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Characterization Of Murine And Human Breast Cancer Stem Cells In Vitro And In Vivo

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CHARACTERIZATION OF MURINE AND HUMAN BREAST CANCER STEM CELLS *IN VITRO* and *IN VIVO*

by

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CHARACTERIZATION OF MURINE AND HUMAN BREAST CANCER STEM CELLS *IN VITRO* BASED ON CELL CYCLE REGULATION

CARNELLA M. LEE

M.S. in BIOLOGY

ABSTRACT

Breast cancer stem cells (BCa-SC) are considered as a rare population of cells which cause resistance to chemotherapy in breast cancer (BCa). This population is characterized in humans and mice by $CD24^{-}$ $CD44^{+}$ $ALDH1^{+}$ phenotype and expresses mesenchymal properties that differ from the normal epithelial $CD24^{+}$ $CD44^{+}$ phenotype expressed in the majority of BCa cells. It has been widely accepted that tumor growth is sustained by BCa-SC that are similar to normal stem cells in which they have the ability to self-renew and differentiate. However, unlike their normal counterparts, BCa-SC are rare cells within tumors with the ability to self-renew and give rise to the phenotypically diverse tumor cell population to drive tumorigenesis instead of organogenesis like normal stem cells.

Based on the pleomorphic properties of BCa-SC within the growing tumor, the present study investigated the plasticity of BCa-SC *in vitro* and *in vivo* using murine and human BCa cell lines. Results of this

study demonstrated that specific changes in the expression of BCa-SC phenotype support a possible reprogramming of these cells based on cell cycle regulation. Since the presence of BCa-SC markers are key factors in promoting resistance to treatments, and based on our preliminary findings regarding tumor progression and growth kinetics of BCa-SC, we hypothesized that the use of cell cycle inhibitors in combination with chemotherapy drugs may serve as an effective treatment regimen to reduce BCa resistance.

We examined the growth kinetics of BCa-SC primarily *in vitro* to determine the expression of phenotypic markers CD24, CD44, and expression levels of ALDH1 based on cell cycle regulation utilizing the murine BCa cell line 4T1 and human BCa cell lines MCF-7 and MDA-MB 231. Results of the present study identified an interesting modulation of the markers during specific time points which suggested a phenotypic reversion from the BCa-SC phenotype to the non-cancer stem cell (NCSC) phenotype.

In an attempt to develop new therapies for BCa, we investigated the phenotypic reversion between the two populations of BCa (SC and NCSC) using cell cycle inhibitors including lovastatin and thymidine to block BCa-SC in the G1 and S phase of the cycle, respectively. Arresting

these cells during the NCSC phenotype blocked the reversion back to the BCa-SC phenotype at later time points. These findings support our hypothesis that use of cell cycle inhibitors to arrest cells during the NCSC phenotype may serve as a better treatment-option.

Developing a combination therapy with cell cycle inhibitor drugs may improve current therapeutic approaches and induce apoptosis in BCa-SC at specific stages of cell cycle while they do not exhibit BCa-SC characteristics. This may bring the field one step closer to designing effective therapies that may eliminate cancer and reduce the rate of relapse.

DEDICATION

I would like to dedicate this thesis to God. Without His strength and guidance this accomplishment would not have been possible. I would also like to dedicate this thesis to my amazing mother, Christine, grandmother, Dorothy, and my sister's, LaTia, Brianna and Anisha. My grandmother and mother have given me a tremendous amount of support and love through this process. I would also like to dedicate my thesis to my loving friends. They have stood by me and supported me through my entire academic career always giving me the confidence I needed.

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LIST OF ABBREIVATIONS

ALDH Aldehyde dehydrogenase

APC Allophycocyanin

BCa Breast cancer

BCa-SC Breast cancer stem cell

bFGF Basic fibroblast growth factor

CD24 Cluster of differentiation 24

CD44 Cluster of differentiation 44

CSC Cancer stem cell

DMEM Dulbecco's modified eagles medium

EGF Epidermal growth factor

EMT Epithelial Mesenchymal Transition

FACS Fluorescence activated cell sorting

FBS Fetal bovine serum

IACUC Institutional Animal Care and Use Committee

NCSC Non cancer stem cells

RT-PCR Real-time polymerase chain reaction

PE Phycoerythrin

PI Propidium Iodide

TGFβ-3 Transforming growth factor beta

CHAPTER 1

INTRODUCTION OF BREAST CANCER AND BREAST CANCER STEM CELL BIOLOGY

Breast Cancer

Breast cancer (BCa) is the second leading cause of cancer-related deaths among women. Despite the advances in therapies, it is estimated there will be approximately 233,000 newly diagnosed cases of BCa and 40,000 deaths in the United States for 2014[1]. Many of the disparities related to BCa death rates are based on race, ethnicity, domicile, and socioeconomic status, with one in eight women being diagnosed with this disease this year. The trend in incidence, mortality, and overall survival continues to increase in life-span and the growth of the population. Current treatments for BCa include: surgery, radiation, chemotherapy and hormonal therapy. Despite the significant progress made in treatments, relapse of this disease occurs more commonly prompting the need to develop new therapies.

The development of BCa can be caused by a series of genetic mutations and molecular changes that can lead to cell transformation [2]. Breast cancer continues to be a substantial cause of mortality for patients and families worldwide. Thus, developing effective therapies may improve the quality of life for women and overall survival. Patients with distant metastasis are associated

with significant morbidity and efficient therapies are limited. Despite the successful methods of treatment, researchers still face a perplexing challenge in which some patients will be cured and some will progress to premature death. Due to the complexity of the disease, chemotherapy for patients with advanced stages of breast cancer is a lofty goal and will require further research to identify efficacious therapies for patients.

Different phenotypes are identified with the stage of the disease and are associated with different biological and clinical behavior. Technological advancement in the fields of genomics and proteomics have allowed better understanding of cancer stem cell biology, identification of biomarkers that are involved in multiple signaling pathways, resistance and metastasis more available to researchers to help improve prognosis and treatment[3]. Classification of the disease stage is entirely based on traditional parameters including histological type, grade, tumor size and biomarkers. Researchers continue to further investigate the mechanisms that promote tumor progression and development which is imperative in understanding the biology of cancer. By exploring these avenues, researchers hope to develop new therapeutic targets to address breast cancer relapse, recurrence and resistance to conventional therapies by isolating and characterizing BCa-SC that are involved in metastasis, invasion, and tumor progression.

Breast cancer stem cells

The cancer stem cell (CSC) theory has become attractive in the realm of BCa and many novel therapies have emerged from this concept. The CSC hypothesis was first proposed over a century ago by Clarke and colleagues, which states that among the cancer cells, a small population acts as stem cells and have the ability to continuously replicate to sustain the cancer[4]. Studies have shown that tumors are organized so that CSC continuously proliferates, driving tumor growth, progression, and metastasis [5]. The clinical relevance of CSC remains controversial despite the advances in understanding their biology. Many of the caveats are primarily due to the limited markers available to identify this rare population of cells. One study demonstrated that BCa-SC possess enhanced metastatic ability, which remains the major cause of cancer related mortality[6]. Exploring the CSC hypothesis will help elucidate the risk associated with recurrence in breast cancer. The involvement of the tumor microenvironment and/or genetic and epigenetic changes could also be fueling factors to this heterogeneous population of cells. Recent studies have shown that within cancer cells, a minority population is capable of self-renewal and differentiation and gives rise to the heterogeneous lineage that resides with the tumor [7]. In BCa, evidence shows that <1% of the population of the cancer cells within the tumor have the ability to drive tumorigenesis and proliferate extensively causing distant metastasis[8]. These cells have been identified by researchers as BCa-SC and are

a crucial component that lead to tumor recurrence, resistance to conventional therapy and relapse [9].

In many cases, the clinical progression is hard to predict and the efficacy of available therapies poorly target the disease. Studies indicate that one possible implication for this problem is the BCa-SC that are refractory to conventional therapies. However, opposed to their normal counterparts, BCa-SC within tumors have the ability to self-renew and give rise to a phenotypically diverse tumor cell population that drives tumorigenesis, instead of organogenesis like normal stem cell[10]. Breast cancer stem cells are characterized by molecular markers CD24- and CD44+ in both humans and mice. Additionally, markers such as aldehyde dehydrogenase 1(ALDH1) and E-Cadherin have also been used to phenotypically describe this population of cells in which their expression is linked to poor prognosis. Breast cancer stem cells are known to promote metastasis and lead to poor prognosis in patients. Thus, further understanding the properties of BCa-SC may lead to the development of novel therapies for treating refractory cancer cells.

Studies have indicated that BCa-SC may arise from normal stem cells, progenitors or more differentiated cells [7, 11]. As previously mentioned, BCa-SC and their normal counterparts have similar characteristics in terms of self-renewal, expression of specific surface markers and common signaling

pathways[12]. To our knowledge, they are not synonymous due to the BCa-SC ability to drive tumorigenesis. Studies have demonstrated that an accumulation of genetic and epigenetic instability increases mutations that enable BCa-SC to acquire the characteristics of self-renewal and tumorigenicity[13]. Many mechanisms that are involved in normal stem cell biology are believed to correlate with BCa-SC biology and have been studied to provide a better understanding of their involvement in disease progression. In BCa, metastasis is known for increasing mortality and is thought to be associated with self-renewal which is a defining trait of BCa-SC [14]. The activation of embryonic pathways such as epithelial to mesenchymal transition (EMT) are implicated in stemness and generates invasive BCa-SC with increased motility enabling them to invade and migrate to distant sites[15]. This transition into mesenchymal cells is imperative for epithelial cells and supports the role of EMT in BCa-SC dissemination. The study of the BCa-SC phenotype along with the pathways that contribute to the plasticity of BCa-SC will further and inhibiting this mechanism could be a promising strategy.

