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The role of HDAC6 in the cellular contractility of metastatic cells

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Abstract

Background: Cancer is the term used to describe a group of diseases, which are all characterised by mutation of normal cells and their uncontrollable growth in different parts of the body. Cancer also has the ability to metastasise, which makes it highly malignant and allows it to quickly spread through the body proving quickly fatal. Mechanobiology is a field of science that studies how differences in the mechanical properties of cells affect cellular properties and disease. Cellular contractility is the ability of cells to exert contraction and generate force by cell-matrix interaction. This cell-matrix interaction results in the exertion of cell traction forces, which is essential for cell migration, maintaining cell homeostasis and cell structure. Histone deacetylase 6 (HDAC6) is an enzyme in humans and HDAC6 gene expression correlates with increased carcinogenesis and metastasis of cancer cells.

Aim: To determine the effect of HDAC6 on contractile forces of metastasising human BJ fibroblasts and the molecular mechanisms that control these contractile forces.

Methods: To measure contractile forces of cells, isogenically matched normal, immortalized, transformed, and metastatic human BJ fibroblast cells were cultured on polyacrylamide gels and cells were imaged using Traction force microscopy (TFM). Contractile forces were also measured after HDAC6 inhibition and HDAC6 siRNA knockdown of metastasising cells. Confocal microscopy was also performed to image the cell-matrix adhesions and cell spreading area between the cellular components and extracellular matrix. Cell migration assays were used to evaluate the effect of HDAC6 inhibition or knockdown on the morphological properties of metastasising fibroblast cells. The intracellular force of the nucleus was also measured in metastasising and HDAC6 inhibited cells.

Results: Metastasising cells have increased cellular contractility than normal BJ fibroblasts, but this contractility is reduced significantly when HDAC6 is inhibited using tubacin in metastasising cells. However, this trend is significantly reversed in siRNA knockdown of HDAC6 since contractile forces increase when compared to untreated metastasising cells. Metastasising cells have decreased nuclear forces than normal BJ fibroblasts and this force decreases further when HDAC6 is inhibited. Metastasising cells have smaller cell-matrix adhesions and decreased cell spreading area compared to normal BJ fibroblasts.

Conclusion: Increased cellular contractility in metastasising cells makes them more invasive. This is dependent on HDAC6 activity via its deacetylase and protein-binding functions. Understanding the molecular mechanisms that control this contractility will allow for the development of drugs that can help reduce metastasis of cancer and mortality rates.

Acknowledgements

The last 3 years have been an exceedingly difficult time for me and everyone around the world due to the Covid-19 pandemic. I personally lost my grandfather (Muhammad Ali) who I was very close to along with other people I knew. There were times that I lost all hope and believed that I could not continue after suffering setbacks but despite all this I am here now at the end of my journey and there are several people who made this possible and I would like to thank them for their support throughout this journey.

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Lastly and most importantly I would like to thank Allah (SWT) for all the abundant blessings in my life and protecting me from any problems and difficulties during this challenging time.

Declaration

I hereby declare that this submission is my own work and to the best of my knowledge, it contains no materials previously published or written by another person, except where due acknowledgement is made in the thesis. The material in this thesis has not previously been submitted for a degree to any other University. I also declare that the intellectual content of this thesis is the product of my own work, except that full assistance from my supervisor in the project's design, conception, execution, presentation, and linguistic expression is acknowledged.

I confirm that I shall abide by the University of Sheffield’s regulations on plagiarism and that all written work shall be my own and will not have been plagiarised from other paper-based or electronic sources. Where used, material gathered from other sources will be clearly cited in the text.

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αTAT	α-tubulin acetyltransferases
ACTA2	α-smooth-muscle actin
AFM	Atomic Force Microscopy
ALL	Acute Lymphoid Leukaemia
AML	Acute Myeloid Leukaemia
BUZ	Ubiquitin-binding zinc-finger domain
CAMs	Cell Adhesion molecules
CD7	Cell Discoverer 7
CDK1/CDC2	Cyclin Dependent Kinase 1/2
CDC42	Cell division cycle 42
CLIP-170	Cytoplasmic Linker Protein 170
CLL	Chronic Lymphocytic Leukaemia
CLSM	Confocal Laser Scanning Microscopy
CRC	Colorectal Cancer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTCL	Cutaneous T-cell Lymphoma
CTFs	Cell Traction Forces
DMB	Dynein motor-binding domain
DMEM	Dulbecco's modified Eagle's Medium
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
ESCC	Esophageal Squamous Cell Carcinoma
FAs	Focal Adhesions
FAK	Focal Adhesion Kinase
FAP	Fibroblast Activation protein
FTTC	Fourier Transform Traction Cytometry
GPCR	G-protein coupled receptor

GRK2	G-protein Coupled Receptor Kinase 2
GTPase	Guanosine Triphosphatases
HDAC6	Histone deacetylase 6
HER2	Human Epidermal Growth Factor Receptor 2
Hsp90	Heat shock protein 90
IFs	Intermediate Filaments
IgSF	Immunoglobulin Superfamily
IGF-1	Insulin-like growth factor 1 receptor
ILK-1	Integrin-linked Kinase 1
LPA	Lysophosphatidic Acid
LSM 980	Light Scanning Microscope 980
MEF	Mouse Embryonic Fibroblasts
MLC	Myosin Light Chain
MLNs	Metastatic Lymph Nodes
MMPs	Matrix Metalloproteinases
mPAD	Microfabricated Post-array Detector
MSD	Mean Square Displacement
MTs	Microtubules
NES	Nuclear export signal
NHL	Non-Hodgkin lymphoma
NLS	Nuclear localization signal
OSCC	Oral Squamous Cell Carcinoma
PA	Polyacrylamide
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PI3k	Phosphoinositide 3-kinases
PIV	Particle Image Velocitometry
qRT-PCR	Real Time Quantitative Reverse Transcription PCR
Rac1	Ras-related C3 botulinum toxin substrate 1
RCC	Renal Cell Carcinoma

RhoA	Ras Homolog family member A
ROCK	Rho-associated protein kinase
SAHA	Suberoylanilide hydroxamic acid
SE14	Ser-Glu-containing tetrapeptide
shRNA	Small hairpin RNA
siRNA	Short interfering RNA/Silencing RNA
STED	Stimulated Emission Depletion
TFM	Traction force microscopy
TGF-β	Transforming growth factor beta
TME	Tumour microenvironment
TSA	Trichostatin A
UV	Ultraviolet
VEGFR-3	Vascular Endothelial Growth Factor Receptor 3
YAP	Yes, associated Protein

Chapter One – INTRODUCTION

Chapter 1- Introduction

1.0 Introduction

General background

The problem: Each cell in the human body has a specific role which is crucial for normal function. In normal physiological conditions cells multiply via cell division and any abnormal cell is usually destroyed by processes such as apoptosis. However, sometimes cell division can cause a normal cell to mutate leading to cancer. Over time some cancer cells can acquire the ability to travel from their original primary site (e.g. breast or prostate) to secondary sites (e.g. lung or bone) in a process known as metastasis. The physical forces that cancer cells use to metastasise, and their underlying mechanisms are currently not fully understood, or how they differ to normal cells with regards to mechanical forces for cell motility and invasiveness. An enzyme that has been studied to be involved in cancer cell metastasis and promoting cell motility is Histone Deacetylase 6 (HDAC6). The aim of the thesis was to understand the mechanisms of HDAC6 in controlling cell contractile forces using the isogenic human BJ fibroblast model to determine if targeting HDAC6 was a potential therapeutic option in cancer.

Current knowledge: Previous work on progression of metastasis and invasive ability of cancer cells has largely focussed on the effect of various therapeutics and signalling pathways, with limited knowledge on the mechanical properties (termed mechanobiology) of cancer cells. This has primarily been due to a lack of modern imaging techniques which are essential for recording and measuring cell movement. Mechanobiology is a new and emerging field of science and an attractive alternative to the traditional methods to study cancer cells. Examples of mechanical properties of cells include contractility, stiffness, viscosity, elasticity and cell adhesion. Since these mechanical properties play important roles in cell function it is important to understand the differences in movement between normal and metastatic cells when they are applied with the same amount of mechanical force.

Contractile forces, also known as cellular contractility, is a mechanical (or physical) property of cells which allows them to self-contract and exert force on their surrounding environment but only if they are adhered to a surface. Cellular contractility is measured in terms of cell displacement (usually in μm) between the cell's initial starting position and its final position after contraction. Cellular contractility is used by cells to divide, to migrate, to heal wounds, and to contract muscles. The interaction between the actin cytoskeleton and myosin-II results

in the generation of tension between cells, which then transmits to the extracellular matrix (ECM) via tension in focal adhesions (FAs) leading to the generation and exertion of cell traction forces and the migration of the cell on the substrate surface.

Cellular stiffness is the most widely researched mechanical property of cells. This has left a gap in knowledge with regards to the understanding of cell contractile forces. Due to this the focus of this thesis was to study cellular contractility instead of cell stiffness. To observe and measure contractile forces in different types of cells an imaging technique known as traction force microscopy (TFM) was used. However, the use of TFM to measure cellular contractility is not exactly correlated with measuring cellular potential for metastasis. There are several factors that need to be considered which can change the cellular contractility of cells. These include the force of stiffness that is exerted by the substrate, which the cells attach to and their surrounding microenvironment. The resultant change to cellular contractility can also affect their cell movement and overall structure. In addition, other factors can influence cell mobility, of particular interest in this thesis was HDAC6, an enzyme in humans, which has several functions in the regulation of transcription and the progress of the cell cycle. Histones are crucial in regulating transcription, the progress of the cell cycle and can also change the chromosome structure of DNA via acetylation or deacetylation.

Previous research has shown that HDAC6 overexpression correlates with increased carcinogenesis and metastasis of cancer cells. HDAC6 also promotes cell motility and is involved in α-tubulin deacetylation. HDAC6 has several different substrates such as alpha-tubulin, heat shock protein 90 (Hsp90), and cortactin (Reference). This makes HDAC6, whose major substrate is alpha tubulin, an important target for developing drugs that can treat cancer. This thesis aimed to clarify the role and mechanisms of HDAC6 in controlling cellular contractile forces.

HDAC6 is very different from other HDACs in terms of its unique structure, location, target receptors, functionality, biological processes and the pathways it uses. HDAC6 regulates several important physiological processes and signalling pathways, which are involved in the various stages of tumour formation and progression including cancer initiation, promotion, progression and metastasis. These factors along with HDAC6's role as an important regulator of several signalling pathways connected to cancer makes HDAC6 a great target for the development of anti-cancer drugs as well as a target for the development of HDAC6 inhibitors to block its function.

Thesis project: This thesis looked at the effect that HDAC6 had on the cellular contractility of metastatic cells when compared to normal cells. The concept of measuring differences in contractile forces between normal and metastatic cells is relatively simple and can be easily achieved using TFM. However, this alone would not be enough as it does not add sufficient scientific value to the topic. Previous literature about research conducted in this field clearly shows that metastatic cells have increased contractile forces when compared to normal cells when all other factors are constant. These previous studies also give several reasons for the increase in contractile forces of metastatic cells and potential mechanisms by which these increase takes place (see section 1.0 for further details). Therefore, this thesis focuses on the effect of HDAC6 on metastatic cells by blocking its action using two different methods (HDAC6 inhibition and HDAC6 siRNA knockdown) and comparing them to the results obtained from normal cells (control). This was done to determine and elucidate the mechanism by which contractile forces are controlled and changed by HDAC6.

There were several reasons for looking at the effect of HDAC6 on contractile forces in this thesis. Firstly, there is limited research in this area and thus presents an exciting opportunity for novel research and to add value to this field of study. Secondly, HDAC6 has become an extremely popular choice recently as a potential target for cancer treatment due to its well-researched role in cancer where several studies have shown increased HDAC6 expression in several cancer types. Therefore, making it even more important to understand the mechanisms by which HDAC6 affects contractile forces in metastatic cancer cells. Thirdly, HDAC6 enzymes are important biomarkers and are intricately linked to tumorigenesis, so by looking at HDAC6 levels in metastatic cells, the severity of different tumour types can be identified. This information could be correlated with the cancer contractile forces for potential early detection and treatment of metastasis.

Thesis Methods The project is based upon a previously used cell model, which uses 4 different types of cells that are all isogenically identical. These 4 types of cells include normal BJ human fibroblasts that were first discovered by the Weinberg scientific team that have gone through different stages of modification including immortalization, transformation and metastasis (Hahn et al, 1999). The cell model consists of primary human foreskin fibroblast cells (BJ) that are immortalized with telomerase reverse transcriptase (BJhTERT), further transformed with the SV40 large-T antigen (BjhTERT-SV40T-variant), and finally metastasised by the introduction of an oncogenic allele of the H-*ras* gene, H-*ras*V12 (BjHTERT-SV40T-Ras).

This model has been successfully used by several different previous studies to replicate the natural steps of metastatic transformation and also allowed me to ensure the validity of the results since the cells are isogenically matched.

Immunofluorescence and confocal microscopy were used to image cell-matrix adhesions and cell spreading area between the cellular components and extracellular matrix, while also measuring the individual sizes and numbers of focal adhesions of normal and metastatic cells. To measure the contractile forces of normal and metastatic cells TFM was the main method used. TFM allowed measurement of contractile forces on an elastic substrate of fixed elasticity value thereby providing us with quantitative information on the value of the contractile forces being exerted by a cell. In TFM, cells were cultured on a gel (primarily made of polyacrylamide) that has fluorescent beads embedded in the gel. First, a cell was allowed to adhere to the gel, then the gel and fluorescently labelled beads were imaged within the gel substrate and recorded as the cell's initial fixed position (U0). Next, the cell was detached, relaxing the gel and allowing the beads to be displaced from their initial positions. Bead displacement was filmed using a microscope (U1). This allowed measurement of cell movement, since the distance moved by the displaced beads from when the gel contracted to when the gel relaxed was equivalent to the contractile force exerted by the cell (Contractile force = U1-U0). Several other techniques were also used in this thesis to obtain results; these will be discussed in detail in subsequent chapters.

Thesis results: Metastatic cells were shown to have increased cellular contractility compared to normal BJ fibroblasts, but this contractility was reduced significantly when HDAC6 was inhibited using tubacin in metastatic cells. This trend was significantly reversed in metastatic cells treated with siRNA knockdown of HDAC6 as contractile forces increased compared to untreated cells. Metastatic cells had decreased nuclear forces than normal BJ fibroblasts and this force decreased further when HDAC6 was inhibited. Metastatic cells had smaller cell-matrix adhesions and decreased cell spreading area compared to normal BJ fibroblasts. This could have been due to increased cellular contractility in metastatic cells making them more invasive; and this was dependent on HDAC6 activity via its deacetylase and protein-binding functions.

1.1 Cancer

1.1.1 Hallmarks of Cancer

Cancer is the term used to describe a group of disorders, which are all characterised by the mutation of normal cells and their uncontrollable growth in different parts of the body. Cancer is a collection of around three hundred diverse types of cancer diseases and is the 2nd highest cause of human deaths worldwide today. In men, lung, prostate, and colon cancers are most prevalent while breast and uterine cancers are most common in women (NCI, Cancer statistics 2024). For children, most cancers occur in the blood and cancers related to the brain (Torre et al., 2023).

Cancer can be divided into several diverse types, however for the ease of classification the most widely used division is based on the cell type that the tumour cells originate from. These include but are not limited to, carcinomas, which originate from epithelial cells, sarcomas, which originate from connective tissue, lymphoma, which originate from cells of the immune system and leukaemia, which originate from cells of the blood (Cancer Research UK, 2024). There are several different cancer types but the most widely spread consist of lung cancer, prostate cancer, colorectal cancer, breast cancer and stomach cancer respectively (Carbone, 2020). Cancer can be caused due to either genetic mutations, lifestyle or environmental factors. These include chemicals which form mutations and cancerous cells. Smoking is also a risk factor due to its carcinogenic ingredients. Environmental factors like radiation and germs like bacteria can also mutate the cytoplasm and nucleus of cells, and lead to genetic disorders and cancers. Cancers from genetic mutations are caused by exposure to chemicals, radiation or heredity factors while other cancers are caused due to diet, obesity, smoking etc (Anand et al., 2008). Cancer is a very prevalent disease with around 18 million cases annually as of 2019 with around 9 million deaths per year (Carbone, 2020).

Mutated cells have the ability to travel from their primary locations, where they originate to spread tumours in different parts of the body. This ability of cancer cells is called metastasis and the fact that cancer can metastasise makes it extremely malignant and allows for tumour to quickly spread throughout the body. Tumour microenvironment (TME) refers to a collective mass of different cancer cell populations along with the extracellular matrix proteins. The tumour microenvironment is also used to describe the cancer cells that are present from the initial stage to the final stage when the cancer progresses and metastasises (Naser et al., 2022). Many hallmarks of cancer such as sustaining proliferation, signalling, metastasis, inducing

angiogenesis are upregulated by the TME (Naser et al., 2022) (Fig.1.1). Cancer progresses via several distinct stages, which means that there are several genes and pathways involved in its progress. These abnormal genes are what leads to mutated cell invasion (Wang et al., 2017). In the past, great progress has been made in cancer research, new studies have tried to understand and improve upon the original idea of the hallmark capabilities of cancer cells that allows them to grow and spread, while highlighting the new mechanistic concepts that were not discovered or discussed in the original study of cancer traits (Hanahan and Weinberg, 2011). Out of all the hallmarks of cancer listed below, invasion and metastasis are the hallmark that this project focuses on (Fig.1.1). Also, while other hallmarks can also be applied to benign tumours, metastasis is a hallmark that is exclusive to malignant tumours (Hanahan and Weinberg, 2011) (Hanahan, 2022).

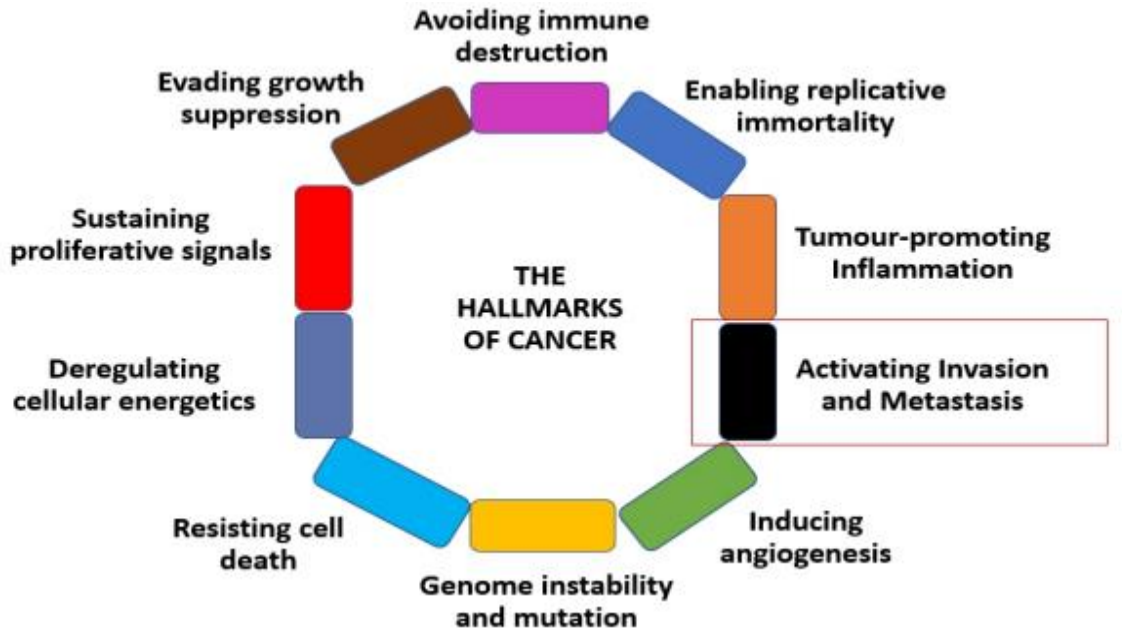


Figure 1.1: Hallmarks of Cancer (Adapted by Azeer Zafar from Hanahan and Weinberg, 2011, Hanahan 2022 and prepared using Microsoft PowerPoint.)
The development of cancer can be divided by the abilities acquired by cancer cells during the development of the tumour that allows them to continue growing.

In previous years, cancer treatment research was mainly focused on studying gene regulation and cell signalling pathways (Ferlier & Coulouarn, 2022). However, in recent times, research has moved towards understanding the role of the mechanical properties exhibited by cancer cells during cancer cell invasion (Mierke et al., 2008). Hormone therapy is another cancer

treatment that involves drugs which alter the effect of certain hormones or disrupt their production. This approach is common with prostate and breast cancers since they deal with hormones (NCI, 2022). Immunotherapy is a treatment which uses drugs to increase the strength of the immune system and promote destruction of cancer cells (Davis., 2003). Precision medicine is a more recent treatment, which tests genes to determine the optimal treatment options for a person's cancer type (Arnedos et al., 2015). Stem cell transplantation is an especially effective treatment for cancer patients with blood-related cancers, such as leukaemia or lymphoma. It involves replacing cells with stem cells that specialize and grow to become new cells which do not have cancer mutations (Dwyer et al., 2010). Finally, surgery is the oldest treatment option for cancer and despite all the latest treatment options currently available, sometimes surgery is the only option that is viable for treatment of cancer for a patient (NCI, *Surgery for cancer* 2024).

The best option that is currently available for cancer treatment is the ability for early detection and diagnosis. Therefore, by using the latest advances in imaging, genetic testing, scanning, and computer informatics critical data can be collected and used for early diagnosis and determining treatment options (Kumar et al., 2024). This data can also be used to determine the effects of drugs and treatments on cancer patients in real time. Successful application of these techniques can improve our understanding of the treatments used in cancer (Hassanpour & Dehghani, 2017).

1.1.2 Carcinogenesis

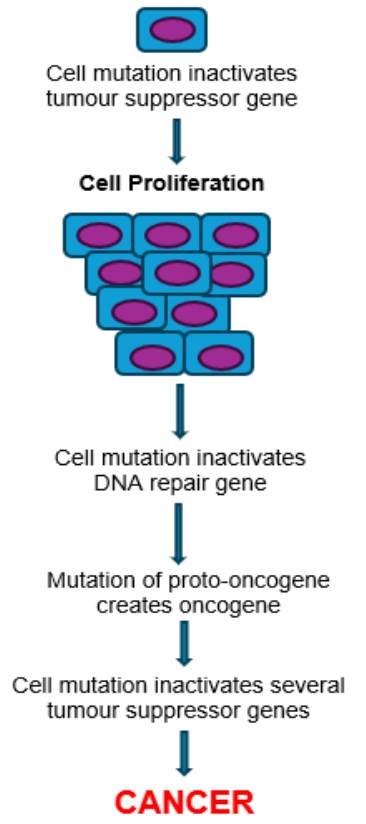


Figure 1.2: Overview of Carcinogenesis (Adapted by Azeer Zafar from the National Cancer Institute and prepared using Microsoft PowerPoint)**.** This figure outlines the different steps that occur and lead normal cells to become cancerous.

Carcinogenesis, also known as tumorigenesis, is the action which transforms normal cells into cancer cells via mutation and cell division. Carcinogenesis occurs via mutation of the cells at their genetic, epigenetic and cellular levels (Majérus, 2022) (Figure 1.2). There are several causes of carcinogenesis which range from genetic mutations, epigenetic changes like gene expression, genome instability, DNA damage etc (Baba, Catoi and Baba, 2007).

DNA mutation and change is accepted as the primary cause of cancer. Therefore, any damage in the DNA results in the DNA repair to stop working which in turn would cause more DNA damage since DNA repair is not taking place and this increases the risk for cancer (Majérus, 2022). Another main cause of cancer is genetic defects. An example of this is research has shown that there are certain genes which when damaged or impaired increase the risk of cancer, with some genetic defects even leading to an absolute certain chance of the person having cancer in his lifetime (Hopkins et al., 2022). Despite this, most cancers are non-hereditary as they can be caused spontaneously. Of these non-hereditary cancers, the majority have no family background while a minority does have some aspect of family background as a cause. In non-hereditary cancers, problems in the repair of DNA are either due to mutations or epigenetic changes that affect genetic expression (Bernstein R., Nfonsam and Bernstein, 2013).

In cancers there are unstable genes known as a "mutator phenotype". Reduction of DNA repair causes damage to increase at a higher level and this increased synthesis of damaged DNA leads to increased mutations and epimutations (Loeb, 2010). Even if DNA repair does take place, any mistake or incomplete repair of the increased damage leads to epimutations and cancer, benefiting cancer invasiveness (Hsieh and Yamane, 2008). Epigenetics is a field of genetics that involves

changes to the DNA which might not be strictly genetic in nature. Examples of these include the methylation of DNA, modification of histones and these changes have also been researched and play a vital role in cancer. (Dupont et al., 2009).

The Cell paper titled ‘‘Hallmarks of Cancer’’ by Hanahan and Weinberg (Hanahan and Weinberg, 2011) (Hanahan, 2022) summarizes that a cell can be classified as a malignant tumour cell when it has the following properties. These include when a cell becomes self-sufficient in controlling signals that influence growth leading to uncontrolled growth, when a cell is able to ignore anti-growth signals, again leading to uncontrolled growth, when cells lose their ability for apoptosis, allowing them to grow even with genetic errors, when the cells lose their ability for senescence, allowing them to replicate uncontrollably, when cells acquire the capacity for sustained angiogenesis, when cells are able to invade their surroundings, becoming an invasive carcinoma and when cells are able to metastasise at different locations (Hanahan and Weinberg, 2011).

According to Lee and Muller, 2010 cells can only lose control over controlled division if there is dysregulation in the genes that control cell cycle and cell proliferation. Proto-oncogenes are genes involved in promoting cell proliferation and replication, while tumour suppressor genes slow down cell proliferation, or stop cell division in order to perform DNA repair. Typically, the conversion of a normal cell to a metastasising cancerous cell is only possible with a cascade of genetic mutations. Uncontrolled division of cells that is a characteristic of cancer is possible only due to a mutation in metastasis (Lee and Muller, 2010).

Proto-oncogenes are any genes that in normal conditions are responsible for basic cellular functions like mitosis and cell proliferation however have the capacity to become cancerous genes, also called oncogenes during genetic mutation and overexpression (Cline, 1987). These oncogenes are the genes that are detected and active during cancer. Also, the lack of tumour suppressor genes leads to uncontrolled cell mitosis. Normally, repair genes are proteins and enzymes with repair capabilities and there are more than 20 types of repair genes that have been detected (Chae et al., 2016). During the process of cell transformation there is a class of genes that represents targets for carcinogenic agents known as oncogenes. Oncogenes are parts of genes that engage in the neoplastic transformation of the cell. These genes change their structure and ability to divide in response to the actions of mutagenic factors or viruses (Dang, 1989).

One of the first oncogenes that was discovered in cancer research was the Ras oncogene. (Tsuchida et al., 2016) The Ras family of proto- oncogenes (consisting of H-Ras, N-Ras and K-Ras) show a high incidence of mutations ranging from 20% to 30% of all human tumours (Prior, Lewis and Mattos, 2012). These mutations can be due to several varied reasons such as induced by UV irradiation for skin tumours, K-Ras mutations due to adduct formation from carcinogens in cigarette smoke in human lung and colon tumours; and specific mutation at codon 249 of the p53 gene in human liver tumours (Croce, 2008). Oncogenes can be formed due to several changes in the genes which range from insertion, deletion, translocation, amplification, point mutation, among others (Dang, 1989).

One example of these genetic changes according to Quintás-Cardama & Cortes, 2009 is in blood cancer caused due to chromosomal translocation between chromosomes 9 and 22. This cancer produces a biomarker called ph1, which has been detected in more than 90% of blood cancer patients and early detection can help with diagnosis and treatment. As mentioned before, proto-oncogenes are normal genes performing basic cellular functions that have the potential to become oncogenes and when any mutations take place, the structure, expression, and function of proto-oncogenes is completely changed becoming oncogenes. These changes in genes disrupt cellular functions and cell cycles allowing for the unchecked proliferation of normal cells that now became cancerous and led to uncontrolled mitosis. An initial idea that was proposed for the treatment of cancer was to remove proto-oncogenes however this idea was immediately dismissed since these genes serve important roles in the body that are irreplaceable. (Quintás-Cardama & Cortes, 2009).

Another class of genes are tumour suppressor genes, which inhibit the process of cell division, mitosis, cell growth and other cancer cell properties (Sherr, 2004). These genes are responsible for DNA repair as they can inhibit the progress of the cell cycle, which helps stop mutations and errors from being transferred to new cells. Unfortunately, tumour suppressor genes are usually impaired by the action of cancer inducing changes (Wang et al., 2018). The p53 protein, is one of the most well-researched tumour suppressor gene, which can be affected and reacts to several external factors like ultraviolet radiation and chemicals, among others. Even though it is believed based on past research that p53 expression is present in almost half of cancers the pathway by which this occurs is not well understood or explained (Velculescu & El-Deiry, 1996) (Soussi, 2000). Since p53 is involved in transcription and in regulating the cell cycle and cell division any mutation in the p53 gene leads to abnormalities during the cell cycle. These

abnormalities eventually lead to the formation of cancer cells making the p53 gene an important target for cancer research (Mantovani et al., 2018).

The normal role of p53 gene in the body involves cell division and differentiation and therefore mutations in p53 occur mostly in the DNA-binding positions (Shaw, 1996). Studies show that the interaction of p53 with CDK1-P2 and CDC2 inhibits the proliferation of cancer cells and prevents them from moving past the G1 and G2 phases of cell cycle (Taylor & Stark, 2001). This occurs due to p53 binding to the DNA and activating the WAF1 gene after DNA mutation. The p53 protein uses its anti-cancer cell proliferation ability via 3 distinct ways that include stimulating the repair proteins, activating apoptosis and blocking the cell cycle at the S phase (Gasco et al., 2002) (Marei et al., 2021). Sometimes, cell mutations also damage the tumour suppressor gene itself, or the signal pathway that activates it, "switching it off". This results in a broken or inhibited DNA repair, which leads to the accumulation of DNA change without repair, that eventually leads to cancer (Duffy et al., 2017). In some instances, cancer cells can move from their primary tumour site to other sites in the body in a process known as metastasis.

1.1.3 Metastasis

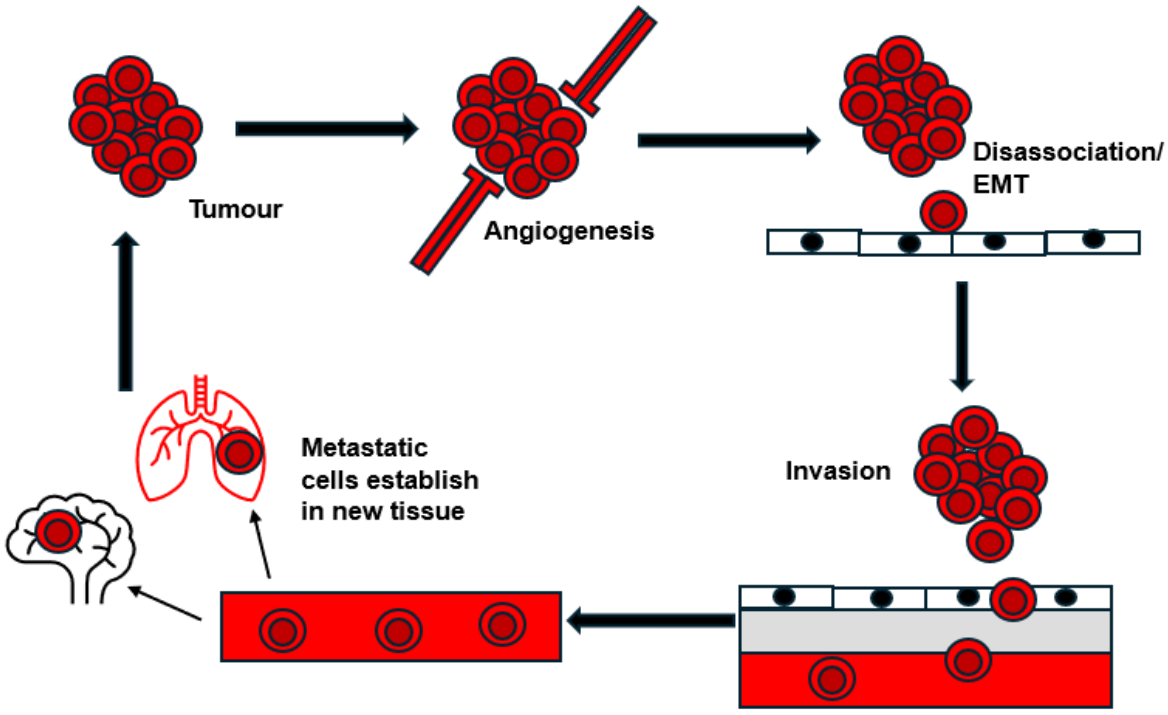


Figure 1.3: Overview of metastasis. (Adapted by Azeer Zafar from De Loso & Yool, 2018 and prepared using Microsoft PowerPoint)
This figure outlines the different stages through which a cancer cell passes before it can be classified as metastatic and metastasis occurs.

Cancer metastasis, also known as the metastatic cascade, is a complicated disease and is described as the spread of cancer cells from their primary location, where they originate to a secondary location in surrounding cells (Klein, 2008). Metastasis is the main cause of mortality in cancer. The movement of cancerous cells from a primary site to a secondary site in the body involves several different mechanisms and pathways. Estimates show that metastasis is responsible for almost 90% of cancer deaths (Chaffer & Weinberg, 2011). Metastasis is complicated and consists of several different mechanisms and pathways which include the division of a secondary tumour from the primary tumour, invasion and spread of the tumour while protecting itself from the immune system and ultimately regulating the environment around itself (Woodhouse et al., 1997). For metastasis to take place, the epithelial–mesenchymal transition (EMT) is essential since this allows cancer cells to spread from a

primary tumour and invade and proliferate in distant organs (Smith & Bhowmick, 2016). The metastasis process is known as the metastatic cascade and it consists of the following steps: Cancer cells disseminate from their original tumour location and spread via the bloodstream or lymph system, to another location in the body. After reaching the next location, cells settle and grow in their new location (Geiger & Peeper, 2009). These cells also have hallmarks mentioned above that allows them to avoid and prevent attacks from the body's immune system. Once these steps take place it is called a metastatic cancer or a stage 4 cancer (Figure 1.3). Metastasis is only considered at stage IV once diagnosis of cancer type has been achieved (Seyfried and Huysentruyt, 2013).

According to Tan, Agarwal and Kaye, 2006, the initial step of metastasis involves the spreading of cancer cells before forming a cascade due to changes in the chromosome such as errors caused during mitosis. The steps that are involved in metastasis are regulated by several different cell types such as endothelial or immune cells. Metastasis proliferates via several different ways such as the proliferation through the different blood streams in the body (Tan, Agarwal and Kaye, 2006).

The cells in a metastatic tumour are the same as those in the primary tumour which means that when a colon cancer metastasises to the thyroid, then the cancer cells in the metastatic colon cancer will be made up of cancerous colon cells, not thyroid cells (Biller & Schrag, 2021). A doctor can determine the type of cell, location where the cell was taken from and if it is a primary or secondary tumour after examining it using a microscope (Huysentruyt and Seyfried, 2010).

Metastasis is a tumour that starts from a cancer cell in a different location of the body. This is because the cells in a metastatic tumour resemble those in the primary tumour. Treatment is determined by the location of the cancer, whether it is localized or metastasised. Unfortunately, the response to a treatment is reduced, and even sometimes just not possible when a cancer has metastasised and become a stage IV cancer (Riggi et al., 2018). One mysterious phenomenon that is unanswered is that some patients lose their cancer tumour after treatment and become disease free for years. However, due to unexplained factors mutation might be triggered again causing the patient to be diagnosed with late-stage cancer and this time with exceedingly high chances of mortality (Mut, 2012). One explanation is that small numbers of cancer cells or micro tumours remain hidden even after an apparent treatment and become dormant so that they do not react and are not detected by the immune response of the body. However, any

trigger in the future causes the tumours to reactivate and continue with their dissemination (Eccles & Welch, 2007). Studies show that all these tumours had disseminated during the preliminary stages of metastasis (Neophytou et al., 2019).

According to Fares et al, 2020 and Park et al, 2022, new research shows that metastasis is not caused by a straight line of steps in linear order but occurs by a mixture of pathways that are occurring at the same time all leading to a metastasis that is spreading through the body. Using the latest technologies and techniques such as live cell imaging, scanning and custom bio-engineered *in vitro* systems have confirmed that parallel mechanisms run simultaneously for cancer cells to disseminate from a primary lesion. For example, cancer cells can mould their structure to fit the space available in their physical microenvironments, however this leads to breaking the chromosomes, change in the expression of genes, and metastasis (Nguyen & Massagué, 2007). This means that there is a greater variety of genes at sites where metastasis has taken place (Fares et al., 2020, Park et al., 2022).

One method of studying metastasis is by creating genetically altered models of animals and using these models to determine the steps which occur during metastasis. This method has several positives and negatives, but they discussed elsewhere (Gomez-Cuadrado et al., 2017; van Marion et al., 2016). Another method of studying metastasis includes the development of creating custom genes which have allowed for *in vivo* studies on a massive scale and understand what promotes cancer growth (Chen et al., 2015; Kalhor et al., 2018). Computer studies and analysing data with information regarding metastasis could provide insight into the mechanisms of this disease (Chen et al., 2015).

Metastasis is a target of current treatment strategies. There are several drugs that are being developed which inhibit the spread of cancer via disrupting the action of certain molecules which are involved in metastasis (Lu et al., 2013). There are several factors such as the rate of metastasis, recurrence of cancer, site of metastasis, receptors among others which are targeted for the treatment of metastasis and this can improve the treatment efficacy and reduce allergic reactions caused due to chemotherapy- in cancer (Jung et al., 2011) (Kalhor et al., 2018).

Unfortunately, the chance that metastasis can recur is still possible and there are still no global treatment methods for patients with severe cancers. This means that if anyone is diagnosed with metastasis, it is considered final and untreatable in most cases. Treatments for this have not yielded results due to failures in developing plans and clinical trials (Weber, 2013). Immunotherapy has increased survival and patient treatments in metastasis. Finally, metastasis

is a complicated multistep problem that requires several simultaneous treatments for effective treatment of metastasis. (Neophytou et al., 2019, Fares et al., 2020). Potentially targeting the mechanical properties of cells may prevent cancer cell movement, therefore the next section describes these mechanisms in detail.

1.2 Mechanical Properties of Cells

1.2.1 Types of Mechanical Properties

Examples of mechanical properties of cells include contractility, stiffness, viscoelasticity, cell adhesion, among others. Among them, contractility and stiffness are important for cell functions. Cells have the ability to change their properties and replicate the structure of their surrounding environment (Moeendarbary and Harris, 2014). Cell mechanics is defined as the response of the cell to the physical forces exerted by the cell's surroundings, including adjacent cells and the ECM (Kumar and Weaver, 2009). The response can be either active or passive. The cytoskeleton is primarily responsible for regulating the various mechanical and chemical properties of cells. This means that any change in the mechanical properties of cells allow us to observe changes in the cytoskeletal structure (Forgacs et al., 2004).

Contractile forces of cells, also known as cellular contractility, is a mechanical (or physical) property of cells which allows them to self-contract and exert force on the surrounding environment but only if they are adhered. Contractile forces in cells are measured by analysing the μm bead displacement, which are presented as a heat map that shows the magnitude of contraction (Sabass et al, 2008). Cell contractility occurs when the actin filaments combine with the myosin II protein and forms two overlapping filaments, which move in opposite directions, and this leads to generation of cellular traction forces (Feld et al., 2020). An important part of this process is carried out by phosphorylating the myosin light chain (MLC) as well as several other important regulators, such as the Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK) (Verin et al., 2001) (Roda et al., 2024). The kinase action of ROCK is activated by combining the small GTPases RhoA with ROCK, which in turns phosphorylates MLC and deactivates the opposite regulator of MLC, which is the myosin light chain phosphatase (Carey et al., 2012).

Cellular stiffness is another mechanical property of cells, which is the ability of cells to resist deformation up to a certain point by exertion of external forces before they lose their structural integrity and collapse. These mechanical properties of cells control several functions of the cell, such as cell adhesion, cell mobility, cell migration, differentiation, and polarization (Nagayama et al., 2004) (Wu et al., 2018). Actomyosin stress fibres play an especially important role in the activation of cellular stiffness and contractility. Stress fibres can be divided into three distinct classes on the basis of their position and composition, which include: ventral, dorsal and transverse fibres (Naumanen et al. 2008). At the subcellular level, stress fibres are made up of interlinking F-actin filaments (between 12–35 actin filaments) and actin-binding proteins combined with non-muscle myosin II (Tojkander et al., 2012).

The physical properties of cells correlate very closely to the physical state of humans since any significant differences in the mechanical properties lead to the disorganization and degradation of main functions of the cells. This is why studying the mechanical properties of cells is a newly developing way to identify, differentiate and determine the conditions of some diseases (Mierke, 2014). Finally, there is cell viscosity, which is also a mechanical cellular property that describes the cytoskeletal status of single cells. Viscosity is the friction provided by a liquid during flow. This means that slow flowing liquids have higher internal friction due to the increased forces between molecules resulting in these liquids being more viscous and having a high viscosity (Encyclopaedia Britannica, *Viscosity* 2025).

Extracellular viscosity is a key physical component that differs in cells between physical conditions, such as cancer disease. Unfortunately, its impact on cancerous cells invasion and the process by which cells react to different conditions in viscosity are not well understood. One study shows that increased mechanical force exerted on the cell induces the action of an actin protein (ARP2/3)-complex-dependent complex actin network, which elevates viscosity (Mullins et al., 1998). Exertion of external force promotes the swelling of the cell and also increases membrane tension. This in turn leads to activating a calcium influx and increases RhoA-dependent cell contractility (Persson, Ambati and Brandman, 2020). The combined action of actin remodelling and RhoA-based contractility increases cell motility at higher viscosities (Bera et al., 2022).

This field of science falls under the category of mechanobiology, which is the study of cells by looking at their mechanical abilities. Mechanobiology is a new and emerging field of science that studies how differences in the mechanical properties of cells affect cell properties and

disease. Mechanobiology is an up-and-coming topic of research in every field, since the concepts derived from mechanobiology can be applied in several fields such diagnostics, immunity, and drug treatment (Hao et al., 2020). Normally, treatment of diseases has been done by focusing on understanding their genetic and biochemical composition. However, research into mechanobiology has shown that the breakdown of cell mechanics or the structure of the ECM is a risk factor for diseases like cancer (Kalluri, 2007).

Understanding nuclear mechanics is important for several types of diseases such as cancer and during cell migration in the experiment, the nucleus deforms when the cell passes through a tight space. The nucleus plays an important role in mechanobiology due to its connection to the cytoskeleton (Denais & Lammerding, 2014). Cells react to mechanical signals via a process known as mechano-transduction; however, the process by which this occurs is not clear (Friedl et al., 2011).

An important aspect that determines the cytoskeletal mechanical abilities of the cytoskeleton is the structure and binding of the links between intermediate filaments, the physical properties of separate polymer fibres and the 3D intermediate filament shape (Fabry et al. 2001; Chen et al. 2010). The cytoskeleton is made up of a compound 3D network of microtubules, actin filaments, intermediate filaments and a group of other proteins and molecules that are all bound together forming links and applying forces (Forgacs et al., 2004).

The earliest example of research done in this field was done by the German surgeon, Dr Julius Wolff who studied the load bearing capacity of the trabecular bone in the 1800s. Dr Wolff devised a method to work on bones so that he could examine their patterns, which was useful since X-rays weren't discovered yet (Brand, 2010). Since then, with the rise of technological research, research into this field of study has vastly increased with research showing that between the period of 1970-2016, over 33,000 studies have been published on mechanobiology (Wall et al., 2017). Mechanical properties are also especially important since they are being used as biomarkers for early cancer diagnosis. This is because cancer cells are softer than adjacent normal cells making them more invasive, and both these properties have an inverse relationship (Di Carlo, 2012). Mechanical properties and their application also show immense potential for drug screening. Even though in the present, there are several challenges that have yet to be overcome before any progress can be made in terms of clinical applications, mechanical properties of single cells are an interesting approach for the treatment of cancers dependent on cellular physical forces (Martinez et al., 2014).

1.2.2 Mechanotransduction

Mechanotransduction is defined as the mechanisms by which cells react to physical forces and change them into biological responses (Ingber, 2006). It is particularly important to understand how mechanotransduction in cells is driven by cellular contractility. Internal mechanotransduction is generated by the transmission of contractile forces from the substrate to the internal cellular structures via differential contractility, which is when the contractility is non-uniform (Wozniak & Chen, 2009). Therefore, it is essential to understand contractile forces, and the impact they have, especially in metastasising cells in order to help understand how metastasis can be controlled and managed (Dunlop, 2019).

Mechanotransduction has been studied using Wolf's Law in the past, which focuses on the mechanical load in tissues but was eventually expanded in the study of the growth and development of cells and tissues. Recently, mechanotransduction is being increasingly studied for its role in several mechanical functions that occur in various processes, which include tissue repair, wound healing, tumorigenesis, and cancer resistance (Wozniak & Chen, 2009). These mechanical functions include pressure, tensile force, stretching force, extracellular matrix (ECM) stiffness, elasticity, viscosity, and cellular contractility.

The above-mentioned mechanical properties affect the movement of messages between the cell-matrix as well as cell signalling. These mechanical properties engage in several different biological processes, including cell development, cell cycle, cell division, cell adhesion, cell migration, cell invasion, and ECM generation (Ingber, 2003). These mechanical properties are also involved in the development of tissues, their repair and tumour invasion, and metastasis (Orr et al., 2006).

The mechanical properties associated with the extracellular matrix are crucial to several biological processes. Stiffness in the extracellular matrix refers to the resistance that is exerted in response to the deformation of tissues, which in turn leads to an increase in tissue elasticity. Stiffness is also controlled by the post-translational changes that are made by several ECM components (Grover et al., 2012). One example of this is when there is a cross-linking of collagen in the ECM that can in turn increase matrix elasticity (Kong et al., 2021). Cellular mechanotransduction of ECM stiffness is an overly complex process. In short, this process occurs via different steps which consist of: Integrins carrying signals from the ECM to different cells. The pathways which facilitate this movement are the RhoA/ROCK pathway, which increases collagen concentration, the Talin/FAK pathway, which assembles F-actin and the

YAP/TAZ pathway, which promotes transcription of genes. These processes in turn leads to increased cell proliferation, migration and invasion (Khatiwala et al., 2009) (Yemanyi et al., 2020).

In the past, research on mechanotransduction focused primarily on the effect of external forces on cell signalling and cell function. However, studies now suggest that contractile forces produced inside the cell via the action of the actomyosin cytoskeleton are also crucial in regulating cell activity suggesting a more important role for mechanotransduction in today's biology. For example, recent studies show that formation of contractile forces due to the actions of actomyosin are also involved in cellular signalling (Moujaber & Stochaj, 2020). Mechanical forces control proliferation primarily via Rho-mediated regulation of cellular contractility. Blocking this pathway also blocks contractility and stops growth regulation (Sanz-Moreno et al., 2011). Mechanotransduction and contractility are regulated in both vitro and in vivo. Therefore, it is important to study the various mechanisms and pathways that engage in the actions of mechanical forces via mechanotransduction (Di et al., 2023)

Despite there being several studies done on understanding the role of mechanotransduction in various cell and mechanical mechanisms, new findings continue to be slow since the tools required to study this are unavailable. An example of this is that although it is well known that different cell types have different stiffness and contractility values in different substrates, what is not well known is the mechanisms by which this stiffness and contractility value is determined. It is not yet clear what factors engage in determining the different values and why different cells react via different integrins (Freyman et al., 2002). This ignorance is compounded by the fact that the techniques used to study mechanotransduction such as migration assays, traction force microscopy, atomic force microscopy among others still hold a certain degree of unreliability due to them being relatively new and untested. Although studies have focused on the structure of focal adhesions and their ability to sense force, it is quite difficult to visualize and to quantify multiple cells at the same time and to understand the mechanism by which they physically interact with each other and in the cytoplasm (An et al., 2009). Mechanical forces and mechanotransduction will play a crucial role in the next few decades in biological studies, especially in the field of life sciences and disease prevention provided that more effective techniques are designed and utilized that provide accurate results.

1.2.3 Cellular Contractility and its Importance in cells

Cellular contractility or the contractile forces of cells is the capacity of cells to exert contraction and generate force via cell and matrix interaction. Cellular contractility is used by cells to divide, to migrate, to heal wounds, and to contract muscles (Rodrigues et al., 2019). Intracellular tension is generated via the actions between the actin cytoskeleton and myosin-II. This intracellular tension is transmitted to the ECM via the action of the focal adhesions (FAs) which leads to the generation and exertion of cell traction forces. These forces allow for the migration of the cell to the substrate surface via the focal adhesions (Aguilar-Cuenca et al., 2013) (Figure 1.4). Cell-matrix interactions result in the exertion of cell traction forces, which is essential for cell migration, maintaining cell homeostasis and cell structure.

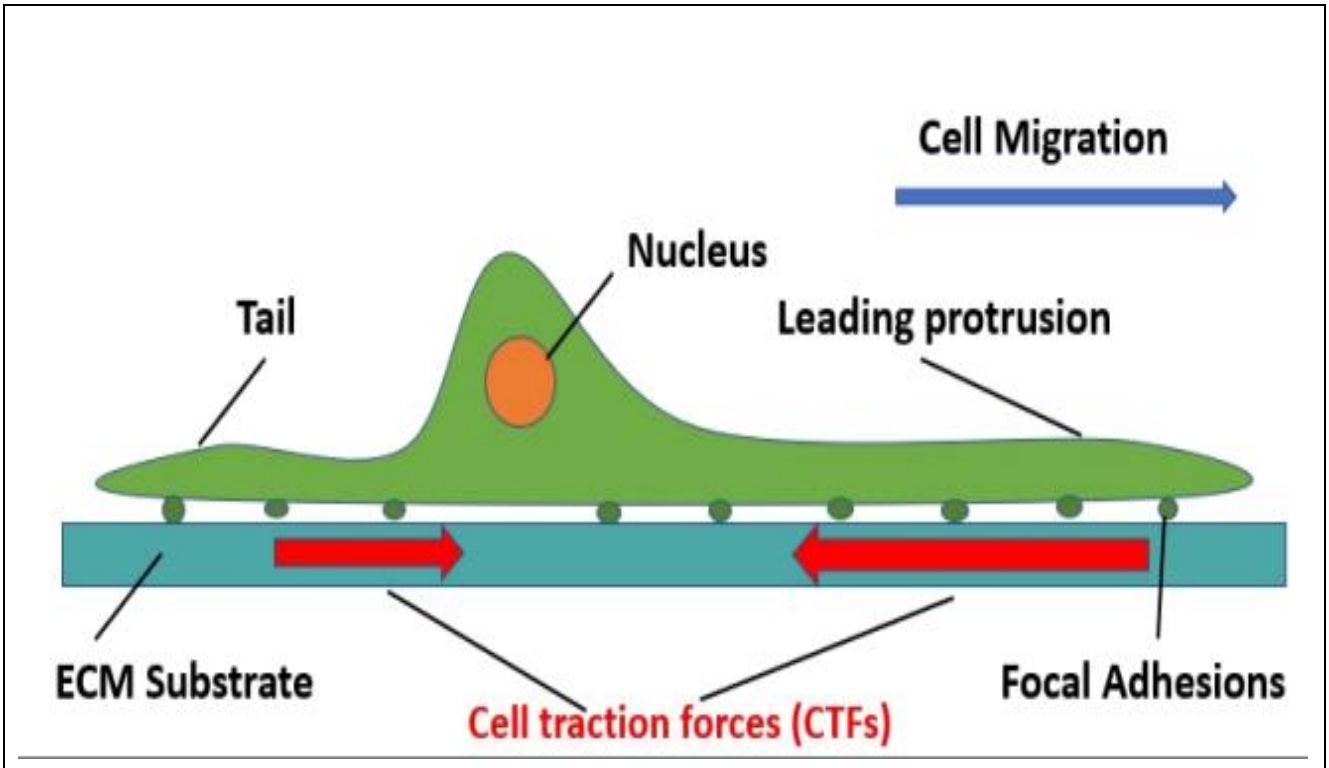


Figure 1.4: Cell Contraction and Generation of Contractile Forces (Adapted by Azeer Zafar from Gavin, *Cytoskeleton methods and protocols* 2010 and made using Microsoft PowerPoint)**.** This figure outlines how a cell attaches to a surface and the contraction of the cell against the surface via FAs generates a force called cell traction forces and allows for migration.

Traditionally according to Janmey and Miller, 2010 it has been cellular stiffness, which is the most commonly studied and understood mechanical property. This project however focuses on only one property specifically, which is cellular contractility or the contractile forces since the

molecular mechanisms that link contractility are not well understood. This leaves a gap in knowledge in the study of contractile forces, which are also important in interpreting the mechanical properties of cells, especially cancerous cells (Janmey and Miller, 2010).

For example, Nagayama et al., 2004showed that cell stiffness was linked to a network of stress fibres. To understand this link between cell stiffness and contractility, cell stiffness was measured when contractile forces were controlled by treating with certain reagents. This study clearly showed the connection between cellular stiffness and contractile forces. Increase in cellular contractility led to increased cell stiffness while decrease in cellular contractility decreases cellular stiffness. The variation in stiffness were induced due to the action of contractile forces and presence of the contractile actin network (Nagayama et al., 2004).

Another study Das, 2013 studied the effect of the extracellular matrix via cellular contractility. Results showed that there is a correlation between cell stiffness and cancer metastasis. In 2D cultures, stiffness of cells increases cell proliferation, which directly correlates to cellular contractility. The study also shows that increased ECM concentration is linked with greater focal adhesions, increased contractile forces, and an increase in ECM breakdown. This means that when ECM density is increased there is an increased cellular contractility that is associated with increased cell spreading in fibroblasts cells. In summary, this study shows a close link between ECM concentration and cellular contractility in metastasising breast cancer cells (Das, 2013).

1.2.4 Effect of Cellular Contractility in Cancer

Metastasis results in the development of a malignant tumour in cancer cells. Mechanical properties of the TME act as important regulators during cancer invasiveness. Studies show that the invasiveness of cancer is associated with increased formation of contractile forces (Mierke, Rosel, Fabry and Brabek, 2008). Contractile forces are generated and allow cells to move through dense spaces and also generate activity by myosin II, and this leads to ECM changes, which is important in cancer metastasis. (Poincloux et al., 2011).

During metastasis, cancer cells are being acted upon by several different mechanical forces which causes their morphological shapes to deform while they are proliferating to neighbouring cells and forming secondary metastasis in different sites (Mierke, 2014). These different mechanical forces consist of contraction, stiffness, adhesion, and internal fraction among others

and form great changes to the tissue structure. Cells interact with these mechanical forces using cell-matrix adhesions with the help of receptors. Metastasis triggers a mixture of biological and mechanical responses which include activating the signals by increasing the cellular contractility of cells, increasing the stiffness of cells and the reorganization of the actin structure, and thereby improving the chances of cell invasion and proliferation. Studies also show that the epithelial cells have increased stiffness in metastasis and begin the epithelial to mesenchymal transition (EMT) when they are metastasising (Gkretsi & Stylianopoulos, 2018) (Wei et al., 2015).

There have been studies conducted that have measured the changes on cells in response to changes in the ECM. The mechanical properties of the tumour microenvironment are known as crucial factors of cell invasion, metastasis and proliferation while also greatly increasing the rate of cancer cell migration and spread. One study Haage and Schneider, 2014 showed that MMP activity is upregulated in response to increased cellular contractility. However, blocking cellular contractility results in MMP downregulation in pancreatic cancer cell lines. Results of the study has shown that a stiffer environment result leads to increased cell migration and invasion along with increased contractile forces. Also, RhoA, which leads to enhanced contractility tends to result in more invasive cancer cells (Haage and Schneider, 2014). Also, in cancer cells measuring the traction forces was a highly effective indicator to distinguish between cancer cell lines of different levels of invasiveness. Peschetola et al., 2013 showed that more invasive cancers have increased cellular contractility on soft substrate. Therefore, if we accept these studies then logic dictates that a decrease in cellular contractility will also inhibit cancer cell metastasis.

The study by Menhofer et al., 2014 measures this same fact that inhibiting the metastasis of breast cancer cells by reducing cellular contractility via treatment with Chondramine, which is an actin targeting compound. The study shows that Chondramine is responsible for inhibiting the migration of cancer cells in an *in vitro* model via the action of different signalling pathways and this affects cell contractile force. Cell migration depends on contractility, which occurs when new focal adhesions are formed in the direction of migration thus making contractility especially important in cancer invasion. The study observed that Chondramine inhibited the contractility of cancer cells, which in turn inhibited the migration and invasion of cancer cells. This shows that cellular contractility is the primary cause for this condition. Therefore, a combination of Chondramine with specific targeted therapies can lead to viable treatment to treat tumour metastasis (Menhofer et al., 2014) (Mierke et al., 2008).

Another traction force study Koch et al., 2012 compared the contractile forces of several tumour cell lines by invading a thick collagen 3D matrix network using a 3D collagen assay to analyse the effect cellular contractility has on cancer cell invasiveness and migration. The study measured the deformations in the matrix around the cell by locating the exact positions of the fluorescent beads located within in the gel matrix. The results of the study showed that high contractility is essential for invasion of cancer through a thick 3D matrix; while unfortunately some non- invasive tumour cells can also have increased contractile forces but cannot exert this to migration and invasion (Koch et al., 2012).

Unfortunately, cellular contractility that is measured by TFM technique is not absolutely exact in measuring the potential for metastasis. There are several factors that need to be considered as shown by Liu et al., 2020 which can change the cellular contractility of cells such as the stiffness of the substrate and the microenvironment. The resultant change to cellular contractility can also affect their cell movement and overall structure (Liu et al., 2020). This means that it is important to determine the exact range of stiffness where cells can exert maximum contractility and where no contractility is exerted. (Pasqualini et al., 2018). Although this is a different cell model than the one being used in my project, the idea might still be useful in other contractile cells including different cancers.

1.2.5 Generation of Contractile forces as Biomarkers of Cancer

Metastasis is currently the biggest hurdle in the treatment of cancer so there is a lot of research that is attempting to find a single or multiple biological markers for metastasis. Although cellular elasticity, cell mobility and contractility of cancer cells have all been identified as biomarkers of the invasive ability of cancer cells in different studies with different cell models (Molter et al., 2022). Unfortunately, there is currently no universal molecular marker to detect metastasis (Kwon, Yang, Moon and Kim, 2020).

