STUDY OF UPREGULATION OF DRUG RESISTANCE IN H460 LUNG CANCER CELLS VIA MICROCHANNEL DEVICE

by

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THESIS

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ABSTRACT

Study of Upregulation of Drug Resistance

in H460 Lung Cancer Cells

via Microchannel Device

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Metastasis is the main reason of high mortality rates in cancer patients. Treating these patients becomes difficult once the cancer has metastasized, especially due to their drug resistance. Drug resistance is a common problem that occurs when treating cancer patients, as in most cancer types, the cells are capable of drug efflux. This makes treatments like chemotherapy ineffective. Therefore, it is important to find alternative methods of treatment that can effectively kill drug resistant cancer cells. Lung cancer is one type of cancer that is the second most diagnosed cancer in the U.S. In this study, microchannels were used to mimic metastasis H460 lung cancer cells. Confined-migrating and non-migrating (2D control cells) were separated. Through Western blotting and some analysis, it was found that there is a high chemo-resistance in cancer cells that is correlated to increased p-glycoproteins in physical confinement. Calcitriol was also used to pre-treat the cells and it was found they are capable of reversing chemo-resistance. This can lead to new therapies being developed with the use of calcitriol. In addition, this study shows the value that microchannels devices can contribute to cancer resistant studies.

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CHAPTER 1

INTRODUCTION

1.1 Lung Cancer

Lung cancer is the second most diagnosed cancer in the United States. The number one cause of lung cancer is smoking, as it damages the lungs [8]. There are two types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). There are three subtypes of NSCLC: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. These three are classified by where the cancer originates in the lungs. For squamous cell carcinoma, the cancer forms in the thin, flat cells that line the lungs. For large cell carcinoma, it begins in several different types of large cells in the lungs. Lastly for adenocarcinoma, the cancer begins in the cells that line the alveoli and secrete mucus. There are a few other subtypes such as pleomorphic, carcinoid tumor, salivary gland carcinoma, and unclassified carcinoma, but these are less common [9]. For SCLC, it spreads more quickly than the NSCLC. It also has subtypes: small cell carcinoma and combined small cell carcinoma. Almost all people who get SCLC get it due to smoking. This cancer type is the most aggressive form as it proliferates quickly and creates large tumors [10].

1.2 Metastatic Process

Cancer has become a leading mortality of death globally around the world. Each year, 10 million new cases of cancer are diagnosed worldwide. There were 8 million deaths caused by cancer worldwide in 2013[3]. With the current unhealthy lifestyle that people live now (diet, exposure to occupational carcinogens, and smoking) cancer cases are rising. In developing countries, a quarter of cancer cases are caused by infections that are linked to cancer [2]. Metastasis is the

main reason for the mortality of patients who are diagnosed with cancer. It is estimated that metastasis is the reason for 90% of cancer deaths [4].

Metastasis is a very lengthy and complicated process. Cancer cells invade other tissues during the process of metastasis, leading to abnormalities in other parts of the body, leading to death of the patient. This whole process is called the metastatic cascade, which broadly consists of these three steps: invasion, intravasation, and extravasation. During the development of the primary tumor, tumor cells are genetically and phenotypically capable of interacting with the environment around them and surviving. They are able to release VEGF, for example, to initiate angiogenesis. The stromal cells surrounding them have protein factors that enhance tumor growth and spreading of the tumor cells. The malignant cancer cells start to stray away from the tumor mass at the primary site and begin to invade the surrounding stroma. This happens as a result of genetic/epigenetic changes within the body. These cells then have the capability to pass through the basement membrane and extracellular matrix or go through the lymphatic or circulatory system. From there, the cancer cells can spread to different organs. Then the process of intravasation begins; angiogenesis is initialized in order for the tumor to survive, as it requires nutrients and removal of waste products. Tumor cells are able to do this by secreting pro-angiogenic factors. Afterwards, biochemical reactions occur between the tumor cells and the endothelial cells of the body creating adhesion to the endothelial cells through the strong bonds formed. This penetrates the endothelium and the basement membrane, which is the final process, extravasation. At the new site, the tumor cells can proliferate and form and secondary tumor mass (Figure 1).

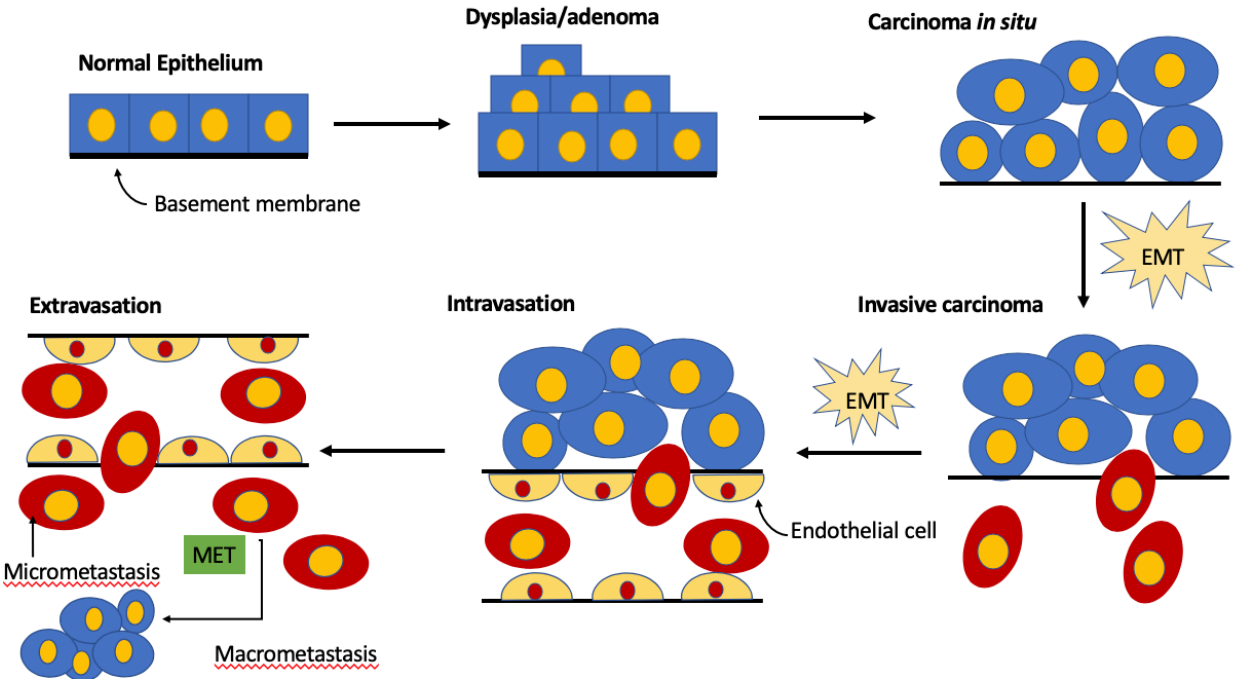

Figure 1.1. This figure shows the whole metastasis cascade process: invasion, intravasation, and extravasation [4].

