invited review

Of mice and models: improved animal models for biomedical research

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Bockamp, Ernesto, Marko Maringer, Christian Spangenberg, Stephan Fees, Stuart Fraser, Leonid Eshkind, Franz Oesch, and Bernhard Zabel. Of mice and models: improved animal models for biomedical research. Physiol Genomics 11: 115–132, 2002; 10.1152/physiolgenomics.00067.2002.—The ability to engineer the mouse genome has profoundly transformed biomedical research. During the last decade, conventional transgenic and gene knockout technologies have become invaluable experimental tools for modeling genetic disorders, assigning functions to genes, evaluating drugs and toxins, and by and large helping to answer fundamental questions in basic and applied research. In addition, the growing demand for more sophisticated murine models has also become increasingly evident. Good state-of-principle knowledge about the enormous potential of second-generation conditional mouse technology will be beneficial for any researcher interested in using these experimental tools. In this review we will focus on practice, pivotal principles, and progress in the rapidly expanding area of conditional mouse technology. The review will also present an internet compilation of available tetracycline-inducible mouse models as tools for biomedical research (http://www.zmg.uni-mainz.de/tetmouse/).

transgenic mice; knockout mice; conditional mouse models; Cre and Flp recombinase; tetracycline; doxycycline; IPTG; isopropyl-β-D-thiogalactosidase; rapamycine; progesterone; RU486; ecdysone

ONE OF THE PRINCIPAL ISSUES facing biomedical research today is to convert analytical data and sequence information into knowledge about function. With continuously expanding databases of sequenced genes and a seemingly unlimited amount of biologically compelling and clinically significant data, the laboratory mouse has become an increasingly important tool for facilitating functional in vivo data on gene function.

During the last two decades fundamental insight into gene function has been efficiently provided through conventional knockout and transgenic experiments. However, these experiments frequently revealed serious limitations intrinsic to conventional gene-targeting and transgenic approaches. Introducing genetic changes to the germ line of a mouse may track down the effects of a particular gene but may also have severe developmental consequences, complicating or even precluding the desired experimental analysis. Good examples illustrating these restrictions are embryonic lethal knockout phenotypes but also include adaptive gene expression, compensating for the absence or hyperexpression of a specific gene, or cytostatic and cytotoxic effects in response to inappropriate gene expression. To overcome these undesired limita-
tions and to precisely control gene expression or extinction of gene function in a spatiotemporal fashion, conditional mouse models are becoming increasingly popular. It is therefore expected that the second generation of mouse models will significantly improve our ability to address the myriad of questions about gene function.

In this review we will examine different conditional transgenic and gene-targeting techniques and also provide a brief overview of conventional mouse transgenesis. In addition, we will present a novel database compiling already established tissue-specific effector mice for conditionally expressing recombinant genes using the “tet on/off” system. It is not intended to be comprehensive, and the reader is encouraged to explore other excellent recent reviews on this topic (50, 84, 117, 131).

Basic Constitutive Transgenic Strategies

Since the first report of transgenic mice generated by injecting DNA into the pronucleus of one-cell mouse embryos, this technique has been immensely useful in creating model organisms for research purposes (44). Normally, the transgenic construct consists of a selected enhancer and/or promoter, which may also direct gene expression to a specific tissue or developmental stage, linked to the sequence to be expressed. A schematic overview illustrating the basic methodology for generating transgenic mice is shown in Fig. 1. Using this approach, one may directly test in the mouse the role of selected gene products, dominant negative mutants, or specifically designed proteins.

Moreover, antisense mRNA expression or expression of ribozymes can diminish a specific endogenous gene function (30, 32, 66, 79, 87). In addition, overexpression of toxin-encoding genes might be used to mimic hypoplasia (82, 126) and expression of a prodrug metabolizing enzyme like the herpes simplex virus type 1 thymidine kinase is useful to conditionally ablate specific cell populations (18, 29, 111, 135, 144). Alternatively, transcriptional elements including promoters, enhancers, silencers, or complete locus control regions can be assayed in transgenic mice (38, 71, 105, 120, 131).

In theory, once integrated into the murine genome, the injected DNA can manifest its function. However, as the insertion occurs at random, positional variegation effects may be considered, and both the function of endogenous genes might be affected by the insertion of a transgene (insertional effects; Ref. 110). As a solution to this recurring problem single-copy integration of the transgene into a selected site or the use of so-called insulator elements has been described (14, 23, 36, 41, 97). Alternatively, transient generation of transgenic mice is often used for studying transcriptional regulatory elements in mice (131). This system is useful in that no stable transgenic mouse lines need to be established; however, a sufficiently large number of founder animals must be generated and examined. A comparison of all founders will ensure that phenotypes due to variegational position effects of the transgene will become obvious. However, such an approach will be expensive, time consuming, and technically challenging.

Conventional Germ Line Gene-Targeting

With the advent of murine embryonic stem (ES) cells in 1981 and the insight that mammalian cells have the enzymatic machinery to appropriately recombine homologous DNA sequences exactly with their counterparts on the chromosome, the necessary experimental tools for engineering genetic modifications in the mouse were in place (34, 90, 134, 155). Indeed, building on these scientific milestones, the first knockout mice were almost immediately generated by homologous recombination in ES cells (28, 137). A general scheme outlining the basic strategy for generating knockout mice is depicted in Fig. 2.

A crucial advantage of gene-targeting approaches over transgenic techniques is that homologous recombination in ES cells clearly defines the site of integration and allows for precisely designing the genetic change to be introduced. As shown in Fig. 2A a gene-targeting construct will usually consist of a core region containing the desired genetic change together with a positive selection cassette (conferring resistance to antibiotics such as neomycin or hygromycin). This core is flanked by two regions of absolute sequence identity with the targeted region (homology arms) required for homologous recombination to take place. Given that
homologous recombination in ES cells is a relatively rare event compared with random integration of DNA, improved strategies to enrich for recombined ES cell clones are needed. These enrichment strategies usually make use of negative selection markers such as the herpes simplex virus type 1 thymidine kinase gene or the diphtheria toxin \textit{A} chain coding region (10, 20, 161). Using a negative selection marker, ES cell clones with correct homologous recombination will not contain this marker, as removal of nonhomologous flanking sequences will take place prior to homologous integration. By contrast most randomly integrated targeting constructs still retain the negative selection marker, thus permitting counterselection against randomly integrated clones. In case of planning more complicated multistep genome engineering strategies, suitable selection markers allowing both positive and negative selection have also been developed (20).

