Enrichment of a Population of Mammary Gland Cells that Form Mammospheres and Have In vivo Repopulating Activity

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Abstract

The identification of mammary gland stem cells (MGSC) or progenitors is important for the study of normal breast development and tumorigenesis. Based on their immunophenotype, we have isolated a population of mouse mammary gland cells that are capable of forming “mammospheres” in vitro. Importantly, mammospheres are enriched for cells that regenerate an entire mammary gland on implantation into a mammary fat pad. We also undertook cytogenetic analyses of mammosphere-forming cells after prolonged culture, which provided preliminary insight into the genomic stability of these cells. Our identification of new cell surface markers for enriching mammosphere-initiating cells, including endoglin and prion protein, will facilitate the elucidation of the cell biology of MGSC. [Cancer Res 2007;67(17):8131–8]

Introduction

Adult stem cells are rare, immature cells capable of self-renewal and generation of many of the distinct differentiated cell types within a given tissue or organ. The existence of self-renewing mammary gland stem cells (MGSC), sometimes termed progenitors, which can differentiate into ductal or alveolar epithelial cells as well as myoepithelial cells, has been suggested by serial transplantation into mammary fat pads and retroviral tagging experiments. Thus, mammary gland–generating cells can be transplanted serially for up to seven generations (1), and a single-tagged cell can give rise to a complete mammary gland on transplantation (2).

Some have proposed that somatic mutations occurring in the MGSCs can accumulate over time, leading to tumorigenesis (3). Accordingly, the enrichment or purification of MGSC should greatly benefit the elucidation of mechanisms underlying normal mammary gland development as well as carcinoma formation. The adaptation of fluorescence-activated cell sorting (FACS) using combinations of cell surface markers has significantly expedited the procedures of stem cell isolation. Recently, a putative human breast cancer stem cell population was identified as CD24–/low Lin–/low CD49f–/low (4). Furthermore, several reports that described the use of mammary fat pad transplantation showed that isolation of cells displaying various combinations of cell surface markers, including Sca-1+ (5), CD24low (6), CD45–/low CD11b+CD29highCD24+ (8), can enrich mouse normal mammary epithelial stem cells from the heterogeneous populations of cells present in the normal mammary gland.

Although these studies significantly advanced the procedures of stem cell isolation, they also raised questions for further exploration. For example, the relationship of CD24 expression to MGSC isolated under different conditions has remained unclear, as are the identities of possible additional stem cell markers. Moreover, it remains unclear whether pure populations of MGSCs can be isolated. These considerations underscore the need to identify additional cell surface markers and to characterize the expression patterns of known markers, such as CD24, by populations of MGSC.

The most definitive proof of cells functioning as MGSCs continues to depend on the ability of such cells to reconstitute a differentiated mammary gland following implantation into cleared mammary gland fat pads (9). In more recent years, an in vitro test of MGSC function has come from the ability of such cells to grow as nonadherent mammospheres (10). Although the majority of cells within the mammospheres are differentiated and thus lack gland-repopulating activity, the spheres do contain small numbers of multipotent cells that can develop into all lineages of the mammary gland and can sustain self-renewal in culture (10). In addition, transplanted human mammospheres have been shown to participate in the ductal/alveolar development in humanized nonobese diabetic–severe combined immunodeficient mammary fat pads (11). This raises questions about the identity of the mammary gland epithelial cells that are capable of forming mammospheres in vitro and whether these cells represent the same cell population that is capable of mammary gland reconstitution in vivo.

We recently identified several new cell surface markers for hematopoietic stem cells (HSCs), including endoglin (12) and prion protein (PrP; ref. 13). By combining these antigens and other markers for immunostaining and cell sorting, we sought to enrich different mouse mammary gland cell populations and to investigate the surface phenotype of mammosphere-initiating cells and the relationship of mammosphere formation in vitro and mammary gland reconstitution in vivo.

