Award Number: W81XWH-06-1-0702

TITLE: Characterization of Human Mammary Epithelial Stem Cells

PRINCIPAL INVESTIGATOR: Peter D. Eirew

CONTRACTING ORGANIZATION: British Columbia Cancer Agency
Vancouver, BC V5Z 1L3 Canada

REPORT DATE: October 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Characterization of Human Mammary Epithelial Stem Cells

The mammary epithelium of normal adult female mice contains stem cells with extensive in vivo regenerative and self-renewal potential. Analogous cells presumably exist in the mammary glands of adult women, and are candidate targets for the first transforming mutations that lead to the evolution of breast cancer stem cells. The objective of this grant is to develop a robust, quantitative and specific assay for these hypothesized normal human mammary stem cells, to enable identification of markers of these stem cells, to develop a method for their purification, and to derive information about their frequency and how they are regulated. In the first 2 years of this grant, we have established conditions that allow human mammary gland structures to be reproducibly generated in subrenal xenografts in highly immunodeficient mice, starting with small innocula of dissociated human mammary cells. The regenerated glands are similar in morphology and cellular organization to normal human mammary glands, bounded by a basement membrane with an outer layer of myo-epithelial cells and an inner layer of polarized luminal cells that can be induced to produce milk. We have also established that the presence of regenerated structures can be determined by detecting the in vitro clonogenic progenitors they contain and this endpoint can serve as an objective indicator of the presence of a primitive stem-like cell in the initial cells transplanted. This retrospective functional assay allows limiting dilution analysis of positive xenograft yields to derive stem cell frequencies in differently manipulated populations. Using this approach we have found the frequency of stem cells in normal human mammary tissue to be ~1 per 5000 cells and their phenotype to be CD49f+/EpCAM-/low CD31-/CD45-. These findings set the stage for further biological and molecular characterization studies of normal human mammary stem cells and their relationship to human breast cancer stem cells.

14. ABSTRACT

Breast Cancer

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:

17. LIMITATION OF ABSTRACT

18. NUMBER OF PAGES

19a. NAME OF RESPONSIBLE PERSON

USAMRMC

19b. TELEPHONE NUMBER (include area code)
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>- Concept</td>
<td></td>
</tr>
<tr>
<td>- Scientific Progress</td>
<td></td>
</tr>
<tr>
<td>- Training Opportunities</td>
<td></td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>7</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>8</td>
</tr>
<tr>
<td>Conclusion</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>9</td>
</tr>
<tr>
<td>Appendix</td>
<td>10</td>
</tr>
</tbody>
</table>

INTRODUCTION

There is growing evidence that the continued propagation of many human tumors, including breast cancers, is driven by a subset of cells within the tumor referred to as “cancer stem cells”. These cells are thought to originate from normal tissue stem cells and evolve over time through the serial acquisition of genetic and epigenetic alterations. While this concept is at an early stage of investigation in solid tumors, there is considerable potential that this line of study may lead to the development of novel therapeutic strategies that target these key malignant cells. It is therefore critical to understand the mechanisms that regulate normal human mammary stem cells, and how dysregulation or abnormal reactivation of these mechanisms may lead to the development of breast cancer stem cells. The objective of the project supported by this training grant is to develop a robust and reproducible method to detect, isolate and characterize mammary stem cells in normal adult mammary tissue. This report covers the second year of the grant, during which substantial progress has been made in developing a stem cell assay as well as obtaining information on the phenotype and frequency of stem cells in normal human breast tissue.

BODY

1. Concept

Functional mammary epithelial cells are generated continually throughout life through a multi-step differentiation process from a pool of long-lived, self-renewing mammary epithelial stem cells. A simplified depiction of this process is shown on the next page, in which differentiated cells of the two mammary lineages (luminal, myoepithelial) are generated from stem cells via intermediate shorter-lived, clonogenic progenitor cells. These latter populations of progenitor cells have been identified, characterized and partially purified in both human and mouse mammary tissue, and are termed “colony-forming cells” (CFCs) because they are detected by their ability to generate clonal colonies in a 2-D adherent culture system. Evidence of a stem cell population in the mouse mammary gland has been obtained based on detecting the ability of individual rare mammary cells to regenerate an entire mammary tree when transplanted into the mammary stroma of congenic recipient mice (hence the term: “mammary repopulating units”, MRUs). We hypothesize that analogous stem cells exist in the human mammary gland, and that they may also be detected by the development of an appropriate in vivo xenograft procedure.

The objective of this grant is to develop a robust and reproducible strategy to detect human mammary stem cells, and to investigate their biological properties in the normal (i.e., non-cancerous) female adult breast tissue. My approach is to exploit a xenotransplant system, originally shown to allow the propagation of human mammary epithelial fragments and recently adapted successfully for use with dissociated mammary cell suspensions. Human mammary cells are combined with fibroblasts in small collagen gels, then implanted under the kidney capsule of highly immunodeficient, hormone-supplemented mice. After a number of weeks, organized human mammary structures are found to have been regenerated in the xenografts and these contain differentiated cells of both lineages as well as the various progenitor cell types. Assuming these structures and the cells they contain originate from primitive stem-like cells (“human MRUs”), we have developed this system into a quantitative assay tool for these rare cells.
2. **Scientific Progress**

The scientific progress is summarized briefly below, with reference to the specific objectives in the Statement of Work. Greater detail is provided in the manuscript attached in the Appendix, recently published in Nature Medicine journal. References given below refer to figures in the manuscript.

