A MULTI-PRONGED MODULATION OF SIGNALING PATHWAYS IN TARGETING BREAST CANCER STEM CELLS

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmaceutical Sciences)
in The University of Michigan
2012

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To my family for their love and unwavering support

ACKNOWLEDGEMENTS

I would like to extend my heartfelt thanks to many people that helped me in this endeavor. First is my advisor, Dr. Duxin Sun, who I regard as one of the most influential people in my graduate study. It is a privilege to be a part of his group and I am immensely thankful for his support in research as well as warm caring he and his wife gave me. I would also like to thank my committee members, Dr. Max S. Wicha, Dr. David E. Smith and Dr. Wei Cheng for their valuable time and thoughtful input. I especially thank Dr. Max S. Wicha for his guidance in research and generosity to provide me with the technique and laboratory resources through the entirety of my dissertation study.

Secondly, I would like to thank my lab mates past and present and peer students in the program, for their friendship and supports, particularly to Bryan Newman for his tremendous help in animal studies and gracious help in proofreading my manuscripts. Also, I appreciate the many types of financial support that allowed me to complete my Ph.D. journey.

Third, I would like to show my appreciation to collaborators, Dr. Hasan Korkaya, Dr. Sulin Liu, Dr. Shawn G. Clouthier and Dr. Sarah J. Conley and Dr. Jian Zhu. I especially thank Dr. Jian Zhu for his help on analyzing microarray data. The following people, Dr. Juhchin Yang and Mrs. Grace Cheng, Dr. Asen Ivanov and Dr.

Shodai Ota have made Ann Arbor feel like my second home and I would like to send my sincere gratitude for giving me the strength to complete my degree.

I would like to thank my parents, my sister and my brother for supporting me in every way they could through this process. Without their support, I would not have been able to study overseas to pursue my dream.

TABLE OF CONTENTS

DEDICATION----------ii

ACKNOWLEDGEMENTS ----------iii

LIST OF TABLES ----------vii

LIST OF FIGURES ----------viii

Chapter 1

Research Objectives ---------- 1

Chapter 2

Background

Breast cancer overview----------6
Cancer stem cells----------8
Identification of cancer stem cells in breast carcinomas----------10
Breast CSC Markers----------12
Origin of CSCs----------13
Resistance of CSCs to chemotherapies----------16
Pathways regulate cancer stem cells----------18
Methodology used in studying breast CSCs----------25
Cancer stem cell therapy----------27
Histone deacetylase (HDAC) ----------28
Heat shock protein 90 (Hsp90) ----------30
References----------36

Chapter 3

Hsp90 inhibitor 17-(Allylamino)-17-Demethoxygeldanamycin Targets Breast Cancer Stem Cells

Abstract----------48
Introduction----------50
Methods----------53
Results----------59
Discussion----------67
Conclusions----------72
Figures----------73

References----------82

Chapter 4

Preclinical evaluation of efficacy of 17AAG in combination with Docetaxel in adjuvant therapy

Abstract----------87
Introduction----------89
Methods ----------92
Results----------96
Discussion----------100
Conclusions----------104
Figures----------105
References----------110

Chapter 5

HDAC inhibitor SAHA targets Breast Cancer Stem Cells

Abstract----------113
Introduction----------115
Methods----------118
Results----------125
Discussion----------133
Conclusions----------140
Tables----------141
Figures—----------143
References----------155

LIST OF TABLES

Table 5.1 Primers used for real-time RT-PCR analyses----------141
Table 5.2 Highly activated genes in Aldefluor-positive cells----------142

LIST OF FIGURES

Figure 2.1 $CD44^{+}CD24^{-}$/$lineage^{-}$ cells recapitulated the phenotypic diversity-------12

Figure 2.2 Roles of CSCs in tumorigenesis and treatment resistance in breast carcinoma----------16

Figure 2.3 Schematic representation of the Notch pathway and putative therapeutic targets----------20

Figure 2.4 Schematic representation of the Hedgehog pathway and putative therapeutic targets----------22

Figure 2.5 Schematic representation of the Wnt pathway and putative therapeutic targets----------24

Figure 2.6 The Hsp90 chaperoning cycle----------33

Figure 3.1 Effect of 17AAG treatment on the Aldefluor-positive cells----------73

Figure 3.2 Treatment with 17AAG inhibits the self-renewal capability of SUM159 and MCF7 Aldefluor-positive cells *in vitro*----------74

Figure 3.3 Treatment with 17AAG inhibits the growth and the self-renewal capability of the Aldefluor-positive population in SUM159 tumor xenografts--------75

Figure 3.4 Treatment with low-dose 17AAG (5 mg/kg) reduced tumor growth and the number of Aldefluor-positive cells in MC1 tumor xenografts----------77

Figure 3.5 Treatment with low-dose 17AAG reduced tumor engraftment efficiency ----------78

Figure 3.6 Treatment with 17AAG reduced the expression levels of the mediators of Akt/β-catenin/Wnt pathway----------79

Figure 3.7 Effect of 17AAG on MCF7 $CD44^{+}$/$CD24^{-}$ cells----------81

Figure 4.1 Efficacy of 17AAG as given in the advanced setting----------105

Figure 4.2 Efficacy of 17AAG as given in the adjuvant setting----------106

Figure 4.3 Effect of 17AAG on reducing Aldefluor-positive cells in advanced and adjuvant setting----------107

Figure 4.4 *In vivo* imaging of tumor development in response to 17AAG in the adjuvant setting----------108

Figure 4.5 Efficacy of combinational therapy of 17AAG and Docetaxel----------109

Figure 5.1 SAHA reduced the number of SUM159 Aldefluor-positive CSCs----------143

Figure 5.2 SAHA reduced the viability of SUM159 cancer cells----------144

Figure 5.3 SAHA treatment inhibited the self-renewal and proliferative capability of SUM159 Aldefluor-positive cells *in vitro*----------145

Figure 5.4 SAHA inhibited the growth of SUM159 and MC1 tumors----------146

Figure 5.5 SAHA reduced the portion of Aldefluor-positive cells in SUM159 and MC1 tumors----------147

Figure 5.6 SAHA impaired the tumor engraftment efficiency of SUM159 cells in secondary mice----------148

Figure 5.7 SAHA impaired the tumor engraftment efficiency of MC1 cells in secondary mice----------149

Figure 5.8 Effect of SAHA on the expression pattern of MYC target genes, and levels of MYC gene and protein----------150

Figure 5.9 SAHA reduced the expression of BMI-1 gene in Aldefluor-positive cells ----------151

Figure 5.10 SAHA reduced the level of ALDH1A1 gene----------152

Figure 5.11 SAHA inhibits MCF7 $CD44^{+}/CD24^{-}$ cells----------153

Figure 5.12 Effect of SAHA on the expression CD44 and CD24 protein----------154

CHAPTER 1

Research Objectives

The major obstacles of cancer treatment are tumor recurrence and metastasis, making most cancers incurable. Recently, the model of cancer stem cells (CSCs) shed light on the causes of recurrence and metastasis. The CSC model suggests that tumor is initiated by a small subset of cancer cells, termed cancer stem cells. CSCs have intrinsic properties similar to those of normal stem cells, including longevity and the ability to self-renew. Normal adult tissues contain a small portion of stem cells that are responsible for the replacement of terminally differentiated cells. During self-renewal, tissue stem cell generates an identical stem cell and a progenitor cell that further gives rise to a number of differentiated cells. Similarly, among heterogeneous cell populations within tumors, only cells expressing CSC-markers have the ability to initiate tumors in immune-deficient mice(1). CSCs were first identified in leukemia and later found in a broad spectrum of solid tumors, including those of breast (2). Breast CSCs are refractory to conventional chemotherapy or radiation, as evidenced by several preclinical and clinical studies (3, 4). As a result, CSCs survive through treatments. Once treatments cease, CSCs may again regenerate tumors and cause tumor relapse. In addition, CSCs may migrate to distal positions of the body and initiate metastasis. In support of the CSC

theory, tumor regression has not correlated reliably with long-term survival (5). The existence of CSCs could explain the high frequency of relapse and resistance of cancers to many currently used therapies. New approaches should be developed to effectively target the CSCs, which could vastly improve cancer therapies and outcome. Despite much effort invested on the discovery of anti-CSC inhibitors, finding an effective compound to eliminate CSCs still remains challenging because CSCs are regulated by multiple pathways and cross-talks occur among these pathways. If we only target a single CSC-pathway, others pathways still exist to serve the same function. In this context, we hypothesize that modulation of multiple oncogenic signaling pathways simultaneously via inhibition of a single common target, such as heat shock protein 90 (Hsp90) or histone deacetylase (HDAC), effectively inhibit breast cancer stem cells. To test this hypothesis, we proposed three specific aims: Aim 1: To investigate efficacy of Hsp90 inhibitors to eliminate breast cancer stem cells *in vitro* and *in vivo* by modulation of multiple oncogenic signaling. Aim 2: To optimize the dose regimen for Hsp90 inhibitors for therapeutics of breast cancer stem cells *in vivo*. Aim 3: To study the efficacy of HDAC inhibitor to eliminate breast cancer stem cells *in vitro* and *in vivo* by multiple epigenetic modulations.

Drugs that are currently available for clinical use were selected for this study because the regulatory requirements would be less stringent when developing these agents for the indication being investigated in our studies. A Hsp90 inhibitor, 17AAG, was selected for its ability to inhibit multiple pathways that ae known to be vital to the maintenance of stem cell function in CSCs. A HDAC inhibitor, SAHA, was

selected as it has been reported to induce differentiation in cancer cells by modulating multiple gene expressions. Study designs to evaluate these two drugs on anti-CSCs and the relevant findings are outlined below.

In Chapter 2, relevant background information is presented to familiarize readers with the breast cancer biology and its relevant therapy, particularly in CSC theory and up-to-date studies in CSCs and the relevant therapies. In addition, clinical evaluations of two drugs (17AAG and SAHA) in this study are reviewed.

Chapter 3 explores 17AAG, a clinically evaluated member of Heat Shock Protein 90 (Hsp90) inhibitors. The rationale to evaluate this class of cancer therapy is that Hsp90 is involved in multiple CSC-associated surviving pathways. Since Wnt/b-catenin, Notch and Hedgehog pathways are emerging targets for anti-CSC treatment, a number of compounds that interfere with these pathways have been evaluated preclinically and clinically for their efficacy of targeting CSCs. However, these pathways display intensive crosstalk, so targeting a single pathway may not be sufficient to achieve the desired efficacy. Inhibiting Hsp90, in this sense, provides an ameliorated approach by simultaneously inhibiting multi-pathways crucial for the survival of CSCs and thereby eliminates CSCs. This chapter provides *in vitro* and *in vivo* data showing 17AAG is effective at targeting CSCs. The mechanism through which 17AAG inhibits CSCs is investigated and reported in this chapter. The clinical relevance and significance of the contribution of our findings are discussed in this chapter.

Chapter 4 explores the efficacy of 17AAG as an adjuvant therapy and as combinational therapy. Since CSCs represent only a very small subset of tumor cells

(<10%), even a complete elimination of CSCs may not translate to tumor shrinkage. In this context, tumor reduction that is typically used to evaluate the efficacy of anti-cancer agents in clinical trials may not be an appropriate endpoint for CSC-targeting agents. Therefore, CSC-targeting agents are suggested to be evaluated in either combinational therapy together with chemotherapy or in adjuvant therapy as a single agent. In this case, the clinical benefit of targeting CSCs to reduce the tumor relapse may not be undermined by the highly proliferated cells.

In this chapter, efficacy of 17AAG to target CSCs in adjuvant- and advanced- settings is assessed in parallel. Mice bearing early-stage tumors were used to simulate the adjuvant setting where treatment is given to eliminate the residual cancer cells right after removing the primary tumor. Mice bearing well-grown tumors were used to simulate the advanced setting where therapy is given to patient with advanced tumors. In addition, efficacy of 17AAG in combination with Docetaxel, a standard chemotherapy for breast cancer, is evaluated and demonstrated.

In Chapter 5, a differentiation approach is applied to target CSCs using SAHA. The reasons to explore SAHA, a histone deacetylase (HDAC) inhibitor, in this context are that (1) SAHA is approved by FDA to treat cutaneous T cell lymphoma and extensively evaluated in clinical trials for breast cancer, and (2) HDAC inhibitors have been shown to induce differentiation in cancer cells. The effect of SAHA on the CSC population was evaluated *in vitro* and in a mouse model. The molecular mechanisms underlying the inhibition of breast CSCs is revealed based on microarray analysis of gene expression. The studies in the chapter show that SAHA partially converts breast CSCs to non-stem cells, evidenced by reduced expression of

stem cells markers such as CD44, CD24 and ALDH1. Also, inhibition of breast CSCs after SAHA treatment may be mediated through BMI-1 and MYC pathways.

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Chapter 2

Background

Breast cancer overview

Breast cancer is the second leading cause of cancer-associated death in women and accounts for 14 % of the cancer deaths in the world (1, 2). Breast cancer incidence rates remain stable among all racial/ethnic groups from 2004 to 2008 (1). Breast cancer death rates have been decreasing since the early 1990s due to the improved screening technique and surgical interventions and therapy. The five-year survival rate for women diagnosed with localized breast cancer that has not spread to lymph nodes or other locations is 98%. The survival rate decreases to 84% or 23% for when tumors spread to nearby lymph nodes or other organs, respectively. The survival rate continues to decline over time. Specifically, the survival rate is 75% 10 years after diagnosis (1, 3). Around 6% of patients with breast cancer are diagnosed with distant metastases, and in 30% of patients with early breast cancer the disease will eventually have recurrent tumors, which is metastatic in most cases (4). Once metastatic disease develops, the possibility of a cure is very low and the five-year survival rate declines to 20% (4).Despite advances in early detection and treatment of breast cancer, local or distant recurrence of tumors fare still unpreventable because the efficacy of current therapies is limited by the emergence of therapy-resistant cancer cells (5, 6). As a result, metastatic breast cancer remains

an incurable disease by current treatment strategies. This highlights the need for novel therapies to improve therapy-resistance.

At present, therapeutic approaches to treat patients with breast cancers include surgery, radiation therapy, chemotherapy, hormone therapy, and targeted therapy. Most prevalent approach consists of surgical removal of tumors, followed by other types of therapy as adjuvant treatment. Chemotherapy and radiation are often used in adjuvant therapy to destroy any residual cancer cells and prevent tumor recurrence. The treatment to patients with breast cancer is selected first based on the hormone receptor status, such as such estrogen and progesterone receptors, and the status human epidermal growth factor receptor type2 (Her 2). Endocrine therapy (e.g. Tamoxifen) is commonly used to treat patients with advanced ER-/PR-positive breast cancer. Patients with Her2 amplified breast cancers are often treated with Herceptin®. For the rest of breast cancer types or tumors resistant to endocrine treatment, chemotherapy is commonly used. In contrast to the localized therapies (e.g. radiation and surgery), Chemotherapy is classified as a systemic therapy because chemotherapy circulates through the whole body and can simultaneously treat the primary tumor and metastasized tumors.

The available chemotherapeutic drugs for breast cancer are categorized into four types: alkylating agents (ex. cyclophosphamide), anthracyclines (ex. doxorubicin and epirubicin), antimetabolites (ex. fluorouracil and methotrexate), and taxanes (ex. Paclitaxel and docetaxel). Chemotherapy given as a combinational treatment with other drugs has been shown to be more effective than any one single

drug (7). For instance, a large, multicenter study reported that patients treated with doxorubicin and cyclophosphamide (AC), followed by Paclitaxel had significantly better disease-free survival and overall survival rates than patients treated with AC only. However, no combinational chemotherapeutic treatments have been demonstrated to cure metastatic breast cancer.

Lastly, targeted therapy based on the status of cellular receptors has been shown to be an effective approach to treat otherwise resistant breast cancers. Based on expression levels of Her2, estrogen receptor (ER), and progesterone receptor (PR), breast cancer can be classified into Her2-positive, ER-/PR-positive or triple-negative groups. The most successful example of targeted therapy is Herceptin®, which is commonly used to treat Her2 over-expressed breast tumors. Her2 overexpression is seen 20% of breast tumors. Breast cancers that overproduce HER2 tend to be more aggressive and are more likely to recur. Herceptin® in conjunction with adjuvant chemotherapy can lower the risk of recurrence of HER2-overproducing breast cancers by about 50% in comparison with chemotherapy alone.

Cancer stem cells

The emergence of cancer stem cell (CSC) hypothesis provides a potential explanation for tumor metastasis and resistance to chemotherapies. The failures observed in current cancer treatments are not usually thought to be due to the lack of tumor response, but due to tumor recurrence or metastasis for which CSCs are thought to be responsible (8). In recent years, a growing body of evidence suggests

that a hierarchy of heterogeneous cell populations may exist in breast tumors (9-11). CSCs are believed to be a small subpopulation of tumor cells that has unlimited capability to proliferate and sustain tumor formation and growth. On the other hand, other tumor cells only have limited proliferation ability (12). This CSC model accounts for the major obstacle of breast cancer-tumor recurrence and metastasis. As with any other stem cells, CSCs have the intrinsic self-renewal properties through which they can give rise to heterogeneous cells comprising the bulk of tumors.

Traditionally, chemotherapies have been developed based on the ability of these agents to cause tumor regression in animal models. Because CSCs are rare population (<10%), tumor regression induced by therapies is expected to mainly result from the elimination of the non-CSC population. However, if therapies fail to target CSCs, CSCs would remain after therapy and be able to regenerate the tumor, resulting in tumor relapse (12). As CSCs can migrate and lodge at distal sites from the primary tumor, the remaining CSCs can lead to metastasis by producing progenitor cells and bulk tumor cells through self-renewal and cell division.

Moreover, normal stem cells have great defensive mechanisms, such as increased expression of BCL-2 family proteins and increased expression of membrane transporters multiple drug resistance (13, 14), that make them relatively resistant to exogenous toxic agents. Similarly, CSCs may also adopt analogous defensive mechanisms and therefore be inherently resistant to chemotherapies (12). Based on the evidence above, the CSC model provides an explanation for a

number of clinical observations including resistance to therapies, metastasis, and tumor relapse (9, 15) .

Identification of cancer stem cells in breast carcinomas

The concept that cancers may arise from CSCs dates back to 1968 in a study by Wodinsky et al. that observed only 0.1-1% of leukemic cells were able to form colonies *in vitro* and *in vivo* in mice spleens (16-18). The findings indicated that some cells are equipped with the ability to propagate disease. The subsets of cells that can develop leukemia in recipients were characterized and described as leukemic stem cells by Dick and Bonnet 30 years later. The evidence by Dick and Bonnet showed that an isolated population of $CD34^+/CD38^-$ cells from human acute myeloid leukemia (AML) possesses tumor-initiating ability. As few as 5,000 $CD34^+/CD38^-$ cells were needed to initiate AML in immunodeficient mice through differentiation into leukemic blasts, while as many as 5,000,000 cells bearing phenotypes other than $CD34^+/CD38^-$ failed to develop leukemia in mice. The capacities of $CD34^+/CD38^-$ cells to self-renew and differentiate are two intrinsic features of stem cells. Therefore, $CD34^+/CD38^-$ phenotype is recognized as markers for leukemic stem cells. Furthermore, evidence suggests that hematological malignancies are very likely to be regulated by a hierarchical system, where a small amount of cells are responsible for the generation of the bulk of tumors and progression of disease (19).

As with blood cancers, solid tumors have also been speculated to harbor CSCs among the heterogeneous populations. A small proportion of cell bearing

specific phenotypes derived from neuroblastomas, pancreatic, colorectal, lung, and ovarian cancers cells have been demonstrated to be tumorigenic and capable of forming colonies *in vitro* and transfer disease *in vivo* (20, 21). The existence of CSCs in breast carcinoma was first discovered by Al-Hajj et al (12). As low as 100 $CD44^{+}/CD24^{-/low}$ lineage cells, derived from primary or metastatic sites in nine patients, were able to form tumors in immunodeficient mice, whereas 10,000 cells without this phenotype failed to form tumors. Moreover, the key feature of stem cells is that they can generate an identical daughter stem cell and a progenitor cell that continuously differentiates to various phenotypic cells that constitute cancerous tumors. When the $CD44^{+}/ CD24^{-/low}$ cells from the above study were injected these cells into the breasts of NOD/SCID mice, tumors formed containing four different phenotypic groups of cells including $CD44^{+}/CD24^{-}$ cells, $CD44^{+}/CD24^{+}$ cells, $CD44^{-}/CD24^{-}$ cells and $CD44^{-}/CD24^{+}$ cells, as seen in primary tumors. The work of Al-Hajj et al. clearly demonstrated that $CD44^{+}/CD24^{-}$ lineage breast cancer cells possess the capability to self-renew and differentiate, much like normal stem cells. Their work also suggests the existence of hierarchy in breast cancer, in which the majority of tumor cells that have limited proliferative ability are derived from the rare CSC population (12).

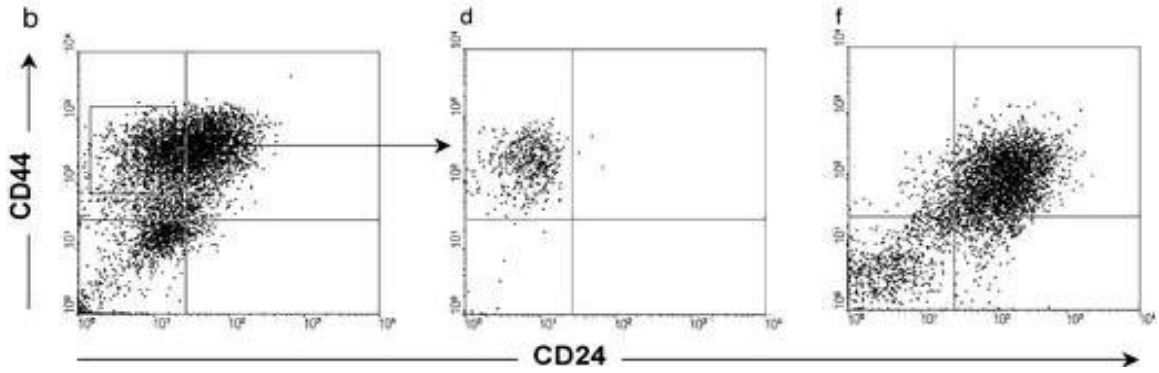

Figure 2.1 $CD44^{+}CD24^{-}$/$lineage^{-}$ cells recapitulated the phenotypic diversity. $CD44^{+}CD24^{-}$/$lineage^{-}$ cells, isolated and injected into the breasts of NOD/SCID mice, formed a tumor that contained heterogeneous cell types (Panel f) similar to those observed in the original tumor (Panel b) (12).

Breast cancer stem cell markers

Identification of reliable markers to differentiate the CSC populations from tumor cells is a key aspect of studying CSCs. Apart from the aforementioned $CD44^{+}/CD24^{-}$ surface antigens, the activity of aldehyde dehydrogenase 1 (ALDH1) is a widely accepted marker to identify breast CSCs. ALDH1 is the enzyme responsible for detoxifying a wide variety of aldehydes to carboxylic acids (22). Cells that have high ALDH activity have been associated with several types of murine and human stem hematopoietic cells as well as neural stem and progenitor cells (23, 24). Therefore, ALDH1 activity has been established as a potential marker for both normal and malignant stem and progenitor cells. ALDH1 activity, assessed using the Aldefluor assay, has been used to isolating stem cells from leukemia and multiple myeloma (24).

In the context of breast carcinoma, Ginestier et al. demonstrated that ALDH1 activity can be a promising marker to isolated stem cells from cancerous and normal

breast cells. Cells with high ALDH1 activity, as assessed by the Aldefluor assay, has been termed ALDH1-positive or Aldefluor-positive cells, and have been characterized to have phenotypic and functional characteristics of stem cells, as demonstrated by their ability to form tumors in mice but not Aldefluor-negative cells (25). Moreover, serial passages of Aldefluor-positive cells generate tumors that recapitulate the phenotypic diversity similar to the initial tumor. This data indicate that Aldefluor-positive cells possess the properties of stem cells. The breast cancer cells having Aldefluor-positive and $CD44^{+}/CD24^{-/low}$ markers have the greatest tumorigenic ability, in which as few as 20 of these cells were capable of generating a tumor in mice (25). *In situ* staining of 577 biopsy specimens of breast tumors for ALDH1 expression indicated a correlation between ALDH1 expression and poor prognosis, suggesting that ALDH1 is a powerful predictor of poor clinical outcome (26). However, Ginestier et al. also reported that the overlap between cells expressing $CD44^{+}/CD24^{-}$ and cells with high ALDH1 activity is only approximately 1%. This raises a question of whether there is a uniform population or heterogeneous populations of breast CSCs in one single tumor (27). Recent studies have shed light on the heterogeneity of CSCs showing that $CD44^{+}/CD24^{-}$ phenotype or high ALDH1 activity is strongly associated with intrinsic molecular subtypes (e.g. luminal or basal) (27, 28). For these reasons, cell markers, $CD44^{+}/CD24^{-}$ and ALDH1 activity, have been widely used to evaluate the ability of drugs to target CSCs.

Origin of cancer stem cells

The Critical role of CSCs in the initiation, metastasis, and relapse of cancers, is widely accepted, and yet the origin of breast CSCs remains unclear. At present, three hypotheses have been proposed on the basis of experimental evidence to explain the origin of CSCs (Figure 2.1). One postulation is that CSCs originate from tissue stem cells. Small amount of multipotent and undifferentiated tissue stem cell are found in bone marrow, adipose tissues, heart, brain, lung, liver, pancreas, skin, retina, breast, ovaries and prostate. (29-39). Deregulating the self-renewal and proliferative pathways is thought to transform tissue stem cells into CSCs. Several lines of evidences support this postulation. First, tissue stem cells and CSCs share common self-renewal pathways that are essential for their cellular function. Moreover, deregulating these pathways cause the expansion of CSCs. In addition, Al-Hajj et al. believed that breast CSCs are likely to originate from basal mammary stem/progenitor cells due the similarities in the cell surface profiles. Both breast CSCs and mammary stem/progenitor cells display $CD24^{-/}CD44^{+}$ surface markers (12). Lastly, normal stem cells have long lifespan, which makes them more susceptible to accumulate mutations that eventually drive oncogenic transformation (40, 41).

An alternate explanation of the origin of CSCs is that CSCs originate from a population of more differentiated progenitor cells. Progenitors, as compared to stem cells, have limited lifespan as well as ability of proliferation before they enter the terminal state. Thus, progenitors need to first acquire the stem-like properties, such as self-renewal, so as to have the opportunity to accumulate additional mutations to become oncogenic [55]. This concept is supported by a number of evidence from

hematopoietic and solid malignancies. First, co-expression of Bcl-2 and Bcr/Abl protein (a fusion protein that causes chronic myelogenous leukemia) in leukemia cells render these cells capable of initiating leukemia in mice [59]. Second, the finding of Molyneux et al. shows that majority of human BRCA1-associated and sporadic basal-like tumors are derived from luminal progenitors rather than from basal stem cells(42).

Another plausible source of where CSCs originate from relative differentiated cells that have acquired the stem-like properties via epithelial–mesenchymal transition (EMT). EMT is a biologic process which enables epithelial cells to regain the mesenchymal stem cell properties such as invasiveness, ability to migrate, and resistance to apoptosis (43). The association between CSCs and EMT is based on the facts that EMT activation is a required process during tumor invasion and metastasis. A recent study showed that immortalized human mammary epithelial (HMLE) cells that have undergone EMT have the gene expression profile similar to CSCs derived from HMLE cells. Moreover, HMLE cells that have undergone EMT have the ability to form tumorsphere *in vitro* and form tumors *in vivo* (44), which is an indication of self-renewal property found in stem cells.

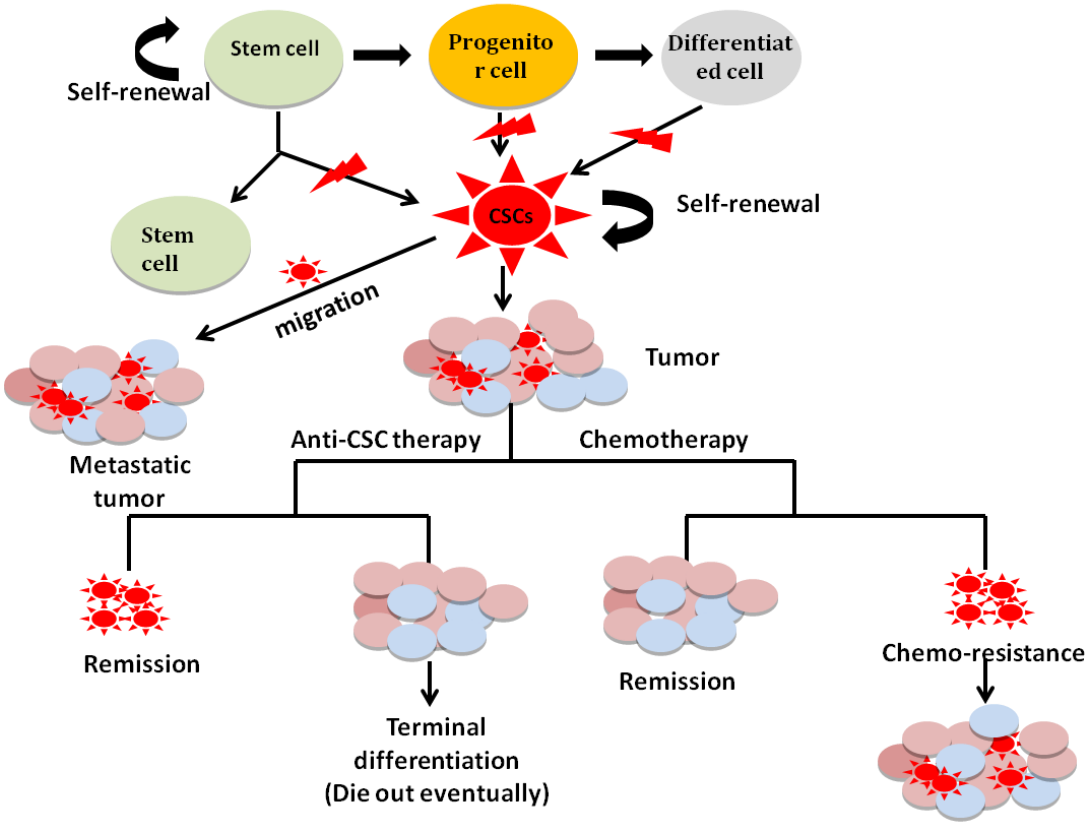

Figure 2.2 Roles of CSCs in tumorigenesis and treatment resistance in breast carcinoma. Mammary stem cells self-renew and can differentiate into progenitor cells, which eventually differentiate into fully differentiated breast cells. CSCs can arise from normal stem cells, progenitor cells and even differentiated hepatocytes when oncogenic events occur during cellular processes. The expansion of CSCs results in the formation of the primary tumor, which is composed of a heterogeneous mass of cancer cells. When CSCs migrate to distal locations, they can give rise to a tumor, which results in distant metastases. When chemotherapy or radiotherapy is used, CSCs play an essential role in treatment resistance and can cause tumor recurrence. In contrast, CSC-targeted therapy can effectively eliminate CSCs so as to prevent tumor relapse (45).

