

Controlling your losses: conditional gene silencing in mammals

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Methods are now widely used in mice, and to a lesser extent in mammalian-cell culture, for the constitutive silencing of target genes in order to assess their function. For a variety of reasons, not least because many genes are essential for viability, it is important that these methods can be adapted to allow the controlled silencing of target genes. Reviewed here are the ways in which gene-silencing methods can be combined with a growing number of genetic control systems to generate cell lines or mice that are, in effect, conditional mutants. These approaches are still being developed and promise to open up key areas of cell and animal biology to genetic analysis.

Geneticists rely on genes that have lost their function. Classically, they start with an interesting heritable phenotype and use genetical and physical mapping to track down the underlying gene and its mutation; more often than not, the gene has lost its function. Modern geneticists, however, are often in the converse position of having a cloned gene with no associated phenotype and, therefore, need to silence or inactivate the gene in its cell or organism of origin to see what happens. The key methods for gene silencing are gene modification by homologous recombination (gene targeting), and the expression of genes for antisense RNA or dominant-negative proteins (Box 1). With the ever-expanding databases of sequenced genes, most of whose functions are unknown (or at least unclear), methods such as these will become increasingly important in the coming years. But what about genes that are essential for viability? Many biologically compelling and clinically important genes fall into this category including, for example, genes that promote and control progress through the cell cycle or prevent apoptosis or, at the multicellular level, genes that coordinate various key events in the developmental programme. By definition, inactivation of an essential gene will be lethal and no cell or animal will be available for mapping experiments or for phenotypic analysis. In 'classical' genetics this problem is solved by the isolation of conditional (e.g. temperature-sensitive) mutants. This solution is particularly powerful in genetically tractable organisms, such as bacteria and yeast, but becomes cumbersome when applied to mammalian cells and cannot be extended to whole animals. Conditional gene silencing is, therefore, a highly desirable goal in mammalian genetics.

The benefits of conditional gene silencing

In somatic-cell genetics the benefits are obvious. Whereas constitutive silencing of an essential gene is entirely unproductive, conditional silencing allows cells to accumulate before the gene is inactivated, so that the

experimenter can observe the nature of the lethality. Furthermore, experiments with conditionally mutant cells are internally controlled; it is not necessary to compare clones, or pools of clones, in which the gene has or has not been silenced. Cells that conditionally express an essential gene can also be used as a conditionally 'null' genetic background in which to test the function of various mutant forms of the gene of interest. In this way, one can ask not only what the gene does, but how it does it.

In the whole animal, constitutive silencing of an essential gene is not entirely uninformative because, for instance, the stage of arrested development and physiology of the non-viable mouse can provide useful clues as to the function of the gene. But the inability to establish viable strains of mutant mice can make further analyses prohibitively expensive or time-consuming.

Box 1. Gene-silencing methods

Gene targeting^{8,12-14}

This refers to homologous recombination (HR) between a specifically designed targeting construct and the chromosomal target gene of interest. Targeting constructs are most commonly used to disrupt a target by inserting a heterologous sequence and/or making a small deletion. More specialized design and use of the targeting constructs can allow a whole range of pre-defined chromosomal alterations to be made, ranging from single base-pair changes to megabase-pair deletions, truncations or translocations. Mammalian somatic-cell lines can be used, but if the target gene is autosomal, two rounds of gene targeting are necessary. If the host cells are totipotent mouse embryonic stem (ES) cells, targeted clones can be reintroduced into a developing blastocyst and contribute to the developing embryo. When the germ cells of the resulting chimeric mouse are ES-cell derived, breeding can be used to generate mice that are heterozygous and homozygous for the desired mutation.

Antisense techniques^{7,15,16}

Expression of a target gene can sometimes be suppressed by the binding of antisense molecules that are complementary to its transcript. The mechanism of action is poorly understood and

can be nuclear or cytoplasmic and affect transcript stability, processing, transport or translation. Stable overexpression of antisense RNA molecules requires the introduction of a gene encoding a transcript that is complementary to all, or part of, the target gene transcript. (Where a transient effect is sufficient, antisense molecules can be introduced in the form of long RNA molecules made by *in vitro* transcription, or short synthetic oligonucleotides.) The effectiveness of antisense RNA can be enhanced by incorporating ribozyme sequences that catalyse endonucleolytic cleavage of the target transcript, or other sequences that promote stability or determine subcellular localization.

