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Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice

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Abstract

Cre-loxP based gene deletion approaches hold great promise to enhance our understanding of molecular pathways controlling mammary development and breast cancer. We reported earlier the generation of transgenic mice that express the Cre recombinase under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). These mice have become a valuable research tool to delete genes specifically in the mammary gland, other secretory organs, and the female germline. We have now characterized in depth the expression of the MMTV-Cre transgene using the ROSA26-lox-Stop-lox-LacZ reporter strain to determine the temporal and spatial activation of Cre on the level of single cells. Our results show that MMTV-mediated Cre-activation is restricted to specific cell types of various secretory tissues and the hematopoietic system. Secondly, the timing of Cre expression varies between tissues and cell types. Some tissues express Cre during embryonic development, while other selected cell types highly activate Cre around puberty, suggesting a strong influence of steroid hormones on the transcriptional activation of the MMTV-LTR. Thirdly, Cre expression in the female germline is restricted to individual mouse lines and is therefore dependent on the site of integration of the transgene. Information provided by this study will guide the researcher to those cell types and developmental stages at which a phenotype can be expected upon deletion of relevant genes.

Introduction

Over the last decade, the gene targeting technique has permitted an unparalleled insight into genetic pathways involved in mouse development and tumorigenesis. Despite these achievements, the role of many genes in development and disease remains elusive, since their deletion from the murine genome is either lethal or does not mimic closely the progression of the disease in humans. Furthermore, many human disorders are the result of sporadic, acquired mutations in a limited number of cells that are surrounded by normal tissues. Hence, targeting gene deletions or modifications precisely to specific cell types at a distinct developmental stage is essential to establish high fidelity mouse models for human diseases. Such defined mutations can now be modeled in a temporal and spatial fashion using the Cre-lox technology.

The Cre-lox technology is a binary system, where the Cre recombinase catalyzes the deletion of a DNA fragment between two directly orientated *loxP* sites (Hoess et al., 1984; Hoess et al., 1987). LoxP

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recognition sites are inserted around the regions to be deleted using homologous recombination, thus creating an allele, which is flanked by *loxP* sites (a floxed allele). The temporal and spatial excision of the floxed allele is achieved by expressing the Cre enzyme under a tissue-specific promoter in the same mouse (Nagy, 2000).

MMTV-Cre transgenic lines were generated in an attempt to obtain consistently high expression of Cre recombinase in mammary epithelium and this has now been established. An analysis of recombination in double transgenic mice carrying a reporter transgene and the MMTV-Cre was initially performed in an attempt to determine the expression pattern of Cre in these animals (Wagner et al., 1997). This study, as well as RT-PCR assays, revealed that Cre was active in many organs of MMTV-Cre mice, although these studies did not permit an analysis at the level of single cells. There was an initial uncertainty as to whether these mice could be beneficial for a temporal and spatial gene deletion approach. However, the MMTV-Cre mice have now been used successfully by many laboratories. The lack of embryonic lethality of offspring from crosses of MMTV-Cre lines with more than a dozen floxed genes has allowed the development of more appropriate, tissue-specific animal models for several human diseases. The inactivation of several genes, including *Brca1*, which had been deleterious in conventional gene deletion approaches, has been successfully performed through a deletion generated by this conditional knockout approach (Xu et al., 1999).

The availability of the ROSA26 LacZ reporter strain has allowed the identification of Cre expression at a cell-specific level (Soriano, 1999). We have used these ROSA26 LacZ reporter mice to reinvestigate the expression pattern of the MMTV-Cre transgenic lines on a single cell level. The MMTV-Cre mice have been distributed to more than 20 laboratories worldwide and are now available at the Jackson Laboratory (Bar Harbor, Maine). The results of the expression patterns observed for the MMTV-Cre transgenic lines using the ROSA26 LacZ reporter strain should be helpful for directing investigators using these animals to identify the specific cells in which Cre is expressed and the precise timing of Cre activation.

