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Immortalized, pre-malignant epithelial cell populations contain long-lived, label-retaining cells that asymmetrically divide and retain their template DNA

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ABSTRACT

Introduction: During selective segregation of DNA, a cell asymmetrically divides and retains its template DNA. Asymmetric division yields daughter cells whose genome reflects that of the parents', simultaneously protecting the parental cell from genetic errors that may occur during DNA replication. We hypothesized that long-lived epithelial cells are present in immortal, premalignant cell populations, undergo asymmetric division, retain their template DNA strands, and cycle both during allometric growth and during pregnancy.

Methods: The glands of 3-week old immune competent Balb/C female mice were utilized intact or cleared of host epithelium and implanted with ductal-limited, lobule-limited, or alveolar-ductal progenitor cells derived from COMMA-D1 pre-malignant epithelial cells. 5-bromo-2-deoxyuridine (5BrdU) was administered to identify those cells which retain their template DNA. Nulliparous mice were then either injected with [^{3}H]-thymidine (^{3}H-TdR) to distinguish 5BrdU-label retaining cells that enter the cell cycle and euthanized, or mated, injected with ^{3}H-TdR, and euthanized at various days post-coitus. Sections were stained for estrogen receptor-α (ER-α) or progesterone receptor (PR) via immunohistochemistry. Cells labelled with both 5BrdU and ^{3}H-TdR were indicative of label-retaining epithelial cells (LREC).

Results: Cells that retained a 5BrdU label and cells labelled with [^{3}H]-thymidine were found in all mice and were typically detected along the branching epithelium of mature mouse mammary glands. Cells containing double-labelled nuclei (LREC) were found in

the intact mammary gland of both pregnant and nulliparous mice, and in mammary glands implanted with pre-malignant cells. Double-labelled cells (^{3}H-TdR/5BrdU) represent a small portion of cells in the mammary gland that cycle and retain their template DNA (5BrdU). Some label-retaining cells were also ER-α or PR positive. LRECs distributed their second label (^{3}H-TdR) to daughter cells; and this effect persisted during pregnancy. LRECs, and small focal hyperplasia, were found in all immortalized premalignant mammary implant groups.

Conclusions: The results indicate that a subpopulation of long-lived, label-retaining epithelial cells (LRECs) is present in immortal premalignant cell populations. These LRECs persist during pregnancy, retain their original DNA, and a small percentage express ER-α and PR. We speculate that LRECs in premalignant hyperplasia represent the long-lived (memory) cells that maintain these populations indefinitely.

INTRODUCTION

In 1975, John Cairns proposed that, during the division of stem/progenitor cells, the template DNA strand of a parent cell is non-randomly retained, while the newly synthesized strand is selectively segregated to a daughter cell [1]. As a result of this mechanism of asymmetric division, any spontaneous mutations or errors that may develop during DNA replication would occur in the newly synthesized strand and be passed along to the daughter cell, reducing the accumulation of genetic errors and

subsequently, cancer risk of the long-lived parent cell. In addition, this type of scheme would allow for the survival and maintenance of progenitor "stem" cells that are capable of producing expendable daughter cells. Since then, many investigations have been carried out providing support for Cairns "immortal DNA strand" hypothesis [2-4] that include, among others, cells of the mammary gland and intestine [5, 6].

The mammary gland is a unique organ that matures in the adult mammal via successive rounds of proliferation and apoptosis [7, 8]. In order to accomplish this feat, a subpopulation of cells with regenerative properties is present in the gland. It was postulated that if mammary "stem" cells were present, these cells would retain an exogenously applied label, being then identified as the "longer-lived" cells of a population either due to mitotic quiescence or selective DNA segregation [9]. While the state of differentiation was not clear (e.g. pluripotent versus multipotent), it was evident that the mouse mammary epithelium contained cells that were the progenitors of the tissue [9]. In fact, it was found that some of these mammary progenitor cells were capable of retaining their label, and thus their template DNA strand, while they traversed the cell cycle [6]. Newly synthesized DNA was found to be distributed to daughter cells as a result of asymmetric cell division [6].

In order to identify progenitor cells as well as determine if asymmetric division occurs, two labels have been applied to cells over the course of mammary gland development [5, 6, 10]. In previous studies, 5-bromo-2-deoxyuridine (5BrdU) was administered during allometric mammary gland growth and was used to identify those long-lived cells that were capable of retaining their label. A second DNA label, tritiated thymidine (^{3}H-TdR) was used to distinguish those cells that were traversing the cell cycle

at various periods during this study. Cells that contain a double-label of both 5BrdU and ^{3}H-TdR in their nucleus were interpreted as long-lived, label retaining, cycling epithelial cells (LRECs).

Data have shown that various tissues within the body utilize asymmetric division for cellular replacement, protection of long-lived cells from mutations that could occur during DNA replication, and consequently, cancer risk [5, 6, 11-13]. However, it is unknown whether pre-cancer or cancer cells themselves utilize this method to maintain their populations. Earlier work in our laboratory demonstrated that retroviral insertions were maintained undisturbed through several transplant generations in non-transformed mammary populations despite the presence of actively replicating retrovirus and the presence of unintegrated retroviral DNA [14, 15]. This scenario was found in mouse mammary tumor virus (MMTV)-induced epithelial hyperplastic outgrowths which are immortal, i.e., capable of unlimited growth during serial transplantation [16]. Therefore, we hypothesized that long-lived epithelial cells present in immortal cell populations undergo asymmetric division, retain their template DNA strands, and cycle both during allometric growth and during pregnancy. We speculate that LRECs present in pre-malignant hyperplasia represent long-lived cells that maintain these pre-neoplastic populations indefinitely.

MATERIALS AND METHODS

Mice

Three-week-old, immune-competent female Balb/C mice were used as hosts for transplantation studies. All mice were housed in Association for Assessment and

Accreditation of Laboratory Animal Care-accredited facilities in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The National Cancer Institute Animal Care and Use Committee approved all experimental procedures.

Cells

Clonal populations of immortalized, pre-malignant cells were derived using flow cytometry for side-population (SP) cells from COMMA-D1 murine mammary epithelial cells (Kittrell et al.: Prospective isolation and characterization of committed and multipotent progenitors from immortalized mouse mammary epithelial cells with morphogenic potential, submitted). COMMA-D1 murine mammary epithelial cells were originally isolated from the mammary gland of midpregnant BALB/c mice [17]. Derived clonal populations included "Non-Side Population 2" ("NSP2"), "Non-Side Population 3" ("NSP3"), and "Side Population 3" ("SP3"). The clonal population NSP2, interpreted to be a ductal progenitor cell, yields a ductal outgrowth that fills the mammary fat pad, but does not differentiate in response to hormones (Kittrell et al.: Prospective isolation and characterization of committed and multipotent progenitors from immortalized mouse mammary epithelial cells with morphogenic potential, submitted). NSP3 cells have poor growth *in vivo* and, when successfully implanted, typically fills less than 10% of the mammary fat pad with a nest of surviving cells (Kittrell et al.: Prospective isolation and characterization of committed and multipotent progenitors from immortalized mouse mammary epithelial cells with morphogenic potential, submitted). Finally, the population SP3 cells typically fill 20-59% of the mammary fat pad upon injection (Kittrell et al.: Prospective isolation and characterization of committed and multipotent

progenitors from immortalized mouse mammary epithelial cells with morphogenic potential, submitted). Cells were grown in Dulbecco's Minimal Essential Growth Medium (Gibco, Carlsbad, CA) mixed with F12 Nutrient Medium (Gibco), supplemented with 2% fetal bovine serum (Gibco), 1% 1 M HEPES Buffer (Sigma, St. Louis, MO), 1% 100x antibiotic antimycotic (Gibco), 5 ng/ml EGF (per 500 ml, Sigma), and 10 μg/ml insulin (per 500 ml, Sigma) at 37°C with 5% CO_2.

