Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands

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Summary

It has been postulated that the stem cells of somatic tissues protect themselves from mutation and cancer risk by selective segregation of their template DNA strands. Self-renewing mammary epithelial stem cells that were originated during allometric growth of the mammary ducts in pubertal females were labeled using [^{3}H]-thymidine (^{3}HTdR). After a prolonged chase during which much of the branching duct morphogenesis was completed, ^{3}HTdR-label retaining epithelial cells (LREC) were detected among the epithelium of the maturing glands. Labeling newly synthesized DNA in these glands with a different marker, 5-bromodeoxyuridine (5BrdU), resulted in the appearance of doubly labeled nuclei in a large percentage of the LREC. By contrast, label-retaining cells within the stroma did not incorporate 5BrdU during the pulse, indicating that they were not traversing the cell cycle. Upon chase, the second label (5BrdU) was distributed from the double-labeled LREC to unlabeled mammary cells while ^{3}HTdR was retained. These results demonstrate that mammary LREC selectively retain their ^{3}HTdR-labeled template DNA strands and pass newly synthesized 5BrdU-labeled DNA to their progeny during asymmetric divisions. Similar results were obtained in mammary transplants containing self-renewing, *lacZ*-positive epithelial cells suggesting that cells capable of expansive self-renewal may repopulate new mammary stem cell niches during the allometric growth of new mammary ducts.

Key words: Mammary, Stem cell, Asymmetric division, Autoradiography

Introduction

It has been suggested that somatic stem cells in epithelia are capable of retaining [^{3}H]-thymidine (^{3}HTdR) administered at their inception over long periods of time (Bickenbach, 1981; Cotsarelis et al., 1990; Morris et al., 1985). In 1975, Cairns (Cairns, 1975), and subsequently Potten (Potten et al., 1978), suggested that one of the reasons for this property is that somatic stem cells selectively segregate their template DNA strands to themselves and pass the newly synthesized chromatids to their daughters during asymmetric divisions. In a recent paper, Potten and his co-workers (Potten et al., 2002) have convincingly demonstrated that stem cells in the crypt of the small intestine do indeed retain their template DNA (^{3}HTdR) and pass the newly synthesized strands marked with 5-bromo-deoxyuridine (5BrdU) to their progeny. This property is claimed to effectively protect long-lived stem cells from mutagenesis related to errors occurring during DNA replication and subsequently explains, in part, why stem cells in the small intestine rarely give rise to intestinal cancers (Potten et al., 2002).

Long label retaining cells (LREC) have been reported among the epithelium of the murine mammary gland using both ^{3}HTdR and 5BrdU (Welm et al., 2002; Zeps et al., 1998; Zeps et al., 1996). It has been reported that as many as 50% of mammary epithelial cells are labeled with ^{3}HTdR after three consecutive injections and much of this label is lost after 2 weeks, consistent with the loss of label by semi-conservative exponential cell divisions. Some cells retained label following this 2-week period and had autoradiographical grain counts similar to cells immediately following ^{3}HTdR injection. A greater number of these cells were obtained when ^{3}HTdR injection was made just at estrus or met-estrus during the estrus cycle (Zeps et al., 1998; Zeps et al., 1996). These authors chased the label for just two weeks and used adult females 9-16 weeks of age. In preliminary studies they determined that no heavily labeled cells were present after 5 weeks. In a very different approach, Welm et al. (Welm et al., 2002) labeled mice with 5BrdU delivered from an implanted Alzet pump for 14 days beginning at 3 weeks of age. Subsequently the pump was removed and the number and location of labeled mammary cells was analyzed at weekly periods for 9 more weeks. These investigators found that the number of labeled epithelial cells decreased quite rapidly reaching <5% by 9 weeks. These label-retaining cells remaining at 9 weeks were variously determined to be expressing progesterone receptor (PR), ~1.5% and keratin K14 or K18, myoepithelial and luminal epithelial cell markers respectively. In addition, these authors found that the LREC epithelial population at 9 weeks was more prevalent in side population (SP) cells after fluorescence-activated-cell sorting (FACS), suggesting that they may represent mammary epithelial stem cells.

To develop LREC among the mammary epithelium,

mammary implants in the cleared mammary fat pads of syngeneic or immune-compromised recipients and the intact host mammary glands were labeled with ^{3}HTdR for a 5-day period in the fifth week of life. Implants, host mammary glands and small intestine were removed on the third day following the last ^{3}HTdR administration to determine the efficiency of labeling. At the 10th week of life, samples were again taken to assess for the presence of ^{3}HTdR-positive LREC. In the 11th week, 5BrdU was administered to determine if mammary LRECs could be labeled simultaneously with ^{3}H and 5BrdU. Subsequently, the mice were treated with various hormonal combinations in an attempt to chase 5BrdU from doubly labeled LREC. Our results indicate that a very large percentage of LREC were doubly labeled with ^{3}HTdR and 5BrdU and that this number dropped precipitously following a 6 day chase providing evidence that a large proportion of mammary LREC are actively traversing the cell cycle and are capable of retaining their template strands during asymmetric cell divisions.

