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Immunologic Applications of Conditional Gene Modification Technology in the Mouse

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Abstract

Since the success of homologous recombination in altering mouse genome and the discovery of Cre-*loxP* system, the combination of these two breakthroughs has created important applications for studying the immune system in the mouse. Here, we briefly summarize the general principles of this technology and its applications in studying immune cell development and responses; such implications include conditional gene knockout and inducible and/or tissue-specific gene over-expression, as well as lineage fate mapping. We then discuss the pros and cons of a few commonly used Cre-expressing mouse lines for studying lymphocyte development and functions. We also raise several general issues, such as efficiency of gene deletion, leaky activity of Cre, and Cre toxicity, all of which may have profound impacts on data interpretation. Finally, we selectively list some useful links to the Web sites as valuable mouse resources.

Keywords

Cre; *loxP*; conditional knockout; transgenic; bacterial artificial chromosome (BAC); hematopoietic cells; fate mapping; inducible gene expression; lymphocyte cell development and differentiation

GENERAL INTRODUCTION TO THE Cre/*LoxP* SYSTEM

By altering the genome at specific gene loci through homologous recombination in mouse embryonic stem (ES) cells (reviewed in Capecchi, 1989a,b), Mario Capecchi and Oliver Smithies pioneered the generation of genetically modified mice in the late 1980s and early 1990s, which resulted in their sharing of the Nobel Prize in 2007 with Martin Evans, who was the first to culture mouse ES cells (Evans and Kaufman, 1981). Since then, hundreds of labs have used such technology to generate a variety of mice, each carrying a modified allele of a particular gene, often referred to as germ-line knockout mice; such knockout mice have provided valuable tools in examining the functions of a particular gene both in vitro and in vivo. However, in some cases, knocking out of a gene in the mouse germ line leads to

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embryonic lethality or a severe developmental defect in early progenitors, preventing the researcher from studying gene function in mature cells. Therefore, a better gene manipulation technology is required.

Cre (Causes recombination), a 38-kDa integrase encoded by bacteriophage P1, mediates site-specific recombination between 34-bp sequences referred to as *loxP* (locus of crossover (x) in P1 bacteriophage) sites (reviewed in Sauer, 1998; Nagy, 2000). A *loxP* site is composed of a nonpalindromic 8-bp sequence (GCATACAT or ATGTATGC) flanked on either side by 13-bp inverted repeats (ATAACTTCGTATA; Hamilton and Abremski, 1984; Hoess et al., 1982). Cre-based recombination between the two *loxP* sites leads to a reciprocal exchange of DNA strands (Fig. 10.34.1). Cre-mediated recombination requires a minimum of 82 bp between two *loxP* sites for efficient recombination, though there is no upper limit (Hoess and Abremski, 1985). Indeed, high deletion efficiency by Cre is still observed between two *loxP* sites that are 400 kb apart (Nagy, 2000). The two *loxP* sites can be in the same orientation on the same chromosome or a different chromosome, or in the opposite orientation, thus leading to deletion, inversion, duplication, or translocation of chromosomes (van der Weyden et al., 2002; Branda and Dymecki, 2004). Sauer and Henderson (1998) created a Cre-expressing mouse cell line and showed that Cre-mediated site-specific recombination occurred in vivo, indicating that the prokaryotic Cre-*loxP* system can function in mammalian cells.

Rajewsky's group was the first to use the Cre-*loxP* technology in generating mouse models, including conditional inactivation of a target gene only in a selected cell population (Gu et al., 1993, 1994; Rajewsky et al., 1996). In a germ-line knockout strain, the target gene is inactivated in all cells throughout all developmental stages, whereas in conditional knockout, gene inactivation is either cell type specific or under temporal control. The specificity and timing of gene deletion are determined by the nature of Cre and its expression pattern. Up to now, there are more than 500 independent Cre mouse lines available (Nagy et al., 2009).

Not only Cre expression can be controlled by cell-type-specific regulatory elements or in an inducible way by tetracycline (or doxycycline; Gossen et al., 1995; Baron and Bujard, 2000; Bockamp et al., 2002) or by poly (I:C) through the production of endogenous interferon (note that this system may not be ideal for studying the immune system because of the involvement of type I interferon (Kuhn et al., 1995). Cre can also be engineered so that its activity is modulated by drugs (Metzger et al., 1995; Feil et al., 1996; Brocard et al., 1997). For example, the Cre protein can be fused to a mutant ligand-binding domain of the estrogen receptor that selectively binds to 17-β-estradiol analogs, e.g., tamoxifen, but not the endogenous estrogen (Feil et al., 1996; Brocard et al., 1997). The CreER fusion protein is normally present in the cytoplasm, but is translocated into the nucleus to induce gene excision upon the addition of the ligand. $CreER^{T2}$, a newer version of CreER, which is 10-fold more sensitive to 4-hydroxytamoxifen (OHT) than CreER (Indra et al., 1999). Therefore, the use of $CreER^{T2}$ is preferred.

