

Identification of Putative Bovine Mammary Epithelial Stem Cells by Their Retention of Labeled DNA Strands

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Stem cells appear to retain labeled DNA for extended periods because of their selective segregation of template DNA strands during mitosis. In this study, proliferating cells in the prepubertal bovine mammary gland were labeled using five daily injections of 5-bromo-2-deoxyuridine (BrdU). Five weeks later, BrdU-labeled mammary epithelial cells were still evident. The percentage of BrdU-labeled epithelial cells was greatest in the lower region of the mammary gland, near the gland cistern, and was decreased toward the periphery of the parenchymal region, where the ducts were invading the mammary fat pad. Increased numbers of BrdU-labeled epithelial cells in basal regions of the gland are likely a consequence of decreased proliferation rates and increased cell cycle arrest in this area. In peripheral regions of mammary parenchyma, the percentage of heavily labeled epithelial cells averaged 0.24%, a number that is consistent with estimates of the frequency of stem cells in the mouse mammary gland. Epithelial label-retaining cells seemingly represent a slowly proliferating population of cells, as 5.4% of heavily labeled cells were positive for the nuclear proliferation antigen Ki67. Because epithelial label-retaining cells contain estrogen receptor (ER)-negative and ER-positive cells, they apparently comprise a mixed population, which I suggest is composed of ER-negative stem cells and ER-positive progenitors. Continuing studies will address the usefulness of this technique to identify bovine mammary stem cells and to facilitate studies of stem cell biology. *Exp Biol Med* 232:1381–1390, 2007

Key words: progenitor cells; estrogen receptor; mammary cell proliferation; label retaining cells; bromodeoxyuridine

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Introduction

Evidence for the existence of mammary stem cells is largely derived from mouse studies. In most species, mammary development during pregnancy and lactation is followed by extensive mammary involution at weaning. The redevelopment of the mammary gland during the next cycle of pregnancy and lactation suggests that a self-renewing population exists within the mammary parenchyma. Transplantation experiments in mice have shown that isolated segments from any portion of the developing or lactating gland are capable of regenerating a complete mammary ductal and alveolar network (1–3). Experiments by Kordon and Smith (4) showed that an entire mammary gland could be regenerated with the progeny of a single cell following transplantation of a tissue fragment into cleared mammary fat pads. Recently, multiparameter cell sorting has been used to isolate murine mammary stem cells, which are capable of regenerating the mammary gland from a single cell (5, 6). In the human breast, the existence of mammary stem cells is further supported by the observations that entire mammary lobules are often comprised of cells showing identical X-inactivation patterns (7) and from cancer studies in which mammary tumors comprised of a variety of cell types are frequently found to be of clonal origin (8–10).

Epithelial stem cells have been shown to retain labeled DNA strands for extended periods of time (11–13). This retention of the label may result from segregation and selective retention of template DNA strands by stem cells undergoing asymmetric division rather than from cell quiescence (13). Selective retention of template DNA strands was subsequently shown to occur during asymmetric division of adult murine intestinal crypt cells (14), muscle satellite cells (15, 16), and mammary epithelial stem cells (17).

Studies of stem cells and progenitor cells in the bovine mammary gland have been limited. Previous studies demonstrated that proliferative cells in prepubertal bovine mammary glands displayed light cytoplasmic staining and were estrogen receptor- α (ER)-negative (18, 19). These studies suggested that the most undifferentiated population of cells likely contains mammary stem cells or primitive

progenitor cells. However, because this population accounts for approximately 10% of mammary epithelial cells prepubertally, it undoubtedly contains more than epithelial stem cells. The objective of this experiment was to identify and to characterize putative bovine mammary stem cells based upon their ability to retain labeled DNA for extended periods. Because they are critically important to mammary development and tissue maintenance, increased knowledge of the biology of bovine mammary stem cells is of practical value to the dairy industry. This knowledge also may be beneficial for human medicine because of similarities between bovine and human mammary tissue (18) with regard to tissue architecture, mammary growth characteristics, the expression of the ER and the progesterone receptor among mammary cells, and the relationship of ER/progesterone receptor status to cell proliferation.

Materials and Methods

Experimental Design. Four Holstein heifers were used in this experiment. At 2 months of age (74 ± 5 days), the heifers were injected intravenously with 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) once daily for 5 consecutive days. BrdU was injected into the jugular vein in a saline solution containing 20 mg BrdU/ml (0.9% sodium chloride; pH 8.2) at a dosage of 5 mg/kg body weight. Thirty-seven days after the last BrdU injection, heifers were killed at the abattoir of the Beltsville Agricultural Research Center. Udders were removed and mammary tissue was collected from three parenchymal regions within the right rear mammary gland (i.e., quarter) of the udder for histologic evaluation. These regions were located along an imaginary line from the teat toward the dorsal surface of the mammary gland and included tissue from the region of the gland cistern; the region approximately midway between the gland cistern and the mammary fat pad; and the parenchyma at the border of the mammary fat pad.

Mammary tissues obtained at slaughter for histologic evaluation were fixed overnight in 10% neutral buffered formalin at 4°C and then were stored in 70% ethanol until further processing. Tissues then were dehydrated and embedded in paraffin according to standard techniques and were sectioned at 5 μ m onto Superfrost Plus slides (Erie Scientific Co., Portsmouth, NH).