Cell transplantation

Smith et al. has previously described in detail the procedure utilized to clear the mammary epithelium from the inguinal fat pad of three-week-old host mice, as well as the subsequent transplantation of cells [18-20]. Briefly, three mice per condition (6 control (thoracic) glands; 6 inguinal fat pads) were anesthetized, both inguinal mammary fat pads cleared and a cell suspension injected. Twenty thousand NSP2 cells, fifty thousand NSP3 cells, or fifty thousand SP3 cells suspended in phosphate buffered saline were inoculated in 10 μl volumes using a Hamilton (Reno, NV) syringe equipped with a 30-gauge needle. Control mammary glands (thoracic) remained intact.

Cell labeling *in vivo*

Two weeks after the implant surgery (mouse age of 5 weeks), 1 mg (100 μl volume), 5-bromo-2-deoxyuridine (5BrdU) was administered via intraperitoneal injection for two consecutive days per week for 5 weeks (Figure 1). The 5BrdU injections were then stopped for three weeks (mouse age of 13 weeks), when some nulliparous mice were inoculated with [^{3}H]-thymidine (^{3}H-TdR) (50 μCi, 100 μl) and euthanized within 90

minutes (Figure 1). Remaining mice (mouse age of 12-13 weeks) were mated with male Balb/C mice. Female mice were subsequently inoculated with ^{3}H-TdR (50 μCi, 100 μl) and euthanized within 90 minutes at either 4-6, 8-10, or 12-15 days post-coitus (Figure 1). Mammary fat pads that were either intact (i.e., control thoracic) or containing the implanted cells were collected, fixed in neutral buffered formalin and prepared for autoradiography and immunohistochemistry. Small intestine from the same mice served as a non-mammary control for autoradiography

Immunohistochemistry

Mammary glands, embedded in paraffin, were cut in 5 μm sections and mounted on positively charged slides. Sections were subsequently cleared in xylenes and rehydrated through a series of graded ethanols, then heated to a boil in a microwave in 10 mM citrate buffer (BioGenex, San Ramon, CA) for antigen retrieval. Endogenous peroxidase activity was blocked using a 0.6% hydrogen peroxide/methanol solution for 15 minutes at room temperature. Slides were blocked with normal goat serum (for estrogen receptor- α (ER-α) and progesterone receptor (PR) staining only) (Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature, then incubated overnight with either rabbit polyclonal anti-human progesterone receptor (1:1600; Catalogue Number A0098; DAKO, Carpinteria, CA), rabbit polyclonal anti-human estrogen receptor-alpha (1:2000; Catalogue Number sc-542; Santa Cruz, Santa Cruz, CA), or biotinylated anti-mouse BrdU (1:50; Catalogue Number A21301MP; Invitrogen). Negative tissue controls were included in all immunohistochemical analyses. For ER-α and PR staining, a secondary antibody of goat anti-rabbit (1:100; Vector Laboratories) was applied for 30 minutes at

room temperature. Sections were then processed for 30 minutes at room temperature using the Vectastain ABC Standard Elite (Vector Laboratories), and visualized using Diaminobenzidine (DAB; Sigma, St. Louis, MO). Slides were counterstained using Gill's hematoxylin (Vector Laboratories).

Autoradiography

Following immunohistochemical processing, slides were transferred to distilled water, then coated with Kodak NTB-2 liquid emulsion (Carestream Health, Inc., Rochester, NY, USA) diluted 1:1 with distilled water. After drying for one hour, slides were stored in lightproof slide boxes at 4°C for 7 days. After exposure, the slides were developed in Kodak D-19 (Carestream Health, Inc., Rochester, NY, USA), washed in distilled water, and fixed in Kodak rapid fixer diluted 1:1 with distilled water. Slides were washed once more in distilled water and dehydrated through a series of graded ethanols and xylenes. Sections were mounted with Permount (Fisher Scientific, Pittsburgh, PA), and slides subsequently observed and evaluated for autoradiographic grains and for immunostaining.

Cell labeling *in vitro*

Cells were plated in 25 mm^2 tissue flasks, cultured as described above, and allowed to incubate overnight. Cells were then treated with culture medium plus 0.5 μM 5-ethynyl-2-deoxyuridine (EdU) diluted in phosphate buffered saline (PBS) for imaging using the Click-iT® EdU Imaging Kit (Invitrogen). Twenty-four hours later, the EdU solution was removed, cells were washed with PBS, and either cells immediately fixed at room

temperature for 15 minutes with 4% paraformaldehyde, washed twice with tris-buffered saline (TBS), and processed for EdU imaging, or culture medium replaced. Unfixed cells were then grown to confluency and serially passaged five times (NSP2 and NSP3 cells serially passaged at 1:3; SP3 cells serially passaged at 1:6). At the fifth passage, cells were washed with PBS, and fixed with 4% paraformaldehyde at room temperature. Fifteen minutes later, the fixative was removed and cells washed twice with TBS. For EdU imaging, cells were permeabilized for 20 minutes at room temperature with 0.2% Triton® X-100 and subsequently washed twice with TBS. Two ml of freshly prepared Click-iT® reaction cocktail (Invitrogen, prepared as described by the manufacturer) per 25 mm^2 surface area was added to the cells, which were then incubated in the dark for 30 minutes. The reaction cocktail was subsequently removed, cells were washed two times with TBS, and nuclei stained for 4 minutes at room temperature with diamno-2-phenylindole (1:1000, DAPI, Invitrogen, Carlsbad, CA). DAPI was removed, cells washed two times with TBS, and flask bottoms mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL). EdU positive cells were visualized using fluorescent microscopy.

RESULTS

Murine implantation experimental design

The mouse mammary gland is a dynamic organ that has been shown to regenerate itself from as little as one cell [14]. Consequently, it is likely that the mammary gland contains stem or progenitor cells allowing for self-renewal to occur. Previously, it has been shown that there is a population of cells within the epithelium of the mouse mammary gland that

retain a ^{3}H-TdR label, deemed label-retaining cells [9]. Utilizing this knowledge, we sought to identify and locate long label retaining mouse mammary epithelial cells *in vivo* as well as determine the presence of long label-retention in immortalized, pre-malignant cell populations maintained in epithelium-divested mammary fat pads. Three-week old Balb/C female mice were anesthetized and their inguinal mammary glands cleared of host epithelium using protocols described by Smith et al. (3 mice per condition; 6 cleared mammary fat pads) [18-20]. Either twenty- or fifty-thousand immortalized, pre-malignant cells (NSP2: 20,000 cells, NSP3 and SP3: 50,000 cells) were inoculated in 10 μl volumes. Control (thoracic) glands remained intact (3 mice per condition; 6 intact glands). Subsequently, in order to identify label-retaining cells present in the mouse mammary gland, 5BrdU was administered to the 5-week old female mice during allometric mammary ductal growth for two consecutive days/week for 5 weeks. The 5BrdU injections were then stopped for three weeks (mouse age of 13 weeks). Some of the nulliparous female mice were then inoculated with ^{3}H-TdR to distinguish 5BrdU-label-retaining cells that remain in the cell cycle, and subsequently euthanized. The remaining 13-week old mice were mated, inoculated with ^{3}H-TdR, and euthanized at either 4-6, 8-10, or 12-15 days post-coitus (Figure 1). Glands were collected and prepared for autoradiography and immunohistochemistry. Sections were further stained for ER-α or PR. Mammary cells within the ducts and alveoli that retain both a 5BrdU and ^{3}H-TdR double-label are indicative of long-lived label retaining cycling epithelial cells (LREC).

Label-retaining cells were found in immortal cell populations

Sections of mouse mammary fat pads implanted with immortalized, pre-malignant cells were examined for evidence of outgrowths containing label-retaining cells. Of the implanted fat pads, 12/16 NSP2, 0/18 NSP3, and 10/18 SP3 yielded outgrowths that filled between 10-50% of the fat pad. In both pregnant and nulliparous mice, outgrowths containing NSP2 implanted cells typically filled 10-40% of the fat pad, yielding ductal structures with blunted side branches. Outgrowths containing SP3 cells filled 20-50% of the fat pad with both ductal and lobular structures. Finally, of specific note, NSP3 cells implanted into the mammary fat pads of athymic nude mice yielded only focal areas of epithelial cells. These results suggest that the NSP3 cells had indeed survived, but did not produce outgrowths consisting of ducts and/or alveoli consistent with that found with NSP2 or SP3 cell implants. These spherical areas of epithelial cell growth typically only filled 3-5% of the fat pad (thus, it was not recorded as an outgrowth). In the NSP2 and SP3 outgrowths, occasional areas of atypical epithelial hyperplasia (~0.1-1.5 mm in diameter), with nuclei that appeared to be normal, were apparent (Table 1).

