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European Journal of Pharmacology 375 (1999) 327–337

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Review

Genetically modified animals in pharmacological research: future trends

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Accepted 30 April 1999

Abstract

The recognition of molecular control elements which govern cell and organ function is essential for the development of novel drug therapies and for an understanding of drug actions. Thus, a major interest is focused on methodologies which permit the identification of novel control elements. This is of particular relevance for the identification of drug targets, the distinction of target isoforms, the differentiation of signalling pathways, the generation of disease models and toxicological testing. In this review, we discuss different classes of genetically modified animals and their potential to elucidate biological processes relevant for pharmacological research including functional genomics. Techniques which permit the time- and tissue-specific inducible regulation of gene expression present an important methodological advance.

Keywords: Transgenic; Gene targeting; Gene knockout; Mutagenesis; Tetracycline; Inducible gene expression

1. Introduction

A major aim in pharmacological research is the identification of molecular control elements which regulate complex physiological functions such as blood pressure, immunoresponsiveness or mental abilities. These molecular elements are of additional interest since they represent potential therapeutic targets for various disease states. A powerful approach to identify the regulatory elements is the identification of the genes which provide the major structural and regulatory functions in an organism. Three different experimental strategies have been developed to assess gene function in terms of providing the determinants for a particular phenotype.

1.1. Analysis of natural variation

Individual non-pathological variations in behaviour or variations in the predisposition to disease are frequently due to the occurrence of naturally polymorphic genes. By breeding a line of animals with a pronounced particular feature, e.g., high blood pressure or fear behaviours, the quantitative trait loci (QTL) can be mapped with the aim of identifying the genes responsible for the particular phenotype. However, due to methodological limitations, this approach has largely failed so far to yield detailed genetic information.

1.2. Random mutagenesis

In this classical approach, deviations in the phenotype of an animal are induced by chemical mutagenesis. A recent example of this strategy was provided by the identification and subsequent positional cloning of the *clock* gene, which controls the circadian timing as shown by the lengthened circadian period and the abolished persistence of rhythmicity in the mutant animal (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997; for review, see Takahashi et al., 1994).

1.3. Targeted mutations and transgenes

In this approach, a known gene is mutated or a new gene is transferred into the animal to assess its contribution to the phenotype. This approach, based on gene technology, has proven to be very powerful in defining biologically essential genes. The present status and future trends in this field are the topic of this review, which is related to another article by the same authors (Rudolph and Möhler,

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00195-8

1999). The use of genetically modified animals as pharmacological tools has also been reviewed by Bürki and Ledermann (1995) and by Wei (1997).

2. Generation of transgenic and targeted mutant animals

Transgenic mice are animals expressing a foreign gene, the 'transgene', which is typically introduced into the mouse germline by microinjection of DNA into fertilized mouse eggs. The DNA will be integrated into the genome at random. Since the location and the level of expression varies depending on the integration site, the tissue distribution and the level of expression of most transgene constructs will vary from mouse line to mouse line, even though the same DNA has been injected. Mice generated using this approach most frequently represent gain of function mutations, exhibiting, for example, constitutive and tissue-specific overexpression. However, when expressing dominant negative, ribozyme or antisense constructs, they may also represent loss of function mutations. The generation of transgenic animals via microinjection of DNA into fertilized eggs has not only been achieved for mice but also for other species including the rat.

In the targeted mutant approach, targeting vectors are constructed that are specifically integrated at a desired genomic location by homologous recombination in murine embryonic stem cells. These cells are subsequently injected into blastocysts, where they contribute to the developing embryo which carries the mutation in the germline. In case of a gene inactivation experiment, the target gene is disrupted by introduction of a neomycin resistance marker and/or by deleting part of the gene ('knockout'). Knockout mutations may be studied in the heterozygous state (to assess potential gene dosage effects) and the homozygous state (to analyze the null phenotpye). The phenotypic changes observed in knockout mice are expected to provide information on the normal function of the respective genes in wild-type animals. Targeted mutagenesis is, however, not limited to gene ablations. Virtually any desired subtle mutation, e.g., point mutations, replacement of one murine gene by another murine gene or replacement of a murine gene by the homologous human gene may be introduced into the mouse genome ('knock-in'). The possibility to generate point mutations permits a more precise modelling of many human disease mutations. Unfortunately, the embryonic stem cell technology is currently feasible only for the mouse.

Major elements in the process of drug discovery in which genetically modified animals play a role are (a) the identification and validation of molecular drug targets, (b) the generation of animal models of disease which enable testing of novel therapeutic strategies and (c) the early recognition of toxicological effects.

3. Identification and validation of drug targets

The precise knowledge of the pharmacological role of receptors which frequently exist as multiple subtypes is particularly important for predicting specific drug actions. Two types of genetic modifications are largely suitable for a functional assessment of drug targets, the knockout and the knock-in strategies.