Isolation and Characterization of BCa-SC

In addition to existing knowledge, further studies on appropriate markers could provide clues that will explain why BCa-SC are resistant to novel chemotherapies. The most common markers used to identify this population of BCa-SC are CD44, CD24 and ALDH1. Although the present study primarily

focuses on BCa-SC, tumors that correlate with the CSC hypothesis are also identified in leukemia[16], bladder[17], ovarian[18] and pancreatic cancer[19]. Hence, a careful observation of these markers is imperative in understanding the mechanisms and the controversial biology of CSC. Therefore, identifying and characterizing BCa-SC could introduce new ideas for treatment strategies that may possibly trigger apoptosis.

The role of CD44 and CD24 as CSC marker in Breast Cancer

Since $CD44^+$ and $CD24^-$ are the more common phenotypic markers used in BCa for both human and mouse studies, we sought to further characterize these cells using flow cytometry analysis and mammosphere culture methods. It was vital to test for stem cell activity in the cell lines used in this study as we tested this concept using the mammosphere formation assay, which allowed undifferentiated cells the ability to form mammospheres of several generations in non-adherent culturing conditions [20]. $CD44^+$ $CD24^-$ cells have been isolated from several different cell lines in our lab using FACS analysis. Studies report that BCa-SC have elevated levels of CD44 as well as elevation of other genes such as TGF-β3, TWIST and SNAIL, which are related to stem cell biology [21]. As previously mentioned, EMT is a phenomenon related to the development of BCa and during normal development is induced by receptor kinases such as TGF-β and signaling pathways such as WNT/β-catenin and Notch[22]. These processes

are also thought to play a role in BCa. Transcriptional factors such as TWIST and SNAIL target proteins such as E-Cadherin and studies show that signaling networks from the tumor microenvironment(TGF-β, WNT/β-catenin,etc) promote their expression and activity in malignant tumor migration[23]. Studies show that changes in cell adhesion promotes migration during tumor invasion which are similar characteristics of EMT during normal development[24]. One of the hallmarks of this process involves loss of cell polarity, which is caused by the repression of the protein E-Cadherin by transcriptional factors TWIST and SNAIL [25]. Expression of these markers has been shown to be directly correlated with poor patient prognosis as well as metastasis, invasion and resistance. The emergence of BCa-SC has led to discoveries, indicating an isolated population that is characterized by $CD44^+$ $CD24^-$ can form tumors with as little as 200 cells when implanted into the mammary fat pad of mice, suggesting that these cells have tumorigenic potential[9]. It is also known that BCa-SC with this phenotype are associated with metastatic potential[26]. We have conducted time kinetic experiments that have shown interesting cycling properties for these markers. Further investigation of the time kinetics of these markers is necessary for us to understand how to trigger BCa-SC for therapeutic studies. Along with adhesion characteristics, CD44 has been implicated in cancer cell migration, invasion and metastasis. Although the mechanisms for bone metastasis is unclear regarding CD44, recent studies show that knocking down CD44 can suppress

tumorigenicity and bone metastasis in nude mice[27]. CD44 is thought to interact with hyaluronan, which has also been implicated to promote invasion and metastasis in cancer cells[28]. From these studies, we have concluded that the role of CD44 plays an important role in the development of bone metastasis and tumor invasion. Using real- time reverse transcriptase polymerase chain reaction (RT-PCR) and FACS analysis, we were able to see a trend over different time points for the expression of both markers. Our data suggests that understanding the involvement of the cell cycle is critical in our understanding of the behavior of CD44/CD24 cancer cells. Once we are able to monitor cell cycle regulation, we hypothesized that if BCa-SC can be arrested during particular phases of the cell cycle, particularly when they possess the NCSC phenotype, we can then use cell cycle inhibitor drugs in combination with chemotherapy agents to prevent the reversion back to the BCa-SC phenotype which is resistant the chemotherapy.

The role of ALDH1 as CSC marker in Breast Cancer

Aldehyde dehydrogenase 1 (ALDH1) is an important detoxifying enzyme that is responsible for metabolizing intracellular aldehydes [29]. This enzyme is highly concentrated in HSC and is believed to be involved in differentiation. There are many forms of the dehydrogenase family but the isoform ALDH1 has shown to enhance stem cell biology characteristics in mammary stem cells and is a well-known predictor of poor clinical outcome [29]. Aldehyde dehydrogenase 1

has a primary role in the liver metabolism of retinol to retinoic acid, which initiates cellular differentiation. More commonly, the function of retinoids are known to be mediated by retinoic acid receptors and retinoic X receptor that are transcriptionally expressed in the nuclei of target cells [30]. There have been proposed mechanisms using in vitro and in vivo studies that show HSC differentiation can be delayed with inhibition of ALDH1 activity and retinoid signaling [31]. Therefore, it is believed that since ALDH1 is highly concentrated in HSC and could possibly play a role in differentiation, this enzyme is more than likely involved in the differentiation of BCa-SC as well.

Many studies show ALDH1 expression provides a more effective marker for BCa-SC identification[32]. Beyond its important metabolizing function, research shows that ALDH1 plays a significant role in chemoresistance when positively expressed [33]. Before its identifying role in CSCs, the role of ALDH 1 in chemoresistance was first identified in a cyclophosphamide- resistance leukemic cell line[34].Studies show that cyclophosphamide are resistant in other cells lines and show elevated levels of ALDH1[34]. These studies confirmed the role of ALDH1 mediated cyclophosphamide resistance and inhibition of its activity sensitizes CSCs to chemotherapeutics [33]. Therefore it is important that specific identification of BCa-SC should include these three markers for evaluation of their biology. Hence, an independent study of BCa-SC kinetics and the influence of the cell cycle by studying CD44, CD24, and ALDH1, may

provide clues to new therapeutic targets that will prevent relapse of BCa-SC population.

Rationale for this dissertation research

Given the need for novel therapies for BCa arising from repopulating BCa-SC and their ability to promote chemotherapy resistance, relapse, tumorigenesis and metastasis, we sought to determine the role of BCa-SC and the basic biology behind its function in BCa. Based on our preliminary results in murine breast cancer cells and tumor progression *in vivo,* we hypothesized that BCa-SC plays a significant role in BCa progression and could serve as a potential target to inhibit tumor progression, chemoresistance and metastasis. To further investigate this hypothesis, BCa-SC were isolated based on the markers CD24, CD44 and ALDH1. To assess the effects of CD24 and CD44 expression and understand the tumor promoting properties of this population *in vivo* and additional growth kinetics *in vitro,* fluorescence-activated cell sorting (FACS), was used to isolate the population of BCa-SC in which specific markers were up regulated and down regulated at specific time points in vitro.

In order understand the plasticity of BCa-SC specifically *in vitro,* we monitored marker expression utilizing mammoshphere culture. We also evaluated the gene expression of other markers specifically associated with stem cell characteristics using real-time reverse transcriptase polymerase chain reaction

(RT-PCR) such as: E-Cadherin, TWIST, SNAIL, and TGF-β3. Breast cancer stem cell expression showed an interesting trend during specific time points which demonstrated a reversion between the BCa-SC phenotype and the NCSC phenotype. This led to further experiments to test our hypothesis that the expression of BCa-SC is based on cell cycle regulation and arresting the cells when the NCSC phenotype is displayed, could allow cells to undergo apoptosis with proper treatment.

After careful observation using the growth kinetics of BCa-SC during specific time points, further *in vitro* experiments were conducted. Results of the *in vitro* experiments demonstrated that there was a possible reprogramming of BCa-SC based on cell cycle regulation, thus inhibition using cell cycle drugs during specific time points. These findings present evidence in favor of our hypothesis that show if differences exist in the stem cell characteristics of BCa-SC by monitoring cell cycle regulation. As a proof of principle, the study demonstrates that arresting cells during NCSC expression using cell cycle inhibitor drugs could serve as a potential therapeutic strategy when used in combination with chemotherapy drugs to combat BCa which results in metastasis, disease recurrence and chemoresistance due to the existence of BCa-SC.

Conclusions

Breast cancer poses a serious threat to mortality and the quality of life worldwide. Although advancements in technology and therapies have emerged, there are a large number of refractory cases due to the existence of BCa-SC that progress to life-threatening stages of the disease. These points demonstrate the need for further understanding of the biology associated with BCa-SC along with more advanced therapies to target this population.

The present study demonstrates that the phenotype associated with BCa-SC provide clues to help elucidate their role in the progression of BCa, thus targeting of this population appears essential to prevent chemoresistance. Monitoring the growth kinetics at specific time points played a pivotal role in correlating the events with the cell cycle and depletion of the BCa-SC phenotype led to a vast discovery for a combination therapy. The present study's finding that the reversion between the BCa-SC phenotype and the NCSC phenotype could be altered to favor the NCSC phenotype using cell cycle inhibitors could serve as a possible therapy. Thus, investigating the reprogramming of BCa-SC based on cell cycle regulation could be the polar principle in eliminating these cells that help maintain the tumor and remain resistant to current therapies.

Chapters 3-5 will provide details and comprehensive discussion of the experimental results, their implications in BCa and therapeutic approaches to the disease.

