NAZARBAYEV UNIVERSITY

SCHOOL OF MEDICINE

Master thesis

TITLE: Screening for Inhibitors of Zeb1, a Key Regulator of Epithelial to Mesenchymal Transition (EMT) in Breast Cancer Cells.

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2023

DECLARATION

I hereby declare that the thesis is my original work, and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

Student name: Abdullateef Abdulsalam

Signature:

Date: 22/04/2024

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Table of Contents

List of Abbreviations

AKT/GSK3: AKT (Protein kinase B) / GSK3 (Glycogen synthase kinase 3)

BC: Breast Cancer

BRCA1: Breast Cancer Gene 1

BRCA2: Breast Cancer Gene 2

CDH1: Cadherin-1

CtBP: C-terminal binding protein

DMEM: Dulbecco's Modified Eagle Medium

EMT: Epithelial-Mesenchymal Transition

EMT-TFs: Epithelial-Mesenchymal Transition Transcription Factors

ER: Estrogen Receptor

ERBB2: Erb-B2 Receptor Tyrosine Kinase 2 (also known as HER2)

FDA: Food and Drug Administration

GFP: Green Fluorescent Protein

GnRH: Gonadotropin-releasing hormone

HDAC1/2: Histone Deacetylases 1 and 2

HER2: Human Epidermal Growth Factor Receptor 2

MET: Mesenchymal-Epithelial Transition

PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PR: Progesterone Receptor

PBS: Phosphate-buffered saline

TP53: Tumor Protein 53 (also known as p53)

TNBC: Triple-Negative Breast Cancer

ZEB1: Zinc Finger E-Box Binding Homeobox 1

List of Figures

Abstract

Background: Breast cancer (BC) has an estimated new cases of about 2.3 million individuals and approximately 685,000 deaths in 2020, thereby making it the most common cause of mortality in women. Different subtypes of BC are categorized breast into three clinical subtypes based on the expression or lack of hormone receptors: progesterone (PR), estrogen (ER), and human epidermal growth factor receptor 2 (Her2). Despite the considerable progress made in the treatment of the various types of BC, more research is still needed to address some major obstacles in breast cancer treatment, especially those associated with poor prognosis and reduced survival rates among BC patients like chemoresistance and cancer metastasis; these processes are mediated by Zeb1, which is the key regulator of the EMT.

Methods: Cell culture was used to propagate MCF7 cell lines and do transfection. Western blot was used to assess the effects on the markers of EMT such as Zeb1 and Cdh1. Cell cycle analysis using flow cytometry was used to examine candidate inhibitors of EMT after induction of Zeb1.

Results: The induced MCF7 cells show a higher percentage of cell cycle arrests at the G1 phase than non-induced cells after treatment with candidate inhibitors.

Conclusion: Out of the three PKC inhibitors tested midostaurin, auranofin, and resveratrol; resveratrol demonstrated a more significant impact on Zeb1-expressing cells than those without expression of Zeb1 by decreasing the percentage of cells at the G1 phase, hence, resveratrol might directly interfere with the activity of Zeb1.

Keywords: Breast Cancer, Chemoresistance, Metastasis, Inhibitors, EMT

CHAPTER ONE

1.0 Introduction

Breast cancer (BC) has an estimated new cases of about 2.3 million individuals and approximately 685,000 deaths in 2020, thereby making it the most common cause of mortality in women (Sung *et al.*, 2021). The global burden of breast cancer shows significant regional disparities, with a disproportionate number of cases occurring in low and middle-income countries. Historically, African and Asian countries have had lower incidence rates, typically less than 40 cases per 100,000 females (Bray, McCarron and Parkin, 2004; Joko-Fru *et al.*, 2020). However, recent reports are showing a rising incidence in these regions. For example, in Kazakhstan, the incidence rate has risen from 39.5 in 2009 to about 49.6 per 100,000 female population in 2018 (Toguzbayeva *et al.*, 2021). In contrast, transitioned countries in Northern America, Europe, and Northern Europe report the highest incidence rate with more than 80 cases per 100,000 females (Ferlay *et al.*, 2019). Despite this, the mortality rate is lower in these countries compared to transitioning countries in West Africa, the Caribbean, Micronesia, and so on (Sung *et al.*, 2021). This disparity in survival rates is often attributed to late-stage diagnoses in transitioning countries.

Projections suggest a concerning trend that by the year 2040, the global incidence of BC is expected to be more than 3 million new cases annually, with about one million deaths annually, this escalation is associated with population growth and an increase in the aging population, and an increase in the prevalence of risk factors in certain regions. Additionally, almost half of these cases will be from among the Asian population (Ferlay *et al.*, 2019). If this remains unchecked, this trend will further exacerbate the health and economic burdens associated with BC. Hence, this underscores the necessity for research efforts to enhance the efficacy of BC treatment, with a particular focus on prevention and effective therapeutic interventions.

The occurrence of BC has been associated with different risks in men and women. For example, in women, some of the unmodifiable risks that may be associated with an increased risk of developing BC include age, familial history, early onset of menstruation, delayed menopause, and mutation of BRCA1 or BRCA2 genes (Sun *et al.*, 2017)(Penn Medicine, 2024.). However, other modifiable risk factors such as obesity, excessive alcohol consumption, physical inactivity, hormonal replacement therapy as well and being nulliparous have been identified as factors that increase the risk of BC occurrence in women (Sun *et al.*, 2017). In men, factors such as ethnicity,

race, age, a mutation in BRCA genes, especially BRCA2, diabetes, Klinefelter syndrome, obesity, gynecomastia, exogenous estrogen or testosterone use and increase in serum estradiol level have been linked with increased risk of men breast cancer development (ACS, 2024.).

1.1 Sub-types of BC

Breast cancer is characterized by a high degree of heterogeneity, arising from the wide array of genetic differences in the mammary epithelial cells. Due to its molecular heterogeneity, an integrated assessment of both the histology of the primary tumor and immunohistochemistry has been used to categorize breast cancer into three clinical subtypes based on the expression or lack of hormone receptors: progesterone (PR), estrogen (ER), and human epidermal growth factor receptor 2 (Her2) (Orrantia-Borunda *et al.*, 2022). Therefore, genomics profiling has enabled further refining of BC into four intrinsic molecular subtypes: HER2-enriched (HER2+), luminal A, luminal B, and triple-negative BC (Orrantia-Borunda *et al.*, 2022).

The luminal A subtype constitutes the majority, around 60-70%, of all BC cases. It is characterized by the presence of hormone receptors, specifically estrogen receptor (ER) and progesterone receptor (PR), coupled with a lack of human epidermal growth factor receptor 2 (HER2) expression and a low level of Ki67. Additionally, Luminal A-like tumors display features associated with low risks, such as low grade and proliferation rate, and often present the most favorable clinical outcomes (ZHANG *et al.*, 2014; Hicks and Lester, 2016). In contrast, luminal B tumors which are of a relatively higher grade more proliferative, and of intermediate prognosis represent 10-20% of BC cases and share similarities with luminal A subtype in ER expression, but may show variable expression of PR and varying levels of Ki67. They may also exhibit positive or negative HER2 status (Orrantia-Borunda *et al.*, 2022). HER2-enriched tumors typically lack ER and PR expression but show positive expression of HER2 and a high Ki67 index, HER2-enriched tumors are high grade with an intermediate prognosis and are typically aggressive and more proliferative than the luminal subtypes (Iqbal and Iqbal, 2014)(Orrantia-Borunda *et al.*, 2022). Conversely, breast cancer subtypes lacking ER, PR, and HER2 expression are commonly referred to as "triple-negative" breast cancers, and they account for about 20% of all BC cases and often overlap with the basal-like category. This subtype is extremely aggressive with poor clinical outcomes (ZHANG *et al.*, 2014; Loibl and Gianni, 2017; Orrantia-Borunda *et al.*, 2022).

Genomic profiling of breast cancer has revealed various genetic alterations beyond the germline mutation in *BRCA1* and *BRCA2* genes among different subtypes of BC with each of the subtypes expressing different degree mutation of *TP53, PIK3CA, TP53 CDH1,* and *ERbB2* genes (Shaath, Elango and Alajez, 2021).

