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PRINCIPAL INVESTIGATOR: Dr. Kay-Uwe Wagner

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

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Dr. Kay-Uwe Wagner

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

E-mail: kuwagner@unmc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Purpose: The goal of this proposal is the cloning of specific cells from the mouse mammary gland that have the capacity for self-renewal. There is overwhelming evidence that these types of cells play an important role in the origin of mammary cancer. Cloning and characterizing these cells would provide with new insights to find unique indicators for self-renewing cells that can be utilized for cell selection techniques or drug targeting.

Major findings: We have identified a new mammary epithelial cell population that originates from differentiating cells during pregnancy. This epithelial population does not undergo cell death during involution and remodeling following a lactation period. We show that these cells can function as alveolar progenitors in subsequent pregnancies, and they can play an important role in functional adaptation. We have studied the growth properties of these cells *in vitro*, and we have clonally amplified these cells in primary mammary epithelial cell cultures. In transplantation studies, this parity-induced epithelial population shows the capacity for self-renewal and contributes significantly to ductal morphogenesis and lobulogenesis (i.e. these cells have important features of mammary stem cells). However, it is still unclear whether these cells can give rise to *all* epithelial subtypes including myoepithelial cells.

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Table of Contents

Introduction:

The **main objective** of this study is the cloning of specific cells from the mouse mammary gland that have the capacity for self-renewal. Using the Cre-lox recombination system that might allow us to genetically label self-renewing cells, we plan to identify and clone cells that show characteristics of stem cells. There is overwhelming evidence that these types of cells play an important role in the origin of mammary cancer (reviewed by Chepko and Smith, 1999). The cloning and characterization of these cell types would provide new tools to find unique indicators for self-renewing cells that can be utilized for cell selection techniques or drug targeting.

Body:

1. Generation of an *in vivo* model that allows the labeling of self-renewing cells and the detection of genetic differences in mammary glands of non-pregnant nulliparous and parous animals.

Initial studies:
As outlined in the research proposal, we wanted to utilize the Cre-loxP recombination system to genetically label self-renewing cells that show specific features of stem cells. We have previously developed various transgenic strains that express Cre recombinase in the epithelial cells of the developing mammary gland (Wagner et al., 1997). To accomplish our goal, we had to first gather preliminary data on the temporal and spatial expression of Cre recombinase in each of the transgenic strains at the level of single cells. Therefore, we crossed all individual Cre-expressing lines into lox reporter strains that carry the Rosa-lox-Stop-lox-LacZ (herein referred as Rosa-LacZ) or Rosa-lox-Stop-lox-βgeo (herein referred as Rosa-βgeo)(Mao et al., 1999; Soriano, 1999). The tissue-specific expression of Cre recombinase catalyzes the excision of the transcriptional *Stop* sequence between two directly oriented *lox* sites, which subsequently leads to the activation of the reporter construct (LacZ or βgeo) under control of the ubiquitously expressed Rosa locus (for more information see Wagner et al., 2000). We first investigated the expression pattern of Cre recombinase driven by the long terminal repeat of the mouse mammary tumor virus (MMTV-LTR). We studied the temporal and spatial expression of the MMTV-Cre transgene in two independent lines, and the results are described in detail in the publication attached (Wagner, K.-U.; K. McAllister; T. Ward; B. Davis; R. Wiseman and L. Hennighausen (2001): Spatial and temporal expression of the Cre gene under the control of the MMTV LTR in different transgenic lines. ***Transgenic Research***, in press). ***In summary***, the two lines examined showed distinct differences in their expression pattern in the developing mammary gland. For instance, the line D activates the Cre transgene much later, and the transcription seems to be more tightly controlled by ovarian hormones. However, both lines exhibited extensive recombination in all epithelial cells including the ductal myoepithelium suggesting that the MMTV-LTR is not specifically expressed in certain epithelial subtypes. Although it has been known from earlier studies that the MMTV-LTR is expressed in mammary stem cells (Kordon and Smith, 1998), the MMTV-Cre transgene is, nonetheless, of limited value to label specifically these self-renewing cells since all descendents of MMTV-Cre expressing stem cells also express the Rosa reporter. Based on our previous findings (Wagner et al., 1997), these preliminary results were expected.

A new strategy to label self-renewing mammary epithelial cells:
As shown above, the Cre-lox technique is less useful in identifying putative, undifferentiated mammary stem cells. However, we have developed a strategy based on the Cre-lox technique that will

allow us to label differentiating cells that maintain certain features of stem cells such as self-renewal. The principle of this strategy is based on the fact that a certain number of cells that express terminal differentiation markers bypass apoptosis during the involution phase following lactation (Wagner et al., 1997). We show later that these cells remain in the parous gland, where they can give rise to a clonal population of alveolar cells during subsequent pregnancies. Therefore, the often-stated paradigm that "*all differentiated cells undergo apoptosis during involution and new secretory epithelial cells arise from undifferentiated alveolar precursors*" is probably incorrect. Beside that dogma, one might expect that our approach would lead to the identification of alveolar progenitor cells. Surprisingly, these self-renewing cells are more than just alveolar precursors since they can contribute to the morphogenesis of primary and secondary ducts in transplants (see chapter 2.). To our knowledge, this is the first time that a trans-differentiation process of cells from an alveolar into a ductal type has been documented in a normal mammary gland.

Background: In mammals, steroid and peptide hormones in synergy with local growth factors control the proliferation and differentiation of mammary epithelial cells (Topper and Freeman, 1980). Mammary gland morphogenesis proceeds in distinct phases. A mammary anlage and rudimentary ducts form during fetal development, but the majority of ductal elongation and branching occurs primarily after the onset of puberty. Pregnancy induces alveolar proliferation (lactogenesis I), and functional differentiation and milk secretion are achieved shortly before parturition (lactogenesis II) (Hennighausen and Robinson, 1998; Neville, 1999). At the end of the lactation period, the mammary alveolar compartment regresses rapidly, and the morphology of the gland resembles that of a mature virgin. This involution process goes through two distinct phases (Lund et al., 1996). The first phase is characterized by an induction of apoptotic signaling pathways in the alveolar compartment without remodeling. During the second phase of involution, the basement membrane and extra-cellular matrix are degraded, and the alveolar compartment is obliterated. It is a paradigm that all terminally differentiated cells undergo apoptosis, and that the alveolar compartment is reconstituted in subsequent pregnancies from undifferentiated mammary stem cells or alveolar precursors.
In each reproductive cycle, proper alveolar differentiation and milk protein synthesis require the synergistic action of lactogenic hormones and local growth factors (Hennighausen and Robinson, 1998). Prolactin seems to play a central role in this differentiation process (Horseman et al., 1997; Ormandy et al., 1997). The signal transducer and activator of transcription 5a (STAT5a) as a component of the prolactin-signaling pathway (Liu et al., 1997) cooperates with other factors such as the glucocorticoid receptor and C/EBPβ and to achieve a maximum of milk protein gene expression (Stocklin et al., 1996; Wyszomierski and Rosen, 2001). Very low levels of milk protein gene expression can also be found in virgins, but their synthesis increases considerably during the second half of pregnancy (Robinson et al., 1995). Interestingly, the transcriptional regulation of milk protein genes varies slightly between caseins and whey proteins. In mice, casein transcription increases rather early during pregnancy, and high levels of expression of the whey acidic protein (WAP) and α-lactalbumin is restricted to the last phase of pregnancy (i.e. mainly in lactogenesis II) (Pittius et al., 1988; Robinson et al., 1995). The differential up-regulation of caseins and whey proteins may reflect a progression toward terminal differentiation. Beside hormones and local growth factors, a proper expression of WAP requires cell-to-cell contact and the formation of a closed lumen (i.e. a correct three-dimensional structure of an alveolus)(Chen and Bissell, 1989). Therefore, the expression profile of WAP is frequently applied as an indicator for advanced differentiation of mammary epithelial cells. High levels of WAP expression are maintained throughout lactation, but its expression declines significantly during the first phase of involution (days 1 and 2 after weaning) and reaches nearly

