LEBANESE AMERICAN UNIVERSITY

The Effect of HuArgI (Co)-PEG5000 on The Migration of Ovarian Cancer

By
Nour El Mais

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

School of Arts and Sciences
April 2020

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Nour El Mais I.D. #: 201502076

Thesis Title: The Effect of HuArgI (Co)-PEG5000 on The Migration of Ovarian Cancer

Program: MS in Biological Science

Department: Natural Sciences

School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approv
it in Partial Fulfillment of the requirements for the degree of:

Master of Science in the major of Molecular Biology

Thesis Advisor's Name: Mirvat El Sibai and Ralph Abi Habib

Signature: [redacted] Date: 22 / 05 / 202(
Day Month Year

Committee Member's Name: Roy Khalaf

Signature: [redacted] Date: 22 / 05 / 202(
Day Month Year

Committee Member's Name: Michella Ghassibe-Sabbagh

Signature: [redacted] Date: 22 / 05 / 202(
Day Month Year

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants the Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Nour El Mais

Signature: [redacted] Date: 4 / 5 / 2020
Day Month Year

iii

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that:

1. I have read and understood LAU's Plagiarism Policy.
2. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.
3. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Nour El Hais

Signature: [redacted] Date: 4 / 5 / 2020
Day Month Year

iv

Dedication Page

To my loving family.

Acknowledgments

First of all, I would like to thank God for giving me the patience and guiding me throughout all of my life paths.

I am wholeheartedly grateful to my family, for their support, unconditional love, and presence beside me at all times.

My sincere recognition and gratitude are addressed to Dr. Mirvat El-Sibai and Dr. Ralph Abi Habib for their advice, guidance, support and patience, throughout those two years, as without their persistence I couldn't have achieved this.

Thank you to Dr. Michella Ghassibe Sabbagh and Dr. Roy Khalaf, my committee members, I would like to express my greatest appreciation for their valuable feedback and positive energy.

In addition, thank you to all my colleagues who added value, love and support to my journey.

Last but not least, I would love to thank my close friends, who were there for me, through the highs and lows. Who always made me remember that there are good things waiting for me at the end of this road.

The Effect of HuArgI (Co)-PEG5000 on the Migration of Ovarian Cancer

Abstract

Ovarian cancer being the first cause of death in gynecological diseases, paves the way towards increasing the search for a targeted therapy that could control the metastasis of a late-stage diagnosed ovarian cancer. HuArgI (Co)-PEG5000 is a potential drug to target cancers that depend on exogenous sources of arginine and cannot synthesize their own. In this study, we hypothesized that HuArgI (Co)-PEG5000 can decrease the migration of ovarian cancer cells. Our results show a decrease in the migratory rate of the cells and their rate in wound closure. The second thing we looked into was the adhesion of those cells and the formation of focal adhesions. The number and area of focal adhesions were quantified and the results show a decrease in the area and number of focal adhesions upon treating with HuArgI (Co)-PEG5000. Finally, we looked at the effect of HuArgI (Co)-PEG5000 on 3D motility of the ovarian cancer cell line, using a collagen based assay. It showed that SKOV3 cell line does not show significant invasiveness to collagen. In conclusion, we noticed an effect on ovarian cancer by treating the cells with HuArgI (Co)-PEG5000 which opens the way for further studies on the effect of arginine deprivation and autophagy induction on the migration of ovarian cancer.

Key Words: HuArgI (Co)-PEG5000, Arginine, Migration, Autophagy, Ovarian Cancer, SKOV3.

Table of Contents

LIST OF FIGURES x

LIST OF ABBREVIATIONS xi

Chapter one 12

Literature review 12

1.1. Cancer Overview 12

1.2. Metastasis and Migration 13

1.3. Ovarian Cancer 16

1.4. Ovarian Cancer Metastasis 16

1.4.1. Ovarian Cancer Classification 17

1.5. Arginine 19

1.5.1. Arginine dependence 19

1.5.2. Arginine in Cancer 21

1.5.3. Arginine and migration 22

1.5.4. Arginine as mTOR substrate 24

1.5.5. Totally or Partially Ariginine Auxotrophic? 25

1.6. Arginase 26

1.6.1. Development as a drug 26

1.6.2. HuArgI (Co)-PEG5000 and death by autophagy 28

1.7. Purpose of this study 31

Chapter Two 32

2. Materials and Methods 32

2.1. Cell cultures 32

2.2. Expression and Purification of HuArg1(CO)-PEG 5000 32

2.3. HuArg1(CO)-PEG 5000 Concentration 32

2.4. Antibodies and reagents 32

2.5. Immunostaining 33

2.6. Motility assay/analyzing 2D motility 33

2.7. Fixed Wound Healing Assay 33

2.8. Adhesion assay 34

2.9. Invasion Assay 34

2.10. Quantitation of focal adhesions 35

Chapter Three 36

3. Results 36

3.1. HuArgI (Co)-PEG5000 decreases 2D motility of SKOV3, human ovarian cancer cell line 36

3.1.1. HuArg1(CO)-PEG 5000 decreases the rate of wound closure of SKOV3. 36

3.2. HuArg1(CO)-PEG 5000 decreases the cell motility of SKOV3. 37

3.3. HuArgI (Co)-PEG5000 decreases the adhesion of SKOV3 cell line to collagen. 39

3.4. HuArgI (Co)-PEG5000 decreases the formation of focal adhesions in SKOV3 cell line. 40

3.5. HuArgI (Co)-PEG5000 has no effect on 3D motility of SKOV3 cell line. 42

Chapter 4 43

Discussion 43

Chapter 5 47

Conclusions **47**
Bibliography **48**

LIST OF FIGURES

Figures		Page Number
Figure 1	The Metastatic Cascade.	17
Figure 2	The Ovarian Cancer Subtypes.	21
Figure 3	The Urea Cycle.	22
Figure 4	Nutrients Starvation.	32
Figure 5	Fixed Wound Healing Assay.	39
Figure 6	HuArgI (Co)-peg5000 and Rapamycin decrease the	40
Figure 7	2D motility of SKOV3 cell line.	
	HuArgI (Co)-PEG5000 demotes cell adhesion to collagen in SKOV3 cells.	41
Figure 8	HuArgI (Co)-PEG5000 decreases focal adhesion formation SKOV3 cell line	43
Figure 9	HuArgI (Co)-PEG5000 has no significant effect on the 3D motility of SKOV3 cell line.	44

LIST OF ABBREVIATIONS

MMPs: Metalloproteinases

HGSC: high grade serous carcinoma

HGSOC: high grade serous ovarian carcinoma

HuArgI (Co)-PEG5000: Human Arginase I Cobalt Pegylated 5000

ASS1: argininosuccinate synthase

ASL: argininosuccinate lyase

OCT: ornithine transcarbamyl transferase

iNOS: inducible nitric oxide synthase

NO: nitric oxide

mTORC1: Mammalian target of rapamycin complex1

FAK: focal adhesion kinase

mTOR: Mammalian target of rapamycin

NOS: nitric oxide synthase

ADI: arginine diaminase

PEG: polyethylene glycol

rhArg: recombinant human arginase

Co: cobalt

OTC: ornithine transcarbamylase

2D: two-dimensional motility

3D: three-dimensional motility

Chapter one

Literature review

1.1. Cancer Overview

A cell that loses control over its replication machinery and proliferates in an uncontrolled pace could lead to a disease known as cancer. Cancer is the second cause of death globally, with around 9.6 million registered deaths in 2018, according to the World Health Organization ("Cancer," 2020) and the second cause of death in the United States with a prediction of 606,520 deaths to take place in 2020, according to the American Cancer Society (Siegel, Miller, & Jemal, 2020).

Those uncontrolled cellular replications will then form a mass of tissue that is called a tumor. A tumor could be benign or malignant and these classifications are made according to whether the cell could migrate and invade or not. However, if the tumor turned out to be malignant, it will metastasize meaning that the cancerous cells will be migrating to different areas of the body. This is when they start to break out of one organ's boarders and metastasize to the other. Cancer tumor is formed from one progenitor cell although not all cancer tumors are alike in behavior (Cooper, 2000). Those cells will be accumulating mutations due to several genetic changes thus giving rise to malignant cells, the later will form the primary tumor (Yokota & Biology Division, 2020). The cells will enter the hyperplasia stage where they are replicating uncontrollably however the mass cannot yet be seen with the naked eye. Further changes affecting this group of cells leads them to the later stage, which is dysplasia, where they no longer look normal and they will

start breaking out. Some of those cells will undergo de-differentiation which will bring them back to primitive cells where they have the ability to differentiate and become a different type of cell ("Cancer Development | CancerQuest,"). These cells will be invading nearby tissue and metastasizing which gives them the label as malignant tumors (Seyfried & Huysentruyt, 2013).

All organs in one's body system can generate cancerous cells. Lung cancer, pancreatic cancer, breast cancer and ovarian cancer are all examples of different cancer types (Idikio, 2011).

1.2. Metastasis and Migration

Cell migration is the process by which the cell undergoes cytoskeleton modifications, in order for it to move. Actin rearrangements take place and detachment and formation of focal adhesions in different parts of the cell occur, for it to change direction and move. Usually, what initiates migration is the presence of a chemo-attractant, which is a chemical molecule that the cell is in need of. After that, an arrangement of the actin cytoskeleton takes place for the cell to form its leading edge, for it to then form focal adhesions at the front of the cell to pull the cell forward. This process includes the formation of filopodia and lamellipodia at the leading edge of the cell (DeFea, 2013; Iglesias & Devreotes, 2008).

Cell migration goes into two extents, either group of cells migrating together, like in gastrulation or in cancer metastasis, or single cells migrating like the one of fibroblasts or immune system cells (Trepat, Chen, & Jacobson, 2012).

Metastasis, *in vivo*, is the process by which the cells invade into the tumor's neighboring tissues, then they can migrate into vessels and survive there. After that

they reach an organ and infiltrate it then localize there, start proliferation and from colonies (Eger & Mikulits, 2005). Stephen Paget, in 1889, describes the process of the primary tumor metastasizing to distant organs as “seed and soil” process. It is proposed that the tumor doesn’t migrate randomly, however it needs an organ with specific characteristics that it’ll migrate to (Paget, 1989). This theory was proven in several types of cancers. For instance, breast cancer preferential site for migration is to the lungs, bones and the brain.

Factors other than the primary tumor influence the site of metastasis, like physiological and other biological constraints. The tumor’s microenvironment control invasion through the regulation of cytokines, chemokines and production of metalloproteinases (MMPs) (van Zijl, Krupitza, & Mikulits, 2011).

As we said earlier, metastasis is described in five steps (Figure 1):

- The first step is invasion and migration where the cancer cell invades the basement membrane and migrates. The cell mechanically changes its shape to form protrusions and contracts then it degrades the matrix using MMPs (Haeger, Krause, Wolf, & Friedl, 2014; Hapach, Mosier, Wang, & Reinhart-King, 2019; Wisdom et al., 2018).
- The second step is angiogenesis and intravasation, where the cancer cells start forming blood vessels to supply the tumor with the needed nutrients and oxygen (Carmeliet & Jain, 2000). Then those blood vessels are used to deliver the cells to the vascular system or the lymph nodes for them to migrate to distant organs (Hapach et al., 2019).
- The third step in this process is surviving in circulation and being able to attach to the endothelium. It is difficult for the cell to survive through circulation due to the presence of immune cells and getting bombarded with

red blood cells (Wirtz, Konstantopoulos, & Searson, 2011). The circulating cell then collide with the endothelium with the help of E-selectin or P-selectin; and then binds the epithelium with the help of intracellular adhesion molecule-1 or vascular cell adhesion molecule-1 (Hapach et al., 2019).

- The fourth and fifth steps here are extravasation and colonization. The cell will leave the vessels into the tissue where colonization will take place. The tissue should be compatible with the tumor for it to be able to exit the vascular system and proliferate (Hapach et al., 2019).

