In Vivo Expansion of the Mammary Stem/Progenitor Cell Population by Xanthosine Infusion

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Mammary stem cells provide for growth and maintenance of the mammary gland and are therefore of considerable interest as determinants of productivity and efficiency of dairy animals and as targets of carcinogenesis in humans. Xanthosine treatment was previously shown to promote expansion of hepatic stem cells *in vitro*. The objective of this study was to determine if *in vivo* treatment with xanthosine can increase the mammary stem cell population. Xanthosine was infused into the right mammary glands of four female Holstein calves for 5 consecutive days. Immediately after each xanthosine treatment, calves were injected intravenously with 5-bromo-2-deoxyuridine (BrdU). Forty days after the final treatment, calves were euthanized and mammary tissue harvested. BrdU-label retaining epithelial cells (LREC) were detected immunohistochemically and quantified. Retention of BrdU was used as a marker for putative bovine mammary stem cells. Infusion of xanthosine into the bovine mammary gland significantly increased the number of LREC in treated glands compared to contralateral control glands ($P < 0.05$). LREC averaged 0.4% of epithelial cells in control glands and 0.8% in xanthosine-treated glands. The increase in LREC in xanthosine-treated glands was supported by a concomitant increase in telomerase activity ($P < 0.01$) and a correlation between LREC and telomerase ($P < 0.05$; $r^2 = 0.7$). Data indicate that *in vivo* treatment with xanthosine can be used to increase the number of mammary stem cells. This is the first demonstration of an *in vivo* treatment to increase the endogenous population of mammary stem cells, with utility for biomedical research and dairy management. Exp Biol Med 234:475–482, 2009

Key words: mammary development; stem cells; progenitor cells; immortal DNA strands

Introduction

Mammary cell proliferation, turnover and tissue regeneration are functions of mammary stem cells. Adult, or somatic, stem cells are capable of self renewal and of differentiating to generate all cell types of the tissue or organ in which they reside. Analogous to other tissues (1–3), it is generally accepted that mammary stem cells are responsible for growth and differentiation of the developing mammary gland and for maintenance of cell populations within the adult mammary gland. The most compelling evidence for the existence of mammary somatic stem cells has been the ability to regenerate an entire mammary gland of a mouse from a single cell implanted into a murine fat pad that was previously cleared of epithelium (4, 5). Mammary stem cells are histologically undifferentiated epithelial cells that can divide symmetrically to produce two identical stem cells, or asymmetrically to produce a stem cell and a progenitor cell of committed cell lineage (ductal, lobular-alveolar, or myoepithelial) (5–7). In contrast to stem cells, mammary progenitor cells have a finite proliferation capacity and restricted differentiation capacity (5, 6). Stem cells appear to be sensitive to mitogenic stimuli and to be present throughout the mammary epithelium, to facilitate the cyclic proliferation, regression and cell turnover that occurs during each lactation cycle. However, this task may be partially fulfilled by a population of quiescent, primitive progenitor cells that are held in reserve for such a purpose (8).

Studies of stem cells and progenitor cells in bovine mammary gland have been limited (9, 10). This is despite the inherent economic importance of the species and the

This work was funded by the United States Department of Agriculture, Agricultural Research Service, CRIS 1265-31000-083-00D.

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Received November 6, 2008.
Accepted January 20, 2009.

DOI: 10.3181/0811-RM-320
1535-3702/09/2344-0475$15.00

similarity of the cytoarchitecture of the prepubertal bovine mammary gland to that of the human breast (11). To identify putative mammary stem cells, we have taken advantage of the capacity of stem cells to retain labeled DNA strands for extended periods of time (10). This retention of label is attributed to segregation and selective retention of template DNA strands by stem cells undergoing asymmetric division, rather than being due to cell quiescence after incorporation of label (10, 12–15). Label-retaining epithelial cells (LREC) were present in quantities (~0.2% of mammary epithelial cells; (10)) that are consistent with the prevalence of stem cells in mouse mammary gland (4, 5, 16).