Materials and Methods

Mice. C57BL/6J, green fluorescent protein (GFP) transgenic C57BL/6J, and FVB mice were purchased from The Jackson Laboratory. Rag1−/− mice were maintained in the Whitehead Institute animal facility.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Mammary cell preparation. Primary mammary epithelial cells were prepared from 9- to 12-week-old virgin female mice as described (14). Mammary glands were chopped with a McIlwain tissue chopper (Mickle Laboratory Engineering Co.) or manually by razor blade. The tissue was washed with PBS and then placed in collagenase-dispase solution (liver digest medium, Invitrogen) at 37°C for 2 h (for experiments in Fig. 1 and Table 1), or the tissue was placed in MM+ medium [DME/F12 supplemented with 2% calf serum, 10 mmol/L HEPES, 10 ng/mL epidermal growth factor (EGF), 10 μg/mL insulin, 5% bovine serum albumin, and 100 μg/mL penicillin/streptomycin] containing 3 mg/mL collagenase A (Roche) at 37°C for 2 h (for experiments in Figs. 2–4 and Table 2). The resultant organoids were either resuspended in 0.25% trypsin-EDTA (Invitrogen) for 10 min before being filtered through a 40- or 70-μm strainer followed by direct immunostaining and sorting (for experiments in Fig. 1 and Table 1) or used for organoid culture followed by immunostaining and sorting (for experiments in Figs. 2–4 and Table 2). The sorted cells were used for mammosphere culture.

Organoid culture. Freshly isolated organoids were plated on tissue culture dishes in MM+ medium. Medium was changed at days 1, 3, and 5. The cells were harvested at day 6 by trypsinizing with 0.05% trypsin for 10 to 15 min before being filtered through a 40- or 70-μm strainer. The cells were then stained with desired antibodies, and the FACS-sorted fractions were used for mammosphere culture.

Mammosphere culture. Single-cell suspensions were grown in 100 μL/well of MM+ medium with 1:50 B27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL basic fibroblast growth factor (bFGF), and 10 μg/mL heparin in Corning Costar 3474 96-well plates at a density of 50,000 or 100,000 cells/mL (for Fig. 1 and Table 1) or 20,000 cells/mL (for Figs. 2–4 and Table 2). Mammospheres were collected by 70-μm strainer and dissociated with 0.05% trypsin for 15 min to obtain single-cell suspension.

Transplantation and mammary gland whole mounts. Three-week-old females of Rag1−/− mice were used as recipients. Their inguinal mammary glands were surgically cleared of the endogenous epithelium as described (14).

Indicated numbers of cells were injected into the cleared fat pads of 21-day-old Rag1−/− females by using a 50-μL Hamilton syringe. After 6 weeks, the glands were dissected and the whole mounting was done as described (14).

Flow cytometry. Anti-PrP monoclonal antibody (mAb; SAF-83, Cayman Chemical) was FITC conjugated using the QuickTag FITC conjugation kit (Roche) as described (13). Alternatively, the staining with anti-PrP was followed by antimouse-FITC. The specificity of anti-PrP was verified by its inability to bind to PrP-null cells and its ability to specifically bind to cell lines expressing transfected PrP (data not shown). When endoglin was detected, cells were stained with biotinylated anti-endoglin mAb (eBioscience) followed by streptavidin-allophycocyanin (APC; BD PharMingen) or streptavidin-phycocerythrin (PE)/CY5.5 (eBioscience). Anti-CD24-FITC and anti-CD49f-PE were from BD PharMingen. For detection of CD24, PrP endoglin, and CD49f, cells were stained with anti-PrP followed in order by antimouse-PE/Cy5.5, biotinylated anti-endoglin, streptavidin-APC, 

Figure 1. Presence of mammosphere-initiating cells in various populations of freshly isolated mammary gland cells. Surface expression of CD24 alone (A), CD24 and PrP (A), CD24 and endoglin (B), CD24 and CD49f (B), or CD24, CD49f, PrP, and endoglin (B) in freshly isolated mammary gland cells and mammospheres formed by cells fractioned based on their respective staining. Representative mammospheres or nonsphere aggregates formed by freshly isolated mouse mammary gland cells. Bar, 100 μm.
and anti-CD24-FITC and anti-CD49f-PE. Sorting was done on a MoFlo instrument.

**Real-time PCR.** Total RNA was isolated from indicated cell populations. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Samples were analyzed in triplicate 25 μl reactions (300 nmol/L of primers, 12.5 μL of Master Mix), which was adapted from the standard protocol provided in SYBR Green PCR Master Mix and reverse transcription-PCR protocol provided by Applied Biosystems. The default PCR protocol was used on an Applied Biosystems Prism 7000 Sequence Detection System.

