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Characterizing the effect of arginine deprivation on breast cancer cell migration and proliferation

By

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Characterizing the effect of arginine deprivation on breast cancer cell migration and proliferation

George El Hajj

Abstract

Depriving cells of arginine, a semi-essential amino acid, is a developing strategy to target various cancer types. However, little is known about the effect of arginine-degrading enzymes in breast cancer, a disease of diverse molecular and phenotypic subtypes. In this project, we utilized a pharmacologically formulated human recombinant arginase (HuArgI(Co)-PEG5000) to evaluate the impact of arginine deprivation on breast cancer. We studied three breast cancer cell lines MCF-7 (a non-metastatic model for luminal breast cancer), UACC-2087 (a slow-growing model of triple negative breast cancer), and MDA-MB-231 (a highly aggressive and metastatic model of triple negative breast cancer). Using wound healing assay, Ki67 immunofluorescence assay, and annexin v pi assay, we respectively found that a 72-hour HuArgI(Co)-PEG5000 treatment mitigated MCF-7 and UACC-2087 cells' migration, proliferation and survival. Interestingly, while treatment of MDA-MB-231 for a shorter time frame (24h) with HuArgI(Co)-PEG5000 did not result in robust anti-migratory or anti-proliferative effects, longer (72h) incubations induced a level of cell death in this cell line superseding that of UACC-2087 and MDA-MB-231. Our findings suggest the presence of differential mechanisms of action for arginine depriving enzymes across breast cancer subtypes, and point towards robust cytotoxic effects in highly proliferative triple negative breast cancer cells.

Key words: Breast Cancer, Arginine, Arginase, Migration, Proliferation, Survival

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List of Abbreviations

ADI: Arginine deiminase

AML: Acute myeloid leukemia

ASL: Arginosuccinate lyase

ASS-1: Arginossucinate synthetase-1

BC: Breast Cancer

DMEM: Dulbecco's Modified Eagle Medium

ER: Estrogen Receptor

FBS: Fetal Bovine Serum

Her2: Human epidermal receptor 2

HuArg1: human pegylated arginase 1

HuArg1(Co)-PEG5000: Human recombinant arginase 1 cobalt PEG 5000

ITS: Insulin Transferrin Selenium

NO: Nitric oxide

NOS: Nitric Oxide synthase

Orn: Ornithine

PBS: Phosphate-Buffered Saline

PR: Progesterone receptor

PS: Penicillin-Streptomycin

RFS: relapse free survival

TNBC: Triple negative breast cancer

Chapter One

Literature Review

1.1 Breast cancer

1.1.1 Definition, Epidemiology and Histological Subtypes

Breast cancer is a malignant tumor that has formed in the breast. Cancer exists as a result of mutations in the genes responsible for regulating the growth of cells. Normally, old cells die to give place for healthy new cells to take over. However, overtime mutations in genes give cells the ability to proliferate without control or regulation, producing more cells and forming a tumor (Martínez-Reyes et al., 2021). It is one of the most commonly diagnosed cancers in women and it is the principal cause of cancer death in women (Sharma et al. 2010). Breast cancer can be divided into two major histological groups: sarcomas and carcinomas. Sarcomas are rare cancers that arise from the stromal component of the breast (Wilson and Balkwill 2002). Carcinomas are cancers that arise from the breast epithelium which develops into ducts and terminal lobules. Breast carcinomas can therefore be subdivided into lobular and ductal carcinomas. Among all histological subtypes of breast cancer, ductal carcinoma is the most common constitutes 80% of all breast cancer cases. While lobular carcinoma constitutes 10% of all breast cancer cases (Bertucci et al., 2008).

1.1.2 Molecular Subtypes

There is a variability in the therapeutic response and patient survival among cancer patients having identical or similar histological and clinicopathologic classifications. That is why gene expression signatures that define molecular subtypes were identified (Perou et al., 2000). Breast cancers can also be classified molecularly based on the expression of erb-b2 receptor tyrosine kinase 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR) (Eliyatkın et al., 2015). There are four subtypes of breast cancer: Luminal A, Luminal B, HER2 and triple negative. Luminal breast is a highly heterogeneous disease with different histologies, gene-expression profiles and mutational patterns. Luminal breast cancer expresses 70% of identified breast cancer cases worldwide. Luminal breast cancer can then be classified into luminal A (ER^+, PR^+ and $HER2^-$) that has low expression of Ki67 protein. Luminal A breast cancer patients have good prognosis with an 85% overall survival rate (Howlader et al., 2018). Luminal breast cancer can also be classified into luminal B (ER^+, PR^- and $HER2^-$) which has high expression of Ki67 protein. Luminal B breast cancer patients have an intermediate prognosis with an 80-85% overall survival rate (Howlader et al., 2018). HER2+ breast cancer (ER^-, PR^- and $HER2^+$) which is the second most common subtype of breast cancer constituting 20% of breast cancer cases with an 82.7% overall survival rate (Howlader et al., 2018). Triple negative breast cancer which is ER negative, PR negative and HER2 negative has the lowest incidence rate (10% of all breast cancers) and the poorest overall survival rate of 62.1% (Howlader et al., 2018).

Molecular Subtypes	Luminal A	Luminal B		HER2+	TN
		(HER2-)	(HER2+)		
Biomarkers	ER+ PR+ HER2- Ki67low	ER+ PR- HER2- Ki67high	ER+ PR-/+ HER2+ Ki67low/high	ER- PR- HER2+ Ki67high	ER- PR- HER2- Ki67high
Frequency of Cases (%)	40–50	20–30		15–20	10–20
Histological Grade	Well Differentiated (Grade I)	Moderately Differentiated (Grade II)		Little Differentiated (Grade III)	Little Differentiated (Grade III)
Prognosis	Good	Intermediate		Poor	Poor
Response to Therapies	Endocrine	Endocrine Chemotherapy	Endocrine Chemotherapy Target Therapy	Target Therapy Chemotherapy	Chemotherapy PARP Inhibitors

Figure 1. Classification of molecular subtypes of breast cancer and therapies. Subtypes of breast cancer and their biomarkers, frequency of cases. Histological grade, prognosis, response to therapies (Weigelt & Reis-Filho, 2009).

1.1.3 Molecular Models of Metastasis

Metastasis is the spread of cancer from its primary site to distant organs and increases the risk of death for breast cancer patients (Chambers et al., 2002). EMT is the transition of cells with epithelial characteristics to those with mesenchymal characteristics and phenotype (Brabletz et al., 2018). This is found in embryonic development. However, EMT is activated in many cancer types. Activation of EMT increases cancer cell motility in collective migration of cancer cell clusters or as single cells which in turn favors invasion and dissemination (Brabletz et al., 2018). Tumor cell clusters also contribute to metastasis and are known to be more efficient than single cells at producing metastases (Cheung & Ewald, 2016). If the metastatic seed (primary tumor) is a single cancer cell, the resulting tumor will be monoclonal. However, if the metastatic seed is a collective cluster then the resulting tumor can be polyclonal in nature (Cheung & Ewald, 2016).