This whole process tends to be inefficient, resulting in a latent period of time for the cancer cells to proliferate at the new site after infiltration. The metastatic process can be slow, such as a couple of years in breast cancer or a few months like for lung cancer. Many factors can affect where the tumor will spread and the severity of metastasis to the organs, such as cellular origin, intrinsic properties of the tumor, circulation patterns, and tissue affinities. There are certain organs that cancer cells prefer to grow. Generally, the organs attacked the most by cancer cells are the lung, liver, brain, and bone. The lungs are the most common site for primary tumors, as it works as a filter for tumor cells as the flow of the venous drainage goes directly through the lungs. The liver is the most common site for metastasis. Certain cancers have their own specific secondary tumor sites. For patients with lung, breast, melanoma, renal, and colorectal tumors, metastasis to the brain is common. This leaves about a median survival of 12 months for the patient to live. For prostate, breast, and lung cancer, metastasis to the bone is also common. Bone

metastasis is either osteolytic or osteoblastic processes that either favor bone resorption or bone formation. This creates multiple complications until the patient's death, such as hypercalcemia, bone pain, pathological fracture, spinal cord compression, and consequent paralysis. The mechanism for how it becomes osteoblastic or osteolytic is unclear. It could be due to excess factors that are produced by cancer cells, such as platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), VEGF, Wingless and NT-1 (WNT1), parathyroid hormone related protein (PTHrP), urokinase-type plasminogen activator (uPA), prostate specific antigen (PSA), endothelin-1 (ET-1) and BMPs. Why metastasis to the bone occurs in these types of cancers is currently unknown. However, the 'seed and soil' theory suggests certain tumor cells were developed during the development of the primary tumor. They have a special genetic phenotype or cytokine and proteases that allow it to later direct its way to the bone. This also allows them to attach to the bone endothelium more effectively. Also, the bone provides the 'soil' for the 'seeds' (tumor cells) since the bone has plenty of cytokines and non-collagen proteins that can allow prostate cancer cells to survive and proliferate in the bone. This therefore emphasizes how metastasis can be a severe cause of death in patients with cancer. There are multiple suppressor and promoters of metastasis that can either enhance or suppress the metastasis process. These can be altered through genetics/epigenetics. There are genes that initiate the metastasis process by promoting cell motility, epithelial mesenchymal transition (EMT), extracellular matrix (ECM) degradation, angiogenesis or avoiding the immune system. The spread of metastasis occurs through the suppression of the non-coding RNA. Also, certain components enhance the invasion process, such as VEGF and HGF. All of these contribute to the severity of metastasis in the body [1].

1.3 Current Therapies

There are several therapies that are implemented to treat cancer. The tumor can be surgically removed from the body in an attempt to get rid of the cancer cells. Though this treatment can be ineffective if metastasis has already occurred in the body or not all of the tumor was removed. Then a tumor can grow in another area in the body or the tumor mass can grow back, respectively. Therefore, the patient is not completely rid of cancer, making this treatment option not a 100% effective cure. Radiation therapy is another treatment used. It uses high-powered energy beams (like X-rays or protons) to kill the cancer cells. However, since it uses intense electromagnetic waves, it can cause side effects to the patient, as it can affect other areas other than the tumor, damaging them in the process. Immunotherapy is another breakthrough in cancer treatment, in which it uses the patient's own immune system to fight back the cancer cells [5]. However, there can be some severe adverse effects that can occur, such as the treatment can contribute to an overactive immune system. As it is still a developing therapy, other long-term effects are still unknown [6]. The most common treatment is chemotherapy. This uses chemicals to kill the cancer cells within the body. However, there can be side effects if the drugs do not target the tumor directly, leading it to spread throughout the whole body and causing adverse effects [5]. Also, the tumor can have a resistance to multiple drugs given for treatment, making them multidrug resistant. Therefore, it is difficult to combat the cancer when the drugs used are not effective. There are multiple mechanisms that contribute to drug resistance (Figure 2).

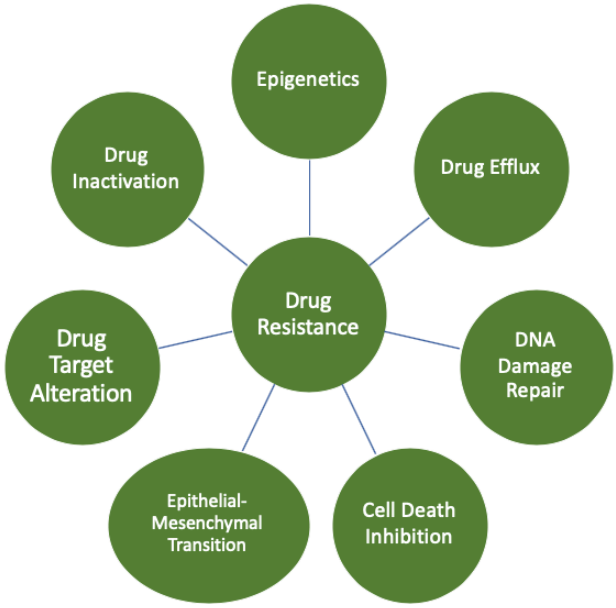

Figure 1.2. Mechanisms that cause drug resistance in cancer cells [7].

1.3.1 Mechanisms of Drug Resistance

In order for drugs to be activated, they must go through a series of pathways and interactions with different molecules. If there is any mutation or down-regulation in those pathways, it will cause the drug to deactivate. Also, most anticancer drugs require metabolic activation.

These mutations or down-regulation of the pathways can be caused by the cancer cells, making any drugs used against it ineffective. For example, p53 is a tumor suppressor protein that promotes apoptosis. However, this protein is mutated by the cancer, making it dysfunctional and therefore causing drug resistance.

The target for the drug can be manipulated by the cancer as well. By modifying the target of the drug, it can create a drug resistance. For example, there are anticancer drugs that target topoisomerase II in an attempt to damage the cancer cells' DNA. However, if the cancer cells mutate the topoisomerase II gene, then it can resist the anticancer drug that targets it. There are

multiple other targets that can become altered by the cancer cells: kinases, signal transduction processes, and modifying enzyme expression levels.

The drug can also be transported out of the body before reaching its target, which is called drug efflux. ATP-binding cassette (ABC) transporters are responsible for the normal physiological function of transporting toxins out of the body to prevent their accumulation. Whenever a substrate binds to the transmembrane domain, there is a change in its conformation that pushes the substrate out of the cell. Cancer cells take advantage of this process of drug efflux by making an overexpression of these ABC transporters, such as MDR1, MRP1, and BCRP.

Cancer cells are able to repair any DNA damage that occurs. Therefore, they are resistance to chemotherapy drugs that damage the DNA by enacting mechanisms that repair DNA damage. Such mechanisms are nucleotide excision repair and homologous recombination. This allows further drug resistance and therefore makes drugs that target DNA ineffective. To make them effective, measures would have to be taken to inhibit pathways that cause DNA repair.

Normally when cells become mutated or there is a failure to meet the checkpoints in a cell's growth, they undergo apoptosis in order to eliminate any cells that don't function properly and keep the cell number in check. However, in many types of cancers, there is a high expression of antiapoptotic proteins like NF-κB. With this high expression of these proteins, the process of apoptosis cannot function and therefore allow the proliferation of the cancer cells.

Drugs are also ineffective because of the epithelial to mesenchymal transition (EMT) that occurs when solid tumors become metastatic. As mentioned previously, metastasis is the movement of the cancer cells from its primary site to a secondary site in the body. When EMT occurs, the cells inside of the tumor start to have a down-regulated expression of cell adhesion receptors, which provide cell-cell attachment. This then increases their motility, allowing them to branch off from

the primary tumor and travel to a different site in the body. Therefore, with chemotherapy, drugs may be able to target the tumor, however it can be difficult to prevent their metastasis. Since cancer cells exist outside of the primary site, the cancer is therefore not cured.

Epigenetics is a large and important factor in cancer cell drug resistance. Two main types of epigenetic changes are DNA methylation and histone modification via acetylation or methylation. DNA methylation is when methyl groups bind to the cytosines at the CG-dinucleotides. When histone modifications are done, they alter the chromatin conformation (acetylation opens the chromatin and deacetylation closes it, preventing it from any genetic modifications). Through these processes, cancer types are able to suppress tumor suppressor genes. Also, cancers can develop by hypermethylation of genes, like ones that allow DNA mismatch repair processes. This can also cause them to become drug resistance as certain hypermethylation of certain genes can create resistance [7].

