Transgenic Mouse Technology in Skin Biology: Inducible Gene Knockout in Mice

Christian Günschmann[1], Elena Chiticariu[2], Bhavuk Garg[3], Merve Meliha Hiz[4], Yora Mostmans[5], Maria Wehner[6] and Lukas Scharfenberger[1]

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INTRODUCTION

Mutations in a variety of genes underlie different skin pathologies, in either monogenic or multigenic diseases. *In vivo* model systems are essential to investigate the contribution of the different proteins encoded by these genes to disease development. Furthermore, these models are necessary to develop and test new treatments for these diseases. Gene-targeting strategies in mice allow investigators to turn genes off or on, either in the whole organism or in a tissue-specific manner (Gu *et al*., 1994). Strategies to inactivate genes using Cre–loxP technology, for example, were introduced in the first part of the "Transgenic Mouse Technology in Skin Biology" Research Techniques Made Simple article (Scharfenberger *et al*., 2014). However, many complete and tissue-specific Cre-mediated knockout mice result in embryonic or early perinatal death, thereby precluding the analysis of gene function in different cell types and in the regulation of skin homeostasis (Lewandoski, 2001). In addition, many skin diseases only manifest in adult stages of life, and this is often accompanied by an altered gene expression. To circumvent early lethality and/or mimic the alterations in gene expression that precede or accompany disease, several transgenic mouse systems have been developed, which allow investigators not only to regulate gene expression in a tissue/cell-specific manner (spatial control) but also to initiate this alteration at a time point that is determined by the researcher (temporal control).

Several inducible systems have recently been developed, each with different advantages and limitations (Garcia and Mills, 2002; Lewandowski, 2001). Some of these inducible systems are based on spatiotemporal control of promoter activity by regulatory elements inserted in front of the promoter, whereas in other systems the spatial localization of the protein of interest (mostly Cre) determines whether the protein is active. Using Cre as an example of a spatiotemporally induced protein, we will discuss the two most common models: the tetracycline/doxycycline binary transactivation model and the tamoxifen–Cre–inducible model.

ADVANTAGES

- Inducible mouse technology enables investigators to determine not only where but also when to turn on or off genes of interest.
- It also allows investigators to assess the functional importance of overexpression or loss of proteins in normal skin homeostasis and at different stages of disease.

LIMITATIONS

- Long-term treatment with the inducer can lead to side effects.
- The system can be "leaky," resulting in undesired expression of the protein of interest.

THE TETRACYCLINE-INDUCIBLE CRE–LOXP SYSTEM

Tetracycline (Tet)-inducible systems use an artificial protein (tetracycline-responsive transactivator (tTA)) to regulate the expression of proteins of interest (Garcia and Mills, 2002; Figure 1). As an example we use the Cre protein, which can control the timing of Cre-mediated recombination of loxP sites. The tTA fusion protein is composed of the tetracycline repressor from bacteria and the viral protein p16 (used by Jaubert *et al*., 2004). tTA is able to bind to a 19 base pair–long DNA sequence (tet Operon (tetO)) and induce the expression of the gene of interest that is inserted after the tetO (in this case the Cre recombinase). In the presence of the antibiotic Tet or its derivative, doxycycline

[1]*Department of Dermatology, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), SFB829 "Molecular Mechanism Regulating Skin Homeostasis," University of Cologne, Cologne, Germany;* [2]*Department of Dermatology, University of Medicine and Pharmacy "Victor Babes," Timisoara, Romania;* [3]*Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, University of Vienna, Vienna, Austria;* [4]*Department of Biology, Faculty of Science & Arts, Çanakkale Onsekiz Mart University, Çanakkale, Turkey;* [5]*Department of Dermatology, University Hospital Brussels, Free University of Brussels, Brussels, Belgium and* [6]*Institute for Human Genetics, University Clinic Bonn, Bonn, Germany*

Correspondence: Christian Günschmann, Department of Dermatology, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), Joseph-Stelzmann-Strasse 26, 50931 Cologne, Germany. E-mail: cguensch@uni-koeln.de

(Dox), tTA changes its confirmation and the tTA is no longer able to bind to the tetO, thus shutting down expression of the Cre recombinase (Garcia and Mills, 2002). Inactivation of a gene at the time point of interest requires the persistent application of Tet/Dox until this time point (Jaubert *et al.*, 2004). Upon clearance of Tet/Dox from the tissue, the Cre recombinase is active, resulting in recombination of the loxP sites and, in the case of deletion, inactivation of the gene (Figure 1a).

