Brain Struct Funct (2010) 214:91–109
DOI 10.1007/s00429-009-0230-8

REVIEW

Animal transgenesis: an overview

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Received: 23 August 2009 / Accepted: 6 November 2009 / Published online: 25 November 2009
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Abstract Transgenic animals are extensively used to study in vivo gene function as well as to model human diseases. The technology for producing transgenic animals exists for a variety of vertebrate and invertebrate species. The mouse is the most utilized organism for research in neurodegenerative diseases. The most commonly used techniques for producing transgenic mice involves either the pronuclear injection of transgenes into fertilized oocytes or embryonic stem cell-mediated gene targeting. Embryonic stem cell technology has been most often used to produce null mutants (gene knockouts) but may also be used to introduce subtle genetic modifications down to the level of making single nucleotide changes in endogenous mouse genes. Methods are also available for inducing conditional gene knockouts as well as inducible control of transgene expression. Here, we review the main strategies for introducing genetic modifications into the mouse, as well as in other vertebrate and invertebrate species. We also review a number of recent methodologies for the production of transgenic animals including retrovirus-mediated gene transfer, RNAi-mediated gene knockdown and somatic cell mutagenesis combined with nuclear transfer, methods that may be more broadly applicable to species where both pronuclear injection and ES cell technology have proven less practical.

Keywords Conditional gene inactivation · Gene targeting · Inducible transgene expression · Pronuclear injection · Transgenic animal

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Introduction

The generation of transgenic animals is essential for the in vivo study of gene function during development, organogenesis and aging. It also permits the evaluation of therapeutic strategies in models of human disease, as well as the investigation of disease progression in a manner not possible in human subjects. Commercial applications include the preparation of recombinant proteins, protection of animals against disease, and introduction of new genetic traits into herds. Transgenic animals have been produced in a variety of species (Table 1). Transgenic vertebrates have been developed in species with both scientific and commercial value including fish, amphibians, birds, and mammals. Transgenic invertebrate species include some widely used in research such as the arthropod fruit fly *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans*, as well as organisms with commercial value

Table 1 Some species in which transgenic animals have been produced

	References
Mammals	
Mice (*Mus musculus)*	Gordon et al. (1980), Joyner and Sedivy (2000)
Rats (*Rattus rattus)*	Hamra et al. (2002), Kato et al. (2004), Hirabayashi et al. (2005), Agca et al. (2008)
Rabbits (*Oryctolagus cuniculus)*	Fan and Watanabe (2003)
Sheep (*Ovis aries)*	McCreath et al. (2000), Denning and Priddle (2003), Wheeler (2003)
Pigs (*Sus domestica)*	Lai et al. (2002), Houdebine (2009), Kragh et al. (2009)
Cattle (*Bos taurus)*	Donovan et al. (2005), Richt et al. (2007), Houdebine (2009)
Goats (*Capra hircus)*	Wheeler (2003), Houdebine (2009)
Dogs (*Canis familiaris)*	Hong et al. (2009)
Marmosets (*Callithrix jacchus)*	Sasaki et al. (2009)
Rhesus monkeys (*Macaca mulatta)*	Yang et al. (2008)
Birds	
Chickens (*Gallus gallus*)	Mozdziak and Petitte (2004)
Japanese quail (*Coturnix japonica*)	Huss et al. (2008)
Amphibians	
Frogs (*Xenopus laevis* and *Xenopus tropicalis)*	Macha et al. (1997), Sinzelle et al. (2006), Ishibashi et al. (2008)
Fish	
Zebra fish (*Danio rerio)*	Zelenin et al. (1991), Davidson et al. (2003), Huang et al. (2008)
Goldfish (*Carassius auratus)*	Houdebine and Chourrout (1991), Wang et al. (1995)
Nile tilapia (*Oreochromis niloticus*)	Martinez et al. (2000), Maclean et al. (2002), Hrytsenko et al. (2009)
Carp (*Cyprinus carpio)*	Yoshizaki et al. (1991)
Channel catfish (*Ictalurus punctatus)*	Dunham et al. (2002)
Atlantic salmon *(Salmo salar)*	Sin et al. (2000), Houdebine (1997)
Invertebrates	
Arthropod fruit fly (*Drosophila melanogaster*)	Rubin and Spradling (1982), Fujioka et al. (2000)
Nematode (*Caenorhabditis elegans)*	Fire (1986), Mello et al. (1991)
Mollusk Japanese abalone (*Haliotis diversicolor suportexta)*	Tsai et al. (1997)
Mollusk Eastern oyster (*Crassostrea virginica)*	Cadoret et al. (1997)
Mollusk dwarf surfclam (*Mulinia lateralis)*	Lu et al. (1996)

including eastern oysters, dwarf surfclams and the Japanese abalone.

For research purposes transgenic models in the invertebrates *D. melanogaster*, and *C. elegans* as well as the vertebrates zebra fish *Danio rerio*, *Xenopus laevis*, and *Xenopus tropicalis* offer advantages of low cost of maintenance and the rapidity with which large numbers of transgenic organisms can be generated. However, their distant phylogenetic relationship to humans may limit their use in modeling human pathological conditions. By contrast, while phylogenetically closer to humans, the generation and maintenance of transgenic mammals such as sheep, cattle, and pigs involves a large investment in time and resources, which prevents their use in most research settings. Currently, the cost of generating a transgenic pig is about US $20,000 and that of a transgenic cow is around US $250,000 (Kind and Schnieke 2008). As such the use of transgenic cattle is largely limited to projects with commercial applications such as generating animals resistant to bovine spongiform encephalopathy by deleting the prion receptor (Richt et al. 2007), or producing cows resistant to staphylococcal mastitis (Donovan et al. 2005, 2006). Transgenic pigs have been generated to produce organs for xenotransplantation (MacKenzie et al. 2003; Sprangers et al. 2008; Ekser et al. 2009). Along with rabbits and cattle, pigs have also been used as protein bioreactors to produce biologically active recombinant proteins (Fan and Watanabe 2000, 2003; Houdebine 2004, 2007, 2009).

Transgenesis in mice is the most often used approach to generating models of human disease. Transgenic mice offer the advantages of relatively low cost, a short gestation time (18.5–21 days, depending on strain), and perhaps most importantly a well developed set of technologies for introducing genetic modifications. The availability of genetically inbred strains and the relatively close evolutionary relationship of mice to humans are additional advantages.

Transgenic pigs have been developed as models of retinitis pigmentosa (Li et al. 1998) and Alzheimer's disease (Kragh et al. 2009) and transgenic models of human diseases have also been generated in rabbits (Fan and Watanabe 2003) and rats (Mashimo and Serikawa 2009). However, transgenic technologies are not as widely available in these species as in mice where the techniques for gene targeting and pronuclear injection are well developed and widely available. Indeed, many of the methods for genetic manipulation used in the mouse are not routine or even presently possible in other mammalian species, although the recent development of rat and pig ES cells creates the potential that gene targeting may be extended to these species (Buehr et al. 2008; Wu et al. 2009). The generation of knockout, knockin and humanized rats is also being pursued using nuclear transplantation of genetically modified somatic cells (Zhou et al. 2003).

