellitus (Ristow et al. 2003). These mice show a slowly progressive reduction of islet mass and number causing an impaired insulin-secretory response to glucose and carbohydrates and accumulating in overt diabetes. Furthermore, the authors observed that this impaired stimulus does not result from an unaltered insulin secretion from isolated islets, suggesting that stimulus-secretion coupling is intact in the frataxin-deficient β cells consistent with the findings in FRDA patients. The exact pathway leading to diabetes in this model is still under investigation, but the frataxin deficient β cells clearly exhibit increased apoptosis and a reduction in proliferation rate.

3.3 Temporally Controlled Neurological FRDA Mouse Models

To obtain specific and progressive neurological models for FRDA, we generated inducible knockout mouse models using two transgenic lines (28.4 and 28.6, having distinct neuronal specificities) expressing the tamoxifen-dependent recombinase (Cre-ER^T) under the mouse Prion protein (Prp) promoter, thus enabling us to spatiotemporally control somatic mutagenesis of conditional alleles of the targeted genes. Both Prp-Cre-ER^T lines express the Cre-ER^T

recombinase in the nervous system, but although the 28.4 line has a wide expression pattern, the Cre-ER^T expression of the 28.6 line is mostly restricted to the hippocampus, the cerebellum, and the dorsal root ganglia (Weber et al. 2001). Both lines developed the most prominent features of the human disease: a slowly progressive mixed cerebellar and sensory ataxia associated with a progressive loss of proprioception and absence of motor involvement (Simon et al. 2004). These mouse models also parallel the human disease at the histopathological level. The models have degeneration of the posterior columns of the spinal cord that appear translucent, because of demyelination and loss of fibers, and severe lesions of neurons in Clarke's columns, hallmarks of FRDA disease. In addition, one of the mutant lines has specific damage to the large sensory neuron cell bodies in the DRG, another distinctive feature of FRDA. The time of occurrence of these lesions suggests that as in patients, the anomalies observed in the neuronal cell bodies of the DRG are a primary event, whereas the neuronal loss in Clarke's column and the degeneration in the posterior column might be secondary events.

The progressive neurodegeneration of the DRG is an excellent model for unraveling the pathological cascade leading to neuronal death in FRDA. Several cell death pathways can be activated during neurodegeneration, including apoptosis and the more recently accepted mechanism involving autophagy (Xue et al. 1999). Although oxidative insult to cultured cells from FRDA patients results mostly in apoptotic for FRDA (Wong et al. 1999; Santos et al. 2001), no evidence for such a cell death mechanism in vivo has been provided thus far. In particular, apoptosis processes could not be detected in the complete frataxin knockout (Cossee et al. 2000) or in the conditional mouse models (Puccio et al. 2001). Surprisingly, we have observed that the degenerative mechanism involved in the dorsal root ganglia neurons is an autophagic process, leading to removal and degradation of damaged cytosolic proteins and organelles (Fig. 2). Autophagy is characterized by the presence of autophagic vacuoles and autophagosomes that are formed by rearrangement of subcellular membranes (rough endoplasmic reticulum or the trans-Golgi system) to sequester cytosolic constituents and organelles and traffic them to lysosomes for degradation (Reggiori and Klionsky 2002). Different steps of the autophagic process were clearly seen in the large myelinated DRG neurons of the Cb mutants. In addition to autophagosomes, lipofuscin accumulation was also observed in these neurons. Lipofuscin is composed of proteins, lipids, carbohydrates, and metals, particularly iron (Brunk and Terman 2002) derived from mitochondria and metalloproteins having incomplete autophagocytosis and lysosomal degradation, thereby leading to their accumulation. As lipofuscin accumulation has been reported both in the DRG and cardiomyocytes of FRDA patients (Lamarche et al. 1980, 1982; Larnaout et al. 1997), this cellular response is certainly a close consequence of frataxin deficiency. Moreover, both processes appear to be cell autonomous and progressive, as the ganglion cell neurons that show autophagosomes and/or lipofuscin accumulation are

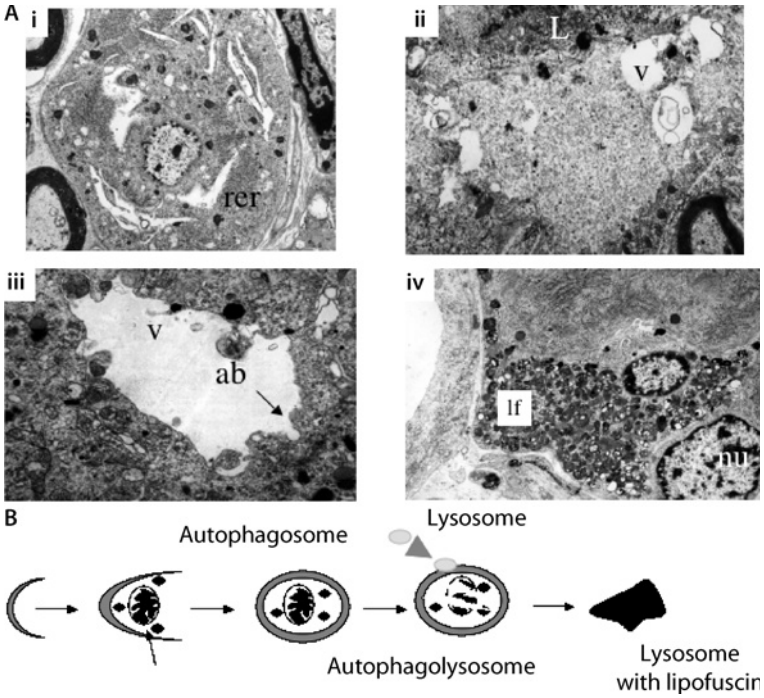

Fig. 2 A,B Autophagic features and lipofuscin accumulation in the dorsal root ganglia of the inducible neurological model. **A** Electron microscopy on CB mutant DRG showing various profiles of autophagy. *i*: A marked dilatation of the RER generates vacuoles containing remnants of ergastoplasm and lysosomes. *ii*: Electron-lucent cytoplasmic area with fibrillary material, delimited by a membrane-like structure. Focal cytoplasmic breakdowns occur, and several autophagic vacuoles develop at the periphery sequestering portions of cytoplasm that contain lipopigments, glycogen, and concentric bodies with membrane remnants. Lysosomes are present at the periphery. *iii*: a large vacuole of autophagy shows membrane activity and pinocytosis (*arrow*) and contains projections of cytoplasmic matrix and ribosomes that are either attached to the inner membrane or freely floating. Autophagic bodies are seen. *iv*: Peripheral massive lipofuscin accumulation, made of numerous dense granules with various shapes.*Ab,* autophagic bodies; *L,* lysosomes; *lf,* lipofuscing; *rer,* rough endoplasmic reticulum; *v,* vacuole; *nu,* nucleus. **B** Schematic diagram of the different steps of autophagy

deleted for frataxin. Furthermore, the concurrent activation of both processes could explain the lack of detectable iron deposits within the DRG.

Finally, both the inducible neurological mouse models (Simon et al. 2004) and the previous FRDA mouse models that we have generated (Puccio et al. 2001) clearly show accumulation of damaged mitochondria as a direct consequence of frataxin deficiency. Interestingly, the cardiomyocytes in the cardiac model appear to compensate for lack of frataxin by mitochondrial proliferation and cellular hypertrophy without entering an autophagic process. In contrast,

DRG neurons appear to survive without frataxin for a longer period by intracellular removal of damaged organelles and proteins through the autophagic and lysosomal pathways. Therefore, the slowly progressive phenotype of the inducible neurological models would reflect the progressive nature of the autophagic process, proposed as a protective mechanism for the elimination of defective mitochondria with dysfunctional inner membranes (Lemasters et al. 1998).

These models are excellent tools to unravel the pathological cascade of FRDA and to test compounds that interfere with the degenerative process, such as antioxidants, which are good pharmacological candidates. Furthermore, these models should prove useful for the investigation of neurodegenerative mechanisms characterized by delayed onset and slow progression over years or decades. The spatiotemporally controlled conditional gene-targeting approach that we have used is particularly adapted to study the mechanisms of late onset and slowly progressive neurodegeneration and is amenable to large experimental flexibility through modulation at will of the timing of induction.

4 Conclusion

In conclusion, murine models have provided valuable insights into the pathological cascades that are associated with the Friedreich ataxia disease, with a particular emphasis on early changes, disease states that are not or rarely accessible from human patients. Particularly, conditional animal models based on homologous recombination have been crucial in developing neurological, diabetic, and cardiac models for Friedreich ataxia as the classical knockout was embryonic lethal. An important challenge will be to discern which of these changes directly affect disease development and how different pathways interact.

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Animal Models in Cardiovascular Diseases: New Insights from Conditional Models

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Abstract Conditional systems have proven to be efficient and powerful to delineate several aspects of cardiac pathophysiology and diseases. The possibility of addressing a particular time point in animal life is certainly an important breakthrough allowed by conditional strategies with temporal control of either transgene expression or gene modifications. The purpose of this review is to present various mouse models for cardiovascular diseases based on conditional approaches.

Defining the role of important actors of cardiovascular pathophysiology in mouse models should help to analyze their potential role in human diseases, more specifically in blood pressure control, heart failure, arrhythmia, and vascular diseases. These animals can be used as living models of human diseases or

for testing therapeutic approaches using new drugs or gene therapy. Moreover, mouse models are useful to either support or suggest clinical investigations in humans. The purpose of this review is to present various mouse models for cardiovascular diseases based on conditional approaches. Comprehensive and complementary reviews have been published recently elsewhere (Heine et al. 2005, Berger 2005; Robbins 2004). Due to space limitations, the aim of the present review is not to be exhaustive but rather to underline benefits and drawbacks of the various possible strategies dedicated to conditional, inducible gene expression or modification, especially in case of models for cardiovascular diseases.

1 Introduction

In vivo analysis of gene function is often based on the production of animal models with specific genetic modifications. These approaches have been successfully applied to several domains in the cardiovascular field and provided numerous and helpful insights in cardiovascular pathophysiology. Such modifications can result in a gain or loss of function. Two strategies are used to assess these goals. First is additive transgenesis, consisting in the introduction into the genome of a transgene composed of a fusion gene between a promoter sequence and a cDNA of interest. The promoter confers widespread or tissue-specific expression of the cDNA. The cDNA of interest can encode for a wild-type protein or a mutated protein acting as a negative dominant protein or mimicking gene mutations observed in human diseases. The second approach is gene targeting. It consists in the introduction of specific mutations into a gene of interest by homologous recombination using the embryonic stem (ES) cell technology. In most cases, the mutation results in the generation of a null allele (so-called knockout, KO), allowing gene inactivation after making the animals homozygotes. Truncated or mutated proteins can also be produced using gene targeting. Finally, using a similar technique (so-called knock-in, KI), expression of a protein of interest can be placed under the control of the endogenous regulatory sequences of a different gene.

In these cases, the expression of the wild-type or mutated protein, as well as the site-directed gene modification, is constitutive. The gene mutation is present in the first cell composing the embryo and thereafter in all cells of the adult animal. Spatiotemporal restriction of the expression of the protein of interest is linked to the properties of the promoter used to make the fusion gene (in the case of additive transgenesis) or by the expression pattern of the gene that has been mutated (in the case of KI). Therefore, the protein is expressed as early as the promoter used in the transgenic construct is turned on and its expression pattern follows the full expression pattern of the promoter used. In order to dissect the function of a gene product more precisely, it may

be deleterious that the transgene or the mutation is expressed early during development or in several organs rather than one of interest. For example, toxic effects or lethality that are related to early expression of the transgene during embryonic development may impair the analysis of the phenotype. Moreover, as often observed in KO experiments, redundancy between related gene products can severely reduce the usefulness of such animal models.

To overcome these limitations, several strategies have been developed in order to predict and to control where and when the transgene or the gene mutation will be expressed. Among these approaches, several have proven to be functional in vivo in transgenic animals, particularly the tetracycline inducible system and the Cre-lox recombinase, either constitutive or inducible (Tables 1–4). As detailed elsewhere in this book, conditional systems are mostly based on a binary system. It is necessary to generate two sets of transgenic animals. One mouse line expresses the activator (tet transactivator, Cre recombinase, Glp65, etc.) under the control of a selected tissue-specific promoter. Another set of genetically modified animals express the acceptor construct, in which the expression of the transgene of interest (or the gene to be modified) is under the control of the target sequence for the tet/Gpl65 transactivators (or is flanked with LoxP sequences), for example. Mating the two strains of mice allows the spatiotemporal control of transgene expression or of the desired gene alteration.

2
The Tetracycline Inducible System

2.1
General Strategy

The tetracycline-dependent regulatory systems (tet systems) developed in Herman Bujard's laboratory permit a stringent control of gene expression over a wide range of cells in culture as well as in transgenic animals (Bockamp et al. 2002; Schonig and Bujard 2003; see also the chapter by R. Sprengel and M.T. Hasan, this volume). The tet system relies on two components: (1) a tetracycline-responsive transactivator (tTA or rtTA) and (2) a tTA/rtTA-dependent promoter, so-called tetO-minimal promoter, that controls expression of a downstream cDNA in a tetracycline-dependent manner. The tet system using tTA is called tet-OFF, since tetracycline or doxycycline allows transcriptional down-regulation (Furth et al. 1994). Since tetracycline or derivatives are not usually present in living animals, exogenous administration of tetracycline or derivatives allows temporal control of transgene expression in vivo. A mutant form of tTA, called rtTA (reverse tTA), has been isolated using random mutagenesis. In contrast to tTA, rtTA is not functional in absence of doxycycline, but needs the presence of the ligand to allow transactivation (Kistner et al. 1996). This tet system is therefore called tet-ON.

The two systems work as a mirror image and are functionally equivalent when transferred into mice. It should be noted that the original tet-ON system needs higher doxycycline concentrations to be active, as compared to tet-OFF to be inactive (Kistner et al. 1996). This may be of importance when used in vivo since bioavailability of doxycycline may differ between tissues. Recently, a second-generation tetON system (based on a mutated rtTA transactivator called rtTA S2-M2) displayed lower background activity and higher Dox sensitivity. One advantage of tet-ON over *tet*-OFF is that expression of the transgene is not present once doxycycline is present and that up-regulation in vivo is faster than down-regulation. A hallmark of the tet systems is the tightness of control, the possibility of regulating gene activity in a tissue-specific manner, the doxycycline dose-dependent response, as well as the possibility to come back to a control situation by stopping transgene expression when desired. However, the major difficulty using the tet system is that the control of the expression of the acceptor construct is sometimes leaky, due to strong positional effects on the tetO minimal promoter. This requires the generation of several acceptor mouse strains in order to find those that do not express the transgene constitutively but in an inducible manner. Several reports have been published recently that described improvement of the tet system (transactivators with less toxicity or different ligand sensitivity, bi-directional tetO minimal promoters, reduced leakiness of the tet system) (Baron et al. 1995; Urlinger et al. 2000).

2.2 Designing Cardiac-Specific Tet Transactivator Mice

Conditional expression has been achieved in cardiomyocytes using specifically designed transactivator mice (Table 1).

Fishman and co-workers were the first to adapt the tet system to the cardiovascular field. They first generated a cardiac-specific transactivator mouse line in which expression of the original tTA transactivator was controlled by 645 bp of the regulatory elements of the rat αMHC gene (Fishman et al. 1994). This allowed tetracycline-dependent expression of Id1, a gene involved in the control of differentiation in the heart only (Passman and Fishman 1994). In order to improve the system, Fishman and co-workers generated a new transactivator strain using 2.9 kB of the rat αMHC regulatory sequences, leading to efficient and reliable expression of luciferase (Yu et al. 1996). Heterogeneous transgene expression in the heart was reported with cell to cell variation of LacZ reporter gene expression (Yu et al. 1996). This may indeed relate to the LacZ responsive strain used here since we and others (Dor et al. 2001; Beggah et al. 2002; Mungrue et al. 2002; Barandon et al. 2005) have not observed this heterogeneity in transgene expression using this particular MHCtetOFF strain. Expression was tightly controlled by tetracycline and

Table 1 Conditional models with tet-dependent cardiac expression

Transgenic line	Promoter	Trans-activator	Inducer	Target locus	Over-expressed gene	Phenotype	Reference
αMHC-tetOFF	645 bp rat αMHC	tTA	Tetracycline PO 2.5 mg/ml	tetO-Id1	Id1		Passman and Fishman 1994
Rat αMHC-tetOFF	2.9 kb rat αMHC	tTA	Tetracycline PO 1 mg/ml Doxycycline PO 0.2 mg/ml	tetO-Luc tetO-β-gal	Luciferase β-gal	Reporter gene expression	Yu et al. 1996
			Doxycycline PO 0.2 mg/ml	TetO-Ro1 + tetO-β-gal	Ro1 β-gal	Decreased heart rate Lethal arrhythmia	Redfern et al. 1999; Redfern et al. 2000
			Tetracycline PO 1 mg/ml	tetO-PKCβ*II*	Constitutively active PKCβ*II*	Cardiac hypertrophy ICaL remodeling	Alden et al. 2002
			Tetracycline PO 100 ug/ml Pellets	tetO-VEGF	VEGF	Congenital heart defect Adult neovascularization	Dor et al. 2001; Dor et al. 2002
			Doxycycline PO 0.2 mg/ml	tetO-ACVI	AC VI	Increased systolic LV function	Gao et al. 2002
			id	tetO-myrAKT	Constitutively active Akt1	Modulation of AMP-activated protein kinase	Kovacic et al. 2003

Table 1 (continued)

Transgenic line	Promoter	Trans-activator	Inducer	Target locus	Over-expressed gene	Phenotype	Reference
			id	tetO-HDAC5	HDAC5	Sudden cardiac death Cardiac mitochondria defects	Czubryt et al. 2003
			id	Bidirectional β-gal-tetO-iNOS	iNOS β-gal	Cardiomyopathy, sudden cardiac death, arrhythmia	Mungrue et al. 2002
			id	Bidirectional	mMR anti-sense mRNA	Dilated	Beggah et al. 2002
				β-gal-tetO-MR antisense	β-gal	cardiomyopathy Interstitial fibrosis	
			id	Bidirectional tetO-hMR	hMR	Ventricular arrhythmia	Ouvrard-Pascaud et al. 2005
			id	Bidirectional β-gal-tetO- ET1	Endothelin 1 β-gal	Inflammation and dilated cardiomyopathy	Yang et al. 2004
			id	Bidirectional β-gal-tetO- FrzA	FrzA β-gal	Altered ischemic preconditioning	Barandon et al. 2005
mouse αMHC-tetOFF	5.5 kb mouse αMHC	tTA	Doxycycline PO 0.2 mg/ml	tetO- β-gal MHCmintetO-ELC1a MHCmintetO-GSK3βCA	β-gal ELC1a Constitutively activated GSK3β	Reporter gene expression ECL1 isoform replacement	Sanbe et al. 2003

Table 1 (continued)

Transgenic line	Promoter	Trans-activator	Inducer	Target locus	Over-expressed gene	Phenotype	Reference
						Attenuation of pressure overload hypertrophy	
			id	MHCmintetO-Nix	Nix	Cardiopathy in neonates but not in adults	Syed et al. 2004
Mouse αMHC-tetON	5.5 kb mouse αMHC	Opt-rtTA	Doxycycline	Bidirectional Luc-tetO-α5 integrin tetO-TACβ1D	Luc α5 integrin TACβ1D	Reporter gene expression α5 Integrin Truncated α1D integrin	Valencik and McDonald 2001
			PO 0.2 mg/ml	tetO-SERCA2a	SERCA 2a	Improved Ca handling Reversal of cardiac hypertrophy	Suarez et al. 2004
Mouse αMHC-tetON	5.5 kb mouse αMHC		Doxycycline PO 2 mg/ml	tetO-dnNFAT (Co-integrated with the transactivator)	Dominant negative NFAT	Atrial morphological alteration	Schubert et al. 2003

tTA, tetracycline transactivator; rtTA, reverse tetracycline transactivator; tetO, tet responsive promoter; αMHC, α-myosin heavy chain; β-gal, β-galactosidase gene; Luc, luciferase; Id1, helix-loop-helix protein-negative regulator of differentiation during skeletal myogenesis; Ro-1, RASSL opioid modified human k opioid receptor; PKCβII, constitutively active protein kinase C βII isoform; VEGF, vascular endothelial growth factor; ACVI, adenylcyclase type VI; myrAKT, consitutively active Akt1; HDAC5, class II histone deacetylase 5; iNOS, inducible nitric oxyde synthase; mMR, mouse mineralocorticoid receptor; hMR, human mineralocorticoid receptor; ET1, endothelin 1; MHCmintetO, tetracycline-responsive myosin heavy chain minimal promoter; ECL1, essential myosin light chain; GSK-3βCA ,consitutively activated glycogen synthase kinase-3β; Nix, hypertrophy-regulated mitochondrial death protein; TACβ1D, Truncated α1D integrin; SERCA 2a, sarco(endo)plasmic reticulum Ca2+-ATPase 2a; dnNFAT, dominant-negative nuclear factor of activated T cells

10 days was enough to completely abolish transgene expression (Yu et al. 1996). Using this MHCtetOFF strain, Dor et al. reported transgene expression as early as E9.5, which could be prevented by doxycycline given to the pregnant mother (Dor et al. 2001). A novel MHCtetOFF strain (called line 55) has been developed by J. Robbins's group using a 5.5-kb mouse cassette (including untranslated 1–3 exons) and the first-generation tTA (Sanbe et al. 2003). Despite very low expression of the tet transactivator protein, this line appears quite efficient for conditional transgene expression in the heart (Sanbe et al. 2003). McDonald and co-workers reported on a MHCtetON transactivator strain with cardiac-specific expression of the first-generation codon-optimized tetON transactivator under the control of the 5.5-kb αMHC mouse promoter (Valencik and McDonald 2001). In order to improve the level of expression of the transgene of interest, which could be critical to affect abundant cardiomyocyte proteins such as structural proteins or to express a dominant negative mutant for example), a chimeric, cardiac-specific responder construct was designed by J. Robbins and co-workers (Sanbe et al. 2003). First, the mouse 5.5-kb αMHC promoter was attenuated by removing GATA sites and thyroid response elements. The tetO responsive moiety was then introduced, allowing both strong cardiac-specific and tet-dependent expression using this so-called MHCmin tetO conditional promoter. Efficiency was demonstrated by conditional overexpression of the atrial isoform of the highly abundant essential myosin light chain protein, leading to 42% replacement of the endogenous ventricular isoform by the atrial isoform (Sanbe et al. 2003).

Concerns have been raised about the influence of cardiac transactivator expression on subsequent molecular and functional phenotypes. Indeed, cardiac expression of the S2M2 tetON transactivator under the control of the 5.5-kb mouse promoter lead to lethal cardiomyopathy within 2 months (Sanbe et al. 2003). This may rely on the high level of rtTA S2-M2 expression and possible toxic effect related to the VP16 moiety, despite the use of attenuated VP16 activation domain. This was not the case for the MHCtetOFF and the MHCtetON lines developed with the first-generation transactivators (Yu et al. 1996; Valencik and McDonald 2001; Sanbe et al. 2003). Careful and extensive analysis of the MHCtetOFF line generated by G.I. Fishman (Yu et al. 1996) indicated that subtle but significant phenotypic changes could be observed, suggesting mild cardiomyopathy associated with an increased in vitro submaximal cardiac contraction and myofilament Ca^{2+} sensitivity (McCloskey et al. 2005). This was accompanied by molecular changes, as assessed by microarray analysis. Of note, doxycycline unexpectedly does not affect these findings, suggesting possible positional effects of the MHCtTA construct. Such alterations have not been reported to date (while less extensively analyzed) in the various models already published (see below), even though adequate controls have usually been done. This may be the result of the various genetic backgrounds used in these studies or to other unknown confounding effects. Nevertheless, this work suggests caution in the use of appropriate

controls and the need to compare the conditional model with littermates of various genotypes, especially with the MHCtetOFF monotransgenic ones or with nonexpressing (Dox-treated) bigenic mice to avoid data misinterpretation.

2.3 New Insights into Cardiac Pathophysiology Using the Tet System

2.3.1 Signaling Pathways

To control G protein signaling in vivo, a modified Gi-coupled κ opioid receptor activated by a synthetic ligand was conditionally expressed in cardiomyocytes. This resulted in a ventricular conduction delay and lethal cardiomyopathy. This could be induced and reversed in adult mice, turning transgene expression on or off, providing a nice opportunity to dissect the role of Gi signaling in causing cardiac pathology (Redfern et al. 1999, 2000). The role of PKCβ was analyzed using conditional expression of a constitutively active PKCβ mutant (Alden et al. 2002). Expression in adults caused mild and progressive ventricular hypertrophy with impaired diastolic relaxation. Expression in newborns resulted in sudden deaths with marked abnormalities in the regulation of intracellular calcium. Molecular and electrophysiological analyses done on cardiomyocytes isolated from newborn transgenic mice indicated that this relies on an increased activity of L-type Ca channels, highlighting the specific role of this PKC isoform in cardiomyocyte Ca homeostasis (Alden et al. 2002).

Conditional transgenesis not only allowed overexpression of a protein of interest but also was used to decrease gene function through targeted expression of dominant negative mutants. The role of NFAT/calcineurin pathway in heart development was examined in a transgenic strain with co-integration of constructs for MHCtetON and a dominant negative mutant of NFAT (dnNFAT) (Schubert et al. 2003). To our knowledge, this is the sole example of co-integration of both activator and responsive constructs in the cardiovascular field. Embryonic expression of dnNFAT resulted in thin atrial myocardium with severe sarcomere disorganization and reduced cardiac troponin l and T expression, indicating that, among other possible functions, NFAT plays a critical role in the structural architecture of the developing myocardium (Schubert et al. 2003). These data extended those obtained with the constitutive KO of NFAT (impaired valve and septum formation, leading to embryonic lethality) and demonstrated temporal requirement of NFAT, a finding that could be obtained with the use of this tet conditional system since constitutive expression of dnNFAT would prevent the generation of the transgenic line and therefore prevent this type of study. The implication of NFAT in adult was not examined here but this conditional model might be

powerful to further delineate the role of NFAT/calcineurin in cardiac diseases.

The involvement of FrzA/sFRP-1 and the Wnt/frizzled pathway in ischemic preconditioning was elegantly addressed recently using a mouse model allowing conditional, cardiac-specific expression of FrzA, a secreted Frizzled-related protein that antagonizes the Wnt/Frizzled pathway (Barandon et al. 2005). FrzA blocks the canonical Wnt pathway by interfering with GSK3β phosphorylation, therefore preventing nuclear β-catenin translocation. Barandon et al. first set up a mouse model of ischemia-reperfusion (IR) with preconditioning (PC) (Barandon et al. 2005). Modulation of FrzA expression during the IR or IR+PC clearly indicated that FrzA overexpression prevented the beneficial effect of PC on areas at risk and cardiac function after IR. Dissection of the AKT-GSKβ signaling pathway indicated that FrzA blocked the PC-induced GSK-3β phosphorylation independently of AKT and JNK pathways (Barandon et al. 2005). These data demonstrated that disruption of the Wnt/frizzled pathway induces activation of GSK-3β and reverses the benefit of preconditioning and suggested a key role of GSK-3β in cardioprotection. The protective role of GSK-3β in cardiac hypertrophy after TAC-induced pressure overload was directly addressed by conditional overexpression of a constitutively active GSK-3β (GSK3-CA) using the MHCmintetO promoter described above (Sanbe et al. 2003). Specificity was confirmed by the manipulation of GSK3-CA expression with Dox during the TAC protocol. GSK3-CA blunted cardiac hypertrophy when expressed before TAC and strongly attenuated hypertrophy when expressed on time with TAC. These two examples highlighted the benefit of the conditional tet system, which allows both delayed expression and/or reversion protocols at the time of pathological challenges.

Conditional increase of inducible nitric oxide synthase (iNOS) expression in cardiomyocytes has been achieved by Mungrue et al. using the MHCtetOFF system and a responder strain allowing tet-dependent iNOS expression (Mungrue et al. 2002). This resulted in increased peroxynitrite generation, heart block, and sudden death. Death was related to bradyarrhythmia caused by severe atrioventricular conduction defects including second- and third-degree AV blocks. These data suggested that increased iNOS observed in various inflammatory-associated cardiac diseases may sensitize the heart to fatal events, synergistically to other deleterious molecular alterations. Interestingly enough, a model with constitutive iNOS expression did not reach the same conclusions (Heger et al. 2002). However, as discussed by Mungrue et al. (2003), it may rather be that constitutive iNOS expression resulted in counter selection mechanisms allowing only low-expressing founders to survive because of the embryonic lethality reported in the conditional model if iNOS expression was allowed during embryogenesis. This underlines the benefit of conditional over constitutive transgene expression, especially in case of embryonic toxicity.

2.3.2 Manipulating cAMP and Ca Homeostasis in the Heart

Type VI adenylate cyclase (ACVI) has been proposed as a potential target for heart failure. Indeed cardiac-specific, but constitutive, expression of ACVI was able to enhance β adrenergic receptor (βAR) responsiveness and cardiac function, abrogate myocardial hypertrophy, and improve survival in a transgenic model of heart failure caused by cardiac Gq overexpression (Tang et al. 2004). In order to test the potential of ACVI expression in animals with fully developed heart disease (reversion protocol), Gao and co-workers developed a conditional, cardiac-specific and Dox-dependent ACVI overexpression model, allowing manipulation of ACVI expression either before or after onset of the pathology (Gao et al. 2002). By taking advantage of the tight regulation provided by the tet-OFF system, the authors showed increased βAR responsiveness and left ventricular contractile function, suggesting that ACVI could be tested as a candidate therapeutic target in heart disease models (Most et al. 2002).

The sarco(endo)plasmic reticulum Ca^{2+-}ATPase (SERCA2a) which pumps cytoplasmic Ca^{2+-} into the lumen of the sarcoplasmic reticulum (SR), is a key player in E-C coupling and contractile dysfunction in heart failure. A decrease in SERCA2a activity is considered a hallmark of cardiac failure, both in humans (dilated cardiomyopathy) and animal models (ischemia or pressure overload) (Periasamy and Huke 2001). Increasing SERCA activity may therefore be a powerful therapeutic approach to increase cardiac performance in such diseases. Indeed, constitutive, cardiac-specific SERCA2a overexpression alleviated contractile dysfunction in animal models of heart failure (Ito et al. 2001). However, this does not indicate whether or how increasing SERCA2a activity would be beneficial as a therapeutic scheme. W.H. Dillman's group designed a conditional model allowing cardiac-specific and inducible expression of SERCA2a (Suarez et al. 2004) using the MHC-tetON strain designed by Valencik and colleagues (Valencik and McDonald 2001). Ca transients showed an accelerated decline and a higher cytosolic peak in cardiomyocytes from DT mice, associated with a 45% increase in SR load. This was associated with an increased contractile function, as determined by echocardiography, at the basal state. Increasing SERCA2a expression after aortic constriction (AC) for 7 days only clearly rescued the diminished cardiac function, which returned to the nonbanded control values (Gao et al. 2002).

2.3.3 Endocrine Control of Cardiac Function

The vascular endothelial growth factor (VEGF) is involved in angiogenic growth and its expression is tightly controlled by hypoxic insults. VEGF is required for proper heart morphogenesis and its role in cardiac development is exquisitely dependent on normal levels and appropriately timed expression.

To model in vivo hypoxia-dependent induction of VEGF and to address the consequences on heart development, a conditional model was designed to achieve in vivo spatiotemporal control of VEGF expression (Dor et al. 2001). A 4.5-fold increase in VEGF embryonic expression was noted at E9.5, a level similar to cardiac hypoxia-induced expression. This led to the disorganization of endocardial cushion development, possibly linking gestational hypoxia to congenital heart defects through VEGF abnormal expression, resulting in embryonic lethality at E11.5 (Dor et al. 2001). Interestingly enough, switching VEGF expression at different stages unmasked VEGF-mediated abnormal phenotypes later on, with the formation of endothelial cell clusters at E12.5. Of course, this kind of study would not be possible without such a precise and powerful conditional system due to the lethal phenotype associated with early embryonic VEGF expression. This model also made it possible to study the role of cardiac VEGF in adulthood, providing new insights into adult neovascularization and possible impact on pro-angiogenic therapy in the heart (Dor et al. 2002).

Neurohormonal control of heart function plays a key role in cardiac pathophysiology. The role of both locally synthetized and systemic hormones have been described in patients and in experimental models of congestive heart failure (CHF). Among these, endothelin and aldosterone gain increasing attention. The role of endothelin (ET1) in the pathogenesis of CHF remains controversial. In order to delineate cardiac-specific consequences of ET1 and avoid deleterious embryonic effects, conditional ET1 overexpression in the heart was achieved using the tet system, breeding a bidirectional tetO ET1/LacZ strain and the MHCtetOFF transactivator strain (Yang et al. 2004). This resulted in a tenfold, inducible and cardiac-specific increase of ET1 levels. Embryonic expression was noted at E10.5 and resulted in embryonic lethality since only one-quarter of the expected tTA/ET1 offspring was obtained. Prevention of ET1 expression during development with Dox allowed the analysis of adult DT animals (Yang et al. 2004). Cardiac expression of ET1 was sufficient to cause increased expression of cytokines and an inflammatory cardiomyopathy, leading to heart failure and death. This was partially prevented by the systemic administration of the ET_A/ET_B receptor blocker LU420627 (Yang et al. 2004). The clear relationship between cardiac ET1 expression and myocarditis-related dilated cardiomyopathy observed in this model suggests that endothelin has a potential deleterious role in cardiac pathophysiology as a proinflammatory molecule, providing insights in the therapeutic potential of blocking ET-1 signaling pathways in heart failure.

The role of the mineralocorticoid hormone aldosterone (aldo) in cardiovascular diseases has recently been highlighted in both experimental models and in clinical trials through pharmacological blockade of the aldosterone receptor, i.e., the mineralocorticoid receptor (MR) (Delcayre and Silvestre 1999; Pitt 2004; Rossi et al. 2005). Spironolactone, an old MR antagonist used, and eplerenone, a more specific MR blocker, both decreased morbidity and mortality in patients with CHF or ischemic cardiopathies (Pitt 2004). Previously

described experimental models, such as nontargeted MR gene inactivation (Berger et al. 1998), widespread MR overexpression (Le Menuet et al. 2001), or pharmacological models (Brilla and Weber 1992), are complex to analyze and a direct relationship between Aldo and/or MR and their effects on the cardiovascular functions is difficult to establish unambiguously.