Dominant-negative mutations

Overexpression of certain mutant forms of the target gene can give rise to a protein that is inactive, but that can form stable complexes with molecules required for the normal protein to function. Such dominant-negative mutations can be deletions or truncations (e.g. removing the DNA-binding domain, but not the *trans*-activating domain, from a transcriptional *trans*-activator) or point mutations (e.g. mutating an activating phosphothreonine residue in a protein kinase without preventing the formation of heterodimers).

Box 2. Gaining control of gene expression in mammalian cells

Endogenous transcriptional switches

The promoter sequences that make genes responsive to various natural inducer molecules can be exploited. For example, sequences from interferon (IFN)-induced genes¹⁷, the metal ion-inducible metallothionein (MT) gene¹⁸ and from the dexamethasone-inducible long terminal repeat of mouse mammary tumour virus (MMTV)¹⁹ can confer inducibility when suitably positioned next to a target gene.

A disadvantage of these systems is that the inducer will switch on various endogenous genes as well as the target gene, and these might have phenotypic consequences that must be distinguished from those of the target gene. Also, the degree of inducibility is often limited by a significant amount of un-induced transcription.

Exogenous transcriptional switches

The advantages of these systems are that the inducers have no known effect on mammalian cells and that high induction ratios are achievable. The disadvantage is that genes encoding the necessary regulatory proteins must be introduced into the host system.

The Lac system

The *lacI* gene of *Escherichia coli* encodes the lactose repressor protein which, when the inducer isopropyl β-D thiogalactoside (IPTG) is absent, binds to its 18 bp recognition sequence (*lacO*) and sterically represses transcription. IPTG-induced transcriptional derepression can be established in mammalian cells if *lacI* is expressed and *lacO* sequences are suitably positioned in the target gene. The optimal number and positioning of *lacO* sequences is uncertain²⁰. Induction factors of up to 60 have been reported²¹. As with the Tet system (see below), the Lac repressor can be converted to an inducible activator²².

The Tet system^{20,23}

This is based on the tetracycline repressor of *E. coli* which binds to its 19 bp recognition sequence (*tetO*) in the absence but not in the presence of tetracycline. Bujard and colleagues have turned the Tet repressor into a transcriptional activator by fusing the *tetR* gene to the VP16 gene of herpes simplex virus. Expressed in mammalian cells, the resulting gene product (tTA) confers

tetracycline (or deoxycycline hydrochloride)-suppressible transcription on target genes with *tetO* sequences in their promoter region. The number and positioning of activating *tetO* sequences is not as critical as for repressing *lacO* sequences. Several variations on this basic system have been developed, including an activator (rtTA) that binds *tetO* only in the presence of inducer. Depending on the host cell, very tight regulation can be achieved (e.g. up to 10⁵-fold induction in HeLa cells^{24,25}).

*The ecdysone system*²⁶

Ecdysone is a steroid hormone that induces transcription in *Drosophila* via a heterodimeric nuclear receptor. The genes for the receptor subunits can be expressed in mammalian cells and will activate hormone-dependent transcription of any gene that has been appropriately linked to ecdysone-response elements. The ecdysone analogue muristerone A is used for induction. Induction factors of over 200 have been reported.

Post-translational switches²⁷

These are mostly based on the oestrogen receptor (ER). ER binds to heat-shock proteins in a hormone-dependent fashion: hormone binding releases the receptor. Proteins can be made functionally hormone-dependent by fusing them to the ER ligand-binding domain. The resulting fusion protein remains in an inactive, sequestered form until released by the addition of oestrogen. A modified ER (ERTTM) has several advantages including more specific, although not completely specific, regulation by the ligand 4-hydroxytamoxifen (4-OHT)²⁸.

Ligand-induced dimerization²⁹

Ligands can be used to promote the interaction of DNA-binding and transcriptional-activation proteins that have been engineered to carry ligand-binding domains. In this way, tight transcriptional regulation of reporter genes linked to appropriate binding sites has recently been demonstrated³⁰⁻³². Although the ligands used to date (rapamycin, mifepristone, FK1012) are not always neutral with respect to cell and animal physiology, this approach has the potential to yield a whole range of useful ligand-inducible systems. For certain target genes, whose products are normally activated by dimerization and can be engineered to bind the appropriate ligands, ligand-induced dimerization can also be used to achieve post-translational control²⁹.