1.2 Treatments/Management

The treatment of BC is clinically multidisciplinary, the established and widely accepted approach for individuals with BC includes a combination of surgery, chemotherapy, and radiotherapy. The choice of treatment option depends largely on the tumor burden and size. Typically, after surgical tumor excision, patients with luminal subtypes frequently get adjuvant endocrine therapy consisting of either an aromatase inhibitor or a gonadotropin-releasing hormone (GnRH) analog in addition to tamoxifen (Goss *et al.*, 2005; Moo *et al.*, 2018). HER2-positive patients received neoadjuvant chemotherapy plus anti-HER2 targeted therapy such as trastuzumab and pertuzumab (Loibl and Gianni, 2017), while in triple-negative breast cancer (TNBC), standard neoadjuvant chemotherapy usually includes an anthracycline and a taxane (Marra and Curigliano, 2021). Recently, pembrolizumab, an anti-PD-L1 has been approved by the FDA in combination with chemotherapy for metastatic and advanced early-stage TNBC (Kwapisz, 2021). Although considerable progress has been made, more research is still needed to address the major obstacles in breast cancer treatment. One of the significant factors contributing to a poor prognosis and reduced survival rates among BC patients is drug resistance and cancer metastasis. Moreover, the toxicity associated with treatment has a detrimental effect on the quality of life experienced by patients

1.3 Cancer Metastasis, Chemoresistance, and the EMT Program

The shift in the program of cell evolution known as the epithelial-mesenchymal transition (EMT) occurs when cells acquire mesenchymal characteristics and lose their epithelial markers. During the transitional phase, the epithelial cells in monolayer culture acquire a spindle-shaped mesenchymal morphology, replacing their cobblestone appearance (Nieto, 2009). The mesenchymal to epithelial transition (MET) is a process that allows these freshly produced mesenchymal cells to change back into epithelial cells **(Figure 1)** (Nieto, 2009). The structural organization is important for the epithelial sheets constitute cells of various tissues characterized

by the apical-basal polarity, held together by the tight junctions, and in the adheren junction by cell surface epithelial cadherin (E-cadherin) molecules. When the EMT process is triggered, E-cadherin expression is suppressed, which causes the epithelial cells to lose their cobblestone shape. The cells express genes linked to the mesenchymal states and simultaneously take on the spindle-shaped mesenchymal appearance. The group of genes that encourage the development of the mesenchymal cell state is expressed while suppressing the expression of genes that uphold the epithelial state **(Figure 2)** (Nieto, 2009) (Dongre and Weinberg, 2018).

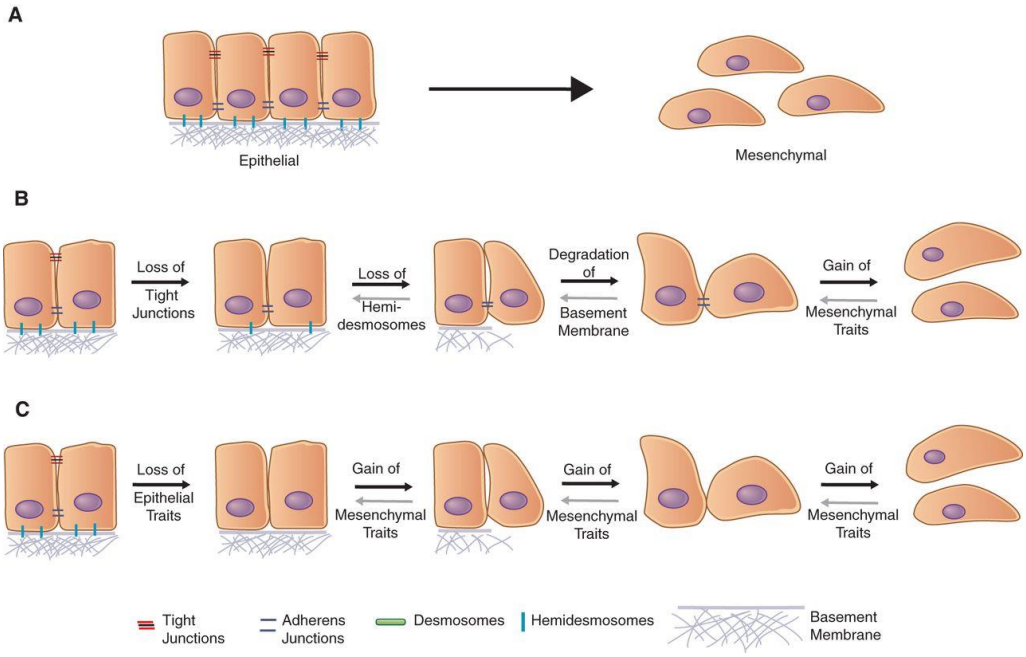

Figure 1: Transitioning of epithelial to mesenchymal cell adopted from (Pattabiraman and Weinberg, 2016)

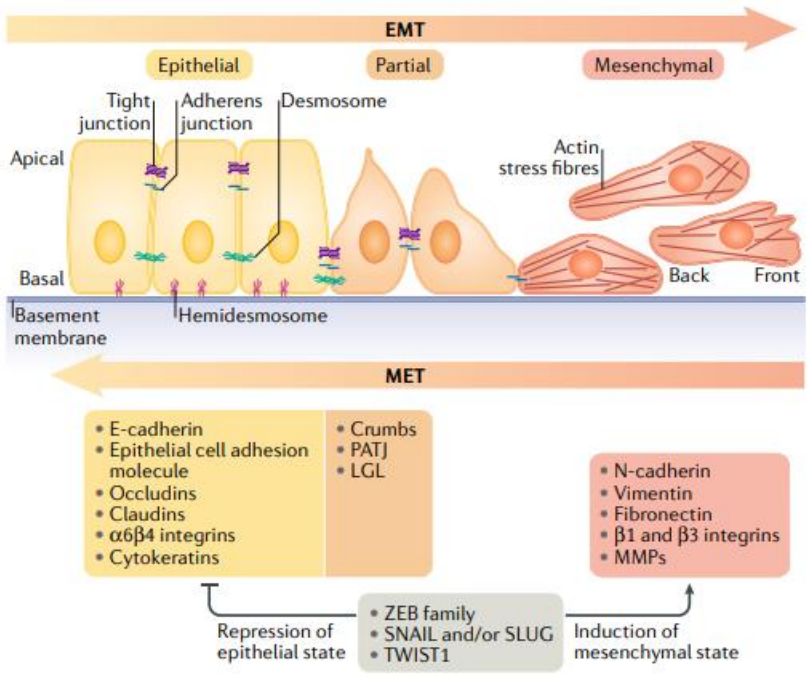

Figure 2: A pathway of a typical EMT program Adopted from (Dongre and Weinberg, 2018)

EMT-associated transcription factors such as the Zeb1 and epigenetic regulator during cell injury promote this complex transition. Through enhancement of motility, invasion, and increased resistance to apoptotic stimuli, the EMT gives cancer cells metastatic properties. This type of EMT tumor-acquiring cells is characterized by plasticity and exhibits intensive chemoresistance (Mittal, 2018)(Dongre and Weinberg, 2018).

The EMT signature was also associated with breast cancer invasiveness and metastasis defined by distinct tumor immune microenvironments (Song *et al.*, 2022). Several genes such as Zeb1, Twist, Chd1, Chd2, MMP9, snail, TGF- β, and so on have been proven to be biomarkers of the EMT process (Aiello and Kang, 2019). The role of the EMT-TFs in BC is therefore important to explore the mechanisms of EMT involved in the tumorigenesis, development, and metastasis of BC.

It has already been proven that the EMT is kept in balance by a double negative feedback loop between microRNAs and EMT-TFs such as Zeb1/2 (Korpal *et al.*, 2008a)**.** The MicroRNAs are key regulators for the mesenchymal-epithelial transition (MET) because of reverse cancer stem cell properties and also increase the sensitivity of the cancer cells to chemotherapy by enhancing

the E-cadherin expression through the direct target of Zeb1 and *Zeb2*, which are known transcription factors of that are overexpressed during the EMT process (Gregory *et al.*, 2008)(Korpal *et al.*, 2008a). Remarkably, Zeb1 in breast cancer has conferred resistance to multiple drugs, for example, tamoxifen resistance through estrogen receptor-alpha hypermethylation, trastuzumab resistance through Zeb1/mir-200, and improved repair of DNA damage resulting from chemotherapy via ATM pathway (Zhang *et al.*, 2017)(Korpal *et al.*, 2008b)(Dent *et al.*, 2011).

Interestingly, although all EMT-TFs play key roles in cancer metastasis, a study shows that the overexpression of Zeb1 is strongly related to the presentation of the mesenchymal phenotype. As Zeb1 is the most important regulator of metastasis in human breast cancer (Addison *et al.*, 2021), while the knockdown of zeb1 inhibits metastasis, and grading, and ensures the expression of epithelial phenotype and loss of cellular plasticity (Krebs *et al.*, 2017). Since Zeb1 causes cell cycle arrest predominantly at the G1 phase, inhibitors of cell cycle arrest are hypothesized to act as chemosensitizers by taking advantage of the alteration of the cell proliferative characteristics and causing a variety of short- and long-term reproductive cell deaths (Dent *et al.*, 2011).

