Chapter 24

Adenoviral and Transgenic Approaches for the Conditional Deletion of Genes from Mammary Tissue

Kay-Uwe Wagner, Edmund B. Rucker III, and Lothar Hennighausen

Abstract. Over the past decade the tools of gene targeting have permitted an unparalleled insight into genetic pathways that control mammary development and tumorigenesis in the mouse. However, the role of many genes in development and disease remains elusive, since their deletion from the mouse genome is either lethal for the mouse or does not mimic human disease progression. Thus, targeting gene deletions or modifications precisely to mammary epithelial cells during distinct time windows is a promising approach to establish high-fidelity mouse models for the study of development and disease.

Abbreviations. adenoviral (Ad); β-lactoglobulin (BLG); cytomegalovirus (CMV); embryonic stem (ES); gene of interest (GOI); long terminal repeat (LTR); mouse mammary tumor virus (MMTV); whey acidic protein (WAP).

INTRODUCTION

For more than a decade, gene targeting and the generation of knockout mice have been powerful genetic tools to evaluate gene functions in the context of the living organism. This approach has provided mechanistic insight into genetic pathways guiding mammary development and function (1). In many cases, however, the deletion of a gene results in embryonic lethality, and more sophisticated approaches have to be employed to study development of the mammary epithelium. If the fetus dies after day E13.5, transplantation of a mutant mammary gland anlagen into the cleared fat pad of a wild-type recipient can be used to study epithelial development (2). However, in those cases in which the fetus dies prior to E13.5 (e.g., deletion of *Brca1*, *Brca2*, *Rad51*, or *Bcl-x*), mammary anlagen cannot be rescued by transplantation. Furthermore, a phenotype observed in the mammary epithelium of viable gene deletion mice may not be the result of cell autonomous mechanisms. The deletion of

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some genes results in the altered synthesis or release of systemic factors, which in turn causes secondary effects on the mammary epithelium (3, 4). To more accurately examine molecular pathways that lead to distinct alterations in the mammary epithelium of certain mouse models, it is necessary to separate primary functions of a protein in the developing tissue from secondary effects. An understanding of the mechanisms becomes even more complex when other exogenous factors, such as aberrant nursing behavior, become modulating factors (5). Furthermore, it has been reported that the genetic background can modulate the penetrance of phenotypes (6, 7). To minimize these limitations new technologies are required that allow for the specific alteration of genes in defined cell types. Not only are developmental biologists in need of such tools, but cancer researchers would also benefit from an improved knockout technology. Some mutations of tumor suppressor genes introduced into the germ line of mice result in different types of tumors than expected from the human situation. For example, mutations in the *Rb* gene cause retinoblastomas in humans, whereas mice lacking *Rb* develop pituitary tumors (8). Besides germ-line mutations, most human cancers are the result of sporadic acquired mutations in a limited number of cells that are surrounded by normal tissues. Such defined mutations can now be modeled in a temporal and spatial fashion with a new technology called *conditional or tissue-specific gene targeting*.

THE CRE-LOXP RECOMBINATION SYSTEM IS A TOOL FOR CONDITIONAL GENE TARGETING

Cre-loxP and Flp-frt are two recombination systems that were adopted from their original hosts (bacteriophage P1 and *Saccharomyces cerevisiae*, respectively) to generate site-specific DNA modifications in tissue culture cells (9) or in transgenic mice (10–12). They are both binary systems, in which the recombinase (Cre or Flp) recognizes its specific target sequence (loxP or frt) and catalyzes the deletion, insertion, or inversion of the DNA sequence located between the target sites. Initially, the Cre-loxP system was shown to be more efficient than the Flp-frt system in embryonic stem (ES) cells or transgenic mice. Despite recent improvements of the Flp-frt system (13), the Cre-loxP recombination system is already more widely used, and a large number of mutant mice exists with either a Cre transgene (for a list of mice see http://www.mshri.on.ca/nagy/cre.htm) or a targeted endogenous gene with inserted loxP sites ("floxed" genes). After legal issues regarding the unrestricted use of the Cre-loxP system in basic research have been settled (14), this system will probably remain the leading technology for conditional gene modifications in the future.

The mechanism underlying the Cre-loxP system is illustrated in Figure 24-1. Briefly, a loxP (locus-of-crossing-over) site is a 34 bp DNA sequence (Figure 24-1A) consisting of two inverted repeats and a nonpalindromic spacer region that determines its orientation. Several monomers of Cre recombinase bind to each of the loxP recognition sites and excise the DNA fragment between two directly orientated loxP sites as a circular segment, leaving one loxP site in the chromosome (Figure 24-1B). To a lesser extent, this recombination event is reversible (integration). Two other excellent features of the Cre-loxP recombination system have been revealed recently: First, the distance between two loxP sites does not seem to be a limiting factor because several cM of intervening sequences between loxP sites have been successfully deleted (15). Second, recombination events between homologous and even non-homologous chromosomes can be achieved (16). The Cre-loxP system will not only be used to delete important parts of a particular gene in an organ of interest, for example the mammary gland, but also to induce cytogenetic modifications, such as chromosomal loss or rearrangements on mouse chromosomes, in a tissue-specific fashion.