With the use of conventional gene-targeting strategies, hundreds of genes have been successfully engineered and the corresponding phenotypes analyzed (see http://tbase.jax.org/ and http://research.bmn.com/mkmd for electronically searchable databases).

In addition, the careful comparison of identical genetic changes in different mouse strains revealed the importance of genetic background in the function or loss of function of a particular gene (106, 121). As the genetic background is the collection of all different genes present in the organism it is not surprising that in many cases the observed phenotype, which was introduced by a single genetic modification, might be strongly influenced by other genes. These modifier genes are often completely unlinked to the targeted locus but are closely related to its function. For example, mice null for \(\gamma\)-protein kinase \(C\) showed a marked difference in sensitivity and tolerance to ethanol solely depending on the genetic background (11). This issue is compounded by the desired intercrossing of several modified lines which may have been generated on genetically distinct backgrounds. For this reason it becomes critical for the interpretation of any knockout and transgenic genotype to distinguish whether the observed phenotypic difference is brought about by the targeted mutation or instead is the result of background genetic variation. Nevertheless, there are a number of strategies available to the researcher, appropriate to minimize any undesired variegational
phenotype as brought about by genetic background differences.

Of course, the "gold standard" for any knockout experiment would be to use genetically identical animals as controls. This can be achieved for example by establishing an 129 isogenic inbred knockout mouse strain (usually 129/Sv, 129/SvEv, or 129/Ola) or by using murine ES cell lines derived from additional mouse strains with better reproductive performance than 129 derivatives (26, 73, 81). As an additional possibility, the analysis of mice from the same litter but showing wild-type, heterozygous, and homozygous modified pups can also help to uncover the influence of genetic background on the phenotype observed. Furthermore, analysis of a large number of litters should help to clarify this issue. A very elegant alternative to exclude genetic background effects is to perform all planned experiments with the same individual mouse prior and after the induction of the reversible phenotype (83). Most importantly, reversible gene-targeting strategies may demonstrate that the induced phenotype is clearly associated with the mutated gene as reversion back to a functional gene should reverse the observed effect. However, the most common practice used to minimize genetic background effects is the time-consuming generation of congenic knockout strains. Congenic animals are genetically identical except for a single region containing the introduced change. In general, 2–3 years of backcrosses to a defined strain are required before the resulting genetic backgrounds are statistically >99% homogeneous (129). The Jackson Laboratory offers a possible shortcut in this time-consuming backcrossing by implementing marker-assisted selection of mice. This technique can reduce the time needed to generate a defined genetic background on backcrossed mice from 10 generations to 5 generations, shortening the time required by half (http://jaxmice.jax.org/html/services/services-speedcongenic.shtml). Commercial suppliers including the Jackson Laboratory may also offer F2 hybrid mice that approximate the mixed strain background under analysis. As a last resort to avoid phenotypic variations, it is possible to examine large numbers of knockout mice with mixed background, thus ensuring that the range of phenotypes due to genetic background are detected.

Although it is not always feasible to use genetically identical mice for analyzing the effect of a given knock-out, and the majority of previously published phenotypes are from genetically mixed backgrounds, it always makes sense to use the most genetically similar background to the knockout as a control.

Finally, gene trap approaches in ES cells have been used for generating entire knockout libraries (33, 132, 152, 166). With this approach, the unique integration site of the transfected vector serves as a signpost for the subsequent identification of the trapped gene. In addition, through the combination of a promoterless splice acceptor sequence together with a reporter gene as part of the gene trapping strategy, information regarding the spatiotemporal expression of the trapped gene can often be obtained in ES cell-derived hemizygous animals. Electronically searchable databases of large-scale public domain and commercial gene trap initiatives can be accessed via http://socrates.berkeley.edu/~skarnes/resource.html, http://tikus.gsf.de/, and http://www.lexgen.com. Taking a closer look at these sites, especially at the noncommercial ones, prior to initiating a planned knockout project is therefore good practice and may also save time and money.

Conditional Switchable Transgenic Mice

In experimental settings, use of conventional transgenic technology control over the onset of transgene expression will strictly depend on positional integration effects and on the nature of the chosen regulatory elements. However, constitutive expression of a transgene is often too inflexible to meet the needs of a specific experimental question. For example, too early or to widespread expression of the transgene may lead to phenotypic or physiological aberrations producing secondary pleiotropic responses as a result of the introduced genetic alteration. Distinguishing effects of the resulting phenotype might turn out to be extremely difficult, as cell autonomous versus cell non-autonomous effects are not clearly divisible and compensatory systemic changes are often concealed. In addition, for many experimental questions it might be necessary to analyze the function of a transgene within a specific developmental window or in a particular cell lineage. Unfortunately, adequate temporal or tissue-restricted promoters may not always be available. For these reasons, the perfect conditional transgenic mouse should, in principle, include the following criteria. First, induced overexpression of the transgene should be tightly controlled such that no leaky background expression precludes the accurate analysis. Second, the inducing compound should be nontoxic and highly specific for the target gene. Third, induction kinetics should be fast and expression levels sufficiently high to produce a rapid and detectable effect. Fourth, the induced switch should be reversible so that defined developmental periods or critical stages in disease can be appropriately monitored.

To date, several transgenic mouse systems have been successfully used for tightly controlling spatiotemporal reversible transgene expression in mice. Nearly all of them share a common denominator in that they hinge on the sequence of three principal events: 1) Ligand-mediated activation of a transcriptional transactivator, 2) DNA binding of the transactivator, and 3) transactivator-induced transcriptional activation (see Fig. 3A). Translating this system into a conditional mouse model usually requires an effector mouse, expressing a ligand-inducible transcriptional transactivator, and, in addition, a responder mouse line, which has the capacity to specifically express a chosen transgene upon stimulation by the transactivator. Subsequent intercrossing of effector mice with the responder line should lead to the birth of bi-transgenic offspring, capable of conditionally expressing the de-
sired transgene (see Fig. 3B for a schematic representation of the underlying strategy).

