UNIVERSITY OF CALIFORNIA, MERCED

Adhesion Gene Regulation in Mammary Cell Proliferation

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Quantitative Systems Biology

by

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2011

The Thesis of Eric HonYui Lau is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced

2011

ACKNOWLEDGMENTS

I would like to acknowledge Professor Maria Pallavicini for her support as my advisor. Her guidance throughout my graduate studies has made this thesis possible.

I would like to thank the Pallavicini Lab undergraduate assistants Elyse Ozamoto, Desiree Sigala, and Jaime Tawney for their diligent work on this project.

To the members of Suite 325, thank you for your input and discussions about troubleshooting protocols and reviewing data.

I dedicate my Thesis to my parents, Frederick and Teresa, for their unyielding love and encouragement.

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ABSTRACT OF THE THESIS

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Professor Maria Pallavicini, Advisor

The mammary gland undergoes dynamic remodeling which requires cell proliferation in order to maintain the gland's form and function. Cell communication plays a critical role during proliferation. Here I show that Comma1D-βgeo (C1D) cells exhibit different rates of proliferation when grown in adherent versus non-

adherent culture. The modulation of substrate attachment during culture presents different environments for C1D cell growth, altering the repertoire of adhesion molecules (AMs) which may affect proliferation. Data show that the CD24 subpopulations have differing proliferation potentials in the different culture conditions. Cells expressing $CD24^{low}$ grown in adherent culture have a higher proliferation rate compared to $CD24^{high}$ cells. Cells expressing $CD24^{high}$ grown in non-adherent culture form more mammospheres compared to $CD24^{low}$ cells. Intracellular analysis showed that C1D cells, along with phenotypes not yet identified in the mammary hierarchy, exhibit predominately basal cells ($K5^{+}\alpha SMA^{+}$ and αSMA^{+}). These basal phenotypes were expressed in cell subpopulations grown in adherent culture while subpopulations in non-adherent culture contained a variety of basal and luminal cells including progenitor populations ($K5^{+}$, $K5^{+}\alpha SMA^{+}$, αSMA^{+}, $K5^{+}K8^{+}$, and $K8^{+}$). Microarray analysis revealed a common upregulated gene Sialophorin (*Spn*, CD43). Categorized by ontology as involved in adhesion and proliferation, sialophorin was upregulated in subpopulations demonstrating higher proliferation rates in both culture conditions ($P < 0.05$). Thought only to be present on hematopoietic cells, *Spn* is has been found to be highly expressed in primary breast tumors and their metastases, affecting proliferative capability. Here, *Spn* may also play an important role in normal mammary development by regulating the proliferation of mammary progenitor cells.

CHAPTER 1. INTRODUCTION

1.1 Mammary Gland Biology

The mammary gland is organized into hollow ducts, comprised of luminal epithelial cells surrounded by a layer of basal myoepithelial cells. The myoepithelial cells secrete proteins and proteoglycans that constitute the basement membrane with which they are adjacent. Basement membrane proteins include laminin, fibronectin, and type IV collagen, which provide structural support as well as contribute to epithelial cell maintenance and growth. The ducts end in a round bulb-like structure called the terminal end bud (TEB) (Figure 1). The mammary gland undergoes many remodeling events in the lifetime of a mammal. These highly regulated cycles, occurring during puberty and pregnancy, involve branching, differentiation, secretion, and apoptosis. At puberty, the terminal ends buds proliferate rapidly, causing ductal elongation. The epithelial cells at the TEB, also known as cap cells, differentiate and form luminal and myoepithelial cell layers. The TEBs then regress and are replaced by alveolar buds which form at the ends of the ducts. These alveolar buds are circular

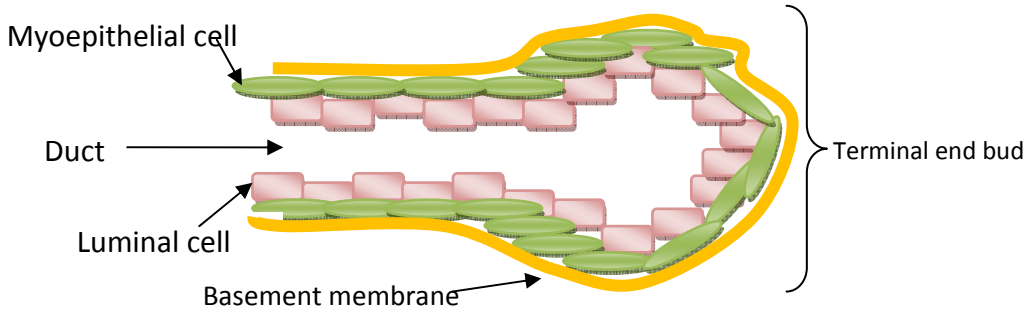

Figure 1: A representation of a mammary gland duct leading to a terminal end bud. These structures are found branched throughout the fatpad.

and have a hollow center surrounded by a layer of epithelial and myoepithelial cells with basement membrane. During pregnancy, ductal branching increases and the alveolar buds differentiate into mature lobules capable of casein synthesis. At parturition, milk fat globules are synthesized in the alveoli and are squeezed out via myoepithelial cell contraction. After weaning, milk stasis initiates the process of involution. The ducts and alveoli which once occupied the whole of the fatpad undergo apoptosis. The alveolar lobules collapse and ductal epithelium flatten out as adipocytes repopulate the majority of the fatpad. Apoptosis and remodeling occur until the gland resembles a pre-pregnant state [1-4].

Development and maintenance of the mammary gland relies on a continuous supply of progenitor cells capable of renewing the cell population. These progenitors must be able to proliferate and then differentiate into the luminal and basal cell types. Intercellular communication during these events is critical for successful remodeling.

1.2 Role of Adhesion Molecules in Development and Disease

While *in vivo* studies have shown that a mammary stem cell (MaSC) is able to generate an entire functional gland [5], the mechanism by which this expansion process occurs is incompletely understood. Cell-cell and cell-microenvironment interactions, often mediated by adhesion molecules (AM), are known to regulate cell behavior in multiple tissue systems. For example, desmosomal components such as desmocollins are involved in epidermal organization and differentiation [6]. Nectin, a component of adherens junctions, has been found to cluster around immature synapses

of cultured hippocampal neurons affecting position and size of synapses during neuron development [7].

Adhesion molecules are also postulated to regulate stem cell behavior in the mammary gland. Mammary duct formation occurs through the disruption in adhesion of differentiated cells to the extracellular matrix (ECM) and intercellular contacts. Integrins, a major component of focal adhesions and hemidesmosomes, are constantly upregulated and downregulated during events such as pregnancy, lactation, and postnatal development [8-9]. Integrins also link cytosolic keratins to ECM components such as laminin [10]. The cell-matrix interaction between the $\alpha_6\beta_1$ integrin and laminin is important for cell differentiation and β-casein production in human mammary development [11]. The $\alpha_6\beta_4$ integrin is found to be part of hemidesmosome formation localized between myoepithelial cells and the basement membrane which confers apoptosis resistance [12]. Deletion of the β_1 subunit from basal cells resulted in a disorganized branching of ducts, fewer side branches, and reduced regenerative potential of the epithelium [13]. The polarity of basal cell division was also affected, causing random symmetric and asymmetric division planes in contrast to symmetric parallel division to the basement membrane. Deletion of the β_1 integrin subunit in alveolar lobules decreased milk production and alveolar cell proliferative capabilities [14]. While these data provide evidence of the role of selected adhesion molecules in mammary stem cell proliferation and differentiation, it is recognized that the mammary environment is complex and that multiple regulators of cell-cell and cell-environment interactions are likely to be occurring. Strategies to

manipulate mammary stem cell behavior in defined environments through modulating AM interactions provide opportunities to define cell-cell and cell-environment interactions that contribute to proliferation.

Disruption of AMs in mammary gland development can result in cancer progression and metastasis. Mammary tumor cells expressing β_4 integrins show resistance to apoptosis, promoting tumor cell proliferation and survival while the expression of a dominant negative mutant of β_4 integrin delayed induction of tumorigenesis and metastasis [15]. Upregulated β_1 integrin signaling induces focal adhesions that enable progression of invasive carcinoma [16]. Ductal carcinoma cell lines studied in transplantation assays displayed high levels of α_6 integrin, which was associated with higher *in vivo* growth fractions along with more lesion containing ductal structures [17]. Abundance of $\alpha_6\beta_4$ integrin in some breast cancer cell lines is associated with an invasive myoepithelial phenotype, which promotes the survival and migration of breast carcinoma cells [18-19]. Loss of $\alpha_6\beta_4$ expression in the SUM-159 breast carcinoma cell line showed a 15% increase in apoptosis in three-dimensional culture and produced 67% fewer colonies in soft agar assays, affecting the tumorigenicity of the cells. *In vivo*, the absence of the $\alpha_6\beta_4$ heterodimer in SUM-159 cells decreased tumor formation by 48% compared to control [20]. Thus, like in normal tissues, AMs have a significant role in influencing behavior of aberrant mammary cells. The proliferation of mammary cell types, including the MaSC, may also be influenced by AM interaction during the dynamic remodeling and regulation of different cell types.

1.3 Tools for Investigating the Role of AMs in Mammary Biology

1.3.1 Comma1D-βgeo Cell Line

Comma1D-βgeo (C1D) cells, derived from the mammary glands of BALB/c mice, are a cell line containing multiple cell populations that can be phenotypically identified. C1D cells are a good model for studying mammary stem cells because they exhibit normal cellular function (non-tumor forming), are heterogeneous, are able to grow structures in 3-D culture, and are able to generate ductal and alveolar-like outgrowths *in vivo* [21]. C1D differentiation can give rise to mammary epithelium consisting of a basal myoepithelial layer and a luminal epithelial layer. C1D cells expressing high levels of Sca1, an antigen found in most stem cell types, were able to generate mammary ductal structures in the cleared fatpads of virgin recipient mice 5 times more readily than unfractionated C1D cells and 3.8 times more readily than the Sca-1$^{neg/low}$ C1D populations [21]. The Sca1 C1D population is also enriched for expression of CD49f (α6 integrin) and CD24 (HSA, heat stable antigen). Structures generated by these populations include ducts and terminal endbuds containing luminal and myoepithelial cell lineages [22].

C1D cells have several desirable features that make it useful and appropriate to examine the signaling pathways involved in mammary stem cell-progenitor transitions. In particular, C1D cells displays typical cuboidal epithelial-like morphology in two-dimensional (2-D) culture, casein synthesis, and duct-like

outgrowths in 3-D culture as well as in cleared fatpads [22]. Upon differentiation, C1D cells predominantly exhibit a basal phenotype, providing a model for basal cell differentiation similar to some of the more common mammary cancer types [21]. The C1D line is considered a pre-malignant breast cancer cell line because it contains mutations in the tumor suppressor protein p53. Sequencing of cDNA from the parent COMMA-1D cell line show mutations in the p53 alleles. On one allele there is a transversion of guanine to cytosine resulting in a missense mutation that replaces cysteine for tryptophan at codon 138. In the second allele, there is a deletion in the first 21 nucleotides of exon 5 [23]. With these mutations present, the daughter C1D cell line show no signs of tumor formation [21]. C1D cells therefore serve as a useful cell line for the study of AMs in mammary biology.

