LEBANESE AMERICAN UNIVERSITY

The Effect of Recombinant Anthrax Lethal Toxin on Breast Cancer Cell Motility, Adhesion, and Invasion

By

Dana El Chami

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology

School of Arts and Sciences
April 2020

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Dana El Chami I.D. #: 201408831

Thesis Title: The Effect of Recombinant Anthrax Lethal Toxin on Breast Cancer Cell Motility, Adhesion and Invasion

Program: MS in Molecular Biology

Department: Natural Sciences

School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approved 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: 18 / 05 / 2020 (Day / Month / Year)

Committee Member's Name: Roy Khalaf

Signature: [redacted] Date: 18 / 05 / 2020 (Day / Month / Year)

Committee Member's Name: Michella Ghassibe-Sabbagh

Signature: [redacted] Date: 18 / 05 / 2020 (Day / Month / Year)

Dedicated to my beloved parents and grandparents

Acknowledgments

First, I thank God for his blessings and the strength to complete this body of work.

I would like to express my deep and sincere gratitude for my supervisors, Dr Mirvat El-Sibai and Dr. Ralph Abi Habib for giving me the opportunity to do research and providing me with invaluable guidance, support, and insight throughout my thesis.

My appreciation also extends to LAU faculty of the Biology department, especially to my committee members, Dr. Roy Khalaf and Dr. Michella Ghassibe-Sabbagh.

I would also like to thank all my colleagues and members of El Sibai lab for their constant encouragement.

Finally, I extend my deepest gratitude to my family and friends for their patience, support, and guidance.

The Effect of Recombinant Anthrax Lethal Toxin on Breast Cancer Cell Motility, Adhesion, and Invasion

Breast cancer-related deaths are mostly due to breast cancer invasion and migration to distant secondary regions. Targeting breast cancer cell metastasis is an important therapeutic approach. The mitogen-activated protein kinase (MAPK) pathway is a key cell signaling pathway that plays a major role in cell invasion and migration. Many studies have targeted the MAPK pathway as a way to target cell survival and motility. In this study, we use Lethal Toxin (LT) a potent MAPK inhibitor that selectively inactivates all the kinases in the MAPK pathway. LT proved to affect breast cancer cell migration, adhesion, and invasion. Cells treated with LT showed a significant decrease in motility as seen in 2D time-lapse and wound healing assays. Additionally, cells treated with LT showed an increase in adhesion, decrease in invasion across a collagen matrix, and in increase in Rho A activation. We speculate that LT inhibited cell migration by deregulating the activity of Rho GTPases, proteins known to play a role in cell migration. In this study, we describe the effect of LT as a potential breast cancer cell invasion and motility inhibitor.

Key words: MAPK, Anthrax lethal toxin, breast cancer, migration, invasion, adhesion

Table of Contents

List of Figures

List of Abbreviations

2D: 2 dimensional
AKT: Protein Kinase B
AML: Acute myeloid leukemia
BCL2: B-cell lymphoma 2
BSA: Bovine serum albumin
CAF: Cancer associated fibroblast
CMG2: Capillary morphogenesis gene 2
CK: Cytokeratin
DMEM: Dulbecco's modified Eagle's medium
ECM: Extracellular matrix
EF: Edema factor
EGFR: Epithelial growth factor receptor
ELK1: Ets-like transcription factor
EMT: Epithelial to mesenchymal transition
ER: Estrogen Receptor
ERBB2: Erb-B2 Receptor Tyrosine Kinase 2
ERK: Extracellular regulated kinase
ET: Edema toxin
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
FRA-1: Fos related antigen 1
GDP: Guanosine diphosphate
GEF: Guanine nucleotide exchange factor
GPCR: G-protein coupled receptor
GRB2: Growth factor receptor bound protein 2
GRP78: Glucose regulated protein
GST: Glutathione S-transferase
GTP: Guanosine triphosphate
HER2: Human Epidermal Growth Factor Receptor 2
hTERT: Human telomerase catalytic subunit gene
JNK: c-Jun NH2-terminal kinase
LF: Lethal factor
LT: Lethal toxin
PI3K: Phosphoinositide 3-kinases
M3K: Mitogen-activated protein kinase kinase kinase
M2K: Mitogen-activated protein kinase kinase
M1K: Mitogen-activated protein kinase
MAPK: Mitogen-activated protein kinase
MEK: Mitogen-activated protein kinase kinase
P27: Cyclin-dependent kinase inhibitor 1B
P53: Tumor protein 53
P63: Transformation-related protein 63
PA: Protective agent

PAK1: p21 activated kinases 1
PBS: Phosphate Buffered Saline
PR: Progesterone receptor
PTEN60: Phosphatase and tensin 60
Rac1: Ras-related C3 botulinum toxin substrate 1
RAF: Rapidly Accelerated Fibrosarcoma
RAS: Rat sarcoma
RBD: Rhotekin binding domain
Rho A: Ras homologous member A
ROI: Region of interest
RSK: Ribosomal serine kinase
SAPK: Stress activated protein kinase
SH2: Src homology 2
SH3: Src homology 3
SHC: SH2-containing collagen
SMA: Smooth muscle actin
SOS: Son of sevenless
TEM8: Tumor endothelial marker 8
TRF2: Telomeric repeat binding factor 2
TRK: Tyrosine Receptor Kinase

Chapter One

Literature Review

1.1. Cancer

1.1.1. Cancer Overview

The underlying abnormality that leads to the development of a tumor is the continuous uncontrolled proliferation of cells, which occurs through the activation of oncogenes and the inhibition of tumor suppressor genes (Cooper, 2000). These changes in the genome lead to cell cycle progression by avoiding cell cycle checkpoints, as well as evasion of apoptotic mechanisms that contribute to cancer development and progression (Pucci, Kasten, & Giordano, 2000). Tumors can be either characterized as benign or malignant based on the tumor cells' abilities to metastasize to different tissues. Malignant tumors detach from the primary tumor, invade through the basement membrane into circulatory and lymphatic systems, escape the immune system, and invade and grow at surrounding tissue sites (Seyfried & Huysentruyt, 2013). Unlike malignant tumors, benign tumors cannot metastasize to other tissues and can be extracted surgically without the possibility of regrowth ("National Breast Cancer Foundation," 2014).

1.1.2. Characterizing Cancer

Malignant tumors are classified by the type of cell from which they arise, and by their tissue of origin (Idikio, 2011). Since malignant growths can arise from any tissue, there have been over a hundred different types of cancers classified, but they all share a set of common characteristics identified as the hallmarks of cancer (Fouad & Aanei, 2017). The ten hallmarks of cancer are summarized as: an increased

proliferative ability through the dependence on self-sustained growth signals, decreased sensitivity to anti-growth signals, escaping apoptosis, sustained angiogenesis, limitless replicative potential, tissue invasion, metastasis, avoiding immune response, genome instability and mutation, tumor-promoting inflammation, and reprogramming of metabolic energetics (Hanahan & Weinberg, 2011).

1.1.3. How Does Cancer Arise?

The clonal expansion theory suggests that tumors are initially formed from a single cell that begins to proliferate creating a tumor mass due to a carcinogenic event; this tumor itself is not malignant. As the cells of this tumor proliferate, errors arising in DNA replication will result in daughter cells that are called subclones. Expanding on Darwin's theory, cells with mutations that are advantageous to the life of the cell will persist whereas mutations that are deleterious to the life of the cell will cause the elimination of this specific cell and its subclones (Stratton, Campbell, & Futreal, 2009). As these distinct subclones continue dividing they give rise to a heterogeneous population of subclones with the same early mutation but different late mutations. The subclone that has acquired all of the genetic hallmarks will be the subclone that prevails giving rise to a malignant tumor capable of metastasizing (Greaves & Maley, 2012). Two specific types of mutations are involved in the discussion of advantageous mutations that affect a tumor's progression into malignancy which are: the gain of function mutation in oncogenes and loss of function mutation in tumor suppressor genes.(Lodish et al., 2000) A subclone that has acquired all the hallmarks of cancer would have sustained these two types of mutations, the order in which these mutations are sustained would depend on the cell type and tissue origin.

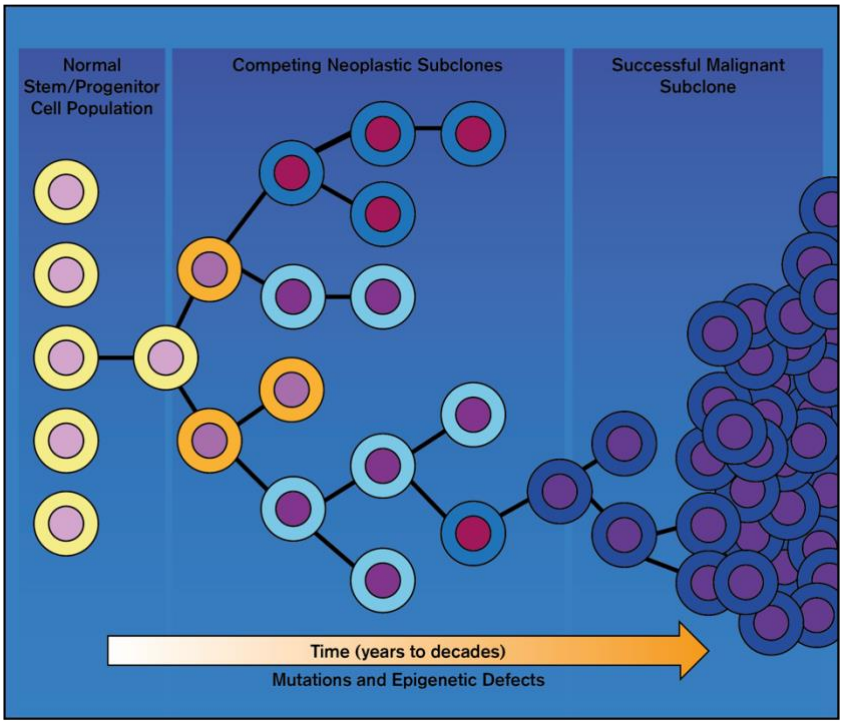


Figure1: Role of clonal selection in tumor development and progression. Adapted and modified from Teh & Fearon (2020)

1.1.4. Cancer Cell Motility

Cancer cell migration and invasion are key processes in cancer cell metastasis; the major cause of cancer-related death. In cancer cell migration the cell follows a chemoattractive path by remodeling its actin cytoskeleton in the direction of the chemoattractant, resulting in protrusion formation at the leading edge. At the leading edge, adhesive molecules known as focal adhesions couple the cell to the underlying membrane, thus preventing the retraction of the leading edge. However, at the trailing edge, the focal adhesions dissolve to the underlying membrane leading to actin filament depolarization, and body contraction towards the leading edge (Ananthakrishnan & Ehrlicher, 2007).

