DETECTION AND CHARACTERIZATION OF HUMAN MAMMARY STEM CELLS

by

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ABSTRACT

The mammary gland of adult female mice contains undifferentiated epithelial stem cells with \textit{in vivo} regenerative and self-renewal properties. A biologically similar population likely exists in the human breast but a specific and quantitative methodology to identify and characterize these cells has been lacking. In this study I show that human mammary structures are reproducibly generated when dissociated suspensions of primary human mammary cells are propagated in collagen gels that have been implanted under the renal capsule of highly immunodeficient, hormone-treated mice. These structures contain differentiated cells of both mammary lineages in a spatial organization similar to normal mammary tissue, and display functional maturation into milk-producing glands when the hosts become pregnant. \textit{In vitro} assays of single cell suspensions prepared from these regenerated glands revealed the consistent presence of mammary progenitor cells able to form adherent bi-lineage as well as pure luminal and myoepithelial colonies. In addition, when these cells are suspended in new gels and transplanted into secondary immunodeficient mice, similar progenitor-containing structures are demonstrable, indicative of a regenerative process that recreates the normal developmental hierarchy. This daughter progenitor production endpoint allows the frequency of these self-renewing human mammary stem cells to be derived from limiting dilution transplant assays as 1 per $10^3$–$10^4$ cells in normal adult human reduction mammoplasty samples, and their phenotype to be established as CD49f$^+\text{EpCAM}^{-/\text{low}}\text{CD31}^-\text{CD45}^-$. I have also developed methodologies to isolate fractions of cells from mammoplasty tissue that are enriched in cells in different phases of the cell cycle (G0/G1/S/G2/M). Application of functional
assays to these fractions indicates that a proportion of stem and progenitor cells in normal adult breast tissue exhibit phenotypes that are associated with actively proliferating cells. These studies support a model of mammary cell production that includes a significant rate of normal turnover of primitive cells, and sets the stage for further work to identify the factors and molecular interactions that regulate this process.
PREFACE

I designed and conducted all the experiments presented in Chapters 2, 3 and 4 and drafted the Thesis manuscript. The xenotransplantation methodology that forms the core of this study built upon work initiated by John Stingl, and also incorporated the in vitro mammary progenitor assay methodology developed by John Stingl and refined by Afshin Raouf. Gulisa Turashvili and Sam Aparicio reviewed the histological preparations. Joanne Emerman helped organize the accrual of the mammoplasty material used. Robert Kay, Aly Karsan and Shoukat Dedhar guided the project as Committee members and reviewed the manuscript. Connie Eaves conceptualized the study, guided the design of experiments and finalized the writing of the manuscript.

Animal experimental protocols were approved by the University of British Columbia Animal Care Committee (certificate: A06-1520).

The following publications arose from this Thesis work and are incorporated into the material presented in Chapters 2 and 3. They are attached as Appendices A and B.


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ABBREVIATIONS

\(^3\text{H}-\text{Tdr}\) methyl-tritiated thymidine
ALDH aldehyde dehydrogenase
AP alkaline phosphatase
APC allophycocyanin
BAA bodipy aminoacetate
BAAA bodipy aminoacetaldehyde
BrdU 5-bromo-2'-deoxyuridine
CFC colony-forming cell
CK cytokeratin
DAB 3,3’-diaminobenzidine
DAPI 4’,6-diamidino-2-phenylindole
DEAB diethylaminobenzaldehyde
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
EMT epithelial to mesenchymal transition
ER estrogen receptor
FBS fetal bovine serum
FGF fibroblast growth factor
FITC fluorescein isothiocyanate
hESC human embryonic stem cell
HF Hanks Buffered Saline Solution supplemented with FBS
HMEC human mammary epithelial cell
HRP horseradish peroxidase
IGF insulin-like growth factor
MP main population
MRU mammary repopulating unit
NOD-SCID non-obese diabetic, severe combined immunodeficient
NOG NOD-SCID interleukin-2 receptor gamma null
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal lobular unit</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
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To my parents
1. INTRODUCTION

1.1 Mammary gland structure and development

The human adult female mammary gland comprises a continuous branching network of hollow epithelial tubes termed “ducts” (Fig. 1-1a). At their ends are clusters of round, secretory “alveoli” which, together with the terminal ducts, are termed “terminal ductal lobular units” (TDLUs) ¹. The ductal-alveolar tree extends radially from the nipple to form 5-10 discrete lobes. These lobes are embedded in a collagen-rich intra-lobular stroma that contains an assortment of cell types, including fibroblasts, adipocytes, endothelial cells and hematopoietic cells, further surrounded by a looser, primarily adipose inter-lobular stroma. There is considerable heterogeneity in the degree of lobular complexity between adults and even within a single breast². In rodents, the number of pairs of mammary glands is greater (6 in rats, 5 in most strains of mice). These comprise less complex branched ductal-alveolar trees and are embedded in a predominantly adipose stroma³ (Fig. 1-1b).

The mammary gland undergoes profound developmental and morphological changes after birth and extending into late adulthood (Fig. 1-2). It develops initially from the embryonic epidermis and then expands to create a rudimentary ductal network during gestation⁴. After birth the organ becomes largely quiescent until the onset of puberty when the ducts extend and penetrate and branch into the stroma, led by specialized multi-layered “terminal end buds” (TEBs) that form at the tips of the major ducts. Pregnancy is associated with a massive burst of proliferation in the mammary epithelium, further ductal side branching, and the appearance of greatly increased numbers of secretory alveolae at the termini of the newly generated ducts. In mice, a 27-fold increase in
epithelial cell number has been measured in comparisons of the mammary gland from
virgin and pregnant females. Following weaning, the epithelial cells in the gland
undergo large scale apoptosis and the gland remodels to resemble its pre-pregnant state.
Similar but much less extensive amplifications and regressions occur during each
menstrual cycle.

In both human and rodents the entire epithelial tree is lined by a bilayered
epithelium enclosing a hollow lumen and surrounded by a basement membrane (Fig. 1-3). An inner layer of differentiated secretory luminal epithelial cells lines the ducts and
lobules. These cells generate milk in the distal alveolae during lactation. An outer layer
of contractile smooth muscle-like myoepithelial cells directs the milk down the ductal
network to the nipple. The myoepithelial layer is continuous in the ducts, with a more
dispersed arrangement in the alveolae. The latter arrangement allows luminal cells in the
alveolae to be in direct contact with the basement membrane. The epithelium is also
presumed to contain primitive cell populations with the regenerative capacity to support
tissue homeostasis and well as the dramatic remodelings that accompany puberty,
menstrual cycles, pregnancy and lactation. In recent years, there has been intensive effort
to identify these cells and understand their regulation.

1.2 Evidence for a stem cell hierarchy in the normal mammary epithelium

The existence of stem cells in the mammary female gland has long been inferred from the
physiological regenerative capacity of its epithelium, and from observations of regions of
contiguous X-chromosome inactivation in normal human breast tissue. Experimentally, the in vivo regenerative capacity of the mouse mammary epithelium has
been extensively studied using the “cleared fat pad” transplantation strategy initially described by DeOme and colleagues\textsuperscript{11}. This procedure involves surgically removing an anatomically-defined portion of the #4 fat pad in pubescent (21–24 day-old) mice, leaving an epithelium-free portion that can be used as a transplantation site to evaluate the regenerative potential of injected test tissue or cell populations. Both normal and premalignant tissue fragments as well as dissociated suspensions of mammary cells have been found to grow in such cleared mammary fat pads within several weeks. Furthermore, if the hosts are subsequently made pregnant, full functional differentiation of the regenerated mammary epithelial cells into milk producing cells can also be obtained.

Early studies using this strategy showed that tissue fragments from any part of the mammary tree are able to regenerate an entire mammary gland following their transplantation. These findings implied that the responsible regenerative “units” are dispersed throughout the mammary tree\textsuperscript{12,13}. The regenerated glands could be serially passaged through many subsequent generations before becoming senescent, and donor age, reproductive history and developmental state did not affect the number of passages that could be performed before senescence was reached. However, senescence was reached earlier when fragments at each passage were taken from the periphery rather than the centre of the mammary tree. This suggested that different regenerative units had different overall lifespans governed by the number of mitotic divisions they had undergone rather than their chronological or metabolic age, the peripheral cells presumed to have undergone more divisions than those at the centre of the gland. More recent studies have identified various molecules that can overcome this transplantation-induced
senescence; for example, by homozygous p53 deletion or activation of oncogenes by mouse mammary tumor virus insertion\textsuperscript{13,14}.

Clonal tracking studies provided the first evidence that the \textit{in vivo} repopulating units could be single cells. Using retrovirally-infected cells and following integration site patterns through serial generations, it was demonstrated that an entire regenerated mammary tree could be the clonal progeny of a single cell within the initial transplant\textsuperscript{15}. This was confirmed directly in recent years when it was shown that an entire functional mammary tree could be regenerated from transplants of single dissociated epithelial cells. Cells with the ability to regenerate mammary trees (termed “mammary repopulating units”, MRUs) were characterized by a CD24\textsuperscript{med}CD49f\textsuperscript{high}CD29\textsuperscript{high}Sca-1\textsuperscript{med}CD45\textsuperscript{–}Ter119\textsuperscript{–}CD31\textsuperscript{–} phenotype\textsuperscript{16,17}. Limiting dilution serial transplants further showed that mouse MRUs have the capacity to undergoing at least 10 symmetrical self-renewing divisions\textsuperscript{16,17}. Based on their capacity for extensive self-renewal and multi-lineage regenerative capacity, MRUs meet current operational criteria used to define a cell as a stem cell.

Quantitation of MRU frequencies using limiting dilution analysis indicated an MRU frequency of \(~1\) per 1,500 cells in dissociated cell suspensions prepared from the mammary tissue of young adult FVB or C56Bl/6 mice. This equates to \(~1,500\) MRUs per mammary fat pad\textsuperscript{16}. Subsequent studies have measured MRU frequencies as high as 1 per 100 total mammary cells\textsuperscript{18}, using a modified protocol in which test cell innocula are suspended in reconstituted basement membrane (Matrigel\textsuperscript{TM}) prior to transplantation. It is not clear whether this makes the MRU assay more sensitive (i.e., improves the detection efficiency) or less selective (i.e., widens the range of cell types that register a
positive readout). In other epithelial systems the presence of Matrigel has been also reported to enhance the in vivo engraftment frequency of cell lines\textsuperscript{19, 20} and primary cells\textsuperscript{21, 22}.

Attempts to develop approaches analogous to the fat pad transplant assay, but suitable for detecting human mammary stem cells, have been hindered by challenges in propagating human mammary cells in vivo. Primary human mammary organoid fragments survive but do not readily proliferate when transplanted into the fat pads of immunodeficient mice\textsuperscript{23, 24}. This has been attributed to inappropriate murine stromal cell signaling to human epithelial grafts, a reflection of the very different mammary stromal composition in the two species. These considerations have encouraged the development of xenotransplant approaches that incorporate additional stromal elements to support the human cells. One involves combining human mammary organoids\textsuperscript{25} or suspensions of dissociated human mammary epithelial cells (HMECs)\textsuperscript{26} with fibroblasts in collagen gels which are then implanted under the renal capsule of immunodeficient hormone-supplemented mice. This site is highly vascular and has successfully supported the engraftment of a variety of normal and neoplastic human epithelial tissues\textsuperscript{27-36}. The second approach relies on pre-colonizing the cleared mouse mammary fat pad with human fibroblasts a few weeks prior to transplanting human tissue fragments or cell suspensions\textsuperscript{37}. The proliferation of transplanted human cells and subsequent regeneration of organized bilayered structures of human mammary cells over a subsequent period of a few weeks has been reported for human mammary cells transplanted into both these sites. These therefore set the stage for the development of a functional assay to quantify and characterize primitive human mammary cells with in vivo regenerative properties.
Culture systems have also been used to identify populations of clonogenic human or mouse mammary progenitor cells that proliferate and differentiate under defined conditions. Dissociated HMECs seeded at low density on irradiated mouse fibroblast layers generate adherent colonies after 6-10 days of incubation in epidermal growth factor (EGF)-supplemented serum-free medium\textsuperscript{38, 39}. These conditions support the formation of three types of colony: “pure” luminal colonies that consist entirely of tightly-organized collections of cells that express luminal epithelial markers (mucin 1 (Muc1), cytokeratin (CK) 8/18 and CK19); “pure” myoepithelial colonies that consist entirely of dispersed elongated cells expressing myoepithelial markers (CK14, CD49f, CD10, p63 and sometimes smooth muscle actin(SMA)); and “mixed” colonies that contain a central core of tightly associated cells with luminal cell features surrounded by a halo of dispersed cells with myoepithelial cell features (Fig. 1-4). These colonies are inferred to be the progeny of three different types of “colony-forming cell” (CFC) with developmental potentials that are luminal lineage-restricted, myoepithelial lineage-restricted, or bi-lineage.

Cell fractionation studies have shown that the luminal CFCs are prospectively separable from myoepithelial and bipotent CFCs in 3-day cultures initiated with HMECs based on the different cell surface markers they express. In addition, this approach can now be used to obtain these functionally distinct, normal human mammary progenitor cell types at very high purities and high yields (20-50% compared with a starting CFC frequency of 1-5% in non-cultured primary tissue): luminal CFCs being CD49f\textsuperscript{+}EpCAM\textsuperscript{+} (Muc1/C133)\textsuperscript{+} (CD10/Thy1)\textsuperscript{−} and myoepithelial and bipotent CFCs being CD49f\textsuperscript{−}EpCAM\textsuperscript{−} (Muc1/C133)\textsuperscript{−} (CD10/Thy1)\textsuperscript{+} \textsuperscript{38-40}.
Subsets of dissociated mouse mammary epithelial cells also form adherent colonies when cultured under similar conditions in serum-free medium containing EGF or EGF plus fibroblast growth factor (FGF). However, the cells within the colonies express promiscuous combinations of lineage markers that are not seen in vivo. This complicates the interpretation of the lineage repertoires of murine CFCs. The expression of lineage markers appears to be more faithfully preserved when primitive mouse mammary cells are cultured in 3-D collagen or Matrigel systems. These conditions also support the process of branching morphogenesis. Murine luminal CFCs have a CD29medCD24highCD61+ phenotype. Since they are phenotypically separable from MRUs and are generated from MRUs in vivo, it can be inferred that these CFCs represent a downstream intermediate (transit-amplifying) population.

Suspension culture systems have also been developed in which human or murine mammary epithelial cells can survive and produce spherical colonies (“mammospheres”). This approach was based on earlier work showing that neural cells can be similarly propagated as “neurospheres”. Serial passaging of mammospheres has been demonstrated, with enrichment for cells with the ability to produce ductal-alveolar structures in 3-D culture and both luminal and myoepithelial progeny in 2-D CFC assays. However, difficulties in separating cell aggregation from growth in mammosphere cultures have hampered the development of this approach as a useful method to rigorously quantify a particular subset of primitive mammary cells.

Taken together, the cell types identified by in vivo (mouse) and in vitro (mouse and human) assay systems suggest that breast epithelial tissue differentiation is a hierarchical multistep process. At the origin of this hierarchy is self-renewing bipotent
stem cell that gives rise to a bipotent CFC, which then generates differentiated luminal epithelial and myoepithelial cells through two intermediate lineage-restricted types of CFC (Fig. 1-5). An alternative model proposes that bipotent progenitors contribute only to specific structural elements of the mammary tree (ducts or alveoli). This was supported by early observations of small outgrowths resembling isolated ducts or alveoli generated in cleared fat pad transplants of limiting numbers of mouse or rat mammary cells\textsuperscript{15,49-51}. This latter model is now less well accepted, as limited outgrowths are no longer frequently observed, and may have been caused by factors inherent to the transplant procedures used or the hormonal status of the recipient rather than providing a correct reflection of the intrinsic developmental properties of different progenitor cell types.

1.3 Pathways that regulate the proliferation of primitive mammary cells

The maintenance of differentiation hierarchies in tissues throughout adult life provides a number of features useful to the ability of the tissue to regulate its output of cells that require replacement at defined intervals. One of these is to enable large expansions to occur upon variable injury-related or physiological demands (e.g. as during pregnancy and lactation in the mammary gland). A second is the ability to fine tune the regulation of cell production analogous to the incorporation of a series of gears of decreasing sizes. A third is the ability to enable multiple different regulators to be involved at different stages thus allowing for further fine control of the ultimate output of mature cells. The most primitive cells, referred to as stem cells are typically assumed to possess an ability to divide without irreversibly changing their potential to generate differentiating progeny
(the definition of self-renewal) as well as generating progeny destined to differentiate. Hierarchies are then defined in terms of co-ordinated changes that segregate successive stages that the cells must undergo to become definitive end cells. These may be large or small depending on the tissue and its biology. The number of cells at each subsequent level in the hierarchy is the outcome of a balance between the rate of proliferation (versus maintenance in a quiescent state) and the rate at which cells are lost through cell death. Dynamic regulation of these processes requires mechanisms by which specific cell types in the hierarchy can differentially receive or process extrinsic signals, as well as feedback control mechanisms that allow the response of the entire system to be coordinated. In the mammary gland, the role of endocrine hormones as master coordinating regulators of mammary morphogenic programs is well established. However, the complex downstream signaling mechanisms that regulate specific compartments of primitive cells are very poorly understood. Indeed even the rates of their turnover are not established.

The ovarian endocrine hormones estrogen and progesterone are master regulators of the morphological changes that occur during puberty, pregnancy and menstrual/estrus cycles in both humans and rodents. These hormones bind members of the nuclear receptor family (estrogen receptor (ER) α and β, and progesterone receptor (PR)), allowing them to act as transcriptional activators of genes with the relevant response elements in promoter and enhancer regions. Estrogen is required for the development of the ductal network during puberty. Thus a severely compromised mammary tree is seen in mice bearing targeted mutations in ERα or aromatase cytochrome P450 (an enzyme involved in the biosynthesis of estrogen). A similar result is obtained in ovariectomized mice and these effects can be partially rescued by exogenous estrogen
administration\(^57\). The compromised mammary trees in ovariectomized and aromatase knockout mice contain markedly reduced numbers of mammary stem cells, as quantified by the MRU assay\(^58\). ER\(\beta\) appears to be less critical to the development of the mammary gland, as the epithelium develops normally through puberty and pregnancy in the ER\(\beta\) null mouse\(^59\).

ER\(\alpha\) and PR expression is restricted to subsets of the luminal cells in the ducts and lobules. Almost all ER\(\alpha^+\) cells are non-dividing, and exert a mitogenic effect on neighbouring cells through paracrine mechanisms\(^60,61\). This separation breaks down in early ER-positive breast lesions, with increased numbers of proliferating ER-expressing cells observed\(^62\). Notably, ER\(\alpha\) and PR expression in the mouse was detected in FACS-sorted “luminal fraction” (phenotype CD49\(^{\text{med}}\)CD24\(^{\text{high}}\)) cells that are enriched for differentiated luminal epithelial cells and highly depleted of MRUs. Conversely, ER\(\alpha\) and PR expressing cells were absent or present at very low frequencies in the “basal fraction” (phenotype CD49\(^{\text{high}}\)CD24\(^{\text{med}}\)) that contains the MRUs\(^63\). This implies that stem cell regulation by these hormones must be via indirect mechanisms (i.e. mediated by effects of these hormones on other cells). Stromal cells in the breast also express ER\(\alpha\), although recombination experiments indicate that expression of ER\(\alpha\) in epithelial is more important for ductal development than expression of ER\(\alpha\) in stromal cells\(^64\). The role of estrogen-mediated signaling in human breast tumorigenesis is evidenced by epidemiological studies that associate cumulative estrogen exposure with increased breast cancer risk and the efficacy of estrogens antagonists or aromatase inhibitors in breast cancer treatment and prevention\(^65-68\). ER\(\alpha\) expression also has significant prognostic implications since ER\(\alpha\)-positive tumors have a more favorable outcome\(^69,70\).
Full development and functional differentiation of mammary tissue through pregnancy is additionally regulated by progesterone and prolactin. PR null mice (specifically the PR-B isoform) develop normal ductal networks during puberty, but these do not undergo tertiary branching or alveologenesis during pregnancy and cannot produce milk\textsuperscript{71-74}. Wnts and RANKL appear to be important intermediate messengers between the PR-expressing luminal cells and the primitive cells in the basal fraction. Supplementing mice with progesterone plus estrogen induces expression of Wnt4 and RANK in luminal fraction cells and expression of their ligands Lrp5 and RANK in basal fraction cells, consistent with a paracrine control mechanism\textsuperscript{58, 75}. A similar induction of RANKL/RANK in luminal/basal cells is observed in the progesterone-elevated environment of pregnancy\textsuperscript{58}. Ectopic Wnt1 expression rescues the side-branching deficiency of PR\textsuperscript{+} mice\textsuperscript{76}. Absolute MRU numbers are expanded in Wnt1-overexpressing transgenic mice but not in mice overexpressing ErbB2. This further suggests that Wnt1 but not ErbB2 plays a significant role in regulating mammary stem cell numbers\textsuperscript{17}. Furthermore, addition of Wnt3a to 3-D Matrigel cultures of normal mouse mammary cells supports the maintenance or expansion of MRU and CFC numbers over many passages\textsuperscript{77}. The Wnt pathway has also been implicated in the regulation of mammary stem cell proliferation and self-renewal in epidermal, intestine and hematopoietic systems. Components of the Wnt pathway are perturbed in many hereditary and sporadic cancers\textsuperscript{78-80}. Numerous growth factors are also involved in regulating the proliferation of mammary cells, acting downstream of and cross-talking with activated endocrine pathways\textsuperscript{81}. These include members of the EGF, insulin-like growth factor (IGF) and
FGF families. EGF and insulin are both key components of media used to optimize human and mouse CFC detection and FGF is additionally used to stimulate the formation of mammospheres and mouse CFCs. It is also notable that ErbB2 (HER-2/neu), one of the receptors for the EGF-related ligands (including EGF, TGF-α, amphiregulin and epiregulin) is overexpressed in 20-30% of human breast cancers. ErbB1 (also called the EGF receptor, EGFR) but not ErbB2 expression is detected by quantitative reverse transcriptase-PCR (rt-PCR) in the MRU-enriched mouse basal cell fraction\textsuperscript{63}. Amphiregulin is the only EGF-related ligand that is transcriptionally upregulated by ectopic estrogen in ovariectomized mice, and has been proposed as an important intermediary in estrogen-induced proliferation during puberty\textsuperscript{82}. Consistent with this, amphiregulin null mice develop highly compromised ductal trees during puberty (similar to ERα null mice). This deficiency is recapitulated when cells from these trees are transplanted into cleared fat pads of wild-type recipients\textsuperscript{82}.

Examples of negative regulators of mammary cell growth have also been reported. Transforming growth factor-β1 (TGF-β1) in its active form is recognized as a negative regulator of mammary proliferation during puberty. The development of mammary ductal trees is compromised when activated TGF-β1 is introduced \textit{in vivo} using slow release implants\textsuperscript{83,84} or expressed in the mammary gland using the mammary-specific MMTV promoter\textsuperscript{85}. In addition, tissue fragments from heterozygous TGF-β1\textsuperscript{+/-} mice show enhanced proliferation compared with wild type tissue when their growth in transplanted cleared fat pads of wild type recipients is compared\textsuperscript{86}. Overexpression of active TGF-β1 also inhibits branching morphogenesis of mammary cells growing in 3-D culture systems\textsuperscript{87}.
The Notch regulators appear to have multiple roles affecting specific aspects of mammary stem and progenitor differentiation. Inhibition of Notch 3 in highly purified populations of human bipotent CFCs (by exposing them to an inhibitor of γ-secretase activity or short hairpin RNA-induced silencing of Notch-3) causes a marked reduction in the proportion of subsequently formed colonies that contain luminal cells without affecting colony formation by luminal-restricted CFCs. These findings indicate that Notch3 is an important positive regulator of the commitment of bipotent cells to the luminal lineage. In mice, cells from the MRU-enriched fraction which also contain a constitutively activated intracellular signaling domain of Notch1 have been found to generate alveolar nodules containing luminal cells only rather than bilineage mammary trees. Activation of Notch4 in mammary cells by proviral integration of mouse mammary tumor virus is oncogenic, and overexpression of Notch1 is often observed in human breast tumors.

Gata-3 and Elf-5 have emerged as important transcription factors involved in the execution of the luminal lineage differentiation program. Gata-3 expression is restricted to body cells of TEBs and to luminally-positioned cells in ducts and alveoli in mature mammary glands. Cre-mediated targeted deletion of Gata-3 in the luminal cell compartment under the MMTV (non-pregnant mice) or WAP (pregnant mice) promoters led to an increase in the proportion of luminal cells that express the CFC marker CD61. Conversely, forced expression of Gata-3 in basal fraction cells in Matrigel cultures resulted in the induction of luminal cell differentiation markers including WAP and β-casein. Elf-5 expression is also restricted to luminal cells, being most strongly expressed during pregnancy. A role in executing the alveolar differentiation program...
is suggested by defects in their formation during pregnancy when a single Elf-5 allele is deleted\textsuperscript{92}. Conversely, precocious formation of alveolae was seen when Elf-5 was overexpressed in virgin mice\textsuperscript{93}.

1.4 Location and nature of a mammary stem cell niche

Stem cells in diverse tissues have been found to occupy specific microenvironments or “niches”. The localization of specific combinatorial signals has been proposed to facilitate a number of key regulatory functions. These include maintenance of self-renewal properties, segregation of factors that regulate the symmetry of stem cell divisions, and the overall regulation of the stem cell population size\textsuperscript{94}. Characterization of a postulated niche for mammary stem cells has been limited by a lack of knowledge regarding the physical location of these cells. The CD49\textsuperscript{high}CD29\textsuperscript{high} MRU phenotype established by transplantation studies\textsuperscript{16, 17} suggests a basal position in the mouse mammary epithelium, as expression of this integrin pair (\(\alpha 6\) and \(\beta 1\)) is highest in basally-located cells\textsuperscript{95}, as reported for other epithelia\textsuperscript{96}. The binding specificity of the integrin pair for laminin (a major basement membrane component) suggests a function for physically anchoring stem cells to the basement membrane as well as a possible role for integrin signaling in stem cell regulation. However, the absence of additional markers that distinguish MRUs from other cell types in the basal cell layer (e.g., mature myoepithelial cells) has so far precluded the precise localization of stem cells on the basis of their immuno-phenotype.

Electron microscopy studies of the ultrastructure of the mouse and rat mammary epithelium have identified a number of putative primitive cell types that lack the
morphological features of either differentiated luminal or myoepithelial cells\textsuperscript{97,98}. These include basally- or supra-basally-positioned “small light cells” (~3% of epithelial cells) characterized by a small size and pale cytoplasm sparse in organelles. Similar light cells have also been identified in cattle and in the human mammary gland\textsuperscript{99,100}. However, because these cells are recognized morphologically in fixed tissue their postulated developmental attributes cannot be tested directly. It is interesting to note, however, that correlative studies demonstrate small light cells to be no longer present in glands that have reached growth senescence through serial transplantation\textsuperscript{101}.

