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Using Short-term Transplantation

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| 14. ABSTRACT <b>Background:</b> Prevailing models maintain that stem cells comprise a minority of epithelial cells. However, some data suggest the percentage of mammary stem cells may be underestimated using common assays. <b>Rationale:</b> Short term transplantation using fragments of mammary duct offer an opportunity to test the prevailing stem cell model. If division-competent stem cells represent a small percentage of all epithelial cells, the initial rate of cell division in transplanted fragments should be low. However, if stem cells can include more differentiated, yet division-competent cells, the initial rate of cell division in fragments should be high <b>Objectives:</b> 1) To determine the range of mammary stem cell types participating in gland regeneration. 2) To develop the short-term transplantation assay as a means by which critical regulators of stem and progenitor cell behavior can be discovered and evaluated. <b>Relevance:</b> Studies will provide a direct test of prevailing stem cell models. |             |                         |                            |                                               |                                           |
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## Introduction

Normal mammary epithelial stem cells are long-lived, self-renewing, and give rise to all progenitor and differentiated epithelial cell types. They are also hypothesized to be the cell of origin for breast cancer (Clarke et al., 2003). In breast cancer, it is thought that a sub-population of “cancer stem cells” having self-renewal and differentiation mechanisms similar to those of normal stem cells, might be resistant to common therapies, and therefore be responsible for treatment failure and disease recurrence. Thus, it is essential to define the full range of division-competent stem/progenitor cell types in the mammary gland and to understand their regulation for successful treatment of breast cancer.

Normal mammary stem cells are distributed throughout the ductal tree. Limiting dilution transplantation and *in vitro* “mammosphere” forming assays using dissociated epithelial cells suggest stem cells express CD29 and CD24 or CD49f and CD24 and represent about 1 in ~1,400 cells (Shackleton et al., 2006; Stingl et al., 2006). Coupled with electron microscopy (EM) studies showing only three division-competent epithelial cell types in the intact mouse mammary gland, these data support prevailing models in which division-competent stem/progenitor cells comprise only a minority of epithelial cells (Smith and Boulanger, 2003).

In conflict with such models, other data suggest the true percentage of division-competent stem/progenitor cells in the mammary gland may be dramatically underestimated using common assays. For example, cell division in mature ducts and in the terminal end bud, a structure enriched in stem/progenitor cells, is dependent on epithelial-epithelial and epithelial-stromal interactions (Wiseman and Werb, 2002). In addition, stem cells may require the presence of a three-dimensional “stem cell niche” for maintenance and self-renewal (Chepko and Dickson, 2003). Other studies show that growth of normal epithelium is inhibited by the presence of nearby or pre-existing epithelial structures (Faulkin and Deome, 1960). Thus, assays using dissociated cells or the intact gland may not allow the developmental potential of otherwise division-competent stem/progenitor cells to be expressed. Perhaps most damaging to these models are elegant genetic and transplantation studies showing that functionally differentiated epithelial cells retain division competence, can give rise to both luminal and myoepithelial cells upon transplantation, and can participate in the development of oncogene-driven mammary cancer (Boulanger et al., 2005; Wagner and Smith, 2005).

Short term transplantation assays using small fragments of intact mammary duct transplanted into epithelium-free fat pads of host mice offer an excellent opportunity to test the prevailing stem/progenitor cell model, and to explore the developmental plasticity of mammary epithelium (Chew and Hoshino, 1970). With small fragments (~1000 cells), three-dimensional structure and cell-cell interactions are largely maintained, while the inhibitory effects of neighboring epithelium are minimized. Thus, stem/progenitor cells should be in an environment more favorable to cell division. In addition, onset of regeneration can be timed precisely.

If division-competent stem/progenitor cells represent a small percentage of all epithelial cells, the initial rate of cell division in transplanted fragments should be low, similar to that of dissociated cells, and limited to relatively undifferentiated epithelial cell types defined previously by EM. However, if stem/progenitor cells can include more differentiated, yet division-competent cells, the initial rate of cell division in fragments should be higher than that observed in transplants of dissociated cells, and include a broader range of epithelial cell types. Preliminary data showing ~15% of epithelial cells proliferate by 24 hours support the latter model.