1.3.2 Mammary Culture

The two *in vitro* culture systems that will be used to grow C1D subpopulations are 2-D adherent tissue culture and mammosphere culture. The adherent culture model uses polystyrene dishes treated with vacuum gas plasma which creates a hydrophilic, negatively charged surface [24]. This surface facilitates adhesion where cells adhere in a monolayer fashion to the bottom of the culture dish. The mammosphere culture model is based on propagation of cells in a dish coated with a hydrophilic, neutrally charged hydrogel layer which will inhibit ionic attachment [25]. Mammosphere culture is inherently a two-

dimensional model designed to select for three-dimensional spheroid formation through the elimination of anchorage dependent cells. In mammosphere culture, cells are suspended in media where no contact is made with the culture plate. The concept of mammospheres came from approaches in neurobiology where neural stem cells cultured in low attachment conditions formed neurospheres capable of proliferation and differentiation when transplanted [26-27]. Undifferentiated cells survived in culture under non-adherent conditions while differentiated cells underwent anoikis [28]. Unlike necrosis where a cell dies of infection, toxin, or trauma, anoikis is a form of apoptosis where cell death is triggered by the lack survival signals resulting from the lack of attachment from a substrate, normally the extracellular matrix [29-30]. In both the mammosphere and neurosphere culture systems, differentiated cells undergo apoptosis due to lack of anchorage [29], whereas the remaining stem/progenitor cells have increased capability to divide and self renew without anchorage dependence [31]. Adhesion molecule mediated interactions take place between cells as a mammosphere forms. The cells that are able to survive without adhering to a substrate are able to proliferate. The propagation of mammary cells under these non-adherent conditions is able to yield spherical structures suspended in liquid media containing cells with high repopulation potential [29]. These cells are able to from functional ductal networks, demonstrating their ability to differentiate into the necessary mammary structures found in normal mammary development and function [32].

The adherent culture and non-adherent mammosphere culture systems will be used to enable exploration of adhesion gene expression profiles of mammary progenitor cells grown in either condition. The major physical difference between the two culture conditions involves AM interactions primarily with either a substrate (adherent culture) or neighboring cells (non-adherent culture). The effects of AMs on cell proliferation can be assessed by culture of mammary progenitor cells in adherent and non-adherent conditions.

1.3.3 Markers to Define Progenitor Subpopulations

Stages of mammary cell differentiation are identified with the presence of intracellular and extracellular markers which can be constructed into a hierarchy based on transitioning subpopulations. While some components of the hierarchy are established, the transitional stages and the underlying molecular regulation of the transitions are incompletely understood and the characterization of the mammary gland hierarchy is still a work in progress [33-34]. Figure 2 shows a diagram of some of the subpopulations known to be included in the mammary hierarchy. Mammary stem cells differentiate into luminal and basal progenitors expressing CD24, CD49f, and keratin 5 (K5) [5, 21, 35]. Luminal progenitors differentiate into luminal cells of the mammary duct, alveolar luminal cells of the alveolar lobule, and estrogen receptor (ER) positive luminal cells; basal progenitor cells differentiate into the myoepithelial cells surrounding the ducts and alveolar lobule [36-40]. Luminal progenitors expressing CD61, CD24, and $CD29^{low}$

differentiate into the luminal cells lining the inner ducts and alveoli [36, 38]. Myoepithelial cells display K5, keratin 14 (K14) and α-smooth muscle actin

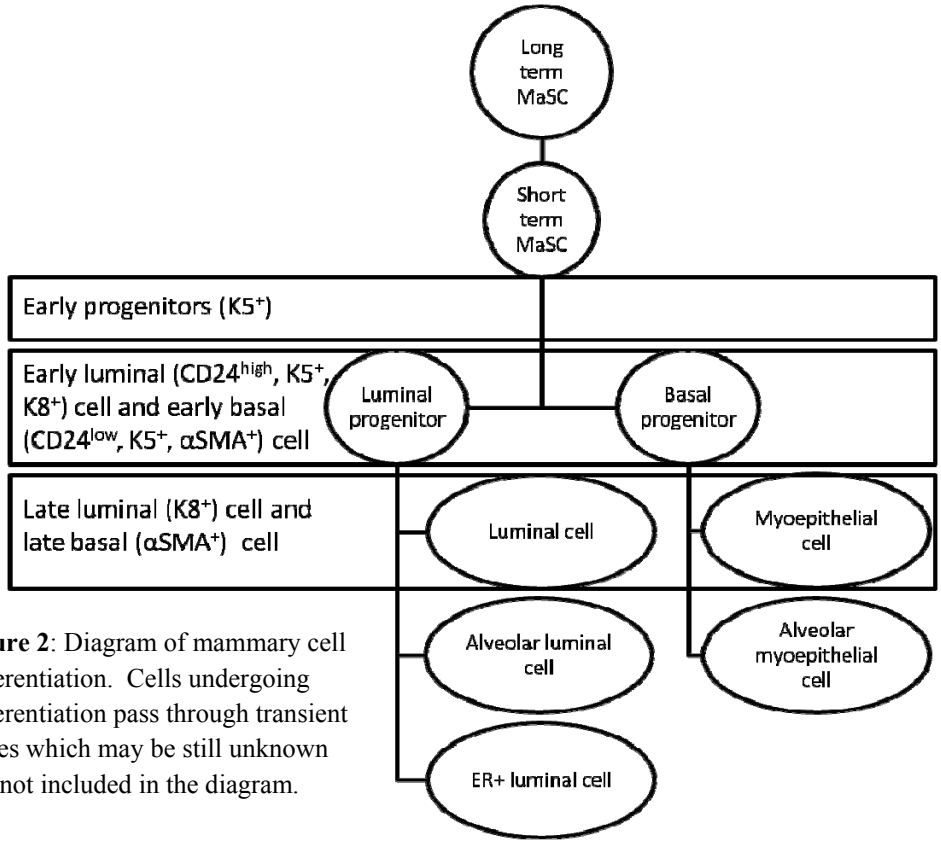

Figure 2: Diagram of mammary cell differentiation. Cells undergoing differentiation pass through transient stages which may be still unknown and not included in the diagram.

(αSMA) while luminal cells show keratin 8 (K8), keratin 18/19, and varies in ER expression [34-36, 38-40]. A list of known markers and proposed subpopulations is contained in Table 1.

The two markers used to identify the C1D subpopulations in my experiments are CD24 and CD49f. CD24, a glycosyl phosphatidylinositol-anchored protein, and CD49f, the integrin alpha 6 protein, have been found to be expressed on mammary progenitor cells as well as breast cancer stem cells.

Additionally, cells with high repopulation potential have been found to express CD24 and are highly enriched for CD49f [5, 36, 41-42]. CD24 has been used to discriminate subpopulations of primary mouse epithelial cells which have been found to express differential lineage markers and possess different repopulation potentials: the $CD24^{high}$ population represents luminal cells and the $CD24^{low}$ population represents basal cells. The $CD24^{low}$ population is also enriched for stem/progenitor activity [43].

Proposed Mammary Cell Type	Known Markers
Long term MaSC	$Sca1^{+}$, $K6^{+}$, $K14^{+}$, $K18^{+}$, $CD29^{hi}$, $CD49f^{hi}$, $CD24^{+/med}$, $CD61^{+}$, $Sca1^{lo}$, ER^{-}, PR^{-}, $ErbB2^{-}$
Sort term MaSC	$Sca1^{+}$, $K6^{+}$, $K14^{+}$, $K18^{+}$, ER^{-}, $CD24^{+/med}$, $Sca1^{lo}$, $CD29^{hi}$, $CD49f^{hi}$
Luminal progenitor	$K5^{+}$, $CD61^{+}$, $CD24^{+}$, $CD29^{lo}$
Luminal cell	$K18^{+}$, $CD61^{-}$, $CD61^{+}$, ER^{+}/ER^{-}, $Sca1^{+/-}$, $CD49f^{+}$, $CD61^{-}$, $K8^{+}$, $K19^{+}$
Alveolar luminal cell	$K18^{+}$, $CD61^{-}$, ER^{+}/ER^{-}, $CD24^{lo}$, $CD49f^{hi}$
ER+ luminal cell	$CD24^{hi}$, $Sca1^{+}$, $CD49f^{-}$, $CD14^{-}$, $CD61^{-}$, $PrlR^{+}$, PR^{+}
Basal progenitor	$K5^{+}$, $CD24^{+}$, αSMA^{+}
Myoepithelial (basal) cell	$K14^{+}$, $ER\alpha^{-}$, $K5^{+}$, $CD24^{lo}$, αSMA^{+}
Alveolar myoepithelial (basal) cell	$ER\alpha^{-}$, $CD24^{lo}$, $CD49f^{hi}$

Table 1: Known markers and groupings in mammary gland differentiation.

1.4 Hypothesis and Specific Aims

I hypothesize that adhesion genes affect the proliferation of mammary progenitors under adherence modulated conditions. C1D progenitor subpopulations discriminated by CD24 and CD49f should show differential adhesion gene expression between cells grown in adherent versus non-adherent culture conditions.

Aim 1: Establish model systems of mammary development to facilitate the investigation of adhesion genes in proliferation. Specifically, I will use CD24 and CD49f as well as flow cytometry to identify and isolate immature C1D subpopulations to determine differential proliferative and differentiation potentials assayed in 2-D adherent culture and non-adherent mammosphere culture.

Aim 2: Determine the adhesion gene repertoire of C1D cells cultured under adherent and non-adherent conditions. Specifically, I will identify differentially expressed adhesion genes in sorted C1D subpopulations by whole murine gene expression microarrays. Subpopulations based on CD24 and CD49f expression will be used to establish the relationship between phenotype and AM gene expression and to establish how these expression profiles are modulated by culture conditions. Bioinformatic approaches will be used to identify candidate signaling pathways assembled on the basis of differential gene expression data in the discriminated subpopulations modulated by each culture model.

1.5 Goal of Research

The goal of this research is to identify adhesion molecules involved in the proliferation of mammary progenitor cells under adherence modulated conditions. The use of C1D cells as a model of mammary development, including its ability to survive in adherent and non-adherent conditions, confers its usefulness and appropriateness for the proposed study to examine differentially expressed AMs taking part in the proliferative capabilities of mammary progenitor cells. The use of

adherent and non-adherent culture conditions subject cells to two different environments that might influence their AM repertoire. In turn, these different AM expression profiles may be the cause of differential rates of proliferation.