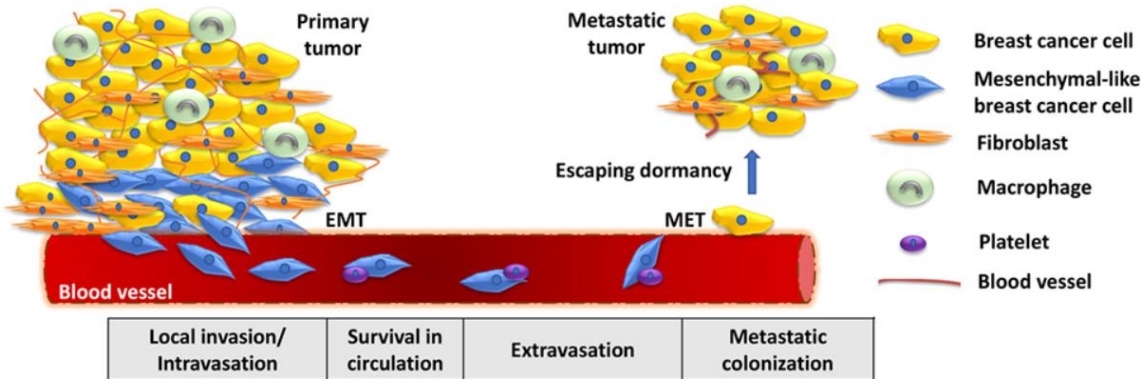

Figure 2. Model of metastasis in breast cancer. Metastasis occurs through invasion, survival in circulation then extravasation and metastatic colonization (Neophytou, Boutsikos et al. 2018).

1.1.3.1 Proliferation

Cellular proliferation has predefined steps throughout the entire cell cycle. It is controlled by protein complexes made of cyclins and cyclin-dependent kinases. Genetic mutations can lead to hyperproliferation of cells into cancer cells through the deregulation of cell-cycle checkpoints, mutations in DNA repair mechanism and mutations in apoptosis. For example mutations of cyclin D1 and Cdk4, are described as oncogenic events (Cordon-Cardo, 1995). The activated oncogenes and loss of tumor suppressors in turn alter metabolism (Dang, 2012). Cancer cells autonomously alter their metabolic pathways due to the increased bioenergetic and biosynthetic demand (Martínez-Reyes & Chandel, 2021). Furthermore, mutations of cell-cycle checkpoints lead to further deregulations that could lead to cancer. Cell-cycle checkpoints are mechanisms where the cell halts progression through the cell cycle until DNA is repaired properly and to make sure that mitosis is complete (Kastan & Bartek, 2004). DNA damage occurs in a variety of ways. Energy released by free O_2 radicals can damage DNA. In addition, alkylating chemicals can alter purine bases of DNA (Kastan & Bartek, 2004). Cells with intact DNA-damage

response arrest or die when DNA damage occurs. This arrest reduces the chance of progression into malignancy. Mutations in apoptosis and checkpoint pathways can facilitate the survival of cells with genetic abnormalities which boosts the chance for malignant transformation (Kastan & Bartek, 2004).

1.1.3.2 Migration

Cell migration beyond the basement membrane and into the stromal extracellular matrix is believed to be an important contributor to metastatic progression. Migration is the directed movement of one cell or a group of cells in response to a chemical attractant or mechanical scaffolds (Trepat et al., 2012). Tumor cell motility is a hallmark of invasion and is the first step in metastasis. Cell migration is regulated by intracellular pathways. The cytoskeleton provides basic infrastructure for the maintenance of cell motility and one of the three types of the cytoskeletal proteins is actin filaments. In cancer cells, actin-binding proteins regulate the assembly and crosslinking of actin filaments (Jiang et al., 2009). Another example of intracellular pathways controlling migration is the Rho GTPase family. Overexpression of Rho GTPases is found in breast tumors while Rho GTPases were barely detectable in normal mammary tissues. Rho GTPases promote cell motility by mediating the formation of stress fibers and focal adhesions (Jiang et al., 2009).

1.1.3.3 Apoptosis

Apoptosis is the process of programmed cell death. Cellular proliferation in an organism is regulated either by increasing or decreasing cell division, or inducing cell death. Apoptosis is important for various physiological processes and for the maintenance of

homeostasis (Kalimuthu & Se-Kwon, 2013). Inhibition of apoptosis leads to the activation of survival factors which promotes cell proliferation in cancer (Kalimuthu & Se-Kwon, 2013). The imbalance between cell survival and apoptosis is necessary in the development of breast cancer which allows the accumulation of genetic mutations (Fernández et al., 2002). Among the hallmarks of cancer are uncontrolled proliferation and reduced apoptosis (Parton et al., 2001). For example, Bcl-2 and Bcl-x_L are expressed in normal levels in a healthy cell but overexpressed in most breast cancers. This overexpression of Bcl-2 and Bcl-x_L allows for more aggressive cell survival and tumor development and drug resistance (Fernández et al., 2002). The mechanisms by which Bcl-2 proteins regulate apoptosis, govern the steps that determine whether specific caspase families, cell death proteases, become active or remain inactive (Krajewski et al., 1999). This is important as it is a main determining factor for whether tumor cells will undergo growth or regression as a result of therapy as many of the therapeutic methods rely on apoptosis. Therefore, it is possible to inspect the molecular and biochemical levels of certain breast cancer cell lines by inspecting apoptosis (Parton et al., 2001).

1.1.3.4 Metabolic Stress, Hypoxia and Angiogenesis

Angiogenesis is a process by which blood vessels are formed. Processes found in angiogenesis include tumor cell migration, proliferation and differentiation of endothelial cells (Almendro & Gascón, 2006). Angiogenesis in tumor cells is crucial since proliferation and metastatic spread depends on sufficient supply of oxygen and nutrients and waste removal. In the absence of vascular support, tumors may become necrotic or even apoptotic. Furthermore, the supply of oxygen and formation of new blood vessels facilitate metastasis for cancer cells (Nishida et al., 2006). Hypoxia induces metastasis.

Hypoxia is the state in which oxygen is not available in enough amounts at the tissue level. Hypoxia in tumor cells causes irregular vascularization, pushes cells into EMT and increases resistance to therapy. Hypoxia also leads to an increase in migration and induces metastasis (Muz et al., 2015). Furthermore, hypoxia aids in increasing blood vessel formation and aggressiveness (Muz et al., 2015). Hypoxia also affects hormone responsiveness of human breast cancer cells. For example, hypoxia promotes estrogen-independent growth of breast cancer. It also affects expression levels of progesterone receptor in some breast cancer cell lines (Kurebayashi et al., 2001).