A number of genetic pathways have been implicated as regulators of mammary stem cell proliferation and differentiation. These have included NOTCH, Hedgehog, Wnt, BRCA1, p21, p63 and Pten (17–20). However, recent *in vitro* experiments indicate that p53 promotes asymmetric proliferation of somatic stem cells through down-regulation of inosine-5′-monophosphate dehydrogenase (IMPDH) (21, 22), the rate limiting enzyme for guanine nucleotide synthesis. This decreases the concentration of guanine ribonucleotides (rGNPs) and promotes asymmetric division of somatic stem cells. Because this function of p53 can be short-circuited by addition of xanthosine, which is readily converted to XMP (xanthosine monophosphate, the product of the IMPDH reaction) by ubiquitous nucleoside kinases, *in vitro* treatment of hepatocytes with xanthosine suppressed asymmetric division, enhanced symmetric division, and caused expansion of the stem cell population (23).

The objective of the current study was to test the ability of xanthosine to expand the mammary stem/progenitor population *in vivo*. Parameters used to assess the impact of treatment were the prevalence of label retaining cells (LREC) and telomerase activity. The mammary gland provides an excellent site to test this action of xanthosine because the nucleoside can be infused into the mammary ductal system or readily injected into the mammary parenchyma. If successful, such treatment would provide an additional tool for biomedical research and for enhancing efficiency of animal production.

Materials and Methods

Experimental Design. Four female Holstein calves were used in this experiment. At three months of age (95 ± 8 d; mean ± SE) the right glands were infused through the teat canal with 1.5 or 2 ml of 10 mM xanthosine in sterile saline for 5 consecutive days. Volume depended upon gland capacity. The right rear gland within each udder received an additional injection of 1 ml of 10 mM xanthosine into mammary parenchyma, to ensure delivery to the peripheral regions of the gland despite the lack of patent canals in distal regions of the ducts (11). Intraductal infusion was performed using a sterile 20 gauge Luer stub adapter after cleaning the teat end with 70% ethanol. Parenchyma was palpated and outer region injected using a 22 gauge, 1 inch hypodermic needle. After each xanthosine treatment, calves were injected intravenously with 5-bromo-2′-deoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO). BrdU was injected into the jugular vein in a sterile saline solution containing 20 mg BrdU/ml (0.9% NaCl, pH 8.2) at a dosage of 5 mg/kg body weight. Forty days after the last BrdU injection, heifer calves were euthanized at the abattoir of the Beltsville Agricultural Research Center. Mammary tissue was collected from a parenchymal region within each mammary gland that was in close proximity to the border with the mammary fat pad.

Mammary tissues that were harvested for histological evaluation were fixed overnight in 10% neutral buffered formalin at 4°C and then stored in 70% ethanol until further processing. Tissues were then dehydrated and embedded in paraffin according to standard techniques and sectioned at 5-μm onto Superfrost™ plus slides (Erie Scientific Co., Portsmouth, NH). Tissues for evaluation of telomerase activity were snap frozen in liquid nitrogen and stored at –80°C.

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 hydrated in a graded series of ethanol to phosphate buffered saline (PBS, pH 7.4). Tissue sections were quenched with 3% H_2O_2 in PBS for 10 min and then washed in PBS (3 × 2 min). Microwave antigen retrieval was then 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 min, remained undisturbed for 5 min, and then were microwaved for an additional 5 min. Slides remained in the buffer for a 30-min cooling period. They were then washed in PBS (3 × 2 min) and blocked with 5% non-immune goat serum in PBS (30 min) 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 (11, 24). Briefly, after the overnight incubation with BrdU-antibody (clone BMC 9318, MAB3424, Chemicon International, Inc., Temecula, CA) used at 2 μg/ml in PBS containing 1% normal goat serum, tissue sections were washed in PBS. They were then stained using the Histostain SP kit (Zymed Laboratories, San Francisco, CA). Slides were incubated at room temperature with biotinylated secondary antibody, washed in PBS and then incubated with a steptavidin-peroxidase conjugate. After washing in PBS, sections were incubated with diaminobenzidine, counterstained with hematoxylin and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

