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An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal

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SUMMARY

Mammary gland biologists have long assumed that differentiated secretory epithelial cells undergo programmed cell death at the end of lactation and that the alveolar compartment is reconstituted from undifferentiated precursor cells in subsequent pregnancies. It is generally agreed that the remodeled gland in a parous animal resembles that of a mature virgin at the morphological level. However, several physiological differences have been noted in comparing the responses of mammary epithelia from nulliparous versus parous females to hormonal stimulation and carcinogenic agents. We present genetic evidence that an involuted mammary gland is fundamentally different from a virgin gland, despite its close morphological resemblance. This difference results from the formation of a new mammary epithelial cell population that originates from differentiating cells during pregnancy. In contrast to the majority of fully committed alveolar cells, this epithelial population does not undergo cell death during involution or remodeling after lactation. We show that these cells can function as alveolar progenitors in subsequent pregnancies and that they can play an important role in functional adaptation in genetically engineered mice, which exhibit a reversion of a lactation-deficient phenotype in multiparous animals. In transplantation studies, this parity-induced epithelial population shows the capacity for self-renewal and contributes significantly to the reconstitution of the resulting mammary outgrowth (i.e. ductal morphogenesis and lobulogenesis). We propose that this parity-induced population contributes importantly to the biological differences between the mammary glands of parous and nulliparous females.

Key words: Mammary gland, Cre recombinase, Epithelium, Parity, Stem cells, Involution, Differentiation, Mouse

INTRODUCTION

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; Hennighausen and Robinson, 2001; 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 extracellular matrix are degraded and the alveolar compartment is obliterated. It is generally agreed that differentiated alveolar 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; Hennighausen and Robinson, 2001). 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 5 (STAT5), as a component of the prolactin-signaling pathway (Liu et al., 1997; Miyoshi et al., 2001), cooperates with other factors such as the glucocorticoid receptor and C/EBPβ to achieve

maximum expression of genes for milk proteins (Stocklin et al., 1996; Wyszomierski and Rosen, 2001). Very low levels of expression of genes for milk proteins 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 genes encoding milk proteins 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 upregulation of caseins and whey proteins may reflect a progression towards terminal differentiation. Beside hormones and local growth factors, proper expression of the *Wap* gene 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 the *Wap* gene is frequently applied as an indicator for advanced differentiation of mammary epithelial cells. High levels of *Wap* gene 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 to 6 days after weaning the litter) (Burdon et al., 1991; McKnight et al., 1992).

On the basis of two paradigms [(1) high levels of WAP gene expression are restricted to differentiated mammary epithelial cells and (2) differentiated alveolar cells undergo apoptosis], it is unclear whether a WAP-promoter driven transgenic mouse model expressing Cre recombinase might be useful for studying 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 generally accepted paradigms on mammary development. Clearly, our observations are not an artifact caused by a deregulated activation of the promoter of our randomly integrated WAP-Cre construct, as Ludwig and co-workers (Ludwig et al., 2001) have recently reported similar observations in mutant mice that express Cre under the endogenous WAP promoter (WAP-Cre knock-in mutants). Based on these findings, we hypothesize that a specific 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 and the Rosa-lox-Stop-lox-*lacZ* (herein referred to as Rosa-*lacZ*) to monitor differentiation and cell survival in the developing and involuting mammary gland on the level of single cells. The main objective was to determine how many WAP-Cre expressing cells (i.e. hormone responsive and differentiated cells) are capable of resisting programmed cell death during involution. We have determined their location and growth properties in multiparous animals, in transplants and in culture. We demonstrate that these partially committed cells are not just alveolar progenitors, but they also share similarities with multipotent mammary stem cells. They can contribute to both ductal and alveolar epithelial cell types in transplants. Moreover, we show that the 'differentiation and survival' process provides a mechanism for cell selection that is important for bypassing genetic pathways in gene deletion models, in order to revert to a mutant phenotype in subsequent pregnancies.

