Chapter 18

Inducible Cre Mice

Susanne Feil, Nadejda Valtcheva, and Robert Feil

Abstract

The Cre/lox site-specific recombination system has emerged as an important tool for the generation of conditional somatic mouse mutants. This method allows one to control gene activity in space and time in almost any tissue of the mouse, thus opening new avenues for studying gene function and for establishing sophisticated animal models of human diseases. A major technical advance in terms of in vivo inducibility was the development of ligand-dependent Cre recombinases that can be activated by administration of tamoxifen to the animal. Here we describe how tamoxifen-dependent Cre recombinases, so-called CreER recombinases, work and how they can be used to generate time- and tissue-specific mouse mutants. The focus will be on the CreER^{T2} recombinase, which is currently the most successful CreER version. We will give an overview of available CreER^{T2} transgenic mouse lines and present protocols that detail the generation of experimental mice for inducible gene knockout studies, the induction of recombination by tamoxifen treatment, and the analysis of the quality and quantity of recombination by reporter gene and target gene studies. Most of the protocols can also be used as general guidelines for the generation and characterization of Cre/lox-mediated genome modifications in mice.

Key words: Transgenic mice, inducible gene knockout, somatic mutagenesis, CreER recombinase, tamoxifen, in utero, ROSA26, R26R, X-Gal staining, mouse models of human disease.

1. Introduction

In order to understand the role of a given gene product in a given cell type at a given developmental stage, genetic techniques have been developed that allow for the introduction of defined mutations into the mouse genome at will, in a specific cell type and at a chosen time *(1, 2)*. Most current conditional gene-targeting systems are based on the use of the site-specific recombinase Cre (*c*yclization *re*combination) which catalyzes recombination between two 34-bp DNA recognition sites named loxP (*lo*cus of

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crossing [*x*-ing]-over of bacteriophage *P*1). The basic strategy for Cre/lox-directed gene knockout experiments is to flank, or "flox", an essential exon of the gene of interest with two loxP sites (by homologous recombination in embryonic stem cells), and then to "deliver" Cre that excises the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. Delivery of Cre can be achieved by crossing mice carrying the floxed target gene with transgenic Cre-expressing mice.

Clearly, key to successful conditional gene targeting is the availability of Cre transgenic mouse strains in which Cre activity is tightly controlled in space and time. To add inducibility to the Cre/lox system, ligand-dependent chimeric Cre recombinases, so-called CreER recombinases, have been developed *(3–6)*. They consist of Cre fused to mutated hormone-binding domains of the estrogen receptor. The CreER recombinases are inactive, but can be activated by the synthetic estrogen receptor ligand 4-hydroxytamoxifen (OHT), therefore allowing for external temporal control of Cre activity. Indeed, by combining tissue-specific expression of a CreER recombinase with its tamoxifen-dependent activity, the excision of floxed chromosomal DNA can be controlled both spatially and temporally by treating the mouse with tamoxifen, which is metabolized to OHT. The operating mode of inducible CreER mice is outlined in **Fig. 18.1**. It is important to note that inducible Cre/lox systems also allow one to limit unwanted Cre activity and associated side effects – for instance, ectopic recombination due to transient Cre expression during development or potential toxic effects due to prolonged high levels of Cre activity *(7, 8)*.

The properties of CreER recombinases were continuously improved to decrease the background activity in the absence of inducer and to increase the sensitivity and efficiency of tamoxifen-induced recombination in mice. The CreER^{T2} recombinase, which contains the human estrogen receptor ligand-binding domain with a G400V/M543A/L544A triple mutation, is currently the sharpest tool in the CreER box and its use is highly recommended for inducible mutagenesis in the mouse *(4, 9, 10)*. **Table 18.1** lists transgenic mouse lines that express CreER^{T2} in specific cell types. Many of them have already proven useful in addressing biological questions. A convenient way to characterize the recombination properties of a given Cre transgenic mouse line is the use of Cre reporter mice, the most popular one being the so-called R26R line that produces β-galactosidase after Cre-mediated excision of a STOP cassette from the broadly expressed ROSA26 locus *(11)*. The following sections describe the generation and analysis of inducible mouse mutants using CreER^{T2} transgenic mice and R26R reporter mice. However, the protocols can readily be adapted to perform Cre/lox-assisted