Gene manipulation may be achieved in a spatiotemporally regulated manner by tissue-specific expression of inducible $CreER^{T2}$. Sometimes, Cre or $CreER^{T2}$ can be introduced

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into the cells by retroviral or adenoviral transduction methods (Rohlmann et al., 1996; Wang et al., 1996; Stec et al., 1999; Zhu et al., 2004). A cell-membrane-permeable form of Cre protein, Tat-Cre, has also been used to delete conditional alleles in vitro (Wadia et al., 2004). The conditional knockout of a gene in a particular cell, tissue, or organ at a specific time point not only avoids the early lethality or severe developmental consequences, if any, but also helps study cell-intrinsic gene functions in particular cell types at certain developmental stages without the interference of a systemic effect, which could be potentially caused by a global deletion or over-expression.

Other similar systems such as FLP-*FRT* are being used in genome engineering in ES cells and transgenic mice (Dymecki et al., 1996a,b). FLP is a 423-amino acid monomeric peptide encoded within the 2-μm yeast plasmid of *Saccharomyces cerevisiae* that uses phosphotyrosine for energy, whereas *FRT* is composed of an 8-bp asymmetric spacer (TCTAGAAA or TTTCTAGA) surrounded by 13-bp repeats (GAAGTTCCTATTC). The asymmetric region dictates whether excision or inversion occurs after recombination. Both Cre and FLP recombinases belong to the tyrosine site–specific recombinase class; thus, they act similarly. However, FLP-mediated gene deletion is less inefficient. Therefore, an enhanced form of FLP, FLPe, has been developed, which makes the FLPe-*FRT* system an alternative to the Cre-*loxP* system (Rodriguez et al., 2000). Before the FLPe-*FRT* system is used, three *loxP* sites are usually employed for making a conditional knockout mouse strain (Gu et al., 1994). A pair of *loxP* sites flanks the DNA sequence encoding a selection marker, whereas another pair flanks the DNA fragment of interest (the two pairs share one *loxP* site). The selection of Cre-mediated partial excision, by EIIa-Cre (Lakso et al., 1996) for example, to remove only the DNA fragment encoding the selection marker, is somewhat difficult, resulting in a lengthy process for generating a conditional allele. Nowadays, the Cre-*loxP* and FLPe-*FRT* systems are often combined for preparing targeting constructs in such a way that FLPe-*FRT* system is responsible for removing the selection marker, whereas the Cre-*loxP* system takes care of the DNA fragment under study. The International Knockout Mouse Consortium (IKMC) often utilizes such a combined strategy for generating new mouse lines.

Besides the Cre-*loxP* and FLPe-*FRT* systems, Dre-*rox*, a related recombinase-DNA pair, has been reported to work in mice (Anastassiadis et al., 2009). Cre mutants and chimeric Cre/FLP may offer another alternative strategy (Hartung and Kisters-Woike, 1998; Shaikh and Sadowski, 2000). In addition, besides inducing recombination between two *loxP* sites, Cre can induce specific recombination between two *lox511* sites; the *lox511* site is an alternative recognition site for Cre that has a different spacer sequence compared to the *loxP* site (Soukharev et al., 1999). Since recombination between *loxP* and *lox511* is very inefficient, the combination of *loxP* and *lox511* pairs has been used for site-specific gene insertion. There are other integrases, such as phiC31 and phiBT1, that belong to the serine site-specific recombinase class and induce directional rather than reversible recombination. These have been reported to function in eukaryotic genome engineering, such as in yeast, Drosophila, and mammalian cells (Thyagarajan et al., 2001; Groth et al., 2004; Keravala and Calos, 2008; Xu et al., 2008). These alternatives may create more applications for sophisticated gene manipulation in the mouse in the future.

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APPLICATIONS OF Cre-*LoxP*

Removal of the selection marker and generation of germ-line knockout

When a specific allele is targeted by an exogenous DNA fragment via homologous recombination, a selectable marker is required for efficiently obtaining the targeted allele. However, such a selectable marker sometimes affects the expression of the neighbor genes (Fiering et al., 1995), which may have related functions to the targeted gene. Therefore, in such cases, it is essential to remove the selectable marker by Cre-*loxP* or FLPe-*FRT* technology before proper investigation on the germ line knockout mouse line can be carried out. Furthermore, for generating a conditional knockout allele, the selection marker must be removed so that the allele flanked by two *loxP* sites (floxed) may function as a wild-type allele before its deletion. For this purpose, one may use a Cre or FLPe mouse line, in which Cre expression is under the control of CMV or β-actin promoters that are active in the germ line (Schwenk et al., 1995; Rodriguez et al., 2000). Such germ-line Cre mouse lines can also be used to generate the germ-line knockout allele from a conditional allele.

Conditional gene deletion

The Cre-*loxP* system has been widely used for conditional gene deletion in the mouse. The first step is to prepare a mouse model in which either the whole gene of interest or a critical gene segment is flanked by two *loxP* sites. The conditional mouse lines containing two *loxP* sites are usually designed to be wild-type mice. Because of such requirements, the selection of the sites to be inserted by *loxP* sequences is important. Usually, large introns with fewer regulatory elements are preferred; non-coding sequences that are conserved among species should be left intact. Occasionally, insertion of *loxP* sites into the gene locus may result in a hypomorphic allele.