Several studies have been performed that have looked at cellular contractility and mechanical forces to study cell properties such as the focal adhesion area and size, cell invasiveness and cell proliferation (Chrzanowska-Wodnicka & Burridge, 1996) (Deshpande et al., 2008). These contractile forces have the potential to act as biomarkers for testing malignant and metastatic cells. An example of this is that in several cancers such as breast and lung cancer models metastasising cells exert increased contractile forces than those exerted in non-metastasising normal cells (Menhofer et al., 2014). However, further research needs to be done in this field in order to draw a definitive conclusion whether if anticancer drugs are effective. Unfortunately, currently when there is a new cancer treatment being tested, the only factors that are being looked at include cell proliferation, cell invasiveness, apoptosis and cell migration. Cellular contractility is not a factor that is currently being addressed when testing cancer treatments (Sleeboom et al., 2024).

A study Kraning-Rush, Califano and Reinhart-King, 2012 quantified the contractile forces of highly metastatic cancer cell lines and compared them to non-tumorigenic epithelial cell lines. This was done to measure the traction force that is generated as a biological marker for metastatic potential. The data from the study suggests that measuring the increase in force generated in cells having high metastatic potential can serve as a character of metastatic cells that could possibly act as a biomarker for metastatic cancer. However, this data alone cannot be used to accept contractile force as a universal marker of metastatic behaviour unless we get the same result from every potential type of cancer. Also, studying additional proteins which increase cellular contractility such as RhoA, ROCK, or decrease it such as myosin light chain phosphatase, might also lead to a biomarker for traction stresses which can directly be used to clinically diagnose metastasis (Kraning-Rush, Califano and Reinhart-King, 2012).

Rho-associated protein kinase (ROCK) is a kinase that belongs to the AGC family of serine threonine kinases, and its primary role is in cell movement and shape regulation and its primary

function is in the cytoplasm (Amin et al., 2013). ROCK is an important factor in several different biological processes. ROCK has a downstream effect on Rho GTPase, and Rho is an important regulator of the cytoskeleton. This makes Ras homolog gene family member A, RhoA-ROCK important in cell migration and promoting cellular contractility (Amano, Nakayama and Kaibuchi, 2010). ROCK is responsible for increasing the activity of the motor protein myosin II and this process is done by 2 different mechanisms. The first mechanism is by phosphorylating the myosin light chain (MLC) which leads to an increase in the myosin II ATPase activity and cause actin filaments to interact with each other leading to the contraction of actin fibres (Figure 1.5). The second mechanism is when ROCK inactivates MLC phosphatase and increases MLC phosphorylation. (Figure 1.5) (Watanabe, Hosoya and Yonemura, 2007).

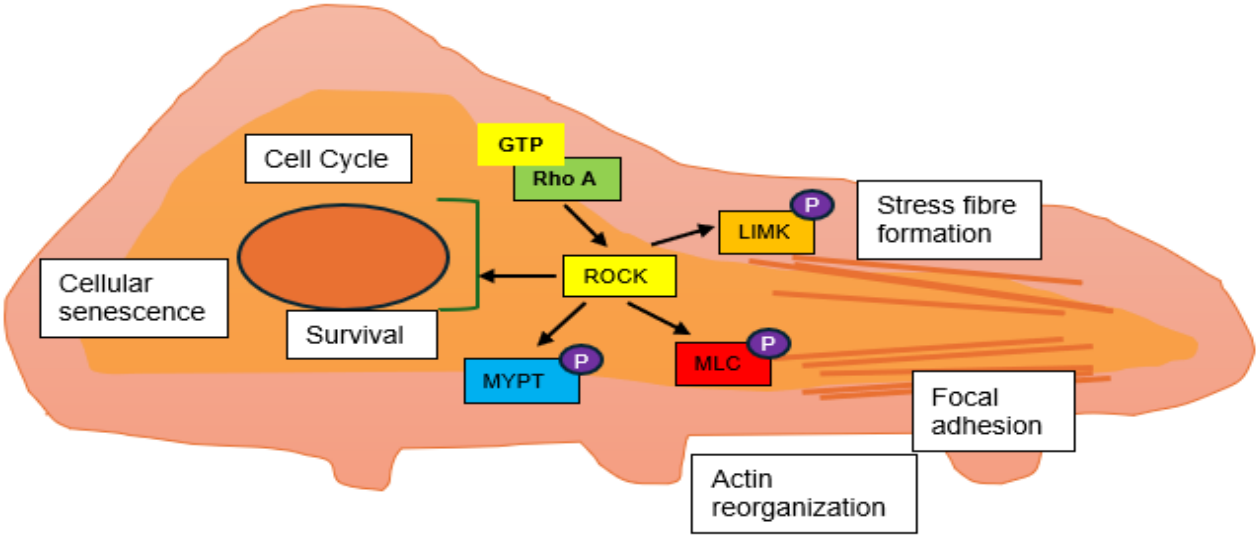


Figure 1.5: Overview of Actin Reorganization (Adapted by Azeer Zafar from Kim et al., 2021 and prepared using Microsoft PowerPoint)**.**

The figure shows how actin reorganization takes place via the actions of RhoA-ROCK pathway using focal adhesions and this generates of cellular forces.

In conclusion, the study shows that metastasising cells exert much higher traction forces than non-metastatic cells in aggressive cancer models (Kraning-Rush, Califano and Reinhart-King, 2012). Therefore, this is a promising avenue to identify a mechanism to therapeutically target contractile force for inhibiting metastatic progression.

Protein filaments are necessary to achieve cell migration and force generation. Some of these filaments include Microtubules, Vimentin, Microfilaments among others and therefore it is absolutely essential to have a clear understanding of these protein filaments (Leduc & Etienne-Manneville, 2015). These filaments are actually part of a structure called the Cytoskeleton.

1.3 Cytoskeleton

The cytoskeleton refers to the interconnected network of fibres which are the primary component of the eukaryotic cells. This network of fibres in eukaryote cells contain several different protein filaments and proteins that assist with cell movement and migration (Birchmeier, 1984) (Wickstead & Gull, 2011). The cytoskeleton provides the structural shape and support to the cell, while also holding all the cell components as well as allowing for cell migration, cell division and cell signalling pathways (Fletcher & Mullins, 2010). The cytoskeleton structure is composed of 3 main types of fibres, which include:

- Microfilaments
- Microtubules (MTs)
- Intermediate Filaments (IFs)

These three main types of fibres are together involved in controlling the shape and structure of cells (Hardin et al., 2015) (Figure 1.6). All three types of fibres cannot exist singularly and thus form complexes which are organized into networks. This makes them stronger and allows them to resist deformation when external force is applied but also allows them to reorganize their internal structure in response to external forces. They are also involved in maintaining the integrity of intracellular structures of cells (Hardin et al., 2015). The polymerization and depolymerization of microfilaments and MTs are involved in generating forces that are directed in one direction and can drive changes in cell shape. This combined with molecular forces that move alongside the actomyosin filaments and microtubules allow for the organization of cellular components (Petrášek & Schwarzerová, 2009).

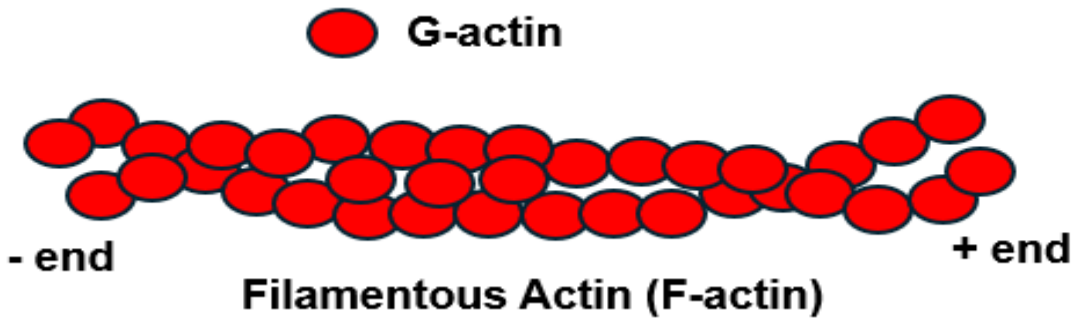
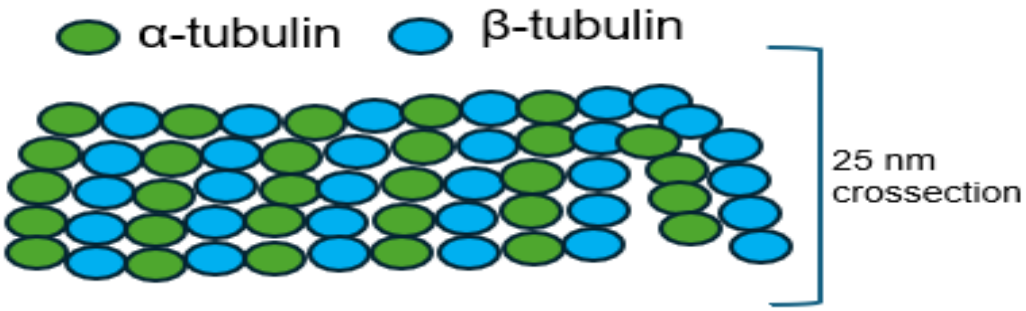
A	
B	
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	Figure 1.6: Overview of External Structures of 3 main Cytoskeleton Fibers: (A) Microfilaments, (B) Microtubules and (C) Intermediate Filaments (Made by Azeer Zafar using Microsoft PowerPoint) This figure shows the structure of all 3 primary cytoskeleton fibres along with how they all link together to form single structures.

Microfilaments (Fig 1.6A), which are also known as actin filaments as they are formed by the interlinking of two strands of actin, which is a globular protein. Microfilaments are very thin protein fibres (2-5 nm in diameter), similar to a string are the smallest components of the cytoskeleton. They are most common in muscle cells and the protein actin with combination

of myosin causes the movement and contraction of muscles. Microfilaments also play a role in cell movement including cytokinesis and contractility (Cooper, 2000) (Gunning et al., 2015).

Microtubules (Fig 1.6B) are made up of a protein called tubulin which is divided into α-tubulin and β-tubulin. These tubulins combine together to form round, hollow in the core tubes that are small in size (25 nm in width). Each tube of a microtubule is composed of 13 different tubulins that link together (Ledbetter & Porter, 1963). Since microtubules are very dynamic structures, they can rapidly change their structure and orientation allowing them to keep growing or shrinking in response to local or foreign conditions. Microtubules are primarily involved in transporting cellular materials through the cell barriers and also chromosome separation during cell division (Yang et al., 2010).

Studies like Al-Rekabi, Haase and Pelling, 2014 have been done on the role of microtubules in fibroblast cells during cellular contractility. They have studied the molecular mechanism of microtubule depolymerization on contractile forces. Previously, it was accepted that a whole actin cytoskeleton is essential for the increase in traction stress regardless of myosin II activity or MT depolymerization. However, Al-Rekabi, et al., 2014 showed that microtubule depolymerization alone is also responsible for inducing an increase in traction forces (Al-Rekabi, Haase and Pelling, 2014).

Rape et al., 2011 carried out nocodazole mediated depolymerization of microtubules in NIH3T3 fibroblast cells followed by treatment of myosin II with blebbistatin. Next, TFM was applied to measure the average traction forces for NIH3T3 cells before and after microtubule depolymerization takes place. Since traction forces are produced via myosin-II-mediated contractility, treatment with blebbistatin, a compound that inhibits myosin II ATPase should have inhibited traction forces. However, there was a six time increase in traction forces after nocodazole treatment, which depolymerized microtubules in cells with the myosin II inhibition, suggesting a myosin-II-independent focal adhesion kinase regulated pathway (Rape, Guo and Wang, 2011).

Kraning-Rush et al., 2011 treated highly metastatic MDA-MB-231 carcinoma cells with nocodazole to inhibit microtubules and analysed for differences in cytoskeleton and cellular contractility in both 2D and 3D. In order to quantify contractile force, TFM was used in both 2D and 3D substrates. Results showed that the contractile forces generated were identical in

2D and 3D matrices, and that disruption of microtubules in either microenvironment increased cellular contractile forces. Therefore, regulating the cytoskeleton of microtubules is essential to suppress contractile forces in both 2D and 3D matrices (Kraning-Rush et al., 2011).

The Intermediate Filaments (Fig 1.6C) are components of the cytoskeleton that are about 12 nm in diameter and provide tensile strength to the cell structure which allows it to resist external force and are also important in the formation of keratin. Examples of IFs include intermediate filaments made of vimentin, keratin, neurofilaments, lamin and desmin (Herrmann et al., 2007).

An important intermediate filament called vimentin is necessary as it allows cells to be able to transition from their epithelial state to a mesenchymal state via a process known as epithelial-mesenchymal transition (EMT) and allow them to migrate as single cells. Vimentin also maintains mechanical integrity in epithelial-mesenchymal transition cancer cells. There are several molecular mechanisms involving vimentin that control cellular contractility (Kidd et al., 2014)

Several studies have studied the effects of vimentin knockdown on the contractile forces of cells. Jiu et al., 2017 used CRISPR and siRNA to knockdown vimentin in human osteosarcoma cancer cells leading to increased actin stress fibre assembly and cellular contractility, which was measured using TFM. It also showed that whole vimentin IFs are necessary to cause changes to the cytoskeleton of actin. Depletion of vimentin led to promotion of activity in RhoA and the assembly of actin stress fibres (Jiu et al., 2017).

De Pascalis et al., 2018 used siRNA mediated knockdown of vimentin in primary astrocytes, which resulted in increased traction forces. Vimentin controlled the distribution and strength of contractile forces as well as collective migration in astrocyte cells. Vimentin intermediate filaments also contribute to the actomyosin cell contractility (De Pascalis et al., 2018).

Liu et al., 2015 knocked down MDA-MB 231 cancer cells via siRNA knockdown to deplete vimentin. This results in reduced cell proliferation, loss of directional migration, reorganization of cytoskeletons and reduction in size of focal adhesions. In turn, this results in reduced mechanical strength and decreased cellular contractile forces. On the other hand, vimentin overexpression in MCF7 cells results in an increase in the stiffness, motility and migration of cells. Therefore, vimentin is crucial in intracellular mechanical properties by maintaining cytoskeleton shape and traction force in cancer cells (Liu et al., 2015).

One study Chen et al., 2016 measured the effect of the disruption of vimentin cytoskeleton on contractile forces. To do this, the study seeded goat articular chondrocytes on a PA gel and disrupted the vimentin cytoskeleton using acrylamide. TFM was used to measure the changes to cellular traction forces after applying a transient compression to cells and results showed that although there was no difference in contractile forces for untreated chondrocytes, there was a major decrease of contractile forces in vimentin-disrupted chondrocytes (Chen et al., 2016).

Vimentin controls the resistance of cells to mechanical stress and has a greater ability to resist stress without breaking in vitro compared with actin or microtubules. To test the effect of vimentin inhibition on contractile forces, Costigliola et al., 2017 fluorescently labelled vimentin using TALEN genome editing, which allowed for vimentin structure to be observed and expressed in non-immortalized human foreskin fibroblasts, shRNA mediated inhibition of vimentin leads to an increase in contractile forces, which was measured by TFM (Costigliola et al., 2017).

Vimentin controls cell motility, cell adhesion and is therefore used to understand the underlying mechanisms active between cell adhesion and contractile forces. Peschetola et al., 2013 showed that measuring the traction forces is an effective method to distinguish between different cancer cell lines invasive potential. In this study, 2 epithelial bladder cancer cell lines, one more invasive (T24), and a less invasive one (RT112) were tested. Results showed that T24 cells show bigger focal complexes, thus allowing for a more efficient migration of cancer. RT112 cells developed a less focal complexes on soft gels and move more slowly. This is measured through the Cell Migration and Mean Displacement (MSD) method and was found that more invasive cancers have increased cell migration and cellular contractility on soft substrate (Peschetola et al., 2013). In Rönnlund et al., 2013, the protein distribution patterns in metastasizing cells were used to compare the spatial distribution of cell adhesion sites and vimentin filaments using ultrahigh resolution stimulated emission depletion (STED) microscopy technique. Results found that cancer cells had a significantly higher density of focal adhesions leading to increased tumour cell metastasis (Rönnlund et al., 2013).

1.4 Actin, Myosin, and the Myosin Cycle

The actin cytoskeleton has an important role in cell mechanobiology and in cellular contractile force generation, in both 2D substrates and 3D matrices. This is in addition to its actions of regulating branching, elongation and crosslinking of actin with contractile filaments. There are several molecular mechanisms influenced by actin and myosin that control contractility (Menhofer et al., 2014).

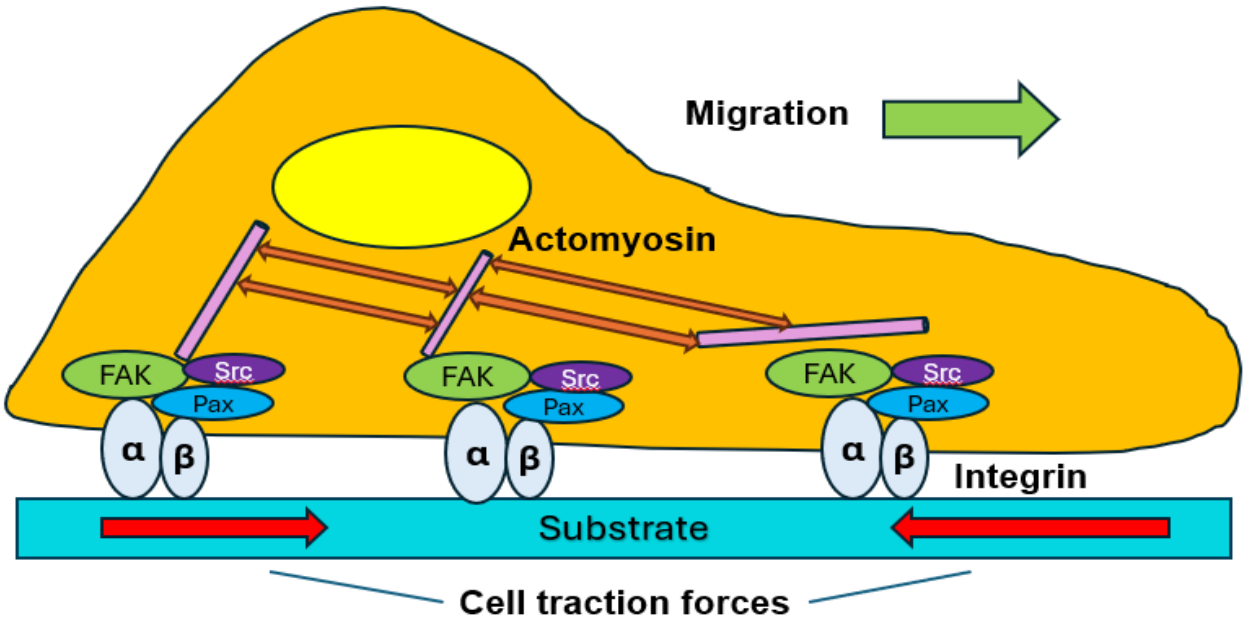


Figure 1.7: Overview of Traction Force Generation via Actomyosin (Adapted by Azeer Zafar from Wang & Li, 2009 and prepared using Microsoft PowerPoint)**.**
The figure shows how the interactions of actomyosin with focal adhesions produces cellular forces.

Traction forces are generated through myosin-actin interactions. Actomyosin is the combination of actin and myosin within the cytoskeleton, which makes it contractile. Myosin is a protein that generates force by converting chemical energy to mechanical energy and can pull on actin filaments. In non-muscle cells, this occurs due to the interaction of actin filaments with the molecular myosin II and produces contraction, which is how traction forces are generated. The force produced by actomyosin in the cytoskeleton is responsible for processes like traction force, cell signalling and cell adhesion (Kollimada et al., 2021) (Weißenbruch & Mayor, 2024) (Wang & Li, 2009) (Figure 1.7). Myosin phosphatase is an enzyme that

dephosphorylates the myosin II light chain, undoing the contraction force generation process (Murrell, Oakes, Lenz and Gardel, 2015).

One mechanism mediates the knockdown of Calponin-3 (Cnn3), an actin-binding protein that is associated with stress fibres. Stress fibres are contractile bundles of actin that are composed of actin and myosin II and these fibres are present in non-muscle cells. These stress fibres promote the assembly and contractility of non-muscle cells. Ciuba et al., 2018 mediated knockdown of Cnn3 using CRISPR, which resulted in an increase in contractility of stress fibres. Therefore, Cnn3 is essential for controlling contractile forces within the stress fibre network. This upregulation of contractility is achieved by Cnn3 restricting the movement of myosin II molecules along the actin filament in stress fibres (Ciuba et al., 2018).

The actin cytoskeleton is an essential component of metastasis as it participates in both cancer cell invasion and migration (Shankar & Nabi, 2015). Another mechanism mediates the polymerization of actin using Chondramine, which is an actin binding compound that acts as an anti-metastatic agent. Chondramine treatment inhibits metastasis in lung cancer. Chondramine mediated polymerization of actin decreases contractile forces of highly invasive cancer cells and decreases the activity of RhoA Signalling. Thus, Chondramine inhibits the pro-contractile signalling cascade (Menhofer et al., 2014).

Joo and Yamada, 2014 proposed that there is an equilibrium between contractility and regulation of myosin II. This equilibrium, which is the effect on contractility by myosin is mediated by myosin phosphatase. Therefore, by using blebbistatin to mediate inhibition of myosin in human foreskin fibroblasts, results showed a decrease of contractile forces as measured by TFM (Joo and Yamada, 2014). Kraning-Rush et al., 2011 treated highly metastatic MDA-MB-231 carcinoma cells with blebbistatin to inhibit myosin and analysed for changes in cellular contractility. In order to quantify contractile force, TFM was used for measurement in both 2D and 3D substrates. Results showed that the inhibition of myosin in either microenvironment decreased cellular contractile forces (Kraning-Rush et al., 2011).

Blebbistatin (myosin inhibitor) mediated inhibition of myosin in Ras transformed NRK cancer cells results in a decrease in contractile forces (Helfman and Pawlak, 2005). Similar results were observed in another experiment that used ML-7, which is also an inhibitor to mediate

decrease in activity of actin, which also resulted in decreased contractile forces (Busche et al., 2008) (Figure 1.8) (Schierbaum et al., 2019).

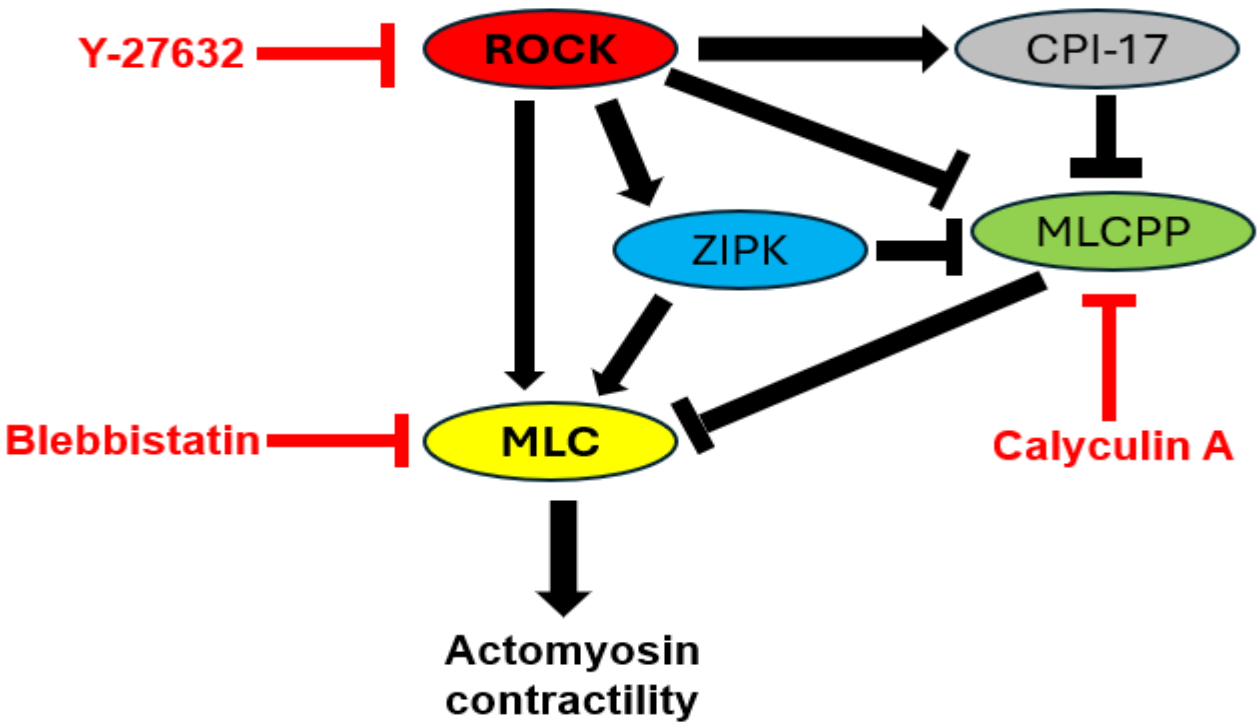


Figure 1.8: Overview of Actomyosin mediated Contractility via MLC (Adapted by Azeer Zafar from Busche et al., 2008 and prepared using Microsoft PowerPoint).

The figure shows the mechanism of the myosin regulatory light chain mediated (MLC) actomyosin generated cell contractility and the effect of blebbistatin on this mechanism.

Contractile forces are generated via the phosphorylating action of the MLC. This phosphorylation leads to actomyosin mediated contractility and myosin activity. Several signalling pathways react with the phosphorylated MLC and generate the formation and increase of contractile forces. The primary pathways of MLC phosphorylation are via MLC kinase and ROCK signalling (Figure 1.8) (Holz, Apel and Hassel, 2020).

1.5 Rho GTPases and Rho-ROCK Pathway

The Rho family of GTPases, is a family of guanine nucleotide-binding proteins involved in the mechanisms that regulate cell contractility (Boureux et al., 2006) (Figure 1.9). A previous study has shown that the contractile forces of cells are increased by RhoA and reduced by Rac1 (Vega and Ridley, 2008). RhoA positively promotes myosin regulatory light chain (MLC), increasing actomyosin mediated contractility with the help of F-actin and activity of myosin II via Rho kinase (ROCK) activity, while Rac1 inhibits MLC. This makes the activities of RhoA and Rac1 mutually inhibitory (Vega and Ridley, 2008).

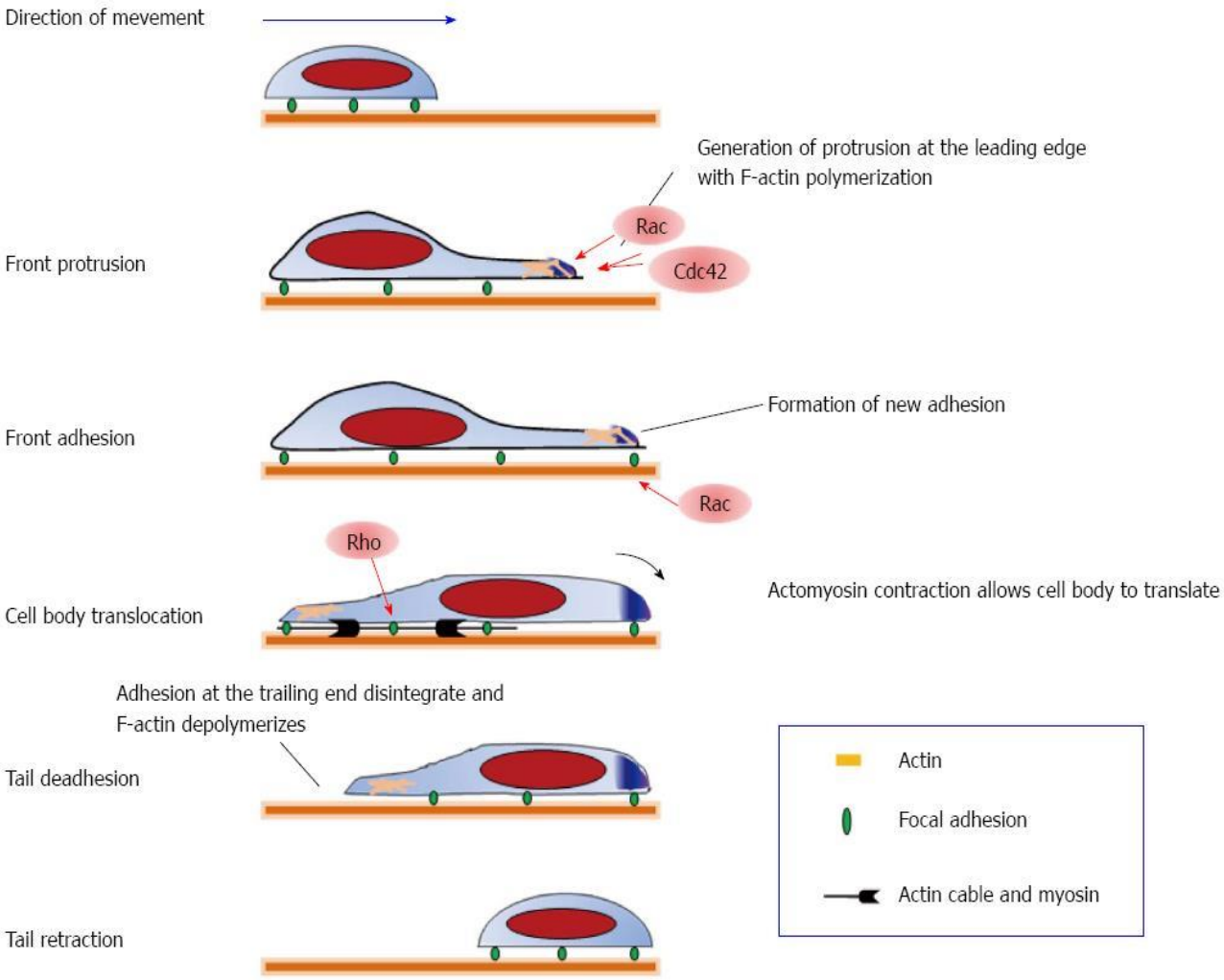


Figure 1.9: Overview of Cell Contraction and Migration via Rho-Rac. (Matsuoka, 2014)
The figure shows how cell migration takes place via the actions of Rho-Rac which generates cell adhesion.

Cells use at least two different methods to exert cellular contractility. As explained by Gad et al., 2012 in the first method, activation of RhoA results in reduced cell spreading, while in the second method Rac1 is activated resulting in increased cell spreading. Active mutant variants, like RhoAV14, Rac1L61 and RhoDV26 were used in the experiment to obtain results. These showed that RhoAV14 produced greater increases in cellular contractility, while Rac1L61 and RhoDV26 show lesser increases when compared to Rho AV14. Also, a more even spread and higher density of focal adhesions results in increased contractile forces of cells (Gad et al., 2012) (Lessey et al., 2012).

Activating the ROCK signalling pathway mechanism in cancer cells is involved in cellular contractility. McGrail et al, 2014 showed that in Metastatic ovarian cancer, cells are very malignant due to increased chemoresistance, and cells prefer to adhere to soft microenvironments. On soft matrices, lysophosphatidic acid (LPA) mediates activation of ROCK in ovarian cancer cells. treatment with Y27632 (ROCK inhibitor) reduces contractile forces, which suggests that the ROCK pathway is essential for cell contraction (McGrail, Kieu and Dawson, 2014).

Another mechanism is the disruption of the cytoskeleton via RhoA-ROCK pathway. Kim et al., 2019 showed that in oral squamous cell carcinoma (OSCC), cancer-associated fibroblasts increase carcinogenesis and disrupt the cytoskeleton via RhoA-ROCK pathway. Comparing the matrix remodelling capacity between YAPS127A and WT of Yes-associated protein in hTERT-hNOFs cells using a 3D ECM-remodelling assay, showed increased cellular contractility, tissue stiffness and promoted the migration and invasion of OSCC cells (Kim et al., 2019).

The primary reason that RhoA activity is so potent in inducing contractile forces in cells is because it has the ability to induce all the three processes, which are polymerization of actin, linking of F-actin, and sliding of actin filament due to actomyosin that are known to control cellular contractile forces (Gad et al., 2012). Actomyosin sliding is the process of driving contraction in actomyosin. Myosin II that is regulated by the ROCK pathway is due to the regulation of contractile forces in fibroblast cells. The Rho-dependent pathway regulates the contractile force output in a cell (Beningo et al., 2006). In addition to the mechanisms and factors described above that play a role in mechanical properties of cells, the tissue surrounding cells also plays a role.

1.6 Extracellular Matrix

The extracellular matrix (ECM) is a group of molecules released by cells, and which then surrounds the cells in tissues. Their primary function is to provide structural support for the cells that they surround while also helping these cells to perform their assigned functions. There are several components of the ECM which all combine together to form a structurally strong structure, which then reacts with mechanical properties allowing for cell migration, cell adhesion, contractility, stiffness among others (Theocharis et al., 2016) (Bonnans et al., 2014) (Figure 1.10).

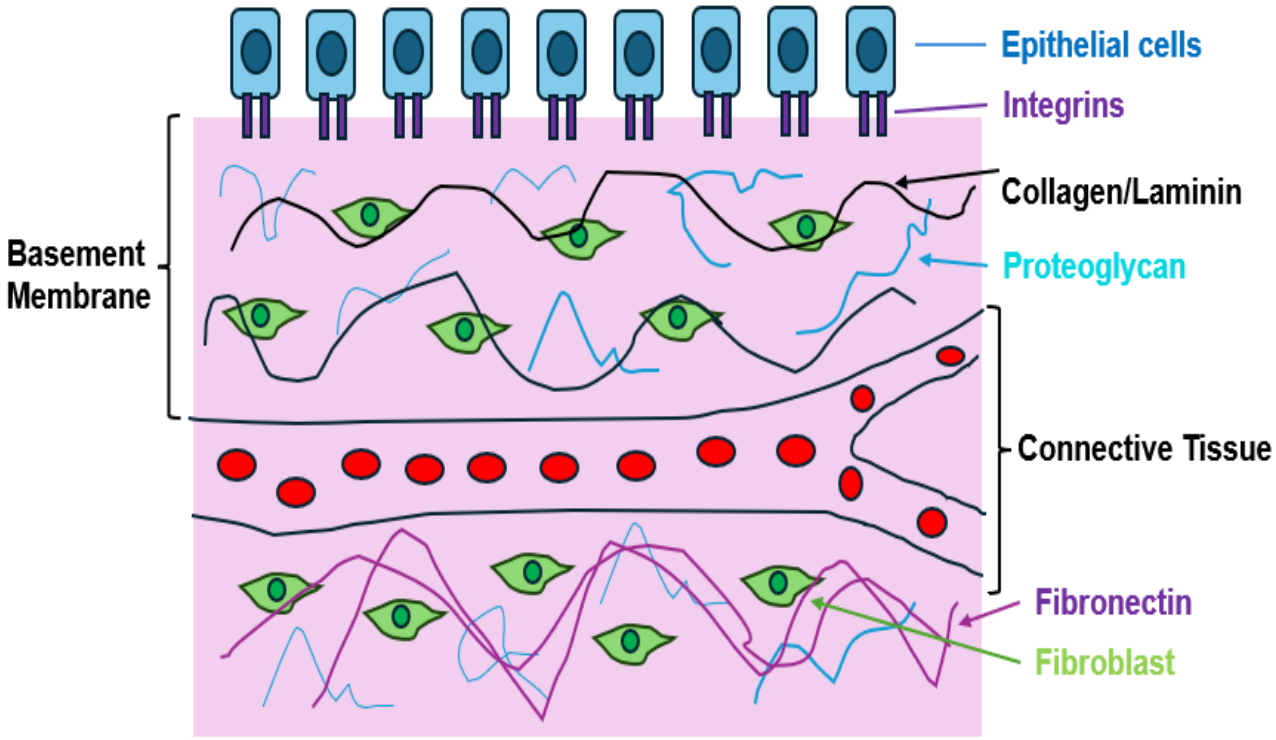


Figure 1.10: Overview of the Extracellular matrix structure (Made by Azeer Zafar using Microsoft PowerPoint)

This figure shows the cross section of the Extracellular matrix as well as all the levels and components that make up the ECM, which are also explained below.

The ECM has two primary components which are (Theocharis et al., 2016):

1. **Basement membrane:** This refers to the ECM present between the epithelial and stromal layers of cells.
2. **Interstitial matrix:** This refers to the layer of ECM that surrounds the cells forming a porous 3D lattice.

The basement membrane (BM) is a thin layer of extracellular matrix that is present between the epithelial and endothelial cell layers. It surrounds the muscle, fat, and nerve cells in the body providing structural support, aiding in migration, cell differentiation and also separates different cells from each other (Pozzi et al., 2017). There are several different types of proteins that make up the basement membrane which include:

Table 1.1: Proteins and Cellular Receptors in Basement Membrane (Pozzi et al., 2017)

Protein Type	**Cellular Receptor**
Collagen IV	Integrins
Collagen XVIII	Heparan Sulphate Proteoglycan
Fibulin	Integrins
Nidogen	Integrins
Laminin	Integrin
Perlecan	Dystroglycan

ECM is composed of a mixture of three main types, which are (Theocharis et al., 2016):

1. Glycosaminoglycans (GAGs) and proteoglycans, which assist the cell in resisting compressive forces.
2. Glycoproteins (laminin, fibronectin)
3. Fibrous proteins, which are involved in providing cell with tensile strength (collagens, elastin)

1.6.1 Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) are linear polysaccharides. They are signalling molecules that are involved in several different cellular procedures such as in the production of cytokines, the recruitment of leukocytes and dealing with an inflammatory response among others that are crucial for cell survival. Examples of GAGs include sulphates such as chondroitin, keratan, dermatan, heparan and hyaluronan (Gandhi & Mancera, 2008).

1.6.2 Glycoproteins

These are cell adhesion proteins, which include Fibronectin, which connect cells with collagen fibres in the ECM, allowing cell movement through the ECM and Laminin proteins, which are present in the basal laminae and assist in cell adhesion (Spiro, 1973).

1.6.3 Fibrous Proteins

Collagen is an especially important structural component of the ECM. It is the most common protein that is present in the skin and bone, making up around 30% of their total protein. Collagen is essential in providing support for laminin, proteoglycans, and cell surface receptors (Lewin, 2007). There are 28 different types of collagens (1-28) have been studied so far with further research being done on collagen (Parry et al., 2017). Collagen are divided based on their structure, which include:

- Fibrillar (1, 2, 3, 5, 11, 24, 27) – Over 90% of collagen in the body is fibrillar in nature. It is mainly present in the bones, skin, tendons, ligaments, and cartilage. This type of collagen has a diameter of between 11-30 nm in diameter and fully formed collagen fibers have a diameter between 400-3000 nm.
- Fibril-associated (9, 12, 14, 16, 19, 20, 21, 22) – These collagens attach themselves to fibrillar collagen and are believed to assist in helping the collagen fibres to maintain their structure within the ECM.
- Beaded filament (6)
- Network-forming collagen (4, 8, 10) – These collagens form net-like structures especially in the basement membrane where they link with anchoring fibres (type 7) linking the basement membrane, ECM and collagen.
- Collagen (4) – This collagen assembles itself and is crucial in the formation of the basement membrane.

- Transmembrane collagens (13, 17, 23, 25) – These collagens have important roles in maintaining structure shape and integrity, cell adhesion, cell differentiation and in development of tissues. (Parry et al., 2017) (Lewin, 2007)

1.7 Cell Migration

Cell migration is defined as the specific movement of cells in one or multiple directions independently by itself or by the actions of an external force or other factor. These factors or factors can be either mechanical or chemical in nature. The migration of cells through the ECM is regulated by the interaction between several different forces and frictional forces. Cell migration is crucial for maintaining the proper structure of multicellular organisms and allowing them to move (Mak et al., 2016). Cells migrate due to the actions of external signals like chemical and mechanical signals. It is important to recognize the mechanism by which cells migrate as this can lead to developing new therapeutic methods such as the control of invasive tumour (Swaney et al., 2010) (Trepat, Chen and Jacobson, 2012). The migration of the cells via the tissue matrix is essential for several different body functions such as healing, division among others but also for the invasiveness and metastasis of cancer cells (Trepat et al., 2012).

Cell migration on 2D surfaces, like plastic tissue culture dishes, involves several processes such as polarization, mechanical force at the leading edge of the cell, generation of contractile forces via the actomyosin, and mechanical forces at the rear edge of the cell. Cell traction forces are essential for the invasiveness of cells and to cross the barrier of the integrin-mediated adhesion. The migration of cells through a thick 3D network of ECM proteins requires a much greater force than migration through a 2D environment and therefore can only occur if the cell produces sufficiently higher traction forces and are able to cross the barrier of the surroundings (Pawluchin & Galic, 2022). The speed of migration of cells in a 3D network is proportional to the maximum traction forces that these cells are able to exert on their surroundings. However, it is important to note that when the contractility of actomyosin is inhibited, cells lose the ability through a 3D network (Yamada & Sixt, 2019). This suggests that the metastasising cancer cells which produce higher traction forces become more invasive and proliferative when compared to those cells with lower traction forces. Most cells have the ability to adhere and migrate on both 2D surfaces in the ECM and 3D networks as long as the network is of a suitable stiffness. (Koch et al., 2012).

Cell traction in 2D surfaces is determined by imaging the movement of fluorescent beads which have been embedded in a flexible polyacrylamide gel of specific stiffness on which the cells are cultured. These same beads can also be spread through a 3D network and used to measure cellular contractility during migration using the same principle as for 2D surfaces (Maskarinec

et al., 2009). However, migration in 3D networks is more complex than in 2D surfaces since in order for cells to migrate through a 3D network, cells must overcome both the adhesion forces like the 2D surface but also the frictional force being exerted in the surrounding matrix of the 3D network. (Wang & Lin, 2007) (Butler et al., 2002).

One study Koch et al., 2012 showed that invasive metastasising cells exerted great forces on their environment. This study showed that the traction force exerted by MDA-MB-231 breast carcinoma cells in a soft (118 Pa) 3D polyacrylamide gel was more than 40 times than that exerted on a cultured on harder (4.8 kPa) polyacrylamide gel. These results were surprising since most studies like (Köser et al., 2011) and (Nagayama et al., 2004) among others listed later on show that most cell types in most cases have increased contractile forces on stiffer surfaces. One potential explanation for this could be that the structure of the surrounding matrices might affect the ability of cells to generate tractions which is not very well understood. However, large contractile forces alone are not enough for cell invasion to take place since studies also show that several non-invasive carcinoma cells also have the ability to generate large contractile forces. In order for greater cell invasion to take place, the direction in which the traction forces are also particularly important and also need to be considered along with the size of the traction forces. Cell invasion is more effective if traction forces are directed in a specific direction (Koch et al., 2012).

The cytoskeletal model describes the process of cell migration. This model states that the cell has a leading edge and a tail. Actin polymerization in the leading protrusion at the front end of the cell results in the generating traction forces involved in cellular motility and cell migration generation. However, the tail counteracts the contractile forces that are needed for forward movement during cell movement. This action results in the formation of cell traction forces and if the force applied by the leading protrusion is greater, cell migration and cell movement will take place (Sun & Zaman, 2016) (Fig.1.11). The basic model used to describe cell migration consists of 3 simple steps: the formation and expansion of a lump at the leading edge of the cell, the sticking of the leading edge to the substrate via focal adhesions, and the contraction of the rear edge of the cell against the substrate to generate traction (Maurin et al., 2008) (Svitkina, 2018). This 3-step cycle was first described by Abercrombie in 1980 and has remained mostly unchanged in all this time.

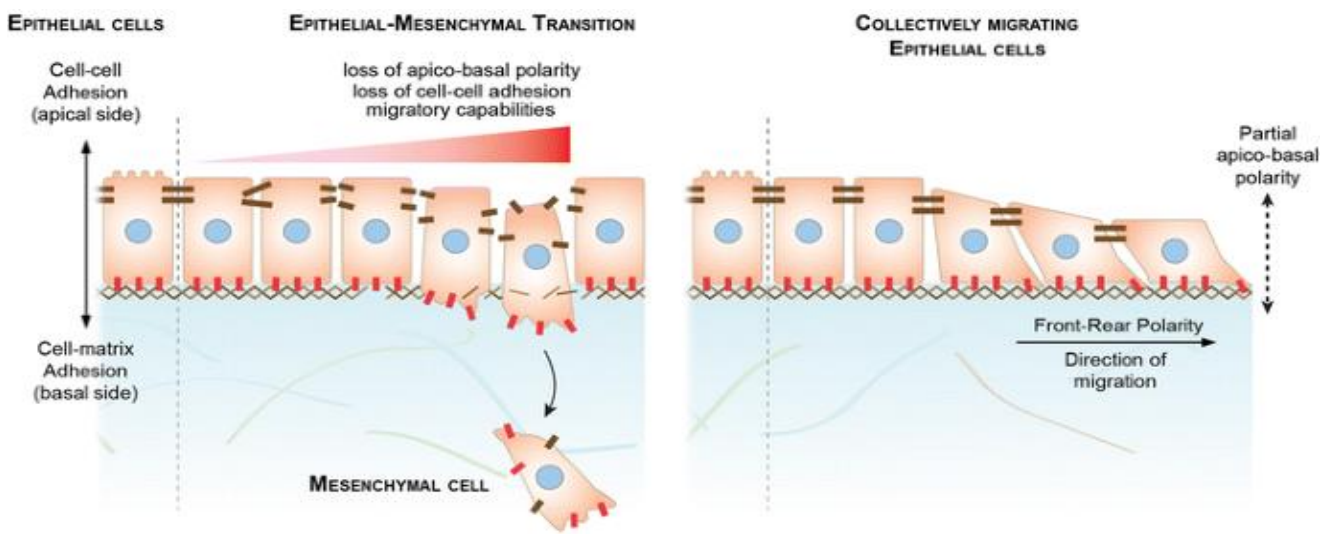


Figure 1.11: Cell Migration (Theveneau and Mayor, 2013)

Figure shows action of the cell traction forces on an ECM substrate surface, allowing for cell migration. The cell has a leading-edge protrusion and a tail. In the leading protrusion, actin polymerization generates force that is counteracted by the tail. This friction results in the formation of cell tractions forces and cell migration if leading force is greater.

Migrating mesenchymal cells express polarity at the front and back edge of the cells. Polarity is essential to determine the direction of travel for a cell and without polarity a cell would begin moving in all directions at once or cell spreading, which is what transformed cells are known to do (Sun & Zaman, 2016). This polarity acts at a molecular level since certain molecules are restricted from moving to specific regions of the inner cell surface. An example of this is that the activated Rac and CDC42 are present at the anterior end of the cell, whereas RhoA is found towards the posterior end of the cell (Ridley, 2003).

It is important to note that the cell migration listed above is mesenchymal migration which is only one type of cell migration. However, there are also other types of cell migration, which are described below in the following sections.

1.7.1 Single Cell Migration

There are 2 different types of migration that involve single cells, which are amoeboid and mesenchymal migration. In amoeboid migration, similar to an amoeba the cells which use this type of migration have a blebby constitution and these round or elliptical cells migrate without the help of any focal adhesions or protrusions such as filopodia (Sadjadi et al., 2022). Without any assistance, these blebby cells cannot migrate via adhesion or attaching on the substrate but instead propel themselves. Examples of this type of migration include leukocytes migrating through ECM (Mak et al., 2016).

Another form of ameboid migration is the pseudopodal ameboid migration. This migration occurs via the action of filopodia present at the leading edge of the cells and the displacement of the cells due to weak interactions between the cell and substrate. Examples of this type of migration include neutrophils and dendritic cells (Sadjadi et al., 2022).

Mesenchymal migration is characterized by migration via the action of numerous focal adhesions and actomyosin contractility of elongated, spindle-like cells. Examples of this type of migration is seen in cells such as fibroblasts and sarcoma cells (Bear & Haugh, 2014).

1.7.2 Collective Cell Migration

This type of cell migration is collective in nature since it involves a collective and united migration of epithelial cells, which are all interacting with each other in order to migrate. Various types of cell adhesions include junctions like adherens, tight, gap and desmosomes among others are essential to facilitate this type of migration and they also manage the movement of cells through the cell barrier (Rørth, 2009).

Examples of collective epithelial cell migration include the angiogenesis of blood vessels via the formation of blood vessel branches as well as the branches that are formed due to the formation of mammary glands. Not all the movement in this migration is collective as seen in *Drosophila* egg chambers or metastasising groups of invasive cancer cells where epithelial cells migrate separately instead of collectively. Other examples include the migration of

epithelial cells via branched strands stretching out of a tissue, which are most commonly seen in invasive cancers (Ilina & Friedl, 2009).

The most well-known form of collective migration in epithelial cells is 2D sheet migration. This involves cells migrating on 2D surfaces that are interconnected. Examples of this type of migration occur during wound healing, both in skin and in other epithelial tissues such as the intestine and cornea. The primary connected mechanism between all these types of collective migrations is that they all function by the mechanical connecting of the cells via stable cell-cell adhesions. These cell adhesions improve cytoskeletal activity and coordination between all the cells that are involved. Due to this migration, the group of cells involved can develop a directional polarity at the collective level, similar to the polarity that is required for migration at the single-cell level (Yamada & Sixt, 2019).

1.8 Focal Adhesions

Focal adhesions (FAs) are structures made up of several different proteins, and they are responsible for binding several different types of cells and actin filaments with the ECM surface. This process of FA linking does not occur in a single step but instead occurs via a series of steps which include the initiation, grouping, growth, spread and disassembly (Chen et al., 2003). In tissue 2D surfaces, FAs are located at the bottom of the cell surface and are more popularly known as the "feet of the cells" due to their positions and also allow for the interaction and exchange of materials between the cells and the ECM. A study by Zamir & Geiger, 2001 shows that new FAs grow and increase in size at the leading edge of the cells. Also, it is important to note that FAs are dynamic in nature and constantly moving since cells are continuously attaching and detaching from the ECM surface and FAs are the contact points that regulate all these processes (Wehrle-Haller, 2012). Some of these processes include cell linking since they allow cells to link with the ECM substrate or cell spreading since they promote traction at the leading edge of the cells and increase invasiveness of metastasising cells (Zamir & Geiger, 2001) (Zaidel-Bar et al., 2004).

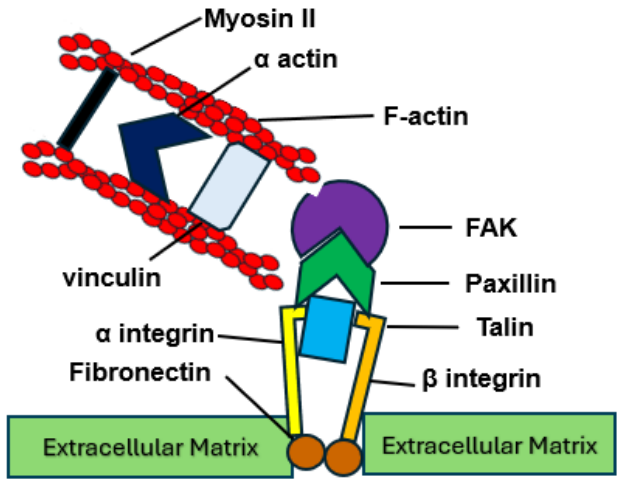


Figure 1.12: Structure of Focal Adhesion (Adapted by Azeer Zafar from Van Tam et al., 2012 and made using Microsoft PowerPoint)**.**
The figure shows the internal structure of the focal adhesion along with all the different components that allow focal adhesions to function.

As mentioned before, FAs are very dynamic in nature since they are constantly growing and shrinking because of the binding of their component proteins (also called as "plaque proteins"). This change in size of FAs occurs due to differences in physical properties such as traction forces, cell adhesion or stiffness. FAs have been observed to move from the edge of the cell towards moving in the centre while the cell also moves over the FAs (Petit & Thiery, 2000). However, despite this movement FAs are still primarily stationary structures when compared relative to the underlying substrate and only become dynamic during attachment, disassembly or sliding based on the nature of the surrounding microenvironment (Besser & Safran, 2006). FAs

form complexes that consist of multiple proteins and perform several functions which occur via the contact of the cells and the ECM. Actin is also involved in this process due to its structure and functionality (Bachir et al., 2017). FAs are always located at the end of stress fibres and are a crucial part of the cytoskeleton (Figure 1.12). Using their positions, FAs transmit forces when they bind cells to the ECM and this force is transmitted through the cytoskeleton network via adhesion receptors.

1.8.1 Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine-based kinase involved in cancer proliferation. It also combines environmental signals from the ECM and the mechanical forces. FAK transmits signals which are derived from several different cell functions such as cell adhesion, cell proliferation and cell migration. Studies show FAK is overexpressed in several cancerous tumours when measured in vivo (Zhou et al., 2019). Since FAK is a scaffolding protein, it interacts and regulates the activity of several other signalling kinases which include p53, IGF-1R, VEGFR-3 and PI3k among others. This regulation then leads to FAK being controlled by other regulators and is an interesting target for cancer treatment and cure research (Zamir & Geiger, 2001).

Studies like Zhang et al., 2022 also show that FAK is essential in cancer metastasis due to its role in the interaction of ECM and integrin. These studies showed that increased expression of FAK leads to higher cancer progression, invasion and infiltration leading to increased mortality in most cancers. Increased expression of FAK has also been measured in cancer cells that are resistant to drugs while inhibiting FAK in these studies has shown to reduce drug resistance in cancer cells making studying FAK an ideal target for a potential biomarker or for the development of cancer treating drugs (Schwock et al., 2009) (Madan et al., 2006). Based on these results, studies have concluded that FAK is involved in making cancer cells drug resistant via the role of mechanical pathways (Zhang et al., 2022).

1.8.2 Role of Focal Adhesions in Cellular Migration

FAs have important roles in cellular migration and are involved in the retrograde flow of actin. This retrograde flow is when actin filaments form a protrusion at the leading edge and use the force that is generated there to flow back towards the centre of the cell structure (Zhao & Guan, 2011). FAs are assembled in a highly compact manner that assists with the retrograde flow of actin. It is this phenomenon that produces traction forces during cell migration and FAs allow

cells to attach to the ECM preventing retrograde flow from taking place, which in turn produces traction forces allowing cell movement to take place. These traction forces can be visualized via TFM technique, and these forces are essential for the growth, movement and spread of focal adhesions (Yamashiro & Watanabe, 2014) (Guo & Wang, 2007) (Burridge & Guilluy, 2016).

Another factor of FAs that is crucial in cell migration is the way in which FAs are assembled. Since FAs are dynamic, their assembly or disassembly during cell migration allows FAs to change their composition and structure. The way that this occurs is first, FAs are formed at the cell's leading edge, but these FAs are small in size. Most FAs are unable to mature and grow causing them to be disassembled, however the few FAs that actually mange to mature assemble into a larger FA (Ezratty et al., 2005). At this stage, more proteins get involved as they are recruited in a straight manner. After all the components come together, FAs fix themselves in a stationary position and are attached to the ECM. This allows the cell to use the FA as an anchor and induce cell migration for back-and-forth movement. Once the cell has moved to its desired location the FA moves to the edge of the cell and is destroyed since its role is complete and the whole cycle is repeated with another FA (Ezratty et al., 2005) (Wu, 2007).

Studies propose that drug-resistant metastasising cells have highly increased stiffness and contractility, and this is linked to the reorganization of the actin fibre structure. Therefore, it is important to research whether these mechanical changes are due to the maturation of FAs, due to their linking of the cells with the ECM. Large, matured FAs lead to the development of increased actin fibres and play a part in the increased mechanical reinforcement of cells.

One study Park, 2018 looked at vinculin, which is a protein that works with FAs and is responsible for the accumulation of integrins. Research shows that if this vinculin is lost, there is inhibition of cell adhesions, cell migration and cell invasion. The study measured that the expression of vinculin in breast cancer cells that were resistant to drugs was double when compared with the normal cells. This could be because of the upregulation of vinculin due to the function of FA, and this made the drug-resistant breast cancer cells less contractile than the normal cells. It could be possible that an increased expression of vinculin would participate in the formation of increased actin stress fibres, and this is what leads to the increased mechanical stiffness and contractility in resistant breast cancer cells (Park, 2018).

1.9 Cellular Adhesion

Cell adhesion can be defined as the method by which cells adhere to each other with the help of different components either directly or indirectly (Alberts et al., 2015). The components involved in cell adhesion consist of cell-adhesion molecules (CAMs) (Lodish, 2008). Cell migration is another cell function that is regulated by cell adhesion. Any changes or problems in cell adhesion disrupts critical bodily functions and can cause diseases like cancer (Alberts et al., 2015).

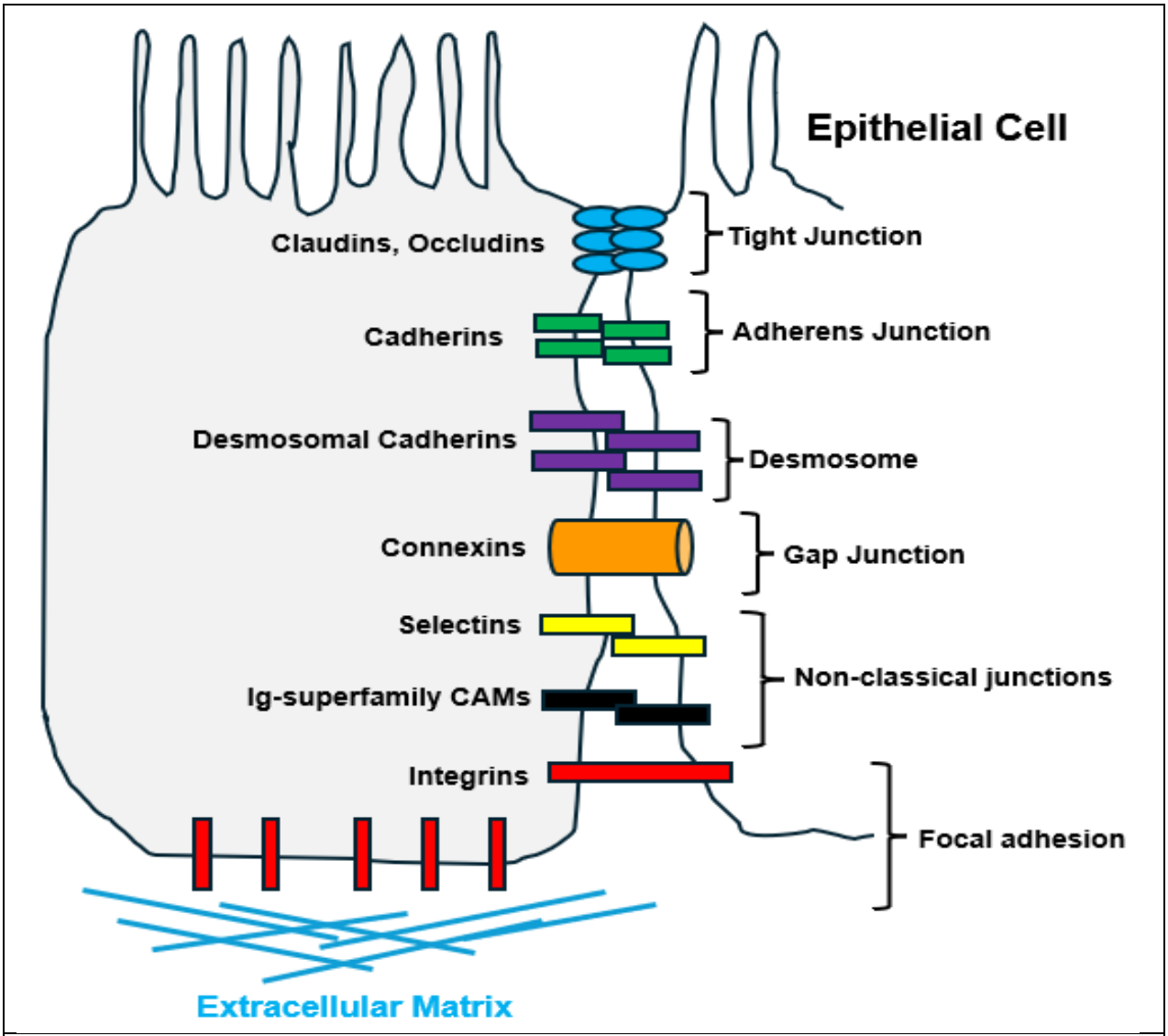


Figure 1.13: Overview of Cell Adhesion Molecules (Adapted by Azeer Zafar from Schnell et al., 2013 and made using Microsoft PowerPoint)**.**

The figure shows a cross section of the different components that are involved in the process of cell adhesion along with all their different categories.

