EXAMINATION OF THE EFFECTS OF CONFINED MIGRATION ON ANDROGEN RESISTANT PROSTATE CANCER AND POTENTIAL TREATMENT OPTION FOR DRUG RESISTANT PC3 CELLS WITH CALCITRIOL

by

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Abstract

EXAMINATION OF THE EFFECTS OF CONFINED MIGRATION ON ANDROGEN RESISTANT PROSTATE CANCER AND THE USE OF CALCITRIOL AS A TREATMENT OPTION FOR DRUG RESISTANT PC3 CELLS

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When localized to the prostate, prostate cancer (PCa) has a near 100% 5-year survival rate, however, when prostate cancer metastasizes the survival rate drastically drops to around 30%. The low death rate of primary PCa is due to the wealth of knowledge we have of non-metastatic cancer and the sufficient treatment regimens. However, this understanding does not apply to metastatic cancer, so therapies are greatly lacking. To gain a better understanding of the invasion process for PCa we utilized microchannel technology to uncover the differences between migratory and non-migratory PCa. Specifically, the following study examines the effects of confined migration on castrate-resistant prostate cancer cell line PC3 in terms of phenotypical shifts and genetic aberrations. Based off our findings and current literature, we believe that migration through a 3D confined space induces epigenetic changes that boost cancer stem cell (CSC) like behavior, expression of efflux and detoxifying proteins and temporarily cause cell cycle arrest which all contribute to a dramatic increase in therapeutic resistance. PC3 cell viability following treatment with doxorubicin was nearly 10 times higher in cells which had undergone migration in comparison to cells which had not. Though the increased resistance is multifactorial, we focus on how the chemotherapy resistance is a result of migration induced escalation in drug deactivation

and drug discharge. Furthermore, we show that PC3 cells which experience confined migration are able to maintain a dormant phenotype for up to 7 days ultimately increasing the cells therapy resistant capabilities. In addition, we show some of the major CSC markers for PCa increased expression after migration including CD133, ALDAH1, Nanog, and EGFR. Due to the increase in resistance we examined if calcitriol (Vitamin D) could enhance the antitumor activity of chemotherapeutic drugs. Our work demonstrates that calcitriol causes migration induced therapy resistant PC3 cells to become greatly more responsive to treatment. With this information we provide a potentially novel method for treatment of therapy resistant prostate cancer using FDA approved calcitriol.

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Chapter 1: Introduction

1.1 Prostate Cancer

1.1.1 Statistics

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer deaths next to lung and bronchus cancers in American men [1]. According to the American Society of Clinical Oncology the 5-year survival rate of men with prostate cancer localized to the prostate is nearly 100%, however, as soon as it metastasizes to other tissues the 5-year survival rate drops to only 30%. There is a shortcoming in treatment for metastatic cancer which is in need of immediate attention because metastatic PCa diagnoses are on the rise and are predicted to increase by 42% over the next decade, with nearly 16,000 new cases of metastatic PCa expected by 2025 [2].

1.1.2 Androgen dependence

There are two categories of prostate cancer, one that is receptive to hormone therapy known as androgen dependent, and those that do not respond to hormone therapy termed castrate-resistant prostate cancer (CRPC). One of the main reasons survival rates are so high is due to effective implementation of androgen depletion therapy, however, this does not work on CRPC cell lines [3]. Currently there are no chemotherapeutic drugs which directly target CRPC cells, leaving a massive gap in treatment for patients who are in the later stages of the disease [4].

1.1.3 Current treatment approaches

Determination of which type of therapy is appropriate for a patient strongly depends on what stage the disease is in. PCa is unique in that it often progresses very slowly; some men go their entire life without knowing they have PCa. If a patient is not experiencing severe symptoms or is already in poor health, physicians may opt to observe the patient and monitor prostate-specific antigen levels at a more frequent rate. A radical prostatectomy, surgery in which the prostate gland and

some surrounding tissues are removed, is a very common treatment option so long as the disease is still localized to the prostate [5]. Radiation therapy, hormone therapy and chemotherapy are often used in conjunction with one another especially in more advanced stages. However it should be noted that chemotherapy is not the standard treatment for PCa in early stages [6] [7]. In the unfortunate, yet not uncommon event that the cancer has metastasized to the bone, there are specific treatments to hinder further progression, prevent fractures, and reduce pain. These treatments include the use of drugs which slow osteoclast activity, corticosteroids, radiopharmaceuticals, and in some cases surgery to stabilize collapsed bone [8] [9].

1.2 Metastatic prostate cancer

1.2.1 Invasion process

The metastatic process for prostate cancer begins with the cells undergoing epithelial-to-mesenchymal transition (EMT), during this process they lose their epithelial phenotype and undergo cytoskeletal shifts and protein expression changes in order to acquire mesenchymal characteristics. When migrating to surrounding tissues, PCa cells can either move as a collective or as a single cell [10]. One of the first changes in PCa cells which induces migration is the reduction in E-cadherin, this allows for cell detachment causing the cell to be more invasive. In order to migrate, cells utilize enzymes which break down the extracellular matrix and the basement membrane then once they have reached the interstitium the cells must either enter the lymphatic or vascular system. Once in the circulatory system PCa cells will attach to the endothelial cells and undergo transendothelial migration, this is a complex process that involves many adhesive interactions involving selectins and integrins which stabilize the binding [11]. It has been shown that PCa cells more readily bind to bone marrow (BM) endothelial cells which is in accordance

with the fact that 80% of all metastatic PCa is observed within bone. Once within the bone the malignant cells stimulate a homing effect for other PCa cells to invade the bone; this phenomenon relies on two factors- the presence of chemokines and the large availability of energy (lipids) in BM. One of the key players in the homing mechanism is the CXC chemokine SDF-1 and its receptor CXCR4. After establishing in the bone, bone remodeling and micro-metastasis occurs, simply put the PCa cells stimulate bone reabsorption by causing osteoclast overactivity and also increase osteoblast activity which increases bone volume and mineralization leading to an overall weaker bone structure [12].

1.2.2 Potential for Dormancy

An interesting characteristic of PCa is its potential for dormancy, this phenomenon causes a patient to appear cancer free only to relapse years later, likely ending with a fatal diagnosis. Despite the mechanism for dormancy still being unclear, there are three classifications of cancer dormancy, cellular dormancy, angiogenic dormancy and immunological dormancy. Cellular dormancy is when an individual cell is in cell cycle arrest usually due to external stress, angiogenic dormancy stems from a lack of proper vasculature causing insufficient nutrients to reach the tumor, lastly, immunological dormancy occurs when immune "surveillance" keeps the tumor at a fixed size by means of constant cytotoxic activity [13]. It is known that PCa disseminated tumor cells (DTCs) have the potential to become dormant once settled in a distant organ, making the cells resistant to chemotherapeutic agents which target actively dividing cells; an important feature of DTCs is maintaining the ability to proliferate while in the quiescent state [14]. One of the key factors in maintaining dormancy or reactivation of the dormant cells is the microenvironment which surrounds the cell, in most PCa cases the environment is BM. DTCs get signals which alter their phenotype (proliferative or dormant) via cross talk from the cells within the bone BM including

BMP7, TGF-β2 and Axl [15] [16]. What remains unclear about dormant cell reactivation is the time component because patients can be in remission for years, but the signals produced in the microenvironment which reactivate the PCa cells are part of normal BM cell function. Since cancer dormancy proves to be a major factor in poor patient prognosis research that sheds light on dormancy and reactivation mechanisms are desperately needed.

1.3 Vitamin D_3

1.3.1 Use in prevention/treatment

Vitamin D_3 (VD_3), also known as calcitriol, is an FDA approved fat-soluble vitamin synthesized when human skin is exposed to UV-radiation and aids in calcium and phosphorus metabolism [17]. Low VD_3 levels (plasma level of <20 ng/ml) can cause weakening of the bones, beyond this, the low levels have also been linked to an increased risk of getting cancer. Many studies have shown that VD_3 can act as preventive for many cancer types by decreasing cancer cell growth, reducing angiogenesis, promoting apoptosis, and it is a natural anti-inflammatory [18] [19] [20]. However, there are issues in studying calcitriol as cancer preventative because studies evaluating VD_3 intake fail to account for VD_3 produced by exposure to UV. Another consideration is that most people who have proper VD_3 levels tend to have an overall healthier lifestyle, so the low risk could be stemming from other factors [21].

Using calcitriol for treatment has also been evaluated due to its ability to moderate inflammation and oxidative stress. In a study with women who had cervical intraepithelial neoplasia grade 1, patients who were supplemented with VD_3 were more likely to go into remission [22]. High levels of calcitriol has also shown to be effective in increasing event-free survival when used in combination with other chemotherapy agents for treating breast, colo-rectal, prostate, B-cell

lymphomas, and various other cancer types [23] [24] [25]. Additionally, VD_3 supplementation has improved patients' quality of life, specifically for patients with advanced stages of oral cancer they experienced ease of swallowing, decrease in pain while chewing and a reduction in chemoradiation-related toxicities [26].

1.3.2 Potential use for PCa

Calcitriol as a preventive and treatment option for men with prostate cancer has been heavily studied. VD_3 inhibits the growth of normal prostate epithelial cells and primary cultures of PCa cells via induction of cell cycle arrest and apoptosis [27]. Men with prostate cancer who also had reduced VD_3 levels exhibited an increased expression of inflammatory mediator compared to patients who didn't have low levels of VD_3 [28]. A study of low-risk prostate cancer patients showed that those who were supplemented with VD_3 for one year had a decrease in their Gleason score and the number of positive cores [29]. The effects of combining calcitriol with other therapies such as, glucocorticoids, nonsteroidal anti-inflammatory drugs, ionizing radiation, cytotoxic agents, and more, has been used in many pre-clinical and clinical PCa studies with varying degrees of results. Due to the inconclusive nature of mainly the clinical trials there is yet to be a consensus on whether VD_3 is effective in treating PCa [30]. While extensively studied in less advanced and primary PCa, the effectiveness of calcitriol has yet to be evaluated against more advanced and chemo-resistant PCa.

