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14. Abstract

The mammary epithelium in normal adult female mice contains undifferentiated stem cells with extensive in vivo regenerative and self-renewal potential. Analogous cells presumably exist in the mammary glands of adult women, and may be the target cells for transforming mutations that lead to the evolution of breast cancer stem cells.
The objective of this grant is to develop a robust, reproducible and specific assay for normal human mammary stem cells, based on a recently-developed xenotransplant methodology. The assay will be applied to identify stem cell markers, develop a methodology to purify stem cells from normal adult breast tissue, and derive information about their frequency and regulation.
Considerable progress has been made in developing a stem cell assay in the first year of this grant. We have established conditions that allow human mammary tissue to be regenerated in subrenal xenografts in highly immunodeficient mice, starting with small innocula of dissociated human mammary cells. Furthermore, we have shown that measuring the number of progenitors present in xenografts after several weeks serves as a sensitive and objective "readout" for the presence of primitive stem-like cells among the transplanted cells, allowing this system to be used as a quantitative stem cell assay. Transplants of FACS-separated sub-populations show that these stem cells have a $CD49f_{high}$ $EpCAM_{-/low}$ $CD31_{-}CD45_{-}$ phenotype.
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.

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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". These may originate in normal tissue stem cells, and develop over time through the acquisition of serial genetic or epigenetic mutations. It is therefore critical to understand the mechanisms that regulate normal human mammary stem cells, and how dysregulation of these mechanisms can lead to the development of breast cancer stem cells. The objective of this training grant is to develop a robust and reproducible methodology to detect, quantify and isolate stem cells in normal human mammary tissue, using a xenotransplantation system. This will enable subsequent characterization of the regulation of these important cells and comparison with breast cancer stem cells. This report covers the first year of the grant, during which substantial progress has been made in the development and validation of the xenotransplantation assay. As a result, preliminary information on the phenotype of human mammary stem cells is emerging.

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[1-4]. A simplified depiction of this process is shown below, in which the two differentiated lineages (luminal, myoepithelial) are generated from stem cells via an intermediate compartment of shorter-lived, clonogenic progenitor cells. We refer to these progenitors as "colony-forming cells" (CFCs), because they are detected based on their ability to generate clonal adherent colonies when cultured in a 2-D tissue culture assay system. We and others have also recently identified and characterized stem cells in the *murine* mammary gland, and coined the operational term "mammary repopulating units" (MRUs) since these cells are detected based on their ability to regenerate an entire mammary tree when transplanted into the mammary stroma of a congenic recipient mouse.
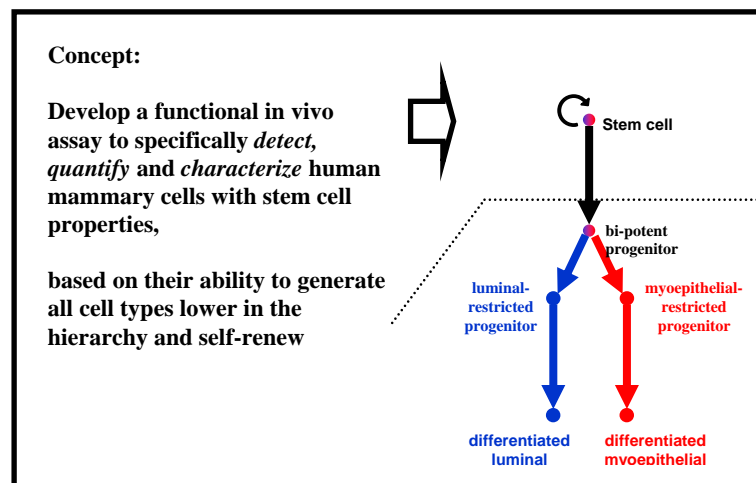
An analogous stem cell population is likely to exist in the *human* mammary gland. It is very important to understand the mechanisms that regulate these cells, investigate how the evolution of a dysregulated development/differentiation process can drive the initiation and progression of human breast cancers, and to identify specific stem cell markers and molecular pathways that can potentially form the basis of novel future 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 in normal human breast tissue. These are the objectives of this grant.

Our strategy to identify human mammary stem cells is based around a xenotransplant system, originally developed to propagate human mammary epithelial fragments[5] and recently adapted for use with dissociated mammary cell suspensions[6]. 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 by 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 on this project to date is presented in detail in the manuscript attached in the Appendix. The manuscript was submitted for review to Nature Medicine journal in October 2007. A later version, incorporating further data, has been accepted for publication in the journal. The scientific progress is summarized briefly below, with reference to the specific objectives in the Statement of Work.

Aim 1: Development and validation of a functional xenograft assay for human mammary stem cells

Experimental conditions have been established that optimize the generation of bi-lineage lobulo-ductal structures in immunodeficient mice, starting with dissociated suspension of cryopreserved human mammary epithelial cells. Furthermore, we have established that measurement of the number of regenerated CFCs in xenografts after a number of weeks in vivo serves as a sensitive and objective readout of the presence or absence of primitive MRUs present in the cells originally transplanted, allowing the frequency of MRUs in any population to be quantified by limiting dilution approaches. Secondary transplants have been carried out, demonstrating that MRUs can self-renew in vivo.

Two further validation steps are planned, to test the assumption that human tissue regenerated in this system is clonally derived from individual MRUs. Firstly, limiting dilution transplant series will be carried out, and the results tested statistically for consistency with a single-hit Poisson process. Secondly, direct tests of the shared clonal origin of multiple regenerated CFCs will be made (by viral marking and/or cell mixing experiments).

