CHARACTERIZATION OF THE CANCER STEM CELL PHENOTYPE IN BONE METASTATIC BREAST CANCER AND ITS TREATMENT WITH GLI2 INHIBITOR

By

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To my Mom and Dad for their unwavering love and support, no matter the distance or time of day,

and

To my late Aunt Linda who passed away from this unfortunate disease. Her story inspires me to expand the knowledge of cancer biology and pave the way for treatments that will help millions of other brave women around the world afflicted by this same disease.

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Chapter 1

Introduction and Significance

1.1 Innovation and Motivation

Recent estimates indicate that approximately one in five people globally will develop cancer within their lifetimes, of which, breast cancer will account for nearly one in four diagnoses in women.[1] Triple-negative breast cancer (TNBC) is an aggressive and heterogenous[2,3] form of cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and abnormal expression of human epidermal growth factor receptor (HER2).[3,4] Tumor-induced bone disease (TIBD) is a collection of morbidities including osteoporosis, fractures, and pain resulting from tumor metastasis, all of which contribute to a poorer quality of life in patients with late-stage TNBC. Taken with the lack of therapeutic targets for TNBC, TIBD patients have an increased risk of recurrence, lower survival rates, and worse overall prognosis compared to those with other forms of breast cancer.[4–8]

Cancer stem cells (CSCs) are a subpopulation of the heterogeneic cancer cells within a tumor with distinct abilities to differentiate, self-renew, and display homeostatic control in response to external stimuli.[9–14] These characteristics are believed to play a vital role in developing the tumorigenic and chemoresistant behaviors associated with aggressive metastatic cancers, particularly relapse chemotherapy.[15–18]

The presence or absence of various markers are used to identify CSC subtypes, namely CD44 and CD24 surface markers, and aldehyde dehydrogenase (ALDH) activity. CD44-expressing cells function in cellular adhesion and orientation, while CD24-expressing cells have immunological importance in B-cell proliferation and differentiation.[19,20] The ALDH enzyme catalyzes the oxidation of aldehydes into carboxylic acids and is a key identifier for the rate of cellular metabolism.[21,22] The presence of $CD44^{high}/CD24^{low}$ and, separately, $ALDH1^{+}$ CSC phenotypes have been identified and correlated with TNBC metastasis.[23–25]

Separately, the expression of Gli2 transcription factor is upregulated in chemoresistant tumors and cancer stem cell markers,[26–29] however, it is unclear whether Gli2 plays a mechanistic role in CSC phenotypes. Indeed, the presence of ALDH has been found to stabilize and enhance Gli2 activity in other forms of cancer.[22]

The objective of this work is to characterize the CSC phenotype of TNBC cells as well as their bone metastasis. Here we will establish potential links with either the $CD44^{high}/CD24^{low}$ and $ALDH1^{+}$ CSC phenotypes and Gli2 as well as evaluate potentially synergistic anti-CSC drug combinations using Gli2 inhibitor, GANT58, and front-line chemotherapeutic, Paclitaxel. By better understanding the mechanistic roles of CSCs in potentiating bone metastasis and TIBD, more effective therapeutics may be developed for clinical translation.

1.2 Specific Aims

The overall goal of this project is to observe the expression of $CD44^{high}/CD24^{low}$ and $ALDH1^{+}$ CSCs, the effect of Gli2-inhibitor on chemosensitivity, and the relation of these CSC subtypes to Gli2 expression in TNBC cell lines and their bone-metastatic derivatives. **The hypothesis is that bone metastasis enriches CSC populations and is driven by Gli2 upregulation, and that Gli2 inhibitor will improve their sensitivity to front-line chemotherapy, reducing overall tumor burden and CSC populations.**

Aim 1: Characterization of the Relative Percentages of CSC Populations in TNBC Cell Lines and their Bone-Metastatic Derivatives

The first aim of this thesis is to understand the role of Gli2 expression in TNBC bone metastasis and their relationships to cancer stem cells. The general strategy was to first quantify the relative expressions of Gli2, CD44/CD24 surface markers and ALDH activity in both human and mouse TNBC cell lines and their bone metastatic derivatives. By directly comparing the relative expressions of CSCs and Gli2 activation before and after bone metastasis, we can determine whether Gli2 upregulation is a driver for bone metastasis, and whether this process bone enriches CSC populations. As such, MDA-MB-231 human and 4T1 mouse breast cancer

cell lines were chosen as the models of interest for this study due to their heterogeneic cell populations. Identification of CD44/CD24 surface markers and, separately, ALDH activity was conducted via fluorescence-activated cell sorting (FACS) on a flow cytometer. Through this method, cancer cells were fluorescently labeled with monoclonal antibody conjugates that are highly specific for the CD44 and CD24 surface markers. These fluorophores are designed to fluoresce only within a specific region of the visible spectrum. As such, when cells are passed through a green laser, we are able to identify the expression of one of the fluorophore-antibody conjugates, and cells passed through a red laser will show the expressions of the other conjugate. For identifying ALDH activity, a separate intracellular assay was used. Cells were suspended in a buffer containing BODIPY-aminoacetaldehyde (BAAA) which freely passes through the cellular membrane. After being catalyzed by the ALDH enzyme, BAAA is converted into a fluorescent form which cannot escape the cell. As such, cells exhibiting high fluorescence intensity are indicative of increased ALDH activity. Finally, western blots are employed to examine the presence of Gli2. By utilizing monoclonal antibodies specific for Gli2 protein, samples that undergo gel electrophoresis are separated based on molecular size and charge. When matched with a ladder control with known sizes, we can determine with certainty the presence of Gli2, and the darkness of such bands are indicative of Gli2 presence.

Aim 2: Effects of PTX, GANT58, and Combination Therapy in CSC Populations in TNBC Cell Lines and their Bone Metastatic Derivatives

The second aim of this thesis investigates the in vitro effect of Gli2 inhibitor, GANT58, with the anticancer drug, Paclitaxel, and establishes a novel method of improving chemosensitivity of bone metastatic cancer cells. Here, the dose-responses of GANT58, paclitaxel and combination were examined in MDA-MB-231 breast cancer cells. The IC50 values for both drugs were determined in both the parental and bone clone cell lines. The MDA-MB-231 bone clones required a paclitaxel dosage three orders of magnitude higher than seen in the parental MDA-MB-231 cell line, indicating that bone clones are much more chemoresistant to frontline chemotherapy. Then we created a library of GANT58 an paclitaxel formulations and to identify

possible synergystic relationships *in vitro*. A synergy heatmap was used to observe the in vitro effects of these formulations, identifying possible regions where synergy is observed, and were quantified by obtaining ZIP scores. Subsequently, the *in vitro* effects of GANT58, PTX, and synergistic combination on CSC expression and Gli2 activity were evaluated through aldefluor assays and western blots. The aldefluor assay was used to identify the relative CSC expressions across treatments for both parental and bone clone cells. Differential expression in treated ALDH CSC populations indicated the ability for each drug to target TNBC CSC populations as well as determine whether Gli2 can be used to promote chemosensitivity of CSCs and inhibit tumor volume. Moreover, relative expressions of ALDH and Gli2 activation were quantified using western blots to corroborate the trends observed from aldefluor assays. Lastly, the effects of these treatments in parental MDA-MB-231 cancer cells were analyzed by qPCR to examine activity of PTHrP, whose regulation plays a key role in potentiating bone metastasis.

1.3 Outline

This thesis focuses on characterizing the relationships between Gli2 regulation and CSC expressions, bone metastasis and tumor potentiation, and chemoresistance in TNBC cells and developing novel CSC targeting strategies in TIBD treatment. Chapter 2 provides an overview on the biology of TNBC, TIBD, and CSCs to shed light on the significance of bone metastasis and clinical need for developing novel targeting strategies. Chapter 3 examines the natural expressions and behaviors of CSC populations within TNBC and bone metastatic derivatives to understand the role of Gli2 in bone metastasis and CSC enrichment. Chapter 4 provides a deeper analysis into these relationships through *in vitro* assays on drug-treated TNBC cells. Chapter 5 discusses the impact of this work and considers the challenges and potential pitfalls encountered in this project to provide guidance for future directions. Each experimental chapter it outlined by a brief introduction followed by the materials and methods, results, and discussion.

Chapter 2

Background

2.1 Introduction

2.1.1 Triple-Negative Breast Cancer and Tumor-Induced Bone Disease

Breast cancer encompasses a diverse range of subtypes with distinct molecular characteristics and clinical behaviors.[2,7] Among these subtypes, triple-negative breast cancer (TNBC) represents a particularly aggressive form that lacks expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[3–5]. TNBC accounts for approximately 15-20% of breast cancer cases and is associated with poor prognosis, high rates of distant metastasis, and limited treatment options.[30–35]

Tumor-induced bone disease (TIBD) refers to a collection of morbidities resulting from advanced late-stage cancers, and 70% of mortalities from metastatic cancers showed evidence of TIBD.[13] TIBD is characterized by cancer cells metastasizing to the bone, resulting in bone destruction, osteoporosis, fractures, and severe pain.[34,36–38] Patients with bone metastases have significantly lower survival rates compared to those without bone involvement.[7,32,39,40] The interplay between cancer cells and the bone microenvironment contributes to the vicious cycle of bone destruction, involving the release of various factors from the bone matrix that further potentiate tumor growth and bone matrix degradation (**Figure 1**).[41,42]

Therefore, there is a significant clinical need for understanding the underlying mechanisms of TIBD in the context of TNBC. By investigating the interactions between TNBC cells and the bone microenvironment, we can gain insights into the specific factors and signaling pathways that drive bone metastasis and TIBD. Furthermore, understanding the impact of TIBD on tumor progression and therapeutic response is essential for developing targeted therapeutic strategies that disrupt this vicious cycle and improve patient outcomes.