1.4 Project Overview

1.4.1 Objective

The main goal of this research is to better understand the effects of confined migration on PCa in hopes of gaining a better understanding of what differentiates non-migratory cells from their

invasive migratory counterparts. This includes investigation into stress induced epigenetic alterations which lead to increased chemotherapy resistance. For this we predominantly focused on the upregulation of cancer stem cell markers because not only are they indicative of therapy resistance, but cancer stem cell markers are also heavily associated with cells that migrate within the body. Through the use of out microchannel technology and the resulting characterization of genetic changes, we could create and/or analyze more effective therapies for migratory and dormant cancer.

1.4.2 Experiment Fundamentals

The cell line used for the following studies is human PC3, which is a highly aggressive prostate cancer cell line known for its androgen resistant nature and high metastatic potential [31] [32]. Cell migration takes place in one of two polydimethylsiloxane (PDMS) microchannel devices which allows for the separation of migratory and non-migratory PC3 cells. The devices were fabricated by pouring uncured PDMS over a silicon wafer imprinted with the desired pattern, silicon wafers were made with soft and photo lithography, and then curing the PDMS, carefully cutting them out and sanitizing them. PDMS is an ideal polymer for this study since it is extremely compatible with cells. PDMS is a very stable polymer, it allows for gas permeation so cells are not experiencing hypoxic conditions, it is non-toxic and PDMS is transparent which allows for easy monitoring and imaging of cells within the channels [33]. The two microchannel devices are a "long channel" device, consisting of 600 5x12μm channels (Figure 1A,B), the "flower device", which has a central reservoir with 6 satellite reservoirs that are connected with either 5x5μm or 15x15μm channels (Figure 1C,D). The long channel device was used in Western Blot studies and any reseeding experiments and the flower device was used for most drug studies. Since the devices

grant us the ability to study the migratory and non-migratory cell populations separately, it allows us make conclusions about the genetic aberrations that result from confined migration.

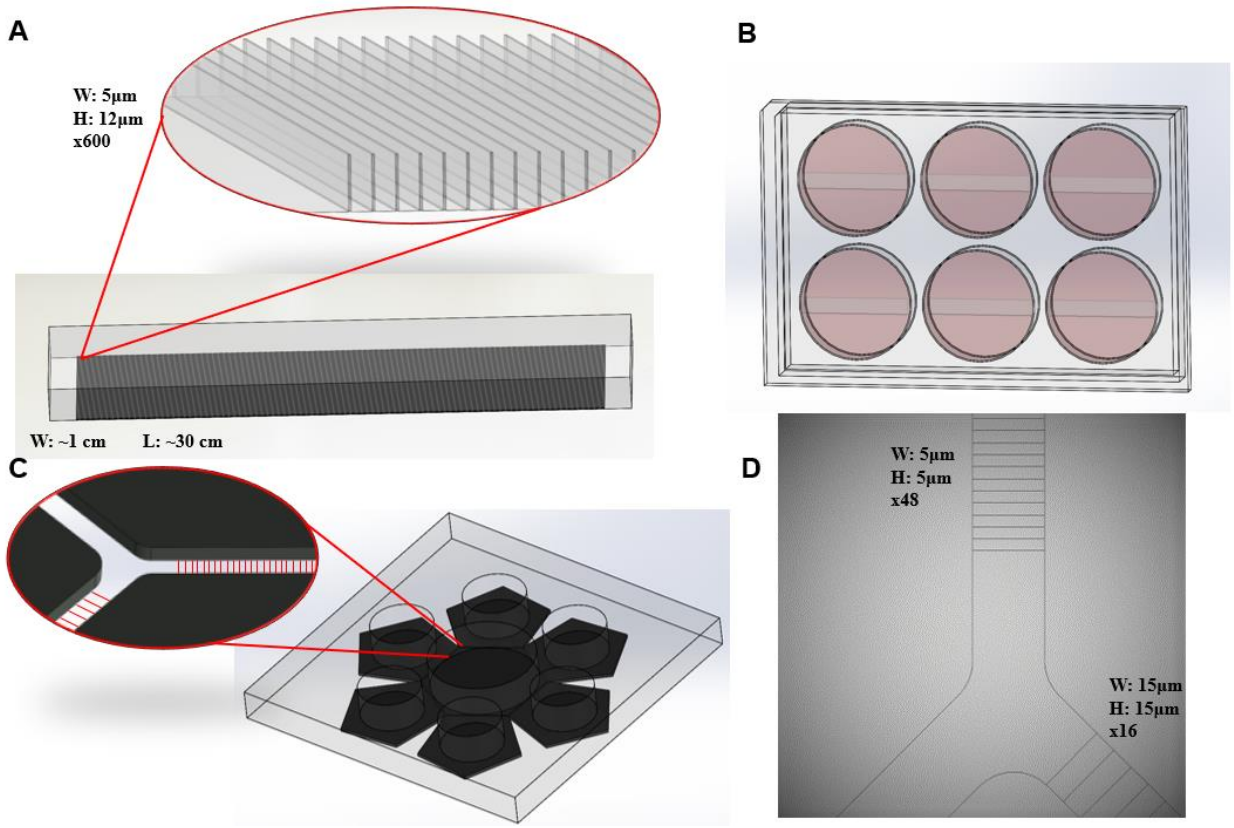

Figure 1. Schematic of PDMS devices utilized. A. PDMS "long channel" microchannel device with 600, 5x12µm channels. **B.** 6-well plate showing PDMS device placed microchannel side-down in each well, $100x10^3$ PC3 cells were seeded in each well and allowed to migrate through channels for 5-7 days. **C.** PDMS "flower" microchannel device with a central reservoir and 6 satellite reservoirs, $20x10^3$ PC3 cells were seeded in in the central reservoir and allowed to migrate to the satellite reservoirs. **D.** There are two different channels widths in the flower device, 5x5µm and 15x15µm, 3 of each per device.

1.4.3 Significance

Prostate cancer development has been well studied in early stages when it is still a primary tumor, so treatments tend to target cells with characteristics of a primary tumor leaving a large gap in treatments for more aggressive forms of the disease, specifically metastatic prostate cancer. Using the microchannel platform to elucidate the factors which contribute to PC3 migration and the accompanying phenotypical changes could lead to the development of more effective therapies. Furthering the understanding of how treatment resistance is acquired and determining the

effectiveness of calcitriol as an anti-tumor agent establishes a potentially novel way of treating aggressive cancers.

1.4.4 Aims

In order to realize the completion of this project with a successful outcome the following aims were carried out.

Aim 1: Characterize genetic mutations that result from confined migration.

Through the use of the microchannel devices, genetic mutations and phenotype alterations of PC3 will be evaluated.

Aim 2: Investigate if vitamin D_3 therapy is a valid option for treatment of chemotherapy resistant prostate cancer.

After determining how migration causes increased therapeutic resistance, we will introduce co-therapy with calcitriol and chemotherapy agents to see if calcitriol enhances anti-tumor effects in therapy resistant PC3 cells.

1.4.5 Successful outcome

Successfully completing this project will result in a better understanding of invasive prostate cancer. The confined migration of PC3 cells resulted in many genetic aberrations which could be further investigated as potential targets for new therapies. Moreover, we can utilize the confined migration platform to study the effects of therapies, such as calcitriol, in a cost and time effective manner. The resulting characterizations and knowledge of migratory PCa has the potential to have a real-world impact by bettering outcomes for advanced-stage prostate cancer patients.

Chapter 2: Phenotypic Changes in Prostate Cancer Resulting from Confined Migration

2.1 Introduction

Prostate cancer (PCa) progression from a primary tumor to metastatic colonization is still not fully elucidated, however, what is known is that PCa preferentially metastasize to bone and that the progression of the disease relies heavily on the dissemination of cancer stem cells (CSCs) [34] [35]. Metastasis causes a significant increase in patient mortality with the 5-year survival rate being about 30%, so there is a need to better understand genetic and phenotypic changes that are caused by migration throughout the body. Along with metastatic progression, the majority of prostate cancer patients, even in early stages of the disease, have disseminated tumor cells (DTCs) without any evidence of metastasis; meaning that cells can escape the primary tumor and remain in a dormant state in other organs, like the bone [14]. Many studies have indicated that post-treatment, these residual dormant tumor cells become increasingly resistant to treatment because standard-of-care treatments often target actively dividing cells [36]. In addition, these cells undergo genetic mutations which contribute to treatment resistance [37]. These residual tumor cells are thought to remain dormant for years before resuming growth, resulting in tumor recurrence which accounts for over 90% of PCa cancer related death.

Current studies report genetic manipulation is necessary to induce dormancy either with mRNA and siRNA, or co-culture of prostate cancer cells with other cell lines known to induce dormancy in these cancer cells [15] [37]. Some major drawbacks of these methods include being very time consuming, costly and methodically complex; additionally, a reasonable assumption would be that these cells are not naturally dormant but are rather being genetically altered to become so. The issue with this is that the genetic changes which induce quiescence may not be the mechanisms

that cause dormancy in the body so any mechanisms and targets discovered could be misleading. If the robust migration of prostate cancer cells through 3D confined space induces quiescence, and thus treatment resistance, such findings could possibly lead to a new, cost efficient, and reliable way to study the process of dormancy in prostate cancer cells. If we can pinpoint primary regulators of dormancy and why this correlates with drug resistance it could lead to the design of more effective prostate cancer treatments.