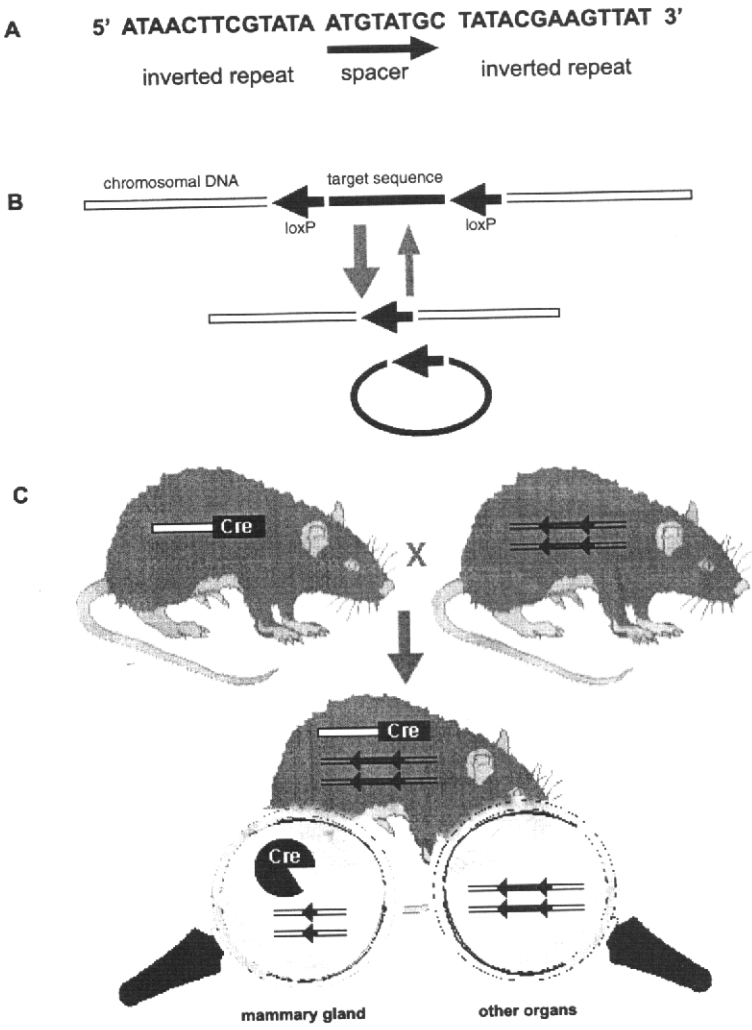

Figure 24-1. Characteristics of the Cre-loxP recombination system. (A) Sequence of a loxP (locus-of-crossing-over) recognition site. (B) Cre-mediated excision of a target sequence flanked by two directly orientated loxP sites. (C) Simplified breeding strategy for a tissue-specific gene deletion in transgenic mice.

Taken together, the alteration of loci that are syntenic to the human genome and that are involved in mammary tumorigenesis will permit the design of high-fidelity mouse models for breast cancer. The first and most important step for mammary gland biologists toward this long-term goal is the development of methods to target the expression of Cre recombinase to specific cell types in the mammary tissue. Once these tools have been established, they can be combined with mouse models that contain one or more floxed loci. In this chapter we describe techniques used to express Cre specifically in the mammary epithelium. Furthermore, we discuss common strategies to insert loxP recognition sites into defined genes by gene targeting.

TECHNIQUES TO EXPRESS Cre RECOMBINASE SPECIFICALLY IN THE MAMMARY GLAND

Transgenic Approach

The transgenic approach requires the generation of transgenic mice that express Cre recombinase specifically in mammary tissue. Upon crossing these mice into strains that contain a floxed gene (gene flanked by loxP sites), the mammary-specific deletion of this gene can be achieved (Figure 24-1C). Depending on the spatial and temporal expression of the Cre transgene, excision of the floxed target gene occurs only in those cells that expressed active recombinase at any time during its ontogeny. The floxed target gene remains unrecombined in all those cells that lacked Cre expression. Since a complete inactivation requires the Cre-mediated deletion of both alleles, it is an advantage to introduce a conventional knockout allele. In progeny that carry the Cre transgene, along with a floxed and a knockout allele, deletion of the floxed gene can be achieved more easily.

Several genetic regulatory elements have been used successfully to target distinct cell types in mammary tissue. The LTR of the mouse mammary tumor virus (MMTV) and promoters of milk protein genes, such as the mouse whey acidic protein gene (WAP) and the ovine β-lactoglobulin gene (BLG), have been used to target growth factors and foreign proteins to various compartments of the mammary epithelium. Other promoters can be utilized for a targeted expression of transgenes in the myoepithelium (keratin 14 promoter) or the adipose stroma (aP2 promoter). Some of these promoters have also been applied to target Cre recombinase to mammary tissue. Formerly, MMTV-Cre, WAP-Cre (17), and BLG-Cre (18) transgenic mice have been reported that express Cre in the ductal and lobuloalveolar epithelium. The aP2-Cre mice can be used to delete genes in adipocytes (19). Transgenic lines with a targeted expression of Cre in the fibrous stroma or myoepithelium have not yet been published. However, no promoters are available to date that permit the expression of Cre exclusively in connective tissues of the mammary gland.

MMTV-Cre Transgenic Lines

The value of a Cre transgenic line for tissue-specific gene deletion is determined by at least two parameters: First, the amount of background recombination in other organs due to a "leakiness" of the regulatory elements in the transgene or the site of integration. Second, the extent of recombination within defined cell types as the result of a mosaic expression pattern of the Cre transgene. Although "tissue-specific" promoters have been used successfully to target growth factors to various cell types, many may not be adequate to regulate Cre recombinase expression tightly (20). Transient activation of the transgene during earlier developmental stages leads to low levels of Cre expression, which in turn can result in significant background recombination. For example, the MMTV–LTR is highly expressed in only a few tissues of adult mice, but MMTV-Cre-mediated recombination was detected in most organs, indicating that the LTR was active at earlier developmental stages or at constant low levels (17). This expression pattern is not dependent on the integration site of the transgene since it was observed in four independent lines. Some MMTV-Cre strains even exhibit extensive recombination in the female germ line, which seems to be integration-site dependent. The germ-line recombination was verified by crossing wild-type males with females that had the MMTV-Cre transgene and one floxed allele of an endogenous locus. The transmission of the floxed allele was analyzed in mice of the subsequent generation that had inherited the

loxP locus but not the Cre transgene (segregated loxP allele). The MMTV-Cre line A shows a 100% recombination efficiency in oocytes, whereas the line D does not show any recombination in germ cells (Wagner, unpublished). Because of this feature, the MMTV-Cre line A was used successfully in several experiments to create complete knockout alleles from floxed loci (Rucker and Wagner, unpublished).