## Results

**Objective 1) *To determine the full range of division-competent stem/progenitor mammary epithelial cell types participating in early gland regeneration.***

**Fragment transplantation:** We have completed a series of ductal fragment transplantation experiments to determine the timing of stem/progenitor cell activation during early regeneration through 48 hours post-transplantation and have initiated transplants to fill in intervening time points to complete this portion of the study rigorously. We have characterized these transplants with a series of markers to begin to define their behavior.

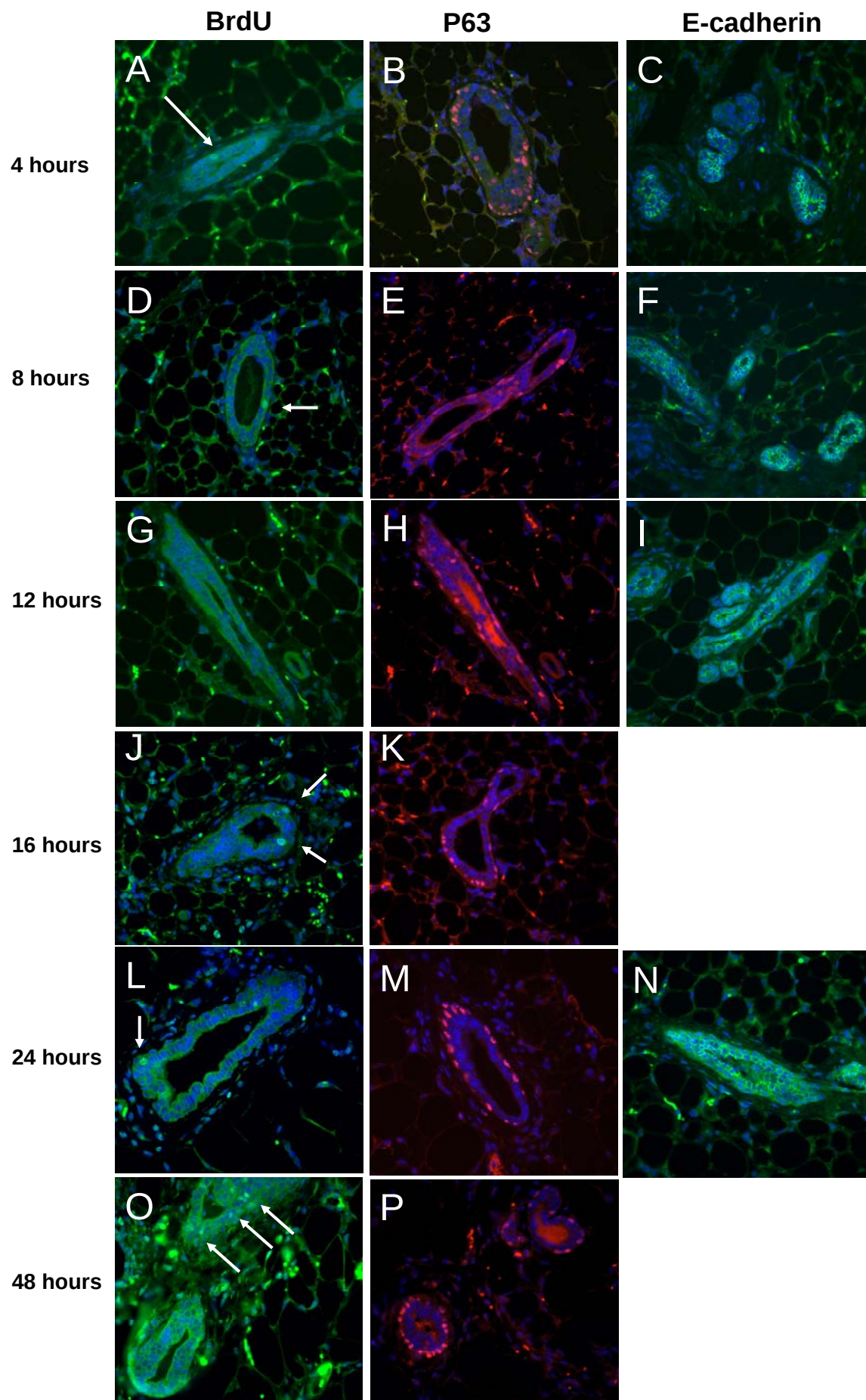
Our initial experimental protocol called for BrdU injection and tissue harvest at 6 hour intervals. We have modified this protocol such that BrdU injection and tissue harvest have been performed at 4 hour intervals after transplantation. This change was instituted because we were concerned that we might miss critical early and/or transient events in the regenerative process. Our concerns appear to be well-founded.