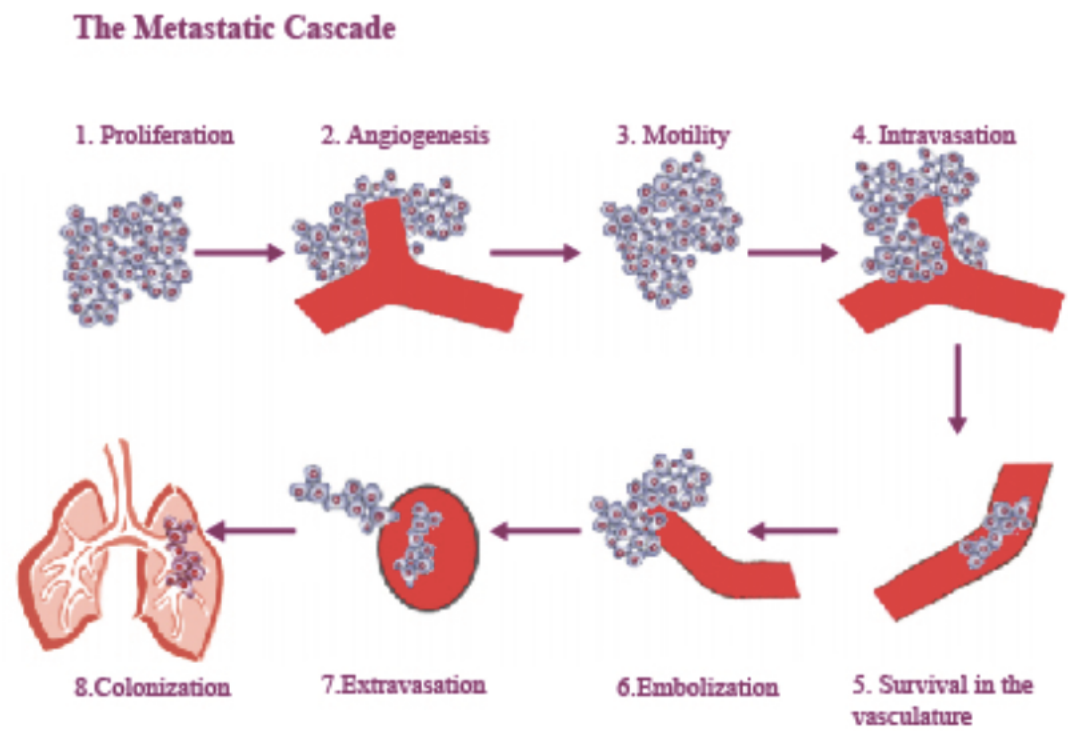

Figure 1: The Metastatic Cacade. The cells undergoing uncontrolled proliferation need an additional supply of nuteints and oxygen. The cells undergo angiogenesis. After the cells have accumelated mutations, they can now invade into the ECM and intravasate into vasculature. After reaching the secondary site, cells adhere to the epithelium and extravasate to form new coloniesin the secondary site (Adapted and modified from: Daves et al. (2007)

1.3. Ovarian Cancer

In 2018, the American Cancer Society predicted 14,070 deaths by ovarian cancer. This type of cancer is known to be very heterogeneous and it is mostly diagnosed at late stages which makes it account for 5% of deaths by cancer (Torre et al., 2018). Ovarian cancer is the leading cause of death when it comes to gynecological diseases (Y. Zhao et al., 2020). It has a low survival rate and high mortality due to the fact that 60% of women are diagnosed in later stages not early ones (stage I and II) (Howlader N, 2015). It is not defined as a single disease, it includes different subtypes and each subtype is dealt with differently in the case of risk, behavior and prognosis. There is still no absolute knowledge about all of the subtypes of cancer since their tissue of origin and development differs. Since it is diagnosed in late stages, this increase in the death rates caused by ovarian cancer, especially the cases diagnosed with high grade ovarian serous carcinoma (HGSOC) (Committee on the State of the Science in Ovarian Cancer, Board on Health Care, Institute of, National Academies of Sciences, & Medicine, 2016).

1.4. Ovarian Cancer Metastasis

Ovarian cancer in women is usually detected after stage I, which means already it had metastasized out of the ovaries and is in higher stages. This makes it harder to target it and treat it. When the ovarian cancer metastasizes to the uterus, fallopian tube, bladder and rectum, it is hard to treat it via chemotherapy or even surgery. This characterizes the tumor to be in stage II, It is easy to metastasize to such organs due to the lack of barriers (Tjhay et al., 2015). When the tumor reaches stage III, its metastasis sites expand to reach the abdominal organs such us the small intestine, retroperitoneal lymph nodes.

When the tumor reaches the final stage, stage IV, it metastasizes to distant organs such as the liver and the lungs (Bast, Hennessy, & Mills, 2009; Bowtell et al., 2015; Motohara et al., 2019; Yap, Carden, & Kaye, 2009). Going back to the "seed and soil" theory, a pattern was shown in ovarian cancer metastasis where they prefer adipose-rich omentum, a layer of peritoneum that covers the intestines in the abdominal cavity, and peritoneal surfaces (Lengyel, 2010; Motohara et al., 2019). It is still not well investigated if ovarian cancer, like other cancers, prepares the secondary niche before reaching it for colonization (Gupta & Massagué, 2006; Lengyel, 2010).

1.4.1. Ovarian Cancer Classification

Ovarian tumors are divided into two types, type I and type II. The basis of this categorization goes back to the morphology and the genetic mutations of those tumors. Type I is characterized as low grade and progresses slowly, however type II is more aggressive and is associated with a TP53 mutation (Levanon, Crum, & Drapkin, 2008). The latter is a HGSOC that is characterized by being aggressive thus associating with late diagnosis and high death rates (Torre et al., 2018). This serous subtype of ovarian cancer accounts for 60% to 80% of death (Levanon et al., 2008; Seidman et al., 2004).

It can be epithelial and non-epithelial. Non- epithelial ovarian cancer is not largely aggressive and accounts for 5% of ovarian cancer cases. On the other hand, epithelial ovarian cancer is more aggressive and accounts for 90% of ovarian cancer cases. It is divided into four subtypes (Figure 2): Serous, endometrioid, mucinous and clear cell. Non-serous tumors include clear cell and endometrioid carcinomas and affect 10% of the female population. They are usually detected at early stages but after they have formed a significant

mass in the ovaries (Hallas-Potts, Dawson, & Herrington, 2019). The ovarian carcinoma subtypes are named according to how much the tumor tissue resembles the normal tissue. Serous endometrioid and mucinous carcinomas resemble the morphology of normal epithelial cells of the fallopian tube, endometrium and endocervix. On the other hand, clear cell carcinomas are similar in morphology to the cells of the gestational endometrium (Selvaggi, 2000).

Previous studies used the cell lines A2780 and SKOV3 as ones for HGSOC. Through out this work, they realized that those cell lines weren't representative for HGSOC and even didn't have the TP53 gene mutation (Domcke, Sinha, Levine, Sander, & Schultz, 2013). In addition, it was requested to re-characterize those cell lines as subtype models because they are not well representative (Domcke et al., 2013). However, according to Beaufort et al. (Beaufort et al., 2014), A2780 and SkoV3 cell lines belonged actually to the non-serous high grade ovarian cancer subtype (Shin, Sangyoon, & Jennifer, 2020), where they expressed high migration abilities and high invasiveness, opposite to what is seen in HGSOC cell lines in vitro (Hallas-Potts et al., 2019).

When it comes to ovarian cancer therapy, some clinical trials showed that chemotherapy when used on such cancer types is promoting metastasis. When the microenvironment of the tumor was studied, scientist found that the tumor became more aggressive after chemotherapy (Y. Zhao et al., 2020). These lead scientists to look for another approach in treating ovarian cancer. One of the

approaches that seemed effective on such heterogeneous types of cancer was amino acid deprivation therapy.

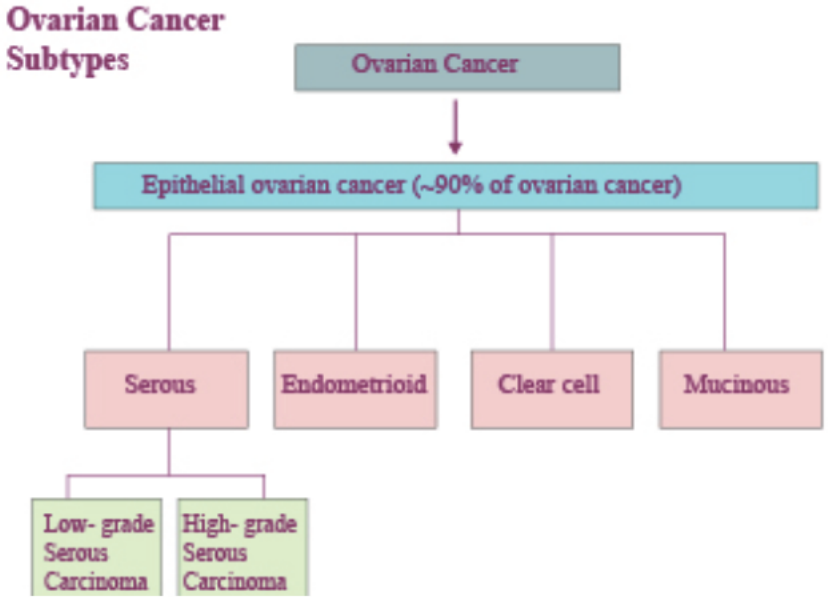

Figure 2: The Ovarian Cancer Subtypes. Epithelium ovarian cancer is subdivided into four subtypes: serous, endometrioid, clear cell and mucinous. The serous subtype is further subdivided into two subtypes: low-grade serous carcinoma and high-grade serous carcinoma.

1.5.1. Arginine Dependence

Arginine is a non-essential amino acid that aids cells in proliferation, survival and other biological processes (Fultang, Vardon, De Santo, & Mussai, 2016). It is acquired from diet, or produced by the body through several pathways (S. M. Morris, Jr., 2007). The small intestines produce citrulline which is then transported into the kidney for arginine production (G. Wu et al., 2009). As shown in Figure 3, it is produced via the urea cycle. The enzyme argininosuccinate synthase (ASS1) catalyzes the conversion of L-citrulline and aspartic acid into argininosuccinate. The later serves as the substrate of ASL to produce L-arginine and fumaric acid, L-arginine could then give rise to ornithine with the help of arginase. Ornithine could be recycled back to L-

citrulline by ornithine transcarbamyl transferase (OCT) (Feun et al., 2008; Shen, Beloussow, & Shen, 2006; Shen, Lin, Beloussow, & Shen, 2003).

Arginine is necessary for spermatogenesis, embryonic survival, fetal and neonatal growth. Also studies show that supplementation with arginine could help in the proper functioning of the reproductive system, the immune system, the renal system, the heart and it plays a role in wound healing (G. Wu et al., 2009). In cancerous cells, it was shown that upon supplying the cells with arginine it was leading to tumor growth and increased proliferation (Delage et al., 2010).

As we previously discussed, arginine could be produced through L-citrulline, by the induction of expression of ASS1 or ASL. This process accompanies the increase in inducible nitric oxide synthase (iNOS) since it can produce L-citrulline and nitric oxide (NO) from arginine and the latter will be recycled back into arginine. This process is known as the Citrulline-NO cycle (S. M. Morris, Jr., 2007).