Other conditional gene manipulations

A clever design of two *loxP* sites flanking a “STOP” cassette, which contains transcriptional and/or translational stop signals, has opened the door to generate many tools for studying the immune system. The general principle is that a *loxP*-STOP-*loxP* cassette is inserted in the front of a gene of interest so that this coded protein is only expressed when Cre deletes the *loxP*-STOP-*loxP* cassette. Depending on the nature of the gene following the STOP cassette, such technology has been utilized for different applications (Fig. 10.34.2). These applications include the following.

Conditional transgenic gene expression—One can insert a gene to be overexpressed downstream of the *loxP*-STOP-*loxP* cassette and then prepare a mouse line. Such a mouse line will not express the transgene until Cre removes the STOP cassette in a tissue-specific or inducible manner. However, once Cre activates the transgene, it is irreversible.

Fate mapping—Because of the irreversible nature of the gene-segment deletion by Cre, the *loxP*-STOP-*loxP* has been used for cell-fate mapping (Jacob and Baltimore, 1999). The commonly used line for immunology research is the *loxP*-STOP-*loxP* conditional reporter line, including PLAP (Jacob and Baltimore, 1999), *lacZ*, *GFP*, and *YFP*, etc., integrated at the *Rosa26* locus (Mao et al., 1999; Soriano, 1999; Novak et al., 2000; Srinivas et al., 2001).

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If a combination of different colors is needed (i.e., with an additional GFP as another marker), other mouse strains such as the ROSA26-STOP-tdTomato line (Madisen et al., 2010), expressing a red fluorescent protein upon Cremediated STOP removal, can be used for fate-mapping studies to minimize the compensation problems.

Cell-type ablation—When the gene following the *loxP*-STOP-*loxP* cassette is a toxic protein, such as diphtheria toxin fragment A (DTA), the resulting mouse line can be used for specific cell–type ablation (Ivanova et al., 2005). Similarly, when diphtheria toxin receptor (DTR) is Cre-inducible and Cre expression is under the control of lineage-specific promoters, Cre-expressing cells can be efficiently eliminated by diphtheria toxin (DT) treatment (Buch et al., 2005). Alternatively, cell ablation can be achieved by inducing a cell surface marker, such as Thy1.1, which can be recognized by a depleting antibody (clone 19E12).

Applications combining the Cre-loxP system and TetR-inducible system

As discussed above, the Cre-*loxP* system is used for a conditional knockout or conditional transgenic so that gene expression is manipulated in a tissue-specific and/or temporally controlled manner. When Cre is fused to ER^{T2}, its activity can be further regulated by tamoxifen. However, Cre-mediated gene deletion or activation is irreversible. Because of this feature, the Cre-*loxP* system is very useful for cell-fate mapping. However, it is not feasible to use this system to turn a particular gene on and off on demand.

The *Escherichia coli* tetracycline repressor (TetR)-based system (Gossen and Bujard, 1992; Gossen et al., 1995; Baron and Bujard, 2000), on the other hand, regulates gene expression in a reversible way (Felsher and Bishop, 1999). TetR proteins bind to antibiotic tetracycline or its derivatives such as doxycycline (Dox); Dox is much more cost effective, available, and efficient in regulating TetR proteins than tetracycline. TetR proteins also bind to the 19-bp operator sequences (tetO), which are built into the promoter of a transgene so that its expression is regulated by Dox and TetR proteins. There are “tet-off” and “tet-on” systems mediated by tetracycline-controlled transactivator (tTA) and reverse tTA (rtTA), respectively. When the tTA protein binds to Dox, it can no longer bind to DNA to activate gene expression (“tet-off”); on the other hand, rtTA only binds to the DNA and activates gene expression in the presence of Dox (“tet-on”).

Due to unique features of the Cre-*loxP* and the TetR-based systems, they can be combined for generating useful research tools (Belteki et al., 2005). When the *loxP*-STOP-*loxP* cassette is placed upstream of the DNA sequences encoding a TetR protein, the expression of tTA or rtTA will be regulated by a tissue-specific Cre. If the Cre is in an inducible form, such as $CreER^{T2}$, the expression of TetR protein can then be limited to the specific tissue cells that are already developed during the period of tamoxifen treatment. Therefore, it may be useful for studying gene functions in fetal cells versus adult cells. If a TetR protein, whose expression is controlled by Cre, is designed to induce a transgene, and at the same time, the endogenous counterpart gene is flanked by two *loxP* sites, the combination of four modified alleles (tissue-specific Cre or inducible Cre; floxed endogenous gene; *loxP*-STOP-*loxP* restricted TetR; and TetR-inducible transgene) offers a perfect system to perform a

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temporal gene rescue experiment after gene deletion in a specific tissue. Since the expression of the transgene is titratable by Dox, this system may be best suited for studying dose effect of a particular gene in vivo.