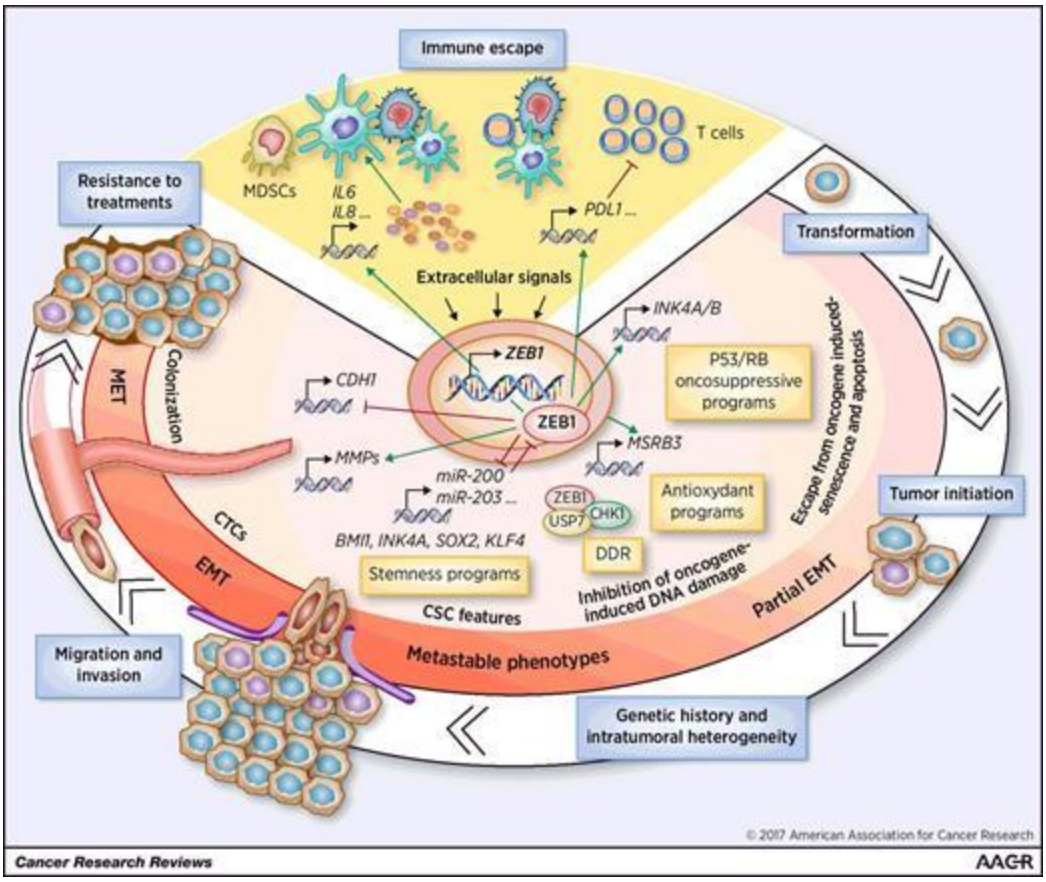

Figure 3: Pathways of Zeb1 in cancer progression adapted from (Caramel, Ligier and Puisieux, 2018)

Most cancer medications that are approved for the treatment of patients with metastatic cancers such as antibody-drug conjugates, tyrosine kinase, and systemic cytotoxic drugs have shown meager survival rates as compared to the efficacy shown from the preclinical results (Ma, Wells and Clark, 2020). Even though immunotherapy has proven to show a long-term remission of cancer and the management of chronic metastases, it has only been effective in a few patients (Wei, Duffy and Allison, 2018). Hence, this highlights the need to find more effective therapies and approaches that will lead to the remission of metastatic cancers by identifying drugs that can inhibit Zeb1 or enhance the E-cadherin by reprogramming cells into the epithelial-like state.

The cost of research and development of a new drug is over a billion dollars and it usually takes an average of ten years before it reaches the market (Berdigaliyev and Aljofan, 2020). To reduce the cost and time of discovery, we want to explore the therapeutic effectiveness of some FDA-approved drugs by repurposing them as Zeb1 inhibitors in reversing EMT processes in BC.

1.4 Statement of Research Problem

BC poses a major health concern, especially in low- and middle-income countries where they usually diagnose cancer at a much later stage of the tumor growth that is characterized by their tendency to metastasize and become resistant to chemotherapy. EMT is an important process in cancer progression, and transcription factors such as Zeb1, Twist, Slug, Chd1, Zeb2, Chd2, and so on play a central role in driving this transition. To develop a more effective therapeutic agent, it is essential to identify and characterize inhibitors of Zeb1, which is the key regulator of cancer metastasis to suppress the EMT process and thereby halt the invasive and metastatic behavior of BC cells. This research seeks to address the need for safe and effective chemical inhibitors targeting Zeb1 to combat the aggressive nature of BC and improve patient outcomes and quality of life.

1.5 Hypothesis

Screening for inhibitors of Zeb1, a key regulator of epithelial-to-mesenchymal transition (EMT) in BC cell line (MCF7-Zeb1), will lead to identifying novel compounds that can effectively suppress EMT, thereby inhibiting breast cancer progression and metastasis.

1.6 Aims

1. To analyze whether some United States Food and Drug Administration FDA-approved drugs are inhibitors of Zeb1
2. Investigate the functional consequences of zeb1 inhibition in the BC cell line.

CHAPTER TWO

2.0 Materials and Methods

2.1 Cell Culture

A genetically modified breast cancer cell line (MCF7) with inducible zeb1 and GFP genes fused was used. The cells were grown in a DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin in an incubator with 5% CO2 at 37 °C. The culture media is often changed twice a week to have optimal cell growth. When the old media is removed by suction, the cells are washed with 5 to 6mls PBS before fresh media of about 11 ml are added. However, if the plate requires passaging, after washing the cell with PBS, 2 ml of 0.125% trypsin is added to the plate and then incubated for about 2 min. To neutralize the trypsin, 4 ml of media was added to the plate and everything was collected in a 15 ml Falcon tube and centrifuged at 243 rcf for 5 minutes. The supernatant was discarded and the pellets were resuspended and shared into new tissue culture plates. 11 ml of the prepared media was added and then incubated at 5% CO2 and 37°C.

2.2 Zeb1 Induction

A stock of 1 mg/ml of doxycycline was used to prepare an induction media containing a final concentration of 0.5 µg/mL to induce the expression of the Zeb1/GFP genes fusion protein. Using a Primovert microscope (Carl Zeiss, Germany), at different time points (0 hours, 48 hours, 72 hours, and 98 hours) the expression of Zeb1 was seen through GFP luminescence. The samples were collected for western blot to check the expression of Zeb1 at this time point.

2.3 Western Blotting

Western blot was used to assess the expression of proteins like Zeb1, E-cadherin, beta-actin, and so on. The samples collected after each time point of induction were lysed with RIPA buffer that contained therein protease inhibitors. 300µL of the sample was diluted with 300µL Laemmli buffer. Samples were vortexed and heated to 95°C for five minutes before being loaded into the wells.

10% running gels were prepared for the separation of proteins. An average of about 9.5 µL was loaded to the 4% stacking gel well after normalization with ImageJ after Comaissee staining. 6 µL of the ladder (PageRuler^{TM}, Lot# 00855291) was uploaded to the first well. The gel was run at 120V for 1 hour in 1x running buffer. To transfer the protein to a nitrocellulose membrane, a

sandwich nitrocellulose membrane between filter paper and sponges was prepared and run with 1x transfer buffer at 100V for 1 hour.

Western blot analysis evaluated the expression levels of proteins such as Zeb1, E-cadherin, and beta-actin. Samples collected at each induction time point were lysed using RIPA buffer containing protease inhibitors. Each sample (300 µL) was then diluted with an equal volume of Laemmli buffer, vortexed, and heated to 95°C for five minutes before loading into the wells.

A 10% polyacrylamide gels were prepared for protein separation, with approximately 9.5 µL of each normalized sample loaded onto the 4% stacking gel well, as determined by ImageJ software following Coomassie staining. A 6 µL volume of protein ladder (PageRuler^{TM}, Lot# 00855291) was loaded into the first well. The gel was electrophoresed at 120V for one hour in 1x running buffer. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane by setting up a sandwich of nitrocellulose membrane between filter paper and sponges. The transfer was performed in a 1x transfer buffer 100V for one hour.

After the transfer of protein to the nitrocellulose membrane, the membrane was washed with PBST and then blocked with 5% non-fat milk for one hour on a shaker. The membrane was cut so that the protein of interest corresponds to the ladder. After washing with PBST, the membrane was incubated with a primary antibody (beta-actin, #8457), (Zeb1, A1500), (Anti-E-cadherin #610181) at 4°C overnight on a shaker. The membrane was collected and washed the next day with PBST and incubated for one hour on a shaker with the corresponding secondary antibodies.

Enhanced chemiluminescence solution was prepared by mixing equal volumes of solution A and B and added to the membrane before visualization. Detection and visualization of bands were carried out with Chemi-Doc Bio-Rad.

2.4 Luciferase Reporter Assay

MCF7-Zeb1 cells were seeded on a 6-well plate and were transfected with lucifer reporter plasmid 384 ng/ml to make a final stock of 6 µg/well, and 5 µg/ml of GFP plasmid as a positive control. The transfected plasmid was diluted in 500 µL of Opti-MEM. The DNA-lipofectamine complex was prepared by adding 500 µL of Opti-MEM with 15 µL of lipofectamine-2000. The mixture was gently vortexed and allowed to incubate for about 10 minutes. The media of the 6-well plate was replaced with 1 mL of DNA-lipofectamine complex and was allowed to incubate for 4 hours

before replacing the lipofectamine media with regular media. The transfected cells were allowed to incubate for 3 days to obtain the highest level of modification. Cytation™ Cell Imaging Reader Multi-Mode Reader (BioTeK) with the software Gen5™ Microplate Reader and Imager Software was used to record the result. The signals were normalized to abcam cell lysis buffers.

2.5 Fixation of Cell Suspension and Flow Cytometry

MCF7-Zeb1 was seeded into a 6-well plate and 2 mL of induction media containing a final concentration of 0.5 µg/ml of doxycycline was used for cell incubation. For cell cycle analysis, fixation was performed using 70% ethanol. At each designated time point (0, 48, 72, and 92 hours), cells were collected into 1.5 mL Eppendorf tubes. The cells were washed with PBS before it was spun down with a centrifuge. The supernatant was discarded while the pellet was resuspended in 271 µL of PBS to disperse cell aggregate. The cell suspension was then added to a precooled 729 µL of 96% ethanol and mixed gently to make the final concentration of the ethanol 70%. The suspension was stored at -20°C, however, it was kept at 4°C for 2 hours before the staining and analysis.