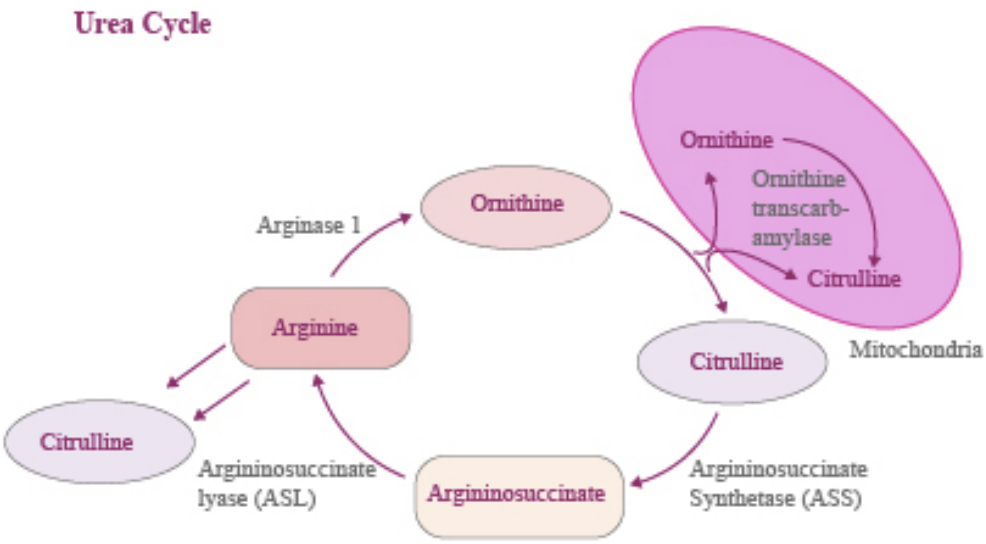

Figure 3: The Urea Cycle. Arginine is converted into ornithine with the help of arginase1. Then ornithine, in the mitochondria, is converted into citrulline with the help of ornithine transcarbamylase. Citrulline will be recycled back to arginine with the help of two enzymes, argininosuccinate synthase and argininosuccinate lyase. Adapted and modified from Patil et al. (2016)

1.5.2. Arginine in Cancer

Cancerous cells, to keep up with massive proliferation with the least energy expenditure possible, it shuts down certain pathways, and one of them is arginine synthesis, and depends on exogenous arginine sources as a supply. Cells that do not have ASS1 are said to be auxotrophic for arginine, which means that the presence of l-citrulline will not help them produce arginine in case of arginine deprivation, this will put the cell under huge stress, and the cells will be dependent on exogenous sources of arginine (Fultang et al., 2016), (Jahani, Noroznezhad, & Mansouri, 2018). Studies proved that depleting arginine from the tumors' microenvironment would result in proliferation inhibition (Jahani et al., 2018). In some cancer types, such as melanoma, studies showed that such cells are metabolizing arginine, with the help of arginase and nitric oxide synthase, to maintain cancerous cells' proliferation and progression while at the same time maintaining an immunosuppression process, to help the cancer cells evade the immune system (S.-H. Kim, Roszik, Grimm, & Ekmekcioglu, 2018). Such information paved the way for choosing arginine deprivation as a therapeutic approach for cancer types that showed arginine auxotrophy i.e. that mostly depend on exogenous sources as an arginine supply.

This helps in therapy where only cancerous cells will be targeted via arginine depletion, because normal cells will be able to synthesize their own arginine however, cancerous cells, auxotrophic for arginine will not (Fultang et al., 2016).

Upon depriving the cells from arginine, there is a down regulation of mTORC-1 protein to initiate the pro-survival autophagy mechanism, as a way for the cell to cope with stress. However, extensive autophagy will lead to cell death (Fultang et al., 2016).

1.5.3. Arginine and Migration

A study by Rhoads et al. (2004) showed that arginine increases migration, in intestinal cells, by increasing the phosphorylation of focal adhesion kinase (Rhoads et al, 2004). In addition to that, the study showed that arginine increased the amount of nitric oxide secreted by the migrating intestinal cells. Arginine is known to be the only precursor for the production of NO and previous studies had shown that NO is one of the main factors inducing malignancy and metastasis of cancer (Choudhari, Chaudhary, Bagde, Gadbail, & Joshi, 2013), (Wang, Li, Chow, Choi, & Leung, 2020). To validate the hypothesis that arginine was promoting migration, excess arginine was present in media, after forming a wound in culture, and western blot was done to measure the levels of tyrosine phosphorylated focal adhesion kinase (FAK). In addition, a NO donor was added to the media. Results showed that the level of phosphorylated FAKs increased upon arginine addition and with the presence of NO donor. To prove the relation of NO to cellular migration, a wound was done and they measure the levels of iNOS. There was a clear increase in the levels of iNOS in the media of the wounded cells and it was correlated with an increase in migration. This provides evidence that arginine is promoting cellular migration by working on phosphorylating FAKs and increasing the production of NO (Rhoads et al., 2004). When FAKs are activated, they promote adhesion and then regulate changes in focal adhesions in terms of size

and are to promote cellular migration (J.-C. Wu et al., 2015). Thus, overexpression of phosphorylated FAKs is said to increase the migration of cells (Cary, Chang, & Guan, 1996).

Previous studies indicated the roles of small RhoGTPases, RhoA, Cdc42 and Rac1, in migration. Through the mediating focal adhesion assembly, promoting formation of filopodia and lamellipodia respectively (Ridley, 2001). To further look into the role of NO in migration, a NO donor was used on human embryonic stem cells and its effect was tested on wound closure. The results illustrated that NO activated RhoA and Rac1 thus promoting the migration of those cells, in vitro and in vivo studies (Rixing Zhan et al., 2016). NO effect on migration was tested also on human keratinocyte cell lines and the results showed an increase in expression of activated RhoA, Cdc42 and Rac1 upon the introduction of NO donor (R. Zhan et al., 2015). This validates the involvement of NO, produced from arginine, in cellular migration, however, the effect of it on Rac1 and Cdc42 in cancerous cell lines is yet to be investigated and validated.

More on arginine and migration, in pancreatic cancer and colorectal cancer, results showed that arginine deprivation decreased the level of MMPs thus decreasing the ability of pancreatic and colorectal cancer cells to break through the extracellular matrix and invade (Wang et al., 2020), (Al-Koussa, Al-Haddad, Abi-Habib, & El-Sibai, 2019). Arginine depletion in colorectal cancer decreased the migration rate of the cells, decreased their ability to adhere and decreased their invasiveness (Al-Koussa et al., 2019).

1.5.4. Arginine as mTOR Substrate

Mammalian target of rapamycin (mTOR), a serine-threonine kinase, regulates the cellular growth and senses nutrient presence. When it is disturbed, it can lead to many diseases such is diabetes, neurodegeneration and cancer (Efeyan, Zoncu, & Sabatini, 2012). mTORC1 complex integrates signals coming from amino acid presence and energy.

When amino acids are not present, mTORC1 becomes inactive, protein synthesis declines and autophagy is induced. However, when amino acids are supplied again, mTOC1 will be reactivated, protein synthesis will resume and autophagy will be inhibited (Nofal, Zhang, Han, & Rabinowitz, 2017; Yu et al., 2010).

This complex showed a relation with the lysosome which is an organelle in the cell responsible for catabolism. So the signals received by amino acid presence sensing, through mTORC1, have a correlation with the decision the cell takes in either growth or breaking down (Efeyan et al., 2012). When amino acids are present, mTORC1 is said to be located at the lysosomes and it inhibits the formation of the phagophore, which is the first organelle needed in the process of autophagy (Bernard & Klionsky, 2013). However, when the cell is under amino acid deprivation, mTORC1 is inhibited which allows the formation of phagophores. The later will promote the fusion of lysosome with autophagosome and proceed to autophagy (Efeyan et al., 2012). To look more into which amino acid could affect the activation and inhibition of mTORC1, a recent study showed that when arginine is abundantly present, it was promoting the phosphorylation of mTOR and ribosomal p70S6K kinase a downstream protein of mTOR, which promotes the anabolic pathways of the

cell, thus activating it. However, upon arginine starvation, the levels of phosphorylated mTOR decreased and phosphorylated ERK1 increased. In addition to that, when the cells were treated with rapamycin, an inhibitor for ribosomal p70S6K kinase, the levels of mTOR decreased and autophagy took place. This indicates the activation of autophagy pathway (catabolic pathway) that was inhibited by active mTOR, since mTOR and ERK1/2 pathway are autophagy regulators (Wang et al., 2020).

1.5.5. Totally or Partially Ariginine Auxotrophic?

Arginine auxotrophy is an opportunity to target cancer cells because those cancerous cells and normal cells differ at this point. It was discovered that most of human cancer types are arginine auxotrophic. The critical genetic mechanism that stands behind this auxotrophy phenomenon is the silencing of ASS1 enzyme (Delage et al., 2012; Riess et al., 2018). As mentioned earlier, when the cell lack ASS1 enzyme or the expression does not fully serve the cell's needs, it will depend on exogenous sources of arginine which will render it totally or partially auxotrophic to arginine. This auxotrophy will make the cell vulnerable and sensitive to arginine deprivation (C.-T. Cheng et al., 2018; S. M. Morris, 2002). When it comes to rescuing those cells by supplying L-citrulline, OCT catalyzes the synthesis of citrulline from ornithine, and this citrulline will be recycled, in the kidneys, to arginine with the help of ASS1 enzyme and ASL. However, we the cell lacks these enzymes, or one of them, citrulline will not be able to rescue any cell and this will render the cell sensitive for arginine deprivation. Hence now it cannot depend on endogenous sources for arginine (P. N. Cheng et al., 2007).

According to The Cancer Genome Atlas pan-cancer analysis, ASS1 levels were low in 12 out of 14 tested cancer types which makes this auxotrophy common and present in several types of cancer not only exclusive to one (Chang et al., 2013). In a study done, the levels of arginine were measured in ASS1 deficient cell lines. In ASS1-lacking MDA cell line, a breast cancer cell line, amino acid levels were measured and the amount of arginine was notably low. Those results indicate that this cell line is auxotrophic for arginine and depends on exogenous sources rather than endogenous ones (C.-T. Cheng et al., 2018).

In two other studies, melanoma and prostate cancer cells that lacked the ASS1 enzyme, meaning that they were auxotrophic for arginine, undergone death by autophagy upon arginine deprivation (R. H. Kim et al., 2009; Savaraj et al., 2010).

1.6. Arginase

1.6.1. Development as a Drug

Studies showed promising results to the usage of arginase or inducible NOS as arginine depriving agents in therapy. This lead to the development of a drug that resembles the human arginase to help depleting arginine and causing the cell to shift from proliferation and migration into death by autophagy and migration inhibition.

This drug was tested and developed gradually to yield the best efficiency in terms of treating tumors ASS1-defficient.

The arginine deprivation therapy started by using argianse1 since it is a human enzyme. However, this did not last long before tests were done and further enzymes started developing on the basics of arginase1. The latter was

not as efficient as it was thought to be when administered to humans due to several reasons; like having a low affinity to arginine, short half life in serum and it did not have the optimal pH value in human's blood stream which was 7.4 since arginase's optimum pH was above 8 (Dillon, Holtsberg, Ensor, Bomalaski, & Clark, 2002; Fultang et al., 2016; Riess et al., 2018).

Recombinant human arginase was a promising replacement for arginine diaminase (ADI), a microbial enzyme extracted from *Mycolplasma* spp. (Sugimura, Ohno, Kusuyama, & Azuma, 1992) and arginase. Recombinant human arginase was developed upon the usage of recombinant DNA technology (P. N. Cheng et al., 2007). To increase its half-life in serum, they enzyme was covalently attached to polyethylene glycol (PEG) of molecular weight 5000. This made it last around 3 days in humans with a sufficient catalytic activity at the physiological pH (P. N. Cheng et al., 2007; Ltd, Yun, Wai, & Bio-Cancer Treatment, 2005).

