INTRODUCTION

Since the majority of breast cancers originate in the epithelial cells lining the duct, it was hypothesized that administration of agents directly into the breast ductal system (intraductal; i.duc) may result in eradication of premalignant disease, and prevention of the development of new lesions. In preclinical models, it was previously demonstrated that the i.duc administration of 4-hydroxytamoxifen or pegylated liposomal doxorubicin (PLD) to carcinogen-induced mammary tumors in rodents was associated with a reduction in mammary tumor volume, eradication of pre-malignant disease, and prevention of new lesions [1]. The i.duc administration of several other chemotherapeutic agents was tested in methylnitrosourea (MNU)-injected rats and both fluorouracil (5-FU) and carboplatin were proved to be effective [2]. We demonstrated that i.duc nanocurcumin prevented the development of mammary tumors efficiently in carcinogen-induced rat models [3].
ported that i.duc PLD injection reduces normal mammary stem cells (MaSCs)’ function, which results in disturbing milk production in lactating mouse mammary gland and inducing malignant tumor [4]. Mouse MaSCs have been reported to be enriched in the subset of mammary cells that are Lin<sup>-</sup>CD49f<sup>hi</sup>CD24<sup>med</sup> or Lin<sup>-</sup>CD29<sup>hi</sup>CD24<sup>med</sup> [5,6]. However, the poor level of stem cell enrichment makes it doubtful that changes in the number of stem cells can be detected by examining these stem cell-enriched populations as a whole. It was suggested that flow cytometry using more specific stem cell markers may solve this problem [7].

Here we have explored i.duc PLD’s effect on stem cell number and function in mouse mammary gland and aldehyde dehydrogenase (ALDH)’s availability as a mouse MaSC marker. We have studied the utility of this stem cell analysis as a screening method to check the safety of i.duc treatment in breast cancer.

**METHODS**

**Animals, animal surgery and treatment**

Six-month-old to 8-month-old postbreeder FVB/N mice were purchased from National Cancer Institute at Frederick, USA and maintained in our animal facility according to institutional guidelines. All experiments were performed with approval of the Animal Care and Use Committee of Johns Hopkins University School of Medicine.

**Intraductal injection**

Mice were anesthetized by isoflurane/oxygen inhalation. Keratin plugs were removed from the surface of the nipple by rubbing gently with gauze soaked in alcohol, revealing the duct orifice. Mammary ducts were cannulated using a 1.0-cm, 34-gauge, blunt-ended needle (Hamilton 7741-01; Reno, NV, USA) attached to a 50 μL tuberculin syringe. PLD (50 μL/duct) was injected slowly into the duct while visualizing the opening under a dissecting microscope.

**Anticancer agent**

PLD was used for experiments. For the stem cell analysis, FVB/N mice were used and 40 μg/duct PLD was injected into all ten ducts at one time. The same injection was repeated 1 week or 4 weeks after the first injection.

**Mammary cell preparation**

Single cell suspensions were made from freshly separated mammary glands of mice by digestion and analyzed by flow cytometry [5]. In brief, mammary glands were digested for 8 hours at 37 °C in serum-free breast tissue digestion medium (StemCell Technologies) plus 2% FBS. Cells (10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup>) were instilled into cleared mammary fat pads of 3-week-old recipient FVB/N female mice. After 5–8 weeks, mammary glands were removed and carmine alum-stained for whole mount analysis. Fat pads were scored as positive or negative based on the presence or absence of mammary ductal outgrowths. Limiting dilution assays used “statmod” software package for the “R” computing environment (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). The frequency of mammary repopulating units (MRUs) in single cell suspensions was calculated using complementary log-log generalized linear model. Two-sided 95% Wald confidence intervals were computed, except in cases of zero outgrowths, when one-sided 95% Clopper-Pearson intervals were used instead. The single-hit assumption was tested as recommended and was not rejected for any dilution series.

**RESULTS**

**Mammary cells from i.duc PLD-treated and normal mouse mammary glands have similar flow cytometry profiles**

MaSCs have been reported to be enriched in the subset of mam-
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Primary mouse mammary epithelial cells were separated into CD49\textsuperscript{high}CD24\textsuperscript{med} and CD49\textsuperscript{high}CD24\textsuperscript{med}-depleted populations using flow cytometry. We observed no change in the proportion of MaSC (CD49\textsuperscript{high}CD24\textsuperscript{med}) fraction in i.duc PLD-treated group compared with untreated group. The percentage of CD49\textsuperscript{high}CD24\textsuperscript{med} cells as a percentage of total epithelium in i.duc PLD-treated mice was 22.4% ± 3.79% (mean ± standard deviation; n = 4 independent preparation), and in control untreated mice the percentage was 21.8% ± 8.59% (n = 4 independent preparation) (Fig. 1B). There was also no significant difference between the proportions of CD49\textsuperscript{high}CD24\textsuperscript{med}-depleted cells in i.duc PLD-treated mouse mammary glands compared with control mice (Fig. 1B).

