Evidence For The Involvement Of Runx1 And Runx2 In Maintenance Of The Breast Cancer Stem Cell Phenotype

Mark Fitzgerald
University of Vermont

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EVIDENCE FOR THE INVOLVEMENT OF RUNX1 AND RUNX2 IN MAINTENANCE OF THE BREAST CANCER STEM CELL PHENOTYPE

A Thesis Presented

by

Mark P. Fitzgerald

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of

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Thesis Examination Committee:

Gary S. Stein, Ph.D., Advisor
David Pederson, Ph.D., Chairperson
Janet Stein, Ph.D.
Seth Frietze, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College
ABSTRACT

In the United States, metastatic breast cancer kills approximately 40,000 women and 400 men annually, and approximately 200,000 new cases of breast cancer are diagnosed each year. Worldwide, breast cancer is the leading cause of cancer deaths among women. Despite advances in the detection and treatment of metastatic breast cancer, mortality rates from this disease remain high because the fact is that once metastatic, it is virtually incurable. It is widely accepted that a major reason breast cancer continues to exhibit recurrence after remission is that current therapies are insufficient for targeting and eliminating therapy-resistant cancer cells. Emerging research has demonstrated that these therapy-resistant cells possess stem cell-like properties and are therefore commonly referred to as breast cancer stem cells (BCSCs). A major hallmark of BCSCs is the cell surface expression of CD44 and lack of expression of CD24, the so-called CD24−/CD44+ phenotype. Research indicates that this dangerous and rare subpopulation of BCSCs may be responsible for cancer onset, recurrence, and ultimately metastasis that leads to death.

Two different model systems were utilized in this research. The first was the MCF7 cell line, a luminal A tumor subtype representative of a mildly invasive breast ductal carcinoma with an ER+/PR+/HER2- immunoprofile. The second was the MCF10A breast cancer progression model, which consists of three cell lines: MCF10A, MCF10AT1, and MCF10CA1a. In this system, spontaneously immortalized, non-malignant MCF10A cells were transfected with constitutively active H-Ras to form pre-malignant MCF10AT1 cells, which were then subcutaneously injected into mice and allowed to metastasize in order to form the oncogenic MCF10ACA1a cell line.

This thesis presents evidence of a CD24low+/CD44+ BCSC subpopulation within the MCF10A breast cancer progression model system. Findings indicate that RUNX1 and RUNX2 expression levels are involved in maintaining the BCSC phenotype. Across two different model systems, qRT-PCR analysis revealed that decreased levels of RUNX1 expression and increased levels of RUNX2 expression are essential for the maintenance of the BCSC subpopulation. It was also shown that low expression levels of RUNX1 and high expression levels of RUNX2 are present in CD24low+/CD44− BCSCs as compared to CD24+/CD44− non-BCSCs. Furthermore, shRNA knockdown of RUNX1 was shown to enhance tumorigenicity, while shRNA knockdown of RUNX2 repressed tumorigenicity in BCSCs, as measured by the tumorsphere-formation assay. This research lays the groundwork for future investigations into the roles of RUNX1 and RUNX2 in regulating stemness in breast cancer.
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CHAPTER 1: LITERATURE SUMMARY

Cancer Stem Cells

The existence of cancer stem cells first came to light back in the 1960’s and 1970’s when researchers studying leukemia and myeloma discovered that only a sub-fraction of cancer cells possessed the ability to proliferate extensively [1, 2]. Over the course of the past fifteen years or so, cancer stem cells have been shown to exist in many different types of solid tumors, including those of the prostate, brain, skin, colon and breast [3–8]. The cancer stem cell theory posits that only a subpopulation of cells within tumors, which have stem cell-like properties, are capable of tumorigenesis. These cells are defined by their ability to self-renew, give rise to differentiated progeny that constitute the bulk of the tumor mass, and form tumors in immunocompromised mice [9, 10]. This theory is in contrast to the traditional, stochastic model of tumorigenesis, which states that all cells within a tumor have tumorigenic potential [11]. It is also known that cancer stem cells are highly resistant to radiation and chemotherapy, and have enhanced metastatic potential [12, 13]. In summary, the cancer stem cell theory posits that only a small subpopulation of cancer cells with stem cell-like properties are able to self-renew and sustain a tumor, similar to the way in which adult stem cells self-renew and sustain tissues and organs. In this theory, non-stem cancer cells are able to cause problems in the body, but it is the cancer
stem cell subpopulation that is responsible for the sustained longevity of the cancer.

There is still debate as to the origin of cancer stem cells. One school of thought suggests that they originate from mutations in adult stem cells, while other possible explanations point to mutations in progenitor cells and transit amplifying cells as being the causative factor of their derivation. There is also speculation that cancer stem cells may arise from mutations in fully differentiated cells that reactivate pathways governing stemness and transformation [17-22]. Furthermore, it has been shown that cellular signaling pathways governing the epithelial to mesenchymal transition (EMT) are involved in the maintenance of the cancer stem cell phenotype [23]. Indeed, it seems possible that cancer cells with stem cell-like characteristics may have more than one method of origin, and it is imperative to identify regulatory molecules responsible for maintaining the cancer stem cell phenotype so that future therapies can target and eliminate this dangerous subpopulation of cells in order to improve patient outcomes.

**Breast Cancer Stem Cells**

In the US, metastatic breast cancer kills approximately 40,000 women and 400 men annually, and approximately 200,000 new cases of breast cancer are diagnosed each year. Worldwide, breast cancer is the leading cause of cancer deaths among women [24]. Despite advances in the detection and treatment of metastatic breast cancer, mortality rates from this disease remain high because
current therapies are insufficient at targeting and eliminating therapy-resistant breast cancer stem cells (BCSCs). A prevailing theory in the field of breast cancer research ascertains that one of the main reasons cancers exhibit recurrence after remission is the existence BCSCs that are chemotherapy and radiotherapy resistant. The resistance that BCSCs have to radiation is attributed to the ability of these cells to transition into and out of a quiescent state, while the chemoresistance of BCSCs is related to their expression of ATP-binding cassette (ABC) transporters, detoxifying transport pumps that normally function to protect cells from environmental toxins. These transporters are up-regulated in BCSCs, and two in particular have been studied extensively: breast cancer resistance protein (ABCG2) and P-glycoprotein (MDR1). ABCG2 is known to efflux doxorubicin, and MDR1 is known to efflux drugs such as paclitaxel. It is the elevated expression of these types of transporters, compared to non-BCSCs, which gives BCSCs enhanced chemoresistance [12,13].