In a study on Hepatocellular carcinoma done by Cheng et al. the tumors that lacked ASS1 and were sensitive to ADI treatment, relapsed and became resistant to ADI as if they became ASS1 positive. Those same resistant tumors were then treated with recombinant human arginase (rhArg) and pegylated rhArg, they showed promising results, where it showed a significance decrease in arginine levels, also it showed specificity were no other amino acids were degraded. Furthermore, the resistant hepatocellular carcinoma were inhibited even though they were ASS1 positive. This lead to the conclusion that maybe the deficiency in OCT is making those cells sensitive to the drug even in the presence of ASS1, because citrulline will not be produced from ornithine thus there is no further production for arginine for citrulline by ASS1 (P. N. Cheng

et al., 2007). To further test the effect of pegylation to rhArg, a study was done by Tsui et al. (Tsui et al., 2009), pegylated rhArg and rhArg were tested *in vitro* and *in vivo*. *In vitro*, both pegylated and un-pegylated rhArg were tested on hepatocellular carcinoma, the pegylated rhArg showed more promising results than those of the un-pegylated. Concerning the *in vivo* trial, both enzymes were injected into rats and the results showed that the pegylated rhArg lasted more in serum, up to 6 days in comparison to the un-peglayted one which lasted for 3 days. Therefore, pegylation increases serum half-life of the enzyme, which means that it increases its solubility and stability.

To further maximize the efficiency of this enzyme, studies showed that replacing the Mn2+, present on the enzyme as co-factor, with Cobalt Co2+, will help in stabilizing the enzyme even more. Replacing Mn2+ with Co2+ showed higher efficiency, on hepatocellular cancer and melanoma, at physiological pH, with higher affinity towards arginine (Stone et al., 2010). This makes the recombinant human arginase 1 Co- Peg 5000 (HuArgI (Co)-PEG5000) the most efficient in targeting auxotrophic tumors, lacking ASS1, or OCT deficient tumors.

1.6.2. HuArgI (Co)-PEG5000 and Death by Autophagy

The degradation of proteins that are present in the cell, when it is under stress, is referred to as autophagy. This process degrades proteins with the aid of lysosomes. The process starts by phagophores formation to degrade cellular organelles, without choosing according to a criterion on which to degrade. After the phagophores close on those proteins, a double membrane structure is formed, the autophagosome. In advance stages of autophagy, the autophagosomes fuse to the lysosomes where enzymes will work on degrading

the cargos to produce amino acids and nutrients needed for the cell. Those metabolites will be sequestered into the cytoplasm for the cell to use (Mizushima, Levine, Cuervo, & Klionsky, 2008; W. K. K. Wu et al., 2012). So in other words, autophagy is a process where the cell eats itself as an energy and nutrient source.

As mentioned in the previous paragraphs, for cells who depend on arginine from exogenous sources, when their microenvironment is depleted from arginine, the cell seeks a rescue mechanism which is autophagy. The cell will start degrading itself, via lysosomal degradation, as a survival mechanism to produce metabolites. Interestingly, it was found that when accompanying arginine deprivation with autophagy inhibitor, such as chloroquine, the cells are showing a huge decrease in viability and count number. Such experiments where done in literature on ovarian cancer cell line, SKOV3. Arginine deprivation showed to induce the autophagolysosomes (Shuvayeva et al., 2014).

In another study done on glioblastoma multiform cell lines. HuArgI (Co)-PEG5000 was used. Its cytotoxicity was higher on totally auxotrophic cells than that of partially auxotrophic cells. To further investigate the role of autophagy in this sensitivity to the enzyme, chloroquine was added to the culture, thus inhibiting autophagy, and increase in the sensitivity to the drug was noticed. This lead to the conclusion that autophagy was activated as a rescue mechanism against arginine depletion that is taking place (Khoury et al., 2015). Furthermore, colorectal cancer cells showed sensitivity to HuArgI (Co)-PEG5000 and a decrease in migration when looking at 2D motility and a decrease in the area of focal adhesion structures was noticed which made the

cell adhere less to collagen. To validate those result, a decrease in RhoA activation was noticed using FRET technique and RhoA is known to mediate adhesion of assembly of focal adhesions (Al-Koussa et al., 2019).

In a different study done on ovarian cancer cell lines, treated with HuArgI (Co)-PEG5000, the 3 cell lines used showed sensitivity to the drug which makes them auxotrophic for arginine. To know the way of death, they stained with Annexin V and PI and the results showed that it was a caspase independent cell death in addition to the cells losing their membrane integrity. This made them look into the possibility of death through autophagy. In SkoV3 cell line, there was an accumulation of autophagosomes upon the treatment with HuArgI (Co)-PEG5000 and this accumulation increased when treated with chloroquine which means those autophagosomes were not reaching their final destination to be processed due to autophagy inhibition with chloroquine. This proved that ovarian cancer cell lines had undergone death by autophagy due to arginine deprivation via HuArgI (Co)-PEG5000 (Nasreddine, El-Sibai, & Abi-Habib, 2020).

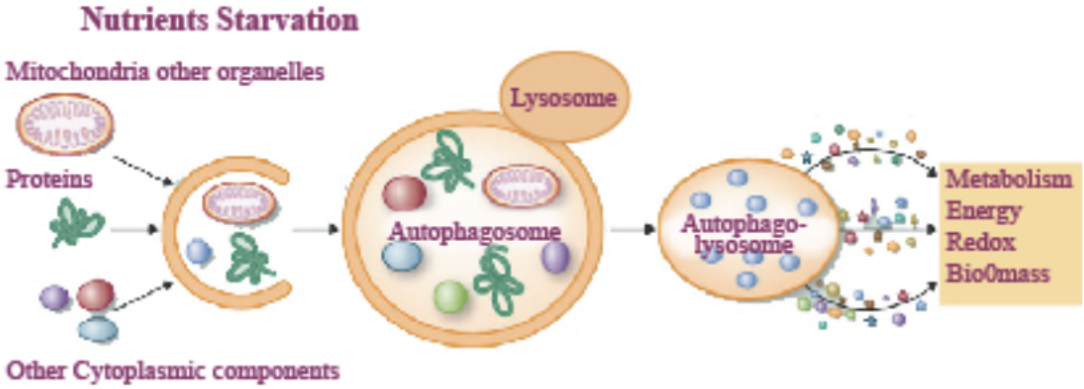

Figure 4: Nutrients Starvation. Under stress, the cell tends to recycle organelles and proteins through a process called autophagy. Where organelles and other cytoplasmic components with be engulfed by atophagosome. The later will fuse to lysosomes and form autophagolysosome where degradation and recyclin will take place. Adapted and modified from the American Association for Cancer Reseach, (2015).

1.7. Purpose of this Study

In this study, we are investigating the effect of HuArgI (Co)-PEG5000 on the migration of SKOV3 cell line. Previous studies were done on SKOV3 using HuArgI (Co)-PEG5000 to determine the cytotoxic concentration of the drug on SKOV3. According to the IC50, the drug concentration was optimized and used in treating the cells (Nasreddine et al., 2020).

The effect on 2D motility was studied by time-lpase and wound closure experiments. In time-lapse, the cells were also treated with rapamycin, an autophagy inducer, to see the effect of autophagy as well on the cells, in comparison to the effect of HuArgI (Co)-PEG5000. To further look into the relation between arginine deprivation and decreasing the cells' motility, an immunostaining assay was done to look at vinculin, which localizes at focal adhesion complexes. Rapamycin was also introduced into the cell culture, to see if autophagy will have an effect on focal adhesions.

To validate the results that were obtained from immunostaining with vinculin, adhesion assay was done while treating the cells with HuArgI (Co)-PEG5000, in the presence and absence of L-citrulline, which rescues the cells that express ASS1 protein.

Chapter Two

Materials and Methods

2.1. Cell Cultures

Non-serous high grade, epithelial ovarian carcinoma (SKOV3) were cultured in DMEM medium supplemented with 10% FBS and 100 U penicillin/streptomycin at 37°C and 5% CO2 in a humidified chamber.

2.2. Expression and Purification of HuArg1(CO)-PEG 5000

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] was expressed and purified as described by Stone et al. (Stone et al., 2010).

2.3. HuArg1(CO)-PEG 5000 Concentration

Previous studies done by Nasreddine et al. (Nasreddine et al., 2020), obtained the IC50 values for HuArg1(CO)-PEG 5000 after treating SKOV3 for 72 hours. The optimized IC50 value used for the experiments in this study is 100 pM.

2.4. Antibodies and reagents

The following primary antibodies were used in this study: Mouse monoclonal anti-Vinculin [VIN-54] (Abcam Inc., Cambridge, UK).

For the secondary antibodies, Fluorescent secondary antibodies (Alexa Fluor 488) was obtained from Invitrogen. To visualize the actin cytoskeleton, cells were stained with Rhodamine-phalloidin (Invitrogen).

2.5. Immunostaining

Cells were plated on glass coverslips while incubated with HuArgI (Co)-PEG5000 and rapamycin. After 48 hours the cells were fixed with 4% paraformaldehyde for 10 minutes at 37^{o}, and permeabilized with 0.5% Triton-X 100 for 15 minutes on ice. For blocking, cells were incubated with 1% filtered BSA in PBS for 1 hour. Samples were then stained with primary antibodies overnight at 4^{o} and with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 63X objective lens on Zeiss Observer Z1 microscope.

2.6. Motility Assay/Analyzing 2D Motility

For motility analysis, images of cells moving randomly in serum were collected every 60 sec for 2 h using a 20x objective. During imaging, the temperature was controlled using a Nikon heating stage which was set at 37˚C. The speed of cell movement was quantified using the ROI tracker plugin in ImageJ software, which was used to calculate the total distance travelled by individual cells. The speed is then calculated by dividing this distance by the time (120 min) and reported in μm/min. The speed of 10 cells for each condition was calculated. The net distance travelled by the cell was calculated by measuring the distance travelled between the first and the last frames.

2.7. Fixed Wound Healing Assay

Cells were grown to confluence on culture plates while incubated with HuArgI (Co)-PEG5000. Then after 24 hours a wound was made in the monolayer with a

sterile pipette tip. Cells were then washed twice with PBS to remove debris and new medium was added. Phase-contrast images of the wounded area were captured at 0, 24 h and 48 h after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated (in μm/h).

2.8. Adhesion Assay

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37 °C washed with washing buffer (0.1% BSA in DMEM). The plates were blocked with 0.5% BSA in DMEM at 37 °C in a CO2 incubator for 1 hour. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to 4x105 cell/ml. 50 μl of cells were added in each well and incubated at 37°C in a CO2 incubator for 30 minutes. The plates were shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining with crystal violet, the plates were washed extensively with water, and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 nm using a Thermo scientific Varioskan Flash Multimode reader (Thermo fisher scientific, USA).

2.9. Invasion Assay

Cells were grown to confluence on culture plates while incubated with to HuArgI (Co)-PEG5000. Invasion assay was performed 48hrs following treatment period using the collagen-based invasion assay (Millipore) according to

manufacturer's instructions. Cells were harvested, centrifuged and re-suspended in quenching medium (without serum). Cells were brought to a concentration of 1x106 cells/ml. In the meantime, inserts were pre-warmed with 300µl of serum free medium for 30 min at room temperature (Corning, New York). After rehydration, 250µl of media was removed from inserts and 250µl of cell suspension was added. Inserts were placed in a 24-well plate, and 500µl of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37˚C in a CO2 incubator. Following 48 hours of incubation period, inserts were stained for 20min at room temperature with 400µl of cell stain provided with the kit. Stain was extracted with extraction buffer (also provided with the kit).100ul of extracted stain was transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was measured at 560µm.

2.10. Quantitation of Focal Adhesions

Image J was used to quantitate focal adhesions. Briefly, two main plugins were used to quantitate focal adhesions, these two plugins are CLAHE and Log3D. CLAHE enhances the local contrast of the image and Log3D filters the image based on user predefined parameters which will allow us to detect and analyze focal adhesions (Horzum, Ozdil, & Pesen-Okvur, 2014).

Chapter Three

Results

3.1. HuArgI (Co)-PEG5000 Decreases 2D Motility of SKOV3, Human Ovarian Cancer Cell Line.

3.1.1. HuArg1(CO)-PEG 5000 Decreases the Rate of Wound Closure of SKOV3.

In both conditions, control and HuArgI (Co)-PEG5000 treated, we can see that there was a migration of cells (Figure 5.A) however, the rate of migration of those cells to establish wound closure decreased after the treatment with HuArgI (Co)-PEG5000 by around 13%, from 7.8 um/hr to 6.8um/hr (Figure 5.C).