Resistance of CSCs to chemotherapies

Increasing studies suggest targeting CSCs may be an approach to overcome the obstacles in cancer treatment including resistance, metastasis and recurrence of

tumors (Figure 2.2). First, CSCs are resistant to currently available chemotherapies despite tumor shrinkage. Secondly, the distinct difference between CSCs and differentiated cancer cells is the unlimited self-renewal potential, through which CSCs can initiate tumors as well as drive tumor progression. Thirdly, conventional chemotherapies, which are designed to target the highly proliferative tumor cells, may have little effect on CSCs because CSCs are thought to be relative quiescent, much like normal stem cells. Consequently, chemotherapies fail to target CSCs, and instead increase the percentage of CSCs in tumors as a result of massive loss of differentiated cells. These statements were supported by a number of studies. Phillips et al. investigated the response of CSCs to both a single dose and a 5-day course of radiation, and found that $CD44^{+}/CD24^{-}$ tumorspheres, derived from MCF7 and MDA-MB-231cell lines, were more resistant to radiation than cells in monolayer culture (46). Furthermore, in an analysis of clinical specimens from patients with breast tumors before and after treatment, chemotherapy increased the percentage of $CD44^{+}CD24^{-/low}$ cells in tumors from a baseline of 4.7% to 13.6% (95% CI = 10.9% to 16.3%) after 12 weeks of chemotherapy. These findings support that chemotherapies enrich CSCs in tumors (47). For the reasons above, CSCs are believed to be responsible for tumor resistance and recurrence.

CSCs are resistant to chemotherapies (e.g paclitaxel and doxorubicin) for various reasons including their slow proliferation, the ATP-binding cassette (ABC) transporters, and the efficient repairing system in response to DNA damage(48). The slow-proliferating CSCs may not be sensitive to many anti-cancer agents that

are designed to target rapidly proliferating cancer cells. For instance, imatinib is designed to target a fused protein, BCR-ABL kinase, which promotes proliferation of multipotent progenitors in Chronic Myelogenous Leukemia (CML). BCR-ABL is essential for the survival of rapidly proliferating progenitor cells, but not the quiescent CML stem cells(8). ABC transporters, including multidrug resistance transporter 1 (MDR1) and breast cancer resistance protein (BCRP) drug transport pumps play an essential role in expelling anticancer drugs from cells, leading to chemo-resistance (49). Apart from chemo-resistant, CSCs are also associated with radiotherapy, evidenced by the work in glioma stem cells by Bao et al. In their study, human glioma cells-derived tumor xenografts after radiation were shown to be enriched with CSCs, characterized as $CD133^{+}$, as compared to non-radiated cells. The enrichment of CSCs in irradiated glioma cells in turn enhanced the frequency of tumor formation in mice. In addition, even though radiation causes equal damages to DNA in CSCs and non-CSCs, CSCs can recover from the damages more quickly than non-CSCs due to their efficient DNA repair system (50). Since CSCs are resistant to chemotherapy and responsible for tumor recurrence and metastasis, there is considerable interest in finding therapeutic agents targeted CSCs.

Pathways regulate cancer stem cells

Identification of the signaling pathways pivotal for the survival of CSCs is of greatest importance for developing drugs to target CSCs. The signaling pathways that are essential to maintain the stem-like trait in normal stem cells are also involved in the regulation of CSCs (51, 52). At present, several major pathways have

been suggested to be involved in the maintenance of CSCs including Wnt/β-catenin, Hedgehog, and Notch, which will be detailed as follows.

1. Notch pathway

Notch signaling is known to regulate cellular proliferation, differentiation and apoptosis to modulate the development of mammary gland development and mammary gland tumorigenesis. Notch signaling is commonly dysregulated in human malignancies with elevated expression of Notch receptors and their ligands in numerous cancers (53-55). Notch dysregulation is also associated with poor prognosis in breast cancer (56-58).

Notch signaling is mediated by Notch receptors and their ligands. At present, four Notch transmembrane receptors have been identified, including Notch1, 2, 3 and 4. Ligands are classified into two distinct families: Delta-like ligands (DLLs) 1, 3, and 4 and Jagged ligands 1 and 2. Once ligands bind to Notch receptors, gamma-secretase and ADAM protease family comes to cleave the intracellular-membrane domain of Notch receptors. Subsequently, the active Notch intracellular domain (NICD) is released into the cytoplasm and translocated to nucleus, where it binds to a transcription complex, to activate a number of genes (e.g. Myc, p21 and Hes) (59).

In cancer stem cells, Notch signaling thought to play a role in the self-renewal function of mammary stem cells and CSCs. Dontu et al. have suggested that up-regulation of Notch signaling can promote the self-renewal of mammary stem cells (60). In their study, activation of Notch pathway by a Notch-activating DSL peptide increased the mammospheres formation of mammary stem cells by 10-fold (60).

Additionally, Notch pathway is believed to be deregulated in CSCs, ultimately leading to uncontrolled CSC self-renewal. This notion is supported by a number of recent studies in breast CSCs. The work of Grudzien et al. demonstrated that breast CSCs have elevated activation of Notch signal, as compared with bulk tumor cells (61). When Notch signaling was blocked by a gamma-secretase inhibitor, MRK033, CSCs lost their ability to form colonies and spheres, suggesting CSCs lost their self-renewal capability (61).

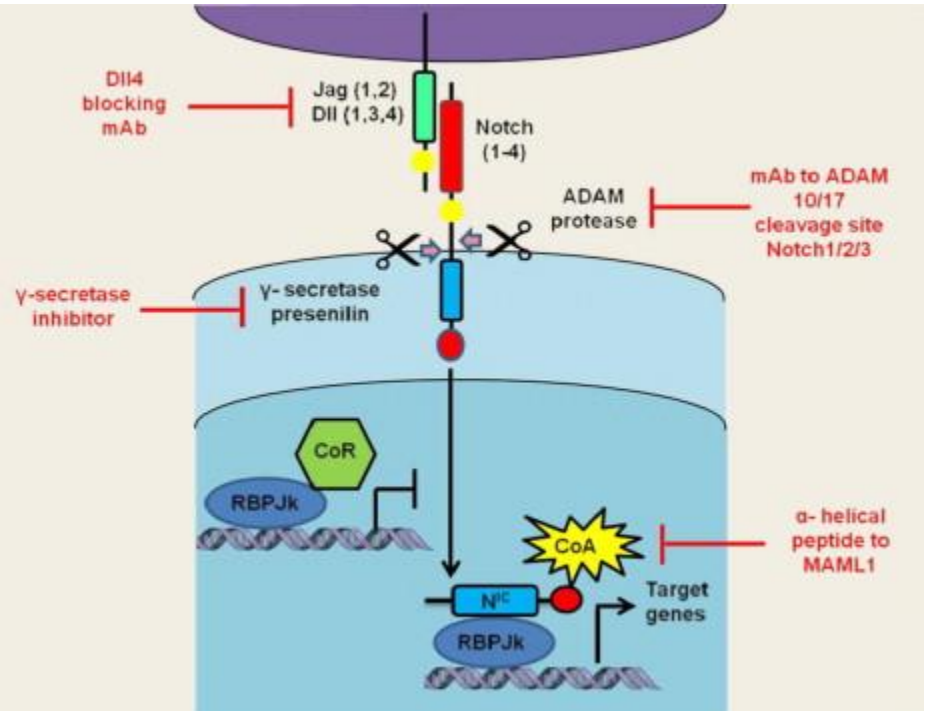

Figure 2.3 Schematic representation of the Notch pathway and putative therapeutic targets (62).

2. Hedgehog pathway

The Hedgehog (Hh) signaling pathway is known to be involved in controlling tissue polarity, patterning maintenance, and stem-cell maintenance during embryonic development (59). Hh signaling is mediated by two transmembrane proteins, Smoothened (Smo) and Patched (Ptc), and three ligands, Sonic Hh (Shh), India Hh (Ihh) and desert Hh (Dhh). Of three ligands, Shh is frequently associated with the development of cancers (ref). Shh is a binding ligand for Ptc, which is a suppressor Smo. When Shh binds to Ptc, Smo is released from Ptc and becomes active. The activated Smo in turn releases transcriptional factors, Gli1, 2 and 3 from a protein complex, SuFu (Fused (Fu) and suppressor of Fused). Upon release, Gli translocates to the nucleus where it activates Hh target genes, such as cyclin D1, cyclin E and Myc (63)

The involvement of Hh signaling in CSCs has been defied in a variety of malignancies including breast, gastric, colon, pancreatic, and prostate cancers as well as leukemia (64-68) and poses as a potential target for drug therapy. For instance, Liu et al. have demonstrated that hedgehog pathway plays a crucial role in regulating self-renewal of human mammary stem cells and breast CSCs (69). Another recent study by Tanaka et al. revealed that Hedgehog signaling is essential for the maintenance of CSCs population, as characterized by $CD44^{+}CD24^{-/low}$ in breast cancer (70). Due to its role in the breast cancer development, Hh pathway has been suggested as promising therapeutic target (70-72).

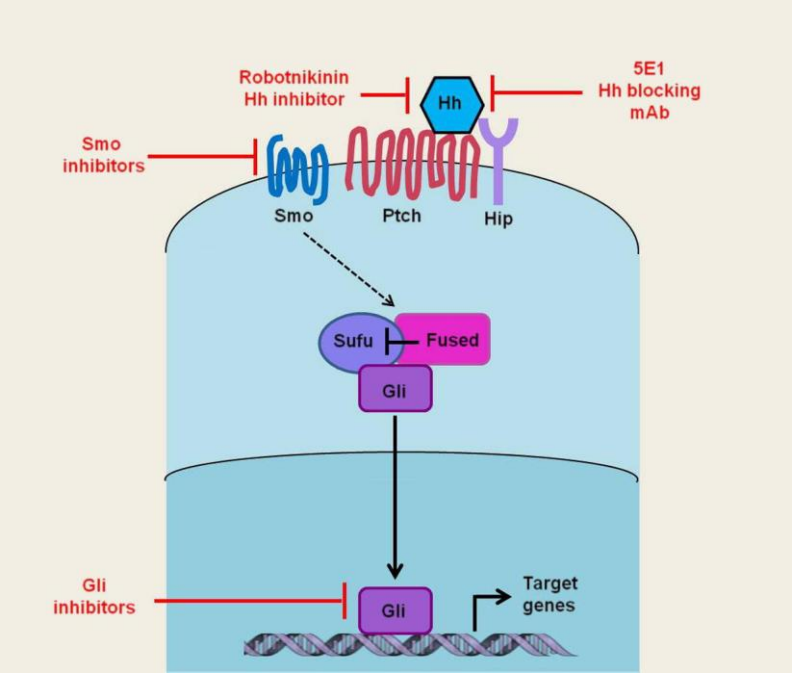

Figure 2.4 Schematic representation of the Hedgehog pathway and putative therapeutic targets (62).

3. Wnt pathway

The highly conserved Wnt pathway is characterized into two categories based on its dependence on β-catenin: canonical (β-catenin-dependent) and non-canonical (β-catenin-independent). In the canonical Wnt/β-catenin signaling, in the absence of Wnt ligands, β-catenin is sequestered in a complex consisting of APC (adenomatous polyposis coli), Axin2, GSK3β (glycogen synthase kinase-3β), and CK1 (casein kinase 1). GSK3β triggers the ubiquitination of β-catenin, which induces degradation of β-catenin by 26S proteasome. In the presence of Wnt, on the other hand, Wnt ligands bind to the complex consisting of Frizzled (Fzd) and Lrp5 (low density lipoprotein receptor-related protein 5) and Lrp6. The interaction will recruit Disheveled (Dvl),

which inhibit the GSK3 β-mediated ubiquitination and degredation of β-catenin. Thus, β-catenin is able to translocate to the nucleus where it binds to TCF/LEF transcription factors (T-cell factor/lymphoid enhancer factor) to activate Wnt target genes such as c-Myc, cyclin D1, and c-Jun.

Aberrant β-catenin pathway has been frequently observed in colon cancer. The role of Wnt/β-catenin signaling was first described in colon cancer, where 90% of tumors harbored mutations that enable constitutive activation of Wnt pathway. Of oncogenic mutations in colon cancer, APC mutation is the most common type, which leads to β-catenin accumulation and then drives constitutive activation of Wnt pathway. The relevance of β-catenin signaling to stem cells is built on the observation that stem-like colon cancer cells with a high level of β-catenin signaling have a much greater tumorigenic potential than non-stem like cancer cells which have less β-catenin activity. Moreover, a growing body of evidence has suggests that deregulation of β-catenin is associated with CSCs in gastric, colon, prostate, liver, lung and breast cancers (73-80).

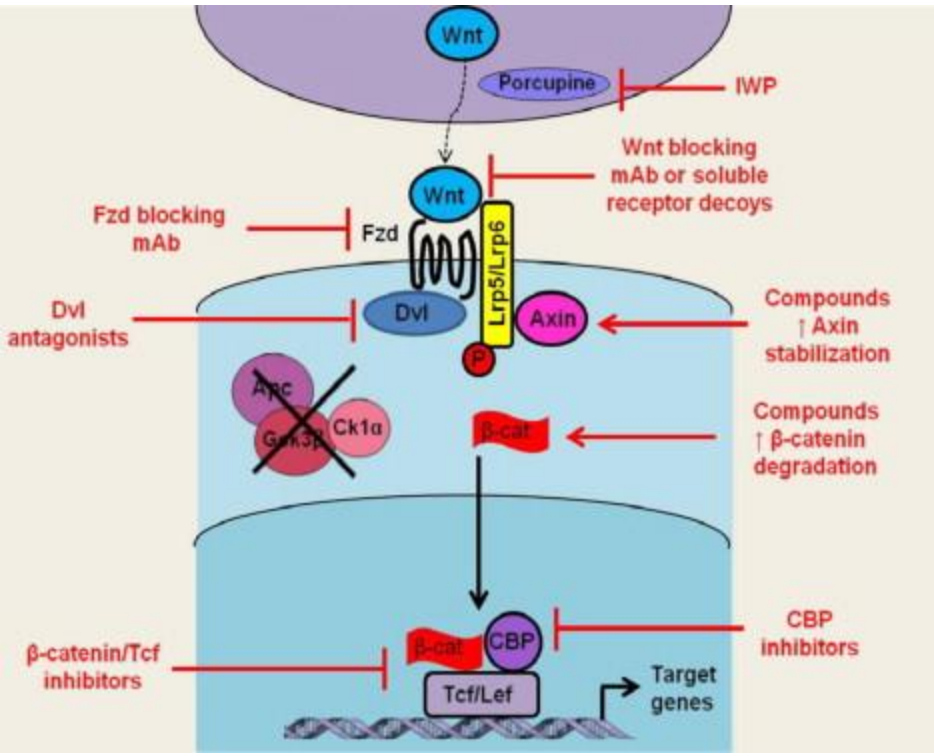

Figure 2.5 Schematic representation of the Wnt pathway and putative therapeutic targets (62).

4. Cytokine network

Apart from the mentioned pathways, cytokine networks have been suggested to regulate CSCs. Cytokines including interleukin-6 (IL-6), -8 (IL-8) and -1ß (IL- Iß) are known key factors to mediate chronic inflammation. The association between inflammation and cancer development was proposed nearly 150 years ago by Virchow when he observed that tumors tend to occur at sites with chronic inflammation (81). Despite the fact that some cytokines (e.g. interferon-γ, IL-1 and tumor necrosis factor-α (TNF-α)) are used as anti-cancer therapies, clinical and epidemiologic evidence support the notion that inflammation increases the risk of cancers. For example, inflammatory bowel diseases and hepatitis-induced

inflammatory increase risks of developing colorectal and hepatocellular carcinoma (82, 83). In breast cancer, the levels of IL-6 in plasma and IL-1α/β in tumors are associated with poor clinical outcome and stimulation of tumor growth, respectively (84-86).

The relevance of cytokine networks in sustaining the growth of breast CSCs is supported by a number of recent studies. For example, Charafe-Jauffret et al. compared the gene expression profiles of CSCs isolated from 33 breast cancer cell lines and showed that IL-8 and its receptor, CXCR1, were preferentially expressed in CSCs (87). Furthermore, the role of IL-8/CXCR1signaling in the regulation of breast CSCs was confirmed by the *in vitro* finding that recombinant IL-8 can promote the stem-like property, as assessed by tumorsphere formation rate. Furthermore, a preclinical study showed that the blockade of CXCR1 by either a small molecule, Repertaxin, or CXCR1-specific antibody can effectively eliminate CSCs and reduce metastasis (88). In light of the identified role of IL-8/CXCR1 and the finding above, cytokine signaling poses as a potential target for CSC-targeted cancer therapy.

Methodology used in studying breast CSCs.

1. Transplantation in NOD/SCID mice

Transplantation assay in immunodeficient mice is prevalently used to evaluate functional activity of CSCs *in vivo*. In this assay, breast cancer cells or human primary breast tumors are first injected into the mammary fat pads of non-obese diabetic/severe combine immunodeficient (NOD/SCID) mice. After treatment,

the dissociated tumor cells are analyzed for the CSC population based on specific CSC markers. Next, the same numbers of dissociated tumors cells from the control or drug-treated mice are orthotopically re-implanted to a second group of NOD/SCID mice who do not receive any treatment. The capability of breast cancer cells derived from the primary NOD/SCID xenografts to form tumors in secondary mice is an indication of self-renewal capability of CSCs. If a drug effectively inhibits this pivotal stem-like feature, CSCs cannot generate various cells to form the bulk of tumors. Therefore, the efficiency of tumor engraftment upon re-implantation is a reasonable surrogate to evaluate the *in vivo* self-renewal capability.

2. Non-adherent sphere culture

Following the isolation of CSCs, the cells are propagated in a non-adherent sphere culture. The sphere culture enables studying CSCs *in vitro* for their self-renewal property and evaluating whether compounds have inhibitory effect on CSCs. Non-adherent sphere culture was first developed by Reynolds and Weiss to isolate and expand putative stem cells from the brain. Brain cells that are cultured in this manner formed a cluster of undifferentiated cells, which were referred to as a neurospheres. Moreover, upon inducing differentiation, the neurospheres derived from the culture was able to give rise to three differentiated cell types of the central neural system, indicating non-adhere culture can maintain the stem cell property of neurospheres (89).

Later on, this sphere culture was successfully adopted to propagate CSCs (also termed tumorsphere formation in cancer cells), derived from brain, breast,

melanoma, lung, ovarian and colon cancers (40, 90-99). The unique feature of stem cells is the ability to grow in serum-free suspension, in contrast to differentiated cells that are typically anchor-dependent and die through anoikis process in suspension (98). The sphere culture takes advantage of this CSC property to propagate CSCs, which leaves differentiated cells to die out in suspension, while maintaining CSCs. The neurosphere culture was later adopted by Dontu et al. who successfully enriched normal breast cells with mammary stem cells (98). Later on, Ponti et al. employed Dontu's mamospshere approach to derive tumorpshere from various breast cancer cell lines (40). They found that the tumorspheres are enriched with $CD44^{+}/CD24^{-}$ cells, which is characteristic of breast CSCs (12) and are capable of generating tumors in immunodeficient mice with injections of as low as 1000 cells derived from tumorspheres. The works above demonstrate that breast cancer cells can be enriched with CSCs and propagated using the non-adherent sphere culture.

Cancer stem cell therapy

According to the CSC model, the occult CSCs are refractory to the chemotherapies due to their dormancy. As a result, once the treatment stops, the surviving CSCs will once again give rise to tumors. Therefore, an effective cancer therapy must be able to not only kill all proliferating tumor cells, but also eliminate or induce differentiation of cancer stem cells.

Therapies could be designed to induce differentiation in cancer stem cells by de-regulating the differentiation-associated signaling, or to eliminate cancer stem

cells by inhibiting the survival pathway of CSCs (100). Tissue stem cells are in charge of replacement of terminally differentiated cells through proliferation of the progenitor cells. Progenitors can further generate a great deal of highly proliferated and well-differentiated tissue-specific progeny cells. The well-differentiated cells only have limited ability to proliferate and eventually enter the terminal death. Likewise, tumor progression heavily relies on CSCs to continuously supply progenitors and differentiated cells. If therapies only target differentiated cancer cells, CSCs can replenish the loss through the self-renewal, leading to tumor recurrence. In contrast, if a therapy can completely wipe out CSCs, the tumors will die out without the support of CSCs. Therefore, de-regulation of differentiation-associated pathway of CSCs, via modulation of proteins such as HDAC and Hsp90, is expected to be of great importance in cancer therapy.

Histone deacetylase (HDAC)

HDACs, along with HAT (histone actyltransferase), regulate gene transcription by controlling the number of acetyl groups on histones. To activate gene transcription, HAT neutralizes positive charges on histones by adding acetyl group on lysine and arginine. The N-terminal tail of histone, which is rich in lysine and arginine, is normally positively charged to bind to DNA, which are negatively charged. Acetylation of histone weakens the interaction between histones and DNA so that the transcriptional machinery consisting of RNA polymerase and transcription factors can access the loose segments of DNA to make the corresponding RNA. In contrast, the removal of acetyl groups by HDAC increases the

positive charges of histone tail, leading to the tight binding of DNA to chromatin. The increased interaction between DNA and histone thus condenses DNA, leading to transcriptional inactivation.

Because activation of HDACs is associated with silencing differentiating genes in stem cells, inhibition of HDACs may reactivate the differentiation signals. This is illustrated and supported in a number of studies. Lee et al. reported that histone acetylation is required for the differentiation of mouse embryonic stem (MES) cells (101). Increase of histone acetylation using a HDAC inhibitor (TSA) promoted MES cells to a more differentiated status. Later, the study by Dovey et al. provide further evidence showing that HDAC1 but not HDAC 2 mediate the differentiation in MES cells (102). Knockout of HDAC1 enhances the differentiation of MES, accompanied by hyper-acetylation in Histone 3. Moreover, the same study reported that increased acetylation of histone 4 was observed during RA-induced Differentiation of mouse embryonic stem cells. In malignant cells, a number of studies support the use of HDAC inhibitors as differentiating therapies to treat various cancers (103-105). For instance, Göttlicher *et al.* illustrated the potential of a HDAC inhibitor, valproic acid (VPA), to inhibit colon, breast, and teratocarcinoma cancer cell lines by prompting differentiation (106). Also, Cinatl et al. reported that VPA induces differentiation of neuroblastomas cells, accompanied by reduced ability for metastasis. In this study, cells that underwent differentiation after treatment with VPA also lost the ability to penetrate endothelium, and thus could potentially reduce the incidence of metastases (107). All together, the capability of

HDAC inhibitors to stimulate differentiation on the basis of these reports warrants evaluating HDAC inhibitors as differentiating therapies against CSCs.

CSCs transformed into non-stem like cancer cells via HDAC inhibition may be effectively targeted by currently available chemotherapies. On the strength of this proposal, the study by Milde et al. supports this poise showing that HDAC inhibitor (Vorinostate) is able to deprive the stem-like properties of ependymoma stem cells by inducing neuronal differentiation (108). Either direct elimination of CSCs or induction of differentiation of CSCs seem very promising and are warranted for more investigation.

Heat shock protein 90 (Hsp90)

1. HSP90 inhibitor in breast cancer

Hsp90 (Heat-shock protein 90) is a chaperone molecule whose function includes regulating the stability and maturation of many oncogenic proteins including Her2, EGFR, mutant ER, Hif-1α, Raf-1, Akt and mutant p53(ref). At present, more than 200 proteins have been identified as Hsp90 client proteins, some of which are involved in development and survival of tumors in various cancers (109). Moreover, many Hsp90 client proteins mediate a number of fundamental cellular processes, such as apoptosis, cell cycle control, cell proliferation, and differentiation (49-51), and most interestingly the regulation of CSC function (110-112). In breast cancer, up-regulation of Hsp90 expression has been observed as compared to nonmalignant breast cells, particularly in poorly differentiated type of

breast carcinomas (113, 114). In addition, elevated Hsp90 expression strongly correlates with lymph node involvement and poor prognosis in patients with breast cancer (113-117). Diehl et al. analyzed specimens, including breast normal tissue, ductal carcinoma in situ, and invasive breast carcinomas using tissue microarray for Hsp90 levels. Their observation showed that the expression of cytoplasmic Hsp90 is significantly higher in ductal carcinoma in situ and invasive breast carcinomas as compared to normal breast tissue. There was no significant difference regarding the levels of nuclear Hsp90 among three types of breast carcinomas. However, significant correlation was found between the level of nuclear Hsp90 expression and the tumor-node-metastasis (TNM). TNM is a system to classify the stage of solid tumors based on the size of primary tumors, lymphatic involvement and the metastatic degree (118). The involvement of Hsp90 in cancer development is evident and presents as a potential target for cancer therapy.

2. Function of Hsp90

Hsp90 is a homo-dimeric protein, consisting of three conserved domains: a C-terminal domain (CTD) responsible for dimerization, a N-terminal domain (NTD) for ATP binding, and a middle domain (MD) for client proteins binding (119), (120). In the chaperon cycle, ATP binding triggers the dynamic alternation of Hsp90 conformation between the open and the close states. When ATP binds to Hsp90, NTD from each monomer come into contact with each other, much like two lids closing. Therefore, ATP-bound Hsp90 is referred as to the “closed” conformation. After ATP is hydrolyzed by Hsp90, NTDs move apart. ADP-bound Hsp90 is thereby referred to as the “open” conformation.

The interaction between Hsp90 and its clients requires other co-factors, such as Hsp70, Hsp40 and Hop (Figure 2.6). A newly synthesized client protein first binds to a complex consisting of Hsp70 and Hsp40, and then Hop mediates the binding of the complex to Hsp90 in the open state. Upon hydrolysis of ATP on Hsp90, Hsp90 re-arranges itself into the closed conformation. Subsequently, Hsp90 complex forms a late complex with other co-chaperones including p23, p50, cdc37 and immunophilins (IP), that catalyzes the conformational maturation of the Hsp90 client proteins (121, 122). Once client proteins are folded correctly, they are released from Hsp90. In addition, the speed of Hsp90 chaperone cycle is regulated by co-factors. For instance, activator of Hsp90 ATPase (Aha1) accelerates the chaperone cycle by promoting the hydrolysis of ATP. The direct binding of Aha1 to MD of Hsp90 enhances the activity of ATPase by stabilizing the interaction between CTD and NTD of Hsp90 (122-125).

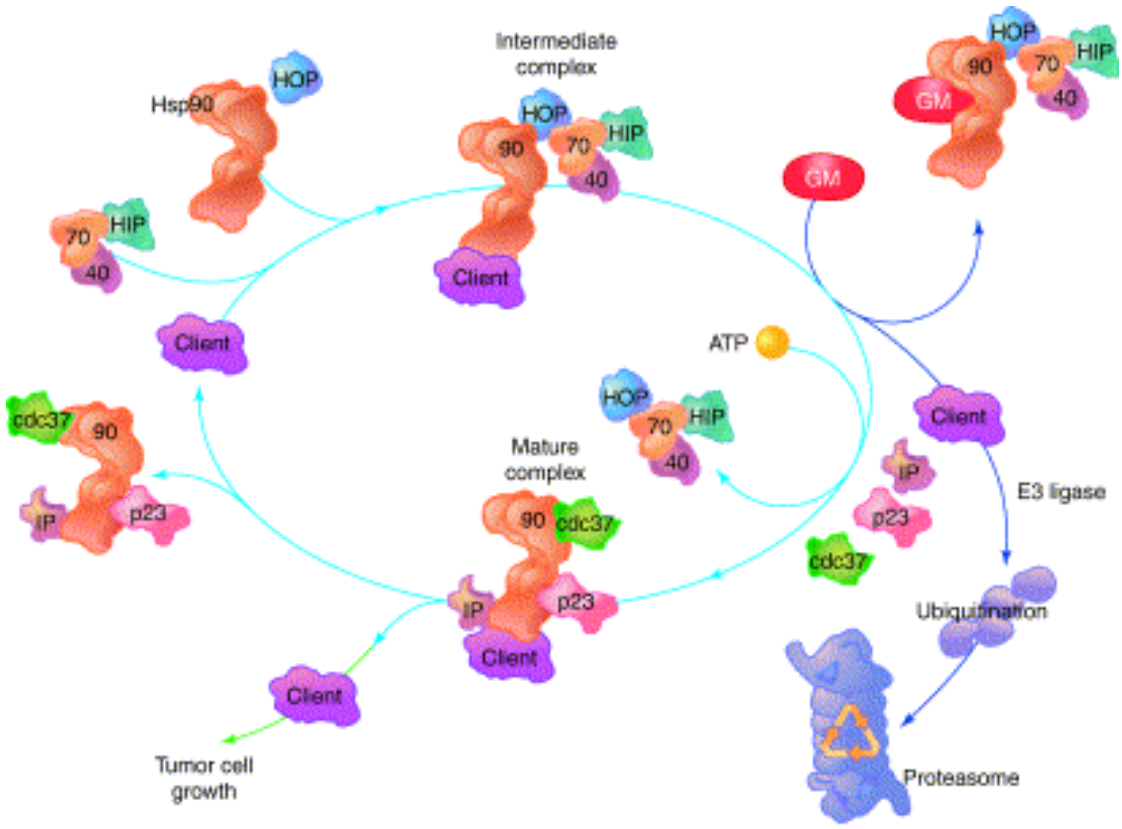

Figure 2.6 The Hsp90 chaperoning cycle. The Hsp90 chaperoning cycle is a dynamic process in which client proteins bind to Hsp90 in an intermediate complex containing the co-chaperones Hsp70, Hsp40, Hip and Hop. Upon ATP binding and hydrolysis, Hsp90 forms a mature complex, containing p23, p50/cdc37 and immunophilins (IP), which catalyzes the conformational maturation of Hsp90 client proteins. Hsp90-inhibitor drugs, such as geldanamycin (GM), bind to the N-terminal ATP-binding pocket of Hsp90 and inhibit ATP binding and hydrolysis, thereby locking Hsp90 in the intermediate complex. The client protein is subsequently ubiquitinated (possibly by a E3 ubiquitin ligase) and targeted to the proteasome for degradation (122)

3. **Hsp90 inhibitors in breast cancer therapy**

Because Hsp90 is involved in stabilizing a broad spectrum of oncogenic proteins, agents that target Hsp90 have become a major focus in cancer research. The reason for much focus is that, by simply targeting a single protein, multiple oncogenic pathways can be inhibited. In particular, Hsp90 inhibition via blocking of ATP

binding has been extensively studied, in both pre-clinical and clinical settings. A natural product, Geldanamycin (GA), is the first such compound (126). GA directly binds to and occupies the ATP-binding pocket in the NTD of Hsp90 to blocks the binding of ATP. As a result, Hsp90 client proteins cannot achieve the proper conformation, which then are degraded by proteasome. In pre-clinical settings, GA proved to be a potent inhibitor of Hsp90 and showed great promise as a novel agent for cancer therapy (127-130).

