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 [3H]-thymidine (3HTdR). After a prolonged chase during which much of the branching duct morphogenesis was completed, 3HTdR-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 3HTdR was retained. These results demonstrate that mammary LREC selectively retain their 3HTdR-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 [3H]-thymidine (3HTdR) 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 (3HTdR) and pass the newly synthesized strands marked with 5-bromodeoxyuridine (5BrdU) to their progeny. This property is claimed to effectively protect long-lived 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 3HTdR 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 3HTdR 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 3HTdR injection. A greater number of these cells were obtained when 3HTdR 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,
Development of Health.

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

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-stop-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 1H-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 1H-thymidine 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.

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Autoradiography and immunohistochemistry

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 A099B, 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, dehydrated, 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 3H-thymidine 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 3H-thymidine 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-stop-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 regenereated 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, ³²PHTdR 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 ³²PHTdR 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 ³²PHTdR, 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 ³²PHTdR injection). On the 5th Friday after ³²PHTdR injection, when the mice were 10 weeks old, tissues from two mice (in each experiment) were harvested to determine the frequency and location of LREC (Fig. 1). On Monday, in the 6th week post ³²PHTdR 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.

**Assessment of labeling efficiency and the number of LREC**

Sections were cut from the tissues taken following the initial pulse of ³²PHTdR 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 ³²PHTdR 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
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 that had been prepared for autoradiography and staining for 5BrdU. Assessment of these sections for 3HTdR-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 3HTdR 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 3HTdR-labeled cells did not decrease; however, the number of LREC doubly positive for 3HTdR 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 3HTdR (Fig. 3K-M) consistent with redistribution of 5BrdU-labeled DNA from 3HTdR-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 3HTdR-label retaining cells
Following the initial 5 day application of 3HTdR, 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

Detection of doubly labeled 5BrdU/3HTdR epithelial cells
The mammary glands, from mice harvested on the day after the second of two 5BrdU injections 4 hours apart, were

![Fig. 2. Blue bars, total percentage of 5BrdU-labeled cells; pink bars, percentage of 3HTdR label-retaining cells; green bars indicate the percentage of double label (3HTdR/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 3HTdR 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 82.8±0.9% of LREC.](Image)
Mammary label-retaining cells divide asymmetrically 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 3HTdR and 5BrdU was conducted as described above. Examination of doubly labeled mammary outgrowths from PI-MEC implants revealed the presence of lacZ-positive, 3HTdR 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 3HTdR 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. 3.** (A-E) Nuclei positive for 3HTdR alone (A,E) or 5BrdU alone (B-D). (F-J) 5BrdU/3HTdR-labeled cell nuclei. Doubly labeled 5BrdU/3HTdR nuclei, singly labeled 3HTdR-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 3HTdR labeled nuclei in 5BrdU-labeled mammary tissues. Scale bar: 10 μm.

**Fig. 4.** LREC (arrows) included cells staining positive for estrogen receptor (A) and progesterone receptor (B). Scale bar: 10 μm.
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 3HTdR marker. This demonstrates that the preponderance of doubly labeled LREC is actively dividing and selectively segregating the old (3HTdR) 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 3HTdR-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 mammmary 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 3HTdR 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 $^{3}$HThDr. These $^{3}$HThDr-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 $^{3}$HThDr 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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