The wound closure, in treated cells, decreased by around 20% in comparison to untreated cells (Figure 5.B). This indicates that HuArgI (Co)-PEG5000 decreases the migration rate of SKOV3 cell line, in 2D.

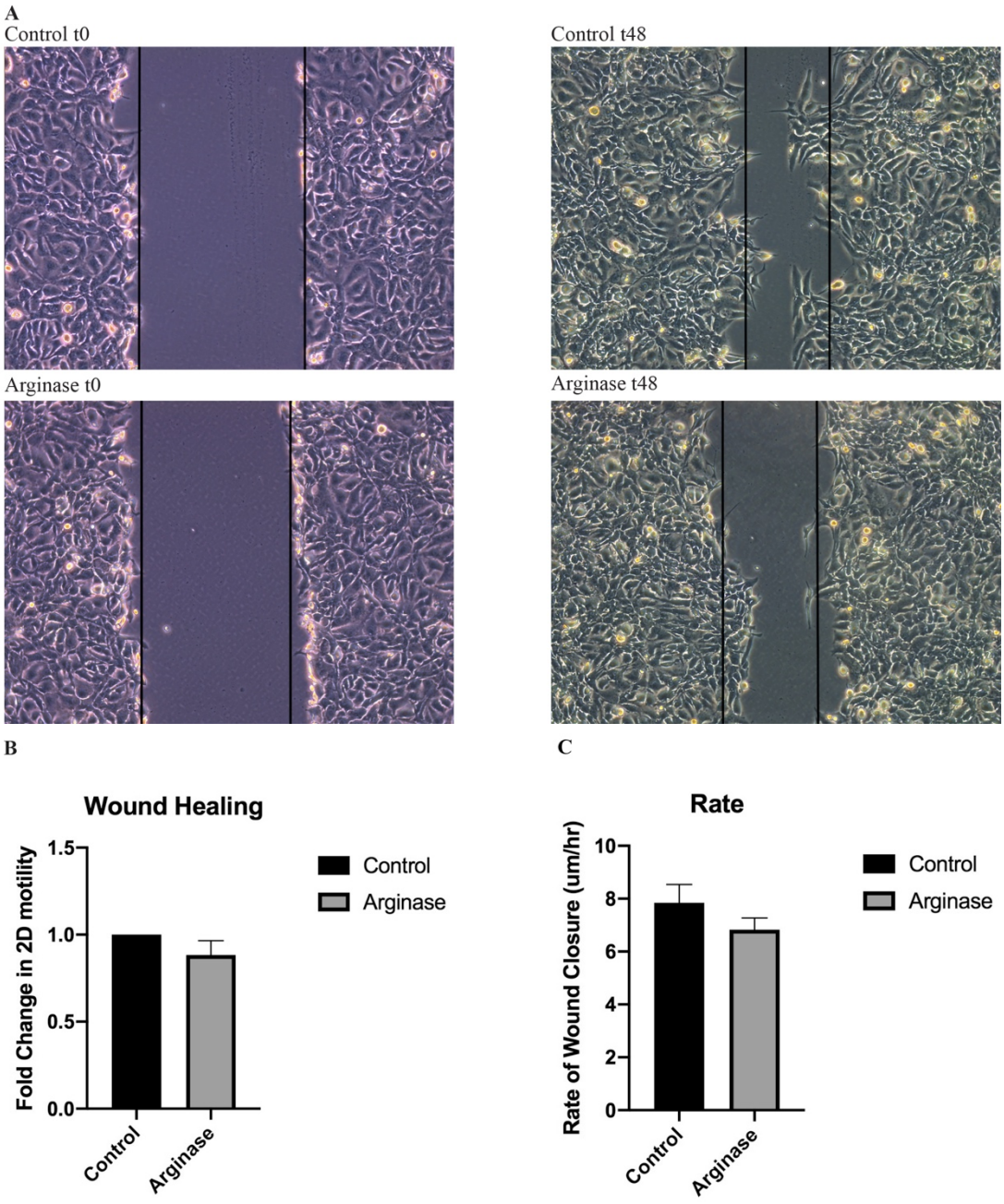

Figure 5: Fixed Wound Healing Assay. (A) SKOV3 cell line was treated with HuArgI (Co)-PEG5000 for 48 hours. (B) Data were measured in fold change of wound closure normalized to the control. (C) Quantification of (A). Data are the mean -/+ SEM from 3 experiments.

3.2. HuArg1(CO)-PEG 5000 decreases the cell motility of SKOV3.

To further validate the results that we got on HuArgI (Co)-PEG5000 decreasing the migration of SKOV3 cell line, a time-lapse assay was done to look at the random motility of those cells. The cells were treated with HuArgI

(Co)-PEG5000 and with rapamycin and imaged for 2 hours. Since HuArgI (Co)-PEG5000 depletes arginine in the cancer cell's culture, this induces autophagy as a rescue mechanism, for further production of metabolites needed by the cell. According to these information, rapamycin here was used to look into the idea of autophagy affecting migration rate as well. The migration rate of cells treated with HuArgI (Co)-PEG5000 decreased by around 40% in comparison to the control, from 1.21 um/min to 0.8um/min. The rate of cells treated with rapamycin also decreased, by 50%, from 1.21um/min to 0.77um/min (Figure 6.A). When looking at the fold change between the 3 conditions, we can observe an additional decrease in migration when treated with HuArgI (Co)-PEG5000 and rapamycin, in comparison to the untreated condition (Figure 6.B).

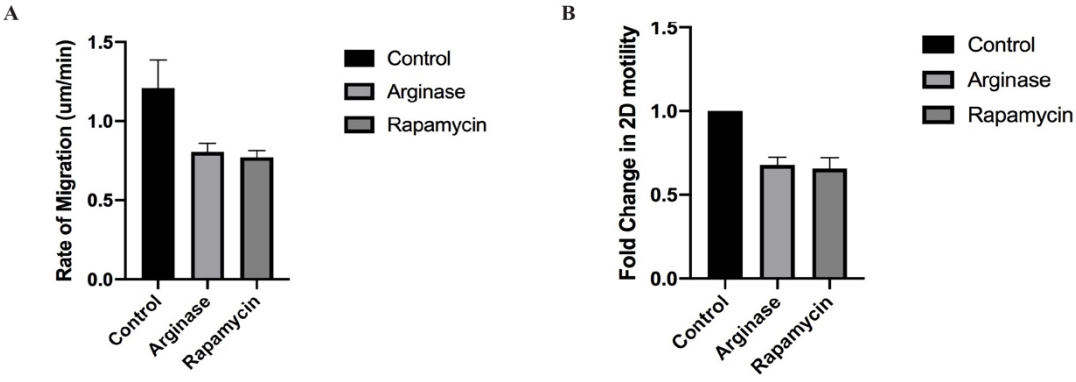

Figure 6: HuArgI (Co)-peg5000 and Rapamycin decrease the 2D motility of SKOV3 cell line. (A) Quantification of the migration rate in um/min, for SKOV3 cells. (B) Quantification of the net speed migration fold in SKOV3, normalized to the control, of projected 120 frames from time lapse movies of cells with an overall duration of 2 hours displaying random cell motility in serum. Data are the mean -/+ SEM taken from 10 randomly selected cells, from 3 experiments.

3.3. HuArgI (Co)-PEG5000 decreases the adhesion of SKOV3 cell line to collagen.

Knowing that the cell adhesion to the extra cellular matrix plays an important role in cell motility, we tested for the adhesion of SKOV3 cells to collagen. After treatment with HuArgI (Co)-PEG5000, the adhesion of the cells to collagen decreased slightly by 16% (from 1 to 0.84). In addition to that, upon the introduction of excess L-citrulline to the treated cells, the adhesion increased by 45% (from 1 to 1.45) (Figure 7.A). This indicates that cells treated with HuArgI (Co)-PEG5000 leading to arginine depletion, were rescued upon the addition of L-citrulline to the media (Figure 7.B).

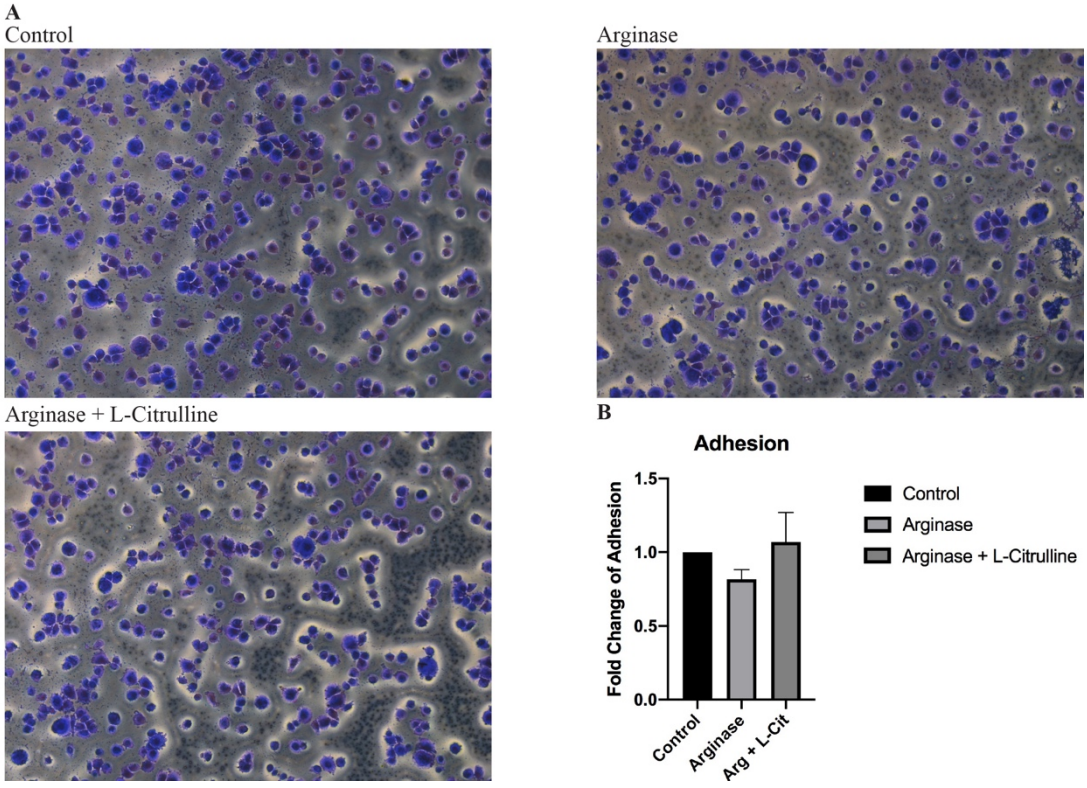

Figure 7: HuArgI (Co)-PEG5000 demotes cell adhesion to collagen in SKOV3 cells. (A) Representative micrographs of SKOV3 cells plated at different conditions control (Upper lane left), SKOV3 plated with HuArgI (Co)-PEG5000 (Upper lane right), HuArgI(Co)-PEG5000 and L-citrulline (lower lane left), fixed and stained with crystal violet (as described in methods). (B) Quantitation of solubilized Crystal violet from SKOV3 plates and the absorption of the plates were measured at 550 nm using an ELISA plate reader. Data were measured in fold change of adhesion normalized to the control. Data are the mean -/+ SEM from 3 experiments.

3.4. HuArgI (Co)-PEG5000 Decreases the Formation of Focal Adhesions in SKOV3 Cell Line.

The adhesion results were further validated by staining with vinculin, a protein which localizes at the focal adhesions. Thus, this immunostaining assay will give us an idea about the formation of focal adhesions in SKOV3 cells (Figure 8.A).