To address the pathophysiological role of MR in the heart or vessels independently of its renal effects, a targeted approach is required and conditional, inducible systems will result in temporal control of MR expression in selected cell targets, but not in other aldosterone-target organs, allowing precise tuning of MR expression over time (Ouvrard-Pascaud and Jaisser 2003). Down-expression of cardiac MR through conditional Dox-dependent expression of an antisense mRNA specific for the endogenous MR leads to heart failure with dilated cardiomyopathy and interstitial fibrosis (Beggah et al. 2002). Interestingly, these molecular and functional phenotypes were fully reversible when endogenous MR expression returned to control levels (Beggah et al. 2002). Both cardiac hypertrophy and interstitial fibrosis regressed, allowing survival of the animals and opening the way to the characterization of Aldo/MR modulated genes as well as signaling pathways that are critical for the clearance of interstitial fibrosis and reversion of dilated cardiomyopathy. A mirror model with conditional hMR overexpression in cardiomyocytes has been subsequently developed (Ouvrard-Pascaud et al. 2005). Cardiac MR overexpression led to major electrocardiographic abnormalities with prolonged ventricular repolarization and spontaneous and triggered ventricular arrhythmias, even when hMR expression occurred in adulthood only (i.e., when time of hMR expression is controlled with dox), pointing to potential MR involvement in acquired disease. This is associated with ion channel remodeling leading to a decrease in Ito K current and an increase in action potential duration and Ca transient amplitude. Associated lethality was prevented by pharmacological MR antagonisms with spironolactone (Ouvrard-Pascaud et al. 2005). Microarray analyses, using hearts from transgenic mice with hMR overexpression and normal circulating aldosterone, are in progress to define genes specifically regulated by MR in the heart. This could allow the validation of drugs that affect function of multiple targets (as opposed to channel-specific drugs) and have a broader spectrum of action, adding novel possibilities for arrhythmia prevention or therapy.

2.4 Conditional Models for Vascular Disease: Use of the Tet System

Conditional vascular expression has been achieved in both endothelium and vascular smooth muscle cells using specifically designed transactivator mice (Table 2). Conditional models are of particular interest for genomic and proteomic analyses.

Table 2 Conditional models with tet-dependent vascular expression

Transgenic line	Promoter	Trans-activator	Inducer	Target locus	Over-expressed	Phenotype	Reference
Tie2-tetOFF	2.1 kb tek/Tie2	tTA	Doxycycline PO 0.2 mg/ml	tetO-β-gal	β-gal	Reporter gene expression	Sarao and Dumont 1998
Tie2tetOFF	id	tTA	id	tetO-TEK	TEK Tie2	Role of TEK/Tie2 in embryo vascularization	Jones et al. 2001
	id		id	tetO-TEK tetO- β-gal	TEK Tie2 β-gal	Epidermal hyperplasia, inflammation, altered angiogenesis	Voskas et al. 2005
Tie2tetON	2.1 kb tek/Tie2+1.7 kB enhancer	rtTA	Doxycycline PO 2 mg/ml	tetO- β-gal (co-integrated with Tie2-tetON)	β-gal	Reporter gene expression	Teng et al. 2002
VE-Cadh tetOFF	Mouse 2.5 kb VE-Cadh	tTA	Tetracycline PO 1.5 mg/ml	tetO-myrAkt	Constitutively active Akt1	Altered retinal vasculature	Sun et al. 2005
id	id	id	id	tetO-endostatin	Tagged endostatin	Tumor growth inhibition	Sund et al. 2005
SM22-tetOFF	3 kb SM22α	tTA	Doxycycline PO 0.2 mg/ml	tetO-RVCH-HA	Rat vascular chymase	Medial thickening hypertension	Ju et al. 2001
id	id	id	id	Bidirectional β-gal-tetO-MEn	Dominant negative Myb-engrailed β-gal	Prevention of arterial remodeling after injury	You et al. 2003

Table 2 (continued)

Transgenic line	Promoter	Trans-activator	Inducer	Target locus	Over-expressed	Phenotype	Reference
id	id	id	id	Bidirectional β-gal -tetO- PMCA	Plasma membrane Ca-ATPase β-gal	Increased vascular reactivity hypertension	Gros et al. 2003
SM22-tetON	Mouse 488 bp SM22α	rtTA	Doxycycline PO food 1 g/Kg	tetO Luc tetO β-gal tetO-dnBMPRII	Luc β-gal Dominant negative BMPRII	Reporter gene expression Pulmonary hypertension	West et al. 2004
SM22-tetON	Mouse 481 bp SM22α	id	Doxycycline PO 2 mg/ml	tetO-UCP1	UCP1	Hypertension atherosclerosis	Bernal-Mizrachi et al. 2005

tTA, tetracycline transactivator; rtTA, reverse tetracycline transactivator; tetO, tet responsive promoter; αSM α, actin smooth muscle; β-gal ,β-galactosidase gene; Luc, luciferase; RVCH-HA, hemagglutinin tagged rat vascular chymase; MEn, dominant negative *MybEngrailed;* PMCA, plasma membrane Ca2+-ATPase; *dn BMPRII,* dominant-negative bone morphogenetic peptide receptor II; UCP1, uncoupling protein 1; VEGF, vascular endothelial growth factor; Tie2, tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2; TEK, tunica interna endothelial cell kinase; myrAKT, constitutively active Akt1; Ck b, actin Chicken b actin promoter; Gsq, sq G protein

2.4.1 Endothelium

The Tie2/TEK promoter has been used to target the endothelium. Sarao and Dumont first described an endothelial-specific transactivator mice using 2.1 kb of the Tie2/TEK promoter (Sarao and Dumont 1998). Conditional expression appeared to be restricted to early embryonic stages. Expression was absent in adult animals probably because of the lack of an enhancer element leading to a progressive loss of TEK promoter activity. This TEKtetOFF line was used to conditionally rescue the lethal phenotype observed in E12.5 TEK-null embryos related to rapid endothelial cell apoptosis and vascular hemorrhage (Jones et al. 2001). Recently, careful examination of a conditional model for Tie2/TEK expression reveals that the system was indeed also functional in adult skin in both endothelial and, surprisingly, in keratinocytes and epithelial cells of the hair follicles (Voskas et al. 2005). This unexpected finding points out to the previously unknown expression of Tie2/TEK in the skin. Targeted overexpression of Tie2 in this conditional model led to a psoriasis-like disease model that is particularly interesting from a pathophysiological point of view. It also point out that careful examination of the transgene expression pattern should be done to avoid misinterpretation of the results.

Weakness of this 2.1 kB Tie2 promoter can be circumvented by using larger regulatory sequences of the Tie2 gene, including 1.7 kb of the intron/exon1 Tie2 cassette (Schlaeger et al. 1997) containing enhancer elements enhancing the Tie2 promoter activity in adult endothelium. A Tie2-tetON mice has been generated and extensively characterized by J.N. Topper's group. Co-integration of both Tie2-rtTA and tetO-nlsLacZ constructs identifies a mouse strain with endothelial-specific and Dox-dependent expression of the reporter gene in the vascular endothelium of all organs examined, i.e., heart-endocardium and coronary arteries, lung, liver, brain, intestine, skeletal muscle, and kidney (in particular in the afferent arterioles and glomerular capillaries) (Teng et al. 2002). Although low background activity was reported in smaller branches of the aorta, the data clearly indicated a robust expression in almost all cells of the vascular tree, without evidence of nonspecific expression in nonendothelial subtypes. Expression was induced after 3 days of oral Dox, sustained for at least 3 months and also observed in E9.5 embryonic vessels if the pregnant mother was treated with Dox (Teng et al. 2002). A similar transactivator mice was generated recently in order to conditionally downregulate Tubedown-1 (Tbdn-1) via overexpression of a 1.4-kb Tbdn-1 antisense RNA in endothelial cells (Wall et al. 2004). The authors focused on the retina and showed a conditional, Dox-dependent decrease, but not quantified, expression of Tbn-1. This resulted in major retinal and choroidal neovascularization and lesions, suggesting that Tbn-1 may be a potential target for treating proliferative retinopathies, although they have not studied the effect in this particular model on pathological lesions of Tbn-1 re-expression once Dox administration is stopped.

Another endothelium-specific promoter has been used to specifically target endothelial cells. The mouse VE-cadherin promoter directed tTA expression in the endothelium (Sun et al. 2005). This VE-Cadh-tetOFF strain was generated in L. Benjamin's group and used to develop a conditional model for myrAkt, a dominant-active form of Akt. The observed embryonic lethality in bigenic mice could be bypassed by giving tetracycline to the pregnant mother, allowing analysis of sustained Akt signaling in adult retinas (Sun et al. 2005). In addition to morphological malformations, the vascular phenotype was consistent with a failure in remodeling and a disturbed patterning and vessel hierarchy in the retina (Sun et al. 2005). Use of the VE-Cadh tetOFF strain allows conditional expression of a secreted form of endostatin, an endogenous inhibitor of angiogenesis. Conditional manipulation of endostatin allowed both temporal control (bypassing the embryonic period) and cell-specific expression. Results indicated that this class of molecule (including tumstatin or thrombospondin-1, for example) acts as an endothelium-specific tumor suppressor (Sund et al. 2005). Cancer growth is therefore not completely dependent on the genetic defects of cancer cells but also depends on the host-derived tumor microenvironment.

2.4.2 Vascular Smooth Muscle

The vascular smooth muscle (VSM) plays a key role in vascular myogenic tone. Among other parameters, this greatly influences blood pressure by modulating peripheral resistance. Several studies have studied how various parameters specifically affect VSM cell properties. Conditional, inducible transgene expression has been obtained with the tet system using a 3-kb SM22α promoter that drives expression of the tetOFF transactivator in VSM cells only (Ju et al. 2001). This strain has been used to conditionally overexpress vascular chymase (Ju et al. 2001), plasma membrane calcium ATPase (PMCA) (Gros et al. 2003), and a dominant negative c-myb transcription factor (*Myb-Engrailed*) (MEn) (You et al. 2003). Conditional overexpression of the vascular chymase led to elevated blood pressure associated with structural remodeling of the aortic wall with increased medial thickening and increased VSM cell proliferation (Ju et al. 2001). This was associated with functional alteration of the vascular properties with increased vasoconstriction to phenylephrine and impaired vasodilatation to cholinergic agents (Ju et al. 2001). These data indicated that this chymase may be a candidate gene in hypertensive patients. Vascular reactivity and blood pressure was also increased in mice with conditional, VSM-targeted expression of PMCA (Gros et al. 2003). The enhanced myogenic tone and sensitivity to vasoconstrictors was related to depressed nNOS activity and reduced cGMP production, both at the basal state and following stimulation. Therefore, this conditional model was particularly useful to demonstrate the relationship between vascular tone and altered expression or function of PMCA in VSM cells, leading to elevated blood pressure.

Factors affecting arterial remodeling after injury need to be identified. They influence subsequent changes in arterial structure and/or flow after angioplasty or atherosclerosis, for example. The cMyb transcription factor has been implicated in the differentiation/proliferation balance of VSM cells and was proposed as a potential target in arterial injury. Husain and his collaborators took advantage of the SM22-tetOFF strain to overexpress a dominant negative form of c-Myb (MEn) in VSM cells (You et al. 2003). They showed that MEn does not affect arterial structure in basal condition but markedly decreased arterial remodeling (VSM proliferation, neointima formation, medial hyperplasia) after mechanical carotid injury. Specificity of the MEn effects was addressed by preventing its expression by Dox administration before injury.

Idiopathic pulmonary artery hypertension (IPH) is a rare disease but reminiscent of iatrogenic (appetite suppressants) or inflammation-related causes. Its pathophysiological basis is not well understood but recent genetic screens revealed that mutations in the receptors of bone morphogenic protein (BMP-R I or II) are clearly associated. An experimental model for pulmonary artery hypertension has recently been generated by West et al. through conditional overexpression of a dominant negative mutant of BMP-RII in VSM cells using a novel SM22-tetON mouse generated with a 488-bp murine SM22 promoter driving the original rtTA transactivator (Bernal-Mizrachi et al. 2005). This SM22-tetON strain allowed transgene expression in the media of large pulmonary artery and aorta. Dox-treated double-transgenic mice, called SM22-tet-BMPRII^{delx4+}, conditionally express BMPRII^{delx4+} in the lung and display increased medial thickness of muscular pulmonary arteries, as well as small pulmonary arteries at the level of alveolar ducts, recapitulating primary lesions seen in humans with pulmonary artery hypertension (West et al. 2004). Interestingly, right ventricular systolic pressure was increased as well as the RV/LV+septum weight ratio, indicating sustained pulmonary hypertension. These data indicated that a loss-of-function of BMP-RII signaling, achieved through targeted expression of a dominant negative mutant of BMP-RII in VSMC, was sufficient to develop pulmonary hypertension, not excluding of course modulatory role of BMP in other cell types; endothelial-specific targeting of BMPRII^{delx4+}, breeding the tet-BMPRII^{delx4+} mice with an endothelial-specific tet transactivator strain, as described earlier, will certainly address this question. The downstream signaling pathway remains to be established but good candidates are LIM-kinase and SMAD-dependent and -independent (p38MAPK, ERK, JNK) pathways (Rabinovitch 2004; West et al. 2004).

Atherosclerosis is a major risk factor for cardiovascular diseases. Disturbances of local metabolism, including uncoupling of respiration and oxidative phosphorylation has been proposed as a mechanism for increased generation of reactive oxygen species (ROS), responsible for oxidative stress implicated in atherosclerosis. To test the hypothesis that inefficient metabolism in blood vessels promotes vascular diseases, targeted, conditional overexpres-

sion of UCP1, a member of the uncoupling proteins (inner mitochondrial membrane anion transporters) family, was obtained in VSMC using a SM22-tetON transactivator strain crossed with a tet-UCP1 strain (Bernal-Mizrachi et al. 2005). First, systolic and diastolic blood pressure was markedly increased upon dox induction of UCP1 expression. Blood pressure returned to baseline after 10 days Dox retrieval. This was associated with an activation of the renin-angiotensin-aldosterone system and urinary Na retention. To examine the effect of VSM UCP1 overexpression on vascular remodeling, bigenic SM22tetON-tetUCP1 mice were bred with apolipoprotein E-deficient mice, a model prone to atherosclerosis. Increased atherosclerotic lesions were noted in SM22tetON-tetUCP1 mice as compared to the various control littermates when fed a Western diet. Increased superoxide production was documented in SM22tetON-tetUCP1 ApoE$^{-/-}$ mice as well as peroxynitrite production resulting from the reaction between superoxide and nitric oxide (Bernal-Mizrachi et al. 2005). Interestingly, blood pressure returned to basal values when animals were treated with the antioxidant Tempol for 5 days. Taken together, these data indicated that local respiratory uncoupling increases oxidative stress, blood pressure, and atherosclerosis, suggesting that enhancing efficient metabolism in the vasculature may be a good target for treating vascular diseases.

3
The Inducible Cre Lox System

3.1
General Strategy

The Cre lox system uses the Cre recombinase isolated from the P1 bacteriophage (Sauer 1998). Cre recombinase catalyses site-specific recombination by crossing over between two distant Cre-recognition sequences, i.e., LoxP sites. The LoxP sites include two 13-bp inverted repeats separated by an 8-bp spacer sequence. Any DNA sequence introduced between the two 34-bp LoxP sequences (called floxed DNA) will be excised due to Cre-mediated recombination. This means that controlling Cre expression in a transgenic animal, either by using spatial control (with a tissue- or cell- specific promoter) or by using temporal control (with an inducible system) would result in the spatio and/or temporal control of the DNA excision between the two LoxP sites. In addition to conditional gene inactivation (conditional KO), this approach can be applied to overexpress a protein. In this case, a floxed stop cassette is inserted between the promoter sequence and the cDNA of interest. Transgenic animals will not express the transgene until Cre is expressed, leading to the excision of the floxed stop cassette.

Inducible Cre recombinases have been developed (Metzger and Feil 1999; see also the chapter by R. Feil, this volume). A prerequisite to allow temporal

control of the Cre-mediated recombination is that the inducible Cre recombinase would not be activated by endogenous ligands, but only by an exogenous ligand that is introduced at a chosen moment. The inducible Cre recombinases are fusion proteins between the original Cre recombinase and a specific ligand-binding domain (LBD). The functional activity of the Cre recombinase depends on an external ligand that is able to bind to this specific domain in the fusion protein. Binding the ligand is intended to allow conformational changes of the fusion protein and/or changes in the intracellular localization associated with targeting of the recombinase to the nucleus (Metzger and Feil 1999).

Initially, fusion proteins were generated with the Cre recombinase and a hormone-binding domain of nuclear receptors, such as receptors for glucocorticoids, estrogens, or progesterone. In order to avoid activation of the engineered Cre recombinases by endogenous ligands such as circulating glucocorticoids, estrogens, or progesterone, mutated hormone ligand-binding domains have been used. In this case, synthetic ligands bind the modified Cre recombinases but endogenous ligands cannot.

3.2 New Insights into Cardiac Pathophysiology Using the Inducible Cre-Lox System

The Cre-Lox system has been successfully used to generate conditional model of cardiovascular diseases. Conditional but constitutive gene deletion in the myocardium has been recently reviewed (Heine et al. 2005) and will not be treated here. Ligand-activated Cre recombinase has been used to allow conditional, inducible gene deletion in the heart (Table 3). Fusion proteins between the Cre recombinase and the mutated ligand binding domains for steroid hormone receptor have been expressed in cardiomyocytes under the control of the 5.5-kb mouse αMHC promoter. The mutated progesterone receptor LBD was fused to Cre and used to generate αMHC-CrePR1 transgenic mice (Minamino et al. 2001). Cre recombinase activity was activated both ex vivo and in vivo by the synthetic antiprogestin RU486 (mifepristone). In vivo, 0.25 mg/day for 5 days was enough to induce recombination-dependent LacZ expression in the Rosa26-LoxLacZ reporter mice crossed with the αMHC-CrePR1 (Minamino et al. 2001). The major drawback, however, was that leakiness of control of Cre activity in the CrePR1 lines allows spontaneous recombination and therefore LacZ expression in absence of induction with RU486. This increased with time, allowing LacZ expression in 25%–80% of the cardiomyocytes. This is a clear disadvantage when conditional deletion is required at a chosen time. This could be useful when tissue heterogeneity is expected to model some chronic disease or arrhythmic disorders (see, for example, the use of mosaic mice with/without Cx43 knock-out; Gutstein et al. 2001). The leakiness of this CrePR1 fusion protein probably relies on post-translational modification of the fusion protein or activation control of the LBD by endogenous

steroids. Because gene inactivation results in an irreversible somatic mutation, cells with the recombined gene will accumulate over time, even if Cre is no longer activated. This problem has been encountered by others and resulted in the generation of Cre fusion with more stringent control of activity, such as the MerCreMer developed by the Reth's group in which mutated murine ER LBD were fused on both the N- and C-terminus of Cre (Zhang et al. 1996). This probably minimizes the consequences of post-translational modifications. This fusion protein provided very tight control by Tamoxifen (Verrou et al. 1999). Tam concentration (which is of course highly dependent on local bioavailability) needs to be high enough to activate the MerCreMer fusion protein (Verrou et al. 1999). A high expression level in a particular cell type would therefore combine tightness and efficiency. Molkentin used this MerCreMer fusion protein to develop a very efficient mouse strain with cardiac-specific expression of a tamoxifen-inducible Cre recombinase allowing temporally regulated gene manipulation in adult and embryonic heart (Sohal et al. 2001). By 3 months of age, contrary to the αMHC-CrePR1 line, almost no leak (less than 1%) is observed. Four intraperitoneal injections of 20 mg/kg/day tamoxifen induced reporter gene expression in at least 80% of the cardiomyocytes. Tam administration from E12 to E16 to the pregnant mother provided robust and uniform LacZ staining, indicating efficient Tam-dependent Cre-mediated recombination in embryonic mouse heart (Sohal et al. 2001). This αMHC-MerCreMer strain has been used to conditionally inactivate the N-cadherin gene in the heart since N-cadherin null mutation is embryonic lethal (Radice et al. 1997). Deleting N-cadherin specifically in heart using a constitutive cardiac-specific Cre recombinase was associated with embryonic death (Radice, unpublished data). Conditional loss of N-cadherin in adult heart resulted in disassembly of the intercalated disk structure, including adherens junction and desmosomes (Kostetskii et al. 2005). Mild cardiopathy was noted, probably related to a decrease in force transmission due to the disruption of cadherin/catenin cytoskeletal linkage. Most animals died after 6 weeks Tam-induced N-cadherin inactivation. This was related to spontaneous ventricular arrhythmia, possibly related to a 60% decrease in Cx43 expression (Li et al. 2005). Gap junction remodeling may be related to the destabilized junctional complexes induced by N-cadherin deletion.

Ligand-activated Cre recombinases can also be used for inducible, but not reversible, transgene expression. Conditional expression of a reporter gene, LacZ for example, is used in the initial characterization of these fusion recombinases. The αMHC-MerCreMer strain has been efficiently used to conditionally express Gαq (Syed et al. 2004) or MKK7D, a constitutively activated upstream activator of c-Jun N-terminal kinases (Petrich et al. 2003). Expression of these proteins is prevented by a floxed stop cassette in bigenic mice obtained after breeding the αMHC-MerCreMer and the conditional Gαq or MKK7D mice. Once Cre is activated by ligand administration, the floxed stop cassette is excised, allowing Gαq or MKK7D expression (Petrich et al. 2003; Syed et al. 2004).

Table 3 Conditional models with inducible Cre-lox system

Transgenic line	Cre recombinase	Ligand-binding domain	Promoter	Inducer	Target locus	Overexpressed/ deleted gene	Phenotype	Reference
αMHC-PR1	Cre PR1	Mutated LBD (641–891) human progesterone receptor	5.5 kb mouse αMHC	RU486 IP 0.25 mg/day/ 5 days)	Rosa26	β-gal	Reporter gene expression Leakiness, progressive LacZ accumulation	Minamino et al. 2001
αMHC-MerCreMer	MerCreMer	Mutated LBD (281–599, G525R) murine estrogen receptor	5.5 kb mouse αMHC	Tam IP (20 mg/kg/day/ 4–6 days)	Rosa 26	β-gal	Reporter gene expression	Sohal et al. 2001
id	id			Tam IP (80 mg/kg/day/ 5 days)	N-Cad flox	N-cadherin	Mild cardiopathy, ventricular arrhythmia	Li et al. 2005
id	id			Tam IP (20 mg/kg/day/ 5 days)	αMHC-flox-MKK7D	MKK7D Upstream activator of c-Jun N-ter kinases	Progressive cardiomyopathy	Petrich et al. 2003 Kostetskii et al. 2005
id	id			Tam IP (15 mg/kg/day/ 5 days	Ck-β actin-flox-Gsq	Gsq		Syed et al. 2004

αMHC, α-myosin heavy chain; cre-PR1, Cre-progesterone receptor binding domain fusion protein; β-gal, β-galactosidase gene; MerCreMer, Cre protein fusion with two mutant estrogen receptor domains; Rosa 26, Cre reporter mice Rosa 26 strain; Tam, Tamoxifen; RU486, antiprogestin (mifepristone); Ncad, N-cadherin; MKK7D, upstream activator of c-Jun N-ter kinases; Ck b actin, Chicken b actin promoter; Gsq, G protein sq

The resulting expression pattern depends on the overlapping expression profile of the αMHC promoter (i.e., cardiomyocytes for αMHC-MerCreMer mice) and of the Ck-β actin or αMHC promoters for the conditional Gαq and MKK7D strains, respectively.

3.3 Conditional System Using the Chimeric Transactivator Glp 65

A mifepristone (RU486) inducible system relying on a chimeric transactivator targeted to the heart has been developed (Table 4). The chimeric transactivator Glp 65 consists of the mutated LBD of the progesterone receptor fused to the GAL4 DNA binding domain and part of the activation domain of the human p65 protein, a component of the NF-κB complex (Burcin et al. 1999). In the presence of mifepristone, but not in its absence, Glp65 exclusively activates the target gene with promoters that contain consensus sequences for Gal4 binding sites (17 mer). Cardiac-specific Glp65 expression was achieved using the 5.5-kb mouse αMHC promoter cassette to control Glp 65 transactivator expression in transgenic mice (Babij et al. 2003; Bo et al. 2005).

Nordstrom's group first demonstrated that conditional cardiac expression of a LacZ reporter gene can be achieved with this system (Babij et al. 2003). Interestingly, the oral route for RU486 administration was preferred to the intraperitoneal one and was shown to be fully efficient, as demonstrated by pharmacokinetics studies of RU486 in FVB/N mice. Sustained expression of LacZ was observed within 7 days of oral RU486 administration (Babij et al. 2003). Of note, despite 10 days RU486 withdrawal, LacZ activity remained detectable, probably because of the long half-life of this particular protein. Indeed, this parameter should be carefully taken into account when reversal protocols are done, whatever the type of conditional system used, since the long half-life of the transgene product could preclude further analysis. Bo and collaborators reported that this system was quite efficient for conditional expression of the human growth hormone (hGH) in bigenic mice obtain when a similar but different αMHC-Gpl65 strain was mated with a responder strain that allowed in vivo Glp65 dependent-hGH expression (Bo et al. 2005). Maximal expression was observed as early as 4 days after RU486 IP administration (250 ug/Kg/day). Sustained expression was observed during chronic RU486 administration (up to 6 weeks) and RU486 withdrawal led to a rapid decrease in circulating hGH after 2 days, in accordance with the metabolism of RU486 in vivo (half-life approximately 20–30 h). It should be noted that the absence of cardiac or general toxicity of either Glp65 or RU486 at the required concentrations make this system useful for various experimental studies.

This αMHC-Glp65 strain has recently been used to study the role of the T-Q92 troponin mutant in the pathogenesis if hypertrophic cardiomyopathy (HCM). Conditional expression of the cardiac troponin T-Q92, responsible for human HCM, was achieved after mating the αMHC-Glp65 mice with a responder 4xGAL4BD-cTnT strain (Lutucuta et al. 2004). cTnT expression was

Table 4 Conditional models with the chimeric Glp65 transactivator

Transgenic line	Chimeric Glp65 transactivator	Ligand-binding domain	Promoter	Inducer	Target locus	Over-expressed gene	Phenotype	Reference
αMHC-GeneSwith regulator	GeneSwith regulator (3.1	Truncated LBD (914 human progesterone receptor	5.5 kb mouse αMHC	RU 486 Oral (100 µ g/kg/day)	6×4x17-GAL4RE-E1b-β-gal	β-gal	Reporter gene expression	Babij et al. 2003
αMHC-GLp65	GLp65	id	id	RU 486 IP (500 µg/kg/day/ 4 days) Pellets (500 µg/kg/ 21 days)	4×17-GAL4RE-tk-hGH	hGH	Reporter gene expression	Bo et al. 2005
id	id	id	id	RU 486 IP 1 mg/kg/day/ 16 days or 70 days)	4×17-GAL4RE-tk-hcTnT	T-Q92 cTnT mutant	Hypertrophic cardiomyopathy	Lutucuta et al. 2004

αMHC, α-myosin heavy chain; β-gal, Glp65; chimeric Glp65 transactivator; RU 486, antiprogestin (mifepristone); hGH, human growth hormone; hcTnT, T-Q92 cardiac troponin T mutant

induced with 1 mg/kg/day RU486 IP daily injection for 16–70 days. Despite relatively low expression of the mutant cTnT protein as compared to endogenous WT cTnT (ratio, approximately 1:10), this resulted in enhanced myocardial systolic function. Long-term expression was associated with reduced A- and B-type natriuretic factors and increased collagen expression as well as increased interstitial fibrosis. Interestingly, switching off mutant cTnT expression due to RU486 withdrawal reversed these functional, molecular, and histological phenotypes. Therefore, this conditional HCM model could be used to specifically address the relevant pathways involved in HCM regression and may help to identify putative targets for HCM therapies.

4 Conclusion

Conditional systems have proven to be efficient and powerful in delineating several aspects of cardiac pathophysiology and diseases. The possibility of addressing a particular time point of the animal life is certainly an important breakthrough provided by conditional strategies with temporal control of either transgene expression or gene modifications. Access to the reversion period due to transgene expression shutoff, using transcriptional regulators like the tet or Glp65-based systems, for example, is a powerful tool to identify putative targets for disease treatment rather than prevention. Indeed, conditional models are of particular interest for genomic and proteomic analyses. Differential gene expression analysis at various time-points before and during onset or remission of the pathology (through controlled up- or down-regulation of transgene expression, for example) should identify altered signaling cascades, preventing interference with long-term compensatory mechanisms that can preclude identification of relevant targets.

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Conditional Animal Models for the Study of Lipid Metabolism and Lipid Disorders

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Abstract The advent of technologies that allow conditional mutagenesis has revolutionized our ability to explore gene functions and to establish animal models of human diseases. Both aspects have proven to be of particular importance in the study of lipid-related disorders.

Classical approaches to gene inactivation by conventional gene targeting strategies have been successfully applied to generate animal models like the LDL receptor- and the apolipoprotein E-knockout mice, which are still widely used to study diverse aspects of atherosclerosis, lipid transport, and neurodegenerative disease. In many cases, however, simply inactivating the gene of interest has resulted in early lethal or complex phenotypes which are difficult to interpret. In recent years, additional tools have therefore been developed that allow the spatiotemporally controlled manipulation of the genome, as described in detail in Part I of this volume. Our aim is to provide an exemplary survey of the application of different conditional mutagenesis techniques in lipid research in order to illustrate their potential to unravel physiological functions of a broad range of genes involved in lipid homeostasis.

Keywords SREBP · PPAR · LRP · Lipoprotein · Atherosclerosis

1
Transcriptional Control of Lipid Metabolism

Lipid homeostasis is crucial for an organism's development, growth, and differentiation and is therefore tightly regulated on different levels, with dysregulation often resulting in developmental disorders or metabolic diseases. Transcriptional regulation of lipid metabolism is accomplished by a network of transcription factors, many of which are controlled by transcriptionally active endproducts or byproducts of metabolic pathways or dietary components. Their sharing of lipophilic ligands and interacting modulatory proteins as well as mutual transcriptional control allow for a stringent control of the abundance and function of these factors. The liver plays a major role in the coordination of lipid metabolism with carbohydrate and energy homeostasis. Consequently, conditional liver-specific mutagenesis in the liver has been described for the majority of genes discussed in this context. Other organs and tissues of interest include adipose tissue, skeletal muscle, and macrophages. Another important aspect of lipid metabolism concerns the turnover of lipid-carrying particles, which will be subject of the second part of this chapter.

1.1
The SREBP Pathway: Transcriptional Regulation of Cholesterol and Fatty Acid Synthesis

The sterol regulatory element-binding proteins (SREBPs) belong to a family of membrane-bound transcription factors that regulate lipid homeostasis (Brown and Goldstein 1997). Three SREBP isoforms, designated SREBP-1a, -1c, and -2, are encoded by two different genes. SREBP-1a induces transcriptional activation of all SREBP-responsive genes. The SREBP-1c isoform, which uses a different first exon, is mainly involved in the regulation of fatty acid synthesis, whereas the role of SREBP-2 is relatively restricted to the regulation of cholesterol synthesis. After their synthesis as membrane-bound precursor proteins at the endoplasmic reticulum (ER) membrane, the SREBPs must travel to

the Golgi apparatus to be proteolytically activated by two membrane-bound proteases, site-1 and site-2 protease (S1P and S2P). The sequential cleavage of SREBP results in the release of the aminoterminal basic helix-loop-helix-leucine zipper (bHLH-Zip) domain, a transcription factor domain that then translocates to the nucleus, where it binds to sterol-responsive elements (SREs) in the promoter/enhancer regions of several dozen target genes, which are involved in the synthesis and uptake of cholesterol and fatty acids. Newly synthesized SREBP forms a complex with the polytopic transmembrane protein SCAP (SREBP cleavage activating protein), which facilitates its incorporation into vesicles that move from the ER to the Golgi, the site of the proteolytic activation of SREBPs. The accumulation of cholesterol in the ER membrane leads to a conformational change of SCAP, which contains a sterol-sensing domain, and its subsequent binding to Insig (insulin-induced gene) proteins. As a result, proteolytic processing of SREBP in the Golgi and accumulation of its mature, transcriptionally active form in the nucleus (nSREBP) are abrogated.

1.1.1 Conditional Mouse Models of Lipid Regulation Through SREBP Signaling

1.1.1.1 Conditional Hepatic Inactivation of Site-1 Protease and SCAP

Tissue culture studies have proven invaluable for the delineation of the SREBP pathway and the molecular mechanisms of its sterol-dependent regulation. However, conditional mutagenesis provided important insights into the complex intertwined relation between the SREBPs and their interacting regulatory proteins as well as the relative roles of the different SREBP isoforms for fatty acid and cholesterol synthesis in the mammalian organism (Horton et al. 2002).

Conventional gene inactivation studies revealed that mice lacking site-1 protease, in which proteolytic activation of all SREBP isoforms is disrupted, die early during embryonic development. To circumvent embryonic lethality, an inducible S1P knockout was generated (Yang et al. 2001). To this end, mice homozygous for a floxed version of the S1P gene and heterozygous for the bacterial Cre recombinase under the control of the interferon-inducible MX1-promoter (MX1-Cre-S1P$^{flox/flox}$ mice) were injected with polyinosinic acid-polycytidylic acid (pI-pC). This synthetic, double-stranded RNA induces an antiviral response in liver and lymphocytes, leading to the expression of Cre recombinase, Cre-mediated recombination between the two loxP sites, and disruption of the floxed gene in the liver within a couple of days (Kuhn et al. 1995). Disruption of hepatic S1P expression led to a marked decline in the levels of all nuclear SREBP isoforms, resulting in significantly reduced rates of cholesterol and fatty acid synthesis due to decreased transcription of genes encoding biosynthetic enzymes. This reduction was less pronounced than in mice with inducible conditional inactivation of the SREBP escort protein, SCAP (MX1-Cre-SCAP$^{flox/flox}$

mice; Matsuda et al. 2001). These mice displayed a robust reduction in SREBP processing, translation of SREBP target genes, and lipid uptake and synthesis, which could not be reversed by hepatic cholesterol depletion with a HMG-CoA reductase inhibitor or insulin-dependent induction of SREBP-1c synthesis through a fasting-refeeding protocol. Triglyceride-rich plasma VLDL particles were significantly lowered due to a decreased secretion by the liver.

The inducible disruption of S1P and SCAP in livers of conditional knockout mice allowed the analysis of hepatic SREBP function in the adult organism, since germline deletion of the *Srebp-1* and *-2* genes resulted in complete or partial embryonic lethality (Shimano et al. 1997b), and conditional inactivation of *Srebp* genes in mice has not been reported. The apparent health of mice with hepatic deficiencies of SCAP or S1P contrasts with the early lethality of mice carrying germline deletions of S1P or SREBP-2, suggesting essential roles of SREBP processing and signaling in extrahepatic tissues or during embryonic development. Indeed, careful examination of mice with conditional hepatic SCAP deficiency revealed that white adipose tissue is able to compensate for the decrease in fatty acid synthesis in the liver by tissue-specific selective upregulation of SREBP-1c transcription (Kuriyama et al. 2005), an important mediator of insulin action (Flier and Hollenberg 1999). Accordingly, an increase in insulin sensitivity of adipose tissue was observed in liver-specific SCAP knockout mice (Kuriyama et al. 2005), raising the possibility that selective inhibition of hepatic fatty acid synthesis in humans might improve the insulin resistance encountered in the metabolic syndrome. It should be mentioned that the proteolytic activators of SREBP, S1P and S2P, have additional functions independent of SCAP, namely in the cellular response to endoplasmic reticulum stress (Ye et al. 2000), which could contribute to the lethal S1P knockout phenotype.

The conditional liver-specific SCAP knockout mouse proved useful in the identification of novel SREBP-dependent target genes. Through comparative microarray analysis of hepatic RNA from mice either overexpressing nuclear SREBP-1a or -2 in their livers (Shimano et al. 1996; Horton et al. 1998) or lacking all three activated isoforms as a result of hepatic SCAP inactivation (Matsuda et al. 2001), 33 genes were filtered out which were upregulated in the two transgenic models and downregulated in the conditional SCAP knockout mice (Horton et al. 2003). One of the identified genes turned out to be proprotein convertase subtilisin/kexin type 9a (*Pcsk9a*) (Park et al. 2004), whose human orthologue is mutated in a rare form of autosomal-dominant hypercholesterolemia (Abifadel et al. 2003).