undetectable levels during the second phase of mammary gland remodeling (i.e. 4-6 days after weaning of the litter)(Burdon et al., 1991 McKnight et al., 1992).
Based on the two paradigms that (I) high levels of WAP expression is restricted to differentiated mammary epithelial cells, and that (II) terminally differentiated cells undergo apoptosis, it was unclear whether a WAP-promoter driven transgenic mouse model expressing Cre recombinase would be useful to study the loss-of-function of genes in the mammary gland, in particular, tumor-susceptibility genes in multiparous and aging mouse models for human breast cancer. Unlike various other WAP-based transgenic strains (Burdon et al., 1991; McKnight et al., 1992), we were able to identify a WAP-Cre expressing line that follows closely the temporal and spatial regulation of the endogenous WAP gene (Wagner et al., 1997). Based on genomic alterations that the Cre recombinase 'engraves' on another reporter transgene (i.e. recombination between loxP sites), we found that a large number of mammary epithelial cells previously expressing the WAP-Cre transgene remained in the mammary gland after complete remodeling (Wagner et al., 1997). More importantly, these cells seem to multiply during subsequent pregnancies, and therefore, our findings contradict the paradigms on mammary development stated above. Clearly, our observations are not an artifact caused by a deregulated activation of the promoter of our randomly integrated WAP-Cre construct since Ludwig and coworkers (2001) have recently reported similar observations in mutant mice that express Cre under the endogenous WAP promoter (WAP-Cre knockin mutants). Based on these findings, we hypothesize that a certain number of WAP-expressing or differentiated cells bypass apoptosis and remain in the parous gland, where they can give rise to a clonal population of alveolar cells during subsequent pregnancies. Indirectly, our hypothesis implies that mammary epithelial cells from parous individuals are different from nulliparous animals in their 'genetic program' despite the close resemblance in their morphological appearance. To address this issue, we have used double transgenic mice carrying the WAP-Cre transgene (Wagner et al., 1997) and the Rosa-LacZ or the Rosa-βgeo reporter constructs (Mao et al., 1999; Soriano, 1999) to monitor differentiation and cell survival in the developing and involuting mammary gland on the level of single cells, and to clone these self-renewing cells *in vitro*.

Basic principle, and specific experimental design for the identification and cloning of self-renewing mammary epithelial cells with partial stem cell character: Figure 1 (see appendices) illustrates the experimental design and basic principle of the transcriptional activation of the WAP-Cre and Rosa reporter transgenes before and during differentiation and in the involution phase. Both transgenes are inactive in undifferentiated alveolar precursors or stem cells in virgin (nulliparous) females. During lactogenesis II, transcription of the endogenous WAP gene and the WAP-Cre transgene is greatly induced by systemic hormones and local growth factors in differentiating alveolar cells. Subsequently, the WAP promoter-driven expression of Cre recombinase permanently activates the transcription of the β-galactosidase reporter gene (LacZ or βgeo) due to the Cre-mediated excision of the floxed transcriptional STOP sequence between Rosa regulatory elements and the LacZ coding sequence. X-Gal staining on histological sections or whole mount tissue samples can be used to identify single cells that express β-galactosidase. Since the Rosa locus is ubiquitously expressed, the transcriptional activation of the reporter gene is independent from the differentiation status of a given cell. This unique feature of our double transgenic mouse model enables us, to monitor not only the differentiation of mammary epithelial cells, but also to permanently label cells that no longer express WAP-Cre during involution and after remodeling is completed. The partially committed cells that remain in the parous and multiparous gland represent a new epithelial population that does not exist in virgin animals. As shown later, these cells have certain features of mammary stem cells such as self-renewal. Cloning of these self-renewing cells can be achieved by establishing primary cell cultures of

involuted WAP-Cre/Rosa-LacZ double transgenics, and subsequent selection of β-galactosidase expressing cells using the FACS-Gal technique (Zambrowicz et al., 1997).

2. Genetic labeling and discovery of a new mammary epithelial cell population that originates from differentiating cells during pregnancy:

We initially analyzed the transcriptional activation of WAP-Cre and the Rosa-LacZ reporter gene during the first pregnancy-lactation-involution cycle of double transgenic nulliparous females and their single transgenic littermates. The inguinal glands (glands #4) were taken at various stages of the reproductive cycle and stained with X-Gal to examine the timing of Cre expression and to monitor the location of WAP-Cre expressing cells during involution (Fig. 2, see appendices). Almost no β-galactosidase expression was detected in virgin and early to mid-pregnant WAP-Cre/Rosa-LacZ double transgenic mice (Fig 2A). Some blue cells could be detected during estrus in virgin mice but they were no longer present at any other stages of the estrus cycle suggesting that these cells do not remain or accumulate in aging nulliparous animals. Cre-mediated excision of the transcriptional STOP sequence and activation of the LacZ reporter was detected in late pregnant animals (>day 16 of gestation), and a maximum of Cre activation is achieved in terminally differentiated cells around parturition (Fig. 2B). Since there is no selective mechanism against Cre-expressing cells, it is not surprising that the vast majority of differentiated alveolar cells express WAP-Cre throughout the entire lactating mammary gland. Therefore, the recombination efficiency was much higher in this particular experimental setting compared to our previous report where WAP-Cre expressing cells were partially eliminated due to the loss of *brca1* (Xu et al., 1999). However, mosaic expression of WAP-Cre or activated Rosa-LacZ was detected in a small percentage of cells in individual alveoli when histological sections were prepared from X-Gal-stained whole mount specimen (data not shown). No X-Gal positive mammary epithelial cells were detected in single transgenic or non-transgenic controls (Fig. 2D to 2F). Programmed cell death of terminally differentiated alveolar cells and irreversible remodeling of the entire mammary gland is initiated 2 to 3 days after removal of the pups from the lactating dam. The remodeling process is usually completed between 5 to 10 days post-weaning in wild type mice with minor variations between different strains. To ensure complete remodeling of the entire gland, we have analyzed mammary glands of involuted double transgenic mice and their controls around 3 to 4 weeks after the removal of the pups. Based on our previous findings (Wagner et al., 1997), we expected that an involuted mammary gland might contain only a moderate or very low number of recombined cells. In contrast, we have observed a significantly greater number of X-Gal positive cells in involuted WAP-Cre/Rosa-LacZ females than anticipated. The blue cells were found on virtually every extremity of the ductal tree (Fig. 2C). A closer examination of histological sections from these whole mounts revealed that the X-Gal positive cells are located primarily in epithelial structures that are similar to that of a terminal ductal lobular unit (TDLU) in humans (Fig. 2H and 2J). Morphologically, these blue cells in involuted dams (parous females) appeared to be similar to cells of the terminal ducts in mature virgins (nulliparous females)(Fig. 2G and 2I). Despite their close resemblance on the histological level these blue cells are different in their 'genetic program' since they had initiated a differentiation program (WAP-expression) as a response to lactgogenic hormones during pregnancy and lactation. However, these cells did not undergo cell death during involution and remodeling. ***In summary***, these X-Gal positive cells represent a new population of epithelial cells that are specific for parous animals. We will demonstrate later that these cells indeed have an altered

genetic program that enables them to quickly adapt to different physiological conditions in mice with a defective hormone-signaling pathway.

3. The parity-induced new epithelial cells serve as lobular progenitors in multiparous animals, and they share certain features with multipotent mammary stem cell.

Based on the location of the X-Gal positive cells at the extremity of the ducts, we hypothesized that these cells might serve as progenitors for the proliferation and differentiation of alveolar cells during subsequent pregnancies in multiparous mice. To address this issue, we examined the growth properties of X-Gal positive cells during early stages of the second pregnancy (day 8 of gestation). As described earlier, alveolar cells highly proliferate at this stage but WAP expression remains low. WAP-Cre/Rosa-LacZ double transgenic dams lactated for 21 days during the first reproductive cycle, and they had at least two weeks of a resting period before they were mated again. At day 8 of the second gestation period, the inguinal glands were taken and stained with X-Gal (Fig 3A to 3C). The majority of the developing alveoli were noticeably X-Gal positive suggesting that these cells are, in fact, descendants of the blue cells that survived during remodeling (Fig. 3B). However, the proliferating population of blue cells was mainly restricted to terminal ducts and developing alveoli. X-Gal positive cells were hardly ever observed in primary or secondary ducts (3C).