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Aim 2: Development of a robust and reproducible methodology for purifying mammary stem cells from normal primary human breast tissue

As an initial application of the MRU assay, we have begun to test candidate stell markers with the aim of identifying a phenotype that can be used to purify MRUs from adult mammary tissue. These experiments involve measuring the MRU content in various subpopulations that are FACS-sorted from reduction mammoplasty samples after staining with antibodies against various candidate stem markers. So far, we have identified an initial set of markers which are expressed by a large majority of MRUs, consistently across 8 mammoplasty samples. The phenotype combines high expression of CD49f (α-6 integrin), a low expression of Epithelial Cell Adhesion Molecule (EpCAM), and a lack of expression of hematopoietic and endothelial markers CD45 and CD31. Sorting by this phenotype allows MRUs to be purified by about 10-fold compared with unsorted cells. We are testing further candidate markers, with the aim of achieving higher isolation purities.

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

This later part of the project has not been tackled yet.

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 also had the opportunity to present this work orally at an international conference on mammary gland biology, as well as give poster presentations at this and other venues. Finally, I have been involved in the preparation of a primary research paper.

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

REPORTABLE OUTCOMES

1.   Peer reviewed papers

As of the end of the period covered by this report, a manuscript was under preparation. This was subsequently accepted (June 2008) for publication in Nature Medicine.

Peter Eirew, John Stingl, Afshin Raouf, Gulisa Turashvili, Sam Aparicio, Joanne T Emerman and Connie J Eaves. A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability. Nat Med. (in press)

2.   Invited talks

<u>Peter Eirew</u>, Characterization of normal human mammary stem cells using a xenotransplant model, delivered at the Gordon Conference on Mammary Gland Biology, Newport RI, July 2007

3.   Abstracts

<u>Peter Eirew</u>, John Stingl Afshin Raouf and Connie Eaves, Characterization of human mammary stem cells using a xenotransplant model, British Columbia Cancer Agency Conference, Vancouver BC, November 2006

<u>Peter Eirew</u>, John Stingl Afshin Raouf and Connie Eaves Characterization of normal human mammary stem cells using a xenotransplant model, Gordon Conference on Mammary Gland Biology, Newport RI, July 2007

CONCLUSION

The work to date on this project is very encouraging, and supports the use of this xenotransplant methodology as a robust and reproducible means to detect and characterize primitive human mammary cells (MRUs) with the hallmark features of stem cells (ability to generate both lineages, ability to generate progenitor CFCs, ability to self-renew). In particular, combining the transplant procedure with an endpoint "readout" of the number of regenerated progenitor CFCs detected after several weeks in vivo provides an objective, quantitative and practical way of assaying for MRUs in any given test populatoin. As a result this approach has allowed us, even at this early stage, to establish a preliminary set of human stem cell markers ($CD49f^+$ $EpCAM^{-/low}$ $CD31^-$ $CD45^-$).

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

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APPENDIX

Manuscript submitted to Nature Medicine in October 2006. A later version of this manuscript, incorporating further experimental data, has been accepted for publication in the journal.

A method for quantifying normal human mammary epithelial stem cells

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ABSTRACT

Previous studies have demonstrated that normal mammary tissue contains a hierarchy of cell types that are ultimately and continuously derived from a self-sustaining mammary stem cell population. However, a suitable assay for detecting, quantifying and characterizing such cells in humans has been lacking. Here we show that histologically normal, bi-layered mammary epithelial structures containing the complete repertoire of luminal and myoepithelial cells as well as their *in vitro* clonogenic progenitors are regenerated in a quantitative fashion in human mammary stem cell-containing collagen gels implanted for 4-6 weeks under the kidney capsule of hormone-treated immunodeficient mice. Using this assay, we show that these human mammary stem cells have a CD49f$^+$EpCAM$^{-/low}$ phenotype and functional self-renewal potential. The development and validation of this assay sets the stage for investigations of normal human mammary stem cell regulation and their relationship to human breast cancer stem cells.

INTRODUCTION

The adult mammary gland is composed of an inner layer of luminal epithelial cells surrounded

by an outer layer of contractile myoepithelial cells. A variety of studies of both mouse and

human mammary tissue indicate that these mature cells are in a state of constant turnover with

continuous replacement from more primitive progenitors. Some of these progenitors can be

detected as colony-forming cells (CFCs) in adherent, serum-free cultures containing epidermal

growth factor (EGF) and an irradiated fibroblast feeder layer[1]. In humans, luminal-restricted,

myoepithelial-restricted and bipotent cells have been clearly defined and distinguished as

separable subsets[1]. 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[2]. In these, virus integration site analysis was used to demonstrate

that some of the serially transplantable mammary structures produced were clonally derived.

More recently, we and the Visvader group showed that such mammary structures are generated

by single $CD49f^+CD29^+CD24^{lo}$ mammary cells that are relatively rare (~1 per 10^3 epithelial

cells) in the normal adult. To emphasize their identification based on a functional endpoint, we

coined the term "mammary repopulating units" (MRUs)[3,4]. We also showed that the

regenerative response of transplanted MRUs included the production of both daughter CFCs and

daughter MRUs - thus showing that the original MRUs recapitulate the entire mammary

epithelial cell hierarchy within each clonally regenerated structure.

In addition to the studies in mice, the existence 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[5], and from subsequent attempts to

generate human mammary glands from cells transplanted into highly immunodeficient mice.

One of these approaches has relied on colonizing the pre-cleared mammary fat pad of the recipients with human fibroblasts in an attempt to create an environment more conducive to the requirements of human mammary epithelial cells[6,7]. However, this protocol has lacked objective endpoints and is also very labor-intensive thus limiting its utility for quantifying and characterizing putative stem cells in test cell suspensions. An alternative strategy has been to suspend dissociated human mammary epithelial cells and irradiated fibroblasts in a collagen gel which is then implanted under the kidney capsule of estrogen and progesterone-treated NOD/SCID mice[8]. This latter approach is based on the finding that viable mammary glands could be maintained when whole fragments of human breast tissue (instead of dispersed cells) were implanted at this site[9]. We now show how this kidney implant protocol as modified for use with dissociated human mammary cell suspensions can be used as a quantitative assay for a rare subset of cells with the cardinal features expected of a mammary epithelial stem cell population.

