Adenovirus-Cre-mediated recombination in mammary epithelial early progenitor cells

Monique Rijnkels and Jeffrey M. Rosen*

Baylor College of Medicine, Department of Molecular and Cellular Biology, One Baylor Plaza, Houston, TX 77030, USA
*Author for correspondence (e-mail: jrosen@bcm.tmc.edu)

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SUMMARY

The transplantation of primary mammary epithelial cells after adenovirus-Cre-mediated recombination provides a new approach for the study of specific gene function during mammary gland development and in breast cancer. Most mammary-gland-specific promoters identified to date are regulated by lactogenic hormones. They are expressed predominantly in lobuloalveolar cells during pregnancy and lactation, but not during early stages of ductal morphogenesis in the mammary epithelial cell progenitors, which are primarily implicated in tumorigenesis. In transgenic mice these promoters will continually or repeatedly express Cre depending on the hormonal environment precluding the definition of cell lineages. To circumvent these limitations, we have taken advantage of the unique regenerative capacity of mammary epithelium to reconstitute a mammary gland in an epithelium-cleared fat pad in conjunction with transient Cre expression using recombinant adenovirus in primary cultures. This approach was validated using mice carrying reporter constructs that exclusively express the *LacZ* gene after Cre-mediated deletion of a floxed DNA fragment. These studies demonstrated that, following recombination, cells that are marked as genetically manipulated contribute to the reconstitution of the mammary gland. The presence of β-galactosidase-expressing cells in serial transplants of the primary outgrowths indicated that early progenitor or stem cells were successfully targeted. With the increased availability of floxed alleles, this approach should greatly facilitate the study of gene function during early stages of mammary gland development and in breast cancer.

Key words: Adenovirus, Cre recombinase, Mammary epithelial cells, Transplantation, Stem cells

INTRODUCTION

A better understanding of the genes and signal transduction pathways involved in normal mammary gland development is needed to elucidate their role in breast cancer. Ideally, gene function should be studied in the context of the stromal-epithelial interactions that are required for normal mammary gland development. However, as genes involved in mammary gland development are often essential in embryonic development, germline knockouts of these genes are frequently lethal or exhibit phenotypes in other tissues. This may restrict the functional analysis of many genes in the mammary gland. To study the role of such genes in mammary gland development new technologies are needed to address these questions.

The mammary gland contains a population of multipotent, tissue-specific stem cells and early progenitor cells throughout development (Chepko and Smith, 1999). These stem cells are thought to be essential for the normal growth, renewal and differentiation of the mammary gland during multiple cycles of pregnancy, lactation and involution. Mammary-specific stem cells may also be important in the etiology of mouse mammary tumors and hyperplasias, which are thought to be of clonal origin (Chepko and Smith, 1999). Thus, to study the effects of gene modification during mammary gland development and tumorigenesis it is desirable to be able to target these modifications to the stem cells or early progenitor cells.

The stem cell/early progenitor cell population is also instrumental to the unique property of mammary epithelial cells to reconstitute a normal mammary gland structure in a fat pad that is cleared of endogenous epithelium (Chepko and Smith, 1999; Edwards et al., 1996; Medina, 1996). This property is retained after primary cells have been in culture for a limited time. The tissue reconstitution approach has provided a powerful tool to study the stromal-epithelial interactions of genes involved in mammary gland development and function either by using null epithelium in wild-type stroma or vice versa, or by introducing genes specifically into the mammary gland epithelium using retroviruses (Brisken et al., 1998; Edwards et al., 1996; Medina, 1996).

Transplantation of the mammary gland primordium can be used to study gene function if mice with germline knockouts survive beyond day E12.5 (Robinson et al., 2000). If, however, gene modification or deletion results in early embryonic lethality, a conditional gene modification approach is required. The Cre/lox recombination system permits tissue-and developmental-stage-specific gene modifications. The expression of bacteriophage P1-derived Cre recombinase induces deletion of DNA fragments flanked by the Cre-recognition sites LoxP (floxed) (Sauer, 1998). This system has been used in numerous tissues to generate specific gene deletions or modifications (Agah et al., 1997; Akagi et al., 1997; Tsien et al., 1996). Conditional Cre expression can be achieved in several different ways: (1) In the majority of the

studies published to date, conventional transgenesis with tissue-specific promoters has been employed (Agah et al., 1997; Akagi et al., 1997; Tsien et al., 1996). (2) In some cases tissue-specific promoters have been combined with inducible/regulatable systems, such as Tet-on/off (St-Onge et al., 1996; Utomo et al., 1999) and Cre-steroid hormone-binding-site fusion proteins (Brocard et al., 1998; Brocard et al., 1997; Kellendonk et al., 1999). (3) Recently, knockin strategies also have been applied to target Cre (Chen et al., 1998; Guo et al., 2000) in order to overcome position effects and mosaic expression of transgenes.