Furthermore, if a gene of interest has essential roles at two or more stages of development, constitutive silencing will reveal only the first. Indeed, the consequences of silencing even non-essential genes can sometimes depend on the developmental stage at which silencing occurs. By delaying gene inactivation until such time as the experimenter chooses, conditional silencing allows such multiple or stage-dependent roles to be revealed. It is also possible that gene silencing in adult animals can be used to model certain human diseases involving the loss of gene or cell function in adults (e.g. cancer and degenerative disorders).

Conditional gene-silencing methods

A variety of methods can be envisaged for achieving conditional gene inactivation. Provided they are specific, inhibitory molecules (e.g. antibodies or antisense oligonucleotides) that are introduced directly into cells can be used for assessing essential gene function. This review, however, focuses on stable gene-silencing methods (Box 1) and how they can be combined with characterized systems for the regulation of gene expression to achieve conditional silencing. I will consider mostly

those regulatory systems that allow the target gene to be controlled by an inducer molecule, either added to the culture medium or administered to the mouse (Box 2). Equally important for studies of gene function in the mouse, although considered in less detail here, is the use of tissue-specific regulatory systems that allow gene silencing to be restricted to specific tissues and, in some cases, to specific times during development.

Conditional gene silencing via gene targeting (conditional targeting)

The use of gene targeting for conditional gene silencing (conditional targeting) is a fairly recent development. Three approaches, for use in cell culture or mice, are illustrated in Fig. 1 and reports of their use are summarized in Tables 1a, 2a and 2b. All involve making the target gene, or a minigene derived from it, responsive to the chosen regulatory system. In the most widely adopted approach this is achieved indirectly by use of the Cre/*lox* system. The gene encoding the site-specific bacteriophage recombinase Cre is introduced as a regulated transgene, while gene targeting is used to 'flox' the target gene, that is, to flank a key region of the target gene

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with *loxP* sites, the 34 bp recognition sequence for Cre. The target gene remains expressed until the *cre* gene is induced; Cre then catalyses site-specific recombination between the *loxP* sites, deleting part of the target gene and thereby silencing it. (Other site-specific recombination systems, such as the *Flp/Frt* system of yeast¹, can be used in a similar way). This 'Cre-based' approach can be envisaged for use in cell culture (Fig. 1a; 2-5) but this has not yet been reported. In mice, it has been achieved by breeding responder mice, carrying the floxed target gene, with regulator mice that bear the regulated Cre transgene² (Fig. 1b; 1, 2). The inducible Cre-based approach has been pioneered in the mouse using the DNA polymerase β gene as the target gene and interferon (IFN) to control *cre* gene expression³. The efficiency of inducible deletion of the target gene was unexpectedly tissue-dependent (e.g. 100% in liver, 8% in brain). Uninduced deletion was detectable but generally less than 5%. In a second example of this approach, a tamoxifen-regulated Cre-ER (oestrogen receptor) fusion protein was used for conditional deletion of a floxed retinoic acid receptor gene in mice⁴. In this study there was no detectable 'background' of uninduced deletions, presumably reflecting more efficient regulation by the tamoxifen system.

So far, the Cre-based approach has been used more for tissue-specific target-gene silencing than for inducible silencing (Table 2). This is usually done by breeding responder mice (carrying an endogenous target gene, or an exogenous reporter gene, with appropriately positioned *loxP* sites) with regulator mice expressing the *cre* gene under the control of a tissue-specific promoter. Alternatively, a viral vector expressing the *cre* gene can be injected into the appropriate tissue of a responder mouse⁵. Because the timing of tissue-specific expression and of virus injection are under developmental and experimental control, respectively, these approaches allow for some temporal as well as spatial control of silencing.