**Aim 1: Development and validation of a functional xenograft assay for human mammary stem cells (months 1-12)**

We have established conditions that reproducibly support the generation of organized human mammary gland structures from dissociated suspensions of previously frozen normal human mammary epithelial cells placed in collagen gels that are then implanted into immunodeficient mice. The regenerated structures resemble normal human mammary tissue and contain both lineages of differentiated mammary cells with the luminal cells showing a normal polarized arrangement surrounded by myoepithelial cells bounded by a basement membrane. The luminal cells can also further mature into milk-secreting cells when the mice undergo pregnancy (Fig. 1a, b).

We have also established that the number of regenerated CFCs detected in xenografts after 4 weeks in vivo serves as a sensitive and objective readout of the presence or absence of primitive MRUs present among the cells originally transplanted, allowing the frequency of MRUs in any population to be quantified by limiting dilution approaches (Fig. 2a-e). Secondary transplants have been carried out, demonstrating that MRUs self-renew in vivo (Supplementary Table 1a, b).

We plan to carry out further validation to test the assumption that human tissue regenerated in this system is clonally derived from individual MRUs. The will involve testing the common origin of multiple regenerated CFCs using input human cells that can be distinguished from one another by genetic (virally-marked) and/or epigenetic (X chromosome inactivation) means.
Aim 2: Development of a robust and reproducible methodology for purifying mammary stem cells from normal primary human breast tissue (months 12-24)

We have screened a number of candidate phenotypic markers for their presence/absence on human MRUs, with the objective of identifying a marker combination that can be used to purify stem cells from normal mammary tissue. These experiments involve measuring the MRU content in subpopulations that are FACS-sorted from reduction mammoplasty samples after staining with antibodies against various candidate stem markers. We have identified an initial set of markers that are expressed by a large majority of MRUs and show a consistent pattern of expression across 9 normal mammoplasty samples. This includes a high expression of CD49f (α6 integrin), low expression of Epithelial Cell Adhesion Molecule (EpCAM), and a lack of expression of hematopoietic and endothelial markers CD45 and CD31 (Fig. 3a-e). Sorting by this phenotype allows MRUs to be purified by about 10-fold compared with unsorted cells and to allow their almost complete separation from luminal restricted progenitors detected as in vitro colony-forming cells (CFCs). We have tested a number of other markers but none of these have proved useful for obtaining further enrichment above what has already been achieved with the markers just named. Additional markers will be tested in the next year with the aim of achieving higher MRU purities.

Aim 3: Biological characterization of normal human mammary stem cells and comparisons with human breast cancer stem cells (months 24-36)

We have used limiting dilution approaches to measure the frequency of MRUs in 5 different normal adult mammary tissue samples. These experiments indicate that MRUs are rare, with measured frequencies of 1 per $10^3 – 10^4$ mammary cells (Supplementary Table 1). Further characterization of the biological properties of MRUs will be carried out during the third year of the grant.

3. Training Opportunities

I have gained hands-on experience of the various in vitro and in vivo techniques used in this project including dissociation of primary mammary tissue, flow cytometry, in vitro mammary progenitor assays and subrenal capsule surgery. I have had the opportunity to present this work orally at 4 scientific conferences, as well as give poster presentations at these and other venues. I have been involved in the preparation of this work as a primary research paper, as well as contributing to the preparation of 4 other primary papers and 1 review paper and in providing instruction to 2 new doctoral students in the methods and concepts that I have learned thus far.

KEY RESEARCH ACCOMPLISHMENTS

- Development and validation of a quantitative methodology to assay for human mammary stem cells
- Identification of a preliminary set of human mammary stem cell markers (CD49f+ EpCAM−/low CD31− CD45−), which allow a stem-cell enriched subset of cells to be isolated from adult human
breast tissue

- Measurement of the stem cell frequency in normal adult human mammary tissue
- Demonstration that normal human mammary stem cells have a phenotype that is distinct from the phenotype of luminal restricted progenitors detectable in colony assays performed in vitro.

REPORTABLE OUTCOMES

1. **Peer reviewed papers**

The following paper was accepted during the reporting period for publication in Nature Medicine journal, and is attached as an Appendix to this report.


