AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF *HOLOTHURIA POLII* EXTRACTS ON PROSTATE CANCER CELLS

by

DIANA HOUSSAM KADI BAHRI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
December 2013

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DIANA HOUSSAM KADI BAHRI

Approved by:

Dr. Marwan El-Sabban, Professor — Advisor
Department of Anatomy, Cell Biology
and Physiological Sciences

Dr. Wassim Abou-Kheir, Assistant Professor — Co-Advisor
Department of Anatomy, Cell Biology
and Physiological Sciences

Dr. Georges Daoud, Assistant Professor — Member of Committee
Department of Anatomy, Cell Biology
and Physiological Sciences

Dr. Abdo Jurjus, Professor — Member of Committee
Department of Anatomy, Cell Biology
and Physiological Sciences

Date of thesis/dissertation defense: December 23, 2013

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ACKNOWLEDGMENTS

Wishing that my words of appreciation would flourish and become acts, and that what was acquired in theory and practice would fruit beyond this present thesis; I start putting in words what made a long journey… short.

My deepest gratitude goes to Dr. Marwan El-Sabban. He, whom with the life he leads, is an example of authenticity, of one being ones' self no matter what the niche induces. Same applies to his teachings; when knowing each student's unique gift, he never hesitates to irrigate it with the best nutrients for it to grow, and manifest itself. For that and for his continuous help, guidance and limitless discussions and advices, I am thankful. His smile and genuine inexplicable inner being made Lebanon feel like home to me.

My gratitude extends to Dr. Wassim Abou-Kheir. His enthusiasm and love for research is reflected throughout this thesis. For his continuous follow up, guidance and meetings that familiarized us with the work and for his generous support, I am grateful.

I would also like to thank the committee members, Dr. Abdo Jurjus and Dr. Georges Daoud, for the time and effort they spent reading and evaluating this work.

Sara Al-Ghadban, whom with her guidance and experiment planning influenced me to manage perfecting my work; for being my indirect umbilical cord from Dr. Sabban, and for making our little heaven available in our movie and matti time in the end of the day or to me sometimes in its beginning, I thank you.

Rabih Merahbi, I've been blessed for being granted your precious friendship, and Hadi Fares, your enthusiasm still beats in the labs. Every shared curiosity fulfilled and every adventure experienced with you two, made my work richer and my stay exquisite.

I am sincerely thankful to Alissar, Jamal, Layal, Fatmeh, Zeina, Jalal, Randa, Rebecca and Zahraa, and to all the friends who contributed directly or indirectly to this work.

This work is dedicated to my *father* and *mother* who have always been the source of every good step made in the way… and my *brothers (Rami & Mazen), twin-soul sister (Laila)* and *Adam,* for living in me always…

‘‘*.. then there are those who plant. They endure storms and all the vicissitudes of the seasons, and they rarely rest. But, unlike a building, a garden never stops growing. And while it requires the gardener’s constant attention, it also allows life for the gardener to be a great adventure.*

Gardeners always recognize one another, because they know that in the history of each plant lies the growth of the whole world.’’

P. Coelho

ABSTRACT OF THE THESIS of

Diana Houssam Kadi Bahri for Master of Science
Major: Physiology

Title: Effect of *Holothuria polii* Extracts on Prostate Cancer Cells

Background: The wealthy and unexplored biodiversity of the Mediterranean Sea has recently attracted the attention of scientists to explore novel bioactive compounds. Prostate cancer is the second most diagnosed cancer in men. Despite progress in the fields of treatment and diagnosis of this disease, incidence and mortality rates are still high.

Aims: The aim of this study is to investigate the effect of the ethanolic sea cucumber (*Holothuria polii*) extract (SCE) and their fractionated material against the proliferation and metastasis of human prostate cancer cells. Their bioactivity is also assessed for targeting an enriched population of prostate cancer stem cells (CSCs).

Methods: The effect of the crude SCE on the viability and proliferation was investigated using trypan blue exclusion assay, MTT assay and Real Time Cell Analysis (RTCA). Effect on cell cycle progression was studied on DNA content using Propidium Iodide (PI) by flow cytometer. Hoechst nuclear staining was performed to assess for apoptotic bodies as a proof of apoptosis. Immunofluorescence (IF) and western blotting (WB) were also performed on apoptotic markers. SCE effect on metastasis was further evaluated by invasion (RTCA, gelatin zymography) and migration assays (RTCA, wound healing). Real-time PCR, WB and IF were performed to assess the effect of SCE on the transcriptional and translational level and cellular localization of EMT markers, a pro-angiogenic factor and a metastatic marker. Sphere-formation assay was conducted to study the effect of SCE on targeting an enriched population of prostate CSCs. Finally, the potential of the aqueous SCE obtained from increased solvent polarity fractionation was also investigated on prostate cancer cells' proliferation using trypan blue exclusion assay, and on its ability to target prostate CSCs using sphere formation assay.

Results: SCE inhibited the proliferation of two prostate cancer cells in a time- and dose-dependent manner. Cell cycle analysis revealed that SCE causes G_0-G_1 arrest and an increase in Pre G_0-G_1 suggesting DNA fragmentation. Hoechst stain, WB and IF showed that apoptosis is the mechanism that SCE is inducing. SCE inhibited the migration and the invasion of prostate cancer cells. No change was observed at the transcriptional level, yet at the translational level, mesenchymal and metastatic markers decreased upon treatment. SCE inhibited the proliferation of an enriched population of prostate cancer stem/progenitor cells indicating that SCE targets CSCs. Furthermore, preliminary data showed that the aqueous fractionated SCE also inhibited the proliferation of prostate cancer cells in 2D and 3D cultures.

Conclusions: Our data suggests that SCE inhibits the proliferation and metastasis of prostate cancer cells, presumably through targeting prostate cancer stem/progenitor cells.

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LIST OF ABBREVIATIONS

ALDH: Aldehhyde dihydrogenase

AR: Androgen receptor

Bcl2: B-cell CLL/lymphoma 2

bFGF: basic fibroblast growth factor

BPH: Benign Prostatic Hyperplasia

CSC: Cancer stem cell

CXCR4: C-X-C chemokine receptor type 4

DAPI: 4-6 diamidino-2-phenylindole

DHT: Dihydrotestosterone

DMSO: dimethyl sulfoxide

ECM: Extracellular Matrix

EGF: epidermal growth factor

EMT: Epithelial to Mesenchymal Transmission

ERK: Extracellular signal-regulated kinases

EZH2: Histone-lysine N-methyltransferase

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IF: Immunofluorescence

MAPK: Mitogen-activated protein kinases

MDR: Multi drug resistance

MM: multiple myeloma

MTT: [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

NSCs: Neural stem cells

PAP: Prostatic acid phosphatase

PARP: Poly ADP ribose polymerase

PBS: Phosphate buffered saline

PC: Prostate cancer

PI: Propidium Iodide

PSA: Prostate-specific antigen

PTEN: Phosphatase and tensin homolog

RTCA: Real time cell analysis

SCE: Sea cucumber extract

SDS: Sodium dodecyl sulfate

SFU: Sphere forming unit

SP: Side population

TMPRSS2: Transmembrane protease serine 2

TSCs: Tissue specific stem cells

WB: Western Blot

γH2AX: Phosphorylation of histone H2AX

INTRODUCTION

Prologue

Cancer is one of the major causes of death in the globe and prostate cancer (PC) is the most frequently diagnosed cancer among men in many western countries. Although early detection of PC is recently attainable; there is still little genuine effective therapy for patients with advanced local PC and/or metastatic one. Most patients with advanced PC respond initially to androgen ablation therapy, however, some of the patients develop androgen independent tumors that are inevitably fatal. A similar response is approached via surgery, chemotherapy and radiotherapy treatments. All this suggest that metastatic PC remains an uncured disease by the present therapeutic approaches (Litwin et al., 1995). The main reason is thought to be the presence of resistant cells in the tumor that are responsible for cancer progression and relapse. Cancer stem cells are the most crucial target in the treatment of cancer. A thorough understanding of cancer stem cells' biology and the way it differs from a normal stem cell facilitates its selective targeting, thus, its elimination, yielding a more promising and improved therapeutic outcome.

Recent studies have shown that marine organisms are a rich source of anti-cancer compounds, thus the rich biodiversity of marine life in the Mediterranean Sea harbor tremendous amounts of unexploited bioactive molecules. In this study, crude extracts of Sea Cucumbers (*Holothuria Polii*) were collected from the Lebanese coast of the Mediterranean Sea and their effect was investigated on prostate cancer cells which are the model of solid malignancy chosen in this study.

A. Cancer

Malignant neoplasm or what is known as cancer, is the result of the accumulation of mutations and epigenetic changes that eventually lead to the down regulation of tumor-suppressor genes and the activation of oncogenes. It is characterized by the uncontrollable division and growth of cells that lead to tumor formation and later on to its invasion into nearby organs. Its heterogeneous nature and its ability to persist, progress and evolve, engaged scientists for decades.

I. Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in men, the second leading cause of cancer death after lung cancer in western countries and the sixth leading cause of cancer mortality among men worldwide with 899 000 new cases and 258 000 new deaths estimated to have occurred in 2008 worldwide (Center et al., 2012). Since PC is a slow growing tumor, its diagnosis usually occurs in its late stages when it is already metastasized. Although early detection of prostate cancer is now feasible; there is still no effective therapy for patients that have gained advanced local PC and/or metastatic one. Most of the patients with advanced PC respond initially to therapy, whether androgen ablation, surgery, chemotherapy or radiotherapy. However, some patients develop androgen independent tumors that are inevitably fatal suggesting that there is a subpopulation of resistant tumor cells that are capable of cancer recidivism; this subpopulation has ‘’stem-like’’ cell characteristics and are called: cancer stem cells (CSCs). All this suggest that locally advanced and/or metastatic PC remains an uncured disease by the present therapeutic approaches.

1. Prostate gland overview

The prostate gland is the largest accessory sex gland of the male reproductive system. Its main function is to store and secrete a clear, alkaline fluid that contains: prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), fibrinolysin, citric acid, bioavailable zinc and mainly polyamines; and contribute to the formation of the seminal fluid (Devens, Weeks, Burns, Carlson, & Brawer, 2000). Furthermore, it plays a fibromuscular role in restricting the urine since it is located in the pelvis, below the urinary bladder, surrounding the urethra (J. E. McNeal, 1980b).

The prostate consists of 30 to 50 tubuloalveolar glands arranged in three concentric layers: an inner mucosal layer, an intermediate submucosal layer and a peripheral layer that contains the main prostatic glands (J. E. McNeal, 1980a). The adult prostatic parenchyma is divided into four morphologically and functionally distinct zones (Figure 1):

- The central zone which contains about 25% of the glandular tissue and is resistant to both carcinoma and inflammation.
- The transitional zone surrounding the prostatic urethra comprises about 5% of the prostatic glandular tissue and contains the mucosal glands. It is in this region where proliferative disorders take place, e.g. Benign Prostatic Hyperplasia (BPH) forming nodular masses of epithelial cells.
- The peripheral zone surrounding the central zone and comprising 70% of the glandular tissue of the prostate. Most prostatic carcinomas arise in this zone and it is also the most susceptible to inflammation
- The periurethral zone containing mucosal and submucosal glands. (J. E. McNeal, 1981).

There is a non-glandular area called the anterior fibromuscular stroma shown also in figure 1. The growth of the epithelium of the glandular tissue is regulated by the hormone dihydrotestosterone (DHT) and consists mainly of secretory luminar epithelial cells, which arranges into a single cell layer synthesizing several prostatic proteins that are secreted into the ductal lumen. These cells are characterized by the expression of androgen receptor (AR), cytokeratins 8 and 18, CD57 and PSA. They require continuous direct androgenic stimulation to maintain their structural and functional viability. The prostate epithelium is also composed of neuroendocrine cells, macrophages, intra-acinar lymphocytes and basal epithelial cells that express cytokeratin 5, cytokeratin 14 and CD44 (Chatterjee, 2003).

When the androgen level drops below a certain threshold, like in the case of surgical or chemical castration, the secretory cells undergo apoptosis, causing glandular involution. Animal studies have shown that there was approximately 90% loss of prostatic secretory epithelial cells through apoptosis after physical castration (Maitland, 2013). On the other hand, basal cells are expected to represent the stem cells of the prostate gland, surviving castration since most of them do not possess AR. Although their survival does not depend on androgen, they require androgens for proliferation and differentiation into secretory cells (Çetintaş, Kaymaz, & Kosova, 2013) under the normal physiological state. Cells with accumulated mutations are removed by apoptosis and a steady state balance is maintained between cell proliferation and apoptosis. However, pathological states trigger the hyper stimulation of androgen and/or growth factors, thus affecting the delicate balance of prostatic cell growth and death, allowing a subset of epithelial cells to evade the normal checkpoint control of cell cycle progression and eventually proliferate abnormally (Chatterjee, 2003).

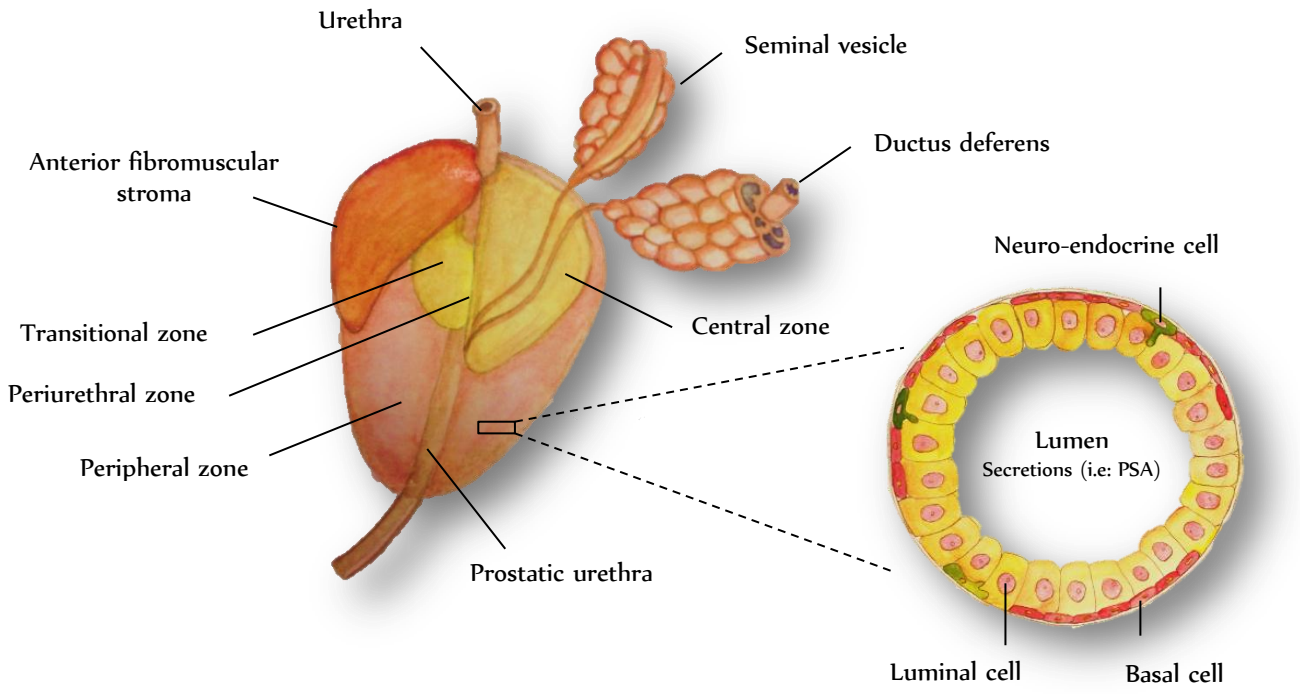


Figure 1. The prostate gland and its cellular components.
Schematic representation showing the different zones that comprise the prostate gland and its cellular composition.

2. <u>Prostate cancer (PC) types and risk factors</u>

Approximately 95% of prostatic tumors are adenocarcinomas (Stamey et al., 2001). There is another type of prostate cancer that does not show the first symptoms that usually adenocarcinomas do, like the increase of prostate-specific antigen (PSA) levels, making it more difficult to detect in its early stages. This other type initiates from specialized cells within the prostate and it is regarded as 'small cell carcinoma'. The wide variation in international PC incidence rates and trends is in part due to the substantial differences worldwide in the diagnosis of latent cancers through prostate-specific antigen (PSA) testing of asymptomatic individuals as well as during prostate surgery.

Additionally, the most established risk factors for PC are:

a) Age: men above the age of 50 have a higher risk in developing PC, and more than three quarters of cases are diagnosed in men over 65 years, yet the largest number of cases are diagnosed in 70-74 age.
b) Race: black men have higher risk than white men, nevertheless, Caucasian have higher risk than Asians. Yet, Asians that lived in the west were more prone to have PC, indicating that life style and environmental factors should be considered as risk factors as well.
c) Family history of the disease: according to epidemiological studies, the risk of sons and brothers of men carrying the disease is augmented and about 5 to 10 percent of all prostate cancers diagnosed are hereditary (Stamey et al., 2001).

3. Diagnosis of prostate cancer

PC can be diagnosed by clinical symptoms or by clinical examination of individuals that are at risk and/or by testing the serum PSA. However, the decisive diagnosis is made by histologically examining the prostatic tissue.

1) Clinical diagnosis:

Symptoms rarely appear in PC's early stages, however more advanced disease may show specific lower urinary tract symptoms including frequent urination, nocturia (increased frequency of urination at night), hematuria (blood in urine) or dysuria (pain during urination), yet the majority of PC cases remain asymptomatic. The clinical presentation of PC changed significantly since 1990s due to increased utilization of PSA and prostatic biopsies, aiming to detect the disease in its early stage (Frankel, Smith, Donovan, & Neal, 2003).

2) Diagnostic tests:

a. Digital Rectal Examination (DRE)

The main limitation of digital rectal examination is the significant variability of the examiner. Since the introduction of PSA testing in late 1980s, digital rectal examination is replaced by PSA testing as the main diagnostic tool (Croswell, Kramer, & Crawford, 2011).

b. Prostate specific Antigen (PSA) testing

PSA is a glycoprotein present in small quantities in the serum of men with healthy prostates. Its level is often raised in prostate disorders including PC. PSA testing was not initially envisioned as a screening strategy rather its first use was for the evaluation of treatment responses in men with PC (Croswell et al., 2011). Since the advent of PSA testing, the spectrum of PC cases has changed in many developed countries. At the population level, significantly more men are now diagnosed with localized and low grade disease. Furthermore, a significant reduction in mortality has also been attributed to PSA screening in the US, where PSA screening was introduced at the population level (Hoffman, 2011).

c. Transrectal ultrasound guided prostatic biopsies

The definitive diagnosis is usually decided through taking a transrectal ultrasound guided biopsy which is a procedure whereby prostatic tissue is obtained for histological evaluation (Terris & Stamey, 1991).

4. Grading prostate cancer stage

The stage of the disease designates the extent of the cancer within the gland and its invasion around the prostate itself and more distally as metastatic lesions.

The TNM classification is utilized to stage PC, with T representing tumor and its invasion into adjacent structures, N representing whether the regional lymph nodes play a role in it or not, and M indicating the presence or absence of distant metastasis.

Gleason sum score is the score that is based on histological pattern of tumor and is widely used to measure the aggressiveness of cancer (J. McNeal & Gleason, 1991). This score is the sum of the assigned grades /ranging from 1 to 5/ to the most common and the second most common morphologic tumor patterns respectively. Gleason score (range 2-10) is considered to be an advantageous prognostic factor for PC patients (Sogani et al., 1985).

Risk stratification of men with localized PC is performed by combining the PSA, Gleason grade and clinical stage. And it is classified as the following (Partin et al., 1993):

- Low risk men (PSA < 10ng/ml and Gleason grade ≤ 6 and clinical stage T1-T2a)
- Intermediate risk men (PSA 10-20ng/ml or Gleason grade = 7 or clinical stage T2b-T2c)
- High risk men (PSA > 20ng/ml or Gleason grade 8-10 or clinical stage T3-T4).

Treatment decisions are made according to the regarded risk stratification.

5. Pathways involved in PC etiology

Etiology of PC is involved in several pathways. They include:

1) ERK/MAPK activation

The ERK/MAPK pathway includes many proteins, primarily MAPK (Mitogen-activated protein kinases, originally called ERK, Extracellular signal-regulated kinases), which communicates through phosphorylation, acting as an "on" or "off" switch. It is also known as the Ras-Raf-MEK-ERK pathway. In prostate cancer this pathway is activated leading to altered transcription of genes that are important for the cell cycle besides altering the

translation of some mRNA to proteins (Abou-Kheir, Hynes, Martin, Pierce, & Kelly, 2010; Pearson et al., 2001).

2) PTEN inactivation

Phosphatase and tensin homolog (PTEN) is a protein that acts as a tumor suppressor gene through the action of its phosphatase protein product. This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly. In prostate cancer this pathway is inactivated (Abou-Kheir et al., 2010).

3) TMPRSS2-ERG fusion

Transmembrane protease serine 2 (TMPRSS2) is an enzyme whose gene is up-regulated by androgenic hormones in prostate cancer cells and down-regulated in androgen-independent prostate cancer tissue. TMPRSS2 protein's function in prostate carcinogenesis relies on the overexpression of ETS (E-twenty six) transcription factors, such as ERG and ETV1, through gene fusion. TMPRSS2-ERG fusion gene is the most frequent as it is found in 40% to 80% of prostate cancers in humans. ERG overexpression contributes to the development of androgen-independence in prostate cancer through a disruption of androgen receptor signaling (Tomlins et al., 2005).

4) NKX3.1 decrease/ Myc increase

Homeobox protein NKX3.1 is encoded by the NKX3-1 gene in humans. The homeodomain-containing transcription factor NKX3A is a recognized prostate tumor suppressor that is expressed in a largely prostate-specific and androgen-regulated manner. Loss of NKX3A protein expression is a commonly found in human prostate cancers. As for Myc, it is activated through several signals such as Wnt, Shh and EGF by the MAPK/ERK pathway. Myc

activation results in increasing the cell proliferation by upregulating cyclins and downregulating p21 and Bcl2. Myc is a very strong proto-oncogene and it is usually found to be upregulated in many types of cancers (Little, Nau, Carney, Gazdar, & Minna, 1983)

5) EZH2 overexpression

Histone-lysine N-methyltransferase (EZH2) is over expressed and advances cancer since it increases the histone methylation that silences the expression of tumor suppressor genes. It has been demonstrated that EZH2-targeting drugs inhibit the progression of brain and prostate cancer in pre-clinical models (Suvà et al., 2009).

6. Therapeutic approaches

The choice of treatment is often influenced by the risk of patients to progress or die of the disease as arbitrated by patient's age, Gleason score, tumor stage and serum PSA levels. For early PC, the chief treatment modalities include surgery, radiotherapy and active surveillance. As for patients that didn't undergo early detection of PC, androgen ablation hormone therapy or PSA monitoring is advised.

1) Active surveillance

Meantime, active surveillance is considered usually for men with low PSA-level, localized disease and Gleason score ≤ 6 (Albertsen, 2010). These patients are monitored with an intention that if there are any signs of disease progression, active treatment or intervention will be started. Follow-up of these men on active surveillance includes PSA-testing, digital rectal examination and sometimes prostatic biopsy (Heidenreich et al., 2008). This approach prevents the serious side effects associated with treatment modalities. Given that patients that have high level

of PSA are diagnosed with small and asymptomatic tumors which may not progress, this approach seems reasonable. However, the main disadvantage of this approach is that men are not cured from the cancer and they have to live with the disease, which may have psychological effects. Some evidence suggests that men on active surveillance had highest quality of life compared with other treatment modalities for localized PC (Hayes et al., 2010). Although, active surveillance is considered to be a good treatment option, yet the prediction of tumor progression of individual patients is still difficult.

2) Radical Prostatectomy

Surgical treatment has significant benefits and it is usually curative. Radical prostatectomy, allows a complete pathological assessment of the tumor (Heidenreich et al., 2008). Following a radical prostatectomy, PSA should not be detectable and any increase in PSA would indicate the relapse of the disease. Side effects still can occur such as impotence and urinary incontinence. Sexual dysfunction can occur in 32% to 53% of cases receiving surgical treatment (Knight & Latini, 2009). Laparoscopic prostatectomy has been introduced as a less invasive surgical approach to minimize the side effects of radical prostatectomy.

3) Radiotherapy

External beam therapy and brachytherapy are the commonly used radiological approaches. External beam therapy has some advantages over surgical therapy, these advantages include: lower rate of urinary incontinence, no risk of anesthesia, no inpatient hospital stay and may even be used in men with co-morbidities (Heidenreich et al., 2008). Sexual dysfunction can also result following radiation

therapy. Yet, the main problem associated with external beam therapy is the risk of radiation induced damage to the rectum, since it is located behind the prostate and the bladder. These complications can be avoided using brachytherapy which is placing radioactive pellets within the prostate.

4) Hormonal therapy

Androgen deprivation therapy was previously used to treat the metastatic PC cases but recently it had become the second most commonly used treatment modality after surgery (Krahn et al., 2011). Some evidence suggested that hormonal therapy prolongs the survival of PC patients but significant side effects are also reported. Because androgens are essential for the physiological activity of other body functions, this therapy has many side effects including loss of libido, erectile dysfunction, fatigue, depression, osteoporosis, new onset diabetes and hypertension and even increase the risk of cardiovascular mortality (Isbarn et al., 2009).

II. Cancer Stem Cells

Decades ago, the hypothesis of cancer stem cells (CSCs) was advocated (Reya et. al, 2001), yet the absence of a direct and evidential method yielded in a delayed approval. Recent research, however, unveiled the fact that tumors are heterogeneous comprising of a variety of cell populations resembling in hierarchy that of a normal tissue, having a small percent of cells that have the ability to self-renew but that are also tumorigenic; these cells are the so called cancer stem cells (CSCs). (T. Reya, S. J. Morrison, M. F. Clarke, & I. L. Weissman, 2001). These findings suggest that further understanding of CSCs is crucial for cancer therapy.

1. Definition of CSCs:

CSCs are a subpopulation of cancer cells with a self-renewal capacity, tumorigenicity, and multipotentiality. Yet, CSCs have also been described as a sparse population of quiescent cells with a capacity to generate an immense number of multilineage progeny, and with the ability to resist conventional therapies and recapitulate the original tumor. These latter descriptions were based on models that may not express the real biology of tumors. The limitations of such models have led to the redefinition of CSCs as tumor cells that exhibit self-renewal capacity and can regrow the tumor of origin without interference of quiescent, origin, resistance to treatments or other traditional characteristics of stem cells, making the term 'cancer-initiating cells' (perhaps more accurately "cancer-originating or maintaining cells") a more appropriate term to use since it would minimize confusion by abolishing the exigency of these cells to meet criteria that define somatic stem cells (Zhou et al., 2009). Other authors may as well prefer to use the term ''stem-like'' cancer cells or ''tumor stem-like cells'' implying 'both a stochastic (clonal) and hierarchical (stem cell) model' involving the concepts of dormancy, DNA repair, high expression of multi drug resistant (MDR)-type membrane transporters and the defensive role of hypoxic niche environments determining the conspicuous resistance towards anti-cancer treatments and the large possibility of recidivism (Kondo, 2012).

2. Origin of CSCs:

Cancer has been thought to arise from either precursor or differentiated cells that have gained oncogenic mutations. Later on, after the discovery of stem cells in adult tissues, it has been proposed that tissue-specific stem cells (TSCs) could be a main target for such mutations that occur through a dysregulation of the normally tightly regulated processes of self-renewal

and differentiation since TSCs survive and continue to proliferate throughout life, making them more prone to accumulate mutations. Furthermore, 80% of cancers arise from epithelia that are in contact with the external environment and that contain a large variety of TSCs.

