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ABSTRACT

The mammary epithelium of normal adult female mice contains stem cells with extensive in vivo regenerative and self-renewal potential. Indirect evidence has suggested that analogous cells exist in the mammary glands of adult women, and are candidate targets for the first transforming mutations that lead to the evolution of breast cancer stem cells. The objective of this grant was to determine whether these hypothesized normal human mammary stem cells might be detected and quantified by a robust and specific assay that could be used to enable the purification and phenotypic properties of these cells, and to derive information about their frequency and how they are regulated. During the 3 years of this grant plus 1 year extension, I established conditions that allow human mammary gland structures to be reproducibly generated in subrenal xenografts in highly immunodeficient mice, starting with small innocula of dissociated human mammary cells. The regenerated glands are similar in morphology and cellular organization to normal human mammary glands, bounded by a basement membrane with an outer layer of myo-epithelial cells and an inner layer of polarized luminal cells that can be induced to produce milk. I also established that the presence of regenerated structures can be determined by detecting the in vitro clonogenic progenitors they contain and this endpoint can serve as an objective indicator of the presence of a primitive stem-like cell in the initial cells transplanted. This retrospective functional assay allows limiting dilution analysis of positive xenograft yields to derive mammary stem cell frequencies in differently manipulated populations. Using this approach I found the frequency of stem cells in normal human mammary tissue to be ~1 per 5000 cells and their phenotype to be $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$. I have found that most human MRUs do not efflux Hoechst 33342 dye (implying a low/absent activity of efflux pump Brcp1/ABCG2) and also do not exhibit high fluorescence in the Aldefluor assay (implying a low activity of aldehyde dehydrogenase). 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 MRUs in normal adult breast tissue exhibit phenotypes that are associated with actively proliferating cells. These findings set the stage for further biological and molecular characterization studies of normal human mammary stem cells and their relationship to human breast cancer stem cells. Detailed findings have been published in Nature Medicine and Nature Protocols journals.

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Eirew,P., Stingl,J., and Eaves,C.J. . Quantitation of human mammary epithelial stem cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay. Nat Protoc. 2010 Dec;5(12):1945-56.

INTRODUCTION

There is growing evidence to support the concept that the proliferation of many human tumors, including breast cancers, is driven by rare subpopulations of "cancer stem cells". The first event(s) that lead to the production of these cancer stem cells may occur in normal tissue stem cells, but later genetic or epigenetic changes that establish full oncogenic activity are likely to be acquired in a complex fashion over extensive periods of time in progeny with stem or progenitor features and may include the reactivation of stem cell properties in cells that had otherwise lost them. It is therefore critical to understand the mechanisms that regulate normal human mammary stem cells, and how dysregulation of these can lead to the development of breast cancer stem cells. The principal objective of this training grant was to develop a robust and reproducible methodology to detect, quantify and isolate stem cells in normal human mammary tissue, using a xenotransplantation system, to enable subsequent characterization of the regulation of these important cells and comparison with breast cancer stem cells. During the three years of the grant, a robust xenotransplantion assay for human stem cells was developed and validated. Furthermore, a number of phenotypic markers were identified that characterize the stem cells that read out in this assay, and that allow these cells to be isolated at enriched purities from some (but not all) other cell types in human breast tissue. This report covers a 12-month no-cost extension period that was approved for the grant to enable further biological characterization of human mammary stem cells (under Aim 3 in the Statement of Work). These included investigation of aldefluor dehydrogenase activity in human mammary stem and progenitor cells, and also characterization of the ex-vivo cytokine response of these cell types and other properties that are informative regarding the cell cycle profile of these cells.

BODY

1. Concept

A number of studies in recent years have established that the mature cells in the mammary epithelium are continually generated by a multi-step differentiation process from a pool of long-lived undifferentiated self-renewing mammary epithelial stem cells via an intermediate compartment of shorter-lived progenitor cells. We refer to the progenitors as "colony-forming cells" (CFCs), because they are detected based on their ability to generate adherent colonies when plated at low densities in 2-D tissue cultures[1, 2]. We and others have identified and characterized more primitive and phenotypically distinct cells in the *murine* mammary gland with extensive self-renewal and proliferative properties[3, 4], and have coined the operational term "mammary repopulating units" (MRUs) for them. This term reflects the method used to detect murine mammary stem cells which is based on their individual ability to regenerate an entire mammary tree when transplanted into the mammary stroma of a congenic recipient mouse.

The present project tests the hypothesis that an analogous stem cell population exists in the *human* mammary gland. It is very important to understand the mechanisms that regulate these cells, to investigate how dysregulation of their development/differentiation can drive the initiation and progression of human breast cancers, and to identify specific stem cell markers and molecular pathways that can potentially form the basis of future novel diagnostic and therapeutic strategies. Achievement of these ends requires the development of tools to selectively identify human

mammary stem cells, as well as methodologies for purifying them from the heterogeneous populations of cells present in normal human breast tissue and eventually understanding how they are normally regulated. The objectives of this grant were to initiate this type of study.

Our strategy to identify human mammary stem cells is based on a xenotransplant system, originally developed to propagate human mammary epithelial fragments and recently adapted for use with dissociated mammary cell suspensions. Human mammary cells are combined with fibroblasts in small collagen gels, then implanted under the kidney capsule of highly immunodeficient, hormone-supplemented mice. After a number of weeks organized human mammary structures are regenerated in the xenografts, and these contain differentiated cells of both lineages, progenitors that can be detected in 2-D in vitro CFC assays as well as daughter cells capable of repeating this process in secondary recipients. Under the assumption that these structures, and the cells they contain, are regenerated from primitive stem-like cells ("human MRUs"), we have developed this system to act as a quantitative assay for these rare and important cells.

2. Scientific Progress

The scientific progress is summarized briefly below, with reference to the specific objectives in the Statement of Work. Greater detail is provided in the two manuscripts attached in the Appendix, recently published in Nature Medicine[7] and Nature Protocols[8] journals. The references given below refer to figures in the Nature Medicine publication[7].

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

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

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

By transplanting small cell doses containing one or fewer MRUs, I have demonstrated that parent MRUs generate multiple daughter CFCs in vivo, and that the assay is therefore detecting a clonal regeneration process. I am still planning additional experiments to validate the clonality of the process, by further testing the common origin of regenerated CFCs using input human cells that can be distinguished from one another by genetic (virally-marked) and/or epigenetic (X chromosome inactivation) means.

Aim 2: Development of a robust and reproducible methodology for purifying mammary stem cells from normal primary human breast tissue (months 12-24)

I have screened a number of candidate phenotypic markers for their presence/absence on human MRUs, with the objective of identifying a combination of markers that can be used to purify stem cells from normal mammary tissue. These experiments involve measuring the MRU content in subpopulations that are FACS-sorted from reduction mammoplasty samples after staining with antibodies against various candidate stem markers. I have identified a combination of markers that are expressed by a large majority of MRUs and show a consistent pattern of expression across 9 normal mammoplasty samples. This includes a high expression of CD49f (α6 integrin), low expression of Epithelial Cell Adhesion Molecule (EpCAM), and a lack of expression of hematopoietic and endothelial markers CD45 and CD31 **(Fig. 3a-e)**. Sorting by this phenotype allows MRUs to be purified by about 10-fold compared with unsorted cells and to allow their almost complete separation from luminal restricted progenitors detected as CFCs.

I have screened other candidate phenotypic markers (CD10, Thy1, CD48, SSEA3, SSEA4, EPCR, CXCR4, KIT, CD49b, CD49d), but none of these has proved useful for obtaining significant incremental enrichment beyond what has already been achieved with the $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$ combination.