2. **Invited talks**


3. **Abstracts**

Peter Eirew, John Stingl, Afshin Raouf, Joanne Emerman and Connie Eaves, “Normal human mammary stem cells detected using a xenotransplant model represent a novel population”, Canadian Stem Cell Network Annual General Meeting, Toronto, ON, November 2007


CONCLUSION

We have made significant progress in the development and validation of a xenotransplant-based methodology to detect primitive human mammary cells (MRUs) with the hallmark features of stem cells (the ability to generate both differentiated lineages, the ability to generate daughter CFCs, and the ability to self-renew). Notably, by combining the transplant procedure with an endpoint “readout” after several weeks in vivo of detectable regenerated CFCs, we have established an objective, quantitative and practical way to detect MRUs in any given test population and to use limiting dilution transplants to quantify their frequency. Such experiments have shown MRUs to be rare cells in normal adult mammary tissue (1 per $10^3$-$10^4$ cells) and to be characterized by a CD49f$^{+}$ EpCAM$^{–/low}$ CD31$^{–}$ CD45$^{-}$ phenotype.

We anticipate a number of benefits from this project, when completed. The establishment of a reproducible methodology to detect human mammary stem cells will be a considerable breakthrough in the field, as none exists at present. When combined with a more developed stem cell purification methodology, it will allow investigators to investigate molecular and cellular mechanisms operating specifically at the level of these important cells, avoiding problems associated with studying bulk populations. Information on how these mechanisms can become dysregulated has the potential to lead to novel therapeutic strategies that specifically target breast cancer stem cells.

Reference List


APPENDIX

Paper published in Nature Medicine journal detailing the scientific results to date from the project funded by this Training Grant:

A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability

Peter Eirew1, John Stingl1,6, Afshin Raouf1, Gulisa Turashvili2, Samuel Aparicio2,3, Joanne T Emerman4 & Connie J Eaves1,5

Previous studies have demonstrated that normal mouse mammary tissue contains a rare subset of mammary stem cells. We now describe a method for detecting an analogous subpopulation in normal human mammary tissue. Dissociated cells are suspended with fibroblasts in collagen gels, which are then implanted under the kidney capsule of hormone-treated immunodeficient mice. After 2–8 weeks, the gels contain bilayered mammary epithelial structures, including luminal and myoepithelial cells, their in vitro clonogenic progenitors and cells that produce similar structures in secondary transplants. The regenerated clonogenic progenitors provide an objective indicator of input mammary stem cell activity and allow the frequency and phenotype of these human mammary stem cells to be determined by limiting-dilution analysis. This new assay procedure sets the stage for investigations of mechanisms regulating normal human mammary stem cells (and possibly stem cells in other tissues) and their relationship to human cancer stem cell populations.

The human mammary gland is a compound tubulo-alveolar structure composed of two lineages of epithelial cells: an inner layer of luminal epithelial cells surrounded by an outer layer of contractile myoepithelial cells. These mature cells are in a state of constant turnover, being continually replaced from more primitive mammary epithelial progenitors. Some of these progenitors can be detected as colony-forming cells (CFCs) in vitro, and, in humans, luminal-restricted, myoepithelial-restricted and bipotent mammary epithelial CFCs can be prospectively isolated as separable subsets1,2. Definitive evidence of more primitive mammary epithelial cells with self-renewal property of stem cells was first provided in mice by mammary fat pad transplantation experiments3. More recently, we and another group showed that the mammary structures produced in this assay are generated from single CD49f+CD29+CD24low mammary repopulating cells (termed mammary repopulating units, or MRUs) that are relatively rare (≈1 per 1 × 105 epithelial cells) in the glands of normal adult virgin female mice4,5.

The presence of mammary stem cells in normal adult women has been inferred from analyses of X-chromosome inactivation patterns indicating a frequent clonal origin of cells in adjacent lobules and ducts6 and from attempts to regenerate mammary gland structures from human mammary epithelial cells (HMECs) transplanted into highly immunodeficient mice. One of these transplantation approaches has relied on colonizing the precleared mammary fat pad of such mice with human fibroblasts to create an environment conducive to the requirements of HMECs7,8. We have been developing an alternative strategy that involves suspending test cells together with irradiated fibroblasts in a collagen gel, which is then implanted under the kidney capsule of estrogen- and progesterone-treated nonobese diabetic severe combined immunodeficient mice (NOD-SCID) mice9, on the basis of previous findings that viable mammary tissue fragments can be maintained at this site10. We now show how this latter protocol, as modified for use with dissociated human mammary cell suspensions, can be used as a quantitative assay for a subset of human mammary cells with stem cell properties and a basal phenotype.

RESULTS
Dissociated HMECs regenerate organized structures in vivo
We initially found that collagen gels seeded with suspensions of normal human mammary cells and irradiated mouse C3H 10T1/2 fibroblasts and then placed under the kidney capsule of hormone-supplemented11 female NOD-SCID mice (or derivative strains) contained regenerated epithelial structures when the gels were removed and examined 2–8 weeks later (Fig. 1a,b). These structures included both round and elongated duct-like arrangements of cells organized as a polarized bilayered stratified epithelium enclosing a lumen and surrounded by a basement membrane containing laminin and collagen IV (Fig. 1b). The cells in the inner and outer layers expressed established markers of differentiated mammary luminal and myoepithelial cells respectively. Cells expressing nuclear estrogen receptor-α and cells expressing progesterone receptors were also present. Overall, the spatial distribution of cellular markers in regenerated structures was similar to that seen in normal human mammary tissue.