3.1. Function of receptor subtypes revealed by gene inactivation (knockout)

A receptor system that has so far proved unsuitable for drug development largely because of its multiple and complex functions has recently emerged as an interesting field for subtype-specific drugs based on a gene inactivation study. In mice lacking the adenosine receptor subtype A_{2A} (Ledent et al., 1997), caffeine fails to display its psychostimulant effect and depresses locomotor activity, suggesting that adenosine A_{2A} receptor antagonists may be suitable as cognitive enhancers. An increased anxiety and a decreased pain response in the A_{2A} knockout mice points to adenosine A_{2A} receptor agonists as potential anxiolytics and antagonists as potential non-addictive analgesics. In the periphery, the increased platelet activation suggested a potential role for adenosine A_{2A} receptor agonists as anticoagulants and for adenosine A_{2A} receptor antagonists in the treatment of clotting defects. Finally, the high blood pressure observed in these animals suggests a potential role of adenosine A_{2A} receptor agonists in the treatment of hypertension, whereas adenosine A_{2A} receptor antagonists may be useful in the treatment of shock. Thus, an attractive drug target with potential for a variety of indications was revealed in a single knockout experiment.

The α_2-adrenoceptor, of which three subtypes (α_{2A}, α_{2B} and α_{2C}) exist, is another example of how genetically modified mice may contribute to our understanding of the biological and pharmacological role of drug targets. α_2-Adrenoceptor agonists are known to produce an initial hypertension and a long-lasting hypotension. However, subtype-selective agents have not been available to determine the role of individual receptor subtypes in the mediation of these vascular effects. In separate experiments, targeted mutations were generated in all three receptor subtypes. Studies of mice lacking functional α_{2A}-adrenoceptors (MacMillan et al., 1996) or α_{2B}-adrenoceptors (Link et al., 1996), respectively, demonstrated that the α_{2A}-adrenoceptor mediates the central hypotensive effect, while the α_{2B}-adrenoceptor mediates the peripheral hypertensive effect. Thus, α_{2A}-adrenoceptor selective agonists would have a more profound hypotensive effect than the nonselective α_2-adrenoceptor agonists currently in use. However, α_{2A}-adrenoceptor agonists would likely retain the sedative side effect of nonselective α_2-adrenoceptor agonists such as clonidine (Lakhlani et al., 1997). The α_{2C}-

adrenoceptor subtype which is expressed in the basal ganglia, hippocampus and neocortex, is linked to cognitive functions. In α_{2C}-adrenoceptor knockout mice, enhanced startle responses, diminished prepulse inhibition and shortened attack latency in the isolation–aggression test were noted (Sallinen et al., 1998), thus indicating that α_{2C}-adrenoceptor specific drugs may be of value in disorders associated with enhanced stress sensitivity and reduced sensorimotor gating, e.g., schizophrenia, attention deficit disorder, post-traumatic stress disorder and drug withdrawal symptoms. Since α_{2C}-adrenoceptors are not or only marginally involved in sedation (Hunter et al., 1997) and in blood pressure regulation (Link et al., 1996), such compounds would not be expected to display sedative or cardiovascular side effects.

3.2. Function of receptor subtypes revealed by point mutations (knock-in)

If the inactivation of a gene is expected to lead to a lethal or disabling phenotype, more refined strategies are required to elucidate the pharmacological significance of its gene product. The introduction of pharmacologically relevant point mutations that leave the physiological function and expression unaltered is exemplified for the $GABA_A$ receptor. This receptor is the target for benzodiazepine drugs which are used clinically as anxiolytics, sedatives, anticonvulsants and myorelaxants. Classical benzodiazepines such as diazepam act on $GABA_A$ receptors containing the α1-, α2-, α3-, or α5-subunit. It is unknown, however, which action of diazepam is mediated by which receptor subtype. This knowledge would be essential if compounds with more specific actions are to be developed. A mouse line has recently been generated which carries a point mutation in the benzodiazepine binding site of the α1-subunit of the $GABA_A$ receptor ($\alpha1^{H101R}$ mice). This point mutation renders the α1-$GABA_A$ receptor diazepam-insensitive, while the sensitivity for the physiological neurotransmitter GABA is essentially unchanged (Wieland et al., 1992; Kleingoor et al., 1993; Benson et al., 1998). In these mice, all remaining diazepam actions are mediated by the α2-, α3- and α5-$GABA_A$ receptors. Initial behavioural studies revealed that the major actions of diazepam are unaltered in $\alpha1^{H101R}$ mice, while other activities are abolished (Rudolph et al., 1998). Thus, the knock-in strategy provides a dissection of the pharmacological spectrum of diazepam. Subtype-specific ligands of the benzodiazepine binding site promise to display selective actions, e.g., anxiolytic action without sedative side effects.