Despite the fact that GA displayed the great potency in Hsp90 inhibition, significant hepatic toxicity (ref) associated with GA lead to the need for less toxic derivatives. Two such derivatives have gained significant interest, which are 17-allylamino-17-demethoxygeldanamycin (17AAG) and the water soluble analogue of 17AAG, 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG). These agents have been formulated for clinical trials, which are Tanespimycin (17-AAG, KOS-953; Bristol-Myers Squibb, New York, NY, USA) and IPI-504 (Infinity Pharmaceuticals, Cambridge, MA, USA). Tanespimycin, considered a first-generation geldanamycin derivative, has demonstrated robust anti-tumor activity in preclinical models of HER2-positive breast cancer (131). In accordance with Tanespimycin, the other formulation of 17AAG, IPI-504, showed antitumor activity in preclinical setting, causing simultaneous degradation of multiple oncogenic proteins (e.g. HER2, Akt, p-Akt, and p-MAPK). IPI-504 has also been found to display antitumor activity against pancreatic cancer xenografts and is a promising compound against gastrointestinal stromal tumors of platelet-derived growth factor receptor-α

mutant. The advent of the less toxic derivatives revived the notion of Hsp90 inhibition as a viable approach to cancer therapy.

More importantly, Hsp90 inhibitors in combination with currently existing therapy have shown to be an effective cancer treatment strategy in recent preclinical studies and clinical trials. IPI-504 in combination with trastuzumab was able to re-sensitize Her2-positive breast cancer that is refractory to Herceptin® (132). Furthermore, a recent phase II trial of tanespimycin (17-AAG) in combination with Herceptin® was conducted in patients with HER2-positive metastatic breast whose disease had previously progressed while on Herceptin® treatment. Interestingly, co-administration re-sensitized these tumors to therapies and showed a great tumor response (133, 134). More Hsp90 inhibitors, such as IPI-504 and AUY922 (Novartis, Cambridge, MA, USA), are currently under evaluation in early-phase clinical trials as single agents or in combination with trastuzumab.

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Chapter 3

Hsp90 inhibitor 17-(Allylamino)-17-Demethoxygeldanamycin Targets Breast Cancer Stem Cells

Abstract

The relative resistance of breast cancer stem cells (CSCs) to chemotherapies highlights the need to develop new agents to target this cell population. The present study suggests that 17-(Allylamino)-17-Demethoxygeldanamycin (17AAG), a Hsp90 inhibitor, targets breast CSCs, identified using the Aldefluor assay, *in vitro* and in mouse xenografts at low doses. Breast Aldefluor-positive cells, which are known to display stem cell properties, exhibited a seven-fold higher sensitivity to Hsp90 inhibition *in vitro* than the bulk population of breast cancer cells. Low concentrations of 17AAG (5-10 nM) reduced the amount of Aldefluor-positive cells by more than 50% and inhibited their tumorsphere formation rate. In mice, low-dose of 17AAG (5 mg/kg) reduced the number of Aldefluor-positive cells in xenografts generated from human primary breast cancer MC1 cells by 60%. In addition, 17AAG was shown to impair the tumorigenicity of residual Aldefluor-positive cells in secondary implantation in NOD/SCID mice. The western blot analysis and LEF-1/TCF reporter assay suggested that the inhibition of Aldefluor-positive cell growth by 17AAG involves the dysregulation of Akt/β-catenin/Wnt

signaling, which is known to be a vital CSC survival pathway. To our knowledge, the current study is the first to investigate the efficacy of 17AAG on the breast CSC-like Aldefluor-positive cells. The findings of this study propose the inhibition of CSC as a new approach to evaluate the clinical importance of Hsp90 inhibitors for breast cancer therapy.

Introduction

Cancer stem cells (CSCs) are defined by their unlimited potential to initiate tumors and are strongly associated with cancer relapse, metastasis, and resistance (1, 2). Conventional chemotherapies designed to eliminate the proliferative and differentiated cancer cells are ineffective against the quiescent and undifferentiated CSCs. As a result, resistant CSCs can potentially reinitiate tumors despite otherwise effective chemotherapies (2-4). The lack of effective treatments against tumor recurrence and metastasis mediated by CSCs clearly demonstrates a need for new therapeutic approaches to target this population (5).

Heat shock protein 90 (Hsp90) is well established as a key target for the treatment of various cancers, due to its involvement in the regulation of multiple oncogenic pathways. Hsp90 over-expression in breast cancer has been associated with both tumor aggressiveness and poor prognosis (6-8). Hsp90 is an essential molecular chaperone for a wide variety of oncogenic proteins, including Akt, Her2, MEK and HIF-1α (9-12). The involvement of Hsp90 in multiple oncogenic pathways provides a key target for drug development in cancer therapy.

17-(Allylamino)-17-Demethoxygeldanamycin (17AAG) is a Hsp90 inhibitor that has been studied extensively as an effective agent against a variety of tumors in preclinical models (13-15). However, 17AAG as a single agent has produced little clinical response in human, as tolerable doses proved inefficient at inducing tumor regression. A recent phase II clinical report by Gartner et al. indicated that 17AAG alone had no tumor response, and induced grade 3 and 4 toxicities in patients with

metastatic or advanced breast cancer (16). As off-target toxicities may result from the quinine moiety in 17AAG, many efforts have been made to develop less toxic and more efficacious Hsp90 inhibitors. For instance PU-H71, a purine-scaffold Hsp90 inhibitor, has been demonstrated to induce complete tumor response in a triple-negative breast cancer without inducing toxicity (17). Therefore, dose limiting toxicities (DLTs) are the major obstacles hindering the clinical development of 17AAG.

Resistance of CSCs to currently available therapies poses tumor reoccurrence as a major challenge in the treatment for cancers. Studies have shown a number of Hsp90 client proteins (Akt, Her2, p53, HIF-1α) to be involved in the regulation of CSC-associated signaling pathways. Through the inhibition of multiple Hsp90-mediated signaling pathways, 17AAG may provide a novel strategy to target CSCs and prevent tumor reoccurrence. This notion was substantiated by several recent studies in bladder cancer (18) and glioblastoma multiforme (11). However, little work has been performed evaluating the efficacy of 17AAG on the CSC population in breast cancer.

In this study, the inhibitory effects of 17AAG on breast CSCs, their self-renewal ability, and the mechanism underlying the 17AAG-mediated CSC inhibition, were evaluated using *in vitro* and *in vivo* assays. The data showed that 17AAG significantly reduced the number of CSC-like cells, as determined by the Aldefluor assay, and damaged their self-renewal capability. Compared to differentiated cells, 17AAG eliminated Aldefluor-positive cells at a 7-fold lower concentration. Lastly, our findings indicate that dysregulation of the Akt/β-catenin/Wnt self-renewal

pathway is associated with the inhibition of Aldefluor-positive cells by 17AAG. Taken together, our findings suggest further investigation is warranted for the use of low doses of 17AAG as a possible treatment option for inhibiting breast CSCs.

Methods

Cell Lines and Reagents

The SUM159 cell line (ER-, PR-, and Her2-) was originally obtained from Dr. Stephen Ethier (Karmanos Cancer Center, Detroit, Michigan); MCF7 cell line (ER+, PR+, and Her2-) was originally purchased from American Type Culture Collection (19). SUM159 cancer cells were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 1% antibiotic/antimycotic (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B), and 20 μg/ml gentamycin. MCF7 cancer cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic, and 5 μg/ml insulin. The cells were maintained in culture at 37 °C and 10% CO_2. 17AAG was purchased from LC Laboratories (Woburn, MA). Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA). BIO was obtained from EMD Biosciences (San Diego, CA). DAPI (4'-6-Diamidino-2-phenylindole) was purchased from Sigma-Aldrich (St. Louis, MO). Matrigel and anti-H2Kd antibody were purchased from BD Biosciences (San Jose, CA). Antibodies against phospho-Akt^{Ser473}, Akt, and phospho-GSK3β^{Ser9} were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-Actin and Cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ALDH1 antibody was obtained from BD Transduction Laboratory (Franklin Lakes, NJ). Active-β-catenin (anti-ABC) clone 8E7 mouse monoclonal antibody was obtained from the Millipore

Corporation (Billerica, MA). Antibodies against CD44 and CD24 were purchased from BD Biosciences (San Jose, CA).

MTS Cell Proliferation Assay

SUM159 cancer cells were seeded into 96 well microplates (3,000 cells per well). Cells were treated with increasing concentrations of 17AAG (0.001–1 μM). After 48 hrs, cell viability was assessed by the MTS assay (Promega, Madison, WI) according to the manufacturer's instructions. Cellular IC_{50} was defined as the concentration of 17AAG necessary to decrease the viability of the cells to 50% compared to DMSO-treated cells (control).

Aldefluor Assay and Flow Cytometry Analysis

The Aldefluor assay was carried out according to the manufacturer's protocol (Stemcell Technologies, Vancouver, BC). For both *in vivo* and *in vitro* studies, an Aldehyde Dehydrogenase 1 (ALDH1) substrate, BODIPY-aminoacetaldehyde (BAAA), was added to a single cell suspension at a concentration of 1.5 μM, which was then incubated for 40 min at 37 °C. Additionally, a portion of cells was incubated with a 10-fold molar excess of an ALDH1 enzyme inhibitor, diethylamino benzaldehyde (DEAB), with BAAA and incubated similarly for 40 min. After Aldefluor staining, cells were washed with HBSS containing 2% fetal bovine serum. Subsequently, cells were stained with 1 μg/ml PI or DAPI to exclude non-viable cells. Flow cytometry was performed at the University of Michigan Cancer Center Flow Cytometry Core.

Data analysis was performed with the software program WEASEL (Walter and Eliza Hall Institute of Medical Research).

CD44/CD24 staining and flow cytometry

To assess the effect of 17AAG on the CSC-like cells, characterizing by CD44+/CD24-, MCF7 were treated with 5-10 nM 17AAG for three days and then subjected to CD44 and CD24 staining according to the manufacture's protocol (20). In brief, cells were re-suspended in phosphate-buffered saline supplemented with 0.5% fetal bovine serum (1×10^6 cells/50 µl). CD24-PE and CD44-APC (BD Biosciences, San Jose, CA) were added to the cell suspension and incubated at 4°C in the dark for 20 min. Subsequently, cells were stained with 1µg/mL DAPI to exclude non-viable cells. The identification was performed using flow cytometry at the University of Michigan Cancer Center Flow Cytometry Core. Data analysis was performed with the software program Weasel (Walter and Eliza Hall Institute of Medical Research).

Tumorsphere Formation

SUM159 and MCF7 Aldefluor-positive cells were plated onto ultralow-attachment 96 well plates (Corning, Corning, NY) at a density of one cell per well. Tumorspheres were treated with 1-10 nM 17AAG and cultured for seven days in a serum-free mammary epithelial basal medium (MEBM) (Cambrex Bio Science Walkersville, Inc.) supplemented with B27 (Invitrogen, Carlsbad, CA), 20 ng/ml EGF (BD Biosciences, San Jose, CA), 1% antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B), 20 µg/ml

Gentamycin, 1 ng/ml hydrocortisone, 5 μg/ml insulin, and 100 μM beta-mercaptoethanol (Gibco Invitrogen, Carlsbad, CA) in a humidified incubator (10% CO_2, 37 °C). Primary tumorspheres were collected, washed, and enzymatically dissociated into single cell suspensions for subsequent passages. Single cell suspensions were plated onto ultralow-attachment 96 well plates at low cellular densities of less than five cells per well, and cultured in the absence of drug treatment. Tumorsphere formation rate was denoted as the ratio of the number of tumorspheres to the number of seeded cells.

Tumor Tissue Dissociation

The excised tumors from mice were enzymatically dissociated to obtain single cell suspensions, as previously described (20). In brief, tumors were minced finely with scalpels and incubated in Medium 199 containing 10% collagenase/hyaluronidase (StemCell Technologies, Vancouver, BC) at 37 °C for 30 minutes. Filtered (40 μm filter, BD Biosciencies, San Jose, CA) single cell suspensions were centrifuged (1500 rpm at 4°C) and pellets were resuspended in PBS for the Aldefluor assay and tumor re-implantation.

Tumor Xenograft Model and Re-implantation

All experiments involving mice were approved by the University Committee on the Use and Care of Animals at the University of Michigan. To evaluate the effect of 17AAG treatment on tumor growth *in vivo*, breast cancer xenografts generated from SUM159 cells and a primary human breast cancer (MC1) were used. MC1 has

previously been characterized as triple-negative (ER-, PR- and Her2-) (20). SUM159 or MC1 cells mixed with Matrigel (50%, v/v) were injected into the fourth mammary fat pads of four-week-old NOD/SCID mice (Jackson Laboratories, Bar Harbor, MI). Mice bearing SUM159 tumors received either the vehicle (1:1 (v/v) mixture of DMSO and ethanol) or a medium-dose 17AAG (35-50 mg/kg) three times a week for two and a half weeks. Mice bearing MC1 tumors received the vehicle or a low-dose 17AAG (5 mg/kg) three times a week for three weeks. Tumor size was measured twice a week.

Tumor re-implantation was conducted as previously described (20, 21). In brief, single cell suspensions were stained with DAPI for viability assessment. The recovered live cells were stained with anti-H2Kd antibody to distinguish human breast cancer cells from mouse cells. Viable SUM159 or MC1 cells derived from mice treated with 17AAG or the vehicle were injected into the fourth inguinal mammary fat pad of secondary NOD/SCID mice. Tumor growth was measured weekly.

Western Blotting

Unsorted SUM159 cells were treated with 0.1-2 μM 17AAG for two days under adherent conditions. Then, Aldefluor-positive SUM159 cells were sorted into 6-well ultralow-attachment plates (Corning) at a cellular density of $1x10^5$ cells/well and cultured in non-adherent conditions for three days in the presence of 5-500 nM 17AAG. At the end of each culture period, total cell lysates were prepared and total protein were quantified with BCA protein assay reagents (Pierce, Rockford, IL). Equal amounts of proteins were separated on a Tris-glycine 4-15% gradient precast

gel (Bio-Rad, Hercules, CA), transferred to a PVDF membrane, and then blocked with 5% BSA. The Immobilized proteins were probed with Akt, phospho-Akt, GSK3β, phospho-GSK3β, Active-form β-catenin (ABC), Cyclin D1, ALDH1, and β-Actin primary antibodies.

LEF-1/TCF TOP-GFP Reporter

The MCF-7 cell line was used for the LEF-1/TCF TOP-GFP Reporter assay. MCF-7 cells transfected with the reporter genes were cultured in the presence of either 10 nM 17AAG, 300 nM BIO, or 10 nM 17AAG plus 300 nM BIO for three days under non-adherent conditions. The tumorspheres were dissociated as described above, stained with DAPI for viability, and analyzed for the portion of GFP-positive cells by flow cytometry. Meanwhile, parental MCF-7 cells were used as a negative control to gate for the GFP-positive cell population.

Statistical Analysis

The Student t-test was used to perform statistical analysis. Data are presented as the mean ± 2SD ($n \geq 3$). All p values are two-tailed.

Results

Aldefluor-positive cells were sensitive to Hsp90 inhibition by 17AAG *in vitro*

The effect of Hsp90 inhibition on CSCs was evaluated by measuring the changes in the Aldefluor-positive cell numbers after 17AAG treatment (Figure 3.1A). Two common approaches to identify breast CSCs are CD44 and CD24 antibody staining and Aldefluor assay. However, SUM159 cells are known to be rich with the CD44+/CD24- phenotype (>90%) regardless of the presence of the stem cell population (22). Therefore, this assay does not effectively identify CSCs in SUM159 cells. In contrast, Aldefluor assay has previously been demonstrated by Ginestier et al. to effectively identify CSC and progenitor cell population in breast carcinomas (20). For this reason, Aldefluor assay was chosen to identify breast CSCs in SUM159 cells. SUM159 cells were cultured for three days in the presence of 1, 5 and 10 nM of 17AAG or DMSO control. Consistent with percentages previously reported for this cell line (9, 23), an average of 5% of the control-treated SUM159 cells were Aldefluor-positive. Strikingly, concentrations as low as 5 nM 17AAG reduced the percentage of Aldefluor-positive cells to 1.88%, a 60% reduction as compared to the control cells ($p<0.05$) (Figure 3.1B). At 10 nM 17AAG, there was a 64% reduction in Aldefluor-positive cells as compared to the controls ($p<0.01$). Importantly, low concentrations of 17AAG (5-10 nM), which effectively inhibited Aldefluor-positive cells, had little effect on the bulk population of cancer cells (IC_{50} = 35 nM for the bulk population of SUM159 cell line) (Figure 3.1C). These findings indicate that

Aldefluor-positive cells from breast cancer are more susceptible to 17AAG treatment than differentiated cancer cells.

17AAG impaired tumorsphere formation and proliferation of Aldefluor-positive cells *in vitro*

We examined the changes in the tumorsphere formation rate and the sphere size of Aldefluor-positive cells in response to 17AAG treatment. Measurement of tumorsphere formation rate and the sphere size via serial passaging of tumorspheres under non-adherent conditions are established surrogate method for evaluating the self-renewal capabilities of mammary stem cells and CSCs and the proliferative capabilities of progenitor cells (9, 24). Furthermore, only CSCs and their progenitor cells have been reported to survive in non-adherent conditions through formation of tumorspheres (25). When Aldefluor-positive SUM159 cells were treated with 5-10 nM 17AAG for seven days, 17AAG reduced the tumorsphere formation rate of SUM159 cells by 50% and 74% (n=3), respectively (Figure 3.2A).

In order to examine whether 17AAG induces permanent self-renewal impairment of CSCs, primary tumorspheres were propagated for an additional passage. Single cell suspensions derived from 17AAG- and DMSO-treated primary tumorspheres were re-plated at a density of less than five cells per well and cultured in the absence of 17AAG or DMSO for seven days. Remarkably, secondary tumorsphere formation rate from 17AAG-treated cells was reduced to an even greater extent (76% and 90% in 5 nM and 10 nM 17AAG, respectively, n=3) than what was observed in the primary passage (50% and 74% in 5 nM and 10 nM,

respectively) (Figure 3.2A). The data suggests that 5-10 nM 17AAG induces long-lasting impairment of self-renewal ability in the Aldefluor-positive cells.

In addition, the sizes of the derived tumorspheres, quantified with ImageJ, were substantially reduced by 60 and 80% in area at 5 nM and 10 nM 17AAG, respectively (n=1) (Figures 3.2B). This suggests that 17AAG treatment reduces the proliferative capability of breast cancer progenitor cells.

Additionally, in order to examine if these results can be extended to breast CSCs found in other phenotypes besides basal breast cancer, Aldefluor-positive cells from the luminal breast cancer cell line MCF7 (ER+/PR+/Her2-) were treated with 17AAG as described above. Consistent with our results using SUM159, 17AAG significantly abrogated primary tumorsphere formation by 60% (n=3) at a concentration of 5 nM (Figure 3.2C). Furthermore, as previously seen with SUM159, reduction in secondary tumorsphere formation rate under drug-free conditions was more pronounced (74% reduction, n=3) than what was seen in the primary passage (60%). Lastly, the sizes of primary and secondary tumorspheres decreased by 72% and 60% in area, respectively, at 5 nM 17AAG (n=1) (Figure 3.2D). Taken together, these data show that 17AAG diminishes the self-renewal capability of Aldefluor-positive cells and their progenitor proliferation regardless of their phenotype.

17AAG treatment reduced the amount of Aldefluor-positive cells and their tumor engraftment efficiency in mouse xenografts

In order to verify the *in vitro* findings of 17AAG inhibition, SUM159 tumor xenografts were generated in NOD/SCID mice (21, 26). Numerous reports have

shown that *in vitro* techniques may not be adequate to estimate the effectiveness of potential therapeutic compounds, likely due to oversimplified culture systems that fail to account for the microenvironment-mediated resistance to treatment (2, 27). The treatment group (n=5) received a medium dose of 35 mg/kg 17AAG via I.P. injection, three times a week for two weeks, followed by 50 mg/kg dose for another half-week.

The choice of dose and schedule were based on previous studies in mouse xeografts bearing various tumor types, where doses ranging from 20 to 100 mg/kg were used. During the course of our experiment, 35 mg/kg 17AAG only modestly reduced tumor growth. For this reason, we increased the dose to 50mg/kg for the last two treatments. Dose was increased to ensure that tumor size reduction would be observed, as tumor size was the only feasible assessment for the efficacy of 17AAG during treatment. The tumor size reduction is expected to reflect the effect of 17AAG on the differentiated cells rather than Aldefluor-positive cells. However, greater sensitivity of Aldefluor-positive cells to the drug *in vitro* suggested that if inhibitory effect is observed on the differentiated cells, then Aldefluor-positive cells are inhibited as well. The control group (n=5) received a vehicle with the same dosing schedule. At the end of the experiment, animals were sacrificed and the tumors were analyzed. A 31% reduction in tumor growth was observed in 17AAG-treated mice as compared to vehicle-treated mice (Figure 3.3A). Aldefluor analysis of the tumors showed that 17AAG-treated mice had a 70% reduction in the percentage of Aldefluor-positive cells compared to vehicle-treated mice (n=5 for

each group) (Figures 3.3B and 3.3C). These data suggest that 17AAG is able to reduce the number of Aldefluor-positive cells *in vivo.*

To confirm the 17AAG-mediated self-renewal impairment in Aldefluor-positive cells *in vivo*, residual tumor cells from drug- or vehicle-treated primary mice were re-implanted into secondary mice to assess tumor engraftment efficiency. Tumor engraftment efficiency is defined as the ability of residual CSCs after drug treatment to initiate tumors in secondary mice and is commonly used to evaluate the *in vivo* self-renewal ability of breast CSCs (26, 28). Tumor engraftment efficiency was evaluated based on the time from cell implantation to tumor detection and the subsequent rate of tumor growth. Re-implantation of 50,000 cells from vehicle-treated mice led to a detectable formation of tumors by week two with a growth rate of 1.63mm/week (Figure 3.3D). In contrast, re-implantation of cells from 17AAG-treated mice took an additional four weeks to develop detectable tumors (week six), showing a two-fold reduction in growth rate (0.77 mm/week). Re-implantation of 5,000 cells from vehicle-treated mice formed palpable tumors in week four with a growth rate of 1.07 mm/week, while those from 17AAG-treated mice did not form tumors in the 10 week period (Figure 3.3D). These findings indicate that 17AAG treatment significantly reduces the tumor engraftment efficiency and tumor growth of the remaining Aldefluor-positive cells.

Low-dose 17AAG reduced the Aldefluor-positive cell population in primary tumor xenografts

Because Aldefluor-positive cells were seven-fold more sensitive to 17AAG as compared to differentiated cells *in vitro*, we further investigated whether 17AAG can inhibit breast Aldefluor-positive cells at lower dose of 5 mg/kg *in vivo*. Primary human breast cancer xenografts were generated in mice using MC1 cells, which are cells derived from a patient with metastatic breast cancer (29). Mice bearing MC1 tumors were treated with 5 mg/kg 17AAG or the vehicle three times a week for three weeks. Compared to the vehicle-treated mice, 17AAG treated mice (n=5) displayed a 60% reduction in tumor volume (Figures 3.4A and 3.4B). After three weeks of treatment, mice were sacrificed and the tumors were analyzed with Aldefluor assay. Treatment with 17AAG resulted in a 60% reduction of Aldefluor-positive cells in the excised tumors (n=5) compared to controls (Figure 3.4C and 3.4D).

To investigate the effect of 17AAG on the self-renewing potential of the residual Aldefluor-positive cells in the primary human breast cancer xenografts, tumor engraftment efficiency was evaluated using the tumor re-implantation assay. Tumor cells derived from vehicle- or 17AAG-treated mice with primary human breast cancer xenografts were injected into the fat pads of secondary NOD/SCID mice at 2,000 or 20,000 cells per re-implantation. All mice (n=4) implanted with tumor cells from vehicle-treated mice (2,000 and 20,000 cells) developed palpable tumors in secondary NOD/SCID mice within four weeks (Figure 3.5A). In contrast, 100% of mice (n=4) receiving 2,000 cells from 17AAG-treated mice remained tumor-free, and 75% of mice receiving 20,000 tumor cells remained tumor-free (Figure 3.5B). These results support our *in vitro* findings, and demonstrate that

doses as low as 5 mg/kg can reduce Aldefluor-positive cells and inhibit the tumor-initiating potential of the residual cells.

Inhibition of Aldefluor-positive cells by 17AAG is mediated by the Akt/β-catenin/Wnt Pathway

The co-chaperone function of Hsp90, which stabilize its client proteins including Akt, is instrumental in cancer progression. Additionally, Akt has been shown to facilitate the activation of the β-catenin/Wnt pathway, which regulates mammary stem cell proliferation and self-renewal (21). We therefore examined the effect of 17AAG treatment on the levels of Akt and its associated proteins in SUM159 cells. Because unsorted SUM159 cells cultured under adherent conditions are mainly differentiated (>90%), the unsorted cells were used to represent the differentiated population. To obtain an accurate representation of the CSC population, sorted Aldefluor-positive cells were cultured under non-adherent conditions to prevent differentiation. To validate that non-adherent culture adequately delayed differentiation, we stained the cultured cells for ALDH1 expression. High expression levels of ALDH1 observed in both DMSO- and 17AAG-treated Aldefluor-positive cells, compared to the differentiated cells, indicated that the Aldefluor-positive cells remained undifferentiated after the three-day treatment period (Figure 3.6A). Strikingly, 10 nM of 17AAG downregulated phospho-Akt, phospho-GSK3β, and active β-catenin by more than 70% in Aldefluor-positive cells compared to DMSO-treated cells (Figure 3.6A). In the differentiated population, 10-fold greater concentration of 17AAG, at 0.1 μM, demonstrated a similar reduction of

these protein levels (33-70%) after a two-day treatment (Figure 3.6B). Cyclin D1, a β-catenin transcriptional target gene, was also reduced by 47% in response to the reduction of active β-catenin in Aldefluor-positive cells. Because β-catenin is an essential component of Wnt signaling, the depletion of β-catenin implies that Hsp90 inhibition with 17AAG suppresses Wnt signaling.

To provide further evidence that the 17AAG-mediated reduction of β-catenin leads to an inhibition of β-catenin/Wnt signaling, MCF7 cells transfected with LEF-1/TCF-driven GFP reporter were utilized to monitor the β-catenin transcriptional activity. The ability of 17AAG to eliminate the CSC-like population of the MCF7 cells and inhibit their self-renewal was verified prior to carrying out the report assay (Figure 3.7 and Figure 3.2C and 3.2D). Cells transfected with the LEF-1/TCF-GFP reporter system were treated with 17AAG in a non-adherent condition for three days to form tumorspheres. Following the dissociation of tumorspheres, the quantity of GFP-positive cells was analyzed by flow cytometry. Upon three-day treatment, 10 nM 17AAG reduced the percentage of GFP-positive cells by 40% as compared to treatment with DMSO, which indicated a reduction in β-catenin (n=3). In addition, this inhibition by 17AAG was reversed using a GSK3β inhibitor, BIO (Figure 3.6C). This data suggests that 17AAG-mediated β-catenin inhibition indeed occurs through upstream Akt regulation.

Discussion

Conventional chemotherapies, which target the bulk tumor population, can cause tumor regression but fail to eradicate CSCs (2-4). As a result, the remaining CSCs can regenerate tumors through self-renewal and contribute to tumor relapse. This may explain the observation that tumor regression correlates poorly with patient survival for breast and many other cancers (30-32). Thus, effective ways to target the CSC population are necessary in order to significantly extend survival in cancer patients. Furthermore, simply diminishing the number of CSCs may not be sufficient to successfully treat cancers, since residual CSCs can still reinitiate tumor growth. Hence, a more comprehensive approach for inhibiting CSCs would be completely eliminate CSCs, or one that reduces the number of CSCs and also diminishes their self-renewal capacity.

The results of the current study provide evidence to suggest 17AAG is an effective therapy to target the CSC-like Aldefluor-positive cells in breast cancer. Treatment with 17AAG significantly decreased the number of Aldefluor-positive cells and impaired their self-renewing capacity in SUM159 and MCF7 cells *in vitro*. The ability of 17AAG to eliminate Aldefluor-positive cells was further verified in SUM159 and MC1 xenografts. The MC1 xenograft model was used in our study because it is triple-negative (ER-, PR- and Her2-), which is similar to SUM159, and has been used in a previous study regarding breast cancer stem cells (20). A 70% reduction of Aldefluor-positive cells was observed after treatment with 17AAG (35-50 mg/kg). More importantly, similar efficacy (60% reduction) was observed with a

low dose treatment (5 mg/kg). Lastly, the capability of 17AAG to impair self-renewal was confirmed *in vivo*, as re-implantation of 17AAG-treated SUM159 and MC1 tumor cells into secondary mice showed a considerable delay of tumor initiation and an increased number of tumor-free mice.

Based on the presented data, 17AAG may be expected to be effective against CSCs at doses that are significantly lower than the previously studied doses. In previous preclinical studies using mouse xenografts, efficacy in a variety of tumor types was observed only at medium to high doses (25-100 mg/kg) (33-35). However, the studies focused only on the reduction of bulk tumor size, rather than inhibition of CSCs. A pharmacokinetic study by Xu et al. (36) reported that the intravenous administration of 40 mg/kg of 17AAG gave rise to peak concentrations of approximately 10 μM and 1 μM in plasma and tumor sites, respectively. According to the above finding, the dose required to reach the concentration effective against CSCs (5-10 nM) can be expected to be much less than the previously studied dose. Illustrating this concept, the result of the present study shows that doses as low as 5mg/kg effectively inhibits Aldefluor-positive cell growth and self-renewal capability in mice. Although the effective doses vary between cell types in mouse xenografts, the study suggests 17AAG effectively targets CSC-like cells at doses that are significantly lower than those reported in literature, which may avoid toxicities in human.