CAMs are divided into 4 separate groups:

1. Integrins
2. Immunoglobulin (Ig) superfamily (IgSF)
3. Cadherins
4. Selectins.

CAMs can be divided into homophilic CAMs, which bind to same kinds on another cell and include Cadherins and IgSF while the second kind is heterophilic CAMs, which bind to different kinds and include integrins and selectins. Every adhesion molecule serves a different function since they bind at different places and any defect in the binding of CAMs or problem in their expression leads to problems in cell adhesions. Since CAMs have so many functions at different levels, they are directly related and form part of a single adhesion network (Lodish, 2008).

CAMs have the ability to attach with one another, and this results in the creation of structures called Cell junctions. These cell junctions are further divided into 4 types based on their functions:

1. Anchoring junctions (some examples of these junctions include adherens, desmosomes and hemidesmosomes). These junctions improve contact between cells and improve adhesion. (Ferreira et al., 2014)
2. Occluding junctions (also known as tight junctions). They form barriers to diffusion via cell-to-cell adhesion. (Schneeberger & Lynch, 1984)
3. Channel-forming junctions (also known as gap junctions). They form bonds between adjacent cells in order for transport of molecules to take place. (Sosinsky & Nicholson, 2005)
4. Signal-relaying junctions. These are present in the nervous system (Alberts et al., 2015).

1.9.1 Cadherins

Cadherins are Ca2+-dependent proteins that are crucial in the development of junctional adhesions and regulate cell-cell adhesions, balancing cell cohesion, and regulating the structure of tissues. Cadherin structure is composed of many different domains, along with one signal sequence, a protein precursor, one transmembrane domain, and five ectodomains. Four domains bind to Ca2+ ions allowing cell folding and the fifth domain contains an N-terminal extracellular domain. All proteins that have one or more than one extracellular domains of cadherin are classified as a member of the cadherin family (Shapiro & Weis, 2009).

1.9.2 Immunoglobulin Superfamily

The immunoglobulin (Ig) superfamily (IgSF) as its name suggests comprises many different cell adhesion molecules (CAMs), all of which are involved in different functions. IgSF comprise of one or more immunoglobulin-like domains and bind with adjacent cells using CA2+ dependent pathway. Examples of IgSF CAMs include, neural CAMs (NCAMs), immunoglobulin-like CAMs (Ig-CAMs), intercellular CAMs (ICAMs) and vascular CAMs (VCAMs). The interactions between different types of IgSF CAMs lead to cell-cell adhesions from the leading edge while the cytoplasmic tail of these CAMs Interactions between the immunoglobulin CAMs leads to cell adhesion, while the tail end of these proteins helps with cell attachment (Janiszewska et al., 2020).

1.9.3 Integrins

Integrins are important receptors that transmit cell signals, make up several transmembrane glycoproteins and mediate cell-ECM adhesions. Integrins are made when the two subunits α and β join together. Subunits α and β are made up of one ectodomain, one transmembrane domain and a short tail domain in the cytoplasm. Both α and β together determine the binding regions for different proteins and the effect of the receptors. Integrins link the cytoskeleton of cells with the ECM microenvironment along with regulating critical cell functions such as migration and some signalling mechanisms (Garcia et al., 2017). Integrins also contribute to tumorigenesis and metastasis by making changes to the extracellular surroundings occurring during tumour proliferation. β1 Integrin and FAK play a critical role in the survival of dormant cells, and they also allow these dormant cells to switch to a proliferative state (Angulo-Urarte et al., 2020, Janiszewska et al., 2020).

1.9.4 Selectins

Selectins are cell adhesion receptors which are primarily present in leukocytes and endothelial cells and play an important role in metastasis. Selectins are made up of 3 different types, which include P-selectins, present in platelets, L-selectins in leukocytes and E-selectins in endothelial cells. Selectins are a family of specialised CAMs involved in transient cell–cell adhesion occurring in the circulatory system and also requires Ca^{2+} ions to function similar to cadherins (Garcia et al., 2017, Janiszewska et al., 2020).

One study showed that during metastasis, tumour cells expressed selectin on platelets, leukocytes, and on the endothelium. When P-selectin was absent, it prevented platelet-tumour cell from accumulating, and this increased metastasis. The study also showed that in addition to P-selectin, platelets also expressed CD40 on their surfaces, which accelerated inflammation and atherosclerosis (Läubli & Borsig, 2010). The study also showed that the deficiency of CD40 in the blood increased lung metastasis expression, which makes CD40 important in cancer. Studies have also shown that E-selectin promotes the adhesion of tumour cells and the spread of metastasis (Janiszewska et al., 2020).

1.9.5 Cell Junctions

Anchoring Junctions: The main CAMs in anchoring junctions are cadherins, which are important for cell migration and cell adhesion (Angulo-Urarte et al., 2020).

Adherens junctions are responsible for maintaining cell shape and ECM interaction through their domains. Proteins link cadherins to actin filaments, which is important for adherens junctions to facilitate cell adhesion. (Garcia et al., 2017).

Desmosomes have a similar structure to adherens but made up of different components and their primary function is to provide resistance against mechanical forces. Some cadherins such as desmogleins and desmocollins act slightly different in that they link themselves to IFs instead of filaments of actin (Angulo-Urarte et al., 2020).

Occluding Junctions (Tight junctions): They are located in epithelial and endothelial tissues, and they control the fluids in these tissues. They are formed by the binding of proteins on adjacent membranes. Just like anchoring junctions, the intracellular domains of these junctions' proteins bind them in clusters and join with actin filaments to maintain the structure of the occluding junction (Angulo-Urarte et al., 2020).

Gap Junctions: They are made up of proteins called connexins. Connexins from neighbouring cells align with each other and form channels. These channels move materials between the cytoplasm of two adjacent cells and also maintain the structure of cells. apart from holding cells together and providing structural stability like anchoring junctions or tight junctions. These junctions only allow the movement of specific materials and manage cell signalling by controlling the movement of molecules involved in cell signalling (Angulo-Urarte et al., 2020).

1.9.6 Effect of Cell Migration and Adhesion on Cancer cell Invasion

Tumour cells can invade the neighbouring tissues due to problems in their mechanical properties. These are controlled by cell surface adhesions and the filament cytoskeleton (Rönnlund et al., 2013). Cell invasion and movement of tumour cells of different levels of invasiveness can be studied based on the principles of TFM on soft substrates. The best targets for development of therapeutic drugs are FAs since they localize upstream however it is also important to consider the effect of downstream signalling since this might negatively impact the efficacy of any potential drugs that are developed (Eke & Cordes, 2015).

Remodelling of cell and ECM interaction along with Epithelial-to-Mesenchymal transition (EMT) results in an increase in motility and cancer invasiveness as well as degradation of ECM during the early stages of cancer progression (Yilmaz & Christofori, 2009). Several studies like Gatenby & Gillies, 2007 and Sousa et al., 2019 have shown that glycolysis is involved in the progress of cancer, with major roles in cancer cell invasion, ECM degradation, motility and increased invasion. By-products of glycolysis such as methylglyoxal and lactate induce EMT and increase cancer progression and invasion by activating, yes-associated protein (YAP) signalling and through the degradation of the ECM respectively (Sousa et al., 2019) (Gatenby & Gillies, 2007) (Akram, 2013).

During malignant invasiveness cells lose their dependence on interactions with the ECM and the signalling pathways that result from this. During this, E-cadherin are lost and since they are essential for cell-cell adhesion it leads to epithelial-mesenchymal transition making metastasising cells more motile and invasive. EMT in this state makes epithelial cells more mesenchymal, increasing cellular migration. This process is essential for the development of organs and healing of injuries, however in cancer, it is essential for metastasis and resistance

to cancer treatment. In tumours, EMT results in increased inflammation in the TME (Parekh & Weaver, 2009) (Mukherjee et al., 2022).

An important aspect of EMT is the "cadherin switching" in which E-cadherin decreases and N-cadherin increases. This results in adhesion to collagen in the ECM, while N-cadherin also activates the Rho GTPase signalling pathway and the Wnt signalling pathway (Loh et al., 2019). All of these pathways contribute to the aggressive tumour phenotype with the capacity to move from the primary tumour location to secondary locations. However, a recent study has shown that E-cadherin improves the survival factor in metastasis of breast cancer by restricting apoptosis. This cadherin switching improves cancer cell motility and also promotes cell proliferation in the blood, improving metastasis formation (Loh et al., 2019).

A study Peschetola et al., 2013 has shown that measuring the traction forces was useful to separate between various cancer cell lines of different invasive levels. In this study, 2 cancer cell lines, one more invasive (T24), and one less invasive (RT112) were evaluated. T24 cells showed larger focal complexes, which led to faster migration of cancer, while RT112 cells developed smaller focal adhesions on soft gels and migrate slower. This is measured through Mean Square Displacement (MSD) method and was found that more invasive cancers have increased cell migration and cellular contractility on soft substrate (Peschetola et al., 2013). Another study Schierbaum, Rheinlaender and Schäffer, 2019 combined atomic force microscopy (AFM) with traction force microscopy (TFM) to measure both elastic properties (using AFM) and contractile force (using TFM) on single cells. This meant that stiffness, fluidity, and net contractile movement could be directly measured using the 2 above techniques and used together give a clearer picture of a cell's mechanical movement (Schierbaum, Rheinlaender and Schäffer, 2019). Thus, the degree of invasiveness of cancer cells can be determined by measuring the mechanical forces of cells. This points to an interesting problem due to their opposite results related to cell migration and adhesion so warrants further research into the matter. It is possible that altering adhesion molecules expression might cause cancer cells to become more metastatic, however, reducing cell motility may prevent metastasis. A molecule known to promote cell motility is HDAC6, which is discussed in detail below.

1.10 Role of HDAC6 in Contractile forces of cells

1.10.1 Introduction to HDAC6

Histone deacetylases present in mammals consist of 18 HDAC isoforms that are divided into 4 classes based on their homology to yeast deacetylases. Class 1 includes 4 isoforms (HDAC I, II, III and VIII), while Class 2 consists of 6 isoforms and is sub-divided into Class 2A (HDAC IV, V, VII and IX) and 2B (HDAC VI and X), while Class 4 has just 1 isoform (HDAC XI). Each of the 11 isoforms depend on zinc since they all have a zinc-binding domain at their active site. (Fig 1.3) (Butler et al., 2010). Histone deacetylase 6 (HDAC6) is involved in transcription and the progress of the cell cycle. HDAC6 overexpression correlates with increased carcinogenesis and metastasis of cancer cells. HDAC also promotes cell motility and is involved in α-tubulin deacetylation (Ruijter et al., 2003). Unlike other HDAC isoforms which are located in the nucleus, HDAC6 is mainly located in the cytoplasm. HDAC6 was first studied in 1999 due to its link with the *Saccharomyces cerevisiae* histone deacetylase, HDAC1. The gene that encodes for HDAC6 is found in chromosome Xp11.22-23 in humans. HDAC6 has both a deacetylase activity and a strong affinity to ubiquitin (Grozinger, Hassig and Schreiber, 1999, Zhang et al., 2014).

Seigneurin-Berny et al., 2001 shows that the human HDAC6 protein consists of 1215 amino acids with a molecular mass of 131 kDa and contains several functional domains (Fig.1.14). HDAC6 has 2 nuclear export signals (NES), which is where the deacetylase activity takes place. HDAC6 also contains a Ser-Glu-containing tetrapeptide (SE14) which prevents HDAC6 from losing their position within the cytoplasm. The N-terminus of HDAC6 also contains a nuclear localization signal (NLS), which allows for the deacetylase activity to move between the nucleus and the cytoplasm. HDAC6 is different from other HDAC isoforms since it possesses two homologous catalytic domains (called DD1 and DD2; located at the N-terminus and at the centre), and both of these domains contribute to the enzymatic activity of HDAC6. However, it is only DD2, which is the domain with α-tubulin deacetylase activity that has been well-studied (Liang & Fang, 2019). All the HDAC6 inhibitors that have been developed function by targeting this domain. However, this can sometimes result in discrepancies between experimental observations, which are made with pharmacological inhibitors and genetic knockout approaches, because genetic knockdown targets both DD1 and DD2. Finally, HDAC6 also has a dynein motor-binding domain (DMB) and at the C-terminus there is a

unique ubiquitin-binding zinc-finger domain (BUZ), which allows HDAC6 to perform cellular functions that are non-enzymatic (Liang & Fang, 2019) (Seigneurin-Berny et al., 2001).

There are also several different substrates of HDAC6 including α-tubulin, heat shock protein 90 (Hsp90) and Cortactin (de Zoeten et al., 2011). Hsp90 is responsible for promoting transcriptional activation as it structurally facilitates the maturation of hormone receptors and some kinases (de Zoeten et al., 2011). HDAC6 is responsible for the activity of Hsp90 since knockdown of HDAC6 results in loss of Hsp90 activity and impaired nuclear translocation and transcriptional activation. Similarly, Cortactin is an acetylated protein that is overexpressed in several carcinomas. Knockdown of HDAC6 leads to increased acetylation of Cortactin, which prevents its translocation and impairs cell motility (Aldana-Masangkay, Sakamoto, 2011).

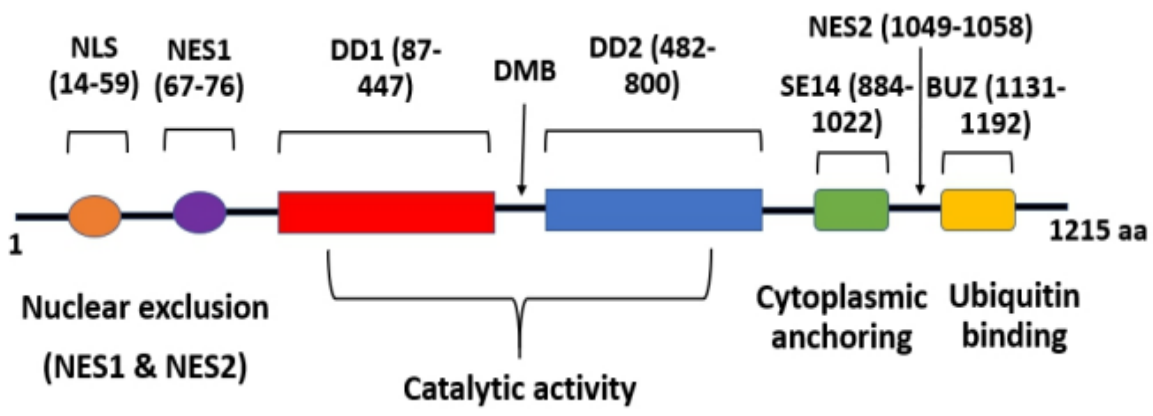


Figure 1.14: Structure of HDAC6 and its Functional Domains (Made by Azeer Zafar using Microsoft PowerPoint).

HDAC6 has two NES (NES1, NES2), a SE14 motif, an NLS at the N-terminal. There are two catalytic domains (DD1 and DD2) and a dynein motor-binding domain (Li, Shin and Kwon, 2012)

1.10.2 Importance of studying HDAC6

Histones are regulators of transcription and the progress of the cell cycle. The acetylation or deacetylation can also change the chromosome structure of the DNA. HDAC6, whose major substrate is α-tubulin, is an important target for drug development to treat cancer due to its major role in cancer cells (Kaur et al., 2022). This is why it is especially important to study the

effects of HDAC6 for cancer and to specifically clarify the role of HDAC6 in controlling contractile forces in cells along with the mechanism used by HDAC6 for this control.

Although other HDACs are responsible for the deacetylation of histones, however HDAC6 also affects nonhistone protein function. Evidence suggests that each HDAC is different from others in terms of substrates that they target and therefore the molecular and signalling pathways by which they function and regulate. However, HDAC6 is special among all the HDACs discovered so far, since it has a different structure and function from the other HDAC types. HDAC6 is quite different from the other HDACs in terms of its unique structure, localization, target receptors, functionality, its biological processes and the pathways it uses. HDAC6 regulates several important physiological processes and signalling pathways, which are involved in the various stages of tumour formation and progression including initiation, promotion, progression and metastasis (Li et al., 2018). These factors along with HDAC6's role as a key regulator of several signal pathways connected to cancer makes HDAC6 makes a great option for developing anti-cancer drugs as well as an option for developing HDAC6 inhibitors to target HDAC6 (Kaur et al., 2022). HDAC6 is an oestrogen-regulated gene and oestrogen-mediated up-regulation of HDAC6 shows a connection between the levels of HDAC6 expression and metastasis of breast cancer, which is important in prognosis of cancer patients. Increased expression of HDAC6 has been measured in several cancer types and mouse tumour models. HDAC6 expression was also measured in mouse embryonic fibroblast (MEF) cells, and results showed that Ras expression in cancer leads to up-regulation of HDAC6. Therefore, increased expression of HDAC6 in different kinds of tumours shows the important role of HDAC6 in cancer (Li et al., 2018) (Aldana-Masangkay and Sakamoto, 2011).

HDAC6 is a regulator in several different signal pathways involved in cellular growth as well as in cancer such as Ras, ERK, and Wnt signalling pathways among others (Jo et al., 2022). A study showed that the downstream activation of epidermal growth factor receptor (EGFR) is essential in cellular proliferation Therefore, cell invasion can be controlled by inhibiting the EGFR signalling. The action of HDAC6 via its deacetylase activity of α-tubulin results in EGFR breakdown. Therefore, HDAC6 inhibition, leads to the increased acetylation of α-tubulin, which leads to the deregulation of microtubule and further increase of EGFR breakdown, further reducing cellular invasion and making HDAC6's role important in reducing cancer proliferation and invasion (Gao et al., 2010).

In order to understand this role of HDAC6, different studies that compared the expression of HDAC6 in different types of cancers were looked at. One study Zhang et al., 2019 measured HDAC6 expression in colon cancer and analysed the difference in HDAC6 expression levels between cancer and adjacent healthy cells. The results of this study showed that cancer cells had higher HDAC6 expression than in the adjacent normal cells. This meant that patients that had a higher expression of HDAC6 had a worse prognosis. However, knockdown of HDAC6 resulted in decreased cell viability and the invasiveness of colon cancer cells. The study concluded that HDAC6 knockdown resulted in the reduction of cancer growth and invasion via the MAPK and vi pathway. (Zhang et al., 2019).

In addition, HDAC6 also regulates several important cellular processes and signalling pathways critical to homeostasis in normal cells as well as in cancer (Figure 1.15). Studies like Zhang et al., 2004 and Passaro et al., 2021 show that knockdown or inhibition of the function of HDAC6 promotes chemotherapy as well as radiation-induced apoptosis, autophagy, and senescence. Studies like de Zoeten et al., 2011 and Deskin et al., 2020 also show that inhibiting HDAC6 with tubacin or its silencing with HDAC6 siRNA knockdown induces Notch-1 signalling. Identifying proteins that interact with HDAC6 can help in understanding the role of HDAC6 in the body. These proteins range from Foxp3 in inflammation, GRK2 in cell signalling, p300 in transcription, Ku70 and PP1 in cell survival, Hsp90 in angiogenesis, tubulin for cell motility and VCP in protein degradation among others (Zhang et al., 2004) (Passaro et al., 2021) (Deskin et al., 2020) (de Zoeten et al., 2011).

HDAC6 is involved in cell migration regulating motility via tubulin and cortactin (Zhang et al., 2007). Microtubules (MTs) are involved in cell motility and are assembled by α-tubulin. The deacetylation of α-tubulin by HDAC6 affects the structure of MTs and this leads to microtubule depolymerization. ERK1 also phosphorylates HDAC6, which enhances the deacetylase activity of tubulin and leading to increased cell migration (Wu et al., 2018). Furthermore, the regulation of MTs and cellular motility by HDAC6 depends on the interaction between HDAC6 and GRK2, which is an HDAC6 stimulator. The regulation of α-tubulin by HDAC6 affects cellular motility since acetylation or deacetylation of HDAC6 affects the structure of MTs (Hubbert et al., 2002).

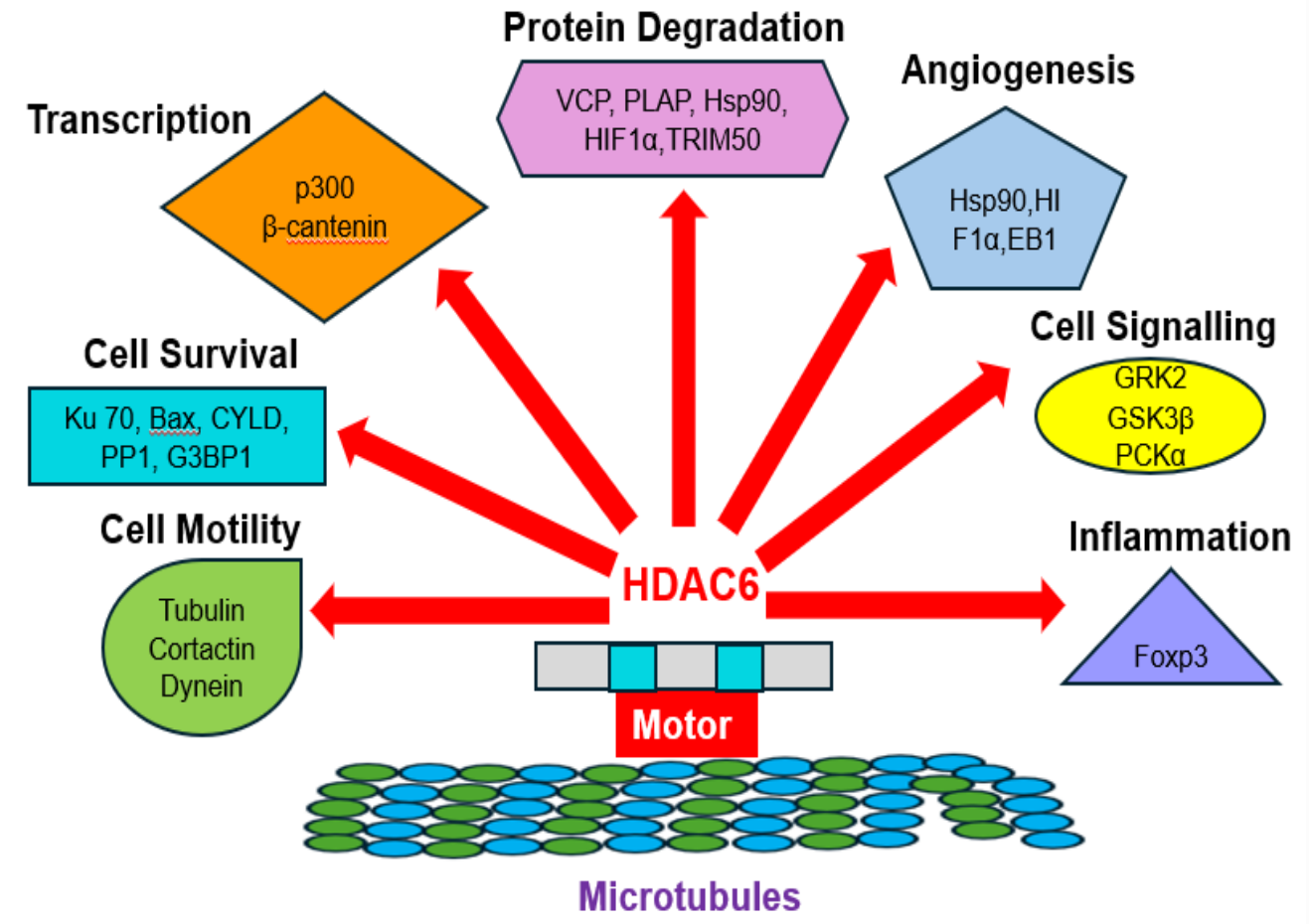


Figure 1.15: Overview of HDAC6 Functions in the Body (Adapted by Azeer Zafar from Li et al., 2012 and made using Microsoft PowerPoint)**.**

This figure shows all the different essential functions that require HDAC6 to function effectively in the human body and the proteins that are involved with HDAC6 to carry out these functions.

1.10.3 HDAC6 in Cancer

HDAC6 is becoming an extremely popular choice recently as a potential option for the treatment of cancer due to its well-researched role in cancer and cancer proliferation. Studies that are referenced in Table 1.2 show increased HDAC6 expression in several cancers including acute myeloid leukaemia (AML), colon cancer, breast cancer, ovarian cancer and human embryonic kidney cancer (HEK), among others and this increased expression correlates with advanced tumour stages and enhanced tumour invasion, increased invasive capacity of tumour cells and higher mortality rates. HDAC6 is involved in cancer since it performs important roles such as cellular proliferation, cell growth and invasion. HDAC6 expression is necessary for oncogenic transformation and tumour growth which are important hallmarks of tumour cells (Figure 1.16). This is because there is upregulation of HDAC6 in several tumour types and is required for faster tumour growth. Therefore, HDAC6 is an important target for developing anti-cancer drugs (Lee et al., 2008).

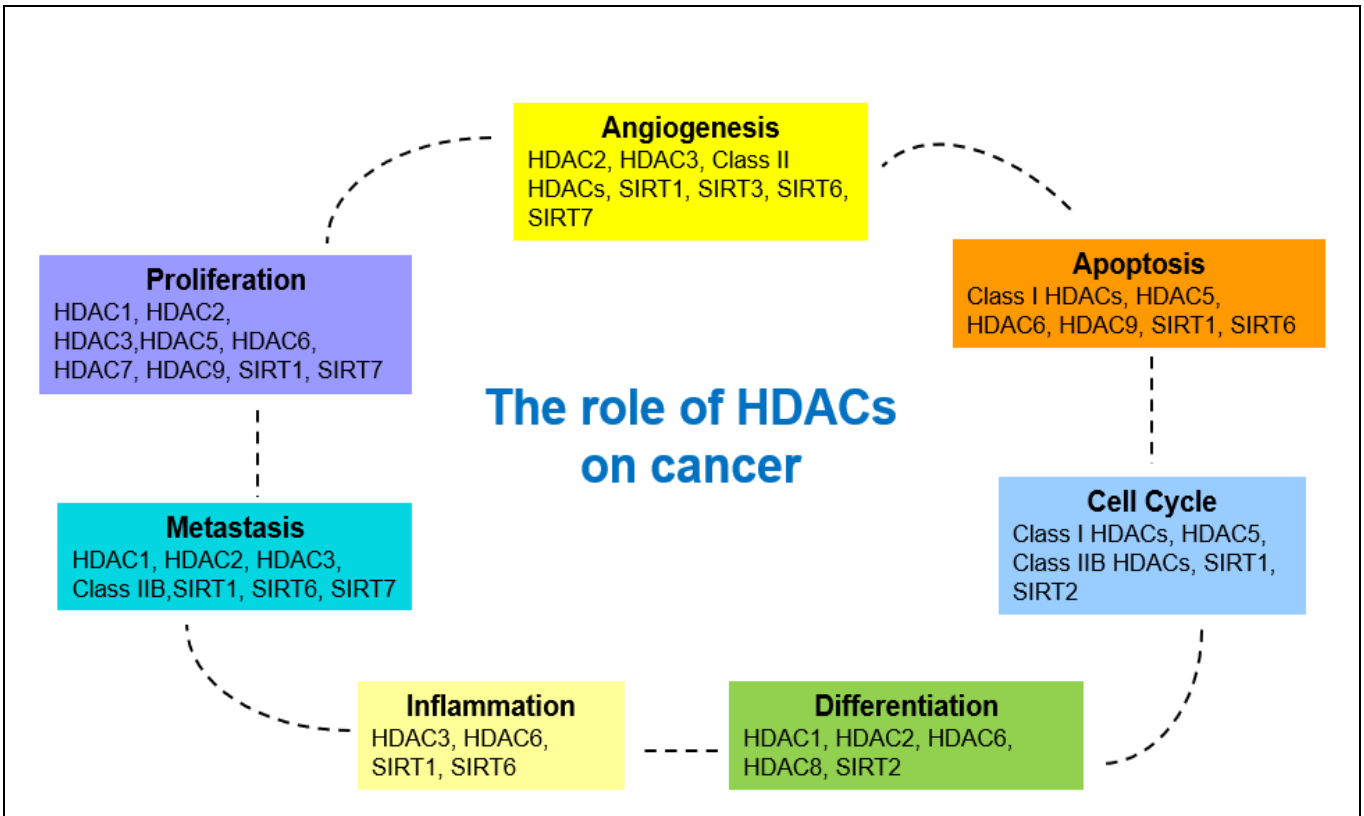


Figure 1.16: Overview of the role of HDACs in cancer (Adapted by Azeer Zafar from Hontecillas-Prieto et al., 2020 and made using Microsoft PowerPoint)

This figure shows the involvement of HDACs in different stages of cancer proliferation along with the different types of HDACs involved in each stage of cancer.

Increased HDAC6 expression is found in several cancers but its role in gastric and liver cancer is not very well understood. More research is required to clearly understand the mechanism of HDAC6 by which it expresses itself in different cancers. Due to the effect of HDAC6 expression in several cancers, HDAC6 is considered as an ideal option for cancer treatment. Although many HDAC6 inhibitors are currently being researched, there are very few that have been applied for clinical trials (Zhou et al., 2021).

Expression of oncogenes results in increased deacetylase activity of tubulin via HDAC6 and changes the spread of acetylated microtubules. The expression of oncogenes also induces increase in cellular stiffness and promotes cancer cell invasiveness. Finally, HDAC6 is crucial for the breakdown of the vimentin matrix leading to increased cell stiffness in cancer cells (Gad et al., 2012).

1.10.3.1 Esophageal cancer

Esophageal cancer is a very malignant cancer present in the digestive tract. A study showed that HDAC6 had increased in ESCC cells when compared with normal epithelial cell (Tao et al. 2018). Another study showed that a lower expression of HDAC6 in lymph cancer mean that patients had a better overall survival chance (Xie et al. 2018). However, some specific siRNA and inhibitors such as MiR-601, selective inhibitor ACY1215 and bortezomib can stop cell growth and decrease cell migration by downregulating HDAC6 and increasing the acetylation of α-tubulin and HSP90 (Cao et al. 2018, Tao et al. 2018) which leads to suppression of proliferation, invasion, and migration Overall, HDAC6 acts as an oncogene in this cancer (Zhou et al., 2021).

1.10.3.2 Colorectal cancer

Colorectal cancer (CRC) is among the most common malignant tumours worldwide (Benson et al., 2017). Studies observed that HDAC6 overexpression in CRC cells corresponded with increased mortality and knockdown of HDAC6 reduces the health and migration of CRC cells. Also, HDAC6 inhibition is involved in tumour suppression, and this is due to increased levels of acetylated α-tubulin. The inhibitor ACY-1215 had been widely used in clinical trials to treat cancer. ACY1215 also inhibited cell proliferation, migration, and invasion (Zhang et al., 2019). One study suggested that using both the drugs ACY-1215 and oxaliplatin could potentially increase their potency rather than either drug being used alone in cancer cells (Lee et al, 2018).

1.10.3.3 Pancreatic adenocarcinoma

Pancreatic adenocarcinoma is a highly metastatic cancer with a survival rate of less than 5% (Ryan et al., 2014). A study showed a higher HDAC6 expression in pancreatic cancer cells than in normal cells. (Li et al. 2014). However, another study showed no difference in the expression of HDAC6 levels of 7 different pancreatic cancer cells and normal human cells (Wang et al. 2012a). Another study shows point mutations are activated in K-Ras in pancreatic cancer, which lead to a poor therapeutic response. This study showed that HDAC6 inhibition reduced the growth of cancer cells which showed how effective this method was in treating pancreatic cancer (Li et al., 2021).

1.10.3.4 Gastric cancer

Gastric cancer (GC) is one of the most dangerous types of cancers in the digestive system and patients with stage 4 gastric cancer have extremely poor prognosis (Bray et al., 2018). A study showed much higher HDAC6 expression in gastric cancer than in precancerous cells, and that HDAC6 acts as an oncogene in gastric cancer (Wang et al. 2012). However, another study reported a higher expression of HDAC6 in normal cells than actual gastric cancer cells (He et al. 2017). Further studies show that increased expression of HDAC6 was associated with higher mortality (Wang et al. 2021).

1.10.3.5 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is among the top 5 most common causes of cancer deaths (Bray et al., 2018). A study showed an increased expression of HDAC6 in HCC cells than in primary liver cells while knocking down HDAC6 significantly decreased the movement and invasion of all HCC cell lines (Kanno et al. 2012). Another study confirmed the same results in more HCC cell lines (Ding et al. 2013). These two studies described the oncogenic role of HDAC6. However, another study reported the decrease of HDAC6 in patients with HCC, which was associated with poor clinical prognosis (Jung et al. 2012).

Overall, inhibiting HDAC6 results in the inactivation of cancer signalling pathways and cancer progression which then results in a decrease in cell proliferation and tumour growth. As discussed previously, HDAC6 is important for mediating cancer signalling pathways resulting in enhanced cell spreading. One of the hallmarks of cancer is metastasis, and HDAC6 is important for movement of tumour cells during metastasis., HDAC6 is also required for cell motility due to its substrate's actin and tubulin. Inhibiting HDAC6 increases α-tubulin

acetylation, improves microtubule stability and reduces migration of cells. Inhibition of HDAC6 via tubacin increases acetylation of microtubules (Zhou et al., 2021).

Other HDAC are also involved in deacetylation and are involved in different tumours. However, my project only focuses on one subtype of HDAC which is HDAC6. But it might be relevant to further study the effect of other HDACs and their expression in cancer (Hassanpour & Dehghani, 2017).

There are several epigenetic mechanisms of HDAC6 in cancer, which include histone modifications, DNA methylation and microRNAs. Methylation a very commonly studied epigenetic modification in the context of pathogenesis and prognosis of various cancer types (Fang et al., 2015). These epigenetic mechanisms are not directly related to my project and cellular contractility. However, they help clarify the effects of epigenetic modifications in terms of real effects in disease. For example, the DNA methylation epigenetic modification is linked to the drug resistance of tumour cells, the use of epigenetic modification agents combined with chemotherapy improves the drug resistance of patients with a dismal prognosis.

1.10.4 Role of HDAC6 as a Cancer Biomarker

HDAC6 expression correlates with a poorer or better survival from cancers depending on upregulation or downregulation of HDAC6 expression. Since HDAC inhibitors show anti-cancer effects therefore it is important to identify biomarkers and study the mechanisms by which HDAC6 expression acts in cancer cells. By definition, a biomarker is any compound that changes, in response to a therapeutic agent and this change can be measured (U.S. Food and Drug Administration, 2020). HDAC6 enzymes are important biomarkers and are intricately linked to tumorigenesis, which means that by measuring HDAC6 levels, the severity of different tumour types can be identified (Li and Seto, 2016).

An effective biomarker is one that can be quantified in response to a change in caner progression or a change of cancer drug. One study Fotheringham et al., 2009 conducted a genome-wide loss-of-function screen using shRNA of over 7000 genes to determine such biomarkers. The study identified genes that when inhibited in cancer cells prevented the death of HDAC inhibitors. An example of these genes was HR23B, which is responsible for moving ubiquitinated proteins to the proteasome as well as regulating the effectiveness of drugs. Measuring the levels of HR23B allowed for quantification of the response of cancer cells to HDAC inhibitors. This means that by identifying patients who had high HR23B levels, they

could be separated into a group that can benefit from HDAC inhibitor therapy (Fotheringham et al., 2009).

Another study Zhang et al., 2017 investigated the link between HDAC6 expression and renal cancer. The study used qRT-PCR and immunohistochemistry to measure HDAC6 expression in cancer and adjacent non-cancerous tissues. The results of the study showed that there was an increased upregulation in HDAC6 mRNA expression among renal cancers as compared with non-cancerous tissues. HDAC6 expression correlated with poor survival chances for RCC patients as those with higher expression had poor survival. Therefore, it was determined that HDAC6 can serve as a biomarker for RCC progression (Zhang et al., 2017).

Also, since there is increased HDAC6 expression in several cancers, evaluating the type of cancer that affects the link between HDAC6 expression and prognosis is especially important. For example, in lung cancer, increased HDAC6 expression correlates with better chance of survival, which might be because HDAC6 is regulated by oestrogen (Batchu, Brijmohan and Advani, 2016).

The table below (Table 1.2) has compiled and displayed the expression of HDAC6 in several different cancer subtypes from various studies. This table shows a clear trend that HDAC6 expression is always higher than normal in cancers.

Table 1.2: HDAC6 expression in various cancer subtypes.

Cancer	Expression	Reference
Acute Lymphoblastic Leukaemia (ALL)	Overexpressed- expression increases in advanced stage	(Bradbury et al., 2005)
Acute Myeloid Leukaemia (AML)	Overexpressed	(Bradbury et al., 2005)
Breast Cancer	Overexpressed	(Yoshida et al., 2004) (Zhang, 2004)
Cutaneous T-cell Lymphoma (CTCL)	Overexpressed- correlates with longer survival	(Marquard et al., 2008)
Hepatocellular carcinoma	Overexpressed- expression increases in advanced stage	(Ding et al., 2013) (Kanno et al., 2012)
Oral Squamous cell carcinoma	Overexpressed- expression increases in advanced stage	(Witt, Deubzer, Milde and Oehme, 2009)
Ovarian cancer	Overexpressed- expression increases in advanced stage	(Bazzaro et al., 2008)
Urothelial cancer	Overexpressed	(Rosik et al., 2014)
Chronic Lymphocytic Leukaemia (CLL)	Overexpressed- correlates with longer survival	(Van Damme et al., 2012)

1.10.5 Role of HDAC6 as a Drug Target

HDACs are an important target for cancer and currently there are several HDAC inhibitors that are being studied in trials as potential cancer drugs (Cairns, 2001). Recently, a new therapy called combination cancer therapy is becoming popular, and this therapy involves developing treatments that prevent drug resistance for the treatment of metastasis (Figure 1.17). As DNA methylation is an epigenetic modification of HDACs, there is a close link to the drug resistance of tumour cells. Combining epigenetic modifications of HDAC6 and microRNAs with chemotherapy improves a patients' resistance to cancer (Li et al., 2018). Since overexpression of HDAC6 correlates with increased metastasis, therefore inhibiting histone deacetylation is an effective treatment for treatment of cancer. HDAC6 specifically is also a key regulator in several signalling pathways involved in cancer, this makes HDAC6 important for therapy (Matthias, Seiser and Yoshida, 2011).

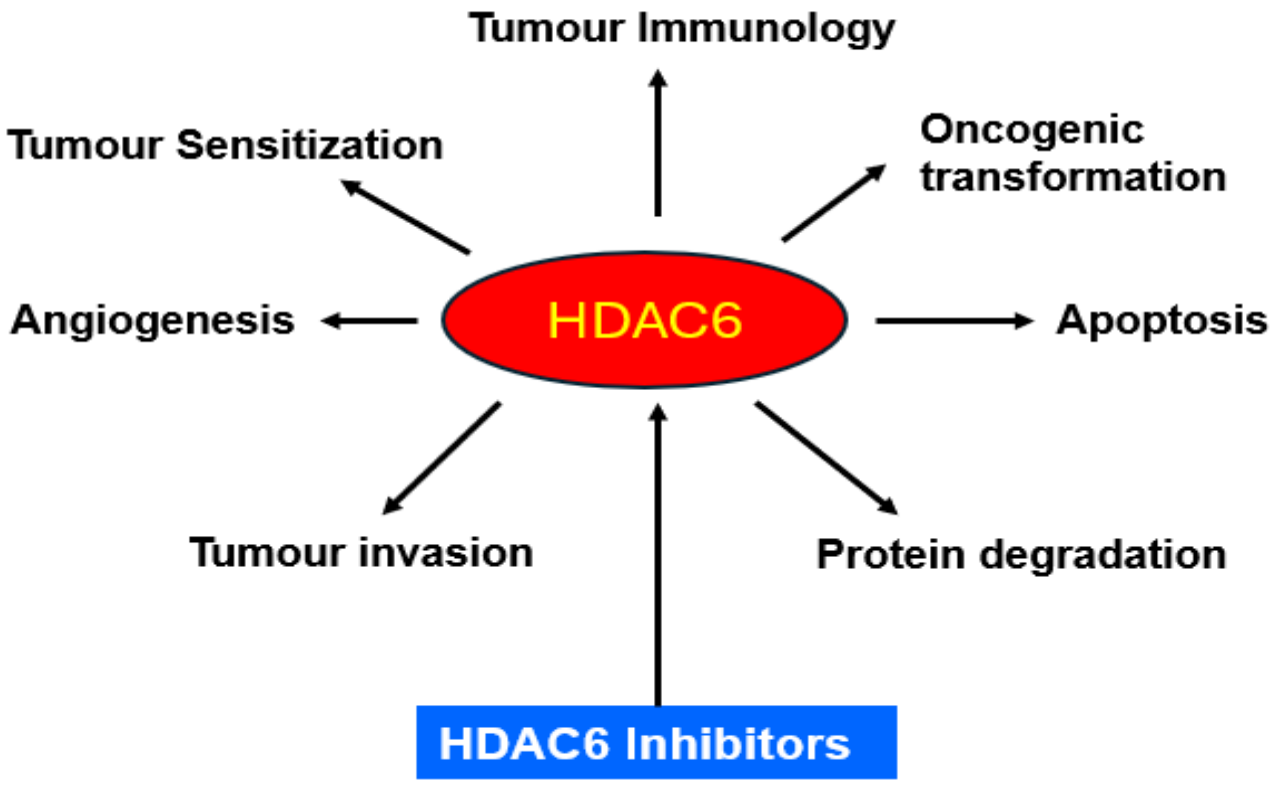


Figure 1.17: Overview of HDAC6 in Cancer and Potential Drug Targets (Adapted by Azeer Zafar from Peng et al., 2023 and made using Microsoft PowerPoint).

HDAC6 is an important target for study as a drug target for treating cancer as seen in the above figure.

Regulating HDAC deacetylation interferes with the acetylation of histones and allows histones to establish a balance leading to the inhibition of tumour cells, cellular invasion, and metastasis. Researching and developing new selective HDAC inhibitors are necessary in controlling the progress of cancers and for anti-cancer treatment (Sengupta & Seto, 2004). The HDAC6-specific inhibitor that was first synthesized was Tubacin, but it cannot be used in vivo since it is very toxic and complex to synthesize. Ricolinostat is the most popular HDAC6 inhibitor in clinical trials since it can inhibit HDAC6 mediated pathway without being toxic (Lee et al., 2013). Research has found that by combining microRNAs and epigenetic drugs, patient drug resistance can be significantly improved (Rastgoo et al., 2017).

HDACs are widely researched cancer treatment targets and HDAC inhibitors have proven to be highly effective for treating cancer, but their side effects prevent them from being used widely. Several studies have been done on the development of HDAC inhibitors along with their specific substrates but less are available on their use in clinical studies. Among the different HDAC subtypes, HDAC6 is more important for my project since it is separate from other HDACs based on its different substrates and structure. The special properties of HDAC6 give it more effective functions from other HDACs (Lee et al., 2013). It is for these reasons that great effort is being made to study the effects of HDAC6-selective inhibitors in various cancers and to reduce their side-effects (Marks, 2010).

HDAC6 inhibitors represent an effective treatment option for cancer patients (Silva, Kalinsky, Quayle and Yang, 2017). Based on this idea, one study Silva, Kalinsky, Quayle and Yang, 2017 researched that HDAC6 expression is highly increased in HDAC6-dependent cancer cells and acts as an important regulator. The study also created an HDAC6-score algorithm in which they profiled mRNA expression to determine the HDAC6 expression of different tumour samples. This HDAC6 score was used as a biomarker to easily identify cancers with high HDAC6 expression and depend on HDAC6 function. The study analysed 2500 primary breast cancers and found that around 20% of these breast cancers had a positive response to HDAC6 inhibitors. This study then confirmed their results by comparing the HDAC6-score with the inhibition response of HDAC6 to the HDAC6 inhibitor, Ricolinostat in breast cancer models in both vitro and in vivo (Silva, Kalinsky, Quayle and Yang, 2017).

Another study Wu et al., 2018 tested a synthesized compound called MPT0B451, which has an inhibitory effect against HDAC6 and tubulin. Results showed that MPT0B451 significantly inhibited cancer cells due to HDAC6 activity inhibition. This was done by MPT0B451

increasing apoptosis in these cancer cells, mitotic arrest and tumour growth inhibition. Thus, MPT0B451 can serve as an anticancer drug treatment in human cancers (Wu et al., 2018).

The treatments for many types of cancers include toxic drugs which reduce the effectiveness of the cancer treatments. Therefore, it is important to develop drugs that can target multiple pathways and reduce toxicity to make the treatments more viable (Cleeland et al., 2012). HDAC6 inhibitors regulate metastasis growth inhibition, cell apoptosis, and reduction in metastasis size making them attractive targets for these multi-target drugs. Studies also show there is an increase in the efficacy of treatment when HDAC6 inhibitors are combined with other anti-cancer drugs to form a cocktail of drug treatments. These multi-target drugs also increase therapeutic effects since they target several different locations and overcome resistance to drugs (Wu et al., 2018).

Another study Jiang & Ma, 2022 looked at the effect of Canagliflozin as a treatment for gastric cancer. Canagliflozin occupies the hydrophobic pocket of HDAC6, and canagliflozin binds directly to HDAC6 at the cellular level. The study looked at HDAC6 because of its importance in gastric cancer. The study showed that canagliflozin blocked the process of EMT and the migration of cells by inactivating HDAC6 in cancer cells. Based on these results, it is worth looking at canagliflozin to research the discovery of new inhibitors (Jiang & Ma, 2022).

However, not all research that is available about the subject is optimistic about the positive effects of HDAC6 as a drug target for cancer. One study Depetter et al., 2019 studied the impact of several HDAC6 inhibitors, in different cancer models. These inhibitors were used to inhibit several all the different cancer cell from multiple lesions in both *in vitro* and *in vivo.* Although some inhibitors resulted in α-tubulin acetylation, most failed to show any serious improvement in cancer treatment. Most of these HDAC6 inhibitors were only effective at high concentrations and resulted in lesser growth of tumours and invasive capacity of cancer cells *in vitro* and *in vivo*. These results show that these HDAC6 inhibitors are not remarkably effective when used alone by themselves but might be useful when combined with other anti-cancer drugs. Therefore, any results obtained regarding this need to be studied very carefully due to the high concentrations that result in lower selectivity and potential side effects. This is because HDAC6 has been studied as a therapeutic target in cancer research and a lot of effort has been made in developing HDAC6 and their use as anti-cancer agents. In conclusion, it is important that the specificity of HDAC6 be considered when testing HDAC6 inhibitors for cancer treatment (Wang et al., 2018) (Depetter et al., 2019).

1.10.6 Effect of HDAC6 on Contractile forces

Increased HDAC6 expression results in the transformation of cancer cells and metastasis (Medler et al., 2016). Also, since HDAC6 is an essential regulator that modulates smooth muscle α-actin activity (Li, Shin and Kwon, 2012), which increases cellular contractile forces and promotes tumour cell morphology and cancer development (Valenzuela-Fernández, Cabrero, Serrador and Sánchez-Madrid, 2008). Based on these results, a hypothesis can be proposed that it is HDAC6 expression which is involved in cellular contractility.

Another study Rathje et al., 2014 measured the HDAC6 expression in cancers and results showed increased levels of HDAC6 expression in cancer cells as compared to control cells. Further tests also showed that an increased expression of HDAC6 in normal cells caused the vimentin intermediate filaments to break down causing the epithelial to mesenchymal transition of normal cells to cancer cells. To ensure that HDAC6 is necessary for the breakdown of vimentin, the paper used tubacin, an HDAC6 inhibitor to see if inhibition of HDAC6 prevented the breakdown of vimentin. Treating HDAC6 with tubacin reduced the amount of vimentin and prevented its breakdown. This confirmed that this breakdown depends on HDAC6 and on microtubule deacetylation. More importantly, results also showed that HDAC6 increased cellular stiffness and invasiveness capacity of the oncogene-expressing cell system. This result is particularly important since it can be reasonably assumed that if HDAC6 increases cell stiffness, then it should also increase contractile forces since both properties increase during increased metastasis (Rathje et al., 2014).

Another study Coleman et al., 2020 measured the effect of increased α-tubulin acetylation on cytoskeletal stiffness using the HDAC6 inhibitor tubacin. Results showed that when tubacin inhibited HDAC6 and increased α-tubulin acetylation, the muscles cells showed decreased rates of cellular contraction and cytoskeletal stiffness. It is again confirmed that HDAC6 inhibition correlates with decreased stiffness, which meant similar results for contractility (Coleman et al., 2020).

Similarly, an increase in acetylation of microtubules results in decreased contractile force and vice versa as seen in Joo and Yamada, 2014. This was done by the interaction of myosin phosphatase with HDAC6, a microtubule deacetylase. This link between contractile forces and acetylation of microtubules is regulated by myosin phosphatase by activating and deactivating myosin II and HDAC6. The study used blebbistatin to increase microtubule acetylation, which

resulted in a decrease in HDAC6 resulting in increased microtubule acetylation and reduced cellular contractility (Joo and Yamada, 2014).

Based on the results from the studies mentioned above, which state that there are increased levels of HDAC6 in cancer cells as compared to control cells, HDAC6 is necessary for the breakdown of the vimentin intermediate filaments, tubacin inhibits HDAC6 and increases α-tubulin acetylation, thereby decreasing rates of cellular contraction, we can make a reasonable assumption that HDAC6 requires further study for its role in the physical properties of cells. Increasing HDAC6 results in increased mechanical forces while inhibiting HDAC6 results in decreased mechanical forces (Saito et al., 2013). Since previous studies that have studied the effects of HDAC6 have deduced that it increases cell stiffness in metastasising cells, therefore we can claim based on knowledge of cellular contractility that contractile forces should also increase in metastasising cells since cell stiffness and cellular contractility have a positive correlation in terms of their effects as they have been shown to increase in other studies that didn't study HDAC6 specifically. Although, this link is not exactly clear now since few studies have been done on the effect of HDAC6 on cellular contractility in cancer cells.

1.11 Molecular Mechanisms affecting Contractile force expression.

The mechanisms that are involved in expressing contractile forces in cancer cells are not well understood. This is because in the past, research for cancer treatment has primarily focused on cell signalling pathways instead of mechanical force pathways. Only recently has this focus shifted to understanding the role played by the mechanical properties in cancer cells during cancer cell invasion.

1.11.1 Role of Rho GTPases and ROCK pathway in contractile forces in cancer

The Rho family of GTPases, are guanine nucleotide-binding proteins regulate cell contractility. A study shows that the contractile forces of cells are increased by RhoA and decreased by Rac1 (Vega and Ridley, 2008). RhoA positively promotes myosin regulatory light chain (MLC), increasing actomyosin contractility with the help of F-actin and myosin II by the kinase activity of ROCK activity, while Rac1 inhibits MLC. This makes the activities of RhoA and Rac1 mutually inhibitory (Vega and Ridley, 2008).

Cells exert contractile forces by at least two different methods as mentioned in Gad et al., 2012. The first method includes activating RhoA, which results in reduced cell spreading, while the second method Rac1 includes the activation and resultant increased cell spreading. Active mutant variants, like RhoAV14, Rac1L61 and RhoDV26 were used in the experiment to obtain results. These showed that RhoAV14 induces greater increases in the cellular contractility of cells, while Rac1L61 and RhoDV26 show smaller increases when compared to Rho AV14. Also, cell surfaces show a more even distribution and greater distribution of nanoscale adhesion particles when contractile forces are higher in cells (Gad et al., 2012).

Activation of the ROCK signalling pathway mechanism in cancer cells is necessary for increased cellular contractility. McGrail et al, 2014 showed that in ovarian cancer, cells displayed increased malignancy due to increased cancer cell migration and proliferation. These cancer cells prefer to adhere to soft microenvironments. On soft matrices, lysophosphatidic acid (LPA) mediates activation of ROCK in ovarian cancer cells. treatment with Y27632 (ROCK inhibitor) and reduces cellular contractility, which suggests that the ROCK pathway is essential for cell contraction (McGrail, Kieu and Dawson, 2014).

Another mechanism is the disruption of the cytoskeletal structure via RhoA-ROCK pathway. Kim et al., 2019 showed that in oral squamous cell carcinoma (OSCC), cancer-associated fibroblasts increase carcinogenesis and disrupt the cytoskeleton via RhoA-ROCK pathway. Comparing the matrix remodelling capacity between YAPS127A and WT of Yes-associated protein in hTERT-hNOFs cells using a 3D ECM-remodelling assay, showed increased cellular contractility, tissue stiffness and promoted the movement and spread of cancer cells (Kim et al., 2019).

The primary reason that RhoA activity is so potent in inducing cellular contractility in cells is because it has the ability to induce all the three processes, which are the polymerization of actin, the cross linking of F-actin and the movement of actin via actomyosin have been studied to control contractile forces (Gad et al., 2012). Actomyosin sliding is the process of driving contraction in actomyosin. The Rho-dependent pathway and Myosin II that is regulated by ROCK pathway is responsible for the regulation of contractile forces in fibroblast cells (Beningo et al., 2006).

1.11.2 Role of Intermediate Filaments in Contractile forces in cancer

Physical forces move from the cell to the nucleus via the interaction between the intermediate filaments (IFs) and microtubules (MTs) and this also controls the mechanical properties within the cytoskeleton (Ndiaye et al., 2022).

Vimentin is a type 3 IF structural protein located in mesenchymal cells. There are several studies that show that vimentin is important for the generation of contractile forces in cells (Chen et al., 2016, Liu et al., 2015).

The IF vimentin is necessary for cells to transition from the epithelial state to the mesenchymal state (EMT) allowing the cells to migrate. Vimentin also maintains mechanical integrity in epithelial-mesenchymal transition cancer cells. There are several molecular mechanisms involving vimentin that control cellular contractility.

Several studies have studied the effects of vimentin knockdown on the contractile forces of cells. Jiu et al., 2017 used CRISPR and siRNA to knockdown vimentin in human osteosarcoma cancer cells leading to increased formation of actin fibres and contractile forces, which was measured using TFM. It also showed that intact vimentin intermediate filaments are required to facilitate the effects on the actin cytoskeleton. Depletion of vimentin led to promotion of RhoA activity and actin stress fibre assembly (Jiu et al., 2017). De Pascalis et al., 2018 used siRNA mediated knockdown of vimentin in primary astrocytes, which resulted in increased traction forces. Vimentin controlled the distribution and strength of contractile forces as well as collective migration in astrocyte cells. Vimentin IFs are also involved in the organization of the actin cytoskeleton as well as actomyosin cell contractility (De Pascalis et al., 2018). Liu et al., 2015 knocked down vimentin via siRNA in MDA-MB 231 cancer cells. This results in reduced cell proliferation, loss of directional migration, reorganization of cytoskeletons and reduction in focal adhesions size. In turn, this results in reduced mechanical strength and decreased cellular contractile forces. On the other hand, vimentin overexpression in MCF7 cells increased the stiffness of cells, their motility and movement. Therefore, vimentin is a regulator which manages intracellular mechanical properties by maintaining the cytoskeleton (Liu et al., 2015).

One study Chen et al., 2016 measured the effect of the disruption of vimentin cytoskeleton on contractile forces. The study seeded chondrocytes on a polyacrylamide gel and used TFM to measure the contractile forces. Results showed that although there was no difference in

contractile forces for untreated chondrocytes, there was a major decrease of contractile forces in vimentin-disrupted chondrocytes (Chen et al., 2016).

Vimentin controls the resistance of cells to mechanical stress and has stronger resistance in vitro when compared to actin or microtubules. To test the effect of vimentin inhibition on contractile forces, Costigliola et al., 2017 fluorescently labelled vimentin was expressed in non-immortalized human foreskin fibroblasts, shRNA mediated inhibition of vimentin leads to an increase in contractile forces, which was measured by TFM (Costigliola et al., 2017).

Vimentin controls cell motility, cell adhesion and is therefore used to understand the underlying mechanisms active between cell adhesion and contractile forces. Peschetola et al., 2013 showed that measuring the traction stresses was an effective indicator to distinguish between cancer cell lines of different levels of invasiveness. In this study, two cancer cell lines, one more invasive (T24), and a less invasive one (RT112) were evaluated. T24 cells showed larger focal complexes, which led to faster migration of cancer, while RT112 cells developed smaller focal adhesions on soft gels and migrate slower. This is measured through Mean Square Displacement (MSD) method and was found that more invasive cancers have increased cell migration and cellular contractility on soft substrate (Peschetola et al., 2013). In Rönnlund et al., 2013, the focal adhesion distribution in metastasizing fibroblast cells were compared to the focal adhesions in normal cells using stimulated emission depletion microscopy (STED) Results found that metastatic cells had significantly higher focal adhesions numbers and distribution leading to increased tumour cell metastasis (Rönnlund et al., 2013).

Research shows that vimentin expression inhibits cellular contractility, while the collapse of the vimentin structure increases cell contractile forces (Jiu et al., 2017, De Pascalis et al., 2018). However, other studies like (Liu et al., 2015 and Chen et al., 2016), showed a decrease in contractile forces despite knocking down of vimentin and disruption of vimentin structure respectively, which does not fit with the expected results. Potential explanations for these different results might be these studies using different potential methods to knockdown vimentin, using different methods to measure contractility, action of vimentin via different pathways or making unsupported assumptions while carrying out experiments. However, none of these reasons is enough to justify having contradictory results to the established understanding of the topic.