\textit{In vivo} DNA labeling approaches have also been used to suggest the location of candidate mammary stem cell populations. The validity of this approach relies on the presumption that mammary stem cells are all proliferating during puberty but proliferate very infrequently thereafter. The studies are analogous to those used to also analyze primitive cells in the skin epidermis, hair follicles, cornea and intestine\textsuperscript{102-104}. The approach involves exposing mice during puberty to a pulse of a labeled thymidine analogue (tritiated thymidine (\textsuperscript{3}H-Tdr) or 5-bromo-2-deoxyuridine (BrdU)) followed by the detection several weeks later of cells that retain the label\textsuperscript{105-108}. This strategy has led to the identification of populations of long-term label-retaining cells exclusively\textsuperscript{108} or predominantly\textsuperscript{107} within the luminal layer of epithelial cells. The DNA labeling techniques do not permit isolation of viable labeled cells, so the developmental potentials of label-retaining mammary cells have thus far not been tested. A recent transgenic approach in which mice express fluorescently tagged histone proteins now offers the technical means to extend this type of analysis more rigorously to viable cells\textsuperscript{109}. Interestingly, in both the intestine and in the hematopoietic system, it has now been
shown that such label-retaining cells are not the stem cells\textsuperscript{110, 111}. It has also been proposed that the mechanism of label retention may involve a selective segregation of DNA in asymmetrically-dividing stem cells, with newly synthesized chromatids being passed onto the daughter cell that does not retain stem cell properties\textsuperscript{112}. While there is some experimental data in the mammary gland and colon to support this concept\textsuperscript{108, 113, 114}, it remains controversial as to whether the observations are pertinent to stem cells in these tissues. Interpretation of these various labeling experiments is further complicated by the recent demonstration that thymidine analogues can be recycled from dead to dividing cells\textsuperscript{115}. The underlying presumption that mammary stem cells enter a quiescent or infrequently proliferating state after puberty is also open to question, as discussed below.

1.5 Are primitive cells quiescent or cycling in the “resting” adult breast?

While the non-pregnant adult female breast is referred to as “resting”, it is well established that this state incorporates some cellular turnover. Attempts to determine the proportion of mammary cells that are proliferating have made use of a number of strategies; e.g., immunodetection of Ki67 expression\textsuperscript{116, 117}, \textit{in vivo} uptake of BrdU\textsuperscript{118} or deuterium-labeled water\textsuperscript{119}, \textit{ex vivo} uptake of tritiated thymidine in organoid cultures\textsuperscript{120-125}, and quantitation of mitotic cells in sections of normal mammary tissue\textsuperscript{126, 127}. These suggest that on average \(~1-2\)% of mammary epithelial cells in premenopausal women and \(~0.2-0.4\)% in postmenopausal women are in cycle. The cycling cells are found throughout the mammary gland, are typically dispersed rather than being grouped in foci, and are predominantly luminally-positioned\textsuperscript{116, 123}. There is considerable variation
throughout the menstrual cycle, with a 3- to 5-fold peak detected in the mid-late luteal phase compared with a trough in the early follicular phase\textsuperscript{117, 120, 122, 124, 126}. A similar cyclical pattern is observed during the 4-6 day rodent estrus cycle\textsuperscript{7, 128-130}. While the mechanisms that regulate proliferation remain to be fully explained, it should be noted that peak mitotic activity coincides with peak serum progesterone levels and suppression of nuclear ERα expression. Similarly, the trough coincides with the lowest serum levels of both estrogen and progesterone\textsuperscript{7, 131, 132}. Apoptosis is also highest during the late luteal phase in humans\textsuperscript{116, 117, 126, 127} and rodents\textsuperscript{7, 129}.

Proliferative activity is also maintained in women taking oral contraceptives, although there is disagreement as to whether or not there are significant changes in the proportion of cycling mammary cells in oral contraceptive users\textsuperscript{133, 134}. The differences in findings may be related to variations in the estrogen and progestogen content of oral contraceptives as well as formulations providing doses that are uniform over time (“monophasic”) or phased to more closely mimic the normal human cycle (“biphasic” or “triphasic”)\textsuperscript{135}.

An important question is the extent to which the proliferative activity observed in the adult breast occurs in stem cells, progenitor cells or cells approaching terminal differentiation. No information has been generated to date regarding the cell cycle profile of primitive human mammary cells although 2 recent studies have suggested that the majority of stem cells may be in cycle in young adult mice. Functional assays of mouse mammary cells fractionated based on their fluorescence after incubation with DNA and RNA binding dyes Hoechst 33342 and Pyronin Y showed almost all (>90%) MRUs and CFCs to be in the Pyronin\textsuperscript{high} subsets\textsuperscript{16}. This would be expected of a cycling population
since proliferative activity is associated with elevated ribosomal RNA levels and cells with >2n DNA content\textsuperscript{136}. Indeed, this approach has been widely and successfully used to determine the cell cycle profile of normal and malignant hematopoietic cell populations\textsuperscript{137-145}. However, a correlation between the Pyronin\textsuperscript{high} phenotype and proliferative status has not been rigorously validated for mammary cells. Separately, a 14-fold increase in absolute MRU number was measured in dioestrus as compared with oestrus mice\textsuperscript{75}. This finding implies that MRU execute extensive self-renewal divisions during the few days that separate these phases of the murine estrus cycle. However, an alternative explanation of these latter findings could be that the MRU assay differentially favours the detection of MRUs in different stages of the estrus cycle in ways that are unrelated to their proliferative status. Further experiments will be needed to address this possibility.

Technical caveats notwithstanding, these studies raise the interesting possibility of a predominantly proliferating primitive cell at the head of a hierarchy that produces a mostly quiescent organ. This concept is of significant importance to understanding the sensitivity of the mammary gland to oncogenesis (lifetime likelihood in women in Canada is 1 in 9\textsuperscript{146}). A continual cell turnover in persistent compartment of cells would potentially be a mechanism for accumulating a sufficiently high number of mutagenic events to initiate an oncogenic process within a lifetime. Such considerations underline the importance of defining the actual rate and mechanisms regulating the proliferative activity of human mammary stem and progenitor cells.
1.6 Thesis objectives

In summary, there is extensive evidence that the regenerative properties of mammary epithelial tissue is confined to primitive populations of mammary epithelial cells that in the mouse show stem cell properties in transplantation assays. These cells differ in their biological properties from the bulk of differentiated cells in the tissue which can be prospectively separated as distinct populations based on various immunophenotypic markers. A number of signaling pathways have emerged as relevant to the regulation of key primitive cell functions such as proliferation, self-renewal and lineage specification. However, details of these mechanisms remain poorly understood. This includes a lack of knowledge of the dynamics with which different primitive cell populations become proliferatively active; a topic of particular interest in the human mammary gland as a basis for understanding how this aspect of mammary cell regulation is perturbed in human breast cancer.

While rodent models (including the cleared fat pad transplant assay) have provided particularly powerful tools, methods to detect, analyze and characterize the properties of stem cells in human mammary tissue have not been available. The need for human studies is further heightened by the differences between mouse and human mammary gland structure and development. These include differences in the stromal composition of the mammary gland, ductal-alveolar tree architecture, length of the estrus cycle, menopausal changes, the pathology of spontaneous tumors and the requirements for cellular transformation. Understanding the hypothesized role of mammary stem cell dysregulation as a driver of the diverse types of human breast cancers also mandates
a need to develop methods to study and characterize the cycling status of normal adult human mammary stem cells.

The overall aims of this investigation were thus to fulfill these needs:

(1) to develop a robust and reproducible methodology to detect and quantify normal human mammary stem cells.

(2) To identify markers that facilitate their purification and allow their prospective isolation from other types of mammary cells, including those with different types of in vitro clonogenic activity.

(3) To develop and apply methods for assessing and comparing the cycling properties of different subsets of primitive mammary epithelial cells obtained from normal breast tissue.
Figure 1-1  Structure of human and mouse mammary glands
Schematic, adapted from Ref\textsuperscript{148}
Figure 1-2 Postnatal development of the mammary epithelial tree
Schematic, adapted from Ref\textsuperscript{149}
Figure 1-3  Cross section through a human mammary duct
Schematic, showing bilayered arrangement of luminal and myoepithelial cells surrounded by a basement membrane and embedded in a fibrous stroma
Figure 1-4  Examples of pure luminal, pure myoepithelial and mixed colonies
Primary HMECs were cultured for 10 days at clonal density in EpicultB medium on a layer of irradiated fibroblasts. Examples of individual colonies are shown, after fixation and staining with Wright’s Giemsa. (a) pure luminal colony, comprising a tight cluster of cells with a smooth boundary, (b) pure myoepithelial colony, comprising an irregular arrangement of dispersed teardrop-shaped cells, (c) mixed colony, comprising both these elements. Bars=1mm.
Figure 1-5 Model of the human mammary epithelial differentiation hierarchy
In this model, a self-renewing bipotent stem cell gives rise to a bipotent CFC which generates differentiated luminal epithelial and myoepithelial cells through two intermediate lineage-restricted types of CFC.
2. DEVELOPMENT AND VALIDATION OF A QUANTITATIVE ASSAY FOR HUMAN MAMMARY STEM CELLS

2.1 Introduction

This chapter describes the development and validation of a functional assay to detect and quantify highly primitive cells in normal adult human breast tissue that display in vivo regenerative properties expected of mammary stem cells. There are a number of important considerations inherent to the design of a clonal regeneration assay. Because the objective is to design a test to analyze the properties of individual cells, the approach must assume that the test cells can be obtained in a viable state and their properties assessed using single cell, limiting dilution or other clonal tracking methods.

Dissociation protocols have been optimized for the isolation of viable epithelial cells from human reduction mammoplasty tissue\textsuperscript{150}. The assay system must then provide the dissociated cells with suitable and saturating exogenous conditions (e.g., growth factors, hormones, extra-cellular matrix, supportive stromal cells, etc.) to allow the cells of interest to demonstrate their maximal intrinsic regenerative potential. Specificity for a given subset of cells is achieved by optimizing a combination of exogenous factors in combination with an appropriate regenerative endpoint.

Assuming that the frequency of the cells of interest would generally be too low for direct analysis of single cells, we chose to evaluate the applicability of standard limiting dilution analysis methodology for this purpose. This requires that replicate assays are carried out at doses containing “limiting” numbers of the cell of interest (i.e. close to 1 per assay) and results analyzed using models that support the assumption that regeneration follows a “single-hit” Poisson process (i.e., a positive regenerative output.
depends solely on the presence of 1 or more of the cells of interest as input)\textsuperscript{151}. Such statistical models can also be used to test whether the readout is consistent with the single hit process expected of clonal regeneration in the presence of saturating exogenous factors.

Both \textit{in vitro} and \textit{in vivo} approaches have been previously developed to detect clonogenic cells in the mouse mammary gland\textsuperscript{16, 17, 38, 39}. Among these, \textit{in vivo} transplantation approaches have shown the greatest specificity for cells with the functional properties of mammary stem cells (MRUs)\textsuperscript{16, 17}. With the objective of detecting an analogous cell population in the human mammary gland, my efforts were therefore focused on the development of a transplantation-based methodology. This used as its basis a subrenal capsule xenotransplantation system, first used to propagate intact mammary epithelial fragments successfully\textsuperscript{25} and subsequently shown to be adaptable for use with dissociated human mammary cells\textsuperscript{26}.

\section*{2.2 Methods}

The protocol paper “Quantitation of human mammary epithelial cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay”\textsuperscript{152}, attached as a Appendix B, contains a detailed description of the methods followed to prepare single-cell suspensions from primary mammoplasty tissue, carry out subrenal capsule transplantation surgery and assay for regenerated structures and CFCs. A brief summary of these methods is included here.
2.2.1 Animals. Female NOD-SCID, NOD-SCID β2-microglobulin–null and NOD-SCID interleukin-2 receptor-γc–null (NOG) mice were bred and housed at the animal facility at the British Columbia Cancer Research Centre. Unless otherwise specified, the data was generated with NOG mice as transplant recipients. Surgery was carried out on mice between the ages of 5 weeks and 10 weeks. All experimental procedures were approved by the University of British Columbia Animal Care Committee.

2.2.2 Dissociation of human mammary tissue. Anonymized discard tissue from normal premenopausal women (ages 19–40) undergoing reduction mammoplasty surgery was collected with informed consent according to procedures approved by the University of British Columbia Research Ethics Board and processed the tissue as previously described\textsuperscript{15}. Briefly, the tissue was transported from the operating room on ice, minced with scalpels and then dissociated for 18 hours in Ham’s F12 and with Dulbecco’s Minimal Eseential Medium (DMEM) (1:1 vol/vol, F12 to DMEM, STEMCELL Technologies, Vancouver, BC) supplemented with 2% wt/vol bovine serum albumin (BSA, Fraction V; Gibco Laboratories, Grand Island, NY), 300 U/ml collagenase (Sigma-Aldrich, St Louis, MO) and 100 U/ml hyaluronidase (Sigma). In some experiments, this medium was supplemented with 10 ng/ml epidermal growth factor (EGF, Sigma), 10 ng/ml cholera toxin (Sigma), 1 µg/ml insulin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and 5% vol/vol fetal bovine serume (FBS, STEMCELL Technologies). An epithelial-rich pellet was obtained by centrifugation at 80 g for 4 minutes and cryopreserved in 6% dimethylsulfoxide (DMSO)-containing medium at –135 °C until use. Single-cell suspensions were subsequently prepared from freshly
thawed pellets by treatment with 2.5 mg/ml trypsin supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) (STEMCELL Technologies), washing once with Hanks Buffered Saline Solution (STEMCELL Technologies) supplemented with 2% vol/vol FBS (referred to as HF), followed by treatment with 5 mg/ml dispase (STEMCELL Technologies) and 100 µg/ml DNase1 (Sigma), after which the cell suspension was passed through a 40-µm filter (BD Biosciences, San Jose, CA) to remove remaining cell aggregates.

2.2.3 *In vitro* mammary CFC assay. 60-mm tissue culture dishes were incubated for 1 hour at 37 °C with a 1:43 dilution of Vitrogen 100 collagen (Collagen Biotechnologies, Palo Alto, CA) in phosphate-buffered saline (PBS, STEMCELL Technologies). Each dish was seeded with test cells obtained from primary tissue or digested collagen gels combined with 2.0 × 10⁵ freshly thawed, previously irradiated (with 50 Gy) NIH 3T3 mouse fibroblast cells in 4 ml of EpiCult-B medium (STEMCELL Technologies) supplemented with 5% FBS and 0.5 µg/ml hydrocortisone. Cultures were incubated at 37 °C and 5% CO₂, with a change to serum-free EpiCult-B plus 0.5 µg/ml hydrocortisone 1 day later. In some experiments, the EpiCult-B medium was replaced with DMEM and F12 supplemented with 0.1% BSA, 10 ng/ml EGF, 10 ng/ml cholera toxin and 1 µg/ml insulin (referred to as SF7 medium). After 7–10 days, the dishes were fixed briefly in a 1:1 vol/vol mixture of methanol and acetone at room temperature, stained with Wright’s Giemsa (Sigma) and the colonies scored visually under a dissecting microscope. Colonies were routinely categorized into the following subtypes: pure luminal colonies composed of tightly-clustered cells with a smooth colony boundary; pure myoepithelial
colonies composed of dispersed teardrop-shaped cells; mixed colonies containing both of these elements and showing a ragged colony boundary. However, in some cases, colonies were stained with antibodies against human Muc1 and CK14 to confirm the presence of luminal and/or myoepithelial cells as defined by expression of these lineage-specific biomarkers.

2.2.4 Preparation of collagen gels. Concentrated rat’s tail collagen was prepared as previously described\textsuperscript{153} and stored at –20 °C. Aliquots were thawed and the pH neutralized immediately before use by making a mixture of concentrated collagen (78% vol/vol), 5×DMEM solution (20% vol/vol) and concentrated sodium hydroxide (2% vol/vol). To prepare gels, C3H 10T½ mouse embryonic fibroblasts (a kind gift from G. Cunha, University of California, San Francisco, CA) were harvested from subconfluent cultures, X-irradiated with 15 Gy, mixed with dissociated human mammary epithelial cells and resuspended in cold neutralized collagen. 25µl aliquots containing $2.2 \times 10^5$ C3H 10T½ fibroblasts and the desired number of HMECSs were pipetted into individual wells of a 24-well plate. The gels were allowed to stiffen in a 37 °C incubator for 10 minutes and then incubated in warm EpiCult-B medium plus 5% FBS for 50 minutes. The plates were kept on ice until all gels had been transplanted. In some of the early experiments, cells from a telomerase-immortalized human adult mammary fibroblast line or primary human mammary fibroblasts were used instead of C3H 10T½ fibroblasts.

2.2.5 Subrenal xenotransplantation surgery. The hair on the backs of anesthetized mice was shaved and the skin swabbed with 70% alcohol. An anterior to posterior
incision approximately 1.5 cm long was made dorsally around the area of the kidneys. A small incision was made in the abdominal wall above one kidney and the kidney exteriorized by applying gentle pressure on either side. Under a dissecting microscope, the kidney capsule was lifted from the parenchyma with fine forceps and a 2-4 mm incision made in the capsule. Up to 4 gels were inserted under the capsule using a fire-polished glass pipette tip. After suturing the incision in the abdominal wall, the procedure was repeated on the contralateral kidney. Finally, a slow-release pellet containing 2 mg $\beta$-estradiol and 4 mg progesterone (both from Sigma) in MED-4011 silicone (NuSil Technology, Carpinteria, CA) was inserted 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$^{154}$. In some experiments, the mice were mated 9 days after the gels were transplanted.

To recover viable cells from the xenografted gels, recipient mice were killed and the gels removed aseptically from the kidneys under a dissecting microscope. The gels were then incubated for 4–4.5 hours at 37 °C in SF7 medium supplemented with 5% FBS, 600 U/ml collagenase and 200 U/ml hyaluronidase. After digestion, the cells were washed once and treated for 5 min with prewarmed trypsin-EDTA with gentle pipetting. In experiments using a histological endpoint, undigested gels were fixed for 1-2 hours in 10% vol/vol paraformaldehyde.

2.2.6 Cell separation. Mammary cell suspensions were preblocked in cold HF supplemented with 10% human serum (Sigma), then labeled with antibodies in cold HF.
Preblock and antibody incubations were for 10 minutes on ice. Propidium iodide (PI) (Sigma) or 4',6-diamidino-2-phenylindole (DAPI, Sigma) was added to 1 µg/ml for live/dead cell discrimination. Sorts were performed on either a FACS VantageSE or a FACSDiva (Becton Dickinson). A list of all antibodies used for flow cytometry in this and subsequent Chapters is provided in Table 2-1.

2.2.7 Immunohistochemistry. Deparaffinized 4µm sections of paraformaldehyde-fixed collagen gels were processed for immunohistochemistry with a Discovery XT automated system (Ventana Medical Systems, Tucson, AZ). Primary antibodies were applied, followed by horseradish peroxidase (HRP)–conjugated Discovery Universal Secondary Antibody (Ventana). Slides were developed with a 3,3’-diaminobenzidine (DAB) Map Kit (Ventana). Some slides were processed manually with primary antibodies followed by alkaline phosphatase (AP)-conjugated Envision-AP (DAKO, Carpinteria, CA) and developed in FastRed (Sigma). All slides were counterstained with hematoxylin. Optimal antibody concentrations were established by titration, and heat-induced antigen retrieval was used where recommended by supplier. For dual-color staining of colonies, 60-mm culture dishes were fixed briefly in 1:1 vol/vol acetone and methanol and preblocked in Tris-buffered saline containing 5% wt/vol BSA and 10% vol/vol FBS. The dishes were then incubated sequentially with a primary antibody to Muc1, AP–conjugated anti-mouse Envision polymer, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development solution (Sigma), biotin-conjugated antibody to cytokeratin-14, HRP–conjugated streptavidin and DAB. A list of all antibodies used for immunohistochemistry in this and subsequent Chapters is provided in Table 2-2.
2.2.8 Statistical analyses. MRU frequencies were calculated using single-hit Poisson statistics and the method of maximum likelihood in the L-Calc (STEMCELL Technologies) or ELDA\textsuperscript{151} (Walter and Elizabeth Hall Institute of Medical Research) software program. The values obtained are shown with the derived 95% confidence interval. Goodness of fit to a single-hit model was tested using standard chi-squared statistics. All other data in this and subsequent chapters are expressed as the arithmetic mean ± standard error of mean (SEM), unless otherwise stated.

2.3 Results

2.3.1 Dissociated HMECs regenerate organized structures in transplanted mice

It was previously reported that organized bilayered epithelial structures are regenerated when dissociated reduction mammoplasty cells are combined with supportive fibroblasts in collagen gels and surgically implanted under the kidney capsule of highly immunodeficient mice supplemented with human estrogen and progesterone\textsuperscript{26} (Fig. 2-1a). Such structures were visualized by removing the collagen implants from mice after several weeks in vivo, then fixing, sectioning and staining with hematoxylin and eosin (H&E) using standard methods. These findings were replicated, with structures generated from every human mammoplasty sample thus tested (n=6) when at least 10\textsuperscript{5} HMECs were transplanted. An example of an H&E-stained section of a 4-week xenograft containing elongated ductal and round acinar structures is shown in Fig. 2-1b (top left panel).
Different sources of fibroblasts were tested for their ability to support the generation of structures from HMECs. Comparable structures were observed using a telomerase-immortalized human fibroblast cell line, primary human fibroblasts obtained from reduction mammoplasty tissue or C3H 10T½ murine embryonic cells. No such structures were observed in control gels transplanted with fibroblasts alone. Since the murine fibroblasts were most readily propagated in vitro they was used as supportive cells in all subsequent experiments.

To determine whether the spatial expression of markers within regenerated structures is similar to what is seen in normal human mammary tissue, sections through 4-week xenografted gels were processed for immunohistochemistry using antibodies against selected markers (Fig. 2-1b). Within the structures, most of the outer layer of cells expressed the differentiated myoepithelial cell markers SMA and CK14, and most of the inner layer of cells expressed the differentiated luminal epithelial markers Mucl and CK18. A basement membrane expressing collagen IV and laminin separated the epithelial structures from the surrounding gel and fibroblasts. The structures also contained some cells (mostly luminally-postioned) that expressed nuclear ERα and PR, with ERα expression also seen in some fibroblasts. Staining with an antibody to human Ki67 showed the presence of cycling cells. In summary, the spatial distribution of cellular markers in regenerated structures was strikingly similar to normal human mammary tissue.
2.3.2 Milk is produced in structures regenerated from HMECs in pregnant mice

To determine whether milk could be produced in xenografts under physiological lactogenic conditions, recipient female mice were mated 1 week following transplant and the gels removed and examined just before parturition. It was necessary to carry out these experiments in the absence of hormonal supplements, since mice receiving the estrogen and progesterone implants did not become pregnant after mating. Within xenografts, positive staining for human β-casein and lactoferrin (2 protein components of milk) was seen prevalently in vacuoles of luminally-positioned cells and in the luminal space (Fig. 2-1c). These cells also had hyperchromic, slightly pleomorphic nuclei, typical of cells in lactating human mammary tissue. Control xenografts grown in hosts that were not mated showed occasional small structures with positive staining for these antibodies. This milk production may be the result of stimulation from endogenous mouse hormones that follow a 5-day estrus cycle, albeit at levels lower than during pregnancy.

2.3.3 Regenerated CFCs serve as a readout of transplanted human MRUs

Use of histological means to discriminate “positive” from “negative” transplants (i.e., gels containing organized structures versus those containing no structures) is time-consuming and expensive, as the entire gel must be sectioned and stained. The criterion of how much of a structure should allow a gel to be scored as positive is also necessarily somewhat subjective. Furthermore, when HMECs were immunomagnetically sorted using antibodies to basal markers CD49f or CD44 or luminal markers CD24, Muc1 and EpCAM and subsequently transplanted, evidence of structure generation was seen in all
subfractions tested (i.e., CD49f+, CD49f−, CD44+, CD44−, CD24+, CD24−, Muc1+, Muc1−, EpCAM+, EpCAM−) (John Stingl, unpublished observations). This suggested that a broad range of human mammary cell types possess the ability to initiate some degree of structure formation in vivo, imposing futher difficulties on making a histological endpoint sufficiently specific to identify the most primitive mammary cells exclusively.

For all of these reasons, the presence of CFCs in the xenografts was investigated as a potentially more useful and specific endpoint. To test for the presence of these, single-cell suspensions were prepared from the removed gel-xenografs and the cells plated into standard in vitro CFC assays (Fig. 2-2a). All types of mammary CFCs (luminal-restricted, myoepithelial-restricted and bipotent) were readily detectable in the xenografts for up to 12 weeks, and these CFCs generated colonies that were indistinguishable from those derived from primary mammoplasty tissue (Fig. 2-2b-d). Hereafter, these regenerated progenitor cells are referred to 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. 2-2e).

2.3.4 Quantitation of MRU frequency in normal adult HMEC suspensions

A series of limiting-dilution transplant experiments was performed 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 (5
separate experiments, Table 2-3). Chi-squared tests showed the results were consistent with a single-hit Poisson model in each of the 5 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 $10^3 - 10^4$ HMECs, 1-2 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, $4.1 \pm 0.6$ daughter CFCs.

2.3.5 MRUs can be serially transplanted

Secondary transplant assays were performed 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. For these experiments, primary grafts containing 1,000–3,000 FACS-sorted CD49f$^{+}$EpCAM$^{-low}$CD31$^{-}$CD45$^{-}$ cells (MRU-enriched and containing limiting numbers of MRUs, see section 3.3.1 for the rationale for this sorting approach) were implanted into a first set of mice. Gels were removed 4 weeks later, single-cell suspensions were prepared from them and then 30% of each suspension was plated in a CFC assay to identify primary gels that contained regenerated (secondary) CFCs. The remaining 70% of the cells from the primary gels were combined with fresh feeder cells and suspended in new gels which were then implanted into secondary recipients (Fig. 2-3a). In most cases, primary gels that contained regenerated CFCs also regenerated detectable CFCs in the secondary gels, from which it was inferred that MRUs had been produced in the primary gels (Fig. 2-3b). 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.

2.4 Discussion

The xenotransplantation system developed here provides a new, robust and objective protocol for determining the frequency of cells that meet the rigorous definition of human mammary epithelial stem cells. The cells detected display both complete lactating mammary gland regenerative potential \textit{in vivo} and self-renewal activity demonstrable in secondary transplants. 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 primary human mammary gland (CFCs of all 3 types, and differentiated cells of both lineages). Notably, during the course of their production in this \textit{in vivo} system, the regenerated and differentiating human mammary cells also self-organize to form a 3-dimensional mammary gland structure that appears similar to normal mammary tissue and is capable of responding to physiological signals that induce the production of milk protein.

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 (LTC-ICs) by virtue of their ability to generate
hematopoietic CFCs detectable after 5–6 weeks in cultures containing stromal feeder layers\textsuperscript{155}.