Understanding the AMs involved in progenitor proliferation decisions can help elucidate the controlled processes of mammary gland repopulation as well as the uncontrolled proliferation in tumorigenic outgrowths. Because remodeling occurs frequently in the mammary gland, cells continually rely on communication for positioning and differentiation into cell types required to from functional mammary ducts and lobules. Identifying AMs based on a cells environment can provide some insight into cues that stimulate mammary progenitor growth. This research is a step toward understanding normal mammary development which can be applied to future therapeutic treatment of abnormal proliferation events.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Culture

Comma1D-βgeo cells (gift from Dr. Daniel Medina, Baylor College of Medicine) were plated at 10,000 cells per well in 6 well tissue culture plates (BD Biosciences, Bedford, MA). Adherent cells were maintained in DMEM/F12 (Thermo Scientific, Hudson, NH) supplemented with 2% fetal bovine serum (Invitrogen, Carlsbad, CA), 5 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), 5 μg/mL epidermal growth factor (Invitrogen), and 50 μg/mL gentamicin (Sigma-Aldrich). Media was filtered through a 0.22 micron filter (Millipore, Billerica, MA). Cultures were maintained in an incubator at 37°C with 5% CO_2. Cells were collected using 0.25% trypsin-EDTA (Cellgro, Manassas, VA) and counted using Coulter Counter Z1 (Beckman Coulter, Brea, CA).

Mammospheres were plated at 15,000 cells per well into ultra-low attachment 6 well plates (Corning, Lowell, MA). Mammospheres were maintained in DMEM/F12 (Thermo Scientific), 20 μg/mL bFGF (Invitrogen), 20 μg/mL EGF (PeproTech, Rocky Hill, NJ), 1x B27 (Invitrogen), 4 μg/mL Heparin (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma-Aldrich) and 50 mg/mL gentamicin (Sigma-Aldrich). Cultures are passaged using 0.25% trypsin-EDTA (Cellgro). Mammospheres were passaged at a 1:1 concentration and imaged using a Micromaster Infinity Optics microscope (Fisher Scientific, Pittsburgh, PA) and measured using Micron™ imaging software (Version 1.09, Westover Scientific, Mill

Creek, WA). The culture of mammospheres is fairly recent with media components and culture techniques varying by laboratory [32, 44-48]. In our mammosphere cultures, concentrations of 100k, 50k, 15k, and 10k C1D cells were grown for seven days per passage. The number of spheres was counted and their area measured from photomicrographs taken at passages two and three. Qualified mammospheres were counted as those that had the area measurement of greater than three cells.

2.2 Immunocytostaining and Flow Cytometry

Protocols for immunostaining were modified from those used by the Pallavicini Lab (Heather Bryan, University of California, Merced) [49]. For surface immunostaining, cells grown on tissue culture were harvested by trypsinization and were incubated with conjugated primary antibodies in a solution of phosphate-buffered saline (PBS, Thermo Scientific) containing 2% bovine serum albumin (Sigma-Aldrich) on ice, in the dark for 45 minutes. Cells were washed and resuspended in PBS with 2% bovine serum albumin (BSA, Sigma-Aldrich) for flow cytometric analysis. Antibodies used were anti-CD24 APC-conjugated (IgG2a, clone M1/69, BioLegend, San Diego, CA) and anti-CD49f FITC-conjugated (IgG2a, clone GoH3, BioLegend), with respective isotype and N-1 controls (BioLegend). Dead cells were detected by adding DAPI to the stained cell suspension at a final concentration of 1 μg/mL.

For detection of intracellular antigens, cells were fixed in 100% methanol (Thermo Scientific) for 10 minutes on ice. Staining and washing were performed in

PBS (Thermo Scientific) containing 2% BSA (Sigma-Aldrich). Primary antibody staining was performed on ice for 45 minutes. Secondary antibody staining was performed on ice, in the dark for 30 minutes. Cells were incubated with primary rabbit anti-keratin 5 (polyclonal, Covance, Princeton, NJ), mouse anti-smooth muscle actin (IgG2a, clone 1A4, Sigma-Aldrich), for 45 minutes on ice and then with appropriate secondary donkey anti-rabbit Cy5 (IgG, Jackson Immunoresearch, West Grove, PA), and rat anti-mouse FITC (IgG2a, BD Biosciences) for 30 minutes on ice. For keratin 8 detection, an anti-keratin 8 Ab (clone Troma-1, Developmental Studies Hybridoma Bank, University of Iowa) conjugated to Alexa Fluor 680 (Invitrogen) was given to us as a gift from Dr. Jennifer Manilay (University of California, Merced). In all cases, flow cytometry was performed on either a BD Biosciences FACSAria II or BD Biosciences LSR II instrument using FACSDiva Version 6. Data was analyzed using Flowjo software version 7.6 (TreeStar Inc., San Carlos, CA).

2.3 Microarray Experiments

Freshly sorted (Day 0), adherent (day 2) and mammosphere (passage 2 day 2) C1D culture samples (1000-5000 cell) were sent to Miltenyi Biotec Genomic Services (Auburn, CA) for cDNA extraction and microarray processing. Briefly, for cDNA generation (SuperAmp Service, Miltenyi Biotec), RNA was extracted from the samples using magnetic beads and reverse transcribed using tagged random and oligo(dT) primers. First strand cDNA was generated by tagging the 5' end using 8 U terminal deoxynucleotidyl transferase (Fermentas) and incubating for 60 minutes at

37°C before heat inactivating at 70°C for 5 minutes. Tagged cDNA was globally amplified (Expand Long Template PCR System DNA Pol Mix, Roche) using primer complementary to the tag sequence by incubation at 78°C for 30 seconds, followed by 20 cycles of 94°C for 15 seconds, 65°C for 30 seconds, and 68°C for 2 minutes. This preamplification was followed by 21 cycles of 94°C for 15 seconds, 65°C for 30 seconds, and 68°C for 2.5 minutes with an extension of 10 seconds/cycle and a final step of 68°C for 10 minutes. The PCR product was purified (NucleoSpin® Extract II, Macherey-Nagel) and cDNA yield measured. Two hundred nanograms of purified PCR product was labelled with Cy3-dCTP (Amersham) in a Klenow Fragment (10 U) reaction for 1.5 hours at 37°C before inactivating through addition of 5 µl 0.5 M EDTA pH 8.0. For each microarray analysis, 1.25 µg of Cy3-labeled and purified (Cy-Scribe GFX Purification Kit, GE Healthcare) cDNAs were used.

The hybridization procedure was performed according to the "One-Color Microarray-Based Gene Expression Analysis protocol (version 5.5, #G4140-90040), using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 1.25 µg Cy3-labeled cDNA in hybridization buffer was hybridized overnight (17 hours, 65 °C) onto Agilent Whole Mouse Genome Oligo Microarrays 4x44K using Agilent's recommended hybridization chamber and oven. Following hybridization, the microarrays were washed once with 6x SSPE buffer containing 0.005% N-lauroylsarcosine for 1 minute at room temperature followed by a second wash with preheated 0.06x SSPE buffer (37 °C) containing 0.005% N-lauroylsarcosine for 1 minute. The last washing step was performed with acetonitrile for 30 seconds.

Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System G2505C (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files.

2.4 Statistical Analysis and Bioinformatics

Two-way ANOVA, t-tests, Bonferroni posttests, Tukey's test, standard deviations (SD) and standard error of the mean (SEM) were carried out using GraphPad Prism (Version 5, GraphPad Software, Inc., La Jolla, CA). SD is used to signify the variation in the sample while SEM is used to signify the mean of the population. The best representation was chosen for each experiment.

For microarray experiments, bioinformatics was done though Miltenyi Biotec GmbH. For normalization of intensity data and determination of differential gene expression, FES derived output data files were further analysed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware). Multiple testing correction was performed with the Benjamini Hochberg method to reduce the false discovery rate (R/bioconductor, multtest package). Ratios were computed for all genes as ratio of log2 intensity value relative to the median log2 intensity value of the control samples. All asterisks denote a *P*-value of less than 0.05 unless otherwise indicated.

CHAPTER 3. RESULTS

3.1 C1D Contain Subpopulations With Mammary Progenitor Phenotypes.

C1D cells are able to generate ductal and alveolar-like outgrowths *in vivo*. Therefore, I hypothesize that C1D cells must contain mammary progenitor cells expressing CD24 [5, 41, 43]. To test this hypothesis, C1D cells were stained with CD24 and CD49f, molecules known to be highly expressed in mammary stem cell populations [41]. Co-staining shows two subpopulations: One comprising $CD24^{high}CD49f^{+}$ cells and another $CD24^{low}CD49f^{-}$ (Figure 3A-B). These populations were sorted and reanalysis confirmed that the sorted cells fall within the quadrant corresponding to their prospective population (Figure 3C). C1D cells do contain a subset of cells that are positive for CD24. CD49f is only expressed on cells with high CD24 expression. Since CD24 is well documented to be a progenitor marker, both $CD24^{high}$ and $CD24^{low}$ populations will be analyzed for growth in adherent and non-adherent cultures.

Sorted cells were grown in either ultra-low attachment plates or adherent tissue culture plates. On ultra-low attachment plates, cells become suspended in media, appearing small and round. Cells then proliferated to form cell aggregates termed mammospheres (Figure 4A). On adherent culture plates, cells appeared flat and elongated with an identifiable nucleus and membrane (Figure 4B). Adherent growth spanned the surface of the dish, forming a monolayer of cells. Protocols for the culture of mammospheres vary by laboratory, differing in length of culture times and