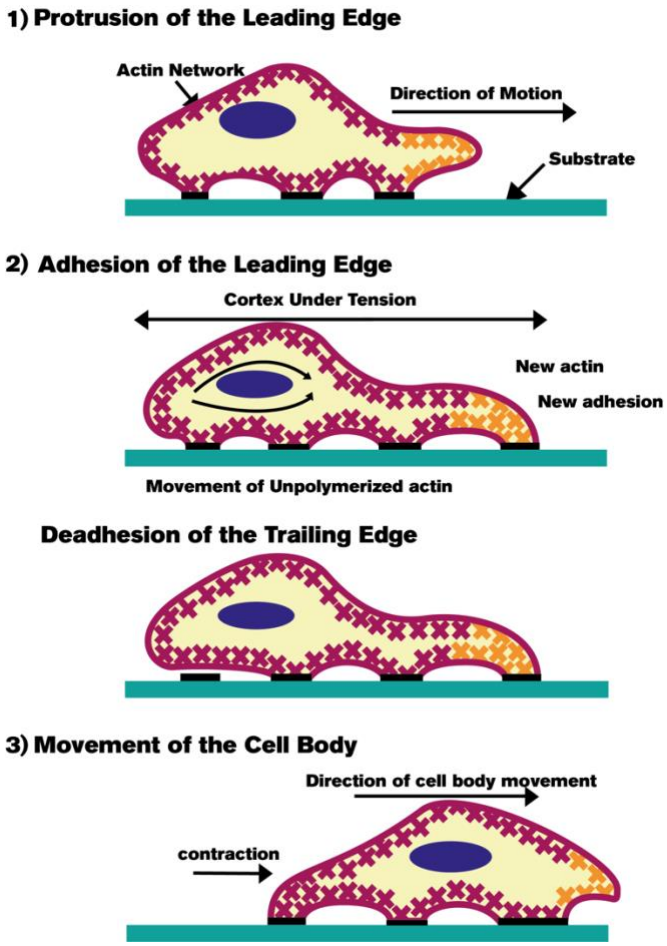


Figure 2: The steps of cell motility. Adapted and modified from Ananthakrishnan & Ehrlicher, (2007)

1.2. Breast Cancer

1.2.1. Statistics and Epidemiology

According to the American Cancer Society, breast cancer is the most common cancer diagnosed among US women and the second leading cause of cancer death among women (DeSantis et al., 2016).

The breast is comprised of two main tissue types; glandular and stromal tissues. Glandular tissue houses the lobules that consist of mammary glands and ducts while the stromal tissue includes connective tissue that consists of adipose, myofibroblasts, and blood vessels (Feng et al., 2018).

Breast cancer is divided into two main types depending on the origin of the cell. Cancers that arise from the epithelium lining the lobules and ducts are called carcinomas, while cancers arising from the stromal tissue of the breast are called sarcomas (Sharma, Dave, Sanadya, Sharma, & Sharma, 2010). It is important to note that breast cancer sarcomas constitute only 1% of primary breast cancers. Breast cancer carcinomas are divided into three subtypes: noninvasive, invasive, and metastatic.

Noninvasive (*in-situ*) breast carcinomas are tumors confined to the ducts of the breast glandular tissue that have still not invaded into the surrounding stromal tissue. Noninvasive carcinomas are called pre-invasive carcinomas as they have the potential to become invasive (Akram, Iqbal, Daniyal, & Khan, 2017).

Invasive or infiltrating breast carcinomas are tumors that originate from the glandular tissue of the breast; either the ducts or lobes; and have invaded into the stromal tissue. Invasive carcinomas can be removed surgically with a low rate of reoccurrence, but they also have the potential to metastasize into surrounding lymph nodes or to distant regions such as the liver or the brain (Akram, Iqbal, Daniyal, & Khan, 2017).

Metastatic breast carcinomas are late-stage carcinomas that not only have invaded into the surrounding tissue but also metastasized to lymph nodes or to different organs such as the liver, lungs, bones, or brain (Sharma, Dave, Sanadya, Sharma, & Sharma, 2010). Metastatic breast cancer has the lowest prognosis and accounts for over 90% of deaths in patients (Jin & Mu, 2015).

1.2.2. Molecular Subtypes of Breast Cancer

Breast tumors are heterogenous, exhibiting great phenotypic diversity. Perou and colleagues proposed that this diversity is accompanied by a corresponding diversity in molecular expression (Perou et al., 2000). Through molecular analysis and gene profiling, Perou and colleagues subclassified breast tumors into three types: HER2 positive, Estrogen receptor (ER) positive, and basal-like breast cancers (Perou et al., 2000).

HER2 positive tumors: The human epithelial receptor 2 (HER2) encoded by the ERBB2 gene is part of the human epidermal growth factor receptor family (Callahan & Hurvitz, 2011). HER2 is a transmembrane tyrosine kinase receptor (TRK), a proto-oncogene, and has no known ligand, which allows it to dimerize with other receptors of the HER family (Asif, Sultana, Ahmed, Akhter, & Tariq, 2016). HER2 regulates internal cell activities such as cell growth, proliferation, and survival. In HER2 positive tumors, there is approximately 100 times overexpression of the HER2 gene (Sauter, Lee, Bartlett, Slamon, & Press, 2009). When HER2 is overexpressed, it leads to the overactivation of cellular pathways such as the PI3K/Akt and the Ras/Raf/MEK/MAPK pathways, which are known to be involved in tumorigenesis. In 1998, Trastuzumab, a monoclonal antibody, was approved for the treatment of metastatic HER2 positive cancers (Hoeferlin, E Chalfant, & Park, 2013). In 2008, Lapatinib was also approved as a treatment for HER2 positive cancers based on its high efficacy in selectively inhibiting the kinase activity of HER2 (Asif, Sultana, Ahmed, Akhter, & Tariq, 2016).

ER-positive tumors: ER is a hormone receptor that belongs to the nuclear hormone receptor family. Once estrogen; a steroid hormone produced mainly in the

ovaries; binds to the ER on the cell surface, it acts as a transcription factor inducing an intracellular cascade of events leading to the transcription of genes involved in cell proliferation and survival (Fragomeni, Sciallis, & Jeruss, 2018).

ER-positive tumors are divided into three types: luminal A, luminal B with her-2 negative, and luminal B with HER2 positive. Luminal A tumors have ER and/or progesterone receptors (PR), whereas luminal B tumors have ER and/or PR in addition to high levels of Ki-67 (Lumachi, Brunello, Maruzzo, & Basso, U. Basso and S. M. M., 2013). Ki-67 is a proliferation marker widely associated with tumor proliferation and growth. Ki-67 has been shown to be expressed in all stages of the cell cycle except for G0 (Li, Jiang, Chen, Zheng, 2015). Luminal B tumors are further divided into HER2 negative and HER2 positive types. Luminal B-HER2 negative tumors do not have HER2 receptors on the cell surface whereas luminal B HER2 positive have HER2 receptors (Lumachi, Brunello, Maruzzo, & Basso, U. Basso and S. M. M., 2013).

Historically, progesterone was found to have a growth inhibitory role in uterine and ovarian tumorigenesis, and recently it has been proven to have a proliferative role in breast tumorigenesis. The mechanism of progesterone in breast cancer tumorigenesis has not yet been elucidated. Graham and colleagues (2009), demonstrated that progesterone increases breast cell proliferation by activation DNA replication (Graham et al., 2009). Other studies have shown progesterone receptor genes to be target genes for estrogen receptors, for that it has been proven that progesterone receptors (PR) share extensive crosstalk between the pathways activated by estrogen receptors (Daniel, Hagan, Lange, 2011). Endocrine therapy is used in the treatment of luminal A and luminal B breast tumors, these drugs block the ER and PR or block the synthesis of estrogen and progesterone. In the early stages of breast cancer

both respond well to endocrine therapy (luminal A having better prognosis than luminal b) due to the tumor relying on both estrogen and progesterone for proliferation and survival (Fragomeni, Sciallis, & Jeruss, 2018). However, in later stages of breast cancer, the tumor can mutate to escape endocrine therapy therefore conferring resistance to therapy (Graham et al., 2009).

Basal-like breast cancers are associated with a lack/low expression of both ER and PR, and no overexpression of the her-2 receptor. The immunohistochemical markers used to identify basal-like breast cancers include cytokeratins, epithelial growth factor receptors (EGFR), p-cadherin, p63, and smooth muscle actin (Badowska-Kozakiewicz & Budzik, 2016).

The molecular significance of cytokeratins (CK) in basal carcinomas is still unclear, but high levels of CK5, CK14, and CK17 are associated with poor patient prognosis (Gusterson, Ross, Heath, & Stein, 2005).