Our findings might suggest that X-Gal positive cells at the extremity of ducts are committed alveolar precursors. The existence of such an epithelial subtype has been demonstrated earlier (Kamiya et al., 1998; Kordon and Smith, 1998; Smith, 1996). One approach to test whether these cells are alveolar precursors is to transplant small pieces of involuted mammary tissue with blue cells from WAP-Cre/Rosa-LacZ dams into the fat pat of immunocompromised recipients (e.g. athymic nude mice). If formerly WAP-Cre expressing cells are committed to differentiate only into alveolar subtypes, then these cells should not contribute to the formation of primary or secondary ducts. The current paradigm is that ductal epithelium develops from ductal progenitors as well as from primary epithelial stem cells (Smith, 1996; Smith and Chepko, 2001). Prior to the transplantation of the involuted WAP-Cre/Rosa-LacZ donor epithelium, the wild type endogenous mammary epithelia had been removed from the virgin recipients. The recipients were maintained as nulliparous females for 8 to 12 weeks to provide sufficient time for the mutant epithelia to penetrate the wild type fat pad and to form a ductal tree. These animals were neither stimulated with exogenous hormones nor mated to avoid a secondary activation of WAP-Cre. We analyzed twenty-two X-Gal stained whole mounts from eleven nulliparous receipints (two #4 glands each). Seven glands did not show any outgrowth and one gland contained wild type epithelium as a result of an incomplete clearing procedure. From fourteen outgrowths only two contained no blue cells, three where partially (50%) stained with X-Gal, and the vast majority (n=9; 75%) exhibited blue staining throughout the entire ductal tree (Fig. 3D and 3E). We repeated this experiment, and again, nine out of ten outgrowths were blue. To verify that the transplantation technique itself does not cause a secondary activation of WAP-Cre, we performed a control experiment where we transplanted mammary epithelium from nulliparous WAP-Cre/Rosa-LacZ double transgenic mice into the cleared fat pad of recipients. We obtained ten outgrowths, and only two exhibited a speckled X-Gal staining in a few parts of the ductal epithelia. Eight out of ten transplants were completely X-Gal negative (Fig. 3F) suggesting that a spontaneous activation of WAP-Cre in transplants from nulliparous donors is a rare event. In addition, the examination of serial sections through the few blue areas revealed that true X-Gal positive cells were not present, and the blue stain was trapped between cells at the edge of the section. Therefore, our findings on the growth properties

of blue cells from involuted WAP-Cre/Rosa-LacZ mice were not an artifact of the transplantation procedure (3F). ***In summary***, using our double transgenic mice to permanently label differentiated and apoptosis-resistant cells we have demonstrated for the first time that cells previously expressing an alveolar differentiation marker (i.e. WAP) can contribute to the formation of primary and secondary ducts. It remains to be determined in a more detailed study whether these cells give rise to all ductal epithelial subtypes including myoepithelial cells. Preliminary studies on histological sections of the transplants show that the blue cells are mainly localized in the luminal epithelium of large ducts (Fig 3G), small ducts (Fig. 3H), and terminal end buds (Fig. 3I).

4. The new parity-induced epithelial population plays a role in functional adaptation.

Transplantation models are powerful tools to study mammary development in mutant mice that exhibit complex phenotypes or embryo lethality after day E12.5 (Robinson et al., 2000). In the majority of experiments that utilize transplantation models, it is desired to study intrinsic effects of a targeted mutation on mammogenesis. Our findings on the selective outgrowth of X-Gal positive cells and their descendants in transplants suggest that there might be differences in transplantation models when mutant mammary epithelial cells originate from nulliparous or parous donors. We postulate that in successive pregnancies a subset of mammary epithelial cells undergo a rigorous selection process. It has been frequently reported that mouse models with a targeted mutation are able to compensate for the loss of an important gene in consecutive lactation periods (e,g. Liu et al., 1998; Ormandy et al., 1997). The genetic pathways involved in the compensation might be different for each mouse model. For instance, the lack of Stat5a can be compensated through upregulation of Stat5b (Liu et al., 1998), or the loss of one functional allele of the prolactin receptor (PRL-R) can be compensated through upregulation of the wild type allele (P. Kelly, personal communication) or through the downregulation of SOCS1 (Lindeman et al., 2001). But, what is the general mechanism for cell selection that results in a functional mammary gland? According to the current paradigm, alveolar self-renewal that originates only from a naïve stem cell population during each pregnancy cycle does not provide a "genetic instruction" for the reversal of a mutant phenotype since differentiated cells that adapt to a mutant situation are lost during the involution phase. We have crossed WAP-Cre/Rosa-LacZ double transgenic mice with heterozygous PRL-R mutants (Ormandy et al., 1997) to address whether our findings on the newly identified mammary epithelial cell population in parous animals provide a general mechanism for alveolar self-renewal and reversal of a mutant phenotype in successive lactations. We have analyzed mammary differentiation and the distribution of X-Gal positive cells in three consecutive lactation and involution periods (Fig. 4). The loss of one functional PRL-R gene inhibited alveolar development (Fig 4A), and lactation could not be established after the first pregnancy cycle. Nevertheless, a limited number of X-Gal positive cells still remained in the mammary gland of involuted WAP-Cre/Rosa-LacZ/PRL-$R^{+/-}$ triple mutant mice (Fig. 4B). A significant increase in the number of differentiated alveolar cells was observed at the end of the second gestation period, and many more blue cells did not undergo apoptosis after remodeling was completed (Fig. 4D). More than 50% of the triple mutant mice were unable to nurse their litter after the second gestation period. Lactation and normal development was restored during the third pregnancy cycle (Figure 4E and 4F). Our observations suggest that the newly identified population of epithelial cells in parous mammary glands might be the basis for a general mechanism that facilitates self-renewal of the alveolar compartment in consecutive lactation cycles. The blue cells that did not undergo apoptosis during the involution phase might serve as the "functional memory" of the mammary epithelium. Again, this

could be a universal mechanism for the positive selection of cells that "learn" how to bypass an altered signaling pathway, but the adaptation of specific compensatory factors to bypass a particular targeted mutation might be different in each mutant mouse strain.

5. In vitro growth characteristics and cloning of the new parity-induced epithelial cell population.

The generation and manipulation of primary mammary epithelial cell cultures is common practice to study signaling pathways, differentiation, gene transcription, cell cycle and cell death, or neoplastic transformation. Generally, a primary epithelial cell line represents a heterogeneous population of epithelial cells. Some cells might be derived from primary ducts, others from secondary ducts or tertiary side branches, alveolar precursors, multipotent stem cells, and some cultures also contain significant amounts of myoepithelial cells. It is difficult to distinguish the origin of each subpopulation *in vitro* since cells change their morphology when grown as monolayers. The potential to repopulate a cleared fat pad of a recipient female is generally maintained after a few passages (Daniel et al., 1971; Young et al., 1971), but it is believed that these outgrowths originate solely from multipotent stem cells (reviewed in Smith and Chepko, 2001). To study morphological features and growth properties of our newly identified epithelial cell population *in vitro* and to compare these features to X-Gal negative epithelial cells, we have derived primary epithelial cell cultures from nulliparous and involuted multiparous WAP-Cre/Rosa-LacZ double transgenic females. Primary cultures were fixed and stained with X-Gal after 48 and 72 hours to assay the relative amount of blue cells among the entire epithelial population. X-Gal positive cells were visible in the majority (61-80%) of epithelial islands derived from organoids of involuted multiparous females (Fig 5A). Some islands, however, remained unstained suggesting that these cells might be descendants from primary ducts since these cells did not express the WAP-Cre transgene (Fig. 3C), alternatively, in some portions of the gland, involution of WAP-Cre expressing cells may be complete. An expansion of X-Gal positive cells was obvious when primary cells were counted after 72 hours in culture (Fig. 5B). At this stage, X-Gal positive epithelial islands contain approximately 20 to 30%, in various cases more than 60% of blue cells that are morphologically indistinguishable from their X-Gal negative counterparts (Fig. 5C). They also actively migrate away from these islands to repopulate the intervening space, and their shape is more fibroblast like (Fig 5D). However, they are morphologically distinct from X-Gal negative fibroblasts that have been separated from these cultures by trypsin treatment (Fig 5I). Epithelial islands from nulliparous WAP-Cre/Rosa-LacZ controls contain no or only very few X-Gal stained cells in 48 or 72-hours-old primary cultures (Fig. 5E to 5G). In addition, the amount of X-Gal positive cells seems to vary when tissues were derived from virgin mice at different stages of the estrus cycle. The percentile rises from diestrus (0.8%) to 4.4% at estrus and declines during metestrus II (0.9%). These findings are consistent with our observations on whole mount stained specimen (see above), and they suggest that these cells undergo apoptosis during the estrus cycle, and they are not a permanent population in the virgin mammary gland. Partial differentiation and WAP expression in the virgin gland as a result of fluctuating levels of hormones during the estrus cycle has been described previously (Robinson et al., 1995). Nevertheless, the amount of X-Gal positive cells in 48-hours-old primary mammary epithelial cell cultures of virgin WAP-Cre/Rosa-LacZ mice (less than 1%) seems to be insignificant in comparison to parous animals (more than 30%). Moreover, the blue cells in the cultures from nulliparous mice do not amplify noticeably when cultures were maintained for 72 or 96 hours (Fig. 5H). Eventually these cells are diluted out in many cultures that have been passaged repeatedly.