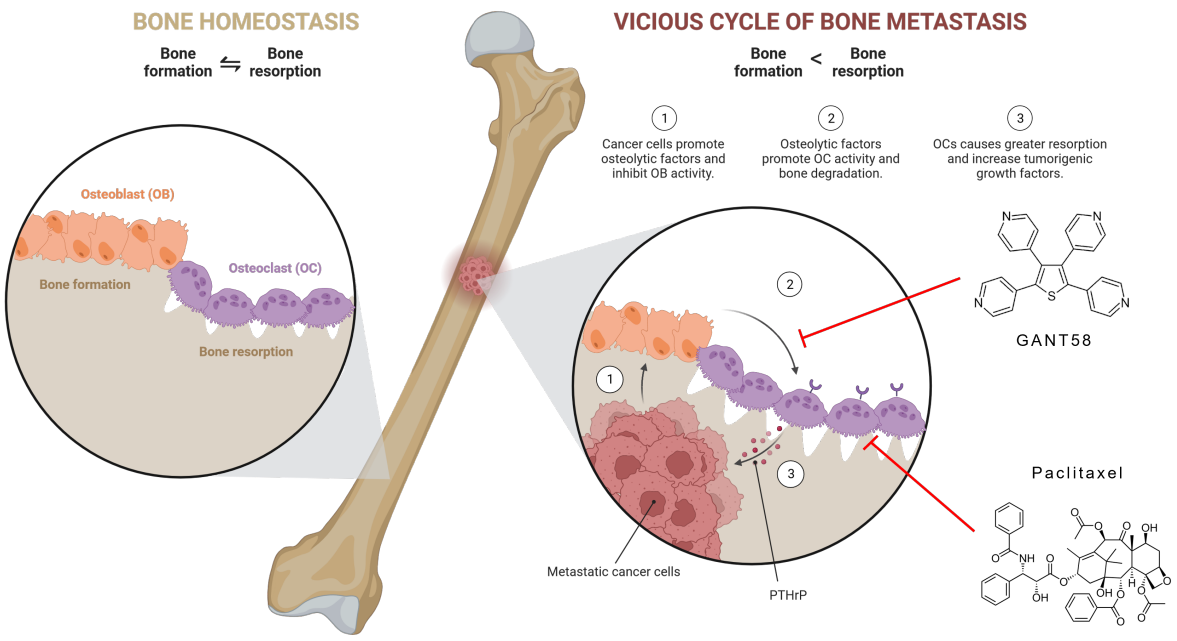

Figure 1: The vicious cycle of tumor-induced bone disease. Created in BioRender.

2.1.2 Cancer Stem Cells

In recent years, cancer stem cells (CSCs) entered the spotlight of cancer research due to their role in tumor initiation, progression, therapy resistance, and metastasis. CSCs are characterized by their self-renewal capacity, ability to differentiate into diverse cell types within the tumor, and their capacity to regenerate the heterogeneous cell population of the tumor.[9,10,43]

In TNBC, specific CSC subpopulations have been identified, particularly the $CD44^{high}/CD24^{low}$ and $ALDH1^{+}$ subtypes. $CD44^{high}/CD24^{low}$cells exhibit enhanced tumor-initiating potential, metastatic ability, and therapy resistance[44,45]. These cells have been associated with a mesenchymal-like phenotype, characterized by the upregulation of mesenchymal markers (such as vimentin, N-cadherin) and downregulation of epithelial markers (such as E-cadherin).[46–48] $CD44^{high}/CD24^{low}$ cells have been shown to possess increased migratory and invasive properties, enabling their dissemination to distant sites, including the bone

microenvironment.

ALDH1, an enzyme involved in cellular detoxification, has also emerged as a marker associated with CSC properties in TNBC.[49] $ALDH1^+$ cells exhibit increased self-renewal capacity, resistance to chemotherapy, and the ability to initiate tumor growth.[12] High ALDH1 expression has been associated with poor clinical outcomes in TNBC patients.[24,50] Moreover, $ALDH1^+$ cells have been shown to have an enriched CSC phenotype, demonstrating increased expression of genes associated with cancer stemness, enhanced sphere-forming capacity, and greater tumor-initiating potential.[12,21,49]

Understanding the characteristics and functional role of $CD44^{high}/CD24^{low}$ and $ALDH1^+$ CSC populations in TNBC is crucial for understanding the underlying mechanisms of tumor initiation, metastasis, and therapy resistance. As such, developing effective targeting strategies aimed at CSC populations may show potential in eradicating TNBC and preventing tumor recurrence.

2.1.3 Gli2 Transcription Factor

Glioma-associated oncogene homolog 2 (Gli2) transcription factor is a key downstream effector of the Hedgehog signaling pathway,[51,52] which plays a crucial role in embryonic development, tissue homeostasis, and stem cell maintenance.[53] Abnormal activation of the Hedgehog pathway has been implicated in various cancers, including breast cancer.[54] Gli2 regulates the expression of target genes involved in cell proliferation, survival, and epithelial-to-mesenchymal transition (EMT).[55]

Recent evidence suggests that Gli2 plays a significant role in the regulation of CSC populations.[28,51,56,57] The upregulation of Gli2 has been associated with enhanced self-renewal capacity, therapy resistance, and metastatic potential in breast cancer.[58,59] However, the precise role of Gli2 in the regulation of CSC populations and its association with bone metastasis in TNBC remain to be fully elucidated.

Investigating the functional role of Gli2 in TNBC, particularly in the context of CSC populations, holds great promise for understanding the molecular mechanisms underlying tumor

initiation, progression, chemoresistance, and bone metastasis. By unraveling the complex interactions between Gli2, CSCs, and the bone microenvironment, we can gain insights into novel therapeutic targets and develop strategies to disrupt these signaling pathways and ultimately improve TIBD patient outcomes.

2.2 State of the Art

2.2.1 TIBD and Metastatic TNBC Therapies

The management of TNBC presents a significant challenge due to the absence of targeted therapies such as hormone receptors or HER2 as therapeutic targets. Consequently, systemic chemotherapy remains the gold-standard for treating TNBC, usually consisting of treatment with anthracyclines and taxanes.[4,60] However, a substantial proportion of TNBC patients develop resistance to chemotherapy, leading to disease progression and poor long-term outcomes.[61–65]

2.2.2 Current Understandings of $CD44^{high}/CD24^{low}$ and $ALDH1^+$ CSC Subtypes

Considerable research efforts have been directed towards understanding the role of $CD44^{high}/CD24^{low}$ and $ALDH1A^+$ subtypes in breast CSC, as they have been associated with the hallmark characteristics of CSCs.[20,44,45,49,66–68] The presence of $CD44^{high}/CD24^{low}$ and $ALDH1^+$ cells in TNBC has been linked to more aggressive tumor behavior, increased risk of metastasis, and poorer patient outcome.[12,50]

Several signaling pathways, including Notch, Wnt, and Hedgehog, have been implicated in CSC self-renewal and chemoresistance.[16,56,69–72] Therefore, targeting these pathways and CSC-specific markers may hold promise for the development of novel therapeutic approaches aimed at eliminating CSCs and preventing disease relapse.

2.2.3 PTHrP, GLI Inhibition, and Chemoresistance

Parathyroid hormone-related protein (PTHrP) is a downstream effector of Gli2 and has been implicated in the development and progression of bone metastasis.[73,74] Elevated PTHrP expression in TNBC cells promotes osteoclast activity and bone resorption, contributing to the vicious cycle

of bone destruction observed in TIBD.[26,27,75] Therefore, targeting PTHrP signaling pathways may offer potential therapeutic strategies for mitigating bone metastasis and associated complications.

GLI inhibition has shown promise as a potential approach in overcoming chemoresistance and inhibit bone metastasis in TNBC.[76–78] By targeting Gli2, it may be possible to disrupt signaling pathways associated with CSC renewal, chemoresistance, and bone metastasis in TNBC.

2.3 Limitations

Despite the recent advancements made in the underlying mechanisms of TNBC and CSCs, the heterogeneic tumor microenvironments continue to pose significant obstacles in developing effective targeted therapies.[4,35,60,79–82] Various molecular subtypes and genetic alterations contribute to the diverse behavior and response to therapies observed in TNBC patients.[4,83] The identification of specific biomarkers and molecular targets that can be effectively exploited for therapy remains an ongoing research endeavor.[36,60,84]

While targeting Gli2 shows promise as a potential therapeutic strategy, several challenges need to be addressed before its clinical translation. The complexity of Gli2 signaling and its diverse functions in normal tissue development and homeostasis necessitate thorough investigation of potential off-target effects and systemic toxicity.[15,53] Moreover, further research is required to unravel the intricate mechanisms underlying Gli2 activation and its association with CSC populations, therapy resistance, and bone metastasis. Combining Gli2 inhibition with other therapeutic strategies, such as front-line chemotherapeutics, or immune-based approaches,[66,85–95] may enhance the efficacy of treatment and overcome potential limitations.