RESULTS

Dissociated human mammary cells can regenerate histologically normal epithelial structures *in vivo*

Figure 1a shows the general experimental design used. Collagen gels containing dissociated single cell suspensions of normal human mammoplasty tissue plus irradiated mouse 10T½ fibroblasts were placed under the kidney capsule of hormone-supplemented non-obese diabetic severe combined immunodeficient (NOD/SCID) mice. From 2-8 weeks later, bi-layered structures typical of the normal human mammary gland were evident both in hematoxylin & eosin-stained preparations (Fig. 1b, upper panels) and in sections stained for Ki67, a marker of proliferating cells (Fig. 1b, lower right panel). The normal histology of these bilayered

structures was further supported by the demonstration that the outer layers stained positively for smooth muscle actin (SMA, Fig. 1b, lower middle panel) and cytokeratin 14 (data not shown), markers expressed by myoepithelial cells[10], and the inner layers expressed Muc1 (Fig. 1b, lower left panel) and cytokeratin 19 (data not shown), markers of mammary luminal cells. Such structures were obtained from every human mammoplasty sample tested when at least 10^5 cells were suspended in a given gel. Flow cytometric analysis of cells harvested from collagenase-treated gels removed from mice further showed that the structures generated contained the same complement of EpCAM$^+$ and CD49f$^+$ cells as primary mammoplasty tissue (Fig. 3d). Similar results were obtained when related strains of mice were used as hosts (i.e., NOD/SCID-*nu/nu*, NOD/SCID-*β2-microglobulin*-null, NOD/SCID-*IL-2 receptor-γ$_c$*-null mice) or when telomerase-immortalized human mammary fibroblasts were used instead of mouse fibroblasts (data not shown).

Production of CFCs in xenografts as an endpoint for detecting input human MRUs

The similarity between the cellular composition of the regenerated structures and normal human mammary tissue prompted us to investigate whether CFCs might also be detected amongst the cells present. Fig. 2 shows the design used for these experiments and the changes in total CFCs detected from 3 to 12 weeks after implanting the xenografts into mice. CFCs were consistently detected at all time points (Fig. 2b). Transplant cell dose-response experiments showed that the number of CFCs present after 4 weeks is linearly related to the number of human mammary cells originally suspended in the gels (Fig. 2c). Based on these results, we seeded additional gels with limiting numbers of cells and used the presence or absence of CFCs to determine the input MRU frequency using single hit Poisson statistics based on the assumption that the CFCs detected are

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the progeny of MRUs initially transplanted. Preliminary results indicate MRU frequencies in organoid-enriched human samples of 1 per 10^3-10^5 cells, and an average yield of 5 regenerated CFCs per MRU 4 weeks post transplant.

Interestingly, the colonies generated from the CFCs obtained from the 4 week-old xenografts included the same 3 types that are produced by CFCs present in freshly dissociated normal human mammoplasty samples and these were also produced in the same ratios (Fig. 2d). Representative examples of a pure luminal colony consisting of tightly-arranged $Muc1^+$ cells, a pure myoepithelial colony consisting of dispersed cytokeratin 14^+ cells, and a mixed colony containing both phenotypes all produced from regenerated CFCs are shown in Fig. 2e.

Human MRUs have a CD49f$^+$ EpCAM$^{-/low}$ phenotype

To investigate whether MRUs defined in this way have a consistent surface phenotype, we transplanted another series of 25 mice with subpopulations of cells from 8 different freshly dissociated human mammoplasty samples after staining the cells with antibodies to CD49f and EpCAM (Fig. 3a,b). In 5 of these experiments, contaminating hematopoietic ($CD45^+$) and endothelial ($CD31^+$) cells were also removed. Almost all grafts in which CFCs were detected 4 weeks later were those initiated with cells from the CD49f$^+$EpCAM$^{-/low}$ fraction (average 91±8% of CFCs detected) and this was consistent for all 8 mammary samples (Fig. 3c). In contrast, direct assays of the different types of CFCs present in the same fractions of primary cells showed that most of the luminal-restricted CFCs were confined to a distinct CD49f$^+$EpCAM^{high} subset (Fig. 3c). However, most of the bipotent and myoepithelial-restricted CFCs were co-enriched in the CD49f$^+$EpCAM$^{-/low}$ fraction.

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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 xenograft assays on cells harvested from primary grafts initiated with low numbers of $CD31^-$ $CD45^-CD49f^+EpCAM^{-/low}$ cells. In these experiments, 30% of the cells recovered from each primary gel were used to determine whether CFCs were present. The remaining 70% of the cells harvested from the primary gels were combined with fresh feeders and suspended in new gels which were then implanted into secondary recipients (Fig. 4a). In most cases, the primary gels that contained CFCs also contained MRUs, i.e., cells able to regenerate CFCs in secondary hosts (Fig. 4b). In contrast, primary gels seeded with the rest of the cells (i.e., everything except the $CD49f^+EpCAM^{-/low}$ population) which produced very few CFCs in primary gels also produced very few cells able to repopulate secondary gels.

DISCUSSION

Here we describe a robust and objective protocol for determining the frequency of normal human mammary epithelial cells that meet the rigorous definition of stem cells with *in vivo* regenerative potential and self-renewal activity demonstrable in secondary transplants. We also show that the structures produced in this assay protocol after 4 weeks are mostly derived from a rare subset of phenotypically distinct $CD49f^+ EpCAM^{-/low}$ cells that regenerate the complete hierarchy of primitive and mature cell types in the same proportions as found in the normal endogenous human mammary gland. Moreover, during the course of their production, the regenerated and

differentiating mammary cells self-organize to form a 3 dimensional mammary gland structure that appears similar to the tissue that is generated in adult females.