Materials and methods

Experimental plan

The experiment was begun when the mice were exactly 3 weeks of age and on a Friday. Three-week-old FVB/N and/or Nu/Nu NCR female mice received a mammary epithelial implant in their surgically cleared (Kordon and Smith, 1998) contralateral #4 and #9 fat pads (Fig. 1). This procedure was repeated in a second experiment with transgenic mammary implants from parous WAP-Cre/Rosa26-flox-Stop-flox-*lacZ* females (Wagner et al., 2002). Sixteen to twenty mice were used for each experiment. Wound clips were removed after 10 days. On the same day, the hosts received injections of 1.0 μg of estradiol, intraperitoneal, daily at 4:00 PM followed by an intraperitoneal injection of ^{3}H-thymidine of 25 μCi at 6:00 PM. This was continued for 5 consecutive days. Two animals were removed for tissue analysis on the Monday following the final ^{3}HTdR injection to determine the number of mammary cells that were labeled. The #3 and #8 host mammary glands were collected and the implanted #4 and #9 mammary fat pads. The small intestine from each animal was excised and bundled to provide a positively labeled control for autoradiography and for 5BrdU incorporation and as an indicator of successful incorporation of the nuclear labels. Subsequently, estradiol (1.0 μg) was given every other day for 3 weeks to promote mammary growth. Upon cessation of estradiol treatment (the 8th week of life), the animals were held for 2 weeks; at the end of the 10th week of life, tissues were removed from two animals to determine the number and location of long-label-retaining mammary cells. The remaining mice were placed in three groups of at least four and treated as follows: group I (1.0 μg estradiol, i.p. at 4:00 PM followed by an i.p. injection of 5BrdU, 1.0 mg in 0.1 ml saline); group II (1.0 μg estradiol and 1.0 mg progesterone i.p. at 4:00 PM and the same dose of 5BrdU; and group III (5BrdU, estradiol, progesterone as per Groups I and II, plus 0.5 μg prolactin per gram body weight twice a day). 5BrdU was given Monday and Tuesday of the 11th week of life. One animal from each group was removed for tissue analysis Wednesday morning. All hormone treatments were maintained for 5 consecutive days. The remaining animals were analyzed 3 days (Monday) following the final hormone treatment.

The protocols and procedures used to perform the experiments upon the animals were reviewed and approved by the Animal Care an Use Committee at the Frederick Cancer Research Center of the National Cancer Institute. Housing and care during the experimental period conformed to the guidelines provided by the National Institutes of Health.

Autoradiography and immunochemistry

All immunohistochemistry was performed after autoradiographical exposure. The sections were deparaffinized and rehydrated and the endogenous peroxidase was inactivated with 1% hydrogen peroxide in methanol for 30 minutes. Antibodies used were anti-5BrdU, 1:500 (DAKO-0744, clone BU20); anti-smooth muscle actin 1:150 (Sigma A2547, clone 1A4); anti-progesterone receptor 1:75 (DAKO A009B, lot 126) and anti-estrogen receptor 1:50 [Santa Cruz Biotech.-Era(MC-20) sc-542, lot 171]. Antigen retrieval was accomplished according to the direction of the manufacturer. Negative tissue controls were included in all immunocytochemical analyses. Sections were counterstained with Hematoxylin or Nuclear Fast Red after immunostaining.

For autoradiography, 5-6 μm sections were cut placed upon slides, dewaxed, rehydrated through ethanol and subsequently dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water. After drying, the slides were stored in lightproof slide boxes at constant humidity and temperature for 20 and 30 days. After exposure, the slides were developed in Kodak D-19, washed in distilled water and fixed in Kodak rapid fixer diluted 1:1 with distilled water. After staining and mounting, the slides were observed and evaluated for autoradiographical grains and for immunostaining under oil with a 63× or 100× objective. Images were recorded with a Kodak digital microscopy documentation system 290.

Determination of autoradiographical grain counts in LREC was made by counting the grains over at least 100 label-retaining epithelial cells in sections from each of the four mammary glands taken from each experimental mouse (2) in each experiment (2). These numbers were compared with the average number of grains found over labeled cells (within the ducts) in the four glands taken from each of two mice (in each experiment), 3 days after the last ^{3}TdR injection was delivered. At least 500 labeled cells were counted in each of these sections (8). These determinations were made upon slides that had been equivalently treated for 5BrdU antigen retrieval, detection of 5BrdU by immunocytochemistry and autoradiography so that any loss of grains caused by these manipulations would be taken into account. In each experiment, the frequency of LREC was determined on the same slides comparing mammary tissues (8) from animals sacrificed following the ^{3}TdR chase with those stained for 5BrdU after introduction of that label (12 glands) and its subsequent chase (36 glands). The frequency of LREC remained essentially unchanged (2.1±0.1%) among all of these tissues. At least 3000 nuclei were examined in each slide. Examination of autoradiographical slides from these tissues that were stained for PR, ER and SMA disclosed similar numbers of autoradiographical grains over LREC nuclei.