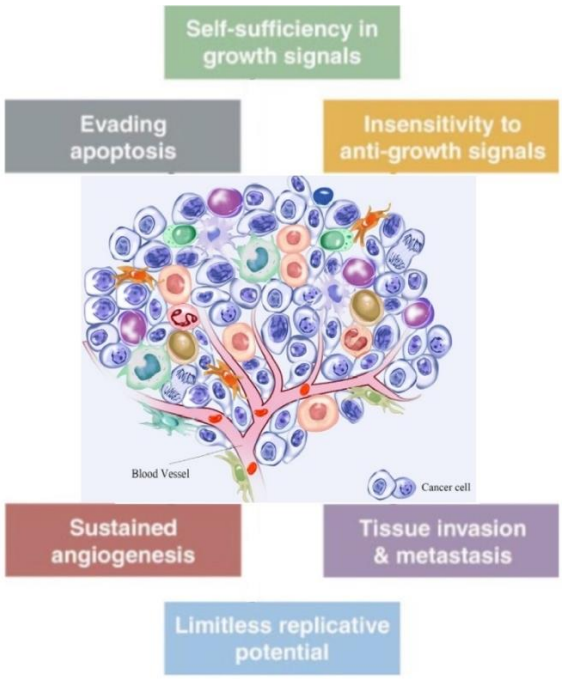

Figure 3. Hallmarks of cancer. Hallmarks of cancer include metastasis, proliferation, angiogenesis, evasion of apoptosis, self-sufficiency in growth signals and insensivity to anti-growth signals (modified from Yan, Xie et al. 2021; Hanahan D, Weinberg RA. 2000).

1.2 Arginine

Arginine is an amino acid that serves as an intermediate in the urea cycle and as a precursor for polyamines, creatine and nitric oxide. Arginine comes from three sources: recycling amino acids from protein turnover (70% of the total arginine production), dietary intake (15%-20% of the total arginine production) and *de novo* synthesis (10% of the total arginine production) from arginine precursor compounds (Albaugh et al., 2017). The main source of *de novo* synthesis is the intestinal-renal axis where the intestines absorb citrulline and the kidneys convert citrulline to arginine. The liver also produces arginine. However, it contains high levels of arginase 1 which breaks down arginine into urea and ornithine (Wu et al., 2009). Arginine is considered a conditionally essential amino acid as it does not need to be obtained from a diet. However, during times of stress, injury, wound healing and rapid growth, the need for arginine elevates (Albaugh et al., 2017). Arginine is also involved in biosynthetic pathways that influence carcinogenesis. For example, arginine is the only precursor for nitric oxide, an essential regulator of normal endothelial cell function. Nitric oxide affects tumor initiation, promotion, progression, apoptosis, and angiogenesis (Morris, 2006).

1.2.1 Arginine Synthesis in the Urea Cycle

De novo synthesis of arginine occurs through the urea cycle by using citrulline as the starting substrate (Wu & Morris, 1998). Ten to fifteen percent of the body's supply of arginine comes from *de novo* synthesis from the small intestine and kidneys. The urea cycle is the process of converting toxic ammonia into urea through a series of steps

(Barmore et al., 2021). Ammonia is first converted to carbamoyl phosphate by the enzyme carbamoyl phosphate synthetase1. Carbamoyl phosphate and ornithine combine to form citrulline. Citrulline alongside aspartate undergo condensation to form argininosuccinate which is carried out by the enzyme argininosuccinate synthetase (ASS). Argininosuccinate is then converted to arginine and fumarate by argininosuccinate lyase (ASL). Arginine is finally cleaved by arginase to form urea and ornithine which is then taken back to the mitochondria to start the urea cycle again (Barmore et al., 2021). Arginine which is nonessential in normal cells becomes necessary in some cancer types in a condition known as arginine auxotrophy. Auxotrophy is the inability of a cell to synthesize a specific organic compound required for the cell's growth (Agrawal et al., 2012). Amino acid auxotrophy has been shown to render cancer incapable of surviving under amino acid depletion. Amino acid auxotrophy is being explored as a possible therapy against cancer (Agrawal et al., 2012).

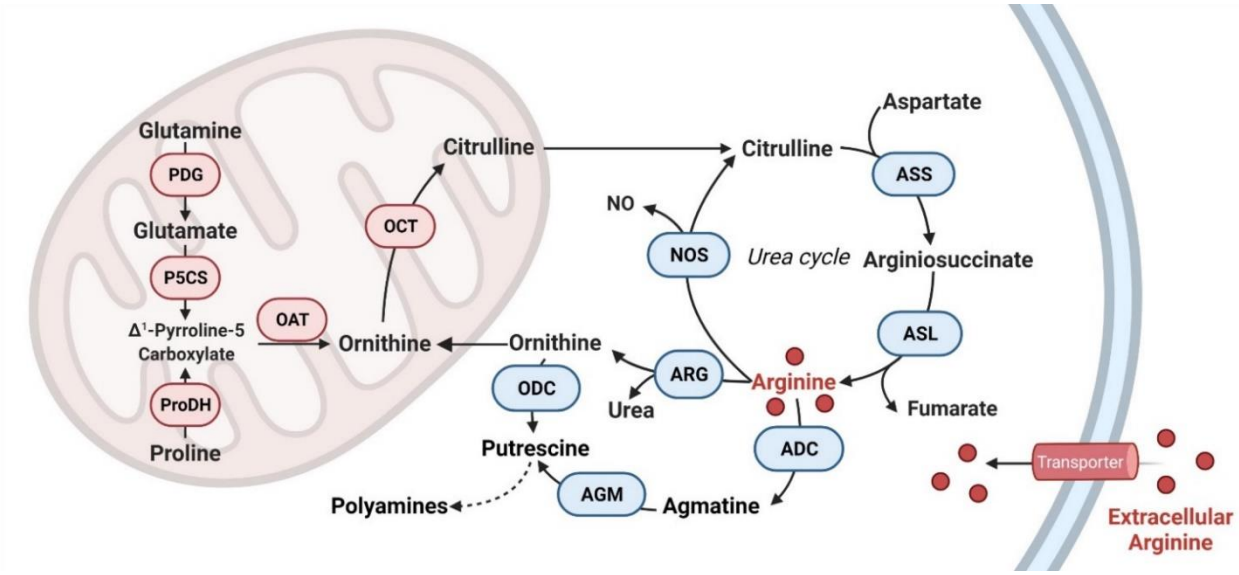

Figure 4. Metabolic pathways of arginine in the urea cycle. Citrulline and aspartate condense to argininosuccinate through ASS. Argininosuccinate turns into arginine and fumarate. through ASL. Arginine is formed into urea and ornithine by arginase (Chen, Hsu et al. 2021).

1.2.2 Abnormal Arginine Metabolism in Cancer Cells

Normal cells are able to synthesize arginine from its primary substrate citrulline through ASS and ASL (Lind, 2004). However, some cancers such as melanoma, hepatocellular carcinoma, and breast carcinoma are auxotrophic for arginine due to an inability to express ASS. Selectively eliminating arginine from the circulation may therefore be a potentially effective anticancer treatment (Dillon et al., 2004). Upregulation and downregulation of the expression levels of ASS and ASL have differing effects on cancer which have been well studied. Loss of ASL expression inhibits cell proliferation and induces apoptosis in specific cancer cell lines (Gong et al., 2019). Importantly, ASS plays a rate-limiting role in arginine production and it's expression varies with cell type, differentiation stage and function (Delage et al., 2010). Upregulation of ASS allows the maintenance of an adequate supply of nitric oxide production that is essential for cancer proliferation and survival (Haines et al., 2011). Conversely, downregulation of ASS leads to certain cancer cells becoming auxotrophic for arginine (McAlpine et al., 2014).