Two distinctive features of the Cre-based approach are its binary nature (the target gene is either on or off, with no intermediate state), and its irreversibility (once inactivated, the target gene cannot be reactivated by reversing the switch). A potential advantage, depending

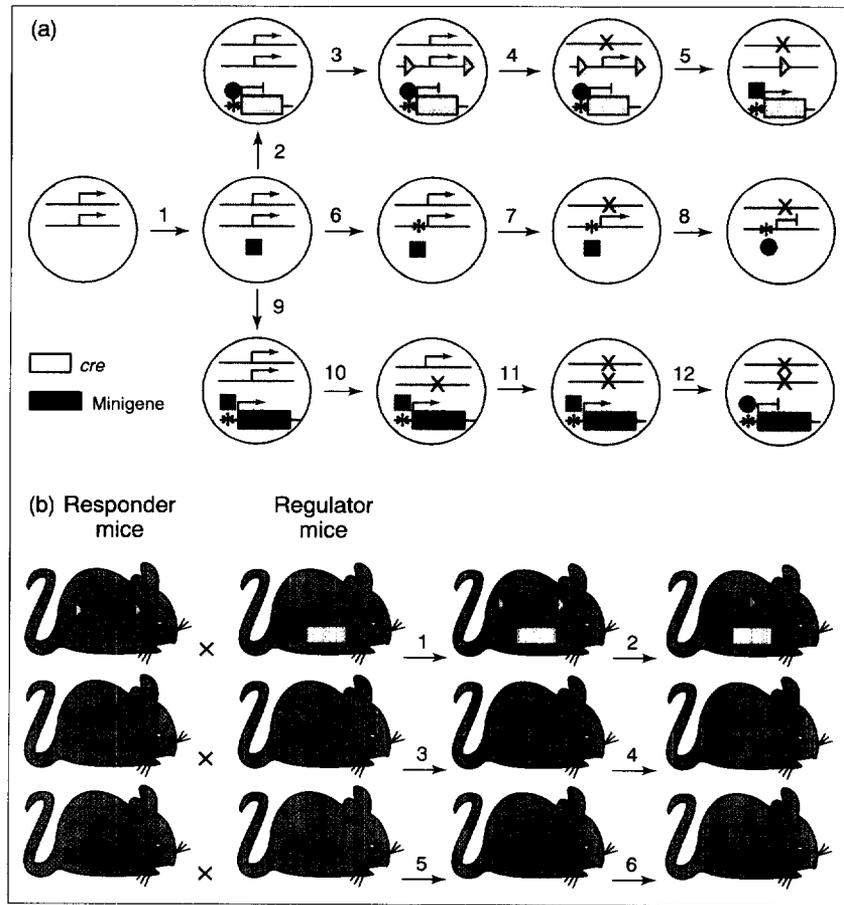


FIGURE 1. Conditional gene silencing by gene targeting in cell lines and mice. All methods rely on one of the regulatory systems described in Box 2. For convenience, a transcriptional regulatory system is shown, but the approaches could equally employ a post-transcriptional system. Regulation is achieved by addition or withdrawal of an inducer molecule that determines whether a transcriptional-regulator protein exists in a form that does (green square), or does not (red circle) allow transcription of any gene controlled by the appropriate regulatory sequence (green asterisk). Yellow triangles represent *loxP* sites. Target alleles (black lines) are expressed (arrows) until disrupted by gene targeting (X). (a) Conditional targeting in cell lines. Cells must first be transfected with the gene encoding the chosen regulatory protein (step 1; clearly this step is unnecessary if an endogenous regulatory system is used). The 'Cre-based' approach (2-5) involves transfection with a regulated Cre minigene (2), 'floxing' of one target allele (3) and targeted disruption of remaining target allele (4). Target gene silencing is then achieved by deletion following upregulation of the *cre* gene (5). The 'direct-control' approaches (6-8 and 9-12) involve the introduction, by gene targeting, of a regulatory sequence into one target allele (6), or the expression of a regulated target minigene (9) and targeted disruption of one (7) or both (10,11) target alleles. Target-gene silencing is then achieved by downregulation of the modified target allele (8) or minigene (12). (b) Conditional targeting in mice. The 'Cre-based' (1,2) and 'direct-control' (3,4 and 5,6) approaches as applied in mice are shown. Each approach involves a genetic cross between a targeted responder mouse and a regulator mouse. Only single alleles are shown because, where necessary, homozygosity for a transgene- or target-gene modification can be achieved by breeding. Once the mice generated in steps 1, 3 and 5 have been made homozygous for their target-gene modification, target-gene silencing can be achieved by administration (or withdrawal) of the relevant inducer (2,4,6).

on the nature of the target gene, is that, as long as the *loxP* sites are positioned with care, target-gene expression is likely to be completely normal until Cre is induced.