Early regeneration appears to occur in a defined series of events. At 4 hours, there is a small amount of DNA synthesis in which ~1.5% of all epithelial cells in the transplant initiate DNA synthesis as determined by incorporation of BrdU (Figure 1A). In addition, other cells enter the cell cycle based on expression of Ki67 alone (Data not shown). At 8 hours, incorporation of BrdU remains at about 1% (Figure 1D). After this point, there is period of relative growth quiescence (Figure 1G), through to the 24 hour timepoint (Figure 1J). However, at 48 hours, there is a significant increase in BrdU incorporation to just over 8% of all epithelial cells.

In addition to changes in BrdU, we also observed transient changes in p63 gene expression in the myoepithelial/stem cell layer. While all myoepithelial cells appear to express p63 strongly (3+ on a scale of subjective scale of 1-3) at 4 hours (Figure 1B and Figure 2), at 8-16 hours, we observed reduction in intensity (1+ to 2+) or slight loss of p63 expression in the myoepithelial cells (Figure 1E, 1H and 1K). p63 intensity begins to recover at the 24 hour timepoint (Figure 1K). However the percentage of p63+ cells remains slightly lower. This loss of p63 expression was not due to caspase-3 mediated apoptosis since we did not observe apoptotic cells in any of the transplanted fragments examined (data not shown).

In the luminal cell layer, while epithelial cells at 4, 8 and 23 hours expressed E-cadherin in a relatively uniform pattern in all luminal epithelial cells (Figure 1C, 1F, and 1L), we believe we observed transient reduction in the percentage of cells expressing E-cadherin, particularly at 12 hours (Figure 1I), with evidence in some sections to suggest reorganization of the epithelial cells within the transplanted fragments at the 12-24 hour timepoints.

Our interpretation of these data is that cells undergoing early DNA synthesis likely represent division-competent cells arrested at the G1/S restriction point of the cell cycle that, upon transplantation, were released from this cell cycle block and allowed to enter S-phase. Cells expressing Ki67 alone may have exited G0, but have not yet initiated DNA synthesis. By 8 hours, most of the Ki67+ cells have entered S-phase. These cells are most likely to be the same ER-progenitor cell types that one observes dividing in the mature gland normally, but are unlikely to represent true regenerative stem cells. Upon cellular reorganization, cells undergoing late DNA synthesis most likely represent regenerative stem cells and division-competent progenitor cells initiating the regeneration process. We are currently evaluating potential markers that might distinguish early and late S-phase cells from one another and from non-dividing cell types.



**Figure 1.** Changes in proliferation and marker expression during early mammary gland regeneration. Timepoint at harvest is denoted to the left of the row to which it applies. Marker assayed is denoted above the column to which it applies. Green arrows denote cells stained positively for BrdU incorporation with co-expression of Ki67. Magenta arrows denote cells expressing Ki67 exclusively. White arrow denotes an area of cells in which E-cadherin expression is reduced or lost.