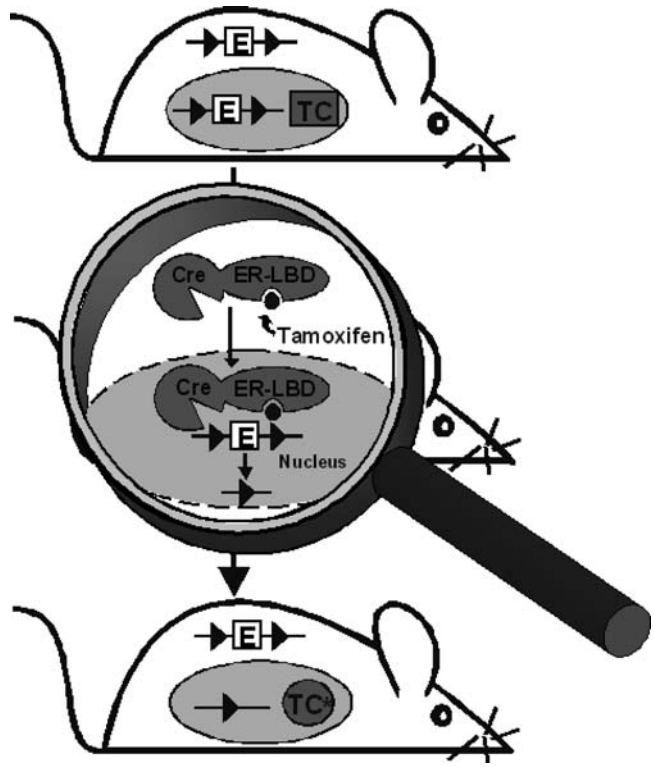

Fig. 18.1. How do CreER recombinases work? Inducible gene inactivation is based on tamoxifen-inducible excision of a loxP (triangle)-flanked exon (E) in cells (shaded oval) expressing a tamoxifen-dependent CreER recombinase (TC). The current model of CreER function is shown under the magnifying glass in the middle part of the diagram. A CreER recombinase consists of Cre fused to a mutated ligand-binding domain (LBD) of the estrogen receptor (ER). In the absence of tamoxifen, CreER is retained in the cytoplasm (boxed TC in the upper mouse). Binding of tamoxifen or OHT to the LBD results in the translocation of the recombinase into the nucleus (circled TC* in the lower mouse), where it can recombine its loxP-flanked DNA substrate; in other words, ligand binding appears to regulate primarily the localization of the recombinase rather than its enzymatic activity per se. Spatiotemporally controlled somatic mutagenesis can be achieved by tissue-specific expression (shaded oval) of a CreER recombinase.

genome modifications with other inducible or tissue-specific Cre mice. Today, hundreds of Cre transgenic mouse lines and about 15 Cre-responsive reporter lines *(1)* are available. Many of them are listed in the Cre mouse line database (http://www.mshri.on.ca/nagy/) and can be purchased from The Jackson Laboratory (http://jaxmice.jax.org/). Further information about ligand-responsive site-specific recombinases as well as about alternative tools for inducible gene manipulation, such as tetracycline-regulated expression systems, can be found elsewhere *(1, 2, 12, 13)*.

Table 18.1.
Examples of mouse lines expressing the CreER^{T2} recombinase

Tissue specificity	Mouse line	Promoter	Reference(s)
Bone			
Osteoblasts and odontoblasts	Col1a1-CreER^{T2}	Collagen 1 α1 chain	*(22)*
Chondrocytes	Col2a1-CreER^{T2}	Collagen 2 α1 chain	*(23)*
Endothelium	Tie2-CreER^{T2}	Tie2 receptor tyrosine kinase	*(24)*
	VECad-CreER^{T2}	Vascular endothelial cadherin	*(21)*
Epithelium			
Intestinal epithelium	Vil-CreER^{T2}	Villin	*(25)*
Internal epithelial organs	K18-CreER^{T2}	Keratin 18	*(26)*
Renal epithelium	KspCad-CreER^{T2}	Kidney-specific cadherin	*(27)*
Fat (adipocytes)	aP2-CreER^{T2}	Adipocyte fatty acid binding protein	*(28)*
Liver (hepatocytes)	SA-CreER^{T2}	Serum albumin	*(29)*
Nervous system			
Astrocytes	GFAP-CreER^{T2}	Glial fibrillary acidic protein	*(30)*
	GFAP-CreER^{T2}	Glial fibrillary acidic protein	*(31)*
	GLAST-CreER^{T2}	Astrocyte-specific glutamate transporter	*(32)*
Neural stem cells	Nes-CreER^{T2}	Nestin	*(33)*
Schwann cells and oligodendrocytes	PLP-CreER^{T2}	Proteolipid protein	*(34)*
Schwann cells	P0Cx-CreER^{T2}	P0 fused to connexin 32	*(34)*
Skeletal muscle	HAS-CreER^{T2}	Skeletal muscle α-actin	*(35)*
Skin			
Keratinocytes	K5-CreER^{T2}	Keratin 5	*(9)*
	K14-CreER^{T2}	Keratin 14	*(36)*
Melanocytes	Tyr-CreER^{T2}	Tyrosinase	*(37)*
	Tyr-CreER^{T2}	Tyrosinase	*(38)*
Smooth Muscle	SM-CreER^{T2}	SM22α	*(10)*
Widespread	Rosa26-CreER^{T2}	Rosa26	*(39)*
	Rosa26-CreER^{T2}	Rosa26	*(40)*
	CMVβactin-CreER^{T2}	CMV enhancer/chicken β-actin	*(41)*
	Ubc-CreER^{T2}	Ubiquitin C	*(42)*

2. Materials

2.1. Generation of Mice

1. At least three mouse lines are required: the CreER^{T2} mouse (*see* **Table 18.1** for a selection), the R26R Cre reporter mouse *(11)*, and the target mouse carrying a loxP-flanked version of

the target gene. In the following these mouse lines are referred to as "Cre mouse", "reporter mouse", and "floxed mouse", respectively. At least 5 male and 5–10 female mice of each mouse line should be available.

2. Mouse facility with standard equipment and room capacity for approx. 30 type III and 50 type II cages, in which approx. 10 and 4 mice can be housed, respectively.

2.2. Induction of Recombination

1. Tamoxifen-free base (Sigma T5648)
2. Ethanol
3. Sunflower oil (supermarket)
4. Syringes (graded in 100-µL intervals) and 22-gauge needles

2.3. Analysis of Recombination

1. CO_2 for euthanasia
2. Perfusion pump (e.g., Perfusor, Braun Medical AG, Germany)
3. 27-gauge needles
4. Dissecting tools (forceps, scissors, etc.)
5. Collecting tray for fixative solution
6. Thermocycler
7. Standard equipment for agarose gel electrophoresis

2.4. Detection of Recombination in Cre Reporter Mice by X-Gal Staining

1. Phosphate-buffered saline (PBS): 135 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4, pH 7.4. Prepare at least 2 L and sterilize by autoclaving; store at RT.
2. Fixative solution: 2% formaldehyde, 0.2% glutaraldehyde in PBS (≈50 mL per mouse); store at 4°C.
3. X-Gal stock solution: 40 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) in dimethylsulfoxide. Prepare 10 mL and store in aliquots of 1 mL at –20°C.
4. X-Gal staining solution: 2 mM $MgCl_2$, 2.5 mM $K_3Fe(CN)_6$, 2.5 mM $K_4Fe(CN)_6$ in PBS; prepare 500 mL and store in the dark at RT. Before use add X-Gal stock solution (40 mg/mL, see above) to a final concentration of 1 mg/mL of X-Gal. Make fresh as required (≈10 mL per mouse).