Among the outgrowths and intact glands, cells labelled with 5BrdU (long label-retaining; template DNA strand) or ^{3}H-TdR (cell cycling; newly synthesized DNA strand) were detected (Figure 2a-f, BrdU : green arrows, ^{3}H-TdR : orange arrows). These single-labelled cells, which included 0.1-23.8% labelled with 5BrdU and 0.4-14.3 % labelled with ^{3}H-TdR, were typically detected along the branching epithelium of mature mouse mammary glands in both pregnant and nulliparous mice (Table 2). Specifically, in the intact glands of mice during early pregnancy (4-6 days post-coitus), we found that approximately 4.5-5.4% intralobular epithelial cells were labelled with ^{3}H-TdR (cell cycling; newly synthesized DNA strand). At 8-10 days post-coitus, the number of

intralobular epithelial cells labelled with ^{3}H-TdR decreased to 1.0-3.4% cells. By 12-15 days post-coitus, alveolar differentiation was substantial, with 5.0-7.5% intralobular epithelial cells labelled with ^{3}H-TdR. These results are consistent with those described by Traurig, who found that the highest rates of mammary epithelial cell proliferation occurred on day 4 post coitus and day 12 (after the onset of placental progestin secretion) [21].

The frequency of epithelial cells labelled with ^{3}H-TdR in fat pads implanted with NSP2 cells (ductal progenitor) was much less in pregnant mice than non-pregnant (1.9 vs 14.3%, respectively; Table 2). These values were in contrast to fat pads implanted with the lobular progenitor cells NSP3 and SP3 where the numbers of ^{3}H-labelled cells increased in response to pregnancy. Cells containing double-labelled nuclei (stained both with 5BrdU and containing autoradiographic grains (^{3}H-TdR; LREC)) were found within all intact glands (Table 3), and were frequently located in mammary outgrowths containing implanted pre-malignant cells (Table 4). Representative images of double-labelled cells are shown in the insets of Figure 2a-f, which were similar for both nulliparous and pregnant mice whose glands were either intact, or fat pads implanted with immortalized pre-malignant cells.

Label-retaining cells found in immortal cell populations asymmetrically divide

Asymmetric division (Figure 3) would be particularly useful for stem and other long-lived cells, whereby a cell's archetype is retained for future generations. Being specifically interested in whether this phenomenon occurred within cells present in immortalized populations, we examined outgrowths from fat pads implanted with pre-

malignant cells and labelled with both 5BrdU and ^{3}H-TdR. Asymmetric division with selective DNA segregation was rare, but occurred in the NSP2, NSP3 and SP3 cells that were implanted into cleared fat pads in both nulliparous (Figure 4a) and pregnant hosts (Figure 4b). Furthermore, it was found that the number of immortalized, pre-malignant cells that contain a single label (either 5BrdU or ^{3}H-TdR) increased up to 108% after chase, providing further support that label-retaining cells within immortalized, pre-malignant populations undergo asymmetric division and pass the newly synthesized DNA strand to their daughters. A similar increase was observed in the intact control glands after the chase period.

Epithelial cells in intact and immortal cell populations occasionally express ER-α or PR and take up ^{3}H-TdR

It was next determined whether a percentage of epithelial cells present in immortalized, pre-malignant cell populations express estrogen receptor-alpha (ER-α) or progesterone receptor (PR). In the fat pads of nulliparous mice implanted with immortalized, pre-malignant cells, ER-α and PR expression was rare, being detected in 0-6% of immortalized cells counted per field of view (Figure 5a, c; Tables 5-6). In the intact glands of nulliparous mice, however, ER-α and PR expression was found in considerably larger cell numbers than implanted fat pads: approximately 17-21% of cells counted per field of view (Supplemental figure S1 in Additional file 1; ER-α (a-c) and PR (d-f), red arrows)(Tables 5-6). These data demonstrate that while occasional cells express ER-α and PR, the number of immortalized, pre-malignant cells that express the steroid receptors is considerably smaller than the number found in normal mammary epithelium.

During pregnancy, ER-α was expressed in approximately 6-7.5% of the cells counted per field of view in fat pads with outgrowths of immortalized, pre-malignant cells (Figure 5b) (Table 5). On the other hand, PR expression in the immortalized, premalignant cells in pregnant mice was substantially less than ER-α expression: 0-1% of cells counted per field of view (Figure 5d) (Table 6). In contrast, 5-7% of epithelial cells present within the intact glands of pregnant mice expressed ER-α and PR (Supplemental Figure 1) (Tables 5-6). Therefore, these data indicate that immortalized, pre-malignant cells implanted into the fat pads of pregnant mice that express PR are present in small numbers.

Cells positive for ER-α (Figures 5a,c, green arrows, inset) or PR (Figures 5b,d, green arrows, inset) and a ^{3}H-TdR label were rare (<1%) in both outgrowths of immortalized, pre-malignant cells and in the intact gland (Tables 7-8). Nevertheless, these results suggest that some of the cells expressing the hormone receptors were also progressing through the cell cycle.

Label-retaining cells were present in non-mammary tissues

While it is evident that label-retaining epithelial cells are present in the regenerating mammary gland, it may be possible that these cell populations are also present in other tissues. Booth et al. described that label-retaining cells were found in several non-epithelial mammary tissues, including nerve, fatty stroma, and endothelial tissue [10]. Consistent with that observation, label-retaining cells, in this study, were also located among cartilage (Supplemental figure 2a in Additional file 2), adipose tissue (Supplemental figure S2b in Additional file 2), skeletal muscle (Supplemental figure S2c

in Additional file 2), and periductal cells (Supplemental figure S2d in Additional file 1). These results indicate that label-retaining cells are present in tissues in addition to the mammary epithelium.

Label-retaining cells were present in pre-malignant cell populations in vitro

In order to verify if label-retaining cells were present in immortalized, pre-malignant cell populations *in vitro*, clones were cultured and treated with 0.5 μM EdU. EdU-labelled cell nuclei were found in all immortalized, pre-malignant cell populations *in vitro* at both passage 0 and at passage 5. In passage 5 cell cultures there was a 2 to 5.5-fold decrease in the number of EdU-label retaining cells present compared to passage 0 (Table 9, Figure 6). One cause for the decrease in number of label retaining cells might be the result of dilution of non-cycling cells during serial passage. At passage 0, approximately 20% of immortalized, pre-malignant cells are EdU-positive. If dilution of non-cycling cells were in play, then, after 5 passages, cells seeded at a 1:3 (NSP2, NSP3) or 1:6 dilution (SP3) would have 1 cell in 243 (3^5) or 1 cell in 7,776 (6^5), respectively, as a label-retaining cell. This was not the case as 5 NSP2 cells of 34 cells (14.7%), 7 NSP3 cells of 157 cells (4%), and 21 SP3 cells of 561 cells (3.8%) were positive for EdU after 5 serial passages (Table 9). These results show that the label-retaining, pre-malignant populations are selectively preserved throughout serial passage *in vitro*. This data implies that label-retaining, pre-malignant cells were not out of the cell cycle during passage, but nevertheless retained their original DNA label. In addition, label-retaining cells in the 5^{th} passage were occasionally found juxtaposed to unlabelled cells suggesting selective label retention during mitosis (Figure 6).

DISCUSSION

Our data demonstrate that there is a subpopulation (<1%) of long-lived cells (LRECs), both in immortalized, pre-malignant cells and in the normal mouse mammary gland, that maintain and protect template DNA through selective strand segregation during cell division. During pregnancy, the LRECs are stimulated to enter the cell cycle and contribute to new progeny through asymmetric divisions. We postulate that these cells represent long-lived cells (LRECs) are the source of regenerative capacity for both normal and premalignant epithelial populations. In a sense, this subpopulation of long-lived cells represents the "memory" or "repository" cells of these populations.