BrdU-Estrogen Receptor-Alpha Dual Labeling. Dual localization of estrogen receptor alpha (ER) and BrdU was performed as described previously (10). The primary antibody and concentration used for BrdU detection in

conjunction with ER labeling was the same mouse monoclonal antibody described above (clone BMC 9318) used at a concentration of 2 μg/ml in PBS containing 1% normal goat serum (11, 24). The BrdU antibody was used in conjunction with ER antibody to determine expression of ER in BrdU-labeled cells. The antibody for localization of ER was rabbit polyclonal antibody sc-7207 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) used at 1:400 dilution. Following microwave antigen retrieval, tissue sections were incubated overnight (4°C) with BrdU antibody and ER antibody. Slides were then washed in PBS and incubated with secondary antibodies for 60 min 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 1:200 dilution. Slides were then washed with PBS, incubated with 2.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) for 2 min and rinsed in PBS. Slides were covered with Prolong Gold anti-fade mounting medium (Molecular Probes), overlain with a glass coverslip and viewed by epifluorescence microscopy (Zeiss Axioskop II, Carl Zeiss Inc., Thornwood, NY).

BrdU-Proliferating Cell Nuclear Antigen (PCNA) Dual Labeling. Dual localization of BrdU and the proliferation marker, proliferating cell nuclear antigen (PCNA), was performed in a manner analogous to that described for BrdU-ER dual labeling. The antibody for localization of PCNA was rabbit polyclonal antibody ab18197 (Abcam Inc., Cambridge, MA) used at 1:300 dilution, in conjunction with the BrdU antibody described above. Antigen retrieval, antibody labeling and detection methods were as described previously.

Quantitation of BrdU Immunohistochemistry. To quantify the number of BrdU-labeled epithelial cells, photographs of tissue sections processed for immunological detection of labeled cells were captured as digital images and saved on the computer. For each slide (3–4 tissue sections per slide), one slide per parenchymal region per heifer, 12 areas of the slide were photographed with a Spot® digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) on a Zeiss Axioskop II microscope with the ×40 objective. The number of BrdU-labeled epithelial cells and total epithelial cells were counted manually. At least 3400 cells were scored per slide (average ± SE = 5016 ± 293).

Tissue sections from the rear mammary glands of all animals were co-labeled for BrdU and ER. These were viewed for the presence of LREC and regions containing LREC were photographed. The ER status and location within the epithelial layer was subsequently determined. A total of 173 LREC were scored (64 from control glands and 109 from Xs-treated glands). Because LREC are present in low abundance, this involved scoring histological sections containing in excess of 22,000 epithelial cells.

Similarly, tissue sections from all animals were co-labeled for BrdU and PCNA. Selected fields containing LREC were photographed and PCNA status of the LREC was determined. A total of 283 LREC, 102 from control glands and 181 from Xs-treated glands, were evaluated.

Telomerase Assay. Mammary tissues previously frozen at –80°C were used to determine telomerase activity with a quantitative real-time PCR-based Telomerase Assay Kit (US Biomax, Rockville, MD) according to the manufacturer's instructions. Briefly, 40 to 100 mg of tissue were homogenized for 20 sec in the provided lysis buffer. Samples were incubated on ice for 30 min and then centrifuged for 30 min at 12,000 × *g* at 4°C. Supernatant was removed and aliquoted. One aliquot was later used for protein determination by the BCA procedure (Pierce Chemical Co., Rockford, IL). Another aliquot was diluted 1:200 with lysis buffer kept at 4°C prior to assay. Additionally, each diluted sample had an aliquot removed for heat inactivation (85°C for 10 min) which was used as a sample negative control. One μl of each of the diluted samples ± heat inactivation was run in the assay along with a serial dilution of the provided standard in lysis buffer. Standard curves typically produced correlation coefficients of 0.998 ± 0.0017 with PCR efficiencies of 94.4 ± 4.3%.

Statistical Analysis. LREC data for front and rear mammary glands were analyzed by two-way analysis of variance and telomerase activity in rear quarters was analyzed by Student's *t* test (Prism, version 4; GraphPad Software, Inc., San Diego, CA).