BCSCs are characterized on the basis of three distinct abilities: self-renewal, differentiation (the ability to form multiple lineages with non-stem cell characteristics), and formation of tumors in immunocompromised mice [14]. There is still a good deal of controversy surrounding the definition of a breast cancer stem cell, and which biomarkers definitively identify this subpopulation of cells. A lack of correlation exists with respect to the biomarkers used to identify and define BCSCs among different breast cancer cell lines and patient tumor samples. Some of the most common biomarkers used to identify BCSCs
are the cluster of differentiation 24/44 (CD24+/CD44+) signature, aldehyde dehydrogenase 1A1 (ALDH+), and SRY-box 2 (Sox2+). It is speculated that each of these populations represents a unique subpopulation of breast cancer cells with stem cell-like properties rather than one common and definitive BCSC population [25]. One of the most common methods used for studying BCSCs is isolation of the CD24+/CD44+ fraction by fluorescence activated cell sorting (FACS).

A major way in which self-renewal ability and tumorigenicity is assessed in the BCSC population is by subjecting BCSCs to the tumorsphere/mammosphere assay. In this assay, single isolated CD24+/CD44+ BCSCs, or subpopulations defined by other common biomarkers, are plated on ultra low-attachment surfaces in serum-free medium supplemented with growth factors. Cells that possess a stem cell-like phenotype will proliferate and form three-dimensional, spherical colonies in suspension called tumorspheres. Cells that lack a stem cell-like phenotype will quickly die from anoikis [26]. It is believed that three-dimensional cell culture systems, such as mammosphere/tumorsphere culture, serve as a more representative model of the in vivo cancer environment than two-dimensional monolayer culture [27]. Furthermore, there is a known association between the ability of breast cancer cells to form tumorsphere in vitro and the in vivo tumorigenicity of tumorsphere-derived cells. In one such study, Grimshaw et al. isolated metastatic cells from pleural effusions of breast cancer patients, and subsequently placed them in tumorsphere culture.
They then dissociated the tumorspheres and injected the tumorsphere-derived cells into SCID mice. They found that cells that formed the largest tumorspheres \textit{in vitro} were also the most tumorigenic when xenografted into immunocompromised mice \cite{28,29}.

Indeed, it is well known that tumorigenicity and differentiation ability can be assessed by injecting isolated BCSCs subcutaneously in the mammary fat pad of immunocompromised mice and observing the formation of primary tumors containing both a BCSC population and a differentiated multi-lineage population. The highly tumorigenic CD24$^-$/CD44$^+$ phenotype of BCSCs was revealed in xenograft experiments conducted by the Wicha Laboratory at the University of Michigan, where they showed that as few as twenty cells with BCSC markers were required to generate primary heterogeneous tumors, whereas anywhere from tens of thousands up to one million non-BCSC cells were needed to generate primary tumors \cite{14,15}.

\textbf{The MCF7 Cell Line and the MCF10A/AT1/CA1a Breast Cancer Progression Model}

The MCF7 cell line is one of the most widely used model systems in breast cancer research. It originated from a pleural effusion of a 69-year-old Caucasian woman. These cells have a luminal epithelial mammary gland phenotype, and represent a luminal A tumor subtype. It is a mildly invasive breast ductal carcinoma, and has an ER$^+$/PR$^+/-$/HER2$^-$ immunoprofile. This cell line has been used widely for over forty years, and there has been extensive research done on
its molecular profile, invasion, migration and proliferation characteristics, and its involvement in lymphangiogenesis and angiogenesis [30,31]. More recently, it has been shown that that MCF7 cells have the ability to form tumorspheres when cultured in serum-free medium in non-adherent conditions. [32]

The MCF10A breast cancer progression model consists of three cell lines: MCF10A, MCF10AT1, and MCF10CA1a. The MCF10A cells are non-malignant breast cells, derived from a patient with benign fibrocystic disease, that were spontaneously immortalized. These cells are not capable of forming tumors [33]. MCF10A cells were transfected with constitutively active H-Ras to form the pre-malignant MCF10AT1 cell line, which is able to form tumors in xenograft experiments with a 25% incidence [34]. MCF10AT1 cells were then subcutaneously injected into mice and allowed to metastasize in order to form the oncogenic MCF10ACA1a cell line, which contains a \textit{PIK3CA} H1047R activating mutation. These cells always result in tumor formation when injected subcutaneously into immunocompromised mice [35]. This model system is widely used to study breast cancer progression.

\textbf{The RUNX Family of Proteins}

The RUNX (runt-related transcription factor) family of proteins is a group of evolutionarily conserved transcription factors that are involved in the regulation of developmental processes. Mammals have three RUNX genes: RUNX1, RUNX2 and RUNX3, and their expression and function vary based on tissue type. RUNX genes are involved in many major pathways associated with
development, such as WNT3, Notch5, transforming growth factor-β (TGFβ), YAP1, and Indian hedgehog4. All three of the RUNX genes in mammals are regulated by dual promoters, which are activated at different times during development to create distinct RUNX isoforms. Isoforms of the RUNX genes, which have distinctive properties, are also formed via alternative splicing events. The Runt domain, a highly conserved DNA-binding domain that contains both activation and inhibitory domains, is present in each of the RUNX proteins, and it binds to CBFβ in order to stabilize the interaction of RUNX with the DNA. The RUNX proteins are not strong transcriptional regulators by themselves, but their interactions with other proteins, such as such as H3K4 methyltransferase mixed-lineage leukaemia (MLL), histone deacetylases (HDACs), and Polycomb repressive complex 1 (PRC1), enhances their transcriptional activity [36-38].

The RUNX Genes and Cancer

Mutations in the RUNX genes are associated with a variety of cancers. Mutations in RUNX1, also known as acute myeloid leukemia 1 protein (AML1), play a significant role in leukemogenesis. RUNX2 is involved in bone development and differentiation. It is a factor specific to the bone lineage, and it is known to play a role in osteosarcoma. Elevated levels of RUNX2 are associated with bone-metastatic breast and prostate cancer, whereas inactivation of RUNX3 is a hallmark of many solid tumors [39-42]. The role that the RUNX genes play in the development of cancer was greatly elucidated when it was discovered that they are MYC-collaborating genes. All of the RUNX genes were
identified as viral insertional targets via proviral insertional mutagenesis experiments in mice genetically engineered to exhibit MYC-overexpression in T-lymphocytes (CD2-Myc transgenic mice). It has also been shown that viral-induced overexpression of RUNX proteins can result in aggressive T-cell lymphomas, and retroviral insertional mutation screens in Arf-knockout mice have revealed a link between RUNX and p53 in both lymphomagenesis and leukaemogenesis. [43-46]. Previous work from our lab using the MMTV-PyMT transgenic mouse model of breast cancer confirmed that RUNX1 is associated with breast cancer progression, and depletion of RUNX1 resulted in the inhibition of breast cancer invasion and migration. In this study, high levels of RUNX1 expression were found in MMTV-derived tumor cells, as well as in lung-metastatic legions of MMTV-PyMT mice. The ultimate conclusion drawn from this work was that breast cancer progression and metastasis is strongly linked to the dysregulation of RUNX1 in tumor epithelial cells [47]. Furthermore, previous studies from our group have repeatedly shown that RUNX2 is involved in breast cancer metastasis, predominantly to bone. [40,48,49]. Lastly, RUNX1 has been shown to be necessary for the development of mammary stem cells [50], which lead us to speculate about its role in breast cancer stem cells, and recent work done by our group has revealed that RUNX1 can function as a tumor suppressor by inhibiting the EMT process and promoting the maintenance of the epithelial phenotype [51].
CHAPTER 2: INVESTIGATING THE ROLE OF RUNX1 IN MCF7 BREAST CANCER STEM CELLS