Targeted gene expression to the mammary gland in transgenic mice has been achieved using milk protein gene promoters such as α-lactalbumin, casein, β-lactoglobulin and whey acidic protein, as well as the mouse mammary tumor virus (MMTV) long terminal repeat. The latter three promoters have been used to direct mammary-gland-specific Cre expression with varying degrees of success (Selbert et al., 1998; Wagner et al., 1997). However, these promoters are all hormone dependent and are predominantly expressed in lobuloalveolar cells during pregnancy and lactation, not during early stages of ductal morphogenesis in the mammary cell progenitors, which are thought to be important in tumorigenesis. Nevertheless, these promoters are useful in the study of mammary gland involution (Chapman et al., 1999). To date, mammary-specific expression in stem/early progenitor cells at early stages of ductal morphogenesis through conventional transgenic technology has not been achieved due to a lack of suitable targeting strategies.

Adenovirus vectors have the potential to deliver Cre-recombinase to the mammary gland at any stage of development. However, systemic virus delivery leads to the predominant infection of liver, lung and heart, but not the mammary gland. In addition, toxicity and immunogenicity are complications often associated with this strategy and are often major obstacles to the successful use of this approach (Agah et al., 1997; Akagi et al., 1997; Rohlmann et al., 1996; Wagner et al., 1997; Wang et al., 1996). Local administration of virus is possible by injecting the virus into the lumen of the gland via the nipple or the primary duct (Jeng et al., 1998; Yang et al., 1995). However, this method is technically challenging in mice and the epithelial cells in closest proximity to the injection site are preferentially infected. Furthermore, mammary stem cells most likely do not border the lumen directly and may, therefore, not be targeted by intraductal virus injection (Chepko and Smith, 1999; Chepko and Smith, unpublished). Potential leakage of virus into the bloodstream might also result in inadvertent immunological responses and possibly recombination events in other tissues.

In the present study the following techniques were combined to generate mammary glands with a modified epithelial genotype. (1) Infection of primary mammary epithelial cell (MEC) cultures with a Cre-expressing adenovirus-construct to induce recombination between LoxP sites and thus the deletion of a floxed DNA fragment resulting in gene modification/inactivation. In the reporter lines used this resulted in the expression of β-galactosidase in the infected MECs. (2) Transplantation of infected MECs, after which both primary and secondary transplants were monitored for the fate of modified cells in the reconstituted mammary gland and the consequence of the gene modification in mammary gland development (see Fig. 1).

In initial studies this system was tested using ROSA26 reporter mice that have a modified ROSA26 locus and express the *LacZ* gene only after Cre-mediated deletion of a floxed DNA fragment (Fig. 2A) (Soriano, 1999). The ROSA26 allele directs ubiquitous expression in embryonic and adult tissue.

MATERIALS AND METHODS

Mice

P. Soriano kindly provided the ROSA 26 reporter (R26R) mice (Soriano, 1999). R26R genotyping on tail tip DNA was performed using the following three primers in a multiplex PCR: R26F2 (5′-AAAGTCGCTCTGAGTTGTTAT-3′), R1295 (5′-GCGAAGA-GTTTGTCCTCAACC-3′), R523 (5′-GGAGCGGGAGAAATG-GATATG-3′). PCR was performed in modified Gitschier's buffer (67mM Tris, pH 8.8, 16.6 mM ammonium sulfate, 6.7 mM $MgCl_2$, 0.001% gelatin) with 10% DMSO. DNA was denatured for 3 minutes at 94°C followed by 40 cycles of 30 seconds at 94°C, 40 seconds at 55°C and 70 seconds at 65°C. 129Sv/C57Bl6 RAG1$^{-/-}$ mice (Mombaerts et al., 1992) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal experiments were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

