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Review:

Conditional gene manipulation: Cre-ating a new biological era^{*}

Jian ZHANG[§], Jing ZHAO[§], Wen-jie JIANG, Xi-wei SHAN, Xiao-mei YANG^{†‡}, Jian-gang GAO^{†‡}

(School of Life Science, Shandong University, Jinan 250100, China)

[†]E-mail: yxm411@sdu.edu.cn; jggao@sdu.edu.cn

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Abstract: To solve the problem of embryonic lethality in conventional gene knockouts, site-specific recombinase (SSR) systems (*Cre-loxP*, *Flp-FRT*, and Φ C31) have been used for tissue-specific gene knockout. With the combination of an SSR system and inducible gene expression systems (tetracycline and tamoxifen), stage-specific knockout and transgenic expression can be achieved. The application of this “SSR+inducible” conditional tool to genomic manipulation can be extended in various ways. Alternatives to conditional gene targeting, such as conditional gene trapping, multipurpose conditional alleles, and conditional gene silencing, have been developed. SSR systems can also be used to construct precise disease models with point mutations and chromosomal abnormalities. With these exciting achievements, we are moving towards a new era in which the whole genome can be manipulated as we wish.

Key words: Site-specific recombinase, Gene targeting, Gene trapping, Inducible systems, Φ C31 system

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1 Introduction

After “the Human Genome Project” (HGP) transcribed the human genome into a huge book of “ATGC” (Adenine, Thymine, Guanine, Cytosine) (Watson and Cook-Deegan, 1991; Abramowicz, 2003), the post-genomic era began. The mission during this era is to interpret the encrypted book into one that makes sense to us. Gene knockout has been the most efficient method for the functional research of these genes, and is based on the simple understanding that “we do not know what we’ve got until we lose it.” However, if the loss is lethal during early embryonic development, all we can learn through conventional knockout is that the product of the gene is essential to the organism. Also, one gene product can function differently within different tissues or cell

types. In this case, the simultaneous knockout of a gene within a whole organism is likely to produce complicated results, with too much noise to understand the specific functions of the gene product. Finally, the gene knockout strategy has been applied to the construction of animal disease models for human genetic disorders, but some human genetic diseases such as cancers develop in certain cell types or at certain developmental stages. A precise model for these diseases has to achieve tissue- and/or stage-specific control of these gene mutations. Thus, conditional gene knockout was developed. Using site-specific recombinase (SSR) systems, we can knock out the target gene within a particular cell type at certain developmental phases (conditional knockout). In this way, we are able to analyze the functions of most gene products. With the expansion of various new technologies in SSR systems, conditional gene manipulation has greatly surpassed conditional gene knockout. Now, we can manipulate the genome freely. In this review, we introduce SSR and inducible systems, and then extend into the applications of new gene knockout methods that have been developed through the effective use of SSR systems. We also

[‡] Corresponding authors

[§] The two authors contributed equally to this work

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discuss other SSR-related gene manipulation methods. Finally, we provide guidelines for gene function studies and a perspective for the future.

2 Tissue-specific knockout using Cre-loxP, Flp-FRT, or ΦC31 system

Some conventional knockout mutations lead to animal embryonic lethality; hence, SSRs and floxed alleles are used to circumvent this problem. SSRs, the enzymes catalyzing site-specific recombination within particular nucleotide sequences, are the key elements for conditional gene knockout. The most widely used systems are the Cre-loxP system from the bacteriophage P1 (Hoess *et al.*, 1982) and the Flp-FRT system from *Saccharomyces cerevisiae* (Sternberg *et al.*, 1981). In the Cre-loxP or Flp-FRT system, Cre or Flp recombinase recognizes the 34-bp nucleotide sequence named loxP or FRT and precisely catalyzes the homologous exchange between the two loxP or the two FRT sites, respectively. No nucleotides are gained or lost in this process, which has sufficient in vivo and in vitro efficiencies (Hoess *et al.*, 1984). In such exchanges, no additional elements, except for some monovalent or divalent cations, are needed (Stark *et al.*, 1992). The size of loxP is neither too large to interrupt the function of the genes, nor too small to occur randomly within the genome.

The Cre-loxP and Flp-FRT systems (Hoess *et al.*, 1982; McLeod *et al.*, 1986) enable the specific manipulation of DNA based on the direction and location of the two loxPs or FRTs (Fig. 1). Cre catalyzes the deletion of the DNA between the two loxPs when the two loxPs are in the same direction on one DNA molecule. When one loxP is on a linear DNA molecule and another loxP is on a circular DNA molecule, the circular DNA integrates into the linear DNA at the target. If two loxPs are in opposite directions, the DNA fragment between them inverts. When one loxP is on a linear DNA and a second loxP is on another linear DNA, the two linear DNA molecules exchange a segment (similar to chromosomal rearrangement). Conditional gene knockout works using the excision function of the SSR systems. The two loxP or two FRT sites are inserted into the genome through gene targeting, flanking the gene of interest or an important exon (Gao *et al.*, 2004). The DNA segment (DNA

flanked by two loxP or two FRT sites) is catalytically excised by the recombinase (Fig. 2). To obtain a tissue-specific cut, a Cre transgenic mouse was produced by placing the Cre recombinase gene downstream of a tissue-specific promoter. By breeding tissue-specific Cre mice with floxed mice (produced by targeting a specific gene), tissue-specific knockout can be obtained. The first tissue-specific Cre line and genomic DNA modifications produced by the Cre-loxP system were developed by Orban *et al.* (1992). With the expansion of the tissue-specific Cre database, controlled gene manipulations can increasingly be achieved (Nagy *et al.*, 2009). Researchers may search databases such as the Jackson Laboratory Cre Repository (<http://cre.jax.org>), the Cre-X-Mice database of the Nagy Laboratory (http://nagy.mshri.on.ca/cre_new/index.php), and the Mutant Mouse Regional Resource Center (<http://www.mmrrc.org>) to obtain access to the desired Cre-expressing lines, and cross these Cre mice with targeted mouse lines. Aside from tissue-specific knockout, the different Cre lines can be used for other types of genetic manipulation.