1Terry Fox Laboratory and 2Molecular Oncology and Breast Cancer Program, British Columbia Cancer Agency, 675 West Tenth Avenue, Vancouver, British Columbia V5Z 1L3, Canada. 3Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Westbrook Mall, Vancouver, British Columbia V6T 2B5, Canada. 4Department of Cellular & Physiological Sciences and 5Department of Medical Genetics, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada. 6Present address: Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 ORE, UK.

Correspondence should be addressed to C.J.E. (ceaves@bccrc.ca).

Received 3 March; accepted 6 June; published online 23 November 2008; doi:10.1038/nm.1791
We also saw many cells that expressed the proliferation marker Ki67 (Fig. 1b) and cells with diffuse chromatin (data not shown), consistent with the interpretation that the structures are produced by an ongoing regenerative process. Some apoptotic cells were also evident (data not shown). These structures were obtained from every human mammary-plasty sample tested when at least 1 × 10^5 cells were transplanted.

When the female hosts were mated 1 week after the gels had been placed in the mice and the structures were examined 3 weeks later, luminal cells with vacuolated cytoplasm that stained positive for human β-casein (a component of human milk) were prevalent (Fig. 1b). They also had hyperchromic, slightly pleomorphic nuclei, typical of cells in human lactating mammary tissue (data not shown).

Regenerated CFCs serve as a read-out of transplanted MRUs
To test for the presence of mammary progenitors in the regenerated structures, we prepared single-cell suspensions from the removed gel-xenografts and plated the cells in vitro in two-dimensional CFC assays (Fig. 2a). We found all types of mammary CFCs (luminal-restricted, myoepithelial-restricted and bipotent) to be readily detectable in the xenografts for up to 12 weeks, and these CFCs grew into colonies that
were indistinguishable from those derived from primary mammoplasty tissue (Fig. 2b–d). Hereafter, we will refer to these regenerated CFCs as secondary CFCs to discriminate them from the primary CFCs present in initial suspensions of dissociated mammoplasty tissue. Transplant cell dose-response experiments further showed that the number of secondary CFCs present in xenografts after 4 weeks is linearly related to the number of human mammary cells originally suspended in the gels (Fig. 2e).

We then performed a series of limiting-dilution transplant experiments to determine the frequency of cells that are responsible for regenerating structures containing secondary CFCs at 4 weeks after transplant. A total of 107 gels were analyzed, each seeded with 500–60,000 cells from freshly thawed, organoid-enriched human mammary tissue (five separate experiments, Supplementary Table 1 online). Chi-squared tests showed the results were consistent with a single-hit Poisson model in each of the five experiments, supporting the interpretation that multiple secondary CFCs are derived from a single common human mammary repopulating cell or unit (human MRU). The frequency of MRUs calculated from these experiments was 1 per $1 \times 10^3$ to $1 \times 10^4$ mammoplasty cells, one to two orders of magnitude lower than the frequency of (primary) bipotent CFCs measured in the same original samples. From the frequency of MRUs determined and the total secondary CFC numbers measured, each MRU was found to generate, on average, 4.1 ± 0.6 daughter CFCs.

**Human MRUs have a CD49f+ EpCAMneg–low phenotype**

We next asked whether these transplantable human MRUs belong to a phenotypically distinct subset of mammary epithelial cells. Accordingly, we isolated various subsets of cells from nine different human mammary samples after staining them with antibodies to CD49f and epithelial cell adhesion molecule (EpCAM, also known as CD326; Fig. 3a,b). In six of the nine experiments, we simultaneously removed contaminating hematopoietic (CD45+) and endothelial (CD31+) cells. We plated an aliquot of each of the subsets shown in Figure 3b into a primary CFC assay and suspended the remaining cells in gels in numbers proportionate to their fractional yields (a total of 119 gels), and we then implanted the gels into mice. Most primary luminal-restricted CFCs (72 ± 10%) were confined to the CD49f+EpCAMneg–low fraction, whereas most primary bipotent (77% ± 11%) and myoepithelial-restricted (97 ± 2%) CFCs were concentrated in the CD49f+EpCAMneg–low fraction (Fig. 3c). The CD49f EpCAMneg–low fraction was mostly devoid of primary CFCs (data not shown). Notably, grafts in which secondary CFCs were detected 4 weeks later were almost exclusively those initiated with cells from the CD49f+EpCAMneg–low fraction (92 ± 3% of the CFCs detected in all xenografts were obtained in gels initially seeded with CD49f+EpCAMneg–low cells). Structures observed in these 4-week-old xenografts showed the same spectrum of CD49f+ and/or EpCAM+ cells detectable by flow cytometry as in primary normal human mammary tissue (Fig. 3d), and, upon immunohistochemical analyses in situ, a polarized organization of cells expressing luminal and myoepithelial markers bounded by a basement membrane (Fig. 3e) was again seen (Fig. 1b).