**Chromosome analyses.** freshly isolated CD24+PrPmed endoglin+ cells, or CD24 PrP+ endoglin+ cells after 6 days of preculturing as described above, were plated on gelatin-coated plates and cultured in MM+ medium for almost 3 weeks until enough of them were available for cytogenetic harvest. Cells were treated overnight with colcemid and chromosome preparations were made according to standard protocols (15). Chromosome hybridization and analysis for spectral karyotyping (SKY) were conducted according to a standard protocol with minor modifications (16). Acquisition of interferograms and subsequent SKY analysis were done with Spectral Imaging 2.6 and SKY View 2.1.1 software (Applied Spectral Imaging, Inc.), respectively, using SpectraCube SD200 (Applied Spectral Imaging) and a Zeiss Axioscope II microscope (Zeiss, Inc.) on a Windows XP Professional workstation (Dell Computer).

**Results**

A population of freshly isolated mouse mammary gland cells forms mammospheres. Based on the established culture condition for human mammospheres (10), we tested various conditions for growing mouse mammospheres and opted to use the optimized medium containing 20 ng/mL EGF and 20 ng/mL bFGF. After 7 days of culture in this medium in a low-attachment culture plate, about 6 mammospheres with diameters >40 μm were formed per 10,000 freshly isolated mammary epithelial cells (Fig. 1; Table 1, Experiment 1). The mammospheres contained an average of ~250 cells, and similar numbers of sphere-forming cells were observed using cells isolated from C57BL/6J and FVB mice.

We attempted to use immunostaining and FACS to identify the fraction of mouse mammary gland cells that could form mammospheres. The tested antigens for immunostaining included CD24, a marker whose absence or expression at low levels characterizes human tumorigenic breast cancer cells (4); PrP and endoglin, both surface markers for mouse HSCs (12, 13, 17); and CD49f, a marker for in vivo repopulating mammary gland epithelial stem cells (7). The surface expression of these antigens in freshly isolated cells was analyzed by flow cytometry (Fig. 1).

A representative FACS plot showed that 39.9% of total cells were negative for CD24 staining (CD24−; Fig. 1, PrD24). Cells staining positively for CD24 (CD24+) could be further fractionated into CD24med (4.2% of total cells) and CD24high (54.0% of total cells) based on the relative levels of CD24 surface expression. The whole-cell population could also be fractionated into PrP− (92.5% of total cells), PrPmed (5.1% of total cells), and PrPhigh (1.8% of total cells) based on PrP staining (Fig. 1, PrP).

We found that 4.2% of total mammary epithelial cells were CD24+CD49f−, of which 8.4% and 20.7% were PrP+ endoglin+ and PrP− endoglin+, respectively (Fig. 1, CD24 PrP Endoglin CD49f). We also obtained the profiles of cells stained for CD24, PrP, endoglin, and CD49f in combination. We found that 4.2% of total mammary epithelial cells were CD24+CD49f−, of which 8.4% and 20.7% were PrP+ endoglin+ and PrP− endoglin−, respectively (Fig. 1, CD24 PrP Endoglin CD49f).

To identify the subpopulations of cells in the mouse mammary glands that were capable of forming mammospheres, we first plated freshly isolated CD24−, CD24med, and CD24high cells in sphere-forming conditions. Only CD24+ cells, including both CD24med and CD24high cells, grew into typical mammospheres (Fig. 1, right; Table 1). The majority of sphere-forming ability resided in the CD24high cells. In one of our experiments (Table 1, Experiment 1), 10,000 CD24high or CD24med cells generated an average of 9.2 or 12 spheres, respectively, whereas the same numbers of CD24− cells formed no spheres. Hence, freshly isolated mammosphere-initiating cells expressed CD24 at readily detectable levels.

We also tested the mammosphere-forming abilities of cells fractionated based on their cell surface expression of either PrP or endoglin. Whereas cells with no or high PrP staining formed none or few spheres, respectively (Table 1), PrPmed cells were capable of generating far more spheres, yielding ~8.5 mammospheres per 10,000 cells in a representative experiment (Fig. 1; Table 1, Experiment 1). Sphere-forming cells also resided in the endoglin− fraction (8.2 ± 1.6 spheres/10,000 cells) but very few in the endoglin− fraction (0 sphere/10,000 cells; Fig. 1; Table 1, Experiment 1) as well as in the CD49f− fraction (4.2 ± 1.5 spheres/10,000 cells; Table 1, Experiment 1); conversely, almost no mammosphere-forming cells were present in the CD49f− population (0.7 ± 0.2 sphere/10,000 cells; Fig. 1; Table 1, Experiment 1). We repeated the mammosphere-forming experiment and observed the same trend (Table 1, Experiment 2).