MATERIALS AND METHODS

Transgenic mice

Male mice carrying the WAP-Cre transgene (Wagner et al., 1997) were crossed with female Rosa-*lacZ* reporter mice (Soriano, 1999). The WAP-Cre transgene gene was identified by PCR as described previously (Wagner et al., 1997). The presence of the Rosa-*lacZ* reporter locus was verified by PCR using the following forward and reverse primers that detect the β-galactosidase gene: 5′-GATCCGCGCTGGCTACCGGC-3′ and 5′-GGATACTGACGA-AACGCCTGCC-3′. Female offspring of these crosses, which carried both the WAP-Cre transgene and the Rosa-*lacZ* reporter, were analyzed for *lacZ* expression along with the offspring carrying only the reporter locus or the WAP-Cre transgene alone (negative control). The mice were analyzed as mature virgins (nulliparous mice; 6 to 12 weeks old), during various stages of the gestation period (day 8, 14, and 16 of pregnancy), during lactation days 1, 3 and 10, and 2 weeks to several months after weaning of the pups. Female WAP-Cre/Rosa-*lacZ* double transgenic mice were crossed with a male mouse carrying a homozygous deletion of the prolactin receptor (Ormandy et al., 1997) to generate WAP-Cre/Rosa-*lacZ*/PRL-$R^{+/-}$ female offspring. The PRL-R knockout mice were a kind gift from Dr P. Kelly to L. H.. Mammary biopsies were taken from the triple transgenic females a few hours after delivering the pups and several weeks later to ensure complete involution and remodeling. All animals used in the described studies were treated humanely and in accordance with Public Health Service policies and federal regulations.

Preparation and X-Gal staining of mammary gland whole mounts

Briefly, the entire inguinal mammary gland (i.e. gland 4) was spread on a glass slide and fixed for 1-2 hours in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP-40 in phosphate-buffered saline (PBS). Tissues were washed repeatedly in 1×PBS and processed for X-Gal staining as described previously (Wagner et al., 1997). Mammary glands were postfixed in 10% formalin, dehydrated in 100% ethanol and placed overnight in xylene before whole-mount analysis. For the analysis of tissue sections, mammary glands were dehydrated following the X-Gal procedure, embedded in paraffin, sectioned and counterstained with Nuclear Fast Red.

Tissue transplantation

DeOme and his colleagues originally devised the technique of tissue fragment transplantation into mammary fat pads cleared from endogenous mammary epithelium (DeOme et al., 1959). The surgical procedures for clearing the fat pad of 3-week-old female mice and the

method of implanting tissue fragments and cell suspensions have been described previously (Daniel et al., 1968; Faulkin and DeOme, 1960; Smith et al., 1980; Smith et al., 1991). Random fragments (~1.0 mm^3) of mammary epithelium were taken from nulliparous and nonpregnant parous WAP-Cre Rosa/*lacZ* females. Immediately after the retrieval of the epithelium from the transgenic donors, the fragments were implanted into the cleared fat pad of 3-week-old recipients (homozygous AthymicNCr-nu females). The recipients were kept as nulliparous virgins for 7 to 12 weeks to provide sufficient time for the transplanted epithelium to penetrate the wild-type fat pad and form a ductal tree. The recipients were neither bred nor treated with hormonal supplements to prevent a secondary activation of the WAP-Cre transgene. After more than 7 weeks, the mammary glands were prepared as whole mounts and stained with X-Gal as described above.

Primary cultures, selection, X-Gal staining

Primary mammary epithelial cultures from nulliparous and parous WAP-Cre/Rosa-*lacZ* double transgenic females were prepared in analogy to methods described previously (Li et al., 2000; Medina and Kittrell, 2000). WAP-Cre/Rosa-*lacZ* cultures were examined by X-Gal staining after 48, 72 and 96 hours. The cells were fixed for 10 to 15 minutes in chilled 0.08% glutaraldehyde in 1×PBS and washed at least three times in 1×PBS before the X-Gal staining procedure described above. The cells were stained for 24 to 48 hours at 30°C and washed twice in 1×PBS before examination.

