Vagaries of conditional gene targeting

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Conditional gene targeting based on excision or inversion of *lox*P-flanked DNA segments by Cre recombinase is a powerful technology for the analysis of gene function, but unexpected expression patterns of cre transgenes, variability of recombination efficiency depending on the target gene and potential toxicity of Cre recombinase represent serious challenges for the experimenter.

Conditional gene targeting in mice allows the introduction of targeted mutations in a cell type–specific and/or inducible way. This is most commonly achieved by flanking a target gene segment with recognition sequences for a site-specific recombinase and expressing the recombinase from a transgene controlled in a cell type–specific or inducible way. Bacteriophage-derived Cre recombinase and its 34–base pair target sequences called *lox*P sites are mostly used in this type of experiment[1–3].

Since its introduction about 10 years ago this approach has become increasingly popular in biomedical research and of course also in immunology. A current search for Cre recombinase transgenic mice in Medline yields over 900 publications, more than half of which were published in 2003 or later. In view of this popularity, it appears particularly important to draw attention to some problems and pitfalls inherent in conditional gene targeting experiments, which when not taken into account can easily lead to incorrect conclusions being drawn from the results. The purpose of this Commentary is to discuss the most important of these problems.

(In-)Fidelity of Cre expression and variability of recombination efficiency

The key feature of conditional gene targeting is its spatial or temporal restriction. This restriction relies on the fidelity of conditional expression of a transgenic or knocked-in cre construct. We use the term 'fidelity' to indicate what the investigator hopes to achieve in terms of the pattern of Cre expression. This hope is based on more or less solid but always incomplete knowledge of the functional importance of the ensemble of regulatory elements surrounding the Cre coding sequence introduced into a particular genomic locus.

One can use multiple strategies to express Cre in a conditional manner. Classical cre transgenes can be equipped with specific promoters and enhancers, cre cDNA can be 'knocked in' to a suitable genomic locus, or the two approaches can be combined by producing transgenic bacterial artificial chromosomes containing a genetic locus engineered to express Cre. None of these approaches, however, guarantees the desired Cre expression pattern. In addition, *lox*P-flanked target genes can differ dramatically with respect to their sensitivity to Cre-mediated recombination. Thus, to be sure about the specificity of a given conditional mutagenesis experiment, one ideally has to analyze recombination of the chosen target gene in various tissues of mice expressing the Cre recombinase construct and the *lox*P-flanked target sequences. In the case of inducible systems, one must also examine recombination before and after Cre induction.

The differential sensitivity of individual alleles to Cre-mediated recombination limits the utility of reporter alleles as they are widely used for the analysis of the fidelity of conditional Cre expression. These reporter alleles consist of genes encoding beacons such as green fluorescent protein or β-galactosidase, whose expression depends on deletion of a nearby *lox*P-flanked STOP cassette. While this approach has the obvious advantage of reporting conditional Cre activity at the single-cell level, the relevance of the results to the 'real' conditional gene-targeting experiment depends on whether the sensitivity of the *lox*P-flanked STOP cassette of the reporter to Cre-mediated recombination is equal to that of the *lox*P-flanked experimental target gene. In addition, one has to make sure that the reporter can in principle be detected in cells throughout the body.

Experiments with the B cell–specific CD19-cre transgene[4] nicely illustrate the importance of these considerations. CD19-cre mediates efficient recombination of most *lox*P-flanked target genes in mature B cells, partial deletion in immature B and pre-B cells and very little recombination in B cell progenitors. A reporter strain expressing yellow fluorescent protein upon Cre-mediated recombination[5] indicated 5% recombination in c-Kit$^+$ pro-B cells and 40% recombination in pre-B cells, respectively[6]. However, conditional ablation of the genes encoding the transcription factors Mcl-1 (ref. 7) and c-Myb[8] resulted in a dramatic block in B cell development at the pro-B cell–to–pre-B cell transition. PCR-based analyses indicated that recombination was nearly complete in cells at this developmental stage in both of these cases[7,8].