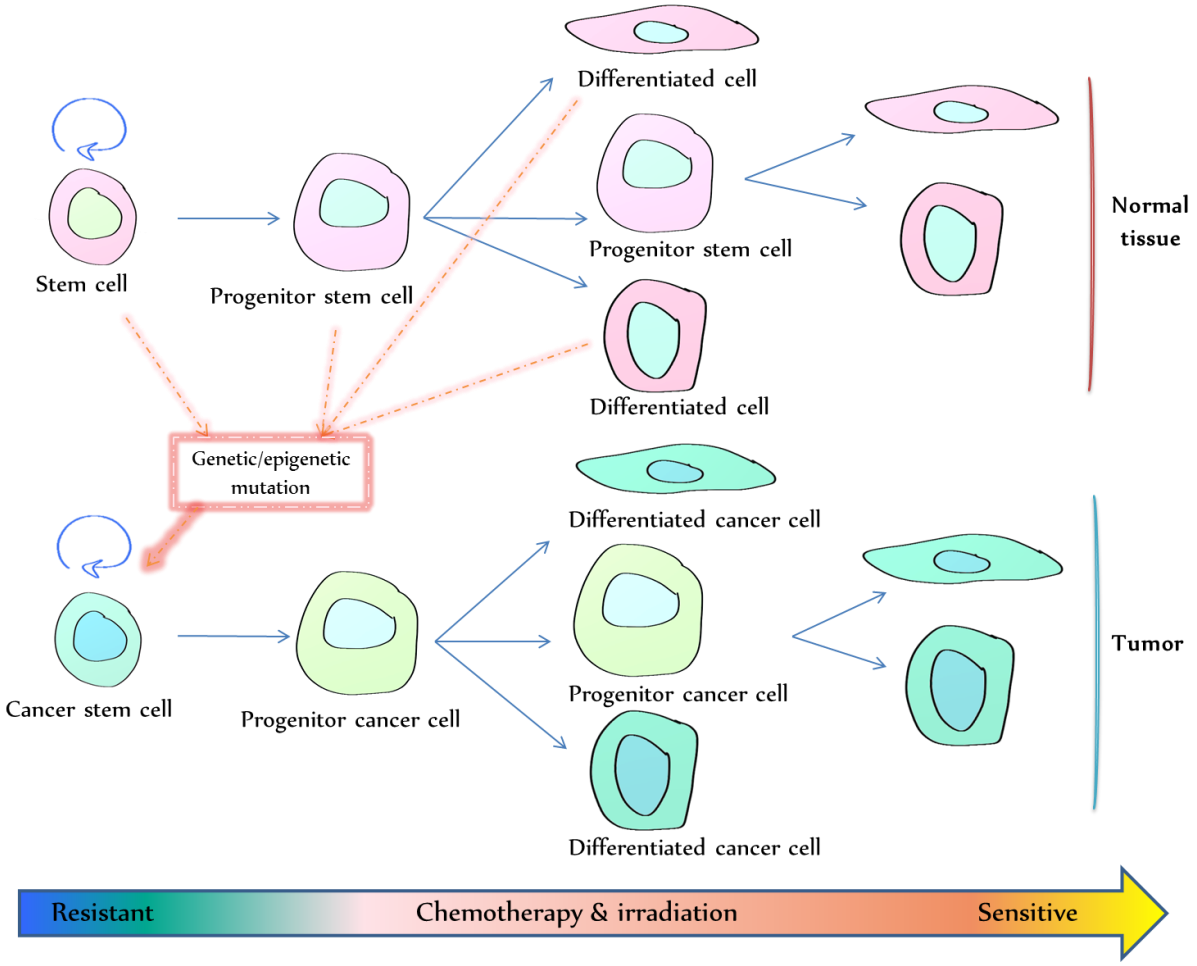


Figure 2. Schematic presentation of the hierarchy of cells in a normal tissue and in a tumor showing the origin of CSCs.
Both tissues consist of differentiated cells, precursor cells that have limited proliferative potency and a small number of stem cells that have self-renewal capability and multipotentiality. CSCs are thought to be generated from either TSCs, precursor cells and/or differentiated cells through genetic/epigenetic mutations. CSCs are more resistant to chemotherapy and irradiation compared with all other cells.

In addition, mutated stem cells and precursor cells have demonstrated that they develop into cancer *in vivo* (Passegué, Jamieson, Ailles, & Weissman, 2003), (Tannishtha Reya, Sean J Morrison, Michael F Clarke, & Irving L Weissman, 2001). Together, these findings suggest

that TSCs, precursor cells and/or differentiated cells can be the origin of malignant tumors (Figure 2).

3. Characteristics

a. Resistance to Chemotherapy

Several anti-cancer drugs attenuate cancer; yet there still remain some resistant cells that induce recurrence, proving the fact that such cells are not only resistant to drugs but are also capable of tumorigenicity (Szakács, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006). Among these drugs are: topoisomerase II, O6-methylguanine-DNA-methyltransferase, glutathione, metallothioneins, dihydrofolate reductase, and various ATP-binding cassette (ABC) transporters, such as the protein encoded by the multidrug resistant gene (MDR), the multidrug resistant protein (MRP), and the breast cancer resistant protein (BCRP1).

It is now recognized that an important mechanism of drug resistance is the expression of active drug efflux pumps in the membranes of cancer cells and CSCs (Kakarala & Wicha, 2008). Additionally, CSCs resistance to cell cycle chemotherapeutic agents may be due to the fact that CSCs are quiescent for extended periods of time. There is further evidence that the intrinsic expression of ABC proteins modulates the resistance of tumors to a wide variety of structurally different chemotherapeutic drugs through their overexpression (Scotto & Johnson, 2001).

Additionally, three transporters belonging to the ABC family, have been implicated as major contributors to MDR in cancer. Discovered over 30 years ago, P-glycoprotein (P-gp; MDR1; ABCB1), MRP-1 (or ABCC1) and, more recently, ABCG2 (alias the BCRP) also appear to function as clinically relevant drug efflux pumps proteins (Gottesman, Fojo, & Bates, 2002).

b. Resistance to Irradiation

Although irradiation is considered to be the most efficient therapy for cancer, a small population of cells still tends to survive leading to recidivism and recurrence, indicating that CSCs are radio-resistant. CSCs also repair the DNA damage caused by radiation more rapidly than non-CSCs due to higher basal levels of phosphorylated checkpoint kinases Chk1 and Chk2 that cause cell cycle arrest until lethal DNA damage can be repaired (Bao et al., 2006).

c. Invasion/Metastatic activity

The fact that human malignancies harbor a subpopulation of cancer stem cells that are responsible of tumor growth, local invasion and distant metastasis has been well established (Hanahan et.al, 2011). Although many researchers have successfully established the relation between the presence of CSCs in the primary tumor and increased metastasis incidence, untill now insufficient studies have shown the relation between CSCs in the primary tumor and their potential role as cell of origin of distant metastasis and whether the cell population responsible for tumor growth is the same one responsible for metastasis. Cell tracking studies in preclinical cancer models are needed to prove that a migrating cellular component of CSCs from within the primary tumor underlies the origin of distant metastasis (Collins, Berry, Hyde, Stower, & Maitland, 2005).

4. Identification

Several methods are commonly used to isolate CSCs from tumors and tumor cell lines using the common characteristics of TSCs, such as cell surface markers, side population (SP), aldehyde dehydrogenase activity (ALDH), and a floating sphere formation.

a. Cell surface Markers

The primary method of stem cells' isolation is Cell Sorting according to cell surface markers by either FACS (Fluorescence Activated Cell Sorting) or MACS (Magnetic-bead separation) (Bradbury et al., 2003).

b. Side population (SP)

Pumps found in the membrane of stem cells efficiently prevent influx of harmful chemicals. Additionally, it was shown that cancer cells, as well as many kinds of normal stem cells, express a number of ABC transporters that exclude the fluorescent dye Hoechst 33342 identifying a SP (Goodell, Brose, Paradis, Conner, & Mulligan, 1996). Nevertheless, some research has shown that TSCs exist in both SP and non-SP and that SP cells do not express stem cell markers (Mitsutake et al., 2007). A number of research groups have found that SP cells – but not non-SP cells – self-renew in culture and are resistant to anti-cancer drugs including Mitoxantrone, and form tumors when transplanted *in vivo* (Ponti et al., 2005). However, since some cancer cell lines do not contain any SP fraction and non-SP cells in many cancer cell lines tend to generate SP fraction during culture, further studies are needed to evaluate whether SP is a suitable method to isolate CSCs.

c. Aldehyde dehydrogenase activity (ALDH)

ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes into carboxylic acids and eventually blocks alkylating agents. Since ALDH increases in TSCs (Jones et al., 1995), using fluorescent substrates of this enzyme along with flow cytometer is used to identify and purify some types of TSCs, like hematopoietic stem cells (HSCs) and neural stem cells (NSCs). Extensive evidence revealed that many types of CSCs highly express ALDH making its purification from tumors and cancer cell lines available (Ginestier et al., 2007).

d. Floating sphere formation

Sphere-formation assay has been widely used to identify stem cells based on its capacity to evaluate self-renewal and differentiation at the single cell level in vitro. Overwhelming evidence has shown that CSCs as well as TSCs, can form floating aggregates called spheres when cultured in serum-free medium with proper mitogens, such as bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor) (Abou-Kheir, Hynes, Martin, Pierce, & Kelly, 2010; Haraguchi et al., 2006). Reynolds and Weiss were the first to use and optimize this method on neurospheres (Reynolds and Weiss, 1992). They enzymatically dissociated the subventricular zone of a human brain tissue to single cells and plated them in non-adherent conditions with serum-free medium in the presence of EGF. A small population of cells began to divide forming spheres of proliferating cells. The majority of cells within these neurospheres expressed nestin which is an intermediate filament present in neuroepithelial stem cells in the embryonic brain (Pastrana et. al, 2011).

Two-dimensional (2D) monolayer cell cultures do not resemble the environment present in 3D tumors, due to the loss of surrounding physiological extracellular matrix (ECM) and high serum concentrations. Yet, Matrigel represents a reconstituted, laminin-rich basement membrane, which supports processes such as cell polarity, cell-cell- and cell-matrix interaction, and re-expression of differentiation markers even in transformed lines. Therefore, Matrigel is used to provide cells with an environment that simulates the *in vivo* environment more. Such three-dimensional cultures constitute the ideal system to assess and manipulate quiescent stem cells *in vitro*. Matrigel addition to only the rim of the wells makes quantification and replenishment of media easier. Density of cells is a critical step to consider mainly when culturing cell lines not primary tissues, since CSCs' invasive characteristics induce MMPs activity that may break the Matrigel eventually. When propagation of spheres

is performed, most of the progenitor cells are expected to differentiate and lose their ability to form spheres, thus an increase in the pool of CSCs is obtained. Generally, the fifth generation of propagation is thought to give a genuine stem cell population (Mansini et. al, 2011).

5. Markers

a. CD molecules

Cluster of differentiation or cluster of designation molecules are a group of special cell surface molecules that provide targets for immunophenotyping of cells. CD molecules play a role in cell adhesion and can act as receptors or ligands, altering the behavior of cells by signal cascade initiation. CD molecules involved in stemness are:

- CD133 also known as PROM1

CD133 molecule is a transmembrane pentaspan protein (Miraglia et al., 1997), and it has been suggested as a surface marker of CSCs in a growing number of cancers, thus there is extensive evidence indicating that CD133 is a specific marker (partly or uniquely) in isolating CSCs in prostate cancer (CD44+/α2β1 ^{hi}/CD133+) (Kasper, 2008)

- CD44

CD44 is a multitasking protein that plays a role in cell adhesion, proliferation, motility, drug resistance, and cell survival (Marhaba & Zöller, 2004). It has also been implicated in wound healing, lymphocyte homing, cell migration, cancer cell growth and metastasis (Ishimoto et al., 2010). It is also a receptor for hyaluronic acid, osteopontin, collagens, and matrix metalloproteinases (MMPs), thus its interaction with hyaluronan (HA) play an essential role in cell signaling in cancer. (Zhou et al., 2009)

- CD24

CD24 is a small glycosylated cell surface molecule that is overexpressed in many human carcinomas. Its overexpression represents an indication of cells gaining invasive abilities (Pirruccello & LeBien, 1986).

- CD138

CSCs in multiple myeloma (MM) have been well defined as CD138-B cells having the capacity to renew and eventually differentiate into malignant CD138+ plasma cells (Matsui et al., 2004). CD138 or syndecan-1 has been identified as a key molecule maintaining the stability of prostate cancer tumor initiating cells (Chen et. al, 2004).

- Other CD molecules that play a role as CSC markers are CD147, CD15, CD166, CD326 among others (Zhong et. al, 2008).

b. ATP-binding cassette (ABC) transporters

ABC transporters enable cancer to escape the cytotoxic effects of chemotherapy. Thus, ABC drug transporters have been shown to protect CSCs from chemotherapeutic agents (Dean, Fojo, & Bates, 2005)

- ABCG2 is a transporter localized predominantly in the plasma membrane unlike other ABC half-transporters, requiring dimerization to become active,. It has been shown that its expression is a conserved feature of stem cells and CSCs (Su et al., 2010).
- ABCB5 is a novel molecular marker for a unique subset of CSCs among melanoma cells (Stuelten et al., 2010)

c. EPCAM

Is a homophilic, calcium independent adhesion molecule comprised of an extracellular domain with epidermal growth factors and thyroglobulin repeat-like domains, a single transmembrane domain and a short 26 amino acid intracellular domain. EPCAM is expressed in a variety of human epithelial tissues, cancers and stem cells (Herlyn, Herlyn, Steplewski, & Koprowski, 1979) and it is one of the markers that identifies tumor cells with high invasive capacities.

d. ALDH1

It has been demonstrated that the overexpression of aldehyde dehydrogenase 1 (ALDH1), which is the enzyme responsible for the oxidation of intracellular aldehydes, is considered to be a marker for normal stem cells and CSCs in MM and leukemia patients (Osta et al., 2004)

e. CXCR4

The human chemokine system includes more than 40 chemokines and 18 chemokine receptors. CXCR4 is one of the best studied chemokine receptors, it selectively binds the CXC chemokine stromal cell-derived factor 1 (SDF-1), also known as CXCL12 (Zlotnik & Yoshie, 2000). CXCR4 is expressed on normal stem cells of various organs and tissues. Interestingly, when CXCR4 is expressed in a variety of cancers, its expression in adjacent normal tissue is minimal or absent, which may explain why some tumor cells express CXCR4 and why many researchers suggest that malignant cells may be derived from CXCR4-expressing normal stem cells (Fredriksson, Lagerström, Lundin, & Schiöth, 2003)

f. SP cells

Althought SP cells identified by efflux of Hoechst dye are present in virtually all normal and malignant tissues, SP cells isolated from tumors have proven to be an attractive alternative strategy to study CSCs (Dou & Gu, 2010)

Other novel biomarkers include DCAMKL-1, Podocalyxin, Piwil2, Nestin and LRCs (Mishra, 2007).

6. Therapeutic modalities for targeting CSCs

Recently, focus of cancer research has shifted from targeting all cancerous cells that form the tumor to targeting a certain subpopulation of cells that have a capacity to self-renew, differentiate into a multilineage progeny, resist chemotherapeutic drugs and initiate neo-tumors. Interesting but debated results yielded from targeting CSCs through their surface markers (as CD133, CD34, and CD24) (Wu & Wu, 2009). Targeting several stem cell signaling pathways such as TKIs and Hh inhibitors appeared to have more promising results (Takebe, Harris, Warren, & Ivy, 2010). Yet, another approach was to target the niche that may have influenced cancer cells to acquire 'stemness' with an antiangiogenic drug that curtails the nutrient supply for cancer cells altering that microenvironment that has been providing cells with what is necessary to acquire stemness and thereby altering quiescency (Kchour et al., 2008). Moreover, a further intriguing aspect of CSCs is yet to be considered, which is: cell metabolism. Genuinely, the unique characteristics of CSCs must be associated with a unique metabolism that differ from that of proliferating cancer cells. This unique metabolism if targeted along with the peculiar genetic plasticity could form further significant targets for the identification and eradication of cancerous cells that may cause recidivism.

The CSC hypothesis has invigorated the research community to find novel approaches to cancer therapy. However, for many cancers, targeting a rare population of tumorigenic cells without considering the large bulk of proliferating cells may not change patient outcomes. A combined therapy that includes both cytoreductive agents together with targeting CSCs populations may provide an effective curative approach with an acceptable toxicity for the treatment of cancer.

III. Metastasis and EMT

Transition between epithelial and mesenchymal (EMT) states contributes to tumor progression and intratumoral heterogeneity (Figure 3). A diverse set of stimuli triggers EMT including growth factor signaling, tumor–stromal cell interactions, and hypoxia. EMT results in cancer cells that have gained CSC-like qualities endowed with a propensity to invade surrounding tissue and are resistant to certain therapeutic interventions. Some characteristics of the niche function to maintain tumor dormancy and render the niche relatively ineffective in order to support CSC proliferation. Then signaling pathways involved in the regulation of CSC function and niche–stem cell interactions can trigger EMT programs establishing and maintaining CSC-like characteristics.

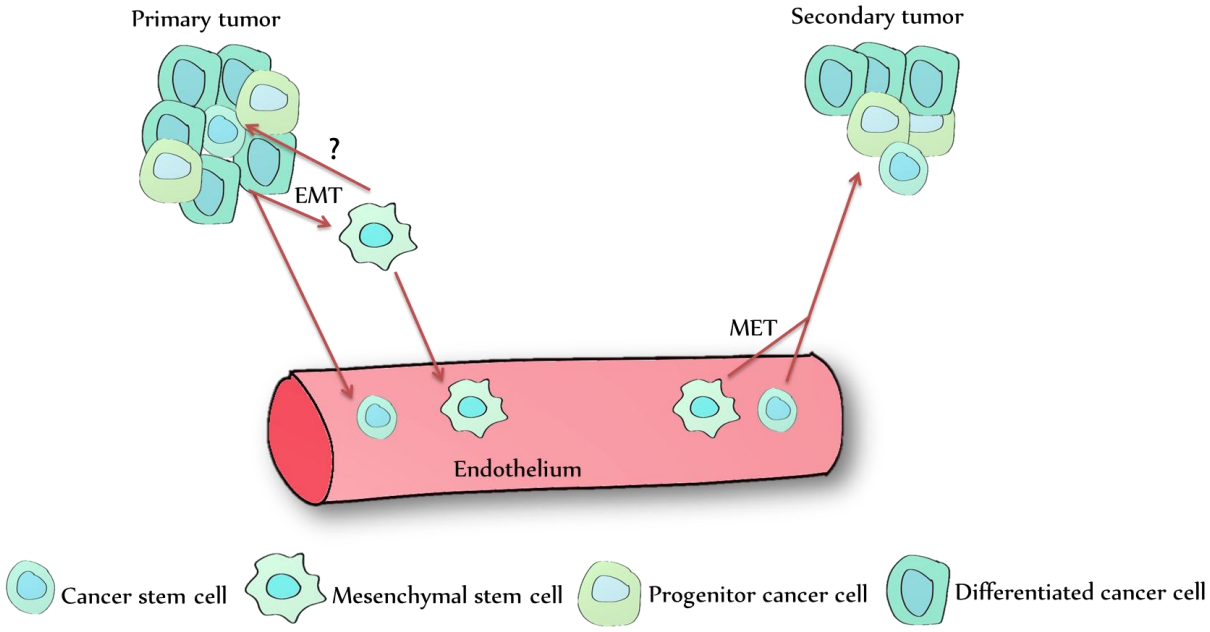


Figure 3. Squematic epithelial to mesenchymal transition and its role in metastasis.

Notch signaling is involved in the regulation of EMT occurring during both embryogenesis and tumorigenesis. It involves multiple receptors, ligands, and downstream mediators (Teng, Zeisberg, & Kalluri, 2007). The outcome of its activation is cell-type specific and can be either oncogenic or tumor suppressive (Pui & Evans, 2006). Due to the parallel expression of both stem cell markers and EMT factors in selected tumor cells at the invasive front, genes that regulate EMT, such as TWIST, SNAI1, and ZEB1 can be misregulated (Mani et al., 2008). ZEB1 links EMT activation, stemness maintenance, and migrating CSCs by suppressing stemness inhibiting micro RNA-200 that reciprocally controls ZEB1 in a feedback loop (Wellner et al., 2009).

Less EMT allows less dissemination, and less expression of stemness maintaining factors results in less tumor- and metastasis-initiating capacity (Yi, Poy, Stoffel, & Fuchs, 2008).

B. Marine Extracts as potential therapeutic compounds

Natural products extracted from terrestrial plants and microbes have been a conventional source of bioactive products for a long period of time, and interest in marine natural products came later due to the need of technological refinements; primarily scuba diving, to collect the source organisms (Bergmann & Feeney, 1951). Lately, focus of natural product researchers shifted to the marine environment due to its rich biodiversity of plants, animals and microorganisms that having adapted to that unique climate, gained the capability to produce a wide variety of primary and secondary metabolites that have shown significant biological activities such as antitumor and anti-inflammatory activities, analgesia, immunomodulation, allergy and antiviral assays (Molinski, Dalisay, Lievens, & Saludes, 2008). Developing drugs from marine sources hold many difficulties since manufacturing quantities of rare compounds ensuring a sustainable supply is not guaranteed. Nevertheless, interest in the remarkable properties of marine natural products remained high enough and inspired innovative solutions, ranging from aquaculture of marine invertebrates to semi-synthesis (Tressler & Lemon, 1951).

I. Marine organisms and their bioactive compounds

According to Blunt and Munro, marine organisms are classified as following:

a) Cyanobacteria (blue-green algae): 7500 species
b) Chlorophyta (green algae): 1000 species
c) Rodophyta (red algae): 4000 species
d) Phaeophyta (brown algae): 1500 species
e) Bacillariophyta, Chrysophyta, Haptophyta: 50000 species
f) Dinoflagellata: 200 species
g) Porifera (sponges): 5000 species
h) Cnidaria (medusae, sea anemones, hydroids, corals): 10000 species

i) Platyhelminthes (flukes, tapeworms, flatworms): 18000 species
j) Annelida (trueworms): 10000 species
k) Bryozoa (moss animals): 5700 species
l) Mollusca (squid, octopus, slugs, mussels, snails, clams, sea hares): 90000 species
m) Echinodermata: 7000 species
n) Crustacea: 60000 species
o) Hemichordata: 100 species
p) Protochordata (tunicates): 3000 species
q) Pisces (fish): 15000 species

[Total: over 230000 species] (Munro et al., 1999)

Some marine bioactive compounds possess some extreme biotoxicity e.g. maitotoxin, brevetoxin B, tetrodotoxin preventing their therapeutic application (Yasumoto & Murata, 1993). Yet some others are used as valuable tools in cell and molecular biology and as drugs (Table 1).

II. Approved drugs derived from marine sources

Interest in the concept of drugs from the sea started in the late-1950s. Beginning in 1951, Werner Bergmann published three reports of unusual arabinoand ribo-pentosyl nucleosides obtained from marine sponges collected in Florida, USA. The compounds eventually led to the development of the chemical derivatives ara-A (vidarabine) and ara-C (cytarabine), two nucleosides with significant anticancer properties that have been in clinical use for decades (Whitley et al., 1977).

The first drug from the sea, ziconotide (ω-conotoxin MVIIA) is a peptide extracted from a tropical marine cone snail, and approved in the United States in 2004 under the trade name Prialt to treat chronic pain in spinal cord injury (Miljanich, 2004; SAMII, BADIE, FU, LUTHER, & HOVDA, 1999) . The second drug from the sea is the antitumor compound trabectedin (yondelis/ecteinascidin-743/ET-743) extracted from a tropical sea-squirt and was

approved by the European Union in 2007 for the treatment of soft-tissue sarcoma (Grosso et al., 2007). Several other candidate compounds and marine natural products are being evaluated in Phase I–III clinical trials for the treatment of various cancers. Examples (Molinski et al., 2008) of potential therapeutic compounds derived from marine sources are classified in Table 1. These drugs are still in clinical trials, except for the two drugs mentioned before, that are already in the market: Yondelis® and Prialt®.

Source organism	Origin	Bioactive Compound	Chemical classification	Therapeutic class
Tunicate	Mediterranean	Aplidine (Aplidin®)	Depsipeptide	Cancer
Bryozoan	Gulf of California	Bryostatin 1	Macrocyclic lactone	Cancer
Cyanobacteria	Caribean	Curacin A	Thiazoline lipid	Cancer
Sponge	Caribean	Cytarabin	Pyrimidine nucleoside	Cancer
Tunicate	Caribean	Didemnin B	Cyclodepsipeptide	Cancer
Sponge	Caribean	Discodermolide	Polyketide	Cancer
Sea Hare	Indian ocean	Dolastatin 10	Peptide	Cancer
Tunicate	Caribean	Ecteinascidin-743 Trabectedin (Yondelis®)	Alkaloid	Cancer
Sponge	Pacific	Girroline	Alkaloid	Cancer
Sponge	Japan	Halichondrin B	Macrolide	Cancer
Molluscs	Pacific	Jorumycin (Zalypsis®)	Alcaloid	Cancer
Gastropod	Hawaii	Kahalaide F	Depsipeptide	Cancer
Sponge	Thailand	Mycaperoxide	Norsesterterpene	Cancer
Actinomycetes	Pacific	Salinosporamide A	γ-Lactam-β-lactone	Cancer
Shark	Atlantic, Pacific	Squalamine	Aminosterol	Cancer
Tunicate	Palau	Cyclodidemniserinol trisulfate	Serinolipid	HIV

Tunicate	Australia	Lamellarin α20 sulfate	Alkaloid	HIV
Sponge	Australia	Dithiocyanates	Dithiocyanate	Nematode infection
Sponge	New Guinea	Contignasterol	Sterol	Asthma
Gastropod	Pacific	Conotoxins, Ziconotide (Prialt®)	Peptide	Pain

Table 1. Approved drugs and potential therapeutic compounds derived from marine sources

C. Sea Cucumber and Cancer

I. Sea Cucumber (SC)

Sea cucumbers belong to the phylum of echinoderms and the class of Holothuroidea, named for their resemblance to the fruit cucumber, they are marine animals with a leathery skin and an elongated body containing a single, branched gonad. Found on the sea floor worldwide with about 1,250 species, the greatest number being in the Asia Pacific region; many are gathered for human consumption and some species are cultivated in aquaculture systems. Sea cucumbers serve a beneficial purpose in the marine ecosystem as they help recycle nutrients, breaking down detritus and other organic matter after which bacteria can continue the degradation process (Conand & Bryne, 1993). Like all echinoderms, sea cucumbers have an endoskeleton located below the skin, calcified structures that are usually reduced to isolated microscopic ossicles (or sclerietes) joined by connective tissue. (Figure 4) (Hamel, Conand, Pawson, & Mercier, 2001).

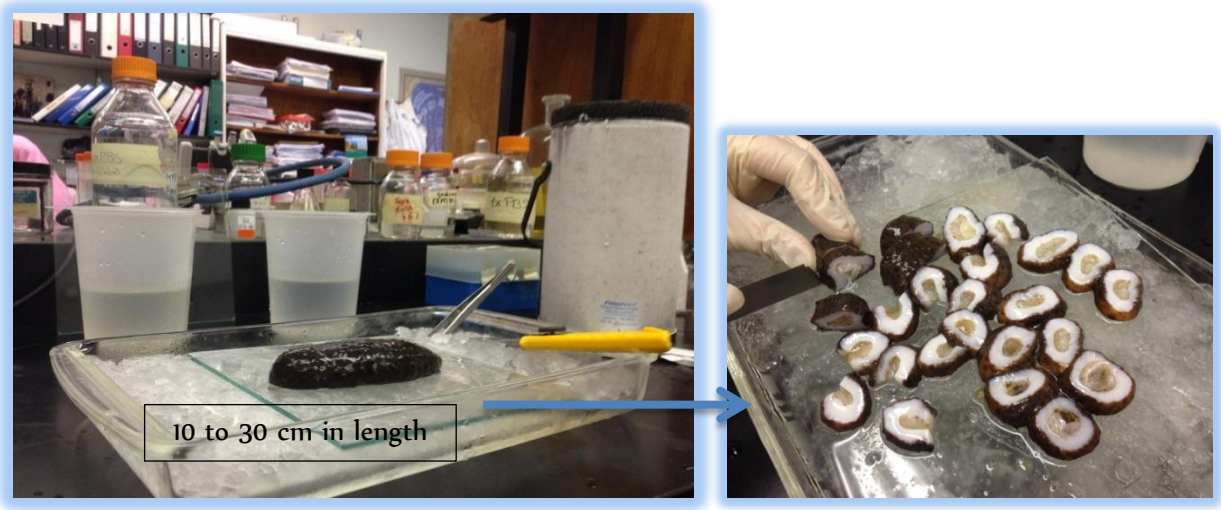


Figure 4. Sea Cucumber collected from the Lebanese coast of the Mediterranean Sea

SCs have long been used for food and folk medicine in the communities of Asia and Middle East, with an impressive profile of valuable nutrients such as Vitamin A, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin), and minerals, especially calcium, magnesium, iron and zinc, a number of unique biological and pharmacological activities including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor and wound healing have been ascribed to various species of sea cucumbers. (Vieira, Mulloy, & Mourao, 1991)

II. Sea Cucumber extract (SCE)

Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives especially triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids. Testing the constituents of sea cucumber extracts of different species

were performed in previous studies, and in distant places like Chile (Sottorff et al., 2013), China (Han, Yi, Liu, Wang, & Pan, 2008; Zhang, Yi, & Tang, 2006) and Italy (Silchenko et al., 2005), all showing that ethanolic extracts of SCs are rich in novel saponins and triterpene glycosides. Some others have shown the presence of collagen proteins (Popov et al., 2011) , GAGs (Zhao et al., 2013), 5-hydroxytryptamine (5-HT) and hypoxanthine (Gregson, Marwood, & Quinn, 1981) in extracts of sea cucumbers. Only two studies have been performed on *Holothuria Polii* species, one by Ismael (Ismael et. al, 2008) in Tunisia and the other by Omran (Omran et. al, 2012) in Egypt; both studies showed that some constituents of the *Holothuria Polii* extracts are triterpene glycosides.

III. Sea Cucumber extract's therapeutic activity

Several investigations on marine organisms from different regions resulted in the identification of several anti-cancer compounds. Sea cucumber extracts of various species showed antifungal, antibacterial, antiparasitic, anticancer and antimetastatic activity *in vivo* and *in vitro*.

Holothuria edulis showed anticancer activity against HL-60 leukemia cells (WAJP, Jeon, Ramasamy, Wahid, & Vairappan, 2013). Extracts of *Holothuria scabra* showed to have anticancer compounds (Ganguly & Roy, 2005). Fractionation of the butanol extract *of Holothuria fuscocinerea Jaege* yielded new triterpene glycosides that showed cytotoxicity upon several tumor cell lines (Zhang et al., 2006). Hypotensive activity was attributed to the presence of 5-hydroxytryptamine and hypoxanthine in the *holothurian Pentacter crassa* (Gregson et al., 1981). Anticoagulant and antitumor effect was addressed due to the presence of Collagen. Ismail et al (2008) demonstrated that the methanolic and aqueous crude extracts along with the pure fractions isolated from *Holothuria polii* cultivated from the Mediterranean

sea coast of Tunisia, caused dose-dependent antifungal activity against some molds and yeasts (Ismail et al., 2008). Omran et al. showed that the ethanolic sea cucumber extract isolated and collected from Mediterranean Sea coast of Egypt has a broad spectrum antifungal activity against Aspergillus niger, Scloretium sp., Candida albicans, Aspergillus flavus and Malessezia furfur. It also has an antibacterial, antiparasitic, antitumor and antimetastatic effects (Omran & Khedr, 2012) (Mona, Omran, Mansoor, & El-Fakharany, 2012).