Summary

The focus of this chapter is an analysis of a putative breast cancer stem cell population within a well-known and extensively researched model system of luminal breast cancer. It was hypothesized that since RUNX1 is known to have tumor suppressor activity, and is also known to be involved in the development of normal mammary stem cells, that depletion of the RUNX1 gene in MCF7 cells would promote the breast cancer stem cell phenotype. An experimental technique, the tumorsphere assay, was established for the first time in our laboratory in order to investigate this hypothesis. In this body of work, we show successful generation of tumorspheres, from the MCF7 cell line, that have increased expression of genes known to be associated with the breast cancer stem cell phenotype. We also show that when RUNX1 is depleted in this model system by an shRNA approach, tumorsphere formation, an indicator of tumorigenic potential, is increased; this outcome lends support to the role of RUNX1 as a tumor suppressor.

Introduction

Tumorspheres are single cell-derived, three-dimensional spherical colonies that grow in suspension when seeded into serum-free medium in ultra low-attachment plates. Tumorsphere culture is an established method used to
enrich for BCSCs, which is evidenced by the fact that tumorsphere forming ability of breast cancer cells in vitro correlates with the ability of tumorsphere cells to form tumors in immunocompromised mice in vivo [28,29]. Additionally, it is known that RUNX1 is involved in the development of normal mammary stem cells [50], and previous work by our group and others has shown that RUNX1 is involved in breast cancer progression [47]. Taken together, this evidence led us to hypothesize that RUNX1 may be involved in the establishment or maintenance of the BCSC population. In the experiments outlined in this chapter, we set out to utilize tumorsphere culture of the MCF7 cell line, a well known, established cell line capable of tumorsphere formation [30-32], as a means to enrich for BCSCs in order to begin investigating the putative role of RUNX1 in the BCSC phenotype.

**Materials and Methods**

**Cell Culture**

MCF7 cells were cultured in 100 mm plates in Dulbecco’s Modified Eagle Medium/Ham’s F-12 50/50 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% (v/v) 50 IU/ml penicillin/50 µg/ml streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were passaged every three to four days with 0.05% trypsin-EDTA, and media was replenished every other day.
**Tumorsphere Culture**

Adherent MCF7 cells were grown to 70 - 80% confluence and enzymatically dissociated into a single-cell suspension with 0.05% trypsin-EDTA. For tumorsphere formation assays, single cells were seeded into 6-well ultra-low attachment plates (Corning, Corning, NY, USA) at a density of either 8,000 or 16,000 cells per well and maintained in Mammocult Medium (STEMCELL Technologies, Vancouver, British Columbia, Canada) for four to five days. For qRT-PCR analysis, cells were seeded at a density of 1 x10^6 cells per plate in 100 mm Petri dishes, for the same duration of time, in order to obtain enough material for analysis. Tumorsphere formation efficiency (TFE) was calculated by dividing the number of tumorspheres formed by the number of single cells seeded, expressed as a percentage. Tumorspheres were manually counted under 100X magnification using an inverted phase-contrast microscope (Leica Microsystems, Wetzlar, DE).

**Quantitative Real-Time PCR**

Adherent MCF7 cell cultures that were approximately 70-80% confluent and Day 4-5 tumorsphere cultures were collected in Trizol (Life Technologies). RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) and subsequently diluted at a 1:15 ratio prior to performing quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems Foster City, CA, USA).
Statistical Analysis

Tumorsphere formation efficiency assays were repeated at least three times. The differences in mean values among groups were calculated, and TFE is expressed as the mean ± SEM. qRT-PCR analyses were conducted on two biological replicates.

Results

MCF7 Tumorsphere Assay

When adherent breast cancer cells are placed into tumorsphere culture conditions in ultra-low attachment plates, these cells are prevented from attaching to a substrate and are forced to grow in suspension. In such conditions, the vast majority of cells die as a result of anoikis, while those that have stem-like properties are able to replicate and form three-dimensional spheroids. The most important aspect of tumorsphere culture is the density, and this must be properly optimized in order to generate robust tumorsphere cultures. If the cultures are seeded at too high of a density, the tumorspheres will begin to fuse together, oftentimes so much so that all of the spheroids in a given culture well or plate will fuse into one continuous string-like structure that quickly becomes necrotic. In these experiments, tumorsphere cultures were successfully harvested for qRT-PCR analysis. Photomicrographs were taken every 24 hours for the duration of culture. Tumorspheres at Day 4 measured
between 100 – 200 μm in diameter. Images shown are representative of at least three biological replicates (Figure 1).

Tumorspheres began to be observable in culture at Day 1 when they were typically between 20 – 50 μm in diameter. During both Day 1 and Day 2 of culture, dying cells, or “ghost cells”, were observed attached to the forming spheroids. These are the cells that lack stem-like properties and are undergoing anoikis. Once the cultures reach Day 3 and Day 4, “ghost cells” were almost non-existent and the tumorspheres exhibited a smooth surface morphology. It is important to carefully determine beforehand the day that cultures reach maturity, for this is when the tumorspheres need to be counted for TFE analysis. The appropriate time to conduct the TFE assay is before the majority of the tumorspheres in culture begin to exhibit dark, necrotic cores. Once this takes place, the tumorspheres will continue to grow in size for several days; however, the necrosis begins to consume the entirety of the spheroids. If the cultures are allowed to go beyond this point, the spheroids will eventually begin to break apart until the point where there are no observable live cells in the culture. Through optimization, it was determined that the point of maturity for MCF7 tumorsphere culture was typically between Day 4 and Day 5.