### Table 1. The frequencies of mammospheres formed by different fractions of freshly isolated mammary gland cells (spheres with diameters >40 μm were counted)

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Frequency (spheres/10,000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfractioned</td>
<td>6.1 ± 1.2</td>
</tr>
<tr>
<td>CD24−</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CD24med</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>CD24high</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>PrP−</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PrPmed</td>
<td>8.5 ± 2.2</td>
</tr>
<tr>
<td>PrPhigh</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Endoglin−</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Endoglin+</td>
<td>8.2 ± 1.6</td>
</tr>
<tr>
<td>CD49f−</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>CD49f+</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>CD24+ CD49f−</td>
<td>19.2 ± 3.4</td>
</tr>
<tr>
<td>CD24+ CD49f−</td>
<td>1.5 spheres/10,000 cells</td>
</tr>
<tr>
<td>CD24med Endoglin+</td>
<td></td>
</tr>
<tr>
<td>CD24med Endoglin−</td>
<td></td>
</tr>
<tr>
<td>CD24high Endoglin+</td>
<td></td>
</tr>
<tr>
<td>CD24high Endoglin−</td>
<td></td>
</tr>
<tr>
<td>CD24med CD49f− Endoglin+</td>
<td></td>
</tr>
<tr>
<td>CD24med CD49f− Endoglin−</td>
<td></td>
</tr>
<tr>
<td>CD24high CD49f− Endoglin+</td>
<td></td>
</tr>
<tr>
<td>CD24high CD49f− Endoglin−</td>
<td></td>
</tr>
</tbody>
</table>

### Experiment 2

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Frequency (spheres/5,000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24−</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>CD24med</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>CD24high</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>PrP−</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>PrPmed</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>PrPhigh</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Endoglin−</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Endoglin+</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>CD49f−</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>CD49f+</td>
<td>3.6 ± 1.3</td>
</tr>
</tbody>
</table>

**Enrichment of Mammosphere-Initiating Cells**
When we fractionated cells based on their cell surface expression of the CD24, PrP, endoglin, and CD49f markers, 10,000 CD24+PrP+endoglin+CD49f+ cells were capable of forming an average of 19.2 spheres (Table 1, Experiment 1). This sphere-initiating activity is higher than that of CD24 high, PrP med, or endoglin+ cells. We noticed that the CD24+endoglin+CD49f+ fraction contains largely PrP med cells (Fig. 1). Overall, this indicated that CD24+PrP med endoglin+CD49f+ mouse mammary epithelial cells are enriched for mammosphere-initiating cells.

Cultured mammary gland cells have altered surface phenotype of mammosphere-initiating cells. We next tested whether in vitro culture of the mouse mammary gland cells could affect their display of critical cell surface markers as well as sphere-forming activity. To this end, mouse mammary glands were isolated and organoids were cultured for 6 days before immunostaining. The surface expression of CD24, PrP, endoglin, CD49f, and CD44 in cells from cultured organoids was then analyzed, as before, by flow cytometry (Fig. 2A).

A representative FACS plot of such precultured mammary gland cells revealed that 12% of total cells were CD24+/PrP− (Fig. 2A, plot 1). In either the CD24− or the CD24+ fraction, about two thirds of the cells were PrP+/PrP− cells (plot 1). PrP+ cells could be further fractionated by endoglin staining (plot 2). In addition, we also examined the surface coexpression of CD44 or CD49f with CD24, PrP, and endoglin. Two thirds of CD44+ cells were CD24− (plot 3). Half of CD49f+ cells were also endoglin− (plot 4). Hence, such
in vitro culture resulted in an increase in those cells expressing certain markers, such as CD24, PrP, and endoglin, and CD49f.

We undertook to investigate the effects of this in vitro culture on the subsequent ability of the mammary cells to generate spheres in vitro. In these experiments, we chose to focus on CD24, PrP, and endoglin as cell surface markers. Using the same sphere-forming conditions as in Fig. 1 and Table 1, we observed that precultured cells formed numbers of mammospheres comparable with those arising from freshly isolated cells. Thus, whereas 10,000 freshly isolated mammary epithelial cells yielded an average of 6.1 spheres, an average of 4.1 mammospheres were generated from 10,000 precultured mammary gland cells (Fig. 2B, left). This indicated that propagation of mouse mammary epithelial cells in monolayer cultures did not yield a significant change in the proportion of sphere-forming cells. The mammospheres formed by precultured cells had an average diameter of 100 μm (Fig. 2B, left).