In this study we utilize our novel microchannel device to study the effects of confined migration in human prostate cancer cell line PC3. After migration, the cells showed in increase in therapy resistance, they acquired a quiescent phenotype which was stable for up to 7 days after migration, and they showed an increase in invasive and CSC marker expression.

2.2 Materials and methods

2.2.1 Device

All the studies used the "long channel device" which has 600 distinct microchannels (5x12 μm), for fabrication we used the same methods as discussed in our previous studies [38]. Briefly, standard negative photolithography and soft lithography were used to fabricate a master silicon wafer. A ratio of 10:1 (v/v) of polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Base, Dow Corning) and curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning) was poured over the completed wafer and baked for 2 min at 75°C, then solidified for 5 min at 150°C. PDMS devices were lifted from the wafer and cut, decontaminated with 70% ethanol, and assembled into 6 well plates.

2.2.2 Cell culture

PC3 cells (ATCC® CRL-1435™) were cultured in a RPMI media supplemented with 10% fetal bovine serum (FBS). Before introducing the cells into the microchannel devices, 6 well plates containing long channel devices were coated with type I collagen overnight, then rinsed three times with phosphate-buffered saline (PBS) for neutralization. PC3 cells were seeded 50 x 10^3 cells on either side of the long channel microchannel device (100 x 10^3 cells per device) they were cultured in supplemented media for 5-7 days. For collection, culture medium was discarded, and cells were washed twice with PBS, then all devices were incubated with 1mL Trypsin-EDTA for 5 minutes, to halt the action of Trypsin 1mL of media was added to each device. The bottom of the well was carefully scraped with Costar® 3008 Cell Lifter (Corning Inc) to remove non-migrated cells, being cautious not to disrupt the PDMS device. After verifying there were no remaining non migrated cells, the PDMS device was peeled up and flipped so the migrating cells were exposed. The wells were once again incubated with Trypsin-EDTA for 5 min and migrating cells were collected from 18 devices (per device estimated to be 5k cells). Cells from both groups were centrifuged for 10 min at 1200rpm. The supernatant was discarded, and the cell pellet was harvested.

2.2.3 Cellular viability

Migrated and non-migrated cells were collected from long channel PDMS devices, counted in order to collect the same amount of non-migrated cells and reseeded and left to incubate for 2-3 days. PC3 cells were treated with 0.01 mg/ml (17µM) Doxorubicin (Dox) (Sigma-Aldrich, St. Louis) for 4 hours and cell viability after drug treatment was determined using the Green Live/Dead Stain (#15J66, ImmunoChemistry) (1:1000 (v/v) in PBS) 50 µl was added to each well and incubated for 10 min at room temperature. The emission was observed by ZEISS LSM 800 fluorescence microscope at a peak absorption of 495nm. Cells were also stained with DAPI,

lyophilized nuclear stain (#6244, ImmunoChemistry) (300nM in PBS) 300 µl was added to each well and incubated for 5 min then rinsed with PBS. The emission was observed by fluorescence microscopy at a peak absorption of 358nm. All images were quantified for fluorescence intensities by ImageJ.

2.2.4 GST Staining

Migrated and non-migrated cells were collected from long channel PDMS devices, counted in order to collect the same amount of non-migrated cells and reseeded and left to incubate for 5-7 days. Cells were either treated with 17µM Dox or left untreated. After removing the Dox and rinsing the cells, 4% paraformaldehyde was added to cells for 15 min to fix the cells, after which they were washed with sterilized PBS 3 times. Blocking solution (4% goat serum in washing solution) was added and left at room temperature for 1 hour. Primary GST (#2624, Cell Signaling Technologies) antibody (1:800, mIgG2a) was added and left overnight at 4°C. Samples were rinsed 3 times with PBS before adding secondary antibody after which they were left for two hours in the dark at room temperature. Samples were rinsed twice with PBS and 14.3µM DAPI (#6244, ImmunoChemistry) was added and left at room temperature for 10 min and then samples were imaged with ZEISS LSM 800 fluorescence microscope, all values were quantified with ImageJ.

2.2.5 BrDU Staining

Migrated and non-migrated cells were collected from long channel PDMS devices, counted in order to collect the same amount of non-migrated cells and reseeded and left to incubate for 5-7 days. The cells were then stained with BrDU (#5292, Cell Signaling Technologies) to identify cells which were proliferating. A washing solution of 0.5% triton and 1x PBS was added to cells and left for 1 hour. When the washing solution was removed 1N ice cold HCl was added for 10 min, the HCl breaks open the DNA structure of the cells, the ice cold HCL was removed and replaced

with 2N HCl and samples were incubated at 37°C for 30 min. Samples were washed 3 times with PBS and blocking solution (4% goat serum in washing solution) was added for 1 hour. Cold primary BrdU antibody (1:500, mIgG1) was added and left overnight at 4°C, the following day the cells were washed 3 times with the washing solution. Secondary antibody was added and left at room temperature, in the dark, for 1 hour then subsequently washed 3 times with washing solution before imaging with ZEISS LSM 800 fluorescence microscope. All values were quantified with ImageJ.

2.2.6 Western Blot

Total cell lysates were obtained by adding RIPA buffer (R0728, Sigma-Aldrich) and protease inhibitor cocktail (P2714, Sigma-Aldrich). Equilibrated protein samples were loaded into a 10% SDS-Page gel and electrophoresed and then electro-transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with a blocking solution containing 5% non-fat milk (Labscientific, M0841). The following monoclonal antibodies were used: EPCAM, EGFR, SOX2, KLF4, uPAR, and ALDAH1. Target proteins were visualized with IgG secondary mouse or rabbit antibodies and a chemiluminescent substrate.

2.3 Results

2.3.1 Cellular viability

To determine if viability increased after migration, the reseeded PC3 cells were treated with 17μM Dox. The cells which had experienced exhibited confined migration exhibited a near 100% viability after treatment whereas reseeded non-migrated cells had approximately a 10% viability (Figure 2A). This indicates that confined migration has a profound effect on the cells ability to resist therapeutic interventions.

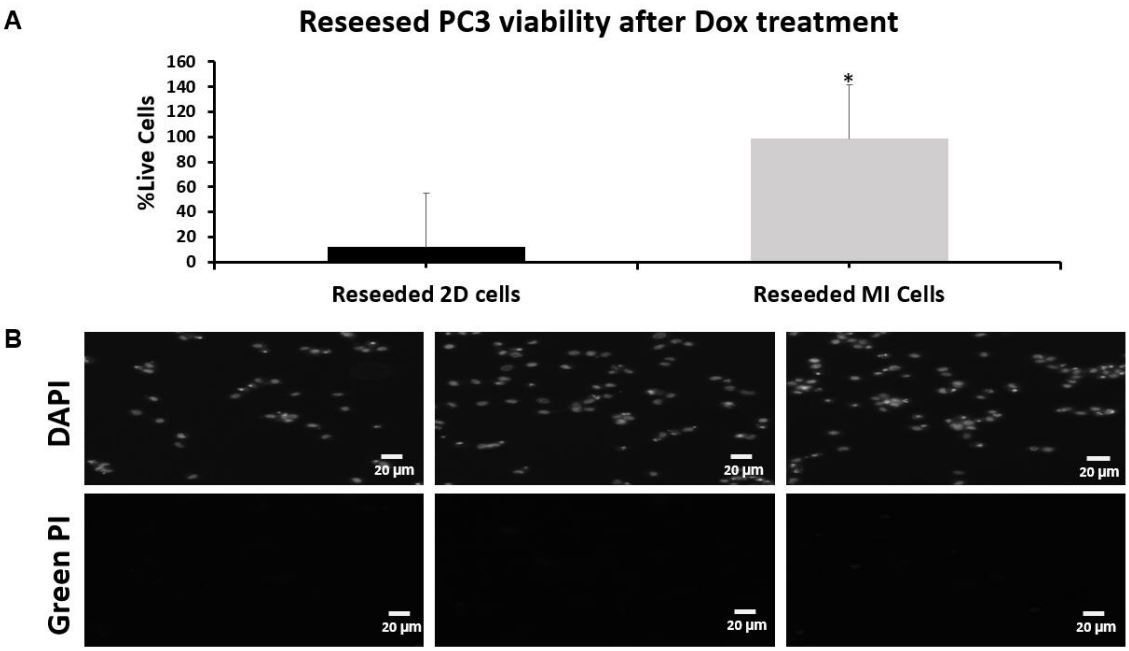

Figure 2. Viability of reseeded PC3 cells after Dox treatment A. PC3 cells were exposed to Dox (17µM) for 4 hours after which they were left to incubate in live image media overnight. They were then treated with DAPI which shows the nucleus and Green PI which indicates if a cell is dead. Reseeded 2D cultured cells had a viability of just 11.72% while reseeded migrated cells had a 98.2% viability. *P <0.05 **B.** Images of reseeded migrated cells after overnight incubation, the top panels show the DAPI stain which indicated the presence of cells where the bottom panels show Green PI stain all panels lack fluorescence indicating all the cells are alive. All results were reproduced, average + S.D.

2.3.2 Drug Deactivating

With increased drug resistance observed in the migrated cells, we wanted to determine what mechanisms were in place leading to the exponential increase in viability, so we evaluated the cells expression of Glutathione-S-transferase (GST). GST is a phase II detoxification enzyme that has been implicated in chemotherapeutic resistance, elevated levels of GST are observed in many tumor types, including prostate tumors. GST detoxifies the cell in two ways, one is through the through glutathione metabolism or by inactivating the MAP kinase pathway [39] [40]. For alkylating agents, drugs that inhibit proliferation through damaging the DNA, GST inactivates them through catalytic conjugation to glutathione; other chemotherapeutic agents target the MAP kinase pathway, which induces apoptosis, however GST can act as an inhibitor for the MAP kinase

pathway [41] [42] [43]. We treated the PC3 cells with Dox and stained for GST after which we observed an overexpression of GST in cells which had migrated and been treated with the Dox (Figure 3). Expression of GST in migrated/non-treated cells compared to non-migrated/non-treated cells was nearly triple, GST expression in migrated/Dox-treated cells compared to non-migrated/Dox-treated cells was more than tripled, and GST expression in migrated/Dox-treated cells increased about 4.5 times compared to non-migrated/non-treated cells.