1.4 Microchannel Devices

Microchannel devices are a key technology that are used in many biomedical engineering applications. Most microchannels are made with polydimethylsiloxane (PDMS) as it is a clear material that makes it easy to see the contents inside of the microchannel. It is also biologically compatible. In order to create the microchannel, a mold is used to create the desired shape and pattern. The mold is a silicon wafer that is spin coated with photoresist for a set duration in order to coat it properly. Then it is set on an even surface to level out the photoresist. Then the mold is ramp heated, which is used to prevent any thermal stress that can cause cracks in the mold. After this, a photomask is placed on the silicon wafer. This process is called photolithography. The photomask is created by the modeling it in the CAD modeling and then printing it onto the silicon wafer using a high-resolution printer. It is necessary that there is not any gap between the

mask and the silicon wafer, otherwise the resulting pattern will be widened on the mold. Then the mold is exposed to UV light and then is post baked in order to crosslink the film. Finally, it is wiped down with isopropyl alcohol.

The microchannel can then be fabricated with this mold. Normally a Dow Corning Slygard 184 silicone elastomer and curing agent is used and mixed in a 10:1 by weight. Then the PDMS is poured on top and heated to a certain temperature. After this, the PDMS is peeled off the mold and the microchannel is imprinted on the PDMS from the mold. Then the microchannel can be placed on a surface, such as a glass slide (Figure 3).

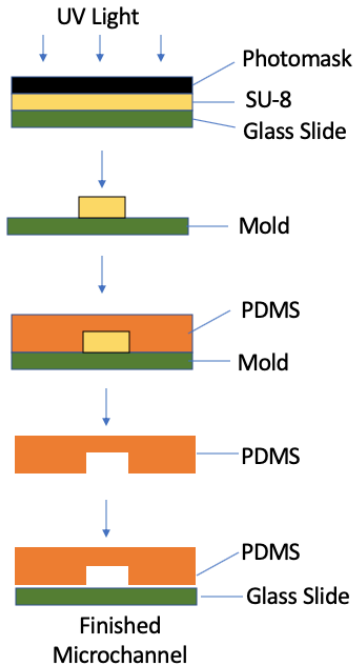

Figure 1.3. Process of mold fabrication and microchannel fabrication [16].

There are multiple biomedical applications that microchannels are used for. Early applications include using them as micropumps, microvalves, microsensors [18]. One way they are used are for drug delivery research. For example, a microchannel can be used to simulate drugs perfusing through the vessels in a particular tissue, such as brain tissue [16]. Microchannels also widely used for fluid flow. Mainly water is used to see the fluid flow and there are inlets and outlets to

see the pressure drop across the microchannel [17]. More recently, microchannels have been used for analyzing biological materials, such as proteins, DNA, cells, embryos, etc. These are analyzed in microreactors, in which two chemical species are mixed together in a reaction chamber. Heat can also be applied to the microchannel to create a microprocessor to study heat transfer [18].

1.5 Western Blot Proteins

CD133 is a transmembrane protein for human hematopoietic stem cells. It is expressed with tumor regeneration, differentiation, and metabolism. It is a key biomarker that is used for characterizing stem cells. CD133 is located on chromosome 4 in humans. It consists of 865 amino acids and 120 kDa in molecular weight. In its structure, it has an N-terminal extracellular domain, five transmembrane domains, and a 59 amino acids cytoplasmic tail. The negative and positive version of the CD133 cells have some similar and some different characteristics. They belong to different cancer stem cell populations and $CD133^+$ glioma cells are from $CD133^-$ cancer stem cells. Both $CD133^+$ and $CD133^-$ CSCs have different expression profiles in transcriptional activities and ECM matrix molecules. CD133 is regulated by many factors, like hypoxia. Under hypoxic conditions, there is a proliferation of $CD133^+$ cells. miRNA also regulate CD133. Some miRNA are capable of enhancing the $CD133^-$ chemoresistance, self-renewal, and tumorigenicity. It can also be influenced by epigenetic factors. Methylation of the CD133 down-regulates gene expression. Demethylation, however, can be seen in many types of cancer, such as colorectal cancer, gastric cancer, and more. CD133 has high metastatic capacities in many cancers. $CD133^+$ are highly chemoresistant. $CD133^+$ lung cancer cells in particular have drug resistance [11].

MDR1(also called ABCB1) is a gene that encodes for p-glycoproteins (P-gp). P-gps are ATP drug efflux pumps that are expressed in places like the blood brain barrier (BBB) by preventing drug permeation into the BBB. They assist in drug efflux of macrocyclic lactones in the nervous system as they would cause high toxicity in the neurological network. It makes multidrug resistance in cancer cells by causing drug efflux of unrelated chemotherapeutic compounds, such as anticancer drugs, immunosuppressants, antiparasitic agents, antimicrobial agents, cardiac drugs, opioids, steroid hormones, and many other types of chemical compounds out of the cell, increasing chemoresistance. P-gp has 170 kDa and its substrates are hydrophobic molecules and are incorporated into the plasma membrane. When the MDR1 gene becomes mutated, which tends to happen in dogs, they can be prone to neurotoxicity since the macrocyclic lactones can penetrate the brain in high numbers [12].

ABCG2 is a member of the ABC transporter family. It also creates multidrug resistance. ABCG2 is able to protect cancer stem cells and its complex oligomeric structure, drug bioavailability, and in predicting both hematopoietic and solid malignancies within the body. The structure of ABCG2 consists of one hydrophilic nucleotide binding domain (NBD) and one hydrophobic membrane-spanning domain (MSD). Based on the arrangement and grouping of these domains, they can be classified as full transporters, half transporters, and non-transporters. ABCG2 in particular is a half transporter, with the MSD consisting of 6 transmembrane segments (Figure 4).

