QATAR UNIVERSITY

COLLEGE OF PHARMACY

MOLECULAR FEATURES OF TRIPLE NEGATIVE BREAST CANCER STEM CELLS:

A GENE EXPRESSION PROFILING ANALYSIS OF MDA-MB-231 CELLS

BY

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ABSTRACT

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Title: Molecular Features of Triple Negative Breast Cancer Stem Cells: A Gene Expression Profiling Analysis of MDA-MB-231 Cells

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Triple negative breast cancer (TNBC) is a chemoresistant subtype of female breast tumors. Chemoresistance is attributed to the presence of long-lived cancer stem cells (CSCs) responsible for therapy failure. This study investigated the differential gene expression of signaling pathways controlling TNBC CSCs. We determined the mRNA and protein expressions of genes responsible for stemness markers, autophagy, apoptosis, CYP450 enzymes, and tumor suppressors TNBC and CSCs. We measured the caspases by fluorescence intensity and quantified cells in LC3 activated cells, reactive oxygen species (ROS), and DNA damaged cells.

CSCs were identified by Aldeflour, side population, and $CD44^{+}/CD24^{-}$ assay. We conducted RNA isolation using TRIZOL and measured the induction by RT-PCR for stemness markers, autophagy, apoptosis, CYP450, and tumor suppressor genes. Proteins were extracted by RIPA and quantitated by Western blot. The caspases fluorescence was detected by immunofluorescence assay. The activated cells in autophagy, oxidative stress, and DNA damage assays were explored by Muse Cell Analyzer. Independent t-test was used to detect statistical significance.

The apoptotic markers BAX, caspase3, caspase8, and caspase9 were downregulated by 53%, 30%, 50%, and 50%, respectively. The cellular content of caspases was diminished in CSCs. However, the anti-apoptotic Bcl-xL was 14% higher in CSC than TNBC. The autophagy p62 gene was 61% upregulated, whereas the ATG and LC3-activated cells were lower by 33% at the protein level and 78.3% by

flowcytometry, respectively. Nonetheless, CYP3A4, and CYP2D6 were lower in CSCs by 75%, 23%, 43%, 23.8%, and 17%, respectively. Moreover, CSCs were localized in G0/G1-phase. Similarly, the tumor suppressor genes BRCA (40%), PTEN (25%), and p53 (36%) were downregulated. The factors, AKT increased by 33%, NF-KB and ki-67 were decreased by 32% at the protein level, and 82.8% at the mRNA level, respectively in CSCs. Finally, CSCs expressed a 100% higher ROS+ than TNBC.

TNBC CSCs are quiescent, with highly functional DNA damage repair mechanism. CSCs induce chemoresistance through downregulating genes responsible for apoptosis, autophagy, and tumor suppression. Targeting dysregulated CSCs pathways could be a potential therapy for overcoming chemoresistance.

Keywords: Triple negative breast cancer (TNBC), MDA-MB-231, MCF-10A, cancer stem cells (CSC), apoptosis, autophagy, tumor suppressor genes.

DEDICATION

This thesis is dedicated to the dearest to my heart, my mom, my dad, and my brothers.

To my husband Khaldoun and little angel Yasmina…Thank you is never enough, I would not have done it without you by my side. I hope I made you proud!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCG2: ATP-binding cassette, subfamily G, member 2

ABCB1: ATP-binding cassette, subfamily B (MDR/TAP), member 1

ABC: ATP-binding cassette

ATCC: American type culture collection

ALDH1A1: Aldehyde dehydrogenase I

APAF1: Apoptotic protease activating factor 1

ATP: Adenosine triphosphate

ATG: Autophagy related gene

AKT: Protein kinase B

ATM: Ataxia telangiectasia mutated

BAAA: BODIPY-amino acetaldehyde

BAA: BODIPY-amino acetate

BAX: BCL-2-associated X protein

BRCA: Breast cancer type 1 susceptibility protein

BMI: Body mass index

BCL-2: B-Cell lymphoma-2

BCSC: Breast cancer stem cells

Bcl-xL: B-cell lymphoma-extra large

Bim: BH3-only protein

BAD: BCL2 associated agonist of cell death

Bid: BH3 interacting-domain death agonist

BSA: Bovine serum albumin

CSC: Cancer stem cells

CARD DOMAINS: Caspase recruitment domains

CYP450: Cytochrome p450

cDNA: Complementary DNA

DNA: Deoxyribonucleic acid

DCIS: Ductal carcinoma in situ

DED: Death-inducing domain

DISC: Death inducing signal complex

DDR: DNA damage response

DMEM: Dulbecco's Modified Eagle's Medium

DEAB: Diethylamino Benzaldehyde

DEPC-H_20: Diethylpyrocarbonate water

dNTP Mix: Deoxynucleotide solution mix

DAPI: 4′,6-diamidino-2-phenylindole

ER: Estrogen receptor

EGFR: Epidermal growth factor receptor

Fas-L: Fas ligand

FBS: Fetal bovine serum

F-primer: Forward primer

GAPDH: Glyceraldehyde 3 phosphate dehydrogenase

HER-2: Human epidermal growth factor receptor 2

HO-1: Hemeoxygenase 1

HCL: Hydrochloric acid

JAK: Janus kinase

LC3-I: Light chain 3 1A/1B

LCIS: Lobular carcinoma in situ

MPT: Mitochondrial permeability transition pores

Mcl-1: Myeloid Cell Leukemia 1

mTOR: Mechanistic target of rapamycin

Mdr-1: Multidrug resistant protein

mRNA: Messenger ribonucleic acid

MSRT: MultiScribe Reverse Transcriptase

MM: Master-mix

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PD-L1: Programmed cell death ligand-1

PUMA: P53 upregulated modulator of apoptosis

PR: Progesterone receptor

PARP: Poly ADP ribose polymerase

PI3K: Phosphoinositide 3-kinase inhibitor

PTEN: Phosphatase and TENsin homolog

PIP3: Phosphatidylinositol 3,4,5-trisphosphate

PBS: Phosphate buffer saline

PI: Propidium Iodide

PVDF: Polyvinylidene fluoride

QNCR: Qatar National Cancer registry

ROS: Reactive oxygen species

RT-PCR: Real time polymerase chain reaction

rRNA: Ribosomal RNA

RT buffer: Reverse transcription buffer

RT Random Primers: Reverse transcription random primers

R-primer: Reverse primer

RIPA: Radioimmunoprecipitation assay

SOX2: SRY-Box Transcription Factor 2

SOX9: SRY-Box Transcription Factor 9

SP: Side population

SDS: Sodium Dodecyl Sulphate

STAT: Signal transducer and activator of transcription

TRAIL: TNF-related apoptosis-inducing ligand

TNF-α: Tumor necrosis factor alpha

TNBC: Triple negative breast cancer

TNFR: Tumour necrosis family receptors

tRNA: Transfer RNA

TEMED: Tetramethylethylenediamine

Tris-buffer: Trisaminomethane buffer

TBST: Tris-buffered saline tween 20

ULK: Unc-51-like autophagy-activating kinase

Chapter 1: Introduction

1.1. Cancer

Cancer has become the second major cause of death worldwide for both males and females after cardiovascular disease. Approximately 9.6 million deaths were accounted to cancer in 2018, in other terms one in every 6 deaths is related to cancer [1]. However, the death rate of cancer in the four most common types (breast, lung, prostate, and colorectal) has declined drastically since the 1900's and this decline has been attributed to the advance in research and medical field [2]. Cancer targets patients of different age groups and affects a broad variety of body organs and cells, it is known as the aberrant growth of tissues leading to the formation of a malignant tumor [1].

This disease has imposed and still imposes a tremendous load on people and healthcare system all over the world. The decrease in cancer mortality is mainly due to the successful combination between a strong preventive strategy, early detection, and patient tailored treatments and advanced medicine [2]. Thus, it has been estimated that the number of cancer survivors will increase from 14.5 million in 2015 to 18.9 million in 2024 [2]. Cancer mainly occurs due to repetitive genetic mutations which eventually lead to abnormal function of the mutated gene [3]. In men the most common cancers are respectively, the prostate, lung, colon, and urinary bladder cancers [3]. However, in women the highest percentages of cancers are in the breast, lung, colon and rectum, and finally the thyroid gland (Fig. 1) [3]. Which implies that the two most prevalent and distinctive cancers between males and females are the prostate cancer and breast cancer, respectively.

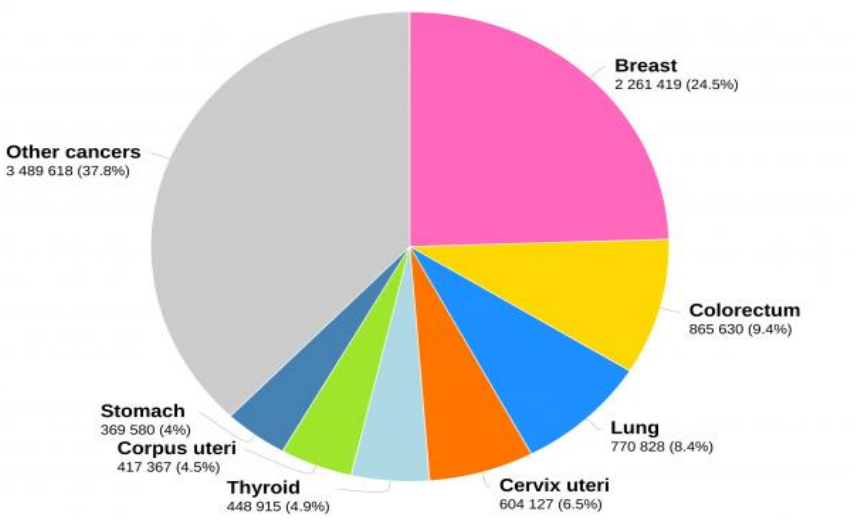


Figure 1. The estimated worldwide number of new cancer cases in females of all ages in 2020. (Globocan 2020)

Cancer progresses through multiple phases known as carcinogenesis. This process is divided into four different stages, (1) Initiation, (2) promotion, (3) progression, and (4) metastasis (Fig. 2) [4]. The initiation step includes genetic alterations or gene mutations that occur spontaneously or induced by certain carcinogenic chemicals. This first step is the most crucial step, since it can lead to dysregulation of the cellular signaling pathways related to cell cycle, apoptosis, differentiation, autophagy, metabolism, and overall survival. However, this step is reversible were DNA repair could still occur and end the carcinogenesis process [4]. The promotion stage is the longest reversible phase, by which the preneoplastic cells, also known as benign neoplasms, proliferate and accumulate, but further progress could still be inhibited by treatments with chemo-preventive agents [5]. The progression phase is the final phase that transfers the preneoplastic cells into fully malignant tumor cells. These fully differentiated cells express new phenotypes, with an increase in tumor size and metastatic potential. The metastasis phase is the final stage were the cancerous cells

spread from the original site to all over the body through transferring in blood and lymph nodes. Chemo-preventive agents are key regulators in the first two phases, where they could reverse the initiation and promotion stages. However, when targeting the progression and metastasis phases, it mainly inhibits further metastasis, angiogenesis, and invasion [4, 5].

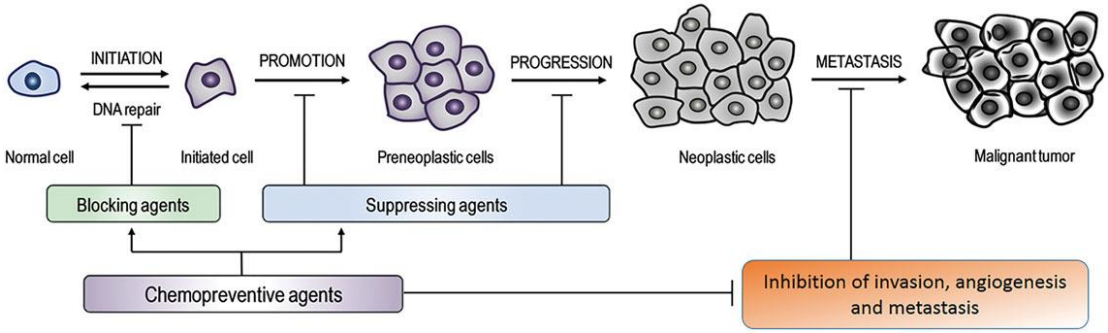


Figure 2. The phases of carcinogenesis: initiation, promotion, progression, and metastasis. (Siddiqui et al. 2015)

1.2. Breast Cancer

The most common cancer present in females, is breast cancer with around 2.09 million cases in 2018 [6]. Breast tumors also rank as the second highest cause of death, were almost 626,679 women die due to cancer every year [7]. Multiple risk factors affect the incidence rate of the heterogenous breast cancer types. Thus, breast tumors are considered as a multifactorial disease, were numerous factors lead to its occurrence [8]. Therefore, its survival, frequency, prevalence, and death vary considerably from a country to another [8].

In Qatar, according to the most recent statistics supplied by the Qatar National Cancer Registry (QNCR) in 2016, around 266 patients were diagnosed with breast cancer, almost 97% were females. The risk to develop breast tumors for Qatari women aged up to 74 years old is 8.4%, which is translated as 1 out of 12 women will develop

breast cancer sometime during their life. However, due to early detection 64% of the cases were diagnosed in either stage1 or stage2 [9].

1.2.1. Breast cancer risk factors.

The most common risk factors are divided into categories: demographic, reproductive, hormonal, hereditary, lifestyle, and other miscellaneous factors.

1.2.1.1.Demographic risk factors.

The demographic factors include gender, age, and blood group. With respect to gender, breast tumors are unique to females and affect them mainly, hence it is rarely diagnosed in men with less than 1% of the total cases [10]. However, these rare cases root to the fact that these men could have had a family history of breast tumors, hormonal imbalance, radiation exposure, or BRCA gene mutation [11]. Moreover, age is the second most important factor after gender. The probability of being diagnosed with cancer increases with age and reaches its maximum in women at menopause. However, also younger females do acquire breast cancer which appears to be more aggressive, larger in size, and with a poorer patient survival [12]. With respect to blood groups, the literature data is still controversial, were some studies suggested that females with rhesus positive and A blood group are at a higher risk of acquiring breast cancer with approximately 45.88% compared to women with AB blood group and rhesus negative with approximately 6.27% [13]. Thus, it is suggested that females diagnosed with breast cancer and are of blood group A should be closely monitored and increased screening and preventive measures should be applied.

1.2.1.2.Reproductive risk factors.

The reproductive factors are also of great importance for the breast cancer incidence. First, the age of menarche or puberty, were females with an early menarche have a two times increased risk of acquiring breast cancer compared to females with

late menarche [14, 15]. Second, the age of menopause is also a key regulator for breast cancer risk were females who acquire menopause at a later age (55 years old) will have a 30% higher risk of breast tumors compared to women who reach menopause at an earlier age of 45 years old [16]. Third, the age of the first childbirth and number of children, were women who had their baby at an older age (>30 years old) had a higher risk of breast cancer compared to women who gave birth to their eldest baby at a younger age [17]. Moreover, every full-term childbirth decreases the risk of ER+ and PR+ for the mother by approximately 10%, on the contrary to nulliparous women who face the highest risk of developing breast tumor later in their life [18].

1.2.1.3.Hormonal risk factors.

The hormonal factors play a significant role as a trigger for breast cancer risk factors, and they are divided between contraceptive methods, ovulation stimulating drugs, and postmenopausal hormonal therapy. Contraceptive methods have been proven to increase the risk of developing breast cancer; however, this risk is diminished after 5 to 10 years of the contraceptive's discontinuation [19, 20]. Similarly, treatment with hormonal therapy after menopause significantly increased the risk for both mortality and developing breast tumors; but the risk decreases after 5 years of hormonal replacement therapy discontinuation [21].

1.2.1.4.Hereditary risk factors.

The hereditary factors play a significant role in the risk of developing breast cancer later in life. Two factors play the critical role in hereditary which are, the genetic factors and a family history of breast cancer. With respect to the genetic aspect, BRCA1 and BRCA2 were found to be the most dominant genes leading to breast tumors in 40% of the hereditary breast tumor cases [22]. It has been shown that, 55-65% of the patients carrying BRCA1 gene mutation and 45% carrying BRCA2 gene mutation have

developed breast tumors by the age of 70 years old [23]. Furthermore, females with a family history of breast tumors are at a higher risk for developing breast cancer at an early stage in their lifetime [24]. However, having only one first degree relative increases the risk by 1.5 times, compared to 2-4 times higher with more than one first degree relative [25, 26].

1.2.1.5.Lifestyle risk factors.

Most known factors causing breast cancer are uncontrollable risk factors a patient could be born with or inherited. However, some factors such as lifestyle risk factors could be controlled and avoided by the patient. These risk factors are known to be, obesity and overweight BMI, alcohol consumption, cigarette smoking, sedentary life style, lack of physical activity, vitamin D deficiency, and irregular sleep patterns [8]. Obesity in females is tremendously correlated to breast cancer and has been discussed as a major risk factor. This correlation is directly related to the fact that androgenic precursors originating from the abundant cholesterol in obese patients is converted by the peripheral aromatase enzymes to estrogen, which in turn increase the hormonal levels and their effect on breast cells [27]. Moreover, the elevated insulin blood levels and insulin resistance increase the growth of cancer cells. Similarly, the body mass index (BMI) is predicator of the disease-free survival, were postmenopausal women with a BMI$\geq$30kg/m^2 when diagnosed with breast cancer had a lower survival duration [28].

With respect to the dietary intake, alcohol consumption has shown to play a significant role in both hormone receptor positive and negative breast tumors through the alcohol carcinogens targeting the breast cells. Moreover, alcoholic women have a 4.2-fold increase in being diagnosed with breast cancer [29]. In addition to alcohol, both active and passive smoking play a characteristic role in increasing the risk of

acquiring ER+/PR+ breast cancer in premenopausal and postmenopausal women. This relation is both dose and time dependent, were the higher the number of smoked cigarettes and longer duration of active smoking increase the risk of breast cancer [30].

Women aged between 50-79 years old with a non-strenuous physical activity routine have shown a decreased risk of acquiring breast cancer [31]. In addition, patients who were diagnosed with breast cancer, but were physically active showed a lower probability of death due to cancer. Thus, 3-5 hours per week of any walking activity decreases both the morbidity and mortality of breast tumors [32].

On the other hand, with respect to vitamin deficiency, the blood level of 25 OH vitamin D has shown to be a critical regulator and the most involved vitamin in cancer diagnosis and prognosis [33]. This relation has shown to be inversely proportional, were lower blood vitamin D levels cause a higher risk of acquiring breast cancer. A case control study has proven that, females with lower vitamin D levels had a 27% higher risk of acquiring breast cancer compared to females with a normal vitamin D level [34]. Therefore, vitamin D supplementation has shown to decrease the incidence of postmenopausal breast cancer. Moreover, a study has shown that most of the patients with breast neoplasms were previously suffering from vitamin D deficiency [35].

1.2.1.6.Miscellaneous risk factors.

Some other risk factors for breast cancer include diabetes, air pollution, and radiation. Diabetes has been highly correlated to breast cancer due to the mitogenic effects of insulin on estrogen regulated breast cells, thus women with type2 diabetes have a higher possibility of acquiring breast cancer [36]. A study in Netherlands have shown that, patients diagnosed with type 2 diabetes mellitus suffered from a more aggressive and advanced breast tumor stage, compared to women without the comorbid diabetic disease. Moreover, the tumors were larger in size and of a higher grade [37].

On the other hand, not only is air pollution a known factor for lung cancer, but also it has been recently correlated to the incidence of breast tumors in postmenopausal women, were a study in nine different countries in Europe have showed that breast cancer is more common in urban areas that are crowded and congested with factories and industries, due to the elevated levels of air pollution [38].

Finally, the risk factor that has shown to be both beneficial and harmful is radiation. Radiation has been a cornerstone therapy for most cancer treatments, such as leukemia, lung cancer, and sarcoma; and, as a method for certain disease diagnosis such as, pneumonia and tuberculosis. However, a large case control study has shown that patients with a previous history of radiation face a 2-3 times fold higher risk of developing breast tumors later in life [39]. Moreover, another study has proven that these patients might develop breast tumors at a younger age, and if they were diagnosed, they will have a higher risk for cancer acquired morbidity and mortality [40, 41].

1.2.2. Breast cancer subtypes.

Breast tumors are divided into different types and subtypes, the two main types are In Situ and Invasive. With respect to the In Situ, it is divided between ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), the latter is a benign tumor that would rarely lead to an aggressive tumor mass [42]. However, the DCIS is a starting point for the growth into an invasive cancer tumor. Almost 20-53% of females with DCIS will be later diagnosed with invasive breast cancer [43, 44]. On the other hand, the invasive breast cancer is the most common type of tumor presenting 81% of the total number of tumors diagnosed, they are known for their ability to invade neighboring and distant cells and organs.

Within this breast cancer types, we have two major groups the histologic subtypes and the molecular subtype [45]. However, the histologic subtype mainly

describes the structure, shape, cell arrangement, and the size of tumor cells. It is constituted from many subtypes which are not very characteristic, but include (1) Infiltrating ductal carcinoma, (2) Infiltrating lobular carcinoma, (3) mixed ductal/lobular carcinoma. The infiltrating ductal carcinoma is the most common subtype that is responsible for 70-80% of the invasive breast tumors, whereas infiltrating lobular carcinoma accounts for 8% and the mixed accounts for 7% of the total cases [46].

The molecular subtype is the most diagnosed and known type of breast tumors, this subtype is categorized into four distinct groups, luminal A or normal like, luminal B, HER-2 positive, and basal-like (Fig. 3) [47]. Within this category the luminal A is the most abundant subtype, that only expresses the estrogen and progesterone hormone receptors were it occupied 68% of the total number of breast cancer cases in 2013-2017 according to the latest statistics in 2020 [48]. Moreover, luminal A subtype is the easiest to treat since it responds very well to the conventional hormonal therapy. This subtype is the least aggressive and has the highest 5 years survival rate (94.1%) compared to the rest of the tumor types [48, 49]. Normal like subtype is very similar to luminal A, except it is less prevalent and has worse prognosis.

The second subtype is the luminal B (11%), which is positive for estrogen, progesterone, and HER-2 receptor. Luminal B 5-year survival rate is 90.4% which makes it more aggressive and associated with worse clinical outcomes. Lately, this type has been correlated to the expression of ki-67 which is a cell proliferation indicator and assists the migration of the cancerous cells [50].

The third molecular subtype occupies 5% of the total breast cancer cases and is the HER-2 receptor positive and hormone receptor negative; this subtype is no longer considered the most aggressive since targeted therapies against HER-2 receptor have been treating the patients successfully and increased its survival rate to 83.6% [51].

The fourth and last type is the basal-like subtype, triple negative breast cancer (TNBC), and is expressed in 15% of the total number of breast cancer cases. This subtype lacks the expression of all the hormone receptors and HER-2 receptor and is extremely aggressive.

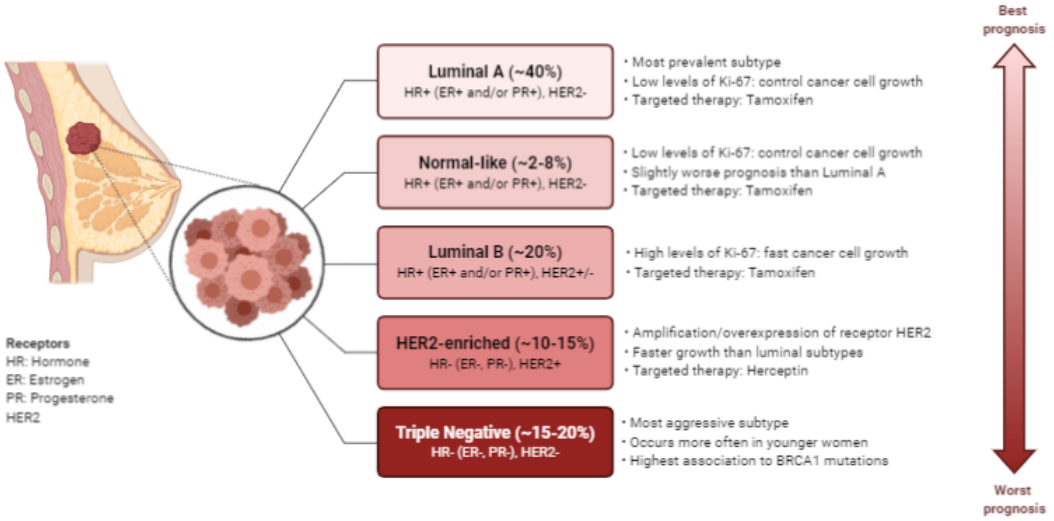


Figure 3. The molecular subtypes of breast cancer.
(BioRender, October 2020)

1.2.3. Triple negative breast cancer.

TNBC is known to be the most aggressive subtype of breast tumors and has the highest rate of recurrence and metastasis [52]. Thus, the five-year survival rate of TNBC is 76.7% compared to 93% of other breast tumors [48, 52]. One of the main reasons for the poor survival rate of this subtype, is the lack of suitable and convenient targeted treatment approaches since there is no specific receptor to regulate or inhibit

[53]. It is considered a critical type of breast tumors, not only because it has a comparatively poor treatment results, but also because women younger than 40 years old have double the risk of acquiring this specific type of cancer [54, 55].

Aside from the regular risk factors of breast cancer, TNBC has unique and distinct risk factors which include the expression of positive BRCA gene mutation that is expressed in 20% of the patients, the African American race, and premenopausal age [56, 57]. The genetic profiling of TNBC, has shown abnormal expression of the tumor suppressor gene p53 and breast cancer susceptibility gene 1 BRCA1 [58]. Therefore, this tumor cannot be targeted with the traditional hormonal endocrine therapy or therapies targeting HER-2 receptor specifically. Moreover, the upregulation of the EGFR protein leads to resistance to the conventional treatment [54].

1.2.4. Triple negative breast cancer treatments.

A specific tailored treatment approach should be designated to each patient diagnosed with TNBC. However, the ideal chemotherapy treatment for TNBC patients has not been identified yet and depends on multiple aspects, but it is suggested that platinum-based drugs have showed the best results [59]. For the most optimum treatment the patients were categorized according to their tumor size or positive lymph nodes, which implies that the breast cancer cells have migrated to the lymph node glands. Thus, the patients were divided and treated according to the breast cancer stage.

Breast cancer is divided into four distinct stages; stage 1 includes cancers that are small and did not spread to any lymph node; stage 2 moderate size and with migration to axillary lymph nodes only; stage 3 includes advanced breast tumors that did not metastasize to distant body organs; finally, stage 4 tumors that have metastasized to distant organs [60].