3.3. Specific functions of diverse post-receptor signalling pathways

Following the activation of a receptor frequently diverse signalling pathways are activated which result in diverse cellular responses. It would therefore be desirable to identify the relevance of a particular signalling pathway for physiological or pharmacological functions. An intriguing example for multiple signalling pathways is provided by the glucocorticoid receptor. Therapeutically, glucocorticoids display metabolic, antiinflammatory and immunosuppressive actions. Depending on the condition to be treated, only some of these actions may be desired (e.g., the antiinflammatory and immunosuppressive actions). Glucocorticoids are known to act via a nuclear glucocorticoid receptor, which mediates transactivation by binding of receptor dimers to glucocorticoid response elements (GREs). In addition, the glucocorticoid receptor mediates transrepression. The repressive effect is likely to be mediated by receptor monomers. Interestingly, a point mutation (A458T) has been identified and introduced into mice which prevents the ability of the glucocorticoid receptor to transactivate gene transcription by cooperative DNA binding, while the repressing function of the glucocorticoid receptor is retained (Reichardt et al., 1998). The initial analysis revealed that transactivation by endogenous glucocorticoids is not required for survival. However, the glucocorticoid-dependent apoptosis of thymocytes and proliferation of erythroblasts is mediated by the transactivation function of the receptor. Further analysis of the point-mutated mice will reveal the functional relevance of the transactivation and transrepression pathways and permit their assessment for specific therapeutic interventions. Indeed, dissociated glucocorticoids, including RU24858, which exert strong transrepression but little or no transactivation, have already been identified. These compounds exert an antiinflammatory and immunosuppressive activity in vivo as potent as that of prednisolone (Vayssiére et al., 1997). The development of glucocorticoid drugs which retain desired activities but lack unwanted side effects would be highly desirable.

3.4. Unexpected drug targets

The analysis of various physiological functions in knockout mice can also lead to the identification of unexpected drug targets. This was recently demonstrated in a mutant mouse lacking the α-subunit of the G-protein G_q. It was noted that the $G\alpha_q$-deficient mice displayed a prolonged bleeding time and were unresponsive to a variety of physiological platelet activators. Correspondingly, the mutant mice survived following an intravenous injection of a mixture of collagen and adrenaline for at least 1 h, whereas wild-type mice invariably died within 5 min. Thus, the mutants were resistant to thrombembolism induced by platelet activation in vivo (Offermanns et al., 1997). These studies identify $G\alpha_q$ in platelets as a potential target for antithrombotic therapy.

3.5. Humanized drug targets in animals

The human and mouse homologues of a specific drug target may display structural differences at the molecular

level up to the point that they display species-specific pharmacological properties. In this case, it is of interest to introduce the respective human gene, for example, into a normal or a knockout animal to obtain a humanized drug target. A case in point is the study of the renin–angiotensin system. It is known that the interaction of human renin and angiotensinogen are species-specific, i.e., they do not react with the respective mouse proteins. In transgenic animals, the specific interactions of the human transgene products can be studied without interference from the host renin–angiotensin system in vivo (Ganten et al., 1992). This is of particular relevance for the development of inhibitors for the human enzyme. When drug efficacy testing is planned on humanized targets, it may in most cases be necessary to remove the endogenous gene to avoid interferences.

Replacement of a murine gene by a human gene is also of interest for establishing animal models of human disease. In one example, the mouse prion gene was replaced by the human prion gene using cre-loxP-mediated gene targeting (Kitamoto et al., 1996). Since the prion-infectivity is species-specific, these mice should be suitable to study the infectivity of the human prion protein. A further example of a humanized gene is the replacement of the mouse α-lactalbumin gene by its human counterpart using the double replacement gene targeting procedure (Stacey et al., 1994). It was found that the human gene was expressed at 16-fold higher levels, but the milk lactose concentration was not affected, although the volume of the milk was slightly increased (Stacey et al., 1995). This replacement technique should also be suitable to manipulate milk composition for pharmaceutical and therapeutic applications. Similarly, humanized antibodies which would be preferred over rodent antibodies as immunotherapeutic agents have been generated by replacing the mouse κ light chain constant (C) region gene with the human C_{κ} gene (Zou et al., 1993). These examples illustrate the feasibility and usefulness offered by genetically modified animals carrying humanized genes.

4. Animal models of human diseases

Pharmacological research can strongly benefit from the availability of suitable animal models to study the pathophysiology of disease and to evaluate novel therapeutic strategies. In case of human hereditary diseases, animal models are based on the strategy to mutate the homologous murine genes. In case of some common disorders, attempts are made to reproduce the molecular hallmarks of the human pathophysiology by transgenic expression of relevant genes. A recent example is Alzheimer's disease. Transgenic mice overexpressing human Alzheimer precursor protein sevenfold (carrying the double mutation KM → NL at positions 670/671 found in patients with early-onset familial Alzheimer's disease) display congophilic plaques, neuritic changes, dystrophic cholinergic fibers and a massive glial reaction indicating inflammatory responses. The plaques are immunoreactive for hyperphosphorylated tau, reminiscent of an early tau pathology in humans (Sturchler-Pierrat et al., 1997). This mouse line as well as similar lines reported previously (Games et al., 1995; Hsiao et al., 1996) replicate some essential features of Alzheimer's disease. They can be used to investigate the role of the Aβ peptide in the pathogenesis of the disease and to test drugs for inhibiting this process.