Even though there is a high expression of EGFR in basal-like carcinomas, these cells are usually resistant to anti-EGFR treatments due to them carrying a mutation in PTEN60(Foulkes, Smith, & Reis-Filho, 2010). Loss of PTEN60 function leads to the hyperphosphorylation of AKT resulting in apoptosis inhibition (Chen et al., 2015).

Smooth muscle actin is a characteristic protein highly expressed by cancer-associated fibroblasts (CAFs) which are fibroblasts of the breast stromal tissue (Fu et al., 2014). CAFs provide physical support to the tumor, assist the tumor in immune system evasion, and immunotherapy resistance (Liu et al., 2019).

P-cadherin is a cell-cell adhesion protein that functions cell processes such as growth, polarity, and migration. The role of p-cadherin is not clear, but it is

upregulated in metastatic tumors (Paredes et al., 2007). Breast cancer cells that have metastasized to the lymph nodes have shown a large increase in the expression of p-cadherin when compared to the primary tumor site, suggesting that p-cadherin functions in breast cancer invasion and migration (Vieira & Paredes, 2015).

P63 is a part of the p53 family of tumor suppressors. P63 has two isoforms; TAp63 and ΔNp63 (Nekulova et al., 2016). TAp63 acts as a tumor suppressor having a role in cell cycle arrest and apoptosis, contrarily ΔNp63 acts as an oncogene playing a role in cell adhesion, invasion, and migration (Melino, 2011). In basal-like carcinomas, the expression of ΔNp63 increases, whereas TAp63 expression decreases (Gatti et al., 2019).

All of the characteristics stated above in addition to other characteristics have given breast cancer an aggressive, invasive, and metastatic profile with limited targeted therapeutic options. Patients with basal-like breast carcinomas have poor prognosis especially if it is in the late stages (Toft & Cryns, 2011).

Anthrax Lethal Toxin

1.3. Composition

Anthrax Lethal toxin is a binary toxin caused by a, rod-shaped, spore forming, gram-positive bacterium, *Bacillus anthracis* (Liu, Moayeri, Leppla, 2014). B. *anthracis c*ontains two virulence encoding plasmids: pXO1 and pXO2. The pXO2 plasmid encodes for the bacterial poly-γ-d-glutamic acid capsule, allowing the bacteria to escape from phagocytosis in the host (Jeon et al., 2015). The second plasmid, PXO1, encodes for three factors: protective agent (PA), lethal factor (LF), and edema factor (EF). These factors alone are not toxic, but a combination of PA and LF or PA and EF

can generate two harmful toxins: lethal toxin (LT) and edema toxin (ET), respectively. A combination of LT and ET generates anthrax lethal toxin causing anthrax and its related symptoms (Bachran & Leppla, 2016).

1.3.1. Mode of Action

Upon being released by B. *anthracis*, PA (83 kDa) binds to the host cell surface at the tumor endothelial marker 8 (TEM8) or the capillary morphogenesis gene 2 (CMG2). Both receptors require divalent cation-dependent interaction essential for PA binding. TEM8 demonstrated increased expression in breast tumor tissue. Upon binding of PA to TEM8 or CMG2, the PA is cleaved by furin-like proteases, releasing a 20 kDa amino-terminal fragment and yielding a 63 kDa carboxy-terminal active PA fragment.

Recent studies have established that PA63 activation and cell surface binding are independent events, the rates of each depend on the accessibility to furin/furin-like proteases and cell surface receptors. The removal of the 20 kDa removes steric hindrance and allows the initiation of oligomerization of the PA63 forming a ring-shaped, pre-pore heptamer or octamer (Liu, Moayeri, Leppla, 2014). The formation of PA63 allows LF binding, 3-4 LF molecules bind to each heptamer or octamer, respectively. This complex will then undergo receptor-mediated endocytosis into the cell. Due to endosome acidification, the PA63 pre-pore complex undergoes a conformational change into a pore complex. This pore complex will create pores in the endosome freeing LF allowing it to translocate into the cytosol, due to PA63 oligomerization only LF can translocate to the cytosol where as PA63 monomers remain at the endosome or cell surface (Agrawal & Pulendran, 2004). Recent studies

have shown that the chaperone GRP78; a molecular chaperone; is essential for LF translocation (Bachran & Leppla, 2016).

LF is a zinc-dependent metalloprotease that functions by cleaving MEKS at their amino terminus and thus inactivating them (Liu, Moayeri, Leppla, 2014).

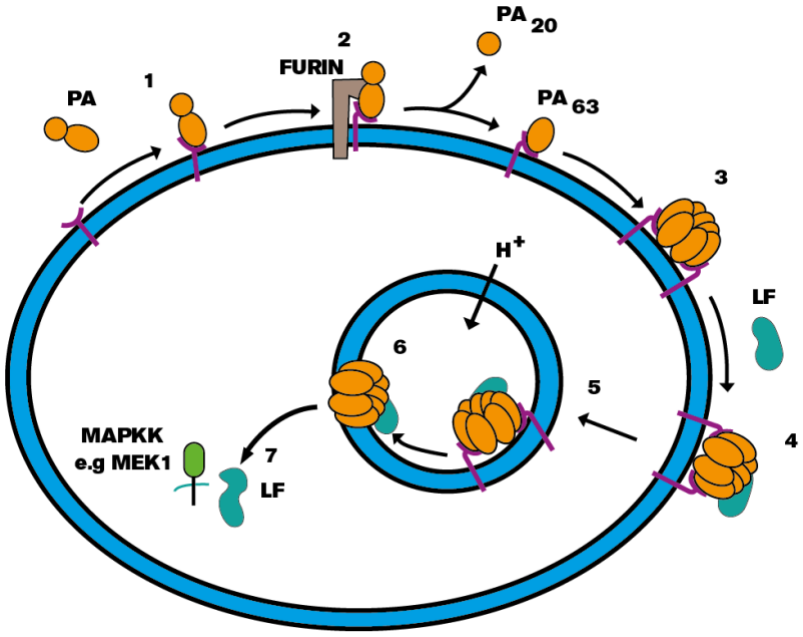


Figure 3: Steps of anthrax in toxification: Steps in anthrax intoxication. 1: PA83 binding to receptor, 2: P83 cleavage by furin proteases releasing P20, 3: PA63 oligomerization; 4, LF binding to octamer, 5: receptor-mediated endocytosis, 6: endosome acidification, 7: translocation of LT into the cytosol Adapted and modified fromWong et al. (2002).

1.3.2. LT and Cancer

In a study done by Kassab et al (2013) the effect of LT was tested on eleven human acute myeloid leukemia (AML) cell lines. Seven out of the eleven cell lines exhibited sensitivity against LT and showed a decrease in cell survival whereas four cell lines did not show any sensitivity to LT because they were PI3K pathway dependent. Also, four out of the seven cell lines that showed sensitivity against LT and one out of the four cell lines that were not sensitive to LT exhibited a decrease in the percentage of cells in G2/M and an increase in the percentage of cells in G0/G1 phase, showing that LT induces cell cycle arrest.

In a study done by Koo et al. (2002), they observed that upon MAPK inhibition with LT, this evoked apoptotic cell death in human melanoma cell lines. Additionally, inhibition of MAPK signaling in human melanoma xenograft models in athymic nude mice using LT resulted in apoptosis, and either complete or significant tumor regression without any obvious cytotoxic side effects on the host animal (Koo et al., 2002).

The effect of LT on glioblastoma motility and invasion was elucidated in a paper done by Al-Dimassi et al. (2016). LT was successful in decreasing cell motility as well as invasion across a collagen matrix. LT treated cells also showed an increase in adhesion and an increase in Rho A activity (Al-Dimassi et al., 2016).

1.4. MAP Kinase Pathway

1.4.1. Mechanism of Cascade Reaction

Ligands such as growth factors, extracellular matrix proteins, and adhesion molecules bind to the transmembrane glycoproteins of a cell surface receptor. Receptors are usually receptor tyrosine kinases (RTK); growth factor receptors; or G-protein-coupled receptors (Molina & Adjei, 2006).

Ligand binding induces RTK dimerization/oligomerization, resulting in receptor autophosphorylation and activation of kinase activity. Autophosphorylation of the RTK leads to the formation of specific binding/docking sites for Src homology domain 2 (Sh2)-containing proteins called adaptor proteins such as SHC and GRB2 (Du & Lovly, 2018).

SHC binds to the RTK at specific phosphotyrosine residues through its Sh2 domain. RTK will then, phosphorylate SHC to create phosphotyrosine residues for the

binding of another adaptor protein; GRB2; Grb2 binds to SHC through its Sh2 domain. Alternatively, Grb2 can also directly bind to the RTK at different phosphotyrosine residues through its SH2 domain. Additionally, Grb2 contains two Src Homology 3 (Sh3) domains, which are domains that recognize proline-rich sequences on proteins. Son of Sevenless (SOS); a guanine nucleotide exchange factor (GEF); recognizes and binds to the SH3 sequences on GRB2 (Liebmann & Bohmer, 2000).

GEFs are nucleotides exchange factors that catalyze the dissociation of GDP from Rho GTPases and its exchange to GTP, leading to the activation of the G-protein activity of the Rho GTPase. Rho GTPases are membrane-associated molecular switches that cycle between their active GTP-bound form and their inactive GDP-bound form. In their active form, Rho GTPases can interact with their downstream effectors, as a result, transmitting signals, causing a change in gene expression. Rho GTPases mediate a multitude of intracellular activities ranging from cytoskeleton rearrangement, cell survival invasion, and metastasis (Rossman, Der, & Sondek, 2005). SOS is a GEF for a specific Rho GTPase, Ras. SOS interacts with inactive GDP-bound Ras catalyzing the exchange of GDP to GTP, activating Ras (Liebmann & Bohmer, 2000). Active Ras can stimulate several downstream signaling pathways such as MAPK pathways and phosphatidylinositol-3 kinase (PI3K) pathways (Shapiro, 2002).

