

Gene targeting: Things go better with Cre

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New technologies are changing the way in which gene targeting experiments are being designed. It is now becoming possible to analyze gene function in defined tissues at specific times during the life of a mouse.

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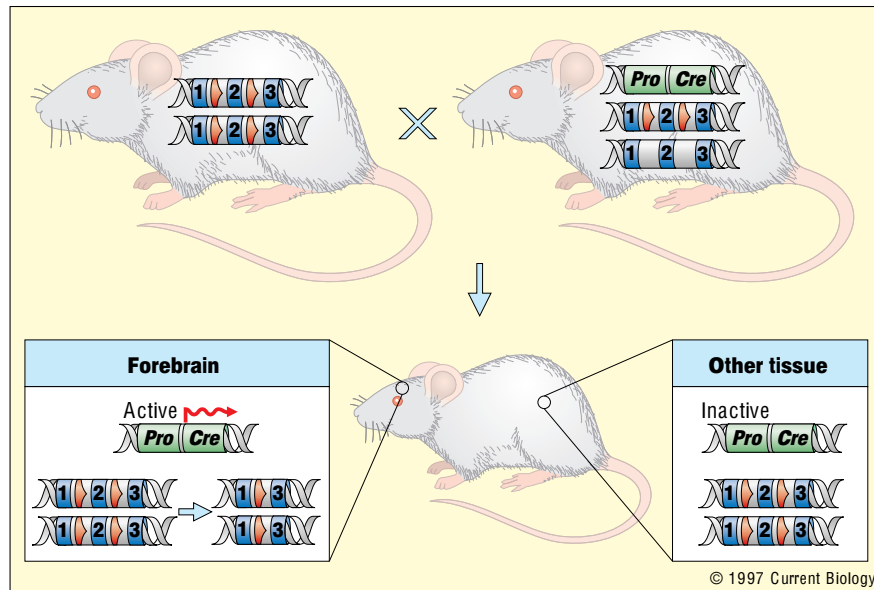
In merely a decade since the first report of targeted gene disruption in mouse embryonic stem (ES) cells [1], hundreds of genes have been mutated using gene targeting techniques. Targeted gene disruption has become so routine that new strains of ‘knockout’ mice are being generated at the rate of at least several hundred per year. A large number of these carry null mutations that lead to obvious developmental defects and frequently cause early embryonic lethality. While these mutations have provided valuable information on gene function in embryonic development, and in some cases on the molecular nature of human inherited diseases, when mutations cause gross developmental defects or early embryonic lethality it is not possible to analyse gene function during later embryonic and postnatal stages. Recently, it has been demonstrated that the Cre/*loxP* site-specific recombination system can be used to mutate genes, and thus analyse their function, at a single developmental stage or in specific cell types or tissues.

Cre is a bacteriophage P1-encoded recombinase that catalyzes sequence-specific DNA recombination between two 34-base-pair repeats, termed *loxP* sites. While Cre’s natural function is to dissociate bacteriophage dimers following replication, it has been shown, in both cultured mammalian cells and transgenic mice, to excise efficiently any DNA flanked by two directly repeated *loxP* sites. In their pioneering study, Rajewsky and colleagues [2] demonstrated that transient Cre expression in ES cells efficiently deletes the DNA between two *loxP* sites that had been incorporated into the genome by homologous recombination. They went on to develop a ‘conditional’ knockout strategy, showing that transgenic mice carrying the Cre gene under the control of a T-cell-specific promoter could delete genomic DNA flanked by two *loxP* sites (termed ‘floxed’) specifically in the T-cell lineage [3]. Although the expected recombination event was not complete in the target tissue, this report demonstrated the feasibility of conditionally inactivating genes in specific cell lineages.

In principle, Cre/*loxP*-mediated gene targeting permits the inactivation of any gene in any tissue at any stage of embryonic or postnatal life (Fig. 1). That substantial progress has already been made in this direction is indicated in three recent papers by Tonegawa and colleagues [4–6], describing the construction and analysis of transgenic mouse lines exhibiting subregion-restricted gene inactivation in the brain. Tsien *et al.* [4] used the promoter from the α -calcium-calmodulin-dependent kinase II (α CaMKII) gene to drive Cre expression exclusively in the forebrain. These animals were then crossed to ‘reporter’ mice containing a *lacZ* gene under the control of the β -actin promoter; transcription and translation of *lacZ* were prevented by an inserted stop sequence, which was flanked by *loxP* sites. Using conditional activation of the *lacZ* reporter transgene — by deletion of the stop sequences — as a functional assay of Cre-mediated recombination, Tsien *et al.* showed that α CaMKII-promoter-driven Cre expression led to activation of *lacZ* expression almost solely in pyramidal cells of the CA1 region of the hippocampus.

Several features of the *lacZ* reporter transgene activation were of particular interest [4]. Analysis of the time-course of *lacZ* activation revealed that Cre-mediated deletion of the stop sequences in the *lacZ* reporter did not occur until the third postnatal week. At this time, neurons in the CA1 region of the hippocampus are in a highly differentiated, postmitotic state. The recombination event was also highly efficient, as close to 100% of the neurons in the CA1 region expressed *lacZ*. This study demonstrated, therefore, that Cre-mediated recombination can occur efficiently in highly differentiated, postmitotic cells. This is an important point to establish, as it greatly increases the potential utility of the technique. Only further work can establish whether Cre-mediated recombination can occur efficiently in all postmitotic cells.