Similarly, animal models for cystic fibrosis have been generated by introducing disease causing human point mutations into the CFTR (cystic fibrosis transmembrane regulator) gene or simply by inactivating this gene. These mice, which exhibit some of the characteristic features of the human disease, have been used to test the therapeutic potential of gene therapeutic approaches. It was demonstrated that the ion transport defect in some tissues can be corrected by liposomal transfer of the CFTR gene (Alton et al., 1993; Hyde et al., 1993). In addition, a second dose of CFTR cDNA–liposome complex was as effective as the first dose (Goddard et al., 1997). Adenoviral gene transfer was inefficient (Grubb et al., 1994).

The effectiveness of gene therapy was also tested in an animal model of hereditary tyrosinemia type I. In this disease, the loss of fumarylacetoacetate hydrolase (FAH) is responsible for acute hepatic failure, renal tubular disease and hepatocarcinoma. In the FAH-deficient mice (Grompe et al., 1993), the defect was corrected pharmacologically (Grompe et al., 1995), with adenovirus-mediated gene therapy (Overturf et al., 1997) and with retrovirus-mediated gene therapy (Overturf et al., 1998). Interestingly, these studies also showed that apparently successfully treated mice have a high incidence of hepatocellular carcinomas derived from remaining non-treated hepatocytes, demonstrating that all enzyme-deficient hepatocytes need to be eliminated for successful long-term therapy. These examples illustrate the usefulness of disease models to probe different strategies for gene therapy.

Though many animal models replicate at least some of the features of a specific disease, there are also examples where the null phenotype in the mouse differs from the phenotype caused by inactivating mutations in humans. One such example is Lesch–Nyhan syndrome, which is caused by inactivation mutations in the hypoxanthine-guanine-phosphoribosyltransferase (HPRT) gene and characterized by hyperuricemia, choreoathetosis, spasticity, mental retardation and self-mutilating behaviour. Mice with a defective HPRT gene (Hooper et al., 1987; Kuehn et al., 1987), however, were essentially normal and healthy with only subtle changes in brain dopamine levels (Finger et al., 1988; Jinnah et al., 1994) Even mice that are deficient for both HPRT and adenine phosphoribosyltransferase (APRT) and thus devoid of any purine salvage pathways are not a model for the behavioural abnormalities associated with Lsch–Nyhan syndrome (Engle et al., 1996). These findings indicate that species differences in biochemical path-

ways or physiological processes or structural differences at the molecular level may have to be taken into account when extrapolating from animal models to human diseases.

Finally, transgenic and gene targeted animals may unexpectedly display a disease phenotype resembling a human condition and thereby provide a clue as to which genes and pathways are involved in the pathogenesis. For instance, inflammatory bowel disease has been observed in knockout mice lacking functional αβ T-cell receptors (Mombaerts et al., 1993), MHC class II (Mombaerts et al., 1993), interleukin 10 (Kühn et al., 1993), interleukin 2 (Sadlack et al., 1993) or the G-protein subunit $G\alpha_{i2}$ (Rudolph et al., 1995).

5. Toxicological testing

Transgenic mouse and rat lines have been developed to allow detection of spontaneous and induced mutations in vivo and in cell cultures and are commercially available as Big Blue (Kohler et al., 1991) and the Muta-mouse (Myhr, 1991) models. They are used for chemical and drug safety assessment, in particular, for the identification of genotoxic carcinogens. These animals contain lambda vectors which can be recovered from the animals and analyzed for the presence of mutations. Similarly, the liability to tumor development may be tested in animals that spontaneously develop tumors such as the p53-deficient mice (Donehower et al., 1992). Some potential for the identification and assessment of nongenotoxic chemicals may be provided by a transgenic mouse line expressing the human growth hormone gene (as a reporter gene) under the control of the human heat shock protein hsp70 promoter (Sacco et al., 1997). Since chemical stressors are able to induce the expression of heat shock proteins, human growth hormone was elevated in the transgenic mice after administration of inorganic toxic chemicals. Similarly, treatment of primary cultures from these transgenic mice with toxic agents lead to the release of human growth hormone into the culture medium. Thus, the effect of individual compounds can be assessed in specific tissues. This mouse model needs further evaluation with respect to sensitivity and specificity for the detection of non-genotoxic agents. Compared with classical toxicological tests, transgenic animals allow toxicological studies to be completed in a shorter period of time and with fewer animals.

6. Tissue-specific gene knockout

Conventional knockout mice are generated by the complete inactivation of the gene of interest. Thus, a mouse homozygous for the mutant allele will lack the gene product already during development. Thus, it may be possible that the adult phenotype is due to the absence of the gene in development (sometimes with compensatory changes) and primarily not due to the absence of the gene in the adult animal itself. In this case, the conventional knockout animal would not be suitable to draw conclusions regarding the function of a particular gene in adulthood. These concerns have led the development of techniques which restrict the knockout to a particular cell type or tissue.