RESULTS

A significant number of differentiated alveolar cells located at the extremity of ducts bypass apoptosis during involution

Genetically engineered mice carrying the WAP-Cre transgene (Wagner et al., 1997) and the floxed Rosa-*lacZ* reporter construct (Soriano, 1999) were used to monitor differentiation and cell survival in the developing and involuting mammary gland. Fig. 1 illustrates the experimental design and basic principle of the transcriptional activation of both transgenes before and during differentiation and involution. 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, owing to the Cre-mediated excision of the floxed transcriptional STOP sequence between Rosa regulatory elements and the *lacZ* coding sequence (Soriano, 1999). X-Gal staining on histological sections or whole mount tissue samples can be used to identify single cells that express β-galactosidase. As 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 not only to monitor the differentiation of mammary epithelial cells, but also to label permanently 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. Whether this new epithelial cell type originates solely from differentiating cells or in addition from fully committed cells

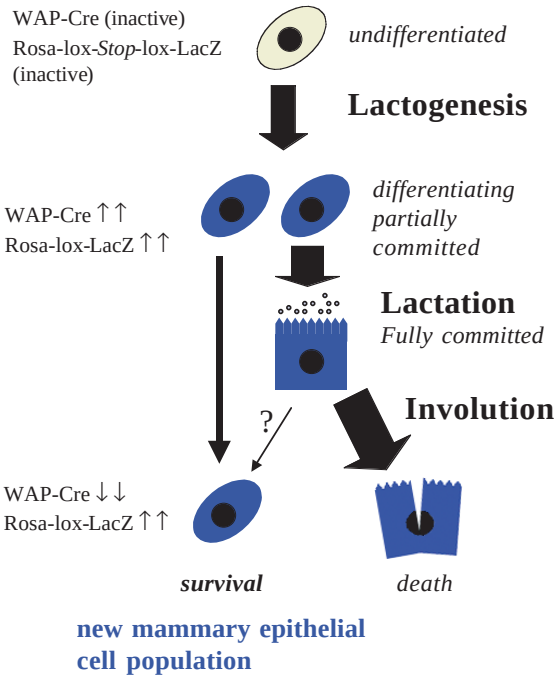

Fig. 1. Experimental design. The basic principle of the genetic labeling of differentiating cells in the developing mammary gland using the Cre-lox technique. In this experimental setting, the WAP-Cre transgene is used solely to monitor the differentiation process of alveolar precursor cells in response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

that de-differentiate during involution cannot be determined in this experimental setting.

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). 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 functionally differentiated cells around parturition (Fig. 2B). As there is no selective mechanism against Cre-expressing