1.1.1.2 Diet-Inducible Overexpression of Nuclear SREBP Isoforms in the Liver

In addition to conditional mouse knockout models, transgenic mice that overexpress the active nuclear forms of SREBPs (tg-nSREBP) under the control of

a diet-responsive rat phosphoenolpyruvate carboxykinase (PEPCK) promoter underlined the distinct but related roles of individual SREBP isoforms in regulating fatty acid and cholesterol synthesis in vivo (Shimano et al. 1996, 1997a; Horton et al. 1998). The PEPCK promoter was chosen because it is active only after birth, is expressed highly in liver relative to other organs, and is inducible by a low-carbohydrate/high-protein diet (Short et al. 1992). Because of the central role of SREBP-1c in mediating the lipogenic action of insulin in the liver, transgenic mice overexpressing the nuclear SREBP-1c isoform driven by the PEPCK promoter (Shimano et al. 1997a) might be useful mouse models for nonalcoholic fatty liver disease, one of the symptoms of the metabolic syndrome, which can lead to liver fibrosis and eventually irreversible liver disease. Interestingly, SREBP-1c can induce transcriptional activation of PPAR-gamma (Kim et al. 1998b; Fajas et al. 1999), another transcription factor involved in the development of hepatic steatosis (Sect. 1.2.1.2).

1.1.2 SREBP-Interacting Proteins and Target Genes

1.1.2.1 Disruption of Hepatic Insig Expression

Insig proteins are polytopic membrane proteins that reside in the endoplasmic reticulum. Besides their function as ER retention proteins for the SCAP-SREBP complex by binding to sterol-loaded SCAP, Insig proteins mediate the proteolytic degradation of HMG-CoA reductase, the key rate limiting enzyme of cholesterol synthesis, upon sterol accumulation in the ER membrane (Sever et al. 2003). Two genes, *Insig-1* and *Insig-2*, encode highly similar proteins with overlapping functions in cell culture systems. Both genes are under reciprocal regulation by insulin in the liver (Yabe et al. 2003; G. Chen et al. 2004). Whereas Insig-1 is induced by SREBP-1c, an insulin target gene, the liver-specific *Insig-2a* transcript contains a regulatory element that mediates its direct repression by insulin. Mice with germline disruption of *Insig-1* mediated by Cre recombinase expression driven from an adenoviral EIIA promoter (Lakso et al. 1996) on a conventional *Insig-2* knockout background did not survive into adulthood (Engelking et al. 2005). In order to investigate the physiological function of both *Insig* genes in the liver, a liver-specific knockout of *Insig-1* mediated by interferon-inducible Cre expression under control of the MX1-promoter was generated in mice lacking *Insig-2*. Lack of either *Insig* gene was not compensated by increased hepatic transcription of the other. In line with results obtained from cell culture studies, lack of both genes in the liver resulted in increased SREBP processing and transcription of target genes, which could not be suppressed by cholesterol feeding, and increased HMG-CoA reductase protein and enzyme activity levels due to a failure to downregulate this protein upon sterol accumulation. As a result, hepatic cholesterol levels were markedly

elevated in these animals even on a chow diet (Engelking et al. 2005). As pointed out by the authors, the combined conventional and conditional inactivation of both *Insig* genes made it possible to track the molecular mechanism for the feedback regulation of cholesterol synthesis in mice discovered by Schoenheimer and Breusch decades ago (Schoenheimer and Breusch 1933) to the sterol-dependent effect of Insig proteins on SREBP processing and HMG-CoA reductase degradation in the liver.

1.1.2.2 Fatty Acid Synthase and HMG-CoA Reductase, Key Enzymes of Lipid Synthesis

Fatty acid synthase (FASN), the central enzyme in fatty acid synthesis, is among the lipogenic enzymes regulated by the SREBP pathway on a transcriptional level. The large cytosolic fatty acid synthase complex converts acetyl-CoA and malonyl-CoA into palmitate, a long-chain saturated fatty acid. Germline disruption of the *Fasn* gene (Chirala et al. 2003) resulted in embryonic lethality, which included most of the heterozygote *Fasn*$^{+/-}$ embryos, indicating partial haploin sufficiency. Supplementation of the dams with a diet rich in saturated fatty acids did not compensate for the loss of homo- and heterozygotes. The same holds true for HMG-CoA reductase, another SREBP target gene and key rate enzyme of cholesterol biosynthesis, where early embryonic lethality of *Hmgcr* knockout mice could not be completely rescued by supplementing mevalonate (Ohashi et al. 2003). Conditional gene targeting strategies will be required to reveal novel functions of these fundamental lipogenic enzymes during mammalian development. Liver-specific inactivation of the fatty acid synthase gene in adult mice using a rat albumin promoter-Cre transgene (Postic and Magnuson 2000) unmasked defects in PPAR-alpha target gene expression (Chakravarthy et al. 2005), which points to a crosstalk between products of the FASN lipogenic pathway and nuclear receptors of the PPAR family (Sect. 1.2.1.2). These mice, which are not able to synthesize fatty acids from carbohydrate, developed hypoglycemia and steatohepatitis when fasted, whereas a PPAR-alpha agonist reversed these effects. The authors concluded that mobilized peripheral fat is not able to sufficiently replace newly synthesized or absorbed fat as endogenous PPAR-alpha ligand, and suggested palmitate, the endproduct of the FASN reaction, or a closely related saturated fatty acid, as important activators of PPAR-alpha (Chakravarthy et al. 2005).

1.2 Transcriptional Regulation of Lipid Metabolism by Nuclear Receptors

The interaction of regulatory transcriptional networks is an important prerequisite for the intricate balance of lipid homeostasis in the mammalian organism. This is accomplished by reciprocal regulation of different classes of transcription factors, convergence on transcriptional coactivators or by lipids

acting as endogenous regulators of transcription factors themselves. For example, SREBP-1c transcription is activated by the nuclear oxysterol receptor LXR (Repa et al. 2000; Schultz et al. 2000), and both SREBP and members of the PPAR family of nuclear receptors interact with the transcriptional co-activator CREB binding protein (CBP)/p300 (Oliner et al. 1996; Mizukami and Taniguchi 1997). Members of the nuclear receptor family of ligand-dependent transcription factors play a central role in controlling lipid physiology (reviewed by Chawla et al. 2001). Nuclear receptors share a common modular architecture including an aminoterminal transcriptional activation region (AF-1), a central DNA-binding domain that binds to highly conserved consensus sequences called hormone response elements, and a large carboxyterminal region that includes the ligand-binding domain. Members of this superfamily, which comprises several dozen genes in mammals, include classical endocrine receptors such as steroid receptors as well as a group of receptors that function as heterodimers with the retinoid X receptor (RXR) (see the chapter by D. Metzger, this volume). Within the last decade, physiological ligands for most of these receptors have been discovered. It turned out that many of these so-called adopted orphan receptors function as nutrient sensors that readjust lipid metabolism in response to dietary challenges by acting on an elaborate network of target genes involved in transport, clearance, and storage of lipids (Chawla et al. 2001). The best-studied of these receptors are the liver X receptors (LXRs), which function as sterol sensors (Peet et al. 1998), the bile acid receptor farnesoid X receptor (FXR) (Tu et al. 2000), and the peroxisome proliferator-activated receptors (PPARs), which can be activated by fatty acids and their metabolic derivatives (Lee et al. 2003). Due to the availability of selective synthetic ligands, the adopted orphan receptors hold great potential for pharmacological treatment of lipid-related disorders. Hence, there is a considerable need for appropriate animal models to study the relative effects of these potential therapeutic targets on lipid and energy homeostasis.

1.2.1 The PPAR Family of Fatty Acid Sensors

Mice carrying germline disruptions of liver X receptors or FXR are viable and will not be discussed here. Instead, we will focus on conditional mouse models of PPAR-gamma and -delta deficiency, which have contributed considerably to our current understanding of how lipid, glucose, and energy metabolism are intertwined (see also the chapter by J.C. Bruning, this volume). The PPARs were originally named after the ability of the founding member of the family, PPAR-alpha, to bind to chemicals that induce proliferation of peroxisomes (Issemann and Green 1990), organelles that are involved in fatty acid oxidation. PPAR-alpha is the molecular target for a class of lipid-lowering agents known as fibrates and is mainly expressed in the liver, where it is involved in regulating lipoprotein synthesis and metabolism. As shown in conventional gene

disruption studies (Lee et al. 1995; Kersten et al. 1999), PPAR-alpha mediates the fasting response by activating fatty acid oxidation, which generates ketone bodies as an energy source for peripheral organs during prolonged starvation. For PPAR-gamma and -delta, mice with conditional genetic deletions have been generated.

1.2.1.1 PPAR-Delta

PPAR-delta (aka PPAR-beta) is ubiquitously expressed and protects mice from diet-induced or genetically induced hyperlipidemia or obesity and hyperlipidemia when an activated form of the receptor is overexpressed in adipose tissue (Wang et al. 2003). To circumvent the problem of frequent embryonic lethality and growth retardation of surviving PPAR-delta-deficient mice (Peters et al. 2000; Michalik et al. 2001), Barak and colleagues chose a Cre/lox-mediated recombination strategy to conditionally disrupt the *Ppard* gene (Barak et al. 2002). Whereas surviving PPAR-delta null mice were lean, selective inactivation in adipose tissue with Cre recombinase expressed under the control of an aP2 (adipocyte fatty acid-binding protein) promoter did not result in hypoadiposity, pointing to a systemic rather than adipocyte-specific effect of PPAR-delta on lipid metabolism. By crossing mice carrying the floxed *Ppard* gene with MyHC-Cre mice (Agah et al. 1997) that express the recombinase driven by the cardiac-specific alpha-myosin heavy-chain promoter, cardiomyocyte-restricted deletion of PPAR-delta was obtained (Cheng et al. 2004). These mice developed lipotoxic cardiomyopathy due to myocardial lipid accumulation as a consequence of decreased myocardial fatty acid oxidation. These conditional knockout studies underline the prominent role of PPAR-delta in fat-burning and energy expenditure by regulating the transcription of genes involved in fatty acid uptake, beta oxidation, and energy uncoupling in muscle and adipose tissue.

1.2.1.2 Involvement of PPAR-Gamma in the Pathogenesis of the Metabolic Syndrome Revealed by Tissue-Specific Inactivation in Fat, Liver, and Muscle

PPAR-gamma, the receptor for the antidiabetic thiazolidinedione drugs (reviewed by Spiegelman 1998), improves insulin sensitivity by inducing genes that are involved in lipid storage and adipocyte differentiation, and can in turn be activated by SREBPs. Like the other PPARs, PPAR-gamma, which exists in two different isoforms, functions as an obligate heterodimer with a retinoid X receptor (see the chapter by D. Metzger, this volume) that interacts with a variety of coactivators and corepressors. Germline disruption of this key regulator of adipogenesis results in embryonic lethality due to placental insufficiency and cardiac defects (Barak et al. 1999; Kubota et al. 1999; Michalik et al. 2001); in mice chimeric for wild-type and PPAR-gamma-deficient cells, null cells did not contribute to adipose tissue (Rosen et al. 1999), a predominant

site of PPAR-gamma expression. Mice with adipose tissue-specific deletions of PPAR-gamma underscored its role in adipogenesis and maintenance of adipocyte function and displayed resistance to diet-induced obesity (He et al. 2003; Imai et al. 2004; Jones et al. 2005). A difference in insulin sensitivity between both models of lipodystrophy in these studies might be related to the use of two different aP2-Cre transgenic lines, which express the recombinase in brown and white adipose tissue to a different extent (Abel et al. 2001). Introduction of a hypomorphic Pro12Ala polymorphism, which is associated with a reduced risk for type 2 diabetes in humans (reviewed in Stumvoll and Haring 2002), into the PPAR-gamma2 isoform by homologous recombination resulted in the disruption of PPAR-gamma2 expression and additional selective absence of the PPAR-gamma1 isoform in white adipose tissue, possibly as a consequence of the introduction of a loxP site upstream of the isoform 2-specific exon B and the downstream presence of a neomycin cassette (Koutnikova et al. 2003). These hypomorphic PPAR-gamma knockdown mice were severely lipodystrophic and displayed a high postnatal mortality rate due to the virtual absence of white adipose tissue. Although PPAR-gamma is expressed at relatively low levels in liver tissue, increased expression levels are observed in steatotic livers. Tissue-specific hepatic disruption was therefore obtained by breeding *Pparg*$^{flox/flox}$ mice to Alb-Cre mice (Yakar et al. 1999), which resulted in reduced hepatic triglyceride levels, hyperlipidemia, and increased insulin resistance in mouse models of hepatosteatosis (Gavrilova et al. 2003; Matsusue et al. 2003), probably due to a delayed uptake of triglycerides by the liver. Partial improvement of these metabolic abnormalities by thiazolidinediones was prevented in mice lacking white adipose tissue (Gavrilova et al. 2003), which points to the importance of this tissue in mediating the insulin-sensitizing effects of this class of drugs. Although PPAR-gamma is expressed at relatively low levels in skeletal muscle, this organ has a major role in mediating the effects of insulin on glucose and lipid metabolism (Moller 2001). Indeed, MCK-Cre^{+}-*Pparg*$^{flox/flox}$ mice with a muscle-specific (Bruning et al. 1998) *Pparg* deletion displayed severe insulin resistance in muscle with subsequent systemic hyperinsulinemia, hyperglycemia, and hypertriglyceridemia (Hevener et al. 2003; Norris et al. 2003). These secondary effects were normalized by thiazolidinediones acting on PPAR-gamma expressed in adipose tissue and liver. Altogether, the tissue-specific knockout studies support a crucial role of PPAR-gamma in the maintenance and action of adipose tissue and point toward its importance in mediating tissue crosstalk between liver, muscle, and fat to coordinate lipid and glucose metabolism.

1.2.1.3 Additional Mouse Models of Conditional PPAR-Gamma Inactivation

Additional mouse models of tissue-specific PPAR-gamma inactivation have been developed; they continue to expand our knowledge of the physiological

functions of this receptor beyond lipid and glucose metabolism. For example, fluid retention and edema, a common side effect of the insulin-sensitizing thiazolidinedione drugs, was abrogated in mice lacking PPAR-gamma in the distal nephron, where it is involved in the regulation of sodium transport (Zhang et al. 2005). This function in fluid homeostasis might contribute to its suggested involvement in the development of hypertension and atherosclerosis (Willson et al. 2001), diseases that are associated with the metabolic syndrome. Other conditional mouse models, which are listed in Table 1, and cannot be discussed in detail due to space considerations, shed light on functions of PPAR-gamma and its ligands in cell cycle progression, inflammation, carcinogenesis, and vascular function.

1.2.1.4
Adipose-Specific Inactivation of PEPCK-C by Deleting Its PPARG-Responsive Element

Mice carrying a null mutation of phosphoenolpyruvate carboxykinase (PEPCK-C), the key rate limiting enzyme of gluconeogenesis in the liver and glyceroneogenesis in fat tissue, die perinatally (She et al. 2000). Interestingly, the absence of PEPCK-C in the liver of Alb-Cre^{+}-*Pepckc*$^{flox/flox}$ - mice did not alter fasting plasma glucose concentrations, but lead to increased levels of free fatty acids in the plasma and hepatic steatosis (She et al. 2000). Olswang and colleagues used an elegant approach to specifically disrupt the expression of the cytosolic form of PEPCK-C in adipose tissue by mutating the binding site for PPAR-gamma (PPARE) in the upstream regulatory region of the encoding gene (Olswang et al. 2002). Expression was abolished in white adipose tissue and markedly decreased in brown adipose tissue, whereas PEPCK-C levels in liver and kidney were not affected. Animals homozygous for the mutated PPARE were viable and lipodystrophic (Olswang et al. 2002).

1.2.2
The PGC-1 Family of Transcriptional Coactivators

Transcriptional coactivators increase the rate of transcription by interacting with transcription factors without binding to DNA themselves. The convergence of a variety of cofactors on a single transcription factor, as well as the action of individual coactivators on different transcription factors, allow for additional levels of complexity in balancing transcriptional responses to environmental stimuli. PGC-1alpha, a member of the PPAR-gamma coactivator-1 (PGC-1) family of transcriptional coactivators, was named after its ability to interact with PPAR-gamma, among other nuclear hormone receptors such as HNF4-alpha (see Sect. 1.2.3). PGC-1alpha and a closely related gene, PGC-1beta, are predominantly expressed in brown adipose tissue and skeletal muscle, key thermogenic tissues. Both coactivators are involved in the up-regulation of genes involved in oxidative metabolism, suggesting a function

Table 1 Additional mouse models of conditional PPAR-gamma inactivation not discussed in the text

Cre transgene (tissues affected)	Phenotype	Reference
Tie-2 (endothelial cells)	Hypertension, tachycardia	Nicol et al. 2005
MX1 (macrophages; liver, spleen, additional organs)	Reduced cholesterol efflux from cholesterol-loaded macrophages	Akiyama et al. 2002
LysM (murine M lysozyme promoter; myeloid cells)	Increased atherosclerotic lesions in atherosclerosis-susceptible mouse models reconstituted with LysM-Cre-PPAR-gamma$^{flox/flox}$ bone marrow	Babaev et al. 2005
Rat insulin promoter (islet beta cells)	Islet hyperplasia; blunted beta diet cell proliferation in response to high-fat	Rosen et al. 2003
MMTV (epithelial cells, B- and T- cells, ovary cells)	Abrogation of conjugated linoleic acid-mediated protection from experimental inflammatory bowel disease	Bassaganya-Riera et al. 2004
MMTV	Impaired female fertility, abrogated mammary gland development secondary to ovarian dysfunction; normal B and T cell development	Cui et al. 2002
Whey acidic protein/WAP (lactating mammary gland)	Normal lactation	Cui et al. 2002
Alpha-myosin heavy-chain (cardiomyocytes)	Cardiac hypertrophy with preserved systolic cardiac function	Duan et al. 2005
Villin (intestinal cells)	Increased susceptibility to experimental inflammatory bowel disease	Adachi et al. 2006

in mitochondrial biogenesis and regulation of energy expenditure. During fasting, hepatic PGC-1alpha is upregulated and contributes to the adaptive beta-oxidation of fatty acids and enhanced gluconeogenesis by induction of PEPCK. A role in glucose and fatty acid homeostasis was further demonstrated through hepatic knockdown of the gene with an intravenously applied RNAi adenovirus targeting PGC-1alpha (Koo et al. 2004), which resulted in fasting hypoglycemia, a fatty liver and enhanced hepatic insulin sensitivity, presumably due to the disrupted action of PGC-1alpha on PPAR-alpha signaling. In contrast, total inactivation of a floxed PGC-1alpha gene through Zp3-driven transgenic expression of Cre recombinase in the female germ line (Lewandoski et al. 1997) resulted in constitutive upregulation of hepatic gluconeogenic gene transcription in the offspring mediated by an increased activity of the transcription factor C/EBP-beta (Lin et al. 2004). Moreover, PGC-1alpha null mice are paradoxically lean and resistant to diet-induced obesity due to hyperactivity, probably as a result of striatal degeneration. These and other open questions will have to be addressed by crossing mice harboring the floxed PGC-1alpha with different tissue-specific transgenic Cre lines. For example, liver-specific ablation revealed that transcriptional upregulation of 5-aminolevulinate synthase (ALAS-1), the key enzyme of heme biosynthesis, during fasting or in response to proporphyrogenic chemicals critically depends on PGC-1alpha (Handschin et al. 2005), establishing a molecular link between nutritional state and hematopoiesis. Adenoviral RNAi-mediated knockdown of hepatic PGC-1beta, which is induced by a high-fat diet along with SREBP-1a and c and interacts with these transcription factors (Sect. 1.1), resulted in a blunted response of lipogenic gene expression after dietary challenge (J. Lin et al. 2005).

1.2.3 HNF4-Alpha, a Master Regulator of Liver Development and Hepatocyte Function

Hepatocyte nuclear factor 4-alpha is a nuclear hormone receptor expressed at high levels in the kidney, pancreas, and liver that binds to DNA as a homodimer. Its activity can be modulated by saturated and unsaturated fatty acid acyl-CoA thioesters in a reciprocal manner (Pegorier et al. 2004). HNF4-alpha plays an important role in the transcriptional regulation of a variety of genes involved in liver development, differentiation, and maintenance of hepatic function. Mutations in the HNF4A gene cause maturity-onset diabetes in the young (MODY), a rare monogenic inherited form of type 2 diabetes mellitus (reviewed by Ryffel 2001), suggesting that HNF4-alpha is also required for the coordination of metabolic networks. While conventional gene disruption of *Hnf4a* resulted in early embryonic lethality prior to the onset of hepatogenesis, postpartal liver-specific inactivation using the Cre-loxP method with an albumin-Cre transgene (Yakar et al. 1999) demonstrated a critical role for hepatic HNF4-alpha in the maintenance of lipid homeostasis (Hayhurst et al. 2001).

In these mice, abnormal lipid and glycogen deposition led to liver hypertrophy, and serum lipid levels were lowered due to a decreased hepatic VLDL secretion. Activation of gluconeogenesis in the fasted state was disrupted, which could be traced to an absolute requirement of hepatic HNF4-alpha for PGC-1alpha-mediated induction (see Sect. 1.2.2.1) of the respective key enzymes, PEPCK and glucose-6-phosphatase (Rhee et al. 2003). Additional defects of hepatocyte-specific metabolic functions in these mice, including ureagenesis and modulation of bile acid homeostasis, have been described (Inoue et al. 2002, 2006). Adenovirus-mediated expression of Cre recombinase in cultured fetal hepatocytes homozygous for the floxed *Hnf4a* allele (Kamiya et al. 2003) led to recognition of the important role of this nuclear receptor for the induction of genes involved in the xenobiotic response during fetal liver development. Indeed, disruption of HNF4-alpha in fetal liver indicated its indispensability for the expression of cytochrome P450 3A4 (Cyp3A4), a key enzyme involved in the biotransformation of xenobiotics. In this study (Tirona et al. 2003), an independently generated mouse model of conditional *Hnf4a* inactivation (Parviz et al. 2002) was crossed with transgenic mice expressing the Cre recombinase under the control of the alpha-fetoprotein (AFP) enhancer and albumin promoter and enhancer (Albafp-Cre), where the floxed allele is deleted in embryonic hepatoblasts between embryonic days 10 and 15 ((Krupczak-Hollis et al. 2004). The same mouse model of fetal hepatic HNF4-alpha inactivation was used to demonstrate the importance of this transcription factor for the epithelial transformation of the liver parenchyma and subsequent generation of normal hepatic tissue architecture (Parviz et al. 2003). These studies exemplify particularly well the value of using different Cre transgenic lines to dissect the diverse functions of pleiotropic genes in the same organ during different developmental stages, thereby allowing study of the metabolic aspects independent of additional roles in organ development and maintenance.

1.3 CCAAT/Enhancer-Binding Proteins and Their Role as Activators of Adipogenesis

1.3.1 C/EBP-Alpha

CCAAT/enhancer-binding protein-alpha (C/EBP-alpha) is the founding member of a family of multifunctional transcription factors and plays a fundamental role in the control of proliferation and differentiation of several cell types, especially of adipocytes, hepatocytes, and hematopoietic stem cells (Ramji and Foka 2002). Mice carrying a germline deletion die perinatally from hypoglycemia as a consequence of a severe dysregulation of glucose homeostasis and display a defect in adipogenesis (Wang et al. 1995). Intravenous injection of a recombinant Cre-expressing adenovirus into mice homozygous for a floxed allele of

the gene made it possible to study the consequences of C/EBP alpha disruption in the adult liver (Lee et al. 1997). These mice developed severe jaundice, which was the result of a reduced expression of the bilirubin-detoxifying enzyme UDP-glucuronosyltransferase expression, and exhibited a downregulation of genes encoding gluconeogenic genes. Since adenovirus infection led to changes in serum lipid levels indicating liver damage, liver-specific C/EBP alpha disruption was obtained by expressing Cre recombinase under the control of an albumin promoter (Inoue et al. 2004). These mice developed age-dependent hepatosteatosis, impaired glucose tolerance, and decreased serum cholesterol levels, resulting from altered expression of enzymes involved in glucose and lipid metabolism. Hyperammonia was also observed. In order to study the effect of a loss-of-function on the differentiation of adipose tissue, Linhart and colleagues rescued the germline knockout by transgenic hepatic expression of C/EBP-alpha under the control of an albumin promoter (Linhart et al. 2001). They observed a selective absence of white vs brown adipose tissue despite high serum lipid levels and hepatic steatosis, which was most likely caused by compensatory lipoprotein uptake by the liver. Another interesting application of conditional gene targeting technology was recently employed to address the role of C/EBP-alpha after birth. Here, a maximal degree of C/EBP-alpha deletion in multiple tissues during postnatal life was obtained by creating mice with a heterozygous composition of the C/EBP-alpha gene locus (Yang et al. 2005). To this end, germline knockout mice were crossed with a line containing a floxed allele (Zhang et al. 2004) expressing Cre recombinase under the control of the inducible MX1 promoter. Injection of pI-pC to induce conditional deletion of the floxed allele resulted in the disruption of C/EBP-alpha in the liver, spleen, adipose tissue, and kidney of these MX1-Cre-Cepba$^{flox/-}$ mice. In contrast to mice with a tissue-selective conditional inactivation of C/EBP-alpha in the liver or in adipose tissue, these animals developed hypophagia and died within 1 month after injection of pI-pC, independent of their age at injection time (neonate or adult). A likely explanation for this lethal phenotype is a severe dysregulation of energy homeostasis, resulting from hypoinsulinemia, depletion of hepatic glycogen stores, loss of triglycerides in white adipose tissue, and impaired liver function. These studies exemplify how the combination of conditional and conventional gene targeting strategies with transgenic tools contributes to the elucidation of complex metabolic interactions.

1.3.2 C/EBP-Beta and Other Family Members

Other members of the C/EBP gene family, which all contain a highly conserved carboxylterminal DNA-binding leucine zipper domain, are also involved in the regulation of lipid and energy homeostasis. For example, surviving compound knockout mice lacking both genes encoding C/EBP-beta and -delta display a defect in terminal adipocyte differentiation (Tanaka et al. 1997). The anal-

ysis of the respective germline mouse knockout models with regard to the regulation of lipid and energy homeostasis has been hampered by pleiotropic effects in different tissues, however. A recently described model of conditional C/EBP beta deletion, which was used to ablate this factor in keratinocytes (Sterneck et al. 2006), will certainly prove useful to address its precise functions in adipocytes, hepatocytes, and other cell types as well.

2 Lipid Transport

Extracellular lipid homeostasis is accomplished by a vast number of proteins that function as lipid carriers, cell surface receptors, or modifiers of lipid-loaded particles. For many of these, conventional germ line disruptions or classical transgenic overexpression of the encoding genes, often in combination, have added important insights into their normal physiological roles and reciprocal interactions during health and disease (Bock et al. 2004). In this section, we will review selected examples of how conditional mouse models have highlighted novel functions of genes involved in different aspects of lipid transport.

2.1 Lipoproteins

Extracellular lipid transport is mediated by lipoproteins, which contain cholesterol, triglycerides, and phospholipids as major lipid components. The proteinaceous component of these particles is comprised of the so-called apolipoproteins, a family of lipid-binding proteins that mediate the interaction of circulating lipoproteins with metabolizing enzymes and cell surface receptors. Apolipoprotein A-I, A-II, and A-IV are derived from different genes and are components of high-density lipoprotein (HDL), which is implicated in reverse cholesterol transport from peripheral organs to the liver and therefore considered anti-atherogenic. The major apolipoprotein component of the proatherogenic low-density lipoprotein (LDL) and of chylomicrons, apolipoprotein B, exists in two isoforms that result from differential splicing of the apoB gene transcript. Covalent linkage of apoB with highly glycosylated apolipoprotein(a) results in atherothrombogenic Lp(a) particles. The different apoC proteins, especially apolipoproteins C-II and C-III, are mainly involved in triglyceride metabolism. Apolipoprotein E is found in all major lipoprotein classes except LDL and can bind to all core members of the family of LDL receptor-related proteins (LRPs) (reviewed by Herz and Bock 2002). The role of other apolipoproteins in lipid metabolism is less well defined. Using high-pressure tail-vein injection of siRNAs directed against the encoding mouse gene, Wolfrum and colleagues showed that the lipocalin apolipoprotein M participates in the formation of pre-beta-HDL and cholesterol efflux to HDL, and that *Apom*-deficient mice are susceptible to atherosclerosis (Wolfrum et al. 2005).

Most of the genes encoding apolipoproteins and lipoprotein-modifying enzymes have been deleted by conventional mutagenesis or overexpressed (reviewed in Bock et al. 2004). However, relatively few examples for the use of conditional mutagenesis techniques to study their physiological functions exist. These models will be discussed in the following sections.

2.1.1 Aplipoprotein(a)

lLp(a) is naturally absent from most mammals except humans and Old World monkeys. Its proatherogenic properties are largely determined by its unique apo(a) component, which is very heterogeneous due to a profound size polymorphism of the encoding gene (reviewed by Berglund and Ramakrishnan 2004). Plasma levels of Lp(a) are positively correlated with an increased risk for atherosclerosis and might be influenced by the presence of a putative transcriptional control region 20 kb upstream of the apo(a) gene. In order to address the physiological significance of this regulatory region, a yeast artificial chromosome (YAC)-transgenic mouse model carrying a hepatically expressed human apo(a) allele containing large up- and downstream DNA regions was developed. LoxP sites flanking the putative transcriptional control region were introduced to allow its Cre-mediated excision (Huby et al. 2003). Indeed, absence of the activator region led to a modest reduction in plasma apo(a) levels, which demonstrates the potential of using conditional mutagenesis to investigate regulatory mechanisms of human genes without a murine orthologue in a physiological context. Effects on plasma lipid levels or atherogenicity were not reported in this study.

2.1.2 A Mouse Model of Familial Hypobetalipoproteinemia

Hypobetalipoproteinemia is defined by plasma levels of apolipoprotein B or LDL cholesterol below the 5th percentile. Among the hereditary causes are truncating mutations of apoB, which lead to reduced production and accelerated clearance rates (Schonfeld 2003). As a consequence of reduced VLDL secretion, triglycerides accumulate in the liver, resulting in hepatosteatosis. In order to model familial hypobetalipoproteinemia in mice, Chen and colleagues (Chen et al. 2000) introduced a single-nucleotide deletion into the mouse *apob* gene, giving rise to a truncated isoform, apoB-38.9. In contrast to previously described mouse models of truncated apoB (Homanics et al. 1993; Kim et al. 1998a), which harbor several copies of the modified gene, the potential influences of foreign DNA sequences in the genome were minimized in these mice by combining a knockin strategy with Cre-mediated removal of the neo cassette in the neighboring intron. A similar approach was used to generate apoB-27.6-producing mice (Chen et al. 2002). These mouse models exhibit

features of human familial hypobetalipoproteinemia (Z. Chen et al. 2000, 2002, 2004; X. Lin et al. 2002, 2005b). Interestingly, homozygosity for the apoB-27.6 encoding allele resulted in a high degree of embryonic lethality, which is reminiscent of *Apob* -deficient mice, whereas the yield of homozygotes was reduced in animals producing apoB-38.9 (Chen et al. 2002). This suggests that the peptide segment between the carboxyl termini of both isoforms (amino acids 1254–1744) is essential for embryonic development.

2.1.3
A Hypomorphic Apolipoprotein E Mouse Model

Apolipoprotein E is an important constituent of lipoproteins in the plasma and in the nervous system that participates in lipid metabolism, cell growth and repair, and modulates cellular signaling cascades mediated by transmembrane receptors (Mahley and Rall 2000). Human apoE is a polymorphic protein encoded by different alleles of a single gene. The reverse genetic association of two major apoE isoforms with hyperlipoproteinemia, cardiovascular and neurodegenerative disease has sparked an enormous interest in the structure–function relationships of this protein, and numerous applications of apoE-deficient, knockin, and transgenic mice have been described (reviewed by Hofker et al. 1998; Meir and Leitersdorf 2004). The apoE4 isoform, which is genetically linked to Alzheimer's disease and atherosclerosis, differs from the two other major human isoforms, apoE2 and apoE3, and from murine apoE in that an intramolecular interaction between its amino- and carboxylterminal domains (Dong and Weisgraber 1996) profoundly alters the structural and functional properties of the molecule. In order to mimic the apoE4 domain interaction in murine apoE, a critical threonine residue at position 61 was exchanged against arginine by a sequence replacement gene targeting strategy, which included a floxed neo cassette close to the mutated site (Raffai et al. 2001). Whereas removal of the neo cassette by crossing homozygous mice with a Cre deleter transgenic mouse resulted in normal levels of apoE expression, its retention yielded a hypomorphic allele with only 2%–5% of regular plasma concentrations (Raffai and Weisgraber 2002). The hypomorphic apoE mice displayed an increased susceptibility to diet-induced hypercholesterolemia, which could be reversed by pI-pC-mediated expression of Cre under the control of the MX1 promoter. Thus, they represent a model of conditional gene repair and provide a useful complement of the available tools to study tissue-specific roles of apoE (Raffai et al. 2003, 2005).

2.1.4
Tissue-Specific Inactivation of Lipoprotein-Modifying Enzymes

A range of different enzymes participate in lipid metabolism by modifying the lipid composition of nascent circulating lipoproteins. Microsomal triglyceride

transfer protein (MTP) is of particular interest because germline inactivation of the encoding gene in mice results in embryonic lethality (Raabe et al. 1998). MTP is a heterodimer with lipid transfer activity that is essential for the assembly and secretion of apoB-containing lipoproteins. Mutations of the large subunit cause abetalipoproteinemia, a rare disease characterized by the near-complete absence of apoB-containing lipoproteins in the plasma, i.e., VLDL, LDL, and chylomicrons. Hepatic inactivation in two different mouse models homozygous for floxed alleles of the *Mttp* gene was either achieved by injection of a Cre-expressing adenovirus (Chang et al. 1999; Raabe et al. 1999) or by crossing them with MX1-Cre-transgenic mice and injecting pI-pC to induce inactivation of the floxed gene (Raabe et al. 1999). Both models displayed a robust decrease in plasma VLDL and HDL as well as drastically reduced apoB-100 levels and moderate hepatic steatosis, whereas differences in the degree of reduction of apoB-48 were reported. Inducible MX1-Cre-mediated inactivation of *Mttp* in atherosclerosis-prone LDL receptor-deficient mice expressing only apoB-100 reversed hypercholesterolemia and development of atherosclerosis (Lieu et al. 2003). This suggests a potential for MTP-inhibiting substances as atheroprotective drugs, although effects on hepatic triglyceride accumulation must be taken into account.