While cost is often prohibitive and the technology less widely available, at times, non-rodent species may offer advantages for modeling human disease. For example, in mice most cholesterol is contained in the form of high-density lipoprotein (HDL) cholesterol, whereas in rabbits most cholesterol is found in low-density lipoproteins (LDL), like in humans. Due to the differing lipoprotein profiles between mice and rabbits the introduction of human transgenes such as apolipoprotein A have resulted in different phenotypes in these two species (Fan and Watanabe 2000). It has also been proposed that the rat may be in general a better species than the mouse for modeling many human disorders (Abbott 2004).

However, as the mouse remains the most widely used species for modeling human neurodegenerative diseases, we will first discuss the techniques that have been widely successful in the mouse. We will then review newer techniques for generating transgenic animals including a number of methods that may be applicable to a broader range of species. We will also briefly discuss transgenic modeling in invertebrates.

Generation of transgenic mice by pronuclear injection

Gordon et al. (1980) first described the introduction of a foreign gene into mice using pronuclear injection into oocytes, an approach that has since been widely employed to study the molecular and cellular functions of many genes. This technology has been so adaptable that core facilities exist in many academic institutions for the generation of transgenic mice. Although the rat is also widely used in neuroscience, pronuclear injection has been less successful in this species and relatively few transgenic rat lines exist. Alternative strategies, such as the use of lentiviral vectors (see below), are currently being explored as means to introduce exogenous transgenes into rats.

Experimentally, when using pronuclear injection most commonly a plasmid is constructed in which the gene/cDNA of interest is placed under the control of a heterologous promoter, whose choice depends upon where and when it is desired that the transgene be expressed (Haruyama et al. 2009). For a protein to be expressed, the cDNA must contain a translational start codon (ATG) with an upstream Kozak sequence [GCCGCC (G/A) NN] (Kozak 1987) to provide for ribosomal recognition of the mRNA start site and an in-frame translation stop codon (UGA, UAG, UAA) for translational termination. Alternatively, tissue and developmental stage-specific expression of a gene can be studied by placing reporter genes such as β-galactosidase (lacZ), or green fluorescent protein (GFP) directly under the control of the gene's promoter and/or enhancer elements.

Inclusion of an intron at the 5′ or 3′ end of the transgene allows splicing of the transgene. Splicing generally results in more stable mRNAs and more efficient RNA translocation from the nucleus to the cytoplasm which typically leads to better transgene expression. Natural introns such as the simian virus 40 (SV40) intron or the rabbit β-globin intron, as well as artificial introns, can be used. In addition, eukaryotic transcriptional stop signals that include the poly (A)-addition sequence (AAUAAA) are usually positioned at the 3′ end of the protein translation sequence. Termination sequences widely used include those from SV40, bovine growth home (BGH), and human growth hormone (HGH) (Sheets et al. 1987; Goodwin and Rottman 1992; Haruyama et al. 2009). Enhancer sequences are genetic control elements that act in position- and orientation-independent manners to control the level and pattern of gene expression. Cell type specific expression of a transgene may be controlled by the inclusion of appropriate enhancer sequences. For example, the intron 2 enhancer of the nestin gene drives expression of transgenes only in neuroepithelial cells (Zimmerman et al. 1994). In order to prevent vector sequences from interfering with transgene expression, the transgenic cassette is typically excised from the plasmid backbone for microinjection.

In mice, transgenic animals can be generated by microinjecting the transgenic cassette into the pronuclei of fertilized oocytes. The foreign DNA integrates randomly and usually in the form of concatemers containing multiple copies of the original fragment ($\sim$70–100 kb). Because there is no corresponding allele on the homologous chromosome opposite the integration site these mice are most appropriately referred to as "hemizygous" rather than "heterozygous." The number of integrated transgenes (the transgene copy number) is generally inversely proportional to fragment size. Therefore, with larger DNA fragments, fewer copies will typically integrate. When multiple DNA fragments are injected simultaneously, they also tend to

integrate into a single site resulting in hybrid concatemeres containing proportionally represented copies of each fragment.

Due to the random nature of transgene integration following pronuclear microinjection, position site-dependent effects may alter transgene expression. These effects may produce transgene silencing, modify the cell and tissue specificity of the transgene or affect overall level of expression. Chromatin-mediated silencing may occur when a transgene integrates into a heterochromatin region while altered expression due to the effects of endogenous enhancers can occur when integration takes place into euchromatic regions. Indeed in the brain, transgene expression can be quite subtly affected by integration site (Elder et al. 1994). Transgene insertion can also alter the expression of endogenous genes at the integration region.

Practically, insertion site effects can be detected by analyzing multiple lines of mice generated from different founders. Insertion site effects can also be minimized by the inclusion of insulator sequences into the transgenic construct. Chromatin insulator elements (Potts et al. 2000; Giraldo et al. 2003; Gaszner and Felsenfeld 2006; Bushey et al. 2008) are DNA sequences that together with their binding proteins block interactions between adjacent chromatin domains (Gaszner and Felsenfeld 2006; Bushey et al. 2008). These elements establish genomic barriers that protect DNA sequences from the effects of neighboring sequences and prevent their interaction with distally located enhancers (Giraldo et al. 2003; Gaszner and Felsenfeld 2006). Among the insulator sequences that have been studied the 5’HS4 chicken β-globin and the mouse tyrosinase locus control region (LCR) insulator elements have both been shown to reduce the variability of transgenic expression when introduced either $5'$, or $5'$ and $3'$ relative to heterologous transgenes (Potts et al. 2000; Giraldo et al. 2003; De Gasperi et al. 2008). The chicken lysozyme locus also has two scaffold/matrix-associated regions (S/MARs) surrounding the gene that have been shown to exhibit boundary-type functions in transgenic mice (Bonifer et al. 1990). Recently, a SINE B2 element of the family of short interspersed repetitive DNA elements has also been reported to function as an insulator at the mouse growth hormone locus (Lunyak et al. 2007).

An alternative to injecting minigenes driven by heterologous promoters is to use artificial chromosomes as transgenes including bacterial artificial chromosomes (BACs), P1 artificial chromosomes (PACs) and yeast artificial chromosomes (YACs) (Schedl et al. 1992; Montoliu et al. 1993; Giraldo and Montoliu 2001; Montoliu 2002). BACs and PACs can accommodate up to 350 kb of insert DNA while YACs can accommodate >1 MB. Due to their large cloning capacity these constructs are likely to include distant regulatory elements, many of which may be unknown but required for proper expression increasing the chance that transgene expression will be optimally regulated and likely to recapitulate the expression pattern of the corresponding endogenous gene in time and space (Giraldo et al. 1999; Giraldo and Montoliu 2001). Some of these elements likely function as insulators like those described above to protect the transgenes from insertion site effects (Montoliu 2002).

BAC transgenesis with fluorescent tags has proven an invaluable tool in making the gene expression atlas of the developing and adult central nervous system in the mouse (GENSAT project, http://www.gensat.org). The GENSAT project has also generated transgenic BAC-EGFP reporter and BAC-Cre recombinase (Cre) driver mouse lines. Similarly, the use of BAC/PAC transgenes expressing fluorescent protein reporters in the optically transparent zebrafish has provided unparalleled visualization of gene expression in a living organism (Yang et al. 2006, 2009). BAC transgenic strategies have also been used to trace neural lineages (Placantonakis et al. 2009), identify sites of synthesis of IL-7 (Repass et al. 2009), create models of Parkinson’s disease using a truncated mutant parkin (Lu et al. 2009), and rescue the Krabbe disease phenotype of the twitcher mouse where cDNA transgenic approaches had proven unsuccessful (De Gasperi et al. 2004). YACs while technically more difficult to work with have allowed transgenesis with extremely large genes such as the $\sim$400 kb amyloid precursor protein (APP) (Lamb et al. 1993). One disadvantage of large DNA constructs such as PACs, BACs and YACs is that they may include other genes that may influence phenotype independent of the gene of interest.