Results and Discussion

In vivo treatment of mammary glands of female Holstein calves with Xs effectively increased ($P < 0.05$) the percentage of mammary epithelial cells that retained BrdU label 40 days later, when treated glands were compared to the within-udder control mammary glands (Fig. 1). Such retention of BrdU labeling by a small number of mammary epithelial cells has been attributed to a feature of asymmetric stem cell division (10, 13, 25, 26), wherein the older parental DNA strand is differentially retained by the daughter stem cell of a mammary stem cell (MaSC) undergoing asymmetric division. This differential strand segregation has been reported in a number of adult tissues (12, 27, 28). Smith demonstrated that LREC constitute a major proliferative component in murine mammary gland, because 80% of the hormone-stimulated LREC incorporated BrdU when administered as two injections 24 hrs apart. To evaluate the proliferative capacity of LREC in bovine mammary gland, we previously examined expression of the Ki67 proliferation antigen and determined that 4% of the LREC expressed Ki67 and were clearly proliferative in absence of exogenous hormones (10). This was deemed an underestimate of proliferative capacity and implied that LREC were proliferative, but not rapidly cycling in the absence of external stimuli (*e.g.*, estrogen stimulation). In the current study (data not shown), we evaluated nuclear expression of PCNA, a cofactor of DNA polymerase delta,

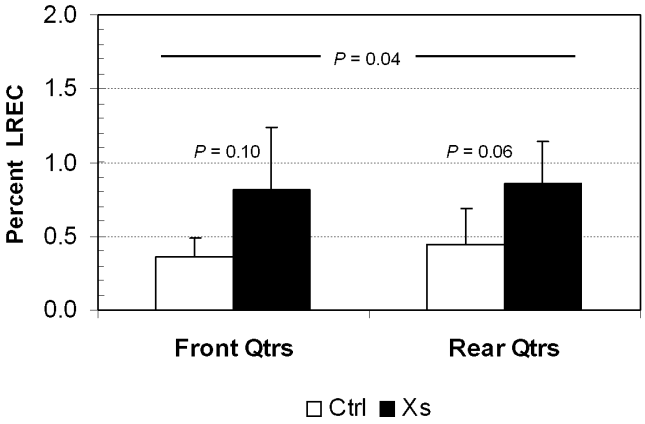

Figure 1. Effect of xanthosine (Xs) treatment on size of the population of label retaining epithelial cells (LREC) in bovine mammary gland. The two mammary glands (quarters, qtrs) on one side of the udder of four Holstein calves were treated with Xs and opposite glands served as controls. The prevalence of LREC is expressed as a percent of total mammary epithelial cells. *P* values for front and rear quarters are reported based upon Student's *t* test and overall significance by two-way analysis of variance.

which has been used as a marker for cells in S phase. Of the 283 LREC evaluated, 14% were clearly PCNA-positive (12% of LREC, $n = 102$, from control glands; 15% of LREC, $n = 181$, from Xs-treated glands) and therefore potentially in S-phase. Because DNA polymerase delta and PCNA play a role in DNA repair, PCNA expression is not conclusive evidence of DNA synthesis linked to cell proliferation. However, our previous Ki67 (10) and current PCNA labeling data indicate that bovine mammary LREC are proliferative. Moreover, LREC retained BrdU label despite being located in epithelial regions that were highly proliferative. We therefore suggest that selective DNA strand segregation is a major factor in label retention and identification of these LREC, and that the observed increase in number of LREC within Xs-treated mammary glands is indicative of an expansion of the stem cell population.

This conclusion is consistent with *in vitro* studies by Sherley and colleagues, who demonstrated that treatment of rat hepatocytes with Xs increased symmetrical stem cell division, thereby expanding the stem cell population and promoting exponential growth kinetics (23, 29). Because LREC represent a mixed population of cells (*e.g.*, ER-negative and ER-positive; (10)) and some progenitor cells may also differentially segregate and retain "immortal" DNA strands (30), we cannot rule out the possibility that increased LREC numbers represent expansion of stem cells and progenitor cells. Indeed we anticipate that an expansion of the progenitor cell population would accompany expansion of the stem cell population.