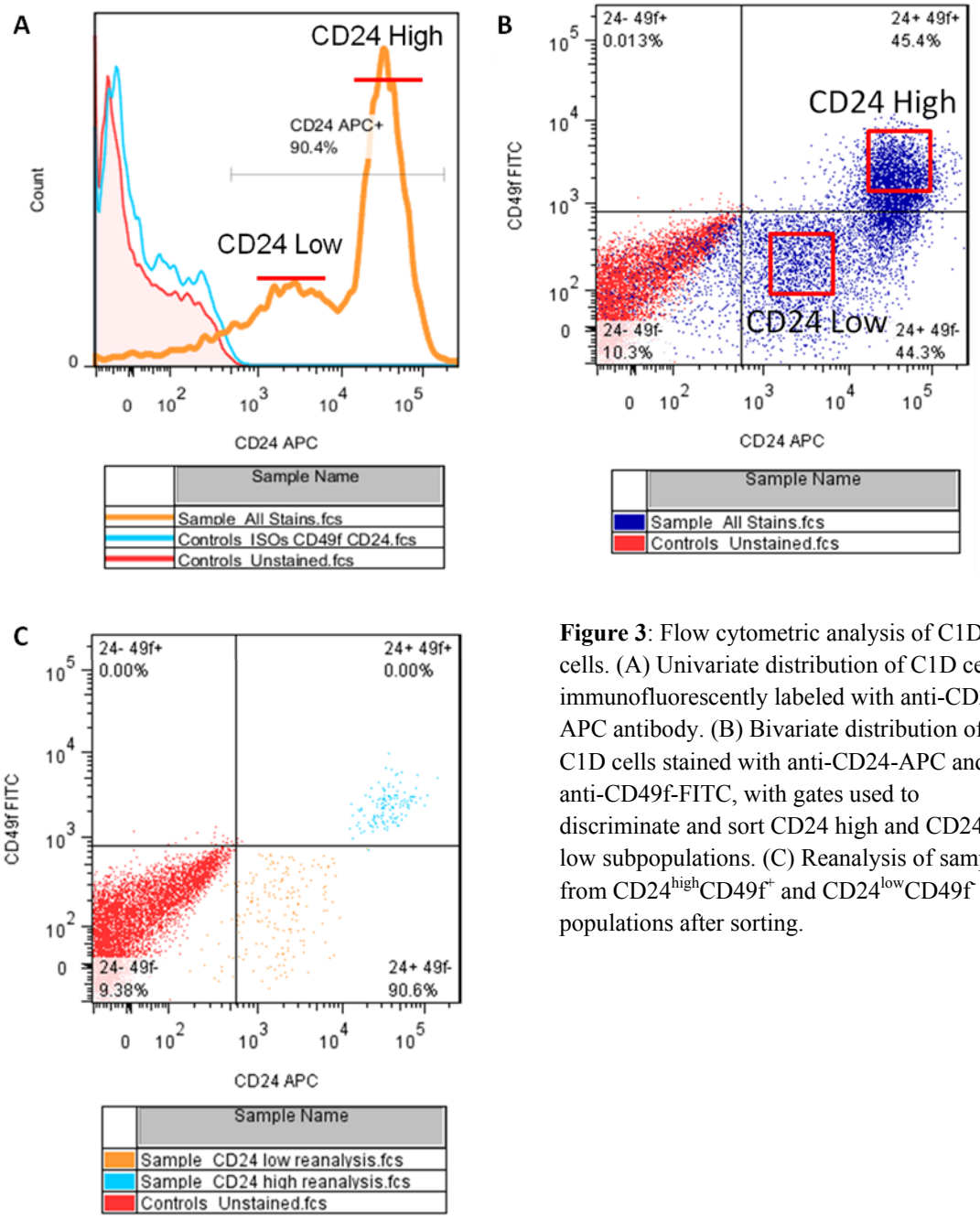

Figure 3: Flow cytometric analysis of C1D cells. (A) Univariate distribution of C1D cells immunofluorescently labeled with anti-CD24-APC antibody. (B) Bivariate distribution of C1D cells stained with anti-CD24-APC and anti-CD49f-FITC, with gates used to discriminate and sort CD24 high and CD24 low subpopulations. (C) Reanalysis of samples from $CD24^{high}CD49f^{+}$ and $CD24^{low}CD49f^{-}$ populations after sorting.

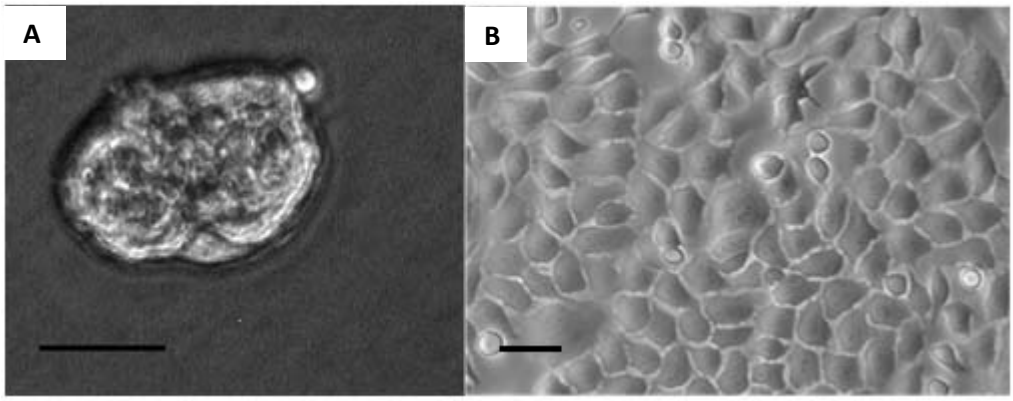

Figure 4: Photomicrographs of C1D cells in mammosphere and adherent culture. (A) Mammosphere taken at passage 2 day 2 of culture in an ultra-low attachment well at 200x magnification. (B) Cells growing in a tissue culture treated dish at 100x magnification. Black bar represents a length of 25 microns.

criteria used to identify a mammosphere [32, 44-48]. Based on these previous methods, C1D cells were tested for optimal mammosphere-forming cell concentrations and culture times. Concentrations of 100,000, 50,000, 15,000, and 10,000 C1D cells were grown for seven days per passage for three passages. The number of mammospheres was counted and their area measured from photomicrographs taken at passages two and three. Cell aggregates were not counted or measured during growth in the first passage due to (a) the cells in the first passage cluster together during plating which makes it difficult to identify a true mammosphere and (b) there is a large amount of cell death that occurs after plating, which will then leave only viable anchorage-independent cells (based on visual inspection of plates, data not shown). These two conditions prevent assessment of mammospheres formation during the first passage. For our analysis, mammospheres were considered to have a minimum area of three single C1D cells.

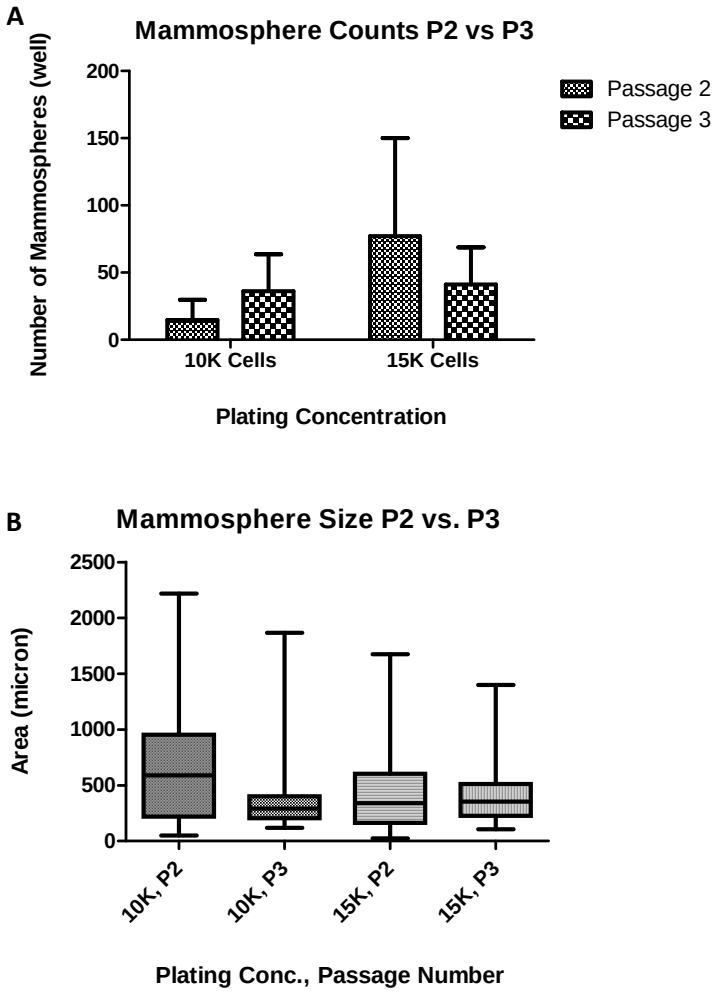

Figure 5: Mammosphere counts and sizes. Unsorted C1D cells were grown at concentrations of 10,000 (10K) and 15,000 (15K) cells per well of ultra-low attachment plates to facilitate the grown of mammospheres. Cells were collected on day 7 of passage 2 and day 7 of passage 3. Photomicrographs of the plates were used for counting and measuring the area of mammospheres. (A) Number of mammospheres in 10K and 15K ultra-low attachment wells during passage 2 (P2) and passage 3 (P3). (B) Area of mammospheres in 10K and 15K ultra-low attachment wells during P2 and P3. Statistical analysis performed with t-test of wells. Experiment done in triplicate. Error bars for mammosphere counts represent SD.

Plates seeded with 100,000 and 50,000 C1D cells showed an extensive amount of cells in suspension as well as debris from cell death. This created overcrowding with large, overlapping cell aggregates and debris which obscured mammospheres from being identified (observation from microscopy, data not shown). Counting and measurement data were collected from only the wells with initial plating of 15,000 and 10,000 cells since mammospheres could be clearly distinguished from these concentrations. There was no significant difference in the number or area of mammospheres when passage 2 and passage 3 cultures were compared (Figure 5).

Based on the apparent higher cell counts in the wells containing 15,000 cells, it was decided that the mammosphere experiments would be done using this plating concentration during the second culture passage.

To determine which day of culture to collect adherent and mammosphere samples, cells should be given enough time to acclimate and respond to their culture conditions. Cells grown in adherent culture take approximately 4-6 hours to adhere to a culture surface and another 12-24 hours to begin proliferation (observation by microscopy, data not shown). C1D cells grown in suspension show signs of proliferation as early as day two of culture (observation by microscopy, data not shown). Cell collection periods were established to be no earlier than day two of culture for adherent cells and day two during the second passage of culture for mammospheres.

$CD24^+$ C1D progenitor cells, able to survive in adherent and non-adherent conditions, should display markers characteristic of mammary basal and luminal cells. In order to identify lineage type, cell populations were collected and analyzed for the presence of keratin 5 (K5), keratin 8 (K8) and α smooth muscle actin (αSMA). Table 2 describes the phenotype of subpopulations based on the aforementioned markers, including undifferentiated bipotent progenitors (K5), luminal progenitors (K5 and K8), basal progenitors (K5 and αSMA), basal cells (αSMA) and luminal cells (K8).

Mammary Gland Cell Types and Corresponding Intracellular Markers	
Cell Type	Marker
Bipotent basal/luminal progenitor	K5
Basal progenitor	K5/αSMA
Luminal progenitor	K5/K8
Basal cell	αSMA
Luminal cell	K8

Table 2: Mammary gland cell lineages and transient cell types with corresponding phenotypic intracellular markers. Intracellular markers are keratin 5 (K5), α smooth muscle actin (αSMA), and keratin 8 (K8).

Day 0 post sort analysis of C1D $CD24^{high}CD49f^{+}$ and $CD24^{low}CD49f^{-}$ populations show high expression of basal progenitor markers and of differentiated basal cells (Figure 6A). This correlates with previous studies showing C1D as a predominately basal cell line [21]. Analysis for the adherent cells at day 2 of culture showed a slight increase in cells expressing the basal cell phenotype along with the emergence of luminal cells in both $CD24^{high}$ (4.8±2.6%) and $CD24^{low}$ (3.6±2.9%) subpopulations (Figure 6B). Mammospheres analyzed at day 2 passage 2 were heterogeneous, containing a decrease in the amount of basal cells compared to day 0 along with the appearance of undifferentiated progenitors as well as luminal and basal progenitor cells (Figure 6C). In all day 2 cultures, the amount of basal progenitors showed a decrease from day 0. Flow cytometric analysis also revealed populations based on marker expression currently not identified as a mammary cell type (data not shown). These populations had either none of the markers present ($K5^{-}/\alpha SMA^{-}/K8^{-}$), had all markers present ($K5^{+}/\alpha SMA^{+}/K8^{+}$), or had both luminal and basal markers present ($K5^{-}/\alpha SMA^{+}/K8^{+}$). A representative FACS plot of the $CD24^{low}CD49f^{-}$ population under mammosphere growth conditions is shown in Figure 7.