Gene targeting in mice

Unlike pronuclear injection where exogenous transgenes integrate randomly, gene targeting makes specific modifications to endogenous genomic sequences (Smithies et al. 1985; Joyner and Sedivy 2000; Notarianni and Evans 2006; Turksen 2006). In the mouse, gene targeting has been refined to the point that a number of commercial entities offer gene-targeting services and some academic centers offer this service through core facilities. Genetic modifications including deletions, point mutations, inversions or translocations can be introduced using this technology. The genetic modifications are first made in ES cells. ES cell lines are derived from early-stage mouse embryos and can be maintained indefinitely in vitro in an undifferentiated state. Yet they retain the capacity that when injected back into an early-stage mouse embryo, they can mix with the endogenous cells of the embryo and contribute to the formation of all tissues in the developing mouse including the germ line.

The gene of interest is modified in the ES cells by homologous recombination using a targeting vector that consists of a modified version of the endogenous gene. For efficient recombination to occur the targeting vector should include >3 kb of DNA homologous to the endogenous mouse gene. As a general rule the greater the length of homology the higher the targeting frequency (Joyner and Sedivy 2000). Targeting vectors are also fitted with a gene conferring drug resistance or sensitivity for the selection of recombinant ES cells. Positive selectable markers allow selection of ES cell clones that have incorporated the targeting vector. Neomycin phosphotransferase (*neo*) resistance is the most commonly used positive selectable marker although the genes for the hygromycin B phosphotransferase (*hph*), puromycin *N*-acetyltransferase (*puro*) and xanthine/guanine phosphoribosyl transferase (*gpt*) have also been used. Counter-selection markers can be included in the flanking regions of the targeting vector to eliminate clones in which random integration rather than homologous recombination has occurred. The *Herpes simplex* thymidine kinase (*HSVtk*) gene has been the most widely used counter-selection marker although diphtheria toxin (*DT*) has been used as well. A positive/negative selection system using hypoxanthine phosphoribosyl transferase (*hprt*) in HPRT deficient ES cells has also been described. Although theoretically appealing, counter-selection does not typically increase the efficiency of gene targeting enough to warrant the increased effort involved in vector construction and is now rarely used.

Since the majority of the integrations are random and do not result in recombination events at the desired locus, ES cells must be cloned and screened by Southern blotting or PCR to identify clones that have been targeted correctly. ES cells containing the correct recombined gene are then injected into a blastocyst-stage mouse embryo from which a chimeric mouse is generated containing endogenous blastocyst as well as ES cells. The resulting chimeras are bred and if successful integration of the ES cells into the germ line has occurred the genetic modification will be propagated as part of the mouse genome creating stable lines harboring the specific genetic modification (Joyner and Sedivy 2000; Notarianni and Evans 2006; Turksen 2006).

Most ES cell lines have been produced from embryos of the 129-mouse strain due to the ease of establishing and the stability of 129 derived ES cell lines. The ES cells are then typically injected in C57BL/6 blastocyst, which allows the identification of chimeric founders due to the agouti coat color derived from the 129 ES cells. However, the 129 background is often not ideal for many studies, necessitating the backcrossing of lines onto the C57BL/6 background. C57BL/6 ES cell lines exist, but have been less widely used than 129 lines.

ES cell technology has been most commonly used to produce null mutants or gene "knockouts." Here, the targeting vector is constructed to allow the precise removal of one or several exons from a gene resulting in the complete abolition of protein production or the production of a non-functional truncated protein (Fig. 1). However, gene targeting can also be used to modify endogenous mouse genes down to the level of creating single nucleotide changes producing what are known as "knockin" mice. In this approach, the mutation is introduced into the region to be homologously recombined, and generally a strategy is included to remove the selectable marker by flanking it with loxP sites allowing subsequent removal by Cre recombinase either in the ES cell or by breeding with a Cre-expressing transgenic mouse line.

In contrast to pronuclear injection, where multiple copies of a transgene insert randomly in the genome, with ES cell-based methods, the native mouse gene is modified in its normal chromosomal location. Therefore, whereas in pronuclear injection a transgene is typically overexpressed and often misexpressed spatially and temporally due to its coupling to a heterologous promoter, with homologous recombination the temporal and spatial expression pattern of the targeted gene mirrors that of the normal gene.

Conditional/inducible gene inactivation

While gene knockout technology has been invaluable in the study of gene function in vivo, there are times when the technology has limitations. For example, gene knockouts may lead to embryonic lethal phenotypes or result in complex multisystem abnormalities. The problem of embryonic lethality precludes the study of gene function in for example adult brain and even if adult animals can be obtained it may be difficult to separate the primary effects of gene inactivation in brain from the secondary effects of abnormalities in other tissues. Fortunately methods are now

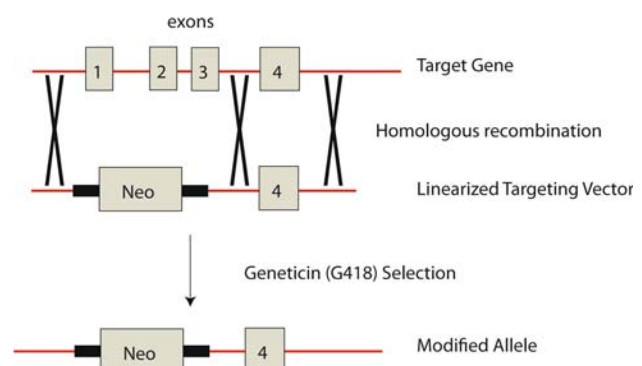


Fig. 1 Homologous recombination. In the example above the homologous recombination results in a gene knockout

available that allow in vivo gene inactivation at defined time points and in a tissue specific manner during development or in adult life. The most widely used approach developed to date makes use of the Cre/loxP recombination system (Nagy 2000).

Cre is a 38 kDa recombinase from the bacteriophage P1 that mediates intramolecular and intermolecular site-specific recombination between loxP sites (Hamilton and Abremski 1984). The loxP consensus sequence of 34 bp consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. Each inverted repeat binds one Cre molecule and recombination occurs in the spacer region with the 8 bp spacer determining the directionality of recombination. Two loxP sequences in opposite orientation invert the intervening DNA while two sites in the same orientation mediate excision of the intervening DNA between the sites after which only one loxP site remains. To introduce loxP sites in the mouse genome homologous recombination in ES cells is again exploited. In this case, the targeting construct is designed to have two loxP sites flanking one or more exons of the gene of interest and positioned in the surrounding introns so as not to disturb gene expression. Floxed mice created by homologous recombination (i.e., mice carrying two loxP sites surrounding the gene of interest) typically show normal expression of the gene as well as a normal phenotype.