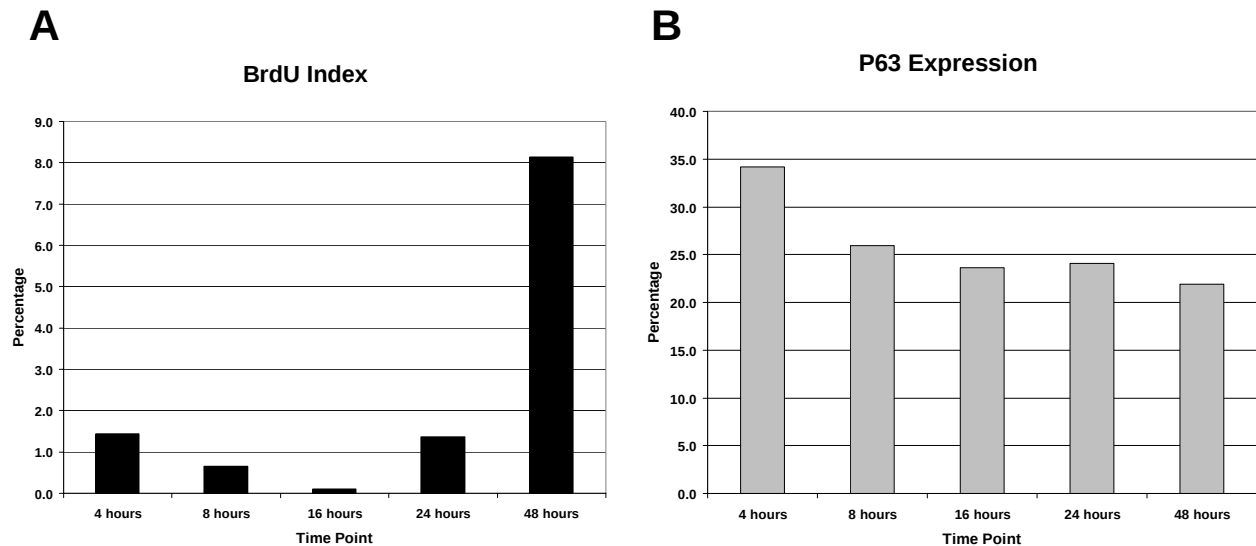


Figure 2. Quantification of BrdU labeling index and p63 expression as a function of time.

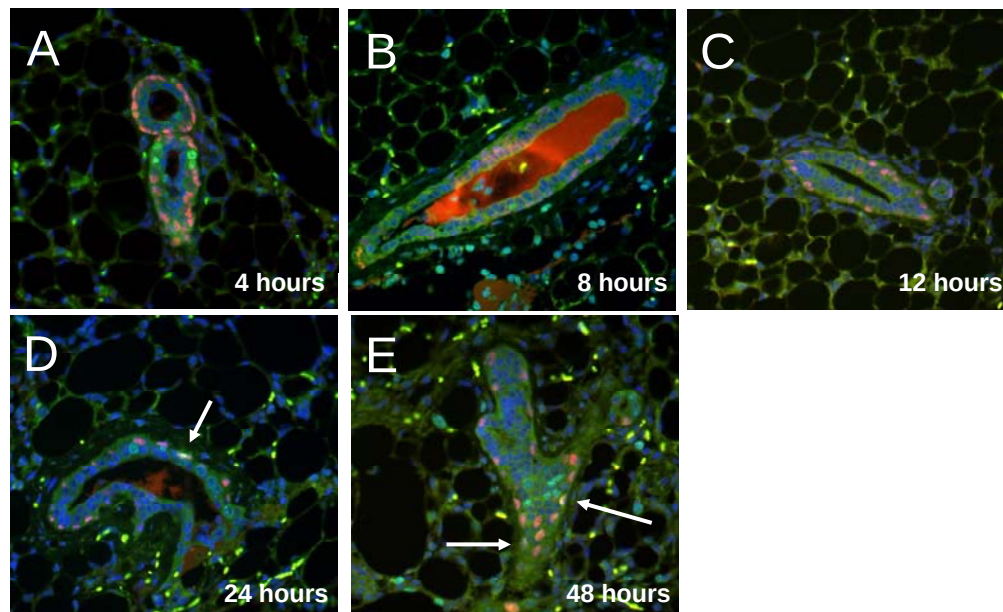


Figure 3. Co-labeling of p63 with BrdU. P63 is postulated to be expressed in mammary epithelial stem cells. These data suggest that mammary epithelial stem cells are activated between 24 and 48 hours posttransplantation

Loss of p63 expression from the myoepithelial cells and reduction/loss of E-cadherin expression from luminal epithelial cells was unexpected and indicates a degree of plasticity of both cell types. It is possible that both events influence the regeneration process. Thus, it would be of interest to see how sustained expression of either protein affected early regeneration. Our prediction is that sustained expression would delay onset of regeneration.