Because CD49f expression has been associated with basally located cells in the mouse mammary gland, we asked whether another marker of basal cells, CD10 (also called common lymphocyte leukemia antigen, or CALLA), would be expressed on the human MRUs detected by our gel transplant assay. The results of two experiments showed that most secondary CFCs (70% in the first experiment and 86% in the second) originated from CD10+ cells. However, 95 ± 3% of the CD49f EpCAMneg–low cells were found to be CD10+, indicating that isolation of CD10+ cells would not yield a purer population of MRUs.

**Human MRUs can be serially transplanted**

To determine whether human mammary cells defined functionally as MRUs on the basis of their in vivo CFC-regenerating activity also have self-renewal ability, we performed secondary transplantation assays. For these experiments, we implanted primary grafts containing...
BFGs initiated with larger numbers (gels, indicating that MRUs had been regenerated in the primary gels regenerated CFCs also regenerated detectable CFCs in the secondary online). In most cases, primary gels that contained with fresh feeder cells and suspended them together in new secondary We combined the remaining 70% of the cells from the primary gels from them and then plated 30% of each suspension in a CFC assay to detectable CFCs in the secondary recipients. (Supplementary Fig. 1a online). In most cases, primary gels that contained regenerated CFCs also regenerated detectable CFCs in the secondary gels, indicating that MRUs had been regenerated in the primary gels (Supplementary Fig. 1b). Of note, similar assays of primary gels initiated with larger numbers (~1 × 10^5) of cells from other (that is, MRU-depleted) fractions produced few or no CFCs in secondary recipients.

**DISCUSSION**

Here we describe a new, robust and objective protocol for determining the frequency of cells that meet the rigorous definition of human mammary epithelial stem cells with both in vivo regenerative potential and self-renewal activity demonstrable in secondary transplants. We also show that the structures that these cells produce after 4 weeks in this assay contain the same hierarchy of primitive and mature epithelial cell types as is found in the normal endogenous human mammary gland and that the regenerated cells are most frequently derived from a rare subset of cells with a distinct CD49f^+EpCAM^−low basal phenotype. Notably, during the course of their production in this in vivo system, the regenerated and differentiating human mammary cells also self-organize to form a three-dimensional mammary gland structure that appears similar to normal mammary tissue and is capable of physiological maturation.

We also show that the number of CFCs in 4-week-old structures serves as a sensitive and quantitative endpoint for human mammary stem cells in the original cell suspension assayed, and their detection as an endpoint avoids the difficulties associated with reliance on a histological approach. This concept is similar to the strategy commonly used to identify very primitive subsets of mouse or human hematopoietic cells referred to as long-term culture-initiating cells by virtue of their ability to generate hematopoietic CFCs detectable after 5–6 weeks in cultures containing stromal feeder layers. In the hematopoietic system, it was shown that the hematopoietic CFCs detected after 5–6 weeks must have originated from a more primitive cell type, as the cells from which they were derived had a different phenotype. In addition, it was shown that the CFCs in the cultures were continuously proliferating and differentiating, making simple persistence an unlikely explanation for their presence. Here we have also shown evidence of proliferative activity within the regenerated structures. In addition, for at least one of the mammary CFC types detected (the luminal-restricted CFCs), it was possible to show a clear difference in phenotype as compared with the cells that produced secondary mammary CFCs detectable 4 weeks later.

The ability to assay the in vivo mammary regenerative activity of dissociated cells is a major advance, as it enables the intrinsic developmental potential of individual cells to be investigated. It also provides renewed support for the concept that the full developmental properties of human mammary stem cells can be expressed in the absence of other cells in the epithelium, in keeping with similar findings for mouse MRUs. EpCAM in the normal resting human breast is highly expressed by luminal epithelial cells and is less expressed by basal cells. In contrast, CD49f (α6 integrin) has an inverse pattern of expression.

Thus, the observed CD49f^+EpCAM^−low phenotype of MRUs suggests a basal location of these cells in situ. Consistent with this expectation is the previous observation that most of the cells in the CD49f^+EpCAM^−low fraction also express cytokeratin-14 (a myo-epithelial marker) and not cytokeratin-19 (a luminal cell marker). In this regard, our present findings for human MRUs mirror those previously reported for mouse MRUs, which also show a basal phenotype. In contrast, we find a marked difference between the phenotype of human MRUs and the reported CD49f^+EpCAM^high phenotype of HMECs that form branched structures in Matrigel, raising concerns that this Matrigel-based readout may not provide a useful surrogate assay for human mammary stem cells.