Limited dilution of primary cells and transplantation into the cleared fat pad is one method to determine the presence of mammary stem cells and to achieve outgrowths that are derived from single or very few stem cells and their antecedents (Kordon and Smith, 1998; Smith, 1996). We have injected single cell suspensions from involuted WAP-Cre/Ros-LacZ epithelial cells into the cleared fat pad of recipients to determine whether X-Gal positive cells alone are capable of reconstructing the entire ductal tree. It was previously shown that 2000 epithelial cells represent an endpoint concentration needed to obtain a positive take in injected epithelium-divested mammary fat pads (Smith, 1996). Therefore, we injected 23,000 epithelial cells from our double transgenic mouse model together with 200,000 fibroblasts as carrier cells into 10 cleared fat pads of recipient mice. This number was used to ensure that at least 2000 of the cells inoculated were X-Gal positive since we determined in this particular population that 10% of the cells expressed β-galactosidase. As described earlier, the animals were kept as virgins and analyzed eight to twelve weeks later. We observed ductal structures in four fat pads (an expected frequency of takes given the low number of epithelial cells injected), and each of these glands contained more than one independent outgrowth. From ten independent epithelial outgrowths, six had X-Gal positive cells (Fig. 5J and 5K). However, none of them appeared to be composed exclusively of blue cells suggesting that the newly discovered epithelial population is not capable of autonomously forming an epithelial structure in non-pregnant females. Therefore, these parity-induced cells do represent a totipotent mammary stem cell population *per se*, but these cells might support stem cell maintenance as discussed in the next section.

In order to better characterize the newly identified epithelial population (e.g. gene expression profiling or transformation assays), it is our goal to establish cell lines that are mainly composed of X-Gal positive cells. We tried various methods to enrich the blue cells in our primary cultures. Several attempts to utilize FACS-Gal (Fiering et al., 1991; Zambrowicz et al., 1997) to separate X-Gal positive cells from unstained epithelial types failed since the viability of the cells was greatly reduced after the sorting procedure. A more successful approach was the use of a newly developed mouse stain (Mao et al., 1999) that expresses a neomycin-LacZ fusion protein (βgeo) under regulatory elements of the Rosa locus (Rosa-lox-Stop-lox-βgeo knockin mice, hereafter referred as Rosa-βgeo transgenics). This Cre-lox reporter strain has the advantage that it can be used for the visualization of the Cre-mediated recombination event (X-Gal staining) in addition to *in vitro* cell selection procedures using G418 in the culture media. Primary mammary epithelial cell cultures of nulliparous and parous WAP-Cre/Rosa-βgeo double transgenic females were generated as described earlier. We began to select the cells after the second passage using 300 μg/ml of G418 in the culture media. No cells survived the selection procedure in cultures from nulliparous animals. These observations support our earlier findings that the few X-Gal positive cells in the virgin gland have different growth properties than their numerous counterparts in parous mammary glands. G418 selection of cells from parous WAP-Cre/Rosa-βgeo mice resulted in cultures with a greatly enriched percentile of X-Gal positive cells (>80%). However, these cultures still contain X-Gal negative cells suggesting that G418 selection is not a perfect procedure to generate a 100% pure population of recombined mammary epithelial cells. Primary mammary epithelial cultures grow more rapidly when cells form connections among each other. However, the formation of junctions limits the G418 selection. The selection process slowed down the growth of X-Gal positive cells, and many of them exhibited signs of senescence after a few more passages. We are currently in the process of immortalizing these cells through infection with retroviral vectors expressing viral proteins (E6, E7) that target cell cycle and tumor surveillance pathways (pRb, p53). We will examine whether these immortalized cells still have the capacity to contribute to ductal morphogenesis and whether these cells can be transformed in vitro. It will be interesting to find out whether the newly identified epithelial population in parous animals contributes to mammary cancer or

whether these cells are resistant to neoplastic transformation, which might explain differences in breast cancer susceptibility between virgin and parous animals.

Key Research Accomplishments:

- Generation of an *in vivo* model that allows the labeling of self-renewing cells and the detection of genetic differences in mammary glands of non-pregnant nulliparous and parous animals.
- Genetic labeling and discovery of a new mammary epithelial cell population that originates from differentiating cells during pregnancy. The newly discovered epithelial subtype in parous animals resists apoptosis during the involution period.
- These parity-induced cells function as alveolar precursors in multiparous animals, and they play a role in functional adaptation. In addition, these cells have certain features of mammary stem cells, for example they have the capacity for self-renewal and they can contribute to both ductal and alveolar morphogenesis.
- Establishment of primary cultures with enriched populations of the parity-induced mammary epithelial cells.

Reportable Outcomes:

Publications:

Wagner, K.-U.; K. McAllister; T. Ward; B. Davis; R. Wiseman and L. Hennighausen (2001): Spatial and temporal expression of the Cre gene under the control of the MMTV LTR in different transgenic lines. ***Transgenic Research***, in press.

Kay-Uwe Wagner, Corinne A. Boulanger, MaLinda D. Henry, Magdalene Sgagias, Lothar Hennighausen, and Gilbert H. Smith (2001): An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *In preparation.*

Conclusions:

We have identified a new mammary epithelial cell population that originates from differentiating cells during pregnancy. This epithelial population does not undergo cell death during involution and remodeling following a lactation period. We show that these cells can function as alveolar progenitors in subsequent pregnancies, and they can play an important role in functional adaptation. We have studied the growth properties of these cells in vitro, and we have clonally amplified these cells in primary mammary epithelial cell cultures. In transplantation studies, this parity-induced epithelial population shows the capacity for self-renewal and contributes significantly to ductal morphogenesis and lobulogenesis (i.e. these cells have important features of mammary stem cells). It is still unclear

whether these cells can give rise to *all* epithelial subtypes including myoepithelial cells. Future studies will determine whether these cells contribute to neoplastic transformation or whether they facilitate for a lower incidence of mammary tumorigenesis in parous animals.

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Appendices:

1. **Figure Legend**
2. **Figures 1 to 5**
3. **Manuscript Wagner et al., 2001 in Transgenic Research**

Figure Legend:

Figure 1: ***Experimental design***. This chart illustrates the basic principle of the genetic labeling of differentiating cells in the developing mammary gland using the Cre-lox technique. In this experimental setting, the WAP-Cre transgene is used solely to monitor the differentiation process of alveolar precursor cells in response to lactogenic hormones. The transient up-regulation of Cre recombinase in differentiating epithelial cells during pregnancy permanently activates a ubiquitously expressed reporter transgene (Rosa-LacZ), whose expression is not dependent of the differentiation status of a given cell. The reporter gene remains active in cells, which no longer require high systemic hormone levels to maintain a functionally differentiated state (i.e. WAP expression). Hence, the permanent activation of the reporter gene genetically labels differentiating cells that bypass apoptosis and remodeling at the conclusion of the reproductive cycle. The X-Gal positive, labeled cells in the remodeled gland represent a new epithelial subtype, which is not present in nulliparous animals.