2.5. Detection of Target Gene Recombination by PCR

1. Lysis buffer: 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% SDS, 200 mM NaCl, 0.5 mg/mL proteinase K (add freshly before use; stock solution: 50 mg/mL in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0)
2. Phenol/chloroform (50%/50%)
3. Ethanol (100%, 70 %)
4. Oligonucleotide primers: each 25 pmol/µL (dissolved in H_2O)

5. 10X PCR buffer (including dNTPs): 500 mM KCl, 100 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 2 mM of each dNTP (dGTP, dATP, dTTP, dCTP)
6. Taq polymerase

3. Methods

3.1. Generation of Mice

For the generation of an inducible tissue-specific knockout mouse at least two basic mouse lines are required: one that expresses the $CreER^{T2}$ recombinase under the control of a tissue-specific promoter (in the following referred to as "Cre mouse"; for a selection of available lines, *see* **Table 18.1**); and the other line carrying loxP sites in the gene of interest (in the following referred to as "floxed mouse"). Besides the generation of the inducible knockout mice of interest (**Section 3.1.2**), it is highly recommended to validate the recombination properties of the Cre mouse, that is, the sites of recombinase expression and tamoxifen-dependent Cre activity, by crossing it to a Cre reporter line (**Section 3.1.1**). Some general guidelines for efficient mouse breeding are presented in **Note 1**.

3.1.1. Generation of Reporter Mice for Detection of Cre Activity

The most popular reporter mouse line is the so-called R26R line (in the following referred to as "reporter mouse") that produces β-galactosidase after Cre-mediated excision of a STOP cassette from the broadly expressed ROSA26 locus *(11)*. The activity of β-galactosidase can easily be detected with single-cell resolution by staining tissues with X-Gal (**Section 3.3.1**). Thereby, Cre activity eventually results in a blue dye precipitate. The Cre mouse is crossed to the reporter mouse to generate mice with the genotype cre/+;+/R, where cre stands for the Cre transgene, R for the R26R reporter allele, and + for the respective wild-type allele. Experimental mice with a single copy of both the Cre and the reporter transgene can be derived in a single breeding step (**Fig. 18.2**). In order to obtain about six experimental animals at least three breeding cages should be set up (*see* **Note 1**). The mice are injected with vehicle or tamoxifen (**Section 3.2**) and analyzed for recombination by X-Gal staining (**Section 3.3.1**).

More breeding steps are required to generate mice with two copies of the reporter gene, which can lead to a stronger X-Gal signal, and/or with two copies of the Cre transgene, which can result in a higher recombination rate. In addition to the characterization of Cre mice, Cre reporter lines are increasingly used to genetically label wild-type or knockout cells in order to perform so-called cell lineage tracing or fate mapping experiments (*see* **Note 2**).

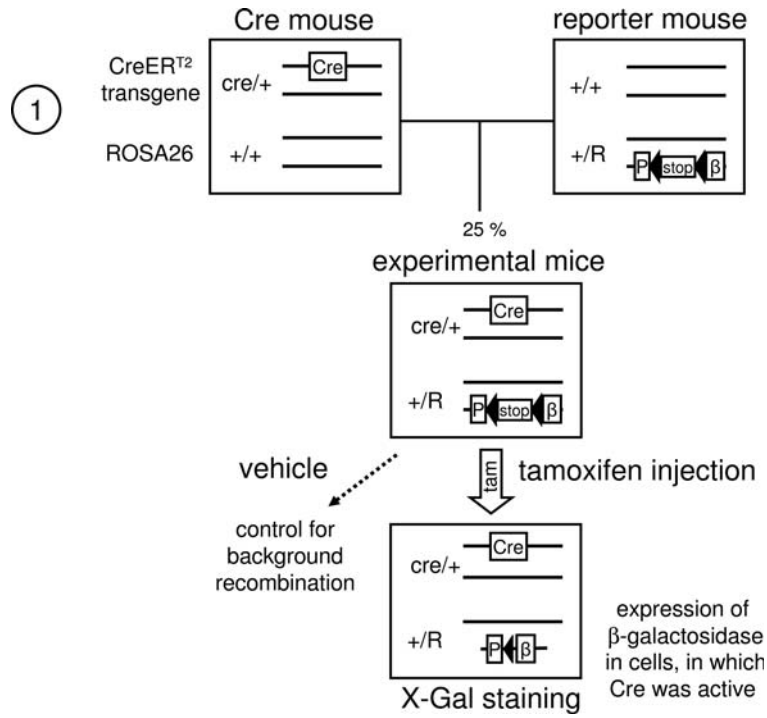

Fig. 18.2. Breeding scheme for the generation of reporter mice for the detection of Cre activity. The genotype of each mouse with respect to the CreER^{T2} transgene and the ROSA26 reporter gene is indicated in the boxes. The number of breeding steps required to derive experimental animals and the expected yield (in percent of offspring) according to Mendelian inheritance is also given. Note that injection of tamoxifen usually results in mosaic activation of the reporter gene in only a subset of the target cells, but not in 100% of the cells as pretended by the scheme. Abbreviations: Cre, CreER^{T2} transgene driven by a tissue-specific promoter; R, R26R Cre-responsive reporter consisting of a β-galactosidase transgene (β) that is expressed from the ROSA26 promoter (P) after excision of a loxP-flanked (triangles) stop cassette (stop); wild-type alleles are denoted by +. For further explanations see text.