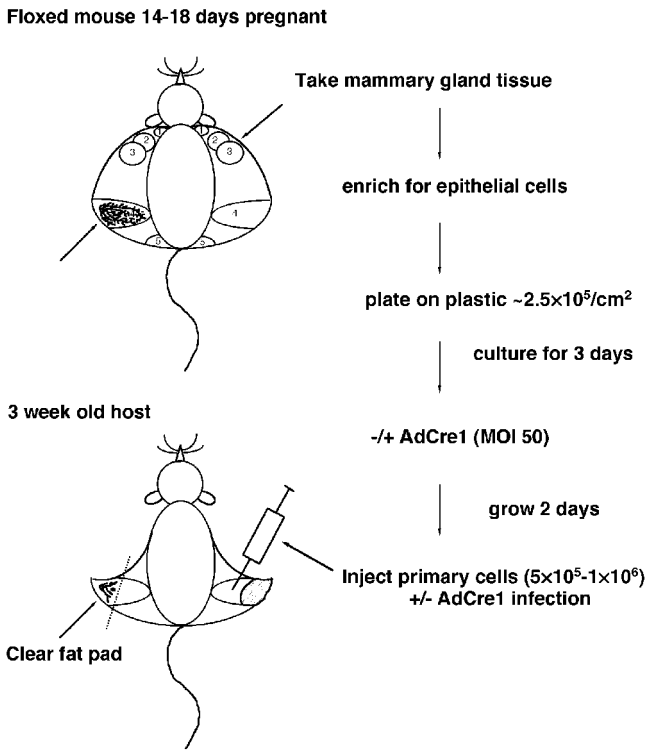

Fig. 1. AdCre1-mediated mammary gland epithelium-specific gene modification. Mammary gland tissue was harvested from all ten glands of late pregnant (14-18 days) animals bearing a floxed allele. Mammary gland tissue was dissociated and enriched for epithelial cells. Those cells were plated on serum/fetuin-coated plastic at a cell density of ~2.5×10^5/cm^2 and cultured for 3 days. Cells were infected with AdCre1 at a MOI 50 and cultured for 2 more days. Cells were injected in epithelium-free fat pads of a 3-week-old host animal on day 5 of culture and allowed to grow out for more than 7 weeks.

Mammary epithelial cell isolation, primary cell culture and AdCre1 infection

All ten mammary glands were isolated from pregnant or >12-week-old virgin $R26R^{+/wt}$ females (5-12 animals per experiment). The epithelial cell fraction was isolated as described previously (Pullan and Streuli, 1996). Primary MECs were plated at a density of about 2.5×10^5 cells/cm^2 in 6-well plates that had been coated using 100 μl/cm^2 serum/fetuin (20% fetal calf serum (FCS, Summit Biotechnology, Fort Collins, CO) and 1 mg/ml fetuin (Sigma, St Louis, MO)). Cells were allowed to plate for 2 days in plating media (F12 medium (Gibco-BRL, Grand Islands, NY), 5 μg/ml insulin, 2 μg/ml hydrocortisone, 5 ng/ml EGF, 50 μg/ml gentamycin, 100 U penicillin/streptomycin and 10% FCS) and switched to growth media (plating media with 5% FCS) for 24 hours. Three days after plating, cells were infected with AdCre1 (Anton and Graham, 1995) at a multiplicity of infection (MOI) of 50 as determined with an adenovirus expressing *E. coli* β-galactosidase (Adβ-gal) (AdCre1 and Adβ-gal were kindly provided by M. Abdelative, Baylor College of Medicine, Houston, TX). Noninfected cells served as a control. After 12 hours cells were washed several times with F12 containing 50 μg/ml gentamycin, and fresh growth media was added. Two days after infection (5 days after plating) cells were harvested by gentle trypsinization and used for transplantation.

Transplantation of MECs from primary culture and tissue from primary outgrowth

The inguinal #4 glands of 21-day-old $RAG1^{-/-}$ females were cleared of mammary epithelium as previously described (DeOme et al., 1959). Trypsinized cells were washed in PBS and resuspended at about 0.5×10^8 cells/ml; 10-20 μl of cell suspension was injected into the cleared fat pad. Transplants were allowed to grow out for 7-20 weeks. Some mice were bred after 12-14 weeks and glands and outgrowths were collected at mid-pregnancy (days 10-16). For secondary transplantation of tissue from the primary outgrowth, small pieces of tissue (~1 mm^3) were transplanted in the cleared inguinal gland as described above.