D. Aim of the study

Overwhelming evidence has suggested that CSCs are at the root of cancer recidivism and progression. The aim of cancer therapies has usually been to terminate all cancerous cells since it was thought that the majority of tumor cells have the capacity to propagate and lead to the growth of the tumor according to the clonal evolution model (Campbell et. al, 2007). This approach has been challenged by the theory of cancer stem cells, that states that only a subpopulation of cells in the tumor has the capacity to self-renew and give rise to a malignant tumor and that targeting these cancer stem cells form a promising approach not only to improve cancer patient's survival but also to cure cancer from its root, preventing its reoccurrence.

Previous work in our laboratory has demonstrated the ability to enrich for a population of prostate cancer stem/progenitor cells by growing cells in 3D spheres forming culture.
In this study, crude extracts of Sea Cucumbers (*Holothuria Polii*) were obtained from the Lebanese coast of the Mediterranean Sea and their effect was investigated on prostate cancer cells that were chosen as a model to study the various effects of this extract. It is believed that this extract could carry a therapeutic role in treating cancer in general, and especially in targeting CSCs.

The aim of this study is to first investigate the therapeutic effect of the crude sea cucumber extract on the proliferation and viability of human prostate cancer cell lines grown in a conventional 2D monolayer model. Furthermore, the effect of the crude SCE on the metastatic ability of prostate cancer cells is assessed, and a focus on an advanced assay (sphere-formation assay) is performed to investigate the effect of SCE in targeting an enriched population of prostate CSCs. Additionally, the therapeutic effect of SCE's aqueous fractioned

phase is assessed, investigating its effect on the proliferation and on targeting CSCs of prostate cancer cells.

MATERIALS AND METHODS

1. Cell lines

DU145 is a human prostate adenocarcinoma cell line derived from brain metastasis and 22RV1 is a non-metastatic cell line that has been derived from a human prostatic carcinoma xenograft, CWR22R that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft.

DU145 and 22RV1 cells were maintained in RPMI-1640 AQ medium (Sigma) and supplemented with 10% of heat-inactivated fetal bovine serum FBS (Sigma), 1% of penicillin-streptomycin P/S (Sigma) and 1% of non-essential amino acids (Sigma), and incubated at 37˚C in a humidified incubator (95% air, 5% CO_2).

2. Sea Cucumber Extracts (SCEs)

a. Sample collection and preparation:

Sea cucumbers were freshly collected from the Lebanese shore and kept in a plastic ventilated container filled with sea water. Sea cucumbers were rinsed with distilled water and cleaned from all visible surface debris. Then they were dissected into 2x2x2 cm pieces, rinsed with distilled water and snap frozen in liquid nitrogen. Afterwards, they were freeze-dried, pulverized (using A11 basic Analytical mill) and stored in 50ml conical tubes at -80°C for further extraction (Figure5).

b. Extraction of SCE:

Eleven grams of pooled powdered material were reconstituted with 80% ethanol, homogenized with hand-held laboratory tissue tearor for 3 min on ice, left at room temperature for 10 min and then centrifuged at 700g for 10 min. The supernatant was

collected on ice and filtered through 100µm nylon mesh. The collected supernatant of sea cucumber samples were immediately frozen in liquid nitrogen, lyophilized and re-constituted with PBS (10% DMSO). The insoluble solid pellet was kept at -20 °C for further extraction (Figure 5).

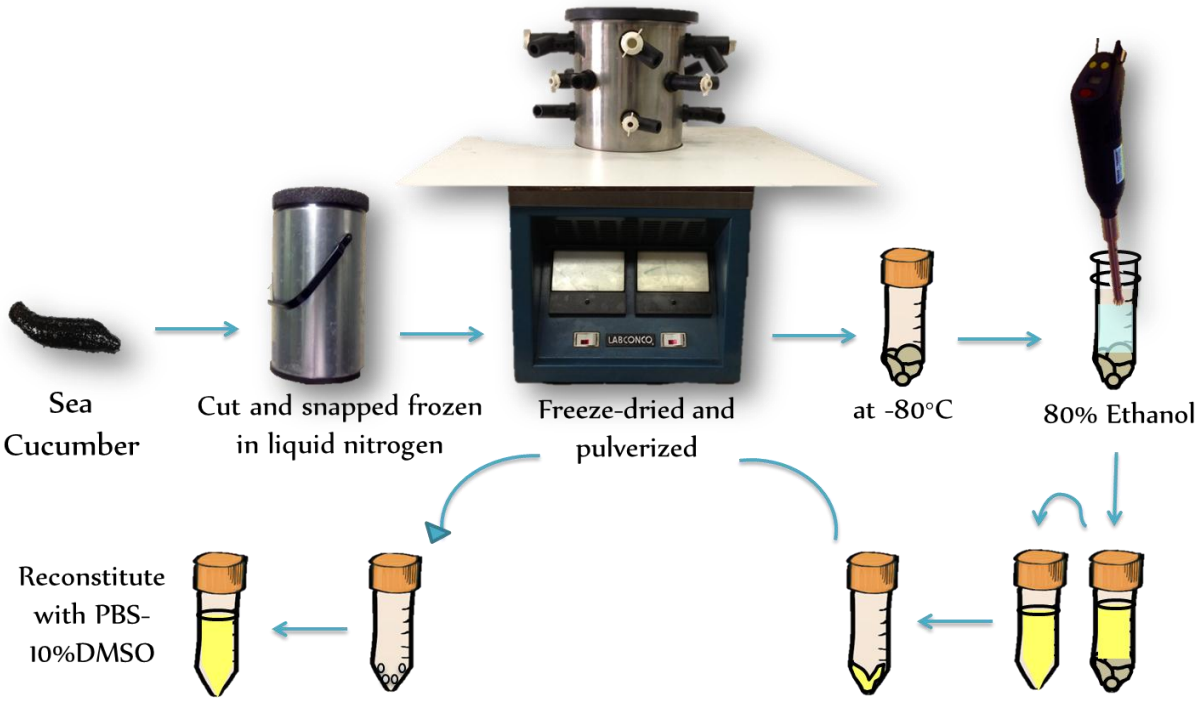


Figure 5. Scheme of the extraction of sea cucumber extracts (SCEs).

c. Analysis of samples:

- Protein/DNA content:

Extracted samples were assessed for protein and DNA content.

Protein determination was performed using both DC Protein Assay and Nanodrop.

Trizol was used to separate the crude SCE into aqueous and organic phases followed by centrifugation at 2500g for 5 minutes at 4Cº. The upper aqueous phase was removed and the lower phase containing the DNA was precipitated with ethanol and sodium acetate.

The sample was mixed by inversion, incubated at room temperature for 2-3 minutes and then centrifuged at 2500g for 30 minutes at 4C°. The supernatant was decanted; pellet was washed with 70% ethanol, and centrifuged 2500g for 5 minutes. DNA pellet was suspended in 1x TE (Tris-EDTA), quantified and stored at -20C°.

d. Fractionation of the crude SCE

Fractionation was performed in favor of identifying the possible target compound(s) responsible for the therapeutic effect. Liquid-liquid extraction using 4 different solvents with increasing polarity was performed as follows: Petroleum ether, chloroform, ethyl-acetate and butanol. Each organic solvent was evaporated and each of the extracted material was dissolved in PBS/DMSO and its bio-activity was assessed on different types of cancer cells to identify the active fraction, which will be later chromatographed on a C18 silica column.

3. Cell Proliferation Assays

a. Trypan-blue Exclusion Assay

Cell proliferation of prostate cancer cell lines, DU145 and 22RV1, upon treatment with SCE, was assessed using trypan blue exclusion assay where viable cells were visually counted using a hematocytometer. Viable cells can be counted repeatedly over a time course, and thus, the rate of cell proliferation can be determined. After seeding prostate cancer cells (DU145: 10000 cell/cm^2, 22RV1: 17500 cell/cm^2) in 24 well plates and treating them with several concentrations of SCE, cells were photographed and harvested in 24, 48 and 72 hour time points. Cell pellets were collected and re-suspended with media, then 50 µl of cell suspension was mixed with 50 µl of trypan blue (1.4 mM Trypan blue, 154 mM NaCl, 500 mM EDTA (ethylenediaminetetra-acid). Cells were counted on the four corner chambers of a hematocytometer. Cell average was multiplied by 2 x 10^4 to find the number of the cells/ml.

Each condition was counted in triplicates, four independent experiments were performed and the average of all experiments was shown as a graph.

b. MTT assay

Antiproliferative and cytotoxic effects of SCE were measured in vitro by using MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay. DU145 and 22RV1 cells were plated in 96-well culture plates (DU145: 10000 cell/cm^2, 22RV1: 17500 cell/cm^2). Cells were incubated overnight then treated in triplicates with various extract concentrations. Three time points; 24, 48 and 72 hrs; 20 µl of 5 mg/ml (in 1 x phosphate buffered saline (PBS) MTT reagent was added to each well and incubated at 37°C for 4 hours. In this step, viable cells had the ability to convert a soluble salt into insoluble precipitate. One hundred microliter of solubilization solution was then added into each well to dissolve the formazan crystals. Finally, the reduced MTT optical density (OD) was measured at a wavelength of 595 nm using an ELISA reader (Multiskan Ex). The percentage cell viability was presented as OD ratio between the treated and untreated cells at indicated concentrations.

c. RTCA

For real-time cell proliferation assays, 100 µl of media cell culture media was added to each well of a 16-well E-Plate to obtain background readings. Seven to ten thousand cells/well in 150 µl of media were then seeded into the E-Plate. The E-Plates containing cells were then incubated for 30 minutes at room temperature and placed on the RTCA station located in a cell culture incubator. The proliferation of the cells was monitored using xCELLigence system at 45-minute interval for 72h.

4. Cell Cycle Analysis

Cell cycle analysis was performed by flow cytometry after cells were permeabilized and stained with propidium iodide (PI) (Molecular Probes ®). 22RV1 and DU145 cells were seeded in duplicates in 6-well plates (DU145: 10000 cell/cm^2, 22RV1: 17500 cell/cm^2) and incubated for 48h prior to SCE treatment. Cells were then harvested at 48h post-treatment, washed with PBS, centrifuged at 450g for 5 min, resuspended in 1ml of cold PBS, fixed with 4ml of cold 100% ethanol and then stored at -20C° for further staining and analysis. Fixed cells were then treated for 1h with 200μg/ml DNase-free RNase A, stained with 1mg/ml PI and incubated for 10min in the dark in a flow tube (BD Flacon). Fluorescence of PI, a measure of DNA content in a cell population, was performed using Flow Cytometry (FACScan, Becton Dickinson). A total of 10,000 events were acquired in order to assess the proportions of cells in different stages of the cell cycle. Analysis of cell cycle distribution was performed using FlowJo Software.

5. Detection of Cell Death (Apoptosis)

Hoechst staining

Hoechst staining was performed to assess the integrity of the nucleus when cells are treated with SCE: this could hint to whether cells are undergoing apoptosis when proliferation is inhibited. Cells were seeded on glass cover slips in 24-well plates. Treatment was performed after 24h. After 48h of treatment, the media was removed; cells were washed with PBS and incubated with 4% formaldehyde at 4°C for further staining. Cells were then washed with PBS and incubated with a concentration of 1 μg/ml of Hoechst-33342 (Molecular Probes ®) for 10 minutes in the dark. Cells were then washed with PBS 3 times for 10 minutes each and the glass cover slip was fixed on a microscope slide covered with a drop of a Prolong® Gold

antifade reagent (Molecular Probes®, Invitrogen) to strongly attach them and preserve the fluorescent signal for a long duration of time. Slides were then visualized under a confocal microscope (Zeiss LSM 710).

6. RTCA cell migration and invasion assays

Invasion and migration assays were measured using xCELLigence RTCA DP instrument (RTC; xCELLigenec Roche Penzberg, Germany). DU145 cells were seeded on a cell invasion/migration plate (CIM plate 16) that uses micro-electronic sensors on the underside of an 8 µm microporous polyethylene terephthalate (PET) membrane of a Boyden-like upper chamber (Figure 6).

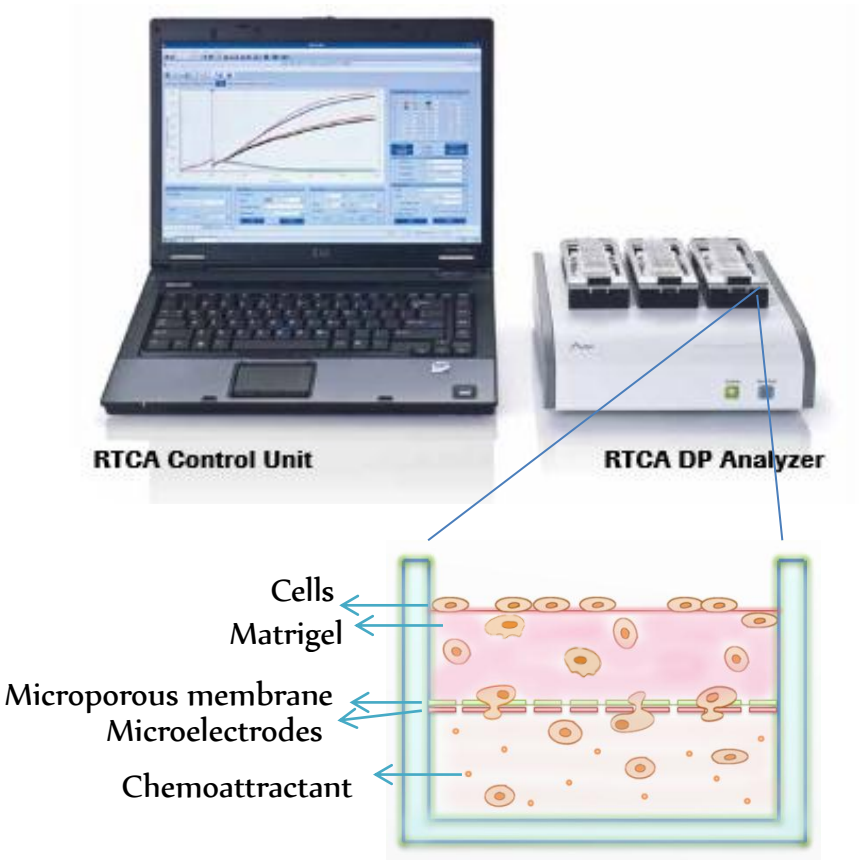


Figure 6. Schematic representation of RTCA.

As cells migrate or invade (when coated) from the upper chamber through the membrane into the bottom chamber in response to a chemo-attractant, they interact and adhere to the electronic sensors, thus causing an increase in electrical impedance. Changes in the impedance correlate with numbers of migrated or invaded cells on the underside of the membrane, therefore allowing automatic and continuous measurement of migration/invasion. For invasion assays, the upper surface of the membrane was pre-coated with 30 µl of growth factor–reduced Matrigel (BD Biosciences, Bedford, MA) diluted in serum-free medium at a ratio of 1:20, incubated at 37°C/5% CO_2 for 4 h, then washed with PBS. For all migration and invasion assays, 160 µl of RPMI medium with 10% FBS was added to the lower chamber of each well (used as a chemo-attractant) and 30 µl of the same medium but without FBS to the upper chamber. DU145 cells (untreated and treated) were seeded at the seeding density of 20000 cells/well into the upper chambers contained in 120µl serum-free medium. Migration and invasion was monitored by taking cell impedance readings every 15 minutes for a minimum of 18 h.

7. Wound Healing

Cells were seeded in 6-well tissue culture plates with the density of 50000 cells/cm^2. When cells are 100% confluent, half the wells were treated with Mitomycin for an hour and a half to prevent the cells from dividing and therefore assess that the wound is not being healed due to inhibition of cell division but through inhibition of cell migration. Gently and slowly a straight scratch is made in the monolayer with a sterile pipette tip across the center of the well. The resulting gap distance therefore equals to the outer diameter of the end of the tip. After scratching, a gentle wash with medium is done to remove the detached cells. The well is

replenished with fresh medium and treatment. Photos were taken at the time of treatment and after 8, 12, 24 and 30 hours. The gap distance is evaluated using Zen software (Zeiss).

8. Transcriptional expression of genes involved in cancer invasion and metastasis upon treatment with SCE using Real-time Polymerase Chain Reaction (rt-PCR)

a. RNA extraction

DU145 cells were seeded in 6-well plates (10000 cell/cm^2) and treated after 48h with SCE. At 48h time point, the media was removed and cells were washed with PBS and stored at -80°C. RNA extraction was performed using the RNAspin Mini kit (GE Healthcare) through a process containing a step of treatment with Deoxyribonuclease I (Fermentas) to remove traces of genomic DNA. Finally, RNA content was quantified and stored at -20°C for subsequent cDNA synthesis.

b. cDNA synthesis and Real-time PCR

Following RNA extraction, cDNA was synthesized from 1 µg of total cellular RNA using the RevertAit™ First Strand cDNA Synthesis Kit (Fermentas) and then it was quantified. Real-time PCR was then performed using the iQ™ SYBR® Green Supermix (Bio-Rad) in a CFX96 system (Bio-Rad). Amplification of Real-time PCR was done using primers (Tib molbiol) listed in table 2. This reaction was performed as follows: precycle of 95°C for 3 minutes for an initial denaturation followed by 40 cycles each consisting of an initial 95°C step for 10 seconds, then x°C for 30 seconds (x being the appropriate annealing temperature for each gene, see table 2), and 72°C for 30 seconds and finally a step of extension after the cycles consisting of 72°C for 5 minutes. The experiment was prepared in duplicates and the results were analyzed to obtain the fold change: each gene has a threshold cycle (Ct) which

was normalized to GAPDH, the housekeeping gene, and the control was used as a reference for comparative analysis.

Gene of interest	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
E-cadherin	F:CAGAAAGTTTTCCACCAAAG R:AAATGTGAGCAATTCTGCTT	58	106
Snail	F: CTTCCAGCAGCCCTACGAC R: CGGTGGGGTTGAGGATCT	58	71
	F: AGCTACGCCTTCTCGGTCT R: CCTTCTCTGGAAACAATGACATC	58	124
VEGF	F:AGGCCCACAGGGATTTTCTT R:ATCAAACCTCACCAAGGCCA	55	111
GAPDH	F:TGGTGCTCAGTGTAGCCCAG R:GGACCTGACCTGCCGTCTAG	58	111

Table 2. Human primer sequences for real time PCR
Primers of genes used in the Real-time PCR experiment with the convenient annealing temperature and amplicon size (retrieved from Primer-Blast®)

9. Translational expression of proteins involved in cancer stem cells maintenance, invasion and metastasis upon treatment with SCE using Western blotting analysis

a. Protein extraction

DU145 cells were treated with SCE, washed with PBS after 48h and stored at -80°C. Protein extraction was performed by adding lysis buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) and 10% glycerol) containing protease inhibitor and phosphatase inhibitor. This was followed by a shearing step using a needle and proteins were quantified using the DC Protein Assay (Bio-Rad) as per manufacturer's recommendations employing bovine serum albumin (BSA) as a standard.

b. Western blotting

Equal amounts of proteins (60µg) suspended in sample buffer which constitutes of lysis buffer and traces of bromophenol blue, were boiled for 10 minutes after addition of β-mercaptoethanol. Samples were loaded into a stacking gel over a 8, 10 or 12% SDS-PAGE

gel depending on the molecular weight of the protein of interest. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) for 2 hours at 4°C after its activation with methanol. The membrane was then stained with Ponceau to verify the quality of the transfer and subsequently destained with distilled water. After that, it was blocked in 5% fat-free milk prepared in 0.1% Tween-20 PBS for 1h at room temperature. The following primary antibodies were used: anti-β Actin or GAPDH primary antibodies to verify equal loading, Vimentin, E-Cadherin, BCL2, PARP, CXCR4, CD44 and EZH2 primary antibody (1:1000, Santa Cruz Biotechnology). The primary antibodies were recognized by a secondary anti-rabbit antibody (1:3000, Santa Cruz Biotechnology). Both primary and secondary antibodies were diluted in the blocking solution. Finally, the membranes were washed and visualized using chemiluminescence by Luminol reagent (ImmunoCruz™ Santa Cruz). Quantification of the resulting bands was achieved by densitometry using the Image J software (version 1.47 t) and the expression of proteins was normalized to a housekeeping gene, and results were plotted accordingly.

c. Gelatin zymography

To assess the activity of matrix metalloproteinases (MMPs) after SCE treatment of DU145 cells, gelatin zymography was performed. 100 µg of proteins extracted from the cells were loaded into a stacking gel with 10% polyacrylamide resolving gel containing gelatin which separated the proteins. 2.5% FBS was used as a positive control to detect MMP-2 and MMP-9 enzymatic activities at different molecular weights. The gel was then washed twice with a washing buffer containing Triton X-100 for 30 min and incubated in a substrate buffer containing Tris-HCl, $CaCl_2$ and sodium azide at 37°C overnight. Gels were then stained with 0.5% Coomassie Brilliant Blue R-250 stain for 1h followed by a destaining step using a destaining buffer (30% ethanol, 10% acetic acid and 60% water). Finally the gels were

visualized by a Bio-imaging system (Visidoc-it): enzymatic activity is shown by white bands on a blue background.

d. Immunofluoroscense:

Cells fixed with 4% formaldehyde were permeabilized with 0.5% Triton X-100 for 4 minutes and blocked with 1X PBS containing 2% BSA for 1 hour at room temperature. Cells were probed with one of the following primary: PARP, Bcl2, CD44, CXCR4, Vimentin, E-Cadherin, γH2AX at a dilution of 1:300 overnight at 4ºC in 1X PBS containing 2% BSA, washed with 1X PBS-Tween, and incubated with a fluorophore-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody at a dilution of 1:200 for 1hr at room temperature. Nuclei were stained with DAPI. Images were taken using LSM 7-10 Confocal Laser microscope at 40X magnification.

10. Sphere forming assay

After counting the single cell suspension of prostate cancer cell lines, 1,000 cells are suspended in cold MatrigelTM/serum free RPMI-1640 (1:1) in a total volume of 50 µl, in duplicate. Cells were plated uniformly in a circular manner around the bottom rim of a well in a 24-well plate and allowed to solidify in the incubator at 37°C for an hour, before 0.5 ml of RPMI-1640 AQ 2% FBS media (with or without treatment) was added gently in the middle of each well. Spheres were replenished with warm media as in the original plating (with or without treatment) every two to three days. Spheres were counted and photographed after 15 days. To passage spheres, the medium was aspirated off and matrigel was digested with 0.5 ml of Dispase solution (Invitrogen, Carlsbad, CA, 1 mg/ml, dissolved in RPMI-1640 medium) for 1 hour at 37°C. Spheres were collected, incubated in 1 ml warm Trypsin/0.05% EDTA at 37°C for 10 minutes, then used media with FBS to deactivate the trypsin and passed through a

27 gauge syringe 5 times. Cells were counted by hematocytometer and replated as before (Figure 7).

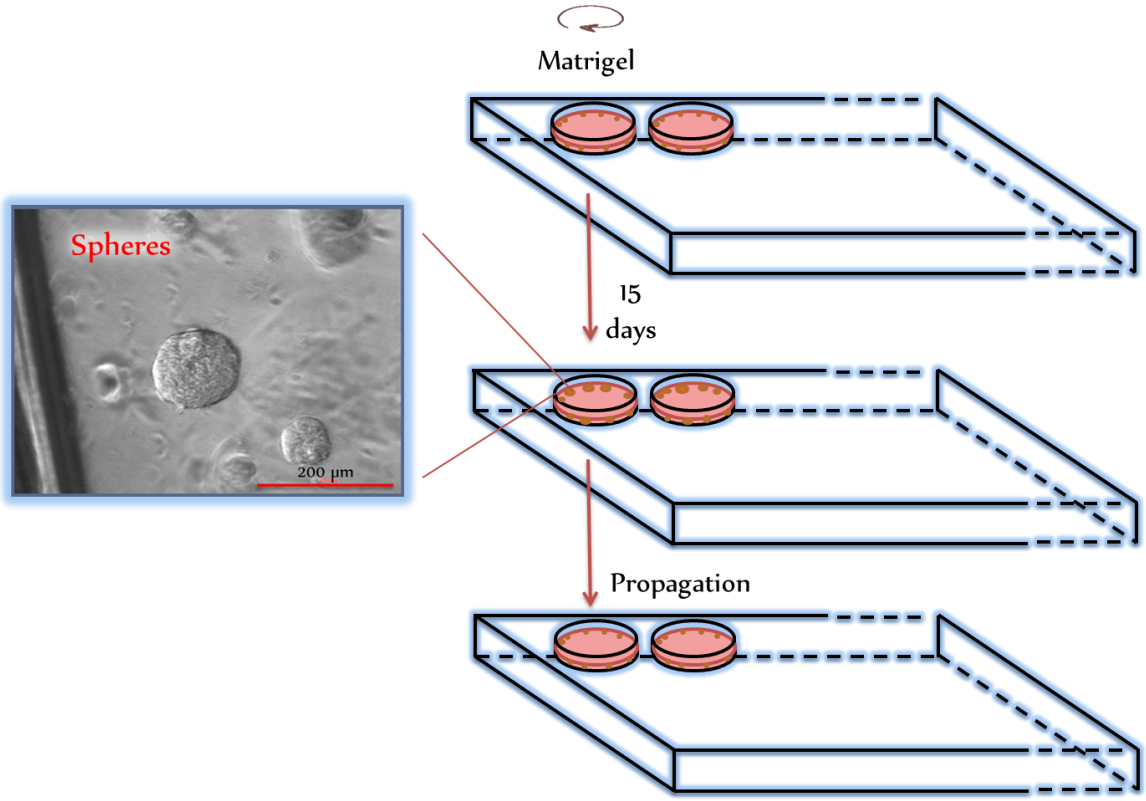


Figure 7. Schematic representation of the spheres forming assay

Statistical analysis

The results are expressed as mean values ± SD and the corresponding error bars are displayed in the graphical plots. Statistical comparisons between groups were carried out by an unpaired Student's t-test. P-value of < 0.05 was considered indicative of a statistically significant difference.

RESULTS

1. Crude SCE analysis

The first step in this study was to investigate SCE proteins concentration using DC Protein Assay. The trendlines obtained from plotting different dilutions of BSA (Bovine Serum Albumin) and SCE were parallel, indicating that the increase in BSA concentration is parallel to the increase in SCE's concentration obtained; i.e. the concentration calculated from the absorbance is an indication of the protein content. At sample solvent ratio, protein concentration was 3 mg/ml. Further experiments were performed to identify the presence of those proteins and their molecular weight, yet data were not shown since further experiments need to be held for confirmation.

As for DNA determination, Nanodrop was used after DNA isolation from SCE. After blanking with TE buffer, DNA concentration was obtained: 0.7 µg/µl with the O.D: 260/280= 1.75 reflecting the purity of the DNA.

2. Effect of the crude SCE on the proliferation of prostate cancer cells in vitro (2D)

a. Trypan blue exclusion assay

To assess the effect of the crude SCE on the viability and proliferation of both, the DU145 and 22RV1 cell lines, trypan blue exclusion assay was performed. Treatment of different concentrations of SCE was applied and tested in three time points: 24, 48 and 72 hours. Number of cells decreased after 72 hours of SCE treatment as shown in figure 8.

Figure 9 demonstrates the effect of SCE on DU145 and 22RV1 cell lines upon treatment with different concentrations of SCE expressed as percentage of control, showing that SCE inhibits the proliferation of DU145 and 22RV1 cells in a time- and dose-dependent manner.

SCE had no significant effect on the proliferation of DU145 cells at concentrations up to 10 µg/ml while it decreased the proliferation of DU415 cells significantly at concentrations ranging between 30 to 100 µg/ml. At 50 µg/ml SCE treatment the decrease obtained reached 38% at 24h, 40% at 48h and 60% at 72h. Inhibition in proliferation is further reflected with a 60% decrease in proliferation at 24h time point when treated with 100 µg/ml, and 80% decrease at 48h remaining the same at 72h time points, suggesting the presence of resistant cells among this population. SCE was toxic to DU145 cells starting from concentration 150 µg/ml and above.

As to 22RV1 cells, SCE had no significant effect on their proliferation at concentrations up to 30 µg/ml while it decreased its proliferation significantly at concentrations ranging between 50 to 100 µg/ml. SCE was toxic to 22RV1 cells starting from 150 µg/ml and above. This toxicity is reflected with an 82% decrease in proliferation at 24h time point and 90% decrease to 100% death in 48h to 72h time points.