X-Gal and immunostaining of mammary gland whole mounts

To identify *lacZ*-positive progeny in WAP-Cre/Rosa26-flox-Stop-flox-*lacZ* mammary outgrowths, whole mounts of the entire implanted gland were fixed and stained as described earlier (Wagner et al., 2002). Briefly, the gland was spread on a glass slide, fixed in paraformaldehyde (4.0%) for 1-2 hours, permeabilized in 0.01% NP-40 in phosphate buffered saline (PBS) overnight at 4°C and subsequently processed for X-Gal as described (Wagner et al., 1997). Stained glands were repeatedly rinsed in PBS, then post-fixed in Carnoy's fixative, cleared in 100% ethanol and the placed in xylene before whole-mount analysis. For histological examination X-Gal-stained whole mounts were embedded in paraffin wax, sectioned at 6 μm and counterstained with nuclear Fast Red.

Results

Experimental plan

All regions of the mouse mammary epithelial tree are fully competent to reproduce an entire gland upon transplantation

into an epithelium-cleared mammary fat pad (Daniel et al., 1968; Kordon and Smith, 1998). Consequently, all regions of mouse mammary glands contain mammary epithelial stem cells (Smith and Chepko, 2001). In addition, all parts of the regenerated gland are likewise capable of recapitulating a new mammary tree upon transplantation to a second round of cleared mammary fat pads. Therefore, mammary stem cells must be self-renewed through expansive symmetric cell divisions during this regenerative process. To access this property of mammary stem cells, ^{3}HTdR was injected into 5-week-old females bearing autochthonous implants of mammary tissue (Fig. 1). At this age, both the intact and implanted mammary tissues would be in the midst of allometric growth when ^{3}HTdR was administered. Complete growth of the mammary ducts in intact glands is attained between 10-12 weeks of age. During active growth, most, if not all, of the dividing epithelial cells are present within or at the outermost boundary of the terminal end buds of the growing ducts. Thus, during the 5-day pulse (a daily injection of ^{3}H-TdR, 24 hours apart) both renewing stem cells and dividing epithelial (transit) cells destined to differentiate along the various epithelial cellular lineages in the gland will be labeled. Thereafter, cells that continue to divide will partition their labeled DNA among daughters in a semi-conservative manner and become progressively free of label. Only cells that immediately go out of cycle, possess very long cell cycles or divide asymmetrically retaining their template strands will maintain significant levels of the label. Zeps et al. (Zeps et al., 1996) have reported that mammary epithelial cell labeling efficiency was greatest during estrus and metestrus in cycling mature virgin female mice. Therefore, we injected the mice with 1.0 μg of estradiol every other weekday during the chase period to mimic estrus and promote epithelial cell proliferation and duct morphogenesis. Estradiol treatment was discontinued after the eighth week of life (3 weeks after ^{3}HTdR injection). On the 5th Friday after ^{3}TdR injection, when the mice were 10 weeks old, tissues from two mice (in each experiment) were harvested to determine the frequency and location of LRECs (Fig. 1). On Monday, in the 6th week post ^{3}HTdR pulse, the remaining mice (12) were placed into three groups and were given 1.0 mg 5BrdU for 2 days; on the 3rd day, tissue was harvested from one mouse in each group to ascertain the level of 5BrdU incorporation. Group I received 1.0 μg estradiol, group II received 1.0 μg estradiol plus 1.0 mg progesterone and group III received estradiol, progesterone and 0.5 μg prolactin. These treatments were given Monday to Friday, and were intended to promote epithelial cell proliferation and duct side branch development. In addition, the degree of mammary epithelial cell proliferation in the fully developed gland varies significantly through the estrus cycle (Zeps et al., 1999). The hormone treatments provide a constant stimulus to epithelial proliferation and therefore reduce variation in the proliferation index among the experimental animals because of the estrus cycle. On the following Monday, the nine animals (three in each group) remaining were sacrificed and the mammary implants, host glands and small intestines were collected and prepared for autoradiography and immunohistochemistry.