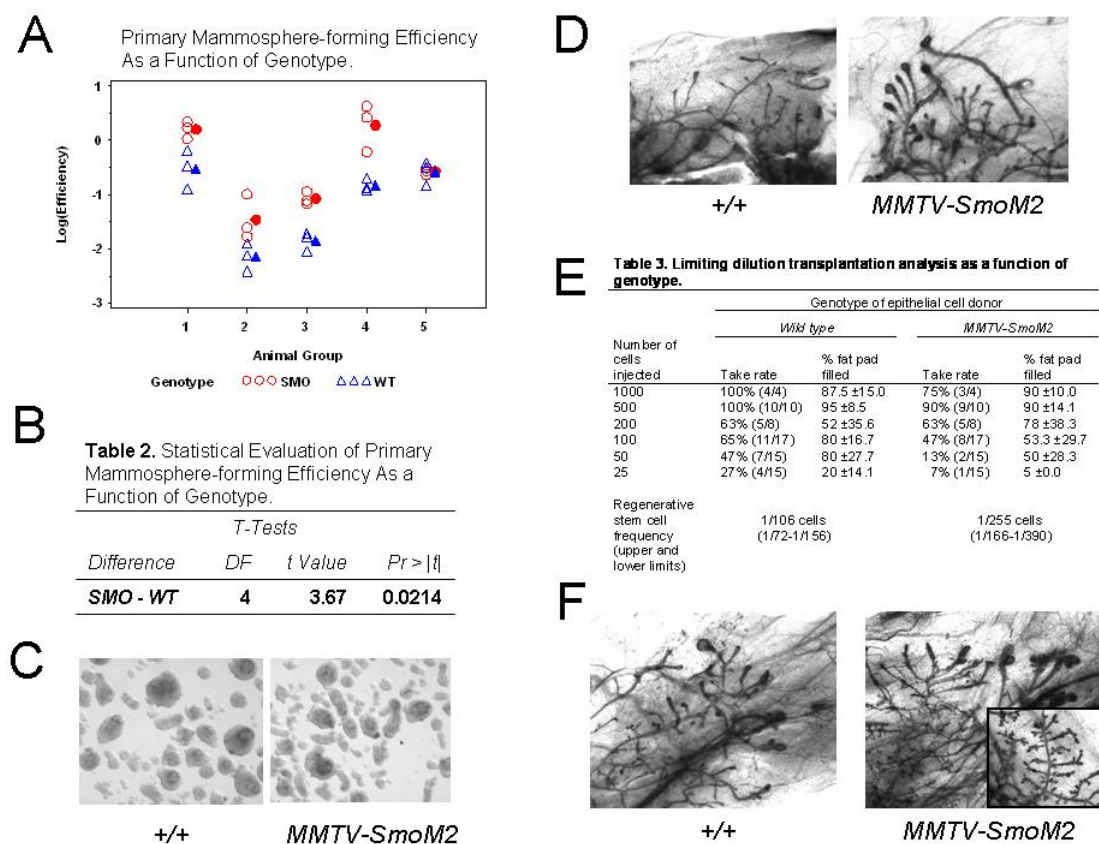
There are a number of technical limitations to these studies that we are attempting to overcome. The primary limitation is the small number of cells present in the transplanted fragments. The small size of the transplants makes them exceptionally difficult to find for conducting immunofluorescence analysis. We are therefore using epithelium genetically tagged to express enhanced cyan fluorescent protein (ECFP), which affords the opportunity to localize the transplanted duct fragment roughly within a given gland. We have begun to use 0.5mm skin biopsy punches to isolate the region containing the transplanted fragment coupled with use of tissue microarrays of multiple fragments from a given timepoint to enhance our ability to characterize regeneration events.

In addition to the difficulty in localizing transplanted epithelium, the small fragment size means that samples are exhausted quickly during microtomy, thus requiring numerous transplants for each timepoint. Finally, while putative stem cell markers have been published recently, all putative stem/progenitor cell markers used currently rely on subtle differences in fluorescence intensity in flow cytometric analysis using thousands of cells. Flow cytometric analysis is not possible for small fragments such as those being analyzed in this study ( $n < 1000$  cells/fragment). Thus, new markers are required that can be used in immunofluorescence analysis. We are attempting to identify such markers using a candidate gene approach.

***Mammosphere-formation and Limiting-dilution transplantation:*** In addition, to the fragment transplantation experiments, we have spent considerable effort to optimize mammosphere-formation and limiting dilution transplantation protocols for analysis of stem cell function in both wild type and mutant mice. We have used these techniques to compare stem cell function in FVB mice and our new *MMTV-SmoM2* mice expressing a constitutively activated form of Smoothened, the main effector of activated hedgehog signaling.