2.1.5 Lipoproteins as Vehicles for Signaling Molecules

Recently, an as yet unrecognized function of lipoprotein particles beyond lipid transfer between cells was described (Panakova et al. 2005). Biochemical co-purification showed that morphogens carrying lipid modifications and lipophilic glycosylphosphatidylinositol (GPI)-linked insect proteins co-purify with lipophorins, insect analogs of vertebrate lipoproteins (Ryan 1990). Inactivation of lipophorins in *Drosophila* larvae was achieved by means of RNA interference. A modified GAL4-UAS (upstream activation sequence) system (Brand and Perrimon 1993) combined with heat-shock dependent expression of the yeast flippase (FLP) recombinase (Ito et al. 1997) allowed the inducible reduction of *apolipophorin* transcript levels by heat shock at 37°C. This resulted not only in a perturbed lipid transport, but also disrupted the long-range movement, possibly the endocytic uptake, and biological action of the morphogens Wingless and Hedgehog (Panakova et al. 2005). These data raise the intriguing possibility that lipoproteins and their receptors might influence morphogen signaling in vertebrates by regulating their transport and availability, as has been suggested for several members of the LRP family (Sect. 2.2.1). Interestingly, soluble amyloid-beta, which is generated by proteolytic cleavage of the amyloid precursor protein APP, binds to apolipoprotein E (reviewed by Selkoe 2002). It remains to be established if and how this interaction contributes to the regulation of its synaptic signaling functions through nicotinic receptors (Snyder et al. 2005).

This novel link between lipoproteins and secreted signaling molecules would provide an additional means of synchronizing nutrient availability with growth and patterning during development.

2.2 Lipid Transport Across the Cell Membrane

The intracellular uptake of lipoproteins is mediated by members of the low-density lipoprotein (LDL) receptor gene family, which consists of seven core members in mammals. The LDL receptor-related proteins (LRPs) are type I transmembrane receptors of considerably different sizes that share a similar structure of their extracellular domains. All LRPs contain at least one NPXY (for asparagine-proline-any amino acid-tyrosine) tetra-amino acid motif in their intracellular domains, which was first described as an endocytosis motif that is important for the assembly of intracellular signaling complexes as well (reviewed by Herz and Bock 2002). Other lipid receptors include the class of scavenger receptors, which are involved in the cellular uptake of chemically modified lipoproteins and of HDL-derived cholesteryl esters. Specialized membrane-associated fatty-acid-binding and transport proteins such as fatty acid translocase and fatty acid transporter protein have been identified as well (reviewed by Dutta-Roy 2000). A fourth group of major transmembrane lipid transport proteins is represented by the large family of ATP-binding cassette (ABC) transporters. These multiple-membrane spanning proteins translocate a multitude of lipophilic and xenobiotic substances across lipid bilayers. One of its members, ABCA1, mediates the cellular efflux of cholesterol and phospholipids to high-density lipoprotein particles and therefore seems to be critical for reverse cholesterol transport. Mutations in this gene cause the rare autosomal-recessive Tangier disease, which is characterized by the near absence of atheroprotective HDL in the plasma and deposition of cholesteryl esters in reticuloendothelial cells (reviewed by Oram and Heinecke 2005). Its expression is regulated by an intricate transcriptional network involving, for example, PPAR and SREBP. Liver-specific ablation of *Abca1* in mice by different technical approaches has been reported by several groups. The analysis of Alb-Cre-*Abca1*$^{flox/flox}$ mice provided evidence for the importance of hepatic ABCA1 in the lipidation of nascent apoA-I, a crucial HDL component, and suggested that the liver is the major source of circulating HDL (Timmins et al. 2005). Similar results were obtained in mice in which *Abca1* was downregulated by injecting an siRNA-mediating adenovirus (Ragozin et al. 2005). Chang and colleagues described a model based on Cre-loxP-induced RNA interference combined with the tetracycline-on system under control of the albumin promoter to obtain spatiotemporally controllable conditional *Abca1* inactivation (Chang et al. 2004).

2.2.1 Conditional Inactivation of LRPs

2.2.1.1 Functions of LRP1 in Lipoprotein Metabolism and Cell Signaling

Mutations in the gene encoding the LDL receptor, the founding member of the LRP family of endocytosis and signaling receptors, were identified as the underlying cause of autosomal-dominant familial hypercholesterolemia. Conventional germline knockout of the Ldlr gene in mice confirmed its crucial role in the turnover of LDL cholesterol and provided a widely used animal model for hypercholesterolemia and related diseases (Ishibashi et al. 1993). LRP1 was cloned based on its close structural homology to the LDL receptor (Herz et al. 1988). Because of its ability to bind to apoE-containing lipoproteins in vitro, it was suggested that LRP1 might function as a hepatic receptor for apoB-48 and apoE-containing chylomicron remnants, which do not accumulate in LDL receptor-deficient humans and animals. Since germline inactivation of LRP1 results in midgestation embryonic lethality, a proof for its identity with the hepatic chylomicron receptor came from its liver-specific inactivation by adenovirus-mediated Cre recombinase gene transfer (Rohlmann et al. 1996) or pI-PC induction of MX1-driven Cre expression in mice carrying floxed LRP1 alleles on an LDL receptor null background (Rohlmann et al. 1998). However, LRP1 is a ubiquitously expressed multifunctional receptor that not only functions as an endocytic receptor for lipoproteins, but also participates in signal transduction pathways (reviewed by May et al. 2005). Its extracellular ligands include proteinases, extracellular matrix proteins, growth factors such as PDGF and the Alzheimer disease gene amyloid precursor protein (APP) (reviewed by Herz and Strickland 2001). Conditional ablation of LRP1 in smooth muscle cells (SMCs) by Cre recombinase expression under the control of the Sm22 promoter (Holtwick et al. 2002) resulted in severe defects of vascular wall integrity, namely smooth muscle cell proliferation, disruption of the elastic layer, and formation of aortic aneurysms (Boucher et al. 2003). Cholesterol feeding markedly increased susceptibility to atherosclerosis without altering the lipid profile of SMC-LRP1-deficient mice, which could be reversed by treating the animals with the PDGF receptor tyrosine kinase inhibitor imatinib, indicating that the observed upregulation of PDGF receptor expression and signaling in vascular smooth muscle cells is crucial for the development of this striking phenotype (Boucher et al. 2003). Tissue-specific knockout of this versatile protein in differentiated neurons of $LRP1^{lox/lox}$ mice by expressing Cre recombinase from a synapsin promoter (Zhu et al. 2001) induced behavioral and motor abnormalities as well as hypoinsulinemia, resulting in premature death at around 9 months of age (May et al. 2004). Since no gross neuropathological abnormalities on a macroscopic or histological level were observed, these symptoms might be attributable to a functional deficit in neurotransmission.

Additional conditional mouse models of cell type-specific LRP1 deletion using the Cre-lox system will be required to analyze the broad physiological spectrum of this receptor in the brain as well as in peripheral tissues (see Table 2).

2.2.1.2 Megalin and Its Role During Embryonic Development

Megalin (LRP2, aka gp330) is a large multi-ligand binding LRP mainly expressed by resorptive epithelia, pointing to a function in transport and endocytosis. Surviving mice carrying a targeted deletion of the *Lrp2* gene (Willnow et al. 1996) display holoprosencephaly similar to the human Smith-Lemli-Opitz syndrome (a recessive defect of cholesterol biosynthesis), a phenotype that is also observed in mice lacking the megalin ligand apoB-100 (Homanics et al. 1993) or the morphogen Sonic hedgehog (Shh), another megalin ligand that is lipid-modified by attachment of a single cholesterol moiety and palmitate (reviewed by McCarthy and Argraves 2003). Because megalin is highly expressed both in the developing neuroepithelium and in the yolk sac endoderm, it was unclear whether the holoprosencephaly is merely the result of an impaired apoB-mediated placental cholesterol transport defect or involves additional receptor activities in the forebrain (discussed by Farese and Herz 1998). This question was directly addressed by selective inactivation of megalin in the epiblast excluding the yolk sac (Spoelgen et al. 2005). To this end, mice carrying floxed megalin alleles (Leheste et al. 2003) were crossed with knockin mice expressing Cre recombinase from the Mox2 locus (Tallquist and Soriano 2000), which means the placental contribution to the knockout phenotype can be determined. This study indicated that sustained expression of megalin in the yolk sac did not rescue the forebrain phenotype of megalin deficiency, and pointed to a role of megalin in morphogen-mediated forebrain patterning (Spoelgen et al. 2005). A similar effect on morphogen function during limb development was recently shown for another LRP member, MegF7 (LRP4) (Johnson et al. 2005). Studies using mouse models of conditional LRP inactivation that have not been mentioned in the text are listed in Table 2.

2.2.2 Disruption of the Sinusoidal Endothelium in the Liver

The hepatic uptake of lipoprotein remnants by the LDL receptor or LRP1 takes place within the space of Disse, which separates hepatocytes from fenestrated sinusoidal endothelial cells lining the sinusoidal vasculature in the liver (Fraser et al. 1995). By temporally downregulating vascular endothelial growth factor (VEGF) signaling in the developing liver (Carpenter et al. 2005), the structure and fenestration of the hepatic sinusoidal endothelium was disrupted. This was accomplished through the tetracycline-repressible expression of a truncated VEGF receptor-2 construct lacking a cytoplasmic tyrosine kinase domain,

Table 2 Studies with mouse models of conditional LRP inactivation not mentioned in the text

Mouse model	Phenotype / conclusion from study	Reference
MX1-Cre-LRP1$^{lox/lox}$	Elevated factor VIII levels, further increase after adenoviral RAP (receptor-associated protein) gene transfer	Bovenschen et al. 2003
MX1-Cre- LRP1$^{lox/lox}$ on different LRP-deficient backgrounds	Participation of LDL receptor in factor VIII clearance	Bovenschen et al. 2005
MX1-Cre-LRP1$^{lox/lox}$ on LDLR$^{-/-}$ ApoE$^{-/-}$ background	Increased atherosclerosis independent of plasma cholesterol	Espirito Santo et al. 2004
MX1-Cre-LRP1$^{lox/lox}$	LRP1 is dispensable for Plasmodium sporozoite infectivity in mice	(Marshall et al. 2000)
Overexpression of RAP in MX1-Cre-LRP1$^{lox/lox}$ mice lacking LDLR on LDLR$^{-/-}$ VLDLR$^{-/-}$ background	Hypertriglyceridemia and increase in plasma VLDL levels	(van Vlijmen et al. 1999)
	Delayed triglyceride clearance	Espirito Santo et al. 2005
ApoE-megalin $^{lox/lox}$	Kidney-specific inactivation resulting in vitamin D deficiency and osteomalacia	Leheste et al. 2003
	Disturbed vitamin A homeostasis	Raila et al. 2005
	Downregulation of the disabled-2 (Dab2) adapter protein in proximal tubule cells	Nagai et al. 2005
	Impaired trafficking of renal inorganic sodium phosphate co-transporter	Bachmann et al. 2004
	Impaired proximal tubular reabsorption of radioactively labeled somatostatin	de Jong et al. 2005
	Defect in renal reabsorption of myoglobin	Gburek et al. 2003

which is important for transduction of the VEGF signal, under control of a C/EBP-beta promoter. As a consequence, lipoprotein uptake was severely impaired, underlining the importance of this anatomical filter for lipoprotein metabolism and lipid homoeostasis (Carpenter et al. 2005).

3 Conclusions and Outlook

The availability of a multitude of conditional mouse models for genes related to lipid metabolism is a powerful resource to study many different aspects of lipid physiology and lipid-related disorders. The examples discussed in this chapter illustrate how the tissue-specific mutagenesis of various genes highlights the prominent role of the liver and adipose tissue, but also of macrophages and skeletal muscle, in the regulation of lipid homeostasis, as well as the intimate connection between lipid and carbohydrate metabolism. The delineation of central nervous pathways exerting metabolic control will bring about an increase in applications of neurobiological tools and techniques, as exemplified by the combination of virus-mediated retrograde neuronal tracing technology with the Cre-lox system to identify higher cortical centers that modulate hypothalamic leptin signaling (DeFalco et al. 2001). Emerging areas of research, such as the function of lipoproteins and their receptors beyond mere lipid transport in cellular signaling and development, or the connection between lipid metabolism and neurodegenerative diseases, will profit from the ongoing technical improvements in generating and analyzing conditional animal models.

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Conditional Mouse Models to Study Developmental and Pathophysiological Gene Function in Muscle

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Abstract This chapter will review conditional mouse model systems that have been developed to study gene function in skeletal, cardiac, and vascular smooth muscle cells in vivo with an emphasis on the utility of these models for investigating developmental and pathophysiological gene function in muscle. In general, these systems have utilized muscle-specific/selective promoter-enhancers in conjunction with site-specific DNA recombinases, e.g., Cre-loxP, and fusion proteins with these recombinases that confer temporal control, such as tamoxifen-inducible CreER systems. A major focus of this chapter will be to discuss unique challenges of studying Cre-mediated mutagenesis/gene targeting in these muscle types during development and in the adult animal, some of which are inherent of the muscle cell type being studied. For example, unlike cardiac and skeletal muscle cells, the vascular SMC is extremely plastic and able to undergo rapid phenotypic modulation to various environmental cues in vivo. Thus, employing SMC marker gene promoter enhancers for conditional gene targeting in SMCs must take into account the possibility and/or certainty

that the particular SMC promoter enhancers used may or may not be transcriptionally active in SMCs of a vessel wall under normal and some pathophysiological conditions. Moreover, individual floxed loci within the same muscle cell type and tissue have different degrees of sensitivity to Cre, most likely dependent on the chromatin state of that particular gene, i.e., closed/condensed state or open/active state. Thus, Cre recombination may be ineffective for specific floxed gene DNA. Lastly, rigorous efforts must be made to confirm the degree of recombination in a tissue, taking into full account the multicellularity of the tissue, to understand the extent of the physiological effect in that organ.

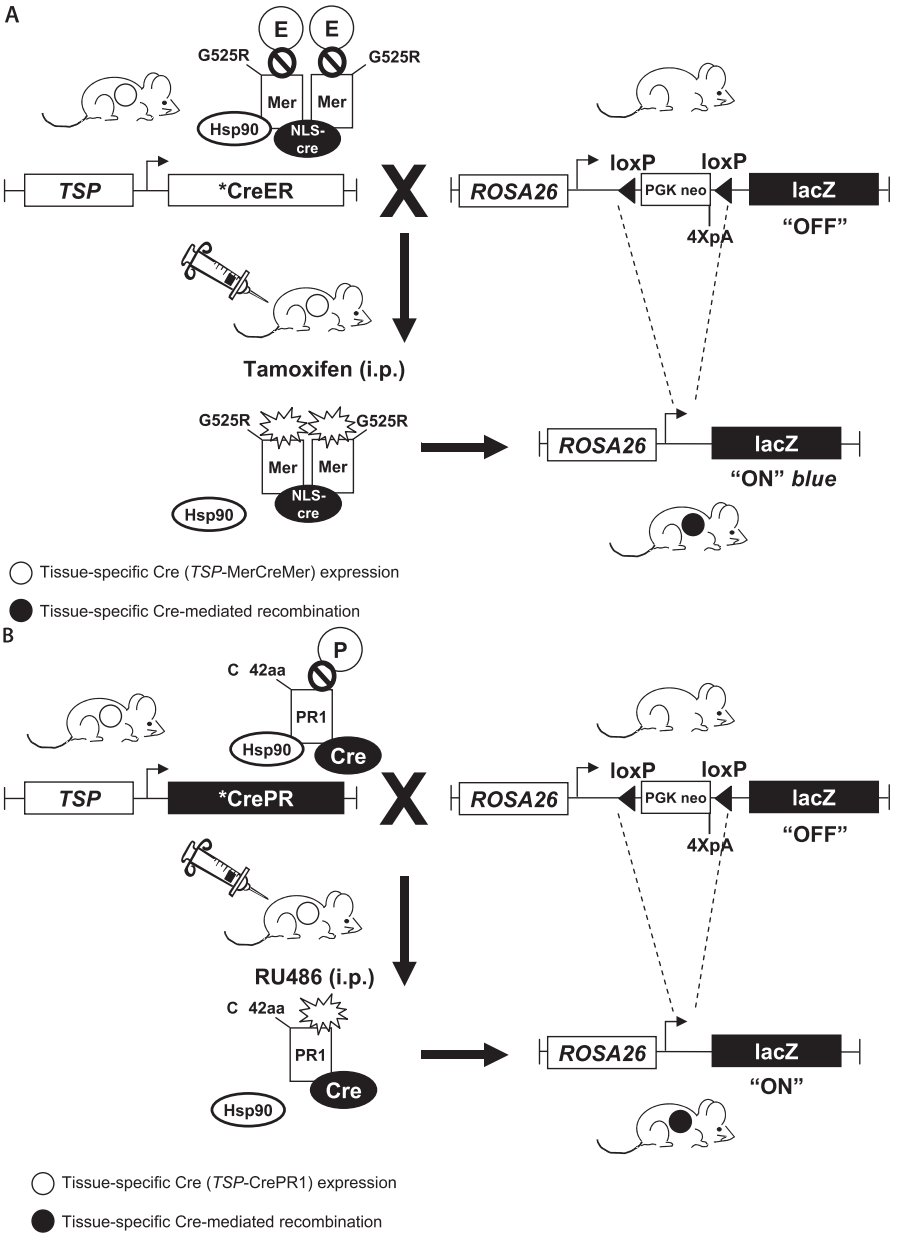
Keywords Skeletal muscle · Cardiac muscle · Smooth muscle · Cre-recombinase · Promoter

1 Introduction

Generation of conventional knockout (KO) mice by homologous recombination in embryonic stem cells has been widely exploited as a powerful tool by biologists over the last decade as a primary means to elucidate the function of candidate genes in vivo. Indeed, there has been remarkable progress in determining whether a gene is dispensable during development and undoubtedly such approaches have greatly advanced our understanding of many genes in the pathogenesis of multiple diseases and maintenance of normal physiological function in the adult animal. However, there are a number of inherent limitations of conventional knockout mice that limit their utility. For example, it is very difficult to ascertain what changes are primary vs secondary. Many investigators have inappropriately assumed that comparison of complex phe-

Fig. 1 A,B Tissue-selective conditional mutagenesis in mice. **A** Tamoxifen-inducible Cre recombination. Using a tamoxifen-responsive mutated estrogen receptor fused to Cre (*CreER*) under control of a tissue selective promoter (*TSP*; *left mouse*), tissue-selective Cre recombination can be induced following a series of i.p. injections of tamoxifen (Sect. 1). *The specific variation of CreER depicted here is the MerCreMer protein described by (Verrou et al. 1999) and in the chapter by R. Feil, this volume. Other variations of CreER include MerCre (Verrou et al. 1999), $CreER^{T}$, and $CreER^{T2}$ (Feil et al. 1997). **B** RU486-inducible Cre recombination. Using a modified RU486 (antiprogesterone) -responsive mutated progesterone receptor fused to Cre (CrePR), tissue-selective Cre recombination can be induced following a series of i.p. injections of RU486 (Sect. 1). *The specific variation of CrePR depicted here is CrePR1 described in (Wunderlich et al. 2001) and in the chapter by R. Feil, this volume. Note that endogenous estrogen (*E*) and progesterone (*P*) do not bind to MerCreMer and CrePR1, respectively. In both **A** and **B**, the *TSP*-CreER or *TSP*-CrePR mouse are crossed to the ROSA26 Cre indicator mouse line (*right mouse*). The ROSA26 Cre reporter mouse contains a *flox*ed PGK neomycin resistance gene with 4 polyA repeats followed by the β-galactosidase/lacZ reporter gene (Soriano 1999). The 4 polyA repeats serve as a transcriptional termination sequence such that only when the loxP-PGKneo 4X polyA-loxP DNA sequence is recombined by Cre is the lacZ gene translated. lacZ expression is typically determined by a colorometric assay using a substrate for the lacZ enzyme (X-gal) that stains lacZ expressing cells *blue*, as shown in Figs. 2 and 3

notypes between KO and wild type (WT) mice involve a single variable, i.e., the absence of a single gene of interest. Perhaps the most explicit examples of this are gene profiling experiments that compare KO vs WT mice showing literally hundreds if not thousands of gene differences. One of the most striking is the



ICAM-1 (intracellular adhesion molecule 1) KO mouse, which shows not only altered leukocyte adherence to endothelial cells, as originally hypothesized (Sligh et al. 1993), but is also grossly obese and hyperinsulinemic at an old age on normal chow and much earlier when fed a high-fat diet (Dong et al. 1997).

Thus, the ideal in vivo gene targeting system would be one that allows for tissue-selective gene targeting in a timed or conditional manner, i.e., tissue-selective conditional mutagenesis/gene targeting. To this end, chimeric proteins have been developed that include Cre recombinase (see the chapter by R. Feil, this volume) fused to a mutated hormone binding domain (HBD) from either the estrogen receptor or the mutated progesterone receptor system (see the chapter by R. Feil, this volume) (Fig. 1). The underlying principle is that tissue selective activity of Cre is silent until the selective ligand binds the mutated hormone binding domain of the fusion protein. Tissue-selective conditional mutagenesis systems require two mouse lines, a tissue-selective HBD-Cre mouse line that is crossed to a mouse that has been genetically engineered to have the endogenous gene of interest *flox*ed by two *loxP* sites (see chapter by V. Brault et al., this volume). Upon activation of the HBDCre complex, tissue-selective Cre recombination and mutagenesis of the *flox*ed gene of interest occurs. Extensive work has gone into modifying the HBDCre fusion proteins and multiple generations of these systems have evolved. In this chapter, we will refer to the two most widely used systems for muscle-selective conditional mutagenesis: the CreER systems (tamoxifen responsive) (Feil et al. 1997;Verrou et al. 1999) (Fig. 1A) and the CrePR systems (RU486 responsive) (Kellendonk et al. 1996) (Fig. 1B). The evolution, principles and multiple variations of these model systems are described in greater detail in the chapters by R. Feil and W. Weber and M. Fussenegger, this volume and schematically in Fig. 1 for reference throughout this chapter.

The overall focus of this chapter will review current tissue-selective conditional mouse models available to study gene regulation in skeletal (Sect. 2), cardiac (Sect. 3) and vascular smooth muscle (Sect. 4) cells with select examples to illustrate the utility of these models for investigating developmental and pathophysiological gene function. Each section contains a table that summarizes available skeletal, cardiac, and smooth muscle conditional gene targeting models and describes the pattern of transgene expression during development and in the adult. We have also included available skeletal-, cardiac-, and smooth muscle-selective Tet-regulatory mouse lines for reference to the chapter by R. Sprengel and M.T. Hasan, this volume, although there is no discussion of these models in this chapter. Given the vast number of available models and limited space, we have chosen select systems within muscle types that illustrate important general concepts and caveats that can be encountered when using Cre systems to study conditional gene targeting in any cell type. We apologize in advance to our colleagues for omitting discussion of many excellent studies in this rapidly expanding field.

2 Gene Targeting in Skeletal Muscle

2.1 Directing Gene Targeting to Skeletal Muscle During Development and/or Differentiated Skeletal Muscle Fibers

Many genes involved in skeletal muscle development, function and regeneration are also involved in other cell lineages. For example, skeletal, cardiac, and smooth muscle are similar in that each is a contractile tissue that modulates force development through calcium-dependent processes; yet these muscle types have distinct embryological origins and specialized functions that are inherent to the expression of unique contractile protein isoforms or transcription factors required for lineage determination and function. However, as shown in Table 1, many of the Cre models that have been developed use promoter enhancers of proteins that are active in both cardiac and skeletal muscle, as well as skeletal muscle alone. Moreover, in skeletal muscle restricted instances, the promoter enhancer construct is not always active in myoblasts during embryogenesis or muscle differentiation but often restricted to the differentiated skeletal muscle fibers. Thus, the purpose of Table 1, as well as Tables 2 and 3, is to depict the selectivity of Cre recombination under control of multiple skeletal muscle selective promoter enhancers. What is also apparent from Table 1 is the disparity of available inducible/conditional Cre models that employ either CreER or CrePR systems to study conditional gene targeting in skeletal muscle (Fig. 1) and thus the major focus of this section will be to review noninducible skeletal muscle promoter enhancer Cre lines.

There are several advantages and disadvantages to each of the Cre lines in Table 1. However, when used appropriately, these lines serve as powerful tools to determine the function of a gene during skeletal muscle development and in differentiated skeletal muscle fibers. For example, the transgenic *MCK*-Cre (muscle creatine kinase promoter enhancer) line is unique in that *MCK* is not expressed in myoblasts but is upregulated in differentiated multinucleated skeletal myotubes (Wang et al. 1999; Andrechek et al. 2002) as well as cardiac myocytes (Table 1). The *MCK*-Cre line can thus be used to ask specific questions pertaining to gene function in the differentiated skeletal muscle fiber, presuming the *flox*ed gene candidate plays a minimal role in cardiac development or function. In contrast, the *Myf5*-Cre^{ki} line, where Cre is knocked into the *Myf5* locus, faithfully recapitulates the expression pattern of the endogenous *Myf5* gene and is uniformly expressed in all proliferating myoblasts (Tallquist et al. 2000). This is not surprising given that *Myf5* is a myogenic regulation factor (MRF). MRFs are bHLH transcriptional activators, and the four family members, Myf5, MRF4 (also called Myf6), MyoD (also called Myod1) and myogenin, are important in skeletal muscle specification and differentiation. Moreover, knockout of *Myf5* is embryonically lethal and muscle differentiation does not

Table 1 Mouse models of conditional mutagenesis in skeletal muscle

Promoter enhancer expression pattern	Promoter	Gene/protein encoded System	Embryogenesis	Adult	Reference
MCK	Muscle creatine kinase	Cre	SKM, cardiac	SKM, cardiac	Wang et al. 1999
		tTA			Ghersa et al. 1998; Ahmad et al. 2000
		rtTA			Grill et al. 2003
HSA	Human SKM α-actin	Cre	Myoblasts, SKM fibers, cardiac	SKM, cardiac	Miniou et al. 1999
		$CreER^{T2}$			Schuler et al. 2005
Myf5	Myogenic regulation factor	$CreER^{ki}$	Myoblasts, SKM	SKM	Tallquist et al. 2000
mic1f	Myosin light chain 1f	Cre, Cre^{A}	SKM (activated late)	SKM fast-twitch fibers	Bothe et al. 2000
mef2c	Myocyte enhancer factor	Cre	Myoblasts, SKM	SKM	Heidt and Black 2005
MyoD	Myogenic regulation factor	Cre	Myoblasts, SKM fibers	SKM (weak), satellite cells (injury)	Chen et al. 2005

SKM,— skeletal muscle; $Cre^{k,i}$ —Cre knock-in, must be carried as +/–; *CreA,*— extrinsic polyA signal; $CreER^{T2,}$ tamoxifen-inducible; *tTA,* —TET-OFF; *rtTA,* —TET-ON

occur, most probably because *Myf5* is the first MDF to be expressed in the developing somite and is restricted to the forming myotome (Montarras et al. 1991). Thus, the *Myf5*-Cre^{ki} line can be used to ask the specific role of a *flox*ed gene candidate in skeletal muscle development, whereas the *MCK*-cre line can be utilized to determine gene loss-of-function in the differentiated muscle fiber.

To illustrate the use of these two lines in determining the role of a *flox*ed candidate gene in myogenic differentiation and function in maintaining differentiated skeletal muscle fibers, Huh and colleagues performed separate crosses with *Myf5*-Cre^{ki} and *MKC*-Cre to a *flox*ed Rb (retinoblastoma protein) gene mouse (Huh et al. 2004). Phosphorylated Rb plays a key role in controlling cell cycle progression through the G1 restriction point for entry into S-phase. During myogenic differentiation, proliferating myoblasts must also exit the cell cycle from the G1 phase, before the restriction point. Therefore, it was hypothesized that Rb plays an analogous role in myoblasts by regulating the switch from proliferation to differentiation. To test the requirement for Rb

in myogenic differentiation, Huh and colleagues examined the proliferation and differentiation potential of primary myoblasts in which a *flox*ed Rb allele was deleted either before (*Myf5*-Cre^{ki}) or after (*MCK*-Cre) differentiation. *Myf5*-Cre^{ki} x *flox*-Rb, lacking Rb in myoblasts, died immediately after birth and exhibited high numbers of apoptotic nuclei and an almost complete absence of myofibers, i.e., severely impaired myogenesis. In contrast, *MCK*-Cre x *flox*-Rb, lacking Rb in differentiated myofibers, were viable and exhibited a normal skeletal muscle phenotype and ability to regenerate. Thus, using this approach, determining the role a gene plays in skeletal muscle differentiation and function in the differentiated muscle fiber can be determined.

Recently, two new models have been developed that allow for selective myoblast Cre recombination and restriction of Cre-recombination to adult skeletal muscle fibers, the *MyoD*-Cre and *mef2c*-Cre mouse lines (Heidt and Black 2005; Chen et al. 2005) (Table 1). The *MyoD*-Cre line is very similar to the *Myf5*-Cre^{ki} line in that MyoD is expressed in skeletal muscle progenitors beginning at mouse embryonic day 9.5 and high levels are maintained throughout development. In adults, MyoD is expressed at low levels in the mature muscle fiber, but is upregulated in satellite cells after muscle injury (Hawke and Garry 2001). Thus, this model system can be used to study the role of a specific gene candidate not only in skeletal muscle differentiation, but also in undifferentiated myogenic precursor cells (satellite cells) in the adult mouse. The *mef2c*-Cre mouse also provides a unique tool in that this line shows complete recombination in all fast and slow twitch muscle fibers, whereas the *mlc1f*-Cre mouse is restricted to fast twitch (Bothe et al. 2000). Activity of the *mef2c* and *mlc1f* promoter enhancers are completely restricted to skeletal muscle throughout development and in adult mice.

Interpreting Cre-recombination in a multicellular tissue, such as muscle, can be a major challenge. The *ROSA26* Cre-reporter mouse (R26R) contains a *flox*ed PGK neomycin resistance gene with 4 polyA repeats followed by the β-galactosidase/lacZ reporter gene (Soriano 1999) (Fig. 1A, B). The four polyA repeats serve as a transcriptional termination sequence such that only when the loxP-PGKneo 4X polyA-loxP DNA sequence is recombined by Cre is the lacZ gene translated. lacZ expression is typically determined by a colorometric assay using a substrate for the lacZ enzyme (X-gal) that stains lacZ expressing cells blue. However, the *ROSA26* Cre-indicator mouse merely serves as a qualitative tool to assess Cre recombination by this colorimetric assay in histological cross-section or whole tissue samples. Further quantification of excision of the *flox*ed allele can be determined by Southern blot analysis. In doing so, one must consider the multicellular composition of the tissue being studied. For example, skeletal muscle tissue contains endothelial cells associated with blood vessels, Schwann cells associated with axons, satellite cells, adipose cells, and fibroblasts, in addition to skeletal muscle fibers. Thus, not all nuclei in skeletal muscle tissue are contained within the muscle fibers.

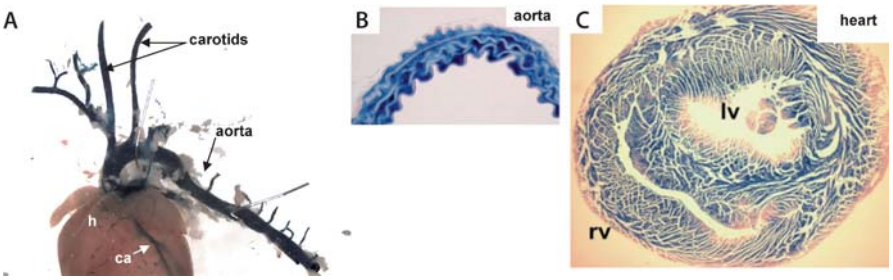
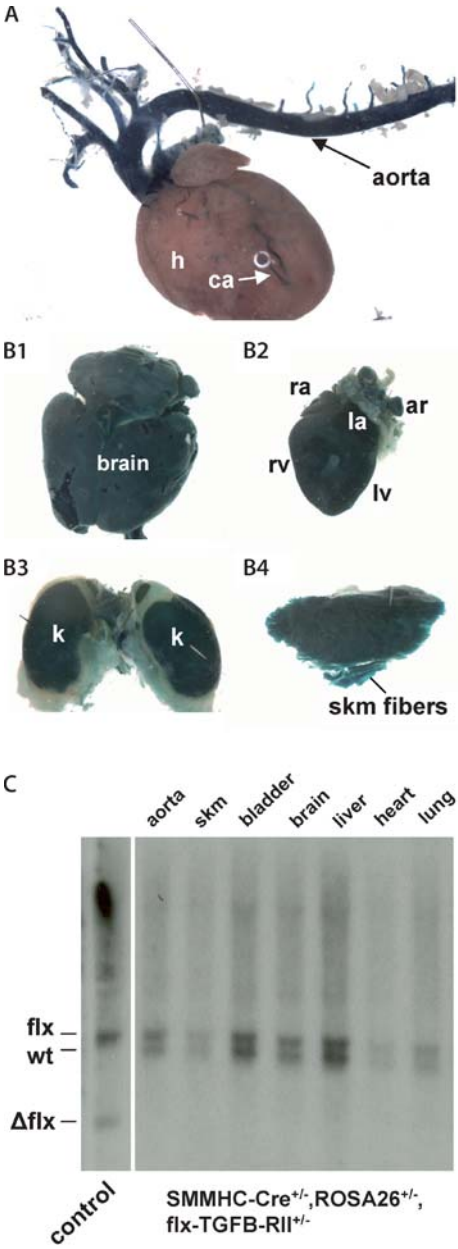


Fig. 2 A–C SMC-selective/specific promoter enhancers are expressed by other cell types during development. As depicted in **A**, expression of the *SM22*α-lacZ transgene (*blue*) is restricted to the coronary arteries (*ca*) of the adult heart (*h*) as well as blood vessels of the aortic tree (unpublished image from our lab). During embryonic development, the SM22α promoter enhancer is also expressed in the embryonic heart. Crossing the *SM22*α-Cre mouse to ROSA26 results in Cre recombination in the arteries (**B**), as predicted, but also the myocardium (**C**) of the adult animal, which occurred during development. Such results make it very difficult to distinguish whether gene targeting in the heart or blood vessels is the primary or secondary cause for the observed phenotype. Images in **B** and **C** represent positive lacZ staining (*blue*) in a cross-section of the aorta and the heart (*rv* right ventricle; *lv* left ventricle), respectively. (**B, C** Reprinted from Lepore et al. 2005)

Detailed histological analysis of skeletal muscle tissues indicated that approximately 45% of the nuclei within skeletal muscle tissue are contained within muscle fibers (Schmalbruch and Hellhammer 1977). Thus, one could reason

Fig. 3 A–C SMMHC-Cre leak and loci sensitivity. As depicted in **A**, crossing the *SMMHC*-Cre line to *ROSA26* results in Cre recombination and lacZ expression (*blue*) restricted to blood vessels of the aortic tree and coronary arteries (*ca*) of the heart (*h*), as previously published by our lab (Regan et al. 2000b). A limitation of the *SMMHC*-Cre transgenic mouse line is the potential occurrence of Cre-leak under control of the SMMHC promoter enhancer, as described in detail in Sect. 4.1.3. We have observed events of Cre-leak in the *ROSA26* locus in Cre-negative offspring from a *SMMHC*-Cre x *ROSA26* cross, as indicated by ubiquitous lacZ expression (*blue*) in non-SMC tissues, brain (**B1**), heart (**B2**), kidney (**B3**), and gastrocnemius (skeletal muscle, **B4**). *lv,* left ventricle; *rv,* right ventricle; *ra*, right atrium; *la,* left atrium; *ar,* aorta; *k,* kidney. Lastly, not all gene loci have the same sensitivity to Cre recombination in SMCs as the ROSA26 locus. We bred the ROSA26 Cre indicator locus into the *flox*ed TGFβ type II receptor (flx-TGFβ-RII) mouse line. This mouse, ROSA26/flx-TGFβ-RII, was then crossed to the *SMMHC*-Cre mouse. In multiple offspring with the genotype of SMMHC-Cre$^{+/-}$/ROSA26$^{+/-}$/flx-TGFβ-RII$^{+/-}$, SMC-selective Cre recombination was noted by lacZ induction in vascular smooth muscle as the result of Cre recombination of the ROSA26 locus, similar to that observed in **A**. However, as depicted by the Southern blot in **C**, in a segment of the same mouse aorta, as well as bladder (a SMC-rich tissue), Cre recombination of the flx-TGFβ-RII locus was absent. In **C**, *flx* denotes nonrecombined flx-TGFβ-RII locus, *wt* denotes endogenous locus, and *Δflx* denotes recombined flx-TGFβ-RII locus; the control lane represents tissue from a SMMHC-Cre$^{+/-}$/flx-TGFβ-RII$^{+/+}$ mouse

that selective and efficient deletion of a *flox*ed allele using a muscle-specific Cre-recombination mouse line would result in approximately 45% deletion of the *flox*ed allele. Indeed, in *mlc1f*-Cre x *flox*ed *erbB4* mice, studies applying this rationale showed recombination of approximately 42%–50% of the *flox*ed



erbB4 sequence in skeletal muscle tissue. This approach, however, is only associative and does not definitively prove Cre recombination is only occurring in skeletal muscle cells.