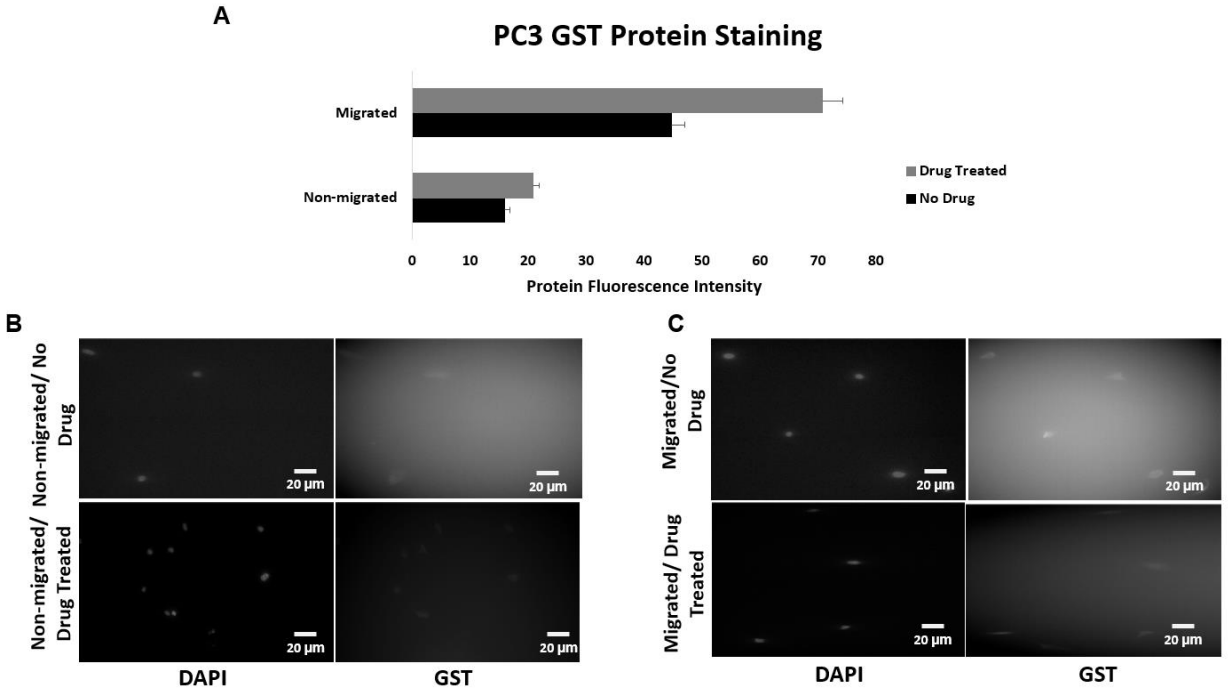

Figure 3. GST staining to determine drug deactivating capabilities of Migrated vs non-migrated PC3 cells. A. Cells which have migrated through physical confinement with no drug treatment produce 2.80 times the amount of glutathione transferase (GST), a known drug deactivating protein, than non-migrated non-treated cells. Migrated cells which were treated with 17µM Dox produce 3.40 times the amount of GST than non-migrated Dox treated cells. Migrated Dox treated cells produced 4.42 times the amount of GST compared to migrated non-treated cells. Fluorescent images of dead cells (DAPI) and GST concentration from the non-migrated cells (**B.**) and migrated cells (**C.**) All results were reproduced, average + S.D.

2.3.3 PC3 Dormancy after Migration

Prostate cancer recurrence is linked to cells that avoid detection because they were in a dormant state and later begin proliferating in tissues which they have metastasized to. We examined cell

proliferation with BrDU staining which is incorporated into cell DNA which is actively dividing. When stained with BrDU the reseeded migrated cells had a significantly weaker signal intensity compared to non-migrated cells (Figure 4). Proliferation in migrated cells was less than half that of cells which had not experienced confined migration, it should be noted that the decrease in proliferation does not stem from the cells being dead as it can be seen that cells are still displaying a healthy, spindle shaped, morphology.

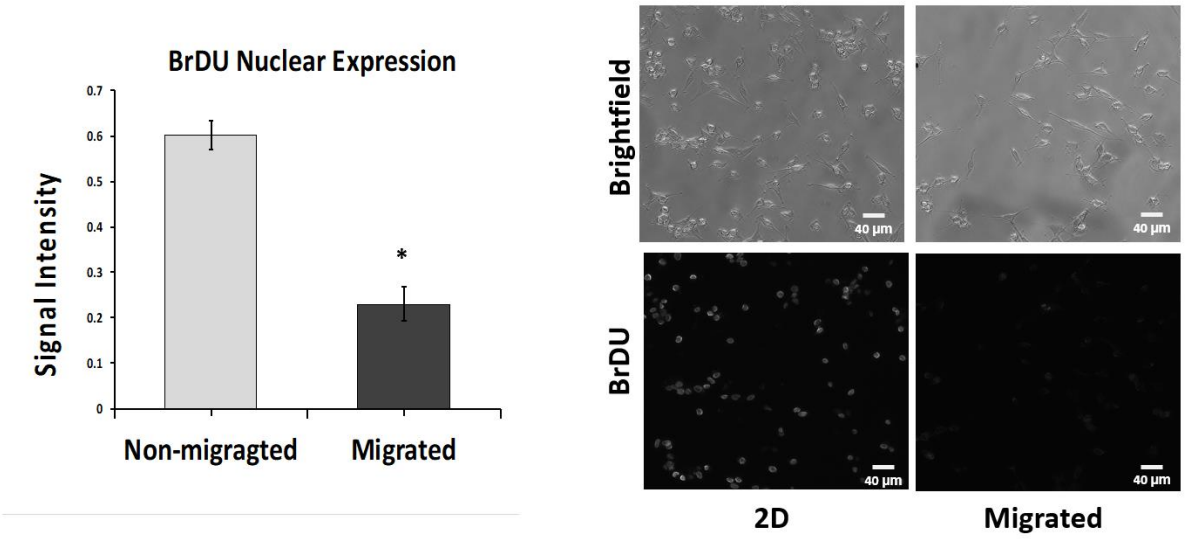

Figure 4. A. Proliferation assay comparison of migrated vs. non-migrated PC3 cells. Cells were stained with BrDU, which stains the nucleus of dividing cells only. Migrated cells show significantly fewer proliferating cells (i.e., dormancy) than non-migrated cells. *P <0.05 **B.** Images of cells after BrDU staining, on the left are 2D cells and on the right are migrated cells that were reseeded for 7 days. The fluorescence of BrDU in Migrated cells is much less than in the 2D cells.

2.3.4 Protein Expression Changes

We examined the protein expression of both migrated and non-migrated PC3 cells since metastatic PCa is known to have a different protein profile compared to cells at the primary site. We wanted to evaluate CSC markers because therapeutic resistant cells and DTCs tend to have increased expression of CSC related proteins. Epithelial cell adhesion molecule (EpCAM) is a CSC marker that is correlated with the progression of low grade prostate cancer to more aggressive stages, we

show here that cells that migrated through the microchannel had a 1.5 increase in EpCAM expression (Figure 5A) [44] [45]. Epidermal growth factor receptor (EGFR) is linked to prostate cancer metastasis and chemotherapeutic resistance, our results demonstrate PC3 cells upregulation of EGFR by more than 1.5 times after confined migration (Figure 5B) [46] [47]. SOX-2 is not only a key marker of metastasis for PCa but it is also associated with CSC properties, migrated PC3 cells more than doubled expression of SOX-2 (Figure 5C) [48] [49] . Krüppel-like factor 4 (KLF4) is a known tumor suppressor for PC3, also, KLF4-null cells have a greater migratory capacity compared to non-altered cancer cells [50] [51]. PC3 cells which experienced confined migration had a drastic drop in KLF4 expression (Figure 5D). Urokinase-type plasminogen activator receptor (uPAR) has been implicated in tumor progression and specifically for PC3 it has been show to increase the cells ability to migrated to the bone, our migrated PC3 cells showed an 8 times elevated expression of uPAR (Figure 5E) [52] [53]. Aldehyde dehydrogenase 1A1 (ALDH1A1) is a known CSC marker for prostate cancer cells, PC3 cells which underwent confined migration increased their expression of ALDH1A1 by around 1.5 times (Figure 5F) [54].