**Gene Expression Analysis of Adherent MCF7 Cells vs. 3-D Tumorsphere Culture**

Gene expression analysis by qRT-PCR was performed on adherent MCF7 cells as well as whole tumorspheres that were harvested on Day 4, and results
were analyzed using GAPDH as a control (Figure 2). Tumorsphere-derived cells showed increased expression of RUNX1 (p = 0.043), RUNX2 (p = 0.044), Oct4 (p = 0.036), Nanog (p = 0.0021), Sox2 (p = 0.0031) and Vimentin (p = 0.024) compared to adherent cells. A very slight increase in CD44 and E-cadherin expression was also observed in tumorspheres compared to adherent cells, though this was not statistically significant, and there was no observable difference in CD24 expression levels between tumorspheres and adherent cells. This gene expression analysis was performed on two biological replicates. Gene expression analysis of RUNX1 and RUNX2 levels was performed because the aim of this study was to gain insight into whether or not altered levels of these transcription factors are associated with the cancer stem cell phenotype. Oct4, Nanog and Sox2 were examined because these are known markers of pluripotency and stemness. Vimentin and E-cadherin were chosen because they are known markers of the epithelial to mesenchymal transition (EMT), and altered expression of EMT markers is often associated with the cancer stem cell phenotype. CD24 and CD44 were examined because one known phenotype associated with breast cancer stem cells is CD24low-/CD44+, however significant differences in these markers were not observed in adherent MCF7 cells compared to whole tumorspheres. The CD24low- fraction is a mixed population of cells containing low or no CD24 expression as determined by FACS analysis. We hypothesized that since BSCSs with this aggressive BCSC phenotype are known to represent only a very small fraction of total breast cancer cells, this
CD24_{low}\text{/}CD44^+ phenotype was not observable when examining whole tumorspheres that were not FACS sorted for these markers.
Figure 1. MCF7 Tumorsphere Assay
MCF7 tumorsphere cultures were successfully established and maintained in culture for a period of four days. Representative images are shown at 100X and 200X magnification. Tumorspheres at Day 4 measured between 100 – 200 µm. Images shown are representative of at least three biological replicates.
Figure 2. Gene Expression Analysis of Adherent MCF7 cells vs. MCF7 Tumorspheres.

MCF7 tumorsphere cells exhibited significantly increased expression of RUNX1, RUNX2, and the pluripotency markers Oct4, Nanog and Sox2, as well as decreased expression of Vimentin, compared to adherent cells. Differences in CD24, CD44 and E-Cadherin expression levels were not significant. Error bars represent mean ± SEM. P-values are shown where differences in expression levels were significant. The expression differences in CD24, CD44 and E-Cadherin were not statistically significant.
To gain further insight into whether or not RUNX1 plays a role in the breast cancer stem cell phenotype, we used a RUNX1-knockdown MCF7 cell line previously created in our lab by Deli Hong [51]. We compared the tumorsphere forming efficiency (TFE) across MCF7 parental cells, two shRNA knockdown MCF7 stable cell lines representing two different shRNA constructs (C1 and C4), as well as a non-silencing shRNA control (NS) (Figure 3). TFE values were as follows: Parental = 1.25 ± 0.05% SEM, NS control = 1.17 ± 0.04% SEM, shRUNX1 C1 = 1.85 ± 0.03% SEM, and shRUNX1 C4 = 2.12 ± 0.05% SEM. These results show that knockdown of RUNX1 in MCF7 cells resulted in increased tumorsphere-forming ability compared to the non-silencing control. This indicated that shRUNX1-depleted MCF7 cells have increased tumorigenic potential, lending support to the notion that RUNX1 has tumor suppressor activity, and providing evidence that knockdown of RUNX1 may promote stemness in MCF7 cells.
Figure 3. Analysis of Tumorsphere Forming Efficiency in MCF7 Parental Cells vs. RUNX1 Knockdown.

shRUNX1 MCF7 cells exhibited an approximately 2-fold greater tumorsphere forming ability compared with the non-silencing control. Results shown are representative of three biological replicates.
Discussion

These experiments represented our first investigations into the cancer stem cell phenotype, as we set out to establish breast cancer stem cell research protocols. Through lengthy optimization and troubleshooting, we were able to successfully establish tumorsphere cultures from the MCF7 cell line. We showed that tumorspheres, a known measure of tumorigenic potential and cancer stemness, created from this model system express genes shown to be essential for pluripotency and known to be associated with the breast cancer stem cell phenotype. Our results also showed that tumorsphere-derived breast cancer stem cells exhibited increased expression of RUNX1 and RUNX2 compared to adherent cells. Furthermore, our results revealed that both shRUNX1 MCF7 cell lines exhibited increased tumorsphere-forming ability (~2-fold) compared with the non-silencing control. This indicated that knockdown of RUNX1 increased tumorigenic potential; lending support to the notion that RUNX1 exhibits tumor suppressor activity.

Interestingly, although loss of RUNX1 increased tumorsphere forming efficiency, the tumorsphere population as a whole exhibited higher RUNX1 expression levels than adherent MCF7 cells. This observation suggested that perhaps there is a subpopulation of cells within tumorspheres that express high levels of RUNX1 that may potentially represent breast cancer stem cells with significantly enhanced tumorigenic potential.
The initial rationale for this investigation strategy was to utilize tumorsphere culture as a means of enriching a breast cancer stem cell population large enough to conduct downstream analyses, since the CD24\(^-\)/CD44\(^+\) fraction in our MCF7 cell line was incredibly scarce (~0.5 – 1.0% of cells). This method proved to be technically unfeasible and prohibitively expensive, and thus this line of investigation was cut short. As a result, some experiments did not include a minimum of three biological replicates. We decided to move on to a different model system, which provided a much larger CD24\(^-\)/CD44\(^+\) population to work with, discussed in the next chapter.
CHAPTER 3: INVESTIGATING THE ROLE OF RUNX1 AND RUNX2 IN THE MAINTENANCE OF THE CANCER STEM CELL PHENOTYPE IN A BREAST CANCER PROGRESSION MODEL

Summary

The focus of this chapter is the identification and analysis of a putative breast cancer stem cell population within a known model system of breast cancer progression. We were able to identify, isolate and investigate a CD24<sup>low</sup>/CD44<sup>+</sup> breast cancer stem cell subpopulation within the pre-malignant, tumorigenic MCF10AT1 cell line of this progression model. We showed that this BCSC subpopulation has decreased levels of RUNX1 expression and increased levels of RUNX2 expression compared to the non-BCSC population, lending further support to the putative role of RUNX1 as a tumor suppressor. Given the fact that elevated levels of RUNX2 are associated with some metastatic breast cancers, and the fact that breast cancer stem cells are known to have increased metastatic potential, we hypothesized that depletion of RUNX2 by an shRNA approach would suppress the breast cancer stem cell phenotype. Our results provided preliminary evidence that depleting RUNX2 decreased the breast cancer stem cell population in this model system of breast cancer progression.

Introduction

The MCF10A model consists of three cell lines that together represent a complete progression system from normal, but immortalized, breast epithelial cells to fully metastatic breast cancer cells. MCF10A cells were derived from
benign breast tissue of a female patient with fibrocystic disease. These cells were subsequently transformed via transfection with T24 Ha-ras to form the premalignant MCF10AT1 cell line. MCF10AT1 cells were then subcutaneously injected into SCID mice and allowed to metastasize in order to generate the metastatic MCF10CA1a cell line [34,35].