Material and methods

Male mice of lines A and D carrying the MMTV-Cre transgene (Wagner et al., 1997) were crossed with female ROSA26 LacZ reporter mice (Soriano, 1999). Offspring of these crosses, which carried both the Cre transgene and the ROSA26 LacZ transgene, were analyzed for LacZ expression along with the offspring carrying only the ROSA26 transgene alone (negative control). Mice carrying the Cre transgene gene were identified by PCR using the following forward and reverse primers: 5′GCCTGCATTACCGGTCGATGC3′ and 5′CAGGGTGTTATAAGCAATCCCC3′. The ROSA LacZ locus was identified by PCR using the following forward and reverse primers: 5′GATCCGCGCTGGCTACCGGC3′ and 5′GGATACTGACGAAACGCCTGCC3′. All tissues were fixed for 1–2 h in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP-40 in PBS and stained for β-galactosidase activity (1 mg/ml X-gal, 30 mM $K_3Fe(CN)_6$, 30 mM $K_4Fe(CN)_6 \cdot 3H_20$, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% NP-40, 1x PBS) overnight at 30°C. Mammary glands were postfixed in 10% formalin, dehydrated to 100% EtOH, and placed overnight in xylene before whole mount analysis. All other tissues were dehydrated to 70% EtOH, embedded in paraffin, sectioned, and counterstained with Nuclear Fast Red.

Both of these MMTV-Cre transgenic lines are now available from the Jackson Laboratory (line A: Stock # 003551, B6129-Tgn(MMTV-Cre)1Mam; line D: Stock # 003553, B6129-Tgn(MMTV-Cre)4Mam. All animals used in the described studies were treated humanely and in accordance with public health service policies and federal regulations.

Results

The MMTV-Cre transgenic lines express the Cre gene under control of the MMTV-LTR (Wagner et al., 1997). The MMTV-LTR has been used extensively to target different genes to mammary tissue. Since many of these studies focused on transforming genes and its effect on mammary epithelium, it was concluded that the MMTV-LTR is expressed preferentially in mammary tissue. However, previous studies did not provide clear information on the cell-specificity and temporal activation of the MMTV-LTR. We addressed these questions through an analysis of two independent transgenic strains (lines A and D) of mice that carry the MMTV-Cre transgene and the ROSA26 LacZ reporter locus. In ROSA26 LacZ reporter mice the lacZ gene within the ROSA locus is silent, but can be activated upon deletion of the floxed Stop sequence by

Table 1. Tissue/cell-type expression profile of two mmtv-cre transgenic lines

	Line A	Line D
Mammary epithelial cells	∗∗∗ (> day 6 pp)	∗∗∗ (> day 22 pp)
Oocytes	∗∗∗	No
Salivary gland (epithelial cells)	∗∗∗	∗∗∗
Skin (epidermis, hair follicles)	∗∗∗	∗∗∗
Leydig cells	∗∗	∗∗
Vas deferens	∗∗	∗∗
Seminal vesicles	∗∗∗	∗∗∗
B and T cells	∗∗∗	∗∗∗
Megakaryocytes	∗∗	∗∗∗
Erythroid cells	∗∗	∗∗∗
Pancreas (acini)	∗∗	∗∗
Liver	∗	∗
Trachea	∗	∗
Brain	∗∗	∗∗
Bronchiolar epithelial cells	∗∗ (in adult only)	No
Adrenal gland	∗∗ (in adult only)	No
Female reproductive tract	∗∗ (in adult only)	No
Kidney	∗	∗
Lung	∗	∗
Stomach	No	No
Intestine	No	No
Heart	No	No
Skeletal muscle	No	No

Expression profile for the MMTV-CRe A and D transgenic lines.
***high; **moderate; *low recombination efficiency; No; Cre-mediated recombination undetectable; pp, post-partum.

Cre recombinase (Soriano, 1999). The activation of the lacZ gene will mark Cre expressing cells and all their descendants, since the ROSA26 promoter is expressed in essentially all embryonic and adult mouse tissues. The Rosa26 locus was discovered by random insertional mutagenesis (gene trap), and its biological function is not known. No overt phenotypes have been reported in heterozygous and homozygous Rosa26 gene trap mutant mice (Zambrowicz et al., 1997).