The most straightforward way to examine the pattern of Cre-mediated recombination in the various tissues of the mouse is Southern blot analysis distinguishing wild-type, *lox*P-flanked and recombined alleles. However, this method requires substantial numbers of cells and is also of limited sensitivity. If insufficient numbers of cells are available, PCR-based methods may be chosen, or conditional alleles can be constructed such that recombination of

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the target gene is accompanied by the activation of a reporter transgene. While these methods are more sensitive, their quantification is not as straightforward as that of Southern blot data, and their evaluation is often based on unproven assumptions, such as one's ability to detect reporter expression in certain or all cell types.

An example of infidelity of Cre-mediated conditional targeted mutagenesis is shown in **Figure 1**, which depicts deletion of a *lox*P-flanked allele of the gene encoding the YY1 transcription factor, as effected by the CD21-cre3A bacterial artificial chromosome–based transgene[9]. CD21 expression is induced in B cells when they differentiate from immature, transitional cells into long-lived follicular and marginal zone B cells. Thus, the CD21-cre3A transgene was developed to allow *in vivo* genetic analysis specifically of mature B cells. Indeed, CD21-cre3A recombines *lox*P-flanked alleles in mature splenic B cells[9]. CD21-cre3A also mediates efficient recombination in follicular dendritic cells, which are known to express CD21[10]. Unexpectedly, substantial deletion is also seen in a variety of other tissues, most spectacularly in forebrain (**Fig. 1**). Indeed, CD21-cre3A transgenic mice homozygous for the *lox*P-flanked *yy1* allele die early and exhibit various developmental abnormalities, among them an almost complete obliteration of the forebrain (H. Liu, M.S.-S., M. Alimzhanov, K.R. and Y. Shi, unpublished data). While this indicates that unintentionally, the CD21-cre3A strain can be used for conditional mutagenesis in the forebrain, it is also a dramatic demonstration of the infidelity of expression cre transgenes may exert, even when they represent knock-ins into a locus whose expression appeared, at least to an immunological mind, to be restricted to mature B cells and follicular dendritic cells[11].

A special case of infidelity: Cre-mediated recombination in the germline

Cre transgenes of all varieties hold the potential to effect efficient recombination of *lox*P sequences in the male and particularly the female germline[12–16]. If this phenomenon is ignored, groups of experimental compound mutant animals may turn out to be inhomogeneous. Some of the animals may carry the desired conditional mutation, whereas in others, a larger fraction and perhaps all cells of the body might be mutated. This non-homogeneity, which can entirely obscure the experimental outcome, depends in most cases on whether a compound mutant animal inherited the *lox*P-flanked allele together with the cre transgene from one of its parents. When working with situations involving Cre-mediated germline recombination, an investigator must set up the breeding in such a way so as to ensure that the cre allele and the *lox*P-flanked allele are inherited from different parents.

To make evident the extent of this problem and that of possible infidelity of conditional gene targeting in general, we list relevant properties of some of the Cre transgenes frequently used in our and other immunological laboratories in **Table 1**.

Cre toxicity

Perhaps the most important potential problem in conditional gene-targeting experiments using Cre recombinase is that of Cre toxicity. Although most cre-transgenic mouse strains seem to develop normally, a study by Capecchi's group strikingly demonstrated that Cre can be toxic for cells, by attacking their genomic DNA. *Prm1* promoter controlled Cre expression in postmitotic spermatids led to immediate abortion of fertilized eggs with 100% penetrance, concomitant with massive DNA damage visualized as 'scrambled DNA' in chromosome spreads of male pronuclei[17].

