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TITLE: A Novel Strategy for Isolation, Molecular and Functional Characterization of Embryonic Mammary Stem Cells Using Molecular Genetics and Microfluidic Sorting

PRINCIPAL INVESTIGATOR: Geoffrey M. Wahl, Ph.D.

CONTRACTING ORGANIZATION: The Salk Institute for Biological Studies
La Jolla, CA 92037-1099

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14. ABSTRACT
   We developed a genetic system to identify, isolate, and characterize mammary stem cells. Our system consists of an activator component that is dependent on Wnt signaling, which is essential for mammary gland development, and on a doxycycline regulated reporter to toggle the system on and off. The reporter component labels the chromatin of cells for direct visualization. We used a modular design to enable the system to be applied to cancer models and other organs. Over the past year we have targeted mouse embryonic stem cells with our modular transgenic system and validated its Wnt responsiveness. We have also established assays for isolation and characterization of small numbers of viable, fluorescently labeled cells. In parallel, we have determined that embryonic mammary rudiments contain concentrated mammary stem cells and are isolating these structures to obtain stem cell specific expression signatures. The molecular reagents and strategies we are developing have broad applications for studies examining the relationship between normal and cancer stem cells, and determining whether they share the same origin.

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INTRODUCTION

Tumors commonly exhibit cellular heterogeneity in situ. Transplantation of certain subsets of cells from the tumor into immune-compromised mice can generate similar heterogeneity, which is also observed upon secondary transplantation [1]. Such observations suggest the existence of a subset of cells within the original tumor that can both self-renew to regenerate the tumor mass and produce cellular complexity. Such heterogeneity could also explain the frequent failure of cancer therapies despite initial reduction of tumor bulk. Since self renewal and the production of cellular complexity through differentiation are also essential properties of normal tissue stem cells, the subset of cells which perpetuate cancer have been termed “cancer stem cells”. It is unclear whether “cancer stem cells” arise through mutations that impart self-renewal to proliferating progenitors or mutations that occur in normal stem cells to render them tumorigenic [2]. In either case, understanding the functional properties of cancer stem cells and how they may be derivative or distinguishable from normal stem cells should enable more effective strategies to detect, prevent, treat, and cure cancer. At the root of understanding whether cancers originate from transformation of stem or progenitor cells, or both, requires effective methods to isolate and characterize normal stem cells. We are using several approaches to identify, purify and functionally characterize mammary stem cells (MSC). First, we hypothesize that MSC concentration will be higher in embryonic than adult mammary glands due to the absence of terminally differentiated cells in early embryogenesis. Second, we developed a genetic, fluorescent chromatin labeling strategy to determine whether MSCs cycle infrequently, and would consequently be more fluorescent than proliferating counterparts. Our strategy enables us to track and localize labeled cells at various stages during embryogenesis and into adulthood, and if MSC cycle frequently, they would lose fluorescence, providing clear distinction between the two prominent stem cell models. Our strategy would also indicate whether there are classes of cycling and non-cycling MSC. The proposed strategy should enable us to isolate MSC using micro-fluidic cell sorting or micro-manipulation and to assay their self renewal and multilineage differentiation potential using transplantation assays, and to profile their gene expression patterns. The expression signatures we obtain can be assayed in human tumors and used for prognosis and as indicators of therapy success while revealing functionally relevant genes and pathways, which may provide novel therapeutic targets and be used to improve existing mouse models of normal and pathological development.

BODY

Task 1: Determine whether there is a population of label retaining cells formed during early mammary gland development