For these reasons, the characterization of CSC expression in TNBC cell lines and their bone metastatic derivatives may prove to be a significant step in developing novel therapeutics for TNBC.

Chapter 3

Characterization of the Relative Percentages of CSC Populations in TNBC Cell Lines and Their Bone Metastatic Derivatives

3.1 Introduction

CSCs have gained significant attention due to their role in tumor initiation, metastasis, and therapeutic resistance, especially in TNBC pathologies.[16–18,76,96–98] CSCs possess self-renewal capabilities and have been implicated in tumor recurrence and treatment failure.[4,7,9,62,99–101] Therefore, understanding the characteristics and behavior of CSCs in TNBC, particularly in the context of bone metastasis, is of great importance in developing targeted therapeutic approaches for treating TIBD.

In this first aim, we sought to investigate the relative percentages of CSC populations in human and murine TNBC cell lines and their bone metastatic derivatives. Specifically, we focused on human-derived MDA-MB-231 cells and 4T1 mouse cells for their distinctly heterogeneous cell populations harboring mesenchymal CSCs[72] Expression of $CD44^+/CD24^-$ cells as well as upregulation of ALDH are known to be reliable markers for identifying breast CSCs[21,22].

Our objective was to address the overarching question of whether bone metastasis enriches CSC populations and whether Gli2 is related to these metastatic cells. By examining the base-level expressions of Gli2 activation, $CD44^+/CD24^-$ CSCs and $ALDH^+$ CSCs in both human TNBC cell lines and their murine bone metastatic derivatives, we sought to determine the differences in the proportions of these CSC populations between the parental TNBC cells and their bone-metastasized counterparts.

3.2 Materials and Methods

3.2.1 Materials

All antibodies and Fc-blocking materials were obtained from BioLegend unless otherwise specified.

3.2.2 CSC Antibody Staining

Cells were cultured at 37°C in DMEM (Gibco) with 10% heat inactivated FBS (Corning) and 1% Penicillin-Streptomycin (Gibco). Cells were collected for the experiment once they reached confluency – approximately 80% of the bottom surface of the T75 flask is covered in a monolayer of adhered cells. Cell media was aspirated and washed with -/- PBS (Corning). The cells were then incubated in Phenol Red-free TrypLE Express dissociation reagent (Fisher Scientific) for 3 minutes and centrifuged at 500 rcf at 4° C for 5 minutes. The resulting pellet was resuspended in -/- PBS and a cell count was performed. $3x10^6$ cells were collected in a separate tube for staining and centrifuged at 500 rcf for 5 minutes. The supernatant was aspirated, and the cell pellet was resuspended in 2 mL of 5% Human TruStain FcX (anti-mouse TruStain FcX PLUS for murine cell lines) and incubated for 10 minutes at room temperature. Separately, antibody dilutions were created in -/- PBS solution, stored on ice and isolated from any light sources. Fc-blocked cells were then centrifuged and resuspended in 700 µL of -/- PBS and divided evenly among the antibody dilution tubes. Cells were incubated in the antibody solutions for 30 minutes at room temperature isolated from any light sources. The antibody-stained cells were then centrifuged at 500 rcf at 4°C for 5 minutes and washed with -/- PBS three times before plating for flow cytometry. Each cell type was gated according to an Fc-blocked, unstained control and their respective isotype controls. The resulting cell population (**Figure 4**) is shown.

Each cell type was gated according to an Fc-blocked, unstained control and their respective isotype controls. The resulting cell population (**Figure 4**) is shown.

3.2.3 Bone Clone Cell Lines

MDA-MB-231 human breast cancer cells and 4T1 murine breast cancer cells were purchased from ATCC (notated throughout as parental cells). Bone metastatic clones (bone clones, BC) of these cell lines were generated by Dr. Julie Rhoades' lab as previously published.[102] Briefly, cells were injected into left cardiac ventricle of athymic nude mice (MDA-MB-231) or Balb/c mice (4T1), and disseminated tumor cells were collected from the bone marrow at sacrifice. Cells were

cultured in DMEM (MDA-MB-231; Gibco) or RPMI 1640 (4T1; Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Corning) and 1% penicillin-streptomycin (Corning).

3.2.4 ALDH$^+$ CSC Assay

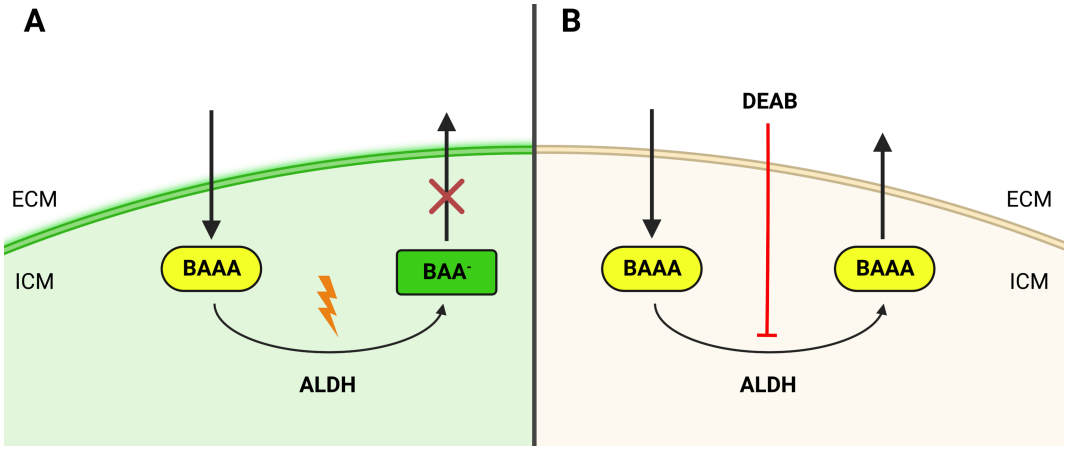

Figure 2: ALDEFLUOR^{TM} assay diagram. **A.** Cellular fluorescence without DEAB inhibitor. **B.** No cellular fluorescence when DEAB inhibitor is present. Made in BioRender.

ALDEFLUOR^{TM} Kit (StemCell Technologies) was utilized for the detection of cells expressing high levels of ALDH1$^+$ activity. DRAQ7^{TM} Dye (Abcam) was utilized for the staining and detection of nucleic acid from non-viable cells in the far-red region of visible light. Since the excitation-emission spectrum of ALDEFLUOR^{TM} falls within the green region, and DRAQ7^{TM} Dye within the red region, this makes the two choices ideal for combining in flow cytometry experiments, as this ensures negligible spectral overlap between channels. Probenecid (Cayman Chemical) was dissolved in 500 µL DMSO and added to ALDEFLUOR^{TM} assay buffer to create a 2.5 mM solution to improve cellular efflux channel inhibition and subsequently increase fluorescence intensity and data reliability. ALDEFLUOR samples were prepared according to the manufacturer-defined protocol for SKBR3 human breast cells.[103] After incubating the ALDEFLUOR-reacted cells at 37°C, additional measures were taken to ensure minimal cellular efflux of the ALDEFLUOR^{TM} reagent by keeping test tubes on ice at all times after the incubation step. The final round-bottom 96-well plate used for the flow cytometry analysis was also kept chilled on ice until analysis was to be performed. A Guava easyCyte (Millipore) was

used for conducting flow cytometry, and data was analyzed using the FlowJo software.

3.2.5 ALDEFLUOR™ Flow Cytometry Gating Method

The gating strategy employed in this study involved sequential gating to exclude debris and dead cells that may interfere with the results. Since the peak emission of the ALDEFLUOR™ dye fluoresces within the green region of the visible spectrum, our population of interest is expected to have an intense green fluorescence. However, special care must be taken in order to isolate the ALDH1$^+$ CSC population. Initially, a gate was set on the Forward Scatter (FSC) vs Side Scatter (SSC) plot to exclude debris and ensure that the remaining events were cells. Then, a gate was applied on the RED-R Hlog (fluorescence emission in the red channel) vs SSC plot. DRAQ7 staining was utilized to identify dead, lysed, or otherwise non-viable cells as they exhibit a high RED-R fluorescence. The final gating step aimed to identify cells with high ALDH activity. A gate was set on the Green-B Hlog (fluorescence emission in the green channel) vs SSC plot, whereby the gating was drawn as close to the main population as possible. By applying this gating strategy, the population of interest exhibiting both high ALDH activity and intact viability were successfully identified and analyzed. Untreated cells with DEAB inhibitor were used as the negative controls for each respective cell line and were used to establish the gates. Each untreated and treated cell sample utilized the gating from "Untreated + DEAB" to determine the relative CSC percentages (**Figure 3**).

3.2.6 Western Blots for Native TNBC CSC Expression

Western blots were performed to assess natural Gli2 activation in MDA-MB-231 parental cells and their bone clone derivatives.