Activation of Ras can also occur through the activation of G-protein coupled receptors (GPCRs) which are seven-transmembrane receptors. GPCRs can activate Ras indirectly through the recruitment and transactivation of RTKs which will then activate Ras through the pathway stated above (Blaukat, Barac, Cross, Offermanns, & Dikic, 2000). Alternatively, GPCRs can activate Ras directly through the activation of

focal adhesion kinases (FAKs) resulting in their autophosphorylation consequently creating phosphotyrosine docking sites for Src and Grb2 which in turn lead to Ras activation (Shapiro, 2002).

The MAPK family includes three kinase types: the extracellular regulated kinases (ERKs), the stress-activated protein kinases p38 (SAPKs), and the c-Jun NH2-terminal kinase (JNKs) (Li et al., 2016). A specific set of extracellular cues activates one of the three kinase types, which, in turn, phosphorylate cytosolic substrates leading to their translocation into the nucleus where they act as transcription factors causing a change in gene expression (Bermudez, Pages, & Gimond, 2010).

Each MAPK cascade is activated by a different set of extracellular ligands. MAP3Kinase (M3K) associates with Ras by binding to it through its Ras binding domain. Binding to Ras activates the serine/threonine kinase activity of M3K, which triggers the sequential phosphorylation and activation of M3K's downstream effector MAP2Kinase (M2K). M2K phosphorylates its downstream effector MAP Kinase (MK) and activates it. MK phosphorylates several downstream effectors such as transcription factors, causing a change in gene expression (Shapiro, 2002).

The Raf/MEK/ERK pathway is a signal transduction pathway that relays signals from cell surface receptors to transcription factors, therefore, regulating gene expression. The ligands activating the RTKs are growth factors and cytokines. After binding to the receptor, and Ras activation, Ras recruits and phosphorylates the first kinase in this pathway which is Raf (MAP3K). Successively, Raf will phosphorylate a second kinase, MEK (MAP2K), which can then phosphorylate two proteins, ERK1 and ERK2. ERK1 and ERK2 are then translocated into the nucleus, where they can

phosphorylate different transcription factors and kinases, causing changes in gene expression. ERK's translocation into the nucleus affects many different cell processes such as cell proliferation, cell cycle progression, cell adhesion, cell invasion, cell survival, metabolism, differentiation, and transcription. The most notable targets of ERK1/2 are c-Myc, c-Fos, and Elk1, and c-Jun (Li et al., 2016).

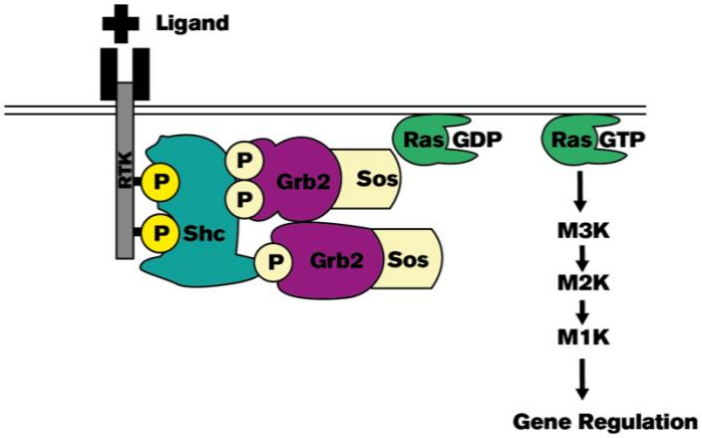


Figure 3. Diagrammatic representation of growth factor induced MAPK pathway. Ligand binds to RTK leading to its phosphorylation, resulting in the formation of protein-protein complex between Shc, GRB2, and SOS. Subsequently to this, GDP Ras is converted to GTP-Ras which activates M3K. M3K activates M2K which then activates M1K leading to gene regulation/expression. Adapted and modified from Yuryev & Wennogle (1998)

1.4.2. MEKs in Tumorigenesis

Mutations in the Ras/Raf/MEK/ERK pathway are closely linked to various mechanisms of tumorigenesis. Mutations in Ras and Raf are mutually exclusive, leading to ERK hyperactivation, causing dysregulation of ERK-signaling-related substrate activation.

1.4.2.1. Cell Survival

Activation of the Ras-ERK pathway promotes cell survival by inducing the expression of anti-apoptotic proteins such as BCL-2 BCL-Xl, and MCL-1(Neuzillet et

al., 2014). Anti-apoptotic proteins bind to and sequester pro-apoptotic proteins, preventing them from inducing apoptosis through mitochondrial outer membrane permeabilization. ERK also activates a family of ribosomal serine/threonine kinases (RSK) which upon activation will translocate to the nucleus catalyzing the phosphorylation and activation of pro-apoptotic proteins. Furthermore, the Ras-ERK pathway promotes cell survival by inhibiting the expression of pro-apoptotic proteins that belong to the BCL-2 family. ERK targets the pro-apoptotic proteins; Bim; for polyubiquitination leading to its degradation. Moreover, ERK phosphorylates the pro-apoptotic protein Bad leading to its sequestration by phosphoserine binding proteins (Mebratu & Tesfaigzi, 2009).

1.4.2.2. Cell Cycle Regulation

The Ras-ERK pathway promotes cell cycle progression by inducing the expression of cyclins and repressing cell senescence proteins. When ERK is translocated into the nucleus, it phosphorylates the transcription factor ELK1. ELK1 induces the expression of a proto-oncogene, c-Fos. ERK then phosphorylates and stabilizes c-Fos, allowing it to function as a transcription factor for cyclin D (Rodríguez et al., 2010). Also, ERK activation can lead to the inhibition of p27; a cyclin-dependent kinase inhibitor; causing cell cycle progression into S phase (Shapiro, 2002).

In addition, ERK activation contributes to telomere stability causing senescence evasion. In a study done by Picco and colleagues (2016), they showed that telomere stability and protection are under the direct control of ERK via the phosphorylation of telomeric repeat binding factor 2 (TRF2). Also, ERK can target a transcription factor, Ets, which can stimulate the expression of a tightly regulated gene

called the “telomerase catalytic subunit gene” (hTERT) (Goueli & Janknecht, 2004). hTERT expression restores telomere repeats leading to aberrant cell proliferation.

1.4.2.3. Epithelial to Mesenchymal Transition (EMT)

EMT is a cellular process that enables cell migration. EMT occurs mainly during embryonic development and wound healing but is also a key process in cancer cell metastasis. During EMT, epithelial cells lose their cell polarity and cell-cell adhesions/junctions, by downregulating E-cadherins and upregulating N-cadherins. Cells also upregulate the expression of matrix metalloproteases in order to degrade the extracellular matrix. To enable EMT the cell changes is signaling cascades by reprogramming its gene expression and inducing the expression of migratory and invasive genes (Lamouille, Xu, & Derynck, 2014). The mechanism of action has not been elucidated, but recent studies have shown that an increase in MEK/ERK signaling led to a downregulation of E-cadherin, and an upregulation of N-cadherin and matrix metalloproteases which renders the cell more motile (Xu, Lamouille, & Derynck, 2009).

1.4.2.4. Rho A

Rho A is a member of the Rho GTPase family. Rho A is a key regulator of intracellular activities such as actomyosin dynamics, focal adhesion dynamics, and stress fiber formation leading to cell motility (Zhou & Zheng, 2013). ERK activation induces the transcription of the Fos-related antigen 1 (Fra-1) gene. Pull-down experiments have shown that Fra-1 indirectly inhibits Rho A activity through the inactivation of b-1 integrin which in return inhibits Rho A activity (Vial, Sahai, & Marshall, 2003; Zhou & Zheng, 2013).

1.4.2.5. Rac 1

Rac1 is a Rho GTPase that belongs to the Rac family of GTPases. Rac1 activation regulates actin polymerization in lamellipodia. After the ERK-dependent depression of Rho A activity, a second ERK-dependent cascade occurs via the receptor urokinase-type plasminogen activator which then activates Rac1 (Vial, Sahai, & Marshall, 2003)

1.5. Purpose of the study

In this study our main purpose was to investigate the effect of LT; a MEK inhibitor; on breast cancer cell motility and invasion. Previous studies have shown an effect of LT on glioblastoma motility and invasion.

First, twe used suitable drug concentrations to examine the effect of LT on cell viability. Then, the effect of LT on 2D motility, adhesion, and invasion in breast cancer was elucidated. Furthermore, the effect of LT on Rho A and Rac1 activation was identified through pull-down assays.

Chapter two

Materials and Methods

2.1. Cell culture

Human epithelial triple negative breast cancer cell line MDA-MB-231 was cultured adherently in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin at 37˚C and 5% CO2 in a humidified chamber.

2.2. Drug Concentrations

Previous studies showed that maximum LT concentrations had no effect on MDA-MB-231 proliferation. For that, a 10^{-9} M LT and 10^{-8} M PA concentrations were used for cell treatment.

2.3. Motility Assay/Analysing 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 at 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.

2.4. Wound Healing Assay

Cells were grown to confluence on culture plates while incubated with LT and 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 and 24 h after wounding for 72 hours. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated (in μm/h).