Despite the fact that MMTV-Cre mice exhibit background recombination in a variety of organs, these strains are still valuable for mammary-specific gene deletion experiments. In contrast to conventional belief, a mosaic deletion of genes that are essential for early embryonic development did not result in prenatal lethality (21; Wagner and Rucker, unpublished). MMTV-Cre transgenic mice can be used to delete genes in the ductal epithelium of virgin females without administration of exogenous hormones (17). This feature is important not only for studying gene function during ductal development but also for modeling specific types of cancer, such as ductal carcinoma *in situ* (21), and for experiments that require treatment of mice with mutagens such as DMBA or radiation. Nevertheless, the amount and timing of MMTV-Cre-mediated recombination in the ductal epithelium of various lines remains to be determined when suitable reporter mice become available.

If MMTV-Cre mice are to be used in mammary-specific gene deletion experiments one needs to be aware of several important limitation. All lines, regardless of their ability to recombine floxed loci in the germ line, exhibit Cre-mediated recombination in many tissues, including the tail. If a PCR assay on DNA of a tail biopsy is used to verify the genotype of a given mouse that has inherited the MMTV-Cre transgene from one parent and a loxP allele from the other parent, it is also possible to identify a recombined locus. Therefore, it is not a PCR artifact if three alleles (wild type, floxed, recombined–null) are detected in these transgenic mice. In fact, the PCR assay for the recombined allele can be used to verify the presence of MMTV-Cre in intercrosses (F1 generation). However, it is practically impossible to determine exactly the genotype by PCR in the subsequent (F2) generation when a recombined–null allele is used in combination with a floxed locus and a MMTV-Cre transgene. The null–recombined allele can be detected in mice that have inherited the MMTV-Cre transgene and either two floxed alleles or one floxed and one null–recombined allele. This problem can be bypassed by using Southern blotting or a null allele from a conventional knockout that can be detected independently from the floxed or recombined locus in a separate PCR assay. Another option to avoid this obstacle is a specific mating strategy. There are no limitations in breeding schemes if one utilizes exclusively floxed alleles in combination with MMTV-Cre lines that do not show any expression of Cre in the germ line, for instance mice of the MMTV-Cre line D. Alternatively, the following mating scheme can be applied if one uses a null–recombined allele of a gene of interest (GOI) to increase the recombination efficiency:

$$\underset{\text{MMTV-Cre(D) GOI}^{\text{null/wild type}}}{\text{Female or male}} \times \underset{\text{MMTV-Cre(D) GOI}^{\text{lox/lox}}}{\text{Male or female}}$$

Homozygous knockout (null) mice could be used to simplify the breeding strategy if this genotype does not lead to early lethality or reproductive problems. The breeding scheme has to be modified if a strain is used that expresses Cre in the female germ line, such as MMTV-Cre line A. For maintaining a nonrecombined loxP allele of the GOI one needs to follow this breeding strategy:

$$\underset{\text{GOI}^{\text{lox/lox}}}{\text{Female}} \times \underset{\text{MMTV-Cre(A) GOI}^{\text{lox/lox}}}{\text{Male}}$$

A breeding strategy with a null allele via Cre-mediated recombination in the female germ line is very simple and can be performed efficiently with this mating scheme:

$$\underset{\text{Female}}{\text{MMTV-Cre(A) GOI}^{\text{lox/lox}}} \times \underset{\text{Male}}{\text{MMTV-Cre(A) GOI}^{\text{lox/lox}}}$$

Since all floxed loci will be recombined in the oocyte, all littermates will inherit uniformly a null–recombined allele from the mother and a floxed locus from the father. This efficient breeding strategy is only suitable when the GOI is not essential for oocyte development. It might be problematic if a deletion of the gene affects the survival of the pups due to the inability of the female to lactate. In that case the pups can be fostered, or the matings have to be performed in a less efficient way by using MMTV-Cre(A) $\text{GOI}^{\text{lox/wild type}}$ or $\text{GOI}^{\text{null/wild type}}$ females.

Cre Expression under Regulatory Elements of Milk Protein Genes

WAP-Cre (17) and BLG-Cre (18) transgenic mice are valuable tools to delete genes specifically from the mammary epithelium. Since WAP and BLG are milk protein genes, it is not surprising that Cre-mediated recombination increased during pregnancy and peaked at lactation. Between 50% and 80% of the epithelium underwent recombination (18, 22), and both strains exhibited very little recombination in other tissues. We have evidence to suggest that these low levels of recombination are of no physiological consequences. For instance, the deletion of the *Bcl-x* gene from the mouse genome results in massive neuronal cell death (23), but the low-level recombination in the brain of WAP-Cre/$\textit{Bcl-x}^{\textit{loxP/null}}$ mice is without apparent physiological consequences (Rucker, unpublished). Since Cre expression in the WAP-Cre and BLG-Cre transgenic mice is highest during pregnancy and lactation, they can be used to delete genes in these stages of mammogenesis. In contrast, the recombination efficiency is significantly lower in virgin mice. Although the secretory epithelium undergoes programmed cell death after the pups are weaned, some recombined cells remain in the gland of WAP-Cre transgenics (17). During subsequent pregnancies those cells probably give rise to a new population of epithelium with pre-recombined alleles, thus explaining the higher recombination efficiency in subsequent pregnancies.