Historically, these binary systems were plagued by leaky transcription of the transgene even in the absence of the inducing ligand (9, 69). Furthermore, as the initial activators were derived from oncogenic viruses, undesirable neoplastic activation of endogenous mouse genes was observed (99). By now, these initial problems have been principally solved, presenting the researcher with several effective options as to how he or she will set up tight “genetic switches” for the projected conditional mouse.

The Tetracycline System

Over the last decade the tetracycline (tet) regulatory system has been extremely useful for generating reversible transgenic mouse models. This has led to a large collection of tet-controlled transgenic mice suitable for studying gene function in many different cell types and also during selected time points (for recent reviews, see Refs. 3 and 88, as well as http://www.zmg.uni-mainz.de/tetmouse/ for an electronically searchable database).

Pioneering work by Gossen and Bujard (45) demonstrated the general usefulness of the Escherichia coli Tn10-derived tet resistance operon for tightly regulating conditional transgene expression in mammalian cells. In this study the tet repressor (TetR) was fused to the VP16 transactivation domain of herpes simplex virus, resulting in the tetracycline controlled transactivator (tTA). In the absence of tetracycline, or the commercially available low-cost alternative, doxycycline (DOX), tTA binds to its specific tetracycline operator consensus sequence (tetO), being able to also activate transcription of a chosen transgene when combined with a synthetic minimal promoter sequence. By contrast, addition of DOX to this system induces a conformational change in the tTA effector, preventing DNA binding and leading to a shutdown of the target gene. In summary, use of the tet system requires two coordinate building blocks: the ligand-dependent transactivator tTA as the effector, and a tetO-CMV minimal promoter cassette governing the expression of the transgene as the responder. Figure 4A demonstrates the underlying mechanism of the tet-off system.

Fig. 3. Conditional transgenesis. A: binary control of conditional gene expression at the molecular level. 1) Ligand-mediated activation of the effector composed of an activation domain (yellow) and a ligand-binding domain (light blue). Ligand is the solid brown box. 2) Specific interaction of ligand-bound effector with its cognate binding site (CBS). 3) Transcriptional induction of the gene of interest. B: implementing binary switches in mice. In general, for the production of conditional transgenic mice, two independent genetically modified mice strains are required: first, a line expressing a ligand-dependent effector, and second, a responder line which conditionally expresses the transgene under the control of the effector. Crossing of the effector with the responder line results in a bi-transgenic mouse containing all necessary elements for conditionally regulating the expression of the gene of interest. Pmin, minimal promoter; pA, polyadenylation signal; Pspec, specific promoter.
The well-established pharmacological properties of tetracycline and many of its derivatives made these compounds very promising candidate regulators for establishing conditional regulatory systems in the mouse. These properties include predictable pharmacokinetics, good tissue distribution, known half-life times, lack of toxicity, and the ability to cross mammalian cell membranes and also the placenta. In actual fact, almost immediately after the initial publication of the tTA tet-off regulatory system in tissue culture cells, the first report of tet-based transgenic mice demonstrated the excellent potential of this system (42). Despite the impressive induction levels, reaching in some tissues five orders of magnitude, and excellent shutdown kinetics which were reported, certain limitations of the tet system also became evident. Initial major problems included repeatedly detectable residual activity of the tetO-CMV minimal promoter, cellular toxicity of the transactivator, low sensitivity to DOX in certain tissue types, internal cryptic splice acceptors within the transactivator coding sequence leading to incorrect translation, instability of the transactivator coding mRNA, and slow in vivo induction kinetics of the transgene. During the last decade these problems have been effectively addressed, and at present an exquisite assortment of “second-generation” tet-based regulatory systems for generating conditional mice are available to the researcher.

One of the first crucial advances building on the original tTA system was the development of the reverse tet-on tetracycline-controlled transactivator (rtTA, tet-on shown in Fig. 4B). This rtTA molecule is induced upon administration of DOX thus complementing the original tTA system and also solving the problem of slow activation kinetics (46). Introducing the rtTA system to transgenic mice increased the speed of transgene expression substantially, in some cases reaching complete activation in about 1 hour compared with the slow activation kinetics of up to 1 week reported for the original tTA (51, 72).

A second recurring drawback of the tet system was the unwanted residual activity of the tetO-CMV responder even in the absence of an effector (42, 58, 68, 72). This problem was tackled by direct transcriptional targeting of the leaky tetO-CMV responder. To this end, tet-dependent transcriptional repressors were designed which do not physically interfere with the tet-transactivators but will repress any residual transcription of the responder during noninduced periods. These tetracycline-controlled repressors are fusion proteins combining the DNA-binding domain for the tetracycline operator consensus sequences (tetO) fused to a

![Fig. 4. The Tet system. The Tet system can be used to conditionally activate gene expression in the mouse. A: the Tet-off system (tTA) will activate expression in the absence of its ligand doxycycline (DOX, shown as brown box). Upon addition of DOX, transcription of the gene of interest is extinguished. B: in contrast, addition of DOX to the Tet-on system (rtTA) results in transcriptional induction of the gene of interest. tTA, tetracycline-dependent transactivator; rtTA, reverse tetracycline-dependent transactivator; DOX, doxycycline (ligand); TRE, Tet-responsive element.](http://physiolgenomics.physiology.org/).
strong transcriptional silencing element. A very elegant strategy to ensure stringent DOX-dependent regulation of the tetO-CMV responder is coexpression of the activator together with the repressor using a bicistronic approach in genetically modified animals (39, 40, 58). Interestingly, first experiments in transgenic mice provided convincing evidence for DOX-mediated repression capable of completely suppressing transcription of the target gene without compromising rTTA-mediated activation (167).

In certain cell types and transgenic animals, stable expression of the original rTA and tTA appeared to be difficult, probably due to toxicity resulting from the VP16 transactivation domain (8). To overcome this problem a set of tetracycline-responsive transactivators, which are tolerated at higher intracellular concentrations, have been developed (1, 5, 139). Finally, remodeling of the original tTA and rTTA transactivators has been efficiently used for substantially improving the activator together with the repressor using a bicistronic approach in genetically modified animals (138, 141, 151).