2.5. Adhesion Assay

Plates (96-well) were coated with collagen using collagen solution, type I from rat tail (Sigma) overnight at 37˚C then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37˚C in a CO2 incubator for 1 h. Then washing the plates and chilling them on ice followed. The cells were trypsinized and counted to 5x105 cell/ml. A total of 50 ul of cells were added in each well and incubated at 37˚C in a CO2 incubator for 30 min. The plates were then shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 min. 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 min. The absorption of the plates was read at 550 mm using a plate reader.

2.6. Boyden Chamber/Invasion Assay

Cells were treated with toxin or left untreated as control, and the invasion assay was performed following the treatment period using the collagen-based invasion assay

(Millipore) according to the manufacturer's instructions. Briefly, 24 h prior to the assay, cells were starved with serum-free medium. Cells were harvested, centrifuged and then resuspended in quenching medium (without serum). Cells were then brought to a concentration of 1x106 cells/ ml. In the meantime, inserts were prewarmed with 300 µl of serum-free medium for 30 min at room temperature. After rehydration, 250 µl of medium was removed from the inserts, and 250 µl of cell suspension was added. Inserts were then placed in a 24-well plate, and 500 µl of complete medium (with 10% serum) was added to the lower wells. Plates were incubated for 48 h at 37˚C in a CO2 incubator. Following the incubation period, inserts were stained for 20 min at room temperature with 400 µl of cell stain provided with the kit. The stain was then extracted with extraction buffer (also provided). The extracted stain (100 µl) was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical density was then measured at 560 nm.

2.7. Pull Down Assay

Cells were lysed and incubated with GST-RBD or GST-PAK1 and the pull-down assay was performed using the RhoA/Rac1/Cdc42 Activation Assay Combo kit (Cell Biolabs Inc., San Diego, CA, USA) following the manufacturer's instructions. Lysates were incubated with GST-RBD (for RhoA) or GST-PAK1 for Rac for 1hour at 4˚C. GTP-RhoA and GTP-Rac were detected by western blotting using the anti-RhoA and the anti-Rac antibodies provided in the kit. Total proteins were collected prior to the incubation with GST beads and used as a loading control.

Chapter Three

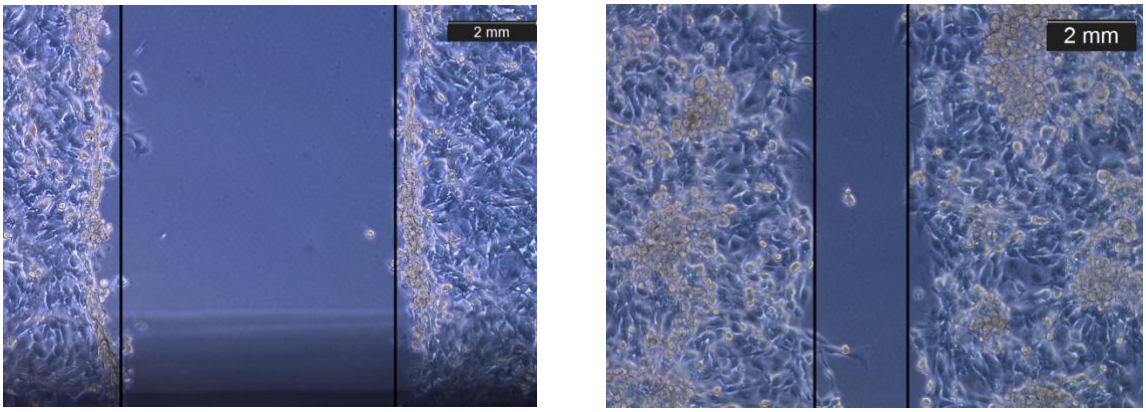
Results

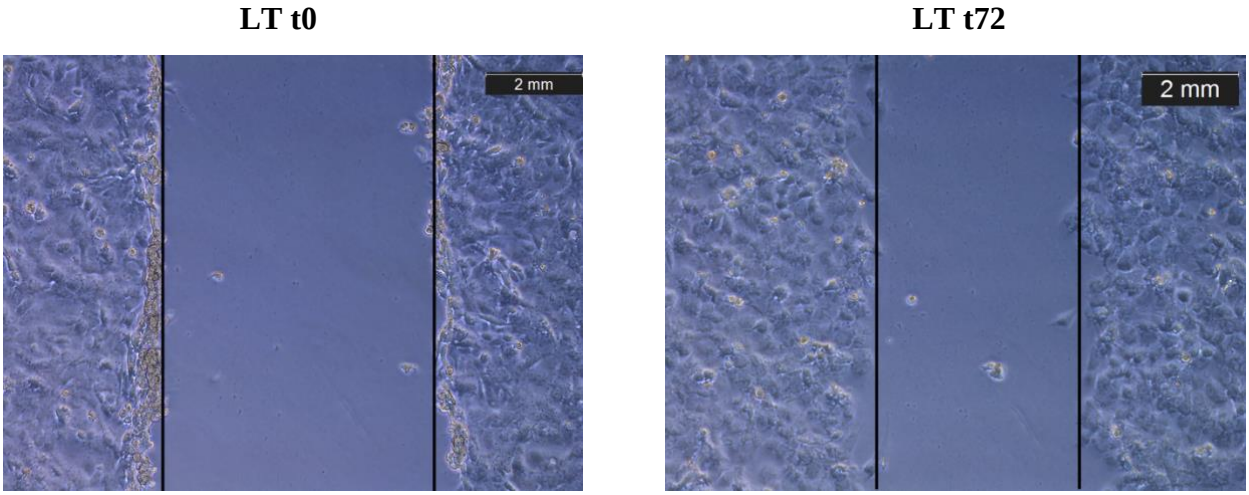
3.1. LT decreases breast cancer cell motility

3.1.1. LT decreases MDA-MB-231 cell migration (wound healing).

In order to study the effect of LT on MDA-MB-231 cells migration, we performed a 2D wound closure assay. The rate of wound closure was calculated over the span of 72 hours. Treated cell exhibited a remarkable 75% decrease in speed when compared to the control, where the wound closure rate decreased from 3.8 to 0.9 um/h.

(A) **Control t0** **Control t72**





(B)

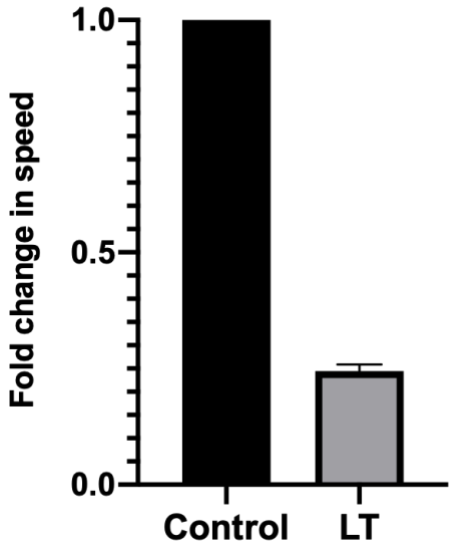


Figure 5. (A) Fixed wound healing assay. MDA-MB-231 cell lines were treated for 72 hours with or without LT (B) Quantitation for (A), wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the cells was calculated in μm/h. Data is the fold change ± SEM from three wounds closure assays from three independent experiments.

3.1.2. LT decreases MDA-MB-231 motility (time lapse assay)

2D time lapse microscopy was performed and the cumulative length/net path of migration of individual cells was quantitated. Treated cells displayed a 30% decrease in the total net path compared to the control cells.

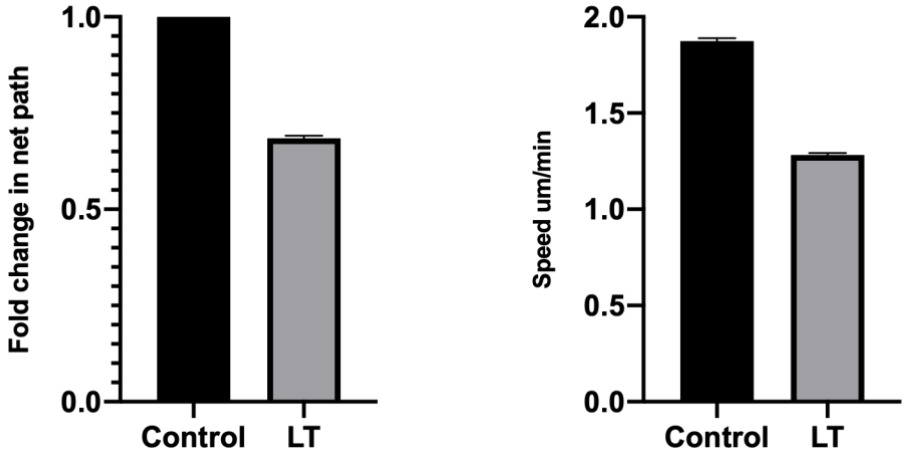


Figure 6: LT decreases MDA-MB-231 cell motility. The fold change (left panel) and the cell speed (right panel) of projected 120 frames from 2 h long time lapse movies were quantitated and expressed in μm and μm/min, respectively. Data are the mean ± SEM from 3 trials, 10 movies per trial.

3.2. LT increases breast cancer cell adhesion to collagen

Since treated cells exhibited a reduction in total net path and speed, we suspected that this reduction in migration is due to an increase in MDA-MB-231 adhesion to the underlying matrix. Cells treated with LT displayed a 43% increase in adhesion.