While we demonstrated 17AAG could target breast CSC-like cells, this study implies that 17AAG may be clinically effective only when used in combination with other therapies. Indeed, clinical trials have found 17AAG has poor tumor responses

at clinically used doses when used by itself due to dose-limiting toxicities (DLTs) (16). However, in clinical trials, efficacy is assessed using Response Evaluation Criteria in Solid Tumors (RECIST), which are based on shrinkage of tumor size. Tumor shrinkage largely reflects changes in the differentiated cell population rather than the rare CSC population. Therefore, the failure of previous clinical trials using 17AAG, which were dependent on RECIST, cannot be used to predict its efficacy against CSCs (37, 38). Consistent with these clinical trials, our study has shown that 17AAG treatment at low to medium doses causes only cytostatic arrest of tumor size, rather than tumor regression. Therefore, the low dose 17AAG therapy would best be used in combination with an effective conventional treatment strategy, which targets the bulk cell population to reduce tumor size. A recent clinical trial combining 17AAG with Herceptin in HER2-positive metastatic breast cancer was successful, illustrating the potential effectiveness of a 17AAG combination therapy (39). Our study suggests a novel treatment method, utilizing conventional chemotherapy in combination with minimal doses of Hsp90 inhibitors.

The present study also provides insight into the inhibitory mechanism of 17AAG on the CSCs, and suggests 17AAG is an inhibitor of Akt/β-catenin signaling. Akt-mediated phosphorylation of GSK3β is known to inhibit the kinase activity of GSK3β (40). On the other hand, the non-phosphorylated active form of GSK3β is known to inhibit the β-catenin/Wnt pathway by inducing β-catenin degradation (41). These observations suggest that inhibition of Akt will lead to an increase in the non-phosphorylated active GSK3β, which in turn leads to degradation of β-catenin and inhibition of the β-catenin/Wnt pathway. Furthermore, a study by Korkaya et al.

has established the role of Akt in regulating Wnt/β-catenin signaling using mammary stem cells (21). This study showed that breast cancer tumorspheres, which are enriched with CSCs, had higher levels of phospho-Akt, phospho-GSK3β (inactive form) and β-catenin, as compared to differentiated cells. When these tumorspheres were treated with an Akt inhibitor, perifosine, reductions in the levels of inactive phospho-GSK3β and nuclear β-catenin levels were observed. To reverse the effects of Akt inhibition by perifosine, the investigator treated the cells with a GSK3β inhibitor, BIO, in addition to perifosine. Addition of BIO resulted in an increased level of nuclear β-catenin, which indicated increased Wnt/β-catenin signaling activity. The findings above are further corroborated by the observation in our study that the expression level of Cyclin D1, a transcriptional target of β-catenin, is reduced in response to 17AAG. In addition, a number of studies have shown that the constitutive activation of Akt is associated with the expansion of CSCs in thyroid (42), breast (9, 21), brain (43), colon (44) and prostate (45) cancers. Also, studies have shown the role of Hsp90 in stabilizing Akt and phospho-Akt (46-48). As Hsp90 inhibition ultimately results in the disruption of multiple pathways, we cannot exclude the possibility that other disrupted pathways contribute to the inhibition of breast CSCs. However, our findings are in agreement with the current understanding of the Akt/β-catenin signaling pathway and implicate 17AAG as an inhibitor of Akt/β-catenin/Wnt signaling.

One limitation to the findings of our study is that in the re-implantation of tumor cells from 17AAG-treated and vehicle-treated mice, the number of re-implanted CSCs was not controlled. Although the total number of re-implanted

tumor cells was controlled, the drug-treated tumors were composed of less Aldefluor-positive cells (1.37%) as compared to the vehicle-treated tumors (3.47%). Therefore, secondary mice receiving the re-implantation from vehicle-treated mice likely received more Aldefluor-positive cells than from 17AAG-treated mice. To address this concern, the number of Aldefluor-positive cells implanted into secondary mice was calculated based on the Aldefluor assay data in Figure 3.4B and compared to the tumor engraftment efficiency. Of 20,000 tumor cells, 694 cells are calculated to be Aldefluor-positive in the vehicle-treated mice and 274 cells are calculated to be Aldefluor-positive in the drug-treated mice. Of 2,000 tumor cells, 69 cells are Aldefluor-positive in vehicle-treated tumors and 27 cells are Aldefluor-positive in drug-treated tumors. The data implies that as low as 69 Aldefluor-positive cells from vehicle-treated mice formed tumors in secondary mice 100% of the time, while as many as 274 Aldefluor-positive cells from drug-treated mice formed tumors only 25% of the time. This indicates that impaired tumor engraftment efficiency observed in the drug-treated tumors is not only due to the difference in the number of re-implanted Aldefluor-positive cells, but also to their self-renewal ability. These findings suggest that 17AAG is able to target Aldefluor-positive CSC-like cells and inhibit their tumorigenic capability with even a low dose.

Conclusion

This study is the first to demonstrate the inhibitory effect of 17AAG on breast CSC-like cells and to suggest the mechanism of the drug's inhibitory action. Administration of 17AAG significantly reduced the number of Aldefluor-positive cells and impaired their tumorigenic potential. These effects may be mediated by Akt degradation and subsequent disruption of the β-catenin/Wnt pathway. Furthermore, low-dose 17AAG (5 mg/kg) was able to achieve efficacy similar to that of a medium dose (35-50 mg/kg) of drug, thereby diminishing the amount of Aldefluor-positive cells and their tumorigenic potential. Despite the fact that 17AAG was unable to completely wipe out all Aldefluor-positive cells, 17AAG substantially reduced the tumorigenicity of Aldefluor-positive cells via inhibition of the self-renewal function. These data suggest that Hsp90 inhibitors should be evaluated as a novel approach to target breast CSC-like cells for therapeutic use.

FIGURES

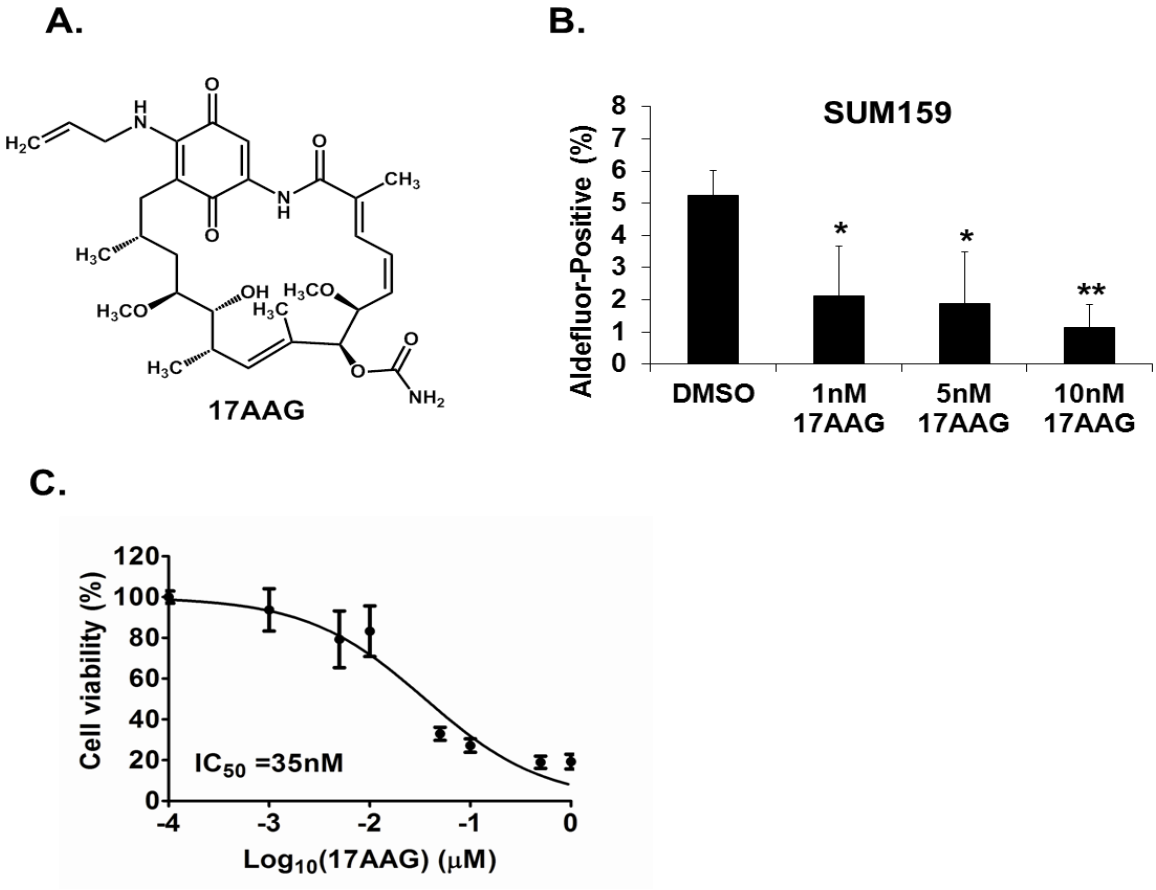

Figure 3.1 Effect of 17AAG treatment on the Aldefluor-positive cells. (A) Chemical structure of 17AAG. (B) Treatment with 1, 5 and 10nM of 17AAG for three days decreased the percentage of Aldefluor-positive cells by 59.6%, 64.2% and 78%, respectively, as compared to the control. (C) SUM159 cells, treated with increasing concentrations of 17AAG for two days, had an IC_{50} of 35 nM as assessed by the MTS assay. All values in (B) are represented as means of 3 independent experiments ± 2SD. All values in (C) are represented as means of 6 independent experiments ± 2SD (*, $p<0.05$ and **, $p<0.01$).

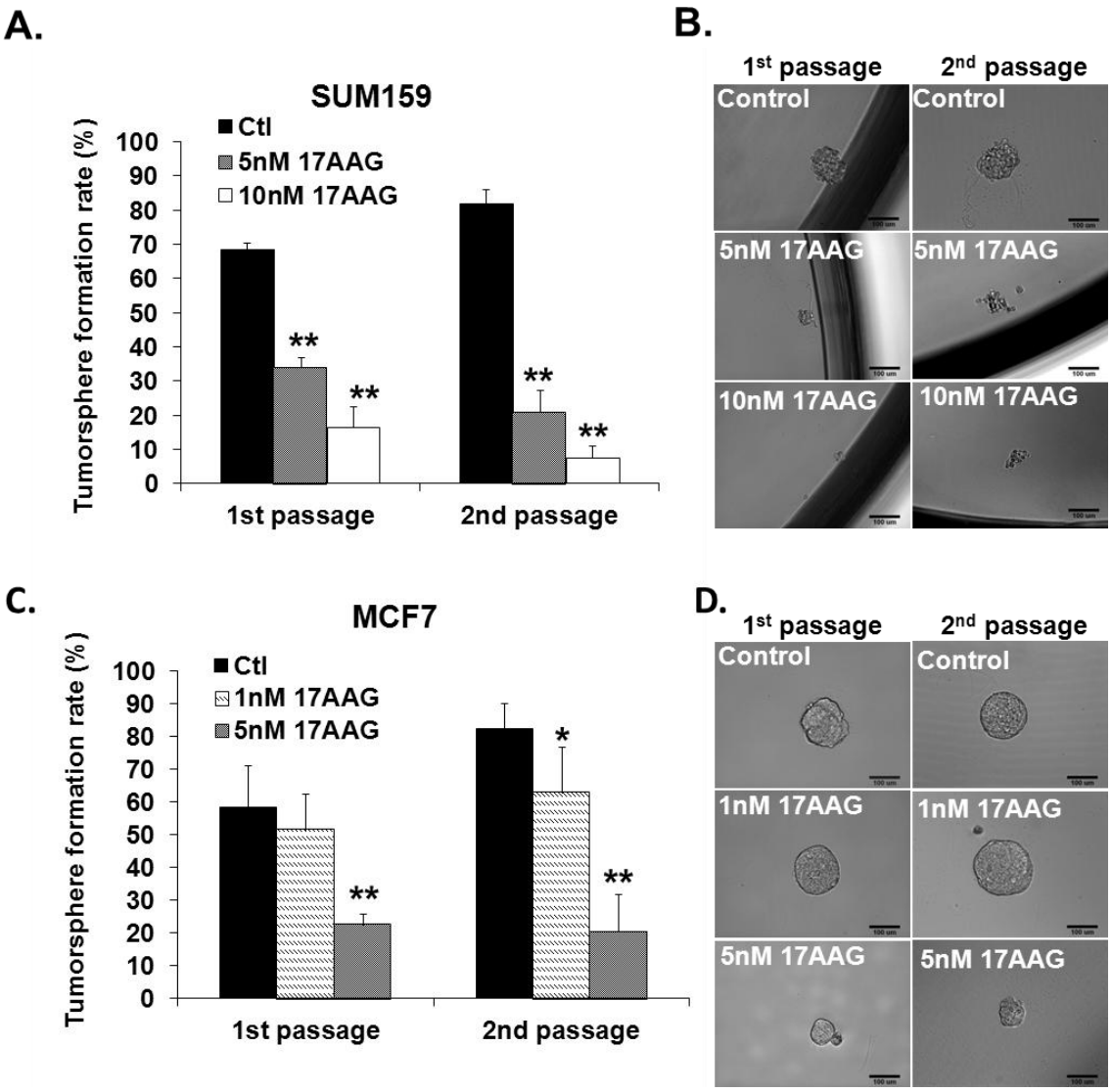

Figure 3.2 Treatment with 17AAG inhibits the self-renewal capability of SUM159 and MCF7 Aldefluor-positive cells *in vitro*. (A) Treatment with 5 nM and 10 nM 17AAG reduced the tumorsphere formation rate by 50% and 74%, respectively. The inhibition was further enhanced in secondary passages in the absence of the drug (76% and 90%, respectively). (B) The diameter of SUM159 tumorspheres was also reduced in response to 17AAG treatment at all concentrations. Additionally, cell morphology became less compact compared to the control. (C) Tumorsphere formation assay of MCF7 Aldefluor-positive cells was performed in the same fashion as for SUM159. Upon exposure to 5 nM of 17AAG, tumorsphere formation rates significantly decreased, by 60%, in the primary passage. Formation of secondary tumorspheres was also reduced to a greater extent (74%) even after the removal of the drug. (D) The diameter of MCF7 tumorspheres was reduced in response to 17AAG treatment at 5nM. All values are represented as means of 3 independent experiments ± 2SD (*, $p<0.05$ and **, $p<0.01$).

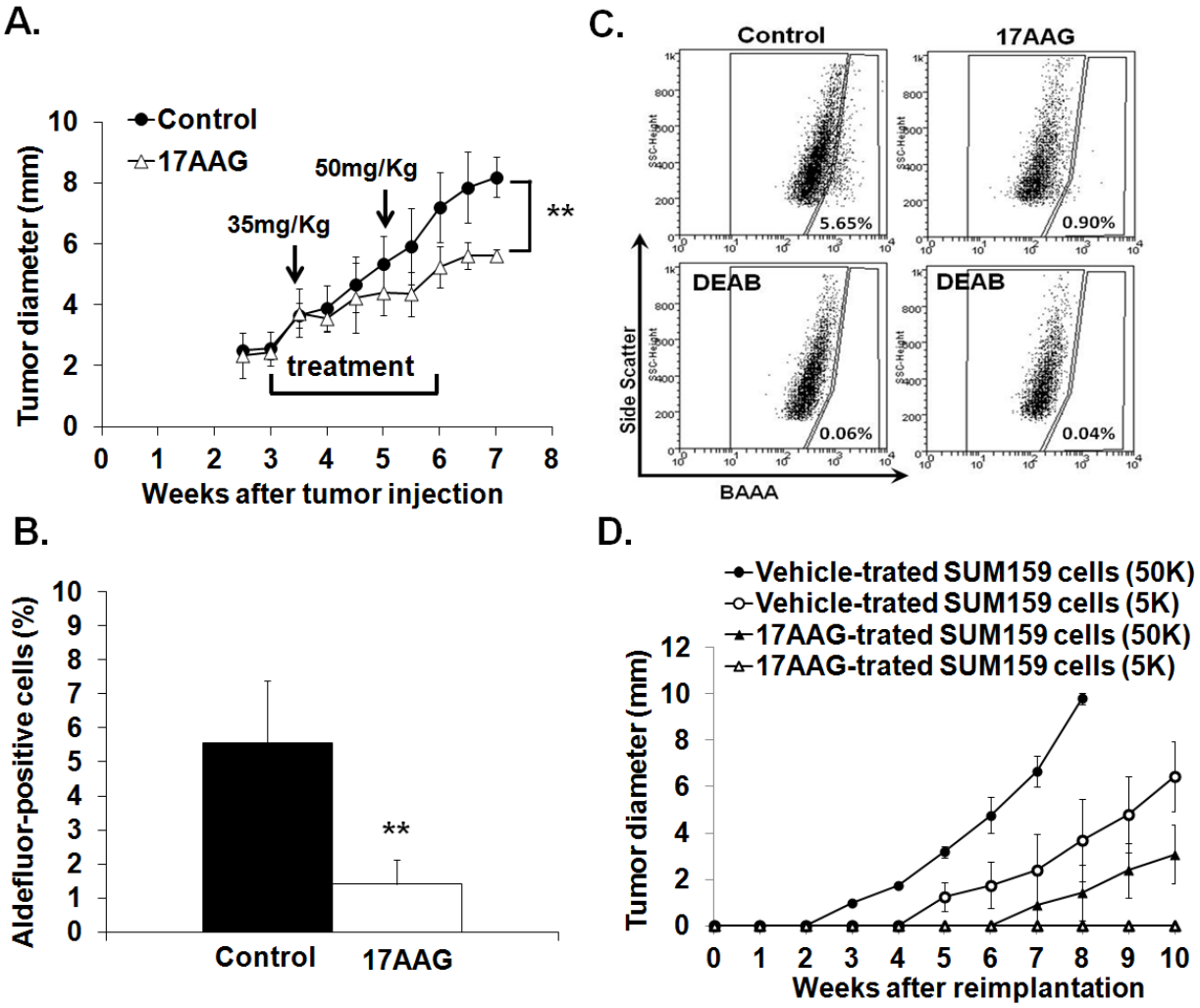

Figure 3.3 Treatment with 17AAG inhibits the growth and the self-renewal capability of the Aldefluor-positive population in SUM159 tumor xenografts. (A) 1×10^6 SUM159 cells were injected into the fat pads of NOD/SCID mice and monitored for tumor growth. When tumors were approximately 2 mm, the mice were treated with 17AAG (35-50 mg/kg) or vehicle. The diameter of tumors treated with 17AAG was 31% smaller compared to vehicle-treated controls. (B and C) At the end of the experiment, tumors were collected and the proportion of Aldefluor-positive population to the bulk population was analyzed using the Aldefluor assay. Treatment with 17AAG reduced the percentage of cells that are Aldefluor-positive by more than 70%. (D) Tumors were collected from 17AAG- or vehicle-treated mice and re-injected into secondary mice. 50,000 cells from vehicle-treated mice formed tumors in secondary mice in week two with a growth rate 1.63 mm/week. In contrast, cells from 17AAG-treated mice took an additional four weeks to form tumors (week six), showing a two-fold reduction in the growth rate (0.77 mm/week). In the re-implantation assay, 5,000 cells from vehicle-treated mice formed palpable tumors in week four with a growth rate 1.07mm/week, while those

from 17AAG-treated did not form tumors in the 10-week monitoring period. All values are represented as means of 5 independent experiments ± 2SD (**, $p<0.01$).

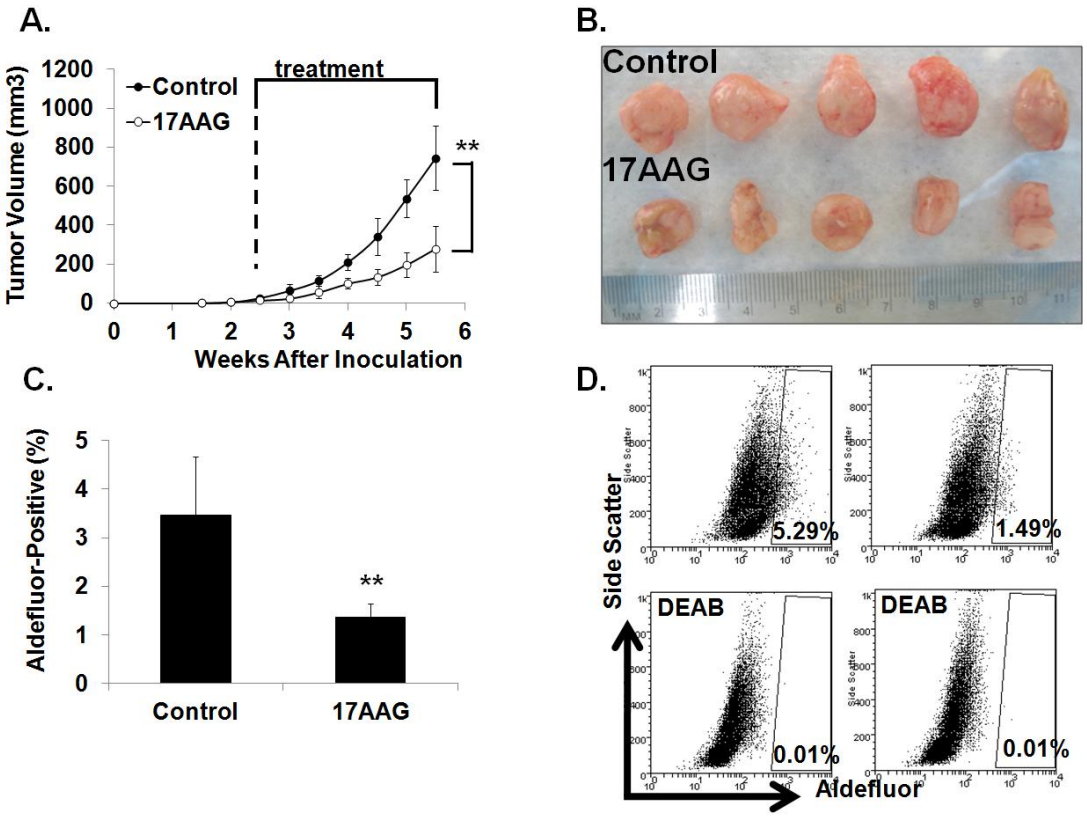

Figure 3.4 Treatment with low-dose 17AAG (5 mg/kg) reduced tumor growth and the number of Aldefluor-positive cells in MC1 tumor xenografts. (A and B) Tumors in mice treated with 17AAG showed a 60% reduction in the tumor growth rate as compared to that in the vehicle-treated group. (C and D) In the drug-treated tumors, 1.37% of the cells were Aldefluor-positive, compared to 3.47% in the vehicle-treated tumors. Treatment of mice with 5 mg/kg 17AAG reduced the fraction of cells that are Aldefluor-positive cells in the tumors by 60%. All values are represented as mean of 5 independent experiments ± 2SD (**, $p<0.01$).

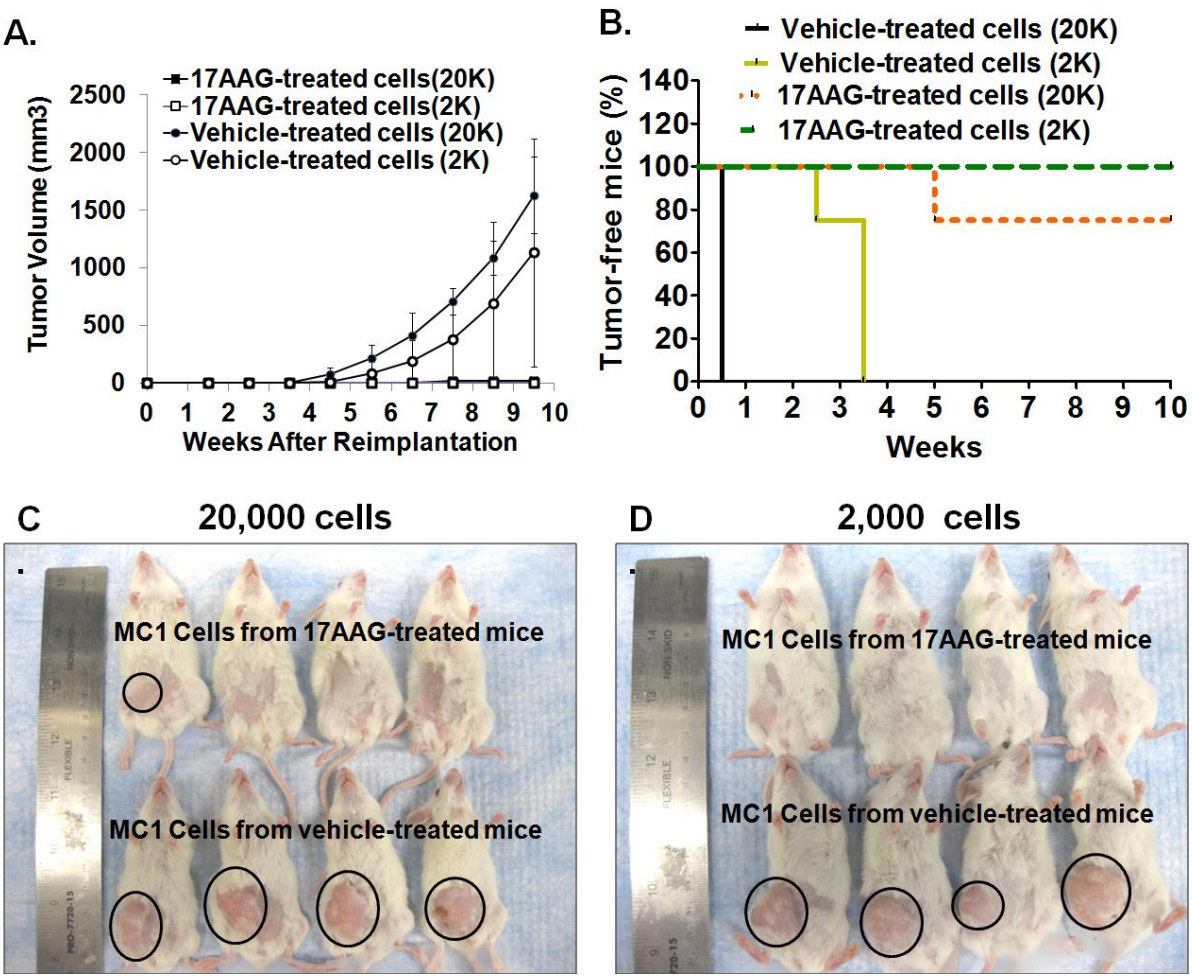

Figure 3.5 Treatment with low-dose 17AAG reduced tumor engraftment efficiency. (A) Mice receiving injections of 2,000 and 20,000 cells derived from vehicle-treated mice formed tumors at week 2.5 and 1.5, respectively, reaching an average volume of 1,133 mm^3 and 1,631 mm^3, respectively, at week 9.5. No significant tumor growth was observed in mice receiving tumor cells from 17AAG-treated mice at either dilution. (B) By week four, all mice receiving cells from vehicle-treated mice harbored tumors at both dilutions. In contrast, 75% of mice receiving 20,000 cells from 17AAG-treated mice remained tumor-free up to the end of the study. All mice receiving 2,000 cells from 17AAG-treated mice remained tumor-free over the 9.5-week monitoring period. (C and D) Images of mice bearing tumors were taken at week 10. The black open circles indicate tumors. All values are represented as means of 4 independent experiments ± 2SD.