Analysis of tissue from MEC transplants

Before transplantation MECs were analyzed for recombination by X-gal staining on coverslips or in separate wells. MEC transplant tissues and as control the host thoracic glands were analyzed for recombination by X-gal staining on the whole gland followed by whole mount staining, or at the DNA level by PCR.

Histochemical analysis

X-gal staining on MECs was performed as described (Sanes et al., 1986). Tissue was fixed in 2% paraformaldehyde in 0.1 M Pipes, pH 6.9 or 1× PBS, pH 7.2 for 1.5 hours, rinsed in wash buffer (2 mM $MgCl_2$ in PBS, pH 7.2) then permeabilized for 2 hours in permeabilization buffer (2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% Nonidet P-40 in PBS, pH 7.2) and incubated in X-gal staining solution (25 mM potassium ferricyanide, 25 mM potassium ferrocyanide in permeabilization buffer with PBS, pH 8.1) for 1-2 hours at 37°C. X-gal (1 mg/ml) was added and tissues were stained for 36-48 hours at 37°C. After X-gal staining, tissues were cleared in three changes of acetone, rehydrated in graded solutions of ethanol and stained overnight as described in http://mammary.nih.gov/tools/histological/Histology/index.html, in 0.2% carmine and 0.5% $AlK(SO_4)$. Tissues were embedded in paraffin and 5 μm tissue sections were stained with hematoxylin and eosin and analyzed by light microscopy.

DNA analysis

DNA for PCR was isolated from fixed glands or sections of paraffin-imbedded tissue scraped from slides essentially as described (Wright and Manos, 1990) and from primary culture cells, nonfixed glands or parts of glands according to standard methods (Ausuble et al., 1987). DNA isolated from fixed tissue or slides is only suitable for PCR amplification of fragments up to 800 bp. For the R26R recombination analysis primers R26F2 (5′-AAAGTCGCTCTGAGTTGTTAT-3′) and Z3 were used (Fig. 2B). Cre1 (5′-GGACTGTTCAGGGATCGCC-AGGCG-3′) and Cre2 (5′-GCATAACCAGTGAAACAGCATTGCT-G-3′) were used to detect the presence of Cre recombinase DNA (data