The main disadvantage of this so-called Tet-off system is the high toxicity of Tet/Dox when used in the long-term treatment of mice. To circumvent this problem the "Tet-on" system was developed, where a reverse tTa (rtTA) protein (Jaubert *et al.*, 2004) only binds to the DNA upon binding to Tet/Dox, which then results in activation of Cre expression (Figure 1b). The advantage of this method is that mice do not have to be treated continuously with Tet/Dox. In addition, expression of the Cre recombinase solely depends on saturation of the drug in the tissue. Using cell/tissue-specific promoters to drive tTA/rtTA fusion protein expression allows spatial control of the turning off or on of genes in the tissue or cells of interest.

THE TAMOXIFEN-INDUCIBLE CRE–LOXP SYSTEM

The second major inducible gene knockout/knockin system is based on nuclear hormone receptors that translocate into the nucleus to regulate gene expression when bound to their corresponding hormone ligand (Garcia and Mills, 2002; Lewandoski, 2001; Figure 2). The estrogen receptor (ER) is such a nuclear hormone receptor that binds estrogen, but also the estrogen antagonist tamoxifen. In the inactive state the ER is bound to heat shock protein 90 (Hsp90) and is thereby excluded from the nucleus. When estrogen or tamoxifen binds the hormone-binding site of the ER, Hsp90 is released and the ER shuttles into the nucleus. This property is used to control the localization of Cre, which can only recombine loxP sites in the nucleus. By fusing Cre to the ER, it is retained in the cytosol by Hsp90 in the absence of hormone. Upon application of tamoxifen, the Cre–ER protein is released from Hsp90 and can now enter the nucleus, where Cre recombines the loxP-flanked (floxed) target gene. This system uses modified versions of the estrogen receptors, which cannot bind endogenous estrogen but are able to bind tamoxifen. The expression of this Cre–ER fusion protein is controlled by tissue-specific promoters (Gu *et al.*, 1994; Denton *et al.*, 2009; Figure 3).

APPLICATION METHODS

Tet/Dox and tamoxifen dissolved in a vehicle, such as ethanol, can be applied topically to a defined area of mammal skin. When a systemic approach is favored, tTA or rtTA mice are usually fed Dox-containing drinking water to induce gene deletion. For tamoxifen-inducible Cre–ER systems, the application methods are more diverse. Similar to Dox treatment, the mice can be fed tamoxifen drinking water or tamoxifen food pellets. This is a convenient method for investigators, and it is considerably less stressful for the mice compared to other procedures. When using these food- and water-based application methods, the gene deletion efficiency depends on the animals' drinking and eating behavior, which directly correlates to the final Dox or tamoxifen dosage in the target tissue.