A staining solution for the flow cytometry was prepared from a stock of (Propidium iodide #3566) 1 mg/mL to make a final concentration of 10 µg/mL and 1 mg/mL of RNAse to make a final concentration of 100 µg/mL. The fixed cells were spun down with a centrifuge, ethanol was removed and the cells were washed with PBS. The suspension was divided into two, and 500 µL of the staining solution was used to resuspend one part of the cells and PBS was used to resuspend the second part. The same procedure was repeated for other time points and then data were collected CYTATION Imagine reader BioTeK.

2.6 Treatment of Cells with Inhibitors

MCF7-Zeb1 cells were seeded into three pieces of 6-well plates. Some wells were induced with 0.5 µg/ml of doxycycline to cause the expression of zeb1, while others were left as positive control non-induced wells. After 24 hours of incubation, inhibitors were prepared in DMEM to make a final concentration of 10 µM from a stock solution of 100 mM. Subsequently, 2 ml of the inhibitor solution was added to each well. As a negative control, 100 mM of Dimethyl sulfoxide (DMSO) was prepared from a stock solution of 14 M, and 2 ml was added to each of the induced and non-

induced wells. The cells were collected after 48 hours for fixation in 70% ethanol and then cell cycle analysis was carried out using a flow cytometer.

CHAPTER THREE

3.0 Results and Discussion

3.1 Morphology Change After EMT Induction, Assessed with Microscopy

The expression of Zeb1 has been proven to suffice the triggering of the EMT (Caramel, Ligier and Puisieux, 2018)(Addison *et al.*, 2021), hence, I triggered the expression of Zeb1 in an epithelial cell line (MCF-7) which causes a change in the phenotype of the cell line from cobblestone to spindle-like feature, and this was most prominent after 72 hours of induction with doxycycline as seen in **(Figure 4).** The morphological change alone is insufficient to confirm the EMT process was mediated by Zeb1, hence, I checked for the fluorescence of the MCF7 after induction.

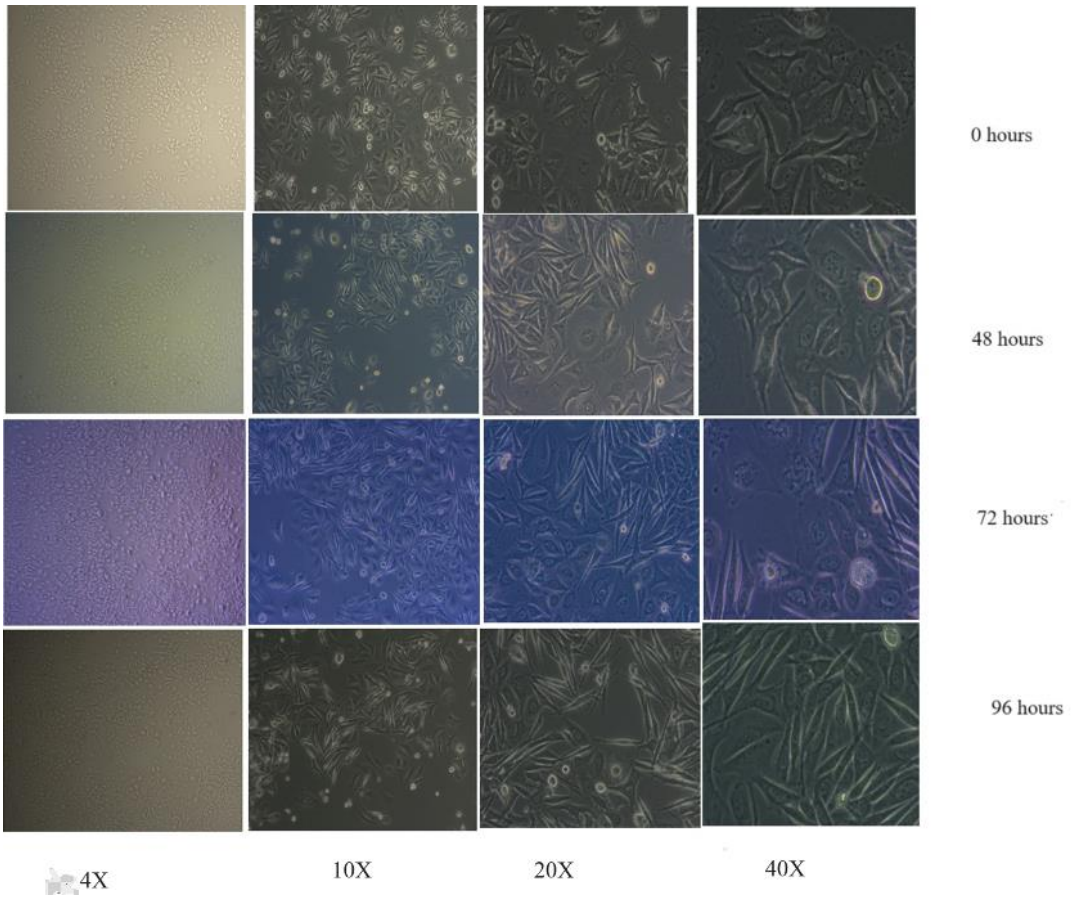

Figure 4: Morphology of mcf7/zeb1 treated with doxycycline by brightfield microscope in magnification 4, 10, 20, and 40 at different time points. Assessed using a Primovert microscope (Carl Zeiss, Germany)

3.2 Flourescence After EMT Induction, Assessed with Microscopy

The induction of Zeb1 with doxycycline in MCF7 was also assessed to confirm the expression of Zeb1/GFP fusion protein as this expression of Zeb1 could be easily detected by the fluorescing of GFP as seen in **(Figure 5).** Zeb1/GFP fusion protein induction was indicated by a progressive increase in GFP fluorescence intensity in the cell nucleus as the time point progressed. Furthermore, the initiation of EMT-like seen through the GFP fluorescence can only confirm the activation of Zeb1 expression indirectly, hence, I explore other forms of additional control to confirm Zeb1 activation such as immunoblot and luciferase assay.

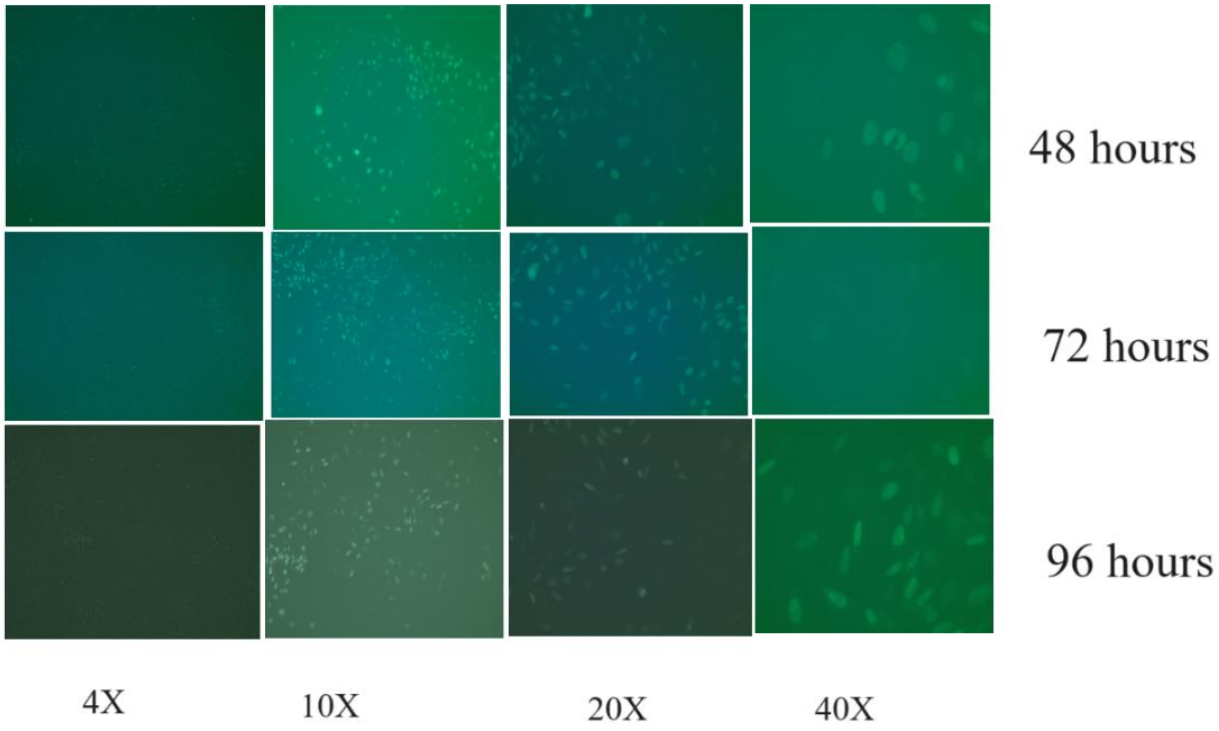

Figure 5: Fluorescence of mcf7/zeb1 treated with doxycycline by fluorescence microscope in magnification 4, 10, 20, and 40 at different time points (48, 72, and 96 hours). Assessed using a Primovert microscope (Carl Zeiss, Germany)