Moreover, the tetO responder cassette has also been significantly improved, allowing the simultaneous expression of two tet-controllable transgenes by virtue of a bidirectional tetO-CMV minimal promoter unit (4). Combining the expression of the transgene with a reporter gene using bidirectional tetO-CMV promoters has already proven to be a formidable experimental tool allowing direct visualization of concurrent transcription of two genes (75). In this respect a recent report demonstrated that it was possible to noninvasively monitor the expression status of a transgene in the living animal (51).

Finally, two groups have reported the successful use of a single construct containing both a tet-responsive transactivator together with the transgene responder on the same plasmid (123, 140). This strategy circumvents the need to generate two independent transgenic lines in the first place and greatly facilitates the mouse breeding strategy as effector and responder will not segregate.

Taken together, during the last years the tet system has seen numerous remarkable improvements. These important advances will help the researcher to better design conditional transgenic models and also to adjust the experimental setup according to his or her specific needs. As the overall number of individual tet-responsive transgenic mouse strains is abundant, the scope of this review does not allow for the detailed description of each and every single study. The reader is, however, encouraged to explore the excellent expert reviews and to also visit the database at http://www.zmg.uni-mainz.de/tetmouse/, which provides direct links to most of the original papers.

**Hormone Receptor-Based Transgensics**

Over the past couple of years, several groups have reported progress in developing conditional mouse models based on nuclear hormone receptor fusions including progesterone-, estrogen-, andecdysone-based transgene switches. In the following sections we will provide an overview of these three similar systems.

**Progesterone and estrogen receptor-based models.** Upon stimulation with an adequate ligand nuclear steroid hormone receptors will form active dimers capable of binding to DNA and activating transcription (19, 35, 109). This property of nuclear receptors makes them amenable as molecular switches in mammalian cells. Indeed, tissue culture experiments suggested that progesterone as well as estrogen nuclear receptor fusions might be suitable effector molecules for regulating target gene expression in mammalian cells (12, 149).

**Progesterone receptor-based models.** Interestingly, COOH-terminal truncated versions of the human progesterone nuclear receptor not capable of binding to endogenous progesterone could nonetheless be activated by RU486 or other synthetic progesterone antagonists (149, 150). As the concentration of synthetic inducers required for the induction of transgene expression turned out to be well below any physiological active threshold, unwanted endogenous anti-glucocorticoid and anti-progesterone effects were not observed.

To establish a suitable system for conditionally expressing a chosen transgene in a mouse, a chimeric fusion protein containing three active domains was constructed. This tripartite polypeptide consisted of the COOH-terminal truncated human progesterone nuclear receptor as the ligand-inducible molecular switch, the GAL4 DNA-binding domain as a specific DNA-anchor [GAL4 binds exclusively to the UAS ("upstream activator sequence"), of Saccharomyces cerevisiae but not to any murine genomic sequence], and the strong transcriptional activation domain of herpes simplex virus VP16. By putting these three elements together, a system for the generation of conditional transgenic mouse models was successfully established, known as GLVP-fusion (for "GAL4/herpes simplex virus VP16 transcriptional activation domain and human truncated progesterone receptor fusion"). Upon binding to a suitable ligand, chimeric GLVPs will associate into active homodimers, capable of specifically binding to GAL4-responsive UAS elements and thus ultimately inducing the desired expression of the transgene. The GLVP system will also allow to deliberately switch between activated and repressed expression of the transgene by simply using a synthetic progesterone antagonist to control the GLVP switch. Good examples for the effectiveness of progesterone-based transgenic mouse models include conditionally switchable lacZ reporter mice (43), tissue-restricted expression of the Cre recombinase (67), the conditional expression of TGFβ3 in the epidermis (147), and the rescue of the lethal α-inhibin phenotype (108).
Estrogen receptor-based models. By means of a similar fusion strategy, the estrogen receptor encoding sequence was fused to a GAL4 DNA binding domain and the VP16 transactivator. In the presence of estrogen the resulting tripartite fusion protein becomes an active dimer, binds to DNA, and will lead to transgene activation through the GAL4 promoter (12). An obvious drawback of estrogen-based systems in the mouse is that estrogen plays an important physiological role in the regulation of cell proliferation and differentiation. As a consequence, other endogenous estrogen-responsive genes will also be induced upon estrogen administration (56, 165). Conversely, endogenous estrogen levels might also activate transcription of the transgene leading to unwanted “leaky” transgene expression. For this reason estrogen receptor fusions to GAL4 and VP16 have not been a preferential choice for generating conditional transgenic mice. By contrast, direct fusions of the estrogen receptor to a variety of heterologous protein coding sequences including transcription factors, oncogenes, RNA-binding proteins, kinases, and the Cre recombinase have been very effective for conditional expression of biologically active fusion proteins (107).

Ecdysone receptor-based models. Ecdysone is a steroid hormone necessary during metamorphosis of insects. The natural ligand for the ecdysone receptor is the 20-OH-ecdysone molting hormone, capable of specifically binding to a heterodimer of the Drosophila ecdysone receptor (EcR) and the product of the ultraspireacle (USP) gene. The activated EcR-USP heterodimeric complex will bind to an ecdysone-responsive element and induce transcription of genes involved in the onset of metamorphosis (162). Several groups have taken advantage of the ecdysone system for conditionally expressing genes in mammalian cells (48, 148, 163). The potential of this system for generating conditional transgenic mice was first shown using a fusion of the VP16 and the glucocorticoid receptor transactivation domains to a truncated version of the ecdysone receptor (100). In addition, it could be shown that the retinoid X receptor, which is the mammalian homolog of the natural ecdysone heterodimer partner USP in invertebrates, will also form functionally active dimers with an engineered ecdysone effector. These studies also demonstrated that transcriptional activation could be selectively targeted to a synthetic ecdysone-responsive element, thus bypassing any unwanted activation through endogenous nuclear hormone receptors (100, 136). As a still simpler alternative to the obligate EcR-USP heterodimeric system, a chimeric Bombyx/Drosophila ecdysone receptor has been created that does not require any retinoid X receptor heterodimeric partners for regulating conditional transgene expression (54). Although the ecdysone system seems very appropriate for the generation of inducible transgenic mouse models, to our knowledge only a small number of additional ecdysone-based transgenic mouse models have been reported in the literature (2, 119).