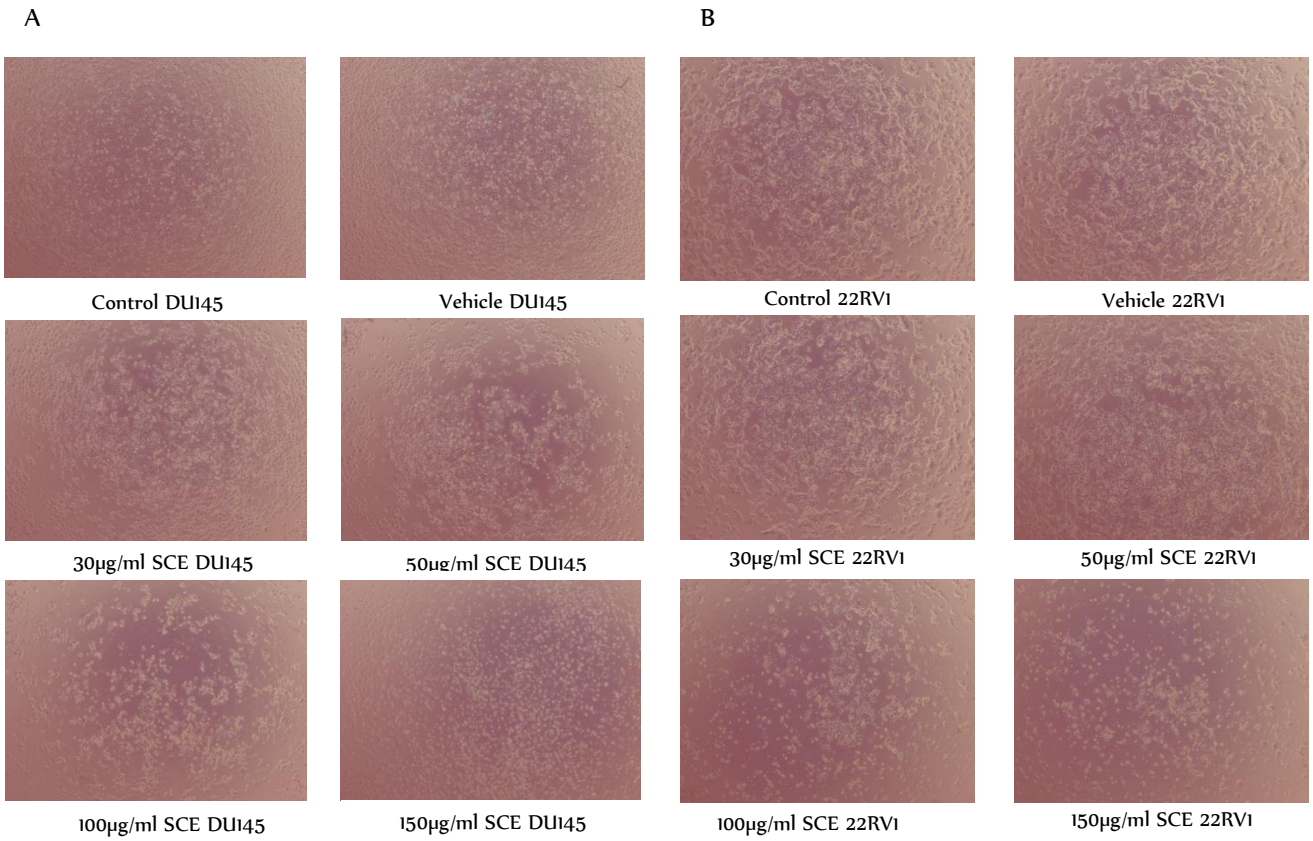


Figure 8. Decrease in living DU145 (A) and 22RV1 (B) cells upon treatment with SCE.
Images were taken after 72 hours of treatment. Cells were visualized by Carl Zeiss microscope at 4x magnification

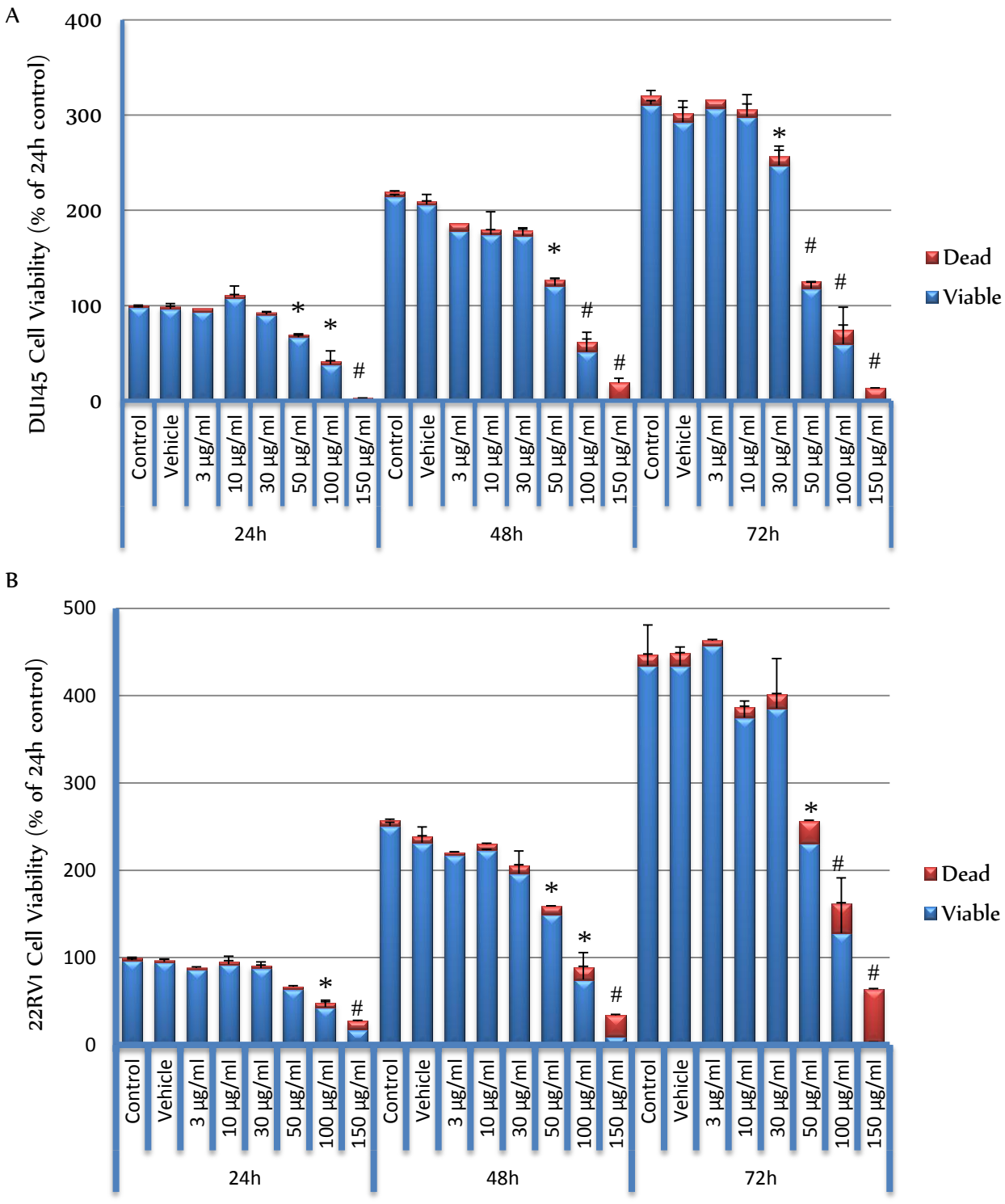


Figure 9. Inhibition of viability and proliferative activity of DU145 (A) and 22RV1 (B) cells in response to treatment with various concentrations of SCE using trypan blue exclusion assay after 24, 48 and 72 hours.

Results are expressed as percentage of total number of cells (viable and dead) of studied group compared to its control. (n=5, 3 wells each ±SD; (* $P < 0.05$; # $P < 0.01$)).

b. MTT assay results

To further confirm the result obtained by the trypan blue exclusion assay, MTT assay was performed. This assay reflects the effect of SCE on the mitochondrial enzymatic activity of cells. The results obtained by MTT assay shown in figure 10, are in accordance with the results obtained using the trypan blue exclusion assay in terms of time and dose dependent effect. However, the inhibition of the mitochondrial enzymatic activity obtained in MTT assay is not as significant as the decrease in cell viability obtained by trypan blue assay. Crude SCE had a significant effect in DU145 cells after 24h of treatment at 150 µg/ml yet at 48 and 72 h it was significant starting from 100 µg/ml. Significant effect in 22RV1 cells was only observed after 48 h of treatment at 150 µg/ml crude SCE concentration.

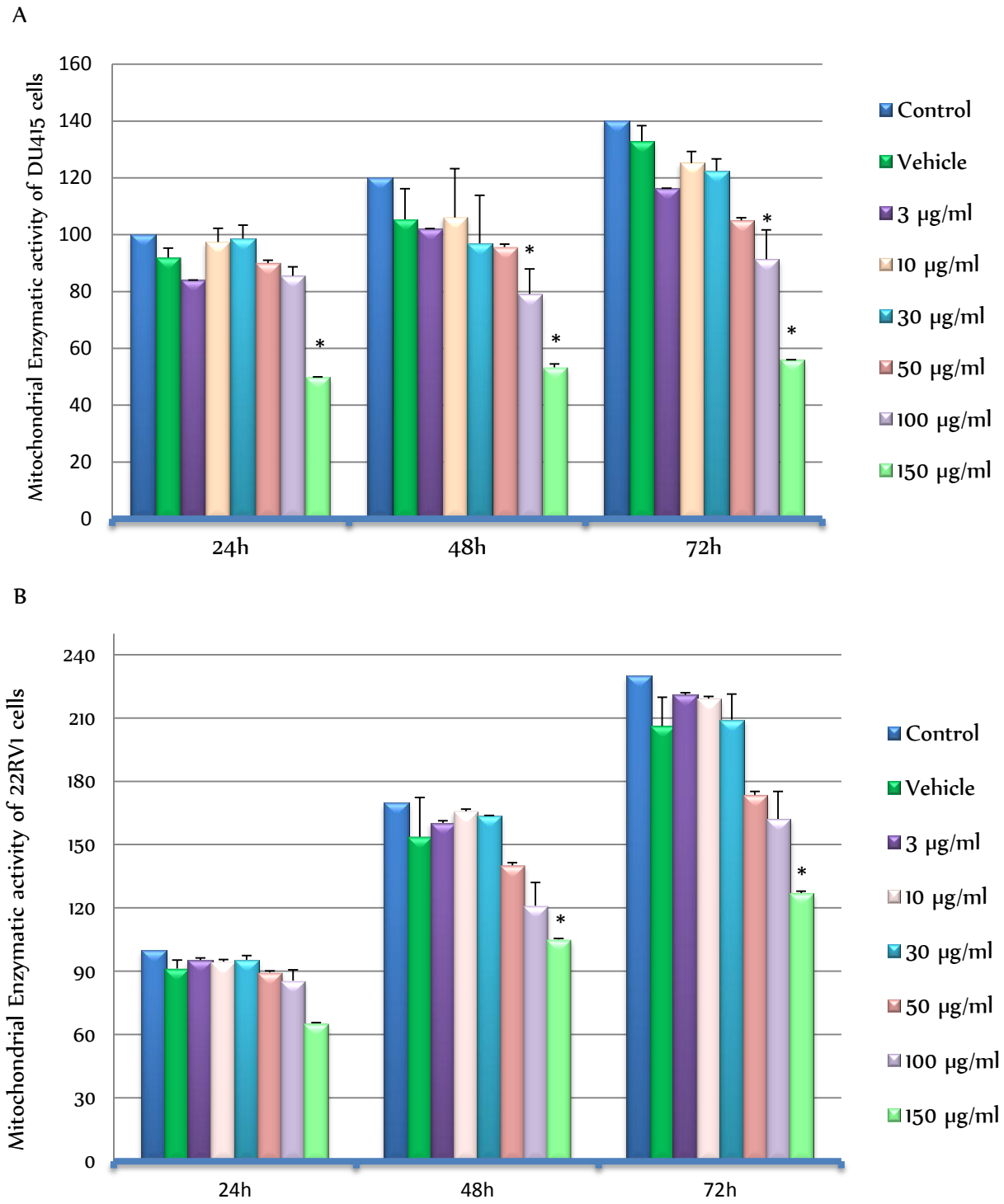
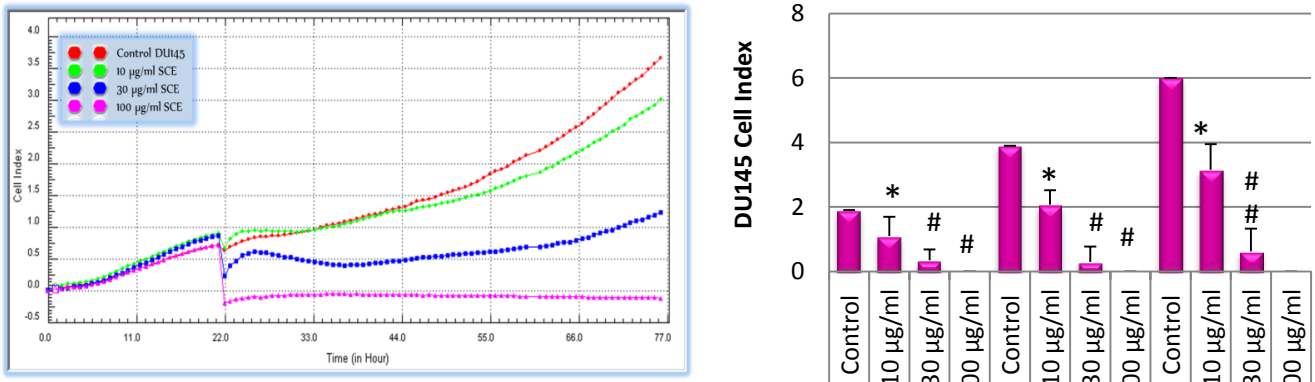


Figure 10. Inhibition in the proliferative activity of DU145 (A) and 22RV1 (B) cells in response to treatment with various concentrations of SCE after 24, 48 and 72 hours, using MTT assay (n=4, 3 wells each ±SD; (*P < 0.05; # P < 0.01)).

c. RTCA results

Cell proliferation (DU145 and 22RV1 prostate cancer cell) was continuously monitored using the xCELLigence RTCA DP Instrument. Featuring a dual-plate (DP) format, the instrument measures impedance-based signals in a cellular assay – without the use of exogenous labels. The higher the number of adherent cells; the higher the impedance signal and accordingly the higher the cell index.

A



B

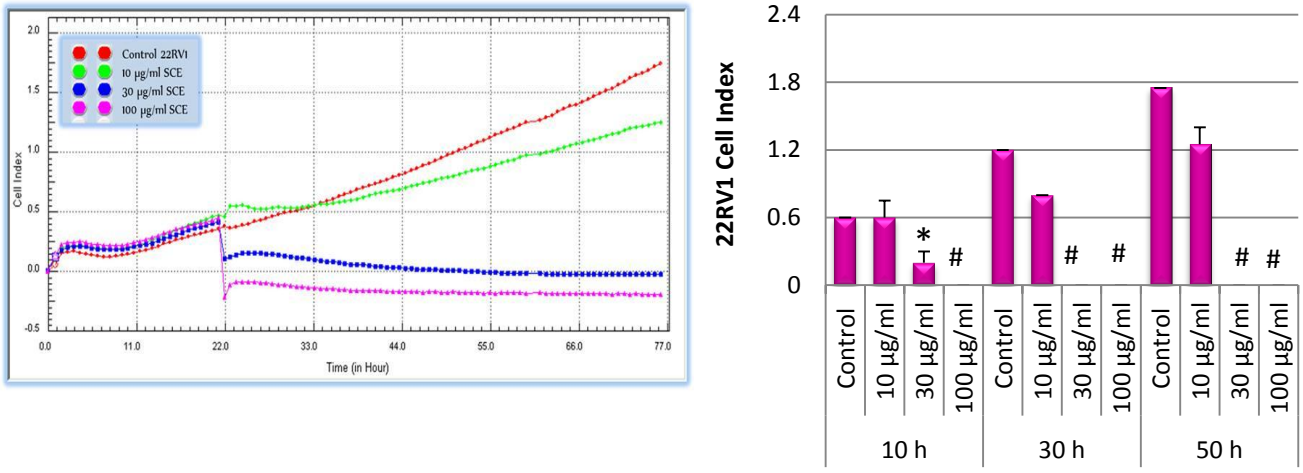
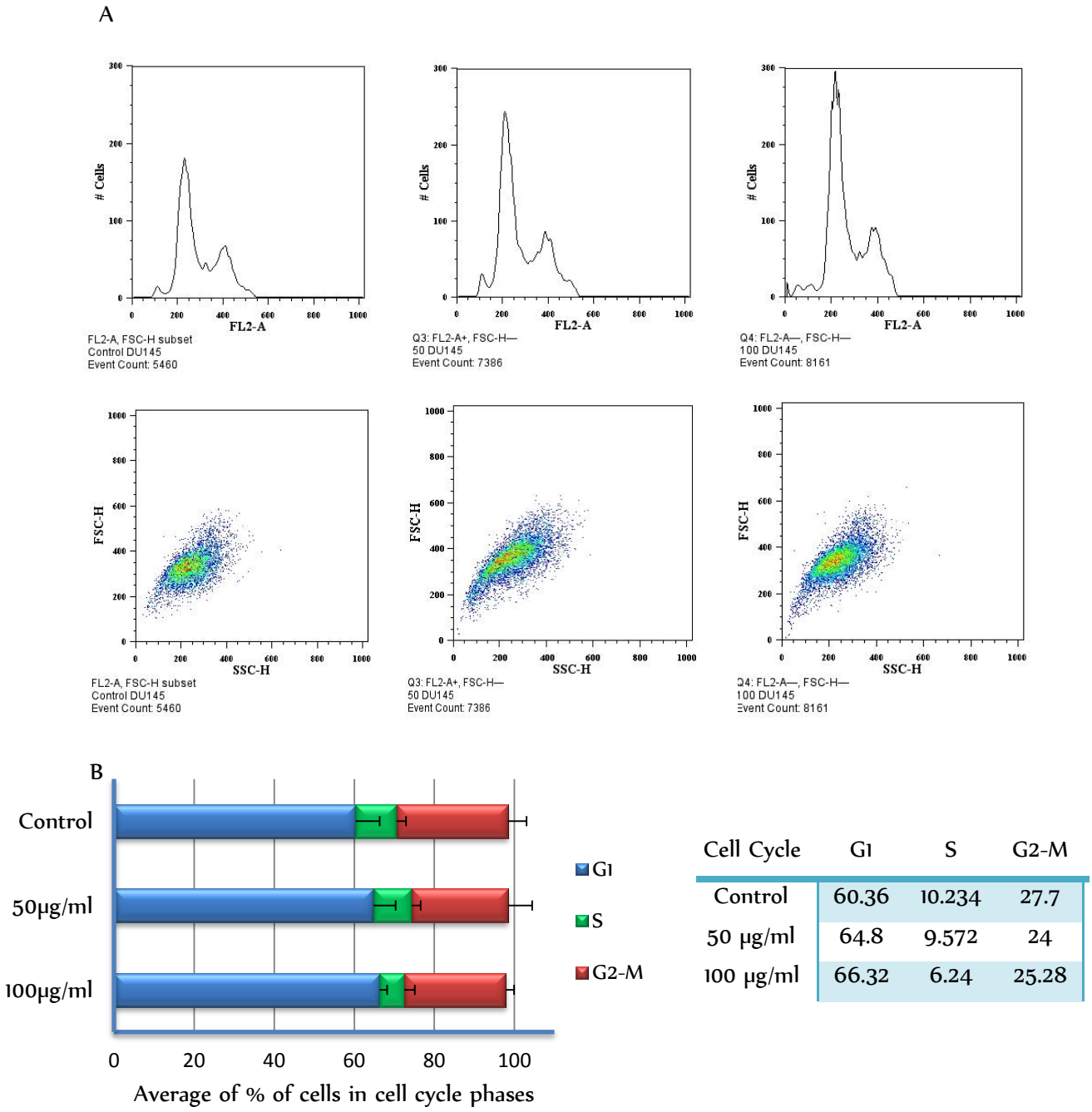


Figure 11. Inhibition of proliferative activity of DU145 (A) and 22RV1 (B) cells in response to treatment with various concentrations of SCE using RTCA.
Cell index vs time are plotted in the left and the corresponding histogram of normalized cell index values relative to control are in the right (n=3, 2 wells each ±SD; (* $P < 0.05$; # $P < 0.01$).

As shown in figure 11, crude SCE inhibited the proliferation of both cell lines in a dose and time dependent manner. The inhibitory effect was significant in DU145 cells starting from 10 µg/ml of SCE in the first hours of treatment and the inhibition proceeded in the next 50 hours as well. As for 22RV1 cells, the inhibitory effect started to be significant at the concentration of 30 µg/ml in the first hours after treatment and the inhibition proceeded in the following 50 hours.

3. Effect of SCE on prostate cancer cell cycle progression

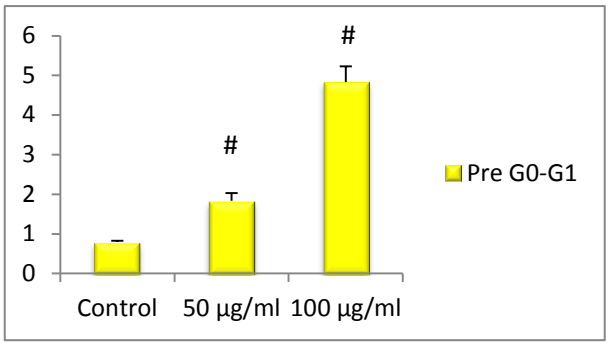
In an attempt to understand how SCE is able to decrease cell count and inhibit cell proliferation, cell cycle analysis was performed by flow cytometer. This will assess the distribution of cells in G_0-G_1, S and G_2-M phases after treatment. As shown in figure 12, 48h post-treatment with crude SCE, a slight cell cycle arrest in G_0-G_1 phase occurred in DU145 cells. SCE increased the percent of cells in this phase by up to 15%, yet this arrest was not statistically significant ($P \leq 0.05$). Furthermore, there was a major increase in Pre G_0-G_1 upon treatment with SCE in both concentrations 50 and 100 µg/ml, indicating that there was an increase in the fragmented DNA upon treatment (Figure 15) suggesting cell death, however the mechanism is still to be determined.



Cell Cycle	G1	S	G2-M
Control	60.36	10.234	27.7
50 µg/ml	64.8	9.572	24
100 µg/ml	66.32	6.24	25.28

Figure 12. Cell Cycle analysis of DU145 cells treated with 50 and 100 µg/ml of SCE after 48 hours showing a slight G_0-G_1 arrest.

(A) Forward scattered -Side scattered of cell cycle distribution (B) Histogram showing the distribution of phases of the cell cycle phases under the effect of SCE at 48h along with its corresponding table (n=5, ±SD; (* $P < 0.05$; # $P < 0.01$)).



Cell Cycle	Pre G0-G1
Control	0.8
50 µg/ml	1.84
100 µg/ml	4.83

Figure 13. Histogram showing the increase of Pre G_0-G_1 phase of DU145 cells 48h of treatment with SCE along with its corresponding table.
Values taken from the flow cytometer analysis (n=5, ±SD; (* P < 0.05; # P < 0.01)).

4. Crude SCE's apoptotic effect on prostate cancer cells

a. Hoechst staining

Hoechst, a DNA intercalating agent, in its excited state allows us to visualize the nucleus of the cells. Hoechst staining of treated vs untreated DU145 cells was performed to assess the effect of SCE (at 50 and 100 µg/ml) on the nucleus and therefore to visualize if any stage of apoptosis is occurring upon treatment. SCE appeared to impact the integrity of the nucleus as shown in figure 14 that demonstrates nucleus shrinkage, fragmentation and the formation of some apoptotic bodies upon treatment with SCE.

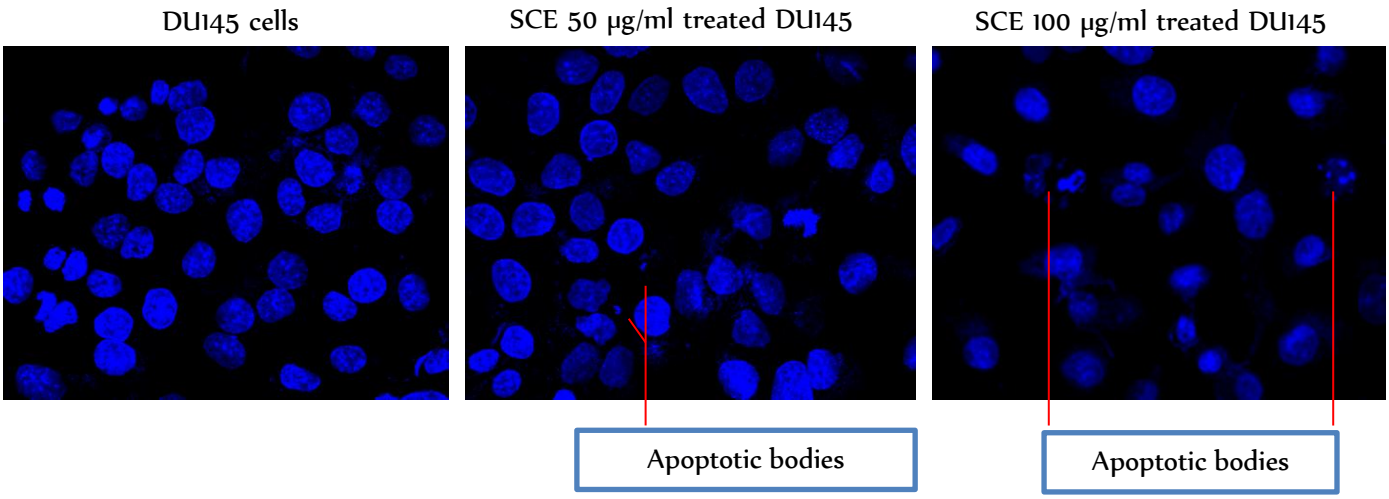


Figure 14. Hoechst nuclear staining showing apoptotic bodies upon 48h SCE treatment of DU145 cells.
Images were taken using 710 LSM Confocal Laser Microscope at 40x magnification.

b. Western Blotting

To further study the effect of SCE on apoptosis, western blotting was performed and the protein expression of BCL2 and PARP was assessed. This experiment was carried out at 48h after treatment with 50 and 100µg/ml SCE. Figure 15 shows that Bcl2, an antiapoptotic regulator protein of cell death which is overexpressed in almost all cancers, was inhibited significantly with SCE treatment and that PARP, playing a role in DNA damage repair and maintaining cell viability, was also decreased in its full length form (116 kD peptide) and cleaved into its 98 kD polypeptide form thus establishing an evidence for the induction of apoptosis.

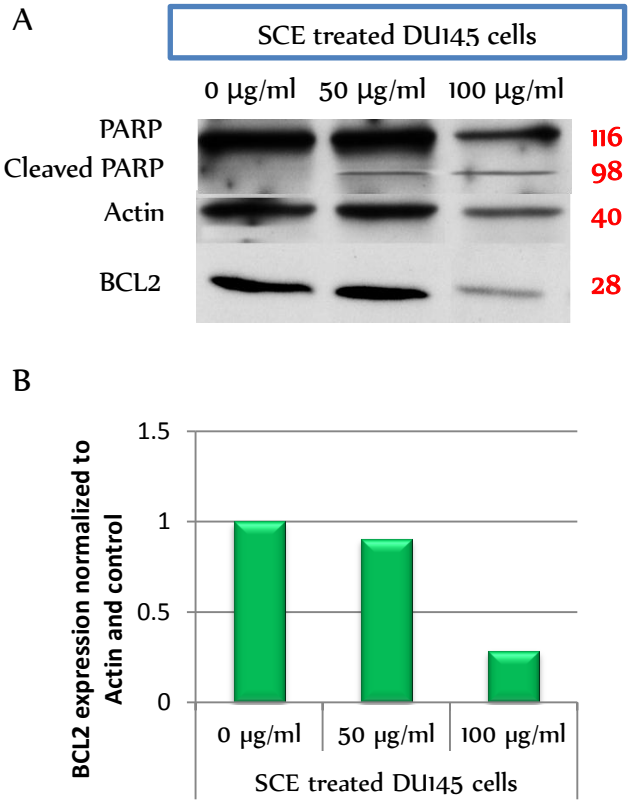


Figure 15. BCL2 protein expression decrease, and PARP cleaveage upon SCE treatment of DU145 cells.

A Western blot of protein extracts from DU145 cells, untreated or SCE-treated (n=1)
B. Histogram representing densitometry analysis of BCL2 expression using Image J software.

c. Immunofluoroscence analysis of PARP, γH2AX and BCL2 in DU145 cells

Immunofluorescence staining of SCE-treated formaldehyde-fixed DU145 cells was assessed for PARP, γH2AX and BCL2 apoptotic markers. As shown in figure 16, the intensity of PARP expression (Red color) increased in SCE treated DU145 cells, indicating that single strand DNA breaks increased, i.e. apoptosis was induced by SCE treatment.