3.*3* Assessing the Protein Expression of EMT-TFs Using Western Blots

Numerous signaling pathways regulate the EMT in a precise manner, however, various epithelial and mesenchymal markers experience a shift in expression patterns upon the Zeb1 induction, which is what causes modifications in cell shape and characteristics as Zeb1 is the key TF in the EMT process in BC (Addison *et al.*, 2021). I carried out a western blot examination of the epithelial marker E-cadherin to verify the start of EMT **(Figure 6)**. and also, to assess the mesenchymal marker Zeb1 to confirm the progression of the EMT process **(Figure 6)**. The levels of protein in the samples (with or without EMT induction) were standardized to the content of actin in order to accurately analyze changes in the content of macromolecules of our interest (Figure 6). According to Wong et al., (2014), epithelial marker E-cadherin is one of the most significant cell adhesion molecules, and it is known to be downregulated with the onset of EMT (Wong, Gao and Chan, 2014). The most significant inducer of EMT, the Zeb1 EMT-TF reduces the expression of the E-cadherin gene by acting on its promoter regions. The western blot showed the appearance of E-cadherin at 0 hours after induction and a gradual increase in the Zeb1 mesenchymal marker with peak expression at 72 hours after induction with doxycycline, therefore, confirming the activation of Zeb1/GFP protein fusion expression **(Figure 6)**.

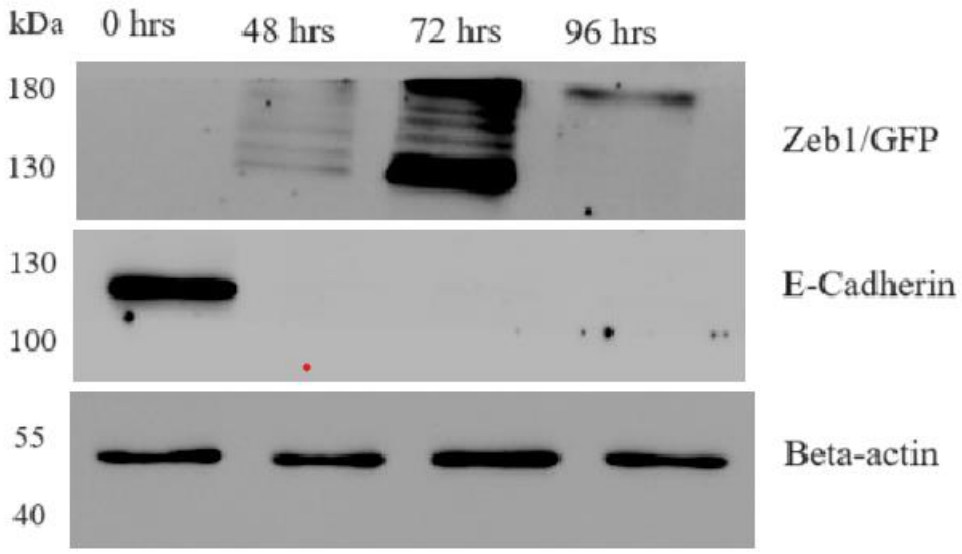

Figure 6**:** The western blot of MCF7/Zeb1 total cell lysates after induction with doxycycline at the time point (0, 48, 72, and 96 hours) with beta-actin, E-cadherin, and Zeb1. The figure indicates the corresponding position of protein molecular weight in kDa.

3.4 Transfection of MCF7 with E-Cadherin promoter Plasmid

To determine whether E-Cadherin can directly cause the suppression of Zeb1, I used the E-cadherin reporter plasmid that contains the luciferin gene and performed the luciferase reporter assay of the MCF7 cells. There was high luminescence at time points 0 and 48 hours **(Figure 9)** which correlates with the time point where Zeb1 is not fully expressed. However, as the induction of Zeb1 progressed, it bound to the E-cadherin promoter causing a decrease in the luminescence and shooting down the luciferase system as depicted in **Figure 9.** The expression of Zeb1 led to the dramatic repression of E-cadherin. The luminescence of the MCF7 is expected to be restored when Zeb1 is inhibited. These findings demonstrate a substantial correlation between the suppression of E-cadherin, a common negative regulator of endogenous Zeb1, and the activation of these genes by ectopic EMT-TFs. The change in morphology and glowing of the Zeb1/GFP fusion protein was monitored after induction of the transfected MCF7 with doxycycline and was assessed with microscopy **(Figures 7 and 8).**

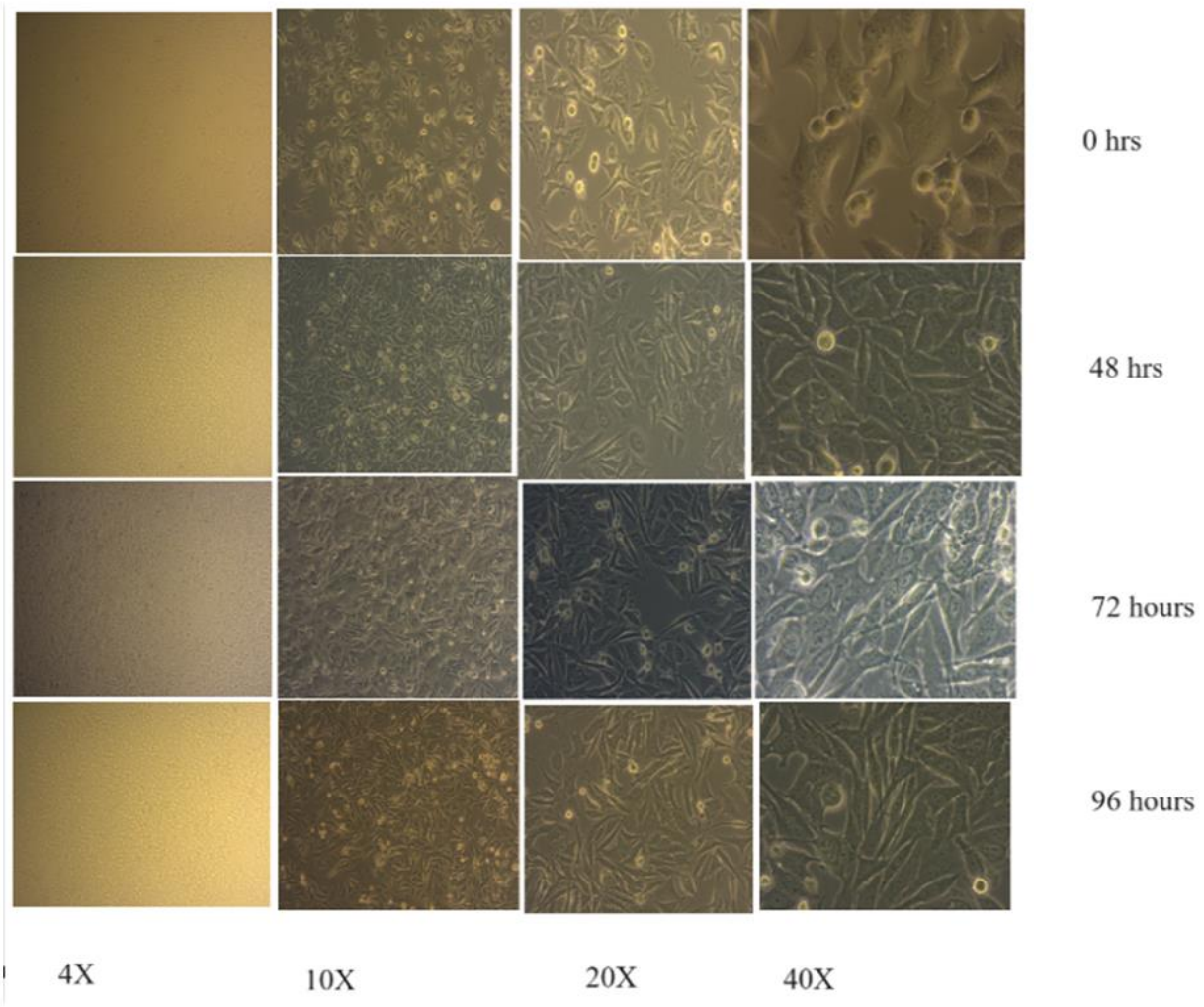

Figure 7: Morphology of mcf7/zeb1 treated with doxycycline and transfected with luciferase promoter of E-cadherin viewed under bright field microscope in magnification 4, 10, 20, and 40 at different time points (48, 72, and 96 hours).