Additional support for the conclusion that Xs treatment increased the number of mammary stem cells was provided by a concomitant increase ($P < 0.01$) in the activity of telomerase in xanthosine-treated glands (Fig. 2A). During DNA replication, the lagging strand cannot be completely copied. This results in shortening of the telomeres, genomic instability, senescence and inability of the cell to proliferate. To overcome this constraint and permit immortality of stem cells, these cells and tumor cells express telomerase (31–33). Telomerase is a reverse transcriptase, consisting of a protein catalytic subunit and a RNA subunit that serves as the template for adding telomeric repeats on the ends of chromosomes to prevent continued truncation of the DNA strands (34, 35). In addition to being increased in Xs-treated quarters, we showed that telomerase activity was correlated with abundance of LREC in the mammary gland (Fig. 2B), lending additional support to the stem cell nature of LREC.

Additional immunohistological analysis of LREC was

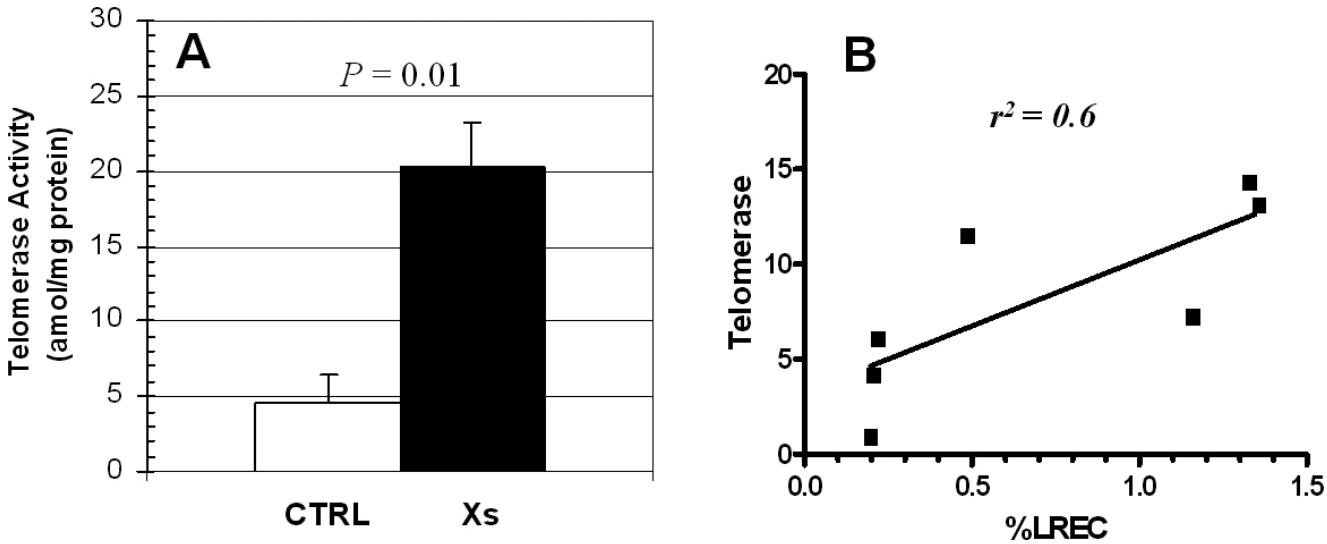

Figure 2. Effect of xanthosine (Xs) treatment on telomerase activity of bovine mammary tissue. The two mammary glands (quarters, Qtrs) on one side of the udder of four Holstein calves were treated with Xs and opposite glands served as controls. (A) Telomerase activity per mg protein in tissue homogenates from Xs-treated and control tissues of the rear mammary glands. (B) Correlation between tissue telomerase activity and LREC content of tissue, expressed as a percent of mammary epithelial cells.