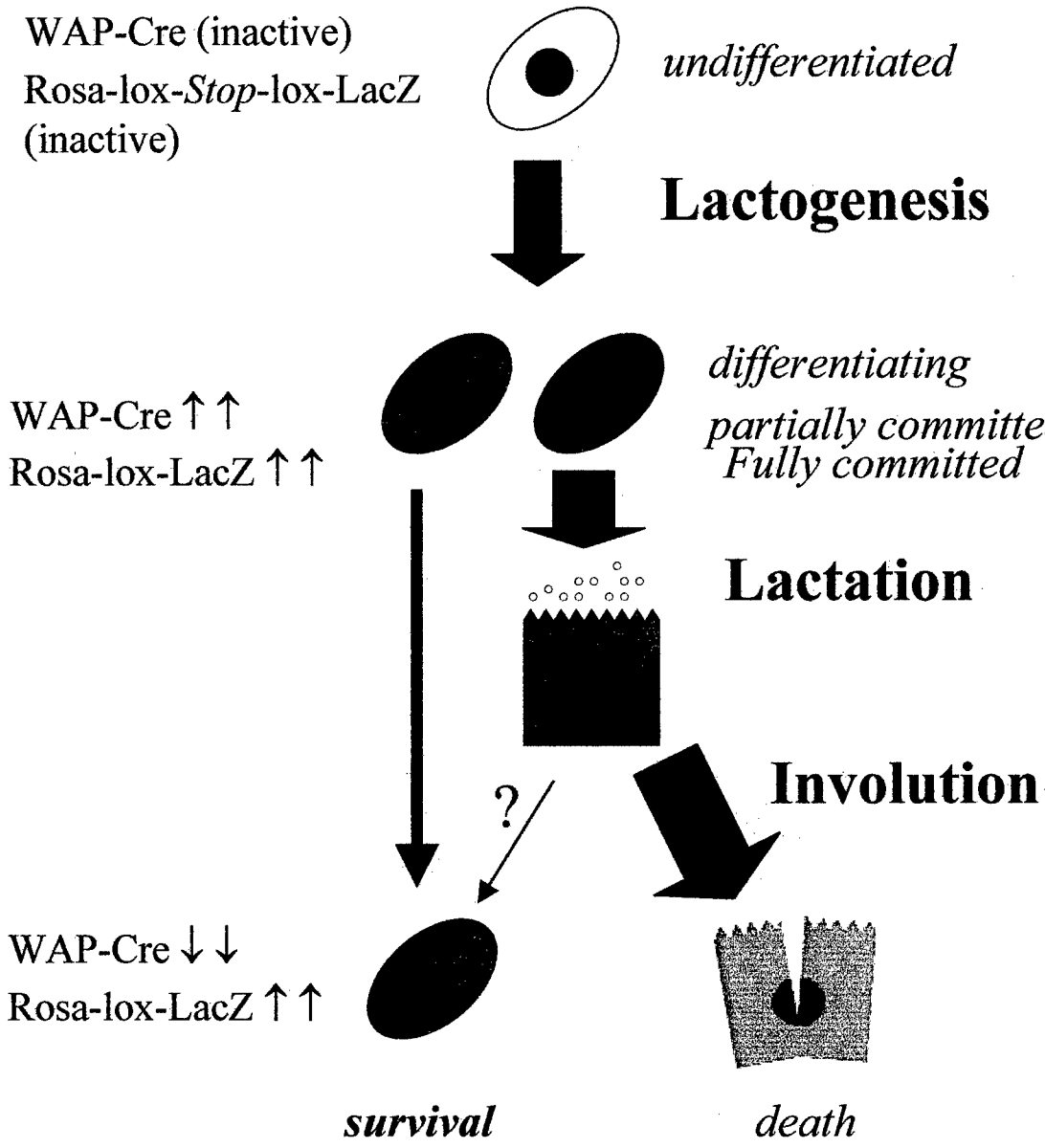
Figure 2: X-Gal stained mammary whole mounts and their histological sections of WAP-Cre/Rosa-LacZ double transgenic females (**A-C; G-J**) and their single transgenic controls (**D-F**). Note that the expression of the WAP-Cre transgene is almost undetectable in virgins and pregnant females until day 14 of gestation (**A**). WAP-Cre and subsequently Rosa-LacZ expression increases sharply thereafter and reaches a maximum of expression at the onset of lactation (**B**). A significant amount of X-Gal positive cells are still present 3 weeks after weaning of the litter (**C**). No X-Gal staining was observed in reporter mice that do not carry the WAP-Cre transgene at 14 days of gestation (**D**), lactation (**E**), or involution (**F**). X-Gal positive cells are not present in histological sections of nulliparous WAP-Cre/Rosa-LacZ mice (**G**; 200x)(**I**; 630x) but abundant in the terminal ducts of parous mice (**H**; 200x)(**J**; 630x).

Figure 3: X-Gal stained mammary whole mounts (**A, D-F**) and their histological sections (**B,C, and G-I**) of WAP-Cre/Rosa-LacZ double transgenic females at day 8 of the second gestation period (**A-C**) and in transplants of parous (**D, E, G, H, I**) and nulliparous (**F**) WAP-Cre/Rosa-LacZ epithelia into nulliparous wild type recipients. Note that the parity-induced epithelial cells in involuted WAP-Cre/Rosa-LacZ animals serve as alveolar precursors in subsequent gestation cycles (**A**; 4x)(**B**; 630x) but they remain essentially absent from larger ducts (**C**; 630x). X-Gal positive cells from involuted double transgenic mice contribute to ductal morphogenesis in transplants (**D, E**; 4x) and they are present in large ducts (**G**; 630x), small ducts (**H**, 630x), and terminal end buds (**I**; 630x). Control transplants from nulliparous double transgenic donors into wild type recipients remain X-Gal negative (**F**; 4x) suggesting that the WAP-Cre transgene cannot be activated by the transplantation technique itself. **LN**, lymph node.

Figure 4: Rescue of the lactation-deficient phenotype in three successive post-partum periods (**A, C, E**; 4x) and corresponding involution phases (**B, D, F**; 4x) in heterozygous prolactin receptor knockout mice that carry in addition the transgenic reporter constructs (WAP-Cre/Rosa-LacZ). Note the simultaneous increase in the number of parity-induced (X-Gal positive; blue) epithelial cells after each involution period and the reversion of the lactation-deficient phenotype and formation of normal secretory lobules after the third pregnancy (**E**). **B**, lower left, magnification of a selected area on the right to demonstrate the presence of a few X-Gal positive cells after the first reproductive cycle.

Figure 5: X-Gal staining of primary cultures of mammary epithelial cells (**A-H, L**), mammary fibroblasts (**I**), and a whole mount (**J**) and its corresponding histological section (**K**) of an outgrowth from transplanted dissociated cells of parous WAP-Cre/Rosa-LacZ mice into the cleared fat pad of nulliparous wild type recipients. Note the high proliferative capacity of blue cells derived from parous WAP-Cre/Rosa-LacZ mice and cultured for 48 (**A**; 100x) and 72 hours (**B**; 100x). Grown as a monolayer, these blue parity-induced cells do not differ in their morphology from other epithelial subtypes (**C**; 630x), and they can actively migrate away from epithelial colonies (**D**; 200x). Epithelial cells derived from nulliparous double transgenic mice and cultured for 48 (**E**; 100x and **F**, 400x) and 72 hours (**G**; 100x) are mostly X-Gal negative. The few blue cells detected in these early cultures of nulliparous controls do not expand even after 96 hours in culture (**H**; 100x). Note also that mammary fibroblasts of parous double transgenic animals are X-Gal negative (**I**, 100x). The injection of single cell suspensions that contain a limited dilution of X-Gal positive cells from parous double transgenic mice into wild type recipients results in the formation of the ductal tree as a result of the re-association of blue cells with mammary stem cells (**J**; 4x; whole mount staining)(**K**; 630x; histological section of an X-Gal stained whole mount). **L**. Primary culture of G418-selected mammary epithelial cells from parous WAP-Cre/Rosa-βgeo females; 200x.