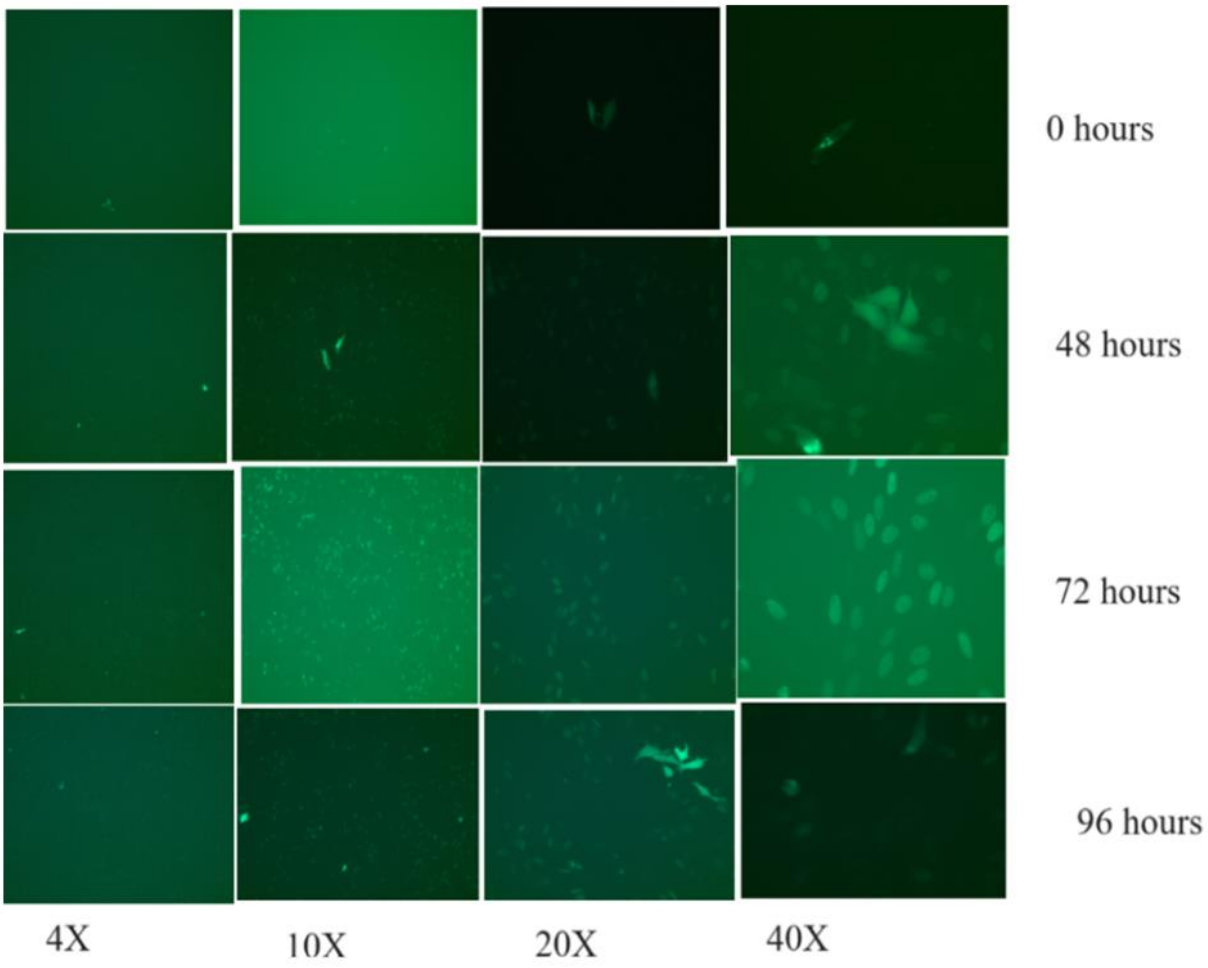

Figure 8**;** Morphology of mcf7/zeb1 treated with doxycycline and transfected with luciferase promoter of E-cadherin by fluorescence microscope in magnification 4, 10, 20, and 40 at different time points (48, 72, and 96 hours).

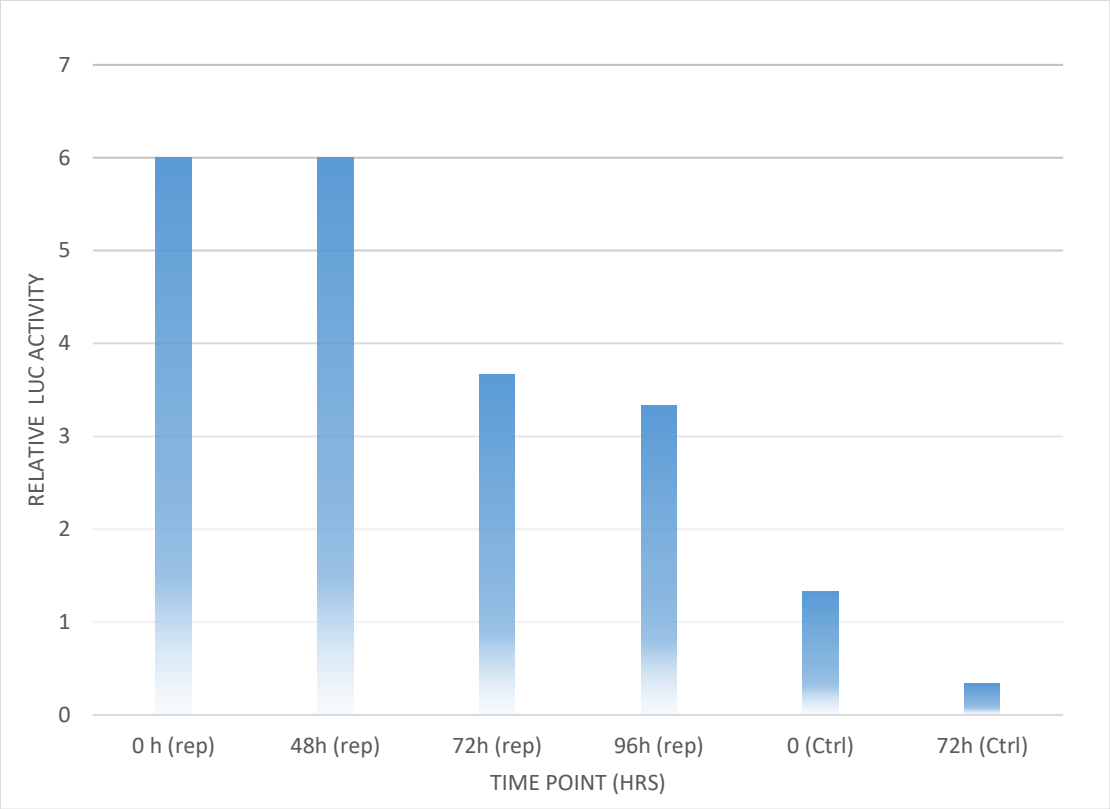

Figure 9**:** Extract of MCF7 cells co-transfected with E-cadherin reporter plasmid to assess the luciferase activities were determined at 0, 48, 72, and 96 hours after transfection using a Cytation^{TM} Cell Imaging Reader Multi-Mode Reader (BioTeK). Luciferase values were normalized to abcam cell lysis buffer.

3.5 Cell Cycle Analysis of Induced MCF7 Using Flow Cytometry

An important feature of EMT is conferring chemoresistance and motility to cancer cells (Singh and Settleman, 2010). Additionally, Aberrant Zeb1 expression causes chemoresistance to the chemotherapeutic used in the treatment of BC, for example, through the ER alpha hypermethylation and several other pathways like Akt/GSK3β/β-catenin in doxorubicin, Zeb1/mir-200 in trastuzumab and so on (Zhang *et al.*, 2017)(Iqbal and Iqbal, 2014). These are in line with my in vitro findings that a metastatic/mesenchymal phenotype of MCF7 is connected to Zeb1 immunoexpression. Furthermore, a recent study theorizes that zeb1 causes cell cycle arrest G1-phase, which is a known hallmark of the EMT process (Sreekumar et al., 2019.). This was confirmed by the cell cycle analysis I conducted using flow cytometry to examine changes in cell

cycle distribution over time, with a particular focus on potential G1 phase arrest induced by Zeb1 after induction of MCF7 with doxycycline as depicted in **(Figure 10).**

Furthermore, I examined the sensitivity of MCF7 cells with the expression of Zeb1 to midostaurin, auranofin, and reservatrol **(Figure 11)**. Since it had been reported that Zeb1-induced EMT usually results to resistance commonly used anti-tumor agent (Sreekumar *et al.*, no date), hence, we induced MCF7 cells and it shows a higher percentage number of cell cycle arrests at the G1 phase than non-induced cells after treatment with potential inhibitors as seen in **(Figure 11; A-D and I-H).**

In principle, cellular cytotoxicity is defined by sub-G1 density (apoptotic/necrotic cells), when cells are arrested in the G1 phase, they try to protect themself from damage inflicted by drugs. Zeb1 plays a role in protecting cancer cells from the effects of genotoxic drugs by eliciting G1 cell cycle arrest, which is an attempt to shield the cells from damage that may be caused by these drugs. Zeb1 inhibitors will counteract this effect and cause a decrease in the number of cells in the G1 phase even when Zeb1 is induced. This effect could occur through mechanisms such as reducing Zeb1 DNA binding activity or interfering with its ability to repress transcription by disrupting its interactions with proteins like C-terminal binding protein (CtBP) and histone deacetylases 1 and 2 (HDAC1/2). To better interpret these findings and refine our therapeutic strategies, future research endeavors should include repeated flow cytometry analyses and the integration of additional methodologies, such as luciferase reporter assays and functional studies.