2.2 Conditional Gene Targeting in Skeletal Muscle

At the time of writing this chapter, we could only identify one inducible Cre model of gene targeting in skeletal muscle, the HSA (human skeletal muscle α-actin) promoter enhancer CreER^{T2} mouse line (Schuler et al. 2005) (Table 1). Initial characterization of the original *HSA*-Cre (Miniou et al. 1999) line by crossing to the *ROSA26* mouse revealed that the HSA promoter enhancer induces Cre recombination in cardiac and skeletal muscle, similar to the *MCK*-Cre mouse line (Wang et al. 1999) described in Sect. 2.1. Thus, utility of this line for gene targeting selectively to skeletal muscle is limited. To circumvent this issue, Metzger and colleagues cloned the tamoxifen-inducible CreER^{T2} transgene under control of the HSA promoter enhancer (Fig. 1A). Crossing this mouse to the *ACZL*-Cre reporter mouse (a similar line to *ROSA26*, where lacZ is activated by recombination of a 5′ *flox*ed stop signal following tamoxifen injection in adult mice) revealed skeletal muscle-restricted Cre recombination with no recombination in cardiac muscle, indicating that the HSA promoter is only active in cardiac cells during cardiogenesis. In this particular instance, 1 mg of tamoxifen was injected IP for 5 days in an adult mouse and tissue harvested for Cre recombination analysis 5 days after the last injection. As the *HSA*-CreER^{T2} mouse line was only recently described in 2005, there are no current examples using this model to study a physiological or pathophysiological function of a gene candidate in adult mice. However, the *HSA*-CreER^{T2} line and future generation of inducible Cre lines using the *mef2c* (Heidt and Black 2005), *myoD* (Chen et al. 2005), *Myf5* (Tallquist et al. 2000), and other promoter enhancers described in Table 1, will undoubtedly allow for controllable gene targeting independently in skeletal muscle progenitor cells, select skeletal muscle fiber types, and satellite cells to address the function and role of specific *flox*ed gene candidates in skeletal muscle development, regeneration, and pathophysiology of multiple disease states. For example, generation of a *MyoD*-CreER mouse line would allow for selective gene targeting of a *flox*ed gene in skeletal muscle satellite cells following skeletal muscle injury to study the role of the candidate gene in satellite cell regeneration of skeletal muscle. Lastly, the *MCK* promoter enhancer has been engineered for both the Tet-ON (rtTA) (Grill et al. 2003) and Tet-OFF (tTA) (Ghersa et al. 1998; Ahmad et al. 2000) systems, (Table 1, see chapter by R. Sprengel and M.T. Hasan, this volume on Tet-systems). The *MCK*-rtTA mouse line is very effective and flexible at providing tightly regulated overexpression of a gene candidate selectively in adult skeletal muscle following doxycycline administration (Grill et al. 2003).

3
Gene Targeting in Cardiac Muscle

3.1
The αMHC Promoter Enhancer: The Gold Standard for Inducible Cre-Mediated Mutagenesis in Cardiac Muscle

Unlike the skeletal muscle Cre systems discussed in Sect. 2.0, there are several inducible Cre cardiac-selective mouse lines. In fact, the selective restriction of the cardiac αMHC (α-myosin heavy chain) promoter enhancer to cardiac atrial and ventricular myocytes in the embryonic, postnatal and adult mouse, with no off-target organ expression, has made this promoter enhancer the tool of choice, as shown in Table 2 (Subramaniam et al. 1991; Agah et al. 1997). The construct which the majority of these mouse lines employ is typically termed the 5.5-kB αMHC promoter enhancer. The αMHC promoter enhancer contains approximately 100 bp of the 3′ end of the βMHC gene, the entire intergenic region (4.0 kB) between the βMHC gene and the αMHC gene, and through the 5′ untranslanted region of the αMHC gene (Gulick et al. 1991; Subramaniam et al. 1991). Multiple *cis* regulatory elements known to bind transcription factors that play a key role in muscle-restricted expression are present near the start site of transcription, including TRE, CArG box, MEF-2, MEF-1, and MCAT elements. In Sect. 4.1.3, we will discuss the importance of identifying and manipulating *cis* regulatory elements to alter tissue selective expression of a transgene. Two other lines of worthy note are the *MLC-2v*-Cre^{ki} mouse line, which is selectively active in ventricular, not atrial myocytes (Chen et al. 1998), and the β*MHC*-Cre mouse line, which has similar expression patterns to the αMHC promoter enhancer (Parlakian et al. 2004) (Table 2).

The α*MHC*-Cre mouse line, as well as the *Nkx2.5*-Cre line (Moses et al. 2001), have been powerful tools to study the role of several *flox*ed gene candidates in cardiogenesis. However, as is the case with any promoter enhancer Cre construct that is active during organ development, gene disruption often results in embryonic, fetal, or neonatal lethality. Thus, the focus of this section will be on tamoxifen-regulated Cre (MerCreMer; Fig. 1A) and RU486-regulated Cre (CrePR1; Fig. 1B) gene targeting under control of the αMHC promoter enhancer (Sohal et al. 2001; Minamino et al. 2001). Although not covered herein, several TET-ON and TET-OFF systems have also been generated (Fishman et al. 1994; Redfern et al. 1999; Valencik and McDonald 2001) (Table 2).

To illustrate cardiac-restricted recombination in embryos, the α*MHC*-MerCreMer mouse was crossed to the *ROSA26* mouse and pregnant females were injected with tamoxifen (20 mg/kg) for 4 days, as illustrated in Fig. 1A. Embryos taken at day 17 showed complete induction of Cre activity in the atria and ventricles of the embryonic heart by X-gal staining for lacZ activity. Moreover, Cre induction was complete and restricted to the heart in postnatal pups that were allowed to live 17 days and 6 weeks. Remarkably, untreated

Table 2 Mouse models of conditional mutagenesis in cardiac muscle

Promoter enhancer expression pattern	Promoter	Gene/protein encoded System	Embryogenesis	Adult	Reference
α*MHC* heavy chain	α Myosin	Cre	Atrial/ ventricular Myocytes	Atrial/ ventricular myocytes	Agah et al. 1997
		MerCreMer CrePR1 rtTa tTa			Sohal et al. 2001 Minamino et al. 2001 Valenick et al. 2001 Fishman et al.1994; Redfern et al. 1999
MLC-2v	Myosin light	$Cre^{(ki)}$	Ventricular myocytes	Ventricular myocytes	Chen et al. JBC1998
MCK creatine kinase	Muscle	(Table 1)	SKM, cardiac	SKM, cardiac	(Table 1)
SM22α	SM22α cytoskeletal protein	(Table 3)	Cardiac, SMC	SMC	(Table 3)
Nkx2.5	NK homebox factor	$Cre^{(ki)}$	Early cardiac lineage marker		Moses et al. 2001
β*MHC*	β Myosin heavy chain	Cre	Atrial/ ventricular myocytes	Atrial/ ventricular myocytes	Parlakian et al. 2004
CaαA	Cardiac α-actin	Cre	Atrial/ ventricular myocytes	Atrial/ ventricular myocytes	Miwa et al. 2000
hSMA	SMC α-actin	Cre	Cardiac, SMCs	SMCs	Miwa et al. 2000

SKM, —skeletal muscle; *SMC,* —smooth muscle cell; $Cre^{k,i}$ —Cre knock-in, must be carried as +/–; *MerCreMer,* —tamoxifen-inducible; *CrePRI,* —RU486-inducible; *tTA,* —TET-OFF; *rtTA* —TET-ON

α*MHC*-MerCreMer/*ROSA26* mice showed no detectable lacZ activity in the ventricles and atria of the heart at embryonic day 17, 17 days postnatally, or 6 weeks of age. Such a result can be rare with inducible Cre models because even low levels of unregulated Cre activity result in irreversible recombination, which can have a cumulative effect on lacZ staining. Whereas 6-week-old untreated α*MHC*-MerCreMer/*ROSA26* showed no detectable recombination, by 3 months of age, approximately 1% spurious recombination within the heart was observed. These results indicate that the MerCreMer transgenic protein (Verrou et al. 1999) is tightly regulated within the heart so that only very low

levels of unregulated recombination are observed in the absence of tamoxifen. In contrast, the α*MHC*-CrePR1 mouse line, which requires IP injections of RU486 (mifepristone or anti-progesterone) shows significant Cre recombination in the absence of RU486 at 6 weeks, although the transgene appears to be more tightly regulated 1 week postnatally (Wunderlich et al. 2001; Minamino et al. 2001), as illustrated in Fig. 1B. Moreover, this leakiness varied between α*MHC*-CrePR founder lines but correlated with the level of CrePR protein in the heart, suggesting that locus-dependent regulation of the transgene plays a critical role in this process. The leakiness of CrePR expression could complicate the interpretation of the physiological vs developmental role of a given gene modification and could also raise the specter of embryonic lethality in the presence of the homozygous *flox*ed allele background. Whether this reflects activation from endogenous circulating progesterone or an inherent leakiness in the construct per se, this is worthy of additional examination. Although significant modifications were made to the original CrePR fusion protein to increase RU496 sensitivity and decrease Cre leakiness in vitro (Wunderlich et al. 2001), it appears that further work is necessary for this system to become mainstream in cardiac gene targeting in vivo.

The α*MHC*-MerCreMer line has allowed investigators to test inducible loss of candidate gene function in the beating myocardium of adult mice for the first time. For example, structural integrity in the heart is maintained by the end-to-end connections between myocytes called the intercalated discs. N-cadherin is the primary protein involved in establishing this connection and is highly expressed in the developing and mature myocardium. However, genetically null N-cadherin mice die during embryogenesis and thus the function of N-cadherin in adult mice was unknown. The *flox*ed N-cadherin mice were crossed to α*MHC*-MerCreMer (Kostetskii et al. 2005). Tamoxifen-treated mice showed complete disassembly of the intercalated disc structure, including adherens junctions and desmosomes. As expected, the mice exhibited modest idiopathic cardiomyopathy and impaired cardiac function, dying within 2 months of gene deletion. What was surprising was that these mice also displayed conduction slowing and arrhythmogenesis, suggesting that gap junction interaction between myocytes was also altered. Indeed, future studies showed that connexins −43 and −40 were decreased in N-cadherin-deficient mice (Li et al. 2005a). These data suggest that perturbation of the N-cadherin/catenin complex in the adult heart may be an underlying cause of disease, leading to the establishment of the arrhythmogenesis by destabilizing gap junctions at the cell surface and sudden death.

There are several open questions that will need to be addressed. Of particular relevance will be the ability to drive the high efficiency of the recombination event in endogenous *flox*ed alleles, which will ultimately require excising both alleles in a sufficient number of myocytes to exert a physiological effect in the intact organ in vivo. To date, the efficiency of the conditional mutations has been a frequent recurring problem, making interpretation of negative results

highly ambiguous although the α*MHC*-MerCreMer mouse appears to have worked well in a small set of studies completed thus far. For example, *R26*-CreER^T mice were crossed to *flox*ed Rb, *flox*ed Brca2, *flox*ed p53, and *ROSA26*. The *R26*-CreER^T line expresses the tamoxifen-inducible Cre fusion protein ubiquitously from the ROSA26 locus (Vooijs et al. 2001). Southern blot analysis showed that tamoxifen-induced recombination of *flox*ed Rb approached 100% in most tissues, whereas moderate recombination was observed with Brca2 and p53 in the same samples. This might be explained by several factors including: (1) variability in Cre sensitivity of different gene loci as a consequence of differences in chromatin structure at floxed gene loci, a factor that can also vary as a function of developmental stage; (2) the heterogeneity of the tissue samples examined; (3) the distance between loxp sites; and/or (4) the tissue selective expression patterns of these genes. To test whether multiple *flox*ed loci within the same mouse show varying recombination patterns, a *R26*-CreER^T/*ROSA26*/*flox*Brca2 mouse was generated. Whereas recombination of both loci behaved similarly in the duodenum and lung, significant differences were seen in the testis where *ROSA26* recombined to a much greater extent than *Brca2*. However, this might be explained by the fact that the R26 locus is expressed ubiquitously in the testis but Brca2 is only expressed in proliferating cells (Blackshear et al. 1998), suggesting that the *Brac2* chromatin locus may be in a state that is condensed or folded in such a way that Cre cannot recombine the loxP sights in nonproliferating cells. *This example illustrates a key issue with all conditional gene knockout systems, i.e., that there needs to be temporal synchrony of expression of the recombinase with sensitivity of a particular floxed gene locus.* Finally, the recent observation that nonspecific cardiac injury and cardiomyopathy can accompany the αMHC-driven overexpression of green fluorescent protein (Huang et al. 2000) and tTA (McCloskey et al. 2005) raises the question as to whether HBD-Cre fusion proteins will result in cardiac injury and dysfunction at baseline. It should be noted that previous studies with animals overexpressing Cre in the heart have indeed documented cardiomyopathy in a subset of lines, additionally emphasizing this point.

4
Gene Targeting in Vascular Smooth Muscle

4.1
Unique Challenges of SMC Gene Targeting

4.1.1
No Single Gene Exclusively Marks SMC Lineage

The vascular smooth muscle cell (SMC) in mature animals is a highly specialized cell whose primary function is contraction and regulation of blood vessel

tone/diameter, blood pressure, and blood flow distribution. SMCs within adult vessels proliferate at an extremely low rate, exhibit very low synthetic activity, express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for this cell's unique form of contraction. However, with the possible exception of smooth muscle myosin heavy chain, there is no single marker gene that is exclusively expressed in SMCs and not other cell types (Owens 1995; Owens et al. 2004). For example, although expression of the most widely studied SMC marker gene SM α-actin is highly restricted to smooth muscle and smooth muscle-like cells in adult organisms, this gene as well as virtually all other SMC marker genes including SM22α, smoothelin, caldesmon, calponin, and metavinculin are all transiently expressed in other cell types including skeletal myoblasts/myotubes and cardiomyocytes during development, as well as in activated myofibroblasts in adult tissues (Owens et al. 2004). To illustrate this point, Fig. 2A shows no *SM22*α-lacZ transgene activity in the adult mouse myocardium but excellent expression in the coronary arteries and vessels of the aortic tree *(blue)*. However, when a *SM22*α-Cre mouse is crossed to the ROSA26 Cre indicator mouse, vascular SMCs (Fig. 2B) and the myocardium (Fig. 2C) show Cre recombination, as indicated by lacZ *(blue)* expression that occurred some point during development of the heart. As such, use of the promoters of these genes for purposes of conditional gene targeting and transgenic gene targeting in smooth muscle must take into account that phenotypes observed may not reflect cell autonomous effects in SMCs and may be secondary effects due to promoter activity in non-SMCs during embryonic development. Indeed, crossing the *SM22*α-Cre mouse to a floxed-SRF mouse (serum response factor) resulted in severe cardiac and vascular SMC ultrastructure defects and embryonic lethality (Miano et al. 2004).

4.1.2 Unlike Skeletal and Cardiac Muscle, SMCs Are Not Terminally Differentiated and Retain Extensive Plasticity Even in Adult Animals

Unlike skeletal muscle fibers and cardiac myocytes, which are terminally differentiated in the adult animal, SMCs retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental cues that normally regulate phenotype (Owens et al. 2004). For example, following vascular injury, e.g., angioplasty, SMCs exhibit high rates of proliferation, migration, and extracellular matrix production critical for repairing the vessel but are also responsible for increased neointimal hyperplasia and luminal narrowing, which is clinically relevant in postangioplasty restenosis (Owens et al. 2004). A hallmark of SMC phenotypic modulation following vessel injury includes suppression of the contractile proteins that define the mature/contractile SMC, SMC differentiation marker genes. Note that this is a normal response that is required for vascular repair as SMCs revert back to the more mature phenotype by reinducing SMC

differentiation marker genes (once the injury has been resolved). Phenotypic modulation of SMCs also plays a key role in development of atherosclerosis due to neointimal migration and proliferation as well as much accelerated production of extracellular matrix (Owens et al. 2004). As such, employing SMC marker gene promoter enhancers for conditional gene targeting in SMCs must take into account the possibility and/or certainty that the particular SMC promoter enhancers used may not be transcriptionally active under some pathophysiological conditions. Indeed, our lab has shown that expression of SMMHC, SM α-actin, and SM22α promoter reporter transgene constructs are profoundly suppressed following vascular injury (Regan et al. 2000a). Moreover, these transgenes mimic the response of endogenous SMC marker genes, and as such, the phenomenon is likely to be true for most if not all other SMCs marker gene promoter enhancers that have been used to drive SMC-selective Cre-mediated gene targeting in mice (Table 3). Similarly, we have shown that the wild-type SM22α promoter is silenced within phenotypically modulated SMCs within intimal lesions as well as underlying medial SMC within aortic arch lesions in the $ApoE^{-/-}$ mouse (Regan et al. 2000a; Wamhoff et al. 2004). Thus, placing an inducible Cre fusion protein under control of either of these promoter enhancers could result in ineffective Cre recombination depending on the phenotypic state of the SMC and activity of the promoter enhancer. There are means to circumvent this issue, yet they have not been completely put into practice, as described subsequently in Sect. 4.1.3. What is also clear in Table 3 is that SMC-targeted inducible Cre systems are truly in their infancy. Whether this is due to the inherent limitations of these very plastic promoter enhancers can only be speculated. However, we will discuss the promise of three currently available SMC-selective Cre recombination model systems: *SMMHC*-Cre (Regan et al. 2000b), *SMMHC*-Cre-eGFP (Xin et al. 2002), and *SM22*α-$CreER^{T2}$ (Kuhbandner et al. 2000).

Systems employing the SMMHC promoter (Regan et al. 2000b) have a distinct theoretical advantage over systems using SM α-actin (Mack and Owens 1999; Miwa et al. 2000) and SM22α (Lepore et al. 2005; Zhang et al. 2006) in that Cre expression and subsequent recombination is restricted to vascular SMCs, whereas these other promoters are robustly expressed in the myocardium and/or skeletal muscle during embryogenesis (Li et al. 1996). As shown in Fig. 3A, we have previously shown that crossing the *SMMHC*-Cre mouse to the ROSA26 Cre indicator mouse resulted in highly efficacious Cre recombination and induction of lacZ that was virtually completely specific for SMC tissues (other than a very small number of atrial myocytes) and highly efficacious (nearly 100%). Similarly, Kotlikoff and co-workers (Xin et al. 2002) have developed a novel *SMMHC*-Cre line using our SMMHC promoter-enhancer that contains an internal ribosomal entry site (IRES) 3′ of the Cre coding sequence to co-express eGFP (i.e., *SMMHC*-Cre-eGFP). This mouse provides a powerful means to achieve both SMC-specific gene targeting but also a simultaneous index as to whether a given cell is expressing the SMMHC gene at that point

Table 3 Mouse models of conditional mutagenes in smooth muscle

Promoter enhancer expression pattern	Promoter	Gene/protein encoded System	Embryogenesis	Adult	Reference
SM22α	SM22α cytoskeletal protein	Cre	Cardiac, SMCs	SMCs	Lepore et al. 2005
		$Cre^{(ki)}$	Cardiac, SMCs	SMCs	Zhang et al. 2006
		$CreER^{T2(ki)}$			Kuhbandner et al. 2000
		$CreER^{T2(tg)}$			Kuhbandner et al. 2000
		tTA			Ju et al. 2001
hSMαA	SM α-actin	Cre	Cardiac, SKM, SMCs	SMCs	Miwa et al. 2000
SMMHC	SM myosin heavy chain	Cre	SMCs	SMCs	Regan et al. 2000
		Cre-eGFP			Xin et al. 2002
Wnt1	Neural	Cre crest-specific protein	Neural crest		Danielian et al. 1998; Jiang et al. 2000
		$CreER^{T}$			Danielian et al. 1998
Pax3	Neural	Cre crest-specific protein	Neural crest		Lee et al. 2000

SKM,— skeletal muscle; *SMC,*— vascular and visceral smooth muscle; $^{(ki}$—$^{)}$, knock-in, must be carried as +/–; $^{(tg}$—$^{)}$, transgenic; $CreER^{T}$—, tamoxifen-inducible; $CreER^{T2}$—, tamoxifen-inducible; *tTA* —TET-OFF

in time, a major advantage for in vivo lineage tracing and assessment of SMC differentiation and/or phenotypic switching. In contrast, results of similar experiments using the SM22α promoter to drive Cre show robust Cre recombination in the heart and SMCs (Fig. 2). Thus, one must be aware that phenotypes observed in mice generated with *SM22α*-Cre systems may be the result of gene targeting in non-SMCs.

4.1.3 Additional Limitations of SMC-Promoter Gene Targeting Systems

There are also several additional inherent limitations of SMMHC- and other SMC-promoter gene targeting systems. *First*, activation of these promoters requires that SMCs first differentiate before gene targeting occurs. Indeed, this

problem is even worse with the SMMHC promoter since it (and the endogenous SMMHC gene) is induced slightly later in SMC development than the SM α-actin or SM22α promoters (e.g., E13.5–14.5 rather than E10.5–12.5 in dorsal aortic SMCs) (Regan et al. 2000b). As such, all of the currently available SMC gene targeting systems are not useful for studying the role of candidate genes in early stages of SMC differentiation or initial formation of SMC lineages (See Sect. 4.2). *Second*, we have observed that certain floxed alleles appear to be susceptible to non-cell autonomous or exogenous Cre derived from SMCs lining the female or male reproductive tracts. Indeed, in *SMMHC*-Cre lines that show very high expression levels (presumably due to insertion in a permissive gene locus), we have observed frequent cases of what appears to be transplacental leakage of Cre recombinase protein even with the ROSA26 Cre indicator mouse line (Fig. 3B). That is, we have observed Cre excision and activation of the floxed ROSA locus *in fetuses and tissues of adult mice that themselves have no Cre gene* (Fig. 3B). This recombination is not restricted to SMCs, and indeed in some cases can result in 100% recombination in all cells and tissues, including brain, heart, kidney, and skeletal muscle (Fig. 3B). Although the precise mechanisms remain to be determined, we postulate this phenomenon is the result of the very high level of expression of *SMMHC*-Cre in the gravid uterus, and the subsequent leakage of Cre protein into the uterus where it has induced recombination in either the fertilized egg or some portion of cells in early blastocysts. This phenomenon appears to be dependent on the combination of the relative Cre recombinase sensitivity of a given floxed gene locus, and the level of expression of Cre in that particular transgenic founder line. Cre sensitivity is likely a function of the chromatin state of the gene locus in question and accessibility of that locus to recombinases in general, a property that is known to vary with developmental stage and cell type, including SMCs (McDonald et al. 2006). We have found that this problem is much more prevalent in circumstances where the female is Cre-positive rather than the male. *Lastly*, as discussed above at the end of Sect. 3.1, particular gene loci have different sensitivities to Cre recombination in vascular SMCs. To show this, we bred the ROSA26 Cre indicator locus into the floxed TGFβ type II receptor (flx-TGFβ-RII) mouse line. This mouse, ROSA26/flx-TGFβ-RII, was then crossed to the *SMMHC*-Cre mouse. In multiple offspring with the genotype of *SMMHC*-Cre$^{+/-}$/ROSA26$^{+/-}$/flx-TGFβ-RII$^{+/-}$, SMC-selective Cre recombination was noted by lacZ induction in vascular smooth muscle as the result of Cre recombination of the ROSA26 locus (similar to Fig. 3A). However, as depicted by the Southern blot in Fig. 3C, distal sections of this same mouse aorta, as well as bladder (a SMC-rich tissue), Cre recombination of the flx-TGFβ-RII locus was absent. *This clearly illustrates the important point that individual floxed loci within the same cell type and tissue have different degrees of sensitivity to Cre, most likely dependent on the chromatin state of that particular gene, i.e., closed/condensed state or open/active state.* In any case, *the preceding issues clearly indicate that there are unique challenges in doing SMC*

gene targeting and that it is absolutely essential that one rigorously validate both the efficacy and specificity of gene targeting in individual gene targeted mice.

Because of these limitations and/or other problems, as yet there is not a single published paper to our knowledge that has successfully employed a (nonconditional) SMC-specific/selective promoter Cre system to achieve highly specific and efficacious knockout of a gene exclusively in SMCs. However, these SMC gene targeting systems have recently proven to be effective in a number of Cre-mediated gene induction/overexpression studies (Petrich et al. 2003; Dikalova et al. 2005; Zhang et al. 2005). In Cre-mediated gene induction studies, the general approach is to engineer a *flox*ed indicator gene, e.g., eGFP, with a polyA signal 3′ of the candidate gene under control of a ubiquitously expressed promoter, such as CMV or CX1, or a tissue selective promoter (Fig. 4). For example, Griendling and colleagues (Dikalova et al. 2005) crossed the *SMMHC*-Cre mouse to a transgenic mouse *CX1*-loxP-eGFP-polyA-loxP-nox1 mouse. The *CX1* promoter is a hybrid promoter consisting of a portion of the chicken β-actin promoter and CMV enhancer sequences to drive high-level ubiquitous gene expression in all mouse cell types in vivo (Okabe et al. 1997). In this example, non-smooth muscle cells express eGFP because the *SMMHC*-Cre transgene is inactive. However, in SMCs expressing *SMMHC*-Cre, effective Cre-recombination is determined by loss of eGFP expression and induction of nox-1 protein expression. There are multiple variations of this system, such as placing an inducible Cre isoform, e.g., $CreER^{T2}$, under control of a tissue-specific promoter in one transgenic mouse and breeding to another transgenic mouse with the inducible overexpression construct under the same promoter (Petrich et al. 2003) (Fig. 4). It has been proposed that restricting the gene candidate to the target tissue of interest using tissue-specific promoter enhancers increases the probability of tissue-selective inducible Cre-mediated recombination of the *flox*ed indicator gene (eGFP/polyA) and induction of the gene of interest.

Another alternative for gene targeting in SMCs is the *SM22*α-$CreER^{T2(ki)}$ mouse line (Kuhbandner et al. 2000), which expresses the tamoxifen-inducible Cre fusion protein from the *SM22*α gene locus as a result of genetic knock-in (ki) of $CreER^{T2}$. The evolution of the *SM22*α-$CreER^{T2(ki)}$ mouse appears to have resulted from the low efficacy of tamoxifen-inducible Cre-expression in the transgenic version, *SM22*α$CreER^{T2(tg)}$, where the transgene is randomly integrated into the mouse genome. Whereas no recombinase activity could be detected in vehicle-treated *SM22*α-$CreER^{T2(ki)}$ mice, administration of tamoxifen induced the excision of a loxP-flanked reporter transgene (*ROSA26* mouse) in vascular SMCs. Indeed, as discussed in further detail in the chapter by S. Moosmang et al., this volume, the *SM22*α-$CreER^{T2(ki)}$ has been an invaluable tool to study the role of ion channels in regulating blood pressure in adult mice, specifically the L-type voltage-gated Ca channel (Moosmang et al.

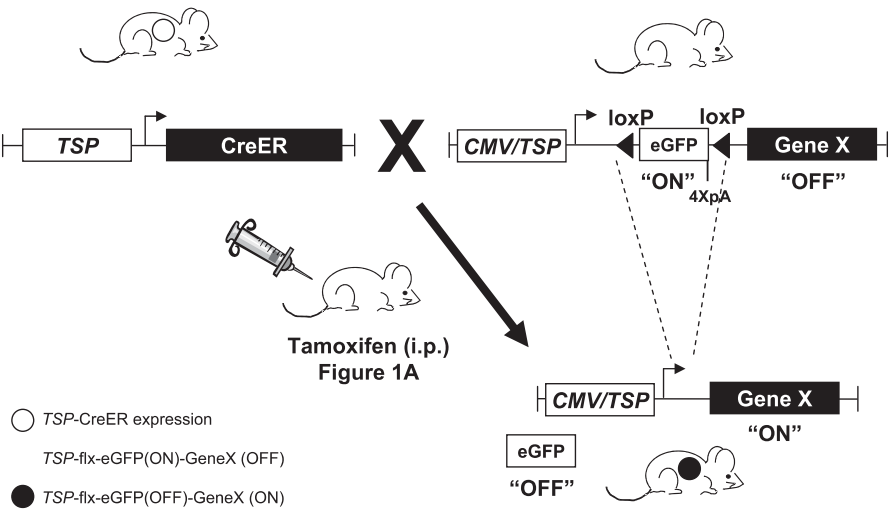


Fig. 4 Cre-mediated gene induction. A transgenic mouse is generated with a floxed indicator gene, e.g., eGFP, followed by the gene of interest under control of a ubiquitously expressed promoter such as *CMV* or a tissue selective promoter (*TSP*) (*right mouse*). The CMV-*flox* eGFP-GeneX mouse is crossed to any *TSP*-Cre line or inducible Cre line, e.g., *TSP*-CreER (*left mouse*). As long as Cre recombination has not occurred, the cell type being targeted will remain eGFP-positive. However, following Cre recombination, these cells now express the gene of interest and are eGFP-negative

2003), which when genetically knocked out in the entire organism is embryonic lethal. The SM22α promoter enhancer has also been used to drive expression of the tTA protein (Tet-OFF) (Ju et al. 2001) (Table 3). This being said, there are several conflicting reports regarding the efficacy of inducible Cre systems and Tet-regulated systems under control of the SM22α promoter enhancer, the most common being incomplete tissue or mosaic transgene expression when crossed to indicator lines. These reports show Cre recombination efficiencies from 0% to 100% (Kuhbandner et al. 2000; Lee et al. 2005; Handa et al. 2005). Several observations may explain this. *First*, as discussed above, the remarkable phenotypic plasticity of the SMC is known to be accompanied by altered levels of smooth muscle differentiation marker expression. For example, there exists a dramatic difference between the *SMMHC*-lacZ (Madsen et al. 1998) transgenic mouse expression pattern vs the integrated signal observed with the *SMMHC*-Cre (Regan et al. 2000b) mouse when crossed to *ROSA26*. That is, the *SMMHC*-lacZ mouse shows a mosaic expression pattern of lacZ in vascular SMCs where there appears to be an equal portion of SMCs expressing and not expressing the lacZ transgene. However, crossing the *SMMHC*-Cre mouse to ROSA26 results in what appears to be 100% recombination in vascular smooth muscle and expression of lacZ in all cells (Fig. 3A). Thus, even within a normal/healthy mouse, the SM22α promoter and other SMC-selective/specific

promoters may mirror in part the spatially and temporally stochastic nature of vascular SMC-specific gene expression. *Second*, spatial and temporal regulation of SMC marker genes may be a result of cell-to-cell stochastic regulation of multiple transcription factors and different promoter *cis* regulatory elements. Indeed, work by our own lab has shown that key *cis* elements within the SM22α promoter are required for suppression of transgene expression in response to vascular injury and atherosclerosis. For example, SMC expression of the *SM22*α-lacZ transgene is suppressed following acute vascular injury of the mouse carotid and in advanced atherosclerotic lesion in vivo (Regan et al. 2000a; Wamhoff et al. 2004). Mutating a G/C-rich Sp1 transcription factor binding site in the promoter enhancer of *SM22*α*G/C*-lacZ mice effectively prevented suppression of the transgene compared to *SM22*α-lacZ mice following acute vascular injury and complex atherosclerotic lesion development. Theoretically, the *SM22*α*G/C* promoter enhancer could be used to drive hormone-sensitive Cre fusion proteins in quiescent vascular SMCs and phenotypically modulated SMCs, i.e., where endogenous *SM22*α expression has been suppressed. *Third*, one cannot rule out silencing these promoters as the mice are interbred for multiple generations, the age of the animal being studied, diet, disease, or strain-dependent repression of these SMC promoter enhancers. Thus, time-consuming, rigorous attempts must be made to validate the level and efficacy of Cre recombination in these all tissue-specific condition gene targeting mouse models.

4.2 Directing SMC Conditional Gene Targeting to Specific SMC Lineages During Development

In all vertebrate embryos, the heart initially forms as a single linear tube located along the ventral midline. Blood flow from the early heart is carried from the primordial ventricle through a single outflow vessel, known as the truncus arteriosus. This vessel bifurcates in a region termed the aortic sac into a series of bilaterally paired dorsal aortae. As development proceeds, a series of morphogenic processes result in the division of the single outflow vessel to become the ascending aorta and pulmonary trunk and in the asymmetric reorganization of the pharyngeal arch arteries to become the left-sided arch of the aorta and associated blood vessels. The *Wnt-1* gene is expressed specifically in the neural plate, in the dorsal neural tube, and in the early migratory neural crest population at all axial levels excluding the forebrain. Expression of Wnt-1 is extinguished as the crest cell lineage migrates away from the neural tube and is not expressed at any other time or in any other place during development. Thus, the *Wnt-1*-Cre and *Wnt-1*-CreER^{T} mouse lines provide a tool to perform lineage tracing of the neural crest cell line (Table 3) (Danielian et al. 1998). Crossing the *Wnt1*-Cre line to *ROSA26* revealed that cells from the neural crest contribute to investment of the cardiac outflow tract

and formation of great vessels, including SMCs of aortic arch, the right and left common carotid arteries, the right subclavian but not the descending aorta or the left subclavian, nor the coronary circulation, which is derived from the proepicardial organ (Jiang et al. 2000). Similar results were found with the *Pax3*-Cre mouse, another neural Crest specific gene (Table 3) (Li et al. 2000). Such tools allow the investigator to ask whether a specific gene candidate is involved in neural crest investment and development of the great arteries, similar to skeletal muscle development studies using the *MyoD*-Cre mouse line.