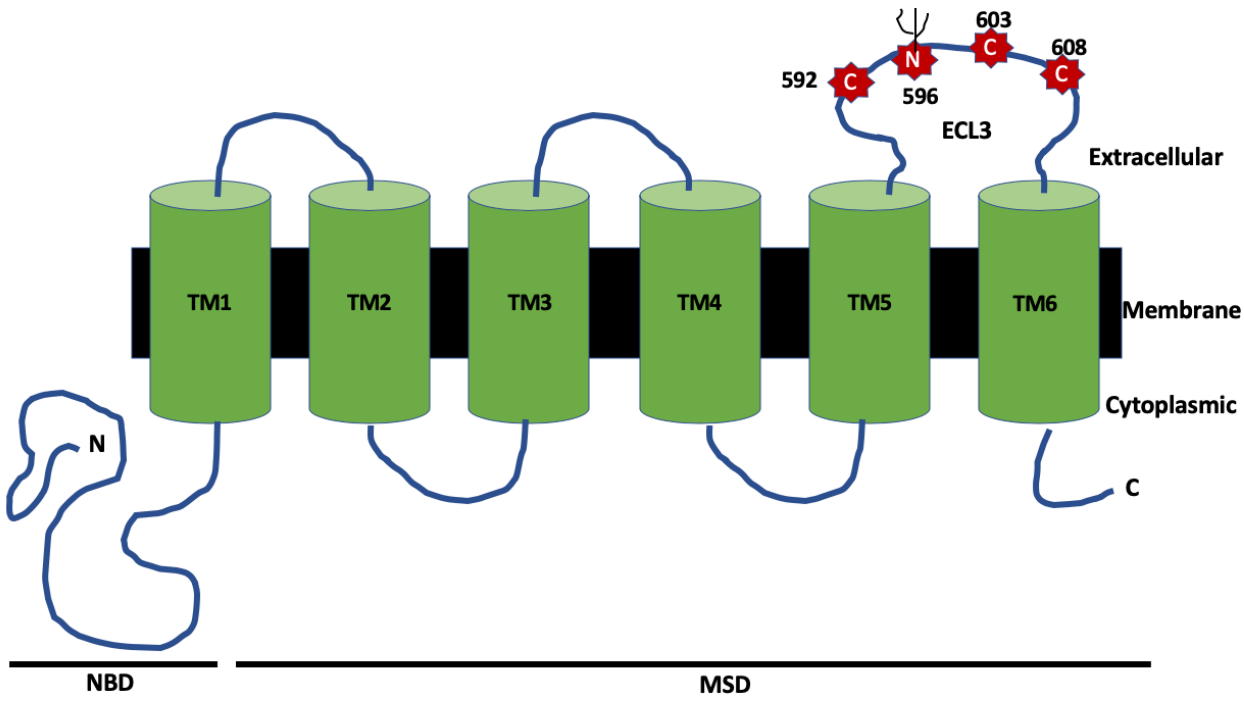

Figure 1.4. The structure of ABCG2 consisting of NBD and MSD. The MSD consists of 6 transmembrane segments located in the membrane [13].

It is hypothesized that due to its half size, ABCG2 can work like a homo-dimer. This was surmised by an experiment done when a co-expression of an ATPase-dead ABCG2 and wild-type ABCG2. This resulted in a reduction of the ABCG2 transport activity. Also, the ABCG2 migrated as a monomer in reducing conditions, but as a dimer in non-reducing conditions. But recent evidence suggests that ABCG2 may actually be higher order of homo-oligomer on plasma membranes. ABCG2 is expressed in several places in the body: the placental syncytiotrophoblasts, epithelium of the small intestines and colon, liver canalicular membranes, and the ducts and lobules of mammary tissue. ABCG2 can also be present in the venous and capillary endothelium. It mainly resides in the plasma membranes of the cells in the tissues mentioned above. The high expression of ABCG2 is seen in cancer cell lines that are drug resistant and in clinical tumor tissues. It is regulated with gene amplification, epigenetic

modifications, transcriptional and posttranscriptional regulation. For epigenetic regulation, demethylation allows increased expression of the ABCG2 gene. The ABCG2 gene has a CCAAT box that is located 274 bases upstream from the transcription start site. If this box is removed, it reduces the transcription activity and therefore the ABCG2's expression. For posttranscriptional regulation of ABCG2, some miRNAs can inhibit ABCG2 expression and even promote differentiation of the stem cells. It also inhibits migration and invasion of pancreatic cancer cells. There is also another inhibitor, PZ-39, that inhibits ABCG2 and also causes conformational changes and accelerates degradation of ABCG2. ABCG2 also makes multidrug resistance. There are small modulators that can bind to ABCG2 and can reduce its half-life. This can be a venue for therapeutic research to reduce the expression of ABCG2 in cancer [13].

Nanog is a homeobox transcription factor that is important in determining the fate of embryonic stem cells and aids in self-renewal of cells. It is a key player in maintaining pluripotency during embryonic development. The Nanog promoter region consists of five CpG-dinucleotides, OCT3/4-SOX2 binding motif, a TATA-box and binding sites for some transcription factors. The region is 299 bp long, from -264 to +35). There are transcription variants of the mRNA encoded by Nanog: Nanog-001 and -002 (both for putatively protein coding), Nanog-003 (for nonsense mediated decay), and Nanog-004 (protein coding) [13].

Nanog is a homeobox gene, which is a group of genes that have a common DNA sequence that function in body segmentation and cell differentiation during the embryonic stage of development. These genes consist of a 180 bp segment which codes a 60 amino acid DNA-binding protein domain that is called the homeobox. It is capable of binding to DNA and regulating a variety of genes [14].

These CD133 and ABCG2 are typical cancer stem cell (CSC) markers. Therefore, these are commonly used in research related to detecting protein expressions in cancer. Also, ABCG2 is an ABC transporter. Since it has the characteristics of drug efflux, it is something that is necessary to study the protein expression of. MDR1 was chosen since it is commonly known as the drug efflux protein since it produces p-glycoproteins. This expression is important to look at in metastasized cancer cells. There are many functional studies that provide evidence that Nanog plays an important role in malignant disease, since it has many harmful properties, like invasiveness, tumorigenicity, and chemoresistance. Therefore, it is important to study its protein expression [24].

There is a myriad of proteins that are expressed in cancer cells, however once the cancer cell metastasized, only a couple of proteins have increased expression after metastasis [24]. CD133, ABCG2, MDR1, and Nanog are among those types of proteins.

1.6 Calcitriol for Drug Resistance

Calcitriol is a type of Vitamin D_3. This molecule is like a steroid that is animal-derived. It is like a prohormone and it is the most predominant form of Vitamin D in humans. It is made from sterol 7-dehydrocholesterol when the skin is exposed to UV B radiation. This can also be acquired through dietary supplements. In order for Vitamin D_3 to become activated, it must undergo an activation process. It is highly lipophilic, so it can only be transported through the blood stream if it is bound to a vitamin D-binding globulin (VDBP), which is a specific plasma protein. Once Vitamin D_3 is made or taken in through dietary uptake, it is activated by the liver or kidney. Inside of the liver, there are multiple cytochrome P450 enzymes, mitochondrias and microsomes that hydroxylate the Vitamin D_3 and release it into the blood stream to the kidney for complete activation. To measure the amount of Vitamin D_3 in the body, it is usually

measured by the amount of serum 25(OH)D_3 level that is produced from the liver. It has a short half-life of 6 hours but a long systemic half-life of 2 months. When there are low levels of serum calcium in the kidney, the CYP27B1 coverts the 25(OH)D_3 to 1,25 dihydroxyvitamin D_3 or calcitriol (Figure 5). The most active form of calcitriol is as a small lipophilic molecule that is capable of cellular penetration.

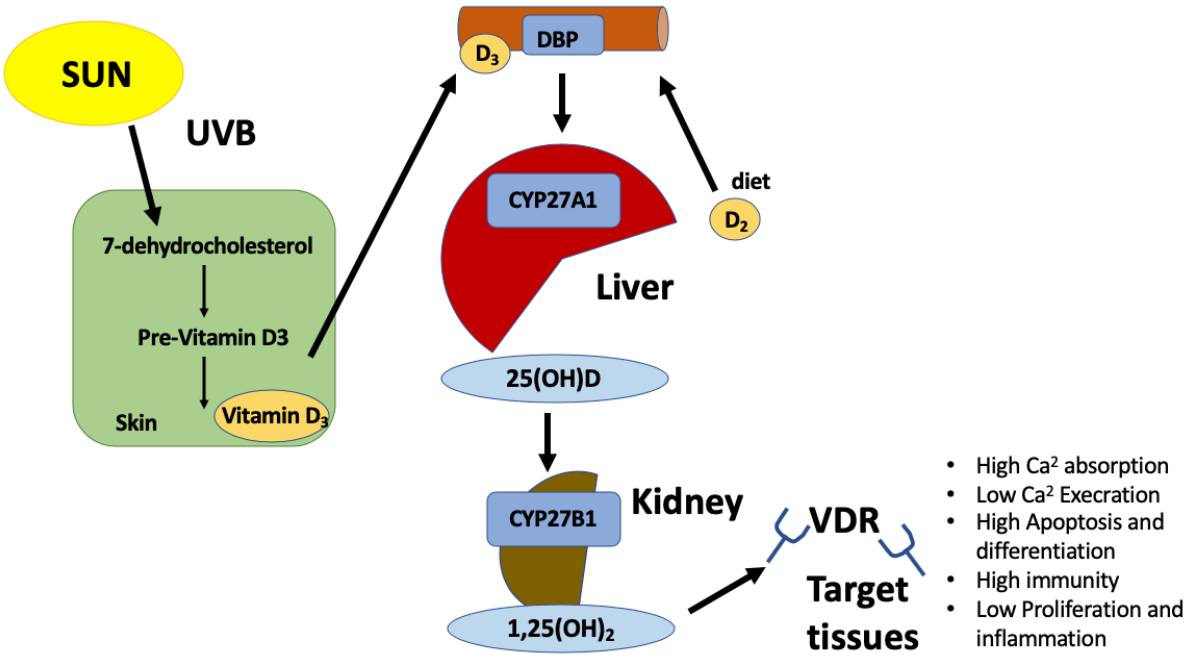

Figure 1.5. The process of biological activation of calcitriol [15].