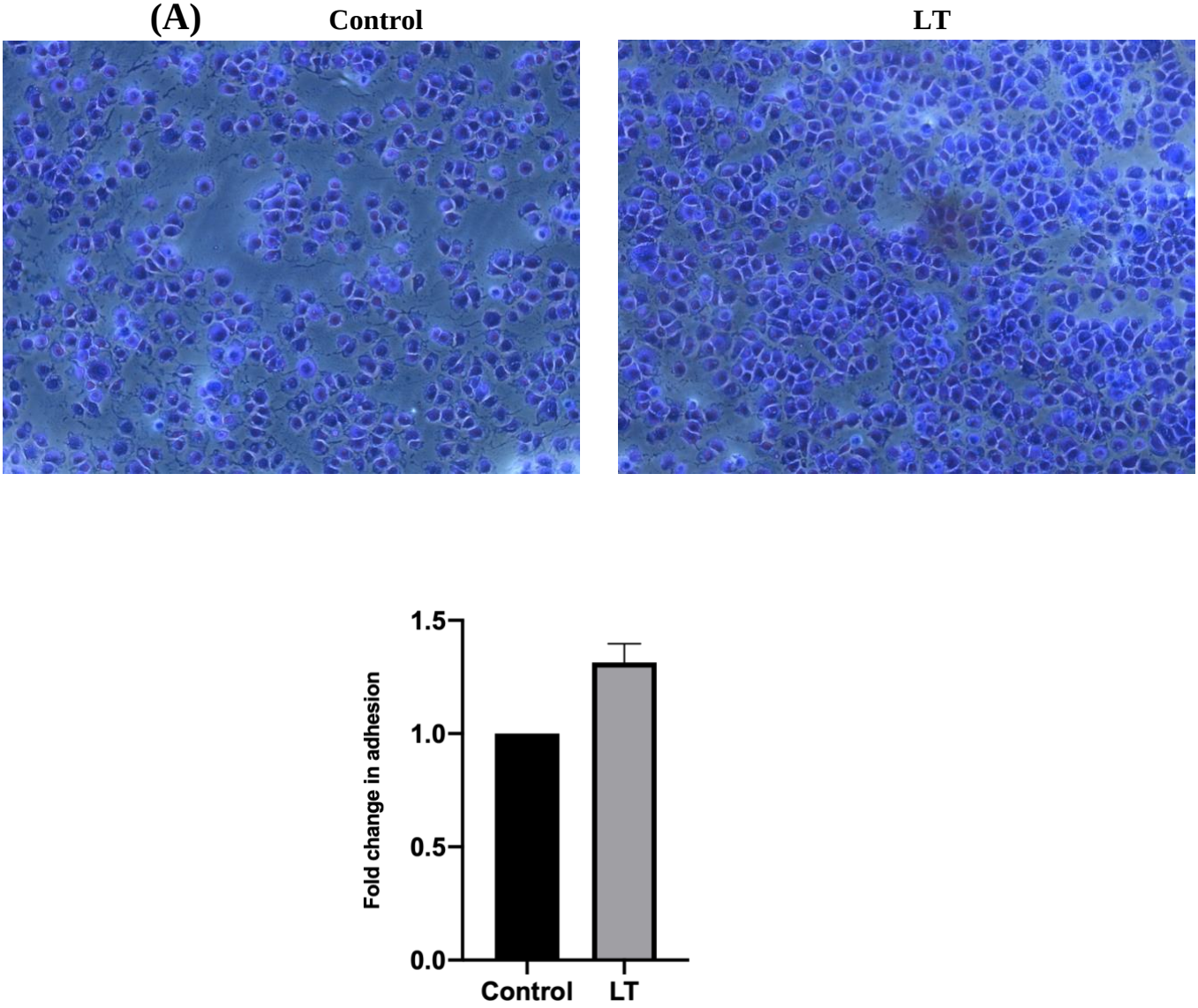


Figure 7: LT increases MDA-MB-231 adhesion to a collagen matrix. (A) Representative micrographs of cells fixed and stained with crystal violet to assess adhesion. (B) The graph is a quantitation whereby crystal violet was solubilized and the absorption of the plates was read at 550 nm using an ELISA plate reader. Data are reported in arbitrary units.

3.3. LT decreases breast cancer cell invasion

Having suspected an inability for the cells to invade due to increased cell adhesion we were interested to look at the effect of LT on cell invasion. We carried out an *invitro* collagen-based invasion assay with FBS as a chemoattractant. The trans-well chambers were filled with serum free media and used as negative controls. The control and treated cells were stained with cell stain, the cell stain was then solubilized using extraction buffer and the absorption of the cell suspension was read on an ELISA at 550nm. The results show a decrease in cellular invasion by 36% when the cells are treated with LT.

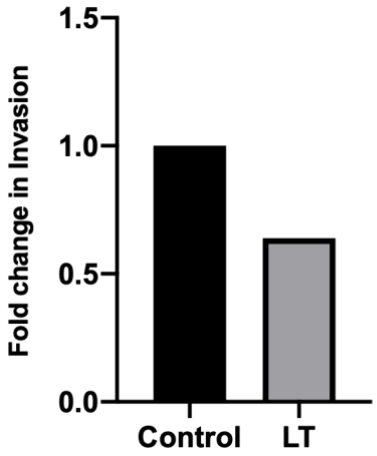


Figure 8. LT decreases MDA-MB-231 invasion across a collagen matrix: Quantitation of the cell suspension absorption at 550 nm

3.4. LT increases Rho A activation

Since our treated cells showed an increase in cell adhesion we wanted to see if this is due to an increase in Rho A activation, its well established in the literature that Rho A plays a major role in cell adhesion and the formation of focal adhesions. Treated cells exhibited a 2.7-fold increase in Rho A activation as compared to the control.

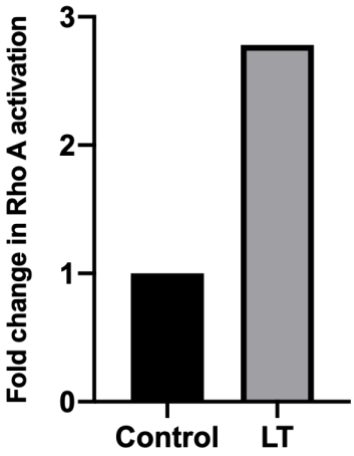


Figure 9: Effect of LT on RhoA activation: MDA-MB-231 cells were treated with LT. The cells were then lysed and incubated with GST-RBD (Rhoteckin binding domain) to pull down active Rho. The samples were then blotted with Rho antibodies.

3.5. LT increases Rac-1 activation

Rac-1 is a major contributor of cell motility and has been shown to drive lamellipodia formation at the leading edge. For that we did a pull-down assay to look Rac-1 activation levels upon LT treatment. Treated cells exhibited a 1.4-fold increase in Rac-1 activation when compared to the control.

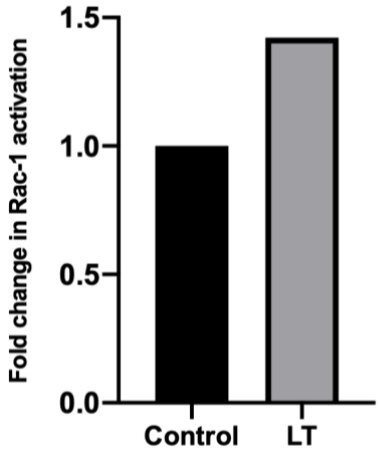


Figure 10: Effect of LT on Rac-1 activation: MDA-MB-231 cells were treated with LT. The cells were then lysed and incubated with GST-PBD (PAK1 binding domain) to pull down active Rac-1. The samples were then blotted with Rac1 antibodies.

Chapter Four
Discussion

Breast cancer metastasis to secondary regions is the main reason behind poor patient prognosis (Jin & Mu, 2015). The MAPK pathway has been implicated in many signaling pathways that play a major role in cell processes such as cell cycle regulation, apoptosis, survival, and EMT (Zhanf & Liu, 2002). Additionally, different studies have shown a relationship (negative or positive) between the MAPK pathway and the different Rho GTPases. Rho GTPases affect different cellular processes such as migration, actomyosin contractility, and lamellipodia formation (Pullikuth & Catling, 2007).

Previous studies have established that inactivation of the MAPK pathway could lead to the inhibition of cancer cell survival as well as the inhibition of migration and invasion (Al-Dimassi et al 2012). Al-Dimassi and colleagues have shown that upon MAPK pathway inhibition using LT led to a significant decrease in astrocytoma migration and invasion (Al-Dimassi et al 2012).

Based on the ability of LT to cause cell death or inhibition of migration and invasion due to MAPK inhibition, breast cancer cells were treated with LT, and the migratory and invasive capabilities of the cells were examined. Previous studies in our lab showed that LT had no cytotoxic effect on MDA-MB-231, for that reason maximum drug concentrations were used. Treating breast cancer cells with LT led to a decrease in 2D cell motility as shown in the quantification of the wound healing assay. Cells treated with LT showed a decrease in wound closure speed as compared to the control. To eliminate the possibility that LT effects wound closure by effecting cell replication and

proliferation instead of migration, we did a time-lapse assay to look at cell speed upon LT treatment. In the time-lapse assay; a random 2D migration assay; we looked at the cumulative lengths taken by individual cells over the span of 120 minutes, and then calculated the speed for each individual cell. Cells treated with LT showed a significant decrease in cell speed, therefore supporting the results obtained in the wound healing assay.

In the time-lapse assay, the treated cells showed a different morphology than the untreated cells. The morphology displayed was previously shown to be associated with an increase in cellular focal adhesions. We suspected a relationship between LT and an increase in cellular adhesion. Treating the cells with LT led to an increase in adhesion as shown in the quantification of the adhesion assay. Previous studies have showed a

Due to the increased adhesion we observed in the treated cells, we suspected that this would also be accompanied by a decrease in invasion since highly adherent cells have a decreased capability of invading the underlying ECM. Additionally, MDA-MB-231 is known to be an invasive cell line and we wanted to test if LT had a negative effect on cellular invasion. We used media with 5% FBS in the lower chamber (since FBS contains growth factors) of the inserts as a chemoattractant and starved the cells in media with 0% FBS to induce cellular invasion. Cells treated with LT showed a significant decrease in invasion through the collagen matrix (imitates the ECM). Previous studies have shown a positive relationship between the MAPK pathway and EMT/invasion, our findings come in parallel with previous studies since MAPK inhibition led to a decrease in invasion. In previous studies, an increase in adhesion was observed in astrocytoma cells when Rho A was overexpressed. We wanted to see if LT was inhibiting breast cancer migration by deregulating the activity of Rho A.