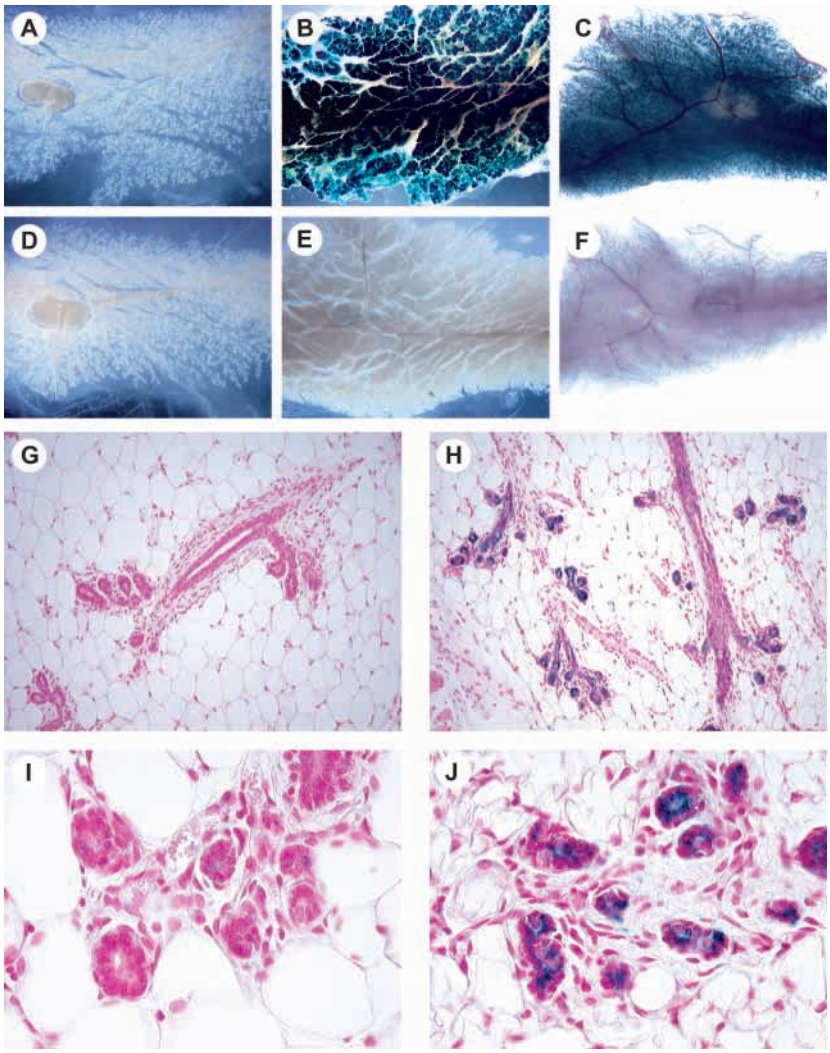

Fig. 2. X-Gal stained mammary whole mounts and their histological sections of WAP-Cre/Rosa-*lacZ* double transgenic females (A-C,G-J) and their single transgenic controls (D-F). (A,D) Whole mount, first pregnancy, day 14 of gestation. (B,E) Whole mount, several hours post partum after the first pregnancy. (C,F) Whole mount, 3 weeks after weaning of the litter following the first gestation cycle. (G,I) Tissue sections from nulliparous (virgin) double transgenic mice counterstained with nuclear Fast Red. (H,J) Tissue sections from parous, non-pregnant (i.e. involuted) double transgenic females counterstained with nuclear Fast Red.

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 with 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-F). Programmed cell death of 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 and 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. By contrast, we 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 were located primarily in epithelial structures that are similar to that of a terminal ductal lobular unit (TDLU) in humans (Fig. 2H,J). Morphologically, these blue cells in involuted dams (parous females) appeared to be similar to cells at terminal ducts in mature virgins (nulliparous females) (Fig. 2G,I). Despite their close resemblance at the histological level, these blue cells are different in their 'genetic program' as they had initiated a differentiation program (WAP expression) as a response to lactogenic 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 adapt quickly to different physiological conditions in mice with a defective hormone-signaling pathway.

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

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 proliferate highly at this stage, but *Wap* gene expression remains low. WAP-Cre/Rosa-*lacZ* double transgenic dams lactated for 21 days during the first reproductive cycle, and they had at least 2 weeks resting 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-C). 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-

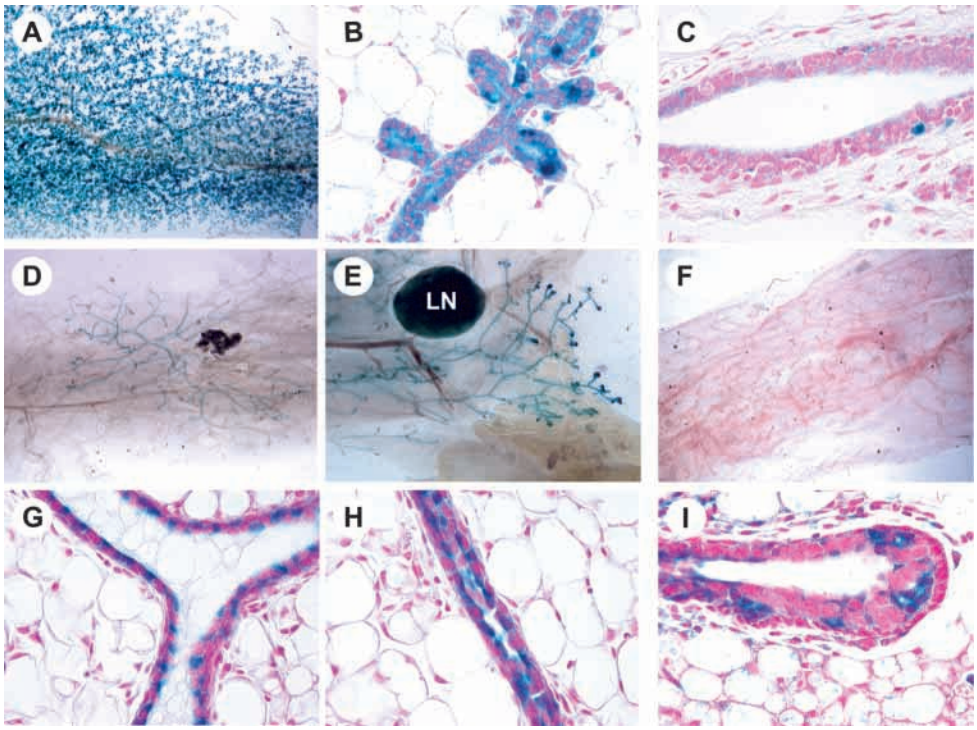

Fig. 3. X-Gal stained mammary whole mounts (A,D-F) and their histological sections (B,C,G-I) of WAP-Cre/Rosa-*lacZ* double transgenic females at day 8 of the second gestation period (A-C) and in transplants of parous (D,E,G,H,I) and nulliparous (F) WAP-Cre/Rosa-*lacZ* epithelia into nulliparous wild-type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