Figure 1



new mammary epithelial cell population

Figure 2

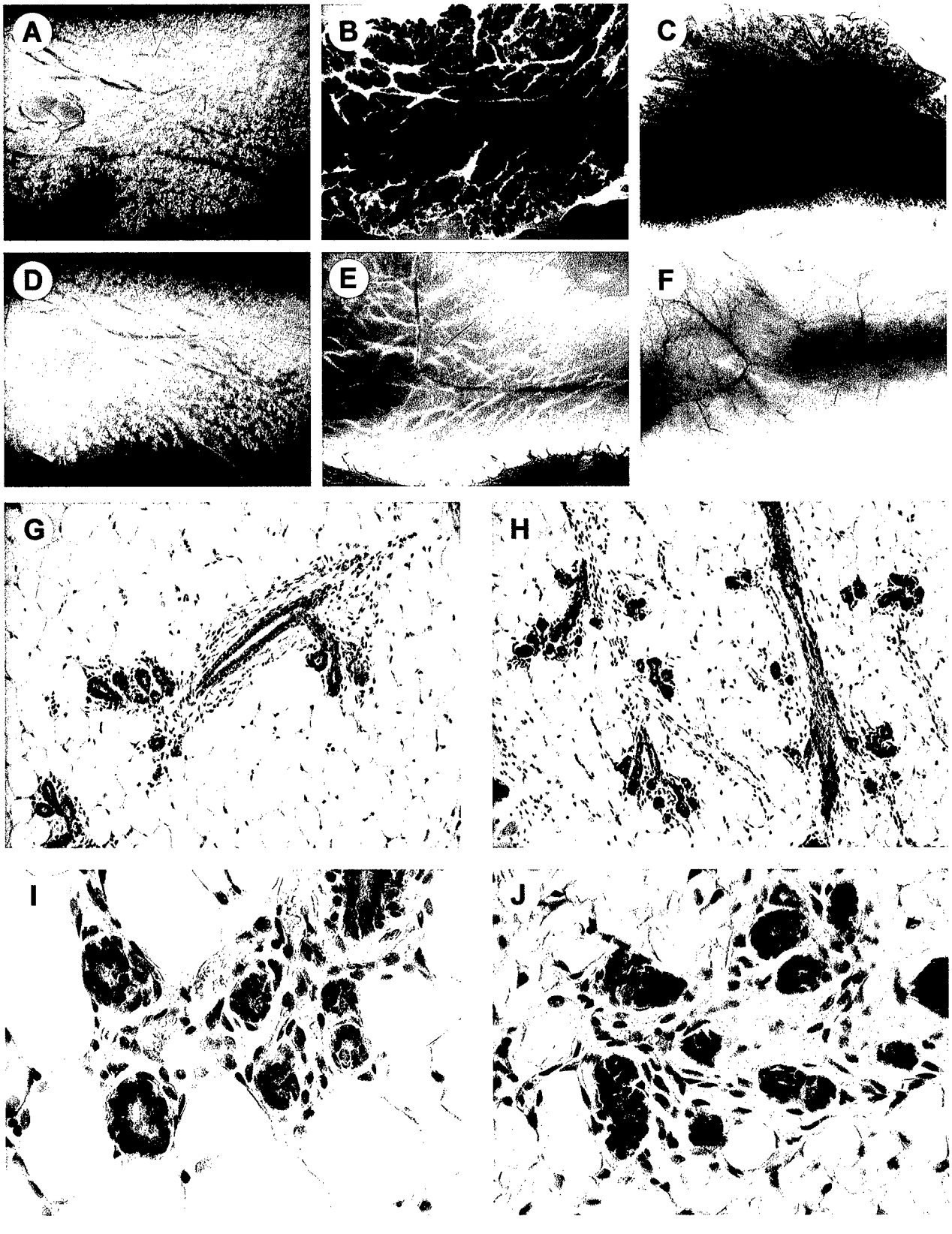


Figure 3

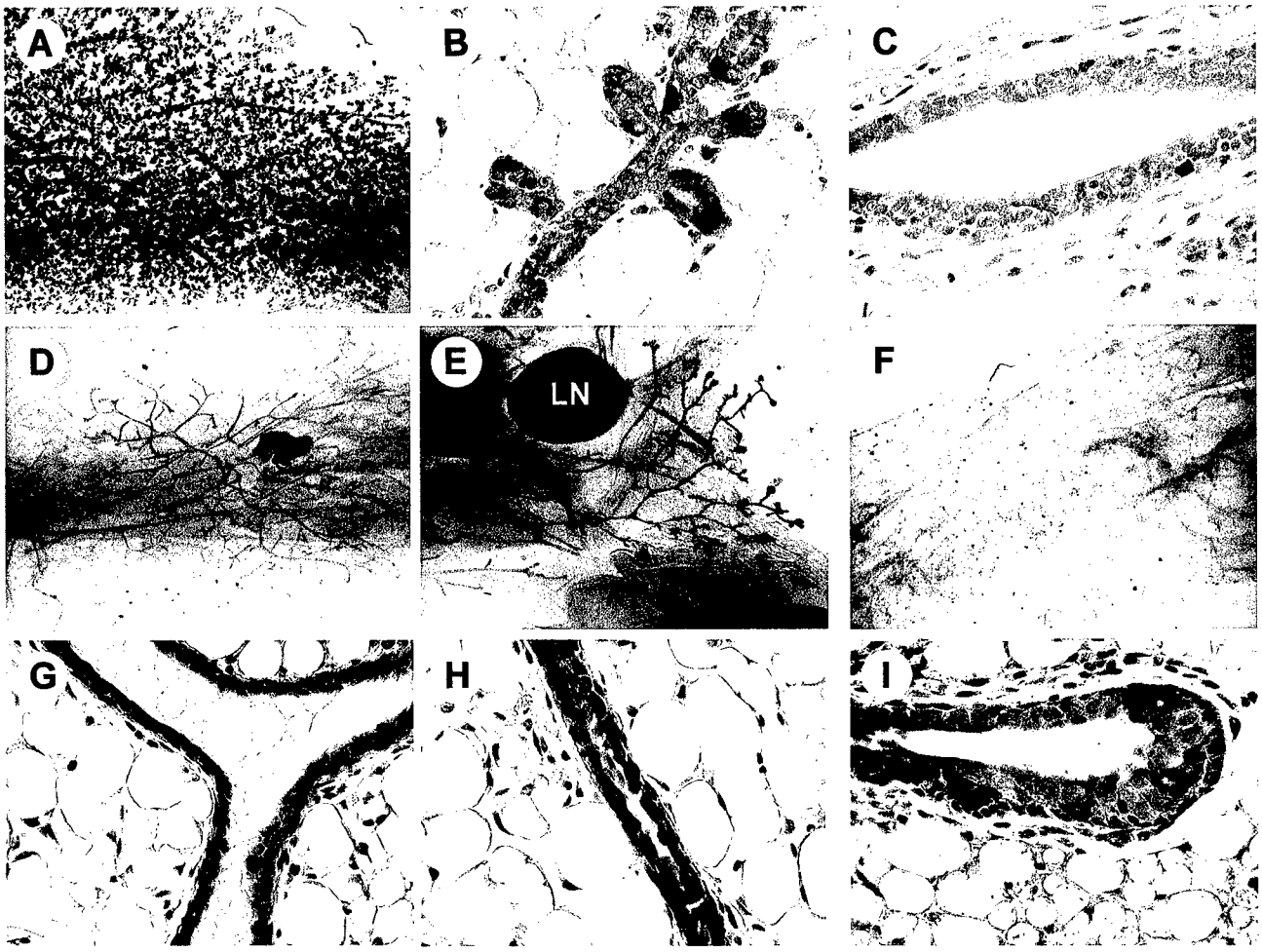


Figure 4

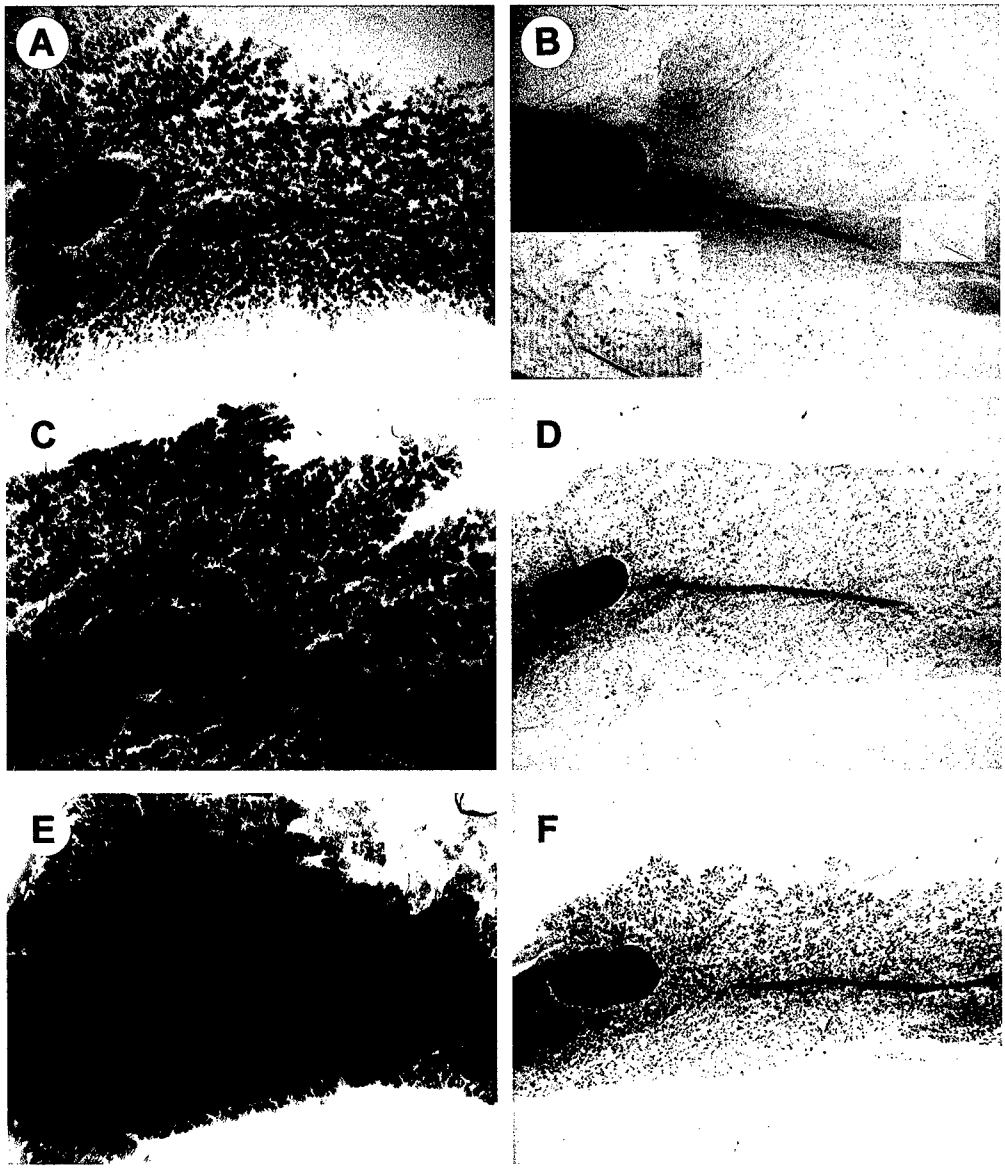
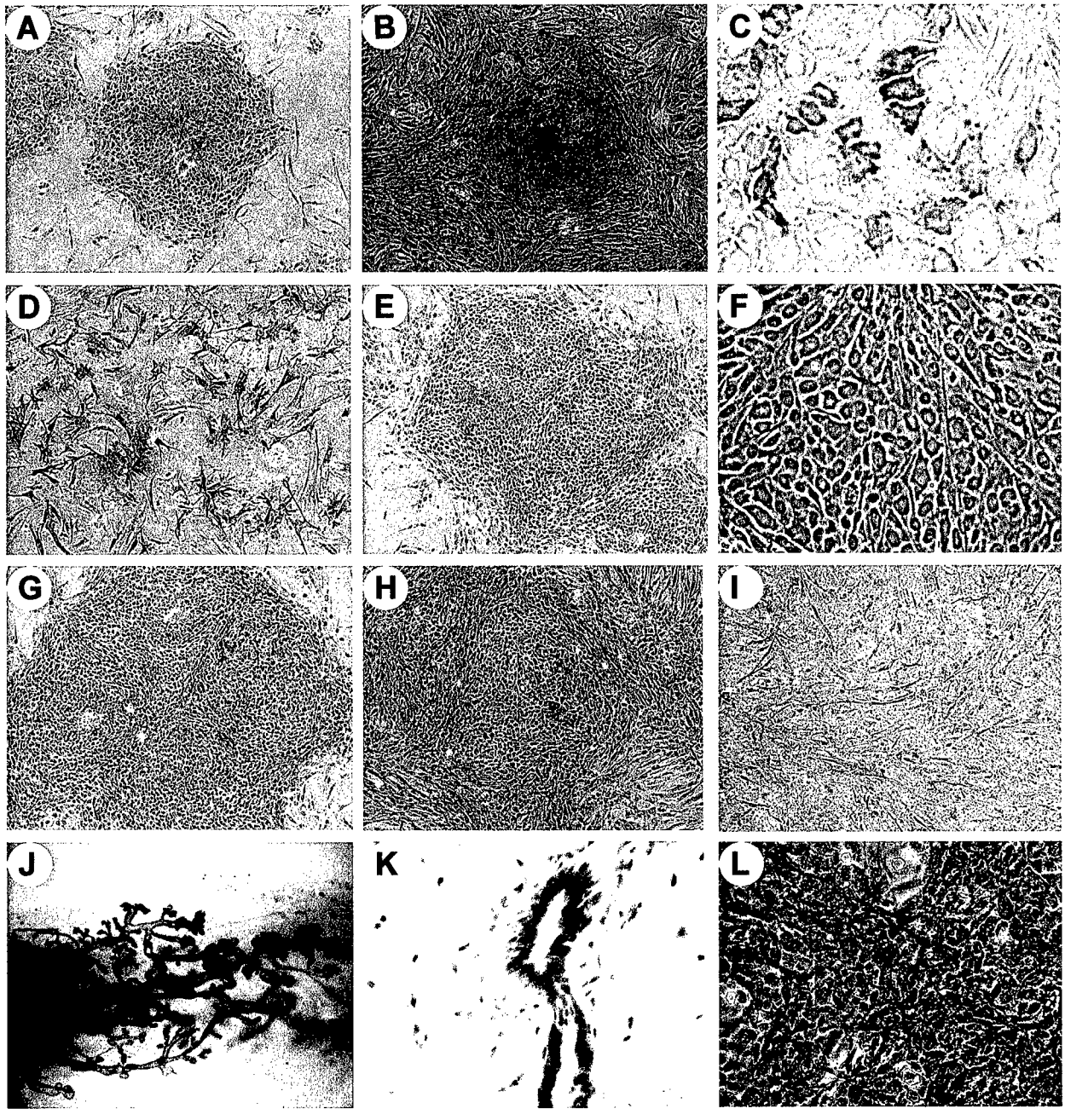


Figure 5



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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

Kay-Uwe Wagner[1,2,*], Kim McAllister[3,*], Toni Ward[3], Barbara Davis[3], Roger Wiseman[3] & Lothar Hennighausen[2]

[1]*Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Room 8009, Omaha, NE 68198-6805*