In a second approach, gene targeting is used to place the target gene directly under the control of the regulatory system and, for cell culture work, to disrupt the remaining target allele (Fig. 1a; 6-8). This 'direct-control' approach has been demonstrated in cell-culture

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TABLE 1. Examples of conditional gene silencing in cell lines

Host	Target gene	Control system	Comments/phenotype	Ref.
(a) Gene targeting (direct-control)				
Human fibrosarcoma (HT1080)	<i>CDC2</i>	IPTG/LacZ	DNA re-replication and apoptosis	6
Chicken B cell (DT40)	<i>ASF/SF2</i>	Tet/tTA	Defective pre-mRNA processing; death	33
Chicken B cell (DT40)	<i>CENP-C</i>	4-OHT/ER	Metaphase/anaphase arrest; apoptosis	59
(b) Antisense				
Mouse fibroblast (NIH3T3 RAS)	<i>Myc</i>	Dex/MMTV promoter	Loss (80–98%) of growth in soft agar, reduced tumorigenicity	34
Rat pheochromocytoma (PC12)	<i>RelA</i>	Dex/MMTV promoter	Reduced adhesion, growth and tumorigenicity and NF- κ B binding	35
Mouse fibroblast (C3H 10t1/2)	<i>Ras</i>	Zn ²⁺ /MT promoter	Decrease in growth rate and TPA response	36
Mouse pituitary (AtT-20)	<i>Pc1</i>	Cd ²⁺ /MT promoter	Loss of chromogranin A and β -endorphin secretion	37
(c) Dominant negative				
Rat pheochromocytoma (PC12)	<i>Ras</i>	Dex/MMTV	Quiescence and decreased apoptosis on serum withdrawal	38
Intestinal epithelial (IEC-18)	<i>Ras</i>	Zn ²⁺ /MT	Loss of activation of ERK1 and reduced growth inhibition by TGF- β	39
Hamster CO60	<i>PARP</i>	Dex/MMTV	After irradiation, 90% reduction in poly(ADP-ribose); reduced viability	40
Human carcinoma (HeLa)	<i>Dynamin</i>	Tet/tTA	Inhibition of transferrin internalization	41
Mouse thymoma (EL4)	<i>Myb</i>	4-OHT/ER	No effect on cell cycle; apoptosis	42
Mouse cytotoxic T-cell (B6.1)	<i>Myb</i>	4-OHT/ER	Accumulation in G1	43
Human ProB	<i>SHC</i>	Zn ²⁺ /MT	Increased IL-3-induced apoptosis	44
Human fibrosarcoma (HT1080)	<i>TRF1</i>	Tet/tTA	Telomere elongation	45

experiments, in which endogenous human *CDC2* gene expression was placed under the control of the Lac regulatory system⁶. The resulting cells were dependent on the inducer isopropyl β -D thiogalactoside (IPTG) for survival, and underwent DNA re-replication and apoptosis in its absence. In principle, the same approach could be used in mice (Fig. 1b; 3, 4). A major difference between this approach and the Cre-based approach is that it allows for intermediate and reversible expression of the target gene, which would clearly be an advantage for studying gene-dosage effects and the consequences of temporary gene silencing. Another advantage of this approach, in common with the Cre-based approach, is that it allows for normal transcription of the target gene before silencing, provided the control system is based on transcriptional repression (e.g. Lac) or on post-translational regulation (e.g. ER). Direct control, based on conditional transcriptional activation, would probably override any subtle (e.g. cell-cycle dependent) transcription of the target gene in the 'on' state. Furthermore, in the 'off' state, the target gene's normal expression is likely to persist, unless gene targeting can be designed to disrupt the normal regulatory signals and introduce the inducible ones simultaneously.

A third approach, also a form of direct control, uses gene targeting to disrupt both endogenous alleles; viability depends on expression of an ectopic, regulated minigene derived from the target gene (Fig. 1a; 9–12 and b; 5, 6). If the minigene includes the target gene's normal transcriptional-regulatory sequences, this approach becomes very similar to the first direct-control approach, with the same advantages and restrictions. It will usually be easier, however, to express the minigene under the control of exogenous transcriptional-regulatory sequences. This will allow a free choice of control system, but will

probably exclude the chance of reproducing normal target-gene transcription in the 'on' state. Use of this method in mammalian cells has yet to be described, but it has been used in chicken DT40 cells, where homologous recombination is particularly efficient (Table 1a).