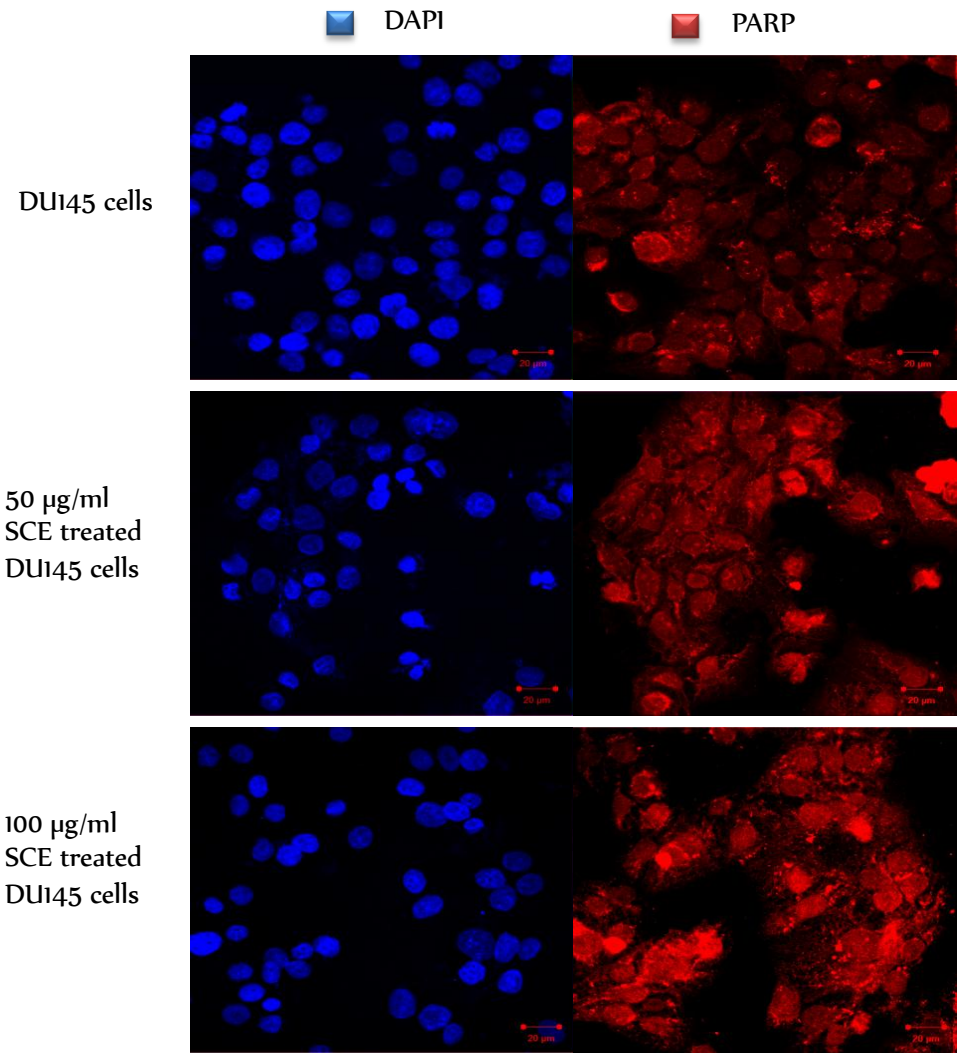


Figure 16. Increase of PARP (Red) expression in DU145 cells after 48h treatment with 50 and 100 µg/ml of SCE using oil lens LSM 710 Confocal Laser microscope at 40x magnification.

Similarly, figure 17 shows that γH2AX intensity (Red color) was augmented, suggesting an increase in double strand DNA breaks upon treatment and thus apoptosis induction. As for the antiapoptotic protein, BCL2 (Green color), the intensity decreased in SCE treated DU145 cells, confirming the western blot results thus apoptosis occurrence.

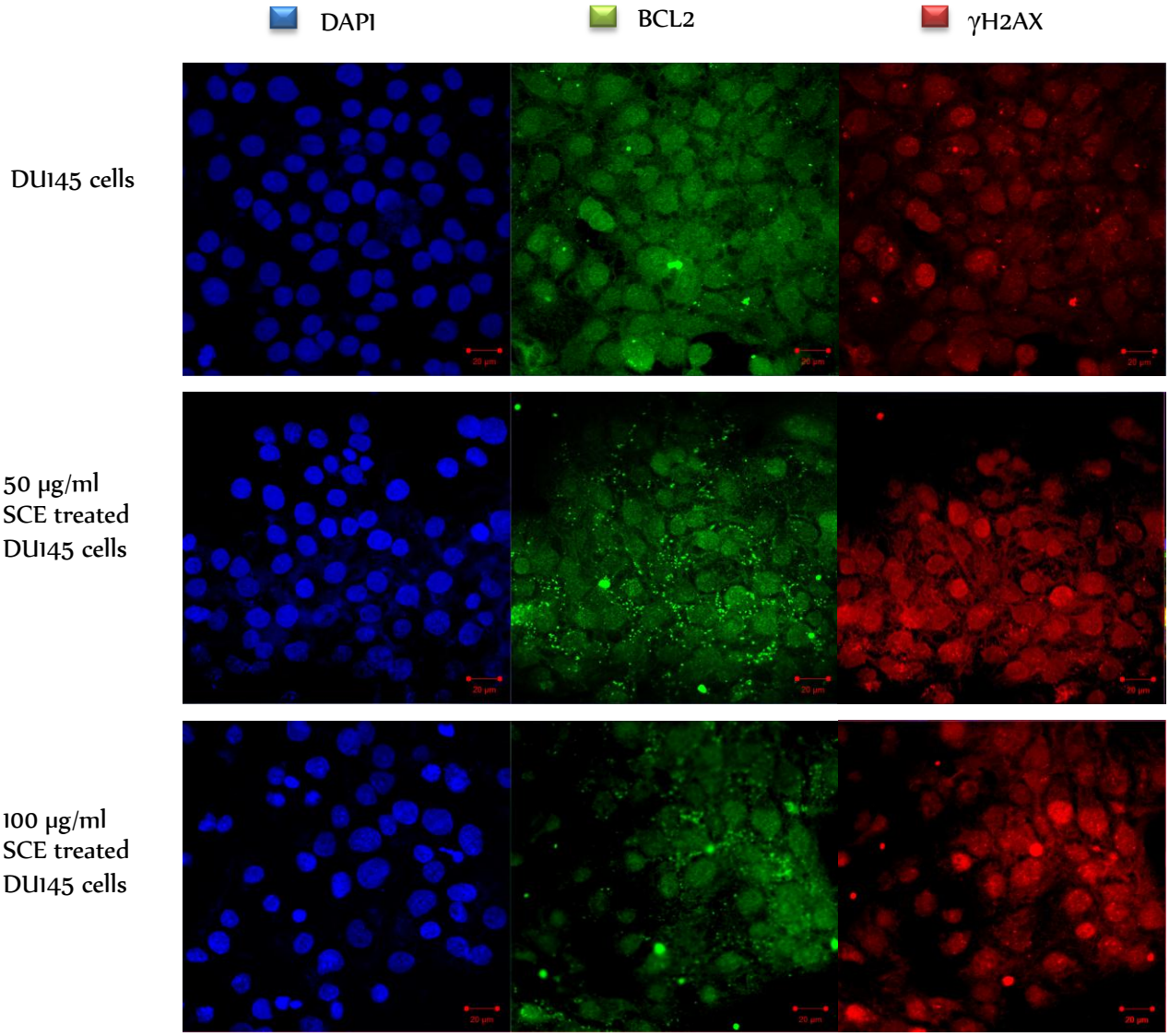


Figure 17. Increase of γH2AX (Red) and decrease of Bcl2 (Green) expression in DU145 cells after 48h treatment with 50 and 100 µg/ml of SCE using oil lens LSM 7-10 Confocal Laser microscope at 40x magnification

5. Effect of the crude SCE on metastasis:

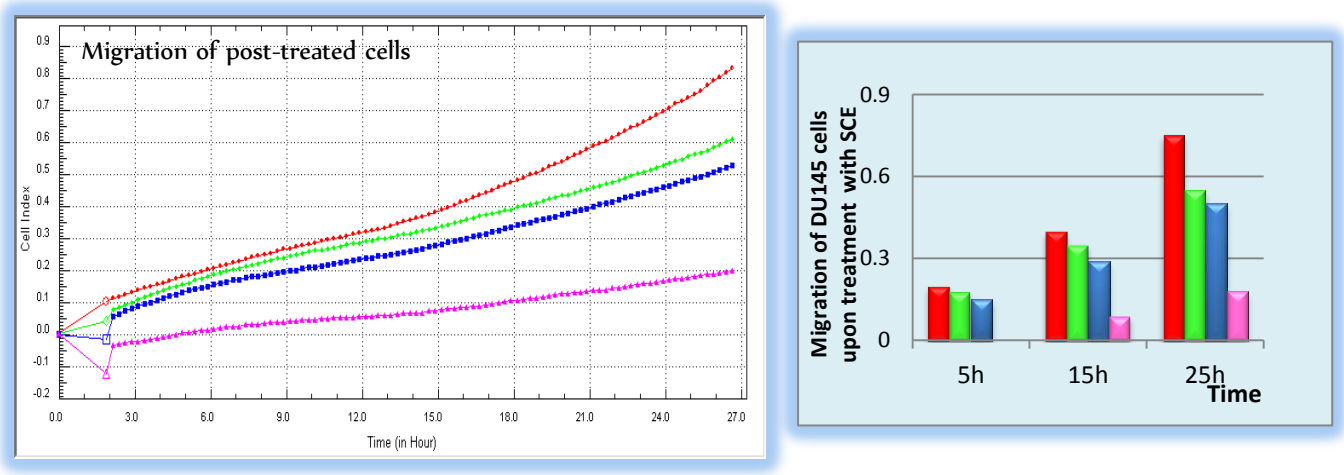
a. Effect of the crude SCE on the migration of DU145 cells

1) Real Time Cell Analysis (RTCA):

Tumor cell migration is an important step in the multistep process of cancer metastasis. Cell migration is the movement of cells from one area to another, generally in response to chemical signals, and is important in diverse physiological and pathological processes. To better understand the effect of SCE on metastasis, RTCA migration assay was conducted. This assay allows monitoring and quantifying, in real-time, the migration of DU145 cells.

Migration of DU145 cells, as indicated by cell-index profiles, decreased by SCE pre-treatment and post-treatment in a time and dose dependent manner. Migration of SCE post-treated DU145 cells decreased compared to the non-treated DU145 cells (Figure 18-A) and migration of pre-treated DU145 cells decreased as well when treated before seeding in RTCA plates (Figure 18-B). A significant decrease was observed using the concentration 50 µg/ml of SCE (75% decrease). Pre-treated and post-treated DU145 cells' proliferation was also assessed along with this experiment (Figure 18-C).

A



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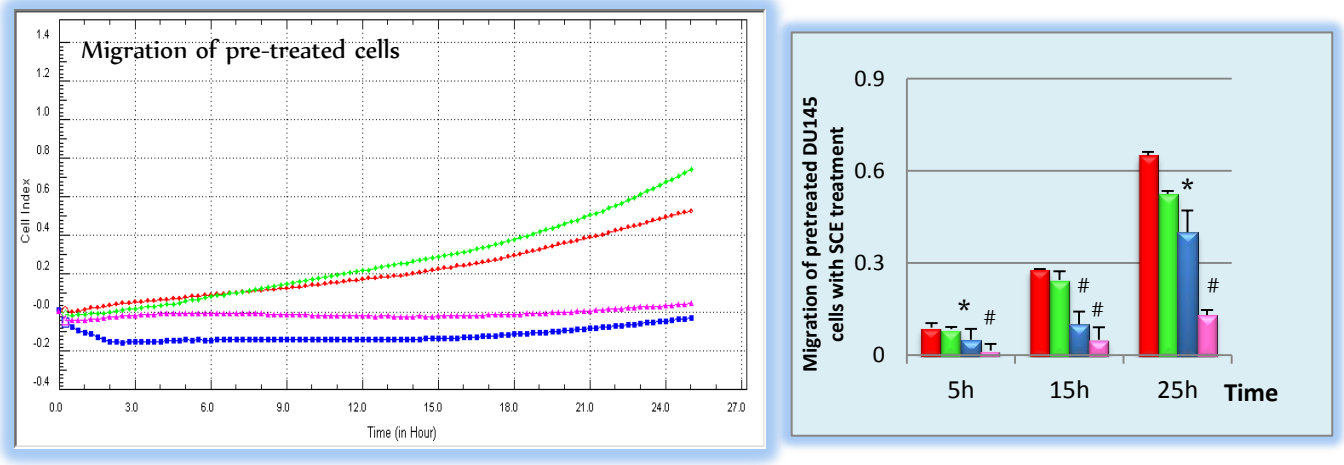

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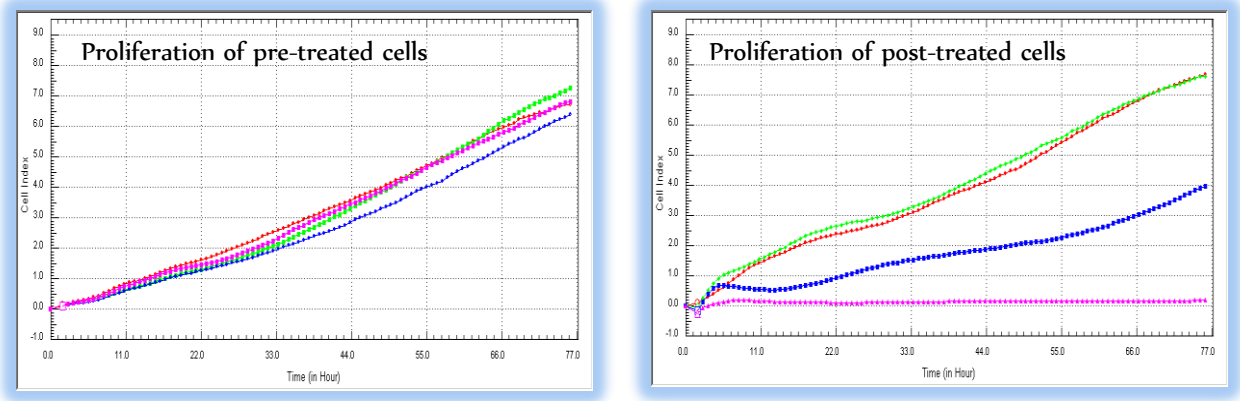
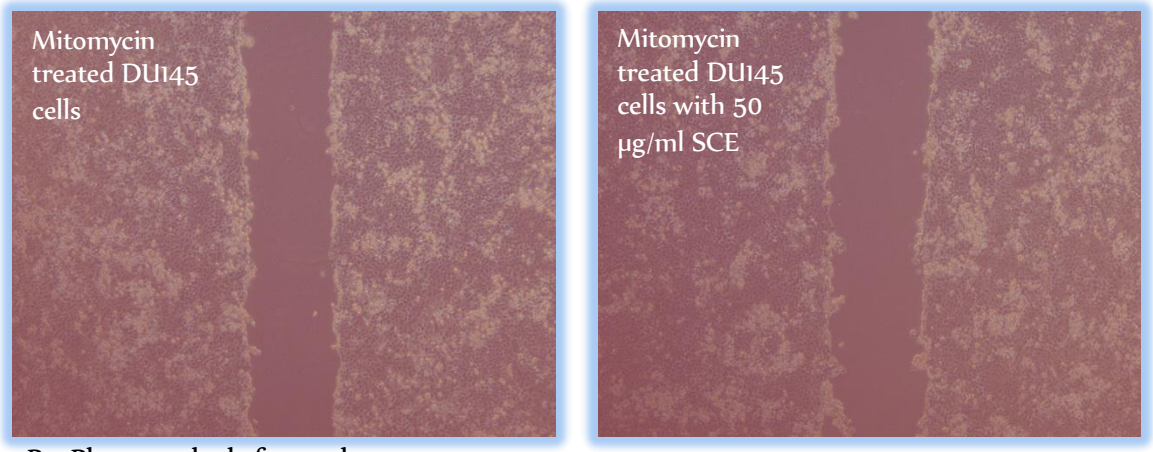


Figure 18. Migration assay by RTCA.
A) Plots of cell index of migrated cells vs time with the corresponding histogram on the right of normalized cell index values of migration relative to control when treated with SCE after seeding. B) Plots of cell index of the migrated cells vs time with the corresponding histogram on the right of normalized cell index values of migration relative to control when treated with SCE in 2D before seeding in RTCA plates. C) Proliferation of DU145 cells with SCE pre-treatment and post treatment (n=2, 2 wells each ±SD; (* $P < 0.05$; # $P < 0.01$))

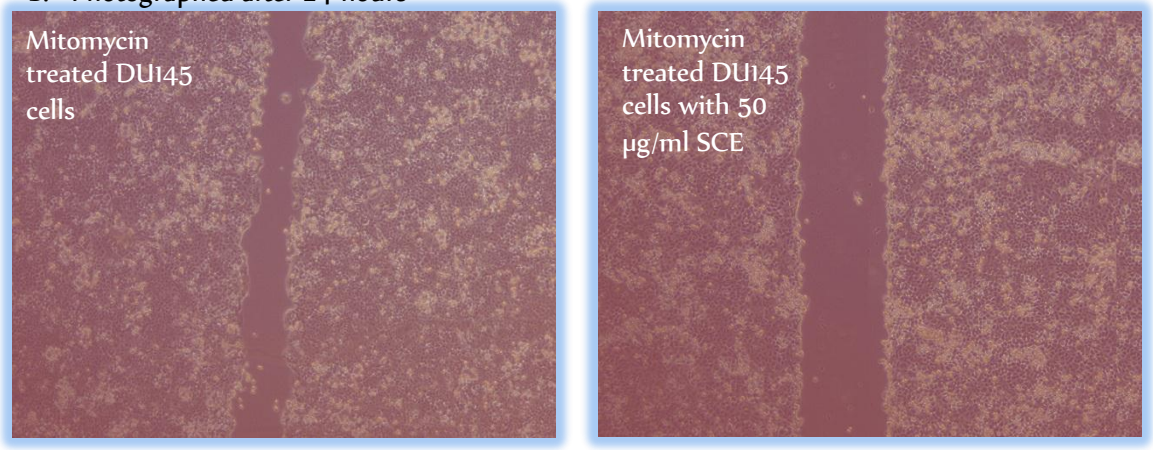
2) Wound Healing assay:

In order to confirm the inhibitory effect of the crude SCE on the migration of DU145 cells, wound healing assay was performed. To assess the effect on migration and eliminate the interference that the division of cells may cause in this assay, Mitomycin was added to stop the division of cells and thus to allow evaluating the effect of SCE on the migration of DU145 cells by visualizing wound healing if migration is not inhibited. As shown in figure 19, when cells were treated with 50 µg/ml of crude SCE, the wound distance remained almost equal to its distance on the time of scratch confirming that crude SCE has an inhibitory effect on the migration of DU145 cells. The wound formed in wells having cells that were treated with Mitomycin alone was smaller after 24 hours, indicating that untreated cells were migrating.

A. Photographed at the time of scratch



B. Photographed after 24 hours



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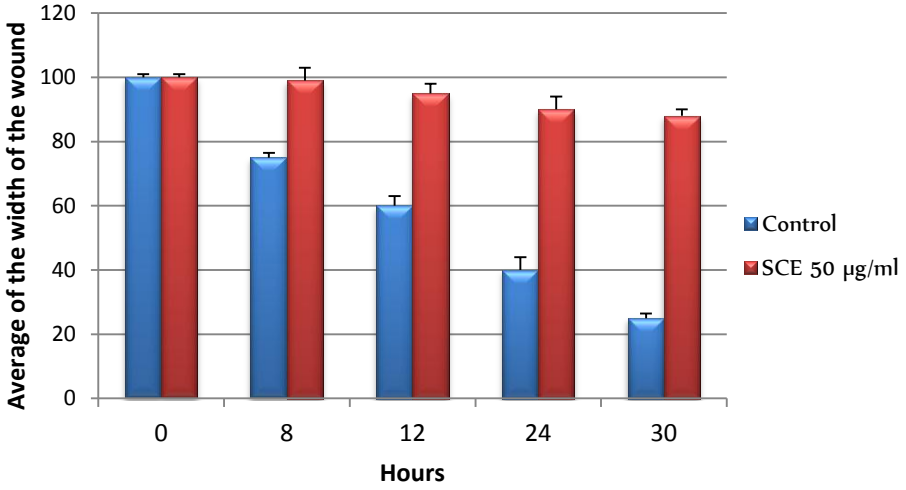


Figure 19. Wound healing assay.
A**)** Images taken at the time of scratch. B) Images taken after 24 hours of wound scratching. Cells were visualized by Carl Zeiss microscope at 4x magnification. C) Histogram of the width of the wound per µm during 30 hours after scratch

b. Effect of crude SCE on the invasion of DU145 cells

1) Real Time Cell Analysis (RTCA)

Cell invasion is the intrusion on and destruction of adjacent tissues, particularly with respect to cancer cells. To better understand the effect of SCE on metastasis, RTCA invasion assay was conducted. This assay allows monitoring and quantifying, in real-time, the migration of DU145 cells. Invasion of DU145 cells, as indicated by cell-index profiles, was slightly induced by SCE pre-treatment (Figure 20-B) and decreased significantly by SCE post-treatment in a time and dose dependent manner (Figure 20-A). This decrease was significant using the concentration 50 µg/ml of SCE (65% decrease). Pretreated and post treated DU145 cells' proliferation was also assessed along with this experiment (Figure 20-C). The slight induce in invasion of pretreated DU145 cells is explained by the increase of the percent of resistant cells among the remaining cells that were seeded post-treatment thus more mesenchymal cells were present that are more invasive in nature, causing that slight increase as a result.

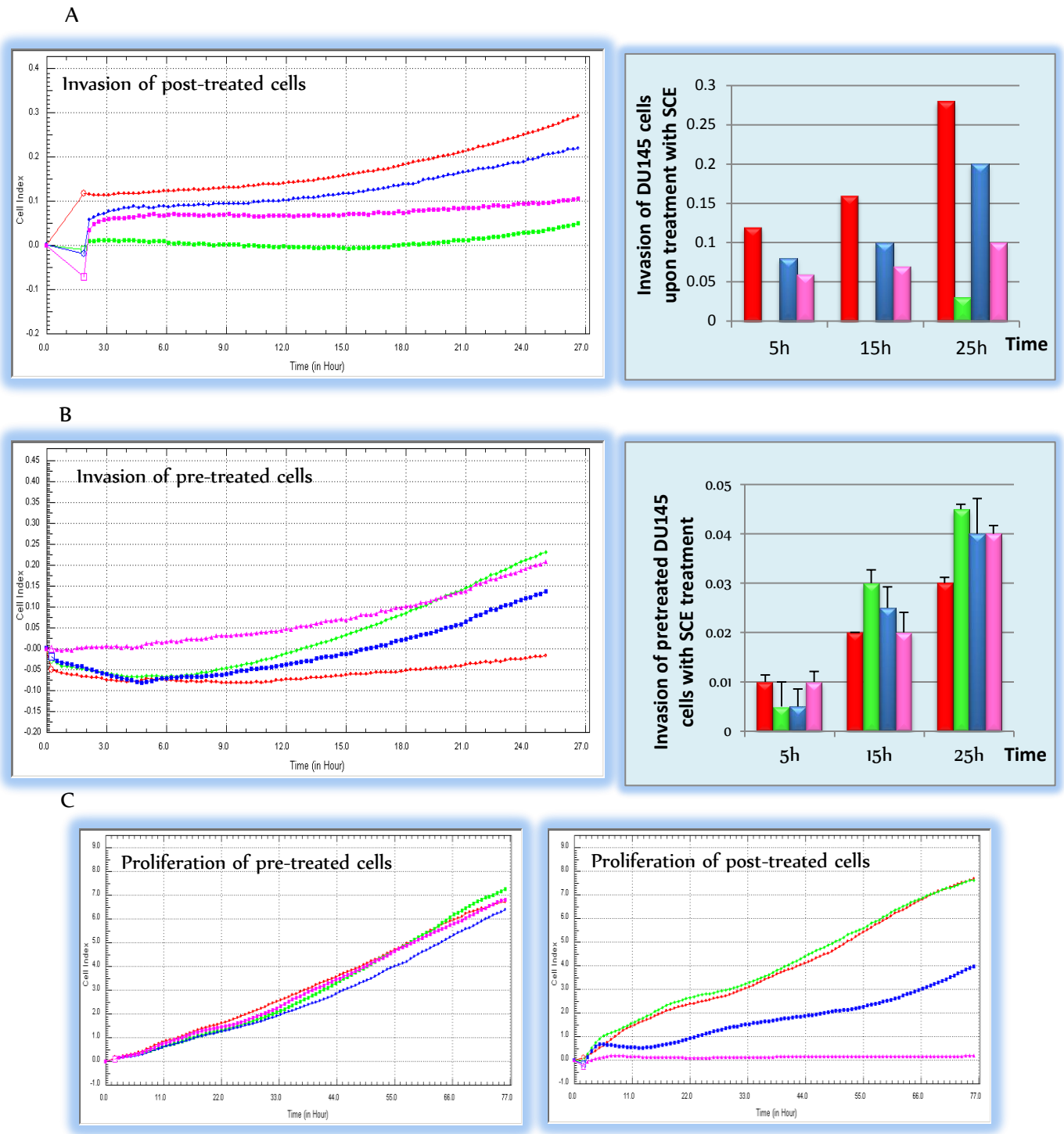


Figure 20. Invasion assay by RTCA.
A) Plots of cell index of the invasive cells vs time with the corresponding histogram on the right of normalized cell index values of invasion relative to control when treated with SCE after seeding over the matrigel. B) Plots of cell index of the invasive cells vs time with the corresponding histogram on the right of normalized cell index values of invasion relative to control when treated with SCE in 2D before seeding over the matrigel C) Proliferation of DU145 cells with SCE pre-treatment and post-treatment (n=2, 2 wells each ±SD)

2) Effect of the crude SCE on invasion factors -metalloproteinases (MMPs)- by Gelatin Zymography

In order to further understand the effect of the crude SCE on the invasion step of metastasis, gelatin zymography, a functional assay was performed to study how SCE can affect the activity of proteinases belonging to the family of MMPs that play an essential role in invasion. As shown in figure 21, the crude SCE increases the activity of MMP-9 and MMP-2 in DU145 cells up to 2 folds when treated with 100 µg/ml in 48h time point.

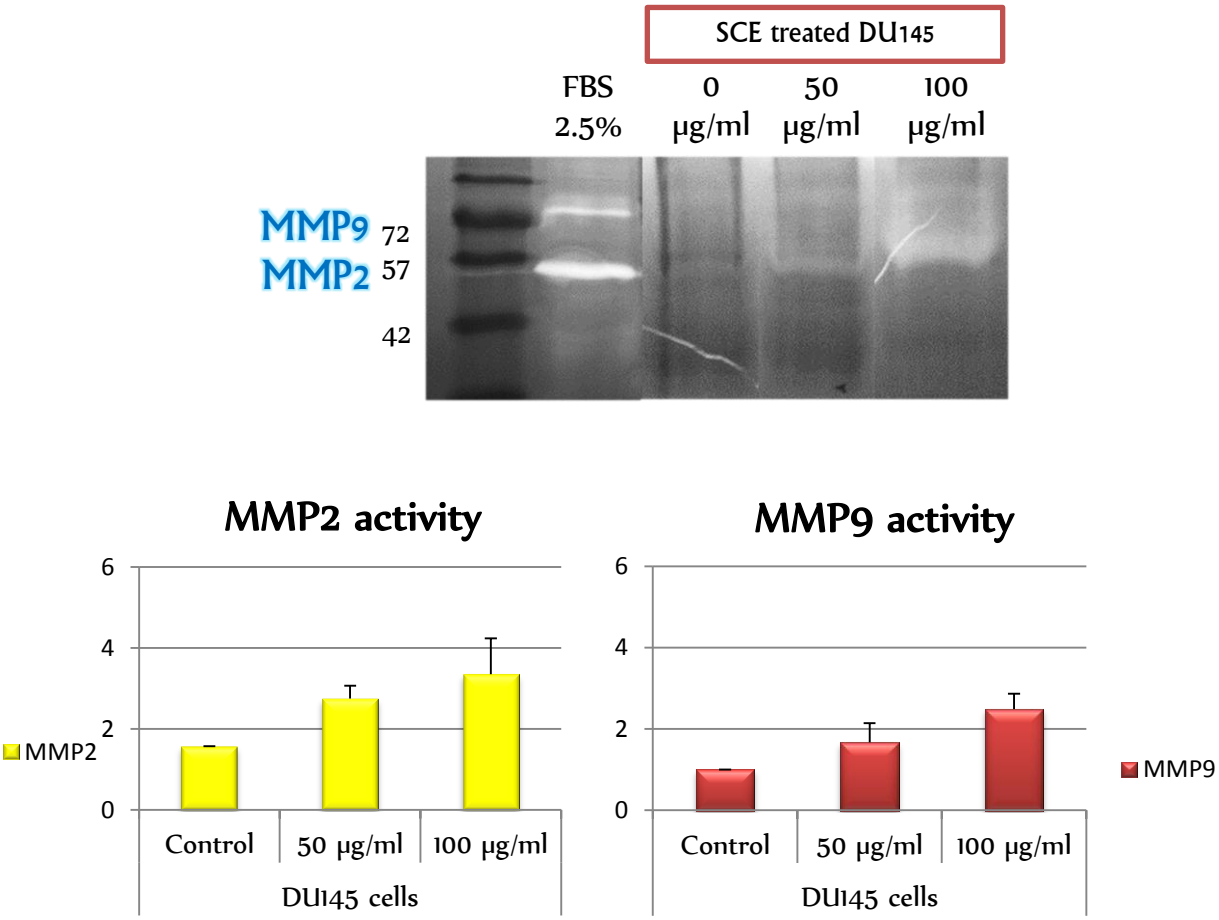


Figure 21. MMP enzymatic activity in DU145 protein extracts following 48h of SCE treatment. Protein extracts were analyzed for MMP2 and MMP9 activity by gelatin zymography. 2.5% FBS was used as a positive control for the MMP2 and MMP9 enzymatic activity.

c. Effect of crude SCE on EMT and other metastatic markers

1) SCE effect on the transcription of EMT markers and VEGF

Since SCE had an inhibitory effect on the migration and the invasion, relevant alterations on the molecular level are studied. Real-time PCR was performed, SCE at 50 and 100 µg/ml after 48h of treatment of DU145 cells effect on EMT markers was assessed. VEGF, a pro-angiogenic factor, was also checked by the same technique: real-time PCR. Figure 22 shows that SCE downregulates EMT markers: E-cadherin, snail and twist, which are transcription factors crucial for the EMT process and VEGF as well.