In all dose-response transplant series, the relationship between the frequency of positively engrafted (i.e., CFC-containing) gels as a function of input cell dose was shown by chi-squared tests to be consistent with a single-hit Poisson process. In biological terms, such a process is compatible with the clonal regeneration of the CFCs detected at limit dilution from individual primitive cells (MRUs) in the presence of saturating exogenous factors. More highly stringent statistical tests have been developed to test consistency with the single-hit model\textsuperscript{156, 157}, although limitations to the size of this dataset precludes their use here\textsuperscript{158}.

The most stringent test of clonality is tissue regeneration from transplants of single isolated cells, as previously demonstrated for murine MRUs\textsuperscript{16, 17}. The low frequency of MRUs in human mammary tissue makes such direct testing impractical at present. This has prompted much effort to seek more highly selective MRU markers or marker combinations (e.g., see Chapter 3) that might solve this problem. An alternative strategy is to test clonality by tracking the progeny of genetically marked HMECs\textsuperscript{159, 160}. Although marker analyses have failed to generate a scheme suitable for single cell transplants of human cells, preliminary studies with mixtures of transduced cells also support the single cell origin of structures obtained from transplants expected to contain limiting numbers of MRUs (L Nguyen and C Eaves, personal communication).
Figure 2-1 Organized structures are generated in vivo from dissociated primary HMECs

(a) Schematic showing xenotransplantation approach with histological endpoint. Single cells obtained by enzymatic dissociation of normal human reduction mamboplasty samples were combined with irradiated fibroblasts in collagen gels, as described in the Methods. Gels were transplanted under the kidney capsule of highly immunodeficient mice given slow-release pellets of human β-estradiol and progesterone (E2 + P). Gels were removed after a number of weeks in vivo, then fixed, sectioned and stained using standard histological methods.

(b) H&E and immunostained sections through 4-week xenografts produced in the gels, showing examples of round and elongated duct-like structures. The immunostained xenograft sections demonstrate a spatial distribution of markers similar to that seen in normal human breast tissue. The sections stained with antibodies to collagen IV and laminin show the presence of a basement membrane separating the epithelial structures from the surrounding gel and fibroblasts. SMA and CK14 are two markers of basally located myoepithelial cells. Muc1 and CK18 are luminal epithelial cell markers. The sections stained with antibodies to ER-α and PR show that some fibroblasts as well as epithelial cells stained positively for ER-α. Ki67 is a marker of cycling cells.

(c) Immunostained sections through gels that was transplanted 4 weeks previously into a female mouse that was made pregnant 9 days after transplant, stained with antibodies to β-casein and lactoferrin. The positive staining provides evidence of human milk production within the regenerated alveolar structures. Scale bars, 50 μm.
CFC production in vivo as an indicator of human MRU repopulating activity

(a) Experimental protocol (as in Fig 2-1a but showing the use of CFC output measurements as an endpoint of human MRU activity).

(b) The number of CFCs detected per gel after various times in vivo. The legend inside the figure shows the number of human cells transplanted per gel in each of the time-course experiments performed (n = 3).

(c) The distribution of different types of CFCs in freshly thawed normal human breast tissue compared with the distribution of these cells in 4-week xenografts generated from the same tissue samples (n = 9).

(d) Representative colonies generated from cells derived from 4-week xenografts after dual-colour immunostaining with antibodies to both Muc1 (blue) and cytokeratin-14 (brown). Left, pure luminal cell colony; middle, pure myoepithelial colony; right, mixed colony containing both lineages. Scale bars, 1 mm.

(e) The CFC output in the gels after 4 weeks was linearly related to the number of human cells transplanted. A representative experiment is shown in which six gels were analyzed per cell dose.
Figure 2-3 MRUs can be serially transplanted

a) Experimental protocol. Cells isolated by FACS were transplanted into primary (1°) recipients. Four weeks later, 30% of the cells from each 1° gel were used to identify those that contained detectable CFCs. The remaining 70% of the cells were transplanted into secondary (2°) recipients. Another 3½ weeks later, CFC assays were performed on the cells harvested from these 2° gels.

b) Results. Data from 3 serial transplant experiments performed as described in (a) are shown. Both the frequency of gels implanted in 1° and 2° hosts in which at least 1 CFC was detected and, in brackets, the average number of CFCs in the assayed portion of 1° and 2° gels are indicated. The cellular fractions tested were “basal” (CD49f+EpCAM−lowCD31−CD45+) and “non-basal” (combined CD49f+EpCAM−CD31−CD45− and CD49f−CD31−CD45−). See section 3.3.1 for the rationale for using these fractions.
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<th>Supplier</th>
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Table 2-1  Antibodies used for flow cytometry
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**Table 2-2** Antibodies used for immunohistochemistry
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<td>1,390 (640-2,960)</td>
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<td>43 4,000 4,000</td>
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Table 2-3 Measurements of MRU frequency using 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, consistent with the clonal origin of the CFCs from MRUs 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 ± 0.6 secondary CFCs per input MRU was determined. NOG mice were used as recipients except for experiment #4 (NOD-SCID-B2M<sup>−/−</sup>) and experiment #5 (NOD-SCID)
3 CHARACTERIZATION OF THE PHENOTYPE OF HUMAN MAMMARY STEM CELLS

3.1 Introduction

The primary objective of the experiments described in this chapter was to characterize MRUs phenotypically in terms of markers they express for which specific identifying reagents exist. Some of these were chosen based on the expectation that they might serve to distinguish MRUs from other more differentiated HMECs and/or different types of mammary CFCs. A second objective was to evaluate potential markers that alone, or in combination might enable the isolation of MRUs at sufficient purities to allow their further biological, gene expression and signaling profiles to be examined directly. This required explicit selection of a list of candidate markers because the MRU assay is labor-intensive and not suited to high-throughput approaches. The additional requirement to be able to couple a specific phenotype with functional MRU activity also restricted the choice of candidate markers; either to proteins with extracellular domains recognized by suitably fluorescently-conjugated antibodies (since antibody labeling of intracellular epitopes cannot be carried out without fixation) or to strategies that generate a fluorescent phenotype in viable cells.

Thirteen candidates fulfilling one or the other of these criteria were selected (see Table 3-1). This list included several cell surface markers previously reported to characterize subsets of murine and human mammary cells (including murine MRUs). Another group included 5 antibodies whose reactivities are (positively or negatively) associated with human embryonic stem cells (hESCs). The rationale for selecting these was based on a recent global gene expression profiling study that identified a hESC gene
expression “signature” in a cohort of basal-type human breast tumors\textsuperscript{162}. Another group of markers were chosen because they had been found to be selectively associated with very primitive cells in other tissue systems, in particular murine hematopoietic stem cells which can now be purified to near homogeneity. These included antibodies to KIT (also known as CD117, c-Kit-ligand receptor, and steel factor receptor)\textsuperscript{163, 164}, EPCR (also known as protein C receptor, PROCR)\textsuperscript{165}, CD48\textsuperscript{166} and CXCR4 (also known as CD184, the stromal-derived factor-1 receptor and fusin), which has been implicated in stem cell homing to the bone marrow\textsuperscript{167}.

Finally, 2 enzyme-mediated phenotypes were chosen, efflux of Hoechst 33342 and high aldehyde dehydrogenase (ALDH) activity. Hoechst 33342 is a substrate of the ABCG2 transporter and this allows ABCG2\textsuperscript{+} cells to display a unique “side population” (SP) phenotype\textsuperscript{168, 169}. An ability to efflux Hoechst 33342 is a discriminating marker of quiescent murine hematopoietic stem cells but the activity is lost when these cells are activated into the cell cycle\textsuperscript{170}. It was previously reported that \~2\% of mouse mammary epithelial cells comprise a detectable but only a small proportion (<10\%) of all the murine MRUs were found to be contained in the SP fraction\textsuperscript{16, 171}. ALDH proteins belong to a large family of enzymes (20 in mouse, 19 in humans) that catalyze the conversion of aldehydes to carboxylic acids. ALDH activity can be detected using the fluorescent substrate bodipy aminoacetaldehyde (BAAA, also known as “Aldefluor”). BAAA diffuses into (and out of) cells but is retained intracellularly when it is converted to the membrane impermeable bodipy aminoacetate (BAA) which can be achieved by a number of ALDH family members. High ALDH characterizes many primitive human hematopoietic sub-populations\textsuperscript{172-174}. In normal human mammary tissue, the subfraction
with high Aldefluor fluorescence was reported to contain CFCs of all types at higher frequency than the Aldefluor low subfraction, as well as higher frequency of cells capable of generating mammospheres in vitro and structures in vivo in fibroblast-colonized fat pads	extsuperscript{175}. Additional markers not expressed on mammary epithelial cells were used to deplete dissociated breast tissue samples of hematopoietic (CD45\textsuperscript{+}) and endothelial (CD31\textsuperscript{+}) cells.

3.2 Methods

3.2.1 Cell Separation. Cryopreserved organoid-enriched human mammary tissue samples were defrosted, dissociated to obtain single cell suspensions, and labeled for flow cytometry as described in section 2.2.6 using antibodies against individual candidate markers (see Table 2-1). Unless otherwise stated, cells were also co-labeled with antibodies against CD31 and CD45, to allow contaminating endothelial and hematopietic cells to be preemptively removed from the analyses or final sorts. In some experiments, cells were also co-labeled with antibodies against EpCAM and CD49f, to allow additional exclusion of stromal (EpCAM\textsuperscript{−} CD49f\textsuperscript{−}) cells, and to allow analysis of the expression of markers in mammary subfractions defined by CD49f and EpCAM. Gates were drawn with reference to a control population of cells that were labeled with the viability dye alone (PI or DAPI) or to a “fluorescence minus one” negative control labeled with all antibodies except the candidate marker under test. For measurement of ALDH activity, cells were incubated for 30-60 minutes at 37 °C in SF7 with BAAA using the ALDEFLUOR kit (STEMCELL Technologies) following the manufacturer’s instructions. Sorting gates were drawn with reference to fluorescence levels in a negative
control incubated with BAAA plus inhibitor diethylaminobenzaldehyde (DEAB). For measurement of ABCG2 activity, cells were incubated in prewarmed SF7 medium supplemented with Hoechst 33342 (10µM, Sigma) for 90 minutes at 37 ºC. The side population of cells effluxing the dye was identified as the subpopulation showing low Hoechst Blue and Hoechst Red emission profiles. Control populations for this analysis were prepared by incubating with Hoechst 33342 plus an ABCG2 inhibitor, either fumitremorgin C (Sigma, 25 µM) or reserpine (Sigma, 5 µM). Sorts were performed on a FACSVantageSE, FACSDiva, FACSaria or Influx machine (all from Beckton Dickenson, San Jose CA).

3.2.2 Functional Assays. Fractions were isolated by FACS based on expression of candidate phenotypic markers (individually or in combination). Aliquots of cells from each subfraction isolated were seeded into (primary) CFC assays. Further aliquots were combined with C3H 10T½ fibroblasts in collagen gels, and implanted subrenally into NOG mice as described in Chapter 2. After 4 weeks, gels were removed, dissociated, and the cells seeded into (secondary) CFC assays. For both primary CFC and MRU assays, the relative number of cells assayed from each subfraction was proportional to the relative size of the subfractions. The relative output of colonies (primary or secondary) was measured to determine the relative distribution of primitive cells (CFCs or MRUs, respectively) in the subfractions tested. In some experiments the cells dissociated from gels were labeled with fluorescent antibodies against CD49f, EpCAM, CD31 and CD45 and analyzed by flow cytometry.
3.3 Results

3.3.1 MRUs have a “basal” CD49f<sup>+</sup> EpCAM<sup>-low</sup>CD31<sup>-</sup>CD45<sup>-</sup> phenotype

Assays were first carried out to test whether transplantable human MRUs belong to a phenotypically distinct subset of mammary epithelial cells defined by expression of CD49f and EpCAM. The in situ cellular distribution of these two markers in the human mammary epithelium is similar to that of murine MRU markers CD49f and CD24 in the mouse mammary gland, with accordingly similar FACS profiles<sup>16</sup>. Various subsets of cells were therefore isolated from 9 different human mammoplasty samples after staining the cells with antibodies to CD49f and EpCAM (Fig. 3-1a, b). In 6 of the 9 experiments, contaminating hematopoietic (CD45<sup>+</sup>) and endothelial (CD31<sup>+</sup>) cells were simultaneously excluded. Aliquots of cells from sorted fractions (A, B and combined C+D as depicted in Fig. 3-1b) were assayed for MRU content by combining them with irradiated 3T3 fibroblasts in collagen gels which were then implanted into mice. Four weeks later, the number of regenerated progenitors in each gel was determined by secondary CFC assay. The number of HMECs transplanted from each fraction was proportional to the total number of cells of that phenotype within the original mammoplasty sample. The relative output of secondary CFCs from gels initiated with HMECs from different fractions then served as a measurement of the relative MRU content of the fractions assayed based on the linear relationship established between MRU input and secondary CFC output (see Chapter 2, Section 2.3.3). Separate aliquots from each sorted fraction were seeded directly (i.e., without prior transplantation) into primary CFC assays, to determine the distribution of CFC in the different fractions assessed.
Most primary luminal-restricted CFCs (72 ± 10%) were confined to the CD49f⁻EpCAM⁺ fraction whereas most primary bipotent plus myoepithelial-restricted CFCs (68 ± 12%) were concentrated in the CD49f⁺EpCAM⁻/low fraction (Fig. 3-1c). The CD49f⁻EpCAM⁻ and CD49f⁻EpCAM⁺ fractions were mostly devoid of primary CFCs. Interestingly, grafts in which secondary CFCs were detected 4 weeks later were almost exclusively those initiated with cells from the CD49f⁺EpCAM⁻/low fraction (92 ± 3% of the CFCs detected in all xenografts were obtained in gels initially seeded with CD49f⁺EpCAM⁻/low cells). This implies that most MRUs have a CD49f⁺EpCAM⁻/low phenotype.

To test whether the MRU-enriched fraction had structure-forming (in addition to CFC-generating) ability in the absence of other cellular fractions, 4-week gels initiated with sorted CD49f⁺EpCAM⁻/lowCD31⁻CD45⁻ cells were also fixed, sectioned and immunostained. The same polarized organization of cells expressing luminal and myoepithelial markers bounded by a basement membrane (Fig. 3-1d) was observed as those generated from unsorted mammary cells. Furthermore the FACS profile of cells dissociated from these gels also showed distinct CD49f⁺EpCAM⁻/low CD49f⁺EpCAM⁺ and CD49f⁺EpCAM⁻ subfractions (compare Fig. 3-1e and Fig. 3-1b).

High expression of CD49f has been associated with basally-positioned cells within the human and mouse mammary epithelium16, 17, 176 and high EpCAM expression with cells with a luminal position38, 177. These combined data demonstrate that MRUs in the human breast share phenotypic markers with basally-positioned cells.

The MRU-enriched fraction (CD49f⁺EpCAM⁻/lowCD31⁻CD45⁻) is therefore referred as the “basal” fraction. The CD49f⁺EpCAM⁺CD31⁻CD45⁻ and CD49f⁻
EpCAM⁺CD31⁻CD45⁻ fractions are referred to as the “luminal” and “mature luminal” fractions respectively, since clonogenic activity was largely absent from the latter. The CD49f⁻EpCAM⁻CD31⁻CD45⁻ cells are referred to as the “stromal” fraction.

3.3.2 Human MRUs are mostly CD10⁺ CD133⁻

In order to test whether MRUs share other phenotypic markers with basally-located cells, functional assays were carried out on subfractions of cells expressing 2 other established markers of basal cells, CD10 (also called common lymphocyte leukemia antigen or CALLA) and Thy1 (also called CD90). FACS analysis confirmed that both these markers were expressed by a majority (>80%) of basal fraction cells and a minority (<10%) of luminal (EpCAM⁺) cells, as well as the majority of stromal cells (Fig. 3-2a,b, 3.3a,b). When FACS-sorted CD10⁺ and CD10⁻ aliquots were transplanted in vivo, most (78±8%, n=2) secondary CFCs were detected in gels seeded with CD10⁺ cells, indicating that most MRUs are CD10⁺ (Fig. 3-2c, right panel). Primary CFC assays also showed that most (84±6%) bipotent plus myoepithelial CFCs are CD10⁺ whereas most (83±5%) luminal CFCs are CD10⁻ (Fig. 3-2c, left panel). Similar results were obtained when Thy1⁺ and Thy1⁻ fractions of HMECs were assayed for their content of primary CFCs (most bipotent and myoepithelial CFCs were Thy1⁺, and most luminal CFCs were Thy1⁻) (Fig 3.3c, left panel). However, MRUs did not show as clear or consistent a Thy1 phenotype, with 24% and 76% of MRUs found to be Thy1⁺ in the 2 samples tested (Fig. 3-3c, right panel).

Similar experiments were carried out using an antibody (AC133) reactive with the carbohydrate portion of the luminal marker CD133 (also known as Prominin 1). CD133
expression has been associated with transplantable tumor forming ability in human glioblastoma, colorectal and prostate tumor samples\textsuperscript{178-180}. FACS analysis showed that within normal human mammary tissue CD133 is primarily a marker of cells in the luminal fraction and not in the basal or stromal fractions (Fig. 3-4a,b). Functional assays demonstrated that MRUs, bipotent CFCs and myoepithelial CFCs are predominantly AC133\textsuperscript{−}, whereas luminal CFCs are predominantly AC133\textsuperscript{+} (Fig. 3-4c).

Overall, these results demonstrate that functionally-defined MRUs, bipotent CFCs and myoepithelial CFCs exhibit additional phenotypic markers of basally-positioned cells, whereas luminal CFCs exhibit markers of luminally-positioned cells. Expression of these markers is highly correlated with CD49f and EpCAM expression; thus, there is only marginal benefit in their inclusion in a purification strategy for MRUs or CFCs beyond what can be achieved using CD49f and EpCAM alone.

3.3.3 Human embryonic stem cell markers SSEA-3 and SSEA-4 are expressed in the mammary gland, but are not useful MRU markers

To investigate whether hESC markers are expressed on normal HMECs, MRUs and/or CFCs, dissociated mammoplasty cells were analyzed by flow cytometry after staining with antibodies against EPCAM and CD49f, plus SSEA-1, SSEA-3, SSEA-4, Tra-1-60 or Tra-1-81. All 5 of the latter group of antibodies reacted with human mammary cells (Figs. 3.5a,b, 3.6a,b, 3.7a,b, 3.8a,b, 3.9a,b). In each case, the proportion of cells expressing the marker was highest in the luminal cell fraction (69±12\% positive for SSEA-4, 10-40\% positive for each of the other 4 markers) and low in the basal fraction (9±4\% positive for SSEA-4, <1\% for the other 4 markers). Functional assays carried out
on sorted fractions from 3 mammoplasty samples stained with SSEA-3 or SSEA-4 expression showed that MRUs and CFCs were SSEA-3⁻ (Fig. 3-5c), most MRUs, myoepithelial and bipotent CFCs were SSEA-4⁻, and luminal CFCs were split between the SSEA4⁻ and SSEA-4⁺ fractions (43±18% SSEA-4⁺) (Fig. 3-6c).

3.3.4 Markers that allow stem cells to be selectively isolated from other tissues do not enrich for MRUs within the EpCAM⁻lowCD49f⁺ fraction of HMECs

Staining HMECS for surface KIT expression showed a sizeable proportion of the luminal (but not mature luminal) fraction cells were positive (72% and 98% in 2 samples) and these included the majority of functionally identified luminal CFCs (87±13%, 3 samples, Fig. 3-10a-c). Within the basal fraction, KIT, CD48, EPCR and CXCR4 were each expressed by a small minority (~1%) of cells (Figs. 3.10–3.13a,b), but these were not found to contain MRUs when the corresponding positive (and negative) subsets of cells were assayed functionally for MRU activity; i.e., MRUs were consistently in the more predominant KITR⁻, CD48⁻, EPCR⁻, and CXCR4⁻ fractions (Figs. 3.10–3.13c).

3.3.5 MRUs do not exhibit a “side population” phenotype

The efflux properties of Hoechst 33342 dye by primitive mammary cells were examined next. Incubation of HMECs for 90 minutes with Hoechst 33342 yielded a small SP fraction (0.13±0.02% of HMECs, 4 samples, Fig. 3-14a, left panel), which included cells from all EpCAM/CD49f subfractions (Fig. 3-14b,c). Moreover, the SP subpopulation persisted when the ABCG2 inhibitors, fumitremorgin C or reserpine, were present during the period of incubation with Hoechst 33342 (Fig. 3-14a, middle panel). This indicates
that this phenotype in normal human mammary cells cannot be interpreted to infer expression of ABCG2. Notably, dissociated HMECs that had been cultured for 3 days (and hence assumed to be cycling, see Chapter 4), also contained a similar proportion of cells displaying the SP phenotype (0.14% and 0.25%, 2 samples, Fig. 3-14a, right panel). When the SP and non-SP (“main population”, MP) cells were isolated by FACS and assayed functionally, MRUs and CFCs were almost exclusively found in the MP fraction, for both non-cultured (Fig. 3-14d, upper panels) and cultured (Fig. 3-14d, lower panels) HMEC preparations. The SP phenotype is therefore not one that is shared by MRUs or CFCs.

### 3.3.6 MRUs have an Aldefluor low phenotype

To compare Aldefluor fluorescence in different human mammary subfractions, HMECs were incubated with BAAA for 30-40 minutes, then immunostained with antibodies against CD49f, EpCAM, CD31 and CD45. The increased level of fluorescence obtained in all fractions was diminished in the presence of the specific ALDH inhibitor DEAB, which is commonly used to define the level of specific activity present (Fig. 13.5a, compare blue lines with grey shaded areas). Aldefluor-based fluorescence was greatest in the luminal and stromal cell fractions (Fig. 3-15a, blue lines) which exhibited median fluorescence intensities that were much higher than those characteristic of the basal cell fraction (15.4±2.2-fold higher, p<0.001; and 14.1±2.7-fold higher, p<0.001, respectively; 7 samples, Fig 3.15b).

These profiles also highlight the difficulty of interpreting Aldefluor\textsuperscript{high} staining results when heterogeneous populations are being stained together because the DEAB
control may show variable levels of inhibition in different cell types. Here, it can be seen that the Aldefluor\textsuperscript{high} subset of cells with fluorescence exceeding a threshold set from the DEAB treated control (Fig. 3-15a, vertical line) comprised a sizeable proportion of the luminal and stromal cells (28.1±2.3% and 31.0±5.4%) along with a small minority of basal and mature luminal fraction cells (1.0±0.3% and 2.8±0.7% respectively, n=7; Fig. 3-15c). When the luminal and basal HMECs were then sorted into their respective Aldefluor\textsuperscript{high} and Aldefluor\textsuperscript{low-med} fractions and then assayed for MRU s or CFCs, almost all MRU activity was found in the Aldefluor\textsuperscript{low-med} fraction (94% and 95% of secondary CFCs were detected in gels seeded with cells from this fraction, 2 samples, Fig. 3-15d, right panel). The Aldefluor\textsuperscript{low-med} fraction also contained 88% of all primary bi-potent plus myoepithelial CFCs and 44% of all luminal CFCs (n=2, Fig. 13.5d, left panel).

A further 3 mammoplasty samples were then analyzed using a strategy that divided cells into Aldefluor\textsuperscript{low}, Aldefluor\textsuperscript{med} and Aldefluor\textsuperscript{high} fractions, using 2 thresholds set from the maximum Aldefluor fluorescence in the DEAB-treated basal cell population and the DEAB-treated luminal cell population (Fig. 3-15e). Luminal and basal cells fractionated in this way were then assayed for relative CFC and MRU activity as above. For the luminal CFCs (which were predominantly detected in the luminal fraction, as expected), the split between Aldefluor low:med:high fractions was on average 5:62:33 (Fig. 3-15f, upper panel). In contrast, the myoepithelial plus bipotent CFCs (predominantly in the basal fraction) were split 75:24:1 and the MRUs 81:18:1 (Fig. 3-15f, lower panel).
Overall, these findings demonstrate that high ALDH activity is a characteristic of cells with luminal features, including luminal-restricted CFCs, but not of myoepithelial-restricted CFCs, bi-potent CFCs or MRUs, which show low ALDH activity.

3.4 Discussion

The phenotype studies described here showed most MRUs (as defined by secondary CFC generation in vivo) to be positive for phenotypes associated with basally located HMECs; i.e. CD49f, CD10 and variably CD90 (Thy1)\(^{40,176,181}\), and low/negative for HMECs that line the lumen of the mammary gland; i.e., EpCAM and CD133\(^{177,182}\). They were also negative for CD31 and CD45, as are all HMECs, which allows the removal of the large numbers of hematopoietic cells and endothelial cells that are present in dissociated suspensions of breast tissue. Assessment of other candidates (SSEA-3 and 4, CD117 (KIT), EPCR, CD48, CXCR4, Hoechst 33342 efflux activity (SP phenotype) and ALDH activity) showed that none of these were positive markers of MRUs and the low ALDH activity of MRUs discriminated them from the luminal CFCs.

The phenotype that consistently identified MRUs with the greatest selectivity was CD49f\(^+\)EpCAM\(^{low}\)CD31\(^-\)CD45\(^-\) (92±3%, n=9 of all detected MRU activity in freshly dissociated cells obtained from cryopreserved organoid-enriched reduction mammoplasty tissue). This cellular fraction represented 15.5±2.3% (n=15) of all the cells, indicating a ~6-fold enrichment in MRU content. This phenotype, however, is not highly specific for MRUs, as the average frequency of MRUs was only ~1 per 850 CD49f\(^+\)EpCAM\(^-\)CD31\(^-\)CD45\(^-\) cells (applying a 6-fold enrichment in purity to an estimated starting
MRU frequency of ~1 per 5000 unfractionated mammary cells, see Section 2.3.4 in Chapter 2).

CD49f together with CD29 constitutes an integrin with specificity for binding to laminin, which is a major component of the basement membrane surrounding the mammary gland. Expression of CD49f by basally located HMECs implicates an attachment role for these cells to the basement membrane which might include MRUs. Consistent with this possibility is the reported finding that a majority of sorted CD49fEpCAM$^{-}\text{low}CD31^{-}\text{CD45}^{-}$ cells express other markers of basally located myoepithelial cells (i.e., vimentin, CK14 and p61) detectable by immunohistochemistry$^{183}$.