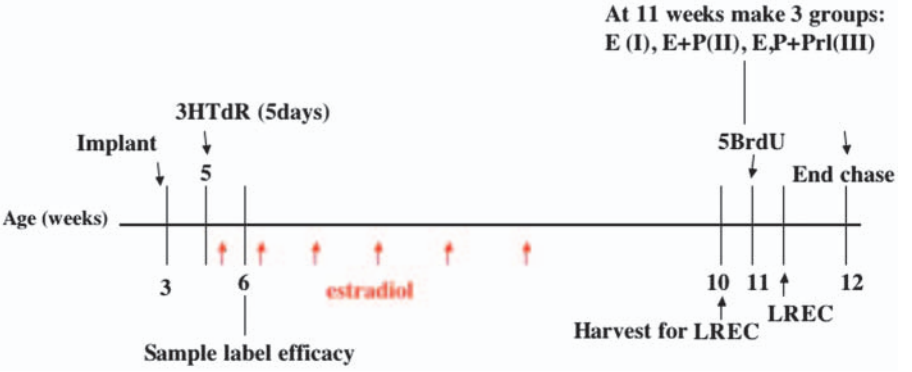

Fig. 1. Diagram showing the experimental procedures. Mice entering puberty and exactly 3 weeks of age on the day (Friday) of surgery were anesthetized, their #4 and #9 mammary fat pads were cleared of endogenous mammary epithelium and mammary fragments were implanted immediately into the cleared pads. The mice were allowed to recover for 10 days then the wound clips were removed (Monday). They were injected with 1.0 μg estradiol and ^{3}HTdR i.p., for 5 days, Monday to Friday. On the following Monday, two mice were removed to provide mammary tissues (#3,4,8,9 fat pads) and small intestine for autoradiographical analysis. Thereafter for the next 3 weeks, the remaining mice received 1.0 mg estradiol each Monday, Wednesday and Friday. Then, when they were 10 weeks old (Friday), an additional two mice were removed to provide tissue samples to determine by autoradiography the number of label-retaining cells (LREC) present. On the following Monday, the mice were placed into three groups receiving different hormone combinations and were inoculated with 5BrdU. i.p. 24 hours apart, on Monday and Tuesday. One mouse from each group (3) was removed Wednesday morning to provide tissues for evaluation of 5BrdU incorporation and ^{3}HTdR retention (LREC arrow). The remaining mice (9) received hormones as indicated until Friday and on the following Monday were euthenized to provide tissues for evaluation of 5BrdU incorporation and ^{3}HTdR retention (End chase arrow).

Assessment of labeling efficiency and the number of LREC

Sections were cut from the tissues taken following the initial pulse of ^{3}HTdR and prepared for immunostaining and autoradiography. Slides were prepared for staining with anti-smooth muscle actin (SMA), anti-estrogen receptor (ER), anti-progesterone receptor (PR) and anti-5BrdU. Several thousand cells were counted from each and the percent of labeled cells in the mammary glands was calculated to be greater than 50%. Mammary cells associated with growing terminal end buds were nearly 70% labeled (not shown). The high frequency of labeled cells was anticipated in the mammary tissues sampled only 3 days following the last thymidine injection. The distribution of labeled cells in the growing ducts was similar to that described by others (Zeps et al., 1998). Mammary epithelial cells in the terminal end buds that were positively labeled with ^{3}H-TdR were the cap cells, body cells and cells in the subtending duct. In addition to the epithelium, periductal cells in the stroma also incorporated label. Subsequent to the 5-week chase period, tissue slides were similarly prepared for staining and autoradiography, and the number of LREC

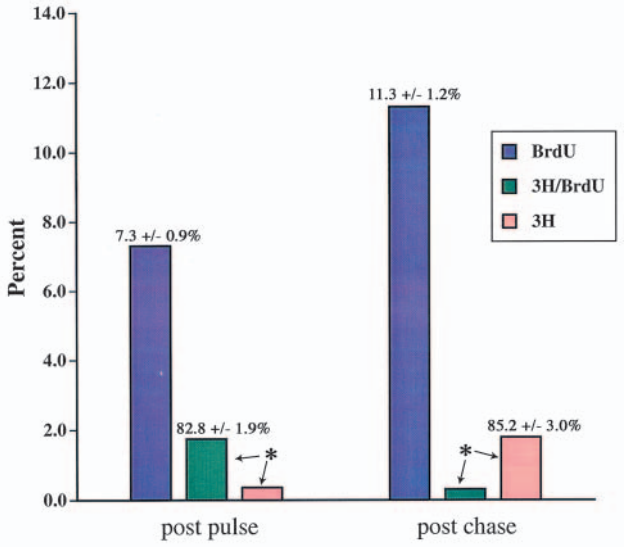

Fig. 2. Blue bars, total percentage of 5BrdU-labeled cells; pink bars, percentage of ^{3}HTdR label-retaining cells; green bars indicate the percentage of double label (^{3}HTdR/5BrdU)-containing nuclei. After the two day 5BrdU pulse (post-pulse), 7.3±0.9% of the nuclei were 5BrdU-positive including ~1.8% of the total number of ^{3}HTdR label-retaining cells (82.8±0.9% of LREC). Following a 5 day chase (post chase), the percentage of 5BrdU-positive nuclei increased to nearly 12%, while the percent of doubly labeled ^{3}HTdR/5BrdU nuclei dropped to 0.6%(14.7±3.0% of LREC) and the singly labeled ^{3}HTdR-retaining cells rose to roughly 1.6% (85.2±3.0% of LREC) of the total nuclei counted. *The percent of LREC in the population after 5BrdU incorporation did not vary significantly, remaining near 2.0%.