After treating the cells with HuArgI (Co)-PEG5000 and staining against vinculin, focal adhesions were counted, a decrease by 34% in the number of FA was noticed. When the cells were treated by rapamycin, there was no significant decrease in the number of FA (Figure 8.B). In addition to that, the area of the FAs was quantified and a significant decrease was noticed upon treating the cells with HuArgI (Co)-PEG5000. The area of FA decreased by 66%, in comparison to the control. However, upon treating with rapamycin, it decreased by 7% (Figure 8.C).

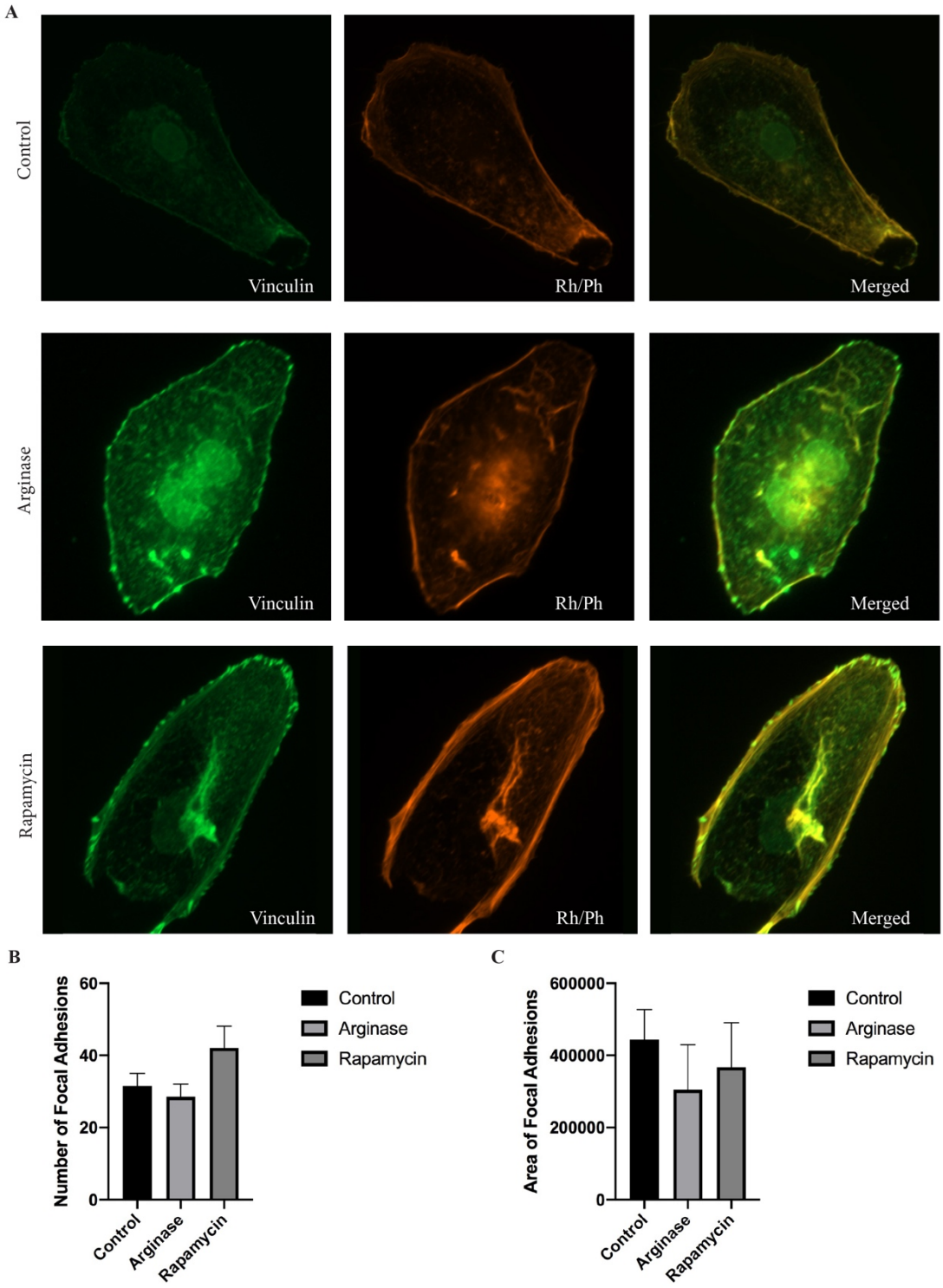

Figure 8: HuArgI (Co)-PEG5000 decreases focal adhesion formation SKOV3 cell line. (A) Representative micrographs of (SKOV3) control (upper line), HuArgI (Co)-PEG5000 (second lane). Rapamycin (lower lane that were fixed and stained with anti-vinculin. Cells were imaged using a 60x objective. (B) Quantitation of number of focal adhesions upon treatment with HuArgI (Co)-PEG5000 and Rapamycin. (C) Quantitation of areas of focal adhesions. Data are the mean -/+ SEM from 3 experiments.

3.5. HuArgI (Co)-PEG5000 has no effect on 3D motility of SKOV3 cell line.

After establishing the effect of HuArgI (Co)-PEG5000 on 2D motility of SKOV3 cells, its effect on 3D motility was investigated. An *in vitro* collagen based assay was done on SKOV3 cell line. The results showed no significance change in invasiveness of the cells upon treatment with HuArgI (Co)-PEG5000 (Figure 9).

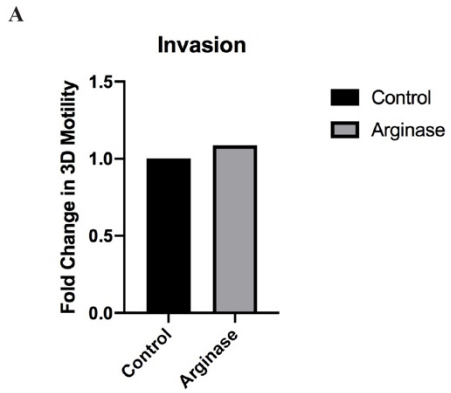

Figure 9: **HuArgI (Co)-PEG5000 has no significant effect on the 3D motility of SKOV3 cell line**. SKOV3 cells were treated by HuArgI(Co)-PEG5000. After 48 hours, cells that invaded to the basement side of the collagen basement membrane towards FBS for 48 hours were stained with cell stain according to manufacturer's specifications. After that, the cell stain was extracted and the absorbance was measured at 560 nm. (A) Quantitation of Optical Density for SKOV3 measured in arbitrary units.

Chapter 4

Discussion

Ovarian cancer being the fifth cause of death in women, leads scientists to look for further kinds of therapy to target it. The fact that its diagnosis is at a later stage makes it hard to control via surgery or chemotherapy. In addition to that, it was proven that ovarian cancer cases that relapsed after treatment with cisplatinum, a chemotherapeutic, were ASS1 deficient, which made them resistant to the chemotherapy introduced (Nicholson et al., 2009). Thus, new approaches were tested as a way to limit the migration and metastasis of ovarian cancer. One of the approaches that reached clinical trials is amino acid deprivation. The drug, HuArgI (Co)-PEG5000, acts on depleting arginine from the tumor's microenvironment in the purpose of limiting proliferation and metastasis.

At first, we hypothesized that HuArgI (Co)-PEG5000 will impair the migration ability of SKOV3 cell line. In the time-lapse experiment, done to observe 2D motility, a decrease in motility was noticed upon the treatment with the drug. When rapamycin was added as well, we noticed the same decrease in migration ability and speed of the cells. This indicates that HuArgI (Co)-PEG5000 is decreasing the migration of the cells and it is influencing downstream effectors of the autophagic pathway, due to lack of arginine. A study done by Shuvayeva et al., proves that autophagy in SKOV3 cell line, was induced upon the deprivation of arginine. They stained against LC3 marker, which indicates the presence of autophagosomes, for LAPM1, which indicates the presence of lysosomes and for BECLIN1 which also plays a role in autophagy regulation. Upon imaging, co-localization of LC3 and LAMP1 was observed which indicates the formation of autophagolysosomes, in the arginine deprived SKOV3 cells.

They studied the localization of BECLIN1, at the Golgi which indicates as well the activation of autophagy (Shuvayeva et al., 2014; Trincheri et al., 2008).

In a study done in 2014, NOS was highly expressed in SKOV3 cell line, knowing that it is a highly aggressive ovarian cancer cell line. They correlated the high expression of NOS to proliferation, aggressiveness and migration of this cell line. Since NO, synthesized from arginine through the help of NOS, contributes to cellular migration in lung cancer and to high proliferation rate in ovarian and breast cancer (Caneba et al., 2014). Thus, the decrease in migration rate and wound closure rate seen in our experiments tackling 2D motility, might be correlated to arginine deprivation, by HuArgI (Co)-PEG5000, and ultimately decreasing the production of NO.

STAT3 protein is correlated with increasing cell motility and invasion of ovarian cancer cells and gastric cancer cells. In gastric cancer, inhibition of expression of ASS1 protein correlates with decreasing the expression of STAT3 thus decreasing motility (Shan et al., 2015). Furthermore, in the study done by Montell et al. (2004), active STAT3 was correlated with invasive and metastatic ovarian cancer, and further experiments, done on SKOV3, showed that active STAT3 localizes in focal adhesions of the cell, this was clear when phosphorylated STAT3 co-localized with focal adhesion marker, Vinculin (Montell, Honami, Jinsong, Wenjun, & Denise, 2004). So it plays a role in cellular adhesion and is involved in changes at the cytoskeletal level. After inhibiting STAT3 in SKOV3 cell line, there was a decrease in the migration ability of the cell line in the presence of a chemo-attractant (Montell et al, 2004). This paves the way to investigate this topic and explain the decrease in adhesion we noticed in our experiments after depleting the cells from arginine through treating the cells with HuArgI (Co)-PEG5000. The fact that the cells were rescued by the addition of L-citrulline could be due to them being partially auxotrophic to arginine thus expressing

ASS1 protein. However, what makes SKOV3 sensitive to arginine depletion and totally depending on exogenous sources of arginine, is that it lacks the expression of ornithine transcarbamylase (OTC), which is an enzyme used for arginine production (Shuvayeva et al., 2014).

In addition to the decrease in adhesion in our experiments, we noticed a decrease in focal adhesions' number and area, in cell treated with HuArgI (Co)-PEG5000, however there was only a decrease in the area of focal adhesions in cells treated with rapamycin, an autophagy inducer, and increase in the number of FA. Similar results were noticed in literature where HuArgI (Co)-PEG5000 decreased the focal adhesion area in colorectal cancer cells. They correlated this decrease in FA maturation to the decrease of RhoA activation due to treatment with HuArgI (Co)-PEG5000 (Al-Koussa et al., 2019).

As for rapamycin decreasing migration rate, and increasing focal adhesion number while decreasing their area, Zhoa et al. proposed that autophagy, in ovarian cancer, decreases the epithelial to mesenchymal transition thus decreases migration and invasion of SKOV3 and A2780 cell lines (Z. Zhao, Zhao, Xue, Zhao, & Liu, 2016). Those results can explain why arginine deprivation, and ultimately activating autophagy, is decreasing adhesion and migration of SKOV3 cell line, upon the treatment with HuArgI (Co)-PEG5000.

Furthermore, upon examining the literature, a correlation between RhoGTPases: Rac1, Cdc42 and RhoA, activation and inactivation and autophagy could be observed. Where autophagy regulation of RhoGTPases affects the cytoskeleton organization and migration (Dower, Wills, Frisch, & Wang, 2018).

While trying to investigate the effect of HuArgI (Co)-PEG5000 on 3D motility, our results did not show any invasive behavior into collagen-coated wells, with or

without HuArgI (Co)-PEG5000. Our results are consistent with the literature were SKOV3 showed high invasive abilities on Matrigel and showed no significant invasive ability when plated onto collagen (Hallas-Potts et al., 2019; Sodek, Brown, & Ringuette, 2008).