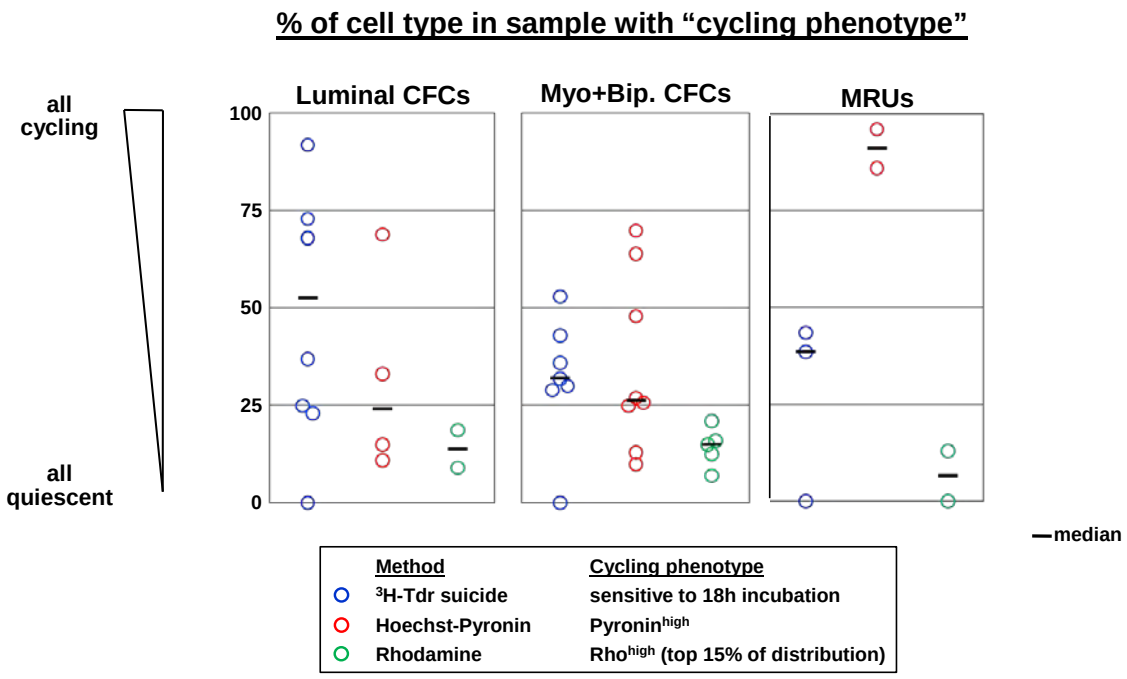
Aim 3: Biological characterization of normal human mammary stem cells and comparisons with human breast cancer stem cells (months 24-48)

I have used limiting dilution approaches to measure the frequency of MRUs in 5 different normal adult mammary tissue samples. These experiments indicate that MRUs are rare, with measured frequencies of approximately 1 per 5000 mammary cells **(Supplementary Table 1)**.

I have also carried out assays to determine whether MRUs efflux the dye Hoescht 33342 to generate a "side population" phenotype. This phenotype is a characteristic of adult murine hematopoietic stem cells but not murine mammary stem cells, and is believed to reflect activity of the efflux pump BCRP1/ABCG2. I found that the large majority of MRUs do *not* efflux this dye (i.e., MRUs are mostly found in the "main population"). I have also investigated the level of aldehyde dehydrogenase activity in MRUs, as measured by the Aldefluor assay, since an $Aldefluor^{high}$ phenotype was suggested in a recent study to characterize xeno-transplantable tumorigenic cells from human breast cancer samples[5]. My results suggest that the large majority of MRUs in normal tissue have an $Aldefluor^{low}$ phenotype, whereas luminal cells (including luminal CFCs) are $Aldefluor^{high}$. This is interesting, as it suggests a marker that may be expressed differently between normal MRUs and the cells that generate breast tumors. I am preparing a manuscript that details these findings. We have recently participated in a study that applies the xenotransplant methodology to dissociated *bovine* mammary tissue, and find that MRUs within this tissue also exhibit an $Aldefluor^{low}$ phenotype[6].

Experimental work during the 12-month no-cost extension period also focused on investigation of the cell cycle profile of MRUs and CFCs in normal adult breast tissue. There is a particular challenge to studying this aspect of cell biology in *functionally-defined* cell types, namely the requirement of the retrospective assay methodologies for *viable* cells as input material. This

precludes the direct application of many "gold-standard" methods of cell cycle analysis that rely on the detection of intra-cellular factors (e.g., cyclins, Ki67, BrdU) in *fixed* cells. I therefore applied three complementary approaches to sort (or select) mammary subfractions based on phenotypic properties that have been shown in other systems to be associated with cell cycle stage (G0, G1, S, G2, M) and can be measured in viable cells. The **thymidine suicide** methodology is based on the concept that quiescent cells when stimulated in vitro enter the cell cycle after a considerable delay and can therefore be distinguished from cycling cells based on their insensitivity to S-phase-specific cytotoxic agents (e.g. high specific activity tritiated thymidine (^{3}H-Tdr)) over a period of time[7]. The **Hoechst-Pyronin** methodology relies on identifying a presumed cycling cell subset based on elevated cellular RNA content (measured by the fluorescence after incubation with RNA-binding dye Pyronin Y) and/or a >2n cellular DNA content (measured by the fluorescence after incubation with DNA-binding dye Hoechst 33342)[8]. The **Rhodamine** methodology relies on identifying a presumed cycling subset of cells based on elevated metabolic activity (measured by the fluorescence after incubation with Rhodamine 123 dye that binds to activated mitochondria)[9], though validation experiments indicated that this approach is less selective for cycling cells in the mammary gland than the other two. These methodologies were applied to a series of ten human mammoplasty tissue samples, and functional assays applied to measure the proportion of different primitive cell types that displayed a "cycling phenotype" (sensitivity to 18h incubation with ^{3}H-Tdr; high Pyronin fluorescence; high Rhodamine fluorescence). As shown in the figure below, significant proportions of CFCs (luminal lineage-restricted, or combined myoepithelial lineage-restricted plus bipotent) and MRUs were found to exhibit phenotypic properties that are associated with actively cycling cells. These findings encourages consideration of models for the normal generation of mammary cells that incorporate a continual level turnover at all levels of the tissue hierarchy. These methodologies will also enable future work to identify the factors and molecular interactions that can alter the cycling status of these different normal human mammary cell types and how their effects may be dysregulated in human breast cancer stem cells.



3. Training Opportunities

I have gained hands-on experience of the various *in vitro* and *in vivo* techniques used in this project including dissociation of primary mammary tissue, flow cytometry, in vitro mammary progenitor assays and subrenal capsule surgery. I have had the opportunity to present this work orally at 4 scientific conferences, as well as give poster presentations at these and other venues. I have been involved in the preparation of a primary research paper describing the findings of this study, a protocol paper describing the methodology in detail, and have also contributed to the preparation of 3 other primary papers. I have provided instruction to 2 new doctoral students in the methods and concepts that I have learned thus far. I have also given a practical demonstration of the method at a workshop on mammary stem cell techniques.

Overall, this outstanding training opportunity has provided both the motivation and the grounding to continue a career as a cancer researcher beyond my PhD studies.

KEY RESEARCH ACCOMPLISHMENTS

- Development and validation of a quantitative methodology to assay for human mammary stem cells
- Identification of a preliminary set of human mammary stem cell markers ($CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$) which allow a stem-cell enriched subset of cells to be isolated from adult human breast tissue
- Measurement of the stem cell frequency in normal adult human mammary tissue
- Demonstration that normal human mammary stem cells have a phenotype that is distinct from the phenotype of luminal restricted progenitors detectable in colony assays performed in vitro.
- Characterization of the activity in MRUs of efflux pumps (Brcp1/ABCG2) responsible for the Hoechst 33342 "side population phenotype" and of aldehyde dehydrogenase activity.
- Development of methodologies that can be used to sort/select subpopulations of human mammary cells that are enriched in cells in particular phases of the cell cycle (G0/G1/S/G2/M).
- Demonstration that a proportion of MRUs and CFCs in normal adult mammary tissue show phenotypic properties that are associated with actively cycling cells.

REPORTABLE OUTCOMES

1. Peer reviewed papers

Eirew,P., Stingl,J., and Eaves,C.J. Quantitation of human mammary epithelial stem cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay. Nat Protoc. 2010 Dec;5(12):1945-56.

Martignani,E., Eirew,P., Accornero,P., Eaves,C.J., Baratta,M. Human milk protein production in xenografts of genetically engineered bovine mammary epithelial stem cells. PLoS One. 2010 Oct 19;5(10):e13372.

2. Abstracts

Eirew,P., Stingl,J., Raouf,A., Turashvili,G., Aparicio,S., Emerman,J. and Eaves,C.J. Characterization of the cell cycle profile of human mammary stem cells with in vivo regenerative properties. AACR Advances in Breast Cancer Research, San Diego, CA, October 2009

3. Completion of doctoral studies

My PhD thesis was defended and accepted by the University of British Columbia in May 2011.

CONCLUSION

This project has resulted in the development and validation of a xenotransplant-based methodology to detect primitive human mammary cells (MRUs) with the hallmark features of stem cells (the ability to generate both differentiated lineages in organized 3-D structures, the ability to generate daughter CFCs, and the ability to self-renew). Notably, by combining the transplant procedure with an endpoint "readout" after several weeks in vivo of detectable regenerated CFCs, we have established an objective, quantitative and practical way to detect MRUs in any given test population and to use limiting dilution transplants to quantify their frequency. Such experiments have shown MRUs to be rare cells in normal adult mammary tissue (1 per 5000 cells) and to be characterized by a $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$ phenotype.

This work has been published in a very high impact journal (Nature Medicine)[7], and presented as invited talks at various international conferences. A detailed description of the methodologies developed has also been published in another high impact journal (Nature Protocols)[8], which provides an excellent means to disseminate the methods to the wider breast cancer research field.