WAP-Cre mice have been used to delete the *Brca1* gene specifically from the mammary epithelium (21). The loss of BRCA1 resulted in abnormal ductal outgrowth and the formation of mammary tumors after a long latency. BRCA1 deficiency led to genetic instability and increased apoptosis. In subsequent pregnancies, WAP-Cre expression initiated numerous recombination events. This was essential for the onset of tumorigenesis in this disease model and is important from a technical perspective. For example, a single recombination event initiated by transient expression of Cre via adenoviral vectors (AdCre) during the first pregnancy did not result in the induction of tumors despite a reasonably large number of recombined cells. Those manipulated cells could be detected by PCR also after several pregnancies, but no tumors originated in AdCre-treated $\textit{Brca1}^{\textit{fl/null}}$ females during a period of 20 months (Wagner and Xu, unpublished). Therefore, we hypothesize that WAP-Cre expression is necessary to generate an increasing number of BRCA1-deficient cells during every pregnancy period. These cells are susceptible to alterations of the DNA since they are rapidly dividing and, coincidentally, lack BRCA1. The BRCA1 protein is a caretaker, not a gatekeeper. Among other functions, BRCA1 has been suggested to act in a complex with proteins (e.g., Rad51) that are essential for DNA repair. In the mammary gland, the expression of BRCA1 is elevated in dividing epithelial cells especially during pregnancy (for a review see Ref. 24).

Hence, WAP-Cre-mediated *Brca1* gene deletion during pregnancy predisposes a large pool of cells to acquire additional mutations. We do not know whether these mutations follow a common pattern. However, some mutations frequently affect gatekeeper genes such as *Trp53* (21).

Improved Cre Transgenic Strains for Mammary-Specific Gene Deletion

Because of the inherent limitations encountered in the MMTV-Cre, WAP-Cre, and BLG-Cre mice, it may be necessary to introduce additional levels of control. First, an inducible Cre recombinase can be utilized either through dual systems, such as the tetracycline inducible system (25), or the fusion of Cre to other regulatory proteins, such as estrogen receptor (26), progesterone receptor (27), or glucocorticoid receptor binding domains (28). Second, the inducible Cre recombinase needs to be targeted to the mammary gland in such a way that the window of Cre expression is expanded and the mosaic expression of the transgene is minimized. Unfortunately, both criteria cannot be fully accomplished with the genetic tools available today. For instance, the efficiency of recombination in binary inducible systems is unsatisfactory (25), and the utilization of hormone binding domains in Cre fusion constructs has limitations since all ligands (estrogen, tamoxifen, dexamethasone, etc.) are also biologically active in the gland. The mosaic expression of a Cre transgene in the mammary epithelial compartment may be eliminated through a knock-in approach, i.e., the insertion of the Cre coding sequence into an endogenous locus that is expressed in the mammary gland. An earlier expression of this locus in other organs is not a limiting factor when it is combined with one of the Cre inducible systems mentioned.

Cre Expression in the Mammary Gland through Adenovirus-Mediated Gene Transfer

Adenovirus-mediated gene transfer is a suitable approach for the temporal expression of Cre. Recombinant adenoviral (Ad) vectors are able to infect nondividing cells without integrating into the genome of the host. Therefore, they are perfect tools to shuttle foreign genes into a variety of tissues. Cre has been targeted successfully with Ad vectors to various cell types *in vitro* (29) and *in vivo* (30, 31). To examine the possible utilization of those vectors for a mammary gland-specific gene deletion, we have delivered an AdCre vector to mammary tissue by surgical injection. Furthermore, we have infected primary mammary epithelial cells with AdCre in an attempt to combine an *in vitro* gene deletion technology with the mammary transplantation method (22).

Direct Injection of AdCre into the Mammary Gland

MATERIALS AND INSTRUMENTATION. Although recombinant adenoviral vectors are replication deficient, special care should be taken to avoid contamination or self-infection. The production and purification of adenovirus require a P2 cell culture facility, and we recommend performing the transfer of the vectors into the mouse in a biosafety hood. Infected instruments should be sterilized, and all nonrecyclable materials should be treated as biological waste. The following instruments and materials should be available: dissecting microscope (e.g., Zeiss Stemi SV11), 50- or 100-µl Hamilton syringe with a 25-gauge ($\frac{5}{8}$ inch) needle (purchased from Thomas Scientific), cotton swab, cork board, wound clips, and other instruments to perform a surgery as described by Robinson *et al.* in Chapter 26 this volume (2).

REAGENTS AND REAGENT PREPARATION

1. A recombinant adenovirus vector containing a CAG-Cre transgene (AxCANCre) was described earlier (29). CAG is a ubiquitously expressed regulatory element containing the cytomegalovirus (CMV) immediate early enhancer and regulatory elements of chicken β-actin promoter.
2. The adenovirus was propagated in 293 cells (Quantum Biotechnology, Canada) and purified according to a procedure described in detail by Li *et al.* in Chapter 21, this volume (32). The purified virus was stored in 20- to 50-μl aliquots (2.8×10^9 pfu) at −70°C. The viral stock was thawed on wet ice, diluted 1 : 10 with chilled 1 × PBS (Gibco BRL), and used instantly for injection. The dilution step is important to adjust the final virus concentration and to lower the glycerol concentration to ensure maximum infectivity.