Floxed mice are then crossed to mice expressing Cre recombinase leading to permanent inactivation of the gene based on the pattern of Cre expression that can be controlled with cell type specific promoters. Regulation can also be programmed in specific temporal patterns if the chosen Cre driver is activated at a certain developmental stage or in a certain physiological situation. An ever-expanding number of Cre deleter lines have been created and a database of these lines exists (Cre-X mice at nagy.mshri.on.ca/cre) (Nagy et al. 2009). In the nervous system, the nestin intron 2 enhancer, which is expressed in neuroepithelial cells including both glial and neuronal progenitor cells, has been widely used to produce pan-CNS knockouts of genes (Dubois et al. 2006). The calcium-calmodulin kinase II (CamKII) promoter, which drives Cre expression postnatally in forebrain neurons including the neocortex and hippocampus, has also been frequently used to produce gene inactivation postnatally in forebrain (Tsien et al. 1996). Cre activity can also be used to turn on a gene that is silenced by the presence of translational stop sequences (loxP-stop-loxP) positioned between a promoter and a regulated gene (De Gasperi et al. 2008).

Inducible systems have also been developed to allow temporal and tissue or cell type specific gene inactivation on a time frame chosen by the investigator rather than dictated by onset of a specific promoter's expression pattern. Currently, the most widely used of these inducible systems is one based on the use of an inducible estrogen receptor and the hormone tamoxifen (Fig. 2) (Metzger et al. 1995; Zhang et al. 1996; Garcia and Mills 2002; Hayashi and McMahon 2002). In this system, gene inactivation is achieved at chosen time points by using a chimeric protein in which Cre has been fused to a mutated nuclear estrogen receptor that has lost its ability to bind endogenous hormones but still binds the estrogen agonist tamoxifen or the synthetic steroid RU486. Without induction the chimeric Cre protein is sequestered in the cytoplasm in a complex with the heat-shock protein, hsp90, and is inactive. Ligand binding disrupts the interaction with hsp90 resulting in migration of Cre into the nucleus and in Cre-mediated ablation of target regions flanked by loxP sites. Examples of the utility of this approach include the introduction of null mutations in the retinoid X receptor alpha in skin (Li et al. 2000) and the conditional inactivation of huntingtin in adult brain (Dragatsis et al. 2000). Although the tamoxifen system is notable for its tightness of regulation, it may not be as efficient at gene excision in all organ systems (Kellendonk et al. 1999).

The analogous *Saccharomyces cerevisiae* Flp/Frt system (Zhu et al. 1995; Zhu and Sadowski 1998) has also been

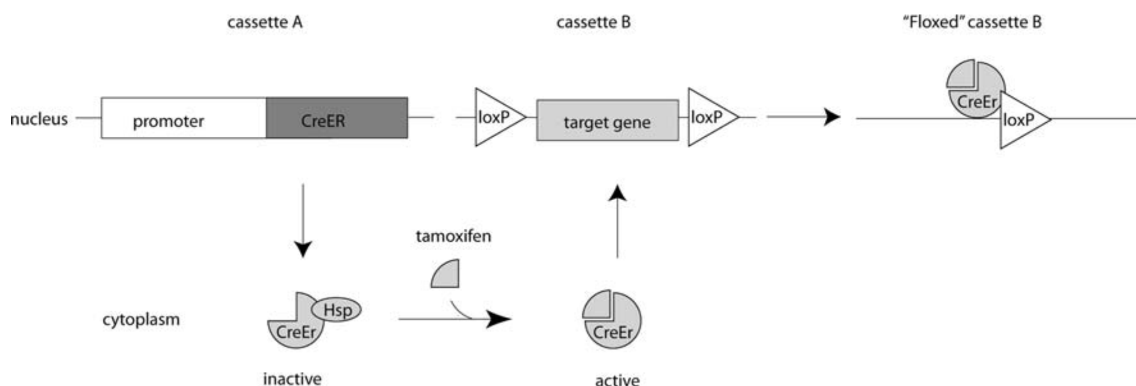


Fig. 2 Tamoxifen-inducible conditional gene knockout. *creEr* chimeric Cre-estrogen receptor protein, *hsp* heat-shock protein 90

used to develop constitutive and inducible transgenic models (Dymecki 1996; Dymecki and Tomasiewicz 1998; Meyers et al. 1998; Takeuchi et al. 2002; Hunter et al. 2005; Wirth et al. 2007; Kim and Dymecki 2009). In the Flp/Frt system the 34 bp flippase recognition target (Frt) sites are recognized by the Flp flippase recombination enzyme. Flp and Cre may also be used in combination (Ryding et al. 2001) to remove, for example, a selectable marker gene from a conditional allele at the ES cell stage with Flp/FRT, and then produce a subsequent deletion in vivo using Cre (Moon and Capecchi 2000). A floxed hypomorphic allele could also be knocked out completely using conditional Cre, or reverted back to wild type using Flp. One can envisage many more situations where multiple simultaneous or sequential conditional mutations could be created using recombinase combinations, either at single or multiple loci (Ryding et al. 2001).

Developments in the use of Cre continue. To provide for tighter in vivo regulation of Cre a newer system was developed which uses ligand-induced dimerization technology (DiCre system) (Jullien et al. 2007). In this approach, two inactive Cre chimeric proteins are generated fused either to the FK506-binding domain of FKBP12 or the binding domain of the FKBP12-rapamycin associated protein FRB. Rapamycin induces heterodimerization of the Cre chimeras leading to the reconstitution of Cre activity. The DiCre system has been used to target Cre activity in ES cells as well as in mouse embryonic and adult tissues. This system could also be exploited to establish conditional Cre deleter mice. Moreover, it offers the possibility of obtaining regulated recombination in a combinatorial manner. This methodology should permit the tight regulation of Cre activity at any desired time point and in a cell type specific manner by driving expression of the chimeric proteins from two different promoters. An additional advantage of this approach is that it should overcome the problem of cryptic loxP sites in mammalian genomes that may result in unwanted Cre-dependent chromosomal rearrangements (Schmidt et al. 2000; Semprini et al. 2007).

Gene regulation systems based on the tetracycline-resistance (tet) operon of *E. coli* have also been widely used to regulate gene expression (see below). Tet has also been used to control Cre activity in transgenic mice (Sun et al. 2007; Nagy et al. 2009). However, the tet system has not been widely adopted as a general method for controlling Cre-mediated gene inactivation likely due to the often leakiness of the tet system.

Inducible transgene expression

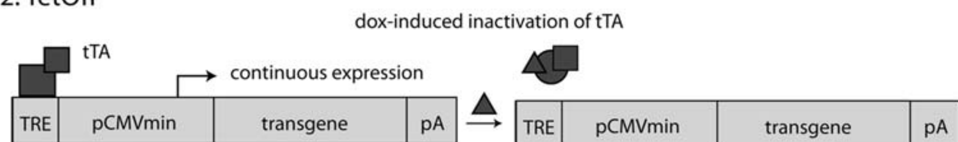
Cre-mediated gene regulation has proven a highly versatile system for gene regulation in the mouse. However, Cre excision is a one-time and irreversible event and there are circumstances in which the ability to switch a gene on and off is desirable and a number of inducible systems have been designed that allow temporal and spatial regulation of transgene expression (Ryding et al. 2001; Yamamoto et al. 2001).

By far the most widely used in transgenic mice is the tet regulatory system which is based on the tet operon of *E. coli* (Gossen and Bujard 1992; Stieger et al. 2009). Activity of the tet operon is regulated by the tet repressor (tetR) which binds to a DNA sequence termed an operator (tetO) with binding functioning as a transcriptional repressor. Transcriptional repression is reversed by tetracycline which prevents binding of tetR to the operator.