Use of animals for these investigations was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Immunohistochemistry. Slides were dewaxed in xylene and were hydrated in a graded series of ethanol to phosphate buffered saline (PBS; pH 7.4). Tissue sections were quenched with 3% hydrogen peroxide in PBS for 10 mins and then were washed in PBS (three washes \times 2 mins). Microwave antigen retrieval then was used. Slides were heated in a microwave at high power (650 W) in 400 ml of 10 mM citrate buffer (pH 6.0) in a covered, glass, staining

dish for 5 mins, remained undisturbed for 5 mins, and then were microwaved for an additional 5 mins. Slides remained in the buffer for a 30-min cooling period. Then they were washed in PBS (3 washes \times 2 mins) and were blocked with 5% nonimmune goat serum in PBS (30 mins) prior to overnight incubation at 4°C with primary antibody.

BrdU Single Labeling. Bright-field microscopic detection of BrdU-labeled cells was performed as described previously (18). Briefly, after the overnight incubation with BrdU-antibody (clone BMC 9318, MAB3424; Chemicon International Inc., Temecula, CA), tissue sections were washed in PBS. Then they were stained using the Histostain SP kit (Zymed Laboratories, San Francisco, CA). Slides were incubated at room temperature with biotinylated secondary antibody, were washed in PBS, and then were incubated with a streptavidin-peroxidase conjugate. After washing in PBS, sections were incubated with diaminobenzidine, were counter-stained with hematoxylin, and were mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

Dual-Label Immunofluorescence. The nuclear proliferation antigen Ki67, the epithelial marker cytokeratin 19 (CK19), and the ER were localized in BrdU-labeled cells using dual-label immunofluorescence. Methods were analogous to those reported previously for dual-label immunofluorescence (18).

BrdU-Ki67 and BrdU-ER Dual Labeling. The primary antibody used for BrdU detection in conjunction with Ki67 or ER labeling was a mouse monoclonal antibody (clone BMC 9318; Chemicon International Inc.) used at a concentration of 2 μ g/ml in PBS that contained 1% normal goat serum (18, 20). The BrdU antibody was used in conjunction with the Ki67 or ER antibody to determine expression of these antigens in BrdU-labeled cells. The Ki67 antibody was rabbit monoclonal SP6 (NeoMarkers, Fremont, CA) and was used at a 1:100 dilution in PBS that contained 1% normal goat serum. The antibody for localization of the ER was rabbit polyclonal antibody sc-7207 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and was used at a 1:400 dilution. Following microwave antigen retrieval, tissue sections were incubated overnight (4°C) with the BrdU antibody and with the Ki67 or ER antibody. Slides then were washed in PBS and were incubated with secondary antibodies for 60 mins at room temperature. Goat anti-mouse secondary antibody conjugated with Alexa 594 and goat anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Inc., Eugene, OR) were used at a 1:200 dilution. Slides then were washed with PBS, were incubated with 2.5 μ g/ml 4',6-diamidino-2-phenylindole for 2 mins, and were rinsed in PBS. Slides were covered with Prolong Gold anti-fade mounting medium (Molecular Probes), were overlain with a glass coverslip, and were viewed by fluorescence microscopy.

BrdU-CK19 Dual Labeling. The primary antibody used for BrdU detection in conjunction with CK-19 labeling was a sheep polyclonal antibody (ab1893; Abcam Inc.,

Cambridge, MA) used at a 1:100 dilution in 1% normal goat serum. Cytokeratin-19 was detected using a mouse monoclonal antibody (NCL-CK19; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) used at a 1:100 dilution in 1% normal goat serum. To facilitate BrdU staining with this antibody, slides were subjected to microwave antigen retrieval followed by incubation in 2 N HCl for 30 mins at room temperature. Slides then were washed with PBS, and sections were incubated with goat anti-mouse secondary antibody conjugated with Alexa 594 and donkey anti-sheep antibody conjugated with Alexa 488 (1:200 dilution; Molecular Probes) for 60 mins at room temperature. Slides then were washed, were counter-stained with 4',6-diamidino-2-phenylindole, and were mounted as previously described.

Quantitation of BrdU Immunohistochemistry. To quantify the number of BrdU-labeled epithelial cells, photographs of tissue sections processed for immunologic detection of labeled cells were captured as digital images and were saved on the computer. For each slide (3–4 tissue sections per slide; one slide per parenchymal region per heifer), 10 areas of the slide were photographed with a Spot digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) on a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY) using the $\times 40$ objective. The numbers of BrdU-labeled epithelial cells and of total epithelial cells were counted manually. At least 3100 cells were scored per slide (average \pm SE: 4936 \pm 291).

Statistical Analysis. Data pertaining to regional effects on the percentage BrdU-labeled cells were analyzed using a one-way ANOVA after arcsine transformation. The Bonferroni multiple comparison test was used for post-ANOVA comparisons (Prism, version 4; GraphPad Software Inc., San Diego, CA).

