

Cre/loxP System for Generating Tissue-specific Knockout Mouse Models

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Editor's note: From time to time, we take the opportunity in *Nutrition Reviews* to highlight a particularly exciting application of sophisticated methodological advances that are relevant to the nutrition research community. In the current issue of *Nutrition Reviews*, Dr. Claudine Kos has provide a brief review of some of the salient features of the Cre/loxP system for generating tissue-specific knockout mouse models. Hopefully, this review will provide additional background to Dr. George Wolf's Brief Critical Review (page 253) of the use of the Cre/loxP technique by investigators to gain further insight into the function of the peroxysome proliferators-activated receptor-gamma (PPAR- γ), as well as promote its further use within experimental nutrition.

Alteration of the mouse genome by conventional transgenic and gene-targeted approaches has greatly facilitated studies of gene function. However, a gene alteration expressed in the germ line may cause an embryonic lethal phenotype resulting in no viable mouse to study gene function. Similarly, a gene alteration may exert its effect in multiple different cell and tissue types, creating a complex phenotype in which it is difficult to distinguish direct function in a particular tissue from secondary effects resulting from altered gene function in other tissues. Therefore, methods have been developed to control conditions such as the timing, cell-type, and tissue specificity of gene activation or repression. This brief review provides an overview of the Cre/LoxP system for generating tissue-specific knockout mouse models.

Key words: Cre/loxP, gene knockout mice, methodology

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Conditional Gene Deletion Using Cre Recombinase and LoxP Sites

The Cre/LoxP system is an approach for generating tissue-specific gene knockout mice (Table 1). The standard method requires two different genetically engineered mouse lines to achieve a tissue-specific gene deletion. In most cases, Cre- and loxP-containing strains of mice are developed independently, then crossed to generate offspring with the tissue-specific gene knockout (Figure 1). The first mouse strain contains a targeted gene flanked by two loxP sites ("floxed gene") in a direct orientation. This mouse strain harboring the floxed gene can be crossed to any other strain of mice expressing Cre recombinase in a specific tissue, cell type, or developmentally regulated manner. The second mouse strain is a conventional transgenic mouse line expressing the Cre recombinase under the control of a promoter that is specific for a particular cell or tissue type. When the floxed mouse and the Cre-expressing mouse are crossed, some offspring will inherit both the floxed gene and the Cre-expressing transgene. In the tissue where the Cre recombinase is expressed, the DNA segment flanked by the loxP sites will be excised, and consequently inactivated. The targeted gene flanked by loxP sites remains active in the cells and tissues that do not express Cre. For example, in the current issue of *Nutrition Reviews*, two recent studies have been highlighted that investigated the function of the peroxysome proliferators-activated receptor gamma (PPAR- γ) in muscle and liver using the Cre/loxP system. In these studies, transgenic mice containing a floxed PPAR- γ gene were crossed with mice harboring Cre recombinase under the control of either a creatine kinase promoter to direct Cre expression exclusively in muscle, or an albumin promoter to direct Cre expression only in the liver.

What Is a LoxP Site?

A loxP site is a 34-base pair (bp) DNA sequence that is composed of an 8-bp core (which determines directionality) flanked on each side by 13 bp of palindromic