Expression during mammary gland development

Overall, the two transgenic lines investigated (lines A and D) displayed similar expression profiles although some differences were noted (Table 1). When the ROSA-lox-STOP-lox-LacZ reporter gene is activated in a cell by Cre-mediated recombination, this cell and all of its descendants will express β-galactosidase and will stain blue with X-gal. All studies were performed comparing multiple sections from multiple animals that carried both the ROSA26 LacZ transgene and the MMTV-Cre transgene (positive sample) compared to mice that contained the ROSA26 LacZ transgene but not the MMTV-Cre transgene (negative control). Cre expression, as monitored by blue staining, was observed in ducts and alveoli in the mammary gland (Figure 1). Whole mount analyzes demonstrated that mammary ductal cells in mice from line A had undergone recombination as early as day 6 after parturition (Figure 1A). In contrast, line D displayed no recombination as observed by whole mount analyzes in day 7–8-day-old pups or 2-week-old animals. Staining in mammary tissue from the D line was first observed at 22 days of age and continued to show consistent expression from this timepoint onwards. By 5 weeks of age, both lines showed intense staining in most ducts and terminal endbuds, and by 6 weeks of age the entire ductal tree had undergone recombination in both lines (Figure 1B and D). As the mammary gland develops, ductal structures elongate and branching occurs. Ductal elongation proceeds from the terminal end buds (TEBs), which consist of