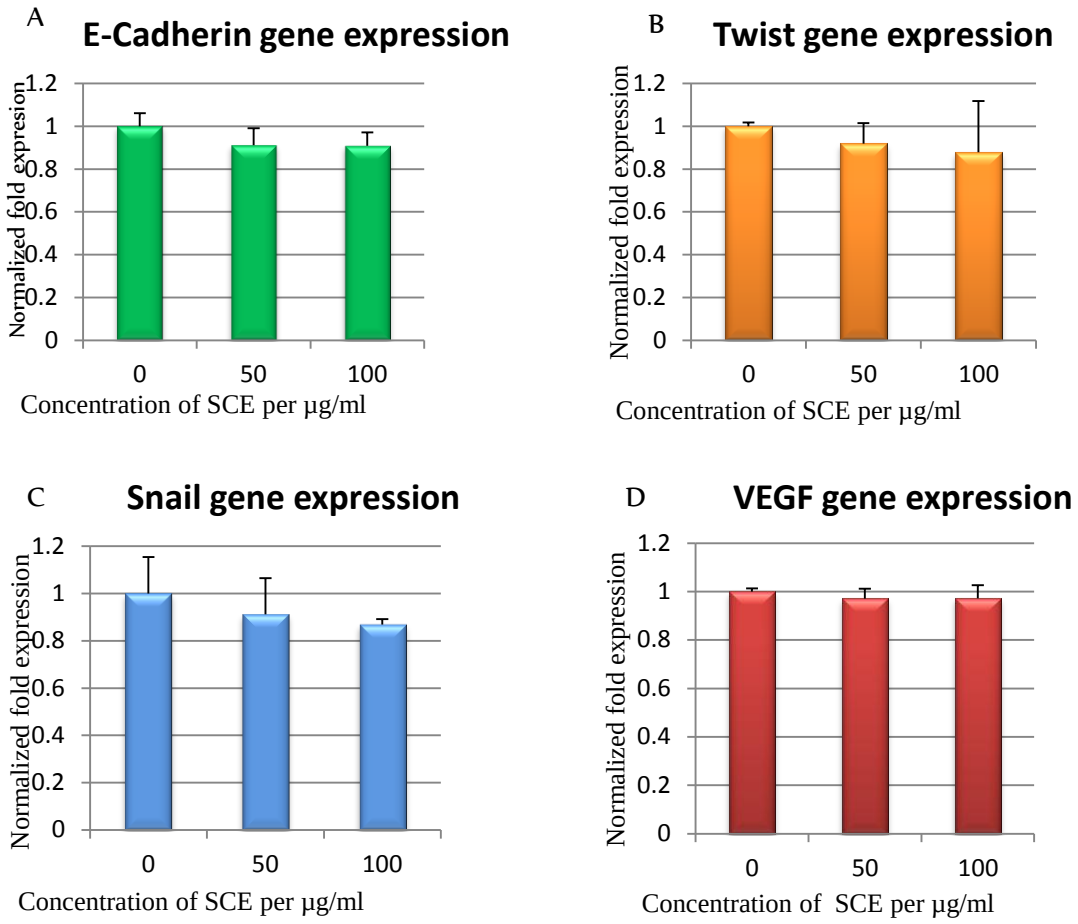


Figure 22. Histograms of the gene expression of EMT markers and VEGF in DU145 cells
Results were obtained by real time PCR. cDNA was obtained from DU145 cells after 48h treatment with SCE (50 and 100 µg/ml**)** A. E-Cadherin gene expression, B. Twist gene expression, C. Snail gene expression, D. VEGF gene expression (n=3, 2 wells each ±SD; (* $P < 0.05$; # $P < 0.01$)).

2) SCE effect on the translational level of DU145 cells

To further study the effect of SCE on EMT markers and invasion, western blotting was performed for E-Cadherin, Vimentin and EZH2. This experiment was carried out at 48h post-treatment with 50 and 100µg/ml SCE. Figure 23 shows the slight downregulation of E-Cadherin and Vimentin that SCE causes when treating DU145 cells.

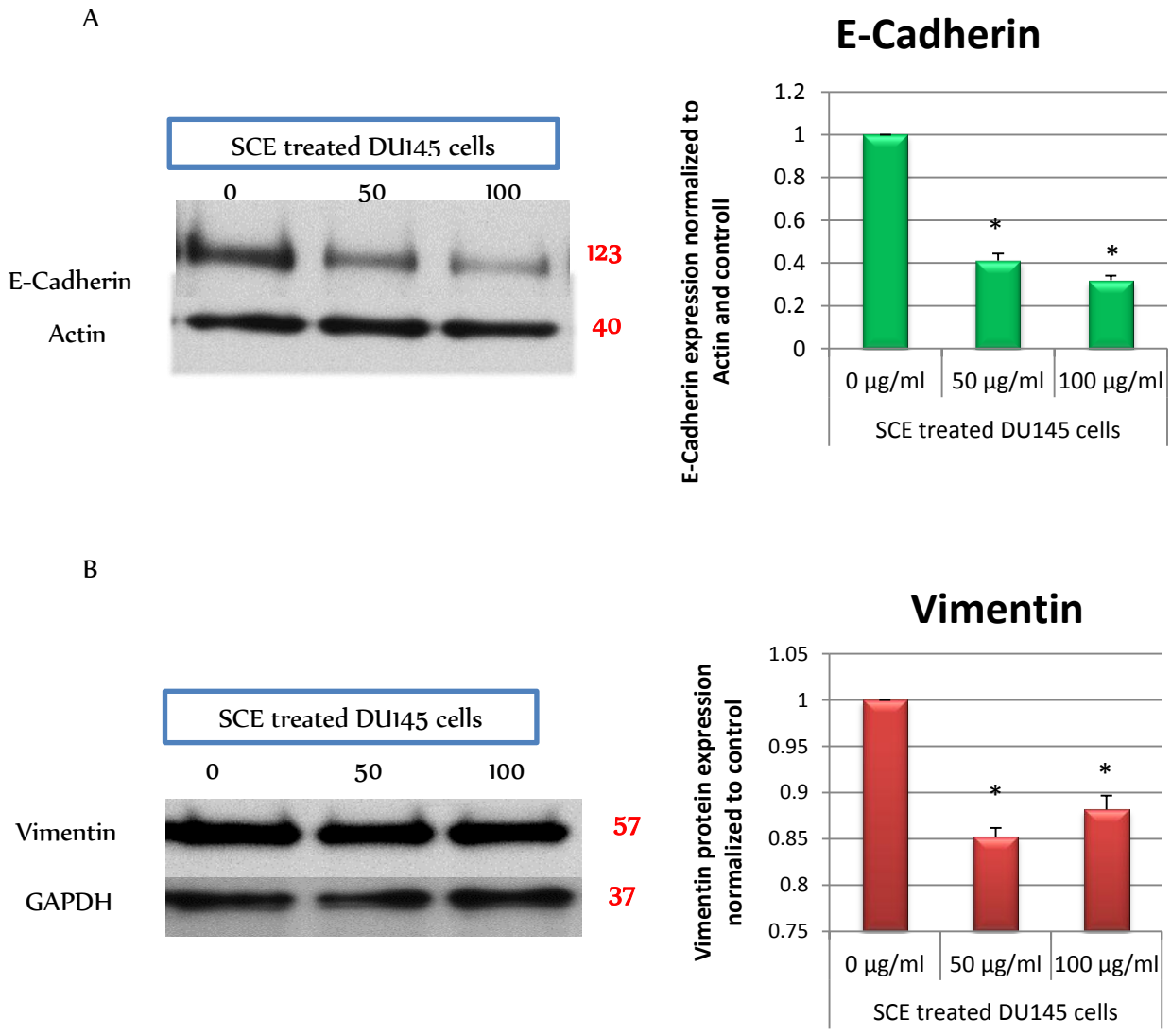


Figure 23. Western Blot analysis of EMT markers.
A) E-Cadherin expression normalized to Actin, B) Vimentin expression normalized to GAPDH. Histograms of densitometry analysis are shown in parallel using Image J software (n=3, ±SD; (* $P < 0.05$; # $P < 0.01$)).

Additionally, western blotting showed that SCE downregulates EZH2 expression with a 30% decrease at 100 µg/ml SCE concentration and 60 % decrease at 150 µg/ml SCE concentration (Figure 24).

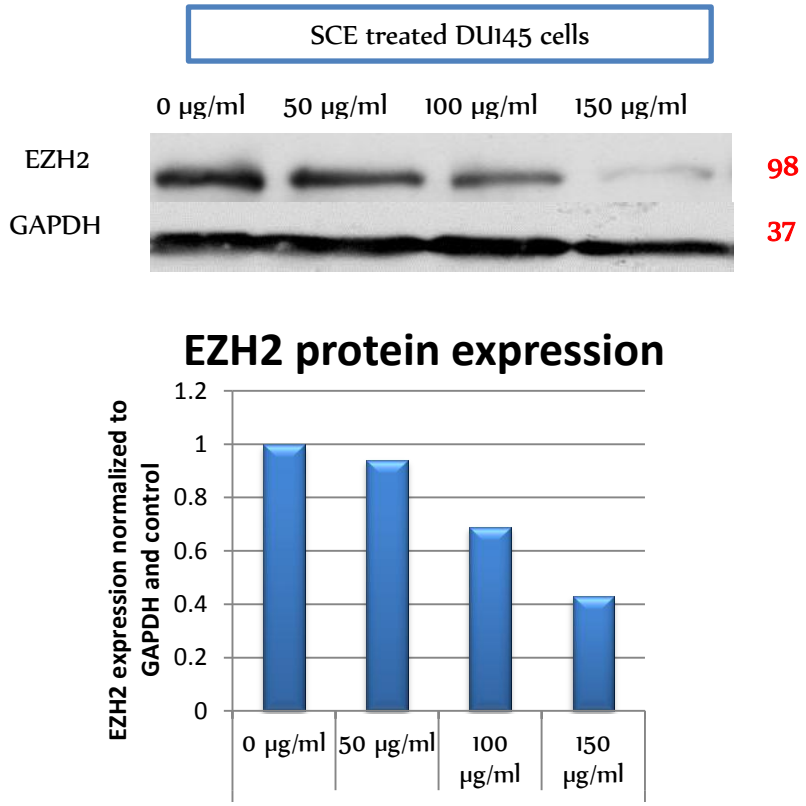


Figure 24. Western Blot analysis of EZH2 expression upon treatment with 3 different concentrations of SCE (50, 100 and 150 g/ml) with its corresponding histogram of densitometry analysis using Image J software.

3) Immunostaining of EMT markers

Vimentin and E-Cadherin expression were further explored by immunostaining. As shown in figure 25, the intensity of Vimentin and E-Cadherin decreases with SCE treatment confirming the results obtained in western blotting.

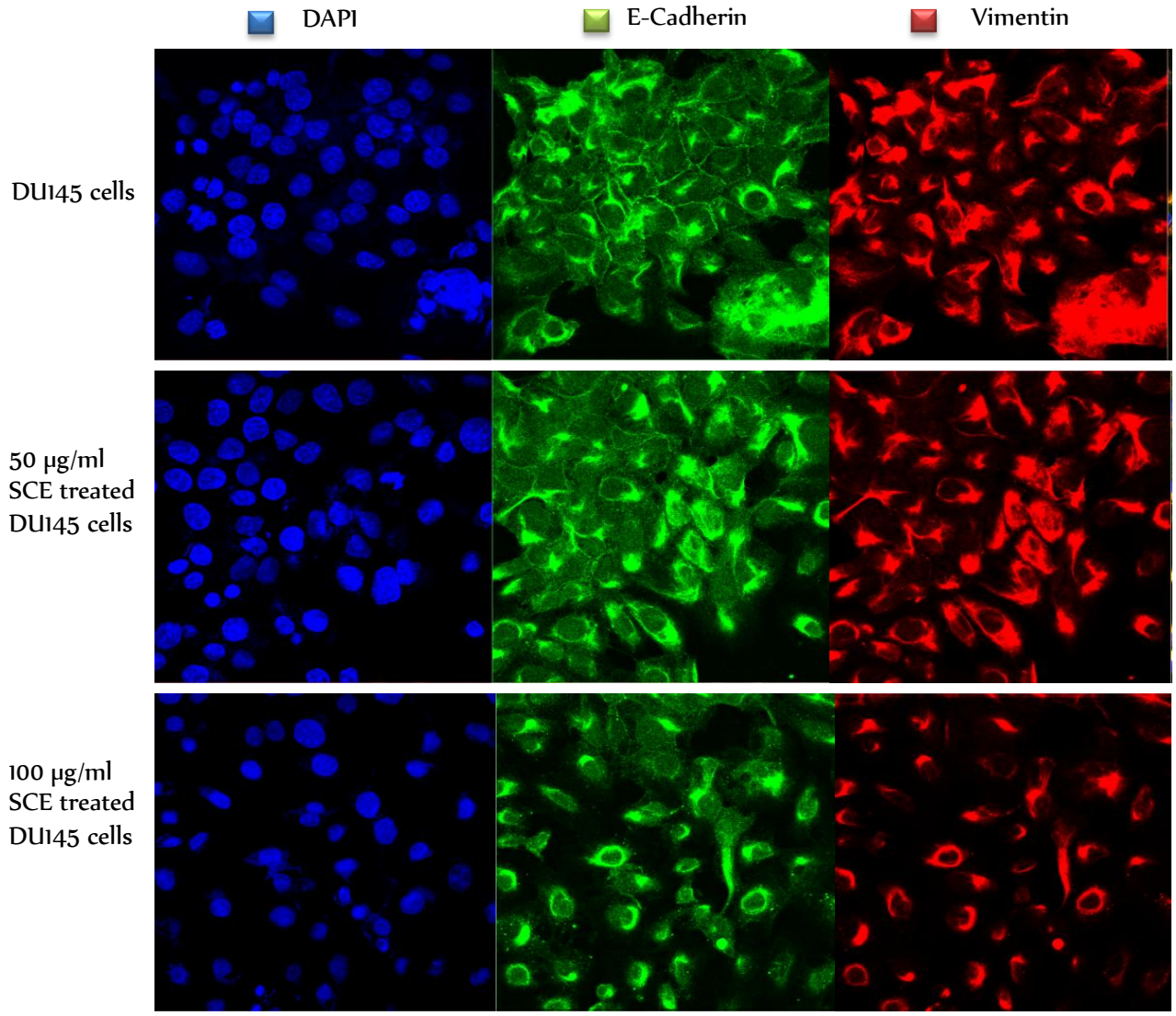


Figure 25. Decrease in the expression of Vimentin (Red) and E-Cadherin (Green) I'n DU145 cells after 48h treatment with 50 and 100 µg/ml of SCE using oil lens LSM 710 Confocal Laser Microscope at 40x magnification

6. Effect of SCE on the proliferation of an enriched population of Prostate Cancer Stem/Progenitor Cells *in vitro*

a. Effect of the crude SCE on the first generation of DU145 and 22RV1 spheres

The ability to form spheres in non-adherent culture is one of the characteristics of prostate CSCs and progenitor cells. To demonstrate the effect of SCE treatment on prostate CSCs, sphere formation of DU145 cells was studied in the presence and absence of SCE and the sphere forming unit (SFU) was calculated according to the following formula: SFU = (number of spheres/number of cells seeded) × 100. As shown in Figure 26 and 27, after culturing DU145 and 22RV1 cells for 15 days in a non-adherent condition using Matrigel, a low number of cells were able to form tumor spheres in untreated conditions (SFU=7 for DU145 cells, SFU=6 For 22RV1 cells). However, no protospheres were able to form in wells treated at the day of seeding (Day 0) with 50μg/ml SCE and above, and no protospheres were able to form in wells treated after two days of seeding (Day 2) with 100μg/ml SCE and above.

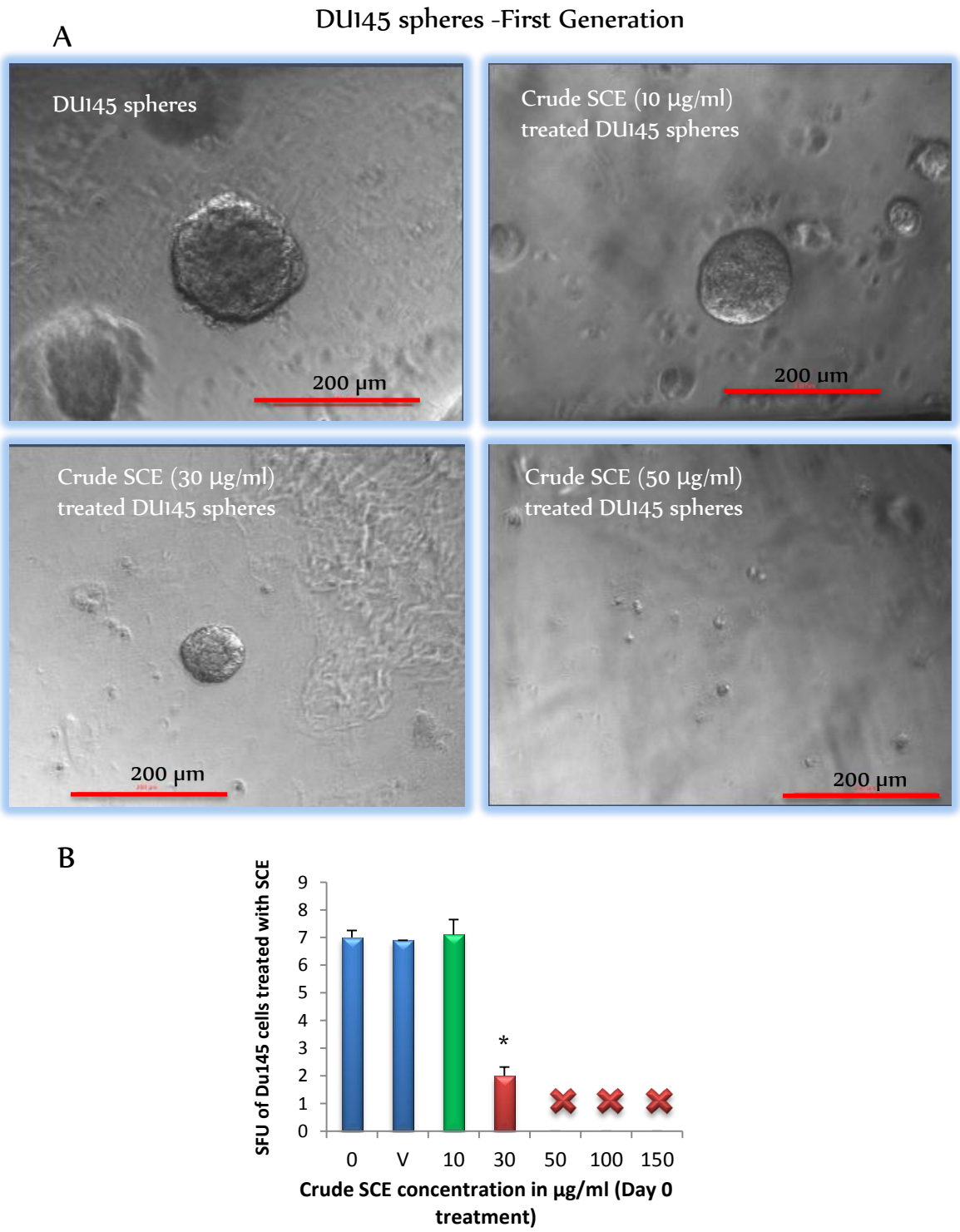
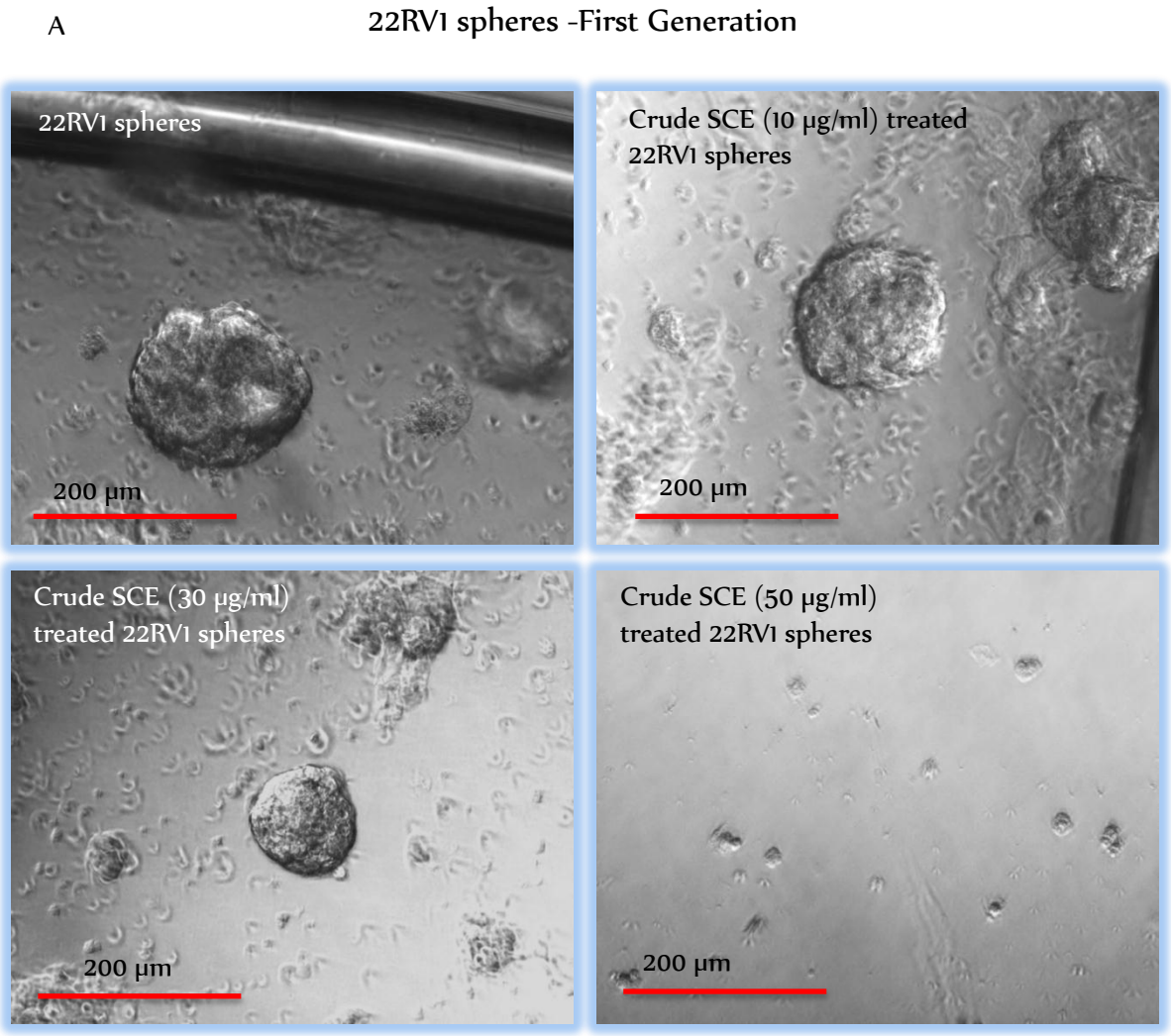


Figure 26. Inhibition of the proliferation and size of the first generation of crude SCE treated DU145 spheres

A) Representative images of DU145 spheres with and without SCE treatment. Images where visualized by Carl Zeiss microscope at 10x magnification and analyzed by Carl Zeiss Zen 2012 image software.

B) Histogram of the SFU is shown with and without SCE treatment (10, 30 and 50µg/ml). Spheres generated are referred to as G1 spheres (n=3, 2 wells each ±SD; (* $P < 0.05$; # $P < 0.01$)).

A



B

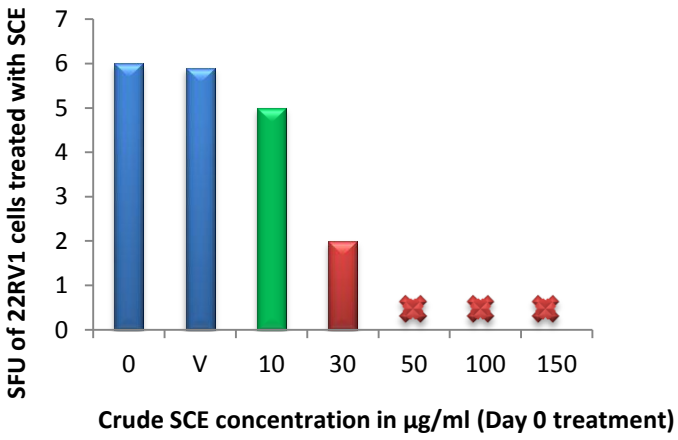


Figure 27. Inhibition of the proliferation and size of the first generation of the crude SCE treated 22RV1 spheres.

A) Representative images of 22RV1spheres with and without SCE treatment. Images where visualized by Carl Zeiss microscope at 10x magnification and analyzed by Carl Zeiss Zen 2012 image software.

B) Histogram of the SFU is shown with and without SCE treatment (10, 30 and 50µg/ml). Spheres generated are referred to as G1 spheres. (n=1)

b. ***Effect of the crude SCE on five generations of DU145 spheres upon propagation***:

DU145 cells that were able to form spheres at lower extract concentrations were propagated further in order to enrich the stem/progenitor pool. Propagation was performed in two conditions, discontinuing treatment and continuing treatment with the same concentration. Upon propagation, protospheres were collected with dispase, dissociated into single cells and seeded again to form spheres of the second generation (G2). The assay was performed till the fifth generation (G5) (Figure 28 & 30). As shown in figure 29 and 31, SFU of nontreated DU145 cells increased with propagation reaching to SFU=15 in the fifth generation, reflecting the enrichment of cancer stem/progenitor cells along the generations.

Figure 29 demonstrates that cells from spheres that were treated at day 0 with 30 μg/ml of crude SCE in the first generation and treated again in the second generation weren't able to form spheres in the second generation. Cells from spheres that were treated at day 0 with 10 μg/ml of crude SCE in the first generation and treated again in the following generations weren't able to form spheres in the fourth generation.

Figure 31 shows that cells from spheres that were treated at day 2 with 50 μg/ml of crude SCE in the first generation and treated again at day 2 in the second generation weren't able to form spheres in the second generation. Cells from spheres that were treated at day 2 with 30 μg/ml of crude SCE in the first generation and treated again at day 2 in the second and third generations weren't able to form spheres in the third generation, and those treated with 10 μg/ml of crude SCE at day 2 in the first generation, stopped forming spheres in the fifth generation.

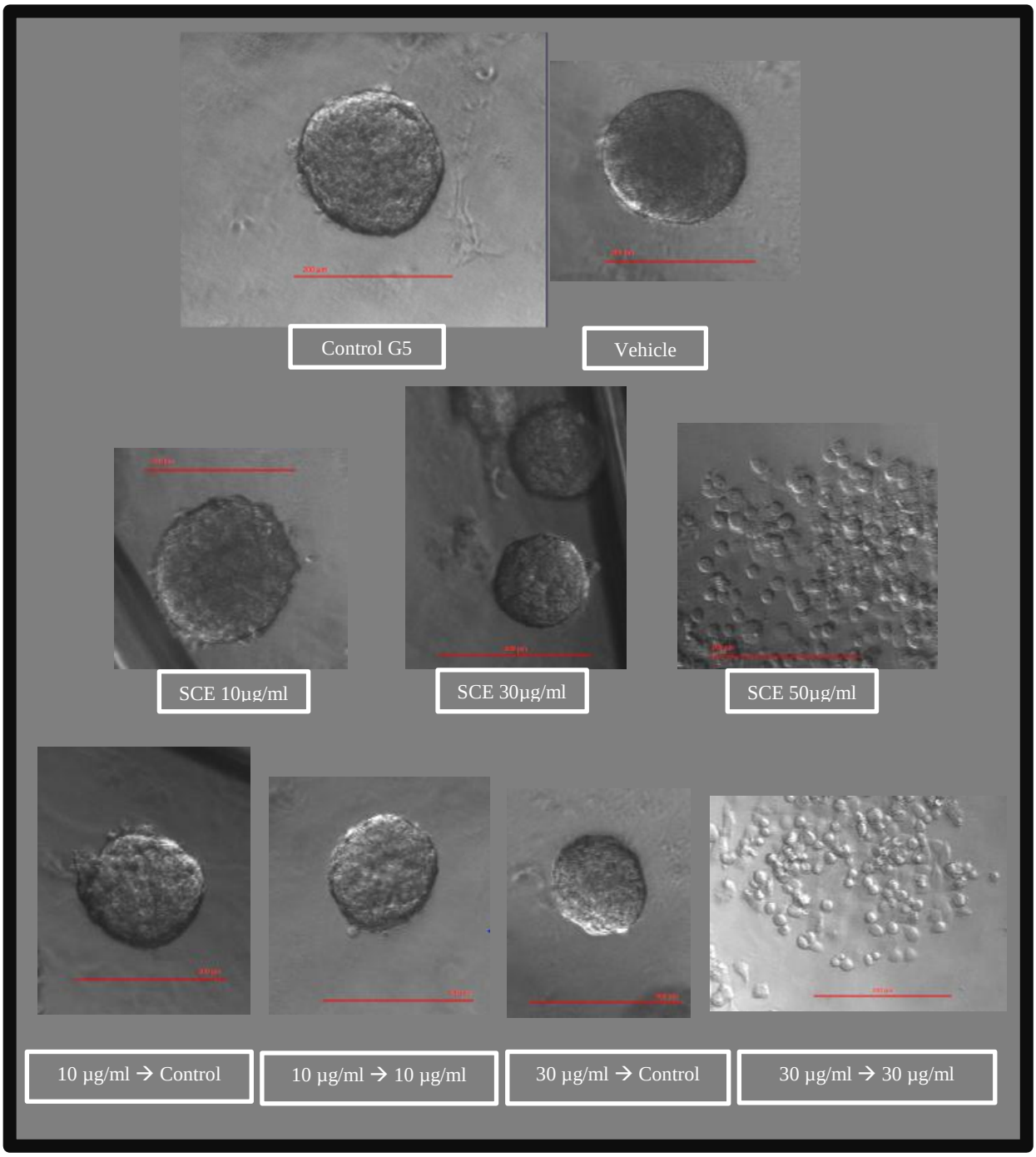


Figure 28. Spheres of the fifth generation treated upon seeding (Day 0)
Representative images of the fifth generation of DU145 spheres with and without SCE treatment. Images where visualized by Carl Zeiss microscope at 10x magnification using Carl Zeiss Zen 2012 image software.