Table 1. Definition of Terms

Transgenic mouse: A mouse into which cloned genetic material has been transferred.
Cre recombinase: A 38-kDa enzyme that recognizes specific nucleotide sequences called loxP sites. Cre recombinase catalyzes DNA recombination between two loxP sites, resulting in either deletion of the intervening DNA segment and one loxP site or inversion (flipping) of the intervening DNA segment and two loxP sites.
Promoter: A DNA sequence that is recognized (directly or indirectly) and bound by a DNA-dependent RNA polymerase during the initiation of RNA transcription.
Bacteriophage: A virus that infects and replicates in a bacterium. There are more than 5000 different types of bacteriophages.
LoxP site: A 34-base pair nucleotide sequence that contains an 8-base pair core sequence (that confers directionality) in the center of two 13-base pair palindromic repeats. Two loxP sites are brought together by the Cre recombinase.
Floxed gene: A gene flanked by loxP sites.
Recombination: The exchange of two portions of DNA segments.
Homologous recombination: The alignment and exchange of a piece of DNA with a similar DNA sequence. Example, a targeting construct (containing stretches of sequences which are similar (homologous) to portions of a gene) aligning with the endogenous gene on a mouse chromosome and exchanging genetic material.
Blastocyst: An early preimplantation stage of a mammalian embryo, made up of the inner cell mass and a thin trophoblast layer that encloses the blastocoel. After embryo implantation, the inner cell mass of the blastocyst becomes the embryonic disc of the developing embryo.
Chimeric mice: A mouse consisting of two or more genetically distinct cell types derived from different zygotes. For example, a mouse arising from a normal blastocyst of the C57B6 strain injected with targeted ES cells of the 129 strain that underwent homologous recombination with a targeting construct.
Reporter gene: A gene whose expression is easily detectable and therefore attached to the end of a promoter and used to study promoter activity.

(complementary) sequences. Although loxP sites are prevalent in the genomes of bacteriophages, this exact 34 bp of sequence is statistically unlikely to occur naturally in the mouse genome. Multiple loxP sites can be introduced into the mouse genome by targeted mutagenesis in embryonic stem (ES) cell lines. Sequences flanked by loxP sites are then said to be “floxed.”

Creation of a Floxed Mouse

Gene targeting experiments have the following three basic steps:

- Construction of a targeting construct containing regions of identity with the mouse chromosome (homology units or arms), a selectable marker (generally a cassette that confers neomycin [G418] resistance), and planned modifications (i.e., loxP sites flanking translation start codon of the gene of interest) that will result in a recombination event near to the region of identity. The loxP sites are placed on either side of a sequence that is required for proper gene expression; absence of the sequence should result in lack of expression. However, it is necessary that the locations of the loxP sites do not adversely effect gene expression once they are incorporated into the mouse genome, so that the gene remains functional in the absence of Cre.
- Homologous recombination of the linearized targeting construct with the endogenous gene of interest in the mouse genome. The stable incorporation of the construct into the genome of pluripotent mouse ES cells allows for subsequent selection and screening in order to identify only those ES clones that have integrated the planned DNA modifications at the appropriate location.
- Aggregation with or microinjection of the targeted ES cells into blastocysts (early embryos). Ideally, all the mouse tissues will be colonized with cells derived from both the targeted ES cells with the floxed gene of interest and the normal host blastocyst cells, resulting in the birth of a chimeric mouse (Figure 2).¹

What is Cre Recombinase?

The bacteriophage P1 encodes the 38-kDa cyclization recombination recombinase enzyme known as Cre (creates recombination), which catalyzes recombination between two specific DNA repeats. Cre is a member of the integrase family of recombinases; it recognizes a specific 34-bp nucleotide sequence motif called a loxP site (“locus of crossover P1”). Cre functions through a transient DNA-protein covalent linkage to bring the two loxP sites together and mediate site-specific recombination. Depending on the orientation of the paired loxP sites, the DNA segment between them will be either excised or inverted. When the two direct repeats are in the same orientation, Cre excises the intervening DNA segment, resulting in a single remaining loxP site. When the repeats are inverted (in opposite orientations), the DNA segment undergoes inversion and the two loxP sites remain. This can also lead to gene inactivation; however, because the segment can flip back (reactivate), it is not used in mouse knockout constructs.