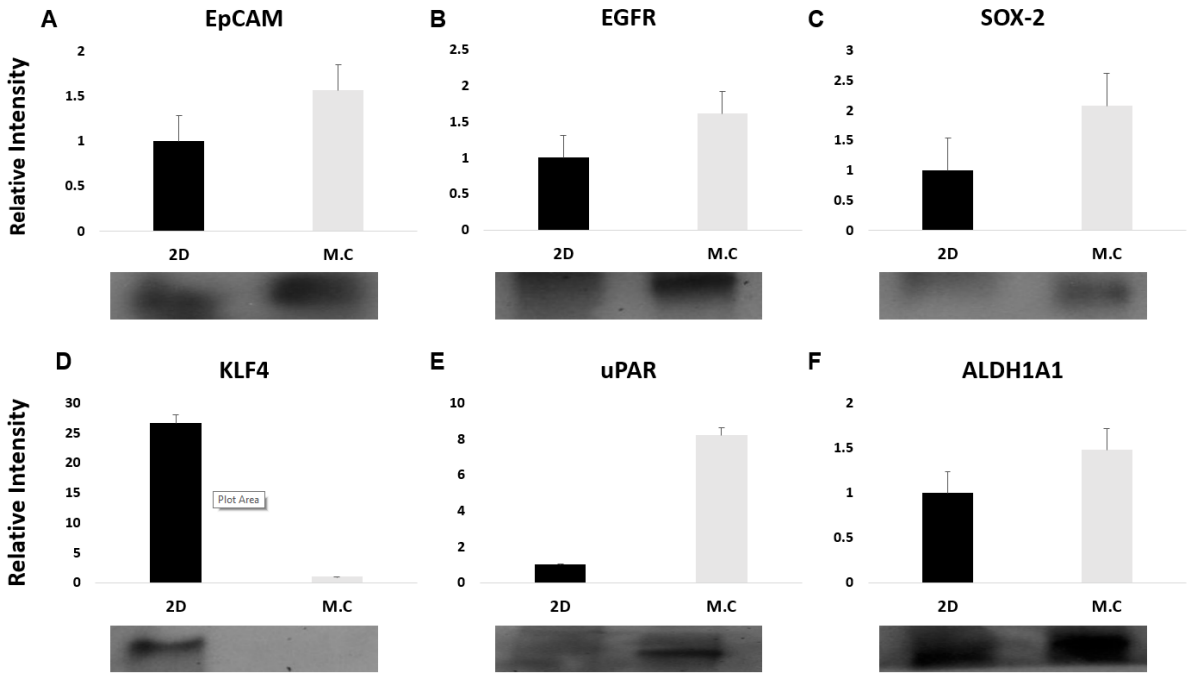

Figure 5. Protein expression changes observed in PC3 cells which have undergone confined migration. Western blot bands and quantification ratios in non-migrating (2D) and migrating (M.C.) PC3 cells. **A.** M.C cells exhibited a 1.57-fold increase in EpCAM expression. **B.** M.C cells increased EGFR expression by 1.62 times. **C.** M.C cells upregulated SOX-2 expression by 2.08 times. **D.** 2D cells had 26.68 times higher expression of KLF4. **E.** M.C cells exhibited an 8.22-fold increase in uPAR expression. **F.** M.C cells upregulated ALDH1A1 expression by 1.48 times. All results were reproduced, average + S.D. Representative blot images of each marker are shown below their respective graph.

2.4 Discussion

It is well established that PCa behaves extraordinarily different once it metastasizes throughout the body, however we as a scientific community are lacking a complete understating of the mechanisms and implications of migration. In this study we used the microchannel platform to examine how prostate cancer behaves under confined migration with the aim of better understanding the correlation between PCa migration and epigenetic changes.

A hallmark of metastatic cancer and what leads to poor patient outcomes is the ineffectiveness of current therapies, specifically high tolerance of chemotherapy. We showed that the migrated PC3 cells had a near 100% viability compared to the almost complete knockdown of non-migrated PC3 cells; drug resistance is a multifactorial and complex feature that results from a response to stress

[55] [56]. The stress factor is the confined migration because the cell is having to change its structure in order to fit through the microchannel, this increases stress proteins which enhances the overall resistance. Previous studies have demonstrated that GST is capable of deactivating drugs, specifically Dox, in multiple cancer lines including prostate and colon cancer [57]. As demonstrated the migrated cells boost GST expression, this information supports the observed viability results and allows us to conclude that the GST is deactivating Dox inside the cells leading to increased survival of migrated PC3 cells.

Another change in PC3 cell behavior is the acquisition of a dormant phenotype after migration. Prostate cancer is known to become dormant when it metastasizes to other locations of the body, it has been heavily correlated with the microenvironment which the DTCs have relocated to, such as the bone, however, in our investigation we found that the migratory process may also be a factor which induces dormancy [58]. After reseeding, cells which had experienced confined migration displayed a decrease in proliferation for up to 7 days post reseeding while still maintaining a healthy morphology. The quiescent phenotype is linked with a cells ability to withstand treatment because most treatments target proliferating cells, so being in a dormant state provides protection against chemotherapeutic agents [59]. Not only does this aid in the explanation of increased viability but migration induced dormancy needs to be explored more; because, other studies which have investigated PCa dormancy have induced dormancy through use of external signals. For example, to obtain the quiescent phenotype PCa cells were co-cultured with cells such as MC3T3-E1 or introduced to external signals such as Wnt5a or TGF-β2 from osteoblasts [60] [15]. Confined migration could provide a more cost-effective way to study the acquisition of a dormant phenotype further elucidating the mechanisms behind PCa quiescence and inevitable relapse.

Lastly, one of the mainstays in metastatic PCa progression is the development of a unique protein expression profile, this is of chief interest for cancer research because it not only allows for a better understanding of the disease, but it also can provide potential therapeutic targets. We observed the upregulation of CSC markers, proteins associated with metastatic progression and the downregulation of the tumor suppressor KLF4. These results are consistent with current literature stating stress induces protein modifications that cause PCa cells to become therapy resistant [61] [55]. More research is needed to create a complete narrative for how migration effects protein expression, but the common theme is that the altered protein expression results in increased therapy resistance.

2.5 Conclusion

Through this study we showed that the actual process of migration induces many observable changes in PC3 cells including an increased resilience to chemotherapy treatments, acquisition of a quiescent phenotype, an upsurge in detoxifying proteins and an overall protein expression profile change including increases CSC markers and decreases in tumor suppressor proteins. Since most prostate cancer deaths are caused by metastatic progression, continued investigation of therapy resistance in PCa through migration holds much promise for the development of more effective treatments.

Chapter 3: Calcitriol as an Effective Treatment Against Migrated, Therapy Resistant Prostate Cancer

3.1 Introduction

Vitamin D_3 (VD_3), specifically, 1,25-dihydroxyvitamin D_3 otherwise known as calcitriol has been extensively studied in multiple forms of cancer including breast, colo-rectal, and prostate; evidence suggests calcitriol is effective for both cancer prevention and treatment [19] [62] [63] [64]. The Vitamin D_3 pathway of prevention and treatment in PCa is not fully understood, however, multiple mechanisms have been suggested. One of the main implications is that calcitriol works as an anti-inflammatory agent by inhibiting the synthesis of prostaglandins which decreases the ability of PCa tumors to develop and progress into more aggressive stages [65]. Numerous studies have shown that calcitriol is linked to prostate cancer prevention because it is known to decrease invasiveness and cause cell G_0/G_1 cycle arrest [65] [66] [67]. Calcitriol has been shown to induce apoptosis in both androgen dependent and castrate-resistant prostate cancer (CRPC) cell lines (LNCaP and PC3, respectively) by downregulating two protective proteins Bcl-2 and Bcl-X_L [68]. Although androgen dependent cell lines are more responsive, calcitriol has also been linked to a decrease in cellular glucose uptake which disrupts the entire glucose mechanism [69]. In prostate cancer progenitor/stem cells VD_3 not only causes cell cycle arrest it also triggers the cells to differentiate into androgen receptive cells through the mediation of interleukin-1 alpha [70]. Furthermore, VD_3 has also been used in combination with other therapies (i.e. Docetaxel, Vitamin A, TBBz, NSAIDs, paclitaxel, etc.) to synergistically increase their antitumor effects through inducing apoptosis, causing cell cycle arrest, decreasing angiogenesis, reducing inflammation and decreasing invasion [4].

In this study we demonstrate that calcitriol can be used to amplify therapeutic effects on cells which have an increased resistance to chemotherapy due to migration. PC3 cells were subjected to migration through channels that are as small as 5µm by 5µm and then either collected for Western Blot studies or evaluated against the non-migrated counterpart to determine viability. The use of FDA approved Vitamin D_3 in combination with chemotherapeutic agents is a unique approach in treating CRPC and migrated prostate cancer, this could potentially fill the void in existing therapy options for the increasing incidence of advanced metastatic PCa.

3.2 Materials and Methods

3.2.1 Cell Culture Cell Harvesting

PC3 cells (ATCC® CRL-1435™) were cultured in a RPMI media supplemented with 10% fetal bovine serum (FBS). Polydimethylsiloxane (PDMS) device fabrication was achieved through the same procedure as our previous studies [38]. Briefly, standard negative photolithography and soft lithography was used to fabricate a silicon wafer. A ratio of 10:1 (v/v) of polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Base, Dow Corning) and curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning) was poured over the completed wafer and baked for 2 min at 75°C, then solidified for 5 min at 150°C. PDMS devices were lifted from the wafer properly cut, decontaminated with 70% ethanol, and assembled into 6 well plates for long channel device or onto glass slides for the flower device. Most cultures were conducted with the long channel microchannel device, which has 600 district microchannels (5x12 µm). Before introducing the cells into the microchannel devices, 6-well plates containing long channel devices were coated with type I collagen overnight, then rinsed three times for neutralization. PC3 cells were seeded 50 x 10^3 cells on either side of the long channel microchannel device (100 x 10^3 cells per device)

they were cultured in supplemented media for 5-7 days. For collection, culture medium was discarded and cells were washed twice with PBS, then all devices were incubated with 1mL Trypsin-EDTA for 5 minutes, to cease the action of Trypsin 1mL of media was added to each device. The bottom of the well was carefully scraped with Costar® 3008 Cell Lifter (Corning Inc) to remove non-migrated cells, being cautious not to disrupt the PDMS device. After verifying there were no remaining no migrated cells, the PDMS device was peeled up and flipped so the migrating cells were exposed. The wells were once again incubated with Trypsin-EDTA for 5 min and migrating cells were collected from 18 devices (per device estimated to be 5k cells). Cells from both groups were centrifuged for 10 min at 1200rpm. The supernatant was discarded, and the cell pellet was harvested.