We anticipate a num ber of benefits from this project. T he establishment of a reproducible methodology to detect human mammary stem cells is considered a major breakthrough in the field, as none previously existed. When combined with markers to purify stem cells from many (though not yet all) other cell types in the breast, it allows investigations of the molecular and cellular mechanisms operating at the level of these important cells to be undertaken, avoiding problems associated with studying bulk populations. The finding that a proportion of stem and progenitor cells exhibit phenotypic properties of cycling cells supports a model of human mammary cell generation that incorporates a level of cellular turnover at all levels of the hierarchy. Information on how these mechanisms can become dysregulated has the potential to lead to novel therapeutic strategies that specifically target breast cancer stem cells.

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APPENDICES

1. Paper published in Nature Medicine journal detailing the development of the human mammary stem cell assay and the identification of MRU markers:

Eirew,P., Stingl,J., Raouf,A., Turashvili,G., Aparicio,S., Emerman,J.T., and Eaves,C.J. A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability. Nat Med. 12:1384-9, 2008.

2. Paper published in Nature Protocols journal, detailing the xenotransplantation methodology to detect and quantify MRUs.

Peter Eirew, John Stingl and Connie Eaves. Quantitation of human mammary epithelial stem cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay. Nat Protoc. 2010 Dec;5(12):1945-56.

nature medicine

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

Peter Eirew[1], John Stingl[1,6], Afshin Raouf[1], Gulisa Turashvili[2], Samuel Aparicio[2,3], Joanne T Emerman[4] & Connie J Eaves[1,5]

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

The human mammary gland is a compound tubulo-alveolar structure composed of two lineages of epithelial cells: an inner layer of luminal epithelial cells surrounded by an outer layer of contractile myoepithelial cells. These mature cells are in a state of constant turnover, being continually replaced from more primitive mammary epithelial progenitors. Some of these progenitors can be detected as colony-forming cells (CFCs) *in vitro*, and, in humans, luminal-restricted, myoepithelial-restricted and bipotent mammary epithelial CFCs can be prospectively isolated as separable subsets[1,2]. Definitive evidence of more primitive mammary epithelial cells with the self-renewal property of stem cells was first provided in mice by mammary fat pad transplantation experiments[3]. More recently, we and another group showed that the mammary structures produced in this assay are generated from single $CD49f^{+}CD29^{+}CD24^{low}$ mammary repopulating cells (termed mammary repopulating units, or MRUs) that are relatively rare ($\sim$1 per 1×10^3 epithelial cells) in the glands of normal adult virgin female mice[4,5].

The presence of mammary stem cells in normal adult women has been inferred from analyses of X-chromosome inactivation patterns indicating a frequent clonal origin of cells in adjacent lobules and ducts[6] and from attempts to regenerate mammary gland structures from human mammary epithelial cells (HMECs) transplanted into highly immunodeficient mice. One of these transplantation approaches has relied on colonizing the precleared mammary fat pad of such mice with human fibroblasts to create an environment conducive to the requirements of HMECs[7,8]. We have been developing an alternative strategy that involves suspending test cells together with irradiated fibroblasts in a collagen gel, which is then implanted under the kidney capsule of estrogen- and progesterone-treated nonobese diabetic severe combined immunodeficient mice (NOD-SCID) mice[9], on the basis of previous findings that viable mammary tissue fragments can be maintained at this site[10]. We now show how this latter protocol, as modified for use with dissociated human mammary cell suspensions, can be used as a quantitative assay for a subset of human mammary cells with stem cell properties and a basal phenotype.

RESULTS

Dissociated HMECs regenerate organized structures *in vivo*

We initially found that collagen gels seeded with suspensions of normal human mammary cells and irradiated mouse C3H $10T^1/_2$ fibroblasts and then placed under the kidney capsule of hormone-supplemented[11] female NOD-SCID mice (or derivative strains) contained regenerated epithelial structures when the gels were removed and examined 2–8 weeks later (**Fig. 1a**,**b**). These structures included both round and elongated duct-like arrangements of cells organized as a polarized bilayered stratified epithelium enclosing a lumen and surrounded by a basement membrane containing laminin and collagen IV (**Fig. 1b**). The cells in the inner and outer layers expressed established markers of differentiated mammary luminal and myoepithelial cells respectively. Cells expressing nuclear estrogen receptor-α and cells expressing progesterone receptors were also present. Overall, the spatial distribution of cellular markers in regenerated structures was similar to that seen in normal human mammary tissue.

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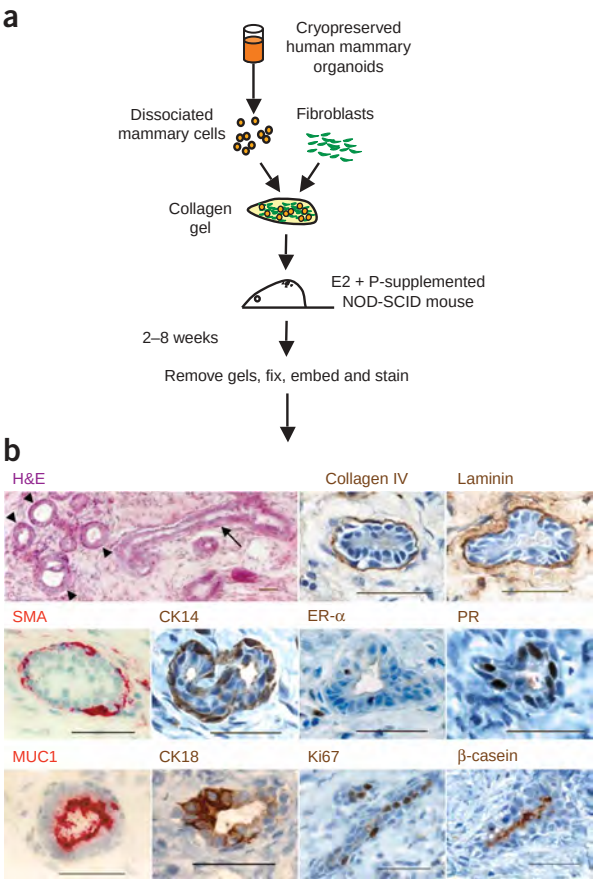

Figure 1 Organized structures are generated *in vivo* from single-cell suspensions of primary human mammary cells. (**a**) Schematic showing xenotransplantation approach. Single cells obtained by enzymatic dissociation of normal human reduction mammoplasty samples were combined with irradiated fibroblasts 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 β-estradiol and progesterone (E2 + 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 luminal epithelial cell markers. The sections stained with antibodies to estrogen receptor-α (ER-α) and progesterone receptor (PR) show that some fibroblasts, as well as epithelial cells, stained positively for ER-α. 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 d 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.

We also saw many cells that expressed the proliferation marker Ki67 (**Fig. 1b**) and cells with diffuse chromatin (data not shown), consistent with the interpretation that the structures are produced by an ongoing regenerative process. Some apoptotic cells were also evident (data not shown). Such structures were obtained from every human mammoplasty sample tested when at least 1×10^5 cells were transplanted.

When the female hosts were mated 1 week after the gels had been placed in the mice and the structures were examined 3 weeks later, luminal cells with vacuolated cytoplasm that stained positive for human β-casein (a 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

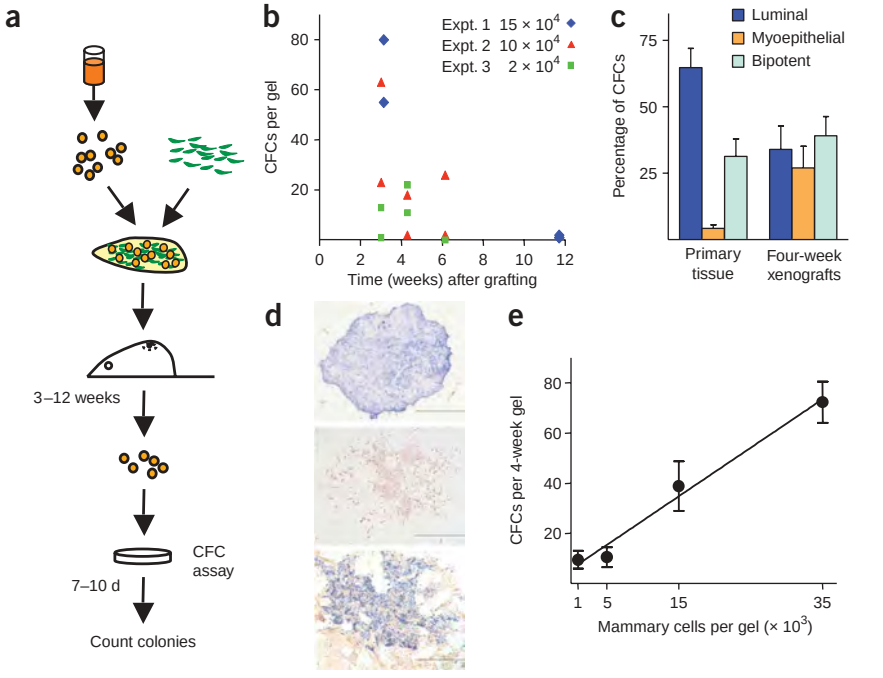

Figure 2 CFC production *in vivo* as an indicator of human MRU repopulating activity. (**a**) Experimental protocol (as in **Fig. 1a** but showing the use of CFC output measurements as an endpoint of MRU activity). (**b**) The number of CFCs detected per gel after various times *in vivo*. The legend 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.