Post-transcriptional conditional gene silencing

Genes encoding antisense RNA or dominant-negative proteins (Box 1) can be used to generate conditional mutants by making their expression dependent on one of the regulatory systems described in Box 2 (or, in mice, on a tissue-specific promoter). Clearly, only transcriptional switches can be used for expressing antisense RNA, but other switches can be used for expressing dominant-negative protein.

There are many reports of inducible antisense experiments in somatic cell lines where expression of the target gene is only modestly (e.g. 50%) suppressed, indicating inefficiencies in the antisense RNA itself, in the control of its expression, or both. Nevertheless, incomplete silencing can sometimes produce an informative phenotype. Some examples of inducible antisense experiments resulting in relatively efficient silencing and/or a clearly inducible phenotype are included in Table 1b.

Antisense and transgenic technologies have been combined with some success⁷, although nothing like the success of combining gene targeting with embryonic stem (ES) cell-based transgenesis. There are, therefore, few examples of conditional antisense expression in mice (Table 2c). Most involve tissue-specific rather than inducible expression.

Reports of conditional phenotypes resulting from the inducible expression of dominant-negative proteins in cell culture are not rare and some recent examples, where informative phenotypes have been obtained, are

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TABLE 2. Examples of conditional gene silencing in ES cells and mice

Host	*Target gene	Control system	Comments/phenotype	Ref.
(a) Gene targeting (inducible Cre)				
ES cells/mice	Floxed <i>DNA polβ</i>	IFN/Mx promoter	IFN-induced <i>DNA polβ</i> deletion. Efficiency variable with tissue.	4
ES cells/mice	Floxed <i>RAR</i>	Tamoxifen/ER	<i>RAR</i> deletion. Efficiency variable with tissue	
ES cells/mice	Infloxed <i>lacZ</i>	Tet/TA	Tet-silenced <i>lacZ</i> expression	46
ES cells	Infloxed <i>lacZ</i>	4-OHT/ER	4-OHT-induced <i>lacZ</i> activation	47
(b) Gene targeting (tissue-specific Cre)				
ES cells/Mice	Infloxed <i>SV40Tag</i>	α-Crystallin promoter	Lens tumours	48
ES cells/Mice	Floxed <i>lacZ</i>	<i>lck</i> promoter	T-cell specific <i>lacZ</i> deletion	49
ES cells/Mice	Floxed <i>DNA polβ</i>	<i>lck</i> promoter	T-cell specific <i>DNA polβ</i> deletion	50
ES cells/Mice	Floxed <i>PolyPep GalNAc-T</i>	<i>lck</i> promoter	T-cell specific <i>PolyPep GalNAc-T</i> deletion. Normal T-cell development	51
ES cells/Mice	Floxed <i>DNA polβ</i>	CMV min promoter	<i>DNA polβ</i> deletion in all tissues	52
ES cells/Mice	Floxed <i>neo</i>	<i>Ella</i> promoter	<i>neo</i> deletion in all early embryo cells	53
ES cells/Mice	(In)floxed <i>lacZ</i>	<i>aCMKII</i> promoter	Forebrain-specific <i>lacZ</i> deletions	54
ES cells/Mice	Floxed <i>DNA polβ</i>	<i>CD19</i> promoter	B-cell specific <i>DNA polβ</i> deletion	55
ES cells/Mice	Infloxed <i>lacZ</i>	<i>PO</i> , <i>POMC</i> and <i>IRBP</i> promoters	Schwann cell, pituitary and retina-specific <i>lacZ</i> expression	5
(c) Antisense (inducible and tissue-specific)				
Transgenic mice	<i>Wnt1</i>	Testis-specific PGK-2 promoter	98% less <i>Wnt1</i> transcript in testes but fertility normal	56
Transgenic mice	<i>MHC classII</i>	Cd ²⁺ /MT	Modest delay of B-cell development in culture	57
Transgenic mice	<i>Gα₁₂</i>	Birth/PEPCK promoter	Delayed development following birth	58
Transgenic mice	<i>Angiotensin</i>	Protein diet/PEPCK promoter	Transient diet-induced loss of liver, plasma angiotensin and its transcript	7

*'Floxed' gene: essential portion is flanked by *loxP* sites, Cre inactivates. 'Infloxed' gene: essential region has insertion with flanking *loxP* sites; Cre activates.

given in Table 1c. The efficiency of target-gene silencing is variable and not always reported, but it is possible to induce a complete loss of activity of the target gene. Despite its restriction to genes in which dominant-negatives can be made, this is clearly a valuable approach. Probably because of the attractions of gene targeting in mice, reports of transgenic mice expressing dominant-negatives are rare, and I know of none where expression is inducible.