Tsien *et al.* [5] then used these α CaMKII-Cre transgenic mice to study the effects of region-restricted inactivation of the *N*-methyl-D-aspartate receptor 1 (*NMDAR1*) gene, which encodes an essential subunit of a postsynaptic ion channel. They crossed the α CaMKII-Cre transgenic mice to mice containing a floxed *NMDAR1* gene, ultimately generating animals heterozygous for the α CaMKII-Cre transgene and homozygous for the floxed *NMDAR1* gene. Whereas mice homozygous for a conventional knockout of the *NMDAR1* gene die neonatally [7,8], mice in which the *NMDAR1* gene is inactivated specifically in CA1 cells — henceforth abbreviated ‘CA1 KO’ mice — grow into adulthood with no obvious pathologies or behavioral

Figure 1

Cre/loxP-mediated gene targeting in transgenic mice. In this model, the expression of Cre is driven by a promoter (*Pro*) that is only active in the forebrain. *loxP* sites (red triangles) have been introduced into introns between exons 1, 2 and 3 of a target gene by conventional homologous recombination (see [3] for details). Mice homozygous for this allele are mated to mice heterozygous for this allele that also contain a Cre-expression transgene (*Pro-Cre*). Some of the progeny of this mating will be homozygous for the *loxP*-containing allele and heterozygous for the *Cre* transgene. These animals will undergo Cre-mediated deletion of DNA between the *loxP* sites in the forebrain. In tissues where the *Pro-Cre* transgene is inactive, no deletion occurs. (Note that, in the *Cre*-transgene parent, the *loxP*-containing allele will also be deleted in the forebrain.)

abnormalities [5]. The CA1 KO mice did, however, exhibit impaired spatial memory, although their performance in nonspatial learning tasks was unimpaired. These results support the view that NMDA receptor-dependent long term potentiation in the CA1 region is crucial for the formation of certain types of memory.

McHugh *et al.* [6] studied freely-moving CA1 KO mice by sophisticated multielectrode recording, in the hope of gaining further insight into the role of synaptic plasticity in hippocampus-dependent spatial learning. Their results indicated that NMDA receptor-mediated synaptic plasticity is necessary for the proper representation of space in the CA1 region of the hippocampus. The experiments described in these two papers [5,6] illustrate the value of region-restricted inactivation of gene expression, as none of the findings obtained with the CA1 KO mice could be studied in animals with a conventional knockout of the *NMDAR1* gene, as they die soon after birth.

While *Cre/loxP*-mediated conditional gene targeting has clear advantages over conventional gene targeting in analyzing gene function, its current use is limited by the availability of well-characterized tissue- or cell-type-specific promoters for making Cre transgenic lines. The versatility of *Cre/loxP*-mediated conditional gene targeting would be increased if it could be made inducible, as well as tissue-specific or region-specific. Two groups have described the successful use of inducible gene targeting systems in mice. Kühn *et al.* [9] used an interferon-responsive promoter from the *Mx1* gene to drive expression of the Cre recombinase. These *Mx1*-Cre mice were then crossed to mice containing a floxed DNA polymerase β

gene. Efficient deletion of the floxed gene was reported in a number of tissues of induced mice, although the rate of background recombination in untreated mice was quite high in some tissues, perhaps due to the endogenous production of interferon in the mice.

Another system for the inducible control of gene expression in transgenic mice is the tetracycline (*tet*) regulatory system developed by Bujard and colleagues [10,11]. The *tet* regulatory system is adapted from the tetracycline-resistance operon of the bacterium *Escherichia coli*, and has been modified for transcriptional activation upon the administration of the antibiotics tetracycline or doxycycline. The *tet* activation cassette can be placed under the control of an organ-specific promoter to obtain tetracycline-dependent, organ-specific transcriptional activation [11]. Thus, it should be possible to combine the *tet* regulatory system with tissue-specific or cell-type-specific promoters to obtain Cre-mediated gene inactivation that is controlled both spatially and temporally.

A second example of inducible gene targeting has been described recently [12]. As the fusion of the ligand-binding domain of steroid hormone receptors to a variety of heterologous proteins can render the activity of these chimeric proteins hormone-dependent, Feil *et al.* [12] designed a chimeric Cre protein whose activity is dependent on a synthetic ligand. They fused a mutated ligand-binding domain of the human estrogen receptor to the Cre recombinase and showed that, when expressed in transgenic mice under the control of a constitutive promoter, the chimeric Cre protein is activated only by the synthetic ligand 4-hydroxytamoxifen, but not by endogenous estradiol. Administration of

4-hydroxytamoxifen induced Cre-mediated excision of a floxed target gene in tissues where Cre was expressed, demonstrating the feasibility of this strategy for inducible gene inactivation. This strategy could be combined with a specific promoter to achieve both tissue-specific and temporally controlled gene inactivation.

Many technical improvements remain to be made in the various Cre-mediated gene inactivation schemes. In most cases reported to date, the efficiency of deletion of floxed genomic DNA is substantially less than 100%, leading to a mosaic inactivation of the target gene. At present, therefore, conditional gene inactivation may be most informative with genes encoding proteins that function cell-autonomously — such as transmembrane receptors, transcription factors and other intracellular proteins. The conditional inactivation of genes whose products function non-autonomously — such as ligands or other secreted proteins — may fail to yield a mutant phenotype unless the efficiency of Cre-mediated deletion is quite high.

The development of gene targeting technology revolutionized the field of mouse genetics a decade ago. Now, the combination of the Cre/*loxP* system, inducible promoters and conventional gene targeting looks set to take the field a stride further. Progress in tissue-specific and temporal-specific regulation of gene expression will be extremely rapid over the next several years, greatly increasing our understanding of the functional roles of the genes being studied. Moreover, the progress of these studies should be fun to observe.

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