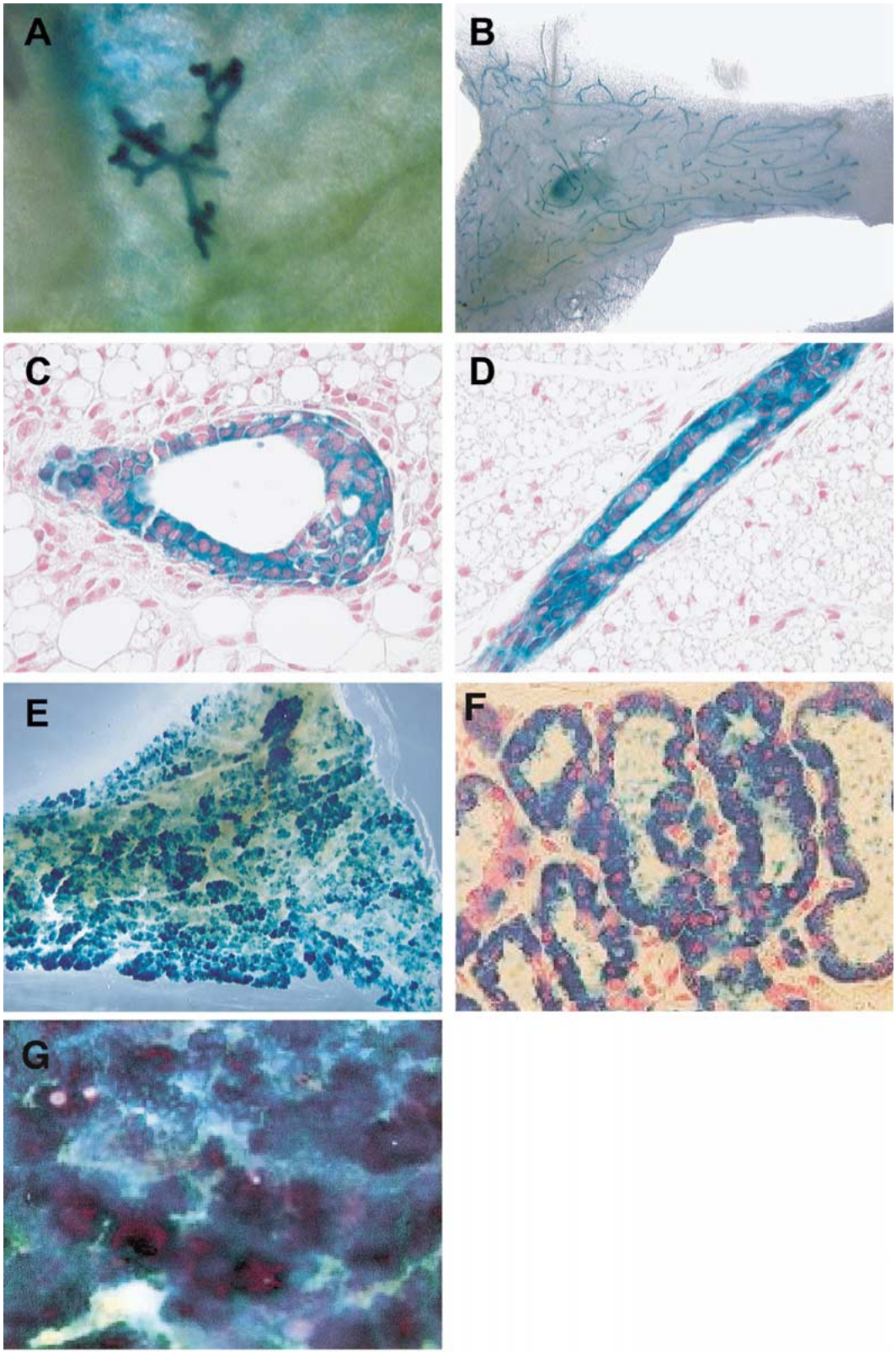

Figure 1.

body cells and cap cells destined to become ductal epithelium and myoepithelium. Both the body cells and CAP cells had undergone recombination in both lines (Figure 1C). Some lymphocytes in the lymph node of the mammary gland had also undergone recombination. Both lines showed extensive expression in epithelial and myoepithelial cells with little or no expression in stroma, fibroblasts, or adipocytes of the mammary gland. Although some mosaicism of staining of luminal epithelial and myoepithelial cells was observed in adult animals (Figure 2A–F), the majority of these cells expressed Cre recombinase.

The lobulo-alveolar mammary epithelium develops during pregnancy, and this process involves extensive cell proliferation and differentiation. Whole mount analysis revealed extensive, although not complete recombination within the lobulo-alveolar compartment (Figure 1E and F). Line A (Figure 1G) exhibited less mosaicism than line D. Some variation was observed between individual mice. This mosaicism suggests that some alveolar progenitor cells did not express the MMTV-Cre transgene. At 3 and 10 days of involution, most but not all of the ducts remained positively stained (data not shown).

MMTV-Cre expression is restricted predominantly to secretory cell types

The MMTV-LTR is active not only in mammary tissue but also in other secretory cell types. In our initial study of the MMTV-Cre mice (Wagner et al., 1997), we used RT-PCR as well as a reporter construct to survey expression and concluded that limited recombination had occurred in every organ. We now analyzed a range of tissues from both lines of transgenic mice on a cellular level (Figure 3). These studies demonstrated that in 3-month-old mice distinct cell types within several organs undergo MMTV-Cre mediated recombination. Both male and female mice at all ages in both lines showed moderate to intense lacZ staining of the submandibular gland (Figure 3A), submaxillary, and parotid glands in the salivary gland. Little recombination was observed in the sublingual gland. Cre-mediated deletion was also detected in secretory cells of the skin epithelium and in hair follicles (Figure 3B), Leydig cells (Figure 3C), epithelial lining of the vas deferens (Figure 3D), and seminal vesicles (Figure 3E). Although we observed some mosaicism, the vast majority of cells in these tissues had undergone a recombination event. In addition to these epithelial cells, we observed recombination in B- and T-cells (Figure 3G and H) and megakaryocytes of spleen in both lines (Figure 3H) and in erythroid cells. Analysis of Ter119-positive erythroid cells revealed that a majority of the cells had undergone Cre-mediated recombination (Wagner et al., 2000). Both lines also showed staining in acini but not islets of pancreas (data not shown).

Other tissues displaying limited Cre expression in 5- and 12-week-old animals from both lines were liver, trachea, and brain neurons. Little or no expression was ever observed in stomach, intestines, adrenal glands, kidney, lung, heart, skeletal muscle, or pituitary. In 3- and 6-month-old animals from line A, we observed additional staining in the bronchiolar epithelial cells, adrenal gland (focal areas of adrenal cortical cells) and numerous areas of female reproductive tract including the endometrial gland and luminal epithelium of the uterus and surface squamous epithelium of the vagina (data not shown). This staining was not observed in younger mice from line A or in mice from line D at any age. Some staining was observed in the prostate for both strains at various ages but this staining was also observed in some control animals and therefore may not represent true positive expression of Cre in this tissue. We further investigated whether recombination can already be observed during fetal development. Recombination in the ectoderm occurs already prior to embryonic day 11.5, as demonstrated by the blue embryos (Figure 3I) and is clearly visible at days 13.5 and 15.5 (Figure 3J and K).