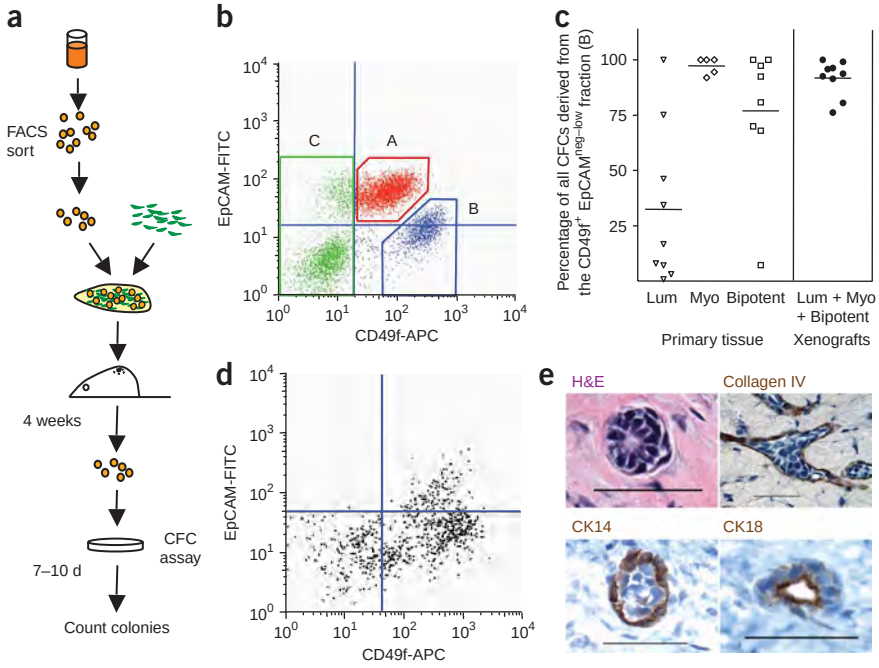

Figure 3 MRUs are CD49f$^+$EpCAM$^{neg–low}$. (**a**) Experimental protocol (as in **Fig. 2a**, but starting with FACS-isolated subsets in the transplanted gels). (**b**) FACS profile of input human mammary cell preparations (depleted of CD45$^+$ and CD31$^+$ cells) showing the gates used to select fractions assayed for MRUs and CFCs. APC, allophycocyanin. (**c**) FACS-separated fractions A, B and C as depicted in **b** were assayed *in vitro* for the presence of primary CFCs and also transplanted *in vivo* to assay for the presence of MRUs as defined by their ability to generate secondary CFCs detectable after 4 weeks. Open symbols show the proportion of each primary CFC type that was present in the original CD49f$^+$EpCAM$^{neg–low}$ fraction B ($n = 9$ mammoplasty samples tested). Lum, luminal-restricted CFCs; Myo, myoepithelial-restricted CFCs; Bipotent, bilineage CFCs. Solid circles show the proportion of subsequently detected xenograft-derived CFCs that were generated in gels originally seeded with CD49f$^+$EpCAM$^{neg–low}$ cells (fraction B). Symbols are absent where samples did not yield a given type of primary CFC. The numbers of cells assayed from each fraction were in proportion to the relative sizes of each fraction. (**d**) A representative FACS profile of cells from a 4-week xenograft initiated with CD49f$^+$EpCAM$^{neg–low}$CD31$^-$CD45$^-$ cells. (**e**) Sections of 4-week-old xenografts in gels initially seeded with purified CD49f$^+$EpCAM$^{neg–low}$CD31$^-$CD45$^-$ cells, stained with H&E (top left) or with antibodies to collagen IV (top right), CK14 (bottom left) or CK18 (bottom right). Scale bars, 50 μm.

were indistinguishable from those derived from primary mammoplasty tissue (**Fig. 2b–d**). Hereafter, we will refer to these regenerated CFCs as secondary CFCs to discriminate them from the primary CFCs present in initial suspensions of dissociated mammoplasty tissue. Transplant cell dose-response experiments further showed that the number of secondary CFCs present in xenografts after 4 weeks is linearly related to the number of human mammary cells originally suspended in the gels (**Fig. 2e**).

We then performed a series of limiting-dilution transplant experiments to determine the frequency of cells that are responsible for regenerating structures containing secondary CFCs at 4 weeks after transplant. A total of 107 gels were analyzed, each seeded with 500–60,000 cells from freshly thawed, organoid-enriched human mammary tissue (five separate experiments, **Supplementary Table 1** online). Chi-squared tests showed the results were consistent with a single-hit Poisson model[12] in each of the five experiments, supporting the interpretation that multiple secondary CFCs are derived from a single common human mammary repopulating cell or unit (human MRU). The frequency of MRUs calculated from these experiments was 1 per 1×10^3 to 1×10^4 mammoplasty cells, one to two orders of magnitude lower than the frequency of (primary) bipotent CFCs measured in the same original samples. From the frequency of MRUs determined and the total secondary CFC numbers measured, each MRU was found to generate, on average, 4.1 ± 0.6 daughter CFCs.

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

Because CD49f expression has been associated with basally located cells in the mouse mammary gland[13], we asked whether another marker of basal cells, CD10 (also called common lymphocyte leukemia antigen, or CALLA)[14], would be expressed on the human MRUs detected by our gel transplant assay. The results of two experiments showed that most secondary CFCs (70% in the first experiment and 86% in the second) originated from CD10$^+$ cells. However, 95 ± 3% of the CD49f$^+$EpCAM$^{neg–low}$ 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 CD49f^{+}EpCAM$^{neg–low}$CD31^{-}CD45^{-} cells (MRU-enriched and containing limiting numbers of MRUs) into a first set of mice. Four weeks later, we removed the gels, prepared single-cell suspensions from them and then plated 30% of each suspension in a CFC assay to identify primary gels that contained regenerated (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 regenerated CFCs also regenerated detectable CFCs in the secondary gels, indicating that MRUs had been regenerated in the primary gels (**Supplementary Fig. 1b**). Of note, similar assays of primary gels initiated with larger numbers ($\sim 1 \times 10^5$) of cells from other (that is, MRU-depleted) fractions produced few or no CFCs in secondary recipients.

DISCUSSION

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

We also show that the number of CFCs in 4-week-old structures serves as a sensitive and quantitative endpoint for human mammary stem cells in the original cell suspension assayed, and their detection as an endpoint avoids the difficulties associated with reliance on a histological approach. This concept is similar to the strategy commonly used to identify very primitive subsets of mouse or human hematopoietic cells referred to as long-term culture-initiating cells by virtue of their ability to generate hematopoietic CFCs detectable after 5–6 weeks in cultures containing stromal feeder layers[15]. 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[16,17]. 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[18]. Here we have also shown evidence of proliferative activity within the regenerated structures. In addition, for at least one of the mammary CFC types detected (the luminal-restricted CFCs), it was possible to show a clear difference in phenotype as compared with the cells that produced secondary mammary CFCs detectable 4 weeks later.

The ability to assay the *in vivo* mammary regenerative activity of dissociated cells is a major advance, as it enables the intrinsic developmental potential of individual cells to be investigated. It also provides renewed support for the concept that the full developmental properties of human mammary stem cells can be expressed in the absence of other cells in the epithelium, in keeping with similar findings for mouse MRUs[4,5].

EpCAM in the normal resting human breast is highly expressed by luminal epithelial cells and is less expressed by basal cells[19,20]. In contrast, CD49f (α_6 integrin) has an inverse pattern of expression[13]. Thus, the observed CD49f^{+}EpCAM$^{neg–low}$ phenotype of MRUs suggests a basal location of these cells *in situ*. Consistent with this expectation is the previous observation that most of the cells in the CD49f^{+}EpCAM$^{neg–low}$ fraction also express cytokeratin-14 (a myoepithelial marker) and not cytokeratin-19 (a luminal cell marker)[21]. In this regard, our present findings for human MRUs mirror those previously reported for mouse MRUs, which also show a basal phenotype[4,5]. In contrast, we find a marked difference between the phenotype of human MRUs and the reported CD49f^{+} EpCAM^{high} phenotype of HMECs that form branched structures in Matrigel[21], raising concerns that this Matrigel-based readout may not provide a useful surrogate assay for human mammary stem cells.