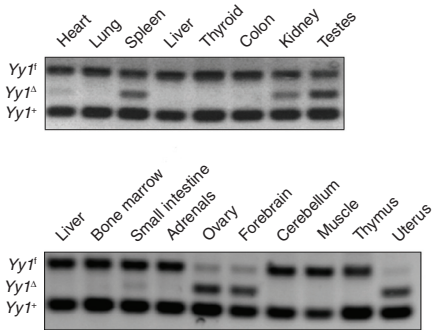

Figure 1 Recombination of a *lox*P-flanked *Yy1* allele effected by the CD21-Cre3A bacterial artificial chromosome transgene in CD21-Cre3A-transgenic mice heterozygous for the *lox*P-flanked *Yy1* allele. f, *lox*P-flanked; Δ, deleted; +, wild-type. Data courtesy of H. Liu and Y. Shi.

Expression of an enzymatically inactive Cre recombinase in similar amounts as wild-type Cre from the same transgenic construct had no effect, demonstrating that the DNA damage resulted from the recombinase activity of Cre.

Mammalian genomes contain cryptic or pseudo *lox*P sites, whose sequence can deviate considerably from the consensus *lox*P site, and which can serve as functional recognition sites for Cre[18]. A recent bioinformatics evaluation estimates that such sites are present in the mouse genome at a frequency of 1.2 per megabase[19]. Although the affinity of Cre for these cryptic recognition sites is much lower than for the consensus *lox*P site[18], DNA damage could arise from Cre-mediated recombination between cryptic *lox*P sites or as a by-product of an unproductive recombination event at a single cryptic *lox*P site when the cellular DNA repair machinery is hindered by Cre, which can form a covalent bond with DNA.

The toxic effects of Cre recombinase activity have been investigated carefully by various groups through retroviral infection of fibroblasts, in which the permanent presence of high levels of this protein induces growth arrest[20–23] and chromosomal abnormalities[21,23]. Transient expression of Cre using a replication-deficient adenovirus uncovered a large range of virus concentrations sufficient for excision of a *lox*P-flanked neomycin-resistance cassette without causing noticeable DNA damage in fibroblasts[24]. This suggests that when the exposure time to the recombinase is limited, Cre can be expressed in amounts that allow efficient recombination of *lox*P-flanked target alleles with minimal toxicity, at least in fibroblasts.

One strategy to minimize the exposure of the target cells to Cre is the use of self-deleting Cre expression vectors[20,21,25], which are flanked by *lox*P sites such that Cre recombinase

Table 1 Fidelity of Cre transgenic mouse strains commonly used in immunology

Cre strain	Desired recombination	Efficiency	Ectopic recombination		Original publication (ref.)
			Somatic cells	Germ cells	
CD19-Cre	B cells	55–95%	-	–	4
Mb1-Cre	B cells	>95%	T cells	Yes	14
CD21-Cre3A	Mature B cells	65–90%	Uterus, ovaries, kidney, testes, forebrain, heart	Yes	9
Cγ1-Cre	GC B cells	>90%	B1 cells[a]	Yes	12
CD4-Cre	T cells	>95%	Dendritic cells[b]	–	32
Lck-Cre	Thymocytes	>95%	Variable[c]	Yes	33
Mx-cre	Inducible	0–100%	Not applicable	–	34
R26-creERT2	Inducible	10–90%	Not applicable	–	29

GC, germinal center; R26, ROSA26

[a]So far only minor recombination of 1–2% have been observed in peritoneal B1 cells. [b]Up to 15% of CD11c$^+$ major histocompatibility complex class II–positive cells in CD4-cre-yellow fluorescent protein reporter mice were yellow fluorescent protein positive by flow cytometry (E. Derudder, unpublished data). [c]http://depts.washington.edu/immunweb/faculty/wilsonlab-reagent.html.