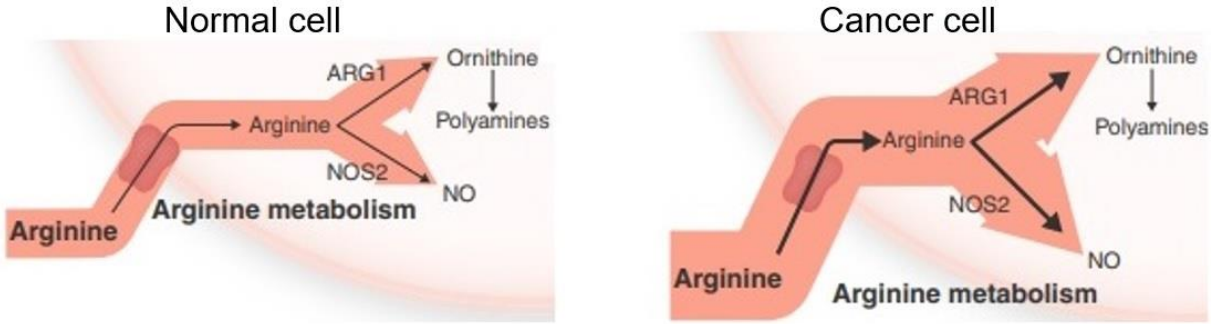

Figure 5. Arginine metabolism in normal and cancer cells. Normal cancer cells have a normal flux of arginine and its products. Cancer cells have an increase in flux of arginine and its products (Riera-Domingo, 2020).

1.2.3 Arginine Transport in Tumor Cells

Arginine is derived primarily from synthesis in the kidney and secondly from dietary sources. Any changes in the dietary intake of arginine may influence conversion of arginine to nitric oxide. At the cellular level, uptake of arginine through the cell membrane is regulated by amino acid transporters. In most mammalian cells, including tumor cells, transport of L-arginine is mediated via the Na^+ independent cationic amino acid transport system (Lind, 2004). Since arginine uptake is the rate limiting factor for cellular nitric oxide production, regulation of arginine may prove useful to influence tumor growth. One possible method to limit arginine use for nitric oxide production is through the systemic treatment with exogenous arginase, an enzyme that catalyzes arginine to ornithine and can thus deplete the circulating pool of arginine and starve arginine auxotrophic tumor cells (Lind, 2004).

1.2.4 Arginine Deprivation

1.2.1.1 Arginine Deprivation as a Cancer Therapy

Tumor progression can be affected by arginine metabolism and its dysregulation. Tumors that lack (ASS) are unable to synthesize arginine and therefore can be targeted using arginine deprivation therapies such as administration of arginine deiminase (ADI) or exogenous arginase (Feun et al., 2015). This leads to normal cells to enter cell cycle (G0) arrest but overcome arginine starvation weeks later after arginase or deiminase was no longer administered. However, malignant cells that were treated with arginase resulted in cell death on a massive scale in three to five days. The malignant cells were not recoverable even in the presence of arginine (Philip et al., 2003). Cells from different

tumor cell lines die quickly *in vitro* after arginine deprivation (Scott et al., 2000). Knockdown of Arginase1 reduces cell migration and induces cell cycle arrest and death *in vitro* in liver cells (Al-Koussa et al., 2020). Arginine deprivation through ADI suppressed acute myeloid leukemia *in vitro* and *in vivo*. ADI-PEG 20 produced a desirable outcome in almost half of acute myeloid leukemia *in vitro* and half of acute myeloid leukemia *in vivo* (Wheatley & Campbell, 2003).

1.2.1.2 Arginine Deiminase

ADI catalyzes the hydrolysis of L-arginine to L-citrulline and ammonia (Park et al., 2003). ADI is generally derived from Mycoplasma due to its high affinity for arginine as compared to human arginase affinity (Dillon et al., 2002). Pegylated arginine deiminase has been used to treat cancers with low levels of ASS activity. How effective ADI is, depends on the level of intracellular ASS which allows tumor cells to recycle citrulline into arginine (Yoon et al., 2007). Even though ADI and arginine decarboxylase can be used for arginine depletion, there are significant drawbacks to using ADI and arginine decarboxylase. Non-cancerous cells treated with arginine decarboxylase are inflicted with severe side effects due to the production of amine agmantine from arginine. (Riess et al., 2018). Furthermore, the use of Arginine Deiminase may be countered by conversion of citrulline back to arginine making a truly arginine-deficient state difficult to achieve (Wheatley & Campbell, 2002).

1.2.1.3 Human Recombinant Arginase 1

Synthesized human arginase 1 (HuArg1) is an arginine deprivation agent. It is used as a therapeutic cancer drug that targets cancer cell lines that are auxotrophic to arginine. Human L-arginase is bound to two Mn^{2+} ion cofactors. Mn^{2+} ions in the serum are lost at

a fast pace and as such it has a short half-life and low saturation constant. Adding Co^{2+} ions to HuArg1 and replacing the Mn^{2+} ions leads to an increase in activity approximately tenfold and leads to a higher stability of the HuArg1 in serum (Khoury et al., 2015). Through PEGylation (addition of polyethylene glycol) there is a higher level of stability. Combining the replacement of Mn^{2+} with Co^{2+} and pegylation, pegylated human arginase (HuArg1 (Co)-PEG5000) is generated (Khoury et al., 2015). HuArg1(Co)-PEG5000 is considered a promising candidate for L-arginine auxotrophic tumors treatment that has reduced immunogenicity (Glazer et al., 2011).

1.3 Study Rationale

In this project, we are studying the effects of arginine deprivation on different breast cancer cell lines using a modified arginase enzyme: HuArgI(Co)-PEG5000. The targeted breast cancer cell lines have different intrinsic molecular and phenotypic features allowing for a broader understanding of the effects of arginine deprivation on migration, proliferation, and survival.

Chapter Two

Materials and Methods

2.1. Cell lines

Human Breast cancer cell lines MCF7 (ATCC HTB-22), MDA-MB-231 (ATCC HTB-26) and UACC-2087 (ATCC HTB-26) were cultured in DMEM (D6429, Sigma, UK) supplemented with 10% FBS (F2442, Sigma-Aldrich, USA), 1% penicillin-streptomycin (L0022, Biowest), 1% Glutamax (Thermo, USA). 0.005mg/ml Insulin-Transferin-Selenium (Gibco, USA) was added to UACC-2087 medium as well. All cell lines were incubated at 37°C with 5% CO_2.

2.2. Pegylated Human Recombinant Arginase 1

HuArgI(Co)-PEG5000 was produced by Aeaglea Biotherapeutics Inc. (Austin, TX, USA) and obtained as a generous gift from Dr. Ralph Abi Habib (Lebanese American University).

2.3. Wound healing assay

Two million cells per well were cultured into in a flat glass-bottom 24-well plate under 3 conditions: 0 nM HuArgI(Co)-PEG5000, 0.5 nM HuArgI(Co)-PEG5000 and 1.5 nM HuArgI(Co)-PEG5000 using MCF-7 and MDA-MB-231 and UACC-2087. After 24 hours, a wound (central line) is cut into each well. The media is removed, then media

containing 0.5 nM of HuArgI(Co)-PEG5000 is added into one well and media containing 1.5 nM of HuArgI(Co)-PEG5000 is added to another well. UACC2087 and MCF7 cells where subsequently imaged every 24 h for 72 h. The more rapidly proliferating MDA-MB-231 cells were imaged at 0 h, 2 h, 6 h, 8 h ,12 h and 24 h time points using an Olympus CKX53 inverted microscope with an Olympus soft imaging solutions GMBH camera.