The basal phenotype found here for human MRUs mirrors the CD49f$^{+}$CD29$^{+}$CD24$^{\text{med}}$EpCAM$^{\text{low}}$CD31$^{-}$$\text{CD45}^{-}$Ter119$^{-}$ phenotype reported for murine MRUs$^{(16, 17 \text{ and J Stingl unpublished data})}$. The ability of sorted basal fraction cells to demonstrate the entire regenerative repertoire in the absence of other cells also mirrors findings in the mouse system. Analogous findings have also recently been obtained using the alternative “humanized fat pad” xenotransplantation system$^{183}$. Human mammoplasty cells with the ability to regenerate macroscopic structures over 8-10 weeks in fibroblast-colonized mouse fat pads were exclusively found in the CD49f$^{+}$EpCAM$^{-}\text{low}CD31^{-}$$\text{CD45}^{-}$ fraction, albeit with a considerably lower frequency of ~1 per 21,500 cells (c.f. ~1 per 850 cells detailed above). It is not possible to say whether the cells that are identified in these 2 xenotransplant assays are distinct or overlapping populations. The order-of-magnitude difference in measured frequencies may indicate that the humanized fat pad
approach is more selective (i.e., detects a rarer subpopulation of primitive cells); or, alternatively, it may be indicative of a technically less efficient assay procedure.

The MRUs were also largely separable from cells of the luminal lineage, as defined by a CD49f EpCAM^+CD31^-CD45^- (“mature luminal fraction”) or a CD49f EpCAM^- (“luminal fraction”) phenotype with luminal CFC activity concentrated in the latter. The separable phenotypes of MRUs and luminal cells, including luminal CFCs, taken together with the observation that luminal CFCs are readily detected in gels seeded with MRUs at limiting dilution from the basal fraction, implies that luminal CFCs lie downstream of MRUs in the human mammary cell hierarchy. Conversely, our failure to identify any markers that distinguish MRUs from bipotent (or myoepithelial) CFCs leaves the precise relationship between these primitive cell types indeterminate. The MRU phenotype also differed from the reported CD49f EpCAM^- phenotype of HMECs that form 3-D branched structures in Matrigel, suggesting that this Matrigel-based readout does not provide a good surrogate assay for human mammary stem cells.

MRUs and bipotent CFCs were found to have an Aldefluor^low phenotype, in contrast to cells of the luminal lineage (including luminal CFCs) which showed an Aldefluor^high phenotype. This mirrors data we recently reported derived from assessments of bovine mammary cells capable of regenerating CFCs in subrenal transplants in NOD-SCID mice, which showed that these cells also have an Aldefluor^low phenotype. On the other hand, these findings appear difficult to reconcile with those reported by Ginestier et al., who reported that the Aldefluor^high cell population contained the majority (~85%) of bipotent CFCs, a minority (~15%) of luminal CFCs, and most cells with the ability to regenerate structures when xenotransplanted into
humanized fat pads. Further experiments will clearly be required to understand the basis of these discrepancies.

On a technical note, it should be noted that all of the MRU data described in this Chapter were derived using a bulk secondary CFC output endpoint. That is, the relative output of secondary CFCs per gel from each fraction was assumed to provide a reasonable quantitative estimate of their relative MRU content. This strategy for relative MRU quantitation is much cheaper and more precise than would be obtained using MRU limiting dilution assays (see Supplement in ref152, attached as Appendix B). However, the utility of data derived using such a bulk approach relies on the validity of the assumption that the average secondary CFC output per MRU is not the major determinant of any differences noted between the populations being compared (an assumption that can, in principle, be tested). To support this assumption, multiple replicate gels were set up to reduce clonal variabilities in the CFC output of individual MRUs using non-saturating concentrations of test cells. A further assumption made here was that the reagents used for fractionating cells did not differentially alter the viability and/or functionality of the cells. While this assumption was not tested explicitly, the consistency with which basal cell surface phenotypes (CD49f+, Thy1+, CD10+, CD133–, EpCAM–/low) gave an association with MRU readout makes it very unlikely that these results are artifacts caused by toxicities to specific antibodies.

In summary, human MRUs show the same basal marker profile as bovine and murine mammary epithelial cells that meet similar or more stringent criteria for defining stem cells functionally. This preservation of basic stem cell properties in the mammary gland across species reinforces expectations that cross-comparisons between these
species will be useful in identifying molecular mechanisms that control their biological behavior. The present findings also underscore the need for continued effort to identify markers that will allow their isolation at greater purity. Such an effort is also important to examine the developmental relationship between MRUs and other basal cell types, including bipotent CFCs, myoepithelial-restricted CFCs and mature myoepithelial cells.
Figure 3-1  MRUs have a CD49f⁺EpCAM⁻flow phenotype

(a) Experimental protocol (as in Fig. 2-2a, but starting with FACS-sorted subsets in the transplants). (b) FACS profile of input human mammary cell preparations (depleted of CD45⁺ and CD31⁺ cells) showing gates used to select fractions assayed for MRUs and CFCs. (c) Left panel: symbols show the proportion of luminal (open red circles) or combined myoepithelial plus bipotent CFCs (solid blue circles) detected in primary in vitro assays that originated in the CD49f⁺EpCAM⁻flow fraction (A in Fig. 3.1b) (n = 9 mammoplasty samples). Symbols are absent where samples did not yield a given type of primary CFC. Right panel: Solid diamonds show the proportion of all subsequently detected xenograft-derived CFCs that were generated in the gels transplanted with cells from the initial CD49f⁺EpCAM⁻flow (A) fraction. The numbers of cells assayed from each fraction were in proportion to the relative sizes of each fraction. (d) Sections of 4-week xenografted gels seeded with FACS-sorted CD49f⁺EpCAM⁻flow CD31⁻CD45⁻ cells, stained with H&E, or with antibodies to collagen IV, CK14 or CK18. Bars = 50 µm. (e) A representative FACS profile of cells from a 4-week xenograft initiated with CD49f⁺EpCAM⁻flow cells.
Figures 3-2 – 3-13  Phenotypic and functional characterization of additional candidate MRU cell surface markers.

See following pages.

(a) Representative FACS profile showing the CD49f and EpCAM profile of human mammary cells that express the candidate marker (blue dots). The grey density plot shows the corresponding profile of all (i.e., marker-positive or -negative) human mammary cells. CD31$^+$ and CD45$^+$ cells were excluded from the profiles by gating. (b) Proportion of cells in phenotypically-defined subfractions that express the candidate marker. Basal= CD49f$^+$EpCAM$^{low}$CD31$^-$$CD45^-$, Luminal= CD49f$^+$EpCAM$^+$CD31$^-$$CD45^-$, Mat.Luminal= CD49f$^+$ EpCAM$^+$CD31$^-$CD45$^-$, Stromal= CD49f$^-$ EpCAM$^-$CD31$^-$CD45$^-$. (c) Results of functional assays. Left panel: proportion of all primary luminal CFCs (empty red circles) and proportion of all primary myoepithelial plus bipotent CFCs (solid blue circles) that originated in the cell fractions indicated. Each symbol represents a different mammoplasty samples, with averages shown as horizontal bars. Right panel: proportion of all regenerated CFCs (luminal plus myoepithelial plus bipotent) detected in 4 week in vivo-propagated gels that originated in the cell fractions indicated, which is a measure of the proportion of all MRUs in each fraction. Solid diamonds represent different mammoplasty samples, with averages shown as horizontal bars. Aliquots from each fraction were seeded in gels in cell numbers proportional to fraction sizes. CD31$^+$CD45$^-$ cells were excluded from sorting gates in all these experiments, and in some cases CD49f$^-$ or CD49f$^-$EpCAM$^-$ cells were additionally excluded.
Figure 3-2  Phenotypic and functional characterization: CD10

(see full legend description following Figure 3-1)
Figure 3-3  Phenotypic and functional characterization: Thy1

(see full legend description following Figure 3-1)
Figure 3-4  Phenotypic and functional characterization: CD133

(see full legend description following Figure 3-1)
Figure 3-5  Phenotypic and functional characterization:  SSEA-3

(see full legend description following Figure 3-1)
Figure 3-6  Phenotypic and functional characterization: SSEA-4

(see full legend description following Figure 3-1)
Figure 3-7  Phenotypic characterization: SSEA-1

(see full legend description following Figure 3-1)
Functional assays not carried out for this marker

Figure 3-8  Phenotypic characterization: Tra-1-60

(see full legend description following Figure 3-1)
Figure 3-9  Phenotypic characterization: Tra-1-81

(see full legend description following Figure 3-1)

Functional assays not carried out for this marker
Figure 3-10 Phenotypic and functional characterization: KIT

(see full legend description following Figure 3-1)
Figure 3-11 Phenotypic and functional characterization: CD48

(see full legend description following Figure 3-1)
Figure 3-12  Phenotypic and functional characterization: EPCR

(see full legend description following Figure 3-1)
Figure 3-13  Phenotypic and functional characterization: CXCR4

(see full legend description following Figure 3-1)
Figure 3-14  MRUs do not have a SP phenotype

(a) Representative FACS profile of mammoplasty cells after 90 minutes incubation with Hoechst 33342, showing a small proportion (~10⁻³) of cells exhibiting a SP phenotype. Left panel: uncultured HMECs. Middle panel: the same HMEC sample incubated with fumitremorgin C (5µM) together with the Hoechst 33342. The proportion of SP cells is not significantly changed. Right panel: The same HMEC sample, cultured for 3 days before Hoechst staining. (b) FACS profile showing the CD49f and EpCAM profile of SP cells in (a). The grey density plot shows the corresponding profile of all human mammary cells in the sample. (c) Proportion of SP cells in phenotypically-defined subfractions that express the candidate marker (see figure legend 3.2-3.12 for fraction definitions). Bars show mean±SEM for 4 uncultured mammoplasty samples. (d) Results of functional assays of SP and non-SP cells. Proportion of CFCs (upper left panel) and MRUs (upper right panel) that originated from SP or non-SP (“main population, MP) cells from freshly dissociated cells from previously cryopreserved mammoplasty samples (n=3). Analogous data is shown in the lower left and lower right panels, using 3-day pre-cultured mammoplasty cells (n=2).
Figure 3-15  MRUs have an Aldefluor low phenotype
(a) Representative Aldefluor fluorescence profiles of different phenotypically defined subsets of HMECs. Dissociated cell suspensions were incubated for 30 minutes with BAAA, then immunostained with antibodies against EpCAM, CD49f, CD31 and CD45. Blue lines show the Aldefluor fluorescence for the starting cell suspension of unfractionated selected only to be viable (upper panel), the 4 subfractions identified in Fig. 3-1b (middle 4 panels), and the hematopoietic/endothelial cells expressing CD31 or CD45 (lower panel). Solid grey profiles show the Aldefluor fluorescence of cells in the corresponding cell fractions when inhibitor DEAB was added during the BAAA incubation. (b) Median Aldefluor fluorescence intensity of cells in each subfraction relative to the median fluorescence intensity of the basal fraction (mean±SEM, n=7 samples). (c) Percentage of cells in each subfraction that exhibit an Aldefluor fluorescence intensity higher than the maximum fluorescence of the DEAB-treated control (vertical line in (a)) (mean±SEM, n=7 samples). As shown, the Aldefluor$^{\text{high}}$ subfraction defined in this way contains a sizeable proportion of luminal and stromal cells, but only a small minority of cells from other fractions. cont…
Figure 3-15 cont.
(d) Results of *in vitro* and *in vivo* functional assays to determine the proportion of CFCs (left panel) and MRUs (right panel) that have an Aldefluor high phenotype. Mammary cells were first sorted into subsets defined as Aldefluor low-med and Aldefluor high with reference to the maximum fluorescence of the DEAB-treated control, and the isolated cells were then assayed for CFC and MRU content. n=2 samples analyzed.
(e) Gating strategy used for more detailed subdivision of fractions. Basal and luminal fractions were each further divided into Aldefluor low, Aldefluor med and Aldefluor high subfractions with reference to gates drawn from the maximum fluorescence of DEAB-treated luminal and basal cells. (f) Results of CFC and MRU assays to determine the proportion of CFCs (upper panel) and MRUs (lower panel) in the 6 subfractions described in (e). n=3 samples analyzed.
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<td>CD49f</td>
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<td>Marker of mouse MRUs\textsuperscript{16,17}, and human luminal, myoepithelial and bipotent CFCs\textsuperscript{38-40}.</td>
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<td>Marker of luminal cells in situ\textsuperscript{177} and CFCs of all types in 3 day precultured HMECs\textsuperscript{38-40}.</td>
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<td>ABCG2</td>
<td>Fluorescence after incubation with Hoechst 33342</td>
<td>Marker of non-activated adult mouse (but not human) hematopoietic stem cells (high transporter activity, low fluorescence “side population” phenotype)\textsuperscript{168,169}.</td>
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<td>ALDH</td>
<td>Fluorescence after incubation with BAAA</td>
<td>Marker of human (but not mouse) hematopoietic stem and progenitor cells (ALDH\textsuperscript{175,186}). Suggested marker of human breast cancer stem cells.</td>
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Table 3-1 Phenotypic markers tested for association with MRU or CFC activity
4 INVESTIGATION OF THE PROLIFERATIVE STATUS OF HUMAN MAMMARY STEM AND PROGENITOR CELLS

4.1 Introduction

The objective of the experiments presented in this chapter was to gain preliminary information as to whether the primitive cells at different levels in the mammary cell differentiation hierarchy (CFCs, MRUs) are quiescent in normal human adult breast tissue or whether a substantial proportion are actively cycling.

A major challenge to obtaining such information is that these primitive cell types are currently identified exclusively by retrospective functional assays that must be initiated with viable cells. Yet the “gold standard” techniques to assess the cycling status of populations of cells rely on the detection of intracellular factors (e.g., cyclins, Ki67, PCNA, BrdU incorporated into DNA) in fixed cells, precluding the simultaneous or subsequent measurement of their functional properties. Nonetheless, there are a number of phenotypic properties that are influenced by or determined by passage of cells through specific stages of the cell cycle and which can be distinguished in viable cells. These latter methods have been successfully used to characterize the cell cycle profile of functionally defined cell types in other tissues.

The thymidine suicide method is one of these, initially developed to examine the cycling status of primitive hematopoietic cells in the bone marrow of adult mice. The technique is based on the principle that the proportion of cells in a fully cycling population will contain a proportion in S-phase that approximates the duration of the S-phase relative to the time required for cells to progress through the entire cell cycle. Thus a pulsed treatment of the population to an agent like high specific activity $^3$H-thymidine
(³H-Tdr) that is uniquely toxic to cells exposed in S-phase will kill that proportion of the
total cycling population. Conversely, if very few cells in the population are actively
cycling, then very few will be killed by such a treatment and the population is inferred to
be in a quiescent state. More recently, the ³H-Tdr methodology has been modified to
enable the more sensitive detection of minor populations of quiescent cells. This has
been accomplished by increasing the exposure of the test cells to the high specific activity
³H-Tdr for a period equivalent to a full cell cycle in order to capture those in G1, G2 and
M as well. This has been facilitated by the finding that, at least in the hematopoietic
system, quiescent cells require a period of stimulation at least equivalent to a full cell
cycle before they will enter cycle S-phase¹⁸⁸. Under these conditions, a more prolonged
incubation of a mixed cell population with high specific activity ³H-Tdr (or other S-
phase-specific cytotoxic agent) for a period of time that is longer than the combined
G2/M/G1 cell cycle time, but shorter than the time taken for a quiescent cell to enter S-
phase, would be expected to kill all initially cycling cells but spare all initially quiescent
cells.

FACS detection or isolation of viable cells stained with Hoechst 33342 and
Pyronin Y staining is another method used to make inferences about the relative
proportion of cycling cells in functionally or phenotypically defined cell populations¹³⁶.
Hoechst 33342 and Pyronin Y are fluorescent dyes that diffuse freely into cells and then
bind stoichiometrically and irreversibly to the DNA and RNA, respectively, that the cells
contain. The use of Hoechst 33342 and Pyronin Y-staining to discriminate cycling and
quiescent cells is based on the fact that cycling cells double their DNA content between
the beginning and ending of S-phase and that cycling cells also typically contain more RNA than their quiescent counterparts (due to their higher content of ribosomes).

Rhodamine-123 (Rho) staining is another strategy that has been used to assess the cycling status of some cell types. Rho is a fluorescent dye that also diffuses freely into cells and then binds to mitochondrial membranes. However, it can also be actively exported by certain energy-dependent cell surface transporters (e.g., ABCB1/MDR/P-glycoprotein) that are selectively expressed on particular cell types. Rho staining may be used to identify cycling cells in the living state based on the assumption that they have more active mitochondria than their quiescent counterparts.

While considerable validation of these methods has been undertaken in connection with their applicability to many types of hematopoietic cells, their applicability for assessing the cell-cycle status of mouse or human mammary populations has not been similarly investigated. This Chapter therefore describes a number of experiments carried out to determine whether and how each of these approaches could be used, before applying them to determine the proportions of MRUs and CFCs in cell suspensions prepared from a series of freshly obtained mammoplasty samples.

4.2 Methods

4.2.1 Cell preparation. All of the human mammary cells used in these studies were derived from mammoplasty tissue that was freshly removed by surgeons, transported to the laboratory on ice, then dissociated rapidly on arrival. The tissue was first mechanically chopped using a McIlwain Tissue Processor (Lafayette Instrument Company, IN). It was then enzymatically dissociated at 37 °C as described in Chapter 2,
but for 4–4.5 hours instead of the usual 18–24 hours, and with 600 U/ml collagenase and 200 U/ml hyaluronidase instead of the usual 300 U/ml and 100 U/ml. Unless otherwise stated, the resulting cell suspensions were analyzed immediately without prior freezing and thawing in order to minimize any change in their cycling state in vivo.

4.2.2 Thymidine suicide assays. Cells to be assayed were suspended in pre-warmed (37 °C) DMEM supplemented with 10 ng/ml EGF, 10 ng/ml cholera toxin, 1 µg/ml insulin, 0.5 µg/ml hydrocortisone and 5% vol/vol dialyzed FBS, and plated in replicates in wells of a 24-well tissue culture plate in 300 µl of medium per well or, for cells numbers > 5x10^5, in wells of a 6-well tissue culture plate in 2ml medium per well. When small numbers of cells (<5x10^4) were assayed, irradiated 3T3 fibroblast cells were also added (4x10^4 fibroblasts per well in 24-well plates). High specific activity (20-25 Ci/mmol) [methyl-^3H]-thymidine (GE Healthcare or Perkin Elmer) or vehicle control (70% ethanol) were added to replicate wells to the desired final concentrations (20 µCi/ml for most experiments) and the plates were incubated at 37 °C for 16 hours – 4 days as indicated. Medium was aspirated at the end of the incubation period, and HF supplemented with 400 µg/ml cold thymidine then added. The cells were then washed once in PBS supplemented with 40 µg/ml cold thymidine. The adherent cells were then harvested using trypsin/EDTA, and aliquots of ^3H-Tdr and vehicle-exposed cells assayed functionally for CFCs and/or MRU activity as described in Chapter 2. For short exposure times (<1 h), the incubations with ^3H-Tdr or vehicle control were carried out in suspension in eppendorf tubes in 300 µl medium rather than under adherent conditions.
4.2.3 Hoechst-Pyronin and Rho staining. The Hoechst-Pyronin staining protocol was adapted from the method of Shapiro\textsuperscript{136}. Cells were suspended at a concentration of $10^6$ cells/ml in pre-warmed (37 °C) SF7 medium supplemented with 1 µg/ml Hoechst 33342 (Sigma) in 15 ml Falcon tubes and incubated for 30 minutes in a 37°C incubator. Pyronin Y (1 µg/ml, Sigma) was added and the tubes returned to the incubator for a further 30 minutes. An equal volume of ice-cold HF supplemented with 1 µg/ml Hoechst and 1 µg/ml Pyronin was added after the incubation and the tubes centrifuged @4°C. The supernatant was removed, and the cell pellet washed in ice-cold HF. The cells were then immunostained with antibodies against cell surface markers following standard procedures, in ice-cold medium throughout. PI (5 µg/ml) was used as viability dye. For FACS analysis and sorting, Hoechst fluorescence was viewed on a linear scale and Pyronin fluorescence on a log scale. Viable cells were identified on a Pyronin-PI plot, suitably compensated. Hoechst-Pyronin profiles were viewed for all viable cells, or gated on specific mammary subfractions. Sorting gates were drawn to identify presumed G0, G1 and S/G2/M subfractions as previously described\textsuperscript{136}; i.e., the cells with >2n DNA content on the Hoechst scale were identified as the S/G2/M subfraction, and the cells with 2n DNA content split into a G0 (low Pyronin) and a G1 (high Pyronin) subfraction with reference to the lowest Pyronin fluorescence of cells within the S/G2/M subfraction. A small Pyronin-low subfraction with 4n DNA content was also observed; this was presumed to comprise G0 cell doublets, and was excluded from sorting gates. For Rho staining, cells were suspended in pre-warmed (37°C) SF7 supplemented with 0.3 µg/ml Rho (Sigma) and incubated for 30 minutes at 37°C. Cells were then washed with ice-cold HF, and immunostained with antibodies against designated cell surface markers.
For some samples, the cell suspensions were cryopreserved and thawed before Hiechst-Pyronin and Rho incubation.

**4.2.4 Immunohistochemistry.** Cells were sorted into cold medium, then cytospun onto microscope slides and immediately fixed with methanol:acetone (1:1 vol/vol) for 5 minutes at room temperature. The slides were stored in PBS @ 4°C for up to 1 week, before immunostaining following standard procedures, using a rabbit anti-human Ki67 primary antibody (clone SP6, Labvision) and rabbit-specific peroxidase-based secondary visualization reagents (Polink 2-plus Rabbit with DAB, Golden Bridge International Inc., Mukiltea, WA).

**4.2.5 Monte Carlo simulations.** For LDA simulations, gels were assumed to have been seeded with exactly the dilution of cells each that would on average contain 1 MRU per gel. For bulk-CFC output simulations, gels were assumed to have been seeded with exactly the dilution of cells that would have produced a mean secondary CFC output of 70 CFCs with a standard deviation of 30, that is consistent with empirical results from experiments in Chapters 2 and 3. Simulations included 20 gels per MRU assay (10 each for tests of control and $^3$H-Tdr treated cells, 10 each for tests of Pyronin$^{\text{high}}$ and Pyronin$^{\text{low}}$ cells, and 10 each for tests of Rho$^{\text{high}}$ and Rho$^{\text{low}}$ cells. Primary CFC simulations assumed a mean colony output of 200 per assay. 1600 experiments were simulated randomly assuming varying proportions (0-100%) of cycling cells, and the 2.5 and 97.5 percentiles of measured proportions of cycling cells were then calculated. Separate calculations were made for each pair of test ($\pm$H-Tdr suicide, Pyronin high and
low cells and Rho high or low cells). The simulations were designed to capture only the uncertainties related to Poisson counting, technical errors or replication, and biological variability in the CFC output per MRU, and not any shortcomings in the selection/sorting methodologies (which were assumed here to be able to distinguish cycling and quiescent cells with 100% accuracy).

4.3 Results

4.3.1 Ki67 measurements identify samples with predominantly quiescent basal fraction CFCs

To determine the frequency of cycling cells in various phenotypically distinguished subsets of normal HMECs, the CD49f\textsuperscript{+}EPCAM\textsuperscript{+}CD31\textsuperscript{−}CD45\textsuperscript{−} (luminal) and the CD49f\textsuperscript{+}EPCAM\textsuperscript{−/low}CD31\textsuperscript{−}CD45\textsuperscript{−} (basal) fractions were isolated by FACS from cells that had been obtained from fresh mammoplasty tissue dissociated using a rapid (4–4.5 hour) chopping and enzymatic digestion procedure. The sorted fractions of cells were then cytospun onto microscope slides and immunostained with an antibody against human Ki67 (Fig. 4-1a). The frequency of Ki67\textsuperscript{+} cells was ~5 times higher in the total luminal (4.3±2.3%) as compared to the total basal fraction of cells (0.8±1.5%, n=5, p=0.03) (Fig. 4-1b). In 3 of 5 samples analyzed, the proportion of Ki67\textsuperscript{+} cells in the basal fraction was lower than the sensitivity of detection (set by a frequency of false positive immunostaining of ~1 per 500 cells). The same sorted fractions were also assayed for their CFC content (Fig. 4-1b). Interestingly, the frequency of CFCs in the basal fraction (>90% of which generate pure myoepithelial colonies or colonies of mixtures of myoepithelial cells and luminal cells) was >10-fold greater than the frequency of basal
fraction Ki67+ cells in 3 of 5 samples, implying that, at least in these samples, 90% were Ki67- (i.e., quiescent). In the luminal fraction, the frequency of CFC was greater than the frequency of Ki67+ cells in 2 of these same 5 samples reinforcing that some of the luminal CFCs were also quiescent.

4.3.2 Development and validation of a thymidine suicide protocol for distinguishing cycling and quiescent HMECs

To develop a 3H-Tdr suicide method applicable to primary HMECs, a first series of experiments were carried out to determine the minimum concentration of high specific activity 3H-Tdr that would kill those in S-phase in a pulse exposure. For this first test, we used 184-htert cells, a telomerase-immortalized human breast epithelial cell line. 184-htert cells were taken from log phase expansion cultures (and hence assumed to contain a high proportion of cycling cell) and were then incubated for 30 minutes in zero or varying concentrations of 3H-Tdr prior to addition of cold Tdr and subsequent assessment of CFC activity. Maximum loss of CFC activity was obtained using a 3H-Tdr concentration of 20 µCi/ml, and this effect was almost reversed by competition with excesses of non-radioactive Tdr (Fig. 4-2a). The 184-htert cells were then exposed to 20 µCi/ml 3H-Tdr for longer periods to determine if and when a maximum would be reached, as might be expected following transit of all the cells through a single cell cycle. Within 24 hours, all CFC activity was lost suggesting the cell cycle of these cells is less than 24 hours. (Fig. 4-2b).

To test the specificity of this 3H-Tdr exposure method for killing initially cycling but not initially quiescent mammary cells, we undertook a second series of experiments with 2 more “control” populations. One was a replicate of the 184-htert cell model but
this time using primary HMECs that had been previously cultured for 4 days in EGF-containing medium. Intracellular Ki67 staining confirmed that >80% of these cells were proliferating (Fig. 4-2c, right panel). The second control population was an “EGF-starved” control, generated by culturing mammary organoids for 4 days in EGF-free medium. Ki67 staining showed most of these cells to be quiescent (Ki67−, Fig 4-2c, left panel). Each of these populations was then cultured for varying lengths of time in EGF-containing medium in the presence or absence of high-specific activity 3H-Tdr (20 μCi/ml), and CFC assays then performed. A 24-hour exposure time again proved highly discriminatory between the predominantly cycling and quiescent populations, with loss of 93±3% of CFCs in the former population but only 11±3% in the latter (Fig. 4-2d).

4.3.3 High Pyronin Y fluorescence selectively identifies cycling human mammary cells, but with high toxicity and low specificity

The ability to discriminate cycling from quiescent HMECs on the basis of their fluorescence profile after incubation with Hoechst 33342 and Pyronin Y was next tested. Hoechst-Pyronin stained cells from the same type of EGF-starved and EGF-stimulated HMEC populations described above displayed markedly different profiles. Most of the EGF-stimulated HMECs had an elevated Pyronin fluorescence and these included a sizeable proportion of cells with a Hoechst fluorescence indicative of a DNA complement of >2n, consistent with the high proportion of cycling cells in this population (Fig 4-3a).