Conversely, Cre expression can be under the control of the TetR system, and TetR expression may be designed to be tissue specific. This combination is identical to a tissue-specific CreER^{T2}, but due to the titratable nature of the TetR system, the activity and toxicity of the Cre is better controlled.

TISSUE-SPECIFIC CRE TRANSGENIC MICE

Most of the existing Cre mouse lines can be found at the CREATE (Coordination of resources for conditional expression of mutated mouse alleles) consortium (http://creline.org/), which includes the Cre mouse database at Mouse Genome Informatics (MGI, http://loxP.creportal.org/). Some commonly used Cre mice for studying the immune system will be briefly discussed below and summarized in Table 10.34.1.

Lymphocyte development and differentiation is an extensively studied area in the immunology field. T cells undergo multiple stages of development in the thymus, and these cells then further differentiate into effector cells in response to antigens in the periphery. Thus, T cell development and differentiation provide a perfect model for investigating fundamental immunological questions.

To apply conditional gene modification technology in T cell research, multiple Cre lines have been prepared to serve different purposes. The Lck-cre mouse line (Lee et al., 2001) is a commonly used tool for studying gene functions during early T cell development. The disadvantage of this Cre model is that the deletion efficiency is low and varies from mouse to mouse.

CD4-cre line (Lee et al., 2001) is frequently used for general purposes due to its high deletion efficiency. However, since CD4-Cre is turned on from late CD4/CD8 double negative (DN) stage and fully expressed at CD4/CD8 double positive (DP) stages, it is not an ideal model for studying early T cell development. Even for T cell development from DP to CD4 or CD8 single positive (SP) mature T cells, caution should be taken since the half-life of a particular mRNA and its protein product may be long enough to obscure the results. On the other hand, deletion of a particular gene by CD4-Cre may result in developmental defects or a complete development block. In such cases, other Cre lines are needed for studying gene functions in mature T cells.

Cre expression driven by the distal promoter of Lck (dLck-cre) becomes an important model to avoid thymic development problems. The dLck-Cre (line 3779) starts to be expressed at the late DP stage and reaches high activity at the SP stage (Wang et al., 2001). Therefore, one may avoid T cell developmental blockage and obtain na¨ıve mature CD4 T cells. For example, *Gata3*$^{fl/fl}$-CD4-Cre mice display severe blockage from DP to SP CD4 T cells (Zhu et al., 2004); residual CD4 T cells developed in these mice show a highly activated phenotype. However, *Gata3*$^{fl/fl}$-dLck-Cre mice show normal T cell development; up to 70% to 80% of na¨ıve CD4 T cells harvested from these mice have gene deletion (Yagi et al.,

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2010); deletion efficiency of dLck-Cre in mature CD8 T cells is somewhat higher, but dLck-Cre does not seem to be active in regulatory T cells. For studying gene functions specifically in regulatory T cells, the Foxp3-YFPCre mouse line is the best choice (Rubtsov et al., 2008).

An alternative choice is to use OX40-cre for studying peripheral T cells (Zhu et al., 2004; Klinger et al., 2009). However, OX40 is up-regulated by TCR-mediated signaling, and its maximum expression is on day 2 to day 3 after T cell activation. Thus, OX40-cre has limitations because it is not useful for studying early events during T cell differentiation. In addition, OX40-Cre is active in regulatory T cells, even in the thymus. Although OX40 is induced by TCR in CD8 T cells, the deletion efficiency in these cells is relatively lower compared to that in activated CD4 T cells. For studying gene function in activated CD8 T cells, granzyme-B-Cre (Jacob and Baltimore, 1999) can be used, although this Cre also deletes floxed genes in activated CD4 T cells.

CD4-Cre deletes floxed sequences in both CD4 and CD8 T cells. To delete floxed genes only in peripheral CD8 but not CD4 T cells, CD8a–Cre (Maekawa et al., 2008), also known as E8I–Cre, may be used. There are several Cre lines, such as Mb1-Cre (Hobeika et al., 2006; expressed at pre-pro B stage) and CD19-Cre (Rickert et al., 1997; expressed at pro-B stage), designed for studying gene functions in B cell development as well as in effector B cells. CD2-Cre (Zhumabekov et al., 1995) mediates DNA excision at the common lymphoid progenitor (CLP) stage, and thus may be used for studying gene function in both T and B cells (de Boer et al., 2003). Other Cre lines have been used for studying gene regulation in innate lymphoid cells (ILCs; Rawlins et al., 2009), macrophages (Clausen et al., 1999), and dendritic cells (DCs; Caton et al., 2007). All the hematopoietic cells are developed from hematopoietic stem cells (HSCs). To study gene functions in hematopoietic lineages, one may consider Vav-Cre (de Boer et al., 2003).