Results and Discussion

Previous studies have demonstrated that epidermal stem cells of the skin (11, 12) and intestine (13), as well as adult muscle satellite cells (16), labeled *in vivo* with ^3H -thymidine during their conception characteristically retain labeled DNA strands for prolonged periods. Similarly, when mammary epithelial cells of prepubertal mice were labeled *in vivo* with ^3H -thymidine, label-retaining epithelial cells (LRECs) were present 5 weeks later (17, 21). This was ascribed to the ability of stem cells to retain template stands of DNA and to pass the newly synthesized strands or chromatids to their daughter cell during asymmetric division (13, 14), a process frequently referred to as the immortal strand hypothesis. Strongest support for this hypothesis is the recent report by Conboy *et al.* (15), who tracked DNA strands during tissue regeneration of adult muscle by employing three different modified nucleotides (bromo-, chloro-, and iodouridine). Other recent studies are identifying potential mechanisms in which new and old DNA strands are segregated (22–24)

This study adapted the method of Smith *et al.* (17) to

identify putative mammary epithelial stem cells based on their retention of BrdU-labeled DNA. Mammary epithelial cells of calves were labeled *in vivo* with five daily injections of the thymidine analogue BrdU, and tissues were obtained 37 days later to identify and characterize the LRECs. Because we had previously demonstrated a very high proliferation index in calves aged 2–3 months (19, 25), and because my intent was to test the suitability of this methodology for future studies addressing the impact of prepubertal treatments on the mammary stem cell population, I did not additionally stimulate cell proliferation by administering steroids during the postlabeling period.

The LRECs were evident in the mammary epithelium of prepubertal calves 37 days after BrdU labeling, at which time the nuclei of these cells could be characterized as heavily or lightly BrdU-labeled (Figure 1A). The percentage of labeled epithelial cells that fell into these categories was evaluated for each of the three parenchymal regions sampled: cisternal, mid, and outer regions. The mean prevalence of BrdU-labeled cells declined from 15% in the cisternal region to 3.2% in the outer region (Figure 1B). Heavily labeled cells declined from 4.7% in the cisternal region to 0.2% in the outer region. Because cell proliferation is slow in the cisternal region, the basal region of the gland closest to the teat (18), the retention of the label by many of the epithelial cells in this area most likely resulted from the growth arrest of many epithelial cells in this region, rather than by selective retention of labeled DNA by stem cells. This was evident when dual-label immunofluorescence was used to identify cells that were positive for the Ki67 nuclear proliferation antigen, or actively cycling cells (26), and cells that were BrdU-labeled (Figure 2). The greatest number of cells retaining a BrdU label was evident in the cisternal region of the mammary gland, where the frequency of actively proliferating cells was lowest. In outer regions, extensive proliferation of the mammary epithelium during the post-labeling period promoted dilution of the BrdU-label during successive rounds of semiconservative DNA replication and promoted random chromatid distribution to daughter cells in all but the epithelial stem cells. Data are consistent with the putative identification of LRECs as mammary epithelial stem cells in the outer regions of the mammary parenchyma, where the epithelium is most rapidly proliferating. The frequency of heavily labeled LRECs in outer parenchymal regions of the prepubertal bovine mammary gland (0.2%) is consistent with estimates of the frequency of mammary epithelial stem cells in mouse, based upon implantation of dispersed cells at limited dilution into cleared (epithelium-free) mammary fat pads ($< 0.1\%$; Ref. 27) or with estimates based upon isolation of mammary epithelial stem cells by sorting and transplantation (0.14%; Ref. 6). Smith (17) identified LRECs at a frequency of 2% of mouse mammary epithelial cells, which is similar to the total LREC frequency (3.2%, heavily plus lightly labeled cells) in outer regions of the bovine mammary epithelium. The frequency of LRECs in the outer