It has previously been established by our group that RUNX1 and RUNX2 are intimately involved in breast cancer progression and metastasis [47-49]. Furthermore, it is known that BCSCs are a highly aggressive and metastatic subpopulation of tumor cells thought to be responsible for cancer onset, metastasis and recurrence following remission [14,15]. Based on the outcomes of the experiments outlined in Chapter 1, we needed to find a way to obtain a greater number of BCSCs in order to further our investigations. Since it is known that human BCSCs can be identified by the CD24\textsuperscript{low/-}/CD44\textsuperscript{+} phenotype [16], and since our preliminary FACS analyses of this model system indicated that there may be a significant CD24\textsuperscript{low/-}/CD44\textsuperscript{+} subpopulation within the MCF10AT1 cell line, we chose this model in order to obtain a more robust CD24\textsuperscript{low/-}/CD44\textsuperscript{+} BCSC population with which to further investigate the putative roles of RUNX1 and RUNX2 in the BCSC phenotype.
Materials and Methods

Cell Culture

MCF10A and MCF10AT1 cells were cultured in 100 and 150 mm plates in Dulbecco’s Modified Eagle Medium/Ham’s F-12 50/50 (Thermo Fisher Scientific) supplemented with 5% (v/v) horse serum (Thermo Fisher Scientific), 100 ng/ml cholera toxin (Sigma Aldrich, St. Louis, MO), 10 µg/ml human insulin (Sigma Aldrich), 20 ng/ml recombinant hEGF (Peprotech, Rocky Hill, NJ), 0.5 µg/ml hydrocortisone (Sigma Aldrich), and 1% (v/v) 50 IU/ml penicillin/50 µg/ml streptomycin (Life Technologies). MCF10CA1a cells were cultured in 100 and 150 mm plates in Dulbecco’s Modified Eagle Medium/Ham’s F-12 50/50 (Thermo Fisher Scientific) supplemented with 5% (v/v) horse serum (Thermo Fisher Scientific) and 1% (v/v) 50 IU/ml penicillin/50 µg/ml streptomycin (Life Technologies). Cells were passaged every three to five days with 0.05% trypsin-EDTA, and media was replenished every other day. The shRUNX2 cell lines were created by Alexandra Ojemann, a Master’s student in the Stein-Lian Laboratory.

Mammosphere and Tumorsphere Culture

Adherent MCF10A, MCF10AT1 and MCF10CA1a cells were grown to 70 – 80% confluence and enzymatically dissociated into single-cell suspensions with 0.05% trypsin-EDTA. Mammosphere are three-dimensional spherical colonies formed from non-transformed mammary cells, while tumorspheres are three-dimensional spherical colonies formed from transformed mammary cells. For
mammosphere (MCF10A) and tumorsphere (MCF10AT1, MCF10CA1a) formation assays, single cells were seeded into 24-well ultra-low attachment plates (Corning) at a density of either 1,000 or 2,000 cells per well and maintained in Mammocult Medium (STEMCELL Technologies) for five to seven days. Tumorsphere formation efficiency (TFE) was calculated by dividing the number of tumorspheres formed by the number of single cells seeded, expressed as a percentage. Tumorspheres were manually counted under 100X magnification using an inverted phase-contrast microscope (Leica Microsystems).

**CD24/CD44 Flow Cytometry**

Adherent monolayer cultures of MCF10A, MCF10AT1 and MCF10CA1a cells were grown to 70 – 80% confluence and gently dissociated with Accutase (MP Biomedicals, Santa Ana, CA, USA). Culture vessels with Accutase were placed at 37°C and checked every 60 seconds to minimize incubation time with the dissociation agent in order to preserve the integrity of the cell surface markers. The Accutase was immediately neutralized with fetal bovine serum (Atlanta Biologicals) and the cells were subsequently washed with 1x phosphate buffered saline (PBS) at a concentration of 10 mM PO₄³⁻, 137 mM NaCl, and 2.7 mM KCl. Cells were then quickly counted on a Countess automated cell counter (Invitrogen/Thermo Fisher Scientific) and 1x10⁶ cells were re-suspended in 100 µl 1x PBS/1%FBS containing 0.64 µg of PE/Cy7 anti-human CD24 (Biolegend, San Diego, CA, USA, cat. # 311120) and 5µl of APC mouse anti-human CD44 (BD Biosciences, San Jose, CA, USA, cat. # 559942). Equivalent amounts of matched
isotype controls, PE/Cy7 Mouse IgG2a, κ (Biolegend, cat. # 400232) and APC mouse IgG2b κ (BD Biosciences, cat. # 555745), were used. Cells were incubated with antibodies for thirty minutes at room temperature, washed twice with 1xPBS/1%FBS, re-suspended in 400 μl 1xPBS/1%FBS and passed through a 40 μm Falcon cell strainer (Thermo Fisher Scientific) in order to obtain a single-cell suspension. Cells were then either analyzed on a BD LSRII flow cytometer (BD Biosciences) or analyzed and sorted into CD24_{low/-}/CD44^{+} breast cancer stem cell (BCSC) and CD24^{+}/CD44^{+} non-BCSC fractions on a BD FACS AriaIII (BD Biosciences) high-speed cell sorter. Analysis of flow cytometry data was performed on FlowJo software version 10.0.8rl

**Quantitative Real-Time PCR**

CD24_{low/-}/CD44^{+} BCSC and CD24^{+}/CD44^{+} non-BCSC fractions were isolated via FACS and subsequently placed in Trizol (Life Technologies). RNA was isolated using the miRNeasy Mini Kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) and subsequently diluted at a 1:15 ratio prior to performing quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems).

**Western Blotting**

Sorted cells were lysed with radioimmunoprecipitation assay buffer (RIPA) consisting of 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris (pH 8.0), as
well as 2X SDS sample buffer consisting of 1 M Tris (pH 6.8), 50% glycerol, 10% SDS, 0.5% bromophenol blue, 0.5% β-mercaptoethanol, 5 μM MG132 proteasome inhibitor (EMD Millipore San Diego, CA, USA), and cOmplete EDTA-free protease inhibitor cocktail tablets (1 tablet per 10 ml) (Roche Diagnostics, Indianapolis, IN, USA). Cell lysates were fractionated in an 8.5% polyacrylamide gel and immunoblotted. Gels were wet-transferred to PVDF membranes (EMD Millipore) using a Bio-Rad transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) and primary antibody incubation was performed overnight at 4°C using the following primary antibodies: rabbit monoclonal RUNX1 (Cell Signaling Technology, Danvers, MA, USA: #4336, 1:1000); rabbit monoclonal RUNX2 (Cell Signaling Technology #12556, 1:1000); rabbit monoclonal Zeb1/TCF8 (Cell Signaling Technology #3396, 1:1000); mouse monoclonal Vimentin (Santa-Cruz Biotechnology, Santa Cruz, CA, USA: sc-6260, 1:1000); mouse monoclonal E-cadherin (Santa Cruz Biotechnology sc21791, 1:1000); mouse monoclonal β-Actin (Cell Signaling Technology #3700, 1:1000). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for immunodetection. Blots were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories) and subsequently imaged on the Chemidoc XRS+ imaging system (Bio-Rad Laboratories).
Statistical Analysis

Tumorsphere formation efficiency assays were repeated at least three times. The differences in mean values among groups were calculated, and TFE is expressed as the mean ± SEM. qRT-PCR analyses were repeated three times. GAPDH was used as a control. Relative mRNA levels were analyzed using a student's t-test. P-values less than 0.05 were considered statistically significant (*P < 0.05, **P < 0.01, ****P < 0.0001).