Cells were cultured in triplicate wells of a six-well plate and harvested using 0.25% Trypsin. A cell count was performed to obtain a concentration of at least 4 x 10^5 cells/mL for adequate protein yield. A BCA protein assay (Pierce) was conducted according to the manufacturer's protocol, and the western blot (Pierce) was conducted according to the manufacturer's protocol (Pierce). 20µg of protein were loaded onto 4-20% Mini-PRTOEAN TGX$^+$ Precast Protein Gels, 10-well, 50 µL

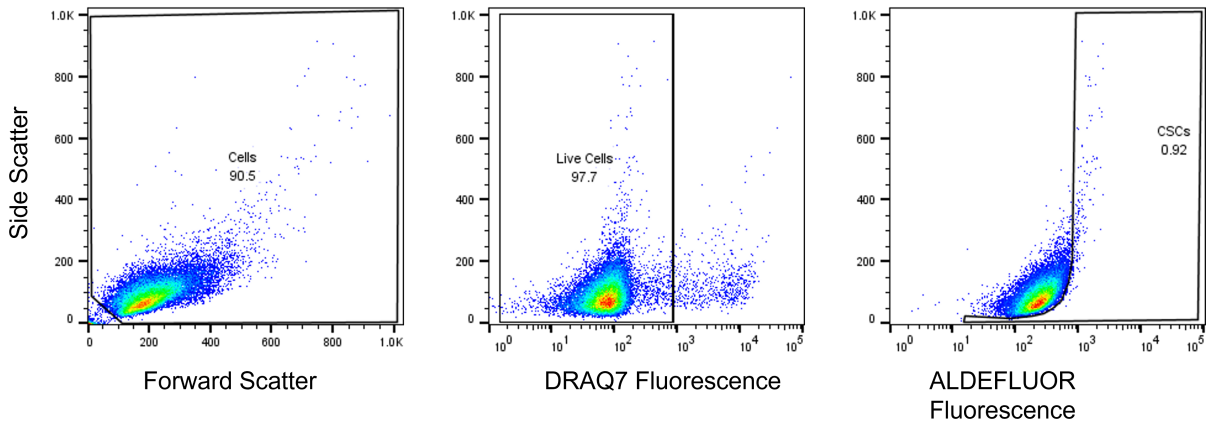

Figure 3: ALDEFLUOR^{TM} assay gating workflow using MDA-MB-231 Bone Clone + DEAB inhibitor cells as a representative example.

(Bio Rad) with Tris-Glycine SDS running buffer ran in 100V. A Mini Protean Tetra Cell 4-gel System (Bio Rad) was used for electrophoresis. The protein transfer was conducted using the iBlot^{TM} 2 Dry Blotting System (Thermo Fischer) onto nitrocellulose membranes according to the manufacturer's protocol.

The membrane was removed and cut in between ladder bands using a razor blade to separate different portions for parallel antibody staining. The membrane was then submerged in 10 mL of TBS Intercept Blocking Buffer (LI-COR Biosciences) and washed at room temperature using a rocker for 1 hour. Separately, 10 mL of a monoclonal primary antibody solution was prepared from a 1:1 TBST and intercept blocking buffer solution, 1:1000 Tween20, and 1:500 of anti-ALDH1A1, anti-Gli2 mAb (Sigma Aldrich) or 1:1000 anti-GAPDH (BioLegend). The membrane was then removed from the blocking buffer and washed with TBST three times for 10 minutes before submerging in the primary antibody solution and probed at 4°C overnight. The following day, the membranes were removed from the primary antibody solution and washed three times with TBST for 10 minutes. Two secondary antibody solutions were prepared; the Gli2 membranes used a 1:5000 solution of IRDye 800 CW Goat anti-Rabbit IgG mAb (LI-COR) or IRDye 800CW Donkey anti-Rat IgG mAb solution (LI-COR). The membranes were submerged in their respective secondary antibody solutions and incubated on a rocker at room temperature for 1 hour, followed

by three washes with TBST for 10 minutes. After the third wash, the membranes were taken for imaging. Western blot signal detection was achieved using Odyssey FC Imaging System (LI-COR) and a ChemiDoc MP Imaging System (Bio-Rad) to visualize the fluorescent bands. Western blots were quantified by densitometry using ImageJ software (National Institutes of Health).

3.3 Results

The $CD44^{high}/CD24^{low}$ phenotype is known to be a reliable marker for identifying CSC phenotypes in breast cancers[83,87,98] Herein, we aimed to quantify the basal expression of $CD44^{high}/CD24^{low}$ CSC populations in parental cells and their bone clone derivatives through fluorescence-activated cell sorting (FACS). We utilized an anti-human/anti-mouse CD44-FITC (Sigma) and anti-human CD24-APC antibody-fluorophore conjugates to identify these particular surface markers. Flow cytometry analysis revealed a noticeable presence of this CSC phenotype in MDA-MB-231 cells compared to the murine 4T1 cells (**Figure 4**). However, there were no observable differences in expression of $CD44^{high}/CD24^{low}$ CSCs between parental and bone clone derivatives.

When evaluating the $ALDH^+$ CSC subtype, obvious differences had emerged between parent and bone clone cell types (**Figure 5**). In both MDA-MB-231 and 4T1 cell lines, bone clones appeared to have a proportionally larger $ALDH^+$ CSC population (MDA: 12.8% vs 0.44%, 4T1: 12.3% vs 6.73%) than the parental counterpart, consistent with the hypothesis that bone metastasis enriches the $ALDH^+$ CSC subtype. Additionally, 4T1 murine cell lines have a larger $ALDH1^+$ than the MDA-MB-231 cells. DEAB inhibitor served as the control for each cell type, since DEAB works by inhibiting the ALDH enzyme. As a result, the BAAA reagent in the assay is not converted into its negatively-charged carboxylic acid form, and thus freely passes through the cellular membrane, leaving no fluorescent molecules to be observed within the cell. The overall effect is that DEAB+ cells will exhibit a significantly smaller $ALDH^+$ population than the DEAB- test samples (**Figure 2**).

After establishing the nominal expression of the $CD44^{high}/CD24^{low}$ and separately the

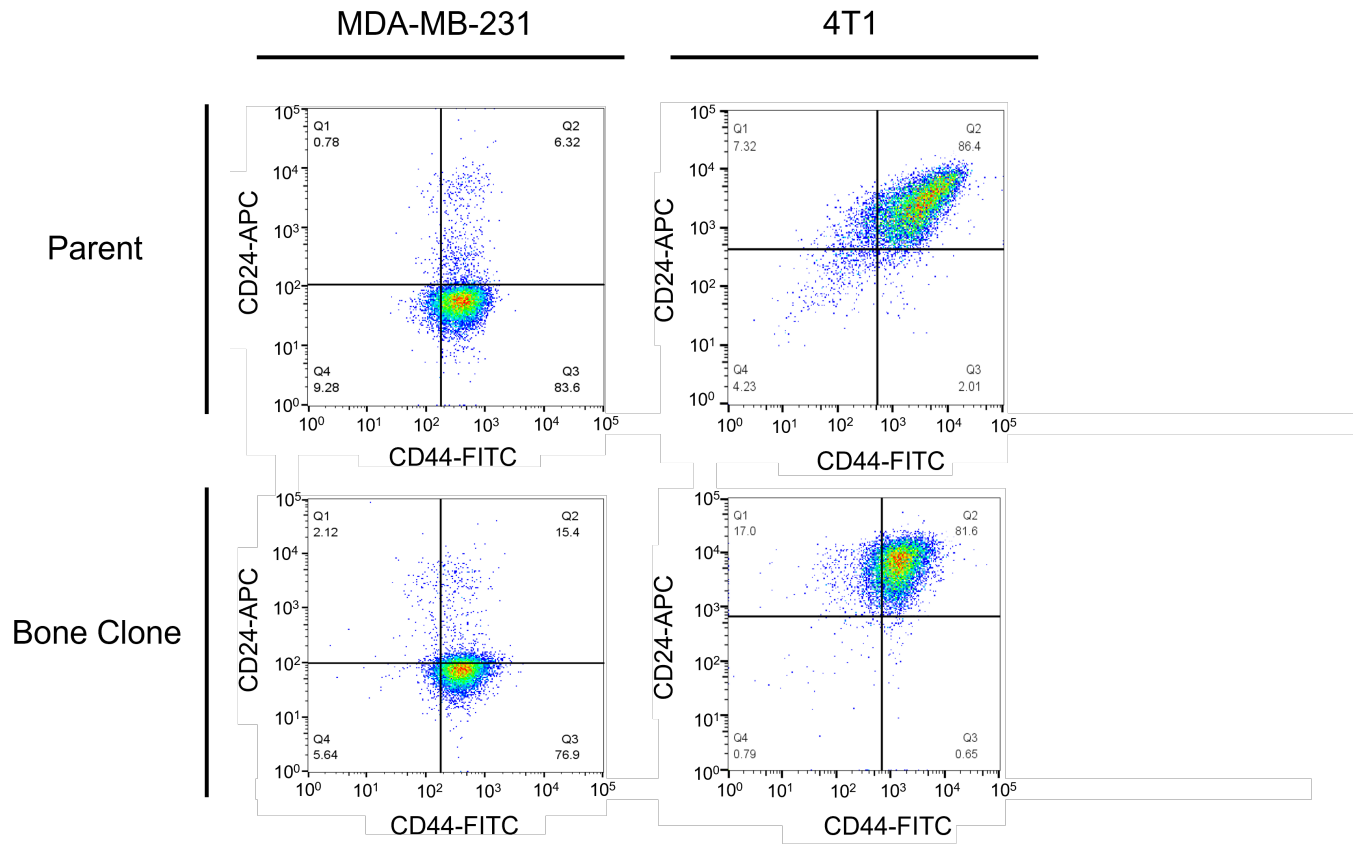

Figure 4: Representative plots quantifying native $CD44^{high}/CD24^{low}$ CSC populations in human and murine TNBC cell lines. n = 1.