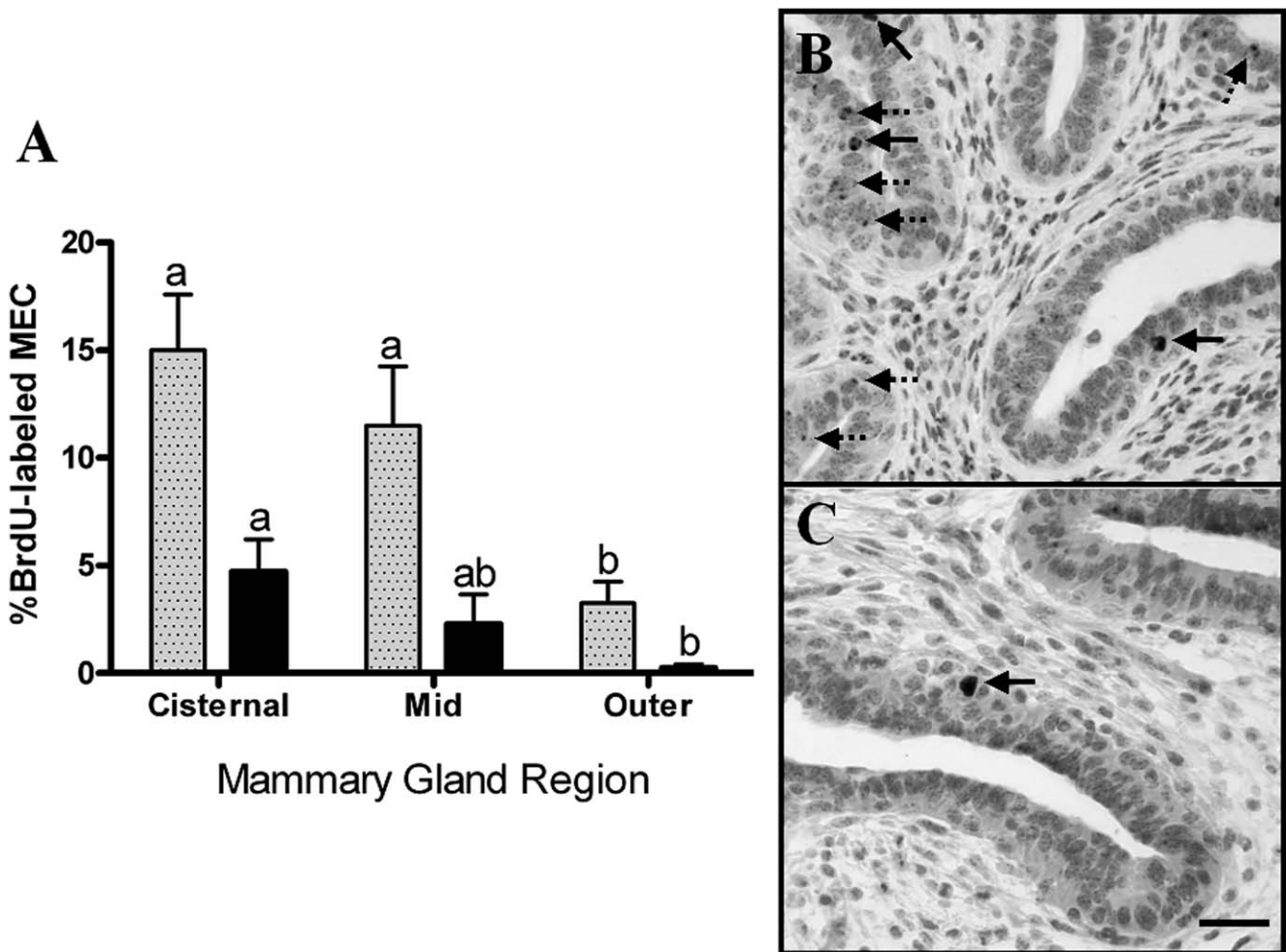


Figure 1. BrdU-labeled mammary epithelial cells (MECs) within regions of the mammary parenchyma. (A) Percent of MECs labeled with BrdU in three parenchymal regions of the gland. Cells are characterized as heavily or lightly labeled, as indicated in B–C. Gray bars indicate the total percentage of labeled cells. Black bars indicate percentages for heavily labeled cells only. Means without a common superscript differ ($P < 0.05$). (B–C) Examples of labeled MECs within the mid-parenchymal region. Solid arrows indicate cells characterized as heavily labeled, and dashed arrows indicate those characterized as lightly labeled. Immunolocalization employed diaminobenzidine staining for light microscopic examination. Scale bar = 50 μ m.

regions of the bovine mammary gland is consistent with previous studies and with the anticipated frequency of mammary stem cells.

Dual labeling for the Ki67 antigen and BrdU was used to evaluate the proliferative capacity of LRECs (Figure 3). Expression of Ki67 in the LRECs of the outer regions of the mammary parenchyma was evaluated, and it suggested that this population of LRECs was proliferative. Of the 162 LRECs that were evaluated (approximately 80,000 epithelial cells), 4% expressed the Ki67 antigen and therefore were clearly actively proliferating, whereas the remaining cells were slowly cycling or were quiescent. Others have shown that epithelial LRECs in the skin (28) and in the mammary gland (17) are proliferative. Dunnwald *et al.* (28) demonstrated that LRECs in the skin are distributed through the cell cycle, with 96% found in G_0/G_1 and 4% found in $S/G_2/M$. Surprisingly, Smith (17) demonstrated that more than 80% of LRECs in the peripubertal mouse mammary gland

incorporated BrdU during the S phase of DNA synthesis when BrdU was administered as two injections at 24 hrs apart, which suggested that the LRECs constitute a major proliferative component of the epithelium. Data in this study appear consistent with the above results, as the Ki67 labeling possibly failed to identify all cycling cells that are not in the S phase. However, these data suggest a LREC-proliferation rate lower than that reported for mouse data (17). To evaluate the ability of Ki67 to identify proliferating cells in bovine mammary gland, BrdU was administered to prepubertal calves as a pulse label. Mammary tissue was obtained when animals were killed, 1–2 hrs after BrdU injection, and histologic sections were subjected to dual-label immunofluorescence staining (data not shown). The Ki67 antigen was evident in cells that were in the S phase (BrdU-labeled) plus an approximately equal number of cells that were in other phases of the cell cycle (not BrdU-labeled). Because proportionately more time is spent in the