The assay described here should allow further enrichment of human MRUs to be achieved. It will also enable related studies of the biological properties and molecular regulation of MRUs of their ability to be transformed by specific oncogenes and of their relationship to cells that propagate various types of spontaneously arising human breast cancers. In this latter regard, it is noteworthy that CD49f is expressed by a subset of cells within the human MCF7 breast cancer cell line that have tumorigenic potential in immunodeficient mice[22]. 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 β2-microglobulin–null and NOD-SCID interleukin-2 receptor-γ_c–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_c–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 discard tissue from normal premenopausal women (ages 19–40) undergoing reduction mammoplasty surgery with informed consent according to procedures approved by the University of British Columbia Research Ethics Board and processed the tissue as previously described[23]. Briefly, we transported the tissue from the operating room on ice, minced it with scalpels and then dissociated it for 18 h in Ham's F12 and DMEM (1:1 vol/vol, F12 to DMEM, StemCell Technologies) supplemented with 2% wt/vol BSA (Fraction V; Gibco Laboratories), 300 U ml^{-1} collagenase (Sigma) and 100 U ml^{-1} hyaluronidase (Sigma). In some experiments, this medium was supplemented with 10 ng ml^{-1} epidermal growth factor (EGF, Sigma), 10 ng ml^{-1} cholera toxin (Sigma), 1 μg ml^{-1} insulin (Sigma), 0.5 μg ml^{-1} hydrocortisone (Sigma) and 5% FBS (StemCell Technologies). We obtained an epithelial-rich pellet by centrifugation at 80*g* for 4 min and cryopreserved it in 6% dimethylsolfoxide-containing medium at –135 °C until use. We subsequently prepared single-cell suspensions from freshly thawed pellets by treatment with 2.5 mg ml^{-1} trypsin supplemented with 1 mM EDTA (StemCell Technologies), washing once with HBSS (StemCell Technologies) supplemented with 2% FBS followed by treatment with 5 mg ml^{-1} dispase (StemCell Technologies) and 100 μg ml^{-1} DNase1 (Sigma), after which we passed the cell suspension through a 40-μm filter (BD Biosciences) to remove remaining cell aggregates.

To recover cells from the xenografted gels, we killed recipient mice and aseptically removed the gels from the kidneys under a dissecting microscope. We then dissected the gels for 4.5 h at 37 °C in EpiCult-B medium (StemCell Technologies) supplemented with 5% FBS, 600 U ml^{-1} collagenase and

200 U ml^{-1} hyaluronidase. After digestion, we washed the cells once and treated them for 5 min with prewarmed tryspin-EDTA with gentle pipetting.

***In vitro* mammary colony-forming cell assay.** We incubated 60-mm tissue culture dishes for 1 h at 37 °C with a 1:43 dilution of Vitrogen 100 collagen (Collagen Biotechnologies) in PBS (StemCell Technologies). We seeded each dish with test cells obtained from primary tissue or digested collagen gels combined with 2.0 × 10^5 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^{-1} hydrocortisone. We incubated cultures at 37 °C and 5% CO_2, with a change to serum-free EpiCult-B plus 0.5 μg ml^{-1} hydrocortisone 1 d later. In some experiments, we replaced the EpiCult-B medium with DMEM and F12 supplemented with 0.1% BSA, 10 ng ml^{-1} EGF, 10 ng ml^{-1} cholera toxin, and 1 μg ml^{-1} insulin. After 7–10 d, we briefly fixed dishes in a 1:1 vol/vol mixture of methanol and acetone at 20 °C, stained them with Wright's Giemsa (Sigma) and visually scored the colonies under a dissecting microscope. We routinely categorized colonies into subtypes as follows: tightly-clustered cells with smooth colony boundary, luminal; dispersed teardrop-shaped cells, myoepithelial; colony containing both these elements and a ragged colony boundary, bi-lineage; although, in some cases, colonies were stained for specific human cytokeratins and human mucin-1 (MUC1) to confirm the presence of either or both of these lineages.

Preparation and assessment of collagen gels. We prepared concentrated rat's tail collagen as previously described[24] and stored it at –20 °C. We thawed aliquots and neutralized the pH immediately before use by adding two parts (vol/vol) concentrated sodium hydroxide to 78 parts concentrated collagen solution and 20 parts 5× DMEM. To prepare gels, we collected C3H 10T$^1/_2$ mouse embryonic fibroblasts (a kind gift from G. Cunha) from subconfluent cultures, X-ray irradiated them (with 15 Gy), mixed them with dissociated human mammary cells and resuspended them in cold neutralized collagen. We added 25-μl aliquots containing 2.2 × 10^5 10T$^1/_2$ cells and the desired number of human test cells into individual wells of a 24-well plate. We allowed the gels to stiffen in a 37 °C incubator for 10 min and then incubated them in warm EpiCult-B medium plus 5% FBS for 50 min. We then kept the plates on ice until all gels had been transplanted. In some of the early experiments, we used cells from a telomerase-immortalized human adult mammary fibroblast line or primary human mammary fibroblasts instead of C3H 10T$^1/_2$ fibroblasts.

Subrenal xenotransplantation surgery. We shaved the hair on the backs of anesthetized mice and swabbed the skin with 70% alcohol. We made an anterior to posterior incision approximately 1.5 cm long dorsally around the area of the kidneys. We also made a small incision in the abdominal wall above one kidney and exteriorized the kidney by applying gentle pressure on either side. Under a dissecting microscope, we lifted the kidney capsule from the parenchyma with fine forceps and made a 2–4-mm incision in the capsule. We inserted up to three gels under the capsule with a fire-polished glass pipette tip. After suturing the incision in the abdominal wall, we repeated the procedure (if required) on the contralateral kidney. Finally, we inserted a slow-release pellet containing 2 mg β-estradiol and 4 mg progesterone (both from Sigma) in MED-4011 silicone (NuSil Technology) subcutaneously in a posterior position before suturing the midline incision. This protocol was previously shown to produce sustained serum levels of these hormones in the mouse approximately equivalent to those at the human midluteal phase peak[11]. In some experiments, we mated the mice 9 d after the gels were transplanted.

Cell separation. We preblocked mammary cell suspensions in HBSS supplemented with 2% FBS and 10% human serum (Sigma), and then labeled them with an allophycocyanin-conjugated rat antibody to human CD49f (clone GOH3, R&D Systems) and FITC-conjugated mouse antibody to human EpCAM (clone VU1-D9, StemCell Technologies). In some experiments, we also labeled hematopoietic and endothelial cells with biotin-conjugated mouse antibodies to human CD45 (clone HI30, Biolegend) and human CD31 (clone WM59, eBiosciences), respectively, followed by R-phycoerythrin–conjugated streptavidin (BD Biosciences). We added propidium iodide (Sigma) at 1 μg ml^{-1} for live/dead cell discrimination. We performed all sorts on either a FACSVantageSE or a FACSDiva (Becton Dickinson).

Immunohistochemistry. We processed deparaffinized 4-μm sections of paraformaldehyde-fixed collagen gels for immunohistochemistry with a Discovery XT automated system (Ventana Medical Systems). We applied primary antibodies to estrogen receptor-α (clone 6F11, Ventana), progesterone receptor (clone 1A6, Ventana), Ki67 (clone K2, Ventana), cytokeratin-14 (clone LL002, ID Labs), cytokeratin-18 (clone Ks 18.04, Progen), laminin (polyclonal, Sigma), collagen IV (clone col94, Sigma) and β-casein (clone F14.20, Harlan Laboratories). We then applied horseradish peroxidase–conjugated Discovery Universal Secondary Antibody (Ventana) and developed the slides with the 3,3′-diaminobenzidine (DAB) Map Kit (Ventana). We processed some slides manually with primary antibodies to MUC1 (clone 214D4, StemCell Technologies) or smooth muscle actin (polyclonal, Abcam), each followed by alkaline phosphatase-conjugated Envision-AP (DAKO) and developed in FastRed (Sigma). We counterstained all slides with hematoxylin. For dual-color staining of colonies, we fixed 60-mm culture dishes briefly in 1:1 vol/vol acetone and methanol and preblocked them in Tris-buffered saline containing 5% wt/vol BSA and 10% FBS. We then incubated the dishes sequentially with an unconjugated antibody to MUC1, alkaline phosphatase–conjugated Envision-AP, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development solution (Sigma), biotin-conjugated antibody to cytokeratin-14 (clone LL002, Labvision), horseradish peroxidase–conjugated streptavidin (Jackson Immunoresearch) and DAB.

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

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

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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. J.T.E. helped organize the accrual of the mammoplasty material used. C.J.E. conceptualized the study and finalized the writing of the manuscript.