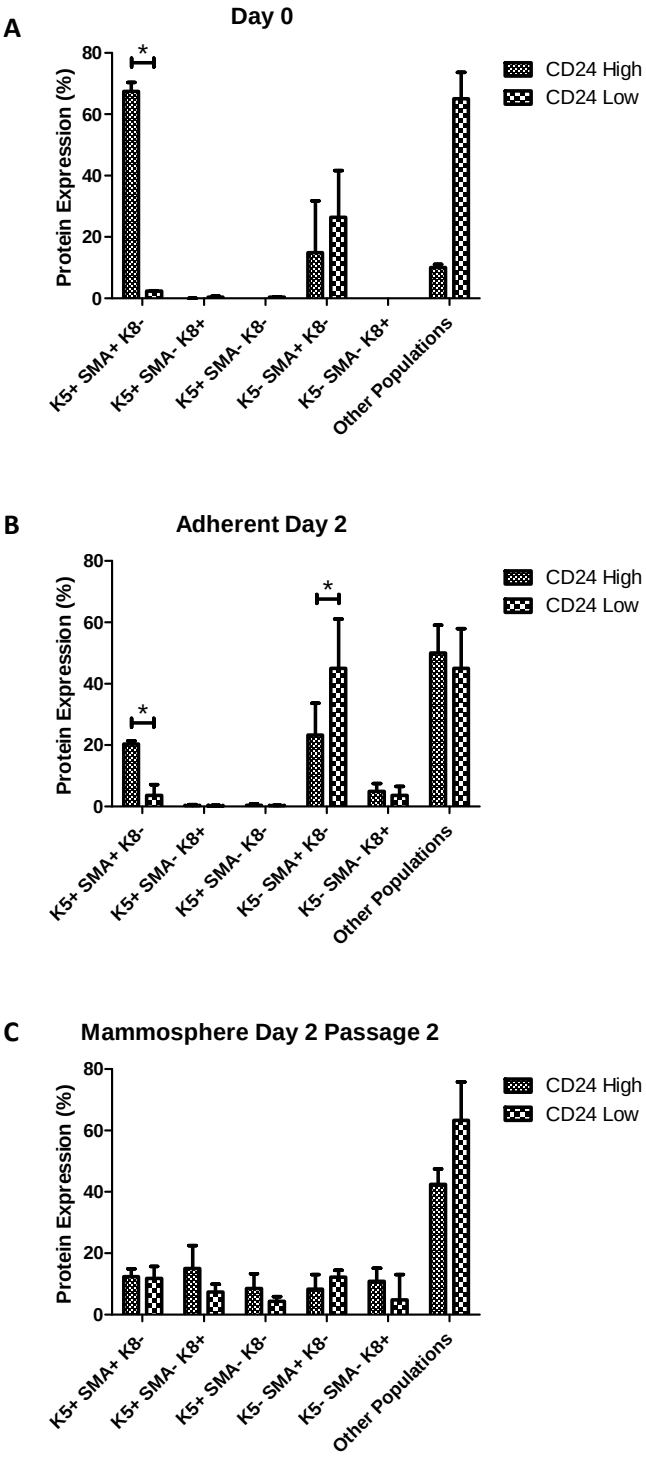

Figure 6: Cytokeratin 5, 8 and alpha smooth muscle actin expression in $CD24^{low}$ and $CD24^{high}$ subpopulations cultured in adherent and non-adherent conditions. Subpopulations were simultaneously labeled with anti-K5-PE, anti-K8-APC-Cy7 and anti-SMA-FITC. (A) Fixation of day 0 cells for keratin analysis was done immediately after flow cytometric sorting. (B) Subpopulations plated in adherent culture were grown for two days and then collected for analysis of lineage markers. (C) Subpopulations plated in mammosphere culture were grown for one passage and then analyzed for lineage markers. Data is averaged from two biological replicates done in triplicate. Statistical analysis performed by 2 way ANOVA followed by Bonferroni post tests. Asterisks denote a *P*-value of less than 0.01. Error bars represent SD.

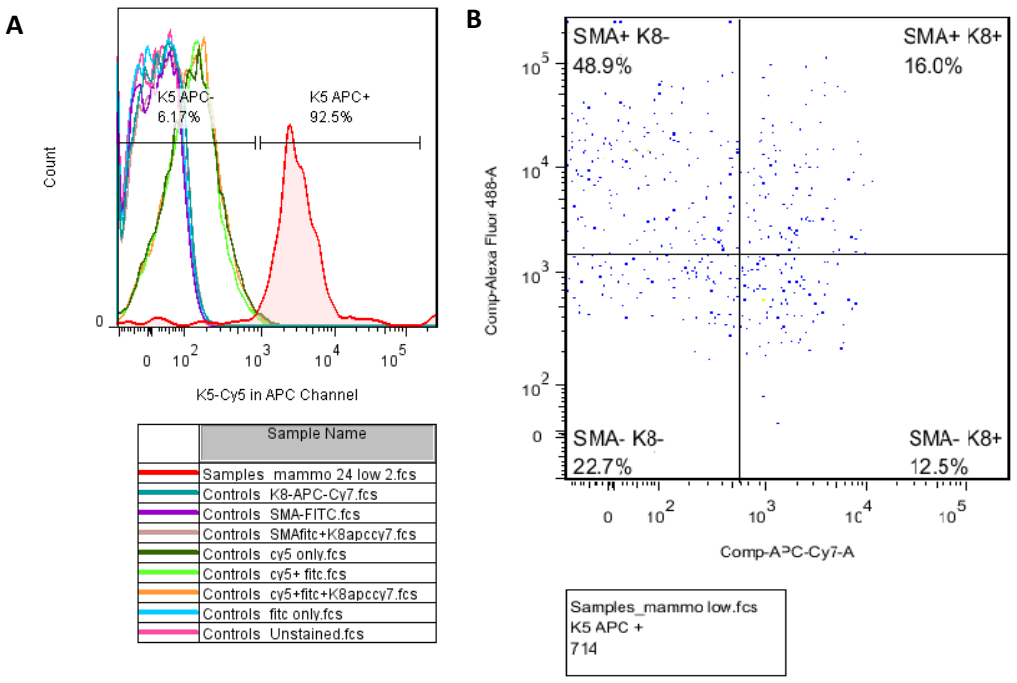

Figure 7: Example of the gating strategies used during intracellular analysis via flow cytometry. Data is from the analysis of the $CD24^{low}CD49f^{+}$ population under mammosphere culture conditions. (A) Gates for keratin 5 (K5) expression are established based on data from an unstained sample as well as a secondary antibody alone controls. A positive control or sample is used to better determine where the positive gate begins. (B) Bivariate of keratin 8 (K8) and α smooth muscle actin (αSMA) expression based on the K5 positive gate.

3.2 C1D Subpopulations Display Variable Proliferation When Placed in Differing Culture Conditions.

The different C1D cells, showing mammary progenitor phenotypes, were analyzed for their ability to propagate in adherent and non-adherent culture. For adherent culture growth, $CD24^{high}CD49f^{+}$ ($CD24^{high}$) and $CD24^{low}CD49f^{+}$ ($CD24^{low}$) cells were sorted into 6-well tissue culture plates and cell growth observed over a period of 10 days. Although both $CD24^{high}$ and $CD24^{low}$ subpopulations showed a steady increase in cell numbers, there were significant differences in the growth rates.

Time course experiments showed that the $CD24^{low}$ population has significantly higher ($P < 0.05$) cell counts compared to the $CD24^{high}$ population grown under the same adherent culture conditions (Figure 8A). This trend of increased cell division correlates with a trend previously described in literature where $CD24^{low}$ cells were found to have a high *in vivo* repopulation potential [43]. The $CD24^{low}$ subpopulation may contain a subset of progenitors that are able to proliferate faster in adherent culture. Cell numbers between $CD24^{low}$ and the unsorted population at each timepoint did not show a significant difference. Table 3 shows the ratio of $CD24^{low}$ cells compared to $CD24^{high}$ cells in adherent culture. Calculating the ratio of growth between the subpopulations based on the average cell counts at each time point, the rate of growth of $CD24^{low}$ increases from day 3 to day 8 and then drops from day 8 to day 10. The decrease in proliferation rate is due to the cells reaching confluency in the culture dish.

Mammary progenitors have the ability to form mammospheres, anchorage dependent spherical structures *in vitro* [32]. $CD24^{high}$ and $CD24^{low}$ C1D subpopulations were sorted and grown under mammosphere forming conditions. Photomicrographs of secondary mammospheres were taken and their area measured at passage 2, day 2 of culture. There is a significant difference ($P = 0.0098$) in mammosphere forming capacity between the $CD24^{high}$ and $CD24^{low}$ subpopulations, with the $CD24^{low}$ cells forming fewer spheres (Figure 8B). This is interesting since the $CD24^{low}$ cells had a higher cell count (proliferation) under adherent culture conditions. There was no significant difference in the mammosphere counts between

Adherent Culture Average Cell Counts			
Timepoint	CD24 Low (cells)	CD24 High (cells)	Growth Ratio (# of CD24 low cells per CD24 high cell)
Day 3	2491	1445	1.72
Day 5	5185	2749	1.89
Day 6	20000	6667	3.00
Day 7	21036	3501	6.01
Day 8	115000	13333	8.63
Day 9	126156	17492	7.21
Day 10	161667	46667	3.46

Table 3: Ratios of $CD24^{low}$ to $CD24^{high}$ cells in adherent culture. Cell counts are averaged from triplicate measurements in three replicate samples.

unsorted C1D cells and the $CD24^{high}$ subpopulation. Comparing cell proliferation of each population between the adherent and mammosphere culture, the $CD24^{high}$ population displays a higher growth rate in the mammosphere culture, but not in the adherent culture. The $CD24^{low}$ population displays a higher growth rate in the adherent culture, but not in the mammosphere culture.