Calcitriol's main functions involving the bone is mineral homeostasis, bone growth, absorption of calcium from the gastrointestinal tract (GI), and stimulation of the absorption of phosphate and magnesium ions. These are all important functions to support the bones and its mineralization. Calcitriol binds to the intracellular vitamin D receptor (VDR) to form the retinoid-X receptor. This then binds to DNA in order to initiate or suppress gene expression. VDR binds best with Vitamin D_3 than another form of Vitamin D. VDR exists in multiple cell types in the body, such as the kidney, heart, muscle, breast, colon, prostate, brain, and immune

cells. But it is mainly expressed in the GI and bone and therefore are physiologically affected the most by calcitriol. In cancer, it is involved in growth and differentiation of the cancer cells as well as their adhesion and apoptosis since calcitriol also regulates the cell cycle. It is highly effective against cancer as it is anti-proliferative, pro-differentiating, pro-apoptotic, and anti-inflammatory. Calcitriol is effective in these ways since it is capable of inhibiting V-H+-ATPase. This enzyme is overexpressed in metastatic cancer by controlling the tumor microenvironments. It does this by extruding protons to the ECM. It also keeps the balance of lactate in cancer cells under hypoxic conditions so the cancer cells will always have a supply of energy. It can also regulate the activation of glycolytic metabolism. It is activated in high levels of HIF-1a or hypoxic conditions, which are the normal conditions of tumor cells. With calcitriol, it can target this V-H+-ATPase and inhibit its expression in the tumor cells [25].

However, as a drug to use against cancer, it has a limitation as it is toxic at higher doses, as it results in hypercalcemic side effects. For use in prostate cancer, calcitriol has been seen to inhibit prostate cancer cell growth in vitro and slows tumor growth in animal models. Therefore, calcitriol can be used in combination with other agents to enhance the effectivity of the drug towards prostate cancer. Doing this combination therapy can kill more cells with low toxicity, can create interactions that allow for optimal treatment of the tumor, slow down drug resistance, lower costs, reduce side effects of the drugs as it is being used a lower dosages, and it can reduce symptoms and increase the patient's life expectancy. To achieve the best combinational therapy, the drugs combined must have different mechanisms of action and should not have any overlapping toxicities. They both should have some effectivity towards the same cancer type. Therefore, the combination drugs need to be chosen carefully and not make unwanted side effects or toxicity which would therefore create low effectiveness in destroying the tumor [15].

1.7 Overview of Paper

In this paper, there will be a discussion of collecting and culturing H460 lung cancer cells in the microchannel device. The cancer cells will be within the microchannel device to imitate the conditions of being in the body: a confined space and metastasis from the primary site to the secondary site. A drug-test study using paclitaxel was then done to see how chemotherapeutic drugs can affect the cancer cells. Lastly, a pre-treatment of calcitriol was done to see if this could provide a solution to reverse drug resistance of the cancer cells. By conducting this study, we hope to provide a new way to carry out drug screening and proteomic studies and therefore create a new way of chemotherapy medication development. Also, new information about the cancer cells' behavior in their invasiveness and resistance can become elucidated through this research. This can therefore pave the way for new chemotherapeutic development that can be effective against lung cancer cells.

CHAPTER 2

METHODS

2.1 Microchannel Device Fabrication

Devices that were designed in the lab with the appropriate microchannel size (5x12 um) was used for the study. To fabricate the devices, the standard negative photolithography and the soft lithography were used. Polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Base, Dow Corning) and curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning) was mixed in with a ratio of 10:1 (v/v). Then the devices were cut and decontaminated with 70% ethanol and assembled onto 6-well plates.

2.2 Migrating Cells and Cell Harvesting

The 6-well plates were coated with Collagen type I (Corning, REF 354236) in order to enhance cell attachment and migration. The devices were washed with PBS three times and then 20 x 10^3 H460 cells were seeded into each well and cultured in 10% serum supplemented medium for 8 days before they were collected. Trypsin-EDTA was used to detach cells. Costar® 3008 Cell Lifter (Corning Inc) was used to remove any cells that were outside of the microchannel. Once it was confirmed with a microscope that there were no more cells outside of the channel (2D control), the PDMS device was then peeled to expose the migrating cells. After using Trypsin-EDTA, the confined-migrating cells were collected from all 24 devices (about 6 x 10^4 cells in total). For the 2D control group, the cells were then collected from the outside microchannel from the same well. The cells from both of the groups were then centrifuged and the cell pellet was harvested.

2.3 Western Blotting

RIPA buffer (R0728, Sigma-Aldrich) and protease inhibitor (P2714, Sigma-Aldrich) cocktail was used to lyse cells. Pre-equilibrated protein samples were loaded into a 10% SDS-Page gel, electrophoresed, and then electro-transferred to a PVDF membrane (Bio-Rad). Transferred membranes were then blocked with a 5% non-fat milk (M0841, Lab scientific). Nanog (D73G4, Cell Signaling), CD133 (D2V8Q, Cell Signaling), MDR1 (E1Y7B, Cell Signaling) antibodies were used for proteomic analysis. All Western blot results were detected following the standard protocol from Bio-Rad and normalized by total protein.

2.4 Viability Analysis

For drug sensitivity analysis, paclitaxel (Sigma-Aldrich), which is a drug commonly used for killing cancer cells, was added to both the 2D control and the confined-migrating groups of cells in varying dosage then incubated. Paclitaxel is also known to slow the spread of cancer cells and its growth. It is capable of promoting assembly of microtubules of repeating subunit polymers α- and β-tubulin heterodimers []. The Green Live/Dead Stain (11J66, ImmunoChemistry; 1:1000 (v/v) in PBS) was used to determine the cell viability after drug treatment. For the calcitriol experiment, all the cells were collected from the microchannel or the 2D cultural condition and were reseeded to the 96 well plate. Then a 5μM calcitriol pretreatment was done for 24 hours and then followed by 24 hours of 1840 nM Dox incubation for both of the groups. The cell viability was then detected by the standard MTT assay (Sigma-Aldrich).

2.5 Statistical Analyses

Microsoft Excel, GraphPad and R studio were used to carry out all of the statistical analyses. A two-tailed, paired student *t*-test were performed to compare the differences between 2D and confined-migrating groups. *P*-value of less than 0.05 was considered statistically significant.

2.6 COMSOL Multiphysics ® 5.3 Simulation

To simulate the microchannel's flow rate, a model was made in SolidWorks ®. One rectangular prism was created, which represents one channel of the microchannel device (it is not necessary to test all 600 microchannel since all the microchannels have the same parameters). The dimensions were the same as one of the channels in the microchannel: 0.05 mm wide x 5 mm length x 0.012 mm height. The inlet and outlet of the microchannel are closed off in order to simulate the flow in COMSOL Multiphysics® 5.3. A project was created in COMSOL Multiphysics ® 5.3. under 3D modeling and laminar flow for the microchannel. The model was then imported into the project. Then the material, PDMS, was selected for the microchannel. After that, the inlet was selected by choosing one end of the microchannel. A 32 μm/hr or 8.889 x 10^{-9} m/s was chosen as the inlet flow. The other end of the microchannel therefore became the outlet, and 1 atm or 101,325 Pa was chosen as the outlet pressure. A normal mesh was done on the model and then the simulation was computed. 50 slices were chosen for the velocity.