$ALDH1^{+}$ CSC expressions, we aimed to see whether Gli2 may be correlated to bone metastasis in TNBC. A western blot was performed to compare the Gli2 activation in MDA-MB-231 parental and bone clone derivative (**Figure 6**) and analyzed by densitometry. Clear Gli2 bands were observed in both parental and bone clone derivative, however, densitometry revealed a higher level of activation in the bone clones. This suggests that Gli2 may be correlated with bone metastasis, linking Gli2 to the observed chemoresistant behaviors observed in TIBD and bone metastatic cells.

3.4 Discussion

Our findings revealed differences in the expression of the $CD44^{high}/CD24^{low}$ CSC phenotype between MDA-MB-231 and 4T1 cells. (**Figure 4**) Both the MDA-MB-231 parental cells and

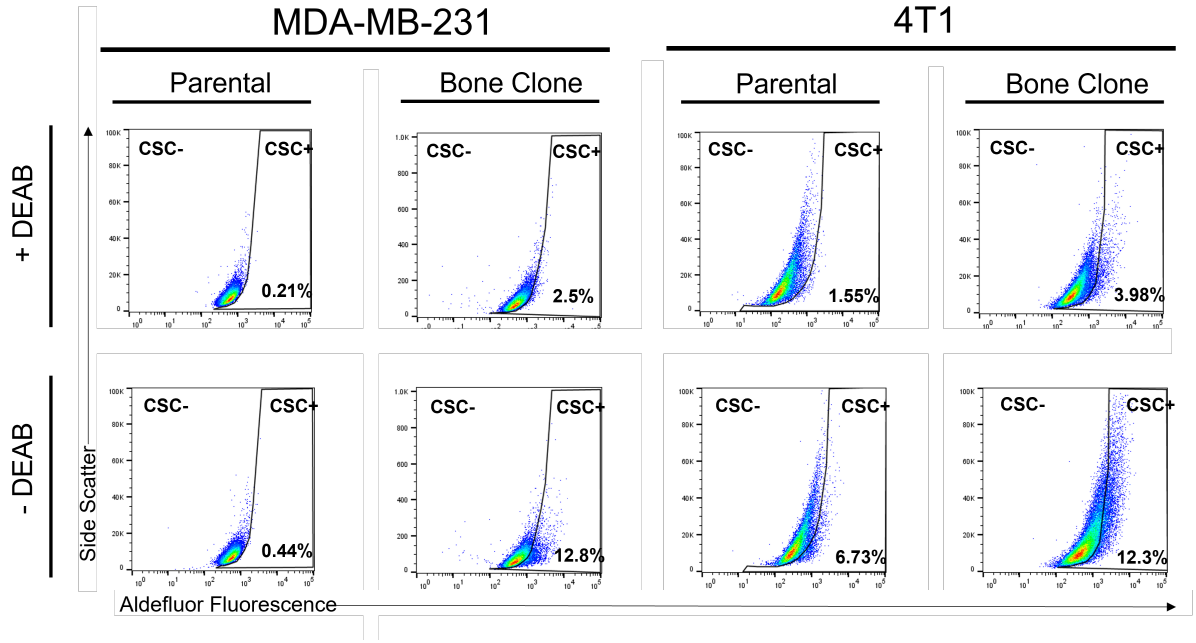

Figure 5: Representative basal ALDH^{+} CSC populations in human and murine TNBC cell lines. n = 1.

MDA-MB-231 bone clone cells demonstrated high expression (Parent = 83.6%, Bone Clone = 76.9%) of this CSC population. This observation aligns with previous studies reporting the presence of CD44^{high}/CD24^{low} CSCs in MDA-MB-231 breast cancer cells, which are associated with aggressive tumor behavior and metastatic potential.[67]

In contrast, the 4T1 parental cells and 4T1 bone clone cells exhibited minimal expression (Parent = 2.01%, Bone Clone = 0.65%) of the CD44^{high}/CD24^{low} CSC phenotype. This unexpected finding suggests a discrepancy in the presence or regulation of this specific CSC population within the 4T1 cell line. These results are in contrast to our initial hypothesis, which proposed that bone metastasis would enrich CSC subpopulations, including CD44^{high}/CD24^{low} cells. However, our data indicates that the bone metastatic 4T1 cells do not exhibit elevated expression of this CSC subtype compared to the parental cells.

Several factors could contribute to the observed differences in CSC expression between MDA-MB-231 human and 4T1 mouse cell lines. One possibility is that the cells in this study were cultured as an adherent monolayer rather than as spheroids, which are known to more accurately

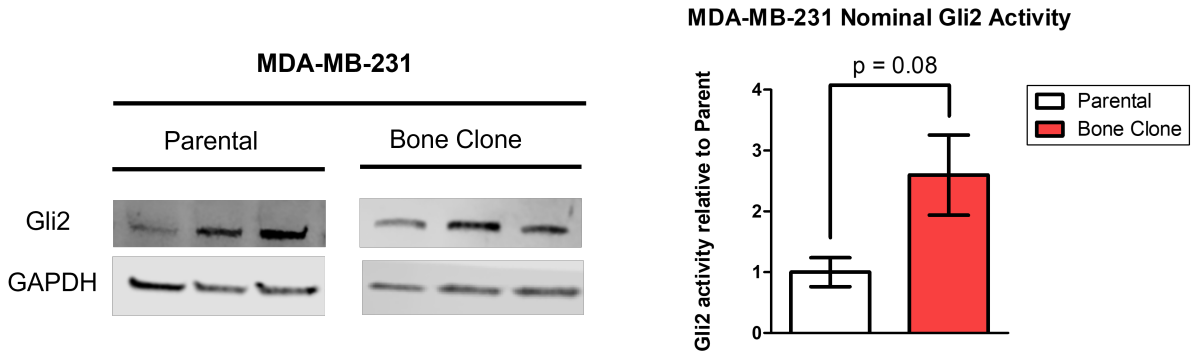

Figure 6: Western Blot analysis of Gli2 activation in MDA-MB-231 cells. (*Left*) Representative western blot images of Gli2 activation in Parental cells and their Bone Clone derivatives. (*Right*) Densitometry analysis of Western Blot images in Parent and Bone Clone MDA-MB-231. Data are presented as the mean ± S.E. p = 0.08 by paired two-tailed Student's *t*-test.

resemble the tumor microenvironment. In addition, spheroids have been shown to induce stemness in cancer cells[3,14,104], which may explain the lack of observed $CD44^{high}/CD24^{low}$ in 4T1 cells as well as the lack of observable differences between parental MDA-MB-231 cells and their bone clone derivatives.

Another factor that may contribute to the observed differences is the influence of the tumor microenvironment. The interactions between cancer cells and the surrounding environment, including stromal cells,[73] extracellular matrix components,[42,52,105] and soluble factors, can modulate the expression of stem cell markers. The microenvironment encountered by MDA-MB-231 cells might be more supportive of the $CD44^{high}/CD24^{low}$ CSC phenotype compared to the microenvironment experienced by 4T1 cells.

3.5 Conclusion

In any case, bone clone derivatives showed no significant differences from the parental cells in terms of $CD44^{+}/CD24^{-}$ CSC expression, suggesting that bone metastasis may not enrich this CSC subtype. As a result, this $CD44^{high}/CD24^{low}$ phenotype was not pursued in Aim 2 for analyzing the effects of drug treatments on Gli2 expression and CSC enrichment. Instead, we focused our efforts on characterizing the expression of the $ALDH1^{+}$ CSC subtype as a reliable CSC marker for

subsequent *in vitro* experiments.

Chapter 4

Effects of PTX, GANT58, and Combination Therapy in CSC Populations in TNBC Cell Lines and their Bone Metastatic Derivatives

4.1 Introduction

The aggressive characteristics associated with TNBC are due in part to the lack of ER, PR, and HER2 expression, leaving virtually no available targets for effective targeted chemotherapy strategies.[3,35] TNBC is associated with increased rates of bone metastasis,[31,38,106] and possible development of TIBD with poor clinical outcomes.[32,34,40] While current treatments for TIBD inhibit destruction of bone matrix through the use of RANKL inhibitors or bisphosphonates[7,107,108], they do not effectively prevent tumor burden or improve patient survival.[7]

The presence of CSCs, characterized by their self-renewal ability and tumor-initiating capacity, contributes to the heterogeneity and therapeutic resistance observed in TNBC.[4] Targeting CSCs has emerged as a promising strategy to improve treatment outcomes and prevent disease recurrence.[2,62,65,82] In our previous aim, we investigated the role of Gli2 transcription factor in bone metastasis and CSC regulation. However, it remains unclear how the combined treatment of chemotherapeutic agents with Gli2 inhibitors impacts CSC populations.

PTX is a frontline chemotherapeutic agent used in TNBC treatment[109,110], but its efficacy is limited by chemoresistance[111] and bone marrow toxicity.[112] GANT58 has shown promise in blocking TIBD and reducing CSC populations.[26] However, the effects of PTX, GANT58, and their combination on CSC populations in TNBC cell lines and their bone metastatic derivatives remain to be elucidated.