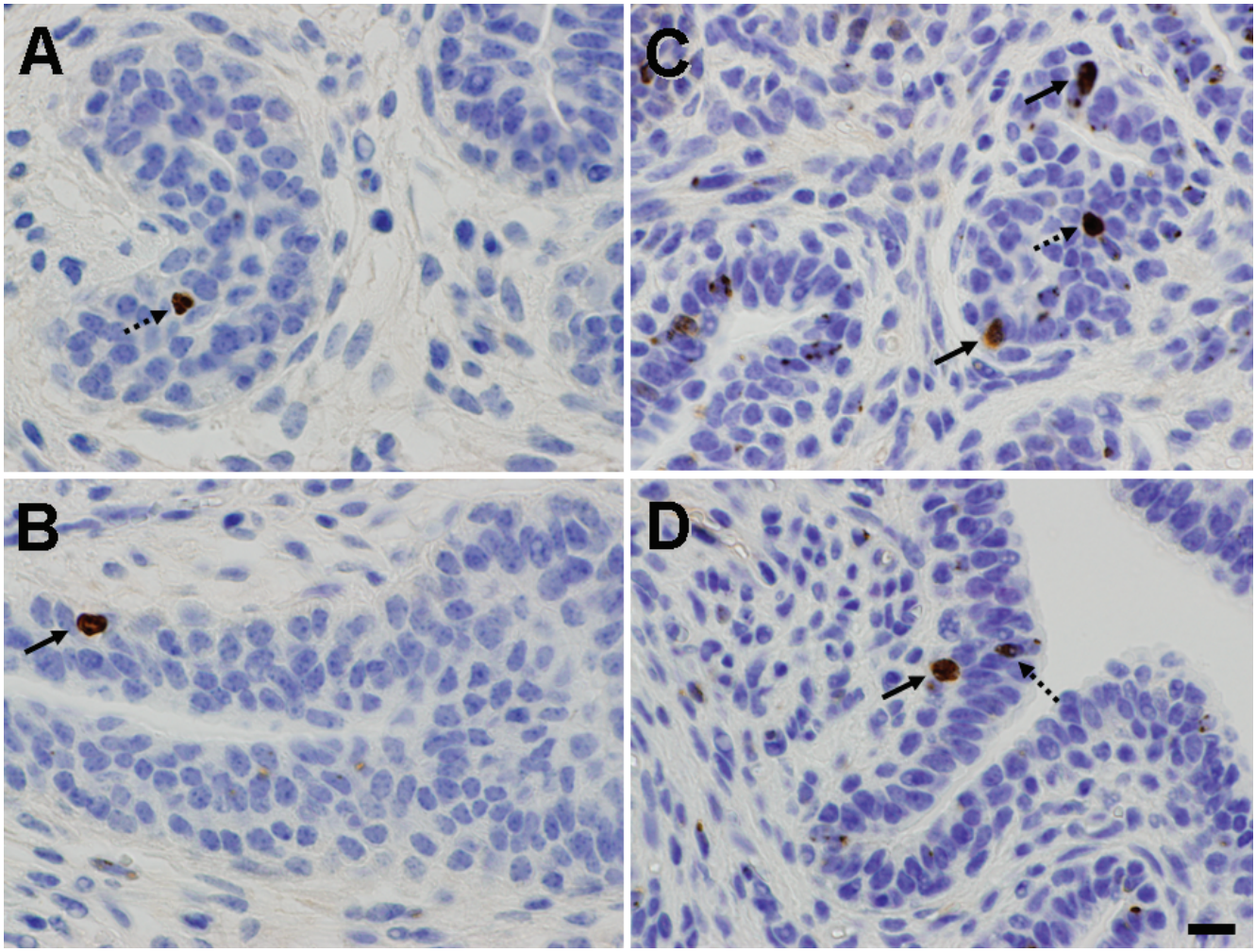

Figure 3. Immunohistochemical localization of BrdU label retaining epithelial cells (LREC) in control and Xs-treated bovine mammary glands. Heavily BrdU-labeled (brown) nuclei were classified as LREC and are identified by arrows. Solid arrows designate basally located LREC and dotted arrows embedded LREC. (A, B) Depict LREC in control glands and (C, D) LREC in Xs-treated glands. Micrographs presented are for tissues from control and Xs-treated glands of the same animal. Scale bar = 10 μm.