2.4. Immunofluorescence staining and widefield fluorescence microscopy

Cells plated for the wound healing assay are fixed using 4% paraformaldehyde and incubated for a duration of 10 minutes at 37°C and 5% CO_2. After fixation is done the cells are permeabilized for 10 minutes in the incubator at 37°C and 5% CO_2 using a permeabilization buffer (0.2% Triton-X-100 in 10% PBS with Mg^{2+} and Ca^{2+}). The cells are then blocked for 30 minutes using a blocking buffer (0.1% Triton-X-100 in PBS with Mg^{2+} and Ca^{2+} with 10% FBS) at room temperature. The blocking buffer is removed and a primary antibody solution (10% Triton-X-100, 10% FBS in PBS with Mg^{2+} and Ca^{2+}) containing Ki-67 (8D5) Mouse mAb#9449 (1:200) is added for 1.5h at room temperature. The cells are washed three times for 5 minutes each with cold PBS. The cold PBS is remoed and a secondary antibody solution (10% Triton-X-100, 10% FBS in PBS with Mg^{2+} and Ca^{2+}) containing Alexa Fluor 568 Phalloidin (1:200) as well as Alexa Fluor 488 goat anti-mouse (1:2000) is added for 1hr at room temperature with no light present. The cells are washed two times with PBS each wash for 10 minutes. The cells are then washed one time with PBS and Hoechst 33342 solution (1:1000) for 10 minutes. The cells are imaged using a widefield fluorescence Zeiss Observer Z1.

2.5. Automated Nuclear Segmentation and Ki67 quantification

Automated nuclear segmentation was performed using Stardist plugin on Fiji (ImageJ). Stardist is a deep-learning tool for nuclei segmentation using thresholding-based methods. Images of cells containing Hoechst staining are used in Stardist plugin on Fiji (ImageJ) using 12.9 percentile low, 96.8 percentile high, 0.3 score threshold, and 0.1 overlap threshold and a versatile fluorescent nuclei model. Using the obtained label images, thresholding is performed with a lower threshold level of 1 and an upper threshold level that is set as the default. After obtaining binary images, image calculator is run on the binary images multiplied by the Hoechst images and the Ki67 images separately. Then a division using image calculator is performed with a ratio of the Ki67 image calculator divided by the Hoechst image calculator. Using the previously obtained ROIs, the mean of each nuclei is obtained in order to calculate the ratio of Ki67/Hoechst.

2.6. Annexin V Pi assay

$200x10^4$ cells per well of MCF-7 and MDA-MB-231 and $300x10^4$ cells per well of UACC-2087 were plated into in a plastic-bottom 6-well plate under 3 conditions: 0 nM HuArgI(Co)-PEG5000, 1.5 nM HuArgI(Co)-PEG5000 and 100 nM HuArgI(Co)-PEG5000 for 3 time points 24 h, 48 h and 72 h. At each time point, the cells are collected and added to 500 μl 10X binding buffer,10 μl Annexin V-FITC and 10 μl propidium iodide for 10 min in the dark. The cells are then run on a C6 Flow Cytometer (BD Accuri).

2.7 Statistical Analysis

Statistical Analysis was performed and graphs were plotted in Prism 8 (GraphPad). We utilized Anderson-Darling, D'Agostino and Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests to assess normality. When the data was not normally distributed, we used the Kruskal-Wallis test (with Dunn correction for multiple comparisons) to compare three datasets. Statistical significance was considered starting from $p < 0.05$.

Chapter Three

Results

3.1 HuArgI(Co)-PEG5000 reduces wound healing in specific breast cancer cell lines

Proliferation and invasion are hallmarks of cancer and targeting these two hallmarks in breast cancer might provide potential therapeutic benefit. In order to analyze the effect of arginine deprivation on migration and proliferation of breast cancer cells using HuArgI(Co)-PEG5000, wound healing assays were conducted. MCF7, UACC-2087 and MDA-MB-231 were grown in serum medium. The cell lines were treated under 3 conditions: 0 nM (control), 0.5 nM HuArgI(Co)-PEG5000 and 1.5 nM HuArgI(Co)-PEG5000 for 72h for MCF-7 and UACC-2087. MDA-MB-231 was treated for 24h. The results showed that (HuArgI(Co)-PEG5000)-induced arginine deprivation led to a decrease in the rate of wound closure when compared to that of the untreated cells, in MCF7 (Figure 6A, A') and UACC-2087 cells (Figure 6A, B'). However, (HuArgI(Co)-PEG5000)-induced arginine deprivation had no effect on the rate of wound closure when compared to untreated cells, in MDA-MB-231 cells (Figure 5C, C'). In our experimental conditions, the lack of effect on MDA-MB-231 cells could be due to the short treatment duration (24h) which we had to use for these cells in a wound healing assay, given the high proliferative and migratory rates of these cells. This 24-h duration may not be sufficient to deplete arginine below a phenotype-relevant level.