As a result, the number of CFCs present in 4 week-old structures serves as a sensitive and quantitative endpoint for human mammary stem cells in the original cell suspension assayed and avoids the difficulties associated with reliance on a histological endpoint. It is, in fact, similar in concept to the identification of a very primitive subset of murine or human hematopoietic cells referred to as long-term culture initiating cells because of their definition as cells that generate hematopoietic CFCs detectable after 5-6 weeks in cultures containing stromal feeder layers[11]. In the hematopoietic system, it was shown that the CFCs detected after 5-6 weeks must have originated from a more primitive cell type since the cells from which they derived had a different phenotype[12,13] and the CFCs present in the cultures were continuously proliferating and differentiating making simple persistence an unlikely explanation for their presence[14]. In the present experiments, evidence of proliferative activity within the regenerated structures was also demonstrated and for at least one of the CFC types detected (the luminal-restricted CFCs) it was possible to show a clear difference in phenotype from the cells that produced the CFCs detected 4 weeks later. The ability to assay the *in vivo* mammary regenerative activity of dissociated cells is an important 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 present in the epithelium, in keeping with similar findings for mouse MRUs[3,4].

EpCAM in the normal resting human breast is expressed at high levels by luminal epithelial cells and at lower levels by basal cells[15,16]. In contrast, CD49f (α6-integrin) has an inverse pattern of expression[17]. Thus the observed CD49f$^+$EpCAM$^{-/low}$ phenotype of MRUs

would be anticipated to be indicative of a basal location of these cells *in situ*. Consistent with this expectation is the observation by Villadsen et al[18] that most of the cells in the CD49f$^+$ EpCAM$^{-/low}$ fraction also express the keratin 14 (a myoepithelial marker) and not keratin 19 (a luminal cell marker). In this regard, our present findings for human MRUs mirror those previously reported for mouse MRUs which were also found to be more closely related phenotypically to the basally located myoepithelial cells[3,4]. On the other hand, our findings point to a marked difference in the CD49f$^+$ EpCAM$^{-/low}$ phenotype of human MRUs and the CD49f$^+$ EpCAM$^{+/hi}$ phenotype of human mammary cells that form branched structures in Matrigel[18], raising caution about using this Matrigel-based readout as a surrogate stem cell assay.

The assay described here should allow further enrichment of human MRUs to be achieved and related studies of their biological properties and molecular regulation, their ability to be transformed by specific oncogenes and their relationship to cells that propagate various types of spontaneously arising human breast cancers. In addition, we anticipate that the xenograft strategy that lies at the heart of this assay will provide a new system to investigate the mechanisms that control normal mammary cell proliferation and differentiation *in vivo* and their sensitivity to agents that promote or interfere with these processes.

METHODS

Animals.

Female NOD/SCID, NOD/SCID-*β2-microglobulin*-null, NOD/SCID-*interleukin-2 receptor-γ$_c$*-null and NOD/SCID-*nu-nu* mice were bred and housed at the animal facility at the British

Columbia Cancer Research Centre. Surgery was carried out on mice between the ages of 5-8

weeks.

Dissociation of human mammary tissue.

Anonymized discard tissue from normal premenopausal women (ages 19-40) undergoing

reduction mammoplasty surgery was collected according to procedures approved by the

University of British Columbia Research Ethics Board and processed as previously described[19].

Briefly, tissue was transported from the operating room on ice, minced with scalpels then

dissociated for 18 hours in Ham's F12/Dulbecco's Modified Eagle medium (1:1 v/v,

F12/DMEM, Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 2% w/v

bovine serum albumin (BSA, Fraction V; Gibco Laboratories, Grand Island, NY, USA), 300

U/ml collagenase (Sigma, St. Louis, MO, USA) and 100 U/ml hyaluronidase (Sigma). In some

experiments, this medium was supplemented with 10 ng/ml EGF (Sigma), 10 ng/ml cholera

toxin (Sigma), 1 µg/ml insulin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and 5% fetal bovine

serum (FBS, Stem Cell Technologies). An epithelial-rich pellet was obtained by centrifugation

at 80 x *g* for 4 minutes, and cryopreserved in dimethylsolfoxide-containing medium at -135ºC

until required. Single cell suspensions were subsequently prepared from freshly-thawed pellets

by treatment with 2.5 mg/ml trypsin supplemented with 1 mM EDTA (Stem Cell Technologies),

washing once with Hank's Buffered Salt Solution (Stem Cell Technologies) supplemented with

2% FBS, (referred to as HF) followed by treatment with 5 mg/ml dispase (StemCell

Technologies) and 100 µg/ml DNase1 (Sigma), after which the cell suspension was passed

through a 40 µm filter (BD Biosciences, Mississauga, ON, Canada) to remove remaining cell

aggregates.

To recover cells from the xenografted gels, recipient mice were sacrificed and gels aseptically removed from the kidneys using a dissecting microscope. Gels were then digested for 4.5 hours at 37ºC in EpiCult-B medium (StemCell Technologies) supplemented with 5% FBS, 600 U/ml collagenase and 200 U/ml hyaluronidase. Following digestion, the cells were washed once and treated for 5 minutes with prewarmed tryspin/EDTA with gentle pipetting .

In vitro **mammary CFC assay.**

60 mm tissue culture dishes were incubated for 1 hour @ 37ºC with 1.5 ml of a 1:43 dilution of Vitrogen 100 collagen (Collagen Biotechnologies, Palo Alto, CA, USA) in phosphate buffered saline (Stem Cell Technologies). Each dish was seeded with test cells obtained from primary tissue or digested collagen gels combined with 2.0 x 10^5 freshly-defrosted previously irradiated (@ 50 Gy) NIH 3T3 murine fibroblast cells in 4 ml of EpiCult-B medium supplemented with 5% FBS and 0.5 μg/ml hydrocortisone. Cultures were incubated at 37ºC and 5% CO_2, with a change to serum-free EpiCult-B plus 0.5 μg/ml hydrocortisone 1 day later. In some experiments, the Epicult-B medium was replaced by DMEM/F12 supplemented with 0.1% BSA, 10 ng/ml EGF, 10 ng/ml cholera toxin, and 1 μg/ml insulin. After 7-10 days, dishes were fixed briefly in 1:1 v/v mixture of methanol:acetone at room temperature, stained with Wright's Giemsa (Sigma), and colonies were scored visually under a dissecting microscope. Colonies were routinely categorized 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.