Understanding the effects of PTX, GANT58, and combination therapy on CSC populations in TNBC is crucial for developing more effective treatment strategies. Specifically, determining the impact of these treatments on the enrichment and chemosensitivity of CSCs in both the parental

TNBC cells and their bone metastatic derivatives will provide valuable insights into their therapeutic potential.

In this second aim, we aimed to evaluate the effects of PTX, GANT58, and their combination treatment on $ALDH^+$ CSC populations in TNBC cell lines and their bone metastatic derivatives. We utilized parent primary tumor MDA-MB-231 TNBC cells along with their bone-metastasized derivatives (bone clones) to assess changes in CSC markers and functional properties in response to treatment. By investigating the effects of these treatments on Gli2 and $ALDH^+$ CSC populations, we aim to uncover potential strategies to target and eradicate CSCs in TNBC, ultimately improving TIBD patient outcomes.

4.2 Materials and Methods

In vitro analysis of $ALDH^+$ expression was performed using the ALDEFLUOR^{TM} assay in conjunction with DRAQ7 dye. Western Blots for assessing Gli2 activation and additional confirmation of ALDH expression were performed. Both methods were performed using the same protocols outlined in Chapter 3.2.

4.2.1 Paclitaxel/GANT58 Drug Synergy

To investigate the effects of Gli2 inhibition on chemosensitivity to paclitaxel, a drug synergy study was performed on MDA-MB-231 parental and bone clone TNBC cells. These cells were cultured at a 1000 cell/well seeding density in 96-well plates with 100 µL of media and allowed to incubate at 37°C overnight. Paclitaxel (LC Laboratories) and GANT58 (MedChem Express) were each dissolved in a dimethyl sulfoxide (DMSO) vehicle in serial dilutions ranging from 300µM to 0.0001µM, and a library of drug combinations ranging therapy from 1:1 PTX to GANT58 to 3,000,000:1 PTX to GANT58. After 24 hours of incubation, the media was removed from each well and replaced with 99 µL of fresh media and 1 µL at the intended concentration. Cells were treated with each drug concentration in triplicate wells and allowed to incubate for another 72 hours, after which each well was replaced with another 100 µL of fresh media. An MTS cell viability assay (Abcam) was conducted. Cells were incubated with MTS reagent at 37°C for 2

hours before being analyzed colorimetrically on a NanoQuant plate reader (Tecan). The readings were analyzed using SynergyFinder[113] to obtain separate dose-response curves for paclitaxel and GANT58 as well as a ZIP synergy heatmap and ZIP score (**Figure 7**).

4.2.2 ALDH1$^+$ CSC Treatment

It was determined in pilot studies that the IC80 doses induced more pronounced ALDH$^+$ phenotypes than IC50 concentrations, and were selected for this experiment. MDA-MB-231 cells were seeded in six-well plates at a seeding density dependent on the paclitaxel, GANT58, and combination IC80 concentrations. The treatment procedures for this experiment were similar to those previously described for the drug synergy analysis, however, volumes of media and drug were scaled up to reflect the size of the wells being utilized. After 72 hours of treatment, each sample well was lifted from the bottom of the plate using 0.5 mL of 0.25% Trypsin-EDTA 1X (Gibco) with a 5 minute incubation at 37°C. Once cells were fully dissociated from the plate, the trypsin was quenched with 1 mL of DMEM and a cell count was performed to obtain a desired concentration of 1 x 10^6 cells/mL. A 1 mL cell suspension at this concentration was placed into a 1.5 mL conical tube (Eppendorf) and centrifuged at 600 rcf for 5 minutes. The subsequent steps for preparing ALDEFLUOR^{TM} samples were similar to those described in Chapter 3.2.4.

4.3 Results

ZIP synergy analysis through SynergyFinder[113] (**Figure 7**) showed that GANT58 has an IC50 of 12.76 µM in parental MDA-MB-231 cells, whereas the bone clone derivative had a slightly higher IC50 of 15.88 µM. In contrast, paclitaxel exhibited a similar IC50 value of 0.003 µM. Whereas in bone clone derivative, GANT58 required a slightly higher concentration to achieve an IC50 and paclitaxel required a significantly higher concentration (10 µM) to achieve the same effect. This suggests that bone clones are significantly more chemoresistant to chemotherapeutic while possessing similar tolerance to GANT58. The ZIP synergy heatmaps display concentrations that exhibit high synergy (red). In the parental cells, a 1000:1 concentration of GANT58 to paclitaxel was shown to be highly synergistic (1 µM GANT58 to 0.001 µM

paclitaxel), however, this dose of paclitaxel is small enough that it falls within the "noisy" region of the heatmap and the resulting synergy output for MDA-MB-231 parental cells may be ignored. Whereas in the bone clones, a ZIP synergy score of 50, indicative of very high synergy, was observed in the 10 µM GANT58 to 1 µM paclitaxel region. Each respective dose of drug was sufficiently large so as to not be influenced by suboptimal treatment normally encountered with more dilute concentrations. As a result, this 10 µM GANT58 to 1 µM paclitaxel combination therapy was chosen for use the *in vitro* drug treatment experiments of Aim 2.

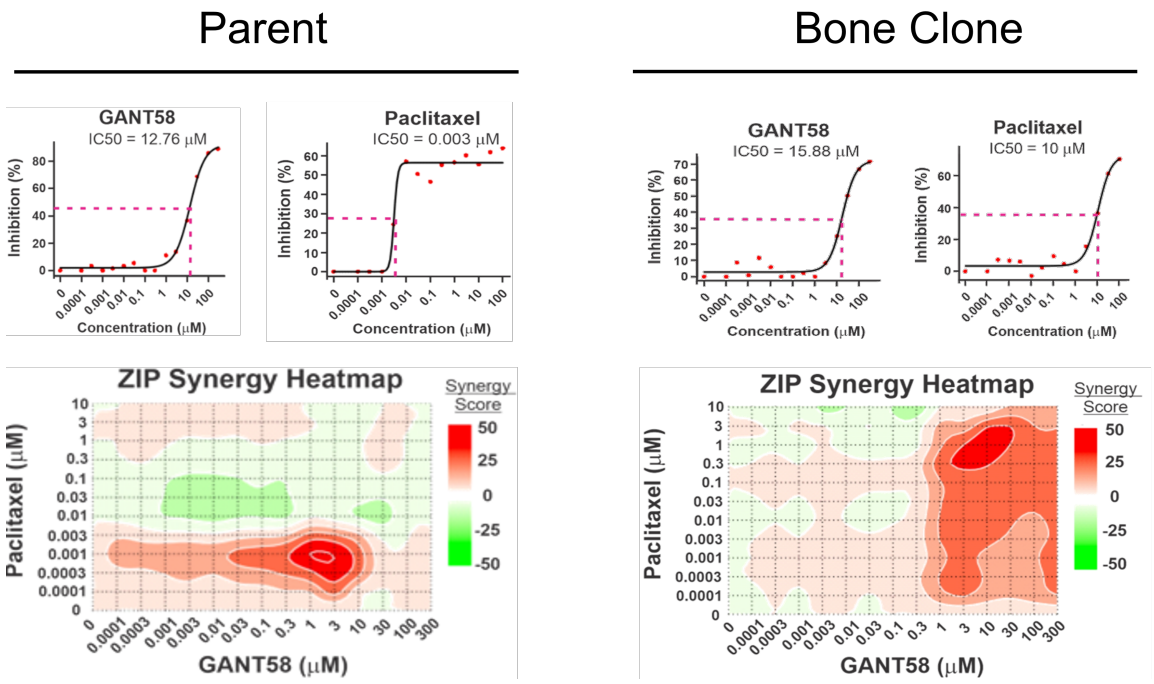

Figure 7: Paclitaxel and GANT58 exhibit strong synergy in MDA-MB-231 cell lines, with a more pronounced effect in the bone clone derivative. (*Left*) Representative dose-response curves (n = 1) for paclitaxel, GANT58 in MDA-MB-231 parent cell line with synergy heatmap. (*Right*) Similar drug synergy analysis and synergy heatmap in MDA-MB-231 bone clone derivatives.

Additional ALDEFLUOR^{TM} assays were conducted on treated parental and bone clone MDA-MB-231 cells to evaluate the effects of Gli2 inhibition, enrichment, and toxicity in the ALDH1^{+} breast CSCs (**Figure 8**). Bone clones have a higher basal ALDH^{+} CSC population than parental MDA-MB-231 cells. Bone clones also have more pronounced enrichment of this CSC subtype with paclitaxel chemotherapy (Bone clone: 17.1%, Parental: 10.4%, n = 1), which falls in line with the

theory that bone metastasis enriches CSC populations. Interestingly, combination treatment seems to improve chemosensitivity of the ALDH$^+$ CSCs as there are markedly smaller populations of this subtype for both parental and bone clone subtypes.