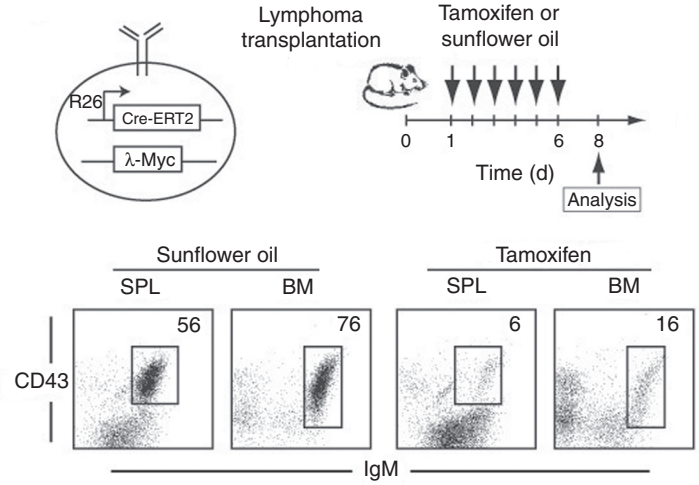

Figure 2 Induction of Cre-mediated recombination from the Cre-ERT2 transgene[29] leads to death of c-Myc-driven primary B cell lymphomas. Numbers above outlined areas (bottom row) indicate percent gated IgM$^+$CD43$^+$ B lymphoma cells among all B220$^+$ B cells in the spleen (SPL) and bone marrow (BM) of mice transplanted with λ-Myc lymphomas and treated with vector (sunflower oil) or 2 mg tamoxifen per day (time, arrow, top right). Tamoxifen treatment had no effect on c-Myc-driven B cell lymphomas lacking the Cre-ERT2 transgene (data not shown). R26, ROSA26 promoter; IgM, immunoglobulin M. Data courtesy of S. Raffel and S. Casola.

terminates its own expression. By using mutant *lox*P sites self-excision can be rendered less efficient than the recombination of the target gene. A second approach is to employ systems of inducible Cre expression. Various such systems have been described, with inducers ranging from type I interferon to tetracycline and tamoxifen[2]. Cre can also be directly delivered to cells bearing a *lox*P-flanked target gene through protein transduction[26,27], but this approach is limited to cell culture or adoptive transfer experiments. In the case of tamoxifen as an inducer, a fusion protein of Cre and the ligand binding domain of a steroid receptor, which keeps Cre in the cytoplasm in the absence of the steroid, is expressed from a transgene in a cell type–specific fashion. Mutant estrogen-binding domains that bind the estrogen analog tamoxifen but not endogenous steroids are mostly used in such experiments[2].

Loonstra and coworkers used fibroblasts expressing a fusion protein of Cre and the ligand-binding domain of the estrogen receptor (CreERT) under control of the endogenous ROSA26 promoter[23]. In this system, efficient excision of the *lox*P-flanked target gene without detectable growth arrest was achieved when the fibroblasts were cultured in low concentrations of the inducer tamoxifen for prolonged periods of time (up to 9 days), although complete excision could not be obtained without compromising fibroblast expansion. The authors also noted that in their system, both recombination and toxicity strongly correlated with cellular proliferation[23]. Interestingly, a positive correlation between proliferation and Cre-induced developmental abnormalities was also found in transgenic flies[28].

In our laboratory, S. Raffel and S. Casola attempted to use tamoxifen-induced CreERT2 (ref. 29) to assess the role of the B cell receptor in the maintenance and progression of c-Myc-transformed B cell lymphomas *in vivo*. These experiments were aborted when control experiments revealed that tamoxifen concentrations necessary for efficient target gene recombination were sufficient to completely wipe out the lymphomas via CreERT2 activity, even when the experimental design precluded B cell receptor ablation (S. Raffel, S. Casola and K.R.; **Fig. 2**). It is important to note that the toxicity toward the tumor cells was not due to tamoxifen *per se* but depended on the presence of CreERT2 (data not shown). Whether the ERT2 ligand-binding domain exacerbates the toxicity of the Cre fusion protein by inducing its recruitment into transcriptional complexes will need to be evaluated in the future.