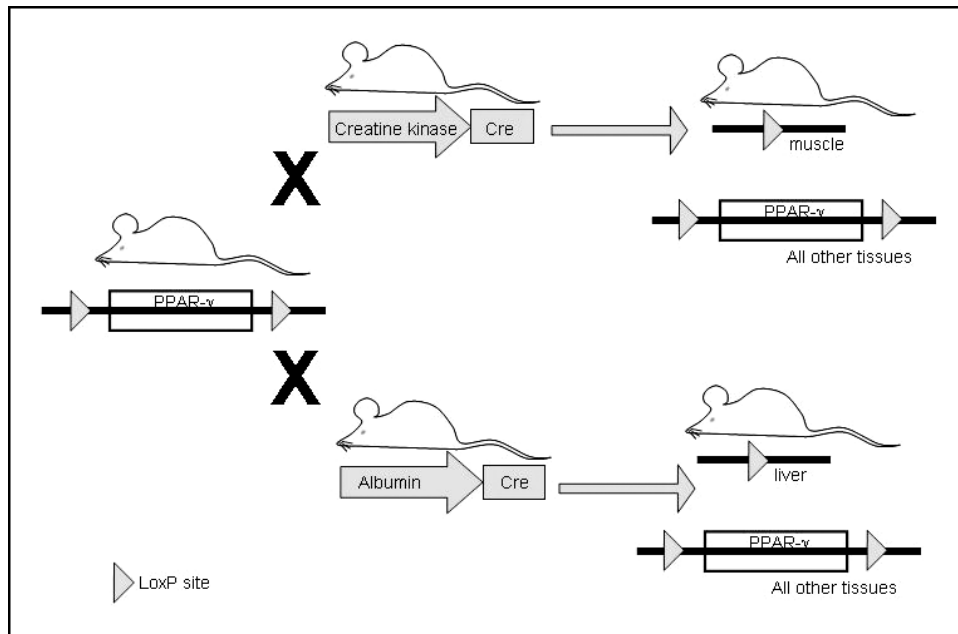


Figure 1. Cre/LoxP system for generating conditional tissue specific knockout mice. The mouse strain harboring the floxed targeted gene can be crossed to various strains of mice expressing Cre recombinase. The targeted gene is inactivated only in tissues expressing Cre and remains active in all other tissue of the body. The Cre expressing strains are described in the text. In this example, a mouse with a floxed PPAR- γ gene is crossed with transgenic mice harboring Cre recombinase under the control of either the creatine kinase promoter to direct Cre expression only in muscle, or the albumin promoter to direct Cre expression only in liver.

Creation of a Cre-expressing Mouse

Cre recombinase is not naturally expressed in mammalian tissues. Thus, it is necessary to create a transgenic mouse line that expresses this enzyme. Moreover, depending upon which promoter is used, Cre expression can be targeted to all tissues or just a specific tissue or cell type. The classic method for the creation and pro-

duction of a transgenic mouse involves the following three steps:

- Isolation and cloning of the gene of interest (in this case, the Cre recombinase gene) in order to design and create a transgenic DNA construct. The transgenic DNA construct contains the Cre gene linked to the end of a promoter that has been selected to generate a particular tissue-specific expression pattern.
- Microinjection of the transgenic DNA construct into the pronucleus of a one-cell mouse embryo, using an extremely fine needle and powerful microscope.
- Implantation of the microinjected embryos into the oviduct of pseudopregnant mouse (Figure 3).¹

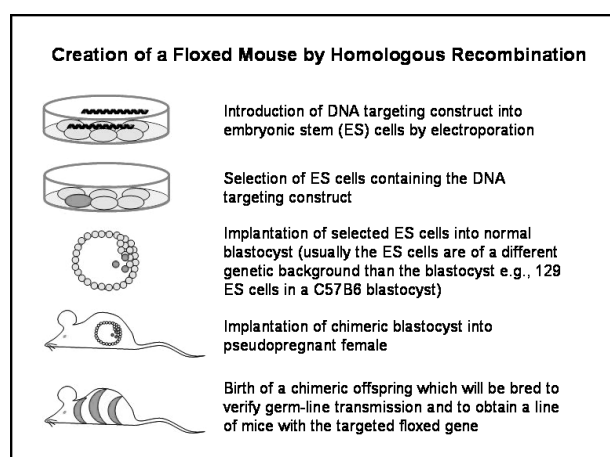


Figure 2. Generation of mouse with a targeted gene alteration by homologous recombination. A targeted DNA construct is electroporated into an embryonic stem cell line. After screening for integration of the construct, positive cells are injected into blastocysts and implanted into a pseudopregnant female. Approximately 21 days later, a chimeric mouse is born.