CHAPTER 2

METHODS

Reagents and Cell Lines

Mouse 4T1 Cells were cultured in serum-free mammary epithelial growth medium (MEGM) containing10ng/ml hEGF, 5µg/ml insulin, 0.5µg/ml hydrocortisone, gentamicin and amphotericin-B. Human breast cancer cell lines were grown in DMEM/F12 and supplemented with non-heat inactivated 10% FBS(GIBCO), Non-essential amino acids; except MCF-7, which are grown without non-essential amino acids. Mammosphere culture medium was supplemented with DMEM/F12, 25 ng/mL EGF and 25 ng/mL bFGF and were placed in ultra- low attachment flasks for determining the optimum expression of CSC and associated cell markers.

FACS analysis for phenotypic characterization of BCa-SC

To assess and analyze marker expression, BCa-SC were harvested at different time points *in vitro* at 0, 12, 24, 36 and 48hrs. Five hundred µl of cells were stained with 1µl of anti-CD24 antibody conjugated with APC and 1µl of CD44 antibody conjugated with PE for 30 minutes at 4°C. Cells were then

washed with sterile phosphate buffer saline (PBS) twice. These cells were resuspended in 100 µl of 4% formaldehyde and analyzed using a FACS Calibur II cell sorter (BD Biosciences) Cells that were $CD24^{-}$ $CD44^{+}$ and ALDH1+ were considered as BCa-SC.

RNA isolation and RT-PCR analysis

To monitor the levels of genes involved in BCa-SC and NCSC, samples were taken, at the same time points as mentioned above, using 500 µl cells to isolate RNA by TRIZOL method. cDNA was prepared using commercially available iScript cDNA kit from BIORAD as per the manufacturer's instructions. The cDNA was then used for RT-PCR analysis using SYBR green (Sigma-Aldrich) to detect expression levels of ALDH1, TWIST, SNAIL, E-Cadherin and TGFβ3. GAPDH was used as the internal control for the RT-PCR analysis.

***In vivo* studies**

Unsorted 4T1 cells, 4T1 BCa-SC and NCSC ($7x\ 10^4$) were implanted subcutaneously into 6 week old BALB/c male mice (n=3) and measured for allograft tumor development starting 10 days post implantation and there after every 14 to 26 days via external measurement in two dimensions using a digital caliper. Tumor progression was followed via non-invasive luciferase imaging using IVIS imaging system to assess tumor growth using ROI measurements.

Cell cycle analysis using propidium iodide staining and FACS analysis

To determine if cell cycle inhibitor drugs could arrest cells during particular phases of the cell cycle, thymidine and lovastatin/mevalonate were used for G1 and S phase synchronization, respectively. 4T1, MDA-MB-231 and MCF-7 cells were synchronized using (4mM and 5mM of thymidine) and (20uM and 40uM of Lovastatin) .Cells ($2x10^4$) were seeded in 6 well culture dishes/mL and allowed to proliferate overnight. Culture medium was replaced with fresh medium containing appropriate concentration of cell cycle drug. At various time points post treatment, cells were washed with PBS. The cells were then treated with 70% cold ethanol drop wise and stored for at least 24hrs at 4°C. Fixed cells were resuspended into PBS followed by staining with propidium iodide (40ug/ml) and incubated for 30 minutes. These cells were resuspended in 100 µl of 4% formaldehyde and analyzed using a FACS Calibur II cell sorter (BD Biosciences)

MTS cell proliferation assay

Cells were seeded in multiple 6 well plates at $4x10^5$ cells/well in replicate cultures. Twenty- four hours after plating, 20 µL/well of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) was added to a plate and incubated for 1 hr at 37°C in a humidified, 5% CO2 incubator, then removed and assayed on a microplate reader at 490 nm to establish baseline readings.

Successive readings were conducted on remaining plates every twenty-four hours post seeding to establish growth curves.

Statistical analysis

Data was analyzed by the Student's t test. Values provided are the Mean ± SEM and the differences were considered significant if $p<0.05$.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF BCa-SC CAN HELP UNDERSTAND WHETER THEIR EXPRESSION PROPERTIES DIFFER DURING GROWTH

Breast cancer stem cells have been implicated as a small population of cancer cells that are similar to normal stem cells and many of the pathways involved in normal stem cell development, when dysregulated, may be utilized by cancer cells for chemoresistance, growth and metastasis. Here, these points will be expanded upon and specifically how the existence of BCa-SC pertains to BCa. These points will be discussed in the context of data from the *in vivo* and *in vitro* studies.

Studies have indicated that BCa can be efficiently propagated in the mammary fat pad of mice and can form tumors [9]. The cancer cells with the phenotype $CD24^{-}$ $CD44^{+}$ make up the bulk of the tumor and are known to be resistant to chemotherapy. Many studies have shown that not all cells within the tumor have the capacity to promote tumorigenesis. Later on, a population with $CD24^{-}$ $CD44^{+}$ phenotype was identified as the small population with characteristics of self- renewal and the ability to regenerate the parental tumor[35]. Breast cancer stem cells are thought to be the driving force behind metastasis, invasion and chemoresistance, therefore, this population serves as a potential target for addressing chemoresistance in BCa[36].

In addition to $CD24^{-}$ $CD44^{+}$ phenotype, other genes will be monitored as well that will help further characterize BCa-SC phenotype. Furthermore, it is well known that EMT plays an important role in normal developmental processes and results in the role of the $CD24^{-}$ $CD44^{+}$ phenotype [37]. Within tumor development, there appears to be a switch in cell adhesion molecules such as E-cadherin. Studies show that down-regulation of this adhesion molecule is expressed in early tumor development causing cells to display mesenchymal phenotype[38]. Towards characterizing the BCa-SC with multiple phenotype and functional markers, we analyzed expression of TWIST, SNAIL, and TGF-β3 to help further characterize BCa-SC. These genes were of interest in our studies because of their association with metastasis and invasion [39, 40]. Using the BALB/c mouse model along with in vitro propagation of cells, we sought to further identify and characterize this subpopulation of cells.

RESULTS

Expression of CD24 and CD44 in murine breast cancer cell line:

Towards understanding whether BCa-SC markers differ in their expression during certain time points and if influence of the cell cycle plays a role, the metastatic BCa cell line 4T1, which is syngeneic to BALB/c mice was used. Stem cell population from 4T1 cells were separated based on $CD24^{-}$ $CD44^{+}$ using flow cytometry. The cells were grown in mammosphere culture method

which allowed the cells to grow in non-adherent conditions and further enrich for BCa-SC phenotype. Results of this study indicated variation in the expression of stem cell markers at different time point's *in vitro* culture. Interestingly, the typical stem cell marker combination $CD24^{-}$ $CD44^{+}$ was high every 12 hours, with a maximum expression at the 48 hour time point (Fig 1). This data indicated a modulation in stem cell markers, possibly correlating with stages of the cell cycle.

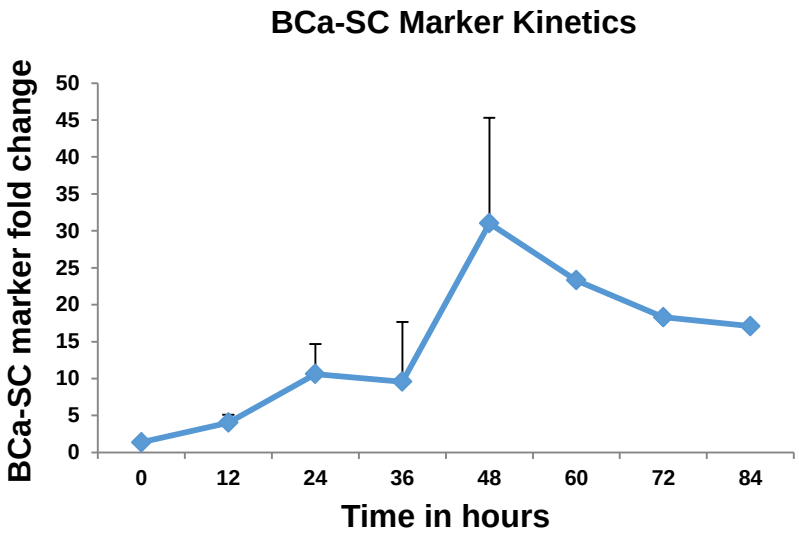


Figure 1: BCa-SC time kinetics for CD44 and CD24 expression. The 4T1 cell line was used to monitor the expression of BCa-SC markers $CD44^{+}/CD24^{-}$. Approximately, 10^{6}/ml murine BCa-SC cells were cultured in cancer stem cell medium in a low attachment flask. Culture supernatants were collected every 12 hours over an 84 hour time period. About 500 µl of cells at each time point were stained with antibodies to CD44 and CD24 and subjected to flow cytometry to detect the presence of BCa-SC markers. Results of this analysis indicated that BCa-SC marker expression peaked every 12 hours. The 48 hour time point shows the maximum expression of cancer stem cells.

As an additional measure of examining the role of BCa-SC markers, cells from replicate cultures were used for total RNA isolation by Trizol method for quantitative RT-PCR analysis. The cDNA was synthesized and used to determine the expression of ALDH1, TGFβ-3, SNAIL, TWIST and E-Cadherin genes involved in cancer stem cell characteristics. Studies show that transcriptional regulation of ALDH1, TGFβ-3, SNAIL, TWIST and E-Cadherin can cause acquisition of a stem-like phenotype and the combination of these genes have been used in previous studies to characterize BCa-SC. From our previous data we were able to conclude that the maximum expression of BCa-SC markers was evident during the 48 hour time point in culture. Furthermore, detection of genes involved in stem cell regulation was also performed using RT-PCR to determine if there was a correlation in the cycling trend in the expression of genes involved in stem cell that followed the same trend that was noted with $CD24^{-}$ $CD44^{+}$ - phenotype. The data suggests that during the 48 hour time point, ALDH1, SNAIL, TWIST and TGFβ-3 were up-regulated and E-Cadherin and CD24 were down regulated (Fig 2). This correlates with previous studies and further confirms possible cell cycle regulation as shown in previous data.