3.2.2 Western Blot

Total cell lysates were obtained by adding RIPA buffer (R0728, Sigma-Aldrich) and protease inhibitor cocktail (P2714, Sigma-Aldrich). Equilibrated protein samples were loaded into a 10% SDS-Page gel and electrophoresed and then electro-transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with a blocking buffer contained 5% non-fat milk (Labscientific, M0841). The following monoclonal antibodies were used: Nanog, CD133, CXCR4 and E-Cadherin. Target proteins were visualized with IgG secondary mouse or rabbit antibodies and a chemiluminescent substrate.

3.2.3 Viability Analysis

Flower PDMS devices with two different microchannel dimensions (5x5µm and 15x15µm) were used to evaluate cells in response to Doxorubicin (Dox). PC3 cells were seeded (20×10^3) in the central reservoir of each device and cultured for up to 5 days. PC3 cells were treated with 0.01

mg/ml (17µM) Dox (Sigma-Aldrich, St. Louis) for 4 hours and cell viability after drug treatment was determined using the Green Live/Dead Stain (#15J66, ImmunoChemistry) (1:1000 (v/v) in PBS) 50 µl was added to each well and incubated for 10 min at room temperature. The emission was observed by fluorescence microscopy at a peak absorption of 495nm. All images were quantified for fluorescence intensities by ImageJ.

3.2.4 Drug Pumping Study

All devices were treated with Dox (17µM) for 4 hours, and then cells were washed with live cell image medium (#1582202, Life Technologies) and immediately imaged. All samples were imaged by a ZEISS LSM 800 fluorescence microscopy using bright field (10ms), and Rhodamine (500ms, Dox). Cells in three different locations were imaged: central reservoir (non-migrated control cells), 5x5 reservoir (cells which migrated through 5x5µm channels), and 15x15 reservoir (cells which migrated through 15x15µm channels). The samples were kept in the image medium overnight and imaged again at the 24-hour time point. The reason we opted for Dox is because it is auto-fluorescent, so it is easy to identify and quantify. The bright field and Dox florescent images were loaded into ImageJ and 3 random cells per image were chosen to measure. Each cell was analyzed by selecting the area of the cell in the bright field image and then the mean Dox fluorescence per pixel was measured for that cell using the dox florescent image, this is a well-established method which allows you to quantify the amount of Dox per cell [71] [72]. To determine the amount of Dox in each compartment, the nucleus, which is visible in the brightfield images as a darker area inside the cell, value was measured and the whole cell -including the nucleus- value was measured and those values were subtracted from each other resulting in the intensity for the cytoplasm.

3.2.5 Calcitriol Study

Migrated PC3 cells were obtained from long channel devices and counted in order to collect the same amount of non-migrated cells. Both migrated and non-migrated cells were reseeded (5K per well) and cultured overnight. Experimental groups were treated with 5µM of calcitriol and left for 24hrs after which the medium was removed, and cells were treated with paclitaxel (0.1nM) for 24hrs. Control groups were treated with either 1% DMSO, 5µM of calcitriol, or 0.1nM paclitaxel and left for 24hrs. To determine viability both an MTS (CellTiter 96® AQueous Non-Radioactive cell proliferation assay, Promega) and an LDH (Cytotoxicity Detection Kit, Takara) assay were performed. Briefly, the MTS assay was performed by adding 100µL of MTS reagent to 96 plate wells containing 100µL of cells in RPMI media, it was left to incubate for approximately 4hrs and absorbance was read at 490nm. The LDH assay was performed by removing 50µL of supernatant and placing it into a prepared 96-well plate with LDH reagent and after 30 min at room temperature the plate was read at 490nm using.

3.3 Results

3.3.1 Migrated vs Non-migrated PC3 Cell Viability

PC3 cells underwent confined migration through the 5x5µm microchannles in the flower device after which they showed a remarkable increased resistance to a lethal dose,17µM (0.01 mg/ml), of Dox. Green PI was utilized to determine which cells were alive, if a cell has died it will florsece. Cells that did not undergo migration total viabilty was under 20%, where as cells which had experinced the confined migration had a viability of nearly 100% (Figure 1A). In figure 1B, it is clear that there is no flourescesnce coming from the migrated cells that were treated with green PI,

where as there is a very bright signal for the non-migrated group. It is also evident that the cells within the migrated group are still exibiting a healthy morphology with very little debris present.

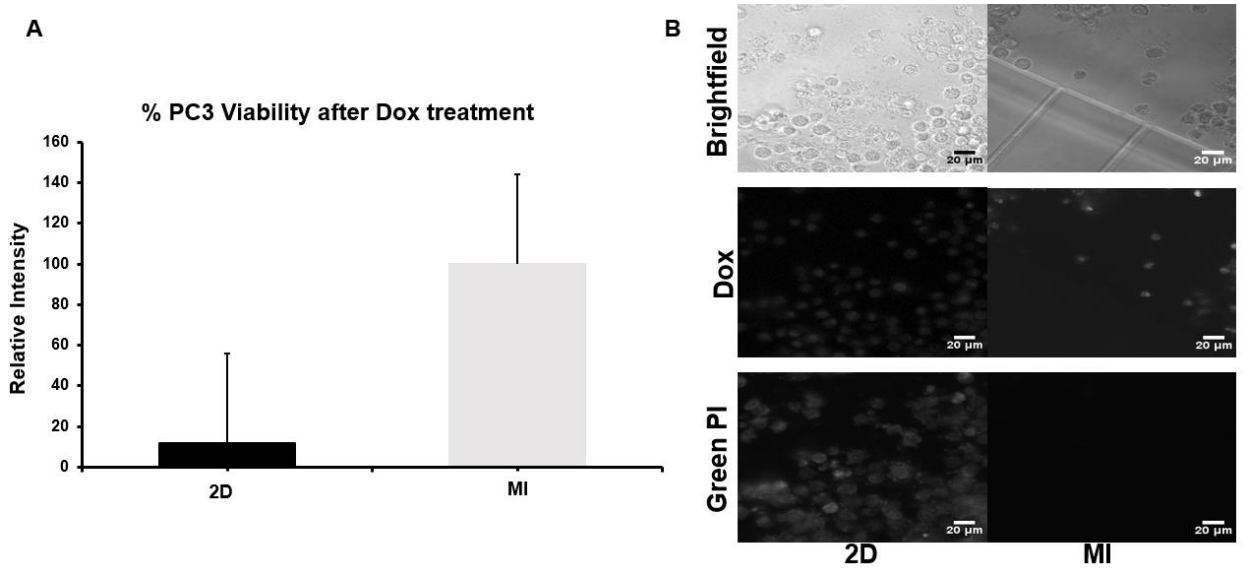

Figure 6. Confined-migrating cells exhibit more chemotherapy resistance than non-migrated cells. A. PC3 cells were exposed to Dox (17µM) for 4 hours after which they were left to incubate in live image media overnight. They were then treated with Green PI which indicates if the cell has died. 2D cultured cells had a viability of less than 20% while migrated cells had nearly 100% viability. **B.** Images of cells after overnight incubation, on the left on non-migrated cells and on the right are migrated cells. The fluorescence of Dox in MI cells is much less than in the 2D cells. While the opposite is true for the Green PI signal which is significantly brighter in the 2D cells. All results were reproduced, average + S.D.

3.3.2 Drug Pumping Study

There is clear evidence that confined migration increases the viability of PC3 cells, so we examined if the cells were capable of drug efflux. For this study, Dox was utilized because of its autoflouresent quality so it is easy to image the concentration in each compartment of the cell (cytoplasm, nucleus, and total cell) [71] [72] [73]. PC3 cells migrated through the 5x5µm and 15x15µm channels of the flower device and then treated with 17µM Dox for 4 hours. The cells were than imaged immediately and then again after 16 hours and Dox concentrations between each group (non-migrated, 5x5µm and 15x15µm) were compared. The amount of Dox in each group varies slighlty after 4 hours but it has the highest concentration in the nucleus for every group

(Figure 2A), however the migrated PC3 cells took up less of the Dox than the non-migrated cells (Figure 2C). After 16 hours there is obvious discharge of the drug from the cells, however the lowest concentration is seen inside the cells is within the 5x5µm group (Figure 2B,D).