DETAILED PROCEDURES. The mice were anesthetized and placed under a dissecting microscope. Initially we tried to inject the viral solution through the nipple by using a fine glass needle. However, we were not able to inject enough solution to penetrate the gland. Instead, we delivered the virus by surgical injection into one inguinal gland (no. 4) as shown in Figure 24-2. For the surgical procedure refer to Chapter 26 in this volume (2). The skin was dissected from the body wall and the gland was exposed by pinning the skin flap to a cork board. A 100-μl Hamilton syringe with a 25-gauge needle was used to administer the viral solution. The preferred injection site was close to the nipple at the base of the ductal tree. The viral

Figure 24-2. Exposure of the left gland no. 4 prior to injection with recombinant adenovirus by surgical means. The arrow indicates the injection site near the ductal base.

solution (10 to 30 µl) was injected very slowly into the gland. This procedure leads quite often to an accumulation of fluids in the form of a small cyst. Most of the viral solution stayed in place after the needle was cautiously withdrawn from the injection site. Occasionally, very small amounts of liquid appeared after retraction, and cotton swabs were used to remove them immediately. After injection, the surgical site was closed with wound clips. All materials used during the procedure were disinfected immediately, autoclaved, and disposed according to safety guidelines for hazardous materials.

IMPORTANT NOTES. A single injection is sufficient to infect a large portion of the mammary gland no. 4 as determined by co-injection of a Cre vector and a loxP reporter virus (22). Since the Cre construct contains the CAG promoter, it expresses transiently in all cell types (epithelium or stroma) that are susceptible to adenoviral infection. We conclude from our co-injection experiment that adenoviral vectors infect predominantly the epithelial compartment and to a lesser degree adipocytes. The virus is able to migrate a considerable distance from the basis of the ductal tree (injection site) through smaller ducts into the alveolar compartment. This suggests that most of the viral solution spreads through the ductal system and less of the suspension migrates through the fat pad. However, we also observed a massive invasion of lymphocytes into all regions of the gland.

Once injected, AdCre can efficiently mediate the recombination between loxP sites on chromosomes *in vivo*. Three days after the viral transfer, the recombination was examined by PCR on a reporter construct (22) and the floxed *Brca1* locus (Wagner and Xu, unpublished). Mammary-specific recombination was achieved in virgin, pregnant, and lactating glands. Among all other organs that were analyzed for background recombination, only the liver showed detectable amounts of Cre-mediated gene deletion. The liver-specific Cre activation was found repeatedly, and we assume that viral particles migrated from the mammary gland via the circulation to that organ. The passage of viral particles into the capillary system of the bloodstream could have been caused by the surgical injection method. As shown previously, the liver is extremely susceptible to adenovirus infection when viral particles were injected intravenously (31).

Furthermore, we could demonstrate that mammary gland remodeling during involution influences the amount of recombination (22). AdCre injection during the first pregnancy resulted in satisfactory recombination efficiencies. However, when analyzed during the second pregnancy cycle, 6 to 8 weeks after virus administration, only a few recombined cells remained in the mammary gland and the recombination efficiency decreased significantly. These observations suggest that mammary gland remodeling during the reproductive phase has a significant influence on the recombination efficiency. This may distinguish the mammary gland from other organs that do not undergo cyclic renewal of various cell types. These data are of particular interest when this technology is applied for studies in the mammary gland that require a high amount of recombination over a long period of time in order to determine a measurable effect of the deleted gene on the physiology of the gland. Under experimental conditions using an inactive floxed transgene, there was also no selective pressure on either the recombined or unrecombined allele. However, the loss of BRCA1 due to Cre-mediated excision leads to programmed cell death because of genomic instability and the subsequent activation of apoptotic pathways. Taken together, there are several intracellular and extracellular mechanisms that can have a profound influence on the efficiency of gene deletion by using AdCre: (1) loss of cells due to immune response against viral proteins in infected tissues; (2) loss of cells due to apoptosis at the end of each consecutive reproductive cycle; (3) loss of cells triggered by apoptotic pathways due to the Cre-mediated excision of the

targeted gene. Nevertheless, a reduced level of recombination in subsequent pregnancies may not be a limiting factor for a mammary-specific knockout of a tumor suppressor gene that is a gatekeeper rather than a caretaker. In that case, the recombination event could lead to a selective amplification of the remaining recombined cells (i.e., tumor formation).

A POTENTIAL COMBINATION OF MAMMARY TRANSPLANTATION AND AdCre-MEDIATED GENE DELETION

The property of mammary epithelium to undergo extensive remodeling after weaning raised experimental problems in utilizing the adenoviral approach to delete genes in the gland. However, this characteristic might serve as the basis for other emerging Cre-loxP technologies. The presence of stem cells in the gland, which give rise to differentiated cell types of the mammary epithelium (ducts, alveoli), allows an *ex vivo* manipulation and subsequent transplantation of modified epithelial cells. A combination of AdCre with cell transplantation (Figure 24-3) could greatly enhance the recombination efficiency. This approach will require isolation of epithelial cells from a donor with a floxed gene, infection with AdCre, and transplantation of the recombined cells into a recipient with a cleared fat pad. These knockout cells will then contribute to the reconstitution of the ductal tree. In initial studies, we were able to show that HC11 cells (22) and primary epithelial cells (32) can be infected very efficiently with adenovirus and that Cre-mediated recombination can be achieved in culture (22). Primary cells from both virgin and lactating animals showed a considerable amount of excision. Potentially, these cells could be transplanted into a cleared fat pad by using a technique similar to that described by Robinson and co-workers in this volume (2). The success of this