The assay described here should allow further enrichment of human MRUs to be achieved. It will also enable related studies of the biological properties and molecular regulation of MRUs of their ability to be transformed by specific oncogenes and of their relationship to cells that propagate various types of spontaneously arising human breast cancers. In this latter regard, it is noteworthy that CD49f is expressed by a subset of cells within the human MCF7 breast cancer cell line that have tumorigenic potential in immunodeficient mice. We thus expect that the xenograft strategy that lies at the heart of our assay will provide a new system to investigate the mechanisms that control normal human mammary stem cell proliferation and differentiation in vivo and the sensitivity of these cells to agents that promote or interfere with these processes. Indeed, it may be anticipated that this in vivo approach will prove useful for the characterization of stem cell populations in other normal human tissues with, where with the exception of the hematopoietic system, a vacuum currently exists.

**METHODS**

**Mice.** We bred and housed female NOD-SCID, NOD-SCID β2-microglobulin-null and NOD-SCID interleukin-2 receptor-γcnull mice at the animal facility at the British Columbia Cancer Research Centre. Unless otherwise specified, the data we present was generated with NOD-SCID interleukin-2 receptor-γcnull mice as transplant recipients. We carried out surgery on mice between the ages of 5 weeks and 8 weeks. All experimental procedures were approved by the University of British Columbia Animal Care Committee.

**Dissociation of human mammary tissue.** We collected anonymized discard tissue from normal premenopausal women (ages 19–40) undergoing reduction mammoplasty surgery with informed consent according to procedures approved by the University of British Columbia Research Ethics Board and processed the tissue as previously described. Briefly, we transported the tissue from the operating room on ice, minced it with scalpels and then dissociated it for 18 h in Ham’s F12 and DMEM (1:1 vol/vol, F12 to DMEM, StemCell Technologies) supplemented with 2% wt/vol BSA (Fraction V; Gibco Laboratories), 300 U ml^-1 collagenase (Sigma) and 100 U ml^-1 hyaluronidase (Sigma). In some experiments, this medium was supplemented with 10 ng ml^-1 epidermal growth factor (EGF, Sigma), 10 ng ml^-1 cholera toxin (Sigma), 1 µg ml^-1 insulin (Sigma), 0.5 µg ml^-1 hydrocortisone (Sigma) and 5% FBS (StemCell Technologies). We obtained an epithelial-rich pellet by centrifugation at 80g for 4 min and cryopreserved it in 6% dimethylsulfoxide-containing medium at −135°C until use. We subsequently prepared single-cell suspensions from freshly thawed pellets by treatment with 2.5 mg ml^-1 trypsin supplemented with 1 mM EDTA (StemCell Technologies), washing once with HBSS (StemCell Technologies) supplemented with 2% FBS followed by treatment with 5 mg ml^-1 dispase (StemCell Technologies) and 100 µg ml^-1 DNase1 (Sigma), after which we passed the cell suspension through a 40-µm filter (BD Biosciences) to remove remaining cell aggregates.

To recover cells from the xenografted gels, we killed recipient mice and aseptically removed the gels from the kidneys under a dissecting microscope. We then dissected the gels for 4.5 h at 37°C in EpiCult-B medium (StemCell Technologies) supplemented with 5% FBS, 600 U ml^-1 collagenase and...
200 U ml−1 hyaluronidase. After digestion, we washed the cells once and treated them for 5 min with prewarmed trypsin-EDTA with gentle pipetting.

**In vitro mammary colony-forming cell assay.** We incubated 60-mm tissue culture dishes for 1 h at 37 °C with a 1:43 dilution of Vitrogen 100 collagen (Collagen Biotechnologies) in PBS (StemCell Technologies). We seeded each dish with test cells obtained from primary tissue or digested collagen gels combined with 2.0 × 10^6 freshly thawed, previously irradiated (with 50 Gy) NIH 3T3 mouse fibroblast cells in 4 ml of Epipart-B medium (StemCell Technologies) supplemented with 5% FBS and 0.5 μg ml−1 hydrocortisone. We incubated cultures at 37 °C and 5% CO_2_, with a change to serum-free Epipart-B plus 0.5 μg ml−1 hydrocortisone 1 d later. In some experiments, we replaced the Epipart-B medium with DMEM and F12 supplemented with 0.1% BSA, 10 ng ml−1 EGF, 10 ng ml−1 cholera toxin, and 1 μg ml−1 insulin. After 7–10 d, we briefly fixed dishes in a 1:1 vol/vol mixture of methanol and acetone at 20 °C, stained them with Wright’s Giemsa (Sigma) and visually scored the colonies under a dissecting microscope. We routinely categorized colonies into subtypes as follows: tightly-clustered cells with smooth colony boundary, luminal; dispersed teardrop-shaped cells, myoepithelial; colony containing both these elements and a ragged colony boundary, bi-lineage; although, in some cases, colonies were stained for specific human cytokertins and human mucin-1 (MUC1) to confirm the presence of either or both of these lineages.