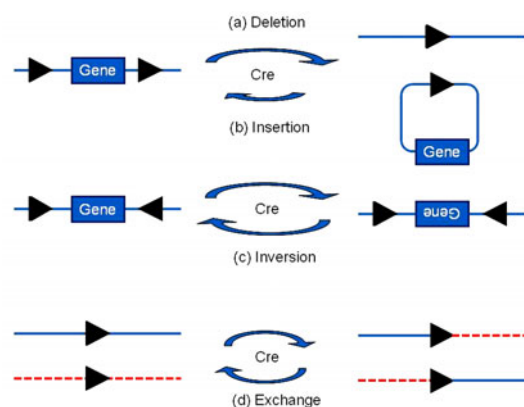


Fig. 1 DNA manipulations controlled by Cre and loxP directions or locations

(a) If the two loxPs are in the same direction on one DNA molecule, the DNA between them will be deleted. (b) When one loxP is on a linear DNA while another is on a circular DNA, the circular DNA will integrate into the linear DNA at the target. Small circular DNA is easily lost in cells; hence, the reaction in (a) happens more easily than that in (b). (c) If two loxPs are opposite, the DNA fragment between them will be inverted. (d) The DNA molecules will exchange a segment if both loxPs lie on linear DNA molecules. The black triangles are loxP sites, and indicate the direction. The length of the arrows indicates the relative tendency of reactions. Modified from Nagy (2000)

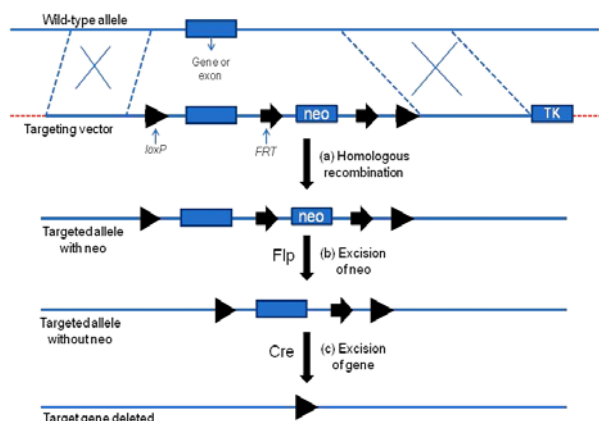


Fig. 2 Conditional gene knockout

(a) In embryonic stem (ES) cells, the wild-type allele recombines with the targeting vectors on two homologous arms. This homologous recombination introduces two *FRT* sites flanking a selection marker neomycin-resistance gene (*neo*) and two *loxP* sites flanking the gene (or exon) of interest. (b) The Flp expression in the ES cells results in the excision of *neo*. (c) At the appropriate stage (usually in mice), the gene (or exon) is excised with the Cre expression. The rectangle (except *TK*) is the gene of interest (or exon). The black triangles indicate the *loxP* sites, and the black arrows indicate the *FRT* sites. *TK*: thymidine kinase gene. Modified from Guan *et al.* (2010)

Although powerful, the *Cre-loxP* and *Flp-FRT* systems remain imperfect. In theory, the recombination catalyzed by Cre or Flp is reversible. Occasionally, the equilibrium favors the undesired direction. For example, in site-specific insertion, the reaction is likely to cut out the already inserted site (Figs. 1a and 1b). Finding ways to control the reaction direction has become a big challenge for the *Cre-loxP* and *Flp-FRT* systems. Thus far, several methods have been developed, one of which involves limiting the reaction time with a heat-shock promoter (Huang *et al.*, 1991; Morris *et al.*, 1991) and introducing subtle mutations into the *loxP* or *FRT* sites to block the reverse reaction (Senecoff *et al.*, 1988; Albert *et al.*, 1995).

To address the reverse reaction problem, a third SSR system, the Φ C31 integrase system, was developed (Thyagarajan *et al.*, 2001). The Φ C31 integrase was derived from the *Streptomyces* phage Φ C31 and catalyzes the recombination between the *attP* site (39 bp minimal size) and the *attB* site (34 bp minimal size), forming *attL* and *attR* sites (Fig. 3) (Groth *et al.*, 2000). The Φ C31 integrase cannot catalyze recombination between the *attL* and *attR* sites (Thorpe and

Smith, 1998; Thorpe *et al.*, 2000). Therefore, the Φ C31 integrase can catalyze recombination only in a strictly controlled direction (Smith and Thorpe, 2002). The Φ C31 system has been successfully applied in site-directed insertion (Sharma *et al.*, 2008) and gene targeting (Lister, 2010; Lu *et al.*, 2011).

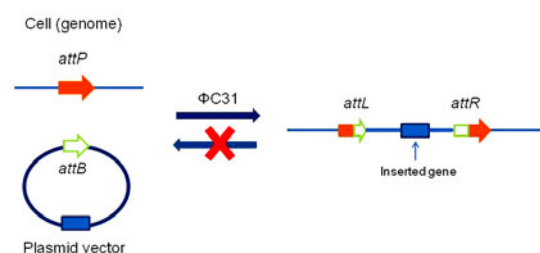


Fig. 3 Φ C31 integrase system

The Φ C31 integrase catalyzes recombination between the *attP* and *attB* sites to form the *attL* and *attR* sites. The *attL* and *attR* sites are the hybrid sites, which are no longer recognized by the Φ C31 integrase. Modified from Groth *et al.* (2000)

In recent years, several pseudo- Φ C31 integrase target (*attP*) sites have been found in human and mouse cells. If a vector with an *attB* site is transfected into mammalian cells, a high frequency of integration occurs at these pseudo-*attP* sites (Thyagarajan *et al.*, 2001; Ma *et al.*, 2006). However, these pseudo sites in mammalian cells may not be a bad thing. Thyagarajan *et al.* (2001) has pointed out that these sites can be used as insertion sites in gene therapy and chromosomal engineering, and pre-clinical applications of the Φ C31 system have also been summarized (Calos, 2006).