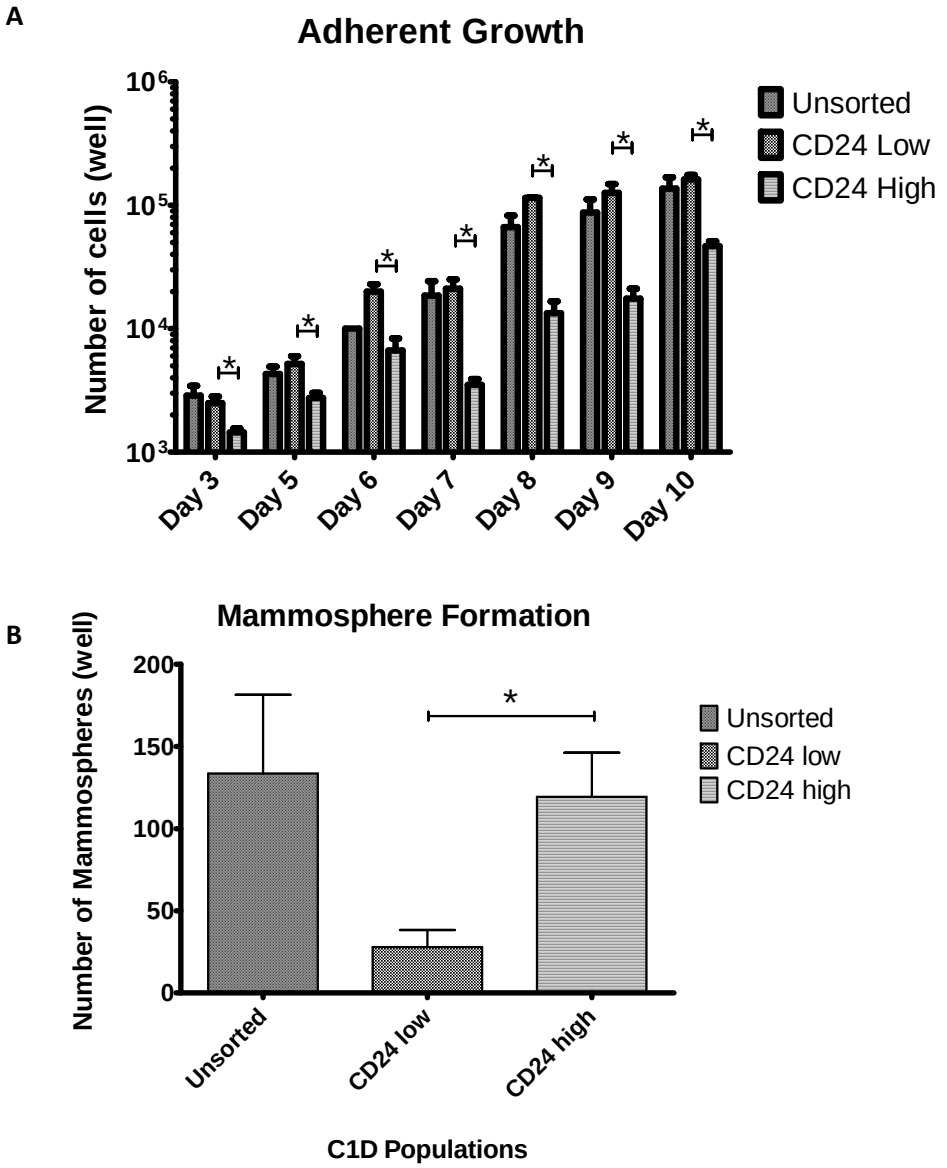

Figure 8: Differential proliferation of CD24 subpopulations cultured in adherent and mammosphere conditions. (A) Sorted CD24 subpopulations were plated in adherent culture for 10 days. Cells were counted by a Z1 Coulter Counter. (B) Mammospheres were enumerated at passage 2, day 2 of culture. Mammospheres were measured and counted from photomicrographs using Micron software. Data represent the average of triplicate measurements in three replicate samples. Asterisks denote a *P*-value of less than 0.05. Statistical testing performed by ANOVA followed by Tukey's multiple comparison tests. Error bars presented in SEM.

3.3 Microarray analysis of C1D subpopulations.

C1D subpopulations have shown differential growth in adherent and mammosphere conditions. Therefore, I hypothesize that adhesion molecules are differentially expressed in adherent and mammosphere culture conditions which contribute to the differential growth rates of the $CD24^{high}CD49f^{+}$ and $CD24^{low}CD49f^{-}$ populations. In order to identify the regulation of adhesion genes within the different culture conditions, gene expression data was gathered on differentially expressed adhesion and proliferation genes between the day 0 populations and the adherent or mammosphere populations. Microarray expression profiles were compared between day 0 and either the adherent or mammosphere sample in both $CD24^{high}CD49f^{+}$ and $CD24^{low}CD49f^{-}$ populations.

Microarray analysis returned expression data for approximately 41,176 genes, which were categorized by their gene ontology of cellular behavior and filtered to find descriptions that matched terms for cell adhesion and cell proliferation. Genes found to be significantly expressed ($P < 0.05$) in all comparisons are listed in Table 4. Comparisons between $CD24^{high}CD49f^{+}$ ($CD24^{high}$) and $CD24^{low}CD49f^{-}$ ($CD24^{low}$) populations in each culture condition yield a common list of significantly differentially expressed genes with exception to *Cfdp1* (craniofacial development protein) and *Itgb3* (β3 integrin). Comparison between the culture conditions with respect to the $CD24^{low}$ population also yields a unique set of genes, but a comparison between the culture conditions and the $CD24^{high}$ population reveals a common set of

Population	Gene Symbol	Fold Expression	*P*-value	Gene Name
Adherent day 2 CD24hi vs. Day 0 CD24hi	*Cd24a*	-8.513	0.023	CD24a antigen
	Cdc42	-4.709	0.018	Cell division cycle 42 homolog
	Cfdp1	-6.676	0.021	Craniofacial development protein 1
	Ctnnb1	-8.043	0.021	Catenin beta 1
	Itgb1	-7.735	0.023	Integrin beta 1
	Rac1	-8.477	0.023	RAS-related C3 botulinum substrate 1
	Sox9	-7.217	0.013	SRY-box containing gene 9
	Bcl2	5.162	0.007	B-cell leukemia/lymphoma 2
	Itgb3	5.852	0.021	Integrin beta 3
Adherent day 2 CD24lo vs. Day 0 CD24lo	*Itgam*	-6.224	0.020	Integrin alpha M
	Cdh13	3.897	0.025	Cadherin 13
	Spn	3.674	0.024	Sialophorin
Mammosphere day 2 CD24hi vs. Day 0 CD24hi	*Cd24a*	-7.564	0.025	CD24a antigen
	Cdc42	-3.565	0.012	Cell division cycle 42 homolog
	Cfdp1	-5.815	0.022	Craniofacial development protein 1
	Ctnnb1	-8.150	0.017	Catenin beta 1
	Itgb1	-8.228	0.017	Integrin beta 1
	Rac1	-10.360	0.014	RAS-related C3 botulinum substrate 1
	Scye1	-10.360	0.014	Small inducible cytokine subfamily E, member 1
	Sox9	-6.648	0.011	SRY-box containing gene 9
	Bcl2	4.509	0.009	B-cell leukemia/lymphoma 2
	Itgb3	6.279	0.018	Integrin beta 3
	Spn	3.286	0.019	Sialophorin
Mammosphere day 2 CD24lo vs. Day 0 CD24lo	*Cd9*	-7.514	0.037	CD9 antigen
	Cdkn2a	-8.365	0.024	Cyclin-dependent kinase inhibitor 2a
	Cfdp1	-8.024	0.035	Craniofacial development protein 1
	Dlg5	-5.886	0.040	Discs, large homolog 5
	Slit2	-8.809	0.029	Slit homolog 2
	Fgf6	5.966	0.038	Fibroblast growth factor 6
	Itgb3	6.060	0.020	Integrin beta 3
	Tnc	3.901	0.020	Tenascin C
Day 0 CD24hi vs. Day 0 CD24lo	--	--	--	--

Table 4: Differentially expressed genes that are identified as both cell adhesion and cell proliferation according to gene ontology. Negative fold expression indicates genes that have been downregulated in the first population compared to the second population. Positive fold expression indicates genes that have been upregulated in the first population compared to the second population. *P*-values were obtained by Miltenyi through individual t-test and Benjamini Hochberg multiple testing correction. Fold expression presented on a $\log_2$ scale.

regulated genes with exception to *Scye1* (small inducible cytokine subfamily E, member 1) and *Spn* (sialophorin). *Spn* was the only gene which showed a significant increase in fold change in the higher proliferating populations, but not in the lower proliferating ones.

Comparing day 0 populations, significant fold changes are expected for the genes encoding CD24 (*Cd24a)*, CD49f (*Itga6*), K5 (*Krt5*), K8 (*Krt8*), and αSMA (*Acta2*). Surprisingly, no significant differences in fold expression were found in genes categorized under cell adhesion and cell proliferation between day 0 controls. Focusing on fold expression changes, I looked at the expression of *Cd24a*, *Itga6*, *Krt5*, *Krt8*, and *Acta2* used to distinguish C1D subpopulations and specific cell types. Table 5 lists fold changes in each gene from the $CD24^{high}CD49f^{+}$ and $CD24^{low}CD49f^{-}$ subpopulations. The $CD24^{high}CD49f^{+}$ subpopulation was used as the baseline for comparison to $CD24^{low}CD49f^{-}$. $CD24^{high}CD49f^{+}$ cells expressing *Cd24a* show a fold expression of -0.22 while $CD24^{low}CD49f^{-}$ cells have a *Cd24a* fold expression of -4.91. This indicates that $CD24^{low}$ cells have a lower expression of *Cd24a* compared to $CD24^{high}$ cells which is what we expect to see. Cells with no CD49f expression had an *Itga6* fold expression of -0.22, which is lower than the -0.07 of CD49f expressing cells. The trend in fold expression of *Cd24a* and *Itga6* complements the expression data from flow cytometric analysis in Figure 3. Looking at intracellular markers in Table 5 and comparing them with literature, the $CD24^{low}CD49f^{-}$ population should express more basal cell markers (*Krt5*, *Acta2*) while the $CD24^{high}CD49f^{+}$ population should express more luminal cell markers (*Krt5*, *Krt8*) [43]. Expression of *Krt5* was

much higher in the $CD24^{high}CD49f^{+}$ population than in the $CD24^{low}CD49f^{-}$ population (0.01 versus −3.87). The basal cell gene *Acta2* also shows a higher expression in

Population	Marker	Gene Symbol	Average fold expression
$CD24^{high}/CD49f^{+}$	CD24	*Cd24a*	-0.22
	CD49f	*Itga6*	-0.07
	K5	*Krt5*	0.01
	K8	*Krt8*	-0.58
	αSMA	*Acta2*	0.18
$CD24^{low}/CD49f^{-}$	CD24	*Cd24a*	-4.91
	CD49f	*Itga6*	-0.22
	K5	*Krt5*	-3.87
	K8	*Krt8*	0.44
	αSMA	*Acta2*	1.79

Table 5: Microarray analysis show gene expression changes of C1D sorted subpopulations. The $CD24^{high}/CD49f^{+}$ population was used as the baseline for comparison of fold change. Data from cells collected at day 0. Average fold expression is calculated from subpopulation samples done in triplicate and presented in log2.