[2]*Laboratory of Genetics and Physiology, National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), 8 Center Drive, Building 8, Room 101, National Institutes of Health, Bethesda, MD 20892*

[3]*Laboratory of Women's Health, Building 101, C438, P.O. Box 12233, MD C4-06, 111 Alexander Drive, NIH, NIEHS, Research Triangle Park, NC 27709*

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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 developed 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

* Author for correspondence: *E-mail:* kuwagner@unmc.edu

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 develop 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

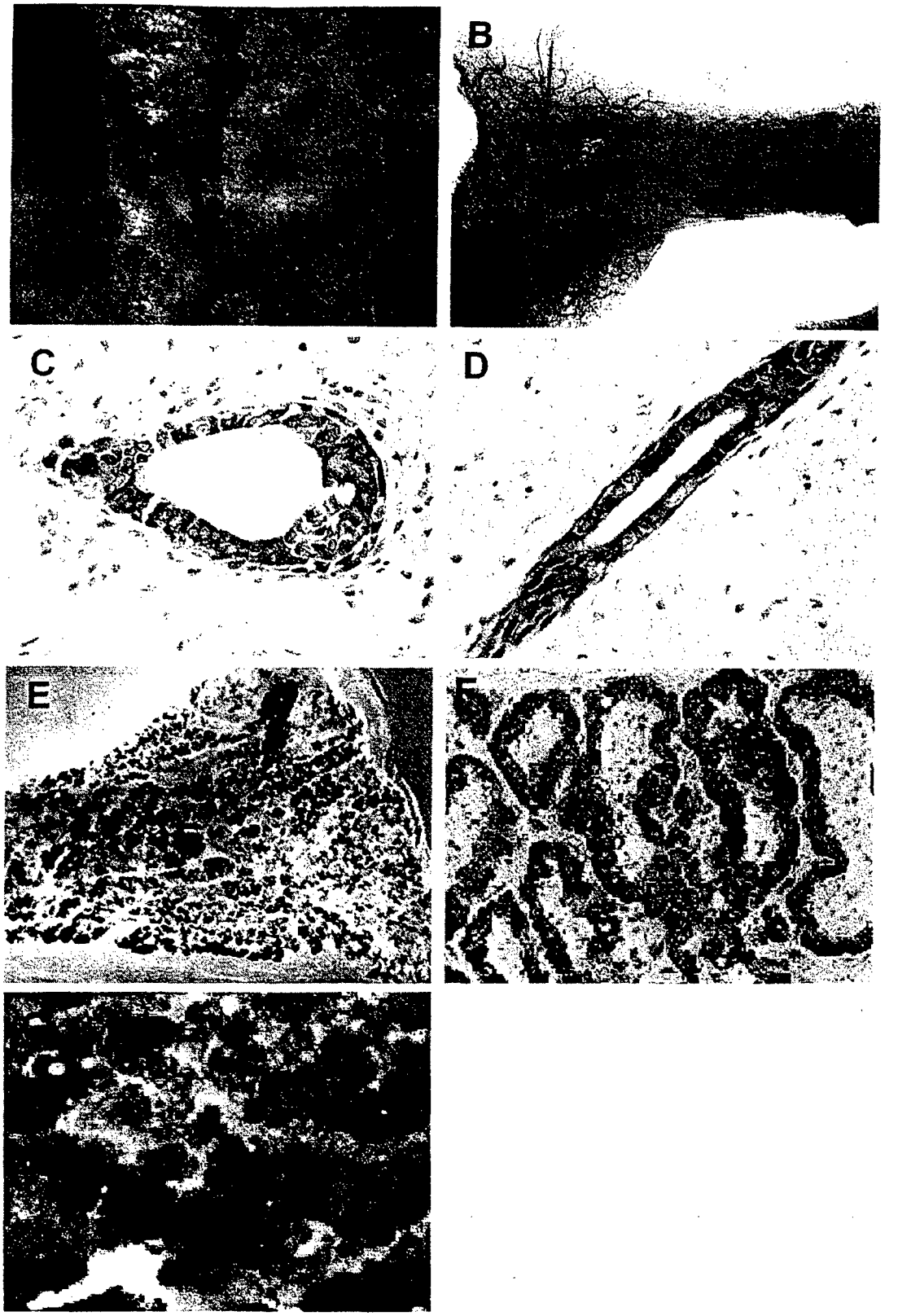

Figure 1.