In non-metastatic breast cancer patients, which are categorized between stages 1-3 with a confined tumor of ≤0.5cm, a mastectomy or breast conserving surgery is the mainstay treatment [61]. In case of a larger tumor size >0.5cm or if patients have a positive lymph node, a neoadjuvant chemotherapy is recommended before surgery to shrink the tumor size and radiation might be an option after the surgery along with adjuvant chemotherapy to target the residual cancer cells. In stage 4 of breast cancer, a combination of chemotherapeutic drugs is used as a first line treatment, which includes anthracyclines (doxorubicin), alkylating agents (cyclophosphamide), and taxanes-based chemotherapies (paclitaxel) [62-64].

However, since TNBC acquires intensive genetic mutations, patients with metastatic tumors should be tested for BRCA1 mutations and for the expression of programmed cell death ligand-1 (PD-L1). Thus, in case of the absence of BRCA1 gene mutation and PD-L1, a single or combination platinum or non-platinum-based chemotherapy is advised depending on the tumor progression [65]. On the other hand, in case of PD-L1 positive tumors adding an immune checkpoint inhibitor to the chemotherapeutic drug is recommended, such as atezolizumab with nab-paclitaxel [61].

However, in case of BRCA1 gene mutation and previous treatment with chemotherapy, it is recommended to administer oral PARP inhibitors which are safer with fewer side effects and higher efficacy. PARP inhibitors have a specific mechanism to target the cancer cells, since PARP is involved in the molecular mechanism that aids in repairing the DNA damage in cancer cells [66]. Thus, when PARP is inhibited the DNA will not be repaired then it will break down and accumulate, which activates the BRCA pathway to repair the damage. Hence, treating patients with both PARP inhibitors and DNA damaging chemotherapeutics will lead to the deuteriation of the tumors [67, 68].

Finally, in patients who are positive for BRCA and PD-L1 but were never treated with chemotherapy; it is recommended to start initial chemotherapy with nab-paclitaxel and atezolizumab. However, for patients only positive for BRCA gene mutation it is recommended to initiate platinum-based chemotherapy and taxanes [61].

1.3. Cancer Stem Cells

Despite all the emerging treatment strategies against cancer including surgery, radiation, and chemotherapy, many patients still face therapy failure and remission. Hence, the poor patient prognosis and survival, and high rate of cancer relapse is a critical and challenging factor. Therefore, commencing from this fact, a critical theory has been developed suggesting that one of the core reasons for chemotherapy failure is the presence of highly chemoresistant type of cells. Thus, the high intensity of resistance and relapse detected in almost all types of cancer is directly provoked by the growth of a regenerative sub-population of carcinogenic cells with stemness properties, known as Cancer Stem Cells (CSCs).

Even with the recent concern in CSCs, very few studies have discussed the association between the deregulated signaling pathways and features of CSCs. These distinctive features are responsible not only for chemoresistance against current tumors, but also for future cancer initiation and recurrence in patients with a history of malignant tumors. Hence, the capability of cancer tumors to proliferate and circulate depends on the small population with stem cell like characteristics.

Accumulating evidence suggests that these cells are the source for tumors and reason for relapse. Not only do these cells give rise to new tumors but are also able to self-renew and differentiate just like normal stem cells. This idea that was once a hypothesis, has been supported by scientific evidence suggesting that CSCs have been identified in multiple tumor types, and that these cells exhibit specific characteristics

such as, (1) when injected in-vivo to animals they are able to generate new tumors, (2) they have enhanced chemo- and radio-resistance compared to non-CSCs, (3) they render the tumor to be very aggressive with high carcinogenicity, metastasis, and recurrence properties, (4) they express unlimited proliferation potentials, self-renewing capacity, and can give rise to new tumor cells within the original tumor or distinct one. [69-73].

Since CSCs are a small, confined and minute population of the cancer cells. Several hypotheses have been suggested on the origin of these CSCs. One hypothesis proposed that these cells originate from genetical alterations in the healthy stem cells of the original tissue line in which the cancer grows. Second hypothesis suggests that they develop initially at the embryonic stage and maintain their dormancy until they give rise to a malignant tumor, however, their exact mechanism by which they originate remains unclear [74-78]. Therefore, CSCs are able to save genetic changes over years and evade the protective control system of the body [79].

CSCs express elevated levels of ATP-binding cassette (ABC) drug transporters such as ABCG2, that actively efflux multiple endogenous and exogenous chemicals and compounds outside the cells opposite to the concentration, which leads to a side population (SP) that presents as a ‘tail’ of cells in the flow cytometry graphs [80-82]. Moreover, CSCs express selective cell surface markers such as, $CD44^{high}$, $CD24^{low}$, and aldehyde dehydrogenase I (ALDH1A1) [80, 81, 83].

The identification and isolation of CSCs depends mainly on the expression of both cell surface markers, $CD44^{+}/CD24^{-}$ and ALDH1A1, in which it has been proven that injecting as little as 200 CSCs with these cell surface marker was able to initiate breast tumors in mice, whereas injecting 20,000 cells lacking these phenotypes failed to initiate breast cancer [84]. Moreover, ALDH1-positive breast CSCs can initiate tumor

formation with a minimum of 500 cells that are chemoresistant [85].

These cells can also be identified depending on some specific transcription proteins such as, SRY-Box Transcription Factor 2 (SOX2), SRY-Box Transcription Factor 9 (SOX9), and Nanog [69, 70, 86]. Numerous studies have found CSCs to be present in different types of cancers including blood cancers [75, 76], breast tumors [84], glioma [87], lung [88, 89], colon [90] and others. Many reports indicate that the most aggressive and recurrent tumors contain a dense population of CSCs [73, 88, 89, 91]. Most of the scientific evidence proposes that patient's death due to cancer invasion and metastasis to neighboring and distant organs, is mediated by chemoresistant CSCs [69].

Therefore, it is currently confirmed that traditional chemotherapy treatments have failed to eradicate the CSC populations, which drastically constrained the performance and effectiveness of many cancer targeted therapies in severely ill patients (Fig. 4). Therefore, it has been concluded that the elimination of CSCs is the cornerstone and most essential criteria to improve treatment outcome, reduce recurrence and relapse, and enhance patient's survival. Thus, improve patients' outcomes and response to therapy, new treatment regimens that directly and specifically target CSCs should be implemented. Thus, further investigation is required for the comprehensive and detailed understanding of CSCs characteristics such as initiation, survival, and differential signaling pathways.

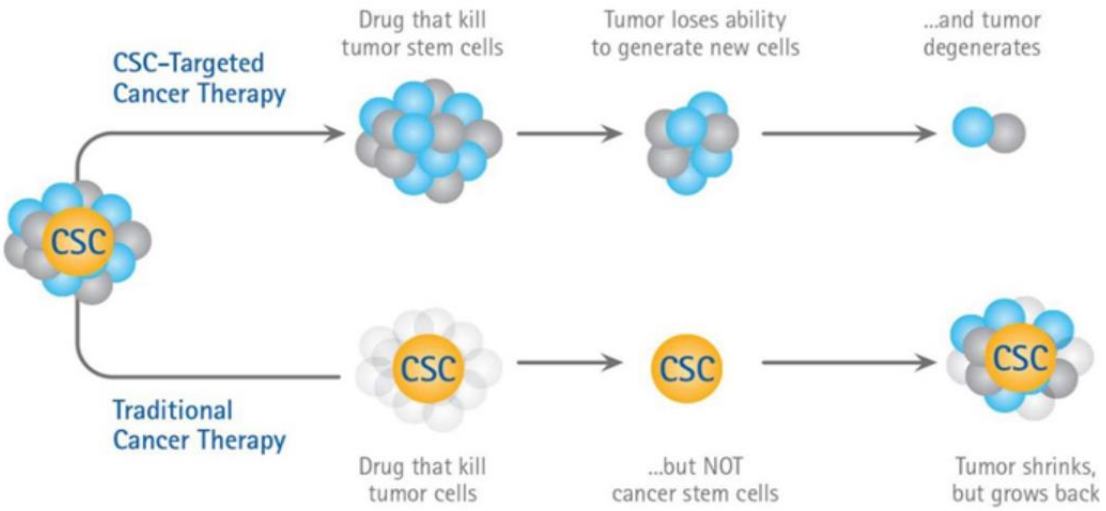


Figure 4. An illustration of the CSC theory. (Sigma al drich, 2020)

In healthy individuals the signaling pathways controlling the function and equilibrium of normal stem cells are very controlled and function in complete harmony [70]. However, in cancer tumors these pathways function abnormally and are completely dysregulated. This lack in harmonized controlled pathways leads to uncontrolled proliferation of tumor cells. These signaling pathways are known to be interwind together; thus, the most studied and tested tracks that manipulate the growth, regeneration, and resistance of CSCs are Wnt/β-catenin, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Notch [10], Hedgehog [23], and phosphoinositide 3-kinase (PI3K)/ Phosphatase and TENsin homolog (PTEN) [92].

Stem cells are critical regulators in the initiation of malignant tumors, due to their long live characteristics and presence during the whole human life span [79]. Treatment failure due to chemoresistance has been linked to the fact that all therapies target only the cancer cells, whereas breast cancer stem cells (BCSC) remain viable [73]. Thus, pose a higher risk of disease relapse after cancer treatment [73]. One of the reasons mediating chemoresistance in cancer cells is the expression of genes responsible for

different signaling pathways. Studies have shown significant variations in the genetic profiling in the CSCs compared to tumor cell population from which the CSCs originated and were isolated from. However, these pathways have not been thoroughly investigated yet.

1.4. Cancer Stem Cells Signaling Pathways

1.4.1. Apoptosis pathway.

1.4.1.1.Apoptosis.

Apoptosis is a mechanism responsible for the removal of impaired cells due to DNA damage [93]. It inhibits the further growth and division of the cells and leads to its shrinkage and death without scattering their content into the cellular environment [94]. In functional and healthy individuals, the body maintains a certain level of homeostatic balance between newly produced cells through mitosis, and the equilibrium between living cells and dead cells available. Thus, the body works in harmony with various pathways to maintain this balance and preserve it [95]. Hence, to produce new healthy cells various regulatory genes are responsible for mitosis, detecting abnormal cellular activity, and apoptosis. Extremes of either apoptosis or mitosis is not recommended since an uncontrolled cell division with minimal cell death leads to cancer [95]. However, minimal mitosis along with excessive cell death leads to multiple degenerative diseases. The apoptosis pathway is divided into an intrinsic pathway and an extrinsic pathway (Fig. 5).

The intrinsic pathway is initiated from within the cell in response to a certain stimulus, thus it is also known as the mitochondrial. Activation could be either through the absence of certain internal signals like hormones, cytokines, and growth factors which activates the inhibited pro-apoptotic molecules BAX, NOXA, and PUMA; or through the exposure to toxins, oxidative stress, radiation, hypoxia, and viruses [96].

Once this pathway becomes initiated, changes occur to the mitochondrial membrane leading to the formation of pores known as mitochondrial permeability transition pores (MPT). These pores allow the leakage of the pro-apoptotic proteins such as cytochrome-C from the mitochondria to the cytoplasm [97]. Afterwards, the cytochrome-C binds to the APAF1 protein and leads to conformational changes which allows it to bind to the deoxy ATP, this binding leads to further conformational changes that will expose the CARD and oligomerization domains of the APAF-1 allowing it to bind to multiple other APAF-1 forming an apoptosome.

Finally, this apoptosome will include multiple exposed CARD domains which in turn will activate numerous procaspase 9 [98]. Once the initiator caspase 9 becomes activated, it in turn activates the executor procaspase 3 into active caspase 3 through binding of cytochrome *c* apoptosome complex [99]. Afterwards, nucleases become activated and lead to chromosomal degradation, chromatin condensation, and finally apoptotic body formation [95].

The second apoptotic pathway is the extrinsic pathway, which is activated by macrophages or immune cells due to the production of death ligands known as Fas ligand (Fas-L), TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF), that eventually bind to the tumor necrosis family receptors (TNFR) [100, 101].

These death ‘ligands in turn bind to the death receptor activating the extrinsic pathway by activating the initiator proteins procaspase 8 and 10 into caspase 8 and 10. The exact activation mechanism for caspase 8, starts with the activation of the death receptor by the death ligand, which will trigger procaspase 8 through its death-inducing domain (DED) to become a death inducing signal complex (DISC) that will recruit multiple procaspases and activate them [102]. Then the active initiator caspases 8 and

10 activate the executor caspases 3,6, and 7 that will start the degradation and cleavage of the cellular proteins and lead to the cell death [103].

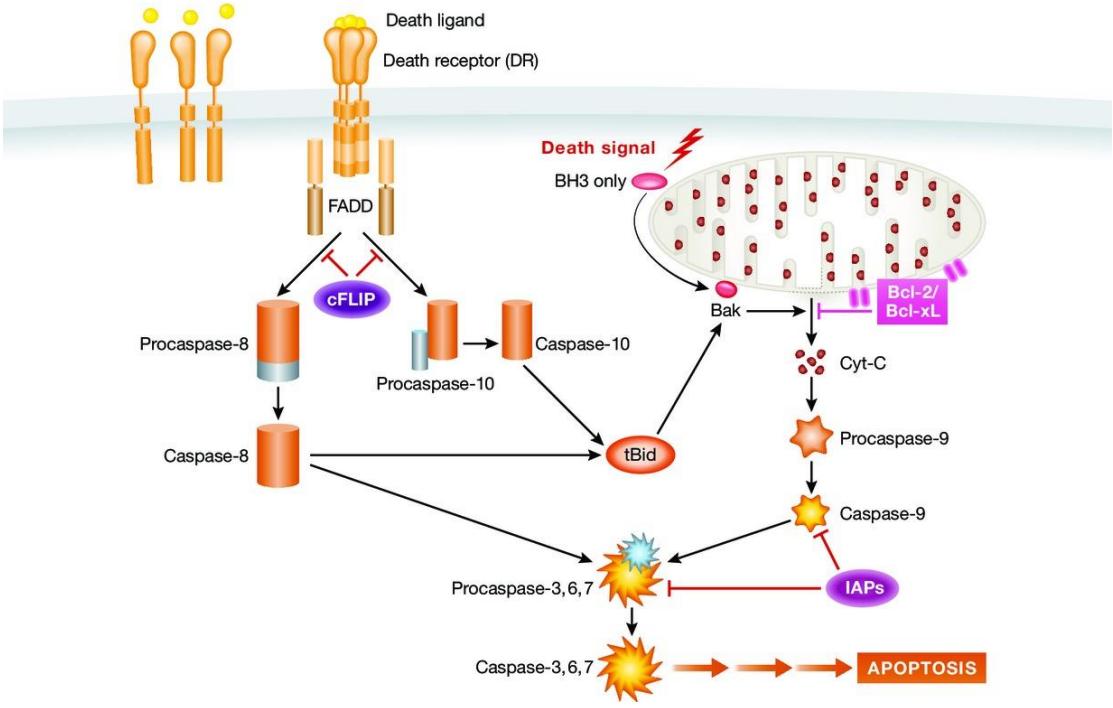


Figure 5. A schematic overview of the intrinsic and extrinsic apoptosis pathways. (Wang et al. 2015)

1.4.1.2.Apoptosis in cancer

In general apoptosis occurs through three different stages, (1) the activation of caspase enzymes, (2) the DNA and protein degradation, and (3) membrane changes and detection by phagocytic cells. At the initiation of apoptosis, the cellular membrane flips and phosphatidylserine become exposed to environment which allows its identification by macrophages leading to phagocytosis. However, in cancer and CSCs the reduced apoptotic signaling pathway or the resistance to this pathway plays a significant role in carcinogenesis (Fig. 6) [104].

However, multiple pathways are used by the cancer cells to overcome apoptosis, the most common mechanisms are: (1) an imbalanced ratio between the proapoptotic and anti-apoptotic proteins, (2) decreased caspases' activity, and (3) defective death receptors interactions [104]. With respect to the disrupted balance in anti-apoptosis and pro-apoptosis regulators the Bcl-2 proteins were noticed to have a critical role in this scheme. The family is divided into three main groups, the anti-apoptotic proteins that contain four BH domains such as Bcl-xL, Bcl-2, and Mcl-1. The second group which is made of only the BH3 and are pro-apoptotic like Bim, BAD, Bid, and PUMA. Finally, the third proapoptotic protein BAD with the four BH3 domains. The pro-survival Bcl-xL and Bcl-2 proteins were not only proven to be upregulated in cancers, but also actively promote cancer cell migration, invasion, and metastasis [105]. Thus, a corner stone treatment could be developed against breast cancer through targeting the apoptosis signaling pathway directly [105].

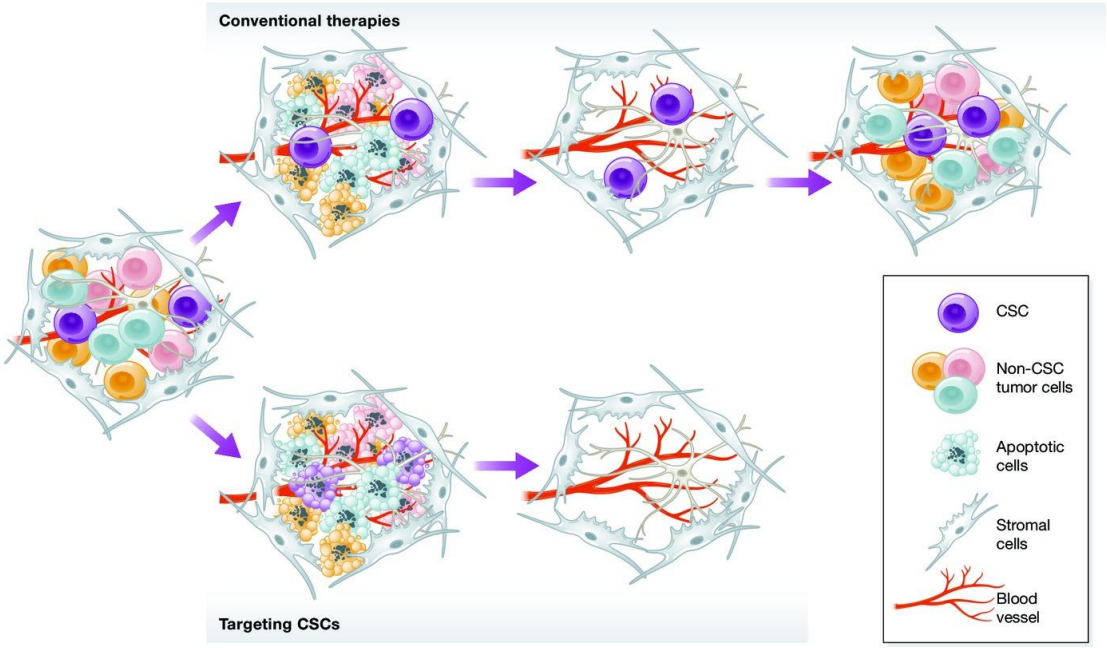


Figure 6. Scheme for treating CSCs by targeted therapy. (Wang et al. 2015)

1.4.2. Autophagy pathway.

1.4.2.1.Autophagy.

Autophagy is a physiological cellular degradative process that occurs in response to stress, organ damage, mutation, and cell starvation. It is responsible for the removal of impaired proteins and organelles in a normal functioning system [106, 107]. The main pathway of autophagy is the intracellular degradation by the double membrane vesicles known as autophagosomes. Autophagy is either general or selective, in case of general autophagy a whole part of the cytoplasm is packed into an autophagosome. However, selective autophagy is specified to certain cellular targets [106]. These autophagosome are responsible for transferring the degraded cellular components to the lysosome for recovery during stressful conditions.

Autophagy is considered as a survival mechanism since it's the primary protective function against damaged cellular proteins and toxins; hence, maintain cellular metabolism homeostasis and enhance cell survival [108]. Autophagy could be considered as a cell survival mechanism or cell death. In case of nutrient deprivation, cells undergo autophagy to maintain constant energy supply levels [109].

The autophagic process is controlled by several proteins and is initiated by mammalian target of rapamycin (mTOR) which is correlated with cell propagation, stress, and cancer progress. mTOR phosphorylates autophagy related genes (ATG) and inhibits the autophagy pathway [110]. In case of stress, mutation, or cellular damage the mTOR protein becomes inactivated, which leads to the initiation of the autophagy pathway. Upon mTOR inhibition, the Unc-51-like autophagy-activating kinase (ULK) complex gets activated through dephosphorylation. The activated ULK protein confines the phagophore and activates PI3K [111]. Thereafter, Beclin-1 and ATG elongate the autophagosome. Then, the light chain-3 (LC3) and ATGs cause the maturation and

elongation of the phagophore. Afterwards, Pro-LC3 becomes transferred into LC3-I and then into active cytosolic LC3-II [112]. Finally, LC3-II enables the binding of autophagosome to be degraded cellular material. Eventually, the mature autophagosome unites with the lysosome to form autolysosomes which will remove the damaged proteins and cellular material (Fig. 7) [113].

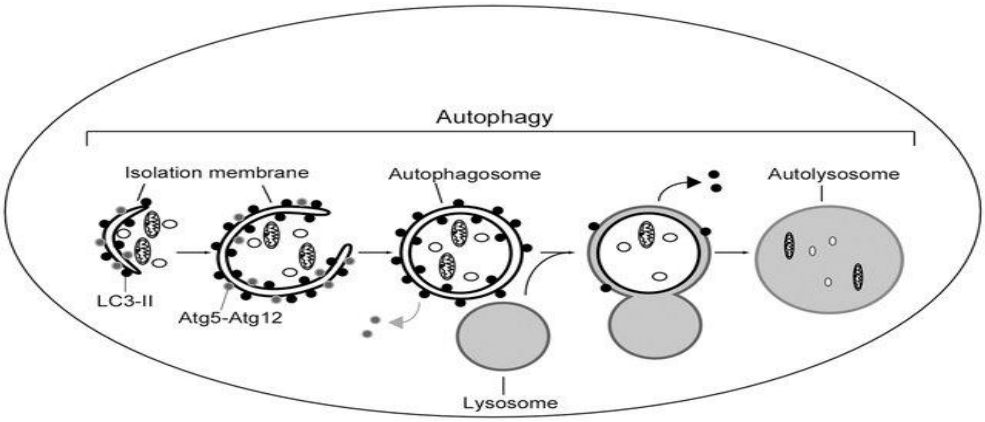


Figure 7. Scheme of autophagy in mammalian cells. (Ohmuraya et al. 2015)

1.4.2.2.Autophagy in breast cancer.

In cancer cells, autophagy plays two distinct roles either by inhibiting the tumorigenesis through inducing the cancer cell death or enhance tumorigenesis by promoting cell proliferation and tumor expansion [114]. In malignancies, autophagy could either act as a tumor enhancer, allowing malignant cells to survive and develop resistance or as a tumor suppressor [106]. In response to hypoxia and nutrient deprivation, breast cancer cells undergo autophagy where they digest their own damaged organelle and cytoplasmic proteins to fulfill the high energetic needs and metabolic demands for metastasis [115]. The mechanism of autophagy is regulated by multiple proteins such as, Beclin1, ATG, p62, and LC3II [106, 116].

1.4.3. Cytochrome P450 enzymes

CYP enzymes are a family of membrane bound protein enzymes, present all over the body but mainly in the liver, intestines, and kidneys. There are 57 different isozymes of the CYP enzymes, however only 6 of them are responsible for 90% of the drug metabolism action [117]. Since response to certain drugs varies enormously between a patient to another, CYP enzymes have been thoroughly studied due to their impact on the patient's clinical outcome. Various drugs affect the activity of CYP enzymes by downregulating or upregulating their expression, however other drugs are either inhibited or activated by the enzyme. Which only makes understanding the cellular function of CYP enzymes crucial for targeting clinical diseases [118]. The three key regulator enzymes in chemotherapeutic drugs metabolism are, CYP3A4, CYP2C19, and CYP2D6. CYP450 enzymes play an essential role in chemoprevention, tumor therapy, metastasis, and carcinogenesis. The CYP3A4 is upregulated in most hormone receptor positive breast tumors, hence the reason why tumor response to treatment has been attributed to the CYP3A4 cellular expression [119].

1.4.4. Tumor suppressor genes.

The p53 gene is a tumor suppressor gene, also known as a stress response protein, since it plays a significant role directed towards correcting DNA damages. This function categorizes p53 as a protective gene towards cancer, thus preventing against mutagenesis and tumorigenesis [120, 121]. However, some studies have shown that p53 protects cancer cells through helping them cope with their environment and overcome the protective stressors, hence aids with tumorigenesis [122]. However, p53 gene is usually inactive in most of the human cancers [123]. On the contrary mutated p53 in cancer cells leads to variable effects on tumors, where the high genomic instability facilitates the tumor evolution aiding in the tumor

adaptation to continuous changes in the tumors system, by providing a certain degree of plasticity [121, 124].

The breast cancer susceptibility gene (BRCA1, BRCA2) is a tumor suppressor gene and is highly predisposed in most cancer types. BRCA gene is responsible for the retainment of genomic integrity and quality during the DNA repair process [125]. Hence, mutations at the BRCA germline level eventually leads to loss of the gene function, which causes the genomic instability and hence ontogenically transfers the pre-malignant cells into tumor initiating cells [126].

The Phosphatase and TENsin homolog (PTEN), is a known tumor suppressor that maintains the homeostatic balance of phosphatidylinositol 3 kinase (PI3K)/AKT pathway. When phosphatidylinositol 3,4,5-trisphosphate (PIP3) becomes dephosphorylated, PTEN reverses the function of PI3K, hence blocking the functions of AKT and mTOR which are reflected on the arrest of the cell cycle advancement, initiation of cell apoptosis, stimulation of metastasis and tumor angiogenesis, cell renewal, and stem cell self-renewal [127-129].

1.4.5. Other Signaling Pathways

The Protein kinase B (AKT) is a serine/threonine-protein kinase that regulates downstream pathways participating in cellular metabolism and proliferation, survival, and angiogenesis. In cancer it is one of the most activated pathways [130]. In almost 40% of the breast cancer, increased AKT1 activity has been recorded [131].

The Nuclear Factor Kappa B (NF-KB) is highly correlated to inflammatory responses. It is initiated in response to DNA mutation and damage, ROS, and cell necrosis. In cancer cells, NFKB is triggered to actively inhibit apoptosis, accelerate cell movement and invasion, and enhance angiogenesis and metastasis of cancerous cells. In autophagy, NF-KB activates BECLIN1 and other autophagy related proteins to

enhance the autophagic process [132].