remaining was determined by counting several thousand (3000-4000) cells from each sample (eight mammary glands in two experiments). The average number of LREC in the mammary tissues after the chase was 2.1±0.1%. This frequency of LREC was not significantly altered in samples taken subsequent to 5BrdU labeling and chase. The average number of grains per labeled nucleus in ^{3}HTdR post pulse samples that had been prepared for 5BrdU antigen retrieval and stained with anti-5BrdU was determined and found to be 4.75±1.15. These slides also served as negative controls for 5BrdU immunostaining. The LREC in the tissue slides (12 mammary glands) evaluated for the combined estimation of nuclear labeling with tritium and 5BrdU following the 5BrdU pulse had an average of 4.12±0.88 grains and those present in the mammary tissues (36 glands) following the 5BrdU chase period had 4.11±0.95 grains per nucleus. At least 80-100 LREC nuclei (~5000 total cells) per experimental animal (n=9) were assessed in the chased mammary tissues to obtain these grain counts. Glands taken the day following the final 5BrdU injection contained 7.3±0.9% 5BrdU-labeled cells; this number increased during the chase period to 11.2±1.2% because of cell division and distribution of the label into the daughter cells (Fig. 2).

Detection of doubly labeled 5BrdU/^{3}HTdR epithelial cells

The mammary glands, from mice harvested on the day after the second of two 5BrdU injections 4 hours apart, were prepared for autoradiography and staining for 5BrdU. Assessment of these sections for ^{3}HTdR-LREC indicated that 2.1±0.1% of the cells counted contained nuclei with autoradiographical grain counts similar to those detected over nuclei after the initial ^{3}HTdR pulse (Fig. 3A,E,N). Surprisingly, 82.8±1.9% of the LREC nuclei were positive for 5BrdU staining (Fig. 2 and Fig. 3F-J). This result indicated that most LREC were actively synthesizing DNA during the administration of 5BrdU. There was no detectable difference in the location or percentage of doubly labeled nuclei among the three experimental groups receiving alternate hormone treatments. Following the 5 day 5BrdU chase, the percentage of ^{3}HTdR-labeled cells did not decrease; however, the number of LREC doubly positive for ^{3}HTdR and 5BrdU dropped to 14.8±3.0% (only 2.1±0.1% of all the epithelial cells were LREC), whereas the number of 5BrdU-positive cells (11.3±1.3%) increased (Fig. 2 and Fig. 3B-D). At the end of the experiment, cells positive for 5BrdU were sometimes juxtaposed to cells positive for ^{3}HTdR (Fig. 3K-M) consistent with redistribution of 5BrdU-labeled DNA from ^{3}HTdR-LRECs to their progeny during mitosis. In contrast to long label-retaining epithelial cells, none of 139 label-retaining stromal cells, observed in the anti-5BrdU stained sections, incorporated 5BrdU during the pulse period providing a strong internal control for distinguishing label retention because of slow or non-cycling cells from those actively proliferating but selectively segregating old and newly labeled DNA.

PR and ER-positive ^{3}HTdR-label retaining cells

Following the initial 5 day application of ^{3}HTdR, both PR-positive and ER-positive epithelial cells were found among the body cells of terminal end buds. Subsequently, following the 5-week and 6-week period during which the tritiated label was chased, LREC with positive staining for PR and ER were still present among the mammary epithelium (Fig. 4). As reported by others, the frequency of association between PR, ER and LREC decreased during the chase period (Welm et al., 2002; Zeps et al., 1999). The association of ER and PR staining in LREC has been previously reported (Zeps et al., 1998). Under our conditions of labeling, we did not encounter the label-retaining myoepithelial cells reported by Zeps et al. (Zeps et al., 1998) following the chase period. However, similar to his report, the presence of stromal LRC closely associated with epithelial structures was quite evident (not shown).

Parity-induced mammary epithelial cells (PI-MEC) and LREC in mammary outgrowths

In whey acidic protein promoter (WAP)-Cre/Rosa26-lox-STOP-lox-*lacZ* primiparous female mice, parity-induced mammary epithelial cells (PI-MEC) were detected in the involuted mammary glands of primiparous WAP-Cre/Rosa26-lox-stop-lox-*lacZ* females by the activation of the *lacZ* reporter gene through WAP promoter-expressed Cre recombinase removal of the floxed transcriptional STOP sequence between the Rosa regulatory elements and the *lacZ*-coding sequence (Soriano, 1999; Wagner et al., 1997). These cells survive the massive cell death during remodeling of the gland following the cessation of lactation and originally represent ~7% of the surviving mammary epithelium, although they increase in frequency upon successive pregnancies. The PI-MEC were shown to be capable of self-renewal upon transplantation and