When this staining protocol was applied to rapidly-dissociated (uncultured) primary HMECs, a >100-fold range of Pyronin fluorescence intensities was observed, with markedly higher fluorescence exhibited by the cells in the luminal as compared to the basal fraction of all samples analyzed (n=8, Fig. 4-3b). To determine whether a
sorting strategy based on the Hoechst-Pyronin profile could accurately separate cycling from non-cycling cells in these samples, gates were drawn around postulated G0, G1 and S/G2/M fractions as previously described\textsuperscript{136, 144}. The S/G2/M fraction was identified as the population of cells with a $>2n$ DNA content on the Hoechst scale. The G0 fraction was identified as the population of remaining cells (with 2n DNA) that showed a Pyronin fluorescence lower than the lowest Pyronin fluorescence of cells within the S/G2/M subfraction. The G1 fraction was identified as the population of remaining cells, with 2n DNA content on the Hoechst scale and a Pyronin fluorescence higher than the G0 subfraction (Fig. 4-3b). Cells from the G0 and combined G1 + S/G2/M fractions of luminal cells were then isolated by FACS, cytospun onto microscope slides and immunostained with an antibody against human Ki67. An average of 70±14% of all of the Ki67$^+$ luminal cells were found in the G1/S/G2M gates (n=3), indicating that this gating strategy offers a reasonable degree of selectivity for cycling cells (Fig. 4-3c, left panel). However, the absolute frequency of Ki67$^+$ cells within the G1/S/G2M gates was only 8±3% (Fig. 4-3c, right panels), confirming that the Hoechst-Pyronin gating strategy used to select for G1/S/G2/M cells, on its own has very poor specificity for cycling mammary cells.

Toxicity of these dyes in viable cells has been previously noted\textsuperscript{190}, raising this effect as a possible concern in interpreting data using this staining strategy to identify the cycling status of different functionally defined subsets of HMECs. Preliminary experiments in which the viability of 184-htert cells was examined by PI staining after their exposure to a range of Hoechst and Pyronin concentrations confirmed the toxicity of these dyes on this cell line (data not shown). Accordingly, experiments were designed to
determine the potential magnitude of this effect on primary human mammary cells.
These involved incubating HMECs in the presence or absence of Hoechst and Pyronin, then immunostaining the cells with cell surface markers plus PI, a viability dye, and finally sorting viable basal and luminal fractions by FACS followed by CFC frequency assessments. Exposure to Hoechst and Pyronin resulted in a marked reduction in the frequency of CFCs within the PI– (i.e., viable) fractions of basal and luminal cells (81±5% and 67±13%, respectively) (Fig. 4-3d).

4.3.4 High Rhodamine fluorescence is not a discriminating property of cycling mammary cells
The ability to discriminate cycling from quiescent mammary cells on the basis of their fluorescence profile after incubation with Rho was next tested. EGF-stimulated control HMECs exhibited a higher (~3-fold) mean fluorescence intensity than the EGF-starved control cells. However, the extent of overlap between the 2 profiles meant that it was not possible to identify a fluorescence threshold level that clearly discriminated between them (Fig. 4-4a). Rapidly dissociated, uncultured HMECs were then incubated with Rho, immunostained with cell surface markers, and the luminal fraction cells were then sorted into Rhohigh and Rholow-med fractions, with the Rhohigh cells defined as those with the highest 15% Rho fluorescence (Fig. 4-4b). Immunohistochemical staining for Ki67 expression revealed that the Rhohigh subfraction contained only 19% of all of the Ki67+ luminal cells (Fig. 4-4c, left panel, n=2), and the frequency of Ki67+ cells within the Rhohigh fraction was 5% (Fig. 4-4c, right panel). These data indicate that high Rho expression is neither a very specific nor a very selective property of cycling mammary cells.
4.3.5 A significant proportion of human mammary CFCs and MRUs in adult breast tissue exhibit cycling phenotypes

Functional assays were then combined with the 3 methods proposed to provide information as to the cycling status of the CFCs and MRUs in 10 freshly isolated HMEC preparations. The experimental design is depicted in Fig. 4-5a. Aliquots of rapidly-dissociated primary HMECs were cultured for 18 hours in the presence or absence of $^3$H-Tdr, and then assayed for CFC and/or MRU content. In the case of the experiments that included MRU assays, the more sensitive “bulk CFC” rather than LDA measurements were used, with large numbers of replicate gels transplanted (total 20-24 gels per sample) to average out biological variability in CFC output per MRU and technical variability between individual gels. Additional aliquots were incubated with Hoechst-Pyronin and/or Rho in combination with cell surface markers, and the luminal and basal fractions were then subdivided into Pyronin$^{\text{high}}$ or Rho$^{\text{high}}$ (cycling) and Pyronin$^{\text{low}}$ or Rho$^{\text{low}}$ (quiescent) subsets which were sorted separated and assayed for CFC and/or MRU frequencies and content.

The results are shown in Fig. 4-5b. The values in the table (upper panel) and in the plot (lower panel) represent the percentage of each functionally-defined cell type (luminal CFCs, combined myoepithelial plus bipotent CFCs, MRUs) in a given sample that had a cycling phenotype according to the criteria established for each methodology (regardless of its validity). For the $^3$H-Tdr suicide methodology, this value is the percentage reduction measured for each progenitor type in the cells exposed to $^3$H-Tdr for 18 hours as compared to those treated the same but without the added $^3$H-Tdr. For the Hoechst-Pyronin staining methodology, the value shown is the number of assayed cells derived from the G1 + S/G2/M gates expressed as a percentage of combined G0 + G1 +
S/G2/M gates. For the Rho staining methodology, this value is the number of cells found in the Rho$^{\text{high}}$ gate (top 10-15% fluorescent Rho-stained cells) expressed as a proportion of the cells captured in the combined Rho$^{\text{low-med}} + \text{Rho}^{\text{high}}$ gates.

Strikingly, for 9 out of 10 samples tested, a sizeable proportion (at least 10%) of primitive cells exhibited a cycling phenotype, albeit with considerable variability between samples and methodologies (Fig. 4-5b). For the combined myoepithelial plus bi-potent CFCs, the median measured proportion of cycling cells ranged from 13%-31% and for the luminal CFCs, the corresponding range was 9%-31%, with the highest values derived from the $^3$H-Tdr assays in both cases. For MRUs, the values for cycling cells ranged even more widely from 6-91% although these were also based on smaller numbers of experiments.

To assess the extent to which the different methodologies gave concordant readings, the proportion of cycling cells determined by the different endpoints used were compared in a pairwise fashion for all samples where 2 or more strategies were applied (Fig. 4-5). Low $r^2$ values were observed from regressions of Hoechst-Pyronin values compared to $^3$H-Tdr suicide ($r^2=0.07$, upper panel) or Rho values ($r^2=0.19$, middle panel). A further test of the slope of the regressions obtained also failed to show either of these outcomes (slopes not significantly different from 0, data not shown). There were too few data points (n=3) to compare the Rho and Hoescht-Pyronin methodologies (lower panel).

4.3.6 In silico simulations to estimate errors for experimentally derived cycling CFC and MRU values

Limitations in available cells precluded repeating the experiments described in section 4.3.4 and hence deriving estimates of the magnitude of technical errors associated with
each measurement. An attempt to estimate the magnitude of these errors was therefore undertaken using a Monte Carlo simulation strategy. These simulations assumed that each primary CFC assay performed carried an associated 10% standard error (combining Poisson counting and technical plating errors) and that each gel-CFC assay had an associated 43% standard error (combining Poisson counting errors, technical seeding errors, along with the biological variability in the CFC output per MRU). The latter figure was derived empirically from the distribution of secondary CFCs obtained from replicate gels in all experiments described in Chapters 2 and 3. MRU assays were simulated using both bulk-CFC and LDA experimental designs, to quantify their relative statistical power. The simulations did not seek to capture shortcomings in the 3 sorting/selection methodologies themselves (each was assumed here to be 100% accurate in distinguishing cycling and quiescent cells).

The results were then plotted to show, for any actual proportion of cycling MRUs or CFCs, the range within which 95% of the measured proportions of cycling MRUs (Fig. 4-6a, b) or CFCs (Fig. 4-6c) would be expected to fall. A number of issues are illustrated by these data. First, the measurement errors are larger for measurements using the $^3$H-Tdr suicide approach (treated-versus-control design, red dashed lines) compared with the Hoechst-Pyronin or Rho staining approaches (2-sorted-population design, blue dashed lines), and these difference are greatest in samples in which most of the cells in question are quiescent. This is because the $^3$H-Tdr suicide assay results are calculated by subtracting 2 numbers (control minus treated), whose combined absolute errors are greatest when both measurements are of similar size (i.e., in samples where most of the cells of interest are quiescent). Second, the statistical errors are considerably (~3-fold)
greater for MRU assays carried out using LDA versus bulk-CFC output endpoints. This is because the LDA approach is informative at cell doses that sample few MRUs per gel, so the experiments carry large Poisson counting errors overall. The bulk CFC approach samples many more MRUs per gel, which more than compensates (in terms of reduced sampling error) for the biological variability in the CFC output per MRU. In fact these considerations dictated our use of bulk-CFC measurements to quantify MRU activity in the experiments described in Chapters 3 and 4. Third, measurement errors of the proportion of cycling CFCs are considerably lower than corresponding measurements of MRUs, as would be expected given the lower technical variability \textit{in vitro} as compared with \textit{in vivo} measurements, and the relative ease with which large numbers of CFCs may be sampled.

Overall, for the 10%-40% range of mean cycling CFCs and MRUs values obtained from the 10 samples examined, the simulations gave estimated errors of ±30% for the $^3$H-Tdr suicide method and ±10% for the other 2 methods for the MRUs (using bulk-CFC output values), and ±15% for the $^3$H-Tdr suicide and ±5% for the other 2 methods for CFCs.

4.3.7 \textbf{A majority of CFCs become quiescent in xenografts}

Finally, the $^3$H-Tdr suicide methodology was also used to assess the cycling behavior of CFCs found in the human mammary structures grown as subrenal xenografts. To perform these measurements, cells were isolated from gels initially seeded with primary HMECs and then removed from the mice 2-4 weeks later. Equal cell aliquots were then incubated \textit{ex vivo} for 24 hours in the presence or absence of $^3$H-Tdr, and the number of CFCs detectable in each arm was then determined. In these experiments, an average of
16±8% of the CFCs obtained from the 2-4 week gels were found to be cycling (Fig. 4-7a). This suggests that the conditions within the subrenal xenograft assay system are highly sub-optimal in terms of their ability to stimulate the proliferation of CFCs.

4.4 Discussion

This Chapter presents an analysis of the distribution among primitive human mammary cells of several biomarkers that serve as indicators of the cycling status of mammalian cells. Each of these was chosen to enable a property of interest to be coupled with a CFC or MRU assay so that inferences could be made about the attributes of these retrospectively and functionally identified cells. The properties assessed were: (i) entry into S-phase (or not) within a period of 18-24 hours of culture under growth stimulating conditions; (ii) the fluorescent profile displayed after incubation with Hoechst 33342 and Pyronin Y; and (iii) the fluorescent profile displayed after incubation with Rho.

Strikingly, 9 of 10 human tissue samples analyzed contained a sizeable proportion (at least 10%) of primitive cell types exhibiting a “cycling phenotype” according to the criteria of each methodology.

There are however many caveats and assumptions that complicate interpretation of the results derived from the use of these methodologies. They fall into 3 categories: (i) the reliability in extrapolating the cycling result obtained as representative of the status of the cells in vivo; (ii) the lack of specificity and selectivity of each method to identify cycling cells (i.e., accuracy of using the “cycling phenotype” to be equated with “cycling cells”); and (iii) the unknown extent of error in the measurements associated with each datapoint.
The extent to which the tissue dissociation process perturbed the population cell cycle profile, either by bringing cells into or out of the cycle, was not quantified. However, efforts were made to minimize such effects through the adoption of a rapid dissociation protocol in which cold tissue was first mechanically chopped, followed by incubation with dissociative enzymes for a relatively short period of time (4–4.5 hours in warm conditions, compared with 18-24 hours in the standard approach). Thus, the time spent at physiological temperatures was considerably shorter than the cell cycle transit time (16-24 hours for most human cell types). All mammary subfractions were successfully isolated using this protocol, though the overall cellular yield was 2-10 fold lower than with longer dissociation approaches. Importantly, the proportion of rapidly dissociated cells that were Ki67+ was consistent with previously published analyses of the proportion of Ki67+ epithelial cells in situ in formalin fixed tissue blocks of normal adult mammary tissue (~1-2% of all epithelial cells\textsuperscript{116, 117}), suggesting that the dissociation protocol did not cause a major shift in the population cell cycle profile.

Two different approaches were used to quantify the accuracy with which the 3 methodologies separated cycling from quiescent cells. The first used primary HMEC populations cultured under conditions of growth factor stimulation or starvation as cycling or quiescent “controls”. The 2 populations registered distinct and high-specific activity $^3$H-Tdr-induced suicide responses (93±3% versus 11±5%, of inactivated CFCs, p<0.01) using a 24 hour $^3$H-Tdr exposure protocol, thus validating the use of this methodology to measure the proportion of cycling cells by their depletion from test cell populations. The Hoechst-Pyronin profiles of the 2 controls were also distinct,
supporting the use of this DNA-RNA content-based approach also. In contrast, the Rho profiles showed a larger degree of overlap, indicating reduced selectivity.

The second strategy used to assess the utility of these biomarkers made use of staining for intracellular Ki67 expression in FACS-sorted fractions of cells in which CFCs and/or MRUs are differentially enriched. Ki67 expression is a precisely regulated protein that is found in cycling cells but not in quiescent cells\textsuperscript{191}. However, although previous studies of the hematopoietic system showed both the Pyronin\textsuperscript{high} and Rho\textsuperscript{high} phenotypes to be both highly selective and sensitive markers of primitive Ki67\textsuperscript{+} cells\textsuperscript{139, 140, 144}, this did not hold up as well for mammary cells. Thus, we found only \(~70\%\) of Ki67\textsuperscript{+} cells were Pyronin\textsuperscript{high}, and as few as \(~20\%\) were Rho\textsuperscript{high}, reinforcing the poorer selectivity of Pyronin and Rho staining to identify cycling mammary cells. Moreover, the frequency of Ki67\textsuperscript{+} cells in either Pyronin\textsuperscript{high} or Rho\textsuperscript{high} fractions was also low (<10\%). This low sensitivity further complicates interpreting results obtained using Pyronin or Rho-based selection strategies, since we have now shown that the Pyronin\textsuperscript{high} and Rhodamine\textsuperscript{high} subfractions contain non-cycling as well as cycling cells.

Exposure to Hoechst and Pyronin was also shown to be toxic, markedly reducing the number of CFC detectable in both the basal and luminal fractions (by 81\% and 67\%, respectively). It is conceivable that the level of toxicity to cycling and quiescent mammary cells is different, given the presumed higher dye uptake by the former, and this may bias results against the detection of cycling mammary progenitors. However, previous work showed that 92\% of young adult \textit{murine} mammary CFCs and 100\% of MRUs were detected in the combined G1 and S/G2/M fractions after Hoechst-Pyronin incubation\textsuperscript{16}, which would argue against selective toxicity to cycling cells.
The expected measurement errors associated with the cycling phenotype data were estimated using Monte Carlo simulations. These aimed to capture Poisson counting errors, plating variability and, in the case of bulk-CFC assays, the biological variability in the CFC output per MRU, but not contributions from any shortcomings in the selection/sorting methodologies themselves (i.e., the latter being assumed to be 100% selective for cycling cells). These errors prove to be largest for cycling MRUs (vs CFCs), and for LDA-based methods vs bulk CFC output measurements for assessing MRU activity, regardless of the cycling property being assessed. In addition, for the \textsuperscript{3}H-Tdr suicide measurements, errors were greatest in samples in which the cell populations of interest were predominantly quiescent.

Overall, the dual considerations of methodology selectivity/specificity combined with estimates of measurement errors indicate greatest confidence in assessment of mammary CFC and MRU cycling activity is obtained from the use of the 3H-Tdr suicide and Hoechst-Pyronin approaches. This serves to reinforce the consistent detection here of a proportion of cycling CFCs and MRU which supports a model of mammary cell production that appears to include a significant rate of normal turnover of their most primitive cells. Clearly, additional studies in the mouse may help to provide more detailed understanding of how proliferation is controlled in the adult mammary gland.
Figure 4-1 Comparison of Ki67\(^+\) cells and CFC frequencies identifies samples with predominantly quiescent basal fraction CFCs

(a) Example of cytospun primary HMECs processed for immunohistochemistry with an antibody against human Ki67. Arrows indicate Ki67\(^+\) cells (nuclear staining, brown).

(b) Limits to the proportion of cycling cells, by comparison of frequencies of CFCs and Ki67\(^+\) cells. FACS-isolated luminal and basal fractions cells were assayed separately for CFC content and for the proportion of cells expressing Ki67. The right hand column in the tables indicate the implied range for the proportion of CFCs in each fractions that was cycling, from zero to \([\%\text{Ki67}^+] / \%\text{CFCs}\), with a maximum value of 100%.

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Figure 4-2  A 24-hour exposure to high specific activity $^{3}$H-Tdr selectively kills cycling but not quiescent CFCs

(a) $^{3}$H-Tdr titration. Plot shows CFC numbers of log-phase 184-htert cells that survived a 30 minute exposure to high specific activity $^{3}$H-Tdr, expressed as a percentage of a control exposed to vehicle only. Optimal CFC kill was achieved with 20 $\mu$Ci/ml $^{3}$H-Tdr, and this was almost entirely reversed with the addition of excess (400 $\mu$g/ml) non-radioactive Tdr during the $^{3}$H-Tdr incubation (hollow diamond). Representative of n=3 experiments.

(b) 184-htert CFCs enter S-phase within 24 hours in adherent culture. Plot shows the numbers of 184-htert cells detected following exposures of varying lengths of time to 20 $\mu$Ci/ml $^{3}$H-Tdr (solid) or vehicle control (dotted). All data are expressed as a percent of the CFC number in the matched control (cells exposed to medium only during the 30 minute incubation). CFCs almost all passed into S-phase within 24 h, as shown by the loss of CFC in the presence of $^{3}$H-Tdr.

(c) Validation of cell cycle profile of predominantly cycling and quiescent primary HMEC controls. A growth factor stimulated ("EGF-stim." ) population was generated by dissociating cells from primary mammaplasty organoids, and culturing for 4 days in EGF-containing SF7 medium. A growth factor starved ("EGF-starved") control population was generated by culturing mammary organoids from the same tissue sample for 4 days in the SF7 medium minus EGF. Representative FACS profiles are shown after staining for intracellular Ki67 expression and DNA content (7-AAD), confirming that the 2 populations contain predominantly cycling and quiescent cells, respectively.

(d) A 24-hour $^{3}$H-Tdr suicide exposure discriminately kills initially cycling cells but spares initially quiescent cells. Plot shows proportions of CFCs in EGF-stim. (solid) and EGF-starved (dotted) control cells that survived incubations of varying lengths of time (30 minutes – 4 days) with 20 $\mu$Ci/ml $^{3}$H-Tdr. Numbers are expressed as a percent of the CFCs detected in the control aliquots at the same timepoint (mean±SEM for 4 tissue samples). The 24-hour time point was highly discriminatory, with the survival of 7±3% versus 89±5% of CFCs in the EGF-stim. and EGF-starved populations.
Figure 4-3  High Pyronin Y fluorescence selectively enriches for cycling mammary cells, but with associated toxicity and low specificity

(a) EGF-stim. and EGF-starved cultured HMEC populations have different Hoechst-Pyronin profiles. Plots show FACS profiles of EGF-stim. and EGF-starved cells after incubation with Hoechst+Pyronin, gated on the basal population of cells (CD49fhighEpCAMmed). The location of presumed G0, G1 and S/G2/M subfractions in the EGF-stim. sample are indicated. The same gates on the EGF-starved sample show a much lower proportion of cycling (G1/S/G2/M) cells compared with the EGF-stim. sample. (b) Identification of presumed G0, G1 and S/G2/M subfractions in primary cells. Left panel shows a representative FACS profile of cells dissociated rapidly (4–4½h) from mammoplasty tissue then incubated with Hoechst+Pyronin. Middle and right panels show the Hoechst-Pyronin profiles of the luminal and basal fractions in this sample (fractions as in Fig. 3-1b). The Pyronin fluorescence profile was higher in luminal compared with basal fraction in all samples analyzed, indicating that G0, G1 and S/G2/M gates should be set at different levels. (c) Pyroninhhigh phenotype is selective but not specific for cycling cells. FACS-sorted G0 and combined G1 + S/G2/M luminal fraction cells were immunostained for Ki67 expression. Left panel shows the distribution of all Ki67+ cells detected across G0 or G1/S/G2/M fractions. Right panel shows the percent of cells in each fraction that were Ki67+. A majority of Ki67+ cells were in the G1/S/G2/M gates but a minority of G1/S/G2/M cells were Ki67+ (n=3). (d) Hoechst-Pyronin incubation is toxic to primary HMECs. Plot shows CFC output of viable (PI–) luminal and basal fraction HMECs sorted after incubation with Hoechst+Pyronin as a percent of CFC detected in the sorted fractions not exposed to the dyes. Hoechst-Pyronin exposure resulted in a marked reduction in CFC number of 67±13% (n=3) and 81±5% (n=7) in luminal and basal fractions.
Figure 4-4  High Rho fluorescence is neither highly selective nor specific for cycling HMECs

(a) EGF-stim. and EGF-starved cultured HMEC populations have overlapping Rho profiles. Plots show FACS profiles of EGF-stim. (left panel) and EGF-starved cells (right panel) after incubation with Rho, gated on the basal population of cells (CD49fhighEpCAMmed).

(b) Identification of Rho\text{high} and Rho\text{low} subfractions in primary mammoplasty cells. Representative FACS profile of cells dissociated rapidly (4–4½h) from mammoplasty tissue then incubated with Rho, gated to show the profiles of luminal (upper panel) and basal (lower panel) fractions. Rho fluorescence profile was higher in luminal fraction compared with basal fraction in all samples analyzed. Sample Rho\text{high} and Rho\text{low} are identified, gating on the highest 15% and lowest 85% Rho-fluorescent cells within each fraction. (c) Rho\text{high} phenotype is neither highly selective nor specific for cycling primary HMECs. Rho\text{low} and Rho\text{high} luminal subfractions were isolated by FACS, cytospun onto microscope slides, and immunostained for intracellular Ki67 expression. Left panel shows the relative distribution of all Ki67+ cells detected in Rho\text{low} and Rho\text{high} fractions. Right panel shows the percentage of cells in each sorted fraction that were Ki67+. Note that Rho\text{high} phenotype was neither highly selective nor specific for cycling (K67+) cells.
Figure 4-5  Analysis of the cycling profile of CFCs and MRUs in adult mammary tissue
(a) Experimental design. Fresh mammoplasty tissue was processed with a tissue chopper then dissociated rapidly (4–4½ h) into a single cell suspension. The $^3$H-Tdr suicide response was measured by culturing aliquots in the presence or absence of $^3$H-Tdr for 18 hours, and comparing their CFC and MRU readouts in functional assays. Further cell aliquots were incubated in Hoechst+ Pyronin or Rho then immunostained with antibodies against cell surface markers. FACS-sorted luminal and basal fraction cells further subdivided into Pyronin$^{high}$, Pyronin$^{low}$ and Rho$^{high}$, Rho$^{low}$ subfractions (as shown in Fig 4-3b, 4-4b) were then assayed for relative CFC and MRU content. (b) Summary of proportion of CFCs and MRUs measured with a “cycling phenotype”. Ten fresh tissue samples were processed using some or all of the methods described in (a). Figures in the tables (upper panels) denote the percentages of each functionally-defined cell type that were measured as having a “cycling phenotype”. For the thymidine suicide methodology, this is percentage decrease in the assayed cell type in the $^3$H-Tdr exposed compared with the control exposed culture. For the Hoechst-Pyronin methodology, this is the assayed cell number in the G1/S/G2/M gate expressed as percentage of the combined numbers in G0 plus G1/S/G2/M gates. For the Rho methodology, this is the assayed cell number in the Rho$^{high}$ gate expressed as percentage of the combined numbers in Rho$^{high}$ plus Rho$^{low-med}$ gates (where the Rho$^{high}$ gate for samples #4, 5, 6, 9, 10 contained respectively the top 10%, 10%, 25%, 15% and 15% of cells by Rho fluorescence). These are displayed graphically in the lower panel. Negative measurements for the $^3$H-Tdr suicide methodology (where the assayed cell number was greater in the $^3$H-Tdr treated compared with control aliquot) are plotted as 0. Bars=median. (c) Low concordance between methodologies. Scatter plots display the cycling proportion measurements (shown in (b)) for pairs of methodologies, for samples and cell types for which more than one methodology was applied. Regression statistics are shown (r$^2$).
### Figure 4-5 cont

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#### Diagrams

- **Table:**
  - Luminal CFCs
  - Myo+Bip CFCs
  - MRUs

- **Graphs:**
  - Luminal CFCs
  - Myo+Bip CFCs
  - MRUs

- **Equations:**
  - \( r^2 = 0.07 \)
  - \( r^2 = 0.19 \)

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**Legend:**
- Luminal CFCs
- Myo+Bip CFCs
- MRUs
Monte Carlo simulations to estimate errors associated with cycling measurements of MRUs and CFCs

Estimated errors in the measured proportion of cycling MRUs and CFCs. Charts show, for varying actual proportions of cycling MRUs (or CFCs) in a tissue sample, the expected range (from Monte Carlo simulations, see Methods) in which 95% of measured proportions would lie. Charts show results from simulated measurements of number of: (a) MRUs using a bulk-CFC experimental design, (b) MRUs using an LDA experimental design, and (c) CFCs. Red dashed lines: 95% range for thymidine suicide approach. Blue dashed lines: 95% range for Hoechst-Pyronin or Rho method. Black line: slope=1, showing a theoretically perfect measurement. The sorting/selection methods were assumed to be 100% accurate in distinguishing cycling and quiescent cells, thus the simulation aimed to capture only the uncertainties related to Poisson counting, technical errors or replication, and biological variability in the CFC output per MRU.
Figure 4-7 Most CFCs become quiescent within 2–4 weeks in subrenal xenografts.
Measurement of the proportion of CFC that are cycling in subrenal xenografts. Suspensions of primary HMECs were combined with irradiated fibroblasts in gels and propagated as subrenal xenografts, as described in Chapter 2. Gels were removed after 2–4 weeks \textit{in vivo}, and viable cells dissociated. Equal cell aliquots were then cultured for 24 hours in the presence of $^3$H-Tdr or vehicle control, and their CFC content determined by the standard \textit{in vitro} assay. Plot shows the percentage reduction in the number of CFCs in the $^3$H-Tdr treated compared with the vehicle treated culture. Four mammoplasty samples were analyzed, each represented by a different symbol.
5. DISCUSSION

The experiments that comprise this thesis work demonstrate that the normal adult female mammary gland contains a rare population of primitive human mammary cells with stem cell properties. We have given these the same operational name of MRU first applied to the murine system because of their detection by virtue of their ability at limit dilution in xenografted immunodeficient mice to regenerate an organized bi-lineage mammary structure with functional milk-producing cells. These regenerated structures also contain all subtypes of progenitor CFC populations. In fact, the detection of these MRU-derived “secondary” CFCs provides a sensitive and objective endpoint of the regenerative activity of transplanted MRUs. Using this approach we found the frequency of MRUs by limiting dilution to be 1 per $10^3 - 10^4$ cells obtained from dissociated normal adult female mammoplasty tissue. Coupling the MRU assay with various sorting and selection techniques enabled the identification of cell surface markers that can be used in combination to isolate MRUs at greater purity. Characterization of other phenotypic properties additionally indicates that a readily detectable but variable proportion of the MRUs and CFCs in normal adult breast tissue are actively cycling.