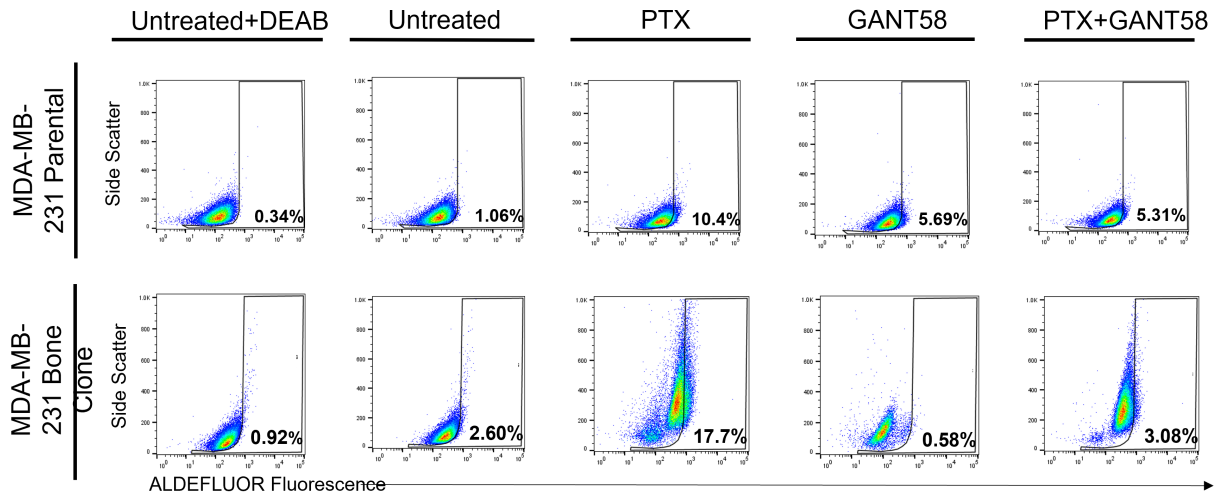

Figure 8: Representative FACS plots of MDA-MB-231 parental and bone clone derivatives subjected to treatment. Data are shown on the green-log vs side scatter plot. Gates for CSCs are shown on the right-hand side of each plot. A single representative treatment group (n = 1) is shown for both parental and bone clone for purposes of showing a direct 1:1 comparison of treatment.

This effect was more pronounced in the bone clone MDA-MB-231 cells, which exhibited a smaller ALDH1$^+$ CSC population than parental cells (Bone clone: 3.1%, Parental: 5.3%). This is likely attributed to the stronger synergistic relationship observed with bone clones (**Figure 7**) for the 10:1 GANT58 to paclitaxel treatment. GANT58-only treatment appeared to have a noticeable effect in targeting the ALDH$^+$ subtype overall since GANT58-only treatment results in a much smaller CSC proportion than either paclitaxel-only or combination therapy.

This treatment experiment was repeated, and the aggregate data was plotted for each treatment group. The data were normalized to the mean untreated DEAB cell from each run. For the MDA-MB-231 parental cells (**Figure 9**), it is observed that the trends of relative CSC-percentage closely matches that of PTHLP expression, indicating that there may be a relationship between regulation of Gli2 expression, ALDH1$^+$ CSC proliferation, and resistance to chemotherapy. Western blot analysis of these cells confirmed that ALDH1$^+$ CSCs were upregulated from

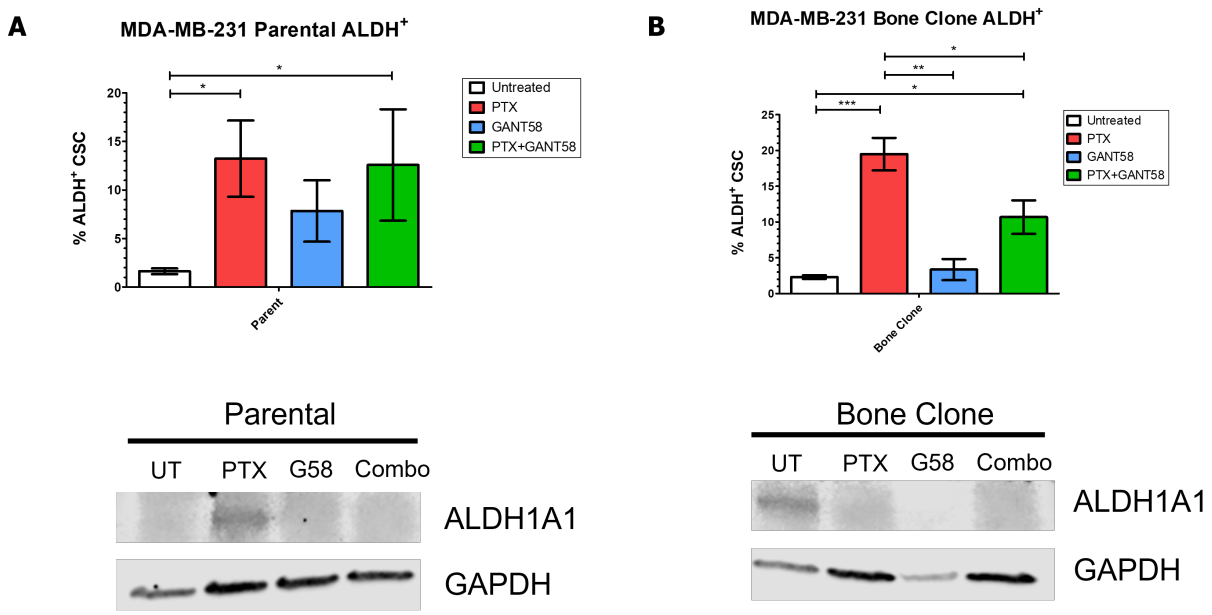

Figure 9: Gli2 activity and ALDH$^+$ CSC activity are correlated with chemosensitivity. Mean CSC percentages $\pm$ S.E. (one-way ANOVA with Tukey's) are presented with treatment conditions normalized to untreated + DEAB controls (not shown). **A.** (*Top*) PTX-only (n = 3, *p $<$ 0.05) and combination treatment (n = 3, *p $<$ 0.05) significantly enriched ALDH$^+$ expression. (*Bottom*) Western blot analysis (n = 1) reveals slight enrichment of ALDH1A1 expression in PTX-only treatment. **B.** (*Top*) PTX-only treatment (n = 6, ***p $<$ 0.0005) and combination (n = 7, *p $<$ 0.02) significantly enrich ALDH$^+$ expression compared to untreated control, while GANT58-only (n = 3, **p $<$ 0.002) and combination (*p $<$ 0.05) reduce this effect compared to PTX-only. (*Bottom*) Western blot images (n = 1) reveal ALDH1A1 activity is present in untreated control but reduced in all treatment conditions.

treatment with paclitaxel-only, confirming that bone metastasis enriches this CSC phenotype.

4.4 Discussion

Prior research indicates that the stemness of cancer cells can be promoted through stress-inducing factors such as drug treatment, hypoxia, exogenous on the tumor microenvironment including drug treatment[8,18,96], and has shown that TIBD can be regulated by Gli inhibition.[26,29,72,72,78,107,114] The previous chapter delineated the link between bone metastasis and upregulated Gli2 activation. This chapter investigated the relationship between the stemness of tumor cells and Gli2 activation by observing the therapeutic effects of paclitaxel, GANT58, and combination therapy separately on parental and bone-metastasized TNBC cells.

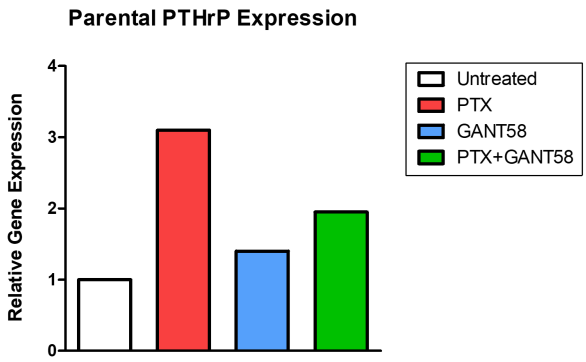

Figure 10: PTHrP expression in parental MDA-MB-231 cells.

Our initial experiments aimed to characterize the degree of chemoresistance within the MDA-MB-231 parental and bone clone cells by obtaining dose-response curves of paclitaxel and GANT58, and further assess the potential benefit in using combination therapy. For this drug synergy study, we created a library of paclitaxel/GANT58 formulations for *in vitro* experiments and found a synergistic ratio between paclitaxel and GANT58 in the treatment of MDA-MB-231 breast cancer cells (**Figure 7**). Firstly, this finding confirmed the benefit in utilizing GANT58 as an adjunct to chemotherapy for a novel strategy in improving therapeutic effect of paclitaxel in chemoresistant bone metastatic cells. Secondly, the benefits of such combination were more pronounced in the MDA-MB-231 bone clones, suggesting that their tolerance to paclitaxel may be linked to the observed upregulation of Gli2 activity (**Figure 6**).

To further investigate the potential link of Gli2 with cancer stemness and chemoresistance, we examined the effects of paclitaxel, GANT58, and synergistic combination on $ALDH^+$ CSCs through FACS. Aldefluor assays were conducted in triplicate wells to identify potential changes in $ALDH^+$ CSC expression after treatment (**Figure 8**). In this representative plot, the $ALDH^+$ CSC subtype is enriched in all treatment conditions when compared to the untreated controls, and this trend is observed in both parental and bone clone cell types (Parental: 10.4% vs 1.1%, Bone Clone: 17.7% vs 2.6%). In repeated trials (**Figure 9**), we found these trends to be statistically significant, suggesting that paclitaxel-only treatment enriches the $ALDH^+$ CSC population. This increase in $ALDH^+$ expression can be explained by two different mechanisms that promote CSC populations.