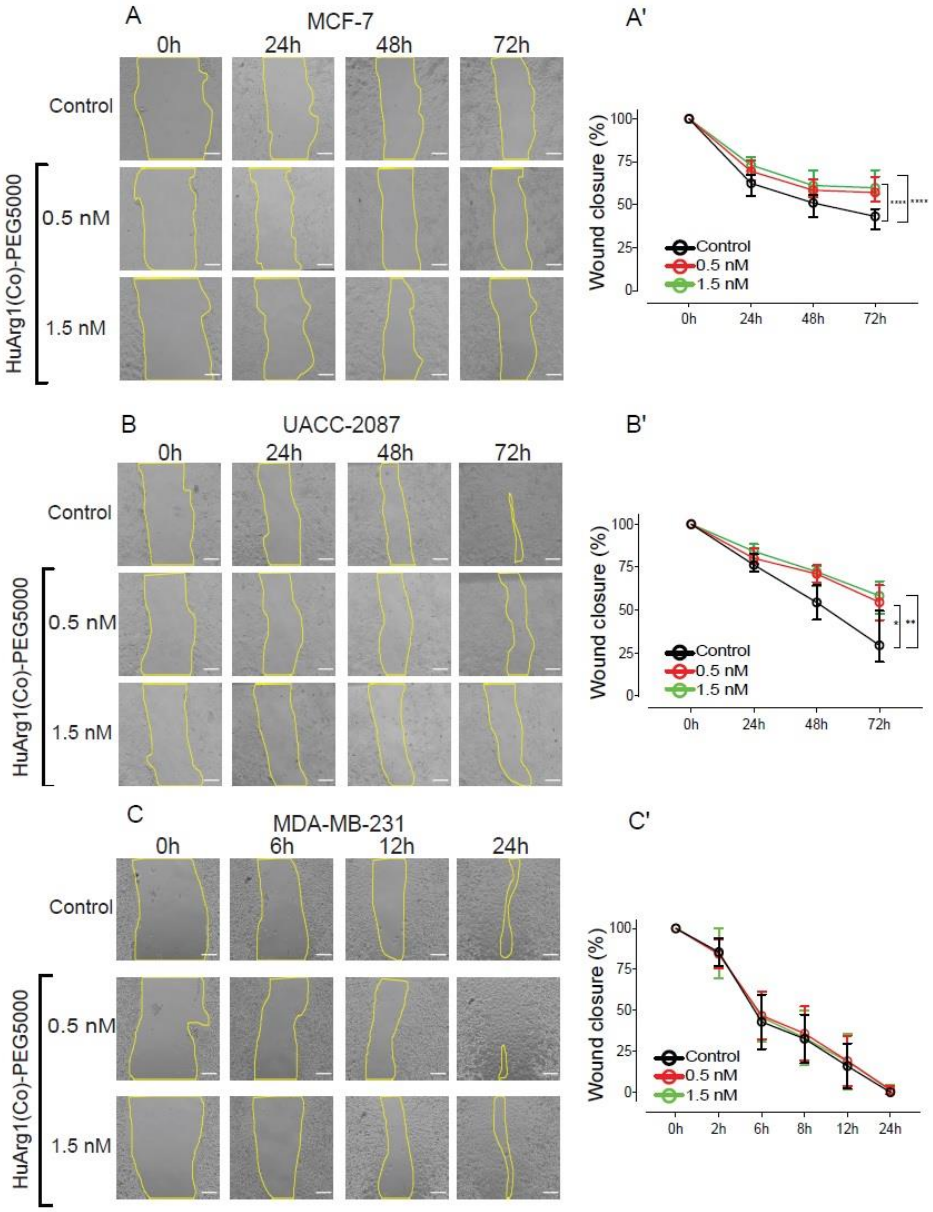

Figure 6. HuArgI(Co)-PEG5000 reduces wound healing in specific breast cancer cell lines. (A, B, C) Representative images of wound healing for MCF-7, UACC-2087, and MDA-MB-231. Cell lines are under conditions of 0 nM HuArgI(Co)-PEG5000 (control), 0.5 nMl HuArgI(Co)-PEG5000 and 1.5 nM HuArgI(Co)-PEG5000 for 72 hours for MCF-7 and UACC-2087 and 24 hours for MDA-MB-231. (A') Quantification of wound closure in MCF7. (B') Quantification of wound closure in UACC-2087. (C') Quantification of wound closure in MDA-MB-231. Five independent wound healing were performed for each cell line.

3.2 HuArgI(Co)-PEG5000 inhibits proliferation of breast cancer cells

Given that the wound healing assay cannot exclude the contribution of cell proliferation from the overall phenotype, we next sought to test more specifically the effect of arginine starvation on proliferation. Therefore, we fixed our cell cultures from the wound healing assay (Fig. 6). We then stained all cell culture for Ki67 (a clinically used marker of active cycling cells) along with pan-nuclear stain Hoechst as an imaging control. We then developed an automated image analysis method for the high-throughput quantification of Ki67 signal at single cell level (Fig. 7). This method consisted of two phases: automated nuclear segmentation, and scoring of Ki67 signal normalized to control nuclear signal (Fig. 7). For automated nuclear segmentation, we trained our own classifiers (one for each cell type) of the machine learning plugin Stardist to accurately detect nuclear boundaries in the Hoechst channel and transform nuclei into individual objects (Fig. 7). After benchmarking, we calculated pixel-to-pixel ratio images of Ki67 over Hoescht and measure the ratio within each segmented nucleus. Cells treated with HuArgI(Co)-PEG5000 showed a significant reduction in Ki67/Hoechst (indicating a decrease in the number of actively cycling cells) in all tested cell lines. However, the decrease in active cycling was least drastic in MDA-MB-231 given the short treatment time (24h) compared to the other two cell types (72h) (Figure 9C, C'). This suggests that a longer arginine-deprivation period (72h) for MDA-MB-231, which we are currently performing, will likely a more robust result.

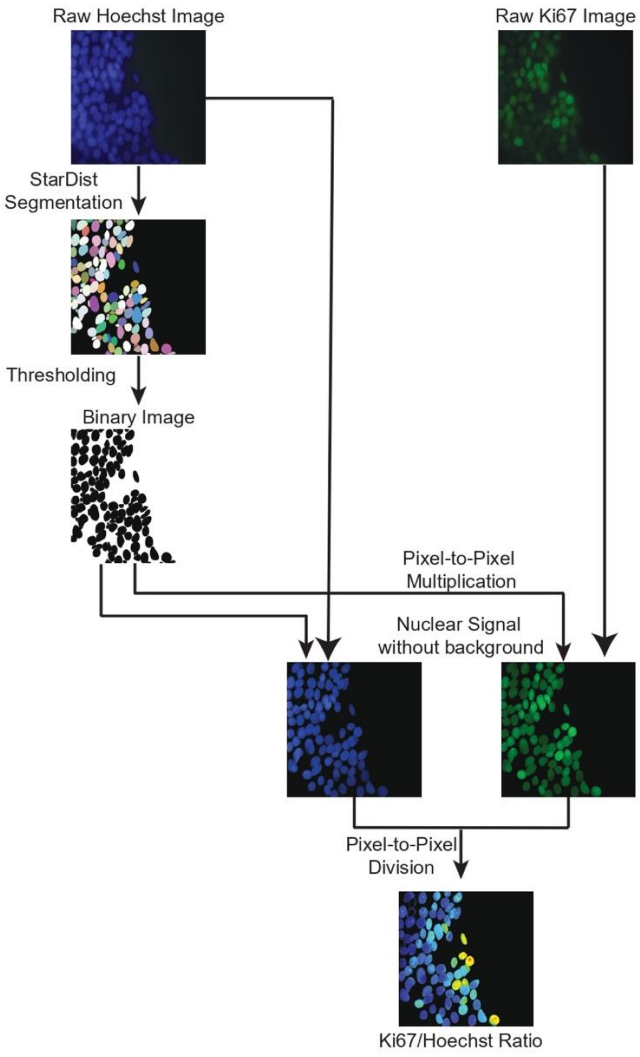

Figure 7. Automated nuclear segmentation method. Automated nuclear segmentation of Hoechst stained MCF7 (A) and UACC-2087 (B) and MDA-MB-231 (C) cell lines. Raw Hoechst and raw Ki67 images are used in StarDist plugin in ImageJ. Thresholding is run on the obtained ROIs (Regions of interest) and label images to obtain binary images. The binary images and the raw Hoechst and raw Ki67 images are put through pixel-to-pixel multiplication to obtain single channel Hoechst and Ki67 that exclude the background. From these segmented background excluded images we generated a Ki67/Hoechst ratio images by pixel to pixel division in ImageJ.

We then benchmarked the accuracy of our machine-learning method against manual segmentation using nuclear area and circularity. We found that, for all three cell types, our automated segmentation was sufficiently accurate in identifying the bulk of the nuclear area (MCF-7 $r \simeq 0.82$; UACC-207 and MDA-MB-231 $r \simeq 0.97$) in order to proceed with fluorescence signal quantification (Figure 8).