$CD24^{low}CD49f^{-}$ cells compared to $CD24^{high}CD49f^{+}$ cells (1.79 versus 0.18). This corresponds to the basal marker expression data for K5 progenitors and basal-only αSMA cells seen at day 0 in Figure 6. Expecting a higher expression of *Krt8* in $CD24^{high}CD49f^{+}$ cells, the gene expression of *Krt8* was actually higher in the $CD24^{low}CD49f^{-}$ population (0.44) compared to the $CD24^{high}CD49f^{+}$ population (−0.58). The gene expression data correlate with flow cytometric analysis of surface and intracellular markers with the exception of *Krt8*. The K8 marker quantified from flow cytometry had the opposite expression pattern compared to the *Krt8* gene quantified by microarray.

These data have shown that C1D cells do contain progenitor populations exhibiting varying levels of CD24 couple with the presence or absence of CD49f. The CD24 subpopulations have differing proliferation potentials in the different culture conditions. Cells expressing $CD24^{low}$ grown in adherent culture have a higher proliferation rate compared to $CD24^{high}$ cells. Cells expressing $CD24^{high}$ grown in non-adherent culture form more mammospheres compared to $CD24^{low}$ cells. Intracellular analysis showed that C1D cells, along with phenotypes not yet identified in the mammary hierarchy, exhibit predominately basal cells ($K5^{+}\alpha SMA^{+}$ and αSMA^{+}). These basal phenotypes were expressed in cell subpopulations grown in adherent culture while subpopulations in non-adherent culture contained a variety of basal and luminal cells including progenitor populations ($K5^{+}$, $K5^{+}\alpha SMA^{+}$, αSMA^{+}, $K5^{+}K8^{+}$, and $K8^{+}$). Microarray analysis of all subpopulations revealed a common upregulated gene Sialophorin (*Spn*, CD43) present in those populations showing higher proliferation.

CHAPTER 4. DISCUSSION

Proliferative changes seen in C1D subpopulations are dependent on adherent and non-adherent culture. The availability of substrate attachment affects gene expression which controls proliferation. In this study, we have seen that there were more cells in $CD24^{low}$ compared to $CD24^{high}$ in adherent conditions, but mammosphere formation is greater in $CD24^{high}$ compared to $CD24^{low}$ in non-adherent conditions. The identification of adhesion molecules from microarray data can give an indication of their effect on the differential growth rates seen in the C1D subpopulations. The adherent and non-adherent cultures differ in the available substrate for cell attachment which can alter the adhesion molecule repertoire and in turn alter proliferation.

Sorting C1D populations for CD24 expression divides the population into luminal ($CD24^{high}$) and basal ($CD24^{low}$) progenitor phenotype [21]. The high enrichment of CD49f in mammary stem cell populations seen in literature was only expressed in our $CD24^{high}$ population. Flow cytometric reanalysis of the sorted populations show the fluorescent expression of CD24 and CD49f shifting to dimmer levels than seen from initial sorting gates. Since fluorescence is quantified by intensity, cells showing a shift in their expression have lost intensity during the time between sorting and reanalysis. This shift seen in the populations is caused by either the continued endocytosis, breakdown, and renewal of surface proteins which can cause the loss of fluorescent markers, or degradation of the fluorescent protein through

time and exposure to light, remain consistently distinct from each other and display appropriate expression of the markers from which they were selected.

Cells grown in mammosphere culture formed aggregates as early as one day after plating. Aggregates did not have uniform shape and individual cells within each aggregate were distinguishable by clearly defined cellular membranes. Apparently, early aggregate formation was due to the clustering of cells when plated. Clustering alone did not promote survival of cells, as wells observed at day seven of culture show an increase in apoptosis indicated by fewer clusters than seen in day 2 (data not shown). Cells were passaged in order to separate aggregates and remove cell debris. On the second passage, there were visually fewer cells with no presence of aggregates carried over from the first passage. Spheres formed on the second passage were analyzed for mammosphere formation.

C1D mammosphere growth was tested with several different cell concentrations (10,000, 15,000, 50,000, and 100,000). The concentrations of 50,000 and 100,000 cells per plate caused overcrowding which resulted in the formation of cell clusters that were indistinguishable from each other. After passaging, large amounts of cell debris remained in the wells making it difficult to see live cells. Viable cells in the second passage were numerous and showed signs of aggregation as seen during initial plating. These high concentrations did not serve as good plating densities due to excessive cell aggregation and debris and were not analyzed for mammosphere formation.

The 10,000 and 15,000 plating concentrations were low enough to have minimal cell debris and distinguishable mammospheres during the second passage. Comparing mammosphere size between concentrations, the 10,000 cell per well concentration seemed to have larger mammospheres compared to 15,000 cells per well concentration at passage 2, but not at passage 3. From visual inspection, wells were not crowded with spheres, making it unlikely that the size difference was dependent on available growth area. While the size of the mammospheres was not a factor in the measurement of sphere formation capability, deviations in size could indicate a cell and its progeny's ability to proliferate more efficiently than others. From these two populations, the 15,000 cells per well plating density had an overall greater number of mammospheres per passage and was used for subsequent cultures.

From intracellular analysis of K5, K8 and αSMA, both $CD24^{high}$ and $CD24^{low}$ C1D subpopulations show an overall basal phenotype when cultured in adherent conditions, but are phenotypically more heterogeneous in mammosphere culture. Progenitors growing in mammosphere culture are able to produce a higher amount of luminal cell types than in adherent culture. Mammosphere culture could be promoting specific cues for progenitor cells to differentiate into both luminal and basal phenotypes as opposed to adherent culture producing a more basal phenotype. Since the mammosphere assay was originally carried out to enrich for progenitor cells by apoptosis of differentiated cell types, the positioning of the progenitor cells in a sphere promote communication required for the differentiation of luminal and basal phenotypes needed for mammary gland development. While previous studies using

primary human mammary cells have given us a snapshot at one timepoint to the position of luminal and basal cell positions [32, 48], multiple time points would can show us spatial changes based on mammosphere growth.

Intracellular analysis in Figure 6 show cell phenotypes contained in the $CD24^{high}$ and $CD24^{low}$ populations. At day 0, $CD24^{high}$ and $CD24^{low}$ cells were fixed immediately after sorting and subsequently stained for K5, K8, and αSMA. Both day 0 CD24 populations express more basal progenitor and basal phenotypes than luminal progenitor and luminal phenotypes as defined in Table 2. Although the C1D cells are described to have an overall basal phenotype, $CD24^{high}$ mammary populations should contain more luminal phenotypes compared to the $CD24^{low}$ populations [21, 43]. The appearance of more basal versus luminal phenotypes in the adherent culture of C1D cells in Figure 6A-B suggests that culture condition contributes to the types of cells we see in the population. Day 0 cells (Figure 6A) were expanded in adherent culture prior to analysis. Adherent day 2 cells (Figure 6B) underwent continued growth in adherent conditions. Constant substrate contact may have resulted in reduced luminal expression compared to a previous study where freshly isolated primary mammary tissues contain luminal phenotypes [43].

Analysis of the adherent day 2 cells show an increase in late basal cell phenotype of approximately 9% in the $CD24^{high}$ population and 19% in the $CD24^{low}$ population compared to the basal progenitor phenotype (Figure 6B). This change may be explained by progenitor cells maturing to basal cells during the culture time. The presence of the luminal phenotype was present during day 2 adherent cultures which

suggest that maturity into a luminal cell is also occurring, but may occur slower than basal maturity; or differentiate at the same rate, but show lower numbers due to the low presence of luminal progenitors at day 0 analysis.

Phenotypes present in mammosphere culture showed more variation between luminal and basal cells, suggesting that the mammosphere culture allows for the presence of luminal progenitors and luminal cells more than in adherent culture. All known mammary phenotypes in the mammosphere culture were under 20%, most likely from compensation for the increase in the number of total cell types present (Figure 6C). Associating either the $CD24^{hi}CD49f^{+}$ or $CD24^{lo}CD49f^{-}$ subpopulation exclusively to a luminal or basal cell type could not be established due to both subpopulations exhibiting phenotypes for both luminal and basal cells. Unlike previous studies from primary mammary cells [41, 43], our intracellular data does not clearly show a division between luminal and basal cell populations in C1D cells using solely CD24 and/or CD49f as distinguishing markers. Microarray analysis of C1D cells present for intracellular basal marker αSMA or luminal marker K8 can be mined for the presence of surface marker proteins which then can be tested to better determine the phenotypic makeup of mammary cell types.

Intracellular data revealed the presence of other cell populations containing none of the specified makers ($K5^{-}\alpha SMA^{-}K8^{-}$), all of the specified markers ($K5^{+}\alpha SMA^{+}K8^{+}$), or markers for both late basal and late luminal cells ($K5^{-}\alpha SMA^{+}K8^{+}$). The presence of these phenotypes has not currently been established in literature for the identification of any particular mammary cell type. The cells with

none of the specified markers ($K5^-\alpha SMA^-K8^-$) could be progenitors that have yet to express K5. These cells may include mammary stem cells which give rise to $K5^+$ progenitors. The cell populations that have all markers for both basal and luminal cells ($K5^+\alpha SMA^+K8^+$ and $K5^-\alpha SMA^+K8^+$) may represent transient populations which have yet to be categorized. Further experiments are necessary to tease out where these populations belong in the mammary differentiation hierarchy.

Mammosphere formation indicated that the $CD24^{low}$ population, showing higher proliferation rates in adherent culture, did not produce as many mammospheres as the $CD24^{high}$ population. In order for a cell cluster to be considered a mammosphere, the area of the sphere had to be at least three times the area of a single cell. Figure 8B shows that the $CD24^{high}$ population had more spheres, but it does not compare how many single cells or smaller clusters were present in the two populations. The data does not measure cell survival, but only cell proliferation. It can be taken from the data that $CD24^{high}$ cells were able to proliferate more than $CD24^{low}$ cells in non-adherent conditions, but it cannot be said that $CD24^{high}$ cells were able to survive better than $CD24^{low}$ cells. It is possible that the $CD24^{low}$ population may have a greater number of single cells that are quiescent rather than having proliferating mammospheres. Interestingly, in both adherent and mammosphere culture, populations that had the higher cell counts were similar in number to their respective controls. It would be expected that in the C1D control population, the cell counts would be somewhere in between the faster and slower growing populations since it contains cells from both subpopulations. For adherent

growth, unsorted C1D cells have counts in between those of the $CD24^{low}$ and $CD24^{high}$ populations. In mammosphere formation, the C1D unsorted population had mammosphere counts higher than the $CD24^{high}$ population. This may suggest that the populations with lower counts ($CD24^{low}$ population) may be somehow suppressed in their proliferation. Another theory could be that the control population, containing both $CD24^{low}$ and $CD24^{high}$ expressing cells, has a synergistic property that increases mammosphere formation.