The first is selective survival and reproduction of CSCs. Regular, non-stemlike TNBC cells that lack chemoresistance and self-renewal capabilities may be more susceptible to the effects of chemotherapy. As a result, these cells are eliminated or significantly reduced in number, while the more chemoresistant cancer cells survive. In a case of natural selection, the selective pressure created by chemotherapy favors the characteristics and traits that enable CSCs to thrive. Specifically, paclitaxel destroys non-CSCs which lack the chemoresistant traits, allowing the $ALDH^+$ CSCs to survive and proliferate, resulting in a proportionally larger CSC population. In contrast, GANT58-only treatment did not significantly enrich $ALDH^+$ CSCs because GANT58 alone does not appear to be an effective chemotherapeutic. Therefore, GANT58 would not be a major selective pressure. Recall that GANT58 is a Gli2-inhibitor that prevents translocation of Gli2 transcription factor to the nucleus. During GANT58 treatment, normal TNBC cells are not being killed off, and the proportion of $ALDH^+$ CSCs is left virtually unaffected. Therefore, it follows that a significant enrichment of $ALDH^+$ CSCs is not observed in the GANT58-only treatment.

Another explanation for the observed increase in proportion of $ALDH^+$ CSCs involves stress responses and epigenetic mutations that potentiate stem-like behaviors including self-renewal and increased resistance to ani-cancer drugs.[95,96,115–118] The tumor microenvironment is dynamic and, when exposed to sublethal doses of chemotherapy drugs, may experience stress responses including E-cadherin knockdown[119], Sp1 activation[96], and epithelial-mesenchymal transition[105,119] amidst other factors.[120]

In both parental and bone clone cells, paclitaxel-only (Parental: $p < 0.03$, Bone clone: $p = 0.0002$) and combination therapy (Parental: $p < 0.04$, Bone clone: $p < 0.02$) generated significant enrichment of the $ALDH^+$ subpopulation, while GANT58-only treatment did not (**Figure 9**) when compared to the untreated control. Interestingly, paclitaxel/GANT58 combination therapy effectively reduced the enrichment of $ALDH^+$ CSCs in both MDA cell types, with a more pronounced effect in bone clones. These results indicate that paclitaxel treatment enriches the CSC populations whereas GANT58 has no such effect. Rather, GANT58 appears to reduce the

$ALDH^+$ CSC expression compared to paclitaxel-only. A similar trend is observed in **Figure 10**, further suggesting a correltation between Gli2 and chemoresistance, considering that PTHrP is a downstream effector of Gli2. When considering the drug synergy data and the Gli2 expressions established in Aim 1, these findings strongly suggest that not only might Gli2 play a mechanistic role in potentiating bone metastasis, but is likely also linked to attenuating chemoresistance in metastatic cell derivatives.[121] Therefore, GANT58 shows considerable potential in $ALDH^+$-targeting therapy in combination with paclitaxel.

4.5 Conclusion

The results acquired in this aim help to establish a novel strategy for treating bone metastatic cancers which lack targeted therapies, exhibit stemlike behaviors that potentiate tumor recurrence and invasiveness, and chemoresistance. GANT58 was shown to successfully attenuate Gli2 expression in the luminal $ALDH^+$ CSCs. Importantly, this attenuation was achieved with a moderate 10 μM dose and did not encounter issues with delivery, suggesting that GANT58 can target Gli2 to efficiently inhibit tumorigenesis at lower concentrations than previously observed.

Cell Line		6-Well	96-Well
Parental MDA-MB-231	**Untreated**	50,000	1,000
	PTX	150,000	3,000
	GANT58	40,000	1,000
	PTX + GANT58	150,000	3,000
Bone Clone MDA-MB-231	**Untreated**	50,000	1,000
	PTX	150,000	3,000
	GANT58	250,000	3,000
	PTX + GANT58	150,000	3,000
Parental 4T1	**Untreated**	35,000	1,000
	PTX	150,000	2,000
	GANT58	80,000	1,000
	PTX + GANT58	150,000	2,000
Bone Clone 4T1	**Untreated**	35,000	1,000
	PTX	105,000	3,000
	GANT58	175,000	5,000
	PTX + GANT58	105,000	3,000

Table 4.1: Cell seeding densities for drug treatments.

Chapter 5

Future Directions

5.1 Concerns, Limitations, and Future Directions

The inherently diverse cellular population of TNBC posed several challenges for this project. One of the main limitations resulted from focusing on a single ALDH1A1 primary antibody for western blot analysis. While the use of ALDH1A1 revealed the enrichment of $ALDH^+$ CSC populations in bone metastatic cells, ALDH1A1 is just one of nineteen isoforms of the ALDH enzyme.[21,122] Recent research indicates that ALDH1A1 in fact may not be the primary contributing isoform for positive aldefluor staining.[22,123,124] Croker et. al investigated the functional roles of ALDH1A1 and ALDH1A3 isozymes in breast cancer cells through siRNA knockdown.[99] They found that ALDH1A3 correlated with overall ALDH activity, cellular adhesion and migration, and colony formation whereas ALDH1A1 was correlated with metastasis and chemoresistance. In the context of research thesis, the ALDH1A1 western blot results may not be sufficient for confirming the $ALDH^+$ enrichment observed in the aldefluor assays (**Figure 9**). As such, the discrepancy between western blot images from Aim 2 and the expected $ALDH^+$ CSC population may be attributed, at least in part, to the limitations of using ALDH1A1 primary antibody as the sole marker for evaluating ALDH activity. Future experiments seeking to compare $ALDH^+$ CSC expressions via aldefluor assay with protein expression via western blot may benefit from pursuing other isoforms, such as ALDH1A3.

In a similar vein, Gli2 belongs to a larger family of GLI transcription factors whose role in non-canonical hedgehog-signaling pathways includes regulating invasiveness and progression of breast cancer cells.[125] In addition to Gli2, Gli1/3 isoforms have been implicated in regulation of osteoclastogenesis and their activity has been shown to be inhibited by GANT58.[71,91,114,126] This project mainly focused on the role of Gli2 transcription factor as a potential means for driving bone metastasis and inhibiting bone resorption. However, our western blot experiments did not

analyze the effects of other Gli isoforms (**Figure 9**). Subsequently, the influence of Gli2 in CSC expression, chemoresistance, and bone metastasis may not represent the true underlying mechanisms in these non-canonical hedgehog signaling pathways. Therefore, it is important that future works investigate of roles of other isoforms such as Gli1 or Gli3 in parallel with Gli2 to obtain a more complete answer.

Perhaps one of the biggest areas of improvement for this project is the in vitro analysis of CSCs as 2D monolayers rather than 3D spheroids, which are widely known to more accurately reflect the complexities of solid tumors.[97,100,104,127,128] More specifically, 3D spheroids portray the spatial arrangement and cell-cell interactions that promote stemlike phenotypes, including activation of signaling pathways like Notch, Oct4, SOX2, or E-cadherin[71,97,119,129], induction of epithelial-mesenchymal transition,[71,130] and activation of hypoxia-inducible factors,[116,131–133] among other factors.[130] For example, Kinoh et. al showed that mesothelioma cells cultured in spheroids showed a four-fold increase in $ALDH^+$ positivity across four separate cell lines.[14] Therefore, the inability to observe differential $CD44^+/CD24^-$ expression in Aim 1 as well as insufficient ALDH expression in Aim 2 may be resolved by culturing the MDA-MB-231 and separately the 4T1 cancer cells as spheroids.

In summary, future studies should aim to investigate the full expression profiles of ALDH and GLI isoforms and employ the use of spheroid cultures in order to more accurately simulate the complex and dynamic TNBC tumor microenvironments.

5.2 Broader Impacts

The research findings from this project shed light on the role of Gli2 in tumor progression and metastasis, chemoresistance, and CSC expression in the context of TIBD. By investigating the expression patterns of known CSC markers $CD44^{high}/CD24^{low}$ and separately $ALDH^+$ in TNBC cell lines, this study showed that Gli2 may be linked to the aggressive characteristics associated with late stage TNBC. Specifically, the identification of Gli2 as a potential regulator of CSC chemoresistance and metastasis, and its downregulation after combination therapy with paclitaxel

and GANT58 offers a new strategy for TNBC targeted therapy when options were limited. TNBC patients with TIBD have a worse prognosis and quality of life than typical TNBC patients, and the promise of new methods for reducing the enrichment of CSC populations to prevent or delay tumor metastasis may improve quality of life and survival outcomes.

Chapter 6

Conclusion

There is a great clinical need to investigate the mechanisms governing tumor bone metastasis and recurrence, CSC proliferation, and the development of chemoresistance in TNBC. By employing the use of Gli2-inhibitors as a method for modulating chemosensitivity of breast cancer cells to front-line chemotherapeutics, we developed an effective strategy for targeting aggressive breast CSC populations and correlated their responsive behaviors to the Gli2 transcription factor associated with the Hedgehog signaling pathway in various cancers. These findings will help guide the future development of cancer treatments, with the hopes of clinical translation to ultimately improve TIBD patient outcomes.

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