Other Inducible Transgenic Models

In addition to the nuclear receptor- and tetracycline-based conditional systems, two different additional innovative approaches have been examined in transgenic mice. Both regulatory systems hold great promise for generating conditional transgenic mouse models but also provide the opportunity to be employed in concert with other switchable systems, thus allowing the control of two and more genes in the same mouse.

The cytochrome P-450 induction system. A prerequisite for most binary switchable systems is the need for establishing two independent transgenic strains. By contrast, the use of ligand inducible endogenous mammalian promoters/enhancers can overcome this necessity, avoiding in some cases the complexity of bi-transgenic animals. However, it is important to bare in mind, that deciding on a mono-transgenic regulatory system might restrict transgene induction to specific tissues and moreover might also preclude the opportunity of crossbreeding the generated effector mouse with other available responder lines and vice versa. The cytochrome P-450 regulatory system is a good example of an effective mono-transgenic approach and has been used to stringently control transgene expression in the mouse and also the rat (17, 65).

Cytochrome P-450 monoxygenases comprise a multigene family whose gene products are essential for detoxifying lipophilic xenobiotics (101, 133). One member of the monoxygenase family, the cytochrome P-450 1A1 (CYP 1A1), is not constitutively expressed in mouse tissues and only becomes activated upon exposure with certain compounds including polycyclic aromatic hydrocarbons (104). In a proof-of-principle experiment, a combination of CYP 1A1 promoter/enhancer elements was successfully utilized to conditionally control the expression of a lacZ reporter gene (17). This study demonstrated tight and reversible regulation, reaching induction levels over four orders of magnitude in certain tissues. Expression was predominantly found in the liver but included several other organs. As the molecular pathway leading to the induction of CYP 1A1 gene activation depends on the amount and chemical nature of the inductor the possibility for dose-dependent fine-tuning of transgene expression was also reported (17). A recent publication by Kantachvesiri and colleagues (65) exquisitely illustrated the enormous potential of CYP 1A1 promoter/enhancer transgene expression to answer fundamental questions in physiology. Using both dose-dependent and reversible transgene expression of renin-2, the authors could tightly control hypertension in the rat. This switchable renin-2 rat model represents an invaluable tool for studying malignant hypertension including accelerated rise in blood pressure, endothelial injury, activation of the renin-angiotensin system, and induced microangiopathy (65).

IPTG-based inducible systems. The recently published isopropyl-β-D-thiogalactosidase (IPTG)-inducible two-compound system for generating conditionally controllable transgenic mice relies on the lactose...
The lac operon switch originally described in a classic paper by Jacob and Monod (61). The *E. coli* lactose operon consists of two main parts the lacI repressor and its cognate DNA binding site lacO. In the absence of lactose the lacI repressor will tightly bind to lacO preventing the transcription of genes important for metabolizing lactose. Only in the presence of lactose does lacI dissociate from lacO and transcription of genes involved in lactose utilization can begin. Initial attempts to utilize the lactose operon in transgenic mice failed as no functional lacI gene was detected as a result of transgene inactivation through DNA methylation (125, 158). To adjust the bacterial lactose operon to eukaryotic transcription and translation standards, Cronin and colleagues (22) meticulously modified the original sequence and gene structure of lacI resulting in the synthetic synlacI repressor. To generate transgenic mice which express the synlacI repressor in a constitutive fashion, the synlacI cDNA was put under the transcriptional control of a human β-actin promoter element. Using tyrosinase, which is an essential enzyme for melanin biosynthesis in the mouse, as a reporter gene they demonstrated that the synlacI repressor system perfectly allowed for conditionally controlling tyrosinase expression. In addition, these experiments showed complete reversibility of transgene expression in the absence of inducer and also, what may be important for developmental experiments, good conditional regulation of tyrosinase in the adult mouse, embryo, and nursing pup. Taken together this first report suggests that the newly developed synlacI IPTG-inducible system provides tight and switchable control of transgene expression in the mouse, making it an excellent candidate tool for generating conditional transgenic models.

**Conditional Gene Targeting**

Each gene-targeting experiment normally involves two basic steps. First, homologous recombination will be used to stably introduce a desired genetic change into the DNA of pluripotent ES cells. After aggregation with or injection into the early embryo, ES cells will be capable of colonizing all its tissues leading eventually to the birth of chimeric offspring. In case the engineered ES cells have contributed to germ cells, the desired genetic modification will be transmitted to future generations (for an excellent hands-on gene-targeting manual see Refs. 53 and 64). With this technique, it has been possible to delete gene function (knockout), to insert novel selected genes or DNA fragments into a given locus (“knock-in”), to introduce subtle changes like point mutations, and to produce chromosomal rearrangements in the germ line of the mouse.

Conventional gene-targeting experiments can be designed to introduce the desired genetic changes into the germ line, thus affecting all tissues during the entire lifespan of the resulting mouse. Several limitations of this germ line gene-targeting approach are obvious. As the mutation will be already present in the first developing cell, an embryonic lethal phenotype might be provoked, precluding any further functional analysis during adulthood. This is particularly true for classic gene knockouts of developmentally important genes. Nearly as undesirable as embryonic lethal phenotypes are pleiotropic effects often observed as a compensatory reaction to the introduced germ line mutation obscuring or preventing a clear-cut analysis. Moreover, the selected gene might have a wide expression pattern, and its general invalidation might thus induce a highly complicated accumulative phenotype involving multiple tissues. Finally, it might be important to only extinguish gene function at a specific developmental time point or during a particular stage in disease. For all these reasons it can be of importance to create mouse models allowing the deletion of genetic material/gene function in selected cells at a specific time. In this manner, one can efficiently avoid embryonic lethality, prevent unwanted pleiotropic side effects and exclude accumulative compensatory developmental changes starting from the earliest developmental stage. The use of site-specific recombinases of the Cre and Flp α-integrase family and the recent development of the first reversibly switchable knockout models have opened an exciting new avenue for creating conditional loss or gain of function mouse models.