An essential first step of this task has been the development of functional molecular genetic tools for the in vivo labeling of cells in the mammary. As described previously in our 2007 progress report, our original transgenic design failed to label cells efficiently in vivo and we determined that this was most likely due to the pervasive problem of position effect variegation associated with traditional transgenesis. We proposed an alternative approach using recombinase mediated targeting into specific open loci and have now successfully implemented this strategy in the Col1A1 and ROSA26 loci. We proposed to label early embryonic mammary precursors, which we show below to contain MSC, using the Wnt signaling pathway as it is essential for mammary development, and an H2B-GFP fusion protein to fluorescently label
used FLP-mediated cassette exchange to introduce STTIL into the frt containing ColA1 locus in ES cells obtained from the Jaenisch lab (Figure 1)[5]. Targeted clones were validated by PCR and cells containing the exchanged cassette were analyzed for Wnt responsiveness in vitro by quantitative RT-PCR following the addition of Wnt3a conditioned media. The induction of Supertop driven tTA2 and LacZ transcripts was in excess of three fold and the fold increase of tTA2 mRNA was statistically similar to the induction of the endogenous Wnt target gene, Axin2 (Figure 2). As expected, there was no statistical difference between the induction of tTA2 and LacZ as they are transcribed as one mRNA with an intervening ribosome entry sequence. These data show that this strategy did generate a Wnt responsive transgene in ES cells. The validated cells were subsequently injected into blastocysts to generate chimeras and breeding of the chimeric progeny to test germline transmission and to generate founder lines is currently underway. We will verify that the STTIL construct in the ColA1 locus responds to Wnt signals as do endogenous Wnt target genes by comparing the spatiotemporal expression pattern of its Supertop driven lacZ gene with existing Axin2-lacZ and LGR5-lacZ mice that we have obtained from EMMA and the laboratory of Hans Clevers [6]. If faithful Wnt signaling is observed, founders will be bred to the TRE-H2B-GFP line for in vivo labeling and label retention studies as originally proposed.

We generated a second targeted open locus by homologous recombination of heterologous loxP (L3, L2) sites into the widely used Rosa26 locus (Figure 1)[7]. Correctly targeted clones were confirmed by Southern blot with external and internal probes. The resulting ES cell line represents a significant improvement over the existing ColA1 line. The heterologous loxP sites prohibit the intramolecular recombination suspected of reducing efficiency at the frt-ColA1 locus, and the deltaTK gene enables negative selection to identify clones with cassette exchange with high efficiency [8]. R26L3-2L ES cell clones are being confirmed for their ability to readily exchange target constructs and produce mice, and will prove a valuable tool to many researchers.

Simultaneously, we are also exchanging a modular dual activator reporter cassette (MARC) into R26L3-2L. Marc contains the Wnt responsive promoter, chromatin [3, 4]. To accomplish this Task, we engineered a targeting construct containing a frt site and the Wnt inducible Supertop promoter followed by the tetracycline transactivator, tTA2, and IRES LacZ (STTIL). We validated this construct in the ColA1 locus by comparing the spatiotemporal expression pattern of its Supertop driven lacZ gene with existing Axin2-lacZ and LGR5-lacZ mice that we have obtained from EMMA and the laboratory of Hans Clevers [6]. If faithful Wnt signaling is observed, founders will be bred to the TRE-H2B-GFP line for in vivo labeling and label retention studies as originally proposed.

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SuperTOP, driving expression of the tetracycline transactivator, tTA2, and an IRES tomato reporter to indicate SuperTOP activation. In the opposite orientation to this activator cassette, we have included the Tetracycline Response Elements promoter (TRE) driving expression of the chromatin label, H2BGFP. This system enables Wnt induced, fluorescent labeling of quiescent, infrequently dividing cells in vivo. By combining the activator and reporter cassette in one transgene, we obviate the need for strain interbreeding and mitigate the potential position effects on TRE-H2BGFP transgene expression that could create unacceptable background. MARC ES cells and mice will be analyzed for Wnt responsiveness as described above.

While developing the Wnt responsive system, we have used an existing cytokeratin 14 (K14) driven tTA gene to validate chromatin labeling in the developing mammary in vivo [9]. Interestingly, our preliminary work using this system demonstrates not only that mammary cells can label brightly in vivo and that the system is sensitive to dox regulation in utero, in young adult and in adult mice, but also that cells may exist which retain bright label from the time of mammary rudiment formation up to 8 weeks of age (Figure 3). Studies are currently under way to determine whether this relatively quiescent pool of cells is enriched for mammary stem cells.