Preparation of collagen gels.

Concentrated rat's tail collagen was prepared as previously described[20] and stored at -20ºC. Aliquots were subsequently defrosted and neutralized immediately before use by mixing in proportions 78% collagen, 20% 5x DMEM and 2% concentrated sodium hydroxide. To prepare gels, 10T½ mouse embryonic fibroblasts (a kind gift from G Cunha, University of California, San Francisco, CA, USA) were harvested from sub-confluent culture, x-irradiated @ 15 Gy, mixed with dissociated human mammary cells and resuspended in cold neutralized collagen. 25 µl aliquots, containing 2.2×10^5 10T½ cells and the desired number of human test cells, were aliquoted into individual wells of a 24-well plate. The gels were allowed to stiffen in a 37ºC incubator for 10 minutes and then floated in warm EpiCult-B plus 5% FBS in the incubator for a further 50 minutes. The plates were kept onto ice until surgery. In some experiments, cells from a telomerase-immortalized human adult mammary fibroblast line were used instead of 10T½ fibroblasts.

Subrenal xenotransplantation surgery.

The hair on the back of anesthetized mice was shaved, and the skin swabbed with 70% alcohol. An anterior to posterior incision approximately 1.5 cm was made dorsally around the area of the kidneys. A small incision was made in the abdominal wall above one kidney, and the kidney exteriorized by applying gentle pressure either side. Viewing under a dissecting microscope, the kidney capsule was lifted from the parenchyma using fine forceps and a 2-4 mm incision made in the capsule. 1-4 collagen gels were inserted under the capsule using a fire polished glass pipette tip. After suturing the incision in the abdominal wall, the procedure was repeated if required on the contralateral kidney. Finally, a slow-release pellet containing 2 mg β-estradiol and 4 mg

progesterone (both from Sigma) in MED-4011 silicone (NuSil Technology, Carpinteria, CA, USA) was positioned subcutaneously in a posterior position before suturing the midline incision.

Cell Separation.

Mammary cell suspensions were pre-blocked in HF supplemented with 10% human serum (Sigma), then labeled with allophycocyanin-conjugated rat anti-human CD49f antibody (clone GOH3, R&D systems, Minneapolis, MN, USA) and fluorescein isothiocyanate-conjugated mouse anti-human EpCAM antibody (clone VU1-D9, Stem Cell Technologies). In some experiments, hematopoietic and endothelial cells were also labeled with biotin-conjugated mouse anti-human CD45 antibody (clone HI30, Biolegend, San Diego, CA, USA) and biotin-conjugated anti-human CD31 antibody (clone WM59, eBiosciences, San Diego, CA, USA), respectively, followed by R-phycoerythrin-conjugated streptavidin (BD Biosciences). 1µg/ml propidium iodide (Sigma) was added at 1 µg/ml as for live/dead cell discrimination. All sorts were performed using either a FACSVantage or a FACSDiva (Becton Dickinson [BD], San Jose, CA, USA).

Immunohistochemistry.

Deparaffinized 5 µm sections of collagen grafts were pre-blocked with 10% goat serum (Sigma), then incubated with mouse anti-human Muc1 antibody (clone 214D4, StemCell Technologies), polyclonal rabbit anti-human smooth muscle actin antibodies (Abcam, Cambridge, MA, USA) or mouse anti-human Ki-67 antibody (BD), followed by incubation in alkaline phosphatase-conjugated polymers Envision-AP (DAKO, Mississauga, ON, Canada) or UltraVisionONE

(Labvision, Fremont, Ca, USA). Slides were developed in FastRed (Sigma) or, for Ki67

staining, with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma)

with counterstaining with Nuclear Fast Red (Biomeda, Foster City, CA, USA). For dual colour

staining of colonies, 60 mm culture dishes were washed with phosphate buffered saline, fixed

briefly in 1:1 v/v acetone:methanol, and pre-blocked in tris-buffered saline containing 5% w/v

BSA and 10% FBS. Plates were incubated in mouse anti-human Muc1 (clone VU1-D9, Stem

Cell Technologies) followed by alkaline phosphatase-conjugated UltraVisionONE and

developed in BCIP/NBT. Dishes were blocked again in 5% BSA and 10% FBS, incubated for

10 minutes in biotin-conjugated mouse anti-human cytokeratin 14 (clone LL002, Labvision,

Cheshire, UK) followed by horseradish peroxidase-conjugated streptavidin (Jackson

Immunoresearch, West Grove, PA, USA) and finally developed using 3,3'-diaminobenzidine

(Sigma). All incubations were for 30 minutes at room temperature, unless otherwise specified.

Statistical analysis.

Data are expressed as mean ± SEM.

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. J.E. helped organize the accrual of the mammoplasty material used. C.E.

conceptualized the study and finalized the writing of the manuscript.

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Competing interests.

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1: Organized structures are generated *in vivo* from single cell suspensions of primary human mammary cells. (**a**) Single cells obtained by enzymatic dissociation of normal human reduction mammoplasty samples were combined with irradiated fibroblasts in a collagen gel as described in the 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**) Sections of 4-week xenografted gels: upper left (low magnification) and right (high magnification), after staining with hematoxylin and eosin (H&E); lower left, after immunostaining using an anti-Muc1 antibody; lower centre, after immunostaining using anti-smooth muscle actin antibodies; lower right, after immunostaining using an anti-Ki67 antibody (blue) and nuclear red counterstain. Bars = 50 µm.