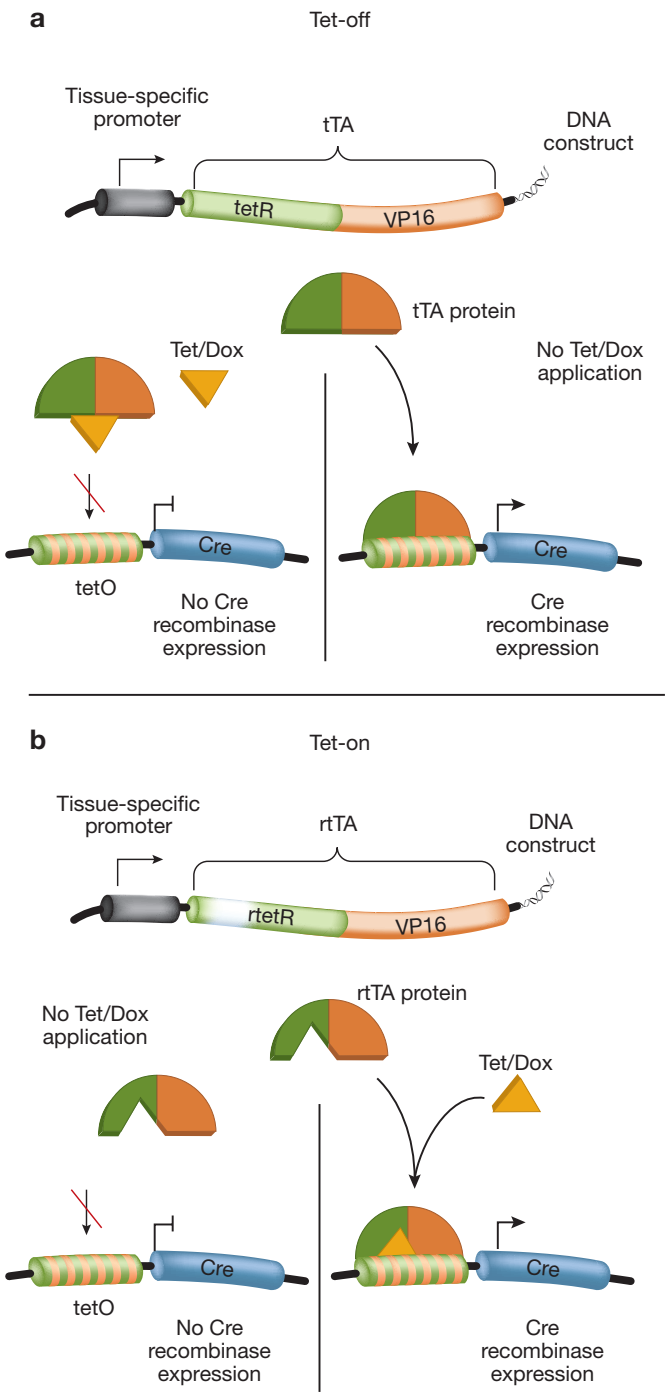

Figure 1. Tetracycline/doxycycline-inducible Cre–loxP system. (**a**) The Tet/Dox-responsive tetracycline-responsive transactivator (tTA) protein, which is expressed under the control of a tissue-specific promoter, can bind Tet/Dox. Upon Tet/Dox application, the tTA is unable to bind the tet Operon (tetO), resulting in an inactive promoter and no expression of the protein of interest, in this case Cre recombinase. When Tet/Dox is removed, tTA is able to bind the tetO element, resulting in activation of the promoter and, thus, expression of Cre (Tet-off). (**b**) The Tet-on system uses a modified reverse tTA (rtTA) protein, which can be activated by Tet/Dox. Upon Tet/Dox application, the rtTA protein is able to bind the tetO and activate the promoter, resulting in expression of the protein of Cre. Subsequently, Cre can then recombine the floxed gene of interest, thus leading to gene inactivation.

In addition, the relatively low water solubility of tamoxifen limits the tamoxifen doses that can be administered via drinking water, making it more difficult to arrive at a dose sufficient for gene knockout. These feeding or drinking variations can be circumvented by the use of a feeding needle, which, however, increases stress for the mice. Intraperitoneal or subcutaneous injections are also common using tamoxifen resolved in corn oil (Figure 3b). The subcutaneous implantation of tamoxifen pellets can also be used in rodents. Often, finding the right method to achieve the best possible deletion efficiency using these systems can be challenging and requires considerable testing.