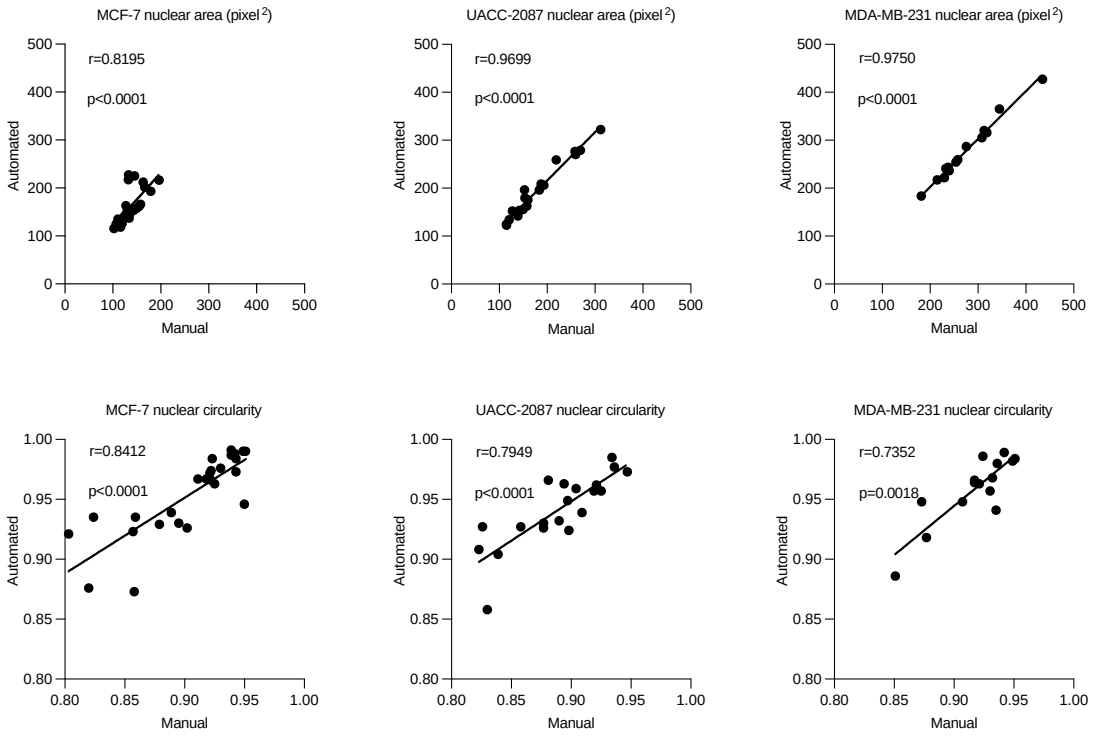

Figure 8. Automated versus manual nuclear segmentation. Automated versus manual nuclear segmentation was applied on MCF-7, UACC-2087 and MDA-MB-231 to compare the area and circularity of nuclei using StarDist plugin on ImageJ.

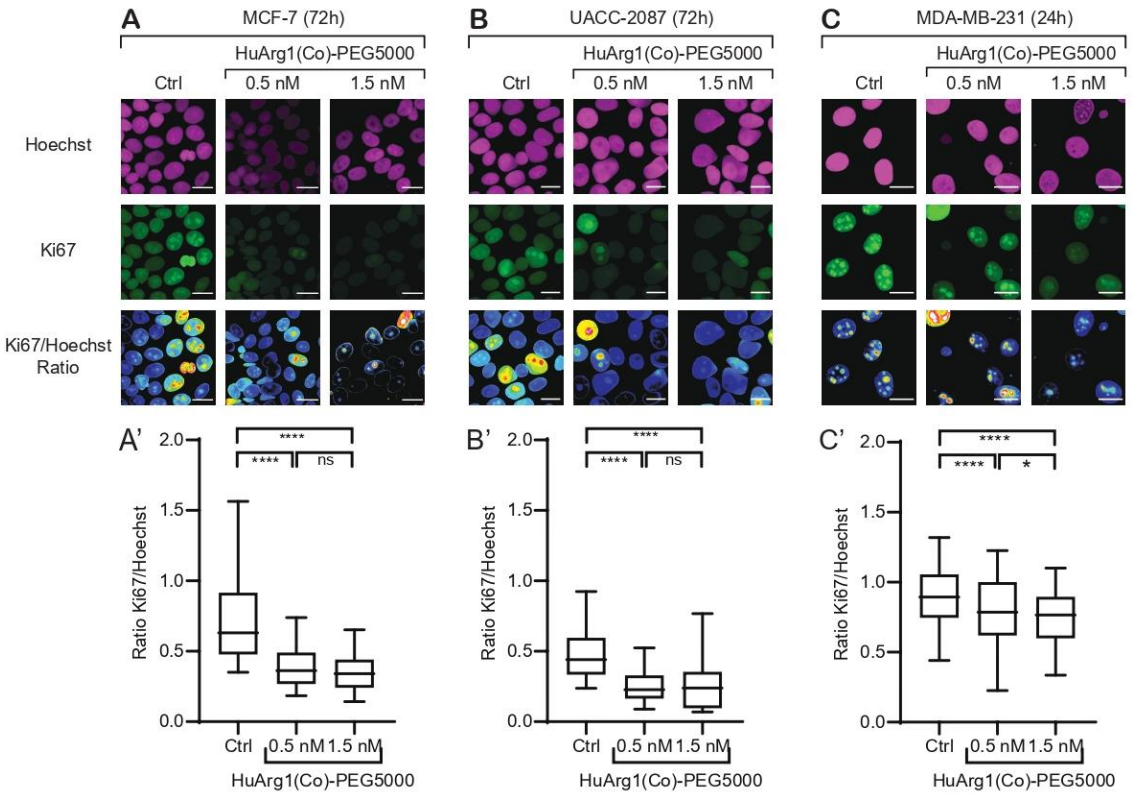

Figure 9. HuArgI(Co)-PEG5000 inhibits proliferation of breast cancer cells. Representative immunofluorescence images of Ki67 and Hoechst and Ki67/Hoechst ratio (A, B and C) for MCF-7 and UACC-2087 and MDA-MB-231 respectively. (A') Quantification of Ki67/Hoechst ratio for MCF-7. (B') Quantification of Ki67/Hoechst ratio for UACC-2087. (C') Quantification of Ki67/Hoechst ratio for MDA-MB-231. Three independent runs were performed on each cell line.

3.3 HuArgI(Co)-PEG5000 induces cell death in breast cancer cells

To characterize the cytotoxic effects of HuArgI(Co)-PEG5000, the Annexin V/Propidium Iodide (PI) apoptosis assay was performed on MCF7 and UACC-2087 and MDA-MB-231. The cell lines were treated under 3 conditions: 0 nM (control), 1.5 nM HuArgI(Co)-PEG5000 and 100 nM HuArgI(Co)-PEG5000 for 24h, 48h, and 72h. The results showed that HuArgI(Co)-PEG5000-induced arginine deprivation increased cell death in all tested cell lines, with the maximal effect observed in MDA-MD-231 cells (Figure 10). However,

this increase in cell death was not consistent with an increase in early apoptosis (percentage of Annex V+/PI- cells) suggesting that observed cell death does not occur with the apoptotic pathway.