**Cleared fat pad transplant analysis with CD49\textsuperscript{high}CD24\textsuperscript{med} mammary cells showed significantly decreased mammary outgrowth potential in i.duc PLD-treated mice compared with control untreated mice**

To evaluate ductal tree regeneration potential, serial dilution transplantation into epithelium cleared mammary fat pad was performed. We used this assay to compare the ability of cells isolated from i.duc PLD-treated mice and control mice to repopulate a cleared mammary fat pad. Limiting dilution assays were used by transplanting CD49\textsuperscript{high}CD24\textsuperscript{med} mammary cells from each group into cleared mammary fat pads of 3-week-old FVB/N recipient mice. The cells were freshly isolated and did not undergo any in vitro culture period prior to transplantation. Transplants were examined after 5–8 weeks and number of outgrowths and the extent to which they filled the mammary fat pads were estimated (Fig. 2). The rate of successful transplants was used to estimate the proportion of MaSC in each population using the L-Calc analysis package [6]. i.duc PLD-treated mammary cells showed a mammary repop-
The extent of fat pad filled by primary mammary epithelial cells from i.duc PLD-treated mammary glands was lesser than that from control untreated mice.

Table 1. Effect of intraductal PLD on mammary repopulation in Lin\(^{\text{CD49}^\text{hi}}\)CD24\(^{\text{med}}\) mammary epithelial cells

| No. of cells injected per mammary fat pad | Control | Intraductal PLD |
|-----------------------------------------|---------|-----------------|
| CD49\(^{\text{hi}}\)CD24\(^{\text{med}}\) cells |         |                 |
| 10\(^5\)                               | 4/12    | 4/27            |
| 10\(^4\)                               | 8/12    | 2/26            |
| 10\(^3\)                               | 2/18    | 1/14            |
| 10                                     | 0/12    | 3/16            |
| Repopulating frequency                  | 1/910   | 1/88,520        |
| 95% CI                                  | (1/1,161–1/199) | (1/162,069–1/48,349) |

Limiting dilution analysis of the repopulating frequency of CD49\(^{\text{hi}}\)CD24\(^{\text{med}}\) mammary epithelial cells from control and intraductal pegylated liposomal doxorubicin (PLD)-treated glands transplanted into wild-type recipients. Cells were injected into cleared mammary fat pads of 3-week-old syngenic recipient mice and collected 5–8 weeks after transplantation. There was 97-fold decrease in absolute number of mammary repopulating unit in intraductal PLD-treated mammary glands.

CI, confidence interval.

\( ^{a}P < 0.0001. \)

Fig. 3. Outgrowth from unsorted cells from intraductal pegylated liposomal doxorubicin (PLD)-treated mammary glands filled less portion of mammary fat pad. (A) A representative image of the extent of the cleared fat pad filled by outgrowths by cells from intraductal PLD-treated and control mammary glands. (B) Bar chart showing percentage fat pad filling by outgrowths from 10\(^4\) unsorted cells from control and intraductal PLD-treated mammary glands.

Fig. 4. ALDH(+) cell population was increased in cells from intraductal pegylated liposomal doxorubicin (PLD)-treated mammary glands compared with control glands. The percentage of cells in the ALDH(+) and ALDH(−) populations in each group are shown. ALDH, aldehyde dehydrogenase.
ALDH(+) cell population was increased in cells isolated from i.duc PLD-treated mammary glands compared with control glands

It was reported that normal and malignant human mammary epithelial cells with increased ALDH activity had stem-/progenitor-like properties and ALDH expression correlated with poor prognosis [10]. To our knowledge, there is no published report defining whether ALDH can function as a mouse MaSC marker in human mammary glands. We next analyzed cells from i.duc PLD-treated and control mammary glands for the expression of ALDH. ALDH(+) population was increased (8.3 ± 5.70 vs. 12.6 ± 6.51) and ALDH(−) population was decreased (64.1 ± 7.31 vs. 55.1 ± 10.81) in cells isolated from i.duc PLD-treated mammary glands compared with control glands (Fig. 4).