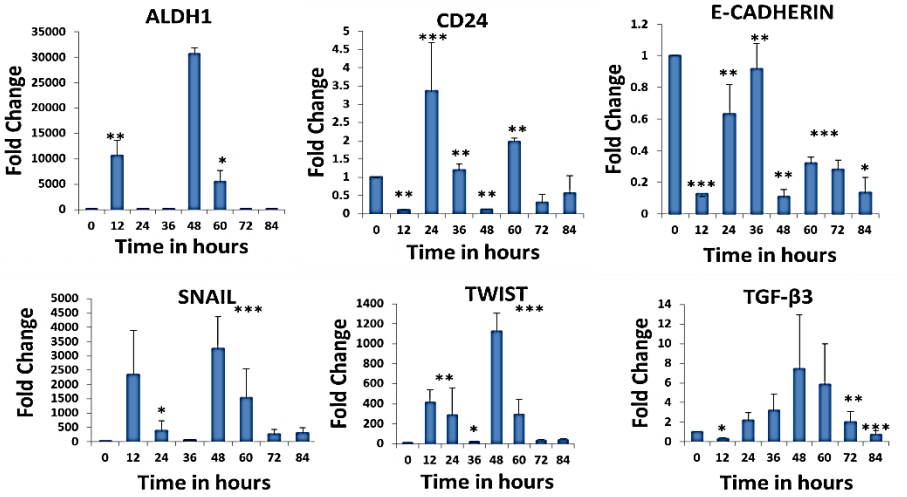


Figure 2: Culturing of BCa-SC results in the expression of stem cell specific functional markers. Expression levels of indicated genes involved in stem cell regulation was followed using time kinetics by RT-PCR to confirm stem cell characteristics. Genes such as ALDH1 ($p < 0.001$), SNAIL ($p < 0.01$), TWIST ($p< 0.05$), TGF-β3 ($p < 0.05$) and E-Cadherin ($p < 0.01$) were used to confirm the stemness of these cells.

To determine the significance of cancer stem cell population in the growth of tumors, monitoring tumor progression in mice will help understand the role of BCa-SC in tumorigenic potential and stem cell biology. To determine the significance of BCa-SC and NCSC in the growth of tumors in vivo, combinations of 4T1 cell populations containing only the stem cells ($CD24^{-}$ $CD44^{+)}$ only the non-stem cells ($CD24^{+}$ $CD44^{+}$) or mixed populations of these two were injected

in mammary fat-pad of 6 weeks old female BALB/c mice. BCa-SC that express $CD24^{-}$ $CD44^{+}$ has tumorigenic potential and assessment of their growth is crucial to understanding the biology of BCa-SC and their role in metastasis and invasion. For this experiment, cells were stained with anti-CD24 and CD44 antibodies followed by cell sorting by flow cytometry. Breast cancer stem cell population $CD24^{-}$ $CD44^{+}$ and NCSC population $CD24^{+}$ $CD44^{+}$ and 4T1, which contain the mixed population, were collected and injected into the mammary fat pad of BALB/c mice. The experiment was performed for days and tumor progression was monitored by non-invasive luciferase imaging to further confirm tumor potential of BCa-SC (Fig 3A). Results of this study, shown in Fig.3B indicated that 4T1 cancer cells show an increase in tumor progression starting at day 10, while 4T1 NCSC show very minimal tumor progression and 4T1 BCa-SC show an increase in tumor progression around day 26. [N= 3]. This helped further confirm the tumorigenic potential of the BCa-SC population and also reveals a possible activity in the microenvironment between the BCa-SC and NCSC population that may contribute to tumor growth. Collectively, the data indicated that compared to 4T1 cells, populations containing only BCa-SC and NCSC exhibited a significantly reduced tumor burden indicating the requirement of both BCa-SC and NCSC for aggressive growth.

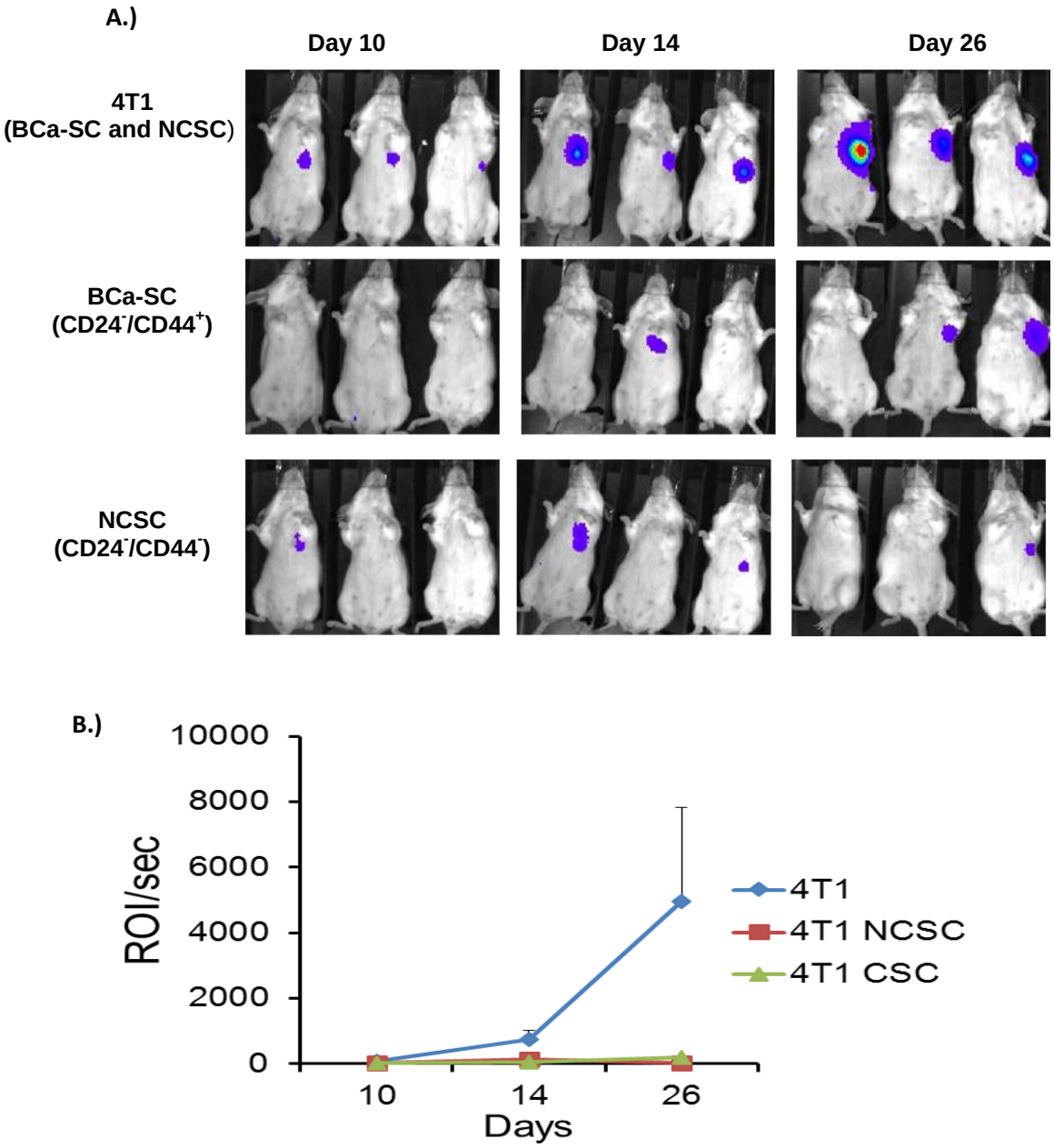


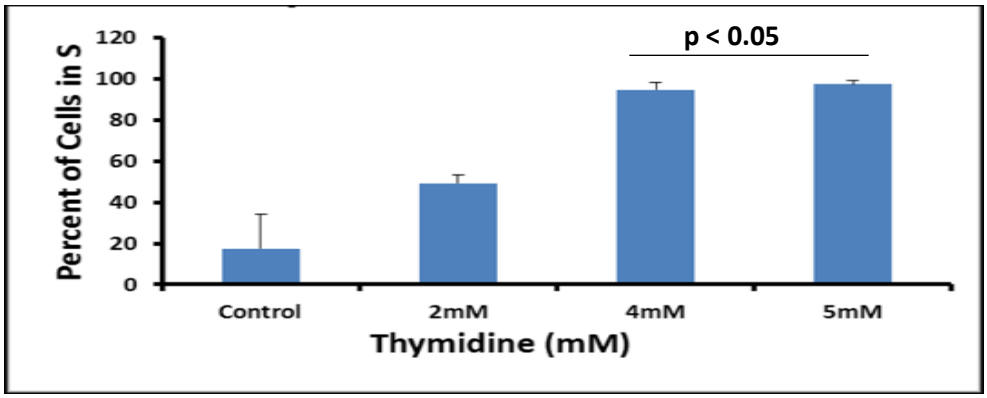
Figure 3: Tumor growth of 4T1 BCa-SC and NCSC post implantation in BALB/c mice. Female BALB/c mice were injected with unsorted 4T1 cells, 4T1 non-CSC or 4T1 BCa-SC ($7x10^4$ cells/ mouse) in mammary fat-pad. Mice were imaged non-invasively based on constitutive expression of firefly luciferase in the cells at regular intervals for bioluminescence to determine tumor progression.