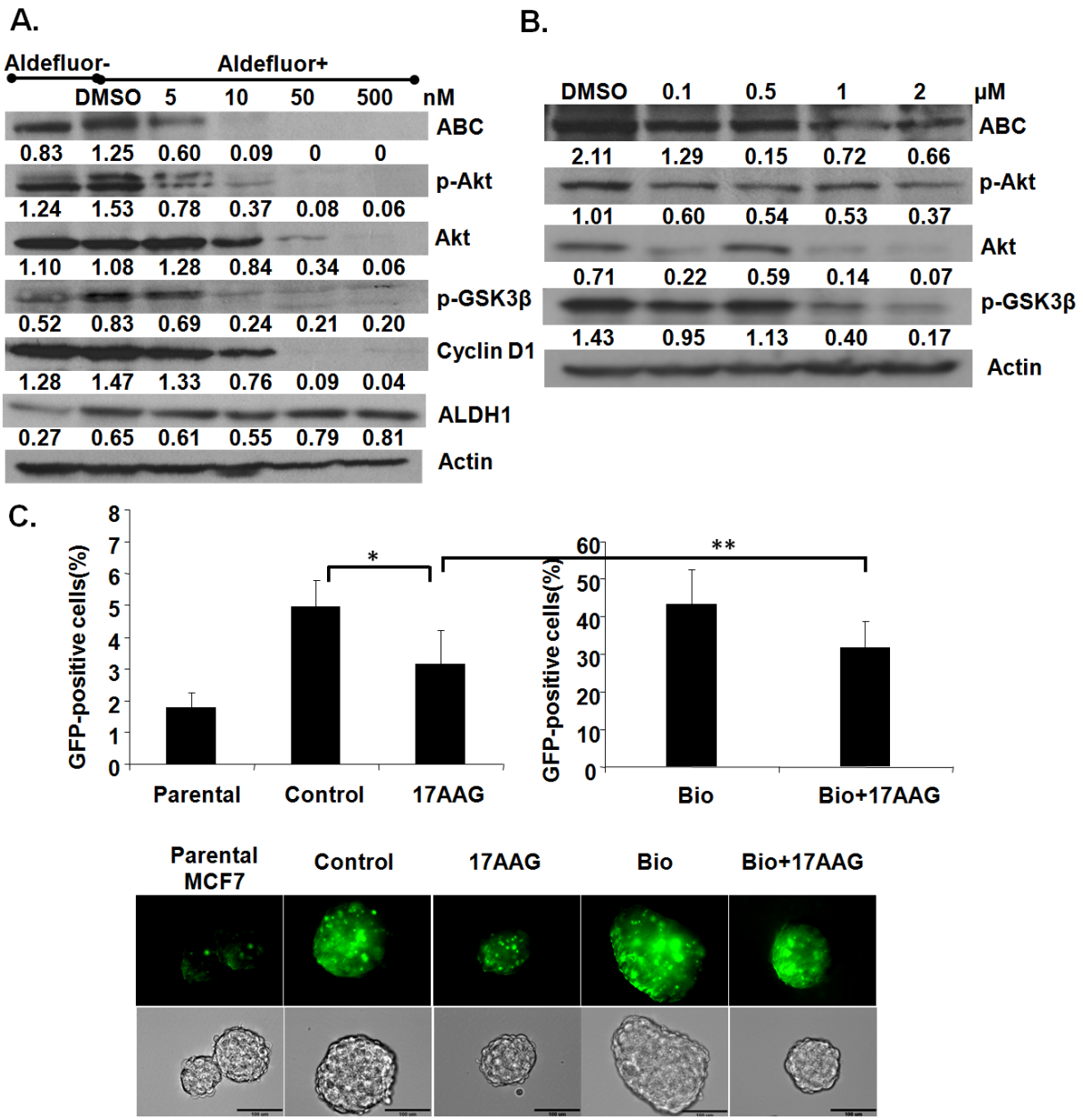

Figure 3.6 Treatment with 17AAG reduced the expression levels of the mediators of Akt/β-catenin/Wnt pathway. (A) Aldefluor-positive cells were isolated and treated with the indicated concentrations (5-500 nM) of 17AAG under non-adherent conditions. The intensities of protein bands, normalized to Actin levels, are listed below each band. As compared to Aldefluor-positive cells, 23-58% higher levels of p-Akt, p-GSK3β and ABC (Active β-catenin) proteins were observed in the DMSO-treated Aldefluor-positive cells. In the 17AAG-treated group, more than 70% reduction in p-Akt, p-GSK3β, and ABC protein levels, and a 47% reduction of Cyclin D1 level were achieved with 17AAG concentrations as low as 10 nM. (B) Treatment with 17AAG decreased the levels of ABC, Akt, p-Akt, and p-GSK3β proteins in a

concentration-dependent manner. More than a 33% reduction in the expression level of these proteins were observed after treatment with 0.1 μM 17AAG. (C) To provide further support that 17AAG exerts its inhibitory effects via dysregulation of β-catenin/Wnt signaling, ABC transcriptional activity was monitored using LEF-1/TCF driven GFP reporter system. Treatment with 17AAG reduced the percentage of cells that are GFP-positive by 40% compared to DMSO treatment. In addition, 17AAG inhibition was reversed with administration of a GSK3β inhibitor, BIO. All values in figures A and B are measurements from a single experiment. All values in figure C are represented as means of 3 independent experiments ± 2SD.

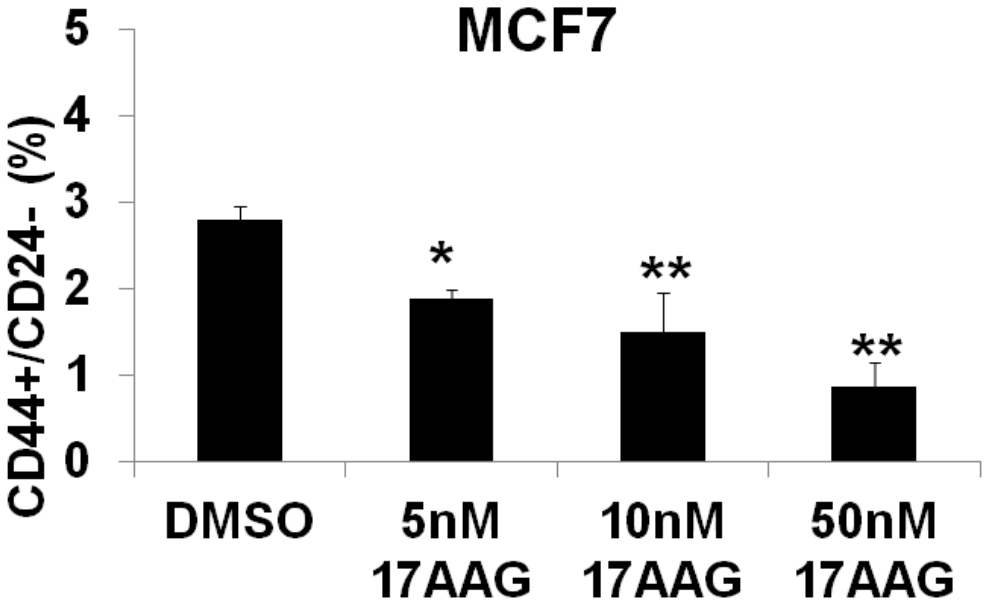

Figure 3.7 Effect of 17AAG on MCF7 $CD44^{+}/CD24^{-}$ cells. A 32%, 46% and 69% reduction in the portion of $CD44^{+}/CD24^{-}$ cells was observed in MCF7 treated with 5, 10 and 50 nM 17AAG, respectively, for three days. All values are represented as means of 3 independent experiments ± SD (*, $p<0.05$ and **, $p<0.01$).

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Chapter 4

Preclinical evaluation of efficacy of 17AAG in combination with Docetaxel in adjuvant therapy

Abstract

The CSC hypothesis suggests that tumors originate from a small subset of cancer cells, termed cancer stem cells (CSCs). CSCs, which are refractory to conventional chemotherapy, have been associated with tumor recurrence. Because anti-CSC agents target a small population, the current clinical endpoint using tumor reduction may be inadequate to assess the efficacy of anti-CSCs agents. Therefore, the use of anti-CSC agents as adjuvant therapy after conventional therapy or in combination with cytotoxic chemotherapy as primary therapy have been proposed as better models to examine the clinical relevance of anti-CSC drugs.

A recent phase II clinical study of 17AAG suggests that 17AAG does not induce tumor response in patients with advanced tumors (1). In contrast, 17AAG in combination with other chemotherapy has a synergistic effect on clinical outcome (2, 3). The discrepancy in clinical outcome of 17AAG raises a question of which models may be appropriate to evaluate the clinical significance of 17AAG, which preferentially targets CSCs at low concentrations. Thus, we employed an animal

model to compare the extent to which 17AAG is able to achieve clinical benefit as a combination or single therapy in adjuvant or advanced settings. In adjuvant therapy setting, we treated mice with 17AAG one day after tumor inoculation. In contrast, we treated mice until 14mm^3 tumors were developed in the advanced tumor growth setting.

17AAG was able to substantially delay tumor progression by 95% in the adjuvant therapy setting compared to only 60% inhibition in the advanced tumor growth setting, despite the similar extent of inhibition on Aldefluor-positive cells (60%) in both experimental settings. These data suggested that 17AAG alone as adjuvant therapy or in combination with chemotherapy has potential to inhibit tumor recurrence and that early treatment has significantly better results when using 17AAG in treating breast cancer. The findings have important clinical implications regarding the use of 17AAG to treat cancers, and warrants further investigation on the novel clinical use of 17AAG.

Introduction

A growing body of evidences supports the existence of CSCs in tumors for various cancers, including breast cancer (4-6). CSCs have been associated with tumor recurrence and metastasis, which are the major obstacles of current cancer therapy. CSCs have shown resistance to conventional therapies (7).Therefore, once the treatment ends, CSCs can again give rise to tumors leading to tumor relapse. Also, CSCs may detach from primary tumors and migrate to a distal location, leading to metastasis. Moreover, on the basis of the CSC model, the complete cure of cancers cannot be achieved unless all CSCs are eliminated. Consequently, a need exists to develop agents that target the rare CSC population.

CSC research has progressed rapidly since the original discovery of CSCs in AML by Dick and Bonnet (8). Recently, advances in understanding the essential pathways involved in the regulation of CSCs, such as Notch, Wnt, Hedgehog, have led to the development of compounds targeting this tumorigenic population, some of which are under clinical evaluation.

Evaluation of clinical efficacy of CSC-specific agents is a major obstacle in clinical development with conventional cancer treatments. Overall survival is an established measurement for clinical efficacy, which requires a large sample size and long follow-up period. Therefore, in Phase II clinical trials that involve much fewer patients, tumor regression as defined by RECIST criteria has been accepted as

an appropriate surrogate for clinical efficacy (9). Yet, the same may not hold true when assessing the clinical efficacy of cancer treatments that target CSCs. CSCs compose a very small proportion of tumors. Therefore, agents that are effective against CSCs may not necessarily induce tumor regression. Hence, endpoints that reflect the functional capability of CSCs should be used in order to assess the clinical benefit of these anti-CSC agents. Better surrogate endpoints are clearly needed for successful clinical development of these agents.

Two approaches have been proposed to evaluate the clinical efficacy of CSC-targeting agents. First, combination therapy of CSC-targeting agents with conventional chemotherapy may eliminate both the bulk of tumor and the resistant CSC population. The merit of combination therapies is that conventional chemotherapies can be used to relieve the tumor burden from the bulk of tumor cells and CSC-targeting agent can be used to eliminate CSCs that are resistant to chemotherapies. In this setting, tumor response based on shrinkage may still be an appropriate primary endpoint. Second, CSC-targeting agents may be an effective treatment when used in the context of adjuvant therapy (10). Adjuvant therapy is the treatment that is given after an initial treatment for patients with a high risk of relapse. For instance, after removing detectable tumors, oncologists use statistical evidence to assess the risk of tumor relapse to evaluate whether patients need adjuvant treatment. In breast cancer, the objective of adjuvant treatment is to wipe out occult tumor cells so as to reduce the risk of recurrence and death. In breast cancer, adjuvant therapy includes chemotherapy (e.g. doxorubicin, paclitaxel, docetaxel, cyclophosphamide, fluorouracil and methotrexate)(11-13), target

therapy (e.g. Herceptin®)(14), radiotherapy, or hormonal therapy (e.g. tamoxifen)(15). In the current paradigm of adjuvant treatment, chemotherapeutic agents are administrated to eliminate the residual cancer cells and prevent relapse after removal of primary tumors. Since conventional chemotherapy is not designed to target CSCs, chemotherapy may be less effective when used in the adjuvant setting. In contrast, agents that targets CSCs may be more effective when administrated in adjuvant than in the advanced setting (referred as to late-staged tumors) since CSCs are the driving force of tumor recurrence.

Even though the use of anti-CSC agents in combined therapy or adjuvant therapy has been proposed as a better approach to evaluate clinical relevance, there is no experimental evidence to support this concept. Therefore, the proposed study employed a preclinical model to evaluate the efficacy of anti-CSC agent, 17AAG, in the combination with Docetaxel or as a single treatment in adjuvant therapy.

Methods

Cell Lines and Reagents

The SUM159 cell line was maintained in Ham's F12 medium supplemented with 5% fetal bovin serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 1% antibiotic/antimycotic (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B) and 20 ug/ml gentamycin. SUM159 cell line was maintained in culture at 37°C and 10% CO_2. 17AAG and Docetaxel (Taxotere®) was purchased from LC Laboratories (Woburn, MA); Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA); DAPI (4'-6-Diamidino-2-phenylindole) was purchased from Sigma-Aldrich (St. Louis, MO); Matrigel and anti-H2Kd antibody were from BD Biosciences (San Jose, CA).

Xenograft Tumor Model

All experiments involving mice were approved by the University Committee on the Use and Care of Animals at the University of Michigan. To evaluate the effect of 17AAG treatment on tumor growth in vivo, we used a primary human breast cancer xenograft generated from a patient (MC1). Injections of 10^5 MC1 cells mixed with Matrigel (50%, v/v) were administered into the fourth mammary fat pads of 4-week-old NOD/SCID mice (Jackson Laboratories, Bar Harbor, MI). In the advanced tumor setting, mice were randomly assigned to one of two groups (n=5, each) at week 2.5, which had similar average tumor sizes, and began treatment. The control group was given the vehicle (1:1 (v/v) mixtures of DMSO and ethanol) and the

treatment group received 5 mg/kg 17AAG three times a week for three weeks. Mice in the adjuvant therapy began treatment one day after tumor inoculation. The control and treatment group were administrated the vehicle and 5 mg/kg 17AAG, respectively, three times a week for three weeks. The tumor size was measured using a caliper twice a week and the tumor volume was calculated by the following formula:

$$\text{Tumor volume} = 1/2\ (\text{length} \times \text{width}^2)$$

Tumor Tissue Dissociation

At the end of treatment, tumors were collected from mice and enzymatically dissociated to obtain single cell suspensions, as previously described (16). In brief, tumors were minced finely with scalpels and incubated in 10% collengenase/hyaluronidase (StemCell Techonologies, Vancouver, BC) in Medium 199 at 37 °C for 30 minutes. Single cell suspensions were obtained by filtering the digested tumor through a 40 μm filter (BD Biosciencies, San Jose, CA). Single cell suspensions were centrifuged at 1500 rpm at 4°C, and pellets were resuspended in PBS for the Aldefluor assay and tumor re-implantation.

Aldefluor Assay and Flow Cytometry Analysis

The Aldefluor assay was carried out according to the manufacturer's protocol (Stemcell Technologies, Vancouver, BC). In brief, an Aldehyde Dehydrogenase 1 (ALDH1) substrate bodipyaminoacetaldehyde (BAAA) was added to a single cell suspension obtained from tumor tissue dissociation, at a concentration of 1.5 uM, which was then incubated for 40 min at 37°C. A portion of dissociated cells was

incubated with a 10-fold molar excess of an ALDH1 enzyme inhibitor, diethylamino benzaldehyde (DEAB) in addition to BAAA. In the presence of DEAB, the ALDH1 enzyme is inhibited from metabolizing BAAA and therefore functions as a negative control. At the end of BAAA-staining, cells were washed once with HBSS containing 2% fetal bovine serum. Subsequently, cells were stained with 1 ug/mL DAPI to exclude non-viable cells. Flow cytometry was performed at the University of Michigan Cancer Center Flow Cytometry Core. Data analysis was performed with the software program WEASEL (Walter and Eliza Hall Institute of Medical Research).

Lentivirus infection

A highly efficient lentiviral expression system (pLentiLox 3.7; http://www.med.umich.edu/vcore/) from the UM Vector Core Facility was used to generate luciferase-expressing SUM159 cells. The cell lines were transfected with the lentiviruses as described previously. In brief, 70% of confluent SUM159 cells were incubated with letiviral particles encoding luciferase overnight. On the following day, the cells were replaced with fresh medium. To verify the expression of luciferase, transfected SUM159 cells were plated in a 96-well plate in a series of dilutions from 500 to 500,000 cells/well, increasing by one-fold after each well. After cell settled, 2 μL D-luciferin 0.0003% (Promega) was added in the culture medium and the counting photon flux was measure by device camera system (Xenogen).

Bioluminescence imaging

Bioluminescence detection procedures were performed as previously described (17). In brief, mice were anesthetized with a 2% isofluorane/air mixture and given a single i.p. dose of 150 mg/kg D-luciferin (Promega) in PBS. A charge-coupled device camera system (Xenogen), equipped with a nose-cone isofluorane delivery system and heated stage for maintaining body temperature, was used to measure photo flux. The images were captured after 5 seconds of exposure and analyzed using Living Image software provided with the Xenogen imaging system. Normalized photon flux represents the ratio of the photon flux detected each week after inoculations and the photon flux detected before injection of tumor cells.

Statistical Analysis

The Student t-test was used to perform statistical analysis. Data are presented as the mean ± SD ($n \geq 3$). All p values are two-tailed.

RESULT

17AAG exhibited pronounced inhibitory effect on the tumor progression and CSCs in adjuvant setting

To demonstrate that the efficacy of CSC-targeting agent, such as 17AAG, on tumor progression may be more pronounced when given during the early development of tumor (a.k.a. adjuvant treatment), two xenograft models were established to represent the adjuvant and advanced treatments. In the adjuvant therapy, 17AAG treatment was initiated one day after MC1 tumor cells were injected to mice. In contrast, drug treatment in the advanced therapy did not start until palpable tumors had developed.

Mice were treated with 5mg/kg 17AAG or the vehicle three times a week for three weeks by i.p. and the tumor growth was measured weekly. By six weeks, a very effective inhibition in the tumor volume, by more than 95%, was observed in the adjuvant treatment setting (Figure 1A and 1B) in contrast to 60% reduction in the tumor volume in the advanced setting (Figure 1C and 1D) (n=5).

At week six, tumors were collected from the control and treated mice and subjected to quantification of CSCs in tumors upon treatment using Aldefluor assay. 17AAG treatments reduced the number of CSCs in drug-treated tumors by 61% as compared to vehicle-treated in the advanced setting (n=5 for each) (Figure 2A). Similarly, a 62% reduction in the number of CSCs in 17AAG-treated tumors was observed as compared to the vehicle-treated in the adjuvant setting (n=5 and 3 for vehicle- and drug-treated groups, respectively) (Figure 2B).

Interestingly, the CSC population in the adjuvant setting did not increase even after stop the drug treatment for three weeks. Unlike conventional chemotherapeutic agents that cause an expansion of CSCs, 17AAG did not increase the pool of CSCs even three weeks after the treatment stops (7, 18). Taken together these studies indicated that the tumor response to 17AAG was more pronounced when the drug was given in the early-stage tumors than in the advanced tumors.

17AAG extend tumor-free period

To further verify that 17AAG used as an adjuvant therapy can prevent tumor relapse, an *in vivo* imaging was employed to monitor tumor initiation. SUM159 breast cancer cell line was transfected with a lentiviral luciferase reporter system. Injections of 50,000 SUM159-luciferase cells were administered into the fat pad of NOD/SCID mice. One day after tumor injection, mice were treated with 5 mg/kg 17AAG or vehicle three times a week for three weeks. The tumor progression was monitored once a week by bioluminescent imaging.

Before treatment, mice were randomly assigned into two groups (e.g. control- and treatment- groups) to obtain a similar average bioluminescent signal. Two mice in the treatment group died in the first two week due to an accident. Two weeks after treatment stop (at week five), the tumor cells developed palpable tumors in the vehicle-treated mice whereas tumor cells gradually died out in the drug-treated mice (Figure 3A). In the period of 10 weeks, all mice treated with the vehicle developed tumors with a rapid growth curve. In contrast, only one out of

three mice in the drug-treated group formed tumors, which did not grow as rapidly as the control.

To quantify the residual CSCs in tumors, tumors derived from the vehicle-treated and the drug-treated mice were collected at the end of week 10 and subjected to Aldefluor assay and flow cytometry. Only 0.59% of cells were Aldefluor-positive in the drug-treated tumor (n=1), while 1.67% of cells were Aldefluor-positive in vehicle-treated tumors (n=5), which equals to a 62% reduction in Aldefluor-positive cells.

Combinatory therapy of 17AAG and Docetaxel prevented tumor recurrence

To evaluate the extent to which co-administration of CSC-targeting and cytotoxic chemotherapy may inhibit recurrence, mice bearing SUM159-luciferase tumors were treated with vehicle alone, 17AAG alone, Docetaxel alone ,or 17AAG and Docetaxel. One day after inoculated with 50,000 SUM159-luciferase cells, mice were randomly divided into four groups (n=5) to achieve similar average of photo flux and treated with either vehicle, 5 mg/kg 17AAG, 10 mg/kg Docetaxel or 5 mg/kg 17AAG plus 10 mg/kg Docetaxel every day for a week. At week three, tumor cells in control mice began growing aggressively, whereas those in other three groups (treated with 17AAG, Docetaxel, or 17AAG plus Docetaxel) did not (Figure 4A). Tumor cells in Docetaxel treated mice did not progress until six week later. Interestingly, tumor cells in 17AAG treated mice or 17AAG plus Docetaxel remained the same over eight weeks. Both 17AAG-treated and 17AAG plus Docetaxel-treated

groups showed significant tumor response as compared to the control group. However, as compared to 17AAG alone and combined with Docetaxel, there is no significant difference in the sense of average photon flux. Regardless, 50% of mice in the combined treatment of 17AAG and Docetaxel had no bioluminescent signals, indicating that tumor cells perished (Figure 4B). This finding suggests that targeting CSCs by 17AAG is able to constrain tumor progression. Looking at 17AAG alone and in combination with Docetaxel, no significant difference was found. The finding is in agreement with our current understanding that in the adjuvant setting where there is little the bulk of cells, targeting CSC by 17AAG is effective in suppressing tumor progression.

Discussion

The CSC hypothesis has shown the clinical discordance between tumor response and long-term survival for many cancers. Specifically in solid tumor, the efficacy of antitumor therapy is usually determined by mainly evaluating tumor shrinkage based on Response Evaluation Criteria in Solid Tumors (RECIST). RECIST defines a minimum of 30% and maximum of 100% shrinkage in tumor size to be considered a partial and complete response, respectively (19). Tumor size increases of 20% compared to the size before treatment are considered as progressive disease. Under RECIST, the assumption is that tumor reduction would result in clinical benefit. However, achieving tumor responses may not be associated with improvements in the duration of overall survival, due to tumor recurrence. For example, a number of large studies in patients with pancreatic or metastatic breast cancers show that the improved tumor response by the optimized combination chemotherapy does not necessarily extend the overall survival period (20, 21). The CSC model suggests that CSCs are cells that initiates tumors, so tumor re-growth following treatment implies that CSCs persist. Along these lines, a number of recent studies in preclinical and clinical settings show that CSCs are relatively resistant to chemotherapy as compared to the bulk of tumor cell, and therefore tumors become enriched with CSCs after chemotherapy. In fact, colorectal and pancreatic tumor xenografts have been found to be enriched with CSCs following chemotherapy (22, 23). In a clinical study involving patients with breast cancer, cells expressing CSC markers were increased after chemotherapy (7). The intrinsic properties of CSCs,

such as quiescence, overexpression of transporters, and high efficiency of repairing cytotoxic damages are thought to confer the resistance to chemotherapy (24). These evidences augment the notion that clinical outcome (e.g. disease-free survival or overall survival) is associated with the amount of CSCs that remain in patients, instead of the tumor response. For this, much effort has been made to develop therapies that can target CSCs.

Despite advances in developing anti-CSC therapies, reliable endpoints to assess the clinical efficacy of these agents are still unavailable. Tumor regression, the current standard for the clinical endpoint, is inadequate to assess the efficacy of anti-CSC agents because CSCs constitute only a small portion of cells within a solid tumor. RECIST is a good indicator of assessing cytotoxic chemotherapies that are designed to target differentiated cells. Because tumors are mainly comprised of differentiated cells, the effective targeting of these cells is reflected by tumor reduction. In contrast, the efficacy of agents targeting CSCs would be underestimated by merely measuring tumor size as an endpoint because CSCs represent only a small subset of tumor cells. Even if CSCs are completely eliminated, the size of tumors may not be greatly influenced. Thus, challenges to assess the therapeutic efficacy of drugs on breast CSCs in patients still remain. Since the readout of eliminating CSCs may not necessarily translate into rapid tumor reduction, the frequency of tumor recurrence may be the most informative endpoint. However, this endpoint may not be feasible in a Phase II clinical trial because it requires long-term long follow-up and a large pool of patients.

To overcome the issue stated above, Lu et al. proposed an alternative to evaluate anti-CSC agents in the adjuvant setting when drugs are given right after the removal of primary tumors (25). Tumor relapse as an endpoint for CSC-targeting agents may be more appropriate because the relapse mainly reflects the tumor-initiating ability of the residual CSCs. In our study, even though a similar reduction of CSCs (60%) by 17AAG treatment was achieved in both early-staged tumors (adjuvant setting) and advanced tumors, the rate of tumor growth in the advanced setting was more rapid than that in the adjuvant setting. The observation in this comparative study clearly supports the notion proposed by Lu et al. that the adjuvant setting may a better model for assessing the efficacy of anti-CSC agents when used as a single therapy. However, the evidence obtained in our study augments their proposed approach. As shown in our data, 17AAG had limited effect in repressing tumor growth, despite its effect on CSCs, because the abundant differentiated cells still continued to proliferate. However, the use of 17AAG in the adjuvant setting still hampered tumor growth greatly, although it did not completely stop the tumor from growing in this case. Because 17AAG is expected to have little effect on differentiated cells at the concentrations used in our study, a possible explanation is that the substantial number of remaining differentiated cells was able to proliferate for several cycles and thus contribute to the tumor growth. Nevertheless, CSC-targeting agents given in early-staged tumors, which resemble adjuvant therapy, display better efficacy in suppressing tumor progression.

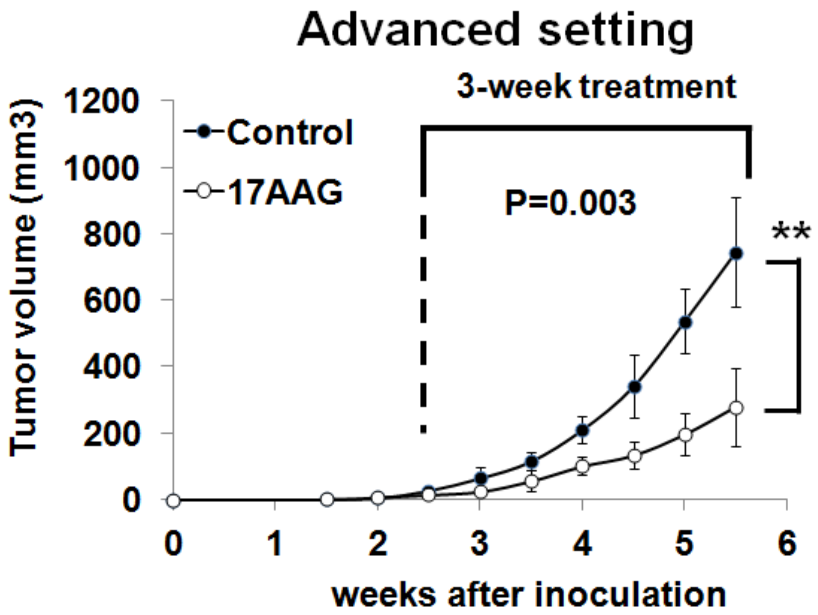
Elimination of both CSCs and differentiated cells is essential in order to achieve the maximal clinical benefit. Even if CSCs are completely wiped out, the majority of remaining cells are differentiated cells, which can proliferate for several cycles and contribute to tumor progression. Therefore, targeting differentiated cells by a cytotoxic chemotherapy to alleviate tumor burden and eliminating CSCs by a CSC-targeting agent to prevent tumor occurrence may be the optimal therapy. The results from combination therapy of 17AAG and Docetaxel in our current study bolster the concept. Mice receiving Docetaxel developed palpable tumors, as assessed by the bioluminescent signal, six weeks post treatment, whereas mice receiving 17AAG or 17AAG and Docetaxel did not. Tumor re-growth in the Docetaxel treated mice may be due to resistant CSCs, which has been identified in a previous clinical study (7). In contrast, co-treatment of 17AAG and Docetaxel completely abolished tumor recurrence. The findings of our study verify the notion that elimination of both CSCs and differentiated cells is crucial for effective cancer therapy.

Conclusion

The present study demonstrates that 17AAG is effective at targeting breast CSCs. Importantly, the use of 17AAG alone as adjuvant therapy or in combination with chemotherapy has shown to potentially inhibit tumor recurrence and improve the overall clinical outcome. Altogether, evidence suggests that 17AAG warrants further clinical evaluation as a CSCs-targeting agent.

Figures

A



B

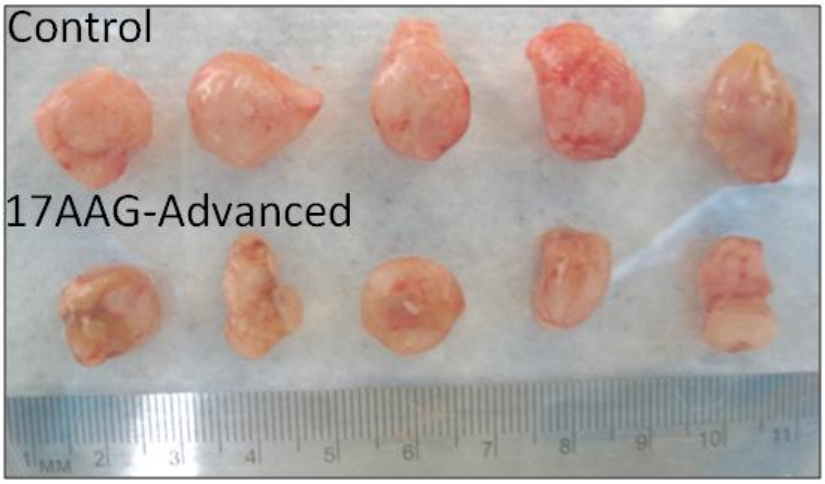
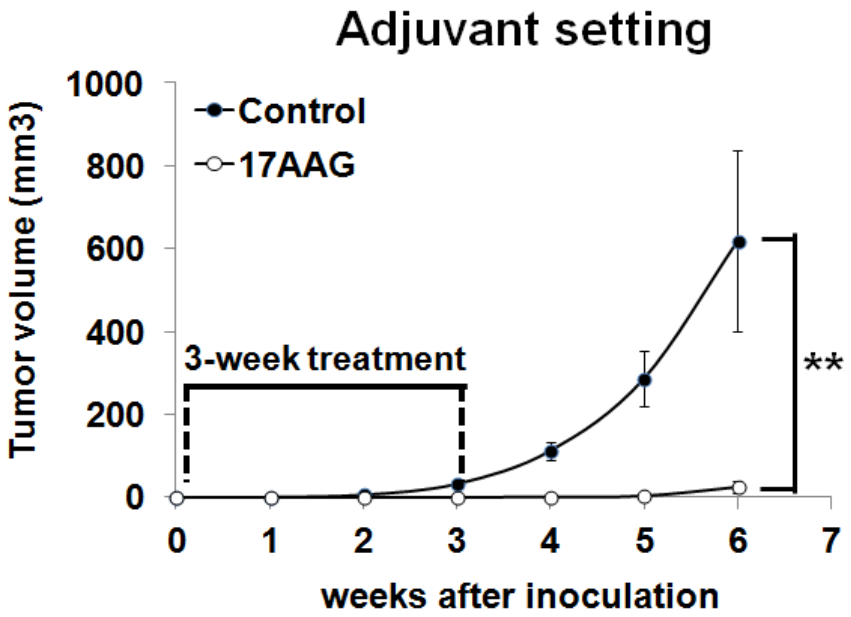


Figure 4.1 Efficacy of 17AAG as given in the advanced setting.