positive cells were hardly ever observed in primary or secondary ducts (Fig. 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 already been demonstrated (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 present theory is that ductal epithelium develops from ductal progenitors as well as from primary epithelial stem cells (Smith, 1996; Smith and Chepko, 2001). Before the transplantation of the involuted WAP-Cre/Rosa-*lacZ* donor epithelium, the wild-type endogenous mammary epithelia was 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, in order to avoid a secondary activation of WAP-Cre. We analyzed 22 X-Gal stained whole mounts from eleven nulliparous recipients (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 14 outgrowths, only two contained no blue cells, three were partially (50%) stained with X-Gal, and the vast majority (n=9; 75%) exhibited blue staining throughout the entire ductal tree (Fig. 3D,E). 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. In summary, our findings on the growth properties of blue cells from involuted WAP-Cre/Rosa-*lacZ* mice were not an artifact of the transplantation procedure (Fig. 3F).

Using our double transgenic mice to label differentiated and apoptosis-resistant cells permanently, 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).

The ‘functional memory’ of the mammary epithelium

Transplantation models are powerful tools with which to study mammary development in mutant mice that exhibit complex phenotypes or embryo lethality after day E12.5 (Robinson et al., 2000; Robinson et al., 2001). In the majority of experiments that use transplantation models, studying the intrinsic effects of a targeted mutation on mammogenesis is required. 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

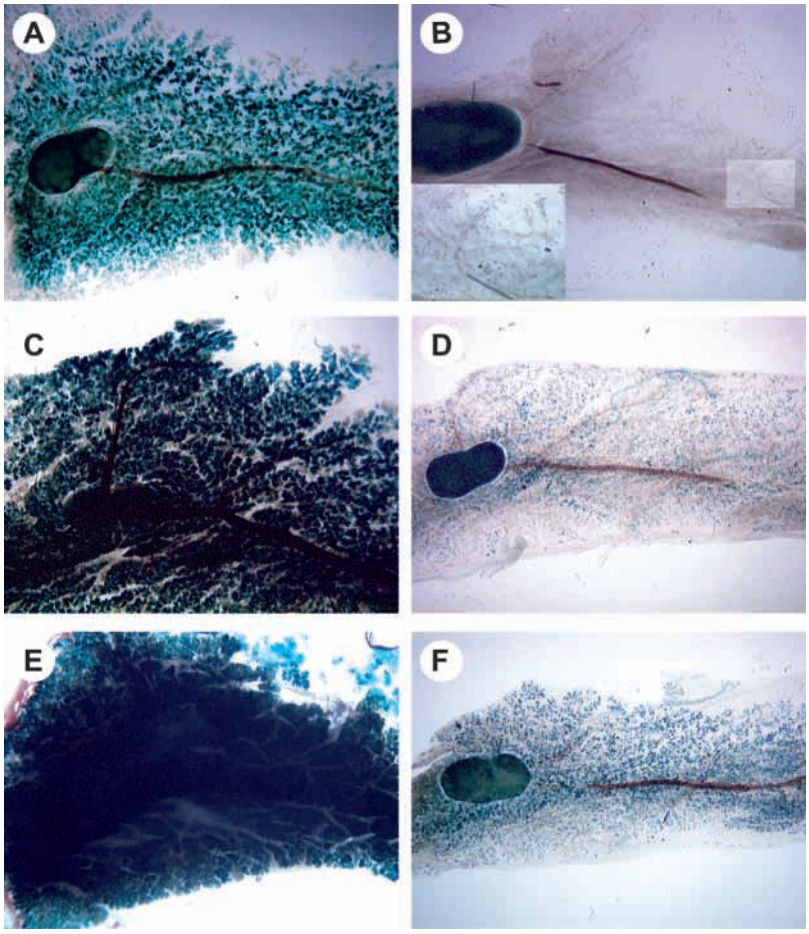

Fig. 4. Rescue of the lactation-deficient phenotype after three successive post-partum periods (A,C,E) and their matching involution phases (B,D,F) in heterozygous prolactin receptor knockout mice that carry in addition the transgenic reporter constructs (WAP-Cre/Rosa-*lacZ*). Note the simultaneous increase 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.