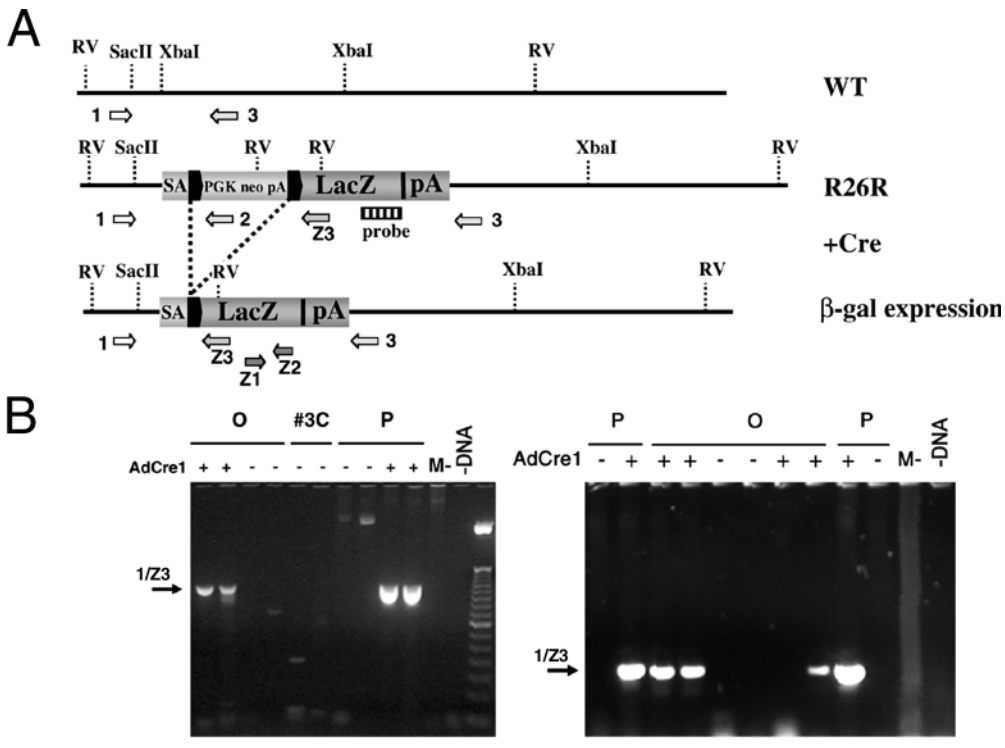

Fig. 2. Reporter construct and recombination analysis by PCR. (A) Map of the wild-type (WT) and R26R alleles, and the recombined R26R allele (Soriano, 1999). The *LacZ* gene was expressed upon Cre-mediated recombination. Primers used for PCR detection of genotype were 1/3 (WT), 1/2 (R26R), detection of recombination 1/Z3 (600 bp fragment) and detection of *LacZ* Z1/Z2 (Araki et al., 1995). (B) PCR analysis for Cre-mediated recombination of the R26R allele in (1) mammary gland epithelium in culture (P): –AdCre1: panel I, lanes 7,8, and panel II, lanes 1,10; +AdCre1: panel I, lanes 9,10 and panel II, lanes 2,9; (2) mammary epithelium outgrowth (O): –AdCre1: panel I, lanes 3,4 and panel II, lanes 5,6; +AdCre1: panel I, lanes 1,2 and panel II, lanes 3,4,7,8; (3) WT mouse tail DNA (M–): panel I, lane 11 and panel II, lane 11; (4) non-DNA control (–DNA): panel I, lane 12 and panel II, lane 12; (5) marker, 50 bp DNA ladder: panel I, lane 13; (6) WT mammary gland DNA (#3C): panel I, lanes 5,6.

not shown). PCR conditions: 3 minutes at 94°, 40 cycles of 1 minute at 94°C, 2 minutes at 58°C (R26R) or 60°C (Cre) and 3 minutes at 72°C, followed by 7 minutes at 72°C.

RESULTS

Adenovirus-Cre-mediated recombination in MECs in primary cultures

Transplantation experiments performed with mouse and rat mammary gland tissue have resulted in estimates of stem cell content ranging between 1 in 100 and 1 in 2500 in the primiparous rat mammary gland (Chepko and Smith, 1999). Based on electron microscopic observations, Chepko and Smith estimated that stem and early progenitor cells comprise approximately 3% of the mammary epithelium at any stage of development (Chepko and Smith, 1999). As the pregnant gland contains a greater number of epithelial cells and, therefore, potentially a higher number of stem cells, 14-18 day pregnant animals were used to isolate mammary gland tissue from all ten mammary glands. Tissue was dissociated with collagenase and trypsin and enriched for epithelial cells as described by Pullan and Streuli (Pullan and Streuli, 1996).

Initial experiments performed using recombinant Ad-β-gal to optimize the conditions of viral infection indicated that, at an MOI of 50, 60-80% of primary MECs were infected (Fig. 3A). Accordingly, AdCre1-infected (Anton and Graham, 1995) MECs derived from R26R mice were analyzed for recombination by X-gal staining 2 days after viral infection (Fig. 3B,C). In agreement with the Adβ-gal infection, 60-80% of the AdCre1 infected cells isolated from mice carrying the R26R reporter construct stained positively for β-galactosidase, indicating that recombination had occurred in 60-80% of these cells. These results indicated that the percentage of β-galactosidase-positive cells after AdCre1 infection was comparable to that observed with Adβ-gal infection.