A



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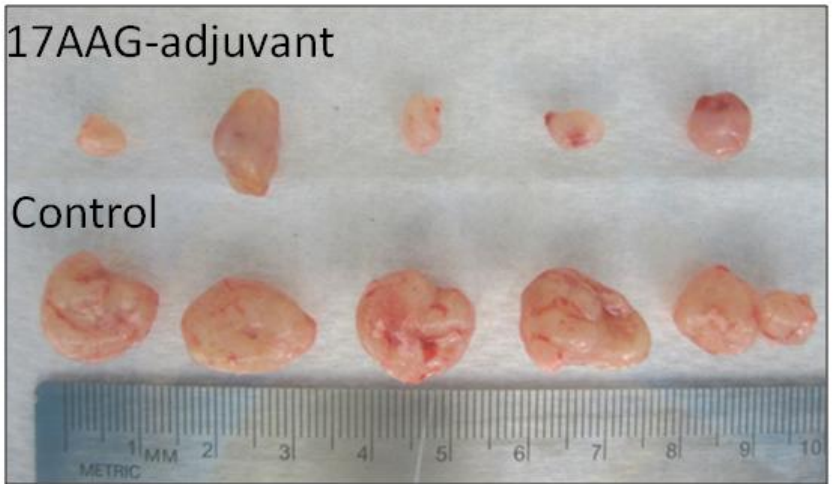
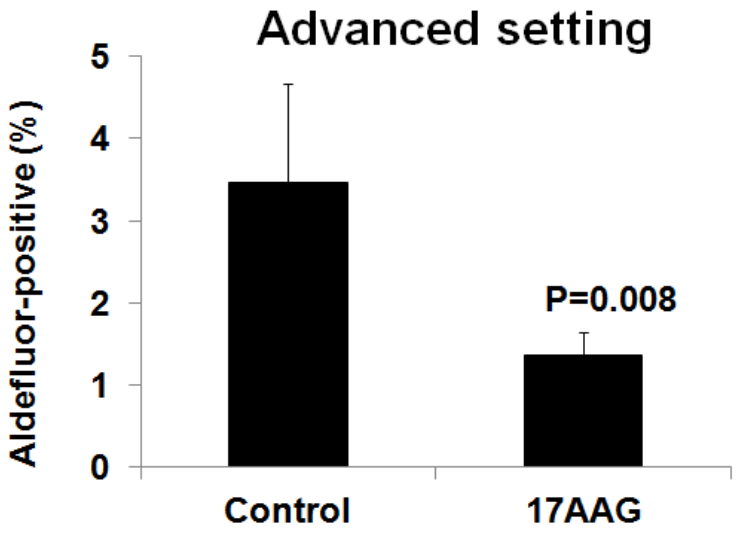


Figure 4.2 Efficacy of 17AAG as given in the adjuvant setting.

A.



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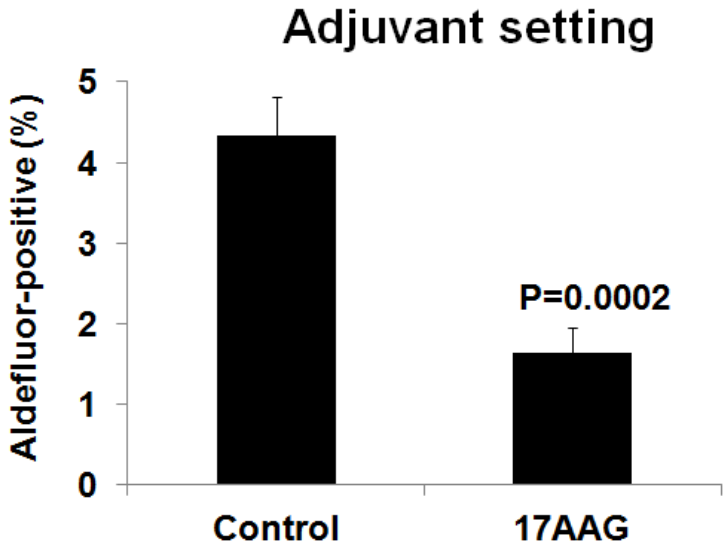

Figure 4.3 Effect of 17AAG on reducing Aldefluor-positive cells in advanced and adjuvant setting.

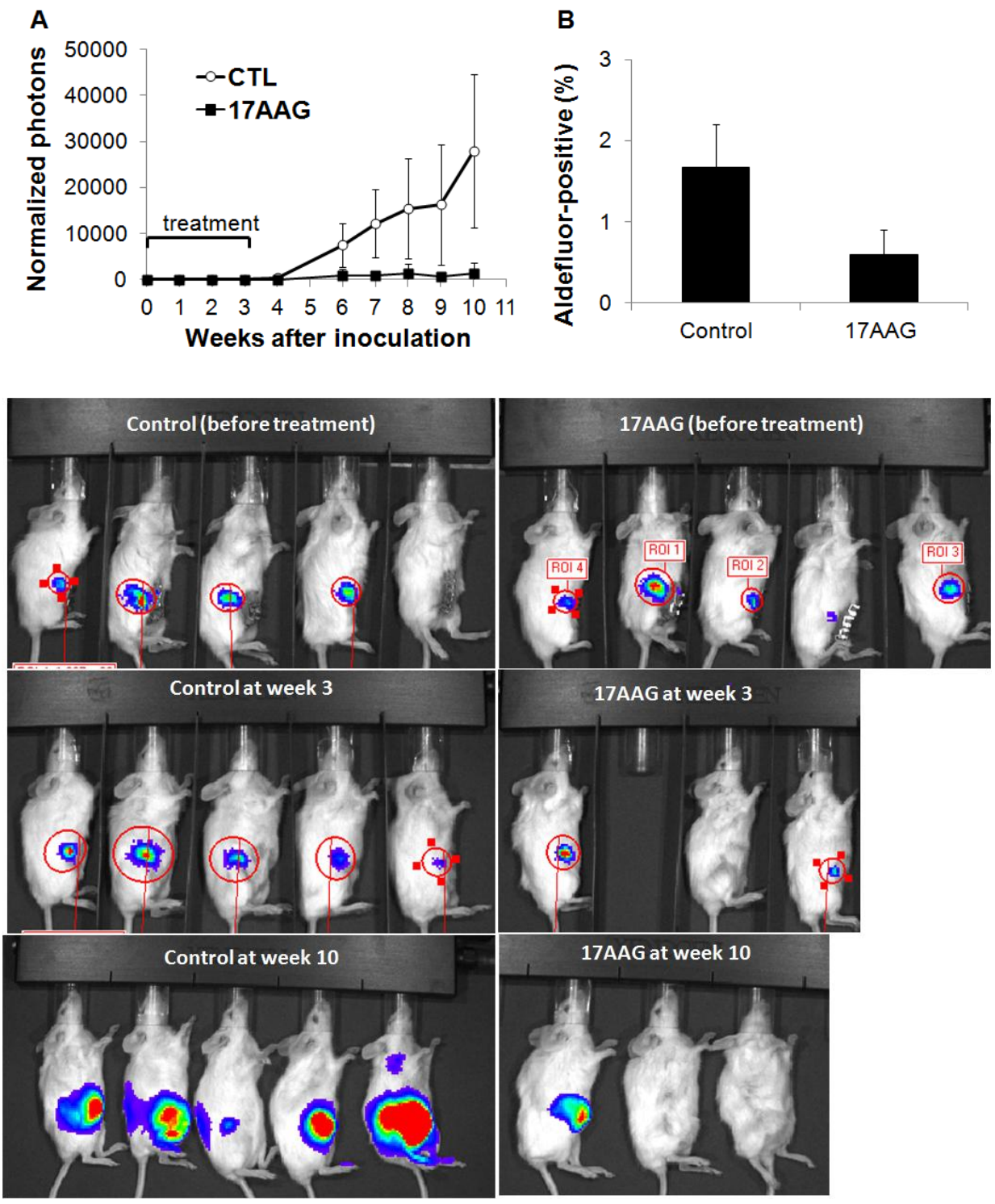

Figure 4.4 *In vivo* imaging of tumor development in response to 17AAG in the adjuvant setting.

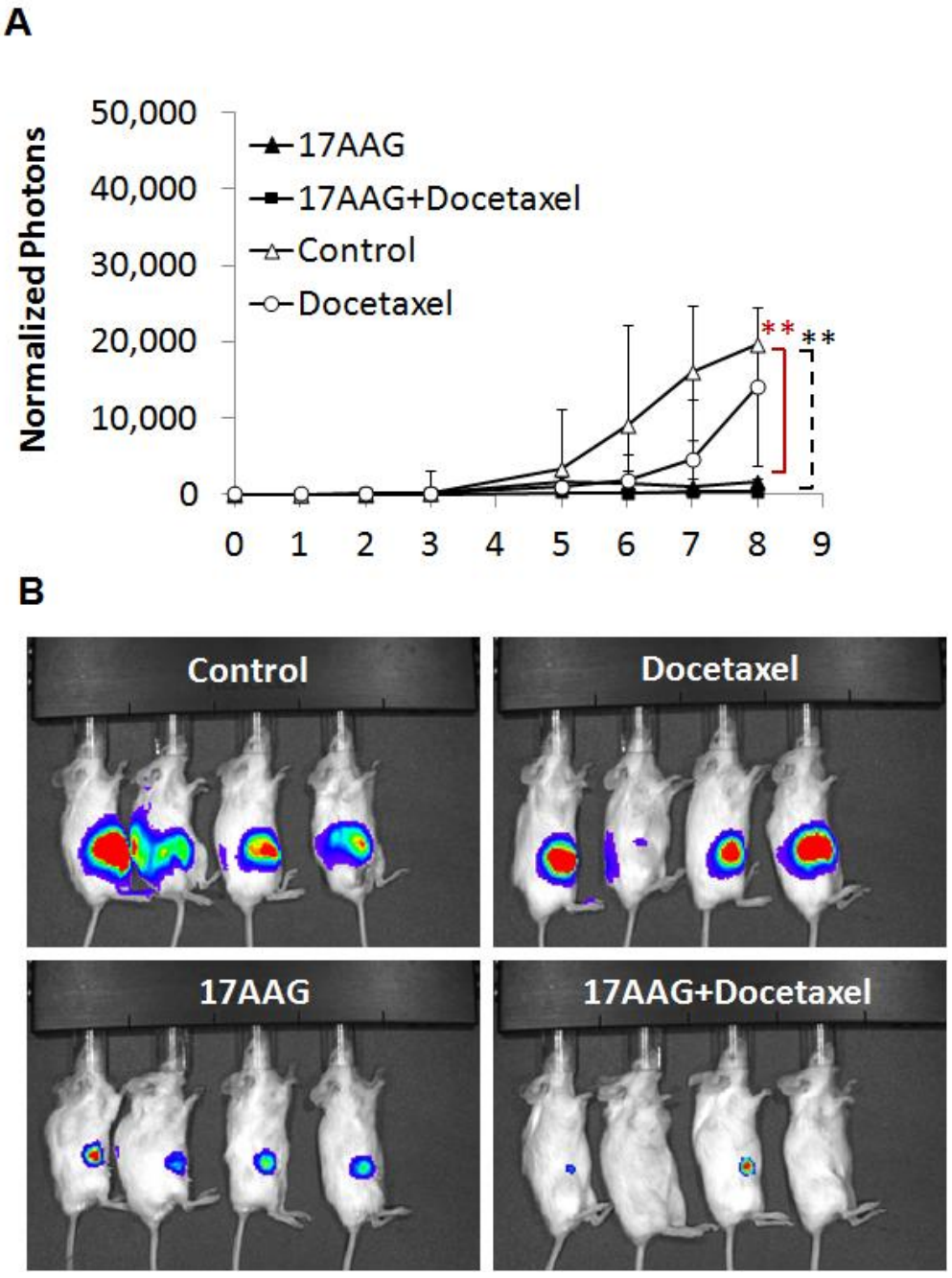

Figure 4.5 Efficacy of combination therapy of 17AAG and Docetaxel.

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Chapter 5

HDAC inhibitor SAHA targets Breast Cancer Stem Cells

Abstract

The cancer stem cell (CSC) model suggests that CSCs confer chemo-resistance, tumor relapse and metastasis. Therefore, effective targeting of CSCs may provide a cure for cancer. Emerging evidence has suggested that epigenetic modification plays an important role in the function of normal and cancer stem cells. Therefore, we investigated the effect of HDAC inhibition by SAHA on breast CSCs.

SAHA reduced the number of cells expressing stem cell markers such as ALDH1 by 90% in SUM159 cell lines. Data from tumor sphere formation assay showed that as little as 0.5μM SAHA was able to inhibit tumorsphere formation efficiency by 77%. Of note, in the absence of drug, the sustained reductions (~80%) were observed in second and third passages, suggesting the impaired self-renewal capability by SAHA is maintained through several passages. The efficacy of SAHA in inhibiting CSCs was further verified in mouse xenografts bearing SUM159 or primary MC1 tumors. SAHA significantly inhibited tumor growth by 45% and 84% in SUM159 and MC1 tumor xenografts, respectively, compared to vehicle-treated groups. Aldefluor-positive cells in SUM159 and MC1 tumors were reduced by 50-

90% after a three-week treatment of SAHA. Tumor re-implantation showed that SAHA greatly abolished the tumor engraftment ability of cells derived from drug-treated mice in secondary mice. This suggested that SAHA was not only able to diminish the CSC population but also to damage the self-renewal ability of residual cells.

Here we demonstrated that SAHA is able to induce depletion of Aldefluor-positive cells and the impairment of their self-renewal potential, mediated through deregulation of two important self-renewal associated genes, Bmi-1 and c-Myc. Our findings have important implications for the utility of SAHA in treating breast cancer.

Introduction

Emerging evidence suggests that breast cancers originate from a small subset of cancer stem cells (CSCs). CSCs were first discovery in acute myeloid leukemia. Since then, they have been indentified in a wide variety of solid tumors including breast(1, 2), lung (3, 4), liver(5, 6), pancreas (7-10), prostate (11, 12),colon (13) and brain(14).

CSCs are endowed with distinctive attributes of stem cells, such as the self-renewal capability and differentiation into multiple cell types. These stem-like properties enable CSCs to generate tumor through self-renewal and differentiation into heterogeneous cell populations. Therefore, they have been strongly associated with relapse, metastasis, and resistance of tumors to chemotherapy (15, 16). Conventional chemotherapies, which target bulk tumor population, can cause tumor regression, but fail to eradicate CSCs. As a result, the remaining CSCs regenerate tumors through self-renewal, which contribute to tumor relapse and apparent resistance to chemotherapy (16-18). The lack of effective treatments against tumor recurrence and metastasis mediated by CSCs clearly demonstrates a need for new therapeutic approaches to target this population (19).

Dysregulation of epigenetic modification by histone acetylation is thought to be a key component of tumor initiation, progression, and differentiation (17). A hallmark of cancer cells is aberrant epigenetic regulation of gene expression, which contributes to tumor initiation and progression. A plethora of studies in hematologic and solid tumors have shown that cancer cells silence tumor suppressor genes to

gain selective growth and invasive advantage. Silencing of the tumor suppressor genes is thought to be mediated by the dysregulation of epigenetic modification by histone acetylation. In addition, epigenetic modifications are important regulators of stem cell function and self-renewal. Histone deacetylases (HDACs) and histone acetyl transferases (HATs) are two enzymes that help maintain homeostasis of histone acetylation.

Epigenetic agents that target HDAC have been extensively studied *in vitro* for their anti-cancer effect. HDAC inhibitors have been shown to have pro-apoptotic and anti-proliferative effect on breast cancer cells. SAHA, also known as Vorinostat, is a pan-HDAC inhibitor that has recently been approved by the FDA for the treatment of cutaneous T cell lymphoma. Several other HDAC inhibitors are currently in various stages of clinical development. Thus, HDACs are clearly important therapeutic targets for treatment of cancers. However, despite widespread acceptance of HDAC as a therapeutic target, the mechanisms of how HDAC inhibition controls cancer growth is not fully understood. In addition, the effect of HDAC inhibitors on breast CSCs is still unknown. Therefore, further investigations on HDAC inhibition is still needed to fully understand the effect and the therapeutic implications of HDAC inhibition for the treatment of cancers.

We thus investigated the effect of SAHA mediated HDAC inhibition on breast cancer stem cells *in vitro* and *in vivo*, and the underlying mechanisms for the observed effects. Our findings showed that HDAC 1, 2 and 3 are highly expressed in the ALDH-positive cells, which are rich in breast CSCs, as compared to ALDH-negative cells. The selective impairment of self-renewal capability of CSCs in

response to treatment with SAHA was demonstrated *in vitro* using tumorsphere formation assay in ALDH-positive cells and *in vivo* using SUM159 and primary MC1 tumor xenografts. We also found data to suggest SAHA targets CSCs and inhibit their self-renewal capability through the dysregulation of stemness-associated genes, c-Myc and BMI-1.

To our knowledge, this is the first time to demonstrate that HDAC inhibition suppresses breast CSCs. Also, our findings support previous studies in other cancer models that demonstrate MYC and BMI-1 are essential for the maintenance of stemness in CSCs. Our findings provide important insight in the context of gene expression profiles in ALDH-negative and ALDH-positive population and the alternation of gene expression in response to SAHA.

Methods

Cell Lines and Reagents

The SUM159 cell line was maintained in Ham's F12 medium supplemented with 5% fetal bovine serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 1% antibiotic/antimycotic (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, 25μg/ml amphotericin B) and 20μg/ml gentamycin. The MCF-7 cell line was maintained in RPMI medium supplemented with 10% fetal bovine serum, 5 μg/ml insulin, and 1% antibiotic-antimycotic. Both cell lines were maintained in culture at 37°C and 10% CO_2. SAHA was purchased from Cayman Chemical (Ann Arbor,MI); Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA); BIO was obtained from EMD Biosciences (San Diego, CA); DAPI (4'-6-Diamidino-2-phenylindole) was purchased from Sigma-Aldrich (St. Louis, MO); Matrigel and anti-H2Kd antibody were purchased from BD Biosciences (San Jose, CA). Antibodies against; Antibodies against BMI-1 was purchased from Upstate (Billerica, MA); ALDH1 antibody was obtained from BD Transduction Laboratory (Franklin Lakes, NJ); Antibodies against CD44 and CD24 were purchased from BD Biosciences (San Jose, CA).

MTS Cell Proliferation Assay

The SUM159 cell line was seeded into 96 well microplates at a density of 5,000 cells per well. Cells were treated with increasing concentrations of SAHA (0.0001–10μM). After 72 hrs, cell viability was assessed by the MTS cell

proliferation assay (Promega, Madison, WI) according to the manufacturer's instruction. Cellular IC50 was shown to be the concentration necessary to decrease viability to 50% of control untreated cells.

Aldefluor Assay, CD44/CD24 Staining and Flow Cytometry Analysis.

A cell population with elevated aldehyde dehydrogenase (ALDH) enzyme activity was reported to enrich in cancer stem/progenitor cells. The Aldefluor assay, used to detect ALDH enzyme activity, was carried out according to the manufacturer's protocol (Stemcell Technologies, Vancouver, BC). For both *in vivo* and *in vitro* studies, the aldehyde dehydrogenase (ALDH) substrate bodipyaminoacetaldehyde (BAAA) was added to the single cell suspension at a concentration of 1.5μM, which was then incubated for 40 min at 37°C. Additionally, a negative control was prepared by adding diethylamino benzaldehyde (DEAB) into a portion of cell suspension containing BAAA. DEAB, ALDH enzyme inhibitor, prevented BAAA from being metabolized into a fluorescent product. Staining for surface markers (CD44 and CD24) was performed according to the manufacturer's protocol (20). Briefly, approximately 10^6 single MCF-7 cells were resuspended in HBSS supplemented with 2% BSA, mouse monoclonal anti-CD44-APC, and anti-CD24-PE, incubated for 20 minutes at 4°C. After BAAA or anti-CD44/CD24 staining, the cells were then washed twice with 10 mL of HBSS containing 2% BSA and resuspended with HBSS supplemented with 2% BSA and DAPI (1μg/mL), and analyzed using the flow cytometry at the University of Michigan Cancer Center Flow Cytometry Core. DAPI was used to exclude non-viable cells. Data analysis was

performed with the software program Weasel (Walter and Eliza Hall Institute of Medical Research).

Tumorsphere Formation

Single SUM159 Aldefluor-positive cells, assessed with Aldefluor assay, were plated onto ultralow-attachment 96 well plates (Corning, Corning, NY) at a density of one cell per well. Tumorspheres were cultured for 7 days in a serum-free mammary epithelial basal medium (MEBM) (Cambrex Bio Science Walkersville, Inc,) supplemented with B27 (Invitrogen, Carlsbad, CA,), 20ng/mL EGF (BD Biosciences, San Jose, CA), 1% antibiotic-antimycotic (100unit/ml penicillin G sodium, 100µg/ml streptomycin sulfate and 0.25µg/ml amphotericin B), 20µg /ml Gentamycin, 1ng/ml Hydrocortisone, 5µg/ml Insulin, and 100µM beta-mercaptoethanol (Gibco Invitrogen, Carlsbad, CA) in a humidified incubator (10% CO_2, 37°C). Primary tumorspheres were collected, and mechanically and enzymatically dissociated into single cell suspensions for subsequent passages. Single cell suspensions were plated onto ultralow-attachment 96 well plates at a low cellular density of less than 5 cells per well, and cultured in the absence of drug treatment. Tumorsphere formation efficiency was denoted as the ratio of the number of tumorspheres to the number of seeded cells.

Tumor Tissue Dissociation

A portion of each tumor was fixed in 10% buffered formalin and paraffin embedded for immunohistochemical staining. The remaining tissue was

mechanically and enzymatically dissociated to obtain single cell suspensions, as previously described (20). In brief, tumors were minced finely with scalpels and incubated in 10% collengenase/hyaluronidase (StemCell Technologies, Vancouver, BC) in Medium 199 at 37 °C for 30 minutes. Single cell suspensions were obtained by filtering the digested tumor through a 40μm filter (BD Biosciencies, San Jose, CA). Single cell suspensions were centrifuged at 1500 rpm at 4°C, and pellets were resuspended in PBS for the Aldefluor assay and tumor reimplantation.

Xenograft Tumor Model and Reimplantation

All experiments involving mice were approved by the University Committee on the Use and Care of Animals at the University of Michigan. To evaluate the effect of SAHA treatment on tumor growth *in vivo*, we used SUM159 cells and a primary human breast cancer xenograft generated from a patient (MC1). SUM159 or MC1 cells mixed with Matrigel (50%, v/v) were injected into the fourth mammary fat pads of 4-week-old NOD/SCID mice (Jackson Laboratories, Bar Harbor, MI). When palpable tumors formed, mice were divided into two groups with the similar average tumor size. The control and treatment group were administrated the vehicle (1:1 mixture of DMSO and ethanol) or SAHA, respectively, three times a week for three weeks. Tumor re-implantation was conducted as previously described (20, 21). In brief, single cell suspensions were stained with DAPI for viability assessment. The recovered live cells were stained with an anti-H2Kd antibody to distinguish breast cancer cells from mouse cells. Viable SUM159 or MC1 cells derived from primary tumors treated with SAHA or the vehicle were injected

into the fourth inguinal mammary fat pad of secondary NOD/SCID mice ($n \geq 3$ for each condition). The tumor growth was measured weekly.

Gene expression profiling with DNA microarrays and analysis

The microarray experiments were conducted using RNA extracts from SUM159 Aldefluor-negative cells and Aldefluor-positive cells (untreated and treated with SAHA) using RNeasy Mini kits, according to the manufacture's instruction (Qiagen). The integrity of rRNA was checked using agarose gel electrophoresis. cDNA was synthesized using oligo-dT primers and Superscript II Reverse Transcriptase(Invitrogen), according to the manufacturer's instructions. The hybridizations were performed using Affymetrix human U133 Plus 2.0 in UM Microarray core facilities. The microarray data (.cel files) was normalized by RMA (Robust Multichip Average) [z1] and subsequently analyzed by the dChip software [z2]. To assess the modulation of MYC activity by SAHA, 247 probesets, representing 192 unique genes, were selected based on the MYC-activation signature. Hierarchical clustering was performed with the average linkage method. Rows were standardized by subtracting the mean and divided by the standard deviation of all samples. Correlation was used as the distance metric. The MYC activation score was defined as the difference in the mean log fold changes between the expected up-regulated genes and the expected down-regulated genes. A positive value indicated activation, and negative one inactivation.

To gain insight into the regulation of gene expression via HDAC activity in Aldefluor-positive and Aldefluor-negative cells, HDAC's target genes were compiled

from multiple CHIP-seq or CHIP-chip experiments. A list of 1000 randomly selected genes was prepared as a control. Observed and expected numbers of up- and down-regulated genes mediated by HDACs in Aldefluor-positive cells were compared against that for Aldefluor-negative cells. Chi-square test was used to assess the difference in observed and expected values. The ratio was calculated as following,

$$\text{Ratio} = (N_{\text{up genes}} / N_{\text{down genes}}) / (N_{\text{exp up genes}} / N_{\text{exp down genes}})$$

A ratio >1 indicated activation and <1 indicated inactivation of HDAC target genes.

Real-Time PCR

The amplification reactions were run at least in triplicate. Reactions were performed in MicroAmp 96 well plates (Applied Biosystems) and contained 5 µl DNA, 3 µl of primers mix (final primer concentration of 300 nM each), and 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), in a final volume of 25 µl. Amplifications were carried out in an ABI PRISM® 7900HT Fast Real-Time. A final dissociation curve was always obtained for each quantitative run. Each sample was tested in triplicated and GAPDH was used as an internal control. Primers for c-MYC, p21, GAPDH, BMI-1, and ALDH1A1 are listed in Table 5.1. The Real-Time PCR data was analyzed using = $2^{-\Delta\Delta Ct}$ relative quantization method following the manufacturer's instructions.

Western Blotting

SUM159 tumorsphere were treated with SAHA for 2 days under non-adherent conditions. Tumorspheres were harvested and lysed in RIPA buffer (PBS,

1% Nonidet P-40, 0.5% deoxycholate, 1% SDS, 1mM sodium orthovanadate, 0.5mM PMSF, 10mg/ml aprotinin, and 20 mg/ml leupeptin) with a protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN) and a phosphotase inhibitor (Cell Signaling Technology, Danvers, MA) for 30 minutes. Supernatant was collected by centrifugation and subjected to protein quantification with BCA protein assay reagents (Pierce, Rockford, IL). Equal amounts of proteins were separated on a Tris-glycine 4-10% gradient precast gel (Bio-Rad, Hercules, CA), transferred to a PVDF membrane, and then blocked with 5% BSA. The Immobilized proteins were probed with c-MYC and BMI-1 antibodies.

Statistical Analysis

The Student t-test was used to perform statistical analysis. Data are presented as the mean ± 95% CI ($n \geq 3$). All p values are two-tailed.

Results

Transcriptional activity of HDAC1, 2 and 3 are highly active in ALDH-positive cells.

To evaluate whether breast CSCs have higher HDACs activities than non-CSCs, we performed computational assessment on the expression profiles of the target genes of HDACs in ALDH-positive and ALDH-negative cells. Numerous studies have reported an enhanced expression of HDACs at mRNA and protein levels in solid tumors from stomach, esophagus, colon, breast, ovaries, lung, pancreas and thyroid (22-24) as compared to the respective tissues of origin. The analysis indicated that the target genes of HDAC1, 2 and 3 are significantly up-regulated in ALDH-positive cells as compared to ALDH-negative cells (Table 5.2). No significant difference in the target genes of the rest of HDAC family members was observed between the Aldefluor-positive and negative populations. This indicates that genes regulated by HDAC1, 2 and 3 may be associated with the maintenance of ALDH-positive cells.

HDAC inhibition by SAHA suppressed the CSC population.

To evaluate whether HDAC inhibition diminishes the amount of CSCs, we examined the changes of the Aldefluor-positive cells in SUM159 breast cancer cell line in response to SAHA treatment. Breast cancer stem/progenitor cells have highly active ALDH enzyme activity (20), which can be detected with Aldefluor assay. Therefore, ALDH1activity is commonly used as a marker to identify the

subpopulation of cells that exhibit CSC properties. SUM159 cells were cultured in the presence of 0.5, 1 or 2 μM of SAHA or DMSO for two days and were analyzed using Aldefluor assay and flow cytometry. The data showed that treatment of bulk SUM159 cells with SAHA reduced the proportion of Aldefluor-positive cells in a concentration-dependent manner (Figure 5.1). At a concentration of 1μM, SAHA significantly reduced the proportion of Aldefluor-positive population by 54%, and 2μM SAHA achieved a 79% reduction of Aldefluor-positive cells as compared to DMSO treated cells. Interestingly, Aldefluor-positive cells showed greater susceptibility to SAHA with IC50 of 1 μM, while that of the bulk cancer cells was 2μM (Figure 5.2). At 1 μM concentration, the number of bulk cancer cells was reduced by only 30%. This suggests that Aldefluor-positive cells are sensitive to HDAC inhibition by SAHA.

SAHA inhibited the tumorsphere formation efficiency of Aldefluor-positive cells

To evaluate whether SAHA suppresses CSC self-renewal capability, tumorsphere formation assay was performed using SUM159 breast cancer cells. Aldefluor-positive cells were plated into a 96-well Ultra-low attachment plate at a density of one cell/well and were treated with SAHA under the suspension condition for 7 days. After 7 more days, tumorspheres were observed under a microscope. Subsequently, the tumorspheres were collected, dissociated to a single-cell suspension, and cultured in the same manner in the absence of SAHA for two more passages. In the first passage, a 76% reduction in tumorsphere formation efficiency was observed in cells treated with 0.5μM SAHA as compared to cells

treated with DMSO (Figure 5.3). Of note, the inhibitory effect on the tumorsphere formation efficiency still remained for the second and third passages after removal of SAHA from the culture medium (74% and 75% reduction, respectively, as compared to DMSO treated cells). The data indicates that SAHA is capable of inhibiting self-renewing potential of CSCs *in vitro*.

SAHA diminished the amount of ALDH-positive cells in vivo.

To verify that SAHA could target breast CSCs *in vivo*, we used SUM159 and primary tumor (MC1) mouse xenograft models established in NOD/SCID mice. Two and half weeks after cancer cell inoculation, mice were treated with 25mg/kg SAHA three times per week by i.p. for 3 weeks. At the end of the study, SUM159 and MC1 tumors in SAHA treated mice exhibited smaller volume, by 45% and 84% respectively, as compared to those in vehicle treated mice (Figure 5.4). At the end of treatment, tumors were harvested and the Aldefluor-positive cells remaining in tumors were assessed with Aldefluor assay and flow cytometry. SAHA reduced the proportion of Aldefluor-positive population by 90% in SUM159 tumors and 52% in MC1 tumors (Figure 5.5). The reduction in tumor volume and the proportion of Aldefluor-positive population after treatment with SAHA suggests SAHA can target CSCs *in vivo*.