employed to determine their localization within the mammary epithelial layer and their estrogen receptor status. Distribution of LREC within the mammary epithelium is depicted in Figures 3 and 4A. The epithelium of the bovine mammary gland, similar to human breast, is composed of multiple layers of epithelial cell. The basal cells that overly the basement membrane in the mature mammary gland are myoepithelial cells, over which there are often several layers of cells in terminal ductular structures (terminal ductular units, TDU). However, in a prepubertal bovine mammary gland, the basal layer does not express significant quantities of α-smooth muscle actin and therefore may be comprised of myoepithelial progenitors and/or other stem/progenitor cells (11). LREC were primarily localized in the basal layer or within one or two cells above the basal layer (Fig. 3). Very seldom were LREC in contact with the lumen (Fig. 4A). Consistent with the proposed MaSC niche (36, 37), LREC were localized near the basement membrane and not in contact with the ductal lumen. Localization of LREC did not appear to differ between control and Xs-treated mammary glands (Figs. 3 and 4A). We previously demonstrated that ER was typically localized in a distinct layer of cells embedded within the mammary epithelium, *i.e.*, a layer that was above the basal layer, but beneath the luminal layer of cells. In the current study, 75% of the LREC were ER-negative (Fig. 4B). Within the limited ability to discern cell borders using immunofluorescence histochemistry of paraffin sections, ER-negative LREC were localized within the basal epithelial layer or in close proximity, as cells that were classified as embedded most frequently were adjacent to the basal layer. Although ER-positive LREC were not luminal, these cells were typically more distant from the basement membrane than were the ER-negative LREC. The distribution and proportion of ER-negative and ER-positive LREC were the same for control and Xs-treated mammary glands (data not shown). Thus, there was no evidence for Xs-induced alteration in the

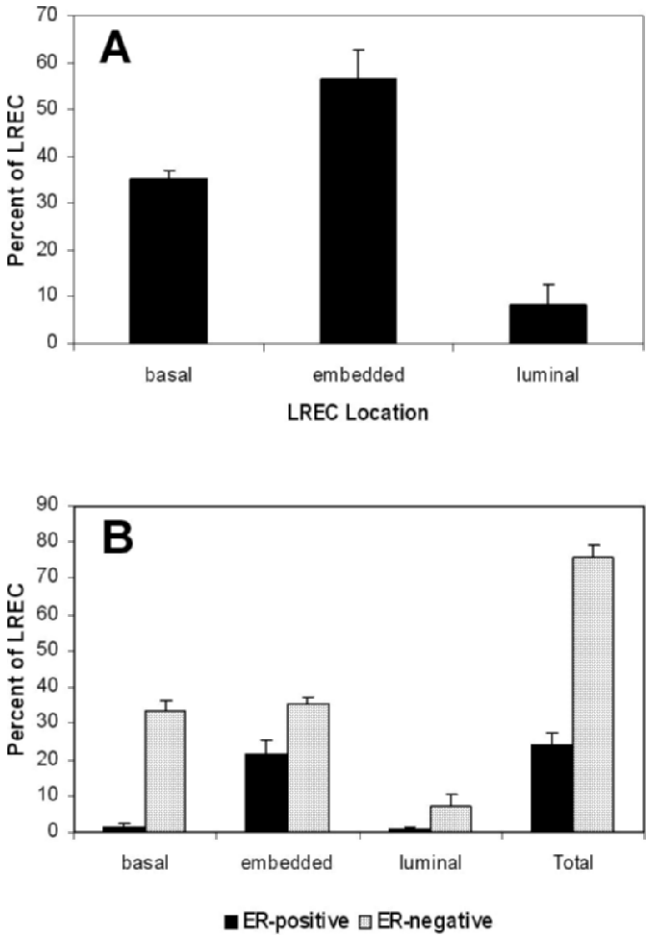

Figure 4. Location and estrogen receptor (ER) status of label retaining epithelial cells (LREC) in control and Xs-treated mammary glands. Because data were not influenced by treatment, means across treatment are presented. A total of 173 LREC, identified by immunofluorescence histochemistry, were scored (64 from control glands and 109 from Xs-treated glands). (A) Location of LREC within the epithelial layer was classified as: basal, overlying basement membrane; luminal adjacent to ductal lumen; embedded, between basal and luminal cells. (B) ER status and locale of LREC. Data are presented as a percentage (± SE) of total LREC.

character of the cell population identified by retention of BrdU label.