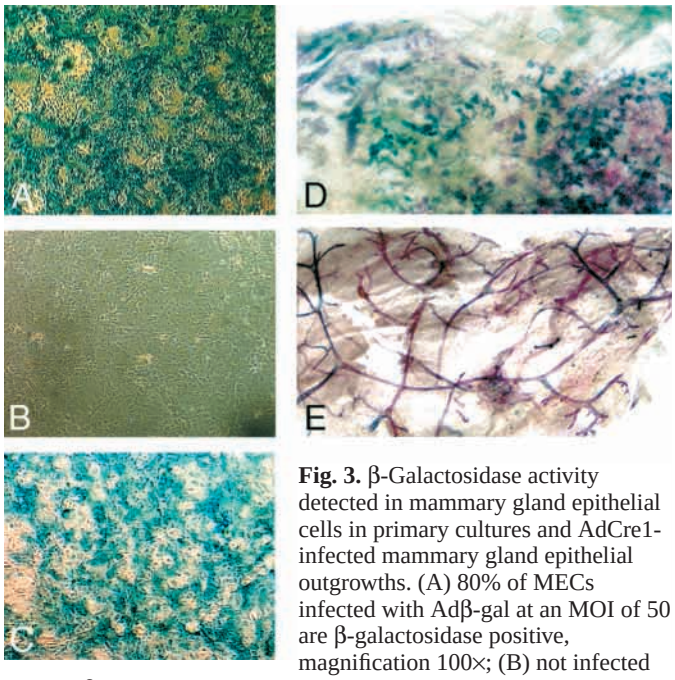

Fig. 3. β-Galactosidase activity detected in mammary gland epithelial cells in primary cultures and AdCre1-infected mammary gland epithelial outgrowths. (A) 80% of MECs infected with Adβ-gal at an MOI of 50 are β-galactosidase positive, magnification 100×; (B) not infected with Adβ-gal, 100×. (C) 71% of R26R MECs infected with AdCre1 at an MOI of 50 are β-galactosidase positive, 100×. (D,E) X-gal and whole mount staining with carmine aluminum of two primary outgrowths of a single primary culture 15 weeks post transplantation. 9472R, 15 day pregnant, 40× (D); 9471R, virgin, 10× (E).

Transplantation of AdCre-infected mammary epithelium and analysis of outgrowths

Having established a protocol for the efficient adenoviral infection of MECs, we next determined whether recombination had occurred in mammary epithelial stem or early progenitor cells. To answer this question, approximately 1×10^6 cells were transplanted following AdCre1 infection into the cleared inguinal (#4) fat pads of 3-week-old RAG1$^{-/-}$ (for the R26R experiments) recipient female mice. Transplants were analyzed at time points ranging between 7 weeks to 10 months post transplantation for the presence of outgrowths, the percentage of the fat pad filled by the outgrowth and for MECs expressing β-galactosidase. Appreciable X-gal staining was observed in two of the five AdCre1-infected R26R MEC outgrowths analyzed at the whole mount level (Fig. 3). PCR analysis detected the presence of recombined cells in seven out of nine (78%) of the R26R outgrowths (Fig. 2B, summarized in Table 1).

The most stringent test for the existence of stem cells is the serial transplantation of tissue fragments or dispersed cells to the autochthonous site in successive hosts. To determine if stem cells were recombined in the original primary cultures, secondary transplants were performed with tissue from two primary R26R outgrowths taken at day 15 post coitum. The thoracic gland (#3) of the donor animal and tissue from a mature virgin ROSA26 were used as controls. At 7 weeks post-transplantation secondary transplants at different stages of mammary gland development (virgin, pregnancy and day 1 of lactation) were analyzed. Some variation in the extent of outgrowth was observed in the fifteen secondary transplants. For example, in three outgrowths an extensive ductal-tree was observed in wholemounts that appeared to be completely β-galactosidase positive (Fig. 4A,B). This indicates that these outgrowths were derived from cells that had undergone recombination in the original primary culture, or their progeny. In some outgrowths only a small cluster of cells (tuft) or a part of the gland exhibited detectable β-galactosidase activity (Fig. 4C,F). Both of these observations suggest that the β-galactosidase-expressing cells were clonally derived. At the cellular level, β-galactosidase activity was detected in both luminal- and myo-epithelial cells, and ductal as well as alveolar epithelial cells displayed the recombined phenotype (Fig. 4D,E,G-I). In some cases, β-galactosidase expression was not uniformly distributed along the ducts or in the alveoli.