Advantages and disadvantages

Just as gene targeting has become the method of choice for constitutive gene silencing in mice, it seems likely that it will become the favoured approach for conditional silencing in mice. Standard gene knockouts in ES cells have become very streamlined and, as has been pointed out², a great attraction of Cre-based conditional targeting is that the construction of floxed responder mice involves little extra effort, compared with standard gene-targeting experiments. (Making responder mice for the direct-control targeting approaches might be a little less straightforward.) As useful lines of transgenic 'regulator' mice (Fig. 1b, Table 2a, b) continue to be developed, conditional gene-inactivation experiments in mice will burgeon. Our present inability to predict whether a given antisense or dominant-negative expression construct will work contrasts with the more reliable outcome of gene-targeting experiments.

The relative merits of the various methods are less clear-cut when one considers their use in cell culture experiments. The antisense and dominant-negative

approaches have the considerable advantage that, once an active construct has been identified, it need only be conditionally overexpressed as a transgene. In contrast, gene targeting in somatic cells, which is less streamlined than in embryonic stem cells, is relatively labour-intensive, especially because both alleles of the target gene must be modified. On the other hand, the predictability of gene-targeting approaches, combined with their potential to silence a target gene with specificity and efficiency, might compensate for the extra effort required.

Specificity and complementation

The specificity of the gene-targeting approaches is likely to be good because the regulatory element (* in Fig. 1) will usually control only the desired gene (*cre*, the target gene or a target minigene). It can be reassuring, however, to confirm that the phenotype is reversed by reintroducing an expression construct for the target gene in question⁶. Such complementation experiments also provide a first step towards structure-function studies in which mutated versions of the target gene are tested for their ability to complement.

Specificity can be achieved in the post-transcriptional approaches, but it can by no means be taken for granted: a given antisense RNA or dominant-negative protein might easily interact with whole families of related transcripts or proteins. Complementation is also more of a challenge because it requires a very efficient target-gene expression construct to overpower the already overexpressed antisense or dominant-negative gene. Even if this can be achieved, the resulting situation is unlikely to be

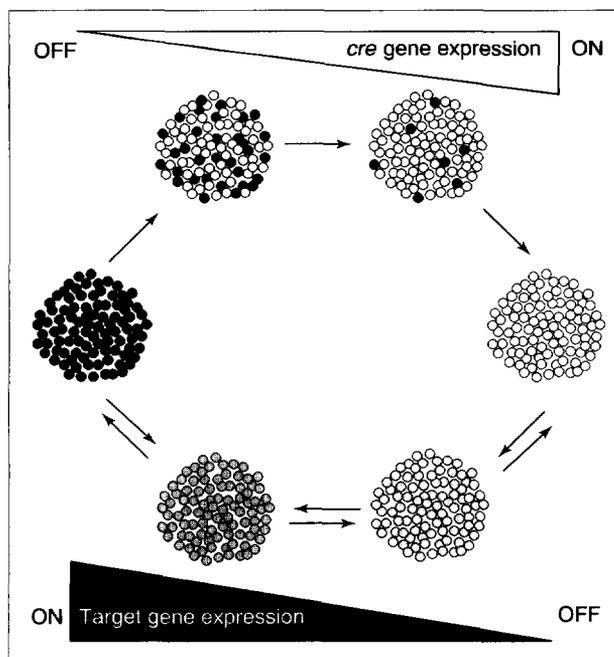


FIGURE 2. Different effects of variable control in Cre-based conditional targeting versus other approaches. Circles represent cells expressing normal (dark grey), intermediate (pale grey) or no (white) target gene product. The effects of tightly silenced (right), normal (left) or leaky (centre) control are shown for the *cre*-based approach (top) and other approaches (bottom). In the *cre*-based conditional targeting, leaky control does not generate intermediate expression, but will generate a heterogeneous cell population. Direct-control conditional targeting and conditional antisense and dominant-negative approaches allow for intermediate target-gene expression and a relatively homogeneous cell population. The *cre*-based approach is irreversible, but other approaches need not be.

physiologically relevant. Conditional mutants generated by post-transcriptional approaches are, therefore, not ideal hosts for structure–function studies.