3 Stage-specific knockout and transgenic expression using inducible gene expression systems

SSR systems are widely used and can be powerful tools for inactivating genes in specific tissues. However, sometimes we want to study gene function at a specific stage. Stage-specific knockout and transgenic expression systems require inducible expression of Cre or Flp. The inducible systems allow turning on the Cre or Flp expression when needed. To achieve stage-specific knockout and transgenic expression, inducible *Cre-loxP* and *Flp-FRT* systems have been developed. The first inducible system

developed was a tetracycline system, the Tet-Off system (Gossen and Bujard, 1992). Other inducible systems were later developed, including the Tet-On system (Gossen *et al.*, 1995), the tamoxifen system (Metzger *et al.*, 1995), and hormone-inducible systems (Braselmann *et al.*, 1993; Yao *et al.*, 1993; Picard, 1994; No *et al.*, 1996; Wang *et al.*, 1999). These systems use an exogenous chemical to control the expression of a target gene. This control is reversible by providing or withdrawing the chemical. The two most frequently used inducible systems in stage-specific knockouts are the tetracycline system and the tamoxifen system.

3.1 Stage-specific knockout using tetracycline-inducible Cre-*loxP* or Flp-*FRT* system

The tetracycline-inducible system consists of the Tet-Off system (tetracycline-controlled transactivator protein (tTA) dependent) and the Tet-On system (reverse tetracycline-controlled transactivator protein (rtTA) dependent) (Gossen and Bujard, 1992; Gossen *et al.*, 1995). In these two systems, expression of the target gene is dependent on the activity of an inducible transcriptional activator. The transcriptional activator is regulated reversibly by the inducing ligand tetracycline or tetracycline derivatives such as doxycycline (Dox).

In the Tet-Off system (Fig. 4a), target gene expression is turned off with the inducing ligand tetracycline or Dox. tTA is an artificial transcription-activating fusion protein of a tetracycline repressor from *Escherichia coli* transposon Tn10 and the activation domain of the herpes simplex virus VP16 protein. tTA binds with a tTA-responsive promoter (Ptet) to drive the expression of a gene in the absence of tetracycline or Dox. After Dox is added, it binds with the transcription factor tTA. The Dox-tTA complex cannot bind with Ptet and target gene expression is stopped. In contrast, in the Tet-On system (Fig. 4b), target gene expression is turned on by the inducing ligand. The transcription factor rtTA does not bind with Ptet without tetracycline or Dox; hence, the gene under study is not expressed. After Dox is added, it binds with rtTA. The Dox-rtTA complex binds with Ptet and target gene expression is initiated.

The Tet-Off and Tet-On systems are complementary (Kistner *et al.*, 1996). Choosing one over the other depends on your strategy.

Stage-specific knockout can be achieved with a combination of SSR and Tet-On systems. Two mouse lines are needed for stage-specific knockout using tetracycline-inducible Cre-*loxP* or Flp-*FRT* system. The floxed animals are produced by targeting a specific gene in embryonic stem (ES) cells and generating

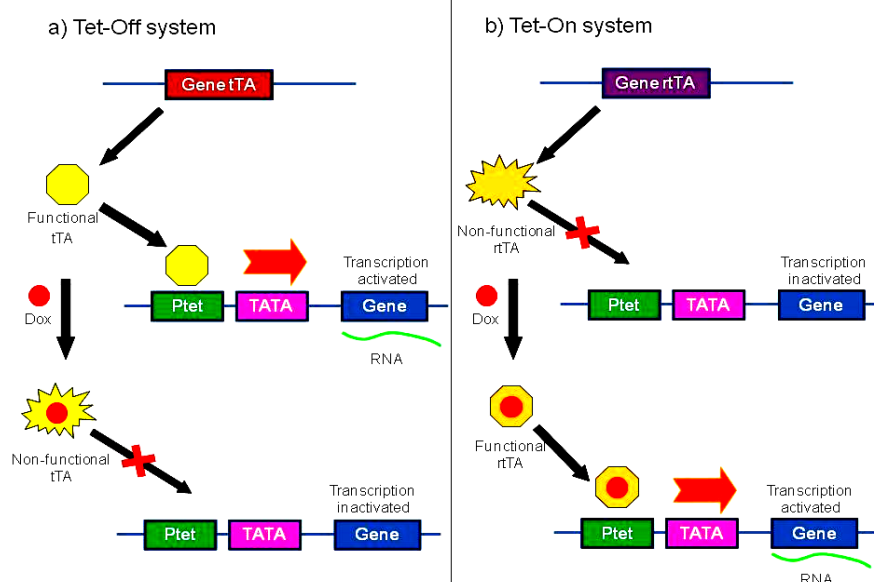


Fig. 4 Tetracycline-inducible system

(a) Tet-Off system. tTA is active without Dox (or tetracycline), and the gene of interest is expressed. With Dox (or tetracycline) treatment, tTA is inactivated, and the gene is no longer expressed. (b) Tet-On system. rtTA is inactive without Dox (or tetracycline), and the gene of interest is not expressed. With Dox (or tetracycline) treatment, rtTA is activated, and the gene is expressed

mice. Another transgenic mouse line is needed in which the expression of Cre (or Flp) can be induced by tetracycline or Dox treatment. By crossing the Tet-On Cre (or Flp) line with a floxed mouse line, the target gene can be knocked out at the specific time when tetracycline or Dox is administered.