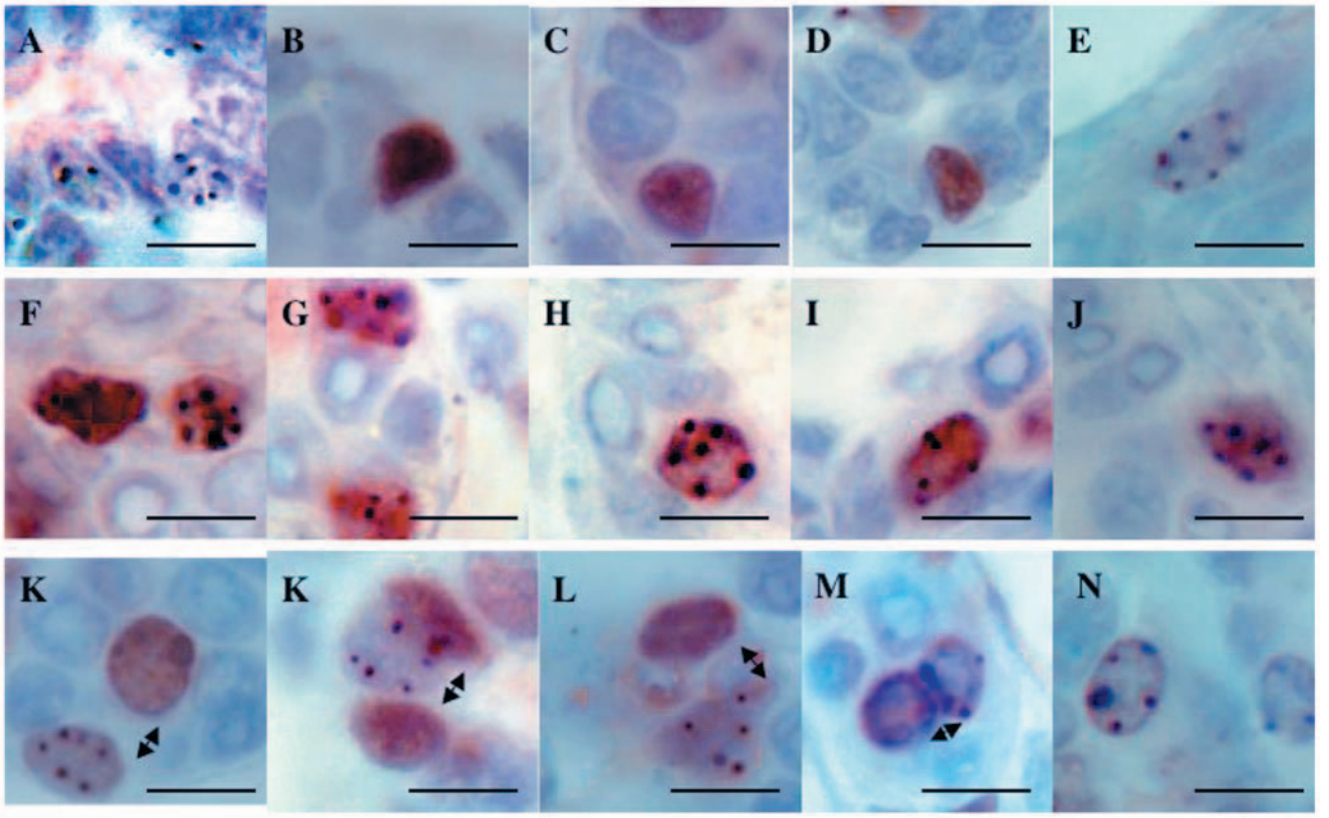

Fig. 3. (A-E) Nuclei positive for ^{3}HtdR alone (A,E) or 5BrdU alone (B-D). (F-J) 5BrdU/^{3}HTdR-labeled cell nuclei. Doubly labeled 5BrdU/^{3}HTdR nuclei, singly labeled ^{3}HTdR-positive nuclei and 5BrdU-labeled nuclei were often juxtaposed, suggesting that their labeling resulted from a recent mitotic event (double arrows in K-M). (E,N) Singly labeled ^{3}HtdR labeled nuclei in 5BrdU-labeled mammary tissues. Scale bar: 10 μm.

to contribute to the population of mammary epithelium found in the resulting mammary outgrowth (Wagner et al., 2002). These cells also acted as secretory lobule-specific progenitors upon subsequent pregnancies. The PI-MEC and their progeny are lineally marked by the constitutive expression of *lacZ* and therefore can be detected by X-gal staining in transplanted mammary outgrowths. To determine if the PI-MEC might become LREC during self-renewal and contribution to mammary transplants in mammary fat pads, mammary fragments containing *lacZ*-positive PI-MEC were implanted in three-week-old Nu/Nu hosts. Labeling with ^{3}HTdR and 5BrdU was conducted as described above. Examination of doubly labeled mammary outgrowths from PI-MEC implants revealed the presence of *lacZ*-positive, ^{3}HTdR and 5BrdU-positive PI-MEC progeny among the epithelium (Fig. 5), demonstrating that certain of the progeny of self-renewing PI-MEC become LREC scattered among other *lacZ*-positive epithelial cells during the process of mammary duct morphogenesis. The observation that the PI-MEC progeny that retain ^{3}HTdR also incorporate 5BrdU following its introduction into the mice suggests that these cells are actively cycling and equivalent to the LREC described above in intact glands. In addition, PI-MEC have been shown to be pluripotent and self-renewing, both in situ and upon transplantation (Boulanger et al., 2004), suggesting that LREC in mammary epithelium represent cells that have the capacity to produce progeny of several epithelial lineages and to possess extensive self-renewal capacity.