Ki-67 is a proliferation marker used mainly to assess the degree and speed of tumor growth and highly correlated with the cell cycle pathway; it is now used as a routine biological marker for the assessment of cancer prognosis in patients [133]. Elevated ki-67 protein expression has been linked to the active phase of the cancerous cell in cell cycle, and associated to invasive high-grade cancers, large tumor size, and lymph node involvement [134].

The multidrug resistant protein (Mdr-1) is a transport protein membrane of the ATP-binding cassette superfamily (ABC), which regulate the hydrophobic molecules. The protein is found all over the body including, liver, kidney, intestines, and adrenal glands. However, its infamous for inducing a significant chemoresistance in different types of cancer and as the main cause for treatment failure [135, 136]. One of the most common reasons for treatment failure in TNBC, is the chemoresistant mechanism adhered by the Mdr-1 protein. Which leads to the failure to eradicate the cancerous cells, due to the efflux of cytotoxic drugs from the site of action [137].

1.5. DNA Damage

DNA Double Strand Breaks (DSBs) are critical DNA lesions, caused by chemical, environmental, and biological elements. If damaged DNA was left unrepaired, it leads to oncogenic rearrangements due to abnormal chromosomes and genomic instability [138, 139]. DNA damage could be caused by ROS, ionizing radiations, UV radiations, and some anticancer drugs.

Once the DNA damage occurs, our body activates the DNA damage response (DDR), which in-turn activates the serine-threonine kinases known as ataxia telangiectasia mutated (ATM). Afterwards, ATM phosphorylates histone H2AX to become gamma-H2AX at the Ser139 position, and activates p53 and BRCA1 genes

through phosphorylation [140]. The detection of elevated gamma-H2AX acts an indicator for the transformation of healthy cells to premalignant and malignant tissues.

It has also been proven that triple negative breast cancer patients with elevated γ-H2AX foci experienced poor survival and prognosis compared to patients with hormone positive breast tumors [141, 142]. However due to variability in ATM and γ-H2AX expression and analysis in different cell types and tissues, the exact interpretation of their function is still quite controversial, since ATM mutations and deletions have resulted in chemotherapy resistance and poor prognosis [143]. Moreover, ATM downregulation in malignant tumors has been correlated to aggressiveness of the tumors (Fig. 8) [144].

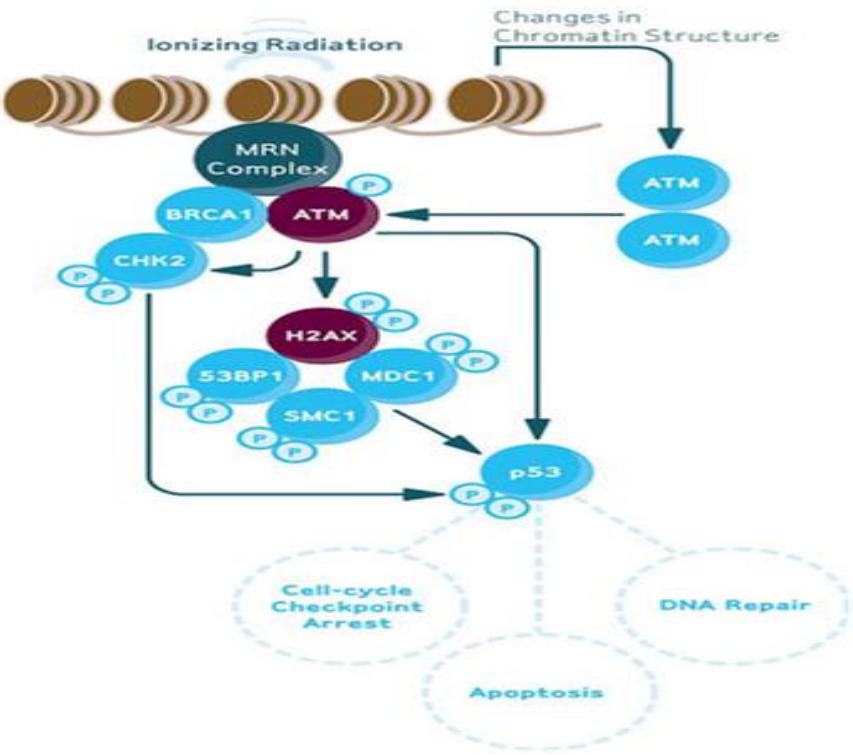


Figure 8. The DNA damage signaling pathway. (www.luminexcorp.com)

1.6. Oxidative Stress

In strictly controlled environments and under physiological conditions, reactive oxygen species are produced during cellular metabolism in inflammatory processes or aerobic aspiration, however the rate of production is increased during states of mitochondrial damage or aging. Moreover, xenobiotics detoxification, macrophages, eosinophils, neutrophils, and endothelial cells are also a secondary source of radical oxygen species.

Under normal conditions our body maintains a balance between the free radical's production and their elimination [145]. The over production of free radicals' imbalance the physiological conditions and affects all organs at different levels. The reactive oxygen species play a critical role in altering DNA sequence structures, inducing chemical modifications, and damaging proteins leading to mutations and abnormalities, causes [146, 147]. The oxygen species do not only affect cellular components, but also leads to alterations in signal transduction, apoptosis initiation, cell differentiation, and necrosis.

Since ROS cause DNA damage, thus it initiates carcinogenesis and tumor formation. However, due to consistent and intense oxidative DNA lesions, the active DNA repair mechanisms fail to repair the DNA alterations. Moreover, the chromosomal damage is not only caused by direct radical attack, but also reaction with cellular components that eventually lead to mutagenicity [148, 149].

In breast cancer, it has been shown that the DNA contains elevated concentration of oxidized bases. The hormone receptor positive showed 3.5-folds higher expression compared to negative hormone breast cancer [150]. Moreover, heme-oxygenase enzyme 1 (HO-1) is involved in regulation of cancer progression through anti-oxidative and anti-inflammatory cellular response to oxidative stress [151]. Where

the HO-1 elevated protein levels were correlated to decreased tumor size, longer patient survival, reduced cancer cell viability, induced apoptosis, cell cycle inhibition, and decrease cancer cells migration and invasion [152].

1.7. Objective, Hypothesis, and Rationale

Even with all the supporting evidence on the role of CSCs in tumor relapse and chemoresistance, the exact characteristics and features of these cells have not been investigated yet. Therefore, many therapy limitations are still present and aggressively hinder the patients' survival rate. Thus, to address this gap in literature we hypothesized that triple negative breast cancer stem cells differentially express genes responsible for chemoresistance. Therefore, we intended to investigate the gene expression of the following signaling pathways controlling the apoptosis, autophagy, CYP450 enzymes, tumor suppressor genes, DNA damage, and oxidative stress in MCF-10A, MDA-MB231, and CSCs. To test and validate our hypothesis, our main objectives:

1. We investigated the quantitative mRNA expression of the genes responsible for apoptosis, autophagy, CYP450 enzymes, tumor suppressor genes, and oxidative stress by real time PCR (RT-PCR).
2. We validated our mRNA data results by measuring the expression of these genes at the protein level using Western blot analysis**.**
3. We measured the expression of caspase 3, caspase 7, caspase 8, and caspase 9 by fluorescence intensity using immunofluorescence assay.
4. We explored the activity of cells in LC3 activated cells in autophagy, ROS+ in oxidative stress, and DNA damage by flow cytometry using Muse Cell Analyzer.

Chapter 2: Materials and Methods

2.1. Materials

4.5g/L Dulbecco's Modified Eagle's Medium (DMEM) - (1X) GlutaMAX™, TRIzol reagent, Propidium Iodide (PI), Countess™ Cell Counting Chamber Slides, and DNase/RNase-Free Distilled Water were bought from Invitrogen Co. (Grand Island, NY). High-Capacity cDNA Reverse Transcription kit and SYBR® Green PCR Master Mix were supplied by Applied Biosystems® (Foster city, CA). The DNA primers were processed and delivered by Integrated DNA Technologies (Coralville, IA). The Aldefluor® kit was supplied by Stem Cell Technologies (Vancouver, Canada). PureZOL™ RNA Isolation Reagent kit, 4X laemmli sample buffer, and ECL Clarity Western Peroxide Reagent/Clarity Western Luminol/Enhancer Reagent were purchased from Bio-Rad Laboratories (Hercules, CA). The Mouse Primary and Horse Radish Peroxidase (HRP)-conjugated secondary antibodies against target proteins, DAPI, and secondary antibody Cruz Marker ™ MW Tag-Alexa Fluor® 488 were purchased from ChemCruz, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The 0.25% Trypsin, Fetal Bovine Serum, 1% Antibiotic-Antimycotic solution, phosphate buffered saline (PBS), StemFlex media, and 2-Mercaptoethanol were ordered from Gibco™, Life Technologies (Gaithersburg, United States). The CD44/CD24 antibodies (PE/Cyanine7 anti-human CD24/CD44 Antibodies were purchased from BioLegend (California, United States). The 4% formaldehyde was supplied by AlliedSignal (Germany). Radioimmunoprecipitation assay (RIPA) buffer, 100X Halt™ Protease Inhibitor Cocktail, Pierce™ Rapid Gold BCA Protein Assay Kit, Pierce™ 10X Western Blot Transfer Buffer Methanol Free, Polyvinylidene fluoride membrane (PVDF membrane), 1% Bovine Serum Albumin, and Pierce™ 20X TBS Buffer were ordered from ThermoFisher Scientific™ (Leicester, UK). Chloroform, EMSURE®

isopropyl alcohol/iso-propranol, and 0.2% triton X-100 were purchased from Sigma-Aldrich (Oakville, Canada). The Muse® Autophagy LC3-antibody based kit, Muse® Multi-Color, DNA Damage Kit, and Muse® Oxidative Stress Kit were purchased from Luminex Corporation (Northbrook, United States).

2.2. Breast Cancer Cell Culture Model

The cell culture technique has been used for many years in laboratories across the globe as a valuable approach for studying and interpreting the molecular biology of cancerous cells. However, the benefits of cell culture models do not only reflect on understanding the cell biology, but also it is of immense importance to evaluate the function of drug effects on cancer cells, process cells for different assays, transfer cells in-vivo, and many other useful assays [153].

In the present research work, normal non-cancerous epithelial breast cells MCF-10A (ATCC® CRL-10317™) (Fig. 9A) and human TNBC MDA-MB-231 (ATCC® HTB-26™) (Fig. 9B), were obtained from the American Type Culture Collection (ATCC) [154] and utilized as a cell culture model. The cells were plated in high glucose 4.5g/L DMEM-(1X)GlutaMAX™ media (Thermo Fisher Scientific, USA) augmented with 10% heat inactivated fetal bovine serum (FBS) (Gibco™, Life Technologies) and 1% Antibiotic-Antimycotic solution (Gibco™, Life Technologies) [155]. The cell maintenance was sustained through plating and re-culturing cells at 80-90% confluency in new T75 cell culture flask with vented cap (Falcon®) at 37°C in 5% carbon dioxide humidified environment [156, 157]. The cell splitting and sub-culturing occurs at a rate of 3-4 days and the media is completely changed every other day.

The MCF10-A and MDA-MB-231 cells were cultured in 75-cm^2 tissue flasks at 37 °C and 5% CO_2 humidified environment and then plated in 6-well plates for RT-PCR, Western blot analysis, immunofluorescence, and flow cytometry assays. The medium was replaced every 2 days and the cells were divided and plated again every 3-5 days at a 3:1 ratio.

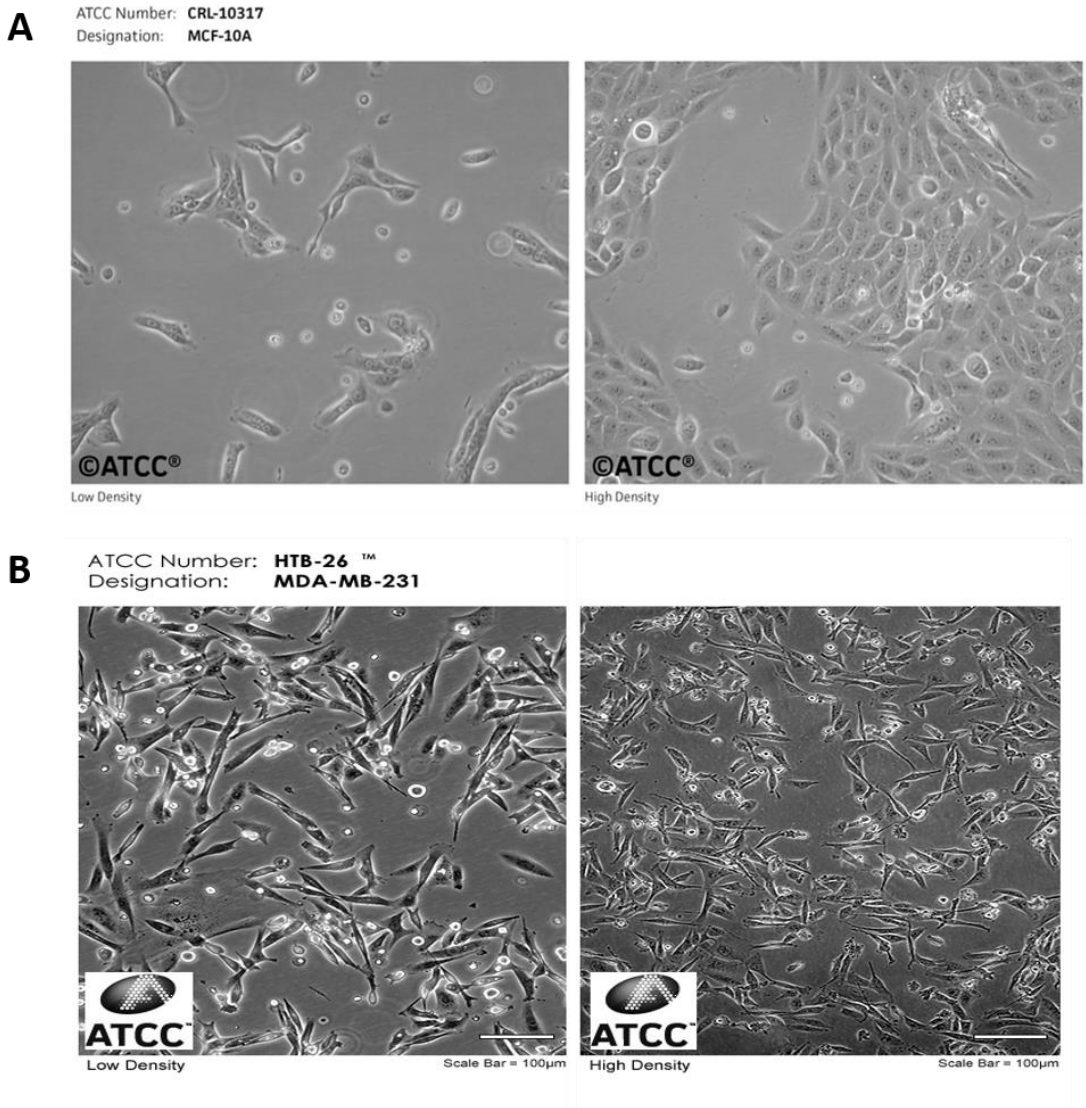


Figure 9. Morphology of MCF-10A (A) and MDA-MB-231 (B) breast cell lines. (www.atcc.org)

2.3. Cancer Stem Cells Formation Assay

Breast CSCs express different behavioral mechanisms at distant levels[158]. Thus, to assess the function and behavior of these cells, we cultured breast cancer cells at low-adherent conditions to give rise to spheroids, which are also known as mammospheres. These spheroids will allow us to study the stem cell features and their signaling pathways [158]. BCSCs of MDA-MB-231 (Fig. 10) were cultured at non-adherent conditions, were the confluent MDA-MB-231 cells were plated in T75 (Falcon®) flasks and trypsinized using 0.25% trypsin (Gibco^{TM}, Life Technologies). The cells were then centrifuged at 1,300 RPM for 5 minutes and the cell pellet was carefully resuspended in StemFlex media (Gibco^{TM}, Life Technologies) and then seeded as single cells into ultralow attachment plates (Nunclon^{TM} Sphera^{TM} Flask, Thermo Scientific^{TM}) [159]. Under such conditions, cells were allowed to grow into non-adherent grape-like spheroids for 14-21 days [160]. The CSCs were supplemented with 4ml fresh media every 3-4 days, and their number and size was determined by Evos® transmitted light microscope. CSCs were then collected for RNA isolation, protein extraction, Aldeflour assay, Side population, $CD44^{high}/CD24^{low}$, Annexin A5/7-ADD, immunofluorescence experiments, and flowcytometry.

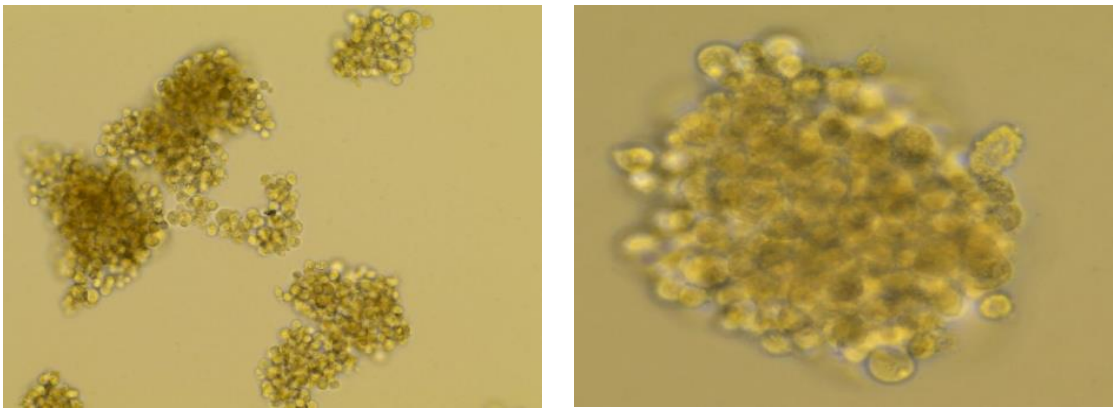


Figure 10. Morphology of MDA-MB-231 CSCs.

2.4. Aldeflour® Assay

ALDEFLOUR™ Kit assay (Stemcell™ Technologies, CANADA) is an original non-immunological fluorescent reagent system that is used to identify, evaluate, and separate stem cells based on the presence of ALDH enzyme activity [156]. For this purpose, $1x10^6$ cells/ml of both MDA-MB-231 cells and CSCs were counted by Countess™ Cell Counting Chamber Slides (Invitrogen™, ThermoFisher SCIENTIFIC) and centrifuged at 300g for 5 minutes, then the pellets were resuspended in assay buffer as per manufacturing instructions. The cells were then incubated for 45 minutes at 37°C with 5ul of ALDEFLOUR reagent which contains ALDH substrate with and without Diethylamino Benzaldehyde (DEAB) reagent, a specific ALDH inhibitor (Fig. 11) [161]. The cells were then centrifuged, and the pellets were resuspended in 300ul of the assay buffer at 4°C. Afterwards, the cells were stained with 3ul Propidium Iodide (PI) (Invitrogen by ThermoFisher Scientific, Vienna, Austria) before running flow cytometry. The percentage of ALDH positive CSC were determined by BD FACS flow cytometer.

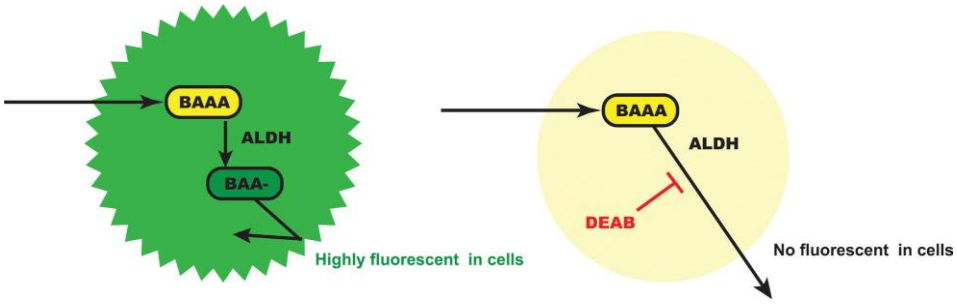


Figure 11. Principle of the Aldeflour assay.
ALDH converts BAAA into the fluorescent product BAA, which is preserved inside living cells. DEAB inhibits ALDH action on BAAA. (Tomita et al. 2016)

2.5. Side Population Assay

The side population (SP) assay is a very essential analysis to detect and isolate CSCs in different tissues based on the ability of CSCs to efflux the fluorescent DNA-binding Hoechst dye via the ATP-binding cassette (ABC) transporters [162]. Confluent cells were collected by trypsinization using 0.25% trypsin (Gibco^{TM}, Life Technologies) followed by spinning. The pelleted cells were then resuspended in a cell-penetrable Hoechst 33342 dye for 90 minutes in water bath [162]. Afterwards, the cells were centrifuged at 300g for 5minutes then resuspended with 2µg/mL of PI in running solution and kept on ice for another 5 minutes [162]. We detected the efflux characteristic by using LSRII flow cytometer from BD Bioscience, where a distinct dark tail appeared to represent the ejection of the dye (Fig. 12) [156].

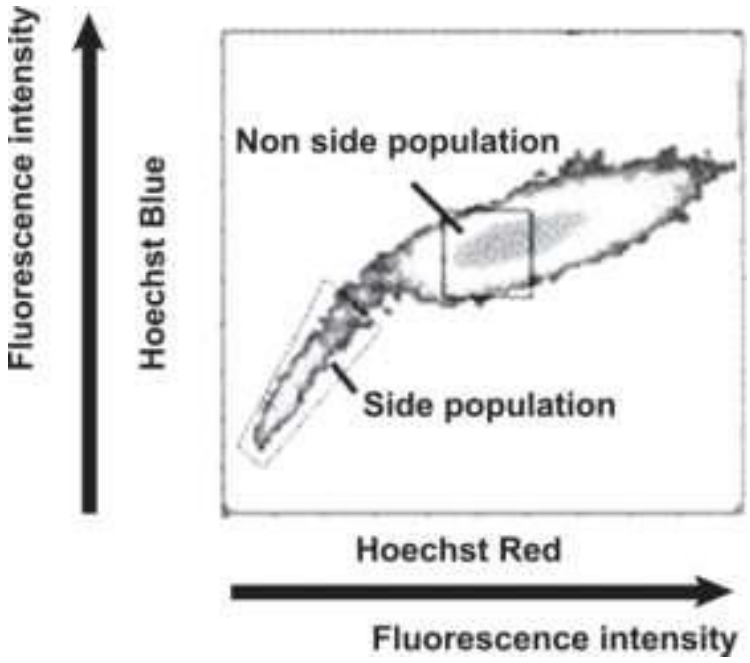


Figure 12. Side population representation analyzed by flow cytometry. (SCR 2010)

2.6. $CD44^{high}/CD24^{low}$ Expression Assay

Overexpression of CD44 receptor ($CD44^{high}$) and low expression of CD24 receptor ($CD24^{low}$) has been proven to be an essential stem cell identification marker [163, 164]. CD44/CD24 proteins are involved in various cellular and molecular mechanisms; in which CD44 is responsible for the growth, expansion, adhesion, and metastasis of cancer cells, whereas CD24 supports the adhesion, progression, and development of tumorigenic cells [165]. Characterization of the CD44/CD24 expression levels were determined using the technique of Li et al. [166].

Confluent MDA-MB-231 cells and the CSCs were cleansed with PBS and then collected by trypsinization using 0.25% trypsin (Gibco™, Life Technologies) followed by spinning at 1,300 RPM for 5 minutes. The pelleted CSCs were incubated with 3ml 0.25% trypsin for 5 minutes to dissociate the spheroids into single cells, which were further centrifuged at 1,300 RPM for another 5 minutes. Approximately $1x10^6$ cells, were counted by Countess™ Cell Counting Chamber Slides (Invitrogen™, ThermoFisher SCIENTIFIC) and incubated with 2ul of 200ug/mL (1:100) CD24 and CD44 antibody (PE/Cyanine7 anti-human CD24 Antibody, BioLegend®) (PE/Cyanine7 anti-human CD44 Antibody, BioLegend®) [166] on ice away from light, respectively. After 30-45 minutes, the cells were pelleted by spinning and the pellets were resuspended in PBS with 0.4% paraformaldehyde (AlliedSignal, Germany) and measured by BD FACS Flowcytometry [167].

2.7. Quantification of mRNA Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

2.7.1. RNA isolation.

For MCF-10A and MDA-MB-231 cells, total RNA was isolated using TRIzol reagent (Invitrogen®, ThermoFisher Scientific). The cells were cultured into 6-well plates until 80-90% confluency, then they were incubated with 500uL of TRIzol reagent [156]. Afterwards, the cells were collected into a sterile 1.5ml tubes (Axygen® 1.5mL MaxyClear Microtubes) and mixed with 200uL of cold chloroform (Sigma-Aldrich) followed by spinning at 12,000 RPM for 15 minutes at 4°C [168]. The upper aqueous colorless supernatant layer, which contains RNA, was transferred to a new centrifuge tube, and mixed with 300ul of isopropyl alcohol/iso-propranol (EMSURE®) for few seconds, followed by spinning at 12,000 RPM for 10 minutes at 4°C to precipitate the RNA. The pellet (RNA) was then purified by washing with 500ul of 75% DEPC-H20 ethanol (EMSURE®) followed by centrifugation at 12,000 RPM for 5 minutes at 4°C. The pellet was then resuspended in 20-50ul of DEPC-H_20 (Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water by life technologies) followed by heating at 55-60°C in a Digital Dry Bath (Bio-Rad) for 10 minutes to ensure total resuspension of the RNA [168].

For CSCs, the total RNA isolation was conducted using PureZOL™ RNA Isolation Reagent kit (Life Science Research, Bio-Rad) according to the origin company's instructions. Single CSCs were mixed with 600uL of the lysis buffer, followed by the addition of 600uL of 70% ethanol (EMSURE®). After mixing, the tube contents were transferred into new spin filter-tubes and centrifuged at 12,000g for 15 seconds, followed by addition of 700ul of wash buffer I. The tubes were centrifuged again, and the pellet was washed by adding wash buffer II and centrifuged. The latter step was repeated multiple times to dry the membrane. After serial centrifugation steps,

30uL of DNAse/RNAse free water were added drop wise to the center of the filter and kept at room temperature for 1 minute followed by spinning at 12,000g for 2 minutes, the resultant filtrate contains the RNA.