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 (Liu et al., 1998; Ormandy et al., 1997). The genetic pathways involved in the compensation might be different for each mouse model. For example, 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 (Prlr) can be compensated through upregulation of the wild-type allele (P. Kelly, personal communication) or through the downregulation of SOCS1 (Lindeman et al., 2001). However, what is the general mechanism for cell selection that results in a functional mammary gland? According to the current theory, 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, as 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 *Prlr* 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 post-partum periods (Fig. 4). The loss of one functional *Prlr* 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*/*Prlr*$^{+/-}$ 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 (Fig. 4E,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.

In vitro growth characteristics 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 culture 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 as 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 by 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 as described in the Materials and Methods. 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 as they did not express the WAP-Cre transgene (Fig. 3C). Alternatively, involution and removal of WAP-Cre-expressing cells may be complete in some areas of the gland, whereas in other region WAP-Cre expression might be mosaic. 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 then 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,G). 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 estrus (4.4%), and declines during metestrus II (0.9%). These findings are consistent with our observations on whole-mount stained specimens, and they suggest that these cells undergo apoptosis during the estrus cycle and that they are not a permanent population in the virgin mammary gland. Partial differentiation and *Wap* gene 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 with 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.

Limiting dilution of primary cells and transplantation into the cleared fat pad is one method for determining the presence of mammary stem cells and for achieving outgrowths that are derived from single or very few stem cells and their antecedents (Kordon and Smith, 1998; Smith, 1996). We have injected single