Figure 2: CFC production as an *in vivo* indicator of human MRU repopulating activity.
(**a**) Experimental protocol (as in Fig. 1 using CFC output as the endpoint). (**b**) Number of CFCs per gel after various times *in vivo*. The figure legend shows the number of human cells transplanted per gel in each experiment. (**c**) CFC output in the gels after 4 weeks is linearly related to the number of human cells transplanted per gel (mean ± SEM from 6 gels per cell dose in a representative experiment). (**d**) The distribution of different types of CFC in structures regenerated in gel xenografts is similar to the distribution of these cells in normal breast tissue (data averaged from 6 samples). (**e**) Colonies generated from 4-week xenografts after immunostaining with anti-Muc1 (blue) and ant-cytokeratin-14 (brown) antibodies. Shown are

representative examples of uni-lineage luminal (upper left), uni-lineage myoepithelial (upper right) and bi-lineage (lower) colonies obtained in these assays. Bars = 1 mm.

Figure 3: MRUs are CD49f$^+$EpCAM$^{-/lo}$. (**a**) Experimental protocol. (**b**) FACS profile of input human mammary cell preparations (depleted of CD45$^+$ and CD31$^+$ cells) showing gates used to select fractions assayed for MRUs and CFCs. (**c**) Solid circles show the proportion of all xenograft-derived CFCs in gels that originated from cells in the original CD49f$^+$EpCAM$^{-/lo}$ fraction (B). Open symbols show the distribution of the CFCs detected directly in the same fractions of the same 8 starting samples. (**d**) FACS profile of cells obtained from a 4-week gel xenograft initiated with CD49f$^+$EpCAM$^{-/lo}$ cells.

Figure 4:   MRUs can be serially transplanted. (**a**) Experimental protocol: 30% of the cells from each 1° gel were assayed directly for their CFC content. The remaining 70% of the cells were transplanted into 2° recipients and CFC assays performed on cells harvested from these gels when they were removed another 3½ weeks later. (**b**) Proportions of gels implanted in 1° and 2° hosts in which at least 1 CFC was detected in the portion of the gel assayed. The fractions indicated correspond to those shown in Fig. 3b.
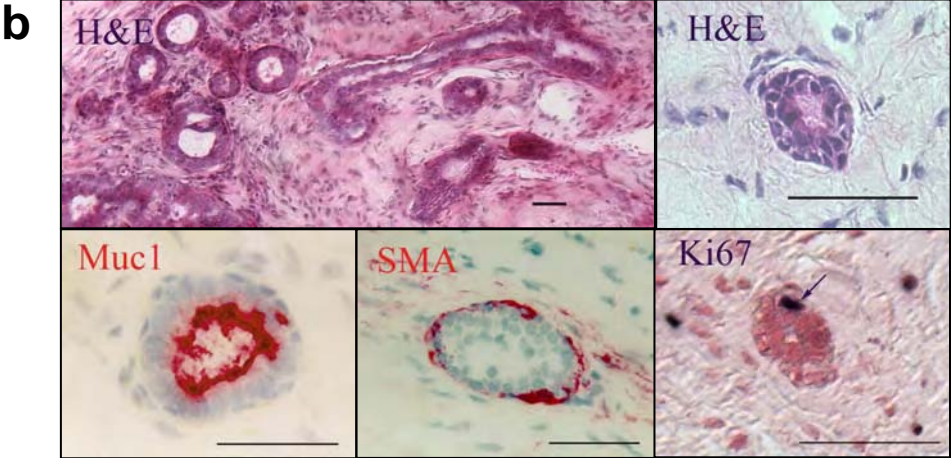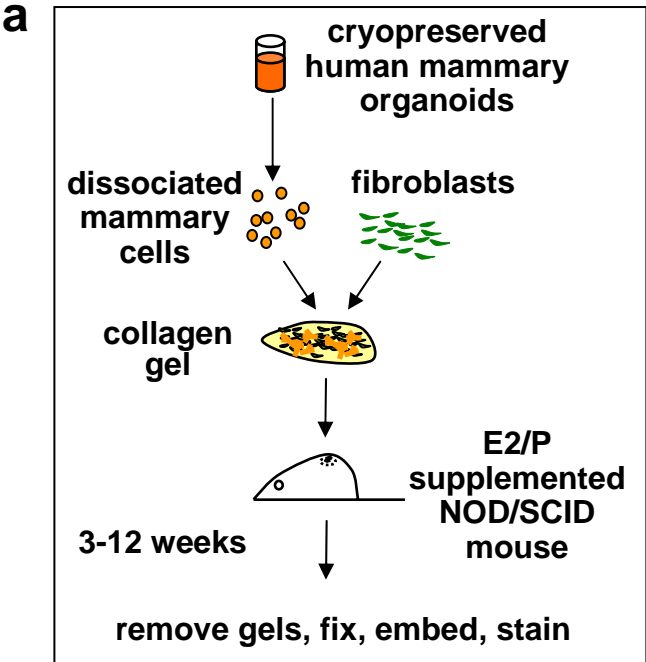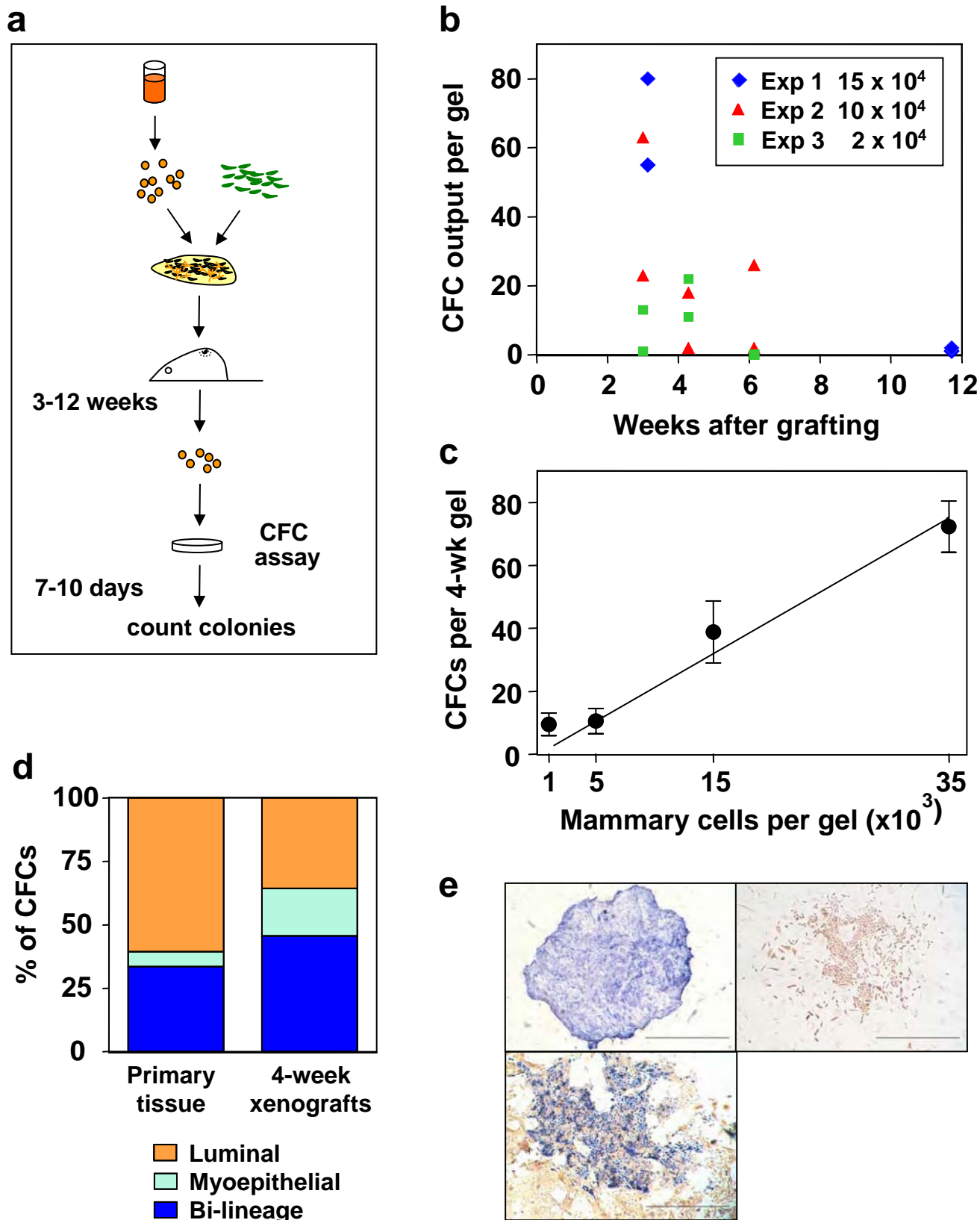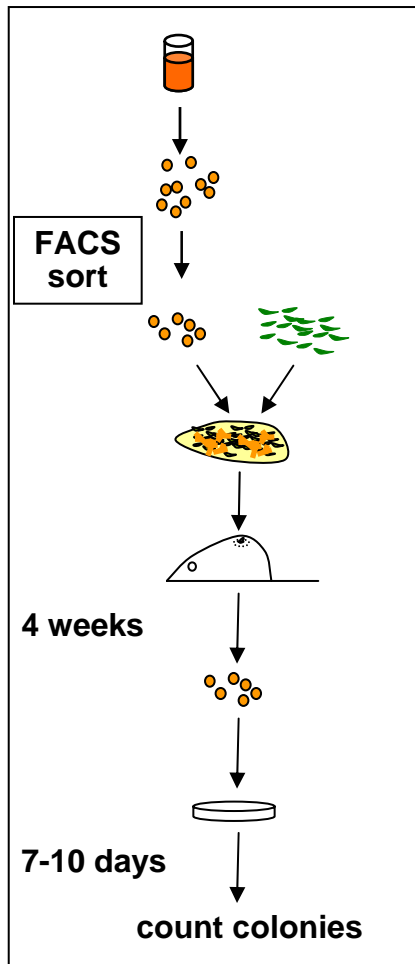
Figure 1