Recent elegant studies by Parmacek and co-workers (Li et al. 2005b) illustrate the utility of the *Wnt-1*-Cre gene targeting system. In brief, they showed that knockout of myocardin-related transcription factor B (MRTFB, or MKL1) resulted in late embryonic lethality due to deformation of the aortic arch and the great arteries (Li et al. 2005b) (Fig. 5). The MRTFB KO mouse was derived from a BayGenomics ESC line (www.baygenomics.ucsf.edu) generated by a gene trap method wherein a *flox*ed genomic sequence was trapped in the MRTFB exon 10 allele. MRTFB is a potent SRF co-activator related to myocardin (Wang et al. 2002). Myocardin is a gene that is exclusively expressed in SMCs and cardiac myocytes that has been shown to potently activate all known CArG-dependent SMC differentiation marker genes, e.g., SMMHC, SM α-actin, and SM22α (Chen et al. 2002; Du et al. 2003; Yoshida et al. 2003) and to be required for SMC development in conventional (Li et al. 2003) but not chimeric knockout mice. The defects in MRTFB knockout mice are evident within brachial arch derivatives as early as E10.5–11.5, whereas no defects were evident in SMC derived from non-neural crest derivatives. As such, these observations suggested that MRTFB may be involved in neural crest cell migration and morphogenesis of these vessels. Indeed, when MRTFB null mice were crossed to *Wnt1*-Cre mice, the βgeo cassette was excised and MRTFB expression was restored in neural crest-derived cells; the mice survived and showed normal development of the aorta and other brachial arch derived blood vessels (Fig. 5).

5 Concluding Remarks

In conclusion, we summarize several important points discussed throughout this chapter regarding conditional gene targeting in muscle and other cell types:

- Tissue-selective conditional gene targeting circumvents, to an extent, secondary phenotypes observed with traditional gene knockout approaches in the entire organism. However, the ability to drive highly efficacious inducible recombination of both endogenous *flox*ed alleles in the intact organ in vivo must be rigorously proven in all systems.

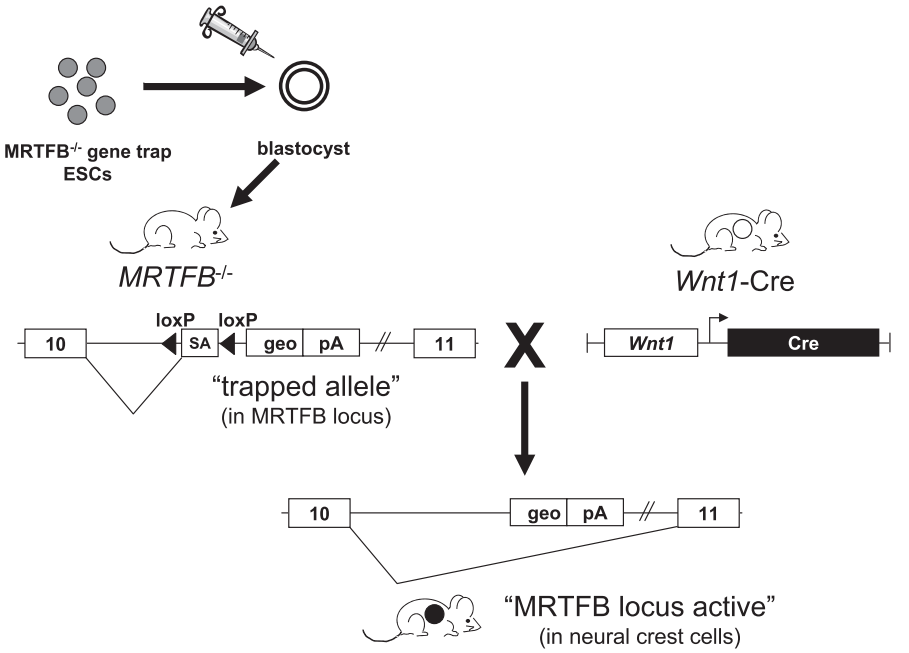


Fig. 5 Gene function recovery. Embryonic stem cells (*ESC*) containing single gene traps can be engineered or purchased from BayGenomics at a minimal cost to generate gene-null mice. In this example, a gene trap was randomly inserted into the MRTFB locus between exons 10 and 11. This gene trap is engineered such that the splice acceptor (SA) is *flox*ed and can be removed by Cre recombination, recovering expression of the gene in a tissue-selective manner. The splice acceptor effectively disrupts translation of the protein of interest. In some instances, the splice acceptor sequence is flanked by two *loxP* sites (e.g., gene trap clone pGT0lxf). In this example, the neural crest *Wnt1*-Cre line (Danielian et al. 1998; Li et al. 2005b) was used to remove the SA from the MTRFB locus and activate the locus, as described in Sect. 4.2. This is a powerful tool that can be employed for any gene in virtually any cell type for gene loss- and recovery-of-function

- Highly selective restriction of the cardiac αMHC promoter enhancer to cardiac atrial and ventricular myocytes in the embryonic, postnatal, and adult mouse, with no documented off-target organ expression, has made this promoter enhancer the tool of choice for cardiac conditional gene targeting.
- The multicellular composition of the tissue being studied must be taken into consideration when interpreting tissue-specific recombination efficacy and physiological effect in the intact organ.
- An excellent repertoire of skeletal muscle promoter enhancers exists to study gene targeting in myoblasts and/or differentiated muscle fibers and satellite cells. However, thus far few experiments have been completed to validate the performance of these promoter enhancers for conditional gene

targeting in the adult animal.

- Individual floxed loci within the same cell type and tissue have different degrees of sensitivity to Cre recombinase, which is likely a function of the chromatin state of that particular gene, i.e., closed/condensed state or open/active state. Of critical importance, it is well established that chromatin structure is developmentally regulated such that the timing of Cre expression and the Cre sensitivity of the target gene loci will be critical in determining the efficacy of Cre recombination.
- Employing SMC marker gene promoter enhancers for conditional gene targeting in SMCs must take into account the possibility and/or certainty that the particular SMC promoter enhancers used may not be transcriptionally active in all SMCs of a vessel under normal and some pathophysiological conditions. In addition, there is some evidence that highly sensitive floxed gene loci may be susceptible to recombination by non-cell autologous Cre derived from SMCs lining the male or female reproductive tracts. Perhaps because of these complications, as yet there is not a single published paper to our knowledge that has successfully employed a (nonconditional) SMC-specific/selective promoter Cre system to achieve highly specific and efficacious knockout of a gene exclusively in SMCs.

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Analysis of Calcium Channels by Conditional Mutagenesis

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Abstract Ca^{2+} influx through various ion channels is an important determinant of the cytosolic Ca^{2+} concentration, which plays a pivotal role in countless cellular processes. The cardiac L-type Ca^{2+} channel, $Ca_v1.2$, represents a major pathway for Ca^{2+} entry and is in many cells expressed together with other high- and low-voltage-activated Ca^{2+} channels. This article will focus on the use of conditional transgenic mouse models to clarify the roles of Ca^{2+} channels in several biological systems. The phenotypes of conditional Ca^{2+} channel transgenic mice have provided novel, and often unexpected, insights into the in vivo function of L-type and T-type Ca^{2+} channels as mediators of signaling between cell membrane and intracellular processes in blood pressure regulation, smooth muscle contractility, insulin secretion, cardiac function, sleep, learning, and memory.

Keywords L-type Ca^{2+} channel · Blood pressure regulation · Sparks · Learning and memory · Insulin secretion

1 Introduction

Rises in intracellular calcium ($[Ca^{2+}]_i$) trigger a multiplicity of processes including gene expression, chemotaxis, muscle contraction, synaptic plasticity, and secretion of hormones and neurotransmitters. Among the many channels and pumps involved in controlling the intracellular Ca^{2+} levels, voltage-gated Ca^{2+} channels play a key role. Voltage-gated Ca^{2+} channels have been classified by their electrophysiological and pharmacological properties, and more recently by their amino acid sequence identity, as either high-voltage-activated (HVA) or low-voltage-activated (LVA). HVA channels include L-, N-, P-/Q-, and R-types, and LVA channels are designated as T-type. There are 11 genes encoding α_1-subunits of voltage-gated Ca^{2+} channels (Yu and Catterall 2004). Alignment of their deduced amino acid sequences suggests that gene duplication and divergence of an ancestral Ca^{2+} channel gene gave rise to LVA and HVA subfamilies (Yu and Catterall 2004). The $Ca_v1.x$ subfamily encodes the four L-type genes *CACNA1S*, *CACNA1C* , *CACNA1D* , *CACNA1F* ($\sim Ca_v1.1–1.4$; α_{1S}, α_{1C}, α_{1D}, α_{1F}), the $Ca_v2.x$ encodes the three neuronal genes *CACNA1A*, *CACNA1B* , *CACNA1E* ($\sim Ca_v2.1–2.3$; α_{1A}, α_{1B}, α_{1E}), and the $Ca_v3.x$ subfamily encodes the three T-type genes *CACNA1G*, *CACNA1H* , *CACNA1I* ($\sim Ca_v3.1–3.3$; α_{1G}, α_{1H}, α_{1I}). The L-type Ca^{2+} channels are blocked by three classes of drugs: the dihydropyridines (DHP), the phenylalkylamines, and the benzothiazepines.

HVA Ca^{2+} channels are heteromeric complexes of five proteins: (a) the pore-forming α_1 subunit of 190–250 kDa, which contains the binding sites for all known Ca^{2+} channel blockers, the voltage sensor, and the selectivity filter; (b) a transmembrane, disulfide-linked dimer of α_2 and δ subunit; (c) an intracellular β subunit; and (d) the transmembrane γ subunit (for details see Hofmann et al. 1999; Catterall 2000). The subunit composition of native LVA T-type channels is unknown. Expression studies of various subunits yielded variable results on an association of the α_2/δ subunit with the cloned T-type α_1-subunit (for review see Lacinova et al. 2000; Perez-Reyes 2003). While these studies indicated that HVA auxiliary subunits may interact with $Ca_v3.x$ subunits and modulate the channel function to some degree, additional biochemical studies are required to establish this interaction in vivo.

Electrophysiological, pharmacological, and physiological experiments yielded a wealth of information on the distribution and function of the various Ca^{2+} channels. However, the channel's significance in vivo is far from clear, because several problems are associated with the currently available pharmacological tools. Many results were based on the use of "specific blockers." However, the "specificity" of these blockers has been questioned, because all known classes of Ca^{2+} channel blockers can also block other voltage-gated channels at higher concentrations (for reviews see Muth et al. 2001; Striessnig 2001; Moosmang et al. 2005b). The most specific and most often used calcium

channel blockers, the DHPs, may in some experiments not effectively block L-type channels opened by physiologically meaningful stimuli, such as action potentials, because of their state-dependent blocking kinetics (Helton et al. 2005). In addition, the calcium channel blockers, e.g., DHPs, do not allow differentiating between the members of the L-type family: they block all four channel subtypes. Moreover, no high-affinity selective T-type Ca^{2+} channel blocker is available. Finally, in physiological in vivo experiments, all L-type Ca^{2+} channel blockers will affect the function of the cardiovascular system to some extent, which may have substantial input on the function of the particular organ system being studied. Conditional mutagenesis of specific genes combined with tissue-specific modulation of the gene expression, therefore, seems to be an ideal way to elucidate the biological function of these channels.

2
Ca^{2+} Channel Alpha Subunit Conditional Knockout and Transgenic Mice

2.1
$Ca_v1.2$ and $Ca_v1.3$ Knockouts

To study the (patho)physiological roles of L-type Ca^{2+} channels in vivo, knockout mice were generated that chronically lack the $Ca_v1.2$ or $Ca_v1.3$ protein in all cells (so-called conventional knockout mice or null mutants). Homozygous $Ca_v1.2^{-/-}$ mice die before day 14.5 p.c. due to the lack of functional $Ca_v1.2$ channels in the heart. Mutations in the *CACNA1C* gene in zebra fish also results in premature death, since $Ca_v1.2$ regulates heart growth independently of contraction in the developing zebra fish (Rottbauer et al. 2001). To overcome this embryonic death, a mouse line has been generated that allows for the time- or tissue-specific conditional inactivation of the *CACNA1C* gene in selected cell types (Seisenberger et al. 2000) using the Cre/lox site-specific recombination system (Rajewsky et al. 1996; Metzger and Feil 1999). In contrast to conventional $Ca_v1.2$ knockout mice, conditional mouse mutants that lack $Ca_v1.2$ selectively in smooth muscle cells, insulin-secreting cells of the pancreas or distinct regions of the CNS (Moosmang et al. 2003; Schulla et al. 2003; Moosmang et al. 2005a) are viable and can be studied as adult animals (Fig. 1; Table 1). Smooth muscle-specific conditional $Ca_v1.2$ knockout mice show several cardiovascular and visceral phenotypes. Conventional $Ca_v1.3^{-/-}$ mutants are viable with no major disturbances of the vascular system and glucose metabolism (Platzer et al. 2000). These animals are deaf due to the complete absence of L-type currents in cochlear inner hair cells and degeneration of outer and inner hair cells (Platzer et al. 2000). Electrocardiogram recordings revealed sinoatrial node dysfunction (bradycardia and arrhythmia) in $Ca_v1.3^{-/-}$ mice (Platzer et al. 2000; Mangoni et al. 2003).

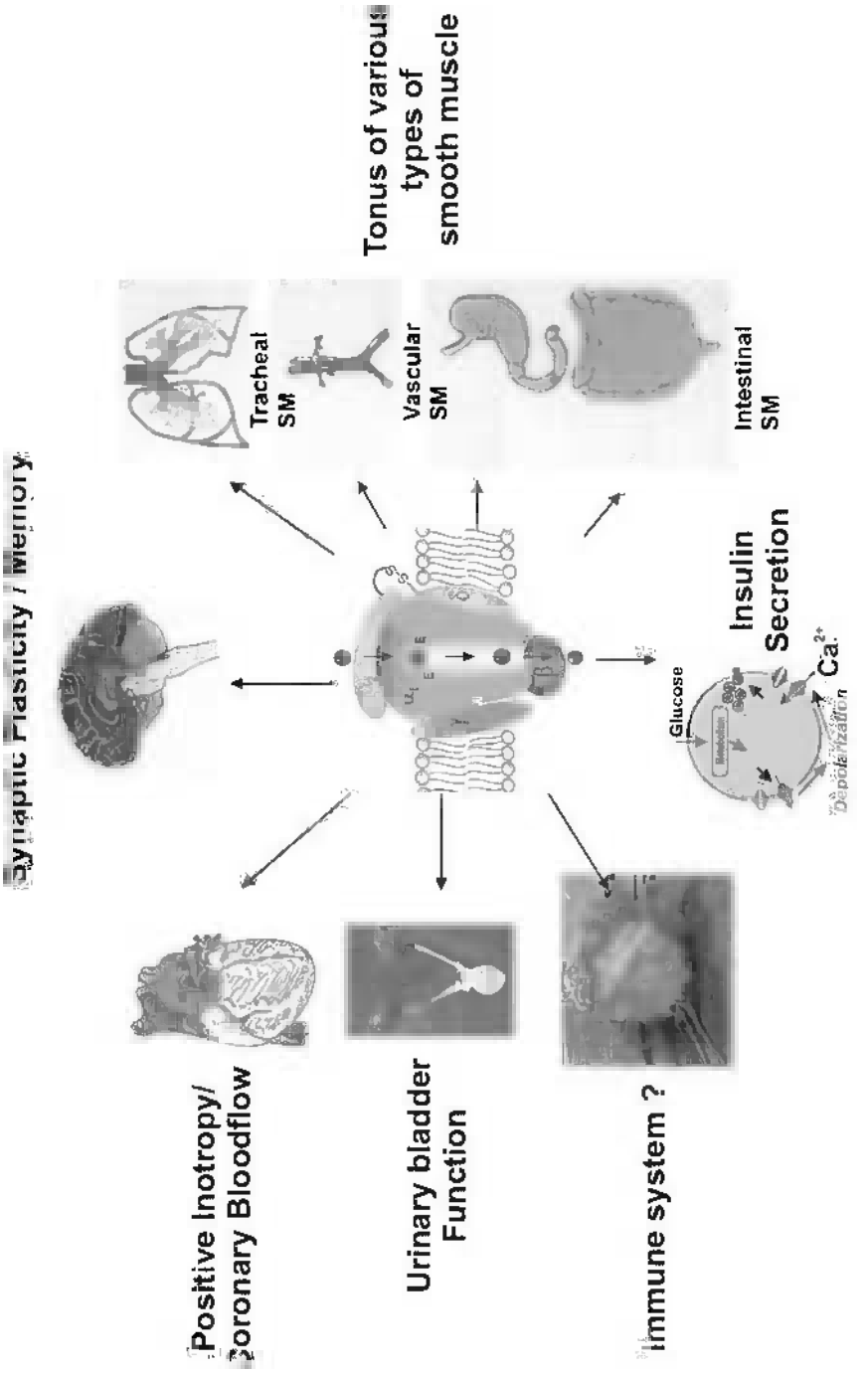

Fig. 1 Physiological functions of the L-type $Ca_v1.2$ Ca^{2+} channel as detected by conditional mutagenesis in the mouse. For details see text

Table 1 Phenotypes of Ca^{2+} channel conditional transgenic animals. For details, see text

Mouse line	Phenotype	Reference
$Ca_v1.2^{SMAKO}$	Blood pressure reduction	Moosmang et al. 2003
(Smooth muscle specific, inducible)	Lack of Bayliss effect/myogenic tone Reduced ATII and phenylephrine effect in vascular smooth muscle cells	
	Absence of Mibefradil effect on blood pressure	Moosmang et al. 2006
	Ileus	Wegener et al. 2006
	Lack of urinary bladder contractions in response to cholinergic stimulation	Wegener et al. 2004
$Ca_v1.2^{HCKO}$	Lack of NMDA receptor-independent LTP	Moosmang et al. 2005a
(Forebrain-specific)	Severely impaired spatial learning	
	Disturbed ERK/MAPK/CREB pathway	
$\beta Ca_v1.2^{-/-}$	Abolished first-phase insulin secretion	Schulla et al. 2003
(Pancreatic B-cell-specific)	Systemic glucose intolerance	
	Disappearance of a rapid component of insulin exocytosis	
Thalamic-$Ca_v3.1$ KO	No prolonged inhibition (>9 s) of action-potential firing in thalamic projection neurons	Anderson et al. 2005
(Thalamus-specific)	Frequent and prolonged arousal, which fragmented and reduced sleep	
$Ca_v1.2DHP^{-/-}$	DHP effects on pancreatic B-cell LTCC currents, insulin secretion, cardiac inotropy, and arterial smooth muscle contractility were lost in $Ca_v1.2DHP^{-/-}$ mice	Sinnegger-Brauns et al. 2004
(Dihydropyridine-insensitive $Ca_v1.2$)	DHPs are mood-modifying agents	

2.2
$Ca_v3.1$ Knockouts

Anderson and co-workers (2005) created a mouse model with a deletion of the *CACNA1G* (~$Ca_v3.1$) gene selective for thalamic relay neurons by using the Cre/loxP system and placing the Cre recombinase gene under the control of the $K_v3.2$ promoter sequence (Table 1). Transcripts of this K^+ channel are highly restricted to thalamic projection neurons (Rudy et al. 1992). In line with this, analysis based on two Cre recombinase-dependent reporter mice confirmed a strong recombination in rostral and midline thalamic nuclei pattern, while outside of the thalamus only a few areas show rather weak recombination.

2.3
Mice with a DHP-Insensitive $Ca_v1.2$ Channel

As mentioned above, mice with a null mutation of the *CACNA1C* (~$Ca_v1.2$) gene are not viable (Seisenberger et al. 2000). Sinnegger-Brauns and co-workers (2004) used an alternative approach to study functions of this channel representing the most prominent L-type channel in the CNS. They created a $Ca_v1.2$ channel knock-in mouse model ($Ca_v1.2DHP^{-/-}$ mice) with Thr1066 in helix IIIS5 replaced by a tyrosine residue, which results in a loss of the high-affinity DHP binding (Wappl et al. 2001). This should eliminate effects of DHPs mediated by the $Ca_v1.2$ channel. The remaining effects can then, in principle, be ascribed to the second major L-type channel in the CNS, the $Ca_v1.3$ channel.

3
Ca^{2+} Channels and the Nervous System

3.1
Function of the $Ca_v1.2$ Channel for Hippocampal Synaptic Plasticity and Learning

Ca^{2+} influx via voltage-dependent Ca^{2+} channels regulates multiple important Ca^{2+}-dependent processes in the nervous system including neurotransmitter release, neuronal excitability and plasticity, excitation-transcription coupling, synaptogenesis, and dendritic growth. The analysis of Ca^{2+} channelopathies in humans as well as in spontaneous and genetically engineered mouse mutants has considerably advanced the knowledge about the role of individual Ca^{2+} channel subtypes and their auxiliary subunits in vivo and is described comprehensively elsewhere (Lorenzon and Beam 2000; Pietrobon 2002, 2005; Arikkath and Campbell 2003; Pietrobon and Striessnig 2003; Striessnig et al. 2004).

It is generally accepted that LTP-like alterations may serve as cellular mechanisms for learning and memory (Kandel 2001; Martin and Morris 2002;

Malenka and Bear 2004). Critical for the induction and maintenance of hippocampal LTP is an activity-dependent rise of the postsynaptic cytosolic Ca^{2+} concentration (Zucker 1999; Malenka and Bear 2004). In the CA1 region, this results mainly from Ca^{2+} influx via NMDA receptors (NMDAR) (Kovalchuk et al. 2000; Sabatini and Svoboda 2000), which requires coincident pre- and postsynaptic activity to relieve a voltage-dependent Mg^{2+} block of NMDAR. This feature intriguingly resembles cellular conditions of a concept for learning established over 50 years ago (Hebb 1949). Therefore, NMDAR-dependent LTP has become the most extensively studied model of synaptic plasticity, and substantial efforts have been made to collect evidence for a functional link to memory acquisition and retention. On the other hand, it has become clear that LTP in Schaffer collateral/CA1 synapses is not homogeneous and comprises NMDAR-independent forms.

An important role for NMDAR-independent LTP and its behavioral correlates has been assigned to L-type voltage-dependent Ca^{2+} channels (VDCC) (Grover and Teyler 1990; Impey et al. 1996; Morgan and Teyler 1999; Borroni et al. 2000; Bauer et al. 2002; Woodside et al. 2004). However, the abovementioned problems associated with systemic application and intracranial infusion of L-type Ca^{2+} channel blockers have given rise to conflicting results (Deyo et al. 1989; Deyo and Hittner 1995; Quartermain 2000). One possible reason is that all L-type Ca^{2+} channel blockers may alter the function of the CNS indirectly, through their effects on the function of the cardiovascular system. In addition, because of the state-dependent nature of inhibition by DHP antagonists (Helton et al. 2005), results from blocker-based experiments do not necessarily exclude the involvement of L-type calcium channels to neuronal processes. It is also difficult to dissociate the function of individual L-type Ca^{2+} channel isoforms using the pharmacological tools available.

Notably, a global deletion of the *CACNA1D* ($\sim Ca_v1.3$) gene had no affect on hippocampal LTP (Clark et al. 2003). A mouse line with a regional inactivation of the *CACNA1C* ($\sim Ca_v1.2$) gene ($Cav1.2^{HCKO}$) (Moosmang et al. 2005a) was created using the Cre/loxP technique and Nex-Cre deleter mice (Schwab et al. 2000). These mice lack the $Ca_v1.2$ protein in the hippocampal formation and cerebral cortex. $Ca_v1.2^{HCKO}$ mice show normal anatomical properties of the brain, normal characteristics of basal synaptic transmission and normal NMDAR-dependent LTP in the hippocampal CA1 region arguing against general, unspecific effects of the neuronal gene knockout. However, $Ca_v1.2^{HCKO}$ mice show striking impairments of NMDAR-independent LTP in the Schaffer collateral/CA1 pathway following very strong tetanic stimulations (e.g., 200 Hz or multiple episodes of 100 Hz). Likewise, mutant mice show strongly reduced NMDAR-independent LTP following brief treatment with the K^+ channel blocker TEA (LTP_K). Remarkably, this defect in LTP_K is associated with the protein synthesis-dependent late phase of LTP (L-LTP): anisomycin, a protein synthesis inhibitor, decreased $L\text{-}LTP_K$ in control mice to the level observed in the mutants, while it did not alter LTP_K in the mu-

tants. The coupling of neuronal excitation and transcription may depend on the activity of $Ca_v1.2$ channels. In line with this view, the defect in protein synthesis-dependent synaptic plasticity in the $Ca_v1.2^{HCKO}$ mice is paralleled by reduced activation of MAP kinase (ERK), reduced CREB phosphorylation and CRE-dependent transcription. Substantial evidence links the activation of these signaling cascades not only to synaptic plasticity, but also to various forms of learning-related behavior (English and Sweatt 1997; Atkins et al. 1998; Hardingham et al. 2001; Kandel 2001; Wu et al. 2001; Pittenger et al. 2002; Thomas and Huganir 2004). Indeed, $Ca_v1.2^{HCKO}$ mice are severely impaired in two spatial learning tasks, a discriminatory water maze (Arns et al. 1999) and a labyrinth maze (Adelsberger et al. 2005).

Taken together, the findings in $Ca_v1.2^{HCKO}$ mice demonstrate the functional relevance of $Ca_v1.2$ channel-mediated Ca^{2+} influx for synaptic plasticity, transcriptional activation and, ultimately, learning and memory. In addition, they suggest a functional link between NMDAR-independent synaptic plasticity in the hippocampus and hippocampus-dependent learning.

3.2 Altered CNS Functions in Mice with a DHP-Insensitive $Ca_v1.2$ Channel

The DHP Ca^{2+} channel agonist BayK8644 activates $Ca_v1.2$ and $Ca_v1.3$ channels with similar efficacy and has no effect on Ba^{2+} currents recorded after heterologous expression of the $Ca_v1.2DHP^{-/-}$ mutant (Sinnegger-Brauns et al. 2004). In wild-type mice, injection of BayK8644 causes neurotoxicity, which results for example in severe motor dysfunction. With increasing doses, BayK8644 can cause even behavioral impairment including self-biting. Remarkably, $Ca_v1.2DHP^{-/-}$ mutants lack these effects, demonstrating that BayK8644-induced neurotoxicity is associated with the $Ca_v1.2$ channel. BayK8644 also causes neuronal activation, which can be assessed by probing Fos expression in many areas of the brain of wild-type mice. In the mutant mice, BayK8644-induced Fos expression is largely diminished in most of these regions linking it to Ca^{2+} influx via $Ca_v1.2$ channels. However, the $Ca_v1.3$ channel may also contribute as Fos expression is maintained in the mutants in specific brain regions including nucleus accumbens, paraventricular hypothalamic nucleus, and amygdala. Similarly, the functional contribution of $Ca_v1.2$ and $Ca_v1.3$ channels to BayK8644-induced neurotransmitter efflux in the ventral striatum differs for glutamate, 5-HT, dopamine, and noradrenaline. A role of the $Ca_v1.2$ channel for BayK8644-induced release of glutamate and 5-HT can be deduced based on the finding that the $Ca_v1.2DHP^{-/-}$ mutants lack the increase observed in the wild-type mice. There is no difference between the genotypes in BayK8644-induced dopamine and noradrenalin release linking it to the $Ca_v1.3$ channel.

Some of the regions activated by BayK8644 (c-Fos expression) belong to anatomical circuits thought to be involved in depression (Manji et al. 2001).

Moreover, nifedipine has antidepressant-like effects (Mogilnicka et al. 1988) in the forced-swim (behavioral despair) test using the time spent floating passively (immobility time) as a measure for depression-like behavior (Porsolt et al. 1977). Remarkably, BayK8644 induced a pronounced increase of the immobility time in $Ca_v1.2DHP^{-/-}$ mutants that lack the BayK8644 effects on motor performance. This supports the view that activation of the $Ca_v1.3$ channel may promote depression-like effects. On the other hand, nifedipine fails to reduce the immobility time in the $Ca_v1.2DHP^{-/-}$ mutant, suggesting that the antidepressant-like effect is associated with the $Ca_v1.2$ channel. It has to be emphasized that, because of the cardiovascular actions of nifedipine, conclusions from this mouse model based on CNS effects of this drug in the wild type still remain ambiguous.

3.3 Function of the $Ca_v3.1$ Low-Threshold T-type Ca^{2+} Channel for Sleep Stabilization

The thalamus functions as an important gate for the flow of sensory-motor information to and from the cerebral cortex. Inhibition of thalamocortical signal transmission, i.e., sensory suppression, may stabilize sleep. Indeed, different states of the sleep-wake cycle and arousal are accompanied by different patterns of neuronal activity in thalamocortical systems (for review see McCormick and Bal 1997; Steriade and Timofeev 2003). During periods of EEG-synchronized sleep, thalamocortical neurons hyperpolarize which triggers characteristic rhythmic discharges with brief burst of action potentials (AP) generated by a Ca^{2+} spike. A major role for this firing pattern has been assigned to low-threshold T-type Ca^{2+} channels (Jahnsen and Llinas 1984) that require strong hyperpolarization to recover from inactivation. Thalamocortical neurons express mainly the $Ca_v3.1$ channel of the low-threshold T-type Ca^{2+} channel family (Talley et al. 1999, 2000). Its role has been demonstrated in mice with a global deletion of the $Ca_v3.1$ channel: thalamic neurons of these animals lack burst firing activity induced by injecting brief hyperpolarizing currents (Kim et al. 2001) or by visceral pain (Kim et al. 2003). Ultimate conclusions about the cell type(s) and brain region(s) involved in the control of AP firing pattern and of possible functional consequences, such as sleep stabilization or generation of spike-and-wave discharges, are difficult to make based on data from a global knockout, because substantial levels of the $Ca_v3.1$ channel are expressed throughout other regions of the brain, including neurons of the thalamocortical network outside the thalamus. Mice with the targeted deletion of the *CACNA1G* ($\sim Ca_v3.1$) gene in thalamocortical neurons were used to study the functional relevance of $Ca_v3.1$ channel-mediated inhibition of nonadapting AP firing for sleep stabilization (Anderson et al. 2005). The corresponding analysis of vigilance states revealed deficits of the mutants in initiating and maintaining sleep despite their reduced activity and possibly

increased homeostatic sleep drive. These findings show that the $Ca_v3.1$ is required in the thalamus, but not the cortex, to stabilize sleep and strengthen the view that sensory suppression prevents disruption of sleep.

4
Smooth Muscle-Specific Deletion of $Ca_v1.2$

4.1
The Cardiovascular System

Chronically elevated arterial blood pressure leads to many fatal diseases such as myocardial infarction or stroke. Vascular tone, i.e., the contractile activity of vascular smooth muscle cells (SMCs) in the walls of small arteries and arterioles, plays a major role in setting arterial blood pressure. Depending on the vascular bed, the contractile state of resistance vessels is regulated by an interplay of vasoconstrictor and vasodilator stimuli from circulating hormones, neurotransmitters, and also directly by intravasal pressure (Bayliss effect) (Bayliss 1902). Vascular smooth muscle contraction is triggered by Ca^{2+}/calmodulin-dependent phosphorylation of the regulatory myosin light chain. The Ca^{2+} needed for activation is mainly provided by Ca^{2+} influx through ion channels and Ca^{2+} release from intracellular stores (Davis and Hill 1999). Particularly myogenic reactivity and tone appear to depend on Ca^{2+} influx through L-type Ca^{2+} channels (Hill et al. 2001). Vascular voltage-gated Ca^{2+} channels are modulated by several signaling systems and may be activated by vasoconstrictors, as described for norepinephrine (Nelson et al. 1988), that stimulate the $G_{q/11}$ signaling pathway. The autoregulatory Bayliss effect (i.e., constriction of the vessel after an increase in transmural pressure) is thought to be based on graded membrane depolarization of vascular smooth muscle in response to pressure. Membrane depolarization would open voltage-gated Ca^{2+} channels and increase vasoconstriction, whereas hyperpolarization would close them and induce vasodilatation (Jaggar et al. 1998).

Tamoxifen-induced, smooth muscle-specific inactivation of the L-type $Ca_v1.2$ Ca^{2+} channel gene in mice ($Ca_v1.2^{smAko}$ mice) reduced mean arterial blood pressure (MAP) in awake, freely moving animals from 120 ± 4.5 to 87 ± 8 mmHg (Moosmang et al. 2003). Depolarization-induced contraction was abolished in tibialis arteries of $Ca_v1.2^{smAko}$ mice and development of myogenic tone in response to intravascular pressure (Bayliss effect) was absent. These results indeed support the concept that the $Ca_v1.2$ channel is required to couple pressure-induced membrane potential changes to the myogenic response. In agreement with this loss of the autoregulation of myogenic tone, basal MAP was reduced by 33 mmHg in $Ca_v1.2^{smAko}$ mice. $Ca_v1.2^{smAko}$ mice still have a functional, albeit attenuated blood pressure regulation. As mentioned above, arterial blood pressure is not only determined by autoregulatory myogenic mechanisms, but also by an interplay of vasoconstrictors (most importantly

angiotensin 2 (AT2) and norepinephrine) and vasodilators such as prostacyclin, NO, and EDHF. The vasoconstricting effect of phenylephrine (PE) and AT2, assessed by MAP increases, was blunted in $Ca_v1.2^{smAko}$ mice. Hind-limb perfusion experiments suggested that 50% of the PE-induced resistance is due to Ca^{2+} influx through the $Ca_v1.2$ channel. Apparently, these hormones use multiple pathways to induce smooth muscle contraction, including $Ca_v1.2$-dependent and -independent pathways. Mechanisms leading to membrane depolarization and activation of L-type Ca^{2+} channels in response to PE, AT2, or increases in intravasal pressure is not clear. Depolarization may be due to activation of a nonspecific cation conductance or a Ca^{2+}-dependent chloride conductance (Nelson et al. 1997). Determining the precise signaling pathways linking stimulation of G protein-coupled receptors to $Ca_v1.2$ channel activation may help to establish new therapies for hypertension and myocardial failure. The principal role of the $Ca_v1.2$ channel in regulation of arterial tone is further stressed by the finding that in $Ca_v1.2DHP^{-/-}$ mice, smooth muscle relaxant effects of the DHP isradipine were completely absent (Sinnegger-Brauns et al. 2004).

4.2 L-Type Ca^{2+} Channels and Sparks

Ca^{2+} sparks are transient local increases in intracellular Ca^{2+} that arise from the opening of a small group of ryanodine receptors (RyR) in the sarcoplasmic reticulum. In arterial smooth muscle, Ca^{2+} released during Ca^{2+} sparks does not affect global Ca^{2+} levels, but couples to Ca^{2+}-sensitive ion channels located nearby in the plasma membrane (for a review see Jaggar et al. 1998). For example, Ca^{2+} sparks can activate BK_{Ca} channels and, thus, an important negative feedback mechanism for the regulation of pressure-induced vasoconstriction (Knot et al. 1998): BK_{Ca} channel-mediated spontaneous transient outward currents (STOCs) cause membrane hyperpolarization, which decreases the activity of voltage-dependent $Ca_v1.2$ channels and in turn global $[Ca^{2+}]_i$. Ultimately, this diminishes vascular contraction and lowers arterial blood pressure. Many vasodilators are now thought to act in part through frequency modulation of Ca^{2+} sparks.

While it is well known that intimate association between the trigger $Ca_v1.x$ channel and target RyR is crucial for the control of Ca^{2+} sparks in heart and skeletal muscle (for a review see Wray et al. 2004), the equivalent coupling process in smooth muscle cells has not been clarified until recently. Using conditional smooth muscle-specific inactivation of the $Ca_v1.2$ channel gene in mice ($Ca_v1.2^{SMAKO}$ mice), it has been demonstrated that the lack of $Ca_v1.2$ channels substantially reduces the frequency and amplitude of Ca^{2+} sparks (Gollasch et al. 2006). Data from these mice further demonstrate that his effect is associated with lower global cytosolic Ca^{2+} levels and reduced SR Ca^{2+} load and can be completely reversed by elevating cytosolic Ca^{2+} levels.