Possible Uses of Conditional Knockout Animals

Why do we want to achieve conditional (i.e., tissue- or cell-type-specific) gene deletion? First, germ-line mutations may be lethal, in which case there is no mouse available to study in vivo gene function. This may be particularly important if the gene of interest can only be investigated in differentiated tissues, or if nutritional factors are being investigated. Second, genes may exert their function at several stages of ontogeny and in different cell types. In the former case, classical gene targeting will allow investigators to identify the initial stage at which the target gene plays a critical role, but not

necessarily later stages. In the latter case, complex phenotypes may result, in which it is difficult to distinguish cell-autonomous from more complex lesions. Finally, conditional gene targeting could allow one to generate models of somatically acquired genetic diseases (such as most forms of cancer) rather than just inherited ones.

Conclusion

Cre-based conditional knockout mice have already proven to be a reliable tool for generating conditional mouse models. Creating targeting constructs that function in a Cre/LoxP system is generally no more difficult than designing classic transgenic and targeting constructs. One of the distinct advantages of the Cre/LoxP

Table 2. Suggested Reading

1. Bockamp E, Maringer M, Spangenberg C, et al. Of mice and models: improved animal models for biomedical research. *Physiol Genom*. 2002;11:115–132.
2. Rajewsky K, Gu H, Kühn R, et al. Conditional gene targeting. *J Clin Invest*. 1996;98:600–603.
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5. Orban P, Chui D, Marth J. Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci USA*. 1992;89:6861–6865.

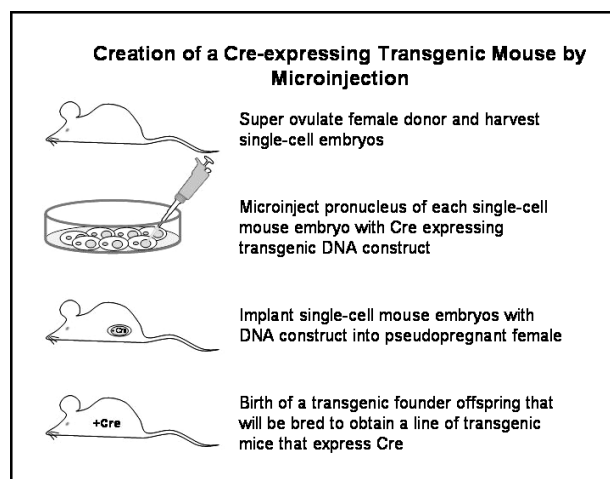


Figure 3. Generation of transgenic mouse by microinjection of transgenic DNA construct into pronucleus of one cell embryo. Embryo is implanted into the oviduct of a pseudopregnant female. Approximately 21 days later, a founder mouse containing the transgene in all somatic and germline cells is born.

system over the classical approach, however, is the flexibility it provides the researcher. Constructs designed with the Cre/LoxP system in mind can be used to generate mice lacking a gene in a particular tissue to avoid early lethality or severe developmental consequences. Multiple Cre mouse strains with diverse, expression-specific promoters can be mated with the same floxed targeted mouse strain based on the particular needs of the experiment. Finally, the increasing availability of transgenic mouse lines expressing Cre recombinase in various tissues and developmental stages offers an exciting opportunity for nutrition researchers to investigate the mechanistic role of diet or specific nutrient supplements under precisely controlled experimental conditions.

Investigators considering Cre/LoxP-based mouse models may want to visit the <http://www.mshri.on.ca/nagy/cre.htm> website, which contains a database of general and tissue-specific deleter Cre mouse strains. A list of suggested reading is included in Table 2.