Transgenic Mouse Technology in Skin Biology: Generation of Complete or Tissue-Specific Knockout Mice

Lukas Scharfenberger[1], Tina Hennerici[2], Gábor Király[3], Sophie Kitzmüller[4], Marigje Vernooij[5] and Julia G. Zielinski[1]

Journal of Investigative Dermatology (2014) **134**, e16; doi:10.1038/jid.2013.457

This article was written as part of the EADV/ESDR summer course Mouse Models in Skin Research, held 22–26 July 2013 at the University of Cologne.

Many human skin diseases are caused by gene mutations that result in either the loss of protein or an altered protein function. To understand how these mutations cause disease and to find possible therapeutic targets, it is crucial to use not only cell culture approaches but also *in vivo* investigations in a complex multicellular organism to assess the contributions of the macro- and microenvironment. Although clinical data provide a wealth of information on disease-causing mutations, it is often difficult to distinguish between cause and consequence in humans. The genetic and environmental variation is another complicating factor in understanding the underlying mechanisms that result in disease. Targeted genetic modifications in related mammals therefore offer great possibilities to investigate molecular mechanisms that underlie mammalian physiology and disease. The mouse is one of the most relevant research organisms because it shares 99% genetic identity with humans, inbred strains exist, and it has been very well characterized on both the genetic and behavioral levels (Paigen, 2003). Most importantly, the groundbreaking work of Mario Capecchi, Martin Evans, and Oliver Smithies in the 1980s, in which they discovered how to introduce specific gene modifications in mice using embryonic stem cells, opened the way to address directly the consequences of specific mutations. For this they received the Nobel Prize in Physiology or Medicine in 2007 (Vogel, 2007). We provide an overview of how to design (conditional) knockout mice and discuss their relevance for understanding human disease.

BENEFITS OF CREATING KNOCKOUT MICE

- A knockout is a mutation in a certain gene of interest resulting in the loss or decrease of function of the gene and the correlating gene product (protein).
- In comparison to whole-body knockouts, conditional knockouts (using the Cre-loxP system) allow the deletion of a certain gene in one specific tissue of interest.
- Via gene targeting, an altered gene can be inserted in the genome of a mouse.

LIMITATIONS

- Complete gene knockout does not always reflect the situation in human disease.
- Redundancy of related gene products may compensate and thus not reveal gene function.
- The *in vivo* situation in mice is not always fully representative of the *in vivo* situation in humans.

HOW TO CREATE A KNOCKOUT MOUSE

A gene knockout is the manipulation of endogenous DNA that results in nonfunctionality or complete loss of the corresponding protein. The knockout of a gene offers the possibility to investigate its usual function by examining the consequences of loss of the encoding protein in a living organism. Capecchi, Evans, and Smithies established the gene knockout strategy by "gene targeting," a method to introduce artificial DNA into mouse embryonic stem (ES) cells (Figure 1). ES cells are isolated from mouse blastocysts and they are pluripotent, which means they can differentiate and give rise to any organ in the mouse. The method of gene targeting uses the natural process of homologous recombination in which two identical (homologous) parts of a DNA sequence are exchanged. The *in vitro* engineered

[1]*Department of Dermatology, Center for Molecular Medicine, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, SFB829 Molecular Mechanism Regulating Skin Homeostasis, University of Cologne, Cologne, Germany;* [2]*Department of Dermatology and Allergy, Philipps University, Marburg, Germany;* [3]*Department of Microbial Biotechnology and Cell Biology, University of Debrecen, Debrecen, Hungary;* [4]*EB House Austria, Department of Dermatology, University Clinic, Salzburg, Austria and* [5]*Department of Dermatology, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands*

Correspondence: Lukas Scharfenberger, Department of Dermatology, Center for Molecular Medicine Cologne, Cologne Excellence Cluster on Cellular Stress Responses in Associated Diseases, Robert Kochstrasse 21, 50931 Cologne, Germany. E-mail: lukas.scharfenberger@gmail.com

DNA (targeting construct) consequently contains sequences that are homologous to a part of the endogenous genomic DNA that flank the target sequence.