After RNA isolation and purification steps, the RNA concentration and pureness were measured using NanoDrop™ 8000 Spectrophotometer (ThermoFisher Scientific). The RNA concentrations were calculated at 260 absorbance using the equation (1 OD_{260} = 40 ug/ul RNA). Whereas the RNA purity was maintained high by keeping the ratio of 260/280 within the range of 2.0 optical density [156]. The RNA samples were then kept at -20 °C for further experiments.

2.7.2. cDNA synthesis.

The cDNA synthesis step was conducted using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems™, ThermoFisher SCIENTIFIC) according to the company's instructions. Approximately 10uL contains 1ug of the RNA from each sample, the RNA and DEPC-H_2O were incubated with 10uL of cDNA master mix (MM) (Tab. 1). The mixture reaction was a total of 20uL volume, which was then incubated in the ProFlex PCR System (Applied Biosystems by life technologies) with the specified cycles protocol (Tab. 2).

Table 1. cDNA mastermix components

Components	**1 reaction**
10X RT buffer (1mL)	2ul
25X dNTP Mix (100mM, 0.2mL)	0.8ul
10X RT Random Primers (1mL)	2ul
MultiScribe Reverse Transcriptase (MSRT) (0.2mL at 50U/uL)	1ul
RNAse inhibitor (2x0.1mL at 20U/uL)	1ul
NF H_20	3.2ul

Table 2. cDNA conversion cycle protocol

Stage	**Cycles**	**Duration**	**Temperature**
Stage 1	1X	10 minutes	25°C
Stage 2	40X	3 minutes	31°C
Stage 3	40X	10 seconds	85°C
Stage 4	infinity	infinity	4°C

2.7.3. Real-time polymerase chain reaction (RT-PCR)

Quantitative polymerase chain reaction (Q-PCR) or Real time polymerase chain reaction (RT-PCR) is a quantitative and sensitive method, that measures the amount of polymerase chain reaction that is translated to the gene expression [169]. It quantifies the gene expression and allows the researcher to detect differential genes expression in different cell lines [170]. The mRNA expression of our focus genes was measured and quantified by QuantStudio® 5 Real-Time PCR System (Applied Biosystems™, ThermoFisher SCIENTIFIC) using SYBR Green Universal Mastermix (ThermoFisher SCIENTIFIC) as outlined previously in Al Dhfyan et al. [156].

The resultant cDNA was amplified by incubation with SYBR green in the presence of forward and reverse primers of each gene (Integrated DNA technologies, Coralville, USA) as illustrated in (Tab. 3) with the specified cycles protocol (Tab. 4).

Table 3. RT-PCR mastermix components

Components	1 reaction
PowerUp™ SYBR™ Green Master Mix	12.5ul
Forward primer (F-primer)	0.07ul
Reverse primer (R-primer)	0.07ul
RNAse/DNAse free water	11.15ul

Table 4. RT-PCR cycle protocol

Stage	Hold	PCR (40cycles)	PCR (40 cycles)
Temperature	95°C	95°C	60°C
Duration	20 seconds	3 seconds	30 seconds

The change in the level of target genes (Tab. 5) were normalized against the housekeeping gene GAPDH levels. Control samples lacking cDNA were added into the same plate, to test for the contamination of any used reagents. The RT-PCR data were studied using the relative gene expression (ΔΔ CT) method, as described, and explained previously [156] using the following equation:

fold change = $2^{-\Delta(\Delta Ct)}$ where $\Delta Ct = Ct_{(target)} - Ct_{(GAPDH)}$ and $\Delta(\Delta Ct) = \Delta Ct_{(CSCs)} - \Delta Ct_{(non\text{-}CSCs)}$ [156].

Table 5. List of primers used in RT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp.
Apoptosis Genes			
Caspase3	CGCAGACCTTGTGATATTCCAG	CGTTTCTTCCATCCTTCCAGG	55.6
Caspase7	TGAGCCACGGAGAAGAGAAT	TTTGCTTACTCCACGGTTCC	55.7-55.2
Caspase8	ACCTTGTGTCTGAGCTGGTCT	GCCCACTGGTATTCCTCAGGC	58.2-60.2
Caspase9	ATGGACGAAGCGGATCGG	CCCTGGCCTTATGATGTT	57.5-51.9
BAX	CCCTTTTGCTTCAGGGTTTC	TCTTCTTCCAGATGGTGAGTG	54.3-54
Bcl-xL	CTGAATCGGAGATGGAGACC	TGGGATGTCAGGTCACTGAA	54.6-55.7
Autophagy Genes			
ATG	CCAACATGGCAATGGGCTAC	ACCGCCAGCATCAGTTTTGG	56.8-58.7
P62	GGGGACTTGGTTGCCTTTT	CAGCCATCGCAGATCACATT	55.9-55.6
LC3II	CATGAGCGAGTTGGTCAAGAT	TCGTCTTTCTCCTGCTCGTAG	55-56.1
Cytochrome P450 Enzymes			
CYP3A4	TACACAAAAGCACCGAGTGG	TGCAGTTTCTGCTGGACATC	55.4-55.5
CYP2D6	TGTGCCCATCACCCAGAT	AAGGTGGAGACGGAGAAGC	56.5-56.8
CYP2C19	GGATTGTAAGCACCCCCTG	TAAAGTCCCGAGGGTTGTTG	55.7-54.7
Stemness Markers Genes			
ALDH1A1	ACTGCTCTCCACGTGGCATCTTTA	TGCCAACCTCTGTTGATCCTGTGA	60.3-60.4
SOX2	TTACGCGCACATGAACGGCT	TGCGAGTAGGACATGCTGTA	60.1-55.7
SOX9	ATGAACGCCTTCATGGTGT	TCTCGCTCTCGTTCAGAAGT	50
Tumor Suppressor Genes			
PTEN	AAGGCACAAGAGGCCCTAGATTTCT	ACTGAGGATTGCAAGTTCCGCCA	60-61.2
p53	ATGGCCTCCCTGTACGACATC-3'	TGTTGCGCTCAATCTCCTCCT	58.9-58.7
BRCA	GCAGGAAATGACATTGTAGGAAAA	TGCCAGAATGAGAAAGAACATCC	53.9-55.2
Other Signaling Genes			
NF-KB	ATGGCTTCTATGAGGCTGAG	GTTGTTGTTGGTCTGGATGC	54.1-54.5
AKT	GAAGGACGGGAGCAGGCGGC	CCTCCTCCAGGCAGCCCCTT	65.9-64.4
TNF-a	CCTGCCCCAATCCCTTTATT	CCCTAAGCCCCCAATTCTCT	55.1-56.6
Ki-67	TCCTTTGGTGGGCACCTAAGACCTG	TGATGGTTGAGGTCGTTCCTTGATG	62.5-58.9
Oxidative Stress			
HO-1	CTCGAAATGGACCCCAACTG	CAGCCCTGGGCACACTTG	55.8
Housekeeping gene			
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	57.6-53.8

2.8. Protein Extraction and Western Blot Analysis

2.8.1. Protein extraction.

Total protein extraction from both MCF-10A and MDA-MB-231 was conducted as outlined previously [171, 172] with several changes for CSCs. Confluent MCF-10A and MDA-MB231 adherent cells in 6 well plates were scrapped in 50µl of Radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher SCIENTIFIC) and Halt™ Protease Inhibitor Cocktail (ThermoFisher SCIENTIFIC). Whereas for pelleted CSCs, they were resuspended in RIPA buffer containing Halt™ Protease Inhibitor Cocktail. The whole proteins from all cells were extracted by keeping the lysates for 1 hour on ice with periodic vortexing every 5-10 minutes, followed by spinning at 12,000g for 20 min at 4◦C [171]. The supernatant, which contains the total extracted proteins, was then collected, and kept at -20◦C for protein quantification.

2.8.2. Protein quantification.

The total proteins were quantified using the Pierce™ Rapid Gold BCA Protein Assay Kit (ThermoFisher Scientific) using bovine serum albumin (BSA) as standard according to the manufacturing instructions. Approximately 10uL from each sample and BSA were incubated with working reagent for 5 minutes at room temperature, thereafter the absorbance at 480nm was reported and the protein concentrations were calculated according to BSA standard curve using spectrophotometer MULTISKAN Sky (ThermoScientific) [171].

2.8.3. Western blot analysis.

Western blot is a laboratory technique used for the identification and separation of the proteins. This method is based on separating proteins depending on their molecular weight by electric current, where the heavier proteins remain at the top of the gel whereas, the lighter proteins move faster towards the end of the gel. Afterwards,

once the proteins are separated, they should be transferred into a specific membrane for probing with the required antibody of the protein of interest (Tab. 6) [173].

Western blot analysis was applied using formerly conducted methods in Korashy et al. in 2004 [171]. Around 30-50µg of proteins from each cell line were diluted with the required volume of H_2O and 4X laemmli sample buffer (BIO-RAD) with 2-Mercaptoethanol (Gibco, ThermoFisher SCIENTIFIC). Thereafter, the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), the samples were run at 60V for 20 minutes followed by 120V for around 2 hours. Then, the proteins were electrophoretically transferred from the gel to the Polyvinylidene fluoride membrane (PVDF membrane) (ThermoFisher Scientific™) in 1X Pierce™ Western Blot Transfer Buffer Methanol Free (ThermoFisher Scientific).

The Protein blots were incubated and blocked overnight at 4 °C in 5% non-fat dairy milk (Regilait), 1% BSA (BSA-Fisher Scientific), and 1X Tween-20 in Tris buffered saline solution (TBST). Afterwards, the solution was disposed, and the blots were washed 3X for 10 minutes interval in a 1X TBST wash buffer successed by overnight incubation with primary antibody (SantaCruz Biotechnology, Dallas, Texas) against target genes at 4 °C in the fridge. On the next day, the primary antibody solution was replaced, and blots were washed 3X with the 1X TBST, followed by addition of secondary antibody (SantaCruz Biotechnology, Dallas, Texas) for 1 hour at room temperature.

The bands were imaged by Chemiluminescence Blot Scanner (ThermoFisher Scientific) using the enhanced ECL Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent (Bio-Rad) according to the company's manual. Membranes were re-probed with GAPDH mouse anti-human primary antibody, which was used as loading control.

Table 6. List of primary antibodies used in Western blot analysis

Primary Mouse Monoclonal IgG Antibody	**Catalogue Number (Santa Cruz Biotechnology)**
Apoptosis Genes	
Caspase3	sc-7272
Caspase7	sc-73424
Caspase8	sc-56070
Caspase9	sc-56076
BAX	sc-20067
Bcl-xL	sc-8392
Autophagy Genes	
ATG-12	sc-271688
Dynactin P62	sc-55603
LC3II (α/β)	Sc-39882
Cytochrome P450 Enzymes	
CYP3A4	sc-53850
CYP2D6	sc-130366
Stemness Markers Genes	
ALDH1A1	sc-374149
Tumor Suppressor Genes	
PTEN	sc-7974
p53	sc-126
BRCA	sc-6954
Other Signaling Genes	
NF-KB	sc-7386
AKT	sc-293125
TNF-a	sc-52746
Mdr-1	sc-55510
Oxidative Stress	
HO-1	sc-136960
Housekeeping gene	
GAPDH	sc-47724

2.9. Immunofluorescence Assay

The immunofluorescence assay is a laboratory technique used to detect the proteins at the cellular levels. This technique can localize the exact place of the proteins through staining the cells with fluorescent antibody that will emit fluorescence when it is exposed to a specific wavelength [174].

The MCF-10A or MDA-MB-231 cells were cultured on glass slides with almost 2-3X10^5 cells/well in 6-well for 3 days until 80% confluence. The cells were then rinsed 2X with PBS and then fixed in 4% formaldehyde (AlliedSignal, Germany) in PBS for 15 minutes at room temperature. Regarding CSCs, the pelleted cells were dispersed and fixed in 70% freshly prepared ice-cold alcohol, then carefully added drop wise on the cover slides. The fixative solutions for all cells were aspirated and discarded, and the cells were rinsed 2X with PBS for 5 minutes interval at room temperature.

The cells were then permeabilized with 0.2% triton X-100 in PBS (Sigma Aldrich) for 10 minutes, followed by washing twice with PBS before they were blocked in 10% FBS in PBS at room temperature. After 1 hour, the fixed cells were rinsed 2X with PBS for 5 minutes interval and then stained with primary antibodies against caspase 3, caspase 7, caspase 8, and caspase 9 proteins overnight at 4◦C in a humidified chamber, followed by incubation with secondary antibody (Cruz Marker ™ MW Tag-Alexa Fluor® 488) in PBS for 2 hours and then with 4′-6-Diamidino-2-phenylindole (DAPI, ChemCruz, Santa Cruz Biotechnology) for nuclear DNA staining for 2 minutes. Each sample cell line was stained three times by the antibody of the targeted protein. The fluorescence staining intensity and cellular localization of the proteins were visualized by the Evos M5000 Imaging System (Invitrogen, ThermoFisher Scientific).

2.11. Autophagy Assay

The cells undergoing autophagy were analyzed by LC3 activation using the Muse® Autophagy LC3-antibody based kit. This kit detects and monitors the amount of lapidated LC3. The permeabilization solution differentiates between the cytosolic LC3 and the autophagic LC3, by solubilizing the cytosolic LC3 and protecting the LC3 in the autophagosome which will further allow the fluorescence measuring by flow cytometer. Moreover, the autophagy reagent A will detect and prevent LC3 degradation by lysosomes.

The MDA-MB-231 cells and CSCs were plated by 40,000 cells/well in a 12-well plate and incubated overnight. Then, the MDA-MB-231 cells and CSCs were incubated for 2 hours with 2,000ul of PBS and 2ul of Autophagy Reagent A (1:1000). Afterwards the cells were trypsinized by 0.25% trypsin, and to the pellet we added 5 uL of Anti-LC3 Alexa Fluor®555 and 95uL of 1X Autophagy Reagent B and incubated on ice for 30 minutes away from light. Then, the samples were washed with 1X Assay Buffer and pellet was resuspended in 200µL 1X Assay Buffer and read by Muse Cell Analyzer at 500 events per uL (Fig. 13).

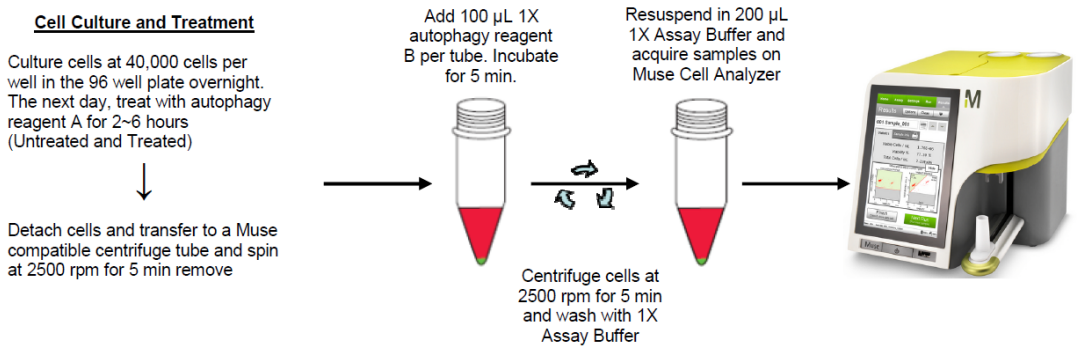


Figure 13. An illustration for the Muse® autophagy LC3-antibody protocol. (www.luminexcorp.com

2.12. DNA Damage Assay

DNA damaged cells were evaluated using Muse® Multi-Color DNA Damage Kit. This kit included two conjugated antibodies that measure the extent of DNA damage through the detection of the phosphorylation of ATM and Histone H2A.X. The MCF-10A, MDA-MB-231, and CSC pellets were resuspended with 500µL of 1X assay buffer per 1,000,000 cells. Then, equal amount (1:1) of Fixation Buffer was added to cell and kept for 10 minutes on ice. Then, the cells were centrifuged at 300 x g for 5 minutes and permeabilized by adding 1ml of ice-cold 1X permeabilization buffer on ice for 10 minutes. Afterwards, cells were reconstituted with 900ul of 1X Assay Buffer + 10µL of the antibody working cocktail solution and kept for 30 minutes away from light at room temperature. Thereafter, 100µL of 1x Assay Buffer was added and centrifuged at 300 x g for 5 minutes. The cells were resuspended with 200µL of 1x Assay Buffer and detected on the Guava Muse Cell Analyzer (Fig. 14).

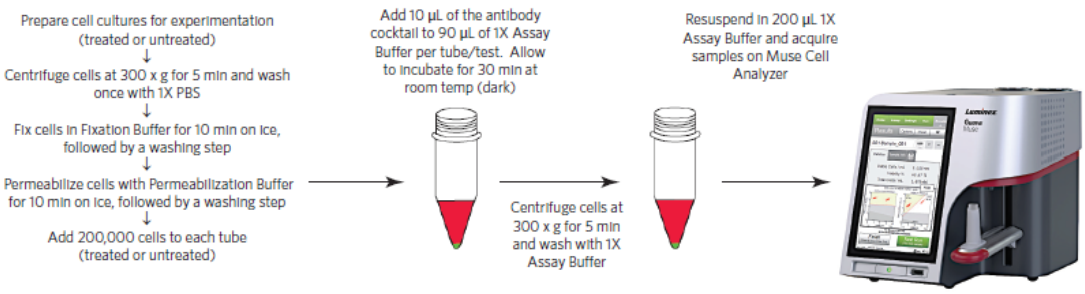


Figure 14. An illustration for the Muse® multi-color DNA damage protocol. (www.luminexcorp.com)

2.13. Oxidative Stress Assay

The reactive oxygen species were analyzed using Muse® oxidative stress kit. This kit allows for the numerical measurement of cells suffering from oxidative stress, depending on the level of reactive oxygen species detected. The dihydroethidium (DHE) reagents enter the cells and interacts with superoxide anions to constitute DNA-binding fluorophore ethidium bromide that binds to DNA and radiate a red fluorescence. The MCF-10A, MDA-MB-231, and CSCs pellets were suspended in 1X assay buffer at 1 x 10^6 cells/mL. Then, 10µL of cells were added into every tube and mixed with 190µL of Muse Oxidative Stress Reagent working solution. Finally, the samples were incubated for 30 minutes at 37°C and measured by Muse Cell Analyzer (Fig. 15).

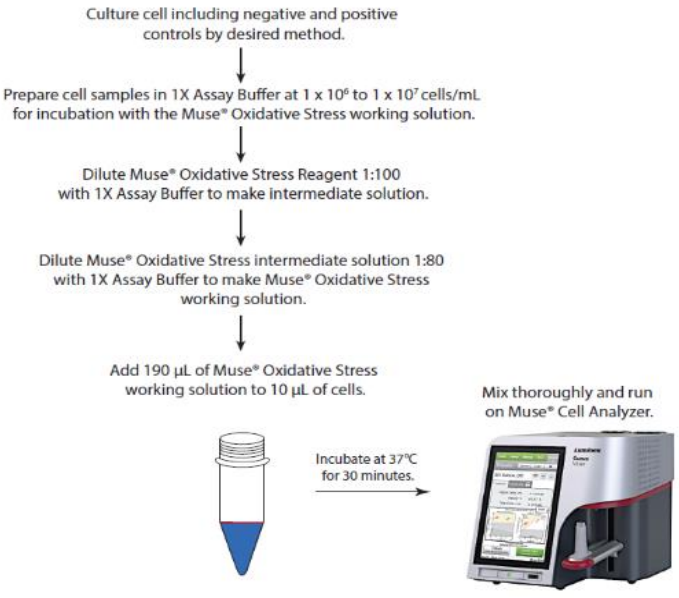


Figure 15. An illustration for the Muse® oxidative stress protocol. (www.luminexcorp.com)

2.14. Statistical Analysis

Statistical analysis of the produced data was performed using SigmaPlot 11 by independent t-test to compare the fold of induction between MCF-10A and MDA-MB-231, and MDA-MB-231 and CSC separately. Difference in the fold of induction between the groups was considered statistically significant at $p<0.05$.

Chapter 3: Results

3.1. Identification and Characterization of Cancer Stem Cells

To explore the stemness features and characteristics of the isolated CSCs, we evaluated the expression and function of 5 different CSC markers; ALDH, $CD44^{high}/CD24^{low}$, SP, SOX2, and SOX9. Therefore, four independent experiments were performed as follows:

3.1.1. Aldehyde dehydrogenase (ALDH) enzyme activity and expression.

ALDH has been considered as a distinctive key marker for the identification, characterization, and isolation of CSCs through endogenous enzyme activity and not cell surface markers [175]. To explore the expression of ALDH, CSCs were incubated with ALDH substrate (BAAA), afterwards the number of ALDH positive cells (CSCs) were detected by Aldeflour assay using flow cytometry (Fig. 16A). However, (Fig. 16B) shows that the number and percentage of ALDH+ cells were higher in CSCs than in differentiating MDA-MB-231 cells (non-CSCs) by approximately 5-folds. The selectivity and specificity of the ADLH+ cells (CSCs) were confirmed by the ability of DEAB, an ALDH inhibitor, to block the conversion of BAAA to its fluorescent substrate BAA.

The results were further confirmed by determining the expression of ALDH1A1 at the mRNA and protein levels in MCF-10A, MDA-MB-231, and CSCs using RT-PCR and Western blot analysis, respectively. The results demonstrated in (Fig. 17B) show a 4-fold increase of ALDH protein expression level in MDA-MB-231 than in MCF-10A cells ($P<0.001$). Importantly, the CSCs expressed higher ALDH mRNA (Mean=13.513, $P<0.001$) (Fig. 17C) and protein (Mean=1.333, $P=0.009$) (Fig. 17D) levels than in MDA-MB-231 cells.

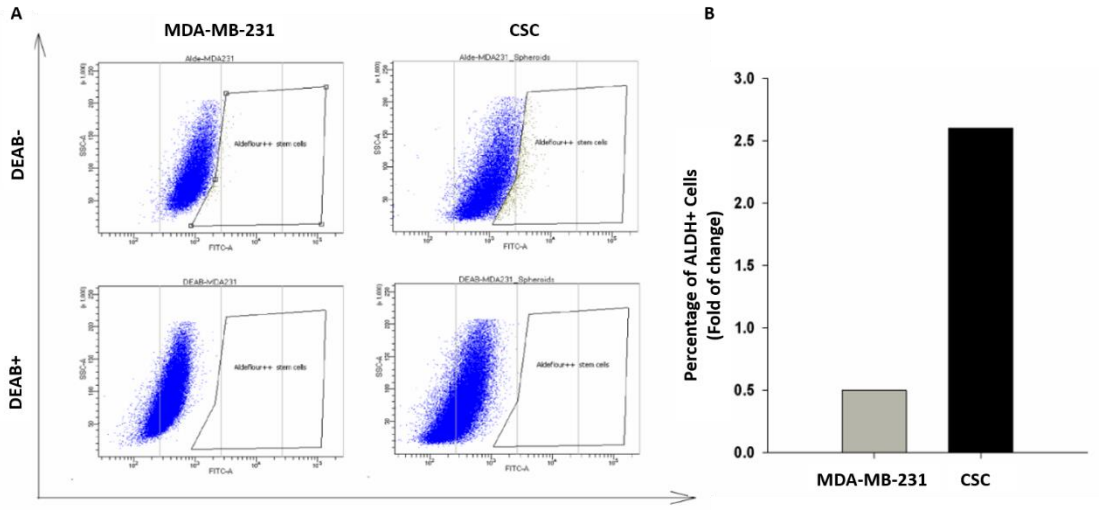
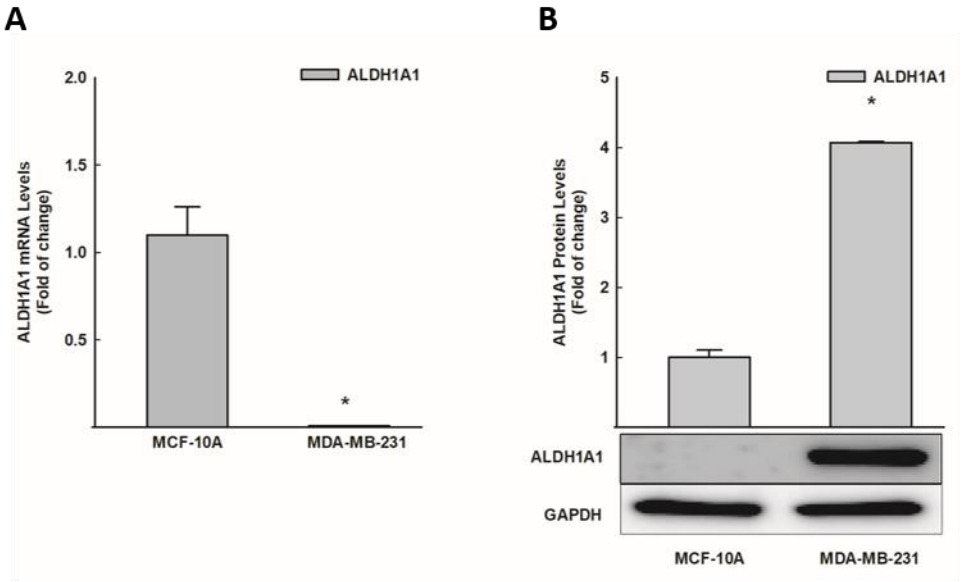


Figure 16. Constitutive expression of ALDH in cancer stem cells versus MDA-MB-231 cells by flowcytometry.
The pelleted MDA-MB-231 cells and CSCs were maintained with ALDH reagent with and without DEAB, an ALDH suppressor (Fig. 16A). Afterwards, the percentage of ALDH positive cells was detected by BD FACSAria® flow cytometer cell sorter (Fig. 16B).