Moreover, buffering Ca^{2+} at relatively large distances from the $Ca_v1.2$ channel pore (>100 nm) disrupts release of Ca^{2+} sparks from RyR. These findings are compatible with the view that, in murine vascular SMCs, RyRs are activated by a global rather than a local $Ca_v1.2$ channel-mediated rise of $[Ca^{2+}]_i$.

4.3 T-Type Ca^{2+} Channels and Blood Pressure Regulation

The significance of $Ca_v3.x$ T-type Ca^{2+} channels for vascular tissue function is unclear. The antihypertensive compound mibefradil blocked T-type Ca^{2+} channels at tenfold lower concentrations than $Ca_v1.2$ and other HVA Ca^{2+} channels (Mehrke et al. 1994; Bezprozvanny and Tsien 1995; Clozel et al. 1997). It was suggested that mibefradil inhibited at therapeutic concentrations in vivo VSM T-type Ca^{2+} channels and thereby produced vasorelaxation (Mishra and Hermsmeyer 1994). However, the functional significance of the effects of mibefradil is difficult to interpret because of the existence of multiple receptors for this drug (for a review see Perez-Reyes 2004).

Moosmang and co-workers used the $Ca_v1.2^{\,smAko}$ mouse line that lacks the $Ca_v1.2$ channel (Moosmang et al. 2003). This channel is blocked in vivo and in vitro by mibefradil. In this model, the pharmacological properties of the putative vascular $Ca_v3.x$ T-type Ca^{2+} channels can be investigated free from interference by $Ca_v1.2$. The major findings were as follows: (1) mibefradil lowers mean arterial blood pressure (MAP) and attenuates peripheral resistance in wild-type but not in SMAKO mice; (2) its effect on MAP and peripheral resistance are mediated by the $Ca_v1.2$ L-type Ca^{2+} channel, and (3) T-type Ca^{2+} channel mRNA expression and SMC membrane potentials are not changed in $Ca_v1.2^{\,SMAKO}$ animals (Moosmang et al. 2006).

These results suggest that the contribution of T-type channels for murine vasoconstriction is minimal. This interpretation is in excellent agreement with the recent finding that deletion of the $Ca_v3.2$ T-type channel impaired coronary relaxation, but not contraction, in mice (Chen et al. 2003). Considering the controversial literature on the role of T-type Ca^{2+} channels for the vasculature (Loirand et al. 1986; Ganitkevich and Isenberg 1990; Mishra and Hermsmeyer 1994; Potocnik et al. 2000; VanBavel et al. 2002; Chen et al. 2003; Perez-Reyes 2004), studies on conditional transgenic mice lacking T-type Ca^{2+} channels, specifically in the vascular smooth muscle, are needed to clarify further the functional role of T-type Ca^{2+} channels.

4.4 The Role of $Ca_v1.2$ L-Type Ca^{2+} Channels in Urinary Bladder and Intestinal Smooth Muscle

DHP-sensitive Ca^{2+} channels are essential for contraction of intestinal smooth muscle such as urinary bladder (Fovaeus et al. 1987; Maggi et al. 1989; Bo

and Burnstock 1990; Schneider et al. 2004) and small intestine (Brading and Sneddon 1980; Blackwood and Bolton 1993). The phenotypes of mutant mice lacking the L-type $Ca_v1.2$ Ca^{2+} channel selectively in smooth muscle have strengthened this view (Wegener et al. 2004). Contraction of urinary bladder smooth muscle elicited by cholinergic stimulation was greatly diminished in $Ca_v1.2^{\ smAko}$ animals. Emptying of intracellular stores by thapsigargin or inhibition of phospholipase C by U73122 had no significant impact on contraction induced by cholinergic stimulation, suggesting that Ca^{2+} release from intracellular stores is of minor importance in murine (Wegener et al. 2004) and human (Masters et al. 1999; Schneider et al. 2004) urinary bladder. Inhibition of the Rho kinase pathway by Y27632 partially attenuated the cholinergic responsiveness of mutant and wild-type muscle, indicating that the muscarinic receptors activate the Ca^{2+} sensitizing pathway in bladder smooth muscle cells (Fleichman et al. 2004; Wegener et al. 2004). These contractility data support a central role of the L-type Ca^{2+} channel in cholinergic signaling in urinary bladder. On the other hand, electrophysiological experiments demonstrate that cholinergic stimulation of isolated detrusor smooth muscle cells reduces or even inhibits the depolarization-induced L-type Ca^{2+} channel current (Yoshino and Yabu 1995; Kajioka et al. 2002). One explanation for this discrepancy may be the use of intact tissue versus enzymatically isolated single cells. Isolation of cells may affect the intracellular signaling pathways. Further studies are needed to unravel how cholinergic receptors couple to L-type Ca^{2+} channel activity.

$Ca_v1.2^{\ smAko}$ mice show a severe intestinal phenotype: a complete ileus (Wegener et al. 2006). Isolated intestinal segments from the mutant mice lack spontaneous contractile activity (Wegener et al. 2006). Presumably, rhythmic contractility of the gut is initiated and triggered by electrical coupling between the interstitial cells of Cajal (ICC) and smooth muscle cells (Horowitz et al. 1999; Cousins et al. 2003). Deletion of the $Ca_v1.2$ channel may disrupt this interaction. Since mild constipation has been reported as an unwanted adverse effect of the treatment with verapamil or nifedipine (Gradman et al. 1992; Bulpitt et al. 2000), a similar role of the $Ca_v1.2$ channel in human intestine is possible.

5 The Function of HVA Ca^{2+} Channels in Insulin Secretion

In pancreatic β cells, glucose metabolism causes an increase in ATP/ADP ratio, which in turn closes ATP-dependent potassium (K_{ATP}) channels. As a consequence, the membrane depolarizes and, in turn, voltage-dependent Ca^{2+} channels activate. The resulting oscillatory increase in $[Ca^{2+}]_i$ directly triggers insulin exocytosis (Ashcroft et al. 1994). Glucose-stimulated insulin secretion is biphasic, with a rapid first-phase that lasts approximately 10 min and a sustained second-phase that can last for several hours (Curry et al. 1968). It is generally accepted that $[Ca^{2+}]_i$ regulates both phases of insulin secretion

(Henquin et al. 2003) and that Ca^{2+}-influx via L-, P/Q-, N-, and R-type channels may be functionally relevant (Komatsu et al. 1989; Ramanadham and Turk 1994). Thus, R-type $Ca_v2.3$-deficient mice showed markedly reduced glucose tolerance and impaired insulin release (Matsuda et al. 2001; Pereverzev et al. 2002). Studies on $Ca_v2.3^{-/-}$ islets and mice suggested that $Ca_v2.3$ is responsible for the second phase of the insulin secretion and that this channel is involved in the glucose-mediated signaling to glucagon release (Jing et al. 2005; Pereverzev et al. 2005). A study on N-type $Ca_v2.2$ channel-deficient mice revealed an increased glucose tolerance and a reduced weight gain without any change in insulin sensitivity under a high-fat diet (Takahashi et al. 2005). The systemic disruption of the $Ca_v\beta_3$ subunit (Murakami et al. 2002), which is abundantly expressed in pancreatic β-cells, improved insulin secretion due to increased glucose-induced $[Ca^{2+}]_i$. This effect was accounted for by enhanced formation of inositol-1,4,5-trisphosphate and increased Ca^{2+} mobilization from intracellular stores (Berggren et al. 2004). The P/Q-type Ca^{2+} channel $Ca_v2.1$ subunit is also expressed in β-cells (Ligon et al. 1998), but the role of the α_{1A} -subunit in glucose homeostasis could not be established, because $Ca_v2.1$-deficient mice showed abnormal behavior and dystonia, and died 3–4 weeks after birth (Jun et al. 1999). This latter finding again points to the drawbacks of conventional gene targeting methods.

L-type Ca^{2+} channels play a dominant role in insulin secretion, since high concentrations of L-type channels blockers inhibit the rise in $[Ca^{2+}]_i$ and insulin secretion in response to various insulin secretagogues (Ashcroft and Rorsman 1989; Mears 2004). Of the four L-type Ca^{2+} channel subtypes, only $Ca_v1.2$ and $Ca_v1.3$ have been identified in β-cells of different species (Iwashima et al. 1993; Seino 1995; Safayhi et al. 1997; Horvath et al. 1998; Yang et al. 1999; Huang et al. 2004). The contribution of these two channels to insulin secretion was discussed controversially until knockout mice became available.

Two mice strains lacking $Ca_v1.3$ subunits ($\alpha_{1D}{}^{-/-}$ mice) were generated (Platzer et al. 2000; Namkung et al. 2001). Platzer and co-workers found no difference between wild-type and $Ca_v1.3^{-/-}$ mice in serum glucose and insulin levels even after i.p. application of glucose, whereas Namkung and co-workers reported that the mutant mice were hypoinsulinemic and glucose intolerant (Namkung et al. 2001). The discrepancies between the two studies were explained by the difference in the genetic background of the embryonic stem cells and different targeting strategies.

To clarify the importance of the $Ca_v1.2$ channel for insulin secretion, Schulla and co-workers established a mouse model in which the *CACNA1C* gene was selectively disrupted in B cells by the use of Cre/loxP recombination (Schulla et al. 2003). The DHP isradipine blocked about 50% of the voltage-gated Ca^{2+} current (I_{Ca}) in wild-type B cells. The L-type Ca^{2+} channel agonist Bay K 8644 as well as isradipine did not affect I_{Ca} in B cells of $Ca_v1.2^{-/-}$ mice ($\beta Ca_v1.2^{-/-}$ cells). Further results demonstrated that $Ca_v1.2$ is the only L-type Ca^{2+} chan-

nel expressed in mouse B cells (Vignali et al. 2006). Interestingly, $[Ca^{2+}]_i$ was unaffected in $\beta Ca_v1.2^{-/-}$ cells and action potential firing was only slightly altered. These findings contrast with the potent inhibition of electrical activity and $[Ca^{2+}]_i$ rises in normal mouse B cells by DHP, and may be the consequence of a compensatory up-regulation of non-L-type channels in B cells. $\beta Ca_v1.2^{-/-}$ mice exhibited a slight hyperglycemia under basal and fasting conditions and an impaired glucose tolerance after i.p. glucose challenge. The first phase of insulin secretion and the rapid component of exocytotic capacitance changes were both attenuated in vivo and vitro. The fact that compensatory up-regulation of other channels restores $[Ca^{2+}]_i$ signaling but not insulin secretion emphasizes the direct and preferential coupling of $Ca_v1.2$ to the exocytotic machinery in B cells. These results were confirmed by the use of the $Ca_v1.2DHP^{-/-}$ mice (Wappl et al. 2001; Sinnegger-Brauns et al. 2004). I_{Ca} of B cells from the $Ca_v1.2DHP^{-/-}$ mice was neither enhanced by Bay K 8644 nor inhibited by isradipine. The glucose-dependent insulin secretion was not DHP-sensitive in this mouse model, confirming that $Ca_v1.3$ does not contribute to Ca^{2+} channel current and insulin secretion in the B cells of mice.

6 Conclusion

Conclusions about the roles of HVA Ca^{2+} channels based on data obtained in conventional Ca^{2+} channel knockout mice are often controversial and speculative. Escaping problems associated with global gene knockout, conditional knockout strategy helped to unveil the role of $Ca_v1.2$ and $Ca_v1.3$ L-type Ca^{2+} channels in insulin secretion and CNS function. Data from the corresponding mouse models show that the $Ca_v1.2$ channel is responsible for the first phase of insulin secretion, for learning and memory and, partially, the control of vascular tone. Certainly, similar knockout models for the non-L-type HVA and LVA Ca^{2+} channels are desired to line out their function in specific tissues.

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Conditional Mutagenesis of G-Protein Coupled Receptors and G-Proteins

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Abstract The G-protein-coupled receptor signaling system, consisting of a huge variety of receptors as well as of many G-proteins and effectors, operates in every cell and is involved in many physiological and pathological processes. The versatility of this system and the involvement of specific components makes G-protein-coupled receptors and their signaling pathways ideal targets for pharmacological interventions. Classical mouse knockout models have often provided important preliminary insights into the biological roles of individual receptors and signaling pathways and they are routinely used in the process of target validation. The recent development of efficient conditional mutagenesis techniques now allows a much more detailed analysis of G-protein-mediated signaling transduction processes. This review summarizes some of the areas in which progress has recently been made by applying conditional mutagenesis of genes coding for G-proteins and G-protein-coupled receptors.

Keywords G-proteins · GPCRs

1 Introduction

G-protein coupled receptors (GPCRs) represent the largest family of transmembrane receptors. The majority of GPCRs in the mammalian organism belong to the group of olfactory, pheromone, and taste receptors, which respond to

exogenous stimuli (e.g., more than 1,000 in mice). In addition to these sensory GPCRs, roughly 450 receptors (without splice variants) have been found. For more than 200 of these GPCRs, endogenous ligands such as hormones, neurotransmitters, or paracrine factors have been identified (Bockaert and Pin 1999; Pierce et al. 2002; Rana and Insel 2002). Binding of an agonist to a GPCR results in a conformational change that allows the receptor to convey the signal to heterotrimeric G-proteins, which in turn regulate the activity of various effector molecules such as enzymes or ion channels.

Heterotrimeric G-proteins consist of an α-subunit and a βγ-complex. More than twenty G-protein α-subunits have been described in the mammalian system, and they can be divided into four subfamilies based on structural and functional homologies (Simon et al. 1991). The main properties of individual G-proteins appear to be primarily determined by the identity of the α-subunit of the heterotrimeric G-protein. An individual cell expresses up to ten different G-protein α-subunits. The βγ-complex of mammalian G-proteins is assembled from a repertoire of five G-protein β-subunits and twelve γ-subunits (Schwindinger and Robishaw 2001). The βγ-complex is not a passive partner of the G-protein α-subunit but plays an important role in the regulation of various effectors (Clapham and Neer 1997).

The cellular and physiological effects induced by activation of GPCRs are determined by the specific coupling of the receptor to subgroups of heterotrimeric G-proteins (G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$). While activated receptors that lead to functionally similar or identical cellular effects usually activate the same G-protein subtypes, it is currently not known whether these receptors use the identical pool of G-proteins and whether the G-protein βγ-subunits involved are identical. Most receptors are able to activate more than one G-protein subtype, which leads to the activation of various signaling cascades. There are some characteristic patterns of G-proteins activated by receptors. At the same time, there are quite a few exceptions in which receptors appear to interact only with very specific G-proteins. Thus the G-protein–receptor interaction in general does not occur in an absolutely specific nor in a very promiscuous way. However, the composition of defined G-protein-mediated signaling pathways may be very specific in a given cellular system.

The most widely expressed $G\alpha_i$-family members are the G_i-type G-proteins (G_{i1}, G_{i2}, and G_{i3}), which have been shown to mediate receptor-dependent inhibition of various types of adenylyl cyclases (Sunahara et al. 1996). Since the cellular levels of these G-proteins are usually relatively high, they also represent an important source for βγ-complexes, which can regulate a variety of cellular effectors.

$G\alpha_q$ family members mediate the pertussis toxin-insensitive regulation of phospholipase C β-isoforms (Exton 1996; Rhee 2001). The G_q family consists of four members whose α-subunits are expressed from individual genes with different expression patterns. $G\alpha_q$ and $G\alpha_{11}$ appear to be expressed more

or less ubiquitously and are primarily responsible for coupling receptors in a pertussis toxin-insensitive manner to phospholipase C β-isoforms (Exton 1996; Rhee 2001). Receptors activating G_q family members in mammalian systems do not discriminate between G_q and G_{11} (Wange et al. 1991; Wu et al. 1992; Offermanns et al. 1994a; Xu et al. 1998), and there appears to be little difference between the abilities of both G-protein α-subunits to regulate phospholipase C β-isoforms (Rhee 2001).

The G-proteins G_{12} and G_{13} constitute the G_{12} family and are expressed ubiquitously (Strathmann and Simon 1990). The guanine nucleotide exchange factors (GEFs) for Rho, p115RhoGEF, PDZ-RhoGEF, and LARG have recently been shown to serve as direct effectors that interact with $G\alpha_{12}$ and $G\alpha_{13}$ (Hart et al. 1998; Fukuhara et al. 2001; Suzuki et al. 2003). Both $G\alpha_{12}$ and $G\alpha_{13}$ interact with the cytoplasmic domain of cadherins and cause the release of the transcriptional activator β-catenin (Meigs et al. 2001, 2002).

Stimulatory regulation of adenylyl cyclases through G-protein-coupled receptors involves G-proteins of the G_s-family of which two main members are known, G_s and G_{olf}. The ubiquitously expressed $G\alpha_s$ gene (*Gnas*) gives rise to several splice variants. Four splice variants, two short forms ($G\alpha_{s\text{-}S}$) and two long forms ($G\alpha_{s\text{-}L}$) are structurally closely related and appear to be functionally indistinguishable (Bray et al. 1986; Mattera et al. 1989; Freissmuth et al. 1991; Kozasa et al. 1998). All known adenylyl cyclase isoforms are activated by $G\alpha_s$ (Sunahara et al. 1996). An additional long splice forms of $G\alpha_s$, termed $XL\alpha_s$, has been described. $XL\alpha_s$ contains a long N-terminal portion that is encoded by a single exon about 35 kb upstream of exon 1 of the $G\alpha_s$ gene, which is spliced to exon 2 of the $G\alpha_s$ gene, thus containing the essential parts of the $G\alpha_s$ protein (Kehlenbach et al. 1994). The *Gnas* gene, which gives rise to additional transcripts, shows a complex imprinting pattern (for review, see Weinstein et al. 2001).

By mediating the effects of numerous hormones, neurotransmitters, metabolites, and other factors, GPCRs are involved in the regulation of all major body functions both under physiological and pathological conditions. Not surprisingly, more than half of the clinically used drugs act via G-protein-coupled receptors, ideal targets since they bind ligands with high specificity and affinity in a reversible manner and often show remarkable specificity with regard to the function or tissue they are regulating. The genes of approximately 165 GPCRs have been inactivated in mice so far (Schöneberg et al. 2004). Interestingly, only 11 of them are pre- or perinatally lethal, considerably less than what is observed for other gene families. This indicates that most GPCRs are involved in rather specialized functions in the adult organism. This review will summarize a number of areas where conditional mutagenesis of GPCRs and/or G-proteins has provided new insights into the biological role of this signal transduction system (Table 1).

Table 1 Conditional alleles and transgenes generated to study the function of GPCRs or G-proteins in mice

Gene	Gene product	Conditionality	Reference
Floxed alleles (GPCRs)			
Adora1	A_1 adenosine receptor	Viral vector (AAV-Cre)	Arrigoni et al. 2005; Scammell et al. 2003
Adora2a	A_{2A} adenosine receptor	Forebrain	Bastia et al. 2005
Cnr1	CB_1 cannabinoid receptor		Marsicano et al. 2003
Crhr1	CRH_1 corticotropin-releasing hormone receptor	Forebrain (CamkII-Cre)	Muller et al. 2003
Drd5	D_5 dopamine receptor		Heyer et al. 2002
Ednra	ET_A endothelin receptor	Cardiomyocyte (αMHC-Cre)	Kedzierski et al. 2003
		Subset of neural crest derived cells (*Dlx5/6*-Cre)	Ruest et al. 2005
Ptger4	EP_4 prostanoid receptor		Schneider et al. 2004
Gabbr1	$GABA_{B(1)}$ receptor		Haller et al. 2004
Edg1	$S1P_1$ sphingosine-1-phosphate receptor	Endothelial cells (*Tie2*-Cre)	Allende et al. 2003
Npy2r	Y_2 Neuropeptide Y receptor	Viral vector	Sainsbury et al. 2002
Sstr5	SST_5 somatostatin receptor	Pancreatic β-cells (RIP-Cre)	Wang et al. 2005
Floxed alleles (G-proteins)			
Gnaq	G_q α-subunit	Cardiomyocyte (MLC2a-Cre)	Wettschureck et al. 2001
		Interferon-responsive cells (Mx1-Cre)	Moers et al. 2004
		Nervous system (nestin-Cre)	Wettschureck et al. 2005
		Neural crest derived cells (P0-Cre)	Dettlaff-Swiercz et al. 2005
		Forebrain (CamkII-Cre)	Wettschureck et al. 2004
Gna13	G_{13} α-subunit	Interferon-responsive cells (Mx1-Cre)	Moers et al. 2003

Table 1 (continued)

Gene	Gene product	Conditionality	Reference
		Endothelial cells (*Tie2*-Cre)	Ruppel et al. 2005
Gnas	G_s α-subunit	Osteoblast/osteocyte (Col1-Cre)	Sakamoto et al. 2005b
		Chondrocytes (Col2-Cre)	Sakamoto et al. 2005a
		Hepatocytes (albumin-Cre)	Chen et al. 2005
Reactivatable null alleles (G-proteins, GPCRs)			
Gnaq	G_q α-subunit	Cardiomyocyte (αMHC-Cre)	Syed et al. 2004
Mc4r	MC_4 melanocortin receptor	Paraventricular hypothalamus (Sim1-Cre)	Balthasar et al. 2005
tTA/rtTA responsive transgenes (GPCRs)			
Ednrb	ET_B endothelin receptor	ET_B expressing cells ($Ednrb^{tTA}$/$Ednrn^{rtTA}$)	Shin et al. 1999
Oprk1[a]	Modified κ-opioid receptor (RASSL)	Cardiomyocyte (αMHC-tTA)	Redfern et al. 1999; Redfern et al. 2000
		Hepatocyte (LAP-tTA)	Redfern et al. 1999
		Bitter sensory taste cells (T2R5-rtTA)	Mueller et al. 2005
		Sweet sensory taste cells (T1R2-rtTA)	Zhao et al. 2003
Htr1b	5-HT_{1B} serotonin receptor	α-CAMKII-tTA	Ghavami et al. 1999
Htr1a	5-HT_{1A} serotonin receptor	α-CAMKII-tTA	Ghavami et al. 1999; Gross et al. 2002
Estrogen receptor-binding domain fusion protein			
Gnaq	G_q α-subunit	Cardiomyocyte specific expression (αMHC; tamoxifen-inducible)	Fan et al. 2005

[a] Mutant κ-opioid receptor with strongly reduced affinity for endogenous ligand but preserved binding to synthetic ligand spiradoline

2 Neural Crest Development

The neural crest is a population of pluripotent cells that originates from the dorsal part of the neural tube. After delamination from the neural tube, neural crest cells migrate to the periphery via characteristic paths. Once they have reached particular sites, they stop moving and differentiate into a wide variety of different cell types. These include cells of the peripheral nervous system, enteric ganglia, endocrine chromaffin cells of the adrenal medulla, melanocytes, most cells of the craniofacial skeletal and connective tissue, as well as part of the heart outflow tract (Le Douarin and Kalcheim 1999). The loss of pluripotency of neural crest cells and their gradual differentiation to particular cell types requires a complex interplay of cell-autonomous processes and the action of environmental signals.

Among the receptors on migrating neural crest cells that receive signals from environmental cues are the G-protein-coupled endothelin ET_A and ET_B receptors, which are involved in the correct differentiation and/or terminal migration of particular neural crest cell subpopulations (Clouthier et al. 2000; Barlow et al. 2003; Lee et al. 2003). Endothelin-1 and ET_A receptor-deficient mice die shortly after birth due to respiratory failure (Kurihara et al. 1994, 1995; Clouthier et al. 1998). Severe skeletal abnormalities could be observed in their craniofacial region, including a homeotic transformation of mandibular arch-derived structures into maxillary-like structures as well as absence of auditory ossicles and tympanic ring (Ozeki et al. 2004; Ruest et al. 2004). In addition, defects in the development of the cardiac outflow tract were present. While findings in mice lacking the ET_A receptor indicated a role in initiating early neural crest cell patterning, it was not clear if continued ET_A receptor signaling was required to maintain patterning mechanisms and whether later differentiation of neural crest derived mesenchyme required ET_A receptor-mediated signaling. To address these questions, Ruest et al. (2005) inactivated the gene encoding the ET_A receptor (*Ednra*) in a subset of cells within the mandibular arch using *Dlx5/6- Cre* transgenic mice (Ruest et al. 2003) that express Cre in the mandibular first arch starting from embryonic day 9.5. Surprisingly, neither $Ednra^{flox/flox}$; *Dlx5/6-Cre* nor $Ednra^{flox/-}$;*Dlx5/6-Cre* embryos develop defects in lower jaw structures as do $Ednra^{-/-}$ mice (Ruest et al. 2005). The authors concluded that once neural crest cell patterning involving ET_A receptors has occurred, the receptor does not appear to be required for subsequent mandible bone development.

The ET-3 and ET_B receptor system has been shown to be involved in the development of neural crest cells taking part in the formation of epidermal melanocytes as well as the myenteric ganglia of the distal colon. In mice lacking endothelin-3 (ET-3) or the endothelin B (ET_B) receptor, this results in white-spotted hair and skin color as well as a dilation of the proximal colon due to defects in the development of neural crest-derived melanocytes and enteric

neurons (Baynash et al. 1994; Hosoda et al. 1994). These defects are very similar to those present in humans suffering from multigenic Hirschsprung disease, which in various cases has been shown to be caused by mutations in ET_B or ET-3 genes (Edery et al. 1996; Gariepy 2001). To determine when ET_B receptor signaling is required during embryogenesis, a mouse line was created in which the endogenous *Ednrb* locus is under the control of the tetracycline-dependent transactivators tTA or rtTA (Shin et al. 1999). Using this system, the ET_B receptor gene could be expressed at different stages of development by administration of doxycylin to the drinking water of pregnant females or by withdrawal of doxycylin, respectively. This study elegantly showed that ET_B receptor function is required for the migration of both melanoblasts and enteric neuroblasts during a restricted period of neural crest development between embryonic days 10 and 12.5.

Activated ET_A receptors signal through the G-proteins G_q/G_{11} and G_{12}/G_{13}, while ET_B receptors couple to G_i/G_o, G_q/G_{11} as well as G_{13}. Mice lacking either $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ die at e9.5 or earlier due to developmental defects of the cardiovascular system (Offermanns et al. 1997; Offermanns et al. 1998; Gu et al. 2002). Although this early embryonic lethality does not allow study of the role of G_q/G_{11} and G_{12}/G_{13} in neural crest cell development, the analysis of e9.5 $G\alpha_q^{-/-};G\alpha_{11}^{-/-}$ animals and of $G\alpha_q^{-/-};G\alpha_{11}^{-/+}$ neonates has provided some evidence that G_q/G_{11} are involved in ET-1/ET_A signaling in the pharyngeal arch mesenchyme (Offermanns et al. 1998; Ivey et al. 2003). To address the question of which G-proteins are involved in endothelin receptor-mediated neural crest development and to study whether the developmental effects of endothelin occur in a cell autonomous manner, mouse lines lacking $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ in neural crest cells were generated using the P0-Cre mouse line (Yamauchi et al. 1999). Mice lacking $G\alpha_q/G\alpha_{11}$ in a neural crest cell-specific manner had craniofacial defects similar to those observed in mice lacking the ET_A receptor or endothelin-1 (ET-1) (Dettlaff-Swiercz et al. 2005). However, in contrast to ET-1/ET_A mutant animals, cardiac outflow tract morphology was intact. Surprisingly, neither $G\alpha_q/G\alpha_{11}$- nor $G\alpha_{12}/G\alpha_{13}$-deficient mice showed developmental defects seen in animals lacking either the ET_B receptor or its ligand endothelin-3 (ET-3). $G\alpha_{12}/G\alpha_{13}$ deficiency in neural crest cell-derived cardiac cells resulted in characteristic cardiac malformations (Dettlaff-Swiercz et al. 2005). These data showed that G_q/G_{11}- but not G_{12}/G_{13}-mediated signaling processes mediate ET-1/ET_A-dependent development of the cephalic neural crest. In contrast, ET-3/ET_B-mediated development of neural crest-derived melanocytes and enteric neurons appears to involve G-proteins different from $G_q/G_{11}/G_{12}/G_{13}$.

3 Vascular Development

$G\alpha_{13}$-deficient mouse embryos show a defective organization of the vascular system that is most prominent in the yolk sac and in the head mesenchyme,

resulting in embryonic lethality at midgestation (Offermanns et al. 1997). Vasculogenic blood vessel formation through the differentiation of progenitor cells into endothelial cells was not affected by the loss of $G\alpha_{13}$. However, angiogenesis that includes sprouting, growth, migration, and remodeling of existing endothelial cells was severely disturbed, and the maintenance of the integrity of newly developed vessels appeared to be defective in $G\alpha_{13}$-deficient embryos. Chemokinetic effects of thrombin, which acts through protease-activated receptors (PARs), were completely abrogated in fibroblasts lacking $G\alpha_{13}$, indicating that $G\alpha_{13}$ is required for full migratory responses of cells to certain stimuli. Interestingly, approximately half of the embryos that lack the protease-activated receptor 1 (PAR-1) also die at midgestation with bleeding from multiple sites (Connolly et al. 1996). This phenotype of embryos lacking PAR-1, which is expressed in endothelial cells, can be rescued by a PAR-1 transgene whose expression is driven by an endothelial-specific promoter (Griffin et al. 2001). This clearly indicates that PAR-1 function is required for proper vascular development. The more severe embryonic defect of $G\alpha_{13}$ compared to PAR-1-deficient embryos suggests that $G\alpha_{13}$ function is not restricted to protease-activated receptor signaling.

The defect in angiogenesis observed in mice lacking $G\alpha_{13}$ raises the question of which cells taking part in blood vessel development requires G_{13}-mediated signaling. An answer was provided by studying mice carrying two floxed $G\alpha_{13}$ alleles as well as a Cre transgene driven by the Tie2 promoter. In these animals $G\alpha_{13}$ deficiency is restricted to endothelial cells and some blood cells. Endothelial-specific $G\alpha_{13}$-deficient embryos showed a phenotype very similar to $G\alpha_{13}^{-/-}$ embryos, and most of the endothelial cell-specific $G\alpha_{13}$-deficient embryos also die at midgestation (Ruppel et al. 2005). This clearly indicates that $G\alpha_{13}$ function in endothelial cells is required for proper embryonic blood vessel development. The embryonic lethal phenotype in endothelium-specific $G\alpha_{13}$-deficient mice could be rescued by transgenic expression of wild-type $G\alpha_{13}$ using the Tie2 promoter. Interestingly, however, these embryos showed intracranial bleeding and exencephaly at later stages of development (Ruppel et al. 2005), which points to additional endothelial cell-independent functions of $G\alpha_{13}$ during development.

In addition to PAR-1 receptors sphingosine-1-phosphate-1 (S1P1) receptors have been shown to be required for vascular development. Mice lacking S1P1 receptors show embryonic hemorrhage leading to intrauterine death between embryonic day 12.5 and 14.5. In contrast to PAR-1-deficient mice, vasculogenesis and angiogenesis appear to be normal. However, vascular maturation was defective due to a deficiency of vascular smooth muscle cells/pericytes (Liu et al. 2000). A detailed analysis of S1P1 receptor-deficient mice showed that maturing blood vessels are unable to recruit smooth muscle cells to the vessel walls. Sphingosine-1-phosphate, which is present in the blood, may act on vascular smooth muscle cells and facilitate their migration to the vessel wall. Alternatively or in addition, sphingosine-1-phosphate could act on S1P1 recep-

tors in endothelial cells, which in turn allows them to recruit vascular smooth muscle cells. Since S1P1 receptors are expressed both in endothelial cells and in vascular smooth muscle cells as well as in other cell types, it was unclear which of the two potential mechanisms are responsible for the phenotype observed in mice lacking S1P1 globally. In a subsequent study, therefore, a mouse line was created in which S1P1 expression was conditionally inactivated in endothelial cells using the Tie2 promoter-driven Cre transgene (Allende et al. 2003). The phenotype of the conditional mutant embryos mimicked the phenotype obtained in embryos with global S1P1 receptor deficiency, clearly showing that the recruitment of vascular smooth muscle cells to developing vessels is regulated via the S1P1 receptor in endothelial cells.

4 Platelet Activation

Platelets are small cell fragments which circulate in the blood and adhere at places of vascular injury to the vessel wall where they become activated, resulting in the formation of a platelet plug that is responsible for primary hemostasis. Platelets can also become activated under pathological conditions, e.g., on ruptured atherosclerotic plaques leading to arterial thrombosis. Platelet adhesion and activation is initiated by their interaction with adhesive macromolecules such as collagen and von Willebrand factor (vWF) at the subendothelial surface (Ruggeri 2002; Jackson et al. 2003). While collagen is able to induce firm adhesion of platelets to the subendothelium (Watson et al. 2001), the recruitment of additional platelets to the growing platelet plaque requires the local accumulation of diffusible mediators. These mediators include ADP/ATP and thromboxane A_2 (TXA_2), which are secreted or released from activated platelets as well as thrombin, which is produced on the surface of activated platelets. These platelet stimuli have in common their action through G-protein-coupled receptors. While ADP induces the activation of G_q and G_i via $P2Y_1$ and $P2Y_{12}$ receptors (Gachet 2001; Kunapuli et al. 2003), the activated TXA_2 receptor (TP) combines with G_q and G_{12}/G_{13} (Offermanns et al. 1994b; Klages et al. 1999). G-protein-coupled protease-activated receptors (PARs), which are activated by thrombin are functionally coupled to G_q, G_{12}/G_{13}, and in some cases to G_i (Coughlin 2002). In response to these secondary mediators of platelet activation, platelets immediately undergo a shape change reaction during which they become spherical and extrude pseudopodia-like structures. In addition, the glycoprotein IIb/IIIa (integrin $\alpha IIb\beta 3$) undergoes a conformational change resulting in binding of fibrinogen/vWF and subsequent platelet aggregation. Finally, the formation and release of TXA_2, thrombin, and ADP is further stimulated. Thus, secondary mediators increase their own formation through G-protein-coupled receptors, resulting in an amplification of their effects, and eventually all G-protein-mediated signaling pathways induced via these receptors become activated.

The requirement of G_q-mediated signaling for agonist-induced platelet activation has been demonstrated by the phenotype of $G\alpha_q$-deficient platelets, which fail to aggregate and to secrete in response to thrombin, ADP, and TXA_2 because of a lack of agonist-induced phospholipase C activation. Despite the central role of G_q in platelet activation, it was recently demonstrated that induction of G_i- and G_{12}/G_{13}-mediated signaling pathways is sufficient to induce integrin αIIbβ3 activation (Dorsam et al. 2002; Nieswandt et al. 2002). To study the role of G_{12}/G_{13} in platelet activation, mice with a conditional allele of the gene encoding $G\alpha_{13}$ were generated (Moers et al. 2003), and conditional mutagenesis was performed using the interferon-inducible Cre transgenic mouse line (Mx1-Cre; Kuhn et al. 1995) in which interferon administration results in complete recombination in the bone marrow. At 2–4 weeks after induction, platelets are deficient in the respective gene product. Interestingly, in $G\alpha_{13}$-deficient platelets, but not in $G\alpha_{12}$-deficient platelets, the potency of various stimuli including TXA_2, thrombin, and collagen to induce platelet shape change and aggregation is markedly reduced (Moers et al. 2003). These defects are accompanied by a defect in the activation of RhoA, and a delayed phosphorylation of the myosin light chain as well as by an inability to form stable platelet thrombi under high sheer stress conditions. In addition, mice carrying platelets that lack $G\alpha_{13}$ have an increased bleeding time and are protected against the formation of arterial thrombi induced in a carotid artery thrombosis model. Thus, G_{13}-mediated signaling is not only involved in the response of platelets to relatively low stimulus concentrations which induce platelet shape change but is also required for normal responsiveness of platelets at higher stimulus concentrations. A reduced potency of platelet activators in the absence of G_{13}-mediated signaling becomes in particular limiting under high flow conditions, which lead to a rapid clearance of soluble stimuli from the site of platelet activation and formation of mediators. In addition, the defective activation of RhoA-mediated signaling in the absence of G_{13} appears to contribute to the observed defect in the stabilization of platelet aggregates under high sheer stress ex vivo as well as in vivo. In fact, RhoA-mediated signaling has been suggested to be required for platelet aggregation under high sheer conditions as well as for the irreversible aggregation of platelets in suspension (Missy et al. 2001; Schoenwaelder et al. 2002). These data indicate that in addition to G_q and G_i, G_{13} is crucially involved in the signaling processes mediating platelet activation via G-protein-coupled receptors both in hemostasis and thrombosis.