As mentioned above, when Cre is fused to a mutant estrogen receptor (ER^{T2}), its activity can be modulated by addition of the estrogen receptor ligand tamoxifen. A mouse line carrying $CreER^{T2}$ under the control of the *Rosa26* locus has been prepared (Seibler et al., 2003). Upon tamoxifen treatment, $CreER^{T2}$ will delete the floxed gene with high efficiency in most tissues except in the brain. Since the deletion is temporally controlled, this method is useful for studying gene function after cells have developed, and for following the cell fate of certain cells that are present at the time of tamoxifen treatment. However, since $CreER^{T2}$ expression is rather ubiquitous, there is a certain limitation to this model. Recently, many groups have generated tissue-specific $CreER^{T2}$ mouse strains, such as CD4-$CreER^{T2}$ and CD19-$CreER^{T2}$, which can be used for inducible deletion of floxed genes specifically in T cells and B cells, respectively (Boross et al., 2009; Aghajani et al., 2012). However, the deletion efficiency is somewhat compromised. Rudensky's lab has developed a Foxp3-GFP-CreERT2 mouse line for the purpose of inducible inactivation of floxed gene in Tregs (Rubtsov et al., 2010). It is a Cre, ERT2, and GFP triple fusion protein. Although it has the advantage of being able to show Cre-expressing cells by GFP, this fusion may further reduce the deletion efficiency. Using the 2A peptide technology (Ryan et al., 1991; Donnelly et al., 2001; Szymczak et al., 2004; Szymczak and Vignali, 2005), our lab has recently developed a Tbx21-ZsGreen-T2A–$CreER^{T2}$ mouse line in which an improved version of GFP (ZsGreen) and $CreER^{T2}$ is connected by a 2A peptide; thus, ZsGreen marks T-bet-expressing cells and

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CreER^{T2} can efficiently delete floxed genes in these cells upon tamoxifen treatment (unpublished data). There are many more tissue-specific CreER^{T2} mouse lines. A database dealing with CreER^{T2} resource created by the Institut Clinique de la Souris (ICS; Illkirch-Strasbourg, France) can be found at http://www.ics-mci.fr/mousecre/.

THINGS TO BE CONSIDERED

Deletion efficiency

An important issue to be considered for conditional knockout using the Cre-*loxP* system is the deletion efficiency. The efficiency is determined by several factors, including Cre activity, the nature of floxed genes, and the function of the floxed genes in cell proliferation and/or survival. The expression levels of a tissue-specific Cre are determined by the activity of similar regulatory elements that also control an endogenous lineage-specific gene. If the Cre expression level is not high enough to mediate recombination, it is possible to modify these regulatory elements during vector construction (such as to remove a silencer); however, such modifications may also result in the loss of cell specificity in Cre expression. A humanized Cre (hCre or iCre), which exhibits higher activity than regular Cre in mammalian cells, has been utilized to improve deletion efficiency (Shimshek et al., 2002). In the case of BAC transgenic–driven Cre expression, there is an opportunity to select for high copy numbers within the transgenic founders. However, the higher the Cre expression, the higher the cell toxicity that may be noticed, while the deletion efficiency is improved.

Gene deletion efficiency is also determined by the nature of the floxed gene to be deleted. Even using a same Cre line, the deletion efficiency may vary from gene to gene. Although it has not been carefully assessed, several possibilities may explain such variation: e.g., epigenetic modifications and DNA looping at the floxed genes may affect Cre-mediated recombination; or if *loxP* sites are inserted near a transcription factor binding element, the accessibility of Cre protein to the *loxP* sequences may be altered. Therefore, it is difficult to predict the exact deletion efficiency for a particular Cre-*loxP* combination.

If the floxed gene is crucial for the development of a specific cell type and this cell type has capacity to undergo tremendous cell expansion before its maturation, it will appear that the deletion efficiency is extremely low due to a counter selection. Similarly, if the gene to be deleted plays a critical role in cell proliferation and/or survival in mature cells, cells that escape deletion may preferentially expand. For example, GATA3 is important for the expansion of Th2 cells. Retroviral expression of Cre resulted in deletion of the floxed Gata3. However, when such cells were maintained in culture for a substantial length of time, i.e., 2 to 3 weeks, cells that had not deleted the *Gata3* dominated the culture.

Therefore, deletion efficiency is a major concern in using conditional knockout mice. It always a good idea to check deletion efficiency at different stages of development either by genotyping or by single-cell analysis, such as flow cytometry, to make sure that the phenotype observed is consistent with the genotype. In some cases, the alteration in phenotype may be underestimated because the cells that have not undergone gene deletion are also included in the analysis.

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Leaky deletion in the germ line

Conditional deletion of a floxed gene segment is dependent on tissue specificity of the Cre expression. Sometimes, Cre expression is leaky, thus resulting in widespread recombination. Therefore, it is not uncommon that a “lineage-specific” Cre line may occasionally delete floxed genes in germ line, and such a knockout allele may then heritably remain in the offspring. This occurs possibly because many cell type–specific genes are transiently expressed during early embryogenesis. Therefore, one should exclude the possibility of germ-line deletion when studying conditional knockout mice. Several lines have been reported to delete genes in the germ line despite the fact that they are designed to be cell-type specific. For example, OX40-Cre, if carried by male mice, may delete floxed gene in the germ line of some offspring. Thus, it is recommended that OX40-Cre should be carried by the female parent. In other cases, the Cre gene may need to be carried by the male parent. It has been noticed that Vav-Cre often induces gene deletion in the germ line possibly due to its expression in the testis and ovaries (de Boer et al., 2003). Therefore, when a new Cre line is established or used, it is better to test whether the female or male mouse is better suited to carry the Cre for breeding purposes. Furthermore, each parent should be screened for germ-line deletion when they are set up for further breeding.