After the targeting construct is delivered into the ES cell (mostly by electroporation), parts of the endogenous gene of interest are exchanged (recombined) with the modified engineered sequence (Figure 1). The targeting construct also contains an antibiotic resistance gene (in general, a "neo" gene that confers resistance to neomycin). Only ES cells that contain the recombined DNA will therefore survive the subsequent neomycin treatment. Because most DNA will integrate randomly into the genome, ES cell clones are then screened for homologous DNA recombination. The "targeted" ES cells are then transferred to the blastocoel cavity of a mouse embryo, which then is implanted in the uterus of a surrogate mother. The recipient embryo in which the manipulated ES cells are injected already contains ES cells. The surrogate mother will give birth to chimeric mice, in which a proportion of the tissues derive from the manipulated ES cell population, whereas the rest derive from the recipient embryo ES cells (so-called chimerism). If the manipulated ES cells contribute to the germline, further breeding of chimeric mice will result in homozygous gene-manipulated mice (Figure 1).

The creation of knockout mice has some drawbacks. First, the resistance gene that selects for positively targeted cells remains in the genome and may have an unpredictable effect on the mutant phenotype. Second, gene inactivation will occur in every cell of the mouse, and its protein product will thus be absent during all important developmental stages. Depending on the significance of the protein of interest in different developmental processes or cell lineages, this may compromise the viability of the knockout mouse. Finally, the protein's absence prevents the investigator from discerning whether the observed effects are tissue autonomous or involve secondary effects of the surrounding tissue. Hence, the removal of the resistance cassette and a specific (conditional) knockout in either a certain tissue or a developmental stage might be beneficial. For example, investigations on the protein Acvr1b were restricted because the total knockout led to embryonic lethality of affected mice. An epidermis-specific Acvr1b knockout was the solution to circumvent the early lethality and continue investigations on this protein in the epidermis (Qiu *et al.*, 2011).

CONDITIONAL GENE KNOCKOUT

Two analogous strategies were developed to overcome the above-mentioned limitations. Both involve site-specific recombination events discovered in bacteria or yeast, respectively (Lewandoski, 2001). In principle, an enzyme with a recombinase activity (Cre recombinase from bacteria or FLP enzyme from yeast) recognizes short (25–50 bp), specific DNA sequences (loxP or FRT sites, respectively) and recombines the DNA region that is flanked by these sequences in a directed way. This means that, depending on the orientation of these small recognition sites, the flanked region is either irreversibly removed or inverted. The discovery that this mechanism works *in vitro* as well as in other organisms, including mammals, has driven the generation of tissue-specific knockout constructs (Gu *et al.*, 1994). Because the Cre-loxP system was developed first and is more widely used than the FLP-FRT system, the Cre-loxP technique will be discussed here.

To create a tissue-specific conditional knockout mouse, two loxP sites, one upstream and one downstream of the functional part or the whole target gene to be inactivated,