3.1.2. Generation of Inducible Knockout Mice

For target genes whose heterozygous (+/–) knockout does not cause a phenotype, which is normally the case, it is recommended to start breeding with the so-called "L– mouse". The L– mouse line can be generated by Cre-mediated excision of the floxed target exon (L2 allele with two loxP sites) to produce an excised knockout gene (L– allele with one loxP site) in germ cells (*see* **Note 3**). Alternative breeding strategies are possible, but might compromise the efficiency of the inducible gene knockout and complicate the interpretation of the experimental results (*see* **Notes 4** and **5**). The standard breeding scheme is depicted in **Fig. 18.3**. In the first step, Cre mice (cre/+) are crossed to L– mice (L–/+). In the second step, their Cre/L– offspring (cre/+; L–/+) is mated with floxed target mice (L2/L2) to generate the experimental mice. In order to obtain sufficient experimental animals for initial analyses

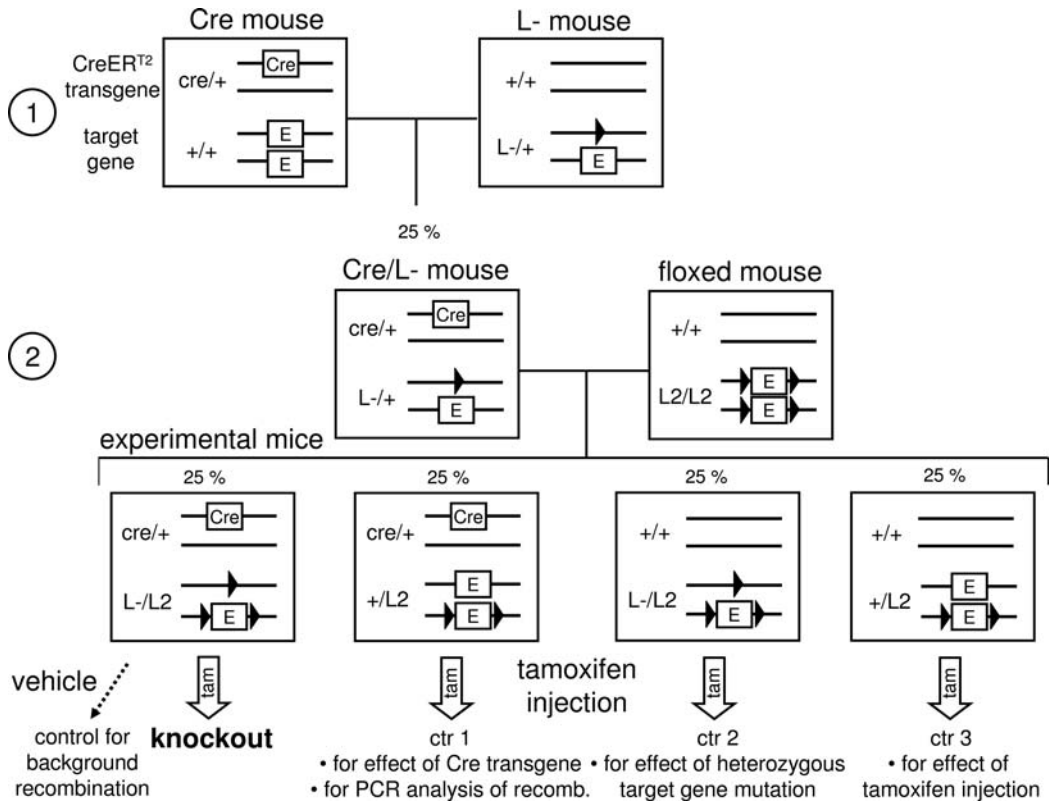

Fig. 18.3. Breeding scheme for the generation of inducible knockout mice and control animals. The genotype of each mouse with respect to the CreER^{T2} transgene and the target gene is indicated in the boxes. The number of breeding steps required to derive experimental animals and the expected yield (in percent of offspring) according to Mendelian inheritance is also given. Injection of pre-mutant mice (lower left box, genotype cre/+;L–/L2) with tamoxifen results in the excision of the floxed exon and, thus, in a gene knockout. Note that the genetic configuration of the experimental mice after tamoxifen treatment is not shown in the diagram. Note also that recombination is usually mosaic, so that only a subset of the target cells, but not 100%, are recombined. Abbreviations: Cre, CreER^{T2} transgene driven by a tissue-specific promoter; E, exon of target gene; triangles, loxP sites; +, L2, and L– denote wild-type, floxed and excised allele, respectively. For further explanations see text.

(approx. six inducible knockout mice plus controls) at least three breeding cages should be set up (*see* **Note 1**). Various useful genotypes are obtained in the same litter (**Fig. 18.3**) and all groups are injected with tamoxifen (**Section 3.2**): "pre-mutant" mice (cre/+;L–/L2) that are supposed to become knockout mice after Cre induction as well as three types of control mice to test for potential phenotypes due to the presence of the Cre transgene (ctr 1), due to the heterozygous loss of the target gene (ctr 2), or due to side effects of tamoxifen treatment which are not related to the gene knockout (ctr 3). Furthermore, some of the pre-mutant animals (cre/+;L–/L2) are injected with vehicle (**Section 3.2**) to estimate the background recombination in the absence of inductor. The initial analyses should include all genotypes and controls; however, the main studies will usually be performed

with the tamoxifen-injected pre-mutant and ctr 1 mice. Some extra vehicle- and tamoxifen-treated ctr 1 mice (cre/+;+/L2) are required for the PCR analysis of recombination (**Section 3.3.2**).