Copy number matters

In some cases, DNA containing *loxP* sites is introduced into the mouse by transgenic technology, which will generate founders that carry an insertion of the DNA fragments in multiple copies. However, to assess the effect of Cre-mediated gene excision between two *loxP* sites, selection of a single copy is required. Otherwise, the observed effect may be resulted from the deletion of excessive copies rather than the deletion of flanked sequence. In addition, some BAC vectors contain a *loxP* site. If the vector sequence is not completely removed before microinjection, the resulting BAC transgenic lines may carry several *loxP* sequences, depending on the copy numbers. Upon Cre activation, the transgene, which is expressed with multiple copies, may no longer be expressed when it becomes a single copy since the remaining copy could represent a partial integration.

Cre toxicity

Cre has cell toxicity when it is expressed at high levels (Loonstra et al., 2001). Pseudo-*loxP* sites are present in the mouse genome, and Cre-mediated recombination between these pseudo-*loxP* sites may occur with low efficiency (Thyagarajan et al., 2000). However, when a codon-optimized Cre with a nuclear localization signal is highly expressed, cell toxicity becomes a major concern. Therefore, it is crucial to include proper controls, i.e., Cre^+ mice without floxed genes, when the Cre-*loxP* system is utilized to study cell survival and proliferation. To minimize the Cre toxicity, it is better to control the Cre expression or activity in an inducible manner. Alternatively, a self-inactivating Cre, i.e., *Cre* gene flanked by two *loxP* sites, may be considered.

USEFUL RESOURCES AND DATABASES RELATED TO MOUSE LINES

The International Knockout Mouse Consortium (IKMC, http://www.knockoutmouse.org/) provides a large collection of mice that carry mutant protein-coding genes. The mouse

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models have been and/or are being prepared through gene trapping and/or gene targeting in ES cells derived from the C57BL/6 mouse strain. A substantial number of the genes in the mouse genome have been targeted, many of which have conditional knockout potential. The IKMC includes the following four programs: Knockout Mouse Project (KOMP) in the U.S. (http://www.nih.gov/science/models/mouse/knockout/); European Conditional Mouse Mutagenesis Program (EUCOMM) in Europe (http://www.knockoutmouse.org/about/eucomm); North American Conditional Mouse Mutagenesis Project (NorCOMM) in Canada (http://www.norcomm.org/index.htm); and Texas A&M Institute for Genomic Medicine (TIGM) in the US (http://www.tigm.org/).

International Mouse Strain Resource (IMSR, http://www.findmice.org/index) and Mouse Genome Informatics (MGI, http://www.informatics.jax.org/) are two valuable resources for finding mutant mice that were either published or deposited into public repositories.

The CREATE (Coordination of resources for conditional expression of mutated mouse alleles) consortium (http://creline.org/) provides a resource for Cre mouse strains. It includes four international databases: CRE mouse database at MGI (http://www.creportal.org/); CRE-X-Mice (http://nagy.mshri.on.ca/cre/); Crezoo at Fleming (http://bioit.fleming.gr/crezoo/); and MouseCre (http://www.ics-mci.fr/mousecre/).

CONCLUSIONS

The conditional gene modification technology, especially the inducible Cre-*loxP* system, has created tremendous opportunities and tools for immunologists to study the principles of the immune systems in the mouse. Now, we have many choices to inactivate or overexpress a specific genes in a tissue-specific and/or temporally controlled manner. Furthermore, we often apply this technology to study lineage relationship and cell-fate mapping. With the advance of new technologies in gene targeting, such as TALEN technology (reviewed in Carlson et al., 2012), it becomes much easier and faster to generate genetically modified alleles. Research in the immunology field will benefit greatly from the development of new tools and technologies.

With the expansion of our knowledge, our questions become more and more sophisticated and precise. For example, we have now realized that some particular cell types can only be defined by a combination of two markers. Thus, it is challenging to manipulate gene expression in these cells in a lineage-specific manner. A plausible solution is to construct the locus of one marker to control FLP expression and the locus of another marker to control *FRT*-STOP-*FRT*-Cre expression. Therefore, only in cells simultaneously expressing both markers will the conditionally modified gene locus flanked by two *loxP* sites or the transgene following a *loxP*-STOP-*loxP* cassette undergo Cre-mediated recombination. With utilization of other, similar recombination systems in combination with doxycycline-inducible systems, gene manipulation specifically in cell subsets co-expressing three or more markers is also possible. Finally, many biological processes are regulated by a combination of several factors in a quantitative way. Thus, combining gene knockout with inducible but titratable gene rescue will be an important future application of conditional gene manipulations in the mouse.