3.2 Stage-specific knockout using tamoxifen-inducible Cre-*loxP* system

The tamoxifen system is the most commonly used inducible system for stage-specific knockout. Unlike the tetracycline system, which regulates the transcription of a gene, the tamoxifen system regulates gene function at the protein level (Logie and Stewart, 1995; Metzger *et al.*, 1995). In this system, a mutated form of the ligand binding domain of the estrogen receptor (ER) is fused with Cre recombinase, forming Cre-ER^T. The Cre-ER^T protein no longer binds to 17 β -estradiol, its natural ligand. Instead, it binds the synthetic ligands tamoxifen or 4-hydroxytamoxifen (Feil *et al.*, 1996; Zhang *et al.*, 1996). In the absence of tamoxifen, the Cre-ER^T fusion protein is located in the cytoplasm and binds to heat-shock proteins such as Hsp90. In the presence of tamoxifen, Hsp90 is displaced from ER, which exposes the nuclear localization signal of the ER. Under the guidance of the nuclear localization signal, the Cre recombinase moves to the nucleus, where it executes its function (Fig. 5) (Chow *et al.*, 2006; Tian *et al.*, 2006). Newer versions of the ligand-dependent recombinase, Cre-ER^{T2} and ER-Cre-ER, have been

developed, which are more efficient than Cre-ER^T (Feil *et al.*, 1997; Indra *et al.*, 1999; Casanova *et al.*, 2002; Matsuda and Cepko, 2007).

As in the tetracycline-inducible system, two mouse lines are needed for making stage-specific knockouts using the tamoxifen-inducible Cre-*loxP* system. In the floxed mouse, the gene of interest is flanked by two *loxP* sites. In the transgenic mouse line, the Cre-ER^T is downstream of a ubiquitous or tissue-specific promoter, and can be activated by tamoxifen at the protein level. By crossing the Cre-ER^T transgenic mouse line with the floxed mouse line, the target gene can be knocked out at the specific time when tamoxifen is administered.

3.3 Stage-specific gene expression in transgenic animals using an inducible Cre-*loxP* system

Transgene technology can be used to introduce a foreign gene into the genome of an organism and overexpress it. It is used mainly to study the function of the inserted gene or to produce large quantities of proteins for medical purposes. The gene transferred into transgenic animals may be controlled to express at a specific time and in a specific tissue using the inducible Cre-*loxP* system. In the construct for stage-specific gene expression in transgenic animals, a STOP is positioned between the ubiquitous promoter (CMV) and the gene of interest. This STOP is flanked by two *loxP* sites. Transgenic animals are produced using the above construct. The transgene is “off” at this stage because of the floxed STOP, but

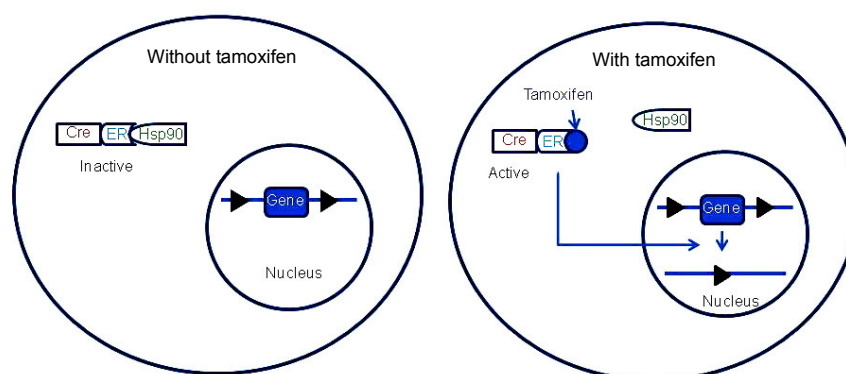


Fig. 5 Tamoxifen-inducible system

In the absence of tamoxifen, Cre-ER binds with Hsp90, and is located in the cytoplasm. When tamoxifen is present, it binds with the ER, displacing Hsp90. The Cre-ER-tamoxifen complex translocates to the nucleus, in which Cre executes its function. The black triangles indicate *loxP* sites. Modified from Tian *et al.* (2006)

once the transgenic mice are crossed with a Cre line, Cre removes the STOP, and the transgene is turned on (Fig. 6) (Dragatsis and Zeitlin, 2001). If an inducible Cre is used, transgenic expression is achieved at a specific time and in a specific tissue.

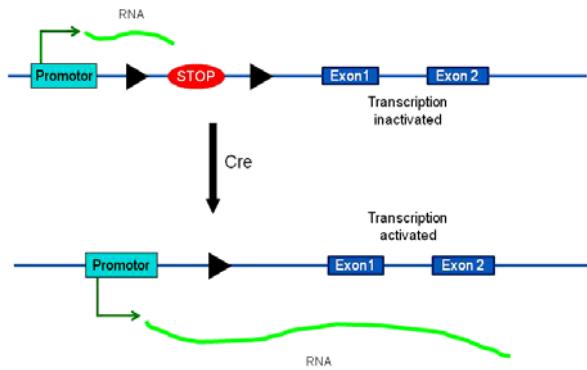


Fig. 6 Stage-specific gene expression in transgenic animals

The floxed STOP cassette is inserted between the promoter and the first exon of the gene. Transgenic animals may be generated using the construct, but exons following the STOP cassette are not transcribed. With the expression of Cre, the STOP cassette is cut out, restoring the normal expression of the gene. The black triangles indicate *loxP* sites. Modified from Dragatsis and Zeitlin (2001)