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 (~~data not shown~~). 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 the MMTV-Cre D transgene. Active β-galactosidase

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.

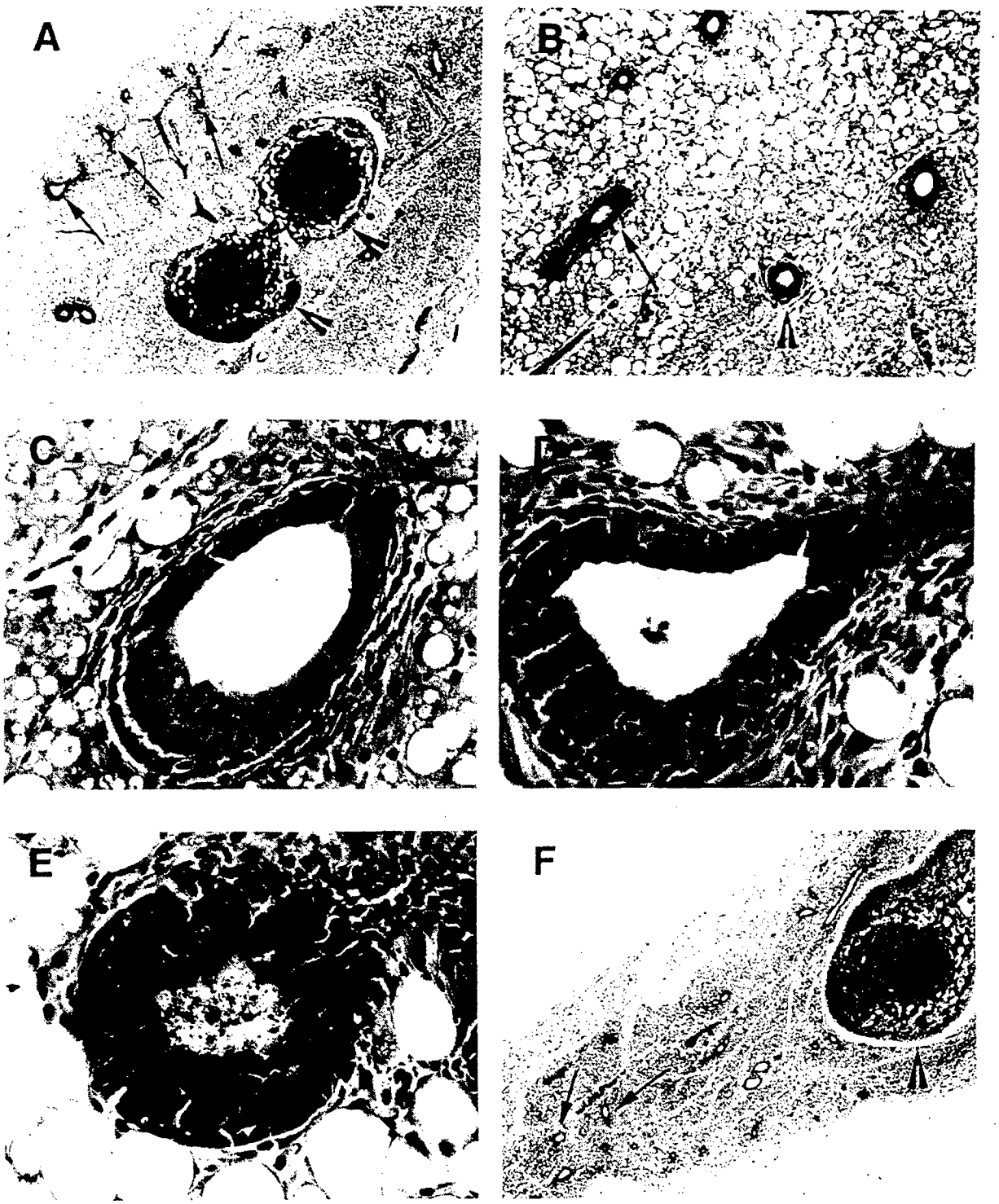

Figure 2. Variable staining of mammary epithelium from an MMTV-Cre line D/ROSA26 reporter mouse. A: 5-week-old mammary gland; low magnification of positively staining ducts. Arrows point to positively staining ducts. Arrowheads point to lymph nodes; B: higher magnification of mammary gland with partial 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.

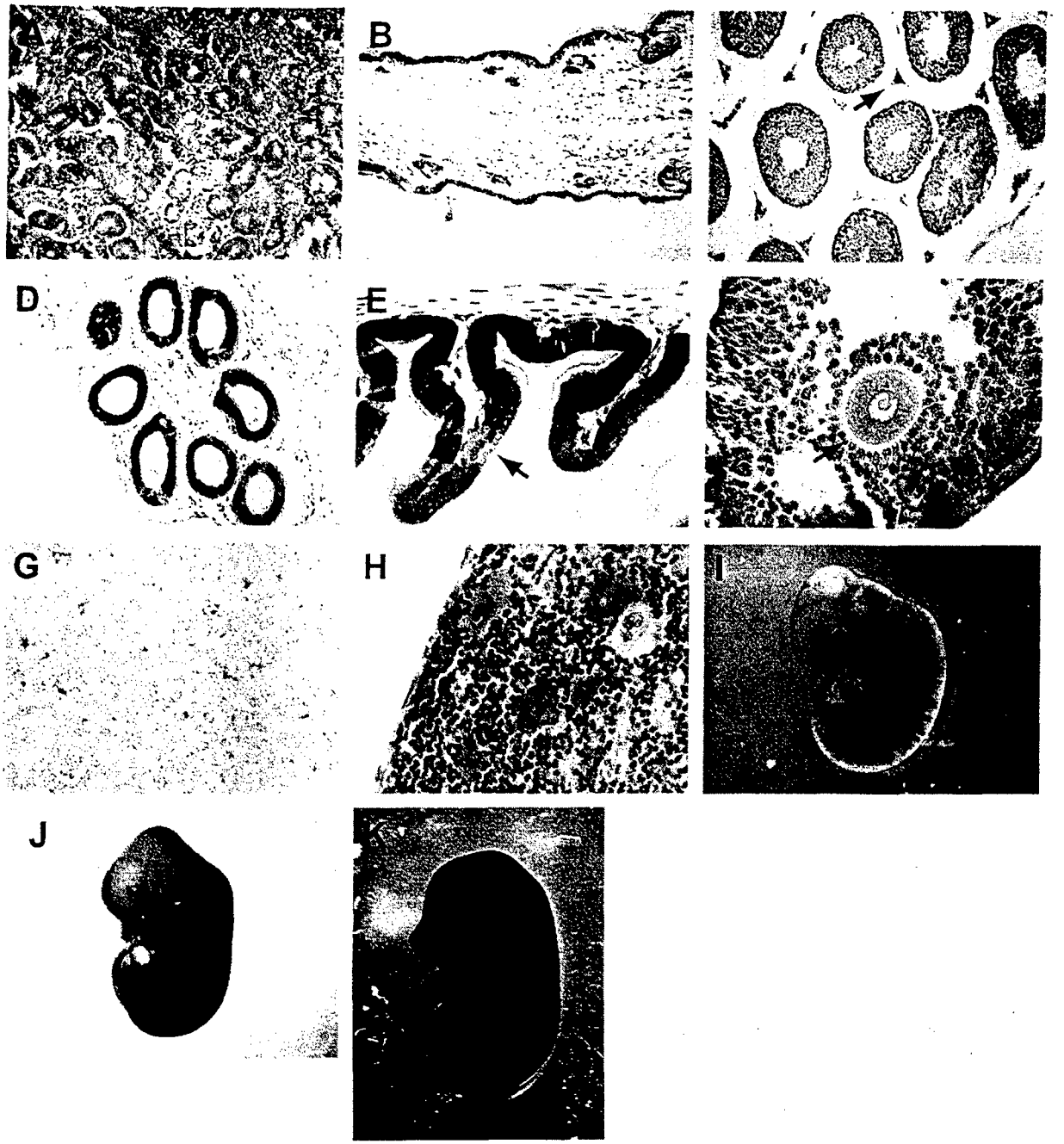

Figure 3. Organ Profile of LacZ expression in double transgenic mice that carry a Rosa26 reporter and the MMTV-Cre line A (A–G) or line D (H–K) transgene. A: submandibular gland; B: skin; C: cross section through adult testis (arrow points to Leydig cells); D: vas deferens; E: seminal vesicle (arrow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

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 stroma 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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