1.11.3 Role of Microtubules in Contractile forces in cancer

Studies like Al-Rekabi, Haase and Pelling, 2014 have been done on the role of microtubules in fibroblast cells during cellular contractility. They have studied the molecular mechanism of microtubule depolymerization on contractile forces. Previously, it was accepted that an entire actin cytoskeleton is essential for the increase in traction stress regardless of myosin II activity or MT depolymerization. However, Al-Rekabi, et al., 2014 showed that microtubule depolymerization alone is also responsible for inducing an increase in traction forces (Al-Rekabi, Haase and Pelling, 2014).

Rape et al., 2011 carried out nocodazole mediated depolymerization of microtubules in NIH3T3 fibroblast cells followed by treatment of myosin II with blebbistatin. Next, TFM was used to measure the contractile forces for cells both before and after microtubules depolymerization. Since traction forces are generated by myosin-II-mediated contractility, therefore treatment with blebbistatin, which is a potent inhibitor of myosin II ATPase will inhibit traction forces. However, there was a 5-fold increase in traction force after nocodazole treatment upon microtubule depolymerization in cells with the myosin II inhibited, suggesting a myosin-II independent focal adhesion kinase regulated pathway (Rape, Guo and Wang, 2011). Kraning-Rush et al., 2011 treated highly metastatic MDA-MB-231 carcinoma cells with nocodazole to inhibit microtubules and analysed for changes in cytoskeletal organization and cellular contractility in both 2D and 3D. In order to quantify contractile force, TFM was used in both 2D and 3D substrates. Results showed that the contractile forces generated were identical in 2D and 3D matrices, and that disruption of microtubules in either microenvironment increased cellular contractile forces. Therefore, dynamic regulation of the MT cytoskeleton is essential to suppress contractility in both 2D and 3D matrices (Kraning-Rush et al., 2011).

1.12 BJ Fibroblast Cell Model

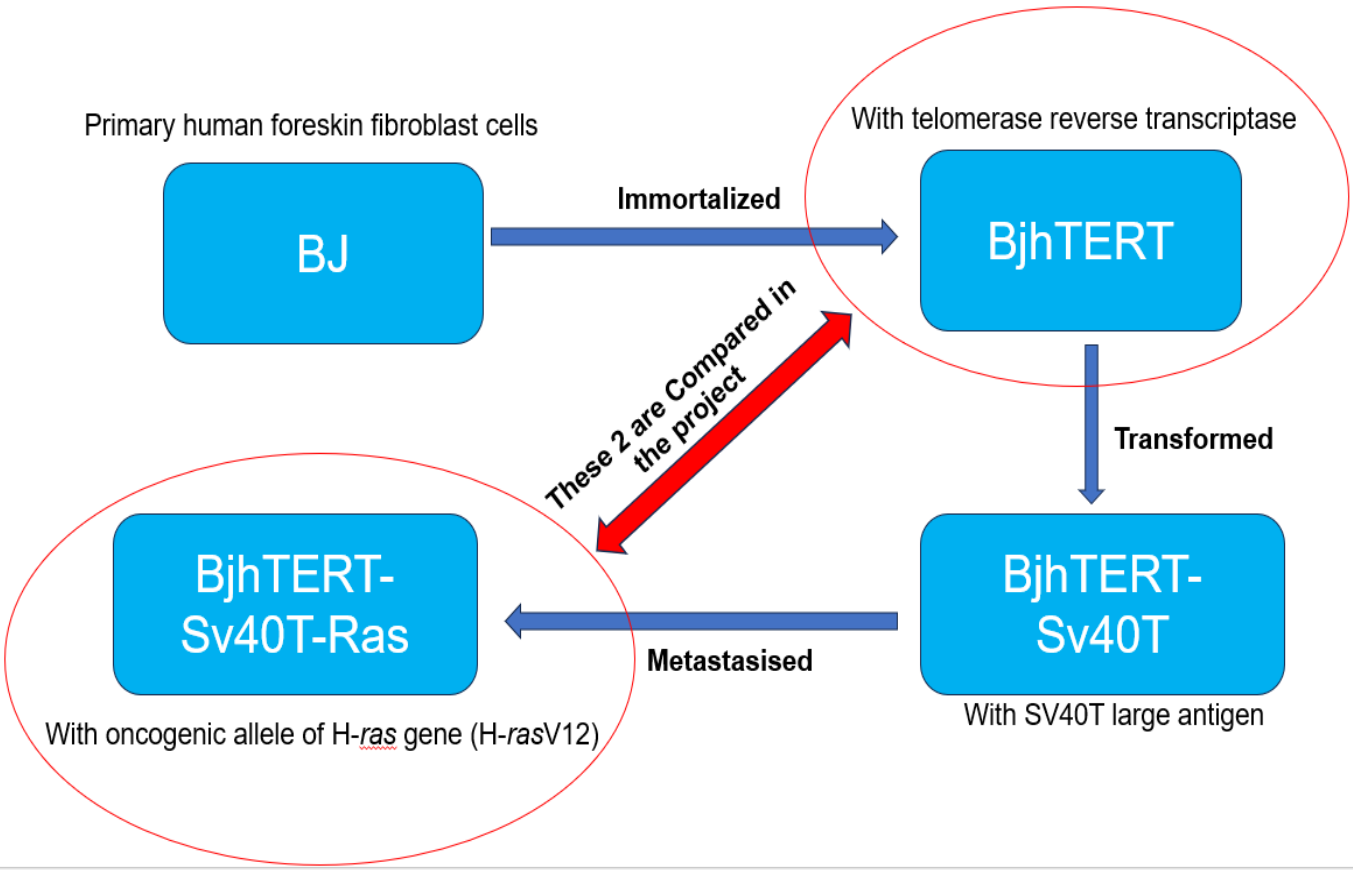


Figure 1.18: Overview of this Project's Cell Model (Prepared by Azeer Zafar using Microsoft PowerPoint)**.** This figure shows the cell model that is used in this project along with all the treatments that were performed to obtain the correct cells for testing in the project.

As explained above, metastasis is not a single step process but one that takes over multiple steps in which normal cells transform gradually to malignant, tumour cells due to genetic changes. My project also is based upon such a cell model, which uses 4 different types of cells that are all isogenically identical. These 4 types of cells include normal BJ human fibroblasts that were first discovered by the Weinberg scientific team that have gone through different stages of modification including immortalization, transformation and metastasis. This cell model uses a cell line, of which the first type is made of primary human foreskin fibroblast cells (referred to as BJ cells), the second type of cells are formed when the BJ cells are immortalized with telomerase reverse transcriptase (referred to as BjhTERT cells), the third

type of cells is when BjhTERT cells are transformed using the SV40 T-antigen (to form BjhTERT-SV40T-variant) and the fourth type of cells are when the BjhTERT-SV40T-variant is artificially metastasized by adding the oncogenic allele of the H-*ras* gene, H-*ras*V12 (referred to as the BjHTERT-SV40T-Ras) (Hahn et al., 1999) (Figure 1.18). This model is effective since several studies have used such models where different genetic components like cancer genes have been introduced to various cell lines and this has allowed us to artificially observe the natural steps of the growth of metastasis in humans in a controlled laboratory setting (MacKenzie et al., 2002).

In terms of morphology, the main BJ cells have a longer clearly defined shape, which breaks down, becomes irregular and cannot be clearly observed when the cells progress to their next stages after transformation and metastasis. This change in shape can also be seen in certain cellular components where some like the microtubules retain their shape all throughout the different cell conditions but others like the golgi apparatus have their shape changed due to a change in phenotype in cells that have been transformed. The process of transformation and metastasis also leads to increased nucleoli in these cells due to their proliferation.

The reason there are large changes in this cell model only at the third stage, which is the large-T antigen transformation (BjhTERT Large-T) is because large-T antigen transformation mediates its actions due to the inhibition of the p53 family of tumour suppressors. Large-T binding reacts with p53, and the transformed cells have inactive p53. This in turn leads to a situation where up to 856 genes are expressed. This also results in the loss of the morphology of the fibroblast cells. These factors in addition to fibroblast activation protein (FAP) and α-smooth-muscle actin (ACTA2) allow for major cellular differentiation after SV40T expression.

Using a 4-stage cell model based on human fibroblasts, we can cover the entire spectrum of cell types present during cancer allowing us to observe and determine the mechanisms and pathways involved in cancer spread as well as studying the other hallmarks of cancer. Ideally, this would be done in vivo however due to practical limitations this model is the best option to study metastasis.

We can therefore use this cell model in our experiments since the cells are isogenically matched and that allows us to ensure the validity of the results obtained when normal and metastasising cells were compared regarding their contractility, cell-matrix adhesions, stiffness and cell spreading area.

1.13 Hypothesis and Aims

1.13.1 Project Hypothesis and Aims

The main aim of this project was to determine the effect of HDAC6 on the contractile forces of oncogenically transformed, isogenically matched, metastasising human BJ fibroblasts and the molecular mechanisms that control these contractile forces in BJ fibroblasts for future development of anti-cancer drugs.

We hypothesized that a higher expression of HDAC6 increases contractile forces in metastasizing BJ fibroblasts. This hypothesis will be tested by the following aims:

Aim 1: Optimize the TFM technique to allow for measurement of contractile forces in cells.

Aim 2: Determine and compare the differences in contractile forces of normal, metastasising and tubacin treated metastasising cells using the BJ model.

Aim 3: Determine and compare the differences in contractile forces of normal, metastasising and HDAC6 siRNA knockdown treated metastasising cells using the BJ model.

Aim 4: Determine and compare the effect of HDAC6 inhibition on the morphological properties and nuclear forces on normal, metastasising and HDAC6 siRNA knockdown treated metastasising cells using the BJ model.

Aim 5: Determine and compare the effect of HDAC6 inhibition on focal adhesions in normal, metastasising and treated metastasising cells to measure cell spreading and invasiveness.

Chapter Two –MATERIALS AND METHODS

Chapter 2 – Materials and Methods

2.1 Cell Culture

The project is based upon a previously used cell model, which uses 4 different types of cells that are all isogenically identical. These 4 types of cells include normal BJ human fibroblasts that were first discovered by the Weinberg scientific team that have gone through different stages of modification including immortalization, transformation and metastasis. This cell model uses a cell line, of which the first type is made of primary human foreskin fibroblast cells (referred to as BJ cells), the second type of cells are formed when the BJ cells are immortalized with telomerase reverse transcriptase (referred to as BjhTERT cells), the third type of cells is when BjhTERT cells are transformed using the SV40 T-antigen (to form BjhTERT-SV40T-variant) and the fourth type of cells are when the BjhTERT-SV40T-variant is artificially metastasized by adding the oncogenic allele of the H-*ras* gene, H-*ras*V12 (referred to as the BjHTERT-SV40T-Ras) (Hahn et al., 1999). This model is effective since several studies have used such models where different genetic components like cancer genes have been introduced to various cell lines and this has allowed us to artificially observe the natural steps of the growth of metastasis in humans in a controlled laboratory setting (MacKenzie et al., 2002). Previous studies have also shown that SV40T and H-Ras oncogenes when introduced to fibroblasts have resulted in cancerous tumours in different organs of the body.

In terms of morphology, the main BJ cells have a longer clearly defined shape, which breaks down, becomes irregular and cannot be clearly observed when the cells progress to their next stages after transformation and metastasis. This change in shape can also be seen in certain cellular components where some like the microtubules retain their shape all throughout the different cell conditions but others like the golgi apparatus have their shape changed due to a change in phenotype in cells that have been transformed. The process of transformation and metastasis also leads to increased nucleoli in these cells due to their proliferation.

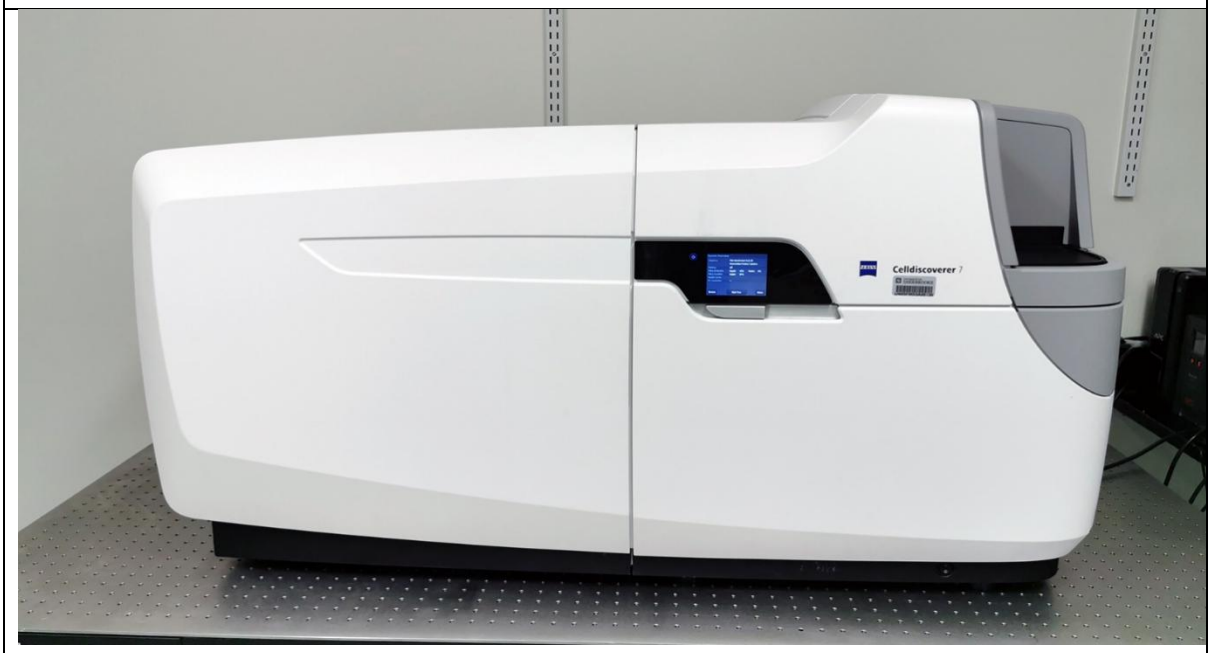
The project used the BjhtertSV40TRasV12 cells (SV40T-RasV12), and the non-transformed, normal, and immortalized Bjhtert cells as control cells (See Section 1.12 and Figure 1.18). Cells that were used were cultured in Dulbecco's modified Eagle's medium (DMEM solution) that had been augmented with 10% foetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin at 37˚C in 5% carbon dioxide. Cells have been processed with DMSO or 10 µM tubacin.

2.2 Microscopes

Two microscopes were used primarily for the research project, which included the Zeiss Cell Discoverer 7 (CD7) Microscope (Figure B) and the Zeiss LSM 980 Microscope (Figure A).

Figure 2.1: Overview of Microscopes Used (Images taken by Azeer Zafar)

A: Zeiss Light Scanning Microscope (LSM 980). This microscope was used primarily for confocal microscopy.



B: Zeiss Cell Discoverer 7 (CD7). This microscope was used to perform TFM and for live cell imaging.

The CD7 is a live-cell imaging microscope that can run the TFM technique once the technique had been set up and optimized. This microscope also had access to temperature control and a CO2 pump that allowed for cells to stay alive while experiments are being performed. The other microscope that was used was the Zeiss Light Scanning Microscope (LSM 980), which is a confocal microscope that allowed the comparison between cells with regards to their cell-matrix focal adhesions and cell spreading area in super resolution without compromising on picture resolution. The LSM 980 also had an incubation system, which made it suitable for working with live cells. This equipment incorporated the Airyscan 2 and Elyra 7 super-resolution systems.

2.3 siRNA Transfection

The knockdown of the HDAC6 protein in the metastasising BjhTERT-SV40T Ras cells was done using siRNA according to the instructions provided by the manufacturer. According to the experimental design that was planned, cells were seeded at 40,000 cells/well in a P12 well plate that were sourced from STARLAB International, Germany and are the CC7682-7512 model. . These cells were incubated for 24 h in DMEM media augmented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin.

The actual process of transfection was performed on BjhTERTSV40TRas cells using the Hiperfect Reagent (model 301704). This transfection reagent used both the Hs_HDAC6_type5 and the Hs_HDAC6_type6 FlexiTube siRNA (model 1027417) for transfection along with the negative control siRNA (model 1022076). These transfection reagents were used at a concentration of 25 nM after 96 hrs of incubation. All the transfection reagents were sourced from Quiagen, Germany.

Initially, the plan was to treat the cells for 24 hours but when the cells were lysed and the amount of HDAC6 proteins was detected via western blotting, it was determined that treatment times had to be increased since HDAC6 had not been knocked down. This was followed by treatment for 48 hrs and 72 hrs before finally choosing 96 hrs because that is when HDAC6 knockdown finally took place by the siRNA and was confirmed by western blot. At the end, the cells that had been successfully transfected were incubated for at least two passages and then knockdown was reconfirmed using western blot analysis.

2.4 Western Blotting

All the cellular proteins (25ug/lane) were separated using Stain free precast polyacrylamide gels (4%–20%) (4568094, Bio-Rad Laboratories Ltd.), and western blot procedures were performed as described previously (Evans et al., 2022).

The list of antibodies which were used are listed as follows:

- anti-mouse-acetylated-α-tubulin (T6793, Sigma Aldrich)
- anti-rabbit-α-tubulin (ab176560, Abcam)
- anti-mouse-GAPDH (60004, Proteintech)
- anti-rabbit-HDAC6 (NB100-56343, Novis Biologicals)
- HRP-conjugated goat anti-mouse/rabbit
- Secondary antibodies (GtxRb-004-DHRPX/GtxRb-003-DHRPX, Immunoreagents).

The bands obtained from the western blots were quantified using the ChemiDoc Imaging System (Type: 12003154) and Image Lab Touch 2.4, version 1709691 software. In addition, to determine the ration between the acetylated-α-tubulin/α-tubulin, the western blots were stripped using a Stripping buffer (Cat: 21059, Thermo Scientific) before they were re-probed. GAPDH has a molecular weight of 36 kD while HDAC6 has a molecular weight of 150-160 kD.

Gel electrophoresis was performed using Stain Free precast polyacrylamide gels (5%–22%) from BioRad. Gels obtained were then treated in an electrophoresis tank that had electrophoresis running buffer (BioRad) that had been diluted by 10 using distilled water. Cell solutions (50-80µg, maximum 50µl) were treated with 6x sample loading buffer and heated to 110°C for 10 minutes and cooled at room temperature. Precision Plus Protein Dual Colour Standards (BioRad) were used to identify the proteins on the bands based on their molecular weights. This was done by running the gels at a constant voltage of 200V for 45-60 minutes depending on the level that each protein needed to be separated for.

2.5 Measurement of Contractile Forces: Traction Force Microscopy

There are several techniques that can be used to measure the contractile forces of cells. These include Traction force microscopy (TFM), microfabricated post array detectors (mPADs), collagen gel contraction assay to name a few. Each technique works on different principles, has different advantages, and disadvantages which needed to be considered before using these

techniques. However, this project used only TFM because it was the most relevant and widely used technique compatible with this project.

Traction force microscopy (TFM) allows us to measure traction forces on an elastic substrate thereby providing us quantitative information on the value of the contractile forces being exerted by a cell. TFM is a very commonly used technique to measure contractile forces in cells. This is because it can be easily performed on a simple laser-scanning confocal microscope (Sabass et al., 2008).

In TFM, cells are seeded on a polyacrylamide gel substrate containing fluorescent beads and is coated with a layer of collagen. First, the cell adheres to the gel and contracts the gel. This cell that is adhered to the gel, exerts force and is in a strained position. Then, the gel is imaged and the position of the fluorescently labelled red beads within the gel substrate are recorded as they are in a fixed position (Figure 2.2A). Next, trypsin is added to detach the cell from the gel which relaxes the gel and allows the beads to be displaced from their initial positions. This displacement of the beads is filmed using a microscope. This allows us to measure the movement of the cells since the distance moved by the displaced beads from when gel is contracted to when the gel relaxes is equivalent to the contractile force exerted by the cell (Figure 2.2B). TFM allows for the quantification in terms of increase or decrease of contractile forces exerted by the cells on gel substrate displacement under contraction. Therefore, measuring bead displacement shows the magnitude of contraction (Plotnikov, Sabass, Schwarz and Waterman, 2014).

The images obtained were analysed using ImageJ/MATLAB software via the following process. First, the gel is deformed by the action of the forces exerted by the cells on the elastic gels. This causes the displacement of fluorescent beads that are embedded in the gel. Then the images of bead positions are created, aligned, and cropped. Next, using the ImageJ plug-in Particle Image Velocitometry (PIV), displacement of beads is measured. Finally, the traction force is measured using the Fourier Transform Traction Cytometry (FTTC) method plug-in, exerted by the cell based on four parameters, which are the Image Pixel Size, Poisson Rate (normally 0.5), Gel Stiffness (the project used 12 kPA gels) and Regularization Factor Plugin (Fig 2.3).

The experiment used pre-prepared 12 kPa Matrigen SoftTrac™ hydrogels that were coated with collagen and were embedded with 0.2 μm green/yellow fluorescent beads sourced from Cell Guidance in Cambridge. Attached cells seeded at 2500 cells/cm2 and fluorescent

embedded beads were imaged on the plates at a 40x magnification using a Zeiss Cell Discoverer 7 Fluorescent Microscope (Figure 2.1B) that was purchased from Carl Zeiss Microscopy.

The images obtained of the fluorescent beads present in a gel at relaxed position were taken after treating the gel for 5 minutes with a solution of 0.5% (w/v) Triton-X-100 and 20 mM NH4OH PBS to remove any other cells present on the gel. Displacement of beads before and after the removal of cells was tracked by PIV followed by FTTC using a modified ImageJ macro that was shared by Dr. S. Lee (Lee and Kumar, 2020) from a previously described method (Martiel et al., 2015). This allowed me to determine the size of the corresponding cell traction force field generated by the cell. Also, the total traction forces (in N) were quantified by adding traction forces over the area occupied by the cell. The elastic energy (in J) stored in the gel to allow it to exert the observed deformation was calculated by summing the products of displacement with the force over the cell area.

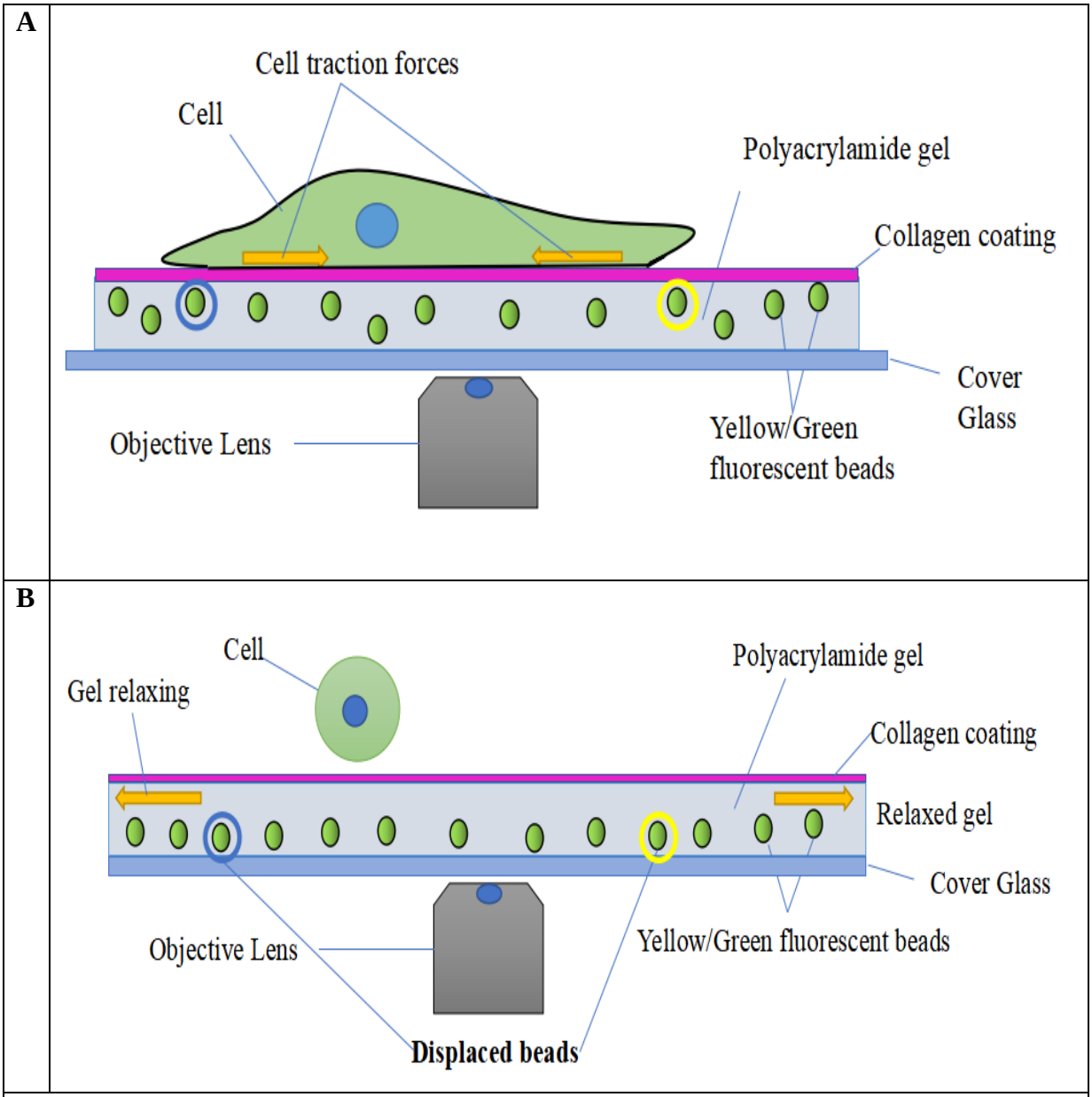


Figure 2.2: Overview of TFM Force Measurement using Microscope. (Made by Azeer Zafar using Microsoft PowerPoint)

This figure shows (A) a cell attached to a polyacrylamide gel by exerting contraction on the gel and holding the fluorescent beads in place while (B) shows the cell detached from the gel causing the beads to relax and spread proportional to the contractile force exerted by that cell. This movement of beads is measured by a CD7 microscope and analysed using FIJI/ImageJ software.

The project involved preparing and designing a custom protocol for TFM since this was the first time TFM was being performed in my research lab. I worked extensively to design the protocol below, which was then successfully applied in my lab and was successful in giving us the data and results for the measurement of contractile forces for the cells that were being tested in my project.

Custom Designed TFM Protocol:

1. **Preparing the sample**- The desired cell line was cultured in the right medium under the correct environmental conditions. The live-cell imaging medium was prepared by adding FBS and L-glutamine to DMEM, which lacks phenol red. On the day of TFM, incubate polyacrylamide gels for 30 min at 37°C with live-cell imaging media before the experiment. Use trypsinization to harvest the transfected cells, suspend in live-cell imaging medium, and plate on TFM substrates for desired time (Plotnikov et al, 2014).

2. **Setting up the microscope chamber for TFM in order to acquire TFM images**- Assemble the perfusion chamber inside the microscope according to the Zeiss CD7, ZEN 2.1 (blue edition) manual from page 17, First steps subtitle. Focus the lens on the yellow-green, fluorescent beads in the gel. Cells can be imaged by locating the yellow-green, fluorescent beads marker under the cell. Take a phase contrast image of the cell, in order to show that there are cells present. Take time-lapse TFM images or films by parallel filming of fluorescently labelled beads or microspheres. Cells were detached from the TFM substrate by perfusing the cells with 5 mL 0.5% trypsin and incubating them on the stage for three minutes to detach the cells. Another phase contrast image of the cell was taken to confirm that the cells have detached. Save the images and films taken in both the czi. format and .tiff format for future analysis (Sabass et al, 2008).

3. **Calculating traction forces with Fourier-transform Traction Cytometry (Fig 2.3)**- Before calculating the traction forces, the images were first adjusted for brightness and improved quality of bead recognition. For calculation with FTTC, the images are interpolated on to a rectangular grid that covers the whole image. The bead displacement is measured by the change of the bead's position between the initial reference (seen as black dots) and in presence of contractile force (seen as coloured dots), in a process called Particle Image Velocitometry (PIV). Bead Displacement is then calculated by equations of linear elasticity via 2 functions, the force exerted by the

cell and the gel mechanical characteristics (like the Young's modulus and Poisson Ratio). The plug-in FTTC is used to measure the contractile force, which is exerted by the cell and results in gel deformation and bead displacement (Gad et al., 2012).

4. **Presenting TFM Data-** Finally, the contractile forces of the cells obtained from measuring the cells displacement were taken from imaging the fluorescent beads embedded inside the hydrogel. After considering the different experimental conditions, high resolution images were taken, and a map of the displacement field was generated. the Contractile force was determined by calculating the force exerted (J), which is the total amount of energy transferred from the contracting cell to the substrate (gel) on which it adheres. The data sets were represented as maps of bead displacements and their corresponding tractions. (Sabass et al, 2008).

This detailed protocol allows for quick and easy application of the TFM technique to measure contractile forces between different cells as well to be able to analyse the results obtained. This is important as being able to set up and run the TFM technique is crucial to the project since this is the main technique which is used to measure the contractile forces of cells and is the main focus of the project.

During TFM analysis, the computational analysis assumes that the polyacrylamide gel on which the cells are cultured, and the experiment is performed is a homogenous, static gel of fixed structure. This means that it is important for the gel to remain constant in order to get accurate results and any changes from these factors will bias the force result that is obtained. There are several factors that determine a part in the quality of the gel that is used and its reproducibility, and these factors include the formulation of the gel, the time taken for gel preparation and the conditions in which the gel is stored. Hydrogels need to be carefully stored since they have a high chance of suffering from drying or aging (Martiel *et al.*, 2015). Nowadays, nearly all the gels that are used for TFM including the ones used in my project are made of polyacrylamide due to its unique properties which include limited stiffening and immediate strain recovery (Dembo and Wang, 1999). Unfortunately, in some cases if the deformation is too high it can cause the polyacrylamide hydrogel to stiffen (Boudou *et al.*, 2009).

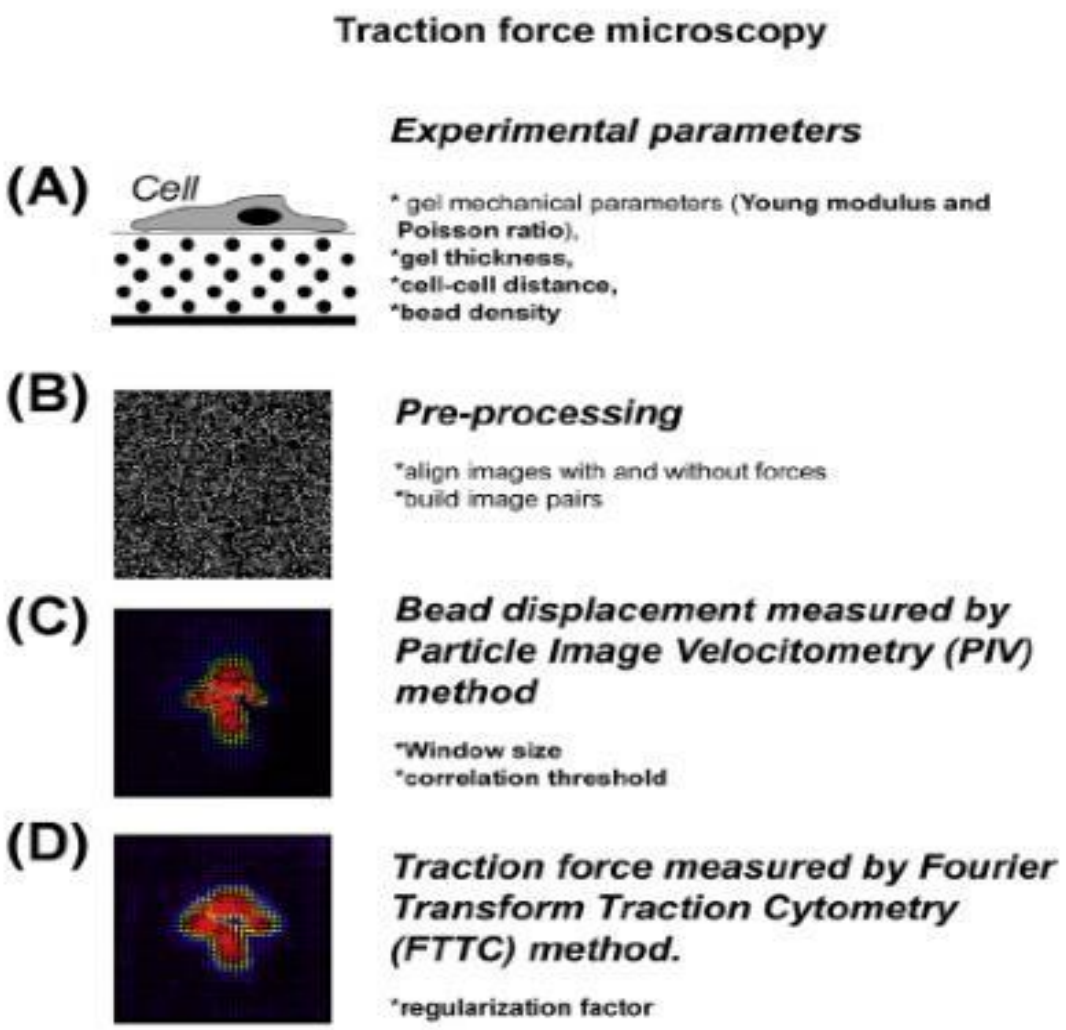


Figure 2.3: TFM Images Analysis Method Overview: (Taken from Paluch, *Biophysical Methods in cell biology* 2015)

(A) Forces exerted on the cells plated on elastic gels deform the gel and cause the displacement of fluorescent beads present in the gel. **(B)** Images are created, aligned, and cropped. **(C)** Using the ImageJ plug-in PIV, displacement of beads is measured. **(D)** The plug-in FTTC is used to measure the traction force, exerted by the cell based on four parameters, which are a) Image Pixel Size b) Poisson Rate (normally 0.5) c) Gel Stiffness d) Regularization Factor Plugin (Martiel et al., 2015)

Depending on the formula that is used to prepare the polyacrylamide gel, there can be variations in the structure of the hydrogels that can cause changes and stiffen the gel over a period of time or due to a change in temperature during storage (Denisin and Pruitt, 2016).

Since the magnitude of traction forces that are being generated are extremely sensitive to changes in substrate stiffness and contractility (Plotnikov *et al.,* 2012; Elosegui-Artola *et al.,* 2016), any changes in the stiffness of the gel's stiffness can affect the range of traction force measurements being recorded. Embedding the gels with fluorescent beads to track contractile forces is another source of variability that needs to be addressed (Lee *et al.,* 2015). In addition, the type of surface coating that is used on the polyacrylamide surface also affects the contractile force generated despite their being no significant change in cell shape (Pompe *et al.,* 2011). Therefore, it is important to consider all variabilities while calculating the contractile forces during TFM.

As mentioned previously, the stiffness of the gels used during TFM is crucial in determining an accurate result for the contractile force of different cells. There are several experiments that require hydrogels to measure the effect of physical forces on cells including cell proliferation, migration, differentiation. However, despite great strides in the field of mechanobiology, preparing hydrogels with specific stiffness consistently remains a particularly challenging issue, which in turn leads to inaccurate results during experiments that cannot be reproduced. Most techniques that are popular today for measuring gel stiffness are expensive and sophisticated making it difficult for more people to adopt these mechanobiology techniques (Gandin et al., 2021). These gel measurement techniques include various kinds of Rheometric Analysis, which measure the change in shape of different materials when they are under stress:

1. Atomic Force Microscopy (AFM): A technique which produces indentations using a cantilever with a spherical tip on the gel surface. The deformation caused by the cantilever is proportional to the stiffness of the substrate, in this case the hydrogel (Whitehead et al., 2021).

2. Uniaxial Compression: A technique in which a continuous force is applied on the gels at variable speeds until the integrity of the gel fails and this point of gel failure is proportional to the stiffness potential of the hydrogel used.

Since these techniques are expensive, difficult to set-up and perform and usually give variable results most experiments including mine use commercially prepared hydrogels of pre-defined stiffness.

2.6 Live Cell Imaging

Live-cell imaging is the technique by which time-lapse microscopy is used to study living cells and is used to better understand biological functions of cells. Most experiments use a combination of fluorescence microscopy with live cell imaging.

Live cell imaging is not a very simple or easy technique and can be a challenge to perform. There are certain factors that need to be considered before performing this technique such as ensuring that the cells are healthy and moving normally when placed under a microscope. The conditions under which cells are maintained are especially important as these conditions are responsible for the whether the experiment is going to be a success or not. These conditions usually include keeping the cells at the correct temperature as well as the right carbon dioxide concentration.

Live cell imaging has several potential benefits in research, which include:

- Allowing for the visualization of transient events while monitoring live cells over time.
- Regulation of external factors ensures that the cells being studied are in their native state.
- This technique prevents the formation of background items which can disturb the quality of images obtained.
- Results obtained from live cell imaging cannot be obtained from any other technique.

It is for these reasons that my project used live cell imaging based on the instructions provided in the paper (Evans et.al 2022).

According to this paper, BjhTERT SV40T Ras cells were seeded at 103 cells/well in 10 well slide chambers that had glass bottoms sourced from Greiner, model 543079. These cells were then allowed to attach and proliferate for 48 hours. Next, the cells were treated with DMSO or tubacin for 3 hrs, along with 0.05 μg/ml Hoechst nuclear stain to stain the nuclei of the cells. Once the staining was complete, the cells were live cell imaged, with images being taken every 45 min for 18–24 h, using a 10× magnification Cell Discoverer 7 Fluorescent Microscope from Carl Zeiss.

In order to live cell image, the siRNA transfected cells, the cells were incubated for 72 hrs after the siRNA transfection took place as this allowed for optimal knockdown efficiency to be reached before the imaging. Analysis of 15 films were performed for both the control and the tubacin inhibited HDAC6 cells for both live cell imaging experiments.

2.7 Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence (IF) microscopy is a commonly used technique of immunostaining and is a type of immunohistochemistry that uses fluorophores to observe and locate the bound antibodies.

IF microscopy can be divided into primary (direct) and secondary (indirect). The primary technique involves using a single antibody that is bound directly to a fluorescent dye, which then binds to the target molecule. Next, immunofluorescence microscopy is used to detect the conjugated fluorescent dye that is bound to the target molecule thereby locating the target. This technique is quicker due to fewer steps during staining and avoiding antibody cross-reactivity or non-specificity which can affect the result due to increased background signal. The secondary technique involves two different antibodies: a primary antibody, which only binds to the target molecule, and a secondary antibody that is bound to the fluorescent dye, which then binds to the primary antibody once it is attached to the target molecule. Although this method is longer than the direct method with more steps, it is used since it is more flexible with regard to experimental design, which results in better detection of the target through amplification of signals and is simpler since the secondary antibody conjugates are widely commercially available.

Confocal microscopy, or confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM) is a technique that uses light to image objects and provides vastly improved resolutions. In confocal microscopy, light from an external source is shone directly through the object and an image is formed. There are a several factors that must be taken into account when using confocal microscopy in experiments such as the magnification used, the size of the sample, the speed with which imaging will take place, the fluorescence being used and the process by which the sample is prepared.

My experiment was carried out by first using cells that had been fixed and permeabilized in a PBS solution that was made up of 3.7% formaldehyde, 0.1% glutaraldehyde and 0.2% Triton-

X100. The actual immunofluorescent staining process was performed exactly as described in a previous paper (Gad et.al 2012). The antibodies used were as follows:

- anti-mouse-vimentin, clone V9 (V6389, Sigma Aldrich)
- anti-rat-vimentin (MAB2105, R&D systems)
- anti-mouse-p-Tyr (sc-7020, Santa Cruz)
- anti-rabbit-β-tubulin (ab6046, Abcam)
- Alexa-Fluor-488 phalloidin (Invitrogen)
- Goat anti-mouse-Alexa-Fluor 555 (Invitrogen)
- Goat anti-rabbit Alexa-Fluor 647 (Invitrogen)
- Goat-anti-rat-Alexa-Fluor 647 (Invitrogen)

After treatment, the cells were imaged using the ZEISS LSM 980.

2.8 Cell Shape Analysis

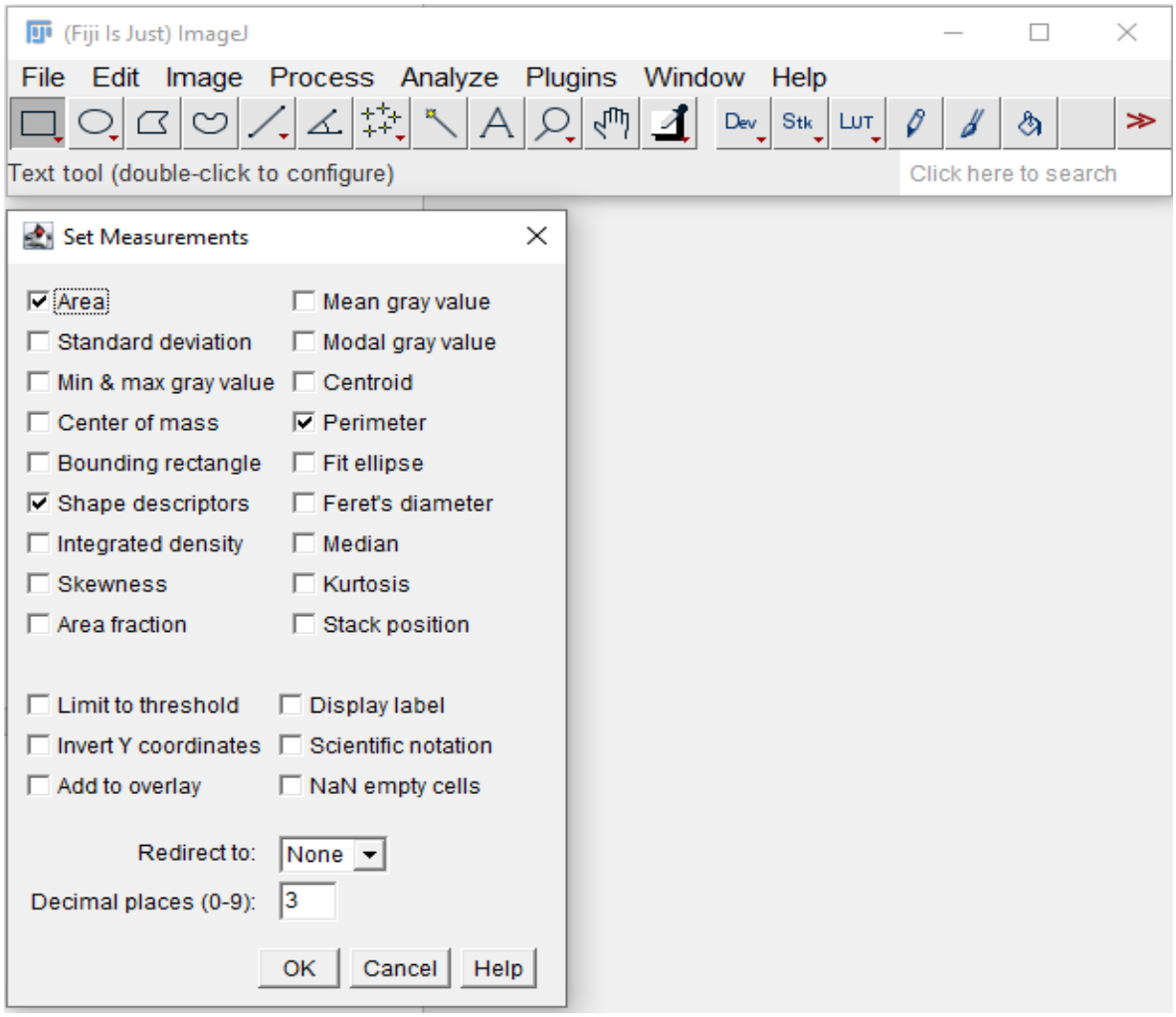


Figure 2.4: Overview of Cell Shape Analysis (Made by Azeer Zafar using FIJI (ImageJ) software)

This figure shows the software interface and the parameters that are selected in order to automatically analyse the shape of the cell and quantify the results.

Cell shape is the large-scale expression of several organized biological processes which are controlled by cytoskeletal interaction, the membrane bound proteins and the ECM. It is especially important that we have the ability to analyse cell morphology now that microscopy becomes more quantitative and higher throughput. It is important that we develop computational tools which have the ability to extract, a quantitative and statistically significant result from lots of data for the analysis of cell shape. Shape factors are used in image analysis and microscopy that numerically describe the shape of a particle, independent of its

size. Shape factors are calculated from measured dimensions, such as diameter, circularity, aspect ratio, area, perimeter, etc.

In my experiment, the shape of the cells was quantified using the ImageJ software. This was done by taking the images of the cells obtained and using a scale bar that allowed me to calibrate the shapes of the cells at different points of time (2 hrs, 8 hrs, 12 hrs and 18 hrs). The calibration allowed for drawing a clear manual border around the imaged cells in all the frames, which then allowed me to measure the area and other physical features of the cell such as its circularity and aspect ratio. During the analysis of the cell shape, the number of cells (shown as treated/control) were as follows, 2 h (220/285), 8 h (196/286), 12 h (177/258) and 18 h (177/224).

2.9 Migration Assay

Cell Migration is an essential property of live cells that is required for the normal growth of cells, their immune response, and in disease progression such as cancer. The migration assay is the most popular technique to measure cell migration, which is essential for biomedical research such as cancer biology, among others. The principle of migration assay method is quite straightforward. It involves a cell culture wound assay in which a tear is made on the top layer of the cell monolayer. Next, the speed with which the wound closes and cell migration takes place is quantified by imaging the wound with a microscope at several time intervals. Studying cell migration is particularly important for cancer as the main cause of mortality in cancer patients is because of metastatic progression. There are also other techniques which can be used to measure migration, including transwell migration assays and invasion assays which are the most widely used today.

Migration assay provides excellent results for cell migration. This technique can also be used to investigate cell invasion by adding a coating of ECM on top of the membrane in order to replicate the process by which the invasion of the ECM takes place. Additionally, results obtained after the migration through the assay, staining of the proteins along with fluorescent microscopy is invaluable for the morphological study of cells during 3D migration and invasion.

In my experiment, the cells were seeded at 1000 cells/well in slide chambers, which were sourced from Greiner, model 543079 and incubated for 24 hrs. 1 hr before the live cell imaging

takes place, the solution was replaced with a fresh solution that had 0.05 g/mL Hoechst nuclear stain, sourced from Invitrogen, type H3570. This was followed by 17 h live-cell imaging on Cell Discoverer 7 (Zeiss, Germany), with images taken every 20 minutes.

2.10 Image Analysis

FIJI/ImageJ, which is an open-source freely available image processing software was used to calculate the morphology of a cell with a defined boundary. FIJI was also used to analyse the contractile forces of cells obtained from TFM images (Schindelin et al., 2012) (Figure 2.5).

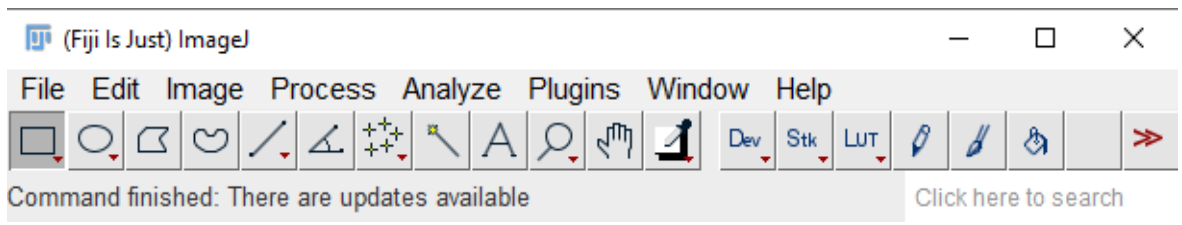


Figure 2.5: Overview of FIJI (ImageJ) Software. (Made by Azeer Zafar)
This figure shows the software interface of the software that is used to detect, measure, analyse and quantify the physical forces of cells along with cell boundaries.

In order to count the cells, the contrast of the image is first increased. This is done using the Brightness-Contrast command but only works when the image is in the 8bit format. Next, the Image-Adjust-Threshold function is used to divide the image into features that are being measured, and the rest go to the background. Objects that are either too small, large or have weird features are removed and then the particles are analysed using the required functions. First, the scale on the image is set either manually or it will be automatically calculated using pre-programmed parameters. The Analyse/Set Measurements are then used to calculate the required information. FIJI also displays a list of measurements criteria that can be used for measurement and analysis. For this task, Area is selected, and the Analyse Particles command are then used as above (Figure 2.6).

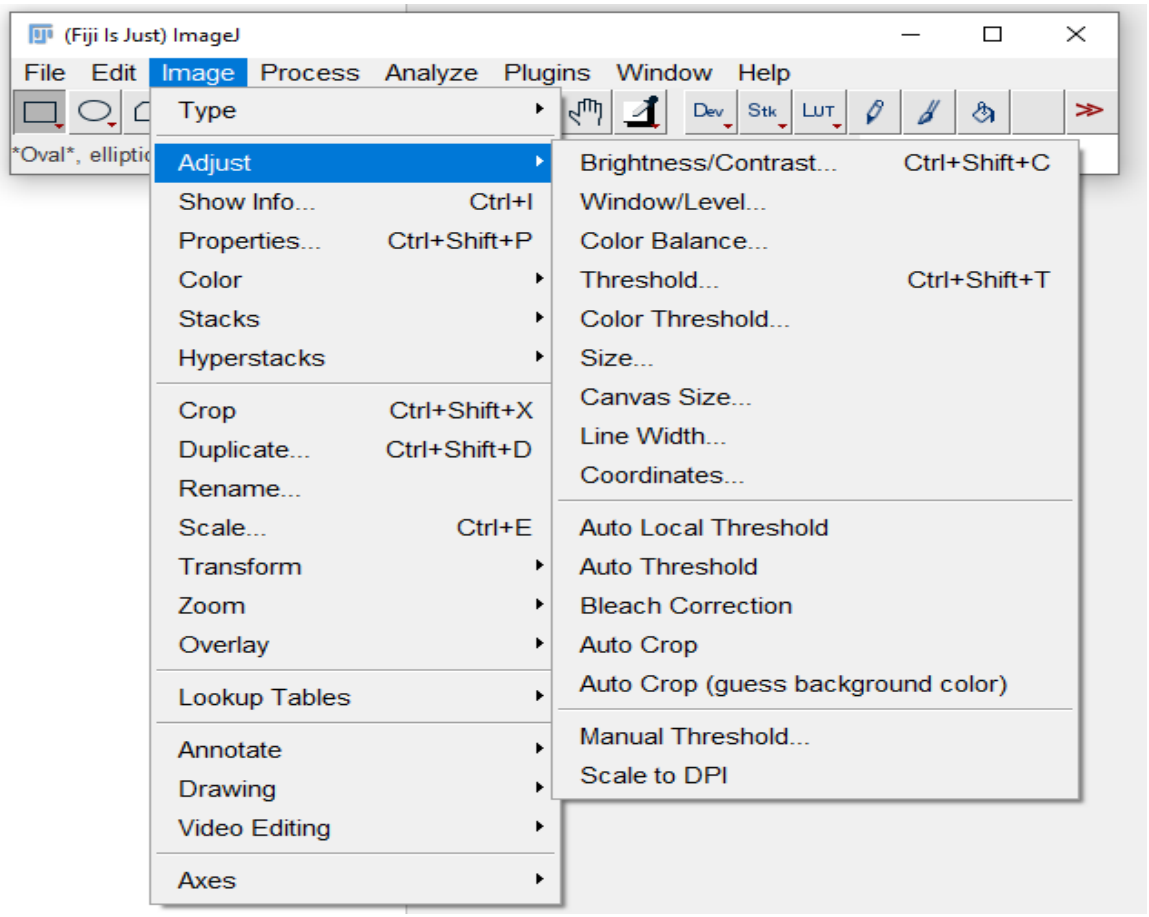


Figure 2.6: Overview of Image Analysis Function (Made by Azeer Zafar using FIJI (ImageJ) software)

This figure shows the software interface and the parameters that are available in order to automatically adjust the image settings in order to quantify image analysis.

FIJI can also be used to measure the area of the nuclei of the cell. Image is adjusted for contrast, followed by a limit being applied. Select the area of the cell which includes the region of interest or even multiple regions of interests. Once all the regions of interests are selected, FIJI automatically calculates the area of the desired cell as long as the scale bar has been accurately set.

In my project, the videos obtained from live cell imaging were processed and the mean cell migration speed and persistence of these cells is recorded using the Track Mate plugin present in FIJI. For my cell shape analysis results, the images obtained after 9 hrs were used, and the individual cells were selected manually and then followed by quantification by shape descriptors by FIJI version 1.53.

Calculating the traction force of a single cell is normally based on measuring the deformation of the cell-culture substrate in response to the force generated by the cell traction force microscopy (TFM; Dembo and Wang, 1999), Different types of readings have been used to determine the mechanical movement of the cells, which include

- The force per cell adhesion (Plotnikov *et al.*, 2012)
- The force produced at cell ends (Rape et al., 2011b)
- The average traction force of the cell (Rape *et al.*, 2011a)
- The integrated absolute forces (Reinhart-King *et al.,* 2005)
- The contractile energy (Butler *et al.,* 2002)

The last is the one which I believe best characterizes the mechanical effort generated by the cell. However, for all of these readouts, the average values of all the individual cells are normally used to represent cell traction forces.

2.11 Nuclear Force Analysis

The project measured the force that was necessary to exert the nuclear deformation that is seen between the attached and detached states of the cells according to a previously described method in another paper (Estabrook et al., 2021). The algorithm that was used in this paper was modified to allow us to analyse the nuclear stained Hoechst nucleus images obtained from the cells when they were imaged during TFM experiments. Briefly, cells were incubated with Hoechst 33342 dilution (0.05μg/mL) for 30 min at 37˚C prior to TFM imagining and nuclear shape images were taken of the attached and detached cell states as described. The total force that was obtained was calculated using a custom R script using the data obtained by combining the total of the force vectors divided by the perimeter of the cells.

2.12 Statistical Analysis

The statistical analysis for the TFM measurements was performed using the data quantification software GraphPad Prism software version 9.3.1. Ordinary 1-way ANOVA and t-tests were used to compare between the different experimental conditions for energy, total force and max force. Data was presented as mean.

The statistical analysis for the focal adhesion sizes was performed using unpaired t-tests in the data quantification software GraphPad Prism software version 9.3.1. The sizes and frequencies of the focal adhesion that were obtained were used to make a histogram (bin size, 1 μm2).

The statistical analysis of western blots was performed using GraphPad Prism software version 9.3.1 and the data was displayed as mean ± standard deviation or mean with SEM.

Also, the differences in results seen between groups were analysed using the Anova and Student's t-test, where $p \leq 0.05$ was taken as statistically significant. The mean values of the aspect ratio, cell migration speed and persistence, cells from which debris is detached and left behind during migration, filament distribution were analysed using the data quantification software GraphPad Prism v.9.3.1.

Finally, for the migration analysis, the statistical analysis was also performed using the data quantification software GraphPad Prism version 9.3.1. Ordinary 1-way ANOVA and t-tests were used to compare between the different experimental conditions for mean speed, linearity and cell shape. Identification of outliers was performed using the ROUT method (Q=1%).

Chapter THREE – EXPERIMENTAL RESULTS

Chapter 3 – Experimental Results

3.1 TFM Optimization

The primary component of the project was to measure the effect of HDAC6 on the contractile forces of metastasising human fibroblasts and compare them to normal fibroblast cells using TFM as the main technique. Therefore, it was important to ensure that the TFM was properly set up and ready to use to get clear and accurate results, especially as TFM was not established in our research lab. I initially wrote a detailed and well researched protocol (Section 2.5), then tested this extensively with multiple experiments to ensure it measured contractile forces of cells.

To optimise TFM technique, metastasising Ras cells were first stained with the Hoechst nuclear stain. The nuclei movement of these cells was filmed over 16 hours using different concentrations of Hoechst stain under the CD7 microscope and with different seeding cell numbers. Filming over 16 hours also allowed me to measure and determine the exact time required for cells to adhere to the polyacrylamide gel surface that contained the fluorescent beads used for TFM (Fig 3.1). Staining with Hoechst was done since the TFM technique was not yet set up and filming for Hoechst stain in cells would help ensure that the metastasising cells were attaching and seeding successfully with the polyacrylamide gel before the actual experiment could begin.

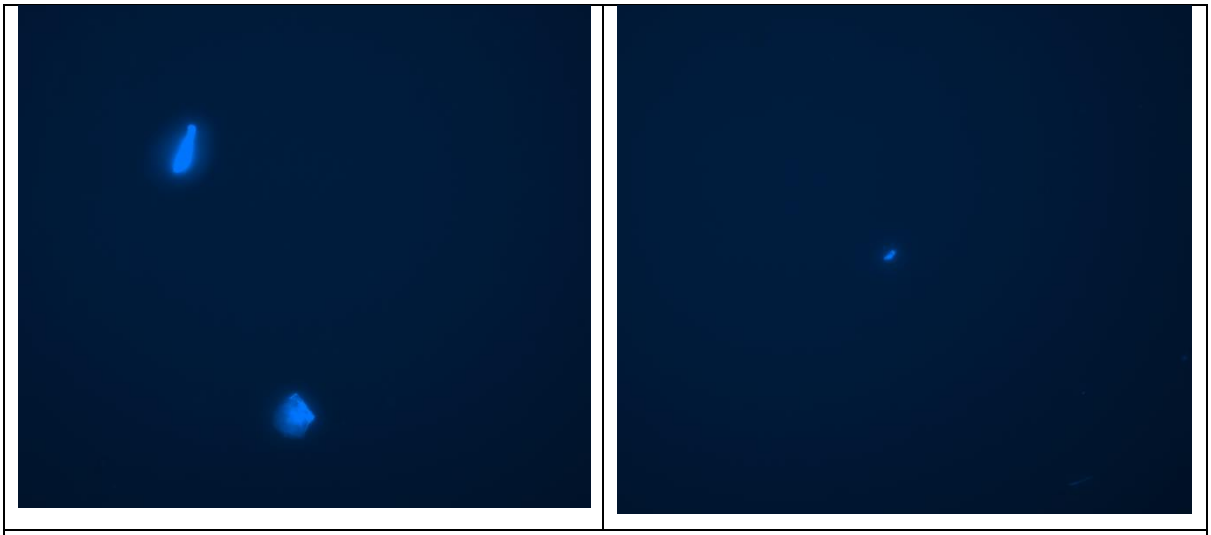


Figure 3.1: Failed Hoechst Staining Images. (Images taken by Azeer Zafar)
Cells were imaged under different conditions and concentrations of Hoechst stain. However, images obtained were not clear and there were several problems. Each experiment gave different image results as can be seen in the two images above.