4 Conditional gene trapping

Aside from gene targeting, gene trapping is a widely used large-scale, high-throughput knockout method. Like conventional knockout via gene targeting, gene trapping also causes embryonic lethality. To circumvent this problem, a conditional gene trapping method was developed by the European Conditional Mouse Mutagenesis (EUCOMM) program. This method is based on the inversion function of the SSR system. The conditional gene trapping vector consists of a selection/reporter cassette flanked by four pairs of SSR sites in opposite orientations (Friedel *et al.*, 2007). *FRT* and *F3* are heterotypic recognition sites for Flp recombinase. *F3* is a mutated version of *FRT*. Flp recombinase catalyzes the recombination between two *FRT* sites or between two *F3* sites, but it cannot catalyze the recombination between an *FRT* site and an *F3* site. *LoxP* and *lox5171* are heterotypic recognition sites for Cre recombinase. Cre recombinase catalyzes the recombination be-

tween two *loxP* sites or between two *lox5171* sites, but not between a *loxP* site and a *lox5171* site. After transfection, this conditional gene trapping vector inserts into the intron of a gene, similar to the conventional trapping vectors, and leads to a mutation because the cassette is placed in the orientation for trapping genes. Knockout mice can be produced using these gene-trapped ES cell lines. Alternatively, this mutated allele can change into a conditional allele at the ES cell level by transiently expressing Flp recombinase in the ES cells. Flp recombinase inverts the selection/reporter cassette at either the *FRT* or the *F3* site. This conditional allele is not mutagenic because the cassette is in the opposite orientation for trapping genes. A mouse line can be made using the ES cells carrying this conditional allele. The trapping vector may be re-inverted into the mutant orientation at *loxP* or *lox5171* sites in some tissues by crossing the above mice with another tissue-specific Cre mouse line. Using this method, a tissue-specific knockout mouse line was generated (Fig. 7) (Schnütgen, 2006). This conditional trapping cassette traps any gene without modification, and may be used directly as a conventional trapping cassette to trap the genes in all tissues from the start of development. For genes that require conditional trapping, ES cells with this trapping vector can be treated with Flp to inactivate the trapping cassette. Mice can be generated using ES cells with this inactive trapping cassette. By crossing these mice with a tissue-specific Cre line, the trapping cassette is functionally restored and the target gene is trapped (knocked out).

Another type of conditional gene trapping is called the “removable gene trap”, in which the trapping vector is flanked by two *loxP* sites (Fig. 8) (Ishida and Leder, 1999). Unlike Schnütgen (2006)’s strategy, Ishida and Leder (1999)’s method traps poly A signal but not promoters. An advantage of this “poly A trap” method is that it can disrupt silent genes in ES cells because the trap cassette has its own promoter. With the controlled expression of the Cre recombinase in certain tissues and at certain time points, the trapping vector can be excised from the gene, resulting in the restoration of normal gene expression. However, Shigeoka *et al.* (2005) found one problem of the “poly A trap” method: inefficient expression of the selection marker by nonsense-mediated messenger RNA (mRNA) decay (NMD).

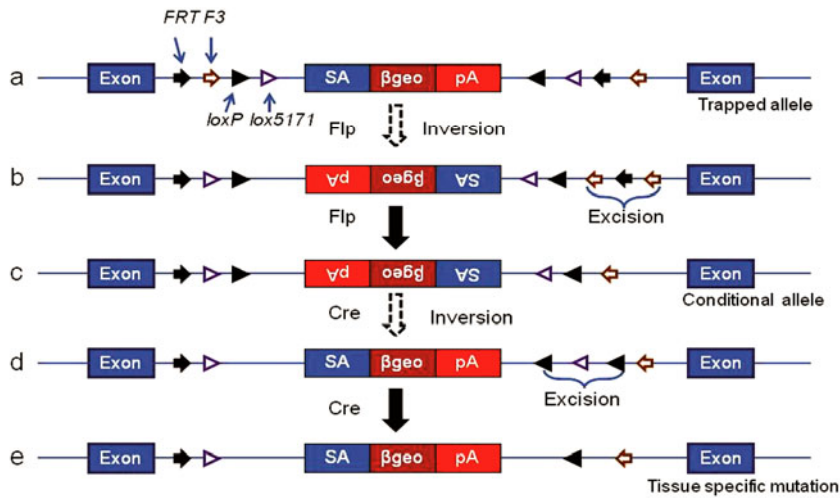


Fig. 7 Conditional gene trapping in the EUComm

(a) Trapped allele. The trapping vector inserts into the genome of the ES cells. With a splice acceptor, a fusion transcript is formed between the upstream exons of the gene and the selection/reporter cassette. The transcription of the downstream exons is terminated, and results in the mutation of an endogenous gene. (b) Transient step for inverting the trapping vector with Flp recombinase. By expressing Flp recombinase in the ES cells, the selection/reporter cassette is inverted from a pair of *F3* or *FRT*. Consequently, a pair of homotypic *F3* or *FRT* sites is placed in the same orientation. Recombination between these two homotypic sites excises the other heterotypic site between them, and the selection/reporter cassette is locked against re-inversion. (c) Conditional allele. The inversion restores the gene function in the ES cells because the selection/reporter cassette is in the opposite orientation for trapping genes. (d, e) Transient step and trapped allele in specific tissues. By breeding the mouse from the ES cell line in (c) with a tissue-specific Cre mouse, the trapping vector is re-inverted, resulting in a conditional mutation in specific tissues depending on the Cre mouse line. The mechanism of re-inversion is the same as that of the inversion in the Flp-*FRT/F3* system in (a) and (b). SA: splice acceptor; β geo: a fusion of neo and β -gal; pA: poly A. Modified from Schnütgen (2006)

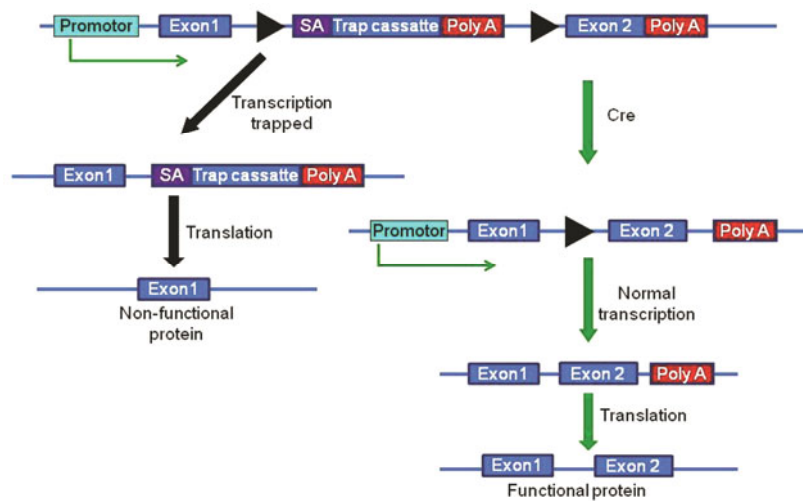


Fig. 8 Removable gene trap

A floxed trapping cassette is inserted within a gene (between exon1 and exon2 in this case). The transcription is “trapped”, and only exon1 is translated, resulting in a non-functional protein. When Cre is expressed, the trapping cassette is excised, and the gene resumes its normal transcription and translation. The black triangles indicate *loxP* sites. Modified from Ishida and Leder (1999)