5.1 Structure of the human mammary stem cell hierarchy

The results described in Chapters 2 and 3 extend previous work to delineate the hierarchy of primitive cell types in human, mouse, and bovine mammary glands. Taken together, these results demonstrate considerable conservation of the differentiation programs between diverse mammalian species. This includes the generation of mature luminal and myoepithelial cells from a pool of self-renewing bi-
lineage stem cells in a multi-step process that includes intermediate bi-lineage and lineage-restricted progenitors (Fig. 1-4). The most primitive cell types currently identified in each species (murine MRUs detected by cleared fat pad transplant assay, bovine and human MRUs detected by subrenal xenotransplant assay) express combinations of cell surface markers that are characteristic of and shared with differentiated myoepithelial cells that occupy a basal position in situ. These cell types are largely separable from cells of the luminal lineage, including luminal CFCs. The human bipotent and myoepithelial progenitors also co-purify with myoepithelial cells. Identification of analogous murine bipotent CFCs has been hampered by challenges in establishing culture conditions that preserve lineage markers, though a candidate population of bipotent CFCs has recently been identified in the basal fraction cells when they are cultured under low oxygen conditions (J. Stingl, M. Makarem, unpublished data).

Whole transcriptome analyses of mammary cells fractionated on the basis of CD49f/EpCAM (human) and CD49f/CD24/CD61 (mouse) demonstrate that many regulatory pathways are also conserved in functionally analogous subpopulations. Receptors for estrogen, progesterone and prolactin receptors are preferentially expressed in the differentiated luminal subsets in both species, consistent with previous reports, and implying that control of MRU and CFC proliferation by these hormones is indirect. Luminal CFC-enriched subsets express Kit, Elf5, CXCR4 and ALDH1A3 (an ALDH family member that is the predominant contributor to the Aldefluor high phenotype in human breast cancer cells). Transcripts overexpressed in both myoepithelial/bipotent CFC/MRU subfractions include p63, Lgr6 (a Wnt receptor that characterizes epidermal stem cells) and EMT markers Slug and Twist2.
The expression of basal cell markers (e.g., CD49f, CD10, Thy1) in the cell types in the MRU→bipotent CFC→myoepithelial-restricted CFC→myoepithelial cell branch of the hierarchy and luminal cell markers (e.g., EpCAM, CD133) in the luminal-restricted CFC→luminal cell branch suggests a physical separation of the 2 differentiation processes in situ. They also suggest a possible role of signaling from the basement membrane (e.g., via integrins) in the maintenance of stem cell properties in a basally-located niche and/or suppression of luminal commitment in stem cells and bipotent CFCs. Consistent with this, the mammary trees that develop from transplanted mouse tissue fragments carrying a conditional deletion of CD29(β1) under the CK5 promotor (predominantly expressed in basal cells) show a disorganized structure, and are profoundly deficient in regenerative properties in secondary transplants, indicating a loss of stem cell activity\textsuperscript{197}. The loss of CD29 also affects the orientation of the mitotic spindle in dividing basal cells in this model, as also observed in the skin\textsuperscript{198}; cell divisions were predominantly parallel to the basement membrane in regenerating wild type mammary tissue whereas perpendicular and parallel divisions were equally frequent in the absence of CD29\textsuperscript{197}. Outgrowths generated from transplants of mouse mammary tissue carrying a targeted deletion of CD49f (α6) do not display an overt ductal-alveolar developmental phenotype\textsuperscript{199}. This is surprising in view of its high expression level on normal mouse and human MRUs, although this may reflect functional redundancy between α-chain integrins.

Interactions between MRUs and basement membrane components may also underlie the marked (~10-fold) improvement in engraftment frequency observed when mouse mammary cells are transplanted in the presence of Matrigel\textsuperscript{18}. Matrigel is rich in
laminin and collagen, though the mechanisms for this remain to be determined.

Interestingly, it has recently been demonstrated that sorted basal fraction mouse mammary cells that are cultured for short periods of time on Matrigel can give rise at higher frequencies than the original cells to mammary trees when transplanted into cleared fat pads (J.Stingl, unpublished data). This apparent acquisition of MRU properties by cells previously thought to be downstream “differentiated” cells raises the possibility of an alternative model of the hierarchical structure in the mammary gland. What may now require consideration is a model in which transitions between MRUs, bipotent CFCs, myoepithelial CFCs and expression of features previously assumed to represent activation of a terminal myoepithelial differentiation program may be more fluid than assumed in earlier models. Examples of differentiated cells “reactivating” primitive programs in normal tissues under physiological conditions are still rare, memory B- and T-cells being perhaps the sole exception. Interestingly, however, forced expression of certain oncogenes or transcription factors\textsuperscript{200} appear to achieve this effect fairly rapidly. In addition, it is noteworthy that a recent study of Src-transformed MCF10A mammary cells demonstrated cells with transplantable tumor-initiating ability and cells that lack this property to exist \textit{in vitro} in a dynamic equilibrium, with a role for interleukin-6 in upregulating the tumor-initiating properties in cells that appear to lack this property\textsuperscript{201}.

5.2 \textbf{Proliferative activity of primitive cells in the normal adult female breast}

The results described in Chapter 4 demonstrate that a detectable proportion of the MRUs and CFCs present in freshly dissociated normal adult female mammoplasty tissue exhibit
phenotypic properties associated with cycling cells. The most definitive of these were a high sensitivity to an 18-24 hour exposure to $^3$H-Tdr which we showed measures S-phase mammary cells with high specificity and sensitivity, and a high Pyronin fluorescence which we showed was also a good marker of cells outside the G0 compartment. Nevertheless, considerable variability was observed in the proportion of primitive cells displaying these cycling-phenotypes in a survey of 10 individual breast samples (Fig. 4-5). Although this likely reflects biological variability between samples, we showed that inherent errors in the quantitative assays also make an important contribution to the variability seen (Fig. 4-6). Technical caveats notwithstanding, the data set the stage for renewed investigation of regulatory mechanisms that govern cell turnover in the normal mammary gland in adult humans and the involvement of MRU and CFC populations in this system.

Under homeostatic conditions in the adult when body growth has stopped, the proportion of stem cells that are in cycle is a function of the rate of loss of differentiated cells, the frequency of stem cells within the tissue, and the average number of differentiated cells that are generated per (non-stem cell) daughter of a stem cell division. Interestingly, however, the extent to which these different control mechanisms are used varies markedly between different tissues. In the case of the hematopoietic system, the stem cells in the adult mouse are rare (~1 per $10^4$–$10^5$ cells)$^{202,203}$ and slowly cycling (~once per month or less)$^{204-208}$. This implies that, on average, relatively large clones must be generated per stem cell division via the multi-level hierarchy of transit amplifying divisions to satisfy the high demand for new differentiated cells. Although the data available for human hematopoietic stem cells are not as detailed or precise, they
support the main features of the situation described for mouse; i.e. rare subset of cells with a very small proportion of cycling components at any given moment in time. Our findings that MRUs are similarly rare members of the human mammary epithelium with a substantial proportion in cycle at any given time implies a much smaller clone size generated per stem cell division given the lower overall cell turnover in the tissue. This in turn suggests a likely role for apoptosis in the amplifying divisions downstream of the MRUs and a short half-life of the mature end cells produced as a mechanism to regulate total cell numbers as exemplified by recent studies in the small intestine of the mouse. Interestingly, a cyclical pattern of apoptosis has been documented in mammary tissue, although their state of differentiation could not be discriminated. Nevertheless, it is notable that a cyclical amplification of MRUs in mouse mammary tissue with progression through the menstrual cycle has also been noted consistent with a possible link between MRU-initiated waves of proliferation and apoptosis.

A further aspect of interest raised by these data is the quiescence apparent in a substantial proportion of mammary CFCs. This finding implies that at least some of these cells can have a considerable lifespan (in chronological time, not necessarily number of mitotic divisions) in the mammary gland. The presence of a large and long-lived pool of mammary progenitors thus raises the interesting possibility that these cells could be mutational targets of relevance to the evolution of particular subsets of human breast cancer. Viral insertion of a combination of oncogenes more readily generated an \textit{in vivo} tumorigenic phenotype in sorted normal EpCAM$^+$ compared with CD10$^+$ HMECs, demonstrating that luminal cells can be the targets of transformation in
experimental systems\textsuperscript{212}. Likewise, Cre-mediated deletion of BCRA1 targeted to luminal cells by the Blg-promotor in mice led to the development of tumors that phenocopied human BRCA1\textsuperscript{−/−} tumors\textsuperscript{213}, whereas targeting the deletion to basal cells by the K14 promotor produced phenotypically different tumor types. In humans the overall gene expression profile of BRCA1-driven tumors and basal-type breast tumors was found to be more closely related to the normal luminal-progenitor fraction than the stem-cell enriched basal fraction\textsuperscript{183}. While cell of origin conclusions remain speculative, these observations encourage work to further investigate the activity of normal luminal progenitor programs\textsuperscript{40, 43, 88, 183, 194, 214} in breast cancers.

If MRU populations exhibit a continual level of proliferative activity through adulthood, a mechanism to counteract the cumulative shortening of telomeres through mitotic divisions would be required. Consistent with this, the transcript level of the catalytic subunit hTERT is detectable by \textit{in situ} hybridization\textsuperscript{215} or rt-PCR\textsuperscript{216} in adult human breast tissue, albeit at low levels. However, functional activity measured by the telomeric repeat and amplification protocol (TRAP) assay is below detection limits\textsuperscript{217, 218}. A more detailed analysis of telomerase expression and activity in MRU-enriched fractions would thus provide a useful test of models that incorporate regular stem cell turnover.

5.3 Future directions

The present findings suggest many opportunities for future research into the mechanisms that control the integrity and physiology of the normal human mammary gland and how these may be altered to cause breast cancer. These include studies to further delineate the
hierarchy of primitive cell types in the mammary gland and their developmental inter-
relationships; and to further define the cellular and molecular mechanisms that regulate
key primitive cell functions such as proliferation, self-renewal, lineage-restriction and
apoptosis.

There are many unanswered questions regarding the structure of the normal
mammary gland. For example, is there heterogeneity in the stem cell compartment in
terms of longevity or cellular output, as already suggested by the variable fat pad filling
in limiting dilution transplants\textsuperscript{16,49,182,192}? Are there cell type(s) intermediate between
stem cells and bi-potent progenitors (i.e., “short term repopulating cells”)? Is the multi-
step differentiation process wholly irreversible or reversible under physiological
conditions? Do the subsets of stem cells that drive tissue expansion during pregnancy
overlap with the cells that contribute to tissue homeostasis in the resting adult breast or
are they a separate reserve of deeply quiescent stem cells? The experimentally more
tractable murine system is clearly better suited to addressing these complex questions
than the human system – even envisaging improvements to current xenotransplant
technologies that may permit human mammary stem cells to execute their developmental
potential more fully.

An important intermediate goal is to identify further phenotypic markers that will
distinguish MRUs from other basal fraction cell types, and that could be used alone or in
combination with other markers to isolate these cells at higher purities and indicate their
physical location \textit{in situ}. The expression profile of FACS-sorted basal fraction cells has
already been determined by hybridization array and/or SAGE in mice\textsuperscript{16,194,219} and
humans. This has served as a preliminary resource to allow a more systematic approach to the identification of candidate markers for functional testing.

The dynamics with which different functionally-defined primitive mammary cell types enter or exit the cell cycle are amenable to study in mice using transgenic reporter approaches. Notably, models that allow fluorescent labeling of cells that pass through S-phase (e.g., using GFP-labeled histones) or that generate stochastic expression of multiple fluorescent transgenes (the “Brainbow” model) have been applied successfully to characterize the proliferative activity of primitive cells and the propagation of their clonal progeny in many tissues, including skin, blood, brain and intestine. Analogous approaches are not yet feasible to study human mammary tissue in situ, although some could be applied to models of human mammary tissue propagated as xenografts.

Understanding the development and evolution of the clones that exist in human breast cancers will require analogous rigorously executed methodologies and assessment. Notably, there are currently no in vitro or in vivo systems in which primary breast cancer tissue can be reliably and reproducibly propagated, though some level of success has been reported for metastatic pleural effusions transplanted into immunodeficient mice. The ability demonstrated here to propagate normal human mammary cells in vivo clearly encourages further studies to determine whether cells from primary breast cancer cells can grow under appropriate conditions as subrenal xenografts (or in the alternative humanized fat pad system). The reported success in growing diverse primary human cancer cells types (prostate, ovarian, lung, skin, colon) under the renal capsule also lend support to the potential application of the subrenal grafting strategy. These
efforts will also benefit from a rapidly growing description of the signaling pathways that drive different molecular subtypes of breast cancers, as revealed by gene expression profiling\textsuperscript{225-229} and tissue microarray\textsuperscript{230-233} studies. Xenotransplantation protocols could thus seek to improve and perhaps tailor conditions for particular subtypes. These might consider the delivery of more stimulatory local signals (e.g., exogenous hormones\textsuperscript{37}, ECM components). Ultimately, the goal would be to provide a system for evaluating and accurately predicting treatment responses.
REFERENCES


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APPENDIX A

Published paper:

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

Peter Eirew1, John Stingl1, 6, Afsin Raouf1, Gulisa Turashvili4, 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, which are then flushed to regenerate mammary gland structures from human mammary epithelial cells (HMECs) transplanted into highly immunodeficient mice. These transplantation approaches have relied on complementing the preceding mammary fat pad of the same mouse with human fibroblasts to create an environment conducive to the requirements of HMECs. 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 nude breast cancer immunodeficient mice (NOD-SCID) mice, allowing for in vivo growth of normal human mammary cells. 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 ducts and from attempts to regenerate mammary gland structures from human mammary epithelial cells transplanted into highly immunodeficient mice. One of these transplantation approaches has relied on complementing the preceding mammary fat pad of the same mouse with human fibroblasts to create an environment conducive to the requirements of HMECs. 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 nude breast cancer immunodeficient mice (NOD-SCID) mice, allowing for in vivo growth of normal human mammary cells. 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 ducts 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 complementing the preceding mammary fat pad of the same mouse with human fibroblasts to create an environment conducive to the requirements of HMECs. 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 nude breast cancer immunodeficient mice (NOD-SCID) mice, allowing for in vivo growth of normal human mammary cells...

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-supplemented female NOD-SCID mice (or derivative strains) contained regenerative 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 extending 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 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.

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Figure 1. Organized structures are generated in vivo from single-cell suspensions of primary human mammary cells. (a) Schematic showing xenotransplantation approach. Single cells obtained by enzymatic dissociation of normal human reduction mammary plast samples were combined with irradiated fibroblasts in a collagen gel as described in Methods. Gels were then transplanted under the kidney capsule of immunodeficient mice given slow-release pellets of human E2 and P. (b) H&E and immunostained sections through 4-week xenografts produced in the gels. The H&E-stained section shows examples of round (arrowheads) and elongated duct-like (arrow) structures. The immunostained xenograft sections show a spatial distribution of markers similar to that seen in normal human breast tissue. The sections stained with antibodies to collagen IV and laminin show the presence of a basement membrane separating the epithelial structures from the surrounding gel and fibroblasts. Smooth muscle actin (SMA) and cytokeratin 14 (CK14) are two markers of basally located myoepithelial cells. MUC1 and cytokeratin 18 (CK18) are lamellar epithelial cell markers. The sections stained with antibodies to estrogen receptor (ER-x) and progesterone receptor (PR) show that some fibroblasts, as well as epithelial cells, stained positively for ER-x. Ki67 is a marker of cycling cells. The section in the bottom row at the far right is from a gel that was transplanted 4 weeks previously into a female mouse that was made pregnant 9 days after transplant. This section was stained with antibodies to β-casein, and the positive staining provides evidence of human milk production within the regenerated alveolar structure. Scale bars, 50 μm.

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, lamellar cells with vacuolated cytoplasm that stained positive for human β-casein (a protein 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

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**Figure 2.** CFC production in vivo as an indicator of human MRU repopulating activity.
(a) Experimental protocol (as in Fig. 1a) but showing the use of CFC output measurements as an endpoint of MRU activity. (b) The number of CFCs detected per gel after various times in vivo. The legend inside the figure shows the number of human cells transplanted per gel in each of the time-course experiments performed (n = 3). (c) The distribution of different types of CFCs in freshly thawed normal human breast tissue compared with the distribution of these cells in 4-week xenografts generated from the same tissue samples (n = 9). (d) Representative colonies generated from cells derived from 4-week xenografts after dual-color immunostaining with antibodies to both MUC1 (blue) and cytokeratin 14 (brown). Top, pure luminal cell colony; middle, pure myoepithelial colony; bottom, mixed colony containing both lineages. Scale bars, 1 mm. (e) The CFC output in the gels after 4 weeks was linearly related to the number of human cells transplanted. Shown is a representative experiment in which six gels were analyzed per cell dose. Error bars represent means ± s.e.m.
were indistinguishable from those derived from primary mammary-plasty 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 mammary-plasty 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 regenerating cell or unit (human MRU). The frequency of MRUs calculated from these experiments was 1 per $1 \times 10^3$ to $1 \times 10^4$ mammary-plasty 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, $4.1 \pm 0.6$ daughter CFCs.

**Human MRUs have a CD49d**

EpcAM$^{high}$-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-plasty samples after staining them with antibodies to CD49d 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 CD49d$^+$EpcAM$^{high}$ fraction, whereas most primary bipotent (77% ± 11%) and myoepithelial-restricted (97 ± 2%) CFCs were concentrated in the CD49d$^+$EpcAM$^{low}$ fraction (Fig. 3c). The CD49d$^+$EpcAM$^+$ fraction was mostly devoid of primary CFCs (data not shown). Notably, gels in which secondary CFCs were detected 4 weeks later were almost exclusively those initiated with cells from the CD49d$^+$EpcAM$^{low}$ fraction (92 ± 3% of the CFCs detected in all xenografts were obtained in gels initially seeded with CD49d$^+$EpcAM$^{low}$ cells). Structures observed in these 4-week-old xenografts showed the same spectrum of CD49d$^+$ 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 CD49d 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 CD49d$^+$EpcAM$^{low}$ 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
1,000-3,000 CD49fEpCAM 

CD31. CD45 

cells (MRU-enriched 

and containing 

limiting numbers of MRUs) into a first set of mice. 

Four weeks later, we removed these gels, prepared single-cell suspensions from them, and then plated 30% of each suspension in a CFC assay to identify primary gels that contained regenerative (secondary) CFCs. 

We combined the remaining 70% of the cells from the primary gels with fresh feeder cells and suspended them together in new secondary gels, which we then implanted into secondary recipients (Supplementary Fig. 1a online). In most cases, primary gels that contained regenerative CFCs also regenerated detectable CFCs in the secondary gels, indicating that MRUs had been regenerates in the primary gels (Supplementary Fig. 1b). Of note, similar assays of primary gels initiated with larger numbers (1 x 10^9) 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 frequency of cells that produce 4 weeks in this assay contain the same hierarchy of primitive and maternal epithelial cell types as is found in the normal endogenous human mammary gland and that the regenerative cells are those that are most frequently derived from a rare subset of cells with a distinct CD49fEpCAM 

EpCAM 

basal phenotype. Notably, during the course of their production in this in vivo system, the regenerates and differentiates 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 regenerates structures. In addition, for at least one of the mammary CFC types detected (the luminal-restricted CFCs), it was possible to show a clear distinction 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, 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 (a.k.a. integrin) has an inverse pattern of expression in situ. Thus, the observed CD49fEpCAM 

EpCAM 

phenotype of MRUs suggests a basal location of these cells in situ. Consistent with this expression is the previous observation that most of the cells in the CD49fEpCAM 

EpCAM 

fraction also express cytokeratin-19 (a myoepithelial 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 CD49fEpCAM 

EpCAM 

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 immunocompetent 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 where, with the exception of the hematopoietic system, a vacuum currently exists.

METHODS

Mice.

We bred and housed female NOD-SCID, NOD-SCID/F2-microglobulin-null and NOD-SCID interleukin-2 receptor-γ-null 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-γ-null 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 discarded tissue from normal premenopausal women (ages 18-40) 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 scalps, 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% washed BSA (Froton V; Gibco Laboratories), 300 U/mL collagenase (Sigma) and 100 U/mL hyaluronidase (Sigma). In some experiments, this medium was supplemented with 10 ng/mL epidermal growth factor (EGF, Sigma), 10 ng/mL cholera toxin (Sigma), 1 ng/mL laminin (Sigma), 0.5 mg/mL hyaluronidase (Sigma) and 5% FBS (StemCell Technologies). We obtained an epithelial-rich pellet by centrifugation at 80g for 2 min and cryopreserved it in 8% 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 trypsin supplemented with 1 mM EDTA (STEM Cell Technologies), washing once with FBSB (StemCell Technologies) supplemented with 2% FBS followed by treatment with 5 mg/mL dispase (STEM Cell Technologies) and 100 U/mL DNase (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 xenograft gels, we killed recipient mice and aseptically removed the gels from the kidneys under a dissecting microscope. We then digested the gels for 45 h at 37°C in Epilclear-B medium (STEM Cell Technologies) supplemented with 5% FBS, 600 U/mL collagenase and
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In vitro mammary cell-fusing assay. We incubated 60-mm tissue culture dishes for 1 h at 37 °C with a 1:4 dilution of Trypan Blue 0.10% collagen (Collagen Biotechnologies) in PBS (StemCell Technologies). We seeded each dish with test cells obtained from primary tissue or digested collagen gel combined with 2.0 × 10⁶ freshly thawed, previously irradiated (with 50 Gy) NIH 3T3 mouse fibroblast cells in 4 ml of Epil-Cult-B medium (StemCell Technologies) supplemented with 5% PBS and 0.05 μg/mL hydrocortisone. We incubated cultures at 37 °C and 5% CO₂, with a change to serum-free Epil-Cult-B plus 0.05 μg/mL hydrocortisone 1 h later. In some experiments, we replaced the Epil-Cult-B medium with DMEM and P1L supplemented with 2% BSA, 10 μg/mL EGF, 10 μg/mL cholera toxin, and 1 μg/mL insulin. After 4-10 d, we briefly fixed dishes in a 1:1 vol/vol mixture of methanol and acetone at 20 °C, stained them with Wright’s Glemsa (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, disordered cells, myoepithelial cells containing both these elements and a ragged colony boundary, or granules, although in some others, colonies were stained for specific marker proteins (see Methods).

Preparation and assessment of collagen gels. We prepared concentrated rat tail collagen as previously described⁶ and sterilized it at 20 °C. We thawed the pellets and neutralized the pH immediately before use by adding two parts (vol/vol) concentrated hydrochloric acid to 7 parts 0.1% collagen solution and 2 parts 5x DMEM. To prepare gels, we collected 3H HEPES mouse embryonic fibroblasts (a kind gift from G. Olschewski) from subconfluent cultures. X-ray irradiated them (with 15 Gy), mixed them with dissociated human mammary cells and reseeded them in cold neutralized collagen. We added 25-μl aliquots containing 2.2 × 10⁶ HEPES cells and the desired number of human test cells in individual wells of a 24-well plate. We allowed the gels to equilibrate at 37 °C incubator for 10 min and then incubated them in warm Epil-Cult-B medium plus 5% FBS for 90 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 HEPES HEPES fibroblasts.

Subrenal xenograft transplantation assay. We shaved the hair on the backs of anesthetized mice and shaved the skin with 70% ethanol. We made an anterior to posterior incision approximately 1.5 cm long dorsally around the area of the kidney. We also made a small incision in the abdominal wall above the kidney and exteriorized the kidney by applying gentle pressure on either side. Under a dissecting microscope, we lifted the kidney capsule from the pararenal fat tissue and made a 2-4-mm incision in the capsule. We inserted up to three gels under the capsule with a fine-pulled 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 containing 2 mg β-estradiol and 1 mg progesterone (both from Sigma) in MDS-4811 silicone (Wilmington Technology) subcutaneously in a posterior position before suturing the skin incision. This protocol was previously shown to produce sustained serum levels of these hormones in the mouse approximately equivalent to those at the human mid-late stage peak.⁷ In some experiments, we stained 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 allophycocyanin-conjugated rat antibody to human CD49f (dose GOBES, R&D Systems) and PTC-conjugated mouse antibody to human EpCam (dose VU1-D9, StemCell Technologies). In some experiments, we also labeled hematopoietic and endothelial cells with biotin-conjugated mouse antibodies to human CD45 (dose HI30, Biogenex) and human CD31 (dose WM69, eBioScience), respectively, followed by alkaline phosphatase-conjugated streptavidin (BD Biosciences). We added propidium iodide (Sigma) at 1 μg/mL for live/dead cell discrimination. We performed all sorts on either a FACSVantage or a FACSDiva (Becton Dickinson).

Immunohistochemistry. We processed deparaffinized 4-μm sections of paraaffin-embedded fixed collagen gels for immunohistochemistry with a Discovery XT automated system (Ventana Medical Systems). We applied primary antibodies to estrogen receptor-α (dose 6F11, Ventana), progesterone receptor (dose 1A6, Ventana), EGF (dose E2, Ventana), cytokinin-14 (dose LL020, ID Labs), androgen receptor (dose KS 18.04, Progris, final dilution 500 μg/mL, Dako), collagen IV (clone 9F3, Sigma), and β-casein (dose F48.20, Harlan Laboratories). We then applied horseradish peroxidase-conjugated Vectorstain Universal Secondary Antibody (Ventana) and developed the slides with the 3,3,5-triaminobenzidin (TMB) Clump Kit (Ventana). We processed some slides manually with primary antibodies to MUC1 (dose 21474, StemCell Technologies) or smooth muscle actin (dycytoplasmic, Abcam), each followed by alkaline phosphatase-conjugated Vectorstain ABC-HRP (Dako) and developed in Fastred (Dako). We counterstained all slides with hematoxylin. For dual-color staining of gels, we fixed 60-mm culture dishes briefly in 1:1 vol/vol acetone and methanol and preblocked them in PBS buffered saline containing 5% w/v gelatin and 1% w/v FBS. We then incubated the slides sequentially with an unconjugated antibody to MUC1, alkaline phosphatase-conjugated Envision AP (Dako), 4-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development solution (Sigma), biotin-conjugated antibody to cytokinin-14 (dose LL010, Vector), horseradish peroxidase-conjugated streptavidin (Jackson Immunoresearch), and DAB.