Next, to optimise TFM and to confirm that the protocol could be used to measure the contractile forces of BJ fibroblast cells on 2D gels, a Pilot experiment without any cells to ensure all the components were working in sync was performed. Fluorescent beads that are necessary to view and measure the movement of the cells were viable at this stage and the filming process, confirming the microscope was effective and could be used to run TFM.

After this a complete pilot TFM experiment with actual cells was then run, this was to check if the movement of the cells could be filmed and the contractile movement of these cells could be measured, since this was the primary purpose of the microscopy technique as well as a crucial aspect of the project.

Once the TFM method was optimised an experiment was set up using the BjhTERT-SV40T-Ras cells, then cells were filmed overnight. This should have been followed by analysing the film obtained to measure the bead displacement and the traction force (contractile force) of the cells used, but unfortunately, several problems were encountered (see Table 3.1 for specific details).

Further troubleshooting and analysis of the procedure determined that the errors that that were being received along with the failure of TFM techniques were due to the cells dying during the trypsinization process because of using a high concentration of trypsin to detach the cells during filming. To resolve this, a Pilot trypsinization experiment was planned that would answer if cells could be trypsinized in a 35 mm dish (which is what we use to seed the cells and film them) for TFM technique (Fig 3.2, Fig 3.3).

Table 3.1: Troubleshooting TFM Process. List of problems faced (left) during set up and running of TFM and the steps taken to resolve the problems (right).

Problems Faced	Troubleshooting Steps
Cells dying while staining with Hoechst.	Initial cell incubation time
Filming cell movement using the microscope	Cell numbers seeded in polyacrylamide gel plate.
Difficulty with focusing on the cells in the microscope	Changing the concentration of the Hoechst stain used to stain cells.

Problems with adjusting the settings on the microscope.	Changing the default magnification settings on the microscope in order to focus better
Death of the cells being filmed (although they were kept in optimum conditions, 37°C and 5% CO2) probably due to the trypsinization process.	Reducing the length of time for which cells were filmed since extended exposure to trypsin can destroy the cell membrane.
Inability to focus on the cells during filming at the required magnification.	Changing the concentration of trypsin being used for detachment of cells
Inadequate growth of the cells despite seeding them under the right conditions and for the required incubation period	Changing the temperature and CO2 concentration in which cells were kept.

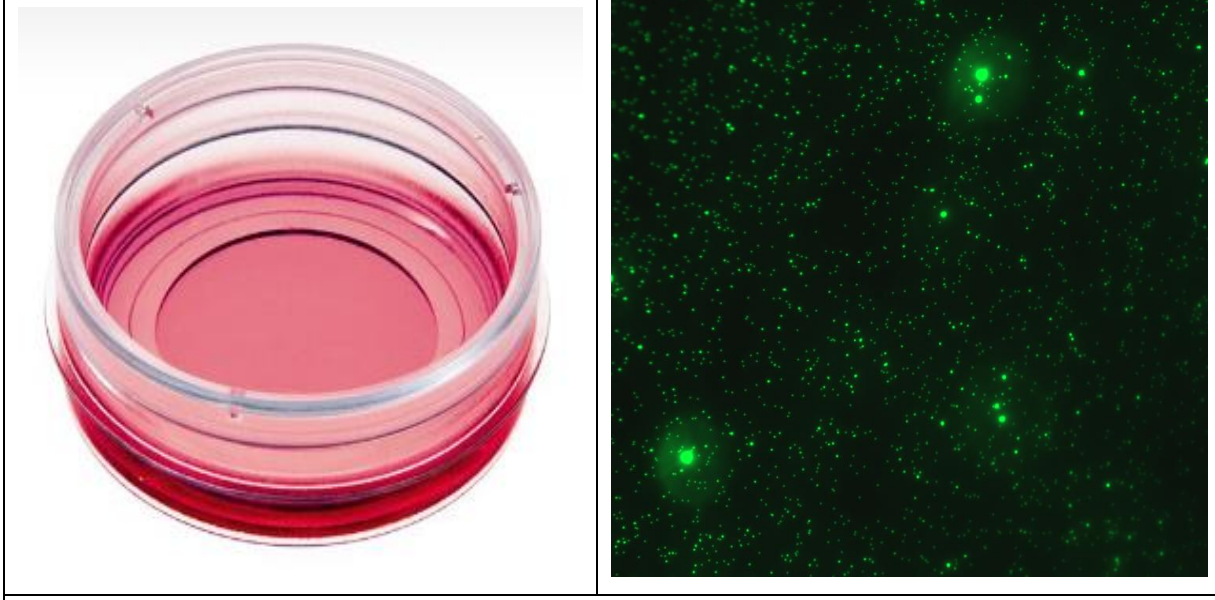


Figure 3.2: Pilot TFM Experiment. (Images taken by Azeer Zafar)
(Left) The 35 mm polyacrylamide gel plate (hydrogel) that contains the yellow-green, fluorescent beads. (Right) Filmed the gel plate at 60x and the experiment was a success since the fluorescent beads (green) that are necessary to determine the contractile forces of the cells were viable and the filming process also ran smoothly.

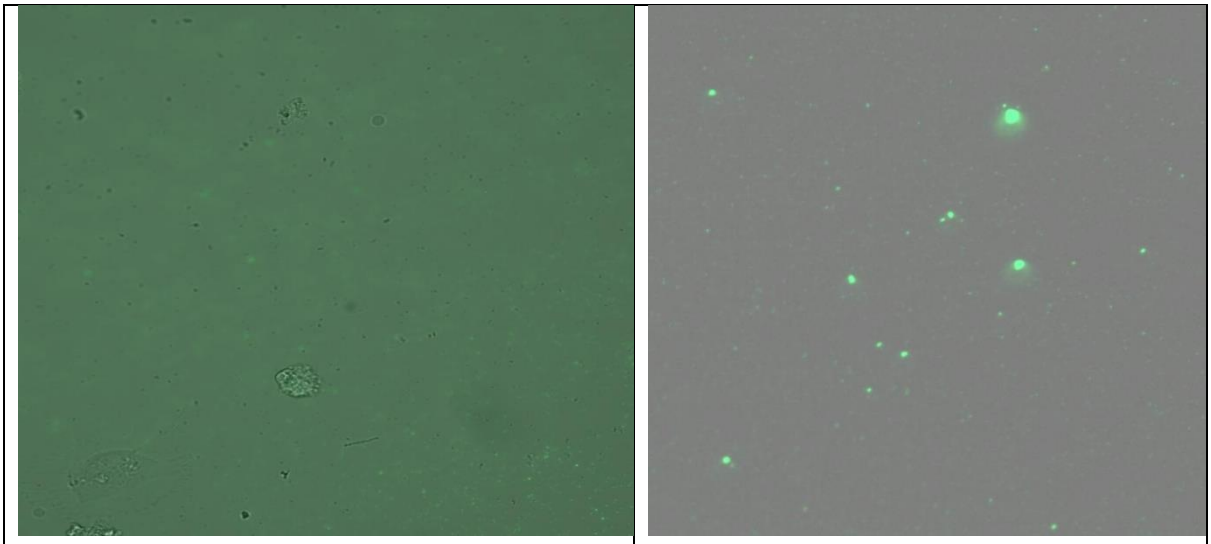


Figure 3.3: Failed Pilot TFM Experiment (Images taken by Azeer Zafar).
(Left) 20x Brightfield Alexa 488 with no significant cells seen. It is unclear what has happened to the cells since they were clearly present during seeding. (Right) 20x Yellow Green fluorescent channel where fluorescent beads are not clearly seen and do not seem viable. Field is also hazy which might be due to changes in the microscope settings.

In this troubleshooting experiment, three different concentrations for trypsin were used and the correct trypsin concentration (1%) was determined, which could be used to detach the cells without killing them. The problem of trypsinizing the cells once they were inside the CD7 microscope was also resolved since this is a closed microscope with only a small inlet at the top from which I had to manually pipette the trypsin into the 35 mm plate. The reason for this is that once the plate is inside the microscope and the initial position of the cells has been filmed and recorded, the next stage is trypsinization in order to detach the cells so that their final position can also be recorded. However, this must be done in the same frame where the single cell is present. If the plate is removed in order to add trypsin, then the frame and position of the single cell is lost, and the traction force cannot be measured any more. Therefore, the trypsin must be added to make from the inlet in the top and because the lid of the 35 mm plate is closed, the lid had to be removed before placing the plate in the microscope to add the trypsin.

Once all the issues had been resolved, the entire TFM experiment was re-run which gave results that could be analysed.

3.2 Focal Adhesions Quantification Analysis Optimization

In addition to measuring contractile forces of cells using TFM, I also measured the cell-matrix focal adhesions and cell spreading area of normal fibroblast and metastasising Ras cells with HDAC6 being inhibited using Tubacin or knocked down with HDAC6 siRNA. To perform these experiments, I designed an experiment that allowed me to fix cells and stain the cells with different antibodies for focal adhesions, vimentin, and actin. I then used the LSM 980 confocal microscope to image these fixed stained cells which were then later quantified using ImageJ/FIJI. This then required focusing further on troubleshooting, which mostly involved repeating the experiment several times but changing certain properties or settings each time (Table 3.2).

Table 3.2: Troubleshooting Focal adhesion quantification analysis. List of problems faced (left) during set up and running of measuring focal adhesions and the steps taken to resolve the problems (right).

Problems Faced	Troubleshooting Steps
Cells dying after seeding them.	Changing initial cell incubation time.
Difficulty with focusing on the cells in the microscope.	Changing the focus settings of the microscope to get better images.
Problems with adjusting the settings on the microscope.	Changing the default magnification and zoom settings on the microscope in order to focus better.
Inability to focus on the cells during filming at the required magnification.	Changing the type of primary and secondary antibodies that I used to stain the cells in order to get better signals.
Breakdown of the microscope while operating it during imaging.	Changing the concentrations and volumes of primary and secondary antibodies that I used to stain the cells in order to get better signals.
Cracking of coverslips on the slides during imaging.	Taking regular break intervals in between imaging to prevent the microscope from overheating and shutting down and stopping the experiment.

3.3 Comparing the difference in contractile forces of normal, metastasising and tubacin treated metastasising cells.

Result: Inhibiting HDAC6 via tubacin leads to a decrease in the maximum traction force exerted by metastasising cells.

Previous studies have shown that metastasising cells have increased contractility than normal non-metastasising cells and it is this factor that makes metastasising cells more invasive in cancer. Previous studies have shown that there is a direct correlation between the force exerted by cells on their environment and their ability to invade their surroundings (Kraning-Rush et al., 2012). I therefore aimed to determine if oncogene-expressing, invasive and metastasising fibroblasts show increased cellular contractile force, as compared to normal control, and if so, if this is mediated by an HDAC6- dependent molecular mechanism. Studies show that this increased contractility can be attributed to HDAC6, and it is important to test this fact. Therefore, an experiment was planned and designed to test the difference in contractile forces between normal and metastasising BjhTERT cell lines. This was further expanded on by inhibiting the HDAC6 in metastasising BjhTERT cells using tubacin to compare how the contractile forces change. The experimental design, designed by me was used to test the conditions is shown below (Fig 3.4).

Before evaluating the contractile forces of the cells, a western blot was performed with the assistance of Dr Ana Lopez, my technical supervisor to measure the expression of HDAC6 in normal BjhTERT and metastasising BjhTERTSv40TRas cells (Fig 3.5A) and the results clearly show an increase of HDAC6 expression in the metastasising cells when compared to normal control cells. These results confirm previous studies (Rathje et al., 2014, Lea Feld, 2020) that claim that HDAC6 expression is overexpressed in metastasising cells when compared to normal cells.

Next, the contractile forces were measured by me, and the results obtained give both quantitative measurements of contractile forces as well as qualitative traction maps that show the contractile force that is being exerted on representative images (Fig 3.5B & Fig 3.6). Traction force heat maps for both cell conditions, as well as metastasising cells that has HDAC6 inhibited with tubacin, were assessed (Fig 3.5B). The yellow lines show the traction force boundaries of all the cells in the images below. The results from these images show a clear increase in traction force in metastasising cells (middle image) when compared to normal cell (left image), which is in line with the results that show increased HDAC6 expression in

metastasising cell. However, when the metastasising cell is inhibited using HDAC6 inhibitor tubacin (Fig 3.5B), the traction force decreases (right image) and becomes closer to the force in normal cells (left image).

Traction forces were then quantified and used to measure cellular contractility in the form of three separate aspects (Fig 3.6). These include the energy that is present in the cell and is required to produce the required contraction of the cell in picojoules (pJ) known as elastic energy (left), the ratio of the force exerted by the cell proportionally divided by the area covered by the gel on the polyacrylamide gel, known as total force in piconewtons (pN) (middle) and finally the highest value of the force (max force) that is exerted by the cell on the gel in pascals (Pa) known as maximum force (right). The measured traction force in metastasising cells (BjhTERTSv40TRas + C) was similar to the traction force as measured in control cells (BjhTERT), however there was an increase in the levels of maximum contractile force ($P \leq 0.01$). However, treating the metastasising cells with HDAC6 inhibitor Tubacin led to a decrease in the elastic energy, the total force, and the maximum force of metastasising cells significantly when compared to untreated metastasising cells and brough contractile forces closer to normal ($P \leq 0.01$). Looking at these results show us that the ability of cells to exert contractile forces on their surrounding environment increases in metastasising fibroblasts, and this is controlled by higher levels and/or activity of HDAC6.

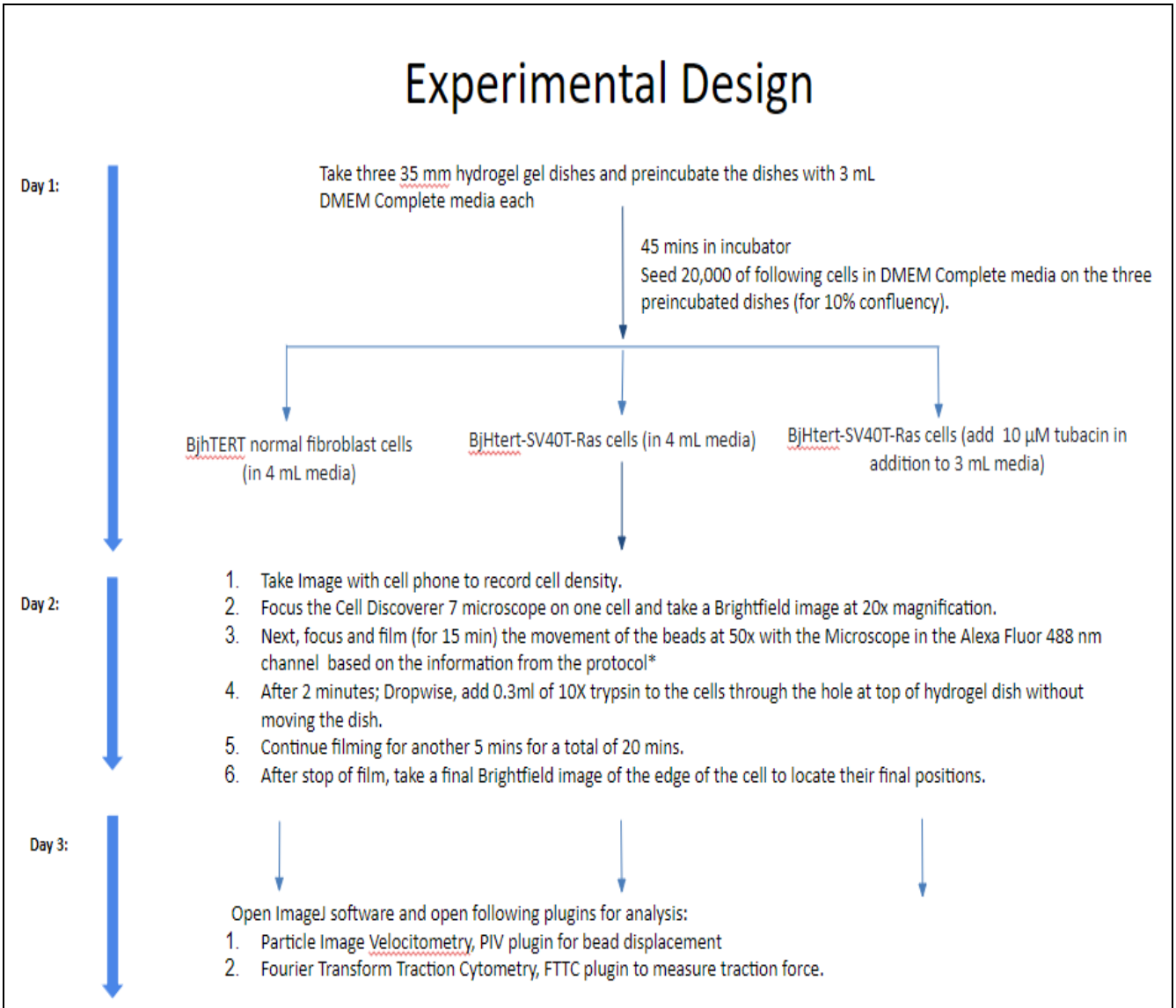


Figure 3.4: Experimental Design of TFM with HDAC6 inhibition. (Made by Azeer Zafar using Microsoft PowerPoint)

The experimental design compares between the differences in contractile forces in all 3 cell conditions (normal BjhTERT, metastasising BjhTERTSv40TRas and tubacin treated metastasising BjhTERTSv40TRas cells.

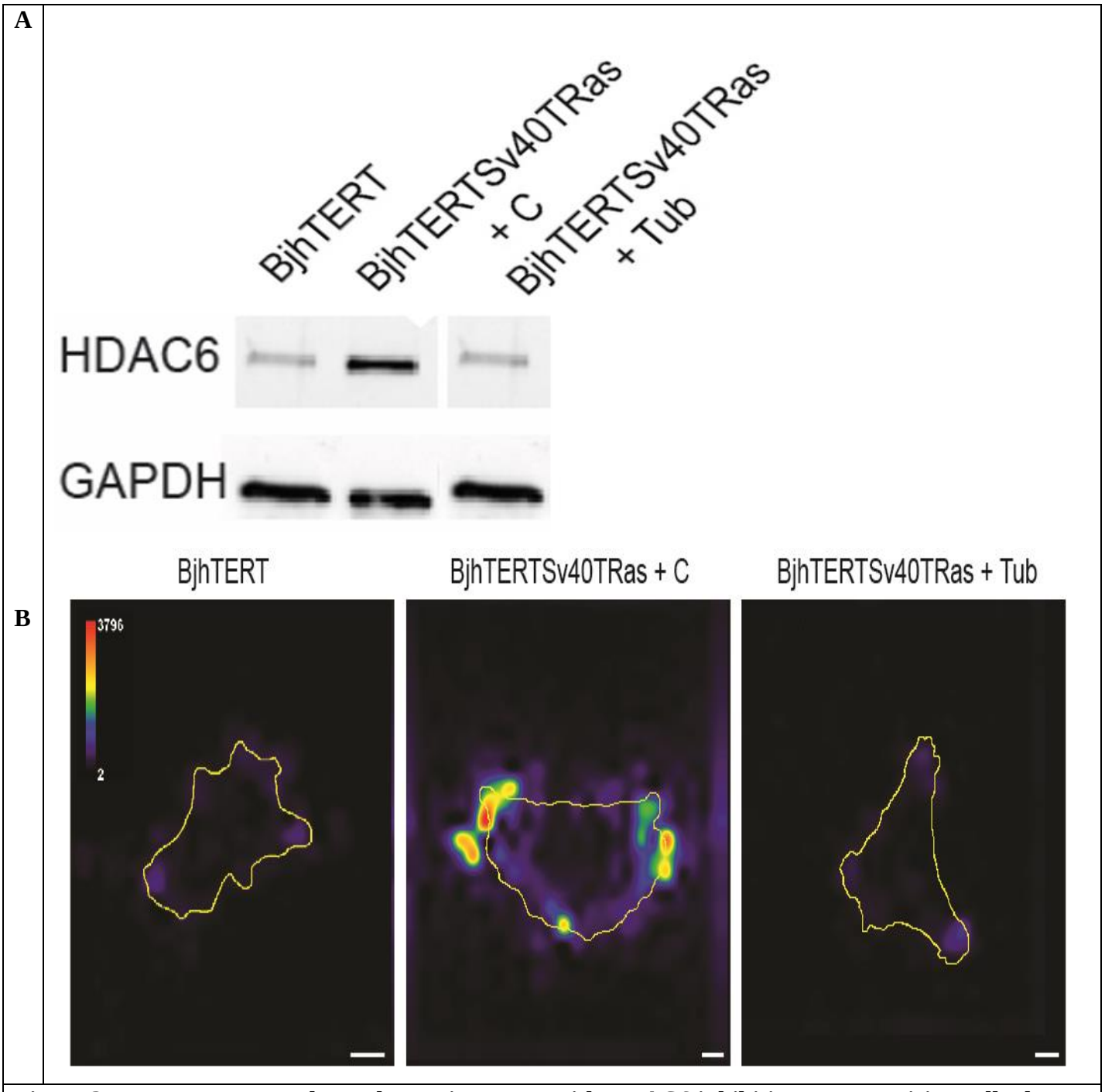


Figure 3.5: TFM Western Blot and Traction Maps with HDAC6 inhibition. Metastasising cells show an increase in the maximum force, which again decreases back to normal levels after treating the metastasising cell with Tubacin to inhibit HDAC6. (Taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

(A) Western Blot shows the amount of HDAC6 protein and GAPDH loading control present in normal BjhTERT cells, metastasising BjhTERTSv40TRas cells and tubacin treated metastasising cells. **(B)** Representative maps of traction force visible in normal control cell, metastasising control cell and tubacin

treated metastasising cell, with coloured scale key that shows the magnitude of traction force, in Pascals. Scale bar: 20 µm. Maximum traction force: 3796 Pascals.

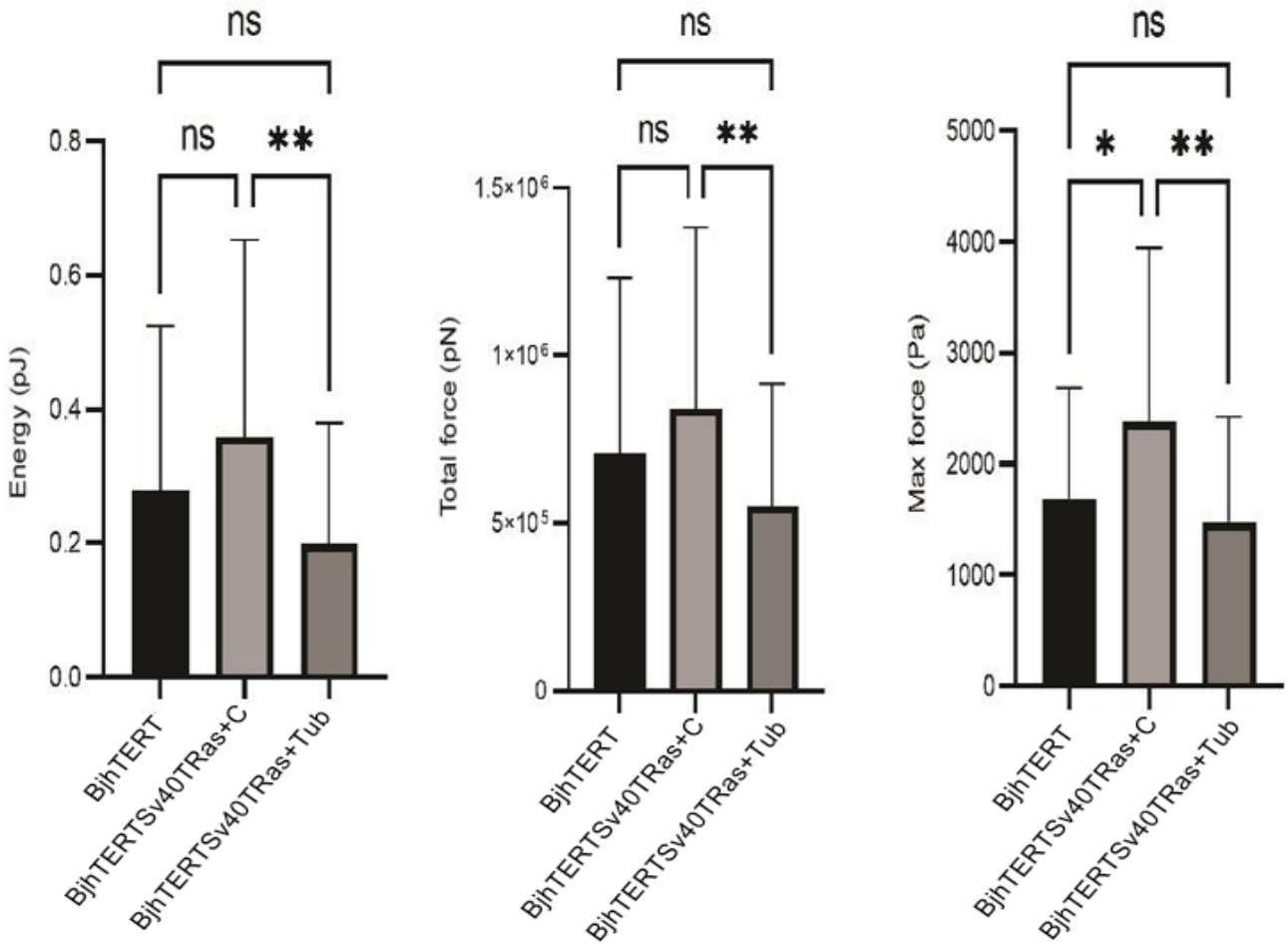


Figure 3.6: Cellular Contractility Quantification with HDAC6 inhibition. Results show a significant increase in max force which is then significantly reduced back and is comparable to max force levels of normal cells after tubacin induced inhibition of HDAC6. (Made by Azeer Zafar using FIJI/ImageJ software and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

The corresponding quantification on the left side shows the traction force spread by a cell over the cell area, known as total traction force, the middle shows the total elastic strain energy, and the right side shows the maximum force exerted by cells. This data has been obtained from at minimum three biological repeats, represented as mean ± SD. *$p \leq 0.05$, **$p \leq 0.01$. **55** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.4 Comparing the difference in contractile forces of normal, metastasising and siRNA HDAC6 knockdown treated metastasising cells.

Result: Knocking down HDAC6 via leads to an increase in the traction force exerted by metastasising cells.

Since this project involved testing the effect of removing HDAC6 from a metastasising cell model on contractile forces and metastasis, it was not enough to merely enough to observe the effect of HDAC6 removal by inhibiting HDAC6 using Tubacin. Therefore, the project was continued by further determining if whether it was only the activity of HDAC6 or also HDAC6 levels that were seen in metastasising cells that were responsible for the increase in contractile forces in metastasising cells. Therefore, another experiment was planned and designed by me to test the difference in contractile forces between normal and metastasising BjhTERT cell lines. This was further expanded on by knocking down HDAC6 with siRNA and analysing the contractile forces in metastasising cells with or without HDAC6 and compared to normal BjhTERT cells. The experimental design used to test the conditions is shown below (Fig 3.7).

Before testing the contractile forces of the cells, another western blot was performed by me and Dr Ana Lopez to measure the expression of HDAC6 in normal BjhTERT and metastasising BjhTERTSv40TRas cells (Fig 3.8 A) and the results clearly show an increase of HDAC6 expression in the metastasising cells when compared to normal control cells. These results confirm previous studies (Rathje et al., 2014, Lea Feld, 2020) that claim that HDAC6 expression is overexpressed in metastasising cells when compared to normal cells.

Next, the contractile forces were measured by me, and the results obtained give both quantitative measurements of contractile forces as well as qualitative traction maps that show the contractile force that is being exerted on representative images (Fig 3.8 B & Fig 3.9). The next figure shows these representative traction force heat maps for both cell conditions as well as metastasising cells that have HDAC6 siRNA knockdown. The yellow lines show the traction force boundaries of all the cells in the images below. The results from these images show a clear decrease in traction force in metastasising HDAC6 siRNA control cells (middle image) when compared to normal BjhTERT cells (left image) but this trend is reversed and there is again an increase in traction forces in treated metastasising cells with HDAC6 siRNA knockdown (right image) (Fig 3.8 B).

Next, the traction forces were quantified by me using FIJI/ImageJ software and used to measure cellular contractility in the form of three separate aspects (Fig 3.9).

These include the energy that is present in the cell and is required to produce the required contraction of the cell in picojoules (pJ) known as elastic energy (left), the ratio of the force exerted by the cell proportionally divided by the area covered by the gel on the polyacrylamide gel, known as total force in piconewtons (pN) (middle) and finally the highest value of the force (max force) that is exerted by the cell on the gel in pascals (Pa) known as maximum force (right). The metastasising siRNA negative control cells (BjhTERTSv40TRas + C siRNA) showed significantly decreased levels of total strain energy, total traction force and max force when compared to normal control cells (BjhTERT). However, this effect is reversed in treated HDAC6 siRNA metastasising cells (BjhTERTSv40TRas + HDAC6 siRNA) and there is a significant increase in levels of total strain energy, total traction force and max force when compared to negative control cells (BjhTERTSv40TRas + C siRNA) and also normal cells (BjhTERT) ($P \leq 0.01$). Looking at these results show us that the contractile forces of cells can be controlled and supressed via the HDAC6 protein levels.

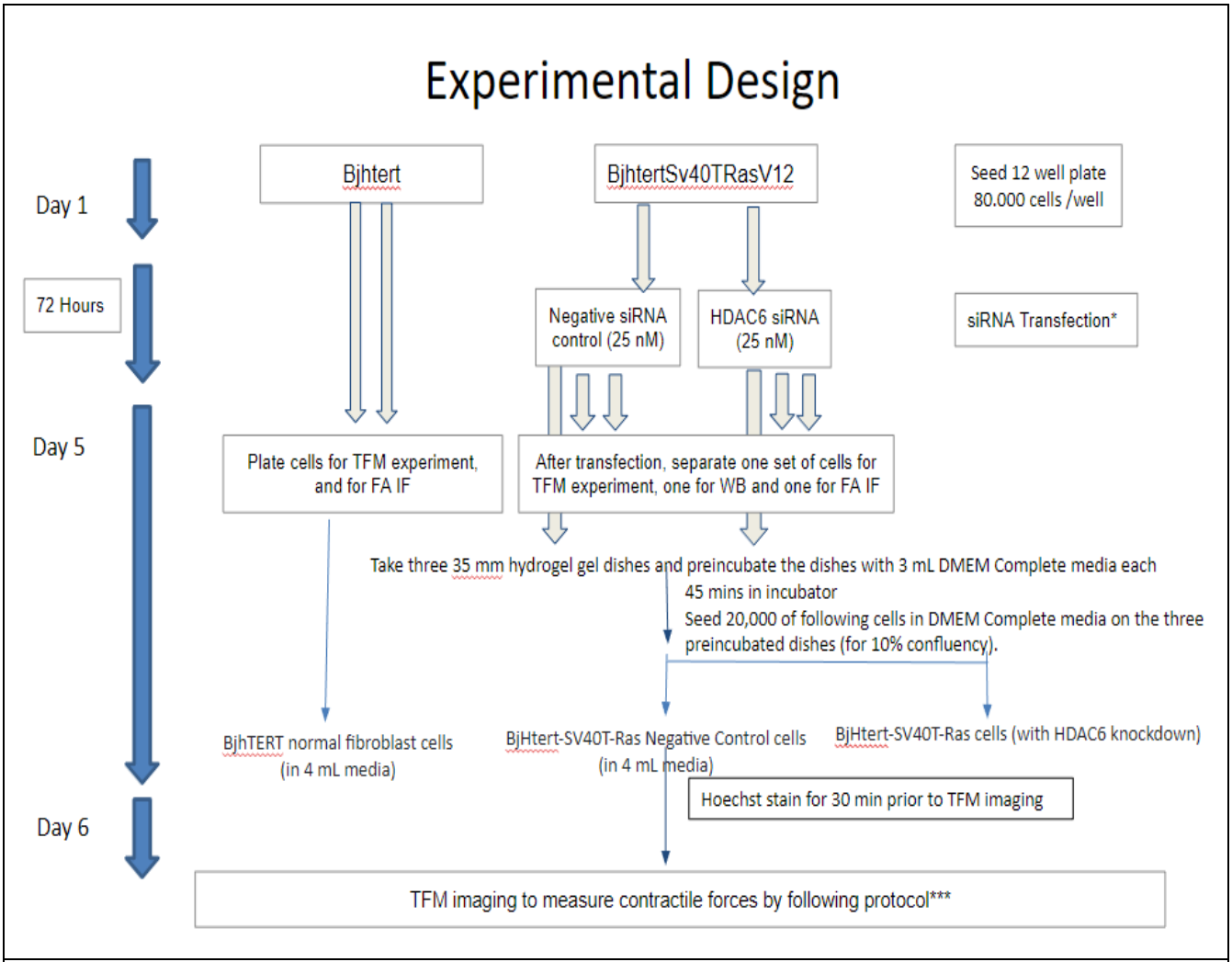


Figure 3.7: Experimental Design of TFM with siRNA HDAC6 knockdown. (Made by Azeer Zafar using Microsoft PowerPoint)

The experimental design compares between the contractile forces in all 3 cell conditions (normal BjhTERT, negative HDAC6 siRNA control metastasising BjhTERTSv40TRas and treated HDAC6 siRNA metastasising BjhTERTSv40TRas cells.

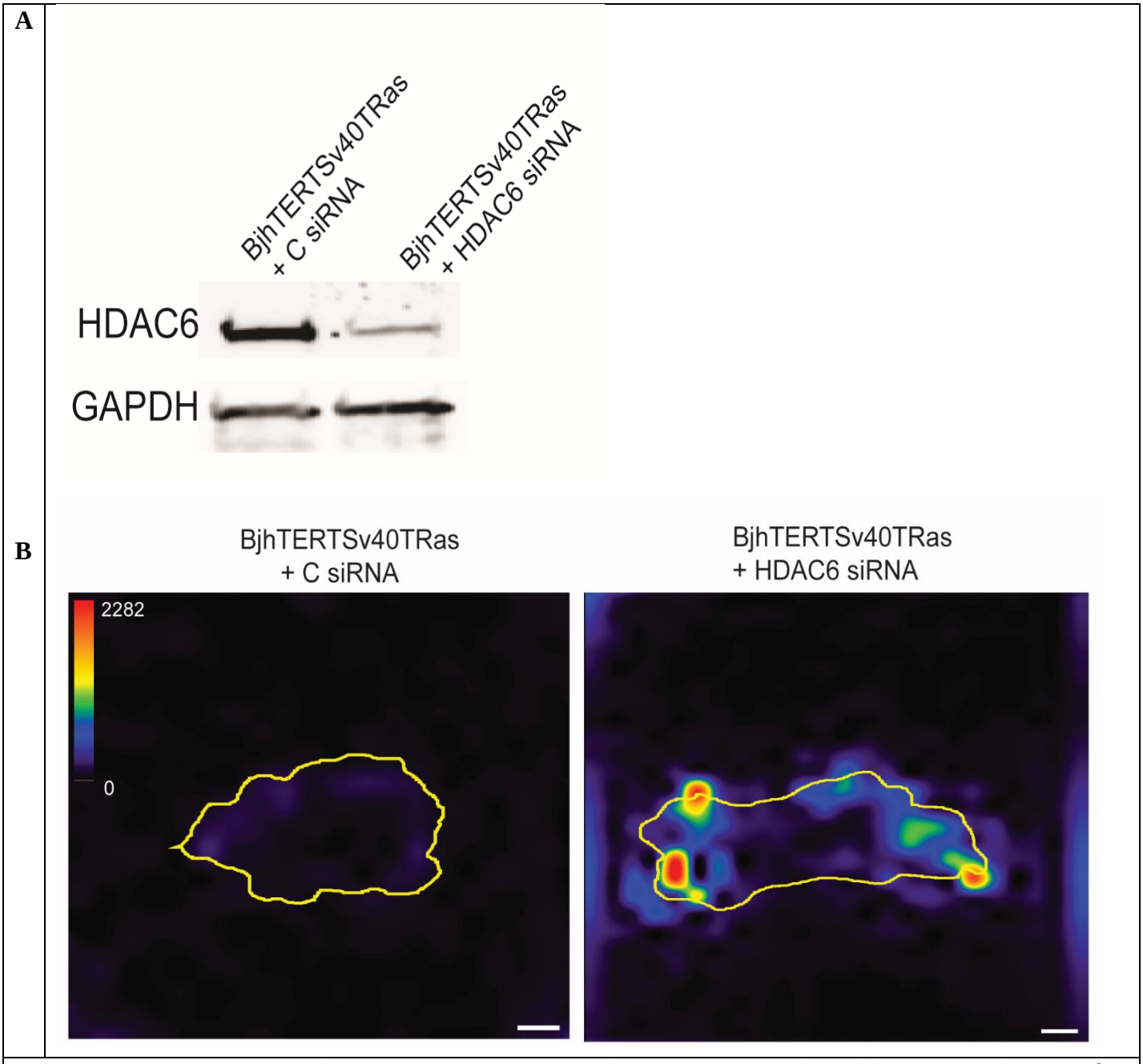


Figure 3.8: TFM Western Blot and Traction Maps with siRNA HDAC6 knockdown. Knockdown of HDAC6 via siRNA leads to an increase in the contractile forces of metastasising cells (Taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

(A) Western Blot shows the amount of HDAC6 protein and GAPDH loading control present in metastasising cells transfected with control siRNA or HDAC6 siRNA **(B)** Representative maps of traction forces, visible in metastasising cells with control siRNA and metastasising cells with HDAC6 siRNA with the coloured scale key that shows the magnitude of traction force in Pascals. Scale bar 20 µm. Maximum traction force: 2282 Pascals.

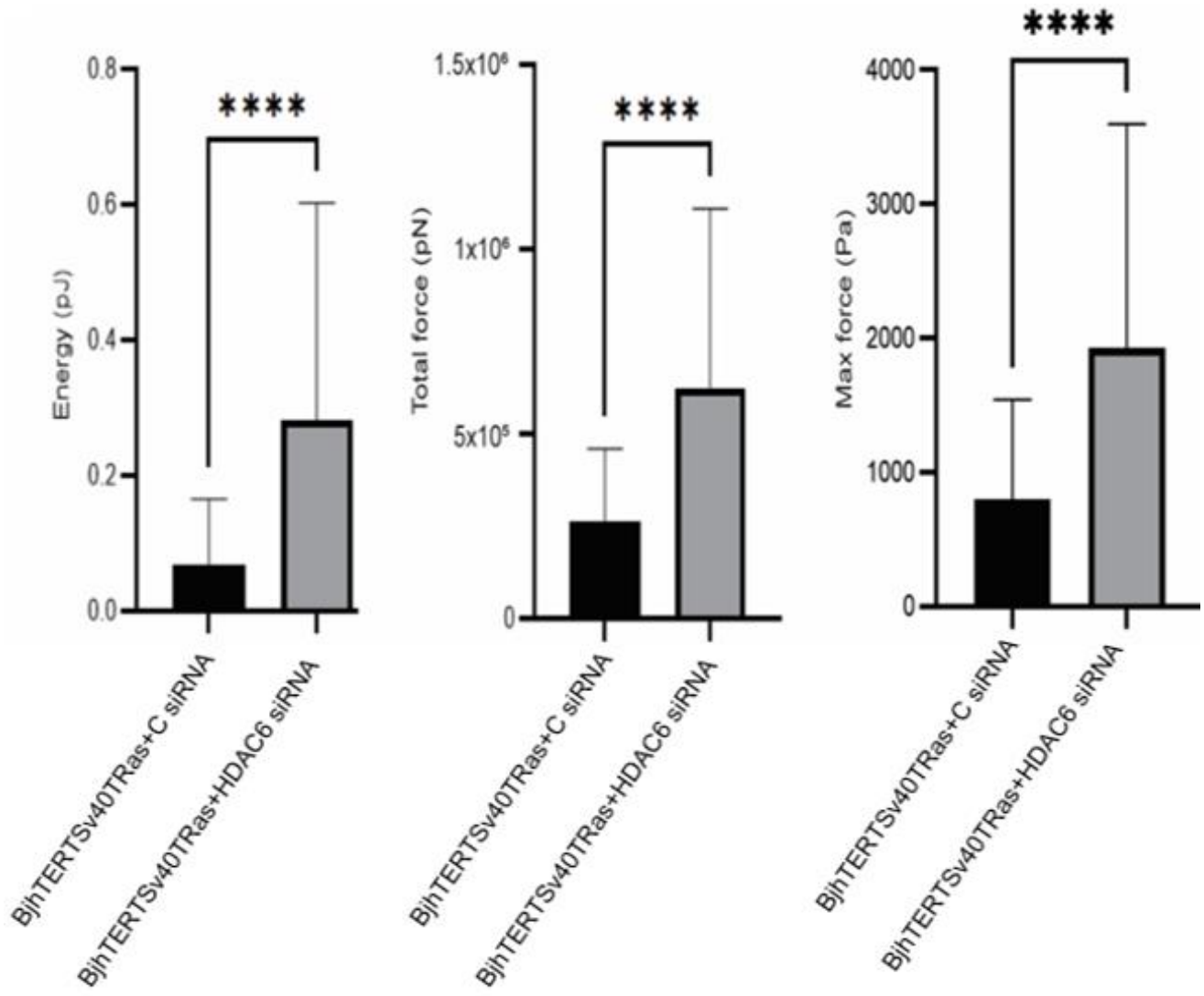


Figure 3.9: Cellular Contractility Quantification with siRNA HDAC6 knockdown. Results show that knocking down HDAC6 via siRNA leads to an increase in the contractile forces of metastasising cells- (Made by Azeer Zafar using FIJI/ImageJ software and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

The corresponding graphs on the left side shows the traction force spread by a cell over the cell area, known as total traction, force the middle shows the total elastic strain energy and the right side shows the maximum force exerted by cells. This data has been obtained from at minimum three biological repeats. Data presented as mean $**p \leq 0.01$. ± SD. $*p \leq 0.05$. **52** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.5 Observing how inhibiting HDAC6 affects the intracellular forces of the nucleus and physical properties of normal, metastasising and tubacin treated metastasising cells.

Result: There is a strong correlation between metastasising cells and a reduction in the intracellular force that is being exerted on the nucleus, with a link to HDAC6 inhibition via tubacin.

The project also measured if there was a change in the intracellular forces that are exerted by cells on their nucleus especially in metastasising cells when treated with HDAC6. Emerging studies show that the cell nucleus has several mechanical properties that are linked to the cytoskeleton and cancer metastasis. During cell migrations, cells change the position of the nucleus, and this maintains polarity and deforms the nucleus allowing cells to pass through small spaces (Wolf et al., 2013). This change in position and shape of the nucleus is normally not possible because of their bigger size and the way they resist deformation. This deformation is overcome by spreading forces that are generated by the polymerization and contraction of cells and transmitted via the cytoskeleton to the nucleus. Previous studies have shown that metastasising cells have a less polymerized and stable cytoskeleton when compared to normal cells, which led to the conclusion that metastasising cells have reduced contractile forces being exerted on their nucleus. The nuclear force is the force that is exerted on the nucleus during cell contraction while nuclear deformation can be described as the change in the shape or size of the nucleus because of any force that acts upon it.

Traction force heat maps for all 3 cell conditions, normal, metastasising and metastasising cells treated with tubacin were assessed by me, Khairat Al Hennawi and Ana Lopez (Fig 3.10). This figure shows the detached and attached images of the cell along with the traction force being exerted on the cell for all 3 conditions. The TFM heat maps allow us to observe where maximum force is being exerted on the cell. The TFM image in the middle has metastasising cells with a higher traction force being exerted than normal cells (top TFM image) and also tubacin treated metastasising cells (bottom TFM image). The TFM images also show that in all 3 cases, the least amount of intracellular force is being exerted on the nuclei of all 3 cells when comparing the position of the nuclei with their positions in the heat maps. This shows that the cells with the lowest intracellular traction force correlates with a higher extracellular traction force.

Next, the qualitative measurement of the deformation and nuclear force for all 3 cell conditions was assessed (Fig 3.11). The top row shows the deformation of the nucleus that occurs during cellular contractility in all 3 cell conditions, normal BjhTERT (left), untreated metastasising BjhTERTSv40T Ras (middle) and tubacin treated metastasising BjhTERTSv40T Ras cells (right) while the bottom row shows the corresponding nuclear force that was exerted during cellular contractility in all 3 cell conditions. These results clearly show that normal BJ cells exert the greatest nuclear force when compared to treated and untreated metastasising cells for a similar nuclear deformation of the nucleus when compared to the other 2 cell conditions. These figures (Figure 3.11) were prepared primarily by Ana Lopez using a custom script provided by Dr Rhoda J. Hawkins and were not directly prepared by me however they were prepared using data obtained from my previous TFM experiments that were performed by me.

Next, the results of the previous images (Fig 3.10) were quantified by me, Khairat Al Hennawi and Ana Lopez showing a graph of the forces that deformed the nuclear shape were reduced in metastasising cells, as compared to control, with no additional change by Tubacin (Fig 3.12) (Chiotaki et al., 2014). Earlier studies have shown that there is a broken and perinuclear localization of vimentin filaments along with smaller F-actin fibres induced due to the expression of oncogenes (Rathje et al., 2014, Ronnlund et al., 2013). It is hypothesised that the intracellular and extracellular contractile forces of cells were less coordinated and correlated in metastasising cells. It was also observed in previous studies that an increased negative correlation between the forces of the nuclei and the total forces exerted on the environment in the metastasising cells, as compared to control. Upon Tubacin-treatment in this study, the correlation between low nuclear and high total cellular forces was reduced to a negligible level. Taken together, these observations indicate that the molecular mechanisms that generate the contractile forces are altered in metastasising cells, which results in that less force is applied to the nucleus when more force is applied on the extracellular environment, and that HDAC6 activity is required for this phenotype.

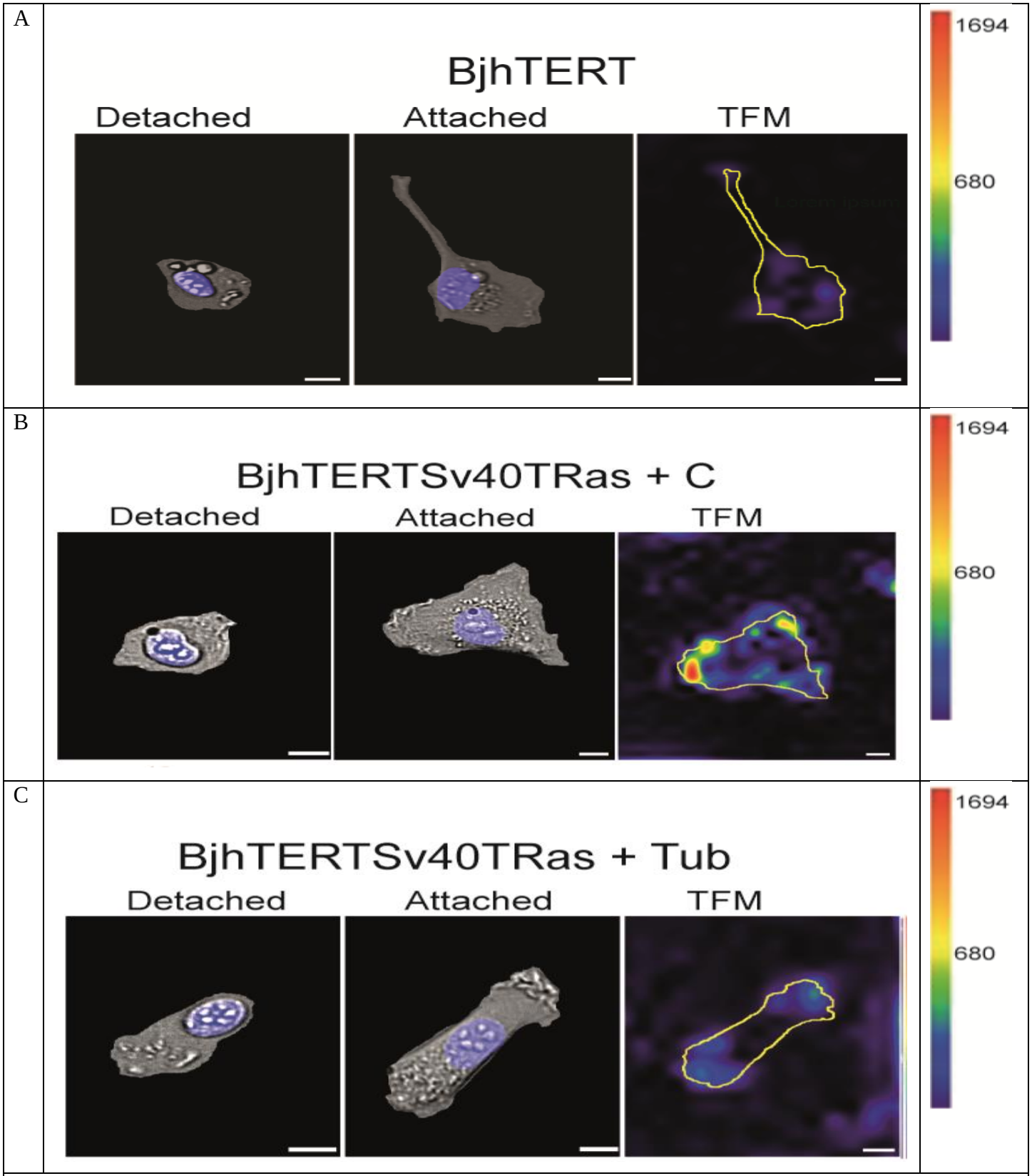


Figure 3.10: TFM images and Traction maps with HDAC6 inhibition. Metastasising cells show reduced intracellular forces exerted on the nuclei which correlates to increased total cellular force in a HDAC6-

dependent manner. (Made by Azeer Zafar, Khairat Al Hennawi and Ana Lopez using ImageJ (FIJI) image analysis software)

Representative images of normal and metastasising cells treated with or without Tubacin (Tub), as seen from top, and left to right: phase contrast (grey) and Hoechst (blue) images of attached (left) and detached (middle) cells, and the corresponding traction forces (right). Scale bars 20 µm. Maximum Force: 1694 pascals.

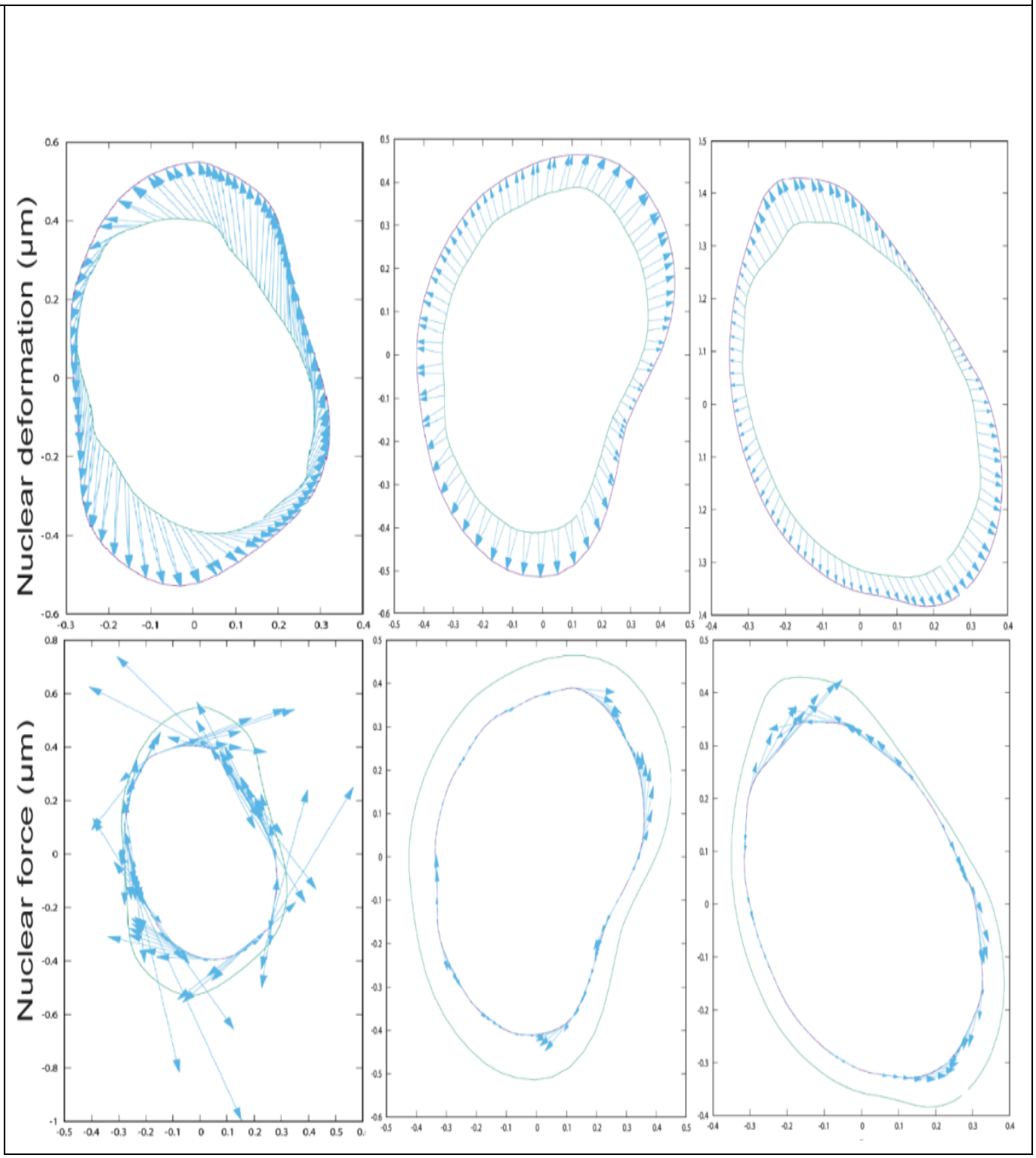


Figure 3.11. Nuclear deformation and nuclear force with HDAC6 inhibition. Metastasising cells exert reduced intracellular forces on the nuclei which correlates to increased total cellular force via HDAC6 reaction. (Prepared by Ana Lopez and Rhoda J Hawkins and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

The corresponding images of the same cell, shows in the top panel the deformation of the nucleus, with the deformed state of the cell in magenta, the incomplete deformed state in green and complete deformation in blue. while the bottom panel shows the nuclear force in the same colours and conditions Normal BjhTERT (left), untreated metastasising BjhTERT Ras (middle) and tubacin treated metastasising BjhTERT Ras cells (right). Scale bars 20 µm. **35** individual cells were checked, measured and analysed in order to obtain the data for this figure.

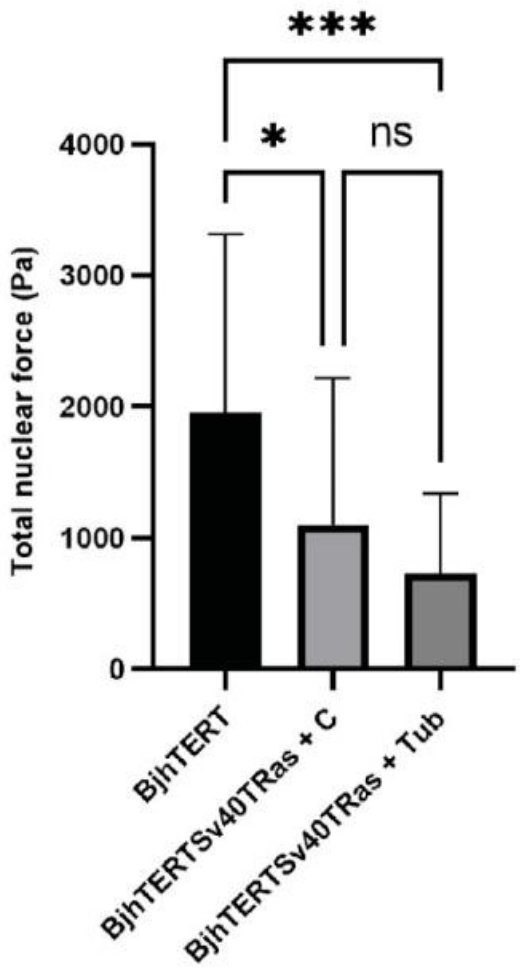


Figure 3.12. Quantification of nuclear force with HDAC6 inhibition. Metastasising cells show reduced intracellular forces exerted on the nuclei which correlates to increased total cellular force in a HDAC6-dependent manner. (Prepared by Azeer Zafar and Ana Lopez and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

Graph of the total nuclear force. This data has been derived from a minimum of three biological repeats and presented as mean ± SD. *$p \leq 0.05$, **$p \leq 0.01$. **35** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.6 Knocking down of HDAC6 and its effect on the morphological properties of normal, metastasising and siRNA HDAC6 knockdown treated cells.

Result: Metastasising cells become larger and more elongated after HDAC6 knockdown via siRNA making them resemble more like normal cells without there being any effect on cells speed and cell direction.

The project also wanted to determine the effect of oncogene-induced changes on the morphology of cells and whether if this change requires the HDAC6 protein. To this end, the project measured the area covered by the spreading of the cell, circularity, the shape of the cell, the cells persistence and the mean speed exerted by the cell during migration of metastasising cells when HDAC6 is knocked down via siRNA and migration assay technique. The experimental design used to test the conditions is shown below (Fig 3.13) and was primarily designed by my colleague Valentina Rossi but was later modified by me based on my requirements. This technique was also primarily performed by my colleagues Dr Ana Lopez and Valentina Rossi, who ran the migration assay to determine the cell's morphological properties. However, I was involved in the seeding and preparation of the cells that were used as well as in the analysis of the data obtained that allowed us to quantify the properties of the cells that were used.

There are several morphological characteristics that are affected in metastatic cells and studying the morphology of these cells when HDAC6 is knockdown using siRNA gives important information about the effect of HDAC6 on the morphology of a cell. Cell spreading is defined when suspended rounded cells that are in a suspension flatten and spread over a flat 2D solid substrate like glass, gels etc. Cell circularity is the measure of the roundness of a cell and is important since metastasising or damaged cells lose their membrane structure affecting their circularity. Cell persistence is the ability of cells to continue moving in one single direction with regards to their migration and polarity. Cell Mean speed is the average speed of the cell with which it travels from one point to another.