ALDH activity may add further selection of MaSCs to CD49f CD24 in mouse mammary glands

Next we investigated the overlap between the ALDH(+) population and CD49f CD24− phenotype. CD49f CD24−MaSCs were significantly smaller than CD49f CD24−ALDH(+) cell population in both control and intraductal PLD-treated groups.
Table 2. Frequency of mammary outgrowths Lin− mammary cells defined by CD49f, CD24, and ALDH expression

| No. of cells injected per mammary fat pad | Control | Intraductal PLD |
|----------------------------------------|---------|----------------|
| CD49f<sup>hi</sup>CD24<sup>med</sup> ALDH(+) cells | | |
| 10<sup>4</sup> | - | 1/2 |
| 10<sup>5</sup> | 4/4 | 4/17 |
| 10<sup>6</sup> | 4/4 | 2/11 |
| 10<sup>7</sup> | 2/10 | 1/11 |
| 10<sup>8</sup> | 0/8 | 2/12 |
| Repopulating frequency | 1/118 (1/280–1/50) | 1/2,119 (1/3,894–1/1,153) |

Frequency of mammary outgrowths of mammary cells defined by CD49f, CD24, and aldehyde dehydrogenase (ALDH) expression. Mammary outgrowth frequency of CD49f<sup>hi</sup>CD24<sup>med</sup> ALDH(+) and CD49f<sup>hi</sup>CD24<sup>med</sup> ALDH(−) mammary epithelial cells from control and intraductal pegylated liposomal doxorubicin (PLD)-treated glands transplanted into wild-type recipients. Cells were injected into cleared mammary fat pads of 3-week-old syngenic recipient mice and collected 8 weeks after transplantation.

CI, confidence interval.

*a* P < 0.0001.

**DISCUSSION**

Adult stem cells are long-lived, generally quiescent cells that produce new stem cells, and thereby maintain the stem cell reservoir, as well as more committed progeny, which repopulate the organ through proliferation [11,12]. When necessary, it can expand to produce a transiently amplified reservoir of progenitors to repopulate tissues.

The activity of MaSCs and their mitotic progeny is essential to normal mammary growth, differentiation and regeneration in consecutive cycles of pregnancy, lactation, and involution. An important feature of the mammary gland is the regenerative capability of its epithelium, which is demonstrated upon successive reproductive cycles [13]. The ductal cells are those that line the ducts of the mammary gland. Lobular cells form secretory acinar structures and, upon pregnancy and lactation, become alveolar cells that produce milk proteins. The capability to recruit the mammary gland through cycles of pregnancy, lactation, and involution throughout a woman’s lifetime is attributed to stem cells that are suggested to reside in the mammary gland [14,15].

Mouse MaSCs have been shown to be enriched in Lin CD49f<sup>hi</sup>CD24<sup>med</sup>, or CD29<sup>hi</sup>CD24<sup>+</sup> mammary cells [5,6]. However, changes in the number of stem cells can hardly be detected by examining these stem cell-enriched populations as a whole because the poor extent of stem cell enrichment [7]. It has been shown that murine and human hematopoietic and neural stem and progenitor cells have high ALDH activity [16,17]. Increased ALDH activity has also been found in stem cell populations in multiple myeloma and acute myeloid leukemia [17,18]. It has been reported that ALDH is a marker of normal and malignant human MaSCs and a predictor of poor clinical outcome [19]. ALDH activity may thus provide a common marker for normal mouse MaSCs. However, little is known about the relation between ALDH activity and mouse MaSCs [19].

Here, we show ALDH activity may add further selection of MaSCs to CD49f CD24 in mouse mammary glands. CD49f<sup>hi</sup>CD24<sup>med</sup>ALDH(+) cell population shows more enrichment of mouse MaSCs than CD49f<sup>hi</sup>CD24<sup>med</sup> cell population. The percentage of ALDH(+) cells in the CD49f<sup>hi</sup>CD24<sup>med</sup> population in-
increased after i.duc PLD treatment (3.6% vs. 5.7%) because i.duc PLD injection is considered to damage a greater portion of normal mammary cells; and stem cell portions increase relatively.

In conclusion, we present that i.duc administration of PLD reduces MaSC function, but not the number, and ALDH activity may add further selection of MaSCs to CD49f CD24 in mouse mammary glands though more study is needed to underpin and strengthen this result. Screening of chemotherapeutic drugs or other natural products by this method of stem cell analysis may provide safe i.duc treatment in breast cancer. More research with various chemotherapeutic agents and adverse effect monitoring is needed.

CONFLICT OF INTEREST
No potential conflict of interest relevant to this article was reported.

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