To determine whether *MMTV-SmoM2* transgenic mice showed a change in the frequency of mammosphere-forming cells relative to wild type controls, we conducted primary mammosphere-formation assays (Dontu et al., 2003; Dontu and Wicha, 2005). Using normal human mammary epithelial cells, mammospheres can be produced both by cells with multilineage differentiation capacity (a surrogate test for stem cells, presumably having regenerative potential), as well as by division-competent cells with either luminal or myoepithelial differentiation capacity (presumably lacking regenerative potential). In four of five independent paired primary cell preparations, cells derived from *MMTV-SmoM2* mice showed a ~2-fold increase in the percentage of cells capable of forming primary mammospheres (mean raw value = 0.76%) relative to cells isolated from wild type littermate control mice (mean raw value = 0.38%) ( $P=0.02$ , paired-t test) (Figure 4A and 4B). There were no differences in mammosphere size or shape noted between the two genotypes (Figure 4C).

To verify that primary mammospheres contained regenerative mammary epithelial stem cells, we transplanted single mammospheres derived from wild type and *MMTV-SmoM2* mice into contralateral cleared fat pads of three-week old host mice. Mammospheres derived from both genotypes showed regenerative potential, with 2/13 (15%) of wild type mammospheres (Figure 2D, left panel), and 5/15 (33%) of *MMTV-SmoM2* mammospheres (Figure 4D, right panel) capable of regenerating ductal trees. These regeneration frequencies were not statistically different from one another ( $P=0.40$ , Fisher's exact test). Duct morphology in *MMTV-SmoM2* outgrowths was consistently altered relative to wild type in a manner consistent with the phenotype observed in intact mice.



**Figure 4.** Effect of *MMTV-SmoM2* on mammosphere formation and regeneration of the mammary gland. A. Pairwise comparison of log-transformed mammosphere-formation efficiency values for five paired sets of primary epithelial cell preparations. *MMTV-SmoM2* cells showed a two-fold increase in mammosphere-forming efficiency relative to wild type cells in four of the five sample pairs. B. Statistical evaluation of mammosphere-forming efficiency including all five paired samples shown in A. C. Photomicrographs of representative mammospheres from *MMTV-SmoM2* and wild type mice. D. Photomicrographs of representative outgrowths of transplanted mammospheres derived from wild type and *MMTV-SmoM2* mice. E. Limiting dilution transplantation analysis as a function of genotype. F. Photomicrographs of representative outgrowths from limiting dilution transplantations (100 cells).

Because mammosphere-formation assays are an indirect measure of the frequency of stem and progenitor cells present in the intact mammary gland, and because single mammosphere transplants showed no difference in regeneration frequency, it was possible that the two-fold increase in mammosphere-forming efficiency in cells derived from *MMTV-SmoM2* mice might not be due to an increased proportion of regenerative stem cells *in vivo*, but rather due to an increase in the survival (or activity) of mammosphere-initiating cells under anchorage-independent growth conditions. To address this possibility, we conducted limiting dilution transplantation analysis designed to detect differences in the proportion of cells with regenerative capacity directly (Figure 4E).

We were fortunate enough to find a protocol quickly that allows for a transplant efficiently approximately one order of magnitude better than any published result (e.g. 1 regenerative stem cell per 1,400-2000 cells) – and is therefore in much better agreement with our fragment transplantation analyses. In this method, single cell suspensions of primary mammary epithelial cells are diluted into phosphate-buffered saline instead of tissue culture medium. After dilution, cells are mixed 1:1 (v/v) with Matrigel and injected in a 10ul volume. Injected glands are harvested 8 weeks post-transplantation and evaluated for growth of mammary glands.

For both genotypes, as few as 25 cells were capable of regenerating ductal trees. However, the rate of successful transplantation, or “take rate”, was lower in cells derived from *MMTV-SmoM2* mice below 200 cells/gland injected. Using a single-hit Poisson distribution model (Bonnefoix et al., 1996), we estimate the frequency of regenerative stem cells in wild type epithelium is 1 stem cell per 106 cells (Figure 4E). The frequency of regenerative stem cells in *MMTV-SmoM2* epithelium was decreased ~2.5 fold, to 1 stem cell per 255 cells. Again, duct morphology in *MMTV-SmoM2* outgrowths was consistently altered relative to wild type (Figure 4F).

A manuscript summarizing these results has been submitted for publication to *Development* as part of our analysis of a new transgenic mouse model for hedgehog signaling activation (*MMTV-SmoM2*) (see below) and is currently under review.