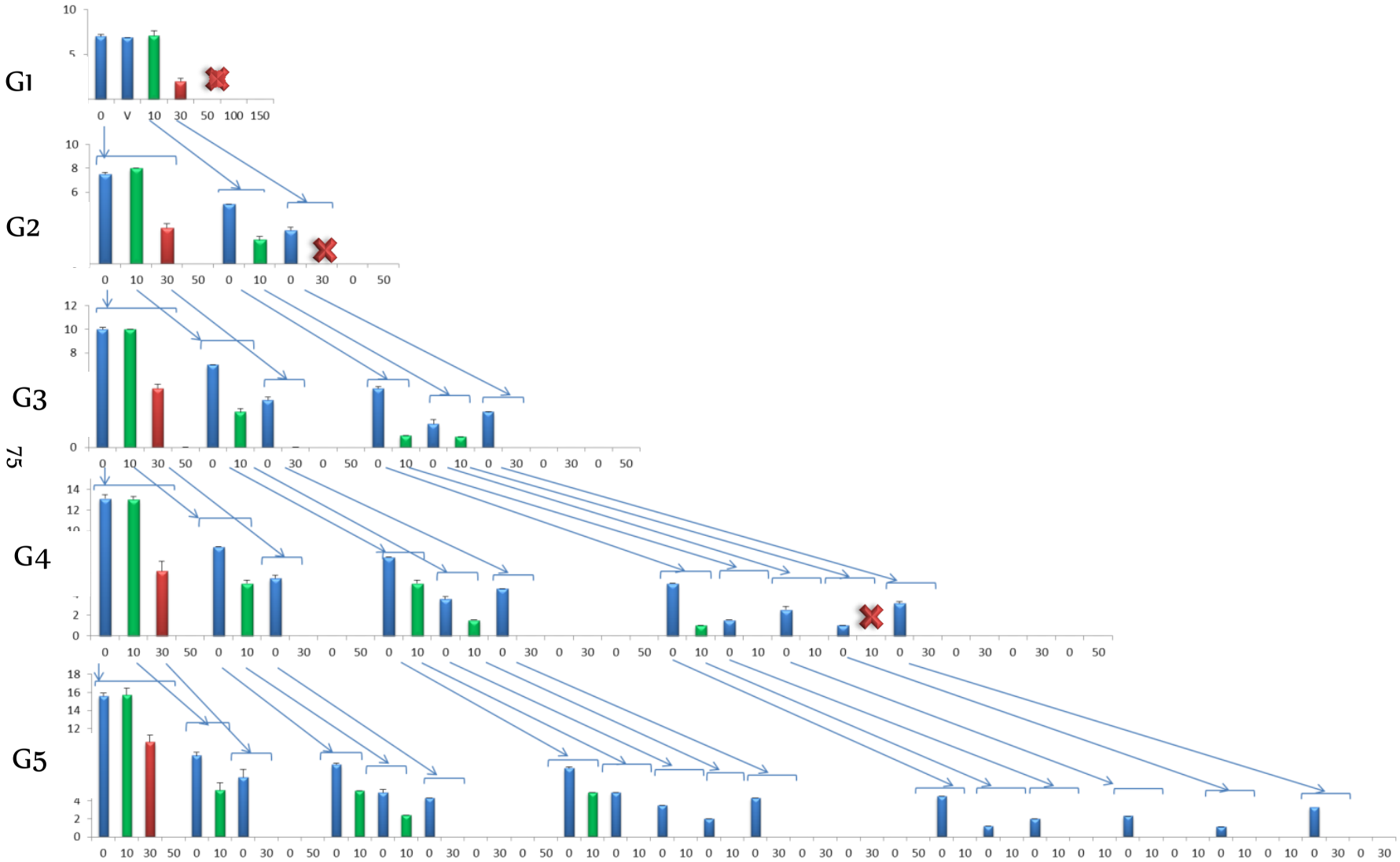


Figure 29. Histogram of the decrease in SFU of five generations of propagated spheres of DU145 cells treated with SCE upon seeding (Day 0).
Spheres were propagated in two conditions: discontinuing SCE treatment and continuing treatment with the same concentration.
Vehicle had no effect on DU145 spheres.

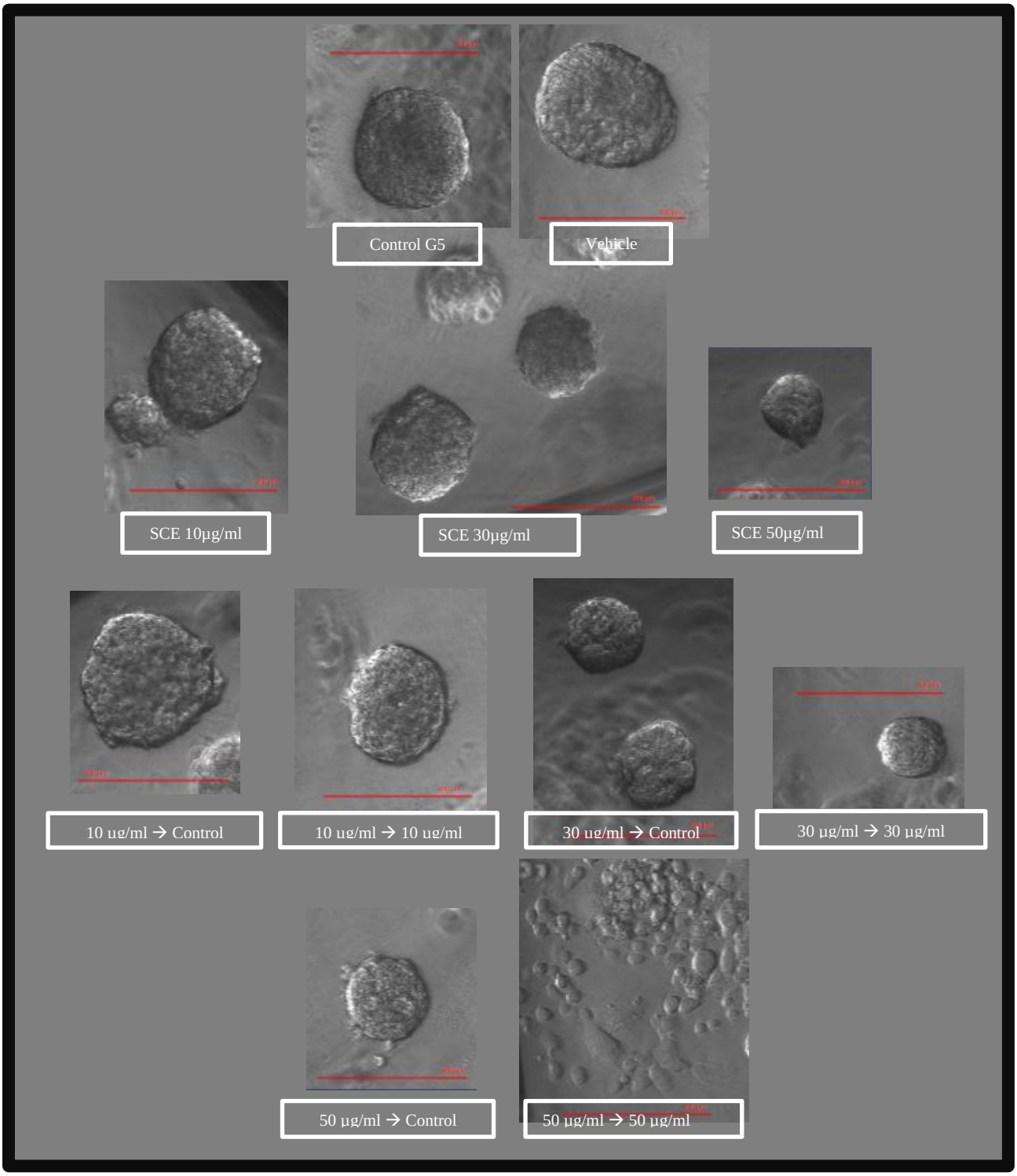


Figure 30. Spheres of the fifth generation treated after two days of seeding (Day 2).
Representative images of the fifth generation of DU145 spheres with and without SCE treatment. Images where visualized by Carl Zeiss microscope at 10x magnification using Carl Zeiss Zen 2012 image software.

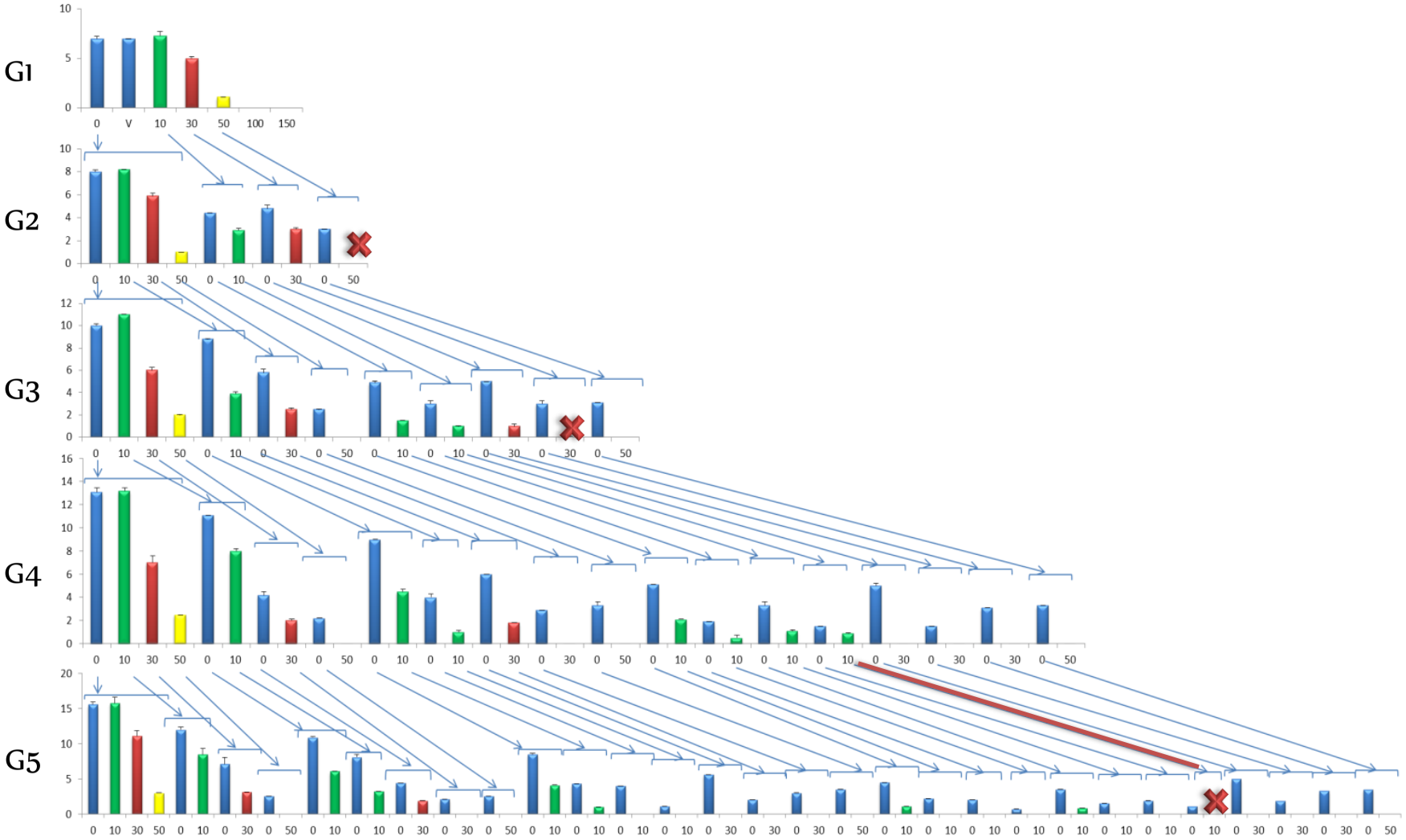


Figure 31. Histogram of the SFU decrease of five generations of propagated spheres of DU145 cells treated with SCE after two days of seeding (Day 2).

Spheres were propagated in two conditions: discontinuing SCE treatment and continuing treatment with same concentration Vehicle had no effect in sphere formation ability.

c. Effect of the crude SCE on the size of DU145 spheres upon propagation

Crude SCE decreased the size of spheres starting from 30µg/ml concentration in the first generation of both DU145 and 22RV1 spheres. Figure 32 shows histograms for the effect of SCE on the size of spheres at the first generation (Figure 32-A) and the fifth generation (Figure 32-B) of DU145 spheres. Another histogram was plotted, showing the number of cells per sphere in order to assess whether the decrease in size is due to an inhibition on the proliferation and self-renewal capacity of stem/progenitor cells or due to a decrease in the size of the cell itself.

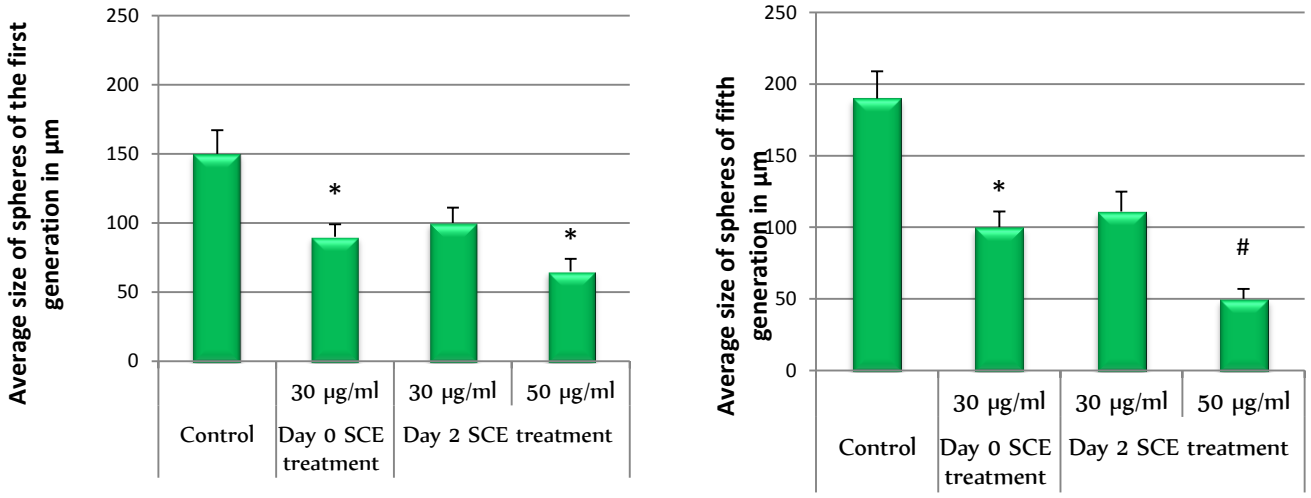


Figure 32. Histograms of the effect of SCE on decreasing the size of spheres in the first (A) and fifth (B) generation of DU145 cells.
Average size of 15 to 30 spheres was calculated from each condition ±SD; (* $P < 0.05$; # $P < 0.01$).

As shown in figure 33, the number of cells per sphere decreased, suggesting that the effect of SCE on the size reflects an inhibition in the proliferation potential of DU145 stem/progenitor cells.

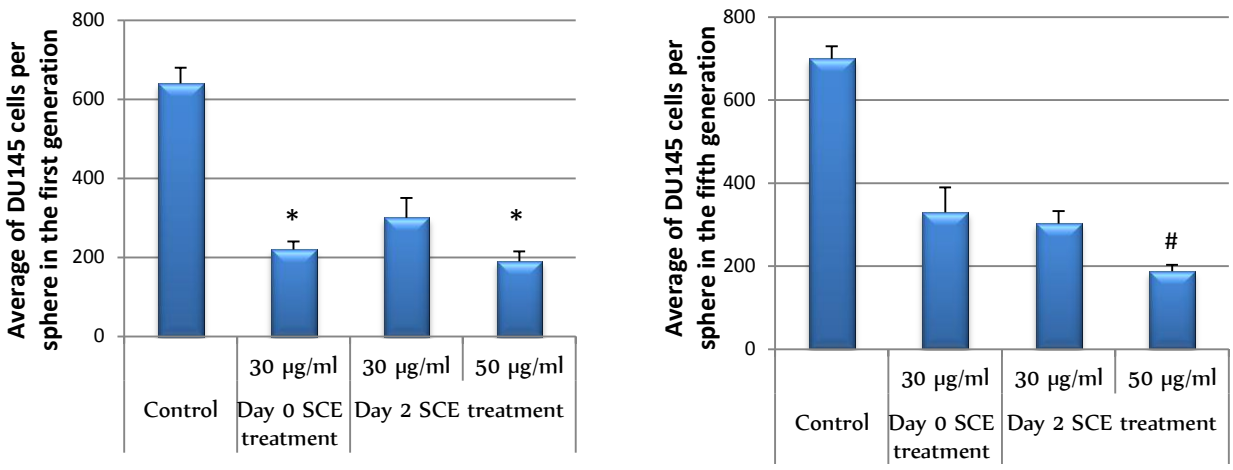


Figure 33. Effect of SCE on decreasing the number of cells per sphere in the first (A) and fifth (B) generation
Number of cells/spheres was calculated in two independent experiments ±SD; (* $p < 0.05$; # $p < 0.01$).

d. Effect of the crude SCE on SCE pre-treated DU145 spheres

DU145 cells were treated in 2D culture with 50 µg/ml crude SCE, 40 % decrease in viability is obtained according to trypan blue exclusion assay (Figure 9), the remaining 60% were collected 48h post-treatment and seeded in a 3D culture (Matrigel around the rim of the wells). Treatment started at day 0 and day 2. As a result, the number of formed spheres (SFU) increased in the un-treated cultures (Figure 34), simulating the results obtained when performing propagation as a tool to enrich for stem/progenitor cells.

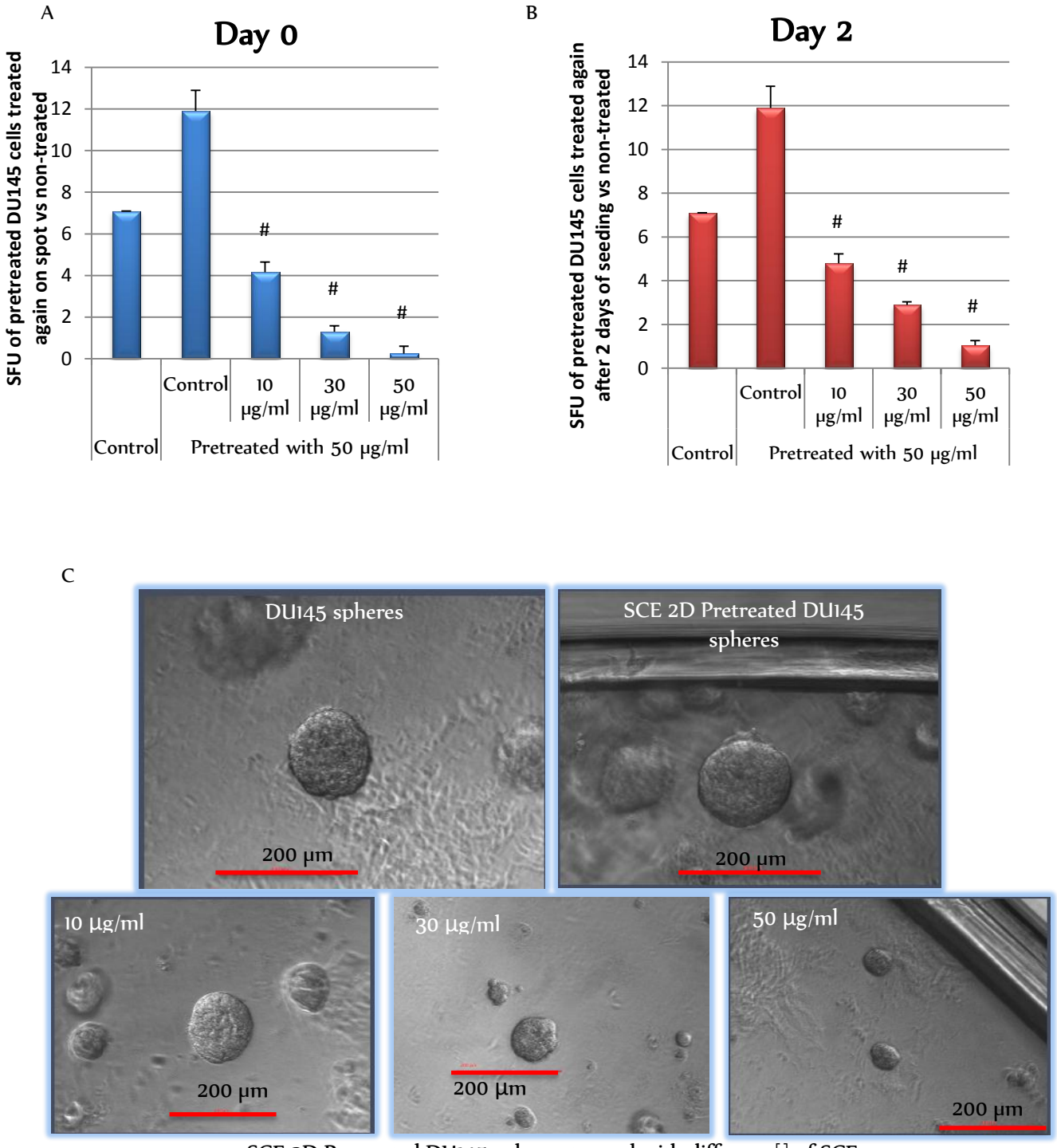


Figure 34. SCE 2D-pretreated DU145 spheres treated with different concentrations of crude SCE. Histograms presenting the effect of crude SCE on the SFU of SCE 2D pre-treated DU145 cells that were post-treated at day 0 (A) and day 2 (B). (C) Representative images of the SCE 2D pre-treated DU145 spheres post-treated at day 0. Images where visualized by Carl Zeiss microscope at 10x magnification using Carl Zeiss Zen image software. (n=3 ±SD; (* $p < 0.05$; # $p < 0.01$))

e. Effect of the crude SCE on cancer stem cells markers

1) Western Blot

The effect of SCE on CSCs was further evaluated by western blotting on the CSC markers: CD44 and CXCR4. Proteins were extracted from the fifth generation of spheres. Figure 35 shows the effect of SCE in 2D and 3D culture. An upregulation of CD44 and CXCR4 was observed in the spheres of the fifth generation, as well as in the 2D treated DU145 cells.

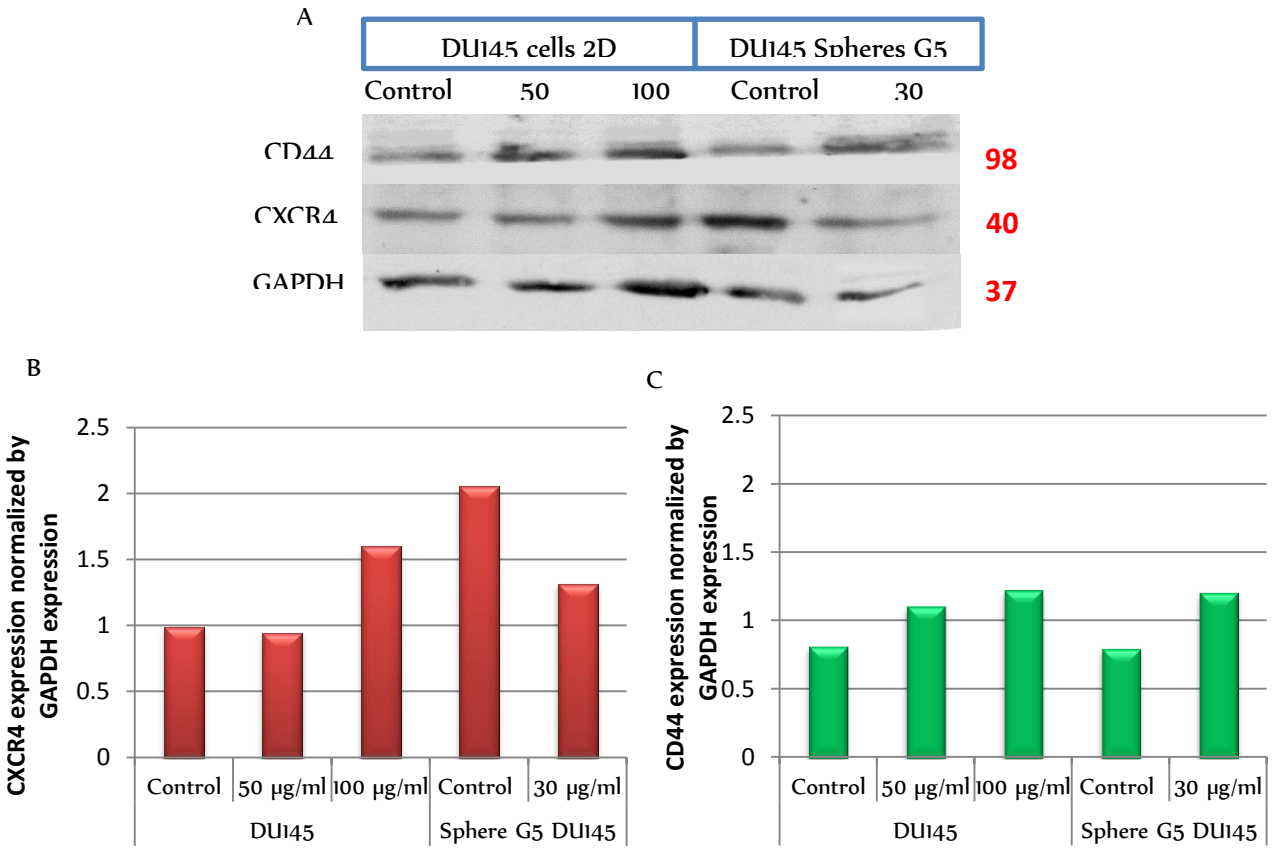


Figure 35. Western blot analysis of CSC markers on DU145 cells in 2D and 3D cultures.

A) Western densitometry analysis
B) Histogram of the expression of CXCR4 normalized by GAPDH
C) Histogram of the expression of CD44 normalized by GAPDH

2) Immunofluorescence

Immunofluorescence staining of SCE-treated DU145 spheres (3D) and SCE-treated DU145 cells (2D) was assessed for CD44 and CXCR4 CSC markers. As shown in figure 36, the intensity of CXCR4 and CD44 expression increased in SCE-treated 2D-DU145 cells, suggesting that the cells that survived the treatment are the resistant cells that hold mesenchymal and stem-like properties. Preliminary data showed that CD44 and CXCR4 were expressed in non-treated spheres; however, in treated spheres CD44 expression was not altered and CXCR4 expression was slightly decreased (Figure 37).