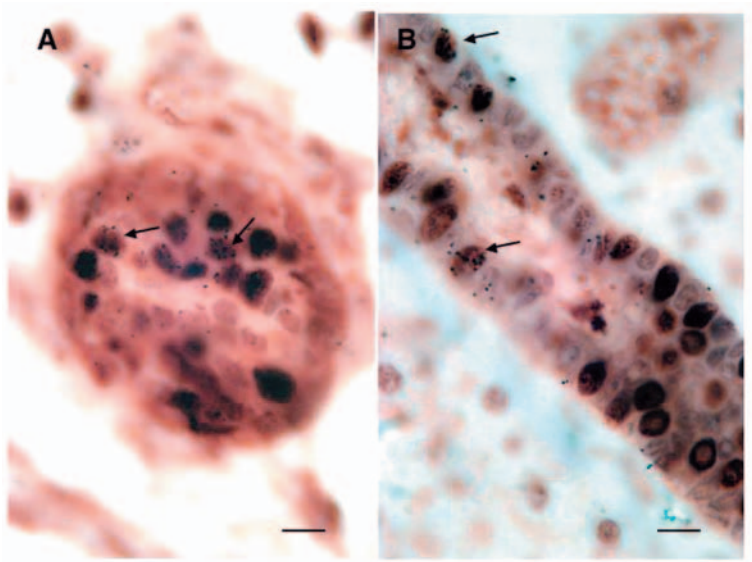

Fig. 4. LREC (arrows) included cells staining positive for estrogen receptor (A) and progesterone receptor (B). Scale bar: 10 μm.

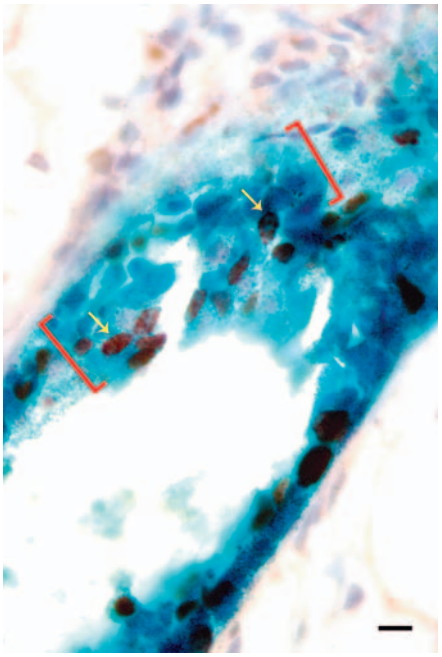

Fig. 5. The progeny of parity-induced mammary epithelial cells marked by the expression of β-galactosidase (blue) included LREC that were doubly labeled by 5BrdU and ^{3}HtdR (arrows) in mammary transplant outgrowths in the cleared fat pads of doubly pulsed female hosts. This indicated that during the process of expansive self-renewal some of these cells become asymmetrically dividing LREC. The red brackets outline the region of the image where the plane of section is tangential to the long axis of the duct, thus exposing the suprabasal epithelial layer. Scale bar: 10 μm.

Discussion

In an earlier study of LREC in adult mouse mammary glands labeled by three consecutive ^{3}HTdR injections given at 8-hour intervals upwards of 50% of the cells were labeled immediately after the injections (Zeps et al., 1996). When contralateral glands were taken from the same mouse 2 weeks later, only 1/50 to 1/1000 cells retained label at the level seen immediately after the ^{3}H pulse. These observations indicate that a considerable amount of cellular turnover occurs in the adult gland. Further study by these authors demonstrated that introducing ^{3}HTdR at estrus and metestrus in cycling virgins provided the greatest number of label retaining cells 2 weeks later. Despite this, mammary glands labeled in this way contained no detectable LREC when examined after 5 weeks (Zeps et al., 1996). This result implies that all the cells labeled by this method were exponentially cycling and distributed the labeled DNA in a semi-conservative manner. It is therefore unlikely that this method detects asymmetrically dividing cells because expansive stem cell renewal is not occurring during maintenance of the fully developed mammary ductal system. Conversely, when glands were labeled for 14 consecutive days during the 3rd to the 5th week of life (during active expansion of mammary stem cells in the allometrically growing ducts), LREC were observed even 9 weeks after the cessation of labeling (Welm et al., 2002). This method, and the one employed here, tags stem cells (sic LREC) at their inception and label is retained in these cells through asymmetric divisions, even though they may often traverse the cell cycle during the weeks subsequent to their derivation.