**Cre and Flp recombinases.** The first site-specific recombination system used for introducing conditionally inducible genetic changes in the mouse was the Cre-lox system (78, 102). Based on the enzymatic activity of the Cre (for “causes recombination”) recombinase, originally isolated from the bacteriophage P1, it is possible to induce site-specific genetic recombination to sequences flanked by so-called loxP sites (“locus of crossover P1”) (49, 52). In a similar fashion the *S. cerevisiae* recombinase Flp will cause recombination between FRT (for “Flp recombination target”) recognition sites (70). LoxP and FRT recognition sequences are 34-bp long DNA motifs consisting of an 8-bp core region flanked by palindromic sequences of 13 bp. As shown in Fig. 5, Cre and Flp recombinases mediate different effects on their DNA target sequences depending exclusively on the orientation of the specific recognition sequence and the number of different DNA molecules involved. Cre- and Flp-induced changes include sequence excision, duplication, integration, inversion, and chromosomal translocation. All of these are extremely useful for tailoring the mouse genome, but as Cre and Flp recombinases have been most extensively used for small scale DNA rearrangements affecting single genes, we will focus in this review on conditional gene knockout strategies. Proof-of-principle experiments have shown that Cre and Flp recombinases will efficiently work in any cellular environment and on any kind of DNA target. In addition, the Cre/loxP and the Flp/FRT systems do not need any additional cofactors or sequence requirements for site-specific recombination in the mouse. Further reading about Cre- and Flp-based strategies for generating chromosomal rearrangements including large deletions and translo-
A gene is achieved by homologous recombination of a specific inducible mouse model. Normally, inducible modifications (47) demonstrated the exquisite potential for creating inductive work from the laboratory of Klaus Rajewsky using ES cell technology with Cre recombinase action, pioneered work from the laboratory of Klaus Rajewsky (47) demonstrating the exquisite potential for creating inductive mouse models. Normally, inducible modification of a specific gene is achieved by homologous recombination in ES cells flanking the sequence to be excised by unidirectional recombination sites. B: inversion requires the sequence of interest to be flanked by bidirectional recombination sites. C: a single recombination site within the genome can be used as a molecular acceptor for inserting a DNA sequence containing a second recombination site. D: interchromosomal translocations can be engineered by placing single recombination sites on different chromosomes. Solid triangles indicate recombination sites. White arrow indicates orientation of the sequence to be excised.

Fig. 5. Possible genetic modifications mediated by Cre or Flp recombinases. Both Cre and Flp recombinases can be used for introducing different molecular alterations into the mouse genome. A: excision of genomic DNA can be mediated by flanking the sequence to be excised by unidirectional recombination sites. B: inversion requires the sequence of interest to be flanked by bidirectional recombination sites. C: a single recombination site within the genome can be used as a molecular acceptor for inserting a DNA sequence containing a second recombination site. D: interchromosomal translocations can be engineered by placing single recombination sites on different chromosomes. Solid triangles indicate recombination sites. White arrow indicates orientation of the sequence to be excised.

Fig. 6. Ubiquitous or tissue-specific recombinase-mediated gene inactivation. A: LoxP site flanked gene locus exhibiting wild-type gene function prior to recombinase excision. B: general deletion of exon 1 using a ubiquitous promoter to drive Cre expression (top) and tissue-specific exon 1 deletion resulting from directed expression of Cre in liver (bottom). P, locus-specific promoter; LoxP, Cre-recognition sites (solid triangles); Pusb, ubiquitous promoter; Pln, liver-specific promoter; Cre, Cre recombinase.
expected to express the recombinase at a high enough level and specificity to introduce the desired alteration. A common problem with many deleter lines is that they will induce incomplete excision of the targeted allele leading to mosaicism of the mouse (145). As the analyzed phenotype might directly reflect the ratio of deleted to wild-type cells, a precise interpretation of the effects might be difficult or impossible. In addition, the often observed lack of control over the spatiotemporal timing of recombination is in many cases due to the nature or leakiness of the promoter and will lead to inappropriate excision of the targeted allele in cell types other than those desired (27). Conversely, for many applications, including those for studying cancer in the mouse, partial inactivation of gene function might be favorable (115, 142).

Overlaying promoter-specific spatiotemporal activity with separately inducible activation of a recombinase promises to further enhance the potential of site-specific recombination. Over the past years several laboratories have developed systems for exogenously controlling Cre activity in mice. These include the control of Cre recombinase activity itself by a synthetic ligand and ligand-dependent transcriptional activation of Cre expression.

The first inducible Cre mouse made use of the Mx1 promoter to drive expression of Cre (76). Since transcriptional activation of the Mx1 promoter is directly dependent on interferon-α, interferon-β, or double-stranded polyinosinic-polycytidilic nucleic acids, expression of Cre can be exogenously controlled. However, the use of this Mx1-Cre deleter strain was essentially limited to excision of alleles in the liver and the immune system since recombination in other tissues was poor (76, 114). As an alternative several groups have developed combined tet-Cre mice allowing Cre expression to be tightly controlled through the tet on/off system. Using DOX-inducible expression of Cre, researchers have reported efficient excision of floxed alleles in the brain and in the intestine (51, 86, 118). In addition, Utomo and colleagues (140) demonstrated tight tissue-specific expression of Cre in transgenic mice with an approach that combined tetO-dependent Cre transcription with rtTA tetracycline transactivator expression on the same construct thus greatly facilitating breeding schemes.

A very different but also effective strategy to exogenously control Cre activity in mice has recently been developed. This strategy required the fusion of Cre proteins to progesterone or ecdysone receptor ligand binding domains (see section on Hormone Receptor-Based Transgenics). Proof-of-principle experiments have shown the versatility of these ligand-dependent recombinase fusions, and several groups have adapted this strategy for temporal control of Cre activity in the mouse (93, 124, 145). The first brand of inducible Cre recombinases employs a truncated progesterone receptor which is directly fused to the Cre recombinase (96, 157), whereas the second approach makes use of a fusion between the Cre recombinase and an estrogen receptor (48, 93, 94, 116, 124, 127). Nevertheless, close inspection of the induced recombination frequencies clearly demonstrated that recombination mediated by some first-generation fusion-recombinases might be mosaic depending very much on the tissue type (13, 24, 67, 124, 143, 145). It is therefore to be assumed that better inducible recombinase molecules are needed to efficiently regulate Cre or Flp activity in the mouse (57, 59, 85, 157).