**Preparation and assessment of collagen gels.** We prepared concentrated rat’s tail collagen as previously described^14 and sterilized it at ~20 °C. We thawed aliquots and neutralized the pH immediately before use by adding two parts (vol/vol) concentrated sodium hydroxide to 78 parts concentrated collagen solution and 20 parts 5 × DMEM. To prepare gels, we collected C3H10T1/2 mouse embryonic fibroblasts (a kind gift from G. Cunha) from subconfluent cultures, X-ray irradiated them (with 15 Gy), mixed them with dissociated human mammary cells and resuspended them in cold neutralized collagen. We added 25-μl aliquots containing 2.2 × 10^5 10T1/2 cells and the desired number of human test cells into individual wells of a 24-well plate. We allowed the gels to stiffen at 37 °C incubator for 10 min and then incubated them in warm Epipart-B medium plus 5% FBS for 50 min. We then kept the plates on ice until all gels had been transplanted. In some of the early experiments, we used cells from a telomerase-immortalized human adult mammary fibroblast line or primary human mammary fibroblasts instead of C3H10T1/2 fibroblasts.

**Subrenal xenotransplantation surgery.** We shaved the hair on the backs of anesthetized mice and snipped the skin with 70% alcohol. We made an anterior to posterior incision approximately 1.5 cm long dorsally around the area of the kidneys. We also made a small incision in the abdominal wall above one kidney and exteriorized the kidney by applying gentle pressure on either side. Under a dissecting microscope, we lifted the kidney capsule from the parenchyma with fine forceps and made a 2–4-mm incision in the capsule. We inserted up to three gels under the capsule with a fire-polished glass pipette tip. After suturing the incision in the abdominal wall, we repeated the procedure (if required) on the contralateral kidney. Finally, we inserted a slow-release pellet (Med-4011 silicone, NuSil Technology) subcutaneously in a posterior position before suturing the midline incision. This protocol was previously shown to produce sustained serum levels of these hormones in the mouse approximately equivalent to those at the human midluteal phase peak^15. In some experiments, we mated the mice 9 d after the gels were transplanted.

**Cell separation.** We preblocked mammary cell suspensions in HBSS supplemented with 2% FBS and 10% human serum (Sigma), and then labeled them with an allop hyocyanin-conjugated rat antibody to human CD49E (clone GOH3, R&D Systems) and FITC-conjugated mouse antibody to human EpCAM (clone VU1-D9, StemCell Technologies). In some experiments, we also labeled hematopoietic and endothelial cells with biotin-conjugated mouse antibodies to human CD45 (clone H16, Biolegend) and human CD31 (clone WM59, eBiosciences), respectively, followed by R-phycocerythrin-conjugated streptavidin (BD Biosciences). We added propidium iodide (Sigma) at 1 μg ml−1 for live/dead cell discrimination. We performed all sorts on either a FACSVantageSE or a FACSDiva (Becton Dickinson).

**Immunohistochemistry.** We processed deparaffinized 4-μm sections of paraformaldehyde-fixed collagen gels for immunohistochemistry with a Discovery XT automated system (Ventana Medical Systems). We applied primary antibodies to estrogen receptor-α (clone 6F11, Ventana), progesterone receptor (clone 1A6, Ventana), Ki67 (clone K2, Ventana), cytokeratin-14 (clone LL002, ID Labs), cytokeratin-18 (clone Ks 18.04, Progen), laminin (polyclonal, Sigma), collagen IV (clone 4C8, Sigma) and β-casein (clone F14.20, Harlan Laboratories). We then applied horseradish peroxidase–conjugated Discovery Universal Secondary Antibody (Ventana) and developed the slides with the 3,3’-diaminobenzidine (DAB) Map Kit (Ventana). We processed some slides manually with primary antibodies to MUC1 (clone 214D4, StemCell Technologies) or smooth muscle actin (polyclonal, Abcam), each followed by alkaline phosphatase-conjugated Envision-AP (Dako) and developed in FastRed (Sigma). We counterstained all slides with hematoylin. For dual-color staining of colonies, we fixed 60-mm culture dishes briefly in 1:1 vol/vol methanol and acetone and preblocked them in Tris-buffered saline containing 5% wt/vol BSA and 10% FBS. We then incubated the dishes sequentially with an unconjugated antibody to MUC1, alkaline phosphatase-conjugated Envision-AP, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development solution (Sigma), biotin–conjugated antibody to cytokeratin-14 (clone LL1002, Labvision), horseradish peroxidase–conjugated streptavidin (Jackson Immunoresearch) and DAB.

**Statistical analyses.** Data are expressed as the arithmetic mean ± s.e.m. except for MRU frequencies. We calculated MRU frequencies with single-hit Poisson statistics and the method of maximum likelihood using L-Calc software (StemCell Technologies), and the values obtained are shown with the derived 95% confidence interval. We tested goodness of fit to a single-hit model using standard chi-squared statistics.