Cre expression in oocytes

Unlike the MMTV-Cre line D, floxed alleles that pass through the female germline of MMTV-Cre line A are deleted in all tissues of the resulting offspring. This suggested that for line A, the integration site of the transgene permitted the MMTV-LTR to be active in oocytes during follicular development. Both 4- and 12-week-old ROSA26 LacZ mice carrying the MMTV-Cre A transgene showed intense lacZ staining of some preantral and antral follicles (Figure 3F) compared both to control littermates not carrying the Cre transgene and to ROSA26 reporter mice carrying

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Figure 1. Mammary epithelium at different developmental stages of MMTV-Cre line A (A, B, G) and line D (C, D, E, F). A: ducts, 6-day-old female; B: 6-week-old virgin; C: cross section through a terminal end bud (TEB); D: cross section through a duct; E: whole mount, lactation; F: cross section through whole mount shown in E; G: whole mount, lactation.

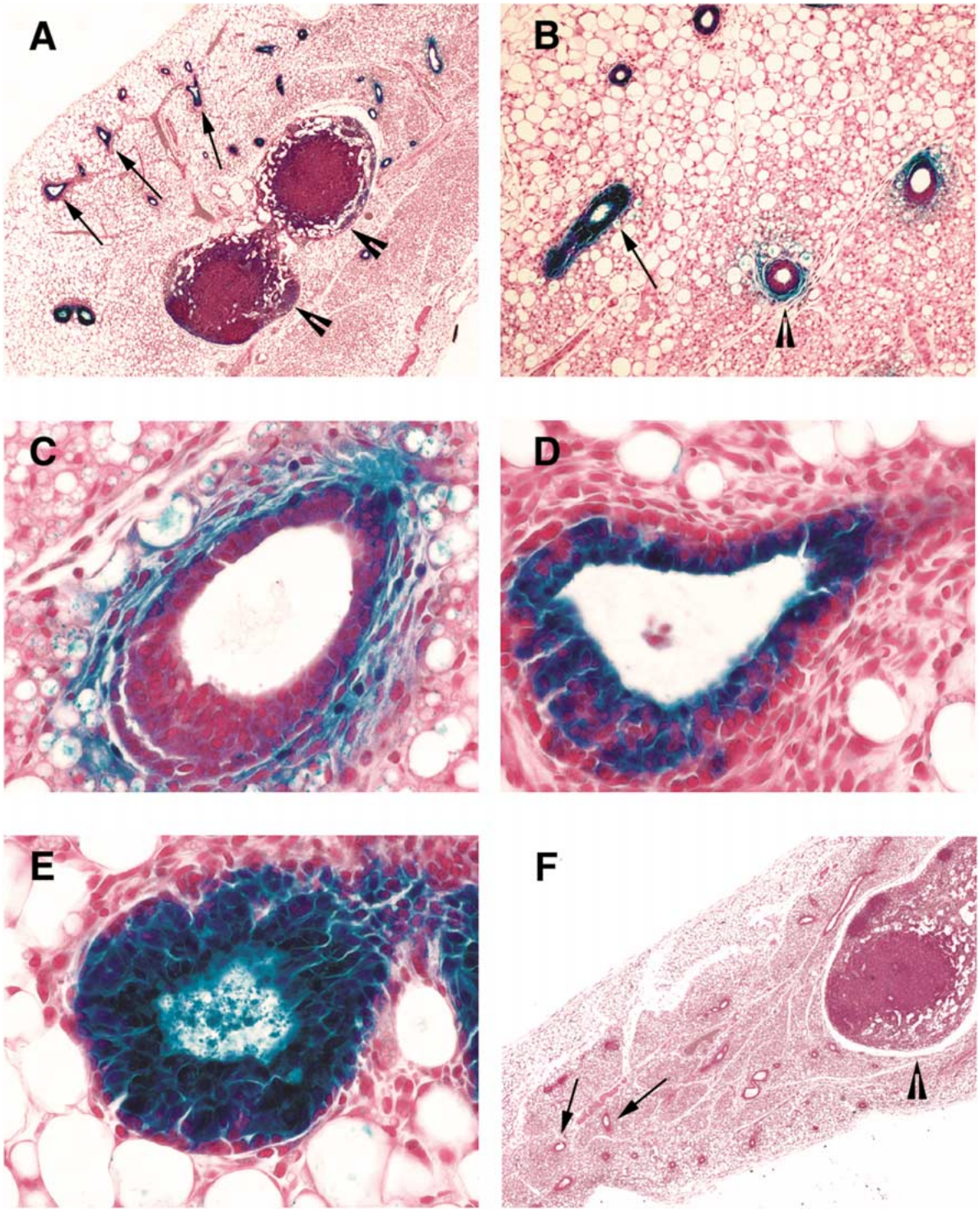

Figure 2. Variable staining of mammary epithelium from an MMTV-Cre line D/ROSA26 reporter mouse. A: 5-week-old mammary gland; low magnification of positively staining ducts. Arrows point to positively staining ducts. Arrowheads point to lymph nodes; B: higher magnification of mammary gland with partial staining of luminal epithelial and myoepithelial cells (arrow and arrowhead); C, D and E are differential staining of individual ducts; F: littermate control; Arrows point to control stained ducts; Arrowhead points to a lymph node.