Protein engineering has also led to the availability of improved second-generation recombinases with novel properties. These changes include codon-improved Cre and Flp recombinases with better in vivo efficiencies (15, 74, 113, 127), engineered recombinases with novel recognition sites (16, 122), and Cre recombinases capable of permeating cell membranes (62, 103).

Despite all existing limitations and the irreversible nature of recombinase-induced genetic changes, Cre- and Flp-based conditional knockout mice have already proven to be a reliable tool for generating conditional mouse models. Investigators considering inducible Cre- and Flp-based mouse models should visit the http://www.mshri.on.ca/nagy/cre.htm database which contains an excellent compilation of general and tissue-restricted deleter strains including also a useful list of different Cre and Flp reporter mice.

Switchable gene knockout. In an ideal mouse model the exogenous control over the knockout phenotype should be reversible. Such a completely “switchable” knockout mouse has significant advantages over “one-way” inducible systems allowing the in vivo analysis of gene function (or loss of gene function) during precisely defined developmental periods and at critical stages of disease. So far, two different groups have been successful in generating such switchable knockout phenotypes. Both approaches made use of restoring gene function by virtue of ligand-mediated overexpression of the switchable gene in an otherwise null background.

One example of a reversibly switchable knockout phenotype in the mouse was the rescue of the α-inhibin null mouse (108). When the inhibin α-subunit is deleted from the germ line, inhibin-deficient mice develop gonadal sex cord-stromal tumors with a penetrance of 100% and ultimately die of a cachexia-like syndrome (91, 92). To be able to exogenously control the expression of α-inhibin in transgenic mice, a progesterone-based binary mouse model was established. In this bi-transgenic mouse, expression of α- and β-inhibin in the liver was strictly dependent on the presence of the inducing ligand mifepristone. By crossing these bi-transgenic mice into an α-inhibin null background, it was possible to completely rescue the lethal phenotype and to also prevent the onset of gonadal sex cord-stromal tumors. Additional experiments with these conditional α-inhibin knockout mice further demonstrated that the system is reversible, as “switching off” α-inhibin gene function did lead to gonadal tumorigenesis. Interestingly, the α-inhibin hormone is not normally produced in the liver but transgenic expression of α-inhibin in hepatic tissues fully rescued the phenotype. This raises important concerns about limitations and general feasibility of switchable knockout mouse
models. Does the induced or rescued phenotype depend on the level of expressed transgene product? How important is the site of transgene expression? How is it possible to exactly direct transgene transcription to the right cells at the right time, thus recapitulating the normal spatiotemporal expression pattern of the endogenous gene?

To some extent these important questions have been addressed using a tet on/off based approach to generate a completely switchable knockout mouse for the G-protein-coupled Endothelin receptor B (Ednrb) (128). To ensure that switchable expression of the Ednrb gene is regulated in a spatiotemporally appropriate manner, a tet-inducible transactivator has been integrated into the endogenous Ednrb locus. With this knock-in strategy two essential objectives are met. First, expression of the inserted tetracycline-dependent transactivator will closely mirror expression of the endogenous Ednrb gene; second, the knock-in leads to complete inactivation of one allele of the Ednrb gene (knock-in effector). A further functional requirement for this approach is a tetO-CMV responder expressing Ednrb in a DOX-dependent fashion. Shin and colleagues (128) introduced this DOX-responsive element into the Ednrb locus which will equally lead to a mono-allelic inactivation of the endogenous Ednrb locus (compare wild-type locus in Fig. 7A with the switchable Ednrb locus in Fig. 7B). Crossing of the knock-in effector with the knock-in responder should lead to mice harboring both the tet-responder and tet-effector in an otherwise Ednrb null background. Analysis of these mice demonstrated that DOX-induced Ednrb expression could fully rescue the null phenotype and that DOX-mediated repression of Ednrb transcription recapitulated the null phenotype (see Fig. 7C for DOX-dependent regulation of Ednrb expression). Moreover, these experiments identified the restricted time periods during which Ednrb gene function is essential for the development of melanoblasts and enteric neurons. This pioneering and elegant experimental approach is not only a good example for workable conditional mouse models but also illustrates the enormous potential of binary systems for generat-

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**A Wildtype Ednrb locus**

![Diagram](attachment:Wildtype_Locus.png)

**B Switchable Ednrb locus**

![Diagram](attachment:Switchable_Locus.png)

**C DOX-dependent regulation of Ednrb expression**

![Diagram](attachment:DOX_dependent_Regulation.png)

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Fig. 7. An example of switchable gene inactivation-endothelin receptor B conditional knockout. A: wild-type locus; endothelin receptor B (Ednrb) single exon locus. B: switchable locus; the engineered Ednrb locus consists of a knocked-in Tet transactivator (tTA) both substituting and inactivating its first allele (effector). In addition, a conditional Ednrb responder knock-in cassette replaces the second allele. This “knock-in/knock-out” strategy results in completely switchable and reversible expression of Ednrb in an otherwise Ednrb null background. C: regulation of Ednrb gene expression is entirely dependent on administration of DOX. Knock-in of the tTA cDNA into the Ednrb locus places the expression of tTA under transcriptional control of the Ednrb locus leading to the precise recapitulation of Ednrb expression by tTA. In the presence of DOX, no Ednrb is expressed from the responder cassette located within the second Ednrb allele. However, in the absence of DOX, Ednrb expression will be resumed. DOX, doxycycline (brown box); P<sub>ednrb</sub>, Ednrb promoter; tTA, tetracycline-dependent transactivator; TRE, Tet-responsive element.
ing completely switchable and appropriately spatio-temporally regulated knockout models.