Note that the two breeding strategies depicted in **Figs. 18.2** and **18.3** can be linked in order to combine the floxed target gene with the reporter gene (*see* **Note 6**). By this means mice are derived, in which Cre activity results in target gene inactivation and reporter gene activation simultaneously in the same cells, so that knockout cells can be easily visualized by X-Gal staining (**Section 3.3.1**). Such a configuration can also be used to follow the fate of knockout cells in a wild-type environment via cell fate mapping (*see* **Note 2**).

3.2. Induction of Recombination

Many reports describe the systemic or local administration of tamoxifen or OHT to induce recombination at defined stages during pre- and postnatal development. Administration schemes that have been used with $CreER^{T2}$ mouse lines are summarized in **Table 18.2**. Below the standard protocol for the systemic administration of tamoxifen to adult mice is described.

1. Suspend 100 mg of tamoxifen-free base (Sigma T5648) in 0.5 mL of ethanol in a 15-mL tube and add 9.5 mL of sunflower oil (do not autoclave). Mix the suspension thoroughly using a vortexer and sonicate for 5 min at 37°C (*see* **Note 7**). In addition, prepare 1.0 mL of sunflower oil containing 5% ethanol for vehicle injection.
2. The tamoxifen stock solution (10 mg/mL) and dilutions thereof in sunflower oil can be stored at –20°C for up to 4 weeks.
3. Before use, thaw and sonicate the tamoxifen solution at 37°C for 5 min.
4. The experimental mice (reporter mice, *see* **Section 3.1.1** and **Fig. 18.2**, or pre-mutant and control animals, *see* **Section 3.1.2** and **Fig. 18.3**) are injected with tamoxifen at an age of 4–6 weeks. Inject each mouse intraperitoneally (i.p.) with 100 μL of tamoxifen stock solution (corresponds to 1 mg of tamoxifen) or with vehicle for five consecutive days (*see* **Note 8**). Do not house the tamoxifen-treated mice together with vehicle-treated or untreated animals in the same cage (*see* **Note 9**).
5. Analyze the mice for recombination (**Section 3.3**) and/or phenotypes at the earliest 3 days after the last injection.

3.3. Analysis of Recombination

Reporter mouse lines are useful tools to analyze the tissue specificity and the recombination efficiency of the Cre mouse line of interest. However, the recombination sensitivity of the Cre reporter must not necessarily correlate with that of the floxed target gene. Therefore, it is strongly recommended to monitor the excision of the target gene at the DNA level and, most importantly, the expression

Table 18.2
Examples of induction schemes for CreER^{T2} transgenic mice

Recombination	Mode of ligand administration	Time of analysis	Target tissue	Reference(s)
In utero in embryos[1]	To pregnant females i.p. 1 mg Tam (at E12.5)	E14.5	CNS	*(34)*
	To pregnant females oral 3-4 mg Tam (at E8.5–E11.5)	E9.5–E16.5	Limb development	*(43)*
	To pregnant females i.p. 1 mg OHT for 3 consecutive days (at E12.5–14.5)	E16.5	Bone	*(22)*
	To pregnant females oral 5-10 mg Tam depending on embryonic stage (max. 3 times 10 mg Tam at E12.5–E13.5)	Neonatal	Skeletal development	*(41)*
Postnatal in pups	To lactating mothers i.p. 1 mg Tam for 5 consecutive days (at P1–P5)	8-week-old offspring	CNS	*(34)*
	To pups (P12–P17) i.p. 0.25-1 mg OHT for 5 consecutive days	P19	Bone	*(22)*
	To pups (P1–P3) oral or intragastric injection of 0.05–0.1 mg Tam topical skin exposure to OHT for 3 consecutive days	1 week after last treatment	Endothelium	*(21)*
Postnatal in adults	To 8–10-week-old mice i.p. 0.01–1 mg OHT for 5 consecutive days	10 days and 25 days after last treatment	Skin	*(9)*
	To 5-week-old mice i.p. 0.01–1 mg Tam or OHT for 5 consecutive days oral 1 mg Tam for 5 consecutive days	3 days-16 weeks after last treatment	Smooth muscle	*(10)*
	To 4–8-week-old mice oral 1–5 mg Tam for 5 consecutive days	3 days after last treatment	Ubiquitous	*(39)*

Local	Perivascular delivery device, 0.1%–10% (w/w) OHT for 7 days or 14 days	1 week post application	Femoral and carotid artery	*(44)*
	Topical exposure to the back skin, 1 mg OHT in 0.2 mL EtOH for 5 consecutive days	Up to 5 weeks after treatment	Skin	*(45)*
	Matrigel plug containing 50 nM OHT	5 days after matrigel implantation	Endothelium of tumor vasculature	*(21)*
In cultured cells	1 μM OHT for 48 h	48 h post application	Vascular smooth muscle cells	*(10)*
	500 nM OHT for 4.5 days	4.5 days post application	Embryonic stem cells	*(39)*
	100 nM OHT for 48 h	48 h post application	Rat cortical collecting duct cells	*(46)*

E, embryonic day; i.p., intraperitoneal; OHT, 4-hydroxytamoxifen; P, postnatal day; Tam, tamoxifen.
[1] Note that treatment of mice during pregnancy with tamoxifen or OHT can impair normal delivery of the pups. To obtain live offspring, caesarean section might be necessary.

of the target protein itself (*see* **Note 10**). The following sections describe the analysis of recombination by reporter gene studies at the cellular level (**Section 3.3.1**) as well as by target gene studies at the DNA level (**Section 3.3.2**). Note that recombination rates are affected by many parameters, including the particular genomic location and distance between the loxP sites and the ability of tamoxifen to reach a given organ (*see* **Note 11**). A thorough analysis of recombination is very important, because the tamoxifen-induced gene knockouts in a given cell type or tissue are not complete in most of the cases; in other words, the induced mice are usually mosaics with varying fractions of co-existing wild-type and knockout cells.