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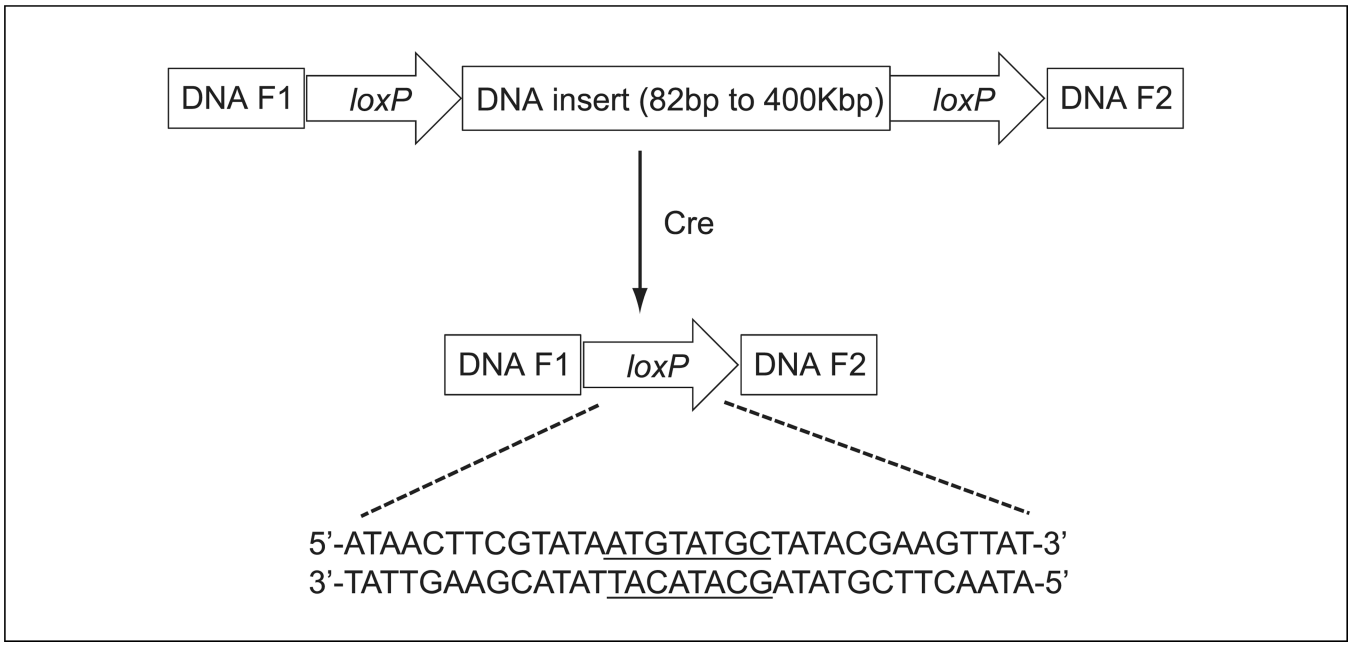

Figure 10.34.1.

Principle of Cre-mediated deletion of DNA that is flanked by two *loxP* sites. DNA insert with its size from 82 bp up to 400 kbp between two *loxP* sites in a same direction is deleted by Cre-mediated DNA recombination. The full DNA sequence of a *loxP* site is shown.

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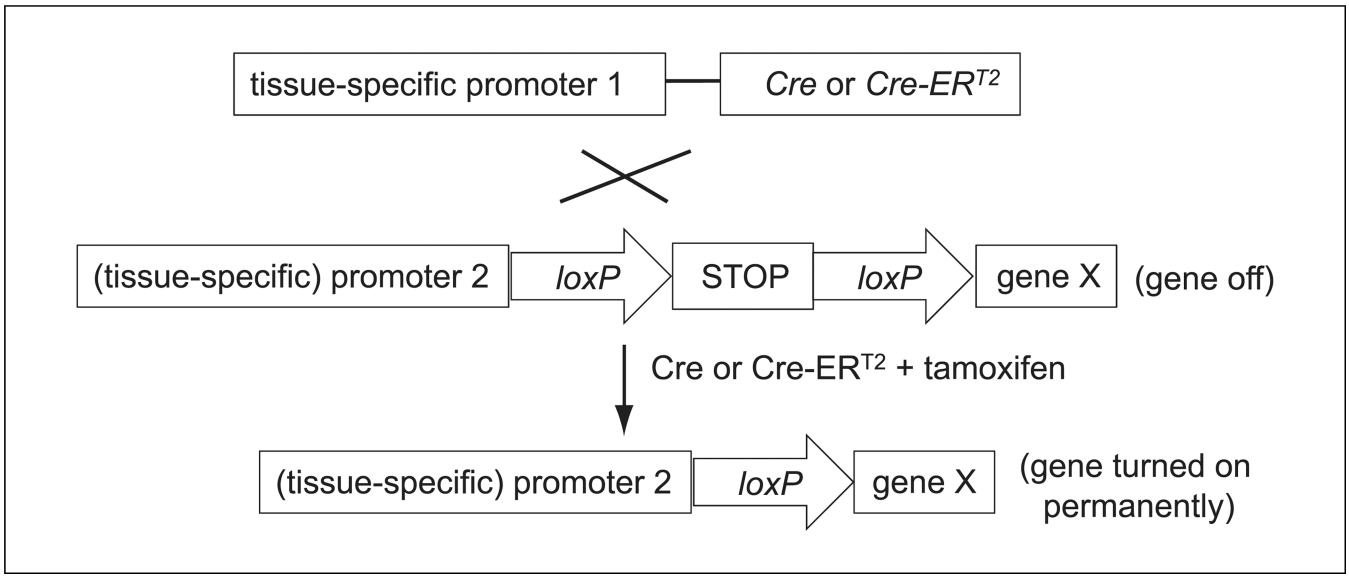