Here, we show that both untransformed mammary epithelial cells as well as immortalized, pre-malignant epithelial cells divide asymmetrically (Figure 4) and are selectively conserved through serial passage *in vitro* (Figure 6), suggesting that a subpopulation of progenitor-like cells resides in each of these populations. Is it the case that these label-retaining epithelial cells may be pluripotent or multipotent stem cells giving rise to progeny committed to a specific lineage? While the answer to that question is presently unknown, in this study, it was shown that LRECs are capable of self-renewal, a characteristic specific to stem cells, as identified by retention of a 5BrdU label. Cells in the small intestine, neural tissue, skeletal muscle, and Drosophila ovarioles, as well as the mammary gland have been shown to selectively retain their template DNA during asymmetric divisions [6, 10, 22]. In these cases, all of the chromatids possessing template DNA are retained. What is the mechanism(s) by which this is accomplished? Several reports have indicated possible mechanisms by which older DNA chromatids

may be recognized and selectively retained. In fission, yeast kinetochore-specfic proteins associate selectively with the older chromatids during meiotic divisions [23]. In mouse colon crypt epithelial cells, sister chromatids were non-randomly segregated during mitotic divisions apparently by recognition of specific DNA sequences [24]. In addition, Armakolas et al. suggests that individual old and new DNA strands may be selectively distributed during mitotic division in differentiating cells of different lineages [25]. For example, selective strand segregation was found in endodermal cells, while random strand segregation was noted in others [25]. Thus, it may be possible that the cell type and/or tissue microenvironment regulates the mode of strand distribution, and therefore the pattern of cellular differentiation.

In addition to the mammary epithelium, label-retaining cells were also found in non-epithelial mammary structures. Booth et al. discovered label-retaining cells in nerve, fatty stroma, and endothelial tissue [10]. Here, label-retaining cells were also found within cartilage, adipose tissue, skeletal muscle, and in periductal cells (Supplemental Figure 2a-d). These results suggest that progenitor cells may be present elsewhere within the body, serving as tissue reserve cells and being utilized only when needed. An example of such a cell can be found in skeletal muscle [13, 26]. Shinin et al. identified a subpopulation of muscle satellite cells that divided asymmetrically and retained their template DNA during muscle regeneration [13]. It was found that these effects persisted during muscle growth, as well as during muscle injury [13]. At the present, though, it is unknown whether a specific physiological event, such as wounding, pregnancy, or cell death, is necessary to trigger the activation of tissue-specific progenitor cells. Nevertheless, their presence in the tissues identified in this study is intriguing.

Cells expressing either ER-α or PR also incorporated ^{3}H-TdR into their nuclei (Tables 7-8), indicating that some steroid receptor positive cells are traversing the cell cycle. These results are consistent with those found by Booth et al. [10], who surmised that the epithelial cells that express either ER-α or PR and incorporate ^{3}H-TdR into their nuclei represent a functionally distinct cell population undergoing asymmetric division [10]. Thus, these results suggest that within the mammary gland, there are distinct cell populations (e.g. ER-α and/or PR positive versus ER-α and/or PR negative), each potentially arising from a different progenitor.

Data presented in this study indicate that only a small number (<1%) of cells present in hyperplasia possess these progenitor-like cell characteristics. We believe it is possible that the lower frequency of double-labelled cells recorded in the present experiments may be due to the relatively short pulse used to introduce the second DNA label into the tissue, especially if cycling LREC pass more slowly through the S phase than symmetrically dividing cells. In past experiments [6], the second label was applied over a 48 hour period (in the presence of estradiol, estradiol plus progesterone, or estradiol, progesterone, plus prolactin), yielding a population of nearly 2% double-labelled cells [6]. Labelling over 48 hours may likely result in labelling both slowly cycling and rapidly cycling cells. Alternatively, it may be the case that immortalized, pre-malignant populations already contain progenitor cells whose template DNA strand was not initially labelled by 5BrdU. In this case, the unlabelled template strand would be selectively retained and the newly synthesized strand, labelled with 5BrdU, would be passed to a daughter cell via asymmetric division. Thus, the number of LRECs (tagged by 5BrdU) would be small. In the data presented here, however, we suppose that

symmetric expansion of the progenitor population occurs, rendering this supposition unlikely.

Conclusions

These findings demonstrate that there is a subpopulation of long-lived cells, characterized by their ability to retain a DNA label, that are present in immortalized, pre-malignant cells. These cells retain their original DNA strands and divide asymmetrically to maintain their cell populations and protect their template DNA. During pregnancy, where these cells persist, they are stimulated to enter the cell cycle and contribute to new progeny through asymmetric divisions. New long label retaining cells, which include those that express ER-α and PR, continue to cycle. We speculate that LRECs present in pre-malignant hyperplasia represent the long-lived (memory) cells that maintain these populations indefinitely.

ABBREVIATIONS

5BrdU: 5-bromo-2-deoxyuridine; DAPI: diamno-2-phenylindole; EdU: 5-ethynyl-2-deoxyuridine; ER-α: estrogen receptor-alpha; ^{3}H-TdR: [^{3}H]-thymidine; LRC: label-retaining cell; LREC: label-retaining epithelial cell; MMTV: mouse mammary tumor virus; Non-SP2: non-side population 2; Non-SP3: non-side population 3; PBS: phosphate buffered saline; PR: progesterone receptor; SP3: side population 3.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

GHS and CAB conceived the study, the design, and interpreted the data. KMB performed data collection, data interpretation, and wrote the manuscript. CAB and GHS performed the mouse surgeries. DM, FB and FSK isolated and characterized the immortalized, pre-malignant COMMA-D cell clones. All authors have read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1: Murine implantation experimental design. The inguinal mammary glands of three-week old Balb/C mice were cleared of host epithelium and inoculated with either twenty- (NSP2) or fifty-thousand (NSP3, SP3) immortalized, pre-malignant cells. Control (thoracic) #3 and #8 glands remained intact. 5BrdU was administered for two consecutive days/week for 5 weeks, followed by a break (chase) period of three weeks. Nulliparous female mice were then inoculated with ^{3}H-TdR and subsequently euthanized within 90 minutes. The remaining mice were mated, inoculated with ^{3}H-TdR, and euthanized within 90 minutes of receiving label at either 4-6, 8-10, or 12-15 days post-coitus.

Figure 2: Immortal cell populations contain LRECs. Glands and fat pads from nulliparous mice either utilized intact or implanted with immortalized, pre-malignant cell clones were labelled with 5BrdU (green arrow) and ^{3}H-TdR (orange arrow). Double-

labelled cells (labelled with both 5BrdU and ^{3}H-TdR, black arrow, inset) were identified using light microscopy and were found in both intact glands **(a, c, e)** and implanted fat pads **(b, d, f)**. LREC's were found in all immortal, pre-malignant cells (b, NSP2; d, NSP3; f, SP3). Some cells had a patch-like 5BrdU-staining pattern (red arrow). Representative images are shown. Scale bars equal 20 μm.

Figure 3: Diagram of symmetric and asymmetric division. Brown background indicates a cell with template DNA; black dots indicate a cell that is cycling. **(a)** During symmetric division, a cycling (black dots) parent cell with template DNA (brown background) divides equally yielding two proliferating (black dots) identical daughter cells that each have one template DNA strand (brown background) and one newly synthesized DNA strand. **(b)** In asymmetric division, a cycling (black dots) parent (stem) cell with template DNA (brown background) undergoes unequal division, retaining its template DNA (brown background), but yielding one proliferating (black dots) daughter cell containing DNA that was newly synthesized from the parent's template strand.

Figure 4: Asymmetric cell division occurred in immortalized, pre-malignant cell populations. Fat pads from mice implanted with immortalized, pre-malignant cells were labelled with 5BrdU and ^{3}H-TdR. Double-labelled cells that undergo asymmetric division and pass a ^{3}H-TdR label on to their daughter cell (red arrow) in **(a)** nulliparous and **(b)** pregnant mice were identified using light microscopy. Scale bars equal 5 μm.