We then examined which subpopulations of precultured cells could form mammospheres. When we plated precultured CD24+ and CD24− cells in sphere-forming conditions, to our surprise, only CD24+ cells grew into mammospheres (Fig. 2B, right). CD24+ cells (10,000) generated an average of 16.7 spheres with a diameter of 162 μm. In contrast, CD24− cells formed only small irregular aggregates composed of several cells (Fig. 2B, middle). Hence, after preculturing (6 days of in vitro growth of organoids before mammosphere culture), mammosphere-initiating cells resided exclusively in the CD24+ fraction. This was in contrast to the behavior of freshly isolated mammary epithelial cells, in which the sphere-initiating activity resided in the CD24− fraction.

We further subfractionated the CD24+ fraction of precultured cells into PrP+ and PrP− populations and tested the sphere-initiating capability of each of these subpopulations. Whereas CD24+ PrP− cells were capable of growing into larger spheres, CD24+ PrP+ cells produced smaller spheres. In a representative experiment (Fig. 2C), 400 precultured CD24+ PrP− cells generated 1 sphere with an average diameter of 201 μm, whereas the same number of precultured CD24+ PrP+ cells generated spheres with an average diameter of 146 μm. When we plated 200, 100, or 50 cells from these CD24+ PrP− and CD24+ PrP+ fractions, they all generated a single sphere. In addition, in all cases, CD24+ PrP− cells formed larger spheres (Fig. 2C). Therefore, in contrast to freshly isolated cells, in which the sphere-initiating cells are enriched in the PrPmed fraction, precultured cells contain PrP− cells as their major sphere-initiating population.

When we fractionated precultured cells based on their cell surface expression of CD24, PrP, and endoglin, the CD24+ PrPmed endoglin− cells formed the greatest numbers of mammospheres; these were also the largest in size. Table 2 summarizes the results of mammosphere formation by these various cell populations in a representative experiment. For example, 2,000 of CD24+ PrPmed endoglin− cells generated an average of 1 sphere with an average diameter of 138 μm. The same number of CD24+ PrP− endoglin− cells produced 1 sphere with a diameter of 70 μm. As expected, CD24+ PrP− endoglin− and CD24+ PrPmed endoglin− cells produced smaller spheres at lower frequencies than CD24+ PrP− endoglin− cells. Therefore, the CD24+ PrPmed endoglin− fraction of the precultured cells is enriched for sphere-initiating cells.

In summary, the surface phenotype of mammosphere-initiating cells changes dramatically following in vitro culture. For freshly isolated cells, CD24+ PrPmed endoglin− cells are enriched for sphere-initiating cells; following culture, sphere-initiating cells are enriched in the CD24+ PrP− endoglin− population.

Table 2 also shows that, as we progressively reduced the number of cells introduced into the sphere culture, we observed fewer and smaller spheres. Nevertheless, a sphere could be generated by as few as five plated precultured CD24+ PrP− cells (Table 2), indicating that this cell population is highly enriched for sphere-forming cells. We were therefore intrigued to know whether a mammosphere can be formed from a single initiating cell introduced into the sphere culture.

Two approaches were adopted to address this possibility. In the first, mammary gland cells isolated from GFP+ and control non-GFP-expressing mice were mixed in a ratio of 1:10 and plated for sphere formation. If the sphere is initiated from a single cell, then ~10% of finally formed spheres should be formed exclusively by GFP+ cells. However, as the result showed, 9% of spheres were composed largely of GFP+ cells, and 10% of these were formed exclusively from GFP+ cells (Fig. 3A), whereas the remainder was a mix of GFP+ and GFP− cells. Therefore, only 0.9% of spheres contained exclusive GFP+ cells. This suggests that the majority of spheres were formed by more than a single initiating cell.