CHAPTER 3

RESULTS

3.1 Assessment of stress when cells migrate inside the microchannel device through simulation testing of flow rate

Since the microchannel would be used to test for metastasis of the cells, understanding the stress the cells undergo when they migrate is important to evaluate to make sure the cells are in fact traveling in a confined environment. The resistance that the cells face is dependent on the microchannel's dimensions (equation in Figure 3.1b). Therefore, simulations were done in COMSOL Multiphysics® 5.3 with the stress indicated as the flow rate inside of the microchannel (Figure 1b). Multiple conditions were set for the simulation. PDMS material is what the microchannel was made out of, so it was the material applied in the COMSOL Multiphysics® 5.3 simulation. The inlet velocity was set as 32 μm/hour or 8.889×10^{-9} m/s which was determined as the migration rate of cancer cells. Also, the outlet pressure was set to 101,325 Pa or 1 atm, which is normal atmospheric pressure. Everything was considered under laminar flow rather than turbulent flow, since the flow rate of cells can be estimated to the flow rate of liquids. There are 100 plane slices that are used to see the velocity flow. By using 100 plane slices, the most accurate reading of the velocity flow rate over the overall microchannel can be obtained. By comparing two differently sized microchannels, one of 100 x 100 μm (which represents a 2D microchannel) and another of 5 x 12 μm, the 2D microchannel has a higher velocity than the 5 x 12 μm microchannel.

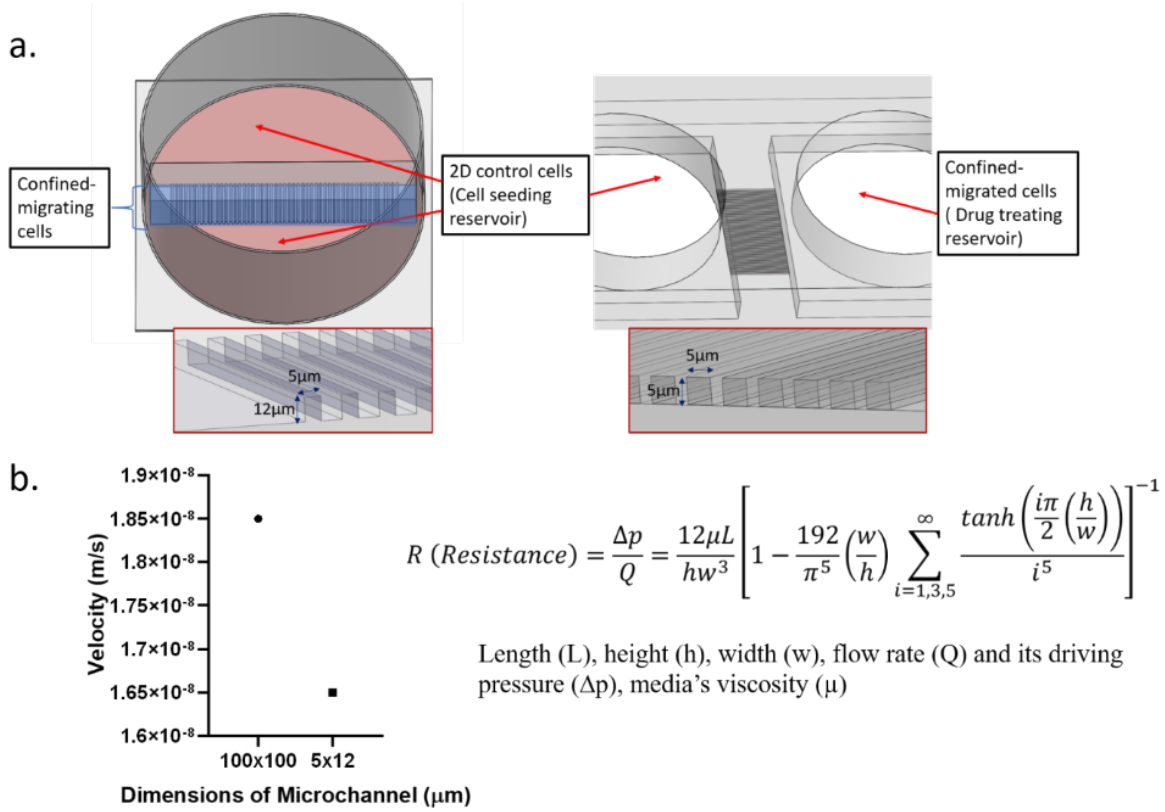

Figure 3.1. Cells receive confinement during migrating in microchannel and is channel size related. a. Two different sized microchannel devices were used in this study to generate confined-migrating H460 cancer cells. Microchannel size labeled in the red window below each device schematic image. confined-migrated cells', confined-migrating cells' and 2D control cells' collecting locations are labeled. **b.** COMSOL Multiphysics® 5.3 simulations of the microchannel demonstrating velocity flow through the microchannel. 100µm X 100µm sized channel was used to simulate 2D conditions. Left equations show the resistance cell needs to overcome when they are migrating inside the microchannel. As the driving pressure (Δp) remaining the same, resistance and moving velocity (in equation showed as flow rate) have an inverse relationship.

3.2 Drug resistant proteins have increased expression in confined-migrating cells

Western blots were done of each protein for both normal cultured cells (2D cells, the control, C) and confined-migrating cells (M). We investigated four proteins: CD133, MDR1, ABCG2, and Nanog. These four proteins were all increased in the confined-migrating cells. A series of three trials of western blotting were conducted to test the protein expression the H460 lung cancer cells. The resulting averages of the data is shown below (Figure 3.2). CD133 is expressed 4

times more or a 300% difference in the confined-migrating cells than the control. ABCG2 is expressed 1.8 times more or a 180% difference in the confined-migrating cells than the control. MDR1 is expressed 5.8 times more or a 580% difference in the confined-migrating cells than the control. Nanog is expressed 2 times more or a 200% difference in the confined-migrating cells than the control. CD133 and Nanog are biomarkers for stem cells and ABCG2 and MDR1 are expressed in drug resistant cancers. Therefore, these expressions indicate the migrating cancer cells have a cancer stem cell property and an increase in chemotherapeutic resistance due to drug efflux after metastasis.