Although Rho A promotes the formation of focal adhesions and induces actomyosin contractility, high levels of Rho A activity inhibit migration by impairing actomyosin contractility (Ridley, 2015).

During cell migration, the levels of Rho A activity need to cycle between high and low. Initially, Rho A needs to be active to form focal adhesions at the leading edge which enable the contraction of the trailing edge, following that Rho A activity needs to decrease for the focal adhesions to dissolve at the trailing edge for the cell to contract forward (Khalil et al., 2014). High levels of Rho A prevent the trailing edge from contracting, therefore, inhibiting cell movement. A pulldown assay was done using Rhotekin RBD agarose beads to observe the levels of Rho A activity by pulling only the active/GTP-bound form of Rho A. The results showed an increase in Rho A activity in LT treated cells, suggesting that LT is preventing cell migration by effecting Rho A activation. Rac-1 activity was also assed using PAK-1 PBD agarose beads that pull down only the active/GTP bound Rac-1. The results showed an increase in Rac-1 activity in LT treated cells.

Chapter Five

Conclusion

In this study we wanted to look at the of LT on breast cancer cell migration, adhesion and invasion. Our data has showed that LT is a potent MAPK inhibitor as it had a significant effect on breast cancer migration, adhesion, and invasion. MDA-MB-231 is a highly aggressive and invasive type of breast cancer, LT was capable of inhibiting its motility and invasion as well as having a positive effect on Rho A activity and therefore increasing cellular adhesion. Further studies should work on deciphering the signaling cascade downstream of MAPK, which led to Rho A activation upon MAPK inhibition.

Bibliography

Agrawal, A., & Pulendran, B. (2004). Anthrax lethal toxin: A weapon of multisystem destruction. *Cellular and Molecular Life Sciences : CMLS, 61*(22), 2859-2865. https://doi.org/10.1007/s00018-004-4251-4

Akram, M., Iqbal, M., Daniyal, M., & Khan, A. U. (2017). Awareness and current knowledge of breast cancer. *Biological Research, 50(1), 33* https://doi org/10 1186/s40659-017-0140-9

Al-Dimassi, S., Salloum, G., Saykali, B., Khoury, O., Liu, S., Leppla, S. H., . . . El-Sibai, M. (2016). Targeting the MAP kinase pathway in astrocytoma cells using a recombinant anthrax lethal toxin as a way to inhibit cell motility and invasion. *International Journal of Oncology, 48*(5), 1913-1920. https://doi.org/10.3892/ijo.2016.3431

Ananthakrishnan, R., & Ehrlicher, A. (2007). The forces behind cell movement. *International Journal of Biological Sciences, 3*(5), 303-317. https://doi org/10 7150/ijbs 3 303

Asif, M., Sultana, S., Ahmed, S., Akhter, N., & Tariq, M. (2016). HER-2 positive breast cancer - a mini-review. Asian Pacific Journal of Cancer Prevention, 17, 1609-1615. https://doi.org/10.7314/APJCP.2016.17.4.1609

Bachran, C., & Leppla, S. H. (2016). Tumor targeting and drug delivery by anthrax toxin. *Toxins, 8*(7), 197. https://doi org/10 3390/toxins8070197

Badowska-Kozakiewicz, A., & Budzik, M. P. (2016). Immunohistochemical characteristics of basal-like breast cancer. *Contemporary Oncology (Poznan, Poland), 20*(6), 436-443. https://doi.org/10.5114/wo.2016.56938

Bermudez, O., Pages, G., & Gimond, C. (2010). The dual-specificity MAP kinase phosphatases: Critical roles in development and cancer. *American Journal of Physiology.Cell Physiology, 299*(2), 189. https://doi org/10 1152/ajpcell 00347 2009

Blaukat, A., Barac, A., Cross, M. J., Offermanns, S., & Dikic, I. (2000). G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of gαq and gαi signals. *Molecular and Cellular Biology, 20*(18), 6837. https://doi.org/10.1128/MCB.20.18.6837-6848.2000

Chen, Y., Shi, Y., Lin, J., Ye, Y., Wang, X., Chen, G., & Guo, Z. (2015). Combined analysis of EGFR and PTEN status in patients with KRAS wild-type metastatic colorectal cancer. *Medicine, 94*(40), e1698. https://doi org/10 1097/MD 0000000000001698

Cooper, G. (2000). *The Cell: A Molecular Approach* (2nd ed.). Sunderland (MA) Sinauer Associates. https://www ncbi nlm nih gov/books/NBK9839/

Daniel, A. R., Hagan, C. R., & Lange, C. A. (2011). Progesterone receptor action: Defining a role in breast cancer. *Expert Review of Endocrinology & Metabolism, 6*(3), 359-369. https://doi org/10 1586/eem 11 25

DeSantis, C. E., Fedewa, S. A., Goding Sauer, A., Kramer, J. L., Smith, R. A., & Jemal, A. (2016). Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. *CA: A Cancer Journal for Clinicians, 66(1), 31-42.* doi:10.3322/caac.21320 Du, Z., & Lovly, C. M. (2018). Mechanisms of receptor tyrosine kinase activation in cancer. *Molecular Cancer, 17*(1), 58. https://doi.org/10.1186/s12943-018-0782-4

Du, Z., & Lovly, C. M. (2018). Mechanisms of receptor tyrosine kinase activation in cancer. *Molecular Cancer, 17*(1), 58. https://doi org/10 1186/s12943-018-0782-4

Feng, Y., Spezia, M., Huang, S., Yuan, C., Zeng, Z., Zhang, L., . . . Ren, G. (2018). *Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis* https://doi.org/10.1016/j.gendis.2018.05.001

Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *American journal of cancer research, 7(5), 1016-1036. Retrieved from* https://pubmed.ncbi.nlm.nih.gov/28560055
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5446472/

Foulkes, W. D., Smith, I. E., & Reis-Filho, J. S. (2010). Triple-negative breast cancer. *The New England Journal of Medicine, 363*(20), 1938-1948. https://doi org/10 1056/NEJMra1001389

Fragomeni, S. M., Sciallis, A., & Jeruss, J. S. (2018). Molecular subtypes and local-regional control of breast cancer. Surgical Oncology Clinics of North America, 27(1), 95-120. https://doi.org/10.1016/j.soc.2017.08.005

Fu, Z., Song, P., Li, D., Yi, C., Chen, H., Ruan, S., . . . Zheng, S. (2014). Cancer-associated fibroblasts from invasive breast cancer have an attenuated capacity to secrete collagens. *International Journal of Oncology, 45*(4), 1479-1488. https://doi.org/10.3892/ijo.2014.2562

Gatti, V., Bongiorno-Borbone, L., Fierro, C., Annicchiarico-Petruzzelli, M., Melino, G., & Peschiaroli, A. (2019). *p63 at the crossroads between stemness and metastasis in breast cancer* https://doi org/10 3390/ijms20112683

Goueli, B. S., & Janknecht, R. (2004). Upregulation of the catalytic telomerase subunit by the transcription factor ER81 and oncogenic HER2/neu, ras, or raf. *Molecular and Cellular Biology, 24*(1), 25-35. https://doi.org/10.1128/mcb.24.1.25-35.2004

Graham, J. D., Mote, P. A., Salagame, U., van Dijk, J.,H., Balleine, R. L., Huschtscha, L. I., . . . Clarke, C. L. (2009). DNA replication licensing and progenitor numbers are increased by progesterone in normal human breast. *Endocrinology, 150*(7), 3318-3326. https://doi org/10 1210/en 2008-163

Greaves, M., & Maley, C. C. (2012). Clonal evolution in cancer. *Nature, 481*(7381), 306-313. https://doi.org/10.1038/nature10762

Gusterson, B. A., Ross, D. T., Heath, V. J., & Stein, T. (2005). Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. *Breast Cancer Research : BCR, 7*(4), 143-148. https://doi org/10 1186/bcr1041

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. https://doi.org/10.1016/j.cell.2011.02.013

Hoeferlin, L. A., E Chalfant, C., & Park, M. A. (2013). Challenges in the treatment of triple negative and HER2-overexpressing breast cancer. *Journal of Surgery and Science, 1*(1), 3-7. Retrieved from https://pubmed.ncbi.nlm.nih.gov/24818173 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4012677/

Idikio, H. A. (2011). Human cancer classification: a systems biology- based model integrating morphology, cancer stem cells, proteomics, and genomics. *Journal of Cancer, 2*, 107-115. https://doi org/10 7150/jca 2 107

Jeon, J. H., Lee, H., Cho, M., Park, O., Park, J., & Rhie, G. (2015). The poly-γ-d-glutamic acid capsule surrogate of the bacillus anthracis capsule is a novel toll-like receptor 2 agonist. *Infection and Immunity, 83*(10), 3847. https://doi.org/10.1128/IAI.00888-15

Jin, X., & Mu, P. (2015). Targeting breast cancer metastasis. *Breast Cancer : Basic and Clinical Research, 9, 23-34.* https://doi org/10 4137/BCBCR S25460