SAHA impaired tumorigenicity of tumor cells.

To demonstrate that the residual Aldefluor-positive cells after SAHA treatment possess poor tumorigenicity, tumor re-implantation assay was conducted. Two dilutions of single-suspension cells obtained from vehicle- or SAHA-treated primary SUM159 tumors were re-injected into secondary NOD/SCID mice.

The tumorigenic capacity was evaluated based on the onset of tumor formation and the rate of tumor growth. All mice (n=5) receiving 100K of SUM159 cells derived from vehicle-treated mice developed tumors at week 1, whereas those (n=5) receiving the same number of cells from SAHA-treated mice took additional two week to form tumors (Figure 5.6). Similarly, mice injected with 10K cells derived from vehicle-treated mice all formed tumors at week 2. However, those inoculated with the same amount of cells from drug-treated mice did not form tumors until week 5. Of mice receiving 100K or 10K of SAHA-treated SUM159 cells, 20% and 60% remained tumor-free, respectively, at the end of 4 to 5-week monitoring period. In contrast, all mice inoculated with cells from vehicle-treated mice had tumors at both dilutions. Moreover, once the tumor developed, the tumor growth rate of SAHA-treated tumor cells was significantly slower at both dilutions (74mm^3/min and 4mm^3/min for 100K and 10K, respectively) than those of vehicle-treated cells (846 mm^3/week and 160mm^3/min for 100K and 10K, respectively).

Similarly, tumor re-implantation assay was conducted using MC1 cells at two dilutions (20K or 2K cells). Correspondingly to the observations in the SUM159 re-implantation data, MC1 cells from drug-treated tumors had longer time to tumor development and slower tumor growth rate, as compared to those from vehicle-treated tumors. At a dilution of 20K cells, all mice (n=4) injected with vehicle-treated tumors formed tumors at week 3 with an average tumor growth rate of 61mmm3/min, whereas mice (n=4) injected with cells from drug-treated tumors took additional 3 weeks to form tumors. Over a 8-week period, only 50% of mice developed tumors with a tumor growth rate of 2mm3/min (Figure 5.7). Of mice

(n=3) receiving 2K cells from vehicle-treated tumors, all began to develop tumors at week 7 with a growth rate of 55mm^3/week, while only 33% of mice receiving cells from drug-treated tumors began to form tumors at week 10 with a growth rate 1.3mm^3/week. Taken together, the findings show that SAHA treatment impaired the tumorigenicity of the remaining Aldefluor-positive cells.

SAHA reduces the expression level of MYC

To investigate the potential mechanisms underlying breast CSC targeting by SAHA, we conducted a microarray gene expression analyses of four cell groups: SUM159 untreated Aldefluor-negative cells, Aldefluor-positive cells treated with DMSO for 12hours, Aldefluor-positive cells treated with SAHA for 12 hours, and Aldefluor-positive cells treated with SAHA for 24 hours. Comparing Aldefluor-positive and Aldefluor-negative cells, a general trend toward opposite gene expression profile was observed. Genes up-regulated in Aldefluor-positive cells were down-regulated in Aldefluor-negative cells and vice versa. Of genes that have been identified to play a role in the maintenance of CSC properties, CD44, ALDH1A1, and the target genes of MYC were identified show Aldefluor-dependent and treatment-dependent expression patterns. The vast difference in the gene expression profile between Aldefluor-positive and Aldefluor-negative cells reflects the phenotypic difference between CSC population of tumor cells and the differentiated tumor cell population.

To assess the effect of SAHA on MYC activity, a genome-wide microarray analysis of MYC-targets was conducted on four subgroups, Aldefluor-negative group, Aldefluor-positive group exposed to DMSO, Aldefluor-positive group exposed

to SAHA for 12 hours and Aldefluor-positive group exposed to SAHA for 24 hours. Interestingly, the expression of MYC gene signature in the Aldefluor-positive subgroup was significantly different from that in Aldefluor-negative subgroup (Figure 5.8A). However, the MYC-signature genes of the Aldefluor-positive cells became similar to that of Aldefluor-negative cells after treatment with SAHA, which was clearly demonstrated at 12hrs and further more at 24 hr (Figure 5.8A).

To quantitatively evaluate MYC activation, signature activation score was calculated from the microarray results above. Signature activation scores were calculated as the differences in the level of gene expression between genes expected to be elevated and genes expected to be decreased in cells with increased MYC activity. In other words, higher positive score is expected to correlate with higher MYC activity, while lower negative score is expected to correlate with decreased MYC activity. The MYC signature score of Aldefluor-positive subgroup was 40, which was significantly higher than the score of -30 in the Aldefluor-negative subgroup (Figure 5.8B). Upon exposure to SAHA for 12 hours or 24 hours, MYC signature activation score of the Aldefluor-positive cells significantly declined to 0 or -20, respectively. Collectively, the data displays treatment duration-dependent decrease in MYC expression after SAHA treatment.

To confirm the results of the signature activation score, Real-Time PCR of the MYC gene was conducted on the four subgroups. A 2.5-fold higher MYC expression level was observed in Aldefluor-positive cells compared to Aldefluor-negative cells (Figure 5.8C). After a 12 hour treatment with SAHA, the gene expression level of

MYC in Aldefluor-positive cells was reduced to a level close to that of Aldefluor-negative cells. The effect of SAHA on MYC expression appeared to be sustained at 24hours. The reduction of MYC protein in response to SAHA treatment was observed as well (Figure 5.8D). Together, SAHA MYC overexpression in Aldefluor-positive cells was reversed by SAHA treatment.

Treatment with SAHA leads to reduction of BMI-1 expression in Aldefluor-positive cells

Studies in embryonic stem cells and cancer stem cells have shown that Bmi-1 is a crucial component for the maintenance of the self-renewal capability [31, 75, 76-80]. Previous studies have reported that BMI-1, a CSC regulator, was highly regulated in the CSC population To know whether the level of BMI-1 gene is up-regulated in the CSC-like cells, the gene levels in four subgroups: Aldefluor-negative cells, DMSO-treated Aldefluor-negative cells, SAHA-treated Aldefluor-positive cells and SAHA -treated Aldefluor-negative cells were quantified with Real-Time PCR. As compared to Aldefluor-negative cells, the expression of BMI-1 genes was significantly 5-fold higher in Aldefluor-positive cells, which can be down-regulated by 2-3 folds upon SAHA treatment (Figure 5.9). Accompany by a decreased gene expression, the level of BMI-1 protein was down-regulated (Figure 5.8D).

Effect of SAHA on CSC-markers: ALDH1A1 and CD44/CD24

As expected in cells with high ALDH activity, microarray analysis showed ALDH1A1 was significantly up-regulated in Aldefluor-positive cells as compared to Aldefluor-negative cells. The microarray data also showed a reduction of ALDH1A1 expression after treatment with SAHA for 12 hours. These observations were

verified by Real-Time PCR, which showed that the gene expression of ALDH1A1 was 3 fold higher in Aldefluor-positive cells than in Aldefluor-negative cells (Figure 5. 10). Furthermore, a 2-fold reduction in ALDH1A1 expression level was observed in response to treatment with SAHA for 12 hours. The effect was sustained at 24 hours.

In addition, SAHA also affected the expression of CD44 and CD24 genes. Cells with the phenotype $CD44^{+}$/ $CD24^{-}$ have been shown to be enriched with breast CSCs. The expression of CD24 gene was lower in Aldefluor-positive cells, as compared to that in Aldefluor-negative cells. However, upon SAHA treatment, CD24 expression in Aldefluor-positive cells was up-regulated (Figure 5.11). In contrast, the expression of CD44 gene was higher in Aldefluor-positive cells as compared to that in Aldefluor-negative cells. Interestingly, two different responses of CD44 expression genes were observed in response to SAHA treatment. One set of probsets, which included at_212063 and at_229221, indicated that CD44 gene was down-regulated after drug treatment, whereas the other set including five CD44 probsets showed the opposite response. The levels of CD44 and CD24 proteins in response to SAHA were detected using CD44 and CD24 antibodies, followed by flow cytometry. SAHA treatment significantly reduced $CD44^{+}$/ $CD24^{-}$ cells (Figure 5.12A), which was accompanied with increases in $CD44^{-}$/ $CD24^{+}$ cells (Figure 5.12B). Together, the findings suggest that SAHA treatment converted cells from the tumorigenic phenotype to non-tumorigenic phenotype.

Discussion

Tumors are thought to originate from a subset of cancer cells termed cancer stem cells (CSCs), which possess the stem cell traits such as self-renewal capability and the ability to give rise to multiple cell lineages (25, 26). Through self-renewal, CSCs drive tumorigenicity, tumor recurrence, and metastasis. Through differentiation, CSCs give rise to heterogeneous populations of cancer cells and perpetuate tumor growth. The existence of CSCs was first demonstrated by Dick et al., where they successfully identified a small population of leukemia cells that drive disease progression (27). The rare tumorigenic CSCs have been identified and isolated from various hematological and solid malignancies, including breast (1, 2), pancreatic (7-10), prostate (11, 12), colon (13), brain(14), skin (28) cancers. The CSC hypothesis describes that only CSCs are able to initiate tumors and maintain tumor growth through self-renewal and CSCs are thought to mediate tumor recurrence and metastasis.

The identification and isolation of CSCs remain key challenges in studying CSCs despite widespread acceptance of the concept. In breast cancer, putative CSCs were first reported in the study of Al-Hajj et al. where breast tumor was demonstrated to contain a small population with stem cell properties bearing the $CD44^{+}/CD24^{-/lineage-}$ phenotype (29). Subsequently, the work of Ginester et al. demonstrated that aldehyde dehydrogenase 1 (ALDH1) is a marker for stem/progenitor cells in healthy and cancerous human breast tissues (20). The study found that cancer cells with elevated ALDH1 activity, as assessed by Aldefluor

assay, exhibited tumorigenic capability because they were able to self-renew and generate heterogeneous cell populations. Moreover, the Aldefluor-positive cells had only approximately 1% of overlap with the previous described $CD44^{+}/CD24^{-/lineage-}$ CSCs (30, 31). Still, increasing evidence indicates that higher percentage of Aldefluor-positive tumor cells may serve as a novel prognostic marker for poor clinical outcome in multiple human solid cancers including breast (20, 32, 33), lung(34), pancreatic(35), bladder (36) and prostate (37) cancers. In spite of the existence of varying findings in literature, ALDH1 activity still presents itself as a strong candidate for the identification of CSCs *in vitro* and *in vivo.*

The analysis of gene expression indicated that the target genes of HDAC-1, 2 and 3 were significantly up-regulated in Aldefluor-positive cells whereas the target genes of other HDAC isoforms were not different, as compared to Aldefluor-negative cells. Our finding is in agreement with previous studies citing increased expression of HDAC1 and 3 in breast cancer (23)and HDAC 1, 6, 8 overexpression is strongly associated with invasive potential of breast cancer (38). Down-regulation of HDAC1 induces cellular differentiation was reported in breast cancer (39). HDAC1 expression has shown to correlate with the degree of tumor differentiation in various cancers (40-42). Although the exact mechanism for how the different HDAC isoforms are involved is unclear, the observations suggest that HDAC1, 2, and 3 may be involved in the regulation of Aldefluor-positive cells.

Since CSCs govern tumor initiation, metastasis, and recurrence through aberrant self-renewal and differentiation to heterogeneous lineages, inhibition of CSC self-renewal is essential to preventing tumor progression. Tumorsphere

formation assay has been widely used to evaluate *in vitro* CSC self-renewal capability (43-49). Our *in vitro* studies demonstrated SAHA is effective against the CSC-rich Aldefluor-positive population within tumors to block the self-renewal. This finding coincides with the findings of Robertson et al. showing that SAHA inhibits the self-renewing ability of inflammatory breast cancer (IBC) cell lines (50). However, they used unsorted cells in tumorsphere formation assay and hence the readout may be a mixed response from both CSC and differentiated populations. To better characterize the effect of SAHA specifically on the CSCs, we sorted the cancer cells based on the Aldefluor assay prior to subjecting the cells to tumorsphere formation assay. Our data provides a more definitive evidence to suggest that HDAC inhibitors such as SAHA are effective at inhibiting self-renewal of breast CSCs.

The damaged self-renewal ability of Aldefluor-positive cells caused by SAHA led to the poor tumorigenic potential. In the re-implantation of tumor cells from SAHA-treated and vehicle-treated mice to assess the tumor engraftment efficiency, the number of re-implanted CSCs was not able to be controlled. Although the total number of re-implanted tumor cells was controlled, the drug-treated tumors were composed of less Aldefluor-positive cells (0.19%) as compared to the vehicle-treated tumors (4.38%). Therefore, secondary mice receiving the re-implantation from vehicle-treated mice likely received more Aldeflour-positive cells than from SAHA-treated mice. To address this concern, the number of Aldefluor-positive cells inoculated into secondary mice was calculated based on the Aldefluor assay data in Figure 3D and compared to the tumor engraftment efficiency. Of 20K tumor cells, 876 cells are calculated to be Aldefluor-positive in the vehicle-treated mice and 420

cells are calculated to be Aldefluor-positive in the drug-treated mice. Of 2K tumor cells, 88 cells are Aldefluor-positive in vehicle-treated tumors and 42 cells are Aldefluor-positive in drug-treated tumors. The data implies that as low as 88 Aldefluor-positive cells from vehicle-treated mice formed tumors in secondary mice 100% of the time in 8 weeks, while as many as 420 Aldefluor-positive cells from drug-treated mice formed tumors only 50% of the time. This indicates that impaired self-renewing potential observed in the drug-treated tumors is not only due to the difference in the number of re-implanted Aldefluor-positive cells, but also their self-renewal ability. These findings suggest that SAHA is able to inhibit the tumorigenicity of Aldefluor-positive cells.

The role Myc plays in promoting self-renewal of stem cells have been established in a variety of stem cells including embryonic stem cells (51-53), neural stem/progenitor cells (54-56), hematopoietic stem cells (57, 58), and glioblastoma stem cells (59). A recent study in glioma malignancy by Wang et al. reported that MYC knockdown in CSCs by siRNA attenuated their ability form neurosphere formation and abolished tumorigenic ability of form tumors in mouse. Their findings highlight the critical role of MYC in the regulation of CSC self-renewal. In accordance with their study, our findings showed that highly activated MYC in Aldefluor-positive cells was attenuated after SAHA treatment. On strength of the role of MYC in self-renewal, inhibition of self-renewal and tumorigenicity of CSCs by SAHA may be mediated through MYC.

Elevated BMI-1 gene expression observed in our study reflects the current understanding of gene expression profile of CSCs. BMI-1, a transcription repressor,

has been shown to play a role in the regulation of hematopoietic and neuronal stem cell self-renewal (25, 60). Recently, elevated expression of Bmi-1 is also increasingly being recognized as an important feature for the maintenance of stemness in CSCs. Ample studies revealed that BMI-1 is highly expressed in cancer cells with high tumor initiating capacities (60-66). Our observation of elevated BMI-1 gene expression in Aldefluor-positive cells in support of the speculation that elevated BMI-1 expression is a feature of cells possessing stem-like properties, including CSCs.

Apart from being a feature of CSCs, BMI-1 may play a role as a mediator for the inhibitory effect of SAHA on CSCs. BMI-1 has been reported as a transcriptional target of HDAC inhibitors (67). Similarly, the findings of our experiment demonstrate that SAHA is capable of suppressing the levels of BMI-1 gene and protein expression. We have also shown both *in vitro* and *in vivo* that SAHA is able to inhibit the self-renewal capability of the CSC-like Aldefluor-positive cells. Furthermore, previous studies have demonstrated the relationship between the level of BMI-1 expression and the ability of CSCs to self-renew. For example, stable knockdown of BMI-1 in CD133-positive glioblastoma multiforme stem cells resulted in the inhibition of clonogenic potential *in vitro* and of brain tumor formation *in vivo*(65). Moreover, induction of BMI-1 over-expression via up-regulating a BMI-1 upstream signal has been shown to promote the self-renewal in breast mammary and cancer stem cells (5). Similar findings have also been reported in prostate cancer (61, 62), head and neck small cell cancer (66), and hepatocelluar carcinoma (68). Taken together, the findings of our study suggest that BMI-1 may be one of the

mediators of the inhibitory action of SAHA on CSCs among numerous genes affected by HDAC inhibitors.

Among varied genes caused by SAHA, genes associated with CSC markers such as ALDH1A1, CD44 and CD24 were changed modulated by SAHA. Higher expression of ALDH1A1 gene was down-regulated in SAHA treated Aldefluor-positive cells as compared to DMSO-treated. Apart from ALDH1A1 gene, the CSC-phenotype of $CD44^{+}/CD24^{-}$ was partially converted to $CD44^{-}/CD24^{+}$. Of note, two distinct responses of CD44 genes to SAHA were observed. One set including 5 gene probes was up-regulated by SAHA, whereas the other set including down-regulated by SAHA. This may due to the variant CD44 isoforms. Despite of the various responses, antibody staining for surface CD44 and CD24 markers confirmed that SAHA indeed reduced the portion of $CD44^{+}/CD24^{-}$ cells and increased CD44-/CD24+ cells. Shipitsin et al. have showed that CD24 is preferentially expressed on more differentiated cells whereas CD44 is expressed on more progenitor-like cells (69). It is unclear whether the changes of ALDH1A1 and CD44 and CD24 transcription are direct response of HDAC inhibition or the outcomes through mediators, such as MYC or BMI-1. Nevertheless, the down-regulated ALDH1A1 and CD44 gene, and up-regulated CD24 upon HDAC inhibition support in vitro and in vivo findings that a significant reduction of Aldefluor-positive cells and impaired self-renewal after SAHA treatment.

Beyond their established roles in the transcriptional regulation, HDACs have been shown to be involved in the post-translational regulation. For instance, HDAC6 has been reported to acetylate many non-histone targets, such as heat shock protein

90 (Hsp90). Upon HDAC inhibition, hyper-acetylation would impair Hsp90's co-chaperon function. As a result, hyper-acetylated Hsp90 is unable to stabilize its client proteins and instead causes the degradation of client proteins (70). In our present study, we did not address whether the non-transcriptional effect of SAHA is associated with breast CSC inhibition, however, we could not exclude the possibility that acetylated non-histone targets might contributed to the suppression of breast CSCs. Several studies, including our previous studies, suggested that HSP90 inhibition would be a promising therapeutic approach to target CSCs in bladder (71) and breast cancers (manuscript is under revision), glioma (72), leukemia (73, 74) and lymphoma (under revision). Thus, apart from gene modulation, HDAC-mediated Hsp90 inhibition might contribute partially to the inhibitory effect on breast CSCs observed in our current study.

Conclusion

In summary, the current study provides evidence to suggest that HDAC inhibition could be a potential approach to target breast CSCs. Gene expression analysis of SUM159 cells indicated that the target genes of HDAC1, 2, and 3 are highly active in the Aldefluor-positive population compared to the Aldefluor-negative population. HDAC inhibition by SAHA reduced the number Aldefluor-positive cells as well as impaired capability of self-renewal, leading to poor tumor engraftment ability in secondary mice. Last, inhibition of Aldefluor-positive cells might be mediated through two critical components, MYC and BMI-1. Along with reduction in BMI-1 and MYC, two breast CSC markers, ALDH1A1 and CD44 were down-regulated, suggests that SAHA converted these cells from stem-like phenotype to more differentiated status. Our study has important implications for the use of HDAC inhibitors as a differential agent in anti-CSCs therapy.

TABLES

Primer		Oligo sequence (5' to 3')
Bmi-1	Forward	GGAGACCAGCAAGTATTGTCCTATTT
Bmi-1	Reverse	CATTGCTGCTGCTGGGCATCGTAAG
c-Myc	Forward	ATGCCCCTCAACGTTAGCTTC
c-Myc	Reverse	CTGAGACGAGGATGTTTTTGATGAAGG
GAPDH	Forward	CTCAGACACCATGGGGAAGGTGA
GAPDH	Reverse	ATGATCTTGAGGCTGTTGTCATA
ALDH1A1	Forward	AGCCTTCACAGGATCAACAGA
ALDH1A1	Reverse	GTCGGCATCAGCTAACACAA
$p21^{WAF1}$	Forward	ATGTCAGAACCGGCTGGGGA
$p21^{WAF1}$	Reverse	GCCGTTTTCGACCCTGAGAG

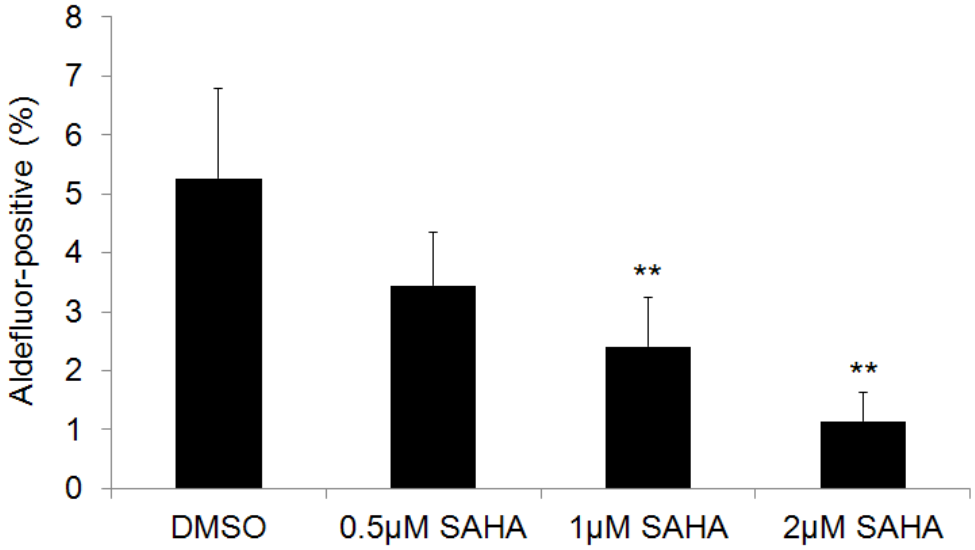
Table 5.1 Primers used for real-time RT-PCR analyses

GeneSet	Pos-obs	Neg-obs	Pos-exp	Neg-exp	ratio	Pvalue
HDAC1_TARGETS_UP	42	16	20	38	4.73	1.22E-09
HDAC2_TARGETS_UP	18	1	6	13	32.45	3.17E-09
HDAC1_AND_HDAC2_TARGETS_UP	31	9	14	26	6.21	1.75E-08
HDAC3_TARGETS_UP	50	27	27	50	3.34	3.95E-08

Table 5.2 Highly activated genes in Aldefluor-positive cells (ratio >1 indicated activation and ratio <1 indicate inactivation)

FIGURES

A



B

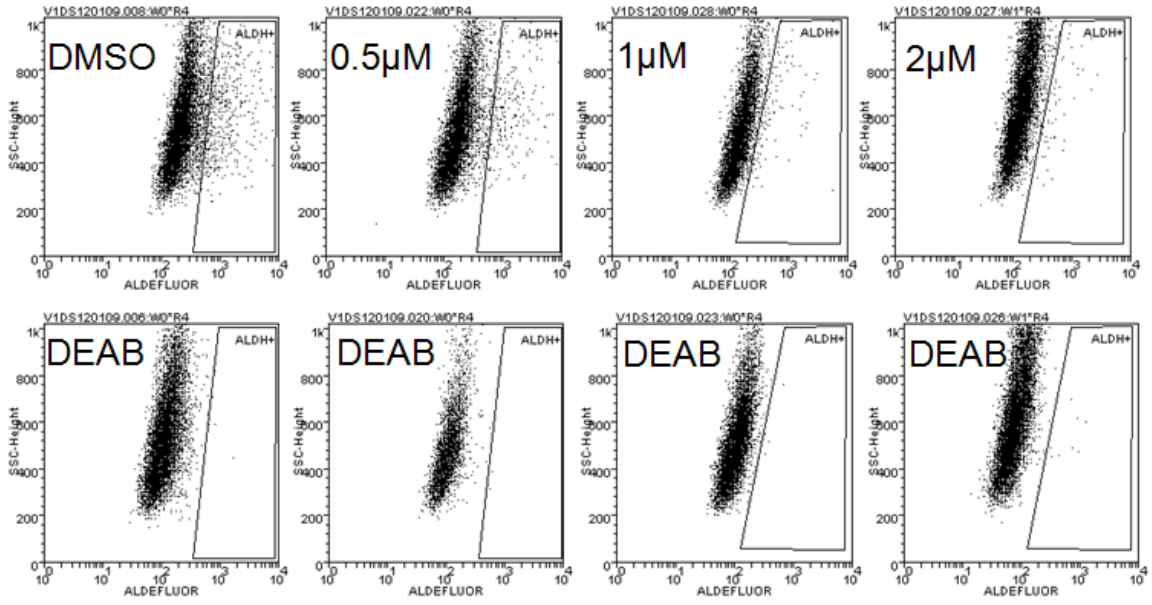


Figure 5.1 SAHA reduced the number of SUM159 Aldefluor-positive CSCs.

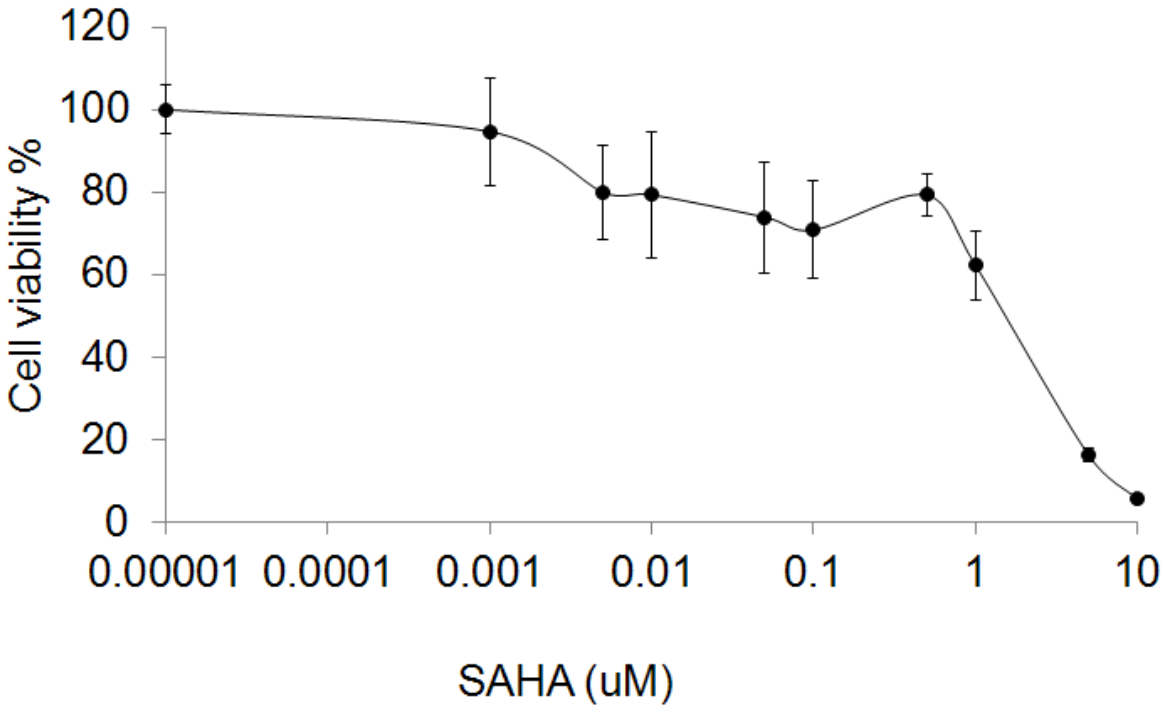


Figure 5.2 SAHA reduced the viability of SUM159 cancer cells.