Platelets from mice with interferon-induced deficiency of both $G\alpha_q$ and $G\alpha_{13}$ were completely unresponsive to diffusible stimuli such as ADP, thromboxane A_2, or thrombin, even when applied at very high concentrations in combination, while all stimuli are able to induce platelet aggregation, shape change, and RhoA activation in platelets lacking only one Gα subunit. This indicates, that G_q or G_{13} are required to induce some platelet activation, whereas the activation of G_i-mediated signaling alone is not sufficient to induce activa-

tion of platelets. In addition, platelets lacking $G\alpha_q$ and $G\alpha_{13}$ adhered normally to collagen under high shear but no longer aggregated in response to collagen (Moers et al. 2004), indicating that collagen-induced platelet activation but not platelet adhesion requires intact G protein-mediated signaling pathways.

5 Myocardial Hypertrophy

Adult cardiomyocytes are terminally differentiated postmitotic cells that respond to stimulatory signals with cell growth rather than proliferation. Myocardial hypertrophy can result from pathological conditions that go along with increased hemodynamic load. These processes require the transduction of mechanical stimuli into a hypertrophic stimulus (Frey and Olson 2003). Evidence has accumulated that the initial phase of myocardial hypertrophy involves the formation of cardiac para-/autocrine factors such as endothelin-1, norepinephrine, or angiotensin II. These ligands are known to activate G_q/G_{11}-coupled receptors, such as the α_1-adrenergic receptor, the angiotensin AT_1 receptor, or the endothelin ET_A receptor (Sokolovsky 1993; LaMorte et al. 1994; Sadoshima et al. 1995). In line with this, cardiomyocyte-specific transgenic overexpression of α_1-adrenergic or angiotensin (AT_1) receptors results in cardiac hypertrophy (Milano et al. 1994; Hein et al. 1997).

In mice that lacked individual receptors such as the AT_1 angiotensin II receptor (Hamawaki et al. 1998; Harada et al. 1998) or the α_{1B} and α_{1A} adrenergic receptors (Cavalli et al. 1997; Rokosh and Simpson 2002), pressure overload myocardial hypertrophy was normal while mice lacking both α_{1A} and α_{1B} adrenergic receptors showed defective physiological hypertrophy (O'Connell et al. 2003). This suggests that the functions of individual G-protein-coupled receptors may be at least partially redundant in this process. The role of the main cardiac endothelin receptor, the ET_A receptor, could not be studied in vivo due to the neonatal lethality of ET_A-deficient mice (Clouthier et al. 1998). In order to be able to study the consequence of ET_A receptor deficiency for myocardial hypertrophy induced by various stimuli, cardiomyocyte-specific ET_A-deficient mice have been generated (Kedzierski et al. 2003). Cardiomyocyte-specific ET_A-deficient mice were viable and had no detectable abnormalities in cardiac anatomy and function. Surprisingly, cardiomyocyte-specific ET_A receptor deficiency had no effect on the hypertrophic response to a 10-day infusion of angiotensin II or isoproterenol, although pharmacological blockade of the receptor had been shown to reduce cardiomyocyte hypertrophy in vitro (Ito et al. 1993). Thus, cardiomyocyte-specific ET_A-receptor deficiency does not recapitulate the pharmacological effect of an ET_A receptor antagonist in myocardial hypertrophy. A possible explanation was provided by the observation that in hearts of ET_A receptor-deficient mice the protein levels of the ET_B receptor were twice as high as in wild-type mice, suggesting that the lack of ET_A receptors may be compensated by an increased expression of the ET_B receptor.

Since the above-mentioned receptors have in common that they act *via* G_q/G_{11}, this signaling pathway has been studied intensively with regard to its role in myocardial hypertrophy. Transgenic expression of wild-type $G\alpha_q$ or of a constitutively active mutant of $G\alpha_q$ in the heart has been shown to result in cardiac hypertrophy (D'Angelo et al. 1997; Mende et al. 1998). These studies were conducted with mice in which the expression of $G\alpha_q$ was driven by the conventional αMHC promoter, which drives transgene expression in the atrium starting perinatally and in the ventricle right after birth. To test whether the time period in which cardiac overexpression of $G\alpha_q$ is induced influences the hypertrophic phenotype, two mouse lines were established that allow for inducible expression of $G\alpha_q$ in adult cardiomyocytes (Syed et al. 2004; Fan et al. 2005). Syed et al. used a binary Cre-lox transgenic system in which cardiomyocyte-specific expression of a tamoxifen-activated version of Cre ensures tissue-specific induction of the expression of a $G\alpha_q$ transgene, which in the absence of Cre is silenced by a floxed cassette in front of the $G\alpha_q$ cDNA. In contrast, Fan et al. achieved inducible expression of a constitutively active mutant of $G\alpha_q$ by cardiomyocyte-specific expression of a fusion protein consisting of an active mutant of $G\alpha_q$ and the hormone-binding domain of the estrogen receptor, which in the absence of tamoxifen appears to be inactive. Induction of cardiac $G\alpha_q$ expression in neonates recapitulated the phenotype of conventional αMHC transgenic $G\alpha_q$ mice (Syed et al. 2004). However, adult $G\alpha_q$ overexpression failed to induce cardiac hypertrophy (Syed et al. 2004; Fan et al. 2005). These data suggest that the transgenic overexpression of $G\alpha_q$ requires a growing heart in order to induce myocardial hypertrophy.

To prove that G_q/G_{11} are required for the induction of cardiac hypertrophy, two genetic approaches were used. G_q/G_{11}-mediated signaling was inhibited by transgenic expression a short fragment of the $G\alpha_q$-C-terminus (Akhter et al. 1998), which resulted in a reduced hypertrophic response. In a different approach, the G_q/G_{11}-mediated pathway was completely abrogated by conditional cardiomyocyte-specific inactivation of the genes encoding $G\alpha_q/G\alpha_{11}$ (Wettschureck et al. 2001). Mice with cardiomyocyte-specific $G\alpha_q/G\alpha_{11}$ double deficiency showed no ventricular hypertrophy in response to pressure overload. This strongly supports the concept that G_q/G_{11}-mediated phospholipase C activation is critically involved in the development of mechanical stress-induced cardiac hypertrophy by coupling receptors of various para- and autocrine factors to the induction of a genetic program that results in the growth of cardiomyocytes.

6 RASSLs

The mutagenesis of G-protein-coupled receptors has revealed receptor mutants that are no longer responsive to their natural ligands, while their activation by synthetic ligands is preserved or sometimes even improved. These modified

receptors are called receptor activated solely by a synthetic ligand (RASSL) (Scearce-Levie et al. 2001). A powerful pharmacological tool for in vivo studies has been generated by the inducible expression of RASSLs in specific organs. Conklin and colleagues used a κ-opioid receptor that contains a sequence of the δ-opioid receptor in the second extracellular loop that causes a 200-fold reduction in binding by the endogenous agonist dynorphin but that maintains normal binding and activation by the small synthetic drug spiradoline. This receptor, called Ro1, was then expressed in an inducible manner in cardiomyocytes using the Tet-system (Redfern et al. 1999). This system can pharmacologically induce G_i-mediated signaling in cardiomyocytes. Activation of Ro1 expressed in the heart by administration of spriadoline led to a decrease in the heart rate by up to 80%. The heart rate changes were observed within less than 1 min after i.p. injection of spiradoline. Once expression of Ro1 was induced for several weeks, animals developed arrhythmias and died within a few months due to severe cardiomyopathy (Redfern et al. 2000). This indicates that the receptor also has some basal signaling activity that is sufficient to induce profound cardiac changes in the absence of an agonist when the receptor is present for longer times.

One possible application of inducible expression of RASSL in mice has recently been shown in the taste system (Zhao et al. 2003; Mueller et al. 2005). To analyze the coding of sweet and bitter pathways that are activated by G-protein-coupled receptors recognizing sweet and bitter tastants, and to test whether activation of sweet and bitter receptors or whether activation of particular cell types expressing the receptors results in the perception of bitterness or sweetness, respectively, the RASSL Ro1 was inducibly expressed either in cells expressing sweet receptors or in cells expressing bitter receptors using the Tet-system. In the absence of doxycyclin when no RASSL is expressed, animals did not show any aversive or attractive responses to spiradoline, as one would expect for perception of bitterness or sweetness, respectively. However, after expression of RASSL was induced by administration of doxycyclin, animals with expression in sweet cells showed attractive behavior towards spiradoline (Zhao et al. 2003), whereas animals with RASSL expression in bitter receptor-expressing cells showed aversive behavior (Mueller et al. 2005). This elegant study clearly shows that defined sensory cells of the tongue act as bitter or sweet sensors, and that their activation via G-protein-coupled receptors induces defined behavioral responses.

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Contribution of Targeted Conditional Somatic Mutagenesis to Deciphering Retinoid X Receptor Functions and to Generating Mouse Models of Human Diseases

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Abstract The last decade has witnessed an enormous rise in the interest for retinoid signalling and its cognate receptors, because of their central role in the coordination of development and homeostasis, through their ability to orchestrate the expression of numerous target genes. These receptors include six nuclear receptor (NR) family members, the retinoic acid receptor (RAR) α, β and γ, and the retinoid X receptor (RXR) α, β and γ, which are expressed in many cell types in mammals. Analysis of the development of mouse embryos bearing retinoid receptor null mutations demonstrated that these receptors transduce the effects of retinoic acid (RA, the active derivative of vitamin A) in vivo, and revealed impressive complexity. However, frequent redundancy in receptor functions and lethality of compound RAR-null mutants, as well as of RXRα-null mutants, precluded the characterisation of the functions of these receptors during late development and postnatally. We illustrate here how recent developments of conditional targeted somatic mutagenesis have opened new avenues in analysing the physiological functions of retinoid X receptor sig-

nalling in a variety of tissues and cell types, as well as in exploring the pathophysiological consequences of their alteration that led to novel mouse models of human diseases.

Keywords RXR · Cre recombinase · Cre-ER^{T2} · Heart · Liver · Prostate · Eye · Adipocytes · Keratinocytes

1 Introduction

Retinoic acids (RAs), the active derivatives of vitamin A (retinol), exert their highly pleiotropic effects through two subfamilies of nuclear receptors (NRs), the retinoic acid receptors (RARα, β and γ) and the retinoid X receptors (RXRα, β and γ), which belong to the NR superfamily of ligand-dependent transcriptional regulators. RARs bind both all-*trans* and 9-*cis* retinoic acid stereo-isomers, whereas RXRs interact exclusively with 9-*cis* RA. RARs, like NRs for thyroid hormone (TRs), vitamin D3 (VDR), peroxisome proliferators (PPARs), bile acids (FXR) and several orphan receptors, require heterodimerisation with RXRs to bind to *cis*-acting response elements located in the regulatory regions of target genes to regulate their expression (Chambon 2005; Evans 2005 and references therein).

RARs and RXRs display specific spatio-temporal expression patterns. Whereas RARα is broadly expressed, the more restricted RARβ expression in the developing embryo and in adult tissues indicated that this receptor could be involved in the differentiation of certain epithelia, as well as in the ontogenesis of the nervous system. RARγ expression is mainly restricted to precartilage and cartilage, and to squamous epithelia. RXRα and RXRβ appear to be diffusely expressed at an early stage of mouse organogenesis; at later stages, expression of RXRα is more restricted and occurs predominantly in liver, adipocytes, digestive tract epithelia and skin, whereas RXRβ remains widely expressed at low levels, even in the adult. RXRγ expression is more restricted at all stages. In the embryo, it is mainly expressed in developing skeletal muscles, where its expression persists throughout life. It is also present in the heart, sensory epithelia, specific structures of the brain, as well as in the thyroid gland (Chambon 1994 and references therein).

To investigate the functions of retinoid receptors in vivo, RAR- and RXR-null mutant mice were generated. Mice lacking RARα, RARβ or RARγ are viable, but display many aspects of the post-natal vitamin A-deficiency syndrome, e.g. RARα-, β- and γ-null mutants are growth-deficient, RARα-null mutants display testis degeneration and RARγ-null mutants squamous metaplasia of the male genital tract. Moreover, compound mutants, lacking a pair of RAR isotypes, altogether display the full spectrum of malformations of the foetal VAD syndrome, and die in utero. Mice bearing RXRα-null mutations also have a number of defects characteristic of the foetal VAD syndrome, such as cardiac and ocular defects, and die during foetal development. In con-

trast, RXRβ/RXRγ double mutants develop normally, indicating that RXRα is the functionally most important RXR during development (Kastner et al. 1995; Mangelsdorf et al. 1995; and references therein). These extensive analyses of RAR- and RXR-null mutant mice confirmed the role of these receptors as transducers of vitamin A effects in vivo, in particular during embryonic and foetal development, and assigned many developmental functions to specific RXR/RAR heterodimers. However, the functional redundancy amongst retinoid receptors, together with the lethality of compound RAR- and of RXRα-germ-line-null mutations, precluded the analysis of many functions of these receptors. This chapter illustrates how Cre-mediated conditional somatic mutagenesis of RXR genes was used to dissect the physiological functions of RXRs in various tissues and to generate mouse models of human diseases.

2
RXRα Is Involved in the Control of Cardiomyocyte Proliferation in a Non-cell-autonomous Manner

Early studies revealed that RXRα-null foetuses display morphological defects that phenocopy human congenital heart diseases, including, notably, double outlet right ventricle. Even though E11.5 RXRα$^{-/-}$ embryos have a normal-sized ventricular chamber, proliferation of the myocardium in the compact zone of the ventricular wall is impaired hereafter, which results in an underdeveloped, thin-walled ventricle, and leads to severely diminished cardiac performance and consequently to embryonic heart failure (Kastner et al. 1994; Sucov et al. 1994; Dyson et al. 1995; Gruber et al. 1996; Kubalak et al. 2002). These results suggested that RXRα might play an important cell-autonomous role in cardiomyocyte proliferation. However, the analysis of chimeric embryos, generated by introduction of RXRα$^{-/-}$ embryonic stem (ES) cells into wild-type recipient blastocysts, revealed that RXRα-null cardiomyocytes proliferate normally (Tran and Sucov 1998). In addition, transgenic expression of RXRα in cardiomyocytes of RXRα$^{-/-}$ embryos did not prevent the myocardial hypoplasia and foetal lethality, even though RXRα was expressed in the ventricles by E10.5 (Subbarayan et al. 2000). Furthermore, conditional selective ablation of RXRα in the ventricular chamber of the heart as early as E8.75, by using transgenic mice expressing the Cre recombinase under the control of the myosin light chain 2v regulatory sequences, did not induce any of the cardiac morphogenic defects observed in RXRα-null foetuses (Chen et al. 1998; see also Table 1). Taken together, these data demonstrate that RXRα is not required in the ventricular cardiomyocyte lineage, and that ventricular muscle cell expansion in the chamber wall is a non-cell-autonomous process. Interestingly, it has been recently shown that ablation of RXRα in epicardial cells in mice expressing Cre under the control of the GATA-5 promoter induces cardiovascular defects, that are, however, less severe than in RXRα-null mice

(Merki et al. 2005). Whether the weaker phenotype results from incomplete RXRα ablation in epicardial cells or the requirement of RXRα in multiple cell types in the heart or in extracardiac tissues remains to be determined. Moreover, further genetic studies should make it possible to identify which RXRα partners are involved in the abnormalities resulting from RXRα ablation in the heart.

3 RXRα Has Important Cell-Autonomous Functions in Hepatocyte Lifespan and Metabolic Functions

The liver, which plays crucial functions in the regulation of energy homeostasis, detoxification and production of serum proteins, is subjected to complex regulations.

Compound RXRβ/RXRγ-null mice are viable and their liver function is not overtly impaired (Krezel et al. 1996). As RXRα is the most abundant of the three RXR isotypes in the liver, it could play a prominent role in this organ. RXRα is apparently not critically required for hepatocyte formation during liver organogenesis, as the liver of RXRα$^{-/-}$ foetuses appears histologically normal, even though its development is delayed by about 24 h compared with WT foetuses (Kastner et al. 1994; Sucov et al. 1994). Moreover, RXRα$^{hep-/-}$ mice, in which RXRα is selectively ablated post-natally in hepatocytes by Cre recombinase expressed under the control of the albumin promoter, are viable, but a number of metabolic events regulated by NRs that heterodimerise with RXRs (e.g. PPARα, CARβ, PXR, LXR and FXR) are impaired (Wan et al. 2000a, 2000b). In agreement with the low contribution of RXRα$^{-/-}$ ES cells to liver formation in chimeric mice, detailed analysis of similar adult RXRα$^{hep-/-}$-mutant mice generated in our laboratory revealed increased serum levels of enzymes known to be released during liver necrosis, and the presence of focal necrotic areas in the liver (Imai et al. 2001b). The hepatocyte proliferative index of such mutant mice was concomitantly increased, most probably reflecting a compensatory hepatocyte proliferation, subsequent to death of hepatocytes lacking RXRα, in order to maintain the liver mass constant. Furthermore, liver regeneration in such adult mutant mice was impaired after partial hepatectomy. Finally, temporally controlled hepatocyte-selective ablation of RXRα, induced by tamoxifen treatment of transgenic mice expressing the ligand-dependent Cre-ER^{T} recombinase (see the chapter by R. Feil, this volume) under the control of the human α1-antitrypsin promoter, confirmed that RXRα has important cell-autonomous functions in mechanisms controlling the lifespan and regenerative capacity of hepatocytes (Imai et al. 2001b).

Interestingly, Huang et al. (2006) have recently shown that bile acids stimulate liver regeneration after partial hepatectomy, by activating the bile acid nuclear receptor FXR (Huang et al. 2006). Thus RXRα/FXR heterodimers might regulate the size of the liver in adults by sensing its functional capacity.

Table 1 Conditional targeted retinoid X receptor ablation mediated by Cre- or ligand-dependent Cre transgenic mouse lines

Floxed target gene	Cre mouse line	Target tissue/ cell type	Name of the mutants	Phenotype	Reference
RXRα (Li et al. 2001; Chen et al. 1998)	MLC2v-creKI (Chen et al. 1998)	Ventricular cardiomyocytes	RXRα flox/flox, MLC2vCreKI/+	No phenotype	Chen et al. 1998
	G5-Cre (Merki et al. 2005)	Epicardium	epi-RXRα	Impaired cardiac morphogenesis	Merki et al. 2005
	alb-Cre (Postic and Magnuson 2000) (Imai et al. 2001b)	Hepatocytes	$RXR\alpha^{hep-/-}$	Altered hepatic gene expression and physiology; hepatocyte necrosis	Wan et al. 2000
	$\alpha AT\text{-}Cre\text{-}ER^{T}$ (Imai et al. 2000)	Hepatocytes (adult stage)	$\alpha AT\text{-}cre\text{-}ER^{T}$/ $RXR\alpha^{L2/L2}$	Hepatocyte necrosis; reduced hepatocyte lifespan and regenerative capacity	Imai et al. 2001b
	PB-Cre4 (Wu et al. 2001)	Prostatic epithelium	Cre+/- $RXR\alpha^{floxed/floxed}$	Increased prostatic ductal branching; prostatic intraepithelial neoplasia	Huang et al. 2002
	Trp1-Cre (Mori et al. 2002)	Retinal pigment epithelium	$RXR\alpha^{rpe-/-}$	Morphological and functional alterations of RPE cells; decreased number and impaired function of photoreceptor cells	Mori et al. 2004
	$aP2\text{-}Cre\text{-}ER^{T2}$ (Imai et al. 2001a)	Mature adipocytes (adult stage)	$RXR\alpha^{ad-/-}$	Resistance to dietary- and chemically induced obesity; impaired fasting-induced lipolysis	Imai et al. 2001
	K14-Cre (Li et al. 2001)	Basal keratinocytes	$RXR\alpha^{ep-/-}$	Alopecia; keratinocyte hyperproliferation, abnormal interfollicular epidermal keratinocyte differentiation, skin inflammation in ageing animals	Li et al. 2001
	$K5\text{-}Cre\text{-}ER^{T}$/ $K14\text{-}Cre\text{-}ER^{T2}$ (Indra et al. 1999; Li et al. 2000)	Basal keratinocytes (adult stage)	$RXR\alpha^{ep-/-(i)}$	Alopecia, keratinocyte hyperproliferation, abnormal interfollicular epidermal keratinocyte differentiation and skin inflammation in ageing animals	Li et al. 2000
RXRα and RXRβ	$K14\text{-}Cre\text{-}ER^{T2}$ (Li et al. 2000)	Basal keratinocytes (adult stage)	$RXR\alpha\beta^{ep-/-(i)}$	Alopecia; atopic dermatitis-like chronic skin inflammation and systemic syndrome	Li et al. 2005, 2006

4
Conditional Ablation of RXRα in the Prostate Epithelium Induces Prostatic Intraepithelial Neoplasia

Vitamin A is known to be involved in prostate organogenesis and homeostasis. Exogenous RA inhibits ductal growth and branching of the four prostatic lobes in mice, and offspring of vitamin A-deficient female mice exhibit a squamous metaplasia or an agenesis of the prostate (Wilson et al. 1953; Aboseif et al. 1997; Seo et al. 1997). Moreover, epidemiological studies have implicated low dietary and serum levels of retinol as a risk factor for the development of human prostate cancer, and RA levels are lower in prostate carcinoma than in normal prostate (Hsing et al. 1990; Reichman et al. 1990). It has also been shown that retinoids reduce the clonal growth and tumorigenic potential of human prostate cancer cell lines and inhibit tumor growth and progression in various chemically induced mouse prostate cancer models (Lasnitzki and Goodman 1974; Chopra and Wilkoff 1977; Pollard et al. 1991; Dahiya et al. 1994; de Vos et al. 1997). Interestingly, the human chromosomal region 9q34.3, encompassing the RXRα gene, is characterised by a high rate of recombination, and the incidence of loss of heterozygosity at this locus has been reported to be 20% in prostate cancer. It has also been recently reported that the nuclear expression of RXRα is often downregulated in human prostate cancer cell lines and specimens, and that overexpression of RXRα in prostate cancer cells induces cell death by apoptosis (reviewed in Roy-Burman et al. 2004).

Mice lacking RARα or RARβ do not exhibit alterations in prostate morphology and function, whereas the prostate of RARɣ-null mutant mice exhibit a squamous metaplasia of the prostate (Lohnes et al. 1993; Lufkin et al. 1993; Mendelsohn et al. 1994). That RXRα could play a critical role in prostate was suggested by the observation that the prostate of single and compound RXRβ/RXRɣ-null mice is not obviously altered (Kastner et al. 1996; Krezel et al. 1996). Interestingly, selective ablation of RXRα in prostatic epithelium, using mice expressing Cre under the control of the probasin promoter, results in increased prostatic ductal branching, and multifocal hyperplasia by 4 months of age. Moreover, 5-month-old mutant mice exhibit low-grade prostatic intraepithelial neoplasia (PIN), while high-grade PIN appear in some of the 10-month-old mutant mice. Monoallelic RXRα ablation also induces prostatic hyperplasia and PIN, but their incidence is delayed by several months (Huang et al. 2002). Such mutant mice should be a valuable resource to investigate the molecular events underlying RXRα signalling in prostate, and be useful to develop therapies or chemoprevention of early stages of prostate tumorigenesis.

5
Ablation of RXRα in the Mouse Retinal Pigment Epithelium Induces Retinal Dystrophy

Vitamin A is critically involved in vision, as a source of 11-*cis* retinaldehyde, the chromophore of opsins in photoreceptors of the mammalian eye (Saari 2000; McBee et al. 2001). The retinal pigment epithelium (RPE), a cell monolayer lying between the choroid and the photoreceptor cells, regenerates this chromophore and phagocytoses distal portions of the continuously growing photoreceptor outer segments (Bok 1993; Nguyen-Legros and Hicks 2000). The pathogenesis of age-related macular degeneration, the major and increasing cause of vision loss among the elderly of the industrialised world, is partly caused by RPE dysfunction, but the underlying molecular mechanisms are largely unknown (Bok 2002). Interestingly, ablation of RXRα in the RPE, using mice expressing Cre under the control of the tyrosinase-related protein 1 promoter, decreased the expression of proteins involved in the visual retinoid cycle, such as cellular retinaldehyde-binding protein, retinal G-protein-coupled receptor and RPE65, and induced morphological and functional alterations of the RPE cells. Moreover, the number of photoreceptor cells was decreased in mutant mice, their outer segment was shortened and disorganised, and the light responses in electroretinograms were reduced (Mori et al. 2004). Thus, RXRα appears to be required for maturation of the RPE, which is known to play essential roles in photoreceptor cell functions and survival. It will be of interest to determine whether altered RXRα signalling pathways in the RPE is causing retinal diseases in humans.

Interestingly, mutant mice lacking both RARβ2 and RARγ2 isoforms (Grondona et al. 1996) exhibit defects similar to those observed in mice lacking RXRα selectively in the RPE, thus indicating that RXRα/RARβ2 and RXRα/RARγ2 heterodimers most probably transduce a retinoid signal required for RPE homeostasis.

6
RXRα Plays a Crucial Role in Adipogenesis and Adipocyte Survival

The adipose tissue is critical for energy homeostasis in vertebrates. Whereas the brown adipose tissue dissipates energy through thermogenesis, the white adipose tissue (WAT) stores excess energy in form of triglycerides when caloric intake exceeds expenditure, and releases free fatty acids when expenditure exceeds intake. The amount of white adipose tissue can be modulated by the formation of new adipocytes from precursor cells (adipocyte differentiation) and/or an increase in adipocyte size (adipocyte hypertrophy). Adipocytes are not only a passive energy storage depot, but also function as an endocrine organ, with far-reaching effects on other tissues (Fasshauer and Paschke 2003).

RXRα is expressed at high levels in white adipose tissue (Tontonoz et al. 1994). The adiposity of RXRα$^{ad-/-}$ mice, in which RXRα is ablated in mature adipocytes by tamoxifen treatment of adult aP2-Cre-ER^{T2} mice bearing loxP-flanked RXRα alleles, is normal when fed a regular diet (Imai et al. 2001a). However, such mutant mice are resistant to dietary-induced obesity, thus demonstrating that adipocytic RXRα is essential for the formation of hypertrophic adipocytes. RXRα$^{ad-/-}$ mice are also resistant to monosodium glutamate-induced obesity, and exhibit an increased number of small adipocytes, indicating that RXRα is not only involved in adipocyte hypertrophy, but could also be involved in adipocyte differentiation. The analysis of RXRα$^{ad-/-}$ mice also revealed that fasting-induced lipolysis was impaired (Imai et al. 2001a).

Surprisingly, the characterisation of mice in which the RXR heterodimeric partner PPARγ is selectively ablated in mature adipocytes revealed that PPARγ-null adipocytes die within a few days (Imai et al. 2004), thus demonstrating that this NR is essential for mature adipocyte survival. Even though adipocyte death was not noted in RXRβ- or RXRγ-null mice, nor in RXRα$^{ad-/-}$ mice, PPARγ most probably exerts its adipocyte vital functions as a heterodimer with RXR, as ablation of RXRα in adipocytes of RXRγ-null mice also results in adipocyte death (our unpublished data). Note that, in keeping with a functional redundancy between RXRα and RXRγ, the expression of RXRγ is enhanced in RXRα$^{ad-/-}$ adipocytes (Imai et al. 2001a). As no such redundancy between RXRα and RXRγ was observed when hypertrophic adipocytes are formed during high-fat diet feeding, the lipogenic and vital functions exerted by PPARγ in mature adipocytes might have different requirements for RXR heterodimeric partners: whereas the lipogenic function requires PPARγ/RXRα heterodimers, the vital function can be mediated by either PPARγ/RXRα or PPARγ/RXRγ heterodimers (Imai et al. 2004). The higher RXRα than RXRγ levels in adipocytes indicate that the threshold level of PPARγ/RXRγ heterodimers required to ensure the survival of adipocytes is lower than that required to trigger lipogenesis. These studies also indicate that high-affinity full antagonists of PPARγ/RXRα heterodimer activity could possibly be used to acutely reduce obesity.

7
Ablation of RXRs in Mouse Epidermal Keratinocytes Generates an Atopic Dermatitis-Like Syndrome

The skin is the largest organ of the body and performs essential functions. It acts as a barrier that restricts the exchange of water-soluble compounds, prevents the entry of infectious micro-organisms, filters out harmful ultraviolet irradiations, and contributes to immunological surveillance. It is composed of the epidermal layer and its appendages (hair follicles), which are separated from the dermal layer by a basement membrane. The epidermis, a stratified

epithelium made principally of keratinocytes, is a highly dynamic structure (Fuchs 1997). The inner most basal layer that is attached to the basement membrane is a proliferative layer, from which keratinocytes periodically withdraw from the cell cycle and commit to terminally differentiate, while migrating outward into the next layers known as the spinous and granular layers, which together represent the suprabasal layers. Terminally differentiated keratinocytes or squames that reach the skin surface, form the cornified layer or corneum. Squamous keratinocytes are lost daily from the surface of the skin, and are continuously replaced by differentiating cells moving vectorially. Hair follicles that develop through a series of mesenchymal–epithelial interactions during embryogenesis are also dynamic structures. They are mostly composed of keratinocytes, and their outer root sheath (ORS) is contiguous with the epidermal basal layer. Once formed, hair follicles periodically undergo cycles of regression (catagen), rest (telogen) and growth (anagen), through which old hairs are eventually replaced by new ones (Hardy 1992; Paus and Cotsarelis 1999).

In the epidermis, RXRα is predominant, RXRβ levels are lower, and RXRγ is undetectable (Fisher and Voorhees 1996; Li et al. 2000). To provide insights into RXRα functions in the skin, RXRα was selectively ablated in epidermal keratinocytes. The foetal development of the skin of RXRα$^{ep-/-}$ mice, in which RXRα-null mutation is generated in epidermal keratinocytes at early stages of their foetal differentiation, by Cre expressed under the control of the keratin K14 promoter, is normal, but postnatal skin maturation and primary hair growth are slightly delayed (Li et al. 2001). However, even though the first hair coat appeared normal, and no defects were observed in the hair follicle at the end of the first hair cycle, mutant mice subsequently lost their hair and developed a progressive alopecia after the 4th week, due to an impaired anagen initiation stage of the hair cycle (Li et al. 2001). Interestingly, VDR mutations in humans and in mice also result in alopecia, which in mice resembles that exhibited in RXRα$^{ep-/-}$ mice; in VDR-null mutants the onset of alopecia also appears to be secondary to defects occurring at the anagen phase of the second hair cycle, but foetal hair morphogenesis is normal (Li et al. 2001 and references therein). Thus, heterodimers of RXR and VDR could play an important role in initiation of anagen during the hair cycle. Cre-mediated RXRα ablation in basal keratinocytes also resulted in hyperproliferation and abnormal differentiation of interfollicular epidermal keratinocytes in adult mice (Li et al. 2001). As these abnormalities are not observed in VDR-null mutants, RXRα may exert functions in epidermal proliferation and differentiation that are distinct from those mediated by RXRα/VDR heterodimers in hair cycling.

Interestingly, RXRα$^{ep-/-(i)}$ mice in which RXRα is selectively ablated in adult keratinocytes, also exhibit altered keratinocyte proliferation and differentiation, and develop progressive alopecia (Li et al. 2000), thus demonstrating that RXRα is important post-natally in processes controlling hair cycling, as well as in proliferation and differentiation of epidermal keratinocytes. Moreover, both RXRα$^{ep-/-}$ and RXRα$^{ep-/-(i)}$ adult mice develop skin inflammatory reactions,

whereas the skin of RXRβ-null mice is apparently normal (Li et al. 2001; Li et al. 2000).

To investigate the origin of the inflammatory reaction induced by RXRα ablation in epidermal keratinocytes and to avoid functional redundancies between keratinocytic RXRα and RXRβ, we generated RXRαβ$^{ep-/-(i)}$ mutants, in which both RXRα and RXRβ are selectively ablated in epidermal keratinocytes of adult mice. These mice develop a phenotype similar to that of human atopic dermatitis (AD), characterised by eczematous-like skin lesions with xerosis and pruritus, associated with a skin inflammatory infiltrate mostly composed of CD4$^+$ T helper (Th) 2 cells, dendritic cells, eosinophils and mast cells, and systemic abnormalities including elevated serum IgE and IgG levels, as well as blood and tissue eosinophilia (Li et al. 2005). These studies not only demonstrate that RXRs play a key role in the control of cutaneous inflammation, but also strongly suggest that keratinocytes could play a key role in the pathogenesis of atopic dermatitis. Moreover, the early and strong induction of thymic stromal lymphopoietin (TSLP) expression in keratinocytes of RXRαβ$^{ep-/-(i)}$ mutants indicates that TSLP produced by keratinocytes might be the cytokine which at the top of a chain of immunological events triggers the atopic syndrome. Transgenic mice overexpressing TSLP in epidermal keratinocytes indeed develop AD-like abnormalities mimicking those seen in RXRαβ$^{ep-/-}$ mice (Li et al. 2005).

Interestingly, topical application to mouse skin of 1α,25-$(OH)_2D_3$ (calcitriol), the physiologically active ligand of VDR, also induces TSLP expression in epidermal keratinocytes, and results in an AD-like syndrome (Li et al. 2006). This induction is a cell-autonomous event most probably mediated by RXR/VDR heterodimers, as it is abolished upon keratinocyte-selective ablation of either VDR or RXRα and β. Moreover, although less efficient than calcitriol, a RARγ-selective ligand also induces TSLP expression, but to a level too low to trigger an overt AD-like phenotype. Thus, under physiological conditions RXRs heterodimerised with VDR and RARγ appears to actively suppress TSLP expression in keratinocytes. It will be interesting to determine whether the association between vitamin D supplementation in infancy and an increased risk of atopy, as well as the link between some VDR genetic variants and atopic diseases in human, are related to increased TSLP production in human epithelial cells (for references see Li et al. 2006). In any event, our data strongly support that dysregulation of Vitamin D signalling is a key contributor to genetic and environmental factors that underlie atopic diseases.

8
Concluding Remarks

The analysis of genetically engineered mice in which retinoid X receptor genes were ablated in various tissues and cell types (Table 1), combined with pharma-

cological approaches, has provided important insights into retinoid signalling pathways, as well as in those controlled by RXR heterodimeric partners. The increasing number of Cre and Cre-ER^{T2} cells specifically expressing transgenic mouse lines will help to further decipher the physiological and pathophysiological functions of these nuclear receptors in the mouse, and will undoubtedly generate many more interesting models of human diseases that will be useful in the future to develop effective therapies.

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