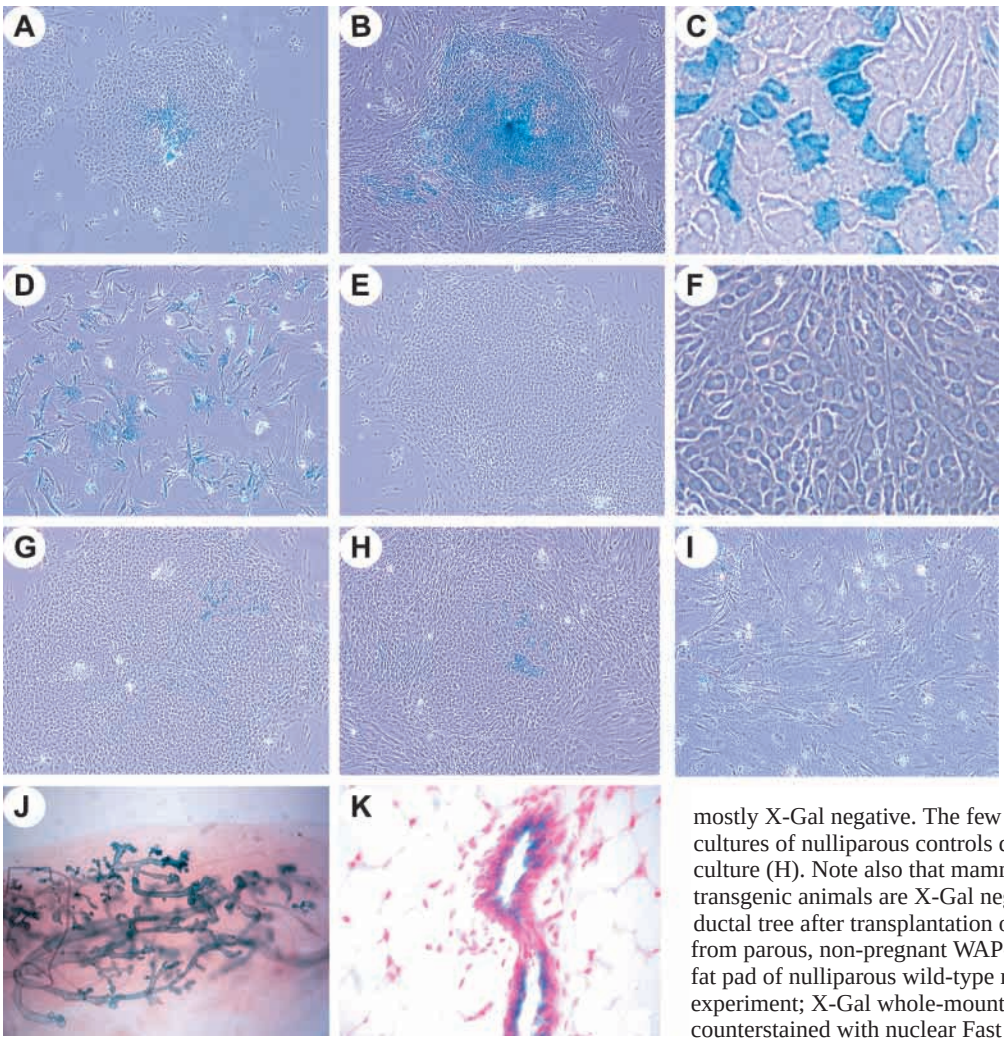

Fig. 5. X-Gal staining of primary cultures of mammary epithelial cells (A-H,L), mammary fibroblasts (I), and a whole mount (J) and its corresponding histological section (K) of an outgrowth from transplanted dissociated cells of parous (involuted) WAP-Cre/Rosa-*lacZ* mice into the cleared fat pad of 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) and 72 hours (B). Grown as a monolayer, these blue parity-induced cells do not differ in their morphology from other epithelial subtypes (C), and they can actively migrate away from epithelial colonies (D). Epithelial cells derived from nulliparous double transgenic mice and cultured for 48 (E,F) and 72 hours (G) 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). Note also that mammary fibroblasts of parous double transgenic animals are X-Gal negative (I). (J) Reconstitution of the ductal tree after transplantation of a small number of epithelial cells from parous, non-pregnant WAP-Cre/Rosa-*lacZ* mice into the cleared fat pad of nulliparous wild-type recipients (limiting dilution experiment; X-Gal whole-mount staining). (K) Histological section of J counterstained with nuclear Fast Red.

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 has previously been 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 ten cleared fat pads of recipient mice. This number was used to ensure that at least 2000 of the cells inoculated were X-Gal positive as 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 8 to 12 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,K); 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.

DISCUSSION

In this report, we establish the emergence of a stable mammary epithelial population following, pregnancy, lactation and involution that is not present in the nulliparous gland. Physiological differences between the mammary epithelium found in nulliparous and in primiparous or parous female mice have been noted previously (Smith, 1987; Smith and Vonderhaar, 1981; Vonderhaar et al., 1978; Vonderhaar and Smith, 1982). In explant cultures, the mammary glands of nulliparous mice must synthesize DNA before they become fully responsive to lactogenic hormonal stimuli. By contrast, explants from the mammary glands of parous females do not require new DNA synthesis before a lactational response. Subsequent studies ruled out completion of the cell cycle and mitosis as an explanation for this difference between the mammary epithelium of parous and virgin mice (Smith and Vonderhaar, 1981). In examining the potential role of the basement membrane collagen synthesis, a further difference was noted between virgin and parous mice in explant cultures. When proline was left out of the medium, virgin explants failed to respond, even to the extent noted in DNA-synthesis inhibited explants, failing either to differentiate cytologically or to produce any milk proteins. Again, by contrast, no reduction in the responsiveness of epithelium from parous, non-pregnant mice occurred in the absence of proline (Smith, 1987). Therefore, in terms of hormone responsiveness in explant cultures, the mammary epithelium from parous females is distinctly different from that in nulliparous mice. Furthermore, in several species, including mice (Medina and Smith, 1999), parous and nulliparous females differ in their propensity to develop mammary cancer. This difference is thought to reflect either systemic changes resulting from pregnancy or more probably the alteration of the mammary tissue itself. The newly identified mammary epithelial population that encompasses a significant region of the entire mammary epithelia in parous animals might be a facilitator of the important physiological changes mentioned above. Therefore, it might be beneficial to discriminate these epithelial subpopulations and evaluate the physiological parameters independently.