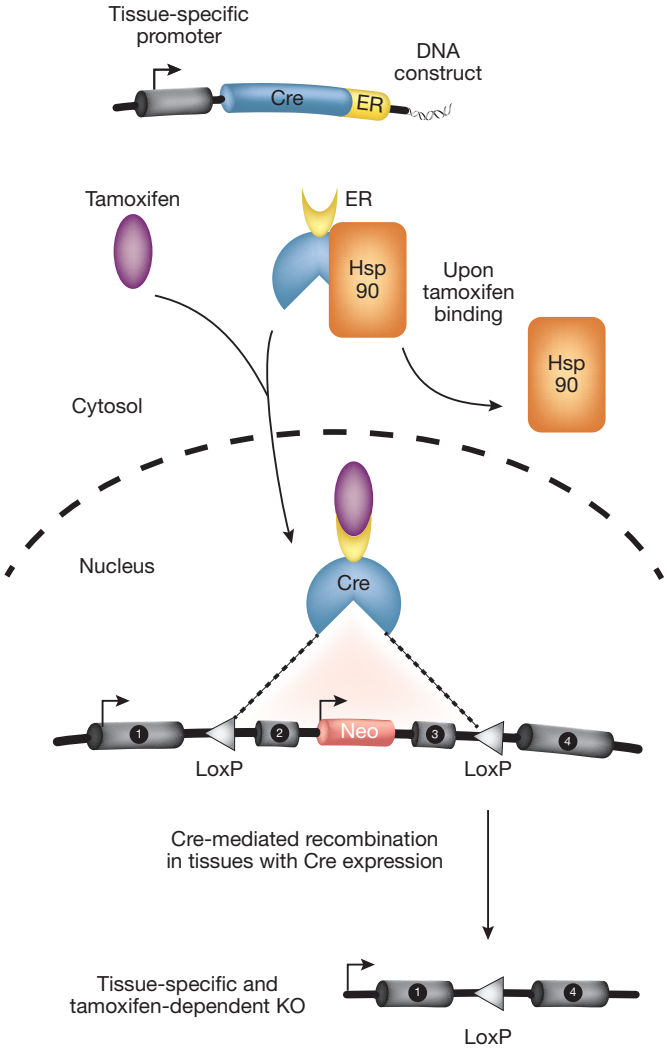

Figure 2. Tamoxifen-inducible Cre–loxP system. The protein of interest (here the Cre recombinase) is fused to a modified estrogen receptor (ER) and controlled by a tissue-specific promoter. In the unactivated state, heat shock protein 90 (Hsp90) binds to the ER, retaining it in the cytosol. Upon tamoxifen treatment, tamoxifen binds the ER, the Hsp90 protein is released, and the Cre–ER fusion protein can translocate in the nucleus. In the nucleus, the Cre recombines the floxed gene, resulting in a knockout.