Chapter 5

Conclusions

In the present study, the effect of HuArgI (Co)-PEG5000 on migration of ovarian cancer was investigated. Results showed that arginine deprivation could be a promising therapeutic approach for cells that depend on external sources of arginine. The fact that L-citrulline was able to rescue the cells deprived from arginine, tells us that SKOV3 is a partially auxotrophic cell line since it expresses ASS1 protein. In addition to that, HuArgI (Co)-PEG5000 was depriving the cells from arginine, which in turn leads them to the autophagic rescue mechanism. This proved to decrease their cellular migration and adhesion abilities.

Future studies should tackle the fact that autophagy plays a role in regulating the small RhoGTPases family, which in turn regulates the cell's cytoskeleton and mediates migration. This will help in understanding more the role of arginine deprivation in decreasing the cell's ability to migrate and will help in orchestrating a therapy that acts on both, autophagy as a cell death mechanism and as a process to inhibit migration. Especially in ovarian cancer, since the cancerous cells have a high ability to metastasize to the peritoneal space before forming a noticeable mass that aids in the prognosis.

Bibliography

Al Koussa, H., Al Haddad, M., Abi Habib, R., & El Sibai, M. (2019). Human Recombinant Arginase I [HuArgI (Co) PEG5000] Induced Arginine Depletion Inhibits Colorectal Cancer Cell Migration and Invasion. *Int J Mol Sci, 20*(23). https://doi.org/10.3390/ijms20236018

Bast, R. C., Jr., Hennessy, B., & Mills, G. B. (2009). The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer, 9*(6), 415 428. https://doi.org/10.1038/nrc2644

Beaufort, C. M., Helmijr, J. C., Piskorz, A. M., Hoogstraat, M., Ruigrok Ritstier, K., Besselink, N., . . . Helleman, J. (2014). Ovarian cancer cell line panel (OCCP): clinical importance of in vitro morphological subtypes. *PLoS One, 9*(9), e103988. https://doi.org/10.1371/journal.pone.0103988

Bernard, A., & Klionsky, D. J. (2013). Autophagosome formation: tracing the source. *Developmental cell, 25*(2), 116 117. doi:10.1016/j.devcel.2013.04.004

Bowtell, D. D., Böhm, S., Ahmed, A. A., Aspuria, P. J., Bast, R. C., Jr., Beral, V., . . . Balkwill, F. R. (2015). Rethinking ovarian cancer II: reducing mortality from high grade serous ovarian cancer. *Nat Rev Cancer, 15*(11), 668 679. https://doi.org/10.1038/nrc4019

Cancer. (2020). Retrieved from https://www.who.int/health topics/cancer tab=tab_1

Cancer Development | CancerQuest. Retrieved from https://www.cancerquest.org/cancer biology/cancer development

Caneba, C. A., Yang, L., Baddour, J., Curtis, R., Win, J., Hartig, S., . . . Nagrath, D. (2014). Nitric oxide is a positive regulator of the Warburg effect in ovarian cancer cells. *Cell Death Dis, 5*(6), e1302 e1302. https://doi.org/10.1038/cddis.2014.264

Carmeliet, P., & Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature, 407*(6801), 249 257. https://doi.org/10.1038/35025220

Cary, L. A., Chang, J. F., & Guan, J. L. (1996). Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J Cell Sci, 109 (Pt 7)*, 1787 1794.

Chang, K., Creighton, C. J., Davis, C., Donehower, L., Drummond, J., Wheeler, D., . . . Biospecimen Core Resource, C. (2013). The Cancer Genome Atlas Pan Cancer analysis project. *Nature Genetics, 45*(10), 1113 1120. https://doi.org/10.1038/ng.2764

Cheng, C. T., Qi, Y., Wang, Y. C., Chi, K. K., Chung, Y., Ouyang, C., . . . Ann, D. K. (2018). Arginine starvation kills tumor cells through aspartate exhaustion and mitochondrial dysfunction. *Communications Biology, 1*(1), 178. https://doi.org/10.1038/s42003 018 0178 4

Cheng, P. N., Lam, T. L., Lam, W. M., Tsui, S. M., Cheng, A. W., Lo, W. H., & Leung, Y. C. (2007). Pegylated recombinant human arginase (rhArg peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res, 67*(1), 309 317. https://doi.org/10.1158/0008 5472.Can 06 1945

Choudhari, S. K., Chaudhary, M., Bagde, S., Gadbail, A. R., & Joshi, V. (2013). Nitric oxide and cancer: a review. *World J Surg Oncol, 11*, 118. https://doi.org/10.1186/1477 7819 11 118

Committee on the State of the Science in Ovarian Cancer, R., Board on Health Care, S., Institute of, M., National Academies of Sciences, E., & Medicine. (2016). Introduction and Background. https://doi.org/https://www.ncbi.nlm.nih.gov/books/NBK367622/

Cooper, G. M. (2000). The Development and Causes of Cancer. doi:https://www.ncbi.nlm.nih.gov/books/NBK9963/

DeFea, K. A. (2013). Chapter Eight Arrestins in Actin Reorganization and Cell Migration. In L. M. Luttrell (Ed.), *Progress in Molecular Biology and Translational Science* (Vol. 118, pp. 205 222): Academic Press.

Delage, B., Fennell, D. A., Nicholson, L., McNeish, I., Lemoine, N. R., Crook, T., & Szlosarek, P. W. (2010). Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. *Int J Cancer, 126*(12), 2762 2772. https://doi.org/10.1002/ijc.25202

Delage, B., Luong, P., Maharaj, L., O'Riain, C., Syed, N., Crook, T., . . . Szlosarek, P. W. (2012). Promoter methylation of argininosuccinate synthetase 1 sensitises lymphomas to arginine deiminase treatment, autophagy and caspase dependent apoptosis. *Cell Death Dis, 3*(7), e342. https://doi.org/10.1038/cddis.2012.83

Dillon, B. J., Holtsberg, F. W., Ensor, C. M., Bomalaski, J. S., & Clark, M. A. (2002). Biochemical characterization of the arginine degrading enzymes arginase

and arginine deiminase and their effect on nitric oxide production. *Med Sci Monit, 8*(7), Br248 253.

Domcke, S., Sinha, R., Levine, D. A., Sander, C., & Schultz, N. (2013). Evaluating cell lines as tumour models by comparison of genomic profiles. *Nature Communications, 4*(1), 2126. https://doi.org/10.1038/ncomms3126

Dower, C. M., Wills, C. A., Frisch, S. M., & Wang, H. G. (2018). Mechanisms and context underlying the role of autophagy in cancer metastasis. *Autophagy, 14*(7), 1110 1128. https://doi.org/10.1080/15548627.2018.1450020

Efeyan, A., Zoncu, R., & Sabatini, D. M. (2012). Amino acids and mTORC1: from lysosomes to disease. *Trends Mol Med, 18*(9), 524 533. https://doi.org/10.1016/j.molmed.2012.05.007

Eger, A., & Mikulits, W. (2005). Models of epithelial–mesenchymal transition. *Drug Discovery Today: Disease Models, 2*(1), 57 63. https://doi.org/10.1016/j.ddmod.2005.04.001

Feun, L., You, M., Wu, C., Kuo, M., Wangpaichitr, M., Spector, S., & Savaraj, N. (2008). Arginine Deprivation as a Targeted Therapy for Cancer. *Curr Pharm Des, 14*(11), 1049 1057. Retrieved from http://dx.doi.org/

Fultang, L., Vardon, A., De Santo, C., & Mussai, F. (2016). Molecular basis and current strategies of therapeutic arginine depletion for cancer. *International Journal of Cancer, 139*(3), 501 509. https://doi.org/10.1002/ijc.30051

Gupta, G. P., & Massagué, J. (2006). Cancer metastasis: building a framework. *Cell, 127*(4), 679 695. https://doi.org/10.1016/j.cell.2006.11.001

Haeger, A., Krause, M., Wolf, K., & Friedl, P. (2014). Cell jamming: Collective invasion of mesenchymal tumor cells imposed by tissue confinement.

Biochimica et Biophysica Acta (BBA) General Subjects, 1840(8), 2386 2395. https://doi.org/10.1016/j.bbagen.2014.03.020

Hallas Potts, A., Dawson, J. C., & Herrington, C. S. (2019). Ovarian cancer cell lines derived from non serous carcinomas migrate and invade more aggressively than those derived from high grade serous carcinomas. *Scientific Reports, 9*(1), 5515. https://doi.org/10.1038/s41598 019 41941 4

Hapach, L. A., Mosier, J. A., Wang, W., & Reinhart King, C. A. (2019). Engineered models to parse apart the metastatic cascade. *npj Precision Oncology, 3*(1), 20. https://doi.org/10.1038/s41698 019 0092 3

Howlader N, N. A. M. K. M. G. J. M. D. A. S. F. K. C. L. Y. M. R. J. T. Z. M. A. (2015). Cancer Statistics Review, 1975 2012 Previous Version SEER Cancer Statistics Review. Retrieved from https://seer.cancer.gov/archive/csr/1975_2012/index.html

Idikio, H. A. (2011). Human Cancer Classification: A Systems Biology Based Model Integrating Morphology, Cancer Stem Cells, Proteomics, and Genomics. In *J Cancer* (Vol. 2, pp. 107 115).

Iglesias, P. A., & Devreotes, P. N. (2008). Navigating through models of chemotaxis. *Curr Opin Cell Biol, 20*(1), 35 40. https://doi.org/10.1016/j.ceb.2007.11.011

Jahani, M., Noroznezhad, F., & Mansouri, K. (2018). Arginine: Challenges and opportunities of this two faced molecule in cancer therapy. *Biomed Pharmacother, 102*, 594 601. https://doi.org/10.1016/j.biopha.2018.02.109

Khoury, O., Ghazale, N., Stone, E., El Sibai, M., Frankel, A. E., & Abi Habib, R. J. (2015). Human recombinant arginase I (Co) PEG5000 [HuArgI (Co) PEG5000] induced arginine depletion is selectively cytotoxic to human

glioblastoma cells. *J Neurooncol, 122*(1), 75 85. https://doi.org/10.1007/s11060 014 1698 5

Kim, R. H., Coates, J. M., Bowles, T. L., McNerney, G. P., Sutcliffe, J., Jung, J. U., . . . Kung, H. J. (2009). Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase independent apoptosis. *Cancer Res, 69*(2), 700 708. https://doi.org/10.1158/0008 5472.Can 08 3157

Kim, S. H., Roszik, J., Grimm, E. A., & Ekmekcioglu, S. (2018). Impact of l Arginine Metabolism on Immune Response and Anticancer Immunotherapy. *Frontiers in Oncology, 8*(67). https://doi.org/10.3389/fonc.2018.00067

Lengyel, E. (2010). Ovarian cancer development and metastasis. *Am J Pathol, 177*(3), 1053 1064. https://doi.org/10.2353/ajpath.2010.100105

Levanon, K., Crum, C., & Drapkin, R. (2008). New Insights Into the Pathogenesis of Serous Ovarian Cancer and Its Clinical Impact. In *J Clin Oncol* (Vol. 26, pp. 5284 5293).