Though there are currently only few reports of cell- or tissue-specific knockouts in the literature, this technique (Fig. 1) will be widely used in the future and is therefore illustrated with two examples from neurobiology and dermatobiology. In the brain, memory formation is mediated by NMDA receptors with the NR1 subunit being an essential component. Since mice deficient for the NR1 subunit gene die perinatally (Li et al., 1994), the role of the NR1

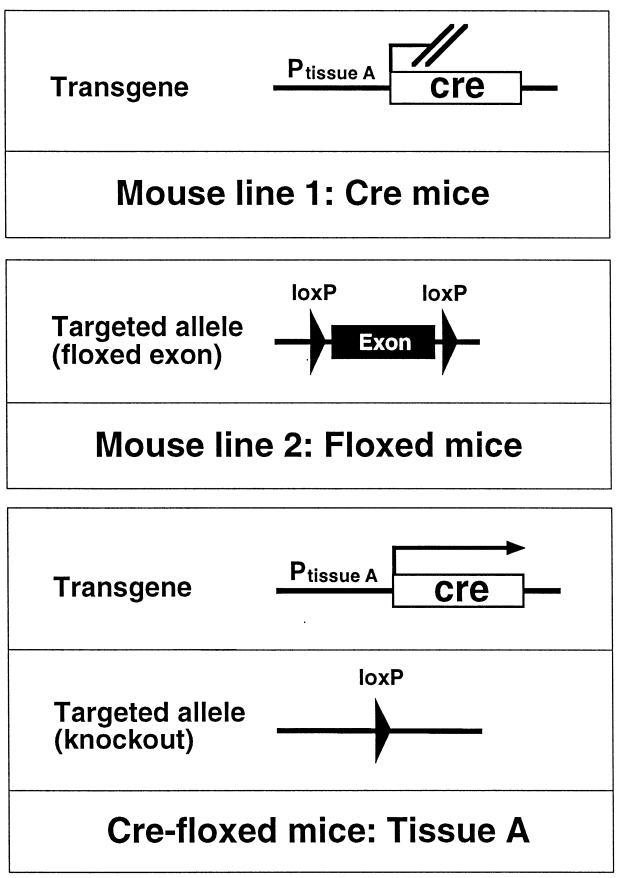

Fig. 1. Tissue-specific knockout using the cre/loxP system. Two lines of mice are generated. One line ('Mouse line 1: Cre mice', top panel) carries a cre transgene driven by a tissue-specific promoter ($P_{tissue\ A}$ = Promoter expressed in tissue A). The other line ('Mouse line 2: Floxed mice', middle panel) carries a targeted mutation, characterized by two parallel loxP sites flanking an exon (or several exons or the entire gene). These two lines are then bred in order to obtain mice which contain the transgenes and are homozygous for the floxed allele ('Cre-floxed mice'). In tissue A, the cre transgene is expressed, leading to cre-mediated excision of the floxed exon and thus to a knockout of the targeted gene in tissue A (bottom panel). In all other tissues, the cre transgene is not expressed, leaving the floxed exon intact. Thus, the gene of interest is specifically inactivated in tissue A.

subunit in adult mice was analyzed with a tissue-specific knockout. To this end, two separate mouse lines were constructed. The first line expressed a cre recombinase transgene under the control of a CaMKII promoter. Probably at least in part as a result of the specific integration site of the transgene, cre expression is highly restricted to the pyramidal cells of the hippocampus. In the second line, part of the NR1 gene was flanked by loxP sites (floxed exons). loxP Sites are not known to affect gene function themselves, although it cannot be excluded that the location of the loxP sites affects regulatory elements in rare cases. After crossing the two lines, the Cre recombinase in hippocampal pyramidal cells recognizes the loxP sites and excises the part of the NR1 gene that is flanked by the loxP sites. Thereby, the NR1 gene is specifically knocked out in the pyramidal cells only. Mice with this cell-specific knockout survive to adulthood without obvious abnormalities apart from an impairment of certain memory functions (Tsien et al., 1996a,b). Another recent example for a tissue-specific knockout is a *Pig-a* knockout in skin which revealed important roles for GPI-anchored proteins in skin development (Tarutani et al., 1997). These two examples demonstrate the feasibility and usefulness of inactivating a gene in a particular tissue. In analogy, the function of potential drug targets may be assessed by a tissue-specific knockout if the phenotype of the complete knockout is lethal. The generation of a floxed allele should be considered even when a conventional knockout is planned. Subsequently, a complete knockout can be achieved by crossing the floxed mouse with transgenic mice expressing the cre transgene in the germline (e.g., EIIa-cre mice, Lakso et al., 1996). Depending on the phenotype observed in the complete knockout, the generation of a tissue-specific knockout can be envisioned by crossing the floxed mouse with a suitable cre-transgenic mouse expressing cre in a tissue-specific fashion. Currently, many researchers who have developed and analyzed complete knockouts are now generating floxed alleles. Though the generation of a floxed allele involves more effort in design and construction of a targeting vector, the significant flexibility offered by this approach should be worth the extra effort for many workers. Floxed alleles are also useful for inducible knockout strategies as described below. Finally, it should also be possible to achieve tissue-specific expression of a gene driven by its endogenous promoter. This may be achieved as a tissue-specific rescue of a knockout mouse. A floxed allele is designed which is a knockout allele in the absence of Cre (e.g., by inclusion of floxed transcriptional and/or translational stop sequences), but it reverts to a functional wild-type allele in the presence of Cre (Zhuang et al., 1998). This rescue may also be designed to be inducible by doxycyline (see below). Whereas a tissue-specific knockout in most cases has to be essentially complete to reveal the null phenotype requiring complete excision of the loxP-flanked sequences, a tissue-specific rescue of a knockout is likely to be successful even if it is incomplete. We therefore expect that cre expression in some transgenic lines may be too weak to achieve a complete knockout but sufficient for a rescue.