Figure 10.34.2.

Applications for the combination of Cre (or inducible Cre) with *loxP*-STOP-*loxP* cassette in front of a gene X. The expression of Cre or inducible Cre such as Cre-ERT2 is usually under the control of a tissue-specific promoter, whereas gene X is under another promoter (tissue-specific or not), but the expression of the gene X is blocked by the *loxP*-STOP-*loxP* cassette until the cassette is removed by Cre-mediated recombination. The system can be used for conditional gene induction if X encodes a general protein, for fate-mapping if X encodes a marker, or for selected cell ablation if X encodes a toxin.

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Table 10.34.1

Commonly Used Cre Mouse Lines for Studying Lymphoid Cells

Name	Tg/KI	Expression in cell types	Note	Reference
ROSA26-CreER^{T2}	KI	Most cells except those in the brain	High deletion efficiency with tamoxifen treatment both in vitro and in vivo	Seibler et al. (2003)
Vav-Cre	Tg	All hematopoietic lineages, testis and ovaries	High deletion efficiency; may cause germ line deletion in some offspring	de Boer et al. (2003)
CD2-Cre	Tg	Common lymphoid progenitors (CLPs)	High deletion efficiency; some modified CD2-Cre lines may only delete genes in T cells but not B cells	Zhumabekok et al. (1995); de Boer et al. (2003)
Lck-Cre	Tg	Early DN stage in the thymus	Deletion efficiency varies	Lee et al. (2001)
CD4-Cre	Tg	Late DN to DP stage, deleting floxed genes in both CD4 and CD8 T cells	High deletion efficiency	Lee et al. (2001)
CD4-CreER^{T2}	Tg	Deleting floxed genes only CD4 but not CD8 T cells in the periphery	Inducible by tamoxifen; deletion efficiency up to 80% in vivo	Aghajani et al. (2012)
dLck-Cre (line 3779)	Tg	Late DP to SP stage	—70% deletion efficiency in CD4 T cells; higher efficiency (80% to 90%) in CD8 T cells; very low in Tregs	Wang et al. (2001)
OX40-Cre	KI	Tregs and activated CD4$^+$ T cells	Endogenous OX40 gene is disrupted by Cre; very low efficiency in activated CD8 T cells	Yagi et al. (2010)
CD8a–Cre	Tg	Mature CD8$^+$ but not CD4$^+$ T cells	Also known as E8I–Cre; Cre expression driven by the core E8I enhancer and Cd8a promoter	Maekawa et al. (2008)
Granzyme-B-Cre	Tg	Activated CD4$^+$ and CD8$^+$ T cells	Cre driven by truncated granzyme B promoter	Jacob and Baltimore (1999)
Mb1-Cre	KI	Starting from Pre-Pro-B stage	Endogenous *Mb1* gene encoding Igα signaling subunit of the BCR is disrupted by Cre; deletion efficiency is better than CD19-Cre	Hobeika et al. (2006)
CD19-Cre	KI	Starting Pro-B stage	Endogenous *Cd19* gene is disrupted by Cre; deletion efficiency is 75% to 95%	Rickert et al. (1997)
CD19-CreER^{T2}	BAC Tg	Similar to CD19-Cre, but its activity requires tamoxifen treatment	Inducible by tamoxifen; deletion efficiency 25% to 60%	Boross et al. (2009)
Foxp3-YFPCre	KI	Only in Foxp3$^+$ Tregs	YFP is dim; endogenous Foxp3 expression intact	Rubtsov et al. (2008)
Foxp3-GFPCreER^{T2}	KI	Only in Foxp3$^+$ Tregs	Inducible but with low deletion efficiency (10% to 20%); endogenous Foxp3 expression intact	Rubtsov et al. (2010)
Id2-CreER^{T2}	KI	Id2-expressing cells: epithelial cells in the lung distal tips as well as progenitor of ILCs and T cells	Inducible but with low deletion efficiency; endogenous *Id2* gene is disrupted by CreER^{T2}	Rawlins et al. (2009)