Most of the inducible strategies discussed above have been already applied for research in physiology. A general overview as to how inducible mouse models serve as experimental tools for answering many of the open questions in diverse research areas such as cancer, metabolic/endocrine dysfunction, cardiovascular disease, and behavioral physiology is outlined in Fig. 8. Applied to experimental physiology, it is clear that switchable mouse models will have a huge advantage over conventional strategies, as they allow for the tight external and in many cases also reversible control of a single gene at a certain time in development or in a certain cell type. For this reason the modeled disease condition will often mirror the clinical situation more appropriately. Very good examples illustrating the power of inducible and reversible mouse models for asking fundamental questions in cancer research have been provided by several groups (21, 25, 37, 55). All of these murine models provided a means to assess the physiological impact of oncogenes in tumorigenesis and for the first time to directly examine the consequences of their extinction after the onset of tumor formation. These model systems have important consequences for any anti-cancer drug development. In this respect experiments performed by Chin and colleagues (21) using a tet-inducible RAS melanoma mouse model (Fig. 8A) showed clearly that extinction of H-RASVAL12 led to regression of tumors in the skin, thus confirming mutant RAS as a principle therapeutic target.

New insights into metabolic/endocrine physiology can also be gained by using inducible mouse models. One of the many published studies illustrates that

![Fig. 8. How can conditional mouse models be applied to research in physiology? Four experimental approaches representing different research areas in physiology are presented to illustrate the power of conditional mouse models. A: cancer physiology. Conditional expression of the RAS oncogene in skin induces formation of melanomas. After extinction of oncogenic RAS progression of the tumor growth stops and is followed by a complete regression (21). B: metabolic/endocrine dysfunction. Tissue-specific disruption of the nuclear gene encoding the mitochondrial transcription factor A (mtTFA) sets off diabetes (130). C: cardiovascular disease. Cardiac fibrosis arises after knocking-down the mineralocorticoid receptor (MR) in cardiomyocytes. Surprisingly, restoring the expression of MR protein was sufficient to completely reverse cardiopathy (7). D: brain dysfunction and learning/memory. To remember or not to remember was the question asked in this spatial and visual memory test. Behavioral experiments carried out in the laboratory of Eric Kandel demonstrated that long-term memory is lost after expression of a truncated dominant negative form of calcineurin in the hippocampus of trained mice. However, the ability to precisely remember the previously learned tasks can be completely restored after extinction of the dominant negative calcineurin (89). Readers interested in exploring further applications using second-generation mouse models in physiology are referred to several additional but more specialized reviews for cardiovascular disease (112), for neurobiology (88, 159), for endocrinology/metabolism (117), and for cancer (60, 63).]
tissue-restricted disruption of the nuclear gene encoding
the mitochondrial transcription factor A (mtTFA) induced diabetes in mice. In this very elegant study Silva and colleagues (130) were able to show that depletion of mtTFA in pancreatic β-cells not only recapitulated the human mitochondrial disease but provided direct evidence for a critical role of the respiratory chain in insulin secretion. This tissue-specific murine diabetes model provides an important tool to further dissect the molecular mechanisms responsible for human mitochondrial diabetes and opens the possibility for testing novel therapeutic approaches in the context of an appropriate animal model (Fig. 8B).

In the area of cardiovascular disease new possibilities for asking old questions and developing novel therapeutic approaches have also emerged. In a recent example of how reversible transgene expression can be applied to gain insight into cardiac disease, Beggah and colleagues (7) developed a reversible mouse model for cardiac fibrosis and heart failure. By conditionally knocking down the expression of the mineralocorticoid receptor (MR) in cardiomyocytes, they could induce cardiac hypertrophy, ventricular dysfunction, interstitial fibrosis, and finally heart failure. Most interestingly, when expression of the MR protein was restored, not only was improvement of cardiac function observed, but surprisingly the cardiomyopathy regressed. This finding identified MR as a candidate therapeutic target, demonstrating for the first time its role in active remodeling of abnormal extracellular matrix deposits.

Last but not least, the area of behavioral neurophysiology has also considerably benefited from the development of second-generation conditional mouse models. For example, to assess the role calcineurin plays in the transition from short- to long-term memory, Mansuy and colleagues (89) trained mice in a maze to learn specific spatial and visual recognition tasks. Induced overexpression of a truncated dominant negative form of calcineurin in the hippocampus of the trained cohort of mice disabled these mice to remember previously learned tasks without affecting their short-term memory. Most strikingly, when expression of the dominant negative calcineurin was extinguished in these mice, long-term memory of the previously learned spatial and visual tasks was completely restored.

As illustrated by the above examples, the use of second-generation mouse models together with the to-be-expected further improvement of these technologies will greatly enhance our possibilities to appropriately address many open but principal questions about the in vivo function of genes in physiology and human disease.

Concluding Remarks

In this review we have summarized the rapidly expanding number of methods to generate spatially and temporally regulated gene expression within the mouse. These massive advances as well as the incremental improvements in established systems present researchers with very powerful tools for analyzing gene function during development, as well as in disease. Today mouse models for a variety of monogenic disorders solely including the modification of a single gene have been generated. However, as many developmental and disease models require the coordinate action of two or more genes, the combination of different switchable systems in one mouse will become increasingly popular. While the technologies presented here are very powerful, it is to be expected that RNA interference-based approaches (31) as well as cloning of mice (146) will soon be additional tools to be applied for building mouse models.

With many groups producing a rapidly expanding number of tet on/off and also Cre/loxP conditional mice, any improvements in the communication and sharing of available strains will be of great help to the scientific community. Toward this end, we now present a comprehensive list of currently available general and tissue-specific tet on/off effector and reporter strains. This compilation can be accessed via http://www.zmg.uni-mainz.de/tetmouse/. If there are any tet on/off strains we unintentionally overlooked or which are currently being tested, we would be delighted to also include these upon notification. As mentioned, an excellent list of Cre- and Flp-based conditional strains is published elsewhere (http://www.mshri.on.ca/nagy/cre.htm).

One very attractive use for the methodologies described above is the genetic marking and tracing of specific cellular populations for fate mapping analysis during development. With the limitations presented by in utero mammalian development (compared to using in ovo developmental models), fate analysis of progenitors is currently technically challenging. We envisage recombinase-mediated cell marking of specific populations such as hematopoietic precursors or stem cells, neural progenitors or germ cells as a very powerful tool for following cell fates during development (156, 160). In the future to come, it will be extremely exciting to see how the further development of conditional mouse technology will transform biomedical research in general.

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We apologize that many significant contributions to this field may have been omitted from this review.

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