Next, the morphological changes in treated and untreated metastasising cells (BjhTERTSv40T Ras) when compared to normal cells (BjhTERT) were quantified by me, Valentina Rossi and Ana Lopez (Fig 3.14). The results observed that the untreated control metastasising cells cover a smaller area as they spread less (Fig 3.14A) along with a more circular shape and less elongation (Fig 3.14B) (Fig 3.14B), when compared to normal cells. Knocking down of HDAC6 via siRNA in the metastasising cells led to a reversal of the above phenotypes when

compared to that of the normal cells. The persistent nature of the migration of the cell is also decreased in metastasising cells (Fig 3.14D), however this does not affect the speed of the cell migration (Fig 3.14E). The results also showed that there was an inverse correlation between the speed of the cell and its persistence (Fig 3.14F). As mentioned previously, the speed and persistence of normal cells remained the same after HDAC6 knockdown but there was a clear reversal of speed and persistence in metastasising cells. Looking at these results, shows that there is a connection of normal cells metastasising to a reduced capacity of cells to expand and elongate allowing for decoupling the speed of cell from its direction and that HDAC6 plays a very important role in this control.

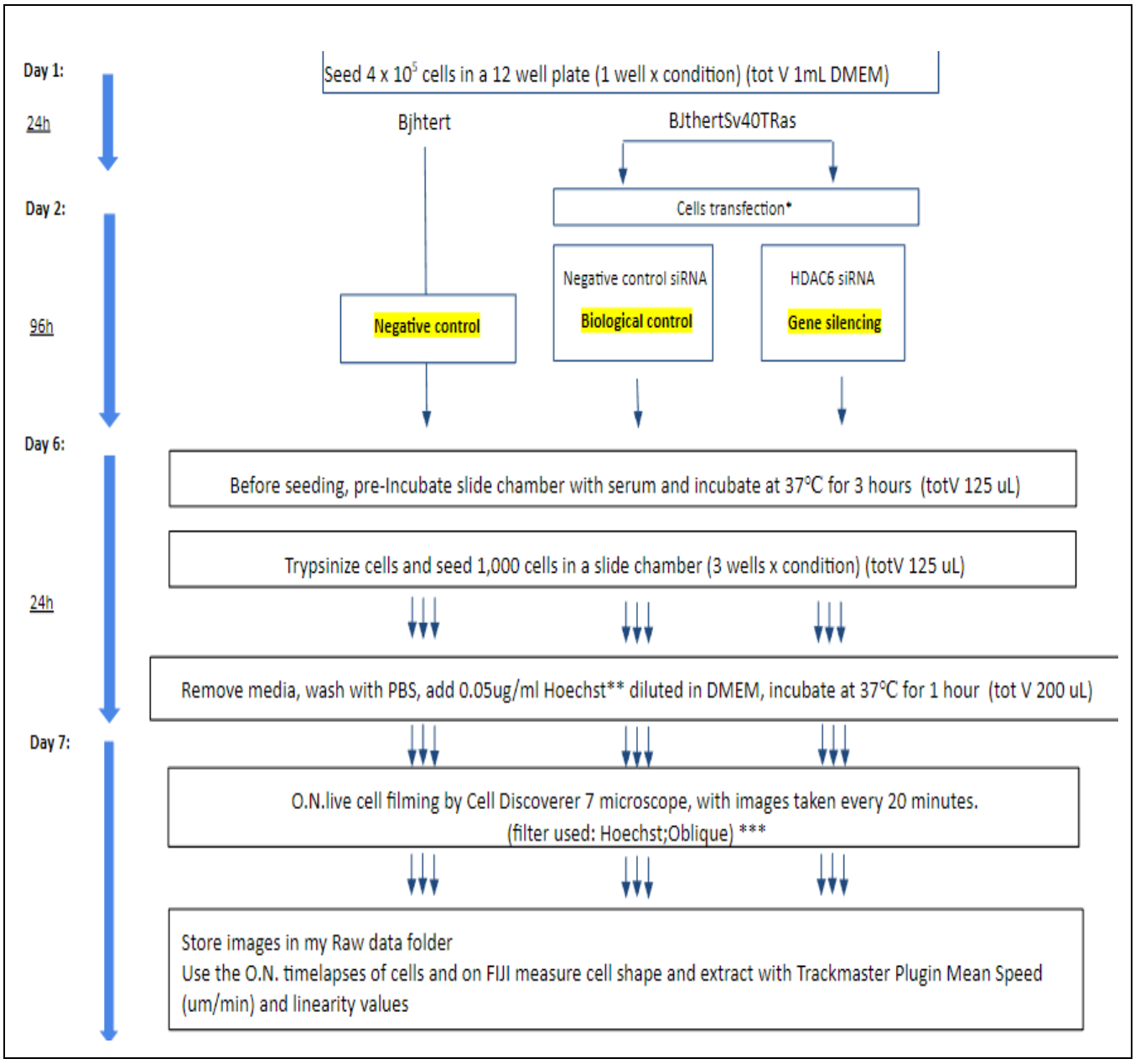


Figure 3.13: Experimental Design of Migration Assay with siRNA HDAC6 knockdown. (Prepared by Valentina Rossi and modified by Azeer Zafar using Microsoft PowerPoint)

The experimental design tests the morphological changes in all 3 cell conditions (normal BjhTERT, negative HDAC6 siRNA control metastasising BjhTERTSv40TRas and treated HDAC6 siRNA metastasising BjhTERTSv40TRas cells.

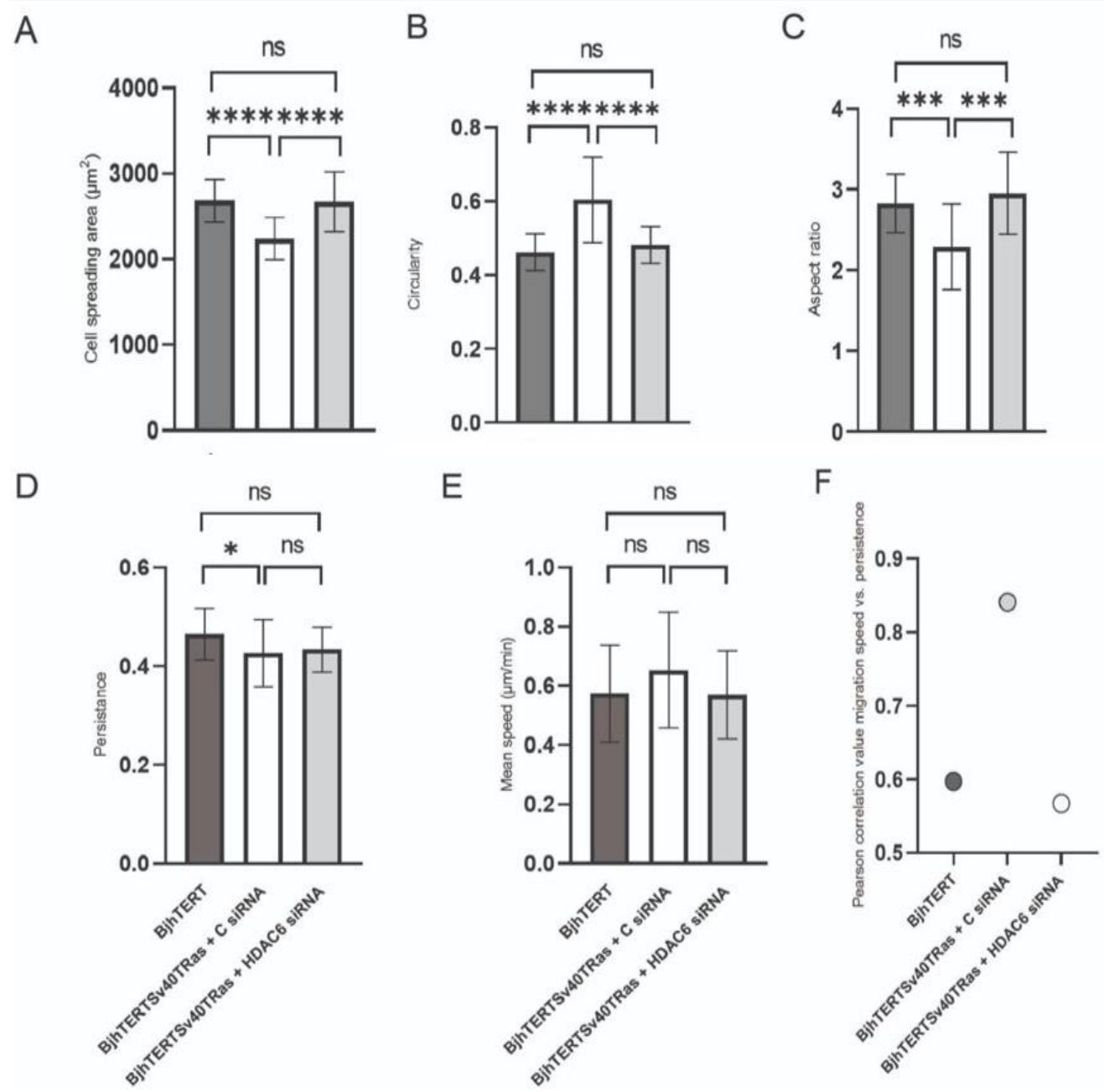


Figure 3.14: Cell Morphology Quantification with siRNA HDAC6 knockdown. HDAC6 knockdown via siRNA reversed the smaller area spread, and the circular shape of metastasising cell back to elongated shape without effecting speed and persistence during cell migration-.

(Prepared by Valentina Rossi, Azeer Zafar, Ana Lopez and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

The **(A)** cell spreading area, **(B)** cell circularity, **(C)** aspect ratio, **(D)** persistence, **(E)** mean speed, and **(F)** Pearson's correlation between mean speed and persistence of fibroblast treated with siRNA or controls, as indicated. Data from three. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (One way ANOVA tests). **42** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.7 Inhibition of HDAC6 and its effect on focal adhesions in normal, metastasising and tubacin treated metastasising cells.

Result: Metastasising cells have a smaller size and more numbers of focal adhesions, and these factors are further increased when HDAC6 activity is inhibited using tubacin.

Cell traction contractile forces are primarily exerted through the interaction of focal adhesions with the substrate they are bound to (Bershadsky et al., 2006). Focal adhesions facilitate the interaction between the cells and the ECM and this link between the ECM and cytoskeleton play an important role as focal adhesions control the shape of the cell in terms of their structure and function. This control allows for cell survival, proliferation, contraction, motility and differentiation. Since HDAC6 is responsible for increased contractility in cells and this contractility affects the cell-matrix adhesions and cell spreading area between cellular components and extracellular matrix. Therefore, inhibition of HDAC6 should affect and decrease the cell-matrix adhesions and cell spreading area between cells and the ECM. Therefore, by staining the focal adhesions, actin and vimentin with immunofluorescent antibodies and taking images of their cell-matrix adhesions before and after HDAC6 is inhibited with tubacin the change in cellular contractility between normal, metastasising and metastasising cells treated with tubacin should be able to be quantified. To understand the effect of HDAC6 on metastasising cells contractile forces, this project compared between the size and numbers of focal adhesions in the metastasising cells to normal control, and after tubacin-mediated inhibition of HDAC6 or HDAC6 knockdown in the metastasising cells. This was done by first fixing and staining the cells for focal adhesions (using phosphotyrosine), for F-actin and vimentin. The stained cells were then imaged using a confocal microscope to determine the structures of the cells including the size and number of the focal adhesions. The

experimental design used to test the conditions is shown below (Fig 3.15) and was designed by me.

Next, cells were stained with phosphotyrosine (first row), actin (second row) or vimentin (third row), as well the merged confocal images (fourth row) for all 3 cell conditions, normal BjhTERT (left), untreated BjhTERTSv40TRas metastasising (middle) and tubacin treated BjhTERTSv40TRas cells metastasising cells (right) with the assistance of Ana Lopez (Fig 3.16). The number and area of focal adhesions for all 3 cell conditions after inhibition of HDAC6 activity using tubacin were quantified (Fig 3.17). There was a significant decrease in the size of focal adhesions for both untreated and treated metastasising BjhTERTSv40T Ras cells when compared to normal BjhTERT cells. Also, the frequency (Fig 3.17C) showed there was a significantly higher number of focal adhesions with a smaller size (0-1 μm^2), while the number of focal adhesions decreases significantly as the size of focal adhesions increased (>1 μm^2). However, there was no significant change or difference in the total number of focal adhesions for all 3 cell conditions.

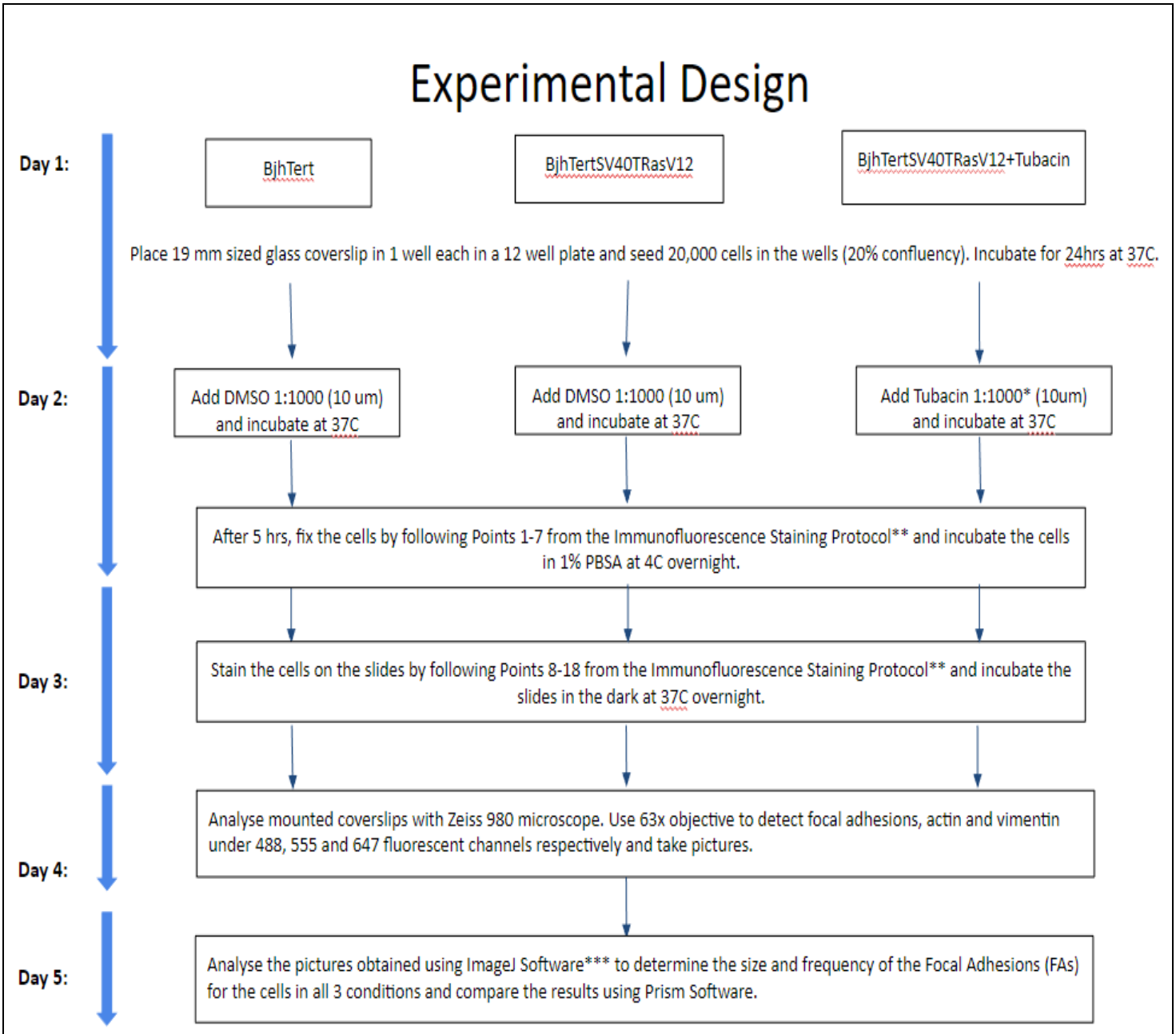


Figure 3.15: Experimental Design of Focal adhesion measurement with HDAC6 inhibition. (Made by Azeer Zafar using Microsoft PowerPoint)

The experimental design stained and imaged the stained cell samples using confocal microscopy for all 3 cell conditions (normal BjhTERT, untreated BjhTERTSv40TRas metastasising cells and tubacin treated BjhTERTSv40TRas cells metastasising cells).

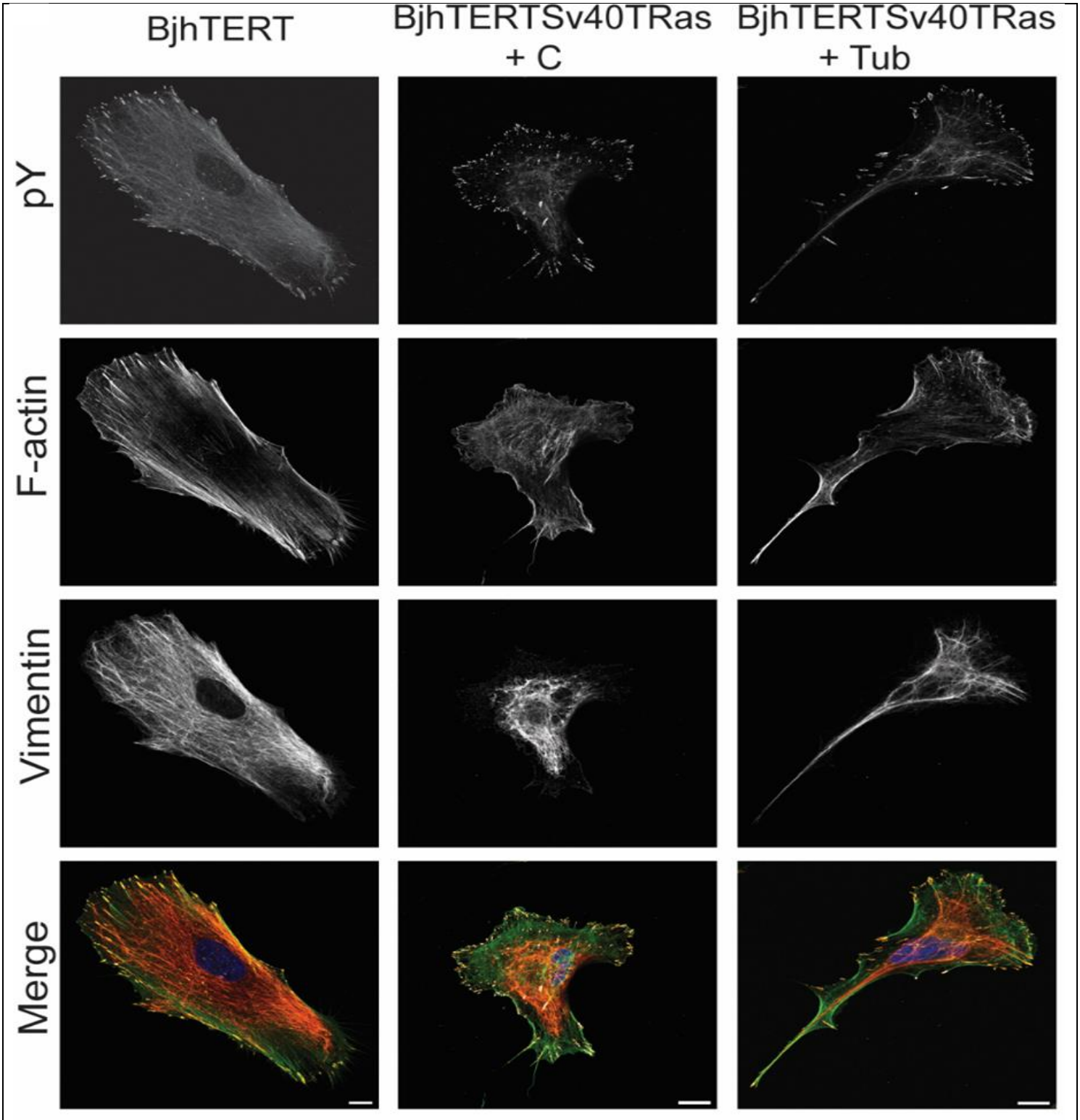


Figure 3.16: Stained Focal adhesion images with HDAC6 inhibition. Metastasising cells treated with tubacin are smaller in size and have a larger number of focal adhesions upon HDAC6 inhibition. (Captured with CD7 microscope by Azeer Zafar and Ana Lopez and Taken from Azeer Zafar's Paper López-Guajardo et al., 2023) Representative images of normal, control or metastasising cells treated with Tubacin (Tub) or DMSO control. showing phosphotyrosine (pY), F-actin, Vimentin, and merged images, as indicated. Scale bars 20 µm.

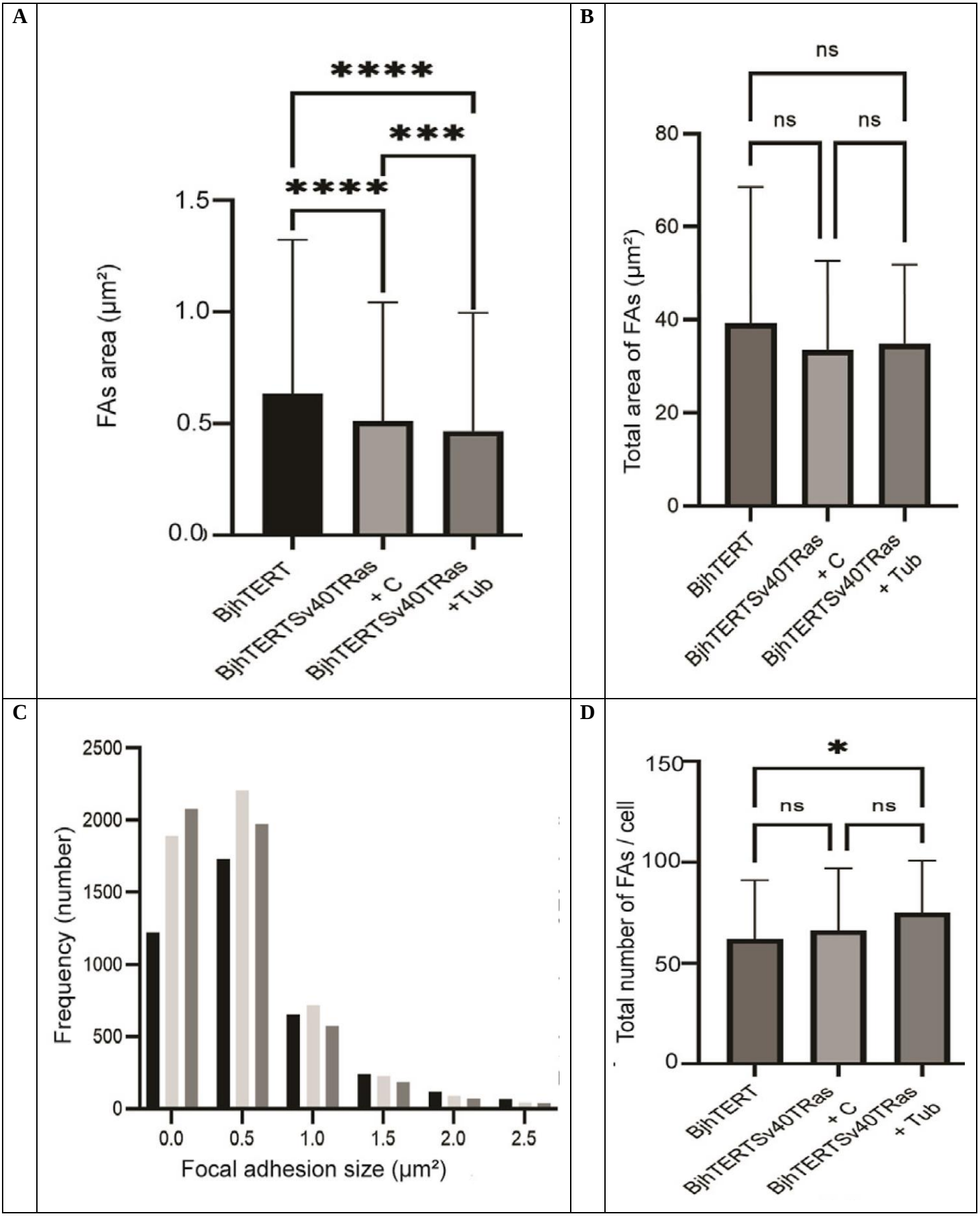

A
****
***
****
FAs area (µm²)
1.5
1.0
0.5
0.0
BjhTERT
BjhTERTSv40TRas
BjhTERTSv40TRas + C
BjhTERTSv40TRas + Tub
B
ns
ns
ns
Total area of FAs (µm²)
80
60
40
20
0
BjhTERT
BjhTERTSv40TRas
BjhTERTSv40TRas + C
BjhTERTSv40TRas + Tub
C
Frequency (number)
2500
2000
1500
1000
500
0
0.0
0.5
1.0
1.5
2.0
2.5
Focal adhesion size (µm²)
D
*
ns
ns
Total number of FAs / cell
150
100
50
0
BjhTERT
BjhTERTSv40TRas
BjhTERTSv40TRas + C
BjhTERTSv40TRas + Tub

Figure 3.17: Quantification of focal adhesions with HDAC6 inhibition. Metastasising cells show reduced sizes and a larger number of focal adhesions, which became even smaller and more in number when HDAC6 was inhibited or knocked down (Prepared by Azeer Zafar using FIJI/ImageJ software and Taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

Quantification of the results for FAs, FA area/cell (top left) of the average size of focal adhesions area in each cell. Total area occupied by FAs for each cell condition (top right). Frequency distribution of focal adhesions numbers based on their sizes (lower left). Total number of FAs/cells in each cell condition (lower right). Data is collected and combined from three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (One way ANOVA tests). **60** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.8 Effect of HDAC6 knockdown on focal adhesions in normal, metastasising and siRNA HDAC6 knockdown treated metastasising cells.

Result: Metastasising cells have smaller sizes and larger number of focal adhesions, and these features are further increased upon HDAC6 knockdown.

The previous experiment (Section 3.7) was repeated with the assistance of Dr Ana Lopez, by staining the focal adhesions, actin and vimentin with immunofluorescent antibodies and taking images of their cell-matrix adhesions before and after HDAC6 siRNA knockdown allowing the change in cellular contractility between normal, siRNA control metastasising and HDAC6 siRNA knockdown treated metastasising cells to be able to be quantified. The experimental design used to test the conditions is shown below (Fig 3.18) and was designed by me.

Cells were stained with phosphotyrosine (first row), actin (second row) and vimentin (third row), as well the merged confocal images (fourth row) for all 3 cell conditions, normal BjhTERT (left), negative control BjhTERTSv40TRas + C siRNA metastasising (middle) and HDAC6 siRNA knockdown treated BjhTERTSv40TRas + HDAC6 siRNA metastasising cells (right) (Fig 3.19). The number and area of focal adhesions for all 3 cell conditions after knockdown of HDAC6 using siRNA were then quantified by me (Fig 3.20). There was a significant decrease in the size of focal adhesions for both untreated and treated metastasising BjhTERTSv40T Ras cells when compared to normal BjhTERT cells (Fig 3.20A). There was also a significant decrease in the total area occupied by the focal adhesions in negative control

and treated HDAC6 siRNA knockdown metastasising cells when compared to normal BjhTERT cells (Fig 3.20B). Also, the frequency (Fig 3.20C) showed that there was a significantly higher number of focal adhesions with a smaller size (0-1 μm^2), while the number of focal adhesions decreased significantly as the size of focal adhesions increase (>1 μm^2). However, there was no significant change or difference in the total number of focal adhesions for all 3 cell conditions (Fig 3.20D).

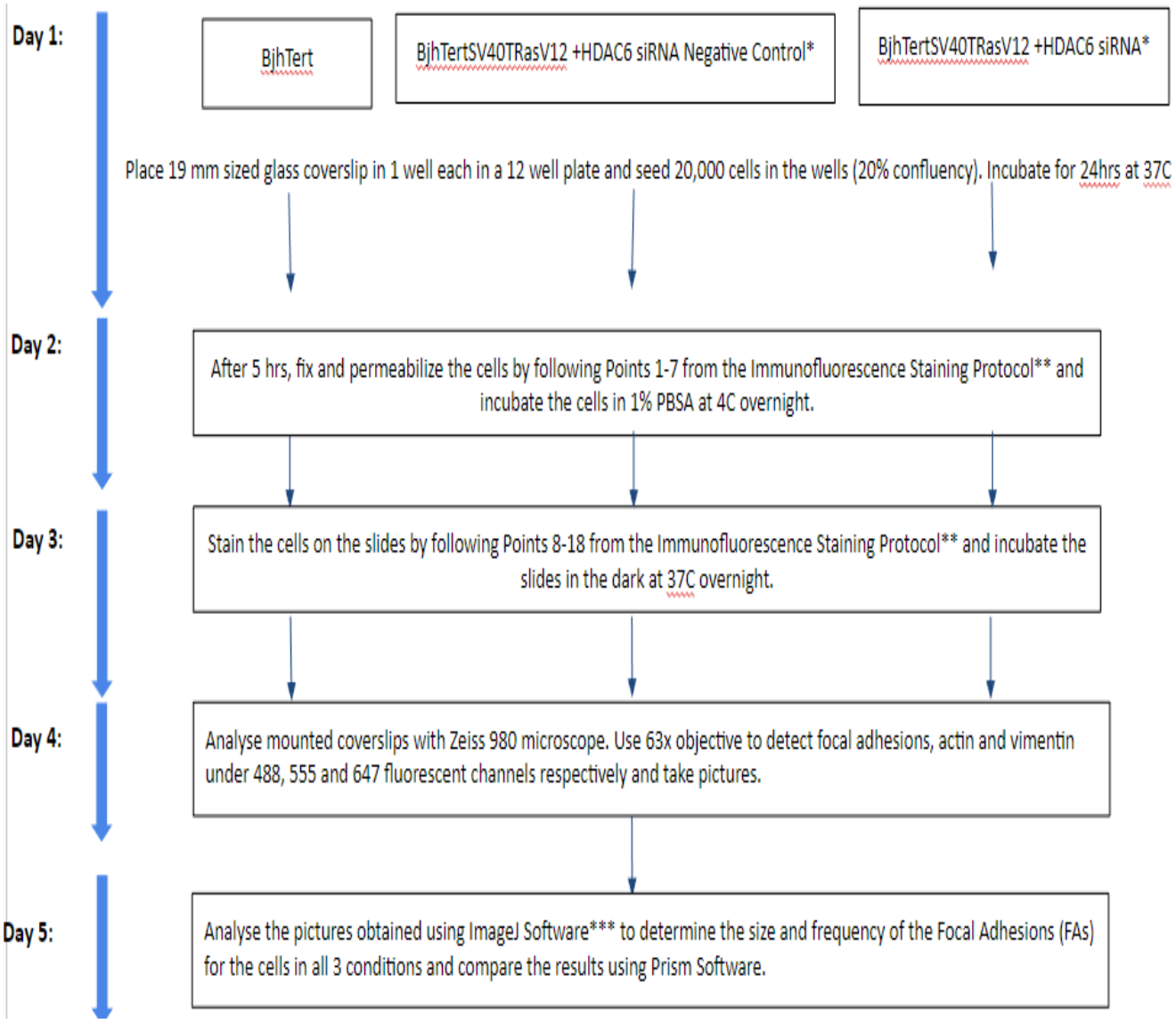


Figure 3.18: Experimental Design of Focal adhesions with siRNA HDAC6 knockdown. (Made by Azeer Zafar using Microsoft PowerPoint)

The experimental design stained and imaged the stained cell samples using confocal microscopy for all 3 cell conditions (normal BjhTERT, negative control BJ Ras metastasising cells and HDAC6 siRNA knockdown treated BJ Ras cells metastasising cells.

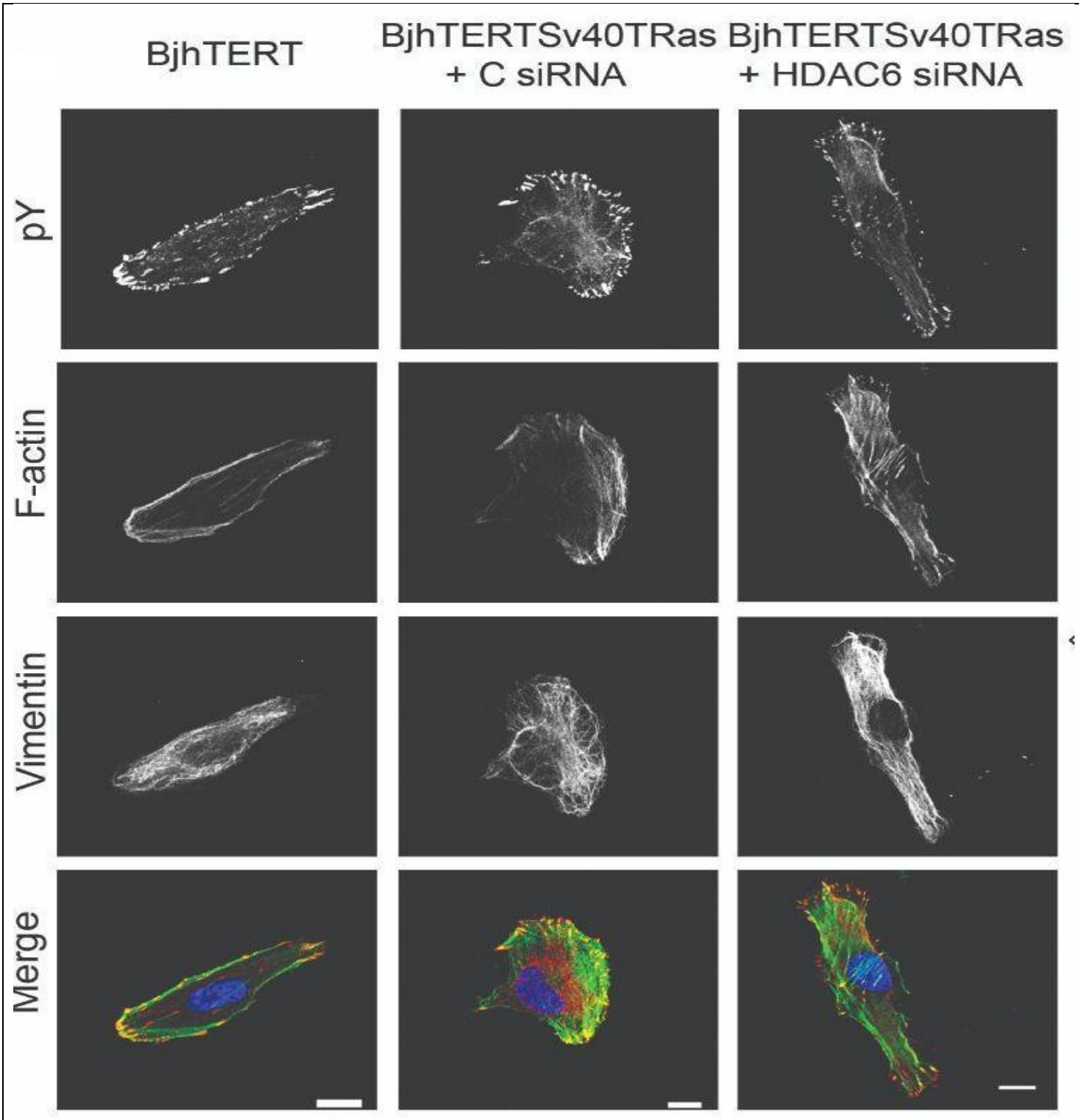


Figure 3.19: Stained Focal adhesion images with siRNA HDAC6 knockdown. Metastasising cells show smaller sizes and a larger number of focal adhesions when HDAC6 is knocked down. (Captured with CD7 microscope by Azeer Zafar and Ana Lopez and Taken from Azeer Zafar's Paper López-Guajardo et al., 2023)
Representative images of normal, metastasising siRNA control or metastasising siRNA HDAC6 knockdown cells. showing phosphotyrosine (pY), F-actin, Vimentin, and merged images, as indicated. Scale bars 20 µm.

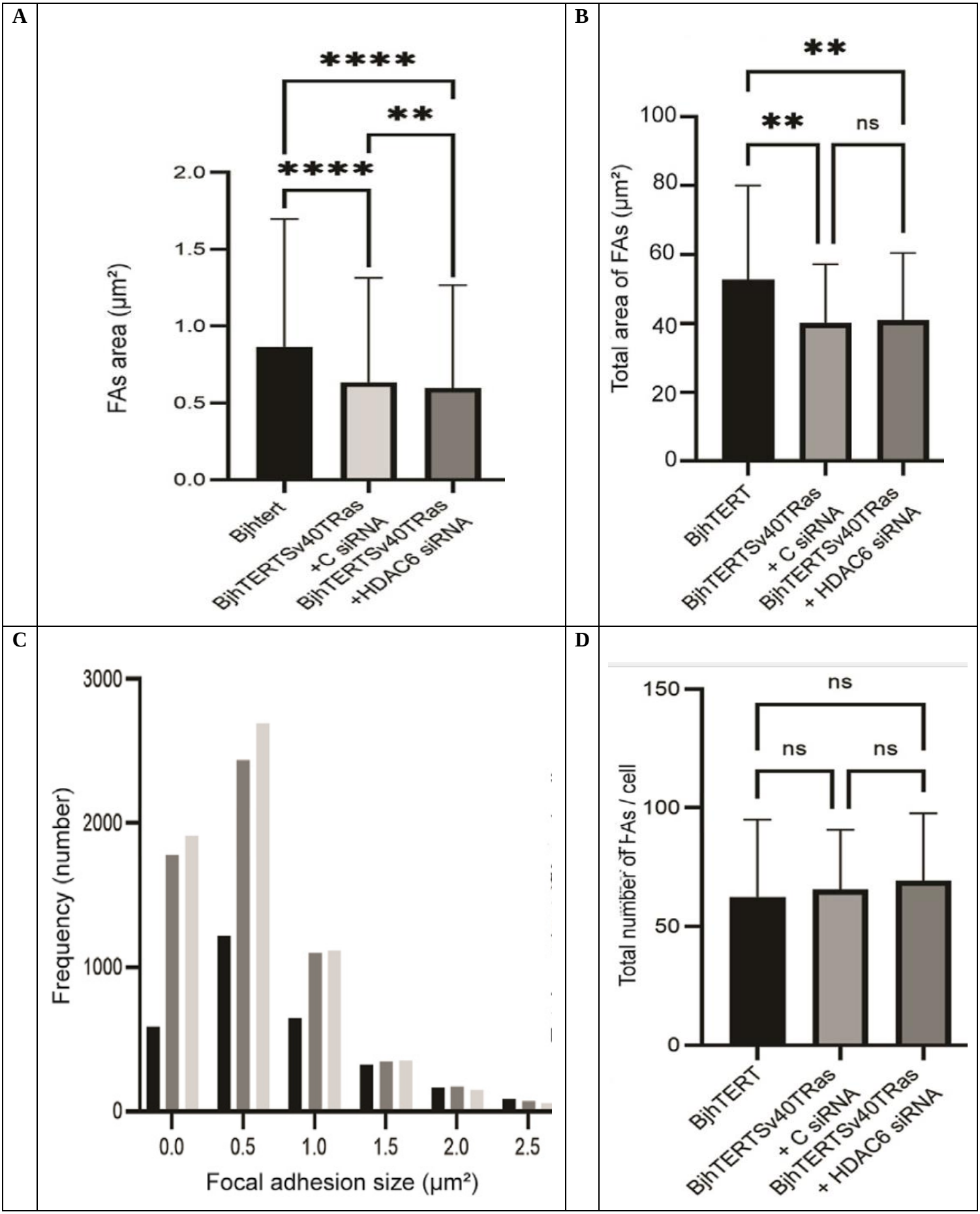

A
****
**
****
FAs area (µm²)
2.0
1.5
1.0
0.5
0.0
Bjhtert
BjhTERTSv40TRas +C siRNA
BjhTERTSv40TRas +HDAC6 siRNA
B
**
**
ns
Total area of FAs (µm²)
100
80
60
40
20
0
BjhTERT
BjhTERTSv40TRas + C siRNA
BjhTERTSv40TRas + HDAC6 siRNA
C
Frequency (number)
3000
2000
1000
0
0.0
0.5
1.0
1.5
2.0
2.5
Focal adhesion size (µm²)
D
ns
ns
ns
Total number of FAs / cell
150
100
50
0
BjhTERT
BjhTERTSv40TRas + C siRNA
BjhTERTSv40TRas + HDAC6 siRNA

Figure 3.20: Quantification of focal adhesion with siRNA HDAC6 knockdown. Metastasising cells are smaller in size with larger numbers of focal adhesions, which become even smaller and more in number when HDAC6 is knockdown. (Prepared by Azeer Zafar using FIJI/ImageJ software and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

Quantification of the results for FAs, FA area/cell (top left) of the average size of focal adhesions area in each cell. Total area occupied by FAs for each cell condition (top right). Frequency distribution of number of focal adhesions based on their sizes (lower left). Total number of FAs/cells in each cell condition (lower right). Data is obtained from three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (One way ANOVA tests). **56** individual cells were checked, measured and analysed in order to obtain the data for this figure.

3.9 Looking at the effect of deacetylase activity of HDAC6 on the α-tubulin acetylation and comparing the difference before and after treatment with HDAC6 inhibition and HDAC6 siRNA knockdown.

Result: Inhibited and Knockdown metastasising cells both show increased acetylation of α-tubulin.

HDAC6 overexpression correlates with increased carcinogenesis and metastasis of cancer cells. HDAC also promotes cell motility and is involved in α-tubulin deacetylation. HDAC6 has both a deacetylase activity and a strong affinity to ubiquitin. The deacetylation of α-tubulin via HDAC6 treatment causes microtubule depolymerization. Overexpressing HDAC6 causes hypoacetylation leading to increased migration while HDAC6 inhibition causes microtubule stabilization. This means that acetylation of α-tubulin affects cell migration. Increased expression of oncogenes results in the upregulation of the tubulin deacetylase HDAC6 activity and alters the distribution of acetylated microtubules. The expression of oncogenes also induces increase in cellular contractility and promotes cancer cell invasiveness. Therefore, theoretically inhibition of HDAC6 activity using tubacin or HDAC6 siRNA knockdown should stop the deacetylase activity of HDAC6 and increase acetylation of α-tubulin which in turn should decrease the migration and invasiveness of metastasising cells.

To test this, it involved conducting two experiments and in both a western blot was performed. The first experiment involved running a western blot for the three cell conditions, normal (BjhTERT), untreated metastasising (BjhTERTSv40TRas) and tubacin treated metastasising

cells (BjhTERTSv40TRas + Tub). These cell conditions were treated with anti-mouse-acetylated-α-tubulin, anti-rabbit-α-tubulin and anti-mouse-GAPDH antibodies to determine if there is an increase in acetylation of α-tubulin once the activity of HDAC6 is inhibited using tubacin. The experimental design used to test the conditions is shown below and was designed by me with the assistance of Dr Ana Lopez (Fig 3.21).

The second experiment involved a similar experiment that is running a western blot for the three cell conditions, except this time the activity of HDAC6 was removed using an siRNA HDAC6 knockdown (Fig 3.22). The three cell conditions were normal (BjhTERT), negative control siRNA metastasising (BjhTERTSv40TRas) and HDAC6 siRNA knockdown treated metastasising cells (BjhTERTSv40TRas + HDAC6 siRNA). These cell conditions were treated with anti-mouse-acetylated-α-tubulin, anti-rabbit-α-tubulin and anti-mouse-GAPDH antibodies to determine if there is an increase in acetylation of α-tubulin once the activity of HDAC6 is inhibited using tubacin. The experimental design used to test the conditions is shown below (Fig 3.22) which was again designed by me with the assistance of Dr Ana Lopez.

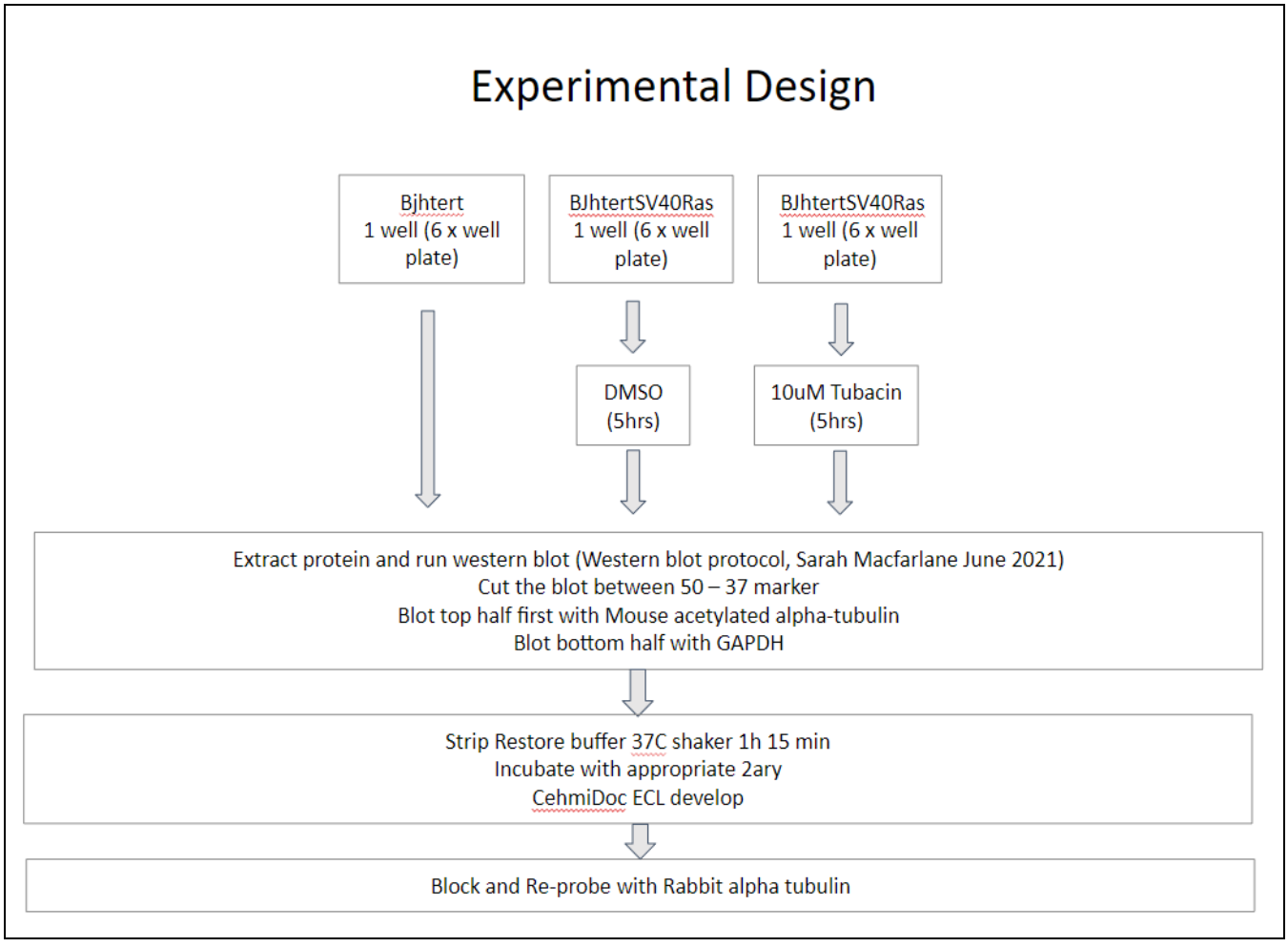


Figure 3.21: Experimental Design to observe HDAC6 Deacetylase Activity under Tubacin mediated HDAC6 inhibition. (Made by Azeer Zafar and Ana Lopez using Microsoft PowerPoint)
The experimental design to perform a western blot after inhibiting HDAC6 activity using tubacin for 3 cell conditions (normal BjhTERT, untreated BjhTERTSv40TRas metastasising cells and tubacin treated BjhTERTSv40TRas cells metastasising cells).

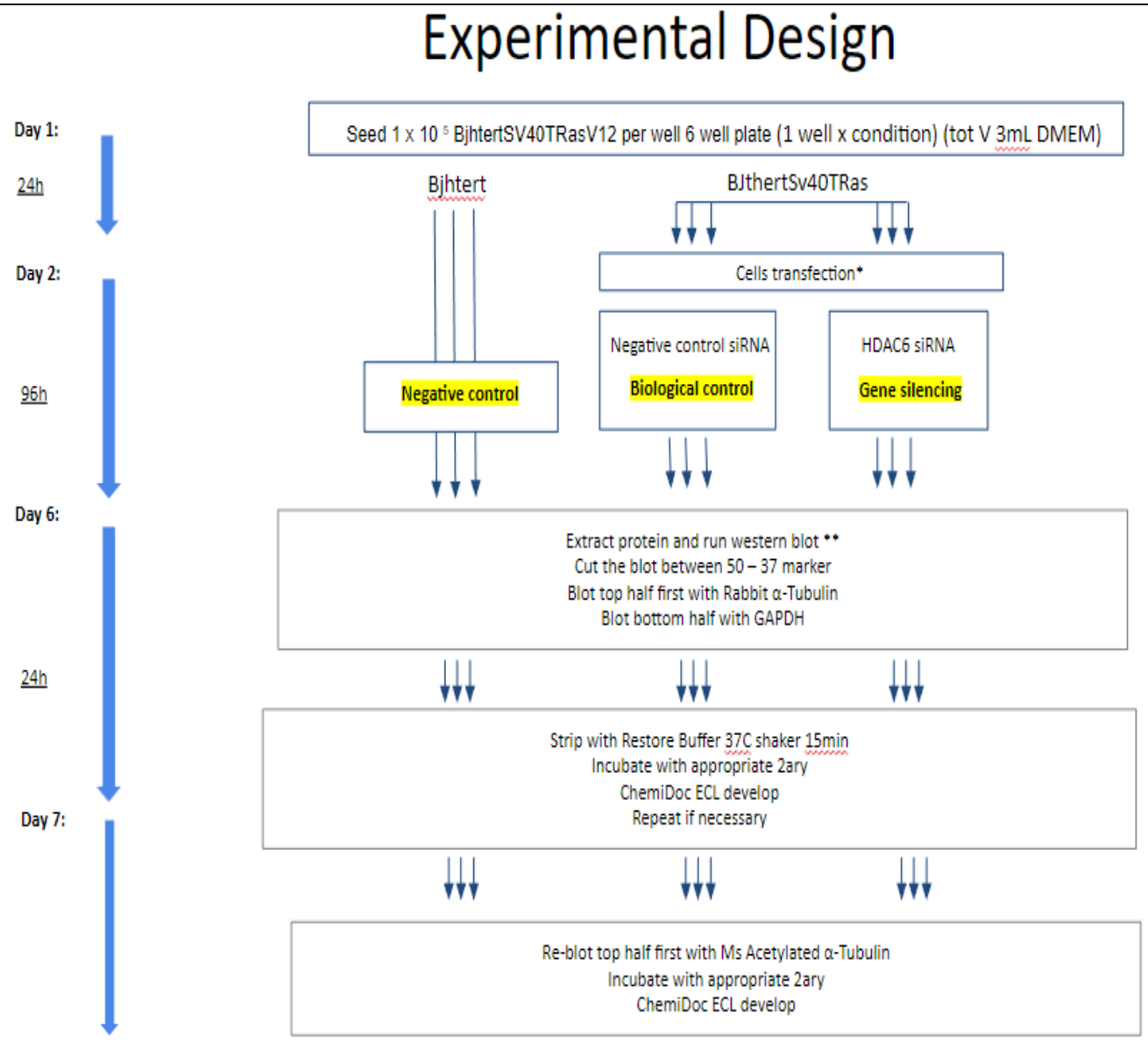


Figure 3.22: Experimental Design of HDAC6 Deacetylase activity Western Blot with siRNA mediated HDAC6 knockdown. (Made by Azeer Zafar and Ana Lopez using Microsoft PowerPoint)
The experimental design to perform a western blot after inhibiting HDAC6 activity using tubacin for 3 cell conditions (normal BjhTERT, negative control BJ Ras metastasising cells and HDAC6 siRNA knockdown treated BJ Ras cells metastasising cells).

The result of this experiment clearly shows that in both cases, when HDAC6 activity is either inhibited or removed completely there is a clear overexpression of acetylation of α-tubulin when compared to normal α-tubulin in the third column of both western blots (Fig 3.23). This proves that inhibition of HDAC6 activity leads to loss of deacetylase activity and increase in acetylation. This in turn will lead to reduced contraction and migration of metastasising cells. Taking the results together, the growth and stability of mature focal adhesions depends on the actions of oncogenes along with the acetylation of tubulin dependent on HDAC6.

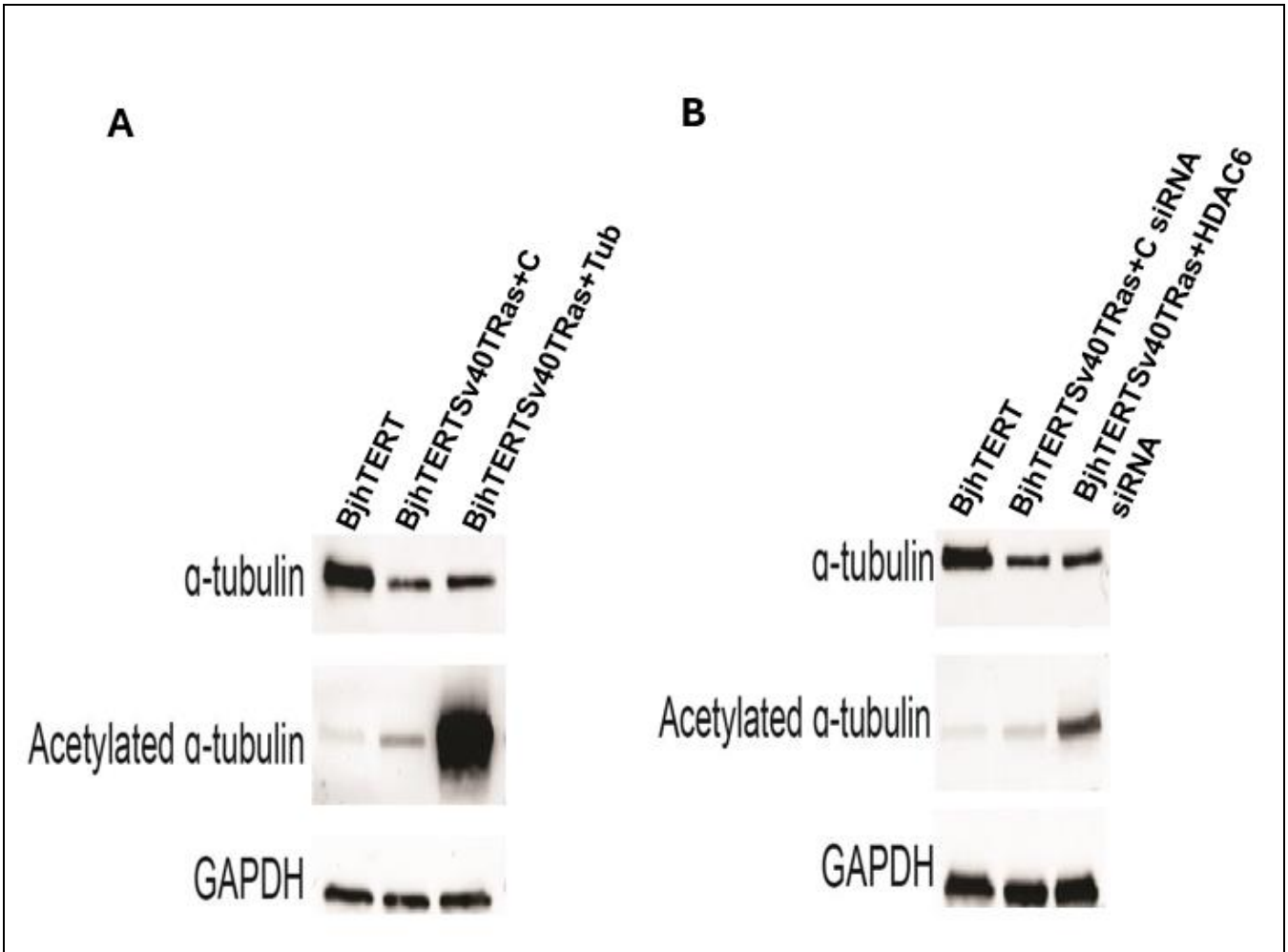


Figure 3.23: Western Blots of HDAC6 Acetylation with HDAC6 inhibition and siRNA HDAC6 knockdown. HDAC6 inhibition and knockdown lead to increased acetylation in metastasising fibroblasts. (Captured by Azeer Zafar and Ana Lopez and taken from Azeer Zafar's Paper López-Guajardo et al., 2023)

Normal and metastasising cells treated without or with Tubacin **(A),** or HDAC6 siRNA **(B),** as indicated, showing levels of total acetylated tubulin and GAPDH loading control.