For use in transgenic mice the tet repressor was converted into a tet-controlled transcriptional activator (tTA) by fusing the tetR DNA binding domain with the activation domain of the herpes simplex virus VP16 transcriptional activation protein (Gossen et al. 1995) (Fig. 3). A tTA

1. Basic Tet-regulated system

2. TetOff



3. TetOn

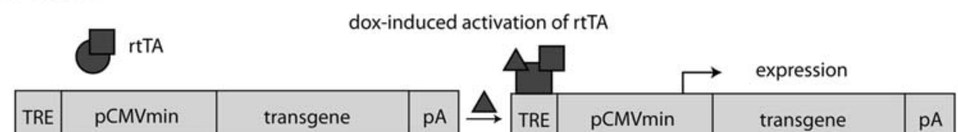


Fig. 3 Schematic representation of Tet-regulated systems. Figure based on Stieger et al. (2009)

regulatable promoter was created by placing a series of tandemly repeated tetO sequences (tet response element, TRE) upstream of a cytomegalovirus (CMV) minimal promoter (P_{min}CMV) with the tetO sequences placed to allow proper alignment of the VP16 activation domain such that tTA binding activates transcription.

In practice, the P_{min}CMV is placed upstream of the gene to be regulated and a transgenic line generated. This line is bred with a second transgenic line in which the tTA is expressed under the control of a tissue specific or other promoter which drives expression of tTA in a spatial/temporal manner. Suppression of transgene expression is achieved by treating mice with tetracycline or more commonly with the more lipophilic analog doxycycline both of which can be given in the drinking water. The system is turned “on” by removing the doxycycline.

This system termed the “TetOff” system is useful in cases that require transgene expression to be maintained in a switched-on state (Furth et al. 1994). However, in order to maintain the system in the “off” state, doxycycline must be administered continuously. This means for example that to restrict gene activation to adult brain, doxycycline treatment must be maintained during embryogenesis and throughout the postnatal developmental period, posing practical challenges. Doxycycline has also been suggested to affect development.

To circumvent the necessity of continuous doxycycline administration to maintain the system “off”, the “TetOn” system was developed (Fig. 3). This system was based on the observation that mutations within the chimeric tTA activator protein (rtTA) reverse its behavior so that it no longer binds spontaneously to the TRE but does so only in the presence of tet or doxycycline (Gossen et al. 1995). Thus, in what is referred to as the TetOn system, the transgene is silent under basal conditions and transcription is induced only in the presence of doxycycline which allows rtTA to bind to the TRE (Kistner et al. 1996). Newer modifications of the tet system, such as the KRAB TetOn in which the original TetR protein is fused with the transrepressing KRAB domain of the zinc-finger proteins Kox1 (human) or Kid-1 (mouse), may provide even tighter regulation by doxycycline (Witzgall et al. 1994; Deuschle et al. 1995; Freundlieb et al. 1999; Szulc et al. 2006; Wiznerowicz et al. 2006).

The components of the *E. coli* lac operon have also been adapted to allow gene regulation in mice. In *E. coli*, transcription of the *lac* structural genes are controlled by the lac repressor protein (lacR) which blocks transcription by binding to an operator sequence (lacO) located between the promoter and the regulated gene. The inducers β-galactoside or isopropyl-b-D-thiogalactopyranoside (IPTG) bind to lacR and allow transcription to proceed. The system requires two transgenes, one that ubiquitously expresses a Lac repressor optimized for eukaryotic expression and the other carrying a transgenic cassette that contains LacO sequences between the promoter and the gene of interest. Reversible induction of the desired gene occurs when double-transgenic animals are exposed to IPTG in the drinking water (Cronin et al. 2001). This system has been used to control expression of tyrosinase in the mouse (Cronin et al. 2001) although it has not been as widely adopted as the tet regulatable systems.

Both insect and mammalian steroid receptor systems have been used for controlled expression of transgenes (Yamamoto et al. 2001). In insects, 20-hydroxyecdysone (Ec), a *Drosophila* steroid hormone involved in metamorphosis, binds to its receptor (the ecdysone receptor, EcR) in the cytoplasm. The complex heterodimerizes with ultraspinacle (USP), the insect homolog of the vertebrate retinoid X receptor (RXR) which leads to nuclear translocation and transcriptional activation of any transgene linked to the EcR/USP DNA binding domain. Ecdysteroids are not normally found in mammals and mammalian steroids do not activate insect ecdysone receptors. However, ecdysteroid-dependent transcriptional activation can occur in mammalian cells when the A/B region of EcR is replaced with a VP16 transactivation domain and the chimeric EcR (VpEcR) is provided with another cassette containing recombinant RXR (rRXR) (Fig. 4). With these modifications the ecdysone system has been successfully used in several studies to reversibly regulate transgene expression in mice (Christopherson et al. 1992; No et al. 1996; Saez et al. 2000; Graham 2002).

In a variation on the VpEcR system to insure that the inverted repeat motif AGGTCA in the ecdysone response element (EcRE) will not be recognized by the mammalian RXR (USP), the EcR DNA-binding domain was mutated so that it could only recognize the half-site sequence AGAACA found in the glucocorticoid response element. Modified in this way, the VgEcR consists of the activation domain of the VP16 protein fused to an EcR that exhibits the DNA-binding specificity of rRXR instead of USP. Upon exposure to ecdysone or the analogs muristerone A (murA) or ponasterone A (ponA) (Saez et al. 2000), the VgEcR/RXR complex binds to the modified hybrid ecdysone response element (E/GRE) and induces expression of the transgene of interest (No et al. 1996; Graham 2002). This system has been used to regulate expression of a luciferase gene, as well as confer ecdysone-inducible regulation of estrogen 2–4-hydrolase β in T cells (No et al. 1996). In other adaptations, ecdysone-inducible systems have been successfully used to regulate expression of a dominant-negative ErbB-1 receptor in cardiomyocytes of young adult mice (Rajagopalan et al. 2008) as well as regulate transgene expression in mammary gland (Albanese et al. 2000), and skin (Saez et al. 2000). Ecdysone-

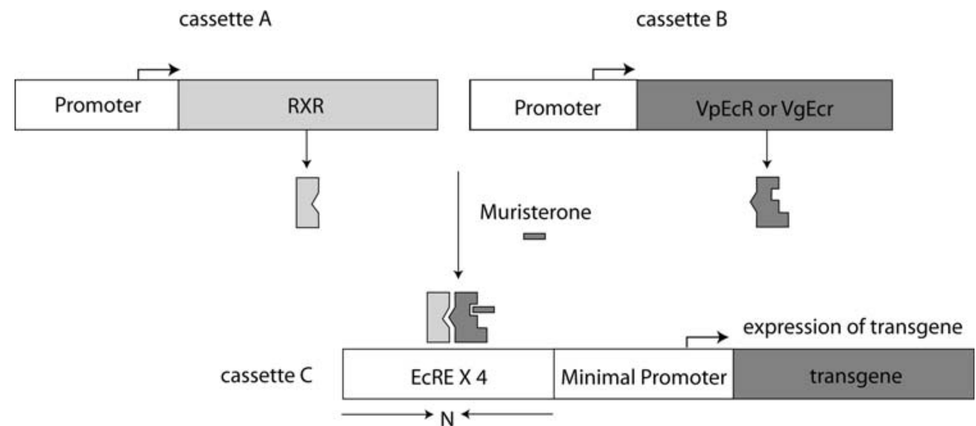


Fig. 4 Ecdysone-inducible system. Four EcREs are placed upstream of a minimal promoter, which can drive transgene expression in the presence of a complex of muristerone-RXR-VpEcR or VgEcR

regulatable lentiviral vectors have also been developed for use in experimental animals (Galimi et al. 2005).