DISCUSSION

To enable the study of gene function in the mammary gland at early stages of postnatal development, a combination of primary culture and transplantation techniques was used to generate mammary glands with a modified or deletion genotype. The levels of recombination reported to date in the ductal epithelium of the mammary gland of virgin mice due to targeted expression of Cre using milk protein gene promoters have been extremely low (Selbert et al., 1998; Utomo et al.,

Table 1. Summary of R26R mammary epithelial outgrowth experiments

	R26R	X-gal	PCR	Growth (%)
Infected	Primary culture	+	+	
	9467L	–	–	30
	9468L	–	+	100
Pregnant	9469L	na*	+	100
Pregnant	9469R	na	+	100
	9470L	na	+	Y‡
	9470R	na	+	Y
	9471R	+	+	100%
Pregnant	9472L	–	na	60%
Pregnant	9472R	+	+	80%
Uninfected	Primary culture	–	–	
	9467R	–	–	0
	9468R	–	–	0
	9471L	–	–	50%
#3§	9468#3	–	–	
	9471#3	–	na	
Pregnant	9472#3	–	na	

*Not analyzed.
‡Outgrowth present but no percentage determined.
§Control thoracic gland of host animal.

1999; Wagner et al., 1997). The timing and patterns of MMTV-Cre expression appear to be highly dependent on the site of transgene integration often resulting in Cre expression both during embryonic development and in a number of other somatic tissues, which may preclude the study of mammary specific gene function (Wagner et al., 1997).

The lack of regulatory sequences that are able to direct expression exclusively to the ductal epithelial cells and their progenitors in the mammary gland at nonpregnant and nonlactating stages of development led to the use of adenovirus-expressed Cre to induce recombination of floxed alleles. Instead of using local administration of the virus, which is technically challenging and might have adverse toxic and immunological effects, adenoviral infection of primary culture MECs was combined with the technique of mammary gland reconstitution, as pioneered four decades ago by DeOme et al. (DeOme et al., 1959). Using this approach, the Cre recombinase was only transiently expressed in these MECs as the nonintegrating viral DNA is diluted during rapid cell division following transplantation, thus genotypically marking