Chapter FOUR – DISCUSSION AND CONCLUSION

Chapter 4 – Discussion and Conclusion

4.1 Discussion

A

Compared	Primary human foreskin fibroblast cells (BJ) **Immortalized** With telomerase reverse transcriptase BjhTERT	BjhTERT **Transformed** with SV40T large antigen and **Metastasized** with oncogenic allele (H-*ras*V12) BjhTERT-Sv40T-Ras	
		BjhTERT-Sv40TRas Control	HDAC6 inhibition via Tubacin
Contractile Forces	=	↑	↓
Focal Adhesion Area	=	↓	↓
Number of Focal Adhesions	=	=	↑

B

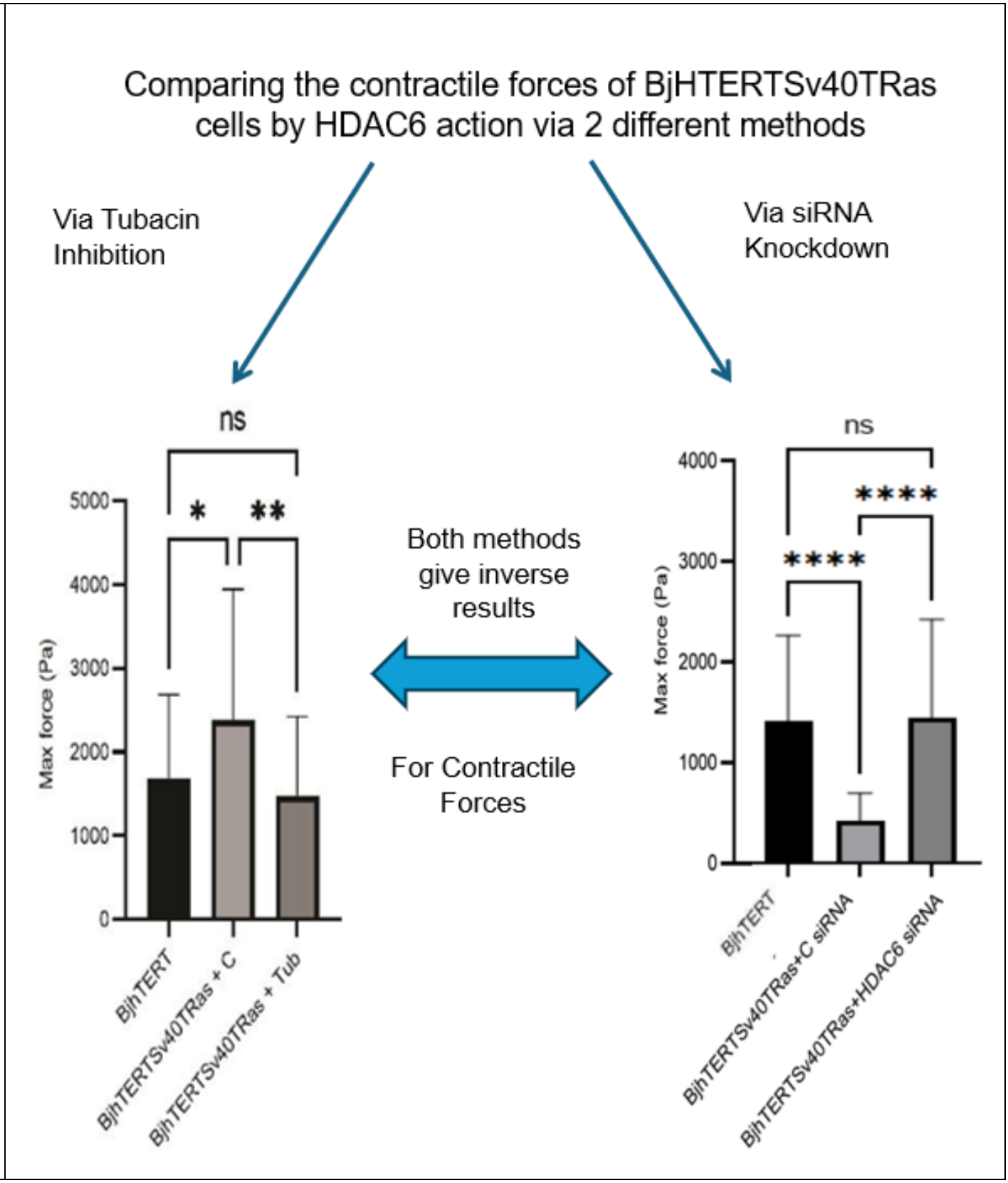
Compared	Primary human foreskin fibroblast cells (BJ) **Immortalized** With telomerase reverse transcriptase BjhTERT	BjhTERT **Transformed** with SV40T large antigen and **Metastasized** with oncogenic allele (H-*ras*V12) BjhTERT-Sv40T-Ras	
		BjhTERT-Sv40TRas Control	HDAC6 knockdown via siRNA
Contractile Forces	=	↓	↑
Focal Adhesion Area	=	↓	↓
Number of Focal Adhesions	=	=	=

Figure 4.1: Overview of the Projects main results. (Made by Azeer Zafar using Microsoft PowerPoint)
This figure summarizes the primary results of the main conditions that were tested (Contractile forces, Focal adhesion area, Number of focal adhesions) in terms of increase, decrease or no change in (A) HDAC6 inhibition via Tubacin or (B) HDAC6 knockdown via siRNA.

A summary of the main results of the experiments is shown in (Fig 4.1) while the figure below (Figure 4.2) shows the graphs of the actual results obtained after performing these experiments under both conditions to measure the contractile forces and focal adhesions.

The physical forces that cancer cells use to metastasise, and their underlying mechanisms are currently not fully understood, or how they differ to normal cells with regards to mechanical forces for cell motility and invasiveness. HDAC6 is an enzyme, which is being studied for its role in cancer cell metastasis and promotion of cell motility. Therefore, the main aim of this thesis was to understand the mechanisms by which HDAC6 controls cell contractile forces using the isogenic human BJ fibroblast model and to determine if targeting HDAC6 was a potential therapeutic option in cancer.

A


Comparing the contractile forces of BjHTERTSv40TRas cells by HDAC6 action via 2 different methods
Via Tubacin Inhibition
Via siRNA Knockdown
ns
*
**
Max force (Pa)
5000
4000
3000
2000
1000
0
BjhTERT
BjhTERTSv40TRas + C
BjhTERTSv40TRas + Tub
Both methods give inverse results
For Contractile Forces
ns
****
****
Max force (Pa)
4000
3000
2000
1000
0
BjhTERT
BjhTERTSv40TRas+C siRNA
BjhTERTSv40TRas+HDAC6 siRNA

B

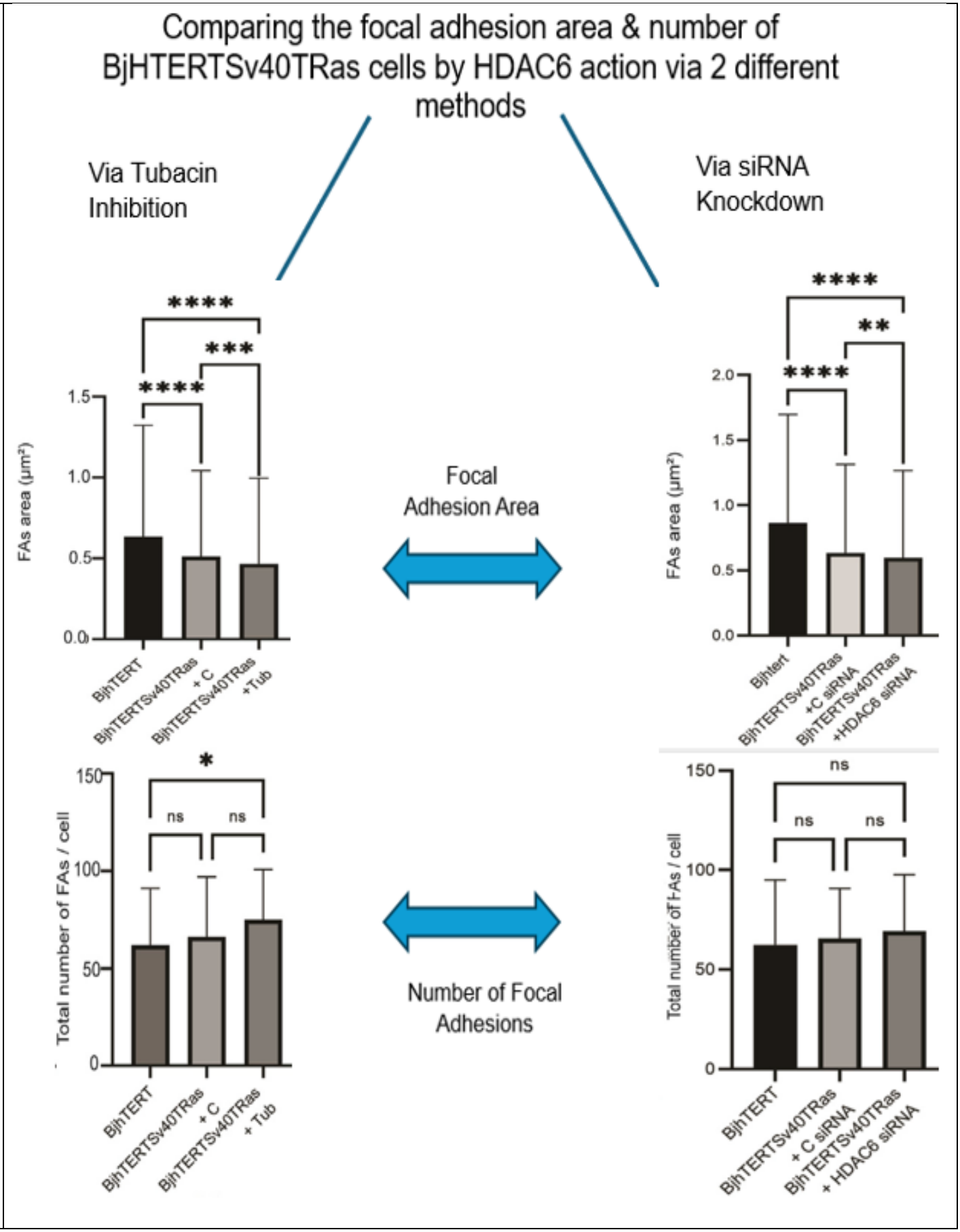

Comparing the focal adhesion area & number of BjHTERTSv40TRas cells by HDAC6 action via 2 different methods
Via Tubacin Inhibition
Via siRNA Knockdown
Focal Adhesion Area
Number of Focal Adhesions
FAs area (µm²)
Total number of FAs / cell
BjhTERT
BjhTERTSv40TRas
BjhTERTSv40TRas +C
BjhTERTSv40TRas +Tub
Bjhtert
BjhTERTSv40TRas +C siRNA
BjhTERTSv40TRas +HDAC6 siRNA
ns
****
***
**
*

Figure 4.2: Schematic figure of the project's main findings. (Made by Azeer Zafar using Microsoft PowerPoint)

A summary of the projects primary results obtained using 2 different techniques (TFM and Confocal Microscopy). (A) compares the contractile forces of BjhTERTSV40TRas cells via HDAC6 action under 2 different conditions (inhibition and knockdown) and (B) compares the number and area of focal adhesions after treating HDAC6 under 2 different conditions (inhibition and knockdown).

Overall, the results obtained after conducting all the experiments described in this thesis showed that the metastasising cells exerted increased contractile forces on their surroundings as compared to normal control BJ cells, while at the same time the forces exerted by these metastasising cells on their nuclei were decreased. There was an inverse correlation in the results which was consistent throughout all the cells but was more strongly observed in metastasising rather than in normal cells. One potential reason for this could be that the forces being exerted in metastasising cells are less structured than normal BJ cells, which could be due to the fact that oncogenic genes result in a weaker cellular environment. These results match results seen in previously conducted experiments and show that cancer cells that are metastasising exert greater forces on their surrounding environment when compared to normal cells. (Kraning-Rush et al., 2012). On the other hand, this exertion of traction forces by cells on their surrounding environment cannot be used as a standalone biomarker for determining cell transformation and metastasis (Mierke et al., 2008). This is because different types of cells have unique compositions and physical structures with regards to their cytoskeletal and cell-matrix adhesions, due to cell-specific cytoskeletal and adhesion differences. This could mean that the results that were recorded are unique to cells that originate from a mesenchymal cell line like those that were used for the project such as fibroblasts. The reduction in the force on the nucleus points to a loss of cellular adhesion, which matches with the well-established idea that cells can only exert force on their surroundings when the cytoskeleton is attached to the extracellular matrix, and this occurs by cell matrix adhesions. This project clearly compares and correlates the difference between the contractile traction forces exerted by cells on their environment compared to the forces that are applied to the cell's nucleus. The results suggest that oncogenes increase the extracellular contractile force and decrease the intracellular force of cells, via HDAC6-mediated deacetylation of microtubules. However, it is not yet clear the exact mechanism by which this HDAC6-mediated deacetylation takes place.

Results also showed that the metastasising cells have increased levels of the HDAC6 enzyme as seen from the Western blots performed and specifically inhibiting HDAC6 activity via the action of the HDAC6 inhibitor Tubacin in these cells undid the increase in force back closer to the force seen in normal cells. This is consistent with results obtained from a previous paper that shows a decrease and reversal of cell shape, cell spreading and cell migration due to tubacin-mediated loss of HDAC6 action in these specific cells (Evans et al., 2022). The results from this project show that the knockdown of HDAC6 via siRNA action give similar or even same results when compared to results seen in earlier experiments, which were that the effect of various oncogenes such as SV40T or c-Myc in normal fibroblast cells results in increased extent of HDAC6 protein. which in turn causes HDAC6-dependent cytoskeletal reorganisation and stiffness of cells (Rathje et al., 2014). However, even though there was clear increase in the acetylation of α-tubulin, increased cell spreading and larger focal adhesions after both the selective inhibition of HDAC6 activity with Tubacin and knockdown by HDAC6 the results on contractile forces were completely opposite. A potential reason for this discrepancy could be that when acetylase-activity of HDAC6 increases the potential protein-binding capacity of HDAC6 decreases which in turn affects the contractility exerted by the cells on their environment. This explanation can be further supported by a similar study which also showed a difference between the results of HDAC6 inhibition and HDAC6 knockdown, especially in terms of the actions of microtubules since the growth and reduction of microtubules only occurred during the inhibition of tubacin but not due to the action of HDAC6 knockdown via siRNA activity. However, on the other hand both processes increased the acetylation levels of tubulin (Zilberman et al., 2009). This study clearly shows that HDAC6 action is crucial for proteins associated with microtubules and that the deacetylation of HDAC6 controls the actions of microtubules. When deacetylation of HDAC6 is inhibited with tubacin, HDAC6 cannot be removed from microtubules, and this prevents the tips of microtubules from growing and in turn blocks the polymerization of microtubules. (Zilberman et al., 2009, Asthana et al., 2013).

Just like my results showed that the increased acetylation of tubulin is accompanied by reduced total contractile force of cells, another study found increased traction forces upon microtubule deacetylation (Seetharaman et al., 2022). There is also evidence that HDAC6 action controls actomyosin mediated contractile forces via substrates like HSP90 and cortactin but not tubulin, (Zhang et al., 2007, Martin and Leibovich, 2005). For example, cortactin is responsible for increasing the polymerization of F-actin, which results in an increase in the movement of the lamellipodia, particularly at the cell leading edges, resulting in increased cancer invasiveness

(Kirkbride et al., 2011), and the production of traction forces in cells (Gad et al., 2012). Hence, a possible reason for the opposite effect of the contractile forces that I observed by inhibiting or knocking down HDAC6 might be that the mechanisms for contractile force are precisely controlled via HDAC6-mediated acetylation but on the other hand the ability of HDAC6 to bind microtubules leads to an imprecise and indirect effect on contractile forces and their generation.

Results also show that the inhibiting HDAC6 activity or knocking it down both reversed the reduced spreading area of cells, less elongated cell shape and defective cell migration of metastasising cells to normal, while showing different results for contractile forces. This could suggest that while the deacetylase activity of HDAC6 controls cell spreading, shape and migration, the tubulin-binding function of HDAC6 controls cell-matrix adhesions and the capacity of these adhesions to transmit forces to the environment (Elosegui-Artola et al., 2016). My results clearly show that the action of oncogenes and HDAC6 exerted on the cytoskeleton and cell-matrix are distinct and can even be counteracting. There are other potential reasons that could account for the discrepancies in the results of the contractile forces obtained via HDAC6 inhibition or siRNA mediated HDAC6 knockdown.

An interesting paper (Chen et al., 2019), which looked at the role of the enzyme endothelial nitric oxide synthase (eNOS) expression and its activity in cardiovascular diseases also treated HUVEC cells with tubacin, which is known as an HDAC6 inhibitor. In line with expectations, tubacin mediated inhibition also inhibited deacetylase activity in the cells and this was seen by relatively increased tubulin acetylation levels. The experiment also exposed HUVEC cells to several other HDAC6 inhibitors, which included tubastatin A, ACY-1215 and SAHA. The experiment also treated HUVEC cells to niltubacin, which is a compound that is similar in structure to tubacin but has not shown any HDAC6-inhibitory activity. The experiment discovered that all the HDAC6 inhibitors except tubacin had a negligible effect on the expression of eNOS in the cells, even though all the inhibitors were united in inhibiting deacetylase activity, as seen by the significant increase in the quantity of tubulin acetylation. The study hypothesized that tubacin action in eNOS might be through HDAC6-independent mechanisms. In order to evaluate this hypothesis, the experiment studied the effect of knocking down HDAC6 via siRNA action on the expression of eNOS cells. Proving the hypothesis, there was a significant reduction in the expression of protein and mRNA in the cells after HDAC6 knockdown via siRNA Furthermore, the study determined that the mRNA and protein levels of eNOS cells were also increased via the action of tubacin in HDAC6 inhibited eNOS cells.,

which supported the idea that it is the action of tubacin which increases eNOS cell expression via mechanisms that are independent of HDAC6. One possible HDAC6-independent mechanism could be due to increased levels of transcription of genes. The study discovered that tubacin does not carry out its actions by inhibiting the levels of HDAC6 but instead this action is performed by mediating an increase in the levels of mRNA. While it cannot be concluded that this is the reason for the discrepancy found in my results between the contractile forces of cells treated with tubacin and siRNA knockdown, greater study and understanding of tubacin's mechanism of action can paint a clearer picture.

Another study (Zilberman et al., 2009), studied the role of HDAC6 in controlling the action of microtubules. After treating cells with tubacin, there was a significant increase in acetylation of microtubules and a significant decrease in the growth of microtubules. On the other hand, treating cells by knocking down HDAC6 with siRNA also led to an increase in the acetylation of microtubules but, surprisingly, did not affect microtubule growth. Also, knockdown of HDAC6 via siRNA removed the effect of tubacin on the growth of microtubules, showing that microtubules growth is affected by tubacin only via HDAC6 inhibition. Therefore, it can be assumed that it is only the presence of an impaired HDAC6 with no catalytic activity, instead of any action by the acetylation of tubulin that led to the increased growth of microtubules in HDAC6 inhibited cells. To support this hypothesis, HDAC6 mutant cells, which have had specific mutations in either one of their two catalytic domains that inactivated them replicated the HDAC6 inhibitors actions on the growth of microtubules. Also, HDAC6 was discovered to be attached with EB1, which is a protein that tracks microtubules and Arp1, which is an important part of dynactin. Both of these proteins are present at the ends of growing microtubules. Therefore, it is hypothesized that the growth of microtubules can be controlled by inhibiting the activity of HDAC6 via tubulin or other proteins involved in microtubules actions. Although, these results do not show that the effects of the tubacin was non-specific. however, the results show that treating cells with tubacin especially at higher amounts doesn't lead to any significant effect on the growth of microtubules, especially in the cells that lack HDAC6, thus proving that tubacin is specific.

Results also show that even without increases in focal adhesions such as their size or number, metastasising cells still show increased contractile forces when compared to normal. Thus, it is possible that focal adhesions could be used as potential markers for measuring contractile forces. However, a previous study has suggested that the increased density of focal adhesions

and actin-fibres is a more important biomarker than looking at the size or number of focal adhesions, (Gad et al., 2012). This makes it important to evaluate if the assembly or disassembly of focal adhesions is what regulates contractile forces. The stiffness of the underlying substrate also affects the cell-matrix adhesions and contractile forces of the cells. Previous research in HDAC6 mediated contractility and migration has consisted of cells being cultured on glass or plastic. Therefore, this project has also carried out all analysis on glass or plastic, except for the TFM measurements which were performed on 12 kPa gels.

Although metastasis is a very dynamic process, metastatic cells at the epigenetic and genetic levels are plastic. The neoplastic cells, which undergo epithelial-mesenchymal transition (EMT) keep interacting with the host environment at all stages of metastasis and at different rates. Therefore, by inhibiting a target that is important in every stage of metastasis, metastatic inhibition can be significantly increased which will reduce resistance to drugs and result in a higher survival rate for the patient. It is this concept that has led to the idea that the most effective approaches towards drug development have been in developing inhibitors of histone deacetylases, especially HDAC6 inhibitors. HDAC6 regulates the levels of acetylation in different non-histone compounds, such as certain proteins that suppress tumour and different oncogenes. Therefore, inhibitors that deacetylate histones (HDIs) are highly effective anti-proliferation agents which target histone deacetylases. HDAC inhibitors can be divided into three main categories, which include broad-spectrum inhibitors, class-specific inhibitors, or isoform-specific inhibitors (Karagiannis and El-Osta, 2006).

HDAC6 inhibitors are different from other histone deacetylases inhibitors as they exclusively deacetylate cytoplasmic proteins. HDAC6 is unique since it is the only histone deacetylase that has its own isoform-specific inhibitors synthesized. One isoform-specific HDAC6 inhibitor, Ricolinostat (ACY-1215), is currently being experimented on to treat patients with relapsed multiple myeloma and lymphoid malignancies. Since all HDAC6 inhibitors that have been developed to date target the DD2 domain, it results in different results being obtained between different experiments that use inhibitors with gene knockout methods, since gene knockout targets both domains (DD1 and DD2) (Kawaguchi et al., 2003).

HDAC6 inhibitors are very effective in different cancers and tumours. They can induce toxic effects on cancer cells and also improve the host anticancer immune response. Several cases have been recorded where cancer treatment was improved by combining with an HDAC6 inhibitor, and studies show that treating with an HDAC6 inhibitor before cancer treatment

begins is highly effective. Combining HDAC6 inhibitors with immune therapy is an effective therapy of several different cancers (Biersack et al., 2022).

Another effective strategy that is being used is changing the purpose of drugs that are already being used for non-cancerous medical conditions to identify new HDAC6 inhibitors. An example of this is the drug carbamazepine, which is an anti-epileptic drug that works via the Her2-targeting mAb drug Trastuzumab (Biersack et al., 2022). During cancer trials that worked with advanced or drug-resistant multiple myeloma patients, the HDAC6 inhibitors Ricolinostat showed effective results that highlighted the potential of selective HDAC6 inhibitors for future anticancer treatments (Vogl et al., 2017). This strategy includes designing individual therapies for cancer patients who have higher HDAC6 levels which leads to decreased side-effects and better prognosis for treated patients. Similarly, looking at the effects of selective HDAC6 inhibitors on CD20, combined with Rituximab, which is currently used for the treatment of non-Hodgkin lymphoma (NHL), give encouraging results for leukaemia and lymphoma patients. (Kaur et al., 2022).

Since the action of cancer is not well understood, it has hindered the research of newer medicines and earlier tumour detection methods that can identify the cancer before the spread of metastasis. Therefore, it is especially important to better understand cancer if we want to reduce the mortality levels associated with cancer. Much more research about the role of mechanotransduction and the effect of contractility in cancer needs to be performed, as there are very few experiments currently available to draw conclusions from. The main challenges facing cancer research in relation to metastasis and mechanical forces are the inability of existing methods for early detection of cancer and the lack of available markers for mechanical forces in cancer cells (Bregenzer et al., 2019).

This inability of early detection methods for cancer limits both the treatment options as well as our understanding of cancer progression. Research studying the invasiveness of the disease has mostly been studied in vitro and results are usually determined from computer models and tumour measurements. In order to make progress in understanding the effect of mechanobiology in cancer it is important to develop in vitro three-dimensional models that can replicate the mechanical forces being exerted in cancer in vivo. Also, as three-dimensional in vitro models are developed and become more accurate, these models will require us to add more cell populations from different groups of people along with the various conditions that they suffer from since the TME change can alter the effect of the cancer on the body. This

affects the results that we obtain in several studies significantly and makes making connections and comparison very difficult (Bregenzer et al., 2019).

More research is now being done on the mechanical properties and the microenvironment of a cancerous cell. As research progresses and understanding of molecular mechanisms increases, better drugs and therapies can be developed to specifically target cancer cells and improve the efficacy of treatments. The results obtained from experiments done for this project, will help in planning a future course of action regarding further research into the topic. In the future, this will allow us to examine and define the exact molecular mechanisms controlling cellular contractility, improve our knowledge, understand the significance and the part played by various components such as vimentin, HDAC6, Rho GTPases, ECM, actin, myosin, microtubules among others in both normal and cancer cells to develop effective treatments against cancer.

4.2 Future Plans

Initially, the project also involved measuring and comparing the contractile forces of normal fibroblasts and metastasising Ras cell lines on 3D dextran fibres in addition to the 2D polyacrylamide gels that were used in the project above. Previous scientific literature has shown that different types of cells have different contractile forces when cultured on 3D fibres when compared with 2D gels, even when the other conditions are all kept constant. Therefore, before drawing any conclusions based on the results of my experiments, it would have been beneficial to compare and ensure that the contractile forces obtained are consistent in both the 2D gels and the 3D fibres microenvironment.

However, due to the Covid-19 pandemic lockdown there was a huge loss of time, and the timeline of the project had to be modified. After the lockdown, once the labs reopened there was insufficient time to work on the 3D fibres and instead priority was given to measuring contractile forces on the 2D polyacrylamide gels. Also, creating 3D fibres (of specific lengths and thickness) is an exceedingly long and complex work that requires extensive experience and is very time consuming, which I did not have. Instead, since I had to continue with my project, I decided to change this aspect of my project and instead focus more on the intracellular forces that are exerted on the nuclei of normal and metastasising cells as well as studying the physical features of these cells such as cell circularity, persistence, migration, spreading area, aspect

ratio etc on 2D gels. I decided to use only pre-prepared commercially available gels which had a stiffness of 12 kPa for all my experiments.

Despite these setbacks, future research on measuring and comparing the contractile forces of normal fibroblasts and metastasising Ras cell lines on 3D dextran fibres is essential since the results obtained from this research will be more relevant to in vivo cell models. The current results obtained from the work on 2D gels are more relevant to in vitro cell models and in vivo cell models would be more effective in human studies.

Another part of the project that had been planned initially was that once the role that HDAC6 plays in controlling contractile forces was established, the next step was to determine the mechanism by which HDAC6 increases contractility. This would have been done by first expressing an HDAC6 variant that lacks deacetylase activity in normal cells. This was supposed to have been done by transiently transfecting normal cells with HDAC6 constructs of either the wild type of protein or the HDAC6 ubiquitin binding region in normal cells and analysing if this would induce a more metastasising adhesive and contractile phenotype. However, owing to a lack of time and technical supervision, it was decided to just knock down the HDAC6 protein from the metastasising cell using an HDAC6 siRNA. This allowed for the comparison between the contractile forces of normal, metastasising, and normal cells expressing HDAC6 variant that lack the deacetylase activity using TFM as well as comparing between these cells with regards to their cell-matrix focal adhesions and spreading area using confocal microscopy. This change would give the same result that was required since knocking down HDAC6 from the cells would also completely remove its effect and therefore would allow for testing the contractile forces of cells without the effect of HDAC6 in the equation.

However, it is still relevant to repeat the experiments to determine the mechanism by which HDAC6 increases contractility but this time by first expressing an HDAC6 variant that lacks deacetylase activity in normal cells. This is done by transiently transfecting normal cells with HDAC6 constructs of either the wild type of protein or the HDAC6 ubiquitin binding region in normal cells and analysing if this would induce a more metastasising adhesive and contractile phenotype.

There was also a plan to repeat these particular experiments with different surfaces and stiffness of gels to compare the results and confirm that the results remain consistent despite the change in substrate conditions, which normally are important factors in mechanobiology.

Another future plan that would be interesting would be to prepare a public database of different kinds of cells along with their measured contractile forces using TFM. All the cells will be cultured on gels of different materials and stiffness. This would be a long-term project that would help future researchers in the field of mechanobiology as it would save time and resources. The researchers who would work on cellular contractility and mechanobiology would be able to save time and resources by just looking up the contractile forces of different types of cells at their desired gel stiffness, instead of having to spend time and resources repeating TFM for each cell that they plan to test for their respective experiments. This publicly available database could then be potentially expanded to include data on other mechanical properties such as cellular stiffness, among others.

4.3 Conclusion

The results of this project show that the physical contractile forces which are exerted by the cells on their surrounding environment are increased, while the intracellular cytoplasmic force on the cell nuclei is decreased in metastasising fibroblasts when compared to normal BJ cells, and that this is dependent on HDAC6 activity. The results show that oncogenes regulate the cytoskeleton and focal adhesions, via 2 distinct pathways, which include the deacetylase dependent pathway and via the cell binding capacity of HDAC6, which is responsible for changing the cell shape, movement, area covered by the cell and the force exerted by external physical factors that lead to increased invasion of metastatic cells. The results obtained also match the hypothesis by way that that increased expression of HDAC6 make metastasising cells more contractile and invasive. Thus, research that targets HDAC6 expression such as HDAC6 inhibitor drugs are particularly important for future treatment of cancer and this project helps with finding the mechanism that HDAC6 uses to increase invasiveness.

Chapter Five – REFERENCES

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Aguilar-Cuenca, R., Juanes-García, A. and Vicente-Manzanares, M. (2013) 'Myosin II in mechanotransduction: Master and commander of cell migration, morphogenesis, and cancer', *Cellular and Molecular Life Sciences*, 71(3), pp. 479–492. doi:10.1007/s00018-013-1439-5.

Ahuja, D., Sáenz-Robles, M. and Pipas, J., 2005. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene*, 24(52), pp.7729-7745.

AK Haris, P Wild and D Stopak (1980) in their article' 'Silicone rubber substrata: a new wrinkle in the study of cell locomotion''.

Akram, M. (2013) 'Mini-review on glycolysis and cancer', *Journal of Cancer Education*, 28(3), pp. 454–457. doi:10.1007/s13187-013-0486-9.

Alberts, B. *et al.* (2015) *Molecular biology of the cell*. New York, NY: Garland Science, Taylor and Francis Group.

Aldana-Masangkay, G. and Sakamoto, K., 2011. The Role of HDAC6 in Cancer. *Journal of Biomedicine and Biotechnology*, 2011, pp.1-10.

ALISAFAEI, F., JOKHUN, D. S., SHIVASHANKAR, G. V. & SHENOY, V. B. 2019. Regulation of nuclear architecture, mechanics, and nucleocytoplasmic shuttling of epigenetic factors by cell geometric constraints. *Proc Natl Acad Sci U S A,* 116**,** 13200-13209.

Al-Rekabi, Z., Haase, K. and Pelling, A. (2014). Microtubules mediate changes in membrane cortical elasticity during contractile activation. *Experimental Cell Research*, 322(1), pp.21-29.

Amano, M., Nakayama, M. and Kaibuchi, K., 2010. Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton*, 67(9), pp.545-554.

Amin, E. *et al.* (2013) 'Rho-kinase: Regulation, (dys)function, and inhibition', *bchm*, 394(11), pp. 1399–1410. doi:10.1515/hsz-2013-0181.

Anand, P. *et al.* (2008) 'Cancer is a preventable disease that requires major lifestyle changes', *Pharmaceutical Research*, 25(9), pp. 2097–2116. doi:10.1007/s11095-008-9661-9.

Angulo-Urarte, A., van der Wal, T. and Huveneers, S. (2020) 'Cell-cell junctions as sensors and transducers of mechanical forces', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1862(9), p. 183316. doi: 10.1016/j.bbamem.2020.183316.

An, S.S. *et al.* (2009) 'Cell stiffness, contractile stress and the role of extracellular matrix', *Biochemical and Biophysical Research Communications*, 382(4), pp. 697–703. doi:10.1016/j.bbrc.2009.03.118.

Arnedos, M., Vicier, C., Loi, S., Lefebvre, C., Michiels, S., Bonnefoi, H. and Andre, F., 2015. Precision medicine for metastatic breast cancer—limitations and solutions. *Nature Reviews Clinical Oncology*, 12(12), pp.693-704.

ASTHANA, J., KAPOOR, S., MOHAN, R. & PANDA, D. 2013. Inhibition of HDAC6 deacetylase activity increases its binding with microtubules and suppresses microtubule dynamic instability in MCF-7 cells. *J Biol Chem,* 288**,** 22516-26.

Baba, A., Catoi, C. and Baba, A., 2007. *Comparative Oncology*. Bucharest: The Publishing House of the Romanian Academy, p. Chapter 2 CARCINOGENESIS

Bachir, A.I. *et al.* (2017) 'Actin-based adhesion modules mediate cell interactions with the extracellular matrix and neighbouring cells', *Cold Spring Harbor Perspectives in Biology*, 9(7). doi:10.1101/cshperspect. a023234.

BANCE, B., SEETHARAMAN, S., LEDUC, C., BOEDA, B. & ETIENNE-MANNEVILLE, S. 2019. Microtubule acetylation but not de tyrosination promotes focal adhesion dynamics and astrocyte migration. *J Cell Sci,* 132.

Baskar, R., Lee, K., Yeo, R. and Yeoh, K., 2012. Cancer and Radiation Therapy: Current Advances and Future Directions. *International Journal of Medical Sciences*, 9(3), pp.193-199.

Batchu, S., Brijmohan, A. and Advani, A., 2016. The therapeutic hope for HDAC6 inhibitors in malignancy and chronic disease. *Clinical Science*, 130(12), pp.987-1003.

Baudino, T., 2015. Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Current Drug Discovery Technologies*, 12(1), pp.3-20.

Bazzaro, M., Lin, Z., Santillan, A., Lee, M., Wang, M., Chan, K., Bristow, R., Mazitschek, R., Bradner, J. and Roden, R., 2008. Ubiquitin Proteasome System Stress Underlies Synergistic Killing of Ovarian Cancer Cells by Bortezomib and a Novel HDAC6 Inhibitor. *Clinical Cancer Research*, 14(22), pp.7340-7347.

Bear, J.E. and Haugh, J.M. (2014) 'Directed migration of mesenchymal cells: Where signaling and the cytoskeleton meet', *Current Opinion in Cell Biology*, 30, pp. 74–82. doi:10.1016/j.ceb.2014.06.005.

Beningo, K., Hamao, K., Dembo, M., Wang, Y. and Hosoya, H. (2006). Traction forces of fibroblasts are regulated by the Rho-dependent kinase but not by the myosin light chain kinase. *Archives of Biochemistry and Biophysics*, 456(2), pp.224-231.

Benson, A.B. *et al.* (2017) 'Colon cancer, version 1.2017, NCCN clinical practice guidelines in oncology', *Journal of the National Comprehensive Cancer Network*, 15(3), pp. 370–398. doi:10.6004/jnccn.2017.0036.

Bera, K., Kiepas, A., Godet, I., Li, Y., Mehta, P., Ifemembi, B., Paul, C.D., Sen, A., Serra, S.A., Stoletov, K., Tao, J., Shatkin, G., Lee, S.J., Zhang, Y., Boen, A., Mistriotis, P., Gilkes, D.M., Lewis, J.D., Fan, C.-M. and Feinberg, A.P. (2022). Extracellular fluid viscosity enhances cell migration and cancer dissemination. *Nature*, [online] 611(7935), pp.365–373. doi:https://doi.org/10.1038/s41586-022-05394-6.

Bernstein, C., R., A., Nfonsam, V. and Bernstei, H., 2013. DNA Damage, DNA Repair and Cancer. *New Research Directions in DNA Repair.*

BERSHADSKY, A. D., BALLESTREM, C., CARRAMUSA, L., ZILBERMAN, Y., GILQUIN, B., KHOCHBIN, S., ALEXANDROVA, A. Y., VERKHOVSKY, A. B., SHEMESH, T. & KOZLOV, M. M. 2006. Assembly and mechanosensory function of focal adhesions: experiments and models. *Eur J Cell Biol,* 85**,** 165-73.

Besser, A. and Safran, S.A. (2006) 'Force-induced adsorption and anisotropic growth of focal adhesions', *Biophysical Journal*, 90(10), pp. 3469–3484. doi:10.1529/biophysj.105.074377.

Biersack, B., Nitzsche, B. and Höpfner, M. (2022) 'Immunomodulatory properties of HDAC6 inhibitors in cancer diseases: New chances for sophisticated drug design and treatment optimization', *Seminars in Cell & Developmental Biology* [Preprint]. doi: 10.1016/j.semcdb.2022.09.009.

Biller, L.H. and Schrag, D. (2021) 'Diagnosis and treatment of metastatic colorectal cancer', *JAMA*, 325(7), p. 669. doi:10.1001/jama.2021.0106.

BINKER, M. G., ZHAO, D. Y., PANG, S. J. & HARRISON, R. E. 2007. Cytoplasmic linker protein- 170 enhances spreading and phagocytosis in activated macrophages by stabilizing microtubules. *J Immunol,* 179**,** 3780-91.

Birchmeier, W. (1984) 'Cytoskeleton structure and function', *Trends in Biochemical Sciences*, 9(4), pp. 192–195. doi:10.1016/0968-0004(84)90137-3.

Boureux, A. *et al.* (2006) 'Evolution of the Rho family of Ras-like GTPases in Eukaryotes', *Molecular Biology and Evolution*, 24(1), pp. 203–216. doi:10.1093/molbev/msl145.

Boyault, C., Sadoul, K., Pabion, M. and Khochbin, S., 2007. HDAC6, at the crossroads between cytoskeleton and cell signalling by acetylation and ubiquitination. *Oncogene*, 26(37), pp.5468-5476.

Bradbury, C., Khanim, F., Hayden, R., Bunce, C., White, D., Drayson, M., Craddock, C. and Turner, B., 2005. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. *Leukemia*, 19(10), pp.1751-1759.

Brand, R., 2010. Biographical Sketch: Julius Wolff, 1836-1902. *Clinical Orthopaedics & Related Research*, 468(4), pp.1047-1049.

Bray, F. *et al.* (2018) 'Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries', *CA: A Cancer Journal for Clinicians*, 68(6), pp. 394–424. doi:10.3322/caac.21492.

Bregenzer, M. *et al.* (2019) 'The role of Cancer Stem Cells and mechanical forces in ovarian cancer metastasis', *Cancers*, 11(7), p. 1008. doi:10.3390/cancers11071008.

Bonnans, C., Chou, J. and Werb, Z. (2014) 'Remodelling the extracellular matrix in development and disease', *Nature Reviews Molecular Cell Biology*, 15(12), pp. 786–801. doi:10.1038/nrm3904.

BURRIDGE, M. C.-W. A. K. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *Journal of Cell Biology*, 133**,** 1403-1415.

Burridge, K. and Guilluy, C. (2016) 'Focal adhesions, stress fibers and mechanical tension', *Experimental Cell Research*, 343(1), pp. 14–20. doi: 10.1016/j.yexcr.2015.10.029.

Butler, J.P. *et al.* (2002) 'Traction fields, moments, and strain energy that cells exert on their surroundings', *American Journal of Physiology-Cell Physiology*, 282(3). doi:10.1152/ajpcell.00270.2001.

Butler, K., Kalin, J., Brochier, C., Vistoli, G., Langley, B. and Kozikowski, A., 2010.

Rational Design and Simple Chemistry Yield a Superior, Neuroprotective HDAC6 Inhibitor, Tubastatin A. *Journal of the American Chemical Society*, 132(31), pp.1084210846.

Cairns, B., 2001. Emerging roles for chromatin remodelling in cancer biology. *Trends in Cell Biology*, 11(11), pp. S15-S21.

Cancer statistics (2024) *NCI*. Available at: https://www.cancer.gov/about cancer /understanding/statistics (Accessed: 28 March 2025).

Cao, J. *et al.* (2018) 'Ricolinostat (acy-1215) suppresses proliferation and promotes apoptosis in esophageal squamous cell carcinoma via mir-30d/PI3K/AKT/motor and ERK PATHWAYS', *Cell Death & Disease*, 9(8). doi:10.1038/s41419-018-0788-2.

Carbone, A. (2020) 'Cancer classification at the Crossroads', *Cancers*, 12(4), p. 980. doi:10.3390/cancers12040980.

Carey, S.P., D'Alfonso, T.M., Shin, S.J. and Reinhart-King, C.A. (2012). Mechanobiology of tumour invasion: Engineering meets oncology. *Critical Reviews in Oncology/Hematology*, 83(2), pp.170–183. doi:https://doi.org/10.1016/j.critrevonc.2011.11.005.

Chae, Y.K. *et al.* (2016) 'Genomic landscape of DNA repair genes in cancer', *Oncotarget*, 7(17), pp. 23312–23321. doi:10.18632/oncotarget.8196.

Chaffer, C.L. and Weinberg, R.A. (2011) 'A perspective on cancer cell metastasis', *Science*, 331(6024), pp. 1559–1564. doi:10.1126/science.1203543.

Chen, C.S. *et al.* (2003) 'Cell shape provides global control of focal adhesion assembly', *Biochemical and Biophysical Research Communications*, 307(2), pp. 355–361. doi:10.1016/s0006-291x (03)01165-3.

Chen, C., Yin, L., Song, X., Yang, H., Ren, X., Gong, X., Wang, F. and Yang, L. (2016). Effects of vimentin disruption on the mechanoresponses of articular chondrocyte. *Biochemical and Biophysical Research Communications*, 469(1), pp.132-137

Chen, J., Zhang, J., Noor Ahmad Shaik, Yi, B., Wei, X., Yang, X., Naik, U.P., Summer, R., Yan, G., Xu, X. and Sun, J. (2019). The histone deacetylase inhibitor tubacin mitigates endothelial dysfunction by up regulating the expression of endothelial nitric oxide synthase. *Journal of Biological Chemistry*, 294(51), pp.19565–19576. doi:https://doi.org/10.1074/jbc.ra119.011317.

CHIOTAKI, R., POLIOUDAKI, H. & THEODOROPOULOS, P. A. 2014. Differential nuclear shape dynamics of invasive andnon-invasive breast cancer cells are associated with actin cytoskeleton organization and stability. *Biochem Cell Biol,* 92**,** 287-95.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) ‘Rho-stimulated contractility drives the formation of stress fibers and focal adhesions.’, *The Journal of cell biology*, 133(6), pp. 1403–1415. doi:10.1083/jcb.133.6.1403.

Ciuba, K., Hawkes, W., Tojkander, S., Kogan, K., Engel, U., Iskratsch, T. and Lappalainen, P. (2018). Calponin-3 is critical for coordinated contractility of actin stress fibers. *Scientific Reports*, 8(1).

Cleeland, C.S. *et al.* (2012) ‘Reducing the toxicity of cancer therapy: Recognizing needs, taking action’, *Nature Reviews Clinical Oncology*, 9(8), pp. 471–478. doi:10.1038/nrclinonc.2012.99.

CLEVERS, J. E. V. A. H. 2016. Tissue-specific designs of stem cell hierarchies. *NATURE CELL BIOLOGY,* 18.

Cline, M.J. (1987) ‘Keynote address: The role of proto-oncogenes in human cancer: Implications for diagnosis and treatment’, *International Journal of Radiation Oncology*Biology*Physics*, 13(9), pp. 1297–1301. doi:10.1016/0360-3016(87)90219-7.

Coleman, A., Cavalcante Joca, H., Shi, G., Lederer, W. and Ward, C., 2020. Microtubule Acetylation Regulates Striated Muscle Mechanotransduction. *Biophysical Journal*, 118(3), p.424a.

Colin-York, H., Eggeling, C. and Fritzsche, M. (2017). Dissection of mechanical force in living cells by super-resolved traction force microscopy. *Nature Protocols*, 12(4), pp.783-796.

Cooper, G.M. (2000) ‘Structure and Organization of Actin Filaments’, in *The Cell, 2nd edition*. Second.

Costigliola, N., Ding, L., Burckhardt, C., Han, S., Gutierrez, E., Mota, A., Groisman, A., Mitchison, T. and Danuser, G. (2017). Vimentin fibers orient traction stress. *Proceedings of the National Academy of Sciences*, 114(20), pp.5195-5200.

Croce, C., 2008. Oncogenes and Cancer. *New England Journal of Medicine*, 358(5), pp.502-511.

Dang, C.V. (1989) ‘Oncogenes and proto-oncogenes: General concepts’, *Cancer Treatment and Research*, pp. 3–24. doi:10.1007/978-1-4613-1599-5_1.

DANIELSSON, F., SKOGS, M., HUSS, M., REXHEPAJ, E., O'HURLEY, G., KLEVEBRING, D., PONTEN, F., GAD, A. K., UHLEN, M. & LUNDBERG, E. 2013.

Majority of differentially expressed genes are down-regulated during malignant transformation in a four-stage model. *Proc Natl Acad Sci U S A,* 110**,** 6853-8.

Das, A., 2013. Extracellular Matrix Density Regulates Extracellular Proteolysis via Modulation of Cellular Contractility. *Journal of Carcinogenesis & Mutagenesis*, S13.

Davis, I., 2003. Rational approaches to human cancer immunotherapy. *Journal of Leukocyte Biology*, 73(1), pp.3-29.

DEB ROY, A., GROSS, E. G., PILLAI, G. S., SEETHARAMAN, S., ETIENNE-MANNEVILLE, S. & INOUE, T. 2022. Non-catalytic allostery in alpha-TAT1 by a phospho-switch drives dynamic microtubule acetylation. *J Cell Biol,* 221.

Dembo, M. and Wang, Y.-L. (1999) 'Stresses at the cell-to-substrate interface during locomotion of fibroblasts', *Biophysical Journal*, 76(4), pp. 2307–2316. doi:10.1016/s0006-3495(99)77386-8.

Denais, C. and Lammerding, J. (2014) 'Nuclear mechanics in cancer', *Advances in Experimental Medicine and Biology*, pp. 435–470. doi:10.1007/978-1-4899-8032-8_20.

De Pascalis, C., Pérez-González, C., Seetharaman, S., Boëda, B., Vianay, B., Burute, M., Leduc, C., Borghi, N., Trepat, X. and Etienne-Manneville, S. (2018). Intermediate filaments control collective migration by restricting traction forces and sustaining cell– cell contacts. *The Journal of Cell Biology*, 217(9), pp.3031-3044.

Depetter, Y., Geurs, S., De Vreese, R., Goethals, S., Vandoorn, E., Laevens, A. Steenbrugge, J., Meyer, E., de Tullio, P., Bracke, M., D'hooghe, M. and De Wever, O., 2019. Selective pharmacological inhibitors of HDAC6 reveal biochemical activity but functional tolerance in cancer models. *International Journal of Cancer*, 145(3), pp.735747.

DESHPANDE, V. *et al.* (2008) 'A bio-mechanical model for coupling cell contractility with focal adhesion formation', *Journal of the Mechanics and Physics of Solids*, 56(4), pp. 1484–1510. doi:10.1016/j.jmps.2007.08.006.

Deskin, B. *et al.* (2020) 'Inhibition of HDAC6 attenuates tumor growth of non-small cell lung cancer', *Translational Oncology*, 13(2), pp. 135–145. doi: 10.1016/j.tranon.2019.11.001.

De Zoeten, E., Wang, L., Butler, K., Beier, U., Akimova, T., Sai, H., Bradner, J., Mazitschek, R., Kozikowski, A., Matthias, P. and Hancock, W., 2011. Histone Deacetylase 6 and Heat

Shock Protein 90 Control the Functions of Foxp3+ T-Regulatory Cells. *Molecular and Cellular Biology*, 31(10), pp.2066-2078.

Di, X., Gao, X., Luo, D., Ai, J., Jin, X., Qi, S., Li, H., Wang, K. and Luo, D. (2023). Cellular mechanotransduction in health and diseases: from molecular mechanism to therapeutic targets. *Signal Transduction and Targeted Therapy*, 8(1). doi:https://doi.org/10.1038/s41392-023-01501-9.

Di Carlo, D. (2012) 'A mechanical biomarker of cell state in medicine', *SLAS Technology*, 17(1), pp. 32–42. doi:10.1177/2211068211431630.

Ding, G., Liu, H., Huang, Q., Liang, H., Ding, Z., Liao, Z. and Huang, G., 2013. HDAC6 promotes hepatocellular carcinoma progression by inhibiting P53 transcriptional activity. *FEBS Letters*, 587(7), pp.880-886.

DOS SANTOS, A., COOK, A. W., GOUGH, R. E., SCHILLING, M., OLSZOK, N. A., BROWN, I., WANG, L., AARON, J., MARTIN-FERNANDEZ, M. L., REHFELDT, F. & TOSELAND, C. P. 2021. DNA damage alters nuclear mechanics through chromatin reorganization. *Nucleic Acids Res,* 49**,** 340-353.

DOS SANTOS, A. & TOSELAND, C. P. 2021. Regulation of Nuclear Mechanics and the Impact on DNA Damage. *Int J Mol Sci,* 22.

Duffy, M.J., Synnott, N.C. and Crown, J. (2017) 'Mutant P53 as a target for cancer treatment', *European Journal of Cancer*, 83, pp. 258–265. doi: 10.1016/j.ejca.2017.06.023.

Dupont, C., Armant, D. and Brenner, C. (2009) 'Epigenetics: Definition, mechanisms and clinical perspective', *Seminars in Reproductive Medicine*, 27(05), pp. 351–357. doi:10.1055/s-0029-1237423.

Dunlop, C., 2019. Differential cellular contractility as a mechanism for stiffness sensing. *New Journal of Physics*, 21(6), p.063005.

Dwyer, R., Khan, S., Barry, F., O'Brien, T. and Kerin, M., 2010. Advances in mesenchymal stem cell-mediated gene therapy for cancer. *Stem Cell Research & Therapy*, 1(3), p.25

Eccles, S.A. and Welch, D.R. (2007) 'Metastasis: Recent discoveries and novel treatment strategies', *The Lancet*, 369(9574), pp. 1742–1757. doi:10.1016/s0140-6736(07)60781-8. Eckschlager, T. *et al.* (2017) 'Histone deacetylase inhibitors as anticancer drugs', *International Journal of Molecular Sciences*, 18(7), p. 1414. doi:10.3390/ijms18071414.

Eke, I. and Cordes, N. (2015) 'Focal adhesion signaling and therapy resistance in cancer', *Seminars in Cancer Biology*, 31, pp. 65–75. doi:10.1016/j.semcancer.2014.07.009.

ELOSEGUI-ARTOLA, A., ORIA, R., CHEN, Y., KOSMALSKA, A., PEREZ-GONZALEZ, C., CASTRO, N., ZHU, C., TREPAT, X. & ROCA-CUSACHS, P. 2016. Mechanical regulation of a molecular clutch defines force transmission and transduction in response to matrix rigidity. *Nat Cell Biol,* 18**,** 540-8.

Estabrook, I.D. *et al.* (2021) 'Calculation of the force field required for nucleus deformation during cell migration through constrictions', *PLOS Computational Biology*, 17(5). doi: 10.1371/journal.pcbi.1008592.

EVANS, C. A., KIM, H. R., MACFARLANE, S. C., NOWICKI, P. I. A., BALTES, C., XU, L., WIDENGREN, J., LAUTENSCHLAGER, F., CORFE, B. M. & GAD, A. K. B. 2022. Metastasising Fibroblasts Show an HDAC6-Dependent Increase in Migration Speed and Loss of Directionality Linked to Major Changes in the Vimentin Interactome. *Int J Mol Sci,* 23.

Ezratty, E.J., Partridge, M.A. and Gundersen, G.G. (2005) 'Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase', *Nature Cell Biology*, 7(6), pp. 581–590. doi:10.1038/ncb1262.

Fang, T. *et al.* (2015) 'Methylation and gene expression of histone deacetylases 6 in systemic lupus erythematosus', *International Journal of Rheumatic Diseases*, 19(10), pp. 968–973. doi:10.1111/1756-185x.12783.

Fares, J. *et al.* (2020) 'Molecular principles of metastasis: A hallmark of cancer revisited', *Signal Transduction and Targeted Therapy*, 5(1). doi:10.1038/s41392-020-0134-x.

Feld, L. *et al.* (2020) 'Cellular contractile forces are nonmechanosensitive', *Science Advances*, 6(17). doi:10.1126/sciadv.aaz6997.

Ferlier, T. and Coulouarn, C. (2022) 'Regulation of gene expression in cancer—an overview', *Cells*, 11(24), p. 4058. doi:10.3390/cells11244058.

Ferreira, A.R., Felgueiras, J. and Fardilha, M. (2014) 'Signaling pathways in anchoring junctions of epithelial cells: Cell-to-cell and cell-to-extracellular matrix interactions', *Journal of Receptors and Signal Transduction*, 35(1), pp. 67–75. doi:10.3109/10799893.2014.931426.

Fletcher, D.A. and Mullins, R.D. (2010) 'Cell Mechanics and the cytoskeleton', *Nature*, 463(7280), pp. 485–492. doi:10.1038/nature08908.

Forgacs, G. *et al.* (2004) 'Role of the cytoskeleton in Signaling Networks', *Journal of Cell Science*, 117(13), pp. 2769–2775. doi:10.1242/jcs.01122.

Fotheringham, S., Epping, M., Stimson, L., Khan, O., Wood, V., Pezzella, F., Bernards, R. and La Thangue, N., 2009. Genome-wide Loss-of-Function Screen Reveals an Important Role for the Proteasome in HDAC Inhibitor-Induced Apoptosis. *Cancer Cell*, 15(1), pp.57-66.

Franck, C., Maskarinec, S., Tirrell, D. and Ravichandran, G. (2011). Three-Dimensional Traction Force Microscopy: A New Tool for Quantifying Cell-Matrix Interactions. *PLoS ONE*, 6(3), p.e17833.

Freyman, T.M. *et al.* (2002) 'Fibroblast contractile force is independent of the stiffness which resists the contraction', *Experimental Cell Research*, 272(2), pp. 153–162. doi:10.1006/excr.2001.5408.

Friedl, P., Wolf, K. and Lammerding, J. (2011) 'Nuclear mechanics during cell migration', *Current Opinion in Cell Biology*, 23(1), pp. 55–64. doi:10.1016/j.ceb.2010.10.015.

GAD, A. K., RONNLUND, D., SPAAR, A., SAVCHENKO, A. A., PETRANYI, G., BLOM, H., SZEKELY, L., WIDENGREN, J. & ASPENSTROM, P. 2012. Rho GTPases link cellular contractile force to the density and distribution of nanoscale adhesions. *FASEB J,* 26**,** 2374-82.

Gandhi, N.S. and Mancera, R.L. (2008) 'The structure of glycosaminoglycans and their interactions with proteins', *Chemical Biology & Drug Design*, 72(6), pp. 455–482. doi:10.1111/j.1747-0285.2008.00741.x.

Gandin, A. *et al.* (2021) 'Simple yet effective methods to probe hydrogel stiffness for Mechanobiology', *Scientific Reports*, 11(1). doi:10.1038/s41598-021-01036-5.

Ganz, A., Lambert, M., Saez, A., Silberzan, P., Buguin, A., Mège, R. and Ladoux, B. (2006). Traction forces exerted through N-cadherin contacts. *Biology of the Cell*, 98(12), pp.721-730.

Gasco, M., Shami, S. and Crook, T. (2002) 'The P53 pathway in breast cancer', *Breast Cancer Research*, 4(2). doi:10.1186/bcr426.

Gatenby, R.A. and Gillies, R.J. (2007) 'Glycolysis in cancer: A potential target for therapy', *The International Journal of Biochemistry & Cell Biology*, 39(7–8), pp. 1358–1366. doi:10.1016/j.biocel.2007.03.021.

Gao, Y., Hubbert, C.C. and Yao, T.-P. (2010) 'The microtubule-associated histone deacetylase 6 (HDAC6) regulates epidermal growth factor receptor (EGFR) endocytic trafficking and

degradation', *Journal of Biological Chemistry*, 285(15), pp. 11219–11226. doi:10.1074/jbc.m109.042754.

Geiger, T.R. and Peeper, D.S. (2009) 'Metastasis mechanisms', *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1796(2), pp. 293–308. doi:10.1016/j.bbcan.2009.07.006.

GHASSEMI, S., MEACCI, G., LIU, S., GONDARENKO, A. A., MATHUR, A., ROCA CUSACHS, P., SHEETZ, M. P. & HONE, J. 2012. Cells test substrate rigidity by local contractions on submicrometer pillars. *Proc Natl Acad Sci U S A,* 109**,** 5328-33.

Gkretsi, V. and Stylianopoulos, T., 2018. Cell adhesion and matrix stiffness: coordinating cancer cell invasion and metastasis. *Frontiers in oncology*, *8*, p.145.

Goedecke, N., Bollhalder, M., Bernet, R., Silvan, U. and Snedeker, J., 2015. Easy and Accurate Mechano-profiling on Micropost Arrays. *Journal of Visualized Experiments*, (105).

Grover, C.N. *et al.* (2012) 'Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films', *Acta Biomaterialia*, 8(8), pp. 3080–3090. doi:10.1016/j.actbio.2012.05.006.

Grozinger, C., Hassig, C. and Schreiber, S., 1999. Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proceedings of the National Academy of Sciences*, 96(9), pp.4868-4873.

Gunning, P.W. *et al.* (2015) 'The evolution of compositionally and functionally distinct actin filaments', *Journal of Cell Science*, 128(11), pp. 2009–2019. doi:10.1242/jcs.165563.

Guo, W. and Wang, Y. (2007) 'Retrograde fluxes of focal adhesion proteins in response to cell migration and mechanical signals', *Molecular Biology of the Cell*, 18(11), pp. 4519–4527. doi:10.1091/mbc.e07-06-0582.

Haage, A. and Schneider, I., 2014. Cellular contractility and extracellular matrix stiffness regulate matrix metalloproteinase activity in pancreatic cancer cells. *The FASEB Journal*, 28(8), pp.3589-3599.

Haase, K. and Pelling, A. (2015). Investigating cell mechanics with atomic force microscopy. *Journal of The Royal Society Interface*, 12(104), p.20140970.

HAGGARTY, S. J., KOELLER, K. M., WONG, J. C., BUTCHER, R. A. & SCHREIBER, S. L. 2003. Multidimensional chemical genetic analysis of diversity-oriented synthesis-derived deacetylase inhibitors using cell-based assays. *Chem Biol,* 10**,** 383-96.

Hahn, W., Counter, C., Lundberg, A., Beijersbergen, R., Brooks, M. and Weinberg, R., 1999. Creation of human tumour cells with defined genetic elements. *Nature*, 400(6743), pp.464-468.

Han, Y., Ronceray, P., Xu, G., Malandrino, A., Kamm, R., Lenz, M., Broedersz, C. and Guo, M., 2018. Cell contraction induces long-ranged stress stiffening in the extracellular matrix. *Proceedings of the National Academy of Sciences*, 115(16), pp.4075-4080.

Hanahan, D. and Weinberg, R., 2011. Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), pp.646-674.

Hanahan, D. (2022) 'Hallmarks of cancer: New dimensions', *Cancer Discovery*, 12(1), pp. 31–46. doi:10.1158/2159-8290.cd-21-1059.

Hardin, J. *et al.* (2015) *Becker's world of the cell*. Boston: Pearson. pp. 422–446.

He, Q. *et al.* (2017) 'A decrease of histone deacetylase 6 expression caused by helicobacter pylori infection is associated with oncogenic transformation in Gastric cancer', *Cellular Physiology and Biochemistry*, 42(4), pp. 1326–1335. doi:10.1159/000478961.

Helfman, D. and Pawlak, G. (2005). Myosin light chain kinase and acto-myosin contractility modulate activation of the ERK cascade downstream of oncogenic Ras. *Journal of Cellular Biochemistry*, 95(5), pp.1069-1080.

Herrmann, H. *et al.* (2007) 'Intermediate filaments: From cell architecture to Nanomechanics', *Nature Reviews Molecular Cell Biology*, 8(7), pp. 562–573. doi:10.1038/nrm2197.

Hao, Y., Cheng, S., Tanaka, Y., Hosokawa, Y., Yalikun, Y. and Li, M., 2020. Mechanical properties of single cells: Measurement methods and applications. *Biotechnology Advances*, 45, p.107648.

Hassanpour, S.H. and Dehghani, M. (2017) 'Review of cancer from perspective of molecular', *Journal of Cancer Research and Practice*, 4(4), pp. 127–129. doi: 10.1016/j.jcrpr.2017.07.001.