Use of cell cycle inhibitors for reversing BCa-SC phenotype:

Based on our previous results where the expression of BCa-SC markers peaked at different time points, we hypothesized that a possible reprogramming of BCa-SC could be influenced by the cell cycle. To determine if the use of cell cycle inhibitors could block cells in particular stages of the cell cycle when the cells do not exhibit stem cell characteristics, we first wanted to ensure that cell cycle inhibitor drugs, thymidine and lovastatin were able to arrest cells in the S and G1 phase, respectively**.** Studies show that BCa-SC are known to recapitulate the tumor when serially transplanted and its ability to self-renew[41]. There are many predictions of the CSC hypothesis that are geared towards our focus of study; however our data suggests that characterizing and isolating BCa-SC using functional markers may help us understand their role in their therapy resistance and relapse in BCa patients. Therefore, it is imperative to understand the biology of BCa-SC in order to find therapies that can fully eradicate their existence. Blocking the BCa-SC population in particular stages of the cell cycle when the cells do no exhibit stem cell characteristics may develop more effective therapies in BCa. In this experiment, 4T1 cells ($2x10^5$ cells/ ml) were used for cell cycle synchronization. The first drug used for this experiment was thymidine, which is known to arrest cells in the S phase of the cell cycle by interfering with DNA synthesis [42, 43]. Cells were incubated for 12 hours and treated with one thymidine block (4 mM and 5 mM, independently) followed by a second block

with same concentrations for an additional 14 hours later to arrest cells in the S-phase. Another common inhibitor drug for cell cycle arrest is lovastatin. Lovastatin is a HMG-CoA reductase inhibitor that inhibits the rate-limiting step in the mevalonate pathway[44]. This inhibitor is involved in the stimulation of apoptosis of proliferating tumor cells, like breast cancer cells, by arresting the cell cycle at the G1 phase [45]. 4T1 cells were treated with concentrations of 20 uM and 40 uM for 36 hours. Post treatment, cells were fixed in 70% ethanol and stored at 4°C followed by staining with propidium iodide for FACS analysis. Propidium iodide is capable of binding to DNA making it possible to evaluate cellular DNA content rapidly. Results of this experiment were obtained using FACS analysis and cells were quantified using FACS analysis for the evaluation of DNA content for cell cycle analysis. Results of this study indicated distribution of the major phases of the cell cycle. This data shows that both drugs are capable of synchronizing cells in respected phases of the cell cycle using appropriate concentrations.

A.)



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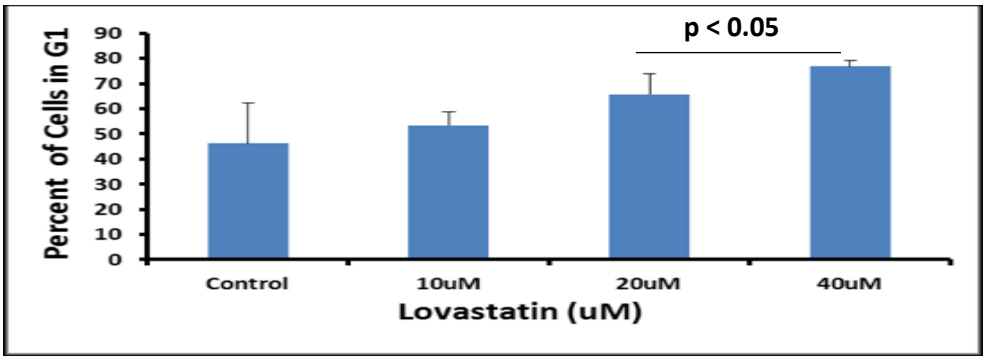

Figure 4: Percent of cell cycle arrest for Thymidine and Lovastatin at different concentrations. After PI staining, cells were tested using FACs analysis to determine optimal concentration for each drug. **A)** Using 4 mM or 5 mM ($p < 0.05$), more than 80% of cells were arrested in S phase using thymidine. **B)** Using 40 uM, more than 70% of cells were arrested in G1 phase using lovastatin ($p < 0.05$).

DISCUSSION

Current understanding of BCa is mainly centered around the hierarchical model and theory of BCa-SC. The BCa-SC are believed to possess enhanced tumor initiating potential and can escape chemotherapy, which eventually lead to

recurrence in patients. In many cases, metastasis, invasion and chemoresistance is thought to result in the survival of BCa-SC, therefore, understanding the mechanisms that are involved in sustaining the BCa-SC population, which drives cancer progression, is crucial in developing more effective therapies to treat BCa. Thus, this work demonstrated increased expression of BCa-SC markers in the 4T1 metastatic BCa cell line during specific time points which suggested the possibility of targeting this population for improved therapy effects. These findings showed an interesting modulation of BCa-SC markers suggesting involvement of the cell cycle and requires further research.

In this study, BCa-SC were evaluated by monitoring the growth kinetics of BCa-SC *in vitro.* Earlier studies demonstrated by Al-Hajj et al, identified and isolated BCa-SC as $CD44^+$ and $CD24^-$ [9]. This work demonstrated that this population expressing $CD44^+$ and $CD24^-$ can form tumors when transplanted in mice. Our studies were in agreement with this previous study and demonstrated that BCa-SC expressing these markers are more tumorigenic than cells that lack these markers. Thus, this further confirmed that BCa-SC are similar to their normal counterparts and supports previous evidence that shows BCa-SC are capable of self-renewal and differentiation. Previous studies show that tumors are comprised of a heterogeneous population of cells which contains subsets of BCa-SC and NCSC. Our findings suggest that there is a possible switch in phenotype from BCa-SC to NCSC during specific time points and targeting the NCSC

population may serve as a potential therapy. There are many proposed mechanisms by which BCa-SC are thought to be the driving force behind resistance. Because chemoresistance is one of the functional characteristics of BCa-SC, it is important to isolate and characterize this population thoroughly. As mentioned earlier, there are many pathways that are thought to contribute to the existence of BCa-SC such as WNT/β-catenin and the Notch signaling pathway and play a role in their resistance[24]. Due to the increasing evidence supporting BCa-SC resistance, we believe there is clinical importance in our observation of this phenotypic switch from BCa-SC to NCSC. This cycling trend observed in our study provided clues to help determine if this phenotypic switch between BCa-SC and NCSC can be altered by arresting the cells at specific time points. So far, there are no studies that test this phenomenon using cell cycle inhibitor drugs. Research using cell cycle inhibitors to arrest the NCSC at specific time points, which can be eradicated using chemotherapy will test this theory.

Additional evidence that BCa-SC have an important role in the progression of BCa, especially in the involvement of metastasis and the ability to form tumors, was provided by our findings that BCa-SC have tumorigenic potential and possible interaction in the microenvironment between the BCa-SC and NCSC contributes to tumor growth. This data also suggest BCa-SC are more aggressive than NCSC and treatment may be useful for targeting this population in BCa.

Although monitoring the growth kinetics of BCa-SC based on CD44 and CD24 expression was useful in determining possible involvement of the cell cycle, utilizing RT-PCR for other genes validated our finding that a pattern exists and compares to the modulation of CD44 and CD24 markers at specific time points as previously described. Taking these findings under consideration, BCa-SC may be involved in reprogramming of the cell cycle that contributes to their proliferation. Further studies are needed to determine the significance of the markers expressed during specific time points and whether the reprogramming can be altered if cells are arrested using cell cycle inhibitors.

In conclusion, these initial experiments established validation for this study to examine the biology of BCa-SC and their tumorigenic role in BCa. *In vitro* experiments confirmed the involvement of the cell cycle in BCa-SC and an observation was made regarding the cycling trend of expression markers at different time points. These studies warrant additional studies to determine if the use of cell cycle inhibitors could play a potential role in blocking the progression of BCa-SC. With this, additional experiments were done to determine if thymidine and lovastatin were suitable drugs to achieve cell cycle arrest. Our studies show that both lovastatin and thymidine are nontoxic and capable of arresting cells in the G1 and S phase of the cell cycle, respectively. Furthermore, characterization of human BCa-SC using time kinetics experiments and RT-PCR analysis will be tested *in vitro*.

Studies in this aim are anticipated to provide information on the significance of BCa-SC and the influence of the cell cycle in their proliferation. Overall, we anticipate the above experiments will provide clues in an effort to understand if the reversal of BCa-SC phenotype to NCSC phenotype is possible by arresting the cells at a specific time point. We plan to evaluate how the use of cell cycle inhibitor drugs will arrest the NCSC population at specific time points, which do not exhibit normal BCa-SC phenotype. We have demonstrated using the 4T1 cells that the expression of BCa-SC markers differs during specific time points suggesting the involvement of the cell cycle. We also successfully blocked 4Tl cells using cell cycle inhibitor drugs in respective phases of the cell cycle.

CHAPTER 4

CELL CYCLE ARREST OF BCa-SC DURING EXPRESSION OF NCSC PHENOTYPE CAN REVERSE STEM CELL CHARACTERISTICS

Upon completion and confirmation of BCa-SC kinetics in the metastatic BCa cell line 4T1, to assess if the expression of BCa-SC differs at specific time points, we sought to test whether a possible reprograming of the cell cycle in human breast cancer cell lines was similar to what was identified in 4T1 cells. In order to possibly translate this to clinical significance, utilizing human BCa cell lines will provide a better understanding on the influence of BCa-SC markers on cell proliferation as related to the cell cycle. The above experiments provided clues in an effort to understand the significance of the reversion between the BCa-SC phenotype and the NCSC phenotype, as it relates to BCa, during specific time points. This postulates that using cell cycle inhibitors to block cells in the NCSC phenotype may prevent reversion back to the BCa-SC phenotype at later time points.