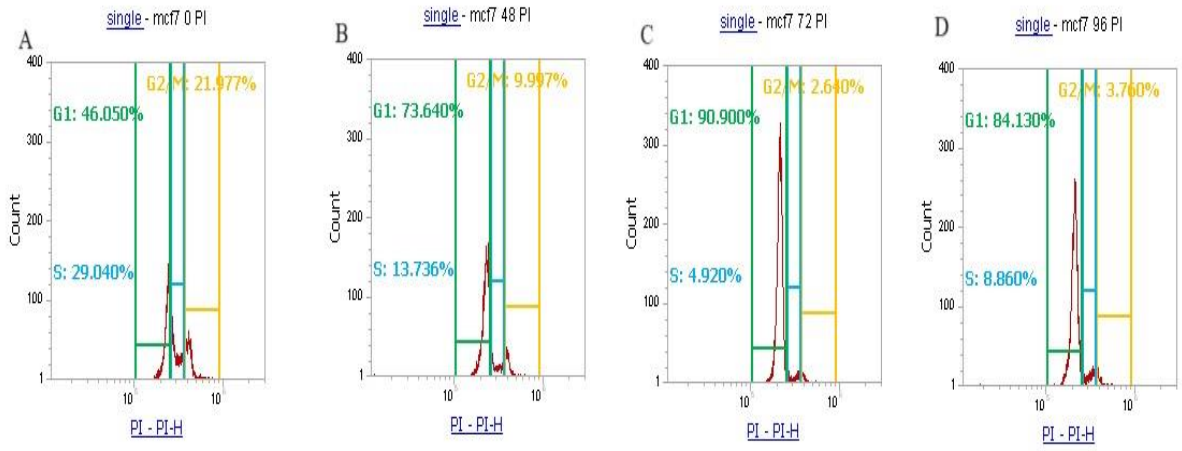

Figure 10**:** MCF7 cells were treated with doxycycline to induce Zeb1 expression and then stained with propidium iodide at different time points (0, 48, 72, and 96 hours). Cell cycle analysis was conducted using flow cytometry to examine changes in cell cycle distribution over time, with a particular focus on potential G1 phase arrest induced by Zeb1. (PI=Propidium iodide)

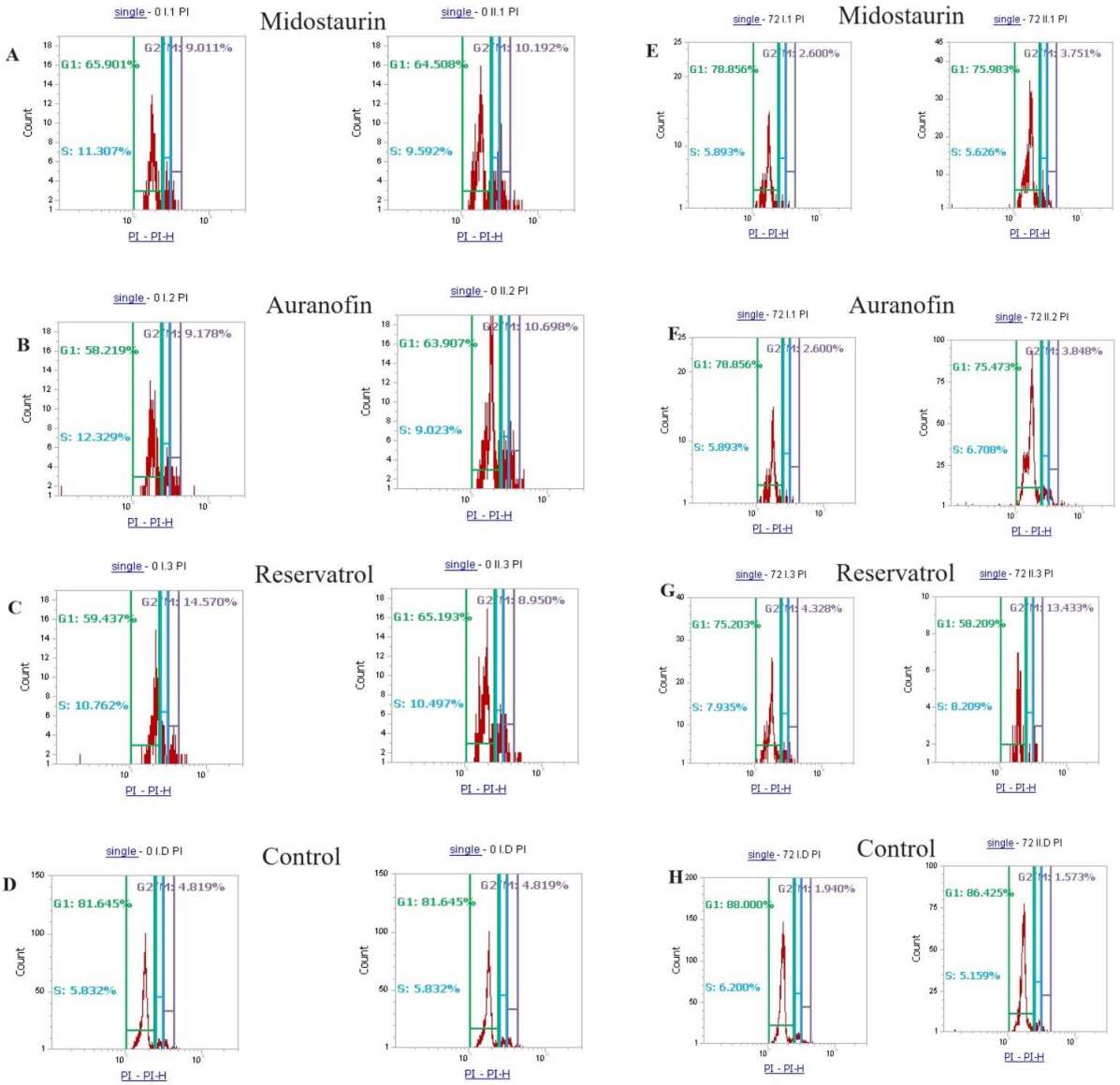

Figure 11**:** Cell Cycle Dynamics of MCF7/Zeb1: Comparative Analysis of Induced **(I-H)** and Non-induced **(A-D)** Cells Treated with reservatrol, midostaurin, and auranofin for 48 Hours Following 24-hour Induction, Assessed via Flow Cytometry and Propidium Iodide Staining

CHAPTER FOUR

4.0 Conclusion

This study has increased our understanding of the role of Zeb1 in the EMT not only in conferring metastatic properties to cancer cells but also affecting their resistance to chemotherapy. However, it is important to conduct a comprehensive investigation into the possibility of proteins or TFs that modulate this pathway either directly or indirectly in their involvement in the EMT, metastatic processes, and chemoresistance activity. The interest in disrupting EMT as a therapeutic approach is growing quickly since EMT and EMT-TFs are implicated in many of these critical elements of cancer progression.

To this end, I assessed for the inhibitors of Zeb1 which are pivotal in improving the quality of life of patients with metastasizing BC, however, my study highlights unexpected outcomes, such as the observed increase in cell density and G1 phase following treatment with midostaurin and auranofin when compared to the non-induced groups. However, only resveratrol shows a decrease in the density of cells at the G1 phase. To better interpret these findings and refine our therapeutic strategies, future research endeavors should include repeated flow cytometry analyses and the integration of additional methodologies, such as luciferase reporter assays and functional studies.

As interest in disrupting EMT and Zeb1 as a therapeutic approach grows, we must continue to advance our understanding of the underlying molecular mechanisms driving cancer progression. By doing so, we can ensure that we can improve the quality of life for patients with metastatic breast cancer and pave the way for more targeted and effective treatments.