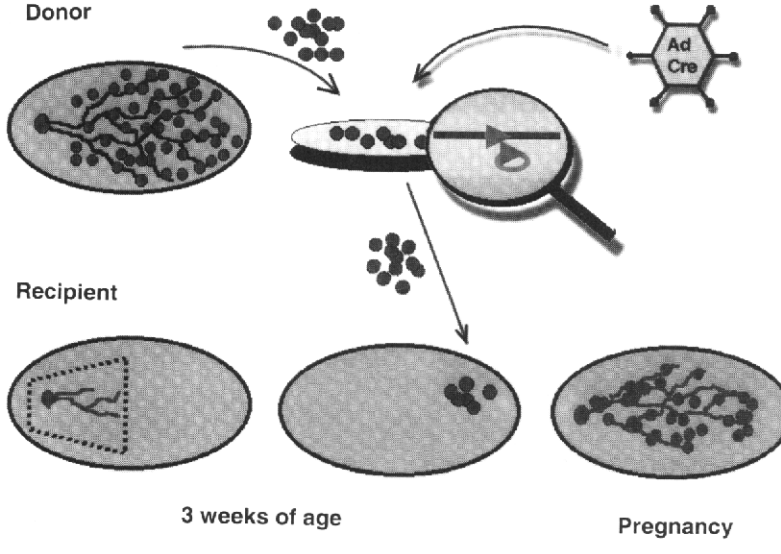

Figure 24-3. Combination of mammary transplantation and AdCre-mediated gene deletion. This approach requires the isolation of epithelial cells from a donor with floxed alleles of a target gene, the infection with AdCre *in vitro*, and the transplantation of the recombined cells into a wild-type recipient. These knockout cells contribute to the reconstitution of the ductal tree if no selection against stem cells with recombined alleles occurs.

technique depends largely on selective mechanisms against recombined stem cells versus unrecombined cells during ductal outgrowth. Nevertheless, when this technology is markedly improved, it will serve as a new tool to study the loss of function of specific genes in the mammary gland.

TRANSGENIC VERSUS ADENOVIRAL APPROACH

An accurate comparison of different techniques for the deletion of genes from mammary tissue is difficult. Generally, it is almost impossible to compare published data about deletion efficiencies between various floxed loci or a particular locus in different organs and simply project these results to the tools used in these specific approaches. The position of the floxed locus and the distance between the loxP sites can greatly influence the recombination efficiency. In addition, selective pressure (positive or negative) on the growth of recombined alleles in different organs may not adequately represent the true extent of recombination. For example, a conditional knockout of *Brca1* in the mammary gland leads to genomic instability and increased apoptosis (21). Therefore, one should expect a different scenario from gene deletions that act in pro-apoptotic pathways. The experimental design will determine whether a transgenic or adenoviral approach should be used. The transgenic approach is clearly the method of choice when high levels of recombination over a longer period of time (e.g., several lactation periods) are required to evaluate physiological effects. For short-term experiments or certain tumorigenicity studies, the adenoviral approach is of particular interest because this method requires less mouse breeding and the recombination event can be achieved at a specific developmental stage (time point of AdCre injection). However, several properties of the AdCre technology need to be improved. First, the immune response against the adenovirus needs to be minimized. The invasion of immune cells into the mammary gland could be significantly lowered through the use of Ad vectors that do not contain viral genes (33) or through the use of lenti viruses. Second, the tissue specificity of Cre expression needs to be improved. AdCre can infect different cell types in the mammary gland. Subsequently, the recombinase excises the floxed gene in all of these cell types since the Cre transgene is under the control of a ubiquitously expressed promoter (e.g., CAG). Therefore, these promoters need to be replaced with regulatory elements for the epithelial compartment. At present, this could be the MMTV–LTR. The utilization of a tissue-specific promoter will also reduce background recombination in other organs.

INSERTION OF LOXP RECOGNITION SITES INTO DEFINED LOCI

Gene targeting is a technique that allows for the intentional alteration of a defined locus within the genome. It can be used to delete, change, or insert additional sequences (e.g., loxP sites) into endogenous genes. The general schematic for gene targeting in ES cells and targeting construct design are based on a positive–negative selection system shown in Figure 24-4. The first step of the system is based on using a positive selectable cassette, the neomycin phosphotransferase gene (*neo*), which is flanked by two regions of homology to the target gene. Therefore, random or homologous integration of this DNA results in the resistance of cells to G418. The second step involves the negative selection cassette, the HSV-thymidine kinase gene (TK), which is placed outside the region of homology in the vector. This TK cassette is integrated into the host chromosome if random integration occurs, but it is