Many studies have been conducted and provide evidence that BCa-SC have profound clinical and pathological relevance[46]. As mentioned above, the existence of BCa-SC can exclusively form tumors that substantiate both disease recurrence and metastasis [9, 26]. Establishing the functional characteristics of

BCa-SC is very imperative for clinical studies, thus further research is needed to circumvent the controversial CSC hypothesis.

We set out to establish the role of BCa-SC using human BCa cell lines MCF-7 and MDA-MB 231 utilizing the isolation methods explained previously. Breast cancer stem cells are capable of proliferation and survival for several passages when grown under appropriate culture conditions. These cells are enriched in progenitor cells that are capable of differentiating into multiple lineages as mammospheres by using non-adherent culture conditions [20]. Hence, propagating the cells under these conditions will enrich sorted cells for BCa-SC population. Previous data shows that the BCa-SC population is not only based on the expression of CD24 and CD44, therefore the use of additional markers are needed to further characterize this population. Based on our preliminary findings and the role of CD44+/CD24- in BCa-SC, this experiment will focus on using additional markers to further characterize the population. Studies show that this population is further refined by high ALDH1 activity. Studies have shown that cells with high ALDH1 expression are enriched for BCa-SC, have sphere forming ability and can form tumors when isolated [29]. This marker was originally used for the isolation of hematopoietic stem cells until its recent use for isolating CSC in many other cancers [29, 47]. As previously mentioned, ALDH1 mainly functions as an oxidizing agent to aldehydes and carboxylic acids; however studies also show that this enzyme is involved in retinoid acid signaling which has

been linked to stemness characteristics of BCa-SC [32, 48]. Although further studies are needed to fully understand the molecular mechanisms associated with ALDH1 activity, the intracellular activity is emerging as an important and reliable CSC marker for many cancers.

Given the aims of the present study to target and elucidate the role of BCa-SC in BCa using *in vitro* experimentation we sought to isolate and monitor CD44 and CD24 expression in comparison with the murine *in vitro* data. For this reason, both human cell lines were propagated under the appropriate culture conditions and the growth kinetics was monitored to determine if there was a possible reprogramming of BCa-SC during specific time points. Recent evidence shows that increased ALDH1 activity helps further characterize CSC population [5]. Our previous findings provide important evidence regarding involvement of cell cycle during the propagation of these cells. Therefore, it is important to test whether cell cycle inhibitor drugs that were used in preliminary studies can accurately arrest the BCa-SC population in the human cell lines. Studies have shown that $CD44^{+}$ $CD24^{-}$ and high ALDH1 expression leads to poor prognosis in patients while showing resistance to chemotherapeutic agents [26, 33, 35]. Repeating the experiments from our previous experiments will allow us to further determine if cell cycle arrest could be achieved during the 24 hour time point and deplete ALDH1 activity. Based on the function of these markers, that causes invasion, metastasis and chemoresistance; studies outlined in this study will determine if

cell cycle inhibitor drugs lovastatin and thymidine can provide cell cycle arrest of human BCa-SC. This data will allow us to further examine the use of cell cycle inhibitor as an effective therapy in targeting the BCa-SC population in conjunction with chemotherapy.

RESULTS

Validation of cell cycle involvement based on BCa-SC expression in BCa cells:

Cancer stem cell populations from human breast cancer cell lines MDA-MB 231 and MCF-7 were sorted using FACS analysis based on $CD44^+$ and $CD24^-$ and then cultured in BCa-SC medium. From our previous data, we hypothesized that BCa-SC marker expression differed during specific time points and this could be due to cell cycle regulation in the human cell lines. Therefore, we first wanted to monitor the time kinetics of CD44 and CD24 expression over an 84 hour time period (Fig.5). Considering the cycling pattern expressed in both cell lines, we predicted that there may be some involvement of the cell cycle that may control the expression of BCa-SC markers. From this data, FACS analysis and RT-PCR validated that during the 24 hour time point in each cell line, NCSC characteristics were present due to lack of CD24 and ALDH1 expression. As previously mentioned, studies have reported that high expression of ALDH1 further confirms BCa-SC phenotype , thus this was critical in our findings[29]. After analysis of BCa-SC kinetics, results showed that during the 24 hour time

point, these cells exhibited NCSC characteristics. Taken together, our results indicate that characterizing cancer stem cells using CD24 and CD44 alone may not correlate with stem cell phenotype. It is believed that high ALDH1 expression in conjunction with the expression of CD44 and CD24 may be crucial to identify the BCa-SC population. It was imperative that we understood the overall cell cycle replication duration in each cell line.

The main phases of the cell cycle are: G0 in which the cell is residing in a quiescent, resting state until triggered by an external signals to start the cell cycle. The majority of the cells in the body reside in this phase. G1 which involves preparation of DNA duplication ,the S phase during which the cell replicates its DNA, G2 phase in which the cell prepares for M phase and cell division and lastly the M phase (Mitosis) which involves the separation of the replicated chromosomes into two daughter cells. Cancer cells are typically described as cells that have acquired features which allow them to override such checkpoints, leading to uncontrolled proliferation so understanding the cell cycle is very important for understanding mechanisms that may have been altered to cause cancer cells to have high proliferative ability and may provide insight on why cancer cells are resistant to therapies. We used two different cell lines due to the difference in doubling time and proliferation abilities with MDA-MB 231 cells having 48 hours and MCF-7 24 hours under normal culture conditions. Using FACS analysis we were able to determine cell cycle phase distribution in the

control groups of each cell line. From this data we saw a variation in each phase that supported our hypothesis that cell cycle involvement could play a role in marker expression at different time points.

Subsequently, the results indicate that in human MDA-MB 231 and MCF-7 cells, ALDH1 expression displayed maximum expression at the 36 hour time point suggesting that this is the BCa-SC population. However, during the 24 hour time point for each cell line, there was no expression of ALDH1 indicating a NCSC population. Therefore, changes in the expression of key cancer stem cell markers in culture could possibly indicate the involvement of events in cell cycle for optimal stemness. Since ALDH1 expression was absent during the 24 hour time point, this implies that cell cycle arrest of BCa-SC during expression of NCSC phenotype can reverse stem cell characteristics and enhance sensitivity to chemotherapy.

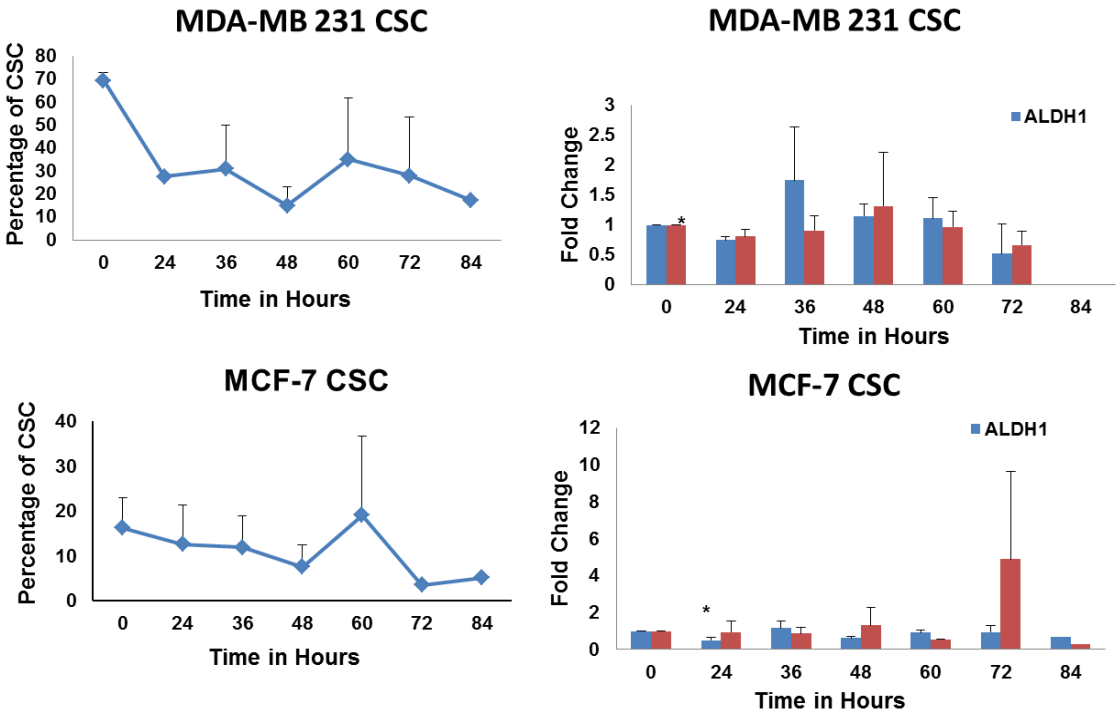

Figure 5: BCa-SC time kinetics for MDA-MB 231 cells and MCF-7 cells. Replicate cultures were harvested and total RNA was isolated by TRIZOL method. cDNA was synthesized using a commercially available iScript cDNA kit from Bio-Rad as per the manufacturer' instructions. The synthesized cDNA was used to determine the expression of ALDH1 and CD24 by real time RT-RCR normalized to GAPDH. Results indicated the expression of ALDH1 was up regulated in BCa-SC while CD24 was down regulated during 36 hour time point. In addition, to further characterize the BCa-SC population, other markers such as E-Cadherin will be used to further define the stemness of these cells. ($p < 0.05$)

Use of cell cycle inhibitors results in depleted activity of ALDH1 in BCa cells:

The next aim of the study was to carefully observe ALDH1 expression after cells were treated with cell cycle inhibitors. As described previously, cells were sorted using flow cytometry based on $CD24^-$ and $CD44^+$ and used for cell cycle inhibitor experiment. Detection of ALDH1 was assessed using RT-PCR analysis. Cells were then seeded in low attachment flasks and treated with desired cell cycle inhibitor drug concentrations (Fig 6). Following treatment of cells with both inhibitor drugs, cells were stained with CD44 and CD24 antibodies followed by 70% alcohol fixation. Cell were also collected during each time point and stained with propidium iodide to determine if cell cycle arrest was achieved. Our results indicate that both MCF-7 and MDA-MB 231 cells were successfully arrested in respective phases of cell cycle. This data demonstrates that treatment of cells with either lovastatin or thymidine at 24 hour time point stably blocks ALDH1 expression at later time points.