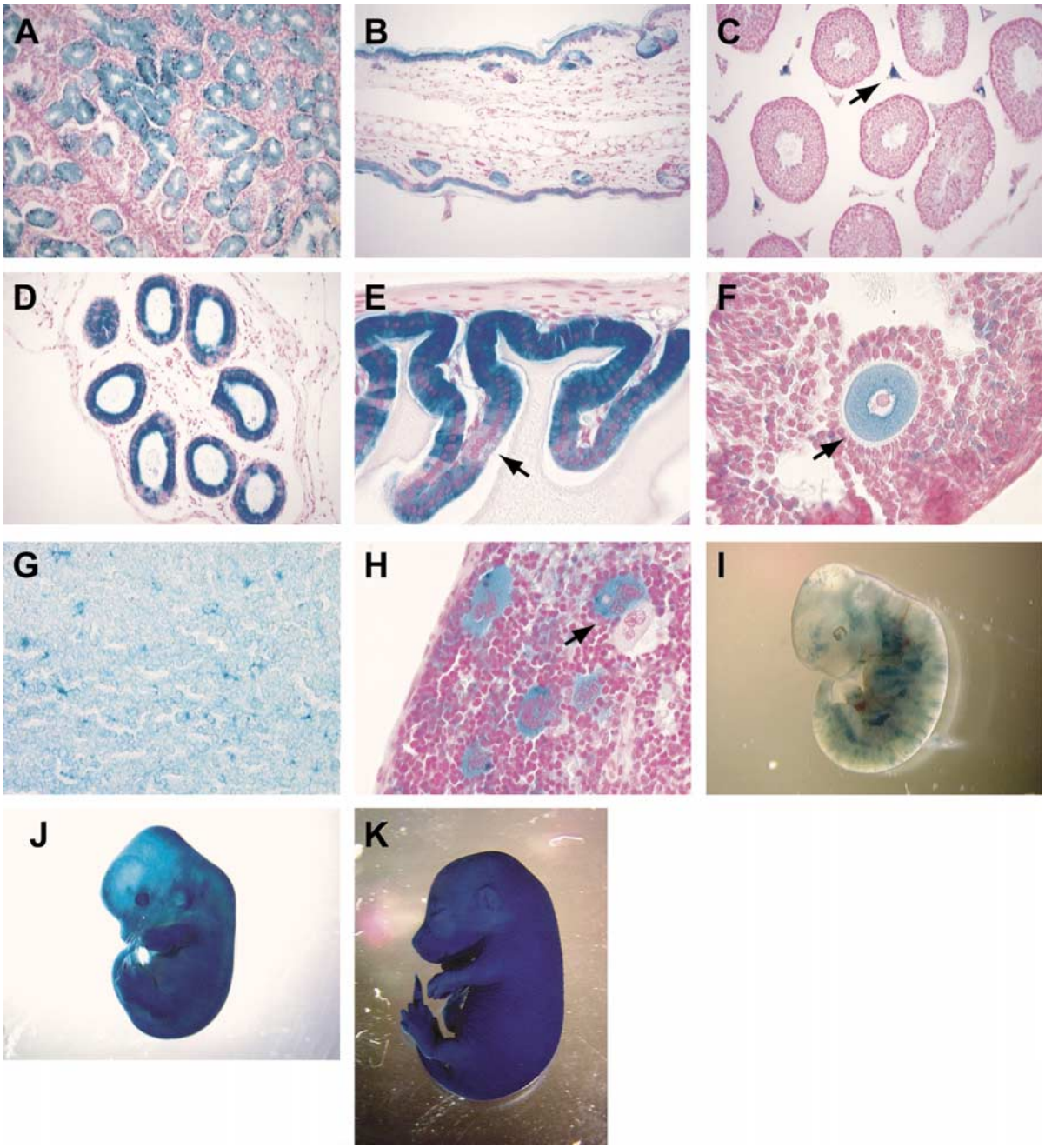

Figure 3. Organ Profile of LacZ expression in double transgenic mice that carry a Rosa26 reporter and the MMTV-Cre line A (A–G) or line D (H–K) transgene. A: submandibular gland; B: skin; C: cross section through adult testis (arrow points to Leydig cells); D: vas deferens; E: seminal vesicle (arrow points to an area of mosaic expression); F: ovary of an adult female (arrow points to the oocyte); G) thymus; H) spleen (arrow points to a megakaryocyte); I) day 11.5 fetus; J) day 13.5 fetus; K) day 15.5 fetus.

the MMTV-Cre D transgene. Active β-galactosidase was also observed in some primordial follicles of 6-day-old MMTV-Cre (A)/Rosa26 double transgenic females (see http://histology.nih.gov; HistoBank accession #1962) suggesting a very early activation of the MMTV-LTR during oocyte maturation. These observations indicate that MMTV-Cre (A) is active in the female germline before meiosis. In fact, MMTV-Cre (A)/Rosa double transgenic females crossed with wild types males generate offspring that carry a recombined Rosa locus (i.e. a ubiquitously activated Rosa-LacZ gene) in the absence of an MMTV-Cre

transgene. An example for the ubiquitous activation of the Rosa locus in the resulting offspring is shown in HistoBank (accession #1999). These animals exhibit extensive β-galactosidase expression in stromal cells of the mammary gland (fat cells, fibroblasts etc.) that is absent in MMTV-Cre (A)/Rosa double transgenic females (see HistoBank #1968).

Conclusions

These studies define the cell-specific expression pattern of the Cre gene under control of the MMTV-LTR in two independent lines of transgenic mice (Table 1). Consistent and most of the uniform recombination has been observed in mammary ductal epithelium, the acinar epithelium of the submandibular gland, skin, Leydig cells, seminal vesicles, megakaryocytes, B-cells, T-cells and hematopoietic cells. Both lines displayed specificity for epithelial cells in the mammary gland but the precise timing of Cre activation appeared to be slightly different for the two lines. In addition, Cre activity in oocytes was only observed in line A.