Results

Identification and Isolation of Breast Cancer Stem Cells

We set out to determine if we could identify and isolate CD24\textsubscript{low/-}/CD44\textsuperscript{+} BCSC populations in the MCF10A progression model. Indeed, our results showed that we were able to identify a CD24\textsubscript{low/-}/CD44\textsuperscript{+} subpopulation in the MCF10AT1 cell line. Both the BCSC subpopulation and the non-BCSC CD24\textsuperscript{+}/CD44\textsuperscript{+} population were isolated by FACS. The BCSC fraction represented, on average, between 17 – 23\% of total live cells gated. Matched isotype controls and single-stained control samples were use to establish gating parameters, and the gating strategy was specifically designed to allow for maximum separation between the BCSC and non-BCSC populations with respect to CD24 expression: one gate was centered around the densest region of the CD24\textsubscript{low/-}/CD44\textsuperscript{+} subpopulation and the other gate was centered around the densest region of the CD24\textsuperscript{+}/CD44\textsuperscript{+} population. (Figure 4).
Figure 4. FACS Gating Strategy for MCF10AT1 CD24<sub>low</sub>-/CD44<sup>+</sup> BCSC and CD24<sup>+</sup>/CD44<sup>+</sup> non-BCSC Populations. A distinct CD24<sub>low</sub>-/CD44<sup>+</sup> BCSC subpopulation was discovered in the MCF10AT1 cells. Gates were used to isolate non-BCSCs from BCSCs. The BCSC subpopulation ranged from ~17–23% of the total cell population.
MCF10A/AT1/CA1a Mammosphere/Tumorsphere Assay

Next, we investigated whether or not all cell lines in this system were capable of forming mammospheres and tumorspheres (see Materials and Methods). Briefly, adherent cells were enzymatically detached and single-cell suspensions were created. Cells were seeded at a density of either 1,000 or 2,000 cells per well in 24-well ultra low-attachments plates and maintained in serum-free culture medium for a period of five to seven days. Our results showed that MCF10A mammosphere as well as MCF10AT1 and MCF10CA1a tumorsphere cultures were successfully established and maintained in culture for a period of five days. Each cell line was cultured in four separate wells in order to have four technical replicates. Photomicrographs were taken every twenty-four hours for the duration of culture. A minimum of four images was taken for each well in order to create an archive of the most representative images possible. MCF10A mammospheres measured ≤ 100 μm in size, while MCF10AT1 and MCF10CA1a tumorspheres measured between 100 - 400 μm in size. Tumorspheres showed a drastically larger morphology compared to mammospheres. MCF10AT1 tumorspheres exhibited a more uneven and rough surface morphology than MCF10CA1a tumorspheres, which exhibited a smooth surface morphology (Figure 5). As we expected, due to the fact that cancer cells divide uncontrollably, the tumorspheres formed by the cancerous cell lines were markedly larger than the mammospheres formed by the MCF10A cells. The unique morphologies associated with the three-dimensional spheroids formed
by each cell line were reproducibly observed in upward of twenty biological replicates.
Figure 5. MCF10A, MCF10AT1 and MCF10CA1a Mammosphere/Tumorsphere Assays. MCF10A (mammosphere), MCF10AT1 and MCF10CA1a (tumorsphere) cultures were successfully established and maintained in culture for a period of 5 days. Tumorspheres exhibited a larger morphology compared to mammospheres, and distinct morphological differences between spheroids from the three different cell lines were observed. Images shown are representative of at least three biological replicates, and are shown at 100X magnification.
Tumorsphere Forming Capacity of MCF10AT1 Subpopulations

In order to gain further insight into the true tumorsphere forming ability of BCSCs versus non-BCSCs from MCF10AT1 cell cultures, we performed three-dimensional tumorsphere assays with FACS-sorted populations. Tumorsphere cultures from CD24\text{low/-}/CD44\text{+} BCSCs and CD24\text{+}/CD44\text{+} non-BCSCs were successfully established and maintained in culture for a period of seven days. Photomicrographs were taken every twenty-four hours for the duration of culture. Tumorspheres formed from CD24\text{+}/CD44\text{+} non-BCSCs measured ≤100 μm in size, while CD24\text{low/-}/CD44\text{+} BCSC tumorspheres measured in excess of 400 μm in size over the course of seven days (Figure 6). In these experiments, there were extreme size and morphological differences in spheroids formed by BCSCs compared to non-BCSCs, and these observations were consistent throughout multiple biological replicates.

In order to quantify the tumorsphere forming ability of both cancer stem cell and non-cancer stem cell populations, we calculated the tumorsphere forming efficiency (TFE) of each population. TFE was compared between MCF10AT1 CD24\text{low/-}/CD44\text{+} BCSCs and the CD24\text{+}/CD44\text{+} non-BCSC population. TFE values were as follows: BCSCs = 3.63 ± 0.15% SEM, and non-BCSCs = 1.4 ± 0.15% SEM (Figure 7). As expected, the cancer stem cell subpopulation had a much higher TFE than the non-stem cell population and the tumorspheres formed by the BCSCs were significantly larger.
Figure 6. MCF10AT1 CD24$^{\text{low/-}}$/CD44$^+$ BCSC and CD24$^+$/CD44$^+$ non-BCSC Tumorsphere Assays.