7. Inducible transgenes

Temporal and spatial expression of transgenes is dependent on the promoter used in the respective construct and the site of DNA integration. In most cases, the expression is constitutive and may thus potentially disturb development. Transgenes may even be lethal. These problems may be overcome by making the expression of the transgene inducible. In this case, the transgenic animals grow up as functional wild-type animals containing a silent transgene. The acute effects of transgene expression can then be reversibly induced in adult animals. A significant advantage of inducible and reversible expression is that the same animal may be studied before and after the expression of the transgene and thus may serve as its own control, particularly in behavioural studies, where questions concerning the genetic background of the animals are particularly important (Anonymous, 1997). Inducible and tissue-specific gene expression in mice has been achieved using a binary tetracycline regulatory system (Kistner et al., 1996). This reverse tetracycline-controlled transactivator (rtTA) system (Fig. 2) consists of a rtTA fusion protein composed

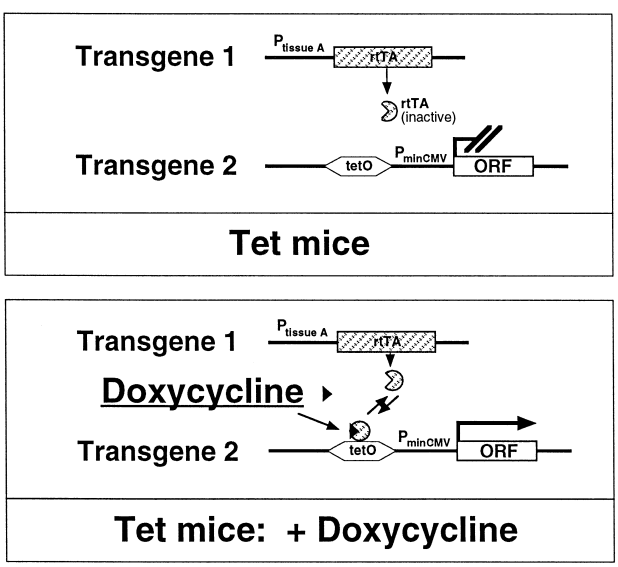

Fig. 2. Inducible transgene expression using the rtTA systems. Transgenic 'Tet mice' are generated which harbour two transgenes: Transgene 1 codes for rtTA, driven by a tissue-specific promoter active in tissue A ($P_{tissue\ A}$). Transgene 2 contains the open reading frame (ORF) of the gene to be inducibly expressed, driven by the minimal human CMV promoter (P_{minCMV}), which is itself inactive but regulated by the tetracycline operator (tetO). In the absence of doxycycline, rtTA will be expressed in tissue A, but does not bind to the tetO (top panel). However, in the presence of doxycycline, a complex of rtTA and doxycycline is formed that binds to tetO, driving the expression of the open reading frame (ORF) of the gene in question in tissue A (bottom panel).

of a mutant version of the Tn10 tetracycline-resistance operon of *Escherichia coli* and a C-terminal portion of protein 16 of herpes simplex virus that functions as a strong transcription activator. The rtTA is placed under the control of a tissue-specific promoter. In the presence of doxycycline (but not in its absence), rtTA binds to the tetO which is placed on a separate construct and activates transcription from a minimal hCMV promoter, which itself is inactive. This minimal promoter drives the open reading frame to be expressed. Thus, when doxycycline is added to the drinking water of the mice, the open reading frame will be expressed in those cells in which rtTA is expressed by means of a tissue-specific promoter. For example, Mansuy et al. (1998a,b) expressed rtTA from the CaMKIIα promoter to achieve inducible and reversible expression of a calcineurin transgene selectively in the forebrain of the mouse and showed that doxycycline-induced calcineurin expression impaired memory processes in the hippocampus. Conversely, it is also possible to shut down the otherwise constitutive expression of a transgene using the tetracycline-controlled transactivator (tTA) system. In this system, a tet repressor is expressed that binds to the tetO in the absence of doxycycline, promoting the expression of the transgene fused to a hCMV minimal promoter. In the presence of doxycycline, the tet repressor fails to bind to the tetO, shutting down transgene expression (Kistner et al., 1996). A refined system by which two genes can be simultaneously regulated in opposite directions is provided by a recent extension of this strategy (Baron et al., 1999).