Inducible systems for gene regulation in transgenic mice have also been devised based on the regulation of cytochrome P-450 (Gonzalez and Nebert 1990; Cheung and Gonzalez 2008), or the mifepristone-inducible system (Ngan et al. 2002). Attempts have also been made to adapt a number of inducible promoters including the heat shock, retinoic acid-induced slow myosin heavy chain 3 gene (SMyHC3), metallothionein, interferon-γ, and C-reactive protein promoters for use in inducible gene regulation in vivo (Gingrich and Roder 1998; Wang et al. 1998). Although these systems have in some instances been successful in vivo their general success has been limited by inefficient gene regulation and high basal levels of expression. In addition, tissue specific effects of some of the inducer molecules have limited their general application.

Recent developments in the generation of transgenic animals

While pronuclear injection and gene targeting in ES cells has been highly successful in mice the basic methods have proven less adaptable to other species, encouraging the search for alternatives. Recently, for example, exogenous DNA has been introduced into oocytes using retroviral transduction. This approach is limited by a requirement for high viral titers to obtain proviral integration. Proviral integration is also random and the insert size that can be accommodated by current vectors is limited to less than 8 kb. However, transgenic mice have been produced by retroviral transduction of male germ-line stem cells (Nagano et al. 2001; Kanatsu-Shinohara et al. 2004) and whereas proviral integration of retroviruses can only occur in actively replicating cells, recombinant lentiviruses can be used to transduce recombinant proviral DNA into the genome of non-dividing cells. Since the zona pellucida constitutes a physical barrier to retroviral/lentiviral infection, recombinant viruses are usually injected into the perivitelline space between the zona pellucida and the cytoplasmic membrane of the embryo or by incubating zona pellucida-free embryos in viral supernatant.

Lentiviral vectors have proven highly efficient in generating transgenic rats and mice (Michalkiewicz et al. 2007; Park 2007; Adams and van der Weyden 2008), farm animals (Fassler 2004; Hofmann et al. 2004, 2006), Japanese quail (Huss et al. 2008), marmoset monkeys (Sasaki et al. 2009), and rhesus monkeys (Yang et al. 2008). For example, transgenic rat models of Alzheimer's disease have been generated via lentiviral vector infection of rat zygotes (Agca et al. 2008). Production of transgenic mice and rats has also been accomplished by retroviral/lentiviral transduction of male germ-line stem cells (Nagano et al. 2001, 2002; Hamra et al. 2002) and pantropic pseudotyped retroviral vectors have been used to prepare transgenic fish (Sarmasik et al. 2002) and crustaceans (Lu et al. 1996).

Sperm-mediated gene transfer is also an effective method for producing transgenic frogs, mice, rats, and pigs with efficiencies of 50–80% or higher (Macha et al. 1997; Ishibashi et al. 2008; Lavitrano et al. 1989, 2003, 2006; Maione et al. 1998; Kato et al. 2004; Hirabayashi et al. 2005). Gene transfer has been achieved using a variety of methods. For example, in one approach, sperm cell membranes were disrupted by repeated freeze–thaw cycles or by exposure to detergents to facilitate introduction of exogenous DNA (Moreira et al. 2006, 2007). Introduction of the Tn5 transposase along with the injected DNA appears to greatly enhance microinjection efficiency including following cytoplasmic injection (Kaneko et al. 2005; Suganuma et al. 2005). In mice and pigs, to facilitate DNA uptake, the recombinant DNA has also been anchored noncovalently to the sperm head with an antibody that recognizes a sperm surface protein (Chang et al. 2002). Others have introduced recombinant DNA into the sperm head by electroporation (Gandolfi 1998, 2000; Sin et al. 2000) or lipofection (Bachiller et al. 1991).

Generation of transgenic animals in non-mammalian vertebrates and invertebrates

Transgenic animals can also be generated in many non-mammalian vertebrates and invertebrates. The arthropod fruit fly *Drosophila melanogaster* and the nematode *C. elegans* have been the most widely used invertebrates. Recently transgenic Zebra fish have also gained much attention as an adaptable experimental model. In non-mammalian vertebrates and invertebrates the pronuclei are not visible, therefore DNA can only be injected into the cytoplasm and processing of the exogenous DNA depends on the host species.

Transgenesis in *Drosophila* currently employs transposable vector systems based on the P element (Rubin and Spradling 1982; Bachmann and Knust 2008). The transgene of interest is subcloned between P element ends and the resulting vector is injected into the posterior cytoplasm of *Drosophila* embryos. In the presence of P element transposase, the transgenic cassette integrates into the pole cell nuclei, which give rise to germ cells. The inclusion of a selectable marker in the transgenic cassette for a trait such as eye color allows identification of transgenic flies as well as genetic mapping of the transgene's chromosomal location (Fujioka et al. 2000). A targeted integration system has been developed by placing the *Streptomyces* phage ϕ31 *attP* target site into the *Drosophila* genome. Co-injection of a transgenic plasmid harboring the donor *attB* sequence and ϕ31 integrase mRNA into *attP* recipient embryos results in site-specific insertion of the transgene into the *attP* site (Fish et al. 2007).

In *C. elegans*, DNA is injected either into maturing oocytes or more commonly into the syncytial gonad which targets the hermaphrodite germline (Berkowitz et al. 2008). Co-injection of a selectable marker such as rol-6 (which affects morphology) or GFP, allows easy identification of transgenic organisms. The germ line will harbor multiple extrachromosomal DNA arrays that if integrated into the host genome will become stably transmitted. To prevent formation of high copy extra chromosomal arrays the *C. elegans* suppressor tRNA gene *sup-7* can be included in the transgenic cassette since *sup-7* tRNA is toxic at high concentrations (Fire 1986). Alternative methods such as particle bombardment (Praitis et al. 2001) and germ line co-injection of the transgenic cassette together with high concentrations of oligonucleotides have also been used for the generation of low-copy number transgenic lines (Mello et al. 1991).

In fish, a tough chorionic membrane surrounds the vitelline membrane, which in certain species makes microinjection practical only through the micropyle, a pore in the chorion through which the sperm penetrates (Houdebine 2007). Treatment of the eggs with reduced glutathione prevents chorion hardening post-fertilization (Yoshizaki et al. 1991; Robles et al. 2007). In chickens and *Xenopus* microinjected DNA remains unintegrated during early development (Mozdziak and Petitte 2004). In fish, the injected DNA is extensively replicated increasing chances for its integration into the host genome. However, the high levels of unintegrated DNA results in multiple integration events during development leading to heavily mosaic founders (Chen and Powers 1990).