(A) Tumorspheres formed from FACS-isolated BCSCs exhibited a vastly different morphology than those formed from (B) FACS-isolated non-BCSCs. Images shown are representative of at least three biological replicates, and are shown at 100X magnification.
Figure 7. Analysis of Tumorsphere Forming Efficiency in MCF10AT1 CD24\textsuperscript{low/-} /CD44\textsuperscript{+} BCSC and CD24\textsuperscript{+}/CD44\textsuperscript{+} non-BCSC Tumorsphere Assays. MCF10AT1 CD24\textsuperscript{low/-} /CD44\textsuperscript{+} BCSCs exhibited increased tumorsphere forming efficiency (p = 0.0019) compared to the CD24\textsuperscript{+}/CD44\textsuperscript{+} non-BCSC population. Results are representative of three biological replicates. n = 3.
RNA and Protein Expression Analysis of MCF10AT1 CD24\textsubscript{low/-}/CD44\textsuperscript{+} BCSCs vs. CD24\textsuperscript{+}/CD44\textsuperscript{+} non-BCSCs

We next examined expression of several genes, including several stem cell markers, in the CD24\textsubscript{low/-}/CD44\textsuperscript{+} breast cancer stem cell population of MCF10AT1 cells compared to the CD24\textsuperscript{+}/CD44\textsuperscript{+} non-breast cancer stem cell population. The CD24\textsubscript{low/-}/CD44\textsuperscript{+} breast cancer stem cell population of MCF10AT1 cells exhibited a statistically significant increase in expression of RUNX2 (p = 0.0068), Vimentin (p = 0.0026), Nanog (p = 0.0034), Zeb1 (p = 0.0022), and Zeb1 lncRNA (p = 0.0063) compared to non-BCSCs, and showed a statistically significant decrease in expression of RUNX1 (p = 0.041), Oct 4 (p = 0.013) and CD24 (p < 0.0001) compared to non-BCSCs (Figure 8). CD44 RNA expression was elevated in BCSCs, though this difference was not statistically significant (Figure 8). RUNX1 decreased slightly (p = 0.041) and RUNX2 increased slightly (p = 0.0068) within the CD24\textsubscript{low/-}/CD44\textsuperscript{+} BCSCs compared to the non-BCSCs. The changes in RUNX1 and RUNX2 expression appeared to decrease and increase, respectively, with CD24 expression. The pluripotency marker Nanog increased in expression (p = 0.0034) in the BCSC population compared to non-BCSCs, and expression of Nanog increased with decreased CD24 expression. Similarly, expression of Zeb1 and Zeb1 lncRNA also increased with decreased CD24 expression.

We observed a reciprocal relationship between RUNX1 and RUNX2 expression in MCF10AT1 BCSCs. We know from our observations in the MCF7
cell line that knockdown of RUNX1 can increase TFE and promote

tumorigenicity. Given our observation that expression of RUNX2 is increased in
BCSCs, and considering the fact that RUNX2 is involved in metastatic cancer, we
hypothesized that RUNX2 is involved in promoting or maintaining stemness in
the BCSC population, and that knockdown of RUNX2 will decrease TFE in the
CD24\text{low-}/CD44^+ population. This hypothesis was tested in subsequent
experiments.
Figure 8. Gene Expression Analysis of MCF10AT1 CD24low/-/CD44+ BCSCs vs. CD24+/CD44+ non-BCSCs.

MCF10AT1 BCSCs exhibited significantly increased expression of RUNX2, Vimentin, Nanog, Zeb1 and Zeb1 IncRNA compared to non-BCSCs, as well as significantly decreased expression of RUNX1, Oct4 and CD24 compared to non-BCSCs. P-values are shown where expression differences were significant. The difference in CD44 expression was not statistically significant.
Consistent with results from RNA expression analysis, MCF10AT1 CD24$^{\text{low/-}}$/CD44$^+$ BCSCs exhibited a marked increase in RUNX2, Zeb1 and Vimentin protein levels, and a decrease in RUNX1, E-Cadherin and CD24 protein levels compared to non-BCSCs. β-actin was used as a loading control. Western blot and protein quantification results are representative of one biological replicate, and thus not statistically significant. However, others in our group have replicated these experiments in triplicate and results are consistent with the data shown (Figure 9).
Figure 9. Western Blots and Protein Quantification of RUNX1, RUNX2, Zeb1, Vimentin and CD24 in MCF10AT1 CD24\textsubscript{low}/-/CD44\textsuperscript{+} BCSCs vs. CD24\textsuperscript{+}/CD44\textsuperscript{+} non-BCSCs.

BCSCs exhibited a marked increase in RUNX2, Zeb1 and Vimentin as well as a decrease in RUNX1, E-Cadherin and CD24 compared to non-BCSCs as indicated by Western blot and associated protein quantification.
CD24/CD44 Flow Cytometric Analysis and Tumorsphere Forming Efficiency of MCF10AT1 shRUNX2 Knockdown Cells

In order to test our hypothesis that knockdown of RUNX2 will decrease the BCSC population, CD24/CD44 flow cytometry was performed on MCF10AT1 parental, shRUNX2 knockdown, and empty vector control cells lines. Results show that the distinct CD24\text{low/-}/CD44\text{+} BCSC subpopulation observed in the parental cells is decreased in shRUNX2 knockdown cells versus empty vector control. According to FACS analysis, the CD24\text{low/-}/CD44\text{+} subpopulation represents ~ 24% of live-gated cells in the parental cell line, ~ 12% of live-gated cells in the EV control, and ~ 4% of live-gated cells in the shRUNX2 cell line. These results are representative of three biological replicates (Figure 10).

In order to gain further evidence in support of our hypothesis, we examined the tumorsphere forming ability of MCF10AT1 shRUNX2 cells, shRUNX2 empty vector control, and the MCF10AT1 parental cell line. The TFE of shRUNX2 MCF10AT1 cells was 0.70 ± 0.12% SEM versus 1.53 ± 0.15% SEM for the empty vector control and 1.83 ± 0.12% SEM for the parental cell line (Figure 11). These results support our hypothesis that knockdown of RUNX2 decreases the BCSC subpopulation in MCF10AT1 cells.
Figure 10. CD24/CD44 Flow Cytometry of MCF10AT1 shRUNX2 Knockdown Cells vs. Parental and Empty Vector Control. MCF10AT1 shRUNX2 knockdown cells showed a marked decrease in the CD24\textsuperscript{low}/CD44\textsuperscript{+} BCSC subpopulation vs. empty vector control and parental cells. The CD24\textsuperscript{low}/CD44\textsuperscript{+} subpopulation represents ~ 24\% of live-gated cells in the parental cell line, ~ 12\% of live-gated cells in the EV control, and ~ 4\% of live-gated cells in the shRUNX2 cell line.
Figure 11. Tumorsphere Assays and TFE Analysis of MCF10AT1 shRUNX2 Knockdown Cells vs. Parental and Empty Vector Control. MCF10AT1 shRUNX2 knockdown cells exhibited decreased tumorsphere forming efficiency compared to empty vector control and parental. Assay was performed in triplicate, and images are shown at 100X magnification.
Discussion

In order to obtain a robust CD24-/CD44+ breast cancer stem cell population, we turned to the MCF10A/AT1/CA1a breast cancer progression model system [30,31]. Initial flow cytometric analysis revealed a distinct CD24low-/CD44+ BCSC subpopulation within the pre-malignant, tumorigenic MCF10AT1 cell line. This subpopulation exhibited decreased levels of RUNX1 and increased levels of RUNX2 compared to the non-BCSC population. Expression levels of Vimentin, Nanog, Zeb1, and Zeb1 lncRNA were also elevated in the BCSC population compared to the non-BCSC population, while expression levels of Oct4 and CD24 were decreased in the BCSC population. We also showed that knockdown of RUNX2 decreased tumorigenicity, as measured by the tumorsphere formation assay. Taken together, these observations suggest that a combination of low expression of RUNX1 and elevated expression of RUNX2 may be involved in the maintenance or development of the breast cancer stem cell phenotype.