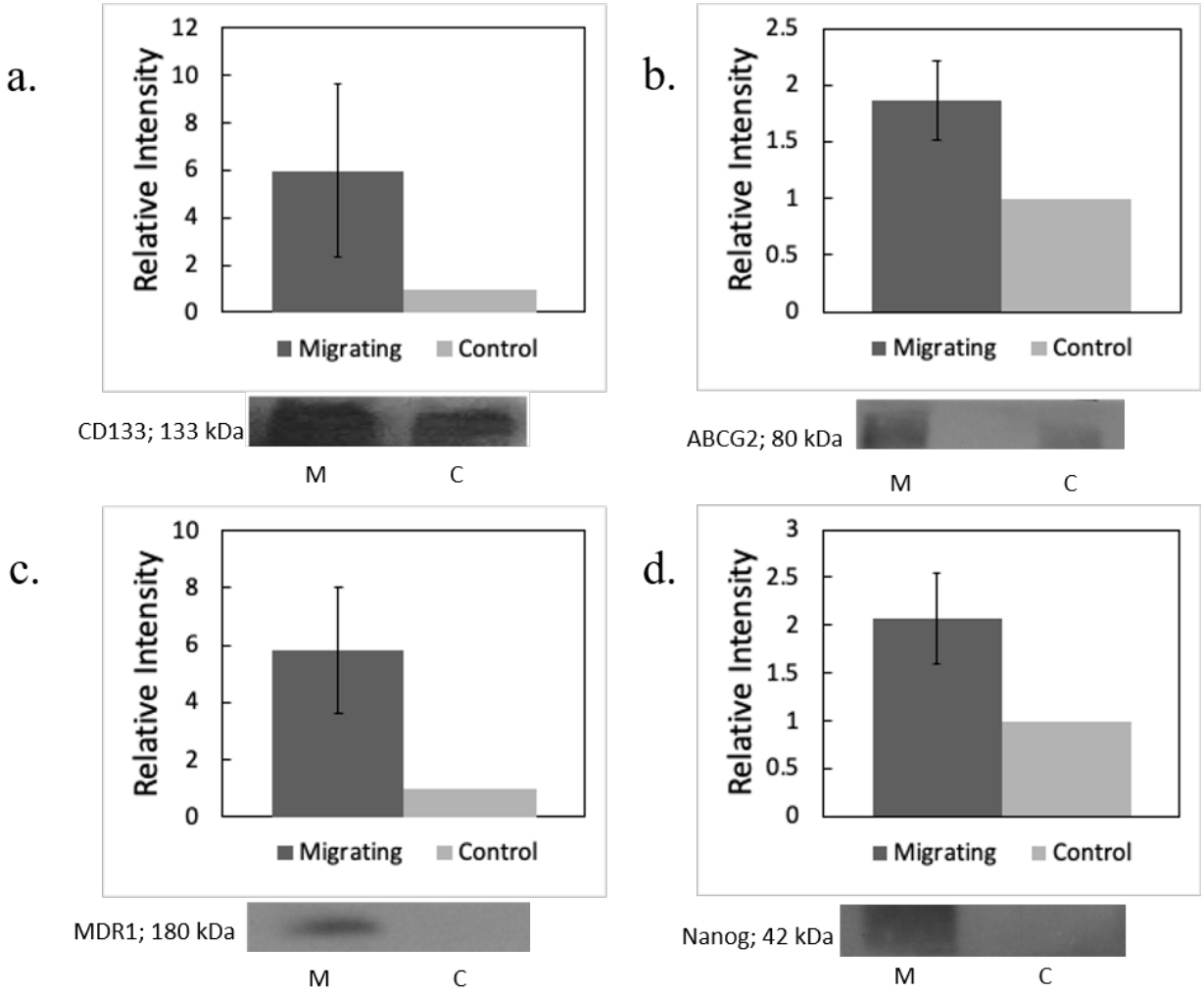

Figure 3.2. Increase cancer stem cell marker and efflux protein expression in H460 cells after confined migration. Several proteins' (A: CD133, B: ABCG2, C: MDR1, and D: Nanog) relative intensities of western blot were compared between confined-migrating and

control groups. Each of them has its corresponding blot beneath the graph. Results are shown as average ± Std. *p<0.05. N=3.

3.3 Chemical sensitivity in confined-migrating H460 cells

Cell viability tests were conducted to test how well migrating H460 cells and normal cultured cells can survive when drugs are applied to them. Paclitaxel was used as the drug and applied to two experimental groups (migrating): one at 10 nM and the other at 50 nM. A control group with normal cultured cells and the same concentrations was done as well for comparison. Images of the 2D space (outside of the microchannels) and inside of the microchannels (5x5 μm). Quantification for the viability was carried out by staining and counting method. All data displayed were normalized to a control. Among the control, the 10 nM and 50 nM Paclitaxel groups for the control, there was a similar amount of Green PI positive cells detected. For the migrating cells, most of the Green PI positive cells were outside of the microchannel (in the 2D area) and fewer positive cells were found inside of the 5x5 microchannel (Figure 3.3a). Overall, the viability of the confined-migrating H460 cells was higher than the 2D cultured cells over both treatment groups (Figure 3.3b). Even though the 2D group had a higher viability than 5x5 group in control group, it will not influence the final results.

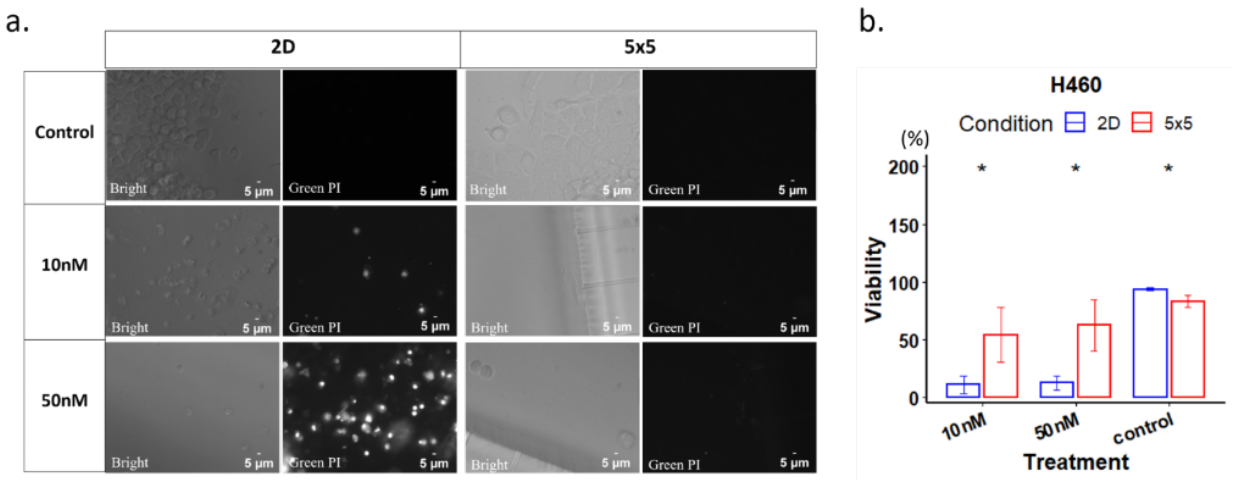

Figure 3.3 Cell Viability after 72hrs incubation of paclitaxel. a. After 72hrs treatment with/without various dosages of paclitaxel (10nM and 50nM), images were taken at 2D and 5x5 for the control and experimental groups. For all groups of images, the left column is bright view images and the right column is Green PI staining images that taken under GFP. The scale bar is shown on the right bottom of each image. **b.** H460 cells throughout microchannels were treated with various dosages (control (0nM), 10nM, and 50nM) of paclitaxel of 72hrs. Cell viability was tested using Green PI staining plus counting. Results showed as average + Std. *p<0.05 between 2D control and 5x5. 2D: normal cultured, 5x5: cells inside 5x5 μm microchannel.

3.4 Resistance reduced by application of calcitriol

To see the effect of calcitriol and Dox on H460 cell viability for both migrating and control, 2D cells, they were pretreated with calcitriol and with different Dox conditions (0 nM, 1840 nM, and 3680 nM). The calcitriol by itself does not have much effect on the H460 2D and confined-migrating cells. However, the confined-migrating cells did show less sensitivity to the 1840 nM Dox treatment compared to the 2D cells. There was a dramatic decrease in cell viability when the migrating cells were pretreated with calcitriol before incubated with Dox 1840 nM (Figure 3.4 right window). For the one that was pretreated with only calcitriol, it was close to 2D level. But when the Dox concentration was increased to 3680 nM, all groups showed similar viability.