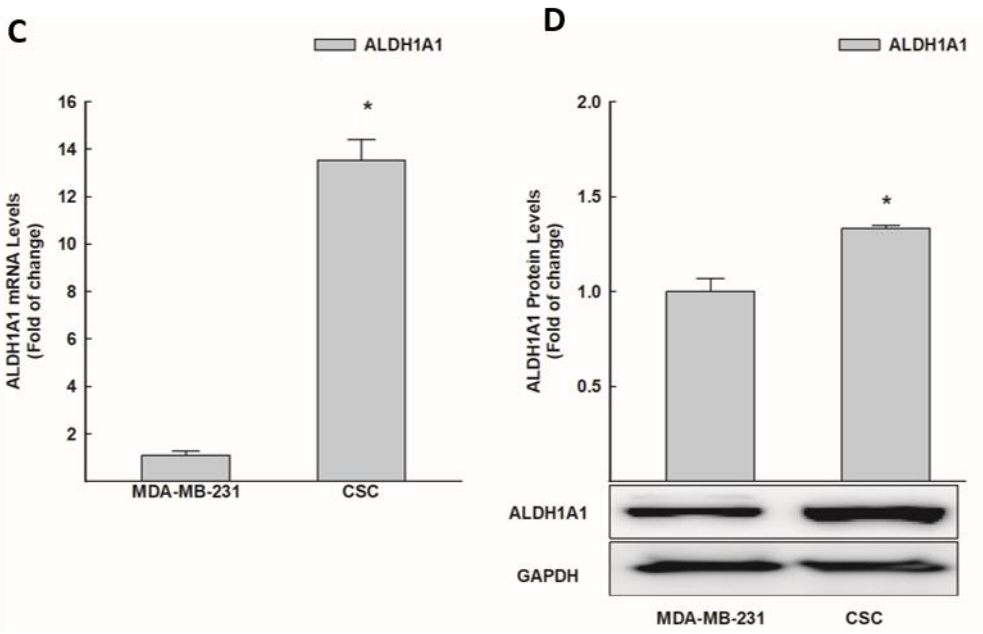


Figure 17. Constitutive expression of ALDH1A1 in MCF-10A, MDA-MB-231, and cancer stem cells.
The basal mRNA and protein expressions of ALDH1A1 in MDA-MB-231 versus MCF-10A (Fig. 17A/B) and CSCs versus MDA-MB-231 (Fig. 17C/D) were determined by RT-PCR and Western blot analysis standardized against GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p<0.05$ The two cell lines were compared using Independent t-test..

3.1.2. Percentage of Side population (SP).

Side population (SP) cells, are a known characteristic for CSCs, they are detected depending on their cellular capability to efflux the fluorescent dye at an increased level than the remaining tumor cells (non-SP), which appears as a single ‘tail’ of cells in the flow cytometry figure [176]. The side population assay has revealed the percentage of side population cells that have actively effluxed the Hoechst 33342 dye through the transporter system (Fig. 18A). Thus, we determined the percentage of SP cells in CSCs and MDA-MB-231 cells using flow cytometry assay. Fig. 18B shows that CSCs expressed a 3.5-fold higher percentage of SP active cells, compared to MDA-MB-231 cells.

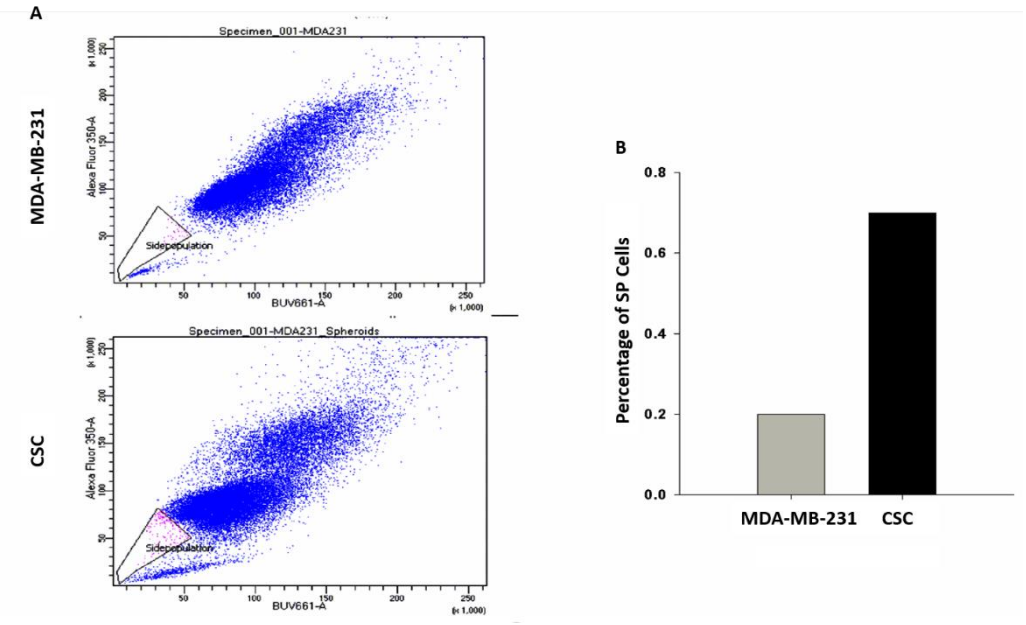


Figure 18. The percentage of side population cells in cancer stem cells versus MDA-MB-231 by flowcytometry.
The MDA-MB-231 cells and CSCs were cultured and pelleted, then they were incubated with Hoechst 33342 dye (Fig. 18A) and the percentage of SP cells was detected using BD FACSAria® flow cytometer (Fig. 18B).

3.1.3. Percentage of $CD44^{high}/CD24^{low}$ expression.

The expression of $CD44^{high}/CD24^{low}$ in CSCs has been proven to be more than 10%, which is an indicator for tumorigenesis and the production of different tumor types [177]. To determine the level of $CD44^{high}/CD24^{low}$ in CSCs as compared to non-CSCs (MDA-MB-231), we kept the cells with CD44 and CD24 antibodies, and measured the percentage of cells expressing $CD44^{high}$ and $CD24^{low}$ on their cell surface using flow cytometry assay (Fig. 19A). However, Fig.19B shows that the percentage of cells expressing high CD44 and low CD24 ($CD44^{high}/CD24^{low}$) were 16-folds higher in CSCs compared to the differentiating non-CSC MDA-MB-231 cancer cells.

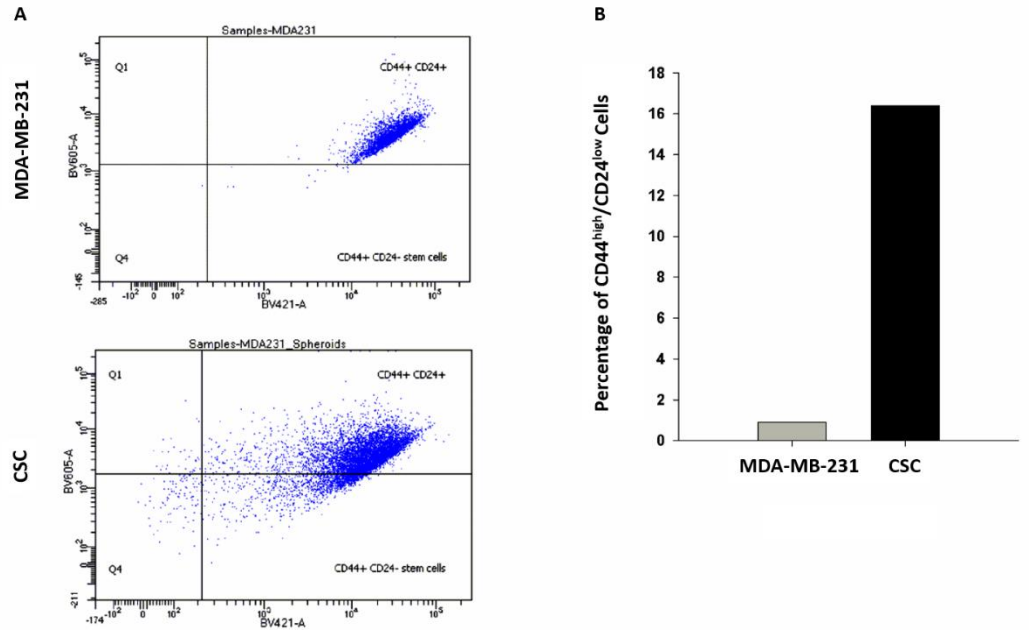


Figure 19. Constitutive expression of $CD44^{high}/CD24^{low}$ in cancer stem cells versus MDA-MB-231by flowcytometry.
The MDA-MB-231 cells and CSCs were cultured and pelleted, then incubated with CD24 and CD44 antibodies to determine the expression of $CD44^{high}/CD24^{low}$ population using BD FACSAria® flow cytometer cell sorter.

3.1.4. Stemness markers SOX2 and SOX9.

The transcription factors SOX2 and SOX9, have been considered as stemness markers, due to their distinctive part in stem cells self-renewal and pluripotency. The expressions of SOX2 and SOX9 were determined at the mRNA level by RT-PCR in MCF-10A, MDA-MB-231, and CSCs. Fig. 20A shows a significant 6-fold increase in SOX9 at the mRNA level in MDA-MB-231 than in MCF-10A. However, the mRNA expression of SOX2 was downregulated by 93.8%. In CSCs, the expression of SOX2 was upregulated by 11-fold compared to MDA-MB-231. However, SOX9 showed an 84.6% decrease in CSCs (Fig. 20B).

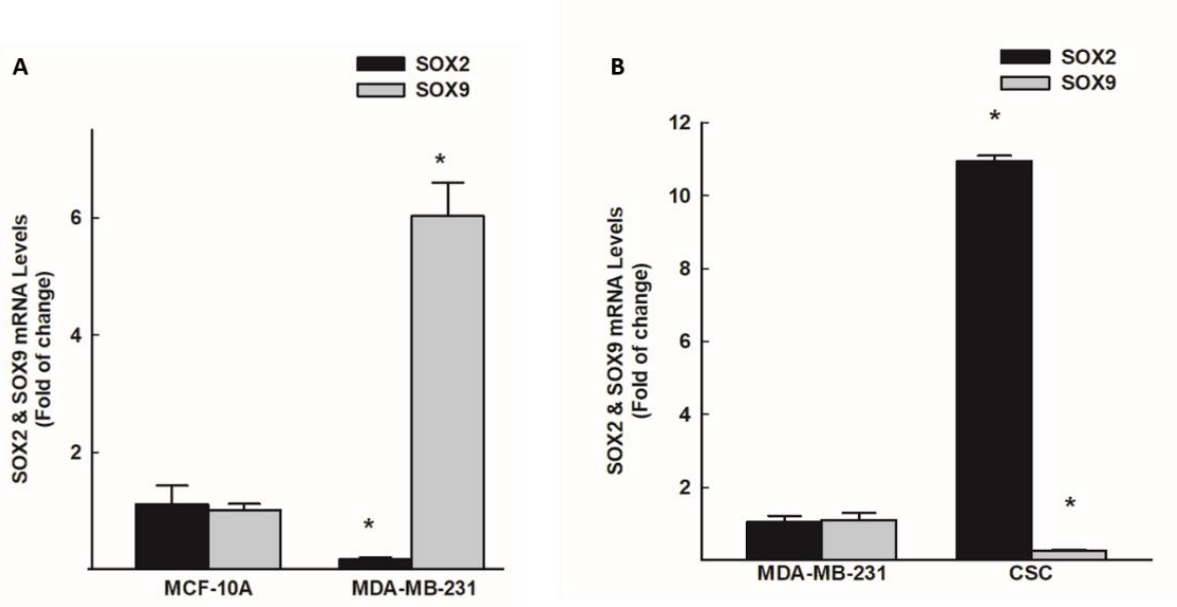


Figure 20. Constitutive expression of SOX2 and SOX9 in MCF-10A, MDA-MB-231, and cancer stem cells.
The basal mRNA expression of SOX2 and SOX9 in MDA-MB-231 versus MCF-10A (Fig.20A) and MDA-MB-231 versus CSCs (Fig. 20B) were determined by RT-PCR standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6). *; $p < 0.05$ The two cell lines were compared using Independent t-test.

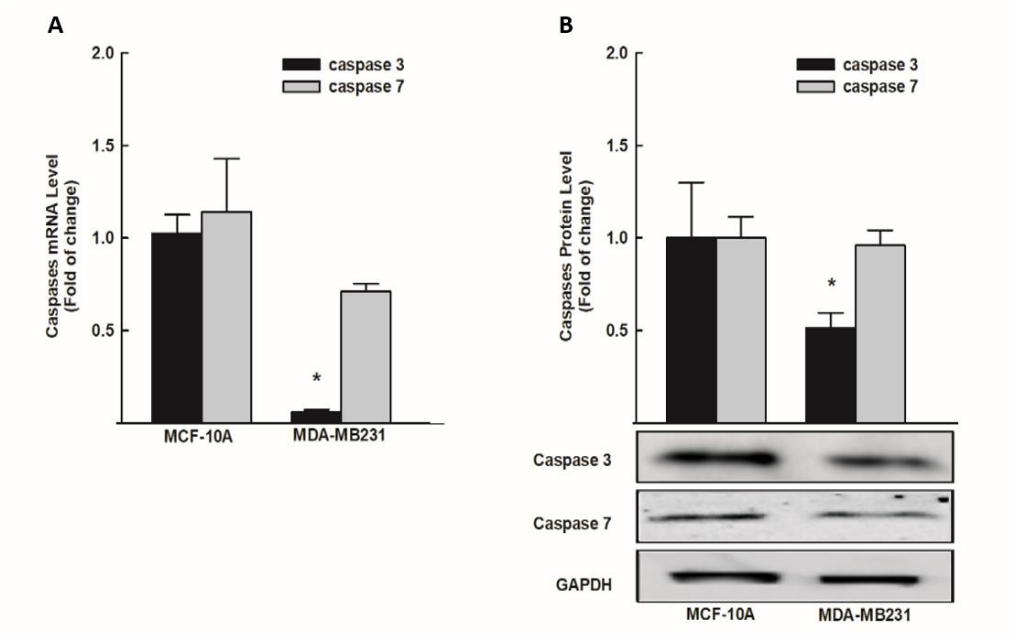
3.2. Differential Expression of Apoptotic Pathways in Cancer Stem Cells Versus Cancer Cells

To further explore the expression and function of apoptosis and its markers' caspase 3, caspase 7, caspase 8, caspase 9, BAX, and Bcl-xL, in CSCs versus non-CSCs, three different experiments were performed as follows:

3.2.1. Expression of apoptotic markers in MCF-10A, MDA-MB-231, and CSCs at the mRNA and protein levels.

To explore the differential expression of the apoptosis markers in normal breast cells, cancerous cells (MDA-MB-231), and CSCs; we first determined the differential expression of the apoptotic markers (caspase 3, caspase 7, caspase 8, caspase 9, BAX, and Bcl-xL) at the mRNA and protein levels in cancerous MDA-MB-231 cells compared to healthy MCF-10A breast cells. Fig. 21 shows differential expression of

caspases 3, 7, 8, and 9 at both levels, the mRNA and protein in MDA-MB-231 cells versus MCF-10A. In which the mRNA and protein levels of caspase 3, caspase 8, and caspase 9 were significantly lower in MDA-MB-231 cells than in MCF-10A. For example, caspase 3 (Fig. 21A), caspase 8, and caspase 9 (Fig. 21C) mRNA levels were significantly downregulated by almost 94.3%,65.4% and 56.9%, respectively, whereas at the protein level, caspase 3 (Fig 21B), caspase 8, and caspase 9 (Fig. 21D) were downregulated by 49%, 35%, and 74%, respectively. On the contrary, caspase 7 did not express any significant variations in both cell lines at both mRNA and protein levels (Fig. 21A/B). On the other hand, expression of Bcl-2 family proteins such as Bcl-xL (anti-apoptotic) and BAX (pro-apoptotic) have shown significant changes in MDA-MB-231 cells versus MCF-10A at the mRNA and protein level. Fig. 21E shows that the mRNA level of the pro-apoptotic BAX was 2-fold higher, whereas anti-apoptotic Bcl-xL gene was 3.5-fold and 2.6-fold upregulated at both the mRNA and protein levels, respectively in MDA-MB-231 cancer cells compared to MCF-10A cells (Fig. 21E/F).



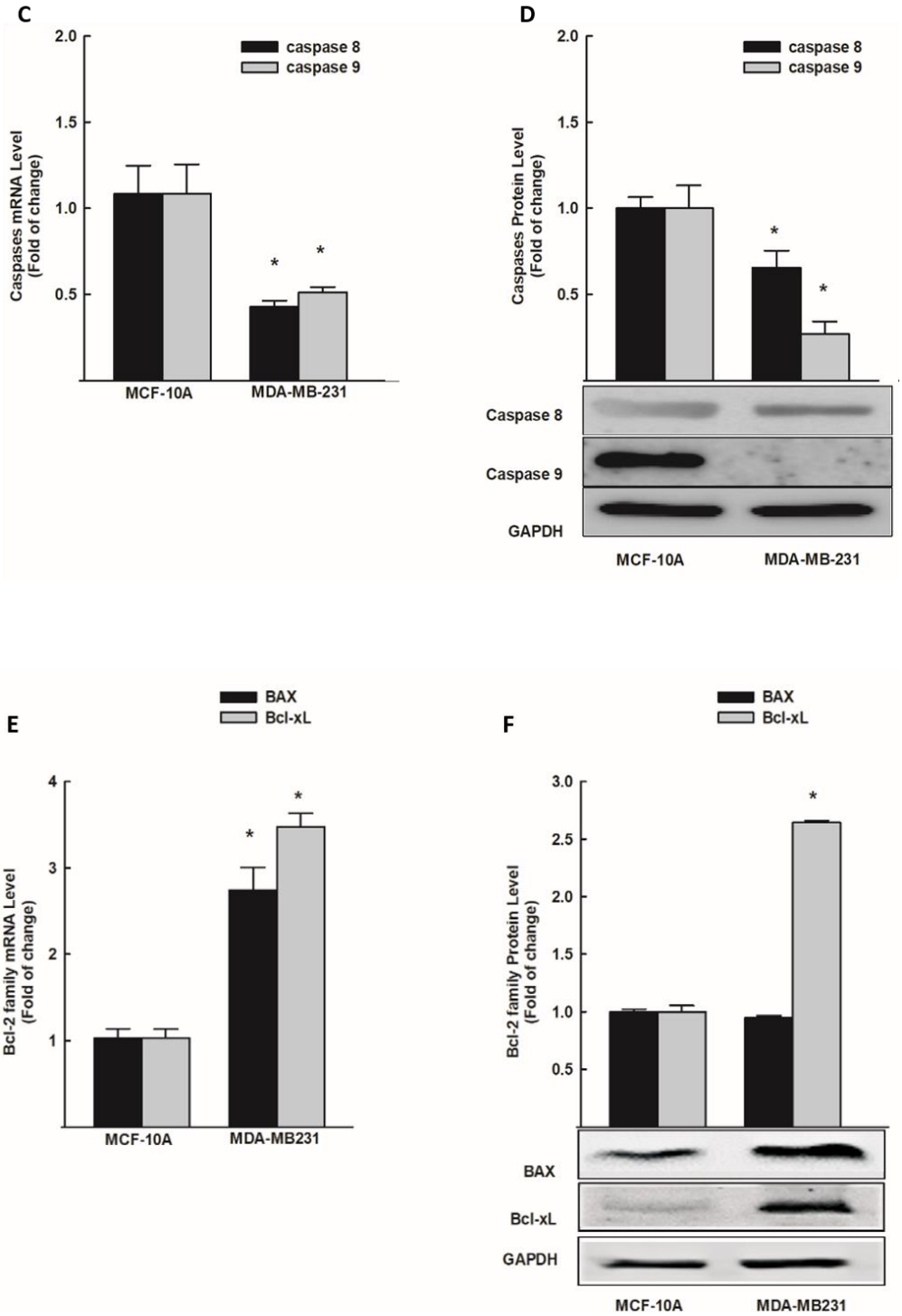
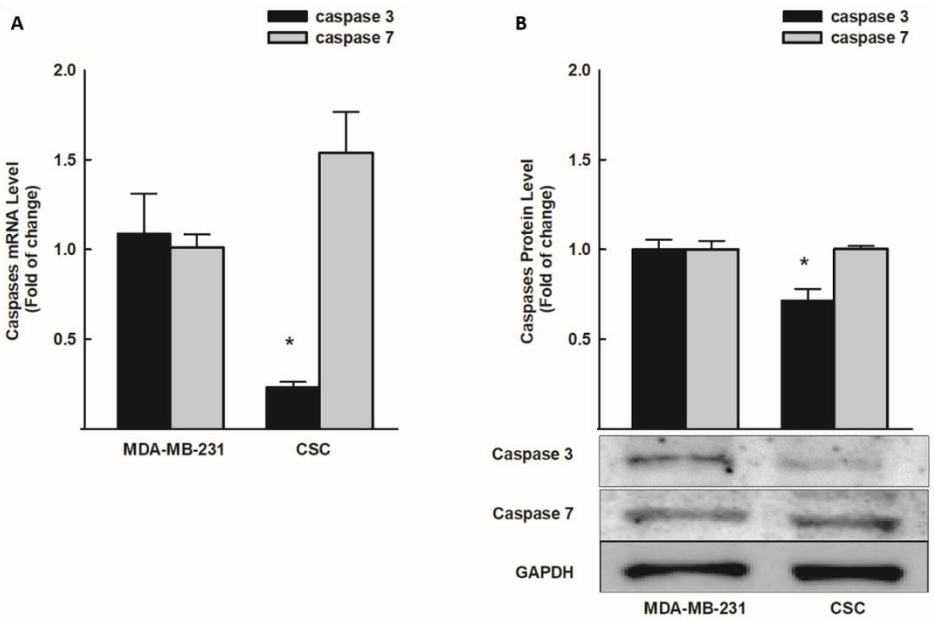


Figure 21. Constitutive expression of caspase 3, caspase 7, caspase 8, caspase 9, BAX, and Bcl-xL in MDA-MB-231 cell line versus MCF-10A cell line.

The basal mRNA and protein expressions of caspase 3 and caspase 7 (Fig. 21A/B), caspase 8 and caspase 9 (Fig. 21C/D), BAX and Bcl-xL (Fig. 21E/F) were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$ The two cell lines were compared using Independent t-test.

In comparison with MDA-MB-231 cells, CSCs showed a lower expression levels of the pro-apoptotic markers indicating resistance to apoptosis. Fig. 22 shows that the mRNA (Fig. 22A) and protein expression (Fig. 22B) levels were significantly downregulated in caspase 3 by 77% and 30%, respectively and caspase 8 by 40% and 50%, respectively (Fig. 22C/D), however in caspase 9 only the protein level (Fig. 22D) expressed a decrease by 50% in CSCs than in MDA-MB-231. On the contrary, caspase 7 expression in CSCs was not significantly different from MDA-MB-231 cells at both the mRNA and protein levels (Fig. 22A/B). With respect to the Bcl-2 family proteins, CSCs significantly expressed lower mRNA and protein expression level of the pro-apoptotic gene BAX by 75% and 53%, respectively, while higher levels of the anti-apoptotic Bcl-xL mRNA (2-fold) and protein (14%) were observed (Fig. 22E/F)