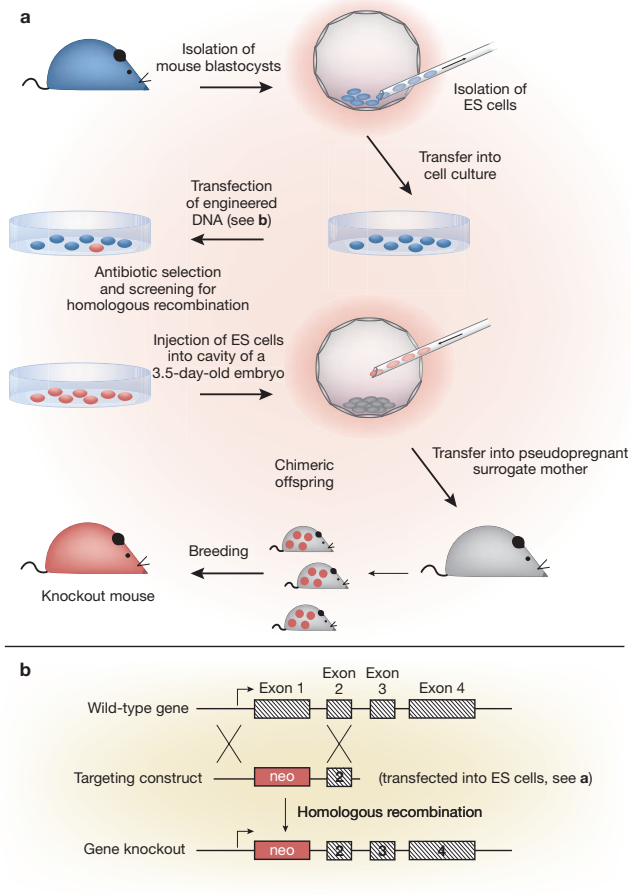

Figure 1. Generation of genetically modified mice. (**a**) Embryonic stem (ES) cells are isolated from blastocysts of pregnant mice. These cells are pluripotent; i.e., they can differentiate to every cell type of the adult organism. The ES cells are taken into cell culture and grown in nutrient medium. The modified DNA (see **b**) is then transfected into the cells by a process called electroporation. Not all of the cells will take up the DNA. Therefore, the cells are treated with an antibiotic, normally neomycin, and only cells containing the modified DNA and thus expressing the neomycin-resistant gene (neo) will survive. The modified ES cells are subsequently injected into a mouse blastocyst embryo, which is implanted into the uterus of a surrogate mother. The surrogate mother will give birth to so-called chimeric mice because their tissues are derived from both the modified ES cells and the recipient embryo's ES cells. Further breeding of the chimeric mice will lead to homozygous genetically modified mice. (**b**) After the targeting construct is transfected into the ES cell, homologous recombination will replace exon 1 with the neo gene, which results in a nonfunctional wild-type gene.

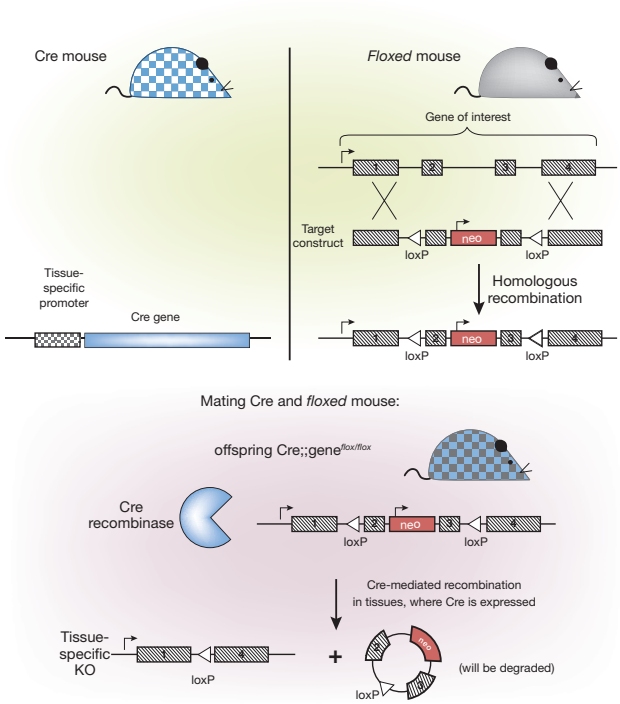

Figure 2. Schematic illustration of the Cre-loxP system. To create a conditional knockout (KO) mouse, two independent mouse strains must be generated and crossed (Figure 1): a "Cre-mouse," expressing the Cre recombinase under a chosen promoter that is specifically active in the tissue of interest, and a "*floxed* mouse." In this example, exons 2 and 3 from the gene of interest are targeted and will be deleted. To this end, a target construct is introduced in embryonic stem cells, in which exons 2 and 3 are flanked by loxP sites, that also includes a neomycin-resistance gene placed in the intron between exons 2 and 3. The construct also contains flanking regions that are homologous to regions of the gene of interest. Upon transfection, homologous recombination results in replacement of the endogenous gene of interest sequences with the target sequence, resulting in *floxed* exons 2 and 3 and hence a floxed "gene X" mouse. Crossing of the Cre mouse with the *floxed* mouse now gives rise to offspring in which the Cre gene is expressed in tissues where the chosen promoter is active. Only in these tissues does loxP site-specific recombination take place, resulting in the deletion of exons 2 and 3 and thus in tissue-specific gene knockout.

must be introduced into the genome. This is achieved by gene targeting with an engineered DNA construct that contains loxP sites at the required positions (Figure 2). A mouse carrying a *floxed* gene (written *gene*$^{flox/flox}$ or *gene*$^{fl/fl}$) will not be a knockout mouse unless the Cre recombinase is expressed and recombines the flanked DNA region. The *floxed* mouse is then crossed with a second mouse that carries the Cre recombinase gene downstream of a tissue-specific promoter. Cre will be expressed in cells in which this promoter is active, recombine at the loxP sites, and accordingly delete the functional part or the whole gene of interest. This will result in a knockout not only in all cells in which Cre is active but, importantly, also in all descendants of these cells. Conversely, no recombination will occur and the expression of the gene of interest remains unaffected in tissues in which the promoter that drives Cre expression is not active.