Ltd, N. C., Yun, L., Wai, L., & Bio Cancer Treatment, I. (2005). Pharmaceutical preparation and method of treatment of human malignancies with arginine deprivation. Retrieved from https://patents.google.com/patent/US20050244398A1/en17

Mizushima, N., Levine, B., Cuervo, A. M., & Klionsky, D. J. (2008). Autophagy fights disease through cellular self digestion. *Nature, 451*(7182), 1069 1075. https://doi.org/10.1038/nature06639

Montell, D. L. S., Honami, N., Jinsong, L., Wenjun, C., & Denise, J. (2004). Activated Signal Transducer and Activator of Transcription (STAT) 3. https://doi.org/10.1158/0008 5472.CAN 03 3959

Morris, S. M. (2002). REGULATION OF ENZYMES OF THE UREA CYCLE AND ARGININE METABOLISM. *Annual Review of Nutrition, 22*(1), 87 105. https://doi.org/10.1146/annurev.nutr.22.110801.140547

Morris, S. M., Jr. (2007). Arginine metabolism: boundaries of our knowledge. *J Nutr, 137*(6 Suppl 2), 1602s 1609s. https://doi.org/10.1093/jn/137.6.1602S

Motohara, T., Masuda, K., Morotti, M., Zheng, Y., El Sahhar, S., Chong, K. Y., . . . Ahmed, A. A. (2019). An evolving story of the metastatic voyage of ovarian cancer cells: cellular and molecular orchestration of the adipose rich metastatic microenvironment. *Oncogene, 38*(16), 2885 2898. https://doi.org/10.1038/s41388 018 0637 x

Nasreddine, G., El Sibai, M., & Abi Habib, R. J. (2020). Cytotoxicity of [HuArgI (co) PEG5000] induced arginine deprivation to ovarian Cancer cells is autophagy dependent. *Invest New Drugs, 38*(1), 10 19. https://doi.org/10.1007/s10637 019 00756 w

Nicholson, L. J., Smith, P. R., Hiller, L., Szlosarek, P. W., Kimberley, C., Sehouli, J., . . . Crook, T. (2009). Epigenetic silencing of argininosuccinate synthetase confers resistance to platinum induced cell death but collateral sensitivity to arginine auxotrophy in ovarian cancer. *Int J Cancer, 125*(6), 1454 1463. https://doi.org/10.1002/ijc.24546

Nofal, M., Zhang, K., Han, S., & Rabinowitz, J. D. (2017). mTOR Inhibition Restores Amino Acid Balance in Cells Dependent on Catabolism of Extracellular Protein. *Mol Cell, 67*(6), 936 946.e935. https://doi.org/10.1016/j.molcel.2017.08.011

Paget, S. (1989). The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev, 8*(2), 98 101.

Rhoads, J. M., Chen, W., Gookin, J., Wu, G. Y., Fu, Q., Blikslager, A. T., . . . Romer, L. H. (2004). Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. *Gut, 53*(4), 514 522. https://doi.org/10.1136/gut.2003.027540

Ridley, A. J. (2001). Rho GTPases and cell migration. *J Cell Sci, 114*(15), 2713. Retrieved from http://jcs.biologists.org/content/114/15/2713.abstract

Riess, C., Shokraie, F., Classen, C. F., Kreikemeyer, B., Fiedler, T., Junghanss, C., & Maletzki, C. (2018). Arginine Depleting Enzymes – An Increasingly Recognized Treatment Strategy for Therapy Refractory Malignancies. *Cellular Physiology and Biochemistry, 51*(2), 854 870. https://doi.org/10.1159/000495382

Savaraj, N., You, M., Wu, C., Wangpaichitr, M., Kuo, M. T., & Feun, L. G. (2010). Arginine deprivation, autophagy, apoptosis (AAA) for the treatment of melanoma. *Current molecular medicine, 10*(4), 405 412. https://doi.org/10.2174/156652410791316995

Seidman, J. D., Horkayne Szakaly, I., Haiba, M., Boice, C. R., Kurman, R. J., & Ronnett, B. M. (2004). The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol, 23*(1), 41 44. https://doi.org/10.1097/01.pgp.0000101080.35393.16

Selvaggi, S. M. (2000). Tumors of the Ovary, Maldeveloped Gonads, Fallopian Tube, and Broad Ligament. *Archives of Pathology & Laboratory Medicine, 124*(3),

474 474. https://doi.org/10.1043/1543 2165(2000)124<474:TOTOMG>2.0.CO;2

Seyfried, T. N., & Huysentruyt, L. C. (2013). On the Origin of Cancer Metastasis. *Crit Rev Oncog, 18*(1 2), 43 73. Retrieved from http://dx.doi.org/

Shan, Y. S., Hsu, H. P., Lai, M. D., Yen, M. C., Chen, W. C., Fang, J. H., . . . Chen, Y. L. (2015). Argininosuccinate synthetase 1 suppression and arginine restriction inhibit cell migration in gastric cancer cell lines. *Scientific Reports, 5*, 9783 9783. https://doi.org/10.1038/srep09783

Shen, L. J., Beloussow, K., & Shen, W. C. (2006). Modulation of arginine metabolic pathways as the potential anti tumor mechanism of recombinant arginine deiminase. *Cancer Lett, 231*(1), 30 35. https://doi.org/10.1016/j.canlet.2005.01.007

Shen, L. J., Lin, W. C., Beloussow, K., & Shen, W. C. (2003). Resistance to the anti proliferative activity of recombinant arginine deiminase in cell culture correlates with the endogenous enzyme, argininosuccinate synthetase. *Cancer Lett, 191*(2), 165 170. https://doi.org/10.1016/s030 43835(02)00693 6

Shin, J. K., Sangyoon, O., & Jennifer, H. (2020). Effect of Varying Mechanical Environment in 2D Culture on Subsequent Metastasis Process of Ovarian Cancer. *Biophysical Journal, 118*(3), 601a. https://doi.org/10.1016/j.bpj.2019.11.3249

Shuvayeva, G., Bobak, Y., Igumentseva, N., Titone, R., Morani, F., Stasyk, O., & Isidoro, C. (2014). Single amino acid arginine deprivation triggers prosurvival

autophagic response in ovarian carcinoma SKOV3. *Biomed Res Int, 2014*, 505041. https://doi.org/10.1155/2014/505041

Siegel, R. L., Miller, K. D., & Jemal, A. (2020). Cancer statistics, 2020. *CA Cancer J Clin, 70*(1), 7 30. https://doi.org/10.3322/caac.21590

Sodek, K. L., Brown, T. J., & Ringuette, M. J. (2008). Collagen I but not Matrigel matrices provide an MMP dependent barrier to ovarian cancer cell penetration. *BMC Cancer, 8*, 223. https://doi.org/10.1186/1471 2407 8 223

Stone, E. M., Glazer, E. S., Chantranupong, L., Cherukuri, P., Breece, R. M., Tierney, D. L., . . . Georgiou, G. (2010). Replacing Mn(2+) with Co(2+) in human arginase i enhances cytotoxicity toward l arginine auxotrophic cancer cell lines. *ACS chemical biology, 5*(3), 333 342. https://doi.org/10.1021/cb900267j

Sugimura, K., Ohno, T., Kusuyama, T., & Azuma, I. (1992). High sensitivity of human melanoma cell lines to the growth inhibitory activity of mycoplasmal arginine deiminase in vitro. *Melanoma Res, 2*(3), 191 196. https://doi.org/10.1097/00008390 199209000 00007

Tjhay, F., Motohara, T., Tayama, S., Narantuya, D., Fujimoto, K., Guo, J., . . . Katabuchi, H. (2015). CD44 variant 6 is correlated with peritoneal dissemination and poor prognosis in patients with advanced epithelial ovarian cancer. *Cancer Sci, 106*(10), 1421 1428. https://doi.org/10.1111/cas.12765

Torre, L. A., Trabert, B., DeSantis, C. E., Miller, K. D., Samimi, G., Runowicz, C. D., . . . Siegel, R. L. (2018). Ovarian cancer statistics, 2018. *CA: a cancer journal for clinicians, 68*(4), 284 296. https://doi.org/10.3322/caac.21456

Trepat, X., Chen, Z., & Jacobson, K. (2012). Cell migration. *Comprehensive Physiology, 2*(4), 2369 2392. https://doi.org/10.1002/cphy.c110012

Trincheri, N. F., Follo, C., Nicotra, G., Peracchio, C., Castino, R., & Isidoro, C. (2008). Resveratrol induced apoptosis depends on the lipid kinase activity of Vps34 and on the formation of autophagolysosomes. *Carcinogenesis, 29*(2), 381 389. https://doi.org/10.1093/carcin/bgm271

Tsui, S. M., Lam, W. M., Lam, T. L., Chong, H. C., So, P. K., Kwok, S. Y., . . . Leung, Y. C. (2009). Pegylated derivatives of recombinant human arginase (rhArg1) for sustained in vivo activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics in vivo and in vitro and action upon hepatocellular carcinoma cell (HCC). *Cancer cell international, 9*, 9 9. https://doi.org/10.1186/1475 2867 9 9

van Zijl, F., Krupitza, G., & Mikulits, W. (2011). Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutation research, 728*(1 2), 23 34. https://doi.org/10.1016/j.mrrev.2011.05.002

Wang, H., Li, Q. F., Chow, H. Y., Choi, S. C., & Leung, Y. C. (2020). Arginine deprivation inhibits pancreatic cancer cell migration, invasion and EMT via the down regulation of Snail, Slug, Twist, and MMP1/9. *Journal of Physiology and Biochemistry, 76*(1), 73 83. https://doi.org/10.1007/s13105 019 00716 1

Wirtz, D., Konstantopoulos, K., & Searson, P. C. (2011). The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat Rev Cancer, 11*(7), 512 522. https://doi.org/10.1038/nrc3080

Wisdom, K. M., Adebowale, K., Chang, J., Lee, J. Y., Nam, S., Desai, R., . . . Chaudhuri, O. (2018). Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. *Nature Communications, 9*(1), 4144. https://doi.org/10.1038/s41467 018 06641 z

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Marc Rhoads, J., . . . Yin, Y. (2009). Arginine metabolism and nutrition in growth, health and disease. *Amino Acids, 37*(1), 153 168. https://doi.org/10.1007/s00726 008 0210 y

Wu, J. C., Chen, Y. C., Kuo, C. T., Wenshin Yu, H., Chen, Y. Q., Chiou, A., & Kuo, J. C. (2015). Focal adhesion kinase dependent focal adhesion recruitment of SH2 domains directs SRC into focal adhesions to regulate cell adhesion and migration. *Scientific Reports, 5*(1), 18476. https://doi.org/10.1038/srep18476

Wu, W. K. K., Coffelt, S. B., Cho, C. H., Wang, X. J., Lee, C. W., Chan, F. K. L., . . . Sung, J. J. Y. (2012). The autophagic paradox in cancer therapy. *Oncogene, 31*(8), 939 953. https://doi.org/10.1038/onc.2011.295

Yap, T. A., Carden, C. P., & Kaye, S. B. (2009). Beyond chemotherapy: targeted therapies in ovarian cancer. *Nat Rev Cancer, 9*(3), 167 181. https://doi.org/10.1038/nrc2583

Yokota, J., & Biology Division, N. C. C. R. I. T. c. C. k. T. J. (2020). Tumor progression and metastasis. *Carcinogenesis, 21*(3), 497 503. doi:10.1093/carcin/21.3.497

Yu, L., McPhee, C. K., Zheng, L., Mardones, G. A., Rong, Y., Peng, J., . . . Lenardo, M. J. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature, 465*(7300), 942 946. https://doi.org/10.1038/nature09076

Zhan, R., He, W., Wang, F., Yao, Z., Tan, J., Xu, R., . . . Luo, G. (2016). Nitric oxide promotes epidermal stem cell migration via cGMP Rho GTPase signalling. *Scientific Reports, 6*, 30687 30687. https://doi.org/10.1038/srep30687

Zhan, R., Yang, S., He, W., Wang, F., Tan, J., Zhou, J., . . . Luo, G. (2015). Nitric oxide enhances keratinocyte cell migration by regulating Rho GTPase via cGMP PKG signalling. *PLoS One, 10*(3), e0121551. https://doi.org/10.1371/journal.pone.0121551

Zhao, Y., He, M., Cui, L., Gao, M., Zhang, M., Yue, F., . . . Chen, L. (2020). Chemotherapy exacerbates ovarian cancer cell migration and cancer stem cell like characteristics through GLI1. *Br J Cancer*. https://doi.org/10.1038/s41416 020 0825 7

Zhao, Z., Zhao, J., Xue, J., Zhao, X., & Liu, P. (2016). Autophagy inhibition promotes epithelial mesenchymal transition through ROS/HO 1 pathway in ovarian cancer cells. *American journal of cancer research, 6*(10), 2162 2177. Retrieved from https://pubmed.ncbi.nlm.nih.gov/27822409 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5088283/