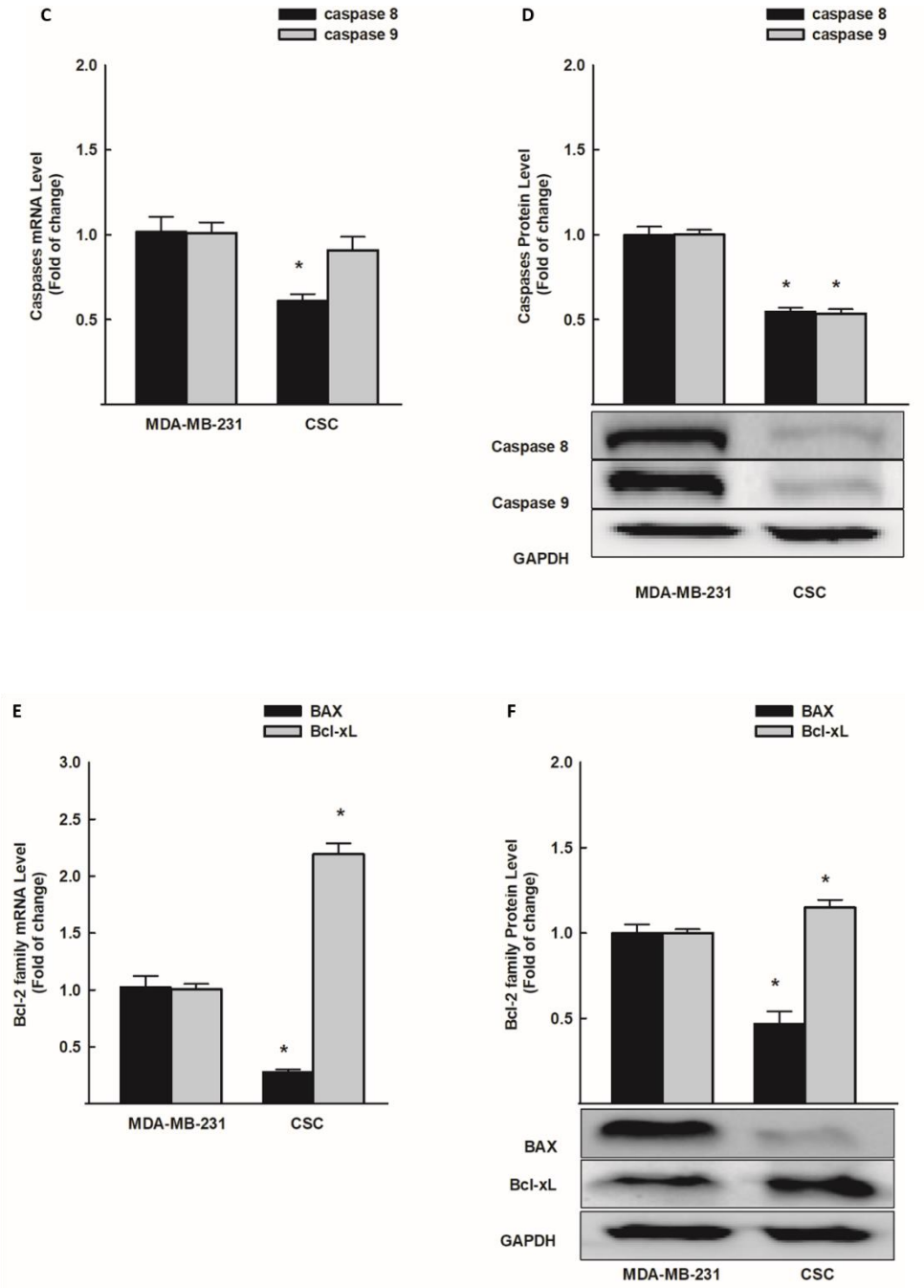
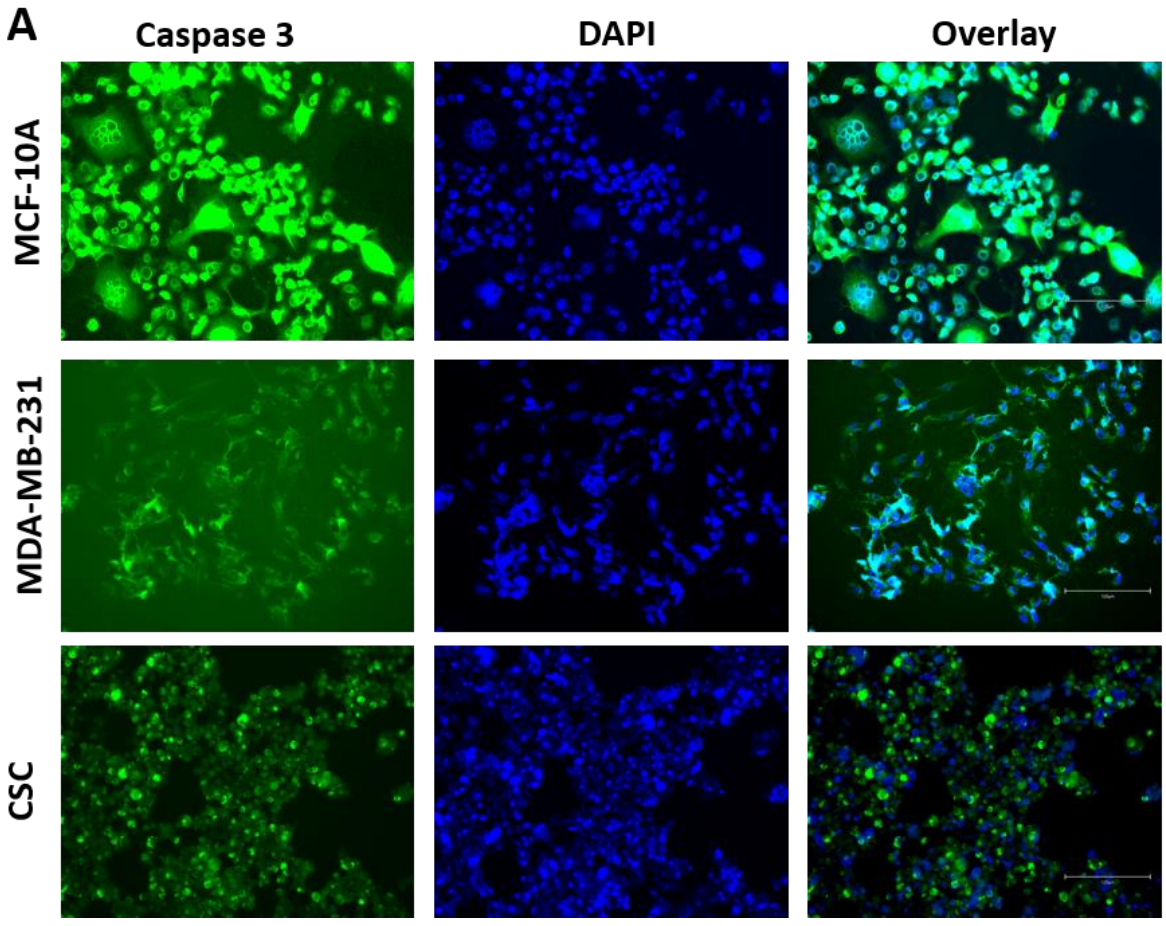
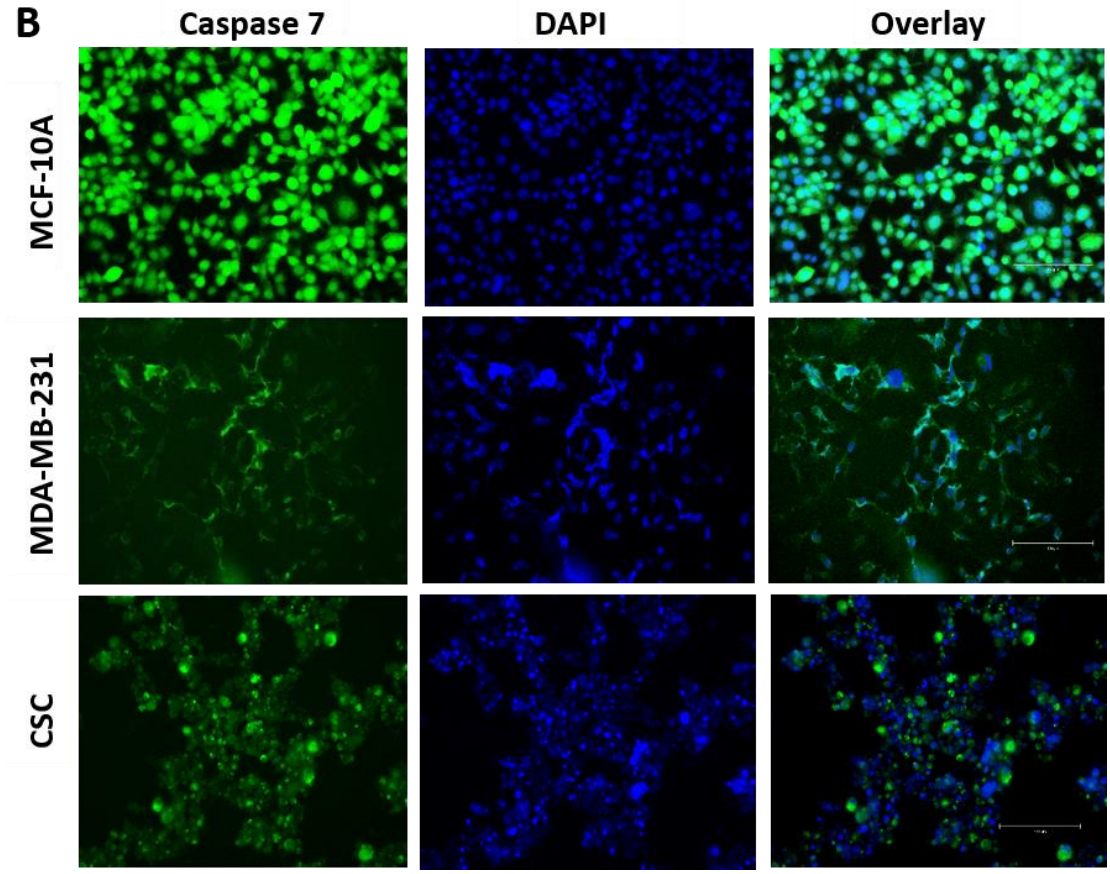


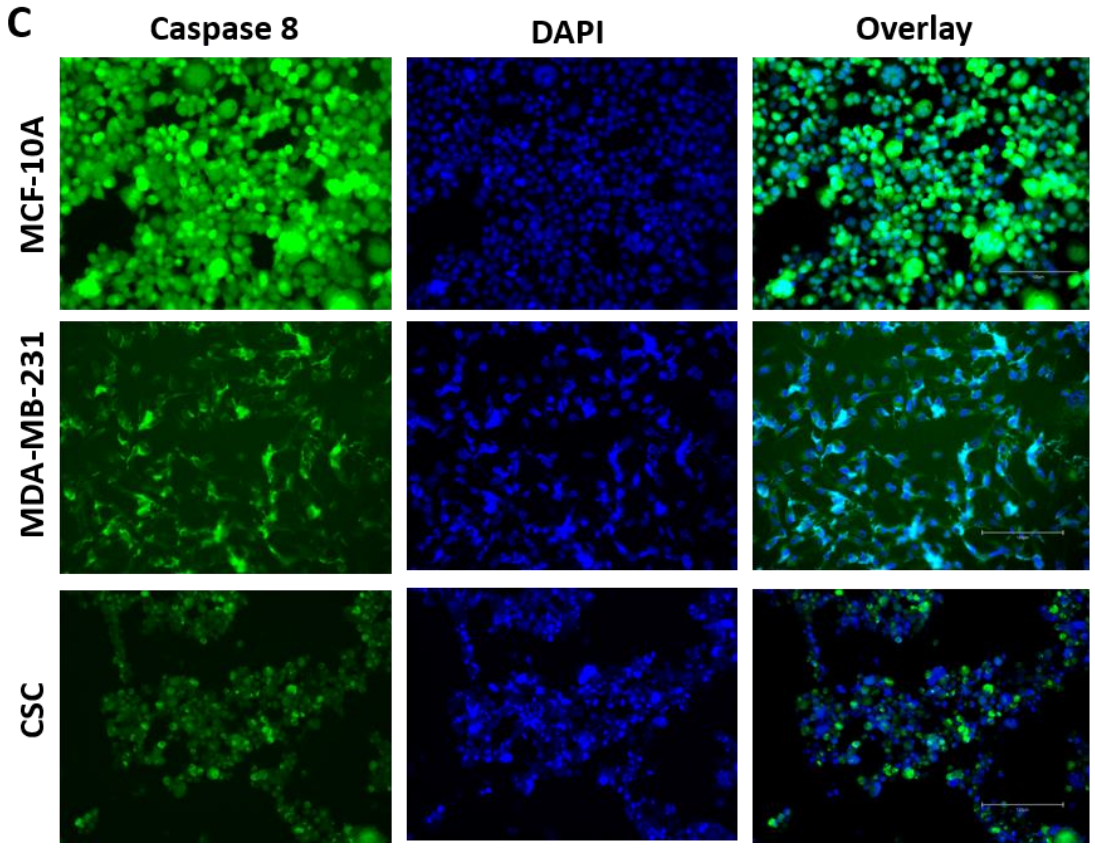
Figure 22. Constitutive expression of caspase 3, caspase 7, caspase 8, caspase 9, BAX, and Bcl-xL in cancer stem cells versus MDA-MB-231 cell line.
The basal mRNA and protein expressions of caspase 3 and caspase 7 (Fig. 22A/B), caspase 8 and caspase 9 (Fig. 22C/D), BAX and Bcl-xL (Fig. 22E/F) were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p<0.05$.The two cell lines were compared using Independent t-test.

3.2.2. Cellular content and localization of the apoptotic markers in MCF-10A, MDA-MB-231, and CSCs by immunofluorescence.

To further confirm the expression pattern of the apoptotic markers in all tested cell models, we determined the cellular content and localization of caspases 3, 7, 8, and 9 using immunofluorescence assay. For this purpose, MCF-10A, MDA-MB-231, and CSCs were incubated with primary antibodies against target proteins followed by DAPI and then the cellular content was visualized by florescence microscope. The immunofluorescence assay results revealed that the expression and activity of caspase 3 (Fig. 23A), caspase 7 (Fig. 23B), caspase 8 (Fig. 23C), and caspase 9 (Fig. 23D) in MDA-MB-231 cells were lower than the control MCF-10A. Whereas, the cellular content of the proteins were completely diminished in CSCs compared to MDA-MB-231 cells.




B
Caspase 7
DAPI
Overlay
MCF-10A
MDA-MB-231
CSC


C
Caspase 8
DAPI
Overlay
MCF-10A
MDA-MB-231
CSC

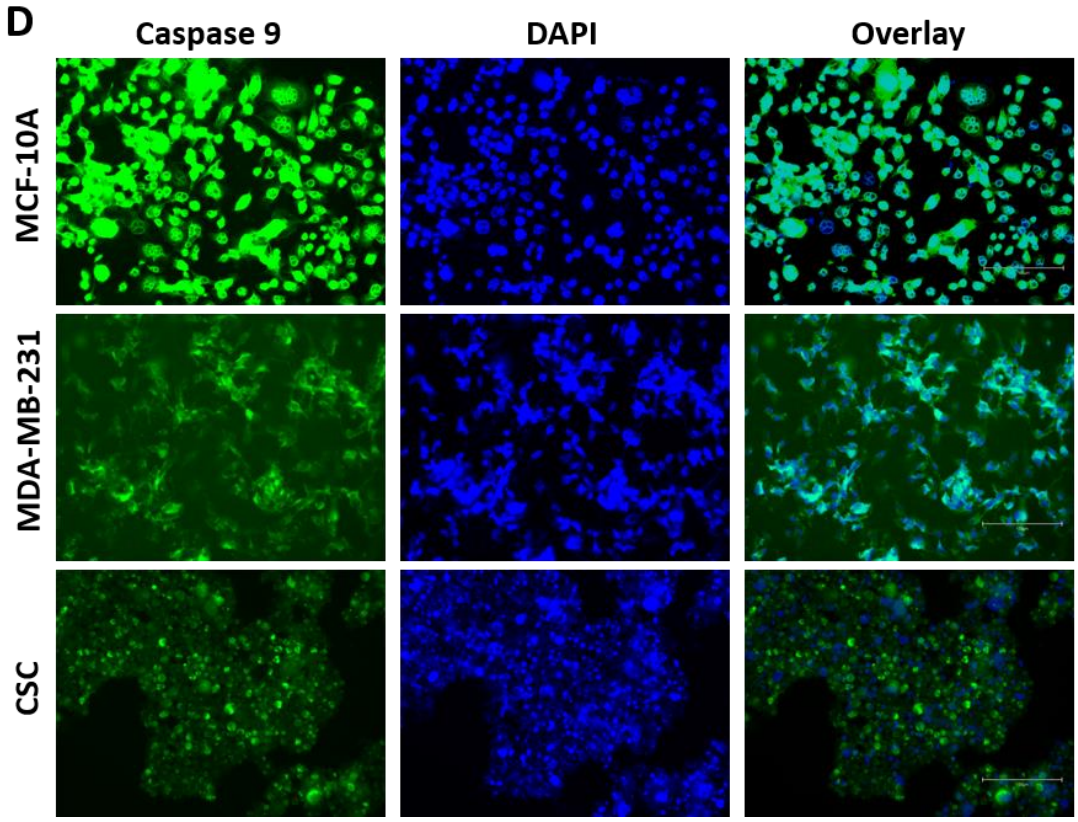


Figure 23. Basal cellular localization and expression of caspase 3, caspase 7, caspase 8, and caspase 9 in MCF-10A, MDA-MB-231, and cancer stem cells.
The MCF-10A, MDA-MB-231, and CSCs cells were stained with primary antibodies against caspase 3 (green) (Fig. 23A), caspase 7 (green) (Fig. 23B), caspase 8 (green) (Fig. 23C), and caspase 9 (green) (Fig. 23D), followed by secondary antibodies (Tag-Alexa Fluor® 488) and DAPI (blue). Thereafter, the cellular localization of the constitute proteins caspase 3, caspase 7, caspase 8, and caspase 9 was detected by Immunofluorescence analysis. Each sample was stained three times against required antibody.

3.4. Differential Expression of Autophagic Pathways in Cancer Stem Cells Versus Cancer Cells

3.4.1. Differential expression of the autophagy pathway regulators in MCF-10A, MDA-MB-231, and CSCs at the mRNA and protein level.

To investigate the differential expression of the autophagy regulators in normal breast cells, breast cancer cells, and CSCs, we first determined their expression between MCF-10A and MDA-MB-231 cells at the mRNA and protein level using RT-PCR and Western blot analysis. In Fig. 24, the main regulators p62 and LC3-II have shown significant changes at the mRNA level, but not at the protein expression level. While the LC3-II gene (Mean=0.680, P=0.002) was downregulated in MDA-MB-231 compared to MCF-10A, p62 gene showed a 4.5-fold increase compared to MCF-10A cell line. However, ATG has shown a significant upregulation at both the mRNA and protein level by 3.6- and 2-fold, respectively. On the other hand, the mRNA and protein expression levels of ATG were significantly higher by 3-fold and 2-fold, respectively.

In CSCs, the autophagy pathway has shown variable regulations of the different genes. In Fig. 25, for example, the expression of LC3-II mRNA and p62 protein levels were significantly higher in CSCs than MDA-MB-231 by approximately 61% and 25%, respectively. On the contrary, the gene expression levels of ATG, were significantly lower at mRNA (62%) and protein levels (33%) in CSCs compared to MDA-MB-231 cells.

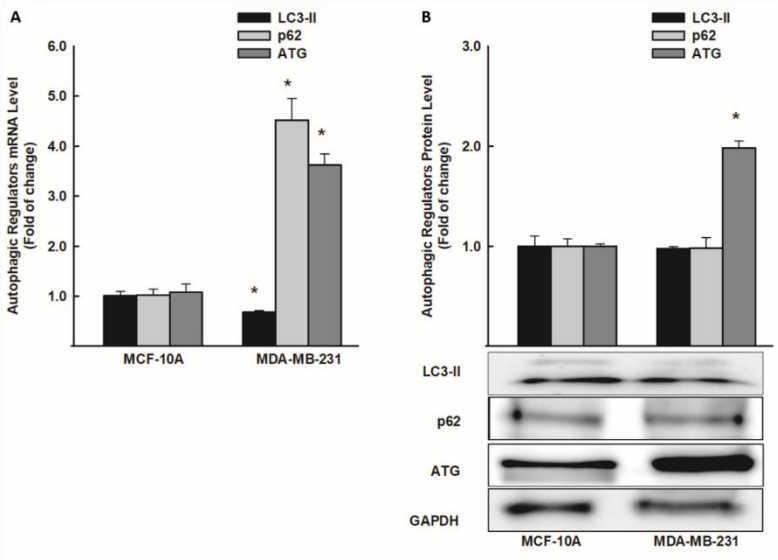


Figure 24. Constitutive expression of LC3-II, p62, and ATG in MDA-MB-231 versus MCF-10A cell line.

The basal mRNA and protein expressions of LC3-II, p62, and ATG were determined by RT-PCR (Fig. 24A) and Western blot analysis (Fig. 24B) standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$ The two cell lines were compared using Independent t-test.

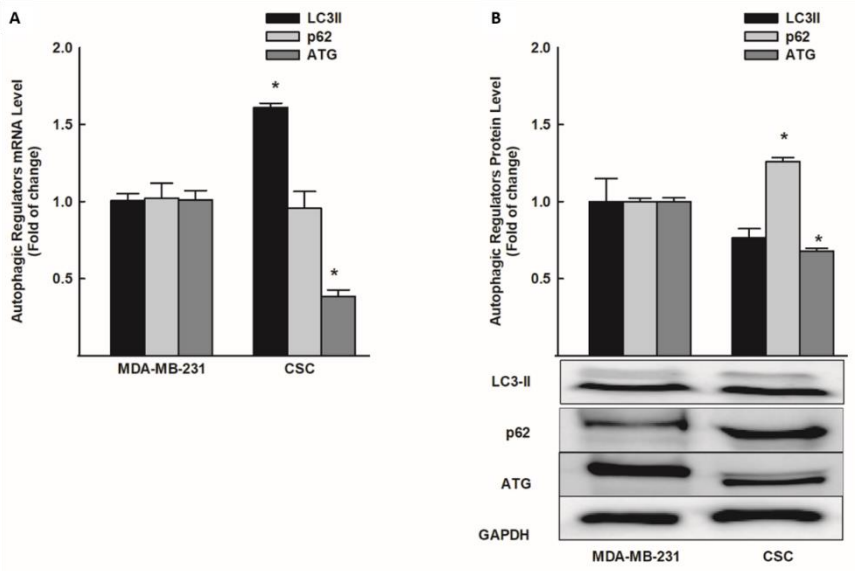


Figure 25. Constitutive expression of LC3-II, p62, and ATG in cancer stem cells versus MDA-MB-231 cell line.

The basal mRNA and protein expressions of LC3-II, p62, and ATG were determined by RT-PCR (Fig. 25A) and Western blot analysis (Fig. 25B) standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

3.4.2. Percentage of autophagic LC3II activated cells in MDA-MB-231 and CSCs by Anti-LC3 Alexa Fluor®555 staining.

To further confirm the differential expression of autophagy markers, we determined the percentage of autophagy induction through LC3II activity in breast cancer cells (MDA-MB-231) and CSCs. For this purpose, the cells were incubated with Anti-LC3 Alexa Fluor®555 antibody and the percentage of autophagic cells was determined through the Muse Cell Analyzer (Luminex Corporation, United States). The MDA-MB-231 cell line (Fig. 26A) demonstrated a higher autophagy activated intensity whereas, the CSCs (Fig. 26B) expressed 78.3% less mean autophagy intensity by activated LC3, and 82% lower autophagy induction ratio compared to MDA-MB-231 (Fig. 26C).

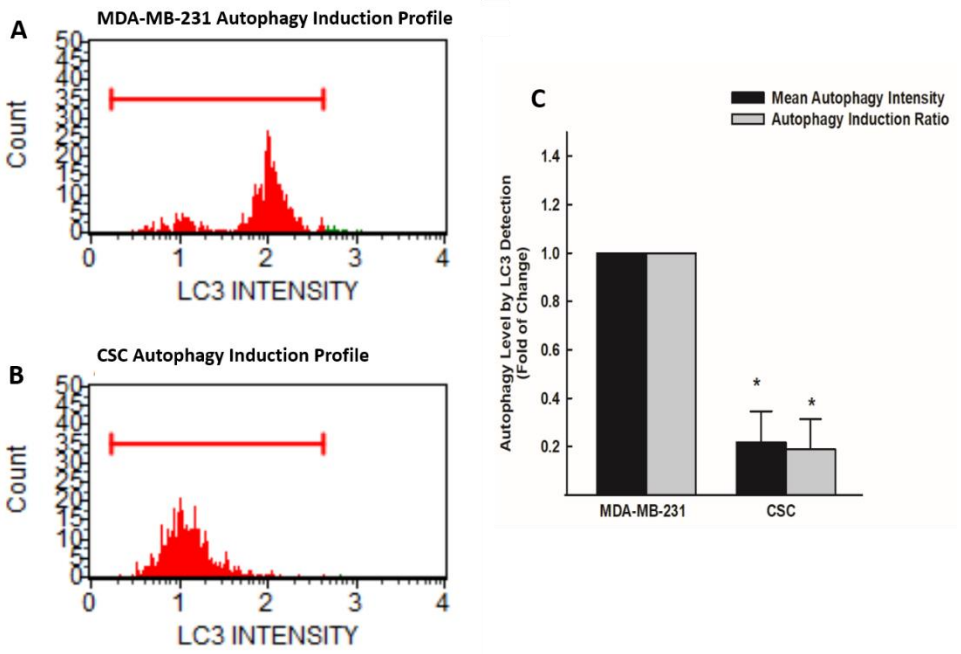


Figure 26. Constitutive autophagy activity by LC3 detection in cancer stem cells versus MDA-MB-231 cell line.
The pelleted CSCs and MDA-MB-231 cells were incubated with Anti-LC3 Alexa Fluor®555 antibody. Then, the value of mean autophagy intensity and autophagy induction ratio in CSCs (Fig. 26B) compared to MDA-MB-231 (Fig. 26A) was detected by the Muse Cell Analyzer (Fig. 26C). The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 3). *; $p < 0.05$. The two cell lines were compared using Independent t-test.

3.5. Differential Expression of Cytochrome P450 Enzymes in MCF-10A, MDA-MB-231, and CSCs at the mRNA and Protein Levels

To further explore the differential expression of the signaling pathways in CSCs, we examined the expression of CYP450 enzymes in healthy breast cells, breast cancer cells, and CSCs. For this purpose, we assessed the mRNA and protein expression levels of CYP3A4, CYP2D6, and CYP2C19 in MCF-10A and MDA-MB-231 cells lines. Fig. 27A shows that CYP3A4, CYP2D6, and CYP2C19 mRNA levels were significantly lower in MDA-MB-231 cells than in MCF-10A cells by approximately 50%, 75%, and 99%, respectively. Whereas the expressions of CYP3A4 and CYP2D6 were upregulated by 17% in both cells (Fig. 27B).

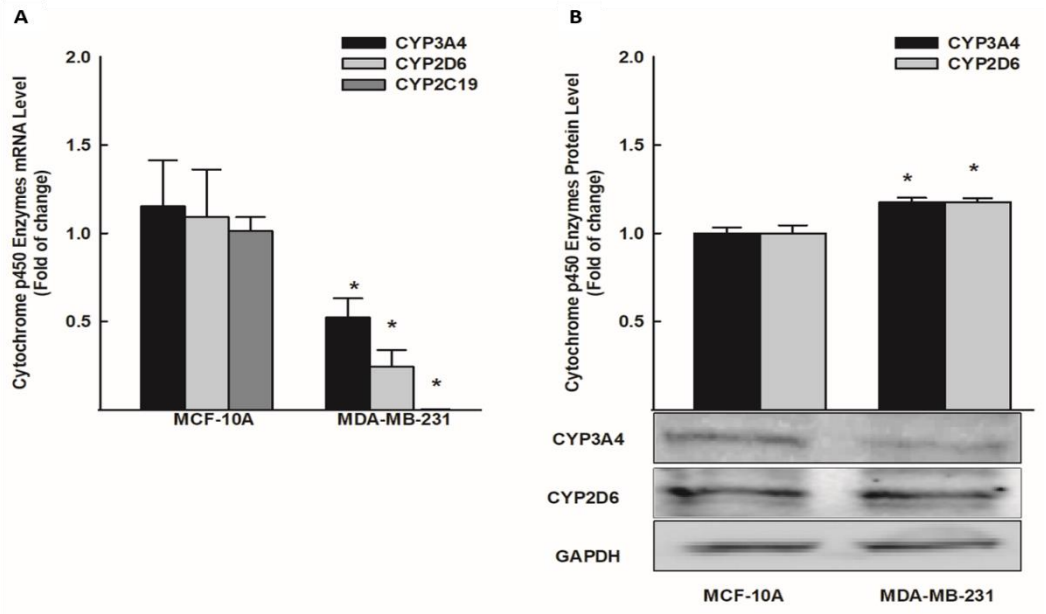


Figure 27. Constitutive expression of CYP3A4, CYP2D6, and CYP2C19 in MDA-MB-231 versus MCF-10A cell line.
The basal mRNA (Fig. 27A) and protein (Fig. 27B) expressions of CYP3A4 and CYP2D6 and the mRNA expression of CYP2C19 were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

In CSCs, Fig. 28A demonstrates the mRNA expression of CYP3A4 and CYP2C19 which showed a significant 9-fold and 3.5-fold upregulation, respectively compared to MDA-MB-231, however the CYP3A4 protein expression was downregulated (Mean=0.762, P=0.011). Similarly, the CYP2D6 mRNA and proteins were significantly downregulated by 81% and 17%, respectively (Fig. 28).