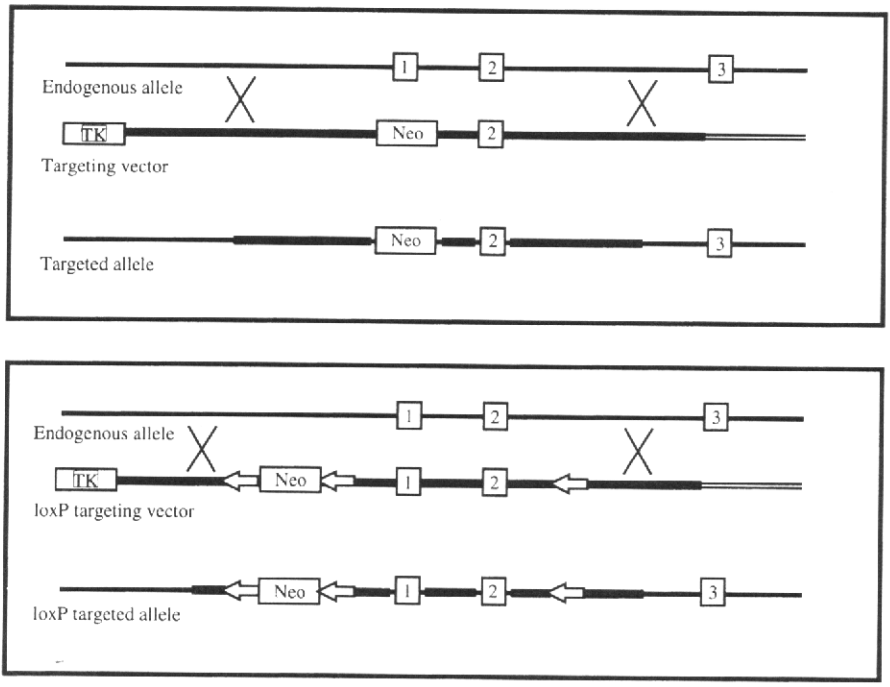

Figure 24-4. Targeting strategy for a conventional gene knockout (A) and the introduction of loxP sites (open arrowhead) for a tissue-specific gene deletion approach (B) based on the positive–negative selection system in embryonic stem cells. (B) The selectable marker, which itself is flanked by loxP sites, is placed in the 5′-upstream region of the gene. The extrinsic loxP site is located 3′ in an intron. Cre-mediated gene deletion can be achieved by recombination between the extrinsic loxP site and either one of the loxP sites of the floxed neomycin cassette. The selectable marker can be removed by partial Cre-mediated recombination between the two loxP sites flanking the neomycin cassette.

removed if homologous recombination takes place. Therefore, adding gancyclovir to the growth medium kills any cells containing the HSV-TK due to random integration.

Figure 24-4A shows a classical gene deletion approach where the promoter and first coding exon of a gene are replaced by a neomycin selectable cassette. In a successful targeting event, crossover occurs between the 5′- and 3′-ends of the homologous DNA sequence and the ES cell genomic DNA. The general targeting strategy is similar in the loxP insertion approach (Figure 24-4B). Here the neomycin selection cassette is placed into a noncoding part of the gene that is also not essential for the regulation of gene expression. The selectable marker is surrounded by loxP sites so that it can be removed by partial recombination if necessary. A third loxP site is inserted on the opposite end of the sequence to be deleted. Excision of the gene is achieved when in the presence of Cre recombinase the outermost loxP site recombines with one of the loxP sites apposed to the selectable marker.

IDENTIFICATION OF TARGETED ES CELLS AND CRE-MEDIATED RECOMBINATION EVENTS *IN VIVO*

In order to distinguish between random integration and homologous recombination in isolated ES cell clones, Southern blot hybridization or PCR is performed. The standard for

target verification is Southern blot hybrization with flanking (external sequence not included in the targeting construct) and internal probes at the 5′- and 3′-ends of the targeting event. Southern blots are performed with external probes to verify targeting and with internal probes to determine whether an additional random integration of the targeting vector has occurred in the ES cell clone.

After verifying by Southern blot that the targeted allele has been transmitted through the germ line of chimeric mice, it is faster to screen progeny by PCR for the presence of the floxed allele. For this purpose, PCR primers can be designed that flank the third loxP site, and amplification will reveal an endogenous and a targeted allele in heterozygous mice (Figure 24-5, primers 4 and 5). After crossing the Cre transgene into the homozygous floxed background it is necessary to analyze the different products expected from the Cre-mediated recombination event. These include the endogenous, floxed, floxed markerless (Figure 24-5, type I deletion), and complete deletion alleles (Figure 24-5, type III deletion). Quantitative PCR or Southern blot can be performed to estimate the extent of recombination (floxed allele versus complete deletion) in the mammary gland in comparison to other organs. The functional down-regulation of the target gene in the mammary gland is verified by a decreased amount of transcript or protein (Northern or Western blot, *in situ* hybridization, immunocytochemistry, etc.).

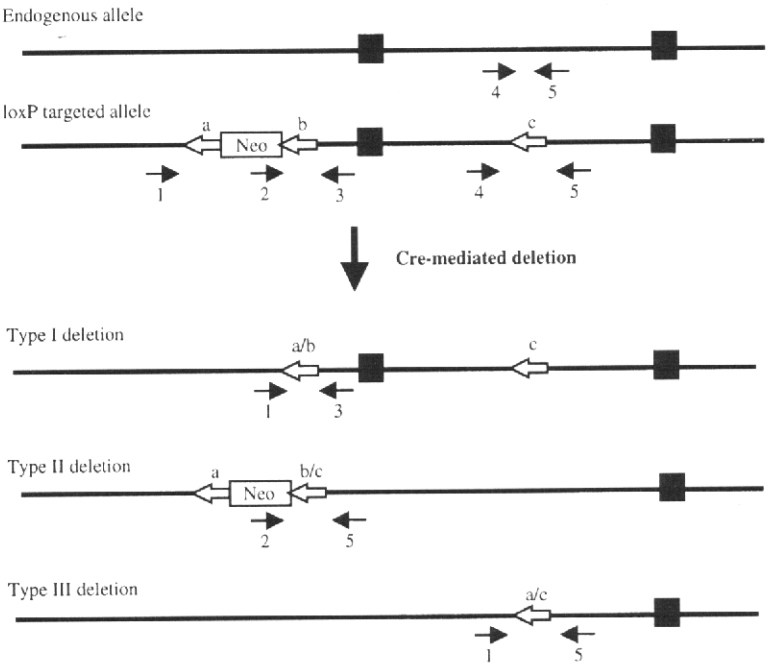

Figure 24-5. Generation of a series of alleles by Cre-mediated recombination following a single targeting event. The introduction of the neomycin selectable marker into various sites of a gene (promoter or intron) can cause hypomorphic or hypermorphic phenotypes. A partial deletion can excise only the selectable marker (type I deletion) or the intervening sequence between marker and exterior loxP site (type II deletion). A complete deletion results in a null allele with only one remaining loxP site in the targeted locus (type III deletion). Solid arrows indicate various PCR primers that can be used in combination to detect the type of Cre-mediated deletion or the presence of a wild type and a floxed allele (primers 4 and 5) for the analysis of the genotype.