In the second approach, we isolated cells from the primary mammospheres and plated individual cells into each well of a 96-well plate by serial dilution. We observed that 5% of the wells

| Table 2. The sizes and frequencies of mammospheres formed by different fractions of cultured mammary gland cells in a representative experiment |

<table>
<thead>
<tr>
<th>Cell fractions</th>
<th>Sphere features</th>
<th>2,000/well</th>
<th>200/well</th>
<th>50/well</th>
<th>20/well</th>
<th>5/well</th>
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<tbody>
<tr>
<td>CD24+ PrP+ endoglin−</td>
<td>Frequency (well)</td>
<td>1</td>
<td>1.04</td>
<td>0.63</td>
<td>0.17</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Diameter (μm)</td>
<td>138 ± 10</td>
<td>75 ± 4</td>
<td>46 ± 4</td>
<td>36 ± 8</td>
<td>35</td>
</tr>
<tr>
<td>CD24+ PrP− endoglin−</td>
<td>Frequency (well)</td>
<td>0.71</td>
<td>0.17</td>
<td>0.13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diameter (μm)</td>
<td>77 ± 42</td>
<td>36 ± 22</td>
<td>27 ± 18</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>CD24+ PrP+ endoglin−</td>
<td>Frequency (well)</td>
<td>1</td>
<td>0.63</td>
<td>0.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diameter (μm)</td>
<td>70 ± 40</td>
<td>40 ± 42</td>
<td>15</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>CD24+ PrP− endoglin+</td>
<td>Frequency (well)</td>
<td>1</td>
<td>0.75</td>
<td>0.17</td>
<td>0.13</td>
<td>0</td>
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<td></td>
<td>Diameter (μm)</td>
<td>118 ± 15</td>
<td>48 ± 8</td>
<td>30 ± 7</td>
<td>27 ± 4</td>
<td>N/D</td>
</tr>
<tr>
<td>Total cells</td>
<td>Frequency (well)</td>
<td>1</td>
<td>0.75</td>
<td>N/D</td>
<td>N/D</td>
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<tr>
<td></td>
<td>Diameter (μm)</td>
<td>93 ± 36</td>
<td>35 ± 42</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
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</table>

Abbreviation: N/D, not determined.
inoculated with single cells formed sphere-like structures (Fig. 3B).
However, these sphere-like structures were generally smaller in size
compared with the mammospheres that we typically observed.
Because of the positive correlation of the size of the sphere structure
and the introduced cell number (Table 2; data not shown), we
propose that single cells generally tend to form smaller spheres or
sphere-like structures and that accessory cells may help to form the
larger mammospheres that we typically observed. Alternatively,
smaller sphere-like structures may adhere to one another and grow
into larger mammospheres.

Mammospheres contain mammary gland–repopulating
cells. We sought to determine whether the cells present in mam-
mospheres could repopulate the entire mammary gland following
engraftment into cleared mammary fat pads. To this end, we
isolated mammospheres by precultured GFP−CD24+PrP−
cells and also collected all the precultured GFP−CD24−PrP−
cells, which only formed the amorphous nonsphere cell aggregates.
We then trypsinized these various cells and transplanted 2,000 of
each of these dissociated cell populations into cleared fat pads of
recipients. Whereas 2,000 cells from mammospheres derived from
the CD24−PrP− fraction generated entire mammary ductal trees,
2,000 of the cultured CD24+PrP− cells failed to do so (Fig. 4A).
This suggests that some mammospheres contain stem cells that have
the in vivo repopulating activity.

In other studies, Mani et al. (ref. 18 and footnote5) suggested that
there is a link between the property of MGSCs and epithelial-
mesenchymal transition (EMT). It was shown that freshly isolated
MGSCs reduced expression of E-cadherin and increased expression
of N-cadherin and Smad-interacting protein 1 (SIP1; ref. 18 and
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mesenchymal transition (EMT). It was shown that freshly isolated
MGSCs reduced expression of E-cadherin and increased expression
of N-cadherin and Smad-interacting protein 1 (SIP1; ref. 18 and
footnote5). We therefore examined the expression of relevant genes
in cultured sphere-forming cells. As Fig. 4B shows, when compared
with freshly isolated nonstem cells, cultured sphere-forming cells
had increased expression of EMT markers N-cadherin, vimentin,
and SIP1 but decreased levels of epithelial-specific transcription
factors, such as E-cadherin, cytokertatin 14, and cytokertatin 18. This
further suggests that cultured sphere-forming cells shared the
similar molecular machinery as freshly isolated stem cells.