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

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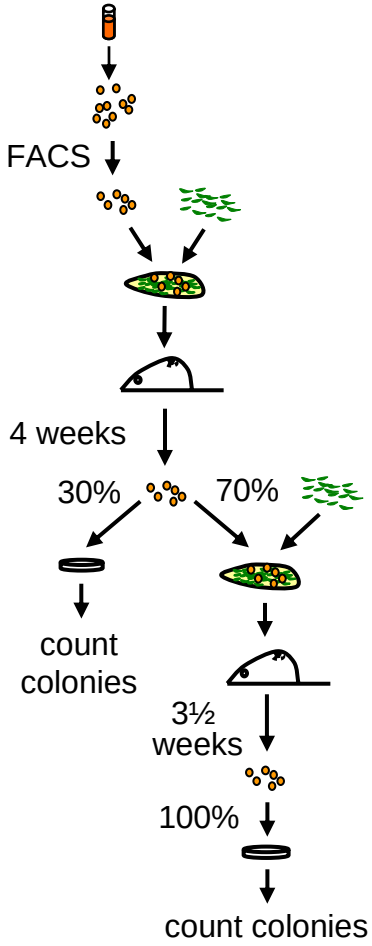
Expt no.	Recipient mouse strain	**Cells per gel**	**+ve gels/ total gels**	**MRU freq. in sample** (95% CI)	Chi squared test for consistency with 1-hit model	Average regenerated (secondary) CFCs per MRU	Primary CFC freq. in sample		
							lum	myo	bi-potent
				1 in	p=		1 in	1 in	1 in
#1	NS/IL-2Rγc$^{-/-}$	**3,000** **10,000** **25,000** **60,000**	**3/6** **5/6** **6/6** **6/6**	**4,890** (2,380-10,080)	0.99	3.7	46	706	145
#2	NS/IL-2Rγc$^{-/-}$	**1,500** **4,500** **12,000** **25,000**	**1/5** **6/6** **6/6** **6/6**	**2,220** (1,060-4,690)	0.45	5.9	38	1,500	59
#3	NS/IL-2Rγc$^{-/-}$	**500** **2,000** **7,000** **18,000**	**1/6** **5/6** **6/6** **6/6**	**1,390** (640-2,960)	0.87	2.3	151	1,059	87
#4	NS/B2m$^{-/-}$	**800** **3,000** **10,000** **22,000**	**0/6** **0/6** **3/4** **6/6**	**9,840** (4,910-19,700)	0.31	3.5	37	194	101
#5	NS	**2,000** **20,000**	**5/7** **7/7**	**1,600** (630-4,060)	1.00	5.2	43	4,000	4,000

Supplementary Table 1. Measurements of MRU frequency by limiting dilution analysis. Results from 5 experiments showing that the regeneration of CFCs in xenografted gels seeded with varying numbers of input human mammary cells 4 weeks previously fits a single hit model, indicating the origin of the CFCs from a single cell (the MRU) whose frequency can be calculated using Poisson statistics. The frequency of MRUs thus quantified was generally one to two orders of magnitude lower than the frequency of CFCs in the same initial sample. From the total number of CFCs detected in gels seeded with such derived numbers of MRUs, an average 4-week yield of 4.1 ± 0.6 secondary CFCs per input MRU was determined.

a



b

Exp't no.	Fraction *	Cells assayed	+ve / total 1ºgels (average CFC/gel)	+ve / total 2º gels (average CFC/gel)
1	B	1,280 12,800	**7/7** (15.0) **3/3** (59.0)	**6/7** (5.1) **3/3** (14.7)
	A+C	84,100	**3/3** (4.0)	**2/3** (1.0)
2	B	2,330 23,300	**7/8** (6.4) **3/3** (50.7)	**5/8** (2.2) **3/3** (61.7)
	A+C	74,800	**3/3** (2.0)	**1/3** (2.0)
3	B	1,430 14,300	**6/7** (3.0) **3/3** (30.0)	**5/6** (3.0) **2/3** (30.0)
	A+C	8,480 84,800	**1/8** (0.1) **0/3** (0.0)	**0/8** (0.0) **0/2** (0.0)

*Fraction A = $CD49f^{+}EpCAM^{+}$, B = $CD49f^{+}EpCAM^{neg–low}$, C = $CD49f^{–}$ all within the $CD45^{–}CD31^{–}$ fraction (see **Fig. 3b**)

Supplementary Figure 1. MRUs can be serially transplanted. (**a**) Experimental protocol. Cells isolated by FACS from the $CD49f^{+}EpCAM^{neg–low}CD31^{-}CD45^{-}$ (MRU-enriched) fraction or other fractions were transplanted into primary (1º) recipients. Four weeks later, 30% of the cells from each 1º gel were used to identify those that contained detectable CFCs. The remaining 70% of the cells were transplanted into secondary (2º) recipients. Another 3½ weeks later, CFC assays were performed on the cells harvested from these 2º gels. (**b**) Results. Data from 3 serial transplant experiments performed as described in (a) are shown. Both the frequency of gels implanted in 1º and 2º hosts in which at least 1 CFC was detected and, in brackets, the average number of CFCs in the assayed portion of 1º and 2º gels are indicated.

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, 4 weeks later, searching for regenerated human cells with *in vitro* 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[1–4]. 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[1,2]. 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[3,4]. 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[5], although how these cancer stem cell 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[6]. This has posed a significant challenge to the development of a transplantation assay protocol for human MRUs analogous to that used to assay mouse MRUs. One approach pioneered by Kuperwasser *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[7,8] or dissociated human mammary cells[9] are subsequently transplanted.

In this paper, we describe a different xenotransplant methodology[10,11] that we adapted from previous studies suggesting the ability of the subrenal capsule site to support the growth of implanted tissue fragments[12]. These earlier studies include the important work of Cunha and colleagues[13], 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 immune-deficient 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

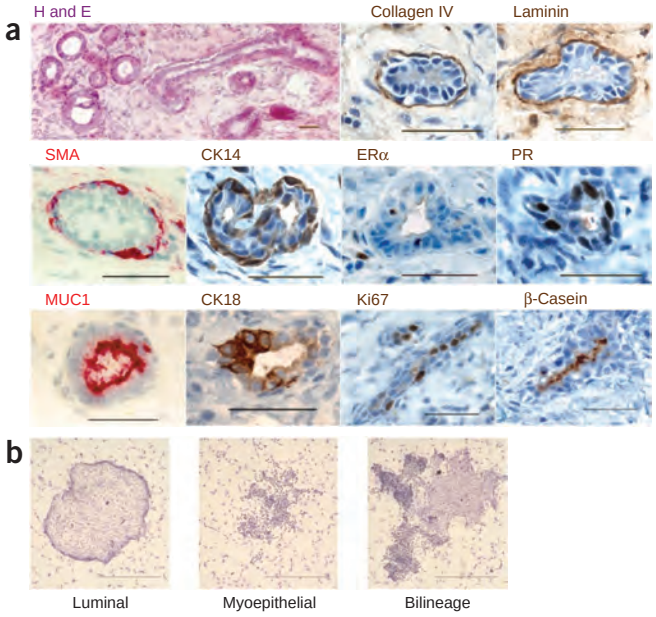


Figure 1 | Examples of human tissue regeneration using histological or CFC endpoints. (**a**) Hematoxylin and eosin (H and E) and immunostained sections of 4-week xenografts. From top left: H and E–stained section showing examples of round and elongated duct-like structures; immunostained sections using antibodies against basement membrane proteins collagen IV and laminin, basal cell markers smooth muscle actin (SMA) and cytokeratin-14 (CK14), hormone receptors estrogen receptor-α (ERα) and progesterone receptor (PR), luminal cell markers mucin 1 (MUC1) and cytokeratin-18 (CK18) and proliferation marker Ki67. The section at the bottom right is from a 4-week xenograft propagated in a donor mouse that was made pregnant 9 d after transplantation. Generation of milk is shown by immunostaining with an antibody against human β-casein. Scale bars, 50 μm. Reprinted with permission from ref. 11. (**b**) Examples of adherent colonies generated *in vitro* from CFCs present among cells dissociated from a 4-week xenograft. Left, pure luminal; middle, pure myoepithelial; right, bilineage. Scale bars, 1 mm.

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[1,11]. Nevertheless, exactly how analogous these two cell subpopulations are placed 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[11] (**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[11], 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[11] (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 transplants obtained 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[14].

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 10^3 to 1 in 10^4 cells and these MRUs are phenotypically distinct from many of the cells detectable *in vitro* as CFCs[11]. 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 gels) 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 mammoplasty 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[14] (see **Box 1** for more details). This requires initially transplanting

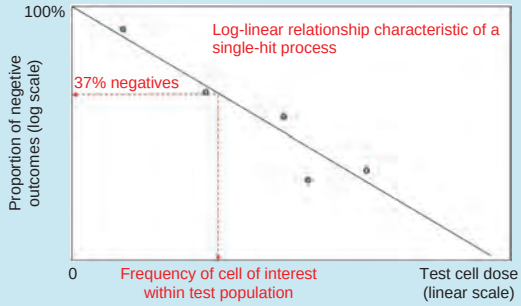
BOX 1 | LIMITING DILUTION ANALYSIS

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[14]. 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 daughter 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 http://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 χ^2-test or, more stringently, a likelihood ratio test derived from the generalized linear model[14].

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 xenografts.
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 18,000 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.

multiple replicate gels containing different numbers of cells and, 4 weeks later, measuring the proportion of positive (at least one secondary CFC detected) to negative (no secondary CFCs detected) gels for each initial cell dose tested. A generalized linear model assuming a single-hit Poisson process is then applied to calculate the frequency of MRUs and the range of uncertainty (confidence limits) associated with this frequency determination.