Figure 2

a

3-12 weeks

7-10 days

CFC
assay

count colonies

b

Legend:
- Exp 1 15×10^4
- Exp 2 10×10^4
- Exp 3 2×10^4

CFC output per gel

Weeks after grafting

c

CFCs per 4-wk gel

Mammary cells per gel ($\times 10^3$)

d

% of CFCs

Primary
tissue

4-week
xenografts

- Luminal
- Myoepithelial
- Bi-lineage

e

Figure 3

Figure 4

a

FACS sort

4 weeks

30% 70%

count colonies

3½ weeks

100%

count colonies
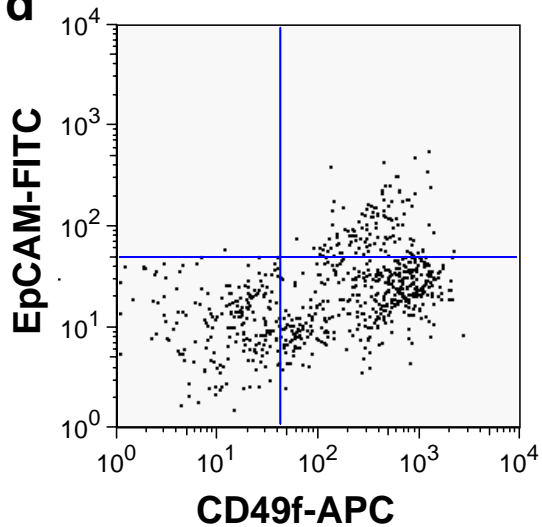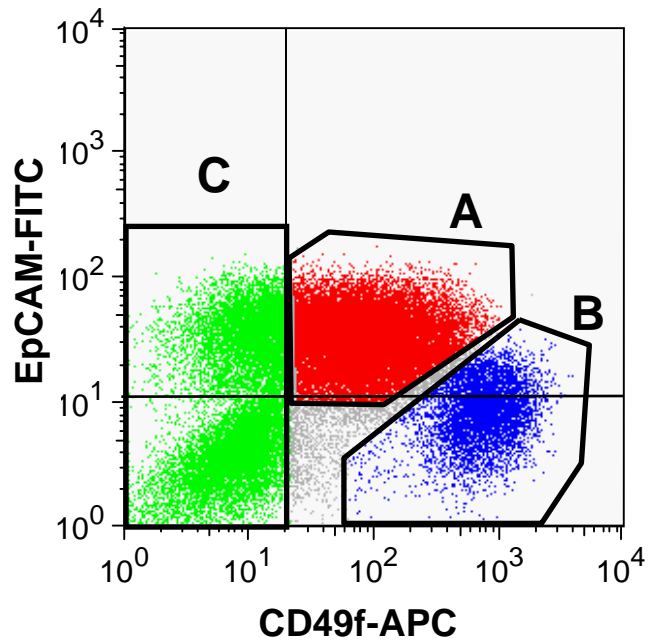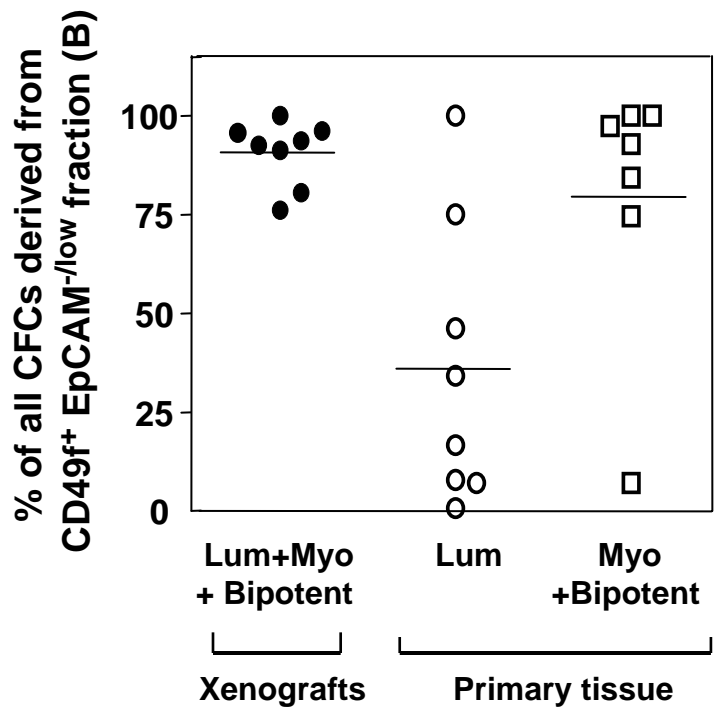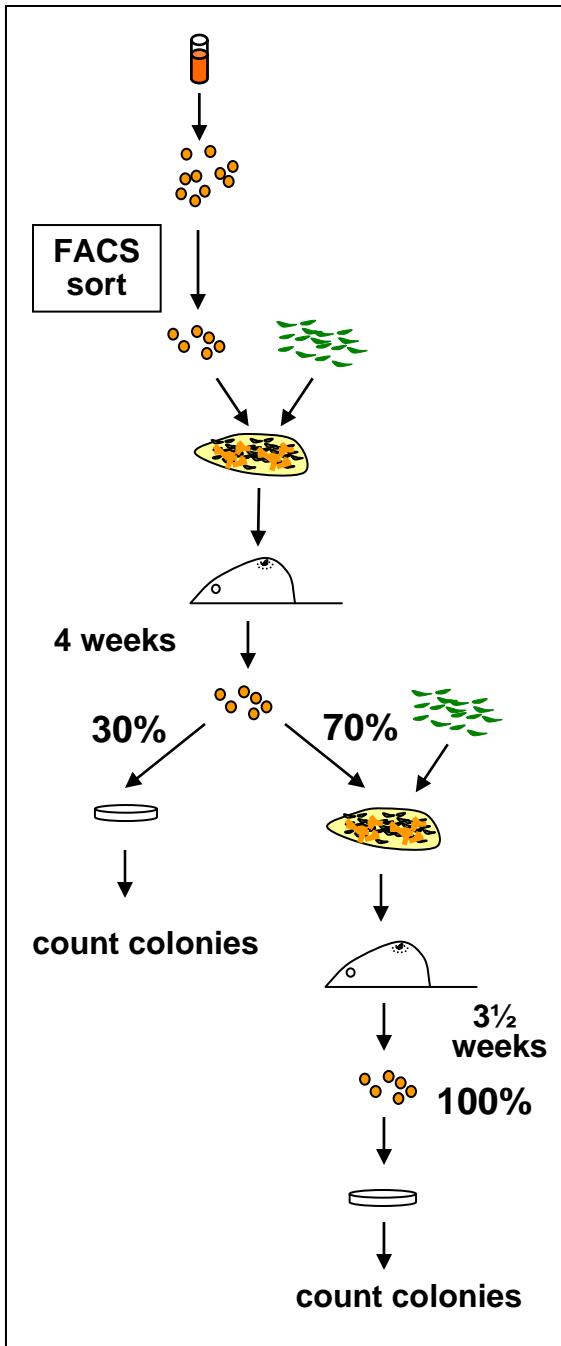
b

Fraction assayed	Cells per gel	+ve gels/ total 1° gels	+ve gels/ total 2° gels
CD49f$^+$ EpCAM$^{-/lo}$ (B)	1,280	7/8	5/8
	12,800	3/3	3/3
CD49f$^+$ EpCAM$^+$ (A) & CD49f$^-$ (C)	84,100	3/3	1/3