For conditional Cre-loxP studies, the timing of Cre activation in the particular transgenic line used can be critical for avoiding embryonic lethality inherent in the germline knockout of many genes and in avoiding non-specific phenotypes unrelated to the target tissue for the human disease one hopes to model. Precise activation of Cre can also be important for conditional studies to determine the precise timing of any treatments used for the conditional animals such as irradiation or carcinogen administration. The specificity of Cre activation observed in these studies in the mammary epithelial cells for both MMTV-Cre line D and MMTV-Cre line A makes this Cre transgenic animal an appropriate model to use when focusing on this key target cell type in the mammary gland.

Although expression of Cre in these MMTV-Cre lines is clearly not restricted solely to mammary epithelial cells, precise mammary gland phenotypes have been successfully generated with these mice. Conditional homozygous *Brca1* animals generated with the MMTV-Cre D line initially displayed a severe inhibition of mammary ductal branching (Xu et al., 1999). These conditional *Brca1* mice went on to develop subsequent tumor formation after a long latency period (10–13 months of age) with a pathology similar to human breast cancer (Xu et al., 1999). Our present study shows that Cre expression in the lobulo-alveolar compartment of the mammary gland exceeded 50% but exhibited mosaicism. Given that the majority of ducts did remain positively stained after lactation and involution in this present analysis, it is highly likely that Cre activation and subsequent inactivation of the conditional knockout gene would not be substantially disrupted with the remodeling of the gland during involution.