3.3.1. Detection of Recombination in Cre Reporter Mice by X-Gal Staining

The R26R reporter transgene *(11)* is highly sensitive to Cre-mediated activation and is thereafter widely expressed; that is, relatively low levels of Cre activity can lead to β-galactosidase expression and X-Gal-stained cells in a wide range of different tissues (*see* **Note 9**). However, note that the ROSA26 promoter that drives β-galactosidase expression in this reporter line might not be active in every tissue and cell type. Thus, it cannot be excluded that the β-galactosidase is not expressed, although the reporter transgene has been recombined at the DNA level, leading to false-negative results of the X-Gal staining. To test whether β-galactosidase can principally be expressed in the tissue of interest, the R26R reporter mouse can be crossed to a "deleter" Cre mouse in order to generate mice that carry the recombined, that is, activated, reporter transgene in every cell. This procedure is analogous to that described for the generation of L– mice (*see* **Note 3**).

1. Sacrifice the mice with CO_2 (*see* **Note 12**).
2. Perfusion fixation (not mandatory):
   - The goal of the perfusion with the fixative solution is to clear the vascular system of blood and to achieve uniform fixation, especially in bigger organs, and to get tissue preparations that are appropriate for sectioning.
   - Prepare the perfusion pump.
   - Place the mouse in a collecting tray and arrange the whole set up under a fume hood (*see* **Note 13**).
   - Open the thoracic cavity and insert a 27-gauge needle about 2 mm into the left ventricle of the heart oriented towards the ascending aorta. Be careful not to pull out the needle throughout the perfusion procedure. Perfuse with the fixative solution at 60 mL/h for 10 min. Effective perfusion is indicated by movements of the tail and legs.
3. Collect the organs and tissues of interest (*see* **Notes 14** and **15**), transfer them into a 15-mL tube with 10 mL of fixative solution and post-fix them for 30 min at RT with gentle shaking. Tissues of the same mouse can be pooled in the same tube.

4. Wash the fixed tissues three times with 10 mL of PBS for 15 min at RT with gentle shaking.
5. Incubate the samples overnight in 10 mL of X-Gal staining solution in the dark at RT with gentle shaking (*see* **Note 16**).
6. Wash the X-Gal-stained tissues three times with 10 mL of PBS for 15 min at RT with gentle shaking.
7. Analyze the samples in a Petri dish using a dissecting microscope (*see* **Note 17**). For photodocumentation different lighting conditions should be explored. Reflections can be avoided by taking pictures from samples totally covered with PBS.
8. Store the X-Gal-stained samples in PBS in the dark at 4°C (*see* **Note 18**).

3.3.2. Detection of Target Gene Recombination by PCR

An essential part of the generation of an inducible knockout mouse is the verification of target gene excision at the DNA level. This method has two advantages over reporter gene studies. First, it tests the recombination of the target gene directly and, second, it is not confounded by the uncertainties associated with the expression profile of the β-galactosidase driven by the ROSA26 promoter (*see* **Section 3.3.1**). PCR analysis is a convenient way to screen the experimental animals for recombination at the DNA level. Three primers (A, B and C) are combined, so that the wild-type (+), floxed (L2), and knockout (L–) version of the target gene can be co-detected in a single reaction (*see* **Fig. 18.4A** and **Note 19**). To analyze the efficiency of tamoxifen-induced recombination, it is recommended to use vehicle- and tamoxifen-treated mice with the genotype cre/+;+/L2 mice (*see* **Fig. 18.4B** and **Note 20**). The same "three-primer PCR" can be employed to genotype the mice during breeding (*see* **Fig. 18.3** and **Note 21**).

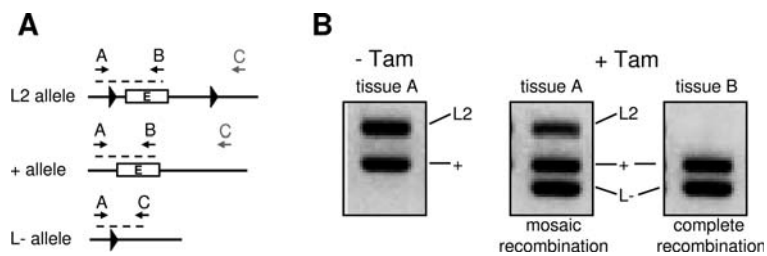

Fig. 18.4. PCR analysis of target gene recombination based on a "three-primer" strategy. (**A**) The positions of the three primers (A, B, and C) used to co-detect the floxed (L2), wild-type (+), and excised (L–) allele of the target gene are indicated. The amplified products are represented by dashed lines; E and triangles denote the target exon and loxP sites, respectively. (**B**) Examples of PCR products detected by agarose gel electrophoresis. Template DNA was obtained from tissues of cre/+;+/L2 mice that have been injected with vehicle (-Tam, *left panel*) or tamoxifen (+Tam, *right panels*). Depending on the tissue analyzed, the tamoxifen-treated mouse shows mosaic or complete recombination as indicated by the level of conversion of the L2 into the L– allele. For further explanations see text.