8. Inducible knockouts

In addition to being specific for a particular cell type or tissue, a knockout can also be designed to be inducible at essentially any desired time point. To make knockouts inducible, a cre transgene can be expressed under the control of the rtTA system (Fig. 3) (Kistner et al., 1996). A tissue-specific promoter will drive rtTA expression, and upon addition of doxycycline rtTA will bind to tetO sequences, thus driving expression of the cre recombinase from the minimal hCMV promoter. In those cells in which cre is expressed, the Cre recombinase will excise floxed (= *f*lank*ed* by *lox*P sites) sequences, leading to a knockout.

Apart from the rtTA system, strategies have been described where cre expression can be induced by interferon (Kühn et al., 1995) and by tamoxifen (Schwenk et al., 1998). For instance, an inducible recombination was achieved specifically in B lymphocytes by expressing a Cre recombinase as a fusion protein under the control of a B cell-specific promoter. Since the Cre recombinase was fused to the ligand binding domain of the human estrogen receptor, the enzyme is inactive in the absence of an estrogen receptor ligand. Upon administration of tamoxifen, the fusion protein is released from its inactive state

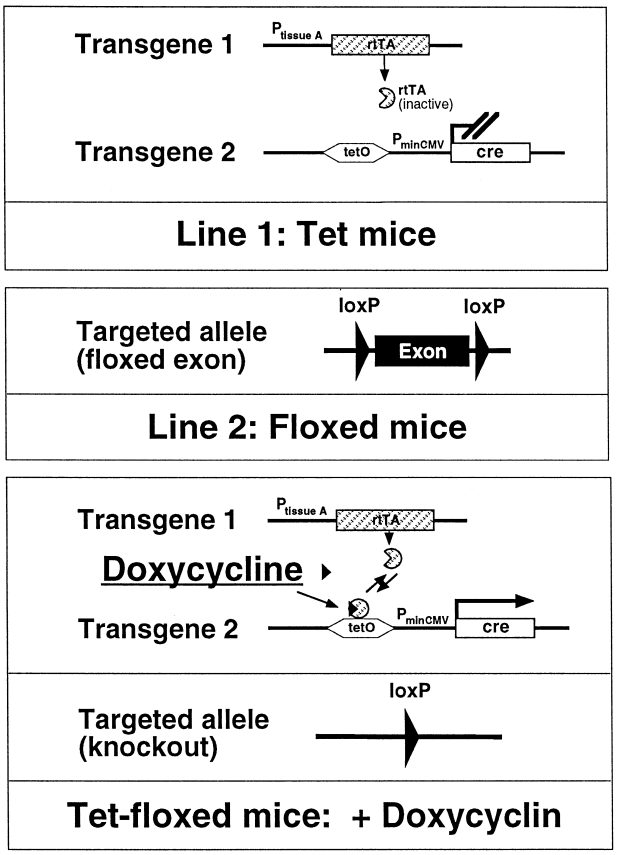

Fig. 3. Inducible tissue-specific knockout using the cre/loxP and rtTA systems. Two mouse lines are developed: transgenic mice which contain the regulatable induction system (see Fig. 2) which regulates cre expression ('Line 1: Tet mice', top panel) and targeted mice in which the gene to be inactivated contains an exon flanked by two loxP sites ('Line 2: Floxed mice', middle panel). The open reading frame of transgene 2 encodes the cre recombinase (cre). The two mouse lines are bred to obtain mice which carry Transgene 1, Transgene 2 and the floxed allele, the latter in the homozygous state ('Tet-floxed mice'). In the absence of doxycycline, the rtTA will be expressed in tissue A. In the presence of doxycycline, a complex of rtTA and doxycycline is formed which binds to tetO, driving the expression of cre in tissue A. Thus, in tissue A, the floxed exon will be excised, resulting in a knockout of the targeted gene in tissue A (bottom panel). In the other tissues the floxed exon (and thus the wild-type function of the gene) will be retained, since the transgenes 1 and 2 are not expressed.

(in which it is bound to the Hsp90 complex), and catalyzes the recombination between two loxP sites present in the genome. Though the recombination efficiency was not greater than ca. 80% (and thus not a real knockout), this study demonstrates that ligand-inducible recombination is feasible (Schwenk et al., 1998). However, insufficient constitutive or inducible expression of the cre transgene appears to be a frequently encountered problem (see also Gu et al., 1994).