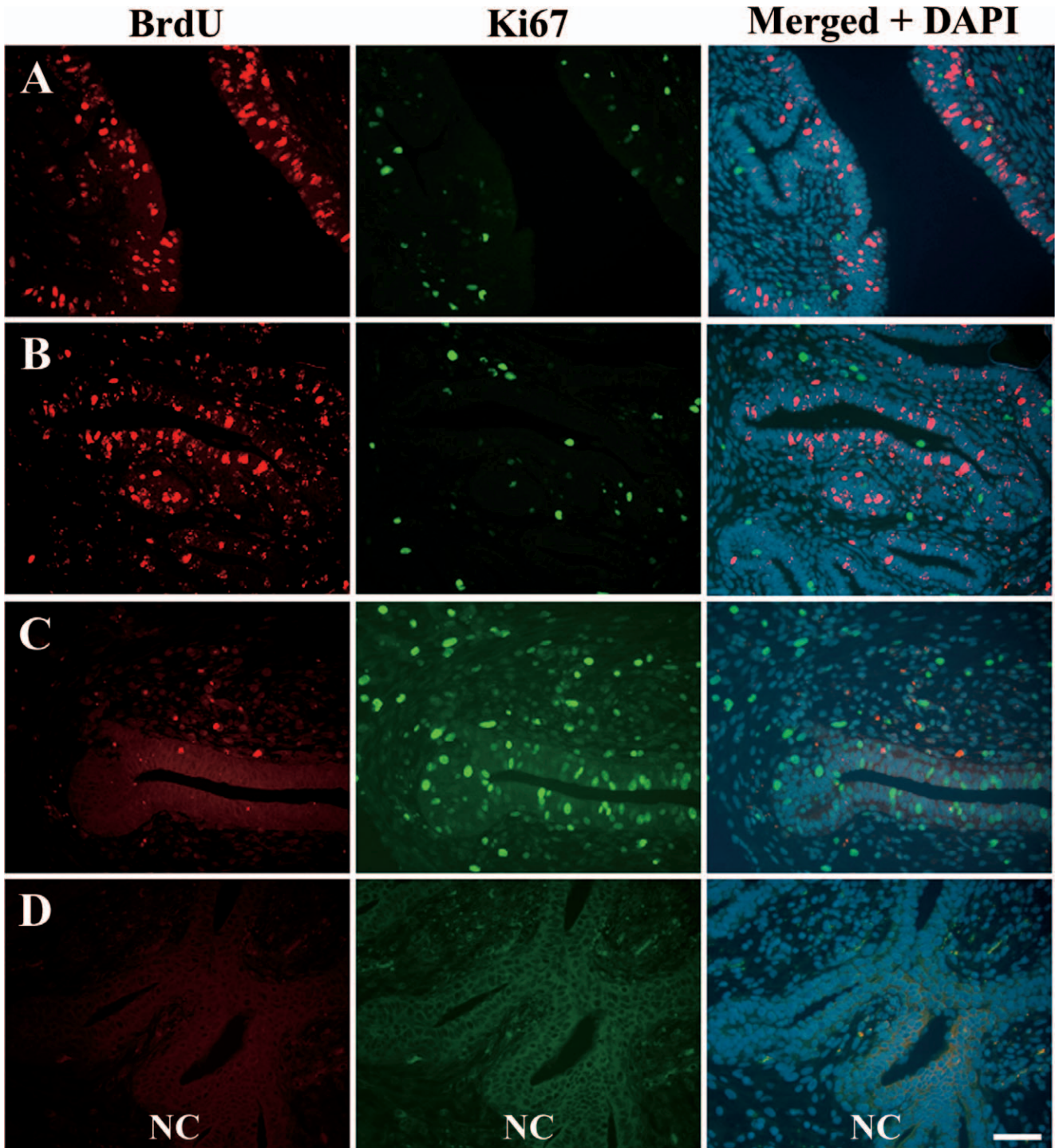


Figure 2. Localization of BrdU-labeled and Ki67-labeled epithelial cells in cisternal- and outer-parenchymal regions of a mammary gland from a single animal. Many BrdU-labeled cells and few Ki67-labeled cells were evident in cisternal regions of the gland, whereas few BrdU-labeled cells and many Ki67-labeled cells were evident in outer regions. (A–B) BrdU and Ki67 staining in two areas from the cisternal region. (C) BrdU and Ki67 staining in the outer-parenchymal region. (D) Nonspecific fluorescence in an outer region of the mammary parenchyma, obtained by substituting control sera for primary antibodies. Scale bar = 50 μ m.

$G_1/M/G_2$ phases than in the S phase, considerably more cells should be Ki67-positive/BrdU-negative than Ki67-positive/BrdU-positive. Thus, Ki67 labeling likely underestimated the true fraction of cells that were actively cycling, which further supports the contention that LRECs actively cycle.

During the period from birth to puberty, the mammary parenchyma undergoes rapid, allometric, hyperplastic growth (18, 29) that is characterized by the proliferation and extension of mammary ducts into the surrounding mammary fat pad. In the absence of estrogen (30–32) or a