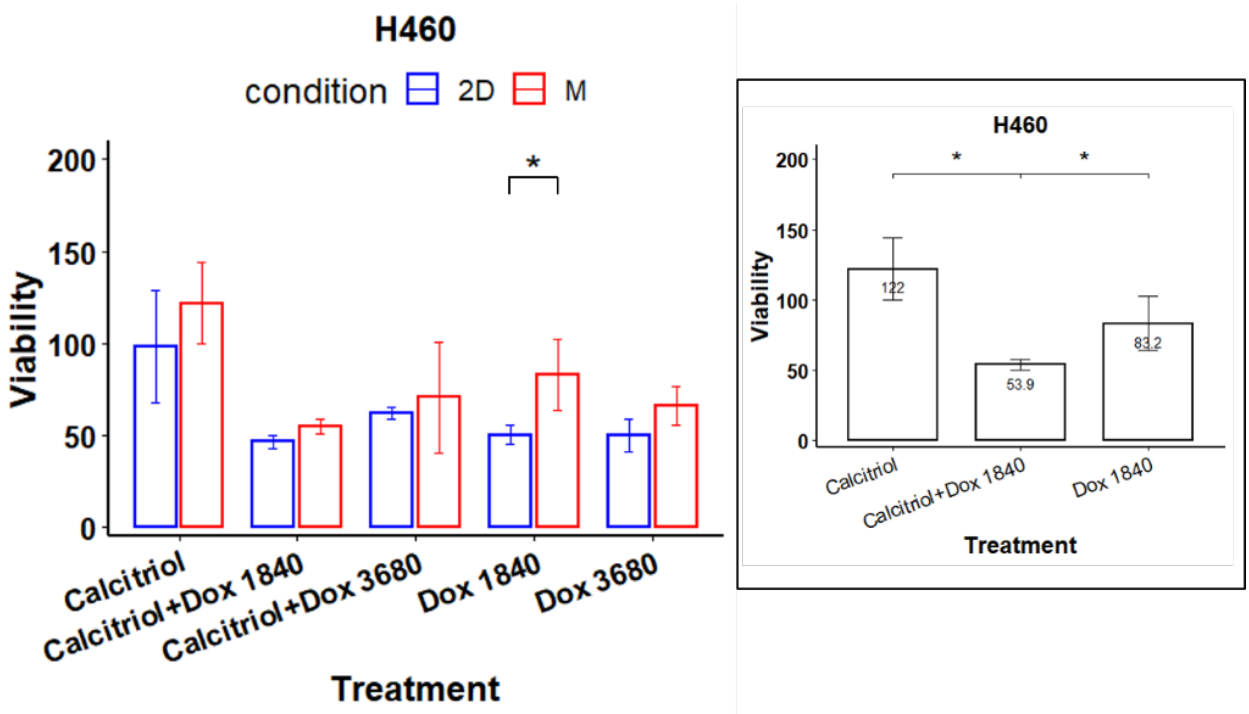

Figure 3.4. Calcitriol pretreatment reduced confined-migrating cells' resistance. Confined-migrating cells and 2D cells were pretreated with 5 μM calcitriol for 24 hours before 24 hours Dox (0nM, 1840nM and 3680nM) treatment. The right window displayed comparison within confined-migrating groups. Results showed as average ± Std. *$p<0.05$. All results were normalized by control. 2D: normal cultured, M: collecting from the microchannel.

CHAPTER 4

DISCUSSION

The major predicament with metastasized cancer cells is their high resistance to chemotherapy, in which anticancer treatments become ineffective. As the cancer cells metastasize from one place in the body to another in physical confinement, their properties change and become different than before they metastasized. To study these changes and how they can affect drug resistance in H460 lung cancer cells, polydimethylsiloxane (PDMS)-based microchannel devices were built to study these lung cancer cells under physical confinement. From these results, we can be able to understand how the cancer cells respond to chemotherapy and the protein changes they undergo.

By the results gathered from the simulation done on the microchannel device, it can be asserted that the stress when cells migrating inside microchannel are higher as the channel size becomes smaller. This increase in the ratios of the wall surface to flow volume creates a high resistance, making a high-pressure drive throughout the microchannel. This then results in low velocities, causing the cancer cells to slow down [12].

The protein analysis that was done via western blot showed increased expression for ABCG2 and MDR1 in confined-migrating H460 cells. ABCG2 and MDR1 both do drug efflux of chemotherapeutic drugs [12 and 13]. Therefore, with an increased expression in both protein in the confined-migrating H460 lung cancer cells, they must acquire some increased drug resistance. This was further verified by the drug sensitivity test since there was a higher cell viability when the cells were treated with paclitaxel (Figure 2.3).

CD133 and Nanog were also two proteins with increased expression. They are commonly used a as biomarkers for cancer stem cells (CSCs) in lung cancer cell lines. The cancer stem cells are

capable of self-renewing themselves and increasing their plasticity. This contributes to their ability of being chemo-resistant [11 and 19].

Therefore, from these findings, traditional chemotherapeutic tactics will not be enough to stop the cancer and other methods must be used to effectively and annihilate the cancer from the body.

Calcitriol is a synthetic version of Vitamin D. Drug cell viability results demonstrate the debilitating effect of Calcitriol 5μM and Dox 1840nM on H460 confined-migrating cells rather than 1840nM Dox alone. This combination allows for an enhanced effect on the cell viability, enabling the cell population to decrease dramatically. Therefore, these drugs can be used as a potential therapy for inhibiting the confined-migrating H460 cancer cell proliferation.

In summary, this study was able to prove physical confinement in the microchannel device through flow rate velocity. This physical confinement and the metastasis of the cancer cells can create different protein expressions, causing increased expressions in drug efflux proteins and CSC related proteins. Since this leads to an increased resistance of chemotherapeutic drugs, they become ineffective to this increased protein expression. Therefore, there must be other therapeutic methods that need to be developed to combat this resistance and would provide a successful solution to stopping metastatic cancer.

In conclusion, metastasis is the leading cause of death in cancer patients. Since lung cancer is the second most diagnosed cancer, it is important to study this cancer type. Cancer cells tend to be drug resistance due to many factors, like epigenetics and drug efflux. Therefore, we studied different proteins through the use of western blots and did a drug viability analysis to see that with the increased expression of these proteins, it led to a drug resistance that would render chemotherapy ineffective. With our calcitriol study, it showed that there was a reduction of cell

viability in migrating cells with the Calcitriol + Dox treatment. This can lead to new therapies that include calcitriol.

Therefore, for future directions, it is important to find ways to combat drug resistance. These can lead to alternative therapies to chemotherapy. Since calcitriol is a promising treatment, it is necessary to do more trials and experiments with calcitriol and see its effect on the cancer cells. Then these results can be not only used on lung cancer, but on other cancer types.

Also, more cell lines should be explored. It could be possible that the results were only characteristic of the H460 cell line. Therefore, another cell line would need to be used to confirm the results that were received from this experiment. One cell line that can be used for comparison is A549 lung cancer cell line. This cell line originates from non-small lung cancer as well [21]. Therefore, it can be used as a comparison to see if the same protein expressions would occur. To further test whether the results are not only characteristic of non-small lung cancer cell lines, a small lung cancer cell line can be tested as well, such as H889, H187, H82, or H69 [22]. Using these cell lines, we can see if the increased protein expression is the same for all lung cancer cell types.

Only four proteins (CD133, ABCG2, MDR1, and Nanog) were used in this experiment. Rather than just these four proteins, more proteins or other proteins other than the ones used in this experiment can be used. A microarray can be created for all of the different antibodies and can be used to determine the makeup of the proteins. Other proteins that can be included could be CD44, CD74, CD98, and NDRG1[23]. These can then be used for future experiment to test out how their expression changes after metastasis.

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