Statistical analyses. Data are expressed as the arithmetic mean ± SEM, except for MEFU 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 99% confidence interval. We tested goodness of fit to a single-hit model using standard chi-square statistic.

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

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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. C.J.E. helped organize the acquisition and storage of the materials used. C.J.E. conceptualized the study and finalized the writing of the manuscript.

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APPENDIX B

Published paper:

Quantitation of human mammary epithelial stem cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay

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Methods to identify and enumerate primitive, and typically rare, undifferentiated cells in normal tissue using functional endpoints are powerful tools for acquiring insights into the mechanisms that regulate normal tissue stem cell turnover and differentiation. In this paper, we describe a xenotransplantation-based protocol that allows mammary stem cells with in vivo tissue regenerative properties to be specifically detected and quantified among the heterogeneous cell populations obtained from dissociated normal human mammary tissue. This methodology involves implanting a collagen gel containing the test cells in combination with supportive fibroblasts under the kidney capsule of highly immune-deficient, hormone-supplemented mice and then, 6 weeks later, searching for regenerated human cells with in vivo clonogenic activity. Quantification of the input human mammary stem cells is achieved using standard limiting dilution transplant approaches. This approach circumvents the need to modify the mouse mammary fat pad, and is objective, rapid (~5 weeks) and economical to perform.

INTRODUCTION

Mammary epithelial stem cells

A number of studies in recent years have established that the mature cells in the mammary epithelium are continually generated by a multistep differentiation process from a pool of undifferentiated self-renewing mammary epithelial stem cells. The key to this progress has been the introduction of specific and quantitative functional assays that detect distinct subsets of cells within the hierarchy on the basis of the longevity and diversity of their particular regenerative abilities. The identification of a mouse mammary stem cell population was based on the development of a transplant assay that reveals the ability of a rare subpopulation of cells (termed 'mammary repopulating units' or MRUs) to individually regenerate an entire mammary tree in the epithelium-cleared mammary fat pad of an immune-compatible recipient mouse. In both mouse and human mammary tissue, a variety of lineage-restricted and bipotent populations of clonogenic mammary progenitor cells (colony-forming cells or CFCs) have also been recognized. The identification of these mammary CFCs is based on detecting their ability to form colonies of plastic-adherent daughter cells of particular lineages when cocultured at low density with irradiated fibroblasts in defined media containing epidermal growth factor. The finding that most mouse MRUs and CFCs can be prospectively isolated as distinct populations is the basis of the concept that mammary cell differentiation is organized as a highly regulated, multistep differentiation process. This concept, in turn, has set the stage for the development of breast cancer models in which tumorigenic 'cancer stem cells' are defined operationally by their ability to regenerate a tumor when transplanted into a suitable recipient, although the tumor cancer stem cells populations relate developmentally to their normal counterparts remains a topic of intense interest.

A key feature of all clonal assays is their restricted applicability to suspensions of viable single cells or to uniquely traceable single cells. A critical aspect of the assay design is therefore to optimize and define exogenous factors (e.g., growth factors, extracellular matrix, supportive stromal cells) that allow the cells of interest to demonstrate their maximal intrinsic regenerative potential.

Xenotransplantation of human mammary epithelial stem cells

Human mammary epithelial cells (HMECs) do not readily proliferate in the adipose environment of the mouse mammary fat pad. This has posed a significant challenge to the development of a transplant assay protocol for human MRUs analogous to that used to assay mouse MRUs. One approach pioneered by Kaperwasser et al. has been to 'humanize' the fat pads of immunodeficient mice by colonizing with fibroblasts. This modification provides a supportive environment in which recognizable human mammary epithelial structures are regenerated when human mammary organoids or dissociated human mammary cells are subsequently transplanted.

In this paper, we describe a different xenotransplantation methodology that we adapted from previous studies suggesting the ability of the subrenal capsule site to support the growth of implanted tissue fragments. These earlier studies include the important work of Cunha and colleagues, who demonstrated the specific applicability of this site to support the propagation of intact fragments of mammary tissue. According to our protocol, dissociated suspensions of cells (or subsets thereof) isolated from normal human mammary tissue are combined with fibroblasts in collagen gels and these gels are then placed under the kidney capsule of highly immunodeficient hormone-supplemented mice. After 4 weeks, the gels are removed and evidence of a regenerated bilayered, normal-appearing human mammary gland structure can be identified (Fig. 1).

Use of in vivo-regenerated mammary CFCs as an endpoint of mammary stem cell activity

Histology can be used to detect evidence of regenerated mammary tissue in the implanted gels (Fig. 1a); however, discrimination of
positive from negative gels by this method is highly subjective and labor intensive. This endpoint is thus poorly suited for extensive limiting dilution assays that are essential for further characterization and purification of the responsible input cells. To circumvent this problem, we chose to assess the production of derivative CFCs as an endpoint of regeneration within xenotransplants (Fig. 1b). This strategy has proven to be highly robust while also providing an objective method for retrospectively detecting the functional activity of the original input mammary stem cells. We use the same operational terminology (MRUs) to refer to the very primitive human cells detected using this approach and the subset of mouse mammary cells that can regenerate a full mammary tree in the cleared fat pad, as both use in vivo regenerative readouts to define highly primitive cells, both produce CFCs, both share a basal phenotype and both display self-renewal activity. Nevertheless, exactly how analogous these two cell subpopulations are located within the respective human and mouse mammary gland cell hierarchies remains to be determined.

To detect human mammary CFCs regenerated in the implanted collagen gels, the removed gels are dissolved enzymatically in a solution containing collagenase and hyaluronidase, followed by treatment with trypsin. These treatments also dissociate any tissue structures present and thereby allow a suspension of viable cells to be obtained. The cells are then plated in vitro to determine whether any CFCs are present (Fig. 1b). Human MRUs are thus operationally defined as the cells that generate CFCs that are detectable in the implanted gels 4 weeks later. Although CFCs can be detected in such xenografted gels for more prolonged periods, we have focused on a 4-week time point because we have noted that the morphology of regenerated structures begins to deviate from normal at later time periods.

The frequency of human MRUs in a test population can then be determined using the presence or absence of CFCs in 4-week gels to distinguish positive from negative outcomes in a standard limiting dilution experimental protocol (see Box 1). This involves transplanting multiple replicate gels containing different numbers of test cells, and then applying single-hit Poisson statistics in a linear model to the measured proportion of positive and negative transplant outcomes for each cell dose tested (wherein positive is defined as ≥1 CFC per gel). The result is a best estimate of the MRU frequency and of the confidence limits of that estimate with respect to the number of transplants and the variability in the results obtained.

Clonal regeneration assays enable the biological properties of specific, functionally defined subsets of regenerative cells to be investigated. This approach has proven to be of considerable interest, because it can detect an input mammary cell type that is typically very rare relative to the more differentiated cells that make up the majority of the cells in the mammary gland and also has different properties. For example, the frequency of the cells detected as human MRUs is ~1 in 10^6 to 1 in 10^5 cells and these MRUs are phenotypically distinct from many of the cells detectable in vitro as CFCs.

The principal limitations of the approach used to detect MRUs are: (i) the requirement to obtain a sterile suspension of dissociated, viable cells, which precludes a simultaneous assessment of the morphology of the tissue from which they have been isolated and (ii) the retrospective nature of the assay used to detect them (i.e., the MRUs are no longer present after they have been identified).

Applications and experimental design

This assay may be used in a number of ways:

1. To discriminate between the presence and absence of human MRUs in a given test cell population (e.g., in a particular subfraction isolated by FACs); transplant test cells as described, retrieve the cells after 4 weeks in vivo and obtain a single cell suspension and then seed into in vitro CFC assays. This “secondary” CFC readout determines whether MRUs were present (colonies generated) or absent (no colonies generated) in the original test cell suspension. Note that this approach is limited by the number of cells initially tested (i.e., put into gel) and also by the number of their progeny ultimately assayed for CFCs. A suitable general positive control is a xenograft initiated with 10^5 unfractionated cells from a normal human mammary gland sample known to contain MRUs (plus the standard supportive fibroblasts). For assays of fractionated cells, it is also useful to assay the unfractionated sample from which the fractions were obtained, to include evaluation of false positives due to fraction impurities and false negatives due to inadequate testing. A suitable negative control is a gel containing no mammary cells (i.e., fibroblasts only).

2. To quantify MRU frequencies in a test population of human mammary cells; use standard limiting dilution approaches (see Box 1 for more details). This requires initially transplanting
**BOX 1 | LIMITING DILUTION ANALYSIS**

Limiting dilution analysis (LDA) is a method of experimental design and analysis used to measure the frequency of cells possessing a biological property of interest within a heterogeneous cell population\(^{2+}\). The approach is applicable in experimental systems, in which the underlying biology can be modeled as a ‘single-hit’ process. That is, a defined biological response is registered if one or more of these cells are present in the input cell population, and a different (or absent) response is registered if none of these cells is present. The approach has been widely used in stem cell biology, immunology and bacterial research, often to detect rare elements or responses that cannot be identified directly in the starting population. In stem cell biology, the objective is typically the detection and quantification of cell type(s) that are able to give rise to clones of daughters cells in a functional regeneration assay (e.g., repopulation of the blood system or production of a tumor).

Key to the design of LDA experiments is the design of a system in which any exogenous factors required to generate the relevant biological response are provided in saturating quantities to the test cells so that the only limiting variable in detecting the cell (or response) of interest is the presence or absence of that cell (or response) in the number of test cells examined. In other words, the limiting factor becomes the presence of at least one cell with the intrinsic ability to generate the response.

The frequency of the cells of interest in the test population is determined by assessing multiple replicates at different cell doses, and then assessing the proportion of tests at each cell dose that results in a positive or negative outcome. A single-hit Poisson linear model and the method of maximum likelihood are then used to derive the frequency of active cells. Software packages including L-Calc (STEMCELL Technologies) and ELDA (Walter and Eliza Hall Institute of Medical Research, free online on https://bioinf.wehi.edu.au/software/elda/index.html) will calculate the frequency of the active cell type, along with confidence intervals around this frequency. They can also be used to test whether experimental data is consistent with an underlying single-hit process using a \(\chi^2\)-test or, more stringently, a likelihood ratio test derived from the generalized linear model\(^{24}\).

Results are often represented graphically. A single-hit process implies that there is a linear relationship between the cell dose and the logarithm of the proportion of tests that result in a negative outcome. The most likely frequency is the cell dose corresponding to 37% negative outcomes.

The LDA approach can be combined with the human MRU assay to calculate the MRU frequency in any population of HMECs, as follows:

1. Prepare a series of collagen gels, with multiple replicates at different cell doses (e.g., 24 gels, 6 containing each of 1,000; 5,000; 20,000; and 60,000 unseparated HMECs, plus a fixed number of feeder cells).
2. Transplant the gels as subrenal xeno grafts.
3. Harvest the gels after 4 weeks in vivo, then seed the dissociated contents of each gel into a CFC assay.
4. For each cell dose, record the proportion of gels that generated ‘positive’ outcomes, defined as those resulting in at least one colony in the CFC assay.
5. Use software (e.g., L-Calc or ELDA) to determine whether a single-hit Poisson model fits the results and, if so, determine the MRU frequency and associated confidence intervals. For example, if the above four cell doses result in 1/6, 3/6, 5/6 and 6/6 positive outcomes, the most likely MRU frequency is 1 per 8,800 cells with a 95% confidence interval of 1 per 4,200–1 per 18,800 cells.

In this example, the 95% confidence interval covers a relatively wide (greater than fourfold) range of frequencies, highlighting that LDA, although powerful, is a relatively low-resolution tool.

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(3) To compare human MRU frequencies (or population content values) based on ‘bulk secondary CFCs yields’, the relative numbers of regenerated CFCs obtained from a defined number of human test cells (referred to as the ‘bulk secondary CFCs value’) in different human samples can be used as a useful approximation of the relative numbers of MRUs contained in the samples. This strategy is much cheaper and more accurate than comparisons of frequencies determined by MRU-limiting dilution assays when the MRU frequencies being compared are similar.

The bulk secondary CFC comparison approach is, however, limited by the implicit assumption that the average secondary CFC output per MRU is not the major determinant of any differences noted between the populations being compared.
**PROTOCOL**

Nonetheless, this is an assumption that can also be tested using
limiting dilution measurements if large differences are
detected in the bulk comparisons. In addition, it is important to
note that experimental designs need to ensure that sufficient
cells are sampled to average out clonal variability in MRU
output while using nonsaturating test cell concentrations in
the xenografts. This strategy is discussed further in
the **Supplementary Methods and Supplementary Figure 1**.

(4) To undertake a rigorous comparison of the MRU frequency (or
content) of two or more human cell populations, perfom
limiting dilution transplant experiments on each human test cell
population of interest to derive MRU frequencies (or derived
yields) for each, and then use standard statistical methods to
establish the significance of any differences seen. Note, however,
that in practice, this method is typically limited to the resolution
of large differences (e.g., several-fold) in human MRU frequency
(or yield) values.

**Comparison with alternative in vivo methodologies**

This methodology uses the subrenal transplant site and fibroblast-
containing collagen implants pioneered by Cunha and colleagues to
propagate intact human mammary tissue fragments in mice. It is
important to note that we have extended this system for use with
dissociated mammary cells as starting material. This modification
is essential for the use of this approach in a clonal regeneration
assay that enables very primitive cells with in vivo regenerative
properties to be quantified and characterized. The further adoption
of an endpoint that detects the regenerated CFC enhances the
sensitivity, cost-effectiveness and practicality of the assay when used
for these purposes.

Our methodology thus differs in a number of respects from the
approach developed by Kuperwasser et al. to propagate human
mammary cells. The differences include the transplant site (subrenal
versus mammary fat pad), the end point used to detect human
mammary stem cell regenerative activity (secondary CFCs versus
macroscopic structures) and the assay time (5–6 weeks versus 8–10
weeks). There are also many similarities between the two
methods. Both involve the use of fibroblasts to improve the stimulated
growth and the differentiation of human mammary epithelial stem
cells (either of which may be manipulated to study the interaction
between these components), both support the production of
polarized bilayered structures from limited numbers of
suspended input cells and both identify a cell of origin with a "basal"
CD49f+ EpCAM+ CD31- CD45+ phenotype. In choosing the
system best suited for a given study, the points in Table 1 should be
considered. Overall, the subrenal methodology is particularly
well suited for quantitative studies and requires fewer mice and
surgical procedures. However, the humanized fat pad methodology
involves a surgically simpler procedure and allows regenerated
structures to be visualized in three dimensions without the need
for histology.

---

**MATERIALS**

**REAGENTS**

**Animals**

- NOD-SCID mice (NOD.CB17-Prkdcsd/sj; Jackson Laboratory) or related
  strains, e.g., NOD-SCID interleukin-2 receptor-γ null (NOD.Cg-Prkdcsd
  Il2rγnull/sj; Jackson Laboratory)

**CAUTION:** All animal experiments should be performed according to applicable ethical guidelines.

**Tissue**

- Discarded tissue from human mammary reduction mammoplasty

**CRITICAL:** Institutional review board approval is required, and appropriate procedures, including informed patient consent, should be followed. Primary tissue should be considered biohazardous and handled with appropriate biohazard precautions.

**General reagents**

- Inhalable anesthetic (Isoflurane; Baxter Healthcare Corporation)
- Buprenorphine (Temgesic; Reckitt Benkiser Pharmaceuticals)

**CAUTION:** Buprenorphine is classed as a narcotic and controlled substance. Adhere to all applicable regulations regarding security and record keeping.

- Meloxicam (inhibitor of nonsteroidal anti-inflammatory; Boehringer Ingelheim)
- Rupivacaine hydrochloride (0.25% [v/vol]; Marcaine, local aesthetic; AstraZeneca)
- Povidone iodine surgical scrub (Parke-Davis; Scientific Supplies, cat. no. MD-144)
- Phosphate-buffered saline solution (PBS; STEMCELL Technologies, cat. no. 37350)
- Hank's balanced salt solution, modified (STEMCELL Technologies, cat. no. 37150)
- Ammonium chloride solution (0.8%, with 0.1 M EDTA; STEMCELL Technologies, cat. no. 07850)
- Collagenase-type II (100 μg/ml, 100 μg/ml; StemCell Technologies, cat. no. 07912)
- Trypsin in citrate saline (STEMCELL Technologies, cat. no. 07400)
- Fetal bovine serum (PBS; STEMCELL Technologies, cat. no. 06100)
- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A-9418)
- EpiCult-B (human mammary epithelial cell culture medium; STEMCELL Technologies, cat. no. 00601)

**CRITICAL:** It is stable for at least 1 year when stored according to the manufacturer’s instructions. It is stable for 2 weeks at 4°C once reconstituted.

- Distilled water
- Mouse Tissue Processor (Lafayette Instrument Company)
- Isopropanol, 70% (v/vol)
- NIH 3T3 fibroblasts

**CRITICAL:** NIH 3T3 fibroblasts

- DMEM medium (STEMCELL Technologies, cat. no. 36250)
- DMEM/F12 medium (STEMCELL Technologies, cat. no. 36254)
- Insulin (Sigma-Aldrich, cat. no. I6841)

**CRITICAL:** Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)

- Bovine collagen, type I (STEMCELL Technologies, cat. no. 04902)
- D-Nase I (Sigma-Aldrich, cat. no. D1N25)
- Disperse (STEMCELL Technologies, cat. no. 07913)
- Acetone (Fisher Scientific, cat. no. A185K)
- Methanol (Fisher Scientific, cat. no. A152K)
- Wright-Giemsa stain (Sigma-Aldrich, cat. no. W532-1L)

**For preparation of concentrated collagen**

- Adult rat tails (Simonsen Laboratories or other supplier)
- Glacial acetic acid (Sigma-Aldrich, cat. no. A8967)
- Ultrasound-modified alginate powder (DMEM; Gibco cat. no. 12100)
- Concentrated sodium hydrosulfide (50% [w/vol] in water; Sigma-Aldrich, cat. no. 415413)

**CRITICAL:** It is highly caustic; avoid contact with skin or eyes

- Penicillin G and streptomycin solution (STEMCELL Technologies, cat. no. 057500)

- Amphoterixin B (anticancerous; Invitrogen, cat. no. 15290-018)

**For preparation of slow-release hormone pellets**

- Silicone elastomer (NuSil Technology, cat. no. MED-4011)
- Polyethylene glycol (PEG)–estradiol powder (Sigma-Aldrich, cat. no. E-2780)

**CRITICAL:** It is considered carcinogenic and teratogenic on chronic exposure; avoid skin or eye contact, ingestion or inhalation

- Progesterone powder (Sigma-Aldrich, cat. no. P-8783)

**CAUTION:** Avoid skin or eye contact, ingestion or inhalation

- Distilled water
### TABLE 1 | Comparison of subrenal and humanized fat pad xenotransplant assay approaches.

<table>
<thead>
<tr>
<th>Assay time</th>
<th>Subrenal method is shorter (approximately 5–6 weeks versus 8–10 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data points per mouse</td>
<td>Subrenal method is more mouse efficient because it allows up to eight grafts per mouse (four per kidney), compared with two for the humanized fat pad protocol (one per fat pad). This is particularly beneficial when quantifying MRU frequencies, as limiting dilution experiments require multiple replicates. It may be possible to perform multiple transplants per humanized fat pad or to use more than two humanized fat pads per mouse, although these have not yet been reported</td>
</tr>
<tr>
<td>Number of surgical procedures</td>
<td>Subrenal method requires fewer surgical procedures (one procedure to implant the gels, as compared with three separate procedures for the humanized fat pad method to first clear, then humanize and finally inoculate the cells). However, adaptations have been reported that condense the humanized fat pad protocol into two or one operation^6</td>
</tr>
<tr>
<td>Quantitation of MRU frequency</td>
<td>Both systems can be used as 'binary' assays, i.e., to distinguish between transplant inocula that contain in vivo repopulating cells from those that do not, and then to use these readouts in limiting dilution transplant experiments to calculate MRU frequencies. The subrenal method gives additional quantitative information about the regenerative quality of the MRUs analyzed through an assessment of the number of secondary CFCs generated (see Supplementary Methods)</td>
</tr>
<tr>
<td>Analysis of structures in three dimensions</td>
<td>Structures formed in the humanized fat pad system can be easily visualized as three-dimensional whole mounts. This is not possible in the subrenal system because of gel opacity and the density of fibroblasts within the gels. Histological analysis of two-dimensional sections is possible and informative in both approaches</td>
</tr>
<tr>
<td>Technical difficulties</td>
<td>The humanized fat pad methodology is surgically simpler to perform. It also does not require the use of specialized magnification equipment (e.g., surgical loupes or a dissecting microscope)</td>
</tr>
<tr>
<td>Heterogeneity of xenograft environment</td>
<td>Subrenal method involves test MRUs being evaluated in a homogeneous environment, because they are randomly dispersed amongst co-suspended fibroblasts. In contrast, Kuperswasser et al.^78 report that the mouse fat pad is typically not homogeneously colonized by fibroblasts and the well-humanized area may not be easily recognizable. Experience is required to subsequently inject human cells into a well-humanized area, to avoid introducing additional variability in the take rate</td>
</tr>
<tr>
<td>Endpoint objectivity</td>
<td>Subrenal method relies on an objectively defined endpoint (secondary CFC detection), compared with the less-well-defined criterion of structure generation. However, there is no reason why secondary CFC formation and its inherent advantages cannot be applied to the humanized fat pad assay</td>
</tr>
</tbody>
</table>

### EQUIPMENT
- Dissecting stereomicroscope (Zeiss SteREO Discovery, Zeiss) or surgical binocular loupe (Eschenbach, model no. 1636-3)
- Small animal hair clipper (Oster A5 with no. 40 blade)
- Surgical tools (autoclaved)
- Iris scissors (angled, 45–5 inches; Fine Science Tools, cat. no. 14063-11)
- Fine spring-loaded scissors (Fine Science Tools, cat. no. 15024-10)
- Curved dressing forceps (Fine Science Tools, cat. no. 11009-16)
- Diamond no. 5 fine forceps (two; Fine Science Tools, cat. no. 11251-20)
- Suturing needle driver (Fine Science Tools, cat. no. 12003-15)
- Absorbable sutures (Johnson & Johnson Medical, cat. no. 3433H)
- Sterile cotton swabs
- Syringe (1 ml)
- Syringe needles (e.g., 30 G)
- Glass Pasteur pipettes
- Glass chamber slide (Lab-Tek, Thermo Scientific, cat. no. 177372)
- Rotatable metal plate or rotatable small animal stage with built-in anesthetic ports (Vet Tech Solutions, cat. no. AN106)
- Small animal heating pad (Fine Science Tools, cat. no. 21061-90)
- Isoflurane vaporizer
- L-Calc (STEMCELL Technologies)
- ELDA (Walter and Eliza Hall Institute of Medical Research, available free online on http://biostat.wehi.edu.au/software/elda/index.html)
- Aluminum foil
- Parafilm (Pechiney Plastic Packaging Company, cat. no. PM996)
- Bunsen burner
- Pipettes and tips (P200)
- Incubator and rotary shaker
- Microcentrifuge tubes
- Falcon conical tubes
- Tissue culture dishes
- Laminar biosafety hood
- Filters (pore size 100 lm and 40 lm; BD Falcon, cat. no. 352350, 352340)
- Hemocytometer

### REAGENT SETUP
Concentrated collagen solution: Following the method described by Nandi and colleagues^8, skin two 25-gm rats using aseptic tools and techniques, cut each in to three pieces, remove the tendons, and weight tendons in a preweighed beaker (expected yield ~1.5 g fibers). Wash the tendons five times in sterile distilled water and transfer to a 250 ml flask. Add 150 ml of a 0.1% (v/v) mixture of glacial acetic acid in distilled sterile water supplemented with penicillin (100 U ml^-1), streptomycin (100 mg ml^-1) and amphotericin B (2.5 mg ml^-1) Add a sterile stir bar, cover the flask opening with sterile aluminum foil, and then with Parafilm. Leave the mixture stirring at 4°C for 5–7 d. Centrifugate the thick collagen solution obtained at 2,000 g for 20 min at 4°C. Remove a small aliquot (~1 ml) to determine the volume of concentrated NaOH required to bring the collagen solution to a neutral pH (7.0). From this aliquot, initially prepare a mixture of 78 µl of cold collagen solution with 20µl of 5X DMEM solution and 2 µl of concentrated NaOH. If the resulting mixture has a neutral pH, the phenol red in the DMEM will acquire a distinct salmon pink color and the mixture will solidify within a few minutes at room temperature (20–24°C). If the mixture is too alkaline (red in color) or too acidic (yellow), repeat using a smaller or larger volume of NaOH, until the neutralizing volumetric ratio of NaOH is achieved. The remaining collagen solution should then be distributed as aliquots to cryovials and can be stored at −20°C for up to 1 year.

**DMEM (5x):** Prepare DMEM solution from the powder following the manufacturer’s instructions, but making one-fifth of the normal final volume. This can be stored at 4°C for up to 1 year.

Hank’s + 2% FBS serum (HF): HF is Hank’s balanced salt solution supplemented with 2% (v/v) FBS. Store at 4°C for up to 3 months.
**PROTOCOL**

**RBC lysate buffer** RBC lysate buffer is a 4:1 (vol/vol) mixture of ammonium chloride solution (pH 7.4). This mixture is stable for 1 year at −20 °C or for 2 months at 4 °C.  

**Slow-release hormone pellets** To prepare 48 pellets that each contain 2 mg of 17β-oestradiol and 4 mg of progesterone, the method previously described by Laidlaw et al. is recommended. Aliquot 1 ml of MED-4011 silicone elastomer (Part A) into a sterile glass chamber microscope slide (or similar sterile glass surface) using a 1-ml syringe (the substance is too viscous to use a micropipette tip). Mix 16.6 mg of the elastomer powder and 192.0 mg of progestosterone powder. Add 100 µl of MED-4011 silicone elastomer (Part B), mix well (avoiding air bubbles); spread the mixture evenly over the surface of the glass slide. Leave to cure for 24 h at room temperature.  

**EQUIPMENT SETUP**  

**Magnification** The subrenal capsule surgery should be performed in a sterile biosafety cabinet with a setup that allows the investigator outside the cabinet to view the procedure under magnification. This can be achieved in two ways:  

- **Option 1:** Perform the surgery under a dissecting stereomicroscope housed in a biosafety cabinet, with the hood safety shield modified to include a small opening through which the microscope eyepieces can pass.  
- **Option 2:** Perform the surgery with the aid of binocular loupes worn by the investigator.  