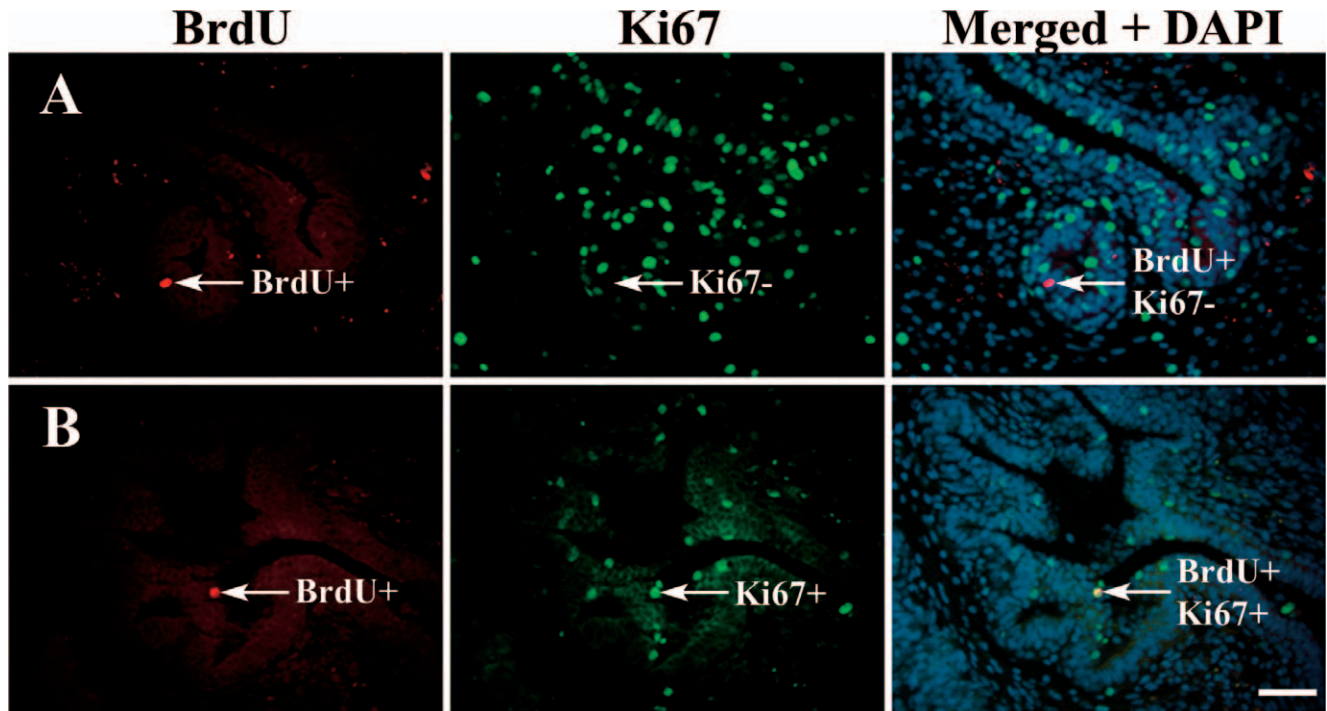


Figure 3. Quantitation of BrdU and Ki67 colocalization. (A) A single BrdU-labeled epithelial cell is evident and is Ki67-negative. (B) A single BrdU-labeled epithelial cell is evident and is Ki67-positive. Scale bar = 50 μ m.

mammary ER (33–35), this early ductal growth does not occur. However, only a portion of mammary cells possess ERs, and the proliferating epithelial cells appear ER-negative in bovine (18), murine (36), and human (37) mammary tissues. Proliferation of mammary epithelial cells seemingly is mediated by paracrine factors derived from cells of the epithelial or stromal compartments of the mammary gland (18, 33, 35, 38), and microarray studies support the importance of paracrine factors and cell-cell communication in driving prepubertal growth of the bovine mammary epithelium (39). Prepubertally, the bovine mammary gland is exquisitely sensitive to mitogenic effects of estrogen, as expression of the ER is greater at this time than during any other phase of mammary development (40), and proliferation in the mammary gland is driven by the very low (1–2 pg/ml) quantities of estrogen circulating prepubertally.

The hormone sensitivity of mammary stem cells is an issue of intense interest. Recent reports indicate that mammary LRECs are likely estrogen targets. Others have shown that mouse (36, 41) and human (42, 43) LRECs express the ER, and we report that ER-positive cells are clearly evident within the bovine LREC population (Figure 4). Quantification of 234 LRECs showed that 57% of LRECs were ER-positive and likely were estrogen targets. Booth and Smith (41) determined that 27% of LRECs in the mouse mammary gland were ER-positive and that the percentage of ER-positive LRECs increased after hormone stimulation. Using ^3H -thymidine labeling of human breast tissue implanted in athymic mice, Clarke *et al.* (43) showed that

LRECs are enriched for putative stem cell markers (p21 and Msi-1) and ER. These reports and the current data suggest that at least a portion of LRECs are estrogen responsive at a given time. Because they comprise a very small percentage of bovine mammary epithelial cells, an association of the proliferation and the ER status of LRECs are not at odds with our previous demonstration that 85%–99% of proliferating mammary epithelial cells are ER-negative (18).

However, recent studies convincingly demonstrate that mammary stem cells are ER-negative. Studies utilizing surface antigens to sort prospective murine mammary stem cells indicate that mammary stem cells are ER-negative (44). Mammary-cell suspensions were depleted of endothelial (CD31⁺) and hematopoietic (CD45⁺, TER119⁺) cells. Remaining cells that expressed high levels of the heat-stable antigen (CD24) and of β_1 - or α_6 -integrin (CD29 or CD49f, respectively) exhibited a high capacity to repopulate cleared mammary fat pads, using epithelial elements capable of morphogenesis and lactogenesis under appropriate physiologic stimuli (5, 6); these cells appear to be ER-negative (44). Similarly, Sleeman *et al.* (45, 46) characterized three fractions of mammary cells based upon expression of CD24. The ability to repopulate an epithelium-cleared fat pad was restricted to CD24 low-expressors, which were ER-negative. *In vitro* clonogenic activity was an attribute of the CD24 strong-expressors, which were ER-positive. Consistent with these results, ER-negative bovine LRECs may represent mammary stem cells.