Figure 5: LRECs present in the mammary fat pads of pregnant and nulliparous mice implanted with immortalized, pre-malignant cell populations express ER-alpha and PR and incorporate ^{3}H-TdR into their nuclei. Immunohistochemistry for ER-alpha (**(a, c)**, red arrow) and PR (**(b, d)**, red arrow), and labelling for ^{3}H-TdR, was performed on mouse mammary glands (inguinal) of nulliparous mice (a-b) or pregnant (c-d) implanted with immortalized, pre-malignant cells. ^{3}H-TdR was incorporated into the nucleus of some cells expressing the steroid receptors (green arrow, inset). Representative images are shown. Scale bars equal 20 μm.

Figure 6: Label-retaining cells were present in immortalized, pre-malignant cells *in vitro*. Immortalized, pre-malignant cell populations NSP2 **(a-b)**, NSP3 **(c-d)**, and SP3 **(e-f)** were cultured *in vitro* as described in the Materials and Methods. Cells were plated, allowed to incubate overnight, treated with 0.5 μM EdU for 24 hours, and fixed, permeabilized, labelled with Alexa Fluor® 488, and counterstained with DAPI either (a, c, e) immediately; or (b, d, f) after serial passage. Representative images are shown. Scale bars equal 40 μm.

TABLES

Table 1: Number of fat pads resulting in small focal hyperplasia of pregnant or nulliparous mice implanted with immortalized, pre-malignant cells

Cell type	Nulliparous	Pregnant
NSP2	6/6	6/10
NSP3	0/6	0/12
SP3	5/6	5/12

The number of fat pads containing small focal hyperplasia, versus those without, was visualized using a light microscope and enumerated.

Table 2: Percent of cells expressing either a 5BrdU or ^{3}H-TdR single label per field of view found in the glands and mammary fat pads of Balb/C female mice that were either intact or implanted with immortalized, pre-malignant cells

Percent (%)

	Nulliparous		**Pregnant**	
Intact (control) Gland: Implant Group	5BrdU	^{3}H-TdR	5BrdU	^{3}H-TdR
Intact: NSP[2]	13.0 : 13.0	8.2 : 14.3	0.6 : 6.8	5.2 : 1.9
Intact: NSP[3]	23.8 : 19.7	3.5 : 2.0	0.8 : 3.5	2.7 : 8.7
Intact: SP[3]	12.0 : 4.4	4.8 : 0.4	0.1 : 4.0	1.9 : 6.5

The number of cells labelled with either 5BrdU or ^{3}H-TdR was counted in three random fields of view (400x magnification). In addition, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification). The total number of cells labelled with either 5BrdU or ^{3}H-TdR was then divided by the total number of cells in a field of view, and the percentage of cells labelled with either 5BrdU or ^{3}H-TdR per field of view obtained.

Table 3: Percent of double-labelled cells per field of view found in the intact glands of nulliparous or pregnant Balb/C female mice

	Percent (%)	
Intact Gland Group	**Nulliparous**	**Pregnant**
Intact Group 1	0.02	0.04
Intact Group 2	0.3	0.004
Intact Group 3	0.1	0.02

The number of double-labelled (5BrdU and ^{3}H-TdR) cells were counted in three random fields of view (400x magnification). Next, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification). The total number of double-labelled cells was divided by the total number of cells, and the percentage of double-labelled cells per field of view obtained. The label "intact group" corresponds to the unoperated (control) #3 and #8 axillary mammary glands in each experimental group.

Table 4: Percent of double-labelled cells per field of view found in the mammary fat pads of nulliparous or pregnant Balb/C female mice implanted with immortalized, pre-malignant cells

	Percent (%)	
Cell Type Group	**Nulliparous**	**Pregnant**
NSP2	0.15	0
NSP3	0.2	0.03
SP3	0	0.01

The number of double-labelled (5BrdU and ^{3}H-TdR) cells were counted in three random fields of view (400x magnification). Next, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification) . The total number of double-labelled cells was divided by the total number of cells, and the percentage of double-labelled cells per field of view obtained.

Table 5: Percent of cells expressing ER-α per field of view found in the glands and mammary fat pads of Balb/C female mice that were either intact or implanted with immortalized, pre-malignant cells

	Percent (%)	
Intact (control) Gland : Implant Group	**Nulliparous**	**Pregnant**
Intact : NSP2	20.4 : 5.8	7.5 : 7.5
Intact : NSP3	21.5 : 6.1	7.4 : 5.9
Intact : SP3	16.4 : 2.3	6.8 : 6.4

The number of cells expressing ER-α was counted in three random fields of view (400x magnification). In addition, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification). The total number of cells expressing ER-α was then divided by the total number of cells in a field of view, and the percentage of cells expressing ER-α per field of view obtained.

Table 6: Percent of cells expressing PR per field of view found in the glands and mammary fat pads of Balb/C female mice that were either intact or implanted with immortalized, pre-malignant cells

	Percent (%)	
Intact (control) Gland : Implant Group	**Nulliparous**	**Pregnant**
Intact : NSP2	21.0 : 5.2	5.2 : 1.0
Intact : NSP3	18.2 : 0.6	5.8 : 0.0
Intact : SP3	17.8 : 0.0	6.8 : 0.8

The number of cells expressing PR was counted in three random fields of view (400x magnification). In addition, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification) . The total number of cells expressing PR was then divided by the number of cells in a field of view, and the percentage of cells expressing PR per field of view obtained.

Table 7: Percent of cells expressing an ER-α and ^{3}H-TdR double-label per field of view found in the glands and mammary fat pads of Balb/C female mice that were either intact or implanted with immortalized, pre-malignant cells

	Percent (%)	
Intact (control) Gland : Implant Group	**Nulliparous**	**Pregnant**
Intact : NSP2	0.20 : 0.10	0.13 : 0.01
Intact : NSP3	0.04 : 0.05	0.08 : 0.03
Intact : SP3	0 : 0.09	0.06 : 0.06

The number of cells expressing an ER-α and ^{3}H-TdR double-label was counted in three random fields of view (400x magnification). In addition, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification). The total number of cells expressing an ER-α and ^{3}H-TdR double-label were then divided by the total number of cells in a field of view, and the percentage of cells expressing an ER-α and ^{3}H-TdR double-label per field of view obtained.

Table 8: Percent of cells expressing a PR and ^{3}H-TdR double-label per field of view found in the glands and mammary fat pads of Balb/C female mice that were either intact or implanted with immortalized, pre-malignant cells

	Percent (%)	
Intact (control) Gland : Implant Group	**Nulliparous**	**Pregnant**
Intact : NSP2	0.20 : 0.02	0.04 : 0.006
Intact : NSP3	0.0 : 0.05	0.17 : 0.0
Intact : SP3	0.10 : 0.0	0.07 : 0.02

The number of cells expressing a PR and ^{3}H-TdR (double-label) were counted in three random fields of view (400x magnification). In addition, the total number of cells in three random fields of view was determined (>1000 cells total counted; 400x magnification). The total number of cells expressing a PR and ^{3}H-TdR double-label were then divided by the total number of cells in a field of view, and the percentage of cells expressing a PR and ^{3}H-TdR double-label per field of view obtained.

Table 9: Percent of label retaining cells *in vitro*

	Percent of Cells Expressing EdU	
Cell type	**Passage = 0**	**Passage = 5**
NSP2	17.4	14.7
NSP3	23.2	4.0
SP3	21.0	3.8

The number of cells expressing EdU was counted in three random fields of view (100x magnification). In addition, the total number of cells in three random fields of view was determined (>100 cells total counted; 100x magnification). The total number of cells expressing EdU was then divided by the total number of cells in a field of view, and the percentage of cells expressing EdU per field of view obtained.

ADDITIONAL FILES

Additional file 1. Supplemental figure S1: LRECs express ER-alpha and PR and incorporate ^{3}H-TdR into their nuclei. Immunohistochemistry for ER-alpha and PR, as well as labelling for ^{3}H-TdR, was performed on the intact mammary glands (thoracic) of female mice. Epithelial cells present in the glands expressed ER-alpha (A-C, red arrow) as well as PR (D-F, red arrow) in the NSP2 (A, D), NSP3 (B, E), and SP3 (C, F) groups.

^{3}H-TdR was incorporated into the nucleus of some cells expressing the steroid receptors (green arrow, inset). Scale bars equal 20 μm.