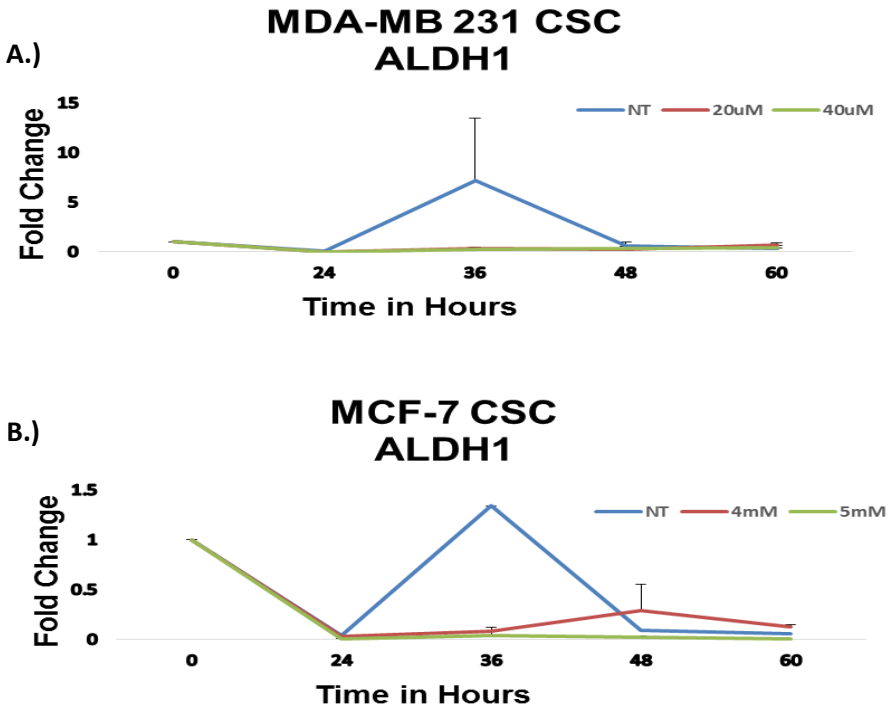

Figure 6: BCa-SC kinetics post treatment with cell cycle inhibitor drugs. A) Lovastatin/Mevalonate was used to block cells in the G1-phase. MCF-7 cells were treated with concentrations of 20 uM and 40 uM for 36 hours. **B)** MDA-MB 231 cells were incubated for 12 hours and treated with a one thymidine block (4 mM and 5 mM) followed by a release of 12 hour to ensure proper synchronization in to the S phase. A second block was administered 12 hours later to arrest cells in the S-phase.

DISCUSSION

During normal physiology and homeostasis, cells in the body are regulated continuously by the cell cycle. The cell cycle is tightly controlled by a series of mechanisms that drive DNA replication and cell division. Studies show that cell cycle dysregulation influences survival in breast cancer and are governed by molecules that when over expressed can lead to high cell proliferation and resistance to therapies[49]. These abnormalities are very common in breast tumors and are used to asses individual tumor markers for therapeutic purposes[50]. Furthermore, we believe that studying BCa along with cell cycle involvement is imperative in understanding the mediators of tumorigenesis and chemoresistance. Overall, the above experiments provided additional evidence supporting the role of BCa-SC markers and the involvement of the cell cycle for proliferation. Using ALDH1 in addition to $CD24^-$ and $CD44^+$ provided a more characterized population in distinguishing BCa-SC from NCSC. Previous studies suggest that limited specificity in characterizing BCa-SC remains a controversial issue, thus it is highly imperative we use the appropriate markers in distinguishing this population.

Although limited markers are used to identify BCa-SC, studies indicate that the markers used in this study are accurate for identifying the BCa- SC population. With this data, we demonstrated that successfully treating BCa-SC with cell cycle inhibitor drugs at 24 hour time point prevents the reversal of

NCSC phenotype back to the BCa-SC phenotype. This implies that a cell cycle inhibitor in combination with chemotherapy agents may lead to apoptosis and may be a therapeutic strategy for treating BCa and due to the absence of the BCa-SC phenotype this therapy will increase the effectiveness of chemotherapy treatment. Since BCa- SC are believed to contribute to the relapse of the disease, we believe this may lower the risk when given novel therapies against the disease and provide apoptosis of BCa cells.

Although studies have previously indicated that only the expression of CD44 and CD24 constitutes the BCa-SC population, more recent studies show high levels of ALDH1 distinguishes BCa-SC population. Interestingly, when ALDH1 is positively expressed, it has been proven to cause chemoresistance in breast cancer[51] and the loss of its function is critical in the treatment of the disease. We have shown here that the loss of ALDH1, along with absence for CD24 expression during the 24 hour time point, suggests that the cells exhibit the NCSC phenotype and should be targeted. Thus, the importance of finding new therapies to target BCa-SC is very imperative for future studies. Due to the heterogeneity of BCa, there are limitations in finding a targeted therapy for the disease and several therapies may be necessary.

In conclusion, we sought out to develop a combination treatment using chemotherapy and cell cycle inhibitor drugs to target the BCa-SC population. We

believe this method will not only eliminate the BCa-SC population, but the remaining cancer cells as well. We have demonstrated that treatment during the 24 hour time point with either lovastatin or thymidine successfully arrests the cells in either the G1or S phase, respectively. To support this finding, the next aim will use cell cycle inhibitor to first arrest breast cancer cells at 24 hour time point followed by treatment with a chemotherapy agent. We anticipate this combination treatment will lead to apoptosis of breast cancer cells and may prove promising as BCa treatment.

CHAPTER 5

SENSITIZATION OF BCa-SC BYCOMBINATION TREATMENT USING CELL CYCLE INHIBITOR AND CHEMOTHERAPY

Developing a novel combination treatment to overcome resistance in BCa has sparked the interest of many researchers. In recent years, lifesaving treatment strategies have advanced for BCa. Treatments for BCa are characterized as surgical, radiation therapy, chemotherapy, hormone therapy and targeted therapies [52]. However, most BCa treatments are often a combination of different therapies to induce a better effect and treatment for patients. Although there are many treatment options available, 40% of BCa cases results in relapse [53].

There are several mechanisms involved in chemoresistance but many studies support the emerging concept of BCa-SC and their involvement in chemoresistance. This small population of cells are characterized as quiescence which drives their resistance to chemotherapy[54]. The stem cell population is correlated with the markers $CD24^{-}$ $CD44^{+}$ $ALDH1^{+}$. Studies have shown that subsets of BCa-SC from the cell line MDA MB 231 are resistant to chemotherapy. Here we will utilize the triple negative BCa cell line to carry out the remainder of our studies due to their display of aggressiveness and metastasis.

Due to the overexpression of BCa-SC markers and their association with resistance to chemotherapy, more effective therapies are needed to eradicate this population. In our previous experiments, we monitored the expression of BCa-SC markers in relation to the cell cycle. Our studies revealed that during the 36 hour time point in the MDA-MB 231 cells line, there was an increase in the expression of ALDH1. Since cell cycle involvement is associated with mechanisms that may provide BCa with proliferation and chemo resistant advantages, it is logical to postulate that different phases of the cell cycle may be responsible for the overexpression of BCa-SC markers. Therefore, the present study pursued this by first examining the expression of ALDH1 during particular time periods and using cell cycle inhibitor drugs to arrest cells during that time point.

RESULTS

For the purposes of understanding whether a combination therapy using cell cycle inhibitors in conjunction with chemotherapy would enhance sensitivity of the BCa-SC population, we tested the monitored the expression of ALDH1 using the MDA-MB 231 cells to further demonstrate the modulation of its activity at different time points. In addition, we postulated that it would be helpful to further characterize the BCa-SC population using additional markers such as E-Cadherin using RT-PCR analysis (Fig 7). The BCa-SC population was sorted using FACS analysis as described previously. We hypothesized that treating the

BCa-SC at the 24 hour time point, when ALDH1 is not present indicating the NCSC population, the BCa-SC population may undergo apoptosis. Based on the expression of ALDH1, our studies further revealed that after cells are treated with 4mM of thymidine during the 24 hour time point, ALDH1 activity is depleted and remains undetectable when compared to the no treatment group.