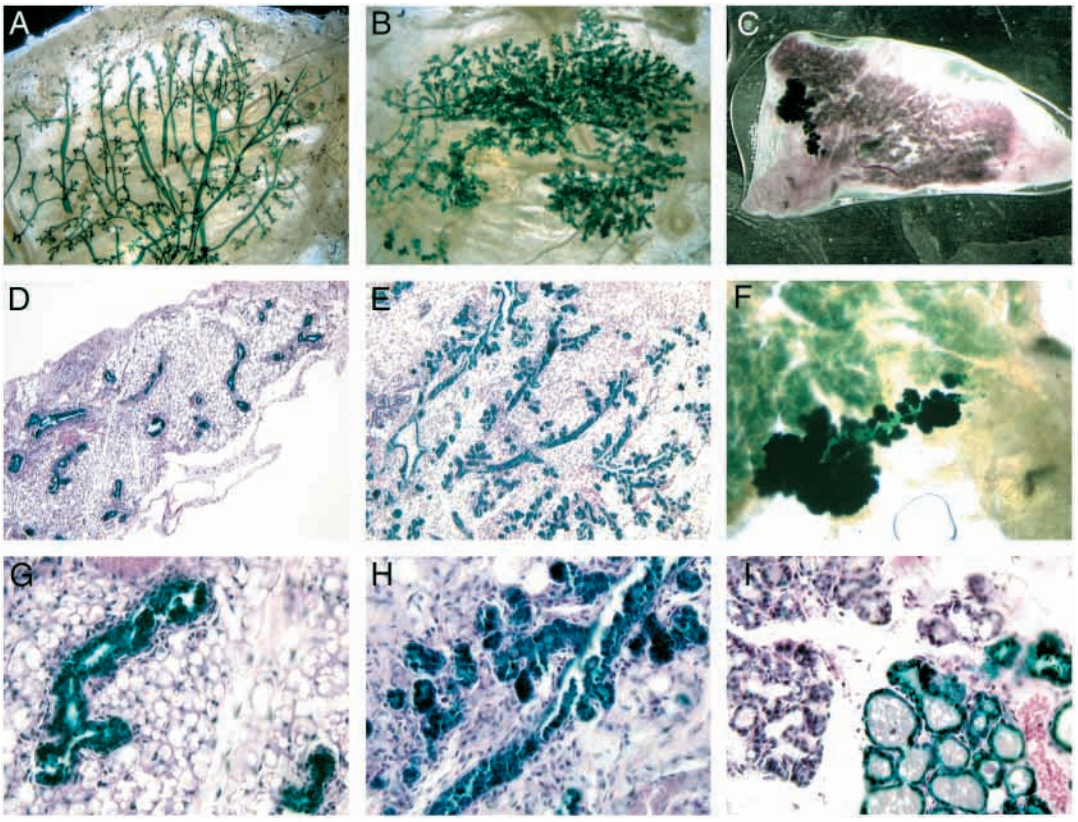

Fig. 4. X-gal staining in secondary transplants of AdCre1-infected mammary epithelium. (A) 2443L, virgin, magnification 20×; (B) 2442L, 12.5 day pregnant, 20×; (F) 2448R, 1 day lactation, 20×; (C) 2448R, 10×, X-gal and carmine whole mount staining. Hematoxylin- and eosin-stained sections of outgrowths are shown in A-C. (D) 2443L, 100×; (G) 400×, 2442L; (E) 100×; (H) 400×; (I) 2448R, 400×.

the cell-lineage and averting potential toxic effects of prolonged Cre expression. This approach restricts the conditional gene modification to mammary epithelial cells, which should enable the study of stromal-epithelial interactions at early stages of mammary gland development. Because the adenovirus was removed from the cells prior to transplantation, potential immunological responses and cytopathic effects known to be associated with the virus were avoided. Using this approach, highly efficient infection and recombination was observed in primary MECs suggesting that recombination occurs in each infected cell. Viral infection, however, did not appear to affect the capacity of MECs to reconstitute the mammary gland. Similar results were obtained for primary cultures derived from mice carrying a transgenic reporter construct consisting of the chicken β-actin promoter and cytomegalovirus (CMV) enhancer driving a *LacZ* gene that is interrupted by a floxed chloramphenicol acetyltransferase (CAT) gene (CAG-CATZ, (Araki et al., 1995)). Recombination of the CATZ allele was detected by PCR in eight out of ten (80%) of the outgrowths (data not shown), a frequency similar to that observed for the R26R outgrowths. However, most likely due to the low levels of expression of the β-actin CMV enhancer-driven transgene in these MECs and outgrowths, X-gal staining was observed in less than 1% of the AdCre1-infected MECs, and only faint staining was detected in one out of eight outgrowths analyzed (data not shown).

The observation that almost all secondary outgrowths exhibited some β-galactosidase-positive cells strongly indicated that stem cells or early progenitor cells were infected in the primary cultures resulting in Ad-Cre mediated recombination. The nonhomogeneous distribution of the β-galactosidase expression in the primary outgrowths suggested that recombination most likely also occurred in some cells that were further along the differentiation pathway. These results suggest that the secondary outgrowths were more clonally derived than the primary outgrowths. Cell sorting for the recombined phenotype might conceivably improve the homogeneity of β-galactosidase expression observed in the primary outgrowths.

The detection of isolated growths or tufts on the extended ductal tree exhibiting the recombined phenotype suggests that there is still considerable heterogeneity in the cell populations comprising the secondary outgrowths. This might also explain the differences in growth potential observed in the secondary outgrowths, that is, abundant growth from stem cells and early progenitors, in contrast to tuft growth from more differentiated progenitors with a more limited division capability.

A mixed population of cells in the outgrowths may in some cases actually provide a distinct advantage in studying the etiology of some cancers, especially if stem cells or early progenitor cells acquire the recombined genotype. Mutations in stem cells at early stages in development might exert a profound effect on cell fate determination resulting in pleiotropic effects during the clonal expansion of cells into different cell lineages. Ideally, to correlate genotype directly with phenotype, a recombination reporter should be included within the floxed construct on a flox/null background. However, most floxed alleles available are not constructed in this manner. Alternatively, the floxed alleles can be bred into a reporter (e.g. R26R) background. Although not all floxed alleles may undergo recombination with the same efficiency, the ability to follow recombination in situ using an easily detected reporter should be a major advantage in correlating observed phenotypic with genotypic changes. Although technically more difficult, laser capture/PCR and/or in situ PCR also provide alternative methods to correlate the extent of recombination with the observed phenotype.

The method described in this study allows for the temporal, spatial and cell-type-specific expression of Cre recombinase to achieve conditional modification of the mammary gland epithelium. This approach should facilitate the definition of stromal-epithelial interactions during ductal morphogenesis in early postnatal mammary gland development, and provides a powerful tool for cell lineage analysis.

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