Additional file 2. Supplemental figure S2: Label-retaining cells are present in non-mammary tissues. Cells expressing 5-bromo-2-deoxyuridine were found in a) cartilage, b) adipose tissue, c) skeletal muscle, and d) periductal cells. Scale bars equal 20 μm.

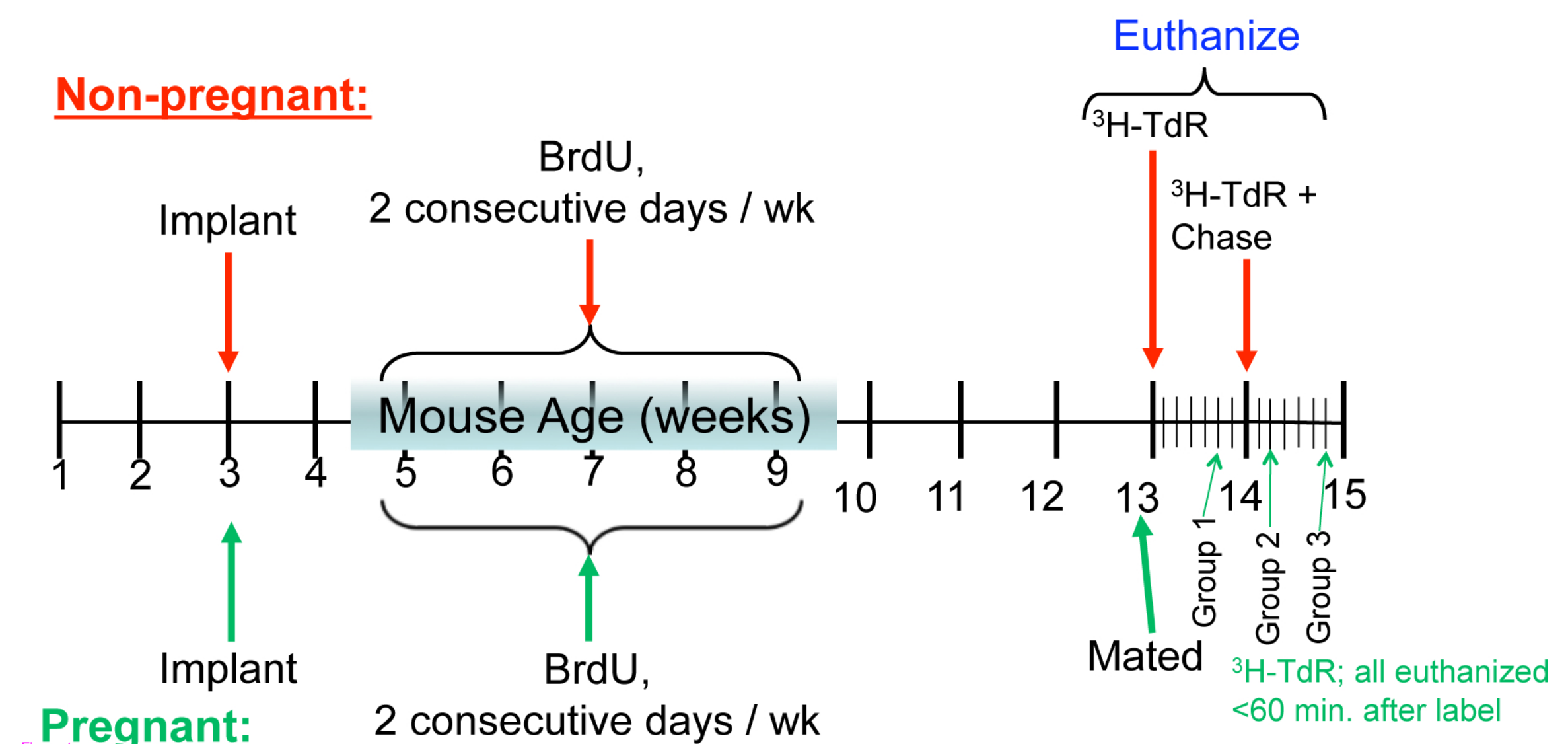
Non-pregnant:
Implant
BrdU,
2 consecutive days / wk
Euthanize
3H-TdR
3H-TdR +
Chase
Mouse Age (weeks)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Group 1
Group 2
Group 3
Mated
3H-TdR; all euthanized
<60 min. after label
Implant
BrdU,
2 consecutive days / wk
Pregnant:

Figure 1

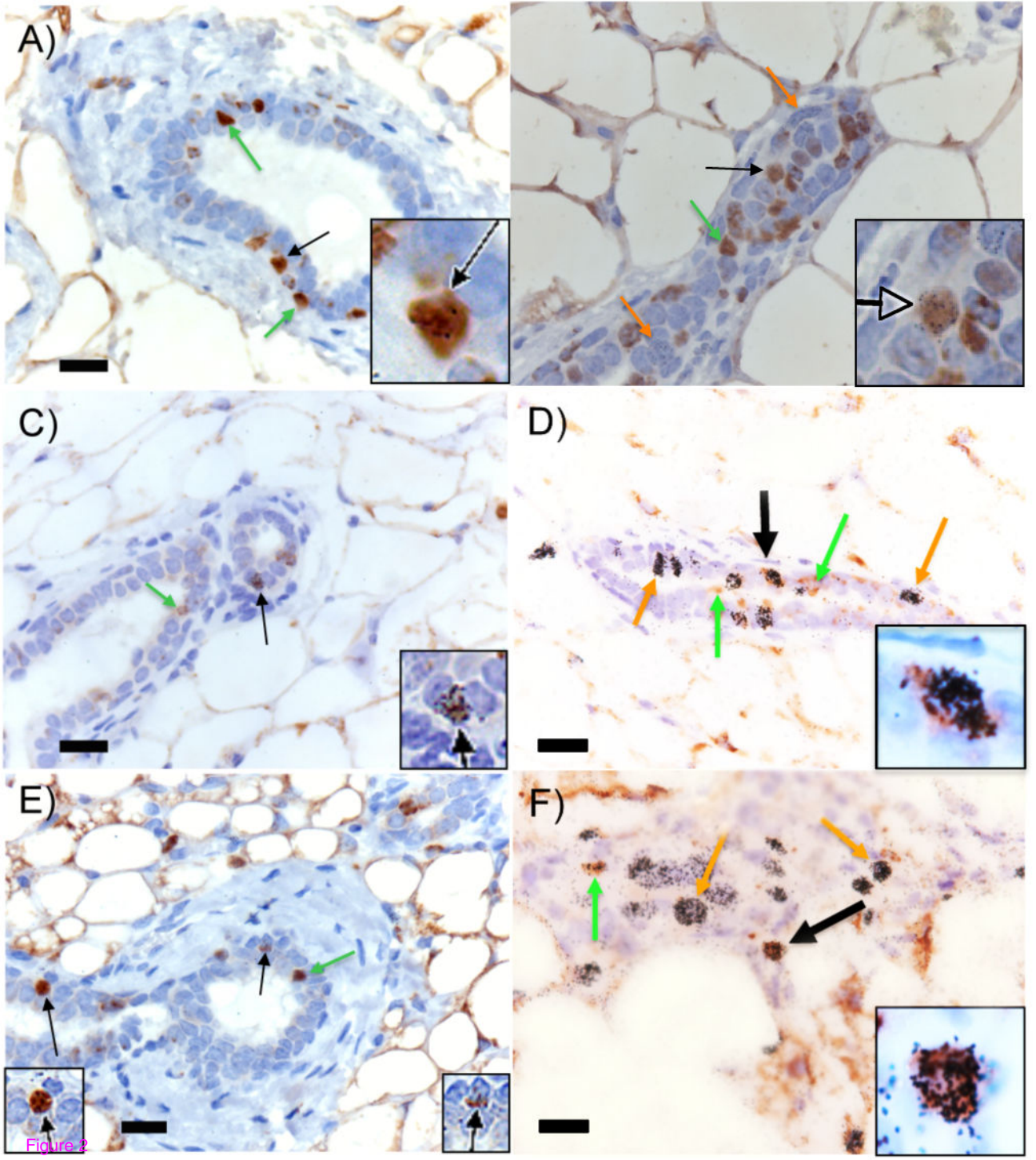

Figure 2

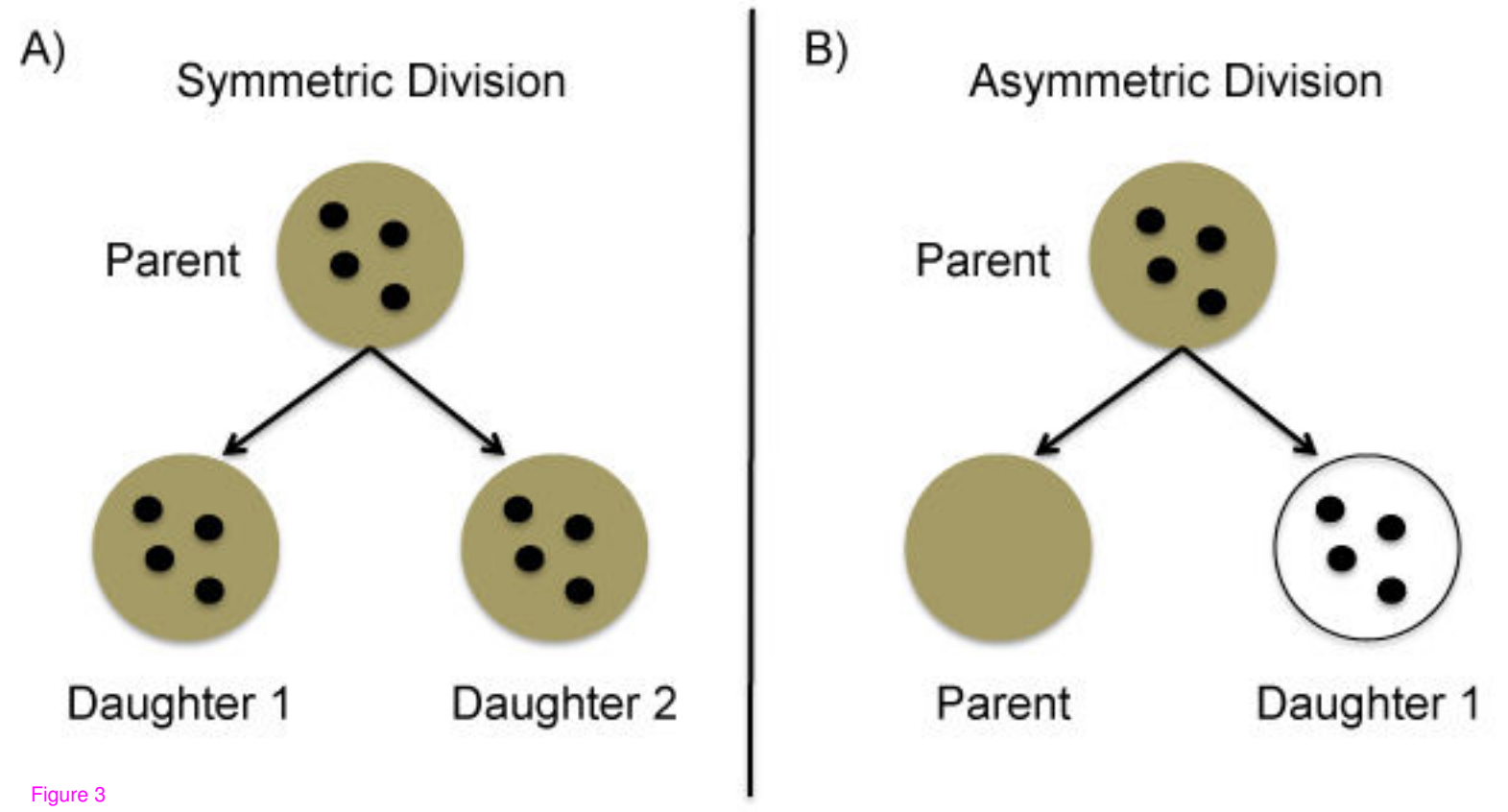
A)
Symmetric Division
Parent
Daughter 1
Daughter 2
B)
Asymmetric Division
Parent
Parent
Daughter 1