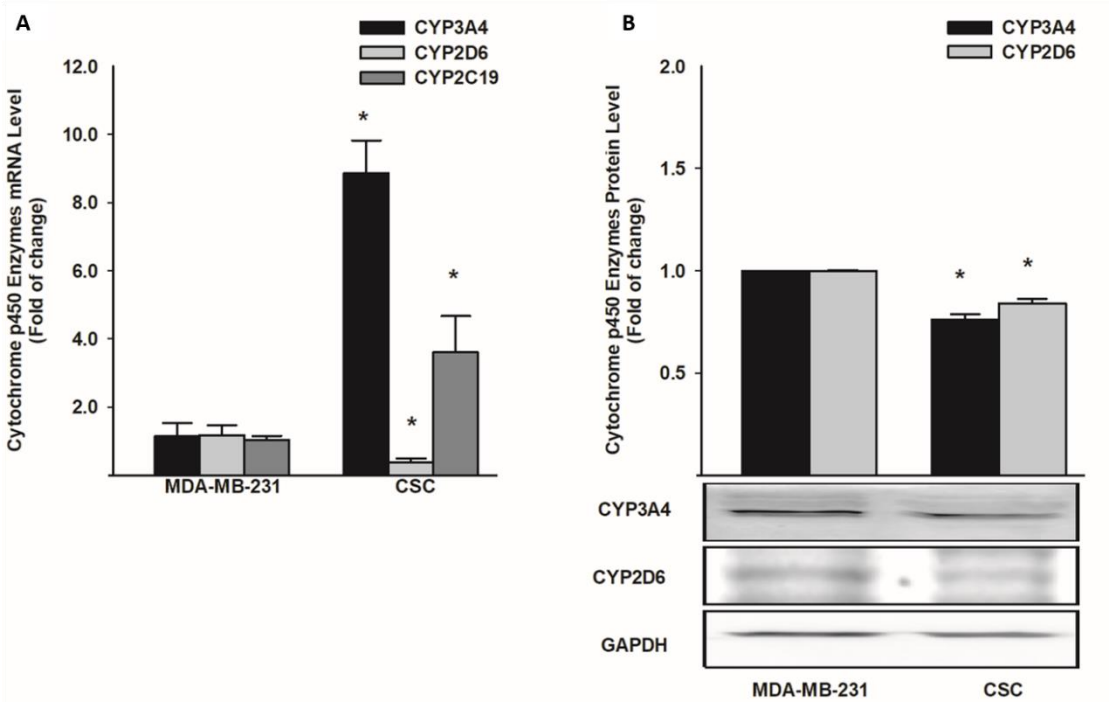


Figure 28. Constitutive expression of CYP3A4, CYP2D6, and CYP2C19 in cancer stem cells versus MDA-MB-231 cell line.
The basal mRNA (Fig. 28A) and protein (Fig. 28B) expressions of CYP3A4 and CYP2D6, and mRNA CYP2C19 were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

3.6. Differential Expression of Tumor Suppressor Genes in MCF-10A, MDA-MB-231, and CSCs at the mRNA and Protein Level

To examine the differential expression of tumor suppressor genes in healthy breast cells, breast cancer cells, and CSCs, we evaluated the expression of BRCA1, PTEN, and p53 genes in MCF-10A and MDA-MB-231 cells by RT-PCR and Western blot analyses, respectively. In MDA-MB-231 cells, the BRCA1 gene was significantly downregulated at the mRNA level (Mean=0.299, P-value=0.001), whereas its protein was upregulated by 1.5-fold in MDA-MB-231 cells. On the contrary, both PTEN and p53 expression levels were upregulated by 2.5-fold and 4.5-fold at the mRNA levels (Fig. 29A), and by about 1.5-fold and 5.5-fold at the protein levels (Fig. 29B), respectively.

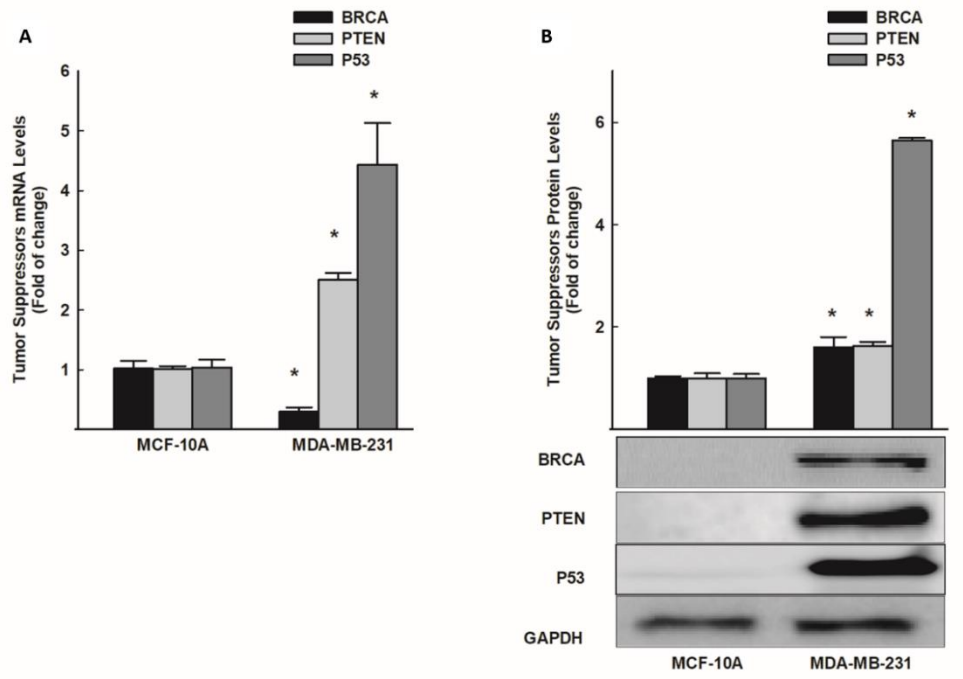


Figure 29. Constitutive expression of BRCA, PTEN, and p53 in MDA-MB-231 versus MCF-10A cell line.
The basal mRNA (Fig. 29A) and protein (Fig. 29B) expressions of BRCA, PTEN, and p53 were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

The results of the tumor suppressor genes in CSCs compared to MDA-MB-231 have displayed a significant upregulation of BRAC1 and PTEN mRNA expression levels by 4.5- and 1.5-fold, respectively, while showed a marked downregulation of p53 by 94% (Fig. 30A). Importantly, at the protein levels, for all the tested tumor suppressor genes BRCA1, PTEN, and p53 were significantly downregulated by 40%, 25%, and 36%, respectively (Fig. 30B).

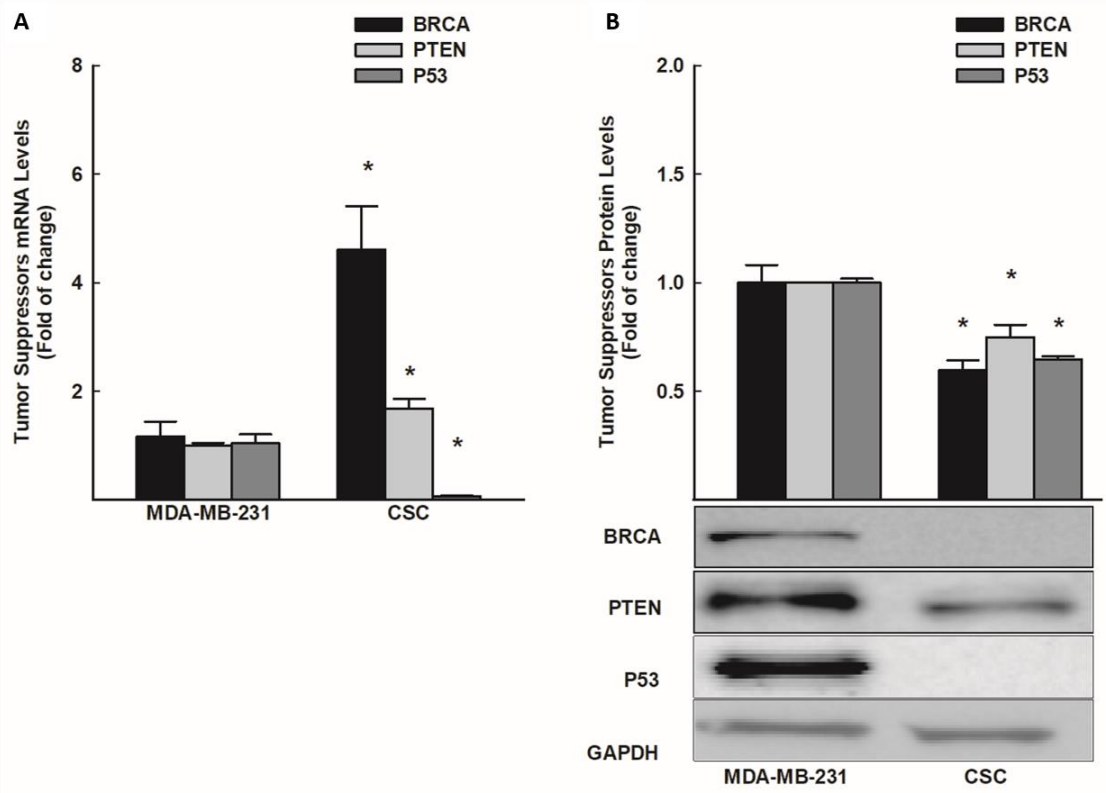


Figure 30. Constitutive expression of BRCA, PTEN, p53 in cancer stem cells versus MDA-MB-231 cell line.
The basal mRNA (Fig. 30A) and protein (Fig. 30B) expressions of BRCA, PTEN, and p53 were determined by RT-PCR and Western blot analysis normalized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$ compared to corresponding adherent cells using Independent t-test.

3.7. Differential Expression of Other Signaling Markers in MCF-10A, MDA-MB-231, and CSCs at the mRNA and Protein Level

To investigate the differential expression of some signaling factors in healthy breast cells, breast cancer cells, and CSCs, we conducted a RT-PCR and Western blot analysis. In MDA-MB-231 versus MCF-10A, according to results in Fig. 39A TNF-a and AKT has shown a significant 99.4% and 46% lower expression at the mRNA level, respectively in MDA-MB-231 than MCF-10A. However, NF-KB (Fig. 39A) and ki-67 (Fig. 32A) expressed a 1.5-fold and 80% increase, respectively. At the protein level both AKT (Fig. 31B) and Mdr-1 (Fig. 32B) were upregulated by 25% and 48%, respectively, however NF-KB was downregulated by 30% in MDA-MB-231 compared to MCF-10A (Fig. 31B).

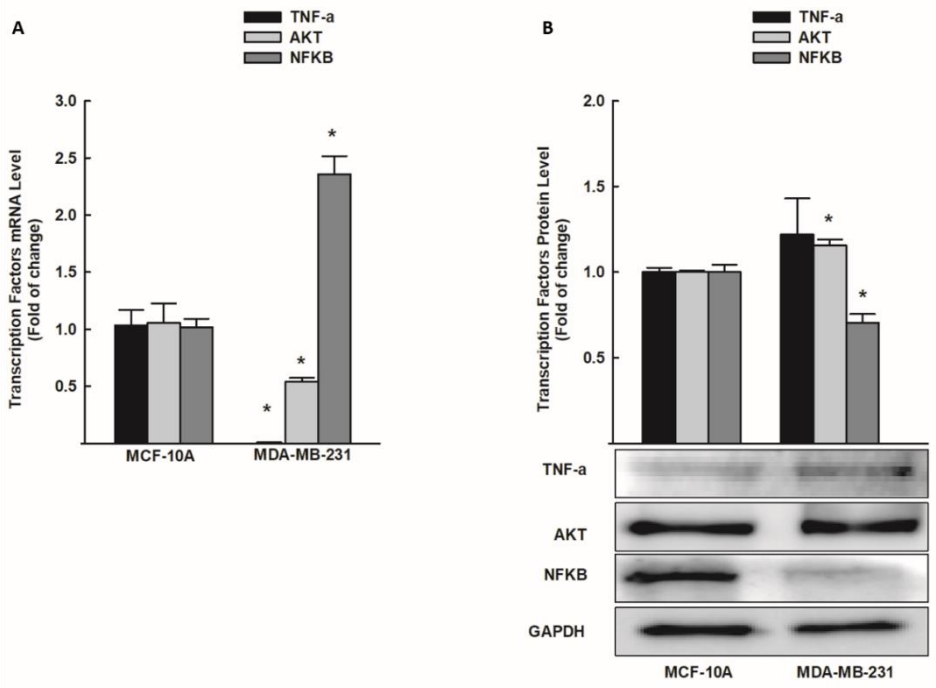


Figure 31. Constitutive expression of TNF-a, AKT, and NFKB in MDA-MB-231 versus MCF-10A cell line.
The basal mRNA (Fig. 31A) and protein (Fig. 31B) expressions of TNF-a, AKT, and NFKB were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

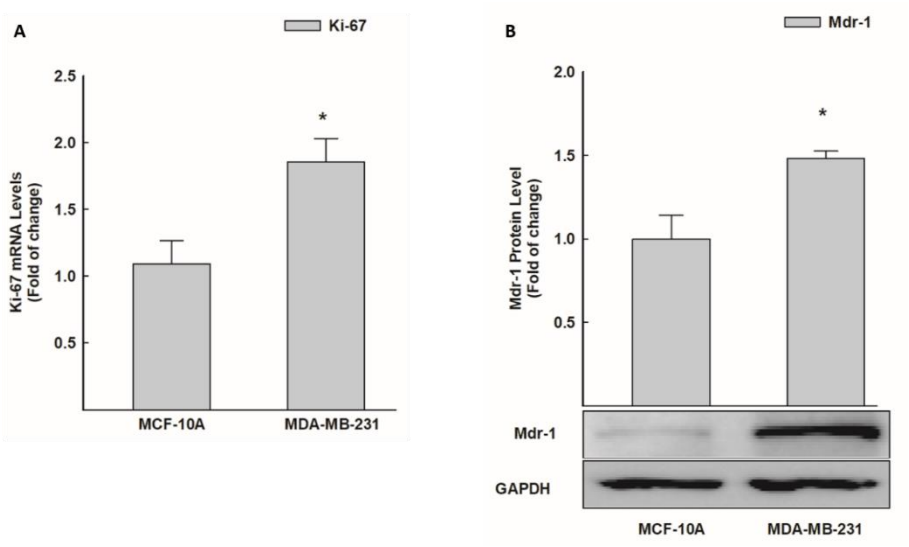


Figure 32. Constitutive expression of Ki-67 and Mdr-1 in MDA-MB-231 versus MCF-10A cell line.
The basal mRNA of Ki-67 (Fig. 32A) and protein expression of Mdr-1 (Fig. 32B) were determined by RT-PCR and Western blot analysis, respectively. The mRNA and protein were standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

In CSCs, TNF-a and NF-KB showed an upregulation at the mRNA level by 15-folds and 50%, respectively (Fig. 33A); however, ki-67 (Fig. 34A) was significantly downregulated (Mean=0.172, $P<0.001$) in CSCs compared to MDA-MB-231. On the other hand, at the protein level only AKT and NF-KB expressed significant changes, by 33% increase in AKT and 32% decrease in NF-KB in CSCs compared to MDA-MB-231 (Fig. 33B).

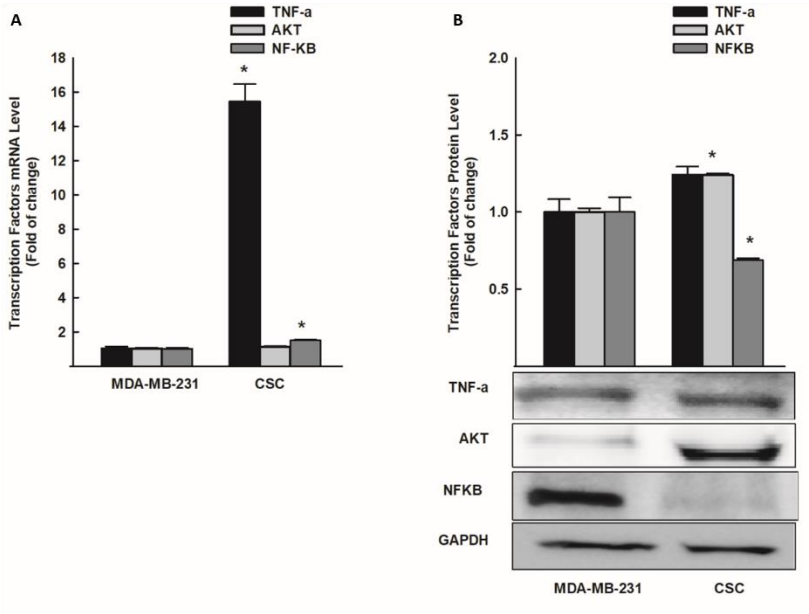


Figure 33. Constitutive expression of TNF-a, AKT, and NFKB in cancer stem cells versus MDA-MB-231 cell line.
The basal mRNA (Fig. 33A) and protein (Fig. 33B) expressions of TNF-a, AKT, and NFKB were determined by RT-PCR and Western blot analysis standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

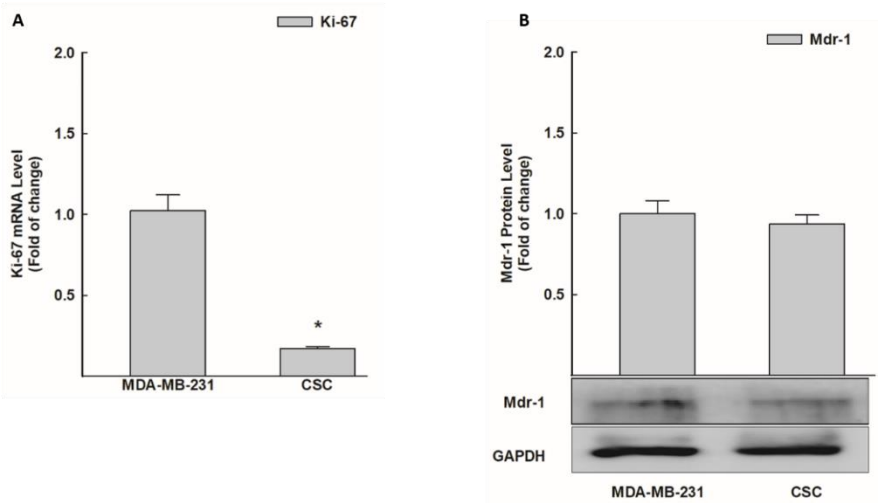


Figure 34. Constitutive expression of Ki-67 and Mdr-1 in cancer stem cells versus MDA-MB-231 cell line.
The basal mRNA of Ki-67 (Fig. 34A) and protein expressions of Mdr-1 (Fig. 34B) were determined by RT-PCR and Western blot analysis, respectively. The mRNA and protein were standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p < 0.05$. The two cell lines were compared using Independent t-test.

3.8. The Levels of DNA Damage by Muse in MCF-10A, MDA-MB-231, and CSCs

To investigate the percentage of double strand DNA damage in MCF-10A, MDA-MB-231, and CSCs we conducted a Muse Multi-Color DNA Damage experiment. The cells were incubated with an antibody working cocktail solution (anti-phospho-ATM (Ser1981), PE and anti-phospho-Histone H2A.X (Ser139), PECy5 conjugated antibodies) and then analyzed by Muse Cell Analyzer (Fig. 35A/B). However, in Fig. 35 MDA-MB-231 showed a lower DNA damage compared to MCF-10A, similarly CSCs showed a lower DNA damage than MDA-MB-231. However, this decrease was not significant (Fig. 36).

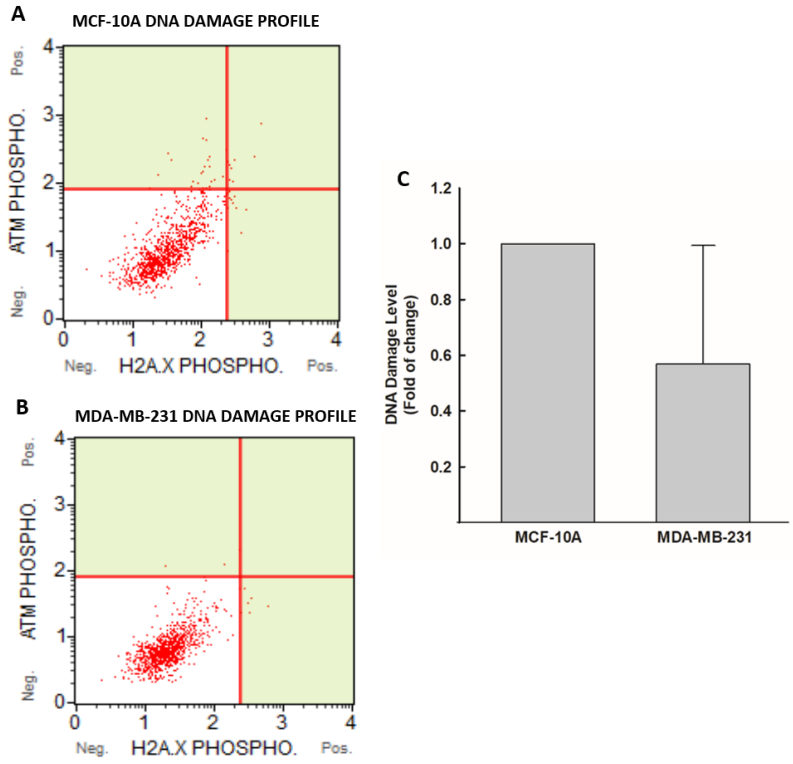


Figure 35. DNA damage levels in MDA-MB-231 versus MCF-10A cell line.
The level of DNA damage in MDA-MB-231 versus MCF-10A was detected by the Muse Cell Analyzer by incubating the cells with anti-phospho-ATM and anti-phospho-Histone H2A.X, PECy5 conjugated antibodies (Fig. 35A/B). The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 3). *; $p < 0.05$. The two cell lines were compared using Independent t-test (Fig. 35C).

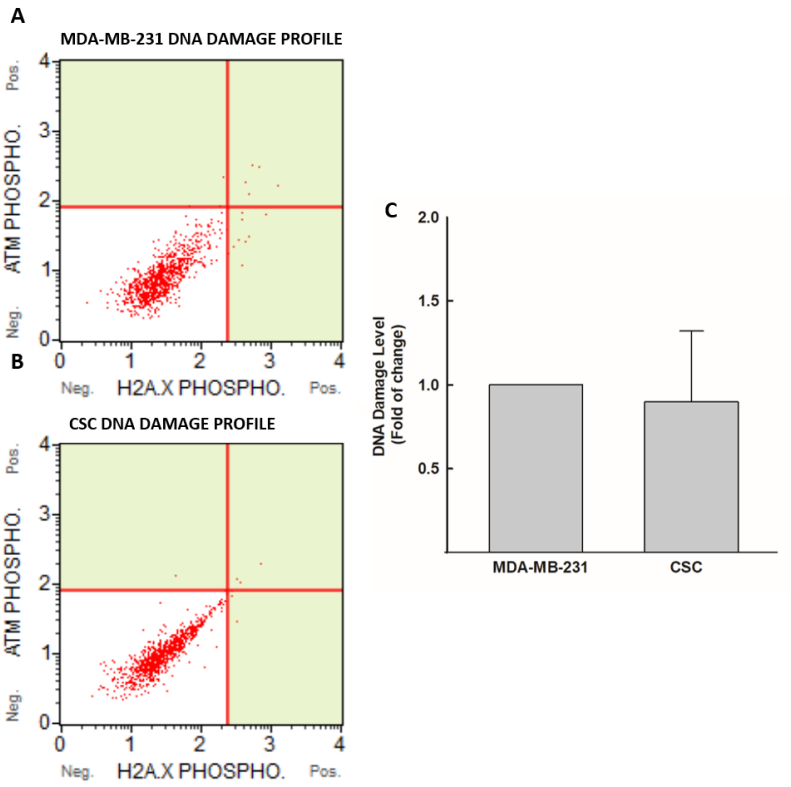


Figure 36. DNA damage levels in cancer stem cells versus MDA-MB-231 cell line. The level of DNA damage in CSCs versus MDA-MB-231 detected by the Muse Cell Analyzer by incubating the cells with anti-phospho-ATM and anti-phospho-Histone H2A.X, PECy5 conjugated antibodies (Fig. 36A/B). The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 3). *; $p < 0.05$. The two cell lines were compared using Independent t-test (Fig. 36C)

3.9. Differential Expression of Oxidative Stress Marker in Cancer Stem Cells Versus Cancer Cells

3.9.1. Expression of hemeoxygenase-1 (HO-1) in MCF-10, MDA-MB-231, and CSCs at the mRNA and protein levels.

To investigate the differential expression of HO-1 gene in MCF-10A, MDA-MB-231, and CSCs. We detected the gene expression through RT-PCR and Western blot analysis. Fig. 37A shows a significant 28-folds upregulation in MDA-MB-231 cells compared to MCF-10A cells at the mRNA level only. Similarly, in CSCs compared to MDA-MB-231 the HO-1 gene expressed a 2.5-folds upregulation at the mRNA level (Fig. 38A).

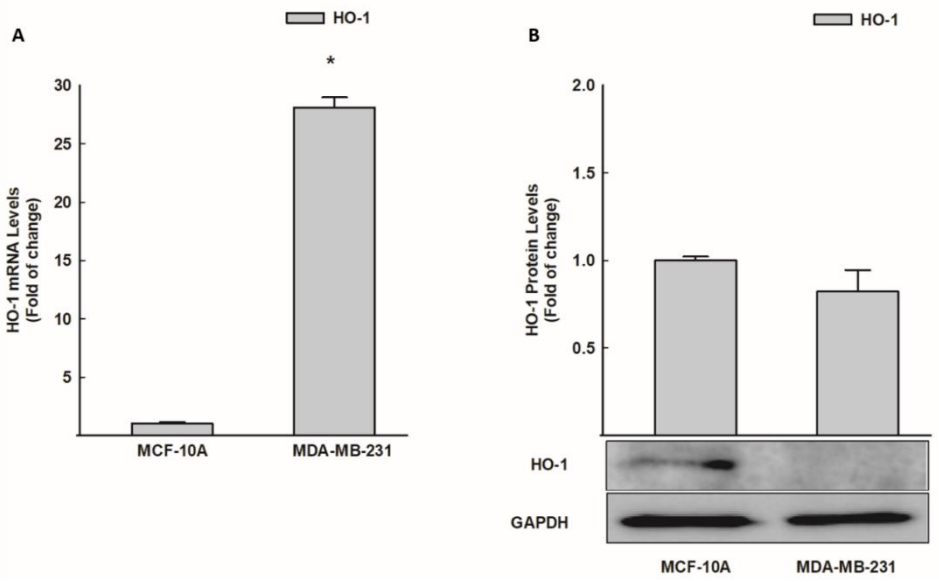


Figure 37. Constitutive expression of HO-1 in MDA-MB-231 versus MCF-10A cell line.