Cytogenetic analysis of mammosphere-forming cells. To get
insight into karyotype constitution of mouse mammary cells after
culture, we sought to conduct spectral karyotyping of freshly and
precultured mouse mammary cells. None of the karyotypes from
freshly isolated cells (Supplementary Fig. S2A and B), regardless of
their ploidy status, contained structurally rearranged chromo-
somes, such as derivatives resulting from deletions or trans-
locations. Precultured cells, on the other side, in addition to diploid
or near-diploid karyotypes without structural rearrangements
(Supplementary Fig. S2C), exhibited sporadic, nonreccurrent
structural chromosomal rearrangements (Supplementary Fig. S2D
and E), such as translocations t(6;19), t(6;12), and t(7;8).

Discussion

We have developed a culture system for growing mouse
mammospheres. Based on this system, we have shown that a
combination of cell surface markers can be used to greatly enrich
mammosphere-initiating cells from the diverse types of cells that
are normally present in the murine mammary epithelium.
Mammospheres are able to regenerate morphologically normal
mammary epithelial ducts on implantation into cleared mammary
fat pads, indicating that at least some of them contain MGSCs.

Our work makes it clear that the mammosphere-initiating cells
show plasticity that becomes apparent when they are introduced
into culture. For example, levels of the CD24 and PrP antigen
expression by mammosphere-initiating cells decrease significantly
following in vitro culture of organoids. Whereas mammosphere-
initiating cells from freshly isolated cells largely reside in the
CD24−PrP1 fraction, the cells from precultured organoids that
can form spheres are enriched in the CD24+PrP− cells. The former
state is more consistent with recent reports showing freshly
isolated MGSCs are CD24+ or CD24med (6–8). A similar shift of
cell surface antigen expression has been well documented in the study
of cultured HSCs (19). For instance, during the ex vivo expansion of
HSCs, several markers for freshly isolated stem cells are partially or
completely lost from the surface of these stem cells. These markers
include PrP. These earlier observations of HSCs indicated that such
shifts in cell surface phenotype do not significantly alter the
marrow-repopulating capability of HSCs; similarly, we found that
in vitro culturing of mammary epithelial cell populations generally

5 S.A. Mani et al., unpublished observation.
maintained the sphere-initiating activity present in these cell populations. More importantly, the spheres formed by the precultured cells could repopulate fat pads. This suggests that the plasticity of cell surface antigen phenotype may be a common phenomenon for a variety of adult stem cells and that such variability is not tightly linked with significant changes in the functioning of stem cell populations.

The expression of PrP by freshly isolated mammosphere-initiating cells was somewhat surprising. PrP is a highly conserved glycosylphosphatidylinositol (GPI)-anchored protein and has been shown to serve as a marker for freshly isolated, long-term repopulating HSCs; its expression is also functionally important for their self-renewal capacity (13). Similar to mammosphere-initiating cells, the expression level of PrP on HSCs is lower than that displayed by certain types of differentiated cells. The expression of PrP in both HSCs and mammosphere-initiating cells suggests that it plays a functional role in multiple types of adult stem cells. Possible related to this is the finding that the infectious prion can be found in the mammary glands of infected animals (20). In addition, Sca-1, another GPI-anchored protein, was also reported to be expressed on both HSCs and MGSC (5). Hence, the physiologic function of PrP and other GPI-anchored proteins displayed by normal MGSC will be an interesting topic for future investigation. Finally, our procedure for enriching mammosphere-initiating cells should prove to be valuable for the elucidation of mammary development and breast cancer carcinogenesis and may be useful for the development of in vitro screening methods for identifying regulators of MGSC growth and differentiation.

We cannot be sure whether the observed aneuploidy in both freshly isolated and precultured mammary gland epithelial cells and sporadic structural rearrangements detected in precultured cells are their intrinsic feature or came as the result of experimental manipulations and prolonged culturing before cytogenetic analysis. It is noteworthy that, due to the limitation of the technique, the originally sorted mammosphere-forming cells underwent significant differentiation after 3 weeks of additional culture before the cytogenetic analyses. Therefore, the karyotyping results might be from both progenitors and differentiated cells. It is known that human embryonic stem cells exhibit chromosomal instability during continuous culturing (21). Yet, the fact that, in spite of sorting and extensive culturing, there still remained a recognizable fraction of karyotypically normal diploid cells is encouraging.

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*6 C.C. Zhang et al., unpublished observation.*
References


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