Mammary progenitor cell survival and proliferation are dependent on a number of regulatory signaling pathways. It has been previously suggested that cadherin/beta-catenin interaction in the Wnt signaling pathway promotes self renewal in mammary progenitors [29]. Differentially regulated genes from the microarray data that fall under or crosstalk in this pathway are *Ctnnb1* (cadherin associated protein beta 1), *Rac1* (Ras-related C3 botulinum substrate 1), and *Cdc42* (cell division cycle 42 GTP binding protein). *Ctnnb1* encodes for β-catenin while CDC42 coupled with RAC1 are involved in cytoskeletal arrangement, acinar structure formation, and in promoting DNA synthesis which can aid in cell proliferation [50-51]. These genes were downregulated in $CD24^{high}$ adherent cultures, which may be linked to $CD24^{high}$ populations exhibiting low proliferation rates. However, the same downregulation was found in $CD24^{high}$ mammospheres which exhibit higher proliferation rates. This could mean that mammospheres have a mechanism different from adherent cells responsible for proliferation.

From the microarray dataset, there were no genes significantly expressed ($P < 0.05$) between $CD24^{high}$ and $CD24^{low}$ day 0 populations. Table 5 compares the fold change in each gene used to distinguish our C1D subpopulations compared with day 0 controls. The changes in fold expression in Table 5 follow the trends seen in the intracellular analysis. From literature, $CD24^{high}$ cells indicate luminal phenotype and should have increased expression of K8 [43]. Microarray data in Table 5 shows that *Krt8* has a lower expression in $CD24^{high}$ cells than in $CD24^{low}$ cells at day 0. Looking at day 0 keratin analysis of K8 populations in Figure 6A, a majority of $CD24^{high}$ cells do have a $K8^{-}$ phenotype. The high percentage of unidentified mammary phenotypes in $CD24^{low}$ cells contain $K8^{+}$ cells and can explain the higher fold K8 expression compared to $CD24^{high}$.

Fold-changes that are seen in the genes between subpopulations may be a result of the culture condition. The culture condition, altering levels of gene expression, in turn influences proliferation rates. In order to find genes possibly involved in proliferation of C1D subpopulations, the similarities and differences in fold expression of genes from each subpopulation should be analyzed. From the filtered subset of adhesion and proliferation genes contained in Table 4, *Bcl2* was found to be commonly upregulated in $CD24^{high}$ populations. BCL2 is an anti-apoptosis protein in the epithelial cells of mammary ducts which is downregulated during involution and upregulated after remodeling events [52]. The log2 fold expression of *Bcl2* increased from day 0 (0.276) to day 2 (5.162) of $CD24^{high}$ adherent culture. The upregulation of *Bcl2*, leading to a decrease in apoptosis, would be one

explanation to why the CD24^{high} population has a greater number of mammospheres (Figure 8B). However, *Bcl2* was also upregulated in CD24^{low} adherent (4.992) and mammosphere (5.697) culture as well as CD24^{high} mammosphere culture (4.509). The lower cell counts in CD24^{high} adherent culture do not explain the role of *Bcl2* in the same context as in the CD24^{high} population, suggesting that BCL2 either does not directly affect cell proliferation or its regulation is obscured by other factors present in the different populations.

Comparing CD24^{low} populations in the different culture conditions ("Adherent day 2 CD24lo vs. Day 0 CD24lo" and "Mammosphere day 2 CD24lo vs. Day 0 CD24lo") resulted in no identical genes being regulated. However, when comparing the CD24^{high} populations in the different culture conditions, *Spn* and *Scye1* were the only two genes significantly regulated ($P > 0.05$) in CD24^{high} mammospheres and not in CD24^{high} adherent.

In the proliferation study, the populations that showed increased proliferation were CD24^{low} in adherent culture and CD24^{high} in mammosphere culture. Contrasting the filtered genes from microarray data, Sialophorin (*Spn*), also known as CD43, is found to be upregulated in both high proliferative subpopulations. The log2 fold change in these conditions were higher than in the lower proliferating subpopulations (1.136 in adherent CD24^{high} and 2.124 in mammosphere CD24^{low}) and much higher than in day 0 (-0.635). Sialophorin is a glycosylated transmembrane protein commonly expressed on the cell surface of lymphocytes, which is involved in altering cell adhesion as well as promoting growth and survival [53-55]. Current research

shows SPN expression to be highly upregulated in granulocytic and myeloid sarcomas in the mammary gland [56-59]. Studies using different non-hematopoietic cancer cell lines demonstrate that CD43 is capable of increasing colony formation and cell growth in the absence of tumor suppressor protein p53 or alternate reading frame (ARF) as cells carrying p53 mutations are unable to trigger ARF-dependent apoptosis [54, 60]. C1D cells are considered a pre-malignant breast cancer cell line because it expresses mutations in p53 [23]. Along with *Spn*, *Scye1* was also significantly regulated in $CD24^{high}$ mammospheres. *Scye1* is induced by apoptosis [61], in which its negative expression may be linked to the positive expression of *Spn*. The upregulation of *Spn* in adherent $CD24^{low}$ and non-adherent $CD24^{high}$ cells, but not in other C1D populations, suggests that it may affect cell proliferation. Further studies are needed to determine the exact effect of SPN on mammary progenitors.

With the limited number of significantly expressed genes categorized under both adhesion and proliferation, none of these genes have been established as a direct link to affecting cell proliferation. From the intracellular analysis of K5, K8, and αSMA, it is difficult to define mammary cell types distinct to either $CD24^{high}$ or $CD24^{low}$ population, but it is clear from the growth data that culture conditions do have an effect on the proliferation of cell populations based on the level of CD24 expression. The changes in proliferation seen between adherent cells and mammosphere formation, combined with microarray analysis, identifies *Spn* as being a likely candidate involved in the regulation of proliferation and survival of these cells.

The research of mammary regulatory proliferation and differentiation events is a long ongoing process. The heterogeneity in subpopulations, the varying growth patterns, and gene expression changes have yet to be understood. This research provides a small glimpse at the complexities inherent of mammary gland development. Understanding regular mammary developmental processes is the critical first step into understanding irregular developmental occurrences such as the formation and metastasis of mammary tumors. The mechanisms occurring in normal development provides the underlying etiology of abnormal events. Continued research in mammary biology is needed to acquire a greater understanding of cell cycle and lineage differentiation as well as creating new therapies in fighting breast cancers.

CHAPTER 5. FUTURE DIRECTIONS

Additional experiments in future studies can be carried out to better explain results as seen in the current study. In intracellular analysis, there were populations that did not fall into a basal, luminal, or progenitor phenotype as described in Table 2. To better identify these cells, we can use additional markers seen in Table 1 which are known to be expressed in the mammary cell hierarchy. For example, the cell population lacking K5, K8, and αSMA expression can be reanalyzed for the expression of keratin 6, a marker expressed by progenitors in early prenatal development of the mammary gland [62-63].

Populations expressing both basal and luminal markers may represent transitional populations that have not yet been categorized. To gain understanding of where these populations are during differentiation, cells may be grown in both adherent and mammosphere cultures and analyzed at multiple time points to examine when K5, K8, and αSMA expression deviates from profiles seen at day 0. The timeline of expression from these populations or of further isolated subpopulations can identify any transient populations occurring before or after our day 2 expression dataset. The results from the temporal data can identify where in the differentiation hierarchy the unknown populations lie.

Data from mammosphere formation experiments could include the area of the sphere along with the number of single cells in culture. Although size will vary with each mammosphere, significant changes in area may be able to indicate the overall

proliferative ability of a subpopulation. Since the mammospheres grow suspended in culture, proliferation increases the width and depth of the sphere. Since no sphere is perfectly round, a measurement of area may not be as accurate as a measurement of volume or cell density to determine sphere size. Flow cytometric techniques are able to measure cell count, size and volume which can help correlate the characteristics of a high or low proliferating mammosphere [64].

In the same non-adherent culture, we can get a better idea of which subpopulations contain quiescent cells. Quiescent populations may contain stem-like progenitors [65-66]. Since all cultures were plated at the same density. The ratio of single cell to mammosphere will indicate a measurement of quiescence found in each subpopulation.

Identifying phenotypes within a mammosphere is important in determining how cellular contact and positioning affects differentiation. Cells in a mammosphere may differentiate depending on the time of culture. Locating which parts of a mammosphere show lineage differentiation to a basal or luminal cells tells us how they have been affected by their position in the mammosphere. Fixation, staining, and analysis using confocal microscopy gives us a three-dimensional image of the mammosphere including where a fluorescent marker is located. Finding trends using multiple mammospheres builds a picture of where differentiation starts and what cell types a certain region produces. A similar study can be done growing mammary progenitors in matrigel where cells have a scaffold environment to from mammary structures.

The regulators of proliferation in normal mammary development are incompletely understood. The microarray analysis identified sialophorin being upregulated in cultures exhibiting high proliferation. Whereas sialophorin was originally thought to be expressed exclusively on hematopoietic cells, it has recently been shown to be upregulated in mammary tumors and are involved in apoptotic regulation in non-hematopoietic cells [54, 56, 67-68]. SPN may have a profound effect on the viability and proliferation potential of cultured cells and mammospheres. To expand the current microarray analysis, the expression of *Spn* should be validated by RT-qPCR while the level of protein could be validated by western blot. These experiments would confirm the presence of SPN in the C1D subpopulations and complement the microarray analysis of fold expression changes.

To determine if SPN plays a role in the proliferative ability of the sorted C1D populations, modulation of SPN can be performed via siRNA knockdown. Proliferation experiments on the SPN modulated subpopulations would indicate if SPN had any effect on their proliferation rates. Moreover, culture of SPN modulated cells under non-adherent conditions would indicate if SPN plays a role in mammosphere formation or if it affects the number of mammospheres generated.

Within the microarray data, additional ontological categories can be analyzed to establish a more extensive list of regulated genes. In Table 4, gene ontology was identified only for those genes that were significantly expressed ($P < 0.05$). Expanding the selection criteria, ontology can be determined for genes over a certain fold expression (e.g. greater than 2.5 fold expression on a $\log_2$ scale) and then filtered

by ontology in each subpopulation. This can also be the first step to identifying signaling pathways by grouping the known genes and then looking at their expression in each subpopulation. While our original analysis was restricted to genes involved in cell adhesion and proliferation, cell behavioral categories such as differentiation, cell-cycle, and cell-death; as well as structural categories such as cytoskeleton and extracellular matrix would create a bigger picture of how regulated genes interact between the subpopulations.

While *in vitro* experiments are easier to standardize, measure, and replicate, using mouse *in vivo* models to study proliferation provides a complex and dynamic environment for mammary cells that provide a more accurate depiction of how cells behave in their natural setting. For example, sorted primary cells labeled with fluorescent nanocrystals can be transplanted into the cleared fatpads of mice and then recollected after 10 days. Fluorescent counting of cells will determine the expansion of cell subpopulations while gene expression analysis can be compared to those obtained by *in vitro* methods to determine if gene expression is similar for in both systems. Cell growth during their time *in vivo* can also lead to differentiation in which expression data can also be compared to *in vitro* analysis.

To further contribute to mammary development research, microarray data combined with the differing culture methods being *in vitro* or *in vivo* prove to be significantly useful in providing additional insight into the genes and associated pathways that may affect mammary progenitor proliferation.

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