Consequences of leaky control systems

All of the conditional silencing approaches described here require tight control systems to avoid unwanted expression, or to ensure adequate expression, of the target gene. In all but the Cre-based approach, leakiness will result in a fairly uniform population of cells in which target-gene silencing is incomplete. The binary nature of the Cre-based approach would appear to be an advantage, in this respect, because intermediate target-gene expression is not possible. Leaky control of *cre* gene expression is a possibility, however, and it is interesting to consider the consequences (Fig. 2). If there is residual expression of the downregulated *cre* gene, then a proportion of cells will inactivate the target gene under ‘permissive’ conditions. Conversely, if there is inadequate expression of the upregulated *cre* gene, a proportion of cells will express the target gene under ‘non-permissive’ conditions. Leakiness is, therefore, to be avoided in all approaches if they are to generate cleanly ‘conditional’ mutants. Nevertheless, in some situations it might be useful to establish leaky or intermediate conditions deliberately, because these might result in an informative phenotype, quite distinct from the tight ‘off’ and ‘on’ phenotypes.

Future developments

Improvements in regulatory systems

All the approaches discussed in this review require regulatable gene expression. To date, most approaches have used transcriptional regulation, either tissue-specific or inducible by glucocorticoids or heavy metal ions. The relatively recent development of more-tightly controlled inducible gene-expression systems (Box 2) has yet to have its full impact on the range of conditional gene-silencing approaches. It seems likely, therefore, that the efficiency and versatility of conditional gene-silencing experiments will improve in the near future.

Improvements in gene-targeting approaches

As already mentioned, the limiting factor for conditional targeting in mice is not gene targeting itself, but the availability of transgenic regulator mice; these are rapidly being developed (Table 2a, b). Efforts to improve the efficiency of gene targeting (discussed in Ref. 8) could, however, make a significant difference to conditional targeting in somatic cells.

Improvements in antisense and dominant-negative approaches

Our present inability to predict whether a given antisense or dominant-negative expression construct will work remains the most significant limitation to these approaches. Progress in antisense gene silencing, conditional or otherwise, therefore requires a better understanding of the factors (e.g. stability and subcellular localization of antisense RNA, or secondary structure in the target transcript) that make one antisense RNA molecule more effective than another. Similarly, dominant-negative approaches will benefit from continuing improvements in the prediction, from primary-sequence information of poorly characterized proteins, of domains or modifications that will act in a dominant-negative way.

An empirical approach to the identification of efficient antisense and dominant-negative genes has been developed⁹. The cDNA of the target gene is randomly fragmented and cloned, in both orientations, into an expression construct. Following transfection into a suitable host cell, a small subset of this library of fragments will silence target-gene expression, by coding either for antisense RNA or dominant-negative peptides. Such negatively acting clones have been dubbed genetic suppressor elements (GSEs). For conditional gene inactivation, the fragments can be cloned into a regulated expression vector. Thus, Pestov and Lau¹⁰ screened an IPTG-inducible library of 19 fragmented growth-related genes and were able to isolate growth-inhibitory GSEs derived from three of them (*fos*, *JunB* and MAPK). The potency of the GSEs was fairly weak, but streamlining this procedure might be a valuable way to identify GSEs that are useful for conditional gene inactivation. (With an appropriate phenotypic screen, the procedure might also be used as a ‘classical’ genetics protocol for isolating anonymous GSEs, capable of conditional gene silencing, from complex libraries). In a related approach¹¹, genes encoding peptide ‘aptamers’ with high affinity for a particular target are identified by screening a randomly generated library expressed in yeast. Active aptamers could become useful reagents for conditional dominant-negative gene silencing.

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Conclusion

The controlled loss of gene function can be achieved by many different combinations of various available methods for gene silencing and gene regulation. Exploration of these combinations has only just begun and future successes in conditional gene silencing now seem to be conditional only on the imagination of the geneticist.

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Conditional progress

Conditional targeting of *RAD51* in DT40 cells (S. Takeda, pers. commun.) and *Apc* in mice¹ have recently been achieved. Direct Tet-controlled downregulation of *RAD51* led to chromosomal breaks and cell death. Inactivation of floxed *Apc* alleles was limited to the colorectal region by injection of a *cre*-expressing adenovirus providing a model for familial adenomatous polyposis.

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