The differences observed in expression pattern between these two distinct lines can be exploited for slightly different uses by individual investigator's needs. The specific activation of the D line seems to coincide more closely with the initiation of puberty in the mammary epithelial cells. In general, this line might be more closely under the control of ovarian hormonal control as one might expect for a 'classic' MMTV-driven transgene. The D line might therefore be more appropriate for use in the context of a conditional knockout where one wants precise inactivation of their gene to occur only with the initiation of ovarian function.

Females from the MMTV-Cre Strain A line which also carry a floxed gene have the particularly useful and unique feature of passing on a null allele to their offspring. This feature allows the investigator to use this unique line for generation of not only conditional but germline deletions of the gene of choice and several laboratories have recently utilized this approach (Gérard et al., 1999; Loots et al., 2000; Rucker et al., 2000). This unique expression pattern in this line is probably due to the integration of the transgene into an as yet unidentified gene active in oocyte development. This has been confirmed in this study by the recombination observed in oocytes from line A, but not in line D. Despite the fact that staining in the oocytes appears mosaic, we have never observed an unrecombined allele when passed through the female germline of an MMTV-Cre line A mouse. It is possible that not all recombined cells always stain for β-galactosidase activity due to staining artifacts or perhaps due to a ROSA promoter expression profile which is not completely ubiquitous in developing oocytes. We have not excluded the possibility that additional recombination occurs at the time of ovulation in this line as well but the activation of Cre appears to occur at least to some extend very early from line A in the oocytes.

In addition to the distinct oocyte expression profile, line A exhibited several other features unique from line D. Although both lines should be appropriate for inactivating genes in mammary epithelial cells, line A may be especially useful for studying the influence of genes on the development of the primary duct of the

mammary gland prior to puberty since activation of Cre in this tissue appears so early in this line. Some differences in the Cre expression pattern in line A appear to vary with age with relatively wider expression in tissue types displayed for older animals. This suggests that activation in some tissues might be time-dependent in this line as well.

The easy availability of the ROSA26 LacZ reporter mouse and the MMTV-Cre Strain A used in combination with the conditional allele of the gene of choice will allow investigators to directly follow the precise cell type specificity of all alterations or phenotypes observed. Since limited mosaicism may influence these experiments in which cell survival molecules are deleted, the inclusion of a marker gene, such as the ROSA26 LacZ reporter mouse, in the experiment to mark those cells that have undergone deletion is highly recommended.

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References

Gérard M, Hernandez L, Wevrick R and Stewart CL (1999) Disruption of the mouse necdin gene results in early post-natal lethality. *Nature Genet* **23**: 199–202.

Hoess R, Abremski K and Sternberg N (1984) The nature of the interaction of the P1 recombinase Cre with the recombining site LoxP. *Cold Spring Harb Sym* **49**: 761–768.

Hoess R, Wierzbicki A and Abremski K (1987) Isolation and characterization of intermediates in site-specific recombination. *Proc Natl Acad Sci USA* **84**: 6840–6844.

Loots GG, Locksley RM, Blakespoor CM, Wang ZE, Miller W, Rubin EM and Frazer KA (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**: 136–140.

Nagy A (2000) Cre Recombinase: The universal reagent for genome tailoring. *Genesis* **26**: 99–109.

Rucker EB, Dierisseau P, Wagner K-U, Garrett L, Wynshaw-Boris A, Flaws JA and Hennighausen L (2000) Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. *Mol Endocrinol* **14**: 1038–1052.

Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature Genet* **21**: 70–71.

Wagner K-U, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li ML, Furth PA and Hennighausen L (1997) Cre-mediated gene deletion in the mammary gland. *Nucl Acids Res* **25**: 4323–4330.

Wagner K-U, Claudio E, Rucker III EB, Riedlinger G, Broussard P, Schwarzberg L, Siebenlist U and Hennighausen L (2000) Conditional deletion of the bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. *Development* **127**: 4949–4958.

Xu XL, Wagner K-U, Larson D, Weaver Z, Li CL, Ried T, Hennighausen L, Wynshaw-Boris A and Deng C-X (1999) Conditional mutation of *Brca1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nature Genet* **22**: 37–43.

Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG and Soriano P (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci USA* **94**: 3789–3794.