Contiguous regions of the mammary epithelium in the human breast are clonally derived as determined by X chromosome inactivation patterns (Tsai et al., 1996), indicating the presence of a pluripotent precursor. This observation has been confirmed and extended to include normal tissues surrounding mammary atypia and tumors in situ (Deng et al., 1996; Lakhani et al., 1999; Rosenberg et al., 1997). In murine mammary glands, considerable evidence supports the existence of locally dispersed multipotent epithelial stem cells (reviewed by Smith and Chepko, 2001). The multipotent epithelial population comprises three subtypes: mammary stem cells (which produce all epithelial cell types, including ducts and alveoli), ductal-limited precursors (which produce only branching ducts) and lobule-limited precursors (which produce only secretory lobules). All of these multipotent epithelial cells are found in clonally derived mammary outgrowths, indicating they arise from a mammary stem cell (Kordon and Smith, 1998). The multipotent epithelial cells in human and murine glands are associated with cellular populations, which express luminal epithelial markers rather than the phenotypic markers associated with myoepithelial cells (Smalley et al., 1999; Stingl et al., 1998; Stingl et al., 2001). These findings are consistent with our observations presented in this report. Some *Wap*-expressing mammary epithelial cells survive post-lactational involution and persist in a luminal niche. Subsequently, upon the succeeding pregnancy, they proliferate to help form new secretory acini. However, in transplants this parity-induced epithelial population shows the property of self-renewal, and these cells maintain themselves at regular intervals among the luminal epithelium of the extending ductal branches. They also orientate themselves within the body of the growing terminal end bud. Thus, these cells express certain features of multipotent stem cells, i.e. the property of self-renewal and the ability to divide symmetrically. The dissociation of involuted mammary glands from WAP-Cre/Rosa-*lacZ* double transgenic animals and the transplantation of the dispersed epithelial cells into the cleared fat pad of recipients demonstrate that this parity-induced epithelial population can re-associate with other cells and produce complete branching mammary ducts. However none of the structures produced in these studies were entirely composed of blue cells, therefore these cells may not be capable of producing all of the epithelial cell types in mammary ducts by themselves. Hence, these parity-induced mammary epithelial cells seem to lack the most important feature of mammary stem cells: the innate capacity to produce diverse progeny such as ductal myoepithelial cells.

The appearance of epithelial cells committed to secretory differentiation and capable of proliferation among the parous mammary epithelium provides a buffer population, which may protect the depletion of primary mammary stem cells from the population as a result of mitotic activity. Serial transplantation shows evidence that mammary epithelium from an aged, multiparous female is equivalent to that from young pubertal females with respect to longevity and growth potential (Young et al., 1971). The ‘rescue’ of normal secretory development and lactation in the prolactin receptor heterozygous knockout mice is an example of the buffering capacity provided by the survival

and proliferative capacity of the parity-induced epithelial population.

A question of interest is the significance of the appearance of partially committed, proliferation-competent cells in the parous mammary gland and the increased resistance to mammary tumorigenesis compared to the nulliparous gland. Women, regardless of ethnicity, who have undergone a full-term pregnancy before 20 years of age have one-half the risk of developing breast cancer compared with nulliparous women (MacMahon et al., 1970). Parous rats and mice also have a greatly reduced susceptibility to chemically induced mammary tumorigenesis when compared with their nulliparous siblings (Medina and Smith, 1999; Russo and Russo, 1996; Welsch, 1985). The mechanism(s) for this protective effect have not been defined. The most widely accepted explanation, offered by Russo and Russo (Russo and Russo, 1996), is that the protection is afforded by the pregnancy-induced differentiation of the target structures for carcinogenesis: terminal end buds and duct termini. More recently, it has been suggested that the hormonal milieu of pregnancy affects the developmental state of a subset of mammary epithelial cells and their progeny, which result in persistent differences in their response to carcinogenic challenge. These changes are reflected in the muted proliferative response to carcinogen exposure by the affected cells and the appearance of a sustained expression of nuclear p53 in the hormone-treated epithelium. The proliferation block and the induction of p53 occur both in rats and in mice and support the generality of this hypothesis (Sivaraman et al., 1998; Sivaraman et al., 2001). The ability to identify and subsequently isolate cells from the parity-induced subpopulation of epithelial cells from multiparous mice offers an opportunity to evaluate various aspects of the differences between parous and nulliparous mouse mammary glands.

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