The experiment was designed to determine whether LREC in mouse mammary gland selectively segregate their template DNA strands to themselves while traversing the cell cycle. The frequency of LREC (~1/50) detectable among the mammary epithelium in these experiments agrees well with the numbers reported by other investigators (Welm et al., 2002; Zeps et al., 1998; Zeps et al., 1999; Zeps et al., 1996). Our data show that >8/10 of mammary LREC become doubly labeled upon the introduction of a secondary DNA synthesis marker (5BrdU). This strongly supports the conclusion that mammary LREC are traversing the cell cycle and are neither out of cycle nor cycling very slowly. In addition, over a chase period of 5-6 days, a large proportion of the doubly labeled LREC become 5BrdU-negative, while retaining the ^{3}HTdR marker. This demonstrates that the preponderance of doubly labeled LREC is actively dividing and selectively segregating the old (^{3}HTdR) DNA to themselves and partitioning the newly labeled (5BrdU) DNA into their daughter cells.

This unexpected result raises several questions regarding the principal functions of LREC in mammary glands and how these may relate to putative stem cell properties. One prospect is that the LREC represent a specific epithelial cell subpopulation whose function is to divide asymmetrically to produce committed transiently amplifying daughters to replace naturally occurring cell loss among the mammary epithelium. Asymmetric cell division is a property of stem cells and particularly of stem cells functioning within a tissue-specific stem cell niche, reviewed by Lin (Lin, 2002). But are LREC multipotent stem cells or simply giving rise to epithelial cells committed to a single epithelial cell lineage? In the current study, it was not possible to determine whether LREC daughters represented epithelial cells committed to one epithelial lineage or to several. In either case, LREC are shown to be self-renewing by retention of the ^{3}HTdR-labeled DNA. This is apparently accomplished by asymmetric distribution of the old and new DNA strands. Therefore mammary LREC possess at least one property commonly ascribed to somatic stem cells. A second property is the ability to divide symmetrically to produce an expanded population of stem cells. To approach this issue, implants of mammary fragments bearing parity-induced mammary epithelial cells (PI-MEC) were examined after the double labeling procedure. PI-MEC marked by constitutive *lacZ* expression expansively self-renew in outgrowths from mammary fragments. We have estimated that each PI-MEC must undergo at least eight doublings during the generation of a complete mammary outgrowth if all are equivalently capable of self-renewal (Wagner et al., 2002). PI-MEC, *lacZ*-positive progeny became LREC in mammary outgrowths and were doubly labeled with ^{3}HTdR and 5BrdU. Therefore, mammary cells (PI-MEC) that are pluripotent and capable of self-renewal and expansion during the allometric growth of mammary ducts can become actively dividing LREC (Boulanger et al., 2004). This observation suggests that certain self-renewing mammary cells might occupy specific micro-environmental locales in the fully developed gland and adopt asymmetric cell division kinetics as defined by retention of a template DNA strand during mitosis.

The observation of long label retaining mammary stromal cells was not reported in the earlier papers, broaching the

subject of LREC in the rodent mammary gland (Welm et al., 2002; Zeps et al., 1996). However, in 1983, a paper was published (Berger and Daniel, 1983) describing the stimulation of DNA synthesis in the proximate mammary stroma associated with actively growing terminal end buds. Here, we also observed DNA synthesis in the mammary stroma surrounding the growing end buds and subsequently the appearance of label-retaining stromal cells following the 5-6 week chase of the ^{3}HTDr. These ^{3}HTdR-labeled cells appeared both in the periductal stroma and in the fat pad stroma. None of these cells incorporated 5BrdU during the 2-day pulse. This result suggests that the label-retaining stromal cells are not cycling or are cycling very slowly in contrast to the LREC.

The significance of strand retention in asymmetrically dividing cells has been implicated in the protection of such cells from mutations resulting from errors during DNA duplication (Cairns, 2002) and thus from cancer risk. The relatively constant turnover of mammary epithelial cells in the cycling female mouse was demonstrated by the very large percentage (>50%) of epithelial cells labeled with ^{3}HTdR in a 24-hour period (Zeps et al., 1996). The rapidity with which this label is diluted through cell divisions in 2 weeks (roughly three estrus cycles) suggests that this strategy would be of selective advantage in preventing the accumulation of mutations in proliferatively competent mammary cells that survive for extended periods. An early pregnancy confers a twofold lifelong protection from mammary cancer risk in rodents and humans. The observation that PI-MEC appear to adopt the strategy of template strand retention during their expansion and self-renewal in mammary transplants offers one possible explanation for this pregnancy-induced refractoriness to carcinogenesis. However, additional studies regarding the susceptibility of PI-MEC to various carcinogenic agents and their capacity to adopt and maintain asymmetric cell kinetics in situ are needed to address this possibility.

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