**Operating stage** Use a flat metal plate that is large enough (e.g., 12 cm × 12 cm) to accommodate a mouse stretched on its back. The nose and mouth cone from the anesthetic vaporizer are securely attached to one side of the plate, and a heated pad is placed on top of the plate underneath the mouse to keep it warm throughout the surgery. If a stereomicroscope is used, the plate should be mobile to allow movement on, off and around the microscope stage. If surgical loupes are worn, the use of a fixed, rotatable small-animal stage and a small heating pad are recommended.  

**Fire-polished glass pipettes** Hold a glass Pasteur pipette horizontally and place the narrow end in the flame of a Bunsen burner for a few seconds. Use metal forceps to grasp the end once it becomes molten and pull in a vertical direction. Return to the flame briefly and manipulate to achieve a 'hockey stick' shape with a fine fire-polished round closed end. Autoclave before use.

**PROCEDURE**

**Preparation of cells**  

**TIMING 1–3 h**  

1. Prepare a single cell suspension from mammary tissue in cold HF medium, as described in Box 2.  

- **CRITICAL STEP** Unless otherwise stated, dissociated primary mammary cell suspensions should be kept cold throughout the protocol to prevent reaggregation.  

- **CRITICAL STEP** Plastic rather than glass pipettes should be used to avoid cell adhesion.  

2. Isolate subfraction(s) of viable cells using standard flow cytometry or immunomagnetic sorting methods (optional).  

3. Remove the culture medium from C3H10T1/2 fibroblasts growing in subconfluent culture in DMEM supplemented with 5% FBS. Add prewarmed (to 37 °C) trypsin and incubate at 37 °C until cells detach from the culture vessel. Add an equal volume of cold HF, transfer to a centrifuge tube and spin for 5 min at 400 g. Resuspend the cell pellet in cold HF at a density no greater than 4 × 10^6 cells per ml, and expose to 15-Gy X-irradiation using an X-ray machine.  

- **CAUTION** Follow machine-specific instructions, dosing and safety procedures.  

- **CRITICAL STEP** Certain other fibroblast sources may be used as alternatives to C3H10T1/2 cells, including primary fibroblasts from human reduction mammoplasty tissue and a telomerase-immortalized human mammary cell line (P.E., J.S. and C.J.E., unpublished observations). The correct X-irradiation dose to deliver to any source of fibroblasts should be determined empirically as the minimum dose that results in cells that survive but that do not proliferate when subsequently cultured in vitro. Note also that mitomycin C treatment can be used as an alternative strategy to render the fibroblasts proliferatively inert.

4. Thaw a 1-ml vial of concentrated collagen solution and transfer it to ice immediately.  

5. Mix the collagen with 5× DMEM and concentrated NaOH (all ice cold) in the volume ratios (e.g., 78:20:2) established previously (see REAGENT SETUP) to bring to a neutral pH. The phenol red in the DMEM should indicate a neutral salmon-pink color.  

- **CRITICAL STEP** Keep the neutralized collagen ice-cold until mixed with cells, as it will set at room temperature.  

6. Aliquot the HMECs plus the irradiated fibroblasts into microfuge tubes in cold HF, in a volume no greater than 1.5 ml. A single microfuge tube can hold the cells required for a single implant (2 × 10^6 fibroblasts plus the desired number of HMECs). Alternatively, cells for multiple replicate implants can be combined in a single microfuge tube (to a maximum of eight to ten replicates per tube).  

7. Spin the microfuge tubes (5 min, 450 g, 4 °C) and place them back on ice.  

8. Starting with one tube, carefully remove as much of the supernatant as possible with a P200 pipette; be careful not to dislodge the cell pellet.
BOX 2 | PREPARATION OF SUSPENSIONS OF DISSOCIATED PRIMARY HUMAN MAMMARY EPITHELIAL CELLS

1. Transport reduction mammaplasty discard tissue from the operating room on ice in sterile specimen cups containing DMEM/F12 medium supplemented with 5% (vol/vol) FBS and penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹). Transfer the tissue into a sterile Petri dish in a laminar biosafety hood.

▲ CRITICAL STEP Tissue specimens should have no skin attached, as this may compromise their sterility.

Mechanical disaggregation
2. Mince the tissue with scalpels.
3. (Optional) Tissue may be more finely mechanically disaggregated using a Mcllwain Tissue Processor (Lafayette Instrument Company), thus allowing the subsequent collagenase-hyaluronidase dissociation time to be reduced to 4–8 h.

Collagenase/hyaluronidase digestion
4. Transfer the minced tissue into sterile dissociation flasks.
5. Add DMEM/F12 medium supplemented with 300 U ml⁻¹ collagenase, 100 U ml⁻¹ hyaluronidase, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 1 μg ml⁻¹ insulin and 2% (wt/vol) BSA. The total volume of the suspended tissue and medium in the dissociation flask should not exceed the widest portion of the flask (e.g., 20 ml volume in a 250 ml flask).
6. Place in a rotary shaker in a 37 °C incubator after sealing the flasks with sterile aluminum foil (plus Parafilm if the incubator is not 5% CO₂ equilibrated).
7. Disassociate in the rotary shaker for ~16–18 h, although longer dissociation time periods may be required for very fibrous samples. Tissue dissociation is complete when the bulk of the cell suspension can be drawn through the bore of a 10-ml plastic serological pipette.
8. Discard fragments of tissue that have not undergone complete digestion, or, if sufficiently numerous, allow them to settle and collect for a second round of digestion with collagenase and hyaluronidase, or pour through a sterile sieve.

Differential centrifugation to enrich for epithelial organoids
9. Transfer the dissociated tissue in dissociation medium to 50-ml centrifuge tubes. Wash the flask vigorously with 5 ml of warm DMEM to liberate organoids still sticking to the glass, and add to the tissue in the centrifuge tubes.
10. Centrifuge using option A or option B.

(A) If all mammary cells are required:
(i) Centrifuge at 450g for 5 min and discard the supernatant. Wash the pellet containing organoids and single cells twice in 10 ml of warm DMEM.

(B) To obtain epithelial-enriched and stromal-enriched fractions:
(ii) Centrifuge at 80g for 30 s. After removing the overlying liquefied fat layer, the pellet will be highly enriched in epithelial organoids.
(iii) If the supernatant is transferred to a new 50-ml centrifuge tube and centrifuged at 200g for 4 min, a second pellet is obtained that contains variable numbers of epithelial cells, stromal cells and red blood cells.
(iv) Transfer the supernatant to a third 50-ml tube and centrifuge it at 450g for 5 min to obtain a pellet particularly enriched for human mammary fibroblasts, hematopoietic and endothelial cells.
(v) Wash each fraction twice in 10 ml of warm DMEM to remove remaining traces of enzymes.

Cryopreservation (optional)
11. Organoid preparations can be frozen in cryovials in freezing medium (50% (vol/vol) DMEM:44% (vol/vol) FBS:6% (vol/vol) DMSO) and stored at −135 °C. When needed, thaw vials rapidly at 37 °C and wash in cold HF.

Further dissociation of organoids to yield a single cell suspension
12. Resuspend pellets in 1–2 ml of cold RBC lysis buffer for 10 min on ice to lyse red blood cells; thereafter, add an equal volume of cold HF, centrifuge at 450g for 5 min and discard the supernatants. (Omit this step when using cryopreserved material, as red blood cells will lyse during the freeze-thaw cycle)
13. Add 1–5 ml of prewarmed 0.25% (wt/vol) trypsin in citrate saline to the pellet and triturate gently for 4 min. Add an equal volume of cold HF and spin at 450g for 5 min. Remove as much of the supernatant as possible, taking care that the pellet is not removed with the supernatant.
14. Resuspend the pellet in 2–5 ml of prewarmed dispase, one-tenth the volume of 1 mg ml⁻¹ DNase I and trypsin gently for 6 min.
15. Dilute the cells with 10 ml of cold HF and pass through a 40-μm filter. (If large clumps of undigested tissue remain, pass through a 100-μm filter before passing through the 40-μm filter).
16. Spin at 450g and wash again in HF.
17. Resuspend cell pellet in 1 ml of HF and count using a hemocytometer.

9] Transfer a volume of neutralized collagen solution equal to 25 μl × the number of replicates in the tube onto the cell pellet and pipette gently to suspend the cells evenly in the collagen. Thereafter, pipette 25-μl aliquots of the mixture into individual wells of a 24-well plate (not tissue culture–treated).

▲ CRITICAL STEP It is necessary to carry out the mixing and aliquoting quickly, as the neutralized collagen will begin to set at room temperature. When suspending cells in collagen, avoid excessive pipetting or introducing bubbles, as this will result in gels that set poorly.

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**PROTOCOL**

10 | Repeat Steps 8 and 9 for further microfuge tubes until all gels have been prepared.

11 | Transfer the plate to a 37 °C incubator. After 10 min, add 1 ml of prewarmed EpiCult-B medium supplemented with 5% (vol/vol) FBS to each well, and float the gels using a P200 pipette tip. Return the plate to the incubator for a further 50–70 min.

12 | Transfer the plate containing the implants onto ice, and store until surgery. The edge of the plate may be sealed with Parafilm to maintain sterility.

**PAUSE POINT** The plate may be kept on ice for several hours.

Surgical implantation of xenografts **TIMING** 2–4 h

13 | Anesthetize a 5- to 10-week-old female NOD-SCID mouse (or derivative strain, e.g., NOD-SCID interleukin-2 receptor-γ, null) using isoflurane gas supplied by a vaporizer to the mouse by a nose cone. Check that the mouse has reached surgical anesthesia by loss of pedal withdrawal reflex.

**CAUTION** Institutional review board approval must be obtained and appropriate guidelines followed for animals and procedures used.

14 | Administer buprenorphine analgesic subcutaneously (dose: 0.05–0.1 mg kg⁻¹). In addition, meloxicam may optionally be administered subcutaneously (dose: 1–2 mg kg⁻¹).

15 | Shave hair from the back of the mouse. Swab the back of the mouse with a cotton swab soaked with a povidone iodine or 70% (vol/vol) isopropanol solution.

16 | Infiltrate a few drops (80–100 μl) of 0.25% (vol/vol) bupivacaine local analgesic subcutaneously under the skin on the back of the mouse where the incision will be made.

17 | Make an anterior-to-posterior incision on the back of the mouse ~1.5 cm in length through the skin over the area of the kidney (Fig. 2a).

18 | Separate the dermis from the underlying body wall by blunt dissection either on both sides of the incision for bilateral grafting or on one side only.

19 | Place the mouse on its side and locate the kidney by viewing it through the body wall. Make a small incision in the abdominal wall over the kidney, slightly longer than the axis of the kidney (Fig. 2b). Thereafter, apply gentle pressure with your forefinger and thumb on either side of the kidney to move the kidney to the exterior of the incision, allowing pressure from the cut sides of the incision to hold the kidney in place (Fig. 2c).

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**Figure 2** | Subrenal capsule surgery. Schematic of the steps undertaken to surgically implant collagen gels under the kidney capsule of mice. Full details are provided in the *PROCEDURE* section (Steps 13–28). (a) Make a dorsal incision in the skin; (b) make an incision in the body wall; (c) exteriorize the kidney; (d) make an incision in the kidney capsule; (e) inject the gel under the kidney capsule; (f) manipulate the gel away from the incision; (g) insert additional gels as required; (h) return the kidney into the abdominal cavity and suture together the incision in the body wall; (i) insert a hormone pellet; (j) suture together the incision in the skin.
**PROTOCOL**

**CRITICAL STEP** If the incision is too small, the kidney will become damaged as it passes through; if it is too large, the kidney will fall back into the abdominal cavity during subsequent steps. It is recommended, therefore, to start with a small incision and then enlarge it in increments. At each increment, attempt to exteriorize the kidney with very gentle pressure. The kidney will move out cleanly when the correct incision size is reached.

**TROUBLESHOOTING**

20] Under magnification (such that the kidney occupies most of the field of view of the microscope or loupes), gently pinch and lift the capsule from the parenchyma of the kidney with jeweler’s forceps and make a 2–4 mm incision in the capsule with fine spring-loaded scissors (Fig. 2d). The capsule should subsequently be kept moist with PBS to avoid tearing.

21] Under magnification, insert the end of a wet (with PBS) fire-polished glass pipette through the incision and create a pocket between the capsule and kidney parenchyma. Great care should be taken not to damage the kidney parenchyma, which will bleed if damaged.

22] Under magnification, lift the cut edge of the kidney capsule with fine forceps, and insert the graft into the pocket under the capsule using the polished glass pipette or a second pair of fine forceps (Fig. 2e–g). Up to four grafts can be placed under a single kidney capsule.

**TROUBLESHOOTING**

23] Gently ease the kidney back into the body cavity (Fig. 2h).

24] Align and suture the body wall incision using absorbable sutures.


26] Insert a sterile slow-release hormone pellet under the skin, position midline and posterior to the incision. The pellet contains 2 mg of β-estradiol and 4 mg of progesterone in a silicone elastomer; approximate size 4 mm x 4 mm x 1 mm (Fig. 2i).

27] Align and suture the anterior-to-posterior incision (Fig. 2j).

28] Allow mouse to recover from anesthesia on a heated pad. Transfer it to its cage when awake. No special conditions are required after surgery, although the mouse should be monitored regularly for the first 48 h for signs of morbidity.

**TROUBLESHOOTING**

**Removal of xenografts and determination of progenitor content by CFC assay**

**TIMING** 6 h, plus 8–10 d for the CFC assay

29] Kill mice 4 weeks after surgery, following institutionally approved procedure (e.g., carbon dioxide asphyxiation and then cervical dislocation).

30] Spray the outside of the mouse liberally with 70% (vol/vol) isopropanol. Make a small posterior dorsal incision in the skin, and then holding on either side of the incision, pull the skin firmly over the mouse’s head to expose the sterile body wall.

31] Using a sterile technique, make an incision in the body wall above one kidney, remove the kidney and place on a sterile dish. Repeat for the contralateral kidney.

32] Use a scalpel to dissect the collagen gels from the kidney parenchyma under a dissecting microscope. Transfer each gel into an individual microfuge tube containing 400 µl of cold EpiCult-B.

**CRITICAL STEP** If a histological endpoint is required instead of a CFC-based endpoint, gels should be fixed in 4% (vol/vol) paraformaldehyde for 1–2 h at this point, then sectioned and stained using standard protocols.

**TROUBLESHOOTING**

33] When all gels have been removed, add 100 µl of collagenase-hyaluronidase solution to each tube (final activity 600 U ml⁻¹ of collagenase and 200 U ml⁻¹ of hyaluronidase). Transfer to a 37 °C incubator for 4–5 h or until gels are fully dissociated. Vortex or triturate with a P200 pipette every 30–60 min during the incubation.

34] Spin tubes at 450g for 5 min at 4 °C and carefully discard the supernatant. Resuspend pellets in 200 µl of prewarmed 0.25% (wt/vol) trypsin in citrate saline, and incubate for 5 min with occasional trituration to dissociate into a single cell suspension.
**PROTOCOL**

**BOX 3 | MAMMARY COLONY-FORMING CELL ASSAY**

This is a well-established functional assay to quantify normal human mammary progenitor cells that show bipotential, luminal-restricted and myoepithelial-restricted differentiation activity when they form colonies in vitro\(^4\). The same CFC assay procedure is used to look for evidence of regenerated mammary progenitors in digested collagen gels harvested from xenotransplanted mice.

CFCs are identified retrospectively as the cells that generate two-dimensional adherent colonies in defined epidermal growth factor-containing culture conditions in the presence of irradiated fibroblast 'feeder' cells. Assays are typically performed in 60 mm tissue culture dishes, which allow objective counting of up to ~200 colonies when appropriate numbers of mammary cells are seeded (~3 × 10^4 to 6 × 10^4) unseeded primary mammary cells or ~0.5 × 10^4 to 2 × 10^4 cells after a few days of initial culture at higher cell densities. If CFC assay dishes are overlaid with too many input CFCs, the colonies become confluent and cannot be reliably scored as distinct entities. The fibroblasts are added to ensure that the number of colonies obtained from cells plated at very low cell densities is not influenced by changes in cell density. The assay may be carried out in collagen-coated or uncoated tissue culture dishes. Collagen coating increases the sensitivity of CFC detection and the size of the colonies produced.

**Preculture dishes (optional)**

1. Incubate 60-mm tissue culture dishes with 1–1.5 ml of a 50–70 μg ml\(^{-1}\) solution of bovine type I collagen in PBS for 1 h at 37 °C; then pipette away the PBS and wash once more with PBS.

**Seed cells**

2. Prepare the required number (0.2 × 10^6 cells per dish) of irradiated NIH 3T3 fibroblast cells. These should be harvested from confluent cultures using 0.25% (wt/vol) trypsin, then X-irradiated with 50 Gy using an X-ray machine. Alternatively, cells irradiated in this way may be cryopreserved and then defrosted just before use.

**CAUTION** Follow machine-specific instructions, dosing and safety procedures.

3. For each 60-mm dish, add 4 ml of prewarmed Epicult-B medium supplemented with 5% (vol/vol) FBS containing 0.2 × 10^6 irradiated NIH 3T3 cells, plus the number of dissociated human mammary cells to be assayed.

**Culture**

4. Incubate dishes for 8–10 d in an incubator at 37 °C, 5% CO_2_.

5. Remove the growth medium after 1 d and replace with 4 ml of serum-free Epicult-B medium.

**CRITICAL STEP** If this is inconvenient to schedule, the medium can be changed after 2 or 3 d instead.

**Fixation**

6. Remove the growth medium from each dish and add 2 ml of acetone/methanol (1:1 (vol/vol)) fixative for 30 s in a fume hood.

7. Rinse dried dishes gently with tap water, then incubate for 1–3 min with 2 ml of Wright-Giemsa stain. Remove the Giemsa stain (it can be reused), then immerse dishes gently in a large beaker of tap water to rinse and allow them to air dry.

**Score**

8. Colonies can then be readily visualized under a microscope at low magnification, and the CFC type (luminal-restricted, myoepithelial-restricted or bipotent) identified on the basis of the morphology of the cells in the colony: colonies derived from luminal-restricted human CFCs have a tightly packed arrangement of cells with a smooth colony boundary; colonies derived from myoepithelial-restricted human CFCs are comprised of isolated teardrop-shaped cells; and colonies derived from bipotent human CFCs contain both these elements with a ragged colony boundary.

9. Only collections of cells containing >50 cells are counted as colonies; sample Giemsa-stained colonies are shown in Figure 2b.

35] Transfer the contents of each tube (cells in trypsin) into a Falcon tube containing 4 ml of Epicult-B supplemented with 5% (vol/vol) FBS plus 0.2 × 10^6 irradiated NIH 3T3 fibroblasts. The serum in the medium will inactivate the trypsin. Seed the contents of each Falcon tube into a 60-mm tissue culture dish.

36] Culture for 8–10 d to allow colonies to form, following the standard mammary CFC assay protocol described in Box 3.

37] Count the number of colonies that develop in each dish. Each colony is derived from a CFC that was present in the gel when it was removed from the mouse. The presence of one or more colonies implies that at least one MRU was present among the cells originally transplanted.

7 TROUBLESHOOTING

38] To quantify the human MRU frequency in a sample, or to compare frequencies between two or more samples, it is necessary to design and carry out experiments that involve multiple xenotransplants. Details are provided in ‘Applications and experimental design’ section above.

Note: for a depiction of the timeline for the entire protocol, see Figure 3.
PROTOCOL

2 TROUBLESHOOTING
Troubleshooting advice can be found in Table 2.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>Bleeding under the kidney capsule</td>
<td>Too much pressure applied while exteriorizing the kidney</td>
<td>Increase the size of the incision in small increments, testing at each time whether the kidney can be exteriorized with gentle pressure from the thumb and forefinger</td>
</tr>
<tr>
<td></td>
<td>Organs other than the kidney come out of the incision in the abdominal wall</td>
<td>Incision in the abdominal cavity is too small</td>
<td>Locate the position of the kidney before making the incision. Any fat that comes out, including the ovarian fat pad, can be left on the exterior during surgery. Any other organs should be carefully manipulated back before proceeding</td>
</tr>
<tr>
<td>22</td>
<td>Collagen gels are insufficiently firm to insert under kidney capsule</td>
<td>Collagen was not properly neutralized</td>
<td>Repeat titration to establish volumetric ratio of NaOH required to neutralize pH</td>
</tr>
<tr>
<td></td>
<td>Collagen stock was too dilute</td>
<td></td>
<td>Prepare a fresh stock of collagen, using a lower volume of liquid per gram of rat tail tendon</td>
</tr>
<tr>
<td>28</td>
<td>Mice show signs of morbidity after surgery</td>
<td>This should not occur, but if so, it would indicate inadequate analgesia or surgical technique</td>
<td>Follow institutional policies for treatment and/or killing</td>
</tr>
<tr>
<td>32</td>
<td>Transplanted gels are not found under the kidney capsule after 4 weeks</td>
<td>Gels squeezed out from under the capsule while the kidney was initially returned into the abdominal cavity</td>
<td>Manipulate gels so that they are as far away as possible from the incision in the kidney capsule. Monitor the position of gels under the microscope while the kidney is being returned into the abdominal cavity</td>
</tr>
<tr>
<td>37</td>
<td>No colonies are generated in secondary CFC assays</td>
<td>No MRUs were present among the cells transplanted</td>
<td>Repeat using a larger cell inoculum, and/or with a positive control population of unfractionated mammary cells</td>
</tr>
<tr>
<td></td>
<td>Problem with CFC assay reagents</td>
<td></td>
<td>Use an aliquot of primary (i.e., nontransplanted) mammary cells as a positive control for colony detection</td>
</tr>
<tr>
<td></td>
<td>Contamination in CFC assays</td>
<td>Contamination acquired while retrieving gels from mice</td>
<td>Sterilize the outside of the killed mouse by spraying alcohol liberally. Use sterile instruments and aseptic technique throughout</td>
</tr>
</tbody>
</table>

● TIMING
The protocol timing is depicted in Figure 3.

Steps 1 and 2, Preparation of cell suspensions: ~1.5 h
Step 2 (Optional), Flow cytometric or immunomagnetic sorting if desired: 1–2 h
Steps 4–28, Surgical implantation: 2–4 h plus 4 weeks of in vivo propagation of cells in xenografted gels
Steps 29–32, Removal of xenografts: 1 h
Steps 33 and 34, Enzymatic dissociation: 4–5 h
Step 35, Seeding of cells into CFC assays: 0.5 h
Steps 36 and 37, Culture, fixation, staining and colony scoring: 8–10 d
Box 1: variable, depending on the experiments conducted with this statistical method
Box 2: 24 h
Box 3: 8–10 d

Figure 3 Timeline for human MRU assay. Human mammary cells are combined with fibroblasts in small collagen gels, which are then implanted under the kidney capsule of highly immune-deficient, hormone-supplemented mice. Xenografts are removed after 4 weeks in vivo, and viable cells dissociated enzymatically. The number of CFCs among the dissociated cells is then determined by plating them in an in vitro clonogenic assay. The detection of one or more CFCs in such assays indicates that at least one human MRU was present among the cells originally transplanted.
ANTICIPATED RESULTS
Positive engraftment (using endpoints of secondary CFC production or histological structure formation) should be consistently obtained in xenografts prepared from dissociated primary mammary tissue if sufficient numbers of mammary cells (10^5–10^6 unseparated cells) are transplanted. We find the frequency of MRUs (defined by secondary CFC generation after 4 weeks and quantified by limiting dilutions analysis) to be ~1 per 10^5–10^6 cells in preparations of previously frozen organoid-enriched primary normal human mammaryplasty tissue samples, although with considerable intersample variability (an order of magnitude)). When xenografts contain limiting or below-limiting numbers of MRUs (which, by definition, a proportion of xenografts generate no detectable CFCs), approximately one to ten in vitro colonies per positive xenograft are obtained.

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AUTHOR CONTRIBUTIONS P.E. and J.S. developed the methodology. P.E. composed the draft of the paper. C.J.E. conceptualized the approach and finalized the writing of the paper.

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SUPPLEMENTARY METHODS

COMPARISON OF THE STATISTICAL RESOLUTION OF FREQUENCY BY LDA VERSUS “BULK CFC” ENDPOINTS

Comparison of the MRU content of 2 or more cell populations is most rigorously carried out by performing separate limiting dilution series to measure the MRU frequency in each (see Box 3). Unfortunately, this approach results in calculations with wide margins of statistical error. Thus, in practice, LDA-based estimates do not allow differences in frequency between populations to be resolved unless these are large (i.e., several-fold) in magnitude. The wide uncertainty arises because transplants are informative in this approach at doses at which relatively few MRUs are present per replicate, therefore the transplant series samples relatively few MRUs overall with a consequent loss of statistical power.

An alternative approach makes use of average bulk output measurements (“bulk CFCs” in the case of MRU frequency comparisons) and offers many advantages for comparing MRU frequencies. This approach utilizes the finding that the number of regenerated CFCs per transplant after 4 weeks in vivo is linearly related over a wide dynamic range to the number of cells (and by implication the number of MRUs) transplanted. Therefore, the relative MRU content of different populations can be approximated by transplanting cell aliquots from each population and measuring the relative numbers of regenerated CFCs per input cell. However, it should be noted that this approach makes the implicit assumption that the average CFC output per MRU is the same (or at least known) for all populations compared. It is also necessary to design experiments in which sufficiently many cells are transplanted (usually in replicate series, each within the dynamic range of the system) so that the heterogeneity of CFC output per MRU is averaged out.

The ability to resolve smaller differences in MRU frequency by this approach is demonstrated in the Monte Carlo simulation shown in Fig. S1. Experiments are simulated in which 20 transplants are carried out (10 transplants for each of 2 hypothetical samples #1 and #2) that are optimally seeded for each of the 2 endpoints. The error bars on the figure show a measure of the limit of resolution of each method – here represented by the range of measured MRU numbers for population #2 that cannot be distinguished at 95% confidence from a measured number of 100 MRUs for population #1. With simulated experiments of this size, limiting dilution experimental design could not resolve frequency differences between samples unless they were more than ~3-fold, compared with ~1.5-fold for comparisons that relied on bulk CFC measurements.


Figure S1. Simulation comparing the ability of LDA or bulk CFC approaches to resolve differences in MRU frequencies between 2 populations.

The bars indicate the range of a measured MRU frequency for hypothetical sample #2 for which the null hypothesis of no statistical difference from measured MRU frequency = 100 for hypothetical sample #1 cannot be rejected with 95% confidence. These are obtained from Monte-Carlo simulations of experiments comprising 20 transplants (10 for each of the 2 samples). For the simulations using the LDA endpoint, transplants were assumed to be made at limiting dilution (i.e., an average of 1 MRU per transplant). Likelihood ratio tests for inequality in frequency between samples were carried out using the ELDA web tool\(^2\), which uses a generalized linear model assuming a single-hit Poisson process. For the simulations using the bulk CFC endpoint, the regenerated CFC output per transplant was assumed to follow a normal distribution with an average of 70 CFCs and a standard deviation of 30 CFCs, which is consistent with technical replicates series carried out by us previously (data not shown). Student t-tests were carried out to test for inequality.