Thus, they developed a novel poly A trap strategy, unbiased poly A trapping (UPATrap), which suppresses NMD of the selectable-marker mRNA and permits the trapping of transcriptionally silent genes without a bias in the vector-integration site (Shigeoka *et al.*, 2005).

5 Multipurpose conditional alleles

Conventional knockout, conditional knockout, and gene trapping are different methods for inducing null gene mutations in the genome. They are quite different in their manipulation. Each of these methods has advantages and disadvantages for studying gene function. Recently, a new method has been developed and used by the EUCOMM and the Knockout Mouse Project (KOMP; <http://www.knockoutmouse.org>). The method generates different alleles (gene trapping, conventional knockout, and conditional knockout) in one manipulation (Friedel *et al.*, 2007; Skarnes *et al.*, 2011). The vector for these multipurpose conditional alleles has a backbone similar to that for conditional knockout, but borrows some elements from the gene trapping vector. The selectable marker neomycin-resistance genes (*neo*) in the vector for conditional knockout was replaced with trapping elements that contain splice acceptor (SA), β geo (a fusion of *neo* and β -gal), and a poly A signal. If the vector inserts into an intron of a gene, SA works to generate fusion transcripts of the selection marker and the exon upstream of the endogenous gene, resulting in a truncated protein from the endogenous gene. When the vector for targeted trapping is transfected into ES cells, homologous recombination occurs, resulting in an insertional mutation that resembles mutations produced through gene trapping. These ES cell lines can be used to produce knockout mice (mutant alleles by gene trapping). When gene trapping causes embryonic lethality, the ES cell lines can be treated with Flp recombinase. Flp deletes the trapping selection cassette flanked by the two *FRT* sites, and generates a conditional allele. The ES cell lines with this conditional allele can be used to produce conditional knockout mice. Alternatively, Cre recombinase can be transfected into the ES cells to delete critical exons, similar to conventional knockout (Fig. 9). However, this strategy appears to have two limitations: (1) in-frame

expression of β geo occurs with a probability of only one third and (2) expression of the selection marker depends on the promoter activity in ES cells, making it impossible to knock out silent genes in ES cells.

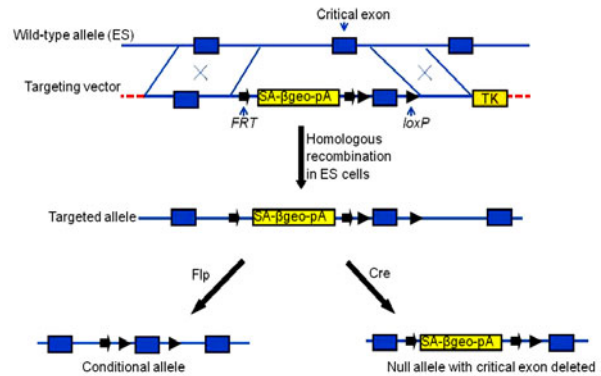


Fig. 9 Multipurpose conditional alleles

The targeting vector contains the floxed gene (or exon) and a trapping cassette flanked by two *FRT* sites. After homologous recombination in ES cells, the gene is targeted. Gene expression is interrupted because of the trapping cassette. If the gene trap causes embryonic lethality, the ES cells can be transfected with Flp, and the trapping cassette is excised, resulting in a conditional allele. Alternatively, the ES cells may be transfected with Cre, and generate null alleles with the critical exon deleted as in the conventional knockout. Modified from Friedel *et al.* (2007)

6 Conditional gene silencing

Following the successful completion of the HGP, the goal of current biological research is to elucidate the function of every gene in the genome and find new therapeutic interventions for human diseases. Gene targeting is powerful for studying gene functions and making mouse models of human diseases. Gene knockout is used to make null mutations and perform loss-of-function studies for specific genes in mammals. However, even with the emergence of high-throughput gene knockout methods and several new resources, such as the International Knockout Mouse Consortium, the production of gene-targeted mice is still time-consuming and labor-intensive.

RNA interference strategies based on gene silencing via short interfering RNA (siRNA) molecules have been thriving in recent years. siRNAs are small, double-stranded segments of RNA that mediate the

degradation of mRNAs transcribed from a certain gene (Montgomery *et al.*, 1998). RNA interference (RNAi), a method for gene knockdown, can be used in both transgenic animals and tissue-cultured cells. An siRNA vector may be introduced into mammalian cells either by pronuclear injection in transgenic animals or by transfection in cultured cells.

By introducing the siRNA into the cells, gene expression can be blocked at the translational level (Montgomery *et al.*, 1998). Its high efficiency in blocking expression and relatively cheap, simple process have established RNAi as an alternative method for studying gene function. However, as in conventional knockout, conventional RNAi cannot be used when the target gene is essential for cellular survival. For conditional gene silencing, the tetracycline-inducible system can be used to drive siRNA expression and knock down the target gene. Alternatively, the floxed STOP/Cre-*loxP* system may be used to down-regulate inducibly the target gene in certain tissues at a specific time.