(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 CFC 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.

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 xenografted gels. 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*: perform 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[13] 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.*[7,8] to propagate human mammary cells. The differences include the transplant site (subrenal versus mammary fat pad), the endpoint 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 limiting numbers of dissociated input cells and both identify a cell of origin with a 'basal' $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$ phenotype[9]. 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-*Prkdc*^{scid}/SzJ; Jackson Laboratory) or related strains, e.g., NOD-SCID interleukin-2 receptor-γc null (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; Jackson Laboratory) ! **CAUTION** All animal experiments should adhere to appropriate national and institutional ethical guidelines.

Tissue

- Discarded tissue from human reduction mammoplasty ▲ **CRITICAL** Institutional review board approval is necessary, and appropriate procedures, including informed patient consent, should be followed ! **CAUTION** Primary tissue should be considered biohazardous and handled with appropriate biosafety precautions.

General reagents

- Inhalable anesthetic (Isoflurane; Baxter Healthcare Corporation)
- Buprenorphine analgesic (Temgesic; Reckitt Benckiser Pharmaceuticals) ! **CAUTION** Buprenorphine is classed as a narcotic and controlled substance. Adhere to all applicable regulations regarding security and record keeping.
- Meloxicam (injectable nonsteroidal anti-inflammatory; Boehringer Ingelheim)
- Bupivacaine hydrochloride (0.25% (vol/vol), Marcaine, local analgesic; AstraZeneca)
- Povidone iodine surgical scrub (Industrial and Scientific Supplies, cat. no. MD344)
- 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 mM EDTA; STEMCELL Technologies, cat. no. 07850)
- Collagenase-hyaluronidase solution (3,000 U ml^{-1} collagenase plus 1,000 U ml^{-1} hyaluronidase; STEMCELL Technologies, cat. no. 07912)
- Trypsin in citrate saline (STEMCELL Technologies, cat. no. 07400)
- Fetal bovine serum (FBS, 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. 05601) ▲ **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
- Mcllwain Tissue Processor (Lafayette Instrument Company)
- Isopropanol, 70% (vol/vol)
- NIH 3T3 fibroblasts
- NIH C3H10T1/2 fibroblasts
- DMEM medium (STEMCELL Technologies, cat. no. 36250)
- DMEM/F12 medium (STEMCELL Technologies, cat. no. 36254)
- Insulin (Sigma-Aldrich, cat. no. I6634)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)
- Bovine collagen, type 1 (STEMCELL Technologies, cat. no. 04902)
- DNase I (Sigma-Aldrich, cat. no. DN25)
- Dispase (STEMCELL Technologies, cat. no. 07913)
- Acetone (Fisher Scientific, cat. no. A18SK)
- Methanol (Fisher Scientific, cat. co. A412SK)
- Wright-Giemsa stain (Sigma-Aldrich, cat. no. WG32-1L)

For preparation of concentrated collagen

- Adult rat tails (Simonsen Laboratories or other supplier)
- Glacial acetic acid (Sigma-Aldrich, cat. no. A9967)
- Dulbecco's modified eagle medium, powder (DMEM; Gibco cat. no.12100)
- Concentrated sodium hydroxide (50% (wt/vol) in water; Sigma-Aldrich, cat. no. 415413) ! **CAUTION** It is highly caustic; avoid contact with skin or eyes
- Penicillin G and streptomycin solution (STEMCELL Technologies, cat. no. 07500)
- Amphotericin B antimycotic (Fungizone; Invitrogen, cat. no. 15290-018)

For preparation of slow-release hormone pellets

- Silicone elastomer (NuSil Technology, cat. no. MED-4011)
- β-Estradiol powder (Sigma-Aldrich, cat. no. E-2758) ! **CAUTION** 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.

Assay time	Subrenal method is shorter (approximately 5–6 weeks versus 8–10 weeks)
Data points per mouse	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
Number of surgical procedures	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[9]
Quantitation of MRU frequency	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**)
Analysis of structures in three dimensions	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
Technical difficulties	The humanized fat pat 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)
Heterogeneity of xenograft environment	Subrenal method involves test MRUs being evaluated in a homogeneous environment, because they are randomly dispersed amongst cosuspended fibroblasts. In contrast, Kuperwasser *et al.*[7,8] 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
Endpoint objectivity	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

EQUIPMENT

- Dissecting stereomicroscope (Zeiss SteREO Discovery, Zeiss) or surgical binocular loupes (Eschenbach, model no. 1636-3)
- Small animal hair clipper (Oster A5 with no. 40 blade)
- Surgical tools (autoclaved)
  Iris scissors (angled, 4½–5 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. 11009-13)
  Dumont 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. J433H)
- 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://bioinf.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 ìm and 40 µm; BD Falcon, cat. no. 352350, 352340)
- Hemocytometer

REAGENT SETUP

Concentrated collagen solution Following the method described by Nandi and colleagues[15], skin two rat tails using aseptic tools and techniques, cut each into three pieces, remove the tendons, and weigh 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% (vol/vol) mixture of glacial acetic acid in distilled sterile water supplemented with penicillin (to 100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and amphotericin B (2.5 µg ml^{-1}). Add a sterile stir bar, cover the flask opening with sterile aluminum foil, then with Parafilm. Leave the mixture stirring at 4 °C for 5–7 d. Centrifuge 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 20 µl of 5× 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 into cryovials and can be stored at −20 °C for up to 1 year.

DMEM (5×) 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% (vol/vol) FBS. Store at 4 °C for up to 3 months.

RBC lysis buffer RBC lysis buffer is a 4:1 (vol/vol) mixture of ammonium chloride solution:HF. 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 β-estradiol and 4 mg of progesterone, the method previously described by Laidlaw *et al.*[16] 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 in 96 mg of β-estradiol powder and 192 mg of progesterone 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. Detach the microscope slide from the chamber; thereafter, use a scalpel to cut the solidified rectangle of silicone/hormones into 48 pellets (each pellet will be ~4 mm × 5.5 mm × 1.25 mm). Store pellets in a sterile tube. These pellets are stable for 1 year 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 sash 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 hose and nose 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[11] (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 450*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 alternative strategy to render the fibroblasts proliferatively inert.

Preparation of collagen gels for xenografts ● TIMING 1–2 h

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 (2×10^5 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 mammoplasty 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 McIlwain 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% CO_2 equilibrated).
7. Dissociate 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 450*g* 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:

(i) Centrifuge at 80*g* for 30 s. After removing the overlying liquefied fat layer, the pellet will be highly enriched in epithelial organoids.

(ii) If the supernatant is transferred to a new 50-ml centrifuge tube and centrifuged at 200*g* for 4 min, a second pellet is obtained that contains variable numbers of epithelial cells, stromal cells and red blood cells.

(iii) Transfer the supernatant to a third 50-ml tube and centrifuge it at 450*g* for 5 min to obtain a pellet particularly enriched for human mammary fibroblasts, hematopoetic and endothelial cells.

(iv) 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 450*g* 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 450*g* 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, add one-tenth the volume of 1 mg ml^{-1} DNase I and triturate gently for 4 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 450*g* 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 aliquotting 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.

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_c 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**).

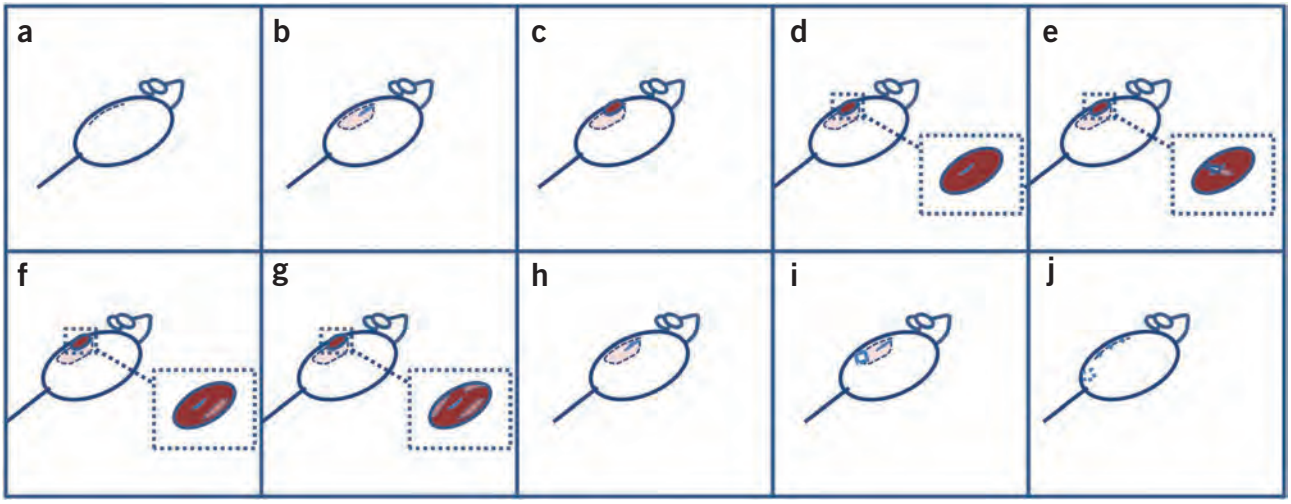


Figure 2 | Subrenal capsule surgery. Schematic of the steps undertaken to surgically implant collagen gels under the kidney capsule of mice. Full details are provided in the PROCEDURE section (Steps 13–28). (**a**) Make a dorsal incision in the skin; (**b**) make an incision in the body wall; (**c**) exteriorize the kidney; (**d**) make an incision in the kidney capsule; (**e**) insert the collagen 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 then suture together the incision in the body wall; (**i**) insert a hormone pellet; (**j**) suture together the incision in the skin.