Kassab, E., Darwish, M., Timsah, Z., Liu, S., Leppla, S. H., Frankel, A. E., & Abi-Habib, R. (2013). Cytotoxicity of anthrax lethal toxin to human acute myeloid leukemia cells is nonapoptotic and dependent on extracellular signal-regulated kinase 1/2 activity. *Translational Oncology, 6*(1), 25-32. doi: https://doi.org/10.1593/tlo.12313

Khalil, B. D., Hanna, S., Saykali, B. A., El-Sitt, S., Nasrallah, A., Marston, D., . . . El-Sibai, M. (2014). The regulation of RhoA at focal adhesions by StarD13 is important for astrocytoma cell motility. *Experimental Cell Research, 321*(2), 109-122. https://doi.org/10.1016/j.yexcr.2013.11.023

Koo, H., VanBrocklin, M., McWilliams, M. J., Leppla, S. H., Duesbery, N. S., & Woude, G. F. V. (2002). Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. *Proc Natl Acad Sci USA, 99*(5), 3052. https://doi org/10 1073/pnas 052707699

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nature Reviews.Molecular Cell Biology, 15*(3), 178-196. https://doi.org/10.1038/nrm3758

Li, L. T., Jiang, G., Chen, Q., & Zheng, J. N. (2015). Ki67 is a promising molecular target in the diagnosis of cancer (review). *Molecular Medicine Reports, 11*(3), 1566-1572. https://doi org/10 3892/mmr 2014 2914

Li, L., Zhao, G., Shi, Z., Qi, L., Zhou, L., & Fu, Z. (2016). The ras/raf/MEK/ERK signaling pathway and its role in the occurrence and development of HCC. *Oncology Letters, 12*(5), 3045-3050. https://doi.org/10.3892/ol.2016.5110

Liebmann, C., & Bohmer, F. D. (2000). Signal transduction pathways of G protein-coupled receptors and their cross-talk with receptor tyrosine kinases: Lessons from bradykinin signaling. *Current Medicinal Chemistry, 7*(9), 911-943. https://doi.org/10.2174/0929867003374589

Liu, S., Moayeri, M., & Leppla, S. H. (2014). Anthrax lethal and edema toxins in anthrax pathogenesis. *Trends in Microbiology, 22*(6), 317-325. https://doi org/10 1016/j tim 2014 02 012

Liu, T., Han, C., Wang, S., Fang, P., Ma, Z., Xu, L., & Yin, R. (2019). Cancer-associated fibroblasts: An emerging target of anti-cancer immunotherapy. *Journal of Hematology & Oncology, 12*(1), 86. https://doi.org/10.1186/s13045-019-0770-1

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). Proto-Oncogenes and Tumor-Suppressor Genes. *Molecular Cell Biology. 4th edition*. Retrieved from https://www ncbi nlm nih gov/books/NBK21662/

Lumachi, F., Brunello, A., Maruzzo, M., & Basso, U. Basso and S. M. M. (2013). *Treatment of estrogen receptor-positive breast cancer* doi: http://dx.doi.org/10.2174/092986713804999303

Mebratu, Y., & Tesfaigzi, Y. (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle (Georgetown, Tex.), 8*(8), 1168-1175. https://doi org/10 4161/cc 8 8 8147

Melino, G. (2011). P63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death & Differentiation, 18*(9), 1487-1499. https://doi.org/10.1038/cdd.2011.81

Molina, J. R., & Adjei, A. A. (2006). The ras/raf/MAPK pathway. *Journal of Thoracic Oncology, 1*(1), 7-9. https://doi org/10 1016/S1556-0864(15)31506-9

National Breast Cancer Foundation. (2014). *National Breast Cancer Foundation*. Retrieved from https://www.nationalbreastcancer.org/breast-tumors/

Nekulova, M., Holcakova, J., Gu, X., Hrabal, V., Galtsidis, S., Orzol, P., . . . Vojtesek, B. (2016). ΔNp63α expression induces loss of cell adhesion in triple-negative breast cancer cells. *BMC Cancer, 16*(1), 782 https://doi org/10 1186/s12885-016-2808-x

Neuzillet, C., Tijeras-Raballand, A., de Mestier, L., Cros, J., Faivre, S., & Raymond, E. (2014). MEK in cancer and cancer therapy. *Pharmacology & Therapeutics, 141*(2), 160-171. doi: https://doi.org/10.1016/j.pharmthera.2013.10.001

Paredes, J., Correia, A. L., Ribeiro, A. S., Albergaria, A., Milanezi, F., & Schmitt, F. C. (2007). P-cadherin expression in breast cancer: A review. *Breast Cancer Research : BCR, 9*(5), 214. https://doi org/10 1186/bcr1774

Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., . . . Botstein, D. (2000). Molecular portraits of human breast tumours. Nature, 406(6797), 747-752. https://doi.org/10.1038/35021093

Picco, V., Coste, I., Giraud-Panis, M., Renno, T., Gilson, E., & Pagès, G. (2016). ERK1/2/MAPK pathway-dependent regulation of the telomeric factor TRF2. *Oncotarget, 7*(29), 46615-46627. https://doi org/10 18632/oncotarget 10316

Pucci, B., Kasten, M., & Giordano, A. (2000). Cell cycle and apoptosis. *Neoplasia (New York, N.Y.), 2*(4), 291-299. https://doi.org/10.1038/sj.neo.7900101

Pullikuth, A. K., & Catling, A. D. (2007). Scaffold mediated regulation of MAPK signaling and cytoskeletal dynamics: A perspective. *Cellular Signalling, 19*(8), 1621-1632. https://doi org/10 1016/j cellsig 2007 04 012

Ridley, A. J. (2015). Rho GTPase signalling in cell migration. *Current Opinion in Cell Biology, 36,* 103-112. https://doi org/10 1016/j ceb 2015 08 005

Rodríguez, J., Calvo, F., González, J.,M., Casar, B., Andrés, V., & Crespo, P. (2010). ERK1/2 MAP kinases promote cell cycle entry by rapid, kinase-independent disruption of retinoblastoma-lamin A complexes. *The Journal of Cell Biology, 191*(5), 967-979. https://doi.org/10.1083/jcb.201004067

Rossman, K. L., Der, C. J., & Sondek, J. (2005). GEF means go: Turning on RHO GTPases with guanine nucleotide-exchange factors. *Nature Reviews Molecular Cell Biology, 6*(2), 167-180. https://doi org/10 1038/nrm1587

Seyfried, T. N., & Huysentruyt, L. C. (2013). On the origin of cancer metastasis. *Critical reviews in oncogenesis, 18*(1-2), 43-73. https://doi.org/10.1615/critrevoncog.v18.i1-2 40

Shapiro, P. (2002). Ras-MAP kinase signaling pathways and control of cell proliferation: Relevance to cancer therapy. *Critical Reviews in Clinical Laboratory Sciences, 39*(4-5), 285-330. https://doi.org/10.1080/10408360290795538

Sharma, G. N., Dave, R., Sanadya, J., Sharma, P., & Sharma, K. K. (2010). Various types and management of breast cancer: An overview. *Journal of Advanced Pharmaceutical Technology & Research, 1(2), 109-126*. Retrieved from https://pubmed ncbi nlm nih gov/22247839 https://www ncbi nlm nih gov/pmc/articles/PMC3255438/

Stratton, M. R., Campbell, P. J., & Futreal, P. A. (2009). The cancer genome. *Nature, 458*(7239), 719-724. https://doi.org/10.1038/nature07943

Sauter, G., Lee, J., Bartlett, J. M. S., Slamon, D. J., & Press, M. F. (2009). Guidelines for human epidermal growth factor receptor 2 testing: Biologic and methodologic considerations. Jco, 27(8), 1323-1333. https://doi org/10 1200/JCO 2007 14 8197

Teh, B. T., & Fearon, E. R. (2020). 14 - genetic and epigenetic alterations in cancer. In J. E. Niederhuber, J. O. Armitage, M. B. Kastan, J. H. Doroshow & J. E. Tepper

(Eds.), *Abeloff's clinical oncology (sixth edition)* (pp. 209-224.e2). Philadelphia: Content Repository Only!. doi: https://doi org/10 1016/B978-0-323-47674-4 00014-

Toft, D. J., & Cryns, V. L. (2011). Minireview: Basal-like breast cancer: From molecular profiles to targeted therapies. *Molecular Endocrinology (Baltimore, Md.), 25*(2), 199-211. https://doi.org/10.1210/me.2010-0164

Vial, E., Sahai, E., & Marshall, C. J. (2003). ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. *Cancer Cell, 4*(1), 67-79. https://doi org/S1535610803001624

Vieira, A. F., & Paredes, J. (2015). P-cadherin and the journey to cancer metastasis. *Molecular Cancer, 14*, 178. https://doi.org/10.1186/s12943-015-0448-4

Wong, T.Y., Schwarzenbacher, R., & Liddington, R. C. (2002). Towards understanding anthrax: Structural basis of target recognition by anthrax lethal factor. Retrieved from https://www-ssrl slac stanford edu/research/highlights_archive/anthrax pdf

Xu, J., Lamouille, S., & Derynck, R. (2009). TGF-beta-induced epithelial to mesenchymal transition. *Cell Research, 19*(2), 156-172. https://doi.org/10.1038/cr.2009.5

Yuryev, A., Wennogle, L. The RAF family: an expanding network of post-translational controls and protein-protein interactions. *Cell Res* **8,** 81–98 (1998). https://doi org/10 1038/cr 1998 9

ZHANG, W., & LIU, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research, 12*(1), 9-18. https://doi.org/10.1038/sj.cr.7290105

Zhou, X., & Zheng, Y. (2013). Cell type-specific signaling function of RhoA GTPase: Lessons from mouse gene targeting. *The Journal of Biological Chemistry, 288*(51), 36179-36188. https://doi org/10 1074/jbc R113 515486