**Note:** Supplementary information is available on the Nature Medicine website.

**ACKNOWLEDGMENTS**

C3H10T1/2 mouse embryonic fibroblasts were a kind gift from G. Cunha, University of California, San Francisco. The authors acknowledge the excellent technical contributions of D. Wilkinson, G. Edin, the staff of the Flow Cytometry Facility of the Terry Fox Laboratory and the Centre for Translational and Applied Genomics. Mammoplasty tissue was obtained with the assistance of J. Sproul, P. Lennox, N. Van laaen and R. Warren. This project was funded by grants from Genome British Columbia and Genome Canada, the Canadian Stem Cell Network and the Canadian Breast Cancer Foundation British Columbia and Yukon Division. P.E. was a recipient of a US Department of Defense Breast Cancer Research Program Studentship, a Terry Fox Foundation Research Studentship from the National Cancer Institute of Canada, a Canadian Imperial Bank of Commerce interdisciplinary award and a Canadian Stem Cell Network Studentship. J.S. held a Canadian Breast Cancer Foundation British Columbia and Yukon Division Fellowship and a Canadian National Science and Engineering Research Council Industrial Fellowship. A.R. held a Canadian Breast Cancer Foundation British Columbia and Yukon Division Fellowship and a Canadian Institutes of Health Research Fellowship. G.T. holds a Canadian Institutes of Health Research Pathology Training Fellowship. S.A. is supported by a Canada Research Council Industrial Fellowship. A.R. held a Canadian Breast Cancer Foundation British Columbia and Yukon Division Fellowship and a Canadian Institutes of Health Research Fellowship.

**AUTHOR CONTRIBUTIONS**

P.E. designed and conducted most of the experiments and drafted the manuscript. J.S. initiated the work that led to the gel implant protocol, undertook preliminary experiments and contributed to the writing of the manuscript. A.R. critiqued the manuscript and participated in discussions of the experiments. G.T. and S.A. reviewed the histological preparations and contributed to the writing of the manuscript. J.T.E. helped organize the accrual of the mammoplasty material used. C.I.E. conceptualized the study and finalized the writing of the manuscript.

**Published online at** http://www.nature.com/naturemedicine/

Reprints and permissions information is available online at http://npg.nature.com/ reprintprintsandpermissions


### Supplementary Table 1. Measurements of MRU frequency by limiting dilution analysis.

Results from 5 experiments showing that the regeneration of CFCs in xenografted gels seeded with varying numbers of input human mammary cells 4 weeks previously fits a single hit model, indicating the origin of the CFCs from a single cell (the MRU) whose frequency can be calculated using Poisson statistics. The frequency of MRUs thus quantified was generally one to two orders of magnitude lower than the frequency of CFCs in the same initial sample. From the total number of CFCs detected in gels seeded with such derived numbers of MRUs, an average 4-week yield of $4.1 \pm 0.6$ secondary CFCs per input MRU was determined.

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Recipient mouse strain</th>
<th>Cells +ve gels/ per total gel gels</th>
<th>MRU freq. in sample (95% CI)</th>
<th>Chi squared test for consistency with 1-hit model</th>
<th>Average regenerated (secondary) CFCs per MRU</th>
<th>Primary CFC freq. in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lum</td>
</tr>
<tr>
<td>#1</td>
<td>NS/IL-2Rγc/-</td>
<td>3,000 3/6 10,000 5/6 25,000 6/6 60,000 6/6</td>
<td>4,890 (2,380-10,080)</td>
<td>0.99</td>
<td>3.7</td>
<td>46</td>
</tr>
<tr>
<td>#2</td>
<td>NS/IL-2Rγc/-</td>
<td>1,500 1/5 4,500 6/6 12,000 6/6 25,000 6/6</td>
<td>2,220 (1,060-4,690)</td>
<td>0.45</td>
<td>5.9</td>
<td>38</td>
</tr>
<tr>
<td>#3</td>
<td>NS/IL-2Rγc/-</td>
<td>500 1/6 2,000 5/6 7,000 6/6 18,000 6/6</td>
<td>1,390 (640-2,960)</td>
<td>0.87</td>
<td>2.3</td>
<td>151</td>
</tr>
<tr>
<td>#4</td>
<td>NS/B2m/-</td>
<td>800 0/6 3,000 0/6 10,000 3/4 22,000 6/6</td>
<td>9,840 (4,910-19,700)</td>
<td>0.31</td>
<td>3.5</td>
<td>37</td>
</tr>
<tr>
<td>#5</td>
<td>NS</td>
<td>2,000 5/7 20,000 7/7</td>
<td>1,600 (630-4,060)</td>
<td>1.00</td>
<td>5.2</td>
<td>43</td>
</tr>
</tbody>
</table>
**Supplementary Figure 1. MRUs can be serially transplanted.** (a) Experimental protocol. Cells isolated by FACS from the CD49fEpCAMneg−lowCD31−CD45− (MRU-enriched) fraction or other fractions were transplanted into primary (1o) recipients. Four weeks later, 30% of the cells from each 1o gel were used to identify those that contained detectable CFCs. The remaining 70% of the cells were transplanted into secondary (2o) recipients. Another 3½ weeks later, CFC assays were performed on the cells harvested from these 2o gels. (b) Results. Data from 3 serial transplant experiments performed as described in (a) are shown. Both the frequency of gels implanted in 1o and 2o hosts in which at least 1 CFC was detected and, in brackets, the average number of CFCs in the assayed portion of 1o and 2o gels are indicated.