**Task 2: Determine whether embryonic mammary precursor cells exhibit functional characteristics of stem cells**

This task was proposed to test the hypothesis that embryonic mammary rudiments provide enriched sources of MSC. The gold standard of stem cell identity in the mammary gland is the ability of a transplanted cell to fully reconstitute a functional mammary gland in a recipient mouse cleared of its endogenous mammary epithelium [10]. We successfully adapted existing transplant methods to assay the existence and concentration of stem cells in the mammary rudiments. Importantly, we validated our hypotheses that embryonic mammary rudiments contain a higher concentration of mammary stem cells than the adult mammary gland. This represents the first quantitative study on the stem cell constitution of the mammary rudiment. Transplanted mammary rudiments from E13.5, E15.5, and E18.5 actin-eGFP transgenic mice generated arborized green-fluorescent outgrowths morphologically indistinguishable from adult mammary glands in recipient mice (Figure 4). Impregnation of recipients induced alveolar development and milk synthesis, two indications of...
Complete multipotency and functionality. These embryonic MSC self renew as dispersed cells from regenerated mammary trees generated new full mammary trees in secondary transplants. Together, these data demonstrate that embryonic mammary rudiments contain cells with the characteristics of mammary stem cells. Very high transplant efficiencies were obtained from embryonic rudiments of all ages (e.g. 92% from E18.5, 100% efficiency from E15.5, and 63% from E13.5 buds). E13.5 buds contain about 1000 cells, implying that their concentration of mammary stem cells is significantly higher than in adult glands (Table 1). We have begun to quantify the MSC frequency in embryonic rudiments at different ages by transplanting limiting dilutions of dissociated cells. Using this approach, the MSC frequency in E18.5 rudiments is approximately 1/1300 (Table 1). We are currently extending these findings by defining the precise concentration of MSC at various stages of embryogenesis. We have preliminary evidence that transplantation efficiency is affected by cellular microenvironment, and we will refine these analyses by co-injecting the dissociated cells in the presence of matrigel or purified matrix components.

**Task 3: Isolate embryonic mammary stem cells to define their gene expression profiles**

The transplant efficiency analysis described above shows that embryonic rudiments are enriched for mammary stem cells, and justify their use for deducing mammary stem cell gene expression signatures. When we evaluated the linearity of RNA amplification from 5x10^5 or 500 cells, we found that linearity was compromised at low cell numbers (Figure 5). We are therefore collecting sufficient mammary buds to enable amplification from 50-100 mammary rudiments (~1-2x10^5 cells at e15.5). We will perform initial studies on dissociated, unlabeled, embryonic mammary cells and will utilize e15.5 mammary buds, as they gave very high transplantation efficiency.

The genetic system described above will enable us to determine whether there are Wnt responsive cells that exhibit characteristics of mammary stem cells in embryonic mammary rudiments, and to analyze their retention of H2BGFP. In some tissues, stem cells are thought to cycle infrequently, while in other tissues they appear to cycle constantly. Mammary stem cells may exhibit different cycling characteristic depending upon their location and stage in development. Our molecular genetic tools will allow us to isolate cells from these diverse

<table>
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<tr>
<th>Table 1</th>
<th>Tissue type</th>
<th>No. of cells per transplant</th>
<th>Successful transplants</th>
<th>Efficiency</th>
<th>Notes</th>
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<tbody>
<tr>
<td>Intact E18.5</td>
<td>&gt;5000</td>
<td>11/12</td>
<td>92%</td>
<td>Competent for alveolar formation and outgrowth in 2ndary transplants.</td>
<td></td>
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<tr>
<td>Intact E15.5</td>
<td>&lt;2000</td>
<td>10/10</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact E13.5</td>
<td>&lt;1000</td>
<td>5/8</td>
<td>63%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult mammary gland (dispersed)</td>
<td>20,000</td>
<td>2/2</td>
<td>100%</td>
<td>MSC frequency: 1 in 6500*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>5/7</td>
<td>71%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>2/4</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1/7</td>
<td>14%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100</td>
<td>0/9</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E18.5 mammary (dispersed)</td>
<td>2,200</td>
<td>4/4</td>
<td>100%</td>
<td>MSC frequency: 1 in 1300*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,100</td>
<td>2/4</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0/4</td>
<td>0%</td>
<td></td>
<td></td>
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<tr>
<td>E15.5 mammary (dispersed)</td>
<td>1,200</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0/10</td>
<td>0%</td>
<td>* determined using L-Calc™ (Stem Cell Technologies)</td>
<td></td>
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Figure 5: Fold induction of p21 in Nutlin treated MEFs. Two independent experiments are labeled #1 and #2.
spatiotemporal contexts and delineate specific gene signatures associated with them.