Previous studies demonstrated the existence of morphologically distinct cell types that are suggestive of stem

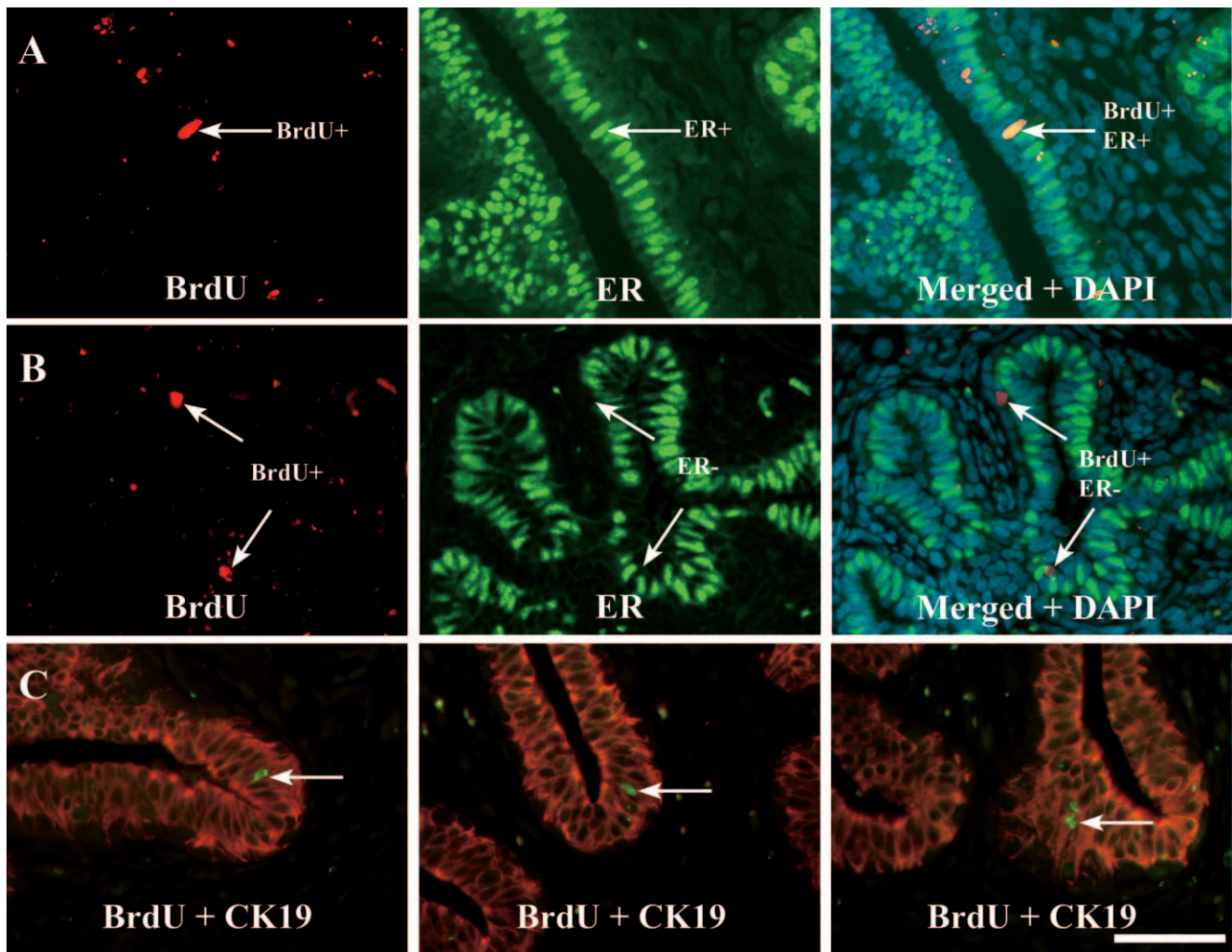


Figure 4. Localization of BrdU-, ER-, and CK19-labeled epithelial cells in the outer region of the mammary parenchyma. (A) A single BrdU-labeled epithelial cell is evident and is ER-positive. (B) Two BrdU-labeled epithelial cells are evident and are ER-negative. (C) Panels in this row depict LRECs that express CK19 in their cytoplasm, which confirms that they are epithelial cells. Scale bar = 50 μ m.