For studies in dermatology the keratin 14 (K14) promoter is often used to drive an epidermis-specific Cre recombinase expression. The murine K14 is only expressed in the basal epidermal keratinocytes (in addition to the tongue, thymus epithelium, and certain cells in the eye; see Figure 3d). Importantly, K14 is expressed in oocytes and therefore must be carried only by males because in females expression would result in gene activation early during development. The mouse line with a K14-Cre;;gene$^{fl/fl}$ genotype will therefore have a gene knockout in which the Cre recombinase is or was expressed, which is the whole skin epidermis, because all epidermal cells derive from the basal keratinocytes (Clayton *et al*., 2007; Qiu *et al*., 2011). Depending on the genotype of the parental mice, the knockout mice will come in a certain Mendelian ratio. Therefore, it is always mandatory to confirm the genotype via PCR and to check whether the gene and thus the protein really is ablated in the targeted tissue (Qiu *et al*., 2011).

ADVANTAGES AND LIMITATIONS

The Cre-loxP system has been very useful to overcome embryonic and early postnatal lethality of classic gene knockout strategies. It also has the advantage of allowing investigations on the function of a gene/protein in a cell- or tissue-specific manner without indirect effects of the surrounding organs. Once a functional Cre line is generated it can be crossed to any *floxed* mouse strain and vice versa, entailing a very large variety of mouse models. There are also mouse lines available in which Cre recombinase expression is driven by a promoter that is already active in the germline, thus resulting in a total body knockout. International consortia and companies nowadays offer the generation of knockout or *floxed* mice for almost every gene (Austin *et al*., 2004) and a large range of *floxed* and Cre mouse lines that have a reported biological relevance are available (e.g., http://informatics.jax.org/recombinase.shtml).

A potential technical limitation of mouse knockout technology could be that deletion of part of the gene does not result in a full loss of protein, as anticipated, but instead results in a truncated protein with different function. Furthermore, the introduction of a transgene (such as Cre) might affect more than just the target protein (Gangoda *et al*., 2012). Perhaps most importantly, the limitations of mouse knockouts to study human diseases mostly involve the disadvantages of the mouse model itself. Not every human disease will have a suitable mouse model and vice versa, and given the complexity of both organisms, a translation from the mouse model to humans will not always be directly possible (Seok *et al*., 2013). To simulate certain diseases, investigate gene functions, or test drug treatment in mice, it may also be inappropriate to knock out a gene. Instead, it might be suitable to use alternative strategies to influence the expression of the gene of interest, such as a gene knockdown to reduce but not completely prevent the gene expression (Wang *et al*., 2007). Further adjustments of the Cre-loxP system, for instance, to not delete but induce gene expression or introduce a point mutation into the gene of interest (so called knockins) or to control the