1. Sacrifice the mice with CO_2 (*see* **Note 12**).
2. Collect the organs and tissues of interest in a Petri dish with PBS (*see* **Note 14** and **20**). Transfer each sample (max. 50 mg) into a 10–15-mL tube (round bottom) with 500 µL of lysis buffer.
3. Homogenize the tissue with an ultraturrax for 1 min and transfer it into a 1.5-mL tube.
4. Incubate overnight at 55°C in a water bath.
5. Add 500 µL of phenol/chloroform (50%/50%) and extract samples by mixing for 30 s with a vortexer.
6. Centrifuge (17,000*g*, 5 min, RT) and transfer the upper aqueous phase that contains the DNA (≈0.5 mL) into a new 1.5-mL tube.
7. Precipitate the DNA by adding 1 mL of 100% ethanol followed by gentle mixing. Centrifuge (17,000*g*, 5 min, RT), wash pellet twice with 1 mL of 70% ethanol, and dry the pellet at RT.
8. Resuspend the pellet in 300 µL of H_2O.
9. Dilute DNA solution 1:100 in H_2O and measure OD_{260} (an OD_{260} of 1.0 corresponds to a DNA concentration of 50 µg/mL). Depending on the tissue, yields of approx. 0.5–10 µg of DNA per mg tissue are expected. Calculate DNA concentration and dilute an aliquot to 0.02 µg/µL. Use 0.5 µg of DNA as template for the PCR.
10. As a starting point use the following standard PCR conditions (*see* **Note 22**):

DNA (0.02 µg/µL)	25 µL
Primer A (25 pmol/µL)	0.5 µL
Primer B (25 pmol/µL)	0.5 µL
Primer C (25 pmol/µL)	0.5 µL
10x PCR buffer	5 µL
H_2O	18 µL
Taq polymerase (5 U/µL)	0.5 µL

- Standard PCR program: Initial denaturation step for 5 min at 95°C; then 35 cycles of denaturation (10 s at 95°C), annealing (30 s at 55°C) and polymerization (30 s at 72°C); and a final polymerization step for 5 min at 72°C; store at 4°C.

11. Analyze PCR products by agarose gel electrophoresis (*see* also **Fig. 18.4B**).

4. Notes

4.1. Generation of Mice

1. In general, breeding cages are set up by placing two female mice (>6-weeks old) to one male mouse (>6-weeks old; if possible use an experienced breeder). The average litter size is 5–10 pups. Note that each breeding step requires 2–3 months, resulting from ≈3 weeks of pregnancy plus 6–9 weeks until the offspring reaches sexual maturity, so that the next breeding step can be started. The basic mouse lines (i.e., the Cre line, the reporter line, and the floxed target line) that are crossed to obtain the experimental animals should be maintained by breeding with wild-type mice of the same genetic background. If the breeding scheme for the generation of inducible knockout mice does not yield pre-mutant animals for tamoxifen injection (*see* **Fig. 18.3**), then this might be related to the fact that both the Cre transgene as well as the target gene are located on the same chromosome. If so, another Cre mouse line which carries the transgene on a different chromosome should be used.
2. Cre reporter mice can also be used to genetically label Cre-expressing cells and their derivatives for cell lineage tracing or fate mapping experiments. Depending on the promoter driving Cre expression, the reporter will be activated only in the respective cells, so that specific cell types can be labeled and their fate can be followed by X-Gal staining, for instance, during embryogenesis, angiogenesis or pathological processes like tumor growth and atherosclerosis *(1, 2, 14–16)*.
3. The L– mouse line can be derived from floxed mice by crossing the latter to a "deleter" Cre mouse that expresses Cre in germ cells, such as the EIIa-Cre mouse *(17)* or the CMV-Cre mouse *(18)*. The Cre-mediated excision of the loxP-flanked exon results in the conversion of the floxed target allele (L2 allele) into an excised null allele (L– allele) in the germ cells of the F1 progeny. After crossing the F1 progeny to wild-type mice, a L– mouse line can be established that does not carry the Cre transgene and transmits the L– allele through the germline. An alternative strategy to obtain a L– allele is the transfection of embryonic stem cell clones that carry the L2 allele with a Cre-expressing plasmid. Last but not least, a mouse line that carries a conventional null allele of the target gene can be used instead of the L– mouse.
4. An alternative strategy to obtain inducible knockout mice is to mate in the first breeding step the floxed L2 mouse (instead of the L– mouse) with the Cre mouse to generate Cre/L2 mice (instead of Cre/L– mice). This strategy should be used

when the heterozygous knockout of the target gene already produces a phenotype or is likely to do so. Compared to the standard breeding scheme as detailed in **Section 3.1.2** and **Fig. 18.3**, the alternative strategy leads to experimental premutant mice that carry two floxed L2 alleles (cre/+;L2/L2 instead of cre/+;L–/L2). Thus, not only one but two L2 alleles must be excised by Cre in order to produce a homozygous gene knockout. The requirement for two recombination events should not pose a problem for experiments with conventional Cre mice, which express a permanently active Cre enzyme. However, it could reduce the knockout efficiency in inducible Cre mice, in which the recombinase is only transiently active during the induction period. Another drawback of the alternative breeding scheme that starts with floxed L2 mice is linked to the potential background activity of the inducible Cre recombinase in the absence of ligand. Recombination background could cause the excision of the floxed exon already in the first generation. If the germline is affected, germ cells carrying the L– allele will be produced. Accordingly, some of the experimental mice obtained after the second breeding step might carry the L–allele in all cells instead of the L2 allele before induction of Cre activity, which might complicate the interpretation of the results. However, it should be noted that the background activity of CreER^{T2} in transgenic mice appears to be very low or virtually absent *(9, 10, 16)*.

5. The breeding schemes shown in **Figs. 18.2** and **18.3** can also be used with conventional tissue-specific Cre mice instead of inducible Cre mice. In this case it is even more important to use L– mice in the first breeding step to avoid uncontrolled germline recombination in the experimental animals.
6. To generate animals, which carry the R26R reporter transgene in addition to the floxed target gene (e.g., for fate mapping experiments), the floxed (L2/L2) mice should first be crossed to reporter (+/R) mice to obtain L2/L2;+/R animals. The latter can then be used instead of the "conventional" floxed (L2/L2) mice in the second breeding step of the scheme outlined in **Fig. 18.3**.