In order to isolate such specific and potentially rare subpopulations of cells, we have validated the use of a micro-volume cell sorter (Celula, Inc.). This instrument is capable of sorting as few as 150 GFP positive cells from a sample size of 5,000 cells. We performed a proof of concept experiment by using the micro-volume sorter to isolate GFP+ cells from adult mammary glands. The viability was >95%, and transplantation of sorted cells produced full mammary outgrowths (Figure 6). The results justify using this cell sorter to isolate H2BGFP-label retaining cells. Alternatively, we may utilize laser capture microscopy to isolate cells and subsequently mRNA from select subpopulations including those that have retained chromatin label in mammary rudiments or adult glands.

Gene expression profiling will be carried out using two complementary approaches. The first is a directed approach employing a TaqMan Low Density Array (TLDA) that quantitatively determines the expression of a set of customizable genes. The array we designed contains validated primer sets for genes in the Wnt, Notch, Hedgehog and TGF-beta signaling pathways, which have previously been implicated in mammary genesis and pathogenesis, a set of mammary stem and lineage markers, genes associated with embryonic stem cell self-renewal and genes associated with mammary cancer (for review see Liu 2005) [11]. The second method is unbiased and interrogates all known genes using a hybridization-based platform such as Nimblegen or Affymetrix arrays. From these data a putative mammary stem cell gene signature will be delineated and the expression of individual genes within the signature will be localized at various developmental stages by immunofluorescence microscopy, in situ hybridization, and/or quantitative RT-PCR.
KEY RESEARCH ACCOMPLISHMENTS

- Targeted ColA1 with a genetic labeling system
- Validated responsiveness of ColA1 targeted ES cells
- Generated chimeric mice
- Designed Rosa26 targeting strategy
- Targeted Rosa26 in ES cells
- Designed and constructed the dual function MARC vector
- Determined the efficacy of doxycycline mediated chase of H2BGFP expression
- Established the presence of some label retaining cells that persist into adult hood.
- Established procedures for micro-volume sorting
- Successful transplantation of dispersed cells from adult mammary glands and embryonic mammary rudiments.
- Established that embryonic mammary rudiments contain stem cells
- Quantification of mammary stem cells in the embryo and the adult.
- Demonstrated the validity of our first hypothesis; mammary stem cells are enriched in the embryonic mammary rudiment compared to the adult mammary gland.
- Determined the effect of cell number on transcript amplification efficiency
- Designed and obtained TaqMan low density array.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We developed and validated a multifaceted approach to the prospective isolation of tissue specific stem cells (Task 1). Our approach is based on a molecular genetic labeling system that uses functionally relevant genetics to label cells in vivo and allows analysis of proliferation and quiescence through chromatin label retention. With this system, we are able to analyze and isolate viable cells at single cell resolution from any spatiotemporal context in the mouse. We have identified the embryonic mammary rudiment as a concentrated source of mammary repopulating cells, which exhibit stem cell capacity in functional assays (Task 2). To accomplish Task 3, we are using the e15.5 mammary bud to derive a MSC expression profile in parallel with our analysis and isolation of mammary cells that label in our Wnt reporter system. Together, these studies will provide a gene signature specific to the MSC, which has not been obtained using traditional surface label screening approaches. The ultimate goal of these studies is to identify definitive functional markers for mammary stem cells such as new candidate molecular pathways in determining self-renewal, or limiting differentiation. The genetic systems we have proposed will then enable direct tests of genes that may be involved in stem cell maintenance, differentiation, and cancer.
REFERENCES