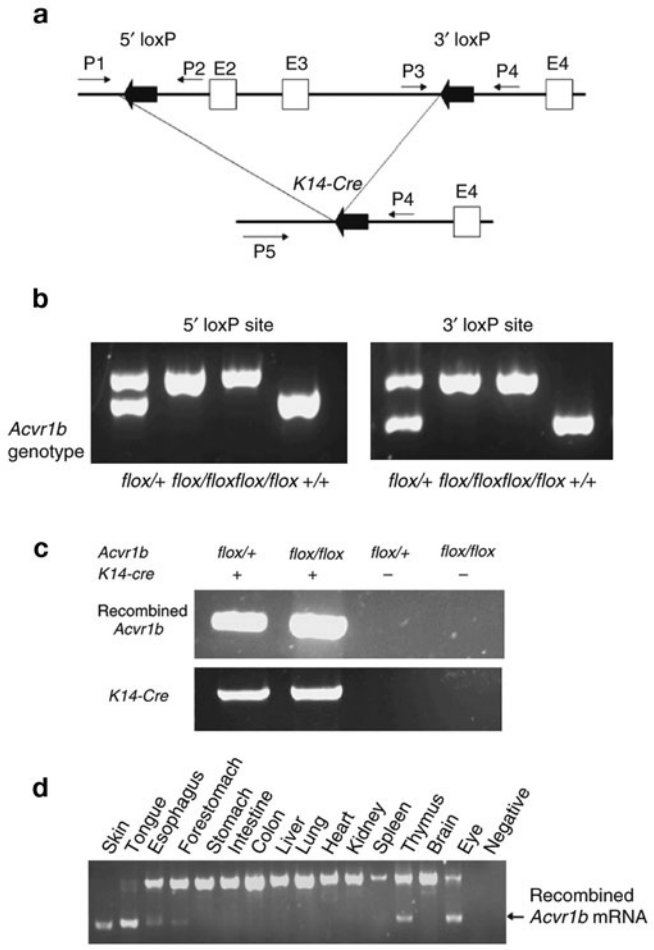

Figure 3. Knockout and genotyping PCR. (**a**) Exons (E) 2 and 3 of the *Acvr1b* gene are flanked by loxP sites. Primers (P) 1–5 were designed for genotyping PCR. (**b**) *Acvr1b* genotyping using P1/P2 or P3/P4. The upper bands include a loxP site and are therefore bigger than the wild-type allele (lower bands). (**c**) PCR with primers P4/P5 detecting the recombined DNA and confirming that it is recombined only when Cre is expressed under the K14 promoter. (**d**) PCR with P4/P5 indicating in which tissue the recombination occurred (Qiu *et al.*, 2011).

induction of Cre expression in a temporal manner (inducible knockout/knockin), harbor diverse opportunities to investigate specific gene functions *in vivo*.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

This CME activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the Joint Sponsorship of ScientiaCME and Educational Review Systems. ScientiaCME is accredited by the ACCME to provide continuing medical education for physicians. ScientiaCME designates this educational activity for a maximum of one (1) AMA PRA Category 1 Credit™. Physicians should claim only credit commensurate with the extent of their participation in the activity.

To take the online quiz, follow the link below:
http://www.classmarker.com/online-test/start/?quiz=rcf52728b592f502

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 determines where a conditional knockout occurs?

A. The promoter that drives Cre expression.

B. The promoter that drives neo expression.

C. The promoter that drives loxP expression.

2. How is a modified DNA construct introduced into the mouse genome?

A. Intradermal injection in adult mice.

B. Via gene targeting and injection into ES cells.

C. Application via drinking water in pregnant females.

3. What is a conditional knockout?

A. The deletion of a gene in every cell of the mouse.

B. The knockout of a gene in only a certain tissue or at a certain developmental stage.

C. A knockout affecting more than one gene.

D. A knockout inducible at a later time point.

4. What is a *floxed* gene?

A. The target sequence that shall be removed surrounded by two loxP sites.

B. A mutated gene that is still producing a functional protein.

C. A gene that has a high natural mutation rate.

5. How can a conditional knockout mouse be created?

A. By crossing a mouse carrying a *floxed* gene with a mouse expressing a Cre recombinase.

B. By crossing mice carrying loxP sites with different orientations.

C. By crossing a mouse carrying a gene for the Cre recombinase with one carrying the gene for FLP.

6. What is a promoter?

A. A gene that stimulates DNA replication in ES cells.

B. A drug that enhances the embryonic growth rate in mice.

C. A DNA fragment in front of a gene that drives its transcription.

ACKNOWLEDGMENTS

L.S. is supported by the German Cancer Aid, and J.G.Z. is supported by the DFG.

SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2013.457.

REFERENCES

Austin CP, Battey JF, Bradley A *et al.* (2004) The knockout mouse project. *Nat Genet* 36:921–4

Clayton E, Doupe DP, Klein AM *et al.* (2007) A single type of progenitor cell maintains normal epidermis. *Nature* 446:185–9

Gangoda L, Doerflinger M, Lee YY *et al.* (2012) Cre transgene results in global attenuation of the cAMP/PKA pathway. *Cell Death Dis* 3:e365

Gu H, Marth JD, Orban PC *et al.* (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type–specific gene targeting. *Science* 265:103–6

Lewandoski M (2001) Conditional control of gene expression in the mouse. *Nat Rev Genet* 2:743–55

Paigen K (2003) One hundred years of mouse genetics: an intellectual history. II. The molecular revolution (1981–2002). *Genetics* 163:1227–35

Qiu W, Li X, Tang H *et al.* (2011) Conditional activin receptor type 1B (Acvr1b) knockout mice reveal hair loss abnormality. *J Invest Dermatol* 131:1067–76

Seok J, Warren HS, Cuenca AG *et al.* (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 110:3507–12

Vogel G (2007) Nobel Prizes. A knockout award in medicine. *Science* 318:178–9

Wang Q, Ilves H, Chu P *et al.* (2007) Delivery and inhibition of reporter genes by small interfering RNAs in a mouse skin model. *J Invest Dermatol* 127:2577–84