Inducible tissue-specific cre expression can also be achieved by introduction of the cre transgene into the tissue of interest by means of an adenoviral vector. In a recent example, after intravenous injection of a cre aden-

ovirus low density lipoprotein receptor-related protein (LRP) expression was abolished in the liver due to the fact that recombinant gene expression from intravenously injected adenovirus is almost exclusively limited to the liver (Rohlmann et al., 1996, 1998). Using the same floxed mouse line, the knockout was induced in the liver but not in the brain when cre was expressed from the interferon-inducible MX1 promoter (Kühn et al., 1995; Rohlmann et al., 1998). These studies demonstrated a role for LRP in the clearance of chylomicron remnants. An adenoviral vector expressing cre has also been injected into specific regions of adult mouse brain with detectable cre expression at the injection site, demonstrating the feasibility of localized delivery of the Cre recombinase (Wang et al., 1996). Thus, inducible tissue-specific knockouts can also be generated by a combination of genetic and virological methods.

9. Allelic series via cre/loxP and flp/FRT systems

In addition to the cre/loxP system derived from bacteriophage P1 (Sauer, 1993), the flp/FRT system from *Saccharomyces cerevisiae* has been employed to achieve genetic recombination in mice (Dymecki, 1996). Though there is in vitro evidence that Flp is thermolabile (Buchholz et al., 1996), effective Flp action at an endogenous locus in somatic tissues in vivo has been demonstrated (Dymecki and Tomasiewicz, 1998). Moreover, a mutant Flp with improved thermostability in vitro and increased recombination efficiency in mammalian cells has been developed (Buchholz et al., 1998). Both systems, cre/loxP and flp/FRT, have recently been combined in a single targeting vector, enabling the generation of an allelic series of mutations in one targeted mouse line by expressing cre and flp either alone or sequentially (Meyers et al., 1998). The neomycin resistance gene in the original targeted mouse generated a hypomorphic allele, which could be reverted to wild-type by Flp-mediated excision of the neo gene. Expression of cre converted the hypomorphic allele to a null allele via excision of floxed sequences (Meyers et al., 1998). The availability of both recombination systems opens the way for novel targeting strategies to achieve regionally restricted gene targeting.

10. Transgenic and knockout mice in functional genomics approaches

Functional genomics, i.e., linking genomic sequence information to biological function, is of major relevance for the identification of novel drug targets. Methods are therefore being developed to facilitate the analysis of gene functions in vivo. Instead of analyzing the function of one gene at a time, entire sets of genes can be analyzed by multiplexing genes using 'in vivo libraries'. To this end, overlapping yeast artificial chromosomes (YACs) covering a specific chromosomal region, which has been implicated in a specific biological function, are introduced into the mouse germline. The various mouse lines will be analyzed with respect to the phenotype in question. In this approach, the phenotype should be expected to be sensitive to gene dosage. If two lines with overlapping YACs display the same desired phenotype, the region of interest in which to look for the responsible gene may be significantly narrowed. Another approach of multiplexing phenotypes is based on the overexpression of cDNAs, which have been derived from expressed sequence tag (EST) databases. Multiple phenotype assays are performed to determine the effect of the overexpressed cDNA on the organism (for a review on multiplexing genes and phenotypes, see Rubin and Smith, 1997). However, we believe that a partial or complete gene inactivation will generally reveal more about normal functions of genes than the overexpression of cDNAs.

11. ES cell knockout libraries

Lexicon Genetics, a biotechnology company based in The Woodlands, TX, has embarked on generating mutations in embryonic stem cells on a large scale by random tagged mutagenesis in such a way that the tagged and thus disrupted genes are easily identified by sequencing. The identification of 2000 tagged genes has recently been reported (Zambrowicz et al., 1998). A database with tagged and thereby inactivated genes ('Omnibank') is being built up and will enable customers to purchase the embryonic stem cell clone carrying the desired gene disruption. This clone can then immediately be injected into blastocysts, thus significantly reducing the time to obtain null mutant mice. Apparently, at least 500 clones are expected to be added to the database each week. Since this approach does not depend on individual genes being cloned and many tags have actually been identified in previously unknown genes, it may also be of help in uncovering the functions of the many genes identified in the course of the human genome project. We expect that the number of potential drug targets will increase significantly by this and other functional genomics approaches. In addition, a broad-based system of access to genetically modified mice would be desirable and is currently being discussed (Anonymous, 1998).

12. Conclusions

Transgenic and gene targeting technologies have made it possible to study the function of individual genes in the context of a living animal. Fundamental insights into biological systems and their pharmacological regulation have

been gained. Tissue-specific and inducible gene expression will greatly increase the selectivity with which these analyses can be performed. Thus, future genetically modified animals will be of even higher value in providing animal models for human diseases and for testing therapeutic strategies. Furthermore, their impact on drug discovery, toxicological testing and on assigning biological functions to novel genes is expected to increase.

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