7 Knocking in point mutations and making mouse models of human diseases

Thus far, knockout remains the most efficient method for studying gene functions and making animal models of human diseases. However, not all gene abnormalities are caused by the total absence of a protein. Point mutations in genes can alter certain amino acids so that normal protein functions are reduced or changed, and such mutations are very common in human diseases. In such circumstances, a simple gene knockout cannot simulate the phenotype of human disease (Cohen-Tannoudji and Babinet, 1998). To make a mouse model of a human disease caused by point mutations, a knock-in mouse line can be generated through gene targeting.

Three steps are needed for knocking a point mutation into a gene (Fig. 10). First, the point mutation is inserted into one homologous arm of the gene-targeting vector. Second, the mutation is introduced into the endogenous gene by homologous recombination in ES cells. Third, the floxed selection markers (neo flanked by two *loxP* sites) are removed by the Cre recombinase activity in the ES cells, or by breeding the mice produced from the targeted ES cells

with another Cre-expressing mouse. In this system, Cre recombinase is used to remove the selectable marker and eliminate the potential side effects on gene function (Cohen-Tannoudji and Babinet, 1998).

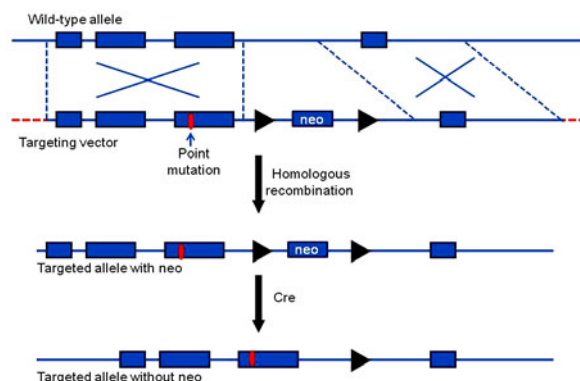


Fig. 10 Introducing point mutations into genes

Similar to gene knockout, the wild-type allele is recombined with the targeting vector with a designed point mutation and a floxed selection marker neo. The selection marker neo can be cut out by Cre, leaving the point mutation and one *loxP* site in the genome. The black triangles indicate *loxP* sites. Modified from Gao *et al.* (2004)

8 Chromosomal engineering

Besides loss-of-function or subtle mutations in a gene, genetic diseases can be caused by large-scale chromosomal abnormalities such as extensive deletions, insertions, translocations, or inversions. Diseases such as Down syndrome, Cri du chat syndrome, and certain cancers are caused by such chromosomal structural abnormalities. Cytogenetic imbalances exist in about 0.6% of newborn infants, 25.0% of miscarriages and stillbirths, and 50.0%–60.0% of first-trimester miscarriages (Shaffer and Lupski, 2000). Therefore, manipulating chromosomes is an important part of constructing genetic disease models.

The SSR system has made possible the generation of mouse models of human diseases associated with chromosomal rearrangements (Yu and Bradley, 2001). The system has been applied to chromosomal rearrangement because of its great potential for manipulating chromosomes: (1) Two *loxP* sites in the same direction cause a deletion; (2) Two *loxP* sites in opposite direction cause an inversion; (3) One *loxP* site on a

chromosome and another on a circular DNA cause duplication; (4) Two *loxP* sites on different chromosomes cause a translocation (Ramirez-Solis *et al.*, 1995; van Deursen *et al.*, 1995).

The Mutagenic Insertion and Chromosome Engineering Resource (MICER; <http://www.sanger.ac.uk/resources/mouse/micer>), a public resource, provides targeting vectors for insertional mutations. This type of vector can be used to create extensive deletions, insertions, translocations, or inversions on chromosomes (Adams *et al.*, 2004). In MICER, two libraries were constructed by inserting genomic DNA fragments into two phage vectors (Fig. 11). The two vectors contain different antibiotic genes that can be used to screen out positive integration in mouse ES cells. Each vector can be used for one targeted integration in ES cells and this integration introduces a *loxP* site into the genome. The two introduced *loxP* sites can be in the same or in opposite orientations, and may be either on the same or on different chromosomes. By expressing Cre in the targeted ES cells with the two *loxP* sites, chromosomal rearrangements will occur. Mice can be generated using these chromosome-engineered ES cells. MICER lists

information on the vector sequences and comprises more than 93 000 constructed, insertional targeting vectors from two libraries (5'*Hprt* and 3'*Hprt*). As well as using the *Hprt* marker, a new chromosome manipulating vector using a pair of fluorescent proteins has been developed (Uemura *et al.*, 2010).

9 Guidelines for functional study of genes

After researchers find a candidate gene for functional analysis, they usually begin by searching the ES cell lines in which the candidate gene has been trapped. Important trapping databases include the International Gene Trap Consortium (IGTC; <http://www.genetrap.org>), EUCOMM (<http://www.eucomm.org>), and the North America Conditional Mouse Mutagenesis Project (NorCOMM; <http://www.norcomm.org>). If no gene-trapped ES cell line is available, researchers may start constructing targeting vectors to target the gene in the ES cells. The conventional method is preferred because the mice with a targeted gene do not have to be crossed with a Cre-expression mouse, saving time for the research.