cells and of amplifying progenitor cell populations for mouse (9) and bovine (19) mammary epithelium. Although stem cells exhibit label retention because of asymmetric segregation of parental DNA strands, and although we have used this attribute to help identify mammary stem cells, this is not a distinguishing characteristic. Progenitor cells may also exhibit this attribute (47), and the mammary gland contains multiple classes of progenitor cells. Even progenitor cells that are responsible for alveolar formation, which occurs during pregnancy, are present in the mammary glands of peripubertal mice (48). The presence of two, nearly equal populations of bovine LRECs, each with a different ER status, may be related to the location within the epithelial layer and the position in epithelial lineage. We suggest that ER-negative LRECs are mammary stem cells, whereas ER-positive LRECs are progenitors. Cells in the basal portion of the epithelial layer are typically ER-negative, whereas a portion of the cells in the luminal layers are ER-positive (18). The ER-negative LRECs primarily are

basal epithelial cells (Figure 4B), which Asselin-Labat *et al.* (44) characterized as positive for β_1 - or α_6 -integrin and which, when also expressing high levels of CD24, represent the population with stem-cell-regenerative properties (44). A significant difference between mouse and bovine mammary growth pertains to the timing and nature of allometric mammary growth. In cattle, the period of allometric mammary growth begins shortly after birth and continues until the onset of puberty. Holstein heifers typically begin ovarian cycles at 7–10 months of age (at approximately 300 kg body weight). The allometric period in mice begins peripubertally at approximately 4 weeks of age and declines at maturity (6–8 weeks of age; Ref. 49). Investigations of LRECs in the mouse mammary gland have utilized mice that are pubertal, whereas the current study used calves that are approximately one-third of the way to the onset of puberty. At this time, the bovine mammary gland does not contain differentiated myoepithelial cells, as assessed by the cell ultrastructure and by the absence of

smooth muscle actin (18). However, the basal cells likely include myoepithelial precursors.

It is clear that mammary ductal growth is ER-dependent but that the proliferating cells are ER-negative. The basal location of ER-negative LRECs suggests that they are stem cells, whereas the more luminal position of ER-positive LRECs, above the ER-negative LRECs, suggests that they may be a category of progenitor cells. Further research is necessary to evaluate the validity of this conclusion and to demonstrate whether bovine mammary stem cells are ER-negative or ER-positive. Because epigenetic changes in the stem cell population appear to occur during development (50), it is also possible that the ER-negative and ER-positive LRECs represent alternative phenotypes of mammary stem cells. Final clarification of the relationship between LRECs, stem cell types, and ER status will necessitate identification of additional markers for epithelial-cell lineage in the bovine. Tools for further elucidation of the epithelial hierarchy in the mouse mammary gland have been developed. However, *in vivo* characterizations of the capacity of isolated cells to recapitulate structures of the human breast and the bovine mammary gland likely will require repopulation of cleared mouse mammary fat pad that is humanized or bovinized with stromal cells from the species of interest (51).

Because LRECs represent a small portion of cells in the epithelial layer, it is important to determine whether these cells are epithelial or are a minor transitory population. Colocalization of cytokeratin-19 in the cytoplasm of cells that were BrdU-labeled (Figure 4C) confirmed that LRECs are of epithelial lineage. A number of investigations have indicated that adult stem cells exhibit a significant degree of plasticity, for in experimental settings these cells are capable of populating an organ or tissue other than the site of origin, and they can seemingly differentiate into cells of developmentally unrelated germ layers (52). Indeed, Boulanger *et al.* (53) recently demonstrated that cells transplanted from adult seminiferous tubules of mice into the developing murine mammary gland contributed to generation of the mammary epithelium. This implies that the tissue micro-environment determines the fate of somatic stem cells. The likelihood that circulating cells can provide a source of tissue regenerative capacity under normal physiologic conditions was recently evaluated (54) using the freemartin calf as an experimental model. A freemartin calf is a female that is born twin to a male calf. Because of placental anastomoses during early gestation, the calves share blood types. The donor cells in the freemartin calf were identified by presence of the Y chromosome, and cell lineage was identified by appropriate cell surface markers. The authors demonstrated that circulating cells contributed less than 1% of cells in nonhematopoietic lineages, with no evidence of clonal seeding from the circulating cells. This provides strong support for the concept that circulating cells exert a minor influence in the development, growth, and turnover of nonhematopoietic tissues in normal conditions. However,

the authors showed that, in response to tissue damage, regenerating granulation tissue (connective tissue) contains a significant contribution of hematopoietic-derived cells. These data are consistent with the finding in this study that bovine LRECs were of mammary epithelial lineage.

Data from the current study demonstrate the feasibility of identifying LRECs in the bovine mammary gland and are supportive of the concept that these LRECs represent putative mammary epithelial stem cells. As such, they are likely targets of manipulation to manage cell growth, cell turnover, and tissue regeneration, thereby improving dairy cow productivity and udder health. The bovine mammary gland also provides an alternative model for biomedical research aimed at evaluating the role of mammary stem cells in mammary gland function and carcinogenesis.

In conclusion, mammary stem cells provide for mammary growth and development and for cell turnover and regeneration of the mammary gland for the life of the organism. This study adapted a procedure to demonstrate the presence of LRECs in the prepubertal bovine mammary gland, and it demonstrated that these cells are cycling and are epithelial in lineage. Because the population contains both ER-positive and ER-negative cells, a mixed population, of which the ER-negative cells are putative mammary stem cells, is likely. The methodology provides a mechanism to further characterize these putative stem cells and to evaluate factors and physiologic events that might alter stem cell numbers. The ability to differentially regulate symmetric and asymmetric division of mammary stem cells would provide an opportunity to influence dairy cattle productivity.

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