In cases when the neomycin selection marker interferes with normal transcription of the gene, the marker should be removed by Cre-mediated recombination either in the ES cells or in the germ line of transgenic mice. Whereas subsequent manipulations on ES cells take the risk that modified ES cells are no longer transmitted through the germ line of chimeric mice, a transgenic approach using EIIa-Cre mice (34) can be employed to excise the floxed neomycin marker *in vivo* (Figure 24-5, type I deletion). The EIIa-Cre transgene exhibits a weak expression in embryonic progenitor cells that contribute to the germ line. This weak Cre expression leads to a partial recombination of the floxed allele, i.e., the elimination of the neomycin cassette. Mice that are heterozygous for the floxed allele and contain the EIIa-Cre transgene should be crossed with wild-type mice for two reasons: first, to verify that recombination has occurred in the germ line and is transmissible; second, to eliminate the Cre transgene that would otherwise cause further gene deletions in subsequent generations. A conditional approach requires breeding to homozygosity of the floxed allele combined with introduction of the Cre transgene. To maximize the extent of recombination it may be necessary to generate mice that carry a null allele (Figure 24-5, type III deletion) and a floxed allele in addition to the Cre transgene. As shown earlier, a null allele can be easily generated by transmitting the floxed allele through the female germline of MMTV-Cre (A) transgenic mice (17).

Although the time and effort are considerable in pursuing the conditional approach, the wealth of information from generating a series of different alleles might outweigh this concern. The altered alleles might display different expression profiles, which then could lead to various phenotypes. For instance, several *N-myc* alleles were generated that gave rise to distinct developmental defects (35). The *Fgf-8* gene, flanked by loxP and frt sites (36), was modified through expression of Cre or Flp recombinase to generate a series of alleles that demonstrated the importance of this gene in gastrulation, cardiac, and brain development. Therefore, a single targeting event can be used to examine a combination of subtle and complex phenotypes in the mouse.

OUTLOOK

A major advantage of the Cre-loxP technology is the interchangeability of its components. Mammary gland researchers will benefit greatly from the numerous mouse lines containing floxed genes previously developed for tissue-specific knockouts in other organs. By simply crossing these floxed alleles into mammary-specific Cre transgenics that are already available, entirely new experimental models can be developed. Nevertheless, the variety of tools for mammary-specific gene deletion needs to be extended, for instance, by combining Cre recombinase with other inducible systems and by using adenoviral Cre vectors in conjunction with epithelial transplantation techniques. It may also be beneficial to generate transgenic lines by targeted insertion of the Cre coding sequence into a milk protein locus to eliminate the mosaic expression of a Cre transgene in the mammary epithelial compartment. However, since milk protein genes and other important loci for mammary gland development and breast cancer are confined to the same chromosome (for example, *Wap*, *Brca1*, and *Trp53* are linked on chromosome 11, and the *Casein* genes and *Brca2* are coupled on chromosome 5), it is in certain cases difficult to achieve the final combination of a Cre transgene, a floxed allele, and a knockout allele by simple mating schemes. Therefore, it is important that different Cre expressing lines including conventional transgenics are accessible to the scientific community.

Cre transgenic mouse strains are useful not only for tissue-specific gene deletions, but also for a tissue-specific activation of transgenes by using a floxed transcriptional stop

sequence between a ubiquitously expressed promoter and the protein coding sequence. Such conditionally activated transgenes can be delivered by standard pronuclear injection into zygotes, gene targeting in ES cells, or viral vectors. To date, the latter approach has only been used in reporter constructs to monitor the recombination event (17), but it could also be utilized to express other biologically relevant molecules. Furthermore, it is of great importance to develop a reporter strain that is highly expressed throughout mammary gland development and that allows for the detection of the Cre-mediated recombination within individual cells. A potentially useful reporter line with a targeted *Rosa26* gene was published recently (37) and should be tested specifically for this purpose. A variety of transgenic reporter strains with other ubiquitously expressed promoters have been published, but so far none of them has been utilized successfully in mammary-specific gene deletion experiments.

Although further refinements are necessary, initial technical hurdles to target the Cre specifically to the mammary gland have been conquered. Mammary-specific tools for gene deletion are currently being tested for their efficacy *in vivo* by deleting floxed genes that are suggested to be essential for mammary epithelial development. The WAP-Cre and MMTV-Cre transgenic lines have recently passed this litmus test, since they were utilized to generate the first mouse model for breast cancer by mammary-specific gene targeting (21). These transgenic tools, now distributed by the Jackson Laboratory, will facilitate new insights into the function of genes during mammary development and tumorigenesis.

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NOTE ADDED IN PROOF

WAP-Cre, MMTV-Cre (A), and MMTV-Cre (D) transgenic mice are now available from the Jackson Laboratory under the following strain codes: B6129-TgN(WapCre)11738Mam, B6129-Tgn(MMTV-Cre)1Mam, and B6129-Tgn(MMTV-Cre)4Mam. Recently, BLG-Cre mice have also been used to delete the Stat3 gene in the lactating mammary gland (Chapman *et al. Genes Dev* 1999 13(19):2604–2616), and the first successful attempt to combine the Cre-lox technique with the tetracycline inducible system in the mammary gland has been reported (Utomo *et al. Nat. Biotechnol.* 1999(11):1091–1096).

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