4.2. Induction of Recombination

7. Tamoxifen is not soluble in water. Oil should be added after suspending tamoxifen in a small volume of ethanol. Note that after sonication the tamoxifen stock solution might still be slightly turbid. Variations of the standard protocol include the use of peanut oil or Miglyol instead of sunflower oil or the use of OHT (minimum 70% of Z isomer, Sigma H6278) instead of tamoxifen. Note that OHT is presumably the actual ligand that binds with high affinity to the estrogen receptor

ligand-binding domain of CreER recombinases. However, because tamoxifen is metabolized to OHT *(19)* and is much cheaper than OHT, it is the drug of choice for most induction protocols that are based on systemic drug administration. OHT should be used directly, whenever limited conversion of tamoxifen to OHT is anticipated – for instance, when recombination is to be induced by locally-restricted drug application or in cultured cells.

8. The injection of mice with tamoxifen is an animal experiment and should be performed in accordance with the local guidelines for animal welfare. The recombination background in the absence of tamoxifen should always be controlled in vehicle (oil/ethanol)-treated mice.
9. Cross-contamination with tamoxifen can take place, if treated and untreated animals are housed in the same cage. Licking of oily tamoxifen suspension, grooming or coprophagous behavior can already cause recombination *(20)*.

4.3. Analysis of Recombination

10. There is no doubt that the disappearance of the gene product, which is in most cases a protein, is crucial for the success of an inducible knockout experiment. Thus, it is mandatory to monitor the expression of the target protein itself, for example, by Western blot analysis of tissue extracts and, if possible, at the cellular level by immunohistochemistry. Note that the half-life of individual mRNAs and proteins can vary greatly, so that it can take days to weeks after tamoxifen injection until the target protein is lost.
11. The conditions for inducible gene inactivation should be optimized for every application. Among the variables that can be changed in order to improve the efficiency of recombination are the mode of drug administration (*see* also **Table 18.2**) as well as the age, gender, and genetic background of the experimental mice. In general, tamoxifen-induced recombination appears to be more efficient in younger than in older mice (SF and RF, unpublished data 2007) *(21)*. Therefore, it is recommended to inject the experimental animals with tamoxifen at an age of 4–6 weeks. Moreover, tamoxifen and OHT, respectively, might not easily cross the blood–brain barrier, thus limiting recombination in the brain.

4.3.1. Detection of Recombination in Cre Reporter Mice by X-Gal Staining

12. Alternatively, other methods can be used to sacrifice the mice. However, the method of choice should be compatible with downstream processing of the tissue samples. For example, cervical dislocation is not recommended before perfusion fixation, because vessels can be destroyed and the fixative solution will not reach every organ.

13. Formaldehyde is carcinogenic! Therefore, the experiment should be performed under a fume hood and the mouse placed in a dish to collect the fixative solution that drips from the animal.
14. Collect not only tissues that are expected to show recombination, but also some not expected to be recombined. Tissues should be obtained from tamoxifen-treated mice as well as from vehicle-treated mice to control for background recombination. An overview of the experimental animals used for X-Gal staining (**Section 3.3.1**) and PCR analysis (**Section 3.3.2**) is shown in **Figs. 18.2** and **18.3**, respectively.
15. In the case of X-Gal staining, it is also important to test organs of wild-type mice for endogenous β-galactosidase activity, which often appears to be greenish-blue. Note that some tissues show a relatively high β-galactosidase background – for instance, some regions of the gut, the intestinal mucosa, and the testes.
16. X-Gal stock solution should always be freshly added to the staining solution. The exact staining time depends on the size of the samples and the level of β-galactosidase activity. A similar protocol can be used for X-Gal staining of mouse embryos and cultured cells, with a fixation time of 15 min and 5 min, respectively. Cultured cells are stained with X-Gal at 37°C without shaking.
17. The X-Gal staining solution penetrates only the outer layer of bigger organs, for example, 2–3 mm of the heart. For uniform staining of deeper regions in organs or embryos the samples can be cut into halves or several slices before X-Gal staining is performed. X-Gal-stained tissues can also be embedded in paraffin and sectioned. It is not recommended to leave the tissues in organic solvents longer than necessary, because the blue precipitate may leach out. It is also feasible to prepare frozen sections at first and to stain them with X-Gal.
18. Unstained tissues may become greenish-blue after 1–2 weeks of storage in PBS.

4.3.2. Detection of Target Gene Recombination by PCR

19. For a successful "three-primer PCR" the following criteria should be met (*see* also **Fig. 18.4A**). The length of the amplified products corresponding to the +, L2, and L– alleles should be in the range of approx. 150–300 bp. Primer C should be located such that the distance between primer A and C on the L– allele is much smaller (<200 bp) than on the + and L2 allele (>500 bp). Thus, the L– allele, if present, is preferentially amplified versus + or L2. These conditions can be fulfilled as soon as the loxP sites flanking the target exon are located more than ≈300 bp apart, which is normally the case.

20. For PCR analysis of recombination it is recommended to use tamoxifen- and vehicle-treated mice with the genotype cre/+;+/L2 (**Fig. 18.3**, ctr 1). The advantage of using these mice rather than the actual pre-mutant mice (cre/+;L–/L2) is that successful recombination is unequivocally indicated by the appearance of an additional PCR product representing the L– allele. Note that the L– allele is already present in untreated pre-mutant mice, which would complicate the recombination analysis.
21. By using the "three-primer PCR" for mouse genotyping, all possible alleles (+, L2, L–) can be detected in the same reaction. Here, every possible genotype, even +/+, results in at least one PCR product. Thus, this PCR strategy excludes the possibility of false-negative results, which can happen when only two primers are used to detect a single allele.
22. The PCR conditions depend on the length of the products and the composition of the primers. The duration and temperature of the PCR steps must be optimized for each particular set of primers and template DNA. Altering the primer concentrations is a particular effective means to adjust the relative abundance of the PCR products.

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