The shRNA RUNX2 knockdown MCF10AT1 cell line was created by Alexandra Ojemann, a Master’s student in the Stein-Lian Laboratory. However, subsequent RNA-seq analysis indicated that this may not have been a complete knockdown. As a result, the observations made here cannot be interpreted with a high degree of certainty, and it would be appropriate to repeat these experiments with a different set of shRUNX2 knockdown cell lines.
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The findings presented in this body of research provide evidence that regulation of, or cellular pathways involved in the regulation of, RUNX1 and RUNX2 may be important for the maintenance of the breast cancer stem cell phenotype. We were able to show, in two different model systems, that low expression levels of RUNX1 and elevated expression levels of RUNX2 are present in CD24\textsuperscript{low/−}/CD44\textsuperscript{+} breast cancer stem cells.

In Chapter 2, we show definitively that knockdown of RUNX1 enhances tumorigenicity in breast cancer cells, as measured by tumorsphere formation efficiency. In Chapter 3, our observations suggest that knockdown of RUNX2 depletes the CD24\textsuperscript{low/−}/CD44\textsuperscript{+} breast cancer stem cell subpopulation and represses tumorigenicity of breast cancer cells, as measured by the tumorsphere formation assay.

Implications and Future Directions

The results presented in this thesis represent preliminary investigations into the putative roles of RUNX1 and RUNX2 in maintenance of the breast cancer stem cell phenotype. In order to lend more credence to these observations, the work with the MCF7 cell line needs to be reproduced and expanded upon, and the work done with the knockdown RUNX2 MCF10AT1 cells needs to be repeated with newly created stable knockdown cell lines. There are several
exciting implications put forth by this body of work that, if expanded upon, have the potential to reveal significant insights into the putative roles of RUNX1 and RUNX2 in regulating the breast cancer stem cell phenotype. This body of work has, for the first time, effectively established three-dimensional cancer stem cell culture protocols for our group. There is no doubt that, with time, our group will expand upon these studies in order to glean further insights into the mechanisms that govern stemness in breast cancer.

Using the mammosphere/ tumorsphere culture protocols for a variety of breast cancer cell lines that have been developed through this work, there are several avenues of investigation that we can now potentially pursue. It would be very exciting to see if we can establish tumorsphere cultures from patient breast cancer tissue samples, isolate and conduct analyses on the CD24\(^{\text{low/}}\)/CD44\(^{+}\) fraction, and compare the results to those obtained through these investigations. A study such as this would enable us to gain further insight into the involvement of RUNX1 and RUNX2 in regulating or maintaining the BCSC phenotype. Furthermore, we now have a three-dimensional cell culture system designed to enrich for BCSCs that can potentially be used for drug screening, toxicity testing, and further knockdown experiments.

Given the fact that traditional cancer therapeutics are insufficient at targeting breast cancer cells with stem cell-like properties, it is of paramount importance that genes and regulatory molecules responsible for governing stemness in breast cancer cells are identified so that novel therapies can be
developed to target and eliminate this incredibly dangerous subpopulation of cells. Indeed, it is widely accepted that breast cancer stem cells are a main causative factor in the initial onset of cancer, and are thought to be responsible for cancer recurrence following remission, as well as metastasis that ultimately leads to death. Findings from this research lay a strong groundwork for future investigations into cellular pathways that govern stemness in breast cancer cells. Gene expression analyses performed here provide interesting starting points for future investigation into genes that are potentially involved in maintaining the breast cancer stem cell phenotype. It would also be very interesting to analyze these BCSC populations at the epigenetic level in order to gain insight into epigenetic regulatory mechanisms involved in governing the breast cancer stem cell phenotype.

Since there still remains much debate over the consensus of population-defining cell-surface markers, and taking into consideration the fact that there is a general lack of cell-surface markers ascribed to them, it continues to be extremely challenging to identify and isolate cancer stem cells (CSCs) from solid tumors. As a result, functional assays are the techniques that are predominantly used to assess the ability of CSCs, or prospective CSC populations, to generate tumors via self-renewal, asymmetric division, and differentiation. Many of these assays are extremely costly and time-consuming because they are based on the formation of tumors in vivo following xenograft transplantations into immunocompromised mice. Since the capability to form spheroids in vitro is an
established method to identify CSC populations, spheroid formation assays represent a relatively low-cost \textit{in vitro} model with which to investigate the CSC phenotype.

Three-dimensional cell culture systems are thought to better mimic the \textit{in vivo} cancer environment compared to traditional two-dimensional monolayer systems. Some of the most popular three-dimensional cell culture systems for studying breast cancer are tumorspheres and organoids, and tumorsphere culture is specifically known to enrich for BCSCs. Future investigations in the field utilizing these two systems will surely help to usher in the discovery of yet to be identified cell-surface markers that will ultimately aid in the development of therapeutics that can target the dangerous BCSC cell populations with enhanced specificity [52-54]. In fact, several developments have occurred since the writing of this manuscript that have served to advance the fields of breast tumorsphere and organoid research. Dr. Hans Clever’s Laboratory has developed a breast cancer organoid biobank consisting of over one hundred organoid lines. Since these organoids are by definition patient-derived three-dimensional cell culture systems, they are highly representative of their \textit{in vitro} breast tumor counterparts, and these models have been adapted for high-throughput screening [55]. The Clevers Lab has also developed organoid systems for gastrointestinal cancers, which they’ve shown to be highly effective models for drug screening. In these patient-derived gastrointestinal organoid cultures, every time that a drug did not work in a patient’s organoids, it also did
not work in the patient. Furthermore, in almost nine out ten cases when drugs were first tested and shown to work in a patient’s organoids, the patient exhibited a response to the drug [56]. Tumorspheres differ from organoids in that they don’t recapitulate the original tissue hierarchy to the same extent, however they are three-dimensional cell culture systems that better mimic the \textit{in vivo} environment than traditional two-dimensional monolayer systems, they are more cost effective and less time-consuming, and tumorsphere cultures can also be readily adapted for high-throughput screening.

It is clear that three-dimensional cell culture systems will play an incredibly important role in the future of cancer research. The protocols established via this body of research have laid the groundwork for our group to further investigate the molecular mechanisms of breast cancer progression and metastasis using three-dimensional tumorsphere culture. This body of work has also provided invaluable insights into the involvement of RUNX1 and RUNX2 in the maintenance of the BCSC phenotype; insights that can be expanded upon to further our understanding of BCSC biology, aid in the discovery of novel BCSC markers, and assist in investigations into the mechanisms of epigenetic regulation and cell-specific nuclear architecture associated with the BCSC phenotype.
BIBLIOGRAPHY