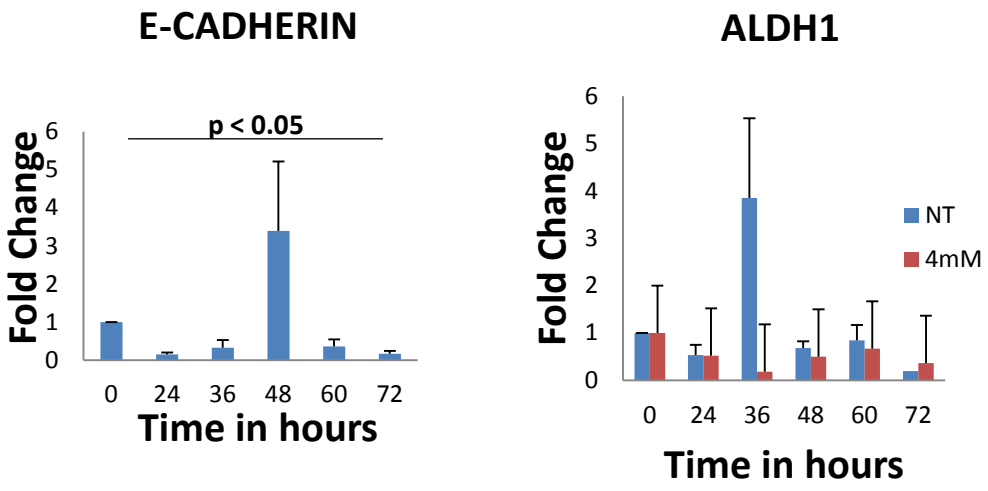

Figure 7: BCa-SC kinetics of E-Cadherin and ALDH1. Replicate cultures were harvested and total RNA was isolated by TRIZOL method. cDNA was synthesized using a commercially available iScript cDNA kit from Bio-Rad as per the manufacturer' instructions. The synthesized cDNA was used to determine the expression of ALDH1 and E-Cadherin by real time RT-RCR normalized to GAPDH. Results indicated the expression of ALDH1 was up regulated at 36 hour time point and down regulated E-Cadherin indicating the BCa-SC population. BCa-SC population was also tested to monitor ALDH1 expression when treated with 4mM of thymidine. ($p < 0.05$)

To determine if a combination treatment is useful in enhancing sensitivity of BCa-SC, we sought out to test our hypothesis using Docetaxel in combination with thymidine. It is well known that docetaxel, one of the taxanes, binds to microtubules in cells and arrest in mitosis by stabilization[55]. Docetaxel is one of the most frequent ant microtubule agents used for BCa chemotherapy. For this

study, we performed a cell viability assay by MTS on four groups (no treatment, docetaxel only, thymidine only and docetaxel plus thymidine) to test the number of viable cells in proliferation. We tested the effects of each drug alone or in combination on the sorted MDA-MB 231 BCa-SC population and the unsorted NCSC population. From previous studies, we showed that treatment of the BC-SC population with thymidine during the 24 hour time point when ALDH1 is not detected stably blocks the expression at later time points. This would allow the cancer cells to be les resistant to chemotherapy since high ALDH1 activity is associated with chemoresistance as stated previously.

Taken together, results of the present study indicate that compared to the docetaxel only group in the BCa-SC population, which are resistant to chemotherapy when arrested with thymidine at the 24 hour time point, over 60% of the cell population underwent apoptosis compared to the control group of NCSC. This supported our hypothesis that using a cell cycle inhibitor in combination with chemotherapy can cause sensitivity of cancer cells (Fig 8).

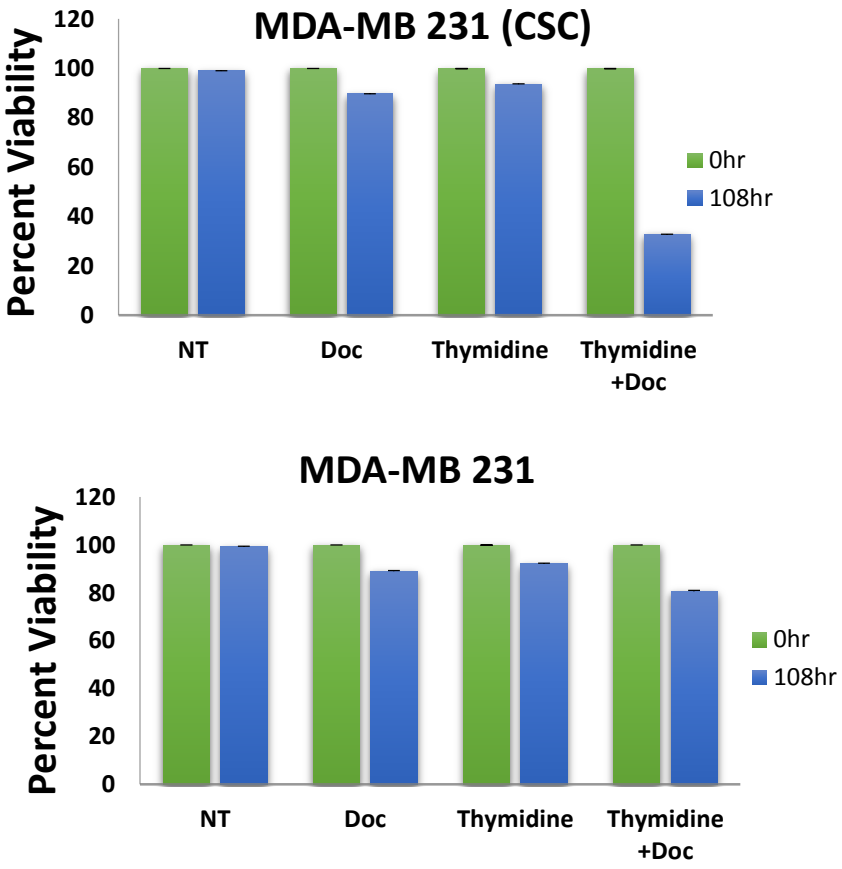

Figure 8: The Effect of Docetaxel and Thymidine on the cell viability in MDA-MB 231 cells by MTS. 4 x10^5 MDA-MB 231 cells and CSC were seeded in 6 well culture dishes 24 hour before exposal to drug treatments of thymidine and docetaxel. The percent viability was presents as mean +/- standard for three replicates. ($p < 0.001$ for all groups)

DICUSSION

Overall, the above studies provide evidence for a combination therapy to enhance BCa-SC sensitivity to chemotherapy. Previous studies suggest that BCa-SC are resistant to chemotherapy which causes relapse in patients. This still remains a limitation, thus it is highly imperative to find an effective therapy for eradicating this BCa-SC population.

We believe this can be achieved by using a combination drug. Although docetaxel is a common drug used for its treatment against BCa, further investigation is needed using a different chemotherapeutic drug. Due to the mechanism of action of docetaxel being cell cycle specific as explained previously[55], a non-cell cycle specific chemotherapy drug may provide useful in combination with the cell cycle drug thymidine. This will provide evidence to insure apoptosis is achieved by chemotherapeutic action and not the action of thymidine. This will further support the non-cytotoxic effects of thymidine.

For future studies we plan to use cisplatin for further experiments. Since 1972, cisplatin is considered the most effective drug in clinical use to treat many cancer types[56]. This alkylating agent forms covalent linkages which blocks DNA replication followed by discontinued RNA replication and protein synthesis[57]. Due to cancer cells having high proliferative ability, this drug specifically targets proliferation and induces apoptotic pathways[58]. Therefore,

we will plan further experiments using this drug to test whether BCa-SC are sensitive using a combination therapy and can undergo apoptosis.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Admission to Candidacy

Research Compliance Verification Form

Instructions
Complete this form, including all applicable forms and the signatures of the student, the student's advisor, and the Graduate Program Director. For research approval forms, contact the Institutional Review Board (IRB) (http://www.uab.edu/irb or 934-3789), or the Institutional Animal Care and Use Committee (IACUC) (http://www.uab.edu/iacuc or 934-7692).

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ☐ Yes (continue below) ☒ No
This research is:
Approved _____ Date _____

IRB Protocol No. _____

Attach a copy of your IRB approval. Your own name must appear on the original approval or on an attached amendment.

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and certain higher invertebrate animals used in research, teaching, or testing at UAB or sponsored through UAB but conducted off-site (i.e., field research or at collaborating institutions, etc.).

Does the research proposed by the student involve animal subjects? ☒ Yes (continue below) ☐ No

This research is:
Approved Yes Date 8-15-2013

IACUC Protocol No. 130808764

Attach a copy of your IACUC Notice of Approval, showing your research subject and the animal project number. If your own name does not appear on the Notice of Approval, take this form to the IACUC office for verification of approval.

The IACUC office verifies that Carnella M. Lee (name of candidate) is covered under the attached approval.

Signature of IACUC representative _____ Date: 1-6-14

NOTE: The student's advisor, the student, and the Graduate Program Director agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards (IRB and/or IACUC) if the proposed thesis or dissertation project requires approval. If approval already exists, this student's name must be added to the existing protocol before candidacy will be approved by the Graduate School. It is the responsibility of the student's advisor and the student to comply with federal and UAB regulations associated with this research. Documentation of continuous, appropriate approval will be required before degree conferral; all required IRB and/or IACUC approvals must be current at the time final versions of theses or dissertations are submitted to the Graduate School.

Student's Signature	Biology Dept.	January 2, 2014 Date
Signature of Student's Advisor	Pathology Dept.	January 2, 2014 Date
Graduate Program Director	Biology Dept.	1-3-14 Date

Updated 10/31/08

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: July 9, 2013

TO: SELVARANGAN PONNAZHAGAN, PhD
SHEL-814
(205) 934-6731

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Role of Innate and Adaptive Immunity in Breast Cancer Bone Metastasis
Sponsor: Internal
Animal Project_Number: 130808764

As of August 15, 2013 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number in Category
Mice	A	750
Mice	C	900

Animal use must be renewed by August 14, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 130808764 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
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