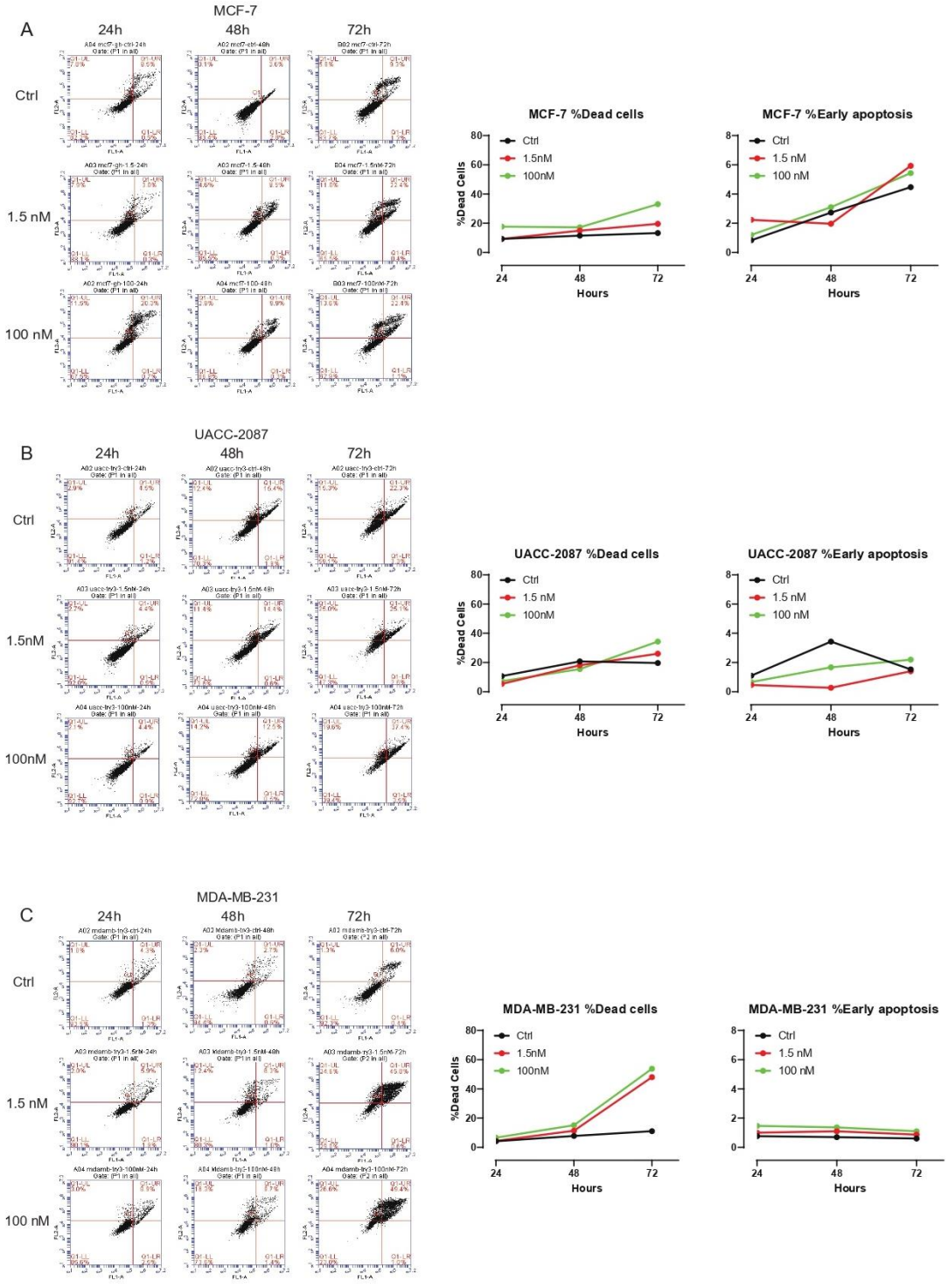

Figure 10. HuArgI(Co)-PEG5000 induces cell death in breast cancer cells.
(A) Quantification of percentage of dead cells and percentage of early apoptosis in MCF-7. (B) Quantification of percentage of dead cells and percentage of early apoptosis in UACC-2087. (C) Quantification of percentage of dead cells and percentage of early

apoptosis in MDA-MB-231. Fl1-A represents the Annexin (X-axis). Fl2-A represents Propidium Iodide (Y-axis).

Chapter Four

Discussion

Downregulation of enzyme ASS1, which is a rate-limiting step in arginine synthesis, in tumor cells results in heavy dependence of the tumor cells on extracellular arginine due to the inability to synthesize arginine for growth. This is known as arginine auxotrophy and characterizes several blood and solid tissue cancers.

MCF7 is a cell line model for luminal subtype breast cancer that expresses estrogen and progesterone receptors. It has low metastatic abilities. UACC-2087 is a triple negative subtype of breast cancer that does not express any hormone receptor with generally mild invasiveness. MDA-MB-231 is also a triple negative subtype of breast cancer that does not express any hormone receptor. In this study, arginine deprivation using HuArgI(Co)-PEG5000 showed that levels proliferation and migration of specific breast cancer cell lines are more vulnerable to HuArgI(Co)-PEG5000 than other cell lines.

First, a wound healing assay showed a decrease in wound closure during arginine deprivation of MCF7 and UACC-2087 with minimal to no change in wound closure in MDA-MB-231. This indicates a decrease in migration, and potentially proliferation, at least in MCF-7 and UACC-2087 after 72-h arginine deprivation. For MDA-MD-231, no effect was observed up a 24-h treatment suggesting this time window is not sufficient for HuArgI(Co)-PEG5000 to deplete the available pool down to a phenotype-causing level. This was further corroborated in an immunofluorescence assay where the ratio of Ki67/Hoechst was studied. There was a significant decrease in Ki67/Hoechst ratio in MCF-7 and UACC-2087 cell lines indicating a decrease in proliferation while MDA-MB-231 had a very slight decrease in Ki67/Hoechst ratio. Finally, an annexin V Pi assay

showed a slight increase in cell death in MCF-7, a moderate increase in cell death in UACC-2087 and a massive increase in cell death in MDA-MB-231 indicating significant cytotoxic effects on highly proliferative triple negative breast cancer cells.

Chapter Five

Conclusion

This study aimed to characterize the effect of arginine deprivation done by HuArgI(Co)-PEG5000 on breast cancer migration and proliferation. Results showed that cell lines from specific breast cancer subtypes are more susceptible to arginine deprivation through the use of HuArgI(Co)-PEG5000 than other cell lines. Migration and proliferation were abrogated in MCF-7 and UACC-2087 breast cancer cell lines treated with HuArgI(Co)-PEG5000. In a shorter time window, there was no apparent effect of HuArgI(Co)-PEG5000 on migration and proliferation of MDA-MB-231 breast cancer cell line. Furthermore, treatment of MCF-7 and UACC-2087 and MDA-MB-231 with HuArgI(Co)-PEG5000 showed an increase in cell death. These findings suggest the presence of mechanisms of action for enzymes inducing arginine deprivation across different breast cancer subtypes. In future studies, the effect of HuArgI(Co)-PEG5000 should be studied on organoids.

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