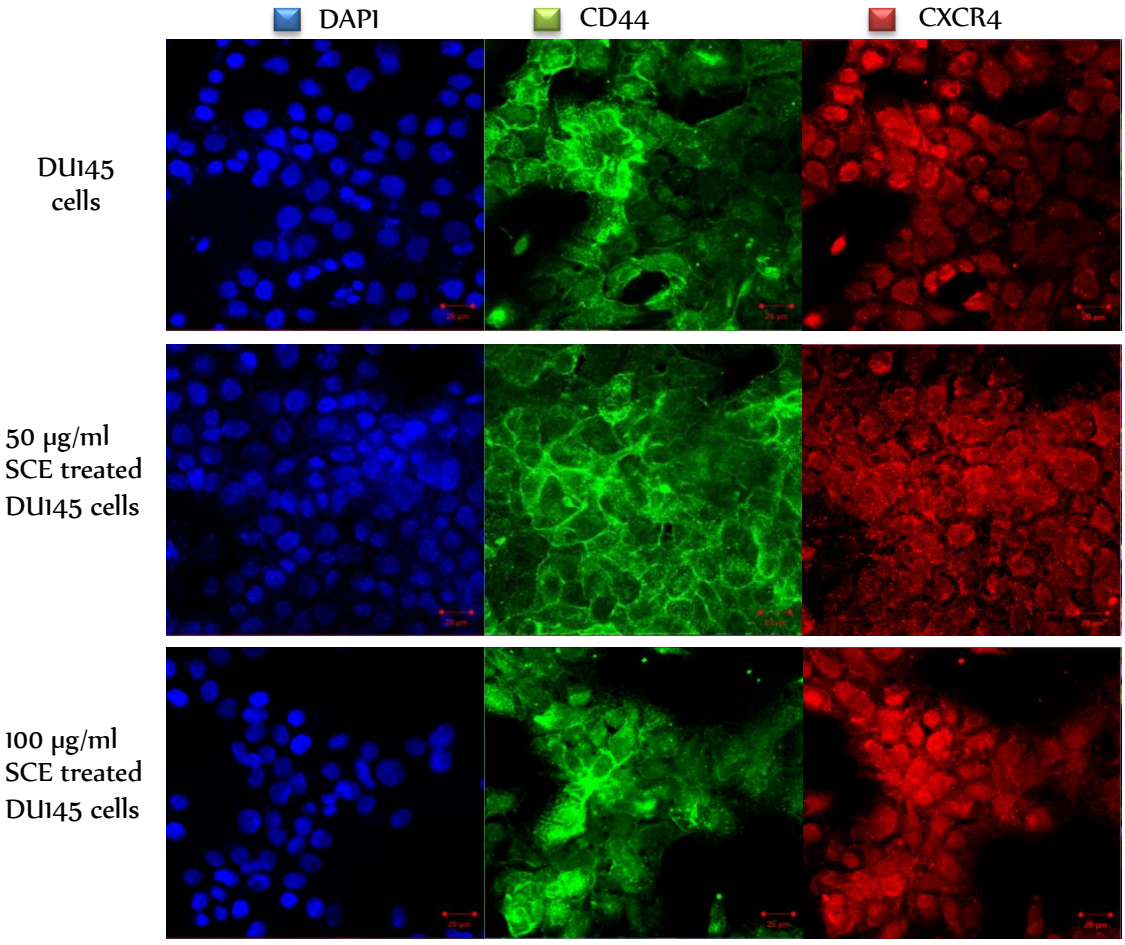


Figure 36. Expression of CD44 (Green) and CXCR4 (Red) on DU145 cells (2D) after 48h treatment with 50 and 100 µg/ml of SCE using oil lens LSM 710 Confocal Laser Microscope at 40x magnification

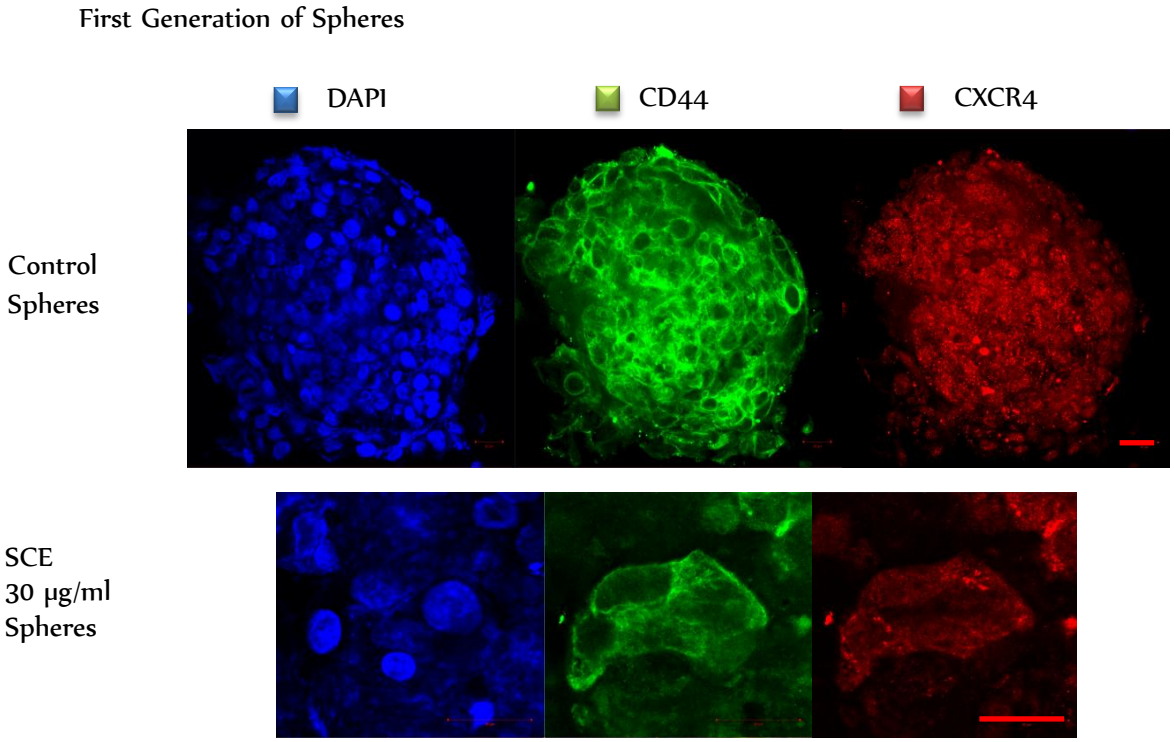


Figure 37. Expression of CD44 (Green) and CXCR4 (Red) on DU145 spheres (3D) after treatment with 30 µg/ml of SCE using oil lens LSM 710 Confocal Laser Microscope at 40x magnification

7. Effect of the aqueous fractioned SCE on DU145 cells:

A schematic representation is shown in figure 38 showing the method utilized to fractionate the crude SCE. Based from data obtained in our laboratory, the aqueous fraction was found to have the most potent bioactive material. The effect of the aqueous fraction of SCE was further assessed on prostate cancer cells.

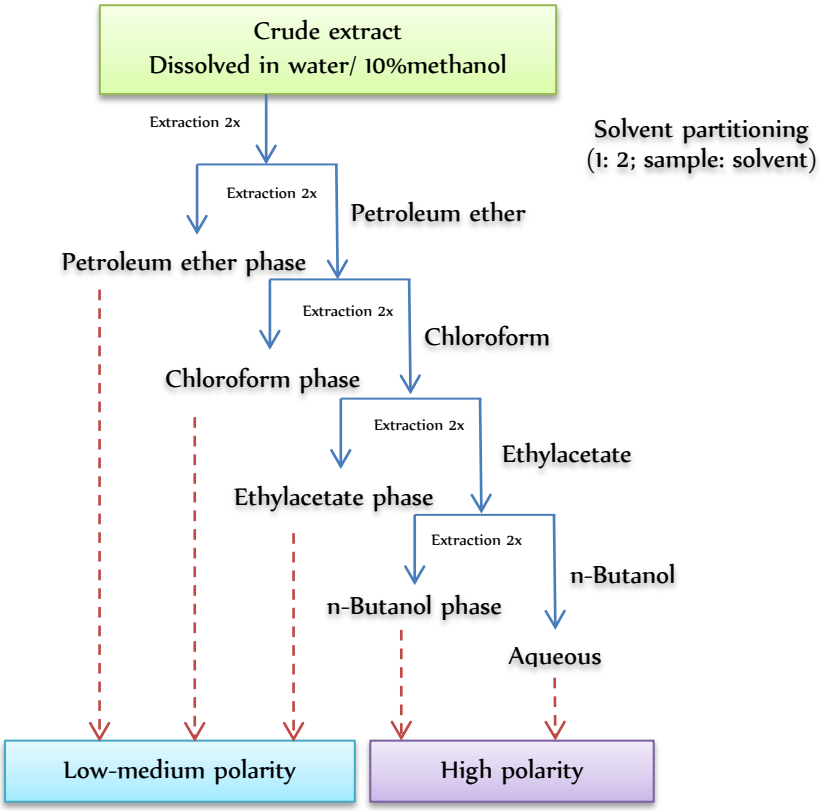


Figure 38. Schematic representation of the fractionation performed on the crude SCE.

a. Effect of the aqueous SCE on the proliferation DU145 cells using trypan blue exclusion assay

To assess the effect of the aqueous SCE on the viability and proliferation of DU145 cells, trypan blue exclusion assay was performed. Treatment of different concentrations of aqueous SCE was applied and tested in three time points: 24, 48 and 72 hours.

Figure 36 demonstrates the effect of different concentrations of the aqueous SCE on DU145 cells expressed as percentage of control, showing that the aqueous SCE inhibits the proliferation of DU145 in a time and dose dependent manner, being more obvious after 72h of treatment.

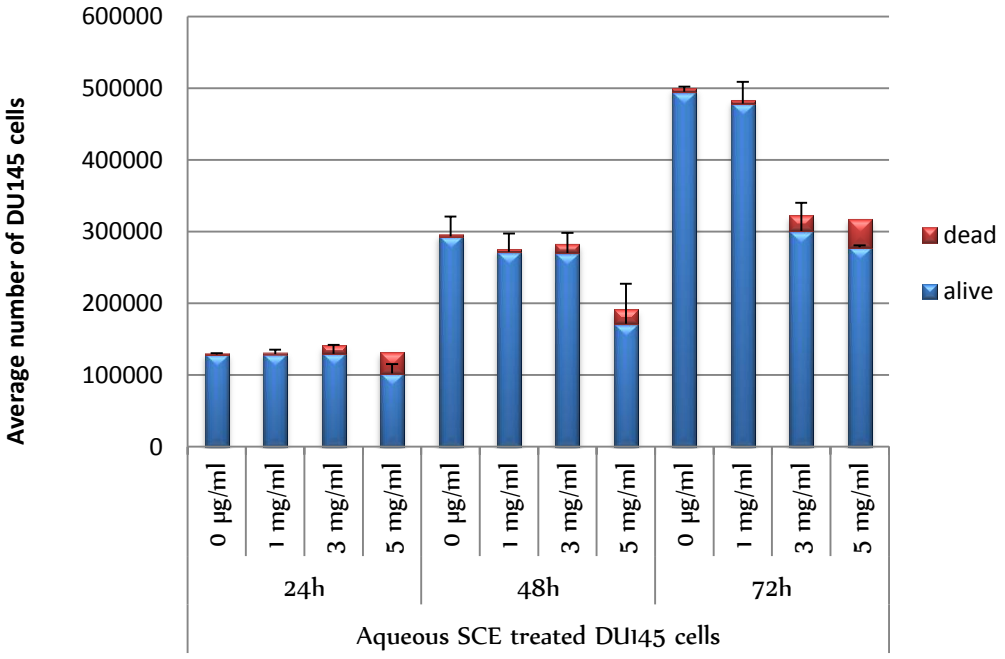


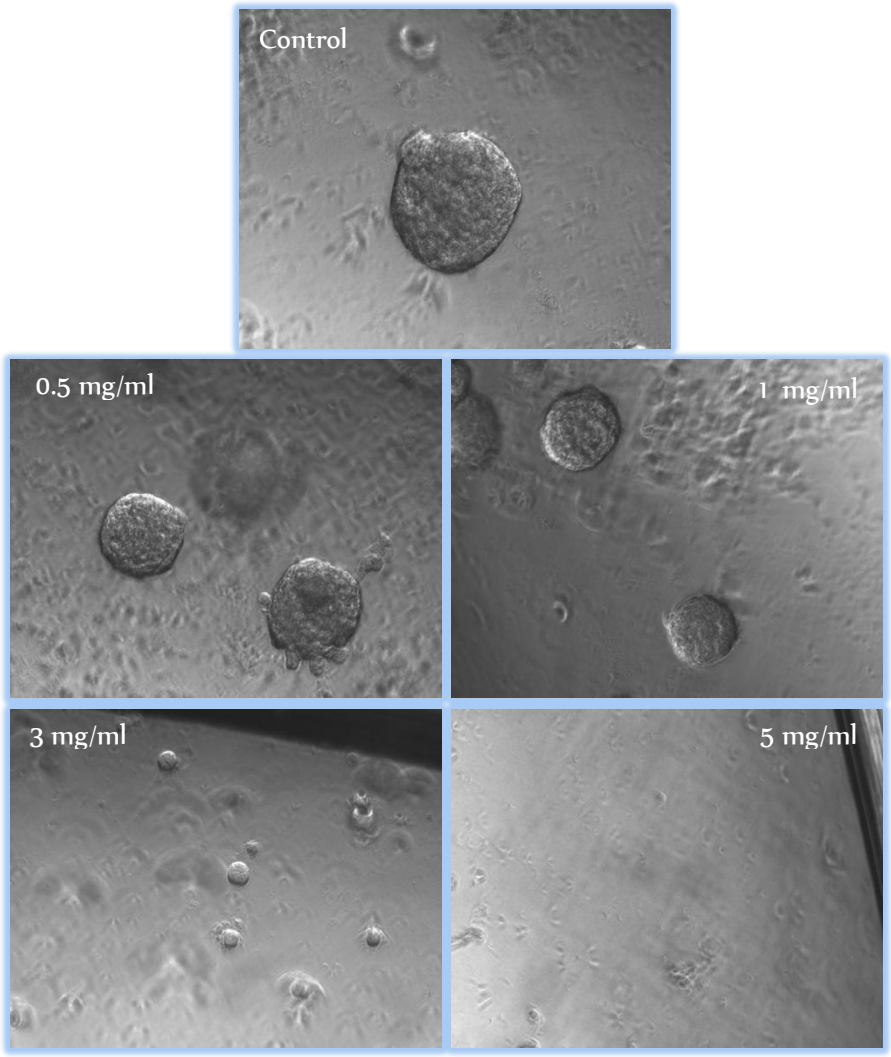
Figure 39. Anti-proliferative activity and reduced viability of DU145 cells in response to treatment with various concentrations of the aqueous SCE.
After 24, 48 and 72 hours, viability was determined by trypan blue exclusion assay. Preliminary results are expressed as total number of cells (viable and dead) of studied group compared to its control.

Aqueous SCE had a slight inhibitory effect after 24 h of treatment with 5 mg/ml and a significant inhibitory effect after 48 h of treating with 5 mg/ml. Aqueous SCE decreases the proliferation of DU415 cells significantly after 72 h of treatment with concentrations ranging between 3 to 5 mg/ml.

b. Effect of the aqueous SCE on an enriched population of stem/progenitor DU145 cells using sphere formation assay

Treatment of DU145 spheres was performed at the day of seeding (Day 0). A small number of cells were able to form spheres in untreated conditions (SFU=6.5).

A



B

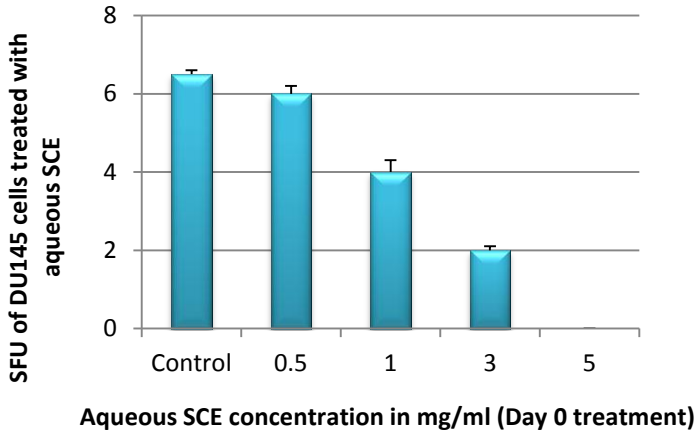


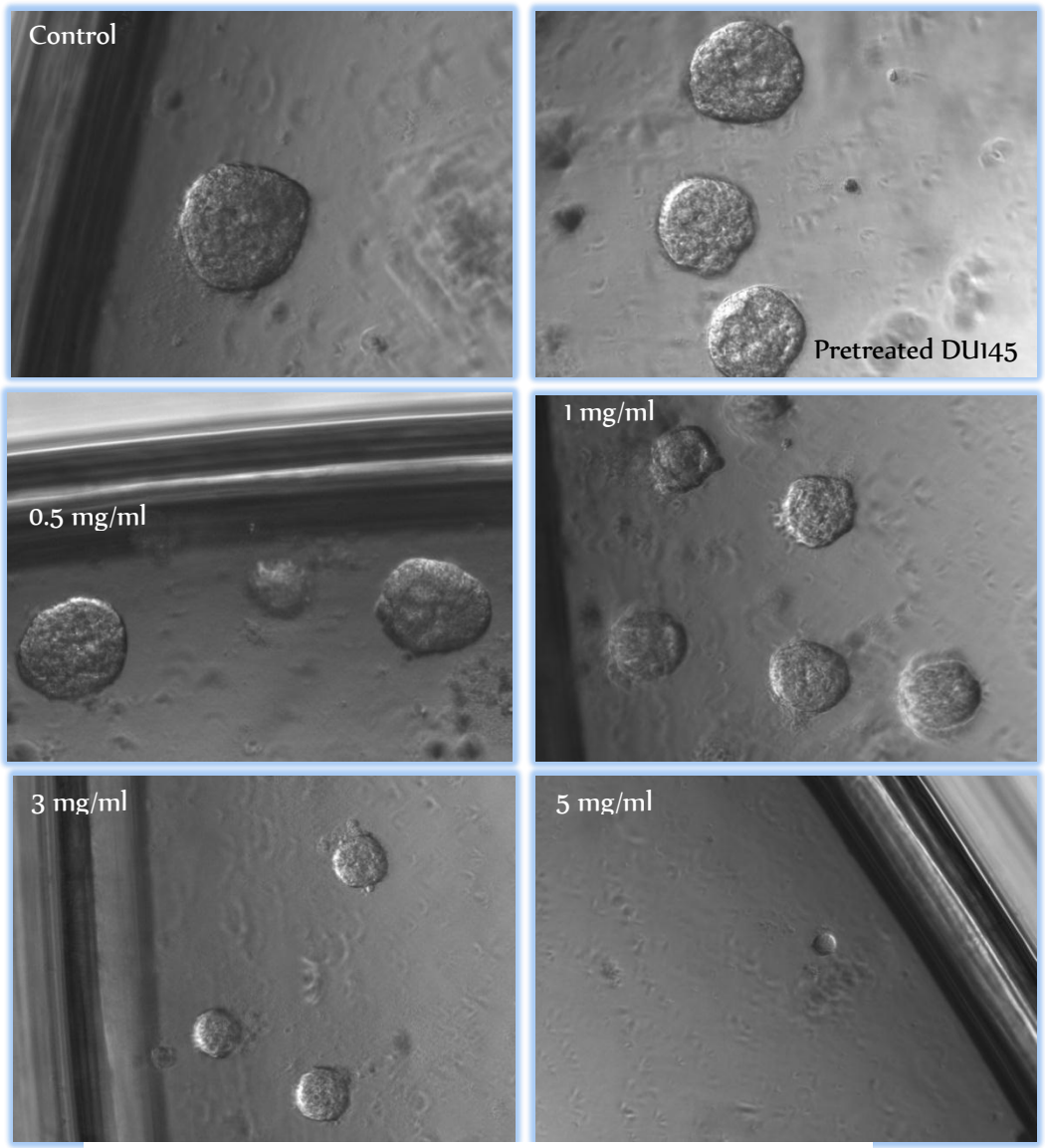
Figure 40. Aqueous SCE effect on DU145 fisrt generation of spheres.

A) Representative images of the first generation of DU145 spheres treated with the aqueous SCE (0.5, 1, 3 and 5 mg/ml)

B) Histogram of the SFU of DU145 spheres after treatment with aqueous SCE (n=1, duplicates ±SD)

As shown in figure 40, the aqueous SCE decreased the size of spheres when treating with 0.5, 1 and 3 mg/ml in a dose-dependent manner. However, no protospheres were able to form when treated with 5 mg/ml aqueous SCE. Furthermore, the remaining DU145 cells in 2D after 72 h treatment with the aqueous SCE were seeded in a 3D culture, and the aqueous SCE was assessed for its ability to target a more enriched pool of stem/progenitor cells. Aqueous SCE showed to decrease the size of pretreated DU145 cells in a dose-dependent manner, preventing any ability to form spheres at 5 mg/ml.

A



B

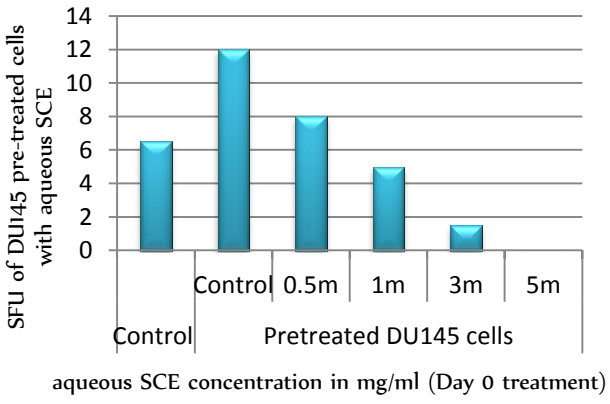


Figure 41. Aqueous SCE effect on pretreated DU145 cells' ability to form spheres.

A) Representative images of the first generation of DU145 spheres treated with the aqueous SCE (0.5, 1, 3 and 5 mg/ml)

B) Histogram of the SFU of DU145 spheres after treatment with aqueous SCE

DISCUSSION

Cancer is a major cause of death around the globe, accounted for 7.6 million deaths (around 13% of all deaths) in 2008. Lung, breast, colorectal, stomach, and prostate cancers are the main cause of cancer deaths. Europe and the America had the highest incidence of all types of cancer. The eastern Mediterranean region had the lowest incidence rates. And because men in the WHO Americas Region had the highest rates of prostate cancer, followed by the WHO Europe Region and the lowest rate of prostate cancer was in the WHO South East Asia Region (Jemal et al., 2008), prostate cancer was used in this study as a model of solid malignancy to investigate the potential of the rich biodiversity of the sea of harboring sources of therapy since they play a major part of the dietary intake of eastern communities. Metastatic PC remains an uncured disease by the present therapeutic approaches due to the presence of resistant cells in the tumor that are responsible for cancer initiation, progression and relapse, these cells are the so-called cancer stem cells; the most crucial target in the treatment of cancer. Several studies have shown that sea cucumbers of different species have anti-cancer and anti-metastatic ability (WAJP et al., 2013) yet very few studies were performed on the species of *Holothuria Polii* that is found mostly in the Mediterrenian sea, with only one showing anticancer activity on MCF7 and HCT116 (Omran & Khedr, 2012). In this study, *Holothuria polii* sea cucumbers were collected from the Mediterranean Sea coast of Lebanon and ethanolic crude extracts were isolated and used to test its potential effect not only on the proliferation of prostate cancer cells *in vitro*, but also on its potential to have an anti-metastatic ability, targeting the core of malignancy in tumors.

Three methodologies were used to first test the effect of crude SCE on the proliferation and viability of two prostate cancer cell lines, DU145 and 22RV1 cells: trypan

blue exclusion assay, MTT assay and RTCA. Crude SCE inhibited the proliferation and viability of both DU145 and 22RV1 cells in a time and dose dependent manner, with RTCA demonstrating a significant inhibitory effect starting with lesser concentrations of SCE than the ones used in trypan blue and MTT assays, since it is the most sensitive method utilized (Limame et al., 2012). The degree of this effect slightly varied from one cell line to another affecting DU145 cells to some extent more than the non-metastatic 22RV1 cells, indicating that SCE effect may be more potent on metastatic cells, so DU145 cells were chosen in the following experiments to further understand the effect of SCE. The inhibitory effect on proliferation theoretically is induced by either halting the proliferation pathway (cell cycle arrest) or by causing cell death to a certain extent. In order to assess how SCE is affecting the proliferation of DU145 cells, cell cycle analysis was performed using flow cytometry. Analysis was performed after 48 hours of treatment with 50 and 100 μg/ml of SCE. Data of five independent experiments revealed a slight cell cycle arrest in G0-G1, and a major increase in Pre G0-G1, suggesting cell death. Hoechst staining was performed, and it appeared that several stages of apoptosis were prominent in SCE-treated DU145 cells, like cell shrinkage and the formation of apoptotic bodies, suggesting that apoptosis is the mechanism of cell death occurring upon treatment with crude SCE. To confirm this conclusion western blot (WB) and immunofluoroscence (IF) were studied on apoptotic markers: Bcl2, γH2AX and PARP. Bcl2 being an anti-apoptotic regulator protein of cell death and overexpressed in almost all cancers, is inhibited with SCE treatment and its translational expression is reduced according to WB and IF results. γH2AX and PARP, both involved in DNA damage repair, have the ability to bind to DNA breaks. In cells undergoing apoptosis, PARP is cleaved from a full length 116 kD peptide into 98 kD and 24 kD polypeptides, thus preventing DNA damage repair. PARP helps cells to maintain their viability; cleavage of

PARP facilitates cellular disassembly and thus serves as a marker of cells undergoing apoptosis. WB showed PARP cleavage upon treatment with SCE, and IF results demonstrated an increase of both γH2AX and PARP, confirming apoptosis occurrence.

The ability of cancer cells to metastasize to distant organs is the most life threatening stage of cancer. Thus despite the ability of SCE to inhibit proliferation through inducing apoptosis, its effect on EMT and metastasis was examined, by first using RTCA to assess the migration and invasion of cells upon SCE treatment. Results of RTCA showed an inhibition in both the migration and invasion of DU145 cells upon treatment with SCE in a time and dose dependent manner. SCE pretreated DU145 cells migration continued to be inhibited; yet a very slight increase in invasion occurred in SCE pretreated DU145 cells, since the percent of resistant cells in the pretreated cells are more than the non-treated DU145 cells, it is logical that they harbor a more potent invasive nature. Wound healing assay was further implemented to confirm the inhibitory effect on migration, and results demonstrated that the wound scratched on cell cultures containing Mitomycin; weren't healed or closed, confirming the inhibition of migration upon SCE treatment. Gelatin Zymography results showed an increase in MMP9 and MMP2 upon SCE treatment, yet further experiments need to be done to better understand if this increase is due to the presence of MMPs in the extract itself or due to an induction of MMPS from DU145 cells favoring invasion. Real time PCR was additionally performed to study the effect of SCE on the transcriptional level of certain metastatic and EMT markers. No significant effect was shown on the gene expression of twist, snail, E-Cadherin and VEGF. Yet on the translational level, WB and IF showed a significant effect in inhibiting Vimentin, a mesenchymal marker. Yet, both also showed a decrease in E-Cadherin, which is an epithelial marker, leading to the conclusion that since SCE is inducing apoptosis,

and apoptosis causes retraction of cells in its early stages, that maybe this is causing the loss of E-Cadherin along with the mesenchymal marker. The effect of SCE on the overexpression of EZH2 in DU145 cells was also assessed using WB. EZH2 being a gene silencer, its overexpression silences the tumor suppressor genes thus promoting tumorigenesis and metastasis; WB results showed that SCE treatment downregultates EZH2 expression in DU145 cells. Thus, crude SCE has an anti-metastatic effect in DU145 cells.

Relapse is unfortunately observed after chemotherapeutic treatments (such as Docetaxel) (Tannock et. al, 2004) because these drugs tend to target the fast proliferative cells in the bulk tumor, potentially missing the quiescent or the slow dividing CSCs. CSCs that survive chemotherapy would have the ability to re-enter the cell cycle and produce highly proliferative-rapidly dividing progenitor cells that can reestablish the tumor. It is even probable that successive cycles of chemotherapy would intensify a tumor by inducing CSCs to produce new therapy resistant cells. Thus it is crucial to find an advanced drug that is effective on the whole tumor, including the CSCs within it. For this, the effect of SCE on this subpopulation of stem/progenitor cells was assessed using sphere formation assay. Data obtained showed an inhibitory effect in the sphere formation ability in a dose dependent manner in response to treatment with SCE which is accomplished with the decrease in the sphere size and the number of cells per sphere. Enrichment for the subpopulation of stem/progenitor cells is accomplished by either propagating or by pretreating DU145 cells with SCE and then seeding them to form spheres (Figure 42). Results from both methods further showed that SCE has an inhibitory effect on the ability of cells to form spheres, suggesting that the crude SCE targets this resistive subpopulation of stem/progenitor cells thus it has the potential to eradicate the whole tumor and prevent recurrence. Previous studies

have also shown the ability of certain compounds to target prostate CSCs using sphere formation assay. Genistein (Zhang. Et al, 2012) and Gamma-tocotrienol (Luk et.al, 2011) are of the thoroughly studied ones.

Additionally, the fractioned SCE in its aqueous phase was further tested and preliminary data showed that it inhibits the proliferation of DU145 cells using trypan blue exclusion assay, and that it also targets the enriched population of stem/progenitor cells by inhibiting their sphere forming ability in the first generation and also in pretreated DU145 cells.

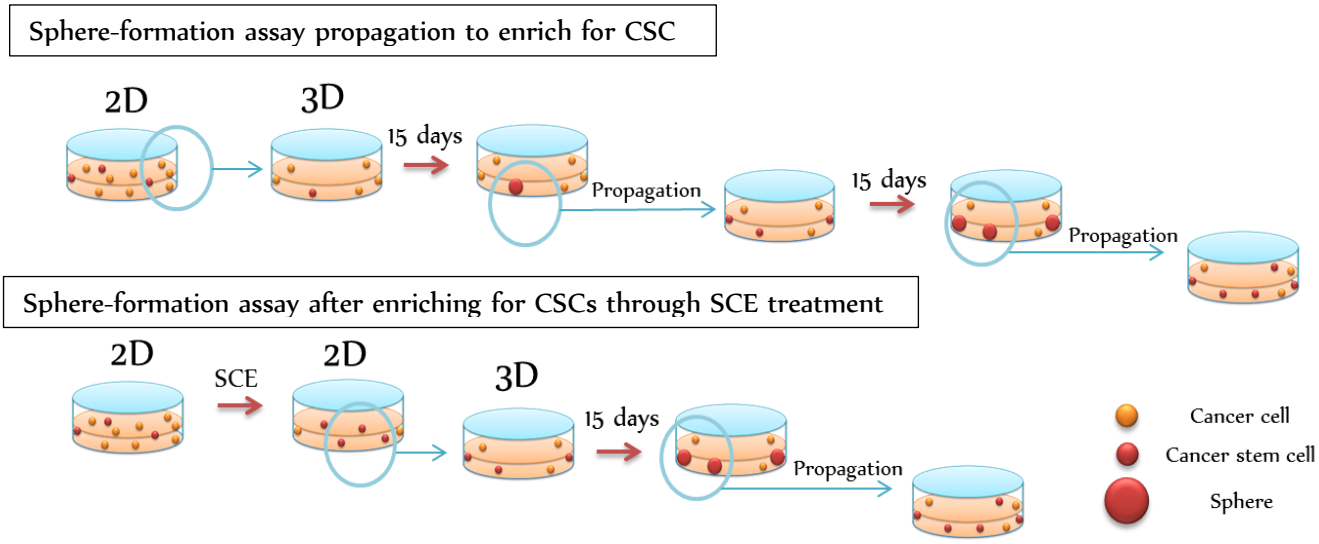


Figure 42. Schematic figure explaining different ways of enriching for CSCs

In summary, our data suggest that SCE has anti-cancer and anti-metastatic potential, and that it also targets prostate cancer stem/progenitor cells. For future prospects, before advancing to clinical trials, the cytotoxicity of this extract should be first tested *in vitro* on normal cell lines and normal stem cells. Furthermore, this study should be complemented with an *in vivo* study to further validate our findings.

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