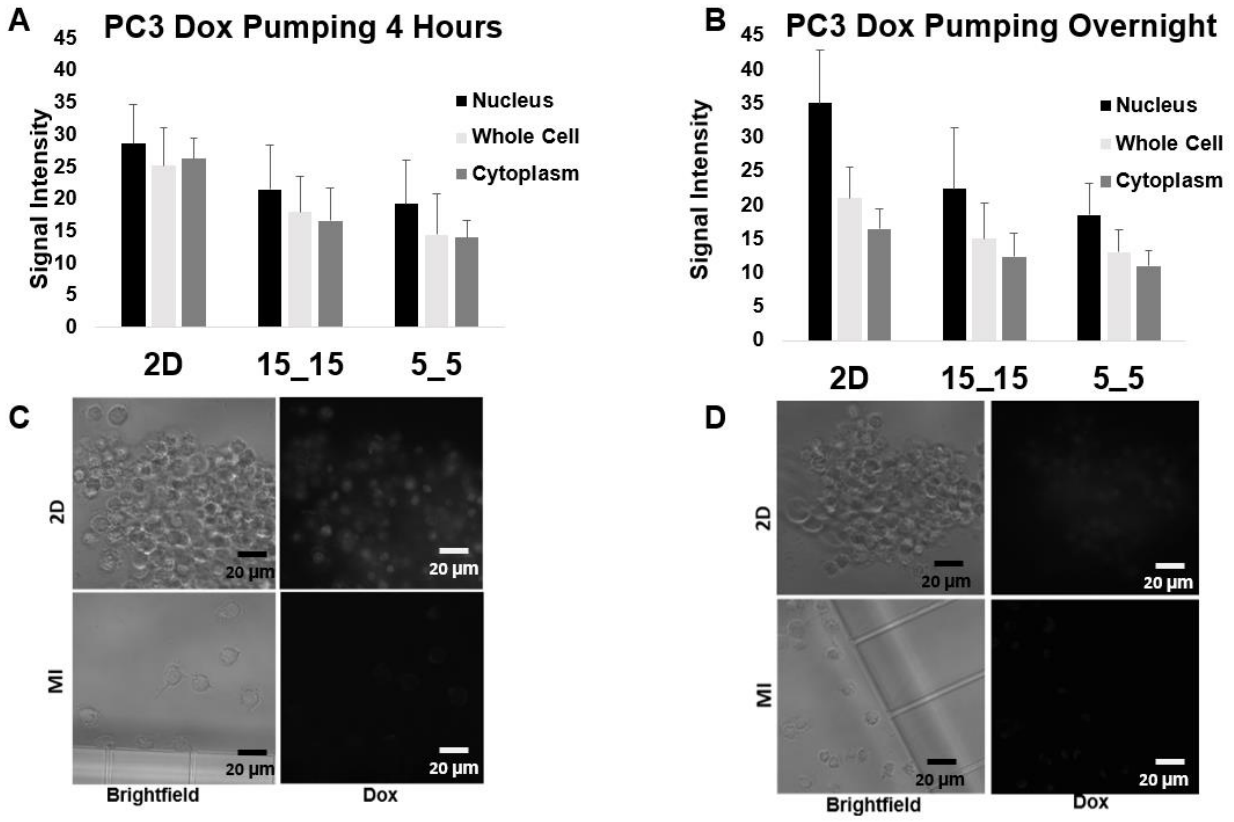

Figure 7. Confined-migrating cancer cells demonstrate an increased drug efflux capability. A.C. Relative concentrations of Dox (17µM) after 4 hours of exposure. The three different conditions quantified are non-migrating cells (2D), cells that migrated through a 15x15µm microchannel (15_15) and 5x5µm microchannel (5_5). The 2D cells took on the most amount of Dox while the 5x5µm cells took on the lowest concentration. **B.D.** Relative concentrations of Dox (17µM) after overnight incubation. All cells demonstrated pumping out of the drug to varying degrees. The 2D cells pumped out the least amount of Dox while the 5x5µm cells have a low concentration of Dox, indicating a significant amount of pumping. All results were reproduced, average + S.D.

3.3.3 Migrated PC3 Cells Exhibit and Increase in Cancer Stem Cell Makers

In order to gain a better understanding of how confined migration effects PC3 cells we investigated to see if the cells which underwent protein expression changes, specifically looking for cancer stem cell markers. CD133 is a bona fide marker of prostate stem/progenitor cells and is related to

cancer metastasis and resilience when exposed to treatment (Figure 3A) migrated cells showed 5 times increase in CD133 expression [74] [75]. The 1.5 decrease of E-Cadherin is suggestive of epithelial-mesenchymal transition (EMT) (Figure 3B) [76]. CXCR4 expression doubled, CXR4 has been shown to be a key regulator of tumor dissemination as well as in the maintenance of prostate cancer stem-like cells (Figure 3C) [34]. Nanog is also a cancer stem cell marker for PC3 cells and tripled in expression after migration (Figure 3D) [74].

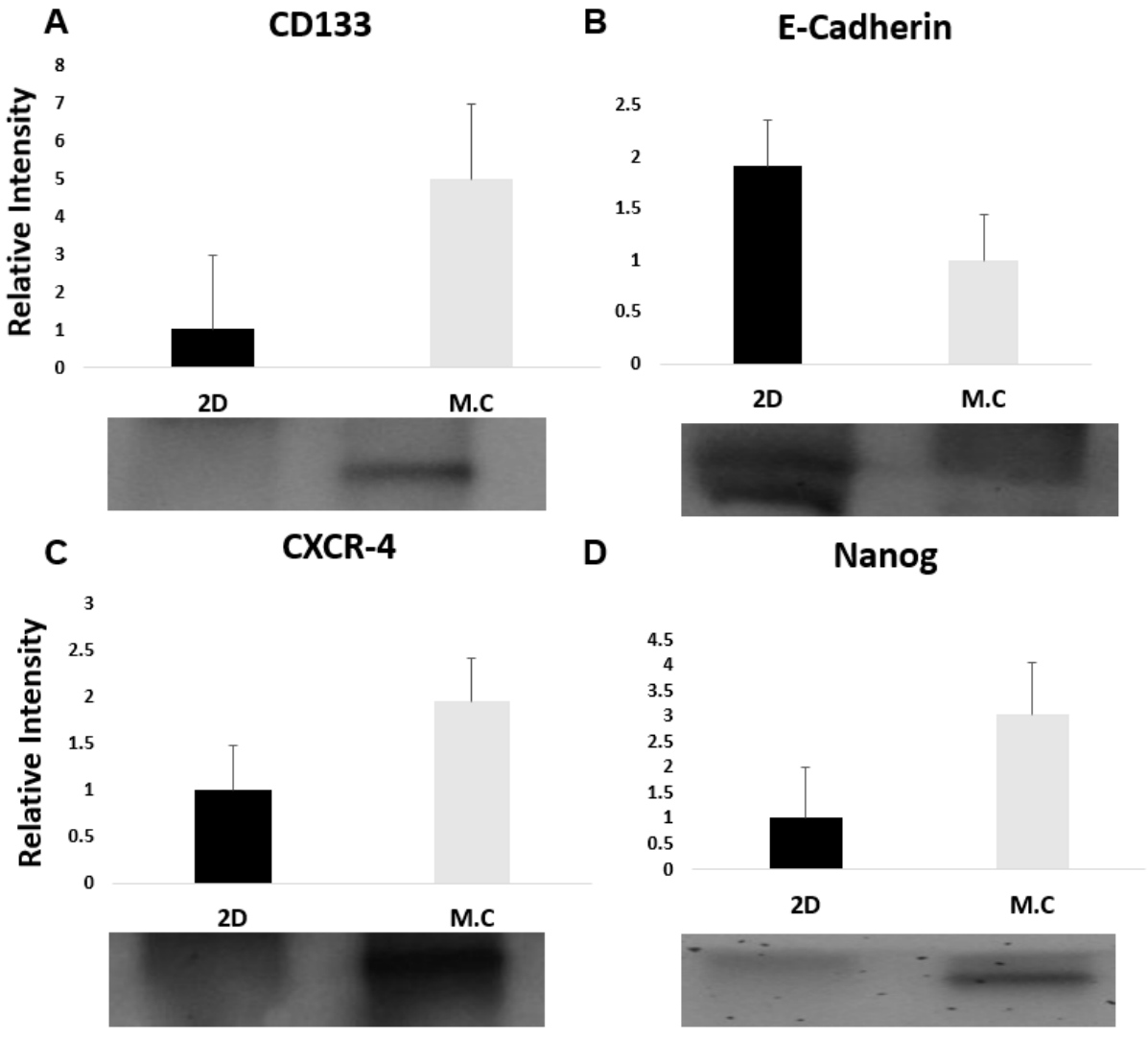

Figure 8. Increased expression of cancer stem cell related markers in confined-migrating cancer cells. Western blot bands and quantification ratios in non-migrating (2D) and migrating (M.C.) PC3 cells. **A.** M.C cells exhibited a 4.98-fold increase in CD133 expression. **B.** 2D cells had 1.91 times higher expression of E-Cadherin. **C.** M.C cells

upregulated CXCR4 expression by 1.95 times. **D.** M.C cells increased Nanog expression by 3.03 times. All results were reproduced, average + S.D. Representative blot images of each marker are shown below their respective graph.

3.3.4 Calcitriol and Paclitaxel as a Combined Therapy for Migrated PC3 Cells

The migrated PC3 cells clearly exhibit excellent therapy resistance so the goal of this study was to determine if there was a treatment that could combat these cells. Calcitriol by itself showed no effect on either the migrated or non-migrated cells. Taxol alone showed a decrease in viability for the non-migrated cells; however, Taxol actually caused an increase in cell viability for the migrated cells (Figure 4B). When cells were pretreated with calcitriol, the migrated group's viability actually dropped below that of the non-migrated cell (Figure 4A,C).

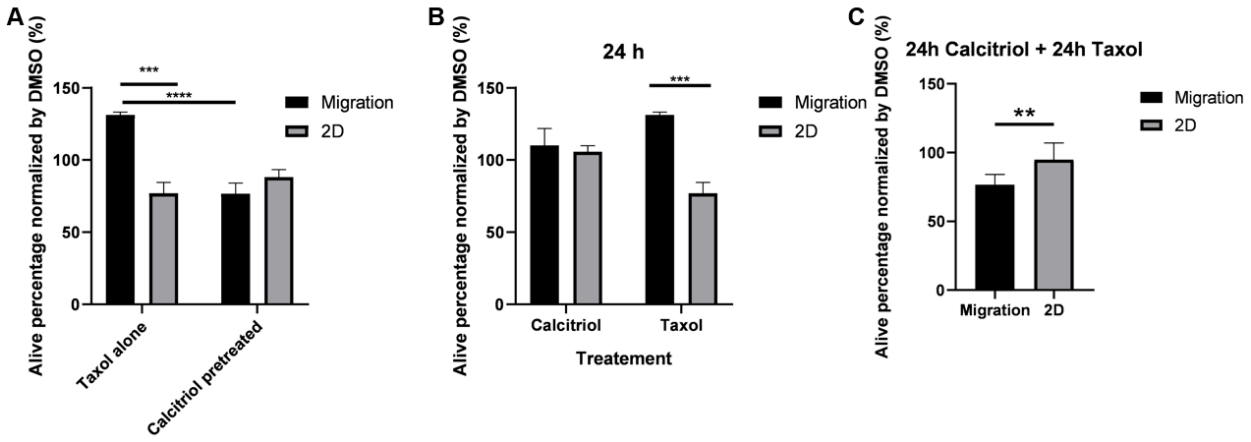

Figure 9. **A. Calcitriol amplifies the cytotoxic effects of Taxol on migrated PC3 Cells.** Viability of migrated and non-migrated cells treated with either just Taxol or pretreated with Calcitriol and then treated with Taxol. **B.** Viability of migrated and non-migrated cells treated with either Taxol or Calcitriol after 24 hours. **C.** Viability of migrated and non-migrated cells treated with Taxol and Calcitriol after 24 hours. All results were reproduced, average + S.D.