The basal mRNA (Fig. 37A) and protein (Fig. 37B) expressions of HO-1 were determined by RT-PCR and Western blot analysis, respectively. The mRNA and protein were standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p<0.05$ The two cell lines were compared using Independent t-test.

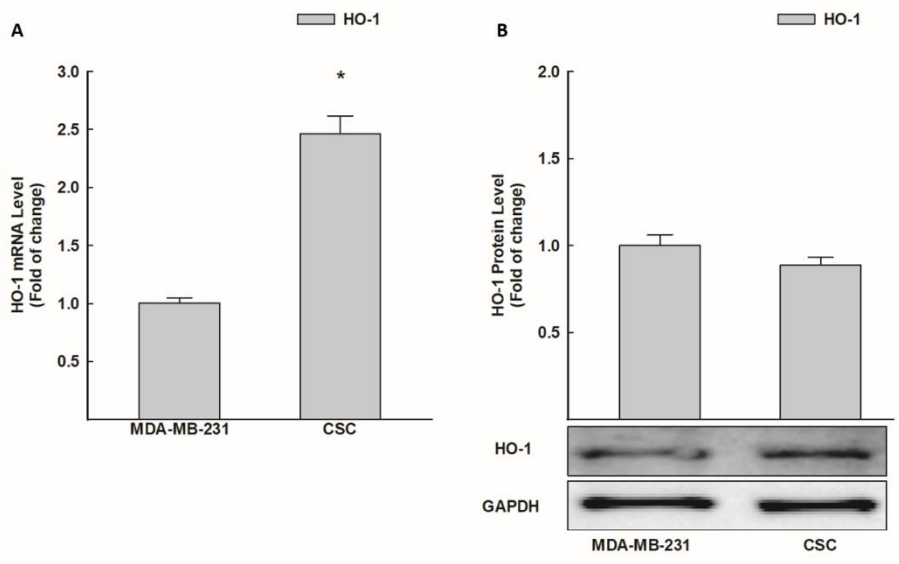


Figure 38. Constitutive expression of HO-1 in cancer stem cells versus MDA-MB-231 cell line.

The basal mRNA (Fig. 38A) and protein (Fig. 38B) expressions of HO-1 were determined by RT-PCR and Western blot analysis, respectively. The mRNA and protein were standardized to GAPDH housekeeping gene. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 6) for RT-PCR and (n=3) for Western blot analysis. *; $p< 0.05$. The two cell lines were compared using Independent t-test.

3.9.2. The levels of reactive oxygen species by Muse cell analyzer in MCF-10, MDA-MB-231, and CSCs.

To further explore the quantity of the cellular population undergoing oxidative stress, we detected the percentages of ROS in MCF-10A, MDA-MB-231, and CSCs through a Muse Oxidative Stress experiment. The cells were incubated with oxidative stress reagent working solution for 30 minutes. Fig. 39C shows that in MDA-MB-231 cells the ROS (+) were significantly less than that of MCF-10A by 34%. However, the CSCs expressed a much higher ROS+ activation compared to MDA-MB-231 by almost 100% (Fig. 40C).

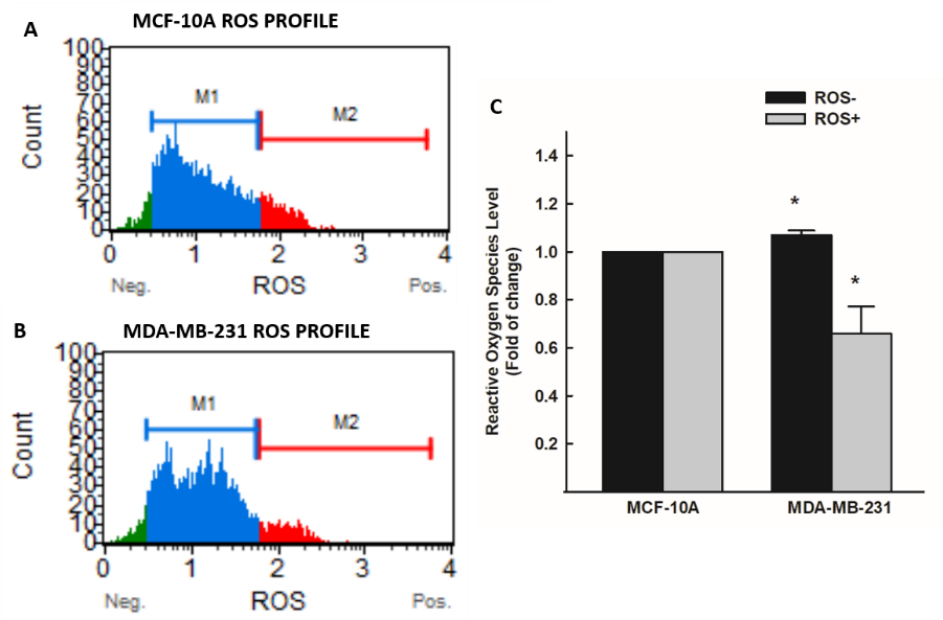


Figure 39. Reactive oxygen species levels in MDA-MB-231 versus MCF-10A cell line. The level of DNA damage in MDA-MB-231 (Fig. 39B) versus MCF-10A (Fig, 39A) was detected by the Muse Cell Analyzer by incubating the samples with Muse oxidative stress reagent. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 3). *; $p < 0.05$. The two cell lines were compared using Independent t-test. M1: ROS^-, M2: ROS^+

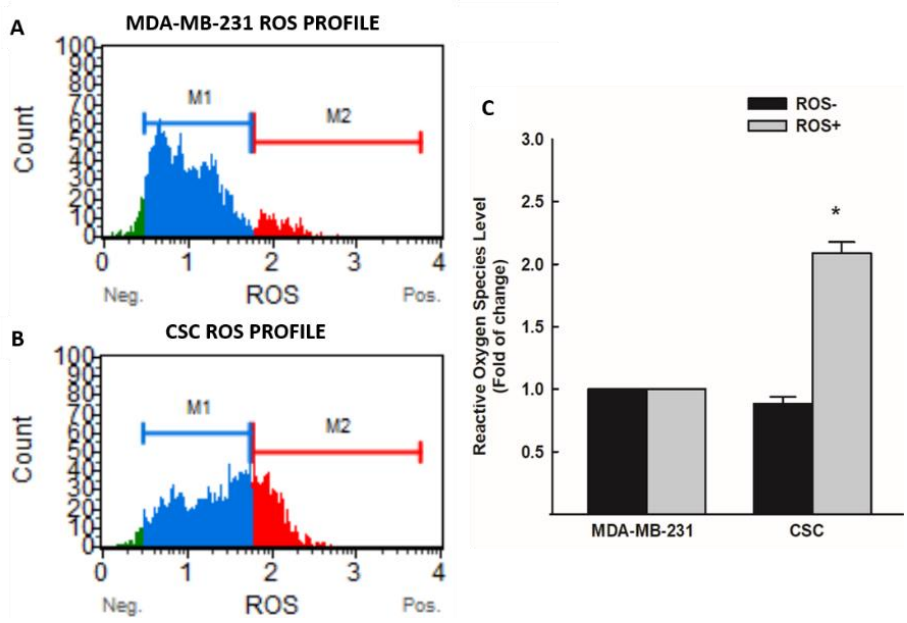


Figure 40. Reactive oxygen species levels in cancer stem cells versus MDA-MB-231 cell line.

The level of DNA damage in CSCs (Fig. 40B) versus MDA-MB-231 cells (Fig. 40A) was detected by the Muse Cell Analyzer by incubating the samples with Muse oxidative stress reagent. The reactions were repeated twice for every experiment and the values are presented as mean ± SEM (n = 3). *; $p < 0.05$. The two cell lines were compared using Independent t-test. M1: ROS^-, M2: ROS^+

Chapter 4: Discussion

Cancer is no longer perceived as a chaotic system disconnected from the body's control pathways [178]. Instead, it proved to be a systematic coordinated structure controlled by progenitor cells and supported by independent dynamics and signaling pathways [178]. Triple negative breast cancer is approximately expressed in 15%-20% of the total number of breast cancer cases [179]. However, it is considered as the most complicated breast cancer subtype, due to the absence of surface receptors that could be directly targeted by specific drugs. Thus, due to its aggressive characteristics and absence of a tailored treatment, TNBC has been ranking as the primary tumor with the highest rate of recurrence and relapse compared to the rest of breast cancer subtypes. However, even with all the emerging novel treatment strategies against TNBC, the five-year survival rate for patients is almost 76%. It was also proven that most patients develop chemoresistance after some time of administering the treatments [180].

Due to the limitations in achieving a better survival rate and lower recurrence and chemoresistance, researchers have emphasized on the need for further understanding of the mechanisms that cause chemoresistance and tumor recurrence. Thus, thorough studies and justifications were provided to understand the reason leading to this treatment failure, tumor recurrence, poor prognosis, and chemoresistance; hence, it was concluded that one of the most common reasons, was the presence of a very small subpopulation of cells, known as the CSCs. These cells are known to share extremely resistant and aggressive characteristics. Several signaling pathways were found to be involved in the functioning of these CSCs [70, 92].

Therefore, better understanding of mechanisms of CSCs chemoresistance and invasiveness is needed. However, there is a big lack in the scientific knowledge of the exact levels of gene expressions signaling pathways that mediate CSCs

chemoresistance. Therefore, due to the lack of literature and enough evidence on CSCs, we hypothesized that the breast cancer stem cells of TBNC are differentially modulating the expression and function of several genes and several transcription factors for the apoptosis, autophagy, CYP450 enzymes, tumor suppressors, stemness markers, and oxidative stress as compared to non-CSCs.

To prove our hypothesis, we a) examined the differential constitutive expression of apoptosis, autophagy, CYP450 enzymes, tumor suppressors, stemness markers, and oxidative stress among the normal breast epithelial cells (MCF-10A), TNBC cells (MDA-MB-231), and CSCs at the mRNA levels using RT-PCR, b) determined the expression of apoptosis, autophagy, CYP450 enzymes, tumor suppressors, stemness markers, and oxidative stress among the normal breast epithelial cells (MCF-10A), TNBC cells (MDA-MB-231), and CSCs at the protein levels using Western blot analysis, c) explored the function, activity, and cellular localization of apoptotic proteins using immunofluorescence, and d) examined the activity of DNA damage and oxidative stress using flow cytometry.

To the best of our knowledge, this is the first study that investigates and discusses the basal differential expression of all the key regulators and pathways responsible for chemoresistance, proliferation, and self-renewal of CSCs compared to both normal and TNBC cells.

Initially, we have conducted a series of experiments to isolate and characterize CSCs. The conducted CSC characterization and identification methods have verified the growth of adequate number of CSCs required for studying the signaling pathways controlling them. Characterization of the stemness features of the CSCs in the current study was confirmed and supported by four selective and specific experimental approaches. First, high expression of ALDH+ cells, in which the selectivity and

specificity of the assay was confirmed by DEAB. ALDH enzymes are responsible for the oxidation of aldehydes to carboxylic acids[181]. This process is a key regulator for the detoxification of the aldehydes produced from environmental toxins and cytotoxic drugs [182]. Hence, cancer cells that express upregulated ALDH enzyme activity are mostly resistant to chemotherapeutic therapies. ALDH enzyme activity does not only lead to chemotherapeutic drug resistance, but also prevent CSCs apoptosis by maintaining a low reactive oxygen species level [182]. Cruzado et al. have concluded that ALDH activity is a useful indication for tracking the tumorigenic potential of CSC subpopulation during disease progression [183].

Second, the high expression of $CD44^{high}/CD24^{low}$ a cell surface glycoprotein receptors, which functions as a unique CSC marker [184]. These results were in agreement with previous studies which showed that CSCs of 8 different breast cancer cell lines, along with colorectal, renal, and lung cancers express high CD44 and low CD24 [185, 186]. The significance of these co-existing surface receptors roots to their function in cell-adhesion and metastasis [186]. Thus, directly assessed as a special indicator for tumor diagnosis, prognosis, and strong proliferative capacity [166]. Moreover, Shao et al. results have demonstrated that cells expressing $ALDH+/CD44^{+}/CD24^{-}$ are capable of self-renewal and generating new carcinomas, thus have stem cell-like characteristics [164].

Third, the increased percentage of SP cells which is recognized by their upregulated expression for certain transmembrane transporters. One of these transporters, is the ABCG2 cell membrane transporter [187]. Thus, the side population experiment characterizes and identifies these group of cells through their unique ability to actively expel the Hoechst dye, a unique feature of CSCs [162]. Similarly, Al-Dhfyan et al. have shown increased SP cells in ER positive MCF-7 cells [156].

Fourth, increased the gene expression of stemness markers such as SOX2 in CSCs more than non-TNBC cells. This aligns with previous studies showed that SOX2 aids in tumor progression, cancer stemness, and chemoresistance [188, 189]. Although the expression of some of these stemness markers SOX2 and SOX9 were downregulated in non-CSCs TNBC compared to healthy breast cells, this could be attributed to the facts that these genes are responsible for poor patient prognosis.

The second stage of the study was to conduct comparative experiments on the three cells, MCF-10A, MDA-MB-231, and CSCs to explore the differential expression of several pathways that play a significant role in chemoresistance, such as apoptosis, autophagy, CYP450 enzymes, tumor suppressors, stemness markers, and oxidative stress.

The regular chemotherapeutic agents are well known initiators of apoptosis and cell death by inducing DNA damage in cancer cells. However, these cells recognize the process and initiate their own protective mechanisms that lead to drug resistance and poor therapeutic response [190]. Therefore, we primarily tackled the most relevant pathway responsible for chemoresistance. The apoptotic markers are known to regulate the TNBC cells survival and escape the regular protective death pathway of our bodies. In this study, we specifically explored the expression levels of pro-apoptotic and anti-apoptotic makers in healthy breast cells, cancerous breast cells, and BCSCs aiming to identify which markers could be mediating the chemoresistance of CSCs. This was performed at two stages, (1) comparing cancerous MDA-MB-231 cells with healthy MCF-10A and (2) comparing CSCs with MDA-MB-231 cells.

Our results indicated that, in comparison with MCF-10A cells, cancerous TNBC express lower levels of all the pro-apoptotic markers; caspase3, caspase 7, caspase 8, caspase 9, and BAX, whereas express higher levels of the anti-apoptotic Bcl-xL. These

differential expressions of apoptotic markers led to the absence of the apoptotic pathway that is considered as one of the main regulators for tumorgenicity. Our observations are in agreement with previous reports showing that inhibition of caspase 9, caspase 3, and caspase 7 in TNBC resulted in inactivation of the apoptosis pathway and increased breast cancer proliferation, metastasis, and invasion [191, 192]. With regard to BCL-2 family, it has been reported by Trisciuoglio et al. and others have shown that Bcl-xL overexpression leads to chemoresistance, invasion, metastasis, and tumor plasticity [193]. It was further proven that the chemical inhibition of Bcl-2 by venetoclax inhibited the growth and metastasis of TNBC by activation of caspase 3, caspase 7, and BAX mediated apoptosis [194].

Perhaps the most interesting results were the observations of CSCs, which showed that the expression levels of apoptotic markers (caspases 3, 7, 8, and 9 and BAX) were much lower in CSCs compared to TNBC, with overexpression of anti-apoptotic Bcl-xL. These results were observed at three levels, the mRNA, protein, and IF assay. Aligned with our results, Safa et al. have shown that the imbalance between elevated ant-apoptotic and decreased pro-apoptotic markers are the reason behind resistance to chemotherapy and CSC survival [195]. Therefore, activating the apoptotic pathway through enhancing caspases function or inhibiting Bcl-2 function could be a novel cancer stem cell therapy [196].

A recent review has addressed the possible connection of cell cycle progress and autophagy pathway, in which induction of autophagy has been well correlated with cell cycle arrest [197]. However, the molecular mechanisms linking them together in CSCs still not clear. In the current study, we reported that CSCs express lower levels of autophagy markers ATG and LC3, but higher p62 compared to TNBC, suggesting that CSCs expressed a much lower autophagy activation. In this context, it has been

reported that increased levels of p62 and impaired autophagy promotes tumor progression and protects the cancer cells against oxidants and detoxifying mechanisms through activating proangiogenic and pro-survival signals [198].

Li et al., have discussed that the controversial function of autophagy in cancers as either a tumor suppressor or tumor promoting role depends on the stage of the cancer [199]. For example, in the early stages of tumorigenesis, ATGs and LC3 act as a salvaging system by suppressing tumor initiation and preventing cancer progression; however, once the tumor forms and progresses autophagy functions as a recycling system that aids the cancer cells survival and growth [199]. Nonetheless, our results were aligned with the results of Claude-Taupin et al. and Zhao et al. who reported that the expression of ATG and LC3 are elevated in TNBC compared to healthy breast cells and this expression was associated with lymph node involvement, shorter disease-free survival, cancer metastasis, and as a marker for the evaluation of cancer prognosis [200, 201].

Our study was the first to show the downregulated expression of autophagy markers and a lower activation of LC3, which could be explained by the fact that, in highly proliferating breast tumors such as TNBC the cells require a high metabolic demand for oxygen and nutrients. Therefore, autophagy aids with supply of these nutrients by the catabolic process that degrades damaged cellular content. Thus, since CSC are dormant cells and in a quiescent state most of their lifespan, they are protected from cytotoxic drugs, then the cells do not require this death pathway [202, 203]. Hence, activating the cell death pathways of apoptosis and autophagy might be a novel approach to target CSCs.

Drug metabolizing enzymes are a major contributing factor to chemoresistance. This is supported by the fact that CYP450 enzymes, such as CYP3A4 and CYP2C, are

expressed in up to 55% of breast cancer tissues and the huge variability in the expression of CYP450 enzymes has been correlated to chemotherapy metabolism such as taxanes [204]. However, there is a lack in the knowledge regarding the expression and function of these enzymes in CSCs. With this regard, we report here that the CYP3A4 and CYP2D6 expression was downregulated in CSCs. For this reason, if we link their expression to the function of CYP3A4 that aids cancer cells in proliferation, migration, and angiogenesis. We can conclude that these functions are not required for dormant quiescent CSCs [205]. However, a recent study reported a correlation between TNBC, tamoxifen treatment, and estrogen receptor beta which is expressed in 60-80% of TNBC. Therefore, a downregulated CYP2D6 will not activate tamoxifen, thus the treatment will fail without adequately targeting the TNBC CSCs [206].

Several signaling markers, such as PI3K/AKT and NFkB, are known to play a crucial role in cancer progression and tumorigenesis. However, whether these factors are differentially expressed in CSCs is still not investigated. Therefore, we have investigated the expression of these factors in CSCs compared to MDA-MB-231 cells. In TNBC, the PI3K/AKT pathway is often activated and essential for tumorigenesis, cancer cell survival, it is considered as an oncogenic pathway [207, 208]. We report here that breast CSCs express the highest levels of AKT expression compared to TNBC MDA-MB-231 and healthy MCF-10A cells, suggesting that overexpression of AKT could be one of the mediators of CSCs development. Our observation was in agreement with Kaboli et al. results who demonstrated that blocking AKT's function inhibits breast cancer cells and CSCs progression, overcomes chemoresistance, and prevents further oncogenic signaling [209].

Another transcription factor that is a known regulator of cancer is the NFkB pathway. In several tumors, NF-kB acts as tumor suppressor through activating various

pathways such as p53[210, 211]. In the current work we measured the expression levels of NFkB gene in three cells, were we found that NF-kB expression level was dramatically downregulated in breast CSCs more than MDA-MB-231 cells which was even lower than healthy MCF-10A cells. It is postulated that downregulation of NFkB decreases apoptotic cell death. This postulation is supported by multiple previous studies reported that activation on NF-KB indirectly enhances apoptosis of cancer cells and inhibits cancer cell proliferation through activating p53 [212-214].

Cell proliferation biomarker ki-67 has been correlated to cancer patient survival and relapse [215], in which high ki-67 expression in TNBC is associated with more aggressive characteristic and a higher risk for recurrence [216]. In the current study, although expression of ki-67 in MDA-MB-231 cells was much higher than healthy cells, unexpectedly, CSCs express lower ki-67 mRNA expression level compared to MDA-MB-231 cells, indicating that CSCs are dormant cells with low proliferating index. Nonetheless, mdr-1 is one of the main reasons for chemoresistance in TNBC due to the efflux of chemotherapeutic drugs, therefore, Abd El Aziz et al. have explained that the overexpression of ATP-binding cassette (ABC) transporter proteins is linked to poor treatment outcomes, chemoresistance, and low survival rate [217].

Our results have shown that, compared to healthy breast cells the TNBC cells expressed an upregulated activity for p53, BRCA, and PTEN. According to a study including 678 TNBC patients 35.8% expressed a high p53 activity. Unlike regular p53 tumor suppressing functions, 80% of TNBC express the highest levels of mutant p53 which stimulate tumors proliferation, migration, chemoresistance, invasion, and poor prognosis [218, 219]. Bae at al. have shown that TNBC patients that lack p53 expression show poorer patient prognosis [220].

Similarly, BRCA gene is known to increase the risk for breast cancer especially

TNBC, which renders the tumor to be more aggressive and chemoresistant. However, a study conducted by Pogoda et al. demonstrated that mutations at the BRCA level did not affect the TNBC patients' outcome [221]. However, on the contrary to TNBC expression the CSC expressed completely inhibited values for the three tumor suppressors. The p53 gene is known for its function in inhibiting the cancer cells growth and proliferation through enhancing apoptosis and DNA repair mechanisms [222]. Fromentel et al. have shown that the loss of function of p53 leads to the survival and proliferation of mature cancer cells and CSCs in human hepatocellular carcinoma [223].

Li et al. have demonstrated that the loss of PTEN expression was associated with larger tumor size, node involvement, metastasis, and worse outcome and prognosis [224]. Moreover, Ciufredda et al. have shown that PTEN loss leads to the increased self-renewal capacity and proliferation of CSCs into CSCs clones [127]. Lastly, Kim et al. have demonstrated that the BRCA1 gene suppresses the CSC characteristics of $CD44^{high}$ expression, however upon BRCA1 gene knockdown the expression was gained again [225]. Therefore, p53, PTEN, and BRCA are considered as molecular players in chemotherapy, since their inhibition allows the CSCs to escape cytotoxic drugs and cause chemoresistance by further downregulating apoptosis and autophagy pathways.

In comparison to healthy breast cells, the TNBC expressed a lower level of ROS species. Since, once cancer forms and progresses, ROS no longer protect against cancer, but act in damaging the tumor's DNA through multiple signaling pathways. Aggarwal et al. have shown that elevated ROS activates a signaling cascade that initiate apoptosis in cancer cells, induce autophagy, and increases tumorigenic cells susceptibility to chemotherapeutic drugs [226]. Therefore, decreased ROS expression protect highly proliferating cancer cells. However, the CSC expressed a high level of ROS compared

to TNBC; an overwhelming number of studies showed that ROS+ aid in tumorigenic cell growth and cancer sustainability [227, 228].

In all three cell lines, there was no difference at the level of DNA damage. This could be explained by the fact that TNBC has a protective mechanism known as the DNA repair pathways which repairs damaged DNA by base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) [229].

Importantly, in CSCs low to minimal DNA damage is expressed. Sacca et al. have shown that CSCs are able to survive stressful conditions by the extensive protection of their DNA, by the rigorous activation of DNA damage sensor and repair system [230]. Thus, it is tempting to elucidate that the elevated oxidative stress and minimal DNA damage cause chemoresistance through upregulating CSC protective mechanisms and cell survival by DNA repair responses and stress responsive proteins which aid in inhibiting cancer cells apoptosis [231].

Chapter 5: Conclusion and Future Directions

Triple negative breast cancer is the most aggressive type of breast tumor that constantly leads to poor patient survival and frequent tumor relapses, due to acquired-chemoresistance and limitations in targeted treatments. However, the core reason behind this failure, is the presence of an extremely resistant and aggressive type of cells known as cancer stem cells. These cells have been found to not only induce chemoresistance, but also lead to future tumor initiation and recurrence in patients with history of malignant tumors.

The significance of this work is two-fold: first, we have uncovered the differential gene expression of the principle signaling pathways controlling the chemoresistant characteristics of CSCs; second, we have identified new pathways and genes that could be a potential future CSCs treatment.

Moreover, these cells possess a highly functional DNA damage repair system that maintains damaged DNA to a minimal. Moreover, the CSCs escape the protective mechanisms of the body through escaping the programmed cell death by impaired apoptotic and autophagic pathways. Finally, CSCs further escape the DNA damage, and programmed cell death by the dysregulated and mutated expression of tumor suppressor genes that are considered as the corner stone for preventing tumorigenesis. In Tab. 7, a summary of the signaling pathways gene expressions in CSCs compared to MDA-MB-231.

Future directions targeting the CSCs is an essential step to reduce recurrence and chemoresistance in most patients. First and foremost, utilizing the quiescent state of CSCs could be a very novel therapy. This could happen by either targeting CSCs in their dormant state or pushing the cells out of the quiescent state and targeting them through activating certain pathways or depleting essential nutrients. Second,

understanding the pathways by which CSCs repair DNA damage may provide therapeutic targets to sensitize CSC to cytotoxic therapies hence improve CSCs treatments. Third, the restoration of the cell death apoptotic and autophagic pathways could be a breakthrough treatment, by activating death receptors through synthetic drugs, antagonize the action of antiapoptotic proteins, and restoration of tumor suppressor gene function. Lastly, it would be of tremendous importance to tackle the microRNA expression in CSCs since they might be key regulators in initiating chemoresistance and poor patient survival.

Table 7. Summary of the gene expression profile in cancer stem cells compared to MDA-MB-231 cells.

Gene	CSCs versus MDA-MB-231
Apoptosis	
Caspase3	↓
Caspase7	↔
Caspase8	↓
Caspase9	↓
BAX	↓
Bcl-xL	↑
Autophagy	
ATG	↓
P62	↑
LC3II	↔
Cytochrome P450 enzymes	
CYP3A4	↓
CYP2D6	↓
CYP2C19	↑
Stemness markers	
ALDH1A1	↑
SOX2	↑
SOX9	↓
Tumor suppressor genes	
PTEN	↓
p53	↓
BRCA	↓
Other signaling genes	
NF-KB	↓
AKT	↑
TNF-a	↔
Ki-67	↓
Mdr-1	↔
Oxidative stress marker	
HO-1	↔

↑: significant upregulation
↓: significant downregulation
↔: no significant change

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