Taken together, these observations clearly demonstrate that depending on its concentration, Cre can be a DNA-damaging agent and therefore toxic for mammalian cells. It seems surprising then that most cre-transgenic mouse strains are described in the literature as undistinguishable from wild-type mice. While it is possible that fibroblasts are more sensitive to Cre toxicity than other cell types such as lymphocytes[22], which have to deal with DNA breaks and nicks as an intrinsic part of their development and may therefore contain a more sophisticated repair machinery, it seems likely that many Cre-expressing mouse strains are not completely normal but may largely overcome Cre toxicity through developmental selection and adaptation processes.

Apart from the toxicity inherent to Cre recombinase, cre transgenes, like any other transgene, may change the expression of genes at and around the site of transgene integration. These alterations might be due to direct disruption of the sequences of these genes or to effects mediated by control elements of the transgenic vector such as promoters, enhancers and sequences regulating splicing and polyadenylation. Some cre-transgenic mouse strains exhibit clear phenotypic abnormalities. For instance, RIP-Cre mice, which are employed to evaluate gene function in pancreatic β–cells, are glucose intolerant[30]. It remains unclear whether this is due to high expression of Cre, transgene insertion effects or a combination of both. On the other hand, most mice generated via knock-in of Cre-encoding sequences into endogenous loci are heterozygous knockouts and gene-dosage effects can come into play. For example, compared to B cells from wild-type mice, B cells from hemizygous CD19-cre mice express half as much CD19. While on first approximation B cell development in these mice appears normal, they contain slightly reduced numbers of peritoneal B1 cells (unpublished observations). These data are in line with findings suggesting that variations in CD19 expression influence B cell selection and function[31].

Controlling conditional gene targeting experiments

We have discussed two basic aspects of conditional gene targeting using Cre-mediated recombination, namely the fidelity of Cre expression from a given Cre transgene and the potential toxicity of Cre recombinase. There are obvious ways to address these issues, which can be translated into critical controls.

The expression patterns of Cre transgenes are often more complex than anticipated, and strict cell type-specificity is the exception rather than the rule. While this problem can be easily assessed, it may nevertheless lead to situations in which a phenotype cannot be conclusively interpreted. For example, if the targeted mutation leads to induced activation of an oncogene or inactivation of a tumor suppressor, outgrowth of rare cells may ensue and it may be impossible to rigorously identify the origin of the selected cells in terms of cell lineage or developmental progression within a lineage. Another quite separate problem relates to the fact that inactivation of a gene does not coincide with the ablation of its product, be it RNA or protein. RNAs and proteins can differ dramatically in terms of their half-lives in cells

and can also maintain their function in the cell over very different ranges of concentrations. It is therefore important to assess the ablation of the target RNA or protein in cells in which Cre-mediated recombination is conditionally induced.

The problem of Cre toxicity, while amply documented in the literature, seems still to be widely ignored in the field. A recent comprehensive meta-analysis of the use of RIP-cre mice revealed that the majority of investigators (12 of 21) had not included the most crucial control, namely mice carrying the cre transgene but not its *lox*P-flanked target[30]. As elaborated above, this omission can lead to serious misinterpretations of experimental data.

While mice or cells expressing the cre transgene alone should be a routine control in conditional gene targeting experiments, such control experiments unfortunately do not completely resolve the issue. Cre toxicity may become apparent only through the introduction of a targeted mutation, which by itself may not have an effect. Thus, Cre-mediated inactivation of an antiapoptotic gene or of a gene involved in DNA repair may lead to apoptosis of the mutant cells because of their inability to deal with Cre toxicity. In the case of the introduction of oncogenic mutations, there is simply no way of excluding a contribution of Cre-mediated DNA lesions to the transformation of rare target cells. As discussed above, attempting to limit the exposure of the cells to Cre recombinase is clearly one worthwhile strategy, and rescue experiments reversing the targeted mutation can further help. But with some uncertainty we will have to live.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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