3.4 Discussion

The standard of care treatments for prostate cancer include surgical resection, chemotherapy, radiotherapy and hormone therapy [77] [78]. However, when PCa becomes increasingly resilient either from being CRPC or due to migration throughout the body, treatments are extremely limited.

In the study we were able to divide therapeutic responsive PC3 cells and therapeutic resistant PC3 cells, this allowed us to not only gain a better understanding of the implications of confined migration on PCa but it also allowed us to test treatment regimens on them.

It is known that cancer cells are able to change the protein expression on the surface of the cell and inside the cell in order to elude treatment, it is this very unique quality that lowers patient survival outcomes [75]. Much like other cancers, this can be heavily attributed to the increase in therapy resistance related to the upregulation in cancer stem cell markers, cell membrane efflux proteins, and drug deactivating proteins [76] [74] [79]. As shown in multiple studies the increase of CSC markers is heavily correlated with therapy resistance. We believe this is the case for PC3 cells too, as PC3 cells behave more and more like CSCs the greater chance they have at survival against chemotherapy or even radiotherapy [80]. Drug efflux pumps are a major component of this study because it is one of the main mechanisms of therapy resistance, as discussed earlier PC3 cells which migrated through confinement greatly increased drug efflux. Our quantification methods, though well-established could be improved, by adding DAPI staining we could better identify the nucleus in each image leading to better and more accurate quantifications. There are multiple types of cellular pumps such as ABC transporters a class of drug efflux pumps and V-H^+-ATPase a proton pump, both of which see increased activity in aggressive cancer cells. Literature has shown that when cells were treated with Dox and a V-H^+-ATPase pump inhibitor, the cells had a buildup of Dox inside the cell leading to decreased cell viability [81]. In a study on breast cancer it was shown that VD_3 inhibits V-H^+-ATPase action making the cancer less aggressive and more susceptible to chemotherapy [82]. So, we can assume this is true for calcitriol treated PC3 cells as well, so since the VD_3 inhibited V-H^+-ATPase action the cell was unable to remove Taxol at a rapid enough rate ultimately leading to cell death.

Calcitriol has shown great promise for many cancers including PCa however it has yet to be shown effective as a treatment against PC3 when it is the soul treatment, it works as a preventative in some cases, but unlike other PCa cell lines the PC3 cells do not respond the same. When calcitriol is combined with CYP24A1 inhibitors, CYP24A1 is an enzyme which is responsible for deactivating calcitriol, treatments with VD_3 have been shown to be effective against PC3 cells [83]. However, these cells did not have an increased resistance like our cells. We demonstrate that using chemotherapeutic reagents in combination with calcitriol is not only effective against non-migrated PC3 cells but also migration included chemotherapy resistant PC3 Cells. Calcitriol holds great promise for treatment against advanced stage PCa because not only is it effective in increasing anti-tumorigenic effects of paclitaxel, the side effects such as hypercalcemia, as long as administered properly, are very minimal [17]. So, with the use of calcitriol we could in fact fill the void which is left for patients with androgen resistant metastatic cancer.

3.5 Conclusion

The observed difference in PC3 cells which experienced confined migration, upregulation of drug efflux and CSC markers resulting in the increased viability, adds to the mounting evidence that primary and migratory cancer cells need to be treated very differently. With the increasing prevalence of metastatic prostate cancer diagnoses, we as a medical community must determine new methods of treatment. We have demonstrated that Taxol and calcitriol is not only an FDA approved drug combination with very minimal side effects stemming from the calcitriol, but it is also a viable option for treatment CRPC and therapeutic resistant PCa.

Chapter 4: Conclusions and Future Outlook

4.1 Conclusions

To recap, this project had the main goal of determining the genetic and phenotypical alterations that occur in PC3 cells as a result of confined migration, and how VD_3 supplementation holds much promise as a treatment method for therapy resistant PCa. The increased viability observed after confined migration is multifactorial and correlates with the upregulation of CSC markers, increased expression of drug deactivation proteins and the cells ability to more readily remove chemotherapeutic reagents.

The first study aimed to characterize what occurs when PC3 cells migrate through confined 5x12μm microchannels. We were able to show not only can they increase their survival by manipulating protein expression, we also demonstrated that a dormant phenotype can be acquired through confined migration. The upregulation of EPCAM, EGFR, SOX2, uPAR, and ALDAH1 all indicate that PC3 cells are able to gain CSC like properties by experiencing external stress. Correlated with this, the observed increase of GST suggests stress also boosts a cells ability to rid itself of toxins. Overall, PC3 cells which underwent confined migration were more robust and had a much higher chance of survival.

For the second study we wanted a better understanding of the increased resilience ascertained previously. What we observed was after migration of PC3 cells there an upregulation of more CSC markers such as, CD133, Nanog, and CXCR4 and an enhanced ability to discharge chemotherapeutic agents from the cell. We utilized this information to determine if there was an uncomplicated way to knockdown therapy resistance. Thus, we studied the combined effects of VD_3 and paclitaxel in attempt to combat the increased resistance observed in migrated PC3 cells.

It proved effective leading us to believe that despite the uncertain evidence in literature, calcitriol could in fact be a viable treatment for advanced PCa.

4.2 Limitations

Granted the findings in these studies led to a better understanding of prostate cancer and how migration changes the phenotype of PCa, there are some limitations.

One of largest limitations of this study is that we only utilized one PCa cell line, the reason we opted for PC3 cells is because it is one of the most aggressive PCa cell lines making it a good model for studying therapy resistance. With that stated there are multiple other cell lines such as DU145 which is also very aggressive and LNCaP which is not a CRPC cell line and not nearly as aggressive; if these same studies were conducted in the other cell lines we could make grander conclusions about PCa as a whole.

Another limitation of the study is that when analyzing calcitriols effectiveness on increasing anti-tumor activity we chose to use paclitaxel which is a great model, but it is not used today during clinical treatments. Chemo drugs used today include docetaxel, cabazitaxel, mitoxantrone, and estramustine [84]; the benefit of using paclitaxel as our model is that both the most commonly used chemotherapeutic agent against PCa, docetaxel, and paclitaxel are in the same family-Taxens. Despite being in the same family they have differing behaviors, including, paclitaxel being schedule dependent and less potent than docetaxel [85].

Lastly, all these studies were *in vitro* studies. While we have shed light on the mechanisms of migration, we would have a better understanding of prostate cancer progression had we used our chemo resistant cells inside an animal model. This does not negate any of our findings with PC3

cells, but we can only conclude about PCa therapy resilience, dormancy and responsiveness to vitamin therapy on a cellular level.

4.3 Future Outlook

Moving forward with the positive results from this work, future studies can home in on a better understanding of confined migration induced genetic aberrations and the practicality of vitamin therapy in treating aggressive cancer. Specifically, it can expand into other PCa cell lines, more clinically relevant drug studies, or into *in vivo* studies where not only are protein and cellular behavior analyzed but the disease can be better characterized as a whole.

Secondly, expanding into microarrays and RNA-sequencing could better help us identify the genetic differences between the migratory and non-migratory cell populations. In prostate cancer, the TGFβRIII-p38MAPK-Rb signaling axis is believed to be a major regulator of dormancy, using RNA sequencing we could determine if the migratory cells are increasing the mRNA output which correlates with axis and then confirm the protein expression through Western Blotting [86]. For confirmation of the VD_3 mechanism we could either us a microarray or RNA sequencing to determine if calcitriol is actually inhibiting the function of drug efflux pumps such as the ABC transporters a drug efflux pump and V-H^+-ATPase pumps. Looking into the correlation between calcitriol and the disruption of the glucose metabolism would also be an ideal future study. Since we mainly focused on CSC markers for our studies, expanding into other genetic alterations which contribute to drug resistance such as expression of MDR-1, MRP-1, or ABCG2 could form an improved story of how migration leads to increased cell viability.

In addition, broadening our understanding of PCa dormancy could massively impact patient survival rates. Here there are two intriguing options, understanding the reactivation mechanism that occurs when DTCs go from quiescent to proliferative or investigating if there is a way to keep

the cells in a permanent state of dormancy. The first option would allow us to have a target for the reactivation process which would hopefully lead to targeted and potent therapy options. The alternative study could allow for patients to live with cancer with no concern of progression or relapse, this may be the safer route because in reawakening dormant cells you risk not being able to properly rid the body of all the active micro-metastasis.

Finally, expansion of the VD_3 studies into how VD_3 deficiencies correlate with PCa bone metastasis and the potential for calcitriol to inhibit bone invasion would gain invaluable information. There are multiple studies that indicated there is a strong correlation between patients who have low VD_3 levels and those who experience bone metastasis [87] [88]. Some studies have even suggested that the deficiency promotes the growth of PCa once in the bone [89]. It is widely understood that calcitriol is a major component of healthy bone metabolism and calcification, so developing an understanding of what mechanisms are in play with bone, vitamin D_3 and prostate cancer could bring more adequate and successful treatments to many patients.

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