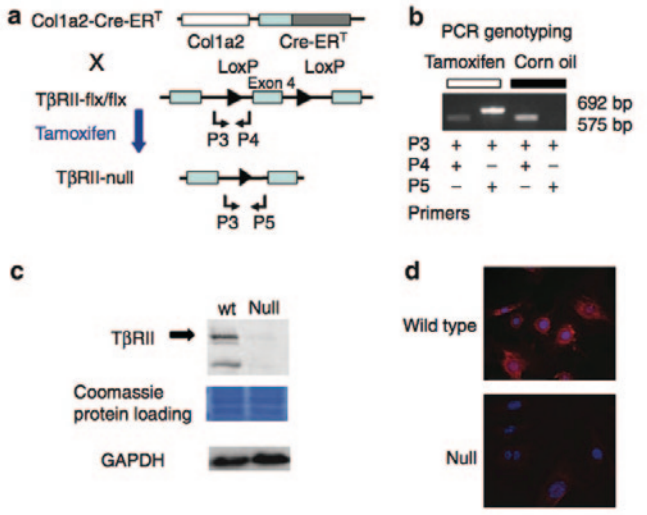

Figure 3. Inducible fibroblast-specific deletion of TβII. The Cre–estrogen receptor (ER) is expressed under the control of the fibroblast-specific Col1a2 promoter. The Col1a2-Cre-ER^T mice were crossed with the TβRII floxed mice. Treatment with tamoxifen leads to a fibroblast-specific deletion of exon 4 of the TβRII protein. (**b**) The successful deletion of exon 4 by intraperitoneal injection of tamoxifen was confirmed by PCR. The 692-bp PCR product from the null allele (P3, P5) is present only after tamoxifen administration. (**c, d**) Lysates from fibroblasts cultured from skin biopsies (**c**) and immunostainings on explanted skin fibroblasts (**d**) confirm the absence of TβRII after tamoxifen treatment. (Adapted from Denton *et al.*, 2009.)

ADVANTAGES AND LIMITATIONS

The main advantage of inducible gene expression models is the temporal control of gene expression or deletion by the external application of a drug. It is an elegant method to overcome problems such as prenatal lethality caused by conventional or tissue-specific inactivation of genes, and it enables the control of gene expression at specific time points. This is especially appealing for studying specific gene function at specific time points during development and homeostasis and, importantly, allows investigators to turn genes on or off at different disease stages, enabling assessment of their importance during the progression of various diseases.

Inducible systems also have several limitations. Beside the problem of choosing the right drug application methods to achieve induction of expression and/or deletion, there are other pitfalls when using these systems. One problem is determining the dose of Dox or tamoxifen. If the dose is too high or the time of treatment is too long, toxic side effects can occur and ruin an experiment. Doses that are too low may result in insufficient induction of the protein of interest (e.g., Cre), resulting in only a partial gene knockout. Furthermore, tamoxifen can interfere with the endogenous ER pathway and might therefore have additional unwanted effects.

As in conditional knockout approaches, the locus of the target gene is important for recombinase efficiency and varies among tissues. In addition, the recombination efficiency of fusion proteins, such as Cre–ER, can vary in different areas of the tissue, resulting in genetic mosaicism (Schwenk *et al.*, 1998;

Lewandoski, 2001). A prominent problem in many inducible knockout methods is the "leakiness" of the system, meaning that the Cre recombinase has an unwanted weak activity independent of the inducing substance. For example, in the tTA system a reduction of Dox levels in the tissue can lead to undesired gene deletion in some cells, leading to an effect before the actual experiment even starts. Similar problems have been observed for the Cre–ER mouse models (Garcia and Mills, 2002).

Despite these disadvantages and the sometimes labor-intensive testing of these mouse models, they offer a great opportunity for spatiotemporal control of gene expression and the assessment of gene function *in vivo*—not only during normal skin homeostasis but also at various stages of skin disease.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for teaching purposes is available at http://dx.doi.org/10.1038/jid.2014.213.

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QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

1. What is a possible disadvantage of total-body knockout mice?

A. The researcher induces the knockout.

B. The knockout is tissue specific.

C. The knockout is embryonic lethal.

2. Which of the following statements is incorrect?

A. The tetracycline-responsive transactivator (tTA or rtTA) binds to the promoter of the target gene (knockout).

B. The tetracycline-responsive transactivator (tTA or rtTA) binds to the tet Operon (tetO).

C. The tetracycline-responsive transactivator (tTA or rtTA) is modulated by Dox.

3. Which protein in the cytoplasm binds the estrogen-fusion protein?

A. Hsp70.

B. Hsp90.

C. BAG-3.

D. NEMO.

4. Which method is *not* preferred for tamoxifen treatment?

A. Feeding with tamoxifen food pellets.

B. Subcutaneous injections.

C. Adding tamoxifen to the drinking water.

D. Applying tamoxifen to the skin.

5. What needs to be checked after treatment of the mice before further investigation?

A. Check for deletion efficiency of the target gene.

B. Check for alterations of related signaling pathways.

C. Check the skin of these mice for a phenotype.

D. Check for behavioral changes of the mice.