▲ 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.

25| If required, repeat Steps 19–24 on the contralateral kidney.

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 × 4 mm × 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 450*g* 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.

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*[3,4]. 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 allows objective counting of up to ~200 colonies when appropriate numbers of mammary cells are seeded (~3×10^3 to 6×10^3 unsorted primary mammoplasty cells or ~0.5×10^3 to 2×10^3 cells after a few days of initial culture at higher cell densities). If CFC assay dishes are overplated 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.

Precoat 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 subconfluent 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. Remove the fixative and allow the dishes to air dry.
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.

? 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**.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
19	Bleeding under the kidney capsule	Too much pressure applied while exteriorizing the kidney	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
		Incision in the abdominal cavity is too small	
	Organs other than the kidney come out of the incision in the abdominal wall	Incision in the abdominal wall is not optimally positioned	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
22	Collagen gels are insufficiently firm to insert under kidney capsule	Collagen was not properly neutralized	Repeat titration to establish volumetric ratio of NaOH required to neutralize pH
		Collagen stock was too dilute	Prepare a fresh stock of collagen, using a lower volume of liquid per gram of rat tail tendon
28	Mice show signs of morbidity after surgery	This should not occur, but if so, it would indicate inadequate analgesia or surgical technique	Follow institutional policies for treatment and/or killing
32	Transplanted gels are not found under the kidney capsule after 4 weeks	Gels squeezed out from under the capsule while the kidney was initially returned into the abdominal cavity	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
37	No colonies are generated in secondary CFC assays	No MRUs were present among the cells transplanted	Repeat using a larger cell inoculum, and/or with a positive control population of unfractionated mammoplasty cells
		Problem with CFC assay reagents	Use an aliquot of primary (i.e., nontransplanted) mammary cells as a positive control for colony generation
	Contamination in CFC assays	Contamination acquired while retrieving gels from mice	Sterilize the outside of the killed mouse by spraying alcohol liberally. Use sterile instruments and aseptic technique throughout
		Contamination in tissue culture reagent(s)	

● TIMING

The protocol timing is depicted in **Figure 3**.
Steps 1 and 3, 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

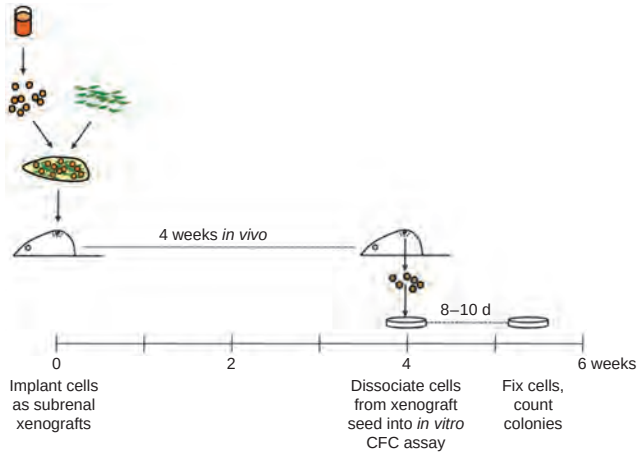


Figure 3 | Timeline for human MRU assay. Human mammary cells are combined with fibroblasts in small collagen gels, which are then implanted under the kidney capsule of highly immune-deficient, hormone-supplemented mice. Xenografts are removed after 4 weeks *in vivo*, and viable cells dissociated enzymatically. 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 (10^3–10^4 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 10^3–10^4 cells in preparations of previously frozen organoid-enriched primary normal human mammoplasty tissue samples, although with considerable intersample variability (an order of magnitude)[11]. When xenografts contain limiting or below-limiting numbers of MRUs (at 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 relative 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[1]. 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.

SUPPLEMENTARY FIGURE LEGEND

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.

References

1. Eirew,P. *et al.* A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability. *Nat. Med.* **14**, 1384-1389 (2008).

2. Hu,Y. and Smyth, G.K. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods.* **15**, 70-8 (2009).

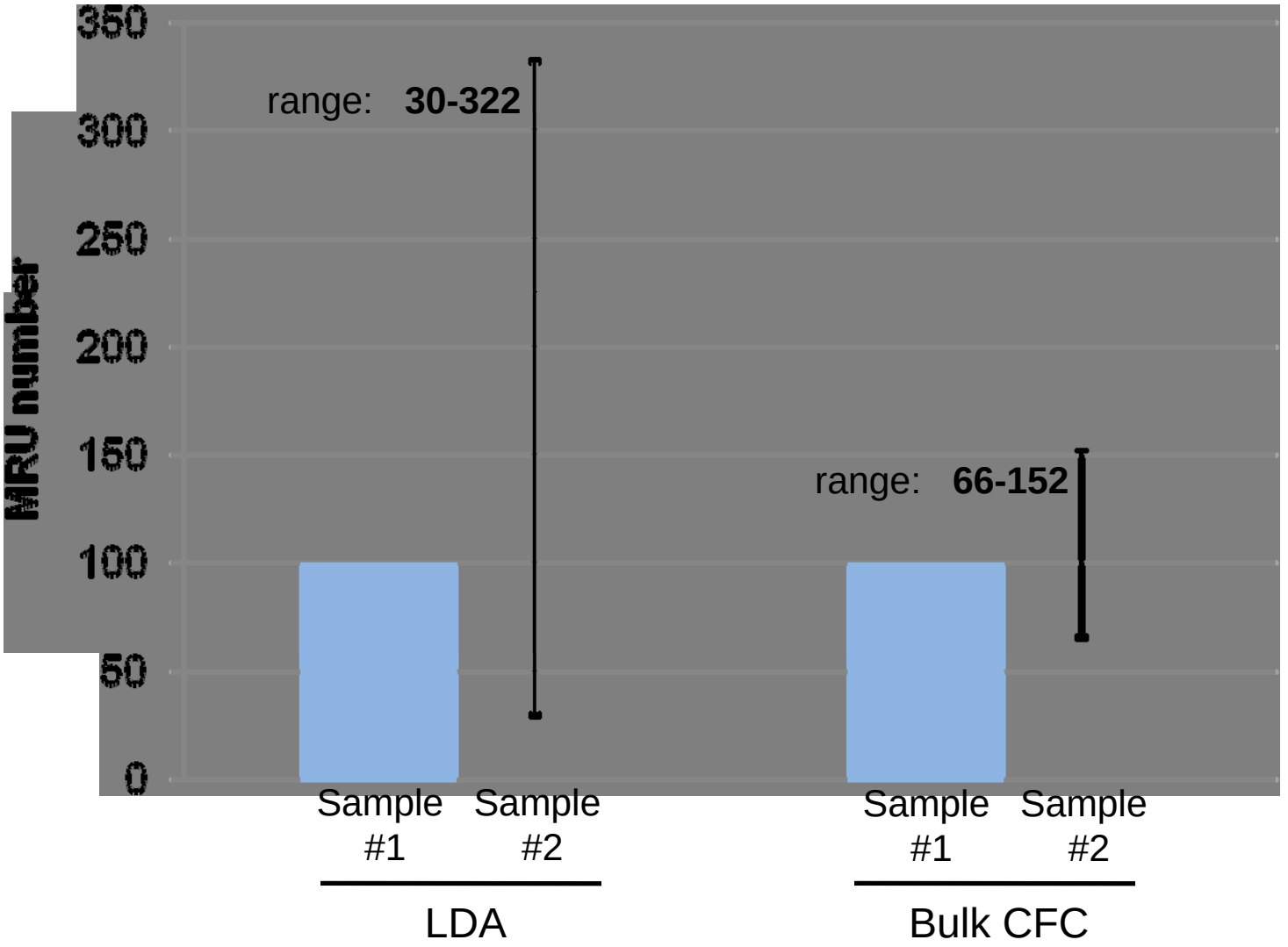
MRU number
350
300
250
200
150
100
50
0
range: 30-322
range: 66-152
Sample #1
Sample #2
Sample #1
Sample #2
LDA
Bulk CFC

Figure S1.