For *Xenopus*, a very efficient system has been developed that involves incubation of permeabilized sperm nuclei with linearized DNA followed by decondensation of the sperm nuclei with an egg extract containing a small amount of a restriction enzyme which stimulates recombination by creating double-strand breaks thus facilitating integration of DNA into the genome. Diluted sperm nuclei are then mixed with unfertilized eggs. One advantage of sperm-mediated transgenesis is that as the transgene integrates into the genome prior to fertilization, the resulting transgenic embryos are not chimeric and no breeding is required to obtain non-mosaic transgenic animals (Ishibashi et al. 2008).

In fish, electroporation is quite suitable for delivery of transgenes into large numbers of sperm/eggs (Sin et al. 2000; Hostetler et al. 2003). Electroporation has also been used to generate non-vertebrate chordate and invertebrate transgenics (Zeller et al. 2006; Correnti et al. 2007). Particle bombardment with DNA-coated metallic beads has been used to deliver DNA into fertilized eggs of eastern oyster, loach, rainbow trout and zebrafish (Zelenin et al. 1991; Cadoret et al. 1997).

Somatic cell mutagenesis combined with nuclear transfer for generation of transgenic animals

ES cell technology has been routinely applied only in the mouse although attempts to develop similar gene targeting approaches in species including rat and pig continue. Another approach being used to bypass the lack of ES cell lines in many species is to make genetic modifications in somatic cells and then introduce these modifications using the nuclear transfer technology that has been developed for animal cloning (Kono et al. 1991; Wilmut et al. 1997). Animal cloning by nuclear transplantation was originally reported by Briggs and King in *Rana pipiens* (Briggs and King 1952) and subsequently by Gurdon in *Xenopus* (Gurdon 1962). More recently this technology has been successfully used for cloning mice (Wakayama et al. 1998; Wakayama et al. 1999; Wakayama and Yanagimachi 1999a, b; Rideout et al. 2000; Wakayama et al. 2000), rats (Zhou et al. 2003), rabbits (Challah-Jacques et al. 2003), felids

(Gomez et al. 2003), and farm animals (Wheeler 2003; Richt et al. 2007).

This technology can be used to produce transgenic animals by first inserting a transgene randomly or performing gene targeting in primary fibroblasts. The somatic cell nucleus of the fibroblast is transferred into an enucleated oocyte that is then activated to initiate development. Gene targeting combined with nuclear transfer has been successful in the generation of transgenic sheep (McCreath et al. 2000; Denning and Priddle 2003), pigs (Lai et al. 2002), and cattle (Richt et al. 2007). Alternatively nuclear transplantation can be achieved through cell fusion mediated by viral envelope glycoproteins (inactivated Sendai virus) or electrofusion (Kono et al. 1988; Pinkert 1994; Gurdon and Melton 2008). The resulting cloned embryo is transferred to a foster mother where it develops into a blastocyst which implants and gives rise to a cloned animal.

Nuclear transplant via cell fusion has been used to prepare a transgenic pig model of Alzheimer's disease expressing the Swedish mutation in the human APP associated with familial Alzheimer's disease (Kragh et al. 2009). More recently, it has become possible to produce induced pluripotent stem cells (iPS cells) from embryonic mouse fibroblasts by reprogramming the cells with the transcription factors Oct4, Sox2, Klf4 and c-Myc (Takahashi et al. 2007a; Takahashi et al. 2007b). iPS cells have been used to create live mice through a process known as tetraploid blastocyst complementation (Kang et al. 2009; Zhao et al. 2009). Although still in its development stage, this technology may provide a powerful method for generating transgenic animals from embryonic fibroblasts that have been genetically modified in vitro.

Other strategies for producing knockout animals

Alternative strategies such as chemical mutagenesis as well as the use of mobile DNA elements (transposons and retrotransposons) or chimeric zinc-finger nucleases have also been used for the generation of knockout animals. Chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) has been used as a method of random mutagenesis in mice and rats (Russell et al. 1979; Hitotsumachi et al. 1985). ENU is an alkylating agent that transfers its ethyl group to nitrogen or oxygen radicals in DNA resulting in base mispairing with consequent base pair substitution. ENU mutagenesis creates one base pair change in any given gene in every 200–700 gametes (Hitotsumachi et al. 1985). Mutant animals are produced by ENU injection into male mice that are bred with wild-type females to produce the mutant offspring. Once a mutant phenotype is recognized, the causative mutation is mapped by positional cloning. Using this approach, dominant mutations causing tumorigenesis (Moser et al. 1990) and affecting circadian rhythm (King et al. 1997) have been identified. ENU mutagenesis is an important tool used by an international consortium whose aim is to establish knockout and conditional mouse strains for all mouse genes (Gondo 2008). It is also employed in a new reverse genetics approach in which the ENU-induced mutations can be identified in gene sequence-based screens (Gondo 2008).

Mobile DNA mutagenesis using class I (retrotransposons) and class II transposons can also be used to produce knockout rodent models (Miskey et al. 2005; Largaespada 2009). Mutations generated through mobile DNA elements are irreversible and are tagged by sequences within the transposon. This approach permits random mutagenesis directly in germ cells with genes stably disrupted at high frequency throughout the genome and a method for identifying the mutation by the transposon tag.

Long interspersed nuclear elements (LINE1, L1) contain an internal promoter within a 5′ untranslated region which controls expression of two open reading frames coding for an RNA-binding protein and a reverse transcriptase with endonuclease nicking activity followed by polyadenylation processing sequences. These retrotransposons mobilize via the reverse transcription of an L1 RNA intermediate using a copy-and-paste mechanism. Integration of the newly synthesized L1 DNA into cellular DNA requires the simple consensus sequence 5′-TTTTA-3′ in the target site. Integrated L1 sequences are often truncated at the 5′ end, with an average total size of 1 kb, many containing only 3′ terminal sequences.

Endogenous L1 retrotransposons have been modified to increase their activity and mutagenic power and have been used for random insertional mutagenesis in rodents (Bushman 2004; Ivics and Izsvak 2005; Ostertag et al. 2007). The reported frequency of germline transmission of de novo L1 insertions has been estimated to be as high as one insertion in every three sperm cells (An et al. 2006). L1 elements are also capable of somatic mutagenesis as has been described in human diseases (Morse et al. 1988; Miki et al. 1992). Human and synthetic mouse L1 sequences can retrotranspose into somatic tissues in transgenic mice (Ostertag et al. 2007). Gene trapping technology has been used to maximize the rate at which retrotransposition insertions interrupt coding exons. A bidirectional gene trap using the human BCL2 gene splice acceptor which is able to splice into exons located more than 100 kb away has been used for gene disruption in the mouse (Seto et al. 1988; Xin et al. 2005; Ostertag et al. 2007).

The Sleeping Beauty (SB) transposon system was derived from inactive Tc1/mariner family transposable elements and uses a cut and paste mechanism shown to be highly efficient in the generation of mutant rodents, amphibians and fish (Davidson et al. 2003; Izsvak and Ivics 2005; Keng et al. 2005; Sinzelle et al. 2006). For germline

mutagenesis in mice, a strain stably expressing the SB transposase is crossed with a separate strain which contains a pool of chromosomally integrated inactive SB transposons, which are devoid of the transposase and contain a mutagenic gene-trap cassette (containing a reporter gene such as GFP) and polyA-trap cassette. Double-transgenic mice are then crossed to wild-type females and the resulting offspring are screened for activation of the gene-trap reporter (Izsvak and Ivics 2005; Keng et al. 2005). In the case of a GFP reporter, expression can be detected only in animals in which the transposon has integrated into a functional gene. The integration loci can be identified by PCR-based techniques, and phenotypic changes associated with the mutations can be examined in homozygous animals (Izsvak and Ivics 2005; Largaespada 2009). Minos, another Tc1/mariner superfamily transposon, has also been shown to have transposon activity in the non-vertebrate chordate *Ciona intestinalis* enabling the creation of stable transgenic lines, enhancer detection, and insertional mutagenesis in this organism (Awazu et al. 2007).