HOLENSTEIN, C. N., HORVATH, A., SCHAR, B., SCHOENENBERGER, A. D., BOLLHALDER, M., GOEDECKE, N., BARTALENA, G., OTTO, O., HERBIG, M., GUCK, J., MULLER, D. A., SNEDEKER, J. G. & SILVAN, U. 2019. The relationship between metastatic potential and in vitro mechanical properties of osteosarcoma cells. *Mol Biol Cell*, 30**,** 887-898.

Holz, O., Apel, D. and Hassel, M. (2020). Alternative pathways control actomyosin contractility in epitheliomuscle cells during morphogenesis and body contraction. *Developmental Biology*, 463(1), pp.88–98. doi:https://doi.org/10.1016/j.ydbio.2020.04.001.

Hopkins, J.L., Lan, L. and Zou, L. (2022) 'DNA repair defects in cancer and therapeutic opportunities', *Genes & Development*, 36(5–6), pp. 278–293. doi:10.1101/gad.349431.122.

Hormone therapy for cancer (2022) *Hormone Therapy for Cancer - NCI*. Available at: https://www.cancer.gov/about-cancer/treatment/types/hormone-therapy (Accessed: 28 March 2025).

HORWITZ, A. R. 2012. The origins of the molecular era of adhesion research. *Nat Rev Mol Cell Biol,* 13**,** 805-11.

Hsieh, P. and Yamane, K., 2008. DNA mismatch repair: Molecular mechanism, cancer, and ageing. *Mechanisms of Ageing and Development*, 129(7-8), pp.391-407.

Hubbert, C. *et al.* (2002) 'HDAC6 is a microtubule-associated deacetylase', *Nature*, 417(6887), pp. 455–458. doi:10.1038/417455a.

Huysentruyt, L. and Seyfried, T., 2010. Perspectives on the mesenchymal origin of metastatic cancer. *Cancer and Metastasis Reviews*, 29(4), pp.695-707.

HU, Z., WEI, F., SU, Y., WANG, Y., SHEN, Y., FANG, Y., DING, J. & CHEN, Y. 2023. Histone deacetylase inhibitors promote breast cancer metastasis by elevating NEDD9 expression. *Signal Transduct Target Ther,* 8**,** 11.

Ilina, O. and Friedl, P. (2009) 'Mechanisms of collective cell migration at a glance', *Journal of Cell Science*, 122(18), pp. 3203–3208. doi:10.1242/jcs.036525.

Ingber, D. (2003) 'Mechanobiology and diseases of Mechanotransduction', *Annals of Medicine*, 35(8), pp. 564–577. doi:10.1080/07853890310016333.

Ingber, D.E. (2006) 'Cellular mechanotransduction: Putting all the pieces together again', *The FASEB Journal*, 20(7), pp. 811–827. doi:10.1096/fj.05-5424rev. JAIN, N., IYER, K. V., KUMAR, A. & SHIVASHANKAR, G. V. 2013. Cell geometric constraints induce modular gene-expression patterns via redistribution of HDAC3 regulated by actomyosin contractility. *Proc Natl Acad Sci U S A,* 110**,** 11349-54.

Janiszewska, M., Primi, M.C. and Izard, T. (2020) 'Cell adhesion in cancer: Beyond the migration of single cells', *Journal of Biological Chemistry*, 295(8), pp. 2495–2505. doi:10.1074/jbc. rev119.007759.

Janmey, P. and Miller, R., 2010. Mechanisms of mechanical signalling in development and disease. *Journal of Cell Science*, 124(1), pp.9-18.

Jiang, D. and Ma, P. (2022) 'Canagliflozin, characterized as a HDAC6 inhibitor, inhibits gastric cancer metastasis', *Frontiers in Oncology*, 12. doi:10.3389/fonc.2022.1057455.

JIU, Y. 2018. Vimentin intermediate filaments function as a physical barrier during intracellular trafficking of caveolin-1. *Biochem Biophys Res Commun,* 507**,** 161-167.

JIU, Y., LEHTIMAKI, J., TOJKANDER, S., CHENG, F., JAALINOJA, H., LIU, X., VARJOSALO, M., ERIKSSON, J. E. & LAPPALAINEN, P. 2015. Bidirectional Interplay between Vimentin Intermediate Filaments and Contractile Actin Stress Fibers. *Cell Rep,* 11**,** 1511-8.

JIU, Y., PERANEN, J., SCHAIBLE, N., CHENG, F., ERIKSSON, J. E., KRISHNAN, R. & LAPPALAINEN, P. 2017. Vimentin intermediate filaments control actin stress fibre assembly through GEF-H1 and RhoA. *J Cell Sci,* 130**,** 892-902.

Jo, H., Shim, K. and Jeoung, D. (2022) 'Targeting HDAC6 to overcome autophagy-promoted anti-cancer drug resistance', *International Journal of Molecular Sciences*, 23(17), p. 9592. doi:10.3390/ijms23179592.

Joo, E. and Yamada, K., 2014. MYPT1 regulates contractility and microtubule acetylation to modulate integrin adhesions and matrix assembly. *Nature Communications*, 5(1).

Jung, S.Y. *et al.* (2011) 'Factors associated with mortality after breast cancer metastasis', *Cancer Causes & Control*, 23(1), pp. 103–112. doi:10.1007/s10552-011-9859-8.

Jung, K.H. *et al.* (2012) 'Histone deacetylase 6 functions as a tumor suppressor by activating c-jun NH2-terminal kinase-mediated beclin 1-dependent autophagic cell death in liver cancer', *Hepatology*, 56(2), pp. 644–657. doi:10.1002/hep.25699.

Kalluri, R., 2007. Tumor microenvironment in cancer progression and metastasis. *The FASEB Journal*, 21(5).

Kanno, K. *et al.* (2012) 'Overexpression of histone deacetylase 6 contributes to accelerated migration and invasion activity of hepatocellular carcinoma cells', *Oncology Reports*, 28(3), pp. 867–873. doi:10.3892/or.2012.1898.

Karagiannis, T. and El-Osta, A., 2006. Clinical Potential of Histone Deacetylase Inhibitors as Stand-Alone Therapeutics and in Combination with other Chemotherapeutics or Radiotherapy for Cancer. *Epigenetics*, 1(3), pp.121-126.

KAREN A. BENINGO, M. D., IRINA KAVERINA, J. VICTOR SMALL, AND YU-LI WANG 2001. Nascent Focal Adhesions Are Responsible for the Generation. *JCB,* 153**,** 881–887.

KATIYAR, A., ZHANG, J., ANTANI, J. D., YU, Y., SCOTT, K. L., LELE, P. P., REINHARTKING, C. A., SNIADECKI, N. J., ROUX, K. J., DICKINSON, R. B. & LELE, T. P. 2022. The Nucleus Bypasses Obstacles by Deforming Like a Drop with Surface Tension Mediated by Lamin A/C. *Adv Sci (Weinh),* 9**,** e2201248.

Kaur, S., Rajoria, P. and Chopra, M. (2022) 'HDAC6: A unique HDAC family member as a cancer target', *Cellular Oncology*, 45(5), pp. 779–829. doi:10.1007/s13402-022-00704-6.

Kawaguchi, Y., Kovacs, J., McLaurin, A., Vance, J., Ito, A. and Yao, T., 2003. The Deacetylase HDAC6 Regulates Aggresomal Formation and Cell Viability in Response to Misfolded Protein Stress. *Cell*, 115(6), pp.727-738.

Khatiwala, C.B. *et al.* (2009) 'ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and Rock', *Journal of Bone and Mineral Research*, 24(5), pp. 886–898. doi:10.1359/jbmr.081240.

Kidd, M.E., Shumaker, D.K. and Ridge, K.M. (2014) 'The role of vimentin intermediate filaments in the progression of lung cancer', *American Journal of Respiratory Cell and Molecular Biology*, 50(1), pp. 1–6. doi:10.1165/rcmb.2013-0314tr.

Kim, D., Kim, E., Jung, D. and Kim, J. (2019). Cytoskeletal alteration modulates cancer cell invasion through RhoA-YAP signalling in stromal fibroblasts. *PLOS ONE*, 14(3), p.e0214553.

KIM, H. R., WARRINGTON, S. J., LOPEZ-GUAJARDO, A., AL HENNAWI, K., COOK, S. L., GRIFFITH, Z. D. J., SYMMES, D., ZHANG, T., QU, Z., XU, Y., CHEN, R. & GAD, A. K. B. 2022. ALD-R491 regulates vimentin filament stability and solubility, cell contractile force, cell migration speed and directionality. *Front Cell Dev Biol,* 10**,** 926283.

KIRKBRIDE, K. C., SUNG, B. H., SINHA, S. & WEAVER, A. M. 2011. Cortactin: a multifunctional regulator of cellular invasiveness. *Cell Adh Migr,* 5**,** 187-98.

Klein, C.A. (2008) 'The Metastasis Cascade', *Science*, 321(5897), pp. 1785–1787. doi:10.1126/science.1164853.

Koch, T.M. *et al.* (2012) '3D traction forces in cancer cell invasion', *PLoS ONE*, 7(3). doi: 10.1371/journal.pone.0033476.

Kollimada, S. *et al.* (2021) 'The biochemical composition of the actomyosin network sets the magnitude of cellular traction forces', *Molecular Biology of the Cell*, 32(18), pp. 1737–1748. doi:10.1091/mbc.e21-03-0109.

Kong, W. *et al.* (2021) 'Collagen crosslinking: Effect on structure, mechanics and fibrosis progression', *Biomedical Materials*, 16(6), p. 062005. doi:10.1088/1748-605x/ac2b79.

Köser, J., Gaiser, S. and Müller, B. (2011) 'Contractile cell forces exerted on rigid substrates', *European Cells and Materials*, 21, pp. 479–487. doi:10.22203/ecm.v021a36.

Kraning-Rush, C., Carey, S., Califano, J., Smith, B. and Reinhart-King, C. (2011). The role of the cytoskeleton in cellular force generation in 2D and 3D environments. *Physical Biology*, 8(1), p.015009.

KRANING-RUSH, C. M., CALIFANO, J. P. & REINHART-KING, C. A. 2012. Cellular traction stresses increase with increasing metastatic potential. *PLoS One,* 7**,** e32572.

KRENDEL, M., ZENKE, F. T. & BOKOCH, G. M. 2002. Nucleotide exchange factor GEF-H1 mediates crosstalk between microtubules and the actin cytoskeleton. *Nat Cell Biol,* 4**,** 294-301.

Kumar, S. and Weaver, V., 2009. Mechanics, malignancy, and metastasis: The force journey of a tumor cell. *Cancer and Metastasis Reviews*, 28(1-2), pp.113-127.

Kumar, Y. *et al.* (2024) 'Automating cancer diagnosis using advanced deep learning techniques for multi-cancer image classification', *Scientific Reports*, 14(1). doi:10.1038/s41598-024-75876-2.

Kwon, S., Yang, W., Moon, D. and Kim, K., 2020. Biomarkers to quantify cell migration characteristics. *Cancer Cell International*, 20(1).

LAFARGA, V., AYMERICH, I., TAPIA, O., MAYOR, F., JR. & PENELA, P. 2012. A novel GRK2/HDAC6 interaction modulates cell spreading and motility. *EMBO J,* 31**,** 856-69.

Läubli, H. and Borsig, L. (2010) 'Selectins promote tumor metastasis', *Seminars in Cancer Biology*, 20(3), pp. 169–177. doi: 10.1016/j.semcancer.2010.04.005.

LEA FELD, L. K., ABHISHEK MUKHERJEE, ARIEL LIVNE, ERAN BOUCHBINDER, HAGUY WOLFENSON1 2020. Cellular contractile forces are non-mechanosensitive. *SCIENCE ADVANCES,* 6.

Ledbetter, M.C. and Porter, K.R. (1963) 'A "microtubule" in Plant Cell Fine Structure', *The Journal of Cell Biology*, 19(1), pp. 239–250. doi:10.1083/jcb.19.1.239.

Leduc, C. and Etienne-Manneville, S. (2015) 'Intermediate filaments in cell migration and invasion: The unusual suspects', *Current Opinion in Cell Biology*, 32, pp. 102–112. doi:10.1016/j.ceb.2015.01.005.

Lee, Y.-S. *et al.* (2008) 'The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis', *Cancer Research*, 68(18), pp. 7561–7569. doi: 10.1158/0008-5472.can-08-0188.

Lee, E. and Muller, W., 2010. Oncogenes and Tumor Suppressor Genes. *Cold Spring Harbor Perspectives in Biology*, 2(10), pp. a003236-a003236.

Lee, J.-H. *et al.* (2013) 'Development of a histone deacetylase 6 inhibitor and its biological effects', *Proceedings of the National Academy of Sciences*, 110(39), pp. 15704–15709. doi:10.1073/pnas.1313893110.

Lee, D.H. *et al.* (2018) 'The HDAC6 inhibitor acy-1215 enhances the anticancer activity of Oxaliplatin in colorectal cancer cells', *International Journal of Oncology* [Preprint]. doi:10.3892/ijo.2018.4405.

LEE, S. & KUMAR, S. 2020. Cofilin is required for polarization of tension in stress fibre networks during migration. *J Cell Sci,* 133.

LEE, Y. S., LIM, K. H., GUO, X., KAWAGUCHI, Y., GAO, Y., BARRIENTOS, T., ORDENTLICH, P., WANG, X. F., COUNTER, C. M. & YAO, T. P. 2008. The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis. *Cancer Res,* 68**,** 7561-9.

Lessey, E.C., Guilluy, C. and Burridge, K. (2012) 'From mechanical force to RhoA activation', *Biochemistry*, 51(38), pp. 7420–7432. doi:10.1021/bi300758e.

Lewin, B. (2007) 'The extracellular matrix and Cell Adhesion', in *Cells*. Sudbury, Mass. : Jones and Bartlett Publishers.

Li, Y. and Seto, E. (2016) 'HDACs and HDAC inhibitors in cancer development and therapy', *Cold Spring Harbor Perspectives in Medicine*, 6(10). doi:10.1101/cshperspect. a026831.

Li, T., Zhang, C., Hassan, S., Liu, X., Song, F., Chen, K., Zhang, W. and Yang, J., 2018. Histone deacetylase 6 in cancer. *Journal of Hematology & Oncology*, 11(1).

Li, Y., Shin, D. and Kwon, S., 2012. Histone deacetylase 6 plays a role as a distinct regulator of diverse cellular processes. *FEBS Journal*, p.n/a-n/a.

Li, D. *et al.* (2014) 'Histone deacetylase 6 and cytoplasmic linker protein 170 function together to regulate the motility of pancreatic cancer cells', *Protein & Cell*, 5(3), pp. 214–223. doi:10.1007/s13238-013-0010-3.

Li, Y. and Seto, E., 2016. HDACs and HDAC Inhibitors in Cancer Development and Therapy. *Cold Spring Harbor Perspectives in Medicine*, 6(10), p.a026831.

LI, D., SUN, X., ZHANG, L., YAN, B., XIE, S., LIU, R., LIU, M. & ZHOU, J. 2014. Histone deacetylase 6 and cytoplasmic linker protein 170 function together to regulate the motility of pancreatic cancer cells. *Protein Cell,* 5**,** 214-23.

Li, Z. *et al.* (2021) 'Gain of LINC00624 enhances liver cancer progression by disrupting the histone deacetylase 6/tripartite motif containing 28/zinc finger protein 354C corepressor complex', *Hepatology*, 73(5), pp. 1764–1782. doi:10.1002/hep.31530.

Liang, T. and Fang, H. (2019) 'Structure, functions and selective inhibitors of HDAC6', *Current Topics in Medicinal Chemistry*, 18(28), pp. 2429–2447. doi:10.2174/1568026619666181129141822.

LIU, C.-Y. *et al.* (2015) 'Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation', *Oncotarget*, 6(18), pp. 15966–15983. doi:10.18632/oncotarget.3862.

Liu, J., Gao, H. and Wang, X., 2015. The role of the Rho/ROCK signalling pathway in inhibiting axonal regeneration in the central nervous system. *Neural Regeneration Research*, 10(11), p.1892.

LING, L., HU, F., YING, X., GE, J. & WANG, Q. 2018. HDAC6 inhibition disrupts maturational progression and meiotic apparatus assembly in mouse oocytes. *Cell Cycle,* 17**,** 550-556.

Lodish, H.F. (2008) 'Cell-Cell Adhesion', in *Molecular cell biology*. New York: W.H. Freeman.

Loeb, L., 2010. Mutator phenotype in cancer: Origin and consequences. *Seminars in Cancer Biology*, 20(5), pp.279-280.

Loh, C.-Y. *et al.* (2019) 'The E-cadherin and N-cadherin switch in epithelial-to-mesenchymal transition: Signalling, therapeutic implications, and challenges', *Cells*, 8(10), p. 1118. doi:10.3390/cells8101118.

López-Fagundo, C., Bar-Kochba, E., Livi, L., Hoffman-Kim, D. and Franck, C. (2014). Three-dimensional traction forces of Schwann cells on compliant substrates. *Journal of The Royal Society Interface*, 11(97), p.20140247.

Lu, D.-Y. *et al.* (2013) 'Cancer metastasis treatments', *Current Drug Therapy*, 8(1), pp. 24–29. doi:10.2174/1574885511308010003.

MacKenzie, K., Franco, S., Naiyer, A., May, C., Sadelain, M., Rafii, S. and Moore, M., 2002. Multiple stages of malignant transformation of human endothelial cells modelled by co-expression of telomerase reverse transcriptase, SV40 T antigen and oncogenic Nras. *Oncogene*, 21(27), pp.4200-4211.

Madan, R. *et al.* (2006) 'Focal adhesion proteins as markers of malignant transformation and prognostic indicators in breast carcinoma', *Human Pathology*, 37(1), pp. 9–15. doi:10.1016/j.humpath.2005.09.024.

Majérus, M.-A. (2022) 'The cause of cancer: The unifying theory', *Advances in Cancer Biology - Metastasis*, 4, p. 100034. doi:10.1016/j.adcanc.2022.100034.

Mak, M. *et al.* (2016) 'Single-cell migration in complex microenvironments: Mechanics and Signaling Dynamics', *Journal of Biomechanical Engineering*, 138(2). doi:10.1115/1.4032188.

Mantovani, F., Collavin, L. and Del Sal, G. (2018) 'Mutant P53 as a guardian of the cancer cell', *Cell Death & Differentiation*, 26(2), pp. 199–212. doi:10.1038/s41418-018-0246-9.

Marei, H.E. *et al.* (2021) 'P53 signaling in cancer progression and therapy', *Cancer Cell International*, 21(1). doi:10.1186/s12935-021-02396-8.

Marks, P.A. (2010). Histone deacetylase inhibitors: A chemical genetics approach to understanding cellular functions. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1799(10-12), pp.717–725. doi:https://doi.org/10.1016/j.bbagrm.2010.05.008.

Marquard, L., Gjerdrum, L., Christensen, I., Jensen, P., Sehested, M. and Ralfkiaer, E., 2008. Prognostic significance of the therapeutic target's histone deacetylase 1, 2, 6 and acetylated histone H4 in cutaneous T-cell lymphoma. *Histopathology*, 53(3), pp.267-277.

MARTIEL, J. L., LEAL, A., KURZAWA, L., BALLAND, M., WANG, I., VIGNAUD, T., TSENG, Q. & THERY, M. 2015. Measurement of cell traction forces with ImageJ. *Methods Cell Biol,* 125**,** 269-87.

MARTIN, P. & LEIBOVICH, S. J. 2005. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol,* 15**,** 599-607.

Martinez, A.W. *et al.* (2014) 'Effects of crosslinking on the mechanical properties, drug release and cytocompatibility of protein polymers', *Acta Biomaterialia*, 10(1), pp. 26–33. doi:10.1016/j.actbio.2013.08.029.

Maskarinec, S.A. *et al.* (2009) 'Quantifying cellular traction forces in three dimensions', *Proceedings of the National Academy of Sciences*, 106(52), pp. 22108–22113. doi:10.1073/pnas.0904565106.

Matoba, S., 2006. p53 Regulates Mitochondrial Respiration. *Science*, 312(5780), pp.1650-1653.

Matsuoka, T. (2014). Rho/ROCK signalling in motility and metastasis of gastric cancer. *World Journal of Gastroenterology*, 20(38), p.13756. doi:https://doi.org/10.3748/wjg.v20.i38.13756.

Matthias, P., Seiser, C. and Yoshida, M., 2011. Protein Acetylation and the Physiological Role of HDACs. *Journal of Biomedicine and Biotechnology*, 2011, pp.1-2.

Maurin, B. *et al.* (2008) 'Mechanical model of cytoskeleton structuration during cell adhesion and spreading', *Journal of Biomechanics*, 41(9), pp. 2036–2041. doi:10.1016/j.jbiomech.2008.03.011.

McGrail, D., Kieu, Q. and Dawson, M. (2014). The malignancy of metastatic ovarian cancer cells is increased on soft matrices through a mechanosensitive Rho-ROCK pathway. *Journal of Cell Science*, 127(12), pp.2621-2626.

Medler, T., Craig, J., Fiorillo, A., Feeney, Y., Harrell, J. and Clevenger, C., 2016. HDAC6 Deacetylates HMGN2 to Regulate Stat5a Activity and Breast Cancer Growth. *Molecular Cancer Research*, 14(10), pp.994-1008.

Menhofer, M., Kubisch, R., Schreiner, L., Zorn, M., Foerster, F., Mueller, R., Raedler, J., Wagner, E., Vollmar, A. and Zahler, S., 2014. The Actin Targeting Compound Chondramine

Inhibits Breast Cancer Metastasis via Reduction of Cellular Contractility. *PLoS ONE*, 9(11), p.e112542.

MIERKE, C. T., ROSEL, D., FABRY, B. & BRABEK, J. 2008. Contractile forces in tumor cell migration. *Eur J Cell Biol,* 87**,** 669-76.

Mierke, C.T. (2014) 'The fundamental role of mechanical properties in the progression of cancer disease and inflammation', *Reports on Progress in Physics*, 77(7), p. 076602. doi:10.1088/0034-4885/77/7/076602.

Moeendarbary, E. and Harris, A., 2014. Cell mechanics: principles, practices, and prospects. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 6(5), pp.371388.

Molter, C.W., Muszynski, E.F., Tao, Y., Trivedi, T., Clouvel, A. and Ehrlicher, A.J., 2022. Prostate cancer cells of increasing metastatic potential exhibit diverse contractile forces, cell stiffness, and motility in a microenvironment stiffness-dependent manner. *Frontiers in cell and developmental biology*, *10*, p.932510.

Moujaber, O. and Stochaj, U. (2020) 'The cytoskeleton as regulator of cell signaling pathways', *Trends in Biochemical Sciences*, 45(2), pp. 96–107. doi:10.1016/j.tibs.2019.11.003.

Mukherjee, A. *et al.* (2022) 'The role of ECM remodeling, EMT, and adhesion molecules in cancerous neural invasion: Changing perspectives', *Advanced Biology*, 6(9). doi:10.1002/adbi.202200039.

Mullins, R.D., Heuser, J.A. and Pollard, T.D. (1998) 'The interaction of arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments', *Proceedings of the National Academy of Sciences*, 95(11), pp. 6181–6186. doi:10.1073/pnas.95.11.6181.

Mut, M. (2012) 'Surgical treatment of brain metastasis: A Review', *Clinical Neurology and Neurosurgery*, 114(1), pp. 1–8. doi:10.1016/j.clineuro.2011.10.013.

Nagayama, M., Haga, H., Takahashi, M., Saitoh, T. and Kawabata, K., 2004. Contribution of cellular contractility to spatial and temporal variations in cellular stiffness. *Experimental Cell Research*, 300(2), pp.396-405.

Naser, R. *et al.* (2022) 'Role of the tumor microenvironment in cancer hallmarks and targeted therapy (review)', *International Journal of Oncology*, 62(2). doi:10.3892/ijo.2022.5471.

NDIAYE, A.-B., KOENDRINK, G.H. and Shemesh, M. (2022) 'Intermediate filaments in cellular mechanoresponsiveness: Mediating cytoskeletal crosstalk from membrane to nucleus and back', *Frontiers in Cell and Developmental Biology*, 10. doi:10.3389/fcell.2022.882037.

Neophytou, C.M., Kyriakou, T.-C. and Papageorgis, P. (2019) ‘Mechanisms of metastatic tumor dormancy and implications for cancer therapy’, *International Journal of Molecular Sciences*, 20(24), p. 6158. doi:10.3390/ijms20246158.

Nguyen, D.X. and Massagué, J. (2007) ‘Genetic determinants of cancer metastasis’, *Nature Reviews Genetics*, 8(5), pp. 341–352. doi:10.1038/nrg2101.

Nobe, K., Nobe, H., Yoshida, H., Kolodney, M., Paul, R. and Honda, K. (2010). Rho A and the Rho kinase pathway regulate fibroblast contraction: Enhanced contraction in constitutively active Rho A fibroblast cells. *Biochemical and Biophysical Research Communications*, 399(2), pp.292299.

Nokin, M.-J. *et al.* (2019) ‘Methylglyoxal, a glycolysis metabolite, triggers metastasis through MEK/ERK/SMAD1 pathway activation in breast cancer’, *Breast Cancer Research*, 21(1). doi:10.1186/s13058-018-1095-7.

Orr, A.W. *et al.* (2006) ‘Mechanisms of mechanotransduction’, *Developmental Cell*, 10(1), pp. 11–20. doi:10.1016/j.devcel.2005.12.006.

Parekh, A. and Weaver, A.M. (2009) ‘Regulation of cancer invasiveness by the physical extracellular matrix environment’, *Cell Adhesion & Migration*, 3(3), pp. 288–292. doi:10.4161/cam.3.3.8888.

Park, S. (2018) ‘Mechanical alteration associated with chemotherapeutic resistance of breast cancer cells’, *Journal of Cancer Prevention*, 23(2), pp. 87–92. doi:10.15430/jcp.2018.23.2.87.

Park, M. *et al.* (2022) ‘Breast cancer metastasis: Mechanisms and therapeutic implications’, *International Journal of Molecular Sciences*, 23(12), p. 6806. doi:10.3390/ijms23126806.

Parry, D.A.D. and Squire, J.M. (2017) *Fibrous proteins: Structures and mechanisms*. Cham: Springer International Publishing.

PARSONS, J. T., HORWITZ, A. R. & SCHWARTZ, M. A. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol*, 11**,** 633-43.

Paluch, E., Nelson, C., Biais, N., Fabry, B., Moeller, J., Pruitt, B., Wollnik, C., Kudryasheva, G., Rehfeldt, F. and Federle, W., 2015. Mechanotransduction: use the force(s). *BMC Biology*, 13(1).

Passaro, E. *et al.* (2021) ‘HDAC6 inhibition extinguishes autophagy in cancer: Recent insights’, *Cancers*, 13(24), p. 6280. doi:10.3390/cancers13246280.

Pawluchin, A. and Galic, M. (2022) ‘Moving through a changing world: Single cell migration in 2D vs. 3D’, *Frontiers in Cell and Developmental Biology*, 10. doi:10.3389/fcell.2022.1080995.

Persson, L.B., Ambati, V.S. and Brandman, O. (2020). Cellular Control of Viscosity Counters Changes in Temperature and Energy Availability. *Cell*, 183(6), pp.1572-1585.e16. doi:https://doi.org/10.1016/j.cell.2020.10.017.

Peschetola, V., Laurent, V., Duperray, A., Michel, R., Ambrosi, D., Preziosi, L. and Verdier, C. (2013). Time-dependent traction force microscopy for cancer cells as a measure of invasiveness. *Cytoskeleton*, 70(4), pp.201-214.

Petit, V. and Thiery, J. (2000) ‘Focal adhesions: Structure and dynamics’, *Biology of the Cell*, 92(7), pp. 477–494. doi:10.1016/s0248-4900(00)01101-1.

Petrášek, J. and Schwarzerová, K. (2009) ‘Actin and microtubule cytoskeleton interactions’, *Current Opinion in Plant Biology*, 12(6), pp. 728–734. doi:10.1016/j.pbi.2009.09.010.

Plotnikov, S., Sabass, B., Schwarz, U. and Waterman, C., 2014. High-Resolution Traction Force Microscopy. *Methods in Cell Biology*, pp.367-394.

Poincloux, R., Collin, O., Lizárraga, F., Romao, M., Debray, M., Piel, M. and Chavrier, P., 2011. Contractility of the cell rear drives invasion of breast tumor cells in 3D Matrigel. *Proceedings of the National Academy of Sciences*, 108(5), pp.1943-1948.

Pozzi, A., Yurchenco, P.D. and Iozzo, R.V. (2017) ‘The nature and biology of basement membranes’, *Matrix Biology*, 57–58, pp. 1–11. doi:10.1016/j.matbio.2016.12.009.

Prior, I., Lewis, P. and Mattos, C., 2012. A Comprehensive Survey of Ras Mutations in Cancer. *Cancer Research*, 72(10), pp.2457-2467.

Putcha, P., Yu, J., Rodriguez-Barrueco, R., Saucedo-Cuevas, L., Villagrasa, P., MurgaPenas, E., Quayle, S., Yang, M., Castro, V., Llobet-Navas, D., Birnbaum, D., Finetti, P., Woodward, W., Bertucci, F., Alpaugh, M., Califano, A. and Silva, J., 2015. HDAC6 activity is a non-oncogene addiction hub for inflammatory breast cancers. *Breast Cancer Research*, 17(1).

Quintás-Cardama, A. and Cortes, J. (2009) ‘Molecular biology of BCR-ABL1–positive chronic myeloid leukaemia’, *Blood*, 113(8), pp. 1619–1630. doi:10.1182/blood-2008-03-144790.

RAFIQ, N. B. M., NISHIMURA, Y., PLOTNIKOV, S. V., THIAGARAJAN, V., ZHANG, Z., SHI, S., NATARAJAN, M., VIASNOFF, V., KANCHANAWONG, P., JONES, G. E. &

BERSHADSKY, A. D. 2019. A mechano-signalling network linking microtubules, myosin IIA filaments and integrin-based adhesions. *Nat Mater,* 18**,** 638-649.

Rape, A., Guo, W. and Wang, Y. (2011). Microtubule depolymerization induces traction force increase through two distinct pathways. *Journal of Cell Science*, 124(24), pp.4233-4240.

Rastgoo, N. *et al.* (2017) 'Role of epigenetics-microrna axis in drug resistance of multiple myeloma', *Journal of Hematology & Oncology*, 10(1). doi:10.1186/s13045-017-0492-1.

RATHJE, L. S., NORDGREN, N., PETTERSSON, T., RONNLUND, D., WIDENGREN, J., ASPENSTROM, P. & GAD, A. K. 2014. Oncogenes induce a vimentin filament collapse mediated by HDAC6 that is linked to cell stiffness. *Proc Natl Acad Sci U S A,* 111**,** 1515-20.

Reinhart-King, C.A., Dembo, M. and Hammer, D.A. (2005) 'The dynamics and mechanics of endothelial cell spreading', *Biophysical Journal*, 89(1), pp. 676–689. doi:10.1529/biophysj.104.054320.

REN, Y., LI, R., ZHENG, Y. & BUSCH, H. 1998. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J Biol Chem,* 273**,** 34954-60.

Ridley, A., 2003. Cell Migration: Integrating Signals from Front to Back. *Science*, 302(5651), pp.1704-1709.

Riggi, N., Aguet, M. and Stamenkovic, I. (2018) 'Cancer metastasis: A reappraisal of its underlying mechanisms and their relevance to treatment', *Annual Review of Pathology: Mechanisms of Disease*, 13(1), pp. 117–140. doi:10.1146/annurev-pathol-020117-044127.

R.H. Gavin (ed.), Cytoskeleton Methods and Protocols, Methods in Molecular Biology, vol. 586).

Roda, V.M. *et al.* (2024) 'Inhibition of rho kinase (rock) impairs cytoskeletal contractility in human Müller glial cells without effects on cell viability, migration, and extracellular matrix production', *Experimental Eye Research*, 238, p. 109745. doi:10.1016/j.exer.2023.109745.

Rodrigues, M. *et al.* (2019) 'Wound healing: A cellular perspective', *Physiological Reviews*, 99(1), pp. 665–706. doi:10.1152/physrev.00067.2017.

RONNLUND, D., GAD, A. K., BLOM, H., ASPENSTROM, P. & WIDENGREN, J. 2013. Spatial organization of proteins in metastasizing cells. *Cytometry A,* 83**,** 855-65.

Rørth, P. (2009) 'Collective cell migration', *Annual Review of Cell and Developmental Biology*, 25(1), pp. 407–429. doi:10.1146/annurev.cellbio.042308.113231.

Rosik, L., Niegisch, G., Fischer, U., Jung, M., Schulz, W. and Hoffmann, M., 2014. Limited efficacy of specific HDAC6 inhibition in urothelial cancer cells. *Cancer Biology & Therapy*, 15(6), pp.742-757.

Ruijter, A.J.M. *et al.* (2003) 'Histone deacetylases (HDACS): Characterization of the classical HDAC family', *Biochemical Journal*, 370(3), pp. 737–749. doi:10.1042/bj20021321.

Sabass, B., Gardel, M., Waterman, C. and Schwarz, U. (2008). High Resolution Traction Force Microscopy Based on Experimental and Computational Advances. *Biophysical Journal*, 94(1), pp.207-220.

Sadjadi, Z. *et al.* (2022) 'Ameboid cell migration through regular arrays of micropillars under confinement', *Biophysical Journal*, 121(23), pp. 4615–4623. doi:10.1016/j.bpj.2022.10.030.

Saito, T., Nishida, K., Furumatsu, T., Yoshida, A., Ozawa, M. and Ozaki, T., 2013. Histone deacetylase inhibitors suppress mechanical stress-induced expression of RUNX2 and ADAMTS-5 through the inhibition of the MAPK signalling pathway in cultured human chondrocytes. *Osteoarthritis and Cartilage*, 21(1), pp.165-174.

Sanz-Moreno, V. *et al.* (2011) 'Rock and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma', *Cancer Cell*, 20(2), pp. 229–245. doi:10.1016/j.ccr.2011.06.018.

Schierbaum, N., Rheinlaender, J. and Schäffer, T. (2019). Combined atomic force microscopy (AFM) and traction force microscopy (TFM) reveals a correlation between viscoelastic material properties and contractile prestress of living cells. *Soft Matter*, 15(8), pp.1721-1729.

Schindelin, J. *et al.* (2012) 'Fiji: An open-source platform for biological-image analysis', *Nature Methods*, 9(7), pp. 676–682. doi:10.1038/nmeth.2019.

SCHAPE, J., PRAUSSE, S., RADMACHER, M. & STICK, R. 2009. Influence of lamin A on the mechanical properties of amphibian oocyte nuclei measured by atomic force microscopy. *Biophys J,* 96**,** 4319-25.

Schneeberger, E.E. and Lynch, R.D. (1984) 'Tight junctions. their structure, composition, and function.', *Circulation Research*, 55(6), pp. 723–733. doi:10.1161/01.res.55.6.723.

Schwock, J., Dhani, N. and Hedley, D.W. (2009) 'Targeting focal adhesion kinase signaling in tumor growth and metastasis', *Expert Opinion on Therapeutic Targets*, 14(1), pp. 77–94. doi:10.1517/14728220903460340.

SCHREINER, S. M., KOO, P. K., ZHAO, Y., MOCHRIE, S. G. & KING, M. C. 2015. The tethering of chromatin to the nuclear envelope supports nuclear mechanics. *Nat Commun,* 6**,** 7159.

SEETHARAMAN, S., VIANAY, B., ROCA, V., FARRUGIA, A. J., DE PASCALIS, C., BOEDA, B., DINGLI, F., LOEW, D., VASSILOPOULOS, S., BERSHADSKY, A., THERY, M. & ETIENNE-MANNEVILLE, S. 2022. Microtubules tune mechanosensitive cell responses. *Nat Mater,* 21**,** 366-377.

Seidel, C., Schnekenburger, M., Dicato, M. and Diederich, M., 2015. Histone deacetylase 6 in health and disease. *Epigenomics*, 7(1), pp.103-118.

Seigneurin-Berny, D., Verdel, A., Curtet, S., Lemercier, C., Garin, J., Rousseaux, S. and Khochbin, S., 2001. Identification of Components of the Murine Histone Deacetylase 6 Complex: Link between Acetylation and Ubiquitination Signalling Pathways. *Molecular and Cellular Biology*, 21(23), pp.8035-8044.

Sengupta, N. and Seto, E. (2004) 'Regulation of histone deacetylase activities', *Journal of Cellular Biochemistry*, 93(1), pp. 57–67. doi:10.1002/jcb.20179.

Seyfried, T. and Huysentruyt, L., 2013. On the Origin of Cancer Metastasis. *Critical Reviews™ in Oncogenesis*, 18(1 - 2), pp.43-73.

Silva, J., Kalinsky, K., Quayle, S. and Yang, M., 2017. Abstract P3-07-06: Inhibition of HDAC6 as targeted therapy for breast cancers. *Poster Session Abstracts.*

Shankar, J. and Nabi, I.R. (2015) 'Actin cytoskeleton regulation of epithelial mesenchymal transition in metastatic cancer cells', *PLOS ONE*, 10(3). doi:10.1371/journal.pone.0119954.

Shapiro, L. and Weis, W.I. (2009) 'Structure and biochemistry of cadherins and Catenins', *Cold Spring Harbor Perspectives in Biology*, 1(3). doi:10.1101/cshperspect. a003053.

Shaw, P.H. (1996) 'The role of p53 in cell cycle regulation', *Pathology - Research and Practice*, 192(7), pp. 669–675. doi:10.1016/s0344-0338(96)80088-4.

Sherr, C.J. (2004) 'Principles of tumor suppression', *Cell*, 116(2), pp. 235–246. doi:10.1016/s0092-8674(03)01075-4.

Sleeboom, J.J. *et al.* (2024) 'The extracellular matrix as hallmark of cancer and metastasis: From biomechanics to therapeutic targets', *Science Translational Medicine*, 16(728). doi:10.1126/scitranslmed.adg3840.

Smith, B. and Bhowmick, N. (2016) 'Role of EMT in metastasis and therapy resistance', *Journal of Clinical Medicine*, 5(2), p. 17. doi:10.3390/jcm5020017.

Sosinsky, G.E. and Nicholson, B.J. (2005) 'Structural Organization of Gap Junction channels', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1711(2), pp. 99–125. doi:10.1016/j.bbamem.2005.04.001.

Sousa, B., Pereira, J. and Paredes, J. (2019) 'The crosstalk between cell adhesion and cancer metabolism', *International Journal of Molecular Sciences*, 20(8), p. 1933. doi:10.3390/ijms20081933

Soussi, T. (2000) 'The P53 tumor suppressor gene: From molecular biology to clinical investigation', *Annals of the New York Academy of Sciences*, 910(1), pp. 121–139. doi:10.1111/j.1749-6632.2000.tb06705.x.

Spiro, R.G. (1973) 'Glycoproteins', *Advances in Protein Chemistry*, pp. 349–467. doi:10.1016/s0065-3233(08)60451-9.

Stachowiak, M., 2020. *Mechanisms Of Actomyosin Contractility in Cells*. [online] Academic Commons.

STEPHEN J. HAGGARTY, K. M. K., JASON C. WONG, CHRISTINA M. GROZINGER, AND STUART L. SCHREIBER 2003. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. *PNAS,* 100**,** 4389–4394.

STEVEN MUNEVAR, Y.-L. W., AND MICAH DEMBO 2001. Traction Force Microscopy of Migrating Normal and H-ras Transformed. *Biophysical Journal,* 80**,** 1744–1757.

STIVAROU, T., STELLAS, D., VARTZI, G., THOMAIDOU, D. & PATSAVOUDI, E. 2016. Targeting highly expressed extracellular HSP90 in breast cancer stem cells inhibits tumor growth in vitro and in vivo. *Cancer Biol Ther,* 17**,** 799-812.

Su, S., Su, S. and Chen, J., 2015. Collagen Gel Contraction Assay. *Protocol Exchange.*

Sun, M. and Zaman, M.H. (2016) 'Modeling, signaling and Cytoskeleton Dynamics: Integrated modeling-experimental frameworks in Cell Migration', *WIREs Systems Biology and Medicine*, 9(1). doi:10.1002/wsbm.1365.

Surgery for cancer (2024) *Surgery for Cancer - NCI*. Available at: https://www.cancer.gov/about-cancer/treatment/types/surgery (Accessed: 28 April 2025).

Svitkina, T. (2018) 'The actin cytoskeleton and actin-based motility', *Cold Spring Harbor Perspectives in Biology*, 10(1). doi:10.1101/cshperspect. a018267.

Swaney, K.F., Huang, C.-H. and Devreotes, P.N. (2010) 'Eukaryotic chemotaxis: A network of signaling pathways controls motility, directional sensing, and polarity', *Annual Review of Biophysics*, 39(1), pp. 265–289. doi:10.1146/annurev.biophys.093008.131228.

SWIFT, J., IVANOVSKA, I. L., BUXBOIM, A., HARADA, T., DINGAL, P. C., PINTER, J., PAJEROWSKI, J. D., SPINLER, K. R., SHIN, J. W., TEWARI, M., REHFELDT, F., SPEICHER, D. W. & DISCHER, D. E. 2013. Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. *Science,* 341**,** 1240104.

Tan, J.L. *et al.* (2003) 'Cells lying on a bed of microneedles: An approach to isolate mechanical force', *Proceedings of the National Academy of Sciences*, 100(4), pp. 1484–1489. doi:10.1073/pnas.0235407100.

Tan, D., Agarwal, R. and Kaye, S., 2006. Mechanisms of transcoelomic metastasis in ovarian cancer. *The Lancet Oncology*, 7(11), pp.925-934.

Taylor, W.R. and Stark, G.R. (2001) 'Regulation of the G2/M transition by P53', *Oncogene*, 20(15), pp. 1803–1815. doi:10.1038/sj.onc.1204252.

Tao, H. *et al.* (2018) '*retracted*: Silence of HDAC6 suppressed esophageal squamous cell carcinoma proliferation and migration by disrupting chaperone function of hsp90', *Journal of Cellular Biochemistry*, 119(8), pp. 6623–6632. doi:10.1002/jcb.26841.

Terriac, E., Coceano, G., Mavajian, Z., Hageman, T., Christ, A., Testa, I., Lautenschläger, F. and Gad, A. (2017). Vimentin Levels and Serine 71 Phosphorylation in the Control of Cell-Matrix Adhesions, Migration Speed, and Shape of Transformed Human Fibroblasts. *Cells*, 6(1), p.2.

Theocharis, A.D. *et al.* (2016) 'Extracellular matrix structure', *Advanced Drug Delivery Reviews*, 97, pp. 4–27. doi:10.1016/j.addr.2015.11.001.

Theveneau, E. and Mayor, R. (2013). Collective cell migration of epithelial and mesenchymal cells. *Cellular and Molecular Life Sciences*, 70(19), pp.3481–3492. doi: https://doi.org/10.1007/s00018-012-1251-7.

Tojkander, S., Gateva, G. and Lappalainen, P. (2012) 'Actin stress fibers – assembly, dynamics and biological roles', *Journal of Cell Science* [Preprint]. doi:10.1242/jcs.098087.

Torre, L.A. *et al.* (2023) *Data from global cancer incidence and mortality rates and trends—an update* [Preprint]. doi:10.1158/1055-9965. c.6516460.v1.

Trepat, X., Chen, Z. and Jacobson, K., 2012. Cell Migration. *Comprehensive Physiology.*

TRICHET, L., LE DIGABEL, J., HAWKINS, R. J., VEDULA, S. R., GUPTA, M., RIBRAULT, C., HERSEN, P., VOITURIEZ, R. & LADOUX, B. 2012. Evidence of a large-scale mechanosensing mechanism for cellular adaptation to substrate stiffness. *Proc Natl Acad Sci U S A,* 109**,** 6933-8.

Trepat, X., Chen, Z. and Jacobson, K. (2012) 'Cell migration', *Comprehensive Physiology*, pp. 2369–2392. doi:10.1002/cphy.c110012.

Tsuchida, N., Murugan, A.K. and Grieco, M. (2016) 'Kirsten Ras* oncogene: Significance of its discovery in human cancer research', *Oncotarget*, 7(29), pp. 46717–46733. doi:10.18632/oncotarget.8773.

Types of cancer (2024) *Cancer Research UK*. Available at: https://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-starts/types-of-cancer (Accessed: 28 March 2025).

Valenzuela-Fernández, A., Cabrero, J., Serrador, J. and Sánchez-Madrid, F., 2008. HDAC6: a key regulator of cytoskeleton, cell migration and cell–cell interactions. *Trends in Cell Biology*, 18(6), pp.291-297.

Van Damme, M., Crompot, E., Meuleman, N., Mineur, P., Bron, D., Lagneaux, L. and Stamatopoulos, B., 2012. HDAC isoenzyme expression is deregulated in chronic lymphocytic leukaemia B-cells and has a complex prognostic significance. *Epigenetics*, 7(12), pp.1403-1412.

Vega, F. and Ridley, A., 2008. Rho GTPases in cancer cell biology. *FEBS Letters*, 582(14), pp.2093-2101.

Velculescu, V.E. and El-Deiry, W.S. (1996) 'Biological and clinical importance of the P53 tumor suppressor gene', *Clinical Chemistry*, 42(6), pp. 858–868. doi:10.1093/clinchem/42.6.858.

Verin, A.D. *et al.* (2001) 'Microtubule disassembly increases endothelial cell barrier dysfunction: Role of MLC Phosphorylation', *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 281(3). doi:10.1152/ajplung.2001.281.3.l565.

Viennet, C., Armbruster, V., Gabiot, A., Gharbi, T., Bride, J. and Humbert, P. (2004). Comparing the contractile properties of human fibroblasts in leg ulcers with normal fibroblasts. *Journal of Wound Care*, 13(9), pp.358-361.

Viscosity (2025) *Encyclopædia Britannica*. Available at: https://www.britannica.com/science/viscosity (Accessed: 29 March 2025).

Vogl, D.T. *et al.* (2017) 'RICOLINOSTAT, the first selective histone deacetylase 6 inhibitor, in combination with bortezomib and dexamethasone for relapsed or refractory multiple myeloma', *Clinical Cancer Research*, 23(13), pp. 3307–3315. doi: 10.1158/1078-0432.ccr-16-2526.

Walker, C., Mojares, E. and del Río Hernández, A., 2018. Role of Extracellular Matrix in Development and Cancer Progression. *International Journal of Molecular Sciences*, 19(10), p.3028.

Wall, M., Butler, D., Haj, A., Bodle, J., Loboa, E. and Banes, A., 2017. Key developments that impacted the field of mechanobiology and mechanotransduction *Journal of Orthopaedic Research.*

Wang, J.H.-C. and Lin, J.-S. (2007) 'Cell Traction Force and measurement methods', *Biomechanics and Modeling in Mechanobiology*, 6(6), pp. 361–371. doi:10.1007/s10237-006-0068-4.

Wang, J., 2010. Cell Traction Forces (CTFs) and CTF Microscopy Applications in Musculoskeletal Research. *Operative Techniques in Orthopaedics*, 20(2), pp.106-109.

Wang, G. *et al.* (2012) 'Class I and class II histone deacetylases are potential therapeutic targets for treating pancreatic cancer', *PLoS ONE*, 7(12). doi: 10.1371/journal.pone.0052095.

Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., Gong, Z., Zhang, S., Zhou, J., Cao, K., Li, X., Xiong, W., Li, G., Zeng, Z. and Guo, C., 2017. Role of tumour microenvironment in tumorigenesis. *Journal of Cancer*, 8(5), pp.761-773.

Wang, X.-X., Wan, R.-Z. and Liu, Z.-P. (2018) 'Recent advances in the discovery of potent and selective HDAC6 inhibitors', *European Journal of Medicinal Chemistry*, 143, pp. 1406–1418. doi:10.1016/j.ejmech.2017.10.040.

Wang, L.-H. *et al.* (2018) 'Loss of tumour suppressor gene function in human cancer: An overview', *Cellular Physiology and Biochemistry*, 51(6), pp. 2647–2693. doi:10.1159/000495956.

Wang, N. *et al.* (2020) 'HDAC6/HNF4α loop mediated by mir-1 promotes bile acids-induced gastric intestinal metaplasia', *Gastric Cancer*, 24(1), pp. 103–116. doi:10.1007/s10120-020-01108-x.

Watanabe, T., Hosoya, H. and Yonemura, S., 2007. Regulation of Myosin II Dynamics by Phosphorylation and Dephosphorylation of Its Light Chain in Epithelial Cells. *Molecular Biology of the Cell*, 18(2), pp.605-616.

Weber, G.F. (2013) 'Why does cancer therapy lack effective anti-metastasis drugs?', *Cancer Letters*, 328(2), pp. 207–211. doi:10.1016/j.canlet.2012.09.025.

Wehrle-Haller, B. (2012) 'Structure and function of focal adhesions', *Current Opinion in Cell Biology*, 24(1), pp. 116–124. doi:10.1016/j.ceb.2011.11.001.

Wei, S.C., Fattet, L. and Yang, J. (2015) 'The forces behind EMT and tumor metastasis', *Cell Cycle*, 14(15), pp. 2387–2388. doi:10.1080/15384101.2015.1063296.

Weißenbruch, K. and Mayor, R. (2024) 'Actomyosin forces in cell migration: Moving beyond cell body retraction', *BioEssays*, 46(10). doi:10.1002/bies.202400055.

Whitehead, A.J., Kirkland, N.J. and Engler, A.J. (2021) 'Atomic Force Microscopy for live-cell and Hydrogel Measurement', *Methods in Molecular Biology*, pp. 217–226. doi:10.1007/978-1-0716-1382-5_16.

Whitmore, W., 1956. Hormone therapy in prostatic cancer. *The American Journal of Medicine*, 21(5), pp.697-713.

Witt, O., Deubzer, H., Milde, T. and Oehme, I., 2009. HDAC family: What is the cancer relevant targets? *Cancer Letters*, 277(1), pp.8-21.

Wee Ma, W. (2011) 'Development of focal adhesion kinase inhibitors in cancer therapy', *Anti-Cancer Agents in Medicinal Chemistry*, 11(7), pp. 638–642. doi:10.2174/187152011796817628.

Wickstead, B. and Gull, K. (2011) 'The evolution of the cytoskeleton', *Journal of Cell Biology*, 194(4), pp. 513–525. doi:10.1083/jcb.201102065.

WILLIAM C. HAHN, C. M. C., ANTE S. LUNDBERG, RODERICK L. BEIJERSBERGEN, MARY W. BROOKS & ROBERT A. WEINBERG 1999. Creation of human tumour cells with defined genetic elements. *NATURE*, 400.

Woodhouse, E.C., Chuaqui, R.F. and Liotta, L.A. (1997) 'General mechanisms of metastasis', *Cancer*, 80(S8), pp. 1529–1537.

Wozniak, M.A. and Chen, C.S. (2009) 'Mechanotransduction in development: A growing role for contractility', *Nature Reviews Molecular Cell Biology*, 10(1), pp. 34–43. doi:10.1038/nrm2592.

Wu, C. (2007) 'Focal adhesion: A focal point in current cell biology and Molecular Medicine', *Cell Adhesion & Migration*, 1(1), pp. 13–18. doi:10.4161/cam.1.1.4081.

Wu, P., Aroush, D., Asnacios, A., Chen, W., Dokukin, M., Doss, B., DurandSmet, P., Ekpenyong, A., Guck, J., Guz, N., Janmey, P., Lee, J., Moore, N., Ott, A., Poh Y., Ros, R., Sander, M., Sokolov, I., Staunton, J., Wang, N., Whyte, G. and Wirtz, D., 2018. A comparison of methods to assess cell mechanical properties. *Nature Methods*, 15(7), pp.491-498.

Wu, Y., Hsu, K., Lee, H., Huang, T., Lin, T., Chen, Y., Sung, T., Liou, J., Hwang-Verslues, W., Pan, S. and HuangFu, W., 2018. A Novel Dual HDAC6 and Tubulin Inhibitor, MPT0B451, Displays Anti-tumor Ability in Human Cancer Cells in Vitro and in Vivo. *Frontiers in Pharmacology*, 9.

Wu, J.-Y. *et al.* (2018) 'Histone deacetylase 6 (HDAC6) deacetylates extracellular signal-regulated kinase 1 (ERK1) and thereby stimulates ERK1 activity', *Journal of Biological Chemistry*, 293(6), pp. 1976–1993. doi:10.1074/jbc.m117.795955.

Xie, Xuan *et al.* (2018) 'Histone deacetylase 6 expression in metastatic lymph nodes is a valuable prognostic marker for resected node-positive esophageal squamous cell cancer', *Cancer Management and Research*, Volume 10, pp. 5451–5460. doi:10.2147/cmar. s178575.

Yamada, K.M. and Sixt, M. (2019) 'Mechanisms of 3D cell migration', *Nature Reviews Molecular Cell Biology*, 20(12), pp. 738–752. doi:10.1038/s41580-019-0172-9.

Yamashiro, S. and Watanabe, N. (2014) 'A new link between the retrograde actin flow and focal adhesions', *The Journal of Biochemistry*, 156(5), pp. 239–248. doi:10.1093/jb/mvu053.

Yang, H., Ganguly, A. and Cabral, F. (2010) 'Inhibition of cell migration and cell division correlates with distinct effects of microtubule inhibiting drugs', *Journal of Biological Chemistry*, 285(42), pp. 32242–32250. doi:10.1074/jbc.m110.160820.

Yemanyi, F., Vranka, J. and Raghunathan, V.K. (2020) 'Crosslinked extracellular matrix stiffens human trabecular meshwork cells via dysregulating β-catenin and Yap/Taz Signaling Pathways', *Investigative Opthalmology & Visual Science*, 61(10), p. 41. doi:10.1167/iovs.61.10.41.

Yilmaz, M. and Christofori, G. (2009) 'EMT, the cytoskeleton, and cancer cell invasion', *Cancer and Metastasis Reviews*, 28(1–2), pp. 15–33. doi:10.1007/s10555-008-9169-0.

Yoshida, N., Omoto, Y., Inoue, A., Eguchi, H., Kobayashi, Y., Kurosumi, M., Saji, S., Suemasu, K., Okazaki, T., Nakachi, K., Fujita, T. and Hayashi, S., 2004. Prediction of prognosis of estrogen receptor-positive breast cancer with combination of selected estrogen-regulated genes. *Cancer Science*, 95(6), pp.496-502.

Yue, B., 2014. Biology of the Extracellular Matrix. *Journal of Glaucoma*, 23, pp. S20-S23

Zaidel-Bar, R. *et al.* (2004) 'Hierarchical Assembly of cell–matrix adhesion complexes', *Biochemical Society Transactions*, 32(3), pp. 416–420. doi:10.1042/bst0320416.

Zamir, E. and Geiger, B. (2001) 'Molecular complexity and dynamics of cell-matrix adhesions', *Journal of Cell Science*, 114(20), pp. 3583–3590. doi:10.1242/jcs.114.20.3583.

Zhang, Z. *et al.* (2004) 'HDAC6 expression is correlated with better survival in breast cancer', *Clinical Cancer Research*, 10(20), pp. 6962–6968. doi:10.1158/1078-0432.ccr-04-0455.

ZHANG, X., YUAN, Z., ZHANG, Y., YONG, S., SALAS-BURGOS, A., KOOMEN, J., OLASHAW, N., PARSONS, J. T., YANG, X. J., DENT, S. R., YAO, T. P., LANE, W. S. & SETO, E. 2007. HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Mol Cell*, 27**,** 197-213.

Zhang, S.-L. *et al.* (2019) 'histone deacetylase 6 is overexpressed and promotes tumor growth of colon cancer through regulation of the MAPK/ERK signal pathway', *OncoTargets and Therapy*, Volume 12, pp. 2409–2419. doi:10.2147/ott. s194986.

Zhang, Z. *et al.* (2022) 'Functional and clinical characteristics of focal adhesion kinases in cancer progression', *Frontiers in Cell and Developmental Biology*, 10. doi:10.3389/fcell.2022.1040311.

Zhang, Z., 2004. HDAC6 Expression Is Correlated with Better Survival in Breast Cancer. *Clinical Cancer Research*, 10(20), pp.6962-6968.

Zhang, Z., Cao, Y., Zhao, W., Guo, L. and Liu, W., 2017. HDAC6 serves as a biomarker for the prognosis of patients with renal cell carcinoma. *Cancer Biomarkers*, 19(2), pp.169-175.

Zhang, S.-L. *et al.* (2019) 'histone deacetylase 6 is overexpressed and promotes tumor growth of colon cancer through regulation of the MAPK/ERK signal pathway', *OncoTargets and Therapy*, Volume 12, pp. 2409–2419. doi:10.2147/ott. s194986.

Zhang, M., Xiang, S., Joo, H., Wang, L., Williams, K., Liu, W., Hu, C., Tong, D.,Haakenson, J., Wang, C., Zhang, S., Pavlovicz, R., Jones, A., Schmidt, K., Tang, J., Dong,H., Shan, B., Fang, B., Radhakrishnan, R., Glazer, P., Matthias, P., Koomen, J., Seto, E.,Bepler, G., Nicosia, S., Chen, J., Li, C., Gu, L., Li, G., Bai, W., Wang, H. and Zhang, X., 2014. HDAC6 Deacetylates and Ubiquitinates MSH2 to Maintain Proper Levels of MutSα. *Molecular Cell*, 55(1), pp.31-46.

Zhao, X. and Guan, J.-L. (2011) 'Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis', *Advanced Drug Delivery Reviews*, 63(8), pp. 610–615. doi:10.1016/j.addr.2010.11.001.

Zhou, J., Yi, Q. and Tang, L. (2019) 'The roles of nuclear focal adhesion kinase (FAK) on Cancer: A focused review', *Journal of Experimental & Clinical Cancer Research*, 38(1). doi:10.1186/s13046-019-1265-1.

Zhou, B., Liu, D. and Tan, Y. (2021) 'Role of HDAC6 and its selective inhibitors in gastrointestinal cancer', *Frontiers in Cell and Developmental Biology*, 9. doi:10.3389/fcell.2021.719390.

Zhu, Y., He, L., Qu, J. and Zhou, Y. (2018). Regulation of Vascular Smooth Muscle Cell Stiffness and Adhesion by [Ca2+] i: An Atomic Force Microscopy-Based Study. *Microscopy and Microanalysis*, 24(6), pp.708-712.

ZILBERMAN, Y., BALLESTREM, C., CARRAMUSA, L., MAZITSCHEK, R., KHOCHBIN, S. & BERSHADSKY, A. 2009. Regulation of microtubule dynamics by inhibition of the tubulin deacetylase HDAC6. *J Cell Sci,* 122**,** 3531-41.