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ANNEXES

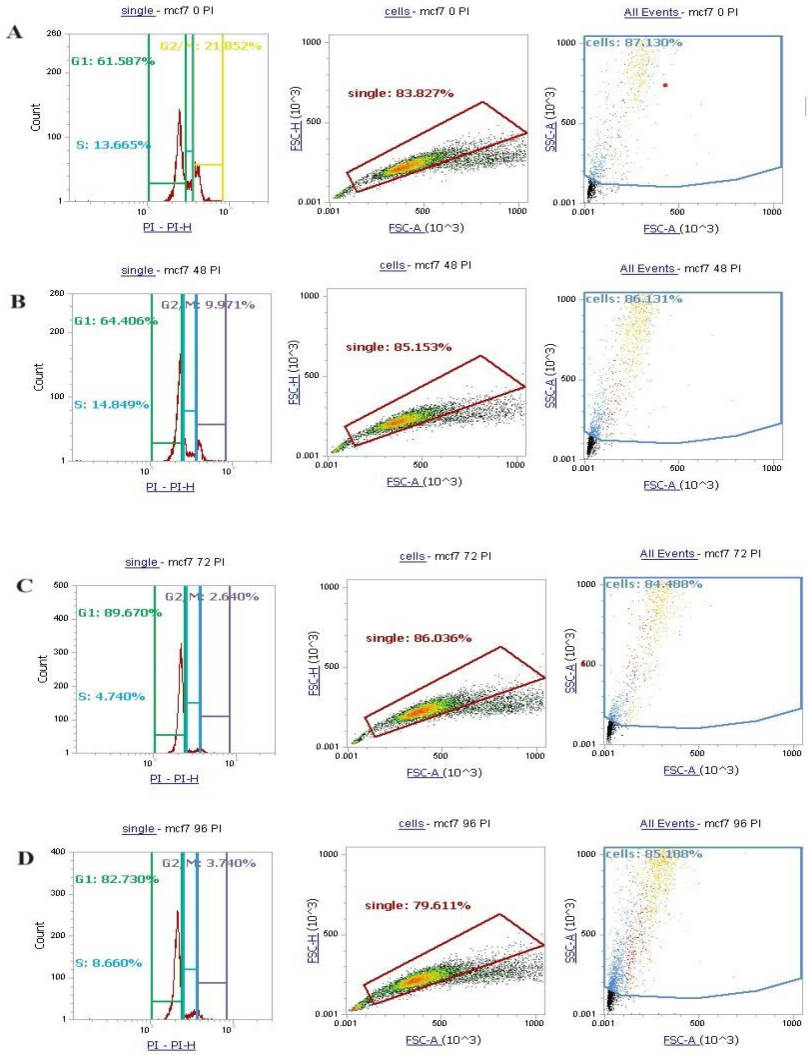
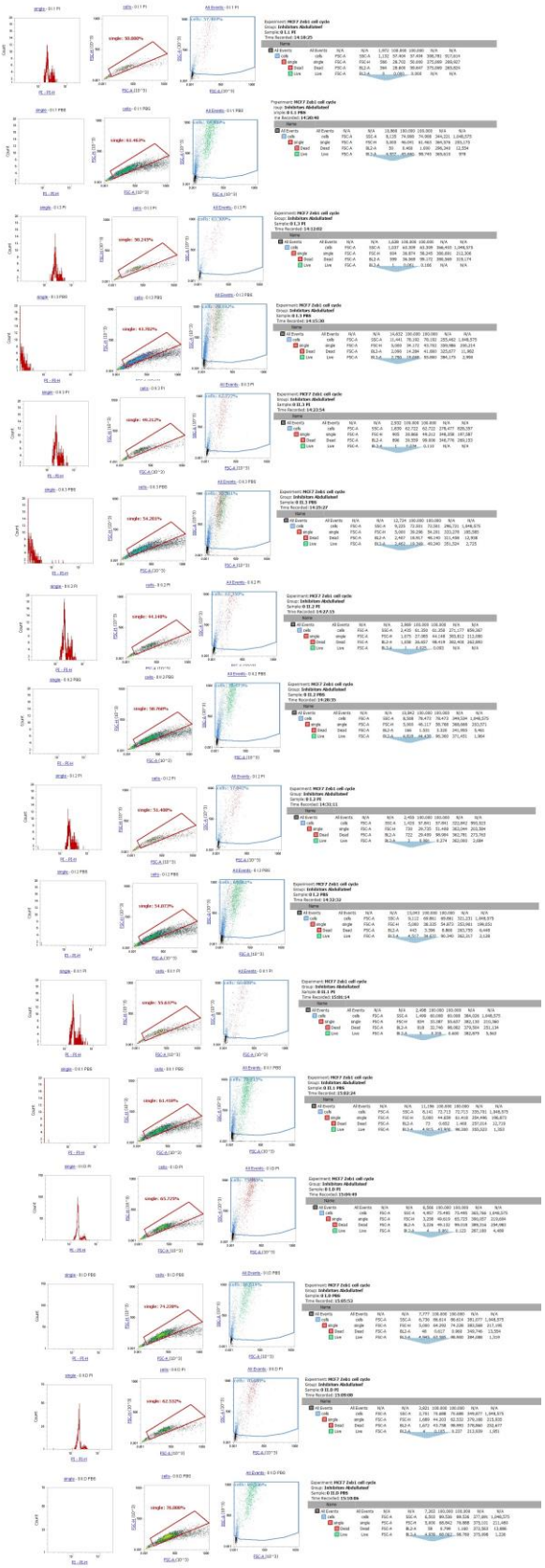


Figure 12: MCF7 cells were treated with doxycycline to induce Zeb1 expression and then stained with propidium iodide at different time points (0, 48, 72, and 96 hours). Cell cycle analysis was conducted using flow cytometry to examine changes in cell cycle distribution over time, with a particular focus on potential G1 phase arrest induced by Zeb1. (PI=Propidium iodide)



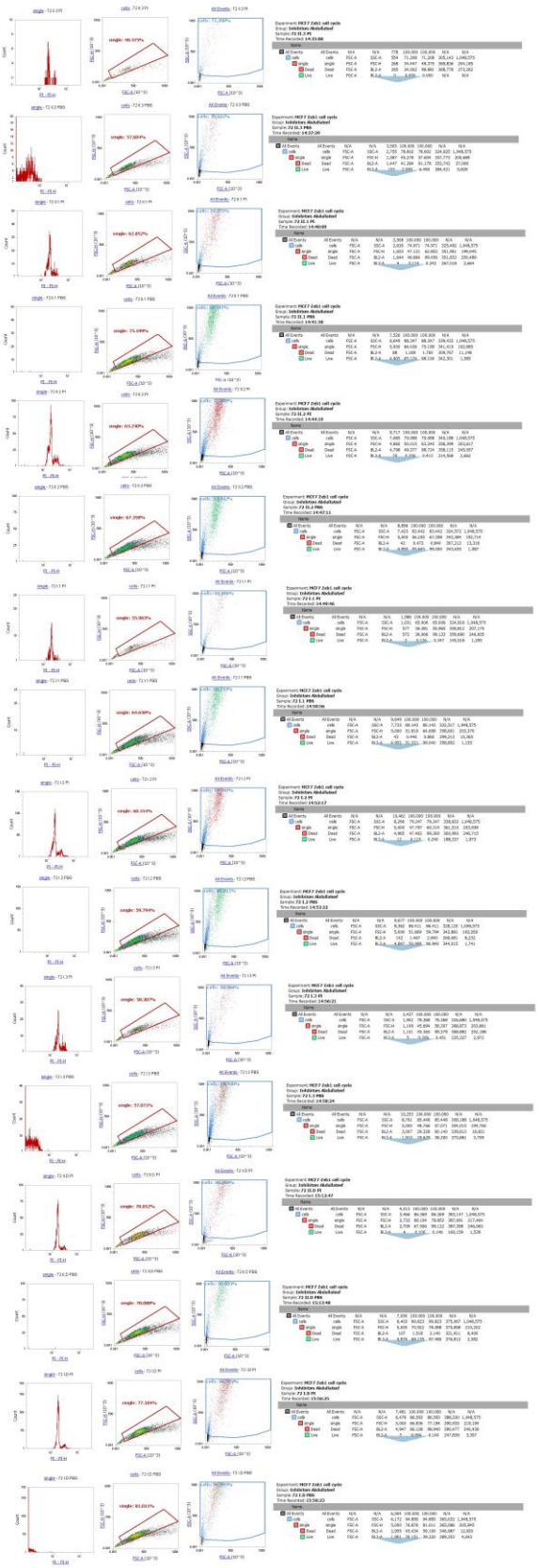


Figure 13; B-actin staining

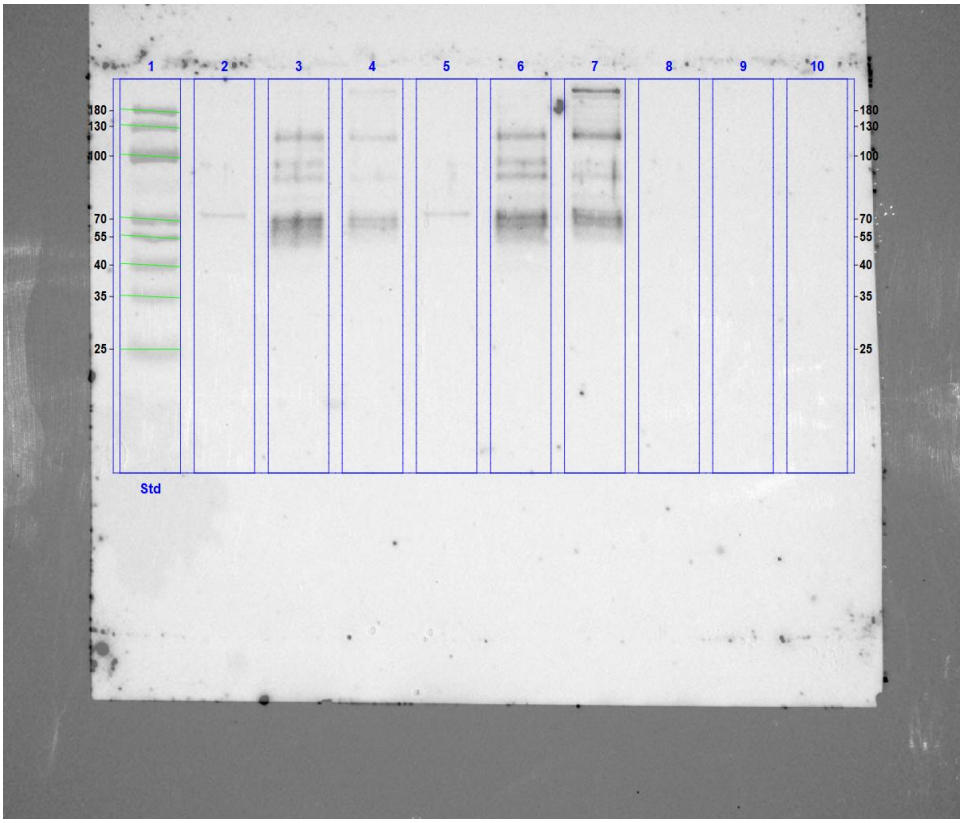


Figure 14: Analysis of Zeb1 Western blot by immunodetection