The ER-status of MaSC has been the subject of considerable investigation, primarily because of implications to mammary tumorigenesis and therapy. Previous investigations based upon identification of stem cells by label retention in mouse mammary gland and in human breast tissue transplanted to immune-compromised mice have suggested that mammary stem cells are ER-positive and therefore directly responsive to estrogen stimulation (25, 26). Application of other presumptive MaSC markers to human breast tissue has similarly suggested that MaSC are ER-positive (38).

However, other recent data provide strong evidence that MaSC are ER-negative. Two mouse studies indicated that after depleting mammary cell suspensions of endothelial ($CD31^+$) and hematopoietic ($CD45^+$, $TER119^+$) cells, a single cell of the epithelial population that expressed high levels of the heat stable antigen (CD24) and β_1- or α_6-integrin (CD29 or CD49f, respectively) was able to generate a functional mammary gland when implanted into epithelium-cleared mammary fat pads (4, 16). These sorted stem cells (39) were ER-negative. Similarly, Sleeman *et al.* characterized three fractions of murine mammary cells based upon expression of CD24 (40, 41) and found that the ability to repopulate a cleared fat pad was essentially restricted to a population of CD24-low expressors that were ER-negative. In human breast, Dontu and co-workers reported that increased aldehyde dehydrogenase-1 (ALDH) activity was associated with a population of human primary mammary epithelial cells that exhibited stem cell properties (42) and were ER-negative. Lineage of these cells appears to be regulated by BRCA1. Knockout of BRCA1 function increased the proportion of cells with MaSC activity, the number of ER-negative epithelial cells and the successful (ER-negative/ALDH1-positive) outgrowths from xenotransplanted cells (17). These data support the previous suggestion by Capuco (10) that in bovine mammary gland the population of ER-negative LREC represent MaSC. However, this awaits further confirmation.

In the mammary gland, proliferating epithelial cells appear to be ER-negative in cow (11) mouse (43) and human (44). Proliferation of mammary epithelial cells is seemingly mediated by paracrine factors derived from cells of the epithelial or stromal compartments of the mammary gland (11, 45–47), and our microarray studies support the importance of paracrine factors and cell-cell communication in driving prepubertal growth of the bovine mammary epithelium (48). Similarly, estrogenic stimulation of mammary stem cells appears to be mediated by paracrine factors emanating from ER-positive mammary epithelial cells. Therefore, we must determine how a single ER-negative stem cell can actively proliferate and give rise to an entire mammary gland. Although unproven, this could result from asymmetric stem cell division to provide either a very limited or more prominent number of ER-positive progenitors, which in turn regulate the MaSC (49, 50).

In the current experiment, intramammary treatment with Xs doubled the number of LREC detected in the bovine mammary gland, expanding upon the seminal *in vitro* studies by Sherley and co-workers (21, 23). Although this increase in a putative MaSC population was evident 40 days after treatment, we anticipate that the effect is not permanent, but temporary. Furthermore, we anticipate that an increase in the number of MaSC will stimulate production of progenitor cells and more differentiated cell types within the gland. Depending upon the physiological status of the animal, this can enhance mammary growth (increase cell number) or enhance cell turnover and tissue repair (increased cell replacement).

In conclusion, we have utilized a simple *in vivo* infusion of Xs into the prepubertal mammary gland to increase the number of putative MaSC. This effect was

evidenced by an increase in the number of LREC and tissue telomerase activity. Furthermore, LREC in Xs-treated glands maintained the same distribution and ER status as those in control quarters, being consistent with localization and ER-status of putative stem cells. To our knowledge, this represents the first non-transgenic technology to seemingly alter mammary stem cell activity. Therefore, this method provides a possible means to manage mammary 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 MaSC in normal mammary function and carcinogenesis. The method is likely applicable to rodent models, thereby providing an additional tool to study the role of MaSC in mammary carcinogenesis.

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