Figure 3

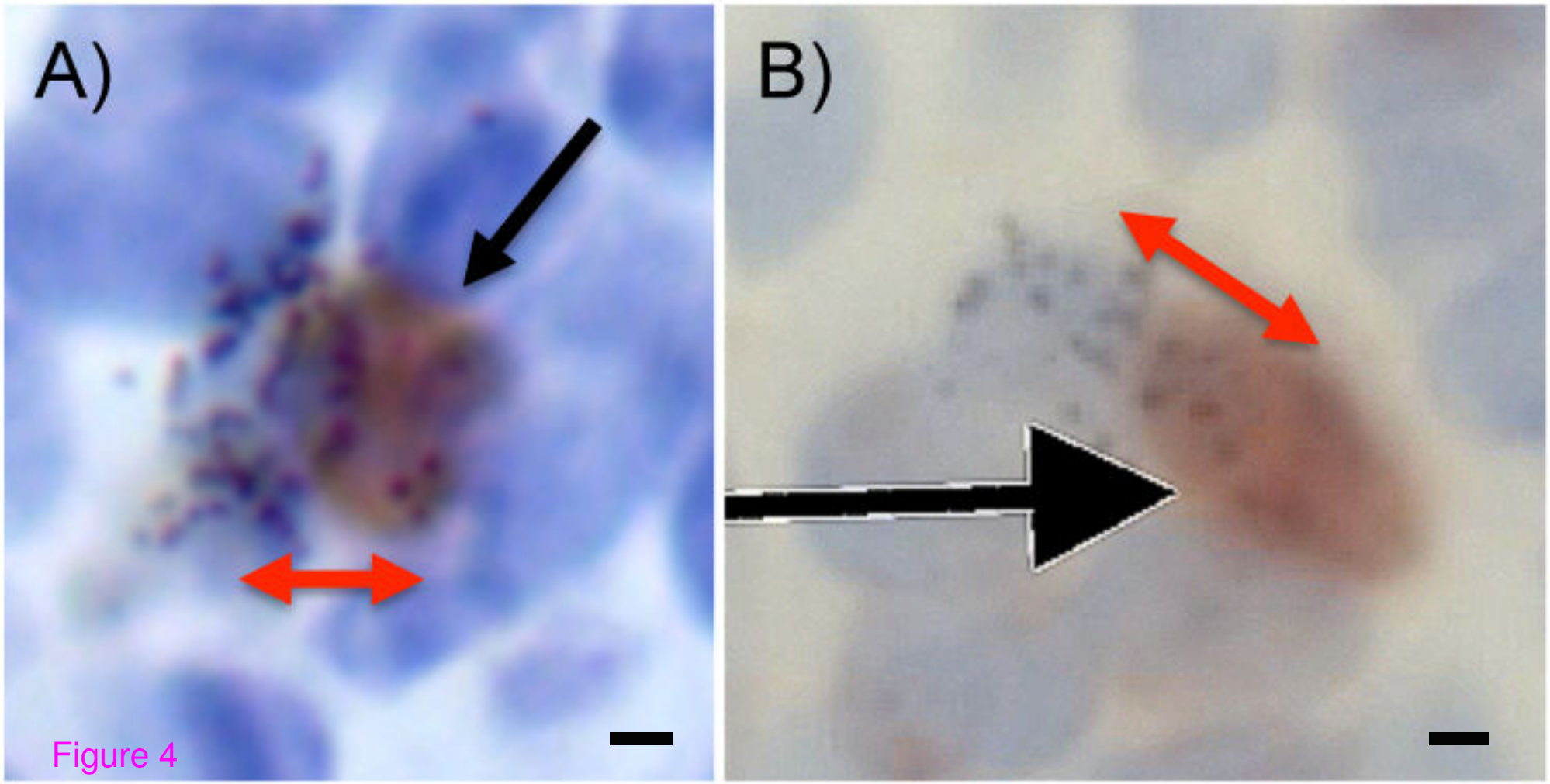

Figure 4

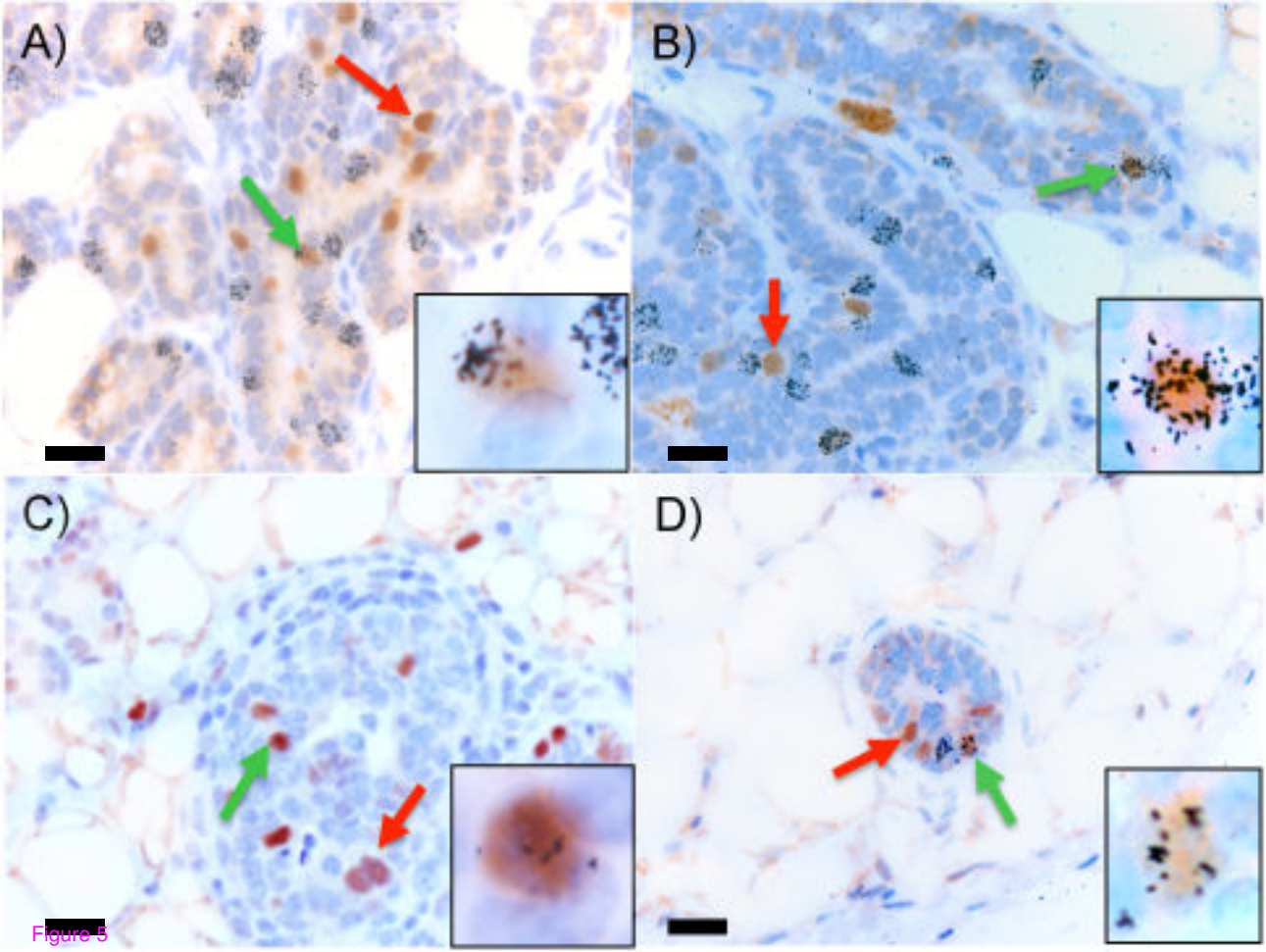

Figure 5

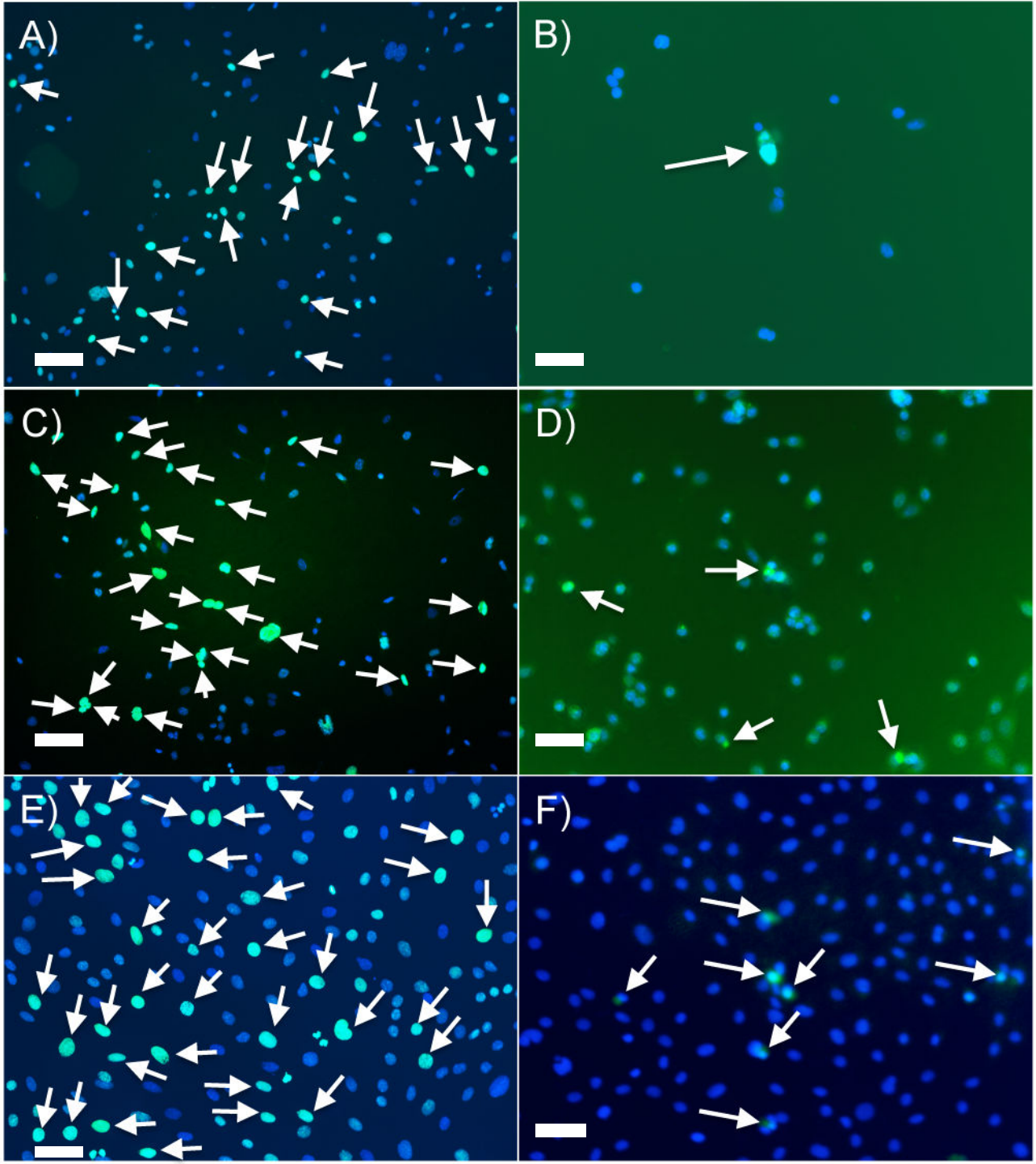

Figure 6

Additional files provided with this submission:

Additional file 1: Bussard et al _Supplemental Figure 1.pdf, 2020K
http://breast-cancer-research.com/imedia/1703046814322343/supp1.pdf
Additional file 2: Bussard et al _Supplemental Figure 2.pdf, 8904K
http://breast-cancer-research.com/imedia/1168761763432234/supp2.pdf