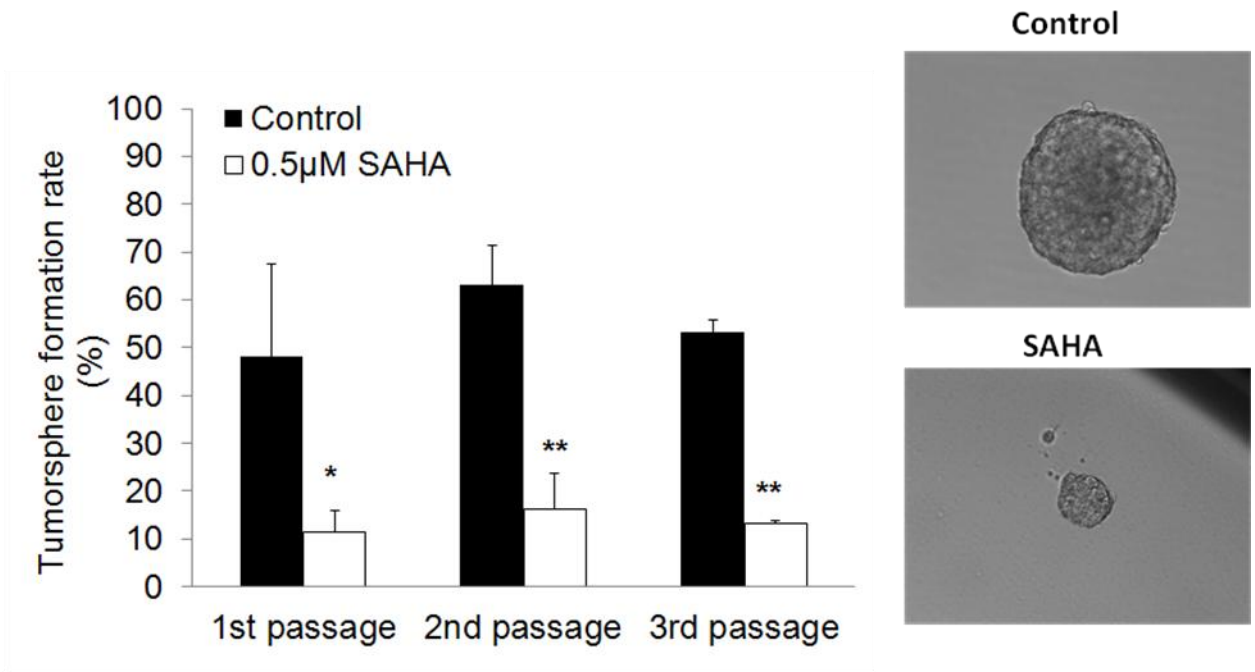
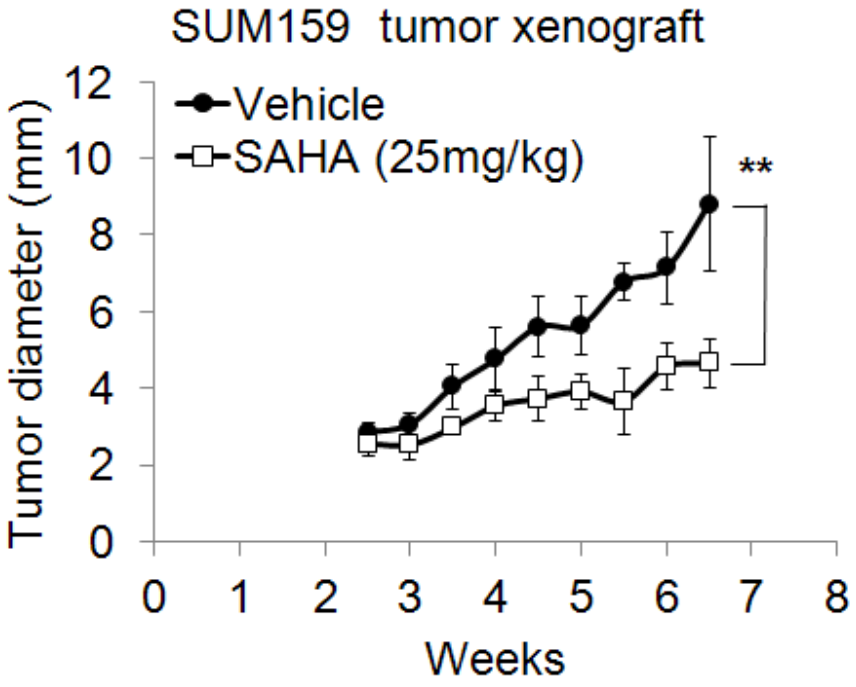


Figure 5.3 SAHA treatment inhibited the self-renewal and proliferative capability of SUM159 Aldefluor-positive cells *in vitro*

A



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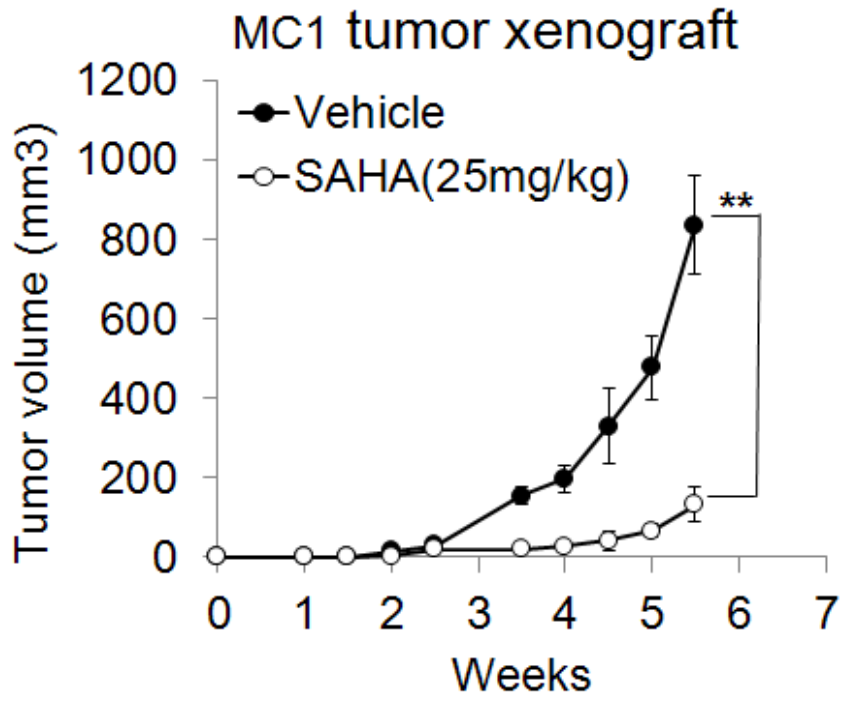
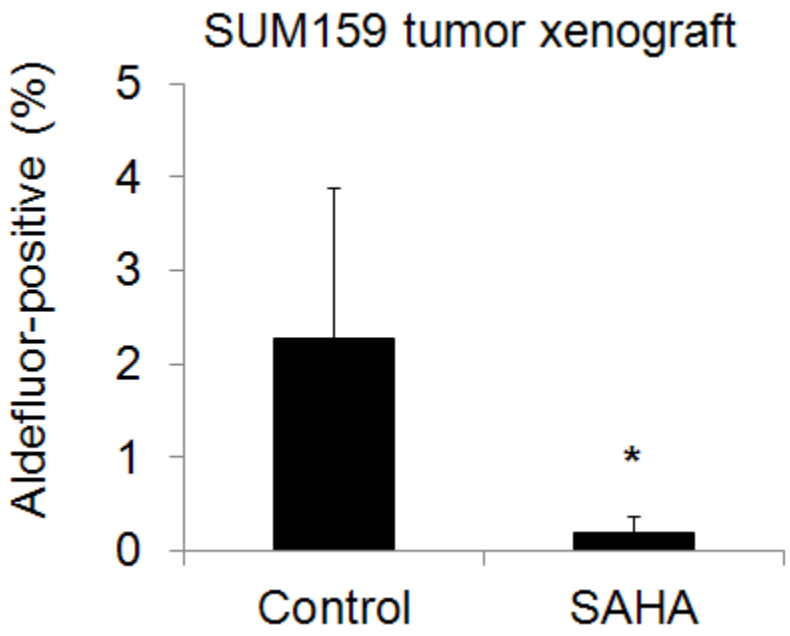


Figure 5.4 SAHA inhibited the growth of SUM159 and MC1 tumors.

A



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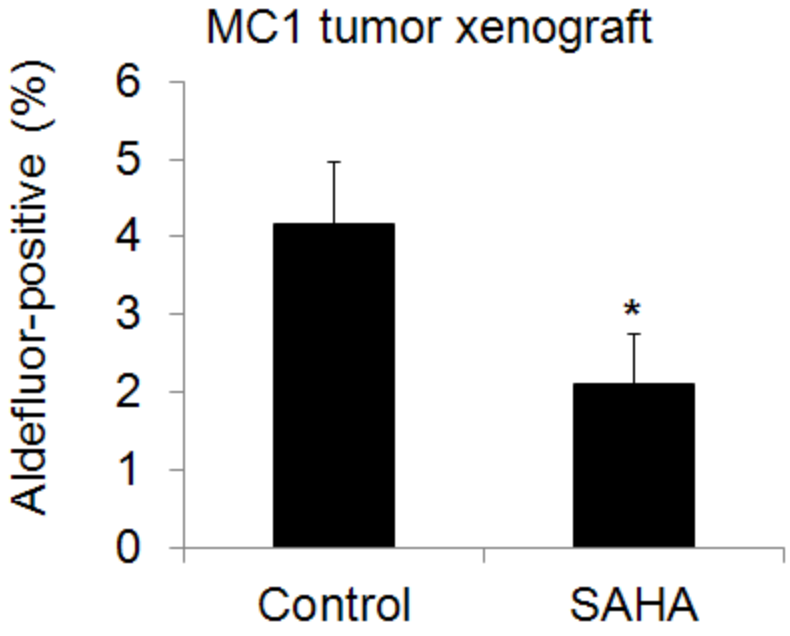


Figure 5.5 SAHA reduced the portion of Aldefluor-positive cells in SUM159 and MC1 tumors.

A

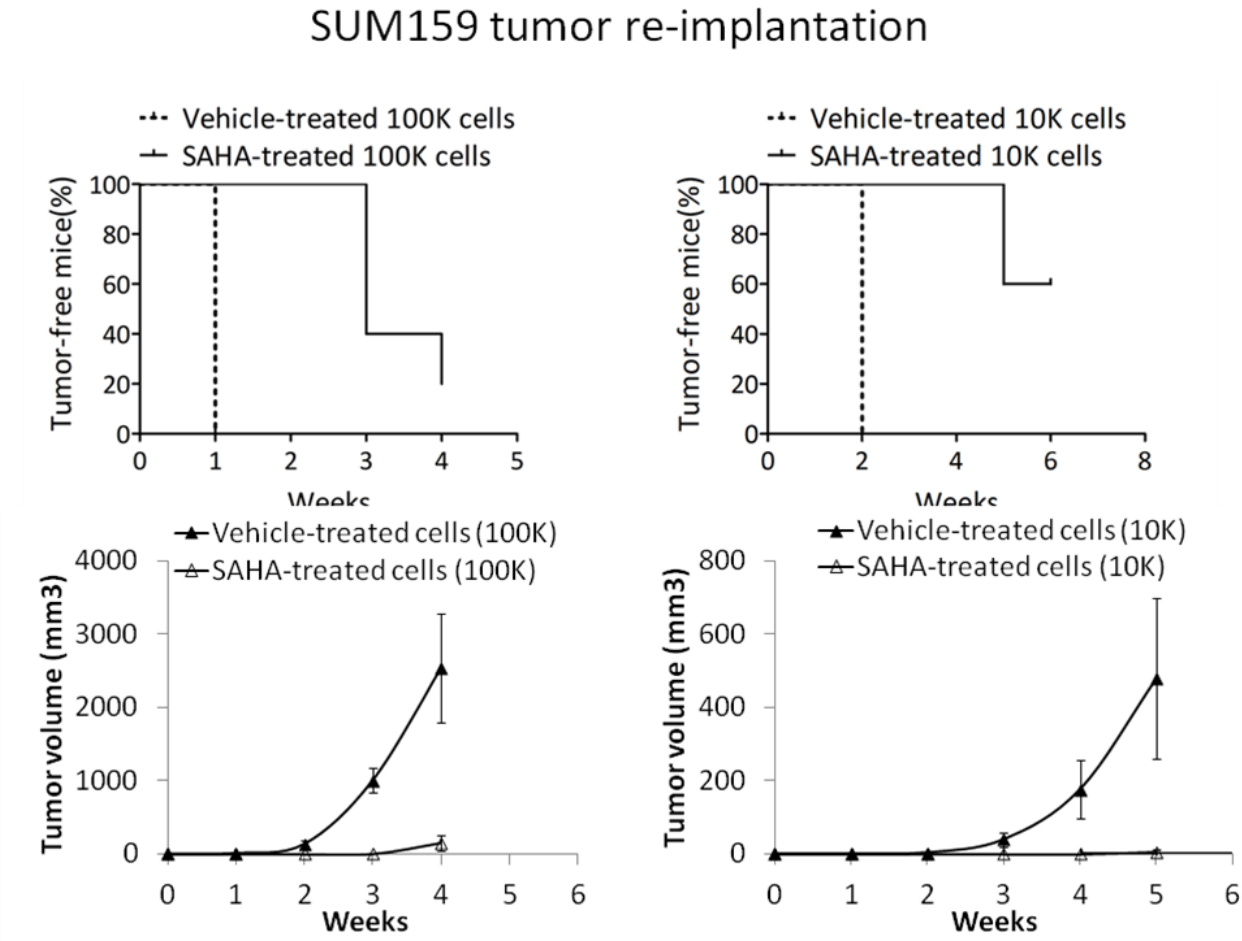


Figure 5.6 SAHA impaired the tumor engraftment efficiency of SUM159 cells in secondary mice

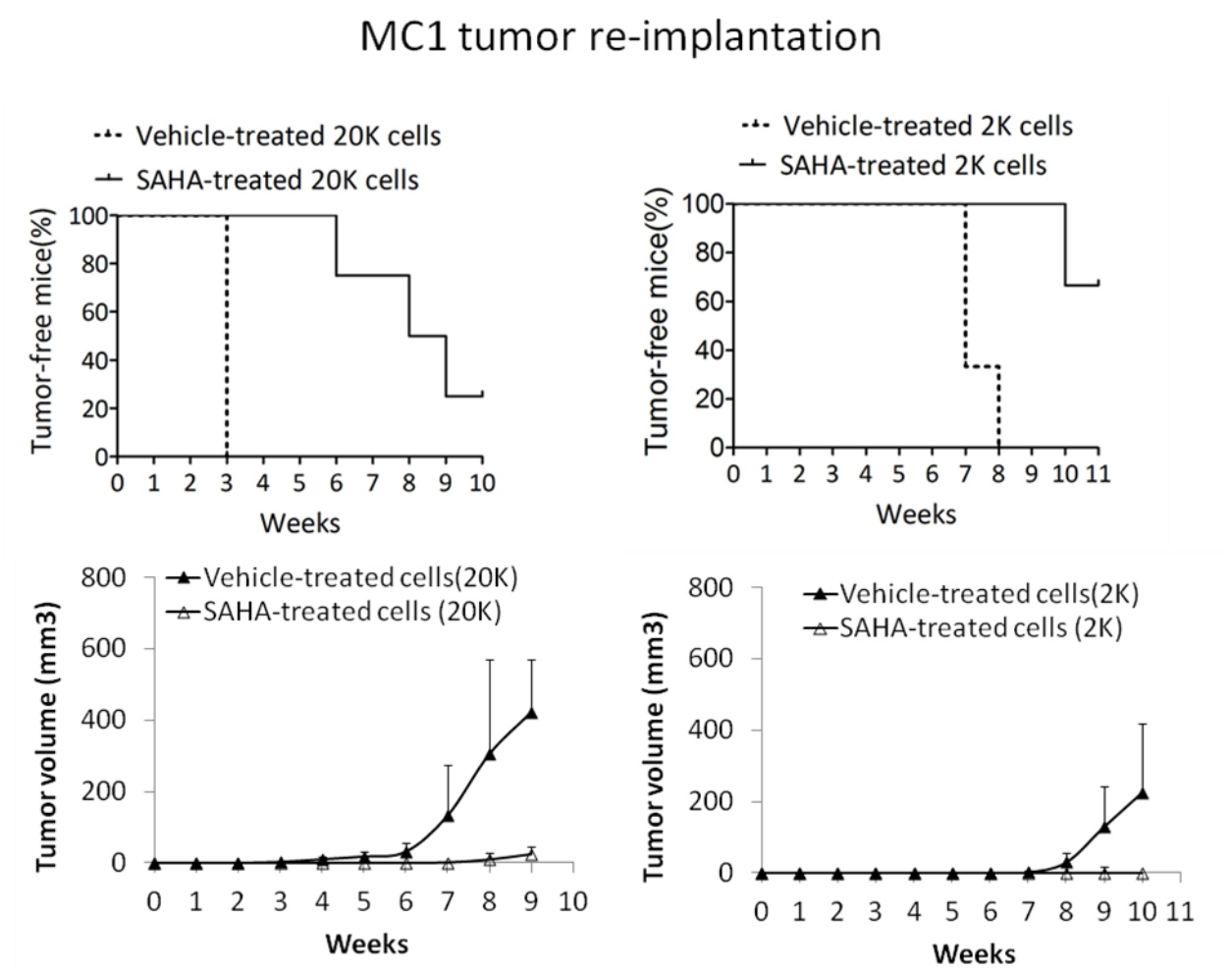


Figure 5.7 SAHA impaired the tumor engraftment efficiency of MC1 cells in secondary mice

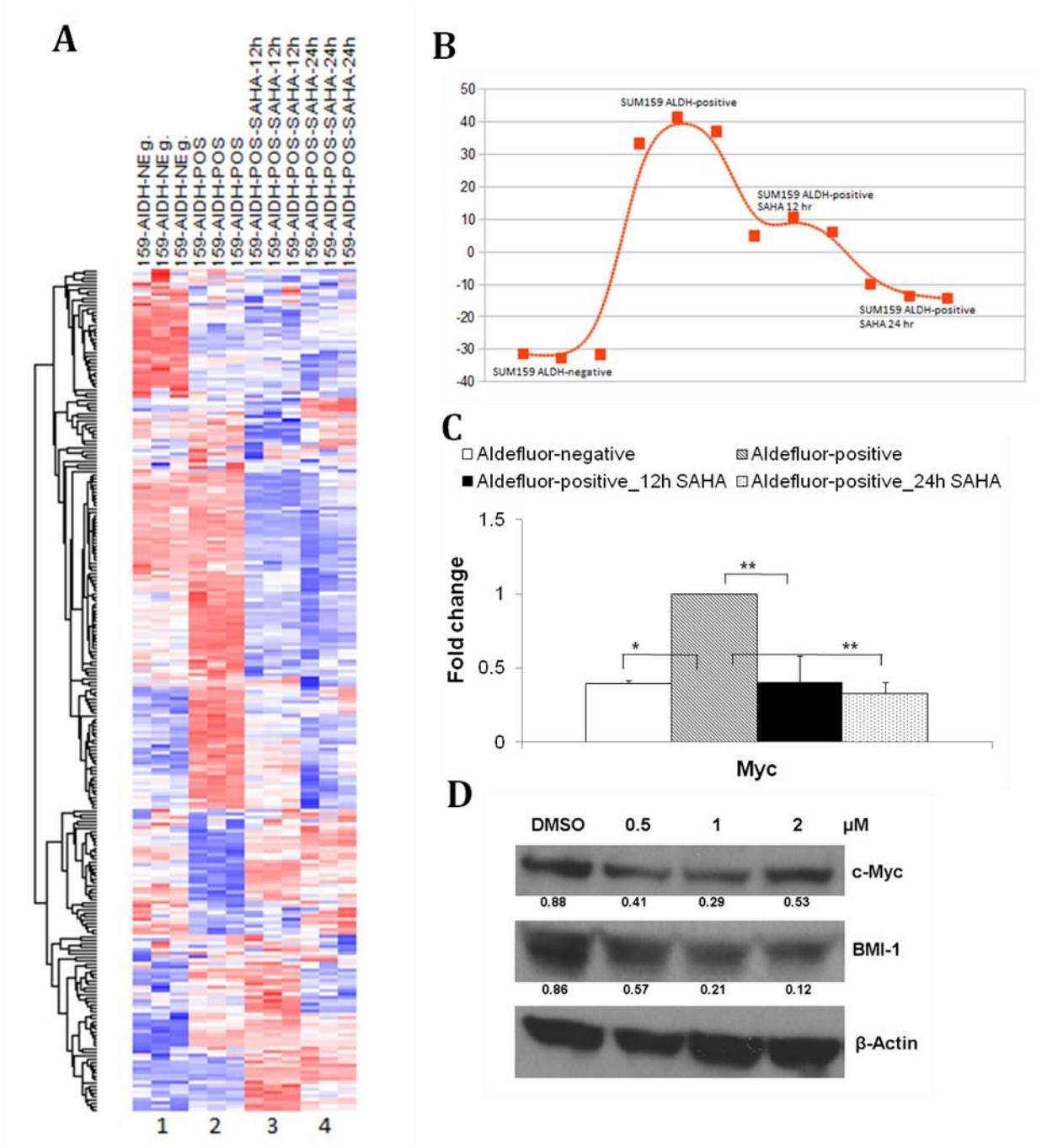


Figure 5.8 Effect of SAHA on the expression pattern of MYC target genes, and levels of MYC gene and protein.

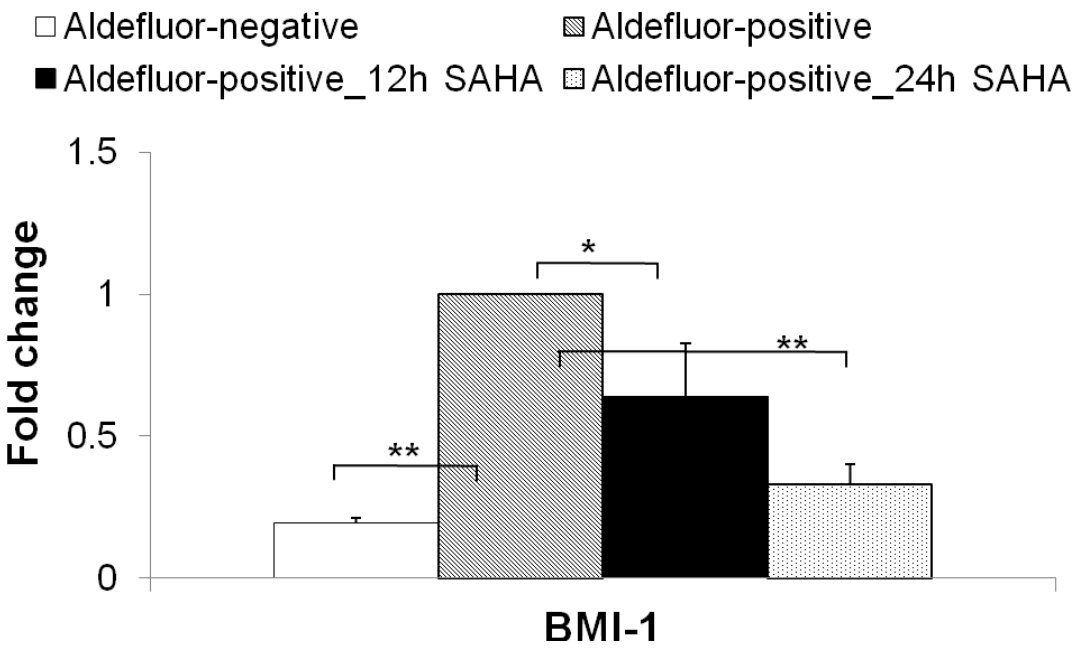


Figure 5.9 SAHA reduced the expression of BMI-1 gene in Aldefluor-positive cells.

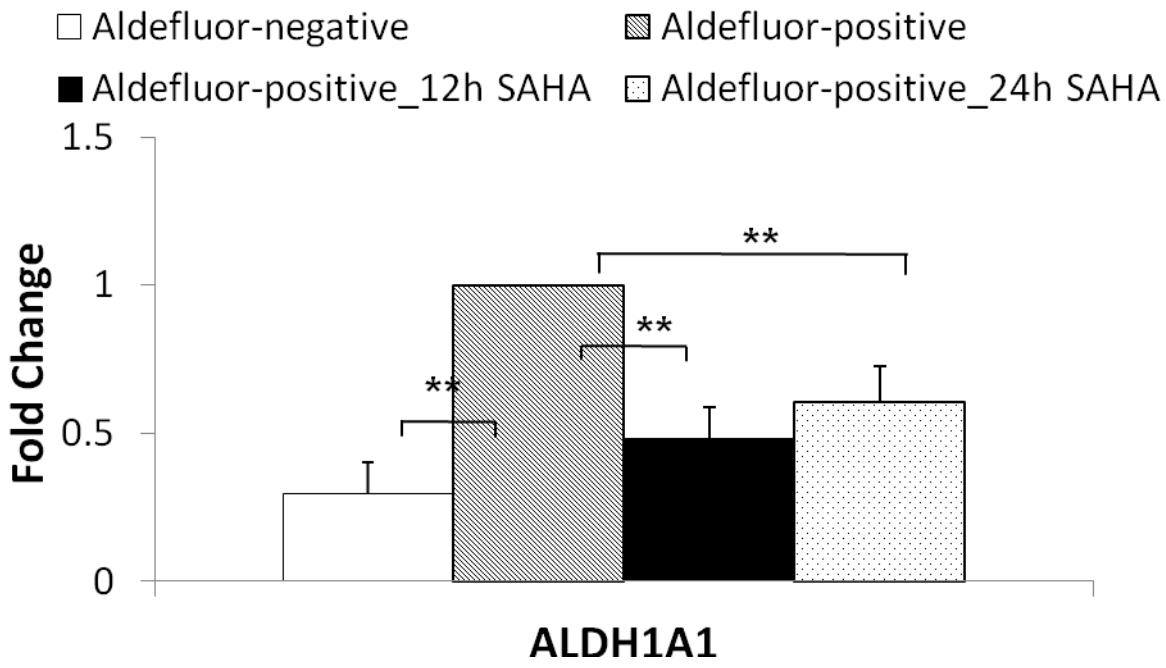


Figure 5.10 SAHA reduced the level of ALDH1A1 gene.

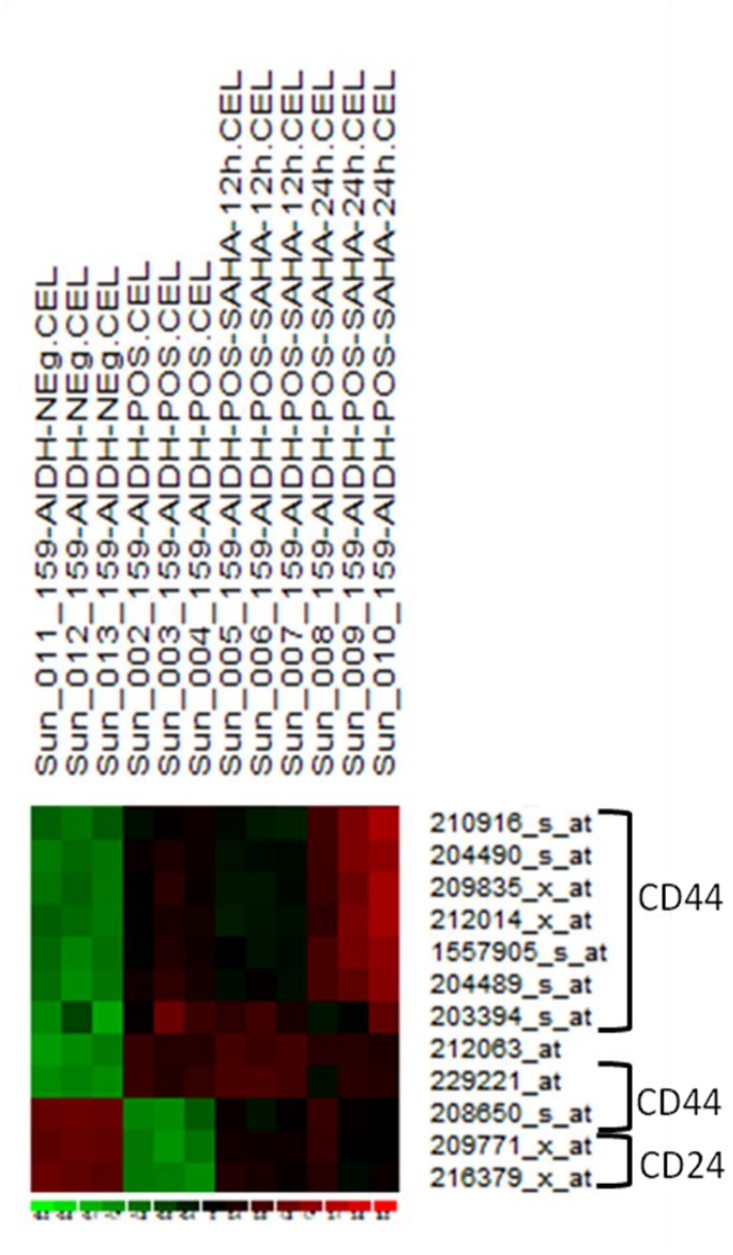
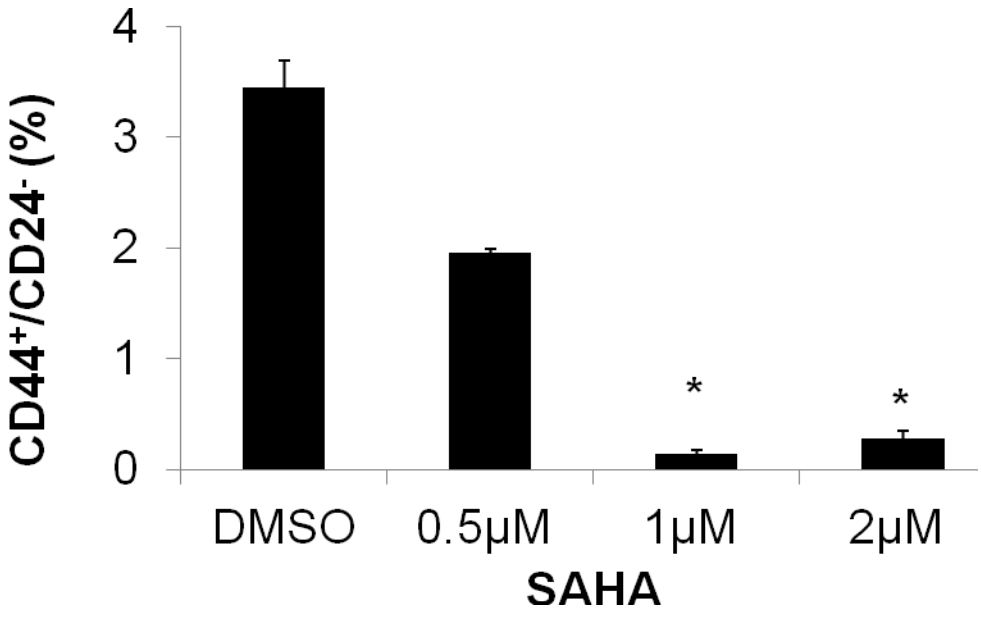


Figure 5.11 SAHA inhibits MCF7 $CD44^{+}/CD24^{-}$ cells

A



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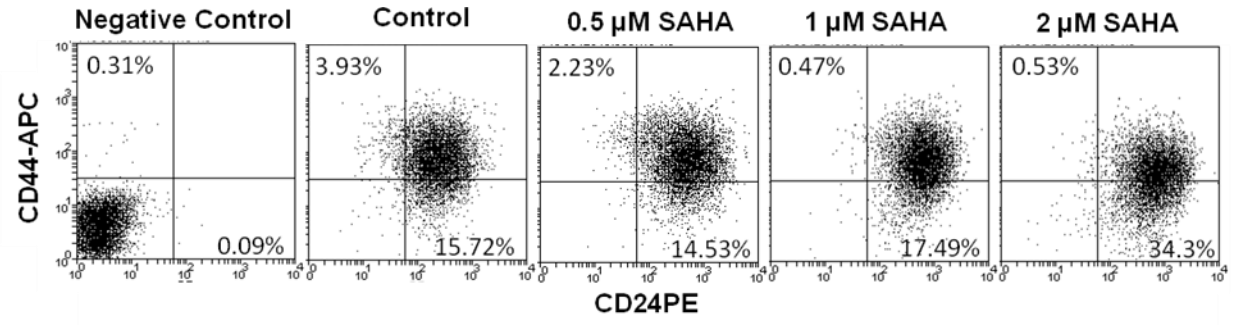

Figure 5.12 Effect of SAHA on the expression CD44 and CD24 protein

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