Clonal Analysis of Mouse Mammary Luminal Epithelial Cell Transplants

Long V. Nguyen,¹ Maisam Makarem,¹ Nagarajan Kannan,¹ Annaick Carles,²
Sneha Balani,¹ Michelle Moksa,² Martin Hirst,²⁻⁴ and Connie J. Eaves^{1,5}

MUCH EFFORT HAS BEEN DEVOTED recently to describing the range of growth and differentiation activities that can be elicited from different phenotypes of normal mammary cells in various conditions [1]. In mice, different genetic lineage-tracing studies have provided evidence of variable self-sustaining properties of cells in the basal and luminal compartments. However, when these cells are prospectively isolated, many mouse basal cells can individually regenerate an entire multibranched mammary gland [1]. In contrast, equivalent regenerative activity has rarely been documented for mouse luminal cells [2], although an ability for it to be activated *in vitro* has been demonstrated [3]. In this study, we demonstrate the utility of cellular barcoding to define the contributions of individual purified luminal cells to the regeneration of two complete gland structures obtained in two cleared fat pads.

EpCAM⁺⁺CD49f^{low/-} (luminal) cells were isolated by fluorescent-activated cell sorting (FACS) from normal virgin

adult female C57Bl/6J mouse mammary glands (Fig. 1A), transduced at 40% efficiency with a library of DNA-barcoded green fluorescent protein-encoding lentiviruses, and 1.3×10^5 cells injected with Matrigel into a single cleared fat pad in each of two mice (Fig. 1B) as previously described for mouse basal cell transplants [4]. The purity of the transplanted cells determined by reanalyzing an aliquot of the sorted cells showed >98% still displayed a luminal phenotype, with a <0.2% chance of being contaminating basal (EpCAM^{low}CD49f⁺) cells that could subsequently contribute to clone formation.

After 8 weeks, whole-mount microscopy revealed complete branched GFP⁺ structures had been regenerated in both mice (Fig. 1C). Isolation of the EpCAM^{low}CD49f⁺ basal cells, EpCAM⁺⁺CD49f^{low/-}Sca1⁻ luminal progenitor cells, and EpCAM⁺⁺CD49f^{low/-}Sca1⁺ terminally differentiated luminal cells from the enzymatically dissociated fat pads (Fig. 1A) revealed that these three subsets were

FIG. 1. (A) FACS plot showing the initial EpCAM⁺⁺CD49f^{low/-} mouse mammary luminal epithelial cells (red circle) that were barcoded and subsequently transplanted. The protocol for enzymatic dissociation and FACS antibodies used are as previously described [4]. The same FACS strategy was used to isolate cells from regenerated glands after 8 weeks *in vivo*. EpCAM^{low}CD49f⁺ basal cells (blue), EpCAM⁺⁺CD49f^{low/-}Sca1⁻ luminal progenitor cells (red), and EpCAM⁺⁺CD49f^{low/-}Sca1⁺ terminally differentiated luminal cells (dark yellow). (B) Flow diagram of the experimental design. Isolated EpCAM⁺⁺CD49f^{low/-} mouse mammary luminal epithelial cells were exposed to a large library of barcoded and GFP-encoding lentiviruses for 4 h followed immediately by their transplantation into the cleared fat pads of prepubertal C57Bl/6J mice [4]. After 8 weeks the regenerated glands were retrieved and dissociated into single-cell suspensions and sorted by FACS into the three subsets indicated in (A). (C) Whole-mount microscopy revealed complete branching GFP⁺ structures had been regenerated after 8 weeks *in vivo*. Scale bar = 1 mm. (D) Table showing the total number of basal, luminal progenitor, and differentiated luminal cells obtained from the regenerated glands. An aliquot of 100 cells was taken from each subset of cells and assayed for CFC activity, as previously described [4]. The CFC frequency in percentage is shown with the mean $\pm$ SD. (E) A heatmap depiction of all 23 clones detected by sequencing the barcodes introduced. The 23 clones are aligned on the horizontal axis, with the vertical axis showing whether they contained basal, luminal progenitor, and/or differentiated luminal cells (blue, red, and yellow, respectively). A gray box means no cells of that type were detected. (F) A ternary plot showing the relative proportions of basal cells, luminal progenitors, and differentiated luminal cells within individual clones, color coded according to whether the clone was bilineage (purple), basal only (blue), or luminal only (red). (G) Distribution of the sizes of bilineage clones (purple), clones of exclusively basal cells (blue), and clones of exclusively luminal cells (red, shown as a percent of the total number of clones of that type). CFC, colony-forming cell; FACS, fluorescent-activated cell sorting; FSC, forward scatter; GFP, green fluorescent protein; SD, standard deviation. Color images are available online.

¹Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada.

²Michael Smith Laboratories, University of British Columbia, Vancouver, Canada.

³Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada.

⁴Canada's Michael Smith Genome Sciences Centre, British Columbia Cancer Agency, Vancouver, Canada.

⁵Department of Medical Genetics, University of British Columbia, Vancouver, Canada.

present in similar proportions in both fat pads (>2-fold more Sca1[−] and Sca1⁺ luminal cells than basal cells, with the Sca1[−] luminal progenitor fraction more prominent; Fig. 1D), despite a 10-fold difference in the total number of mammary cells recovered from each fat pad (3.1×10^4 vs. 3.2×10^3). A normal adult mammary gland would have a higher proportion of differentiated luminal cells compared with luminal progenitor cells [2], suggesting these regenerated glands may be less mature.

The colony-forming cell (CFC) content in each harvested subset was also largely restricted to the basal and luminal progenitor fractions (frequencies of ~36% and ~25%, respectively), compared with the EpCAM⁺⁺CD49^{low}−Sca1⁺ cells (~14%; Fig. 1D). Thus, despite large differences in the overall size of the glands generated from the transplanted cells, their progeny distributions were similar to one another, and also to those found in the normal adult mouse mammary gland [3].

DNA sequencing and barcode analysis [4] detected 14 and 9 clones in the 2 regenerated structures (Fig. 1E). FACS isolation of the different phenotypes in each structure before DNA extraction from them showed that 10 clones (7 and 3 clones in the 2 regenerated structures) were bilineage and had a mean size of $\sim 2 \times 10^3$ cells each. Five clones contained only basal cells and eight only luminal cells (Fig. 1F) with smaller mean sizes of ~300 and ~500 cells per clone, respectively; Fig. 1G).

These findings illustrate the power of cellular barcoding to analyze the clonal outputs of cells with limited growth potential—here applied to rare ($\sim 10^{-4}$) transplantable cells isolated from the luminal compartment of the normal adult mouse mammary gland.

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Address correspondence to:

Dr. Connie J. Eaves

Terry Fox Laboratory

British Columbia Cancer Agency

Vancouver, BC V5Z 1L3

Canada

E-mail: ceaves@bccrc.ca

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