The major advantage of transposon-mediated insertional mutagenesis in rodents is the ability to generate and maintain easily large libraries of insertional mutants in the sperm of founder animals. Effects of the mutations can be analyzed directly by breeding founder animals. Thus, the additional step of breeding germline chimeras required for ES cell-based gene targeting is avoided.

Recently, in *Drosophila*, zebra fish, and rats, direct embryo injection of engineered zinc-finger nuclease (ZFN) encoding mRNA or DNA has been used to generate heritable knockout mutations at specific loci (Carroll 2008; Geurts et al. 2009). Chimeric molecules are used that consist of a DNA-binding zinc-finger domain and a restriction endonuclease. The specificity of DNA cleavage is conferred by varying the zinc-finger domains, as each zinc-finger interacts with a particular triplet of DNA base pairs. Combining different zing fingers permits specific binding to 9–12 bp motifs. Double-stranded breaks occur when two ZFNs bind to the target DNA bringing their nuclease domains together. Repair can occur by homologous recombination if a template is available or simply by non-homologous end joining with the addition or deletion of bases (Carroll 2008; Woods and Schier 2008). In the rat, ZFN-mediated genetic disruptions have been targeted to the endogenous immunoglobulin M (IgM) and Rab38 genes, as well as to an integrated GFP reporter gene (Geurts et al. 2009).

RNAi-mediated transgene knockdown

Constitutive and conditional knockdown of transgene expression in vivo has also been accomplished through RNA interference (RNAi) (Tavernarakis et al. 2000; Hitz et al. 2007, 2009; Kuhn et al. 2007). RNAi is a sequence-specific gene-silencing process that functions at the mRNA level. In invertebrates, long double-stranded RNAs (dsRNAs) are processed into short interfering RNAs (siRNAs) by the Dicer ribonuclease. The siRNA antisense strand serves as a template for the RNA-induced silencing complex (RISC) which recognizes and cleaves the complementary mRNA leading to its rapid degradation. In mammals, dsRNAs (>30 bp) elicit an interferon response resulting in global inhibition of protein synthesis and non-specific mRNA degradation. However, short synthetic dsRNAs can trigger the specific knockdown of mRNAs in mammalian cells without interferon induction if their length is below 30 bp (Elbashir et al. 2001).

Expression vectors have been designed to contain sense and antisense regions that are complementary to a selected mRNA target. These transcripts, which have a stem-loop structure, can fold back and form short hairpin RNAs (shRNAs) that are processed by Dicer into siRNAs. Since these vectors can stably integrate into the genome, they allow permanent gene silencing in transgenic organisms (Hitz et al. 2009). Transgenic shRNA mice have been produced by pronuclear injection (Hasuwa et al. 2002), infection of zygotes or ES cells with lentiviral vectors (Rubinson et al. 2003; Dillon et al. 2005), random integration into ES cells (Lickert et al. 2004), and knockin targeting via recombinase-mediated cassette exchange (RMCE) or homologous recombination (Hitz et al. 2007, 2009; Kuhn et al. 2007; Oberdoerffer et al. 2005). The efficiency of target knockdown can be as high as 90% or greater (Hitz et al. 2009).

Using this technology, spatiotemporal and conditional transgene knockdown has been used to prepare mouse models of Tangier disease (ABCA-1 deficiency) (Chang et al. 2004), diabetes mellitus (Kotnik et al. 2009), and for the conditional brain-specific knockdown of mitogen activated protein kinase (Hitz et al. 2007). Moreover, this technology has been applied successfully in zebra fish (Huang et al. 2008), *Drosophila* (Haley et al. 2008) and *C. elegans* the later the first species in which successful use of transgenic RNAi was reported (Tavernarakis et al. 2000).

Analysis of transgenic animals

The identification and analysis of transgenic animals is first performed on genomic DNA isolated from biopsy tissue, which in case of mice can be a small piece of tail or a piece of ear tissue. The analysis must establish the presence of the transgene, the zygosity, whether transgene rearrangement or deletion have occurred, and transgene copy number. This can be done by polymerase chain reaction (PCR)

and/or Southern blotting. Southern blotting, although more laborious, allows determination of copy number, and detects major rearrangements of the transgene. Transgene copy number can also be determined using real-time quantitative PCR (qPCR) based assays. Transgenic integration can be visualized microscopically using fluorescence in situ hybridization (FISH). This technique allows detection of transgenic animals (Swiger et al. 1995), determination of the chromosomal integration site (Shi et al. 1994), the local chromatin structure, and the effect of the integration site on gene expression (Schmidt et al. 1998). It can also be used to detect germline mosaicism in transgenic male founders (Ibanez et al. 2001), chromosomal rearrangements (Yu and Bradley 2001) and regional chromosomal deletions induced by Cre recombination (Gregoire and Kmita 2008).

The next level of analysis involves determining the pattern and level of transgene transcription or the absence of expression in animals in which a gene target has been silenced or deleted. Transcription analysis can be performed by Northern blotting, ribonuclease (RNase) protection assays and reverse transcriptase (RT)-PCR. As quantitative RT-PCR (qRT-PCR) has become widely available and is easy to perform, it is becoming the technique of choice to analyze expression, due to the precise quantitation that is possible. In situ hybridization can also be used to assess the pattern of mRNA expression at a tissue and cellular level.

Finally, characterization should include analysis of the protein product and its level of expression, which can be correlated with any phenotype that the animals exhibit. Most methods of protein analysis require use of specific antibodies against the gene product of interest. These techniques include Western blotting, enzyme-linked immunosorbent assays (ELISA), radioimmunoassay (RIA), and immunohistochemistry. If the transgenic product is well characterized, it can be analyzed by High Performance Liquid Chromatography (HPLC) as well. Coupled with N-terminal sequencing and mass spectroscopy, this technique can be used to completely characterize the expressed protein.

Concluding remarks

Transgenic technologies have had a tremendous impact on biomedical research and human welfare. Transgenic animals provide valuable experimental models to analyze gene function and regulation as well as facilitating identification of new target genes of therapeutic value. They are also being used to develop and test new therapeutic strategies for human diseases. Transgenic technologies may revolutionize the production of biopharmaceutical products with efficiencies far greater than any conventional microbial or cell-culture production systems. For example, it has been estimated that only 16 transgenic cows would satisfy world needs for HGH (Redwan el 2009). With the development of newer technologies, transgenic technology has become available in many animal species including species of interest not only to the biomedical community, but also to species of commercial and agricultural value, including invertebrates, fish, and livestock. Therefore, it is fair to predict that a prudent use of transgenic technologies has the potential to yield in the very near future major discoveries that will improve health and the quality of life.

Acknowledgments Work in the authors' laboratories has been supported by the National Institute on Aging (grants AG02219, AG05138, AG20139, and AG029361), the Alzheimer's Association (IIRG-07-57318), and a Merit Award from the Department of Veterans Affairs (1I01BX000342).

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