***Objective 2) To develop the short-term transplantation assay as a means by which critical regulators of stem and progenitor cell behavior can be discovered and evaluated.***

Based on our optimized results from Objective 1, we are now in the position to be able to test our hypothesis rigorously using tissue fragments and cells derived from the *MMTV-Wnt1* transgenic mouse model, as well as our new *MMTV-SmoM2* transgenic mouse model (constitutively activated hedgehog signaling) (Moraes et al., 2007). The *MMTV-Wnt1* model was shown to possess an increased number of regenerative stem cells by limiting-dilution transplantation (though using a different method than that developed by us). Our *MMTV-SmoM2* model shows an increase in the proportion of mammosphere-initiating cells (stem and progenitor cells), but a decrease in the proportion of regenerative stem cells in our optimized limiting-dilution transplantation assay (Moraes et al., 2007). Thus, we will have two bona-fide mouse models with which to test our initial hypothesis – one that increases regenerative capacity, and one that decreases regenerative capacity.

## **Key research accomplishments**

1. Temporal characterization of early mammary gland regeneration through 48 hours with respect to BrdU incorporation and Ki67 expression.
2. Discovery of phenotypic plasticity of both luminal and myoepithelial cells during early regeneration with respect to p63 expression (myoepithelium) and E-cadherin expression (luminal epithelium), which offers some mechanistic insight into the early regeneration process since p63 is essential for mammary gland growth.
3. Optimization of limiting dilution transplantation and refinement of the estimate for the frequency of regenerative stem cells in the wild type gland relative to a new transgenic mouse line, *MMTV-SmoM2*, which shows a two-fold depletion in the frequency of regenerative stem cells.
4. Formal demonstration that about 1/3 of mammospheres contain, and therefore arise from, regenerative stem cells.
5. Formal demonstration that the mammosphere assay underestimates the true frequency of regenerative stem cells in the mouse mammary gland.

## **Reportable outcomes**

### **Manuscripts**

Moraes, R.C., Zhang, X., Harrington, N., Fung, J.Y., Wu, M.F., Hilsenbeck, S.G., Allred, D.C., and **Lewis, M.T.** (2007) “Constitutive activation of *Smoothed (Smo)* in mammary glands of transgenic mice leads to increased proliferation, altered differentiation, and ductal dysplasia.” *Development* 134:1231-42.

Zhang X., Harrington N., Moraes R., Wu M., Hilsenbeck S., **Lewis M.T.** (2008) "Cyclopamine inhibition of human breast cancer cell growth independent of *Smoothed (Smo)*." Breast Cancer Research and Treatment (In press)

### **Invited Presentations**

- 2005 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer" 15<sup>th</sup> Annual Breast Cancer Think Tank Meeting, Curacao, Netherlands
- 2005 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" Cold Spring Harbor Workshop on Cancer Stem Cells. Banbury, NY
- 2006 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" 16<sup>th</sup> Annual Breast Cancer Think Tank Meeting, Grand Cayman Islands
- 2006 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" University of Texas Medical Branch, Galveston TX
- 2006 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" University of Alabama, Birmingham. Birmingham AL
- 2006 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" University of Texas MD Anderson Cancer Center, Houston TX
- 2006 "Hedgehog network regulation of stem cells in mammary gland development and breast cancer: the importance of absence" Schering Foundation Research Foundation Workshop on Stem Cells in Cancer. Berlin, Germany
- 2007 "Hedgehog signaling, stem cells, and breast cancer: connections and controversies" University of Miami, Miami FL
- 2007 "Non-canonical Hedgehog Signaling in Mammary Stem/Progenitor Cell Regulation. Gordon Research Conference on Mammary Gland Biology, Salve Regina University, Providence, Rhode Island

### **Conclusions**

Short-term transplantation appears to give a better estimate of the frequency of division-competent cells present in the gland than traditional assays. If suitable markers can be identified that can distinguish regenerative stem cells from downstream progenitor cells, the short-term transplantation assay may be a useful and rapid method for quantifying changes in the relative proportion of these cells due to mutation or treatment with bioactive compounds.

Analysis of the first events of mammary gland regeneration should allow identification of regulatory signaling networks and other regulatory mechanisms that govern stem and progenitor cell self-renewal.

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