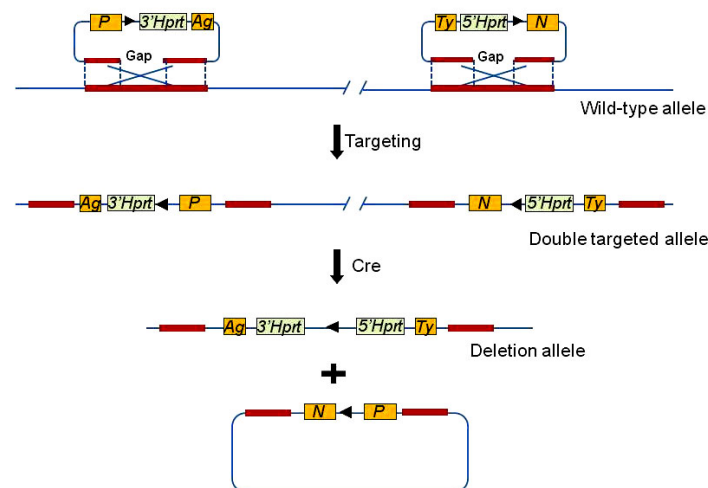


Fig. 11 Generation of large-scale deletions using the two vectors in the MICER

The 5'*Hprt* and 3'*Hprt* vectors are used in two targeting steps to introduce the two *loxP* sites into the mouse genome. The 5'*Hprt* and 3'*Hprt* fragments are kept in the same orientation if a large-scale deletion is to be performed. The DNA between the two *loxP* sites is deleted by the excision of the Cre recombinase in the ES cells. After the excision, the 5'*Hprt* and 3'*Hprt* will form an intact *Hprt* gene, and positive ES cell clones can be selected by hypoxanthine, aminopterin, and thymidine (HAT). *P*: puromycin resistance gene; *Ag*: *K14-Agouti* transgene; *N*: neomycin resistance gene; *Ty*: tyrosinase minigene. The black triangles indicate *loxP* sites. Modified from Yu and Bradley (2001) and Klysik (2002)

If the knockout mutation happens to be lethal, they have to use a conditional strategy. Researchers can still choose between gene targeting and trapping. Conditional gene targeting is a good choice because it features precision and a better chance of a true null allele. However, it has the disadvantage of a complicated procedure, and is time-consuming. Considerable time and effort could be saved if researchers can find the ES cell lines with the gene trapped with the conditional trapping cassette (EUCOMM). However, not every gene has been trapped conditionally despite the fast expansion of conditional trapping databases.

Researchers may choose the multipurpose targeted trapping if conventional gene knockout is likely to be lethal, the trapping ES cell is not available, or the risk of time-consuming conditional gene targeting is not acceptable. The targeted trapping ES cells can be used to produce mice directly as in conventional gene targeting. If the mice are born alive without a null allele, researchers may proceed with knocking out the targeted exon to ensure the complete loss of the gene. If the mice die during the embryonic stage, the targeted trapping ES cells may be transfected with Flp to change the allele into a conditional one. The mice produced with this conditional allele may be crossed with another Cre line to remove the targeted exon in some tissues or at certain stages. Another choice is gene silencing, which is fast for vector construction and mouse breeding. However, RNAi cannot silence a gene completely, and can knock down the expression of the targeted gene only to a certain extent.

10 Prospects

The HGP, one of the greatest accomplishments in the past century, has provided us with the sequence information of our genome. Presently, we are in a new stage of biological research, the post-genomic era. The main work in this era is to elucidate the function of each gene in our genome, and find new therapeutic interventions for human diseases. Comparative genomics has shown high homology between the mouse and human genomes. Hence, mice serve as ideal models for functional research of human genes. Currently, gene knockout is the most efficient way of studying gene functions. Information about the func-

tion of each gene is obtained by observing the phenotype of the whole animal. Knockout mice also serve as animal models of human diseases. We may use these models to study the mechanisms of the diseases and screen drugs for treatment.

Gene targeting and gene trapping are the two main methods for producing knockout mice. The first knockout mouse ES cells made by gene targeting were produced in 1987 (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987). In the last 20 years, different laboratories worldwide have generated a large number of knockout mice, which serve as crucial resources for biological research. Gene trapping is an alternative way to produce knockout mice. It is a random mutation technique (Kothary *et al.*, 1988; Gossler *et al.*, 1989; Abuin *et al.*, 2007) and therefore not as specific as gene targeting. However, it has a high throughput and a large number of genes may be knocked out in a short period of time.

In 2003, the KOMP was proposed at a meeting in Banbury, Cold Spring Harbor, USA. Scientists discussed objectives following the completion of the HGP (Check, 2002; Marshall, 2002), and agreed unanimously to focus on discovering the functions of all mammalian genes. Thus, a genome-wide knockout mouse project is as important as the HGP (Austin *et al.*, 2004). With the fast expansion of gene targeting and trapping programs (Guan *et al.*, 2010), genetic functional studies based on gene targeting and trapping will reach saturation; i.e., most genes within our knowledge will be targeted or trapped, and their function analyzed. However, this saturation does not complete this endeavor, and much work has to be done after all genes in the mammalian genome have been knocked out. SSR systems will play critical roles in various conditional gene manipulations in the biological research after saturation is reached.

In the beginning, SSR systems were used for conditional gene knockout; now, they have more applications. Based on SSR systems, a method was developed for conditional gene silencing in mice. Conditional gene silencing can be used not only for knocking down gene expression but also for gene therapy. In addition to traditional gene therapy, conditional gene silencing has potential in treating cancers and viral infections (Kappel *et al.*, 2006; Lee and Kumar, 2009). Using SSR systems, we may introduce point mutations into genes or engineer chromosomes

so that mouse models for most human genetic diseases can be generated. SSR systems have other applications as well. For example, researchers may conveniently analyze gene interactions using inducible systems. By introducing different drug cocktails to an organism in which different genes are targeted by different systems, researchers can evaluate the interaction of the two genes with various drug formulations. For example, we may produce a mouse line in which gene *A* is controlled by the Tet-On system and gene *B* by the tamoxifen system. When either tetracycline or tamoxifen is used alone, the mouse does not exhibit a certain phenotype, whereas when used together, the mouse shows an obvious deficiency. Therefore, gene *A* and gene *B* work cooperatively, or have similar functions. In another case, when either tetracycline or tamoxifen is used alone, the mouse shows the same deficiency, genes *A* and *B* may function at different steps in the same catalytic pathway. Compared with the Cre-*loxP* and FLP-*FRT* systems, the ΦC31 integrase system has several unique applications. It is a powerful tool for site-directed insertion and shows potential for future use.

The SSR system was developed originally to avoid the problem of embryonic lethality in gene knockouts. Currently, the applications of this technology are expanding beyond this area, and are leading us to a “Cre-ative” era, in which the genes in the genome can be manipulated as desired.

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